Mutational Analysis of Myxoma Virus T1 Protein Reveals Essential Functional Domains

By

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Abstract

Poxviruses have evolved a number of intricate mechanisms that allow them to replicate in the presence of an active host immune response. One way they have accomplished this is by encoding a variety of intracellular and secretory proteins, which function as immunomodulators. The myxoma virus T1 protein (M-T1) and the vaccinia virus 35kDa protein are members of the T1/35kDa family of poxvirus chemokine binding proteins, also known as the chemokine binding protein – II (CBP-II) family, that have been proven to be potent inhibitors of the host inflammatory response. Although lacking similarity to any seven transmembrane chemokine receptors, these proteins have been shown to bind a variety of CC-chemokines with high affinity, resulting in the inhibition of chemotaxis and calcium mobilization in response to CC- chemokines. Here it is demonstrated that purified M-T1 and vv35kDa inhibits the binding of [I^{125}]MIP-1α and [I^{125}] MCP-1 to their cognate receptors on human primary monocytes and THP-1 cells, a human promonocytic cell line, yielding K_i values in the range of 0.31 $\pm$ 0.20nM to 6.4nM. Alternatively, neither viral protein was able to inhibit the binding of [I^{125}]IL-8, a CXC-chemokine, to its receptors on neutrophils. To identify domains necessary for functional inhibition of M-T1, a variety of deletion mutants were created along the length of the gene, cloned and expressed using a baculovirus expression system, and tested for their ability to inhibit the binding of [I^{125}]MIP-1α to its cognate receptors on THP-1 cells. Wild type BacT1 protein inhibited binding with a Ki of 4.0 $\pm$ 1.2nM, whereas mutant viral proteins BacT1DEL23-53, BacT1DEL23-102, BacT1DEL53-102, and BacT1DEL53-170, all lacking one or more cysteine residue or cluster of negative charges, were not able to inhibit binding. These results suggest the importance of

structure in the ability of M-T1 to function in chemokine binding. Furthermore, a specific role in maintaining this inhibitory function may be implicated for the conserved cysteine residues found in M-T1 and vv35kDa.

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Abbreviations

AcNPV	Autographa californica Nucleopolyhedrovirus
BLC	B lymphocyte chemoattractant
BSA	bovine serum albumin
CBP	chemokine binding protein
DARC	duffy antigen receptor for chemokines
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EBI1	Epstein-Barr virus-induced gene 1
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetra-acetic acid
ENA	epithelial-cell-derived neutrophil-activating peptide
ELR	glutamic acid-leucine-arginine
GAG	glycosaminoglycan
GCP	granulocyte chemotactic protein
GPCR	G-protein coupled cell receptor
GRO	growth-regulated oncogene
HCMV	human cytomegalovirus
HHV-	human herpesvirus
HIV	human immunodeficiency virus
HVS	herpesvirus saimiri

ICTV	International Committee for the Taxonomy of Viruses
IL	interleukin
IFN	interferon
kbp	kilobase pairs
kDa	kilodalton
KSHV	Kaposi's sarcoma associated herpesvirus
LARC	liver and activation-regulated chemokine
LB	luria broth
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MGF	myxoma growth factor
MGSA	melanoma growth stimulatory activity
MHC	major histocompatability complex
MCP	monocyte chemoattractant protein
MCV	Molluscum contagiosum virus
MDC	macrophage-derived chemokine
MIG	monokine induced by interferon-γ
MIP	macrophage inflammatory protein
M.M.	molecular mass
mRNA	messenger ribonucleic acid
NAP-2	neutrophil-activating peptide 2
NEB	New England Biolabs
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
RANTES	regulated upon activation normal T-cell expressed and secreted
SD	standard deviation
SDF	stromal-cell-derived factor
SDS	sodium dodecyl sulphate
SLC	secondary lymphoid-tissue chemokine
TAE	Tris-acetate EDTA
TARC	thymus and activation regulated chemokine
TIR	terminal inverted repeat
UV	ultraviolet
UWO	University of Western Ontario
vv	vaccinia virus
wt	wild type

Chapter 1: General Introduction

1.1. Poxviruses

Poxviruses comprise a large family of eukaryotic viruses well documented throughout history. Studies with this family of virus began in the late 18th and early 19th centuries with scientific analysis of the lethal smallpox disease that plagued millions and remains a dark shadow in our history. The causative agent of smallpox, variola virus, and the virus eventually used as its vaccine, vaccinia virus, are classified as members of the poxvirus family belonging to the orthopoxvirus genus. In the 1980's, vaccinia virus was identified as a useful tool for laboratory investigations. These studies lead to several observations of fundamental importance to virology, and consequently set the groundwork for insight into poxvirus structure and function (Fenner *et al.*, 1989).

Poxviruses consist structurally of a large oval or brick shaped virion (250-300 x 250 x 200 nm) containing a nucleoprotein core, which consists of viral DNA and proteins organized as a nucleosome, as is shown in Figure 1.1 (Fenner *et al.*, 1989). The viral DNA is a linear double stranded (ds) DNA molecule with a genome that ranges from 130-300 kilobase pairs (kbp) between poxvirus members. The dsDNA contains covalently closed hairpin termini and terminal inverted repeats, and encodes over 100 polypeptides (Moss, 1990; Esposito, 1991). The core is surrounded by a lipoprotein bilayer membrane and forms a dumbbell shape due to the two lateral bodies present on each side. The core and lateral bodies are engulfed by an outer membrane, approximately 12nm thick, and in some cases by an envelope. Only virions released naturally from the

cell contain this envelope, composed of host cell lipids and virus specified polypeptides. Virions released by cellular disruption, however, lack an envelope (Fenner *et al.*, 1989).

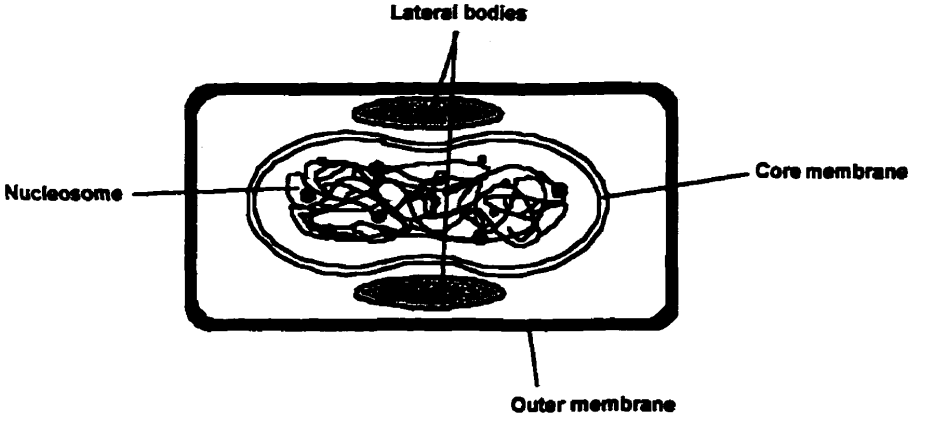


Figure 1.1 – The structure of a poxvirus virion. (Modified from Fenner *et al.*, 1989)

The poxvirus life cycle is initiated upon receptor mediated entry of virions into the host cell, followed by uncoating of the core in the cytoplasm to allow for DNA replication in virus produced factories known as "virosomes" (Moss, 1990). Viral genes expressed prior to capsid assembly and viral DNA replication are termed early genes, and are expressed immediately after entry into the cell. Late viral genes are expressed during replicative and post-replicative processes, and they encode proteins involved in capsid structure and assembly of the virus. The mRNAs formed are 7-methylguanosine capped and 5' polyadenylated by virus encoded early gene products. Splicing is a process that

occurs in the nucleus of cells, exclusively, hence cytoplasmic viral mRNAs are not spliced. Mature virus particles may exit the infected cell via exocytosis, or extrusion via microvilli (Fenner *et al.,* 1989).

Replication occurs exclusively in the cytoplasm of host cells, thereby distinguishing poxviruses from other large DNA viruses. In general, genes that are highly conserved amongst poxvirus members, and essential for virus structure and replication, are located within the central region of the viral genome (Turner and Moyer, 1990). They encode enzymes, such as factors required for mRNA synthesis, replication, and regulators of gene expression. Because poxviruses replicate and disseminate in the presence of an active immune system within an animal host, they also encode a multitude of virulence factors that ensure virus survival in the threat of an aggressive host immune response. Genes encoding these virulence factors, which differ significantly among poxviruses, are often found near or within the terminal inverted repeats (TIRs) and are generally dispensable for growth in tissue culture (McFadden *et al.,* 1995; Spriggs, 1996).

The family *Poxvirinae* contains two subfamilies that each contain several genera, as shown in Table 1.1 (Fenner *et al.,* 1989; Esposito, 1991). Members of the *Chordopoxvirinae* subfamily infect vertebrate hosts, including prototypic viruses from different genera that infect a wide range of species from swine to humans. Those belonging to the *Entomopoxvirinae* subfamily infect arthropod hosts, with prototypic viruses infecting mainly insects. One genus in the subfamily *Chordopoxviridae,* is the *Leporipoxviridae* defined by the Latin word "leporis" meaning hare or rabbit.

Table 1.1 – Classification of poxviruses *

Subfamily	Genus	Prototype virus
Chordopoxvirinae	*Orthopoxvirus*	Vaccinia virus
	Parapoxvirus	Pseudocowpox virus
	Capripoxvirus	Sheep pox virus
	Suipoxvirus	Swinepox virus
	Leporipoxvirus	Myxoma virus
	Avipoxvirus	Fowlpox virus
	Yatapoxvirus	Tanapox virus
	Molluscipoxvirus	Molluscum contagiousum virus
Entomopoxvirinae	*Entomopoxvirus A*	Poxvirus of Melolontha
	Entomopoxvirus B	Poxvirus of Lepidoptera
	Entomopoxvirus C	Poxvirus of Diptera

- Classification adopted by the International Committee for the Taxonomy of Viruses (ICTV) (Adapted from Fenner *et al.*, 1989; Esposito, 1991)

Leporipoxviruses have a very specific host range restricted to rabbits, hares, and squirrels. The principle mode of virus transmission is through biting arthropods (ie. mosquitoes), and infection of the natural host is usually specific for the epidermis or subdermis, with rare occurances of progression to secondary sites (McFadden, 1994). Genetically, the leporipoxviruses contain double stranded DNA genomes of 160-163kbp, terminal inverted repeats (TIR) of 10-13kbp, and a GC content of approximately 40%. Members show serological cross-reactivity; often encoding similar virulence proteins, such as growth factors and inhibitors (McFadden, 1994; Fenner *et al.*, 1989). One of the most extensively studied members of the leporipoxvirus genus is myxoma virus.

1.1.1.Myxoma virus

The history of myxoma virus began with its discovery in the late 19th century, when a group of European rabbits imported into Uruguay were suddenly stricken with a previously undescribed disease (Fenner and Ratcliffe, 1965). The causative agent was

identified as a novel infectious rabbit pathogen called myxoma virus. In 1927, a scientist by the name Aragão classified it as a member of the poxvirus family, whose natural hosts were populations of the North and South American rabbits of the *Sylvilagus sp.*, existing in a non-pathogenic symbiotic relationship (McFadden, 1994). It was not until the introduction of a new rabbit species, the European rabbit *Oryctolagus cuniculus,* that myxoma virus was assigned its new title as a highly pathogenic, almost 100% lethal infectious agent (Fenner and Myers, 1978; McFadden, 1988; McFadden, 1994).

The interest in myxoma virus increased in 1950, with the introduction of the virus into the Australian countryside for the purpose of eradication of a feral European rabbit population (Fenner and Myers, 1978). Myxoma was the first viral agent to be purposefully introduced into the wild for the elimination of a vertebrate pest, which resulted in the popularity of the virus and a spot on the cover of an Australian edition of *Time* magazine. Although initial success resulted in massive reductions in rabbit populations, progressive attenuation of the virus and the emergence of resistant rabbits through natural selection eventually resulted in rabbit repopulation, to levels approaching those prior to introduction of the myxoma virus (Fenner and Myers, 1978). The opportunity to study the evolution of the virus in the wild, however, has lead to the utilization of myxoma virus as a model system for studying virus/host interactions.

The myxoma virus genome is similar to other leporipoxviruses in that it contains a single linear dsDNA molecule of approximately 160kbp, with 11kbp TIRs (McFadden and Graham, 1994). Several of the proteins involved in the pathogenesis of the virus that are not necessary for virus replication, *in vitro*, have been characterized, including M-T1, the subject of this thesis. The *Bam*HI map outlined in Figure 1.1.1 demonstrates the

position of these virulence genes, which encode proteins that function as immunomodulators (Nash *et al.*, 1999).

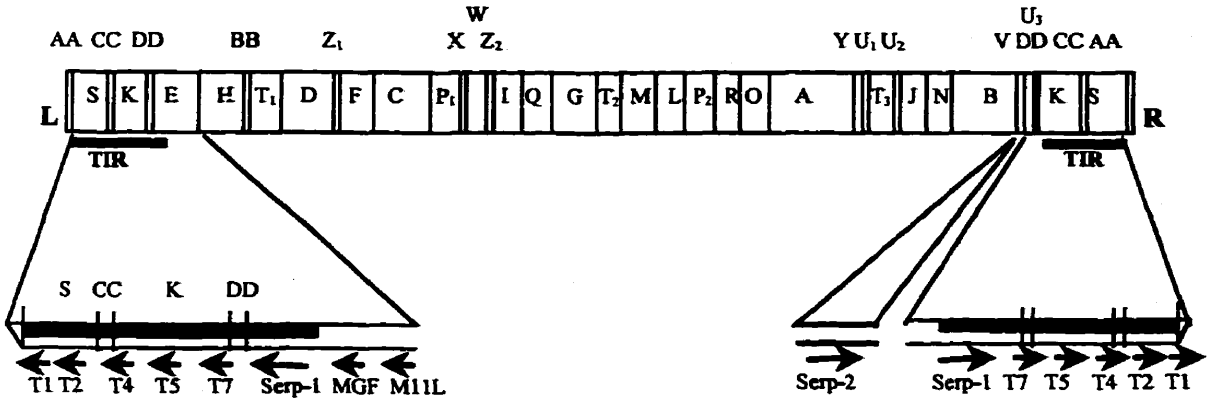


Figure 1.1.1 Myxoma virus genome – The *Bam*HI map of the myxoma genome is shown with vertical lines indicating BamHI restriction enzyme sites and arrows designating the locations of several characterized virulence proteins, including M-T1. Letters are used to identify BamHI restriction fragments. The terminal inverted repeats (TIR) are designated by areas covered in bold lines. (Figure obtained from Nash *et al.*, 1999, see Appendix A for copyright release)

These immunomodulatory proteins can be categorized according to their mode of action and general structure. Two classifications include "viromimetics" and "virostealth", which represent strategies used by virus encoded proteins to modulate the host immune response (Nash *et al.*, 1999). The viromimetics class consists of extracellular viral proteins that mimic host immune factors, resulting in the disruption of a normal immune response during infection. Viral proteins that mimic cellular receptors are termed *viroceptors*; exemplified by myxoma M-T1, M-T2, and M-T7 proteins that function by competitively binding cytokine ligands, thereby blocking the inflammatory signal. In contrast, secreted viral proteins that mimic cellular cytokines or inhibitors, such as myxoma growth factor (MGF) and serine proteinase inhibitor-1 (SERP-1), are termed

virokines. These proteins interfere with immune function by promoting growth, or inhibiting the normal inflammatory cascade leading to an immune response beneficial to the virus (McFadden, 1995; Nash *et al.*, 1999).

The virostealth class includes intracellular proteins that prevent the infected cell from undergoing apoptosis or being recognized by the immune system. Those proteins involved in the disruption of signal transduction, such as SERP-2, M11L, M-T2, M-T4 and M-T5, allow virus infection to proceed within infected cells through the prevention of apoptosis and are termed *viromitigators*. Proteins involved with virostealth hide the virus from the immune system, for example by downregulation of specific cell surface markers such as MHC class I or CD4 molecules (Nash *et al.*, 1999).

