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Mechanisms of Inflammatory Cytokine Secretion by Mast Cells

By

Fu-Gang Zhu

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

at

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Halifax, Nova Scotia
December 1999

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FACULTY OF GRADUATE STUDIES

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by Fu-Gang Zhu

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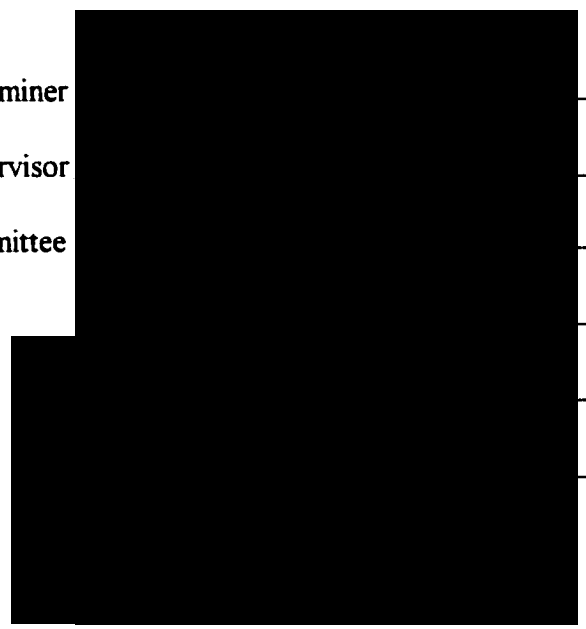

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Signature of Author

To

Lina and Chen

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LIST OF ABBREVIATIONS AND SYMBOLS

APC: antigen presenting cell
BFA: brefeldin A
C5aR: complement 5a receptor
CD: clusters of
CR: complement receptor
CTMC: connective tissue mast cell
DAG: diacylglycerol
DNP-HSA: dinitrophenyl-human serum albumin
FcεRI: high affinity IgE receptor
FcγR: IgG receptor
FCS: fetal calf serum
GM-CSF: granulocyte-macrophage colony-stimulating factor
IFN: interferon
IL: interleukin
IP3: inositol 1,4,5-trisphosphate
ITAM: immunoreceptor tyrosine-based activation motif
KIR: killer cell inhibitory receptor
LPS: lipopolysaccharide
LT: leukotriene
MAP-K: mitogen-activated protein kinase
mBMMC: mouse bone marrow derived mast cell
MHC: major histocompatibility complex
MMC: mucosal mast cell
MMCP: mouse mast cell protease
MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium)
M-CSF: macrophage colony-stimulating factor
NGF: never growth factor
ODN: oligodeoxynucleotide
PAF: platelet-activating factor
PAR: protease activated receptors
PG: prostaglandin
PI3-K: phosphatidylinositol 3 kinase
PKC: protein kinase C
PLCγ1: phospholipase C-γ1.
RMCP: rat mast cell protease
SCF: stem cell factor
STI: soybean trypsin inhibitor
TCR: T cell receptor
TGN: *trans* Golgi network
TNF: tumor necrotic factor
VLA: very later antigen
MAFA: mast cell function-associated antigen

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AUTHOR'S STATEMENT

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- Parts of the work related to DNA-induced cytokine production from mast cells are currently submitted to the Journal of Immunology.

CHAPTER 1

GENERAL INTRODUCTION

Since their discovery over 100 hundred years ago, mast cells have fascinated generations of investigators. Some people consider mast cells "beautiful" (1), others think these cells are "enigmatic" (2) or "versatile" (3). Most investigators, however, would agree on one thing, that mast cell is "one of the best understood and one of the least understood components of the immune system" (4). Mast cells have a great variety of biological capacities, including releasing vasoactive mediators, secreting cytokines, phagocytosis and presenting antigens. For a long time, mast cells have been viewed by many as "trouble makers" for their involvement in allergic disorders. However, studies from recent years have provided strong evidence to show that mast cells can be "protectors" against bacterial infection (5, 6). Mast cells as we know them today may have positive effects in many other situations. It is now high time to "redeem" the mast cells' good reputation so that we can have a balanced view of the mast cells' merits (3).

Mast cells are widely distributed throughout the body, and urogenital tracts, and it is estimated that the total volume of mast cells in the body can amount to the size of the individual's spleen (7). With over 400 million years' evolution, mast cells appeared in the cartilaginous fish approxamately at the same time as T and B lymphocytes (8, 9). Mast cells have a wide range of cell surface receptors, capable of releasing a variety of preformed and newly synthesized mediators, including histamine, proteases, leukotrienes, prostaglandins, cytokines such as IL-3, IL-4, IL-6, IL-10, IL-13, TNF-α, IFN-γ, and GM-CSF, and chemokines, including IL-8, RANTES, MIP-1α, MIP-1β and eotaxin (10). Studies of cytokine secretion by mast cells may provide us with key clues to

understanding mast cells, which are increasingly recognized as essential for our well being and yet about which we have so much to learn.

1. Characteristics of Mast cells

Mast cells were first described in 1878 by Paul Ehrlich, while a medical student, as a group of connective tissue-resident cells that were stained purple with basic blue aniline dyes (11). Since these cells appeared over-full of granules, he named the cell "mastzellen", meaning, "overfed cell" in German. Over the years, people have found mast cells to be invariably present in many species from primitive animals to human. Mast cells are a group of unique immune effector cells, which exhibit complex heterogeneity between different species and different tissue locations. All mast cells have prominent metachromatic granules described by Ehrlich more than 100 years ago. Many lower vertebrates have more mast cells than mammals, and large clusters of mast cells can be observed in some fish and amphibians (12). Mast cells are often strategically located at the interfaces beween the internal and external environments, including the skin, gastrointestinal mucosa and airways

Mast cells vary in size, with diameters between 10 and 18 microns, and appear as round, oval or elongated spindle-shaped cells with many long, thin projections on the cell surface. The single, eccentric nucleus is generally round, with occasional multiple lobules. Each mast cell contains approximately 50 to 200 secretory granules with a size of 0.2-0.5 μm in diameter (13). There are four types of granules observed in human mast cells under an electron microscope, based on granule structures including scroll, crystal,

particle or combination of all three (14). It has been shown that human mast cells from the lung and gut mainly contain the scroll type granules and numerous lipid bodies, while human mast cells from the skin predominantly contained crystal type granules and very few lipid bodies (15).

Cultured mast cells from the bone marrow or other sources of either human or rodent origin are immature mast cells, which are characterized by larger and more heterogeneous granules and numerous small vesicles (**Figure 1A**). With the process of maturation, the mast cell granules become more condensed and more homogeneous in size and shape (**Figure 1B**).

1-1. Rodent mast cells: classification, distribution, development, and mediators

It is fair to say that a great proportion of our knowledge of mast cells comes from studies of rodent mast cells. Pioneered by Enerback in the 60's, morphological and biochemical studies have shown there is notable heterogeneity among rodent mast cells (16). Based on their staining characteristics, cellular content and location, rodent mast cell are divided into two main subgroups, connective tissue mast cells (CTMC) and mucosal mast cells (MMC) (**Table 1**). These may represent extremes of a number of different mast cell sub-types. The sizes and shapes of the connective types of rodent mast cells are usually more uniform than those of the mucosal type. Mast cells located in the skin are most CTMC, while mast cells in the intestinal mucosa are mainly mucosal mast cells. The granules of MMC will lose or have weak staining properties if these mast cells

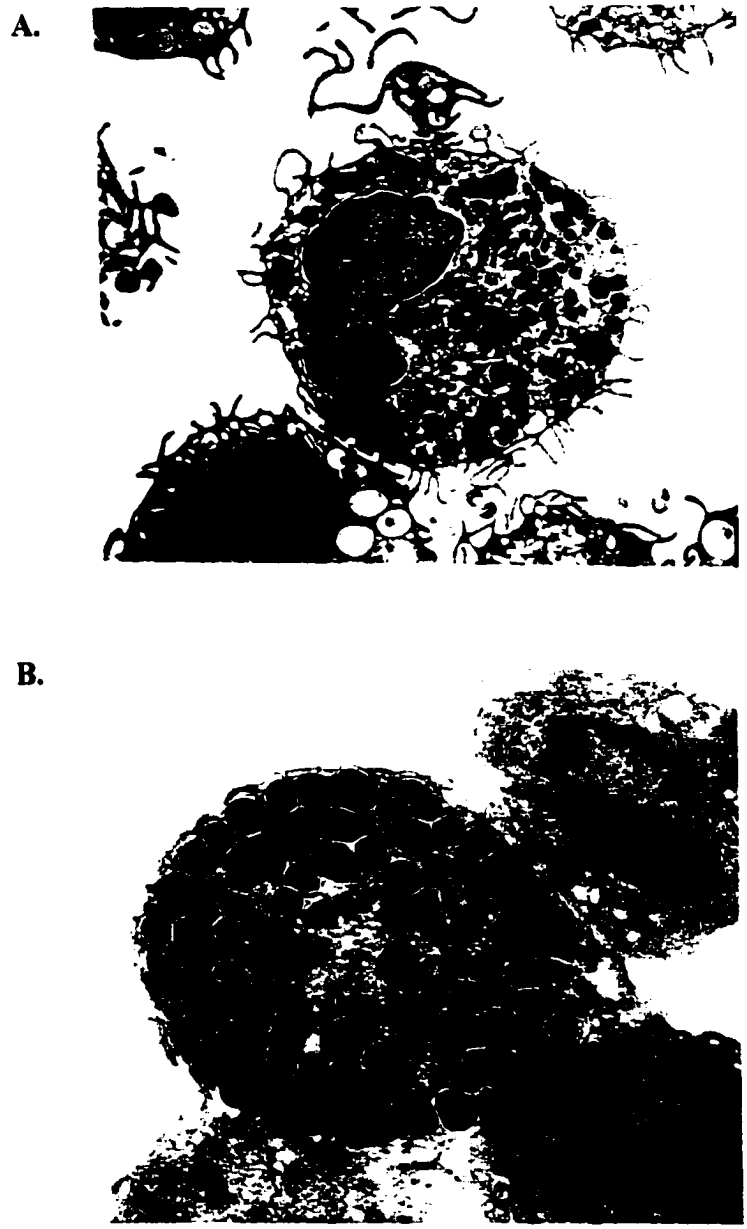


Figure 1. Electron microscopy of **A.**) Immature mast cell: mouse bone marrow-derived mast cells 10000X, in our studies; and **B.**) mature mast cell: a freshly isolated mouse peritoneal mast cell, 12000X. (15).

Table 1. Characteristics of rodent MMC and CTMC

Features	MMC	CTMC
Location	Intestinal mucosa	Peritoneal cavity, intestine submucosa, skin
Staining properties		
Formalin fixation	Affected	Not affected
Alcian blue/safranin O	Blue	Red
Berberine sulphate	Negative	Positive
Neutral protease	RMCP-II (rat) MMCP-1, -2 (mouse)	RMCP-I (rat) MMCP-3, -4, -5, -6, -7 (mouse)
Histamine content	Low (< 2pg/cell)	High (~35 pg/ cell)
Compound 48/80	No degranulation	Degranulation
Migration	Yes	No
Development	T cell dependent	Fibroblast dependent
Life span	Short (half-life < 40 days)	Long (half-life > 6 months)

(References: (16, 17, 21))

are fixed in formalin perhaps as a result of formaldehyde in the solution blocking dye-binding to granule-associated proteoglycans or due to extraction of the proteoglycans from mast cells by the fixative (16). The two types of mast cells also stain different colours with Alcian blue/safranin O staining solution due to the difference in their proteoglycan contents, with chondroitin sulphate E dominant in MMC and heparin prominent in CTMC (1, 16). The preferential staining of MMC with Alcian blue dye is believed to result from the relatively low degree of sulphation of chondroitin sulphate E compared with heparin in CTMC (16). CTMC stain positive with berberine sulphate fluorescent staining due to the presence of highly sulphated heparin in the cells, while MMC do not stain as strongly as CTMC (16).

Progress over the last 20 years has made it possible to classify rodent mast cells using more accurate biochemical criteria, combined with immunohistochemistry to identify different proteases inside their granules (17). Rat CTMC contain rat mast cell protease I (RMCP-I), while rat MMC contain RMCP-II. Mouse mast cell protease 1 and 2 (MMCP-1, -2) are predominantly present in mouse MMC, while MMCP-3 to MMCP-7 are usually found in mouse CTMC (17, 18).

Rodent mast cells develop from hematopoietic stem cells, and leave the bone marrow tissue as "committed precursors", finishing their differentiation in the connective tissue. Mast cell numbers are dramatically increased as the result of helminth infection. However, although a resident mast cell population is observed, such increases are absent in the lamina propria of intestine taken from nematode infected athymic nude mice and rats (19, 20). In contrast, mast cell numbers in the skin from infected or uninfected T cell-

deficient mice are increased similarly to those found in normal mice (19). As the mast cells in the skin are predominantly connective tissue type, and the mast cells in the intestinal lamina propria are typically of the mucosal type, it is proposed that the development of rodent CTMC is fibroblast dependent, and that of rodent MMC in response to infection is more T cell dependent (22). T cell derived cytokines, particularly IL-3, are thought to be responsible for the massive growth of mast cells in the intestines during parasite infection (23). Another piece of evidence to support the differentiating role of IL-3 on mast cell development came from an *in vivo* study, in which IL-3 was given to normal or athymic nude mice, and an increase of mucosal type of mast cells was induced (24). On the other hand, SCF may be mainly responsible for rodent CTMC growth on fibroblasts, as SCF drives the maturation of CTMC in preference to MMC in rodents (25).

Since their successful culture in the early 80's, murine bone marrow-derived mast cells (BMMC) from rodents perhaps are the most popular mast cell model used by investigators throughout the world (21, 26). This is because that BMMC are primary cells behaving more like "real" mast cells when compared with transformed mast cell lines, and they are easily obtained with high purity (>95%) within a relatively short time (~ 4 weeks). The cell contaminants in the final stages of BMMC culture are mainly residual macrophages.

BMMC of either mouse or rat origin are very similar to rodent MMC in many ways, and are generally regarded as immature MMC for they have fewer intracellular granules than freshly isolated tissue mast cells (21, 26). Like MMC, BMMC stain blue

with Alcian blue / safranin O, have chondroitin sulphate as their major proteoglycan and RMCP-II (rat) or MMCP-1, -2 (mouse) as their dominant protease, and fail to respond to 48/80 stimulation.

As described above, BMMC development is primarily dependent on IL-3, but other factors also influence the growth of mast cells in bone marrow culture, such factors include IL-10, IL-9, IL-4 and stem cell factor (27). Rottem *et al.* (28) tested the effects of more than 10 different cytokines on the development of murine BMMC, and found little or no mast cells grown in bone marrow cell cultures in the presence of any of the cytokines other than IL-3 at day 14 of culture. These results demonstrate that IL-3 alone, but not IL-4, -5, -7, -9, -10, M-CSF, GM-CSF, IFN-α, -γ, NGF or SCF alone can promote BMMC development.

1-2. Human mast cells: classification, distribution, development, and mediators

Human mast cells, like rodent mast cells, are a group of heterogeneous cells residing in tissues throughout the body, particularly in the skin, lungs, and gastrointestinal tract. It is estimated that there are 7,000 mast cells per mm^3 in human skin (29), and 20,000 mast cells per mm^3 in the human duodenum (30). Mainly based on their neutral protease content, human mast cells are divided in two major subgroups, MC_T containing tryptase only, and MC_{TC} containing tryptase and chymase (**Table 2**). However, unlike rodent mast cells, human mast cell types, defined in this way, do not have consistent differences in their granule staining properties. Distinguishing the two types of human mast cells in the laboratory is primarily carried out by immunohistochemical staining for tryptase and

chymase as has been employed extensively by Schwartz's group (31), who are credited for the discovery of the two types of human mast cell for their work in the mid- 80's (32).

Table 2. Characteristics of human MC_T and MC_{TC}

	MC_T	MC_{TC}
Main location	Intestinal mucosa Lung alveolar wall	Intestinal submucosa Skin
Protease	Tryptase	Tryptase & chymase
Development	T cell dependent	T cell independent
Major function Proposed	Th2 immune response Host defense	Angiogenesis Tissue remodelling
Inhibition by sodium cromoglycate	Yes	No
Granule morphology	Scroll rich	Scroll rich, grating / lattice

(References: 1, 32)

Like their counterparts in the rodent, human mast cells develop from multipotent hematopoietic progenitors, with a $CD34^+$ phenotype (17, 33). As is the case for rodent mast cells, human mast cells leave the bone marrow as immature cells, and continue to differentiate and mature in peripheral connective tissue. Basophils are also believed to

develop from $CD34^+$ committed progenitors, and enter the blood circulation as terminal differentiated granulocytes (17). Basophils share many properties and cellular contents with mast cells (17).

Unlike the rodent mast cells that employ IL-3 as their major growth factor, human mast cells are primarily dependent on stem cell factor (SCF) for their development. SCF alone or with the further addition of IL-6 has been shown to promote human mast cell development from the bone marrow, fetal liver and cord blood (33, 34). Today, SCF plus IL-6 is commonly used in media for the development of human mast cells from the bone marrow, fetal liver or cord blood (> 10 weeks), while IL-3 supplementation is employed to induce the development of human basophils (> 3 weeks).

1-3. Characteristics of KU812 cells

KU812, generally regarded as a human pre-basophilic cell line, was established from a chronic leukemic patient (35). However, under certain culture condition, KU812 cells will express some features of human mast cells (36), closely resembling MC_T type human mast cells (37). Together with the human mast cell line 1(HMC-1), KU812 is one of very few widely used cell lines by investigators throughout the world in studying human basophil / mast cell development and function (**Table 3**).

Studies over the last few years have greatly increased our understanding of this human cell line (38, 39). In the original report by Kishi *et al.* (35), 0.5 to 40% of KU812 cells could be stained positive with Toludine blue and they contained a low level of histamine at approximately 5 fg / cell. Considerable efforts have been made to promote

Table 3. Major features of KU812 cells

Surface marker/contents	KU812 cells
Preformed mediators	Histamine, tryptase, chymase, heparin, chondroitin and β-hexosominidase
Mast cell-specific surface markers	c-kit, FcεRI
Major surface markers	CD14 (–), MHC-1(+), MHC-II (–).
Immunoglobubin receptors	FcγRII (+), FcγRIII (–), FcεRI (+)
Complement receptors	CR1, CR3, CR4, and C5aR
Adhesion molecules	β1 integrins: VLA-2 (CD49b/29),VLA-4 (CD49d/29), and VLA-5 (CD49e/29) β2 integrins:LFA-1 (CD11a/CD18), Mac-1 (CD11b /CD18), and p150,95 (CD11c/CD18), ICAM-1, LFA-3
Cytokines produced	IL-4, IL-6, IL-8, IL-13, TNF-α and GM-CSF at protein level, IL-5 and SCF at mRNA level.
Cytokine receptors	IL-2, 3, 4, 8, TNF-α, IFN-α, -β, -γ, and GM-CSF

Note: (+) denotes presence of the surface marker, (–) denotes absence of the surface marker; otherwise, all the items in the right column are present on the KU812 cells.

(References: (36, 39, 45, 46))

KU812 cells to differentiate into more mature basophils or mast cells (40-42). An early study showed that differentiation of KU812 cells could be induced by Ara-C, sodium butyrate or a conditioned medium from the T-leukemic cell line, Mo, but not by dimethyl sulphoxide (DMSO) or retinoic acid (40). More recent studies have shown that IL-4, IL-6 and TNF-α, but not IL-1β, IL-2, IL-5, IL-8 and GM-CSF, induced KU812 cell differentiation (41, 42). One very significant finding from these studies is that IL-4 can up-regulate FcεRI expression on the cell surfaces of KU812 cells, resulting in IgE-mediated histamine release from these cells (42). This finding is very significant, because our groups as well as others have dificulties to activate KU812 cells through IgE depedent pathway. If this scheme of differentiation for KU812 cells can be confirmed and well established, KU812 would become a more useful cell line for studying mast cell biology. Magnusson *et al.* (43) demonstrated that 50-60% of the IgE binding described in KU812 cells was through specific high-affinity FcεRI receptors. Both IL-3 and IL-4 have beenshown to increase the expression of FcεRI receptors on KU812 cells (42, 44), and IgE/anti-IgE induced increased histamine release from KU812 cells cultured with IL-4 for 21 days (42).

HMC-1 is the most commonly used human mast cell line, with a higher percentage of positive Alcian blue and toluidine blue staining, and higher mast cell tryptase and histamine content than that described for KU812 cells (**Table 4**) (45). However, HMC-1 cells lack some surface receptors, which put these cells at a disadvantage when being considered as an cell source for studying mast cells when compared with KU812 cells, which are more like basophilic cells without proper differentiation treatment.

Table 4. Comparison between KU812, HMC-1 and human Basophils, Mast Cells

	KU812	HMC-1	Mast cells	Basophils
c-kit	+ (subset)	+	+	–
FcεRI	+	–	+	+
Histamine (pg/cell)	0.01-0.1	$< 1 X10^{-3}$	1-3	1-2
Tryptase (pg/cell)	0. 01	0.1-0.6	10-35	0.04
Alcian blue	+ weak	+	+	+
Toluidine blue	+ weak	+	+	+
CR1, CR3, C5aR	+	–	–	+
Tryptase	+ low	+ high	+	+ trace
Carboxypeptidase A	+	+	+	–
Major basic protein	–	–	–	+

(References: (39, 45, 46, 49, 52))

Nilsson *et al.* (45) reported that the FcεRI α chain was detected on the cell surface of KU812 but not HMC-1 cells, and mRNA for FcεRI α, β, and γ chains was expressed in KU812 cells, while only the mRNA for FcεRI γ chains was found in HMC-1 cells. The FcεRI α chains contain IgE binding sites, and lack of these structures rendered HMC-1 unable to bind IgE (47). Therefore HMC-1 cells fail to respond to stimulation with IgE

followed by IgE receptor cross-linking (48). Another defect in HMC-1 is its mutated c-kit, the SCF receptor, while probably responsible for the uncontrolled growth of HMC-1, and this mutation has the consequence that SCF treatment is unable to mediate the differentiating effects of SCF on this human mast cell line (49).

2. Mast cell mediators

Mast cells have numerous mediators, which are traditionally divided into two groups: preformedmediators,including histamine, serotonin, neutral proteases, and newly synthesised mediators, including prostaglandins, and leukotrienes (50, 51). The third group is an ever-expanding list of protein molecules and peptides with a wide range of biological activities, known as cytokines and chemokines. With rare exceptions, there is a lack of consensus as to whether these mast cell cytokines and chemokines are primarily, preformed and stored, or newly synthesized and released following cell activation. It should be pointed out that some of the mast cell mediators, including cytokines, prostaglandins, and leukotrienes, are also produced by a wide range of other immune and non-immune cells. The amounts of these mediators from non-mast cell sources could be more than that from mast cells under many circumstances. This is perhaps one reason that some people ignore the potential importance of mast cells as a source of cytokines and chemokines in a variety of patho-physiologic processes.

2-1. Preformed mast cell mediators

Unlike most other cells, mast cells contain abundant cytoplasmic granules, within which they store a variety of preformed mediators, including histamine, proteoglycans, neutral proteases and other enzymes. Release of preformed mediators may occur within a few seconds after mast cell activation through either IgE receptor cross-linking or calcium ionophore stimulation and is usually completed within minutes. Such short term release by mast cells is known as degranulation, a process that involves granules rapidly moving toward and fusing with the plasma membrane, resulting in the discharge of granular contents out of mast cells. It has been generally accepted that the mediators released within 20 or 30 min of mast cell activation are likely to be the result of release of preformed, granule-associated stores.

2-1-1. Histamine

Histamine, systematically termed β-imidazolylethylamine, was one of the first mediators found in mast cells back in 1953 (53). Histamine is synthesised from the precursor amino acid, histidine, and stored in the mast granules in ionic association with core heparin or related proteoglycan. Rat peritoneal mast cells contain 10 to 30 pg of histamine per cell, while rat mucosal mast cells have 1 to 3 pg histamine per cell. Human mast cells contain approximately 4 pg histamine per cell, and there is no great difference in histamine content between MC_T and MC_{TC}.

Histamine has a long history as one of the most notorious mast cell mediators associated with acute allergic reactions. It was demonstrated as early as 1910 that histamine had bronchospatic and vasodilator activity when injected to animals (54).

Histamine's effects on smooth muscle in the bronchi and blood vessels are mediated through H1 and H2 receptors (1). Today, a central therapy for asthma and other immediate hypersensitivity reactions is inhibition of the effects of histamine with β-adrenergic agonists and H1 or H2 receptor antagonists.

Beside histamine, 5-hydoxytryptamine (5-HT) is the only other biogenic amine present in substantial amounts in mast cells of rodents, but in much lower amounts in those of human origin. Both mucosal type and connective tissue type mast cells in rodents contain 5-HT at a concentration of around 1pg per cell for CTMC and a much lower concentration for MMC (14). 5-HT exerts its function by binding to different 5-HT receptors, and has bioactivity similar to histamine in many respects.

2-1-2. Neutral proteases

The major portion of the protein content of mast cell granules is made up of mast cell specific neutral proteases, upon which the more updated classification systems for the human and rodent mast cell types are based. The mast cell neutral proteases are a group of proteolytic enzymes, mainly divided into two subgroups, chymases and tryptases. Chymases are the dominant neutral proteases in rodent mast cells, while tryptases are the major neutral proteases in human mast cells (14).

Mast cell tryptases and chymases are traditionally believed to act primarily as degradative enzymes. More recent studies, however, have shown that some of the biological effects of these neutral proteases are mediated by cleaving and triggering protease activated receptors (PARs), which compose a new family of G protein coupled

receptors activated by proteolysis. Three members of this family have been identified, PAR-1, a receptor for mast cell chymase and thrombin, PAR-2, a receptor for mast cell tryptase and trypsin, and PAR-3, another receptor for thrombin. PARs are expressed in a wide variety of cells, and are involved in several pathophysiological processes, including growth and development, tissue modelling, and inflammation (55, 56). We can expect our knowledge about the function of mast cell neutral proteases will be greatly expanded with progress in studies of the PAR family.

2-1-2-1 Chymase

Mast cell chymases are a group of monomeric enzymes stored in the active form as proteins of 26-30 kDa. Human mast cell chymase is present in MC_{TC} type of mast cells. Chymases are the predominant neutral proteases in rat mast cells, with rat mast cell protease I (RMCP-1) associated with CTMC, and RMCP-II related to MMC. At least seven neutral proteases have been identified in mouse mast cells, mouse mast cell protease 1 (MMCP-I) to 7, with MMCP-1 to –5 being chymases, and MMCP-6 and –7 being tryptases. Chymases have a number of potential biological function, including cleavage of neuropeptides, and conversion of relatively inactive angiotensin I into active angiotensin II.

2-1-2-2. Tryptase

Tryptases are the most abundant proteins found in the secretory granules of human mast cells, constituting about 20 % of the total cellular protein in human mast cells. The

tryptase concentration in human MC_T mast cells is approximately 10 pg / cell, while in MC_{CT} the tryptase level is around 35 pg / cell (57). The enzyme is a tetrametric serine protease with a molecular weight of 134 kDa. Like histamine, tryptase is stored inside mast cell granules associated with heparin or other proteoglycans, which stabilise tryptase in its storage form. Once released into the extracellular environment, at an optimal neutral pH, tryptase becomes active. Active tryptases can cleave C3, a number of neuropeptides and other bioactive proteins, and activate fibroblasts.

Recent studies have shown that mast cell tryptases may selectively induce leukocyte influx into local tissue sites. When purified tryptase from human lung tissue was injected into the skin of guinea pigs, accumulation of large numbers of neutrophil and eosinophils was observed within 6 h of injection (58). Injection of tryptase into the peritoneum of mice also stimulated the influx of neutrophils by over 400-fold and of eosinophils at the later time points by up to 10-fold. Another report from Richard Stevens' group demonstrated that i.p. injection of recombinant tryptase mMCP-6, but not the closely related recombinant mMCP-7, into the peritoneal cavities of several strains of mice significantly increased the number of neutrophils in by more than 50-fold (59). Mouse MCP-6 did not directly induce neutrophil chemotaxis, however, the tryptase induced endothelial cells to produce large amounts of IL-8 continually over a 40-h period. These two reports suggest that tryptase may provide an important stimulus for granulocyte recruitment in allergic disorders or other inflammation processes.

2-1-3. Acid hydrolases

Acid hydrolases in mast cells have their optimal activity at an acid pH, which is usually present in inflammatory process. These enzymes play an important role for the lysosomal degradation of ingested particles in phagocytic leukocytes, they may have a similar function in mast cells. The most well known acid hydrolase is β-hexosaminidase, which is released in parallel with histamine upon mast cell activation and has been widely used to follow mast cell degranulation. A synthetic substrate *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide can be cleaved by β-hexosaminidase to release *p*-nitrophenol chromophore that can be measured by reading absorbance at 405 nm. Compared to histamine assay, β-hexosaminidase assay is a faster, safer, and simpler method to measure mast cell degranulation.

2-1-4. Proteoglycans

Proteoglycans are a group of highly acidic macromolecules, including heparin and chondroitin sulphate, which constitute the central protein cores inside cytoplasmic granules of human and rodent mast cells (1, 60). Proteoglycans consist of a single chain protein core convalently linked to glycosaminoglycan side chains that contain repeating unbranched disaccharide units with 1 to 3 sulphate residues for each disaccharide unit. The physicochemical properties of proteoglycans are important for mast cells' characteristic metachromatic staining with basic dyes such as toluidine blue, and also allow proteoglycans to serve as storage matrices for neutral proteases and histamine. Both types of human mast cells, MC_T and MC_{TC} contain heparin, while only the connective tissue type of rat and mouse mast cells have heparin within their granules. The

proteoglycan chondroitin sulphate is present in human MC_T and MC_{TC}, but is only observed in the mucosal type of rodent mast cells (1). Heparin and to a lesser extent, chondroitin sulphate, can have anticoagulant, anticomplement and other effects. Recently two reports published in *Nature* provided some new evidence to confirm the function of heparin in the storage of granule-associated mediators (61, 62). Two groups of investigators from the U.S.A. and Sweden independently established transgenic mice lacking fully sulfated heparin, and found decreased contents of histamine and MMCP-4, -5, and –6 in the mast cells isolated or cultured from those heparin-deficient mice. Microscopic examination revealed reduced numbers and sizes of metachromatic granules in those mast cells (61, 62). It would be interesting to know if any other phenotypic and functional abnormalities are present in such heparin-lacking mast cells.

2-2. Newly synthesized lipid mediators

Within minutes of activation, mast cells not only release preformed mediators but also synthesize *de novo* mediators derived from phospholipids in the cytoplasmic membrane and intracellular lipid bodies (14, 50). The first step is the liberation of 20-carbon fatty acid called arachidonic acid from phospholipids by the activation of phopholipase A_2. Arachidonic acid is then metabolised along either the cyclooxygenase pathway to produce prostaglandins (PGs) and thromboxanes or the lipoxygenase pathway to release 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is subsequently converted into leukotrienes (LTs), LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4. PGD_2 is the predominant cyclooxygenase product, while LTC_4 is the prominent lipoxygenase product

of activated human mast cells. PGD_2 and LTC_4 are also the major cyclooxygenase and lipoxygenase products, respectively, of activated rodent mast cells. However, LTC_4 is only produced in large amounts by the mucosal type, and not the connective tissue type, of rodent mast cells (60). There is no obvious difference between the two types of human mast cells in their synthesis of PGD_2 and LTC_4. In human mast cells, the amount of released PGD_2 is more than 20 time higher than the amount of LTC_4 following IgE mediated activation.

Both PG's and LT's have been shown to play important role in acute allergic reactions (1). PGD_2 induces bronchoconstriction, potentiates airways to respond to histamine, and is chemotactic for neutrophils. Leukotrienes (LTC_4, LTD_4 and LTE_4) seem to have a similar but broader range of bioactivities than PGD_2, i.e., they induce neutrophil influx, to constrict bronchioles and arterioles, and to increase venous permeability. The effects of LT's can last for several hours, and they are 100 fold more potent than histamine. The functions of both PG's and LT's are mediated through specific receptors (46). One recent medical breakthrough is to effectively treat some asthma patients, who show poor response or tolerance to the traditional steroid treatment, with LTD_4-receptor antagonists, such as Zafirlukast and Montelukast (63).

Platelet-activating factor (PAF) is another lipid-derived mast cell mediator that is generated from alkyl acyl-glycerylphosphorylcholine in a process different from that for PG's and LT's. PAF is present in several forms and is quickly inactivated after release, therefore, its bioactivities are not well-defined. However, it is generally agreed that PAF

plays a role in immediate hypersensitivity reactions via its strong chemotactic activity for both neutrophils and eosinophils (14).

2-3. Mast cell cytokines and chemokines

Cytokines are a group of small protein molecules and peptides with a wide range of biological activities, including chemotactic properties. The cytokines capable of attracting and activating leukocytes are further classified as a separate subgroup called chemokines. Cytokines are relatively new members of the mast cell mediator group, but the list of mast cell cytokines has been expanding since the cytokine, GM-CSF, was first detected in a transformed murine mast cell line in 1986 (64). Many of the cytokines and chemokines identified so far can be found in the list for mast cell cytokines and chemokines, which include interleukin (IL)-1, -3, -4, -5, -6, -8, -9, -10, -12, -13, -16, tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1α (MIP-1α), MIP-1β and monocyte chemotactic protein-1 (MCP-1) (10). This list shows that mast cells not only can produce TH1-type cytokines (e.g. IFN-γ) but also TH2-type cytokines (e.g. IL-4 and IL-5), as well as pro-inflammatory cytokines (e.g. TNF-α, IL-1 and IL-6). However, specific mast cell cytokines are differentially induced with different stimulants (65), and cytokine production and histamine release may also be separately induced events (66, 67).

IL-6, TNF-α and GM-CSF have all been shown to be produced by mast cells in substantial amounts. These cytokines each play important roles in allergic and non-allergic inflammation and host defence, and all have their gene expression controlled by

the transcription factor, nuclear factor (NF)-κB (68). These three cytokines will be the focus for studies described in this thesis, therefore, a more detailed description will be given in the following sections.

2-3-1. IL-6

The cytokine IL-6 has had many different names since its first discovery as a B cell differentiation factor (BSF-2) in 1985 (69). The numerous titles relate to its functional pleotropism and diversity of cell sources. One of the best known functions of IL-6 is its critical role during the early stages of infection, trauma or burns when it is responsible for inducing acute phase protein production by hepatocytes (70). Other important functions of IL-6 include promoting the growth and differentiation of B cells and T cells, inducing maturation and activation of neutrophils, eosinophils and macrophages, mediating the pyrogenic effects of LPS, and inducing neurite outgrowth (71). Based on these activities, IL-6 is thought to play an important role in host defense as well as in a variety of inflammatory disorders, including allergic diseases and autoimmune diseases.

Human and mouse IL-6 genes are located on chromosome 7 and 5, respectively (70). IL-6 is a glycoprotein of 212 amino acid residues from human, and of 211 amino acid residues from mouse, with 42 % homology between the two sources of the cytokine. The IL-6 receptor is a heterodimer with one shorter ligand-binding α chain and a longer signal-transducer β chain, and belongs to the cytokine receptor superfamily type I. The β chain is also known as gp130, a common signal transducer shared by other cytokines,

including IL-11, leukemia-inhibitory factor (LIF) and oncostatin M (OSM). Mouse IL-6 receptor α and β chains have 54% and 77%, of homology with human IL-6 receptor α and β chains, respectively. Human IL-6 can activate mouse cells, however mouse IL-6 can not activate human cells (71). For this reason, the murine B cell hybridoma (B-9) cell line is commonly used to measure IL-6 from both mouse and human origins. Since the amino acid sequences of mouse and rat IL-6 are 93% identical and they share bioactivity, B9 cell proliferation bioassay is also used to detect rat IL-6.

The information available at the initiation of these thesis studies suggested that IL-6 was synthesized and secreted upon mast cell activation, although mast cell storage of IL-6 was also suggested by immunohistochemical studies (72, 73). IL-6 has been shown to be generated by many non-hematopoietic and hematopoietic cells, including mast cells. In fact, IL-6 is one of the most common mast cell associated cytokines, produced by mast cells from almost all sources (4, 10). Mast cell-derived IL-6 may play an important role in inflammatory disorders, especially allergic diseases (74). In some cases, such as in the nasal mucosa of rhinitic patients, mast cells have been suggested to be the predominant source of IL-6 (75). On the other hand, IL-6 has been shown to be an important mast cells growth factor that enhances SCF-dependent human mast cell growth and IL-3-dependent mouse mast cell growth (33, 76).

2-3-2. TNF-α

The name "tumor necrosis factor" (TNF) was first put forward by Carswell *et al.* in 1975 to describe an LPS-induced serum factor capable of causing hemorrhagic necrosis

of tumors (77). TNF-α has been the research focus for a generation of investigators, and today the interest in this cytokine is strong as ever. TNF-α plays a central role in many forms of inflammation, host defence and other immune responses (78, 79). TNF-α activates monocytes / macrophages, neutrophils and lymphocytes, induces IL-1, IL-6, IL-8 and MCP-1, and up-regulates endothelial expression of vascular adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin, resulting in recruitment of leukoctes to areas of inflammation (71).

The location of human TNF-α gene is on chromosome 6 linked to MHC genes, while the mouse TNF-α gene is located within the major histocompatibility complex (MHC) on chromosome 17 (80, 81). The amino acid sequence of TNF-α is well conserved between species, with 79% homology between human and mouse (82). TNF-α is synthesized as a 26 kDa precursor protein of 233 amino acids, from which a 17kDa subunit with 157 amino acids is released after proteolytic cleavage. The bioactive form of TNF-α can be either a secreted or membrane-associated homotrimer that is comprised of noncovalently bound three 17kDa subunits (71). An L929 cytotoxicity bioassay is routinely used to measure TNF-α in the homotrimer form, but other methods, such as enzyme-linked immunosorbent assay (ELISA), are required to detect the inactive monomeric form of TNF-α resulting from dissociation of the trimeric form.

The effects of TNF-α are mediated through membrane bound receptors, TNF-α I (TNFR-I, p55) and TNFR-II (p75), both of which are type 1 transmembrane protein with cysteine-rich repeats in their extracellular domains, and belong to TNF-nerve growth factor (NGF) receptor superfamily (68). It is believed that most cellular responses to

TNF-α are mediated by TNFR-I, while the role of TNFR-II remains to be clarified. TNF-α has multiple functions, however, its most prominent profile is as a proinflammatory cytokine.

TNF-α was the first cytokine found to be associated with normal mast cells (4) after it was detected in murine peritoneal mast cells (83). More recent studies have demonstrated TNF-α at mRNA or/and protein levels in human and mouse bone marrow-derived mast cells, purified rat and mouse peritoneal mast cells and several mast cell lines (60). Based on studies of freshly isolated and cultured mouse mast cells by Gordon and Galli, mast cells are thought to be the only cells to have substantial amount of preformed TNF-α stored in the secretory granules ready for rapid release (84). Although their observation has not been widely confirmed by other laboratories or in similar experiments using other sources of mast cells, some immunohistochemical studies have demonstrated positive staining of TNF-α in cytoplasmic granules of human and rat mast cells (85, 86). Other TNF-α producing cells, including monocytes and macrophages (major TNF-α producers) and T and B cells have little or no preformed TNF-α. Gordon and Galli demonstrated that mouse peritoneal mast cells in a "resting" state contained twice as much TNF-α as activated mouse peritoneal macrophages (84). It remains possible that mast cell may provide the most important initial source of TNF-α in the case of acute bacterial infection, and mast cell-derived TNF-α may be a key factor in the development of septic shock. TNF-α from mast cells may also be cactively involved in chronic inflammation by persistent secretion of newly synthesised TNF-α as well as other cytokines. TNF-α has also been shown able to promote human mast cell differentiation

and mouse mast cell development by a mechanism which may involve intermediates such as PGE_2 or IL-6 (41, 76).

2-3-3. GM-CSF

GM-CSF was first purified from conditioned medium of lung tissue from mice injected with endotoxin in 1977 (87). GM-CSF is a glycoprotein with 118 or 144 amino acid residues, for mouse and human, respectively (71). The gene for GM-CSF is clustered with the genes for IL-3, IL-4, IL-5, IL-9 and IL-13 on human chromosome 5q and on mouse chromosome 11 (88), all of these cytokines are known for their function as hemopoietic growth factors (68, 71). The receptor for GM-CSF is a heterodimer comprising a ligand binding α chain and a signal transducing β chain that is shared with IL-3 and IL-5 receptors, and is a member of the type 1 cytokine receptor superfamily. GM-CSF receptors are expressed on neutrophils, eosinophils and monocytes/macrophages. There is 54% homology between human and murine GM-CSF amino acid sequences, but there is not any cross-bioactivity between the two molecules (89).

The most notable functions of GM-CSF are its roles in normal hematopoiesis, including stimulating the proliferation and differentiation of myeloid and erythroid progenitor cells, and megakaryyocytes (89). The other major bioactivities of GM-CSF include enhancing the function of monocytes/macrophages, neutrophils and eosinophils, acting as a chemoattractant for neutrophils and prolonging their survival (71). Based on these functions, it is not surprising that Metcalfe emphasized the important role of GM-

CSF in inflammation (90). The findings that gene transfer of GM-CSF induced *in vivo* lung inflammation and GM-CSF gene knockout mice showed defective immune responses to *Listeria* infection further strengthen the crucial role of GM-CSF in inflammation (91, 92). Like IL-6 and TNF-α, GM-CSF can be produced by mast cells from different origins, and a recent study showed that freshly isolated human mast cells produced a similar amount of GM-CSF as macrophages (93).

