

The Role of Toll-like Receptors in Mast Cell Activation

by

Jeffrey D. McCurdy

Submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

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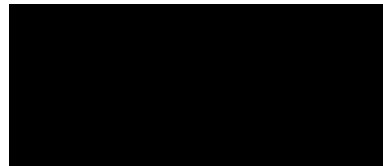
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My family

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Abstract

Mast cells are critical effector cells in allergic responses and play a protective role in host defense against bacterial infections. Cellular responses to a variety of pathogens are dependent on a superfamily of pattern recognition receptors known as Toll-like receptors (TLR). We therefore hypothesized that mast cells express a number of TLRs and require their expression for specific cellular responses to certain pathogen products. Murine bone marrow derived mast cells (BMMC) expressed mRNA for TLR2, TLR4 and TLR6 but not TLR5, while human cord blood derived mast cells (CBMC) expressed mRNA for TLR1, TLR2 and TLR6 but not TLR4. Consistent with the profiles of TLR expression murine BMMC but not human CBMC produced significant levels of NF κ B-regulated cytokines in response to *E. coli* LPS. Functional TLR4 expression was required for BMMC IL-6 and TNF production in response to LPS since BMMC from wild type but not TLR4-deficient mice (C3H/HeJ and C57Bl/10ScCr) responded to LPS challenge. Both human and murine mast cells responded to the TLR2 activator *S. aureus* PGN. PGN induced substantial levels of CBMC GM-CSF and IL-1 β production despite also inducing moderate levels of apoptosis as assessed by caspase activation and a dissipation of mitochondrial membrane potential. Two additional TLR2 activators, zymosan and Pam₃CSK₄ also activated CBMC. To determine if the different TLR2 activators induce distinct profiles of mediators we analyzed CBMC degranulation and LTC₄ generation. PGN and zymosan, which are known to activate a TLR2/TLR6 dimer dependent pathway induced potent levels of LTC₄ (12 (p<0.001) and 23 (p<0.001) fold increase respectively over media controls, n=16) but failed to significantly enhance degranulation. In contrast, the synthetic lipopeptide (Pam₃CysSerLys₄), which is known to activate a TLR1/TLR2 dimer dependent pathway, induced significant levels of CBMC degranulation (3 fold increase over media control p<0.001, n=5) without inducing LTC₄. These findings have important implications for the mechanism of mast cell responses to pathogens and their products and suggest novel mechanisms for generating selective mast cell mediator responses.

Abbreviations

5-Lo	5 lipoxygenase
Ab	antibody
IF	immunofluorescence buffer
Ag	antigen
APP	acute phase protein
BAL	bronchoalveolar lavage
BMMC	bone marrow derived mast cell
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
C	C chemokine
C3a	degradation production from the third component of complement
C3aR	complement 3a receptor
C5a	degradation production from the fifth component of complement
C5aR	complement 5a receptor (CD88)
Ca ²⁺	calcium
Camp	camptothecin
CBMC	cord blood derived mast cells
CC	CC chemokine
CCR	CC chemokine receptor
CD	cluster determinant
RT	reverse transcription
ckit	SCF receptor
cPLA ₂	intracellular phospholipase A ₂
CR	complement receptor
CTMC	connective tissue mast cell
CXCR	CXC chemokine receptor
DAG	diacylglycerol
DC	dendritic cell

DiOC6	dihexyloxacarbocyanine iodide
DNA	deoxyribonucleic acid
DNaseI	deoxyribonuclease I
dsRNA	double-stranded RNA
EDA	extracellular domain A
ELAM	endothelial leukocyte-adhesion molecule
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FADD	fas associated death domain
FcR	Fc receptor
FcR α	FcR α subunit
FcR β	FcR β subunit
FcR γ	FcR γ subunit
FCS	fetal calf serum
Fc ϵ RI	high affinity IgE receptor
FLAP	5-LO activating protein
FLIP	fas-associated death domain-like IL-1-converting enzyme inhibitory protein
FRET	fluorescence resonance energy transfer
GM-CSF	granulocyte-macrophage colony stimulating factor
GPI	glycosylphosphatidylinosityl
HEK	human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid
HMC-1	human mast cell line
hr	hour
Hsp	heat shock protein
<i>i.p.</i>	intraperitoneal
<i>i.v.</i>	intravenous
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IgE	immunoglobulin E

IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IP-10	interferon inducible protein 10
IP3	inositol 1,4,5-triphosphate
IRAK	interleukin-1 receptor associated kinase
IRF3	interferon regulatory factor
ITAMs	immunoreceptor tyrosine-based activation motif
JNK	c-jun NH ₂ -terminal kinase
kDa	kilodalton
KU812	human mast cell/basophilic cell line
LAT	linker of activation of T-cells
LBP	LPS binding protein
LPS	lipopolysaccharide
LT	leukotriene
LTA	lipoteichoic acid
mAb	monoclonal antibody
MAL	MyD88 adaptor-like protein
MALP	mycoplasma associate lipoprotein
MAP-K	mitogen-activate protein kinase
MC ^{CT}	human mast cell subtype containing chymase and tryptase
MC ^T	human mast cell subtype containing tryptase
MDP	muramyl dipeptide
ME	mercaptoethanol
MHC	major histocompatibility complex
min	minute
MIP	macrophage inflammatory protein
MMC	mucosal mast cell
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation factor 88

NFkB	nuclear factor kappa beta
NGF	nerve growth factor
NK	natural killer
NO	nitric oxide
PAF	platelet-activating factor
Pam ₃ CSK ₄	synthetic tripalmitoyl lipopeptide
PAMP	pathogen associated molecular pattern
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mast cell
PCR	polymerase chain reaction
PG	prostaglandin
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PI3P	phosphoinositol-3,4,5-triphosphate
PKC	protein kinase C
PL	phospholipase
PMC	peritoneal mast cell
Poly(I:C)	polyinosine-polycytidylic acid
PTK	protein tyrosine kinase
RANTES	regulated upon activate normal T cell expressed and (presumed) secreted
RIL	recombinant interleukin
RMCP	rat mast cell protease
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
s.c.	subcutaneous
SCF	stem cell factor
SD	standard deviation
SDF	stromal cell derived factor
SEB	staphylococcal enterotoxin B
SEM	standard error of the mean
SH2	Src homology-2 domain

SLP-76	SH2-containing leukocyte phosphoprotein of 76 kDa
STI	soybean trypsin inhibitor
Tec	Tec family of tyrosine kinases
Th	T helper
TIR	toll-interleukin-1-like receptor
TIRAP	toll-interleukin-1 receptor associated protein
TLR	toll-like receptor
TNF	tumor necrosis factor
TRIF	toll-interleukin-1 receptor interferon inducing factor
VCAM	vascular cell adhesion molecule
W/W ^v	WBB6F1-Kit ^W /Kit ^{W^v} (ckit deficient mice)

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1.1 Mast cells

Mast cells are highly granulated immunological effector cells found in nearly every tissue of the body. They are closely related to basophils, but unlike these cells mature mast cells are found primarily in peripheral tissues (Metcalf *et al.*, 1997). Since the initial discovery of the mast cell by Paul Ehrlich in 1878, our understanding of these cells has expanded considerably (Ehrlich, 1878). Much of the early work focused on the detrimental role of these cells in various pathological conditions such as allergic disorders and chronic inflammatory diseases. However, more recently the beneficial roles of the mast cell are becoming apparent. In 1996, two groups simultaneously published groundbreaking work demonstrating a critical protective role for mast cells in host defense against bacterial infections (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996b). This work has opened the doors to an extensive area of research in the field of mast cell biology. Additional research is now focusing on the ability of these cells to regulate tissue remodeling, angiogenesis and the establishment of the adaptive immune response (Levi-Schaffer and Pe'er, 2001). Although these areas of research are only in their infancy, future studies will undoubtedly help to clarify the precise role of the mast cell in these processes.

Mast cells are located in close proximity to blood vessels and nerves and at contact sites with the external environment (Metcalf *et al.*, 1997). These strategic locations place the mast cell in an excellent position to respond to pathogenic challenge, blood born antigens and signals from the nervous system. Upon activation, mast cells have the potential to rapidly release a number of inflammatory mediators as well as the ability to synthesize a wide range of cytokines and chemokines. These mediators are

highly regulated and are selectively produced and/or released depending on the type of stimulation (Leal-Berumen *et al.*, 1994). These features coupled with their role in a broad range of immune responses, has resulted in the mast cell being designated a sentinel cell of the immune system (Galli, 1999). Therefore, studies that help to dissect the mechanisms of mast cell activation are important for understanding the role of these cells in various pathological conditions

1.2 Mast cell development and distribution

Mast cells arise from hematopoietic progenitor cells in the bone marrow. Unique from other cell types such as basophils, their differentiation is not complete until they establish residence in peripheral tissues (Nilsson and Metcalfe, 1996). They initially begin their differentiation in the bone marrow under the influence of growth factors such as stem cell factor (SCF) and interleukin (IL)-3. They then enter the blood as CD34⁺ precursors and eventually migrate into vascularized tissues where they complete their maturation (Rodewald *et al.*, 1996). Locally produced growth factors such as SCF and IL-3 also play a role in terminally differentiating mast cells. The requirement of SCF is demonstrated in mice deficient in the receptor for SCF, *ckit* (WBB6F1-Kit^W/Kit^{Wv} (W/W^v) or mice deficient in SCF itself (Sl/Sl). Kitamura and colleagues demonstrated that both of these murine strains almost completely lack mature mast cells (Kitamura Y, 1978; Kitamura Y, 1979; Kitamura and Hatanaka, 1978).

Mast cells are abundant at host-environment contact sites including tissues such as skin, lungs, nasal mucosa and the gastrointestinal and genitourinary tracts. In the skin, mast cells are primarily found in the dermis, near blood vessels, nerves, hair follicles and

sweat glands with numbers sometimes reaching as high as 10,000 mast cells per cubic millimeter (Kitamura and Hatanaka, 1978; Prussin and Metcalfe, 2003). Although the numbers of mast cells vary from site to site, the highest numbers are associated with sites of greatest nerve density such as the face (Marshall and Waserman, 1995). In the lungs, mast cells can be found in bronchial airway connective tissues and in peripheral intra-alveolar spaces. These locations provide mast cells with increased exposure to pathogens and environmental antigens

1.2.1 Mast cell heterogeneity

It has been appreciated for more than 35 years that mast cells from different anatomical sites even within the same species exhibit substantial heterogeneity. Enerback first demonstrated in the mid 1960's through morphological and histological studies that substantial heterogeneity exists among rodent mast cells (Enerback *et al.*, 1986). Since this initial observation, detailed analysis of human and rodent mast cells resulted in distinct classification schemes.

1.2.2 Classification of rodent mast cell subtypes

In the rodent, mast cells are divided into two major subgroups, connective tissue mast cells (CTMC) and mucosal mast cells (MMC). These subtypes differ considerably in shape, granule content and are located in distinct anatomical locations. MMC are smaller, contain less histamine and are generally located in mucosal sites such as the intestinal submucosa and lung parenchyma. In contrast, CTMC are more granulated and are abundant in connective tissues such as the peritoneal cavity and skin (Schwartz and Huff,

1998). These subsets can also be distinguished on the basis of granule proteoglycan content such as chondroitin sulfate and heparin found in MMC and CTMC respectively. Distinguishing these mast cell sub-types on the basis of proteoglycan content alone can be misleading, especially with immature CTMC which often contain a mixture of highly sulfated proteoglycans.

A more accurate criterion for identification of mast cell sub-types is based upon the chymotrypsin-like neutral protease composition of mast cell granules, with rat CTMC containing rat mast cell protease I (RMCP-1) and rat MMC contain RMCP-II (Forsythe and Ennis, 2000). Mouse mast cell subtypes can also be distinguished by protease content with MMC preferentially expressing mouse mast cell protease (mMCP)-1 and mMCP-2 while CTMC expressing mMCP-5 and mMCP-6 (Gurish *et al.*, 1993; McNeil *et al.*, 1991; Reynolds *et al.*, 1991; Reynolds *et al.*, 1989; Serafin *et al.*, 1991).

Mouse mast cells can be generated *in vitro* by culturing bone marrow progenitor cells in the presence of IL-3 (Tertian *et al.*, 1981). These cells express many of the characteristics associated with MMC (Marshall and Bienenstock, 1990). However, such cultured mast cells usually represent a mixture of mast cell subtypes, the composition of which may be altered by culture conditions and supplements (Ghildyal *et al.*, 1992a; Ghildyal *et al.*, 1992b; Gurish *et al.*, 1992).

1.2.3 Classification of human mast cell subtypes

In contrast to rodent mast cells, human sub-types are less restricted to particular anatomical locations. Both subsets can be found in most tissues, although one subset generally predominates. Therefore, Irani and Schwartz proposed a new classification

scheme for human mast cells according to their neutral protease content. MC^T and MC^{TC} subtypes are distinguished depending on whether they contain only tryptase or both tryptase and chymase in their respective granules (Irani and Schwartz, 1994; Schwartz, 1993). MC^{TC} are generally located in the skin and the intestinal submucosa while MC^T are predominantly found in the lung and intestinal lamina propria. The distribution of MC^T and MC^{TC} is generally analogous to the rodent CTMC and MMC respectively. However, more recent studies have suggested that the majority of enteric mast cells do in fact contain chymase (Aldenberg and Enerback, 1994; Beil *et al.*, 1997). Although the levels of chymase relative to tryptase were very low, the phenotype of human intestinal mast cells is not completely analogous to the murine mucosal mast cell. Multiple tryptase isoforms have since been described which may allow finer differentiation of specific human mast cell types (Peng *et al.*, 2003).

The heterogeneity of mast cells is not only restricted to characteristics of morphology and granule composition but also the response of mast cells to a variety of stimuli. Notably, human skin mast cells release significant amounts of histamine when stimulated with compound 48/80, substance P or the complement degradation product C5a while human lung mast cells are relatively unresponsive to these stimuli (Forsythe and Ennis, 2000).

Human mast cells are commonly generated *in vitro* by culturing umbilical cord blood mononuclear cells in the presence of mast cell growth factors such as SCF, IL-6 and PGE₂ (Figure 1). Using this method, highly pure mast cell preparations (>95% pure) that closely resemble the MC^T phenotype can be obtained (Saito *et al.*, 1996). An alternate method employs SCF, IL-6 and IL-10 as growth factors (Ochi *et al.*, 1999).

Human mast cells have also been developed from peripheral blood CD34⁺ cells and from bone marrow progenitor cells. These cells are routinely grown in the presence of SCF, IL-6 and IL-3 for 8-10 weeks (Rottem *et al.*, 1994).

1.3 Classical forms of mast cell activation

A wide range of activators including immunological stimuli, synthetic compounds, pathogenic organisms and environmental antigens can elicit mast cell responses. Frequently considered types of immunological stimuli include products from the complement cascade such as C3a and C5a or neuropeptides such as substance P (Marshall and Wasserman, 1995; Nilsson *et al.*, 1996). A number of basic peptides such as Magainin also have activating effects on some mast cell types. The ability of synthetic compounds such as the calcium ionophore, A23187 to induce mast cell activation have been well described. Moreover, mast cells respond to a large number of pathogens and pathogen products (Lin *et al.*, 2000b). Perhaps the most widely studied form of mast cell activation involves aggregation of the high affinity IgE receptor (FcεRI) by multivalent environmental antigens.

1.3.1 IgE-dependent mast cell activation

FcεRI has a high affinity for the Fc portion of monomeric IgE (Kent *et al.*, 1994). Aggregation of bound IgE molecules by multivalent antigens results in the rapid release of mast cell mediators and the production of newly sensitized ones. FcεRI is expressed in tetrameric form ($\alpha\beta\gamma_2$) by mast cells and basophils and is expressed in trimeric form

($\alpha\gamma_2$) by Langerhans and peripheral blood dendritic cells (Gounni *et al.*, 1994a; Gounni *et al.*, 1994b; Maurer *et al.*, 1996). A low affinity receptor for IgE, Fc ϵ RII, CD23 has also been documented and is expressed by B cells (Almerigogna *et al.*, 1989).

The tetrameric form of Fc ϵ RI is composed of single copies of α and β chains and two copies of a γ chain. The α chain is responsible for binding IgE through a long extracellular portion containing two Ig-like domains. The β chain is a transmembrane protein that spans the membrane four times with both the NH₂- and the COOH-terminals inside the cell. The γ chains are also transmembrane proteins with short extracellular domains and long intracellular tails. The β and γ chains each contain two immunoreceptor tyrosine based activation motifs (ITAMs) which are important for signaling.

Mast cell activation by Fc ϵ RI aggregation initiates a complex network of signaling pathways involving numerous phosphorylation events and the recruitment and activation of appropriate adaptor and signaling molecules. These events have been reviewed extensively (Nadler and Kinet, 2002; Nadler *et al.*, 2000; Rivera, 2002) and are outlined in figure 2. One of the initial events to occur is the phosphorylation of the receptor itself. However, since Fc ϵ RI does not contain any intrinsic kinase activity it requires the function of various protein tyrosine kinases (PTK). Lyn was the first PTK identified in this signaling network and was shown to phosphorylate the Fc ϵ R β and Fc ϵ R γ ITAMs (Eiseman and Bolen, 1992a; Eiseman and Bolen, 1992b; Jouvin *et al.*, 1994). This initial event occurs rapidly because Lyn is constitutively positioned in close association with the β chain of Fc ϵ RI (Wilson *et al.*, 2000). The phosphorylated ITAMs

function as docking sites for the recruitment of Src homology 2 (SH2) domain-containing signaling molecules such as Syk. Once Syk associates with the phosphorylated ITAMs of FcεRγ it becomes phosphorylated by Lyn. Syk subsequently activates a number of substrates including linker of activation of T cells (LAT) which forms a high molecular weight complex with SH2-containing leukocyte phosphoprotein of 76 kDa (SLP-76), Bruton's tyrosine kinase (Btk) and phospholipase Cγ1 and Cγ2. Activation of the γ phospholipases acts on phosphatidylinositol-3,4,5-triphosphate (PI3P) to generate diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 is responsible for inducing a sustained influx of calcium while DAG activates the serine/threonine protein kinase C (PKC).

A critical role for many of these molecules including, Syk, SLP-76, Grb2 and LAT, in mast cell degranulation have been confirmed using knockout mice (Costello *et al.*, 1996; Zhang *et al.*, 1996) (Pivniouk *et al.*, 1999);(Saitoh *et al.*, 2000). Interestingly, although Lyn-deficient mice have defects in tyrosine phosphorylation and calcium mobilization, degranulation is not impaired (Kawakami *et al.*, 2000; Nishizumi and Yamamoto, 1997). This apparent nonessential function of Lyn suggests that alternate PTKs are required for mast cell degranulation. Recently, an additional PTK, Fyn was shown to associate with FcεRI and become activated following receptor aggregation (Parravicini *et al.*, 2002). Moreover, mast cells from Fyn-null mice have an impaired degranulation response following FcεRI aggregation but not in response to phorbol 12-myristate 13-acetate and the calcium ionophore A23187 (Parravicini *et al.*, 2002). These findings coupled with previous studies strongly suggest a critical role for Fyn but not Lyn

in mast cell degranulation. Fyn works in concert with the adaptor molecule Gab2 to activate phosphoinositol 3 kinase (PI3K) and it is through this interaction that Fyn is able to regulate mast cell degranulation. It is apparent that down stream events of Lyn and Fyn converge at various points. However, our understanding of the interplay between Lyn and Fyn signaling pathways is far from complete. The coordinated efforts of Lyn and Fyn result in an increase in intracellular calcium and activation of protein kinase C, both of which are essential for mast cell degranulation (Ozawa *et al.*, 1993a; Ozawa *et al.*, 1993b).

1.4 Mast cell mediators

To appreciate the potency of the mast cell you need not look further than anaphylactic reactions to food, drugs and insect venom. These reactions result in the sudden and massive release of mast cell granule associated products and generation of lipid-derived mediators, both of which have profound effects on the airway and vasculature. These reactions are often followed by the synthesis of a large number of cytokines and chemokines which may occur hours after the initial stimulation (Gordon *et al.*, 1990; Schwartz, 1994). Interestingly, mast cell cytokine production can be induced in the absence of granule exocytosis (Leal-Berumen *et al.*, 1995), indicating that certain forms of activation may result in the production of distinct profiles of mast cell mediators. The precise role of mast cell derived mediators is often hard to assess in pathological conditions because of the contributions of other cell types. Therefore the production of mast cell mediators and how they are regulated has been a subject of intense focus in

recent literature. The following sections will discuss in detail some of the mast cell derived mediators which are most relevant to this study.

1.4.1 Preformed mediators

The highly granulated nature of mature mast cells can be easily observed by electron microscopy or light microscopy using selective granule stains (Figure 1). The granules appear as electron dense structures and occupy much of the cytoplasmic space of mature mast cells. Upon appropriate activation such as FcεRI aggregation, mast cell granules fuse with the plasma membrane resulting in the release of their contents into the extracellular environment. This process is initiated within seconds (Metcalfe *et al.*, 1997). A slower process involving vesicular transport of pre-formed mediators from secretory granules to the plasma membrane has also been documented and is termed "piecemeal" degranulation (Dvorak *et al.*, 1998; Dvorak and Dvorak, 1975)

Stored in the granules are numerous preformed mediators such as histamine, serotonin, the proteoglycan heparin, numerous neutral proteases and other enzymes such as the acid hydrolase β-hexosaminidase (Schwartz and Austen, 1984). Interestingly, the cytokine TNF has been identified in mast cell secretory granules (Gordon and Galli 1990; (Gordon and Galli, 1991). Importantly the mast cell is the only known source of preformed TNF raising the possibility that mast cell-derived TNF may play an important role in the early stages of mast cell-dependent responses.

Tryptases

Human mast cells express at least five different members of the tryptase family of neutral proteases (Miller and Schwartz, 1989; Miller *et al.*, 1989; Vanderslice *et al.*, 1990). Recent studies have reported a role for members of this family in allergic diseases and potentially in host defense against bacterial infections (Clark *et al.*, 1995; Huang *et al.*, 2001; Krishna *et al.*, 2001). Among the members of this family, α and β tryptases are released by mast cells during inflammatory events and exhibit distinct functions (Huang *et al.*, 2001). Of particular interest, Huang *et al.*, (2001) determined that β 1 tryptase, but not α tryptase injected into the lungs of mast cell deficient mice (W/W^v) provided protection against pulmonary infection by *Klebsiella pneumoniae*. In this model, β 1 tryptase induced neutrophil recruitment and resulted in fewer viable bacteria in the lungs of these mice (Huang *et al.*, 2001). Moreover, β 1 treatment did not alter normal airway responsiveness suggesting that different isoforms of tryptase, although highly conserved may induce different types of mast cell responses.

Histamine

Histamine is one of the predominant mediators contained within mast cell granules. Moreover, the mast cell appears to be the major cellular source of this mediator (Schwartz and Austen, 1984). Histamine directs its effects by binding to the cell surface receptors H1 and H2 (Ash and Schild, 1997). Engagement of H1 results in bronchial constriction and increased vascular permeability which present as symptoms of wheezing and local edema respectively (Schwartz and Austen, 1984). Recently histamine was also shown to up-regulate expression of the pattern recognition receptors TLR2 and TLR4,

suggesting that it may also enhance mechanisms of pathogen recognition (Talreja *et al.*, personal communication). It has also been suggested that histamine may enhance the maturation of immature dendritic cells (Mazzoni *et al.*, 2001).

1.4.2 Newly synthesized mediators

The types of lipid mediators produced by mast cells are related to their heterogeneity. In general, mast cells derived from mucosal sites are thought to produce predominantly leukotriene C₄ while those at connective tissue sites mainly produce prostaglandin D₂ (Lawrence *et al.*, 1987). Mast cells are also capable of producing PGE₂ under certain circumstances (Mencia-Huerta *et al.*, 1983) and are a good source of platelet activating factor (Hogaboam *et al.*, 1992; Lewis and Austen, 1984; Marshall and Waserman, 1995)

Leukotrienes

Leukotrienes (LTs) were first described in 1938 as slow reacting substance of anaphylaxis (Fedberg and Kelloway, 1938). Their release is somewhat delayed compared to histamine however, their effects are much more potent and longer in duration (Drazen, 1998). LT production occurs within minutes of mast cell activation and their effects can persist for hours. LTs are divided into two groups, LTB₄ and the cysteinyl LTs consisting of LTC₄ and its degradation products LTD₄ and LTE₄.

Cysteinyl LTs and histamine have many features in common including the ability to induce bronchoconstriction, vasodilatation and increase vasculature permeability (McMillan, 2001). These similarities are reflected by studies where histamine receptor blockers were unable to completely inhibit the airway contractile activity in allergic

inflammation of the airways (Brocklehurst, 1960). LTB₄ also induces similar effects on the vasculature but does not substantially induce airway smooth muscle contraction (McMillan *et al.*, 2001). Although LTs and histamine share similar effects on the airway and vasculature, LTs are at least 1000-fold more potent (Drazen, 1998). Moreover, a unique function of LTs is their chemotactic capability with LTB₄ predominantly inducing the recruitment of neutrophils and cysteinyl LTs involved in the recruitment of eosinophils.

Because of the involvement of LTs in allergic diseases, a thorough understanding of the regulation of these mediators is important.

1.4.3 Regulation of Leukotriene generation

The steps involved in the generation of LTs and prostaglandins have been reviewed extensively (Henderson, 1991; Leff, 2001) and are depicted in figure 3. Briefly the process begins when arachidonic acid is liberated from membrane phospholipids by the enzyme intracellular phospholipase A₂ (cPLA₂) a process which may be induced by specific receptor-mediated signaling events by cell damage. Free arachidonic acid is then acted upon by 5-lipoxygenase (5-LO) or cyclooxygenase which is important for the generation of leukotrienes and prostaglandins respectively. In the biosynthesis of leukotrienes 5-LO works in concert with the 5-LO activating protein (FLAP) resulting in the conversion of arachidonic acid to 5-HPETE and then to LTA₄. LTA₄ is rapidly converted to LTC₄ by LTC₄ synthase or to LTB₄ by LTA₄ hydrolase. The pathway of leukotriene biosynthesis is highly regulated by multiple intracellular signaling events.

cPLA₂ rapidly translocates to the nuclear envelope following activation and functions as critical regulatory molecule in LT production. The essential role for cPLA₂ was demonstrated by (Nakatani *et al.*, 2000) using cPLA₂^{-/-} mice. Disruption of the cPLA₂ gene resulted in a complete abrogation of leukotriene production in murine bone marrow derived mast cells activated by an IgE-dependent mechanism. PLA₂ activation can be regulated by intracellular calcium and phosphorylation events at critical serine residues. The precise pathways required for the phosphorylation of PLA₂ are not well understood, however a role for p38 and protein kinase C have been demonstrated (Borsch-Haubold, 1998; Borsch-Haubold *et al.*, 1999; Borsch-Haubold *et al.*, 1997; Xu *et al.*, 2000).

Similar to cPLA₂, 5-LO is rapidly recruited to the nuclear envelope following activation. Moreover, 5-LO can also be regulated by Ca²⁺ signals which aid to position 5-LO close to its substrate and activating protein, FLAP. This has been demonstrated using Ca²⁺ chelators which efficiently inhibited the association of 5-LO with the nuclear envelope (Covin *et al.*, 1998).

The Rho GTPase, Rac1 is also suspected to play a key role in the generation of LTs. In support of this concept, Rac1 activation is associated with arachidonic acid release and mutant forms of this molecule can efficiently block 5-LO translocation to the nuclear envelope (Peppelenbosch *et al.*, 1995). Rac1 combined with PI3 kinase is also known to induce a Ca²⁺ flux which explains, in part, the ability of this molecule to regulate LT production. Interestingly, other studies have suggested calcium-independent mechanisms of 5-LO activation. These studies demonstrated the importance of phosphorylation events (Werz *et al.*, 2002) and inherent nuclear localization signals

expressed by 5-LO (Hanaka *et al.*, 2002). It is likely that distinct regulatory mechanisms exist for different activators. Further studies are needed to determine the differences in pathways for LT generation depending on whether the stimuli is delivered by FcεRI aggregation, growth factor or pathogen products.

1.4.4 Cytokines & chemokines

Mast cells have the capacity to generate a large number of cytokines and chemokines as first described by (Metcalfe *et al.*, 1997; Plaut *et al.*, 1989). The production of these mediators may vary depending on the growth environment, the pathological conditions and the mode of mast cell activation. Lists of mast cell cytokines and chemokines have been tabulated and include IL-1, 3, 4, 5, 6, 8, 10, 12, 13, 16, 25, IFN γ , TNF, and GM-CSF RANTES (CCL6), MIP-1 α (CCL3), MIP-1 β (CCL4), MIP-3 α (CCL20), SDF-1 (CXCL12) and MCP-1 (CCL2) (Burd *et al.*, 1989; Gordon *et al.*, 1990; Lin *et al.*, 2002; Selvan *et al.*, 1994). Mast cells are a substantial source of IL-1 β , GM-CSF, IL-6 and TNF, all of which are regulated, in part by the transcription factor NF κ B. A brief description of each of these cytokines is presented below.

TNF

TNF is a pleiotropic cytokine with a clear role in host defense, and a large number of inflammatory diseases (Beutler, 1995; Vassalli, 1992). The importance of TNF in host defense has been demonstrated using TNF receptor knockout mice which were highly susceptible to *Listeria monocytogenes* (Pfeffer *et al.*, 1993). Additional studies have

demonstrated that administration of recombinant TNF can protect mice against gram-negative bacterial infections (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996). However, excessive production of TNF can lead to severe inflammatory reactions and tissue damage which may occur in various chronic inflammatory diseases and in septic shock (Standiford and Strieter, 1992). Therapeutic strategies which target TNF have provided significant clinical benefit to patients with rheumatoid arthritis and a number of other inflammatory disorders. Interestingly, this treatment provided little or no benefit to patients with septic shock (Abraham, 1998; Eigler *et al.*, 1997). These findings are likely due to the redundant nature of various pro-inflammatory mediators of the immune system and the difficulty in treating patients in the end stages of septic shock.

TNF is produced early during inflammatory conditions and exerts numerous biological effects. Central to its role in the inflammatory response is its ability to locally initiate a pro-inflammatory cytokine and chemokine cascade and to induce the upregulation of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1 and E-selectin (Curfs *et al.*, 1997; Rothlein *et al.*, 1988; Walsh *et al.*, 1991). TNF can also modulate systemic effects by inducing fever and sometimes irreversible organ damage associated with intravascular coagulation (Curfs *et al.*, 1997). As a result, production of this cytokine must be highly regulated for effective anti-microbial inflammatory reactions.

TNF is initially synthesized as a transmembrane protein and can be cleaved into the extracellular environment where it functions as a homotrimer. TNF exerts its effects through two membrane bound receptors designated TNF receptor 1 (TNFR1, p55) and TNFR2, p75). TNFR1 is ubiquitously expressed and is believed to mediate most of the

biological effects of TNF while TNFR2 is predominately expressed on endothelial cells and hematopoietic cells and its biological functions are less well understood (Curfs *et al.*, 1997; Papadakis and Targan, 2000)

An important feature of the mast cell is its ability to store preformed pools of TNF in its granules. This was initially described using mouse peritoneal mast cells and more recently with human and rat mast cells (Gordon and Galli *et al.*, 1990; (Beil *et al.*, 1996; Beil *et al.*, 1995). Interestingly other cell types such as macrophages, T cells and B cells do not contain detectable levels of preformed TNF (Beutler and Cerami, 1989; Cuturi *et al.*, 1987; Sung *et al.*, 1988a; Sung *et al.*, 1988b). Moreover, the levels of granule-associated TNF in mouse peritoneal mast cells were substantially higher than the levels observed from LPS stimulated macrophages (Gordon and Galli, 1990). Collectively, these studies have lead investigators to suggest that the mast cell is the only cell to have substantial preformed stores of this cytokine (Gordon and Galli, 1990).

Mast cells also have the ability to generate newly synthesized TNF suggesting that the biological effects of mast cell-derived TNF can be immediate and may persist for hours following activation (Plaut *et al.*, 1989; Gordon and Galli, 1990). Studies *in vivo* have identified an important role for mast cell-derived TNF in IgE-dependent responses (Wershil *et al.*, 1991) and in host defense against certain gram-negative bacterial infections (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996).

IL-6

IL-6 is typically produced in nearly all-inflammatory conditions including those induced by acute infections, chronic inflammatory diseases and tissue injury. The major cellular

sources of this cytokine include macrophages, T cells, B cells, fibroblasts, keratinocytes, endothelial cells and mast cells (Akira *et al.*, 1990). Moreover, IL-6 is one of the major cytokines produced by mast cells from almost all sources (Costa *et al.*, 1998). A role for IL-6 has been well established in the development and differentiation of a range of cell types. IL-6 is closely connected with hematopoiesis, the induction of T cell development, and the differentiation of B cells into antibody-secreting plasma cells (Kishimoto, 1989; Van Snick *et al.*, 1989). In addition, IL-6 is most well recognized for its role in regulating the production of acute phase proteins (APP) by hepatocytes (Korf, 1995; Stadnyk and Gauldie, 1991). This is clearly demonstrated using IL-6^{-/-} mice where the levels of APP such as serum amyloid A are reduced ≈100-fold following challenge with *Lysteria monocytogenes* (Kopf *et al.*, 1994; Kopf *et al.*, 1995). Interestingly, this trend is not observed when IL-6^{-/-} mice are activated by LPS. This may suggest that APP production in response to certain types of bacteria require mechanisms other than IL-6.

The precise nature of IL-6 as a pro-inflammatory or anti-inflammatory cytokine has been a topic of intense controversy. The levels of IL-6 correlate well with toxic shock (Waage, 1998). However, it is not well understood if IL-6 is a major player or is produced as a consequence of this response. Since many of the APP induced by IL-6 are thought to be involved in the regulation of normal homeostasis it is believed that IL-6 may have a role in controlling acute inflammatory responses. In support of this concept, IL-6^{-/-} mice produce substantially greater levels of pro-inflammatory cytokines such as TNF, GM-CSF and IFN γ during endotoxemia as compared to wild type control mice (Xing *et al.*, 1998). Moreover, IL-6^{-/-} mice suffered an increased mortality rate during endotoxemia (Xing *et al.*, 1998). In contrast to these findings, (Kopf *et al.*, 1995)

demonstrated that IL-6^{-/-} mice were less susceptible to the lethal effects of endotoxin administration. The reasons for these controversial findings are currently unknown and demonstrate the necessity for further studies to clarify these issues.

IL-1 β

IL-1 β is a multifunctional cytokine that exerts its effects on virtually every immune cell and a large number of non-immune cells. The extensive role of this cytokine, in health and disease, has been reviewed in detail by (Dinarello, 1996). IL-1 β is a potent stimulator of the cytokine cascade due to its ability to induce the production of a large number of cytokines and chemokines. Local production of this cytokine is also responsible for the generation of inflammatory mediators such as PGE₂ and nitric oxide. Moreover, IL-1 β is able to upregulate the expression of adhesion molecules on endothelial cells such as vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1 and endothelial leukocyte-adhesion molecule (ELAM)-1. Collectively, these events lead to an increase in vascular permeability and cellular infiltration into the site of inflammation. Systemically, IL-1 β can cause fever and may induce significant alterations in normal homeostatic processes (Dinarello, 1996). IL-1 β exerts its effects through the type-1 IL-1 receptor (IL-1RI) (Sims *et al.*, 1993).

Lessons from IL-1 β ^{-/-} or IL-1 receptor (IL-1RI)^{-/-} mice have provided clues toward the biological function of this cytokine. Interestingly, IL-1RI^{-/-} mice were highly susceptible to infection with *Listeria monocytogenes* (Labow *et al.*, 1997). However, IL-1

did not appear to play a significant role in LPS-induced toxicity. These studies may reflect the overlapping roles of IL-1 β with other cytokines such as TNF.

IL-1 β is synthesized as a precursor (proIL-1 β) and requires enzymatic digestion by the IL-1 converting enzyme to produce the biologically active form. When IL-1 β associates with IL-1RI a rapid association occurs between IL-1RI and the IL-1 receptor associated protein resulting in high affinity binding and initiation of a signaling cascade. The intracellular region of IL-1R is highly homologous to the intracellular region of the newly describe Toll-like receptor family and as a result the two receptor systems initiate similar signaling pathways (O'Neill and Dinarello, 2000).

Regulation of IL-1 β production is important to limit excessive tissue damage. The host has also developed additional mechanisms to control the effects of this potent pro-inflammatory cytokine. A second receptor, IL-1R type-II, has also been identified, and although it does not possess signaling capability it functions as a regulatory "decoy" receptor. IL-1 β is also controlled through the actions of a naturally occurring antagonist, designated the IL-1 receptor antagonist (IL-1Ra) (Arend, 1993; Carter *et al.*, 1990; Eisenberg *et al.*, 1990). IL-1Ra itself is structurally related to IL-1 but has the ability to specifically block the binding of IL-1 α and IL-1 β to cell surface receptors without itself activating target cells (Arend *et al.*, 1989; Dripps *et al.*, 1991a; Dripps *et al.*, 1991b; Eisenberg *et al.*, 1990)

GMCSF

GM-CSF was first described based on its ability to stimulate the clonal proliferation of myeloid precursors (Burgess and Metcalf, 1980). Since this initial discovery, our understanding of the role of GM-CSF has expanded considerably. GM-CSF has a range of effects on mature effector cells including neutrophils, monocytes, macrophages and eosinophils. Each of these cells responds to GM-CSF by a cell surface receptor composed of two distinct subunits, α and a common β chain (Hayashida *et al.*, 1990). The α subunit alone mediates low affinity binding, while both subunits are required for high affinity binding.