Summarized, the immunomodulation caused by this plethora of proteins, each having different targets and mechanisms of action, define myxoma virus as an infectious pathogenic agent in the European rabbit. To date, there have been several strains of myxoma virus identified, ranging from relatively nonpathogenic (i.e. neuromyxoma, Nottingham strains) to highly virulent (i.e. Moses, Lausanne strains). The disease-state caused by myxoma virus infection, described initially by Sanarelli in 1896, is known as myxomatosis. (McFadden, 1994)

1.1.2.Myxomatosis

Myxoma virus pathogenesis in rabbits results in the syndrome known as myxomatosis, which varies in severity among virus strains and hosts. For both the *Sylvilagus sp.* and *Oryctolagus cuniculus* there are compromises to the infected host's immune system, however infection of the latter species is far more devastating with up to 100% mortality. Infection of the *Sylvilagus* species results in benign, localized subdermal

lesions at the primary site of virus infection, with only moderate levels of infectious virus, that persist for many months. The virus infects fibroblasts and epithelial cells, with lesions occurring only at the site of inoculation. Although there is slow virus clearance suggesting a slight dysfunction of cellular immunity, the rabbits completely recover (Fenner and Ratcliffe, 1965).

In contrast, infection of *Oryctolagus cuniculus* results in full blown, lethal myxomatosis characterized by multiple external necrotic cutaneous tumors and internal lesions in the stomach, intestines and heart. Infectious virus, present in high titres in the blood, migrates through the lymphatic channel to secondary sites such as the spleen and lymph nodes via infected leukocytes. Secondary infection of conjunctival and pulmonary alveolar epithelia cells causes disruption of the ciliary architecture, which may be a factor in the facilitation of extensive Gram-negative bacterial infections of the eyes, nose and respiratory tract. Although circulating antibody can be detected against the virions and soluble antigens, there is overall immune dysfunction with multiple alterations in cytokine and lymphocyte function. The combination of generalized immunosuppression, internal and external necrotic tumors, and bacterial colonization of the respiratory tract, is believed to be the cause of death (Fenner and Ratcliffe, 1965; Fenner and Myers, 1978; DiFiacomo and Mare, 1994; McFadden, 1994).

The host differences responsible for the two distinct disease phenotypes observed have not yet been defined. It is clear, however, that the overall cellular immune dysfunction seen in both cases can be attributed in part to the several encoded virulence factors functioning as viroceptors, virokines, virostealth, and viromitigators. Together,

these proteins act against the host immune system, at the level of the inflammatory response, to ensure virus survival in the threat of an aggressive host immune response.

1.2. The immune response

The essence of immunology lies in the understanding of how "self" is distinguished from "non-self" (Benjamini and Leskowitz, 1991). It is based on the principle of recognition mechanisms, whose origins date back in evolutionary history, involving cell surface molecules able to specifically bind and adhere to other molecules on opposing cell surfaces. This evolved over time into the complex system of the immune response, which can be loosely divided into innate and aquired immunity (Janeway and Travers, 1994). The former is conferred by external elements or internal substances that are available at short notice to serve as a first line of defense against challenges by foreign invaders, whereas the latter is based on the clonal selection of lymphocytes that proliferate and differentiate into effector cells to eliminate pathogens. A major component of the host's defense mechanism, which incorporates these two forms of immunity, is inflammation (Janeway and Travers, 1994; Benjamini and Leskowitz, 1991).

1.2.1.Inflammation

Inflammation is a complex process often initiated by tissue damage from endogenous factors, such as tissue necrosis or bone fracture, or exogenous factors, such as infection by microorganisms. Tell tale signs of inflammation include swelling, redness, heat, pain, and loss of function in the inflamed area (Benjamini and Leskowitz, 1991).

Generally, the inflammatory response begins with the dilation of blood vessels and enhanced capillary permeability, which leads to edema, and accumulation of leukocytic cells in the area of injury. These cells include polymorphonuclear leukocytes, which are short-lived phagocytic cells that contain lysosomes filled with hydrolytic enzymes. They also produce peroxide and superoxide radicals and bactericidal proteins, which act as defense agents against the invading microorganisms. These cells accumulate within 30-60 min. after infection and are followed by mononuclear cells such as macrophages and lymphocytes, which infiltrate the area within 5-6 hrs if the infection is not resolved. The macrophages maintain phagocytic activity in the area of inflammation/infection, function in the presentation of antigen on their surface, and eliminate the injury or foreign agent through induction of the acquired immune response (Benjamini and Leskowitz, 1991).

If injury or invasion persists, the inflammatory response becomes continuously augmented with a positive feedback cycle of antibody production and cell-mediated immunity. Although the leukocytes are capable of eliminating foreign agents, a majority of their function involves the secretion of soluble, biologically active substances, known as cytokines. These molecules act to attract and activate more cells, resulting in a complex network of signaling and activation which inevitably leads to the eradication of the injury or pathogen.

Cytokines are glycoproteins synthesized and rapidly secreted in response to a stimulus (Rose-John and Heinrich, 1994). Cells secrete a number of different cytokines, and conversely may be activated by a variety of them as well. Cytokines may also act as agonists or antagonists towards each other in context of a target cell. One group of

cytokines, which have been and continue to be actively studied as mediators of the inflammatory response, are *chemo*tactic cyto*kines* known as chemokines (Rollins, 1998; Luster, 1998; Pease and Murphy, 1998).

1.2.2.Chemokines

Up until about 10 years ago, very little was known about the chemotactic proteins that may act as traffic controllers in the recruitment of specialized leukocyte subpopulations to sites of inflammation (Ahuja *et al.,* 1994). Studies of inflammatory diseases lead to the initial discovery of interleukin–8 (IL-8) (Yoshimura *et al.,* 1987) and monocyte chemoattractant protein–1 (MCP-1) (Matsushima *et al.,* 1989); two chemokines with common structural features and the ability to attract different subsets of leukocytes. IL-8 was identified as a chemoattractant for neutrophils, whereas MCP-1 was mainly a monocyte and T-cell chemoattractant. Amino acid sequencing of these two molecules showed that each chemokine contained a different pattern of four conserved cysteine residues; the first two N-terminal cysteines of IL-8 being separated by one amino acid, forming a CXC motif, and the first two N-terminal cysteines of MCP-1 being consecutive and adjacent, forming a CC motif (Baggolini *et al.,* 1997). The motifs described by these proteins became the basis for the classification of chemokines, shown in Table 1.2; the two initial subclasses being the CC-chemokines (also known as β-chemokines) and the CXC-chemokines (also known as α-chemokines). The CXC-chemokines can also be divided into two subgroups, based on the presence or absence of an ELR (glutamic acid-leucine-arginine) motif at the N-terminus of the protein preceding the first cysteine residue. The presence of this motif loosely dictates the chemokine's

function; those containing the motif are chemotactic for neutrophils, whereas those lacking it are mainly chemotactic for lymphocytes (Wells *et al.*, 1998).

In the past few years, interest in chemokines has increased enormously resulting in the discovery of now over 26 members of the CC-chemokine subclass, as well as 17 of the CXC-subclass. Two new classes of chemokines, namely the C-chemokine and CX_3C-chemokine subclasses, have been assigned for the newly discovered chemokines lymphotactin and fractalkine, respectively, based on the presence and positioning of highly conserved cysteine residues (Kelner *et al.*, 1994; Bazan *et al.*, 1997).

Fractalkine, also known as neurotactin, is perhaps the most unique chemokine yet discovered. It is a membrane bound glycoprotein with the first two cysteines in the protein separated by three amino acids, yielding a CXXXC motif, which is the basis for its classification (Bazan *et al.*, 1997). The chemokine domain of the protein is similar in structure to members of CC-chemokine subclass, however it contains a transmembrane domain that is joined onto the chemokine domain by a mucin-like stalk. The protein is predicted to be a type 1 membrane protein, however the molecule in its soluble form, resulting from protease cleavage of basic residues proceeding the chemokine domain, is required for chemotactic function of the protein (Pan *et al.*, 1997). Lymphotactin, another unique chemokine, contains only two of the highly conserved cysteine residues, equivalent to cysteines 2 and 4, compared to other chemokine subfamilies. Despite this fact, lymphotactin maintains the secondary and tertiary structure that is common for all chemokines (Hedrick and Zlotnick, 1998).

The tertiary structure of several chemokines has been solved to date, confirming their similarity (Baggolini *et al.*, 1997). The basic structure, shown in Figure 1.2.2,

includes a flexible amino terminus containing disulphide bond linkages of the two N-terminal cysteines, which is the region responsible for receptor binding. This is followed by an extended loop that leads into a Greek key motif (three anti-parallel β-pleated sheets). These pleated sheets provide a flat base over which a C-terminal α-helix, encoding a heparin-binding domain, extends (Clore *et al.*, 1990; Handel and Domaille, 1996). Almost all chemokines form dimers under crystallizing conditions, and it is in the quaternary form that different subclasses of chemokines exert their gross structural differences. For example, CC-chemokine dimers are more extended and cylindrical than compact CXC-chemokine dimers. It is still unclear whether or not receptor activation requires dimer formation, or if chemokine dimers are formed under physiologic conditions (Burrows *et al.*, 1994; Paolini *et al.*, 1994).

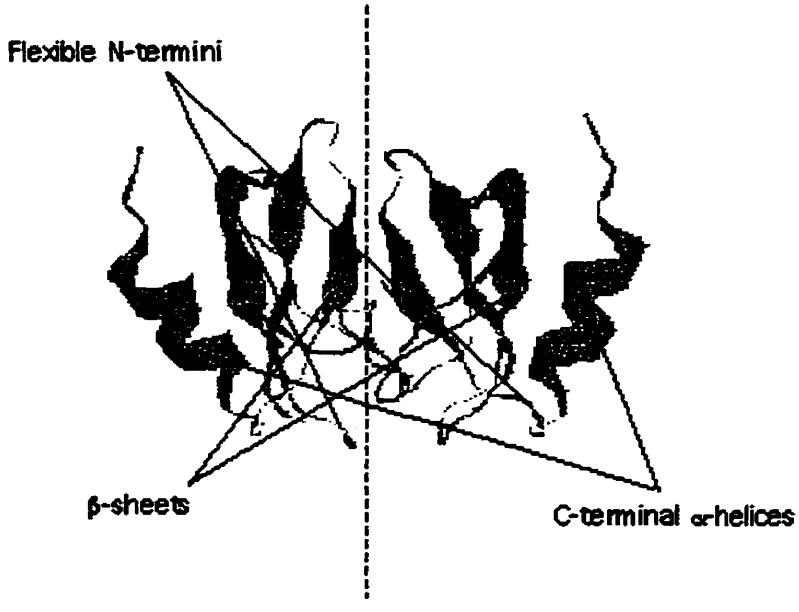


Figure 1.2.2- Structure of RANTES chemokine dimer. Figure designed using the program RASMOL 2.6, based on coordinates of the published structure (Skelton *et al.*, 1995). Monomers represented by molecules on either side of the dotted line.

Several roles have now been assigned to chemokines, some of which involve the regulation of diverse biologic processes, including inflammation, lymphocyte homing, angiogenesis, and development (Baggolini *et al.*, 1994; Baggolini *et al.*, 1997). The ability of chemokines to regulate the homeostatic circulation of leukocytes from the blood to tissues is exemplified by the selective recruitment of leukocytes into inflamed areas caused by tissue damage or invasion by microorganisms (Butcher, 1991). Chemokines are thought to be involved in the extravasation of leukocytes from the blood into tissues, through switching from selectin mediated interactions to integrin mediated interactions (Butcher, 1991; Springer, 1994). Given the correct stimulus a variety of cells, such as leukocytes and lymphocytes, can secrete chemokines, increasing the gradient to which leukocytes may migrate. The stimulus comes from pro-inflammatory cytokines such as IL-1 and TNF-α, lymphocyte secreted IFN-γ and IL-4, and/or bacterial products such as lipopolysaccharide (LPS). Therefore, leukocyte infiltration is a multistep process, in which chemokines participate in conjunction with other cytokines, to increase the pool and responsiveness of a given leukocyte to chemokines (Luster, 1998).

Several chemokines have been identified that are involved in the regulation of lymphocytes rather than the recruitment of leukocytes into inflamed tissues (Dieu *et al.*, 1998). These chemokines are constitutively expressed in secondary lymphoid tissues and include secondary lymphoid tissue chemokine (SLC), B lymphocyte chemoattractant (BLC), and Mip-3β, which strongly attract naïve, resting lymphocytes. Recent studies suggest a role for these chemokines in the promotion of naïve T-cell encounters with antigen-presenting dendritic cells and attraction of antigen-binding B lymphocytes into the T zone of secondary lymphoid tissues (Legler *et al.*, 1998; Ngo *et al.*, 1998). These

findings demonstrate that chemokines not only play a role in inflammatory and immunological responses, but also in normal lymphocyte recirculation and homing.

Chemokines are also capable of modulating angiogenesis and tumor growth. For example, IL-8 promotes angiogenesis and tumor metastasis, whereas platelet factor-4 and IP-10 inhibit these functions (Luster and Leder, 1993; Strieter *et al.*, 1995). Inhibition of angiogenesis and tumor growth may be caused by the displacement of growth factors by certain chemokines, from heparan sulfate sites on endothelial cell surfaces (Baggolini *et al.*, 1997). The corollary of this is that other chemokines may promote growth through the signaling of tumor associated leukocytes, supplying growth factors and promoting angiogenesis. The different chemokines identified and classified to date, along with their chemokine receptor specificities, are listed in Table 1.2.2 (Luster, 1998; Pease and Murphy, 1998).

In order for chemokines to impart their biological activity, they must first bind to their appropriate cell surface receptors. These receptors belong to a class of G-protein coupled cell surface receptors (GPCR).

1.2.3.Chemokine receptors

Cells respond to a variety of signals, such as those conveyed by hormones, neurotransmitters, and growth factors. In order for this to occur, the soluble ligand must interact with its cell surface receptors. Transmembrane signaling processes may be governed by a variety of mechanisms, however many signaling molecules bind to receptors embedded in membranes that are part of a three component signaling system capable of functioning sequentially and reversibly. This signaling system includes the

large family of G-protein coupled cell surface receptors known as serpentine receptors (Donnelly, and Findlay, 1994).

Table 1.2– Chemokine and chemokine receptor classification and specificity

Chemokine Class	Chemokine Receptor Subtype	Chemokine Ligands	Ligand Responsive Cell Types
CC	CCR1	MIP-1α, RANTES, MCP-2, -3,-4, leukotactin-1	Eosinophil,Monocyte Activated T-cell, Dendritic cell
	CCR2	MCP-1,-2,-3,-4,-5	Basophil, Monocyte, Activated T- cell, Dendritic cell, Natural killer cell
	CCR3	Eotaxin, eotaxin-2, RANTES MCP-2,-3,-4,-5, leukotactin-1	Eosinophil, Basophil, Dendritic cell
	CCR4	TARC, MDC	Activated T-cell, Dendritic cell
	CCR5	MIP-1α, MIP-1β, RANTES, MCP-2	Monocyte, Activated T-cell, Dendritic cell, Natural killer cell
	CCR6	LARC	Dendritic cell
	CCR7	ELC, SLC	Activated T-cell
	CCR8	I-309, MIP-1β, TARC	Monocytes
	D6	MIP-1α, MCP-1,-3	Not determined
CXC	CXCR1	IL-8, GCP-2	Neutrophils
	CXCR2	IL-8, GRO α, β, γ, NAP-2 ENA-78, GCP-2	Neutrophils
	CXCR3	Mig, IP-10	Activated T-cell, Natural killer cell
	CXCR4	SDF-1	Monocyte, Resting T -cell, Dendritic cell
	CXCR5	BCA-1	B-cells
C	Not determined	Lymphotactin	Resting T-cell
CX_3C	CX_3CR1	Fractalkine	Monocyte, Activated T-cell, Natural killer cell

Signaling occurs upon binding of ligand to receptor, proceeded by the activation of heterotrimeric guanine nucleotide proteins (G-proteins) (Gudermann *et al.*, 1997). Upon activation, these G-proteins then modulate the activities of a variety of effectors, such as enzymes, resulting in the amplification of a second message (Birnbaumer and Birnbaumer, 1995; Neer, 1995).