3. Mast cell activation

3-1. IgE receptor mediated activation

FcεRI, the high-affinity receptor for IgE, is almost exclusively expressed on mast cells and basophils. One murine mast cell has approximately 10^5 to 10^6 FcεRI, which bind IgE with a very high affinity (*Ka*) of about $1X10^{10}$ M^{-1} (60). One IgE molecule binds to one IgE receptor, but monomeric IgE binding to FcεRI will not trigger mast cell activation. Mast cells can be activated by IgE receptor aggregation through either cross-linking FcεRI by a multivalent antigen or a bivalent antibody to FcεRI. The IgE receptor has a tetrameric structure consisting of a single α chain, and single β chain, and two identical γ chains. The α chain of FcεRI contains a long extracellular segment with two immunoglobulin-like domains, a single transmembrane domain, and a short intracellular tail. The β chain transverses the membrane four times with both C- and N-terminals inside the cell. The γ chain consists of a short extracellular portion, a single transmembrane domain, and a long intracellular tail. The α chain provides an IgE binding site, while both β and the γ chains function primarily in signal transduction (60, 94). The

γ chains seem to play a more important role than the β chain in transducing FcεRI-associated signals as slower and smaller signalling responses were observed in the cells transfected with FcεRI αγ, but not αβ, chains (95). The β and γ chains each have one "immunoreceptor tyrosine-based activation motif" (ITAM), which allow association of the β chains with tyrosine kinase Lyn and of γ chains with the tyrosine kinase Syk.

We still do not have a complete picture of the FcεRI-related signal pathways, but one model has gained considerable acceptance in describing the signal transduction after IgE receptor cross-linking (**Figure 2**) (60, 94). According to this model, the tyrosine kinase Lyn that is constitutively associated with β chain becomes activated within seconds of IgE receptor aggregation, inducing phosphorylation of ITAMs in both β and γ chains. The phosphorylated ITAMs in the γ chains bind to and activate another tyrosine kinase Syk via its src homology-2 (SH2) domain-containing proteins. Activated Syk, in turn, induces phosphorylation and activation of phospholipase C-γ1 (PLCγ1), leading to rapid generation of diacylglycerol (DAG) and inositol 1,4,5- trisphosphate (IP3). DAG activates protein kinase C (PKC), which stimulates the activation of mitogen-activated protein kinase (MAP-K). Both PKC and MAP-K are involved in the later steps of signalling leading to mast cell degranulation, although details have not been clearly clarified.

Among the "early events" following IgE receptor aggregation is the activation of phosphatidylinositol 3 kinase (PI3-K), which is probably induced by activated Syk (60). PI3-K, together with PLCγ1, catalyzes the production of IP3, which trigger the release of Ca^{2+} ions stored in the endoplasmic reticulum (ER) by binding to the IP3 receptor on ER. As a result, the intracellular Ca^{2+} level is elevated, promoting mast cell activation,

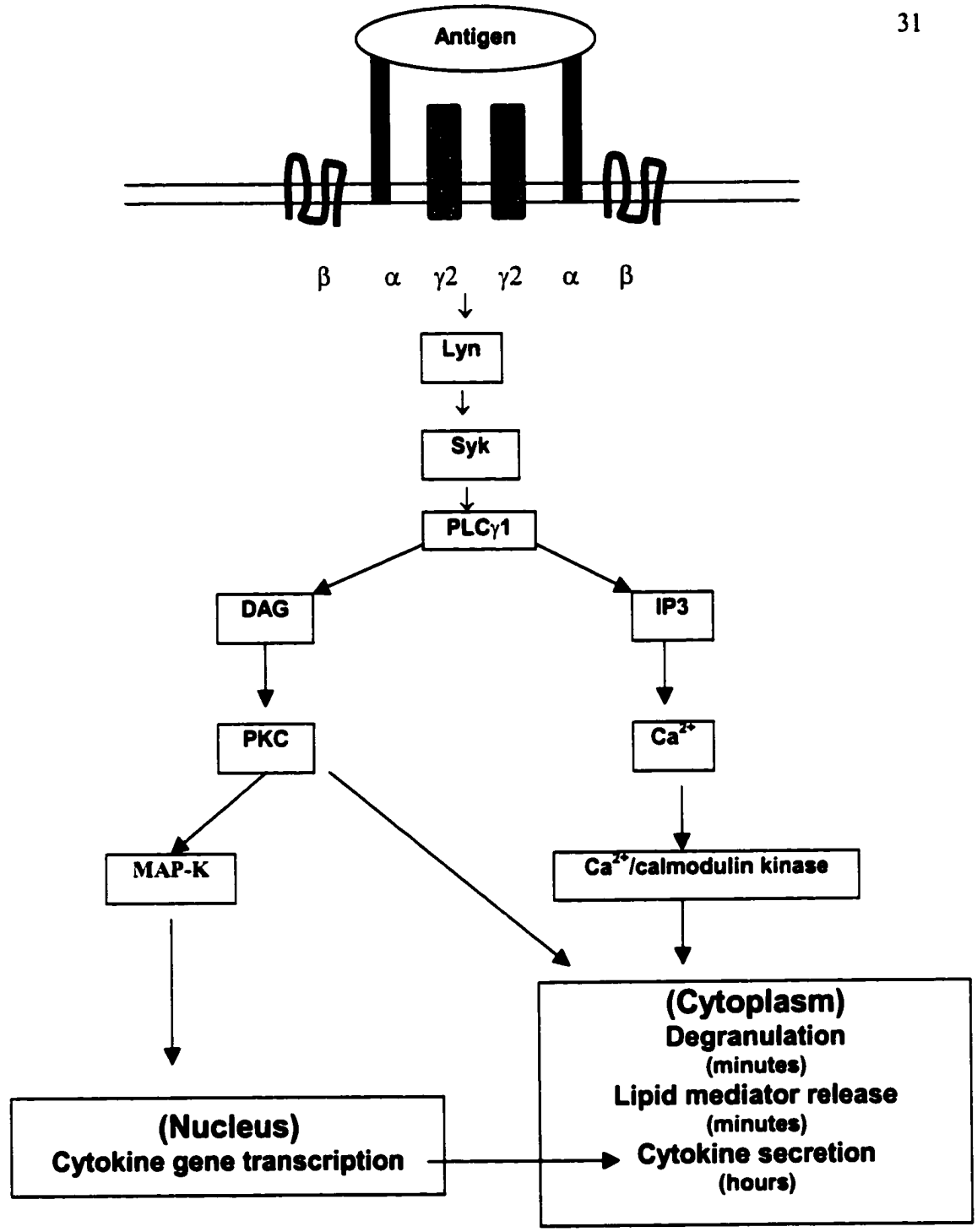


Figure 2. Model of FcεRI-related signal transduction pathways in mast cells

DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; MAP-K, mitogen-activated protein kinase; PI3-K, phosphatidylinositol 3 kinase; PKC, protein kinase C; PLCγ1, phospholipase C-γ1.

(References: (60, 94)

primarily in the form of degranulation (96). GDP-binding protein (G protein) related pathways have long been associated with the upstream portion of FcεRI-related signal transduction, but some investigators suggested that the involvement of G proteins is downstream of the PLC and calcium signal (60). Two types of G proteins, the inhibitory G protein 3 (Gi3) and small Ras-like G protein, rab3, are believed to be involved in the later stages of mast cell degranulation. Both Gi3 and rab3, found in mast cells, are believed to mediate the fusion of granules with the plasma membrane (97-99).

IgE has been demonstrated to up-regulate FcεRI expression on mast cells *in vitro* and *in vivo*, and has been shown to enhance IgE-mediated mast cell activation in term of degranulation and cytokine production (100-102). This new finding seems to provide one explanation for the positive relationship between serum IgE levels and FcεRI expression on circulating human basophils (103), and also lends some support to the approach of reducing IgE levels for allergic disease treatment (104).

IgE receptors are also present in other types of cells, including Langerhans' cells, monocytes, lymphocytes, and eosinophils (46, 105). These IgE receptors include FcεRI with high affinity for IgE, and FcεRII (CD23), with low affinity (*Ka*) for IgE at approximately $4X10^6$ M^{-1}. IgE binding or antibody to FcεRII have been shown to inhibit IgE production *in vivo*, therefore, it is believed that ligation of FcεRII can serve as a negative feedback to the IgE production (106).

IgE receptor FcεRI function is inhibited by several membrane molecules, including gp49 family molecules (107), mast cell function-associated antigen (MAFA) (108), and CD81 (109). gp49B1, a member of Ig superfamily was the first recognized mast cell surface

receptor capable of inhibiting FcεRI-mediated mast cell activation, a finding made by Harvard Katz *et al.* in 1983 on murine mast cells (107). It is proposed that a gp49 ligand coligates FcεRI and gp49B1, resulting in inhibition of the signal transduction cascade initiated from the IgE receptor cross-linking (110). MAFA, found in rat mast cell line RBL-2H3 with a similar structure to that of the mouse killer cell inhibitory receptor (KIR), belongs to the type II transmembrane protein family, and blocks FcεRI-induced activation by inhibiting its initial signal transduction, possible by recruiting tyrosine phosphatase (108). CD81, structurally different from both gp49B1 and MAFA, and is the latest recognized mast cell inhibitory receptor. CD81 inhibited FcεRI-mediated degranulation from RBL-2H3 cell line *in vitro* and also inhibited mast cell degranulation *in vivo* in a rat model of passive cutaneous anaphylaxis (109). Little information is available for CD81-related inhibition, except that such inhibition was calcium independent, different from two other mast cell inhibitory receptors, gp49 and MAFA (109).

3-2. IgE receptor independent activation

One of the most common mast cell stimulants is the calcium ionophore, A23187, which can directly activate mast cells of different species, including human, rat and mouse. This is widely used as a positive control in the following and many other published studies. Another recognised group of non-FcεRI dependent stimuli are basic compounds, including compound 48/80 and mastoparan, both of which can induce degranulation from mast cells through activating G proteins on the membrane. Complement fragments, C3a, C4a and C5a, commonly known as anaphylatoxins, cause

histamine release from either human or rodent mast cells through specific complement receptors. This suggests that mast cells may play a role in immune complex-associated diseases. However, it is not clear how important the anaphylatoxin-induced mast cell degranulation is in comparison with that mediated through FcεRI cross-linking. Both human and mouse mast cells can be activated by SCF, which can also enhance the mast cell response to IgE receptor dependent activation (10).

It has been suspected for a long time that there could be some interaction between mast cells and the nervous system based on the anatomical proximity and the effects of nerve/neuron cell-derived substances on mast cells and of mast cell mediators on the nervous system (111-113). Substance P, a peptide well known as a neurotransmitter has been shown to induce histamine release and TNF-α production from both human and rodent mast cells (111, 114). Mast cell-derived histamine and NGF have been shown to modulate the function and growth, respectively, of peripheral nerve system (60, 111).

In some cases, IgE-independent mechanisms can mimic IgE dependent events, for example, some common allergens, including bee venom and house dust mite can induce mast cell degranulation in the absence of antigen specific IgE (115). Such stimulatory effects are observed in activation of mast cells by phospholipase A2 from bee venom or protease activity by house dust mites.

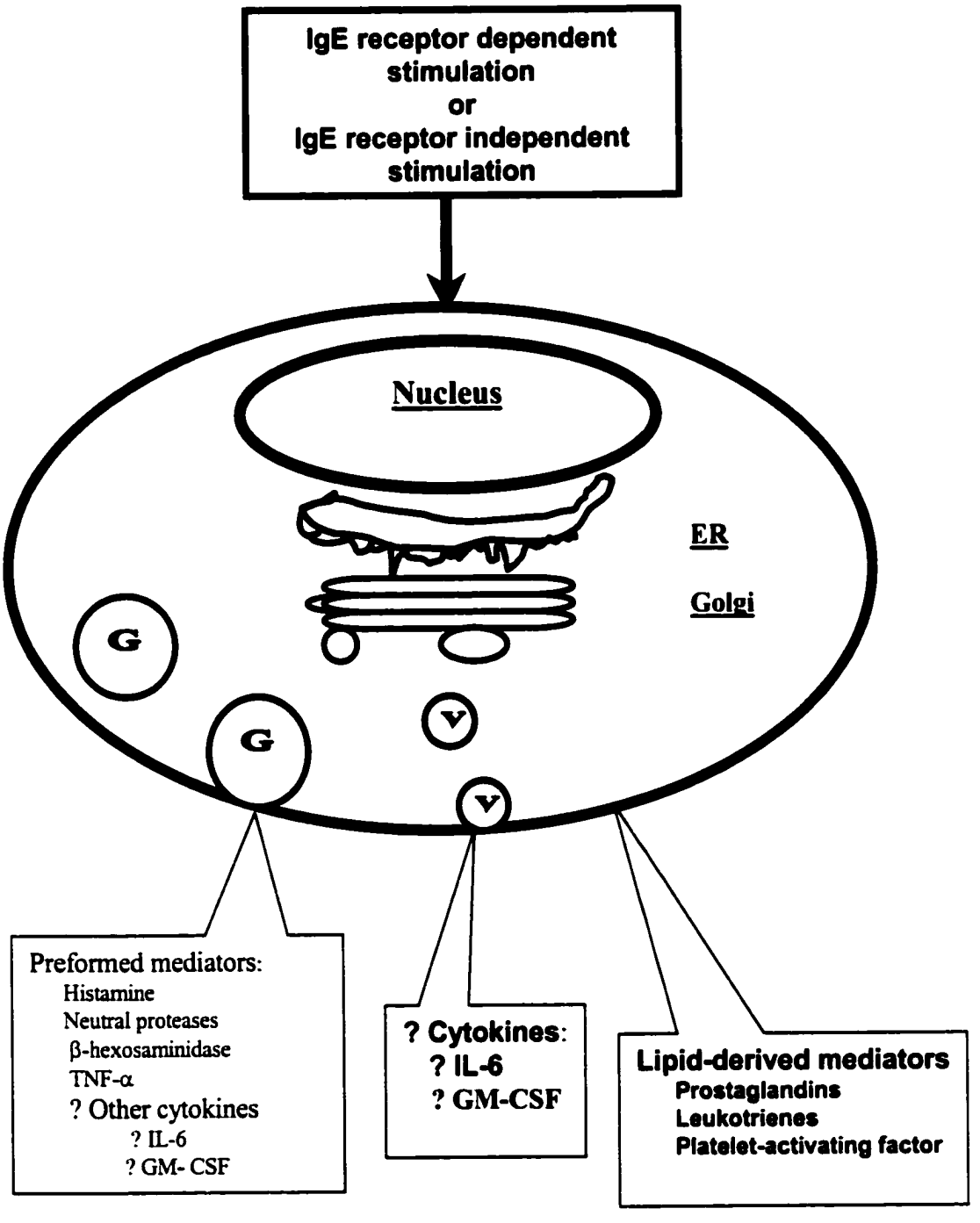
4. Mast cell mediator release

Most cells are capable of secreting proteins and other products, and this is especially true for mast cells. Rapidly released mediators from mast cells contribute to the

initiation of immediate hypersensitivity reaction, or the more severe, potentially lethal anaphylactic shock. In general, large numbers of proteins in cells (including many cytokines) are synthesized in the rough endoplasmic reticulum, then processed and packed in the Golgi complex, finally transported to the cytoplasmic membrane to be secreted. There are two classic secretion pathways, the regulated secretory pathway with the requirement for extracellular stimulus and the constitutive secretory pathway without such stimulus requirement (116). Most cells have a constitutive secretory pathway, a few types of cells, including mast cells, also use a regulated secretory pathway (**Figure 3**). The regulated secretion by mast cells is better known as degranulation. Mast cell degranulation and cytokine secretion (possible through constitutive secretory pathway) may be simitaneously triggered by stimulation via IgE depedent pathway or with calcium ionophore. The two types of mast cell secretion, however, may also be sperate events as shown in previeous studies in Jean Marshall's laboratory, in which LPS and chlera toxin induced IL-6 and TNF-α without degranulation from peritoneal mast cells (65, 66).

4-1. Regulated secretion pathway

For cells possessing a regulated secretion pathway, synthesized proteins and other products are stored at high concentration (>300 mg/ml) inside large cytoplsmic granules (117). Initially, secretory granules form by budding out of the trans-Golgi network (TGN). These membrane-bound granules are so-called immature secretory granules, which become mature by further concentration and condensation of the stored products. The final concentration of mediators, in mature mast cell granules, can be 200 fold higher than in the



V=vesicles
G=granules

Figure 3. Model of Cytokine secretion pathways in mast cells.

original granules. The mature secretory granules can remain inside cells for long periods of time, and are only rapidly discharged from the cells upon activation with secretagogues. In a sense, mast cells are specialized secretory cells, possessing one of the most prominent features of a regulated secretion pathway, i.e., rapidly released stored mediators. After appropriate stimulation, mast cell granules quickly move toward and fuse with the cytoplasmic membrane, and release their contents. Prior to this study, only TNF-α had been described to be released from mast cells through a regulated secretion pathway, i.e. degranulation (84).

4-2. Constitutive secretion pathway

The constitutive secretion pathway involves small transport vesicles that are constantly budding from and leaving the TGN. No stimulation of the cells is required, but the pace of constitutive secretion can be enhanced in activated cells. However, under either condition, protein secretion will be at approximately the rate of protein production (118). The transport vesicles are continuously moving toward and fusing with the cytoplasmic membrane, resulting in a steady secretion of protein products. It is estimated that the time for vesicles delivered from TGN to cell surface is only about 20 min in some cells (116). There is little information about the constitutive secretion pathways in mast cells, however, spontaneous secretion of the cytokines, including IL-1, IL-6 and TNF-α, from mast cells suggests that some cytokines may be released from mast cells through a constitutive secretion pathway (119). More detailed studies are needed to confirm that these cytokines are secreted from mast cells through a vesicle dependent, constitutive secretion pathway.

5. Mast cells in inflammatory responses

Mast cells are critical effector cells in the inflammation related to IgE mediated allergic disorders, including asthma, allergic rhinitis, allergic gastroenteritis and contact dermatitis. Many studies have also suggested the involvement of mast cells in a variety of "non-allergic" diseases, including inflammatory bowel disease (IBD), arthritis, fibrosis, cancer and parasitic infection. The evidence for a role of mast cells in asthma, rhinitis and parasite infection are particularly convincing and will therefore be described in more details.

Table 5. Comparison between regulated and constitutive secretion pathways

	Regulated pathway	Constitutive pathway
Transport vehicle	Large granules, with high density core	Small clear vesicles
Prior storage	Yes	No
Secretagogues	Required	Not required
Secretion kinetics	Not continuous	Continuous
Maturation	Required	Not required

(Reference: (116))

5-1. Asthma and rhinitis

Among the many proposed roles of mast cells one of the least controversial is perhaps the involvement of mast cells in allergic airway inflammation. It is widely agreed that mast cells play a critical role in acute and chronic IgE-dependent inflammation and hypersensitivity of the airways (3, 120). Mast cells become sensitized with bound IgE

specific to a wide range of allergens, including pollens and dust mites. Secondary exposure to the same allergens can trigger mast cells to immediately release preformed mediators followed by newly synthesized mediators. A large amount of evidence has suggested that mast cells may initiate acute airway inflammation by releasing preformed histamine, proteases and possibly TNF-α, and newly synthesized prostaglandins and leukotrienes. As has been described before, each of these mast cell mediators can, to a different extent, cause bronchoconstriction and the influx of neutrophil and eosinophils. Studies over recent years have indicated that mast cells may also contribute to the maintainence of chronic inflammation and hyperresponsiveness of the airways by continuous secretion of cytokines, especially IL-3, IL-4, IL-5, IL-6 and TNF-α. IL-4 increases mast cell numbers and supports mast cell survival, and also enhances IgE production and FcεRI expression. IL-3 and IL5 promote eosinophil growth, differentiation and activity. All of these cytokine effects plus those of TNF-α and IL-6, as described previously, could contribute significantly to the development of chronic asthmatic conditions.

5-2. Parasitic infection

The involvement of mast cells in host defense against helminth parasite infection has long been suspected, based mainly on mast cell accumulation in the intestine, signs of mast cell activation and elevated levels of mast cell mediators. The mechanisms for mast cell-associated anti-parasitic effects are not completely understood, but many studies have strongly suggested that mast cell dependent parasite resistance is primarily mediated

through the acquired immune response (71). There is a close relationship between serum IgE levels and mast cell hyperplasia in the intestine infected with helminths. Mast cells, sensitized with IgE specific for helminth antigens, may explosively release preformed mediators when challenged with the same parasites. Mast cell mediators can cause direct damage to the parasites or recruit/activate eosinophils, which can cause even greater damage to the parasite by releasing their own mediators. Epithelial damage and increased permeability may enhance these processes. Hyperresponsive intestinal smooth muscle and elevated mucus secretion also contribute to the accelerated expulsion of helminths, particularly in secondary infection.

6. Mast cells and innate immunity against bacteria

Although mast cells are not the most prominent members of the immune effector cell club, they possess a unique capability in both innate and acquired immune responses (121). The role of mast cells in acquired immunity, mainly in the form of IgE-dependent allergic reactions, has been intensively studied and is well documented. But information about the mast cells' role in innate immunity is scarce, and the mast cells' proven beneficial function against bacterial infection (5, 6) is not well recognised in the biological / medical community. In the light of a renewed interest in the older, perhaps more mature system of innate immunity (122), it is high time to investigate the roles played by mast cells in these defence processes. There are two key questions that need to be answered: 1.) Are mast cells fighting in the front line against invading pathogens, and therefore active in innate immune function? 2.) How are mast cells interacting with

bacteria or bacterial products? More studies are needed to answer these questions. New studies in this area would certainly broaden our understanding of mast cell biology and may even reveal some unique host defence mechanisms associated with mast cell function.

6-1. Mast cell responses to whole bacteria

Two recently published papers have provided the most convincing evidence, so far, that mast cells play a crucial role in host defense against bacterial infection (5, 6). Both papers reported that mast cell deficient W/W^v mice had a higher fatality and lower pathogen clearance than wild type mice when infected with Gram negative bacteria. Reconstitution of W/W^v mice with bone marrow-derived mast cells was able to restore an effective defence against bacterial infection primarily by recruiting neutrophils to the infected sites. Mast cell-derived TNF-α was credited as the key element in mast cell associated host defence, as the TNF-α levels in bacterium-infected W/W^v mice significantly increased after mast cell reconstitution, and neutralising anti-TNF-α antibody abolished mast cell induced neutrophil influx. However, there are some important questions need to be answered about the results from these studies, that employed W/W^v mice reconstituted with mast cells. Were there a significant number of contaminating hematopoietic cells that could to some degree restore other cell types in which the mice are deficient (W/W^v mice are known to have decreased numbers of other blood cell types)? Could these reconstituting cells other than mast cells count for the improved clearance of infected bacteria, or as pointed out by Erb *et al.* (123), contribute

to the elevated TNF-α level? These reports only dealt with the role of mast cells in host defence against Gram negative bacteria. What about Gram positive bacteria, which are equally important as Gram negative bacteria as pathogens?

In previous studies by Malaviya *et al.* (124, 125), mouse peritoneal and bone marrow-derived mast cells were reported to be able to bind type 1 fimbriae on *E. coli* and other enterobacteria, leading to histamine release and phagocytosis of the bacteria. The same group has also shown that mouse mast cells are able to process enterobacterial antigens and to present them to T cells through class I MHC (6). These observations have yet to be confirmed by other groups, and the potential problems with contaminating cells in the mast cell cultures need to be vigorously addressed. In another study, in which rat footpads were injected with live *Staphylococcus aureus* Cowan 1 (SAC), the mast cell numbers in the regional lymph nodes were decreased, suggesting that the mast cell response to bacterial infection might also involve mast cell migration into the infected sites (126).

6-2. Mast cell responses to bacterial components

Over the past 20 years, many investigators have studied the response by mast cells to stimulation with a variety of bacterial components (**Table 6**), notably lipopolysaccharides (LPS). Two reports from Jean Marshall's laboratory, a few years ago, have gained wide recognition in the mast cell research community because of their unique observations (65, 66). The authors reported an increased IL-6 production, but not histamine release, from rat peritoneal mast cells treated with either LPS or cholera toxin.

Table 6. Mast cell mediator release in response to bacteria and their products

Bacteria/bacterial product	Mast cell mediator release	Mast cell source
Whole bacteria		
Escherichia coli (FimH)	Histamine, TNF-α phagocytosis, antigen presentation	Mouse bone marrow
Klebsiella pneumoniae (FimH)	TNF-α, phagocytosis	Mouse bone marrow
Staphylococcus aureus (protein A)	Histamine	Human lung, tonsil
Streptococcus faecium (?)	TNF-α, phagocytosis	Human cord blood
Pseudomonas aeruginosa (?)	Histamine	Rat peritoneum
Salmonella typhimurium (?)	Phagocytosis, antigen presentation	Mouse bone marrow
Bacterial wall components		
LPS (*Escherichia coli*)	IL-6	Rat peritoneum
LPS (*Bacteroides oralis*)	Histamine	Rat peritoneum
Bacterium secreted products		
Enterotoxin (*Vibria cholerae*)	IL-6	Rat peritoneum
Toxin A (*Clostridium difficile*)	TNF	Rat peritoneum
SEB (*Staphylococcus aureus*)	5-HT	Mouse peritoneum
Hemolysin (*Listeria monocytogenes*	Histamine	Rat peritoneum

References: (2, 127, 129, 131-133)

After above mentioned reports another group using a different bacterial product, toxin A from Clostridium difficile has made similar observations (127). They observed a significant increase of TNF-α production, but without histamine release, in rat peritoneal mast cells treated with toxin A for 4 h. These observations clearly showed that cytokine secretion and degranulation can be separate events.

Protein A and non-protein A component(s) of *Staphylococcus aureus* cell walls have been shown to be able to stimulate histamine release from freshly isolated human basophilic and mast cells (128, 129). The stimulation by protein A is contributed by its binding to $F(ab)_2$ regions of small percent (~10%) of IgE on the cell surface, the binding ability of protein A to Fcγ region of IgG seems to be not related to it stimulatory effcts on mast cells (128). Staphylococcal enterotoxin B (SEB) has been regarded as a T cell specific superantigen capable of activating the majority of T cells by binding to MHC class II molecules and TCR (130). One report showed that SEB was able to induce 5-HT release from murine peritoneal mast cells and RBL-2H3 cells by binding directly to unknown receptors, which were not MHC class II molecules since such molecules were not detected on the cell surface (131).

Although an increasing number of bacteria and bacterial products have been shown to activate mast cells from different sources, the information on the mechanisms by which mast cells recognise and respond to these bacteria or bacterial products has been slow to develop. This area certainly provides new challenges for researchers interested in studying mast cell biology. With more people turning attention to this area of study, we

can expect that we will soon have a better understanding of the mechanisms, by which mast cells play a role in host defense against bacterial infection.

7. Bacterial DNA and immune responses

7-1. Discovery of the immunostimulatory effects of bacterial DNA

It is generally agreed (134) that the immunostimulatory effects of bacterial DNA were discovered by Tokunaga *et al.* In early 80's (135). However, the roots of this discovery go back to the late 1950's when investigators found that mycobacterium BCG (Bacillus Calmette-Guerin) could be used as a general immune stimulant in cancer therapy (see review by Bast *et al.*, (136)). Old *et al.* were among the first to demonstrate the anti-tumor activity of BCG by showing that inoculation of mice with BCG seven days before injecting tumor cells would prevent growth of the tumors transplanted in the mice (136). Subsequent studies have confirmed the anti-tumor activity of BCG in man as well as in other animals, including rats, hamsters and guinea pigs (136). Furthermore, treatment with BCG has been shown to inhibit or delay the growth of tumors induced by chemicals, viruses, or radiation in a number of animal models (136). Different fractions of BCG, particularly cell wall components, have been shown to be responsible for the anti-tumor activity of BCG through either the "local immune response" or " adjuvant effects" by bacterial products (137, 138).

The link between the immunostimulatory effects of BCG and bacterial DNA was first established by a group led by Tokunaga (135), who discovered an anti-tumor activity in the DNA fraction of BCG. This group obtained a nucleic acid-rich fraction from BCG, which contained 28% RNA and 70% DNA, and the DNA was single-stranded with 70% of

guanine-cytosine content. When this fraction was injected into mice four days post-carcinoma transplantation, 9 out of 12 mice were tumor free after 35 days, while control mice treated with PBS all had developed carcinoma. Digestion of the DNA with the enzyme DNase I completely abolished such protection as carcinomas were found in all 12 mice treated with DNase-digested BCG fraction. These results suggested that DNA components were essential for the anti-tumor activity induced by the BCG fraction. In subsequent *in vitro* and *in vivo* studies by the same group, the DNA-rich fraction from BCG was found to induce the production of IFN -α/β and -γ, enhance natural killer (NK) activity of mouse spleen cells (139), and to increase the infiltration of NK cells into tumor tissues (140).

Based on the information, that most of the DNA molecules in the nucleic acid-rich fraction from BCG were oligonucleotides in the 45-mer range, Tokunaga's group synthesised a variety of oligonucleotides with lengths of 15 to 45 bases, and tested their immunostimulatory activities (141, 142). The sequences of these oligonucleotides were chosen from the complementary DNA encoding a variety of BCG proteins. Their studies showed that the synthetic oligonucleotides containing unique palindromic sequences were able to induce IFN-γ production and to enhance NK activity in both in *vitro* and *in vivo* experiments. Three hexamers were identified to be the active palindromic sequences present in oligonucleotides, they included GACGTC, AGCGCT, and AACGTT (142). Noticeably, all these hexamers contain one cytosine-guanine dinucleotide ("CpG" motif), which was flanked by two 5'purines, adenine (A) or guanine (G), and two 3'pyrimidines, cytosine (C) or thymine (T). The authors also showed that the oligonucleotides containing a palindrome, ACCGGT, had no immunostimulatory effects. Although this

hexamer has one cytosine-guanine dinucleotide, like the other three active hexamers mentioned, it is flanked by one purine and one pyrimidine on both the 5'- and 3'-ends rather than the two purines at 5'-end and two pyrimidines at 3'-end. All this evidence suggested that a hexamer with a CpG motif flanked by two 5'purines and two 3'pyrimidines is the key structure rendering an oligonucleotide with immunostimulatory activity. This concept was not clearly put forward by the authors at that time but was delineated a few years later by Krieg's group (143).

Tokunaga's group further examined the biological activities of DNA samples from a variety of animals and plants, and compared the results with those from bacterial DNA (144). DNA preparations from ten species of vertebrates, including three from fish and five from mammals, showed no stimulatory activities on mouse spleen cells, and DNA from 2 species of plants were also inactive. However, DNA from each of five species of bacterium, two types of virus and four species of invertebrates enhanced NK activity and induced IFN-γ production by the cells. The specific structures of DNA from different species have determined whether they will induce response from mammalian cells (**Table7**). The authors pointed out that synthetic oligonucleotides need to be at least 30-mer long before they possessed activity, as a 15-mer oligonucleotide containing active palindromic sequences used in their study did not induce IFN or enhance NK activity in mouse spleen cells (142). According to a later study by Krieg's group, the minimum length of oligonucleotides with mitogenic activity on mouse B cells could be 8 bases long (143). Nevertheless, 20-mer oligonucleotides are the most often used DNA prodcts to activate variety of immune cells.

Table 7. Comparison of bacterial and mammalian DNA

	Bacterial DNA	Mammalian DNA
CpG frequency	High (1/16)	Low (1/60)
Cytosine	Unmethylated	Methylated
AACGTT* frequency	High (0.347)	Low (0.056)
B cell mitogenesis	Yes	No
Immunostimulation	Yes	No

* The hexamer (AACGTT) is one of several palindromic sequences that render an oligodeoxynucleotides the ability of immunostimulation.

(References: (143, 146)

We owe a lot of our today's knowledge about DNA-induced immune responses to Krieg and his colleagues, which is currently the most active group in the world in studying the immune activities of bacterial DNA, and specially oligodeoxynucleotides of around 20 bases long. The bulk of the experiments in Krieg's group were carried with synthetic oligodeoxynucleotides, hundreds of them. Krieg's group was the first to clearly demonstrate that the CpG motif is the essential element present in the oligodeoxynucleotides that have immunostimulatory activity, and the CpG motif needs to be flanked by two 5' purines and two 3' pyrimidines. They also showed that the cytosine in the CpG motif is to be unmethylated (143). They have provided evidence to show that oligodeoxynucleotides having multiple CpG motifs could have greater stimulatory capacity than those having only one CpG motif (143, 145). Krieg *et al.* have broadened

the earlier studies by Tokunaga's group by including B cells, T cells, monocytes and macrophages for their investigation, and by investigating a variety of cytokines, immunoglobulins, and cell surface molecules critical to the immune response. One recent contribution by this group is the concept of inhibitory/neutralising oligodeoxynucleotides that have a CpG motif flanked by one 5' pyrimidine or/and one 3' purines. The oligodeoxynucleotides containing such sequences (e.g., CCG, CGG) not only have no immune activity, but also neutralise the immunostimulatory effects by DNA containing the stimulatory sequence (5' purine-purine-CG-pyrimidine-pyrimidine-3') (146).

7-2. Factors determining the immunostimulatory effects of DNA products

To summarize our current knowledge, it is generally agreed that bacterial or synthetic DNA that can activate immune cells need to have the following features:

1. Contain at least one CpG-dinucleotide (CpG motif).
2. The cytosine in the CpG motif is to be unmethylated
3. The CpG motif must be flanked by two 5' purines and two 3' pyrimidines (the location of which can be in the middle, or at either end of the oligodeoxynucleotides) (142).
4. The length of CpG-containing oligodeoxynucleotides must be at least 8 bases long to be able to activate B cells (143) and to be able to activate other immune cells to secrete cytokines (147).

5. Phosphorothioate CpG-containing oligodeoxynucleotides are much more potent than phosphodiester CpG-containing oligodeoxynucleotides in activating immune cells (2 log difference in the optimal concentration) (143).

However, a recently published report has put forward some data directly challenging some of the core features of immunostimulatory oligodeoxynucleotides (148). This report showed that plasmid DNA treated with CpG methylase had the similar effects as intact plasmid DNA in inducing MHC-I and –II expression on a non-immune cell line of rat thyroid origin. They have shown that neither CpG-ODN nor non-CpG-ODN induced these MHC molecules, and that only double stranded, but not single stranded, oligodeoxynucleotides of the same sequences induced MHC-I and –II expression. Both bacterial and vertebrate (calf thymus and salmon sperm) DNA induced MHC-I and –II expression. These data seem to indicate that activation of "non-immune" cells may be CpG motif independent, and sequence independent, and mainly dependent on the double stranded form of oligodeoxynucleotides. More vigorous investigation is needed to verify such dramatic differences between traditional immune effector and non-immune cells in their response to the natural and synthetic DNA.

7-3. *In vitro* immune response to bacterial and synthetic DNA

In early studies on bacterial and CpG-ODN induced activation of the immune system, *in vitro* experiments were mainly conducted with spleen cells. Only over the last few years did people start to investigate the stimulatory effects of DNA on highly purified different types of cells. Both bacterial DNA and CpG-ODN have been repeatedly shown to

directly activate B cells, monocytes, and macrophages of both mouse and human sources. Although increased activation of T cells and NK cells has been observed after treatment with bacterial DNA and CpG-ODN, there is some controversy concerning whether such activation is the result of direct stimulation or more indirect effects.

7-3-1. B cells

7-3-1-1. Induction of B cell proliferation

So far, the most clear cut evidence for the immunostimulatory effects of bacterial and synthetic DNA is from studies on B cells. Pisetsky *et al.* were perhaps the first group of researchers to demonstrate that bacterial DNA was able to directly activate B cells (149). A few years later, Wang *et al.* (150) further demonstrated that bacterial DNA and CpG-ODN induced DBA/2 mouse B cells to proliferate and to secrete IgM in a dose-dependent fashion. Over 95% of B cells were driven into the cell cycle by either a 15-mer or a 20-mer CpG-ODN. Such a percentage is greater than that induced by optimal concentrations of LPS, therefore, the CpG-containing oligodeoxynucleotides are regarded, by some, as complete mitogens for B cells. The activating effects of CpG-ODN on human B cells were demonstrated by Liang *et al.* (151), who reported that over 95% of human blood B cells showed activation markers, CD25 and CD86, after treatment with CpG-ODN for 48 h. They also observed CpG-ODN-induced production of IgM, IgG, and IgA in the human B cells.

Bacterial DNA-induced B cell proliferation could have an important role in host immunity against bacterial infection, but could also be implicated in pathological conditions associated with imbalances in B cell activation, including SLE. Bacterial DNA

products have been implicated as triggering factors responsible for systemic lupus erythematosus (SLE), an autoimmune disease with anti-DNA antibody as a prominent diagnostic feature (152). DNA isolated from blood plasma of patients with SLE is CpG rich and hypomethylated, suggesting this DNA may be of bacterial DNA origin (153, 154). Prolonged survival of B cells induced by bacterial DNA could increase the opportunity for B cells to produce autoimmune antibodies, including anti-DNA. Furthermore, CpG-ODN have been shown to have synergistic effects with antigens or anti-IgM in stimulating B cells, resulting in B cell proliferation and immunoglobulin secretion (143). These results add more support to the notion that bacterial DNA could contribute to the production of autoantibodies in the body (155).

7-3-1-2. Protection of B cells against apoptosis

Krieg's group was the first to discover that unmethylated CpG-containing oligodeoxynucleotide (CpG-ODN) can rescue WEHI-231 murine B lymphoma cells from anti-IgM induced apoptosis (156). WEHI-231 has been widely used as model to study B cell apoptosis induced by cross-linking of membrane immunoglobulin receptors. This group of investigators had shown that a 20-mer CpG-ODN, but not non-CpG-ODN, had a reduced apoptotic rate from 59% in WEHI-231 cells treated with anti-IgM to 13% in the cells treated with anti-IgM plus CpG-ODN. Moreover, the authors investigated the effects of CpG-ODN on the expression of several proto-oncogenes, including c-myc, bcl2, and bcl-x, whose down-regulation is known to be associated with B cell growth arrest and / or apoptosis (157). They found that CpG-ODN up-regulated bcl2, and bcl-x

mRNA expression in WEHI-231 cells, and reversed anti-IgM-induced down-regulation of c-myc expression. These results suggest that CpG-ODN may rescue B cell apoptosis by reversing the down-regulation of c-myc, bcl2, and bcl-x in anti-IgM treated B cells.

Another group of investigators studied the effects of CpG-ODN on B cell cycle by using primary B cells isolated from mouse spleens, and found a different mechanism by which CpG-ODN protect B cells against apoptosis (150). This group treated mouse B cells with CD40L-CD8, a fusion protein, which caused the B cell to up-regulate Fas expression, and therefore, to become highly susceptible to Fas-mediated apoptosis by anti-Fas antibody, or by CD4+ Th1 effector cells that express CD40 ligand. However, when treated with CD40L-CD8 proteins in combination with CpG-ODN, the B cells were much more resistant to apoptosis induced either by anti-Fas antibody or by CD4+ Th1 effector cells. These findings provide further clues to link the bacterial DNA-induced down-regulation of Fas on B cells with increased production of autoantibodies in autoimmune diseases. Defective Fas-mediated apoptosis has been observed in some SLE patients with mutated Fas ligand gene and in animal SLE models, including MRL/lpr mice with a mutated Fas gene (150).

7-3-2. T cells

Krieg *et al.* reported that CpG-ODN could not induce proliferation of freshly isolated mouse T cells, although the same CpG-ODN induced proliferation in most mouse B cells (143). However, Klinman *et al.* (147) demonstrated that bacterial DNA was able to induce IL-6 and IFN-γ from purified CD4+, but not CD8+, T cells. As the purity of these T

cell types was 87-94%, there was a possibility that cytokine production from the CD4+ T cells was derived from other contaminated cells. Further studies, from another group, lend support to these observations. Sun *et al.* (158) reported that CpG-ODN failed to activate highly purified (99.9%) mouse T cells unless antigen presenting cells (APCs) were added to these cells, and APC-derived IFN-β was believed to be responsible for T cell activation by CpG-ODN. In a recent report CpG-ODN were, however, able to co-stimulate T cells that were triggered via their T cell receptors (TCR) in the absence of antigen presenting cells, resulting in IL-2 production and T cell proliferation (159).

7-3-3. Monocytes/macrophages and Dendritic cells

Both bacterial DNA and CpG-ODN are able to directly activate murine macrophages (160-162). Sparwasser *et al.* reported that TNF-α was induced in a murine macrophage cell line, ANA-1, and murine peritoneal macrophages treated with DNA from Gram negative bacteria (*E. coli*) and Gram positive bacteria, (*Staphylococcus aureus* and *Streptococcus faecalis*) as well as CpG-ODN (160). Calf thymus DNA and non-CpG-ODN did not produce TNF-α. In another study, proinflammatory cytokines, including IL-1, IL-6, IL-12 and TNF-α were induced in a murine macrophage cell line J774 treated with CpG-ODN (163). Murine bone marrow-derived macrophages and murine RAW 264 macrophages have also been shown to produce TNF-α and nitric oxide in response to bacterial DNA and CpG-ODN (162).

Sparwasser *et al.* reported that bacterial DNA and CpG-ODN induced maturation of murine bone marrow-derived dendritic cells by up-regulating MHC-II, CD40 and CD86,

and directly activated these cells to secrete IL-6, IL-12 and TNF-α (164). This report awaits confirmation.

7-3-4. NK cells

Increased NK cell activity was among the first immunostimulatory effects observed with bacterial DNA. Yamamoto *et al.* (139) found that a group of 30-mer CpG-ODN increased mouse spleen cell NK activity against Yac-1 target cells, and such augmented NK activity was abolished by anti-IFN-α/β antibody. Chace *et al.* (165) found that bacterial DNA induced IFN-γ secretion by unfractioned mouse spleen cells, however, the IFN-γ levels sharply dropped to a minimal level when NK cells were depleted from the spleen cells, suggesting that NK cells were responsible for most of this IFN-γ production. However, when highly purified (> 98%) NK cells were treated with bacterial DNA, there was no detectable induction of IFN-γ secretion. When increased numbers of macrophages were added to the NK cells, there was a gradual increase of IFN-γ secretion in the mixed cell cultures receiving the same DNA treatment. These data suggest that bacterial DNA can not directly activate NK cells, and IFN-γ production by NK cells is macrophage-dependent. These authors had demonstrated that purified macrophages secreted IL-12 in response to bacterial DNA treatment, therefore, they proposed that bacterial DNA stimulated macrophages to secrete IL-12, which in turn, induced NK cell to produce IFN-γ. Augmentation of NK activity was also observed in human PBMC treated with *E.coli.* DNA and CpG-ODN, but not with calf thymus DNA or non-CpG-ODN (166).

