ANALYSIS OF MAST CELL MEDIATED IMMUNE RESPONSE TO *LISTERIA MONOCYTOGENES*

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LIST OF ABBREVIATIONS

APCs ..... Antigen presenting cells

BMMC ..... Bone marrow mast cell

BFA ..... Brefeldin A

bFGF ..... basic fibroblast growth factor

BSA ..... Bovine serum albumin

CCR ..... CC Chemokine Receptor

CFU ..... Colony forming units

CR ..... Complement receptor

CTMC ..... Connective tissue mast cell

CXCR ..... CXC Chemokine Receptor

ECM ..... Extracellular matrix

FAK ..... Focal adhesion kinase

FcεR ..... High affinity receptor for IgE

LFA-1 ..... Lymphocyte function associated antigen-1

FSMC ..... Fetal skin mast cells

GPVI ..... Glycoprotein VI

HBSS ..... Hanks balanced salt solution

HGF ..... Hepatocyte growth factor

HGF-R ..... Hepatocyte growth factor receptor

HIV ..... Human immunodeficiency virus

HKLM ..... Heat killed *Listeria* monocytogenes

ICAM-1 ..... Intracellular adhesion molecule-1

IFN-γ ..... Interferon gamma

IL ..... Interleukin

InlA ..... Internalin

InlB ..... Internalin B

IONO ..... Ionomycin

LA ..... *Listeria* plus antibody

LAS ..... *Listeria* plus antibody and serum

LPS ..... Lipopolysaccaride

LT ..... Leukotriene

MCp ..... Mast cell progenitor

MMC ..... Mucosal Mast cell

MMP ..... Matrix metalloprotease

NFκB ..... Nueclear Factor kappa B

NK cell ..... Natural killer cell

PamCys ..... $Pam_3CysSerLys_4$

PBS ..... Phosphate buffered saline

PDGF ..... Platelet derived growth factor

PG ..... Prostiglandin

PMC ..... Peritoneal mast cell

PMN ..... Polymorphonuclear cells

SCF ..... Stem cell factor

TLR ..... Toll Like Receptor

TGF-β ..... Transforming growth factor-beta

TNF-α ..... Tumor Necrosis Factor- alpha

VLA ..... Very late antigen

VEGF ..... Vascular endothelial growth factor

WT ....Wild type

CHAPTER I

INTRODUCTION

Topic Overview

The innate immune system comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. Upon detection of infection, the first lines of host defense are quickly initiated. Cellular mediators of innate immunity migrate toward the source of the infection to produce high levels of cytokines and chemokines that send "danger signals" alerting the host to the infection (Figure 1). Cells of the innate immune system generically respond to pathogens, but unlike the adaptive immune system, it does not confer long lasting, protective immunity to the host. The cells of the innate immune system mediate the initial host-pathogen interaction through binding of pattern recognition receptors (PRRs) and opsins on the pathogen surface. Immediate changes at the site of infection include increased vascular permeability, increased expression of adhesion markers, and recruitment of leukocytes.

Innate immune cells can, in general, be divided into two groups, phagocytes and granulocytes. The phagocytes i.e., macrophages, neutrophils and dendritic cells, engulf and kill the invading pathogen by fusing the pathogen-containing endosome with an acidified lysosome containing strong oxidizing agents. Following phagocytosis, the cells process peptides for presentation on major histocompatability complex (MHC) II for recognition by CD4 T cells. The granulocytes of the innate immune system are basophils, eosinophils, mast cells as well as neutrophils. These cells respond to pathogen by

releasing mediators that are stored in their granules such as histamine, growth factors, and cytokines.

Production of cytokines and chemokines is a highly coordinated and important feature of the innate immune response. Acute phase production of TNF-α, IL-1β, IL-6, and IL-12 result in changes in temperature, blood flow, cell trafficking, and serum protein levels that are critical for survival. However, exacerbation of the acute phase response, characterized by hypotension, vascular collapse, and multiorgan dysfunction, is the cause of host injury during Gram-negative septic shock. Awareness of the pathogen and direction of innate immune cells toward the source of infection is a critical process in limiting infection. It is well known that the nature of the pathogen determines the type of cell that respond to the site of infection. Generally, bacterial infection causes infiltration of neutrophils, viral infections result in mononuclear leukocyte recruitment and helminth infections induce eosinophils and mast cell recruitment. This suggests that resident cells within the environment exposed to the pathogen dictate the specific responses to infections.

Although the innate immune system cannot respond in an antigen specific manner to a pathogen, PRRs allow innate immune cells to make general responses to a pathogen. PRRs recognize molecules that are unique to groups of related microorganisms and are not found on host cells. There are several categories of PPRs that function to recognize and respond to foreign molecules. Endocytic PPRs are found on the surface of

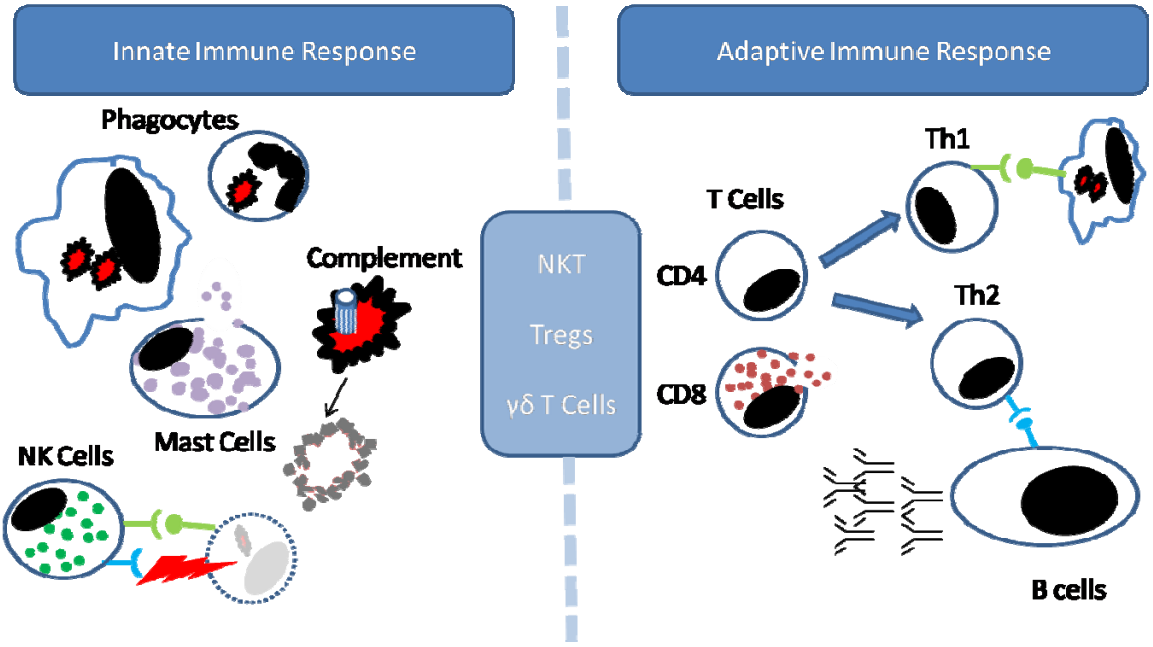

Figure 1. Cells of the immune system. The cells of the innate and adaptive immune response coordinate in order to clear pathogens.

phagocytes and promote attachment and subsequent engulfment of pathogens. Opsin receptors bind the soluble opsin molecules that coat pathogens and prepare them for phagocytosis. The toll-like receptor (TLR) family and CD14 are PPRs induce activation of downstream activation pathways resulting in production of cytokines. In addition to pattern recognition receptors, many pathogens have evolved to utilize host cell receptors, such as integrins to interact with and gain entry into cells.

Integrins

Integrins are heterodimers consisting of non- covalently associated α- and β-subunits. They mediate both cell-extracellular matrix (ECM) and cell-cell adhesion (1). In higher vertebrates, the integrin family is composed of 18 α subunits and 8 β subunits (1). Although, theoretically, they could heterodimerize to form over 100 integrins, only 24 integrins have been identified (2). At least one member of the integrin family has been found on every cell or tissue studied, and most cells express several different integrins(1). Although integrins are often segregated based on their ligand recognition, it should be noted that individual integrins can often bind to more than one ligand (2). Additionally, ligands are often recognized by more than one integrin (2). Nevertheless, distinct integrin families were defined based on ligand specificity, including the integrin collagen receptor family (2-4).

Integrins have been implicated in normal development as well as in many diseases from cancer and atherosclerosis to inflammation and innate immunity. Genetic deletion of some integrin receptor subunits, including the α4, α5 and αv subunits resulted in early embryonic lethality due to vascular and cardiac defects (5-8). Deletion of the α3 and α8

subunit genes results in perinatal lethality due to defects in organogenesis (9, 10). The β1-null animals that lack multiple αβ integrin receptor heterodimers demonstrated peri-implantation lethality (11). This embryonic lethality was predictable based on the expression of the integrin subunit during embryonic development. In other cases, the null phenotype revealed an unexpected role for the integrin, such as the roles of the collagen receptor integrins in innate immunity and inflammation (12, 13).

Integrins as Pathogen Receptors

The expression of at least one integrin receptor on nearly every cell has provided pathogens with an ideal target to hijack and gain entry into target cells and confers pathogen-specific tissue tropism (Table 1). A number of viruses use the β1 integrin family to gain entry into cells (14-20). In some incidences the viral receptor recognition motif mimics the amino acid sequence used for ligand recognition. For instance, the VP1 protein of murine polyomavirus contains a surface loop, DE with an LDV (Leu-Asp-Val) motif (20). This motif is the recognition sequence in fibronectin that allows for adhesion to the α4β1 and α4β7 integrins. Similarly, the Ross River virus E2 sequence may fold to mimic collagen and allow for binding to α1β1 integrin (19). Binding of West Nile virus to αvβ3 integrin is in part mediated by the peptide sequence RGD (Arg-Gly-Asp) since this peptide inhibited infectivity by 30-40% (21). However, elimination the αvβ3 integrin by gene silencing reduced infectivity by ~60% indicating that the αvβ3 integrin may recognize the viral receptor through another site in addition to the RGD motif (21).

Several bacterial receptors also use β1 integrins to invade host cells (22-25). The Scl1 and Scl2 proteins of group A *Streptococcus*, *S. pyogenes* form a collagen-like triple

helix and bind the α2β1 integrin (25). *Staphylococcus aureus* requires the fibronectin binding protein (FnBP) to bind heat shock protein 60, the β1 integrin and fibronectin for maximal infectivity (22, 24). The integrin not only serves as an inactive conduit for bacterial entry into cells but also is involved in pathogenicity (25, 26). FimH from type-1 piliated *Escherichia coli* utilizes α3β1 integrin to enter the cell and to activate Src and FAK (23). In fact, loss of signaling of Src and FAK diminishes the ability of *E. coli* to enter the cell indicating a role for integrin signaling in pathogen invasion (23).
The hijacking of integrins demonstrates that pathogens have evolved to access cells through this pathway. In most cases this is detrimental to the host. By mimicking host motifs, intracellular pathogens can gain entry into host cells. This may represent a selective evolution of pathogen molecules to bind integrins in order to promote immune evasion. Although pathogen recognition by integrins may lead to immune evasion, integrins also play important roles in mediating host protective responses to invading pathogens.

Leukocyte Integrins

Since immune cells are the most motile cells in the body, the regulation of adhesive interactions is integrated into the immunological process. Integrin receptors play pivotal roles in this process. The leukocyte integrins share the common β2 subunit, which is normally expressed exclusively on leukocytes and dimerizes with either the αL, αM, αX, or αD subunit to form LFA-1, Mac-1, p150/95 and αDβ2, respectively (27, 28). piliated *Escherichia coli* utilizes α3β1 integrin to enter the cell and to activate Src and

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Each of the leukocyte integrin α subunits contains an αI domain which is the major site of ligand binding (29). LFA-1 plays important roles in binding to the vascular endothelium during leukocyte extravasation, in homing to specific sites of inflammation or secondary lymphoid tissues, and in the formation of the immunological synapse between T cells and antigen presenting cells (28, 30). The ligands for LFA-1, intracellular adhesion molecule-1, -2, -3 and junctional adhesion molecule-A, are expressed on both the vascular endothelium and on APCs. The αMβ2 and αXβ2 integrins, also known as complement receptor-3 and -4, respectively, bind to complement iC3b opsonized particles and mediate phagocytosis (31). Both the αMβ2 and αXβ2 integrins also bind to ICAM-1 (31). Vascular cell adhesion molecule-1 serves as the ligand for αDβ2, but also for the α4β1 and α4β7 integrins (31). Many other integrins including, but not limited to

Table 1. Bacterial and viral pathogens utilize integrins in order to gain entry into host cells. By mimicking natural integrin ligand protein structure or sequence pathogen receptors hijack integrin receptors in order to gain entry into cells and stimulate signaling pathways that increase cell motility, thereby providing a mechanism for spreading infections.

INTEGRIN	ORAGNISM	PATHOGEN LIGAND
α1β1	Alpha virus	E2[53]
α2β1	Echovirus Rotavirus Group A Streptococcus	VP5[48,51] Streptococcal collagen-like proteins[59]
α3β1	E. Coli	FimH[57]
α4β1	Murine Polyomavirus Rotavirus	VP1 or VP2[54] VP7[49]
α5β1	Human parovirus B19	Unknown[52] (P antigen is a cellular co-receptor)
b1-integrins	Y. pseudotuberculosis, S. Aureus Reovirus	Fibronectin binding protein,[56] Invasin[58,60] Invasin[62] Sigma1[51]
αvβ3	West Nile Virus	Unknown[55]

the α1β1, α2β1, α4β1, α6β1, αVβ1 and α5β7 integrins are also expressed on subsets of leukocytes and inflammatory cells (31).

Hereditary defects in the β2 subunit that impair expression or function of leukocyte integrins or defects in β2 ligands causes life-threatening immunodeficiency, the leukocyte-adhesion deficiency (LAD) syndrome (31, 32). There are three forms of LAD which result in persistent and recurrent infections. LAD-I results from the lack of β2 integrin expression due to missense mutations that prevent localization of β2 integrins to the cell surface or heterodimerization with an α subunit (33). Defects in the synthesis of the sugars on their selectin partners that are required for integrin binding during leukocyte migration results in LAD-II (34). Genetic mutations resulting in the inability of integrins to be activated in response to stimuli from endothelial cells results in LAD-III (35). In addition to the leukocyte receptors, two collagen receptor integrins, α1β1 and α2β1 integrins are expressed on leukocytes and are important in mediating pro-inflammatory responses.

Collagen Receptor Integrins

Collagens are the most abundant proteins in mammals. They are a component of the ECM that not only support tissues, but are also required for cell adhesion and migration during growth, differentiation, morphogenesis, and wound healing. All collagens consist of three polypeptide α chains that are characterized by repeating Gly-X-Y sequences where X is typically occupied by proline and Y is 4-hydroxyproline (36). The human genome contains at least 43 distinct α chains that are assembled into 28 collagen types. Although collagen is abundant, only 4 of the 24 known integrins have

been shown to bind collagens. These collagen receptor integrins share the β1 subunit which heterodimerizes with either the α1, α2, α10 and α11 subunit (37-39). All of the collagen receptor integrins also contain an I domain that is responsible for collagen recognition (29). The collagen receptors are differentially expressed in distinct tissue sites and bind differentially to separate types of collagen.

The α1β1 integrin is abundant on smooth muscle cells, endothelial cells, neural crest cells and activated T cells. It preferentially binds the monomeric collagens, including type IV, type VI and type XII collagen, that in part compose the basement membrane (40). The α2β1 integrin is widely expressed on a number of cell types including endothelial cells, epithelial cells, as well as immune cells including natural killer (NK) cells, T cells and mast cells (12, 41, 42). The α2β1 integrin preferentially binds the fibrillar collagens (I-III) (29, 43). Subtle differences in I domain structure among the collagen integrin receptors account for their ability to recognize distinct collagen subtypes (44). Less is known about the collagen receptors, the α10β1 and α11β1 integrins. The α10β1 integrin is a collagen type II-binding integrin on chondrocytes (45). The α10 I domain displays similar ligand properties as α1 I domain underlining their similarity in ligand recognition of collagen IV and VI (46). Expression of α11 subunit was demonstrated by specialized fibroblasts and ectomesenchymally derived cells (47).

Structure

Crystal structures of integrin I domains have been resolved in the active, intermediate and inactive states for α1I and α2I (48). All I domains have a classic

Rossmann fold in which 6 α-helices surround five parallel and one anti-parallel β-strand (29). Metal ion binding sites are present in I domains and divalent cations, Mg2+ and Mn2+ are required for high affinity binding to collagen (49). The divalent cation mediated ECM adhesion and signaling functions are regulated by large conformational changes between the active and inactive conformations (29). The conformation changes allow signal transduction from the ECM into the cell. The inactive integrin conformation occurs when the α and β subunits are bound in a bent conformation with the head region in close proximity to the cell membrane (50). In this conformation, the I-domain is inaccessible to the ECM. Following inside-out signaling, the integrin adopts an active conformation and the α and β subunits are released exposing the ligand recognition site for interaction with the ECM.

There are structural differences between collagen binding integrins that may explain their different biological functions. While the majority of the backbone sequences of these integrins are predicted to be quite similar due to high sequence homology, the main difference appears to lie in the loops at the edges of highly conserved secondary structural elements (29). For instance, the α1 subunit has a large loop inserted in the β-propeller domain and more potential glycosylation sites compared to other collagen receptors (46). Residue R218 in α1I and α10I may confer additional ligand specificity in those subunits while D219 plays a role in recognition in the α2I (46).

α1β1 Integrin

A recurring theme in the study of α1β1 integrin is that expression on the cell surface is upregulated following inflammation. NK cells, T cells, monocytes and

macrophages all upregulate α1β1 integrin expression following activation by antigen, superantigen, cytokines or chemokines (51-53). The α1β1 integrin was originally defined as very late antigen-1 (VLA-1) because it becomes expressed on T cells more than 6 days following activation (54). Interactions between the α1β1 integrin and collagen modulate cytokine secretion and proliferation following stimulation through the T cell receptor (TCR) (55). T cells that express α1β1 integrin in the peripheral blood are restricted to a small population of CD45RO+ T cells that represent a population of activated T cells (56). This population of α1β1+CD45RO+ cells are antigen-specific memory CD4+ T cells and largely Th1-type memory cells (56).

Although resting monocytes have no detectable α1β1 integrin at the protein or mRNA levels, α1β1 integrin is expressed on monocytes following stimulation with either LPS or IFN-γ (57). Expression is delayed as observed in T cells, but only for 12-16 hours following stimulation (57). Similarly, NK cell activation results in expression of the α1β1 integrin, increased expression of the α4β1 and α5β1 integrins and downregulation of expression of the α6β1 integrin (52). The α1β1 integrin on macrophages is a crucial receptor for semaphorin 7A (Sema7A, CD108) on T cells (58). Semaphorins are a highly conserved family of receptors that are involved in neuronal development, organogenesis, vascularization, angiogenesis, neuronal apoptosis, neoplastic transformation, and more recently immune function (59). Binding of α1β1 integrin to Sema7A fused with the Fc portion of human IgG results in monocyte and macrophage adhesion and activation characterized by IL-6 production by mouse bone-marrow-derived macrophages (58). Loss of the α1β1 integrin results in defective cytokine production following Sema7A stimulation (58). Sema7A is highly expressed on activated T cells, which are responsible

for stimulating macrophage activation. Following T cell-macrophage interaction Sema7A induces clustering of the α1β1 in the immunological synapse(58). Thus, the interaction between Sema7A expressed on T cells and α1β1 integrin expressed on macrophages forms part of the antigen-presenting immunologic synapse and is involved in macrophage activation (58).

α2β1 Integrin

The role of the α2β1 integrin in inflammation has been studied over the past 30 years. Similar to the nomenclature of α1β1 integrin, the α2β1 integrin was also named VLA-2 due to its expression at the late stages of T cell activation (42). Naïve T cells fail to express the integrin; however, activated T cells in a number of chronic inflammatory settings express the integrin (60). In mice transgenic for a T cell receptor recognizing ovalbumin (OVA), activation of T cells with a TCR-specific peptide results in the upregulation of α2β1 integrin expression in Th1 but not Th2 cells (61). The integrin is functional on Th1 cells and enhances TCR-mediated proliferation and cytokine secretion upon α2β1 integrin-dependent adhesion to collagen (61). Additionally, ligand binding of α2β1 integrin by T cells inhibits Fas ligand expression and reduces cell death (62). Similar to α1β1, it has been demonstrated that inhibitory antibodies recognizing the α2β1 integrin can inhibit DTH, CHS and collagen-induced arthritis (13, 63, 64). However, it was not explicitly proven, that α2β1 integrin expression by T cells is required to mediate these responses.

Expression of α2β1 integrin is not limited to activated T cells. In fact, through studies in our lab, we have expanded the expression of α2β1 integrin to subsets of T cells

(CD3+, CD4+, or CD8+), B cells (B220+), NK cells (CD122+), neutrophils (Gr1+), and monocytes/macrophages (F4/80+) (unpublished data) and mast cells (c-kit+) (12). Although the α2β1 integrin is expressed on a number of hematopoietic cells, α2β1 integrin-null mouse showed no change in the relative percentage and absolute number of any cell type (unpublished data). The pan-NK cell monoclonal antibody, DX5 was demonstrated to recognize α2β1 integrin (41). The role for α2β1 integrin on monocytes and neutrophils may serve as a regulator of leukocyte adhesion and extravastation from the vasculature into peripheral tissues.

Many of the studies examining the role of α2β1 integrin in inflammation have utilized inhibitory antibodies to the integrin (13, 61, 62). In some cases, antibody binding of integrins, may actually result in activation of the integrin due to clustering rather than inhibition. To distinguish the specific effects resulting from a lack of α2β1 integrin and its signaling, the α2-null mouse was generated. The α2 integrin subunit knock-out mouse was created by two laboratories, including our own (65). The α2-null mice are viable, fertile and developed normally. It was hypothesized that α2β1 integrin was a critical mediator of platelet adhesion to collagen within the vessel wall after vascular injury. No defects in tail vein bleeding times were initially reported, however, these animals demonstrate profound prolongation in thrombus formation following carotid artery injury (66-68). In addition, defects in the mammary gland branching morphogenesis and wound healing in the α2-null mouse were reported (65). Although there was no overt phenotype in the hematopoetic compartment of α2-null mice, the expression of the α2β1 integrin on a number of immune cells suggested that it may play a role in the immune response to bacterial challenge. To test the role of the α2β1innate immunity, wild-type (WT) and α2-

null mice were challenged with *Listeria monocytogenes*, a Gram-positive intracellular bacterium, and assessed the ability to mount effective immune response following infection (12).

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive bacterium responsible for human listeriosis, a disease characterized by gastroenteritis, meningitis, encephalitis and materno-fetal infection (69). Humans are exposed to *Listeria* by ingesting contaminated food, such as unpasteurized diary products and incompletely cooked meats (70). Immunocompromised individuals are particularly vulnerable to infection and can develop septicaemia and meningitis (71, 72). Women who are pregnant can develop infection of the fetus, leading to septic abortion (73). The incidence of listeriosis varies between 0.1 and $11.3/10^5$ in different countries (74). Infection can spread to the cerebralspinal fluid resulting in meningitis with a devastating mortality rate of 25% (75). Although *Listeria* is not a highly pathogenic organism for most individuals, it is a well characterized tool for the study of the mammalian immune system.

The murine model of listeriosis has been pivotal in the understanding the immune response to *Listeria*. Innate immune responses are essential for early control of *Listeria* infection by inhibiting bacterial growth and dissemination, preventing the spread into systemic, lethal infection (76). In studies using severe combined immunodeficient (SCID) mice and nude mice that lack both T-cell and humoral immunity, mice were able to control *Listeria* infection (77, 78). One of the first cells involved in the innate immune response to *Listeria* is the neutrophil that engulfs and kill bacteria by generation of

reactive oxygen intermediates (79, 80). Macrophages are infected by *Listeria* where the organism is exposed to lysosomal enzymes and degraded. In response to infection, macrophages secrete TNF-α and IL-12, which induce NK cells to produce INF-γ, leading to increased activation of the macrophages and increased bactericidal activity (81)

Cytokines also play a significant role in controlling *Listeria* infection and mice deficient in cytokines or their receptors are highly susceptible to *Listeria* infection. Mice deficient in IFN-γ, CCR2 or the TNF-α receptor p55 display early lethality following *Listeria* infection (82-84). IL-6 deficient mice display decreased resistance to *Listeria* as a result of decreased systemic neutrophilia (85). Type I interferons, usually associated with anti-viral immune responses are also induced following *Listeria* infection. However, interferon-α and -β promote bacterial infection by dampening the immune response by inducing T cell apoptosis and secretion of the immune suppressive cytokine, IL-10 (86). Mice that lack the interferon-α/β receptor display increased resistance to bacterial infection demonstrating that regulation of type I interferons is important in the pathogenesis of *Listeria* (86).

Recognition of *Listeria* by the members of the innate immune response occurs through interaction with TLRs. The most important TLR for *Listeria* recognition appears to be TLR2 (87). Macrophages deficient in TLR2 display decreased production of cytokines, TNF-α, INF-γ, IL-1β and IL-12 following *in vitro* infection with *Listeria* (87). However, *Listeria* infection in mice deficient in TLR2 does not result in a drastic impairment of the immune response, indicating that other receptors may be important in controlling infection (88). Mice deficient in the key adaptor molecule, MyD88, which is important for signaling from several TLRs, are highly susceptible to *Listeria* infection

(88). Several other TLRs may contribute to innate immune recognition of *Listeria* such as, TLR9 which recognizes CpG motifs found within bacterial DNA and TLR5 which recognizes flagellin (89, 90). The role of these TLRs in *Listeria* infection *in vivo* remains to be demonstrated.