The first evidence regarding the nature of chemokine receptors was gathered in the late eighties, where it was demonstrated that the action of the CXC-chemokine IL-8 was blocked by pertussis toxin (Holmes *et al.*, 1991). Since pertussis toxin was known to interact and inactivate G_i –proteins, this lead to the suggestion that G-protein coupled serpentine receptors were involved in chemokine signal transduction. These and later studies lead to the characterization and cloning of several chemokine receptors in a variety of species (Neote *et al.*, 1993; Ahuja *et al.*, 1992; Prado *et al.*, 1994; Kitaura *et al.*, 1996; Murphy, 1996). Chemokine receptors maintain a structure similar to all GPCRs, characterized by seven transmembrane α-helices connected by internal and external loops, with an external N-terminus and a C-terminus extending into the cytoplasm (Murphy, 1994).

Since chemokines are implicated in a variety of biological processes, there must be some mechanism of signal sorting to distinguish the different biological effects imparted by different chemokines binding to a specific receptor. This is accomplished by the variety of G-proteins coupled by the receptor and the effector molecules that are targeted upon G-protein activation. Mutagenesis studies with chemokine receptors define the N-terminal portion of the chemokine receptor as the determinant for ligand specificity (Leong *et al.*, 1994), however *in vivo* ligand receptor interactions demonstrate the

necessity for at least four components (Bokoch *et al.,* 1995). This includes a presentation module, such as a proteoglycan surface, which chemokines may bind via their heparin - binding domain, forming a gradient to which leukocytes may migrate. Also required is a functional chemokine ligand, a chemokine receptor on the target cell surface, and finally a receptor linked cytoplasmic G-protein complex.

Chemokine binding to cognate receptors on cells leads to phosphorylation of the receptor, by G-protein-coupled receptor kinases, followed by association with members of the arrestin family of proteins (Zuker and Ranganathan, 1999). This results in a decreased affinity of the receptor for G-proteins through steric hindrance, quenching the catalytic activity of the receptor. This process is known as homologous desensitization, and is agonist dependent (Ali *et al.,* 1999). Following phosphorylation, members of the arrestin family of proteins bind the receptor and association of the arrestin/receptor complex with clathrin mediates internalization via clathrin coated pits (Zuker and Ranganathan, 1999). Internalization of receptors to endosomal compartments leads to one of two processes: degradation or recycling (Ferguson *et al.,* 1998). The exact process of resensitization has yet to be defined, however receptors do reappear on the cell surface shortly after desensitization, to allow for continuous signaling by the agonist (Ferguson *et al.,* 1996). Another form of desensitization, which involves loss of responsiveness of G-protein coupled receptors, is known as heterologous desensitization (Ali *et al.,* 1999). Here, arrestin-mediated internalization does not occur, agonist occupancy is not necessary, and responsiveness is lost following phosphorylation by second messenger-activated kinases, which have been activated by different receptors or signaling processes (Ali *et al.,* 1999). Much insight remains to be discovered as to G-protein selectivity and

signal transduction, however it is evident that it involves a complex web of activation and signaling pathways (L'Heureux *et al.,* 1995; Myers *et al.,* 1995; Murphy, 1996; Premack and Schall, 1996).

The selectivity of chemokine receptors for chemokines can be classified on the basis that they may be shared, specific, promiscuous or viral. As demonstrated in Table 1.2, shared chemokine receptors refer to those receptors that bind more than one member of a particular subfamily of chemokine, whereas specific chemokine receptors are those that bind only one chemokine. (Murphy, 1994; Premack and Schall, 1996; Murphy, 1996) The other two classifications of receptors, which are not identified in Table 1.2, are promiscuous and viral chemokine receptors which are characterized by molecules that are often widely divergent from members of the chemokine receptor family. The promiscuous Duffy blood group antigen receptor complex (DARC) is an erythrocyte chemokine receptor capable of binding members of both the C- and CXC-chemokine families (Neote *et al.,* 1993; Zhao-hai *et al.,* 1995; Szabo *et al.,* 1995). The final classification of chemokine receptors includes those produced by viruses, which are often shared receptors capable of binding several members of a particular subfamily of chemokines (Pease and Murphy, 1998). These viroceptors are representative members of the viromimetics class of immunomodulators and play a key role in the subversion of the host's immune response.

1.3. Viruses and the immune system

Many viruses have evolved intricate and ingenious mechanisms for evading the immune system of their respective hosts. Chemokines and chemokine receptors exemplify a prime target for many viruses, which may corrupt the system through the exploitation of chemokine receptors and/or subversion of chemokine action (Pease and Murphy, 1998).

Perhaps one of the more studied examples of chemokine receptor exploitation is the usage of cellular chemokine receptors for the purpose of entry into the cell. This strategy was identified for HIV-1 isolates, which gain entry into a cell through the utilization of a chemokine and CD4 receptor (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Deng *et al.*, 1997). Chemokine receptors that function as coreceptors for HIV-1 entry, described *in vitro*, include CCR3, CCR5, and CXCR4 (Dragic *et al.*, 1996; Doranz *et al.*, 1996; Feng *et al.*, 1996; Bleuel *et al.*, 1996).

Another method of exploitation by viruses is through the up-regulation of cellular chemokine receptors. For example, the CC-chemokine receptor –7 (CCR7), initially designated Epstein-Barr virus (EBV)-induced gene 1 (EBI1), is significantly up-regulated in B lymphocytes upon infection with EBV (Ngo *et al.*, 1998). Similarily, CCR7 is also up-regulated in $CD4^+$ T lymphocytes upon infection with human herpesvirus 6 (HHV-6) and HHV-7. The ligand for CCR7, Mip-3β, has been shown to be involved in lymphocyte homing and recirculation (Ngo *et al.*, 1998). Exploitation of the chemokine system by these viruses may have biological activities on infected cells such as growth promotion, protection from apoptosis, and migration into specific anatomical locations *in vivo* (Yoshida *et al.*, 1997).

Although viral exploitation often arises through the utilization of the cellular chemokine system, viruses may also corrupt the system by encoding molecules that have parallel functions to cellular molecules, which is accomplished by the viromimetics class of immunomodulators.

1.3.1. Viral chemokine and chemokine receptor homologues

Many of the large DNA viruses, such as the herpesviruses and poxviruses, encode immunomodulatory proteins that function as virokines and viroceptors (Barry and McFadden, 1997). These proteins exhibit obvious amino acid sequence similarities to host chemokines and chemokine receptors, making conceivable an evolutionary tale of theft and forgery, resulting in the formation of possible agonists and/or antagonists of these host molecules.

To date, there have been at least eight viral chemokine receptor homologues identified (Smith, 1996). Several of these viroceptors, that mimic chemokine receptors from both the CCR and CXCR chemokine receptor classes, are serpentine receptors resembling the cellular family of G-protein coupled receptors and often exhibiting similar functions. These viroceptors include ORF US28 of human cytomegalovirus (HCMV) (Neote *et al.*, 1993; Gao and Murphy, 1994), U12 of human herpesvirus-6 (HHV-6) (Isegawa *et al.*, 1998), ORF ECRF3 of Herpesvirus saimiri (HVS) (Ahuja and Murphy, 1993), and ORF 74 of human herpesvirus-8 (HHV-8) (Arvanitakis *et al.*, 1997).

US28 of HCMV is approximately 30% identical to human CCR1 and has been shown to bind several CC-chemokines, such as MIP-1α, MIP-1β, MCP-1, and RANTES (Kuhn *et al.*, 1995), but not CXC-chemokines. Cells transfected with US28 are also

capable of intracellular calcium signaling in response to CC-chemokine binding, although the importance of a functional virus encoded G-protein-coupled receptor remains elusive (Gao and Murphy, 1994). US28 has also been described as a functional coreceptor for the entry of certain HIV-1 isolates (Pleskoff *et al.*, 1997). Similarly, U12 from HHV-6 resembles CCR3 and also binds a number of CC-chemokines, with its highest affinity for RANTES, and is capable of calcium mobilization (Isegawa *et al.*, 1998).

ECRF3 of HVS is closely related to CXCR2 (approx. 30% amino acid identity) and consequently is capable of binding CXC-chemokine ligands IL-8, MGSA and NAP-2 with calcium mobilization (Ahuja and Murphy, 1993). Like the CXCR2 receptor, no CC-chemokines are bound by ECRF3. ORF 74 of HHV-8 closely resembles ECRF3 and CXCR2, however it differs in its ability to bind both CXC- and CC-chemokines with high affinity (Arvanitakis *et al.*, 1997). ORF 74 is also unique in that it is constitutively active, therefore it does not need ligand binding for downstream signaling to occur in infected cells.

Other viral chemokine receptor homologues identified include ORF US27 and UL33 of HCMV (Margulies *et al.*, 1996), ORF M33 of murine cytomegalovirus (MCMV) (Davis-Poynter *et al.*, 1997), ORF Q2 of capripoxvirus (Cao *et al.*, 1995), ORF K2R of swinepox virus (Massung *et al.*, 1993), and ORF E1 of equine herpesvirus 2 (Telford *et al.*, 1995). ORF UL33 of HCMV and ORF M33 of MCMV are colinear, with a low level of conservation at the N-terminus. Both proteins resemble CCR1, however neither have been described as functional receptors. Nonetheless, UL33 has been shown to localize to virus envelope particles (Margulies *et al.*, 1997), and M33 has been proven necessary for replication in the salivary glands of mice (Davis-Poynter *et al.*, 1997). The

two poxviral proteins, ORF Q2 and ORF K2R, share amino acid identity to each other as well as the HCMV US28 and CCR1. Again, no function has yet been assigned, however a role in prevention of the normal immune response may be speculated. These poxviral proteins may act to bind cellular chemokines, preventing the signaling of inflammatory cells, or they may cause a disruption in normal signal transduction of the cell. Future studies are needed to clarify the exact role of these proteins in virus-host interactions.

Chemokine receptors are not the sole target of the chemokine system by viruses; it has been shown that previously described viruses and virus families also encode a number of virokines that are similar to cellular chemokines. KSHV, for example, contains three ORFs encoding the proteins vMIP-I, vMIP-II, and vMIP-III that have 25%-40% amino acid identity to the CC-chemokine subfamily, especially MIP-1α (Moore *et al.*, 1996; Kledal *et al.*, 1997; Nicholas *et al.*, 1997).

vMIP-II is a functionally potent antagonist of chemokine/chemokine receptor binding for a wide spectrum of receptors including CCR1, CCR2, CCR5, CXCR4, as well as for the viroceptor US28 from HCMV (Kledal *et al.*, 1997). Although binding of vMIP-II to each of these receptors does not result in signal transduction, it blocks the stimulatory effect and chemoattractant ability of human CC-chemokines *in vitro* (Boshoff *et al.*, 1997). In addition, both vMIP-I and vMIP-II have been shown to block the entry of HIV-1 isolates in cells containing CD4 and the chemokine receptors CCR3, CCR5 and CXCR4. Interestingly, both vMIP-I and vMIP-II act as agonists as well; having been described, using an *in vitro* chick egg membrane model of angiogenesis, as potent angiogenic factors (Boshoff *et al.*, 1997).

The only poxviral chemokine homologue described to date, MC148R (Senkevich *et al.*, 1996), functions in a manner similar to vMIP-II of KSHV (Damon *et al.*, 1998). MC148R from Molluscum contagiosum virus (MCV) shows 30% amino acid identity to CC-chemokines, very high similarity to MIP-1β, and retains the disulphide bonding pattern and general structure of chemokines. However, it lacks five amino acids from the amino-terminal that are necessary for receptor activation (Senkevich *et al.*, 1996). Recent studies comprising competition binding, chemotaxis, and calcium mobilization, have defined MC148R as a potent antagonist of monocyte, lymphocyte, and neutrophil signaling in response to members of the CC- and CXC- families of chemokines (Krathwohl *et al.*, 1997). MC148R inhibits the growth of human hematopoietic cells more potently than MIP-1α, its close cellular homologue. Interestingly, MC148R is the only chemokine, other than the cellular chemokine I-309, shown to interact with CCR8 (Damon *et al.*, 1998). This further emphasizes the broad-spectrum antagonistic activity of this viral chemokine towards several members of the chemokine superfamilies.

The examples described above demonstrate the importance of the chemokine/chemokine receptor system in viral pathogenesis, and highlight the use of molecular mimicry by viruses to promote survival within the host. Another group of viral proteins that interfere with the chemokine system, without bearing homology to the cellular molecules they antagonize, have been identified. These proteins are defined by their ability to bind a variety of chemokines, and are therefore referred to as chemokine binding proteins (CBPs). To date, only members of the poxvirus family have been shown to encode CBPs, which have been classified into two families, CBP-I and CBP-II (Barry and McFadden, 1997). Several poxviruses, such as Shope fibroma virus and myxoma

virus, encode functional members of both families. Other poxviruses, such as cowpox, rabbitpox and vaccinia virus (strain Lister), encode proteins from both families; however, only members of the CBP-II family have been functionally identified (Lalani and McFadden, 1997).

There is only one functional member of the CBP-I family, identified in myxoma virus, which is M-T7. It is approximately 37kDa in size, as defined by SDS PAGE and gel filtration, and is the most abundant secreted protein from myxoma virus infection in culture (Upton and McFadden, 1992). It was originally characterized as an IFN-γ receptor homologue (Mossman *et al.*, 1995), however incubation of high concentrations of purified M-T7 with radiolabeled chemokines, from the CXC-, CC-, and C-chemokine families, in the presence of cross-linking agents, demonstrated that M-T7 could also bind chemokines (Lalani *et al.*, 1997). The interaction is not species-specific, and assays using IL-8 C-terminal truncations suggest that binding occurs at the heparin binding domain of the chemokine (Lalani *et al.*, 1997).

There have been several members of the CBP-II family identified to date, encoded by members of both the orthopoxvirus and leporipoxvirus families, including the myxoma virus M-T1 protein (Graham *et al.*, 1997; Barry and McFadden, 1997). They are all 35-40kDa soluble glycoproteins and hence are also described under the classification of the T1/35kDa family of poxvirus chemokine binding proteins. Members of this family have been shown to bind to CC-chemokines with high affinity and very weakly to members of the CXC-chemokine family (Graham *et al.*, 1997; Smith *et al.*, 1997; Lalani *et al.*, 1998; Alcamí *et al.*, 1998).

The effects of chemokine binding *in vitro* have been determined for vaccinia virus 35kDa protein, and other orthopoxvirus 35kDa chemokine-binding proteins, such as cowpox virus p32, variola virus p35 protein, rabbitpox virus 35kDa, and camelpox virus 35kDa proteins (Lalani *et al.*, 1998; Alcamí *et al.*, 1998; Smith *et al.*, 1997). Studies describing the high affinity binding of several of these proteins to CC-chemokines yield K_d values between 0.1-15nM for different CC-chemokines (Alcamí *et al.*, 1998). Other studies have revealed a potent inhibitory function for these proteins in the prevention of CC-chemokine binding to cellular receptors, blockage of intracellular calcium release, and the inhibition of chemotaxis, yielding K_i values between 0.07-1.02nM for different CC-chemokines (Lalani *et al.*, 1998; Smith *et al.*, 1997).

1.3.2.Myxoma virus T1 protein

The myxoma virus T1 protein is the gene product of the first open reading frame within the myxoma virus genome, present in duplicate copies in the terminal inverted repeats (Graham *et al.*, 1997). The purified protein has an apparent molecular mass of 43kDa under reducing and denaturing conditions, and is post-translationally N-linked glycosylated (Graham *et al.*, 1997). M-T1 shares approximately 40% identity with other members of the 35kDa family of poxvirus chemokine binding proteins, with conservation of eight cysteine residues in the protein (Graham *et al.*, 1997). M-T1 is expressed at two to four hours post infection and continues to be secreted during the expression of late genes (Lalani *et al.*, unpublished).