Through this receptor, GM-CSF is able to promote cellular survival, proliferation and function of myeloid progenitors and mature granulocytes (Armitage, 1998; Hamilton, 2002). Studies *in vitro* and *in vivo* have demonstrated that the administration of GM-CSF enhances microbicidal activity to a wide range of bacterial and fungal pathogens (Armitage, 1998). Moreover, GM-CSF^{-/-} mice have a defective pulmonary clearance of bacterial and fungal pathogens (LeVine *et al.*, 1999).

It is believed that GM-CSF regulates microbicidal activity by enhancing the effector function of granulocytes. Studies *in vitro* have shown that GM-CSF can exert an effect on monocytes, neutrophils and macrophages by enhancing phagocytosis, increasing oxidative metabolism and priming cells for enhanced cytokine production (Coleman *et al.*, 1988; Handman and Burgess, 1979). GM-CSF alone is not a potent inducer of many other cytokines but in conjunction with other stimuli such as bacterial products it significantly enhances TNF production (Hamilton *et al.*, 1993).

1.5 Physiological roles of mast cells in health and disease

Increased numbers of mast cells are associated with a number of inflammatory conditions such as rheumatoid arthritis (Gotis-Graham *et al.*, 1998), allergic asthma (Koshino *et al.*, 1996), certain types of tumors (Lauria de Cidre and Sacerdote de Lustig, 1990) and during helminth infections (Befus and Bienenstock, 1979; Nawa and Korenaga, 1983; Ruitenbergh and Elgersma, 1976; Woodbury *et al.*, 1984). In many cases mast cell increases are associated with chronic tissue remodeling especially the angiogenic process in which mast cells have been strongly implicated (Levi-Schaffer and Pe'er, 2001). The evidence for a role of mast cells in allergic diseases is particularly convincing and will be described below.

1.5.1 Role of mast cells in allergic diseases

Mast cells have long been associated with many allergic disorders such as asthma, rhinitis, allergic gastroenteritis and contact dermatitis (Holgate, 1991). Asthma is of particular interest because of the associated morbidity, the enormous economic cost and the reported increasing incidences in many developed nations (Evans *et al.*, 1996; Goldstein *et al.*, 1994). Therefore it is increasingly important to determine the precise role of mast cells in this disorder.

Asthma is a multifaceted disorder typically characterized by three principal features, reversible airway obstruction, airway hyperresponsiveness and airway inflammation that are influenced by both genetic and environmental factors (Peden, 2002). The disease presents as a biphasic disorder with distinct early and late stages, both of which can be triggered following inhalation of allergens by appropriately sensitized individuals. Some individuals however experience only early or late stage responses.

Numerous cell types and a wide range of mediators are believed to be involved, some of which have distinct functions while many provide similar overlapping roles. As a result it is exceedingly difficult to assess the role of an individual cell type.

Despite these major hurdles, a central role for the mast cell in the induction of IgE-dependent acute reversible airway obstruction (early phase) has been established using mast cell deficient mice (W/W^v) (Ando *et al.*, 1993; Takizawa *et al.*, 1989). Moreover, examination of bronchial biopsies from human asthmatic subjects revealed evidence of mast cell degranulation, suggesting the involvement of these cells in the disorder (Bradley *et al.*, 1991). Mast cells contribute to the early phases by rapidly releasing and/or generating potent inflammatory mediators following cross-linking of membrane-bound IgE molecules by specific allergens (Kinet *et al.*, 1991). Among the numerous mast cell derived mediators, histamine, leukotrienes and prostaglandins are believed to be major players and indeed antagonism of their effects attenuates many of the early symptoms of asthma (Wasserman, 1994). LTC₄ derived from resident mast cells plays a key role as the major inducing mediator of early and late phase bronchoconstriction (O'Byrne, 1997). Although there is much less experimental evidence, mast cells are also believed to play a role in the late, chronic phase of asthma through the actions of various cytokines (Bradding and Holgate, 1996).

1.5.2 Role of mast cells in host defense

The strategic distribution of mast cells at common entry points for pathogens (e.g., skin, linings of the gastrointestinal, respiratory, and urinary tracts) and their ability to generate potent inflammatory reactions has suggested a potential role for these cells in host

defense. Evidence from a large number of studies has supported a role for mast cells in host defense against various classes of pathogens.

1.5.3 Role of mast cells in parasitic and viral infections

Intestinal mucosal mastocytosis and signs of mast cell activation are characteristic of certain types of parasitic infections (Befus and Bienenstock, 1979; Nawa and Korenaga, 1983; Ruitenbergh and Elgersma, 1976; Woodbury *et al.*, 1984). Initial studies determined that repeated injection of IL-3 increased mast cell numbers and resulted in a more effective clearance of *Stongyloides ratti* (Abe and Nawa, 1988). Moreover, strategies to inhibit local mast cell hyperplasia in models of *Nippostrongylus brasiliensis*-induced intestinal inflammation correlated with a delay in nematode expulsion (Issekutz *et al.*, 2001). From these studies it was speculated that mast cells play a protective role against parasitic infections. To address this, a number of groups turned to mast cell deficient mice, W/W^v.

In support of a beneficial role, W/W^v mice were much less effective in clearing some intestinal nematode infections than wild type control mice. Expulsion of *Haemaohysalis longicornis*, *S. ratti* or *S. venezulensis* in W/W^v mice had much slower kinetics (Kessler, 1995). Moreover, reconstitution of these mice with bone marrow derived mast cells from wild-type mice restored the protective effect similar to that of the wild-type control mice. Despite these findings, nematode expulsion ultimately occurred regardless of the numbers of mast cells. Moreover, an additional experiment determined that W/W^v mice were not impaired in their ability to expel *N. brasiliensis* (Reed *et al.*, 1984). Collectively, these studies suggest that mast cells play an early role in host defense

against some types of parasitic infections and that additional immune effector cells may play a more important role at later time points.

It has long been recognized that viral infections of the upper respiratory tract can exacerbate the symptoms of asthma (Corne and Holgate, 1997; Johnston, 1997). This is suggestive of potential mast cell-virus interactions. Several *in vitro* studies have begun to examine the response of human mast cells to viral infection (King *et al.*, 2002; King *et al.*, 2000). From these studies, Dengue virus infection of human mast cells resulted in the production of IL-6 and RANTES (CCL5) but not IL-1 β or GM-CSF. Viral infections of the respiratory system have also been associated with increased mast cell density and evidence of mast cell activation (Everard *et al.*, 1995; Sorden and Castleman, 1995a; Sorden and Castleman, 1995b). However, formal experiments examining the precise function of mast cells in anti-viral responses *in vivo* are lacking. Therefore it remains to be determined if the mast cell serves as an effector cell in host defense against viral infections or as a reservoir for viral pathogenesis.

1.5.4 Role of mast cells in bacterial infections

Echtenacher *et al.*, (1996) and Malaviya *et al.*, (1996) performed elaborate studies demonstrating the protective role of mast cells against bacterial infections. In these studies, mast cell deficient mice (W/W^v) were highly susceptible to acute septic peritonitis induced by caecal ligation and puncture (Echtenacher *et al.*, 1996) or by intraperitoneal instillation of a mouse-virulent bacteria, *Klebsiella pneumoniae* (Malaviya *et al.*, 1996). W/W^v mice exhibited almost complete mortality compared to minimal mortality in the wild type control mice. Correction of the mast cell deficiency by

reconstitution of W/W^v mice with BMDC restored survival to levels observed in wild type control mice. A further study demonstrated that increasing the number of mast cells by long-term injection of SCF, correlated with an improved survival rate of these mice in a model of acute septic peritonitis (Maurer *et al.*, 1998).

The mechanism accounting for the protective effects of mast cells is beginning to be defined. Microscopic examination of infected tissues in these *in vivo* models, revealed a significantly elevated bacterial burden in W/W^v mice compared to mast cell reconstituted W/W^v or wild type control mice. These findings correlated with a substantial decrease in neutrophil recruitment to the site of infection in W/W^v mice. Mast cells produce a number of neutrophil chemoattractant molecules when stimulated *in vitro* with bacteria (Lin *et al.*, 2000b; Malaviya and Abraham, 2000; Malaviya *et al.*, 1999). Two of these chemoattractant molecules, TNF and LTB₄ are primarily responsible for the mast cell protective effect in the Gram-negative bacterial models used so far as determined using anti-TNF monoclonal antibody (mAb) or a specific LTB₄ inhibitor which significantly blocked bacterial clearance and host survival (Echtenacher *et al.*, 1996; *et al.*, 1996; Malaviya and Abraham, 2000).

Although these studies have identified the importance of mast cell-derived mediators in host defense, it cannot be ruled out that mast cells induce these mediators from other cell types such as resident macrophages. Moreover, the precise mechanism of mast cell activation by bacteria or bacterial products also remains to be determined.

1.6 Mast cell receptors for pathogen recognition

In general, mast cell activation by bacteria can be achieved through direct or indirect mechanisms. Direct interactions occur via mast cell surface receptors while indirect interactions are facilitated by host derived products such as antibody, lipid mediators or degradation products from the complement system (Galli, 2000). Mast cell-bacterial interactions sometimes involves phagocytosis which may even result in the uptake of multiple organisms. This process, under certain circumstances, can be greatly enhanced by specific host antibody, since human mast cells express a variety of Fc receptors (Woolhiser *et al.*, 2001). Organisms demonstrated to be taken up by mast cells include *Bordetella pertussis* (Mielcarek *et al.*, 2001) and Fim-H expressing *E.coli* (Malaviya *et al.*, 1994), *Pseudomonas aeruginosa* (Lin *et al.*, 2002) as well as a number of Gram-positive organisms (Arock *et al.*, 1998). Cellular caveolae are thought to play a critical role in the mast cell bacterial uptake process (Shin *et al.*, 2000). In some cases, phagocytosis is associated with the initiation of a cytokine response (Malaviya *et al.*, 1994), while in other situations, mast cells are thought to produce cytokines and chemokines in response to bacteria and their products without a phagocytic response. There are a variety of receptor systems that have been implicated in mast cell responses to bacteria, the most studied of which will be reviewed below. Some of these systems may also be important in responses to other pathogens such as viruses, protozoa and fungi. Figure 4 illustrates some of the major receptor systems known to be employed by mast cells in response to pathogens.

1.6.1 Complement and complement receptors

The complement system is widely regarded as an essential component of host defence and has been shown to be an important regulator of mast cell function (Gommerman *et al.*, 2000). The complement system acts at multiple levels including bacterial opsonization by C3b and iC3b components, direct cellular activation by the anaphylatoxins C3a and C5a as well as bacterial lysis via the C5b-C9 membrane attack complex. *In vivo* models have provided direct evidence for a role of complement in bacterial clearance and host survival. (Prodeus *et al.*, 1997) demonstrated using a model of acute bacterial peritonitis achieved by caecal ligation and puncture that mice deficient in the complement components C3 or C4 resulted in significantly higher levels of mortality compared to wild type mice. Interestingly, C3 and C4 were also shown to be important for mast cell activation since reduced levels of histamine and TNF were observed in the peritoneum of C3^{-/-} or C4^{-/-} mice. These findings correlated with reduced neutrophil infiltration and reduced bacterial clearance. Administration of purified C3 protein to C3^{-/-} mice enhanced activation of peritoneal mast cells and restored host survival to levels comparable to wild type mice (Prodeus *et al.*, 1997).

Mast cells express a variety of receptors for complement products suggesting that mast cell function can be modulated at multiple levels by the complement system (Fureder *et al.*, 1995). There is considerable heterogeneity in the expression pattern of complement receptors and the responses to complement products by human and murine mast cells (Fureder *et al.*, 1995). Human skin mast cells but not lung mast cells expressed the receptor for C5a, CD88 and activation of these cells with C5a resulted in histamine release by human skin mast cells but not human lung mast cells (el-Lati *et al.*, 1994; Schulman *et al.*, 1988)

Mast cells also readily bind and phagocytose pathogens that have been opsonized by complement products. Complement coated *Salmonella typhimurium* or the helminth *Schistosoma mansoni* effectively bound to mast cells and this binding was blocked using anti-C3 antibodies suggesting the involvement of complement receptor 3 (CR3) (Sher, 1976; Sher *et al.*, 1979). Insights into this interaction have been provided by studies using iC3b receptor (CR3, Mac-1, CD11a/CD18) deficient mice subjected to caecal ligation and puncture. Reduced mast cell activation and neutrophil recruitment was observed in such animals, which was associated with reduced bacterial clearance and host survival (Rosenkranz *et al.*, 1998). Although reduced neutrophil recruitment into the peritoneum is likely to involve a relative inability of CR3^{-/-} (Mac-1^{-/-}, CD11/CD18^{-/-}) neutrophils to adhere to the endothelium, the observed lack of mast cell activation, in these mice, may also have contributed to impaired host defence.

1.6.2 Fc receptors

Mast cells express a variety of Fc receptors, as previously mentioned, including the high affinity IgE receptor (FcεRI) this providing additional methods for interactions between mast cells and bacteria. Several reports have documented that patients with peptic ulcers and atopic dermatitis have IgE specific antibodies toward *Helicobacter pylori* and *Staphylococcus aureus* respectively (Abramson *et al.*, 1982; Leung *et al.*, 1987). These findings suggest that bacteria-IgE interactions presumably lead to the aggregation of the high affinity IgE receptor, FcεRI and subsequent mast cell activation.

Fcγ receptors may also play a critical role in mast cell interactions, with pathogens especially for recognition of opsonized bacteria. For example, *Borrelia*

burgdorferi, the causative agent of Lyme disease, induces mast cell activation using a mechanism that is thought to involve Fc γ RII and Fc γ RIII (Talkington and Nickell, 1999). *B. burgdorferi* induces low levels of mast cell degranulation and the production of a number of pro-inflammatory cytokines and chemokines including TNF and IL-6 as well as increased mRNA for MIP-1 α (CCL3) and MIP-1 β (CCL3) (Talkington and Nickell, 2001; Talkington and Nickell, 1999). A role for Fc receptors was demonstrated using the neutralizing Ab (Mab2.4G2) which targets Fc γ RII and Fc γ RIII and effectively inhibits *B. burgdorferi* induced mast cell TNF production (Talkington and Nickell, 2001). Moreover, pre-treatment of mast cells with mouse IgG1 or IgG2a also effectively blocked the response (Talkington and Nickell, 2001).

Additional immunoglobulin-dependent mast cell-bacteria interactions have been reported which lead to mast cell activation but utilize mechanisms other than classic antibody-antigen interactions. For example, Protein A from *S. aureus* and protein L from *Peptostreptococcus magnus* have been designated immunoglobulin superantigens for their ability to bind non-specifically to immunoglobulins. *S. aureus* induces human mast cell degranulation and LTC₄ production (Genovese *et al.*, 2000) using a mechanism that appears to be dependent on the presence of protein A since a strain of *S. aureus* deficient in protein A (*S. aureus* Wood strain) failed to induce mast cell activation. Protein A has been reported to bind to either the constant domain of certain immunoglobulins (IgG1, 2 and 4) (Forsgren and Sjoquist, 1966) or a structure located in the Fab region of a number of immunoglobulins (Ingnas *et al.*, 1980; Ingnas *et al.*, 1981). It has been suggested that the Fab but not the Fc binding region of protein A is responsible for mast cell activation (Genovese *et al.*, 2000). Furthermore, monoclonal IgM V_H3⁺ but not

monoclonal V_H6^+ blocked protein A-mediated histamine release by human mast cells suggesting that protein A can interact, directly or indirectly, with the V_H3 region of IgE on human mast cells (Genovese *et al.*, 2000). *P. magnus* also induces IgE-dependant mast cell mediator release, which appears to be dependant on protein L since strain 644 peptostreptococci which does not synthesize protein L failed to induce mast cell histamine release. Protein L interacts with the κ light chain of human IgE, since IgE molecules expressing κ chains but not λ chains blocked mast cell activation (Genovese *et al.*, 2000).

1.6.3 MHC-II interactions with superantigens

Classical bacterial superantigens are a group of proteins or glycoproteins that are able to activate a large subset of T-cells without prior antigen exposure. The mechanism of T cell activation by these agents is markedly different from conventional antigens, which require processing, presentation followed by activation of only specific, clonotypic T cells. Instead, superantigens bind major histocompatibility complex (MHC) class II molecules on antigen-presenting cells outside the peptide groove, and induce massive proliferation of virtually all T cells bearing specific $V\beta$ determinants. In addition to affecting T cells, superantigens are able to modulate cellular responses of a large number of MHC-II-positive cells, potentially including mast cells. Since it has been reported that mast cells are capable of presenting antigen through an MHC-II dependent mechanism (Frاندji *et al.*, 1996; Tkaczyk *et al.*, 1999).

Anti-superantigen IgE antibodies have been reported in patients with peptic ulcers and atopic dermatitis (Abramson *et al.*, 1982; Leung *et al.*, 1993) suggesting that IgE-dependent mechanisms may be involved in bacterial-mast cell interactions involving superantigens. However, the ability of bacterial superantigens to directly induce mast cell activation, independent of IgE and the mechanisms involved is highly controversial. Scheuber *et al.* reported that *Staphylococcus* enterotoxin B (SEB), one of the best-studied bacterial superantigens, evoked immediate-type reactions in the skin of unsensitized monkeys which resulted in rapid mast cell activation and leukotriene generation (Scheuber *et al.*, 1987; Scheuber *et al.*, 1985). In contrast, a subsequent report demonstrated delayed mast cell degranulation following cutaneous injection of SEB into mice suggesting that mast cell activation by SEB in this model occurs through an indirect mechanism (Saloga *et al.*, 1996).

In vitro, SEB induces serotonin release by rat RBL-2H3 mast cells (Komisar *et al.*, 1992). Although these cells directly bind SEB (Olenick *et al.*, 1991), they do not express detectable levels of MHC-II and anti-MHC-II antibody failed to inhibit serotonin release (Komisar *et al.*, 1992). As a result, it has been proposed that SEB might activate an alternate receptor system. Recently, (Calkins *et al.*, 2002) demonstrated that *in vivo* chemokine production in response to SEB challenge was significantly abrogated in toll-like receptor 4 (TLR4)-mutated mice compared to wild type control mice. This study suggests that TLR4 may play an important role in cellular responses to SEB. Further studies are required to determine if TLR4 is involved in the direct recognition of SEB or if TLR4 indirectly regulates SEB responses by modulating MHC-II expression.

In contrast to the findings in the rodent system, it has been reported, by some, that SEB or SEA do not induce degranulation by the human mast cell line MHC-1, although inhibition of mast cell IL-4 production was observed (Ackermann *et al.*, 1998). SEA was, however, shown to bind to MHC-1 cells via MHC-II and elicit significant ultrastructural changes by another group (Dimitriadou *et al.*, 1998). The lack of degranulation in some studies may be a consequence of inadequate levels of MHC-II expression on resting MHC-1 cells. In support of this, a recent study by Vincent-Schneider *et al.* demonstrated that resting rodent mast cells contain MHC-II molecules localized to intracellular granules and require activation for prominent surface expression (Vincent-Schneider *et al.*, 2001). Additionally, (Poncet *et al.*, 1999) demonstrated that resting HMC-1 cells only express low levels of MHC-II and that treatment with IFN- γ or GM-CSF could significantly upregulate its expression. Moreover, MHC-1 were only effective in presenting superantigens to CD4+ T cell hybridomas after IFN- γ treatment, supporting the concept that functional MHC-II is not constitutively expressed by human mast cells and requires upregulation. Further experiments are required to determine if upregulation of MHC-II enables MHC-1 or other human mast cells to degranulate in response to superantigens. Collectively, these findings suggest that mast cells require prior activation to become responsive to superantigens. The requirement for MHC-II up-regulation may prevent aberrant activation of the acquired immune response and prevent mast cell activation unless additional stimuli are present. Therefore it is likely that superantigens are more important activators of mast cells during extensive infections when multiple pathogen-derived or inflammatory stimuli are available or in the context of chronic inflammatory disease.

1.6.4 CD48

Several enteric bacteria have the capacity to bind avidly to mast cells in an opsonin-independent manner. Binding of *E. coli* to murine mast cells results in phagocytosis and mediator production including, TNF release, LTB₄ production and limited degranulation. The interactions between mast cells and a number of enteric bacteria such as *E. coli*, *K. pneumonia*, *Enterobacter cloacae* and *Serratia marcescenes* are dependent on the expression of type 1 fimbriae by these bacteria (Malaviya *et al.*, 1994). The critical component for mast cell-bacteria interactions is a mannose binding lectin denoted Fim-H which is located on the distal tips of fimbrial filaments. In support of this, Fim-H expressing *E. coli* induced significant histamine release from peritoneal mast cells and bladder mast cells *in vivo*. A similar response has been observed *in vitro* using human mast cells (Arock *et al.*, 1998; Lin *et al.*, 1999), Fim-H binds mannosyl residues of glycoproteins on host cell and therefore the receptor for Fim-H varies depending on the host cell. Isolation and sequencing of the mast cell receptor for Fim-H revealed a 45-kDa protein designated CD48. Pre-treatment of mast cells with antibodies directed toward CD48 effectively blocked mast cell adherence to bacteria as well as TNF production confirming that CD48 is the putative receptor for FimH on mast cells (Malaviya *et al.*, 1999).

A recent study demonstrated that CD48 is also important for the recognition of *Mycobacterium tuberculosis* (Munoz *et al.*, 2003). Anti-CD48 antibodies efficiently blocked *M. tuberculosis* induced histamine release from rat peritoneal mast cells.

CD48 is a glycosylphosphatidylinositol (GPI)-anchored protein which attaches to the exoplasmic leaflet of the cell plasma membrane. Since CD48 does not cross the plasma membrane it is unlikely that CD48 itself can initiate signalling pathways. Recently it has been shown that signal transduction by other GPI-anchored proteins such as CD14 requires association with members of the TLR family. Currently it is not known if CD48 associates with members of the TLR family or with other signalling molecules but this is an area of active investigation. It also remains possible that CD48 engagement initiates signaling pathways through the aggregation of lipid rafts, as in the case of Thy-1 signaling (Draber and Draberova, 2002).

These studies have identified a number of strategies for pathogen recognition by mast cells. Aside from the direct mast cell bacterial interactions involving CD48, many of these strategies involve the recognition of opsonized pathogens suggesting a necessity for the acquired immune system or the complement cascade. Studies described in this dissertation and other recent studies described below examine if the recognition of PAMPs through classical innate receptor systems are utilized by mast cells for microbial recognition.

1.7 Innate Immunity and classes of innate receptors

Innate immunity is an ancient defense mechanism that has been evolutionarily conserved to provide the host with a first line of protection against infectious agents. Central to this system is the ability of the host to rapidly mobilize effector mechanisms against a wide range of pathogenic organisms without the requirement of prior exposure. The innate immune system is critical for keeping pathogenic organisms in check and in higher

organisms also functions to initiate the development of the adaptive immune response (Fearon and Locksley, 1996; Janeway, 1998; Medzhitov and Janeway, 1997; Medzhitov *et al.*, 1997).

In order to function efficiently the innate immune system must discriminate pathogenic agents from non-pathogenic host molecules. This is accomplished primarily using a limited number of germ-line encoded receptors know as pattern recognition receptors (PRRs). Examples of defined PRRs include, CD14, β 2-integrins (CD11/CD18), c-type lectins, macrophage scavenger receptors and complement receptors. Most recently, a novel family of mammalian PRRs was identified and collectively termed Toll-like receptors (TLRs) (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002).

1.8 Toll-like Receptors

TLRs are a highly conserved superfamily of PRRs which play an essential role in recognition of microbial products from virtually all classes of pathogenic organisms (Akira *et al.*, 2000; Akira *et al.*, 2001; Qureshi and Medzhitov, 2003; Takeuchi and Akira, 2001). The first TLR was identified in *Drosophila melanogaster* and was designated Toll. Initial studies implicated this molecule in the development of normal dorsal ventral polarity of *Drosophila melanogaster* embryos (Hashimoto *et al.*, 1988). Toll and 18-Wheeler, a closely related family member, were later identified as important participants in anti-fungal and anti-bacterial immune responses respectively in adult flies (Lemaitre *et al.*, 1996; Williams *et al.*, 1997). Since *Drosophila* do not have acquired immune responses they rely almost exclusively on innate immunity for protection against microbial challenge (Medzhitov and Janeway, 1998). Therefore, these initial findings

suggested that the TLR family might be a critical component of the innate immune system for protection against pathogenic organisms.

1.8.1 Structural features

Currently ten mammalian members have been grouped into this superfamily based on a number of conserved structural features, some of which are illustrated in figure 5. All are type 1 transmembrane proteins with an ectodomain containing leucine rich repeats and one or two cysteine rich regions proximal to the plasma membrane. A critical feature of all these receptors is the presence of a highly conserved cytoplasmic domain termed the toll-interleukin-1 receptor like (TIR) domain. As the name indicates this region shares substantial homology with the corresponding region of the IL-1 receptor (O'Neill, 2000). Additional human proteins express this domain such as the intracellular adaptor molecules MyD88, TIRAP, TRIF and the IL-18 receptor (O'Neill, 2000). This domain provides the necessary internal scaffold for the association of well-defined signaling molecules. The TIR domain is highly conserved throughout all members of the human TLR family and is even substantially conserved across species (O'Neill, 2000). Minor changes in the amino acid sequence of critical residues in the TIR domain render these molecules nonfunctional. A well-defined example is a highly conserved proline at position 712 for TLR4 and at position 681 for TLR2. When these residues are mutated the recruitment of MyD88 is significantly abrogated (O'Neill, 2002; Poltorak *et al.*, 1998a; Poltorak *et al.*, 1998b; Takeuchi and Akira, 2002). Interestingly, TLR3 is the only member of the TLR/IL-1 receptor family that does not have this conserved proline

residue (O'Neill 2002). It will be interesting to determine why MyD88 can still be recruited to this receptor.

1.8.2 TLR Expression

TLR expression varies drastically between different immunological cell types. Early studies demonstrated that TLR1 mRNA was ubiquitously expressed by human monocytes, neutrophils, T and B cells, monocytes-derived dendritic cells and natural killer (NK) cells (Muzio *et al.*, 2000b). Moreover, TLR2, TLR4 and TLR5 were restricted to monocytes, neutrophils and dendritic cells while TLR3 was specific for dendritic cells. Further studies using quantitative real time PCR demonstrated that monocytes expressed a broad range of TLRs including high levels of TLR1, TLR2 and TLR4, moderate levels of TLR5, TLR6 and TLR8 and low levels of TLR9. B cells and plasmacytoid dendritic cells expressed TLR1, TLR6, TLR7, TLR9 and TLR10 (Hornung *et al.*, 2002; Muzio *et al.*, 2000b; Zarembler and Godowski, 2002). Moreover, these cells expressed the highest levels of TLR9 and TLR10 while monocytes expressed the highest levels of TLR2 and TLR4. Interestingly, NK cells were shown to express mRNA for TLR3 suggesting that this molecule may not be restricted to dendritic cells. Expression of TLR2 protein was detected on monocytes and eosinophils but not by B cells, T cells and NK cells (Hornung *et al.*, 2002).

A large number of studies have also examined TLR expression by intestinal epithelial cells. These cells are of particular interest because of their constant exposure to microbial products and because of their role in providing a barrier at the host/environment interface. Most intestinal epithelial cell lines examined expressed little

or no TLR4 (Abreu *et al.*, 2002; Abreu *et al.*, 2001; Melmed *et al.*, 2003). Other reports identified constitutive levels of TLR2 and TLR4 near the apical surface (Cario *et al.*, 2002) while TLR5 was selectively expressed on the basolateral surface of the epithelium (Gewirtz *et al.*, 2001). Although epithelial cells express low levels of certain TLRs, these molecules can be rapidly up-regulated following activation with specific stimuli. A wide range of pathogen products, pathological conditions and immunological stimuli can modulate their expression (Cario and Podolsky, 2000; Nomura *et al.*, 2000; Suzuki *et al.*, 2003).

Collectively these studies suggest that TLR expression is not limited to classic immune cells. Moreover, it is evident that innate immune cells, express a broader range of TLR molecules than adaptive immune cells. This is consistent with the notion that the innate immune system provides the first line of defense against microbial infections. The expression of TLRs by adaptive immune cells has been a topic of recent interest as it suggests that pathogen products may play an important role in directly regulating adaptive responses.

1.9 TLR Ligands

It is becoming increasingly clear that all four of the major classes of human pathogens including: virus, bacteria, yeast and protozoa have the capacity to induce cellular responses via TLRs as illustrated in figure 6 (Barton and Medzhitov, 2002). Many of these microorganisms have the ability to undergo rapid mutation resulting in changes in surface molecules. As a result, TLRs and other PRRs have been evolutionarily selected to recognize the most highly conserved and essential structures of these microorganisms

known as pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). In addition to these infectious agents, TLRs are also able to respond to endogenous host proteins that are produced during inflammatory conditions but are absent in healthy tissue. Defining the precise microbial or endogenous components that activate TLRs has proven challenging due to the lack of commercially available reagents and the difficulty in obtaining purified preparations of PAMPs void of contaminants. Although in many cases it is not known if TLR activators bind directly to their respective TLRs, I will still refer to these molecules as receptors. Table 1 represents a detailed list of many of the TLR agonists.

1.9.1 LPS a TLR4 ligand

The first discovery of a suspected TLR agonist came from two independent groups demonstrating that the LPS resistant cell line, HEK-293 could become responsive to LPS after being transfected with TLR2 (Kirschning *et al.*, 1998; Yang *et al.*, 1998). The requirement for TLR2 appeared to be selective since transfection with other TLRs including TLR1 and TLR4 did not confer LPS responsiveness. A subsequent study demonstrating that macrophages from hamsters could respond normally to LPS despite carrying a non-functional TLR2 protein (Heine *et al.*, 1999) challenged these findings. Moreover, when commercial LPS preparations were re-purified by phenol extraction to deplete protein contaminants, TLR4-transfected, but not TLR2-transfected, cell lines were able to respond to LPS (Hirschfeld *et al.*, 2000).

Poltorak and colleagues (1998) helped to clarify that TLR4 but not TLR2 was the receptor for LPS. A genetic approach using physical mapping techniques revealed that

mutations in TLR4 were responsible for the LPS-deficient phenotype in C3H/HeJ and C57Bl/10ScCr mice. C3H/HeJ mice contained a single point mutation in the third exon of TLR4 resulting in a predicted proline to histidine substitution at position 712 of the amino acid sequence. This region is located in the cytoplasmic domain and is highly conserved among most TLRs. In mice of the C57Bl/10ScCr strain, a TLR4 null mutation was identified resulting in the deletion of all three exons of the gene. To further confirm these findings the TLR4 gene was disrupted in a wild-type, murine strain 129/SvJ. Disruption of the TLR4 gene resulted in a phenotype similar to the C3H/HeJ and C57Bl/10ScCr strains including a lack of monocytes TNF and NO₂⁻ production following LPS challenge. Moreover, disruption of the TLR2 gene did not abrogate the LPS responses (Takeuchi *et al.*, 1999).

Despite the convincing genetic data suggesting TLR4 as the receptor for LPS, human embryonic kidney-293 cells transfected with TLR4 remained unresponsive to LPS challenge (Kirschning *et al.*, 1999). This discrepancy indicated that an additional molecule absent in HEK-293 cells was required for LPS responsiveness. (Shimazu *et al.*, 1999) identified a molecule termed MD-2 which could be immunoprecipitated with TLR4 and was shown to physically associate with the ectodomain of human (Shimazu *et al.*, 1999) and mouse (Akashi *et al.*, 2000) TLR4. MD-2 was required for maximal expression of TLR4 and coexpression of both molecules rendered HEK-293 cells responsive to LPS (Shimazu *et al.*, 1999). MD-2 was essential for LPS responsiveness since mice containing a point mutation in this molecule were hyporesponsive to LPS challenge. Moreover, a mAb specific for the murine TLR4-MD-2 complex inhibited LPS responses by peritoneal macrophages.

1.9.2 TLR2 Ligands

Most of the TLR family members appear to have a limited number of ligands. TLR2 in contrast appears to be exceedingly promiscuous and may be important as a general sensor of all major classes of pathogens (Kirschning and Schumann, 2002).

Initially it was demonstrated that whole bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Mycobacterium avium*, *Bacillus subtilis* and *Borrelia burgdorferi* had the ability to activate cells when TLR2 was ectopically over-expressed (Lien *et al.*, 1999; Schwandner *et al.*, 1999a; Yoshimura *et al.*, 1999). These studies also demonstrated that application of highly purified components of these bacteria such as peptidoglycan (PGN), commercially available *Bacillus* lipoteichoic acid (LTA) or synthetic lipopeptide could induced TLR2-dependent responses. Moreover, application of neutralizing TLR2 mAbs inhibited cellular responses to the indicated activators. This finding demonstrated that expression of physiological levels of TLR2 conferred responsiveness to these bacterial products.

Additional experiments using TLR2^{-/-} mice demonstrated the biological significance of these findings. Cytokine and NO²⁻ production by macrophages in response to whole bacteria, PGN, LTA and lipopeptide were significantly impaired when the cells were obtained from TLR2^{-/-} mice (Takeuchi *et al.*, 1999). Moreover, *i.v.* administration of *S. aureus* and *Streptococcus pneumoniae* resulted in significantly greater mortality and higher levels of colonized bacteria in TLR2^{-/-} mice compared to wild type controls. Interestingly, TNF and IL-6 production by *S. aureus* treated macrophages was not completely abrogated in cells from TLR2^{-/-} mice. In contrast, macrophages from MyD88^{-/-}

$^{-/-}$ mice were completely unresponsive to *S. aureus* challenge suggesting that a TLR(s) in addition to TLR2 is responsible for the recognition of the whole bacteria (Takeuchi *et al.*, 2000b). These findings were further complemented by work from Lorenz *et al.*, (2002) who identified a polymorphism in a highly conserved region of the cytoplasmic domain of TLR2 which correlated with increased susceptibility to *S. aureus* infection in humans.

LPS from the spirochetes *Leptospira interrogans* and *Phorphyromonas gingivalis* are structurally different from gram-negative LPS. Interestingly, purified, protein free, preparations of this LPS activates cells via TLR2 but not TLR4 (Werts *et al.*, 2001b). Approaches using cells from TLR2 $^{-/-}$ mice and TLR2 mAbs provide substantial evidence to support the role of TLR2 in recognition of this atypical LPS (Werts *et al.*, 2001b). Although unlikely, the possibility still exists that a minor protein contaminant co-purified with LPS was responsible for the TLR2 dependent signal.

Bacterial and mycoplasma lipopeptides represent an additional group of PAMPs which are able to activate TLR2. A variety of microbial lipopeptides including the outer surface protein A from *Borrelia burgdorferi* (Hirschfeld *et al.*, 1999), *Mycoplasma fermentans* macrophage-activating lipopeptide-2 (MALP-2) (Nishiguchi *et al.*, 2001), the 10-kdal lipoprotein from *Mycobacterium tuberculosis* (Lien *et al.*, 1999) as well as the soluble bacterial lipopeptide analogue, synthetic tripalmytoyl lipopetide (Pam₃CSK₄) are known to activate TLR2. These findings were established through the use of TLR2-transfected cell lines, TLR2 mAbs and TLR2 $^{-/-}$ mice.

Immunostimulatory lipoproteins contain critical NH₂-terminal modification either tripalmytilation or diacylation at a highly conserved cysteine residue (Figure 8). These NH₂-terminal modifications as well as the peptide moieties are critical for cell activation

(Bessler *et al.*, 1990). In addition to the essential role of these modifications for their immunostimulatory properties they are also known to be important for TLR2-dependent recognition (Lien *et al.*, 1999). Subtle changes even in the stereochemistry can significantly diminish the stimulatory properties of these lipoproteins (Takeuchi *et al.*, 2000).

PAMPs from additional classes of pathogens such as fungi and pathogenic protozoa have been identified as TLR2 agonists. It was demonstrated that TLR2 is required for macrophage cytokine production in response to zymosan, a cell wall component of yeast (Underhill *et al.*, 1999b). Glycosylphosphatidyl (GPI) anchors from *Trypanosoma cruzi*, are abundant molecules in the membrane of these parasitic protozoa and contain immunostimulatory properties (Ferguson, 1999). Recently, these molecules were shown to induce macrophage cytokine responses via a TLR2-dependent mechanism (Campos *et al.*, 2001). Additional studies are required to determine the biological role of TLR2 in anti-fungal and anti-parasitic immunity.

