University of Alberta

Analysis of Inflammatory Responses of Macrophages Induced by
Elastase-modified Transferrin
by

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This thesis is dedicated to all the people who lost their lives during the Lebanese war
(April 13, 1975 - October 13, 1990)

ABSTRACT

Modified endogenous proteins have been shown to activate macrophages and alter innate immune responses of the host. In this thesis I examined the immunomodulating activities of peptides generated by elastase digestion of transferrin. The transferrin peptides resolved by FPLC size exclusion chromatography induced nitric oxide response in $P388D_1$ macrophage cell line, murine peritoneal and bone marrow-derived macrophages and goldfish macrophages. A 31 amino acid transferrin peptide identified by mass spectrometry was synthesized because it resembled a defensin-like molecule. This peptide, named TMAP, induced nitric oxide response in different macrophage populations. In addition, TMAP induced other inflammatory responses in activated macrophages including chemotaxis, the production of pro-inflammatory cytokines TNF-α and IL-6, and panel of chemokines MCP5, MIP2, MIP1-α, MIP1-γ, KC and RANTES. Certain elastase-generated transferrin fragments also had anti-inflammatory properties indicated by suppression of the nitric oxide response of macrophages. These peptides remain to be fully characterized. Thus, transferrin-derived fragments may play an important role in host defense by modulating the inflammatory responses of higher and lower vertebrates.

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LIST OF ABBREVIATIONS

AGE:	Advanced glycation end product
AP-1:	Activator protein-1
ASC:	Apoptosis-associated speck-like protein
ATCC:	American typing culture collection
ATP:	Adenosine triphosphate
BAL:	Bronchoalveolar lavage
b-apoTf:	Bovine apotransferrin
BCA:	Bicinchoninic acid assay
BMDM:	Bone marrow-derived macrophages
β-ME:	Beta-mercaptoethanol
CD:	Cluster of differentiation
CARD:	Caspase recruitment domain
CF:	Cystic fibrosis
CR3:	Complement receptor 3
Casp:	Caspase
CTCK:	C-terminal cysteine knot
DAMPs:	Damage associated molecular patterns
DCs:	Dendritic cells
DMEM:	Dulbecco's modified Eagle's medium
DNA:	Deoxyribonucleic acid
EDA:	Extra domain A
EDTA:	Ethylene diamine tetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
eNOS:	Endothelial nitric oxide synthase
ERKs:	Extracellular signal regulated kinases
FPLC:	Fast performance liquid chromatography
Gadd45α:	Growth arrest and DNA-damage-inducible, alpha
G-CSF:	Granulocyte-colony stimulating factor
Glyt1:	Glycine transporter 1
gfsTF:	Goldfish serum transferrin
GSH:	Reduced glutathione
GSSG:	Oxidized glutathione
Hells:	Helicase lymphoid specific
HMGB1:	High mobility group box 1
HSPs:	Heat shock proteins
Hyal:	Hyaluronidase
ICs:	Immune complexes
IDO:	Indoleamine2, 3-dioxygenase
IFNs:	Interferons
ILs:	Interleukins
iNOS:	Inducible nitric oxide synthase
IRAK1:	Interleukin-1 receptor-associated kinase 1
IVET:	*in vivo* expression technologies

JNKs: C-jun N-terminal kinases
KC: Keratinocytes cytokine
kDa: Kilo Dalton
LC/MS/MS: Liquid chromatography/mass spectrometry/mass spectrometry
LPS: Lipopolysaccharide
LRR: Leucine rich repeat
Mal: MyD88-adaptor-like
MAPKs: Mitogen activating protein kinases
m-apoTf: murine apo-transferrin
MCPs: Monocytes chemoattractant proteins
MHC: Major histocompatibility complex
MIPs: Macrophage inflammatory proteins
mmLDL: Minimally modified low density lipoprotein
MMPs: Metalloproteinases
M_r: Molecular weight
Mrps: Myeloid related proteins
MSU: Monosodium urate
MyD88: Myeloid differentiation factor 88
NADPH: Nicotinamide adenine dinucleotide phosphate
NALP3: NACHT-domain, leucine-rich-repeat, and PYD-containing protein 3
NF-κB: Nuclear factor kappa B
NGAL: Neutrophil gelatinase associated lipocalin
NLRs: NOD-like receptors
nNOS: Neuronal nitric oxide synthase
NO: Nitric oxide
NOD: Nucleotide-binding oligomerization domain
NRAMP: Natural resistance associated macrophage protein
oxLDLs: Oxidized low-density lipoproteins
oxPL: Oxidized phospholipids
PAMPs: Pathogen associated molecular patterns
PBSA: Phosphate buffer saline with antibiotics
PBST: Phosphate buffer saline with Tween 20
PCR: Polymerase chain reaction
PDGF: Platelet-derived growth factor
PKC: Protein kinase C
PMSF: phenylmethylsulfonylfluoride
PSGL-1: P-selectin glycoprotein ligand-1
PRRs: Pattern recognition receptors
RAGE: Receptor binding advanced glycation end products
RANTES: Regulated upon activation, normal T cells expressed and secreted
RGD: Arginine-glycine-aspartic acid
RICK: Receptor-interacting serine/threonine kinase
rmIFN-γ: Recombinant murine interferon-gamma
ROCK: Rho-associated coiled-coil-containing protein kinase
ROK: RhoA-binding kinase
SARMs: Selective androgen receptor modulators

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLE: Systemic lupus erythematosus
Smarca5: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
SOD: Superoxide dismutase
STM: Signature-tagged mutagenesis
TICAM1: TIR domain-containing adaptor molecule-1
TGF-β: Transforming growth factor beta
TfR: Transferrin receptor
TIR: Toll/interleukin-1 response domain
TIRAP: TIR domain-containing adaptor protein
TLRs: Toll-like receptors
TMAP: Transferrin macrophage-activating peptide
TNFα: Tumor necrosis factor alpha
TRAM: TRIF-related adaptor molecule
TRIF TIR domain-containing adaptor inducing IFN-β
Trx: Thioredoxin
VEGF: Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

1.0 General introduction

The immune system provides remarkable protection against harmful pathogens that are plentiful in the external environment by generating innate and adaptive responses. In the early 1960s, Medawar and Burnet [1, 2] proposed the "self-non-self" model of immunity. The hallmark of this model is that the immune system can make the subtle distinctions between self (normal host compounds) and non-self (foreign invader compounds). This model has become the cornerstone of immunology for over 50 years. This model, however, does not address central immunological questions of autoimmunity, and "changed self" that occur in puberty, metamorphosis, pregnancy and aging [3].

Another model proposed by Janeway [4] emphasizes the importance of innate immunity in host defense. The central premise of this model of immunity is that the innate immune response can distinguish "infectious non-self" from "non-infectious self". However this model left several important questions unanswered such as why pattern recognition receptors (PRRs) do not distinguish between harmful and non-harmful microorganisms [5] and the reported activation of the immune system in the a absence of a pathogens (e.g. allograft rejection and autoimmunity) [6].

In the early 1990s, Polly Matzinger proposed an ingenious model of immunity, called "danger model", which was provocative and revolutionized the way scientists viewed the immune system [7]. The cornerstone of the "danger model" is that the

immune system recognizes danger signals emitted from damaged "self" tissues due to injury or infection. The exposure of immune cells to the so-called pathogen associated molecular patterns (PAMPs), as proposed by Janeway, is not required. The "danger model" provides flexibility regarding initiation of immune responses particularly in neoplasia-induced autoimmunity and allograft rejection [7]. Recently Seong, and Matzinger [8] modified the "danger model" to include PAMPs, which are displayed by pathogens after significant host-induced stress and not by "healthy" pathogens [9], and endogenous danger signals called damage-associated molecular patterns (DAMPs).

The endogenous danger signals can be released in response to different types of tissue damage caused by burns, radiation, nutrient and oxygen deprivation, neoplasia, autoimmunity, and xenobiotics [10]. The danger signals are collectively known as "alarmins", which function by alerting the immune system to tissue damage or infection [11]. Recent studies have shown that there is a significant number of endogenous proteins such as heat-shock proteins, hyaluronan fragments, high-mobility group box 1, fibronectin fragments, lactoferrin fragments, modified low-density lipoproteins, extracellular ATP, and myeloid-related protein-8 and -14, that have the capacity to activate the immune system [10].

Transferrin is an important endogenous protein that can be considered as an "alarmin". Current evidence suggests that modified transferrin plays an important role in host defense. Transferrin fragments were detected in the bronchoalveolar lavage (BAL) from cystic fibrosis patients also infected with *Pseudomonas aeruginosa* [12]. Immunostimulatory transferrin fragments were also identified in the supernatants of mitogen-activated goldfish leukocytes cultures [13], and more recently a 7.6 kDa

transferrin fragment was detected in the medium conditioned by the invasive metastatic cancer cell line MDA-MB-435S-F/Taxol10p4pSI [14].

That transferrin may be an important alarmin is supported by the following: (1) transferrin is an abundant plasma protein with concentration 2-5 mg/mL in mammals [15]; (2) because of its role in iron transport, transferrin can sequester this essential element from growing pathogens [16], and is consequently a prime target for pathogens to "attack" in order to steal the essential iron ions by means of siderophores [17], or by enzymatic cleavage [18]; (3) in addition to its synthesis in the liver, transferrin is produced by the major phagocytic cell of the body, the macrophage [19]; and (4) transferrin is an acute phase protein [20].

The detection of danger signals and PAMPs by immune cells leads to cells activation and development of inflammatory response, which is a coordinated series of host defense events characterized by increased vascular permeability that facilitates a rapid translocation of cells and effector molecules to the site of infection. Increased vascular permeability allows soluble immune proteins such as antibody and complement, as well as serum proteins such as fibronectin and transferrin, to translocate to the inflammatory site. Increased expression of vascular adhesion molecules and chemokines attract and focus leukocytes such as neutrophils, macrophages, and eventually lymphocytes to the inflammatory site. Once at the site of the infection, inflammatory cells attack pathogens using an elaborate armamentarium of antimicrobial mechanisms. The inflammatory response generally subsides once the infectious agents are contained, giving way to another complex and coordinated process called tissue repair [21].

1.1 Objectives of the thesis

Recent discovery of Toll-like receptors has brought the innate immune host defense mechanisms to the forefront of modern immunology. In particular, the awareness that the innate immunity can discriminate self- from non-self by recognizing PAMPs expressed by pathogens or released by injured "self" molecules has allowed for multitude of studies examining the "bridge" between innate and adaptive immune responses [10]. Transferrin is an important host molecule because of its roles in iron binding and delivery [22] and its involvement in the innate immune response as an acute phase protein [20] and iron sequestration [16] as well as the activation of immune cells.

The main objective of my thesis was to examine the immunomodulatory role of transferrin fragments with specific reference to the regulation of macrophage inflammatory responses of the host. The specific aims of my research were:
(a) To determine the potential enzyme that can cleave transferrin of different species into fragments and identify immunomodulatory peptide(s); and (b) To characterize the immunomodulatory role of the identified transferrin fragment(s) or peptide(s) in the regulation of pro-inflammatory functions of macrophages.

1.2 Outline of thesis

In chapter 2, I review the current literature with respect to activation of immune system by endogenous danger signals, focusing on the induction of inflammatory responses. Chapter 3 contains a detailed description of materials and methods used in different experiments that comprise my thesis. In Chapter 4, the results of the enzymatic

digestion, size and charge separation of the fragments of transferrin from different species and their effects on activating or deactivating macrophages are presented. Chapter 5 describes the characterization of transferrin-derived macrophage activating peptide. Chapter 6 is the general discussion of the findings of my thesis research and future research directions that I believe should be undertaken to further characterize the contribution of modified transferrin in regulation of inflammation.

CHAPTER II

LITERATURE REVIEW

2.0 Overview

The concept of the "self and non-self" recognition by the immune system has recently been expanded by the evidence that in addition pathogens, stressed or modified endogenous molecules are also recognized as "non-self" by pattern recognition receptors (PRRs). The recognition of pathogen-associated molecular patterns (PAMPs) by the PRRs results in the activation of the immune system and provides a bridge between innate and adaptive immunity normally generated in response to pathogen insult or tissue injury.

In this review, I provide description and discussion of the modified endogenous proteins that have been reported to both induce and regulate diverse immune responses of the host. The primary focus of the review is on the role of monocytes/macrophages in surveillance and detection of exogenous and/or endogenous danger signals that lead to competent inflammatory responses of the host.

2.1 Endogenous danger signals

The endogenous stress signals are inflammatory mediators that are not cytokines and are produced by the host during infection or tissue injury. These mediators are known as endogenous danger signals or "alarmins" [11]. According to Bianchi [23] the alarmins have one or more of the following characteristics: (1) they are released by necrotic cells

and not by cells undergoing apoptosis; (2) they are produced by cells of the immune system via a special secretion mechanism or through endoplasmic reticulum-Golgi apparatus pathway; (3) alarmins recruit and activate immune cells such as macrophages and promote innate and adaptive immune responses; and (4) the alarmins aid in the attenuation of the inflammatory response by promoting tissue repair and restoration of homeostasis.

2.1.1 Heat-shock proteins (HSPs)

HSPs are highly conserved proteins present in all eukaryotic and prokaryotic organisms [24]. HSPs can be constitutively expressed or they can be induced by heat, infection, and cellular differentiation [25]. The main function of the HSPs is to serve as molecular chaperones; they bind newly formed and partially folded proteins preventing their aggregation and misfolding [25]. HSPs can also act as chaperonins that mediate protein folding directly [26]. In addition, HSPs play an important role in antigen presentation, cross presentation [27, 28] and tumor immunity [28]. Cytoplasmic HSPs such as Hsp70, Hsp90, and gp96 bind antigenic peptides during their processing inside the cell and promote their traffic and display by the major histocompatibility complex (MHC) class I molecules [29]. HSPs also chaperone peptides released outside the cell that are taken up by antigen-presenting cells via α2-macroglobulin receptor (CD91) [30].

Purified HSPs such as Hsp60, Hsp70, Hsp90, and gp96 from bacteria and mammals, have been shown to induce potent immune responses characterized by the release of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-12. HSPs also promote the discharge of nitric oxide and CC-

chemokines by monocytes, macrophages, and dendritic cells [31, 32, 33]. Surprisingly, the concentrations of the recombinant Hsp60 and Hsp70 required to induce an immune response are lower (< 1 μg/mL), than those purified from mouse liver (10-100 μg/mL) [34, 35].

The reported cytokine-like activities of HSPs are likely mediated through Toll-like receptors-2 and Toll like receptor-4 and a signal transduction pathways leading to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) such as ERKs (p42 and p44), JNK and p38 kinase [34, 35, 36]. HSPs also participate in the propagation and exacerbation of autoimmune diseases. For example, HSP B8 (Hsp22) is commonly expressed in the synovial tissue of rheumatoid arthritis patients [37].

2.1.2 Hyaluronan

Hyaluronan is a major component of the extracellular matrix and exists as a high-molecular-weight-polymer (~ 10^3 kDa), composed of non-sulfated repeating disaccharides of *N*-acetyl-glucosamine and *D*-glucuronic acid, which are connected exclusively by β-linkages [38]. Hyaluronan undergoes active regulation that results in the accumulation of lower-molecular-weight fragments during tissue injury and inflammation [39]. Hyaluronan is degraded through three different pathways which involve the enzyme hyaluronidase (Hyal) family [38]. The removal of hyaluronan fragments is essential for resolution of lung inflammation, a process that is dependent on the hyaluronan receptor CD44 [40]. However, in the absence of CD44 (CD44 - null mice), the stimulation of CD44 - null macrophages *in vitro* by a 135 kDa hyaluronan

fragment induces the expression of macrophage inflammatory protein (MIP)-2 in peritoneal and bone marrow-derived macrophages, suggesting that hyaluronan fragments may also signal through a pathway independent of CD44 [41]. Recent evidence suggest that hyaluronan fragment signals through TLR-2 and TLR-4 because the MIP-2 expression was completely abolished in macrophages from double-knockout mice (TLR-$2^{-/-}$, TLR-$4^{-/-}$) [41].

Another extracellular matrix protein that participates in macrophage activation is biglycan. Biglycan is a small proteoglycan rich in leucine and is released during the degradation of the matrix. Biglycan induced macrophages to release TNF-α and MIP-2, a process dependent on TLR-2 and TLR-4. The induction of the cytokine production in activated macrophages by biglycan was diminished in either TLR-$2^{-/-}$ or TLR-$4^{-/-}$ knockout mice and was completely abrogated in TLR-$2^{-/-}$ TLR-$4^{-/-}$ double knockout mice [42].

2.1.3 Fibronectin

Fibronectin is a high molecular weight glycoprotein, composed of two nearly identical subunits with an M_r of ~ 240 kDa. The two subunits are encoded by the same gene, and through alternative splicing, 20 variants of fibronectin can be generated. Fibronectin mediates cell attachment to the extracellular matrix by binding via the arginine-glycine-aspartate (RGD) tripeptide motif to several integrins, particularly, α5β1 and α4β1, which are the principal receptors for fibronectin [43]. The integrins link the extracellular matrix and the cell cytoskeleton, which facilitates cell migration, differentiation, proliferation, and maintenance of cellular morphology [44].

Fibronectin fragments are generated during the extravasation process of monocytes from the blood vessels during an inflammatory response. Monocytes release metalloproteinases (MMPs), which are capable of degrading all components of the extracellular matrix, which facilitates their migration to the inflammatory sites. One important MMP of monocytes/macrophages involved in the generation of fibronectin fragments is MMP-9, which is induced strongly by pro-inflammatory cytokines, particularly TNF-α [45].

Fibronectin fragmentation can occur during tissue injury and in conditions such as rheumatoid arthritis, epithelial fibrosis, wound healing, and inflammation. The most characterized fibronectin fragment of M_r of 110-120 kDa was shown to bind to macrophages through α5β1 integrin receptor, which resulted in signal transduction pathway that either activates macrophages directly or primes them for activation by other molecules [46].

Another biologically important fragment of fibronectin is the extra domain A (EDA) fragment. EDA is recognized by integrin receptors which in turn associate with other receptors expressed on the surface of macrophages to induce a potent macrophage inflammatory responses [47]. Signaling by the EDA fragment activates NF-κB through TLR-4-MD-2 complex and induces cytotoxic T-cell responses, a process that exacerbates the inflammatory response in patients with rheumatoid arthritis [48, 49].

2.1.4 High-mobility group box I (HMGB1)

HMGB1, also known as amphoterin, is a nuclear protein present in almost all eukaryotic cells and its main biological role is to stabilize nucleosome formation and the

expression of several genes such as glycine transporter Glyt1 and genes involved in repair of UV-induced DNA damage (Gadd45a, Rad 21, Apex1, Hells, Smarca5, Casp12, Casp3, Tial) [50]. HMGB1 has also an important extracellular role as a danger signal and is produced by activated macrophages, dendritic cells, and natural killer cells, or released by necrotic cells in response to infection or tissue injury [51]. HMBG1 is chemotactic to monocytes/macrophages, neutrophils and dendritic cells, and has been reported to promote the maturation of myeloid and plasmacytoid dendritic cells [52]. HMGB1 signals through several Toll-like receptors including TLR-4, TLR-2, and TLR-9 or it may signal by associating with other ligands [53]. For example, HMGB1 bind to the immunoglobulin superfamily member RAGE, which in association with TLR-9 activates plasmacytoid dendritic cells to release IFN-α [54]

HMGB1 participates in the recruitment of stem cells and promotes their proliferation. This was demonstrated by injection of HMGB1 into the infarcted area of the heart, resulting in tissue regeneration and significant recovery of cardiac performance [55].

2.1.5 Modified low-density lipoproteins

Accumulation of low-density lipoprotein (LDL) aids in formation of plaques and contributes to the pathogenesis of atherosclerosis because they are recognized by scavenger receptor expressed on macrophages, and this interaction leads to the formation of foam cells and visible lesions [56]. In addition, oxidized phospholipids (oxPL) formed on oxidized LDL (oxLDL) during LDL oxidation and significantly contribute to oxLDL immunogenicity. The oxLDL induces production of the pro-inflammatory

cytokine/chemokine TNF-α and IL-8, respectively, in monocytes and primes macrophages for antimicrobial functions [57] .

Minimally modified LDL (mmLDL) also contributes to atherosclerosis. However, macrophage recognition of mmLDL is independent of scavenger receptors. Similar to oxLDL, mmLDL is a potent pro-atherogenic and pro-inflammatory lipoprotein. mmLDL is recognized by CD14-TLR-4-MD-2 complex expressed on macrophages, which leads to the induction of actin polymerization and spreading of macrophages, a process that results in the inhibition of phagocytosis of apoptotic cells [58].

2.1.6 Extracellular ATP

During inflammation, platelets and necrotic cells can release large amounts of ATP. Short term stimulation (5 minutes) of macrophages with ATP leads to the rapid secretion of IL-1β 15 minutes post stimulation [59]. Extracellular ATP induces several physiological responses through G protein-coupled P2Y7 receptor, as well as through pattern recognition receptors, NACHT-domain, leucine-rich-repeat, and PYD-containing protein 3 (NALP3) [60]. LPS priming of macrophages is also required for activation of caspase-1 by ATP-gated P2X7 receptor. Interaction of extracellular ATP with NALP3 leads to activation of caspase-1, and secretion of the pro-inflammatory cytokines IL-1β and IL-18. NALP3 and the adaptor molecule Apoptosis-associated speck-like protein (ASC) are essential components for assembly of inflammasomes, cytoplasmic proteins complexes that trigger activation of caspase-1 and eventual release of pro-inflammatory cytokines [61].