Listeria interacts with host cells using a variety of receptors that hijack host cell machinery to gain entry into cells and cross host barriers (e.g. intestinal, blood-brain and maternofetal). Internalin (InlA) and InlB were the first surface proteins of *Listeria* identified to promote host cell invasion (Figure 2) (91). InlA is responsible for the entry of *Listeria* into human epithelial cells through interaction with the host receptor E-cadherin (92). The recognition of InlA by E-cadherin is specific for humans and guinea pigs (93). A single point mutation at residue 16 (proline to glutamic acid) of mouse and rat E-cadherin prevents recognition of InlA (93). This lack of interaction between InlA and E-cadherin prevents the organism from crossing the intestinal epithelial barrier and is responsible for the lack of infection by oral inoculation in mice and rats (93). Oral infection of guinea pigs with *Listeria* induces gastroenteritis (93). *Listeria* crosses the intestinal barrier and induces dissemination and lethality in an InlA-dependent manner (93). Entry of *Listeria* into most other cell types requires InlB which binds to c-met and/or gC1qR/p32 ubiquitously expressed proteins that confer wide spread tropism (94, 95).

Two other virulence factors, ActA and listeriolysin O (LLO), act in the host cell compartment and are important in early stages of bacterial internalization (96, 97). ActA is a bacterial surface protein that enables bacterial propulsion in the cytosol leading to the

invasion of uninfected neighboring cells in a process called cell-to-cell spreading (98). However, in addition to its role in bacterial spreading, *Listeria* defective in ActA are also significantly impaired in cellular attachment and entry (98). The host cell receptor mediating this interaction has yet to be identified. LLO is a toxin that allows *Listeria* to escape from endocytic vacuoles following phagocytosis (99). In the absence of LLO *Listeria* remain trapped in the endocytic vacuoles and are, therefore, non-pathogenic (99). *Listeria* has proven to be an excellent system for *in vivo* analysis of the immune response. Several molecular mechanisms governing the development of innate and adaptive immunity have been uncovered using *Listeria monocytogenes* as a model. However, there are still many questions that can be answered using *Listeria* infections as a model of immunity to intracellular bacteria. These responses include, understanding the signals mediating leukocyte migration to infectious foci and defining the interaction of *Listeria* with host cells. The expression of the α2β1 integrin on many immune cells as well as the previous defined roles of the integrin in immunity suggested that the α2-null mice may display a defect in innate immune responses. Therefore, *Listeria* was used to determine the role of the α2β1 integrin in host defense.

Interperitoneal infection of WT mice with *Listeria* results in rapid IL-6 production at 1 hour post infection and subsequent neutrophil recruitment at 6 hours post infection (12). The α2-null mice were incapable of recruiting neutrophils to the site of infection (peritoneal cavity) at 6 hours following infection, a hallmark of the initial responses to bacterial challenge in the WT mice (12). Although the integrin is expressed on neutrophils, it was not expressed on infiltrating neutrophils in the wild-type mice (WT) (63). One of the earliest steps in the inflammatory process is recruitment of neutrophils to

the site of inflammation. The α2β1 integrin is not expressed on circulating blood PMNs. However, in one study the integrin was shown to be expressed on extravasated neutrophils (63).

In a model of inflammatory colitis, inhibitory antibodies directed against the α2β1 integrin prevented neutrophil accumulation in the colon (100). Using a broad spectrum inflammatory stimulant, thioglycolate, both WT and α2-null mice were capable of recruiting neutrophils, indicating that there is not a defect in neutrophil recruitment or migration in the α2-null animals (12). Although α2β1 integrin is not required for neutrophil recruitment in response to *Listeria* infection, Ridger et al. showed that inhibitory anti-α2β1 integrin antibodies decreased KC- (a CXC chemokine) but not LPS-induced neutrophil migration into mouse lungs, indicating a role for the α2β1 integrin in neutrophil migration in some inflammatory circumstances (101). In addition to the defect in peritonitis in the α2-null mouse at 6 hours post infection, decreased production of the pro-inflammatory cytokine, IL-6, occurred 1 hour following infection (12).

The peritoneum contains a number of innate immune cells poised to respond to incoming challenge. Of all cells in the peritoneal fluid, the c-kit^{pos} mast cell expressed the highest levels of the α2β1 integrin, suggesting that α2β1 integrin expression on the mast cell may play a role in the innate immune response to *Listeria* (12). The role of mast cells in the innate immune response to *Listeria* was confirmed by infecting the mast cell deficient mouse model, W/W^{v} with *Listeria* and assaying for cytokine production and neutrophil recruitment. Reconstitution with α2β1 integrin-expressing PMCs rescued neutrophil recruitment and IL-6 release, while reconstitution with α2-null PMCs did not (12).

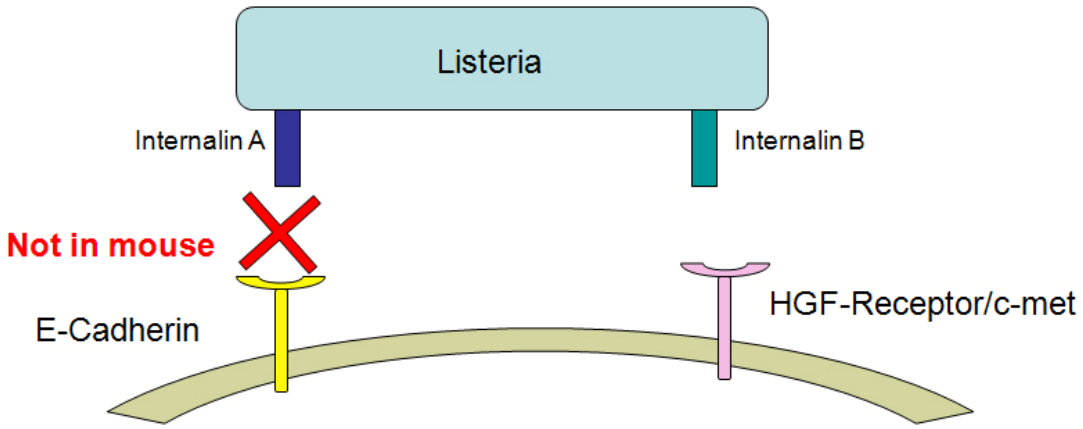

Figure 2. *Listeria* receptors involved in cell entry. Internalin A (InlA) and B (InlB) bind to their host receptors for entry into cells. The interaction between InlA and mouse E-cadherin does not occur due to a single point mutation.

Therefore, α2β1 integrin expression on mast cells and not on other inflammatory cells was responsible for the defect in the early innate immune response to *Listeria* observed in the α2–null mouse (Figure 3) (12). Mast cells are traditionally thought of in terms of IgE-mediated allergic responses and immunity to helmenths, however, mast cells may have a role in immune responses to bacterial and viral pathogens.

Mast Cells

Mast cells are multifunctional, hematopoietic, tissue-resident cells that are ubiquitously expressed throughout the body. They were originally described by Paul Ehrlich in 1879 as "mastzellen", meaning "well-fed cells." Mast cells reside in the connective tissue (skin and peritoneal cavity) and mucosal areas (gastrointestinal and respiratory tracts) that are exposed the external environment. Mast cells were originally thought to primarily serve as mediators of IgE-mediated immediate hypersensitivity known in general as allergy. However, in the past 20 years there has been an increase in the study of mast cells in the pathogenesis of a variety of IgE-independent clinical disease processes including cancer, ulcerative colitis, innate immunity and neuronal disease.

Mast Cell Deficient Mouse Models

An understanding of the *in vivo* molecular basis of mast cell development and function has come mainly from use of mast cell deficient mice. Genetically mast cell-deficient c-*kit* mutant mice are a powerful tool for identifying and quantifying contributions of mast cells in many biological responses. Mice carrying spontaneous loss-of-function mutations at both alleles of the dominant *white spotting* (*W*) locus (i.e., c-*kit*), exhibit a marked

reduction in c-kit tyrosine kinase-dependent signaling, resulting in disrupted normal mast cell development, survival, and function, as well as many other phenotypic abnormalities that are unrelated to the mast cell deficiency (102). The KBB6F-*Kit*$^{W/W\text{-}v}$ (W/Wv) mouse arises from mutations affecting the c-kit tyrosine kinase receptor resulting in few (<1% of normal levels) detectable mast cells in the peritoneal cavity, respiratory system, heart, brain, skeletal muscle and spleen (102). However, they also have other defects not related to their mast cell phenotype including macrocytic anemia, impaired melanogenesis. The C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ model has an inversion mutation in the transcriptional regulatory elements upstream of the c-kit transcription start site (103). These mice are beneficial because they have fewer developmental abnormalities and are fertile, allowing for additional genetic crosses into the mast cell deficient phenotype (103).

These mice have been useful due to the ability to generate what Dr. Steven Galli has termed "mast cell knock-in mice" where the mast cell population is selectively reconstituted with normal mast cells or those containing genetic mutations. Intravenous, intraperitoneal, and subdermal injections of *ex vivo* cultured mast cells results in varied levels of reconstitution of mature mast cells into the mast cell compartments (102, 104). For instance, intravenous injection does not result in reconstitution into the peritoneum and skin of *Kit*$^{W\text{-}sh}$ mice (103). Direct injection into the specific site is required for full reconstitution, indicating that certain chemokines may be required for migration and homing to specific sites (103, 104). This model has allowed many investigators to determine the specific contributions of mast cell specific expression of various cytokines and receptors.

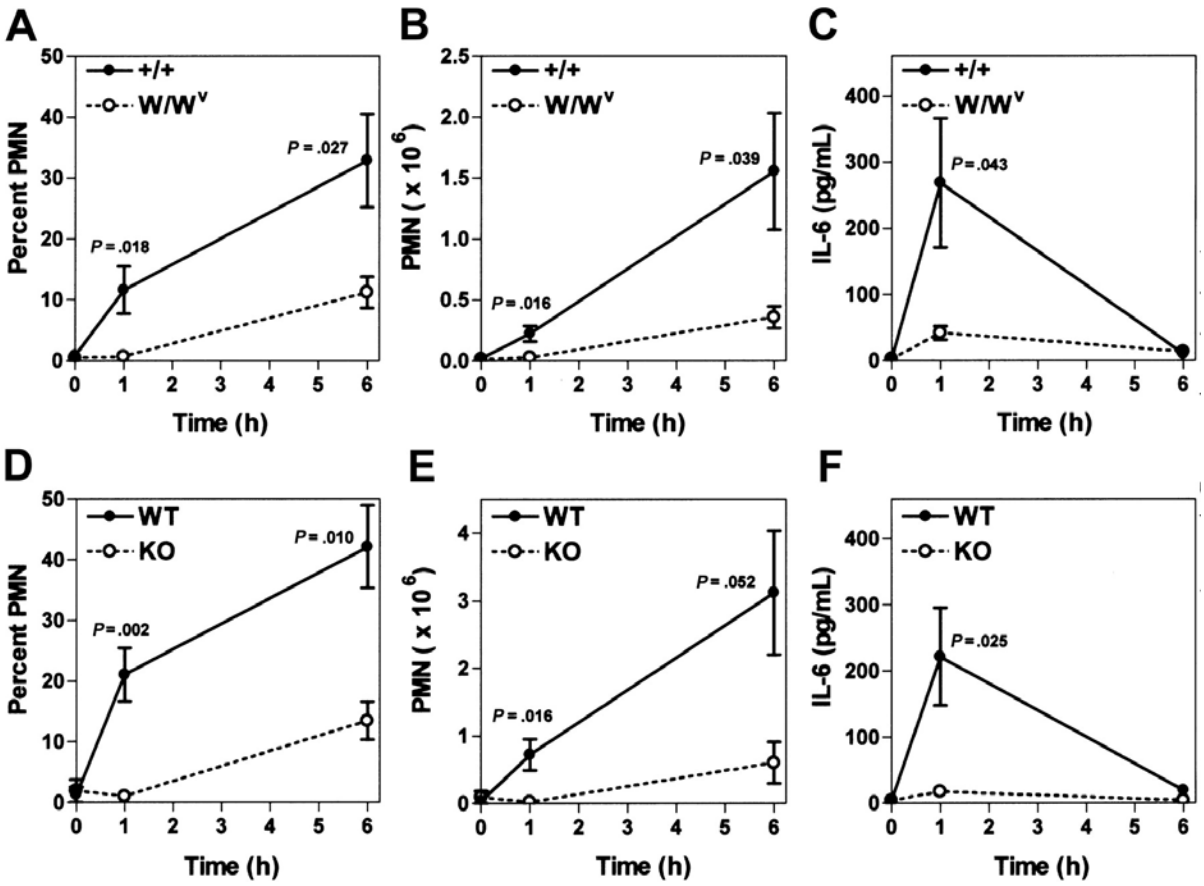

Figure 3. The inflammatory response to *Listeria* infection is both mast cell– and $\alpha_2\beta_1$ integrin–dependent. (A-C) WBB6F1-+/+ mast cell–sufficient (+/+) and WBB6F1-W/W^v mast cell–deficient (W/W^v) mice were infected with 5 x 10^4 *Listeria* intraperitoneally. At indicated times after infection, the percentage of PMN, the absolute PMN number, and the IL-6 concentration in peritoneal fluid were determined. Shown is the combination of 2 experiments (mean ± SEM), with each point representing 5-6 mice (time 0 hours, 2 mice). (D-F) Wild-type (WT) and α_2 integrin–deficient (KO) mice were infected with 5 x 10^4 *Listeria* intraperitoneally. At indicated times after infection, the percentage of PMN, the absolute PMN number, and the IL-6 concentration in peritoneal fluid were determined. Shown is the combination of 2 experiments (mean ± SEM), with each point representing 4-5 mice (time 0 hours, 2 mice). Originally published in Edelson et al 2004 ©American Society for Hematology

Mast Cells in Host Defense

Mast cells have been implicated in host defense against a large number of parasites and, for many years, the role of mast cells in host defense was thought to be limited to parasitic infections. However, over the past 15 years there have been a large number of studies demonstrating the role of mast cells in host defense against bacterial and viral pathogens. The mast cell deficient mouse models have been excellent tools to study the mast cell specific roles in these infection models. Using W/W*v* mice, it has been demonstrated that lack of mast cells results in decreased survival following infection with *Citrobacter rodentium*, *Klebsiella pneumoniae*, Fimbrated *E.* coli (105, 106). A model of sepsis, ceacal ligation and puncture (CLP), can be overcome by adoptive transfer of mast cells into the mast cell deficient animals (105). These studies demonstrated that mast cells initiate the recruitment of neutrophils through release of TNF-α and LTB_4 or LTC_4. One study showed that increasing local mast cell numbers by injection of SCF results in enhanced survival of WT mice following CLP (107).

Although there have been fewer studies in this area, mast cell may have a role in host defense against viral pathogens. Mast cells or their precursors can be infected with human immunodeficiency virus (HIV), dengue virus, cytomegalovirus and adenovirus (108, 109). Infection of mast cell deficient mice with Sindbis virus has demonstrated that, in large part, the inflammatory response in the brain is mast cell dependent (110). In animal models of infection with respiratory syncytial virus or Sendai virus, local increases of mast cell numbers concomitant with lymphocytic infiltrates may be indicative of pathogen-induced mast cell activation (111). Many of the cytokines released from stimulation of mast cells with viral components leads to responses that are

consistent with a role in selective recruitment of T cells and NK cells to the site of infection.

Mast Cell Ontogeny and Homing

Mast cells originate in the bone marrow and differentiate into mature cells once they reach their destination. In the context of inflammation, mast cells are recruited in even greater numbers indicating that mast cells must arise from a progenitor cell that can differentiate into a mature mast cell under specific stimuli. Mast cell maturation only occurs following exit from the circulation and homing to the peripheral site. Significant heterogeneity in mast cell maturation is based on location. In rodents, mature, tissue-resident mast cells are subdivided into two classes: connective tissue mast cells (CTMCs) present in the skin, stomach and peritoneal cavity or mucosal mast cells (MMC) present in the lamina propria or the gastrointestinal mucosa, respiratory tract and nasal mucosa (112, 113). In humans, mast cells are characterized by their protease content. Mast cells containing tryptase (MC_T) are found in the mucosa and mast cells containing both tryptase and chymase (MC_{TC}) are found in the connective tissue. Although the nomenclature differs between rodent and human mast cells, the characteristics of the CTMC/MC_{TC} and MMC/MC_T populations are similar.

Despite differences in surface markers and granule contents, there is plasticity between the mast cell subsets. In fact, transfer of cultured bone marrow derived mast cells (BMMC) (which are characteristically similar to MMCs) or peritoneal cavity derived mast cell colonies (which are CTMCs) into mast cell deficient animals allows for maturation and dissemination of mast cells into the host animal. In the same animal,

CTMCs were identified in the peritoneal cavity, spleen, skin and muscularis propria of the stomach and MMCs were identified in the stomach mucosa (114, 115). The ability of mast cells to reach their target sites and differentiate into distinct subsets indicates that there are differential chemokine migration signals and adhesion molecules that are involved in mast cell trafficking and homing.

Mast Cell Receptors

There are a number of receptors expressed on mast cells that are involved in allergy, asthma, inflammation and host defense. Among them, the longest known has been the high affinity receptor for IgE (FcεR), which is the receptor responsible for binding allergen-specific IgE. The FcεR belongs to the family of multi-subunit immune response receptors composed of an α, a β and two γ chains (116). The α chain constitutes the extracellular component, the β and γ chains contain the receptor activation sites. Aggregation of the FcεR by contact of cell bound IgE with a multivalent antigen results in stimulation of immunoreceptor tyrosine-based activation motifs (ITAMs) (116). Activation of the receptor results in the production and release of a wide variety of preformed mediators (including histamine and mast cell proteases), *de novo* synthesis of lipids (leukotrienes and prostaglandins) and many proinflammatory chemokines and cytokines such as TNF-α, IL-1, IL-2, IL-3, IL-4, IL-6, IL-9 and IL-13 (117). This array of mediators leads to increased vascular permeability, tissue edema, bronchoconstriction, massive leukocyte recruitment and inflammation in the mucosa (116).

Although FcεR stimulation was thought to be the primary function of mast cells, they express a wide array of receptors that demonstrate their involvement in direct

contact with pathogens and host defense. Mast cells are poised at the interface of the internal and external environment and function as the sentinels of host defense (117). Direct interactions between pathogens and immune effectors cells are essential for the generation of early innate immune responses as well as the generation of appropriate acquired immunity (118). The activation of these pathogen receptors results in the initiation of "danger signals" that trigger subsequent rapid and selective response.

One of the most important families of cell-surface receptors in pathogen recognition is the TLR family. The highly conserved TLR family of pattern recognition receptors has been demonstrated to have a pivotal role in many host defense mechanisms. To date, 13 TLR family members have been identified in the human and murine genome and mast cells express TLR1, -2, -3, -4, -6, -7 and -9 but not -5 (119-122). The TLR complex usually consists of a TLR homodimer or heterodimer, a number of co-receptors, and intracellular and extracellular adaptor molecules. Different TLR family member are activated by different pathogen-associated or endogenous proteins. A variety of TLR activators have been defined, which include proteins from all classes of mammalian pathogens, as well as endogenous proteins. TLR9 is an intracellular receptor for CpG motifs found within bacterial DNA (123, 124). TLR4 is a homodimeric TLR and has been shown to mediate responses to LPS, as well to a number of other gram negative pathogen products and to heat shock protein 60. Activation of TLR4 results in degranulation-independent release of TNF-α, IL-1β, IL-6, and IL-13 (125). In contrast, TLR2 is a heterodimeric TLR and mediates responses to peptidoglycan from many gram-positive bacteria and to the yeast cell-wall component, zymosan. Peptidoglycan and zymosan utilize the TLR2/TLR6 hetrodimer resulting in production of IL-4, IL-5, GM-

CSF, IL-1β and LTC_4 (125, 126). The synthetic bacterial lipoprotein $Pam_3CysSerLys_4$ utilizes the TLR2/TLR1 heterodimer and results in production of GM-CSF and IL-1β without release of LTC_4 (126).

In addition to the TLRs, there are several receptors that are involved in direct recognition of specific pathogens. CD48 recognizes FimH which is found of fimbriated *Escherichia coli* resulting in degranulation and TNF-α production (127, 128). Activation of mast cell CD48 also results in phagocytosis and superoxide anion-dependent bacterial killing, whereas, FimH-negative organisms are less susceptible to mast cell induced bacterial killing (129). Another mannose-binding protein from *Schistosoma mansoni* induces mast cell degranulation through an unknown receptor (130). Human mast cells can be activated by *Staphylococcus aureus* derived protein A, which binds IgG or IgE antibodies and in turn will bind the high affinity FcγRI (131).

In a surprising series of studies, it was demonstrated that mast cells bind and serve as a reservoir for latent HIV infection (132). HIV-1 gp120, is an immunoglobin superantigen family member and binds non-specifically with immunoglobin V_H3 gene products (133-135). The interaction with mast cells occurs through binding of gp120 with IgE which, in turn, stimulates FcεR1 to induce histamine and LTC_4 release from mast cells (132). Th2 cytokines, IL-4, IL-13 are also released upon FcεR1 stimulation, upregulating HIV co-receptors, CXCR4 and CCR3 on mast cells, which already express low levels of CD4 (70). HIV-infected mast cells have been resistant to highly active anti-retroviral therapy and the long lived nature of the mast cells provides a good cellular host for latent HIV infection (108). The harboring of virus within the mast cell may serve as a reservoir for HIV in individuals who relapse following therapy (108).

Mast cell expression of the α2β1 integrin is responsible for the early innate immune response to *Listeria*. The α2β1 integrin is a high affinity receptor for collagen; no collagen-like motifs have been described on *Listeria* and α2β1 does not bind *Listeria*. However, the collectin family of proteins, including mannose-binding lectin (MBL), surfactant protein A (SP-A) and D (SP-D), and the ficolins as well as the C1q complement protein are all involved in innate immune responses and all contain a triple helical collagen-like domain which could potentially serve as the binding site for the α2β1 integrin (136-138). Additionally, these proteins function to coat the surfaces of microbes. C1q, MBL and SP-A were identified as novel, divalent-cation dependent ligands for the α2β1 integrin (139). Adhesion of the α2β1 integrin to the collectin molecules was mediated by the α2I domain. The identification of this novel group of ligands suggested a model by which the α2β1 integrin may interface with the innate immune response. *In vitro* activation of WT PMCs, but not α2−null PMCs with *Listeria* required immune complexes containing *Listeria*, anti-*Listeria* antibody and an α2β1 ligand, either C1q or type I collagen (139). Stimulation of WT PMCs with the immune complex resulted in IL-6 secretion after one hour, similar to the α2β1 integrin-dependent mast cell response observed *in vivo* (139).

Mast Cell Structure

Mast cells are characterized by cytoplasmic electron dense granules, dendritic-like processes emanating from the plasma membrane, and multi-lobed nuclei. The secretory granules are rich with a number of different proteoglycans, depending on the anatomical location of the mast cell. CTMCs are rich in heparin proteoglycans which allows them to

be stained by safranin or berberine sulfate. MMCs contain less proteoglycan, fail to stain with safranin, but react with alcian blue dye. In addition to being useful for identification, proteoglycans are required for storage and packaging of mast cell granule mediators. Mast cells produce three main classes of mediators: preformed, granule-associated products, newly generated lipid mediators, and many chemokines and cytokines (Figure 4).

Proteoglycans are thought to provide the major structural base of the mast cell granules. In mast cells, serglycin is the major type of proteoglycan core protein to which either heparin or chondroitin sulfate glycosaminoglycans is attached. Serglycin proteoglycan is required for storage of serotonin and histamine (140). The highly acidic proteoglycans form complexes with basically charged proteases, as well as histamine and β-hexosaminidase to generate the molecular complexes that form the electron dense granules. Upon exocytosis, the complexes are exposed to the extracellular environment at neutral pH, and the associated proteins are released from the proteoglycan core into the extracellular space.

The synthesis of eicosanoid inflammatory mediators, leukotriene (LT) C_4, LTB_4, and prostaglandin (PG)D_2 from arachidonic acid stores occurs in mast cells following various stimuli including crosslinking of the high affinity FcεR and bacterial ligands (141-143). These mediators are important in the allergic inflammation that occurs in the lung during allergy and asthma reactions. The leukotrienes bind the G-protein coupled receptor, Cys-LT-1, resulting in bronchoconstriction, vascular permeability, mucus secretion and eosinophil recruitment. LTB_4 is also a potent mediator of neutrophil

recruitment. PGD_2 is a bronchoconstrictor and also results in constriction of coronary arteries.

Mast cells produce both pro-inflammatory mediators tumor necrosis factor- α (TNF-α), IL-1β and IL-6 as well as anti-inflammatory mediators IL-10 and TGF-β (144-147). Mast cells are often thought to be the major producers of (T helper 1) Th1 and Th2 cytokines, including IL-4, IL-5, IL-13, IFN- γ, IL-12, and IL-18 (148, 149). In addition to cytokines, mast cells are also an important source of growth factors, SCF, granulocyte/macrophage- colony stimulating factor (GM-CSF), basic-fibroblast growth factor, nerve growth factor and VEGF (118, 150, 151). Recently, both human and rodent mast cells have also been shown to produce several antimicrobial peptides (152). Given this vast array of mediator production, mast cells receptor/ligand interactions are important in many biological responses. The mechanism of mast cell response to unique to receptor activation is important in understanding the role of mast cells in biologic responses.