1.9.3 Cooperation between TLRs for maximal responses

Despite the large number of TLR2 agonists, specificity appears to be regulated through heterodimeric interactions with other TLRs, such as TLR1 and TLR6 (Figure 9). Although TLR2 homodimers have not been shown to induce NF κ B activation, dimerization of TLR2 with either TLR1 or TLR6 results in cytokine production (Ozinsky *et al.*, 2000a; Ozinsky *et al.*, 2000b; Zhang *et al.*, 2002). TLR1 was demonstrated to enhance TLR2-dependent responses to soluble factors from *Nisseria meningitidis* (Wyllie *et al.*, 2000). While, dominant negative versions of either TLR2 or TLR6

significantly inhibited responses to PGN, zymosan and MALP-2 but not Pam₃CSK₄ (Ozinsky *et al.*, 2000). Despite these findings, responses to PGN in cells from TLR6^{-/-} are not completely abolished (Takeuchi *et al.*, 2001). This apparent discrepancy may result from impurities in PGN preparations, or may suggest that TLR6 is not obligatory but serves to amplify TLR2-mediated responses.

TLR heterodimers are sensitive enough to recognize subtle differences in microbial structure as demonstrated by bacterial lipoproteins in which the triacylated form is recognized by a TLR1/TLR2 heterodimer and the diacylated form is recognized by a TLR2/TLR6 heterodimer (Morr *et al.*, 2002; Takeuchi *et al.*, 2002). Although the sensitive nature of these receptor systems is apparent in this example, TLR2/TLR6 heterodimers, in addition to diacylated lipoproteins, also recognize PGN and zymosan which do not appear to have substantial structural or amino acid similarity. For this reason, distinct co-receptors, some of which have been identified, may be required for providing increased specificity for these innate receptor systems.

1.9.4 TLR5 Ligand

(Hayashi *et al.*, 2001) demonstrated that CHO cells transiently expressing TLR5 were highly responsive to *Listeria monocytogenes* culture supernatants. The TLR5 stimulatory component was identified by tandem mass spectrometry as flagellin, a 55-kD structural protein of bacterial flagella, important for motility. Flagellin is a highly conserved protein, presumably due to the structural constraints necessary for its function and is therefore a great candidate for a PAMP. Disruption of the flagellin gene in *Listeria monocytogenes* abrogated the TLR5 stimulatory activity of this bacterium. Moreover,

when flagellin was expressed in the nonflagellated bacteria *E. coli*, this conferred the ability of *E. coli* to activate TLR5.

1.9.5 TLR7 and TLR8 Ligands

Currently there are no known natural ligands for TLR7 or TLR8. However, several groups have recently demonstrated that imidazoquinolines, synthetic anti-viral compounds have the capacity to activate these TLRs (Hemmi *et al.*, 2002; Jurk *et al.*, 2002). Interestingly, species difference exist in the ligand specificity of these receptors. In the human, macrophages and DC respond to these agonists via TLR7 and TLR8, while in the mouse only TLR7 appears to be involved (Jurk *et al.*, 2002).

1.9.6 TLR9 Ligands

Bacterial DNA contains higher frequencies of unmethylated CpG nucleotides compared to eukaryotic DNA. Interestingly, bacterial DNA or the analogous synthetic unmethylated CpG oligonucleotides but not eukaryotic DNA has potent immunostimulatory properties. These characteristics make bacterial DNA a likely candidate for a TLR agonist. Shizuo Akira's group first demonstrated that cells from MyD88^{-/-} mice lack responses to unmethylated CpG oligonucleotides suggesting the requirement for a TLR dependent pathway (Hacker *et al.*, 2000; Schnare *et al.*, 2000). Further studies using individual TLR^{-/-} mice revealed that TLR9 but not TLR2 or TLR4 was required for CpG-mediated activation (Hemmi *et al.*, 2000). These findings are of great importance because CpG oligonucleotides have been extensively studied as adjuvants for vaccine therapy and as therapeutic tools for protection against allergic diseases (Kline *et al.*, 1998).

1.9.7 Viral components as TLR agonists

Double stranded RNA (dsRNA) is the signature genetic material of an entire class of viruses and can even be produced during replication by additional classes of viruses. DsRNA is immunostimulatory and therefore it exhibits the properties of a classic PAMP. Previous findings suggested that the protein kinase PKR is involved in the intracellular recognition of dsRNA, resulting in the shut-down of translation (Williams, 1999). Interestingly cells from PKR^{-/-} mice are still able to respond to dsRNA, suggesting the involvement of additional receptors. These findings prompted Alexopoulou and colleagues (Alexopoulou *et al.*, 2001) to examine if dsRNA activated TLRs. 293T cells transfected with individual TLRs demonstrated that TLR3 but not other TLRs was required for cellular responses to the synthetic dsRNA analogue polyinosine-polycytidylic acid (Poly(I:C)) and reovirus genomic dsRNA. Moreover, a neutralizing TLR3 mAb significantly inhibited IFN- β production by a human fibroblast cell line in response to Poly(I:C) treatment (Matsumoto *et al.*, 2002).

Additional viral products are also believed to activate TLRs. Recently, uncharacterized surface protein(s) from cytomegalovirus and several retrovirus strains were shown to be agonists of TLR2 and TLR4 respectively (Compton *et al.*, 2003; Rassa *et al.*, 2002). Moreover, the hemagglutinin protein from measles virus activates TLR2 (Bieback *et al.*, 2002) and the fusion protein from respiratory syncytial virus activates TLR4 (Kurt-Jones *et al.*, 2000b). In these examples it remains to be determined if the recognition of viral components by TLRs has a beneficial role in host defense or a detrimental role by promoting viral pathogenesis. In some cases TLR dependent signals

generate appropriate antiviral responses as demonstrated by TLR4-deficient mice which are less efficient in clearing respiratory syncytial virus than wild type control mice (Haeberle *et al.*, 2002). In contrast, the hemagglutinin protein from measles virus induces the upregulation of its receptor, CD150, on macrophages, suggesting that TLR4 activation may aid in viral entry (Bieback *et al.*, 2002).

1.9.8 Endogenous TLR Ligands

According to the danger theory proposed by P. Matzinger, “stressed” or “damaged” cells can initiate inflammatory responses even in the absence of infection (Matzinger, 2002). This is accomplished through signals emanating from aberrantly expressed protein or lipid molecules, not normally expressed by healthy tissue. During inflammatory conditions, endogenous molecules such as heat shock proteins are upregulated while others that are normally sequestered in cellular membranes or extracellular matrices such as heparin sulfate are released as degradation products. Both of these molecules elicit immune response and are only highly expressed during inflammatory conditions. For these reasons aberrantly expressed host molecules are believed to function as agonists for TLRs.

Heat shock protein (Hsp) 60 and the extra domain A (EDA) of fibronectin are both generated during tissue damage. The immunostimulatory properties of these molecules require a TLR4-dependent mechanism as supported by the findings that responses induced by Hsp60 and the EDA of fibronectin could be completely ablated using macrophages from TLR4-deficient mice (Okamura *et al.*, 2001). Moreover, 293T

cells which are inherently unresponsive to these activators gain responsiveness when transiently transfected with TLR4 and MD-2 (Okamura *et al.*, 2001).

It is suspected that these molecules are released from necrotic cells. Interestingly however, Li and colleagues (Li *et al.*, 2001) demonstrated that necrotic cells induced chemokine responses using a mechanism that was dependent on TLR2 but not TLR4. These findings bring into question the biological significance of hsp60 and EDA as immunostimulatory molecules. Moreover, these studies suggest that an additional unidentified molecule(s), expressed by necrotic cells are able to activate TLR2.

Oligosaccharides from hyaluronic acid and fragments from heparan sulfate are rapidly shed from the extracellular matrix during tissue damage. Both induce cellular responses through TLR4 as determined using TLR4-deficient mice (Johnson *et al.*, 2002; Termeer *et al.*, 2002). Moreover, a neutralizing TLR4 mAb blocked TNF production from monocytes-derived dendritic cells treated with oligosaccharides of hyaluronan (Termeer *et al.*, 2002). These findings suggest that host proteins normally sequestered in cellular or extracellular membranes have the capacity to generate TLR-dependent immune responses.

1.10 Cellular Responses induced by TLRs

TLR activation results in a wide range of biologically important events, including the induction of innate defense mechanisms, modulation of the adaptive immune response and regulation of cell survival. Critical events associated with the innate defense system includes phagocytosis, gene modulation and effector cell recruitment. TLRs actively mobilize to phagosomes following stimulation (Underhill *et al.*, 1999) and have recently

been shown to modulate the maturation of phagosomal-lysosomal compartments (Medzhitov, personal communication). TLRs also induce the upregulation of adhesion molecules on endothelial cells, suggesting a potential mechanism for leukocyte recruitment (Liaudet *et al.*, 2003; Zeuke *et al.*, 2002). Moreover, TLRs are able to induce a wide range of inflammatory mediators including ones that have direct antimicrobial activity such as β -defensins and reactive oxygen intermediates, and ones that have an indirect effect such as cytokines and chemokines.

Among the long list of cytokines induced by TLRs, including those that are regulated by NF κ B, are IL-1 β , IL-6, IL-8 (CXCL-8), TNF and GM-CSF (Akira *et al.*, 2001; (Medzhitov and Janeway, 2002). The interferon-regulated pathway characterized by IP-10 (CXCL10) and RANTES (CCL5) production can also be induced by TLR activation (Barton and Medzhitov, 2002; Imler and Hoffmann, 2003; O'Neill, 2002). Interestingly, all TLRs appear to activate NF κ B regulated genes while only a select group are capable of inducing the IFN-pathway. Interestingly, TLRs can induce a different profile of inflammatory genes depending on the cell type. Therefore, studies examining the response of specific cell types to TLR activation may provide important clues about the function of these cells during inflammatory conditions.

Recent experiments using TLR^{-/-} or MyD88^{-/-} mice have begun to reveal the importance of the TLR family in the induction of adaptive immunity (Eisenbarth *et al.*, 2002; Schnare *et al.*, 2001). T-cell proliferation and IFN- γ production by activated T cells, are characteristic markers of adaptive immunity when mice are immunized with Complete Freund's Adjuvant. However, these responses were not observed in MyD88^{-/-} mice (Schnare *et al.*, 2001). Moreover, classic markers of allergic inflammation, induced

by OVA sensitization and challenge, were not observed using TLR4-deficient mice (Eisenbarth *et al.*, 2002). The explanation for these findings is likely to involve the dendritic cell. TLR activation of immature dendritic cells results in the upregulation of co-stimulatory molecules and maturation markers (reviewed by Akira *et al.*, 2001). Without these events, dendritic cells are unable to stimulate adaptive immune responses. Therefore, direct activation by PAMPs results in dendritic cell maturation and represents an important link between the innate and adaptive immune responses. It is also possible that local inflammatory mediators produced by other cells, in response to TLR agonists are responsible for dendritic cell maturation.

TLR activation is also believed to induce mechanisms to control inflammatory responses. One such mechanism involves the production of anti-inflammatory mediators such as IL-10 and IL-1ra. Moreover, TLR2 dependent activation by bacterial lipoproteins can induce apoptosis suggesting a potential mechanism to prevent excessive cytokine production (Aliprantis *et al.*, 2001; Aliprantis *et al.*, 1999; Lopez *et al.*, 2003). The mechanism of TLR2-mediated apoptosis by bacterial lipoproteins was mediated largely by the recruitment of the Fas-associated death domain protein (FADD) to the TLR2 receptor complex by the intracellular adaptor molecule MyD88 (Aliprantis *et al.*, 2000). Down stream signaling events were dependent on caspase-8 and caspase-3 since apoptosis could be significantly blocked using specific inhibitors to these caspases (Lopez *et al.*, 2003).

1.10.1 Differential responses induced by TLRs

It is becoming apparent that unique signaling cascades can be induced by different TLRs. Studies using RNase protection assays and microarray technology are beginning to unravel these differences. (Hirschfeld *et al.*, 2001b) demonstrated that *E. coli* LPS (TLR4 agonist) was able to induce a unique profile of cytokines compared to *P. gingivalis* LPS (TLR2 agonist) including IFN- γ , IL-6, bioactive IL-12 (IL-12 p70, consisting of p40 and p35 subunits) and MCP-5. Despite these differences, both activators produced abundant levels of a core set of genes including, IL-1 β and MIP-1 α (CCL3). These findings were extended by a subsequent study which used additional TLR2 agonists. The TLR2 activators PGN, Pam₃CSK₄ and zymosan failed to induce high levels of mRNA for RANTES (CCL5), IP-10 (CXCL10) and IL-12 p70, while *E. coli* LPS was able to induce high levels of these genes (Re and Strominger, 2001).

Differences in cytokine production were also observed *in vivo* (Pulendran *et al.*, 2001). When OVA-sensitized mice were co-injected with OVA and LPS from *E. coli* or *P. gingivalis*, differences in antigen-specific CD4⁺ Th and CD8⁺ T cell responses were observed. The TLR4 activator, *E. coli* LPS, evoked a strong Th1-like response characterized by abundant IFN- γ , and little or no IL-4, IL-13 and IL-5 while the TLR2 activator, *P. gingivalis* LPS, induced a strong Th2-like response characterized by abundant levels of IL-5, IL-10 and IL-13 without much IFN- γ (Pulendran *et al.*, 2001). Although these findings point towards a model where different TLRs are able to polarize T cell responses, other studies have shown that *E. coli* LPS is able to induce both types of responses depending on the doses used (Eisenbarth *et al.*, 2002). Therefore, these results may simply reflect differences in the potency of various preparations of LPS.

Despite the questions surrounding the *in vivo* studies, the *in vitro* studies clearly illustrate qualitative differences in the responses induced by distinct TLRs. These observations have a significant impact on our understanding of the immune system as they suggest that our innate defense system has evolved to tailor its response to specific types of microbial infections. These studies also raise an important question, what differences exist in the intracellular signaling pathways to account for these unique TLR responses?

1.11 TLR Signaling Pathways

Since TLRs and the IL-1 receptor family share significant intracellular sequence homology, it is not surprising that these receptors induce a common core of signaling events (O'Neill, 2002; Takeuchi and Akira, 2002). The major events in these pathways have been reviewed extensively and are depicted in figure 9 (O'Neill, 2002; O'Neill and Greene, 1998; Takeuchi and Akira, 2001; Wesche *et al.*, 1997). Central to these pathways is the intracellular adaptor molecule MyD88 which binds to the intracellular domain of these receptors and functions as a scaffold for the association of additional signaling molecules (Wesche *et al.*, 1997). MyD88 contains a toll/interleukin-1 like (TIR) domain, homologous to those expressed by TLRs and IL-1 receptor family members, required for the formations of homotypic interactions between MyD88 and TLRs or IL-1 receptor family members. MyD88 also contains an NH₂-terminal death domain which undergoes homotypic aggregation with the corresponding death domain of the serine/threonine protein kinase, interleukin-1 receptor associated kinase (IRAK) (Wesche *et al.*, 1997). The precise details involved in IRAK activation and the down stream events

remain unclear. However, it is suspected that IRAK becomes activated through an autophosphorylation event resulting in IRAKs dissociation from the receptor complex and subsequently interacting with TNF receptor associated factor 6 (TRAF6) (Janssens and Beyaert, 2003). TRAF6 induces two major signaling pathways involving the mitogen-activated protein (MAP) kinases c-jun NH2-terminal kinase (JNK) and p38 as well as the REL family transcription factor NFκB. The major events associated with these pathways are depicted in figure 9. A more detailed review of these signaling events has been previously documented (Takeuchi and Akira, 2002; O'Neill, 2002; Janssens and Beyaert, 2003).

1.11.1 MyD88-independent pathways

MyD88 is an essential adaptor molecule for many aspects of TLR signaling (Takeuchi and Akira, 2002). However, studies examining signaling pathways in MyD88^{-/-} mice reported surprising findings. When cells from MyD88^{-/-} mice were stimulated with LPS they failed to produce pro-inflammatory cytokines such as IL-1β, IL-6 and TNF but retained the ability to activate NFκB, and members of the MAPK family such as JNK and p38, although with delayed kinetics (Kawai *et al.*, 1999). These transcription factors and signaling molecules were not however activated in the absence of TLR4 suggesting that LPS induced signaling cascades originate from TLR4 and can then use adaptor molecules other than MyD88. These findings appear to be selective to TLR4 agonists since activation of NFκB, and members of the MAPK family did not occur in response to MALP-2 and CpG oligonucleotides, activators of a TLR2/TLR6 heterodimer and TLR9 respectively (Takeuchi *et al.*, 2000).

To explore the functional significance of these findings, cells from MyD88^{-/-} mice were examined for a panel of readouts. Although the production of classic pro-inflammatory cytokines was not induced, dendritic cells from MyD88^{-/-} retained the ability to undergo maturation in response to LPS (Kaisho and Akira, 2001). Interestingly, CpG DNA, which is also known to induce maturation, failed to do so in dendritic cells from MyD88^{-/-} mice, further illustrating the selective nature of the MyD88-independent pathway (Kaisho *et al.*, 2001).

Analysis of MyD88^{-/-} mice also revealed the existence of a MyD88-independent pathway downstream of TLR3 (Alexopoulou *et al.*, 2001; Sato *et al.*, 2002). Induction of dendritic cell maturation markers such as CD80, CD86 and MHC-II, were not affected when cells from MyD88^{-/-} mice were stimulated with Poly(I:C). Moreover, NFκB and MAP kinase activation was induced although with delayed kinetics, similar to that of LPS stimulation (Alexopoulou *et al.*, 2001).

To identify potential genes induced by the MyD88-independent pathway, subtractive hybridization cloning and gene chip microarray techniques were employed. These approaches led to the discovery of a group of genes that were upregulated in macrophages from MyD88^{-/-} mice including, IP-10 (CXCL10), RANTES (CCL5) and IFN-β (Kawai *et al.*, 2001). Interestingly, all of these genes are induced by the interferon pathway which is regulated by the transcription factor interferon regulatory factor 3 (IRF3). Moreover, these genes were selectively induced by TLR3 and TLR4 (Alexopoulou *et al.*, 2001; Doyle *et al.*, 2002; Matsumoto *et al.*, 2002). These data suggest that an additional adaptor molecule must exist which is selectively recruited to

TLR3 and TLR4 and results in the activation of IRF3. These findings led to the search for additional TIR-containing adaptor molecules.

1.11.2 TIRAP/MAL

Two independent groups led by (Fitzgerald *et al.*, 2001) and (Horng *et al.*, 2001) simultaneously discovered a second TIR-containing adaptor molecule which they called the TIR domain containing adaptor protein (TIRAP) and the MyD88-like adaptor (MAL) respectively. Functional analysis initially led to the belief that this protein was responsible for the MyD88-independent pathway. TIRAP was shown to associate with TLR4 and a cell-permeable TIRAP inhibitory peptide efficiently abrogated dendritic cell maturation by LPS but not CpG DNA (Horng *et al.*, 2001).

Despite these eloquent *in vitro* studies, TIRAP^{-/-} mice revealed opposing data. Induction of the IRF3 pathway by LPS was not abolished when using cells from these mice (Horng *et al.*, 2001; Yamamoto *et al.*, 2002a). Moreover, dendritic cell maturation was also intact suggesting that TIRAP is not responsible for the MyD88-independent pathway. Induction of IRF3 in MyD88/TIRAP doubly deficient mice was also intact suggesting that the mechanism of IRF3 induction is not by one adaptor molecule compensating for the other (Yamamoto *et al.*, 2002).

Further characterization revealed that TIRAP is functionally very similar to MyD88 in that cells from TIRAP^{-/-} mice failed to produce pro-inflammatory cytokines including TNF, IL-6 and IL-12p40 and NFκB activation was delayed. TIRAP^{-/-} mice were also unresponsive to TLR2 agonists, including the TLR1/TLR2 agonist Pam₃CSK₄ and the TLR2/TLR6 agonists PGN and MALP-2. In contrast, cells from these mice had

similar responses to cells from wild type controls when activated with Poly (I:C) (TLR3), flagellin (TLR5), imidazoquinolines (TLR7) and CpG DNA (TLR9), suggesting that TIRAP has a restricted role in TLR2 and TLR4 signaling (Horng *et al.*, 2002; Yamamoto *et al.*, 2002a). TIRAP can form heterodimers with MyD88, which lends belief that TIRAP acts in concert with MyD88 (Horng *et al.*, 2001). Further studies are needed to determine why TLR2 and TLR4 require two adaptor molecules, while other TLRs appear to require MyD88 alone. It is possible that MyD88/TIRAP interactions induce a unique profile of genes compared to MyD88 alone.

1.11.3 TICAM-1/TRIF

A significant advance toward the mechanism of the MyD88-independent pathway came from the discovery of a third TIR-containing adaptor molecule called the TIR containing adaptor molecule (TICAM-1) or the TIR containing adaptor inducing IFN β (TRIF) by an independent group (Yamamoto *et al.*, 2002b). A dominant negative version of this molecule significantly abrogated dsRNA induced IFN β induction (Yamamoto *et al.*, 2002). Moreover, inhibition of either TLR3 or TICAM-1 but not MyD88 or TIRAP by RNA interference technology abolished IFN β production by dsRNA (Oshiumi *et al.*, 2003). TICAM-1 was not completely restricted to TLR3 signaling since a dominant negative version of this molecule also inhibited responses to agonists of TLR2, TLR4 and TLR7 (Yamamoto *et al.*, 2002). TICAM-1 associated with TLR2 and TLR3 as assessed by western blot analysis. Interestingly, an independent study observed a selective association of TICAM-1 with TLR3 (Oshiumi *et al.*, 2003). Further experiments using

cells from TICAM-1^{-/-} mice will help to clarify these discrepancies and will help to establish the role of TICAM-1 in response to other TLRs.

1.11.4 Rho GTPase 1/ Phosphatidylinositol-3 kinase

The RhoGTPase, Rac1/Phosphatidylinositol-3 kinase (PI3K) complex associates with TLR2 (Arbibe *et al.*, 2000). Specific inhibitors of PI3K or Rac1 can significantly block NFκB activation by TLR2 activation (Arbibe *et al.*, 2000). These findings are significant as they suggest that molecules lacking a classical TIR domain are still capable of participating in TLR-dependent signaling. However, the precise role of these molecules remains to be determined since these Rac1/PI3K-dependent responses to TLR2 activation are completely abrogated in MyD88^{-/-} mice. This may suggest that Rac1/PI3K is necessary but not sufficient to initiate TLR2-dependent signals. The association of Rac1/PI3K with TLR2 is dependent on cell activation by TLR2 agonists such as *S. aureus*. The cytoplasmic domain of TLR2 contains a binding motif (YXXM) for the p85 fragment of PI3K. This domain is also present in TLR1 and TLR6 but absent in TLR3, TLR4 and TLR5 (Arbibe *et al.*, 2000). Although these data suggest a specific role for PI3K/Rac1 downstream of TLR2/TLR1 or TLR2/TLR6 receptor systems, cells from PI3K-deficient mice are deficient in some responses to LPS (Fruman *et al.*, 1999; Ojaniemi *et al.*, 2003). MyD88 also contains a PI3K binding motif and therefore it is believed that LPS can activate PI3K in a MyD88-dependent manner. The Rac1/PI3 kinase pathway may be selectively activated by TLR2 and TLR4 agonists. However, further studies are needed to address this.

1.12 TLR accessory receptors and adaptor molecules

Studies examining TLR-ligands interactions have determined that the affinity is very weak suggesting that additional molecules are required to stabilize the interactions (Poltorak *et al.*, 2000). A picture is now emerging where multiple accessory receptors and adaptor molecules are required for efficient TLR recognition of their ligands.

1.12.1 Accessory receptors for LPS

One of the first host molecules to interact with LPS is a serum acute-phase protein known as, the LPS binding protein (LBP) (Fenton and Golenbock, 1998). LBP is required to transfer LPS monomers from aggregates to CD14 (Hailman *et al.*, 1994). Cells from either LBP^{-/-} or CD14^{-/-} mice have drastically reduced responses to LPS *in vitro* (Haziot *et al.*, 1995; Wurfel and Wright, 1997). Moreover, very-high concentrations of LPS, several logs higher, are required to activate these cells as compared to cells from wild type littermates. *in vivo*, CD14^{-/-} mice had significantly impaired responses to LPS whereas LBP^{-/-} mice responded almost identically as control littermates. These results suggest a critical role for CD14 and that an additional host protein(s) may play a compensatory role for LBP.

CD14 is a GPI anchored protein lacking a cytoplasmic domain and therefore does not contain intrinsic signaling capability. As a result a signaling receptor, represented by TLR4 is required to initiate appropriate signaling cascades. The exact molecular events leading to the activation of TLR4 are not clear. However, it is believed that CD14 acts as a shuttle to bring LPS in close proximity to TLR4. Support for this model is provided by Fluorescence resonance energy transfer (FRET) technology which demonstrated that a

close association between CD14 and TLR4 only occurs after administration of LPS (Jiang *et al.*, 2000).

Several lines of evidence suggest that CD14 is not the only receptor involved in LPS recognition. Anti-CD14 mAb significantly but not completely inhibited responses to LPS. Moreover similar findings were observed using cells from CD14^{-/-} mice. β 2 integrins (CD11/CD18) have also been shown to bind LPS however further studies are required to assess the biological significance of this interaction. Four additional molecules have recently been discovered with LPS binding ability including Hsp 70, Hsp 90, the chemokine receptor CXCR4 and GDF5 (Triantafilou *et al.*, 2002a; Triantafilou *et al.*, 2002b; Triantafilou and Triantafilou, 2002). These receptors were found in close association with LPS as assessed by FRET technology (Triantafilou *et al.*, 2002). Moreover, monoclonal antibodies to these receptors efficiently blocked LPS induced TNF production. Collectively, these studies suggest the existence of a large receptor cluster required for LPS signaling.

It appears that LPS receptor clusters among different cell types are not completely identical, with some cells containing proteins that are not expressed by others (Triantafilou *et al.*, 2002). Further studies are required to assess the biological significance of this receptor heterogeneity. These findings may have important therapeutic potential by offering potential targets to selectively block the LPS responses of specific cell types.

1.12.2 Additional receptors for PGN and zymosan

Recently (Liu *et al.*, 2000) discovered a PGN binding protein which they termed peptidoglycan recognition protein (PGRP). PGRP bound PGN with a very high binding affinity and was selectively expressed by neutrophils. Unlike, CD14 and most TLRs, PGRP was not expressed on the cell surface but was restricted to neutrophil granules. The biological function of PGRP is currently unknown, however addition of PGRP in concert with gram-positive bacteria inhibited macrophage phagocytosis and cytokine production. These findings suggest PGRP may act to regulate inflammatory conditions induced by gram-positive infections.

PGN consists of chains of repeating disaccharide units composed of N-acetylglucosamine β -1-4 linked to N-acetylmuramic acid. N-acetylmuramic acid residues are substituted with short amino acid peptides and commonly called muramyl dipeptide (MDP). Two independent groups recently demonstrated that the MDP portion of PGN could be recognized by the PRR, NOD2 which has also recently been identified as a susceptibility gene for Crohn's disease (Girardin *et al.*, 2003; Hugot *et al.*, 2001; Inohara and Nunez, 2003). Interestingly, MDP failed to activate TLR2 or TLR2 co-expressed with TLR1 or TLR6, suggesting that components of PGN distinct from MDP are required for TLR2-mediated recognition.

Zymosan, a cell wall preparation from *S. cerevisiae* and other yeast species, is composed primarily of β -glucans, mannans and chitin. Recently, a C-type lectin called dectin-1 was implicated in the recognition of the β -glucan component of zymosan (Brown and Gordon, 2001). Functional studies of this receptor revealed that dectin-1 is responsible for phagocytosis of zymosan particles and induction of reactive oxygen species (Gantner *et al.*, 2003). Interestingly, TLR2 recognizes a β -glucan-independent

portion of zymosan. Moreover, TLR2 is responsible for the induction of pro-inflammatory cytokines but not phagocytosis in response to zymosan activation (Gantner *et al.*, 2003). These studies further suggest that a complex network of receptors are involved in the innate recognition of pathogen products.

1.13 Modulation of allergic diseases by microbial infections

The role of bacterial infections in the development or protection of allergic diseases has been an area of intense interest in recent years. Epidemiological studies have identified an inverse relationship between the prevalence of allergic diseases and socioeconomic status. Particularly convincing data arose from a Swiss study which determined that asthma prevalence was reduced among children growing up on a farm than children from the same villages but not in farming families (Braun-Fahrlander *et al.*, 1999). These findings lend support to the “hygiene” hypothesis, which suggests that reduced incident of exposure to microbial infections, especially during allergen sensitization may be responsible for increased disease prevalence (Holt and Sly, 1997; Holt *et al.*, 1997).

Pathogen infections, although potentially protective during allergen sensitization, are believed to exacerbate the severity of existing allergic diseases. In support of this, respiratory viruses are a major trigger of acute exacerbations of asthma (Corne and Holgate, 1997; Johnston, 1997). Moreover, it was demonstrated that airway reactivity is significantly increased after LPS inhalation in asthmatics and epidemiological evidence has shown that environmental levels of LPS correlate positively with the incidence and severity of asthma (Michel *et al.*, 1991). A strong association also exists between pathogen infections and the severity of atopic dermatitis (Leung, 1999). Superficial

fungal infections are 3-fold more frequent in the skin lesions of patients with this disorder as compared to non-atopic control patients. *Staphylococcus aureus* infections are believed to be of even greater importance, as they can be found in greater than 90% of patients with atopic dermatitis compared to only 5% of control subjects. Moreover, patients demonstrate substantial clinical improvements when receiving antifungal agents or anti-staphylococcal antibiotics (Kolmer *et al.*, 1996; Lever *et al.*, 1988). Collectively, these studies suggest a dual role for pathogen infections: an early protective role against establishing allergic diseases and a detrimental role in disease severity once the disease has developed.

In support of these ideas (Tulic *et al.*, 2002) demonstrated that exposure to LPS early in the sensitization process protected against the development of OVA-specific IgE and abolished the OVA-induced airway hyperreactivity, consistent with the hygiene hypothesis. Moreover, when LPS was administered at later time points >6 days post antigen sensitization, airway hyperreactivity and cellular infiltration were both enhanced. These findings further support the dual role of LPS in modulating allergic inflammation with the time of administration the crucial determinant in the type of immune response elicited. Interestingly, (Strohmeier *et al.*, 2001) demonstrated that LBP^{-/-} mice were resistant to the development of allergic inflammation, suggesting a role for LPS in the sensitization stages of allergic inflammation. The apparent discrepancy may result from differences in the dose of LPS administered. Recently it was demonstrated that low doses of LPS during allergen challenge resulted in a Th2-dominated inflammatory responses, while high doses of LPS induced a Th1 phenotype (Eisenbarth *et al.*, 2002). Interestingly, Th2 inflammatory responses were significantly abolished in TLR4 deficient mice

suggesting the requirement for LPS (Eisenbarth *et al.*, 2001). Collectively these studies indicate an important role for LPS as an adjuvant in the sensitization stage and in exacerbating the symptoms of allergic diseases.

1.14 Objectives

Mast cells play a critical protective role in host defense against bacterial infections. A number of studies have identified potential mechanisms for mast cell recognition of bacterial products (Figure 4). However, many of these are indirect, facilitated by host proteins such as complement by-products or pathogen-specific antibodies. Moreover, they do not represent classic innate defense mechanisms because the host proteins must be generated before the mast cells can make use of them. In order for the mast cell to play an “early” role in host defense it is critical that they respond directly to bacterial challenge, potentially through the use of pattern recognition receptors such as members of the TLR family. As a result we hypothesized that mast cells express a number of TLRs and require their expression for specific cellular responses to certain pathogen products.

The following chapters will discuss the experimental procedures, and provide a detailed analysis of the results obtained from this research. The results are presented as three independent chapters and are followed by a general discussion to bring together the major findings from all three sections. The results reported in chapter 3 entitled “Toll-like receptor 4-mediated activation of murine mast cells”, were published in the *Journal of Leukocyte Biology* (December 2001). This manuscript examined the TLR expression by murine mast cells and investigated the requirement of TLR4 for mast cell responses to LPS. The results reported in chapter 4, entitled “Distinct Toll-like receptor 2 activators

selectively induce different classes of mediator production from human mast cells”, was published by the Journal of Immunology: Cutting Edge (February, 2003). This research examined the TLR expression by human mast cells and investigated whether different TLR2 activators could induce distinct classes of human mast cell mediators. Notably, this was the first publication to demonstrate the profile of TLRs expressed by human mast cells and was also the first to formally demonstrate that distinct TLR2 activators can induce different profiles of mediators. The third results section, chapter 5, is entitled “PGN induces apoptosis and enhances FcεRI-mediated GM-CSF and IL-1β production by human mast cells.” This study focused on the observation that the FcεRI-cross linking could enhance TLR2 activation of human mast cells. The mechanism responsible for this phenomenon was investigated.

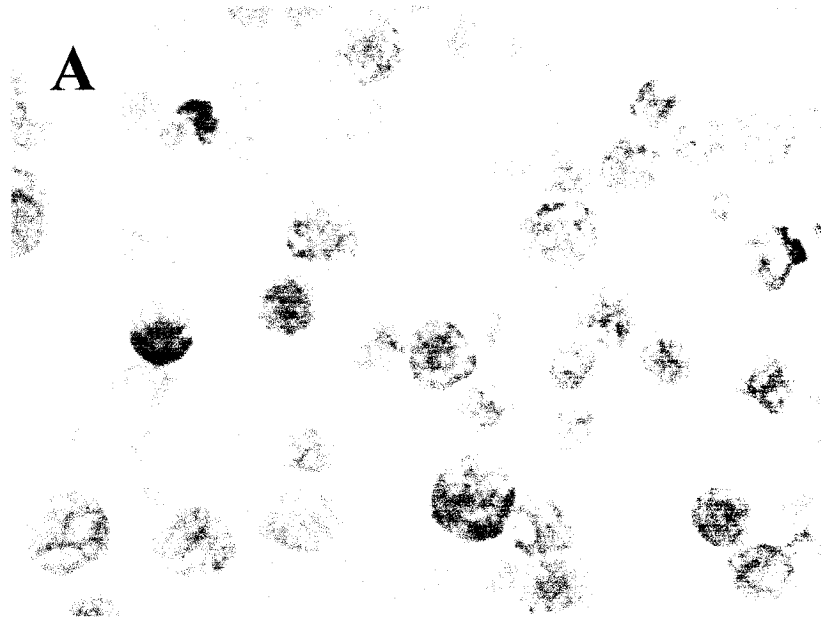


Figure 1: (A) Toluidine blue stained human CBMC
40 X magnification

Figure 2: Signaling pathways induced by FcεRI aggregation. FcεRI-aggregation results in the phosphorylation of ITAMs followed by a complex series of signaling pathways mediated largely by the protein tyrosine kinases (PTKs) Lyn and Fyn. This schematic is a vastly simplified depiction of the major adaptors and signaling molecules essential for the induction of protein kinase C and calcium flux. Although many of these events occur in association with the cellular membrane, and in some cases distinct microdomains for ease of presentation purposes they are depicted in the cytosol. The pathways induced by Fyn and Lyn are integrated at several positions in the pathways including activation of PKC and calcium signals.