7-4. *In vivo* immune responses to bacterial and synthetic DNA

Although the earliest *in vivo* experiments were focused on anti-tumor activity induced by bacterial DNA, the research activities in this area have been advancing in a rather slow pace. However, two recent reports have shown that CpG-containing oligonucleotides can enhance the anti-tumor response in the body (167, 168). In tumor therapy studies on mice transplanted with lymphoma cells, injection of the mice with CpG-ODN alone prolonged mouse survival (167). More significantly when the CpG-ODN was injected in combination with an anti-tumor antibody, over 80% of the tumor-transplanted mice survived up to 60 days, while control mice without any treatment all died within 25 days of receiving tumor transplantation. In similar studies employing the same murine lymphoma model, Liu *et al.* tested the potent adjuvant effects of CpG-ODN on vaccination with a tumor antigen-GM-CSF fusion protein (168). They demonstrated that administration of CpG-ODN plus the tumor antigen-GM-CSF fusion protein prevented tumor growth and increased the survival rate to 70% at day 60 after tumor inoculation, in contrast to the 30% survival rate in the mice receiving tumor antigen-GM-CSF fusion protein and 0% survival rate in the mice without any treatment.

There are further reports dealing with a wide range of other *in vivo* immune responses to bacterial and synthetic DNA. Injection of CpG-ODN, but not non-CpG-ODN, induced increased levels of TNF-α, IL-6 and IL-12 in several strains of mice, including C57BL/6, BALB/c and SCID mice (163). SCID mice are deficient in B, T, and NK cells, therefore, the possible sources of these cytokines may be macrophages or mast

cells, as both types of cells could produce large amounts of TNF-α in response to whole bacteria or bacterial products (6).

Another prominent feature of bacterial DNA and CpG-ODN-induced responses is the induction of Th1 type cytokine IFN-γ and IL-12, and failure to induce Th2 type cytokines IL-4 and IL-5 (145, 147). Based on such information, some investigators have tried to test the notion of using bacterial DNA and CpG-ODN to prevent or treat allergic conditions, which are generally agreed to be Th2-type dominant immune disorders (169). Raz *et al.* (169) reported that injection of plasmid DNA to mice resulted in a Th1 type response with the induction of IFN-γ and IgG2a, and injection of *E. coli* β-galactosidase (β-gal) led to Th2 type response with the appearance of IL-4, IL-5 and IgE, IgG1. When the plasmid DNA was co-injected with β-gal, the mice developed a Th1 type dominant immune response, with decreased levels of β-gal-specific IgE and IgG1, and IL-4, IL-5, and increased IgG2a and IFN-γ. Similar results are also achieved in animals injected with plasmid encoding latex allergens Hev b5 (170) and the house dust mite allergen Der p5 (171). Decreased IgE levels have been observed in these animal models. More interesting is that CpG-ODN could have therapeutic effects on animals with established allergic conditions (172). When OVA-sensitized mice were injected with CpG-ODN 6 days before OVA challenge, the eosinophil infiltration in the BAL fluid dropped to 7 % of the level observed in the mice not receiving CpG-ODN treatment (172).

Several studies have demonstrated the protective effects of of CPG-ODN on potentially lethal infection with intracellular bacteria *Listeria monocytogenes* (173, 174) and intracellular parasite *Leishmania major* (175, 176). The protection in all these murine

models was contributed by the Th1 type cytokines IL-12 and IFN-γ induced by bacterial DNA and CpG-ODN (173, 175).

One of the most prominent functions of bacterial DNA and CpG-ODN is their immune adjuvant property, which has been a major focus for *in vivo* studies over the last few years. Soluble protein ovalbumin (OVA) induces a poor humoral and cellular response due to its poor immunogenicity, however, when co-administrated with CpG-ODN, OVA induced strong B cell and cytotoxic T cell responses (163). Therefore, the authors proposed to use CpG-containing oligodeoxynucleotides as inexpensive and safe vaccine adjuvants. The immune adjuvant properties of bacterial DNA and CpG-ODN have been utilized to enhance vaccination against hepatitis B surface antigen (177) and tumor antigens (178). In another study, Cowdery *et al.* (179) reported that bacterial DNA increased the toxicity of LPS in mice. The mice which received *E. coli* DNA four days before administration of 100 μg/ml of LPS had a morbidity of 59%, while all the mice who received calf thymus DNA treatment survived LPS challenge four days later.

7-5. Bacterial DNA and CpG-ODN induced cytokine production

Cytokine production is one of the most common events occurring to immune cells activated by bacterial DNA or CpG-ODN (**Figure 4**). The information gathered over recent years has pointed out that cytokine induction by bacterial DNA and CpG-ODN is selective rather than random, i.e., these DNA products primarily induce proinflammatory cytokines, including IL-1, TNF-α, and IL-6, and Th1-type cytokines, including IFN-γ and IL-12 (145). However, some cytokines, including IL-2, IL-3, IL-10 and Th2-type cytokine

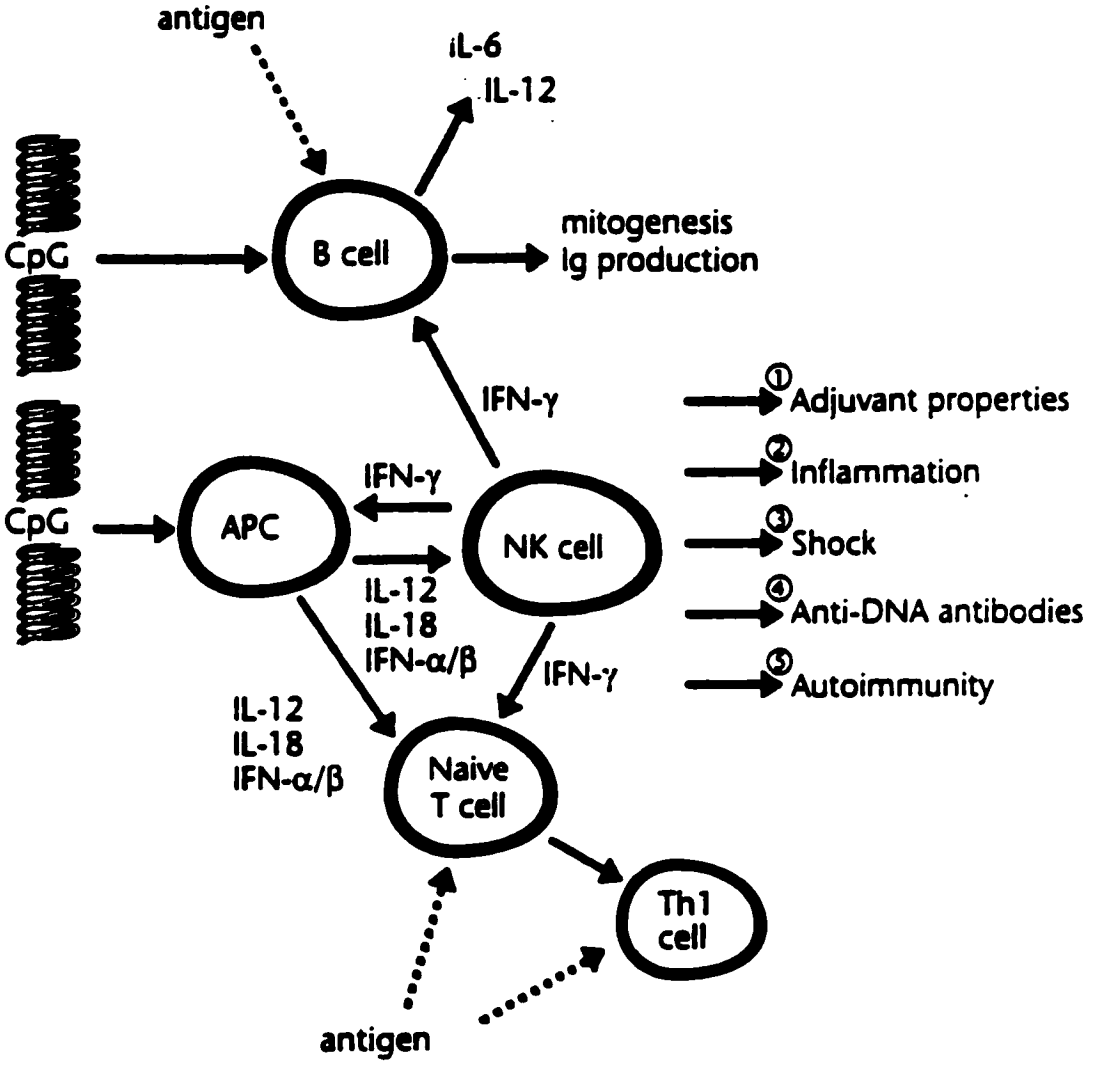


Figure 4. Effects of bacterial DNA and CpG-ODN on immune system.

(Reference: 184)

IFN-α and -β were perhaps the first cytokines shown to be induced in immune cells exposed to bacterial DNA (144). Over subsequent years, both IFN-α and -β production was induced in human monocytes treated with plasmid DNA and CpG-ODN (134). In another study, IFN-γ was induced in mouse spleen cells treated with *E.coli* DNA and CpG-ODN, but not by calf thymus DNA or non-CpG-ODN (147, 180). Both *in vitro* and *in vivo* experiments with mice have shown that NK cells are the major source of this IFN-γ (147).

DNA from both Gram positive and Gram negative bacteria and CpG-ODN have been shown to induce TNF-α from freshly isolated murine macrophages and murine macrophage cell lines (162, 181). In several other studies, IL-6 production was observed in highly purified mouse B cells, the B cell line CH12.LX (182), the murine macrophage cell line J774 (163), and mouse bone marrow-derived dendritic cells (164), all of which had been treated with CpG-ODN (165, 180). IL-6, which like TNF-α, plays an important role in both acute phase of inflammation and chronic inflammation, was induced in B cells treated with bacterial DNA (182). One interesting observation was that DNA-induced IL-6 production was enhanced by simultaneous stimulation through antigen receptors on the surface of B cells (182). This is another example revealing a close interplay between innate and acquired immunity in response to invading pathogens. Induction of the proinflammatory cytokine IL-1β at the mRNA level has been observed in murine RAW 264 macrophages treated with plasmid DNA (183).

IL-12 production was induced in mouse spleen cells treated with CpG-ODN and *E. coli* DNA but not with calf thymus DNA (147). However, there was no detectable IL-12

induction in DNA-activated mouse spleen cells depleted of macrophages, indicating that macrophages were the major cell source of IL-12. In one *in vitro* experiment, bacterial DNA directly activated purified mouse macrophages to produce IL-12 (165). Increased mRNA expression of IL-12 p40 was also found in human monocytes stimulated with plasmid DNA and CpG-ODN (134). Enhanced IL-12 production, in turn, induces IFN-γ from immune cells, especially NK cells. Both IL-12 and IFN-γ greatly enhance classical cellular immune responses.

7-6. Possible mechanisms for DNA- induced immune responses

Although CpG-ODN drove more than 95% of mouse B cells into proliferation cycle, there was no detectable tyrosine phosphorylation, inositol triphosphate generation, or Ca^{2+} mobilization observed in B cells within 10 min of treatment with such synthetic oligodeoxynucleotides (143). In another study, CpG-ODN-induced IL-6 production from mouse B cells was suppressed by antioxidants, but was not affected by the inhibitors of either protein kinase C or protein kinase A (182). This study also showed increased intracellular levels of reactive oxygen species inside mouse B cells treated with CpG-ODN, but not in the B cells treated with non-CpG-ODN, suggesting that CpG-ODN induced B cell activation was mediated through a reactive oxygen intermediate-dependent pathway.

Apparently, bacterial DNA and CpG-ODN can by-pass several important signal pathways to activate immune cells. However, like LPS, bacterial DNA and CpG-ODN are able to activate transcription factor nuclear factor-κB (NF-κB) (183). Stancey *et al.*

reported that NF-κB activity was increased in a murine bone marrow-derived macrophage treated with plasmid DNA, and the extent of the nuclear factor activation was comparable to that induced by LPS (183). Nuclear translocation of NF-κB was also observed in murine peritoneal macrophages and a murine macrophage cell line (ANA-1) treated with *E. coli* DNA and CpG-ODN (160).

A major driving force behind the studies of immune stimulation by CpG-ODN are efforts to explore antisense oligonucleotide therapy, in which the mRNA for specific genes is targeted (185). Understanding CpG-ODN uptake and activating mechanisms may provide new insights into how antisense treatment can be improved. Based on the immune properties of bacterial DNA and random sequences of CpG-ODN, as well as the consistent results obtained from very diverse DNA products, the immunostimulatory effects of CpG containing DNA are most likely exerted through non-antisense mechanisms.

7-7. DNA membrane receptors

There is not yet convincing evidence to confirm the existence of CpG-specific receptors present on the cell surface. However, some studies, particularly those carried out by groups focusing on the mechanisms of oligonucleotide uptake, have suggested that there are cell surface receptors for oligonucleotides (186, 187). Kimura *et al.* (186) reported that a 30-mer CpG-containing oligonucleotide enhanced NK activity and IFN production in murine spleen cells by binding to a cell surface molecule with a MW similar to that of the mouse scavenger receptors (220 kDa). Such CpG-ODN-induced immune activity could be

blocked by negatively charged compounds, including dextran sulfate and polyvinyl sulfate, both of which are also able to bind to the scavenger receptors. Furthermore, the 30-mer oligodeoxynucleotides inhibited the binding of acetyl-LDL, a well-known natural ligand for scavenger receptors, to the cell surface. Based on these observations, the authors suggested that augmentation of NK activity and IFN production by oligodeoxynucleotides was mediated through the scavenger receptors on the spleen cells. In another *in vitro* study, Benimetskaya *et al.* (187) found that the binding of a 15-mer oligodeoxynucleotide to human peripheral blood neutrophils was inhibited by antibodies to Mac-1 (CD11b/CD18; αMβ2), an integrin family member present mainly on the surface of macrophages, neutrophils and NK cells. Fibrinogen, a natural ligand for Mac-1, was shown to block the binding of the oligodeoxynucleotides to the human neutrophils and human myeloid precursor cell line HL-60. They proposed that Mac-1 is a cell surface receptor for oligodeoxynucleotides. However, the observations by the above two groups need to be verified by other laboratories and on other types of immune cells. One way to do this would be to carry out *in vivo* studies on Mac-1 or scavenger receptor deficient or knockout animals, or to conduct *in vitro* experiments on cells with or without expression of these surface molecules.

Despite these studies, an increasing body of evidence indicates that there are no CpG-specific membrane receptors. Krieg et al. (143) pointed out that there was no CpG-specific receptor as both fluorescent-labeled CpG-ODN and non-CpG-ODN had similar binding to mouse B cells, even though only the CpG-ODN had a strong stimulating effect on the freshly isolated B cell. But they did report that there was no stimulatory effect

observed in B cells exposed to CpG-ODN linked to a solid support, suggesting that internalization of CpG-ODN is required for immunostimulatory effects.

8. Rationale for the thesis studies

In the studies to be described in this thesis we sought to examine two major hypotheses related to the regulation of mast cell cytokine expression. The first portion of the work described is focused on the cytokine secretion pathways of mast cells, and the second portion of the studies concentrates on novel inducers of cytokine expression in mast cells.

Mast cells and basophils are unique immune effector cells that exert their function through released mediators, including cytokines. Although an increasing number of cytokines have been shown to be produced by mast cells (4, 7), few studies have examined the secretion routes of mast cell cytokines (84, 188). Investigating the events involved in the secretion of mast cell mediators, particularly cytokines, will help us to better understand the role of mast cells in physiological, pathological, and immunological processes. We have investigated secretion pathways of IL-6 and GM-CSF from KU812 and mBMMC using brefeldin A (BFA) and monensin, two well-characterized protein secretion inhibitors with defined mechanisms of action on Golgi mediated vesicular transport. Both KU812 cells and mBMMC are capable of producing substantial amounts of IL-6 and GM-CSF, which are involved in a wide range of immunologic activities, including acute and chronic inflammation. The main goal for this portion of our studies was to determine whether the cytokines, IL-6 and GM-CSF, are secreted from mBMMC

and KU812 cells through a degranulation-related pathway or a vesicle dependent pathway. It is our hope that the results from our studies would be useful to help designing better ways to modify the secretion of pro-inflammatory cytokines in allergic diseases or other inflammatory conditions.

On the basis of these studies we sought to explore novel inducers of mast cell cytokine expression and chose to examine the second hypothesis that mBMMC and KU812 cells could recognize and respond to bacterial DNA and CpG-ODN by releasing cytokines, particularly IL-6 and TNF-α, which are known to be associated with inflammation and can be produced by activated rodent mast cells with or without concurrent degranulation (65-67, 189). To gain further insights into the mechanisms, by which CpG-ODN exerts its effects, we examined oligodeoxynucleotide uptake by mBMMC using flow cytometry and confocal microscopy. It was hoped that this portion of our studies could shed some new light on mechanisms by which mast cells respond to bacteria, and provide fresh evidence to support the role of mast cells in DNA vaccination.

CHAPTER 2

METHODOLOGY

1. Cell source

1-1. mBMMC

mBMMC were cultured from male C57BL/6 mice, 6 to 8 weeks old (**Figure 5**), housed in the Animal Facility at either McMaster University, Hamilton, Ontario, or Dalhousie University, Halifax, Nova Scotia, Canada. All experimental procedures were approved by the Animal Research Ethics Board of McMaster University and Dalhousie University. The mice were sacrificed by anesthesia or CO_2 inhalation. Intact femurs and tibias were removed from mice. The bone marrow cells were harvested by repeated flushing of the bone shaft with endotoxin-free RPMI 1640 medium (Life technologies, Grand Island, NY). The bone marrow cell culture was established at a concentration of 1 X 10^6/ml in medium consisting of RPMI 1640 (Life technologies), 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (2-ME), supplemented with WEHI-conditioned medium as a source of IL-3 (190). During studies of cytokine secretion pathways in mast cells, the WEHI-conditioned medium was added as 10% of 10 X WEHI-conditioned medium. For convenience, the WEHI-conditioned medium was added as 20% v/v of unconcentrated supernatant from WEHI-3B cells during the period of studies of the DNA and oligodeoxynucleotide-induced cytokine production in mast cells. Both ways of adding WEHI-conditioned medium are equally efficient for culturing mBMMC with high purity. Non-adherent cells were transferred to fresh culture medium once a week. After 4 - 6 weeks of culture, the purity of mast cells from mouse bone marrow cultures was > 95%, as confirmed by toluidine blue and Alcian blue/safranin O staining of cytocentrifuge preparations and by flow cytometri c analysis

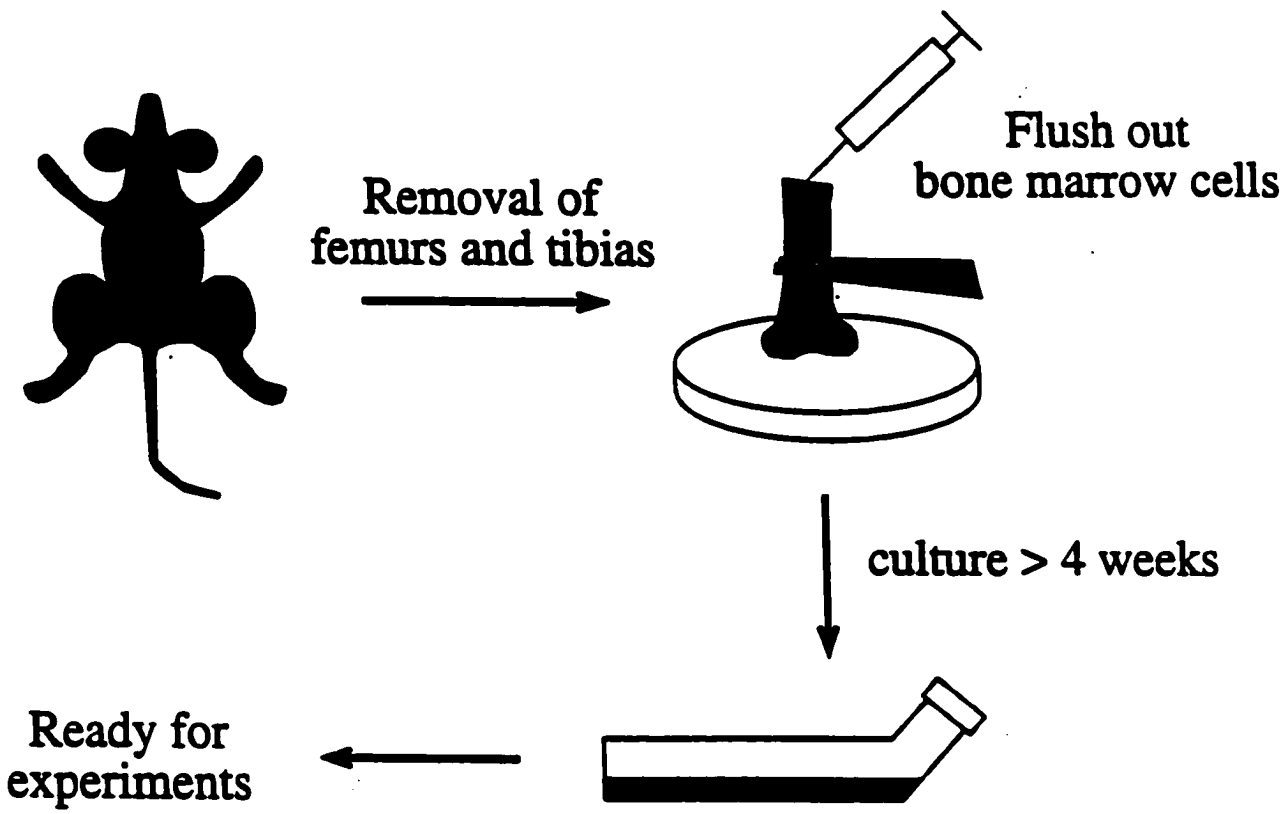


Figure 5. Procedures for murine bone marrow mast cell culture.

of mBMMC that were fluorescently labeled with antibody against mouse c-kit (Cedarlane Laboratories Ltd, clone ACK4).

1-2. KU812

The human basophilic cell line, KU812, was maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. The cells were passaged twice a week. For further differentiation the cells were cultured in the above medium further supplemented with 0.3 mM sodium butyrate (Sigma, St Louis, MO) for 3 days, followed by 3 or 4 days of culture in the sodium butyrate-containing medium with the addition of 50 U/ml recombinant human IFN-γ (Genzyme, Cambridge, MA). Such differentiation conditions are very similar to those which have previously been reported to lead to a more differentiated phenotype (41) and which lead to enhanced expression of both GM-CSF and IL-6 following stimulation (Marshall *et al.* unpublished), To verify the differentiation of KU812 cells, human mast cell tryptase was measured in the cells using a radioimmunoassay (Pharmacia, Uppsala, Sweden). The average concentration was 6 ± 0.6 $\mu g/10^6$ cells (mean ± SEM, n=8), less than the normal tryptase concentration of human mast cells (10 to 35 $\mu g/10^6$ cells) (191) but greater than the amounts observed in normal basophils (192). Therefore, KU812 cells differentiated under the above conditions are considered to be mast cell-like cells, and the term "mast cells" will be used to refer to these "KU812 cells" in this thesis with the author's full awareness of the many differences between the differentiated KU812 cells and normal human mast cells.

1-3. Accessory cell lines

1-3-1. WEHI-3B

WEHI-3B is a BALB/c myelomonocytic cell line that constitutively produces high levels of IL-3 (193). WEHI-3 cells have been cultured in many laboratories to provide a relatively inexpensive source of IL-3 used for mast cell culture mainly from bone marrow stem cells (190). WEHI-3B cells were cultured at a concentration of 0.2 X 10^6 cell/ml in RPMI 1640, supplemented with 5% FCS, 1 mM MEM non-essential amino acids (Life technologies), 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (2-ME). After two days' culture, the cell culture supernatant was collected and stored in –20 ^{O}C after the supernatant was passed though a 0.45 μm filter to remove any residual cells. To obtain 10 X concentrated WEHI-conditioned medium, the supernatant was transferred into dialysis tubing (VWR cat.# 25218-129, Missisauga, ON, Canada), then the tubing was put in a plastic bag and surrounded with aquicide II powder (Calbiochem-Novabiochem, La Jolla, CA), and set at 4 ^{O}C until about 1/10 of the original volume was left in the tubing. This process usually took about 1 to 2 days. The concentrated WEHI-conditioned medium was harvested and stored at –20 ^{O}C for later use.

1-3-2. B-9 cell

The B-9 cell is a growth factor (IL-6)-dependent B cell hybridoma cell line obtained from a fusion of a murine myeloma cell line with BALB/c spleen cells (194). This cell is widely used for measure the bioactivity of IL-6 of either human or mouse

origin (195). B-9 cells were maintained at around 0.1 X 10^6 cell/ml in RPMI-1640 medium, supplemented with 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μM 2-ME, and a supernatant source of IL-6, and passaged twice a week. The IL-6 containing supernatant was collected from 48-h culture of either normal primary human lung fibroblasts or a murine macrophage cell line J774 treated with 2.5 ng/ml of recombinant human IL-1β. The confluent B-9 cells are ready for IL-6 bioassay if the cell viability is over 85% as determined by trypan blue vital staining.

1-3-3. L929 cell

The L929 cell (ATCC# CRL-2148) is a BALB/c fibroblast cell line, highly sensitive to TNF-α, widely used to measure the cytotoxic bioactivity of mouse and human TNF-α (196). The cells were maintained at a concentration of approximately 0.2 X 10^6 cell/ml in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, and passaged twice per week.

2. Major reagents

2-1. Calcium ionophore (A23187).

A23187 is a metabolite of *Streptomyces chartreusensis* with a molecular weight of 523 (**Figure 6**), and belongs to group of hydrophobic molecules known as ionophores that selectively increase the membrane permeability of inorganic ions (197). A23187 is highly selective for divalent over monovalent cations, capable of facilitating a Ca^{2+}-for-H^+ exchange across biological membrane without disturbing the pre-existing balance of

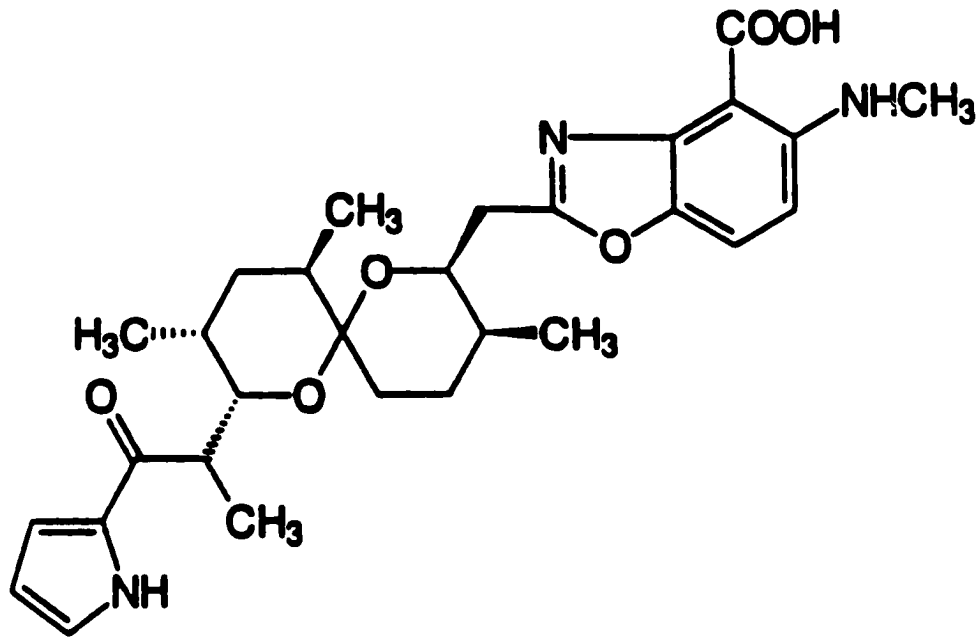


Figure 6. Chemical structure of A23187

(Reference: 198)

Na^+ and K^+ (198). It has been shown that A23187 mediated Ca^{2+} entry into cells, and also released Ca^{2+} from intracellular organelles, including the sarcoplasmic reticulum, resulting elevated intracellular Ca^{2+} levels, most often accompanied by cell activation (199, 200). The effects of A23187 on intracellular Ca^{2+} levels have prompted investigators to use calcium ionophore to study a wide range of cell biologic activities, including secretion. Foreman *et al.* were among the earliest researchers to examine the stimulating effects of A23187 on mast cells, and found that A23187 was able to induce histamine release from mast cells (201). Today A23187 is one of most commonly used stimulants to activate numerous types of cells, especially mast cells; quite often it is used as a positive control to compare with other mast cell stimulants. A23187 is usually dissolved in DMSO or ethanol at a stock concentration of 1 mM and used at a concentration between 0.1 to 1 μM. For the present studies, A23187 (Sigma) was dissolved in DMSO at a stock concentration of 1 mM, and stored at –20 ^{O}C before use. A23187 had no significant cytotoxic effects on either KU812 cells or mBMMC at the levels used in present studies, as assessed by trypan blue vital staining for cell viability.

2-2. Brefeldin A (BFA)

BFA is a macrocyclic lactone with a molecular weight of 280, first isolated from a species of *Penicilium* in 1958, later also found in a variety of other fungi (**Figure 7**) (202). Today BFA is widely used by investigators for its rapid and reversible inhibition of protein secretion, while it has minimal effects on protein synthesis, in eukaryotic cells (203). Within a few minutes of BFA treatment, the Golgi complex disintegrates and

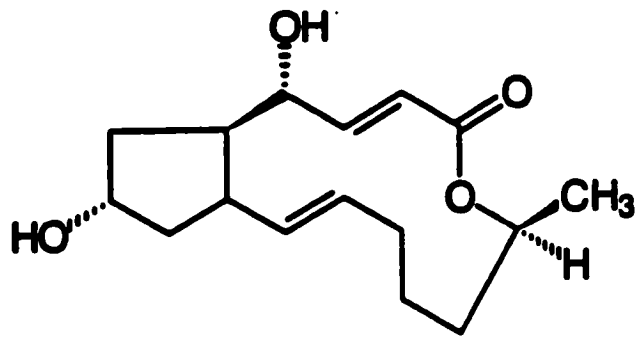


Figure 7. Chemical structure of brefeldin A (BFA).

(Reference: 202)

redistributes into the endoplamic reticulum. As a result, the intracellular transport of synthesised proteins to the Golgi complex is obstructed and the protein secretion out of cells is blocked. Remarkably, the intracellular structures are fully restored and protein secretion is reassumed when BFA is removed.

The mechanism of action of BFA is related to its specific inhibition of guanine nucleotide exchange activity for the small GTP-binding protein ADP-ribosylation factor 1 (ARF1) in the cytosol (204). GTP-bound ARF1 proteins become activated and bind to the Golgi membrane, from where the ARF1 proteins promote recruitment of coat proteins (CP) to form coat protein complexes (COP1). The Golgi-bound COP1 is essential for the budding and forming of transport vesicles, which control the retrograde trafficking between Golgi complex and ER. As the result of BFA-induced inhibition of ARF1, the COP1 coat protein complexes become dissociated from the Golgi membrane, leading to disassembly of Golgi apparatus and its fusing with endoplasmic reticulum (204, 205).

For the present study, a stock solution (Sigma) was prepared of 1 mg/ml BFA in ethanol, and stored at -20°C until use. In order to make sure that the inhibitory effects of BFA on protein secretion occur before cell activation takes place, BFA was added to mast cells 5 min before stimulation with A23187 or through IgE receptor cross-linking. Based on trypan blue vital staining results, no significant cytotoxicity was observed in either KU812 cells or mBMMC treated with BFA at the doses used in the current studies.

2-3. Monensin

Monensin (**Figure 8**) is another commonly used protein secretion inhibitor with a mechanism of action different from that of BFA. Originally purified as a metabolite of *Streptomyces cinnamonensis* in 1967, monensin is an open chain molecule with a molecular weight of 693 (206). Monensin is a sodium ionophore that facilitates the entry of Na^+ into cells, causing collapse of Na^+ and H^+ gradients in the Golgi complex (207). As a result, the Golgi structure and function are disrupted, leading to a failure of secretory vesicle formation and transport from the Golgi complex to the cytoplasmic membrane but with little effect on protein synthesis. In contrast to BFA that initiates its effects from the proximal portion of the Golgi complex, monensin exerts its most profound effects on the distal portion of the Golgi complex. The effects of monensin are rapid and reversible, as studies showed that the swelling *trans* cisternae of the Golgi complex appeared only a few minutes after cells were treated with monensin, and normal protein secretion would resume 20 minutes after the withdrawal of monensin from culture medium (207). For the present study, a 10 mM monensin (Sigma) stock solution was in ethanol, and stored at -20°C until use. As in the case for BFA, monensin was added to mast cells 5 min before adding stimuli. Monensin had no significant cytotoxic effects on either KU812 cells or mBMMC at the doses used in the present studies, as assessed by trypan blue vital staining for cell viability.

2-4. Synthetic oligodeoxynucleotides

Synthetic oligodeoxynucleotides were all 20-base pairs long and phosphorothioate-modified and purified by reverse phase HPLC by the manufacturer (Research Genetics, Huntsville, AL). The sequences of the oligodeoxynucleotides (**Table 8**) used in this study

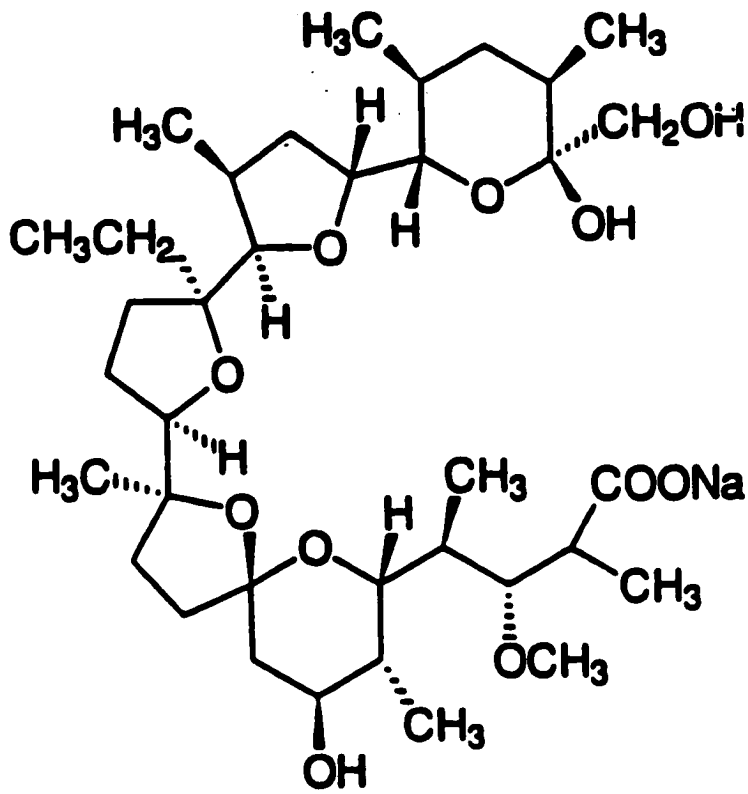


Figure 8. Chemical structure of monensin.

(Reference: 206)

are taken from published reports, in which the CpG-ODN were shown to be able to activate murine B lymphocytes (143) and natural killer (NK) cells (208). The endotoxin content was less than 0.03 ng/mg for all the synthetic oligodeoxynucleotides used in present studies. All the synthetic oligodeoxynucleotides were dissolved in saline at a stock concentration of 2 mg/ml, and were used as single straded by heating at 92 °C for 10 min, and chilling on ice for 5 min before use.

Table 8. Sequences of synthetic oligodeoxynucleotides used in present study

Oligodeoxynucleotides	Sequences
CpG-ODN	5'-TCCATGACGTTCCTGATGCT-3'♣
Control-ODN	5'-ΘΘΘΘΘΘΘGCΘΘΘΘΘΘΘΘΘΘ-3'♣
1CpG-ODN	5'-GAGAACGCTGGACCTTCCAT-3'
2CpG-ODN	5'-ΘΘΘΘΘΘΘΘΘCGΘΘΘΘΘΘΘΘΘ-3'
3CpG-ODN	5'-ΘΘΘΘΘΘΘΘΘCGΘΘΘΘΘCGΘΘ-3'
3GpC-ODN	5'-ΘΘΘΘΘGCΘΘGCΘΘΘΘΘGCΘΘ-3' Ψ

Notes: Θ Indicates identity.

♣ These sequences are taken from Reference (208).

These sequences are taken from Reference (143)

Ψ This sequence was designed by present authors as a control for 3CpG-ODN

2-5. Bacterial DNA

DNA products from *Escherichia coli*, *Micrococcus lysodeikticus* and calf thymus were purchased from Sigma. The bacterial DNA products were dissolved in saline at a stock concentration of 2 mg/ml, and fragmented to 100 to 600 base pairs long by sonication before being stored at –20 ^{O}C. For some experiments bacterial DNA products prepared in this Department were used: *Proteus* and *Streptococcal* DNA preparations were gifts from Dr. Paul Hoffman's laboratory, and plasmid (Bluescrept) purified with QIAGEN mid-kit (QIAGEN, Valencia, CA) was a gift from Dr. Roy Duncan's laboratory. The endotoxin content was less than 0.03 ng/mg for all these bacterial preparations as determined by the Limulus amebocyte lysate assay (Sigma). The endotoxin in the DNA products would be further diluted to a concentration far below the levels (>1 μg/ml) at which mBMMC would be activated to produce substantial amount of cytokines in the present study (**Appendix I**). All the bacterial DNA preparations were heat-denatured at 95 ^{O}C for 10 min, then chilled on ice for 5 min before use.

3. Mast cell activation

Traditionally, mediators that are released from mast cells within 30 min of exposure to stimuli are regarded as preformed mediators discharged through degranulation with an exception for lipid mediators, including prostaglandins and leukotrienes, which are released as newly synthesized products. The mediators secreted from mast cells a few hours after stimulation are usually considered to be newly synthesized products, such as cytokines. For simplicity to describe the following

experiments, the phrase "short term" release is used for the secretion occurring within 20 min of mast cell activation, while the phrase "long term" secretion is arbitrarily designed to refer the secretion over a period of time from 1 h up to 24 h after mast cells have been stimulated.

3-1. Short term release experiments

To examine the effects of BFA or monensin on mast cell degranulation, KU812 or mBMMC cells at $1x10^6$ cell per ml in modified Hepes-Tyrodes buffer were pre-treated with BFA or monensin at the designated concentrations for 5 min at 37°C, followed by a 20 min-period of incubation at 37°C in the presence or absence of 1 μM A23187. At the end of the incubation period, the cells were centrifuged at 300 g for 10 min at 4°C to separate supernatant and cell pellets. After collection of supernatant, the pellets were resuspended in the original volume of medium and disrupted by sonication. The modified Hepes-Tyrodes buffer was prepared as following (in mM): Na, 137; glucose, 5.6; KCl, 2.7; NaH_2PO_4, 0.5; $CaCl_2$, 1; Hepes, 10; plus 0.1% BSA, pH 7.3.

To examine the effects of DNA products on mBMMC degranulation, mBMMC at $1x10^6$ cells per ml in modified HEPES-Tyrode's buffer were incubated with bacterial DNA or CpG-ODN at designated concentrations at 37 °C for 20 min, with calf thymus DNA and non CpG containing ODN as a negative control, and 0.5 μM A23187 as a positive control. After incubation, the cells were centrifuged at 300 g for 10 min at 4 °C to separate supernatant and cell pellets. After collection of supernatant, the pellets were resuspended in the original volume of the buffer and disrupted by sonication.

For histamine assay, both supernatant and pellets were boiled for 5 min to inactivate histaminase and stored at -20°C for later measurement. For β-hexosaminidase assay and cytokine measurement, the supernatant and pellets were stored at -20°C without boiling.

3-2. Long-term secretion experiments on mBMMC activated through FcεRI

For IgE dependent stimulation, mBMMC were sensitized for 24 hours in bone marrow culture medium supplemented with a hybridoma cell line supernatant containing dinitrophenyl (DNP)-specific mouse IgE (209). The cells were washed four times to remove any unbound IgE, then incubated in experimental medium with 1 μg/ml BFA or control medium for 5 min at 37°C, followed by incubation with DNP-human serum albumin (DNP-HSA; Sigma) at a range of concentrations for 24 hours. Supernatant and pellet samples were collected for cytokine assays using a similar methodology to the short term release studies described above. The experimental medium consisted of RPMI 1640, 5% FCS (v/v), 10 mM Hepes, 100 U/ml penicillin, 100 ug/ml streptomycin, 1 mM $CaCl_2$, and 100 μg/ml soybean trypsin inhibitor (STI) (Sigma).

3-3. Long term secretion experiments on A23187-activated mast cells

Prior to experiments, the mBMMC or KU812 cells were washed twice and resuspended in the experimental medium. Either KU812 cells or mBMMC at $1X10^6$ cell/ml were activated with calcium ionophore, A23187, at a range of concentrations from 0.1 μM to 1 μM at 37 °C for either 20 min or for times up to 24 hours (as described in the

Results section for individual experiments), and were then centrifuged at 300 g for 10 min. After the supernatant was collected, the pellets were resuspended in the original volume of experimental medium, and disrupted by repeated freezing and thawing, or sonication. Both supernatant and pellet samples were stored at –20 °C for later analysis.

3-4. Long term secretion experiments on DNA-activated mast cells

mBMMC at $1X10^6$ cells/ml were exposed to bacterial DNA or synthetic oligodeoxynucleotides at the designated concentrations, or medium alone for 20 minutes or for times up to 24 hours at 37 °C. Calcium ionophore, A23187 was used at a range of concentrations (see the Results section for individual experiments) as controls. The supernatants were harvested at different time points, and IL-6 and TNF-α levels were measured by B9 bioassay and L929 bioassay, respectively. Some of the samples were further examined for the levels of IL-4, IL-12, IFN-γ, and GM-CSF by ELISA.

4. Inhibition of mast cell secretion

Either differentiated KU812 cells or mBMMC at $1X10^6$ cell/ml were exposed to BFA or monensin for five minutes at 37 °C at the designated concentrations (0.1 to 1 μg/ml for BFA, and 0.1 to 1 μM for monensin) before being activated with A23187. For mBMMC sensitised with DNP-specific IgE, DNP-HSA was added five minutes after the mast cells were treated with 1 μg/ml BFA at 37 °C. BFA or monensin at the designated concentrations remained in the cell culture during the entire period of experiments, for

BFA and monensin are both reversible secretion inhibitors and their inhibitory effects on cells would be quickly lost if the reagents were withdrawn.