Preliminary studies with M-T1 demonstrated that it was a non-species specific chemokine binding protein, through cross-linking assays with a variety of CC-

chemokines. Scatchard analysis of M-T1 binding with the human CC-chemokine RANTES revealed a K_d of approximately 73nM (Graham *et al.*, 1997). Binding studies with orthopoxvirus members of the CBP-II family, however, yielded K_d values nearly 100 fold smaller. The K_i values obtained for the orthopoxvirus proteins, from inhibition binding assays, were in the same range as those obtained for M-T1. M-T1, however, is a leporipoxvirus protein, which may account for the discrepancies seen between its K_d value and those obtained for the orthopoxviruses. Studies testing the ability of several CBP-II members to crosslink the CXC-chemokine IL-8, revealed a lower affinity (K_d=50nM for vaccinia virus 35kDa protein) compared to the CC-chemokines tested, suggesting only low affinity binding (Graham *et al.*, 1997; Smith *et al.*, 1997).

The isolation and purification of the M-T1 protein allowed for further experimentation with the protein, revealing its capability as a potent chemokine inhibitor (Lalani *et al.*, 1998). In calcium mobilization assays, M-T1 was shown to inhibit the induction of intracellular calcium release in THP-1 cells, a human pro-monocytic cell line, by human CC-chemokines with a K_i of 0.456nM for MIP-1α, and 0.188nM for MCP-1 (Lalani *et al.*, 1998). Interestingly, no inhibition of a calcium response in HL-60 cells, a promyelocytic leukemic cell line expressing CXC-chemokine receptors, was seen during co-incubation of M-T1 with the CXC-chemokine IL-8 (Lalani *et al.*, 1998). Similarly, M-T1 was shown to inhibit the chemotaxis of human primary monocytes in response to the human CC-chemokine MIP-1α,with a 50% inhibitory constant of 10.5nM (Lalani *et al.*, 1998). M-T1 did not, however, functionally inhibit the chemotaxis of human primary neutrophils in response to the human CXC-chemokine IL-8 (Lalani *et al.*, 1998).

To further examine the role of M-T1 in virulence, *in vivo* studies were performed with an M-T1 knockout myxoma virus construct (Lalani *et al.*, 1999). European rabbits infected with the knockout virus contained an increased number of infiltrating leukocytes in tissue lesions around the site of primary infection compared to the numbers observed during wild type infection (Lalani *et al.*, 1999). Summarized, this data suggests a role for M-T1 in mediating and inhibiting the normal trafficking of leukocytes during the inflammatory response to myxoma virus infection. It is clear that M-T1 and members of the 35kDa family have similar functions, which may be linked to areas of conservation among the proteins.

1.4. Thesis objective

The hypothesis that provided the basis for this thesis was that M-T1 altered the function of chemokines. The goal of the thesis study was to determine what domain(s) of the viral protein were/are necessary for this inhibitory function.

To accomplish this goal, the *in vitro* assay of radiolabeled chemokine binding to cognate cell surface receptors was utilized as a model for testing chemokine function. The inhibitory capacity of M-T1, obtained through the calculation of inhibitory constants, was determined by adding it to this system. A variety of M-T1 internal deletion mutants were created, expressed by the baculovirus system, and tested for inhibition of chemokine binding, resulting in the identification of regions necessary for M-T1 function.

Chapter 2: Materials and Methods

2.1. Materials

The pAlter vector and Altered Sites II in vitro Mutagenesis Systems kit were purchased from Promega. Oligonucleotides used in site directed mutagenesis were purchased from and prepared by the laboratory of Dr.David Kelvin, London, Ontario. PCR primers were purchased from Promega. Restriction enzymes and T4 DNA Ligase were purchased from New England Biolabs (NEB). DH5α cells were purchased from GIBCO BRL. The TNT Coupled Reticulocyte Lysate Systems kit was purchased from Promega. The pSP6-2 vector was obtained from Dr. Alex Yu, MBI Fermentas. Cell culture media RPMI1640, penicillin/streptomycin, and L-glutamine were purchased from BioWhittaker. Grace's cell culture medium and fetal bovine serum were purchased from GIBCO BRL. Percoll, dextran, and Histopaque-1077 were purchased from Sigma Chemical Co. pBacPak vector and BacPAK6 DNA were purchased from CLONTECH. Sf-900 SFM (serum-free media) and CELLFECTIN reagent were purchased from GibcoBRL. Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. [^{125}I] MIP-1α and [^{125}I] MCP-1, (2200 Ci/mmol), were purhased from NEN™ Life Science Products, Inc. L-[^{35}S] Methionine (1000 Ci/mmol) was purchased from Amersham. Tween was purchased from FisherBiotech, and phosphate buffered saline (PBS) was purchased from Gibco BRL. Horseradish peroxidase (HRP) – conjugated goat anti-rabbit antibody and X-Omat Blue XB-1 film was purchased from Bio-Rad and Kodak, respectfully.

2.2. Site directed mutagenesis

The parental plasmid used in these studies was the pAlterT1 plasmid which contains a 1kbp fragment of the myxoma virus genome, obtained from the plasmid pMJT1 (Graham, 1997) and sub-cloned in the pAlter vector (Promega), by using *Bam*HI (NEB) as shown in Figure 2.2. Orientation of M-T1 was confirmed by *Pst*I (NEB) restriction endonuclease analysis and sequencing reviewed in section 2.2.2 of Materials and Methods.

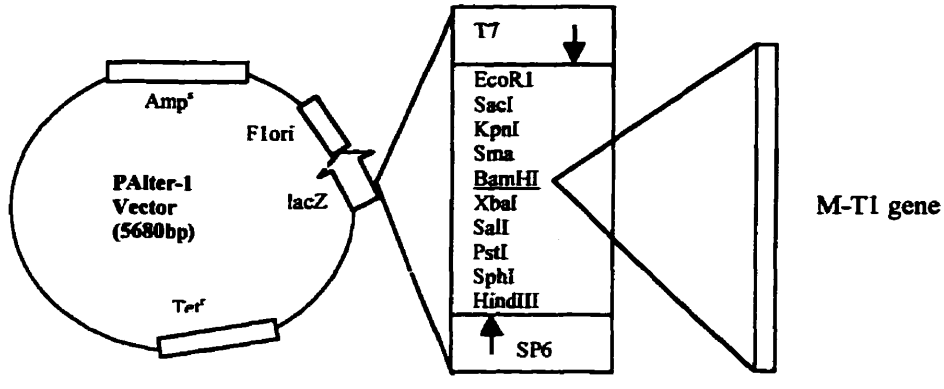


Figure 2.2: pAlter-1 vector-In vitro site directed mutagenesis using the pAlter-1 vector containing the M-T1 gene. See Materials and Methods for sub-cloning details. (Modified from Promega (1994) Technical Manual. Altered Sites II *in vitro* Mutagenesis Systems)

A series of mutant plasmids were constructed in pAlterT1 using the Altered Sites II *in vitro* Mutagenesis Systems (Promega) using the custom oligonucleotides described in Table 2.2. The plasmids were designated pAlterT1m1 through pAlterT1m8 based on the oligonucleotide annealed to each pAlterT1 plasmid to form the mutant plasmids.

2.2.1.Oligonucleotides

Oligomers M1-M7, were designed to introduce *Bgl*II restriction sites at various locations along the length of the M-T1 gene through the introduction of small base mutations. Oligomer M8 was constructed to introduce a *Nco*I site, as shown in Table 2.2. The mutations were created in such a way to cause only in-frame conserved amino acid substitutions.

Table 2.2: M-T1 gene mutations constructed by oligonucleotide-directed mutagenesis. Underlined nucleotides denote changes from the published M-T1 sequence (Graham *et al.*, 1997)

Mutation	Oligonucleotide	M-T1 codon(s) Altered	Amino Acid substitution(s)
M1	GGCATCTGCAGATCTGGCGAAGATGTC	23	Q to S
M2	TGTCAGGGTCTGAGATCTACGACTATTGAA	53,54	RT to RS
M3	GGAAGTACTTACAGATCTATCGTCGGAGGA	103,104	HT to RS
M4	GTGTCTATGAACAGATCTGAGGCGCTCGCC	154,155	RT to RS
M5	CCGTAGATATCAGATCTAGTCGCGTCAAC	171,172	KC to RS
M6	CGGAATGTGTGAGATCTCTAGACATAAC	212,213	KS to RS
M7	GTCTTAAGGTAAGATCTGGCAAACTACTC	258,259	KN to RS
M8	GGCCGCGACCATGGCGACGAAGG	15	L to M

2.2.2. Isolation and verification of mutant plasmid DNA

Mutant plasmid colonies growing on LB/Agar plates containing 100µg/ml of ampicillin, were isolated and grown overnight in 10ml of LB broth containing ampicillin (100µg/ml) in a 37°C shaker (Sambrooke *et al.*, 1992). Plasmid DNA was isolated using Qiagen mini prep columns (Qiagen, Inc.). The presence of each mutation was initially verified by restriction enzyme digestion to ensure that the appropriate restriction enzyme site had been introduced. The sequence of each mutant plasmid was then confirmed by

sequencing through the mutated areas, in both directions, using T7 and SP6 primers (Promega) described by Figure 2.2. The clones were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on an automated sequencing apparatus (Perkin-Elmer Applied Biosystems) at the Robart's Research Institute Sequencing facility.

2.3. Deletion mutant formation

2.3.1.Digestion Strategies

pAlterT1m1-7 mutant plasmids were strategically cut with restriction enzymes to yield 5' and/or 3'-fragments. 5'-fragments were ligated with 3'-fragments and inserted into the appropriately digested parental plasmid, pAlter, to form an intact plasmid. The restriction enzyme digestion strategy chosen for each mutant was based on the formation of a DNA fragment of sufficient size to allow for efficient isolation and ligation as shown in Table 2.3.

Table 2.3: Digestion strategies for pAlterT1 mutants

Mutant	5' Fragment Restriction Digest Strategy	Fragment Size(bp)	3' Fragment Restriction Digest Strategy	Fragment Size(bp)
PAlterT1m1	**AatII/BglII*	1046	*BglII/SphI**	902
PAlterT1m2	**AatII/BglII*	1136	*BglII/SphI**	817
PAlterT1m3	**AatII/BglII*	1290	*BglII/SphI**	663
PAlterT1m4	N/A	N/A	*BglII/SphI**	511
PAlterT1m5	N/A	N/A	*BglII/SphI**	460
PAlterT1m6	N/A	N/A	*BglII/SphI**	337
PAlterT1m7	N/A	N/A	*BglII/SphI**	272
PAlterT1	**AatII*		*SphI**	4687

N/A – Isolation of 5' restriction fragments from a variety of digestion strategies was not possible.

* *Enzyme site present in pAlter vector, not in M-T1 gene*

2.3.2. Ligation of restriction fragments and transformation of mutant plasmids

The restriction enzyme digests were loaded on a 1% agarose gel prior to electrophoresis in 1x Tris-Acetate/ethylenediaminetetra-acetic acid (EDTA) (TAE) at 100V. The bands were visualized under long wave ultraviolet light following gel staining with ethidium bromide. Using an UV transilluminator (VWR Scientific) at 302nm, the band of interest for each digest was excised using a razor blade and placed into appropriately labeled tubes. The DNA was eluted from the agarose using a Gene Clean kit (Bio/Can Scientific), and a small amount was once again electrophoresed through a 1% agarose gel in 1x TAE at 100V, to confirm the size of the DNA fragment and to determine its concentration. Ligation of different 5' and 3' restriction fragments together with the digested parental vector to yield a panel of deletion mutant plasmids (Figure 3.2) was accomplished using T4 DNA Ligase. (NEB) Deletion mutant plasmid constructs were then transformed by electroporation (Bio-Rad) into prepared electrocompetent *E.coli* DH5α cells (GIBCO BRL).

2.3.3. PCR analysis

Colonies were screened for the presence of deletion mutant plasmids through the use of the polymerase chain reaction. In short, T7 and SP6 primers were annealed to the plasmid DNA in the presence of Taq polymerase and the appropriate buffer solutions in a Minicycler^{TM} PCR machine (MJ Research) under the following conditions: 94°C for 5min., followed by 24 cycles of 1min. at 94°C, 1min. at 45°C, and 2min. at 72°C, then 72°C for 10min., and finally 4°C. The PCR products were loaded on a 1 % agarose gel prior to electorphoresis in 1x TAE at 100V and verified for band size corresponding to

the approximate size of the deletion mutant gene. Positive clones containing deletion mutant DNA were isolated and verified by sequencing as described in Section 2.2.2 of Materials and Methods. See Appendix B for an example of sequenced deletion mutant clone.

2.4. *In vitro* transcription/translation

2.4.1 Cloning into pSP6 vector

M-T1 deletion mutants created in pAlterT1 were subcloned into the pSP6-2 vector to perform coupled *in vitro* transcription/translation expression using the TNT Coupled Reticulocyte Lysate Systems kit (Promega). This was accomplished by subcloning several MT1DEL fragments (see Figure 3.2) from pAlterT1 into the pAlterT1m8 plasmid, which contains an oligonucleotide site directed *Nco*I site (CCATGG) after the signal sequence of the otherwise intact M-T1 gene (see section 2.2.1, Table 2.2). MT1DEL fragments were excised from pAlterT1 deletion mutant plasmids by digestion with restriction endonucleases *Pst*I and *BamH*I (NEB) and placed into pAlterT1m8. The corrected pAlterMT1DEL mutant plasmids, containing an *Nco*I site after the M-T1 signal sequence in the MT1DEL fragments, were then used for subcloning into the pSP6-2 vector by digestion with *Nco*I and *Sph*I (NEB) creating pSP6MT1DEL plasmids. The *Nco*I site containing an ATG codon in frame with the remainder of the M-T1 inserted gene would then function as the initiating methionine,

effectively removing the signal sequence from each deletion mutant being expressed *in vitro*, in attempts to mimic the *in vivo* occurrence of signal sequence cleavage.

2.4.2 Detection of pSP6MT1DEL mutants

pSP6MT1DEL mutant plasmids were isolated as described in Section 2.2.2 of Materials and Methods and expressed by coupled *in vitro* transcription/translation. In short, pSP6MT1DEL mutant DNA (0.5µg/µl) was mixed with TNT SP6 RNA Polymerase, TNT Rabbit Reticulocyte Lysate, Amino Acid Mixture Minus Methionine (1mM), Ribonuclease Inhibitor (40u/µl), [^{35}S] methionine at 1,200Ci/mmol (Amersham), TNT Reaction Buffer, and Nuclease-Free water in the proportions dictated by the general protocol for TNT Lysate Coupled Transcription/Translation Reactions (Promega).

Translated products were detected by following the incorporated [^{35}S] methionine (Amersham) through SDS-PAGE analysis. A percentage of the reaction (5µl) was added with an equivalent volume of SDS gel loading buffer, containing 100mM dithiothreitol (DTT) and 2% SDS, and boiled to denature the proteins prior to electrophoresis on a 12% SDS-polyacrylamide gel at 140V, using the Laemmli buffer system (Laemmli, 1970). Gels were dessicated using a BIORAD MODEL 583 Gel Dryer, and exposed to X-Omat Blue XB-1 film (Kodak) in a cassette containing an intensifier screen for approximately 2 days at 37°C. Bands were examined by developing the film in a RGII FUJI x-ray film processor.

2.5. Cell lines and isolation of human primary cells

THP-1 cells (generously donated by the laboratory of Dr. David Kelvin at the Robart's Research Institute) were used in the inhibition of binding experiments described in Section 2.7 of Materials and Methods. This cell line is a human promonocytic cell line, and was maintained at 37°C and 5% CO_2 in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum (GIBCO BRL), 100u/ml penicillin, 100µg/ml streptomycin (BioWhittaker), and 2mM L-glutamine (BioWhittaker).

Spodoptera frugiperda-21 (SF-21) cells (Vaugh *et al.*, 1977) were maintained at 27°C in Grace's media (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL), 100u/ml penicillin, 100µg/ml streptomycin (BioWhittaker), and 2mM L-glutamine (BioWhittaker). These cells were used for expression assays with baculovirus pBacPakMT1 deletion mutants.