2.1.7 Myeloid-related protein- (Mrp8) and (Mrp14)

Mrp-8 and Mrp-14 are members of the calgranulin family of S100 proteins and are the most abundant proteins in the cytoplasm of neutrophils [62]. Secretion of Mrp-8 and Mrp-14 is induced during contact of phagocytes with activated endothelium [63]. Both S100 proteins lack structural requirements for classical transport via the endoplasmic reticulum and Golgi complex. However, release of Mrp-8 and Mrp-14 by human monocytes is a specific and energy-dependent process, which involves activation of protein kinase C (PKC) and active metabolism of the microtubule network [64]. The formation of Mrp-8 and Mrp-14 complexes is a feature of a number of inflammatory diseases including, sepsis, rheumatoid arthritis, inflammatory bowel disease and cancer [62, 65].

TLR-4-MD-2 complex is implicated in the binding of and signaling by Mrp-8 and Mrp-14. The downstream signaling involves the adaptor molecules MyD88 and IRAK-1 that leads to the activation of the transcription factor NF-κB and the transcriptional up regulation of pro-inflammatory cytokines such as TNF-α. For example, Mrp-8 and Mrp-14 knockout mice are more resistant to LPS and galactosamine challenges as measured by a decrease in TNF-α production [66].

2.1.8 Uric acid

Uric acid is the final oxidation product of purine catabolism. It is soluble inside the cell; however, it readily precipitates outside the cell to form monosodium urate (MSU) microcrystals. Uric acid is released by necrotic cells and it stimulates the

maturation of dendritic cells, and when co-injected with antigen *in vivo* enhances the generation of CD8+ T cell responses [67]. In hyperuricemia, MSU crystals can precipitate in joints leading to a type of arthritis known as gout [68]. In addition, very high serum concentration of uric acid is associated with Lesch-Nyhan syndrome [69], cardiovascular disease [70], and diabetes [71].

During an inflammatory response, the MSU crystals stimulate the monocytes/macrophages and endothelial cells to release IL-1β and IL-18 [72]. MSU crystals appear to engage caspase-1 and activate NALP3 (cryopyrin) inflammasome. However, macrophages from mice deficient in IL-1 receptor or components of the inflammasome complex, such as caspase-1, ASC or NALP3, have impaired MSU-induced cytokines production and ultimately reduced inflammatory response [73].

2.1.9 Nucleic acids of mammalian origin in the form of immune complexes

Systemic lupus erythematosus (SLE) is characterized by the production of pathogenic auto-antibodies to nucleoproteins and DNA. The level of anti-DNA antibodies correlates with disease severity, and the deposition of these immune complexes (ICs) in the kidneys contributes to pathogenesis. Recent evidence suggests that the DNA component of immune complexes purified from SLE patients (SLE DNA-ICs) contributes to the development of SLE pathology [74]. In addition, it has been shown that mammalian DNA and RNA, in the form of ICs are endogenous ligands for TLR-9 and TLR-7, respectively. The binding of these ICs, to TLR9 or TLR7 induces potent IFN-α production by plasmacytoid pre-dendritic cells [75].

2.1.10 Lactoferrin

Lactoferrin is a member of the transferrin family of iron-binding proteins and found in most bodily fluids, such as milk, saliva, nasal exudate, bronchial mucus, gastrointestinal fluid, and tears. Lactoferrin is stored in the secondary granules of neutrophils and contribute to innate immunity by sequestering iron from invading pathogens, in addition to its bactericidal activity against both Gram-positive and Gram-negative bacteria. The bactericidal activity of lactoferrin is attributed to a region located in the N-lobe of the protein called lactoferricin [76].

Small lactoferrin peptides (32, 23, 22, and 19 kDa) were identified in the parotid saliva of chronic periodontitis patients. The Con A low-affinity saliva peptides and two synthetic lactoferrin peptides induced the production of IL-6, monocyte chemoattractant protein 1 (MCP-1), and IL-8 [77]. The severity of the clinical symptoms also correlated with the increase of lactoferrin fragments in the parotid saliva of periodontitis patients [77].

2.1.11 Transferrin

Modified transferrin also belongs to the alarmin family of endogenous danger signals. Since modified endogenous transferrin is the focus of this thesis, transferrin's role in the innate immunity of the host will be discussed in the upcoming chapters.

2.2 Alarmin receptors

Exogenous or endogenous danger signals are detected by the immune cells through a set of limited number of germ line-encoded pattern recognition receptors (PRRs). The PRRs are found in the extracellular space (secreted), in the cell cytoplasm or in vesicles, as well as in the plasma membrane. The secreted PRRs can mediate opsonization and activation of the complement pathways. The intracellular PRRs can induce apoptosis and the secretion of cytokines. The PRRs in the plasma membrane enhance phagocytosis of pathogens and facilitate antigen presentation [78].

2.2.1 Toll-like receptors (TLRs)

The TLR family is characterized by an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic domain that is homologous to that of interleukin-1 receptor and is referred to as Toll/IL-1R (TIR) domain [78]. The TIR domain is conserved in all TLRs except for TLR-3. TIR domain interacts with other adaptor molecules containing the TIR domains that mediate TLR signaling. There are five well-characterized activating adaptor molecules with TIR domain including, myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal), TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR domain-containing adaptor molecule-1 (TICAM-1), TRIF-related adaptor molecule (TRAM), and TIR domain-containing protein and HEAT/Armadillo motifs-containing protein (SARM). MyD88 is a very important adaptor molecule and is used in signaling by all TLRs with the exception TLR3. TLR activation in MyD88-knockout mice results in defective

production of IL-12 and TNF-α. TIRAP/Mal is primarily needed for TLR-2 signaling but is also required for TLR-4 signaling, via the MyD88-dependent pathway. TRIF/TICAM-1 is required for TLR-3 signaling as well as for TLR-4 MyD88-independent pathway [79]. On the other hand, SARM is primarily expressed in neurons where it regulates neuronal survival through interaction with Janus kinase 3 (JNK3) in mitochondria [80]. SARM also negatively regulates TLR signaling via TRIF pathway [81]. The activation of the adaptor proteins generally leads to the activation of NF-κB and MAPK pathways, which are important for the competent inflammatory response. As already indicated in the preceding sections, many alarmins bind to TLRs, resulting in immune cell activation, while the recruitment of specific adaptor molecules determines the nature and the magnitude of cellular signaling [10].

2.2.2 The NOD-like receptors (NLRs)

The intracellular Nod-like proteins or receptors are a family of sensors of intracellularly encountered microbial motifs and danger signals that have emerged as being critical components of the innate immune responses and of inflammation in mammals. The NLR family includes both nucleotide-binding oligomerization domains (NODs) and neuronal apoptosis inhibitory protein/MHC class II transcription activator/incompatibility locus protein from *Podospora anserina*/telomerase-associated protein (NALPs/NACHT). Several NLRs, including NOD1, NOD2, NALP3, Ipaf and Naip, participate in host responses to intracellular bacteria or to the presence of specific bacterial products inside the cells. Upon ligand binding to NOD1 or NOD2, serine/threonine kinase receptor-interacting serine/threonine kinase (RICK) is recruited

through CARD-CARD interaction. RICK is then cross activated, which leads to downstream signaling resulting in the activation of NF-κB and MAPK pathways [82].

An additional major function of NOD-like receptors is during inflammation, which has been emphasized by the identification of several different mutations in the genes encoding NOD1, NOD2 and NALP3 commonly seen in patients with inflammatory disorders [83].

2.2.3 Receptor binding advanced glycation end products (RAGE)

RAGE is a transmembrane receptor of the immunoglobulin superfamily. It is capable of binding multiple extracellular ligands, including advanced glycation end products (AGEs), some S100 proteins, amyloid peptide and HMGB1. It is implicated in the pathogenesis of many inflammatory diseases and plays an important role in innate immunity [84]. Elevated levels of RAGE and its ligands are commonly seen during the initiation phase of inflammation [85].

2.3. Inflammatory response

The inflammatory response is a highly conserved host defense mechanism and the study of inflammation dates back 2000 years, where Cornelius Celsus is credited for the description of the hallmarks of inflammation, redness, heat, swelling, and pain [86]. The inflammatory response is important not only for host defense against pathogens but also for the maintenance of homeostasis [86].

The inflammatory response is a cascade of physiological reactions occurring at the cell and tissue levels, involving dilation of arterioles and venules, as well as increased

blood vessels permeability and blood flow, followed by stasis and thrombosis. These physiological changes trigger plasma protein and leukocyte translocation to the inflammatory site. The effector cells at the inflammatory site efficiently attack and remove the invading pathogens and eventually undergo either necrosis or apoptosis. The inflammatory response continues with the removal of cellular debris by phagocytic cells, and tissue repair through the generation of new humoral mediators of cell growth, and the regeneration of new functional and connective tissues [87, 88].

At the onset of inflammation, pre-formed and *de novo* synthesized mediators released by tissue mast cells or platelets [histamine, serotonin, arachidonic acid metabolites, platelet factor 4, platelet-derived growth factor (PDGF)], induce increased vascular permeability [89]. This is accompanied by separation of vasculoendothelial-cadherins at intercellular junctions leading to the rapid formation of pores caused by actin depolymerization, allowing cells and plasma proteins to translocate to the inflammatory site [90]. An important group of soluble host proteins that can be activated by pathogens are β-globulins that comprise the complement system. Nine complement proteins (C1-9) are constitutively present in the plasma and the proteolytic complement fragments, that are generated during complement activation at the inflammatory site and collectively known as anaphylatoxins (C3a, C4a, and C5a), are central chemoattractants for inflammatory cells such as neutrophils and macrophages [91].

The translocation of leukocytes across the endothelium of blood vessels requires proteolytic breakdown of the basement membrane. Upon activation, neutrophils in the blood bind to the endothelium using their PSGL-1 receptor that recognizes P-selectin expressed on the surface of endothelial cells. This interaction causes neutrophils to

secrete elastase and matrix metalloproteinases that breaks down the basement membrane [92, 93]. Through the process of diapedesis, initiated by the chemotactic gradients established by anaphylatoxins and other inflammatory chemoattractants, the neutrophils translocate into tissues and are the predominant cell type at the site of inflammation 24 hours after initiation of the response. Neutrophil migration to the inflammatory site is followed by the migration of the monocytes, which upon reaching tissues undergo a final differentiation step and are known as tissue macrophages. Monocytes utilize β1 and β2 integrins to attach to the endothelial cells and are induced to translocate to the inflammatory site in a similar manner to that of neutrophils. The translocation of macrophages to tissues results in major physiological changes in these cells; they acquire a formidable armamentarium of antimicrobial functions, including enhanced phagocytosis, production of reactive oxygen and nitrogen intermediates, and pro-inflammatory cytokines [94]. Representative mediators of inflammation are listed in Table 2.1.

The inflammatory response is regulated by a delicate balance between the pro-inflammatory cytokines such as IL1β, TNFα, IFNγ, IL8 and IL6, and the anti-inflammatory cytokines including IL1rα, TGFβ, IL10, IL11 and IL6. The regulation of inflammation by pro- and anti-inflammatory cytokines is a complex process because several of the regulatory pathways are redundant. For example, some anti-inflammatory cytokines can have pro-inflammatory properties, further adding to the complexity of the system. The net effect of any pro- or anti-inflammatory cytokine depends on the timing of its release, the microenvironment, the presence of synergistic elements, cytokine receptor density and the target cell responsiveness [95, 96, 97].

2.3.1 Monocytes/macrophage response to danger signals and pathogens

The monocytes originate in the bone marrow from myeloid progenitor cells and are released in the peripheral blood, where they circulate for several days before migrating into tissues and differentiate further into macrophages and dendritic cells, replenishing the pool of resident tissue macrophages [98]. Circulating human monocyte subsets can be distinguished based on their surface markers; the $CD14^+$ are large ~ 18 μm and represent ~80 % of circulating monocytes, and the $CD16^+$ cells are smaller, ~14 μm, and represent ~10 % of the circulating monocytes and their number increases during an infection. Other minor monocytes subsets include the highly phagocytic $CD14^+CD16^+$ $CD64^+$, and the CD56-expressing monocytes, which represent 1-2 % of the mononuclear cells in the blood. The number of CD56-monocytes is elevated in patients with inflammatory bowel disease [99].

During an infection or tissue injury, circulating blood monocytes enter the inflammatory site where they rapidly differentiate into macrophages. The newly formed inflammatory macrophages are typically $CD14^{high}CD16^-CX_3CR1^{low}CCR2^+$ $CD62L^+$ in humans [100]. At the inflammatory site, newly differentiated macrophages become professional phagocytes as they undergo distinct physiological changes induced by the tissue microenvironment, the inflammatory stimuli and by the cytokines that are released at the inflammatory site. Among many physiological changes that occur during monocyte to macrophage differentiation is the up regulation of MHC class II molecules important in antigen presentation as well as co-stimulatory molecules CD80 and CD86, that are required for T cells activation [101].

2.3.1.1 Detection of danger signals

The macrophages display an array of surface receptors that regulate various cellular functions, including differentiation, growth, survival, migration, phagocytosis, activation, adhesion, and cytotoxicity [102]. Macrophages and other antigen presenting cells recognize the conserved microbial structures PAMPs and the danger-associated molecular patterns DAMPs by a limited set of germ-line encoded pattern recognition receptors (PRRs) [5, 6]. The PRRs involved in danger recognition have been discussed earlier in this chapter.

Macrophages recognize and mount an immune response in the event of inflammation caused by tissue damage, mechanical, thermal, and chemical distress, as well as pathogen products [103]. Once they enter the tissue, the macrophages are "primed", mainly, but not exclusively, by interferon-γ (IFN-γ). Initially, IFN-γ is secreted by NK cells and NKT cells, and following the initiation of the adaptive immune response, the major source of IFNγ are T helper 1 cells [104]. The primed macrophages respond to secondary stimuli, such as PAMPs and DAMPs, and are "triggered" by these molecules to become fully activated. The fully activated macrophages exhibit enhanced MHC class II expression, acquire strong microbicidal activity, and secrete a plethora of cytotoxic and inflammatory mediators [105]. Activated macrophages are capable of eliminating pathogens by a variety of different mechanisms including, phagocytosis, release of reactive oxygen and nitrogen intermediates, nutrient deprivation, or by the release of antimicrobial peptides and degradative enzymes.

2.3.2 Antimicrobial functions of macrophages

2.3.2.1 Phagocytosis

Phagocytosis is the process of engulfment of extracellular particles including dead cells, immune complexes and invading pathogens, and is therefore an important process in both host defense as well as homeostasis. There are different types of phagocytic mechanisms that can be generally subdivided into two groups: clathrin-independent and clathrin-dependent internalization of particles [106].

Clathrin-independent internalization of particles is dependent on Rho GTPase and is driven by actin polymerization. This process is separated into phagocytosis type I and II. Type I phagocytosis is mediated by Fc receptors that recognize immunoglobulin-bound particles [107] that results in Rac and Cdc42 recruitment to the site of internalization. The activation of Cdc42 results in the extension of filopodial projections around the particle. Rac is essential for the internalization process [108]. Cdc42 and Rac also recruit Arp2-Arp3 complex along with the regulatory protein WASP. Arp2 and Arp3 promote actin nucleation and polymerization, thereby increasing the efficiency of particle internalization [109].

Type II phagocytosis involves particles opsonized with the complement molecule iC3b that are recognized by the CR3 complement receptor expressed on the surface of phagocytic cells. Unlike phagocytosis type I, this mechanism is dependent on RhoA, and the process is morphologically different. The particles appears to sink inside the cell in the absence of membrane protrusions [110]. However, Arp2 and Arp3 are also required for this process, and their recruitment is dependent on the activation of RhoA [109]. The RhoA effector kinase, ROCK/ROK (Rho-associated coiled-coil-containing protein

kinase/RhoA-binding kinase), is involved in type II phagocytosis because it regulates actinomyosin contractility, which may contribute to the process of engulfment [111].

Clathrin-dependent endocytosis is the best-studied mechanism of particles internalization. The process depends on a cluster of receptors in clathrin-rich area at the cell surface, which then invaginate to form cup. The clathrin-coated vesicles then pinch off and are internalized by the phagocyte, a process mediated by dynamin GTPase. Actin filaments and Rho GTPases are not essential for this mechanism of particle internalization. However, they appear to contribute to the organization, the regulation, and the efficiency of the process [112].

2.3.2.2 Production of reactive oxygen intermediates

Phagocytosis is generally accompanied by a remarkable increase in the consumption of oxygen, a process called respiratory burst. The respiratory burst is one of the main microbicidal responses of phagocytic cells. The respiratory burst depends on the assembly and activation of NADPH oxidase. Assembly of this oxidase requires phosphorylation of its subunits and translocation of cytosolic components composed of $p47^{phox}$/$p67^{phox}$/$p40^{phox}$ and GTPase Rac1/Rac2 to the plasma membrane, forming a new complex with $gp91^{phox}$ and $p22^{phox}$ subunits of the flavocytochrome located on the membrane [113]. This assembly is followed by an increase in intracellular calcium, IP3-mediated signaling, and protein kinase C activation [114]. The activation of the NADPH oxidase, which utilizes cytosolic NADPH, leads to the reduction of extracellular O_2 and generation of superoxide anion ($\bullet O_2-$). The superoxide anion is then enzymatically converted to hydrogen peroxide by an enzyme called superoxide dismutase (SOD).

Subsequently, hydrogen peroxide is a precursor of highly microbicidal hydroxyl radical (•OH) which is generated by the phagocyte via a Fenton-like reaction [113]. Studies using antioxidants, such as superoxide dismutase (SOD), catalase, glutathione (GSH/GSSG), and thioredoxin (Trx), have shown prevention of the toxic effects of $•O_2-$, H_2O_2 and •OH, through processes that restore the normal redox status of the cell [114].

2.3.2.3 Production of reactive nitrogen intermediates

Nitric oxide (NO) is a very versatile molecule and plays an important role not only in the immune system but also in other physiological systems. In innate immunity, NO functions as a tumoricidal and antimicrobial molecule both *in vitro* and *in vivo* [115]. NO production is catalyzed by three forms nitric oxide synthases (NOS): endothelial (eNOS or NOS3), neuronal (nNOS or NOS1) that are constitutively expressed, and inducible (iNOS or NOS2) which is responsible for NO generation during an immune response. Unlike iNOS, eNOS and nNOS are mainly regulated by Ca^{2+} fluxes and the subsequent binding of calmodulin [116]. The NOS isoforms catalyze nitric oxide reaction by converting L-arginine and O_2 to the intermediate molecule N^{ω}–hydroxyl-L-arginine that is further oxidized into L-citrulline and NO [117]. The reactive nitrogen intermediates (RNI) includes the immediate products NO· radical, NO^-, NO^+, and the secondary products, NO_2, NO_2^-, NO_3^-, N_2O_3, N_2O_4, S-nitrothiols (S-NO), peroxynitrite ($ONOO^-$) and nitrosyl-metal complexes [118].

The expression of iNOS is regulated by cytokines and determined mainly by the *do novo* synthesis and stability of iNOS mRNA and protein [119, 120]. Depending on the cytokine or microbial stimulus and the cell type, iNOS gene may be regulated by

different transcription factors involved in the pro-inflammatory responses, such as NF-κB, AP-1, STAT-1α, IRF-1[121], NF-IL-6 [122]. Furthermore, the down regulation of HMG-1(Y) protein leads to the inhibition of iNOS by TGF-β1 [123]. Interestingly, NO exerts a biphasic effect on the transcription of iNOS. For instance, low concentration of NO lead to the activation of NF-kB and the up regulation of iNOS [124], while high concentration of NO down regulates iNOS transcription [125]. Another factor that affects iNOS activity is the availability of arginine. Increased NO production is dependent on the availability of extracellular L-arginine even when the intracellular concentration is adequate [126]. L-arginine can be regenerated by macrophages and vascular smooth muscle cells, by a reaction that is catalyzed by an enzyme called argininosuccinate synthetase, providing ample substrate for the generation of NO [127].

2.3.2.4 Nutrient deprivation

In addition to the generation of reactive oxygen and nitrogen intermediates, macrophages are capable of depriving intracellular pathogens from essential nutrients thereby preventing their growth. There are a number of mechanisms that phagocytes use to deprive the microbes from essential nutrients. For example, modulation of plasma membrane transporters may remove nutrients from vacuolar compartments. The modulation of trafficking of nutrient-rich vesicles may prevent their fusion to those containing pathogens. Furthermore, number of molecules, such as transferrin and lactoferrin can sequester the essential iron from invading pathogens [128].

The examination of nutrient sequestration is usually measured indirectly. These methods evaluation of specific gene expression, the analysis of growth substances that

are required for sustenance of pathogens (natural auxotrophy), or by comparison of the growth of auxotrophic mutants to that of wild-type microbes [128].