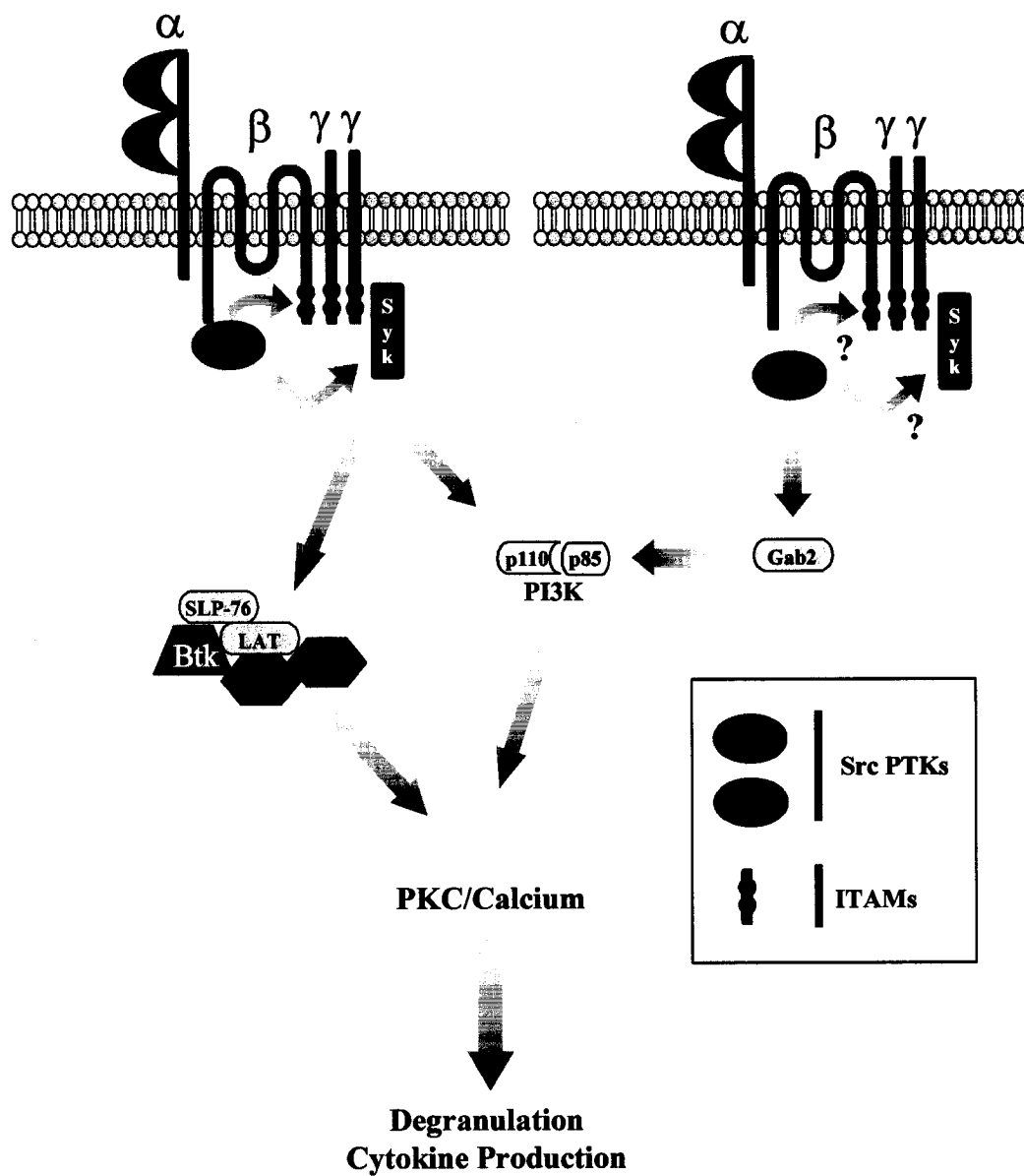


Figure 2: FcεRI signaling pathways
 (Adapted from Rivera, 2002; Nadler and Kinet, 2002)

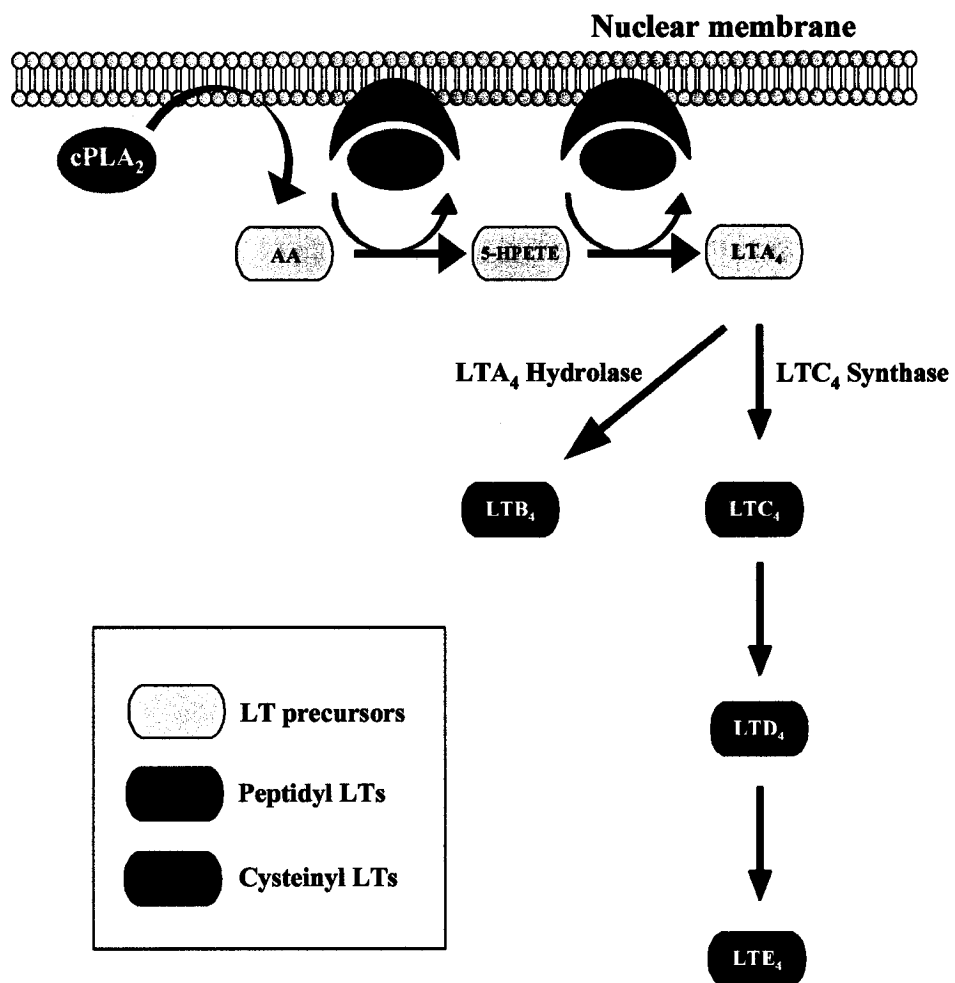


Figure 3: Regulation of leukotriene production
(Adapted from Fisher *et al.*, 1997)

Figure 4: Receptor systems involved in mast cell-bacterial interactions. Mast cells express a wide variety of receptors involved in either direct recognition of bacteria or bacterial products and indirect recognition of bacteria facilitated by host derived products such as complement byproducts and antigen/antibody complexes. Only a select group of the listed complement receptors, CD88 and the C3aR, have the seven transmembrane structure as indicated in the diagram. The (+) and (-) symbols represent positive and negative expression of the receptor while (+/-) indicates low levels of constitutive receptor expression. There is a high degree of receptor heterogeneity within different types of mast cells such as the receptor for C5a, CD88, which is expressed in human skin mast cells but not lung mast cells. MHC-II molecules are expressed at very low levels constitutively but can be up-regulated. Receptor expression were examined at the protein level.

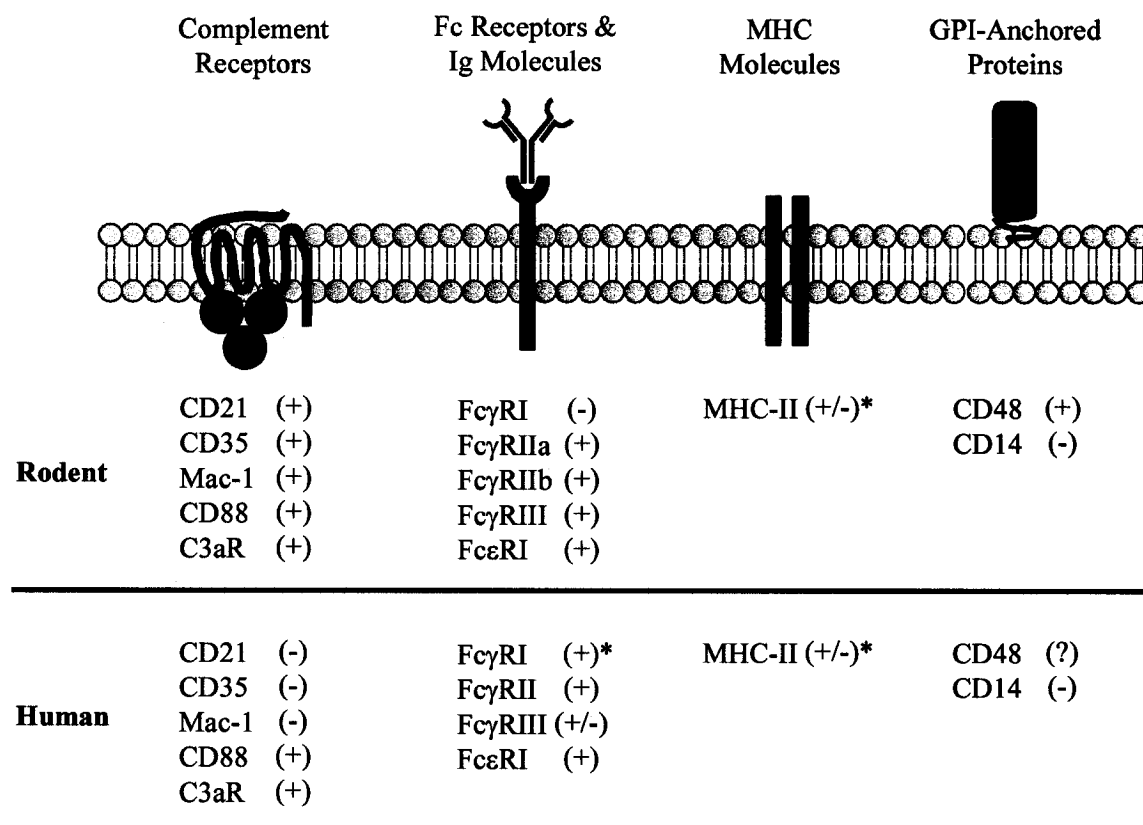


Figure 4: Receptor systems involved in mast cell-bacterial interactions
(Reproduced from Marshall *et al.*, 2003)

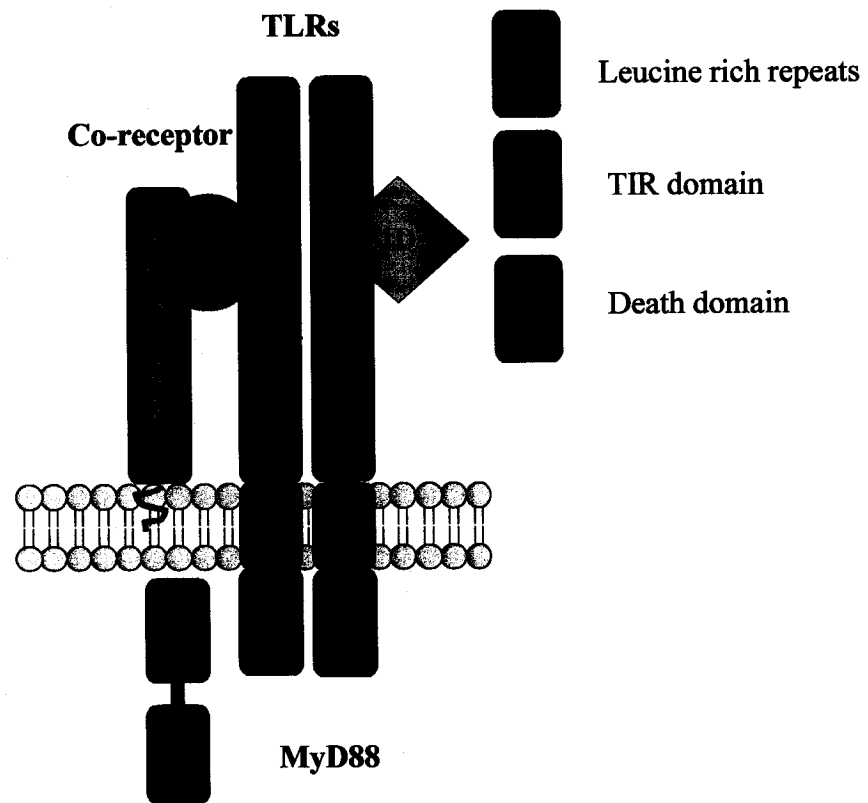
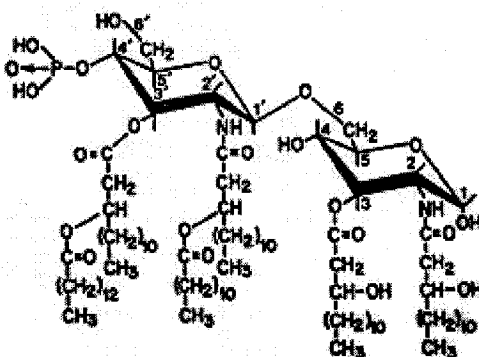


Figure 5: Structure of Toll-like receptors
(Adapted from Medzhitov *et al.*, 2003; Akira *et al.*, 2001)

Lipid A from *E.coli* LPS

PGN and MDP component

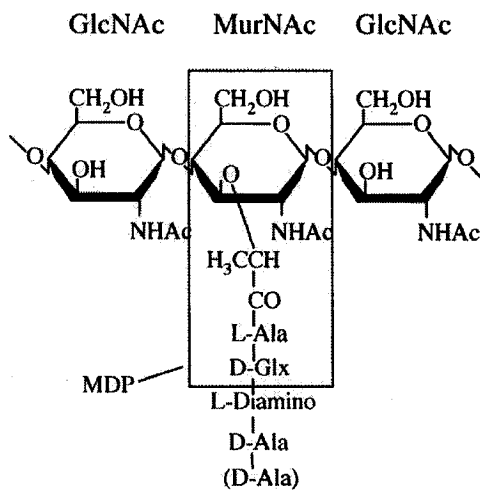
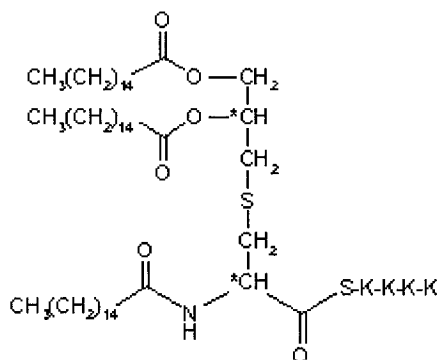


Figure 6: Chemical Structure of the lipid A portion of LPS and the MDP portion of PGN
(Adapted from Girardin *et al.*, 2003)

Pam₃CSK₄

MALP-2

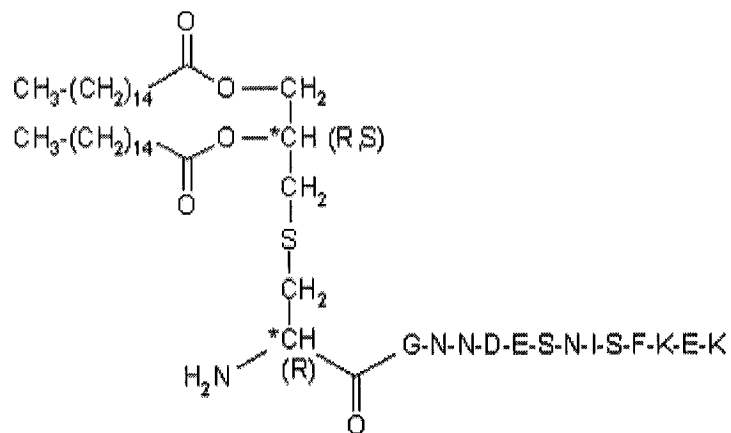


Figure 7: Chemical Structure of Pam₃CSK₄ and MALP-2
(Obtained from EMC Microcollections GmbH Tuebingen, Germany)

Table 1: TLRs and Their Ligands

Ligand	TLR
Whole organisms	
<i>Staphylococcus aureus</i>	TLR2
<i>Streptococcus pneumoniae</i>	TLR2
<i>Mycobacterium avium</i>	TLR2
<i>Bacillus subtilis</i>	TLR2
<i>Borrelia burgdorferi</i>	TLR2
Purified or semi-purified PAMPs	
Peptidoglycan	TLR2 + TLR6
Saccharomyces cerevisiae zymosan	TLR2 + TLR6
Lipoteichoic acid (LTA)	TLR2
LPS (<i>Leptospira interrogans</i>)	TLR2
LPS (<i>Porphyromonas gingivitis</i>)	TLR2
LPS (gram negative bacteria)	TLR4
GPI anchors from parasitic protozoa	TLR2
Flagellin	TLR5
Unmethylated CpG DNA	TLR9
imidazoquinoline compounds	TLR7 or TLR8
Bacterial or mycoplasma lipoproteins	
<i>Borrelia burgdorferi</i> outer surface protein A	TLR2
MALP-2 (diacylated)	TLR2 + TLR6
Pam3CSK4 (triacylated)	TLR2 + TLR1
<i>Mycobacterium tuberculosis</i> 10-kDa lipoprotein	TLR2
Viral PAMPs	
Poly I:C (dsRNA)	TLR3
RSV F Protein	TLR4
Cytomegalovirus (uncharacterized proteins)	TLR2
Retrovirus (uncharacterized proteins)	TLR4
Measles virus hemagglutinin protein	TLR2
Endogenous host molecules	
Hsp60 (human)	TLR4
Fibronectin extra domain A (human)	TLR4
necrotic cells	TLR2
hyaluronic acid oligosaccharides	TLR4
heparine sulfate	TLR4

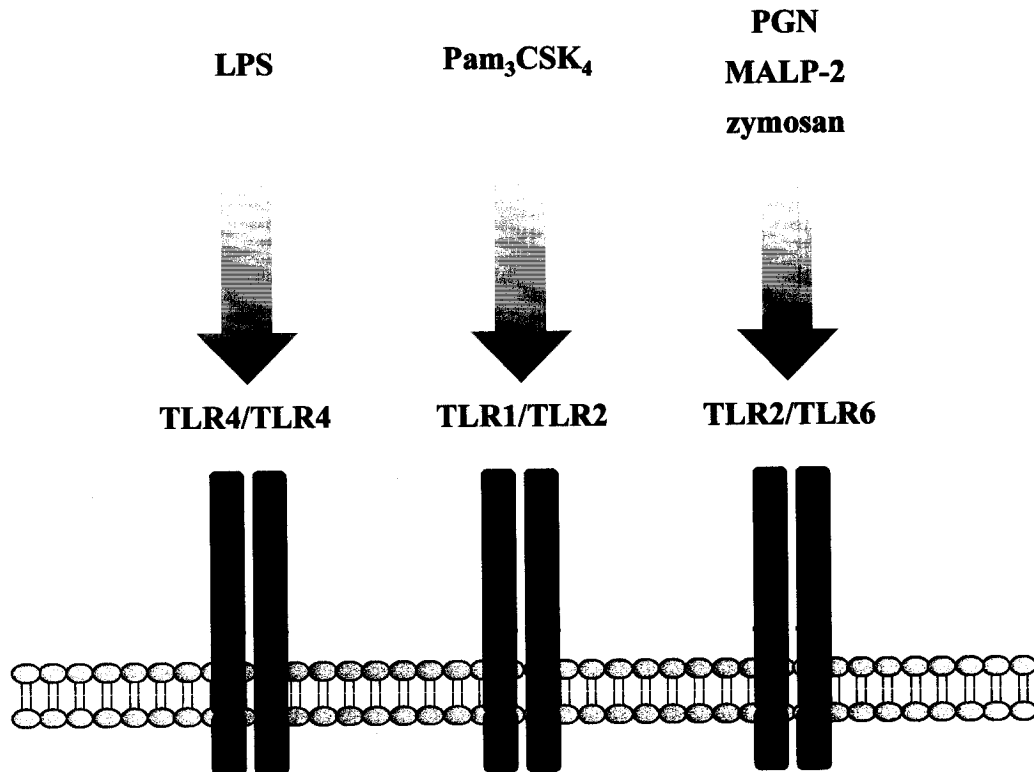


Figure 8: Cooperation between TLRs

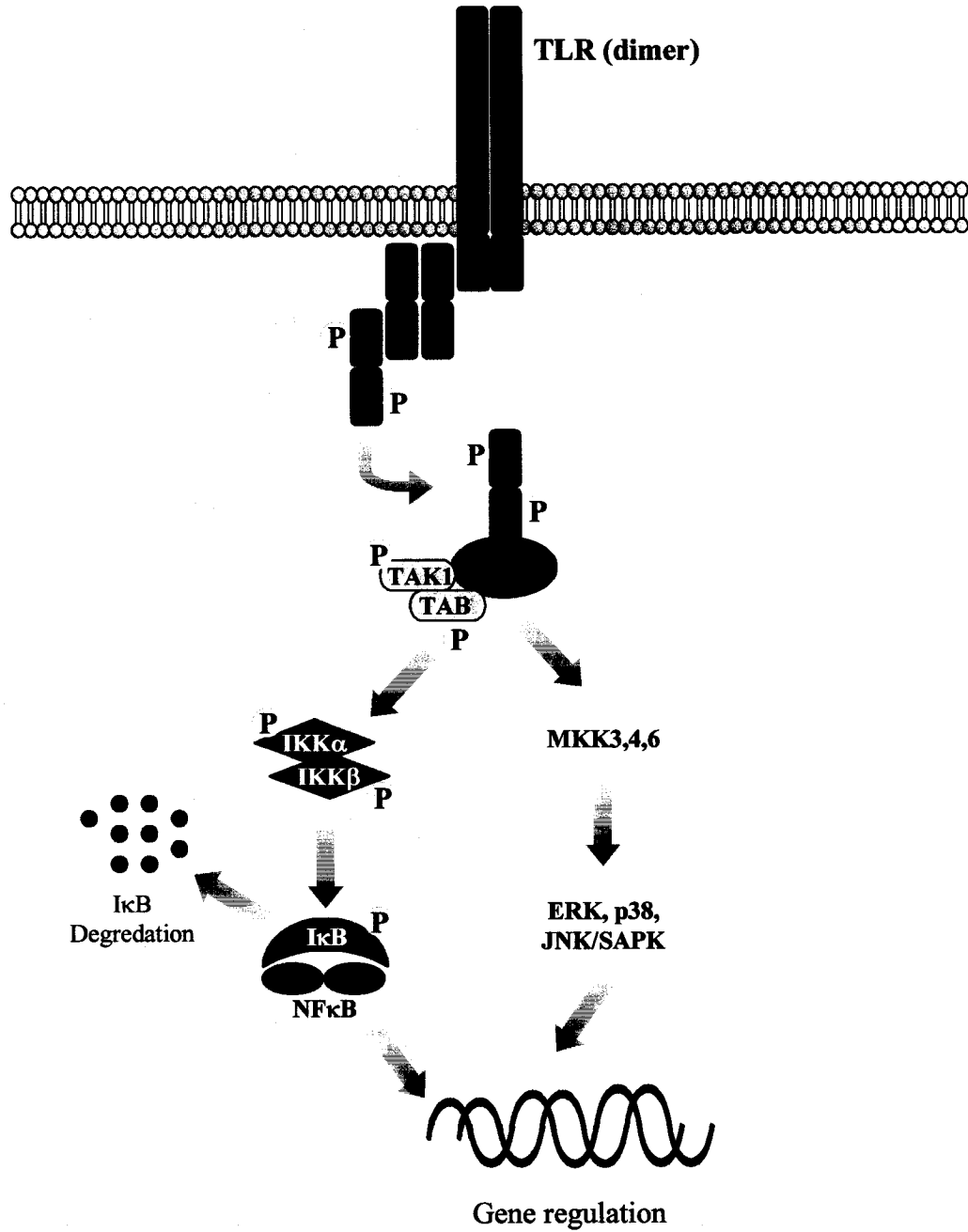


Figure 9: TLR signaling pathway
 (Adapted from Aderem *et al.*, 2000;
 O'Neill, 2001)

Figure 10: Intracellular TLR adaptor molecules. A current model depicting how distinct TLR-dependent responses can be elicited through the recruitment of different combinations of TIR domain containing adaptor molecules. Activation of TLRs by PAMPs such as bacterial lipoproteins (BLP), double stranded RNA (dsRNA), LPS, bacterial flagelin and CpG DNA results in the recruitment of different adaptor molecules including, MyD88, TIRAP and TICAM-1/TRIF. Different combinations of these molecules are required for the induction the proinflammatory cytokines TNF and IL-6, or the costimulatory molecules CD80 and CD86 on dendritic cells or interferon regulated genes such as IFN β .

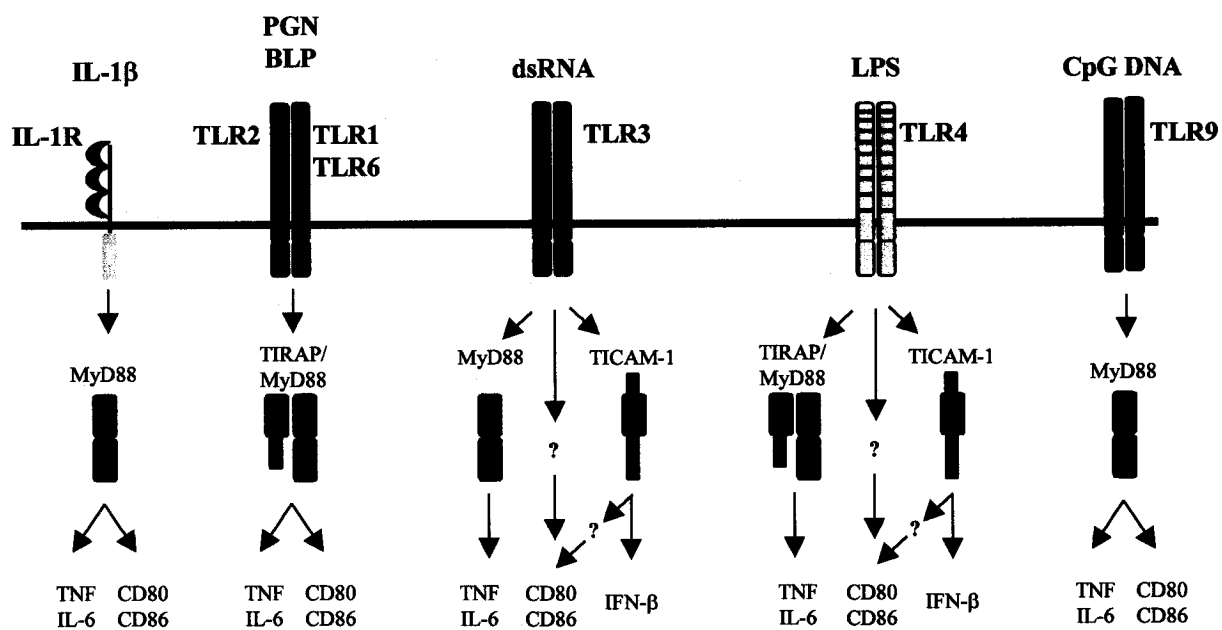


Figure 10: TLR Adaptor molecules
(Adapted from Imler and Hoffmann, 2003)

Chapter 2

MATERIALS AND METHODS

Reagents

Escherichia coli (*E. coli*) LPS (serotype O55:B5) was purchased from Sigma (St.Louis, MO). *Pseudomonas aeruginosa* LPS (serotype 10) Sigma. Peptidoglycan (PGN) purified from *Staphylococcus aureus* Fluka BioChemika distributed by Sigma. *S. aureus* PGN contained < 0.0025 ng/μl endotoxin as assessed using the *Limulus ameobocyte* lysate test purchased from Sigma. *Saccharomyces cerevisiae* zymosan was obtained from Sigma and the synthetic tripalmytoyl lipopeptide, Pam₃CysSerLys₄ (Pam₃CSK₄) was obtained from Prof. Dr. G. Jung University of Tübingen (Tübingen, Germany). The topoisomerase I inhibitor, Camptothecin was obtained from Sigma. Purified rat anti-mouse CD14 mAb (clone rmC5-3, IgG1 κ), purified rat IgG1κ (R3-34) isotype control Ab and goat anti-rat Ig-FITC polyclonal Ab were all purchased from PharMingen (San Diego, CA). Anti-TLR2 polyclonal antibody was purchased from Santa Cruz, CA and normal goat IgG from DAKO (Carpinteria, CA).

Mice

C57BL/6, C3H/HeJ, C3H/HeO_uJ, C57BL/10ScSn mice (The Jackson Laboratory, Bar Harbor, MN) and C57BL/10ScNCr (National Cancer Institute, Bethesda, MD) were housed in sterilized, filter-hooded cages and provided food and water *ad libitum*. All experiments were approved by the Animal Research Ethics Boards of Dalhousie University.

Murine mast cells

MC/9 cells (ATCC CRL 8306), were routinely grown in modified Dulbecco's modified Eagle's medium (Life Technologies, Burlington, ON) containing 36 mg/ml L-aspartate, 0.1 mM non essential amino acids, 50 μ M 2-mercaptoethanol (ME), 10% FCS, and 3 ng/ml rmIL-3 (Pepro Tech, Inc., Rocky Hill, N.J.) in 10% CO₂. Bone marrow-derived mast cells (BMMC) were generated from the bone marrow of C57BL/6, C3H/HeJ, C3H/HeOuj, C57BL/10ScSn and C57Bl/10ScNcr mice according to the method of (Tertian *et al.*, 1981). Briefly, mice were sacrificed, and intact femurs and tibias were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe, and the bone marrow cells were passed through a sterile wire screen to remove any bone fragments. The cell suspension was centrifuged at 320 x g for 20 min at 4°C, and cultured at a concentration of 0.5-1 x 10⁶ nucleated cells/ml in RPMI 1640 (Life Technologies) supplemented with 10% FCS (SIGMA-Aldrich, ON, Canada), 10% v/v concentrated WEHI-3 cell conditioned medium as a source of IL-3, 1% penicillin/streptomycin (Life Technologies), 10⁻⁷ M PGE₂ and 50 μ M 2-mercapto ethanol (BMMC medium). The medium was replaced three times a week with fresh BMMC medium. BMMC were monitored for purity after 4 weeks by Alcian blue (pH 0.3) staining of fixed cytocentrifuge preparations. Once the BMMC population reached a purity of greater than 98% (5-8 weeks) they were used in subsequent experiments.

Human mast cells

Cord blood-derived mast cells (CBMC) were obtained by long-term culture of cord blood progenitor cells as previously described (Lin *et al.*, 2000a), using a modification of the method described (Saito *et al.*, 1996). Briefly, heparinized cord blood, obtained after informed consent, was centrifuged over a Ficoll-Paque separating solution (Seromed, Berlin, Germany). Mononuclear cells, including the progenitors, were cultured at a starting density of 10^6 cells/ml in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, 10% fetal calf serum (all from Life Technologies, Grand Island, N.Y.), 1% (wt/vol) bovine serum albumin (BSA) (Sigma, St. Louis, Mo.), 50 μ M 2-mercaptoethanol (Sigma), 100 ng of human recombinant stem cell factor (Pepto-Tech)/ml, and 20% CCL-204 (American Type Culture Collection) and normal human skin fibroblast supernatant as a source of IL-6. The medium was renewed every 7 days. Following 5–8 wk, mast cells were determined by morphologically and by the presence of metachromatic granules (toluidine staining). Only >95% of pure mast cells was used in experiments except for short-term β -hexosaminidase release studies where >90% mast cell purity was sometimes used. Representative CBMC cultures were also analyzed by flow cytometry for the mast cell marker *c-kit* and the monocyte marker CD14. Less than 2% of the cells expressed CD14 ($n = 6$) while >95% of the cells expressed *c-kit* ($n = 8$). Mast cells were placed in mast cell growth medium devoid of PGE₂ for >16 h before use unless otherwise indicated.

The human basophilic/mast cell line KU812 (Kishi, 1985) was grown in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 10 mM HEPES, 100 U of penicillin per ml, and 100 μ g of streptomycin (Life Technologies) per ml. The human monocytic cell line U937 was maintained in

RPMI 1640 (Life Technologies) supplemented with 10% FCS, 100 U of penicillin per ml, and 100 µg of streptomycin (Life Technologies) per ml. All cell lines were passaged two to three times per week.

RT-PCR for murine gene products

Total RNA was isolated from MC/9 cells, BMMC and the murine macrophage like cell line J774 using Trizol Reagent (Life Technologies), according to the manufacturer's instructions. To remove possible genomic DNA contamination RNA samples were treated with deoxyribonuclease I (DNase I) (Life Technologies). Briefly, 1 µg RNA was incubated with 1 µg DNase I, 1 µl DNase reaction buffer and 7 µl H₂O for 15 min at room temperature, after which time DNase I activity was inactivated by the addition of 2 mM EDTA and heating between 60-65°C for 20 min. Reverse transcription of the RNA samples was achieved using MMLV transcriptase (Life Technologies) and oligo (dT) primers as random templates. Primers used for PCR amplification of MD-2, MyD88, GAPDH, TLR2, TLR4, TLR5 and TLR6 were purchased from Research Genetics, Inc. (AL, USA) and sequences were as follows: 1) mouse MD-2: sense, 5'-GAG AAG CAA CAG TGG TTC TG-3'; antisense, 5'-CTC CTT TCA GAG CTC TGC AA-3'; 2) MyD88: sense, 5'-CGA GTT TGT GCA GGA GAT GA-3'; antisense, 5'-GGA TAC TGG GAA AGT CC T TC-3'; 3) GAPDH: sense, 5'-ACT CAC GGC AAA TTC AAC GGC-3'; antisense, 5'-ATC ACA AAC ATG GGG GCA TCG-3'; 4) TLR2: sense, 5'-GCC TTG ACC TGT CTT TCA AC-3'; antisense, 5'-GGA CTG ATA ATT CCG GAG AC-3'; 5) mouse TLR4: sense, 5'-CAG CTT CAA TGG TGC CAT CA-3'; antisense, 5'-CTG CAA TCA AGA GTG CTG AG-3'; 6) TLR5: sense, 5'-AGT GCT CAG TGC

CTG TAC TA-3'; antisense, 5'-GCA TGT GCTAGG TTC TAG GT-3'; 7) TLR6: sense, 5'-GGC ATC TAG ACC TCT CAT TC-3'; antisense, 5'-ATG GAT AAC GGT GGT ATT GG-3'. The PCR products for murine of MD-2, MyD88, GAPDH, TLR2, TLR4, TLR5 and TLR6, were 277bp, 287bp 245, 407bp, 438bp, 305bp and 230bp respectively. PCR was performed in a 50 μ l reaction mixture comprised of 1 μ M (final concentration) of each forward and reverse oligonucleotide primer, 50 mM MgCl₂, 5 mM of the four deoxynucleotide triphosphates, 5 μ l cDNA preparation, and 0.02 U/ μ l (final concentration) Taq DNA polymerase. Thirty four cycles were used (94°C for 1 min, 1 min at 56°C, and 2 min at 72°C) followed by 7 min at 72°C. Products were run out on a 1.8 % agarose gel containing ethidium bromide. Gel images were captured and visualized using Gel Doc Image Analysis system (Bio-Rad, Hercules, CA).

RT-PCR for human gene products

Total RNA was isolated using TRIzol reagent (Life Technologies) as indicated for RT-PCR of murine gene products. RNA samples were treated with DNase I (Life Technologies). Primers used for PCR amplification of reverse-transcribed RNA samples for MD-2, myeloid differentiation factor 88 (MyD88), β -actin, TLR1, TLR2, TLR4, and TLR6 were purchased from Research Genetics (Huntsville, AL), and sequences were as follows: 1) human MD-2: sense, 5'-GCA CTC ATC CGA TGC AAG T-3'; antisense, 5'-GTT GTA TTG ACA GTC TCT CC-3'; 2) MyD88: sense, 5'-GGG AGG AGA TGG ACT TTG AGT-3'; antisense, 5'-GCA ATA GAC CAG ACA CAG GT-3'; 3) TLR1: sense, 5'-TT CTG GCA CTT CCT TGA AGG-3'; antisense, 5'-GTC TCC AAC TCA

GTA AGG TG-3'; 4) TLR2: sense, 5'-GCC AAA GTC TTG ATT GAT TGG-3'; antisense, 5'-TTG AAG TTC TCC AGC TCC TG-3'; 5) TLR4: sense, 5'-TGG ATA CGT TTC CTT ATAAG-3'; antisense, 5'-GAA ATG GAG GCA CCC CTT C-3'; and 6) TLR6: sense, 5'-ATCA GAA CTC ACC AGA GGT C-3'; antisense, 5'-CAT GAG GAC ACA GCA TGT GT-3'. Thirty-four PCR cycles were used.

Flow cytometric analysis

In 96-well U-bottom plates (Nunc), cells (5×10^5 cells/well) were incubated with purified rat anti-mouse CD14 monoclonal antibody (Ab) (clone rmC5-3, IgG1 κ , PharMingen) or purified rat IgG1 κ (R3-34) isotype control Ab (PharMingen) in immunofluorescence (IF) buffer (PBS, 1% BSA, and 0.2% sodium azide) for 1 h at 4°C. After washing, cells were further incubated for 1 h with goat anti-rat Ig-FITC polyclonal Ab (PharMingen). Cells were washed three times (with IF buffer) and resuspended in 400 μ l of 1% paraformaldehyde (in PBS), and 10,000 cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). The results obtained with specific antibodies were compared with those using isotype matched control antibodies in parallel.