Human primary monocytes and neutrophils, isolated from the blood of healthy donors according to the methods of Wang *et al.*, (1993), were used in the inhibition of binding experiments described in Section 2.7. Human blood was kindly provided by donors recruited by the laboratory of Dr. Ross Feldman at the Robart's Research Institute. Blood was fractionated over Histopaque-1077 (Sigma Chemical Co.) by density centrifugation. The interface layer containing peripheral blood mononuclear cells was further fractionated over a Percoll (Sigma Chemical CO.) gradient to yield a layer containing a >90% pure population of monocytes as assessed by morphological criteria. The lower layer was treated with 3% dextran (Sigma Chemical Co.) to isolate the human primary neutrophils and further clarified by hypotonic lysis with NaCl to remove red blood cells. Cells were finally washed and resuspended in phosphate buffered saline.

2.6. Baculovirus expression

2.6.1. Cloning into pBacPak1

Several pSP6MT1DEL mutants were subcloned into pBacPak1 (CLONTECH Laboratories Inc.) by *Pst*I and *Xho*I (NEB) restriction enzyme digest of MT1DEL fragments from the pSP6MT1DEL mutants. The fragments were then isolated and inserted into the *Pst*I and *Xho*I (NEB) digestion of the intact M-T1 gene present in pBacPakMT1 (generously donated by Rajkumari Singh), as described in Section 2.3.2 of Materials and Methods. pBacPakMT1DEL clones were verified by PCR analysis described in Section 2.3.3 using the following primers:

5'-primer Bac 1: ACCATCTCGCAAATAAATAAG and 3'-primer Bac 2: GCGATCTAAGACACGCAACA. Mutant pBacPakMT1DEL DNA from positive clones was isolated and purified as described in Section 2.1.2 of Materials and Methods.

2.6.2. Transfection of pBacPakMT1DEL mutants

The expression of pBacPakMT1DEL mutants was accomplished by co-transfecting SF21 insect cells with pBacPakMT1DEL mutant DNA and BacPAK6 DNA (CLONTECH). BacPAK6 DNA is linearized (*Bsu*36I) *Autographa Californica Nucleopolyhedrovirus* (AcNPV) DNA specifically designed and prepared to give a high proportion of recombinant viral expression vectors. Briefly, SF21 cells were seeded in a six-well tissue culture plate (Becton Dickinson and Co.) at 9 x 10^5 cells/well in Sf-900SFM (GibcoBRL) and allowed to attach for at least one hour. For each transfection, two solutions were prepared in sterile polystyrene tubes, one containing 2μg of

pBacPakMT1DEL mutant DNA, 5µl of BacPAK6 DNA (CLONTECH) up to 100µl of Sf-900SFM and the other containing 5µl of CELLFECTIN (GibcoBRL) up to 100µl with Sf-900SFM. The two solutions were then combined and left at room temperature for 30min. An additional 800µl of Sf-900SFM was added to each of the lipid-DNA transfection solutions, which were then overlayed onto the SF21 cells that had been washed once with 2ml Sf-900SFM (one transfection solution per well). The cells were incubated for 5hrs at 27°C, after which the transfection solution was removed and replaced with 2ml of Sf-900SFM. Cells were placed at 27°C and assayed for protein expression 48hrs, 72hrs, and 8 days post transfection.

2.6.3 Western Blot analysis of pBacPakMT1DEL mutants

Supernatant from pBacPakMT1DEL transfected cells were removed and placed into appropriately labeled tubes. To 20µl of each supernatant, an equivalent volume of SDS gel loading buffer containing 100mM DTT and 2% SDS was added. The samples were boiled for 5min prior to electrophoresis of samples through a 12% SDS-polyacrylamide gel using the Laemmli buffer system (Laemmli *et al.*, 1970) at 140V. Proteins were transferred to Hybond-C (Amersham Pharmacia Biotech) nitrocellulose membrane by electroblotting at 14V for 1 hour. Blots were blocked in TBS (150mM NaCl, 2.5mM KCl, 25mM Tris-HCl, pH 7.4) containing 5% skim milk powder (w/v) and 0.1% Tween 20 (v/v) overnight at 4°C. Proteins were detected by incubating with 0.02% rabbit anti-M-T1 (v/v) in blocking buffer, at room temperature with agitation. The polyclonal anti-M-T1 is directed at the C-terminal decapeptide (-LRTPTLKACN) at residues 251-260 in the intact M-T1 protein (Graham *et al.*, 1997). After washing 3 times

with TBS containing 0.1% Tween 20 (v/v), membranes were incubated in 0.01% horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (v/v) (Bio-Rad) in blocking buffer, for an additional hour at room temperature with agitation. After washing three more times with TBS/Tween, expressed proteins were visualized by ECL detection (Amersham Pharmacia Biotech) on XB-1 Kodak x-ray film.

2.6.4 Determination of protein concentration

To determine the approximate concentration of each baculovirus expressed protein, a 5µl and 10µl sample of each protein along with samples of purified M-T1 (pM-T1) of known concentration (50ng, 250ng, 500ng) were electrophoresed on a 12% SDS polyacrylamide gel and analyzed by Western blot, as described in Section 2.6.3 of Materials and Methods. Bands were examined using the BioRad Model GS-700 Imaging Densitometer and the Multi-Analyst™ (Bio-Rad) software. The optical density within a selected area, which remained constant for each sample, encompassing the band of interest was measured. Linear regression for the pM-T1 standards was applied (Microsoft Excel) yielding a slope for determination of unknown protein concentration from the equation of the line whereby: $y=mx+b$ and

Y= Adjusted Volume (OD x mm x mm) –The optical density of object (OD) within identified area (mm^2) adjusted for background removal
m=Slope
x=Concentration of unknown protein (ng)
b=intercept

Densities for all baculovirus expressed proteins fell within the linear range of the pM-T1 standard curve, and concentration/volume for each sample (5,10µl) was calculated yielding an average concentration for each baculovirus expressed protein.

2.7. Inhibition of binding

2.7.1. Inhibition of chemokine binding to cell surface receptors

THP-1 cells or human primary cells isolated from human blood, as described in Section 2.5, were washed with binding buffer (RPMI-1640 supplemented with 1mg/ml BSA (EM Science) and resuspended at 10^7 cells/ml. All sample reactions were prepared in triplicate on ice.

The total amount of radiolabeled ligand capable of binding expressed cognate receptors on the surface of cells, in the absence of competitor, was determined by preparing a sample reaction containing 50,000cpm of [^{125}I]chemokine (NEN™ Life Science Products, Inc.) per tube diluted to 100µl with binding buffer. To assay the inhibitory properties of proteins, M-T1 and vv35K, sample reactions were prepared consisting of different concentrations of inhibitor to radiolabeled chemokine (determined by its specific activity). This was accomplished by adding the viral proteins in a variety of fold molar excess to sample tubes containing 50,000cpm of [^{125}I]chemokine, each to a final volume of 100µl with binding buffer. The amount of competition caused by a cold chemokine competitor was also assayed by preparing a sample reaction containing 400ng of the appropriate chemokine added to 50,000cpm of the [^{125}I]chemokine to a final volume of 100µl with binding buffer.

All samples were incubated at 37°C for 30min., whereupon 200µl of the binding buffer cell suspension was added to each of the sample reactions and incubated at 4°C for an additional hour with agitation. The tubes were centrifuged at 15,000rpm for 10min in a microcentrifuge, the supernatants aspirated, and the cells resuspended in 800µl of 10% sucrose (w/v) in PBS (Gibco BRL). The tubes were re-centrifuged at 15,000cpm for 10min, and the supernatant removed. The tops of the tubes were removed carefully using needle-nose pliers, and the Eppendorf tube bottoms containing the cell pellet placed in vials. Counts from the radiolabelled ligand bound to the cellular pellet were measured on a 1272 CliniGamma (LKB Wallac) gamma-counter.

2.7.2 Calculation of % binding, inhibitory constants, and statistical analysis

To determine a protein's efficiency in the inhibition of chemokine binding to the cell surface, % binding was calculated using the following formula:

$$\% \text{ Binding} = \frac{(\text{total cpm bound in the presence of protein}) - (\text{total cpm bound in cold competition})}{(\text{total cpm bound in the absence of protein}) - (\text{total cpm bound in cold competition})} \times 100$$

A mean value (±SD) was obtained for all triplicate samples, yielding an overall percentage binding value (±SD). For inhibition of binding experiments involving baculovirus expressed proteins, it was necessary to normalize the % binding to account for the inhibition resulting from proteins present in the supernatant. To accomplish this the % binding of pBacPakWT protein supernatants (produced by the wild type plasmid lacking M-T1 gene) were assigned 100% binding, and the values obtained for the remaining proteins in each experiment were normalized to this value, yielding a corrected value for binding (*%Binding). The total error was calculated using variances of the mean and propagated according to the rules of uncertainties (Armitage, 1994)

Where applicable, the inhibition constant K_i, was determined according to the equation:

$$\text{\% Binding} = \frac{\text{maximal \% binding}}{([\text{protein}]/ K_i)}$$

or *%Binding

from curve fits of the data % Binding vs. Concentration (nM). (Data not shown)

Chapter 3: Results

3.1. Inhibition of binding by pM-T1 and vv35K

3.1.1. Inhibition of select CC-chemokine binding to human primary monocytes

As shown in Figure 3.1A, binding of [^{125}I]MIP-1α and [^{125}I]MCP-1 to their appropriate receptors on human primary monocytes was effectively inhibited by molar excess amounts of pM-T1 in a dose dependant manner. In fact, almost complete inhibition of chemokine binding (<20%) was seen at 100 fold molar excess of the pM-T1 protein to chemokine. We can compare these observations with those seen in Figure 3.1D whereby 75 to 150 fold molar excess amounts of vv35K also effectively inhibit [^{125}I]MIP-1α and [^{125}I]MCP-1 binding to their cognate receptors on human primary monocytes, to less than 20% binding. Inhibition of binding of MIP-1α,by both inhibitors, is more efficient compared to MCP-1 on human primary monocytes.

3.1.2. Inhibition of select CC-chemokine binding to THP-1 cells

The binding of [^{125}I]MIP-1α and [^{125}I]MCP-1 to their appropriate receptors on THP-1 cells, a human promonocytic cell line, was also quantitatively inhibited by molar excess amounts of pM-T1 and vv35K to chemokine, as shown in Figure 3.1B and Figure 3.1E respectively. Almost complete blockage of receptor binding (<10%) by both chemokines was seen between 50-100 fold excess of pM-T1, and 75-150 fold excess of vv35K. Inhibition of binding of MCP-1, by both inhibitors, is more efficient compared to MIP-1α on THP-1 cells.

3.1.3. Inhibition of IL-8 binding to neutrophils

The binding of the CXC-chemokine [^{125}I]IL-8 to its appropriate receptor on human primary neutrophils was not effectively inhibited by molar excess amounts of pM-T1 as shown in Figure 3.1C. Greater than 90% of the chemokine IL-8 was able to bind its cognate receptors on neutrophils, even in the presence of 100 fold molar excess of pM-T1 to chemokine. Similarly, up to 300 fold molar excess amounts of vv35K also did not prevent [^{125}I]IL-8 binding to human primary neutrophils, with greater than 80% of the chemokine binding to its cognate receptors on neutrophils, as is shown in Figure 3.1F.

Taken together, these results suggest that pM-T1 and vv35K are capable of inhibiting the binding of at least two members of the CC-chemokine family to their cognate receptors on monocytes, whereas they cannot inhibit the binding of the CXC-chemokine, IL-8, to its receptors CXCR1 and CXCR2 on primary human neutrophils.

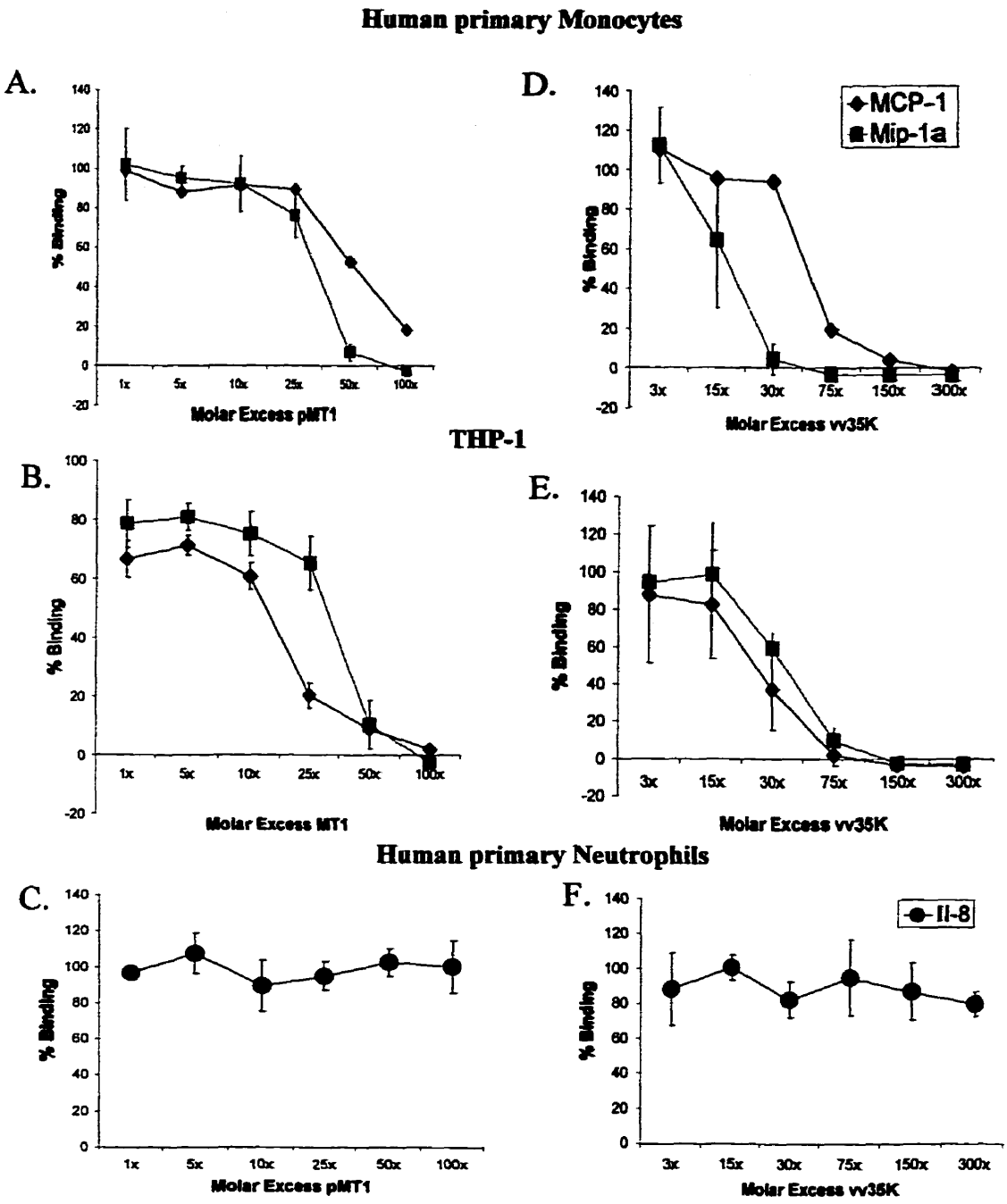


Figure 3.1 – Inhibition of binding by pM-T1 and vv35K. Pre-incubation of molar excess amounts of pure myxoma T1 protein with [^{125}I] labeled MCP-1 (diamonds) or [^{125}I]MIP-1α (squares) effectively inhibits binding of these chemokines to their appropriate receptors on human primary monocytes (panel A) and THP-1 cells (panel B). Pre-incubation with molar excess amounts of vaccinia virus 35K with [^{125}I]MCP-1 (diamonds) and [^{125}I]MIP-1α (squares) also effectively inhibits binding of these chemokines to their appropriate receptors on human primary monocytes (panel D) and THP-1 cells (Panel E). Experiments for M-T1 and vv35K inhibition of MCP-1 binding to primary human monocytes were only performed once, hence error bars were not obtained (Panel A & D). Pre-incubation with pM-T1 (panel C) and vv35K (panel F) did not effectively inhibit the binding of [^{125}I] Il-8 (circles) to its appropriate cells on neutrophils. % Binding (± SD) was determined as outlined in Materials and Methods.