Several approaches were used to study the genes that are regulated during phagocytosis of prokaryotes, including *in vivo* expression technologies (IVET), and signature-tagged mutagenesis (STM). For example, examination of *Slamonella typhimurium* infection by IVET, showed an environment depleted of iron, magnesium, and copper [129]. Field *et al.* [130], using avirulent auxotrophic *Salmonella typhimurium* that could not survive inside murine macrophages, showed that purine, pyrimidine, aromatic amino acids, histidine, and methionine were essential for the survival of the pathogen. Leung and Finlay [131] employed several *S. typhimurium* mutants and showed that purine, uracil, isoleucine, and valine were essential for the survival of the pathogen *in vivo*. However, these same mutants survived in cultured macrophages, suggesting that nutrient microenvironments and/or deprivation mechanisms were different [131].

Membrane transporters may contribute to nutrient deprivation mechanism. An example is, the transporter encoded by gene *Nramp1* (now classified as *Slc11a1*), which plays an important role in iron depletion. This transporter is found in the endosome and in the phagosome membrane of macrophages. Mutation to this transporter renders the macrophages more susceptible to infection by *Mycobacteria avium, Salmonella*, and some species of *Leishmania* [132].

In addition to isoleucine and valine, L-tryptophan is another essential amino acid that is absolutely required for pathogen survival. It has been shown that the pro-inflammatory cytokine IFN-γ induced L-tryptophan degradation that caused the

inhibition of growth of *Toxoplasma gondii* in human fibroblasts [133]. Tryptophan is catalyzed by indoleamine2, 3-dioxygenase (IDO), which is present in phagocytes, and is the first and rate-limiting enzyme of tryptophan degradation via the kynurenine pathway. IDO is active in the ferrous form (Fe^{2+}), but in the absence of the reducing agents, dihydroflavin mononucleotide and tetrahydropteridine, may act as cofactors *in vitro*. However, the role of dihydroflavin mononucleotide and tetrahydropteridine *in vivo* has not been determined [134].

Iron is another essential element that may be sequestered from invading pathogens [16]. Iron is the most abundant metal in humans and the majority of iron is bound to the oxygen transporting molecules hemoglobin and myoglobin, or is stored intracellularly by ferritin and hemosiderin. Relatively little iron is used by enzymes and redox proteins or transported by transferrin (< 1 %) [135]. Iron metabolism is tightly regulated and free iron is almost absent in normal human plasma (concentration of ~ 10^{-18} M). The majority of the iron is bound to serum transferrin, which has extremely high affinity to ferric iron Fe^{3+} with an affinity constant of 10^{-36}M [136].

Invading pathogens have evolved mechanisms by which they can "steal" bound-iron from the host proteins by means of high affinity iron-binding particles collectively known as siderophores or by producing proteolytic enzymes in conjunction with siderophores [137, 138]. Siderophores are highly electronegative particles of small molecular weight (< 1000 Da) produced by Gram-negative and Gram-positive bacteria. They chelate ferric iron from most chemical and organic complexes simply by equilibrium displacement. Iron-bound siderophores are recognized at the bacterial surface by specific membrane receptors and the entire complex is internalized [17].

However, not all bacteria produce extremely high affinity siderophores, for instance, *Pseudomonas areuginosa* siderophores, pyoverdin and pyochelin, require the release of extracellular proteases, one of which is elastase, that cleaves transferrin and releases iron to be picked up by the siderophores [18].

Siderophores released by invading pathogens are counteracted by the host's release of lipocalin 2 or neutrophil gelatinase associated lipocalin (NGAL), which binds siderophores preventing bacterial iron acquisition [139]. Lipochalins are family of small-secreted proteins that normally bind small hydrophobic molecules and interact with cell surface receptors [140]. They are characterized by highly conserved structure, which consists of an eight-stranded antiparallel β-barrel that resembles a cup with ligand binding site [139]. The transcription of lipocalin 2 greatly increases in macrophages activated by LPS, and lipocalin 2-deficient mice are highly susceptible to bacterial pathogens [141].

Recent studies reported that a number of bacterial species have acquired lipocalin-evading mechanisms. For example, salmochelins, siderophores produced by *Salmonella enterica* and *Escherichia coli*, were capable of acquiring iron even in the presence of lipocalin 2 [142].

2.4 Summary

There has been an increased focus on identifying endogenous stress or danger molecules. The endogenous danger signal molecules are generally extracellular modified proteins or intracellular molecules that are released by necrotic and not apoptotic cells. The growing number of these molecules prompted the creation of new class of proteins

called alarmins, or DAMPs. Heat shock proteins induce a potent immune response characterized by the release of pro-inflammatory cytokines. Hyaluronan and fibronectin are two extracellular matrix proteins that are proteolytically cleaved and their degradation products induce immune responses. On the other hand, HMGB1, low-density lipoprotein, extracellular ATP, Mrp-8 and Mrp-14, uric acid, and nucleic acid are all intracellular molecules released by necrotic cells that can activate a number of different immune cells, leading to the production of pro-inflammatory cytokines. Growing evidence has associated alarmins or DAMPs signaling through PRRs which are a limited set of germ-line encoded receptors displayed by immune cells, such as macrophages, either on the surface or intracellularly. The binding of PAMPs and DAMPs to PRRs lead to the activation of macrophages resulting in the deployment of their antimicrobial killing mechanisms, such as phagocytosis, release of nitrogen and oxygen intermediates, nutrient deprivation, and release of pro-inflammatory cytokines. The inflammatory response eventually subsides once the pathogen is eliminated, giving way to the coordinated mechanism of tissue repair.

Table 2.1: Representative mediators of inflammation

Mediator	Source	Effects
Histamine	Mast cells, basophils	Increase vascular permeability, mucus production, smooth muscle contraction
Serotonin	Mast cells, platelets	Increase vascular permeability, smooth muscle contraction
Bradykinin	Kininogen (by proteolytic cleavage)	Vasodilation, production of pain sensation, smooth muscle contraction
C3a, C5a	C3 complement protein	Degranulation of mast cells, smooth muscle contraction
Vasoactive intestinal peptide	Mast cells, neutrophils	Vasodilation
Prostaglandin E_2 (PGE_2)	Arachidonic acid (cyclooxygenase pathway)	Vasodilation, potentiate permeability effects of histamine, bradykinin, and leukotrienes
Leukotriene B_4	Arachidonic acid (lipoxygenase pathway)	Chemotaxis of neutrophils, increase vascular permeability in the presence of PGE_2
Leukotriene D_4	Arachidonic acid (lipoxygenase pathway)	Smooth muscle contraction, increase vascular permeability
Platelet-activating factor	Basophils, neutrophils, monocytes, macrophages	Release platelets' mediators, neutrophils secretion, production of reactive oxygen intermediates by neutrophils, increase vascular permeability, smooth muscle contraction
Pro-inflammatory cytokines: IL-1, IL-6, IL-8, IL-18, TNF-α, IFN-γ,	T cells, NK cells, monocytes, macrophages, dendritic cells, and other cell types	Activation of various immune cells and the induction of the inflammatory response
Chemokines	Various cells	Chemoattractant to various immune cells
Anti-inflammatory cytokines: TGF-β, IL-6, IL-1ra, IL-10	Macrophages, T cells, and other cell types	Modulation of the inflammatory response

CHAPTER III

MATERIALS AND METHODS

3.0 Animals

Four-to-six week old C57BL/6 female mice were purchased from Charles River (Wilmington, MA) and maintained according to Canadian Council for Animal Care (CCAC) guidelines in filter-top cages in the Biological Sciences Animal Facility, University of Alberta.

The goldfish were obtained from Mt. Parnell Fisheries Inc. (Mercersburg, PA). The fish were maintained in the aquatic facility of the Department of Biological Sciences, University of Alberta. The goldfish were kept at 20°C using a flow-through water system on a simulated natural photoperiod (Edmonton, AB). The fish were acclimated to this environment for at least 3 weeks before used in experiments, and were fed trout pellets to satiation daily.

3.1 Antibodies

The following are the antibodies used throughout this thesis: rabbit anti mouse β-actin (Genetex: GTX30632); rabbit anti mouse iNOS (Assay Designs: KAS-NO001); rat monoclonal anti mouse RANTES (Santa Cruz: sc-57422); goat anti mouse MCP5 (R&Systems: AF428); rat anti mouse KC (R&D Systems: MAB4531); rat monoclonal anti mouse MIP-1α (R&D Systems: MAB450).

3.2 Macrophages

The murine bone marrow-derived macrophages (BMDM were prepared as described in [141] and cultured in complete DMEM (15 mM HEPES, 1% non-essential amino acid, 1% sodium pyruvate, 10 % fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin), and supplemented with 20 % L929 supernatant (source of M-CSF).

Murine peritoneal macrophages were isolated according to a protocol described in Paulnock [143]. The peritoneal macrophages were cultured in complete DMEM for 24 hrs at 37 °C, 5% CO_2, in 75 cm^2 tissue culture flasks (Corning) prior to use in the assays. Murine macrophage-like cell line $P388D_1$ was purchased from ATCC and maintained at 37°C in 5% CO_2 and grown in complete DMEM.

BMDM were cultured for 7 to 8 days prior to use and $P388D_1$ cells were used when confluent growth in cultures was observed. Macrophages were detached from culture vessels by trypsin/EDTA solution (0.25 g trypsin (Sigma), 38 mg EDTA in 100 mL 1X PBS, pH 7.4, supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin; PBSA). The cells were suspended in complete DMEM and seeded at 5 x 10^4 and 3 x 10^6 cells/well in 96-well and six-well tissue culture plates (for cytokine array experiments), respectively. The cells were incubated for 24 hrs at 37 °C in 5% CO_2 atmosphere. The next day the medium was removed, the cells washed 2 times with PBSA, and re-cultured in fresh medium containing 100 U/mL of murine recombinant IFN-γ (mrIFNγ) and incubated at 37 °C for a further 16 hrs. After incubation, the cells were washed 2 times with 1X PBSA and 100 μL of fresh medium was added containing the appropriate concentrations of reagents for experimental and control groups, which

will be described in details in chapter 4 and 5 of this thesis. The cells were incubated for a further 24 hrs at 37 °C in 5% CO_2 atmosphere. After incubation, the plates were centrifuged at 430 x *g* for 5 min and the supernatants harvested and used in nitric oxide assay, TNF-α ELISA, Western blot and cytokine array experiments.

Kidney leukocytes were isolated from goldfish as previously described [144] and cultured in a medium containing 5% carp serum, 10 % calf serum, 50 μg/mL gentamycin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 % cell-conditioned medium (supernatants from previous macrophage cultures). Fish macrophages were cultured for 7 to 9 days, harvested using a rubber policeman, and centrifuged at 430 x *g* for 5 min. The cells were re-suspended in fresh complete culture medium. One hundred μL containing 5 x 10^4 cells were seeded into each well of a 96-well tissue culture plate. The appropriate concentrations of reagents for experimental and control groups were added to the cells as described in chapters 4 and 5. The plates were incubated for 72 hrs at 21 °C. After incubation, the plates were centrifuged at 430 x *g* for 5 min, and the supernatants harvested and used for determination of nitrite concentration using the Griess reaction.

3.3 Transferrin

3.3.1 Commercial transferrin

Commercially available murine and bovine apo-transferrin were purchased from Sigma (catalog numbers, T0523, and T1428, respectively). The iron-saturated recombinant human N-lobe of transferrin was produced in HEK 293 cells and was a kind gift from Dr. Ross MacGillivray (University of British Columbia).

3.3.2 Purification of goldfish serum transferrin

Isolation and purification of goldfish serum transferrin was carried out according to the method described previously [145, 146]. Briefly, the goldfish were bled and the serum was kept at -20 °C. Three mL of filtered (Sterivex 0.2 μm) serum were mixed with buffer A (1.2 M ammonium sulfate, 0.5 M sodium citrate, pH 6) and loaded onto phenyl-Sepharose CL-4B column (Sigma) after it was calibrated with buffer A. The purification was done at room temperature at 0.5 mL/min flow rate. The column was washed with 6-column volumes of buffer A after loading the serum sample, followed by 3-column volumes of buffer B (0.6 M ammonium sulfate, 0.25 M sodium citrate, pH 6) were passed through. Transferrin was eluted with buffer C (0.3 M ammonium sulfate, 0.125 M sodium citrate, pH 6). The elution fractions were analyzed using SDS-PAGE and the transferrin fractions were pooled and dialyzed against 8 L of 1X PBS. The protein concentration was determined by BCA method (Pierce) according to the manufacturer's instructions and the fractions stored at 4°C until used in the experiments.

3.4 Enzymatic digestion of transferrin

One mg of mouse apo-transferrin, bovine transferrin, goldfish serum transferrin (gfsTF), and human N-lobe of transferrin (hN-lobe) were digested using a modified protocol described previously [147]. Briefly, transferrin was reconstituted in 1X PBS (pH 8.5), and denatured with 0.2 % (v/v) β-mercaptoethanol for 30 min at 37°C. Transferrin was digested using porcine elastase IV, at 1:50 enzyme/protein ratio at 37°C for ~16 hours. The digestion was stopped by the addition of 5 mM of phenylmethylsulfonylfluoride (PMSF). The digestion of transferrin was confirmed by

non-reducing SDS-PAGE and coomassie blue staining by loading 30 μg/lane of digested transferrin and full-length transferrin (control). All reagents were purchased from Sigma-Aldridge Chemicals, USA.

3.5 Size-exclusion chromatography (Superose 12 and Superdex 75) and anion-exchange chromatography (Mono-Q)

The digested transferrins (1 mg/mL) were separated using fast performance liquid chromatography (FPLC) system (LKB, Amersham/Pharmacia) and Superose 12 size exclusion column. One mL fractions were collected at a flow rate 0.5 mL/min at room temperature in 1X PBSA, pH 7.4, and stored at 4°C prior to use in the experiments. Elastase-digested transferrin was also separated using FPLC (AKTA Explorer 100 A, Amersham/Pharmacia) and Superdex 75 column. The samples subjected to a dual wavelengths (280 and 215 nm) enhanced protein detection system, based on the presence of aromatic amino acids and the peptide backbone. The fractions were collected at a flow rate of 1 mL/min at 4°C in 1X PBSA, pH 7.4, and stored at 4°C prior to use in the experiments.

Pooled fractions from the Superdex 75 size separation were further separated using FPLC (AKTA Explorer 100 A, Amersham/Pharmacia) anion-exchange chromatography (Mono-Q) (Amersham/Pharmacia). The binding buffer used consisted of 50 mM TRIS, pH 8.0. One mL fractions were collected at a flow rate 1 mL/min at 4 °C using elution buffer 50 mM Tris and 1 M NaCl, pH: 8.0. Every fifteen fractions were grouped together and labeled A1 to A15, B1 to B15 and so on.

3.6 Mass Spectrometry

The analysis of murine apo-transferrin digested with elastase was analyzed by reverse-phase high-pressure liquid chromatography and tandem mass spectrometers (LC/MS/MS) at the Institute of Bio-molecular Design, Medical Sciences Building at the University of Alberta, Edmonton, Canada. The peptides were analyzed by MASCOT (Matrix Science) protein search engine.

3.7 Transferrin macrophage-activating peptide (TMAP) synthesis

The transferrin peptide was synthesized by Quality Control Biochemicals (Hopkinton, MA) and Bio Basic Inc. (Markham, ON). The refolding buffer contained 50 mM Tris, 1 mM EDTA, 0.1 M L-arginine, 1 mM glutathione (reduced), and 0.8 mM glutathione (oxidized). The peptide was dialyzed against 8 L 1X PBS using Spectra/Por dialysis membrane (2000 molecular weight cut-off) (Cole-Palmer, Montreal). After dialysis, the peptide was concentrated and filter-sterilized using 0.22 μm filter (Pall Life Sciences), and the presence of the peptide was confirmed by SDS-PAGE and silver staining. The synthetic TMAP was analyzed using Limulus Amebocyte Lysate Endosafe® kit (LAL) (Charles River) according to manufacturer's instructions and found to be endotoxin-free.

3.8 Nitric oxide assay

Fifty thousand cells were seeded in a 96-well tissue culture plate (Costar) and allowed to adhere overnight. The next day the medium was removed and the wells washed 2 times with PBSA to remove any dead cells and debris. The cells were then cultured in fresh medium containing 100 U/mL rmINF-γ (Genentech) for 16 hrs. The medium containing rmINF-γ was removed and 100 μL of fresh medium was added containing the following treatments: non-treated cell or cells treated with one of the following, 1 μg/mL LPS (Sigma), 50 μg/mL murine apo-transferrin, 1 to 75 μg/mL TMAP or 25 μL PBSA and the cells were incubated for 24 hrs at 37°C in 5% CO_2 atmosphere. The assay using *in vitro*-derived goldfish macrophages was identical to that used for mammalian macrophages except that no rmIFN-γ pre-treatment was required and different medium (see above) was employed and the incubation was for 72 hrs at 21°C. The nitric oxide response was determined indirectly by measuring the nitrite accumulation in the culture supernatants using the Griess reaction [148] and a nitrite standard curve (Fig. 3.1). The results were normalized using medium alone values, to account for the presence of nitrite in the culture medium.

3.9 Enzyme-linked Immunosorbent Assay (ELISA)

Ready-Set-Go ELISA (eBioscience; cat. No. 88-7324-22) was used as per manufacturer's directions to measure TNF-α secretion by or TMAP-treated different macrophage populations. Briefly, Corning Costar 9018 96-well ELISA plates were coated using 100 μL/well of capture antibody in coating buffer. The plates were

incubated overnight at 4 °C. After incubation, the plates were washed 5 times with 250 μL/well 1X PBST. The plates were blocked using 1X assay diluent (200 μL/well) and incubated at room temperature for 1 hour. The plates were washed 5 times with 250 μL PBST. One hundred μL of the test samples or standards were added to the wells and the plates incubated overnight at 4 °C. After incubation the plates were washed 5 times using 250 μL PBST and 100 μL/well of Avidin-HRP-antibody were added to the wells and the plates were incubated at room temperature for 30 min. The plates were then washed 7 times using 250 μL PBST, followed by the addition of the substrate solution 1X TMB and the plates were incubated for another 15 min at room temperature. The reaction was stopped by the addition of 50 μL of 2 N H_2SO_4. The absorbance values were determined at 450 and 570 nm, and then the values of 570 nm were subtracted from those of 450 nm.

3.10 SDS-PAGE and Western blot

For Western blot of murine inducible nitric oxide synthase (iNOS) and β-actin, SDS-PAGE was done using 25 μg of cell lysate (cell lysis buffer: 50 mM TRIS-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5% Triton X-100, 1mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and a protease inhibitor cocktail). For determination of chemokines, SDS-PAGE was done using 50 μg of BMDM supernatants resolved using 12.5 % gels under native conditions at 120 Volts for 30 min and then 180 Volts for 20 min. Proteins were transferred onto nitrocellulose membrane (BioRad) at 120 Volts for 40 min and the membranes probed using the following primary antibodies: rabbit anti-iNOS at 1:1000;

rat anti-RANTES at 1:200; rat anti-MIP-1α at 1:500, goat anti-MCP-5 at 1:500; and rat anti-KC at 1:500. The membranes were washed 5 times with PBST and probed with appropriate HRP labeled anti-species secondary antibodies (Jackson Laboratories) for 1 hr. The membranes were washed 5 times with PBST and were developed using ECL kit (BioRad).

3.11 Chemotaxis assay

Chemotaxis of macrophages was assessed using blind-well chemotaxis chambers; the upper and lower chambers were separated using a 0.5 μm pore size polycarbonate membrane (Neuro Probe Inc.). Two hundred μL containing 5 x 10^4 cells suspended in complete DMEM in duplicate were placed in the top well and 200 μL of DMEM containing one of 10 ng/mL f-Met-Leu-Phe (fMLP; positive control), 100 ng/mL murine apo-transferrin, 1-500 ng/mL TMAP (final concentrations) or medium control. After incubation for 2 hrs at 37 °C, the filters were removed, fixed with methanol and stained using Giemsa staining solution (BDH). Cells that migrated through 0.5 μm pores and were located on the underside of the polycarbonate membrane were counted in 20 randomly selected fields of view using a microscope fitted with a 100X objective lens. Chemokinesis was performed to eliminate the concentration gradient between the two wells of the blind-well chemotaxis apparatus. This was accomplished by placing 100 ng/mL TMAP in 200 μL in both upper and lower wells of the chemotaxis apparatus. The results were expressed as the mean ± SEM of number of migrated macrophages/20 fields of view.

3.12 Cytokine array

Four 62-G III series cytokine arrays were purchased from Raybiotech (Norcross, GA; Cat. No. AAM-CYT-G3) and prepared as per manufacturer's instructions. BMDM (3 x 10^6/well) were seeded in a 6-well tissue culture plate and supernatants harvested as described in section 3.2. Cells were either left in medium only (negative control) or treated with 50 μg/mL TMAP. One hundred μL of the cells supernatants were analyzed using array procedure described by the manufacturer. The arrays were quantified using GenePix (Molecular Devices, California) fluorescence scanner and the data analyzed using Gene Pix Pro 6.0 and RayBiotech mouse cytokine G series software.

3.13 Quantitative PCR

Real time PCR was performed using SYBR green method and Fast 7500 thermocycler (Applied Biosytems). The relative quantitations of the mRNA expression levels were determined using the ΔΔCt method. Three μg of total RNA were reversed transcribed into cDNA using cDNA synthesis kit (Invitrogen) and the primers were designed using PrimerExpress 3.0 (Applied Biosystems) software and purchased from IDT (Toronto, ON). The list of primers used in this thesis are presented in Table 3.1.