Murine mast cell activation

Mast cell activation was conducted using an endotoxin free system. Prior to BMHC activation, cells were washed several times in PGE₂-free BMHC medium and incubated for >16 hr. MC/9 cells were washed several times in experimental medium. The experimental media for MC/9 cells consisted of culture media with the addition 100 μ g/ml soybean trypsin inhibitor (SIGMA-Aldrich Canada Ltd, reconstituted in saline)

while the experimental medium for BMDC consisted of RPMI 1640 (Life Technologies) supplemented with 10% FCS (SIGMA-Aldrich, ON, Canada), 1% penicillin/streptomycin (Life Technologies), 50 μ M 2-ME (BMDC medium), 1.5 ng/ml rIL-3 and 100 μ g/ml soybean trypsin inhibitor (SIGMA). In some experiments, cells were activated in the absence of FCS. For these experiments BMDC were washed four times in serum-free medium (AIM V medium, Sigma) supplemented with 1% penicillin/streptomycin (Life Technologies), 50 μ M 2-ME (BMDC medium), 1.5 ng/ml rIL-3 and 100 μ g/ml soybean trypsin inhibitor (SIGMA-Aldrich Canada Ltd.; reconstituted in saline). For control medium, 10% FCS was added to this medium. Cells were incubated at 1×10^6 cells/ml for 6 and 24 h at 37°C with the following reagents *E. coli* LPS (serotype O55:B5), *Pseudomonas aeruginosa* (*P. aeruginosa*) LPS (serotype 10) and calcium ionophore (A23187). Samples were stored at -20°C until assayed.

Human mast cell activation

Mast cell activation was conducted using an endotoxin free system. For most experiments CBMC were initially washed several times in PGE₂-free CBMC culture medium and incubated for >16 hr. In some experiments CBMC cells were activated by IgE-mediated aggregation of Fc ϵ RI. For these experiments CBMC were cultured >48 hr in PGE₂-free CBMC culture medium containing 5 μ g/ml human IgE (Chemicon). The experimental media for CBMC consisted of RPMI 1640 medium (Life Technologies) supplemented with 10% FBS (Life Technologies), 10 mM HEPES, 100 U of penicillin per ml, and 100 μ g of streptomycin (Life Technologies) per ml, 100 μ g/ml soybean

trypsin inhibitor (SIGMA-Aldrich Canada Ltd, reconstituted in saline) and 10 ng/ml human recombinant stem cell factor (Pepto-Tech)/ml. Cells were incubated at 1×10^6 cells/ml for 24 h at 37°C with the following reagents calcium ionophore (A23187), *E. coli* LPS (serotype O55:B5), *S. aureus* PGN (Fluka), *S. cerevisiae* zymosan (Sigma) and Pam₃CSK₄ (Tubingen). Samples were stored at -20°C until assayed.

For IgE-mediated activation, CBMC were washed four times by centrifugation (15 min at 210g and 4°C) after which time cells were re-suspended at a final concentration of 1×10^6 cells/ml in RPMI medium, containing 10% FCS, 100 µg/ml STI and 10 ng/ml SCF. CBMC were activated with 17.5 µg/ml rabbit anti-human IgE (DAKO, Denmark) or 17.5 µg/ml purified rabbit IgG as a control (Cedarlane). Additional samples were activated with PGN alone or PGN with either anti-IgE or purified rabbit IgG. For the combined activation experiments PGN was added simultaneously with anti-IgE or rabbit IgG.

In some experiments CBMC were treated with the pan-caspase inhibitor, z-VAD-fmk (Calbiochem) which inhibits caspase 1, 3, 4 and 7. For these experiments, CBMC were pretreated with 25 µg/ml z-VAD-fmk at 37°C and 5% CO₂ for 30 min. After such time CBMC were activated with various concentrations of *S. aureus* PGN. Following a 24-hr incubation at 37°C and 5% CO₂, cell free supernatants were harvested and analyzed for GM-CSF production by ELISA.

ELISAs for murine cytokines

Murine IL-6 and TNF was measured by "in house" sandwich ELISAs. For IL-6, maxisorp ELISA plates (Nunc/Inter Med, ON, Canada) were coated for 18-24 h at 4°C

with 100 μ l/well of 2 μ g/ml anti-mouse IL-6 capture antibody (Endogen) diluted in 0.1M bicarbonate buffer (0.1M NaHCO₃, 0.5M NaCl, dH₂O). The wells were washed 3 times, and incubated for 1 h with 200 μ l/well blocking solution (1% BSA in PBS). The blocking solution was decanted and the wells were washed four times with Phosphate-buffered saline containing 0.05% Tween 20. Standards (Genzyme) and samples were added to the plate at 50 μ l/well and incubated at 4°C overnight. The wells were washed as described above, and secondary biotinylated anti-mouse IL-6 antibody (Endogen) at 0.5 μ g/ml in assay solution (0.3% BSA, 0.05% Tween 20 in PBS), was added at 50 μ l/well. After 2 h, the wells were washed and 50 μ l/well of streptavidin-alkaline phosphatase (Life Technologies) prepared in blocking solution, was added to the plates for an hour. The wells were washed, and bound secondary antibody was detected with the GIBCO ELISA Amplification System (Life Technologies). The colored product was read at 492 nm. Sensitivity of the IL-6 ELISA was 300 pg/ml.

TNF was measured using a similar protocol. All incubations were carried out at room temperature. Plates were coated with TNF coating antibody, polyclonal goat anti-mTNF Ab (R&D) as mentioned above. Plates were blocked for 2 h followed by the administration of samples and standards. Biotinylated, secondary Ab, anti-murine TNF (MM35000-B Endogen) was added next for 2 h. Subsequent steps were the same as the IL-6 ELISA protocol. The sensitivity of this TNF α ELISA was 30 pg/ml unless otherwise indicated.

ELISAs for human cytokines

The following human cytokines, GM-CSF, IL-1 β , RANTES (CCL5) and IL-6 were measured in the supernatants of CBMC using an in-house ELISA as previously described (Lin *et al.*, 2000a). Briefly, 96-well plates were coated with, anti-human GM-CSF Ab (Genzyme, P-230-E), anti-human IL-1 β (Endogen, Woburn, MA), anti-human RANTES (CCL5) (Endogen) and anti-human IL-6 (Endogen) at 1 μ g/ml for 16–20 h at 4°C. Nonspecific binding to the plates was blocked using a 1% BSA/0.1% Tween-20 solution in PBS for 1 h at 37°C. A total of 50 μ l/well of samples and standards for, GM-CSF (human rGM-CSF; R&D Systems), IL-1 β (human rIL-1 β ; Endogen), RANTES (CCL5) (R&D Systems, BAF-278) and IL-6 (Endogen, M-621-B) were added to the plate and incubated for 18–20 h at 4°C. Biotinylated anti-human GM-CSF (Endogen), anti-human IL-1 β (Endogen; 0.2 μ g/ml), anti-human RANTES (CCL5) (Endogen) and anti-human IL-6 (Endogen) was added to each well and incubated for 1 h at 37°C. After washing, 50 μ l/well of a 1/2000 dilution of streptavidin-alkaline phosphatase (Life Technologies) was added according to the manufacturer's instructions. Sensitivity of the IL-6 and IL-1 β assay was 1.95 pg/ml, that of RANTES (CCL5) was 30 pg/ml, and that of GM-CSF was 3 pg/ml.

Short-term mediator release and β -hexosaminidase assay

CBMC (0.5×10^6 /ml) in modified HEPES-Tyrode's buffer, supplemented with 1% FBS as a source of soluble CD14 and LPS-binding protein, were activated with the positive control A23187 (0.5 mM) or various TLR activators. After a 20-min incubation, supernatants and pellet fractions were each analyzed for β -hexosaminidase according to the method of (Schwartz *et al.*, 1979). Briefly, 50 μ l of each sample was incubated with

50 μ l of 1 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma) dissolved in 0.1 M citrate buffer, pH 5, in a 96-well microtiter plate at 37°C for 1 h. The reaction was stopped with 200 μ l of 0.1 M carbonate buffer/well, pH 10.5. The plate was read at 405 nm in an enzyme-linked immunosorbent assay (ELISA) reader.

Cysteinyl leukotriene assay

CBMC (1×10^6 /ml) were incubated for a predetermined optimal time of 20 min at 37°C with activating agents or controls in RPMI 1640 medium supplemented with 1% FCS and 10 ng/ml stem cell factor. Supernatants were harvested by centrifugation, snap frozen, and later analyzed by an enzyme immunoassay using a commercial kit (Amersham, Montreal, Quebec, Canada), which detected cysteinyl leukotrienes including leukotriene C₄ and its degradation products D₄ and E₄. The sensitivity of this assay was <30 pg/ml.

Immunohistochemistry

Nasal polyp tissues were obtained, after informed consent, from subjects requiring routine polypectomy. Only tissues from subjects who had not received topical steroid therapy in the 3 wk before surgery were used. Frozen tissue sections (5 μ m) were fixed with absolute ethanol for 10 min at room temperature followed by Alcian blue (pH 1.0) staining for 15 min at room temperature. Endogenous peroxidase activity was quenched by subjecting tissue section to 0.5% H₂O₂ for 15 min at room temperature. Samples were washed three times with PBS and blocked with 3% BSA, 0.1% Saponin, 0.1% Tween 20 and 5% normal human serum for 1 hr at room temperature. Slides were stained using anti-TLR2 polyclonal antisera (Santa Cruz Biotechnology, Santa Cruz, CA) or normal

goat IgG at the same dilution (DAKO, Carpinteria, CA) over-night at room temperature. Ab binding was detected using biotinylated swine anti-goat Ab (Cedarlane Laboratories, Hornby, Ontario, Canada) and a commercial development system (Company). Slides were mounted using glycerol gelatin mounting media and covered with a coverslip.

Detection of caspase activation by flow cytometry

Caspase activation was analyzed using FAM-VAD-FMK (Caspatag) according to the manufacturer's instructions (Intergen, Purchase, NY). Briefly, CBMC (>95% pure) were harvested directly from cultures and plated in 24 well plates (Nunc) at a concentration of 0.5×10^6 cells/ml. Cells were then activated at 37°C with various concentrations of *Staphylococcus aureus* PGN (Fluka). As a positive control, cells were stimulated with 5 µg/ml of the topoisomerase I inhibitor, camptothecin (Sigma) and as a negative control cells were incubated with medium alone. Cells were harvested and incubated with Caspatag at 37°C, 5% CO₂. After a 1-hr incubation, cells were washed twice by centrifugation, fixed with 1% paraformaldehyde (in PBS) and analyzed by flow cytometry.

Detection of changes in mitochondrial membrane potential

3,3'-dihexyloxacarbocyanine iodide (DiOC₆) (Molecular Probes, Eugene, OR) was used to assess changes in mitochondrial membrane potential ($\Delta\Psi_m$) as previously described (Kagi *et al.*, 1996). CBMC (>95% pure) were plated in 24 well plates (Nunc) at a concentration of 0.5×10^6 and were activated with various doses of *S. aureus* PGN (Fluka). As a positive control, cells were stimulated with 5 µg/ml camptothecin (Sigma)

and as a negative control cells were incubated with medium alone. 15 min prior to the end-point of the apoptosis assay, samples were treated with 40nM DiOC₆ and maintained for an additional 15 min at 37°C. Cells were harvested and analyzed by flow cytometry.

Statistics

Comparison of data sets for experiments in chapter 3 were performed using ANOVA and Students T Test. Comparison of data sets for experiments in chapter 4 were performed using ANOVA and Dunnett's post-test. Comparison of data sets for experiments in chapter 5 were performed using Students T Test and paired-analysis.

Chapter 3

TOLL-LIKE RECEPTOR 4-MEDIATED ACTIVATION OF MURINE MAST CELLS

3.1 Introduction

A number of lines of evidence suggest that mast cells play a protective role in host defense against bacteria. Mast cells are found in high numbers in vascularized tissues at the host-environment interface (Wasserman, 1989; Galli *et al.*, 1999). This strategic location facilitates their exposure to invading organisms. By virtue of their ability to synthesize large numbers of inflammatory mediators mast cells are able to generate potent inflammatory reactions (Plaut *et al.*, 1989; Burd *et al.*, 1989; Gupta *et al.*, 1996) and numerous accounts have shown that mast cells are responsive to a large number of bacteria and bacterial products (Malaviya *et al.*, 1999; Leal-Berumen *et al.*, 1996; Leal-Berumen *et al.*, 1994; Ackerman *et al.*, 1998). Studies using mast cell deficient mice have provided direct evidence for a protective role of mast cells in host defence against bacterial infections. Echtenacher *et al.* 1996 demonstrated that mast cell-deficient mice ($\text{Kit}^{\text{W}}/\text{Kit}^{\text{W-v}}$) displayed significantly increased mortality, compared to wild-type mice in a model of acute septic peritonitis. Correction of this mast cell deficiency restored survival close to the level observed in wild-type mice (Echtenacher *et al.*, 1996). Similarly, enhanced mortality in $\text{Kit}^{\text{W}}/\text{Kit}^{\text{W-v}}$ mice following intraperitoneal instillation of an enterobacteria could be prevented by correction of the mast cell deficiency (Malaviya *et al.*, 1996). These studies clearly demonstrate that mast cells are required for host defence in some murine models of bacterial infection, however the mechanisms utilized remain largely undefined.

Both Gram-positive and Gram-negative bacteria elicit a number of pro-inflammatory responses in mammalian cells. Many of these responses are induced by cell wall components including, peptidoglycan (PGN) and Lipopolysaccharide (LPS)

from Gram-positive and Gram-negative bacteria respectively. Production of pro-inflammatory cytokines, such as TNF, IL-6 and IL-1 β , play an indirect role in eliminating bacterial infection. However, excessive cytokine production may also lead to endotoxic shock. Determining the molecular mechanism of LPS recognition by responsive cells has been a priority in the development of strategies to artificially modulate the LPS response.

In most cells of the myeloid lineage, including the macrophage, LPS is captured by a serum protein, LPS binding protein (LBP) and subsequently transferred to CD14, a GPI-anchored membrane protein (Hailman *et al.*, 1994). Cells that lack membrane expression of CD14, including endothelial and epithelial cells utilize soluble CD14 (sCD14) for efficient activation (Haziot *et al.*, 1993). Despite the known function of CD14, this receptor lacks an intracellular signaling domain. Therefore, additional molecules with intracellular signaling capacity are required. Members of the Toll-like receptor (TLR) superfamily, have been demonstrated to serve this function.

Among the rapidly expanding family of TLR (Janeway and Medzhitov, 1999; Aderem and Ulevitch, 2000; Takeuchi *et al.*, 1999; Chuang and Ulevitch, 2000; Du *et al.*, 2000; Chuang and Ulevitch, 2001), TLR4, has been most closely linked with LPS signaling. Initially, a number of reports suggested that TLR2 was the signaling receptor for LPS in macrophages (Kirschning *et al.*, 1998; Yang *et al.*, 1998). However protein contamination in commercial preparations of LPS may be responsible for such signals through TLR2 (Hirschfeld *et al.*, 2000). Convincing evidence provided by Poltorak *et al.*, 1998; Hoshino *et al.*, 1999 and Qureshi *et al.*, 1999 demonstrated that mutations in the *Lps* gene, found in hyporesponsive murine strains including C3H/HeJ and C57Bl/10ScCr mapped to TLR4. Sequence analysis of the TLR4 gene in C3H/HeJ and C57Bl/10ScCr

revealed a single point mutation at amino acid 712 and a null mutation respectively (Poltorak *et al.*, 1998). Later, Takeuchi and colleagues (Hoshino *et al.*, 1999) used TLR2 and TLR4 knockout mice to confirm that TLR4 but not TLR2 is the major receptor for LPS *in vivo*.

Although LPS preparations from most species of Gram-negative bacteria appear to activate cells via TLR4, recent studies (Hirschfeld *et al.*, 2001a; Werts *et al.*, 2001a) determined that LPS from *Leptospira interrogans* and *Prophyromonas gingivalis* respectively signal through TLR2 suggesting that LPS from different species of bacteria may be recognized by distinct TLR molecules. However, synthetic P. *gingivalis*-type as well as E.coli lipid A did not activate peritoneal macrophages from C3H/HeJ mice in a further report (Ogawa *et al.*, 2000). Even though LPS responses mediated by TLR2 have been controversial, it is well accepted that products from Gram-positive bacteria such as peptidoglycan signal via TLR2. In addition to the number of *in vitro* studies (Takeuchi *et al.*, 1999) studies using TLR2-deficient mice have confirmed that TLR2 is important for survival and cytokine responsiveness *in vivo* (Takeuchi *et al.*, 2000a). The role of mast cells in the *in vivo* response to LPS and other TLR-ligands is not known.

TLRs and members of the IL-1 receptor family share a highly homologous intracellular domain which has been designated the Toll/IL-1R like region (TIR) (O'Neill, 2002). The TIR domain is important for recruitment of the intracellular adaptor molecule myeloid differentiation factor 88 (MyD88). Association of these receptors with MyD88 occurs through a homotypic interaction which is dependant on a homologous TIR domain in the C-terminus of MyD88 (Muzio *et al.*, 1997; Wesche *et al.*, 1997). MyD88 is critical for IL-1 receptor signaling (Medzhitov *et al.*, 1998), and many

TLR mediated responses (Kawai *et al.*, 1999; Takeuchi *et al.*, 2000b). Studies conducted by Shimazu *et al.*, (Shimazu *et al.*, 1999) identified that human TLR4 alone, is not capable of sensing the presence of LPS. An additional molecule, MD-2 forms a complex with the extracellular domain of TLR4 for effective LPS recognition. Although TLR2 signaling is not dependant on MD-2, responses to a number of bacterial products including peptidoglycan are significantly enhanced by the expression of MD-2 (Dziarski *et al.*, 2001).

We have previously shown that LPS activates mast cells to produce IL-6 and TNF without degranulation (Marshall *et al.*, 1996). However, efficient stimulation of such mast cells requires much higher doses of LPS than classical LPS-responsive cells such as monocytes and macrophages (Gagari *et al.*, 1997). The ability of TLRs to mediate activation of mast cells has not been previously determined, we therefore examined both the expression of TLRs and associated molecules and the ability of LPS to utilize TLR4 to activate mast cells.

3.2 Results

Cytokine production by murine mast cells is induced by high doses of LPS and by *S. aureus* derived peptidoglycan.

BMMC were cultured from C57BL/6 mice (>98% purity) and used as a murine mast cell model. In light of the possible contribution of non-mast cell contaminants the IL-3 dependent murine mast cell line MC/9 was also employed. Cells were stimulated with *E. coli* derived LPS at concentrations ranging from 0.05 µg/ml-5 µg/ml. After 24 h, cell-free supernatants were harvested and analyzed for IL-6 production by ELISA. Activation of BMMC and MC/9 cells with 0.5 and 5 µg/ml but not 0.05 µg/ml *E. coli* LPS resulted in significantly higher levels of IL-6 compared to unstimulated cells (Figure 1A & B). Levels of IL-6 were greatest following stimulation with 5 µg/ml LPS. Using similar activation conditions C57BL/6 BMMC were stimulated with the known TLR2-activator, peptidoglycan (PGN) purified from *Staphylococcus aureus*. After 24 h, PGN treatment induced significantly higher levels of IL-6 compared to unstimulated cells (Figure 1C). Similarly C57BL/6 BMMC stimulated with 100 µg/ml PGN for 6 h produced levels of TNF that were > 8 fold higher than unstimulated cells (mean values of 42 ± 26 pg/ml and < 5 pg/ml TNF for PGN stimulated and unstimulated cells respectively, representative of two independent experiments). To ensure that the IL-6 and TNF responses of BMMC to PGN stimulation were not the result of LPS contamination, BMMC cultured from TLR4 deficient mice, C57BL/10ScNCr, were activated with PGN. Significant IL-6 (Figure 1D) and TNF (Data not shown) production were observed compared to unstimulated control cells.

CD14 Expression by murine mast cells

It is well documented that CD14 is a primary LPS binding molecule on myeloid cells such as, macrophages, monocytes and neutrophils. However, previous studies of human mast cells have suggested that it may not be expressed on this cell type (Escribano *et al.*, 1998; Fureder *et al.*, 1997; Sperr *et al.*, 1994). Therefore, the murine mast cell expression of CD14 was analysed. To determine if these cells express CD14 protein on the cell surface, BMMC (>98% pure) were incubated with anti-CD14 Ab for 1 h followed by a FITC labelled secondary Ab. Fluorescence of cells stained with anti-CD14 Ab was compared to cells stained with an irrelevant isotype control Ab. Using the mouse macrophage-like cell line J774 as a positive control an increase in fluorescence was observed with anti-CD14 Ab stained-J774 cells compared to isotype control stained cells (Figure 2A). The fluorescence distribution of BMMC stained with anti-CD14 Ab was identical to cells stained with the isotype control Ab (Figure 2A) demonstrating a lack of CD14 protein expression on the surface of mast cells.

Mast cell LPS-responsiveness requires the presence of serum

It has been previously reported that surface CD14 negative cells such as endothelial cells (Haziot *et al.*, 1993; Yang *et al.*, 1996) and epithelial cells (Tapping and Tobias, 2000) require soluble CD14, an abundant serum protein in order to respond to LPS. Since mast cells do not express detectable levels of surface CD14 we analyzed the requirement of serum factors for mast cell responses to LPS. C57Bl/6 BMMC were stimulated with LPS or the positive control calcium ionophore, A23187 in the presence or absence of serum.

In the presence of serum, BMMC produced significant levels of IL-6 when challenged with 0.5 and 5 $\mu\text{g/ml}$ *E. coli* LPS (Figure 3). In contrast, IL-6 remained below detectable levels (<0.3 ng/ml, $n=3$) when C57Bl/6 BMMC were stimulated with 0.5 and 5 $\mu\text{g/ml}$ LPS in the absence of serum (Figure 3).

Expression of TLRs by murine mast cells

TLR mRNA expression by murine mast cells was examined by RT-PCR. Two proteins implicated in the LPS response, TLR2 and TLR4, along with two less well characterized TLRs, TLR5 and TLR6 were chosen for analysis. The murine mast cell line MC/9 and BMMC cultured from C57BL/6 mice ($>98\%$ pure) were used as mast cell models while the mouse macrophage-like cell line, J774 was used as a positive control source of TLR mRNA. cDNA was amplified using primers specific for TLR2, TLR4, TLR5 and TLR6. PCR products of the appropriate size were obtained using J774 cDNA (Figure 4). Appropriate PCR products from BMMC and MC/9 cell cDNA were also observed using primers for TLR2, TLR4 and TLR6 but not TLR5 (Figure 4). All PCR products were absent when the reverse transcription step was omitted (Figure 4).

TLR4 function in murine mast cells

We next sought to determine if the induction of TNF and IL-6 by LPS in mast cells required a functional TLR4 for efficient activation. To address this, we compared the LPS responsiveness of BMMC derived from TLR4 deficient mice to control BMMC from a similar genetic background. The TLR deficient murine strains employed were C3H/HeJ and C57Bl/10ScNcr, characterized by a point mutation and a null mutation

respectively in the TLR4 gene. BMMC were cultured from these mice and their respective wild-type, congenic littermates C3H/HeOuJ and C57Bl/10ScSn respectively. BMMC (>97% pure) were stimulated with a range of doses of *E. coli* LPS for a period of 6 and 24 h after which time cell free supernatants were harvested and analyzed for cytokines. Time points of 6 and 24 h were chosen, to measure TNF and IL-6 respectively, based on earlier time course studies in control (C57Bl/6) mast cells (data not shown). BMMC cultured from TLR4 deficient mice were non-responsive to stimulation by *E. coli* LPS (Figure 5). Despite, high levels of cytokine production following stimulation with the positive control, calcium ionophore (A23187), levels of TNF and IL-6 production following LPS stimulation were consistently below the limit of detection of each ELISA, 30 pg/ml and 300 pg/ml respectively. In contrast, control mice responded rigorously to the highest concentrations of *E. coli* LPS and produced markedly increased levels of IL-6 and TNF compared to unstimulated cells.

We further analyzed the requirement of TLR4 for responsiveness of BMMC to LPS from an additional source, *Pseudomonas aeruginosa*. Similar to our previous findings, BMMC cultured from C3H/HeJ and C57Bl/10ScNcr mice were unresponsive to stimulation by *P. aeruginosa* LPS. Levels of IL-6 (Figure 6) production and TNF remained below the limit of detection of the ELISA despite high levels induced following A23187 stimulation. In contrast *P. aeruginosa* LPS markedly upregulated IL-6 (Figure 6) from control BMMC. Similar results were obtained with TNF as a readout (data not shown).

MD-2 and MyD88 mRNA expression by murine mast cells

The mast cell expression of two additional proteins implicated in the LPS response, MD-2 (Akashi *et al.*, 2000; Kawasaki *et al.*, 2000) and MyD88 (Kawai *et al.*, 1999) was characterized, using the murine mast cell line MC/9 and C57Bl/6 BMMC as mast cell models and the mouse macrophage-like cell line, J774, as a positive control. After assessing a positive PCR product for GAPDH, cDNA was amplified using primers specific for MD-2 and MyD88. Prominent bands were observed for MD-2 and MyD88 (Figure 7) using cDNA from all cell types. All PCR products were absent when the reverse transcription step was omitted (Figure 7).

Figure 1: Both the TLR4 activator LPS and TLR2 activator *S. aureus* PGN induce IL-6 production by murine mast cells. (A) C57Bl/6 BMMC and (B) the murine mast cell line, MC/9 were stimulated with a range of doses of *E.coli* LPS in the presence of FCS and 100 µg/ml soybean trypsin inhibitor. Using similar conditions (C) C57Bl/6 and (D) C57BL/10ScNcr BMMC were stimulated with *S. aureus* PGN (1 µg/ml-100 µg/ml). Unstimulated cells were analyzed for constitutive IL-6 production. Cell-free supernatants from each condition were harvested after 24 h and analyzed for IL-6 by ELISA. C57Bl/6 BMMC data are representative of 3 independent experiments from separate BMMC cultures. ND denotes IL-6 levels that were below the level of detection of the ELISA system employed. *** represents $p < 0.001$ compared to medium control.

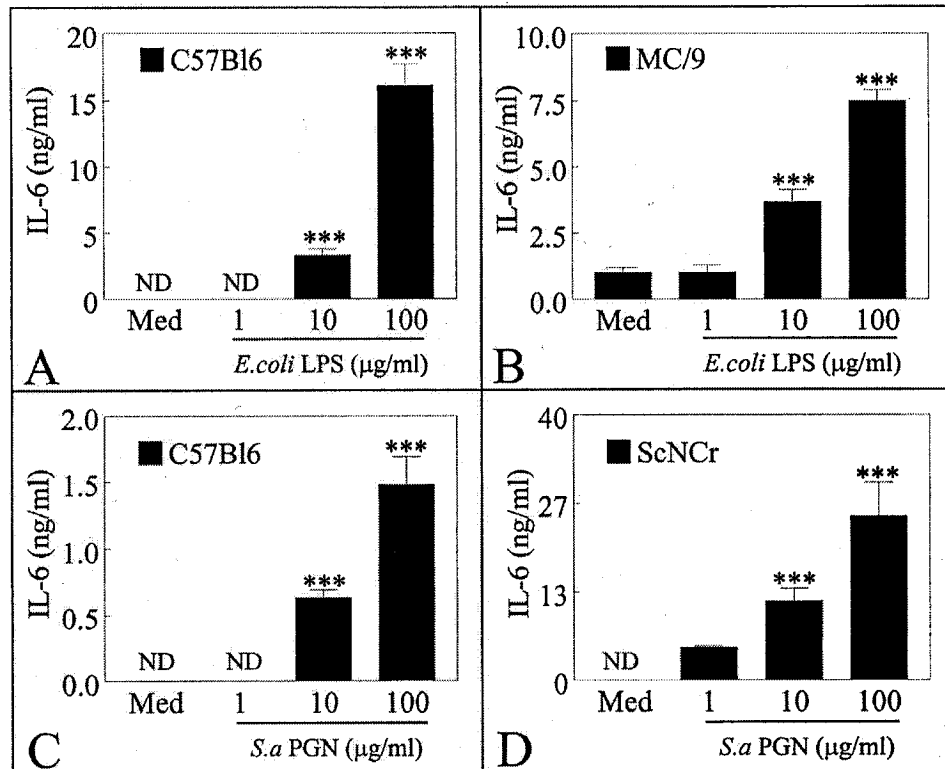


Figure 1

Figure 2: Flow cytometric analysis of murine mast cells for surface CD14 expression. (A) The murine macrophage-like cell line, J774 and (B) C57Bl/6 BMDC (>98% purity) were stained with purified rat anti-mouse CD14 mAb (black line histogram) or purified rat IgG1 isotype control Ab (shaded, histogram). Cells were then stained with a FITC labeled secondary Ab and analyzed for fluorescence. The data presented are representative of two and three independent experiments for BMDC and J774 cells respectively.

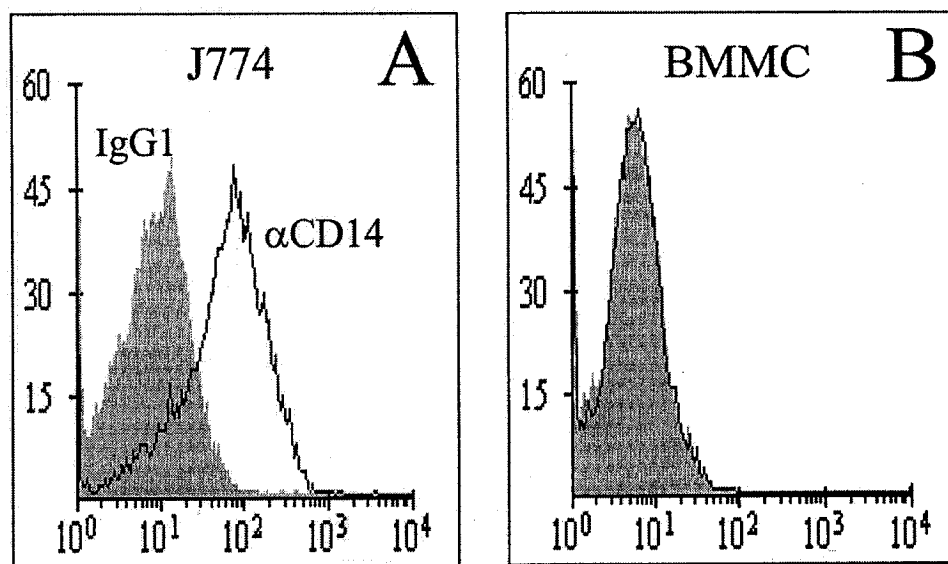


Figure 2

Figure 3: Murine mast cell-responsiveness to LPS requires the presence of FCS. C57Bl/6 (>98% purity) BMMC were stimulated with *E. coli* LPS (0.5 and 5 µg/ml) in the presence (black bars) or absence (shaded bars) of FCS. Unstimulated cells were analyzed for constitutive IL-6 production and 0.5 µM A23187 (A23) was used as the positive control. Cell-free supernatants from each condition were harvested after 24 hrs and analyzed for IL-6 by ELISA. C57Bl/6 BMMC data are representative of 3 independent experiments from separate BMMC cultures. ND denotes IL-6 levels that were below the level of detection of the ELISA system employed. *** represents $p < 0.001$ compared to medium control.

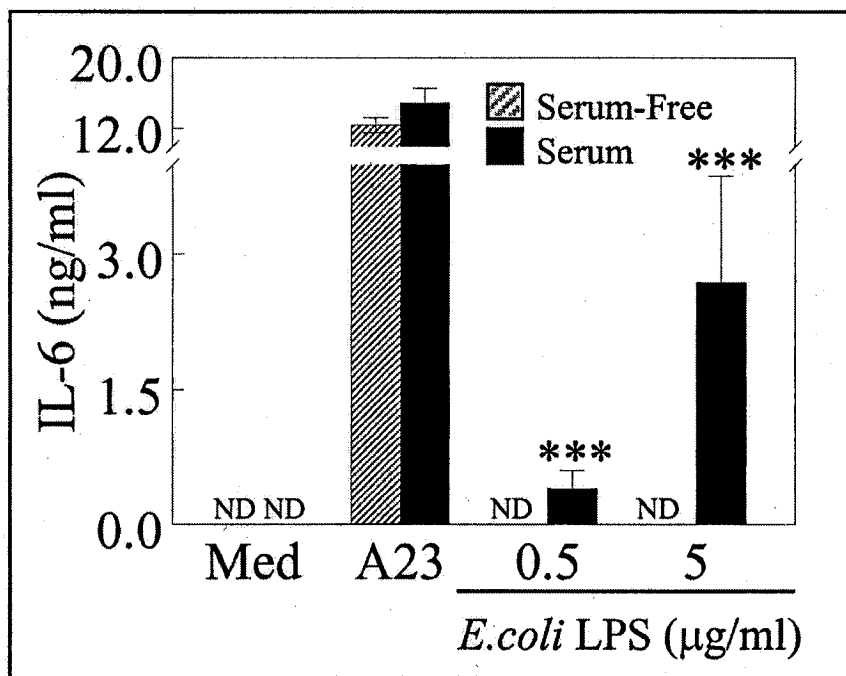


Figure 3

Figure 4: RT-PCR analysis of murine mast cells for TLR2, TLR4, TLR5 and TLR6 expression. Total RNA extracted from C57Bl/6 BMMC and the mast cell line, MC/9 was treated with DNase followed by reverse transcription and PCR amplification using specific primers for 34 cycles. RNA isolated from the macrophage-like cell line, J774 was used as a positive source of murine TLR RNA. RNA without reverse transcription was used as a negative control. The data shown are representative of three independent experiments.

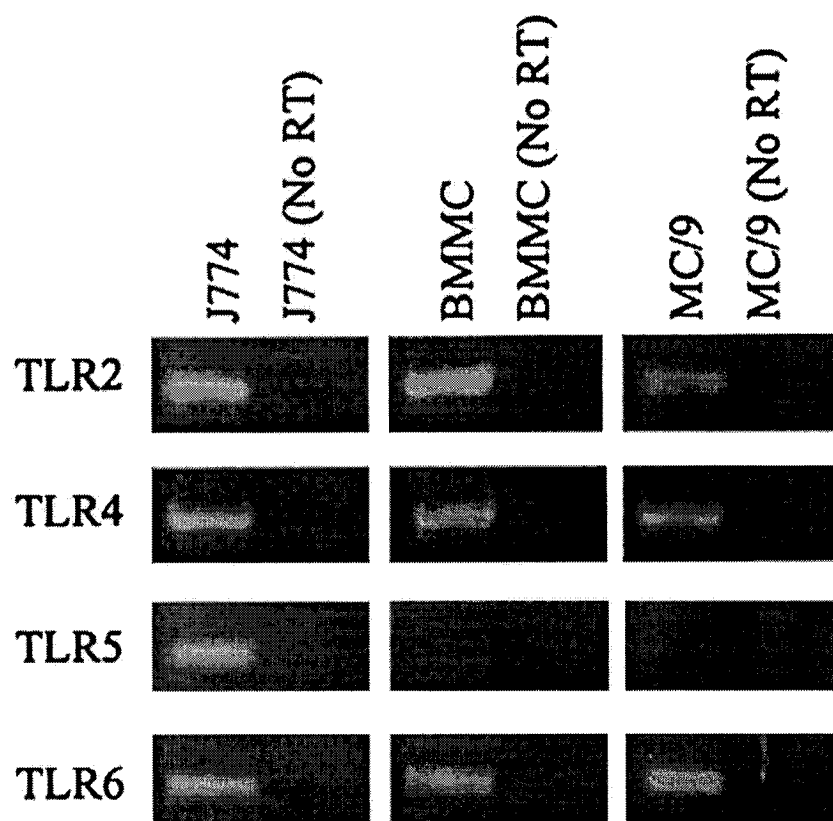


Figure 4

Figure 5: BMMC cultured from wild type mice but not TLR4 deficient mice produce IL-6 and TNF in response to *E.coli* LPS stimulation. BMMC (1×10^6 cells/ml) cultured from C3H/HeJ and C3H/HeOuJ mice (A, C) or C57Bl/10ScNcr and C57Bl/10ScSn mice (B, D) were stimulated for 24 h with a range of doses of *E. coli* LPS. Cell free supernatants were harvested and analyzed for IL-6 (A, B) and TNF (C, D) by ELISA after incubation periods of 24 h and 6 h respectively. ND denotes samples that were below 300 pg/ml, and 30 pg/ml, the limits of detection of the IL-6 and TNF ELISA assays respectively. *** represents $p < 0.001$ compared to medium control. The data presented are representative of two independent experiments for the C3H/HeJ model and three independent experiments for the C57Bl/10 model.