5. Examination of mast cell granule associated markers

5-1. Beta-hexosaminidase enzymatic assays

Beta-hexosaminidase assays were carried out using a previously reported method with minor modification (**Appendix 2**) (210). Supernatant and pellet samples obtained as described above were examined for β-hexosaminidase activity. Fifty μl of samples in duplicates were incubated with 50 μl of 1 mM p-nitrophenyl-N-acetyl-beta-D-glucosaminide (Sigma) dissolved in 0.1 M citrate buffer, pH 5.0 in a 96-well microtiter plate for 1 hour at 37°C. The reaction was stopped with 200 μl/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm in an ELISA reader. The net percent of beta-hexosaminidase release was calculated as following: β-hexosaminidase in supernatant / (β-hexosaminidase in supernatant + β-hexosaminidase in pellet) X 100.

5-2. Histamine radioenzymatic assays

Histamine radioenzymatic assays were performed to measure histamine levels in mBMMC and KU812 samples as previously described (211). Histamine release was expressed as the percent of the total cellular histamine content calculated by the following formula: histamine in supernatant / (histamine in supernatant + histamine in pellet) X 100.

6. Measurement of cytokines

6-1. B-9 cell proliferation assay for IL-6 bioactivity

IL-6 bioactivity in supernatant and pellet samples from mBMMC and KU812 cell experiments was measured by B-9 hybridoma proliferation assay (195). The IL-6 assay was performed in triplicate for each sample and standards in microtiter plates (Nunclon Inter-Med, Nunc, Roskilde, Denmark). After a 72 hour culture of B-9 cells (2500/well) with samples and standards, 10 μl/well of 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; Sigma) was added, After incubation at 37 °C for 4 h, 50 μl/well of 10% Triton/HCl.was added The plates were read at 570 nm on an ELISA reader. IL-6 values were expressed as units/ml where one unit is equivalent to approximately 0.45 pg/ml IL-6. The limit of detection for IL-6 was 10U/ml.

A23187 at the concentrations in this study was previously confirmed to have no significant effects on the IL-6 bioassay (66). BFA and monensin were checked for their effects on B-9 cell proliferation in the presence of IL-6 standards. BFA at the concentrations used in this study had no significant effects on B-9 bioassay. The B-9 bioassay was not applied to examine IL-6 levels in the samples from experiments involving monensin treatment due to its cytotoxic effect on B-9 cells. Bacterial DNA and synthetic oligodeoxynucleotides were checked for their effects on B-9 cell proliferation in the presence and absence of IL-6 standards. These nucleic acid products, at the concentrations used in this study, had no significant effects on the B-9 bioassay.

6-2. L929 cytotoxicity assay for TNF-α bioactivity

TNF-α was measured by a cytotoxicity bioassay with the use of the TNF-α sensitive, mouse fibroblast cell line, L929 (ATCC#CRL-2148). The method is a modification of that in a previous report (212), and has been described in previous studies (66). Briefly, 50 µl/well of 5 X 10^5 L929 cells/ml in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, were added to a 96-well flat bottom plate (Costar, Corning, NY), and incubated at 37 °C for 18 h. The medium was discarded by suction and replaced with 50 µl/well of fresh medium containing 20 µg/ml of cycloheximide (Sigma) and 100 µg/ml of soybean trypsin inhibitor (Sigma). Recombinant mouse TNF-α (PharMingen, San Diego, CA) was used as a standard with seven 10-fold dilutions from 20,000 pg/ml in the same medium. Fifty µl/well of either standards or samples were added in duplicates, and the plates were incubated at 37 °C. After an 18 h incubation, 10 µl/well of MTT (5 mg/ml) was added for further 4 h of incubation of the plates. Then, 50 µl/well of PBS solution, pH 7.4, containing 50% N, N-dimethylformamide (Caledon Laboratories, Edmonton, Canada) and 20% SDS (Bio Rad, Mississauga, Canada) was added, and the plate was read at 550 nm after overnight incubation at 37 °C. The concentration of TNF-α in the samples was calculated with SoftMaxPro (Molecular Devices) based on the standard curves. Preincubation of the samples with neutralizing anti-mouse TNF-α antibody (Genzyme) completely abrogated their cytotoxicity, confirming the specificity of the bioassay for TNF-α present in the samples. The addition of bacterial DNA or synthetic oligodeoxynucleotides at the concentrations used in our experiments did not significantly alter the TNF-α standard curves.

6-3. ELISA assays for cytokines

6-3-1. IL-6.

In the case of KU812 cells treated with monensin, the human IL-6 assay was carried out with purchased ELISA kits (Amersham Life Science, Buckinghamshire, UK), since the growth of B-9 cells was sensitive to monensin inhibition. The minimum detectable level of IL-6 using this system was 1 pg/ml. To verify the B-9 bioassay on mouse IL-6 for use with mBMMC treated with synthetic DNA, an ELISA assay for mouse IL-6 was conducted with commercial kits according to manufacturer's instructions (R&D System, Minneapolis, MN). The minimum detectable level of IL-6 using this system was 3 pg/ml.

6-3-2. GM-CSF

Human GM-CSF levels in supernatant and pellets were measured using either commercially available ELISA kits (R&D Systems) for experiments examining release of this cytokine over 20 min (minimum detection level 2.8pg/ml for human and 1.0 pg/ml for mouse) or with an 'in house" ELISA assay (***Appendix 3***) for GM-CSF production in other experiments. Briefly, the "in house" ELISA involved coating wells of a 96-well ELISA plate with anti-human GM-CSF antibody (Genzyme, Cambridge MA) at 1μg/ml for 16-20h at 4 °C. Non specific binding to the plates was blocked using a 1% BSA, 0.1% Tween 20 solution in PBS for 1h at 37 °C. Fifty μl/well of GM-CSF standards (human recombinant GM-CSF, R&D systems) and samples were added to the plate and incubated for 18-20h at 4 °C. Biotinylated anti-human GM-CSF (0.2 μg/ml) (Endogen, Woburn,

MA) was added to each well and incubated 1h at 37 °C. This was followed with 50 µl/well of a 1/2000 dilution of streptavidin-alkaline phosphatase solution (Life Technologies) incubation for 30 min at room temperature and detection of alkaline phosphatase signal using a commercial ELISA amplification system (Life Technologies) according to manufacturer's instructions. The minimum detectable level was 3.0 pg/ml for human GM-CSF using this system. Mouse GM-CSF levels in the samples were measured with commercially available ELISA kits (R&D System, Minneapolis, MN). The minimum detectable levels of mouse GM-CSF were 8 pg/ml.

6-3-3. IL-4, IL-12 and IFN-γ ELISA assays

Mouse IL-4 levels were measured with commercially available ELISA kits (R&D System, Minneapolis, MN), and mouse IL-12 (p70) levels were measured with ELISA kits from Amersham Pharmacia Biotech (Little Chalfont, UK). The minimum detectable levels of mouse IL-4 and IL-12 were 8 pg/ml and 12 pg/ml respectively. Mouse IFN-γ levels in the experimental samples were measured using an in-house ELISA assay method with paired antibodies purchased from PharMingen (San Diego, CA). The ELISA assay for IFN-γ followed a modified method by Gupta *et al.* (213). Briefly, wells of a 96-well NUNC-Immuno™ plate (Nalge Nunc International, Nunc, Roskilde, Denmark) were coated with anti-mouse cytokine antibody at 2 µg/ml for 16-20 h at 4 °C. Non specific binding to the plates was blocked using 1% BSA, 0.1% Tween 20 in PBS for 1h at room temperature. Fifty µl/well of recombinant cytokine standards and samples were added to the plate and incubated for 18-20 h at 4 °C. Biotinylated anti-mouse cytokines at 0.5

μg/ml were added to each well and incubated for 2 h at 37 °C. This was followed by incubation of 50 μl/well of a 1/2000 dilution of streptavidin-alkaline phosphatase solution (Life Technologies) for 30 min at room temperature and detection of alkaline phosphatase signal using a commercial ELISA amplification system (Life Technologies) according to manufacturer's instructions. Using this system, the minimum detectable level for mouse IFN-γ was 16 pg/ml.

7. CpG-ODN binding and internalization assays

CpG-ODN binding assays - Fifty μl of mBMMC at a concentration of $1X10^7$ cells/ml were incubated at 4 °C with 2 μM of Texas red-labeled 3CpG-ODN and 3GpC-ODN in RPMI-1640/10% FCS with or without 40 μM of unlabeled 3CpG-ODN (**Appendix 4**). Texas red-labeled 3CpG-ODN and 3GpC-ODN were synthesized by Life Technologies, with the same sequences as described for unlabeled 3CpG-ODN and 3GpC-ODN. To minimize internalization of the oligodeoxynucleotides, the incubation was carried out in medium containing 15 mM NaN_3.. After 30 min incubation, the mast cells were washed three times with cold PBS/2%BSA by centrifugation at 300 g at 4 °C 8 min. Cells were fixed in 1% paraformaldehyde / PBS/0.1%NaN_3, and examined for cell surface-bound oligodeoxynucleotides by flow cytometry..

CpG-ODN internalization assays - Fifty μl of mBMMC at a concentration of $1X10^7$ cells/ml were incubated at 37 °C with 0.2 to 2 μM of Texas red-labeled 3CpG-ODN and 3GpC-ODN in RPMI-1640/10% FCS with or without 200 μM of unlabeled 3CpG-ODN, or 20 mM of EDTA (**Appendix 5**). After 6 h incubation, the un-internalized

ligands were stripped by incubating the mast cells in 0.2 M acetic acid (pH2.5) on ice for 10 min, and washed three times in cold PBS/2%BSA by centrifugation at 300 g at 4 °C for 8 min. Cells were fixed in 1% paraformaldehyde/PBS/0.1%NaN_3, and examined by flow cytometry and confocal microscopy

8. Flow cytometry and confocal microscopy

The binding or internalization of Texas red-labeled oligodeoxynucleotides by mBMMC after either 20 minute binding assay or 6 h internalization assay was examined on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The acquisition was done with 10,000 events per sample. The list mode data were corrected for autofluorescence and analyzed by using Winlist 3.0 software packages (Verity Software House, Topsham, ME). Cytocentrifuge preparations from the same internalization assays were viewed for intracellular distribution of Texas red-labeled oligodeoxynucleotides under a confocal microscope (Zeiss LSM 410, Carl Zeiss, Oberkochen, Germany).

9. Pulse-chase experiments

Pulse chase experiments were performed according to an established protocol (**Appendix 6**) (214). Briefly, differentiated KU812 cells at a concentration of 1 x 10^6/ml were starved for 1h at 37 °C in L-leucine deficient RPMI 1640 medium (Life Technologies) with or without 0.1 μg/ml of cyclohexamide. The cells were washed once and pulsed for 1 h with 20 μCi [^{3}H] L-leucine (Amersham, Buckinghamshire, U.K.) in

cold L-leucine deficient RPMI 1640 medium. BFA at the concentrations stated in the Results section was present throughout the pulse-chase period. The cells were washed once with cold 0.02% NaN_3/0.01%BSA/PBS prior to precipitation of total cellular proteins with cold 10% trichloroacetic acid in PBS for 30 min. The radioactivity associated with the cellular proteins was assessed using a scintillation counter.

10. Semi-quantitative RT-PCR

KU812 cells at a concentration of 1 x 10^6/ml were incubated at 37 °C with or without 0.1 μg/ml BFA or 1 μg/ml actinomycin D for either 6 or 12 hours. Total RNA was extracted from the cells using TRIzol reagent (Life Technologies) according to manufacturer's instructions. RT-PCR procedure was performed according to the method of Sorg *et al* (215) with minor modifications (**Appendix 7**). Primer sequences for human IL-6 and GM-CSF were as reported in the literature (31). β-actin primer sequences were; forword primer 5'-ACA TCC GCA AAG ACC TGT ACG-3' and reverse primer 5'-TTG CTG ATC CAC ATC TGC TGG-3'. One μg of total RNA was used as a template for cDNA synthesis in the presence of 5 μM of random hexamer and 200U of Moloney Leukemia virus-reverse transcriptase (Life Technologies) in a final volume of 20 μl at 37 °C for 1h. PCR was carried out with 5 μl of cDNA product in the presence of 1U of *Taq* polymerase (Life Technologies) and 2 μM of specific primers in a final volume of 50 μl as follows: 94 °C for 1min, 57 °C for 2min and 72 °C for 2 min with 36 cycles for IL-6, 38 cycles for GM-CSF and 24 cycles for β-actin in a PTC-100 thermocycler (MJ Research Inc, Waterdown. MA). These cycle numbers were previously determined to

generate PCR products at the exponential phase of amplification. Ten μl of PCR products were run on 1.8% agarose gels in the presence of ethidium bromide and visualised with a transilluminator (Doc 1000, Bio-RAD, Hercules, CA). To measure and analyze the PCR products we used the Gel Document System (Bio-RAD). The optical densities of the PCR product bands were normalised against that of β-actin in each sample.

11. Electron microscopic examination

mBMMC with over 95% purity were washed three times with bone marrow culture medium, then resuspended in experimental medium followed by the addition of either further experimental medium as control or one of the following; 1 μM A23187, 0.5 μg/ml BFA, or 1 μM A23187 plus 0.5 μg/ml BFA, and incubated at 37°C for 3 hours. BFA was added 5 min before A23187. The final cell concentration was 1 X 10^6/ml. After incubation, the supernatant was collected for cytokine assays, and the cells were washed three times with the bone marrow culture medium, and finally resuspended in 0.5% glutaraldehyde, and sent to the electron microscopy laboratory at McMaster University for routine processing. Electronic microscopic examination was carried with a 1200EX Biosystem (JEOL, Tokyo, Japan).

12. Statistical analysis

The response of samples of the same initial preparations to different treatments was compared using Student's t-test for β-hexosaminidase and histamine release, levels of IL-6, GM-CSF, TNF-α and other cytokines.

CHAPTER 3

SECRETION PATHWAYS OF IL-6 AND GM-CSF IN mBMMC AND KU812 CELLS

1. INTRODUCTION

Mast cells and basophils are unique immune effector cells that exert their function through released mediators, such as histamine, proteases, arachidonic acid metabolites, and cytokines. Investigating the events involved in the secretion of these mediators will help us to better understand, and potentially modify, the role of such cells in physiological and immunological processes.

Traditionally, mediators produced by mast cell and basophils were grouped into two categories: preformed, such as histamine, serotonin, neutral proteases, and newly synthesized, such as prostaglandins, and leukotrienes (30, 50, 51, 98, 99). An increasing number of cytokines have been shown to be produced by mast cells (4, 7); however, few studies have examined the secretion routes of mast cell cytokines (84). For many years, whenever considering the potential modulation of mast cell activity in disease, the focus has been on the mechanisms controlling degranulation (22, 84). However, degranulation does not represent the whole picture for mast cell secretion. Previous studies in Dr. Marshall's laboratory demonstrated that IL-6 or IFN-γ secretion and histamine release by mast cells are separate cellular events (65-67, 213, 216), and a number of studies have suggested this may be true for other mast cell systems (132, 217, 218).

The present studies have focused on two cytokines, IL-6 and GM-CSF, both of which are produced in large amounts by rodent mast cells and human mast cells, and are able to enhance proliferation, differentiation and function of T and B lymphocytes (89, 219). IL-6 plays an important role in inducing the acute phase response (220) and mast cells can be the major source of IL-6 involved in local inflammation in atopic patients

(75). GM-CSF promotes inflammation by supporting the growth and function of eosinophils and neutrophils (221, 222) as well as the development of macrophages with a specific cytokine profile (223).

Our main goal, in this study, was to investigate the secretion pathways of IL-6 and GM-CSF from a human basophilic/mast cell line, known as KU812 (35), and murine bone marrow-derived mast cells (mBMMC) using brefeldin A (BFA), and monensin, two well characterized protein secretion inhibitors with defined mechanisms of action on Golgi mediated vesicular transport. These agents have been widely used for the investigation of mechanisms of cellular protein secretion (35, 224). It is hoped that our study will be useful in designing better ways to modify the secretion of pro-inflammatory cytokines by mast cells in allergic diseases.

2. RESULTS

2-1. BFA and monensin inhibit IL-6 release at 20 min post-activation, but have no effect on degranulation of KU812 cells

To examine the effects of BFA and monensin on degranulation and cytokine release, KU812 cells at a concentration of 1 X 10^6/ml were activated with 1 μM A23187 with or without a range of doses of BFA or monensin. KU812 cells were cultured in parallel with medium alone as negative controls. BFA at doses of 0.01 to 1 μg/ml alone or in combination with A23187 had no significant effect on either

histamine (**Figure 9a**) or β-hexosaminidase (**Figure 9b**) release from KU812 cells ($p > 0.05$). Monensin (at 0.01μM to 1 μM) also did not exhibit significant inhibition of KU812 cell degranulation induced by A23187, e.g., cells treated with 0.01 to 1 μM monensin plus 1 μM A23187 had a similar percentage β-hexosaminidase release (34 ± 0.8%) to that of cells treated with 1 μM A23187 alone (36 ± 0.8%, $p > 0.05$, n=4). When cytokine release at 20 min was assessed by B-9 bioassay, A23187 was found to significantly enhance IL-6 release ($p < 0.001$) from KU812 cells that endogenously produce a substantial amount of IL-6, while BFA alone significantly decreased spontaneous IL-6 release ($p < 0.01$) (**Figure 10**). When BFA and A23187 were used in combination, there was a dose dependent response to BFA with a significant reduction of IL-6 release at the dose of 1μg/ml ($p < 0.001$) and 0.1μg/ml ($p < 0.01$), but not at 0.01μg/ml ($p > 0.05$). There was also a dose dependent increase in IL-6 remaining within KU812 cells treated with 0.1 μg/ml and 1 μg/ml BFA in combination with A23187 ($p < 0.\ 0.001$, $p < 0.05$).

Close examination of the data obtained suggested an inhibitory effect of BFA on IL-6 release over the first 20 minutes post-activation from KU812 cells. To confirm that this was due to vesicular transport blockade as well as to compare the effect of BFA with that of monensin, KU812 cells were treated in parallel with either 1 μg/ml BFA or 1μM monensin alone or in the presence of 1 μM A23187 for 20 min as described above. The cell supernatants and pellets were collected for the measurement of β-hexosaminidase activity and IL-6 levels. Monensin had no significant effect on β-hexosaminidase release, but significantly inhibited IL-6 release from KU812 cells either alone or in combination with A23187 ($p < 0.001$, $P < 0.05$, respectively, **Table 9**). Comparison of the effects of

Figure 9. Effects of BFA on KU812 cell short term release of *A.*) histamine; and *B.*) β-hexosaminidase in response to stimulation with the calcium ionophore, A23187. Differentiated KU812 cells were activated with 1 μM A23187 alone or in the presence of various concentrations of BFA. As a negative control, the cells were incubated in parallel with medium alone. Supernatant and pellets were collected separately, and assessed for histamine levels by radioenzymatic assay, and for β-hexosaminidase levels by enzymatic assay. BFA did not induce histamine and β-hexosaminidase release at 20 min, or have a significant effect on A23187-stimulated histamine and β-hexosaminidase release.

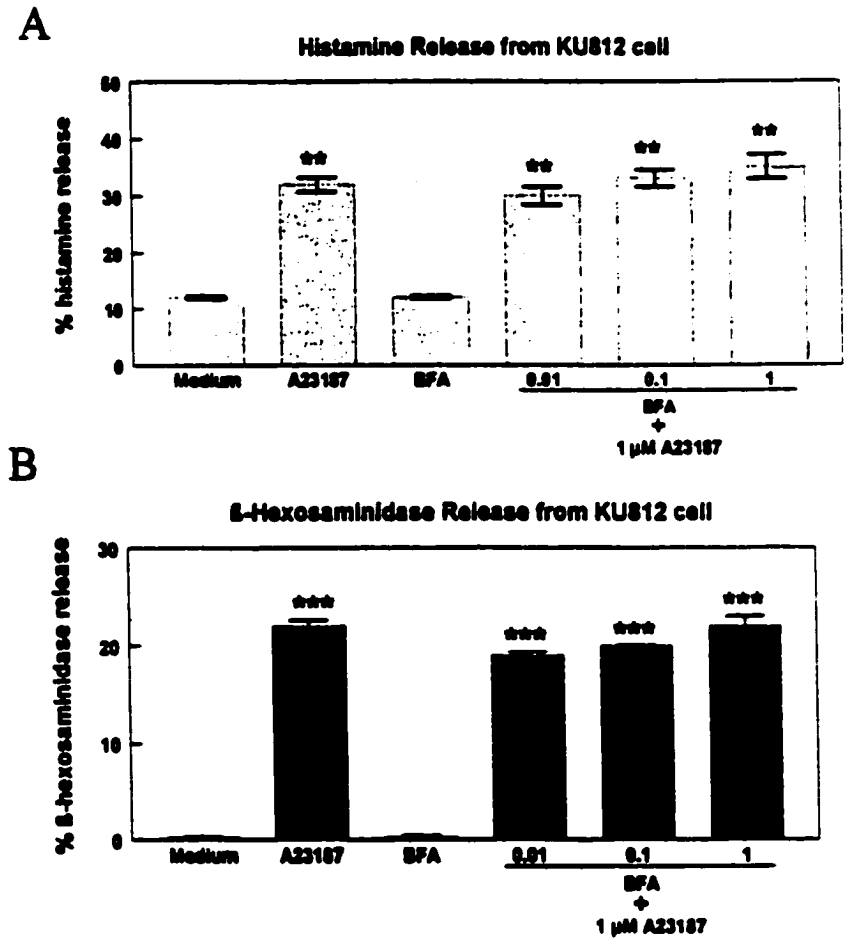

A
Histamine Release from KU812 cell
% histamine release
Medium
A23187
BFA
0.01
0.1
1
BFA
+
1 μM A23187
B
β-Hexosaminidase Release from KU812 cell
% β-hexosaminidase release
Medium
A23187
BFA
0.01
0.1
1
BFA
+
1 μM A23187

Figure 9

Figure 10. The effect of BFA on KU812 cell short term IL-6 release in response to stimulation with calcium ionophore, A23187. Differentiated KU812 cells were activated with 1 μM A23187 alone or in the presence of various concentrations of BFA. As a negative control, the cells were incubated in parallel with medium alone. Supernatant and pellets were collected separately, and assessed for IL-6 levels using B-9 bioassay (the limit of detection for IL-6 was 10U/ml). BFA inhibited short term release of IL-6, and showed a dose-dependent inhibitory effect on A23187-induced IL-6 release. Each point represents the mean data ± SEM. n = 4.

+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$ when compared with Medium group

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ when compared with A23187 group.

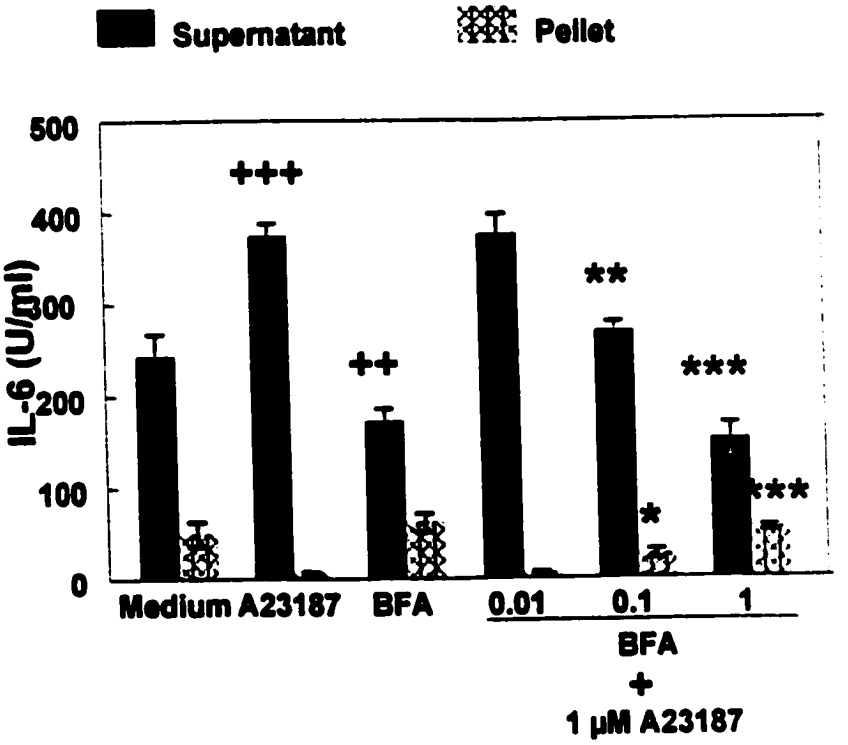

Supernatant
Pellet
IL-6 (U/ml)
500
400
300
200
100
0
+++
++
**
***
*
***
Medium
A23187
BFA
0.01
0.1
1
BFA
+
1 µM A23187

Figure 10

BFA with those of monensin on 20 min IL-6 release showed that BFA was more effective than monensin in inhibiting IL-6 release from KU812 cells either in the presence ($p < 0.001$) or absence of A23187 ($p < 0.001$).

Comparison of the effects of BFA on IL-6 release from unstimulated cells with the effects of BFA on IL-6 release from A23187 stimulated cells suggested that BFA not only inhibited baseline IL-6 release but also A23187 induced IL-6 release from KU812 cells. Based on the data of Table 1, the inhibition of baseline release of IL-6 by BFA was 22%, as calculated from 39% (Medium control release) minus 17% (BFA-treated group release). BFA treatment decreased the IL-6 release rate from 80% as seen in cells treated with A23187 alone to 48% as seen in cells treated with BFA and A23187 together, the difference was 32%, significantly more than the inhibition rate (22%) by BFA alone ($p < 0.05$). A similar comparison of the effect of monensin on A23187 induced and baseline IL-6 release suggested that this agent only significantly inhibited the baseline release of IL-6 over 20 minutes. As shown in **Table 9**, the difference between the amount of IL-6 release from the A23187 group (80%) and that from the monensin plus A23187 treatment group (66%) was 16%, which was similar to the inhibition rate by monensin alone (14%), calculated from 39% (Medium group) minus 23% (Monensin group).

2-2. BFA and monensin have no effect on the initial 20 minute release of granule associated mediators from mBMMC.

mBMMC were treated similarly to KU812 cells, i.e., the cells were incubated with 1 μg/ml BFA for 5 min, followed by 1 μM A23187 for another 20 min at 37°C. Superna-

Table 9. Release of β-hexosaminidase and IL-6 from KU812 cells over 20 min (mean ± SEM)[1]

	Medium	BFA 1μg/ml	Monensin 1μM	A23187 1μM	BFA & A23187 1μg/ml + 1μM	Monensin & A23187 1μM + 1μM
% β-Hex release	6 ± 0.5	6 ± 0.1	6 ± 0.2	40 ± 0.5	41± 0.4	41 ± 0.3
IL-6 (pg/10^6cells)						
Supernatant	7 ± 0.5	3 ± 0.2 $^{++}$	4 ± 0.4 $^{++}$	24 ± 1.2	9 ± 0.3***	13 ± 1.2**
Pellet	11 ± 0.3	17 ± 1.3 $^{+}$	13 ± 1.3	6 ± 0.1	9± 0.3***	7 ± 0.6
% IL-6 Release	39 ± 1.1	17 ± 1.8	23 ± 2.2	80 ± 1.7	48 ± 2.6	66 ± 2.8

1. IL-6 levels were measured with commercial ELISA kit (n=4)

$^{+}$ $p < 0.05$, ++ $p < 0.01$ when compared with Medium group

** $p < 0.01$, *** $p < 0.001$ when compared with A23187 group

tant and pellets were collected from each sample, and were examined for histamine, β-hexosaminidase, and cytokines. There were no significant inhibitory effects on mBMMC release of β-hexosaminidase by BFA treatment alone or in combination with A23187, e.g., mBMMC treated with 1 μg/ml BFA alone had a similar percentage of β-hexosaminidase release (4 ± 2.0) to that of medium control (2 ± 0.5), and the cells treated with 1 μg/ml BFA plus 1 μM A23187 had similar % β-hexosaminidase release (20 ± 2.3) to that with 1 μM A23187 alone (18 ± 1.5).

These results indicated that BFA is an appropriate agent to use to examine vesicle transport dependent mechanisms as distinct from degranulation in mBMMC. The alternate inhibitor of vesicular transport, monensin, also did not alter preformed mediator release from BMMC. A mean β-hexosaminidase release of 19.5 ± 0.14% was observed in cells treated with 0.5 μM A23187 alone while 19.2 ± 0.20 % was released in the presence of A23187 plus 1μM monensin.

Without appropriate stimulation, mBMMC produce only very low levels of cytokines, including IL-6 and GM-CSF, over either a 20 min or 24 h period. Therefore, we did not pursue examining the effects of BFA or monensin on short term release of any cytokines from mBMMC.

2-3. BFA treatment induces changes in IL-6 secretion and increases the amount of cell associated IL-6 over up to 24 hours of treatment of KU812 cells and mBMMC

KU812 cells were treated concurrently with 0.1 μg/ml BFA and 0.5 μM A23187 or with either agent alone for up to 24 hours, supernatant and pellet samples were

collected at different time intervals, and IL-6 measured. BFA significantly inhibited IL-6 secretion from KU812 cells in the presence or absence of A23187 activation at all the time intervals examined (**Figure 11**). In contrast, BFA alone or in combination with A23187 significantly increased IL-6 storage in KU812 cell pellets taken over the full 24 hour time course (**Figure 12**).

mBMMC were treated with 0.1 μM A23187 and 1 μg/ml BFA in the same fashion as with KU812 cells. While there was no detectable IL-6 release or cell associated IL-6 observed in inactivated cells, BFA significantly inhibited IL-6 secretion from mBMMC activated with A23187 from 6 h time up to 24 h (**Figure 13**). Analysis of pellet samples demonstrated that BFA significantly enhanced IL-6 accumulation in the BFA-treated mBMMC with A23187 activation at all time points, with or without A23187 activation from 6 h up to 24 h (**Figure 14**).

2-4. Monensin inhibits IL-6 secretion from KU812 cells over 24 hours

In order to confirm that the inhibitory effect of BFA on the 24 hour secretion of mast cell IL-6 was the result of vesicular transport blockade, monensin was used in parallel with BFA on KU812 cells. KU812 cells were treated with 0.1 μg/ml BFA or 0.1 μM monensin alone or in combination with 0.5 μM A23187 at 37 °C for 24 hours. BFA and monensin alone significantly inhibited IL-6 secretion from KU812 cells ($p<0.001$; and $p < 0.001$, respectively), and increased IL-6 storage inside the cells ($p <0.001$, and $p < 0.05$, respectively) (**Table 10**). BFA and monensin also significantly inhibited IL-6 secretion ($p < 0.001$, and $p <0.001$, respectively), and increased IL-6 storage by A23187

Figure 11. Time course of IL-6 production by KU812 cells treated with 0.1 μg/ml BFA alone or in combination with secretagogue, 0.5 μM A23187 . As controls, the cells were incubated in parallel with medium alone, or with 0.5 μM A23187 alone, respectively. Samples were taken at different intervals of time, and the supernatants and cell pellets were separated and assessed for IL-6 levels using the B-9 bioassay. The supernatant levels of IL-6 were significantly lower at all time intervals in the BFA group than in the Medium group, and the IL-6 release into the supernatant was also significantly less by the cells treated with BFA and A23187 than by cells treated with A23187 alone at all the but 12 h time interval.

+ $p < 0.05$, ++ $p < 0.01$, and +++ $p < 0.001$ when compared with Medium group.

* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ when compared with A23187 group

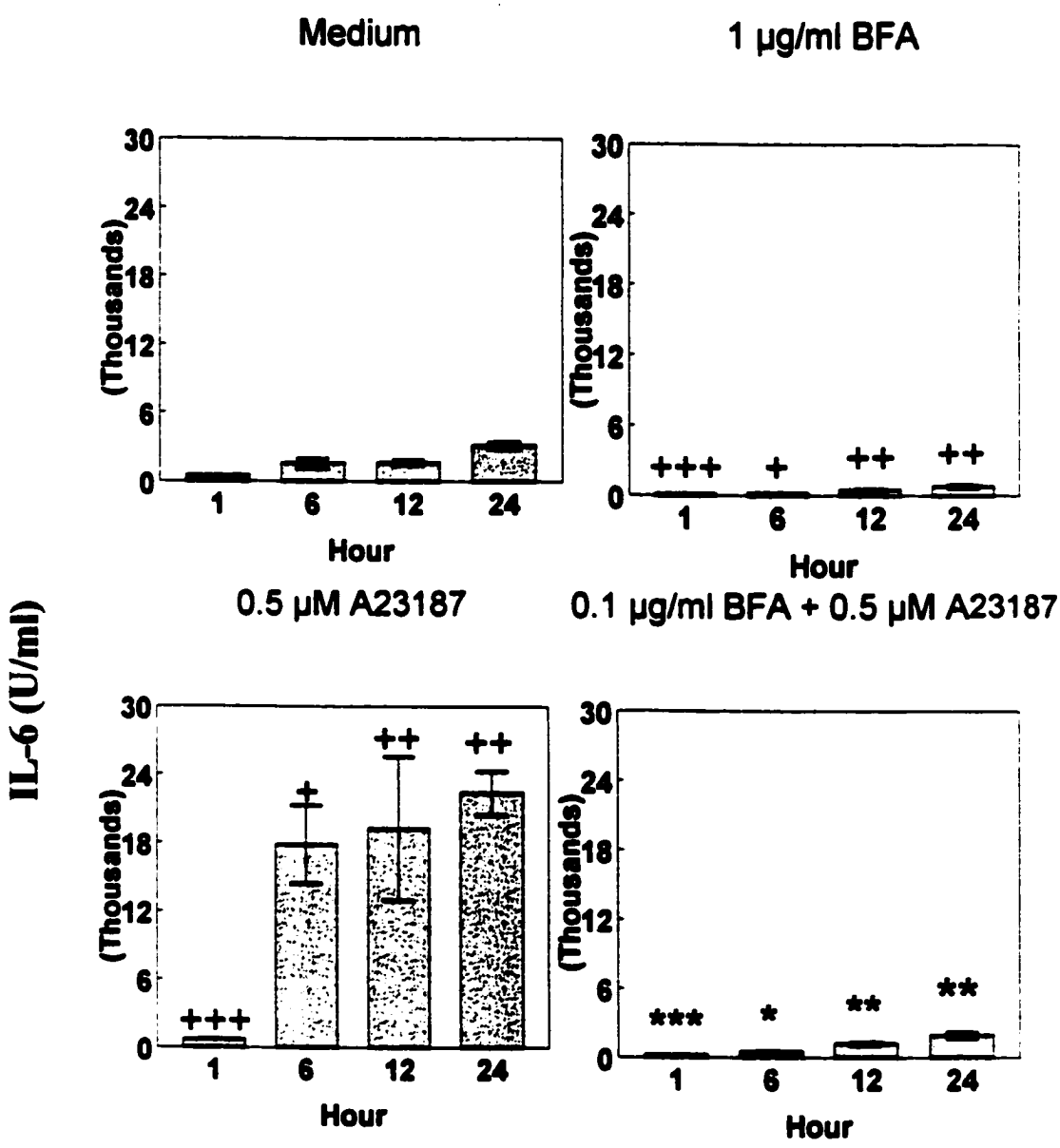

Medium
1 µg/ml BFA
0.5 µM A23187
0.1 µg/ml BFA + 0.5 µM A23187
IL-6 (U/ml)
(Thousands)
30
24
18
12
6
0
1
6
12
24
Hour
+++
+
++
++
+++
++
++
***
*
**
**

Figure 11

Figure 12. Time course of IL-6 production by KU812 cells treated with 0.1 μg/ml BFA alone or in combination with secretagogue, 0.5 μM A23187. As controls, the cells were incubated in parallel with medium alone, or with 0.5 μM A23187 alone, respectively. Samples were taken at different intervals of time, and the supernatants and cell pellets were separated and assessed for IL-6 levels using the B-9 bioassay. IL-6 accumulation in the cell pellets was compared between Medium and BFA groups, and between A23187 and BFA + A23187 groups. Significantly higher levels of IL-6 were present in the cells treated with BFA than with medium (control) at all time intervals, and significantly higher levels of IL-6 were also present in the cells treated with BFA + A23187 than with A23187 alone at all time intervals. Bars represent mean data ± SEM. n = 4.

+ $p < 0.05$, ++ $p < 0.01$, and +++ $p < 0.001$ when compared with Medium group.

* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ when compared with A23187 group.

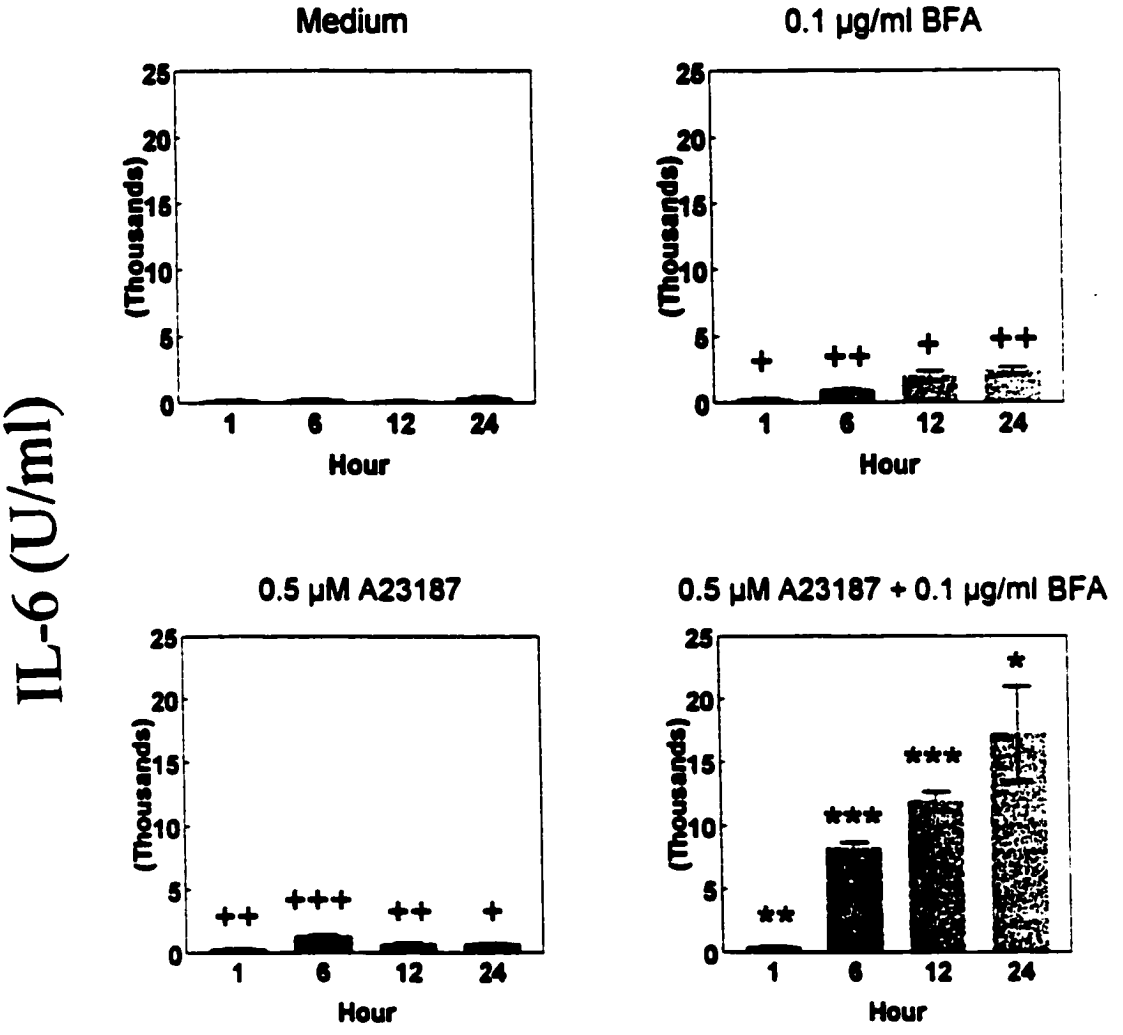

IL-6 (U/ml)
Medium
0.1 µg/ml BFA
0.5 µM A23187
0.5 µM A23187 + 0.1 µg/ml BFA
(Thousands)
25
20
15
10
5
0
1
6
12
24
Hour
+
++
+++
*
**
***

Figure 12

Figure 13. Time course of IL-6 production by murine BMMC treated with 1 μg/ml BFA alone or in combination with secretagogue, 0.1 μM A23187. As controls, the cells were incubated in parallel with medium alone, or with 0.1 μM A23187 alone, respectively. Samples were taken at different intervals of time, and the supernatants and cell pellets were separated and assessed for IL-6 levels. IL-6 release in the supernatants was compared between A23187 and BFA + A23187 groups. BFA significantly inhibited A23187-induced IL-6 release at all but the 1 h time interval. No IL-6 was detected in the Medium and BFA groups any time intervals. The bars represent mean ± SEM. n = 4.

+++ $p < 0.001$ when compared with Medium group.

* $p < 0.05$; ** $p < 0.01$ when compared with A23187 group.