3.14 Statistical Analysis

The statistical analysis was done using one-way ANOVA comparing experimental and control groups. Probability level of $P < 0.05$ was considered significant and $P < 0.1$ was considered marginally significant.

Table 3.1: List of the quantitative PCR primers used in this thesis

Gene	Forward	Reverse
iNOS	5'-CATGGCTTCCACGGGTCAGA-3'	5'-AGGTCCCTGGCTAGTGCTTCA-3'
TNF-α	5'-GCCGATGGGTTGTACCTTGT-3'	5'-GTGGGTGAGGAGCACGTAGTC-3'
IL-1β	5'-TCAGGCAGGCAGTATCACTCA-3'	5'-GGAAGGTCCACGGGAAAGAC-3'
NF-κB	5'-GCCACAGAGATGGAGGAGTTG-3'	5'-GGTCCGGGCATTCACATTAG-3'
Smad4	5'-GGGAGGAGATCGCTTTTGCT-3'	5'-TGCAACCTCGCTCTCTCAATC-3'
IL-6	5'-CAACCACGGCCTTCCCTACT-3'	5'-AGACAGGTCTGTTGGGAGTGGTA-3'
MCP-5	5'-GAGAATCACAAGCAGCCAGTGT-3'	5'-TCAGCACAGATCTCCTTATCCAGTA-3'
MIP-1α	5'-CTCCCAAGCCAGGTGTCATTT-3'	5'-TGGACCCAGGTCTCTTTGGA-3'
MIP-2	5'-GGCTGTTGTGGCCAGTGAA-3'	5'-TGTTCAGTATCTTTTGGATGATTTTCTG-3'
KC	5'-CACTGCACCCAAACCGAAGT-3'	5'-CAAGGGAGCTTCAGGGTCAA-3'
RANTES	5'-CAGCAGCAAGTGCTCCAATC-3'	5'-CTTCTCTGGGTTGGCACACA-3'
MIP-1γ	5'-CTGCCCTCTCCTTCCTCATTC-3'	5'-CTTTTGTCTCTGTTGCATGTGTGA-3'
G-CSF	5'-AGCTGCTGCTGTGGCAAAGT-3'	5'-GTGGCAGAGCGCTGACAGT-3'
VEGF	5'-TCGTCCAACTTCTGGGCTCTT-3'	5'-CAGCTCCGATCGGTTTGTCT-3'
β-actin	5'-CAGCAAGCAGGAGTACGATGAGT-3'	5'-GCTCAGTAACAGTCCGCCTAGAA-3'

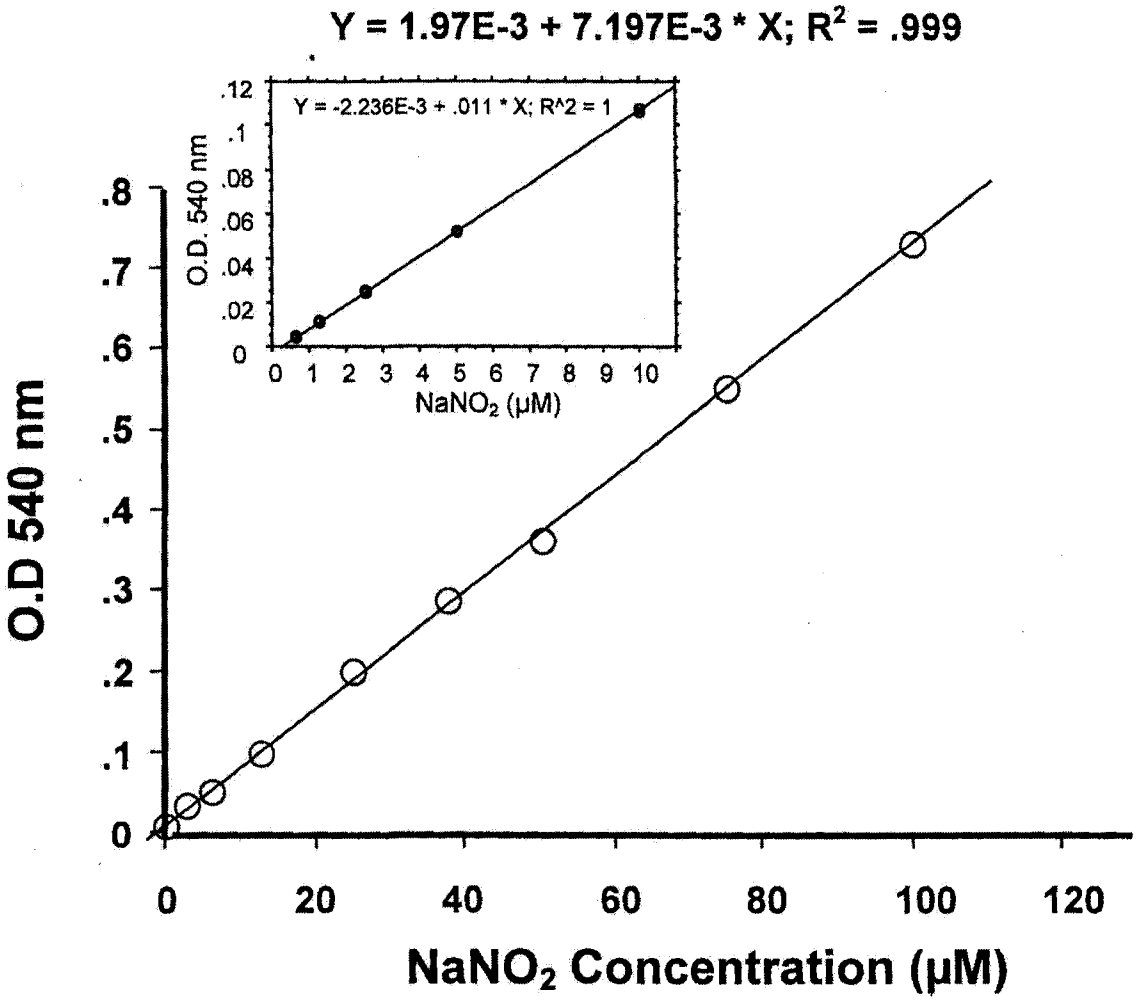

Figure 3.1: Sodium nitrite standard curve for the determination of nitrite production using the Griess reaction. Inset: Sodium nitrite standard curve for low values of nitrite.

CHAPTER IV

ENZYME-MODIFIED TRANSFERRIN FROM DIFFERENT HOSTS MODULATES PRO-INFLAMMATORY RESPONSES OF MACROPHAGES

4.0 Introduction

Transferrins are a family of 75-80 kDa iron-binding proteins that are common across many phyla [149]. Due to the highly conserved biological function of transferrin, it is not surprising that transferrins from diverse taxa are highly homologous. For example, the percent amino acid identity between mouse transferrin and that of goldfish, bovine and human molecules is 41%, 63% and 72%, respectively, and that of the transferrin homologue lactoferrin is 60% [150]. The transferrin molecule is composed of two relatively homologous lobes (C- and N-lobe; ~ 40% sequence identity), with a single iron-binding site in each lobe. In mammals, transferrin is mostly synthesized in the liver, and its main function is the delivery of iron to cells that express transferrin receptor-I and -II (TfRI or CD71 and TfR-II) via receptor-mediated endocytosis. After endocytosis, iron is released from the transferrin N- and C-lobes in the acidic environment of the endosome, and the transferrin-receptor complex is recycled back to the surface where at the physiological pH (pH 7.4) transferrin is released [151]. Transferrin is also synthesized by macrophages, suggesting a possible role for this molecule in host defense, particularly in inflammatory microenvironments [19].

The central role of transferrin as an iron transporting protein has been extended by observations that modified versions of this protein also participate in the regulation of innate immunity. For example, it has been reported that transferrin acts as an acute phase

protein [20] and that it can create a bacteriostatic environment by sequestering free iron from invading pathogens [16]. Transferrin fragments are frequently observed in bronchoalveolar lavage (BAL) of cystic fibrosis patients [12], and in the supernatants of mitogen-activated macrophage cultures of lower vertebrates such as bony fish [13], suggesting that transferrin fragments may act as danger signals "warning" the immune system of the presence of pathogens or of tissue injury.

Recent evidence suggests that modified transferrin is involved in induction of antimicrobial function of macrophages, since immunopurified transferrin fragments present in the supernatants of mitogen-stimulated goldfish leukocytes induce significant production of reactive nitrogen intermediates in goldfish *in vitro*-derived macrophages [13]. Similarly, lactoferrin fragments, found in parotid saliva of periodontitis patients, induce the production of interleukin-6 (IL-6), monocytes chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) [77].

At inflammatory sites, activated neutrophils release a large number of proteases that can degrade endogenous proteins and membrane bound host proteins that can influence the progression of inflammation. One serine protease produced in copious amounts by activated neutrophils is elastase. Activated neutrophils degranulate and release elastase at the inflammatory site [103], suggesting that immunomodulatory transferrin and lactoferrin fragments may be generated by a process mediated by this enzyme.

In this chapter, I describe the results of experiments designed to determine whether elastase can cleave transferrin from different hosts into fragments that induce pro-inflammatory function of different macrophage populations.

4.1 Results

4.1.1 Detection of nitric oxide activity in Superose 12 fractions of elastase-digested transferrins

Mouse apo-transferrin (m-apoTf) was digested with porcine elastase overnight. The digested products were separated by FPLC using a Superose 12 size-exclusion column. The sixty FPLC fractions obtained were then tested for their ability to induce nitric oxide response in murine macrophage-like P388D_1 cells. Figure 4.1A shows the pooled (10 fractions) mean ± SEM nitrite production by P388D_1 cells 24 hours after exposure to 25 μL obtained from individual fractions. The nitric oxide-inducing activity was observed in fractions 31 to 40 and 41 to 50 where the nitrite produced by treated cells were significantly higher than that of the medium control. Shown in Fig. 4.1B are the results for different control groups tested to ensure that the nitric oxide-inducing activity observed in the FPLC fractions was due to the presence of transferrin fragments. Macrophages treated with 1 μg/mL LPS (positive control) produced ~ 22 μM nitrite indicating the P388D_1 cells were responsive to stimulation. Treatments of P388D_1 with either 300 ng of m-apoTf, 10 μg/mL elastase, 25 μL PBSA, 5 mM PMSF, 0.2 % β-ME, or 100 U/mL IFN-γ did not induce a nitric oxide response in P388D_1 cells (Fig. 4.1B).

The fragments in the fractions of bovine transferrin digested with elastase and resolved using Superose 12 column also induced a nitric oxide response in P388D_1 cells. Each pooled Superose 12 fraction was tested for the ability to induce nitrite production by P388D_1 cells. To reduce the number of samples to be assayed each ten consecutive fractions were pooled, and the nitrite levels (mean ± SEM) after 24 hours of cultivation

were determined by the Griess reaction (Fig. 4.2A). Pooled fractions 21 to 30, 31 to 40, and 41 to 50 induced a significant nitric oxide response in $P388D_1$ cells, compared to medium control. Interestingly, 300 ng/mL of non-digested bovine transferrin did not induce nitrite production by $P388D_1$ macrophages (Fig, 4.2B). Similarly, FPLC fractions of human N-lobe of transferrin (hN-lobe) digested with elastase and resolved using Superose 12 column induced a significant nitric oxide response in $P388D_1$ cells (Fig. 4.3A). Like the non-digested bovine transferrin, the non-digested hN-lobe at concentration 300 ng/mL did not have the capacity to induce nitrite production by $P388D_1$ macrophages (Fig. 4.3B).

The goldfish serum transferrin (gfsTf) was isolated and purified using Phenyl-Sepharose CL-4B column. The 1 mg of gfsTf was digested with elastase and fractionated using FPLC and the Superose 12 size exclusion column. Each FPLC fraction was tested for nitric oxide-inducing activity using cells from four primary kidney macrophage cultures established from individual goldfish. The nitrite production of activated goldfish macrophages (mean ± SEM) of pooled fractions is presented in Fig. 4.4A. The nitrite concentrations in cell cultures treated with fractions 31 to 40, and 41 to 50 were marginally significant when compared to medium control ($P < 0.07$ and $P < 0.06$, respectively, one-way ANOVA). The treatment of cells with 300 ng/mL of non-digested gfsTf did not induce a nitric oxide response in goldfish macrophages (Fig. 4.4B).

Interestingly, elastase-digested m-apoTf also induced nitric oxide response in goldfish macrophages, where pooled fractions 31 to 40 and 41 to 50 induced significantly higher nitrite production compared to the medium control, suggesting that this may be a highly conserved mechanism of activation of macrophages (Fig. 4.5A). Notably,

treatment of goldfish macrophages with 300 ng/mL of non-digested m-apoTf did not induce nitrite production by goldfish macrophages (Fig 4.5B).

4.1.2 Detection of nitric oxide activity in Superdex 75 fractions of elastase-digested murine apo-transferrin

The acquisition of a more advanced FPLC system and a size exclusion column (Superdex 75) with peptides resolving capacity between M_r 3 kDa and 75 kDa, allowed me to examine the elastase-digested transferrin fragments using dual wavelengths, at 280 nm which detects aromatic amino acids, and at 215 nm which detects peptide bonds.

The FPLC fractions generated using Superdex 75 size-exclusion column of elastase-digested murine transferrin were tested for their ability to induce nitric oxide response in P388D_1 macrophages. Figure 4.6A shows the FPLC-generated chromatogram of digested transferrin resolved by Superdex 75 column, where a multitude of fragments was present in fractions B1 to C10. Figure 4.6B shows an activity profile of fractions B8 to C3 for induction of nitric oxide response in P388D_1 macrophages, indicating the presence of digested transferrin fragments in these fractions that have immunostimulatory activity. Figure 4.6C shows silver stained SDS-PAGE gel of active fractions B8 to C3, demonstrating the presence of multiple digested transferrin fragments in every FPLC fraction. Due to the presence multiple digested of transferrin fragments following elastase digestion, it was very difficult, if not impossible, to identify individual peptides. Notably, the nitric oxide response induced in P388D_1 macrophages by elastase-digested m-apoTf and resolved using Superdex 75 column was 10 times

higher than that of elastase-digested m-apoTf separated using Superose 12 size exclusion column.

4.1.3 Detection of nitric oxide activity in Mono-Q fractions of elastase-digested murine apo-transferrin

The active fractions observed in Figure 4.6A were pooled B7 to B11 and B12 to B15 based on their nitric oxide-inducing activity. The pooled fractions were separated using anion-exchange chromatography (Mono-Q) and the resulting fractions were tested for the induction of nitric oxide response in P388D_1 macrophages.

Figure 4.7A shows the profile of the Mono-Q fractions of Superdex 75 pooled fractions B7 to B11. The Mono-Q resolved peptides that induced nitric oxide response in P388D_1 macrophages were found in fractions A1 to A10 and B2 to C12. Figure 4.7B shows the results obtained for the different control groups; these being, medium control, or cells treated with one of the following: 1 μg/mL LPS, 300 ng/mL m-apoTf, 100 U/mL IFNγ, 25 μL PBSA, 25 μL Mono-Q buffer 1, and 25 μL Mono-Q buffer 2 used in the experiment to ensure that the observed induction of nitrite production by activated macrophages was due to transferrin fragments.

Figure 4.8A shows the profile the nitric oxide-inducing activity of Mono-Q resolved fragments present in pooled Superdex 75 fractions B12 to B15. The nitric oxide-inducing activity was observed in Mono-Q fractions F5 to G12.

Figures 4.7C and 4.8B show the silver stain of SDS-PAGE gels of the Mono-Q nitric oxide-inducing peptides in fractions B10 to C6 and F12 to G7, respectively. Similar to what was observed for size-exclusion separation of nitric oxide-inducing

fragments, the anion-exchange separation failed to resolve individual fragments that had immunomodulatory activity.

4.1.4 Mass spectrometry analysis of peptides obtained by elastase digestion of murine transferrin

The elastase-generated transferrin peptides were analyzed using LC/MS/MS and the Mascot peptide search engine (Matrix Science) that confirmed the presence of murine transferrin peptides. The mass spectrometry analysis identified a plethora of elastase-cleaved transferrin peptides (Table 4.1), many of which had overlapping sequences demonstrating the non-specific digestion of transferrin by elastase.

4.1.5 Identification of nitric oxide-suppressing transferrin peptides from Superdex 75 fractions of elastase-digested murine apo-transferrin

The FPLC-Superdex 75 chromatogram of elastase digested m-apoTf revealed the presence of transferrin fragments in fractions C4 to C10 (Fig 4.6A). However, the transferrin fragments in these fractions did not possess nitric oxide-inducing activity (Fig. 4.6B). Consequently, experiments were designed to determine whether those fragments could have either nitric oxide inhibitory activity or whether they were fragments that simply did not have the capacity to activate P388D_1 macrophages. The results showed that transferrin fragments present in fractions C6, C7, and C8 had nitric oxide suppressing activity, and that the most pronounce inhibition of the nitric oxide response was induced by transferrin fragments in fraction C7. Figure 4.9A shows the control groups used for this experiment, where nitric oxide response was induced in cells after

treatment with 1 μg/mL LPS, or transferrin fragments present in the Superdex 75 fraction B8 to B15 (shown as the mean ± SEM nitrite response of eight fractions). The non-treated macrophages, or those treated with 300 ng/mL m-apoTf, or 25 μL of Superdex 75 fraction C7, had negligible nitrite production compared to that generated by macrophages treated with LPS or fragments in fractions B8 to B15. Figure 4.9B shows the nitric oxide suppressing activity of transferrin fragments present in fraction C7. The $P388D_1$ macrophages were treated either with 25 μL of nitric oxide-inducing fragments in fractions B8 to B15, or with 25 μL of peptides from fractions B8 to B15 in conjunction with 25 μL of fragments from fraction C7. The nitric oxide inhibiting effect of transferrin fragments present in fraction C7 was notable, as the nitrite concentration generated by macrophages treated with B8 to B15 active fractions in conjunction with C7 was reduced in all experimental groups when compared to the macrophages treated with only fractions B8 to B15.

The Superdex 75 fractions C6 to C8 were pooled and resolved further using anion-exchange chromatography (Mono-Q column). The results presented below, indicate that fragments with both nitric oxide-inducing and nitric oxide-suppressing activities were present in these fractions. Figure 4.10A shows the nitric oxide response to $P388D_1$ macrophages treated with Mono-Q fractions. Surprisingly, a number of C6 to C8 Mono-Q fractions induced a strong nitric oxide response in $P388D_1$ macrophages. The activity of nitric oxide-inducing transferrin fragments present in C6 to C8 appeared to be masked by the presence of nitric oxide-suppressing transferrin fragments in the same fractions. Figure 4.10B shows the silver stained SDS-PAGE gel of non-inducing nitric oxide fragments present in Mono-Q fractions I1 to I12. A large number of transferrin

fragments were present in each fraction. The results of experiments designed to identify the presence of nitric oxide-inhibiting fragments in Mono-Q fractions are shown in figure 4.11A. $P388D_1$ macrophages were treated either with Mono-Q fraction with nitric oxide-inducing activity J13, or treated with fraction J13 in combination with one of the Mono-Q fractions that did not exhibit the nitric oxide-inducing capability I9 to I15 or J1. The nitrite concentration generated by J13 fraction was reduced after addition of equal volume of the non-inducing nitric oxide fraction I9 to J1. To ensure that these results were not due to a dilution effect was supported by data presented in Figure 4.11B where the addition of 25 μL of TRIS-NaCl buffer to cell cultures did not alter the nitric oxide response of $P388D_1$ macrophages treated with 25 μL of nitric oxide-inducing Mono-Q fractions J12 to J15.

4.1.6 Expression of interleukin-1β, inducible nitric oxide synthase, Smad 4 and NF-κB after treatment of $P388D_1$ macrophages with peptides generated by elastase-digested transferrin

To further examine the pro-inflammatory effects of elastase-generated transferrin peptides, I measured the mRNA levels of pro-inflammatory cytokine IL-1β, the enzyme that catalyzes the production of nitric oxide (iNOS), and the transcription factors NF-κB and Smad4 after treatment of $P388D_1$ macrophages with fragments found in fractions that both enhanced nitric oxide response, fractions B11 and B12, and those that suppressed nitric oxide response, fractions C6 and C7. The mRNA expression levels in macrophages treated with fraction B11 were one fold higher for IL-1β and NF-κB and two fold higher for iNOS when compared to the medium control (Fig. 4.12). Similarly,

the mRNA levels of iNOS in the cells treated with B12 fraction were two-fold higher than those of medium control, although the expression of IL-1β and NF-κB in cells treated with B12 fraction were only slightly higher than medium control (Fig. 4.12). In contrast, the expression of IL-1β, in macrophages treated with fragments from Superdex 75 fractions that had nitric oxide-suppressing activity (fractions C6 and C7), were lower than those observed in medium controls and ~ one fold less than the mRNA levels detected in macrophages treated with nitric oxide-inducing fragments from fractions B11 or B12. Importantly, the iNOS mRNA levels in macrophages treated with fragments from fractions C6 or C7 were ~ 2 to 3 fold lower than those in macrophages treated with fractions B11 and B12, respectively (Fig. 4.12), supporting earlier findings that the nitrite production was significantly suppressed by fragments present in fraction C6 and C7 (Fig. 4.9A).