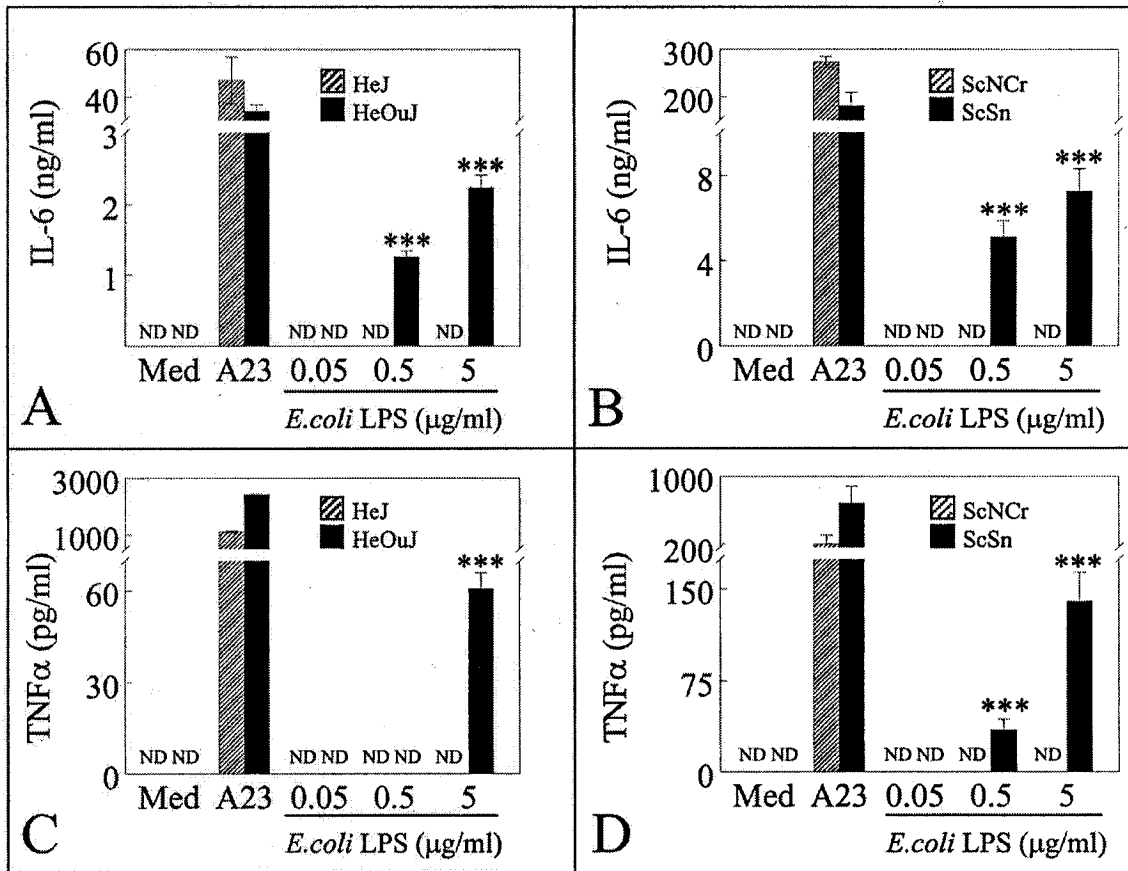


Figure 5

Figure 6: BMMC cultured from wild type mice but not TLR4 deficient mice produce IL-6 in response to *Pseudomonas aeruginosa* LPS stimulation. BMMC (1×10^6 cells/ml) cultured from C3H/HeJ and C3H/HeOuJ control mice (A) or C57Bl/10ScNCr and C57Bl/10ScJn control mice (B) were stimulated for 24 h with 0.05, 0.5 or 5 $\mu\text{g/ml}$ *P. aeruginosa* LPS in the presence of 100 $\mu\text{g/ml}$ soybean trypsin inhibitor. Cell free supernatants were harvested after 24 hours and analyzed for IL-6 by ELISA. ND denotes samples that were below 300 pg/ml, the limit of detection of the IL-6 ELISA. *** represents $p < 0.001$ compared to medium control. The data presented are representative of three independent experiments.

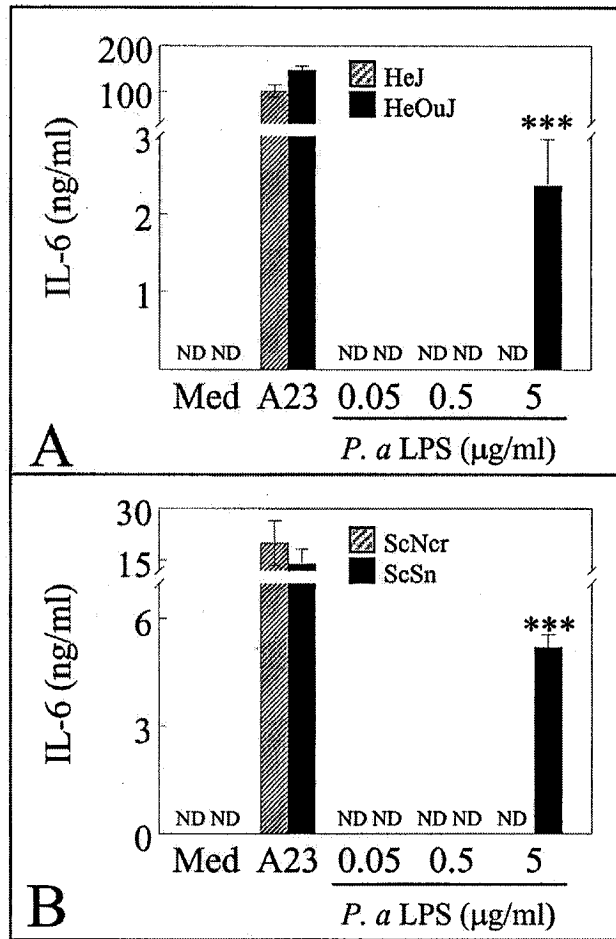


Figure 6

Figure 7: RT-PCR analysis of murine mast cell RNA for MD-2 and MyD-88. Total RNA extracted from C57Bl/6 BMMC and MC/9 cells was treated with DNase followed by reverse transcription and PCR amplification using specific primers for 34 cycles. RNA isolated from J774 cells was used as a positive source of murine MD-2 and MyD88 RNA. RNA without reverse transcription was used as a negative control. Note the expression of both MD-2 and MyD88 by both BMMC and MC/9 cells. The data shown are representative of three independent experiments.

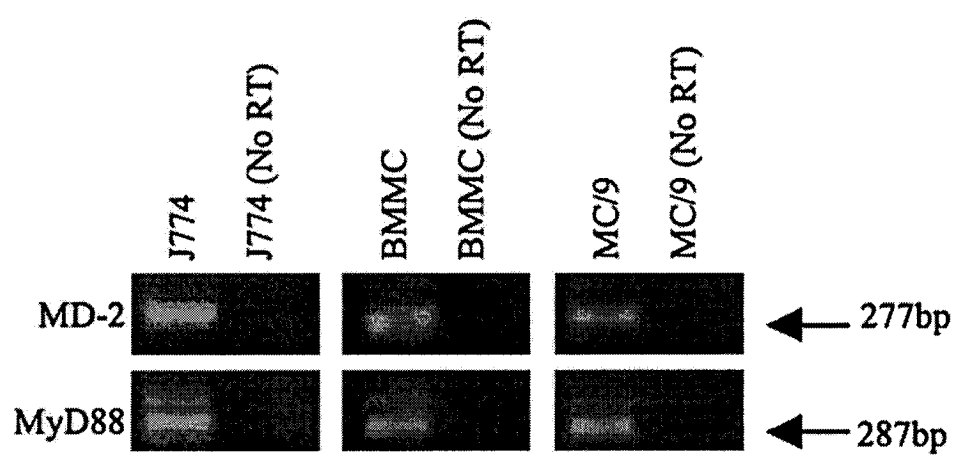


Figure 7

3.3 Discussion

Mast cells have a critical role in host defence against bacterial infections (Etchtenacher *et al.*, 1996; Malaviya *et al.*, 1996). However, the essential mediators derived from mast cell and the mechanism by which these cells respond to infection remain largely undefined. Mast cells may respond through receptor-mediated mechanisms involving direct contact with the bacteria, or mechanisms induced as a consequence of bacterial internalization (Arock *et al.*, 1998; Lin *et al.*, 1999; Sher *et al.*, 1979). In addition, mast cells may be activated through indirect mechanisms involving mediators, such as cytokines, produced by other cells, complement products (Prodeus *et al.*, 1997) or lipid mediators (Leal-Berumen *et al.*, 1995).

This study demonstrates that mast cells express mRNA for several TLRs including TLR2, TLR4 and TLR6 but not TLR5. These findings suggest that mast cells have the potential to respond directly to a variety of invading pathogens through TLR-ligation. The expression of TLR2, TLR4 and TLR6 are particularly important because previous studies have shown that these receptors mediate responsiveness to a number of pathogen products. TLR4 has been implicated in cellular responses to LPS (Faure *et al.*, 2000; Lien *et al.*, 2000), heat shock protein 60 (Ohashi *et al.*, 2000) and the F protein from respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000a). TLR2 is required for responses to products from Gram-positive bacteria (Means *et al.*, 1999a; Takeuchi *et al.*, 1999), *Mycobacterium tuberculosis* (Means *et al.*, 1999b), Spirocheates (Hirschfeld *et al.*, 1999) and Yeast (Underhill *et al.*, 1999b). Furthermore, TLR6 has been shown to enhance the responsiveness of cells to a number of these TLR2 activators (Hajjar *et al.*, 2001; Ozinsky *et al.*, 2000b). It has previously been shown that particular cell types may

preferentially express certain TLRs. For example, TLR3 has been reported to be selectively expressed by dendritic cells but not monocytes/macrophages (Muzio *et al.*, 2000a). The lack of TLR5 expression by mast cells is intriguing and may provide a further marker to distinguish mast cells from other myeloid cells. TLR5 has recently been shown to mediate cellular responses to flagellin, a principal component of bacterial flagella (Hayashi *et al.*, 2001). The innate immune response to bacterial flagellin is mediated by TLR5. In light of this finding it would be predicted that BMMC would not respond to purified flagellin. Moreover, flagellin activation of mast cells may not play a major role in host defense during infection caused by organisms such as *Listeria monocytogenes*. The vigorous cytokine responses that can be induced when murine mast cells are treated with either LPS (TLR4-activator) or PGN (TLR2-activator) suggest multiple TLRs may be functional on mast cells.

The current study demonstrates that TLR4 is essential for LPS responsiveness in murine mast cells. BMMC from TLR4 mutated mice, C3H/HeJ and C57Bl/10ScNcr were unresponsive to LPS stimulation while BMMC from the control mice, C3H/HeOuJ and C57Bl/10ScSn exhibited vigorous responses when stimulated with high doses of *E. coli* or *P. aeruginosa* derived LPS. Commercial LPS preparations may be contaminated with bioactive protein which activates cells via a TLR4-independent pathway. Mast cells did not respond to this contamination however, since neither the C3H/HeJ or C57Bl/10ScNcr mice responded to LPS. *P. aeruginosa* was employed as a second independent LPS source and produced similar results to those observed with *E. coli* derived LPS. Expression of a functional TLR4 by murine mast cells is significant because it provides the mast cells with a further means of directly identifying and responding to

bacterial challenge. Mast cells are resident in large numbers at most major tissues that interface directly with the external environment, including the skin, airways and gastrointestinal tract. This brings the mast cell into contact with bacteria in the first line of host defence. The localization of mast cells close to nerves and blood vessels adds to the potential of these cells for initiating effective immune responses against bacterial infection (Galli *et al.*, 1999).

High doses of LPS are required for activation of the murine mast cell compared to the macrophage. BMMC required 0.5-5 $\mu\text{g/ml}$ of LPS for effective cytokine induction. These findings are consistent with previous experiments employing either BMMC (Gagari *et al.*, 1997) or rat peritoneal mast cells (Leal-Berumen *et al.*, 1994). In light of these observations, the mast cell expression and function of a number of molecules that have been previously implicated in LPS responsiveness were examined. As previously reported for other mast cell types (Beil *et al.*, 1998; Fureder *et al.*, 1997), BMMC do not express cell surface CD14, although the soluble form of this receptor and LPS binding protein (LBP) would be available from the fetal bovine serum in which most of the experiments were performed. When mast cells were activated with LPS, in the absence of serum, no significant IL-6 responses were observed (Figure 3) suggesting that CD14, LBP or other serum factors were essential for the mast cell LPS responses. mRNA for both the adapter molecule MD-2 and the signaling molecule MyD88, which are critical for LPS responses in other cell types (Muzio *et al.*, 1997; Burns *et al.*, 1998), were expressed by BMMC. A number of factors may account for the mast cells' reduced responses to these TLR ligands including decreased receptor expression, reduced receptor homo or heterodimer formation or a deficiency in the intracellular signaling mechanisms.

However, mRNA expression of both the MD-2 and MyD88 molecules known to be critical for LPS signalling was observed in BMDC. Interestingly, we have previously shown that mast cell activation with the TLR9 ligand, CpG oligonucleotides also requires higher doses than those required to activate other cell types (Zhu and Marshall, 2001). However, the doses of PGN required for mast cell activation were very similar to those reported for macrophage (Takeuchi *et al.*, 1999). It is notable that human intestinal epithelial cells, which also lack surface CD14 expression, produce cytokines in response to much lower doses of LPS than the amounts required for BMDC activation (Cario *et al.*, 2000).

These findings have important implications for our understanding of the responses of mast cells to bacterial products. The mast cells' expression of a range of TLRs as well as MD-2 and MyD88 suggests that mast cells may respond to a wide variety of TLR-mediated signals, some such potential responses have already been reported (Lin *et al.*, 2000b). The requirement for a high dose of LPS suggests that even if LPS signals are induced primarily through a shared TLR4 dependent mechanism, alternate cell types may have different thresholds for activation. The BMDC has previously been considered as a model of the mast cells which reside at mucosal surfaces such as the intestine. In these sites, a higher threshold of activation for mast cells may help to distinguish tissue invasion and infection from the background levels of bacterial products associated with the microenvironment.

Overall, these studies demonstrate both cytokine response to a known TLR2 ligand and TLR4-mediated activation of murine mast cells in response to LPS. They also demonstrate the mRNA expression of a number of TLR and necessary accessory

molecules. If mast cells truly serve as “sentinel” cells in host defence (Galli *et al.*, 1999) pattern recognition receptors such as the TLR will have many important functions on mast cells, which we are only beginning to elucidate.

Chapter 4

DISTINCT TOLL-LIKE RECEPTOR 2 ACTIVATORS SELECTIVELY INDUCE DIFFERENT CLASSES OF MEDIATOR PRODUCTION FROM HUMAN MAST CELLS

4.1 Introduction

Mast cells have long been known to contribute to allergic diseases and chronic inflammatory processes and, more recently, have been shown to play an important role in host defense against bacterial infections (Echtenacher *et al.*, 1996; Galli *et al.*, 1999; Malaviya and Abraham, 2001). Mast cells are well equipped to participate in these reactions based on their strategic tissue distribution and ability to selectively produce a wide range of pro-inflammatory mediators. Mast cells are a potent source of preformed, granule-associated mediators, newly synthesized lipid mediators, as well as a large number of cytokines and chemokines. Human mast cells produce the NF κ B-regulated cytokines, GM-CSF and IL-1 β , which have been implicated in the mobilization, recruitment, and enhanced survival of effector cells, such as neutrophils, at the site of infections (Hamilton, 2002). Cysteinyl leukotriene production by mast cells is of particular interest because of their potent bronchoconstrictive and chemotactic effects (O'Byrne, 2000).

Toll-like receptors (TLR) are a family of pattern recognition receptors known to play an important role in host defense (reviewed by: Akira *et al.*, 2001) TLR2 is critical for responses to bacterial-derived lipopeptides (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999; Hirschfeld *et al.*, 1999) and peptidoglycan (PGN) (Schwandner *et al.*, 1999b; Underhill *et al.*, 1999a; Yoshimura *et al.*, 1999) as well as zymosan (Underhill *et al.*, 1999), the cell wall component of yeast, while TLR4 is the major signaling molecule for most types of LPS (Akira *et al.*, 2001). Ligand specificity for a number of TLR2 activators is thought to require heterodimerization with additional TLR molecules, TLR1 and TLR6 (Takeuchi *et al.*, 2002; Ozinsky *et al.*, 2000). Studies suggest that TLR2/TLR6

heterodimers mediate responses to PGN or zymosan, while TLR1/TLR2 heterodimers mediate responses to the synthetic tripalmitoyl lipopeptide Pam₃Cys-Ser-(Lys)₄ (Pam₃CSK₄) in the mouse (Takeuchi *et al.*, 2002; Ozinsky *et al.*, 2000). Distinct TLR molecules are capable of inducing differential cellular responses (Hirschfeld *et al.*, 2001b; Jones *et al.*, 2001). Rodent mast cells as well as murine mast cell lines produce several cytokines, including IL-6 and TNF, in response to activation with the TLR activators LPS and PGN (Leal-Berumen *et al.*, 1994; McCurdy *et al.*, 2001; Supajatura *et al.*, 2002; Supajatura *et al.*, 2001). However, human mast cell responses to TLR activators are poorly defined.

In the current study, human mast cells are demonstrated to express a distinct profile of TLRs compared with murine mast cells and other leukocytes. Differences in TLR expression are reflected by selective mast cell stimulation by TLR activators. Furthermore, distinct TLR2 activators are shown to induce differential mediator production including, the novel selective induction of substantial cysteinyl leukotriene generation and cytokine production without evidence of classical degranulation.

4.2 Results & discussion

TLR mRNA expression by CBMC

It has been previously reported that murine mast cells express mRNA for a number of TLRs including: TLR2, TLR4, TLR6, and TLR8 (McCurdy *et al.*, 2001; Supajatura *et al.*, 2001). To determine the human mast cell expression of TLRs, CBMC and the mast cell/basophil line KU812 were analyzed for TLR mRNA expression by RT-PCR. Prominent PCR products of the appropriate size were expressed for TLR1, TLR2, and TLR6 but not TLR4 by both CBMC and KU812 (Fig. 1A). In comparison, the human macrophage cell line U937 expressed bands for all TLRs examined, including TLR4. All cells examined expressed mRNA for the adapter molecules MyD88 and MD-2.

Immunohistochemical analysis was used to assess the protein expression of TLR2 by human tissue mast cells. The majority of nasal polyp human mast cells, staining positive with Alcian blue, at low pH, also expressed low levels of TLR2 (Fig. 1B and C). TLR2-positive mast cells were also observed in human lung tissue (data not shown). The staining pattern for TLR2 was consistent with surface expression on mast cells. These data demonstrate that human mast cells express a unique profile of TLRs including TLR2 and TLR6, which may suggest a specialized role for in host defense. The lack of TLR4 expression provides an additional marker to distinguish human mast cells from other myeloid cells.

Selective stimulation of CBMC with TLR2 activators

The responses of human mast cells to a number of classic TLR activators were examined. In initial experiments, CBMC (>95% pure) were stimulated with various TLR activators including: *S. aureus* PGN or *E. coli* LPS. When stimulated with the TLR2 activator *S. aureus* PGN (Schwandner *et al.*, 1999a; Yoshimura *et al.*, 1999) for 24 h, CBMC produced significant levels of GM-CSF (Fig. 2A) and IL-1 β (Fig. 2B) in a dose-dependent manner. Levels of GM-CSF were 17- to 85-fold higher than unstimulated control cells when CBMC were stimulated with 10 μ g/ml PGN ($n = 15$ CBMC donors). PGN induced substantially higher levels of GM-CSF than IgE-mediated activation (PGN, $5075 \pm 1260\%$ medium control $n = 4$ and IgE mediated, $350 \pm 191\%$ med control $n = 4$). These cytokines were not detected in sonicated CBMC samples or in the supernatant following 30-min activation with PGN, suggesting that CBMC do not contain or release substantial preformed stores (data not shown). Levels of GM-CSF remained low after a 6-h incubation (41 ± 4.9 pg/ml, $n = 2$) and increased in a time-dependent manner with 430 ± 39 pg/ml ($n = 2$) GM-CSF produced following a 12-h incubation. CBMC from six donors did not show a significant IL-6 response to PGN when considered as a group, although cells from some individual donors exhibited a marginal response. In contrast, production of RANTES (CCL5) was consistently enhanced following activation with PGN (mean, 542 ± 128 pg/ml following treatment with 10 μ g/ml PGN compared with 163 ± 52 pg/ml unstimulated controls, $n = 4$). Using conditions shown to be effective for TLR-mediated activation of murine bone marrow-derived mast cells (McCurdy *et al.*, 2001), CBMC were activated with a high dose of the known TLR4 activator *E. coli* LPS (5 μ g/ml). LPS treatment did not significantly increase GM-CSF (Fig. 2C) or IL-1 β (Fig. 2D) production.

The levels of cytokines produced following LPS challenge did not exceed a 2-fold increase compared with unstimulated control cells ($n > 4$; Fig. 2, C and D).

The selective responsiveness of CBMC to the TLR2 activator (PGN) but not to the TLR4 activator *E. coli* LPS is consistent with the TLR expression pattern of human mast cells (TLR2 but not TLR4). These findings are notably different from the rodent system whereby bone marrow-derived mast cells were shown to express mRNA for both TLR2 and TLR4 and to respond to both PGN and LPS (McCurdy *et al.*, 2001; Supajatura *et al.*, 2001). These results suggest that studies of murine mast cell responses to pathogen products may not be good predictors of human mast cell function.

Differential CBMC mediator production by TLR2 activators

CBMC were activated with either the putative TLR2/TLR6 activators, PGN, and zymosan or the putative TLR2/TLR1 activator Pam₃Cys and analyzed for the short-term release of classical preformed mediators, cytokine production, and cysteinyl leukotriene generation. Stimulation of CBMC for 24 h with zymosan resulted in the production of significant levels of GM-CSF (Fig. 3A) and IL- β (Fig. 3B) in a dose-dependent manner. Significant cytokine production was observed with doses of zymosan as low as 0.01% (w/v) in all CBMC donors examined, with GM-CSF production at least 15-fold higher than in unstimulated control cells ($n = 4$). This profile of cytokines is consistent with that produced by CBMC following activation with PGN. Moreover, the levels of GM-CSF and IL-1 β produced by CBMC were similar to those produced by freshly isolated peripheral blood leukocytes treated with these activators (data not shown). Stimulation of CBMC with Pam₃Cys resulted in a marginal increase in GM-CSF (Fig. 3C) and IL-1 β

(Fig. 3D) at the highest dose of Pam₃Cys examined (100 µg/ml). CBMC were analyzed for cysteinyl leukotriene production after stimulation for 20 min (optimal) with TLR activators. The TLR2/TLR6 activators PGN and zymosan were potent inducers of cysteinyl leukotriene production whereas Pam₃Cys was ineffective (Fig. 4, B–E). Stimulation with either PGN or zymosan resulted in a dose-dependent increase in cysteinyl leukotrienes. The levels of cysteinyl leukotriene generated were similar to other stimuli in human mast cells, including IgE-dependent activation, and were almost equivalent to parallel calcium ionophore (A23187)-induced responses of CBMC. CBMC were also analyzed for β-hexosaminidase release as a marker of mast cell degranulation. Both PGN and zymosan failed to induce significant levels of CBMC degranulation (Fig. 4, A and C; *n* = 4). High doses of PGN may have marginally enhanced mast cell degranulation (≈2-fold greater than spontaneous release, *n* = 5); however, these levels failed to reach statistical significance. In contrast, Pam₃Cys induced significant levels of degranulation in a dose-dependent manner (Fig. 4E). CBMC degranulation in response to Pam₃Cys was evident at concentrations as low as 10 µg/ml.

Taken together, these data suggest that TLR2 is capable of eliciting distinct mediator responses in human mast cells. This may be occurring either through the use of discrete TLR heterodimers (Takeuchi *et al.*, 2002; Ozinsky *et al.*, 2000) or via alternate signaling pathways. Additional receptors have been identified for zymosan including dectin-1 (Brown *et al.*, 2001). Further studies are required to determine whether human mast cells express dectin-1 and whether this molecule functions in cooperation with various TLRs.

It is well established that known TLR activators, such as LPS, can induce production of PGs such as PGE₂ via NFκB-dependent cyclooxygenase 2 generation. However, leukotriene production by human mast cells in response to TLR2 activators has not been previously described. Current models for TLR-mediated signaling do not include a clear mechanism by which leukotriene generation or degranulation would be initiated. A potential mechanism for leukotriene production may involve Rac-1-dependent activation of phosphatidylinositol 3-kinase which has recently been proposed as an important pathway in TLR2 signaling (Aribe *et al.*, 2000). The low yield of CBMC from individual donors and lack of GM-CSF and IL-1β responses to TLR activators in mast cell lines limits the ability to directly address these issues in this system.

Mast cells are well recognized as an important source of leukotrienes in allergic disease. In a number of animal models and disease situations, it has been suggested that local leukotriene generation is dysregulated or may be enhanced by pathogen products under circumstances where there is minimal evidence of mast cell degranulation. LTC₄ and its breakdown products are known to be critical for the bronchoconstriction, edema, and mucous secretion observed in atopic asthma and to be a potent chemoattractant for a number of inflammatory cells as well as dendritic cells. The ability of human mast cells to produce cysteinyl leukotrienes in a degranulation-independent manner in response to TLR activators opens up new possibilities for the mechanisms by which infection exacerbates allergic asthma. The long-term production of RANTES (CCL5), GM-CSF, and IL-1β by mast cells in response to PGN and zymosan may further enhance host responses to pathogen infection and contribute to the chronicity of inflammatory disease at mast cell-rich sites such as the skin and lung.

Figure 1. Human mast cell TLR expression. *A*, RT-PCR analysis of human mast cell TLRs. The reverse transcription step was omitted in some samples (Δ RT) as a control where primers did not span an intron. RNA isolated from the human macrophage cell line U937 was used as a positive control. Note the strong expression of TLR1 and TLR6 and lack of TLR4. *B–D*, Immunohistochemical analysis of human mast cell TLR2 expression. Alcian blue-stained nasal polyp frozen tissue sections were further stained with anti-TLR2 Ab (note red color) (*B* and *C*), or normal goat IgG (*D*). The data shown are representative of three independent experiments for RT-PCR and two independent experiments for immunohistochemistry.

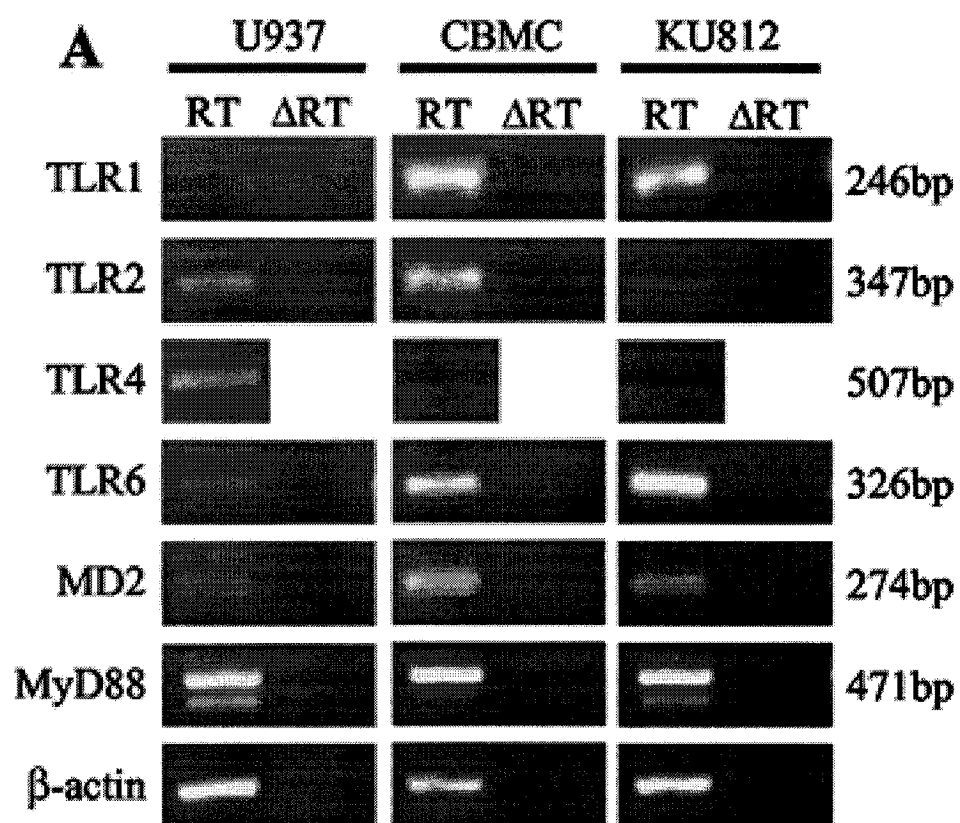


Figure 1A

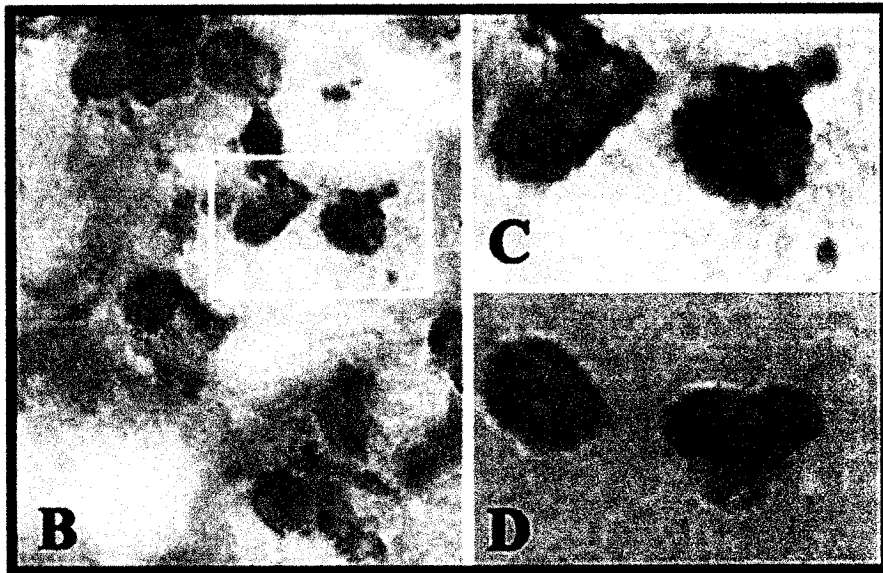


Figure 1B-D

Figure 2. GM-CSF and IL-1 β production by CBMC in response to TLR activators. CBMC (>95% pure) were stimulated with *S. aureus* PGN (*A* and *B*) or *E. coli* LPS (*C* and *D*) for 24 h. Cell-free supernatants were analyzed for GM-CSF (*A* and *C*) or IL-1 β (*B* and *D*) by ELISA. **, $p < 0.01$ compared with medium control. Constitutive cytokine production varied between donors; therefore, representative experiments from CBMC from separate donors are presented. Experiments were conducted in triplicate and expressed as mean values \pm SD. Results are representative of those obtained from six separate donors.

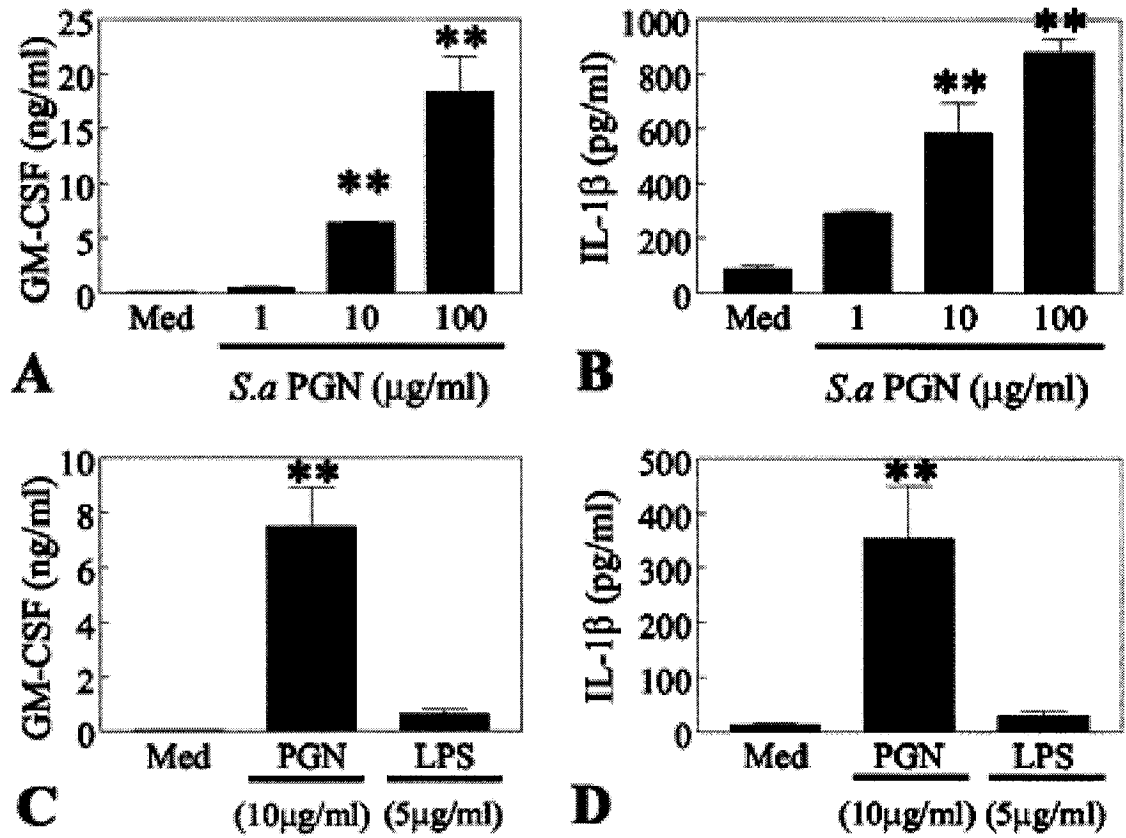


Figure 2

Figure 3. GM-CSF and IL-1 β production by CBMC in response to TLR activators. CBMC (>95% pure) were stimulated with zymosan (*A* and *B*) or Pam₃Cys (*C* and *D*) for 24 h. Cell-free supernatants were analyzed for GM-CSF (*A* and *C*) or IL-1 β (*B* and *D*) by ELISA. **, $p < 0.01$ compared with medium control. Experiments were conducted in triplicate and expressed as mean values \pm SD. Results are representative of those obtained from five donors for zymosan activation and three donors for Pam₃Cys.

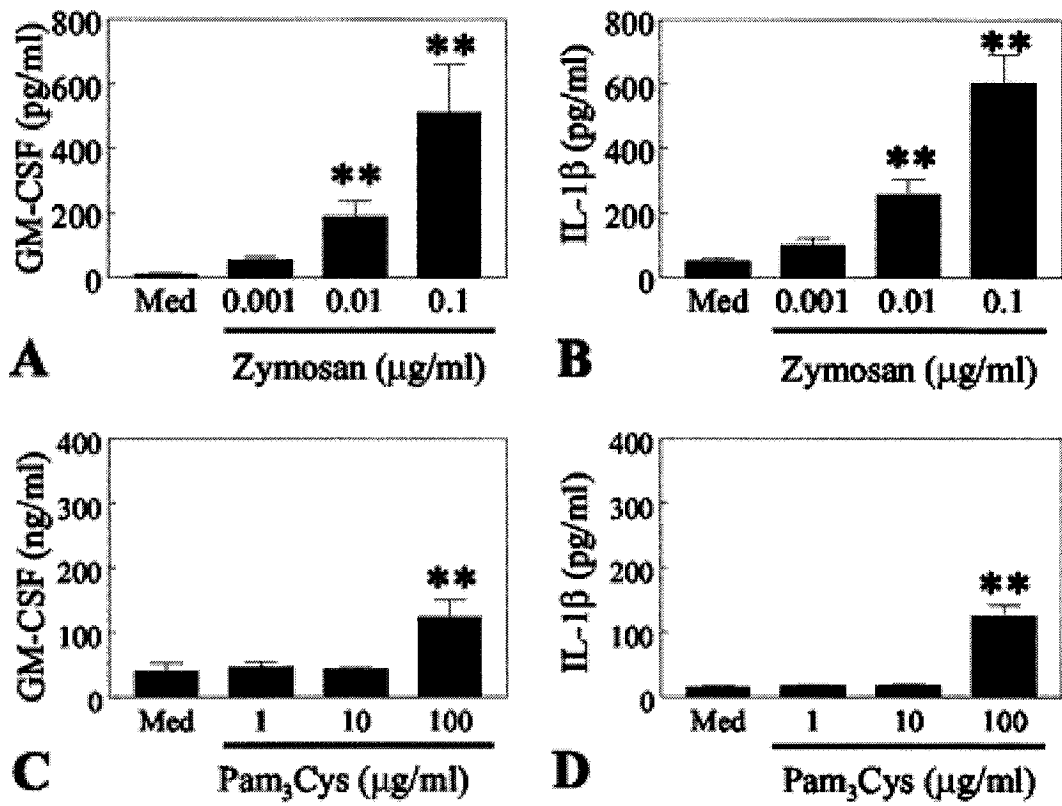


Figure 3

Figure 4. Short-term cysteinyl leukotriene generation and β -hexosaminidase release by CBMC in response to TLR2 activators. CBMC were stimulated for 20 min with *S. aureus* PGN (*A* and *B*), zymosan (*C* and *D*), or Pam₃Cys (*E* and *F*) for 24 h. Cell-free supernatants were assayed for cysteinyl leukotriene generation (*B*, *D*, and *F*) or β -hexosaminidase release (*A*, *C*, and *E*). Calcium ionophore A23187 (A23) was used at an optimal dose of 0.5 μ M. **, $p < 0.001$ compared with medium control. The data presented are the mean values \pm SEM from experiments using CBMC from five separate donors.