3.1.4 Inhibitory constants for M-T1 and vv35K

Table 3.1 contains K_i values derived from the inhibition of binding data of MIP-1α and MCP-1 to their appropriate receptors on human primary monocytes and THP-1 cells by pM-T1 and vv35K. The K_i values were obtained by applying curve fits to the data shown in Figure 3.1, as described in section 2.7.2 of Materials and Methods.

Table 3.1 – Inhibitory constants for M-T1 and vv35K

Cells	Ligand	M-T1 K_i	vv35kDa K_i
1° Monocytes	MIP-1α	1.7 ± 0.50 nM	0.31 ± 0.20 nM
	MCP-1	6.4 nM*	2.5nM*
THP-1 Cells	MIP-1α	1.1 ± 0.33 nM	0.60 ± 0.26 nM
	MCP-1	1.1 ± 0.20 nM	0.69 ± 0.61 nM
1° Neutrophils	IL-8	-----	-----

*Insufficient number of experimental trials for determination of SD
No inhibition of Il-8 binding was seen hence K_i was not determined

3.2. Myxoma virus T1 deletion mutants

pAlterT1m1-7 mutant plasmid DNA was the initial DNA used to create a variety of internal and selected C-terminal deletions through utilization of inserted *Bgl* II restriction enzyme sites. The internal deletions were created in such a way as to span the length of the M-T1 gene, beginning with large deletions and progressing to smaller ones, as shown in Figure 3.2. The deletions were constructed in the pAlter vector and subsequently cloned into pSP6 and pBacPaK1 expression vectors for further experimentation.

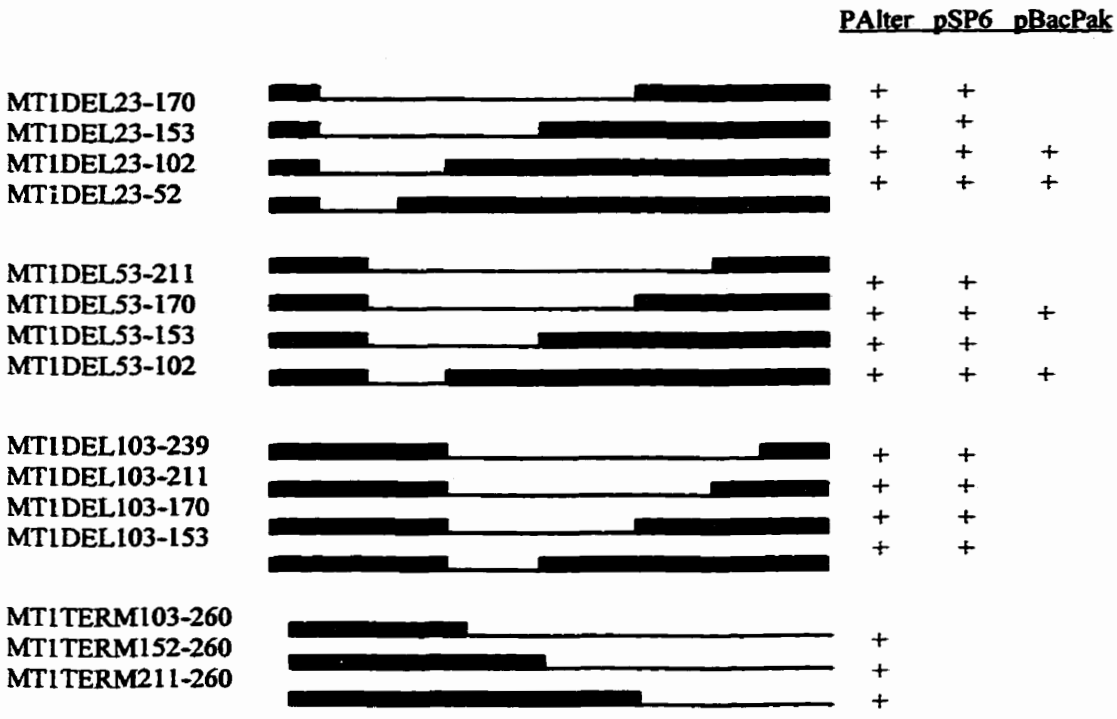


Figure 3.2 – Schematic diagram of myxoma virus T1 deletion mutants. Internal and C-terminal mutants of the M-T1 gene (260aa) were constructed in the pAlter plasmid and several were cloned into the two expression vectors pSP6 and pBacPak1 as illustrated. Lines represent areas deleted whereas boxes represent intact portions of the M-T1 gene. Plus signs identify presence of corresponding deletion mutants into designated plasmids.

3.2.1. Predicted molecular mass

Table 3.2.1. lists the predicted molecular masses of deletion mutants described in Figure 3.2. The molecular mass for each mutant protein was also calculated without the signal sequence to more accurately predict the true size of the expressed proteins. This is based on the absence of a signal sequence in the DNA constructs of those

mutants expressed *in vitro,* and signal sequence cleavage of baculovirus expressed mutants upon secretion *in vivo*. The number of remaining glycosylation sites was also determined to enhance analysis of the deletion mutants, based on their putative assignments in the intact M-T1 gene. (Graham *et al.*, 1997)

Table 3.2.1 –Predicted molecular mass of M-T1 mutants. Based on M-T1 sequence, the predicted molecular mass of each mutant was determined using the MacVector program. The number of amino acids present, as well as the putative number of glycosylation sites, are also described. The molecular masses of mutants were calculated without the signal sequence to properly analyze *in vitro* transcription/translation and baculovirus expressed mutants.

Mutants	A.A	Glycosylation Sites	M.M. (-Sig.Seq.) kDa	M.M. kDa
M-T1	246	2	26.8*/**	28.3
MT1DEL23-52	215	2	23.46**	24.96
MT1DEL23-102	165	2	17.85**	19.35
MT1DEL23-153	114	1	12.37	13.87
MT1DEL23-170	97	1	10.56	12.06
MT1DEL53-102	196	2	21.2**	22.7
MT1DEL53-153	145	1	15.72	17.02
MT1DEL53-170	128	1	13.93**	15.43
MT1DEL53-211	87	-	9.49	10.99
MT1DEL103-153	195	1	21.35	22.85
MT1DEL103-170	174	-	19.56*	21.06
MT1DEL103-211	136	-	15.13*	16.63
MT1DEL103-239	110	-	12.14*	13.64
MT1TERM103-260	250	-	26.79*	28.29
MT1TERM152-260	301	-	32.25	33.75
MT1TERM211-260	359	2	38.52	40.02

**Estimated M.W. for proteins shown in figure 3.3.1*
*** Estimated M.W. for proteins shown in figure 3.4.1.*

3.3. *In vitro* transcription/translation expression

In vitro expression by coupled transcription/translation was performed using pSP6MT1DEL DNA, resulting in mutant proteins containing incorporated [^{35}S] methionine detectable by autoradiography.

3.3.1. Autoradiograph of selected [^{35}S] labeled deletion mutants

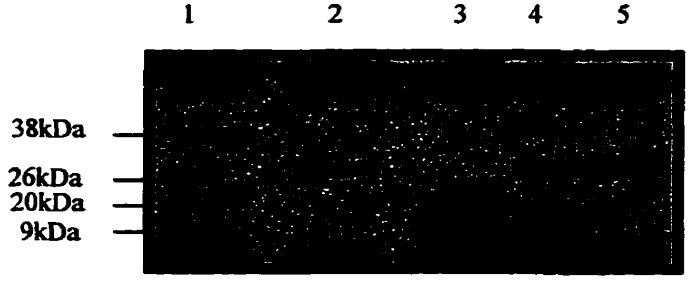
Autoradiography of several [^{35}S]-labeled M-T1 mutants is shown in Figure 3.3.1. (Panel A). The apparent molecular mass of mutant proteins was determined and their deviation from the apparent molecular mass of wt M-T1 was calculated (Panel B). The observed reduction in molecular mass for each mutant protein is also listed and comparison to the predicted reduction in molecular mass reveals only slight discrepancies. One reason for this may be due to the arrangement of amino acids in the proteins, which cause them to migrate slower on SDS-PAGE gels. Another possibility is that the samples were not fully denatured during boiling, or that even in the presence of dithiothreitol (DTT), the reducing process was not complete.

3.3.2. Inhibition of binding by pSP6T1 expressed by *in vitro* transcription/ translation.

The ability of pM-T1 to inhibit the binding of selected CC-chemokines to their cognate receptors on monocytes was shown in Results Section 3.1. To properly analyze the mutants expressed by *in vitro* transcription/translation, the properties of the wild type protein expressed by this method were initially tested. Figure 3.3.2 shows preliminary data collected for the binding of [^{125}I]MIP-1α to its appropriate CCR receptors on THP-1 cells in the presence of lysate containing pSP6MT1. Non-purified M-T1 expressed by *in*

vitro transcription/translation significantly inhibits the binding of [^{125}I]MIP-1α to its receptors on THP-1 cells, however an equal and perhaps greater inhibition of binding is observed for the reticulocyte lysate alone, which was the tentative negative control. This result implies that the lysate itself is participating in the inhibition of binding of [^{125}I] MIP-1α to its receptors on THP-1 cells. Therefore, no conclusions can be made as to the inhibitory properties of M-T1 expressed by this method, since the lysate itself inhibits the binding of [^{125}I]MIP-1α to its cognate receptors on THP-1 cells.

A.

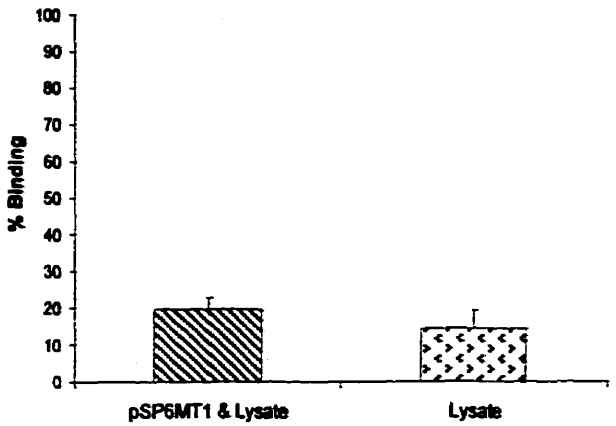


B.

Protein	Observed M.M.	Observed Reduction of M.M.	Predicted M.M.	Predicted Reduction of M.M.
PSP6MT1	41kDa	--------	26.80kDa	--------
PSP6MT1TERM103-260	33kDa	**8.0kDa**	26.79kDa	**0.01kDa**
PSP6MT1DEL103-239	24kDa	**17.0kDa**	12.14kDa	**14.66kDa**
PSP6MT1DEL103-170	34kDa	**7.0kDa**	19.56kDa	**7.24kDa**
PSP6MT1DEL103-153	36kDa	**5.0kDa**	21.35kDa	**5.45kDa**

Figure 3.3.1. Autoradiograph of selected [^{35}S]-labeled pSP6MT1 mutants. Panel A: Selected mutants were expressed and [^{35}S]-labeled by coupled in vitro transcription/translation (Promega). The proteins were run on a 12% SDS PAGE gel and detected by X-ray film overnight. Lane1: pSP6MT1, Lane2: pSP6MT1TERM103-260, Lane3: pSP6MT1DEL103-239, Lane4: pSP6MT1DEL103-170, Lane5: pSP6MT1DEL103-153. **Panel B:** Evaluation of the apparent molecular mass of mutant proteins, approximated from Panel A, in comparison to the predicted molecular masses listed in table 3.2.1. The observed reduction of molecular mass for mutant proteins is compared to that predicted, revealing a slight discrepancy.

Figure 3.3.2 - Inhibition of binding by M-T1 expressed by *in vitro* transcription / translation. Pre-incubation of pSP6MT1 expressed by *in vitro* t/t and lysates with [^{125}I]MIP-1α resulted in inhibition of chemokine binding to its appropriate receptors on THP-1 cells for both conditions suggesting interference of the lysate with the normal inhibitory function of M-T1. SD is based on values obtained from triplicate samples.



3.4. Baculovirus expression of T1 mutants

Expression of deletion mutant proteins by the baculovirus system was chosen as an alternative method for expression due to the several advantages offered by the system. These advantages include the production of large amounts of protein in a relatively short period of time, without the requirement for purification of recombinant virus, and the simple detection of expressed protein by Western Blot analysis.

3.4.1. Western blot analysis using the C-term α-M-T1 antibody

Supernatants were collected from transfected cells at 48hrs and 72hrs post-transfection. Panel A of Figure 3.4.1 is a Western blot of supernatants collected at these two time points. An apparent increase in M-T1 secretion at 72 hrs (lane 3) is observed, similar to the amount of protein expressed from supernatants of recombinant M-T1 baculovirus (lane 1), compared to the amount of viral protein being expressed at 48 hrs

(lane 6). Mock infected cell supernatants (lanes 2 & 5) and BacWT transfected supernatants (lane 4) were also assayed at these time points with no resulting bands, confirming the specificity of the antibody for M-T1 with no cross-reacting species in the supernatants at either time point.

Baculovirus expression is a larger scale process than *in vitro* transcription/ translation, hence only certain mutants cloned into pBacPak1 were chosen for expression by this system. Those chosen were based on the decision to test both the larger deletion mutants as well as those with a much more specific focus, in attempts to see differences in their inhibitory capacity. It was also necessary to express mutants containing the immunogenic decapeptide, allowing identification using the anti-M-T1 C-terminal antibody. The deletion mutants cloned into pBacPak1 and expressed are shown in Panel B of Figure 3.4.1. Once again the apparent molecular masses differ from the predicted ones in Table 3.2.1, possibly due to aggregates of the protein formed in the absence of completely denaturing or reducing conditions. Another possibility to explain the higher apparent molecular masses observed is post-translational processing, such as N-linked glycosylation. pM-T1 from myxoma virus has an apparent molecular mass of 43kDa under SDS-PAGE conditions, however it has been shown that treatment with the enzyme N-glycosidase F results in deglycosylation of the protein to 37-40kDa (Lalani *et al.*, 1998). This suggests that for pM-T1 at least one post-translational modification includes the linkage of N-linked oligosaccharides, hence it is possible that similar modifications are occuring in the baculovirus system.

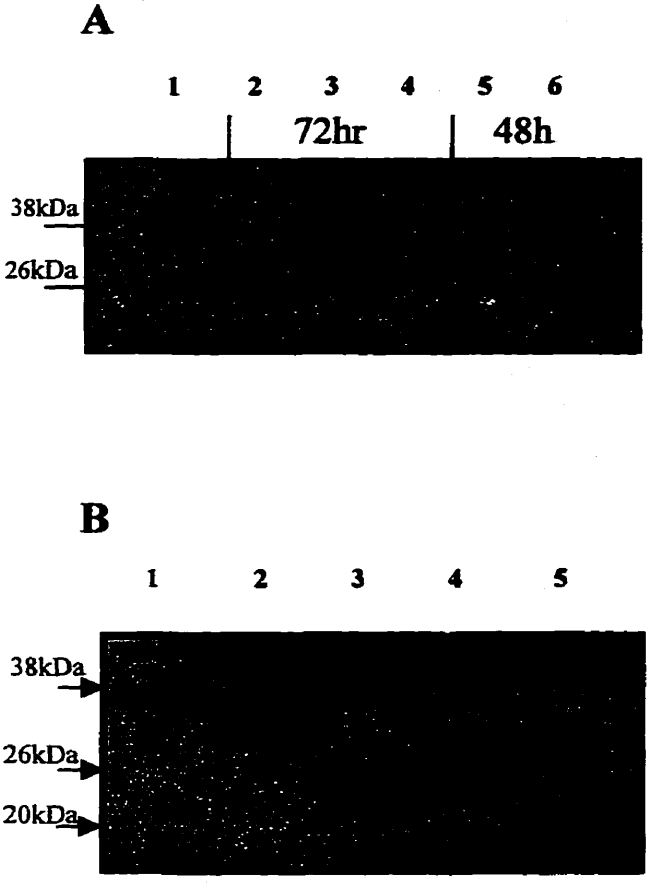


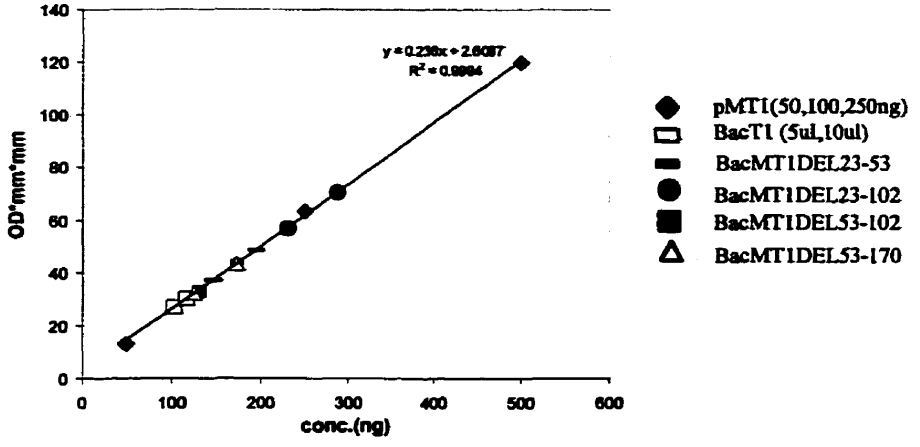
Figure 3.4.1 - Western Blot analysis of baculovirus expressed M-T1 & deletion mutants. Panel A: Secreted mutant proteins were collected post transfection, electrophoresed, transfered to nitrocellulose, and detected by an α-M-T1 antibody directed at the C-term. Supernatants of mock infected, BacT1 and BacWT s (lanes 2 , 3, 4, respectively) collected at 72h revealed an increase in secretion compared to mock infected and BacMT1 (lanes 5 and 6) yet similar to that of a Stable BacMT1 transfectant (lane 1). **Panel B:** Supernatants from BacT1(lane1), BacT1DEL23-52 (lane 2),BacT1DEL23-102(lane3), BacT1DEL53-102 (lane4), and BacT1DEL53-170(lane 5) were collected 8 days post transfection, electrophoresed, and transferred to nitrocellulose for detection by the C-term α-M-T1 antibody. Higher molecular weight bands (above 38kDa) present in lanes 3 and 4 may be a result of SDS-resistant dimer formation.