The first hundred years of mast cell research, from the discovery of mast cells in 1879 until the pivotal reports describing the requirement for mast cells in host defense in 1996, focused on the role of the mast cell in the allergic response. The next hundred will likely continue to focus on the role of the mast cell as a sentinel of host defense. The investigations that occurred during the first decade of mast cell-pathogen interactions have focused on receptors that are involved in pathogen recognition by the mast cell. The next frontiers in this field will likely focus on the biochemical aspects of mast cell activation in response to pathogens and ways to modify these properties for the benefit of the host.

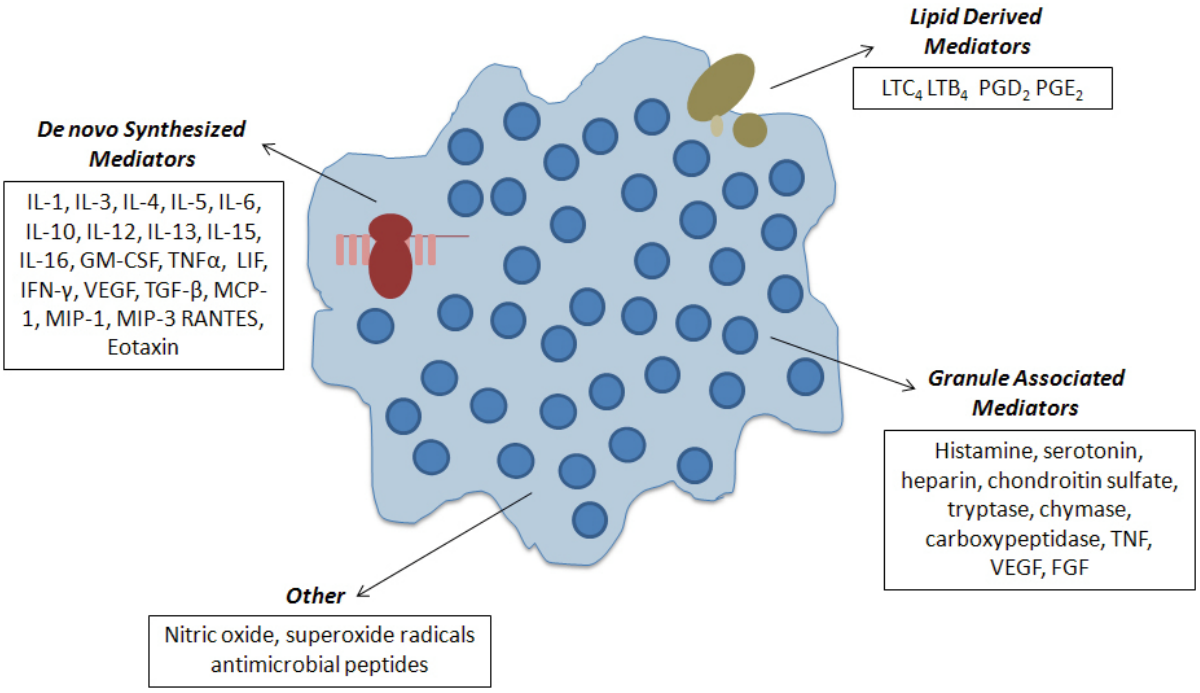

Figure 4. Mast Cell Mediators. Mast cells can produce several classes of mediators. (1) Newly synthesized cytokines, chemokines and growth factors, (2) newly synthesized lipid mediators and (3) granule associated mediators. They are also the source of several factors involved in bacterial killing such as nitric oxide and antimicrobial peptides.

Statement of Aims

The expression of the α2β1 integrin on mast cells is required for the innate immune response to *Listeria monocytogenes* predicted to interact via C1q contained within an immune complex. However, ligation of the integrin is not sufficient to induce activation of mast cells, indicating that a co-receptor is required for activation. In another model of α2β1 integrin-mediated activation, platelets utilize glycoprotein VI (GPVI) as a co-receptor for activation upon adhesion to collagen. We propose three models for co-receptor induced mast cell activation in response to immune complex, FcγR-, complement- or pathogen-mediated co-receptor activation. Identification of the co-receptor that synergizes with the α2β1 integrin to activate mast cells is important for understanding mechanisms of mast cell interaction with pathogens and immune complexes. Additionally, demonstrating a novel co-receptor synergy with the α2β1 integrin may give insight into the role of the integrin in different integrin mediated responses.

In vivo and in vitro, α2β1 integrin mediates activation of mast cells to release IL-6 at 1 hour following infection. The characteristic feature of mast cells is the presence of electron dense granules that contain mediators poised for release. Mast cells are capable of releasing their mediators via compound degranulation, piecemeal degranulation and de novo protein synthesis. Mechanisms underlying IgE-mediated compound degranulation and de novo synthesis and release have been well defined. However, the receptor/ligand interactions mediating piecemeal degranulation have not been well defined. We propose a mechanism of pre-formed IL-6 release following stimulation by *Listeria* immune

complex that is distinct from IgE-mediated compound degranulation. Through this aim, we will demonstrate the mechanism of α2β1 integrin-dependent IL-6 release.

The in vivo innate immune response to *Listeria* has been demonstrated to be α2β1 integrin-dependent at the initiation phase between 1 and 6 hours following infection. An effective innate immune response is required for the initiation of the adaptive immune response. The immune response to *Listeria* results in a cytokine milieu that primes an effective adaptive immune response. The goal of this aim is to determine if the defect in the innate immune response to *Listeria* observed in the initiation phase affects additional immune responses. Our hypothesis is that initiation of the innate immune response and subsequent adaptive immune response is delayed in the α2-null mouse.

The role of the mast cell as a critical mediator of innate and adaptive immunity is rapidly growing. Our laboratory has demonstrated that the α2β1 integrin is able to activate mast cells through ligation of C1q in *Listeria* immune complexes. The experiments presented here will contribute to our understanding of how mast cells are able to recognize and specifically respond to pathogens in ways that are independent of IgE. Additionally, we demonstrate differential mast cell granule components that are distinctly released upon specific stimuli. The very early signals following infection is crititcal in directing the innate immune response. These studies will aid in defining a clear receptor/ligand interaction and cell type that is intricately involved in transmitting these early "danger signals" that initiate the adaptive immune response.

CHAPTER II

MATERIALS AND METHODS

Mice and *Listeria*

α2 integrin-subunit (α2-/-), FcRγ (FcRγ-/-), C1q (C1q-/-)-deficient mice and WT littermate controls on a C57BL/6 x 129/Sv background were used at 6-20 weeks of age. FcRγ-deficient mice were obtained from Dr. Paul Allen (Washington University School of Medicine). C1q-deficent mice, originally generated by Drs. Walport and Botto, were obtained on a pure C57BL/6 background from Drs. Michael Diamond (Washington University School of Medicine, St. Louis, MO) and Gregory L. Stahl (Harvard University School of Medicine, Boston, MA) (153). Mice were maintained under specific pathogen-free conditions in the Vanderbilt University School of Medicine (Nashville, TN) mouse facilities. Within individual experiments, mice were age and sex matched. WT *Listeria* (EGD) and its isogenic mutants, ΔInlA and ΔInlB (provided by Emil Unanue from Washington University, St. Louis, MO) were cultured in brain heart infusion broth (BD, Sparks, MD) at 37^{o}C**.**

In vivo Model of Peritonitis

Listeria strain EGD and its isogenic mutants were stored at mid-log growth as glycerol stocks at -80 ^{o}C and diluted in pyrogen free saline for injection into mice. Bacteria were injected at a dose of 5 x 10^4 *Listeria*/mouse intraperitoneally in 500 μL. At indicated time points after injection, mice were killed and peritoneal exudates were

collected by lavage in 10 mL RPMI. Cell-free supernatants were stored at -20 ^{o}C and later used for the determination of IL-6, TNF- α, INF-γ and IL-1β by ELISA (BD Biosciences). Total peritoneal exudate cell number was determined for each mouse, and cells were cytospun onto slides and stained with the Hem 3 staining kit (Fisher Scientific, Pittsburgh, PA). The percent PMN was determined by differential cell counting. In some experiments, mice were infected for 7 days and serum was collected at post-infection day 0, 2 and 4. Spleens and livers from some mice were harvested at 5 days to determine CFUs. Antigen specific T cells were analyzed as previously described (154).

Mast Cell Preparations

PMCs were isolated from residential peritoneal exudates using percoll gradient centrifugation (~85% purity) (12). Fetal skin-derived mast cells (FSMC) were generated as described previously (155). Single cell suspensions of day 16 fetal trunk skin were generated by incubation in 0.25% trypsin in Hanks Balanced Salt Solution (HBSS) for 20 min at 37^{o}C. After erythrocyte lysis with lysing buffer (0.15 mM NH_4C1, 1.0mM $KHCO_3$, 0.1mM Na_2EDTA), cells were washed and seeded at 2 x 10^4 cells/mL in FSMC media [RPMI1640, 10% FBS,10 mM NEAA, 10mM sodium pyruvate, 0.01% Penicillin-Streptomycin, 25 mM HEPES buffer, 50 uM 2-mercaptoethanol, and 10 ng/mL IL-3 and SCF (both from Prepro Tech, Rocky Hill, NJ). After 10-14 days, nonadherant cells were assessed for the expression of c-kit and expression of the α2β1 integrin. Cultures of FSMCs were used if greater than 85% of the WT cells co-expressed c-kit and the α2β1 integrin. Expression of c-kit, α2β1 integrin or c-met was carried out by flow cytometric analysis using the following antibodies (all from BD Biosciences, San Diego, CA): FITC

(fluorescein isothiocyanate)–anti-CD117 (c-kit; 2B8), PE (phycoerythrin)–anti–CD49b (integrin subunit; HMα2).

In vitro Adhesion Assays

Adhesion assays were performed as previously described (139). Static adhesion assays were performed in 96-well plates (Immulon 2HB; Thermo Labsystems, Franklin, MA) (156, 157). Wells were coated with bovine serum albumin (BSA) (5 μg/mL; Sigma-Aldrich, St Louis, MO), type 1 collagen (25 μg/mL rat tail; BD Biosciences), human C1q (25 μg/mL; Calbiochem, San Diego, CA), a matrix of *Listeria monocytogenes*, anti-*Listeria* antibody, and serum, or a matrix of BSA, anti-BSA, and serum. The *Listeria* or BSA matrix was formed by allowing *Listeria* (strain EGD, 1 x 10^8 organisms/mL in 0.1 M carbonate buffer, pH 8.5) or BSA (5 μg/mL in PBS) to adhere to wells of a 96-well plate overnight. Unattached *Listeria* or BSA was removed and polyclonal anti-*Listeria* antibody (1:200 dilution in PBS; Difco, Detroit, MI) or anti-BSA antibody (1:1000 dilution in PBS; Invitrogen Life Technologies, Carlsbad, CA) was added and incubated at 37°C for 1 hour. Fresh mouse serum from WT, C1q -/-, C3-/-, C4-/-, C5-/- or Factor B-/- mice (sera from C3-/-, C4-/-, C5-/- and Factor B-/- kindly provided by Michael Diamond, Washington University, St. Louis, MO) (50%) was added for 1 hour at 37°C. PMCs (2000 cells/well) were allowed to adhere for 1hour at 37°C in the presence of 2 mM $MgCl_2$ or 2 mM EDTA. Non-adherent cells were removed and adherent cells were quantitated as previously described (157).

In vitro Activation Assays

For *in vitro* mast cell activation by *Listeria*, purified PMCs (5 x 10^4 cells/well) were incubated for 1 hour at 37°C with a washed suspension of *Listeria* (1 x 10^7 organisms), incubated with rabbit anti-*Listeria* antibody, and 50% serum from either WT, C1q -/-, C3-/-, C4-/-, C5-/- or Factor B-/- mice. For *in vitro* mast cell activation by BSA immune complexes, purified PMCs (5 x 10^4 cells/well) were incubated with a washed suspension of latex beads (Polyscineces, Warrington, PA) coated with BSA (3mg/ml), anti-BSA antibody, and serum (50%) alone or in the presence of lipopolysaccharide (LPS) (100ng/ml, Sigma-Aldrich, St. Louis, MO), Pam_3Cys-Ser-$(Lys)_4$ x 3HCl (Pam_3Cys) (100ng/ml, EMC Microcollections, Tuebingen, Germany), *Listeria* (1 x 10^8), heat-killed *Listeria* (HKLM) (1 x 10^8 heated for 30 min at 60°C), or HGF (2 mg/ml, R&D Systems Minneapolis, MN).

To determine the activation by IgE crosslinking, cells (5 x 10^4) were preloaded for 18 h with anti-DNP IgE (1 μg/ml, SPE-7, Sigma Aldrich) in Tyrodes buffer (137 mM NaCl/11.9 mM $NaHCO_3$/0.4 mM Na_2HPO_4/2.7 mM KCl/1.1 mM $MgCl_2$/5.6 mM glucose, pH 7.3). The sensitized cells were washed twice in Tyrodes buffer and stimulated with 100 ng/ml DNP-HSA (Sigma Aldrich) for the indicated time points. In some experiments cells were inhibited by pre-treating the cells with goat-anti-c-met (R&D Systems), actinomycin D (2 μg/ml) cyclohexamide (20 μM), Brefeldin A (1 μg/ml) or monensin (1 μM) (all from Sigma Aldrich) prior to stimulation of FSMCs. Cell-free supernatants were analyzed by ELISA per manufacturer's instructions for IL-6 (BD Biosciences, San Diego, CA), Histamine and Serotonin (both from Fitzgerald Industries). The degree of degranulation was determined by measuring the release of ß-

hexosaminidase. The enzymatic activity of ß-hexosaminidase in supernatants and cell pellets solubilized with 1% Triton X-100 in Tyrode's buffer was measured with *p*-nitrophenyl *N*-acetyl-ß-D-glucosaminide (Sigma-Aldrich) in 0.1 M sodium citrate (pH 4.5) for 60 min at 37°C. The reaction was stopped by the addition of 0.2 M glycine (pH 10.7). The release of the product, 4-*p*-nitrophenol, was detected by absorbance at 405 nm. The extent of degranulation was calculated by dividing the 4-*p*-nitrophenol absorbance in the supernatant by the sum of the absorbance in the supernatant and detergent-solubilized cell pellet.

Mast Cell Fractionation

Fractionation of mast cells to isolate mast cell granules was performed by using a washed preparation of $1x10^7$ mast cells. The pellet was resuspended in PBS and submitted to 3 freeze/thaw cycles. The homogenate was then sonicated at level 4 for 5 bursts then centrifuged at 3000 rpm for 10 min to pellet nuclei. The post nuclear supernatant (PNS) was collected and applied on a two layer Percoll gradient. The Percoll was diluted with 10X sucrose and water and gradient densities were 1.05 and 1.12 (2 ml/layer). The gradient was layered in 5 ml polycarbonate ultracentrifugation tubes (Beckman, Fullerton, CA). After applying the PNS on top of the gradient, the samples were spun at 40,000 rpm for 50 min in a Sorvall Discovery 90SE ultracentrifuge using the AH650 rotor. Fractions of 200 µl were collected starting from the top of the gradient. The fractions were divided in half. 100 µl was used to determine IL-6, Histamine and Serotonin by ELISA, 100 µl was used for western blot analysis.

Immunofluorescence

FSMCs were washed with PBS and fixed with 3% paraformaldehyde for 20 mins, washed and permiabilized with 0.05% Tween-20/PBS for 15 min. Cells were cytospun onto glass slides and blocked with 3% horse serum/PBS for 1 hour at room temperature. Primary antibodies rabbit anti-mouse IL-6 (US Biologicals) and mouse anti-5-HT serotonin overnight at 4 °C. Cells were then washed and treated with Goat-anti mouse AlexaFlour 488 and Goat-anti-rabbit AlexaFlour 633 (Molecular Probes) for 1 hour at room temperature. Cells were mounted and imaged with an LSM 510 META confocal microscope (Carl Zeiss Microimaging, Germany) and the images were processed by using LSM ImageBrowser (Carl Zeiss Microimaging) software.

CHAPTER III

CROSS-TALK BETWEEN THE α2β1 INTEGRIN AND C-MET/HGF-R REGULATES INNATE IMMUNITY

Introduction

The role of the α2β1 integrin in the innate and acquired immune response is an area of active investigation. We initially reported that the α2β1 integrin–deficient mice exhibited markedly diminished inflammatory responses to *Listeria monocytogenes* due to a requirement for α2β1 integrin expression on PMC for mast cell activation and cytokine release *in vivo* (12). Although the α2β1 integrin serves as a receptor for a number of matrix and non-matrix ligands, the integrin ligand during the PMC response to infection is unknown (158, 159). We demonstrated that C1q complement protein and collectin family members, including mannose-binding lectin (MBL) and surfactant protein A (SP-A) all served as ligands for the integrin (139). In addition, the α2β1 integrin is required for mast cell activation *in vitro* in response to *Listeria*. However, ligation of the α2β1 integrin alone is insufficient to activate cytokine secretion because mast cell adhesion to collagen or C1q alone fails to support cytokine secretion (139).

We hypothesize that one or more additional signals emanating from an additional receptor is required to activate mast cell cytokine secretion in response to immune complexes. There are several models by which α2β1 integrin–ligand interactions may stimulate mast cell activation and cytokine secretion (Figure 5A). First, ligation of the α2β1 integrin simultaneously with a second, co-stimulatory receptor might elicit mast cell activation, in a manner reminiscent of the role proposed for the α2β1 integrin and the

GPVI/FcRγ during platelet adhesion to collagen (38, 160). Second, binding of the α2β1 integrin to an immune complex containing C1q may directly activate the complement cascade, resulting in the deposition of C3b or iC3b and generation of complement byproducts such as C3a or C5a which would subsequently stimulate mast cell activation (118, 161, 162).

In this chapter, I describe that neither the FcRγ nor components of the complement cascade are required in α2β1 integrin-dependent mast cell activation. Instead, we describe a novel co-receptor required for mast cell activation, HGF-R/c-met. The *Listeria* specific molecule, InlB, binds to its host cell receptor, c-met to mediate internalization into epithelial and hepatic cells (94). I demonstrate that activation of mature PMCs by *Listeria* plus immune complex requires co-stimulatory signals from α2β1 integrin ligation to either type I collagen or C1q and c-met binding to either InlB or HGF. The synergistic signals from the two co-receptors result in mast cell activation and the release of the pro-inflammatory cytokine IL-6 that induce the early innate immune responses to *Listeria monocytogenes*.

Results

α2β1 integrin-C1q-immune complex interaction was required, but not sufficient, for mast cell activation in response to *Listeria monocytogenes* (139). These data suggested that one or more additional signals emanating from an additional receptor, such as the FcRγ chain, is required to activate cytokine secretion (Figure 5) (12). FcRγ is required to initiate the downstream signals from T-cell receptor and B-cell receptor

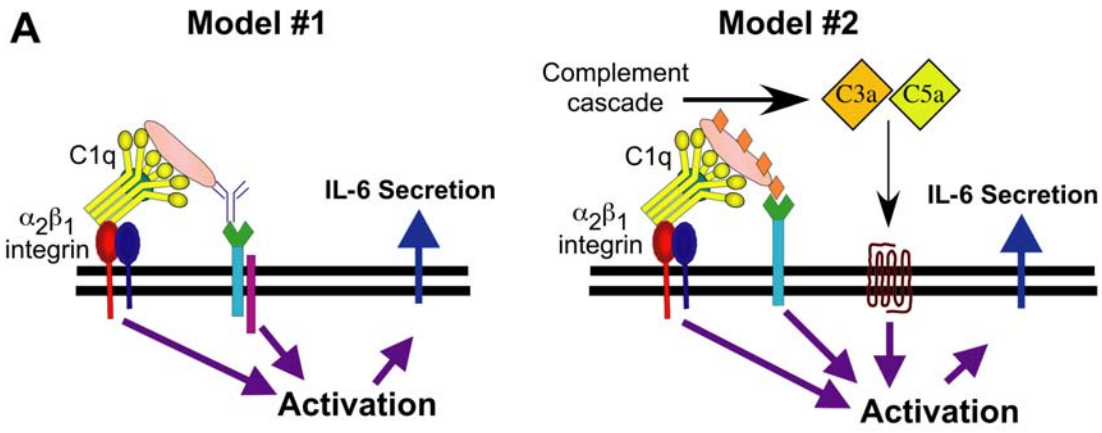

Figure 5. Hypothesized models of mast cell activation by *Listeria* immune complex . (A) A proposed model is a 2-site, 2-receptor model in which concurrent activation of the α2β1 integrin and secondary receptor (complement receptor, FcRγ, *Listeria* receptor) stimulates mast cell activation. Adapted from **Edelson et.al.** Blood, 2006 Jan 1;107(1):143-50**.**

signaling through activation of the (immunoregulated tyrosine activation motifs) ITAM domains. Additionally, the α2β1 integrin co-receptor for platelet activation in response to collagen, GPVI, utilizes the common FcRγ chain to initiate signaling. To determine if the FcRγ chain is required for mast cell activation in response to *Listeria* immune complex stimulation, we compared the ability of purified PMCs from WT or FcRγ-/- mice to bind to a matrix of either *Listeria*-containing immune complexes or type I collagen. PMCs from both, WT and FcRγ$^{-/-}$ mice adhered to collagen and *Listeria* containing immune complexes in an α2β1 integrin-mediated, divalent cation-dependent manner (Figure 6A). These data demonstrate that PMC adhesion to immune complex via the α2β1 integrin does not require FcR

To determine if α2β1 integrin-dependent PMC activation requires signaling downstream of the FcRγ, we measured the release of IL-6 from WT and FcRγ$^{-/-}$ PMCs after stimulation with a complex of *Listeria* plus anti-*Listeria* IgG antibody or an immune complex of *Listeria* plus anti-*Listeria* IgG antibody plus serum. Neither WT nor FcRγ$^{-/-}$ PMCs were activated in the presence of *Listeria* and antibody alone. In contrast, WT and FcRγ$^{-/-}$ mice released similarly high levels of IL-6 in response to *Listeria* plus antibody plus serum (Figure 6B). These results indicate that FcRγ is not required *in vitro* for the α2β1 integrin-dependent activation of PMC by *Listeria* plus immune complex. In addition, these data suggest that the FcRγ does not serve as a co-receptor for the α2β1 integrin in immune complex-stimulated mast cell activation.

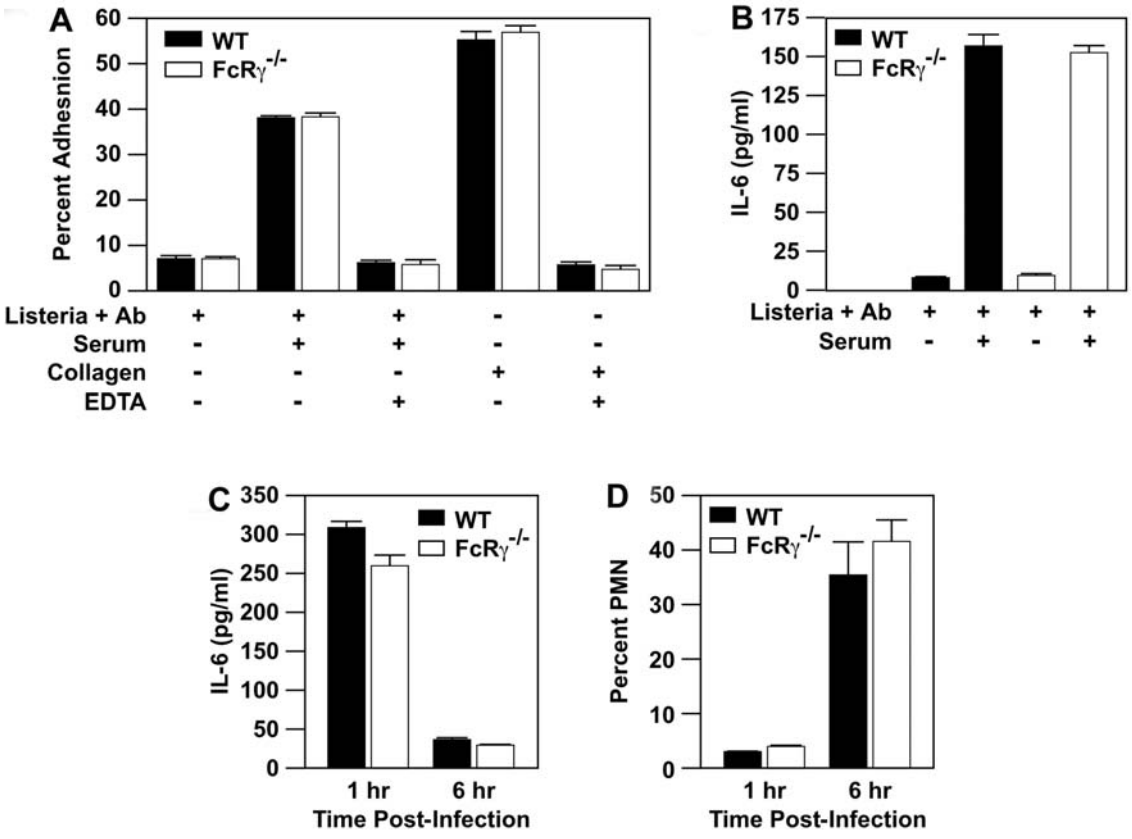

Figure 6. Mast cell activation by *Listeria* immune complex does not require FcRγ
(A) Purified PMCs (2 x 10^4) from either wild-type (WT) mice or from mice lacking FcRγ (FcRγ $^{-/-}$) were assayed for adhesion to a matrix consisting of (1) *Listeria* plus anti-*Listeria* antibody alone, (2) *Listeria*, anti-*Listeria* antibody and 50% murine serum or (3) type I collagen in the presence or absence of 1mM EDTA. (B) Purified PMCs (5 x 10^4) from WT and FcRγ $^{-/-}$ mice were incubated for 1 hour with a washed suspension of *Listeria*, anti-*Listeria* antibody and 50% murine serum. Supernatants were collected and analyzed for IL-6 production by ELISA. All adhesion and activation experiments were carried out in the presence of 2mM $MgCl_2$ All results are represented as mean ±SEM from triplicate wells of a single experiment and represent 1 of at least 3 experiments demonstrating similar results. (C and D) 3 of each, WT and FcRγ $^{-/-}$ mice were infected for 1 or 6 hours with 5 x 10^4 *Listeria* intraperitoneally. At the indicated time points, the percentage PMN and IL-6 in the peritoneal fluid were determined. Shown are representative of at least 3 experiments (mean ±SEM), all carried out in triplicate.