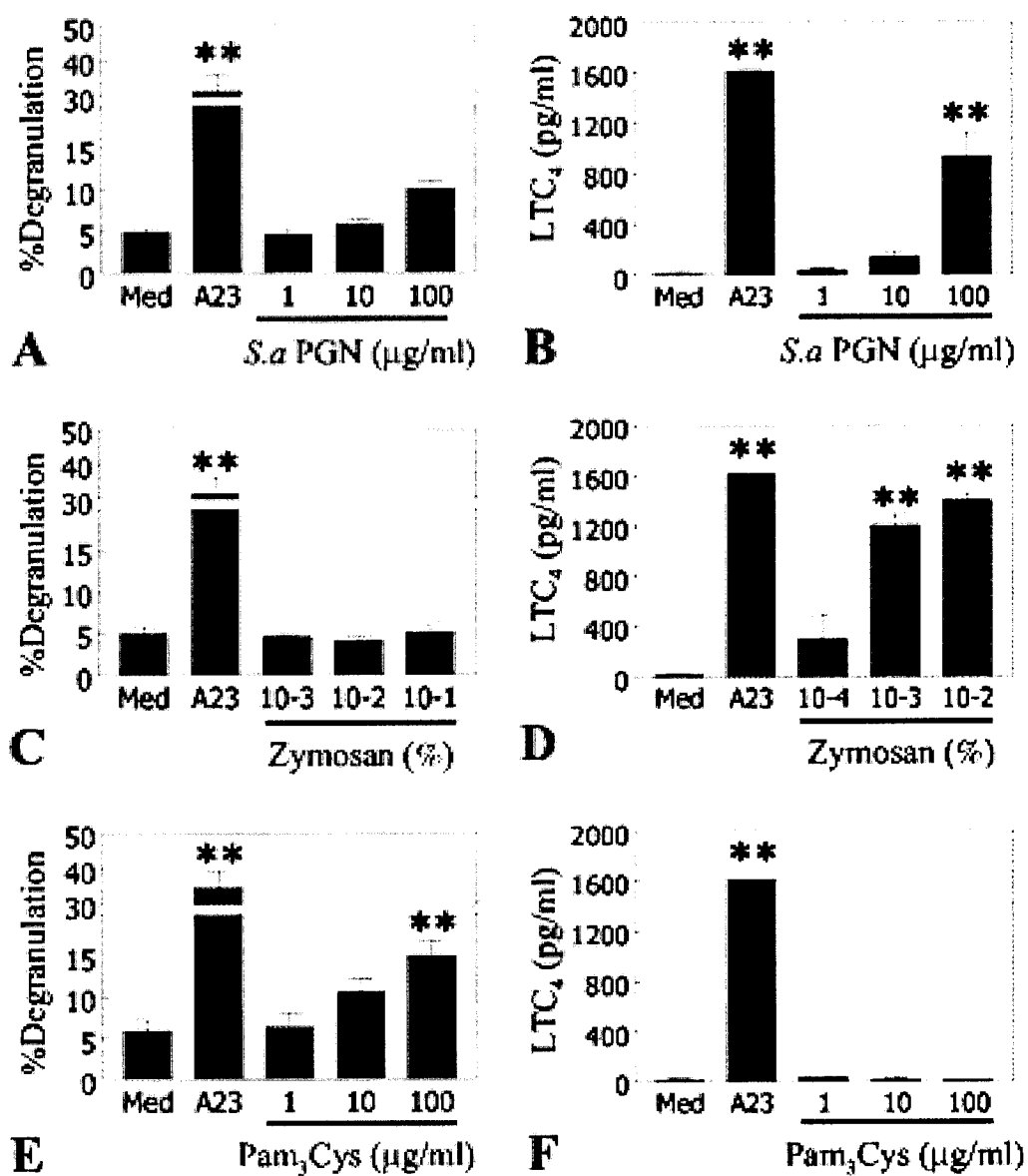


Figure 4

Chapter 5

**PGN induces apoptosis and enhances FcεRI-mediated
GM-CSF and IL-1β production by human mast cells**

5.1 Introduction

Mast cells are recognized as sentinel cells due to their involvement in a broad range of immunological processes and their ability to elicit selective mediator responses (Leal-Burmen *et al.*, 1995) to an extensive range of stimuli (Galli *et al.*, 1999; Lin *et al.*, 2000; (Marshall *et al.*, 2003). Mast cell responses to environmental allergens are closely associated with allergic diseases while mast cell responses to certain bacterial infections are involved in host defense (Holgate, 1991; Etchancher *et al.*, 1996; Malaviya *et al.*, 1996). Interestingly, a number of lines of evidence suggest that pathogen products might also lead to exacerbations of allergic diseases. Airway hyperactivity among asthmatics is significantly increased after LPS inhalation (Michel *et al.*, 1989) and respiratory viruses are a major trigger of acute exacerbation of asthma (Corne and Holgate, 1997; Johnston, 1997). Moreover, *Staphylococcus aureus* infections colonize more than 90% of patients with atopic dermatitis and are believed to contribute to the severity of the disorder (Damsgaard *et al.*, 1997; Mihm *et al.*, 1976). These observations suggest that although a protective role for mast cells against certain types of bacterial infections has been established (Etenchatcher *et al.*, 1996; of Malaviya *et al.*, 1996; Maurer *et al.*, 1997), in other situations, mast cell activation by pathogen products might lead to exacerbation of allergic diseases.

Pathogen recognition is mediated, largely by pattern recognition receptors (PRRs) such as the Toll-like receptor (TLR) family which have been shown to activate the production of NF κ B regulated pro-inflammatory cytokines in response to pathogen associated molecular patterns (PAMPs) (Akira *et al.*, 2001; Janeway and Medzhitov, 2002; Qureshi and Medzhitov, 2003). Peptidoglycan (PGN) from *Staphylococcus aureus*,

bacterial lipoproteins and lipopolysaccharide (LPS) from gram-negative bacteria are well-defined examples of PAMPs and are known to activate TLR2/TLR6 heterodimers, TLR1/TLR2 heterodimers and TLR4/TLR4 homodimers respectively (Bulut *et al.*, 2001; Hajjar *et al.*, 2001; Ozinsky *et al.*, 2000a; Ozinsky *et al.*, 2000b). CBMC constitutively express TLR1, TLR2 and TLR6 and produce a range of mediators in response to PGN, zymosan and the synthetic lipoprotein, Pam₃CSK₄ (McCurdy *et al.*, 2003). Of particular interest, PGN and zymosan induce an early leukotriene C₄ response and sustained cytokine responses including IL-1 β and GM-CSF as well as the Th2-associated cytokines, IL-5, IL-10 and IL-13 from CBMC (McCurdy *et al.*, 2003; Varadaradjalou *et al.*, 2003).

TLR2 activation has recently been shown to also induce an apoptotic pathway in some cell types (Aliprantis *et al.*, 1999; Lopez *et al.*, 2003). Bacterial lipoprotein activation lead to the recruitment of the Fas-associated death domain protein (FADD) to the TLR2 receptor complex by MyD88, a TLR adaptor molecule (Aliprantis *et al.*, 2000). Down stream signaling events were dependant on a classical caspase dependent pathway since apoptosis could be significantly blocked using selective caspase-8 and caspase-3 inhibitors. (Lopez *et al.*, 2003). The ability of bacterial products to induce apoptosis may represent an important regulatory mechanism to prevent excessive inflammation by bacterial infections.

In view of the involvement of mast cells in allergic disorders and host defense, factors their numbers in local tissues are of considerable importance. Changes in mast cell numbers are likely dependent on the balance between signals that promote survival or recruitment and those that induce apoptosis or emigration. Survival signals are

regulated by growth factors such as IL-3 in the mouse (Lantz *et al.*, 1998; Nabel *et al.*, 1981; Tertian *et al.*, 1981) and stem cell factor (SCF) in both humans and mouse (Galli *et al.*, 1999) or by signaling through FcεRI (Asai *et al.*, 2001; Xiang *et al.*, 2001). In contrast, mast cell apoptosis can be induced by the withdrawal of such growth factors, corticosteroids and potentially through the action of cytokines (Piliponsky and Levi-Schaffer, 2000).

The ability of classic TLR activators to regulate mast cell apoptosis has not been formally examined. Moreover, the interplay between TLR- and FcεRI-mediated activation of mast cells with respect to mediator production and cell survival has also not been addressed. In the current study we determined that selective TLR2 activators are capable of inducing markers of apoptosis in mast cells and that FcεRI activation significantly enhanced human mast cell responses to PGN.

5.2 Results

FcεRI-mediated activation enhances human mast cell responses to PGN

We have previously shown that the TLR2 activator, *Staphylococcus aureus* PGN can up-regulate IL-1 β and GM-CSF production by CBMC (McCurdy *et al.*, 2003). Here we investigated if FcεRI-mediated activation could further enhance CBMC cytokine production by PGN. To address this, CBMC were initially sensitized with human IgE for 48 hrs. Previous studies have determined that this sensitization time results in the up-regulation of FcεRI and is required for optimal responses to IgE-mediated activation (Hsu and MacGlashan, 1996; Lantz *et al.*, 1997; MacGlashan *et al.*, 1998; Yamaguchi *et al.*, 1997; Yamaguchi *et al.*, 1999). Sensitized CBMC were then treated simultaneously with anti-IgE and a sub-optimal dose of PGN (10 μ g/ml). Cell free supernatants were harvested after 24 hr and analyzed for GM-CSF and IL-1 β production by ELISA.

PGN induced significant levels of GM-CSF and IL-1 β production from CBMC as previously reported (McCurdy *et al.*, 2003). IgE-mediated activation also induced substantial levels of GM-CSF production compared to IgE sensitized cells treated with the purified rabbit IgG control (3.57 \pm 1.78 fold increase, n=4) (Figure 1). Together, anti-IgE and PGN induced a substantial increase in the levels of GM-CSF in four separate experiments compared to either of the stimuli alone or PGN together with purified IgG as a control (Figure 1). Interestingly, IgE-mediated activation alone did not consistently induce a significant increase in the levels of IL-1 β production. However, when treated with anti-IgE in combination with PGN, CBMC produced substantially greater levels of IL-1 β than when treated with PGN alone (Figure 2). The increased levels of GM-CSF

and IL-1 β by combined PGN and anti-IgE mediated activation were observed in all experiments conducted.

Previous studies have shown that IgE-activation can protect mast cells from apoptosis (Asai *et al.*, 2001; Xiang *et al.*, 2001). Moreover, TLR2 activation by bacterial lipoproteins has previously been shown to induce apoptosis (Aliprantis *et al.*, 1999; Lopez *et al.*, 2003). Therefore a potential explanation for the findings was that, IgE-activation protects mast cells from TLR2-mediated apoptosis resulting in enhanced cytokine production.

Treatment of CBMC with PGN induces markers of CBMC apoptosis

To examine the potential induction of apoptosis by bacterial products we initially used PGN as a model system. A hallmark feature of apoptosis is the induction of the caspase cascade which can be regulated by bacterial products (Thornberry and Lazebnik, 1998). Therefore, a fluorescent pan-caspase detector, FAM-VAD-fmk (Caspatag), which binds to the active sites of caspase 1, 3, 4 and 7 was used to measure caspase activation. When CBMC were activated with 50 $\mu\text{g/ml}$ PGN for 24 hr a significant increase in apoptosis compared to unstimulated control cells was observed (Figure 3). The levels of apoptosis increased ≈ 3 fold. Moreover, the levels of apoptosis induced by PGN were comparable to the levels obtained when cells were activated with the positive control apoptosis inducer camptothecin (Figure 3). A lower dose of PGN (5 $\mu\text{g/ml}$) also consistently induced higher levels of apoptosis than untreated control cells.

Previous studies have demonstrated that a caspase-8 inhibitor could significantly, but not completely inhibit TLR2 mediated apoptosis (Aliprantis *et al.*, 2000; López *et al.*, 2003). This suggests that signaling pathways other than a classical caspase cascade may

be involved in TLR2-mediated apoptosis. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) are an early apoptotic event and have been shown, in some cases, to function independently of the classical caspase cascade (Kroemer *et al.*, 1997; Green *et al.*, 1998; Green and Kroemer, 1998). Therefore, we investigated if PGN could induce this pathway. CBMC were treated with various concentrations of PGN for 24 hours after which time the cells were subjected to DiOC₆. In healthy cells, DiOC₆ rapidly associates with the negatively charged environment of the mitochondrial matrix. Following apoptosis, a dissipation in the mitochondrial potential occurs and results in the leakage of DiOC₆ which can be readily detected by flow cytometry as a decrease in fluorescence intensity (DiOC₆-negative cells) (Kroemer *et al.*, 1997; Zoratti and Szabo, 1995). When CBMC were treated with 50 $\mu\text{g/ml}$ PGN a significant increase in the number of DiOC₆-negative cells was observed compared to the unstimulated control cells (Figure 4). The percentage of DiOC₆-negative cells in the PGN treated group were comparable to the levels observed with the positive control camptothecin. Moreover, treatment of CBMC with a lower dose of PGN (5 $\mu\text{g/ml}$) also induced a substantial increase in the numbers of DiOC₆-negative cells.

Differential effects of a broad-spectrum caspase inhibitor on CBMC GM-CSF production induced by distinct TLR2 activators

To determine if apoptosis of mast cells is preventing maximal cytokine responses, we pre-treated CBMC with a broad-spectrum caspase inhibitor and examined GM-CSF production. When CBMC were pre-treated with z-VAD-FMK and later challenged with PGN, slight increases in the levels of GM-CSF were observed in four out of six

experiments compared to CBMC without the caspase inhibitor (Table 1). Despite this slight trend, the increases in cytokine production in the presence of the caspase inhibitor were not statistically significant ($P>0.3$). To confirm that z-VAD-fmk was bioactive we measured IL-1 β production, since z-VAD-fmk is able to inhibit caspase-1, which is required for processing pro-IL-1 β into its mature secreted form. Consistent with this, z-VAD-fmk significantly abrogated CBMC IL-1 β production (data not shown).

Interestingly, in some cell preparations the caspase inhibitor appeared to have a greater effect on CBMC GM-CSF production in response to other TLR2 activators including Pam₃CSK₄ or zymosan (Figure 4), although there was substantial donor variability. In cells from one donor, z-VAD-FMK strongly enhanced Pam₃CSK₄ and zymosan induced CBMC GM-CSF production (Figure 5A). Interestingly, z-VAD-FMK appeared to have a greater effect on the lower dose of zymosan compared to the higher dose. Using cells from a second donor however, z-VAD-FMK only marginally enhanced Pam₃CSK₄ and zymosan induced CBMC GM-CSF production (Figure 5B). Moreover, in this experiment z-VAD-fmk did not appear to have a greater effect on the lower dose of zymosan.

Figure 1: FcεRI-mediated activation enhances human mast cell GM-CSF production in responses to PGN. IgE-sensitized CBMC from four separate donors were stimulated with 17.5 μg/ml rabbit anti-IgE (αIgE), 17.5 μg/ml purified rabbit IgG (IgG) as a control or 10 μg/ml *S. aureus* PGN. As a negative control CBMC were incubated with medium alone (Med). CBMC were also activated simultaneously with 10 μg/ml PGN and 17.5 μg/ml anti-IgE or 17.5 μg/ml purified rabbit IgG. After 24 hr, cell-free supernatants were analyzed for GM-CSF production by ELISA. Experiments were conducted in triplicate and expressed as mean ± SD.

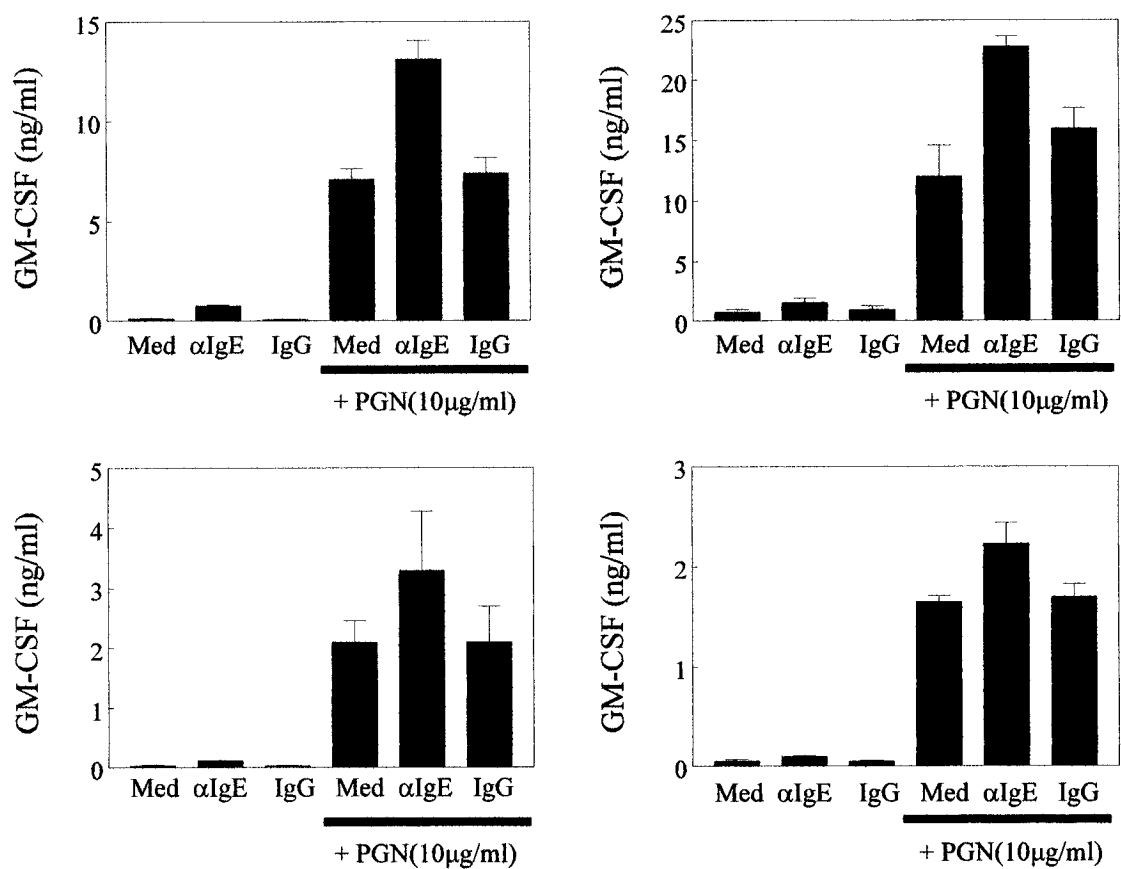


Figure 1

Figure 2: FcεRI-mediated activation enhances human mast cell IL-1β production in responses to PGN. IgE-sensitized CBMC from four separate donors were stimulated with 17.5 μg/ml rabbit anti-IgE (αIgE), 17.5 μg/ml purified rabbit IgG (IgG) as a control or 10 μg/ml *S. aureus* PGN. As a negative control CBMC were incubated with medium alone (Med). CBMC were also activated simultaneously with 10 μg/ml PGN and 17.5 μg/ml anti-IgE or 17.5 μg/ml purified rabbit IgG (IgG). After 24 hr, cell-free supernatants were analyzed for IL-1β production by ELISA. Experiments were conducted in triplicate and expressed as mean ± SD.

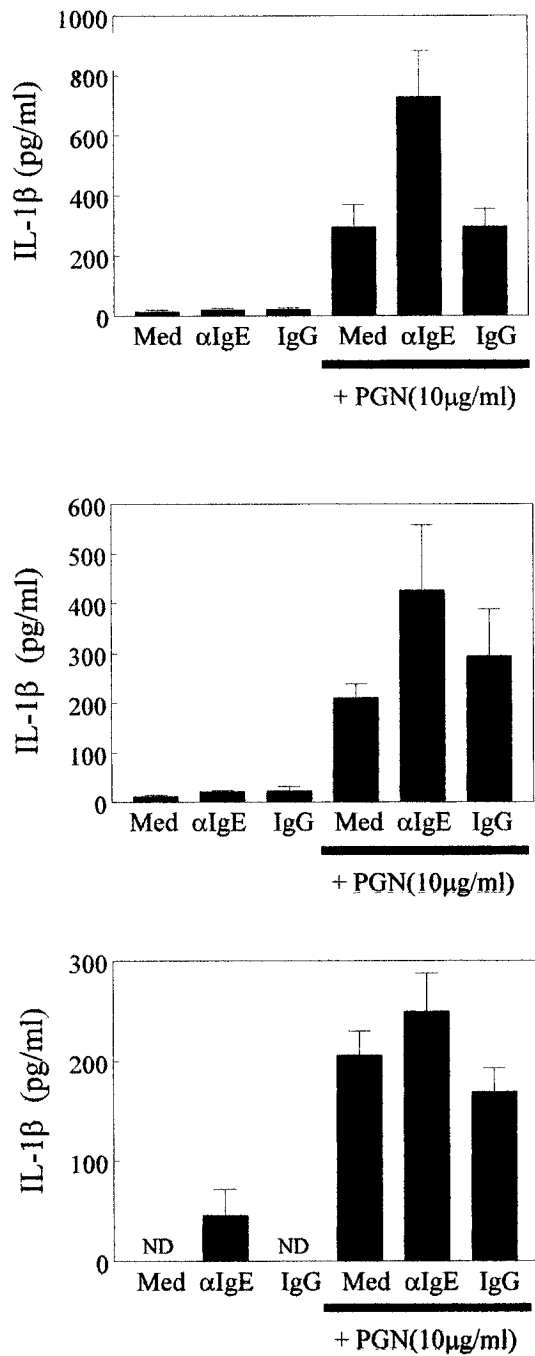


Figure 2

Figure 3: Treatment of CBMC with *S. aureus* PGN induces caspase activation. CBMC (0.5×10^6 /ml) were treated for 24 hr with medium alone (med) as a negative control (A and C) or with *S. aureus* PGN (B and C). Cells were also treated with 5 μ g/ml camptothecin (Camp) as a positive control. To measure caspase activation, cells were stained with FAM-VAD-FMK and visualized by flow cytometry. Increases in caspase activation were detected as forward shifts in fluorescence. Gates were set according to the unstimulated control cells and the % Caspatag positive cells were quantified. Data are presented as representative histograms (A and B) and as quantified data (C) comprised of the mean \pm SEM from at least three independent experiments. * and ** denote significance $p < 0.05$ and $p < 0.005$ respectively.

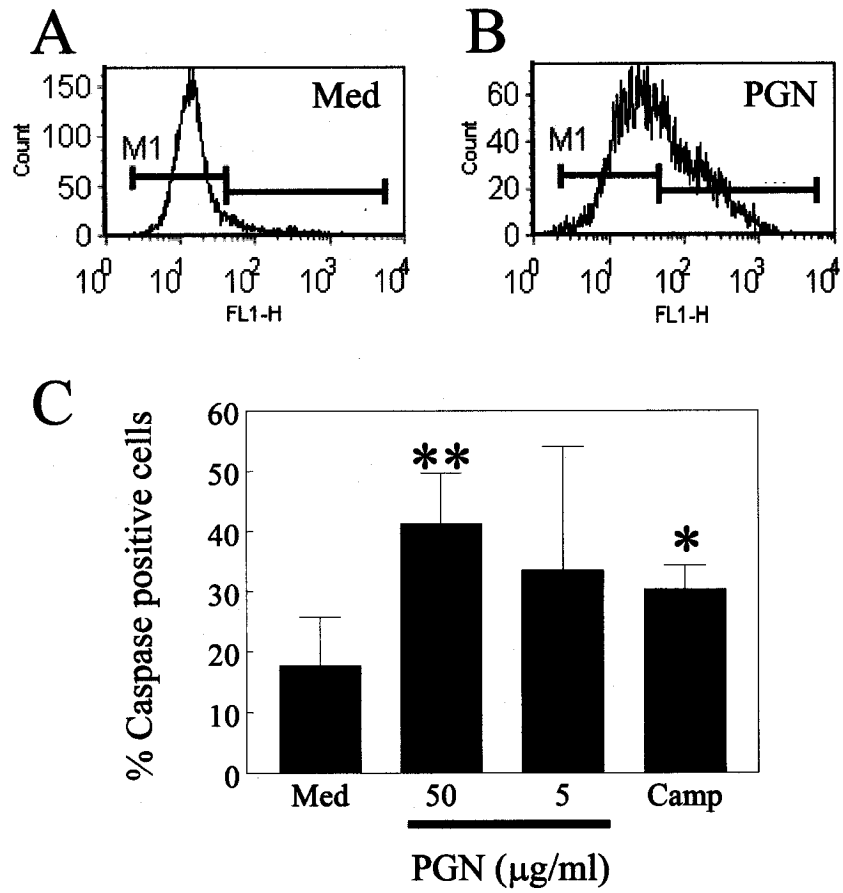


Figure 3

Figure 4: Treatment of CBMC with *S. aureus* PGN results in a loss of mitochondrial membrane potential. CBMC (0.5×10^6 /ml) were treated for 24 hr with medium alone (med) as a negative control (A and C) or with *S. aureus* PGN (B and C). 5 μ g/ml camptothecin (Camp) was used as a positive control (C). 40 mM DiOC₆ was used to assess a loss of mitochondrial membrane potential and was monitored by flow cytometry as a decrease in fluorescence (DiOC₆-negative cells). Gates were set according to the unstimulated control cells and the % DiOC₆-negative cells were quantified. Data are presented as representative histograms (A and B) and as quantified data (C) comprising the mean \pm SEM from five independent experiments. * and ** denote significance $p < 0.05$ and $p < 0.005$ respectively.

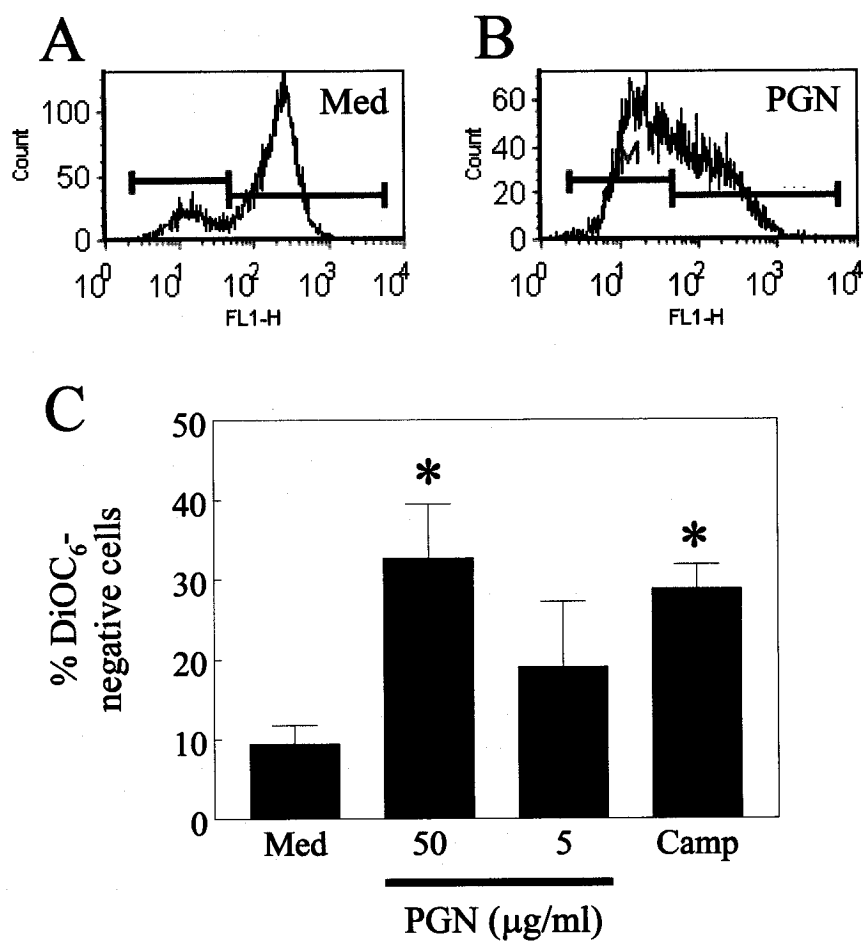


Figure 4

Table 1: The broad spectrum caspase inhibitor, z-VAD-fmk does not significantly modulate PGN-mediated CBMC GM-CSF* production

CBMC Donor	No Inhibitor		Caspase Inhibitor	
	Med [#]	PGN ^{\$}	Med [#]	PGN ^{\$}
1	140 ± 33	4927 ± 485	137 ± 270	6164 ± 1377
2	53 ± 42	2482 ± 637	77 ± 58	3369 ± 1235
3	104 ± 29	2534 ± 1359	67 ± 12	4381 ± 1715
4	5.3 ± 18	1927 ± 219	17.8 ± 4.9	1585 ± 517
5	3.3 ± 1.76	307 ± 249	13.6 ± 5.8	661 ± 456
6	26.9 ± 11	6323 ± 672	23 ± 8.2	5193 ± 419

* GM-CSF production (pg/ml)

Unstimulated cells (Med)

\$ 10 µg/ml *S. aureus* PGN

Figure 5: Treatment with a broad-spectrum caspase inhibitor enhances CBMC GM-CSF production in response to Pam₃CSK₄ and zymosan. CBMC (>95% purity) were pre-treated for 30 min with (black bars) and without (hatched bars) 5μM of the broad-spectrum caspase inhibitor z-VAD-fmk. CBMC were subsequently activated with Pam₃CSK₄ or zymosan. After 24 hr, cell-free supernatants were analyzed for GM-CSF production by ELISA. (A) and (B) represent experiments using CBMC from separate donors. The experiments were conducted in triplicate and expressed as mean ± SD.

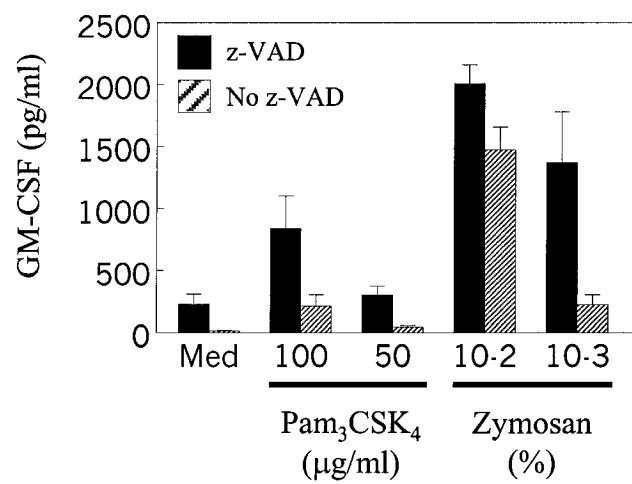
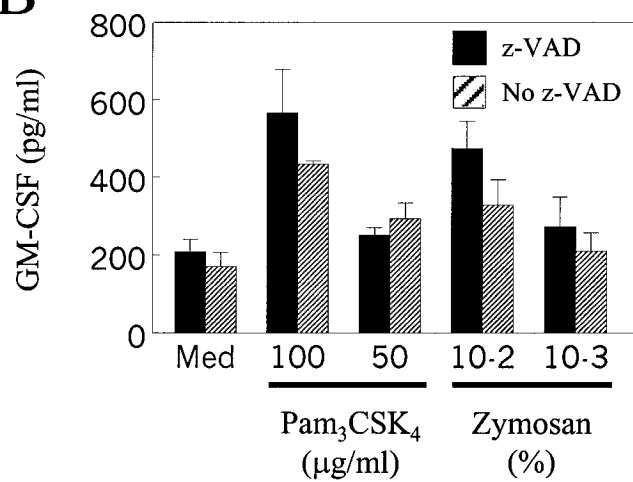
A**B**

Figure 5

5.3 Discussion

Mast cell responses to *S. aureus* PGN are of particular interest because of the association of *S. aureus* infections with atopic dermatitis. Although *S. aureus* infections are believed to contribute to the severity of this disorder the mechanism is not well understood (Damsgaard *et al.*, 1997; Mihm *et al.*, 1976). Here we report that IgE-mediated activation enhances CBMC cytokine responses to the TLR2 activator *S. aureus* PGN. This may contribute to the pathogen-associated exacerbations of allergic disorders. Since IgE-mediated activation is known to protect against mast cell apoptosis caused by growth factor withdrawal or death receptor activation (Finotto *et al.*, 1997; Mekori *et al.*, 1993; Yoshikawa *et al.*, 2000), we were interested in determining if a survival mechanism was responsible for the enhanced cytokine responses. In order to address this, we first examined if PGN could induce CBMC apoptosis.

Apoptosis is regulated through caspase- and/or mitochondrial-dependent mechanisms (Green and Kroemer, 1998; Thornberry and Lazebnik, 1998). Both of these pathways can be induced through distinct mechanisms or can be regulated by each other as in the case of the mitochondrial pathway inducing caspase-3 (Kuwana *et al.*, 1998) or mature caspases initiating the mitochondrial pathway (Susin *et al.*, 1997; Marzo *et al.*, 1998). In this study, PGN induced both caspase activation and dissipation in mitochondrial membrane potential. The ability of PGN to induce caspase activation is consistent with previous studies, which determined that, Pam₃CSK₄ and purified bacterial lipoproteins, additional TLR2 activators, could induce apoptosis in monocytes through a caspase-dependent mechanism (Aliprantis *et al.*, 2000; Lopez *et al.*, 2003). Interestingly, the ability of PGN to also induce changes in mitochondrial membrane potential may

reflect differences between these activators. However, it is not clear if PGN directly induces each pathway or if one is induced as a consequence of the other. Moreover, the relative importance of each pathway in our system has not been addressed. Further studies using specific caspase inhibitors and downstream markers of apoptosis such as DNA fragmentation will help resolve these issues. For example, if a caspase-8 inhibitor strongly inhibits apoptosis while a caspase-9 inhibitor has little or no effect it would provide strong evidence for a classic caspase-dependent mechanism.

We reasoned that if IgE-mediated activation could enhance PGN-mediated cytokine production by preventing apoptosis, then the broad-spectrum caspase inhibitor, z-VAD-fmk should also enhance CBMC cytokine production. However, z-VAD-fmk only marginally enhanced CBMC GM-CSF production in four out of six experiments. In light of this, it is unlikely that the enhanced cytokine responses were due exclusively to an IgE-dependent anti-apoptotic mechanism, although we cannot rule out the possibility that a caspase-independent pathway of apoptosis is involved. Experiments investigating if IgE-activation can directly prevent PGN-mediated apoptosis will begin to clarify this issue.

From these experiments we do not have any formal evidence implicating a survival mechanism for the enhanced CBMC cytokine responses to PGN and anti-IgE mediated activation. As a result, future experiments will also examine other mechanisms such as the ability of PGN and anti-IgE-mediated activation to synergistically enhance transcription factors such as NF κ B.

Preliminary experiments suggested that z-VAD-fmk might have a more pronounced effect on modulating CBMC cytokine production in response to Pam₃CSK₄

and zymosan in cells from some donors. Although experimental variability and a lack of sufficient n have prevented us from drawing strong conclusions, these preliminary findings may suggest that Pam₃CSK₄ and zymosan are stronger activators of apoptosis. We have recently attempted to examine if zymosan induces CBMC apoptosis. However, zymosan particle interference in our flow cytometric readouts from apoptotic cells has hindered us from generating reliable data.

The apparent disparity in CBMC sensitivity to z-VAD-fmk following activation with each of the TLR2 activators and different doses of TLR2 activators may reflect differences in the ability of these activators to disrupt the balance between pro- and anti-apoptotic signals. Anti-apoptotic signals are often regulated through the transcription factors NFκB and JNK (Beg and Baltimore, 1996), which can be activated by TLR agonists (O'Neill, 2002). Therefore, the degree of NFκB and JNK activation by each of the TLR agonists at the doses employed is likely to impact the degree of apoptosis and affect the sensitivity to the apoptosis inhibitor.

The ability of mast cells to undergo apoptosis following PGN treatment might represent an important mechanism for preventing excessive inflammation. We predict that during a bacterial infection, the mast cell would initially mobilize host defense mechanisms through the production of pro-inflammatory mediators and then would undergo apoptosis to help turn off the immune response. In support of this model, CBMC are able to produce substantial levels of IL-1β, GM-CSF and LTC₄ despite undergoing apoptosis. Preliminary experiments demonstrated that CBMC begin to produce IL-1β and GM-CSF around three hours and the levels of these cytokines increased over a 24 hr

period. (Appendix, figure 1). Further experiments are required to determine if the levels of these cytokines reach a plateau and if this correlates with the kinetics of apoptosis.

Inappropriate or excessive mast cell mediator responses are a hallmark feature of allergic disorders. Therefore the ability of PGN to enhance IgE-mediated mast cell cytokine production may represent a mechanism for pathogen-induced exacerbations of allergic diseases. GM-CSF is an important factor for the survival and mobilization of eosinophils while IL-1 β has multiple pro-inflammatory effects. Although our findings suggest that PGN can also mediate apoptosis, future experiments are required to determine if this process is inhibited during IgE-dependent allergic disorders. Disruption of apoptosis might block a natural mechanism for preventing excessive inflammation. Therefore, the potential anti-apoptotic effect of IgE-mediated activation is of particular interest in the context of chronic allergic diseases. This work may identify novel targets for designing therapeutic strategies to regulate mast cell function, particularly in the context of chronic allergic diseases.