3.4.2. Estimation of protein concentration

The amount of protein present in the supernatants of each transfected mutant was crudely calculated using densitometry analysis of the protein bands identified by Western blot. As is shown in Panel A of Figure 3.4.2, a regression line was obtained based on known concentrations of pM-T1 standards. Supernatant samples at two volumes, 5 and 10μl, were applied to the regression analysis yielding concentration values. The densitometry readings of each supernatant sampled fell within the linear range dictated by the pM-T1 standards and an average concentration was determined for each protein, as is

shown in Panel B of this figure. The concentrations obtained demonstrate the abundance of protein that can be obtained through expression by this method.

A.



B.

Protein	Predicted Concentrations
BacMT1	16ng/µl
BacMT1DEL23-52	24ng/µl
BacMT1DEL23-102	37ng/µl
BacMT1DEL53-102	22ng/µl
BacMT1DEL53-170	21ng/µl

Figure 3.4.2: Estimation of protein concentration - Baculovirus expresed MT1 and deletion mutants were detected by Western blot along with pure MT1 standard dilutions of known concentrations. **Panel A:** Densitometry readings were taken using Multi-Analyst (Bio-Rad) software, and linear regression for the pMT1 standards was applied yielding a slope for determination of protein concentration. **Panel B:** Using densitometry values of the baculovirus expressed proteins falling within the linear range, protein concentrations were estimated based on the slope given by the equation of the line defined in panel A.

3.5. Inhibition of binding by baculovirus expressed M-T1 and select deletion mutants

Once the secretion of baculovirus expressed T1 and selected mutants was confirmed and their approximate concentrations in the supernatants estimated, the analysis of their function was performed. To accomplish this, inhibition of binding experiments were used to determine the inhibitory capacity of each baculovirus expressed protein in the binding of CC-chemokines to their appropriate CC-chemokine receptors.

3.5.1. Inhibition of MIP-1α binding to THP-1 cells

The inhibition of binding of the CC-chemokine MIP-1α to its appropriate CC-chemokine receptors on THP-1 cells was tested for baculovirus expressed T1 protein and selected mutants. Panel A of Figure 3.5.1 shows the ability of BacT1 to effectively inhibit the binding of MIP-1α to THP-1 cells in a dose dependant manner. Almost complete blockage of chemokine-receptor binding is seen between 120-175 fold molar excess, and application of a curve fit to the data shown in this panel, as described in Section 3.1.4, yields an inhibitory constant of 4.0 $\pm$ 1.2nM (data not shown). In comparison, T1 deletion mutants BacT1DEL23-53, BacT1DEL23-102, and BacT1DEL53-170 shown in Panel B, C, and E respectively, did not effectively inhibit the binding of MIP-1α to THP-1 cells even in the presence of 1000 fold excess of the protein. In fact, these proteins apparently increased the binding of MIP-1α to THP-1 cells with % binding values ranging between 100-150%. Only one mutant BacT1DEL53-102, shown in Panel D, did not apparently increase nor significantly inhibit MIP-1α binding. Even at 2000 fold excess of the protein, binding was reduced only to 75%. These results suggest that although T1 expressed by baculovirus retains its inhibitory capacity, the deletions

present in all T1 mutants abolish this inhibitory capacity. In the case of BacT1DEL53-102 some functional inhibition may be retained by the protein, but only in the presence of extreme molar excess. This suggests a much larger K_i value than those found for pM-T1 or BacT1, however further studies are needed to reinforce this finding.

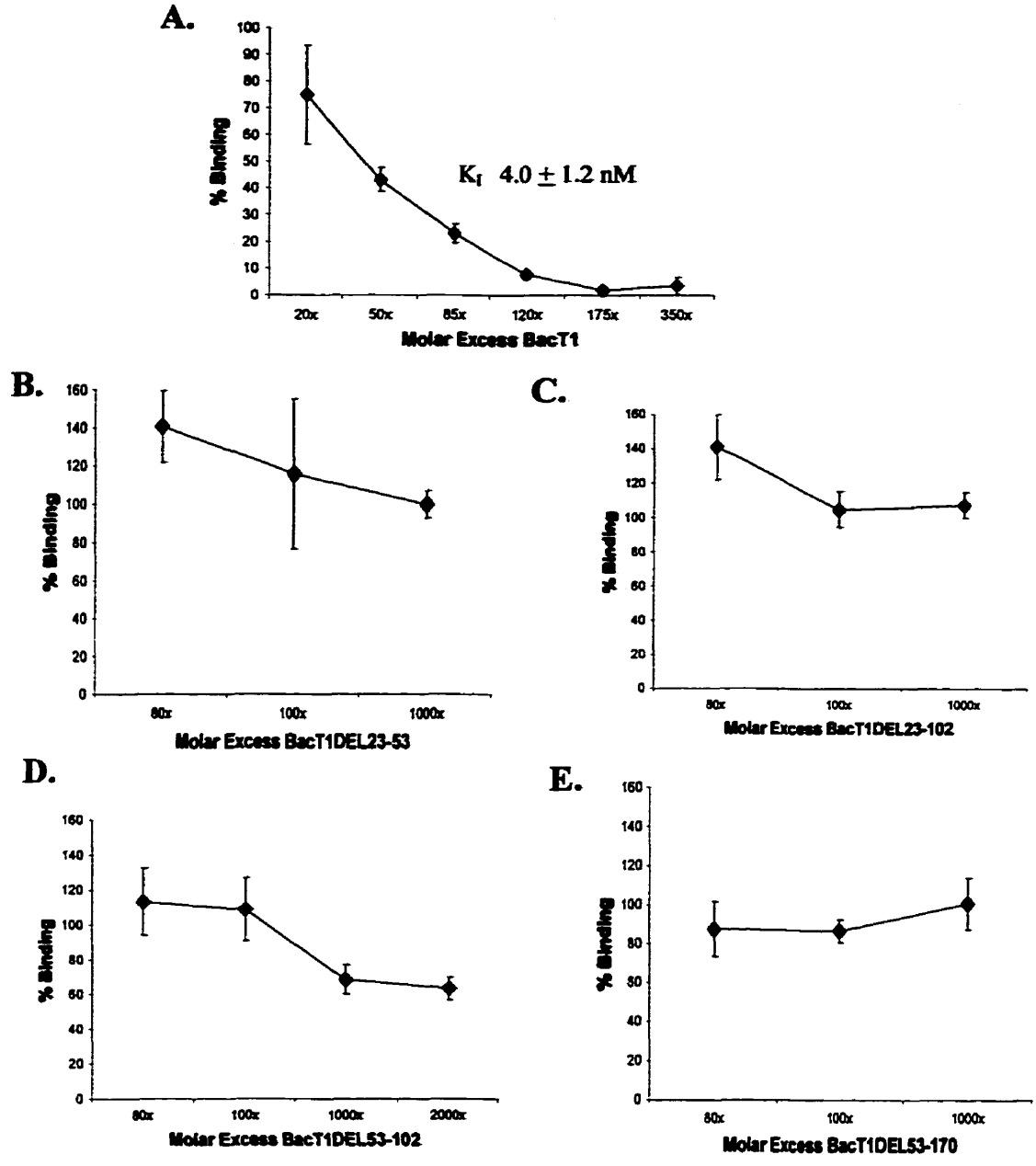


Figure 3.5 - Inhibition of binding of [^{125}I]MIP-1α to THP-1 cells by baculovirus expressed M-T1 and select deletion mutants. Pre- incubation with molar excess amounts of baculovirus expressed T1 with [^{125}I]MIP-1α demonstrates effective inhibition of chemokine binding to its receptors on THP-1 cells with a K_i of 4.0 ± 1.2 nM (panel A). Pre-incubation with molar excess amounts of baculovirus expressed mutants BacT1DEL23-52 (panel B), BacT1DEL23-102 (panel C), BacT1DEL53-102 (panel D), and BacT1DEL53-170 (panel E) with [^{125}I]MIP-1α did not result in any effective inhibition of chemokine binding to its receptors on THP-1 cells. % Binding (± SD), and K_i was determined as outlined in Materials and Methods.

Chapter 4: Discussion

4.1. Inhibition of binding by M-T1 and vv35K

Examination of virus-host interactions has lead to the identification of viral proteins that enable virus replication and dissemination in the presence of an active host immune response. Whether they function as viromimetic or virostealth proteins (Nash *et al.*, 1999), each has its role in the protection of the virus to ensure its propagation within the host. Members of the CBP-II family of chemokine binding proteins are prime examples of viral immunomodulators that act at the level of the inflammatory response. Several members of the T1/35kDa family of poxvirus chemokine binding proteins, including M-T1 and vv35K, cause disruption of the normal inflammatory response through the binding of members of the CC-chemokine family. Recent studies have revealed that this high affinity binding results in the inhibition of several CC-chemokine functions, such as the chemotaxis and cell signaling of target cells *in vitro* (Lalani *et al.*, 1998; Smith *et al.*, 1998; Alcami *et al.*, 1998).

Here, we demonstrate that M-T1 and vv35K proteins inhibit an additional function of chemokines; namely binding to their cognate receptors on cells. The analysis of [^{125}I]MIP-1α and [^{125}I]MCP-1 binding to their appropriate receptors on human primary monocytes and THP-1 cells in the presence of M-T1 and vv35K revealed potent inhibitory properties of both proteins in a dose dependent manner. It was also shown, in the analysis of [^{125}I]IL-8 binding to human primary neutrophils in the presence of M-T1 and vv35K, that these proteins do not interfere with CXC-chemokine binding. Inhibitory constants were calculated from the inhibition of binding data with human primary

monocytes and THP-1 cells, yielding sub-nanomolar to nanomolar K_i values for M-T1 and vv35K. These values were consistently lower for vv35K compared to M-T1, which may reflect the effects of species-specificity, since only recombinant human chemokines were used in these experiments.

Together this data supports the hypothesis that the predominant mechanism by which these proteins function is by inhibiting CC-chemokine induced chemotaxis by competitively binding CC-chemokines in solution away from their cognate receptors, as is shown in figure 4.1(Nash *et al.*, 1999). Binding of M-T1 and vv35K to the N-terminal high affinity receptor-binding domain of CC-chemokines, thus preventing the interaction of the CC-chemokine with its receptors on the cell, can be postulated. Alternatively, M-T1 and vv35K may not bind to the high affinity receptor-binding domain, but instead to another domain on the chemokine, resulting in a conformational change in the chemokine which would also block receptor binding. This hypothesis suggests that the binding domains on chemokines are not independent of one another, but structurally linked. Previous studies have supported this idea, for example mutation of the low affinity heparin binding domain of MIP-1α also inhibits the high affinity binding of the protein to the receptor CCR1 and the ability to cause monocyte chemotaxis (Graham *et al.*, 1996). Finally, M-T1and vv35K may be acting at the cell surface causing, directly or indirectly, the downregulation of chemokine receptors resulting in an apparent reduction in radiolabeled chemokine binding. Future studies will clarify whether one, or a combination of these models, are in effect to prevent the binding of CC-chemokines to their receptors on cells, as shown in this study.

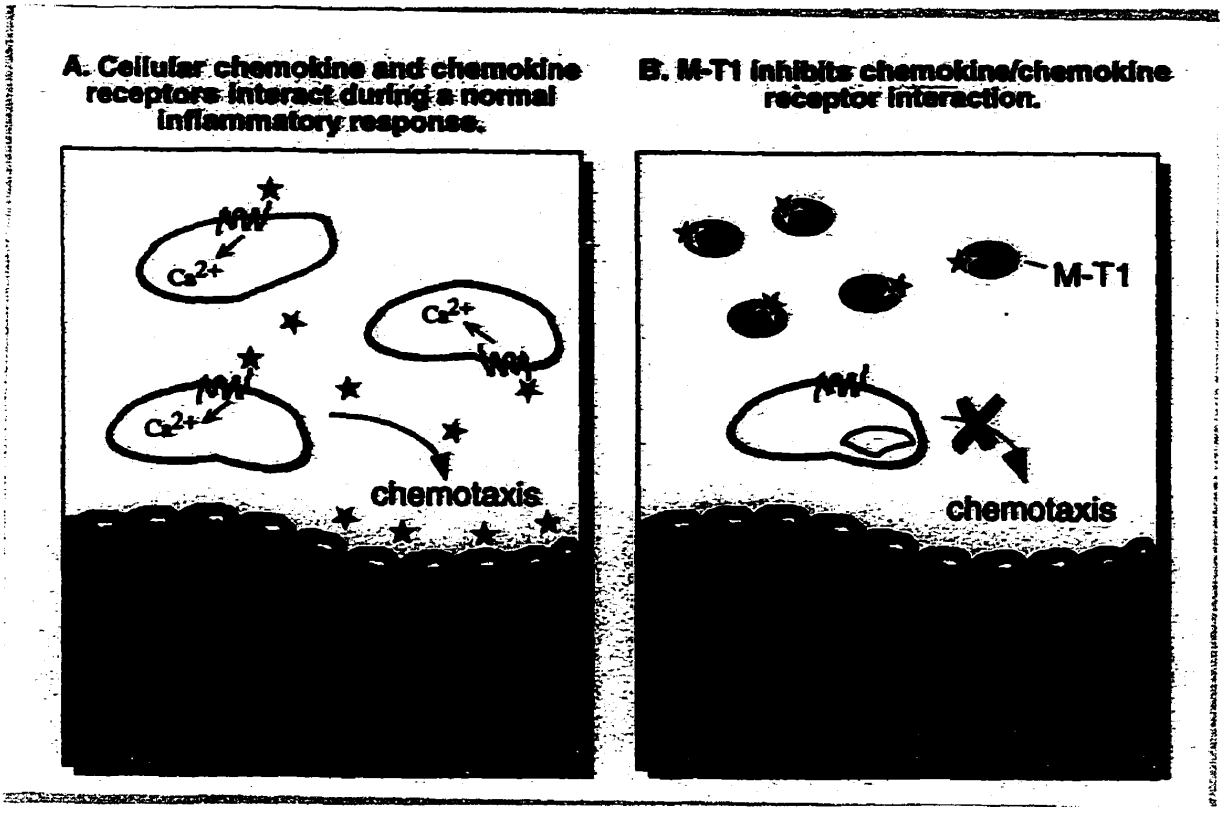


Figure 4.1 Model for M-T1 inhibitory function – Panel A: Recruitment of leukocytes to the site of tissue damage or infection, via a chemokine gradient (stars), during a normal inflammatory response. **Panel B :** Binding of myxoma virus T1 protein (ovals) to host chemokines (stars), preventing association with their cognate receptors on cells and formation of a chemokine gradient, and therefore blocking chemotaxis and calcium signaling. (Figure taken from Nash *et al.*, 1999, see Appendix A for copyright release)

The inhibition of chemokine-receptor binding, by any of the models postulated, is the presumed cause for the inhibition of monocyte chemotaxis and cell signaling studied previously (Lalani *et al.*, 1998; Smith *et al.*, 1998; Alcamí *et al.*, 1998). Together, *in vitro* results with these immunomodulators suggest an alteration of the early inflammatory response by retarding the influx of leukocytes to sites of infection, preventing virus clearance. Preliminary *in vivo* studies with a myxoma virus M-T1 knockout demonstrate an increased number of migrating leukocytes, in the first few days post infection,

compared to the wild type virus (Lalani *et al.*, 1999). This supports the hypothesis that in the presence of M-T1, there is a reduction in the amount of migrating leukocytes. The members of this CBP-II family may also be working in conjunction with other immunomodulators with complementary functions. The exact mode of function of these proteins remains to be discovered, however studies identifying domains responsible for the anti-inflammatory properties of these proteins have large implications in the prevention of disease. This provides the rationale for this thesis, which was to identify the domains necessary for the inhibitory function observed by myxoma virus M-T1 protein.