In light of the importance of the FcRγ in the immune response, I sought to determine if the FcRγ was required for the early neutrophil recruitment in response to *Listeria*. To determine the role of the FcRγ *in vivo* in response to *Listeria*, PMN influx and IL-6 secretion into the peritoneal cavity was evaluated in WT and FcRγ$^{-/-}$ mice. Both WT and mice deficient in the FcRγ exhibited maximal IL-6 secretion at 1 hour and a robust neutrophil response at 6 hours (Figure 6C and D). These data demonstrate that signals downstream of the FcRγ are not required for the early innate immune response to *Listeria*.

I therefore hypothesized that mast cell binding via the α2β1 integrin to an immune complex containing C1q may directly activate the complement cascade, resulting in the deposition of C3b or iC3b and the generation of complement byproducts such as C3a or C5a. In turn, binding of complement components to their cognate receptors on mast cells (complement receptor 1 [CR1], CR3, CR4, C3aR or C5aR) would then stimulate mast cell activation, as has previously been shown (118, 161, 162).

To determine whether complement activation provided the co-stimulatory signal for IL-6 release, I evaluated adhesion and activation of WT PMCs in response to immune complex formed from *Listeria* plus anti-*Listeria* antibody and murine serum from WT mice or mice deficient in classical complement cascade components, C1q, C3, C4 or C5, or the alternate cascade component, Factor B. *Listeria* plus anti-*Listeria* antibody plus serum from WT mice and mice deficient in C3, C4, C5 and Factor B resulted in formation of an adhesive substrate (Figure 7A). As previously demonstrated, C1q-deficient serum failed to form an adhesive substrate for the α2β1 integrin (139). To evaluate PMC activation, I measured secretion of IL-6 by PMCs after stimulation with

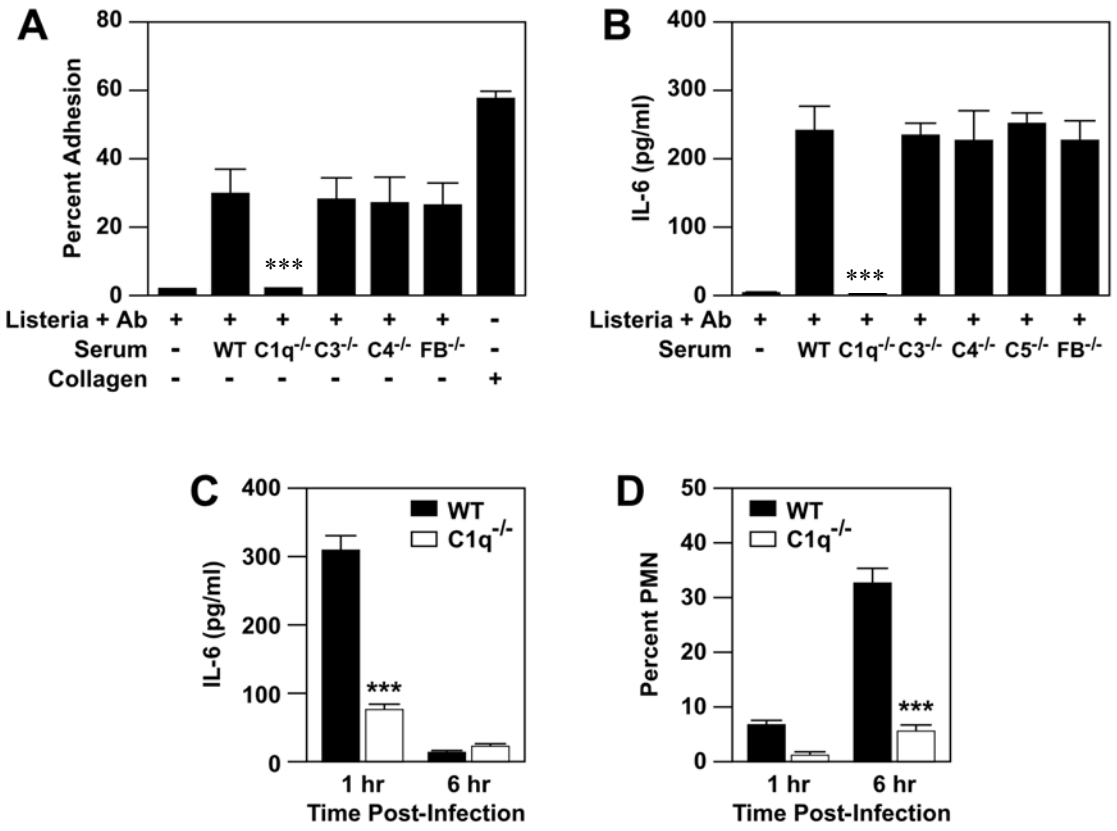

Figure 7. C1q, but not other complement components supplies stimulatory signal for mast cell activation by *Listeria*-immune complex. (A) Purified PMCs (2 x 10^4 cells/well) isolated from WT (WT) mice were assayed for adhesion to a matrix consisting of *Listeria* plus anti-*Listeria* antibody, *Listeria*, anti-*Listeria* antibody alone and 50% murine serum obtained from either WT mice or mice deficient in the complement components, C1q, C3, C4, C5, or Factor B (C1q $^{-/-}$, C3$^{-/-}$, C4$^{-/-}$, C5$^{-/-}$, FB$^{-/-}$) or type I collagen. (B) Purified PMCs (5 x 10^4) from WT and mice were incubated for 1 hour with a washed suspension of *Listeria*, anti-*Listeria* antibody and 50% murine serum from either WT or mice deficient in the complement components C1q, C3, C4, C5, Factor B. Supernatants were collected and analyzed for IL-6 production by ELISA. All adhesion and activation experiments were carried out in the presence of 2 mM $MgCl_2$. All results are represented as mean ±SEM from triplicate wells of a single experiment and represent 1 of at least 3 experiments demonstrating similar results. (C-D) WT and C1q$^{-/-}$ mice were infected for 1 or 6 hours with 5 x 10^4 *Listeria* intraperitoneally. At the indicated time points, the percentage PMN and IL-6 in the peritoneal fluid were determined. Shown are representative of at least 3 experiments (mean ±SEM), all carried out in triplicate. Statistics were performed using unpaired student's *t*-test (***$p<0.001$)

Listeria plus anti-*Listeria* antibody plus WT serum or serum deficient in C1q, C3, C4, C5 or Factor B. WT PMCs secreted high levels of IL-6 in response to immune complexes formed with WT serum, as well as with serum deficient in C3, C4, C5 or Factor B. As expected from an earlier report, (139) immune complexes from C1q deficient serum failed to activate WT PMCs (Figure 7B). Therefore, C1q, but not other complement components, is required for mast cell activation. These results indicate that neither FcRγ nor complement components other than C1q act as co-stimulatory signals for PMC activation by *Listeria* immune complexes.

These studies demonstrated a role for C1q in response to *Listeria in vitro*. To demonstrate a requirement for C1q *in vivo*, we infected WT and C1q-/- mice with *Listeria*. In WT mice, IL-6 was released into the peritoneal cavity at 1 hour post infection. In contrast, C1q-/- mice failed to respond with IL-6 in response to *Listeria* (Figure 7C). Additionally, there was a significant decrease in the number and percentage of PMNs that infiltrated the peritoneal cavity of C1q-/- mice at 6 hours as compared to WT mice (Figure 7D). These results support the hypothesis that C1q is Important in the mast cell dependent response to peritoneal *Listeria* infection. Furthermore, C1q binding to α2β1 integrin provided a signal that could not be duplicated by peritoneal matrix components such as collagen.

WT PMCs adhere to plate-bound immune complexes formed between BSA, anti-BSA antibody, and serum in an α2β1 integrin-dependent manner (139). To determine if binding of WT PMCs to an immune complex alone, without *Listeria*, was sufficient to mediate cytokine secretion, IL-6 secretion by PMCs was analyzed after 1-hour stimulation with immune complexes consisting of BSA-coated latex beads plus anti-BSA

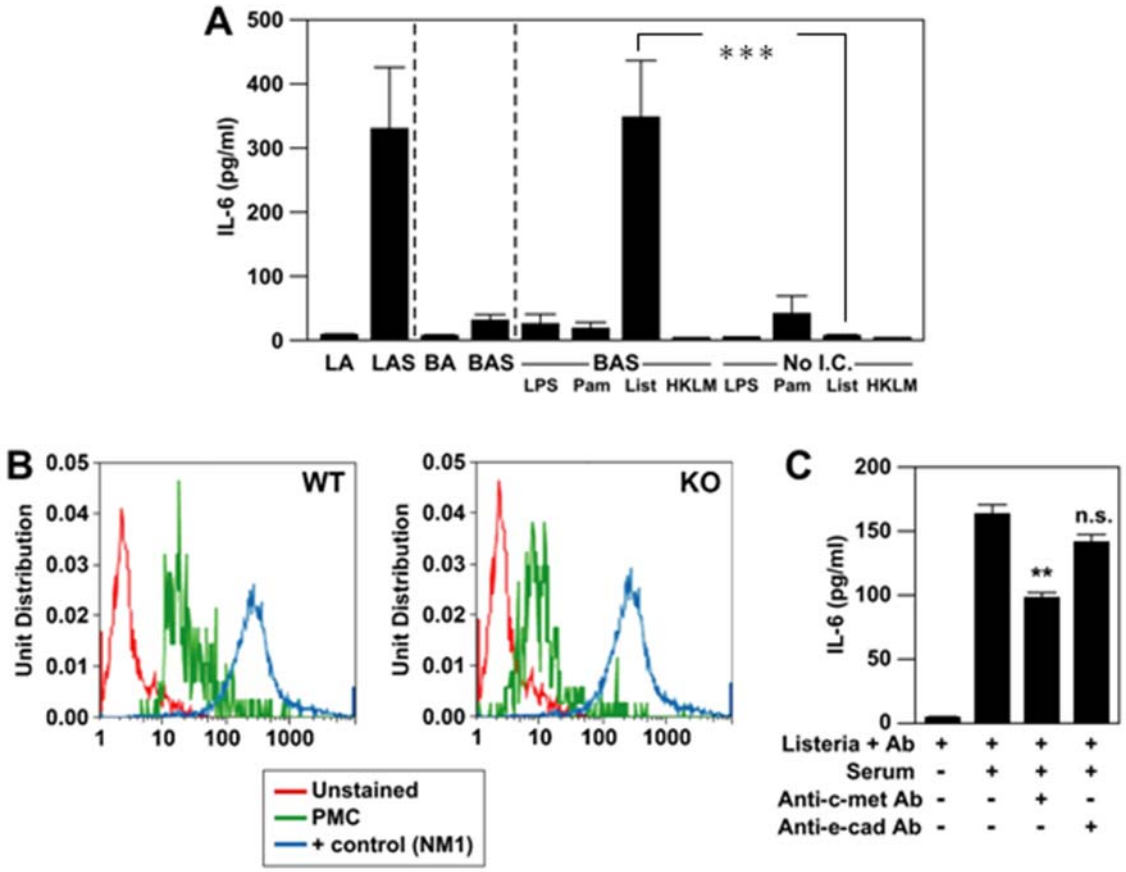

Figure 8. HGF-R/c-met is the receptor providing the co-stimulatory signal for mast cell activation. (A) Purified PMCs (5 x10^4) isolated from WT mice were incubated for 1 hour with a washed suspension of *Listeria* and anti-*Listeria* antibody alone (LA), *Listeria*, anti-*Listeria* antibody plus 50% serum from WT mice (LAS), latex beads coated with bovine serum albumin (BSA) plus anti-BSA antibody alone (BA), BSA plus anti-BSA antibody and 50% WT murine serum (BAS), BSA plus anti-BSA antibody and 50% WT murine serum (BAS) with either LPS (100 ng/ml), Pam3Cys (100 µg/ml), *Listeria* (1 x 10^8), heat-killed *Listeria* (1 x 10^8 organisms), or LPS (100 ng/ml), Pam3Cys (100 µg/ml), *Listeria* (1 x 10^8), heat-killed *Listeria* (1 x 10^8 organisms) alone. Supernatants were collected and analyzed for IL-6 production by ELISA. (B) Representative flow cytometric histograms of PMCs stained with c-met PMCs from WT (panel A) and α2β1 integrin-deficient (KO, panel B) were stained with PE-anti-c-kit and APC-anti c-met and assessed by flow cytometry. Mast cells were identified as c-kit^{high}-staining cells and represented 1% - 3% of resident peritoneal cells in both WT and KO mice. (C) Purified PMCs isolated from WT mice were pretreated with inhibitory antibodies toward E-cadherin, c-met or irrelevant control antibody for 1 hour prior to stimulation with a washed suspension of *Listeria*, anti-*Listeria* antibody alone, or *Listeria*, anti-*Listeria* antibody and 50% WT murine serum. Supernatants were collected and analyzed for IL-6 production by ELISA.

antibody and serum or immune complexes consisting *Listeria* plus anti-*Listeria* antibody plus serum. WT PMCs secreted abundant IL-6 in response to *Listeria* plus immune complex, but failed to secrete IL-6 in response to BSA plus immune complex. Therefore, the immune complex alone was not sufficient to stimulate PMC activation (Figure 8A). The addition of *Listeria* alone to the BSA-immune complex restored IL-6 secretion to levels similar to activation with *Listeria* containing immune complex (Figure 8A). This result suggested that interactions of the PMC with *Listeria* were providing the additional activation signal required following α2β1 integrin-ligation to immune complex.

TLRs induce mast cell activation both *in vivo* and *in vitro*. Since *Listeria monocytogenes* is a Gram-positive bacterium, I hypothesized TLR2 may serve as the necessary co-receptor required to mediate the α2β1 integrin-dependent mast cell activation. To examine the role of *Listeria* and TLRs in immune complex mediated mast cell activation, IL-6 secretion from PMCs was compared after stimulation with BSA-immune complexes plus either live *Listeria*, as shown above, heat-killed *Listeria monocytogenes* (HKLM), the TLR2 agonists Pam_3Cys or the TLR4 agonist, LPS. The addition of heat-killed *Listeria*, TLR2, or TLR4 agonist to the BSA immune complexes failed to result in activation of PMCs (Figure 8A). These results suggest that a heat-labile protein serves as the co-receptor for mast cell activation and that TLR2 and 4 do not provide co-stimulatory activity.

Listeria is an intracellular pathogen that infects humans using two bacterial surface receptors for bacterial internalization, InlA and InlB that bind to E-cadherin and c-met, respectively, on the host cell (91, 94, 163). Mouse E-cadherin does not bind to InlA due to a single point mutation in its binding site on mouse E-cadherin (93). We

evaluated the expression of c-met on mature peritoneal mast cells by flow cytometric analysis. As shown in Figure 8B, WT and α2-null PMCs express high levels of c-met. To determine if *Listeria*-c-met interaction is required, inhibitory anti-c-met or anti-E-cadherin antibodies were used to block PMC-*Listeria* interactions. Preincubation of WT PMCs with anti-c-met antibody prior to stimulation with *Listeria*-containing immune complexes significantly diminished the IL-6 release by 40% ($p=0.03$), suggesting that c-met serves as a co-receptor. As expected, the inhibitory anti-E-cadherin did not significantly alter IL-6 release (Figure 8C).

The best defined ligand for c-met is HGF. We hypothesized that *Listeria*-stimulated PMC activation would be mimicked by HGF binding to c-met. To determine whether HGF binding alone was sufficient to activate WT PMCs when the α2β1 integrin was ligated, we compared secretion of IL-6 by WT PMCs following stimulation by BSA-immune complex plus *Listeria,* BSA-immune complex plus HGF, or *Listeria*-immune complex. The level of IL-6 produced by WT PMCs stimulated by *Listeria*-immune complex, BSA-immune complex plus *Listeria* and BSA-immune complex plus HGF was equivalent (Figure 9A). HGF failed to stimulate IL-6 secretion when incubated with PMCs in the absence of BSA-immune complex (Figure 9A). These data suggest that stimulation of c-met by either *Listeria* or HGF mediates WT PMC activation in the presence of ligated α2β1 integrin.

To determine if coactivation of the α2β1 integrin and c-met is sufficient for PMC activation, we stimulated WT PMCs adherent via the α2β1 integrin to either C1q or type I collagen with either *Listeria* or HGF. α2β1 integrin-dependent adhesion of WT PMCs to

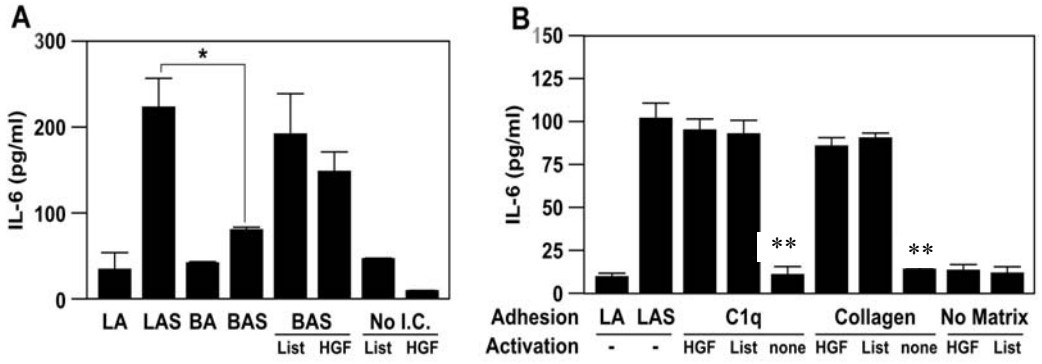

Figure 9. Mast cell activation in the absence of immune complex. (A) Purified PMCs (5 x10^4) isolated from WT mice were incubated with *Listeria* and anti-*Listeria* antibody alone (LA), *Listeria,* and anti-*Listeria* antibody plus 50% serum from WT mice (LAS), latex beads coated with BSA, plus anti-BSA antibody alone (BA), or latex beads coated with BSA, anti-BSA antibody, plus 50% serum (BAS) plus or minus the addition of *Listeria* (1 x 10^8) or HGF (2 mg/ml), or *Listeria* (1 x 10^8) or HGF (2 mg/ml) alone. Supernatants were collected and analyzed for the concentration of IL-6 by ELISA. (B) Purified PMCs (5 x10^4) isolated from WT mice were allowed to adhere to a matrix of *Listeria* and anti-*Listeria* antibody (LA), *Listeria*, anti-*Listeria* antibody plus serum (LAS), type I collagen (25 mg/ml), C1q (25 mg/ml), or tissue culture plastic (No Matrix) with or without either *Listeria* (1 x 10^8) or HGF (2 mg/ml). Supernatants were collected and analyzed for IL-6 production by ELISA. All experiments were carried out in the presence of 2 mM $MgCl_2$. All results are presented as mean ±SEM from triplicate wells of a single experiment and represent 1 of at least 3 experiments demonstrating similar results. The p-values were determined by unpaired student's *t*-test (* $p<0.05$, ** $p<0.01$)

either collagen or C1q alone failed to result in IL-6 release, as previously shown (139). c-met activation of PMCs by *Listeria* or HGF alone failed to result in IL-6 secretion. However, ligation of both the α2β1 integrin and c-met resulted in PMC activation and IL-6 release (Figure 9B). These data indicate that co-stimulation of c- met and the α2β1 integrin is sufficient to induce mast cell activation in the absence of additional stimuli such as immune complexes.

InlB and InlA promote *Listeria* internalization into host cells. The c-met receptor on the host cell binds to InlB on *Listeria*; E-cadherin binds to InlA. To determine the role of InlB/c-met interaction in PMC activation, we quantitated IL-6 secretion by WT PMCs after stimulation with immune complexes formed by WT *Listeria* or mutant *Listeria* containing mutations in either InlA (ΔInlA) or InlB (ΔInlB). Activation of WT PMCs with ΔInlA *Listeria*-immune complex resulted in secretion of IL-6 at similar levels to that secreted by PMCs stimulated by WT *Listeria*-immune complex. In contrast, activation of WT PMCs with ΔInIB *Listeria*-immune complex resulted in significantly reduced secretion of IL-6 to baseline levels (Figure 10A).

Since the early α2β1-dependent innate immune responses to *Listeria* mediated by PMC secretion of IL-6 resulted in neutrophil recruitment at 6 hours (12), we hypothesized that the c-met-InlB interaction was required not only *in vitro* but also for α2β1 integrin-dependent response *in vivo*. WT and α2-null mice were infected intraperitoneally with either WT, ΔInlA, or ΔInlB *Listeria*. As reported, WT mice but not α2-null mice, when infected with WT *Listeria* demonstrate high levels of IL-6 in the peritoneal cavity 1 hour post-infection and robust neutrophil recruitment at 6 hours post-

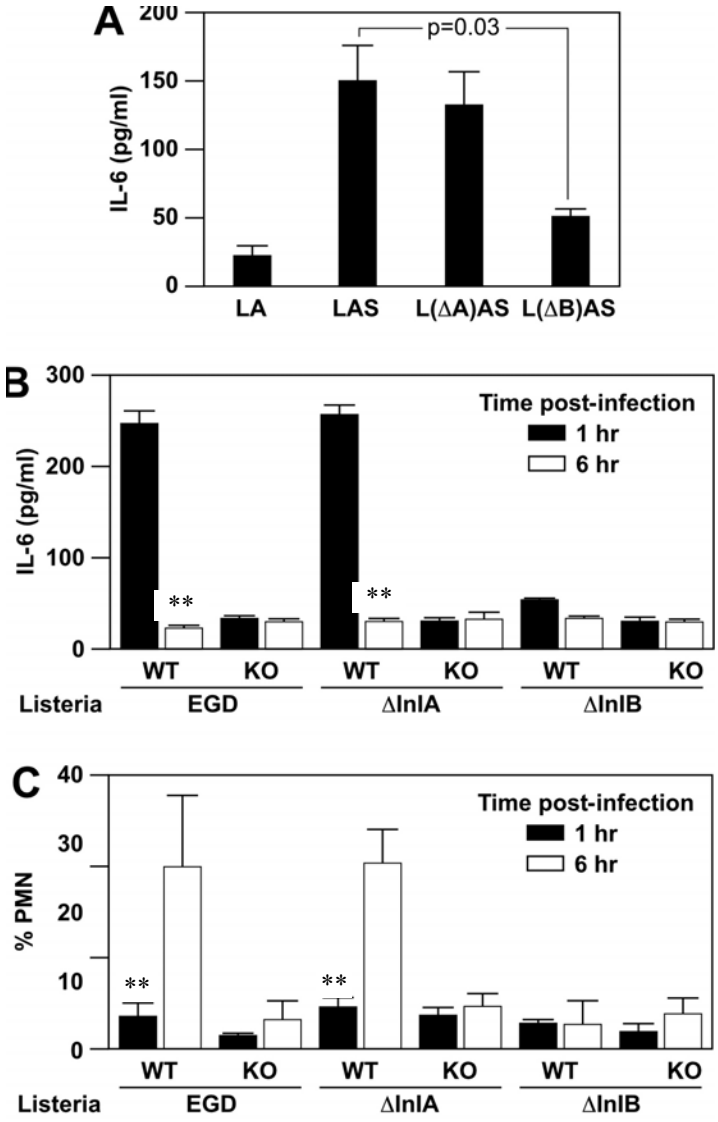

Figure 10. Internalin B is required for mast cell mediated innate and adaptive immune responses Purified PMCs (5 x 10^4) from WT and α2-null mice were incubated for 1 hour with a washed suspension of *Listeria* and anti-*Listeria* antibody (LA), or using WT *Listeria* EGD, (LAS), *Listeria* ΔInlA (L(ΔA)AS), or *Listeria* (L(ΔB)AS), anti-*Listeria* antibody and 50% murine serum. Supernatants were collected and assayed for IL-6 by ELISA. (B-C) WT and α2-null mice were infected for 1 or 6 hours with 5 x 10^4 *Listeria* (EGD), *Listeria* (ΔInlA), or *Listeria* (ΔInlB) intraperitoneally. At the indicated time points, the percentage PMN and IL-6 in the peritoneal fluid were determined. Shown is representative of 2 experiments (mean ±SEM), with each point representing 5-6 mice. Statistical analysis was performed using the Students T test (** $p<0.01$)

infection (12). WT, but not α2-null mice responded with a rapid cytokine secretion and a similar robust neutrophil response to ΔInlA *Listeria*. In contrast, ΔInlB *Listeria* failed to elicit IL-6 secretion or neutrophil recruitment in either WT or α2-null mice (Figure 10B and C). These data demonstrate that binding of c-met to InlB cooperates with α2β1 integrin binding to C1q or collagen in the early mast cell- dependent innate immune response to *Listeria* both *in vivo* and *in vitro*.

Conclusions

I have identified c-met as a novel co-receptor required for α2β1 integrin-dependent mast cell activation. The synergistic contributions of α2β1 integrin and c-met receptor in immune modulation were entirely unexpected. We initially reported that the α2β1 integrin–deficient mouse demonstrated a profound and surprising defect in the innate immune response to *Listeria monocytogenes* (12). α2β1 integrin expression on the PMCs was required for activation and cytokine secretion *in vivo* (12). Previous studies demonstrate a requirement for α2β1 integrin ligation for PMC activation (139). However, α2β1 integrin-ligand interactions alone are insufficient for PMC activation since α2β1 integrin dependent adhesion to collagen or C1q alone failed to support cytokine secretion (139). I hypothesized that one or more additional signals emanating from an additional receptor, similar to that downstream of glycoprotein VI/FcRγ on platelets, was required for PMC activation (38, 160). Alternatively, α2β1 integrin binding to C1q may result in activation of the complement cascade.