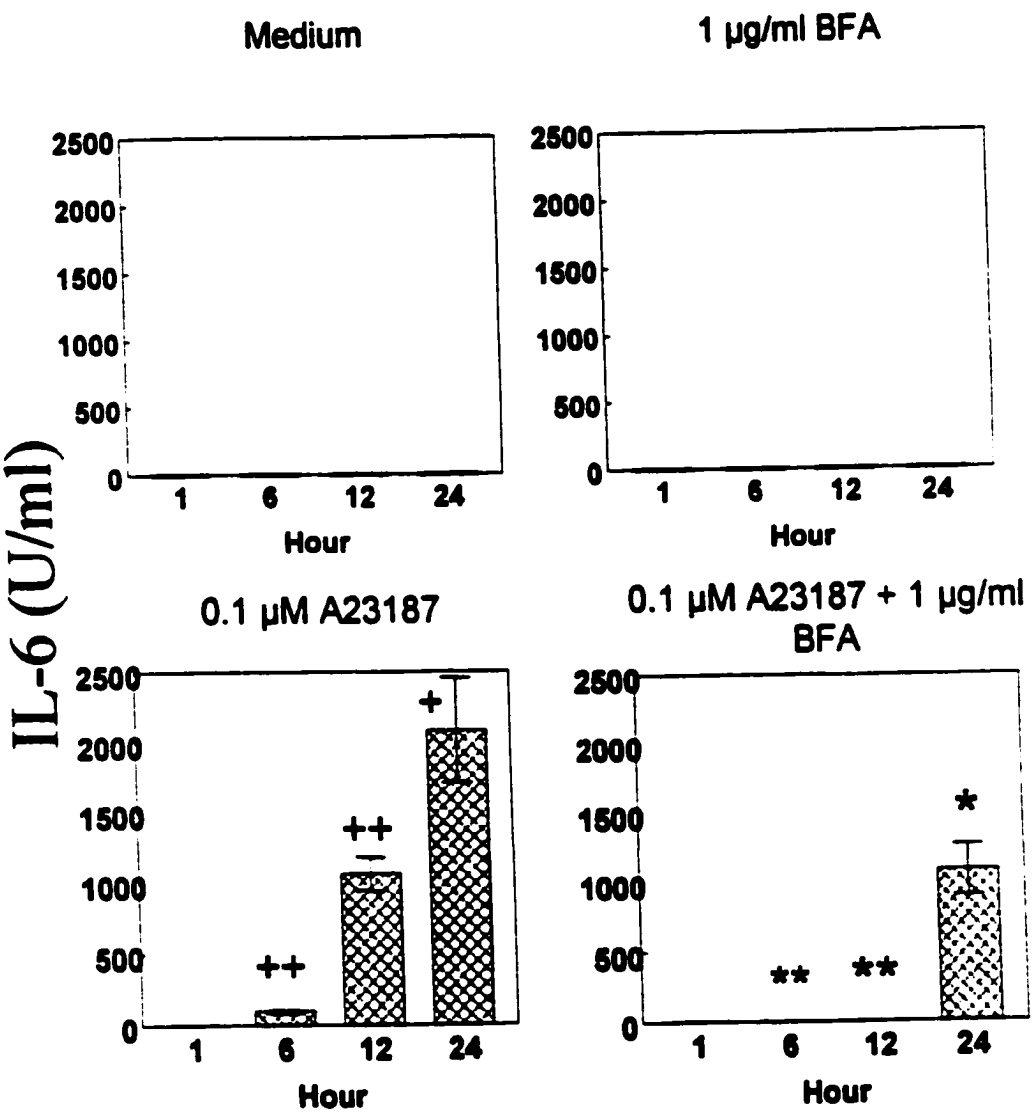

Medium
1 µg/ml BFA
0.1 µM A23187
0.1 µM A23187 + 1 µg/ml BFA
IL-6 (U/ml)
2500
2000
1500
1000
500
0
1
6
12
24
Hour
++
++
+
*
**
**

Figure 13.

Figure 14. Time course of IL-6 production by murine BMMC treated with 1 μg/ml BFA alone or in combination with secretagogue, 0.1 μM A23187. As controls, the cells were incubated in parallel with medium alone, or with 0.1 μM A23187 alone, respectively. Samples were taken at different intervals of time, and the supernatants and cell pellets were separated and assessed for IL-6 levels. IL-6 accumulation in the cell pellets was compared between Medium and BFA groups, and between A23187 and BFA+A23187 groups. No IL-6 was detected in the cells treated with medium alone at any time intervals. The bars represent mean ± SEM. n = 4.

+++$p < 0.001$ when compared with Medium group.

*$p < 0.05$; **$p < 0.01$ when compared with A23187 group.

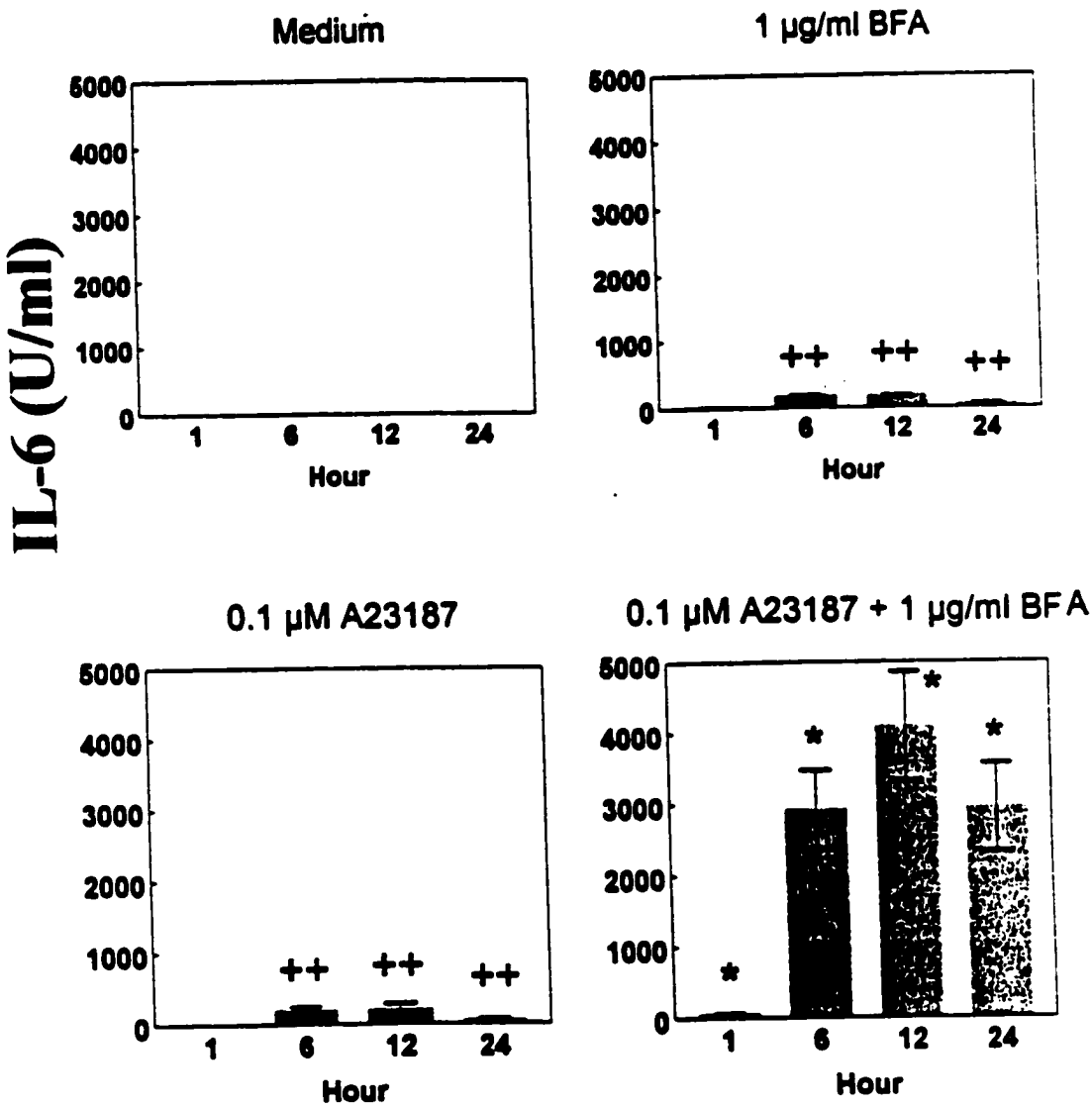

Medium
1 µg/ml BFA
0.1 µM A23187
0.1 µM A23187 + 1 µg/ml BFA
IL-6 (U/ml)
5000
4000
3000
2000
1000
0
1
6
12
24
Hour
++
*

Figure 14

Table 10. Twenty-four hour IL-6 release from KU812 cells (mean ± SEM)[1]

	Medium	BFA 0.1μg/ml	Monensin 0.1μM	A23187 0.5μM	BFA & A23187 0.1μg/ml + 0.5μM	Monensin & A23187 0.1μM+0.5μM
Supernatant (pg/10^6cells)	982 ± 27	272 ± 15^{+++}	177 ± 6^{+++}	3437 ± 136	562 ± 75***	282 ± 28***
Pellet (pg/10^6cells)	130 ± 9	435 ± 9^{+++}	160 ± 7^{+}	172 ± 5	847 ± 118**	220 ± 11*

1. IL-6 levels were measured with commercial ELISA kit (n=4)

$^{+}$ $p < 0.05$, $^{+++}$ $p < 0.001$ when compared with Medium group

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with A23187 group

activated KU812 cells ($p < 0.01$, and $p < 0.05$, respectively).

2-5. BFA blocks antigen-induced IL-6 secretion from mBMMC

Immunological activation of mast cells induces a number of cellular signaling processes not observed following ionophore stimulation. In order to examine the effects of BFA on cells activated through IgE receptor cross-linking, mBMMC were sensitized with DNP-specific IgE for 24 hours, then activated by incubating with DNP-HSA at 37°C for 24 hours. Without stimulation, sensitized mBMMC produced very little IL-6, but they produced up to 40,000 units of IL-6 per 10^6 cells following stimulation with 10 ng/ml DNP-HSA. BFA at a dose of 1 μg/ml significantly inhibited IL-6 secretion ($p < 0.01$) and increased the amount of IL-6 that remained cell-associated ($p < 0.01$) in mBMMC activated with DNP-HSA (**Figure 15**).

2-6. BFA inhibits the secretion of GM-CSF from KU812 cells and mBMMC

The ability of BFA to alter GM-CSF secretion over 20 min was examined for both mBMMC and KU812 cells. Neither BFA nor A23187 treatment had any significant effects on the extremely small amount of GM-CSF secretion or storage by KU812 cells (on average less than 5 pg/10^6 cells in either supernatant or pellets was found in any of the groups). Very low levels (1.5 to 4.5 pg/ml) of GM-CSF were detected in both supernatant and pellets of mBMMC cultured in either medium or treated with A23187 and BFA alone or in combination for 20 min. There was no significant difference in GM-CSF levels among different treatment groups. Given the lack of a detectable GM-CSF

Figure 15. The effect of BFA on long term release of IL-6 from murine BMMC in response to IgE receptor-mediated stimulation. Murine BMMC were sensitized with DNP-specific IgE for 24 h, then were incubated with medium or 10 ng/ml DNP-HSA alone, and with 1 μg/ml BFA in the presence or absence of 10 ng/ml DNP-HSA for 24 h. IL-6 levels were measured in both supernatants and cell pellets. In the absence of IgE mediated antigen activation, there was little IL-6 released or stored by the cells, but large amounts of IL-6 were produced following DNP-HSA treatment. BFA significantly inhibited the release and elevated the accumulation of IL-6 in the cells activated with DNP-HSA. Each point represents the mean data ± SEM. n =4 .

+$p < 0.05$, and ++$p < 0.01$ when compared with Medium group.

**$p < 0.01$ when compared with DNP-HSA group.

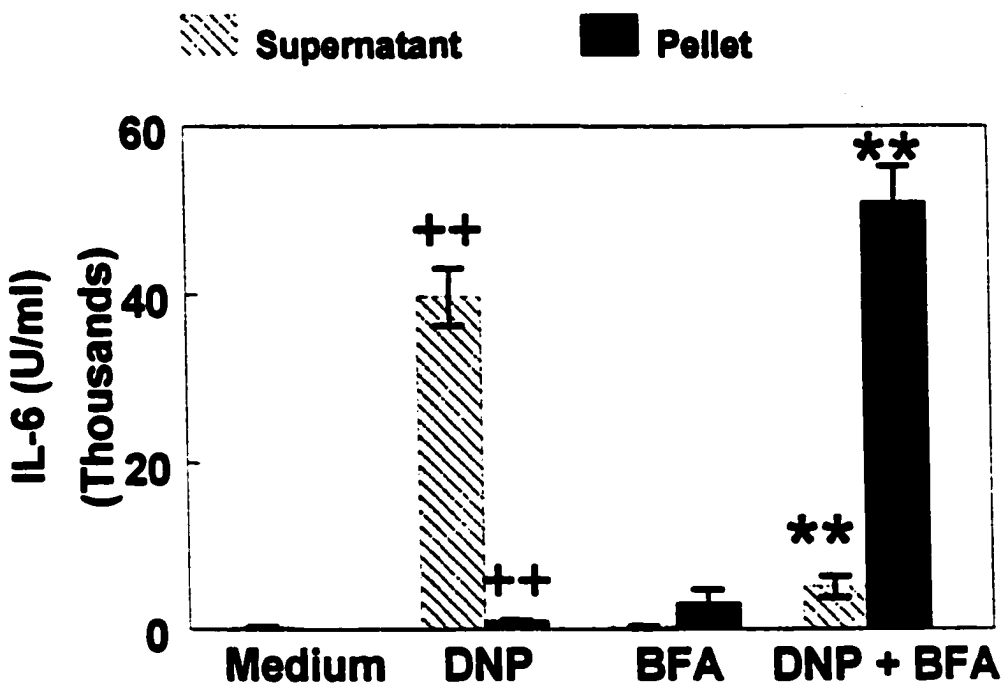

Supernatant
Pellet
IL-6 (U/ml)
(Thousands)
60
40
20
0
++
++
**
**
Medium
DNP
BFA
DNP + BFA

Figure 15

response to ionophore treatment over the initial 20 minute activation, it is difficult to assess the effects of BFA for this cytokine over this time period on mBMMC and KU812 cells.

After a 24 hour incubation of KU812 cells, low levels of GM-CSF were detected in both supernatant and pellet samples of control cells or cells exposed to 0.1 μg/ml BFA, the difference between the two groups of cells in GM-CSF secretion or storage was not significant. There was a striking increase in GM-CSF secretion from the A23187 treated cells ($p < 0.001$). BFA significantly decreased GM-CSF secretion ($p < 0.001$) (**Figure 16**) and increased the amount of cell associated GM-CSF ($p < 0.001$) in KU812 cells stimulated with A23187 (**Figure 17**).

mBMMC produced little or no GM-CSF after a 24 hour incubation with or without 1 μg/ml BFA. BFA significantly reduced GM-CSF secretion from mBMMC that were sensitized with anti-DNP IgE and activated with DNP-HSA for 24 hours (56 ± 6 vs 149 ± 29 pg/10^6 cells, $p < 0.01$) (**Figure 18**). BFA was also able to significantly increase GM-CSF associated with sensitized mBMMC activated with DNP-HSA (188 ± 31 vs. 12 ± 3-pg/10^6 cells, $p < 0.01$).

2-7. The effect of BFA on protein synthesis

BFA has previously been shown to inhibit cellular protein secretion while having little effect on protein synthesis. In the present study, the effects of BFA on total protein synthesis by KU812 cells were investigated using pulse chase experiments. KU812 cells were treated with or without 0.1μg/ml BFA for 6 or 12 hours respectively and were pulse

Figure 16. Time course of GM-CSF production by KU812 cells treated with 0.1 μg/ml BFA alone or in combination with secretagogue, 0.5 μM A23187. As controls, the cells were incubated in parallel with medium alone, or with 0.5 μM A23187 alone, respectively. Samples were taken at different of time intervals, and the supernatants and cell pellets were separated and assessed for GM-CSF levels using the in-house ELISA method. The figures are plotted in log scales due to the large difference in the GM-CSF concentration among different treatment groups. The supernatant levels of GM-CSF were significantly elevated from 6 h up to 24 h time points in A23187 treated cells, but were barely detectable all time intervals in both the Medium group and BFA groups. The GM-CSF release into the supernatant was significantly less by the cells treated with BFA and A23187 than by cells treated with A23187 alone at all the but 1 h time interval. Bars represent mean data ± SEM. n = 4.

+$p < 0.05$, ++$p < 0.01$, and +++$p < 0.001$ when compared with Medium group.

* $p < 0.05$, **$p < 0.01$; ***$p < 0.001$ when compared with A23187 group.

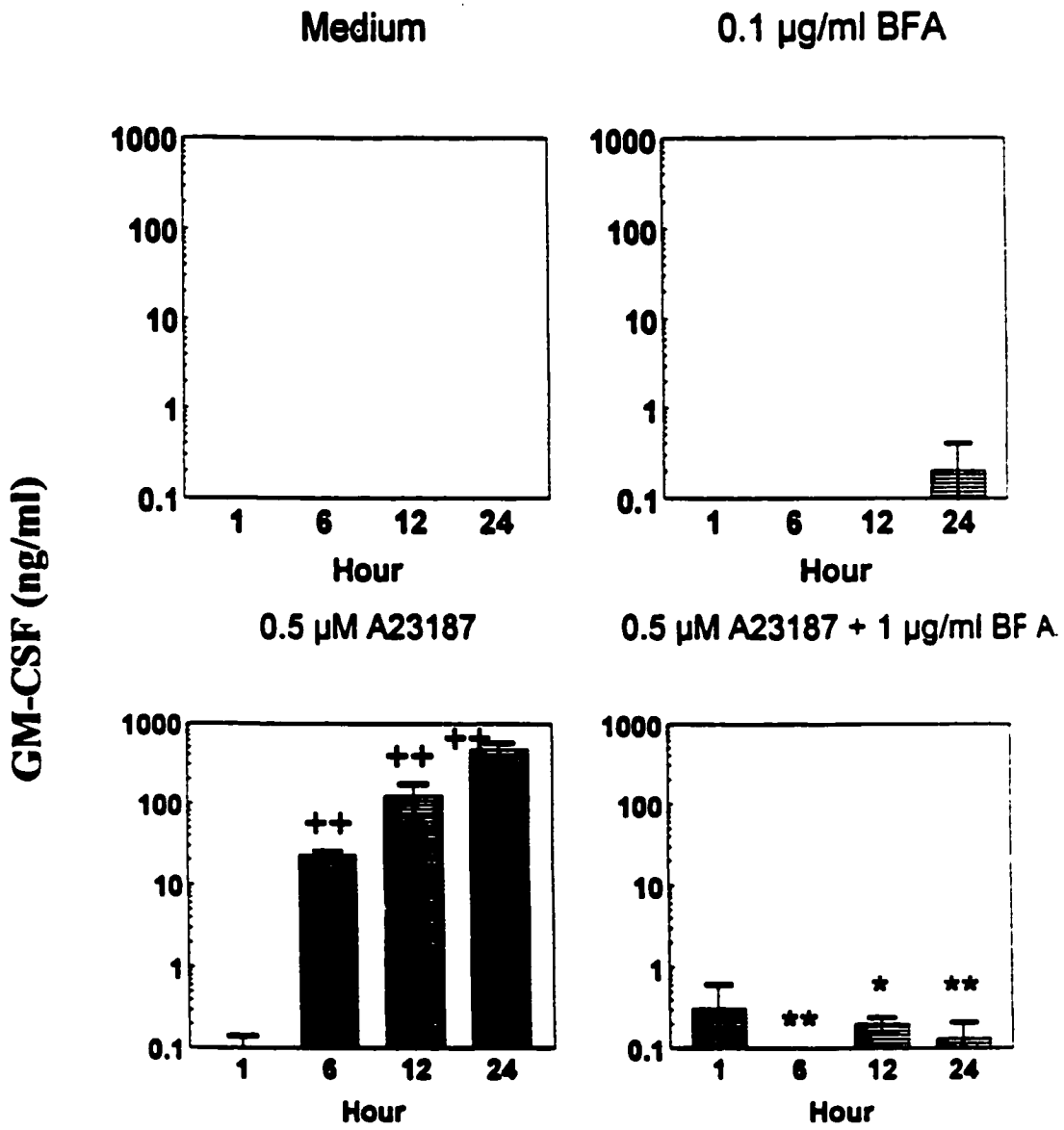

Medium
0.1 µg/ml BFA
GM-CSF (ng/ml)
1000
100
10
1
0.1
1
6
12
24
Hour
0.5 µM A23187
0.5 µM A23187 + 1 µg/ml BF A
++
*
**

Figure 16.

Figure 17. . Time course of GM-CSF production by KU812 cells treated with 0.1 μg/ml BFA alone or in combination with secretagogue, 0.5 μM A23187. As controls, the cells were incubated in parallel with medium alone, or with 0.5 μM A23187 alone, respectively. Samples were taken at different of time intervals, and the supernatants and cell pellets were separated and assessed for GM-CSF levels using the in-house ELISA method. The figures are plotted in log scales due to great difference in the GM-CSF concentration among different treatment groups. GM-CSF levels in the cell pellets were hardly detectable in neither the Medium nor BFA group, but were significantly higher in A23187 and BFA + A23187 groups at all the but 1 h time intervals. Significantly higher levels of IL-6 were present in the cells treated with BFA + A23187 than with A23187 alone at all 1 h time intervals. Bars represent mean data ± SEM. n = 4.

$+p < 0.05$, $++p < 0.01$, and $+++p < 0.001$ when compared with Medium group.

$*p < 0.05$, $**p < 0.01$; $***p < 0.001$ when compared with A23187 group.

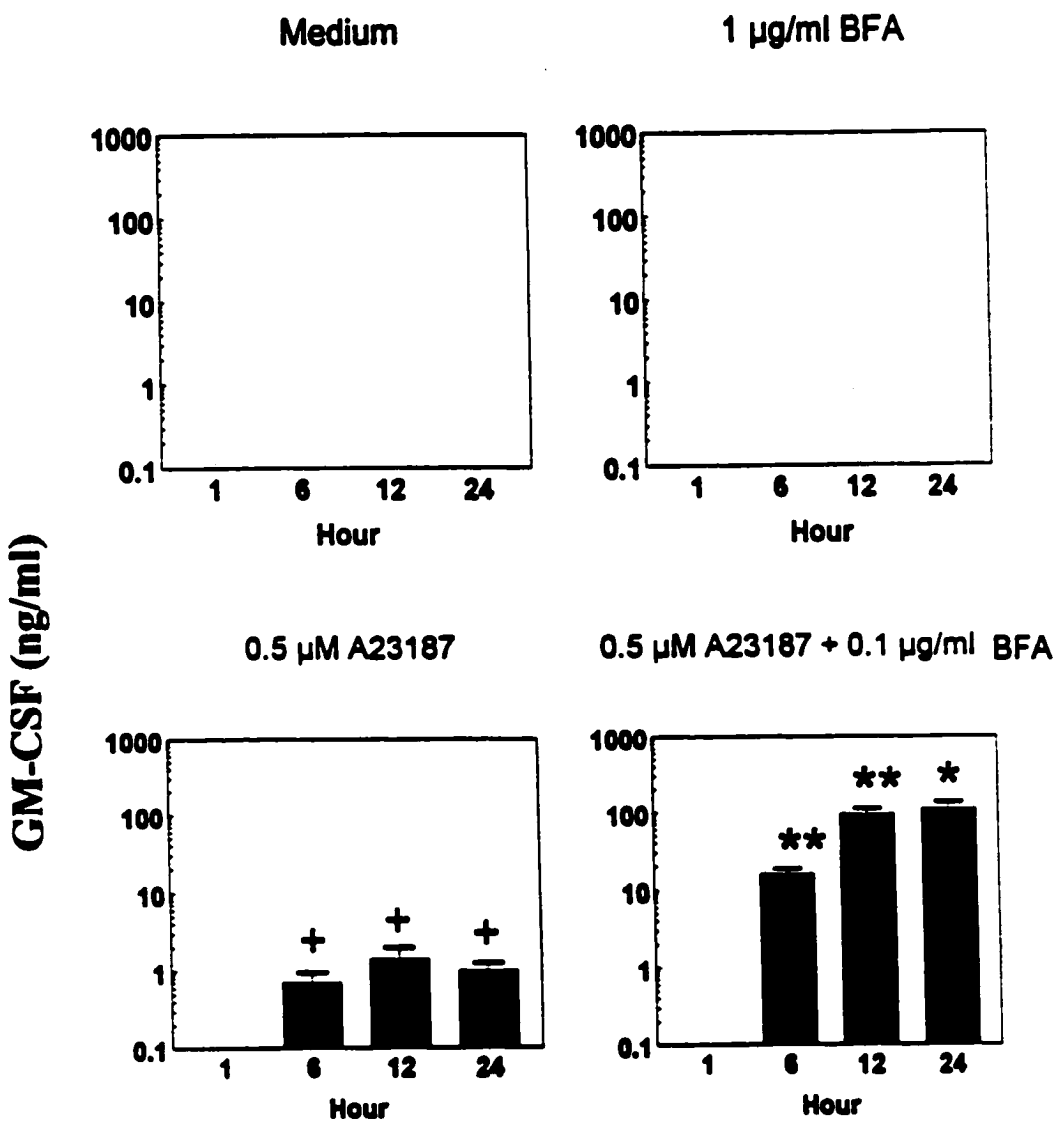

Medium
1 µg/ml BFA
0.5 µM A23187
0.5 µM A23187 + 0.1 µg/ml BFA
GM-CSF (ng/ml)
1000
100
10
1
0.1
1
6
12
24
Hour
+
**
*

Figure 17

Figure 18. The effect of BFA on long term release of GM-CSF from murine BMMC in response to IgE receptor-mediated activation. Murine BMMC were sensitized with DNP-specific IgE for 24 h, then were incubated with medium, 10 ng/ml DNP-HSA alone, 1 μg/ml BFA alone, or with BFA and DNP-HSA in combination at the same concentration for 24 h. GM-CSF levels were measured in both supernatant and cell pellets. Without stimulation with DNP-HSA, there was little GM-CSF present in either supernatant or pellets, but DNP-HSA-treated sensitized cells produced a large amount of GM-CSF. BFA significantly blocked release and promoted the accumulation of GM-CSF in the cells activated with DNP-HSA. Each point represents the mean data ± SEM. n = 4.

+$p < 0.05$ when compared with Medium group.

*$p < 0.05$, **$p < 0.01$ when compared with DNP-HSA group.

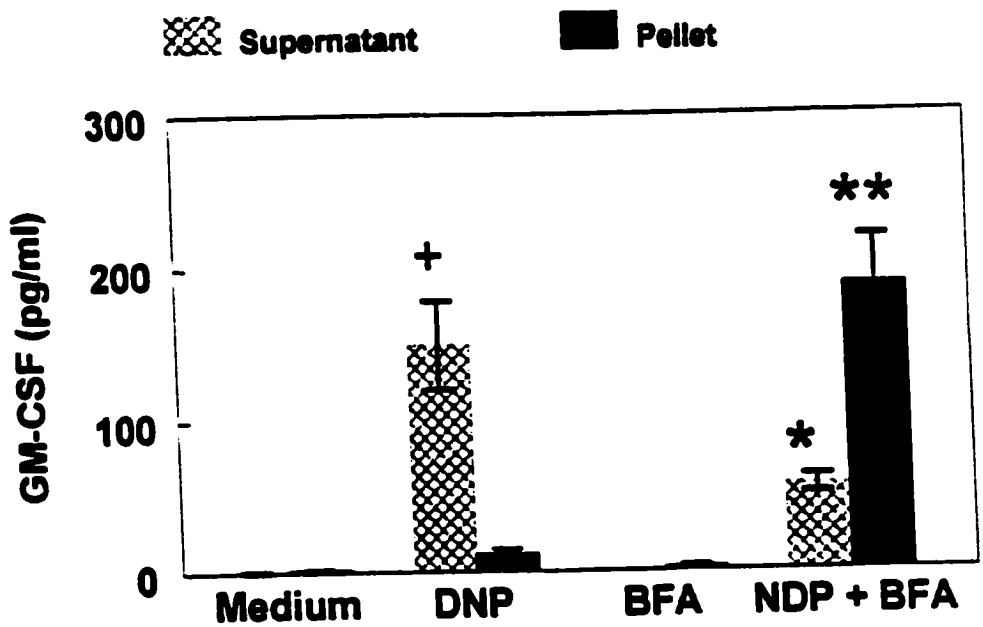

Supernatant
Pellet
GM-CSF (pg/ml)
300
200
100
0
+
*
**
Medium
DNP
BFA
NDP + BFA

Figure 18.

labeled with [^{3}H] leucine for 1h. The BFA dose employed was the same as that used in the other time course experiments. At a 6 hour time point, BFA had no significant inhibitory effect on total protein synthesis in terms of [^{3}H] leucine incorporation (**Table 11**) although substantial inhibition of cytokine secretion was observed at this time point (**Figure 11** and **Figure 16**). At 12 h, significant inhibition of protein synthesis was observed in the presence of BFA (mean 36% inhibition) (**Table 11**), although not sufficient to account for the profound inhibition of cytokine secretion observed at this time point (mean inhibition 94% for IL-6, and >99% for GM-CSF).

2-8. Effects of BFA on IL-6 and GM-CSF mRNA levels in KU812 cells

Semi-quantitative RT-PCR was conducted to examine the effects of BFA on IL-6 and GM-CSF gene transcription in KU812 cells. In a time course study, the IL-6 and GM-CSF mRNA levels were assessed in cells treated with medium, A23187 or BFA alone for different length of time (from 0 to 24 h) by densitometry of electrophoresis gel bands (**Figure 19**). Based on the observation of the gel bands, there was no substantial inhibition of either IL-6 or GM-CSF mRNA levels in BFA-treated KU812 cells in all the time points. Instead, BFA seems to

Table 11. Percentage of [^{3}H] Leucine incorporation into KU812 cells

Duration of treatment prior to pulse-chase	Medium	0.1 μg/ml BFA
6h (n=8)	12.7 ± 0.70 %[#]	11.7 ± 0.99 %
12h (n=12)	16.6 ± 2.19 %	10.6 ± 1.20 %***

[#] Mean values ± S.E.M *** Denotes $p < 0.001$ compared with medium incubated cells

Figure 19. Time course of IL-6 and GM-CSF mRNA expression in KU812 cells. 5 X 10^6 KU812 cells were treated with medium alone (*M*), or with 0.5 μM A23187 (*A*), or 0.1 μg/ml BFA (*B*) for up to 24 hrs, then harvested at different time intervals for RT-PCR. Each gel band represents the RT-PCR product from KU812 cells with different treatment taken at different time points. BFA did not inhibit neither IL-6 nor GM-CSF mRNA expression at different time intervals, while A23187 significantly increased the expression of GM-CSF but not IL-6 mRNA.

slightly enhance the transcription of IL-6 and GM-CSF genes when the bands of BFA treatment are compared with those of medium control at different time points.

Figures 20A and 20B illustrate the results obtained from 4 separate experiments after a 12 h incubation of KU812 cells in the presence or absence of A23187 alone or A23187 plus BFA. Total RNA from the cells with different treatments were isolated and subjected to RT-PCR for IL-6, GM-CSF and β-actin in parallel. The ratios of mean pixel density (MPD) of the bands for each cytokine over that of β-actin was compared among the different treatment groups. For IL-6 RT-PCR products, there is no significant difference in the MPD ratios between A23187 group and A23187 plus BFA group ($p > 0.05$), as well as between A23187 group and Medium group ($p > 0.05$) (**Figure 20A**). These results indicate that neither BFA nor A23187 had notable effects on IL-6 gene transcription at the12 h time point. The results for GM-CSF bands are slightly different (**Figure 20B**). The MPD ratio for GM-CSF band of A23187 group is significant higher than that of Medium group ($p < 0.05$), suggesting that increased GM-CSF mRNA levels were present in the cells stimulated with A23187 for 12 hours. However, there is no significant difference between A23187 group and A23187 plus BFA group in the MPD ratios ($p > 0.05$), indicating BFA, did not affect A23187-induced elevation of GM-CSF gene transcription.

2-9. Ultrastructural analysis confirms that BFA does not induce degranulation

Electron microscopic examination of mBMMC demonstrated that mBMMC cultured in medium alone contained a large number of mature and immature granules,

and smaller-sized vesicles in the cytoplasm, with an intact Golgi apparatus (**Figure 21A**). The Golgi apparatus was not visible in mBMMC treated with BFA, as has been reported by other groups working with the same compound (225). The degree of granulation in BFA treated cells was similar to that of untreated cells (**Figure 21B**).

A23187 induced marked degranulation, but had little effect on the appearance of Golgi apparatus, as few granules were present inside mBMMC following treatment with A23187, while intact Golgi complexes were readily observed in these cells (**Figure 21C**). The Golgi apparatus and the majority of granules were not observable in mBMMC treated with both BFA plus A23187, revealing the profound effects of BFA on Golgi structure and those of A23187 on degranulation by mBMMC (**Figure 21D**).

3. DISCUSSION

There are two classical pathways for protein secretion from cells, the constitutive and regulated pathways (116, 117). The constitutive pathway is spontaneous, characterized by continuous and low level protein secretion in a distinct set of small vesicles delivered from the Golgi apparatus to the cell surface. The regulated pathway is induced by secretagogues, characterized by immediate and massive release of contents in the granules, which after being stored in the cell cytoplasm for a period of time quickly move to fuse with the cytoplasmic membrane and release the granular contents outside of the cell in response to stimulation. Mast cells have long been recognized for their characteristic regulated secretion pathway, known as degranulation, by which the cells release

Figure 20. BFA effects on IL-6 and GM-CF mRNA expression. Experimental conditions and measurements were as stated in Figure 19.

A.) IL-6 mRNA levels were determined with semi-quantitative RT-PCR in KU812 cells treated with or without A23187, or with BFA plus A23187 for 12 h. The relative amount of IL-6 levels in each treatment condition was expressed as the ratio of mean pixel density for the IL-6 bands in relation to the β-actin bands.

B.) GM-CSF mRNA levels were determined with semi-quantitative RT-PCR in KU812 cells treated with or without A23187, or with BFA plus A23187 for 12 h. The relative amount of GM-CSF levels in each treatment condition was expressed as the ratio of mean pixel density for the GM-CSF bands in relation to the β-actin bands. The average ratio of mean pixel density for both IL-6 and GM-CSF RT-PCR products were calculated from 4 separate experiments. * $p < 0.05$ when compared with Medium group.

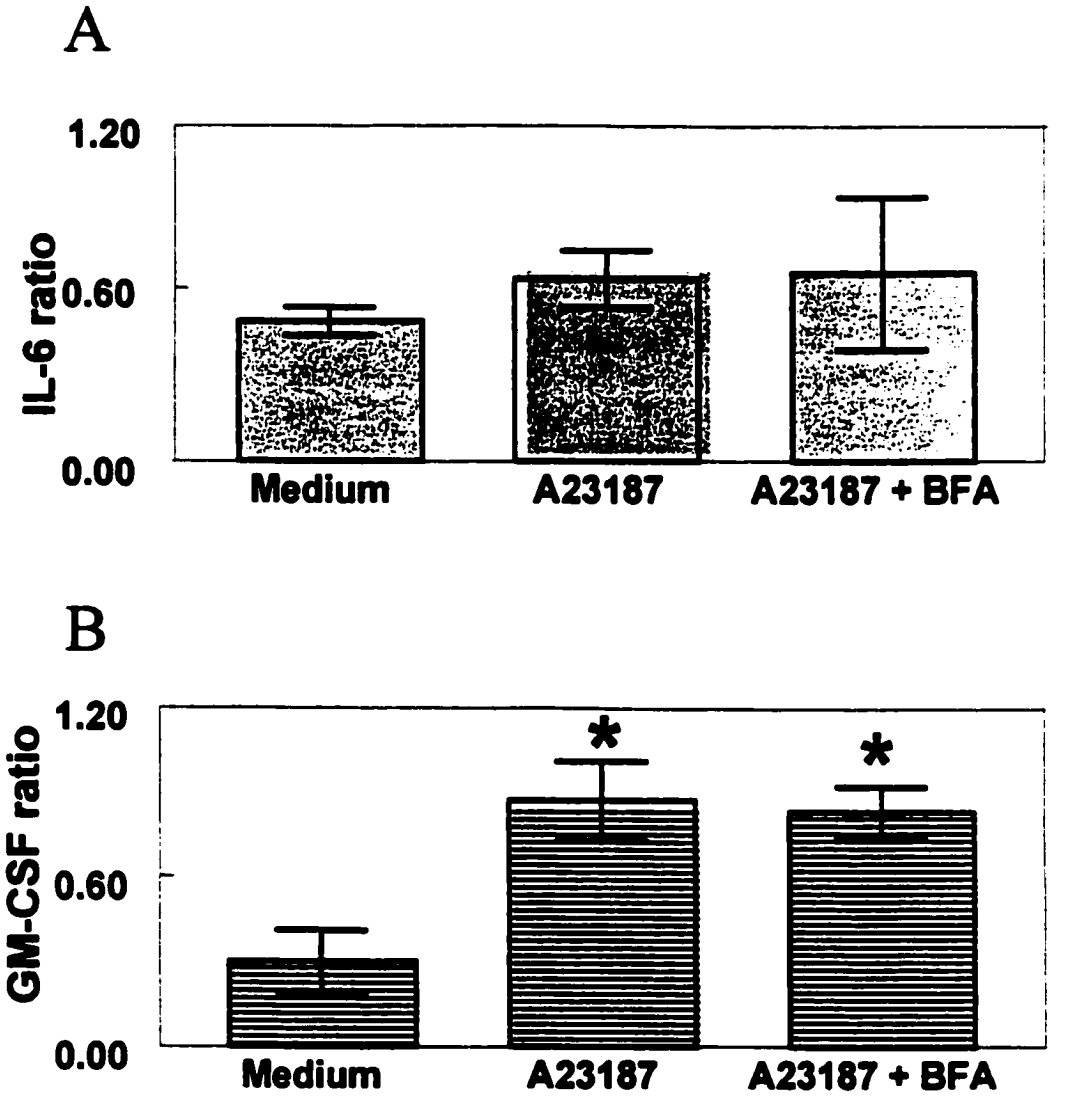

A
1.20
0.60
0.00
IL-6 ratio
Medium
A23187
A23187 + BFA
B
1.20
0.60
0.00
GM-CSF ratio
*
*
Medium
A23187
A23187 + BFA

Figure 20.

Figure 21. Typical ultrastructural changes in murine BMMC incubated for 3 hr under four different conditions: *A*, Medium alone; *B*, treated with 0.5 μg/ml BFA alone; *C* ,with 1 μM A23187 alone; and *D*, treated with 0.5 μg/ml BFA plus 1 μM A23187. Intact Golgi apparatus and large numbers of high-density granules are located in untreated KU812 cells(A). BFA treatment resulted in the disappearance of classic Golgi structure without effects on the granulation of the cells (B). A23187-treated cells lost most of their granules, while retained the Golgi apparatus (C). No Golgi apparatus was visible, and decreased granulation was observed in the cells treated with both BFA and A23187 (D).

A=Medium alone

B=BFA

C=A23187

D=BFA+A23187

Figure 21.

preformed mediators, such as histamine, proteoglycans and a variety of enzymes.

In order to avoid confusion of the term "constitutive production" with what has been known as "constitutive secretory pathway", the term "vesicle dependent transport pathway" will be used to refer to the intracellular vesicles mediated release of mast cell mediators. The term "degranulation-related pathway" is to be used to describe the mediator release associated with degranulation.

The main goal for this study was to determine whether the cytokines, IL-6 and GM-CSF, are secreted from mBMMC and KU812 cells through a degranulation-related pathway or a vesicle dependent pathway. KU812 cells without any stimulation, consistently produced and released most of their IL-6 without substantial cytokine storage. The observations that very little IL-6 is normally cell-associated and that this cytokine is spontaneously released, indicate that IL-6 secretion is unlikely to be due to release by a degranulation-related pathway. However, since ongoing degranulation processes such as "piecemeal" degranulation have been described (226), it is important to examine the cytokine secretion routes more specifically.

BFA and monensin are fungal metabolites able to rapidly and reversibly block intracellular vesicle transport from endoplasmic reticulum to Golgi apparatus, and therefore, inhibit protein secretion with minimal effect on protein synthesis (203, 224). Both BFA and monensin are Golgi-specific agents, but the former mainly affects the proximal compartments, the latter mainly affects the distal compartments of the Golgi apparatus. BFA causes disassociation of the coat protein, β-COP from Golgi membranes, which then are redistributed into endoplasmic reticulum, while monensin, as a Na^+

ionophore, disrupts the ion gradients in the intracellular compartments (203, 207). It has been demonstrated that BFA blocks the release of newly synthesized protein, but has no effect on preformed granule-bound proteins (227).

Although BFA and monensin have been widely used as inhibitors of protein secretion, the information concerning their effects on cytokine release, especially from granulated cells is scarce. It has been previously reported that BFA inhibited the release of TNF-α and TGF-β from a rat mast cell line, RBL-2H3 (217, 228). In these studies, only the effect of BFA on long term cytokine release was determined. Given the well characterized properties of BFA and monensin, it is reasonable to conclude that a cytokine is secreted through the vesicle transport pathway if its release from the cell is inhibited by BFA or monensin, while a cytokine is more likely to be secreted via a degranulation related pathway if its immediate or long term release is not blocked by BFA or monensin, and is temporally related to the release of histamine or ß-hexosaminidase, two well-known mediators stored within the mast cell granules.

3-1. IL-6 short term release is not related to mast cell degranulation

Initially, the effects of BFA on degranulation were examined on KU812 cells and mBMMC. There was no significant inhibition of the initial 20 min release of the granule-bound mediators, histamine and β-hexosaminidase, from either KU812 cells or mBMMC. These findings are in agreement with early reports that BFA would not interfere with the degranulation by a rat mast cell line RBL-2H3 cells (217, 227). However, BFA was shown to significantly inhibit the initial 20 min release from KU812

cells treated with or without A23187. This suggested that the increased initial 20 min release of IL-6 in response to A23187, that could be measured in KU812 cells, was not the result of degranulation but rather due to a vesicular transport dependent pathway initiated rapidly upon cell activation which could be inhibited by BFA.

To further confirm the effects of BFA on cytokine release from KU812 cells, the cells were treated with monensin in parallel with BFA for 20 min. Monensin, like BFA, did not inhibit β-hexosaminidase release from KU812 cells induced by calcium ionophore, A23187, but it significantly inhibited 20 min IL-6 release from KU812, providing further evidence that IL-6 is secreted via a vesicle dependent pathway.