4.2. M-T1 deletion mutant formation

M-T1 and several members of the CBP-II family of poxvirus chemokine binding proteins have comparable sequences, with 81-99% amino acid identity between orthopoxvirus members, 70% amino acid identity between leporipoxvirus members, and 40% amino acid identity between leporipoxvirus and orthopoxvirus members (Graham *et al.*, 1997). Despite the varying identities, each protein maintains conservation of eight cysteine residues and also contains several acidic segments that are often 5-6 amino acids long. Studies examining the involvement of cysteine residues and disulphide linkages in proteins have revealed their importance in the proper functioning of proteins (Stuber *et al.*, 1993; Tournamille *et al.*, 1997). Here, we hypothesized that areas of conservation with the CBP-II members are likely to be involved in the function of the protein, having withstood the evolutionary pressures that lead to the divergence of each poxvirus.

In order to identify the exact determinants for M-T1 function, a variety of internal deletion mutants were created that spanned the length of the gene. One set of deletion mutants created, lacked one or more conserved cysteine residues; whereas another set of deletion mutants created, eliminated groups of acidic amino acids. The strategy chosen to examine the different domains was one of nested deletions. This involved creating large internal deletions, which were progressively narrowed down in each mutant, in an attempt to pinpoint the regions necessary for T1 function. The deletion mutants were initially created in the pAlter vector, through the digestion and ligation of engineered restriction enzyme sites created by site directed mutagenesis. Several mutants were then cloned into two separate expression vectors to enable testing; namely the pSP6 vector for in vitro transcription/translation, and the pBacPak1 vector for baculovirus expression.

4.3. Wild type M-T1 and M-T1 deletion mutant expression

In vitro transcription/translation was chosen initially as the ideal method for mutant protein expression due to its efficiency, though this method produces relatively small quantities of recombinant proteins. Binding studies presented in this thesis confirm that only a small quantity of purified M-T1 protein is necessary for inhibition, making *in vitro* transcription/translation a fast and easy candidate for mutant expression. Several deletion mutants were cloned into the pSP6 transcription/translation vector. The mutants were cloned in such a way as to remove the M-T1 signal sequence upon insertion into the vector, to mimic signal sequence cleavage *in vivo*. Restriction enzyme digest and ligation

at the *Nco*I site was performed to provide the AUG necessary for initiation of translation at the first codon following the signal sequence in the intact M-T1 gene. Expressed mutants containing incorporated [^{35}S]-methionine were viewed by autoradiography, revealing M-T1 and mutant proteins. It was demonstrated that the reticulocyte lysate, alone, was just as effective as an equal volume of wild type M-T1 expressed in reticulocyte lysate, in the inhibition of binding of [^{125}I]MIP-1α to its appropriate receptors on THP-1 cells. In an attempt to eliminate components of the lysate, following translation, molecular mass exclusion spin columns were used to partially clear the lysate of molecular components of 100kDa or more. The flow-through, supernatant, and filter from the column were examined by autoradiography revealing that M-T1, although sufficiently small to pass through the filter, was retained in the supernatant, and within the filter itself (data not shown). This suggests that M-T1 binds a component of the lysate, creating a complex too large to pass through the filter, or that M-T1 itself is aggregating to form a complex of larger molecular mass. Although treatment of the lysate with 1% NP40 resulted in the release of M-T1 from the filter, another method for M-T1 and mutant expression was chosen to avoid any further problems.

Therefore, several mutants were subcloned into a baculovirus expression vector, pBacPakMT1, already containing the M-T1 gene from an initial cloning step. Expression of these mutants was accomplished through transfection with linear viral DNA in SF21 cells. Expression was detected at 72 hours post transfection for M-T1, and at various times post-transfection for a variety of deletion mutants. Deletion mutants BacT1DEL23-53, BacT1DEL23-102, BacT1DEL53-102, and BacT1DEL53-170 were

successfully expressed and analyzed by Western blot analysis using an α-M-T1 antibody directed at the C-terminus (Lalani *et al.*, 1998).

4.4. Inhibition of binding by baculovirus expressed M-T1 and deletion mutants

To ensure that supernatants containing essential molecules expressed from the pBacPak vector did not interfere with the binding experiments, baculovirus-expressed M-T1 was initially tested for its ability to inhibit CC-chemokine binding, as was the supernatant isolated from transfection with wild type plasmid. These studies reveal that wild type supernatants enhance the binding of [^{125}I] MIP-1α to THP-1 cells, however in the presence of secreted M-T1, inhibition of binding is seen in a dose dependent manner. To account for this accentuated binding, percentage binding of wild type supernatant, determined for each quantity of M-T1 supernatant tested, was normalized to 100%, yielding a corrected value for the percentage binding in the presence of M-T1. Here we show that baculovirus expressed M-T1 potently inhibits the binding of [^{125}I]MIP-1α to THP-1 cells with a K*i* of 4.0 ± 1.2 nM. Comparison of this value with that observed for myxoma virus expressed purified T1 (1.1 ± 0.33 nM), suggests that expression from baculovirus might yield a T1 protein that differs functionally from myxoma virus expressed T1. Such differences might be due to differences in the post-translational modification that each system imparts to its proteins, such as glycosylation differences. Myxoma virus expressed M-T1 is sialylated *in vivo*, however baculoviruses and the cells

they infect lack a sialyl transferase. Baculovirus expressed M-T1 may also interact with molecules present in the supernatant, which may interfere with its potency as an inhibitor.

The inhibitory properties of baculovirus expressed M-T1 deletion mutants were then tested for their ability to inhibit the binding of [^{125}I]MIP-1α to THP-1 cells. Once again, an equal quantity of wild type supernatant was tested in parallel to each supernatant containing mutant protein, to obtain a corrected value for percentage binding, as was done for M-T1. Deletion mutants BacT1DEL23-53, BacT1DEL23-102, BacT1DEL53-102, and BacT1DEL53-170 were tested and shown to be unable to inhibit the binding of [^{125}I]MIP-1α to THP-1 cells at any concentration, suggesting loss of function.

These results demonstrate these mutant proteins as lacking any of the inhibitory properties seen in the native M-T1 protein. The percentage binding values for BacT1DEL23-53, BacT1DEL23-102, and BacT1DEL53-170 are greater than 100% suggesting that these proteins are contributing to, as opposed to inhibiting, [^{125}I]MIP-1α binding to the cell surface. This may be due to structural changes and charge differences in the mutant protein that may make them adherent, causing them to have affinity for both the CC-chemokine, and the cell surface, resulting in accentuated binding. Only one mutant, BacT1DEL53-102, suggests the possibility of any residual inhibitory function. Values of percentage binding at 1000x molar excess were somewhat diminished (75%), however whether this is true inhibition is questionable seeing as at 2000x, no further inhibition is observed. This does not exclude the possibility that at doses far exceeding that available by our current mode of expression, that a dose dependent inhibition cannot be achieved. Nevertheless, the large K_i value expected may preclude

this mutant from being considered an inhibitor at all. What it does suggest is the importance of areas that possess cysteines, as well as patches of highly negatively charged amino acids, for M-T1 function. BacT1DEL53-102, was the only mutant not lacking one or more of the eight conserved cysteine residues in the intact M-T1 protein. However, a large cluster of negative charges was deleted in the mutant protein, which may result in the almost complete, but not absolute, loss of function observed. Other possible reasons for the lack of function observed in each mutant were verified. The possibility that the proteins may have been degraded in the supernatants prior to or during testing in the binding experiments was verified by Western blot analysis of the supernatants after the binding experiments were completed. A similar profile as that initially observed for protein expression was obtained, eliminating this possibility. Another possibility is that improper structural folding, which may be expected for those mutants lacking cysteine residues present in the wild type M-T1 protein or even small deletions, prevented the active site of the protein from being exposed and hence affected its function. All mutant proteins were recognized by the α-M-T1 antibody, which suggests that gross structural changes at the C-terminus are unlikely. Still, other methods to demonstrate structural integrity of the proteins, including NMR and circular dichroism, may further clarify the folded nature of the mutant proteins.

4.5. Conclusions and suggestions for future mutagenic analysis

A hypothesis may be made that the proper functioning of the M-T1 protein is dependent on the conservation of domains consisting of negative charge clusters and

cysteine residues, which were absent from the deletion mutants tested. There exists a variety of mutants created in the pAlter vector which span the length of the M-T1 gene, and include N- and C-terminal truncations, that could be helpful in the confirmation of this hypothesis. Future work, supported by the data already obtained, would include the cloning of these pAlter mutants in into pBacPak, and the creation of stable transfectants and virus for those mutants showing varying degrees of loss of function. This would provide large quantities of mutant protein that could be purified and tested in greater detail. Furthermore, a more extensive study involving the mutation of distinct residues would help identify the exact amino acids involved in M-T1 function. This may be accomplished by the individual alanine or serine scanning of the eight conserved cysteine residues present in the protein, as well as making small deletions of 4-5 amino acids in length that encompass only acidic clusters.

Preliminary evidence provided by Bruce T. Seet in the laboratory of Dr. McFadden, at the Robart's Research Institute, indicate that pM-T1 unexpectedly binds heparin-Sepharose, and that this interaction can be competed with increasing amounts of heparin. Members of the fibroblast growth factor family, a number of protease inhibitors, as well as chemokines have been shown to be ligands for glycosaminoglycans (GAGs) such as heparan sulphate and chondroitin sulphate (Koopmann and Krangel, 1997; Kjellen and Lindahl, 1991). More important to this discussion, however, is the interaction of the low affinity domain of chemokines, which is a GAG binding domain. This binding is believed to be necessary for the formation of a solid phase chemokine gradient for the haptotaxis of target cells *in vivo* (Wiedermann *et al.,* 1993). A few models of inhibition can be speculated based on this data of M-T1 interacting with heparin-Sepharose. For

one, M-T1 may bind GAG on the endothelial cell surface, blocking the formation of a solid phase chemokine gradient by competitively binding GAGs, which would prevent the migration and extravasation of target cells. M-T1 may also bind GAG on the cell surface, while at the same time binding chemokines, bringing the two together yet preventing signaling. This would also prevent the formation of any soluble chemokine gradient to which target cells could migrate in response to. Studies have identified a heparin binding motif that consists of basic residues separated by intermittent amino acids in heparin-binding proteins such as antithrombin III and platelet factor 4 (Cardin and Weintraub, 1998). M-T1 has regions of similarity to these motifs, near the C-terminus at amino acids 196-199 and amino acids 236-243. Future studies would also involve mutation of these basic residues to analyze heparin binding, and the impact loss of heparin binding may or may not have on CC-chemokine binding and inhibition.

The data presented in this thesis indicates that several domains in the M-T1 protein are necessary for the inhibition of CC-chemokine binding to cells. The mechanism underlying the modulation of chemokine function by M-T1, however, remains to be determined. Models describing interference of leukocyte migration and disruption of chemokine gradients in inflamed tissues have been postulated. On a larger scale, in terms of pathophysiological significance, M-T1 may play a role in chemokine subversion by interfering not only with the host inflammatory response, but also in the normal regulation and migration of lymphocytes in secondary tissues and throughout the immune system. Exploitation of the chemokine system in this manner has already been described for several herpesviruses (Ngo *et al.*, 1988). The data collected to date for M-

T1, therefore, may serve as a starting point in the design of a larger picture of overall immune modulation caused by M-T1 together with the multitude of virulence factors encoded by myxoma virus.

Appendix A: Copyright Release

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Appendix B: Deletion Mutant Sequence

The following figure lists the sequence obtained for the deletion mutant pAlterMT1DEL53-170, aligned with the intact M-T1 sequence to confirm proper cloning and restriction endonuclease diagnostics during deletion mutant cloning. Underlined bases denote areas of point mutations designed to create Bgl II restriction site. Dotted line denotes area of deletion.

```
M-T1                ATGAAACGCCTGTGTGTATTATTCGCGTGCCTGGCCGCGACCCTCGCG
pAlterMT1DEL53-170  ATGAAACGCCTGTGTGTATTATTCGCGTGCCTGGCCGCGACCATGGCG

ACGAAGGGCATCTGCAGACAAGGCGAAGATGTCCGATACATGGGAATAGACGCCGTGGCCAA
ACGAAGGGCATCTGCAGACAAGGCGAAGATGTCCGATACATGGGAATAGACGCCGTGGCCAA

AATTACAAAGAGGACTACCGGAAGCGACACGCCGTGTCAGGGTCTGCGTACGACTATTGAAT
AATTACAAAGAGGACTACCGGAAGCGACACGCCGTGTCAGGGTCTGAGA----------

CCGCGTATACAGAAGACGAAAACGAAGACGATGGCGCGACGGGTACGGAGCAGCCCGACGA
------------------------------------------------------------

TCCTAGCGAGGAATACGAGTACGACGAAAACGACGAATCGTTTCTAACCGGTTTCGTGATCGG
------------------------------------------------------------

AAGTACTTACCACACGATCGTCGGAGGAGGACTCTCCACGTTCGGAGTTACGGGATGTCCTAC
------------------------------------------------------------

CGTTAAGGCGATATCCGAACACGTCAAAGGACGCCACGTCTACGTCCGACTGTCCAGCGACG
------------------------------------------------------------

CTCCTTGGAGAGATACGAATCCCGTGTCTATGAACCGTACAGAGGCGCTCGCCCTACTCGACA
------------------------------------------------------------

CGTGTGAAGTGTCCGTAGATATCAAATGCAGTCGCGTCAACGTAACCGAAACGACGTACGGA
----------------------TCTAGTCGCGTCAACGTAACCGAAACGACGTACGGA

ACCGCGGCGCTTGTCCCGCGTATAACTCAAGCGACGAGACGCAGTCATATTATCGGATCTACC
ACCGCGGCGCTTGTCCCGCGTATAACTCAAGCGACGAGACGCAGTCATATTATCGGATCTACC

CTGGTCGACACGGAATGTGTGAAGAGTCTAGACATAACCGTCCAAGTGGGTGAAATGTGTAA
CTGGTCGACACGGAATGTGTGAAGAGTCTAGACATAACCGTCCAAGTGGGTGAAATGTGTAA

GAGAACGTCTGATCTCTCGGCGAGAGACAGTCTTAAGGTAAAGAACGGCAAACTACTCGAGG
GAGAACGTCTGATCTCTCGGCGAGAGACAGTCTTAAGGTAAAGAACGGCAAACTACTCGAGG

ACGATATCCTTGTCCTTCGTACGCCTACCCTCAAGGCGTGTAAC
ACGATATCCTTGTCCTTCGTACGCCTACCCTCAAGGCGTGTAAC
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