The mRNA levels of the transcription factor Smad4 were similar in all experimental groups (Fig. 4.12), suggesting that the anti-inflammatory cytokine transforming growth factor-β (TGF-β) which was commonly associated with deactivation of macrophages [150], may not be involved in the observed down regulation of the nitric oxide response by suppression-inducing fragments present in fractions C6 and C7.

4.2 Discussion

At the inflammatory site, different enzymes are released by the immune cells including metalloproteases and serine proteases that have been shown to modify proteins of both pathogens and hosts [103]. Elastase was one of the prominent enzymes identified at the inflammatory sites, and was shown to be secreted by neutrophils early (first 24

hours) in the inflammatory response [12]. Previous studies have shown that host elastase can cleave transferrin in inflammatory microenvironment since transferrin fragments were detected in bronchoalveolar lavage (BAL) of cystic fibrosis patients [12]. Although there are no reports regarding the possible physiological role of elastase-derived transferrin fragments in mammals, it was previously shown that cleaved affinity-purified goldfish transferrin induced nitric oxide response in *in vitro*-derived goldfish macrophages [13]. Based on these observations, I hypothesized that the elastase-generated transferrin fragments may play a role in the regulation of host inflammatory responses in mammals by specifically up regulating pro-inflammatory responses of macrophages.

Due to the relatively low nitric oxide response generated by elastase-digested transferrin fragments obtained using FPLC (LKB) and the Superose 12 column, I repeated the experiments using a different FPLC apparatus and Superdex 75 column, which had better resolution capacity for peptides ranging in M_r between 3 kDa and 75 kDa. Although this experimental setup resulted in higher nitric oxide-inducing activity by the transferrin fragments, however, the separation of individual peptides present in each fraction (as seen in the silver stained SDS-PAGE gel) proved to be difficult. Since mass spectrometry analysis of the peptides in individual fractions would have been very expensive, I elected to use mass spectrometry to analyze non-fractionated enzymatic digest. As presented in Table 4.1, a large number of transferrin fragments were generated by elastase digestion and several were overlapping fragments. The mass spectrometry results demonstrated the non-specific cleaving capacity of elastase, such that the

establishment of consistent and predictable peptide pool after digestion of transferrin with this enzyme was not possible.

Elastase is a serine protease with broad substrate specificity. Elastase is released by neutrophils during an inflammatory response [12], and previous studies have shown that host and bacterial elastase can cleave transferrin [18]. Both bacterial and neutrophil elastase were detected in the bronchoalveolar lavage (BAL) of cystic fibrosis (CF) patients in conjunction with transferrin peptides [12]. Furthermore, it has been shown that the cleavage of transferrin by *Pseudomonas aeruginosa* elastase in the lungs of CF patients enhanced the removal of iron by bacterial siderophores pyoverdin and pyochelin [12]. It was suggested that transferrin cleavage led to an increase in free-iron concentration, which contributed to formation of toxic hydroxyl radical that exacerbated the tissue damage in the lungs of CF patients [12,172].

The presence of peptides from modified host proteins in inflammatory microenvironment that are immunostimulatory was not unique to transferrin. For example, the stimulation of macrophages *in vitro* by a 135 kDa hyaluronan fragment resulted in the expression of macrophage-inflammatory protein (MIP)-2 in peritoneal and bone marrow-derived macrophages [41]. Similarly, fibronectin peptides were generated during the extravasation process of monocytes from the blood vessels through the endothelial basement membrane and the extracellular matrix during an inflammatory response [45]. Small lactoferrin peptides were also identified in the parotid saliva of chronic periodontitis patients. The Con A low-affinity saliva peptides and two synthetic lactoferrin peptides induced the production of IL-6, monocyte chemoattractant protein 1 (MCP-1), and IL-8 in human epithelial HSC-2 cells [77].

Surprisingly, the non-digested human N-lobe of transferrin did not possess immunostimulatory activity, which contradicts the findings of Stafford et al. (2004) [152], where goldfish recombinant C-lobe and N-lobe induced a potent nitric oxide response in both goldfish macrophages as well as $P388D_1$ murine macrophages. Given the conserved nature of immunomodulatory activities of transferrin moieties from different species, one would expect from the results presented above that transferrin fragments of different sizes would be immunostimulatory. In addition to the fact that the transferrin lobes were from different species, hN-lobe was produced using a eukaryotic expression system whereas goldfish transferrin N- and C-lobes were produced using a prokaryotic system. As a result, the hN-lobe native conformation was determined by protein crystallography [153], and the molecule was demonstrated to be a fully functional protein capable of binding iron and delivering it to cells through transferrin receptor I or CD71 [154]. In contrast, the prokaryotic expression of goldfish C-and N-lobes may not have resulted in proper folding of the recombinant peptides, especially a cysteine-rich protein like transferrin, which may have increased the susceptibility of these recombinants to possible proteolytic digestion after they have been added to the macrophage cultures. This is possible since I have demonstrated (see chapter V) that very small transferrin fragments possess immunomodulatory activity. Although Stafford et al. (2004) reported that the recombinant N- and C-lobes were free of endotoxin, there is still a possibility of low-level of endotoxin contamination, (beyond the limits of detection indicated by the *Limulus* amoebocyte assay), in these preparations that could have influenced their findings.

The mRNA expression levels of IL-1β and iNOS were examined in $P388D_1$ macrophages treated with nitric oxide-inducing fragments present in Superdex 75 fractions B11 and B12, as well as in macrophages treated with nitric oxide-suppressing fragments present in Superdex 75 fractions C6 and C7. As expected, the mRNA expression levels of IL-1β and iNOS in macrophages treated with B11 and B12 were up regulated, since the transferrin fragments found in fractions B11 and B12 induced a strong nitric oxide response. It is well established that macrophages release the pro-inflammatory cytokine IL-β [96] and up regulate the inducible form of nitric oxide synthase [118] in response to various stimuli including bacterial LPS [155], as well as in response to the modified endogenous proteins such as lactoferrin fragments [77]. The activation of macrophage antimicrobial response generally involves a signal transduction pathway that leads to activation of the transcription factor NF-κB [156]. The mRNA level of NF-κB was upregulated in P388D1 macrophages treated with nitric oxide-inducing fragments present in fractions B11 and B12. On the other hand, macrophages treated with nitric oxide-suppressing fragments present in fractions C6 and C7 had lower expression of NF-κB than that of cells treated with fractions B11 and B12 and non-treated macrophages. It is therefore possible that the transferrin peptides found in the nitric oxide-suppressing fractions C6 and C7 signal through a signal transduction pathway that leads to the regulation of pro-inflammatory genes at the transcription level. More studies are needed to further explore this possibility.

In addition to macrophage activation, the digest of elastase-treated murine transferrin also had fragments that had macrophage-deactivating properties. I hypothesized that that some elastase-generated transferrin peptides may possess anti-

inflammatory properties, because the search for conserved domains in transferrin using the SMART search engine for conserved protein domains (Heidelberg, Germany) suggested the presence of C-terminal cysteine-knot like domain (CTCK), also present in TGF-β, which has been shown to deactivate goldfish and murine macrophages [157, 158]. However, the real-time PCR results suggested that the inhibition of the nitric oxide response by transferrin fragments was mediated by process that was independent of Smad 4 transcription factor, whose activation was shown to be essential in macrophage deactivation response mediated by TGF-β [159]. The other possibility was that elastase digestion may have also generated fragments that did not have biological activity but had the ability to compete with the active fragments for the receptor that mediated the induction of macrophage activation. The generation of transferrin fragments that have deactivation properties may play an important role in limiting the damaging effects of a prolonged inflammatory response.

In this chapter, I have shown that elastase cleaved transferrin from different species and that the resulting transferrin fragments had both immunostimulatory and immunosuppressing activities. In the next chapter, I report on the identification and characterization of a transferrin-derived fragments with significant pro-inflammatory properties.

Table 1: The identified peptide sequences by mass spectrometry of elastase-digested murine apo-transferrin.

Residue	Amino acid sequence	Residue	Amino acid sequence
8-17	DAMTLDGGWVYD	302-319	TKCDEWSIISEGKIECESAE
28-38	KPVAAEFYGSVEH	303-314	KCDEWSIISEGKIE
30-38	VAAEFYGSVEH	311-318	SEGKIECESA
33-42	EFYGSVEHPQTY	328-343	EKIVNGEADAMTLDGGHA
45-55	YYAVAVVKKGTDF	332-346	NGEADAMTLDGGHAYIA
49-60	AVVKKGTDFQLNQL	333-341	GEADAMTLDGG
53-66	KGTDFQLNQLEGKKSC	336-345	DAMTLDGGHAYI
93-106	RSPLEKAVSSFFSGSC	337-356	AMTLDGGHAYIAGQCGLVPVMA
104-112	SGSCVPCADPVAFPKLCQLCPGCGCSSTQPF	340-350	LDGGHAYIAGQCG
106-114	GSCVPCADPVA	342-354	GGHAYIAGQCGLVPV
116-123	AFPKLCPGCGC	355-362	CGLVPVMAEYYES
118-127	PKLCQLCPGCGC	365-376	NCAIPSQQGIFPKG
120-126	LCQLCPGCG	370-376	SQQGIFPKG
125-134	PGCGCSSTQPF	377-385	KGYYAVAVVKA
131-144	STQPFFGYVGAFKCLK	386-394	KASDTSITWNN
132-141	TQPFFGYVGAFK	395-404	NNLKGKKSCHTG
137-152	GYVGAFKCLKDGGGDVAF	407-414	TAGWNIPMGMLYNRINHCKF
147-159	DGGGDVAFVKHTTIF	413-421	WNIPMGMLYNR
158-167	TTIFEVLPEKAD	415-429	IPMGMLYNRINHCKFDE
167-174	KARDQYELL	418-427	GMLYNRINHCKF
168-175	ADRDQYELLC	420-428	LYNRINHCKFD
172-181	QYELLCLDNTRK	423-434	RINHCKFDEFFSQG
184-190	PVDQYEDCY	436-445	GCAPGYEKNSTL
197-206	PSHAVVARKNNG	441-455	YEKNSTLCDLCIGPLKC
203-212	ARKNNGKEDLIW	451-463	CIGPLKCAPNNKEEY
208-215	GKEDLIWEIL	457-466	NNKEEYNGYT
215-222	EILKVAQEHF	464-476	EYNGYTGAFRCLVEK
235-253	FSSPLGKDLLFKDSAFGLIRV	475-483	LVEKGDVAFVK
241-251	KDLLFKDSAFGLL	488-498	TVLDNTEGKNPAE
246-265	KDSAFGLLRVPPRMDYRLYLGH	502-511	AKNLKQEDFELL
248-261	SAFGLLRVPPRMDYRL	507-516	QEDFELLCPDGT
257-266	PRMDYRLYLGHN	534-542	PNHVVVSRKEK
260-270	DYRLYLGHNYVTA	559-569	FGGSDCTGNFCLF
263-274	LYLGHNYVTAIRNQ	565-573	TGNFCLFKSTT
264-272	YLGHNYVTAIR	586-597	CFVKLPEGTTPEKY
274-285	RNQQEGVCPEGSI	592-604	EGTTPEKYLGAEYMQ
281-289	CPEGSIDNSPV	599-607	YLGAEYMQSVG
282-296	PEGSIDNSPVKWCALSH	600-610	LGAEYMQSVGNMR
291-306	VKWCALSHLERTKCDEWS	620-627	LEACTFHKH

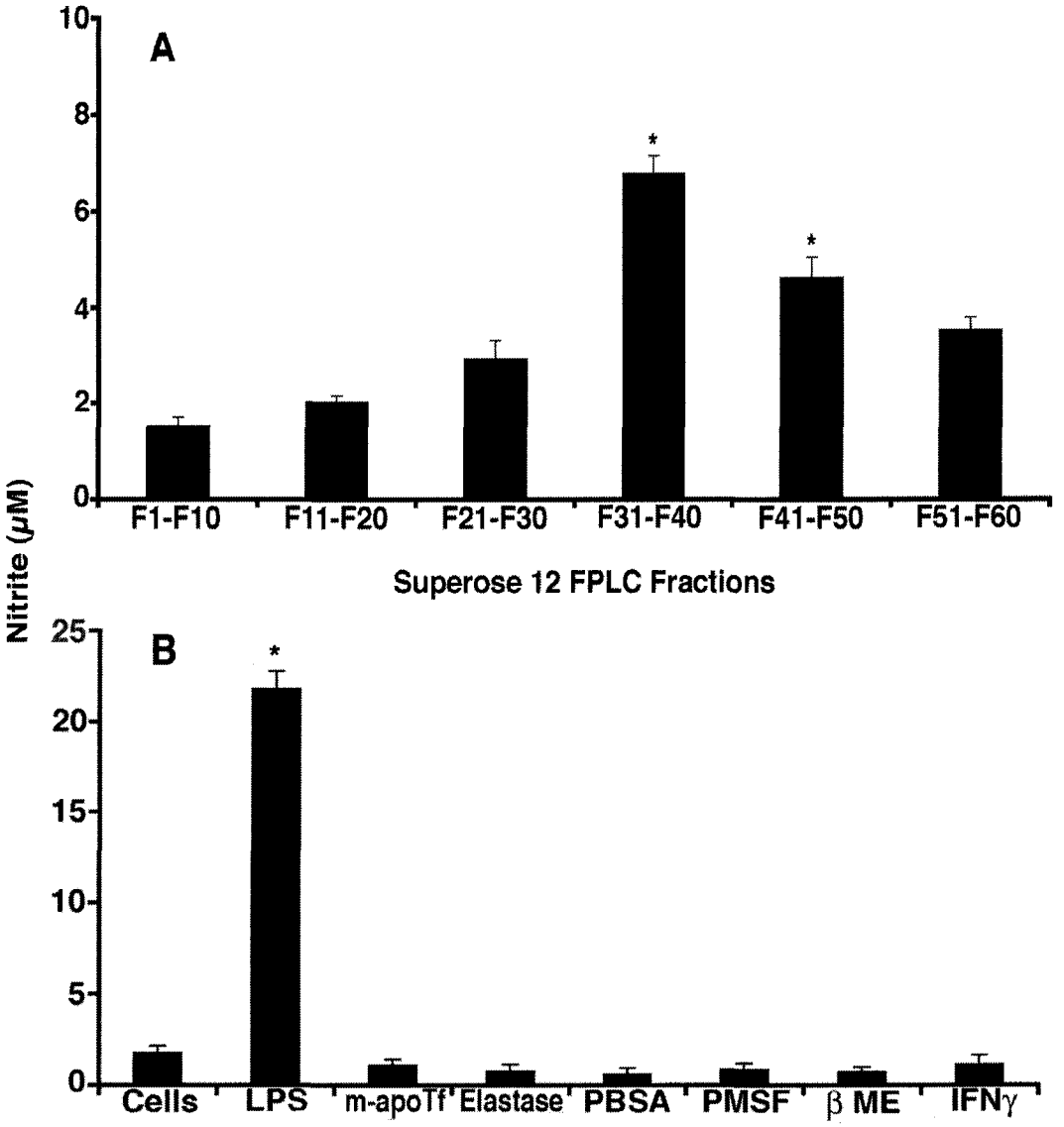

Figure 4.1: The nitric oxide response generated by elastase-digested murine apo-transferrin in P388D$_1$ macrophages. A= the nitric oxide response from pooled FPLC Superose 12 fractions; B= the nitric oxide response of the various control groups used in the experiments: 1 μg/mL LPS, 300 ng/mL m-apoTf, 10 μg/mL elastase, 25 μL PBSA, 5 mM PMSF, 0.2 % β-ME, and 100 U/mL IFN-γ. The nitrite concentrations were determined 24 hours after treatment with different reagents using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate cultures and the data are from two independent experiments that were performed (n=4). (*) $P<0.05$ vs. cells alone.

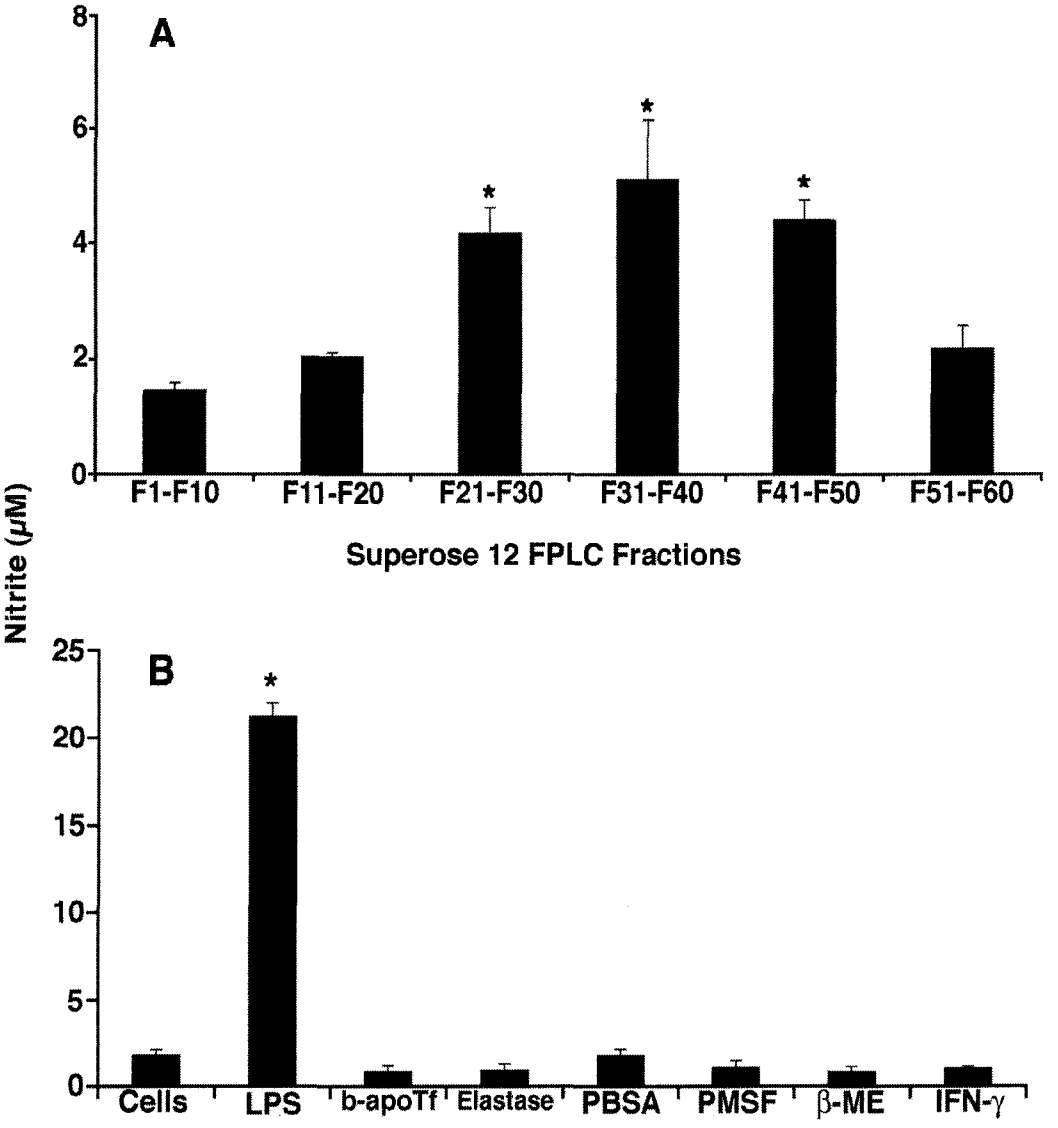

Figure 4.2: The nitric oxide response generated by elastase-digested bovine apo-transferrin (b-apoTf) in P388D$_1$ macrophages. A= the nitric oxide response from pooled FPLC Superose 12 fractions; B= the nitric oxide response of the various control groups used in the experiments: 1 μg/mL LPS, 300 ng/mL b-apoTf, 10 μg/mL elastase, 25 μL PBSA, 5 mM PMSF, 0.2 % β-ME, and 100 U/mL IFN-γ. The nitrite concentrations were determined 24 hours after treatment with different reagents using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate cultures and the data are from two independent experiments that were performed (n=4). (*) $P<0.05$ vs. cells alone.