Although BFA and monensin had similar effects on mast cell degranulation and cytokine release, there were some differences between the two agents. BFA was a more efficient inhibitor of initial 20 min IL-6 release from KU812 cells, and it significantly inhibited both constitutive and ionophore induced initial 20 min release of IL-6 from the mast cells, while monensin significantly inhibited only constitutive but not ionophore induced IL-6 release. These different effects of BFA and monensin may be due to the fact that BFA disturbs intracellular transport between the endoplasmic reticulum and the Golgi apparatus, while monensin acts on more distant parts, between the medial and trans cisternae of the Golgi complex (35, 207). A23187 induced IL-6 release in mast cells may occur at the more proximal compartments of Golgi apparatus.

In most systems studied, secretion of proteins through the vesicle dependent pathway is a spontaneous process, but our data indicates that this process can be promoted, if not initiated, by A23187. This observation could have important

implications in that it suggests another level of regulation of the vesicle dependent secretion pathway in KU812 cells. It is generally believed that the vesicle dependent secretion (constitutive pathway) is regulated primarily at the synthetic level (229), but our data suggests that regulation of the secretion pathway employed for the initial 20 min release of IL-6 by mast cells may also occur at the vesicular transport level as has been demonstrated in pancreatic exocrine cells (229) and colonic epithelial cells (230).

It has sometimes been assumed that short term (within 30 minutes), induced release of mediators, e.g., cytokines, from mast cells is caused by degranulation. Depending on the mediator being studied, however, such early mediator release could represent a combination of newly synthesized and preformed granule associated mediators. Our observations suggest that the initial 20 minute release of IL-6 induced by A23187 treatment of KU812 cells is not predominately from a granule associated source. This observation is consistent with a recent report that the rate of ^{35}S-labelled glycosaminoglycan transport from the Golgi apparatus to the cell surface was increased by IgE receptor stimulation in rat basophilic cells (231).

3-2. IL-6 and GM-CSF secretion is through a vesicle transport dependent pathway

Twenty-four hour activation of mBMMC and KU812 cells in the presence or absence of BFA revealed that BFA alone or in combination with A23187 significantly inhibited IL-6 release. At the same time, BFA significantly increased the amount of IL-6 remaining inside these cells. The secretion of GM-CSF was significantly inhibited in the similar fashion by BFA in both mBMMC and KU812 cells. These data strongly suggest

that vesicle transport dependent mechanism is responsible for the ongoing 24 hour secretion of both IL-GM-CSF by mast cells.

To further confirm the effects of BFA on cytokine release from KU812 cells, the cells were treated with monensin for different periods of time. Monensin, like BFA, did not inhibit 20 min β-hexosaminidase release from KU812 cells induced by calcium ionophore, A23187, but it significantly inhibited 24 hour IL-6 release from KU812, providing further evidence that IL-6 is secreted via a vesicle dependent pathway.

The data from our observations with mBMMC show that BFA not only blocked A23187-induced but also antigen / IgE-induced secretion of IL-6 and GM-CSF over 24 hours. This suggests that BFA has similar effects on cells activated by other physiological stimuli such as IgE receptor cross-linking as with ionophore activation. Another interesting point emerging from the experiments with mBMMC is that the results dispute a common assumption that elevated intracellular Ca^{2+} level would have similar effects on mast cells as those induced by cross-linking IgE receptors. Although both A23187 and treatment of IgE sensitized cells with IgE specific antigen, DNP-HSA, significantly increased the production and secretion of IL-6 and GM-CSF by mBMMC, stimulation via the FcεRI pathway was more than 10 fold stronger than the calcium ionophore in inducing the secretion and production of either cytokine. Such difference may be due the fact that IgE receptor crossing-linking induced mast cell activation is a natural biological event that has all the normal signal transduction elements in place, therefore, result in much stronger response than A23187, which bypasses the usual signal transduction pathways in a intrusive way, possible adversely affect other functions of the cells.

3-3. The effects of BFA on protein synthesis, mRNA expression and intracellular structures

In order to confirm that decreased IL-6 and GM-CSF secretion from BFA treated KU812 cells is due to an effect of BFA on protein secretion rather than on protein synthesis or mRNA expression, pulse chase experiments with [^{3}H] leucine and semiquantitative RT-PCR analysis were performed. Our data demonstrate a non-significant 8% inhibition of overall protein synthesis in cells treated with BFA for 6 h and a significant 36% inhibition at 12h. While the latter result might suggest some overall inhibitory effect of long term BFA treatment this could not account for the over 98% inhibition of GM-CSF production and 94% inhibition of IL-6 production observed in A23187 activated cells in the presence of BFA. It should also be noted that over 80% of the secreted IL-6 or GM-CSF response to ionophore activation alone in KU812 cells was complete after 6 hours of treatment, further reducing any possibility that inhibition of protein synthesis at later time points could account for our observations.

The RT-PCR analysis of both IL-6 and GM-CSF mRNA levels did not demonstrate any significant inhibition of cytokine expression in BFA treated cells at any of the time points examined up to 24h. Notably, GM-CSF mRNA levels but not IL-6 mRNA levels were increased in cells treated with A23187 compared with control cells. This finding is in keeping with previous observations from Northern blot analysis of rat peritoneal mast cells, which suggest a high degree of post transcriptional regulation of expression of IL-6 (216). The results from the pulse chase experiments and mRNA

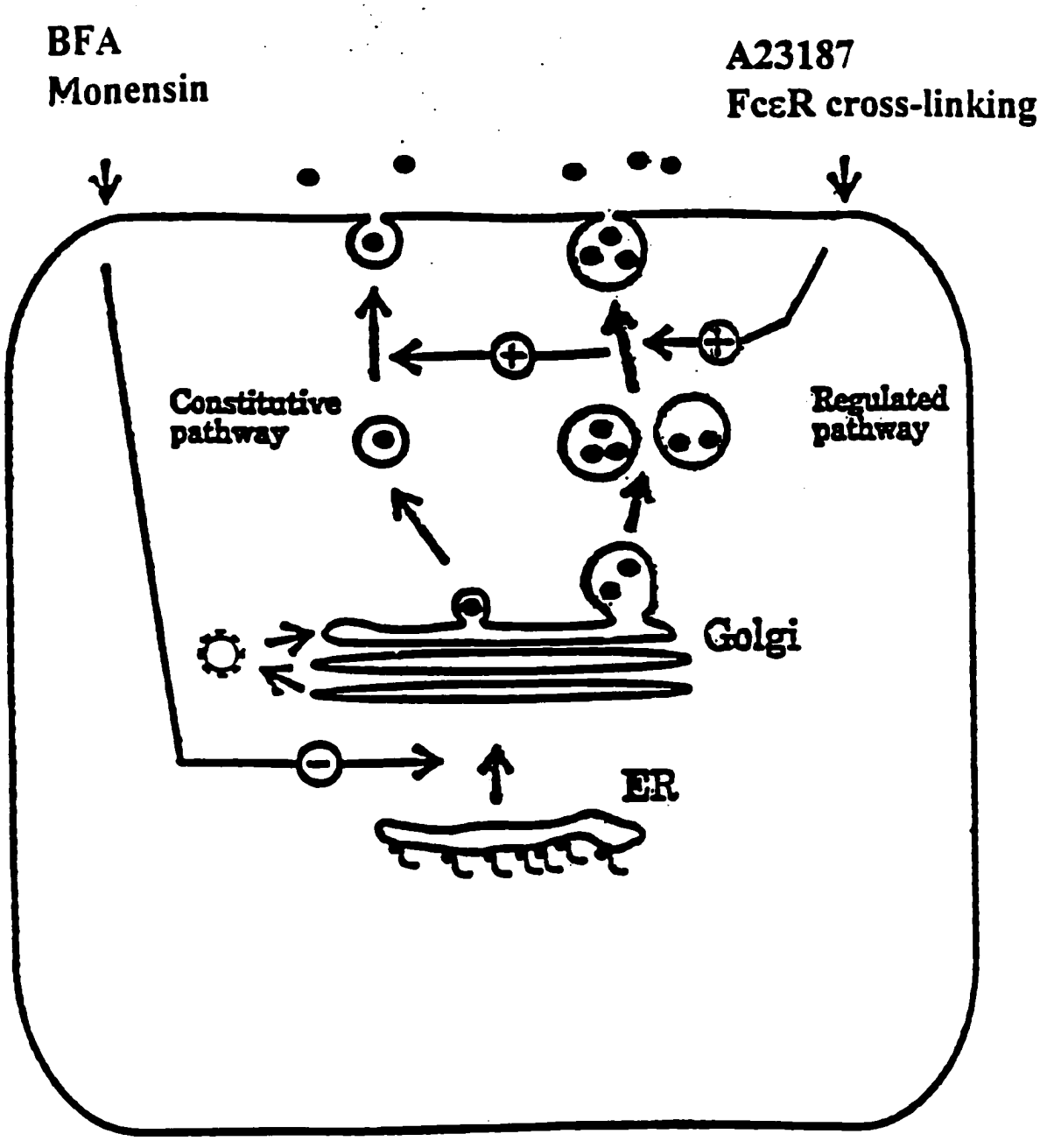


Figure 22. Model of the effects of BFA and monensin on mast cell cytokine secretion. {Modified from R.D. Burgoyne & C.L. Wilde (232}

BFA acts on proximal, and monensin on distal Golgi coplex to block ntracellular trafficking of vesicle dependent transport pathway, but not degranulation of mast cells. A23187 and IgE receptor cross-linking triger mast cell degranulation and promote vesicle dependent transport.

analysis confirm that, as demonstrated in many other systems, BFA appears to be acting mainly at the level of protein secretion in blockade of GM-CSF and IL-6 secretion.

Electron microscopic examination of mBMMC further provided morphological evidence to support the selective effects of BFA on the intracellular transport system. The Golgi apparatus was not detectable in the cells treated with BFA. This is in agreement with the observation by other groups working with BFA (225). Notably, the electron micrographs confirm that BFA does not inhibit the release of granules from mast cells.

In summary, we have demonstrated that initial 20-min release of IL-6 from differentiated KU812 cells was inhibited by BFA and monensin, two protein secretion blockers that had no effect on degranulation, suggesting that a mechanism exists for an initial 20-min induced release of IL-6 that is not degranulation dependent. We have also shown that the ongoing up to 24 h release of both GM-CSF and IL-6 from a human mast cell/basophilic cell line and murine mast cells was inhibited by BFA and monensin, suggesting that the major secretion route for these two cytokines is a vesicular transport-dependent pathway (Figure 22). These results will help us to have a better understanding of the mechanisms involved in cytokine secretion by mast cells, and may be useful for designing pharmacological regulation of mast cell cytokines in the future.

CHAPTER 4

INDUCTION OF IL-6 AND TNF-α BY BACTERIAL DNA AND CpG-CONTAINING OLIGODEOXYNUCLEOTIDES IN mBMMC AND KU812 CELLS

1. INTRODUCTION

Mast cells play unique roles in both innate and acquired immunity (121). It is estimated that the total mast cells in the body can form a mass of the size of the spleen (7), and these cells are widely distributed throughout the body, particularly at mucosal sites and the skin, at the front line of host defense. The strategic location of mast cells makes them ideal sentinel cells to initiate host response mechanisms (233). Animal models of bacterial infection of mast cell deficient W/W^V and control mice have provided convincing evidence that mast cells play a crucial role in host defense against bacterial infection (5, 6). Certain bacteria have been shown to be able to directly stimulate mast cells to release histamine (129), while complement mediated mechanisms account for further mast cell activation in response to bacteria (234). Previous work from this laboratory has demonstrated that bacterial products, such as lipopolysaccharides (LPS) and cholera toxin can induce cytokine production by rat mast cells (65, 66). As important effector cells of the immune system, mast cells are likely involved in the host response to bacterial DNA and CpG-containing oligodeoxynucleotides. However, the effects of bacterial DNA and CpG-containing oligodeoxynucleotides on mast cells have not been previously reported.

The first portion of the thesis studies has demonstrated that mast cell cytokine secretion is under complex control. Degranulation independent cytokine release is most likely to be involved in host defense against bacteria where mast cells are known to be critical but degranulation is not a hallmark of pathology. In the present study, we have examined the hypothesis that mast cells can recognize and respond to bacterial DNA and CpG-ODN by releasing inflammatory mediators. The stimulatory effects of bacterial DNA

and CpG-ODN on murine bone marrow-derived mast cells (mBMMC) have been determined by examining degranulation through measurement of the release of β-hexosaminidase and through evaluation of the secretion of cytokines. Cytokine studies have focused primarily on IL-6 and TNF-α, which are known to be associated with inflammation and can be produced by activated rodent mast cells with or without concurrent degranulation (65, 66, 84). To gain further insight into the mechanisms, by which CpG-ODN exerts their effects, we examined oligodeoxynucleotide uptake by mBMMC using flow cytometry and confocal microscopy.

2. RESULTS

2-1. CpG-ODN induced TNF-α and IL-6 production from mBMMC

To examine the stimulatory effects of CpG-ODN on mBMMC, cytokine secretion from these cells was measured. mBMMC at $1x10^6$ cells /ml were treated with CpG-ODN (5'-TCC ATG ACG TTC CTG ATG CT-3') or control-ODN (5'-TCC ATG AGC TTC CTG ATG CT-3') at a range of doses from 0.1 to 100 μg/ml. Supernatants were harvested after 24 h culture, and examined for IL-6 levels by B-9 proliferation assay. There was a dose dependent increase in IL-6 production by mBMMC in response to CpG-ODN but not to control-ODN (**Figure 23**). IL-6 production by mBMMC treated with 10 μg/ml (1490 ± 128 U/ml, $p < 0.05$) and 100 μg/ml (3122 ± 670 U/ml, $p < 0.01$) was significantly higher than that from the cells treated with medium alone (980 ± 118 U/ml). mBMMC treated with 100 μg/ml of control-ODN produced a similar amount of IL-6 (900 ± 95 U/ml) as under control conditions. The time course of IL-6 production by

Figure 23. IL-6 production by mBMMC in response to stimulation with CpG-ODN. mBMMC were incubated at 37 ^{O}C in the presence of 0.1 to 100 μg/ml of synthetic oligodeoxynucleotides with (*CpG-ODN*) or without 1 CpG motif (*Control-ODN*). As a negative control, the cells were incubated with medium alone (*Medium*). Supernatants were collected at 24 h and assessed for IL-6 by B9 bioassay. CpG-ODN induced significantly higher levels of IL-6 production compared with medium control at the doses of 10 μg/ml ($p < 0.05$) and 100 μg/ml ($p < 0.01$), while Control-ODN did not. Bars represent mean data ± SEM. n = 4. * $p < 0.05$ when compared with Medium group. ** $p < 0.01$ when compared with the Medium group.

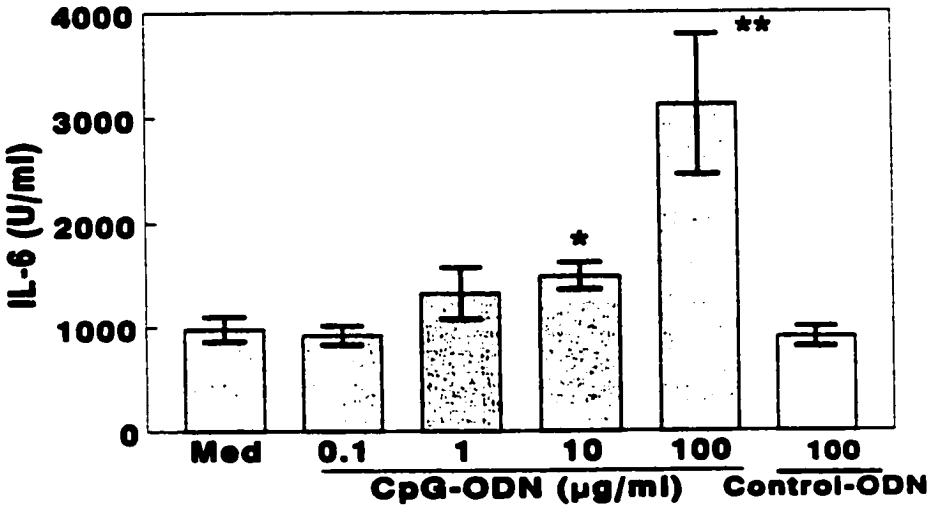

4000
3000
2000
1000
0
IL-6 (U/ml)
**
*
Med
0.1
1
10
100
100
CpG-ODN (μg/ml)
Control-ODN

Figure 23

Figure 24. Time course of IL-6 production by mBMMC treated with 50 μg/ml of oligodeoxynucleotides with (*CpG-ODN*) or without 1 CpG motif (*Control-ODN*). As control, the cells were incubated in parallel with medium alone (*Medium*). Supernatant samples were taken at different intervals of time, and assessed for IL-6 levels using the B-9 bioassay. Significantly higher levels of IL-6 were produced by the cells treated with CpG-ODN than with Control-ODN or medium alone at 24 h time point, but not at earlier time intervals. Bars represent mean data ± SEM. n = 4. * $p < 0.05$ when compared with Medium group. *** $p < 0.001$ when compared with Medium group. ### $p < 0.001$ when compared with ODN group.

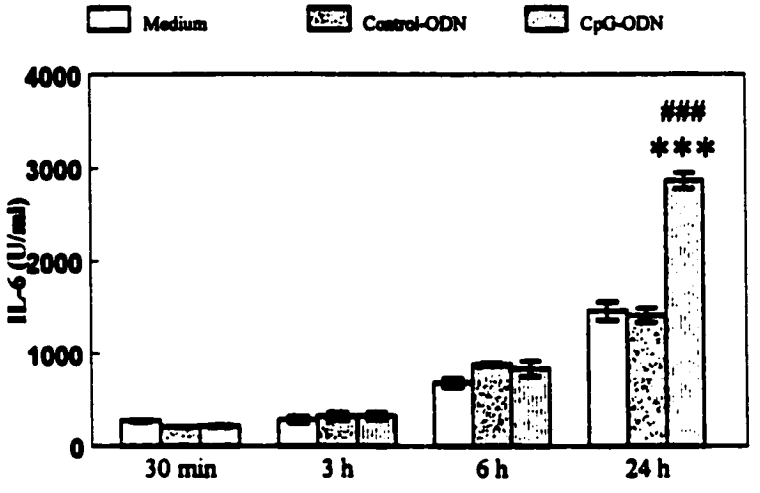

Medium
Control-ODN
CpG-ODN
IL-6 (U/ml)
4000
3000
2000
1000
0
30 min
3 h
6 h
24 h
###
***

Figure 24

mBMMC treated with CpG-ODN or control-ODN was then examined. As shown in **Figure 24**, there was a significant elevation of IL-6 production by CpG-ODN treated mBMMC at the 24 h time point ($p < 0.001$). IL-6 levels from cells treated with control-ODN were similar to those of cells treated with medium alone ($p > 0.05$) at all time points examined.

Supernatants from CpG-ODN and control ODN treated mBMMC were also taken to measure the TNF-α levels by L929 assay. To verify the specificity of L929 assay for murine TNF-α, neutralizing anti-murine TNF-α was added to the samples from mBMMC treated with 3CpG-ODN for 6 hr. The anti-TNF-α antibody had completely abolished the cytotoxicity bioactivity of TNF-α present in these samples (**Appendix 8**). As was the case for IL-6, there was a dose dependent increase in TNF-α production by mBMMC in response to CpG-ODN but not to control-ODN (**Figure 25**). CpG-ODN significantly increased TNF-α production by mBMMC at doses of 1, 10, and 100 μg/ml ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively), while control-ODN at 100 μg/ml did not induce increased TNF-α production ($p > 0.05$). The time course of TNF-α production by mBMMC treated with CpG-ODN was slightly different from that of IL-6 under the same treatment (**Figure 26**). The peak levels of TNF-α bioactivity in supernatants were reached at the 6 h time point, in contrast to the 24 h time point, at which IL-6 reached peak levels.

2-2. The amount of IL-6 and TNF-α produced was related to the CpG motif numbers in oligonucleotides

Figure 25. TNF-α production by mBMMC in response to stimulation of CpG-ODN. mBMMC were incubated at 37 ^{o}C in the presence of 0.1 to 100 μg/ml of synthetic oligodeoxynucleotides with (*CpG-ODN*) or without 1 CpG motif (*Control-ODN*). As a negative control, the cells were incubated with medium alone (*Medium*). Supernatants were collected at 24 h and assessed for TNF-α levels by L929 cytotoxicity assay. CpG-ODN induced significantly higher levels of TNF-α production at the doses of 1 μg/ml ($p < 0.05$), 10 μg/ml ($p < 0.01$), and 100 μg/ml ($p < 0.05$), while Control-ODN at 100 μg/ml did not induce production of this cytokine ($p > 0.05$). Bars represent mean data ± SEM. $n = 4$. * $p < 0.05$, ** $p < 0.01$ when compared with Medium group.

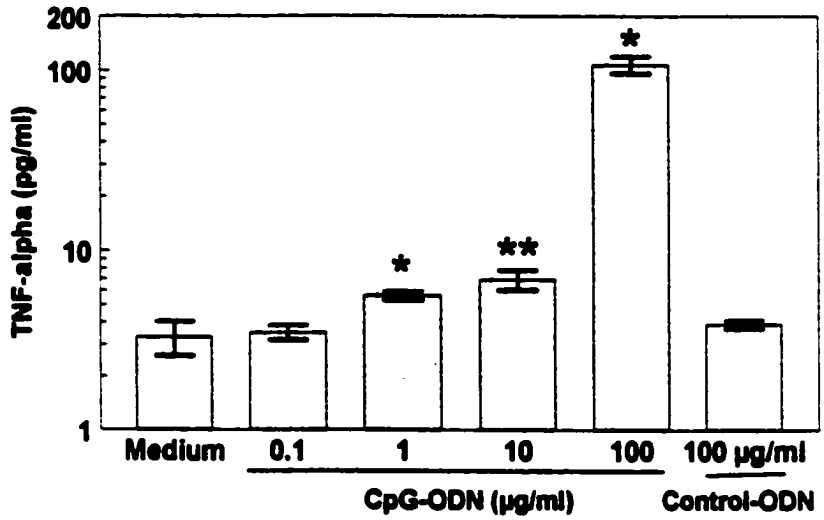

200
100
10
1
TNF-alpha (pg/ml)
*
**
*
Medium
0.1
1
10
100
100 µg/ml
CpG-ODN (µg/ml)
Control-ODN

Figure 25

Figure 26. Time course of TNF-α production by mBMMC treated with 50 μg/ml of oligodeoxynucleotides with (*CpG-ODN*) or without CpG motifs (*Control-ODN*). As controls, the cells were incubated in parallel with medium alone (*Medium*). Supernatant samples were taken at different intervals of time, and assessed for TNF-α bioactivity by L929 cytotoxicity assay. Significantly higher levels of TNF-α were detected in supernatants of the cells treated with CpG-ODN than with Control-ODN or medium alone at 6h time point, but not at other time intervals. Bars represent mean data ± SEM. $n = 4$. * $p < 0.05$ when compared with Medium group. # $p < 0.05$ when compared with Control-ODN group.

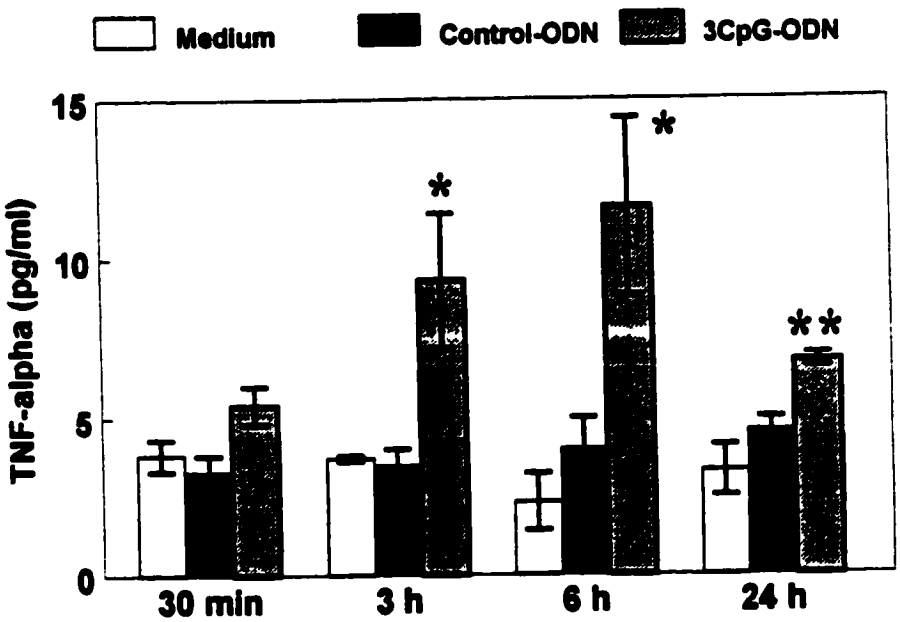

Medium
Control-ODN
3CpG-ODN
TNF-alpha (pg/ml)
15
10
5
0
*
*
**
30 min
3 h
6 h
24 h

Figure 26

Since published data by other investigators (143) indicated that the number of CpG motifs in oligodeoxynucleotides may contribute to the stimulatory effects of CpG-ODN, we sought to examine the cytokine production of mBMMC in response to CpG-ODN of same length but with different numbers of CpG motifs in their sequences. mBMMC at $1X10^6$ cells/ml were treated for 24 h with 50 μg/ml of CpG-ODN with 1, 2, and 3 CpG motifs (1CpG-ODN, 2CpG-ODN and 3CpG-ODN, respectively), and supernatants were collected for IL-6 and TNF-α measurement. As shown in **Figure 27**, all three CpG-containing oligodeoxynucleotides substantially increased IL-6 levels from mBMMC, while control-ODN at the same dose had no effects on the cytokine production. The increase in IL-6 levels was directly proportional to the number of CpG motifs. IL-6 production from mBMMC. IL-6 production from cells treated with 2CpG-ODN was slightly higher than that from cells treated with 1CpG-ODN, although this difference was not statistically significant. IL-6 production from cells treated with 3CpG-ODN was significantly higher than that from mBMMC treated with 1CpG-ODN ($p < 0.01$) or with 2CpG-ODN ($p < 0.01$).

A similar trend was also observed in the pattern of TNF-α production by mBMMC treated with the same ODN with or without 1, 2, or 3 CpG motifs. All the CpG-containing ODN induced significantly higher TNF-α levels from mBMMC than was produced by cells treated with medium alone ($p < 0.01$ for 1CpG-ODN, $P < 0.001$ for 2CpG-ODN, and $p < 0.001$ for 3CpG-ODN). The control-ODN at the same dose did not have a significant effect on TNF-α production by mBMMC (**Figure 28**). Furthermore, TNF-α production from cells treated with 3CpG-ODN was significantly higher than that

from cells treated with either 2CpG-ODN or 1CpG-ODN ($p < 0.05$, and $P < 0.01$, respectively). These data demonstrate that the number of CpG motifs in oligodeoxynucleotides is an important factor in determining the stimulatory effects of the DNA products on mast cells.

Our previous studies have shown that a substantial amount of GM-CSF was produced by mBMMC activated through IgE receptor cross-linking (see Chapter 3 in this thesis), and significantly higher levels of IL-4, IL-12 and IFN-γ were induced from mBMMC treated with killed *Staphylococcus aureus* whole bacteria or with IL-12, respectively (**Appendix 9**). However, in the present study, neither CpG-ODN nor control ODN induced IL-4, IFN-γ or GM-CSF, although there was slight increase of IL-12 from mBMMC treated with CpG-ODN for 24 h in the present study.

2-3. Bacterial DNA induced IL-6 and TNF-α production from mBMMC

The ability of CpG containing oligodeoxynucleotide sequences to induce cytokine production from mast cells suggests a role for normal bacterial DNA in mast cell activation. In order to examine this issue we treated mast cells with various DNA preparations from bacterial or control mammalian sources. While bacterial DNA induced both IL-6 and TNF-α production from mBMMC, calf thymus DNA did not. mBMMC treated with 10 μg/ml of *E. coli* DNA for 24 h produced an IL-6 level of 1305 ± 75 U/ml (mean ± S.D), significantly higher than that from cells treated with medium alone (712 ± 126 U/ml) ($p < 0.01$), while the cells treated with the same dose of calf thymus DNA had an IL-6 level of 652 ± 57 U/ml, comparable to that from cells treated with medium alone

Figure 27. The amount of Il-6 production induced by synthetic oligodeoxynucleotides are related to the numbers of CpG motifs present in the sequences of the oligodeoxynucleotides. mBMMC were treated for 24 h with 50 μg/ml oligodeoxynucleotides of with 1, 2 or 3 CpG motifs or without CpG motifs (*No CpG-ODN*). The CpG-containing oligodeoxynucleotides have the same length and similar sequences but different numbers of CpG motifs. Oligodeoxynucleotides containing one, two, and three CpG motifs (*1CpG-ODN, 2CpG-ODN, and 3CpG-ODN*, respectively) all induced significantly higher levels of IL-6 production from mBMMC than that from cells treated with medium alone (*Medium*) ($p < 0.01$, $P < 0.001$, $P < 0.001$, respectively), while non-CpG-containing oligodeoxynucleotides did not. IL-6 production from 3CpG-ODN treated cells was significantly higher than from 1CpG- ODN, and 2CpG-ODN treated cells ($p < 0.01$, and $P < 0.01$, respectively). Each point represents the mean data ± SEM. n = 4.

** $p < 0.01$, *** $p < 0.001$ when compared with Medium group.

$p < 0.01$ when compared with 1CpG.

$ $p < 0.05$, $$ $p < 0.01$ when compared with 2 CpG.

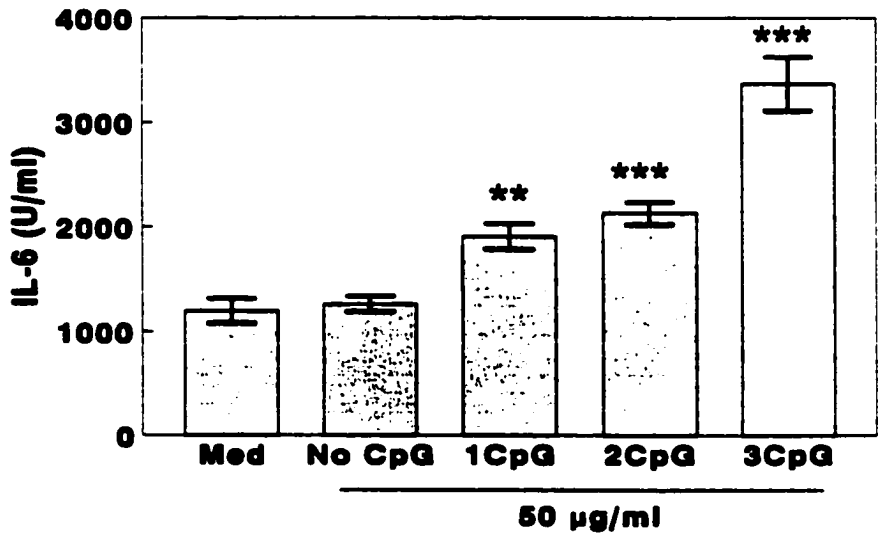


Figure 27

Figure 28. The amount of TNF-α production induced by synthetic oligodeoxynucleotides is related to the number of CpG motifs present in the sequences of the oligodeoxynucleotides. mBMMC were treated for 24 h with 50 μg/ml oligodeoxynucleotides with 1, 2 or 3 CpG motifs or without CpG motifs (*No CpG-ODN*). The CpG-containing oligodeoxynucleotides have the same length and similar sequences but different numbers of CpG motifs. Oligodeoxynucleotides containing one, two, and three CpG motifs (*1CpG, 2CpG*, and *3CpG*, respectively) all induced significantly higher levels of TNF-α production from mBMMC than from cells treated with medium alone (*Medium*) ($p < 0.01$, $P < 0.001$, $P < 0.001$, respectively), while non-CpG-containing oligodeoxynucleotides did not. TNF-α production from 3CpG-ODN treated cells was significantly higher than from 1CpG-ODN, and 2CpG-ODN treated cells ($p < 0.01$, and $P < 0.05$, respectively). Each point represents the mean data ± SEM. n = 4.

** $p < 0.01$, *** $p < 0.001$ when compared with Medium group.

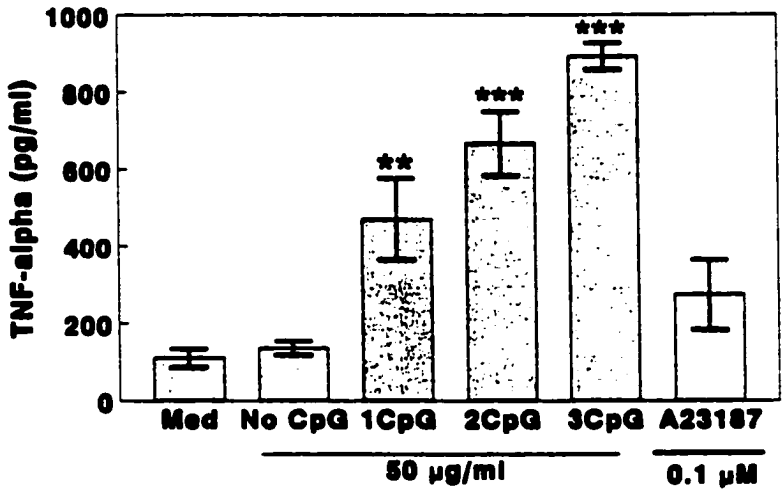

1000
800
600
400
200
0
TNF-alpha (pg/ml)
**
***
***
Med
No CpG
1CpG
2CpG
3CpG
A23187
50 µg/ml
0.1 µM

Figure 28.

($p > 0.05$). Significantly higher levels of IL-6 were observed from mBMMC treated for 24 h with 5 μg/ml *Proteus* DNA ($p < 0.05$) and plasmid DNA ($p < 0.05$) than with medium alone. Significantly higher levels of TNF-α were observed in mBMMC treated for 24 h with 5 μg/ml of plasmid DNA (**Figure 29**).

Methylation of bacterial DNA or CpG-containing oligodeoxynucleotides has been shown to abolish the stimulatory effects of the DNA on murine B cell and macrophages, and was employed as a further control to exclude the possibility that contaminants such as LPS in DNA preparations might be responsible for the observed effects on cytokine production (143, 208). mBMMC were cultured with 10 μg/ml methylase-treated or untreated *E.coli* DNA for 24 hours, and B-9 assay results showed that methylation of *E.coli* DNA completely abolished its stimulatory effects on IL-6 production by mBMMC. The IL-6 production from cells treated with methylase-treated *E.coli* DNA was 1297± 57 U/ml, similar to that from cells incubated with medium alone (1387±48 U/ml) but significantly lower than that from cells incubated with un-treated *E. coli* DNA (2054±246 U/ml) ($p < 0.05$) (**Figure 30**).

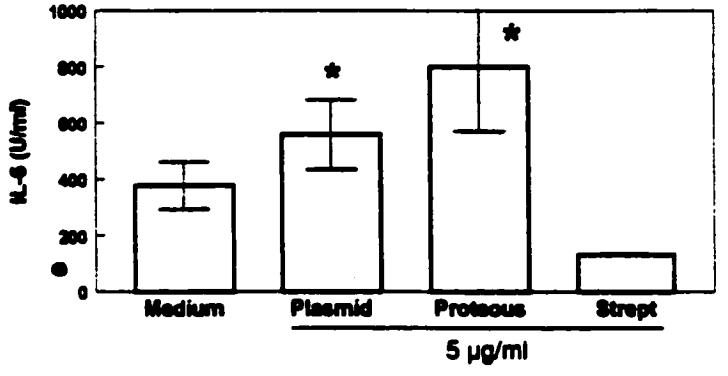
2-4. Neither bacterial DNA nor CpG-ODN induced degranulation from mBMMC

Both bacterial DNA and CpG-ODN have been shown, in our study, to activate mBMMC to secrete cytokines, however, it is also important to know that if these DNA products are capable of inducing degranulation, an important event for early mast cell activation. We investigated the effects of synthetic ODN on short term release of β-hexosaminidase, a for 20 min with either CpG-ODN or control-ODN at 100 μg/ml, the

Figure 29. mBMMC IL-6 and TNF-α production by induced by bacterial DNA. mBMMC were treated for 24 h with 5 μg/ml DNA preparation from *Proteus mirabilis*, *Streptococcus faecalis* and Bluescript plasmids. Significantly higher levels of IL-6 were observed from mBMMC treated with *Proteus* DNA ($p < 0.05$) and plasmid DNA ($p < 0.05$) than with medium alone. Significantly higher levels of TNF-α were observed in mBMMC treated with 5 μg/ml of plasmid DNA ($p < 0.05$). Each point represents the mean data ± SEM. n = 4.

* $p < 0.05$ when compared with Medium group.

A


1000
800
600
400
200
0
IL-6 (U/ml)
*
*
Medium
Plasmid
Proteous
Strept
5 μg/ml

B

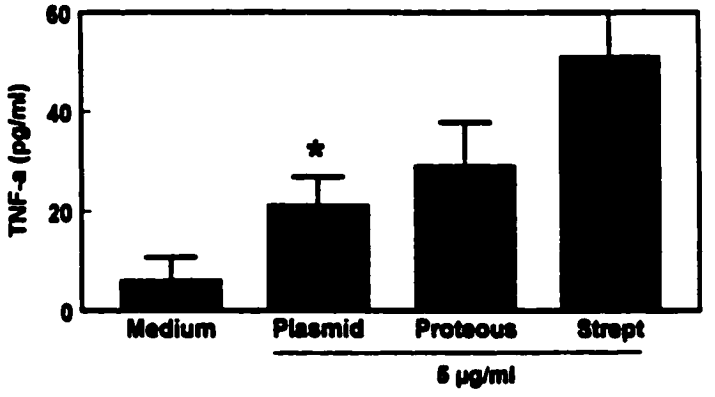

60
40
20
0
TNF-α (pg/ml)
*
Medium
Plasmid
Proteous
Strept
5 μg/ml

Figure 29

Figure 30. Methylation of *E. coli* DNA abolished increased IL-6 production from mBMMC untreated with *E. coli* DNA. mBMMC were cultured with 10 μg/ml methylase-treated or untreated *E .coli* DNA for 24 hours, supernatant samples were taken to measure IL-6 levels using B-9 assay. The IL-6 production from cells treated with methylase-treated *E. coli* DNA (Meth. Ec-DNA) was similar to that from cells incubated with medium alone, but significantly lower than that from cells incubated with untreated *E. coli* DNA (Ec-DNA) ($p < 0.05$). Each point represents the mean data ± SEM. $n = 4$.

`* $p < 0.05$ when compared with Medium group.

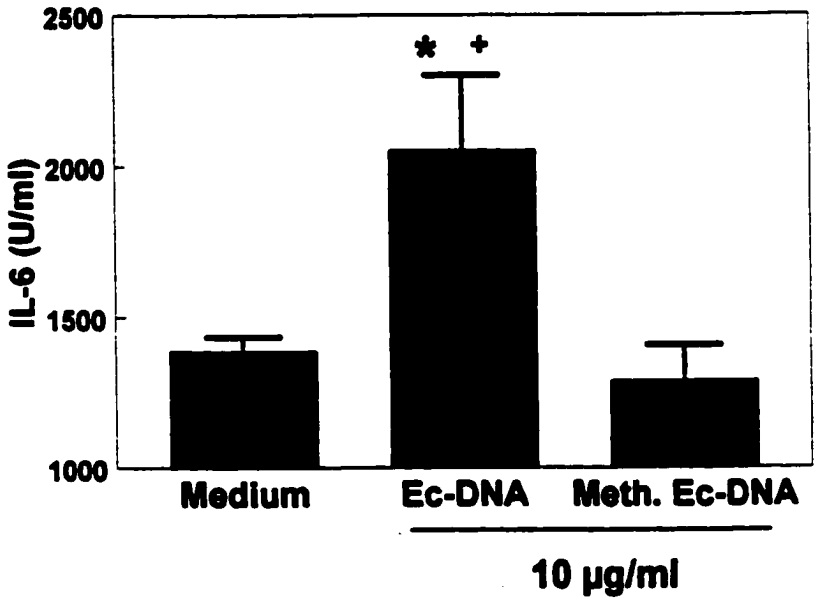


Figure 30

well-known marker for mast cell degranulation. mBMMC at 1 x 10^6 cells/ml were treated highest dose used in this study which induced substantial levels of cytokine production from mBMMC. Neither CpG-ODN nor control ODN had any significant effect on degranulation of mBMMC as measured by β-hexosaminidase release assay following a 20 min activation, while calcium ionophore, A23187, a commonly used stimulant for mast cells, induced significant release of this mediator (**Figure 31**). To examine the effects of bacterial DNA on mBMMC degranulation, mBMMC were treated with 100 μg/ml of *E. coli* DNA or calf thymus DNA, respectively. Neither of the two bacterial DNA preparations nor the calf thymus DNA induced degranulation from mBMMC. The percentage of β-hexosaminidase release from mBMMC treated with these natural DNA preparations were 9 ± 1.2 %, 9 ± 1.5 %, and 8 ± 0.3%, respectively, comparable spontaneous release (10 ± 1.2%) from mast cells incubated with medium alone. The positive control, A23187-treated mBMMC had significantly higher levels (21 ± 1.2%) of β-hexosaminidase release ($p < 0.001$). The observations that no increased IL-6 and TNF-α levels were found at the 30 minute time point further support the notion that DNA-induced cytokine secretion is not related to mast cell degranulation.

2-5. Bacterial DNA and CpG-ODN induced IL-6 but not degranulation from KU812 cells

Although the focus for the current studies on DNA-induced cytokines has been on mBMMC, we also carried out some experiments on KU812 cells. The results from these

Figure 31. The effect of CpG-ODN and ODN on mBMMC short term release of β-hexosaminidase. mBMMC were incubated at 37 ^{o}C for 20 min in the presence of 100 μg/ml of synthetic oligodeoxynucleotides with one or three CpG motifs (*1CpG* and *3CpG*, respectively), or without CpG motif (*No CpG*). As a positive control, the cells were incubated in parallel with 0.5 μM of calcium ionophore, A23187 (*A23187*), and as a negative control, the cells were incubated with medium alone (*Medium*). Supernatant and pellets were collected separately, and assessed for β-hexosaminidase levels. Neither CpG-containing oligodeoxynucleotides nor non-CpG-containing oligodeoxynucleotides induced β-hexosaminidase release at 20 min, while A23187 induced significantly higher levels of β-hexosaminidase release. Each point represents the mean data ± SEM. n = 4. *** $p < 0.001$ when compared with Medium group.