I describe a mechanism of innate immune response mediated by interaction with α2β1 integrin with a soluble, non-matrix factor, C1q. We previously demonstrated that

the α2β1 integrin is a cellular receptor for C1q, the first component in the complement cascade and mediator of innate immune response (139). Interaction in vitro between α2β1 and C1q on mast cells resulted in activation and cytokine secretion. Here I show, through in vivo models, that the C1q/ α2β1 integrin interaction is, in fact, required for innate immune response to *Listeria*. C1q-deficient mice do not release IL-6 or recruit PMNs into the peritoneum following *Listeria* infections. Although collagen and other ECM components are abundant in the peritoneum, collagen binding either does not occur or is not sufficient to mediate mast cell activation following *Listeria* infection. C1q and collagen have very similar sequences and are both capable of providing the α2β1 integrin co-stimulatory signal in response to *Listeria*. However, it appears that in vivo they may induce different responses. This may provide further insight into the role of the α2β1 integrin and its ability to mediate innate immune responses through interaction with C1q.

These studies indicate, neither complement receptors nor FcRγ provide the necessary co-stimulatory signal for α2β1 integrin-dependent IL-6 release *in vivo* or *in vitro*. In fact, the FcRγ is not required for the early innate immune response to *Listeria* either *in vitro* or *in vivo*. Instead, we identified c-met as a novel α2β1 integrin co-receptor that is essential for activation of the innate immune response to *Listeria*. In addition, we demonstrated cooperation between the α2β1 integrin and HGF-R/c-met in immune modulation. In the innate immune response to *Listeria*, the surface receptor InlB, a ligand for the c-met, provides the co-stimulatory signal. In our initial *in vitro* observations, mast cell activation required *Listeria*-generated immune complexes. Since α2β1 integrin-dependent adhesion to either type I collagen or C1q alone was required, but

not sufficient for mast cell activation, we suggested that perhaps the correct orientation of the immune complex was required for recognition (139).

We have demonstrated that the orientation of the immune complex is not required for activation. Simple co-ligation of the integrin with either type I collagen or C1q and c-met with either *Listeria* organisms or c-met's natural ligand HGF is sufficient to induce activation. InlB binding to host receptor, c-met is essential for infectivity and internalization into epithelial cells and hepatocytes. However, because stimulation with HGF and an α2β1 integrin ligand is sufficient for activation we propose that internalization is not required for mast cell activation (163). Our data now support a role for the α2β1 integrin/c-met interaction for the innate inflammatory response to *Listeria*.

CHAPTER IV

SELECTIVE AND DIFFERENTIAL RELEASE OF MAST CELL PREFORMED IL-6 IN RESPONSE TO *LISTERIA* IMMUNE COMPLEX REQUIRES THE α2β1 INTEGRIN

Introduction

Mast cells are versatile cells of the immune system, contributing to both the innate and adaptive defense against external insults. In response to stimulation, three major classes of proinflammatory mediators are released by activated mast cells: preformed granule-associated chemical mediators, newly synthesized arachidonic acid metabolites, and proinflammatory cytokines (164). The release of stored mediators from inflammatory cells can occur by compound exocytosis (degranulation) or piecemeal degranulation. Mast cells can release newly synthesized mediators by classical exocytosis. Other cells such as neurons, pancreatic beta cells, natural killer cells, cytotoxic T lymphocytes, and platelets also undergo degranulation to release their granule-associated mediators. Mast cell secretory granules contain a wide array of preformed mediators, the first to be described was histamine (165). Mast cell granules have subsequently been demonstrated to contain TNF-α and serotonin (166, 167).

The best defined mechanism of mast cell degranulation has been the compound degranulation observed following crosslinking of the high affinity FcεRI with IgE antibodies and specific antigen. IgE crosslinking is a calcium dependent event that initiates a sequence of downstream intracellular signals that result in cytoskeletal reorganization allowing several granule-granule fusion events and subsequent docking

with the plasma membrane and release of soluble mediators (168, 169). This mechanism of widespread granule release is called compound exocytosis or degranulaton. IgE crosslinking is a critical mechanism of mast cell activation. However, mast cells are capable of responding to stimuli independent of IgE crosslinking.

In Chapter III we reported a novel co-receptor activation system for mast cells mediated through crosstalk of the α2β1 integrin and c-met receptors. Immune complexes formed by *Listeria* bound to antibody and C1q activates mast cells. This interaction occurs via the α2β1 integrin binding to C1q and Internalin B (InlB) expressed by *Listeria* binding to c-met. The engagement of both receptors was required *in vivo* for mast cell activation and initiation of immune response to *Listeria.* This co-receptor engagement results in release of interleukin-6 (IL-6) from mast cells following 1 hour of stimulation both *in vivo* and *in vitro*. The mechanism/s by which mast cells initiated this rapid release of mast cell IL-6 was not known.

To understand molecular mechanisms by which α2β1 integrin/c-met binding to *Listeria* immune complex stimulated IL-6 secretion, we compared mast cell activation through *Listeria*-IC and IgE crosslinking. Surprisingly, there were few similarities in terms of mechanism or mediator release. In fact, mast cells contain distinct subsets of granules that can be differentially released, much like platelets, neuronal and pancreatic beta cells. The FcRγ common chain is not required for secretion of IL-6, although previous studies have demonstrated that the FcRγ is required for IgE crosslinked activation of mast cells. These studies suggest that signals leading to IL-6 secretion are distinct from the traditional FcR ITAMs that occur during stimulation of the FcεR. Stimulation via the *Listeria* immune complex results in a unique mechanism of mast cell

activation. These studies demonstrate a mechanism of secretory cell degranulation and define a novel role for mast cell mediator release in host defense.

Results

The study of mechanisms of mast cell degranulation requires a large number of cells. Therefore, we generated fetal skin mast cell (FSMCs) cultures which generate large numbers of mast cells that are mature and morphologically similar to PMCs and express the α2β1 integrin by flow cytometry. Since all of our previous work was carried out using PMCs, we compared the response of WT and α2-null PMCs to FSMCs following *Listeria* immune complex activation to determine if there was a difference in the ability of these two mast cell model systems in expression of α2β1 integrin and in response to stimulation (Figure 11A and B). WT and α2-null FSMCs and PMCs and respond to *Listeria* immune complex-mediated signaling in an α2-integrin subunit dependent manner and released of IL-6 (Figure 11C). Therefore, FSMCs were used in the remainder of the studies to analyze the mechanism mast cell activation and secretion.

To evaluate the α2β1 integrin/c-met engagement in mast cell activation we compared IL-6 secretion and β-hexosaminidase release (a measure of degranulation) following the activation of WT and α2-null FSMCs stimulated with either *Listeria* immune complex or IgE cross-linking (Figure 12). *Listeria* immune complex stimulated IL-6 secretion from WT FSMC, but not α2-null FSMCs, as previously reported (139). In contrast, WT and α2-null FSMCs released similar, low levels of β-hexosaminidase in response to *Listeria* immune complex. IgE- crosslinking, failed to stimulate IL-6

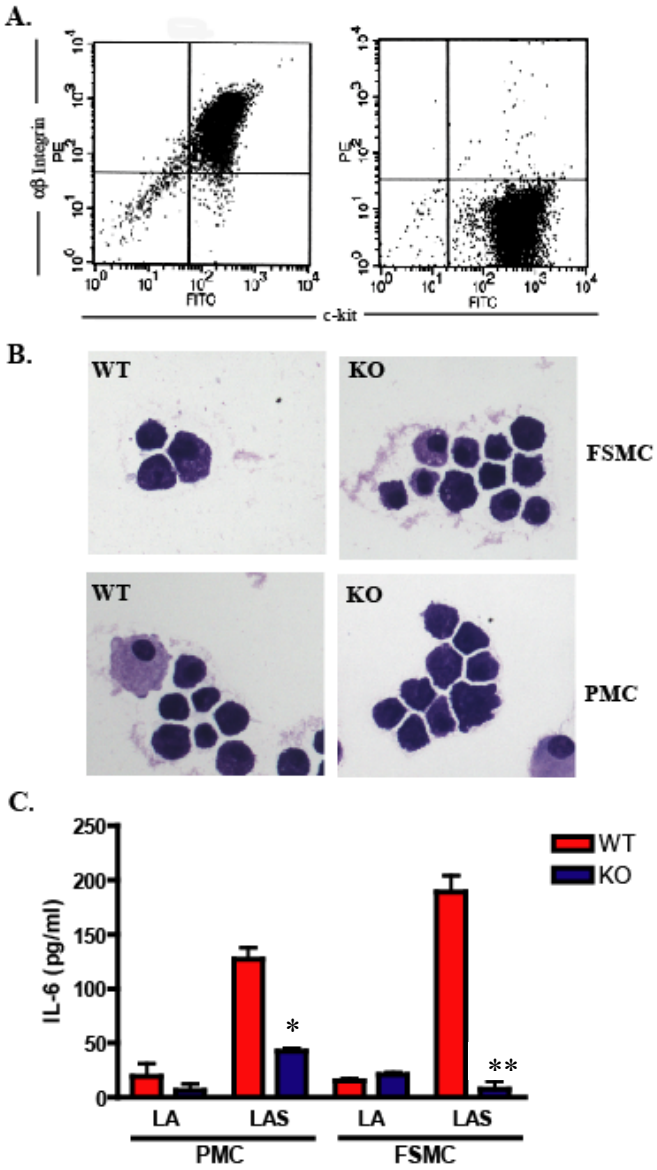

Figure 11. Fetal skin mast cells represent a culture of connective tissue mast cells that express the α2β1 integrin. FSMCs were compared to primary PMCs, connective tissue type mast cells. (A) The expression of the α2-integrin subunit (CD49b) and c-kit was determined by flow cytometry. The FSMCs were >85% positive for c-kit-FITC and CD49b-PE (B) FSMCs and PMCs show similar morphology. (D) WT and α2-null FSMCs and PMCs were stimulated with *Listeria* plus anti-*Listeria* antibody (LA) or *Listeria* plus anti-*Listeria* antibody and serum (LAS) for 1 hour at 37 ºC. The cell-free supernatants were analyzed for IL-6 release by ELISA. IL-6 concentrations are expressed as mean ±SEM. All data are representative of at least 3 similar experiments.

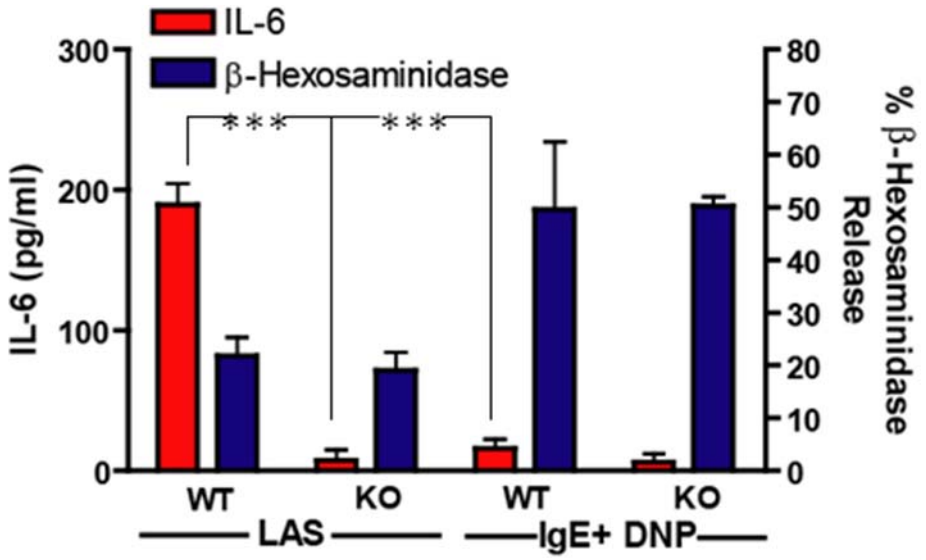

Figure 12. *Listeria* immune complex stimulation results in IL-6 release without degranulation. WT and α2-null FSMCs (5 x 10^4 cells/well) were stimulated with *Listeria* plus anti-*Listeria* antibody and serum (LAS) for 1 hour or anti-DNP IgE overnight followed by 1 hour stimulation with DNP-HSA (IgE + DNP). The levels of IL-6 (white bars) and β-hexosaminidase (black bars) were quantified as described in the Methods. P values were determined using the unpaired students t-test. All experiments were performed in duplicate. Data is expressed as mean ±SEM and is representative of at least 3 similar experiments.

secretion from WT and α2-null FSMCs at one hour, but resulted in similar, robust levels of β-hexosaminidase. These results indicate that IL-6 secretion in response to *Listeria* immune complex requires the α2β1 integrin. In contrast, there is no defect in the ability of α2-null FSMCs to respond to IgE-crosslinking.

One of the classic features of degranulation in response to IgE crosslinking is the requirement of calcium mobilization, which is a crucial second messenger in downstream signaling events. We examined the ability of WT and α2-null FSMCs to mobilize calcium in response to *Listeria* immune complex stimulation, IgE crosslinking or the calcium ionophore, ionomycin. But the WT and α2-null FSMCs mobilized calcium in a similar manner following stimulation with IgE crosslinking or ionomycin (Figure 13A and B). In contrast, neither WT nor α2-null FSMCs failed to mobilize calcium following *Listeria* immune complex stimulation (Figure 13B). To further examine the requirement of extracellular calcium mobilization we evaluated in calcium-free and calcium-containing Hanks Balanced Salt Solution (HBSS) stimulation of FSMCs by either IgE-crosslinking or *Listeria* immune complex. *Listeria* immune complex stimulation resulted in IL-6 release regardless of the presence or absence of calcium in the media (Figure 13C). However, as previously demonstrated, calcium-free media inhibited histamine release via IgE cross-linking (Figure 13C). These results demonstrate that extracellular calcium mobilization is not required for the immune complex stimulation of IL-6 secretion but is required for degranulation in response to IgE crosslinking. To determine the effect of intracellular calcium signaling in degranulation,

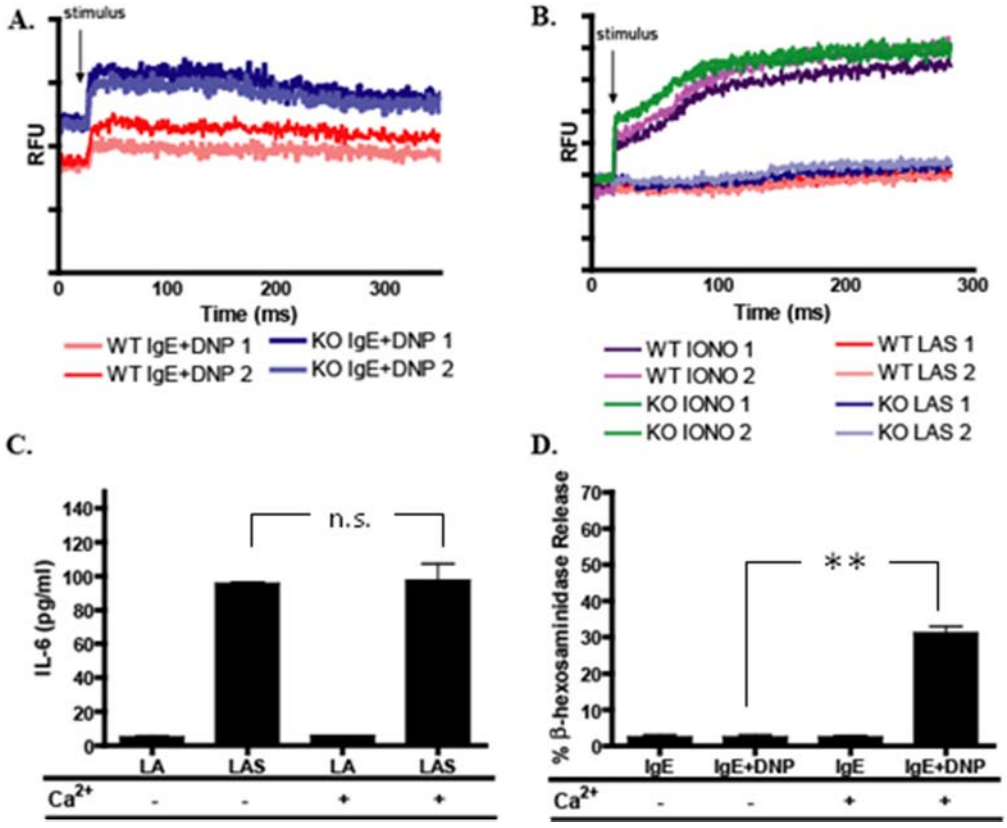

Figure 13. Calcium mobilization is not required for IL-6 release. WT and α2-null FSMCs (1 x 10^6) were treated with (A) anti-DNP IgE overnight and then stimulated with DNP-HAS or (B) *Listeria* plus anti-*Listeria* antibody and serum (LAS) or ionomycin (2μm) over the indicated time course. The results of two independent experiments are shown in each graph. (C-F) WT FSMCs were treated with anti-DNP IgE alone or anti-DNP IgE overnight and then stimulated with either DNP-HSA or cells were treated with *Listeria* plus anti-body (LA), or *Listeria* plus antibody and serum (LAS) for 1 hour in HBSS with Mg^{2+} in the presence (white bars) or absence of Ca^{2+} (black bars). IL-6 (C) β-hexosaminidase (D) and histamine (E) were analyzed in cell free supernatants by ELISA. (F) WT FSMCs were treated with anti-DNP IgE alone or anti-DNP IgE overnight and then stimulated with DNP-HSA or cells were treated with *Listeria* plus anti-body (LA) or *Listeria* plus antibody and serum (LAS) for 1 hour in the presence or absence of the calcium chelator, BAPTA (2.5μM). Cell free supernatants were analyzed for IL-6 and β-hexosaminidase release. Experiments were performed in duplicate and are expressed as mean ± SEM. P values were determined by the Students t test (***$p<0.001$)

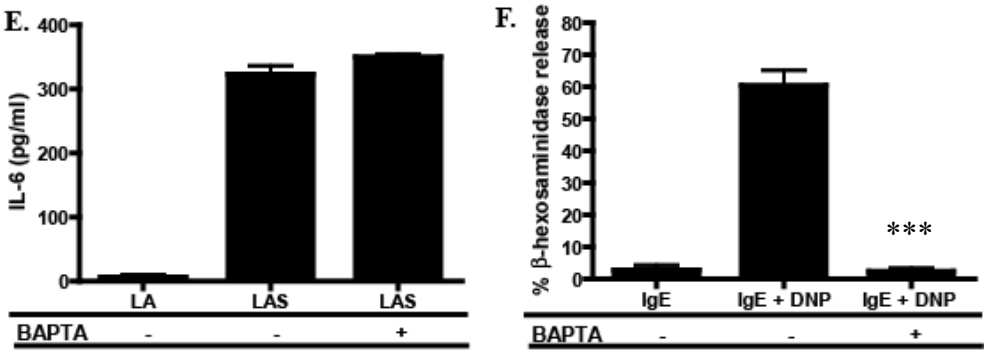

Figure 13. Continued

intracellular calcium signaling was inhibited with the calcium chelator, BAPTA. There was no defect in *Listeria* immune complex induced IL-6 release from WT FSMCs in the presence of BAPTA. But IgE cross-linking mediated degranulation is reduced (Figure 13D). These results further indicate that calcium mediated signaling events are not required for *Listeria* immune complex stimulation of mast cells but are essential for degranulation following IgE crosslinking.

Mast cell degranulation in response to IgE-crosslinking is known to be very rapid (0-10 min). β-hexosaminidase is a marker of the extent of degranulation. However, histamine and serotonin are granule associated mediators that are responsible for the physiologic responses following IgE-mediated degranulation. Our studies have evaluated IL-6 secretion 1 hour following stimulation To determine whether IL-6 is released at earlier time points following stimulation with *Listeria* immune complex or IgE crosslinking, we analyzed the levels of IL-6, histamine, and serotonin at 0, 5, 15, 30, and 60 min. following stimulation. WT but not α2-null FSMC stimulation by *Listeria* immune complex resulted in high levels of IL-6 release as early as 30 min post-stimulation (Figure 14C). IL-6 levels increased until 60 min. Neither histamine nor serotonin was detected following stimulation with *Listeria* immune complex WT or α2-null FSMCs (Figure 14A and B). However, both WT and α2-null FSMCs released high levels of histamine and serotonin within 5-10 min after FcεR stimulation (Figure 14D and E). The magnitude and kinetics of release were identical. IL-6 was not observed over the time course following IgE crosslinking (Figure 14F). These results demonstrate that IgE crosslinking and *Listeria* immune complex result in non-overlapping release profiles with distinct kinetics and secreted mediators.

Mast cells contain a number of preformed mediators that can be rapidly released upon stimulation. Although previous reports demonstrated that IL-6 was synthesized de novo by mast cells, the rapid nature of IL-6 release in response to *Listeria* immune complex suggests that it may be pre-formed in mast cells. To determine if IL-6 is preformed or rapidly synthesized, transcription and translation were inhibited using actinomycin D and cyclohexamide, respectively. Treatment of FSMCs with actinomycin D or cyclohexamide failed to inhibit IL-6 release by WT FSMCs after 1 hour of immune complex stimulation (Figure 15). The lack of inhibition by actinomycin D and cyclohexaminde demonstrates that IL-6 is preformed and not synthesized in the mast cell and that α2β1 integrin-mediated mast cell activation results in release of the preformed IL-6 granule pool.

Although IL-6 is not released at 1 hour following IgE crosslinking, IL-6 is synthesized and released 12 hours following IgE crosslinking as previously reported (170). Therefore, we compared the *de novo* production of IL-6 mediated by either *Listeria* immune complex activation or IgE crosslinking for 12 hours. As previously demonstrated IL-6 is synthesized following 12 hours of IgE crosslinking but not *Listeria* immune complex. The *de novo* synthesis of IL-6 was inhibited by actinomycin D and cyclohexamide. These data suggest mast cell secretion of IL-6 is regulated by two distinct pathways. In one, IL-6 preformed and stored in mast cell granules and ready for rapid release in response to specific stimulation. In the other, IL-6 is transcriptionally regulated and secreted at later time points in response to IgE crosslinking. In addition, these data define at least two distinct pathways leading to either rapid mast cell secretion of distinct granule components in response to *Listeria* immune complex activation or

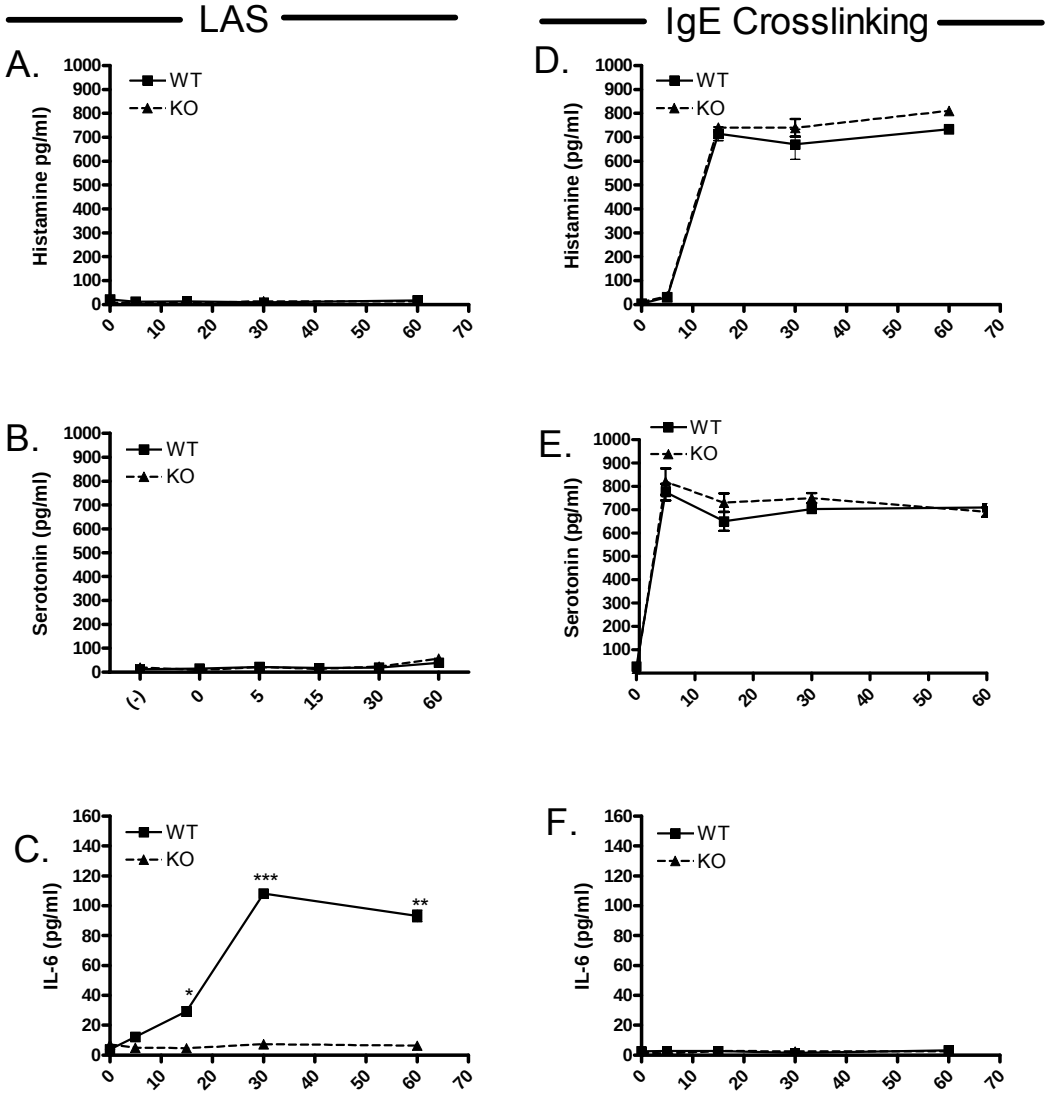

Figure 14. Different stimuli result in selective mast cell mediator release over time. WT and α2-null FSMCs (5 x10^4/well) were treated overnight with either anti-DNP IgE followed by stimulation with DNP-HSA (IgE + DNP) or with *Listeria* plus antibody and serum (LAS) for the indicated time points. Cell free supernatants were collected and analyzed for (A) histamine (B) serotonin or (C) IL-6. Data represent mean ± SEM and are representative of at least 3 similar experiments. P values were determined by performing statistical analysis using the Students t-test (*$p<0.05$, **$p<0.01$,***$p<0.001$)

IgE induced degranulation of mast cells. IL-6 secretion is released in an α2β1 integrin manner via a mechanism different from IgE stimulated degranulation.