CHAPTER 6

GENERAL DISCUSSION

6.1 Discussion

The role of mast cells in host defense has been a subject of interest for more than 30 years. Initial studies provided evidence that mast cells might play a protective role against certain types of parasitic infections (Nawa *et al.*, 1985; Oku *et al.*, 1984). More recently, the mast cell has been demonstrated to respond directly to bacterial products such as LPS (Leal-Berumen *et al.*, 1994) and implicated as an integral component of host defenses against bacterial infections (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996). A number of elaborate studies provided substantial evidence that mast cell-derived mediators such as TNF are critical for protection against certain types of gram-negative bacterial infections (Echtenacher *et al.*, 1995; Malaviya *et al.*, 1996a). Despite these groundbreaking findings, the mechanism of mast cell-bacteria interactions remained poorly understood. A number of studies demonstrated that host proteins such as complement products and antibody could facilitate mast cell-bacterial interactions (reviewed by: Marshall *et al.*, 2003). Specifically the role of classic PRRs as a mechanism for mast cell activation remained poorly defined. The groundbreaking work from a number of groups who identified and characterized TLRs opened up new opportunities to study the potential mechanism of mast cell responses to pathogens. Therefore we hypothesized that mast cells express a number of these receptors and that they are important for selective mast cell responses to pathogen products.

6.1 Summary of the major findings

We identified that murine BMDC express mRNA for TLR2, TLR4 and TLR6 but not TLR5 while human CBMC express mRNA for TLR1, TLR2 and TLR6 but not TLR4

(summarized in table 1). Moreover, mast cells from both species express mRNA for the adaptor molecule MD-2 and the signaling molecule MyD88.

Consistent with the mRNA expression data, murine BMMC but not human CBMC produced significant levels of NF κ B-regulated cytokines in response to LPS. Functional TLR4 expression was required for BMMC IL-6 and TNF production in response to LPS since BMMC from wild type but not TLR4-deficient mice (C3H/HeJ and C57Bl/10ScCr) responded to LPS challenge. In keeping with the mRNA expression data both human and murine mast cells responded to the TLR2 activator PGN. Moreover, two additional TLR2 activators, zymosan and Pam₃CSK₄ also activated CBMC. Interestingly, each of the TLR2 activators induced GM-CSF and IL-1 β production from CBMC while also inducing moderate levels of apoptosis. Finally, we demonstrated that CBMC activation by PGN and zymosan induce significant levels of LTC₄ generation without modulating mast cell degranulation, while Pam₃CSK₄ selectively induced CBMC degranulation without inducing LTC₄ generation. These findings are of particular interest because PGN and zymosan are known to utilise a distinct TLR receptor dimer from Pam₃CSK₄. These data along with those from other groups, who have examined TLR expression and function in mast cells, are summarized in table 2 and 3.

Table 1: TLR expression by human and murine mast cells

TLR	Human		Murine	
	CBMC	KU812	BMMC	MC/9
TLR1	(+) m	(+) m	(ND)	(ND)
TLR2	(+) m, w & I	(+) m	(+) m	(+) m
TLR3	(ND)	(ND)	(ND)	(ND)
TLR4	(-) * m & w	(-)	(+) m	(+) m
TLR5	(ND)	(ND)	(-) m	(-) m
TLR6	(+) m	(+) m	(ND)	(ND)
TLR8	(ND)	(ND)	(+) m	(ND)
MD2	(+) m	(+) m	(+) m	(+) m
MyD88	(+) m	(+) m	(+) m	(+) m

m = mRNA

f= flow cytometry

w = western blot

I = immunohistochemistry

CBMC = Cord blood derived mast cells

BMMC = Bone marrow derived mast cells

* Undetectable baseline expression but can be upregulated with IL-4 treatment

ND: not determined

Table 2: BMBC responses to TLR agonists

Agonist	IL-1 β	IL-5	IL-6	IL-10	IL-13	TNF α	GM-CSF	LTC ₄	β -Hex
LPS	(+)	(-)	(+/-)	(+)	(+)	(+)	(-)	(ND)	(-)
PGN	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(ND)	(+/-)

Table 3: CBMC responses to TLR agonists

Agonist	IL-1 β	IL-5	IL-6	IL-10	IL-13	TNF α	GM-CSF	LTC ₄	β -Hex
LPS	(-)	(ND)	(-)	(ND)	(ND)	(-)	(-)	(-)	(-)
LPS *	(+)	(+)	(ND)	(+)	(+)	(+)	(ND)	(ND)	(-)
PamsCSK4	(+)	(ND)	(ND)	(ND)	(ND)	(ND)	(+)	(-)	(+)
Zymosan	(+)	(ND)	(ND)	(ND)	(ND)	(ND)	(+)	(+)	(-)
PGN	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+/-)

(+/-) conflicting data from different groups

* LPS activation of CBMC primed with IL-4 for five days

ND: not determined

6.2 Changes in TLR expression by growth factors or cytokine exposure

Several recent studies demonstrated that human mast cells are capable of expressing TLR4. Varadaradjalou *et al.*, (2003) recently demonstrated that human CBMC express TLR4 by RT-PCR and Western blot analysis and Okumura *et al.*, (personal communication) demonstrated that human peripheral blood derived mast cells (PBMC) express TLR4 by real time PCR. Although these data initially appeared to contradict our findings, it is likely that distinct culture conditions are responsible for the difference. Varadaradjalou *et al.*, primed CBMC with rhIL-4 for five days while Okumura *et al.*, treated their cells with IFN γ prior to analyzing TLR4 expression and function. Both IL-4 and IFN γ have been shown to up-regulate the expression of TLR4 expression and enhance cellular responses to LPS (Faure *et al.*, 2001; Mita *et al.*, 2002; Mita *et al.*, 2001). Interestingly, CBMC and PBMC cytokine responses to *E. coli* LPS treatment were only induced when these cells were primed with rhIL-4 and IFN γ respectively and consistent with our findings no significant responses were observed in the absence of these treatments (Varadaradjalou *et al.*, 2003). Collectively, these data suggest that CBMC express very low or undetectable levels of TLR4 constitutively and require specific cytokines or growth factors to up-regulate their expression.

The apparent inducible nature of human mast cell TLR4 suggests that other factors might also regulate expression. Previous reports, using other cell systems, have shown that TLR4 can be up-regulated in ischemic tissues and in inflammatory disorders such as inflammatory bowel disease or by pro-inflammatory mediators such as histamine and certain cytokines (Cario *et al.*, 2000; Wolfs *et al.*, 2002). It will be interesting to determine if TLR expression is modulated in allergic disorders where mast cells are

known to play a central role and pathogen infections have been shown to play an exacerbating role.

Collectively, these findings point towards a model where human mast cells constitutively lack TLR4 but may up-regulate expression under appropriate signals from the local microenvironment. A lack of constitutive TLR4 expression or the requirement for high doses of LPS for mast cell activation, as observed in mast cells from the human and mouse respectively, may be an evolutionary advantage to prevent exaggerated cytokine responses to the normal microbial flora of the host. This coupled with the fact that high numbers of mast cells are positioned at contact sites with the external environment such as the lungs, skin and gastrointestinal tract suggest that mast cell responses to TLR activators may be closely regulated to prevent constant, unnecessary inflammatory reactions. Other cell types may share this strategy such as intestinal epithelial cells, which constitutively express low or undetectable levels of TLR2 and TLR4, but can rapidly up-regulate their expression under appropriate conditions (reviewed by: Gewirtz, 2003).

6.3 Mechanisms for mast cell mediator production in response to TLR activators

6.3.1 Cytokine production

The major cytokines analyzed in these studies were IL-6 and TNF in the mouse and IL-1 β and GM-CSF in the human. Although the mechanism involved in the TLR-mediated production of these NF κ B-regulated cytokines has not been formally investigated in the mast cell, other cell types provide important insights (reviewed by: O'Neill, 2002).

Recently, it was demonstrated that stimulation of murine mast cells by agonists of TLR2 and TLR4 result in the activation of NF κ B suggesting that some pathways might be conserved in mast cells (Supajatura *et al.*, 2001 and Supajatura *et al.*, 2002). TLRs also induce the activation of various MAPK such JNKs, ERKs and p38 (Hambelton *et al.*, 1995; Hambelton *et al.*, 1996; Ham *et al.*, 1994) and these are known to play a role in the regulation of these cytokines (Swantek *et al.*, 1997; Lee *et al.*, 1994; (Scherle *et al.*, 1998). Masuda *et al.*, (2002) recently demonstrated that ERK1/2, JNK and p38 were activated in BMMC following stimulation with LPS. Moreover, inhibitors of JNK and p38 significantly abrogated BMMC cytokine production.

The need for much greater amounts of certain TLR activators, such as LPS, to activate mast cells compared with monocytes, macrophages and other cell types suggests differences in receptor or co-receptor usage or cell-specific regulatory events. Moreover, high concentrations of other TLR activators such as CpG-containing oligonucleotides are also required to induce BMMC cytokine production (Zhu and Marshall, 2001), suggesting that common signaling components might be lacking or expressed in insufficient quantities to induce maximal cytokine responses. Therefore, further studies are required to examine the precise signaling events leading to NF κ B and MAPK activation by these TLR activators.

6.3.2 LTC₄

We have reported, for the first time, that TLR activators can directly induce mast cell LTC₄ production. Moreover, human CBMC are a particularly potent source of this mediator when stimulated with the TLR2 ligands PGN or zymosan. Although a number

of studies have examined the intracellular signaling pathways induced by TLRs (reviewed by O'Neill, 2002), it is not clear how TLR activation would result in LTC₄ generation. It is generally accepted that LTC₄ production requires the activation of calcium dependant phospholipase A₂ (cPLA₂) and 5-lipoxygenase (5-LO) through Ca²⁺-dependent signals and through phosphorylation events (Leff, 2001). Recently, Supajatura *et al.*, (2002) demonstrated that PGN could induce a calcium flux in murine BMMC. However, the mechanism regulating this calcium signal is also currently unknown. It is likely that PI3 kinase is involved. In macrophages, PI3 kinase is rapidly recruited and associates with the intracellular region of TLR2 (Arbibe *et al.*, 2000). Moreover, PI3K is known to induce a calcium flux by liberating IP₃ from lipid membranes. Phosphorylation by protein tyrosine kinases such as p38 are also believed to play a role in the activation of 5-LO and cPLA₂ and it also has been shown that TLR agonists can activate this MAP kinases. Therefore, further studies using specific inhibitors of PI3K and p38 will help to clarify their role in the induction of LTC₄ by TLR2 activators.

6.3.3 Degranulation

Several forms of mast cell degranulation have been reported (reviewed by Logan *et al.*, 2003). Classical and compound degranulation occur rapidly and involve the fusion of secretory granules with the plasma membrane resulting in the release of granule contents into the external environment. Classical degranulation is characterized by individual secretory granules fusing with the plasma membrane, while compound degranulation involves multiple intracellular granule-granule fusion events prior to secretion by a single fusion pore (Moqbel *et al.*, 1999; Sceppek *et al.*, 1994). A slower form of degranulation

known as piecemeal degranulation may occur and involves vesicular transport of select pre-formed mediators from secretory granules to the plasma membrane (Dvorak *et al.*, 1975; Dvorak, 1998). Finally, necrosis has also been associated with the release of granules and coincides with cell death (Erjefalt *et al.*, 1998).

In our experiments, Pam₃CSK₄ selectively induced CBMC degranulation within 20 min suggesting that piece meal and necrotic mechanisms of degranulation were not involved. Therefore it is likely that classical or compound degranulation account for these findings. Additional experiments using control lipopeptides, either lacking the NH₂-acyl groups or the COOH-peptide will verify the specific nature of the response to Pam₃CSK₄ and help to confirm that compound toxicity is not responsible for or contributing to the apparent degranulation response.

Exocytosis of secretory granules is a highly regulated process, involving translocation and tethering of granules to the cell membrane, followed by a fusion event and subsequent release of granules into the external environment (reviewed by Logan *et al.*, 2003). Although Ca²⁺ signals and PKC (Logan *et al.*, 2003) are known to regulate these events, the mechanism by which TLR activation induces these regulatory factors has not been established. Therefore, further studies are needed to examine the TLR adaptor molecule(s) and the precise signaling pathways involved. The Rac1/PI3 kinase complex is a likely candidate since it has been shown to associate with TLR2 and is known to induce IP3 and PKC (Arbibe *et al.*, 2000).

In the human CBMC system, we demonstrated that PGN induces only a slight increase in CBMC degranulation which was not statistically significant (\approx 2-fold increase compared to medium control, n=3). Although it appears that PGN does not induce

classical or compound degranulation, we cannot rule out the possibility of piecemeal degranulation. Morphological analysis by electron microscopy has traditionally been used to examine piecemeal degranulation (Dvorak *et al.*, 1975; Dvorak, 1998). Therefore, this approach would be appropriate to determine if PGN and zymosan are able to induce CBMC piecemeal degranulation.

Interestingly, a recent study by Varadaradjalou *et al.*, (2003) demonstrated that PGN could induce CBMC degranulation. Although this study measured histamine instead of β -hexosaminidase, which was assayed in our experiments, both are common mast cell granule markers and are released with similar kinetics (Schwartz *et al.*, 1987). Moreover, both are routinely used to measure mast cell degranulation making it unlikely that the method of detection is responsible for the differences in these studies. A more glaring discrepancy is the differences in culture conditions. Varadaradjalou *et al.*, (2003) only observed substantial levels of mast cell degranulation when mast cells were primed with IL-4. This is consistent with other studies demonstrating an enhanced CBMC degranulation response when cells were primed with IL-4 for five days (Lantz *et al.*, 1997).

In the murine system Supajatura and colleagues (2002) demonstrated that PGN could induce BMDC degranulation. They also demonstrated that intradermal injection of PGN induced an acute cutaneous response within 5 min. Despite using similar protocols, we were unable to replicate their findings (data not shown). Although minor differences in BMDC culture and activation conditions could potentially explain the discrepancies in the *in vitro* data, the protocols used for the *in vivo* skin reactions were virtually identical.

6.3.4 Apoptosis

Apoptosis is induced primarily through a caspase- or a mitochondrial-dependent mechanism (reviewed by: Green and Kroeger, 1998). Previous studies in other cell systems demonstrated that TLR2 activation by purified bacterial lipoproteins and Pam₃CSK₄ induce apoptosis by the caspase pathway (Aliprantis *et al.*, 1999; Lopez *et al.*, 2003;). In these studies it was demonstrated that the Fas-associated death domain protein (FADD) was recruited to the TLR2 receptor complex by the intracellular adaptor molecule MyD88 and resulted in the activation of caspase-8 and subsequently caspase-3 (Aliprantis *et al.*, 2000). In our studies, PGN induced caspase activation and a loss of mitochondrial membrane permeability suggesting the potential involvement of the caspase and mitochondrial pathways. Further experiments are required to determine if both pathways are induced directly by PGN or if one pathway is induced as a consequence of the other. In some situations, when insufficient levels of caspase-8 are induced, caspase-8 is still able to induce apoptosis through activation of the mitochondrial pathway (reviewed by Green and Reed, 1998). Future studies using specific caspase inhibitors will help to clarify these issues.

6.4 Differential mediator production by TLR2 activators: a working model

Our understanding of the innate immune system has changed considerably over the past few years. The identification of an entire family of pattern recognition receptors suggests that our innate immune system is much more complex than initially thought. Furthermore, the ability of the TLR family to form different combinations of heterodimers allows for an even greater degree of flexibility. Recent literature has

investigated whether this increased complexity translates into different cellular responses (Hirschfeld *et al.*, 2001; Re and Strominger, 2001; Eisenbart *et al.*, 2002). These reports identified qualitative differences between TLR2 and TLR4 activators. Notably, TLR4 but not TLR2 induced the interferon-regulated pathway resulting in the production of IFN β as well as IP-10 (CXCL10). Here we demonstrated that PGN and zymosan induce potent LTC₄ production without significantly modulating mast cell degranulation while Pam₃CSK₄ induced substantial degranulation without inducing LTC₄ generation. Based on these findings we propose a model whereby activation of a TLR2/TLR6 heterodimer induces substantial levels of LTC₄ without modulating mast cell degranulation and the TLR1/TLR2 heterodimer induces mast cell degranulation without inducing LTC₄ generation. These findings demonstrate for the first time that subtle differences in TLR heterodimers may result in unique cellular responses. Moreover, these results also suggest that leukotriene generation can occur in the absence of mast cell degranulation.

A possible explanation for these observations may involve the recruitment of distinct intracellular adapter molecules to each of the receptor heterodimers. A number of TIR-containing adaptor molecules have been identified including MyD88, TIRAP/MAL and TICAM-1/TRIF (Medizhotou *et al.*, 2001; Fitzgerald *et al.*, 2001; Oshiumi *et al.*, 2002; Akira *et al.*, 2003). MyD88 and TIRAP/Mal have been shown to play a role in both TLR1/TLR2 and TLR2/TLR6 signaling pathways while TRIF/TICAM-1 is not believed to participate in either of these pathways (Oshiumi *et al.*, 2003; Yamamoto *et al.*, 2002b). Therefore, these adapter molecules are unlikely to account for our findings. The Rac1-PI3K complex which does not contain a classic TIR domain, has been shown to selectively associate with TLR2 in response to heat killed *S. aureus* (Arbibe *et al.*, 2000).

It is likely that heat killed *S. aureus* is a TLR2/TLR6 activator because of the high content of PGN in the outer membrane of this bacterium. Therefore, an attractive model would involve the selective recruitment of the Rac1-PI3K complex to the TLR2/TLR6 heterodimer but not to the TLR1/TLR2 heterodimer. This would explain why PGN and zymosan selectively induce LTC₄ production.

Although, the proposed mechanism appears attractive, there are two major issues that must be considered. First, if the Rac1-PI3K complex induces LT production by generating calcium signals than why is this Ca²⁺ signal not inducing some detectable level of mast cell degranulation? Second, if the Rac1-PI3K complex is not involved in the TLR1/TLR2 pathway than how does Pam₃CSK₄ induce mast cell degranulation? As a result of these confounding issues an alternate model might better explain our findings.

It is possible that additional receptor systems function, in concert with TLRs to induce different classes of mast cell mediators or indeed that some of the responses we are examining in the human system are TLR-independent. The ability PGN and zymosan to activate multiple receptors is particularly relevant because of the complex nature of these activators. PGN consists of chains of repeating disaccharide units composed of N-acetylglucosamine β-1-4 linked to N-acetylmuramic acid while zymosan, a cell wall preparation from *S. cerevisiae* and other yeast species, is composed primarily of β-glucans, mannans and chitin (Brown and Gordon, 2001; Gantner *et al.*, 2003). As a result, it is not surprising that TLR-independent receptor systems including members of the NOD family and dectin-1 have also been shown to bind PGN and zymosan respectively (Hugot *et al.*, 2001; Girardin *et al.*, 2003; Inohara *et al.*, 2003; Brown and Brown 2001; Brown, Herre *et al.* 2003). It is unlikely however, that members of the NOD family are

involved in "early" LT generation since they are intracellular molecules and would require time for their ligands to become internalized (Hugot *et al.*, 2001; Girardin *et al.*, 2003; Inohara *et al.*, 2003). Dectin-1 on the other hand is a transmembrane protein from the C-type lectin family and therefore represents a potential candidate to modulate early LT production (Brown and Brown 2001; Garnet *et al.*, 2003).

6.5 What is the *in vivo* significance of mast cells and TLRs?

6.5.1 Role for mast cells in host defense against bacterial infections

Echtanacher *et al.*, (1996) and Malaviya *et al.*, (1996) initially demonstrated a protective role for mast cells against bacterial infections. These studies also demonstrated that mast cells have the ability to induce neutrophil recruitment through the production of pro-inflammatory mediators such as TNF. Our findings suggest that mast cells can directly respond to bacterial challenge through the use of TLRs. Moreover, elaborate studies by Supajatura and colleagues (2001 and 2002), using mast cell deficient mice (W/W^v) reconstituted with various TLR-deficient BMDC or wild type BMDC have formally demonstrated the importance of TLR4 *in vivo*. Using this model it was determined that mast cells required the expression of TLR4 to effectively protect against acute septic peritonitis induced by cecal ligation and puncture. Moreover, our *in vitro* findings demonstrate that LPS induces pro-inflammatory mediators, such as TNF, from BMDC in a TLR4-dependent manner. Collectively, these studies suggest that TLR4 activation of murine mast cells results in the production of pro-inflammatory cytokines and subsequent neutrophil recruitment for effective host defense. Interestingly, W/W^v mice reconstituted

with TLR4^{-/-} BMDC had a slightly improved rate of survival as compared to unconstituted W/W^v mice (Supajatura *et al.*, 2001; Supajatura *et al.*, 2002). These findings suggest that alternate mechanisms of mast cell-bacterial interactions, potentially involving other TLRs, complement receptors and/or CD48 are important for host defense in mice (reviewed by: Marshall *et al.*, 2003).

Although these studies clearly demonstrate an important role for rodent mast cells and TLR4, they do not necessarily mean that human mast cells will function in a similar manner. This issue requires careful consideration since human mast cells may lack constitutive TLR4 expression. The extent to which human mast cells express TLRs in the context of acute inflammation as associated with early infections remains to be determined. However, the apparent constitutive expression of other TLRs such as TLR1, TLR2 and TLR6 by mast cells suggests that these cells could be more important in gram-positive infections. Future studies of *ex vivo* human mast cells at sites of infection may be particularly important to validate current *in vitro* studies.

6.5.2 Emerging model for role of mast cells in host defense

The protective role of mast cells is not restricted to anti-bacterial responses. A large number of studies identified a role for these cells in host defense against certain types of parasitic infections (reviewed by Else and Finkelman, 1998). Moreover, work from our laboratory has demonstrated that mast cells are responsive to yeast products and dengue virus suggesting a potential role in anti-fungal and anti-viral infections. Interestingly, unique profiles of mast cell mediators are induced by different types of pathogens. PGN and zymosan induce LTC₄, IL-1 β and GM-CSF without substantial amounts of IL-6. In

contrast, dengue virus infection induces significant levels of IL-1 β and IL-6 but not GM-CSF (King *et al.*, 2000). Moreover, dengue virus infection also induces various chemokines such as RANTES (CCL5), MIP-1 α (CCL3) and MIP-1 β (CCL4) but not IL-8 (CXCL8) or ENA-78 (CXCL-5).

From these studies, a picture is emerging which suggests that mast cells have the ability to generate selective responses to different classes of pathogens. In addition to the mast cells' apparent ability to distinguish between broad groups of pathogens such as virus, yeast and bacteria, our findings and those from others (Supajatura *et al.*, 2001; Supajatura *et al.*, 2002) suggest that mast cells can also discriminate more subtle differences between different subtypes of bacteria. Further studies *in vivo* are required to determine if mast cells can mobilize specific types of defense mechanisms depending on the type of infection and if these mechanisms allow for more efficient clearance of pathogen.

Most of these studies examining the role of TLRs in mast cell activation have focused on bacterial products as activators. Therefore, it is of interest to determine if mast cells use similar mechanisms to respond to parasitic and viral challenge. Studies have identified that mast cells are activated directly by parasite-derived antigens suggesting that classic PRRs may indeed be involved (Jarrett *et al.*, 1982; Nutmann *et al.*, 1993). Moreover, studies in other cell systems determined that TLRs are important for cellular responses to certain types of viral infections such as RSV and CMV (Compton *et al.*, 2003; Kurt-Jones *et al.*, 2000a; Rassa *et al.*, 2002). The use of BMDC derived from MyD88^{-/-} mice and individual TLR blocking antibodies will help identify if mast cells require TLRs to respond to these additional types of pathogens.

6.5.3 Potential role of mast cell/TLR interactions in allergic disorders

The ability of mast cells to produce LTC₄ in response to pathogen products has important implications in the ability of pathogens to exacerbate allergic disorders. Similar to histamine, LTC₄ is a potent bronchoconstrictor and has strong vascular inflammatory actions (McMillan, 2001). LTC₄ is also involved in eosinophil recruitment (McMillan, 2001). Collectively, these processes contribute to the early phase of asthmatic reactions (McMillan, 2001). Although LTC₄ has a slower onset than histamine its effects are longer in duration and 100-1000 X more potent (Leff, 2001). Therefore, mast cell LTC₄ generation by PGN and zymosan might contribute to allergic diseases of the airways. Rhinovirus and respiratory syncytial virus (RSV) are classic examples of pathogens that exacerbate allergic airway inflammation. Interestingly, RSV has also been shown to activate a TLR-dependent pathway, suggesting the possibility that these pathogens might also directly induce mast cell LTC₄ generation.

Atopic dermatitis is also of particular interest because more than 90% of patients with this allergic disorder have *S. aureus* infections in their skin lesions while only 5% of normal individuals harbor this organism (Hauser *et al.*, 1985). Evidence for a role of *S. aureus* in atopic dermatitis is supported by the observation that treatment of individuals with anti-staphylococcal antibiotics and topical corticosteroids offer greater clinical benefit than corticosteroids alone (reviewed by: Leung, 1999). Despite these observations, the mechanisms by which *S. aureus* infections exacerbate and/or maintain skin inflammation is not well understood. Studies suggested the involvement of *S. aureus* superantigens through classic FcεRI-crosslinking since more than 50% of patients with

atopic dermatitis make IgE antibodies directed against *S. aureus* superantigens (Leung *et al.*, 1993; Nissen *et al.*, 1997). Moreover, the potential effects of protein A mediated cross-linking of receptor bound antibody must also be considered.

Here we demonstrated that *S. aureus* PGN directly activates CBMC resulting in the rapid generation of LTC₄ and the prolonged production of GM-CSF and IL-1 β . LTC₄ has potent effects on the vasculature and is chemotactic for eosinophils, both of which are associated with atopic dermatitis. IL-1 β is best known for its pro-inflammatory capacity while GM-CSF is known for its ability to modulate granulocyte effector function (Hamilton, 2002). Therefore, it is possible that mast cell derived LTC₄ contributes to the acute and chronic stages of atopic dermatitis while GM-CSF and IL-1 β primarily add to the chronicity of the disorder. We have also demonstrated that IgE-mediated activation enhances mast cell GM-CSF and IL-1 β production in response to PGN suggesting an additional mechanism where bacterial infections can exacerbate allergic disorders.

Fungal infections are also strongly associated with atopic dermatitis and are believed to exacerbate asthma (Leung, 1999). Our findings that zymosan can evoke similar mast cell responses to PGN suggests that these TLR activators may both contribute to the severity of these allergic disorders.

6.6 Major limitations of the current study

6.6.1 Lack of formal proof for TLR involvement in human mast cell responses

PGN, zymosan and Pam₃CSK₄ have all been identified as classic TLR2 activators (Kirschning and Schumann, 2002). This has been established using multiple approaches

including dominant negative constructs, TLR2-blocking antibodies and TLR2^{-/-} mice (Kirschning and Schumann, 2002). In these studies, virtually all of the cellular responses examined were dependent on the TLR2-signaling pathway. Consistent with these findings, PGN is unable to induce human or murine mast cell cytokine production when the TLR2 pathway is blocked (Supajatura *et al.*, 2002; Varadaradjalou *et al.*, 2003). Despite these convincing findings we have yet to formally demonstrate the requirement of TLR2 for mast cell degranulation and LTC₄ generation. This has proven very difficult because of a lack of adequate, commercially available TLR blocking antibodies and because of the inherent difficulty in transfecting primary cells. Although Varadaradjalou *et al.*, (2003) successfully used a commercially available TLR2 blocking antibody to inhibit CBMC cytokine production in response to PGN, we have not been able to consistently reproduce these findings (data not shown). Moreover, we were also unable to block PGN mediated cytokine responses by peripheral blood mononuclear cells as a positive control (data not shown). As a result, our laboratory is currently developing anti-human TLR2 monoclonal antibodies with the hope of acquiring better functional blocking antibodies. Preliminary data suggests that several TLR2 binding clones may have substantial blocking function (data not shown).

TLR2 requires additional TLRs, such as TLR1 or TLR6 to induce maximal mediator responses. Since these findings were concluded using other cell systems, it remains to be determined if mast cells utilize similar dimer dependent mechanisms. Currently, no commercially available TLR1 or TLR6 blocking antibodies exist making it difficult to address these issues.

6.6.2 Lack of formal proof for involvement of co-receptors

Much work remains to determine the precise cell-surface receptors required for mast cell responses to TLR activators. Although we and others (Supajatura *et al.*, 2002; Supajatura *et al.*, 2001; Varadaradjalou *et al.*, 2003) have demonstrated the requirement of TLRs for mast cell responses to various pathogen products, a number of lines of evidence suggest that additional co-receptors and accessory molecules are likely involved. First, FRET analysis has demonstrated a consistent association between between TLRs and various adaptor molecules (Triantafidou *et al.*, 2002b). Second, studies that investigated the binding between TLRs and their ligands concluded that the interaction is very weak. Finally, due to the large number of ligands for some TLRs such as TLR2 it is likely that additional co-receptors are required for increased flexibility of binding.

CD14 is one such co-receptor that is known to be involved in the TLR4-response to LPS (Haziot *et al.*, 1995; Ingalls *et al.*, 1999). Interestingly, we reported that murine BMDC do not express surface CD14, although the soluble form of this receptor would be available from the fetal bovine serum in which most of the experiments were performed. Other cell types such as endothelial cells and epithelial cells also lack surface CD14 and are dependent on serum as a source of soluble CD14 (Haziot *et al.*, 1993; Yang *et al.*, 1996). Although mast cells did not produce significant levels of cytokine in the absence of serum, we cannot rule out the possibility that other serum components such as the LPS binding protein (LBP) are involved in mast cells responses to LPS. Recently, Varadaradjalou *et al.*, (2003) demonstrated that BMDC responses to PGN are not affected by the lack of serum suggesting that PGN might use alternate co-receptor(s). Although additional molecules are known to bind PGN including members of the PGN recognition

proteins (PGRPs) and NOD family, both are restricted to intracellular compartments suggesting they are unlikely to be involved in the membrane proximal events required for ligand binding (Hugot *et al.*, 2001; Inohara and Nunez, 2003; Liu *et al.*, 2000).

Dectin-1 represents a possible candidate as a TLR co-receptor for responses to zymosan. Recent studies have determined that this molecule is expressed on the cell surface and is involved in the phagocytosis of zymosan particles (Brown and Gordon, 2001; Gantner *et al.*, 2003). Therefore, it is possible that dectin-1 aids to stabilize the interaction between TLR2/TLR6. Further experiments are needed to assess the expression and function of dectin-1 by mast cells.

It is likely that the accessory molecules have multiple functions. First it is likely they increase the binding potential of TLRs by bringing the ligand in close proximity. Second it is possible that accessory molecules provide for an increased range of activities for a relatively limited number of TLRs. Some accessory receptors such as dectin-1 are even believed to play a role in transporting ligands to intracellular compartments where they can then interact with intracellular TLRs. The complexity of these systems is becoming increasingly apparent. Further studies using various inhibitors of these accessory molecules and co-receptors are required to determine the precise mechanism of pathogen recognition by mast cells.

6.6.3 Have our conditions for IgE-mediated activation been optimized?

In these experiments, IgE-mediated activation of CBMC resulted in only moderate increases in GM-CSF production and a lack of substantial increases in IL-1 β . Moreover, despite generating substantial levels of LTC₄, IgE-mediated activation consistently did

not induce substantial CBMC degranulation assessed by β -hexosaminidase release (data not shown). These findings clearly suggest that our conditions for IgE-mediated activation have not been fully optimized. Although we incubated our CBMC with IgE for >48 hr which has been shown to up-regulate Fc ϵ RI surface expression, it is possible that the levels of Fc ϵ RI expression remained too low for efficient activation. Other groups have also primed CBMC with IL-4 for enhanced responses to IgE-mediated activation (Varadaradjalou *et al.*, 2003). Therefore, formal studies are required to determine the best culture conditions for Fc ϵ RI surface expression and for maximal mediator responses following Fc ϵ RI aggregation. These studies are of utmost importance especially when studying the ability of IgE-mediated activation to potentiate CBMC responses to PGN. Until our system is fully optimized we will not be able to draw firm conclusions regarding the interplay between PGN and IgE-mediated mechanisms of mast cell activation.

6.6.4 Lack of *in vivo* models and responses to whole bacterium

Additional confounding issues from this research are the lack of data regarding mast cell responses to whole bacteria and the lack of *in vivo* models. The central reason for not using whole bacteria was because we wanted to use a simplified system to examine various TLR activators. Whole bacteria express a large number of PAMPs and other inducers of mediator responses therefore making it difficult to analyze specific TLR-ligand interactions.

This research is also lacking an *in vivo* model to examine the importance of mast cell activation by PGN in the context of cutaneous skin reactions. However, we are

hesitant to use any murine models for such studies because of the differences between human and murine mast cells in their response to TLR activators.

6.7 Concluding remarks

Overall the data presented in this thesis provides new insights into the mechanism of pathogen recognition by mast cells. We have determined that mast cells from human and murine origin express pattern recognition receptors from the TLR superfamily which are known to play a role in mobilizing innate defense mechanisms. Our findings are consistent with this concept, in that mast cells are able to produce a number of pro-inflammatory cytokines following stimulation with TLR activators. The production of TNF by BMDC is of particular interest because of the clear role of this cytokine in mast cell dependent anti-bacterial responses (Etchtancher *et al.*, 1996; Malaviya *et al.*, 1996).

Human mast cells displayed a different profile of TLRs from murine mast cells and this was reflected by the responses of these cells to selective TLR activators. PGN and zymosan, putative activators of a TLR2/TLR6 heterodimer were of particular interest because of their ability to induce potent CBMC responses. The profile of mediators generated in response to these activators included, IL-1 β , GM-CSF and LTC₄. Pam₃CSK₄ a putative TLR1/TLR2 agonist also induced IL-1 β and GM-CSF but unlike the other TLR2 activators it induced mast cell degranulation and failed to generate LTC₄. These notable differences suggest that mast cells can generate different types of immune responses depending on the type of infection.

The profile of mast cell mediators generated in response to PGN is of considerable interest because of the potential role in the context of allergic disorders such

as atopic dermatitis. LTC_4 production is likely to have an important role in the early stages while IL-1 β and GM-CSF are like to contribute to the chronicity of the disorder. Moreover, the ability of PGN to potentiate IgE-mediated mast cell activation further lends credence for a role in exacerbating allergic disorders. Although other groups have implicated TLRs in allergic diseases (Arbour *et al.*, 2000; Blease *et al.*, 2001), further work *in vivo* is required to clarify the precise role of TLR2 mediated activation of mast cells in this context.

Our understanding of TLRs has expanded well beyond the scope of innate immunity and is now believed to participate in aspects of acquired immune responses, autoimmune diseases and inflammatory disorders. Not only does the expression of these receptors provide new insights into how mast cells might contribute to these reactions they also represent targets for novel therapeutic strategies to modulate mast cell function.

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Appendix

Figure 1: TLR2 activators induce human CBMC GM-CSF production in a time dependent manner. CBMC (> 95% pure) were stimulated with 0.01% zymosan, 10 µg/ml *S. aureus* PGN or 100 µg/ml Pam₃Cys₄ in the presence of FCS and 100 µg/ml soybean trypsin inhibitor for times ranging from 0.5-24 h. Supernatants were harvested at each time point and analyzed for GM-CSF by ELISA.

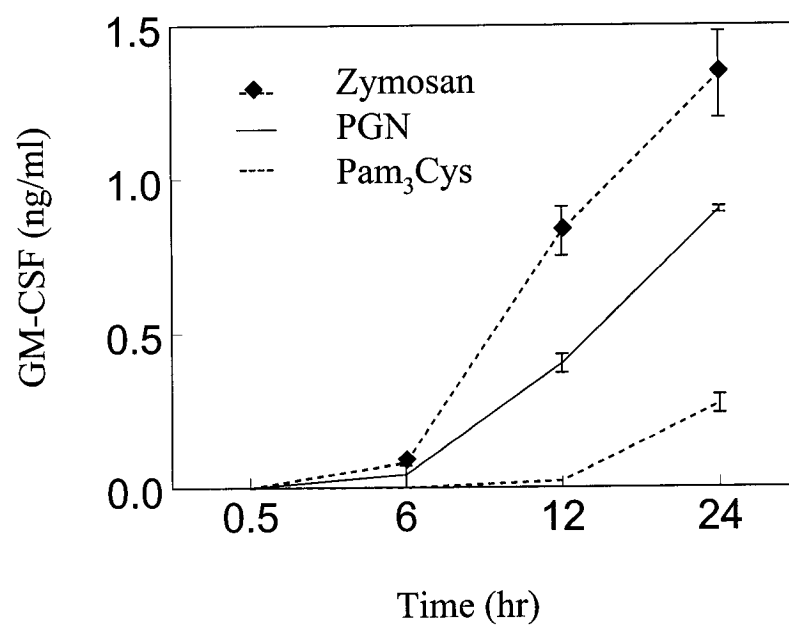


Figure 1

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J Leukoc Biol. 2001 Dec;70(6):977-84

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