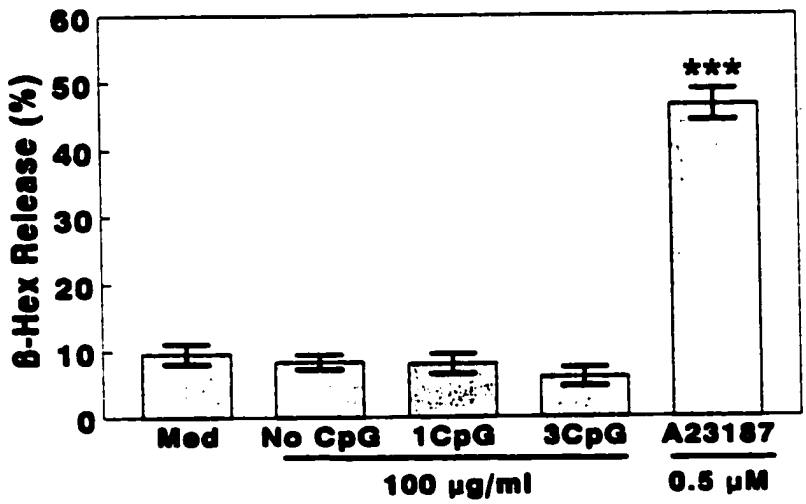

60
50
40
30
20
10
0
β-Hex Release (%)
***
Med
No CpG
1CpG
3CpG
A23187
100 µg/ml
0.5 µM

Figure 31

experiments showed that both CpG-ODN (Figure 32) and *E. coli* DNA (Figure 33) were able to induce significant amounts of IL-6 from KU812 cells, while control-ODN and calf thymus DNA could not. As shown in **Figure 32**, significantly higher levels of IL-6 were induced in KU812 cells treated with either 5 or 50 μg/ml of CpG-ODN over a period of 24 h ($p < 0.05$, $p < 0.05$, respectively), however, the IL-6 levels in a parallel experiment with control-ODN at 50 μg/ml were similar to those treated with medium alone. Like mBMMC, KU812 cells responded to *E. coli* DNA at 100 μg/ml by secreting IL-6 at higher levels ($p < 0.01$) than cells treated with medium alone or with calf thymus DNA at the same dose for 24 h **(Figure 33)**. One interesting observation from these studies was that DNA from the Gram positive bacterium *Micrococcus lysodeikticus* did not significantly induced IL-6 from KU812 cells, in contrast to the DNA from the Gram negative bacterium *E. coli*. This is perhaps due the differences in DNA sequences or due to different residual cellular components in these two DNA preparations. Our earlier studies had shown that killed *Staphylococcus aureus* whole bacteria induced substantial amount of GM-CSF from KU812 cells over 24 h, however, in the current studies 24 h treatment of CpG-ODN and *E. coli* DNA did not induced GM-CSF from KU812 cells. As in the case for mBMMC, neither CpG-ODN nor *E. coli* DNA induced degranulation as measured by β-hexosaminidase release from KU812 cells.

2-6. Mast cells can internalize CpG-ODN

The mechanism by which bacterial DNA and CpG-ODN stimulate immune effector cells is currently unknown, but some studies suggest that CpG-ODN exert their

Figure 32. IL-6 production by KU812 cells in response to stimulation with CpG-ODN. KU812 cells were incubated at 37 ^{O}C for 24 h in the presence of 5 and 50 μg/ml of oligodeoxynucleotides with 1 CpG motif (*CpG-ODN*) or without CpG motif (*Control-ODN*). As a negative control, the cells were incubated with medium alone (*Medium*). Supernatants were collected at 24 h and assessed for IL-6 by B9 bioassay. CpG-ODN induced significantly higher levels of IL-6 production compared with medium control at the doses of 5 μg/ml ($p < 0.01$) and 50 μg/ml ($p < 0.01$), while Control-ODN at 50 μg/ml did not. Bars represent mean data ± SEM. n = 4. * $p < 0.05$ when compared with Medium group.

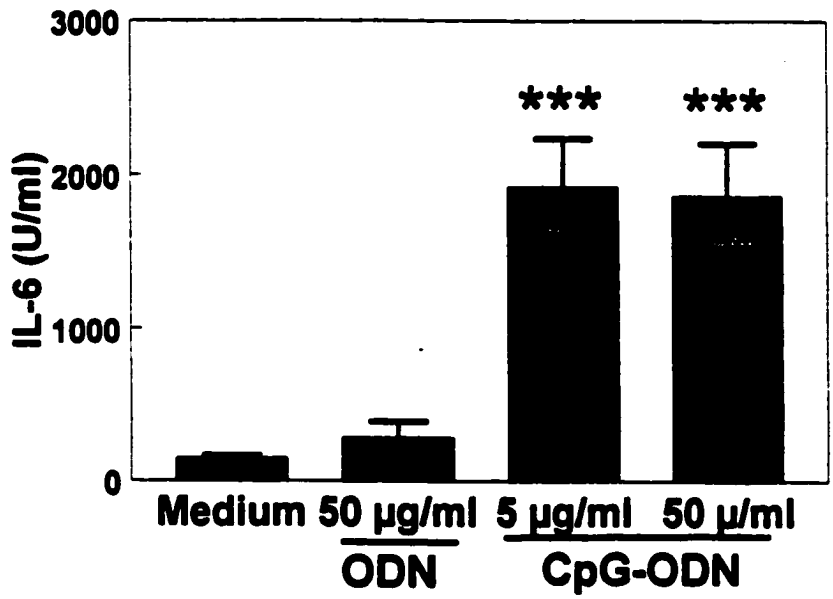

3000
2000
1000
0
IL-6 (U/ml)
***
***
Medium
50 µg/ml
5 µg/ml
50 µ/ml
ODN
CpG-ODN

Figure 32

Figure 33. IL-6 production by KU812 cells in response to bacterial DNA. KU812 cells were incubated at 37 ^{o}C in the presence or absence of 100 μg/ml of to *E. coli* DNA, *Micrococcus lysodeikticus* DNA or calf thymus DNA for 24 h. *E. coli* DNA induced significant higher levels of IL-6 from KU812 cells than those cells treated with medium alone ($p < 0.01$), while calf thymus DNA as well as DNA from the Gram positive bacterium *M. lysodeikticus* did not ($p > 0.05$). Bars represent mean data ± SEM. n = 4.

** $p < 0.01$ when compared with Medium group.

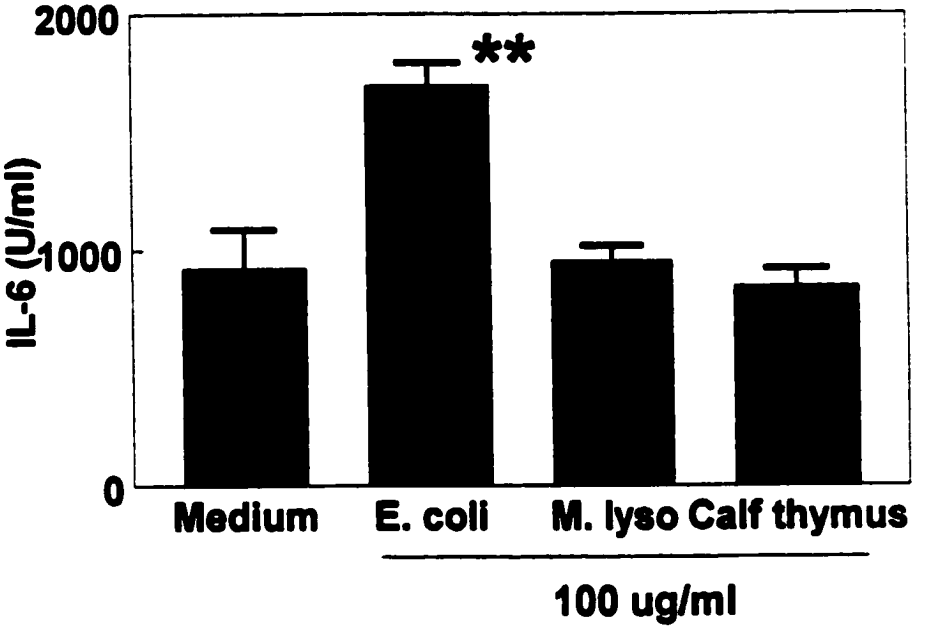


Figure 33

effects after being taken up by immune cells (143). Understanding how ODN are taken-up by different populations of immune effector cells may assist in the design of better methodology to selectively deliver DNA products. The present study examined the binding or entering of Texas red labeled 3CpG-ODN and 3GpC-ODN to mBMMC, and demonstrated that both immunostimulatory and control-ODN were bound to and taken up by mast cells with similar efficiency. mBMMC were examined by flow cytometry after incubation with Texas red labeled 3CpG-ODN and control-ODN for 30 min at 4 ^{O}C (binding assay) or for 6 h at 37 ^{o}C (internalization assay). In a binding assay, both Texas red labeled 3CpG-ODN (TR-3CpG) and control-ODN (TR-3GpC) had a similar percentage of positively labeled mBMMC (57.1% and 56.6%) and a similar mean channel fluorescence intensity data (7.95 and 7.96), indicating a close resemblance between 3CpG-ODN and the control-ODN in their binding to the mast cell surface.

Similarly, in internalization assays, no significant difference was observed between 3CpG-ODN and control-ODN. As shown in **Figure 34**, 3CpG-ODN uptake by mast cells was very similar to that of control-ODN. More detailed studies revealed a dose dependent increase in internalization of Texas red-labeled 3CpG-ODN and control 3GpC-ODN by mBMMC (**Table 12**). mBMMC incubated with 0.2 μM of TR-3CpG showed a positive labeling of 11 % and an arbitrary mean fluorescence intensity (MFI) of 8, in contrast to the positive labeling of 84 % and MFI of 27 for the cells incubated with 2 μM of TR-3CpG. Also, mBMMC incubated with 0.2 μM of Texas red-labeled control-ODN, TR-3GpC, showed a positive labeling of 8 % and an MFI of 7, in contrast to the positive labeling of 71 % and MFI of 23 for the cells incubated with 2 μM of TR-3GpC.

Figure 34. Typical graphs of flow cytometry results on mBMMC uptake of Texas red-conjugated oligodeoxynucleotides. 3CpG-ODN and 3GpC-ODN. mBMMC at 10 X 10^7/ml were incubated for 6 h at 37 ^{O}C with 2 μM of Texas red-conjugated 3CpG-ODN (*TR-CpG*) or Texas red-conjugated 3Gpc-ODN (*TR-GpC*) in the presence of 200 μM of unlabelled 3CpG-ODN (*TR-GpC-ODN+ CpG-ODN*). As a fluorescent background control, the cells were incubated in parallel in medium alone. mBMMC incubated with TR-CpG and TR-GpC show a marked shift of fluorescent intensity over the fluorescent background control in a very similar pattern. These are the representative data of four separate experiments.

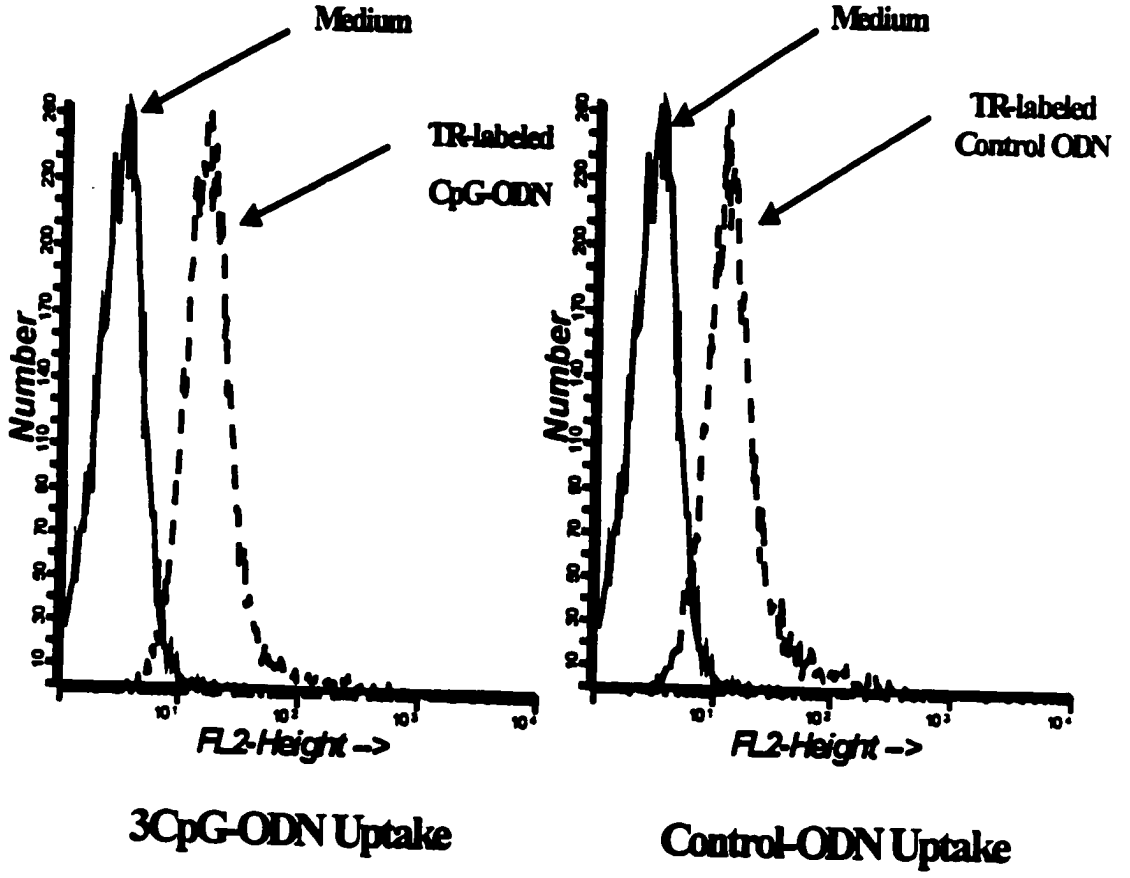

Medium
TR-labeled
CpG-ODN
Number
FL2-Height ->
3CpG-ODN Uptake
Medium
TR-labeled
Control ODN
Number
FL2-Height ->
Control-ODN Uptake

Figure 34

Furthermore, there was no substantial difference between Texas red-labeled 3CpG-ODN and 3GpC-ODN in their internalization by mBMMC over 6 h, as judged by the percent of positively labeled cells (11 % vs. 8%), the MFI (8 vs. 7) at a dose of 0.2 μM, the percent of positively labeled cells (84 % vs. 71%) and the MFI (27 vs. 25) at the dose of 2 μM. To explore the major pathways involved in the internalization of DNA by mast cells, we incubated mBMMC at 37 ^{o}C for 6 h with Texas red-labeled CpG-ODN in the presence or absence of excess unlabeled CpG-ODN. Uptake of Texas red-labeled CpG-ODN was not substantially blocked by a 100 fold excess of unlabeled-ODN based on the percentage of positive labeling (84% vs. 71%) and the MFI (27 vs. 28) (**Table 12**).

Table 12 . Uptake of oligodeoxynucleotides conjugated with Texas red by mBMMC*

	0.2 μM TR-3CpG	2 μM TR-3CpG	0.2 μM TR-Control	2 μM TR-Control	200 μM 3CpG + 2 μM TR-3CpG
% Positive cells	11	84	8	71	71
Mean Fluorescence Intensity	8	27	7	23	25

Note:

* These are representative data of flow cytometry on one of four separate experiments with mBMMC treated as in Figure 34.

TR-3CpG denotes Texas red-conjugated 3CpG-ODN.

TR-Control denotes Texas red-conjugated 3GpC-ODN.

Confocal microscopy of the mBMMC which had taken up either 3CPG-ODN or control-ODN revealed a similar pattern of cytoplasmic staining consistent with uptake by

Figure 35. A typical photograph of confocal microscopic examination of mBMMC after incubation with for 6 h at 37 ^{O}C with 2 μM of Texas red-conjugated 3CpG-ODN or Texas red-conjugated 3GpC-ODN as described in Figure 34. The uptake by mast cells was very similar for the two types of labeled oligodeoxynucleotides. The fluorescence intensity and intracellular distribution vary from cell to cell. The majority of the cells display patches of fluorescence in the peripheral areas of the cells, and small portion of the cells showed a strong and more even distribution of fluorescence over the whole cell.

Figure 35

a pinocytotic / endocytotic pathway (**Figure 35**). Most positively labeled cells showed a patchy peripheral distribution of fluorescence, suggesting an intracellular localization of these oligodeoxynucleotides, likely in the endosomes or lysosomes. A small number of cells showed an intense, but heterogeneous fluorescence covering almost the whole cells, indicating a possible additional nuclear distribution of the labeled oligodeoxynucleotides in these mast cells.

3. DISCUSSION

3-1. Mast cells contribute to innate immunity against bacterial infection

During the evolution of immunity, hosts have developed an efficient innate defense system to quickly recognize and respond to invading pathogens. This is done through discrimination of non-self pathogens from self by recognizing certain characteristics or patterns common to infectious agents (235). Immune cells, including macrophages, monocytes and neutrophils, are equipped with "pattern recognition receptors" (PRRs) on their cell surfaces capable of recognizing common structures present on the majority of pathogens. One example of such a structural pattern is LPS, which is present in all Gram negative bacteria. According to the "danger" theory proposed by Matzinger (236), LPS can serve as a signal to host defense systems, resulting in rapid mobilization of immune responses, namely innate immunity. The most well known PRRs for LPS are CD14 molecules, which are predominantly present on the macrophages, monocytes and neutrophils, and mediate immune responses by specifically binding to LPS (237). The rapid response to the invading pathogens is usually in the form of acute inflammation. Indeed,

LPS has been well documented to induce host cells, mainly monocytes/macrophages and neutrophils, to release numerous inflammatory mediators, including TNF-α, IL-1, IL-6, IL-8, nitric oxide, superoxide anions, and lipid derivatives (238).

Recently, increasing evidence suggests that unmethylated CpG motifs, characteristically present in bacterial DNA, can be recognized by the vertebrate immune system as a danger signal, resulting in an immediate immune response (239, 240). Synthetic oligodeoxynucleotides (ODN) with immune stimulatory effects are characterized by an unmethylated dinucleotide, cytosine followed by guanosine (CpG), flanked by two 5' purines and two 3' pyrimidines, a structure commonly seen in bacterial DNA, but rarely present in mammalian DNA (141, 143). These unique DNA structures are now regarded as a newly recognized common pattern structure associated with bacteria and other microbes (161, 241). It has been proposed that recognition and response to the CpG motifs by immune cells are mediated through "nucleic acid receptors", which can differentiate bacterial from "self" DNA and induce responses from immune cells, in a similar fashion as CD14 mediated response to LPS (161).

The results of from our studies clearly demonstrate that mast cells could participate in innate immunity against bacterial infection through a response to CpG motifs and the production of two cytokines associated with inflammation, TNF-α and IL-6 (**Figure 36**). TNF-α is known to enhance adhesion molecule expression on the vascular endothelium and thus enhance inflammatory cell recruitment. IL-6 is a critical cytokine in the initiation of the acute phase response and also critical to antibody formation through effects on plasma cell differentiation. Our results support the newly proposed concept that CpG motifs, like LPS,

are common pattern structures associated with bacteria, which can be recognized by immune effector cells, including mast cells.

3-2. Similar to other bacterial products, bacterial DNA and CpG-ODN induce cytokines but no degranulation from mast cells.

The time course of CpG-ODN induced cytokine production and the lack of preformed mediator release strongly suggests that bacterial DNA sequences can induce mast cells to produce IL-6 and TNF-α without the necessity for degranulation. These observations are in agreement with previous studies by Dr. Marshall's group that described degranulation independent of cytokine expression by mast cells in response to bacterial products (65, 66). In those studies, LPS and cholera toxin were shown to induce IL-6 but not histamine release from rat peritoneal mast cells (65, 66). These results together with current studies suggest that bacterial products are a group of unique biological stimuli for mast cells across several species, and can be useful tools for us to learn more about mast cell biology, especially in their relationship with bacteria. In the context of host defense to bacterial infection, our data suggest that mast cells may respond to invading bacteria mainly through releasing cytokines to recruit and activate other more "professional" phagocytes, particularly neutrophils and macrophages, and these events are independent of degranulation. However, mast cells may also respond to certain bacteria by releasing granule-associated mediators as reported by other researchers (2).

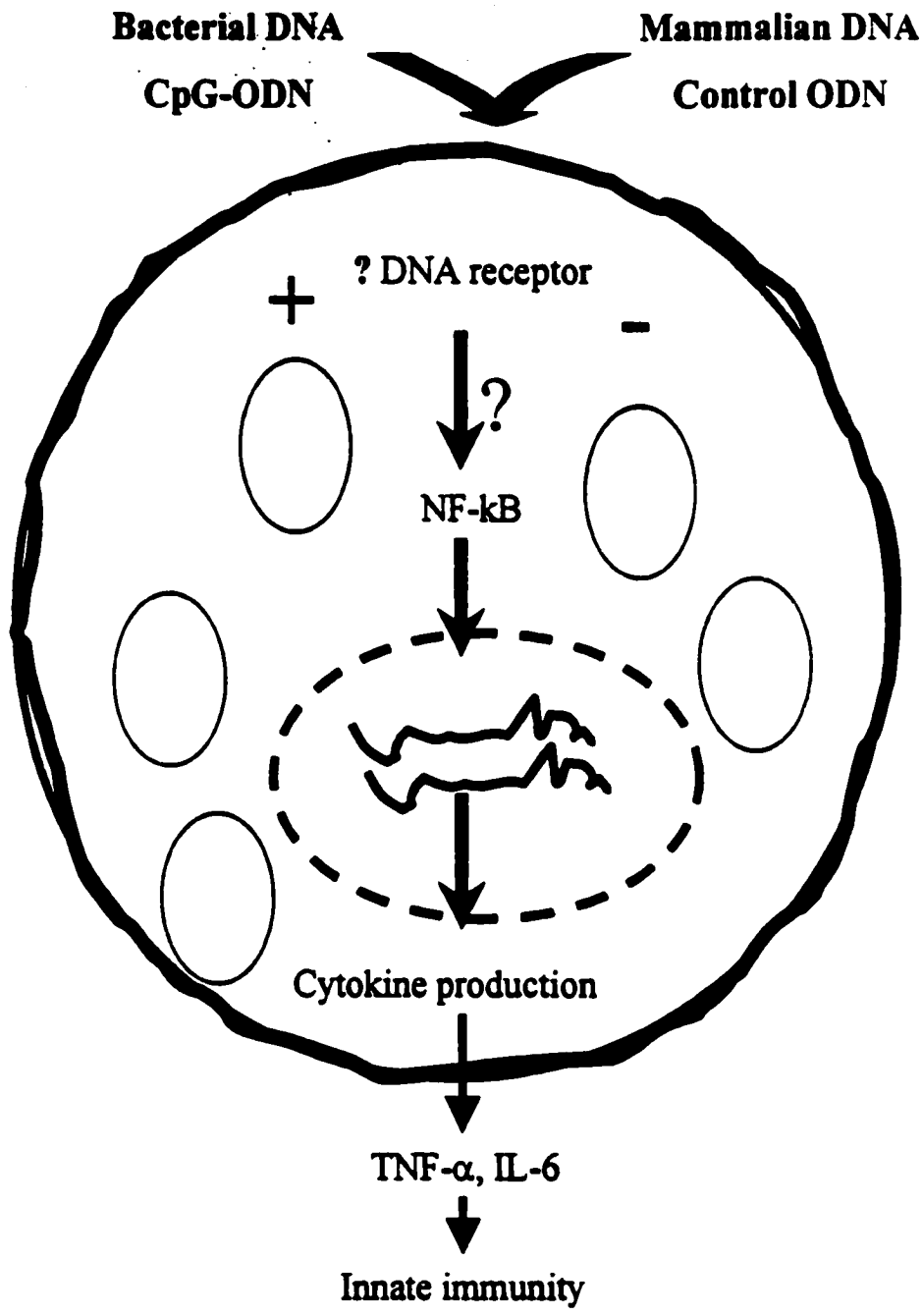


Figure 36. Model of DNA Induced Mast Cell Activation

Both bacterial and mammalian DNA, CpG-ODN and Control ODN can enter mast cells, but only bacterial DNA and CpG-ODN can be recognized as "danger" signals, possible by yet to be identified "DNA receptors". Through unknown steps of intracellular sigalling transduction, NF-κB is translocated to nucleus, leading transcription and translation of TNF-α and IL-6.

3-3. IL-6 and TNF-α are selectively induced in mast cells by CpG-OND and bacterial DNA

Based on the cytokine production we have measured, the effects of CpG-ODN on mBMMC are discriminatory rather than undifferentiated. We demonstrated that CpG-ODN induced significantly higher levels of IL-6 and TNF-α production from mBMMC, but not IL-4, IL-12, IFN-γ and GM-CSF, while previous studies by others and us have shown that activated mBMMC can produce substantial amount of all these cytokines (242). We also observed increased IL-6 but not GM-CSF production from KU812 cells activated with either CpG-ODN or bacterial DNA, while both cytokines were induced in this cell line by either A23187 or killed whole *Staphylococcus aureus* in our previous studies. On the other hand, bacterial DNA and CpG-containing oligonucleotides have been shown to induce IL-6, IL-12, IFN-γ and TNF-α, but not IL-2, IL-4, IL-5, and IL-10, production from murine spleen cells (147, 180). These results suggest that stimulatory DNA mainly induces pro-inflammatory cytokines (TNF-α and IL-6) and Th1 type cytokines (IL-2 and IFN-γ), but not Th2 type cytokines (IL-4, IL-5). Our data partly confirmed their results, that is, CpG-ODN induced mBMMC to produce proinflammatory cytokines, IL-6 and TNF-α but without particular bias toward either Th1 type (IFN-γ) or Th2 type (IL-4) cytokines.

Another major finding from the current studies was that the levels of induced IL-6 and TNF-α from mBMMC were directly proportional to the numbers of CpG motifs in CpG-ODN. The close relationship between the numbers of CpG motifs within a given sequence and its ability to selectively induce the production of TNF-α and IL-6 and the

complete lack of cytokine response from sequences containing GpC as controls suggests a highly specific and closely regulated cytokine induction mechanism.

3-4. DNA-induced mast cell activation may be primarily mediated through intracellular factors

The data from flow cytometry and confocal microscopic observation have shown that there is no significant difference between CpG-ODN and control-ODN in terms of their uptake by mBMMC, although there is significant difference between such sequences in term of their induction of cytokine production. These observations support the early prediction that the major signaling mechanisms mediating the immunostimulatory effects of bacterial and synthetic DNA occur following their uptake (239) and suggest that mast cells follow a similar pattern of activation by DNA to that observed in other cell types.

Uptake of oligodeoxynucleotides has been studied in cell types other than mast cells, however, there is conflicting data on whether the uptake of oligodeoxynucleotides is mediated by specific receptors on the cell surface (243-245). Two groups reported that radio- and fluorescence-labeled oligodeoxynucleotides were internalized into cells in a concentration and time dependent manner consistent with a pinocytotic mechanism, independent of cell surface receptors (243, 245). Another group reported that the internalization of oligodeoxynucleotides was mediated through "DNA receptors" on the cell surface (244). Our study showed a dose dependent uptake of CpG-ODN which could not be inhibited by a large excess of unlabeled ODN. These data suggest that mast cells may take

up ODN through a fluid phase pinocytotic mechanism rather than a receptor-mediated endocytotic pathway.

We did not observe marked differences in the degree of uptake of CpG-containing ODN and control ODN by flow cytometry. These observations suggest that the level at which the cell discriminates between immunostimulatory and irrelevant sequences is intracellular, potentially through interaction with transcription factors such as NF κB (246). To our knowledge this is the first observation to show that a similar level of internalization exists for both CpG-containing and non-CpG containing oligodeoxynucleotides, even though there was a marked difference in term of their potential to induce cytokine production.

Confocal microscopy further confirmed the intracellular localization of Texas red-labeled oligodeoxynucleotides. The distribution of the fluorescence in most cells was in a typical cytoplasmic pattern, especially in the peripheral areas of the cells. The observed intracellular localization was consistent with that of endosomes and lysosomes. However, a small proportion of the cells showed nuclear localization of the labeled oligodeoxynucleotides. The patterns of fluorescence distribution are similar for both CpG-containing and GpC-containing oligodeoxynucleotides. These morphological findings are complementary to those from flow cytometry, providing us with further evidence that mBMMC can internalize oligodeoxynucleotides, and there is no significant difference between CpG-containing and non GpC-containing oligodeoxynucleotides in their uptake by the mast cells.

In summary, we have examined the ability of immunostimulatory CpG-containing ODN and bacterial DNA to activate mouse bone marrow derived mast cells (mBMMC). Mast cells were treated with a range of doses of CpG-containing oligodeoxynucleotides or control oligodeoxynucleotides without CpG motifs. There was a dose-dependent increase in the production of both IL-6 and TNF-α by mast cells. The enhanced cytokine production was directly related to the number of CpG motifs within a given length of sequence. Neither CpG containing oligodeoxynucleotides nor non-CpG containing oligodeoxynucleotides induced degranulation of mast cells. Examination of the uptake of Texas red labeled CpG and non-CpG containing oligodeoxynucleotides revealed that they were both similarly taken up by the mBMMC despite the large differences in cytokine response.

CHAPTER 5

GENERAL DISCUSSION

Mast cells are major effector cells in allergic diseases, but also play a critical role in host defense against bacterial infection. Mast cells have also been shown to be involved in a number of other immunologic and inflammatory responses. Mounting evidence has indicated that mast cells play a prominent role in both innate and acquired immunity. Mast cells have many faces in terms of both their phenotypes and their functions. Despite an increasing awareness of the importance of mast cells for the body and an ever growing interest in studying these unique immune effector cells, we still have so much to learn about mast cells, especially the regulation of their cytokine expression and secretion. The current thesis studies sought to probe the mechanisms of inflammatory cytokine secretion by human and murine mast cells with two related approaches. The research focus, for the first stage of the research, was on the secretion routes, and for the second stage, on the induction of mast cell cytokines.

In our studies of mast cell secretion pathways, we found that KU812 cells and mBMMC secreted IL-6 and GM-CSF predominantly via a vesicular transport dependent pathway rather than a degranulation associated pathway. The human basophil/mast cell line KU812 produced a considerable amount of IL-6 without any stimulation, and this cytokine production was increased by more than 10 fold in cells activated with calcium ionophore A23187. Most spontaneously produced and A23187-induced IL-6 was secreted out of the cells, with only low levels of IL-6 remaining associated with cells. KU812 cells produce little GM-CSF without stimulation, but produce significantly higher levels of this cytokine when activated with A23187. Both IL-6 and GM-CSF, either spontaneously produced or A23187-induced, were blocked in their secretion by a protein

secretion inhibitor, BFA, which had no effects on the release of granule-associated histamine and β-hexosaminidase. These results indicate that IL-6 and GM-CSF were secreted by KU812 cells through a vesicular transport dependent pathway. Similar observations were also made in mBMMC, which produce no or little IL-6 and GM-CSF in their "resting' state but a large amount of the two cytokines when stimulated either by A23187 or the more physiologically relevant approach of IgE receptor cross-linking. BFA inhibited secretion of both the cytokines from mBMMC, suggesting IL-6 and GM-CSF were secreted by mBMMC through a similar pathway.

Of particular interest, from these initial studies, we have provided evidence to dispute a widely held concept that short term (20 min) release of mast cell mediators are always related to degranulation dependent processes. Increased IL-6 release, within 20 minutes of A23187 treatment, from KU812 cells was apparently not directly linked to degranulation and could be inhibited by BFA treatment. These studies have increased our understanding of the IL-6 and GM-CSF secretion pathways in mast cells, providing us with the suggestion that some of the other mast cell cytokines may also be secreted through similar pathways (**Figure 36**). The observations from these studies, and previous studies from our group, suggest that mast cell-derived cytokines may be released and play a role in immune responses, which are not commonly associated with mast cell degranulation. One such immune response is that of mast cells stimulated by bacterial components.

The second part of the studies described in this thesis were focused on cytokine induction in mast cells activated with bacterial DNA and CpG motif-containing

oligodeoxynucleotides. These studies have shown that mast cells can recognize and respond to bacterial and synthetic DNA by selective induction of IL-6 and TNF-α without degranulation. There are no similar observations made for mast cells in the literature, however, many published papers have reported that bacterial DNA and CpG-ODN directly induce a variety of cytokines, particular IL-6 and TNF-α, from other immune cells including macrophages, monocytes, and dendritic cells (239, 240). Mast cells share some key functions with these APCs, such as phagocytosis of bacteria and antigen presentation (247). It is not surprising that mast cells will respond to bacterium-related products such as DNA. However, in keeping with the observation that the ability for phagocytosis and antigen presentation by mast cells is weaker than that of "professional" APCs, higher doses of bacterial DNA and CpG-ODN were needed for mast cells than for macrophages and monocytes to induce similar levels of cytokines. We can speculate that since mast cells are in the front line of host defense at mucosal and skin sites, they may be in contact with a higher local concentration of bacterial products than some other immune effector cell populations.

The unmethylated CpG motif is the most critical structural component in the DNA that can activate mast cells, as the immunostimulatory effects were abolished with either methylation or replacement of the CpG sequence with a GpC sequence in the DNA products. The observations of DNA-induced cytokine production without degranulation are in line with reports that LPS induced IL-6 and TNF-α production but not histamine release from rat mast cells (65, 66). Stimulation of immune cells by both bacterial DNA and LPS involves activation of the transcription factor NF-κB (183, 248), underlining the

similarity in bioactivity between the two bacterial products. As natural products of bacterial breakdown, bacterial DNA and LPS may remain in the host and induce similar harmful effects, including septic shock (160, 248).

The length, backbone structure and concentration of oligodeoxynucleotides are contributing factors in DNA uptake (249), however, there is a lack of evidence to show any difference between immunostimulatory and non-immunostimulatory oligodeoxynucleotides in their rate of uptake by cells. Our current studies have shown that there was a dose dependent internalization of oligodeoxynucleotides by mBMMC, but there was no significant difference between CpG-ODN and control ODN in their binding or uptaking by those mast cells. These observations that the major factor(s) mediating DNA-induced mast cell activation and discriminating pathogen associated "CpG" sequences from irrelevant sequences may be inside rather on the surface of mast cells.

1. MAJOR LIMITATIONS TO THESE STUDIES

1-1. Methodical limitations in studying cytokine secretion studies

Beside the two classical secretion pathways (regulated and constitutive), a third secretion pathway has been proposed by Dvorak *et al*, termed "piecemeal degranulation" (250). This unique secretion model features decreased condensation of the cytoplasmic granules and has been observed in basophilic and mast cells from a variety of species based on morphological evidence (250, 251). In contrast to the explosive release of the granule contents in the event of typical mast cell degranulation, piecemeal degranulation

is a slow and often incomplete release of the granule contents. Unlike the vesicle dependent secretion pathway, piecemeal degranulation is induced by stimulants, such as phorbol ester. Since BFA appears to have no effect on the release of preformed granule mediators at either 20 minutes post activation or at later time points, it is unlikely that piecemeal degranulation is responsible for the observed cytokine release. However, without formal studies of the effect of BFA on the piecemeal degranulation process, the possibility cannot be excluded, that some cytokines may be secreted through this pathway. Although it is has been suggested that each secretory pathway carries different products (116, 227), a small proportion of IL-6 in KU812 cells may possibly be secreted via non-vesicle dependent pathways as IL-6 release was not completely inhibited by either BFA or monensin treatment.

Phosphatidylinositol 3-kinase (PI3-kinase) is an important enzyme for intracellular trafficking, and its products have been shown to increase after mast cell activation. Wortmannin, a PI3-kinase inhibitor, has been shown to inhibit mast cell degranulation from mBMMC and RBL-2H3, without effects on secretion of IL-6 and leukotriene C4 from mBMMC (218, 252). These results indicated that mast cell degranulation and cytokine secretion could be controlled by different mechanisms, and possible through different secretion pathways. This agent was used in some preliminary experiments by us to examine its effects on IL-6 secretion and degranulation by KU812 cells. Compared to BFA, wortmannin has much broader effects on cellular activities other than secretion, including actin polymerization, proliferation and several signaling pathways (253). These effects lead to problems with data interpretation and with IL-6

bioassay inhibition. However, further information concerning the precise mechanisms of cytokine secretion would be an important objective in future studies in this area.

The current studies of mast cell cytokine secretion are related to some very important biological functions of the cell, i.e. intracellular transport and secretion of protein products. The research in these areas in other cell types is making rapid progress, leading to new knowledge and new questions to be answered. According to one of the new models for intracellular trafficking of transport vesicles, there are two main types of coating proteins to cover these membrane-bound vesicles, clathrins and coatomers. Clathrin-coated vesicles primarily mediate the protein transport between Golgi complex and cytoplasmic membrane, and coatomer (COP1 or II)-coated vesicles mainly mediate the trafficking between Golgi complex and ER (254). BFA affects the formation of COPI-coated vesicles, leading to the collapsing of Golgi complex into ER (255). No definite evidence is available on whether the cytoplasmic granules in mast cells are coated with clathrins. Different coating proteins are under different controlling mechanisms (254), however, these mechanisms were not vigorously investigated in our studies of the cytokine secretion by mast cells.

Immunohistochemistry in combination with electron microscopy is a useful tool to demonstrate the intracellular location of cytokines in mast cells. Based on this method, TNF-α and IL-6 have been found present in the cytoplasmic granules, providing morphologic evidence for the existence of preformed cytokines stored in mast cells (72, 256). It would provide collaborating evidence to the data from current studies if IL-6 and TNF-α could be immunohistochemically localized in the small transport vesicles but not

in the granules in KU812 cells and mBMMC at ultrastructural level. However, immunoelectron microscopy is technically challenging for many investigators, including myself. The major potential difficulty for this method is the loss of immunoreactivity of intracellular contents due to the harsh fixation and cell processing procedures. However, such problems may be overcome with proper techniques. A recent report has shown that microwave-assisted fixation in standard glutaraldehyde greatly enhances detection of TNF-α in mast cells by immunoelectron microscopy (256). It would certainly worth trying to use this simple fixation method for any future studies with immunoelectron microscopy on mast cells.

1-2. Limitations in studies of DNA-induced cytokine production studies

1-2-1. Cell source and scope of experiments

Most of the data from the current studies were from experiments on mBMMC, but these murine bone marrow derived mast cells are not 100% pure. Contaminating cells could also produce cytokines and confuse interpretation of our data. The easiest solution to this problem is to use mast cell lines, such as RBL-2H3 and MC/9, both of which are rodent mast cell lines, showing characteristic mast cell bioactivity, i.e., activation via an IgE dependent pathway. In addition to their purity, another advantage for using cell lines is an easy supply of mast cells for study. Human cord blood cultured mast cells and human mast cell line HMC-1 could be used to confirm the observations made in the current studies of DNA-induced activation of KU812 cells. Data from the cord blood-derived mast cell would be especially helpful to confirm the mast cell response to

bacterial and synthetic DNA in the human system. Freshly isolated and purified rat or mouse peritoneal mast cells could also be used for studying a DNA-induced mast cell response. Satisfactory levels of purity of mast cells may be achieved by combining culture methods with routine Percoll purifying methods, magnetic activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS).

Although mBMMC are primary cultured cells very similar to naturally developed mast cells residing in the tissues, they are an immature mast cell type. Mast cell lines, such as the one used in the current studies, KU812, are further away from resembling "real" mast cells due to their tumor nature. Ideally experiments on mast cells like the ones in current thesis studies should be carried out on freshly isolated, highly purified mast cells. But freshly isolated mast cells have their inherent problems, i.e., contamination with other cells and possible damage or changes of the mast cell surface structures and the state of activity resulting from the isolation process. However the results from the studies on these cells do provide us with clues as to what might happen to normal mast cells in the tissue. A further approach may be to use human cord blood derived mast cells.

Due to time limits, the results from our current *in vitro* studies on DNA-induced cytokine production from mast cells are not yet confirmed by *in vivo* studies. The mast cell deficient W/W^V mouse may provide an appropriate model, by which the role of mast cells in the response to bacterial DNA could be assessed in vivo in future studies in this area.

1-2-2. Oligonucleotide uptake

There is controversy concerning whether the uptake of oligodeoxynucleotides is Ca^{2+} dependent (257). Stein *et al.* (245) demonstrated that the binding of a 15-mer phosphodiester oligodeoxynucleotides to a human cell line HL60 was Ca^{2+} dependent, while its phosphorothioate analogue of the same sequence was not. Hartmann *et al.* (245) reported that the uptake of phosphorothioate oligodeoxynucleotides into whole blood granulocytes was strongly dependent on extracellular calcium. In the present study, repeated experiments have been shown a trend that the internalization of the 20-mer phosphorothioate oligodeoxynucleotides is likely Ca^{2+} dependent (**Appendix 12**), however more vigorous tests are needed to verify this issue with more certainty.

One group of researchers reported that oligodeoxynucleotides uptake by human peripheral blood cells was mediated via Mac-1 (CD11b/CD18, αM/β2), a member of leukocyte integrin family (187). They showed that antibodies to Mac-1 prevent 15-mer oligodeoxynucleotides from binding to the human leukocytes at 4 °C for 20 min and from being internalized by the cells at 37 °C for 6 hours. Our preliminary data have consistently showed that a hamster anti-mouse Mac-1 monoclonal antibody (a gift from Dr. Thomas Issuketz, Dalhousie University, Canada) inhibited internalization of oligodeoxynucleotides into mBMMC (**Appendix 12**), however, the appropriate control IgG also showed considerable inhibition. This might be due to the presence of other antagonists for DNA uptake in the antibody preparations. With the availability of anti-Mac-I antibodies and control immunoglobulins from other sources, this issue should be further studied with reference to the observations of Benimetskaya *et al.* (187). Mac-1 has

been shown to be expressed on bone marrow-derived mast cells from a C57Bl/129Sv mice by flow cytometry (258), with properly designed experiments an answer can be found as to whether Mac-1 molecules are indeed receptors for oligodeoxynucleotides in mast cells.