I have now demonstrated that mast cells contain pre-formed IL-6 and are capable of calcium-independent, selective release of IL-6 in response to *Listeria* immune complex. IgE-mediated mast cell degranulation is independent of the classical mechanisms of protein exocytosis via the trans-Golgi network (170). To determine the mechanism by which mast cells release pre-formed IL-6, we inhibited the trans-Golgi network protein transport mechanisms with brefeldin A (BFA) or monensin. The *Listeria* immune complex stimulated release of IL-6 at 1 hour was inhibited by both BFA and monensin (Figure 16A). In contrast IgE crosslinking mediated release of histamine is not significantly inhibited by BFA or monensin (Figure 16B). These results suggest that IL-6 and histamine are stored in vesicles that utilize distinct mechanisms for release. Previous data demonstrated that histamine granules do not utilize the trans-Golgi network protein transport mechanism and are not BFA/monensin sensitive. However, IL-6 is stored in vesicles that are sensitive to BFA and monensin and therefore require ER-Golgi transport mechanisms.

We predict that IL-6 and histamine are preformed and stored in different mast cell granule pools. I collected fractions of mast cell post nuclear supernatant (PNS) isolated over a 2 layer Percoll gradient that separated the cellular contents by size. 200ul fractions were collected and analyzed them for IL-6 and histamine (Figure 17A and B). In both WT and α2-null FSMCs there is a similar distribution of IL-6 and histamine in the granule fractions. IL-6 is exclusively stored in subset of small sized fractions (5-7) with only baseline levels of IL-6 in the remaining larger fractions. The major histamine

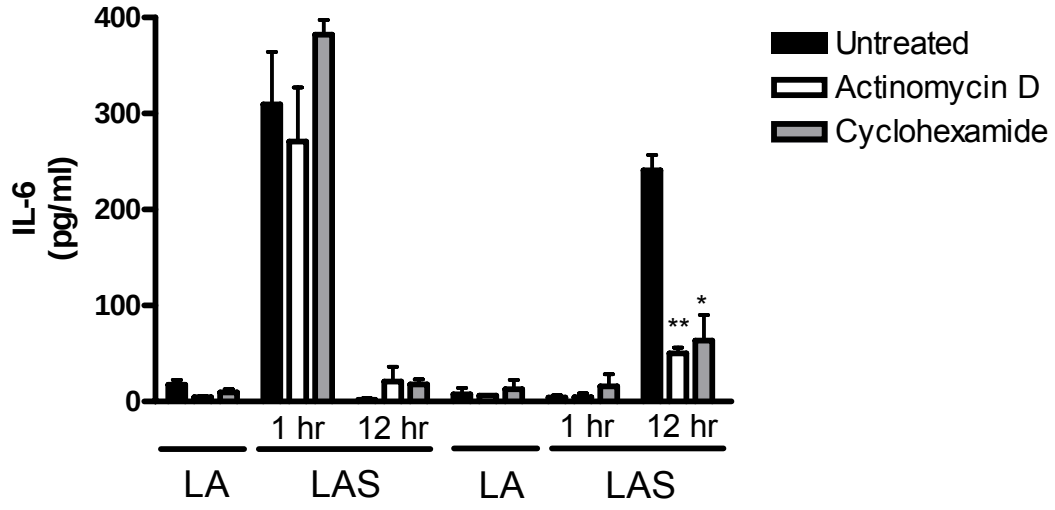

Figure 15. Pre-formed IL-6 is released following immune complex stimulation. WT FSMCs ($5x10^4$/well) were stimulated with *Listeria* plus antibody (LA), *Listeria* plus anti-*Listeria* antibody and serum (LAS) or anti-DNP IgE overnight (IgE) plus DNP-HSA (IgE + DNP) for 1 or 12 hours at 37 ºC in the presence or absence of actinomycin D or cyclohexamide. Cell free supernatants were collected and analyzed for IL-6 by ELISA. Results are represented as mean ± SEM and are representative of one of at least 3 separate experiments. P values were determined by the Students t test (*$p< 0.05$, **$p<0.01$)

fraction occurs within the high density fractions (16-19). These results demonstrate that IL-6 and histamine are not only selectively released; they are also differentially stored in mast cells.

Mast cell granules store histamine and serotonin, which can be visualized by immunofluorescence. Using serotonin as a marker of mast cell granules, we employed confocal immunofluorescence to determine the subcellular localization of IL-6 and mast cell granules. IL-6 (green) is stored in small granules or vesicles throughout the cell (Figure 18). Additionally, there is an aggregation of IL-6 positive small vesicles located in proximity to the plasma membrane. Serotonin (blue), on the other hand, is stored in large granules throughout the cell. In the merged image there is no overlap between the IL-6 and serotonin granules (Figure 18). In addition, smaller, possibly serotonin granules are identified. Serotonin and IL-6 are not co-localized even in small granules further indicating that IL-6 is not stored with histamine or serotonin, markers of classic mast cell granules. Taken together, these results demonstrate that mast cells differentially store IL-6 and histamine and these preformed mediators can be selectively released in response to different stimuli.

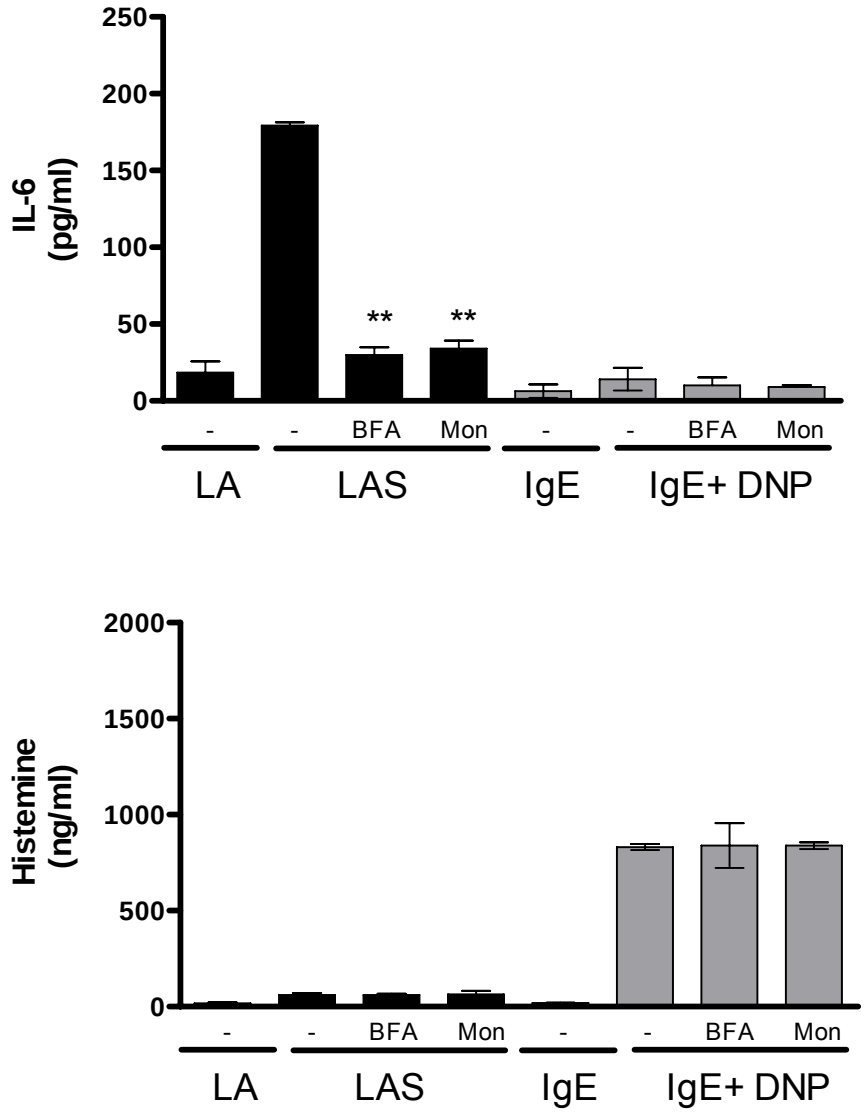

Figure 16. IL-6 but not histamine release requires functional endosomal trafficking. WT FSMCs ($5x10^4$/well) were stimulated with *Listeria* plus antibody (LA), *Listeria* plus anti-*Listeria* antibody and serum (LAS), or anti-DNP IgE overnight (IgE) plus DNP-HSA (IgE + DNP) for 1 or 12 hours at 37 ºC in the presence or absence of monensin (Mon) or brefeldin A (BFA) . Cell free supernatants were collected and analyzed for (A) IL-6 and (B) histamine by ELISA. Results are represented as mean ± SEM and are representative of one of at least 3 separate experiments. P values were determined by the student t test (**$p<0.01$).

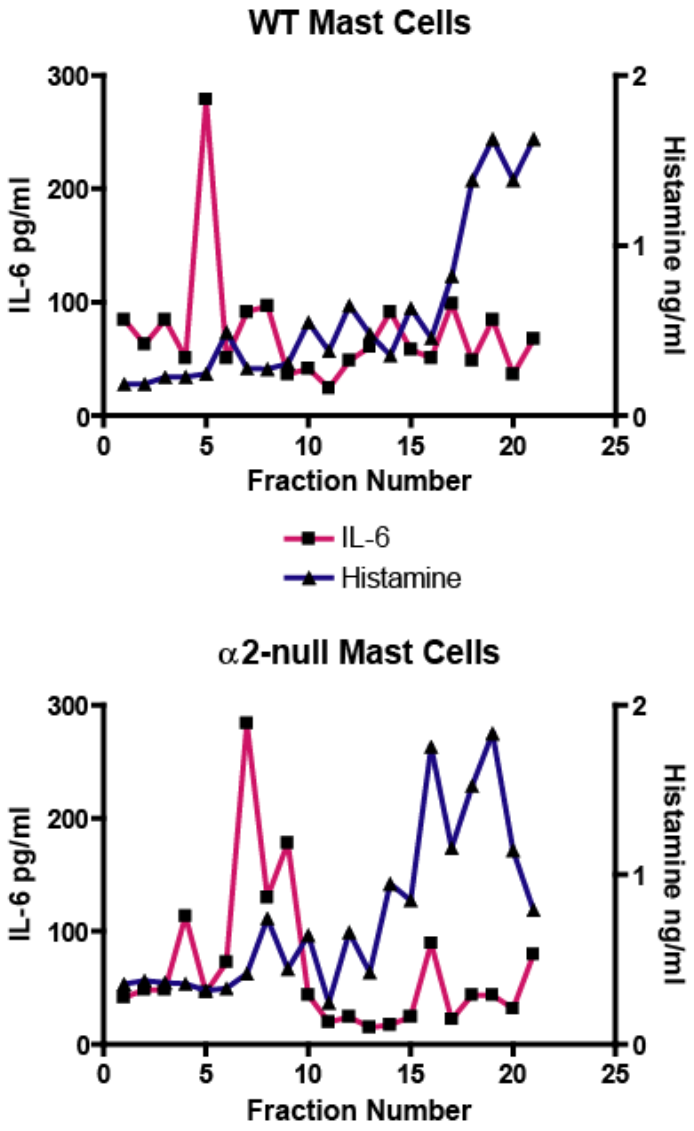

Figure 17. IL-6 and histamine are differentially stored in mast cell granules. Post-nuclear supernatants from WT (A) and α2-null (B) FSMCs (1 x 10^7) were separated over a 2-layer Percoll gradient (1.05 g/ml and 1.12 g/ml). 200 µl fractions were collected from the top of the gradient. Each fraction was analyzed by ELISA for IL-6 (solid line) and histamine (dashed line). Data were combined from 4 separate experiments and represent mean ± SD.

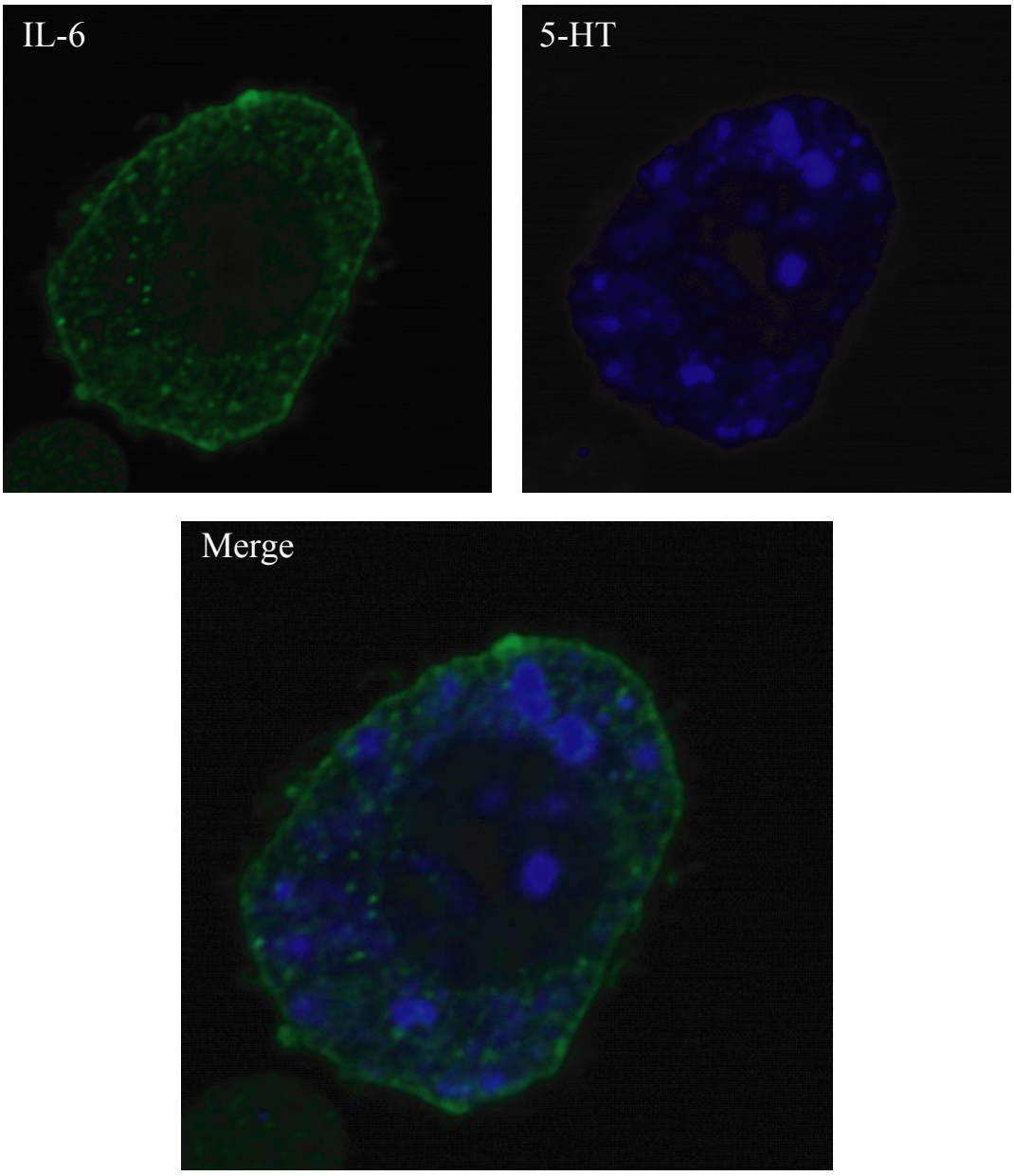

Figure 18. IL-6 is not localized in mast cell granules. Confocal immunoflorescence was used to identify (C) IL-6 (green) and (D) serotonin (blue) and (E) the merged image localization within mast cell granules. The image is representative of 3 separate experiments.

Conclusions

The α2β1 integrin is required for mast cell activation in response to *Listeria* immune complex (139). To understand differential mechanisms of mast cell activation, we compared α2β1 integrin stimulation via *Listeria* immune complex and FcεRI stimulation by IgE crosslinking. Stimulation with *Listeria* immune complex results in release of IL-6 without concomitant release of histamine or serotonin. In response to IgE crosslinking, histamine and serotonin are released in the absence of IL-6. β-hexosaminidase was released but significantly lower levels than the amount observed following IgE crosslinking. β-hexosaminidase is a lysosomal protein marker that to this point does not have known biological function in mast cell responses. It is used as a marker of the *extent* of degranulation and mast cell activation. We hypothesize that the lower level of β-hexosaminidase observed in response to *Listeria* immune complex is a measure of the less intense activation state of the mast cell.

IL-6 released in response to *Listeria* immune complex was insensitive to inhibition by actinomycin D or cyclohexamide and therefore preformed and stored within the mast cell. There is no immediate release of IL-6 in response to IgE crosslinking, FcεRI is known to induce transcriptionally regulate IL-6 release via NF-κB (171). Although IL-6 is not synthesized *de novo* following α2β1 integrin mediated activation, other proinflammatory molecules may be synthesized and subsequently released following *Listeria* immune complex stimulation. This suggests that in addition to the rapid, early events following IgE crosslinking and *Listeria* immune complex activation, there are differences in the later events of activation that regulate the secretion of IL-6 and possibly other cytokines.

α2β1 integrin mediated IL-6, but not FcεRI mediated histamine release is sensitive to inhibition by BFA or monensin, two well characterized protein secretion inhibitors with defined mechanisms of action on TGN vesicular transport. BFA inhibits vesicle movement through the cis-Golgi network, while monensin inhibits transport through the distal compartment of the Golgi apparatus (172). Confocal microscopy demonstrated that IL-6 is not stored in large mast cell granules, but rather is stored in smaller granules that are somewhat distributed throughout the cell but are also focused in proximity to the plasma membrane. In contrast, granules containing serotonin are larger and localized throughout the cell. IL-6 and histamine granules are expressed in different populations that can be separated on a 2-layer Percoll gradient. In accordance with our observations using confocal microscopy, IL-6 granules separate to the less dense fractions, while the larger histamine granules are in the more dense fractions. We hypothesize that in addition to histamine or IL-6, these granule populations likely express other molecules that are important in initiating the specific response to these differential stimuli.

CHAPTER V

α2β1 INTEGRIN MEDIATED REGULATION OF EARLY ADAPTIVE IMMUNE RESPONSE TO *LISTERIA MONOCYTOGENESE*

Introduction

The adaptive immune response to *Listeria* is T cell–mediated and required for complete resolution of the infection. When infected with *Listeria*, nearly 80% of SCID mice, which lack T and B cells, survive (76). However, sterilizing immunity is not reached until weeks or months following infection. In *Listeria* infection the innate immune response is sufficient to control the infection, but the adaptive immune response is important for sterilizing immunity. Between 7–10 days post-infection the number of *Listeria*-specific effector T cells peaks, eliminating remaining infected cells, and then retract, leaving long-lived memory cells. Both $CD4^+$ and $CD8^+$ T cells contribute to bacterial clearance through destruction of infected cells and cytokine secretion, which directs macrophage activation and granuloma formation (173-175)

We have demonstrated that expression of α2β1 integrin is required for 1 hr production of IL-6 and 6 hour neutrophil recruitment following intraperitoneal *Listeria* infection. This cytokine contributes to the cytokine milieu that governs the innate immune response to *Listeria* infection. However, other cytokines are also important in mediating the inflammatory response to *Listeria*. In the α2-null mice, there was a delay in bacterial clearance at 2 days post infection, by 7 days there was no difference in bacterial load (12). In addition to controlling bacterial load, the innate immune response directs the development of an appropriate antigen specific adaptive immune response.

Th1 cells develop from naïve T-lymphocytes and produce IFN-γ to activate macrophages. $CD8^+$ T cells kill infected cells by secreting their cytolytic proteins, granzyme and perforin. Both of these cell types express α2β1 (VLA-2) integrin in their effector stages (60). Therefore, we examined the role of the α2β1 integrin in the innate, adaptive and secondary immune response to *Listeria* infection.

Results

α2β1 integrin expression on mast cells is required for IL-6 production at 1 hour and neutrophil recruitment at 6 hours following infection with *Listeria*. However, IL-6 is not a chemoattractant. To define chemoattractants responsible for neutrophil influx following *Listeria* infection, we measured the levels of IL-1β, leukotriene B_4 (LTB_4), and TNF-α. in the peritoneal fluid of WT and α2-null mice. WT mice, but not α2-null mice demonstrated a marked increase of IL-1β and IL-6 levels at 1 hour post-infection with *Listeria* that was diminished by 6 hours post infection (Figure 19 A and B) (176). In addition, there was a small increase in the level of TNF-α in the WT, but not α2-null mice at 1 and 6 hours (Figure 19C). WT mice, but not α2-null mice manifested a robust influx of PMNs into the peritoneal cavity that peaked at 6 hours after infection, as previously reported (Figure 19D) (12). The level of IL-1β that we observe is consistent with several reports demonstrating the neutrophil chemotactic properties of IL-1β. Although LTB_4 is secreted by mast cells and is a potent stimulator of neutrophil migration, I did not observe increased LTB_4 above baseline at the timepoints tested (data not shown). Therefore, in

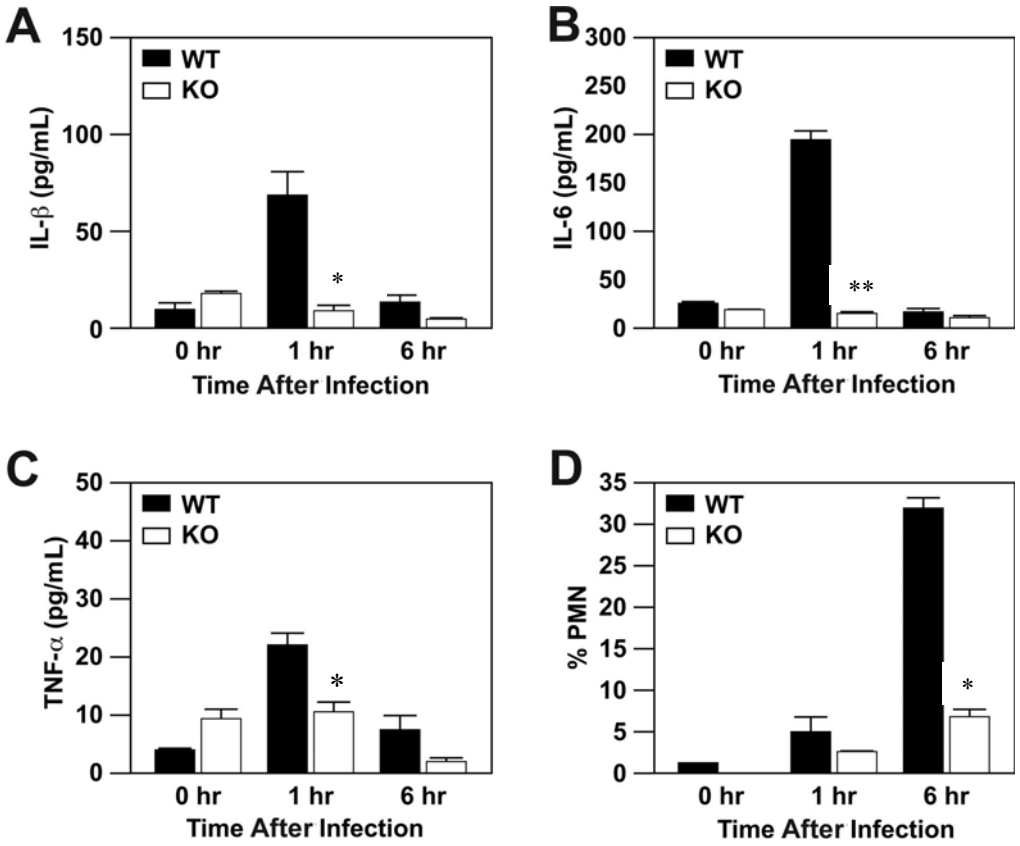

Figure 19. WT mice, but not α2β1 integrin-deficient mice display increased levels of IL-1β, IL-6, and TNF-α in response to *Listeria* infection. WT and α2β1 integrin–deficient mice were infected with $5x10^4$ *Listeria*, interperitoneally. At the indicated time points after infection the concentration of IL-1β (A), IL-6 (B), TNF-α (C) and the percentage of neutrophils (D) were determined. Shown is a combination of experiments (mean +/- SEM) with each time point representing 3 mice. Statistical analysis was performed using the Students t-test (*$p<0.05$, **$p<0.01$)

addition to IL-6, several chemoattractants are secreted in an α2β1 integrin-dependent manner that may contribute to the acute inflammatory response to *Listeria monocytogenes.*

Expression of the α2β1 integrin on mast cells is required for IL-6 production and neutrophil recruitment in response to intraperitoneal *Listeria* infection (12). To compare the cytokine response to *Listeria* infection, we analyzed serum cytokine levels between 1 and 4 days after infection in WT and α2-null mice. The production of IL-6 and TNF-α in response to *Listeria* was α2β1 integrin dependent (Figure 20A and B). The levels of IL-6 in the serum peaked at 1-2 days post infection in WT mice and returned to baseline levels by 4 days post infection. No detectable IL-6 was observed in the α2-null mice. The time course for release of TNF-α was different from IL-6. Serum TNF-α was not detected until 4 days post infection in WT mice. No TNF-α was detected in α2-null mice. In contrast to IL-6 and TNF-α, IFN-γ was increased at 2 days post-infection with *Listeria* in both WT and α2-null mice (Figure 20C).