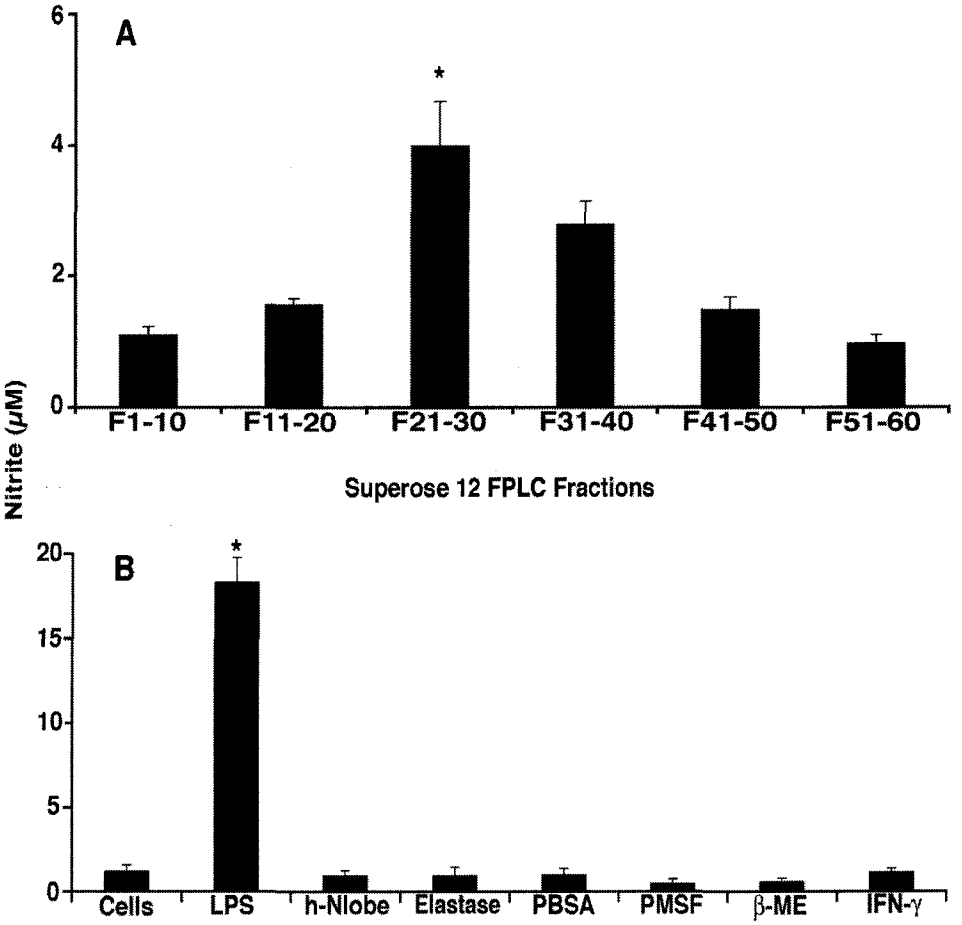

Figure 4.3: The nitric oxide response generated by elastase-digested human N-lobe of transferrin in P388D$_1$ macrophages. A= the nitric oxide response from pooled FPLC and Superose 12 fractions; B= the nitric oxide response of the various control groups used in the experiments: 1 μg/mL LPS, 300 ng/mL h-Nlobe, 10 μg/mL elastase, 25 μL PBSA, 5 mM PMSF, 0.2 % β-ME, and 100 U/mL IFN-γ . The nitrite concentrations were determined 24 hours after treatment with different reagents using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate cultures and the data are from two independent experiments that were performed (n=4). (*) $P<0.05$ vs. cells alone.

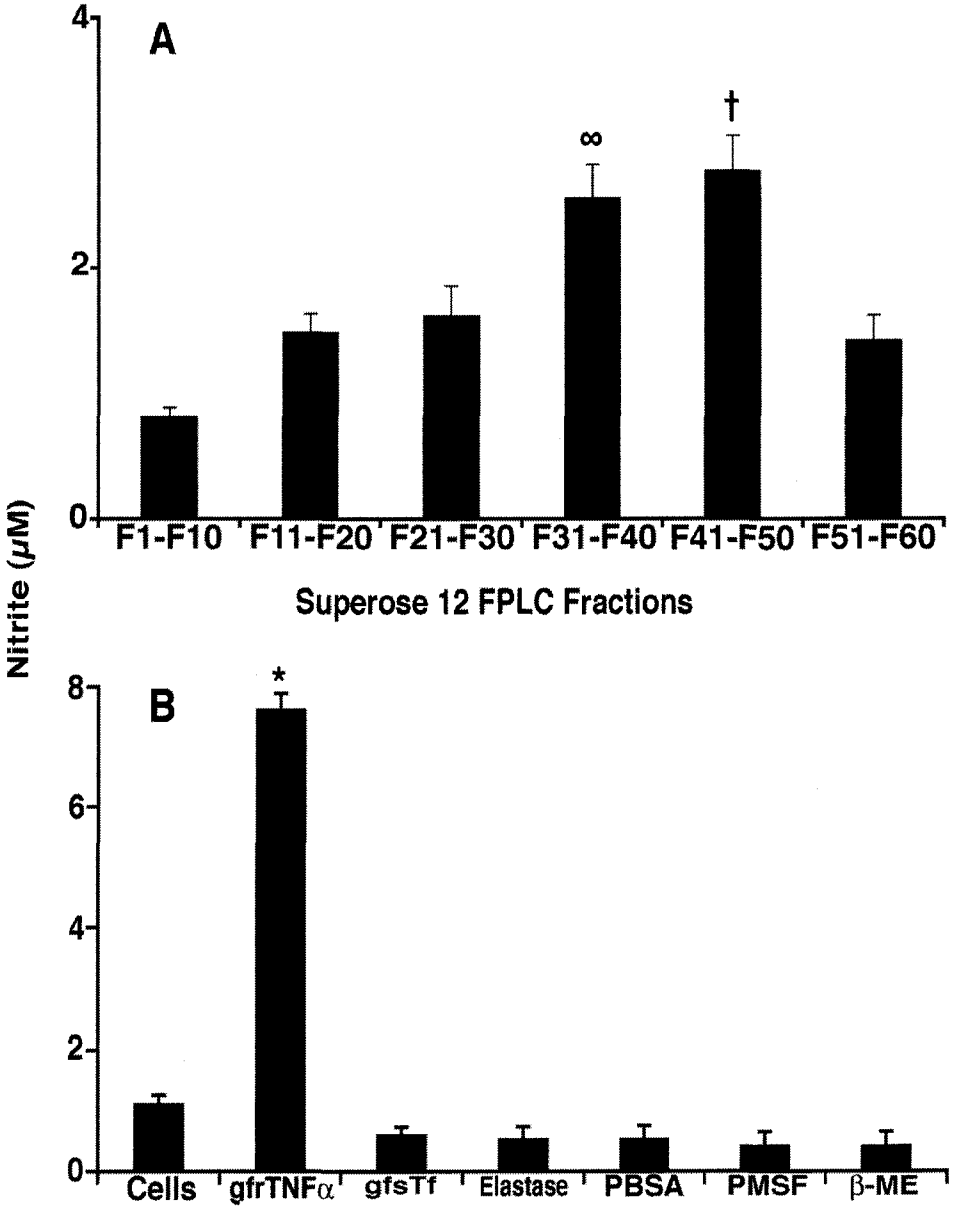

Figure 4.4: The nitric oxide response generated by elastase-digested goldfish serum transferrin in goldfish *in vitro*-derived macrophages. A= the nitric oxide response from pooled FPLC Superose 12 fractions; B= the nitric oxide response of the various control groups used in the experiment: 200 ng/mL gfrTNFa, 300 ng/mL gfsTf, 10 μg/mL elastase, 25 μL PBSA, 5 mM PMSF, 0.2 % β-ME . The nitrite concentrations were determined 72 hours after treatment with different reagents using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate cultures of four fish cultures (n=4). (∞) $P<0.07$; (†) $P<0.06$ vs cells alone.

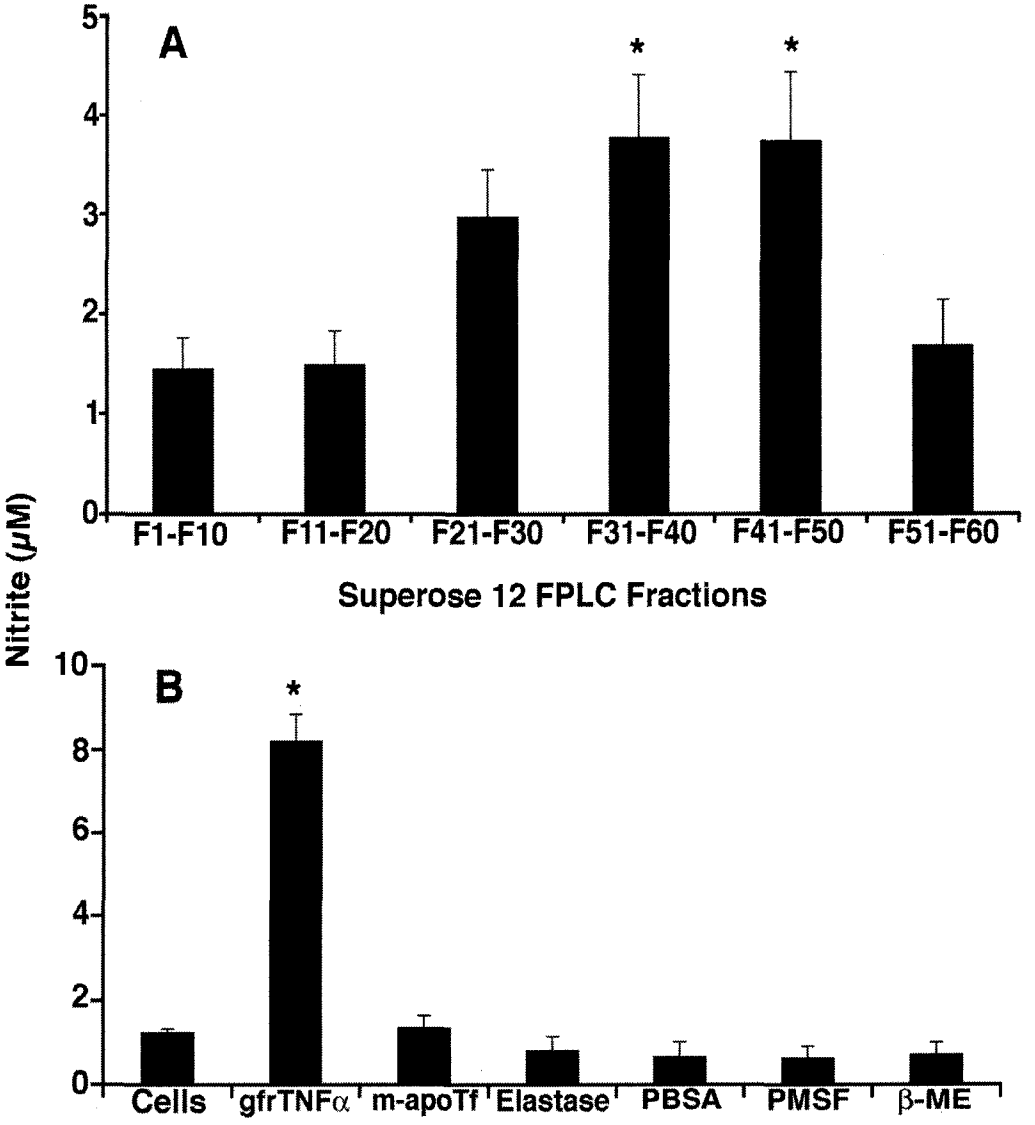

Figure 4.5: The nitric oxide response generated by elastase-digested murine apo-transferrin in goldfish *in vitro*-derived macrophages. A= the nitric oxide response from pooled FPLC Superose 12 fractions; B= the nitric oxide response of the various control groups used in the experiments: 200 ng/mL gfrTNFa, 300 ng/mL m-apoTf, 10 μg/mL elastase, 25 μL PBSA, 5 mM PMSF, 0.2 % β-ME. The nitrite concentrations were determined 72 hours after treatment with different reagents using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate determinations using cells from three cultures established from individual fish (n=3). (*) $P<0.05$ vs. cells alone.

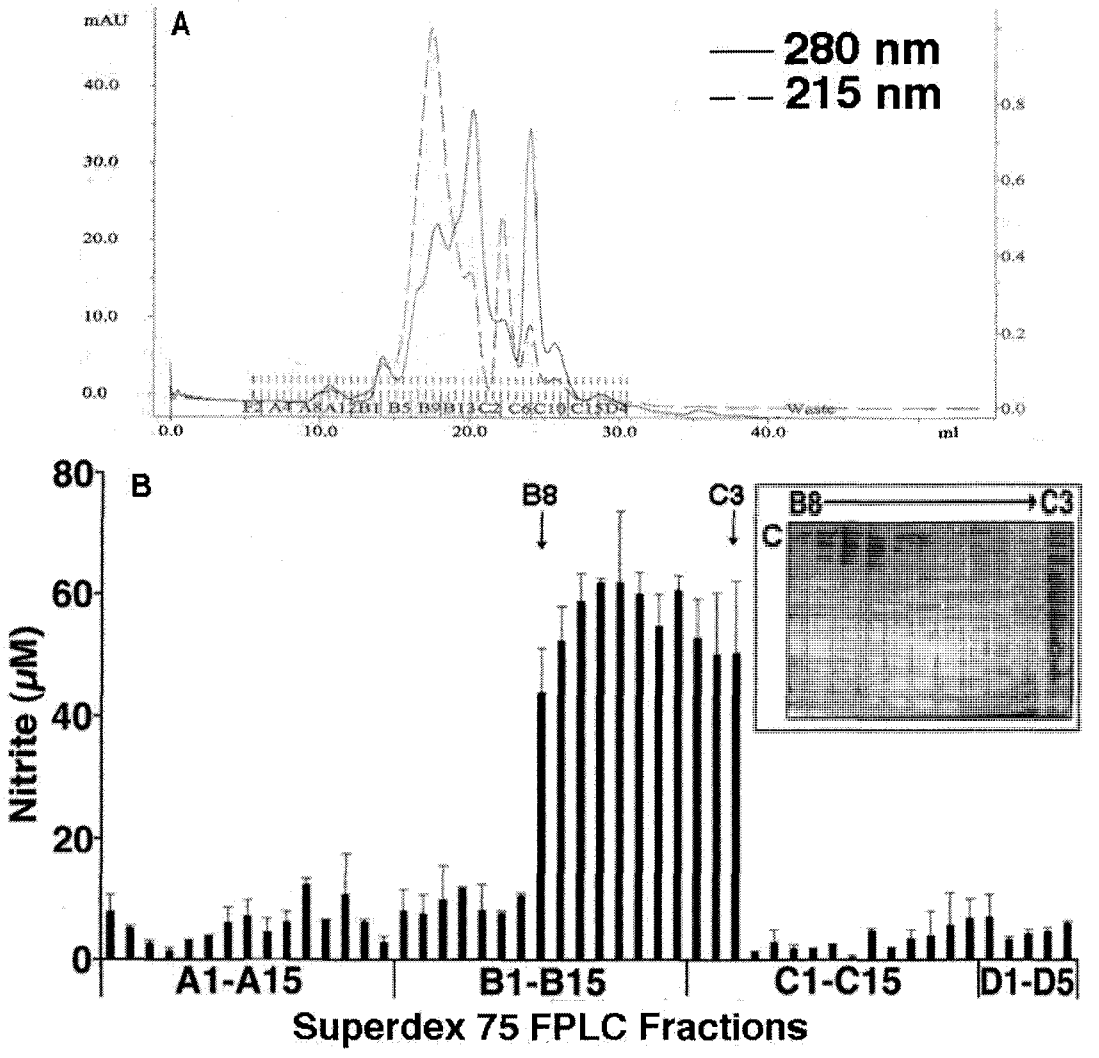

Figure 4.6: The nitric oxide response induced by elastase-generated transferrin fragments in P338D$_1$ macrophages obtained from FPLC Superdex 75 fractions. A= the FPLC generated graph indicating the location of the peptides and their corresponding fractions. B = twenty-five microliters of each fraction were tested for nitric oxide-inducing activity and nitrite concentration was determined using the Griess reaction. C = SDS-PAGE gel of fractions B8 to C3 stained with silver stain. Each point on the graph represents the mean ± SEM of duplicate cultures of two independent experiments that were performed (n=4).

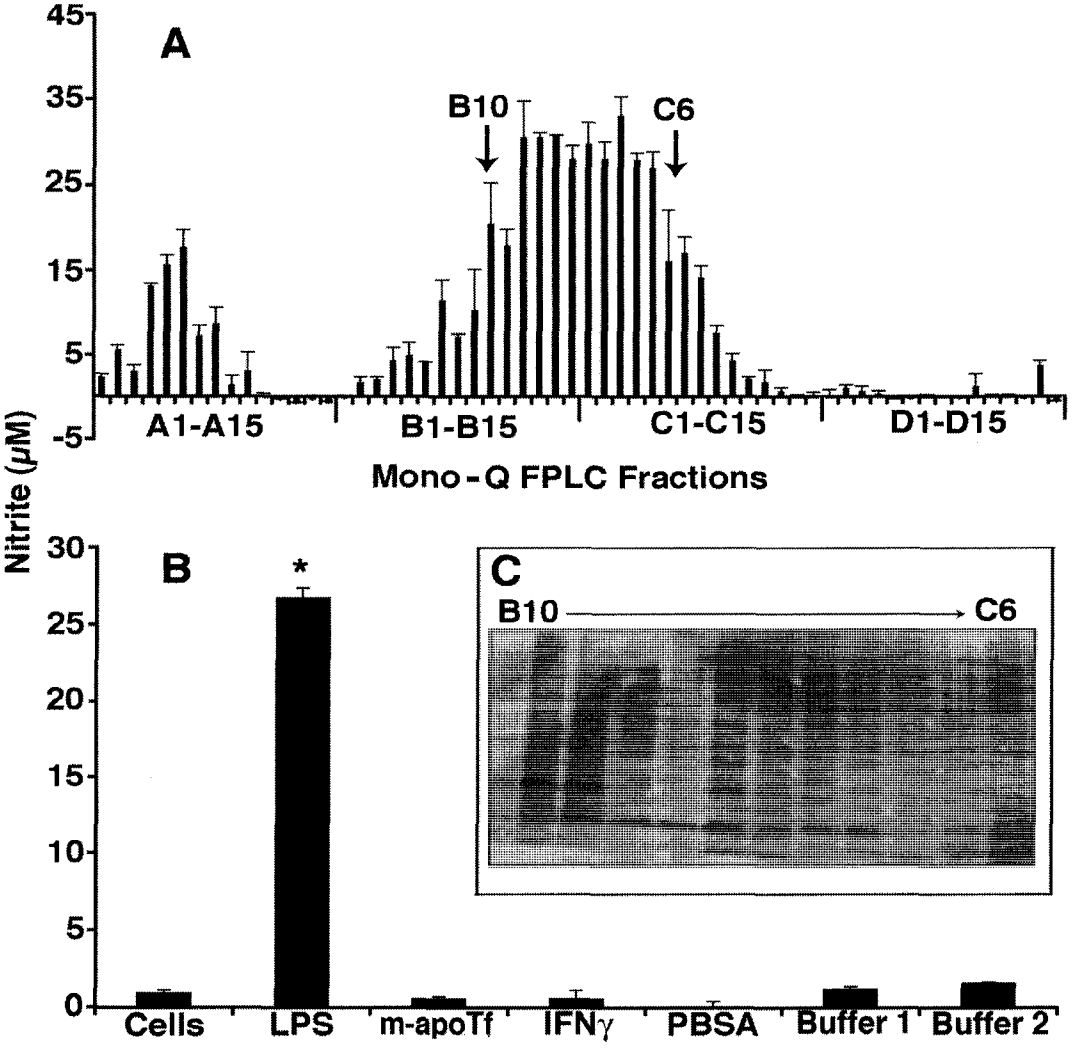

Figure 4.7: The induction of nitric oxide response in P388D$_1$ macrophages by elastase-digested transferrin pooled Superdex 75 fractions B7 to B11 resolved using FPLC Mono-Q column. A= the nitrite production induced by transferrin peptides present in Mono-Q fractions; B = the nitric oxide response induced by various control groups used in the experiments; medium control, cells treated with 1 μg/mL LPS, 300 ng/mL m-apoTf, 100 U/mL IFNγ, 25 μL PBSA, 25 μL Mono-Q buffer 1, 25 μL Mono-Q buffer 2; C= SDS-PAGE gel stained with silver stain, fractions B10 to C6. The nitric oxide response was determined using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM nitrite produced of duplicate determinations and the data are from two experiments that were performed (n=4). (*) $P<0.05$ vs. cells alone.

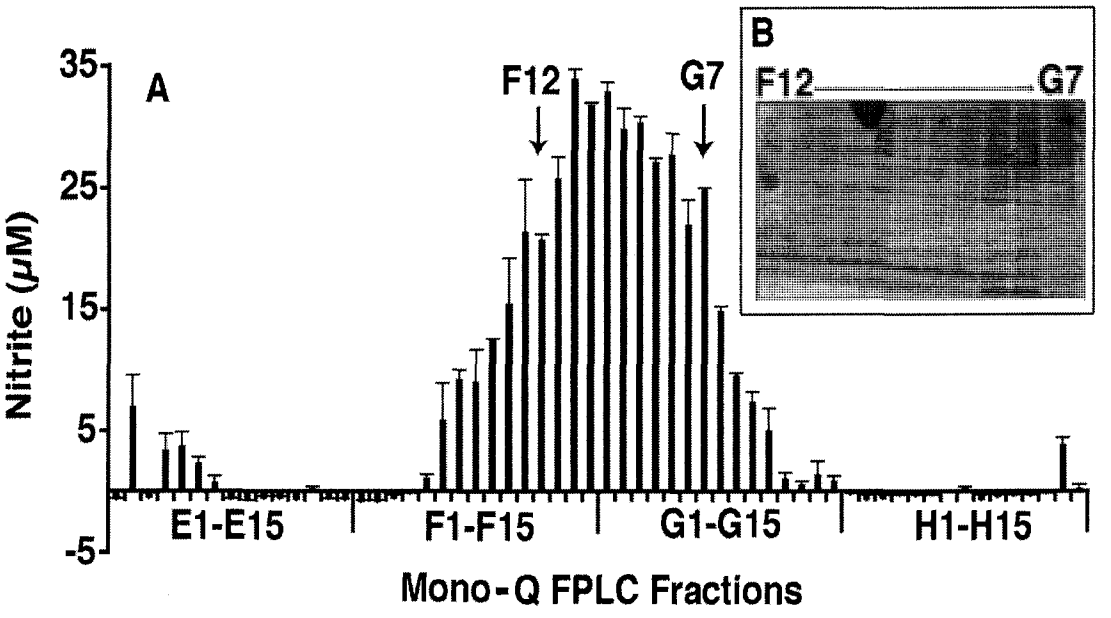

Figure 4.8: Induction of nitric oxide response in P388D$_1$ macrophages by elastase-digested transferrin pooled Superdex 75 fractions B12 to B15 resolved by FPLC Mono-Q column. A = the nitrite production induced by peptides present in individual Mono-Q fractions. B = SDS-PAGE gel stained with silver, fractions F12 to G7. The nitrite concentrations were determined 24 hours after treatment with different reagents using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate determinations and the data are from two experiments that were performed (n=4).