2. CLINICAL IMPLICATIONS

2-1. Modulation of mast cell cytokine production

Several strategies have been proposed to suppress the adverse effects of cytokines: 1.) to inhibit synthesis; 2.) to block secretion; 3.) to neutralize cytokines; 4.) to block cytokine receptors; and 5.) Inhibit signal transduction (259). Current thesis studies have shown that mast cell cytokine production can be blocked by reagents that specifically targeted to their secretion routes. This could have therapeutic potential in situations such as chronic allergic airway inflammation, where mast cell-derived cytokines may play an important role in the development of disease pathology. The same treatment could also be used to block cytokine secretion from other types of immune cells, because the vesicle transport pathway is universal across all kinds of mammalian cells. However, broad blockade of this type is highly toxic to cells over hours or days of treatment. Sodium cromoglycate and nedocromil sodium, known as "mast cell stabilizers", are important drugs used in treatment of allergic diseases. These compounds have been shown to block cytokine secretion as well as degranulation by mast cells (260). The challenge will be to develop selective non-toxic drugs that target cytokine secretion pathways.

2-2. Mast cells' role in innate immunity

The present studies have shown that bacterial DNA and CpG-ODN induced cytokine secretion but not degranulation from mast cells, in a similar fashion as observed in response to a more commonly studied bacterial product LPS, which induced mast cell IL-6 and TNF-α production without degranulation. Mast cells have been shown to play a central role in host defense against bacterial infection in two recent reports published in *Nature* (5, 6). One key component in such mast cell-initiated protective mechanisms is the proinflammatory cytokine, TNF-α, released from mast cells shortly after they are exposed to bacteria. TNF-α functions to recruit and activate "professional" phagocytes (macrophages and neutrophils) and other immune effector cells (T cells and B cells). IL-6, another inflammation-related cytokine, also plays a significant role in fighting bacterial infection. In our studies, both IL-6 and TNF-α have been induced from mast cells by bacterial DNA and CpG-ODN. The mast cells' response to CpG containing oligodeoxynucleotides could play a role in the mast cell response to bacterial infection along with other potent mast cell activators such as complement components and LPS. It remains to be determined what role CpG sequences might play in responses to viral, protozoal pathogens for which the mechanisms of immune mobilization are less well delineated.

2-3. Role of mast cells in DNA based vaccination

Mast cells are strategically located in the body at the interface between external and internal environment. The ability to target local, resident mast cell populations

through CpG activation may allow more localized therapeutic strategies to be developed in mast cell rich sites such as the skin and airways. In the context of DNA-vaccination, the observations of CpG induced mast cell cytokine production may explain some of the tissue specific differences in responses to DNA vaccination approaches. It is notable that mast cell rich sites such as the skin have been shown to be particularly good sites for DNA vaccination protocols (261). In the context of immunomodulation of allergic inflammation, mast cells may contribute redirection of immune response to allergens to a non-IgE dominant response. In an *in vivo* study with a murine model of asthma, Kline *et al.* have demonstrated that co-administration of *Schstosoma mansoni* egg antigens and CpG-ODN could suppress IgE production, eosinophil recruitment to the airways, and bronchial hyperreactivity (262). In another murine model of asthma, Sur *et al.* showed that ragweed-induced IgE production and allergic airway inflammation could be prevented by intratracheally injecting the sensitized animals with CpG-ODN, and such prevention could last over 6 weeks (263). The fact that a large number of mast cells reside in the lung tissue give us reason to believe that mast cells may be involved in CpG-ODN mediated immunomodulation in those animal models, although neither of these groups looked into the role specifically played by mast cells.

3. FUTURE POTENTIAL RESEARCH WORK

3-1. Confirmation of the observations on DNA-induced responses in mast cells with *in vivo* experiments

In vivo experiments have been frequently carried out to confirm the findings from *in vitro* experiments. One logical choice (but not the only one) for the next stage of current studies is to examine the response in animal models treated with DNA products. One common approach to assess the mast cell function is to compare responses in normal and mast cell deficient mice or rats, and to compare the results from mast cell deficient mice with those from mast cell deficient mice reconstituted with bone marrow-derived mast cell (264). Two widely used mast cell deficient mice are W/W^V mice and Sl/Sl^d mice with mutations at both copies of genes for c-kit receptor and stem cell factor, respectively. Parallel experiments can be performed in mast cell deficient W/W^V mice, wild type control mice and mast cell-reconstituted W/W^V mice, and the profiles of DNA-induced cytokine expression can be compared among the three types of mice. Based on the results of our studies, it may be predicted that TNF-α and IL-6 levels would be lower in W/W^V mice than in normal control mice, and the levels of the two cytokines in mast cell-reconstituted W/W^V mice would be comparable to those in the control mice.

3-2. Further investigation of cytokine secretion mechanisms in mast cells

The study of cytokine secretion mechanism in mast cells has been a hot subject for at least 10 years, and is still been vigorously pursued in many laboratories specialized in mast cell research in the world. Confocal microscopy combined with fluorescent immunostaining is a powerful and increasingly popular technique that could be very useful in studying cytokine secretion by mast cells. Fluorescence labeled or unlabeled antibodies to a wide range of cytokines are easily available, can be used to detect intracellular contents of

cytokines in the mast cells. Confocal microscopy study can provide direct evidence of the presence or absence of the cytokine in question. This method is easy to conduct and quick to obtain both qualitative and quantitative data about mast cell cytokine. BFA and monensin used in current thesis studies can be used in the experiments to facilitate identifying intracellular cytokine. These two protein inhibitors have been shown by us and others to increase accumulation of cytokines inside cells while having minimal effects on cytokine synthesis. Flow cytometry on intracellular cytokines, another relatively new technique, may also be used to examine cytokine secretion by mast cells. One advantage for this method over confocal microscopy is that large numbers of samples / cells can be examined at the same time. Another advantage is the feasibility for double or multiple labeling of cells. This is particularly useful when examining samples containing mixed cell populations.

In our current studies, we found an ionophore-induced increase in the short term (20 min) release of IL-6 from KU812 cells, which was not related to granule release. Such an observation is in contrast with a common concept regarding degranulation-associated release of mast cell mediators, and is also in conflict with the traditional view that "regulated", but not "constitutive" secretion, can be accelerated with stimuli. It would be worthwhile to confirm our observation in other cytokines in different types of mast cells. We may find some novel secretion mechanisms operate in unique cells like mast cells.

3-3. Investigation of the possible role of mast cells in DNA based vaccination

Bacterial DNA and CpG-ODN have been used as adjuvants to boost immune response to tumors and pathogens, including viruses and parasites (265). However, there

is no information available on the role played by mast cells in these enhanced immune responses. Considering the large numbers of mast cells throughout the body, residing near the vaccination routes, in the skin, airways and gastrointestinal mucosa, and the increased numbers of mast cells around many tumors including breast cancer and colon cancer (266, 267). It is reasonable to speculate that mast cells may play a significant role in tumor and pathogen vaccination. The proof for such speculation may come from *in vivo* experiments. One simple experimental design would be injection of CpG-ODN together with tumor or pathogen antigens into skin of W/W^V, W/W^+ and mast cell-reconstituted W/W^V mice, then examine cytokine and immunoglobulin levels in the serum after rechallenge with the same antigens 3 to 4 weeks later. A close relationship between any particular cytokine or immunoglobulins and the deficiency or abundance of mast cells in the experimental mice would indicate a possible role of mast cells in DNA-induced or enhanced immune responses.

3-4. Oligodeoxylnucleotide uptake

There is a general consensus in the literature that oligodeoxynucleotides are taken up by cells through endocytosis. However, the agreement ends here. There are conflicting data on whether oligodeoxynucleotide uptake is mediated through specific DNA receptors on the cell surface, and whether extracellular Ca^{2+} and Mg^{2+} ions are needed. Bennett *et al.* reported that internalization of oligodeoxynucleotides by human leukocytes was mediated through a putative DNA receptor of 30 kD molecular weight (268). In contrast, Yakubov *et al.* suggested that oligodeoxynucleotides at high concentration were

internalized mainly through liquid phase endocytosis, known as pinocytosis, and oligodeoxynucleotides at low concentration were primarily taken up via absorptive endocytosis (243). The integrin molecule Mac-1 has been demonstrated by one group to mediate the uptake of oligodeoxynucleotides by human PBMC (187) and by rat liver endothelial cells (269). Scavenger receptors have been suggested to facilitate the uptake of oligodeoxynucleotides by rat kidney parachymal cells (270). By using specific antibodies to Mac-1 or scavenger receptor, it would be possible to determine whether either of these receptors mediates oligodeoxynucleotide uptake by mast cells, which can also be examined by flow cytometry after incubation with labeled oligodeoxynucleotides for up to several hours.

3-5. Possible signaling mechanisms involved in DNA-induced activation of mast cells

The immunostimulatory effects of CpG-ODN and bacterial DNA have become increasingly recognized over the last few years. Investigation of the mechanisms of action by DNA products is just beginning. Some studies have suggested that CpG-mediated activation of immune cells is independent of protein tyrosine kinases, protein kinase A and protein kinase C, but could be blocked by reagents that interrupt the intracellular pH balance and by the inhibitors to the generation of reactive oxygen species (246, 271). Both bacterial DNA and CpG-ODN have been demonstrated to activate transcription factor NF-κB (183). But there is a lack of evidence linking the preceding events triggered by DNA to NF-κB activation. Therefore, it is important in the next stage of studies in this area to investigate the signaling pathways leading to mast cell activation by CpG-ODN or

bacterial DNA. The targets for such studies could include PLC, IP3 and MAP-K, all of which have been involved in signal transduction of IgE dependent mast cell activation (see **Figure 2**).

4. CONCLUSIONS

The present studies have demonstrated that the initial 20 min release of IL-6 from ionophore activated differentiated KU812 cells was inhibited by BFA and monensin, two protein secretion blockers which had no effect on degranulation, suggesting that a mechanism exists for an initial 20 min induced release of IL-6 that is not degranulation dependent. The studies have also shown that the ongoing release of both GM-CSF and IL-6 over up to 24 h from a human mast cell/basophilic cell line and murine bone marrow derived mast cells was inhibited by BFA and monensin. These observations suggest that the major secretion route for these two cytokines is a vesicular transport dependent pathway, the traditional protein secretion route, rather than through the degranulation-related pathway. GM-CSF and IL-6 secretion mechanisms appeared to be similar in IgE receptor mediated activated cells and ionophore activated cells. The present work further supports the growing evidence that mast cells' secretion of cytokines is under complex regulation and that pharmacological regulation of such cytokine expression in disease will require a different approach from the regulation of mast cell degranulation.

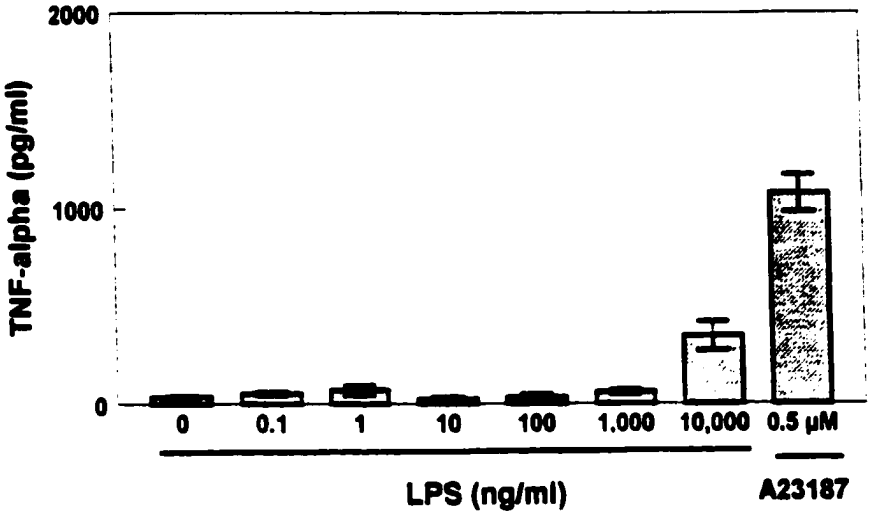
We have also examined the ability of immunostimulatory DNA sequences known as CpG motifs, which are found in increased quantities in bacterial DNA, to activate mBMMC and differentiated KU812 cells. Mast cells were treated with a range of doses of CpG-

containing oligodeoxynucleotides or control oligodeoxynucleotides without CpG motifs. There was a dose-dependent increase in the production of both IL-6 and TNF-α by mast cells treated with the CpG-containing oligodeoxynucleotides. The cytokine levels induced were directly related to the number of CpG motifs within a given length of sequence. Neither CpG containing oligodeoxynucleotides nor non-CpG containing oligodeoxynucleotides induced degranulation of mast cells. Examination of the uptake of Texas red labeled CpG and non-CpG containing oligodeoxynucleotides revealed that they were both similarly taken up by the mBMMC. Our studies have demonstrated that mast cells can recognize and response to CpG motifs presents in bacterial and synthetic DNA by selective induction of IL-6 and TNF-α without degranulation. These results have important implications for the mechanism by which mast cells respond to bacteria and for the role of mast cells in response to DNA vaccination.

Overall, these studies have provided new information concerning the mechanisms of inflammatory cytokine secretion and identified a novel stimulus for the selective induction of production of these mast cell cytokines. Although many further studies remain to be done, it is hoped that these findings will contribute to our future understanding of the role and regulation of the mast cells in host defense and disease.

APPENDICES

Appendix 1. TNF-alpha production by mBMMC in response to LPS stimulation



mBMMC at $1X10^6$ cell/ml were treated with different doses of LPS at 37 °C for 6 h, using culture conditions and supplemented media as described for CpG-ODN activation of mast cells in Chapter 2. Supernatants were collected to measure TNF-α bioactivity by L929 cytotoxicity assay. N=4. Note the dose dependent increase in TNF in response to LPS activation at high doses.

Appendix 2. Protocol for β-hexosaminidase assay

1. Wash cells in culture medium 2 times by centrifuging @ 300 g at 4 ^{0}C for 10 min.
2. Resuspend the cells with Hepes Tyrode buffer (HTB), put on ice.
3. Prepare all reagent solutions with HTB, transfer the solutions to the designated tubes
4. Add cell suspension to tubes with the final concentration of 10^6 cell/ml
5. Incubate the tubes in 37°C water bath for 20 minutes
6. Spin the tubes @ 300 g for 5 min, collect supernatant, resuspend the pellets with HTB
7. Disrupt the pellets by repeated freeze/thawing or sonication (check under microscope for complete cell disruption), spin down cell debris @500 g for 3 min.
8. Transfer 50μl/well of supernatant/pellet samples in duplicate into a 96-well plate, and transfer 50μl/well of HTB in duplicate as control
9. Add 50μl/well of 1mM p-nitrophenyl-N-acetyl-β-D-glucosaminide dissolved in 0.1M citrate buffer
10. Incubate the plate in 37 °C for 1 hr
11. Add 200μl/well of 0.1M carbonate buffer to stop the reaction
12. Read the plate at 405 nm
13. Calculate release rate by the following formula:

$$\% \text{ release} = \frac{\text{O.D. supernatant} - \text{O.D. control}}{(\text{O.D. supernatant} - \text{O.D. control}) + (\text{O.D.pellet-O.D.control})} \times 100$$

(References: Schwarz LB et al. J Immunol 123: 1445-1450,1979)

Solution preparation

1. 0.1 M citrate buffer (pH4.5)

   1.114g Citrate acid (MW=230.14)

   1.368g sodium citrate (MW=294.12

   100ml dH_20

   -Mix and adjust pH to 4.5

2. 1mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NAG)

   34.2mg p-NAG (Cat. # N9376, Sigma)

   100ml 0.1 M citrate buffer

   Stir to dissolve; cover the bottle with aluminum foil, and store in the cold room after use

3. 0.1M carbonate buffer (`pH 10.5)

   1.060g Na_2CO_3 (MW=105.99)

   0.840g $NaHCO_3$ (MW=84.01)

   100ml dH_20

4. Hepes Tyrode Buffer (pH7.35)

   4.0g NaCl

   0.5g glucose (aka Dextrose)

   0.5g BSA (bovine serum albumin)

   1.19g Hepes

   0.1005g KCl

   0.0735 $CaCl_2.2H_20$ (dihydrate)

300ml dH_20

-Stir to dissolve, then add the following:

0.276g $NaH_2PO_4H_20$ (monobasic)

100ml dH_20

-Stir to dissolve, and adjust pH

-Adjust osmolality to 300 3m0sm by adding more dH_20 calculated as following:

-Use the buffer within one week of preparation

-1.24 ml of dH_20 for each m0sm over the required osmolality

Appendix 3. Protocol for ELISA of human GM-CSF

Day one

1. Coat 96-well plate with 50 µl/well of 1µg/ml mAb X hGM-CSF, incubate at 4°C O/N.

    5 µl mAb against hGM-CSF (ZM-213, Genzyme)

    5 ml Borate buffer (pH8.35)

Day two

2. Wash plate with PBS (pH7.4) X4

3. Block with 50 µl/well of 1% BSA/0.1%TW20/PBS at 37°C X 1 hr

4. Wash plate with PBS X 4

5. Add 50 µl/well of samples and standards, incubate at 4°C overnight (13-18h)

    Standards prepared as following:

Standard #	Concentration (pg/ml)	Preparation
1	2000	1µl GM-CSF stock + 500µl 0.2%BSA/0.005%TW20/PBS
2	500	100µl std #1 + 300µl 0.2%BSA/0.005%TW20/PBS
3	125	100µl std #2 + 300µl 0.2%BSA/0.005%TW20/PBS
4	31	100µl std #3 + 300µl 0.2%BSA/0.005%TW20/PBS
5	8	100µl std #4 + 300µl 0.2%BSA/0.005%TW20/PBS
Blank	0	300µl 0.2%BSA/0.005%TW20/PBS

Day three

6. Wash plate with PBS X 4

7. Add 50 µl/well of 0.1 µg/ml biotinylated mAB against hGM-CSF, incubate at 37°C 1 hr.

    (25 µl biotinylated mAb against hGM-CSF (M-501-B, ENDOGEN)

    5 ml 0.3%BSA/0.02%TW20/PBS)

8. Wash plate with PBS X 4

9. Add 50 µl/well of 1/2000 streptavidin-alkaline phosphotase, incubate at RT X 30 min.

(2.5 µl strep-AP (19542-018, GIBCOBRL)

5 ml 0.3%BSA/0.02%TW20/PBS)

10. Wash plate with 0.05M TBS (pH 7.5) X 4

11. Add 50 µl/well substrate solution, incubate at RT X 30min

12. Add 50 µl/well of Amplifier solution, incubate at RT X 10 min 13. Add 50 µl/well of stopping solution, 0.3 M H_2SO_4.

14. Scan plate on an ELISA reader at 495 nm.

Appendix 4. Protocol for Binding Assays with Texas-Red Labeled Oligonucleotides

1. Collect mast cells from culture flask, spin down cells at 300g at 4 ^{O}C for 15 min.
2. Resuspend cells in cold 0.2%NaN_3/2%BSA/PBS at a concentration of 10^7 cell/ml, set on ice.
3. Add 50 μl of the cell suspension to each of a 1.5 ml Eppendorf tube, and add 1.3 μl of Texas-red labeled oligonucleotide stock solution (conc.=500 μg/ml) to each tube (working conc.=12.8 μg/ml or 2 μM).
4. Mix by finger flipping the Eppendorf tubes, incubate the tubes at 4 ^{O}C for 30 min.
5. Add 0.5 ml cold 0.2%NaN_3/2%BSA/PBS per tube, and wash cells at 1000 g at 4 ^{O}C for 5 min, followed by washing two more times.
6. Fix cells with 250 μl of cold 1% paraformaldehyde/PBS per tube for 30 min. The cells can be examined with flow cytometry or confocal microscopy right away or can be kept at 4 ^{O}C for up to 3 days before the examination.

Note.

- For inhibition treatment, including excess unlabeled oligonucleotides, EDTA, anti-CD18, and Cytochalasin D, the cells were incubated with these inhibitors at 37 ^{O}C for 30 min before adding Texas red-labeled oligonucleotides.
- For flow cytometry examination, cytospin slides can be mounted with PBS and the coverslip can be sealed with nail-polish.

Solution preparation:

1. 0.2%NaN_3/2%BSA/PBS

NaN_3	100 mg
BSA	1 g
PBS	50 ml

2. 0.4 M acetic acid/1M NaCl (pH 2.5)

Glacial acetic acid	2.3 ml
NaCl	4 g
H_2O	98 ml

3. 1% paraformadelhyde/PBS

Paraformaldehyde	0.5 g
PBS	50 ml

Appendix 5. Protocol of Internalization Assay with Texas-Red Labeled Oligonucleotides

1. Collect mast cells from culture flask and spin down cells at 300 g at 4 ^{O}C for 10 min.
2. Resuspend cells in cold 10%BFS/RPMI-1640 at a concentration of 10^7 cell/ml, set on ice.
3. Add 50 μl of the cell suspension to each of a 1.5 ml Eppendorf tube, and add 1.3 μl of Texas-red labeled oligonucleotide stock solution (conc.=500 μg/ml) to each tube (working conc.=12.8 μg/ml or 2 μM).
4. Mix by finger flipping the Eppendorf tubes, incubate the tubes at 37 ^{O}C for 6 h.
5. Add 150 μl of cold 0.2%NaN_3/2%BSA/PBS to each tube, followed by adding 200 μl of cold 0.4 M acetic acid/1M NaCl in each tube, set on ice for 10 min.
6. Spin at 1000 g at 4 ^{O}C for 5 min, followed by washing two more times in 0.5 ml of cold 0.2%NaN_3/2%BSA/PBS per tube at 1000 g at 4 ^{O}C for 5 min. Discard the solution.
7. Fix cells with 250 μl of cold 1% paraformaldehyde/PBS per tube for 30 min. Then the cells can be examined with flow cytometry or confocal microscopy right away or can be kept at 4 ^{O}C for up to 3 days before the examination.

Appendix 6. Protocol for Pulse-Chase Experiment

1. Harvest ~ 50 X 10^6 differentiated KU812 cells by spinning at 300 g X 10 min @RT
2. Re-suspend pellet with complete RPMI 1640 medium as 2 X 10^6 /ml, put on ice.
3. Set up 3 treatment groups (n=4) in complete RPMI (Experimental) medium at 3 X 10^6 cells / 3 ml / tube, and incubate at 37°C for 6 or 12 hrs as following: a.) Medium; b.) BFA 0.1μg/ml; c.) cyclohexamide 10μg/ml
4. Mix with frequent inversion of tubes. Group 2 contains 0.1μg/ml BFA, and Group 3 contains 1 μg/ml cyclohexamide.
5. Spin at 300 g X 10 min @ 4°C, re-suspend pellets with 1.5 ml L-leucine-deficient-RPMI medium, transfer the suspension to a 1.5 ml Eppendor tube, and incubate at 37°C water-bath for 1 hr.
6. Spin with 300 g X 5 min at RT, re-suspend cells in 0.5 ml / tube of RPMI medium plus 20 μCi / ml [^{3}H] L-leucine, incubate in 37°C water-bath for 1 hr. Mix with frequent reversion of tubes. Prepare solutions as below: a.) 2.5 ml medium + 10μl stock [^{3}H] L-leucine; b.) 2.5 ml 0.1μg/ml BFA + 10μl stock [^{3}H] L-leucine; c.) 2.5 ml 10μg/ml cyclohexamide + 10μl stock [^{3}H] L-leucine
7. Spin at 300 g X 5 min @ RT, re-suspend cells in 1 ml / tube complete RPMI medium, incubate in 37°C water-bath for 30 min. Mix with frequent reversion of tubers. Group 2 contains 0.1μg/ml BFA, and Group 3 contains 10 μg/ml cyclohexamide.
8. Spin at 300 g X 5 min @ RT, collect supernatant to be stored at □20°C, and re-suspend cells in 1 ml / tube complete RPMI medium
9. Spin at 300 g X 5 min @ RT, discard supernatant, resuspend with 1ml/tube cold PBS.

10. Add 50 µl of pellet suspension to a 1.5 ml Eppendorf tube with 0.5 ml 0.01% BSA / 0.02 NaN_3,. Set up two 1.5 ml Eppendorf tubes with 0.5 ml 0.01% BSA / 0.02% $NaN_{3,\ and}$ transfer 50 µl of leftover of [^{3}H] L-leucine containing medium to the two tubes as [^{3}H] background counting. Set all tubes on ice.

11. Add 0.5 ml cold 20% TCA, mix well by vortex, and put on ice for 30 min.

12. Spin at 1,000 g for 10 min @ RT; discard supernatant, and re-suspend pellets with 1 ml cold PBS.

13. Repeat step 11 once, but re-suspend pellets with 1 ml / tube Ecolit scintillation fluid, and transfer suspension to scintillation vials. Rinse the Eppendorf tubes twice with 1 ml / tube scintillation fluid, and transfer the fluid to corresponding vials (final volume = 3ml/tube).

14. Add 50 µl of leftover of [^{3}H] L-leucine containing medium to 3 ml / vial scintillation fluid in 2 vials as 100% radioactivity samples. Measure radioactivity on the scintillation counter.

Appendix 7. RT-PCR Protocol for Human Cytokine mRNA Expression

Synthesize cDNA by R T (20 μl reaction)

1. Prepare RT master-mix in a 1.5 ml Eppendorf tube as following:

		For 11 samples
H_2O	9 μl	99 μl
5 mM 4dNTP (GIBCO, 10297-018)	2 μl	22 μl
100 μM hexamer (GIBCO, 48190-011)	1 μl	11 μl
5 X RT buffer (GIBCO, 28025-013)	4 μl	44 μl
0.1 M DTT	2 μl	22 μl
200 U/μl M-MLV-RT (GIBCO, 28025-013)	1 μl	11 μl
	Total 19 μl	

2. Spin tubes at 500 g for 10 sec, transfer 19 μl of the RT master-mix to each of 1.5 ml Eppendorf tubes (save the remaining master-mix at -20 °C for later use).

3. Add ~**1 μl** (=1 μg) total RNA to each of five 1.5 ml Eppendorf tubes.

4. Spin tubes at 500 g for 10 sec, then incubate tubes in a 37 °C water bath for 1 hr.

5. Put the tubes on a 65°C heat-blocker for 10 min, and chill quickly on ice.

Amplify cDNA by PCR (50 μl reaction)

6. Prepare PCR master-mix in a 1.5 ml Eppendorf tube as following:

		For 13 samples
5 U/μl *Tag* polymerase (GIBCO, 8038-018)	0.2 μl	2.6 μl
10X PCR buffer	5 μl	65 μl
50 mM $MgCl_2$	3 μl	39 μl

5mM 4dNTP (GIBCO, 10297-018)	5 μl	65 μl
5' primers (100 μM)	1 μl	13 μl
3' primers (100 μM)	1 μl	13 μl
H_2O	30 μl	390 μl
	Total 45.2 μl	

7. Spin to mix at 500 g for 10 sec, transfer 45 μl of the PCR master-mix to each of six 0.5 ml Eppendorf tubes set on ice (save the remaining PCR master-mix at -20 °C for next time to use).

8. Add 5 μl of RT product to each of 5 tubes.

9. Add 5 μl ddH_2O to Negative control tube.

10. Spin to mix at 500 g for 10 sec, add 50 μl mineral oil to each tube.

11. Incubate tubes for 24 (β-actin), 36 (IL-6), or 38 (GM-CSF) cycles in the PCR machine on 7^{th} or 10^{th} floor:

Denature at 94°C for 3 min, followed by 1min for each cycle

Anneal at 57°C for 2 min for each cycle

Extend at 72°C for 2 min for each cycle, with 7 min for the last cycle.

Analyze PCR product by electrophoresis

12. Prepare 35 ml of 1.8 % agarose in a 125 ml Erlenmeyer flask, boil in microwave oven for 30 sec, take out the flask, and swirl by hand to mix, microwave another 10 sec, pure gel into a mini-gel box when it is about 60°C.

Agarose (GIBCO, 15510-019) 0.63 g

1X TAE buffer	35 ml
1mg/ml ethidium bromide	18 μl

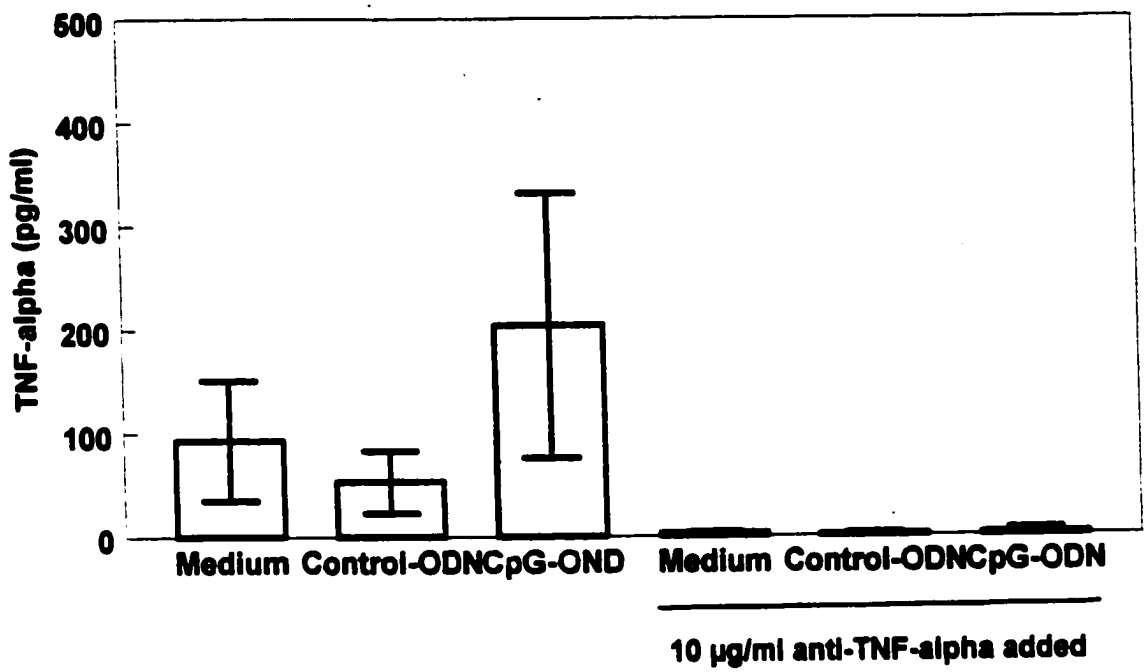
13. Prepare gel-running samples in 1.5 ml Eppendorf tubes as following:

Sample	H_2O	DNA	Loading buffer
β-actin/cytokine	8 μl	PCR product 10 μl	2 μl
Neg. cal	8 μl	PCR product 10 μl	2 μl
Ladder	50 μl	DNA marker 5 μl (For 4 usage's)	5 μl

14. Load the samples to the gel wells, and run the gel at 100 V for ~40 min.

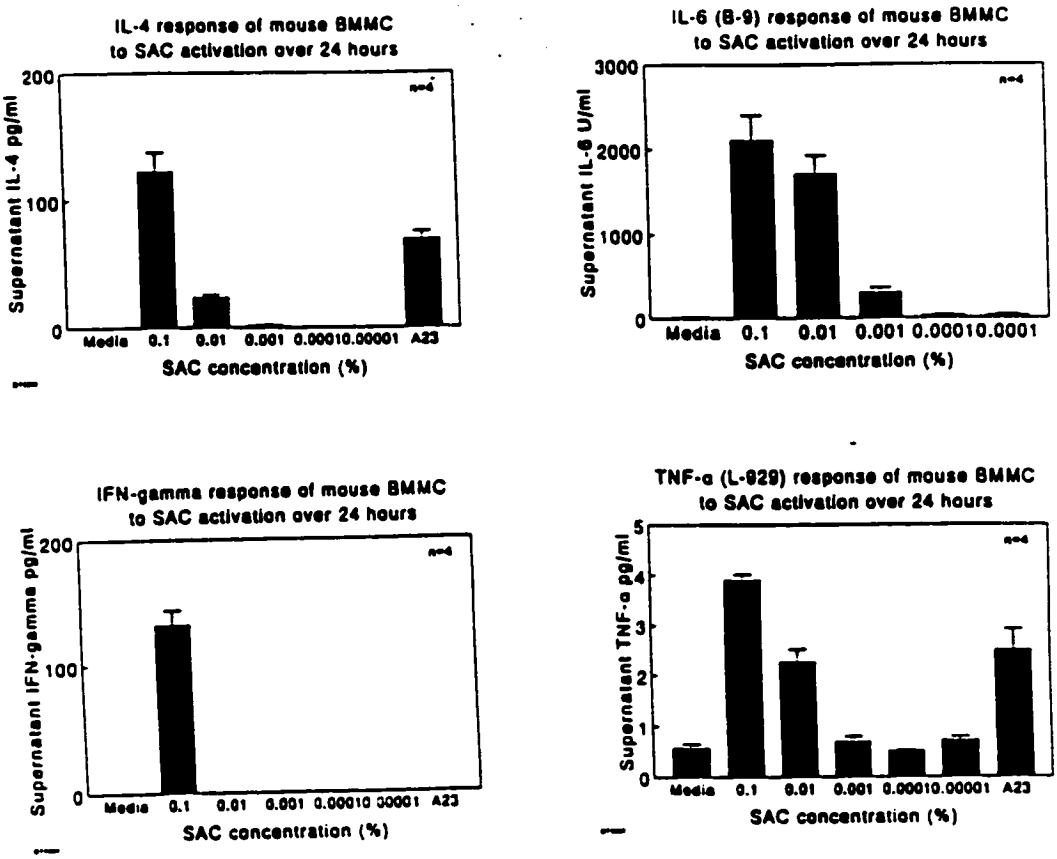
15. Visualize and analyze the PCR product with the ***Gel Document System*** in room 10-3H.

Appendix 8. Neutralization of TNF-α from mBMMC in L929 assay



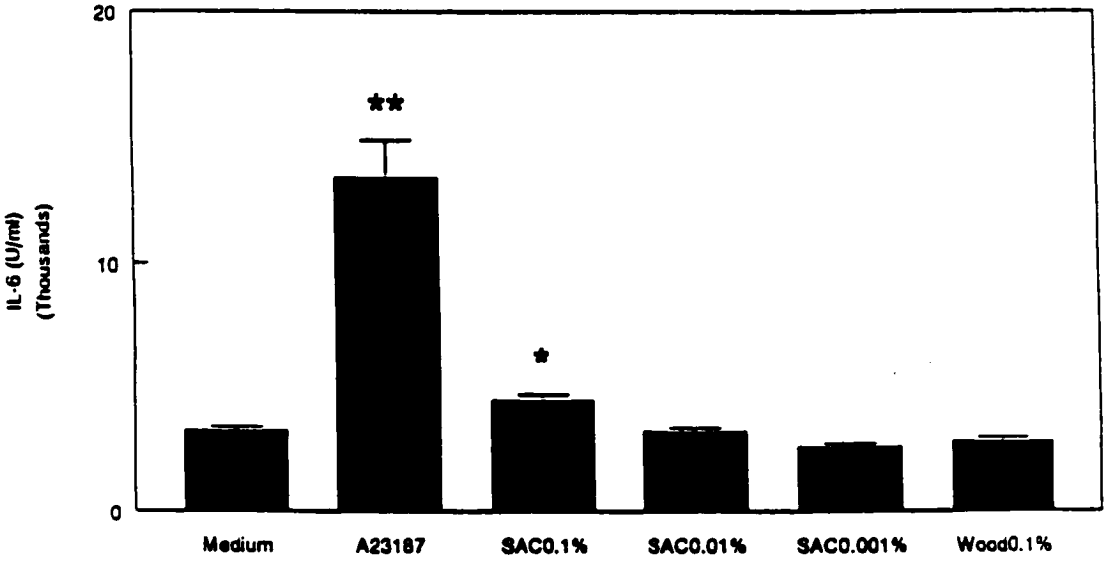
Neutralizing anti-murine TNF-α was added to the samples from mBMMC treated with 3CpG-ODN for 6 hr were before L929 assay. The anti-TNF-α antibody had completely abolished the cytotoxicity bioactivity of TNF-α present in these samples.

Appendix 9. SAC-induced cytokine production from mBMMC



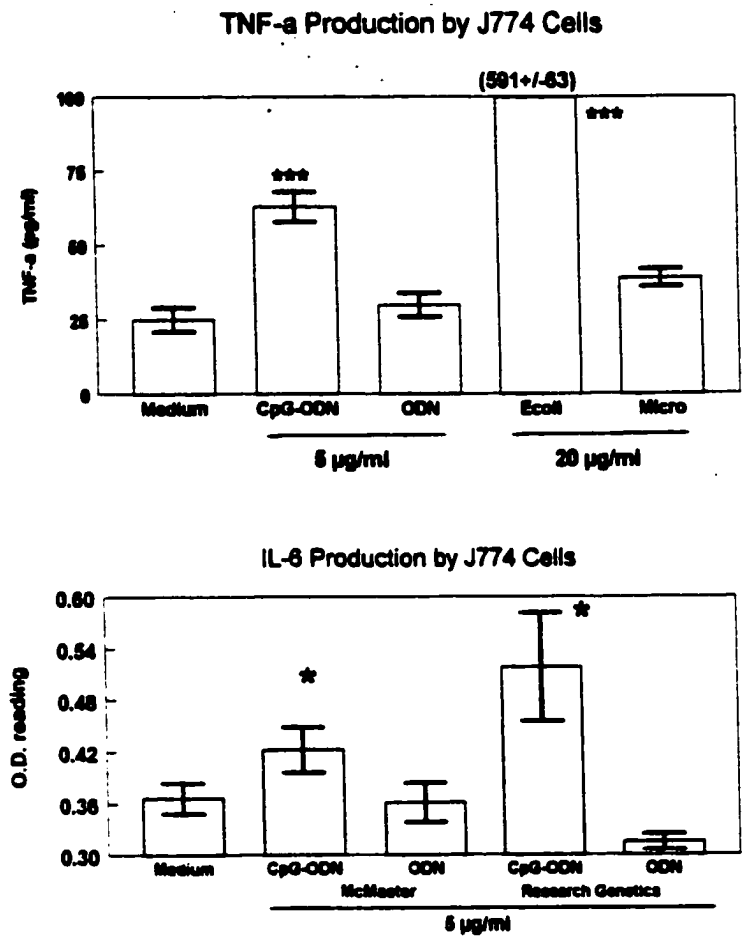
mBMMC at 10^6 cell/ml were treated with killed whole bacterium Staphylococcus aureus Cowan strain 1 (SAC) in a series of dilutions of the stock (Sigama) for 24 h. Supernatant samples were examined for IL-4, IL-6, IFN-γ and TNF-α levels by ELISA, L929 assay or B9 assay as appropriate. Note the increase in expression of each of the cytokines by cells activated with SAC.

Appendix 10. SAC-induced cytokine production from KU812 cells



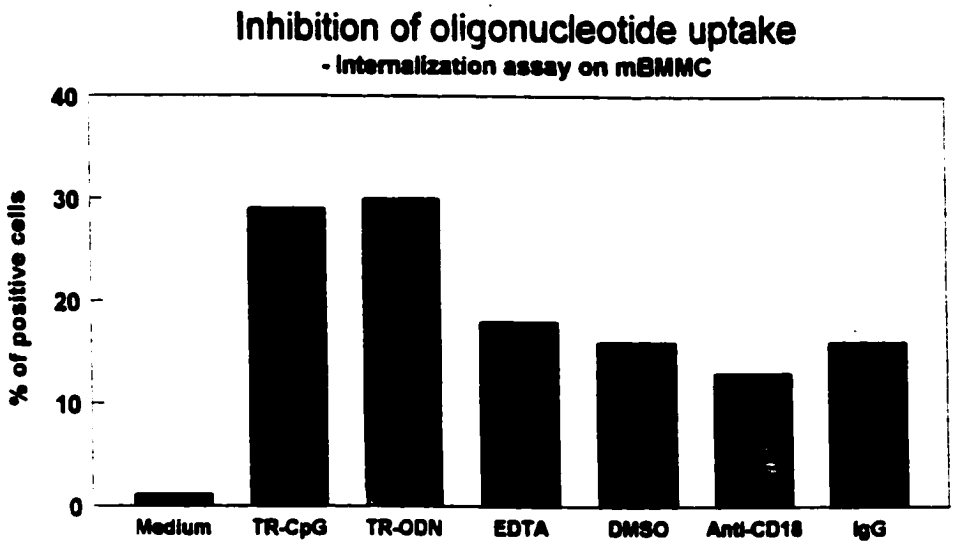
KU812 cells at 10^6 cell/ml were treated with killed whole bacterium *Staphylococcus aureus* Cowan strain 1 (SAC) in a series dilution of the stock (Sigma) for 24 h. Supernatant samples were examined for IL-6 levels with B-9 bioassay. SAC possesses protein A on the bacterial cell surface, while the Wood strain of *Staphylococcus aureus* does not.

Appendix 11. Murine macrophage cell line J774 response to DNA



Murine macrophage cell line J774 cells were treated with 1CpG-ODN (CpG-ODN) or control ODN (ODN), or bacterial DNA from *E. coli* (Ecoli) or *M. lysodeikticus* (Micro) at lower doses than used in mBMMC. Supernatant samples from for 6 h culture were examined for TNF-α levels with L929 cytotoxicity assay, and those from 24 h culture were examined for IL-6 levels with B-9 bioassay (note the O.D. reading scale used for IL-6 content due to high concentration of IL-6 outside the standard curve range). IL-6 data also showed that oligonucleotides synthesized by McMaster University or by Research Genetics had similar effects on J774 cells.

Appendix 12. Oligodeoxynucleotide uptake by mBMMC



mBMMC at the concentration of $1X10^7$ cells/ml were incubated at 37 °C for 6 h with 2 μM of Texas red-labeled 3CpG-ODN in 10% FCS/ RPMI-1640 with or without 20 mM of EDTA, 10 μg/ml anti-mouse CD18 or isotype IgG, or 1%DMSO. 2 μM of Texas red-labeled ODN were used as control. This is representative flow cytometry data from one of four separate experiments. EDTA (to delete extracellular Ca^{2+}) and DMSO (to disrupt cytoskeleton) partially inhibited DNA uptake by mBMMC. Anti-CD18 blocked DNA uptake to about half of the control levels, but control isotype IgG also showed some inhibition, suggesting certain components in the sera products are inhibitory to DNA uptake.

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