We previously demonstrated that the α2β1 integrin on mast cells is required for the early innate immune response to *Listeria*. In these studies, mast cell deficient, W/W^v mice failed to mount an early innate immune response with IL-6 production and neutrophil recruitment. The defect was identical to that observed in experiments using α2-null mice. Both IL-6 secretion and neutrophil recruitment is rescued in the W/W^v mice by reconstitution of WT but not α2-null mast cells. To determine if the defect in serum cytokine levels in the α2-null mice were due to mast cells, W/W^v mice and littermate controls were infected with *Listeria* and serum cytokine levels were analyzed over 4 days. W/W^v mice failed to produce either IL-6 or TNF-α. Littermate control

generated peak levels of IL-6 at day 1 and TNF-α at day4 (Figure 20A-C). These results suggest that mast cells and the α2β1 integrin contribute to both the early immediate innate immune response and to the important cytokine milieu that regulates the immune response to *Listeria*.

An effective innate immune response is critical for the development of an effective adaptive immune response. Although *Listeria* is cleared at 7 days in α2-null mice, we chose this model to evaluate the contribution of the α2β1 integrin to adaptive immunity. $CD4^+$ Th1 cells are the effector cell of the adaptive immune response required for clearance of *Listeria* infection. In response to stimulation with specific antigen, Th1 cells produce INF-γ which enhances the cell-mediated immune response by activating marcrophages. We characterized the antigen-specific $CD4^+$ and $CD8^+$ response in the peritoneal cavity, spleen and lymph nodes of mice infected with *Listeria* for 7 days by stimulating T cells with HKLM. In the spleen, draining lymph node and peritoneal fluid, there was a strong antigen specific $CD4^+$/IFN-γ^+ Th1 response to HKLM in the WT, but this was significantly lower in the α2-null mouse. (Figure 21A-C) In contrast, the antigen-specific $CD8^+$ T cell response was similar in the lymph node, spleen, and peritoneal fluid of both the WT and α2-null mouse (Figure 21D- F). These results suggest that the α2β1 integrin may be important for development of antigen specific T cells in some immune compartments.

Although α2-null mice cleared *Listeria* at 7 days, the role of the integrin in developing a memory response was evaluated. Mice primed and then infected with *Listeria* 4 weeks later, there was no defect in the ability of α2-null mice to clear the

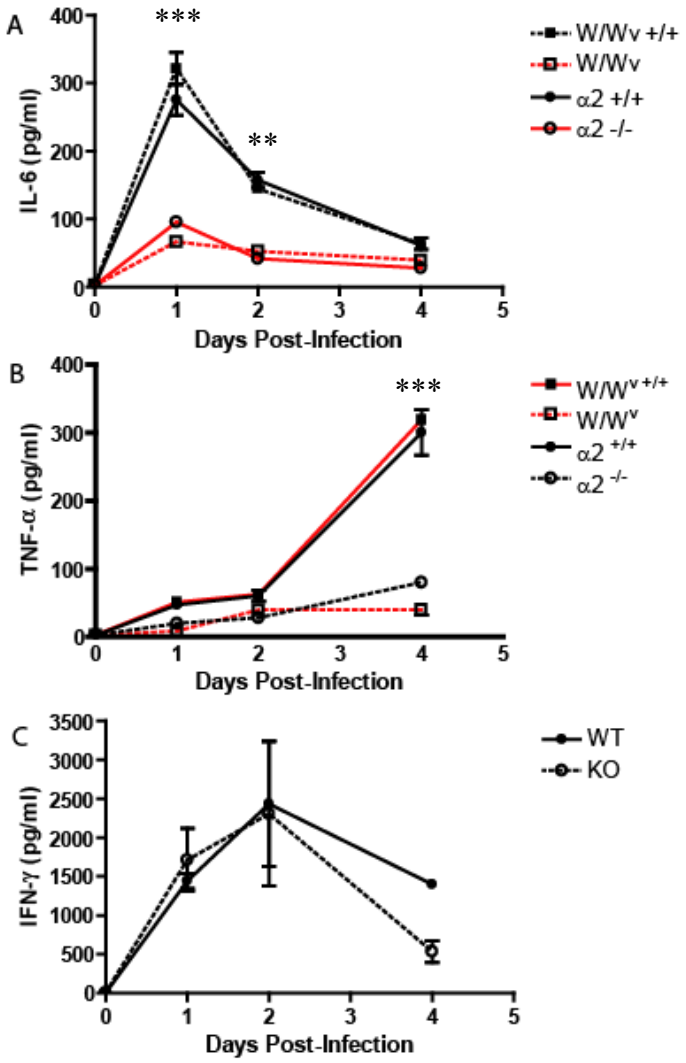

Figure 20. Cytokine milieu following *Listeria* infection. Mice were infection interperitoneally with 5 x 10^4 *Listeria* for 4 days. Serum from WT, α2-null, W/W^v or WT littermate control mice collected and analyzed for (A) IL-6 (B) TNF-α or (C) IFN-γ (WT and α2-null mice only). Each data point represents the mean ± SEM of at least 3 mice and is representative of 2 similar experiments. Statistical analysis was performed using the Students t-test (**p< 0.01, ***p<0.001).

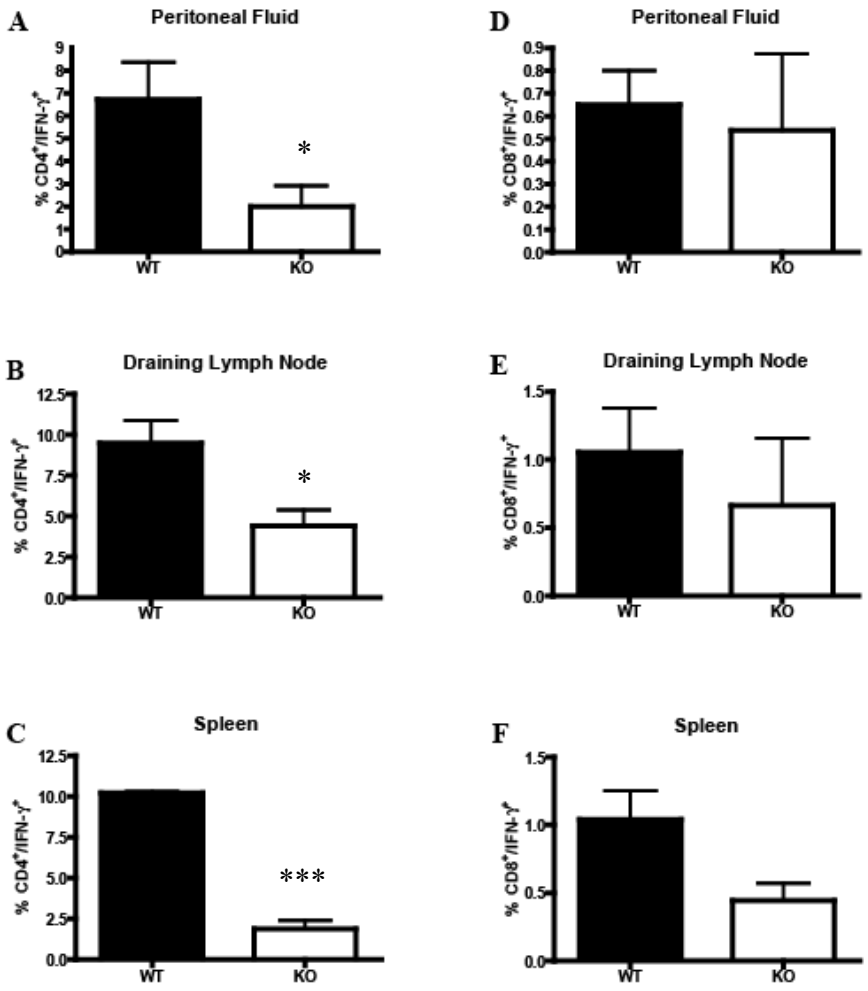

Figure 21. Characterization of Th1 and CTL response to *Listeria* infection. 3-4 mice (WT and α2-null) were infected interperitoneally with 5 x 10^4 *Listeria* for 7 days. Cells from spleens, draining lymph nodes or peritoneal fluid were harvested and restimulated for 5 hours in the presence of GolgiStop with HKLM. Cells were analyzed by flow cytometry for CD4-FITC, CD8-PerCP, IFN-γ-PE. Data represents the mean ± SEM of at least 3 mice. Statistical analysis was performed using the Students t-test (*$p<0.05$, ***$p<0.001$).

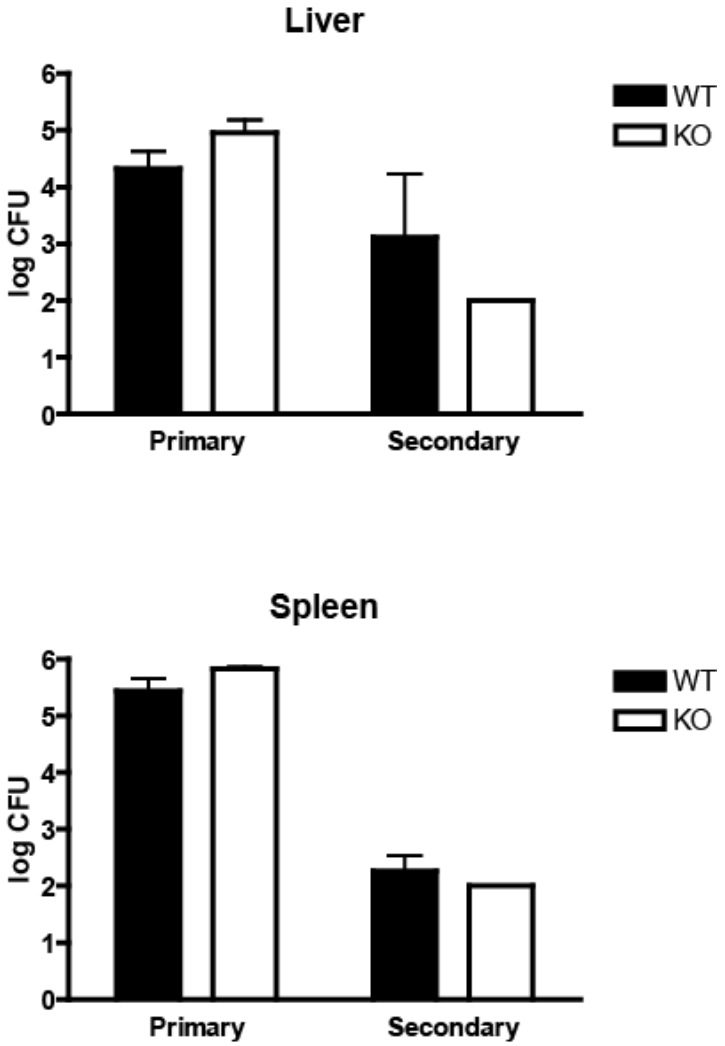

Figure 22. Memory response to *Listeria* infection. Mice (WT and α2-null) were infected interperitoneally with 5 x 10^5 *Listeria*. 5 mice from each genotype were sacrificed at day 3 post-infection, 5 mice were given a second does of 5 x 10^5 *Listeria* 14 days later. Spleens and livers were harvested for logCFU. Data represents mean ± SEM. Results are representative of 2 similar experiments.

infection from the spleen and liver. In naïve mice, 3 days post-infection there was a bacterial burden of 5-6 log CFU in the spleen and 4-5 log CFU in the liver in both WT and α2-null mice (Figure 22A and B). However, in *Listeria* primed mice, 3 days following challenge, the bacterial burden in the spleen was 2-3 log CFU in the liver and spleen in both WT and α2-null mice. These results suggest that there is no role for the α2β1 integrin in the development of the memory response to *Listeria* infection.

Conclusions

The α2β1 integrin-dependent immune response to *Listeria* is characterized by IL-6, IL-β and low level TNF-α release at 1 hour post-infection, followed by neutrophil recruitment at 6 hours. IL-6, IL-1β and TNF-α have roles in response to *Listeria* infection (84, 85, 177-179). In fact, nearly all TNF-αR deficient mice die by day 5 following intravenous infection with 5×10^4 organisms and 40-50% of IL-6 deficient animals die by day 6 following i.v. infection with 10^4 organisms (84, 85). Although IL-1β is not required for survival in response to *Listeria* infection, IL-1β enhances bacterial clearance (177, 178, 180). IL-1β is known to have neutrophil chemotactic properties at doses as low as 50pg/ml; no such chemotactic abilities are known for IL-6 (176). However, IL-6 deficient animals display decreased neutrophilia following *Listeria* infection, indicating that IL-6 may play an indirect role in recruiting neutrophils. We do not observe TNF-α release at the time points tested. However, several reports indicate that TNF-α levels appear at 2-4 days following infection, consistent with macrophage activation and priming of the adaptive immune response (177, 180). These studies demonstrate a role

for the α2β1 integrin to stimulate the generation of specific cytokine milieu for initiation the innate immune response.

The α2β1 integrin and mast cells are important in generating the 2-4 day cytokine response against *Listeria* through production of IL-6 and TNF-α but not IFN-γ. We also observed decreased antigen-specific Th1 cells in the α2-null mouse, indicating that the integrin may be important in Th1 cell differentiation or survival. However, there were no differences in the ability of WT or α2-null mice to clear a secondary infection, indicating that in the *Listeria* model, the memory response is intact.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

Mast cells are granulated cells of the immune system that reside in tissues that are closely associated with the external environment. Upon stimulation, mast cells can release several classes of mediators, de novo synthesized mediators, lipid derived mediators, and preformed mediators contained within mast cell granules. Multiple cytokines, chemokines, and growth factors can be de novo synthesized by mast cells. However, mast cell granules are far more limited in their content. Although mast cells were traditionally considered required solely for IgE mediated immune responses to allergens and helmenths, in the past 10 years the role of mast cells has expanded to include immunity to bacterial and viral pathogens. Previous studies from our laboratory demonstrated that infection of mice with *Listeria* requires mast cells expression of the α2β1 integrin for early immune responses.

Our studies have focused on the role of *Listeria* immune complex mediated activation of mast cells. An immune complex, containing *Listeria*, anti-*Listeria* antibody and C1q containing serum, results in α2β1 integrin-dependent activation of mast cells. This activation requires binding of the co-receptor, c-met, by *Listeria* InlB. However, this activation is not limited to *Listeria* responses. Co-stimulation of mast cells with an α2β1 integrin ligand (Type I Collagen or C1q) and a c-met ligand (HGF) results in activation. These results indicate that the role for the α2β1 integrin and c-met in mast cell activation

can extend past the innate immune response observed in *Listeria* infections and to several different disease models.

The co-receptor stimulation of mast cells results in release of preformed IL-6 from mast cells. The secretory mechanism of IL-6 is distinct from either IgE-crosslinking induced release of histamine and serotonin or IgE induced de novo synthesis of IL-6. In contrast to histamine and serotonin release, the release of IL-6 is independent of calcium mobilization but is dependent on a functional trans-Golgi network trafficking system. IL-6 is not stored in a classical mast cell granule containing histamine or serotonin. Rather, it is stored in small mast cell vesicles that are expressed throughout the cell and associated with the cell membrane. These studies predict an α2β1 integrin-dependent mechanism of piecemeal degranulation for IL-6 release.

Due to the defect in the innate immune response to *Listeria* and the fact that α2β1 integrin is expressed on T cells, it was predicted that the α2β1 integrin would be important in the development of the adaptive immune response to *Listeria* infection. Decreases in pro-inflammatory cytokine production and numbers of Th1 cells were observed in response to *Listeria* infection in the α2-null mouse, but this did not result in differences in bacterial clearance or secondary immune response. These results suggest that in response to *Listeria* infection, there is no defect in the adaptive immune response in α2-null mice. However, the α2β1 integrin may play a role in development of adaptive immunity to other microorganisms or in other disease models.

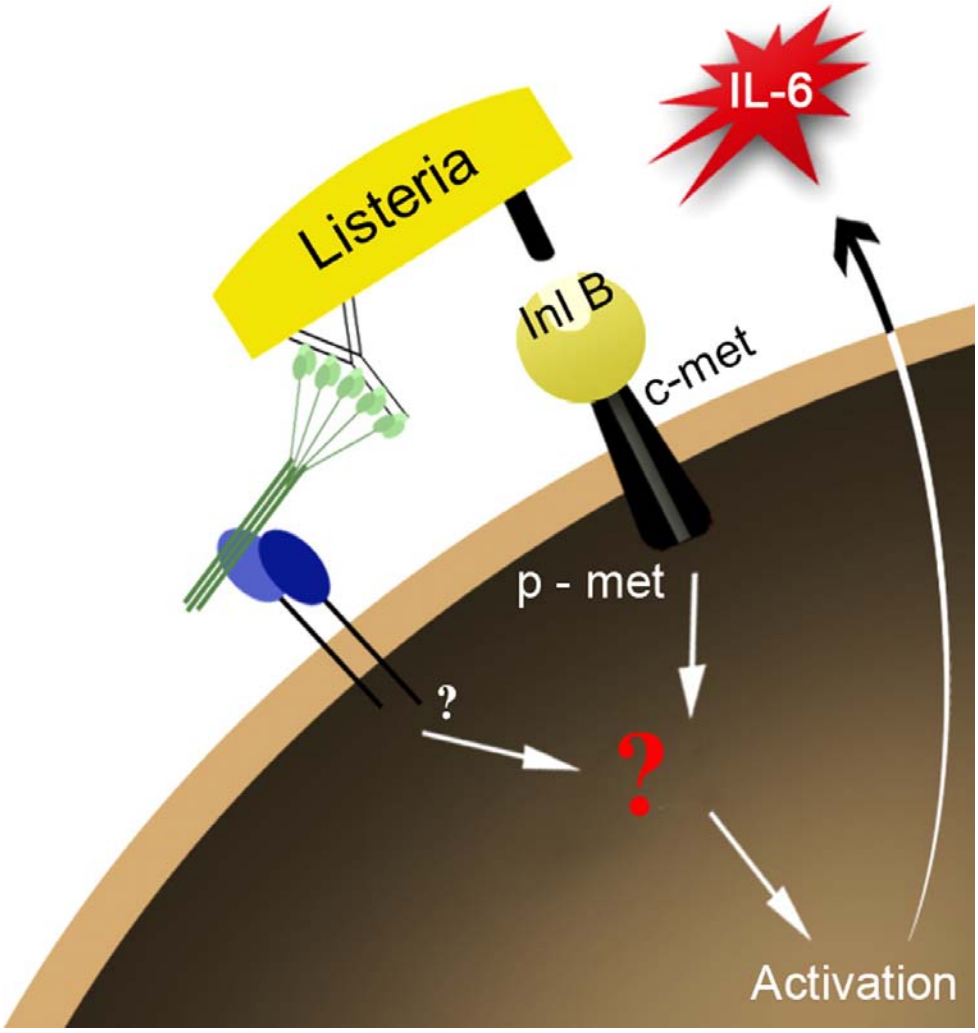

Figure 23. Predicted model of mast-cell activation through c-met and the α2β1 integrin. Mast cell stimulation through c-met and the α2β1 integrin results in cross talk between the 2 receptors, resulting in the activation of the mast cell leading to release of the pro-inflammatory cytokine, IL-6. Originally published in McCall-Culbreath et al ©American Society for Hematology

Implications for α2β1 Integrin/c-met Co-Stimulation

My studies have focused on the role of *Listeria* immune complex mediated activation of mast cells. Immune complex, containing *Listeria*, anti-*Listeria* antibody and C1q containing serum, results in α2β1 integrin-dependent activation of mast cells. This activation requires binding of the co-receptor, c-met, by *Listeria* InlB (Figure 23). However, it is not limited to *Listeria* responses. I demonstrated that co-stimulation of mast cells with an α2β1 integrin ligand (Type I Collagen or C1q) and a c-met ligand (HGF) results in activation.

HGF was originally identified as a mitogen for hepatocytes and as a scatter factor for a number of cell types (181). Although the role of the HGF/c-met interaction in tumor progression and tissue fibrosis has been extensively studied, the role of HGF and c-met in immunity is less well-defined. Data from several groups suggest that HGF promotes B and T cell migration, (182-184) counters the immunosuppressive effects of TGF-β, (185-187) suppresses dendritic cell function, (188) and reduces acute and chronic rejection (189). In this model, synergistic stimulation of mast cells by HGF or InlB and the α2β1 integrin results in activation of the innate immune response and the recruitment of neutrophils to the site of *Listeria* infection. Mast cells also secrete chymases that hydrolyze HGF to generate an HGF antagonist (190). This may serve as a negative feed-back loop to inhibit subsequent immune activation.

I now describe an example of cooperation between the α2β1 integrin and a receptor for either a critical growth factor, HGF, or for a *Listeria* protein required for internalization. This co-stimulation then leads to activation of the mast cell, a cell critical to the innate immune system. The cross-talk between integrins and c-met in endothelial

and epithelial cells regulates cell migration and invasion downstream of signaling pathways that activate FAK which transduces signals to adaptor molecules such as Src family kinases, PI3K, Phospholipase C and Grb2 (191). Following HGF or InlB binding, c-met is phosphorylated on Y1349 and 1356 which serve as docking sites for multiple signal transducers such as Grb2-associated binder1 (Gab1) and multiple Src homology 2 domains (192-194). Although HGF and InlB are both ligands for c-met, they lack sequence and structural similarity (195, 196). In addition, the data illustrate that these two ligands bind separate sites on c-met (94, 197, 198). In spite of their differences, both HGF and InlB activate the intrinsic phosphorylation activity of met and induce tyrosine phosphorylation at Y1349 (94) as well as cellular changes such as scattering and DNA synthesis (198). Isolated ligands of α2β1 integrin and c-met can stimulate mast cell activation in the absence of immune complex. Therefore, I propose that the α2β1integrin/c-met crosstalk occurs via downstream signaling pathways rather than by direct interaction at the cell membrane.

The ability of mast cells to release a pro-inflammatory cytokine in response to stimulation through the α2β1 integrin and c-met may give insight into poorly understood mechanisms of mast cell contribution to various diseases. Mast cells play both positive and negative roles in cancer progression, invasion and angiogenesis (199-203). Identification of the receptor/ligand interactions that activate mast cells in these diseases has been elusive. Mast cells can be found in the peri-tumoral stroma which is largely composed of type I collagen and fibroblasts that can be induced to produce HGF by tumor cell by secretion of IL-1β, bFGF, PDGF, prostaglandin E2 and TGF-β (204). A combination of HGF release and collagen interaction on the mast cells may result in mast

cell activation within the tumor microenvironment to induce tumor growth, differentiation or metastasis (Figure 24). To determine if mast cell α2β1 integrin/c-met interaction is important in tumor progression, tumor cells could be injected into mast cell deficient mice to determine the role of mast cells in tumor development. To determine the role of the α2β1 integrin and c-met, mast cell deficient mice should be reconstituted with WT and α2-null mast cells that have reduced levels of c-met using siRNA. Using the mast cell knock-in model will demonstrate a precise role for the α2β1 integrin and c-met on mast cells in modulating tumor development.

Several different cell surface receptors for C1q and other collectin family members have been reported, including the C1q receptor for phagocytosis enhancement (C1qRp), CR1, calreticulin (CRT), and binding protein for the globular head of C1q (gC1qbp) (205-218). The precise role of each receptor remains an area of active investigation. Although the ability of InlB to interact with the cellular glycoprotein gC1q-R/p32 (95) raised questions concerning whether gC1q-R was involved in the α2β1 and c-met-dependent PMC response. The findings presented here suggest that gC1q-R cannot replace the α2β1 integrin in initiating the innate immune response. Using isolated components in an *in vitro* system, PMC activation requires α2β1 integrin binding to either C1q or type I collagen plus the additional interaction of c-met with either *Listeria* or HGF. Although C1q and *Listeria* both interact with gC1q-R through either the globular head of C1q or InlB of *Listeria*, neither type I collagen nor HGF interact with gC1q-R. IL-6 secretion is similar regardless of whether the α2β1 integrin interacts with C1q or type I collagen and whether c-met is bound by *Listeria* or HGF. These data suggest that

although gC1q-R may play a role in *Listeria* internalization, there is no role for gC1q-R in α2β1 integrin/c-met induced mast cell activation.

I describe an innate immune response mediated by the α2β1 integrin binding C1q, a soluble, non-matrix factor. The α2β1 integrin is a cellular receptor for C1q, the first component in the complement cascade and mediator of innate immune response (139). *In vitro* interaction between α2β1 and C1q on mast cells resulted in activation and cytokine secretion. Using *in vivo* models, the C1q/α2β1 integrin interaction is required for innate immune response to *Listeria*. C1q-deficient mice do not release IL-6 or recruit PMNs into the peritoneum following *Listeria* infections. Because the homology between C1q and the integrin binding site of collagen is high, the importance of the integrin and C1q interaction is likely significant given the low amount of genetic drift between these two sequences over time. Although collagen and other ECM components are abundant in the peritoneum, collagen binding either does not occur, or is not sufficient to mediate mast cell activation following *Listeria* infection.