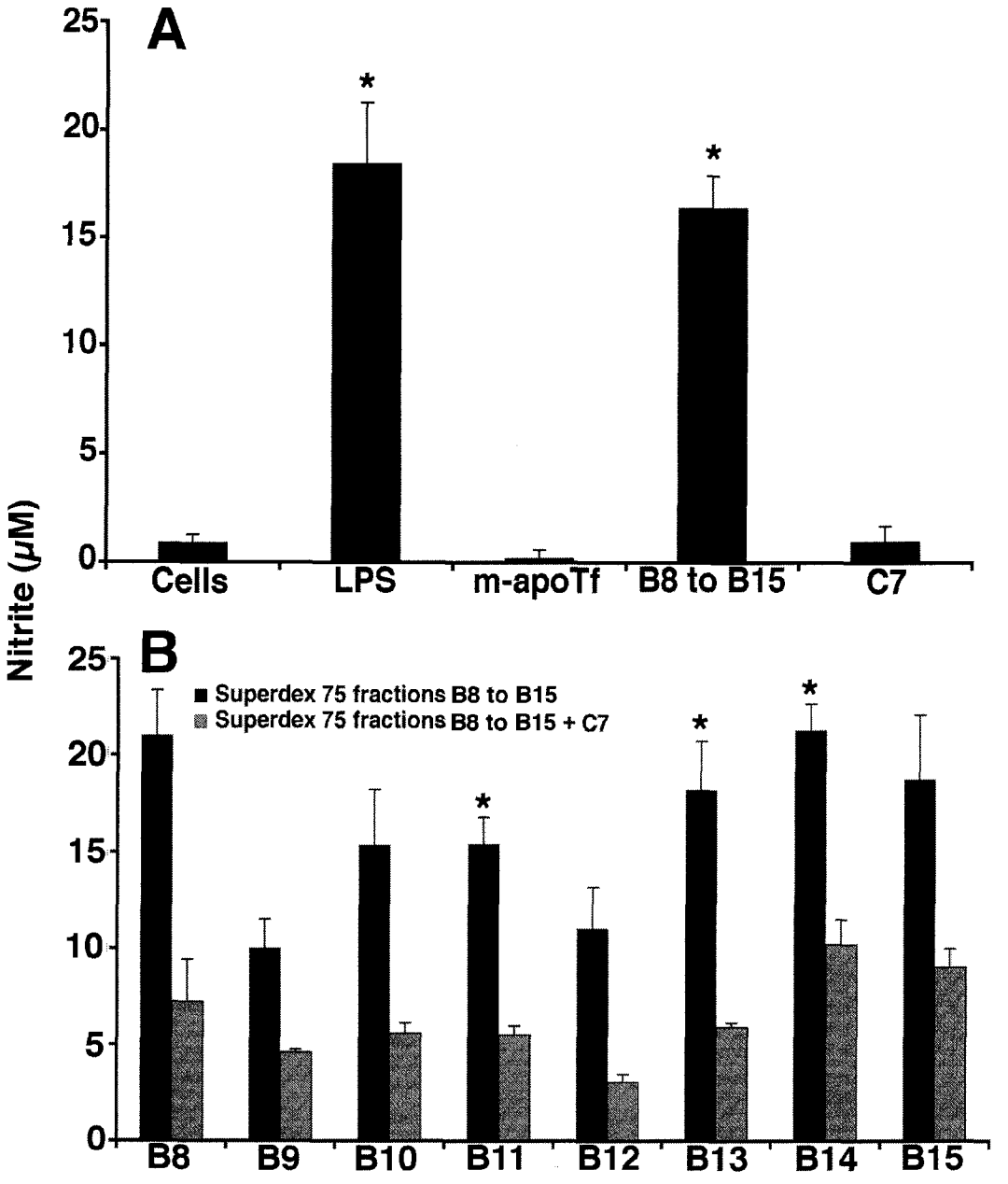

Figure 4.9: Down regulation of the nitric oxide response by Superdex 75 fractions. A = the suppressing activity of transferrin peptides present in Superdex 75 fraction C7; B= the nitric oxide response of various control groups used in this experiments. The nitrite concentrations were determined 24 hours after treatment with different reagents using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate determinations and the data are from two experiments that were performed (n=4). (*) $P<0.05$ vs. B8 to B15 and C7 fractions.

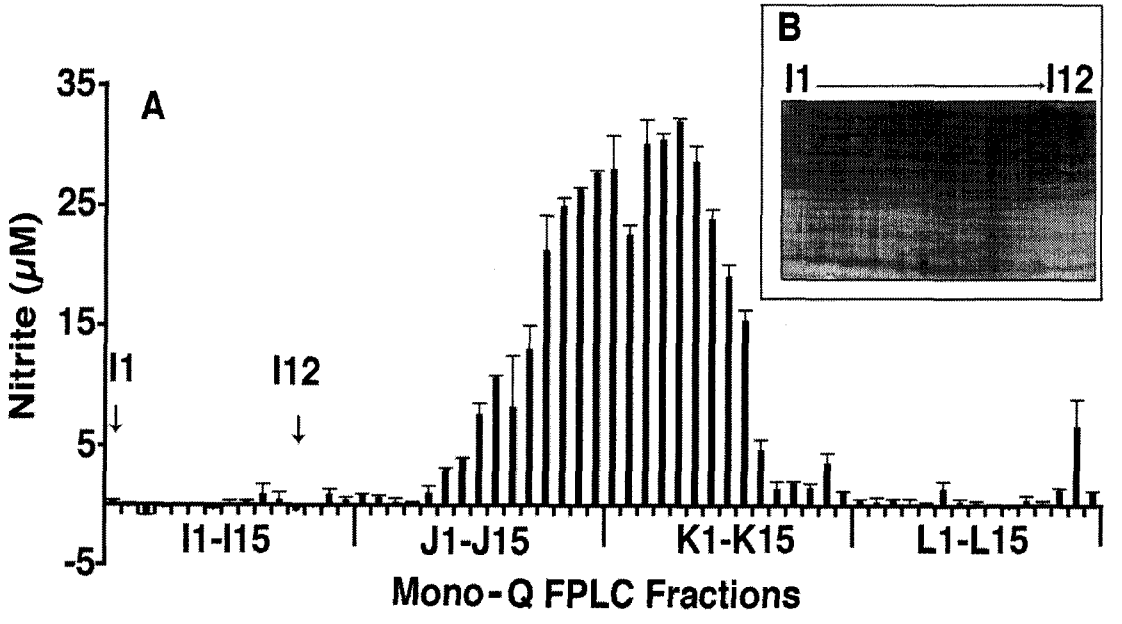

Figure 4.10: Induction of nitric oxide response of pooled Superdex 75 fractions of elastase-digested transferrin, C6 to C8 separated by FPLC and Mono-Q column. A= the nitric oxide response induced by peptides present in Mono-Q fractions; B = SDS-PAGE gel stained with silver, fractions I1 to I12. The nitrite concentrations were determined 24 hours after treatment with different fractions using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate determinations and the data are from two experiments that were performed (n=4).

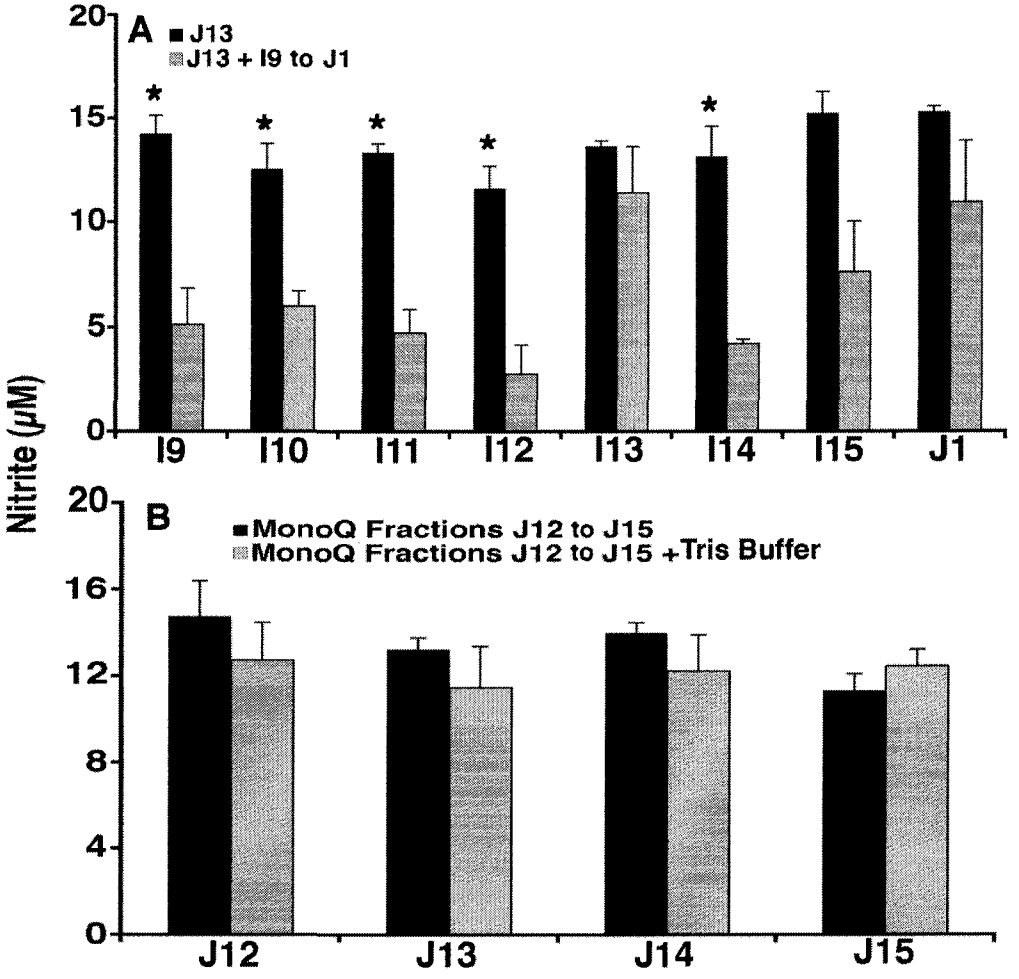

Figure 4.11: The suppression of nitric oxide response of P388D$_1$ macrophages by elastase-digested transferrin peptides present in Superdex 75 pooled fractions C6 to C8 resolved by FPLC Mono-Q column. A= the nitric oxide inhibiting activity of Mono-Q fractions tested, I9 to J1. B= control experiment designed to demonstrate that the addition of 25 μL Tris buffer, does not have an effect on the nitric oxide response generated by the transferrin peptides found in the nitric oxide-inducing fractions. The nitrite response was determined 24 hours after treatment with different fractions using the Griess reaction and a nitrite standard curve. Each bar represents mean ± SEM of duplicate determinations and the data are from two experiments that were performed (n=4). (*P) <0.05 vs. J13 and I9 to J1.

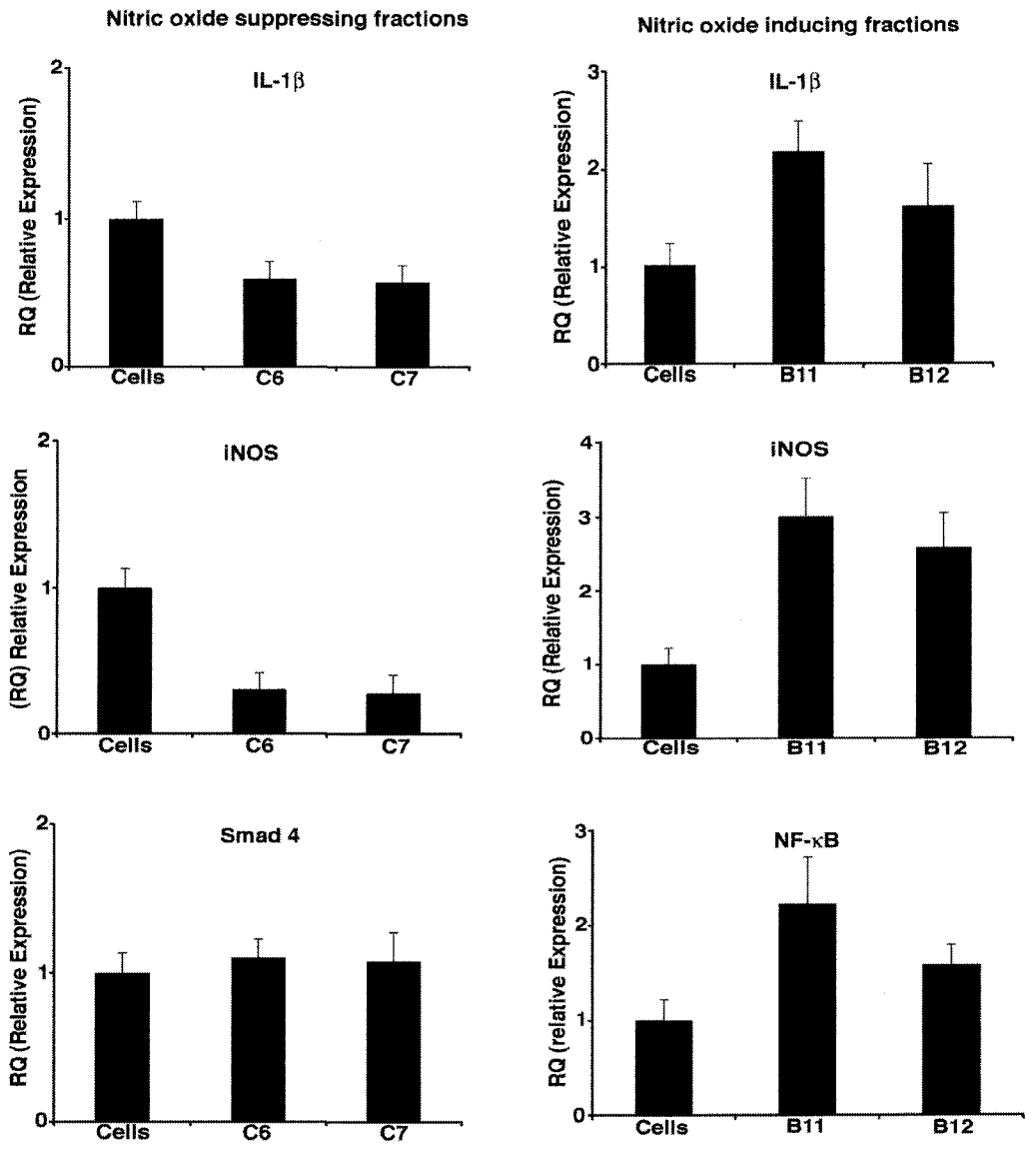

Figure 4.12: Expression of select genes in P388D$_1$ macrophages after treatment with elastase-digested transferrin fragments present in Superdex 75 nitric oxide-suppressing fractions C6 and C7, and nitric oxide-inducing fractions, B11 and B12. Each bar represents one of two independent quantitative PCR determinations done in triplicate (mean ± SEM of triplicate determinations).

CHAPTER V

THE IDENTIFICATION AND CHARACTERIZATION OF TRANSFERRIN-DERIVED MACROPHAGE-ACTIVATING PEPTIDE

5.0 Introduction

The immune system of multicellular animals must distinguish between live and dead cells, as well as recognize invading pathogens. To this end, a number of different mechanisms have evolved for surveillance, defense, and tissue repair. In order for the immune system to respond to injury or infection, warning signals are required for the activation of immune cells via receptors and signaling pathways leading to induction of a competent immune response [23].

Since Polly Matzinger proposed the "Danger Model" of immunity, there is growing evidence that modified endogenous molecules of the host, termed "alarmins", alert the immune system to the presence of tissue and/or cell damage. As described in chapter 4 of this thesis, transferrin is one of the molecules that, when modified, can induce macrophage pro-inflammatory responses.

That modified transferrin is a major host defense protein for delivery of danger signals that can jump-start an immune response is supported by the evidence that it is an abundant plasma protein with concentration 2-5 mg/mL in mammals [15], and by its role in binding iron and sequestering it from invading pathogens [16].

In this chapter, I describe the identification and the characterization of transferrin-derived macrophage-activating peptide. I report on the identification of a

31 amino acid peptide generated by elastase digestion of murine transferrin that was identified using mass spectrometry analysis. The peptide termed TMAP (Transferrin Macrophage-Activating Peptide) was synthetically produced and shown to induce activation of different macrophage populations as measured by nitric oxide production, the release of the pro-inflammatory cytokine TNFα, chemotaxis, quantitative PCR gene expression, and the secretion of a panel of cytokine/chemokines in the supernatant of TMAP-activated macrophages.

5.1 Results

5.1.1 Mass spectrometry analysis of murine transferrin digested with elastase (TMAP)

The elastase digestion of transferrin resulted in the generation of a large number of transferrin peptides (Table 4.1). One of the peptide sequences identified, SGS**C**VP**C**ADPVAFPKL**C**QL**C**PG**C**G**C**SSTQPF, was of great interest since it resembled a defensin-like molecule. We produced this peptide synthetically, and named it TMAP for Transferrin Macrophage-Activating Peptide (Fig. 5.1A) and have refolded the molecule using glutathione refolding buffer (Fig. 5.1B). However, neither the linear nor the refolded forms had direct anti-bacterial effect, suggesting that, at least functionally, the synthetic peptide was not a defensin (data not shown). The crystal structure of recombinant human N-lobe (NCBI, PDB:1A8F) (18) is shown in Fig. 5.1C. TMAP is located in the N2 sub-domain of the N-lobe of transferrin molecule (boxed region).

5.1.2 Induction of macrophage activation by TMAP

The TMAP was tested for its ability to induce macrophage activation in P388D$_1$ macrophages, murine peritoneal macrophages, bone-marrow derived murine macrophages (BMDM), and *in vitro*-derived goldfish macrophages. We measured the nitric oxide response in treated cells using the Griess reaction in all macrophage populations, and the production of pro-inflammatory cytokine TNFα, and induction of chemotactic response in murine macrophages by TMAP.

Although TMAP in the linear form did not induce a nitric oxide response in P388D$_1$, resident peritoneal macrophages, or BMDM (Figs. 5.2, 5.3 and 5.4), however, when properly folded, the peptide induced a strong nitric oxide response in different murine macrophage populations (Figs. 5.2, 5.3 and 5.4). The TMAP induced a dose-dependent nitric oxide response in P388D$_1$ macrophages, which was maximal when the cells were treated with 50 μg/mL of TMAP and was comparable to the nitric oxide response induced by LPS (Fig. 5.2). As little as 10 μg/mL TMAP induced a significantly higher nitric oxide response in P388D$_1$ cells compared to non-treated cells ($P < 0.05$, one-way ANOVA). In contrast, addition of 50 μg/mL full-length murine apo-transferrin (m-apoTf) to the P388D$_1$ cell cultures did not induce a nitric oxide response (Fig. 5.2). To ensure that the nitric oxide response was solely due to TMAP, we also tested the refolding buffer, the dialysis buffer (PBS), and cells treated with rmIFNγ, and found that none of these treatments induced nitric oxide response in P388D$_1$ cells.

Figure 5.3 shows the nitric oxide response induced by TMAP in resident murine peritoneal macrophages. The treatments of cells with 1 μg/mL LPS or 50 μg/mL TMAP induced a relatively strong nitric oxide responses that were significantly different from that of medium control ($P < 0.05$, one-way ANOVA). Like for P388D_1 macrophages, treatment of peritoneal macrophages with 50 μg/mL of full-length m-apoTf or 100 U/mL of rmIFN-γ did not induce a nitric oxide response (Fig. 5.3).

TMAP also induced a potent nitric oxide response in BMDM (Fig. 5.4). Treatment of BMDM with 50 μg/mL of TMAP induced nitrite production in BMDM that was significantly higher than that of cells treated with m-apoTf, those treated with rmIFN-γ or untreated cells (Fig. 5.4). Interestingly, TMAP also induced nitric oxide response in *in vitro*-derived goldfish macrophages (Fig. 5.5). The fish macrophages up regulated nitrite production after treatment with 200 ng/mL recombinant goldfish TNF-α (~ 11 μM nitrite) and 50 μg/mL TMAP (~ 16 μM nitrite), that was significantly different from the nitrite production observed in non-treated medium control cell cultures or fish macrophages treated with m-apoTf.