The α2β1 integrin in expressed on numerous cells, including cells of the innate immune system such as mast cells and NK cells as well as cells of the adaptive immune system such as a subset of activated T cells (28, 41, 60). The α2β1 integrin in these cells has been thought to act primarily through its ability to bind collagen in the extracellular matrix thereby affecting cellular localization. Our work is the first to show that α2β1 integrin can bind C1q and transduce a signal that is absolutely necessary, but not sufficient, to initiate a pro-inflammatory cytokine response. C1q is known to play an important role in immune complex diseases such as systemic lupus erythematosus,

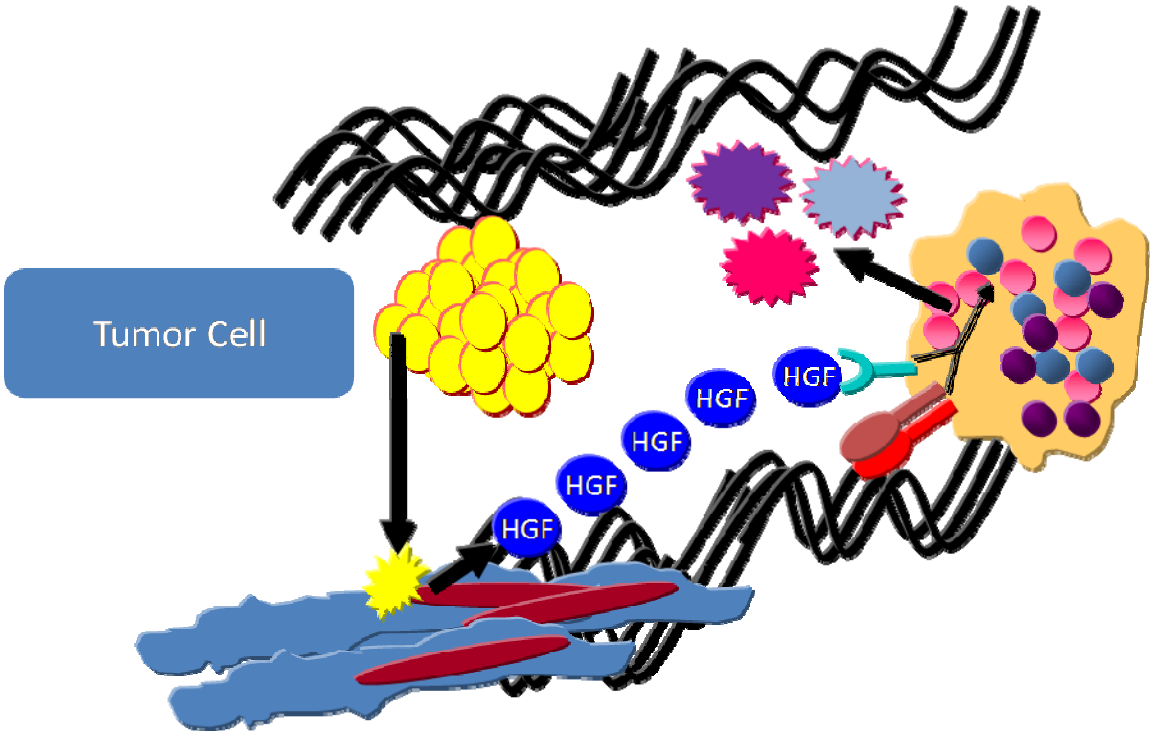

Figure 24. Proposed model for α2β1 integrin/c-met interaction in tumor models. Mast cells are expressed within the stromal space, rich with collagen, a ligand for the α2β1 integrin. Tumor cells induce HGF production by fibroblasts. The presence of both α2β1 integrin and c-met ligands may activate mast cells and induce the release of IL-6 which can modulate tumor proliferation and recruitment of inflammatory cells.

Arthus reaction, autoantibody-induced arthritis, glomerulonephritis and experimental autoimmune encephalitis (153, 219-221). Additionally, mast cells have been associated with these diseases (207, 222-225). Our findings provide a molecular mechanism linking C1q and activation of these inflammatory cells. Mast cells act as sentinels for the immune system at the body's boundaries. C1q binding of the α2β1 integrin is an initiating event of mast cell degranulation and, hence, an initial trigger for the subsequent inflammatory response.

There are several models of autoimmune disease that can be used to determine if the C1q-α2β1 interaction is important in autoimmune diseases. NZM2410 mice spontaneously develop a lupus like syndrome that is similar to the human disease resulting in production of anti-nucleic acid antibodies, proteinuria and glomerulonephritis (226). To determine the role of the α2β1 integrin in immune complex disease, the NZM2410 mice can be bred to α2-null or C1q-null mice and analyzed for markers of disease. Additionally, to determine the role of mast cells in this lupus model, the C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mast cell deficient mice can be bred to the NZM2410 mice. Lack of the α2β1 integrin may prevent the activation of mast cells and release of cytokines that would recruit inflammatory cells.

The role for mast cells in immunity has traditionally been limited to their role in IgE mediated allergic responses and immunity to parasites. Mast cells are known to play an important role in the early response of the immune system and can be stimulated through a range of receptors important in the early immune response such as TLRs 1, 3, 4, 6, and 9, complement receptors (CR2, 4, 5, C3aR, C5aR), and cytokine/chemokine receptors (IL-1R, IL-10R, IL-12R and IFN-gR) (117). I have demonstrated a novel α2β1

integrin-dependent and c-met-dependent pathway for mast cell activation that results in the rapid release of IL-6 and occurs independently of signals downstream of FcRγ. IgE crosslinking is the best described mechanism for mast cell mediator release and requires signaling FcRγ resulting in rapid degranulation and release of histamine and β-hexosamidase (116).

Implications of α2β1 Integrin Mediated IL-6 Release

There are three ways that mast cells release mediators following stimulation. Classical exocytosis is the mechanism which all cells, including mast cells release newly synthesized molecules through trafficking of Golgi-derived vesicles to the plasma membrane for docking and release. Compound degranulation occurs only in granulated cells and characterized by complete granule emptying through multiple intracellular granule-granule fusion events that precede their secretion (227). Piecemeal degranulation is characterized by partially emptying of single granules that do not fuse to one another or the plasma membrane (228). The precise molecular mechanisms and receptors involved in classical exocytosis and compound degranulation have been well defined. However, only very few studies have examined the molecular mechanisms responsible for piecemeal degranulation.

Stimulation of mast cells with *Listeria* immune complex results in release of preformed IL-6. The secretory mechanism of IL-6 is distinct from either IgE-crosslinking induced release of histamine and serotonin or de novo synthesis of IL-6. In contrast to histamine and serotonin release, the release of IL-6 is independent of calcium mobilization but is dependent on a functional trans-Golgi network trafficking system. IL-

6 is not stored in a classical mast cell granule containing histamine or serotonin. Rather, it is stored in small mast cell vesicles that are expressed throughout the cell and associated with the cell membrane. These studies predict an α2β1 integrin-dependent mechanism of piecemeal degranulation for IL-6 release.

Human mast cell secretory granules range in size 800-1000 nm (229). In a study using immature human cord blood mast cells, stimulation with IL-1 induced the localization of newly synthesized IL-6 to small 40-80nm vesicles (230). Due to the size and sensitivity to Golgi-transport inhibitors, IL-6-containing granules observed in my studies may be recently derived from the trans-Golgi network. In a variety of cell types, newly synthesized granule contents are preferentially released. Therefore, immature granules may be an important pool for granule release in mast cells as well. Or, similar to neurons, IL-6 may be stored in a mature organelle that is differentially regulated for release.

formation of immature granules occurs at the *trans*-Golgi network (TGN), and subsequent maturation steps occur beyond this compartment (231, 232). Once formed, immature secretory granules must be processed and remodeled to form mature secretory granules. Fusion of immature secretory granules contributes to the increased size and density of mature granules (233). In neuroendocrine cells, immature granules can undergo regulated exocytosis through the TGN, however fully mature granules become insensitive to this mechanism of release (234). Previous work has shown that rapid IL-6 release in response to the non-specific calcium ionophore A23187 was sensitive to the TGN inhibitors, BFA and monensin (170). IL-6 containing granules are reminiscent of immature granules based on size and sensitivity to TGN inhibitors. However, IL-6 is not

observed in fully mature granules suggesting that IL-6 granules represent a novel population of mast cell granules. BMMCs contain immature granules and may be used to determine if IL-6 is localized in these immature granules.

Conventionally, compound degranulation, a full all-or-nothing exocytotic response, is considered to be the primary mechanism of mast cell preformed mediator release. However, mast cells can undergo ultrastructural alterations of their electron-dense core indicative of secretion without degranulation which has been termed piecemeal degranulation (228, 235, 236). In many cell types (such as neuroendocrine, endocrine, and exocrine cells), a large reserve pool exists and only a small portion of the granules (~1%) are initially rapidly releasable (233). Unlike mast cells, neuroendocine cells, such as pancreatic islet β-cells, chromaffin cells, neurons rarely release the full content of their granule pool but rather undergo ultrastructural changes characteristic of piecemeal degranulation (234, 237). The granules in these cells exist in a range of states from being nonreleasable or in a pool that becomes releasable following a recruitment step, thought to be part of a so-called "ready-releasable" pool. (233)

Signaling events required for degranulation and cytokine secretion are distinct and depend on particular regulatory requirements of each pathway. Mast cell granules are known to contain histamine and serotonin as well as lysosomal proteins, β-hexosaminidase and cathepin D. Because of the expression of lysosomal proteins as well as specific granule markers, these organelles have also been called “secretory lysosomes.” They differ from conventional lysosomes by their electron density and expression of membrane markers (238). Conventional mast cell granules express VAMP2, 3, 8, and 7 which may govern the trafficking of mast cell granules through the

cell (164). It will be important to determine if IL-6 granules contain similar or different vesicle markers. These studies may give insight into the granule/vesicle trafficking mechanisms that govern mediator release in response to differential stimuli.

Several cell types, in addition to mast cells, contain secretory granules that are rich in preformed mediators and poised for release. Cells of the immune system, platelets, NK cells, cytotoxic T cells and neutrophils contain secretory granules that function to kill infected cells (NK and cytotoxic T cells) or aid in destroying phagocytosed pathogens (neutrophils). In many of these cells, the granules have been divided into subsets that have been characterized for their mediator content and stimulation for release (233). In contrast, only recently have mast cell granule pools been characterized to determine the nature of specific granule populations. Using mass spectrometry the content of the different subsets of neutrophil granules has been determined. Based on the subcellular fractionation of mast cell granule populations, other mediators contained within the distinct granule populations may be identified.

Mast cells were considered to contain a homogenous population of granules mainly comprised of histamine and serotonin. This would group them with pancreatic β-cells (insulin) and CD8+ T cells (perforin and granzyme) which contain a single granule population. In contrast, other granulated cells express multiple granule populations such as neutrophils (azurophil, gelatinase, specific) and platelets (α granules, dense granules) (233). I now propose that mast cells contain multiple granule populations that are differentially regulated for release. My results identify the α2β1 integrin as the crucial regulator of mast cell piecemeal degranulation in response to *Listeria* immune complex. In addition to mast cells, the integrin is expressed on platelets, NK cells and $CD8^+$ T

cells, all of which are granulated cells that exhibit varying degrees of degranulation following stimulation.

Platelets are activated upon stimulation with collagen through the α2β1 integrin and its co-receptor, GPVI (66). On $CD4^+$ T cells, adhesion to Type I Collagen via α2β1 integrin enhances IFN-γ production in response to activation with anti-CD3 antibody (239). In a similar manner, the α2β1 integrin may also serve to enhance the release of cytotoxic granules in $CD8^+$ T cells and NK cells. The α2β1 integrin is highly expressed on NK cells, but its function is currently unknown (41). One function of NK cells is antibody dependent cell-mediated cytotoxicity (ADCC) which results in release of cytotoxic granules. Fc receptors bind antibody to activate the NK cell. However, there may be a role for the α2β1 integrin in NK cell activation and degranulation serving as a co-activator of Fc-mediated ADCC activity. Aside from the ADCC, NK cells express receptors that serve to either activate or suppress their cytolytic activity. These receptors bind to ligands on target cells and have an important role in regulating the NK cytotoxic activity. It has been reported that crosslinking of α2β1 integrin on NK cells resulted in decreased cell motility and diminished cytotoxicity (240) (Figure 25). To determine the role of the α2β1 integrin in these granulocytes functional analysis of platelets, NK cells and $CD8^+$ T cells in α2-null mice should be performed.

Piecemeal degranulation of mast cells was indentified in electron micrographs of a number of human diseases and conditions, including contact hypersensitivity, delayed hypersensitivity, urticaria pigmentosa, bullous pemphgoid, melanoma, chronic

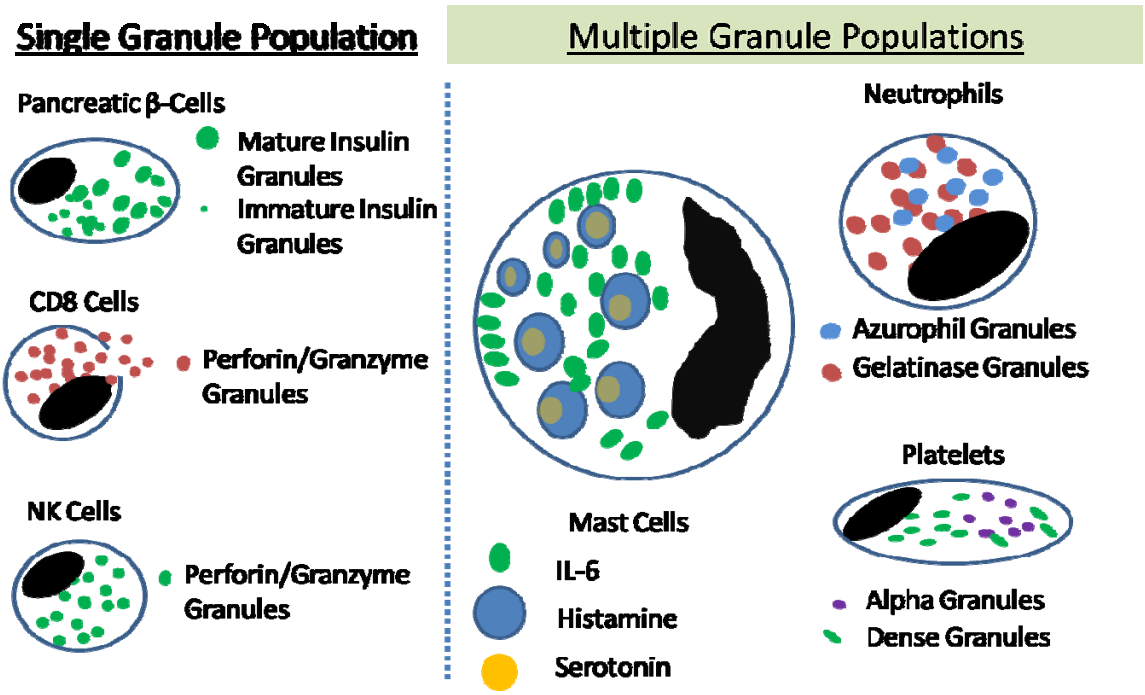

Figure 25. Populations of granules in granulocytes. Granulated cells can be grouped by the expression of multiple granule pools.

glomerulosclerosis, bullous pemphigoid, Crohn's disease, primary and metastatic tumors, skin wound healing and angiogenesis (227, 228). The receptor/ligand interaction in many of these responses has not yet been identified. It is interesting to note that in several of these disease models the α2β1 integrin (contact and delayed hypersensitivity, tumor development and angiogenesis) or immune complex (chronic glomerulosclerosis, bullous pemphigoid) has been described (13, 241-243). I hypothesize that immune complexes may interact with the α2β1 integrin on mast cells to induce piecemeal degranulation and affect disease pathogenesis.

In the present study I describe differential release of *preformed* mast cell mediators in response to different, biologically relevant stimuli. There are several IgE-independent disease processes in which mast cells play a vital role. For instance, mast cells, which have been demonstrated to be influential in a number of autoimmune and inflammatory disorders, are rarely seen to undergo compound degranulation during autoimmune or inflammatory processes (235). This suggests that mast cells employ selective or differential release of mediators through piecemeal degranulation. In response to immune complex, mast cells release α2β1-dependent IL-6 in the absence of compound degranulation. These results suggest that the α2β1 may be an important molecule in regulating mast cell responses in immune complex disease through modulating mediator release from mast cells.

Mast cells have been considered to be the sentinels of the innate immune response. They are poised at the surfaces first exposed to assault by the external environment. Activation by different stimuli can result in responses ranging from anaphylactic shock to anti-tumor responses. Since mast cells contain a number of stored

mediators and numerous other cytokines, chemokines and lipid derived molecules that can be *de novo* synthesized, it is imperative that specific mediator release is a tightly coordinated process. Regulation of mediator release ensures that the appropriate response to the specific stimuli is initiated. For many years it was thought that mast cells were simply on a hair trigger ready to be stimulated for rapid degranulation resulting in anaphylaxis. These studies add to the growing body of evidence that in addition to compound degranulation, mast cells are capable of selective release of specific mediators, without degranulation, in response to different stimuli.

Implications for α2β1 Integrin in Immune Response to *Listeria*

The α2β1 integrin-dependent immune response is characterized by IL-6, IL-β and low level TNF-α release at 1 hour post-infection, followed by neutrophil recruitment at 6 hours. IL-6, IL-1β and TNF-α have been described to have roles in response to *Listeria* infection (84, 85, 177-179). In fact, nearly all TNF-αR deficient mice die by day 5 following intravenous infection with 5 x 10^4 organisms and 40-50% of IL-6 deficient animals die by day 6 following i.v. infection with 10^4 organisms (84, 85). Although IL-1β is not required for survival in response to *Listeria* infection, IL-1β enhances bacterial clearance (177, 178, 180). IL-1β is known to have neutrophil chemotactic properties at doses as low as 50pg/ml; no such chemotactic abilities are known for IL-6 (176). However, IL-6 deficient animals display decreased neutrophilia following *Listeria* infection, indicating that IL-6 may play an indirect role in recruiting neutrophils. We do not observe TNF-α release at the time points tested, however several reports indicate that TNF-α levels appear at 2-4 days following infection, consistent with macrophage

activation and priming of the adaptive immune response (177, 180). These studies demonstrate a novel role for the α2β1 integrin to stimulate the generation of specific cytokine milieu for initiation the innate immune response.

Macrophages are considered to be the initiator of the cytokine response following *Listeria* infection (76). Experiments using genetically modified mice have identified a cytokine milieu required for innate and adaptive resistance to primary *Listeria* infection. Mice deficient in the TNF-α receptor, TNFRp55, are highly susceptible to *Listeria* infection and have >85% mortality rate while WT mice are resistant to infection (84). These mice were unable to clear the bacteria and showed 1000 fold higher titers of *Listeria* in the spleen and liver compared to WT controls. Deficiency in IL-6 results in 40-50% mortality in *Listeria* infections that is likely due to a defect in the ability of neutrophils to migrate from the circulation into the site of infection (85). Mice expressing a dominant negative form of the IFN-γ Receptor α chain selectively in macrophages displayed 33% mortality and higher CFUs in liver and spleen compared to WT mice (82). In addition to these cytokines, IL-1, IL-10 and IL-12 have significant but less potent effects on the innate immune response to *Listeria* (244, 245).

The studies presented here suggest that mast cells may actually be the first cell to encounter *Listeria* and, either directly or indirectly, influence the production of the cytokines following *Listeria* infection. Although macrophages are present in the peritoneal cavities of W/W^v mice, they display decreased local and systemic cytokine production. This suggests that mast cell cytokine production may initiate macrophages and other cells to produce cytokines (Figure 26).

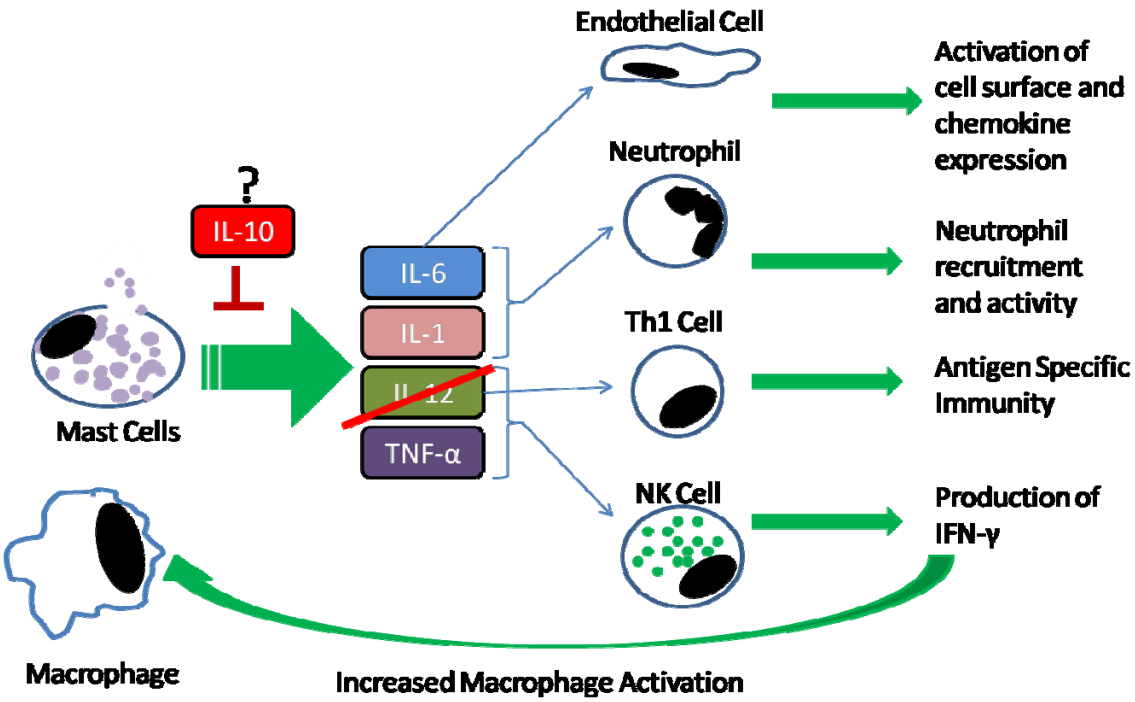

Figure 27. Mast cells induce the cytokine milieu in response to *Listeria* infection. Mast cell α2β1 integrin expression is responsible for the production of the early cytokines in response to *Listeria* infection that mediate further inflammatory responses.

There is a defect in serum IL-6 and TNF-α in both α2-null and W/W^v mice. However, there is not increased lethality in these mice. Although there are systemic defects in these cytokines, local production of these cytokines may be normal in the α2-null mouse and sufficient to control infection and prevent death. Our early reports indicated that the expression of the α2β1 integrin on mast cells is required for IL-6 production and neutrophil recruitment in response to *Listeria* infection and bacterial loads are 10-100 fold higher in spleens and liver in the α2-null mouse at 2 days post-infection (12). However, by 5 days post-infection, there is no difference in bacterial load in either of these organs. I hypothesize that in the α2-null mouse, lack of IL-6 and TFN-α may be responsible for the delay in bacterial clearance at day 2 that is overcome by IFN-γ production by day 5.

The lack of IL-6 and IFN-γ in the mast cell deficient mouse indicates that mast cells are either producing these cytokines or other mast cell mediators are inducing cytokine production from other cells. Mast cells have been reported to be critical in mediating survival to several Gram-negative bacterial pathogens including *K. pneumoniae* and *E. coli* (105, 128). However, the role for mast cells in Gram-positive infections has been less studied. Mast cell deficient mice infected with Gram-positive *Listeria* results in a decrease in inflammatory cytokine production but not mortality during the course of infection. Longer infection courses with *Listeria* or other organisms such as *S. aureus*, to determine if mast cells are required for survival in infections with Gram-positive organisms.

The innate immune system is required for control of the initial infection however; the adaptive immune system must be functional for sterilizing immunity to *Listeria*. We

demonstrate that in α2-null mice, antigen specific $CD4^+$/IFN-γ^+ Th1 cells are decreased at 7 days post-infection. This decrease in Th1 cells does not reflect an inability to mount an effective memory response. Expression of the α2β1 integrin has been shown on activated Th1, but not naïve or Th2 cells. It will be interesting to examine the role of the α2β1 integrin in Th1 vs. Th2 driven immune responses.

There is decreased Th1 response to *Listeria* infection in the α2-null mouse. This defect in Th1 response to *Listeria* may be due to the alterations in the normal cytokine milieu that prevents priming of the adaptive immune response. On the other hand, it has been reported that ligation of the α2β1 integrin enhances IFN-γ production by Th1 cells and inhibits activation induced cell death (AICD) (246). In the absence of the α2β1 integrin, enhanced AICD may account for the decrease in Th1 cells in the α2-null mouse. Taken together, our results suggest that the α2β1 integrin may be an important receptor in regulating Th1 driven innate to adaptive immune transition.

Conclusion

The work presented in this dissertation demonstrates a mechanism of IgE-independent mast cell activation that results in the release of a distinct subset of preformed mediators that requires the α2β1 integrin. Although the α2-null mast cell is unable to be stimulated with *Listeria* immune complex, their response to IgE crosslinking remains unaffected. The α2β1 integrin expression on mast cells plays a role in innate and adaptive immune responses to *Listeria* through regulation of selective release of pre-formed mast cell mediators and Th1 activity. We propose a model by which activation of mast cells through the α2β1 integrin and c-met may occur independently of *Listeria*

immune complex, thereby expanding the role of the integrin and mast cells beyond *Listeria* infections. The role of mast cells in autoimmunity, infection and cancer has been an area of active study. We have defined a novel, biologically relevant mechanism of mast cell activation that may be involved in these disease models. These studies contribute to the growing body of evidence that mast cells and integrins serve as critical mediators of inflammation and disease.

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