TMAP induced significant production and secretion of the pro-inflammatory cytokine TNFα in P338D1, murine peritoneal macrophages and BMDM (Fig. 5.6). The treatment with 1 μg/mL LPS (positive control) or 50 μg/mL TMAP caused a significant secretion of TNF-α in P388D_1 cells measured by ELISA (Fig. 5.6A), peritoneal macrophages (Fig. 5.6B) and BMDM (Fig. 5.6C), compared to non-treated cells. In contrast, 50 μg/mL of m-apoTf did not induce the production or secretion of TNFα in all three macrophage populations tested (Fig. 5.6A, B, C).

TMAP was also found to be a significant chemoattractant for both $P388D_1$ cells and BMDM (Fig. 5.7A, B). TMAP induced a dose-dependent chemotactic response in $P388D_1$ cells, with the highest response observed when 100 ng/mL of TMAP was used as the chemoattractant. As little as 10 ng/mL of TMAP was sufficient to induce a significant chemotactic response in $P388D_1$ cells, when compared to that of medium control. It should be noted that the concentration of TMAP required to induce a maximal chemotactic response was 10 times higher than the concentration of f-MLP (positive control) (Fig. 5.7A). The chemotactic response of BMDM to either f-MLP or TMAP was lower when compared to that of $P388D_1$ cells, but still significantly greater compared to that of non-treated cells or those treated with full-length m-apoTf (Fig. 5.7B). As expected, the chemotactic response was abrogated when the concentration gradient was eliminated (chemokinesis) for both $P388D_1$ and BMDM cells (Fig. 5.7A, B).

5.1.3 TMAP induces secretion of pro-inflammatory cytokines and chemokines in macrophages

To further characterize the pro-inflammatory nature of TMAP, a cytokine antibody array was performed using supernatants of BMDM cultures treated with 50 μg/mL TMAP. This cytokine array had the capacity to simultaneously detect 62 cytokine/chemokines that could be potentially produced by activated BMDM. Shown in Table 5.1 is the array map of cytokines/chemokines measured, and those that were up regulated due to TMAP treatment were boldfaced. Similarly, Figure 5.8A depicts nine cytokines/chemokines (IL-6, MCP-5, G-CSF, MIP-1α, MIP-1γ, MIP-2, KC,

VEGF, and RANTES) that were up regulated in BMDM after treatment with TMAP. The quantitative analysis of the array spots using GenePix Pro 6.0 had showed that MIP-1γ and RANTES had six and five times higher light intensity compared to the other seven cytokines/chemokines that were up regulated by TMAP treatment, respectively (Fig. 5.8B).

The presence of secreted chemokines in the medium of TMAP-activated BMDM indicated by the cytokine array experiments was confirmed by Western blot for RANTES, MIP-1α, MCP-5, and KC (Fig. 5.9). Since TMAP also induced nitric oxide response in BMDM, we also confirmed that iNOS protein was up regulated and present in lysates of TMAP-treated cells (Fig. 5.9).

5.1.4 TMAP increases the expression of pro-inflammatory cytokines and chemokines in macrophages

The mRNA expression level of the nine cytokines/chemokines that were up regulated following treatment with TMAP as well as those of iNOS and TNFα were determined using quantitative PCR. The mRNA levels of IL-6, MIP-1α, MCP-5, MIP-2, KC, RANTES, G-CSF, and VEGF were higher in macrophages treated with TMAP compared to non-treated cells after 24 hrs incubation (Fig. 5.10). However, the mRNA copy number of MIP-1γ was lower in the TMAP-treated cells compared to non-treated cells. The mRNA expression level for cells treated with 50 μg/mL TMAP was about 4-5 folds higher for RANTES, MIP-1α, MCP-5, G-CSF, and VEGF, and ~20-30 folds higher for IL-6, MIP-2 and KC, compared to non-treated cells. Interestingly, the addition of high concentration of apo-transferrin (50 μg/mL)

induced enhanced mRNA expression levels of G-CSF and VEGF (Fig. 5.10). TMAP treated BMDM exhibited significant up regulation, a ~30-fold and ~3-fold increase for iNOS and TNFα mRNA, respectively, compared to that in non-treated cells (Fig.5.10).

5.2 Discussion

As demonstrated in chapter 4, elastase-digested mouse, bovine, human N-lobe and goldfish transferrin fragments induced nitric oxide response of macrophages. To further examine the immunomodulatory role of elastase-derived transferrin fragments, I examined the effects of transferrin-derived synthetic peptide (TMAP) using three murine macrophage populations: P388D_1 macrophages, mouse peritoneal macrophages, and BMDM. That elastase-digested mouse apo-transferrin induced potent nitric oxide response in macrophages while non-digested apo-transferrin did not, suggests that transferrin peptides were immunostimulatory (see chapter 4). Mass spectrometry analysis of elastase-digested transferrin showed that numerous fragments of the protein were present in the digest. Among these was a 31 amino acids fragment containing six cysteine residues that resembled a defensin-like molecule, which was synthesized and named TMAP.

Defensins are antimicrobial peptides with cationic and amphiphilic sequences of 12-50 amino acids with six conserved cysteine residues. In humans, the α-defensins are produced by neutrophils and Paneth cells of the small intestine, whereas β-defensins are produced by leukocytes and epithelial cells in mucosal tissues and skin [160]. In addition, human neutrophil-derived defensins were shown to induce

production of TNFα and IL-1β in monocytes activated with PMA or *Staphylococcus aureus* [161]. Since TMAP did not exhibit antimicrobial activity against Gram (+) or Gram (-) bacterial species tested, we do not believe that this transferrin-derived peptide is a defensin.

TMAP had significant pro-inflammatory properties. It induced the production and secretion of the pro-inflammatory cytokine TNFα in $P388D_1$ cells, peritoneal macrophages and BMDM, and induced chemotaxis of $P388D_1$ and BMDM. Moreover, TMAP treatment of BMDM induced the production of nine different cytokines/chemokines, in particular MIP-1γ and RANTES, and to a lesser extent VEGF, KC, G-CSF, MCP-5, IL-6, MIP-1α, and MIP-2. That TMAP had the capacity to induce secretion of a number of diverse pro-inflammatory molecules suggests that this peptide may play a significant role in the regulation of inflammation.

My results suggest that TMAP, or more broadly, transferrin fragments, may be involved in an array of inflammatory processes such as, defense against infectious agents, tissue injury, and in allergic responses. The cytokine/chemokine profile generated by the cytokine antibody array showed the extensive production of different chemokines that have a plethora of immunological roles. For example, MIP-1γ, RANTES, KC, MIP-2, MCP-5 and MIP-1α have been shown to induce chemotactic responses in diverse cell types and under a variety of different conditions [162, 163, 164, 165, 166, 167]. IL-6 was identified as one of the central pro-inflammatory cytokines whose major roles include the regulation of the immune response in burn patients and tissue repair, as well as resistance to pathogens such as *Streptococcus pneumoniae* [168]. G-CSF and VEGF are growth factors that regulate

neutrophil proliferation and differentiation and angiogenesis, respectively [169, 170].

That enzyme-modified transferrin fragments may be major participants in delivering "danger" signals and jump-starting the innate immune response is supported by the following: (a) transferrin is an abundant plasma protein with concentration 2-5 mg/mL in mammals [15]; (b) due to its role as an iron-binding protein transferrin, sequesters this essential growth ingredient from invading pathogens [16], making it a prime target for pathogen "attack" and subsequent liberation of essential iron by means of siderophores [17] or by enzymatic cleavage [18]; (c) transferrin is primarily synthesized in the liver, but it is also produced by macrophages, and is therefore present at the inflammatory sites during early inflammatory response [19]; (d) the fact that transferrin is an acute phase protein [20]; and (e) transferrin fragments are commonly found in the inflammatory microenvironment such as the lungs of the cystic fibrosis patients [12].

My results suggest that transferrin belongs to the ever-growing family of endogenous danger signal proteins ("alarmins"). The alarmin family includes heat-shock proteins, high-mobility group box1 (HMGB1), low-density lipoprotein, myeloid-related proteins 8 and 14, and many others. Purified HSPs such as Hsp60, Hsp70, Hsp90, and gp96 from bacteria, have been shown to induce a potent immune responses characterized by the release of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), IL-6, and IL-12 in addition to the production of nitric oxide and CC-chemokines by activated monocytes, macrophages and dendritic cells [31,32, 33]. HMGB1 has an important extracellular role as a

danger signal as it is produced by activated macrophages, dendritic cells, and natural killer cells, or released by necrotic cells in response to infection or tissue injury [51]. HMBG1 is chemotactic to monocytes/macrophages, neutrophils and dendritic cells, and has potent immunostimulatory actions in addition to promoting the maturation of myeloid and plasmacytoid dendritic cells [52]. Oxidized low-density lipoprotein (oxLDL) binds macrophages through scavenger receptor that leads to the formation of foam cells and visible lesions. In addition, oxLDL induces the production of the pro-inflammatory cytokine TNF-α and IL-8 in monocytes and primes macrophages [57]. The formation of Mrp-8 and Mrp-14 complexes are generally correlated with a number of inflammatory diseases including, sepsis, rheumatoid arthritis, inflammatory bowel disease and cancer [62, 65]. It has been reported that MRp-8 and Mrp-14 bind TLR-4-MD-2 complex that leads to downstream signaling pathway and the transcriptional up regulation of pro-inflammatory cytokines such as TNF-α [66].

Transferrin fragments may exert their immunostimulatory activity in the early stages of inflammation. It is known that transferrin and lactoferrin fragments are present in BAL of cystic fibrosis patients infected with *Pseudomonas aeruginosa* [6]. It has also been shown that bacterial elastase may be used to release iron from iron-binding proteins and facilitate their uptake by the bacterial siderophores (pyoverdin and pyochelin) [138]. As the inflammatory response progresses, activated neutrophils and macrophages present at the inflammatory site are probably major sources of proteolytic enzymes, including elastase, which I have shown can cleave transferrin into immunomodulatory fragments [138].

The transferrin-derived synthetic peptide TMAP characterized in this chapter, induced potent pro-inflammatory responses of different macrophage populations obtained from different host species, suggests that this macrophage activation pathway may be highly conserved through evolution.

Table 5.1: Reagent map of RayBio ® mouse cytokine array G series 3 (62 cytokines).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS1	POS2	POS3	NEG	NEG	Axl	BLC	CD30L	CD30T	CD40	CRG2	CTACK	CXCL16	Eotaxin
2	POS1	POS2	POS3	NEG	NEG	Axl	BLC	CD30L	CD30T	CD40	CRG2	CTACK	CXCL16	Eotaxin
3	Eotaxin2	FasL	Fractalkine	**GCSF**	GM-CSF	IFN-γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1β	IL-2	IL-3	IL-3Rβ
4	Eotaxin2	FasL	Fractalkine	**GCSF**	GM-CSF	IFN-γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1β	IL-2	IL-3	IL-3Rβ
5	IL-4	IL-5	**IL-6**	IL-9	IL-10	IL12p40/p70	IL12p70	IL-13	IL-17	**KC**	LeptinR	Leptin	LIX	L-Selectin
6	IL-4	IL-5	**IL-6**	IL-9	IL-10	IL12p40/p70	IL12p70	IL-13	IL-17	**KC**	LeptinR	Leptin	LIX	L-Selectin
7	Lympho-tactin	MCP1	**MCP5**	MCSF	MIG	**MIP1α**	**MIP1γ**	**MIP2**	MIP3β	MIP3α	PF4	P-Selectin	**RANTES**	SCF
8	Lympho-tactin	MCP1	**MCP5**	MCSF	MIG	**MIP1α**	**MIP1γ**	**MIP2**	MIP3β	MIP3α	PF4	P-Selectin	**RANTES**	SCF
9	SDF1α	TARC	TCA3	TECK	TIMP1	TNFα	sTNF-RI	sTNF-RII	TPO	VCAM1	**VEGF**	NEG	NEG	NEG
10	SDF1α	TARC	TCA3	TECK	TIMP1	TNFα	sTNF-RI	sTNF-RII	TPO	VCAM1	**VEGF**	NEG	NEG	NEG

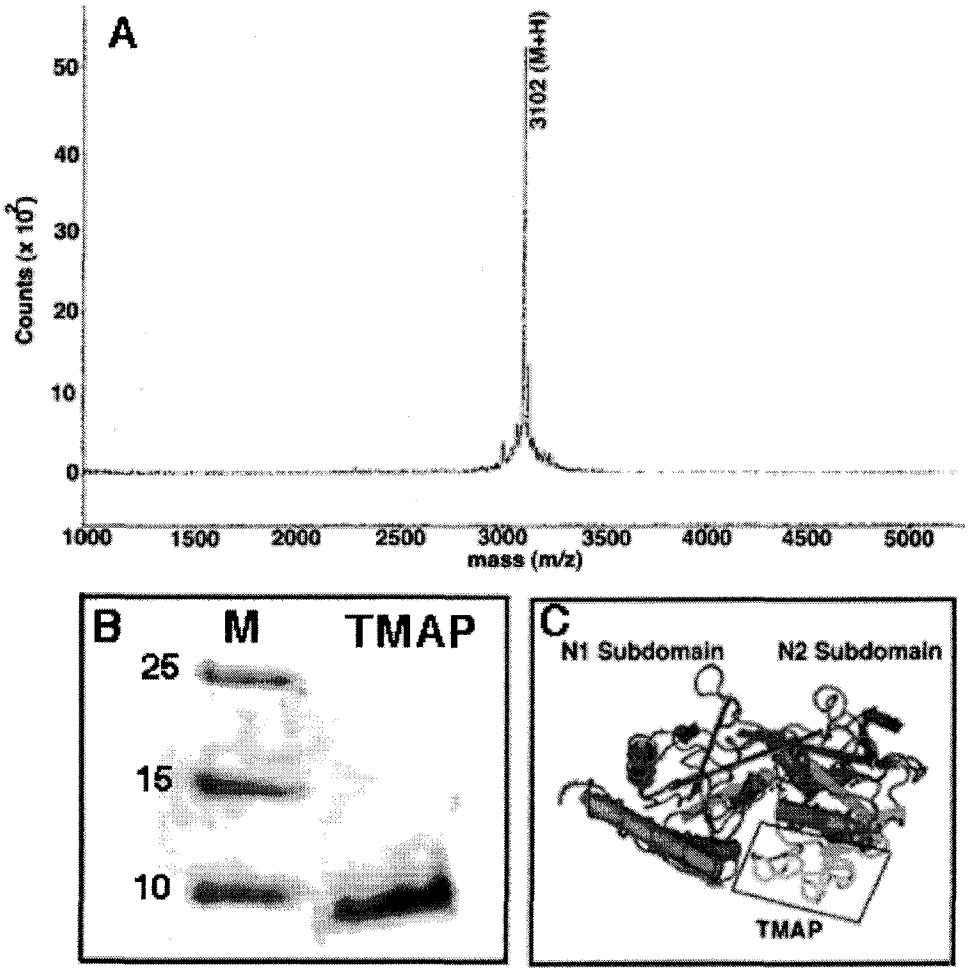

Figure 5.1: Mass spectrometry of the synthetic transferrin macrophage-activating peptide (TMAP) showing the molecular weight of the peptide (A). B = silver-stained SDS-PAGE gel of 5 μg/mL of refolded TMAP resolved under non-reducing conditions; C = model of human N-lobe of transferrin showing the position (boxed) of TMAP in the N2 sub domain of the transferrin molecule. M = molecular marker.

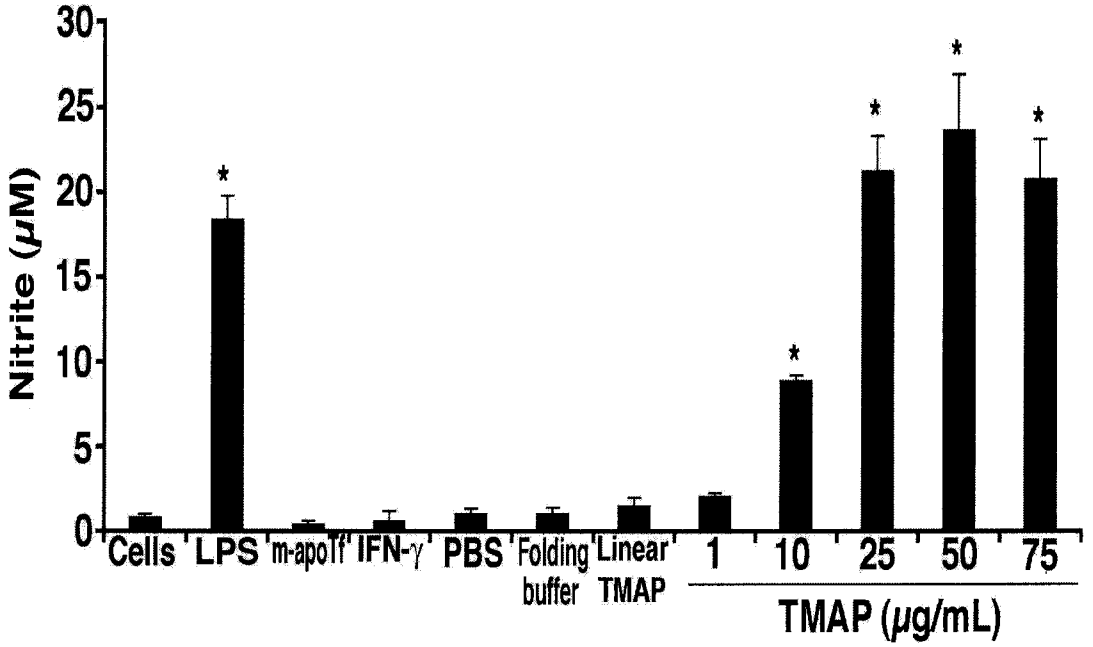

Figure 5.2: Dose-dependent nitric oxide response of macrophages treated with the synthetic transferrin macrophage-activating peptide (TMAP) in P388D_1 murine macrophages. The nitrite production was determined by the Griess reaction and nitrite standard curve. The cells were either left alone or treated with one of the following: 1 μg/mL LPS, 50 μg/mL m-apoTf, 100 U/mL mrIFN-γ, 25 μL PBSA, 25 μL refolding buffer, 50 μg/mL linear TMAP, or various concentrations of TMAP. Each point on the graph represents the mean ± SEM of triplicate cultures of two independent experiments that were done (n=6). * $P < 0.05$ vs. cells alone.

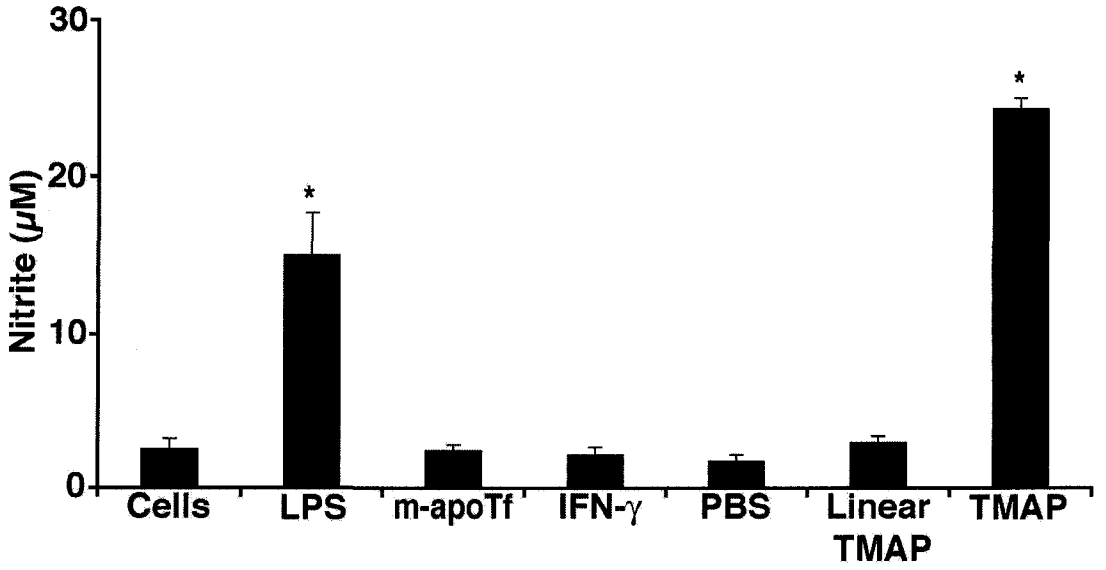

Figure 5.3: The nitric oxide response of resident murine peritoneal macrophages treated with the synthetic transferrin macrophage-activating peptide (TMAP) The nitrite production was determined by the Griess reaction and nitrite standard curve. The cells were either left alone or treated with one of the following, 1 μg/mL LPS, 50 μg/mL m-apoTf, 100 U/mL mrIFN-γ, 25 μL PBSA, 50 μg/mL linear TMAP, or 50 μg/mL TMAP. Each point on the graph represents the mean ± SEM of triplicate cultures of two independent experiments that were performed (n=6). * $P < 0.05$ vs. cells alone.

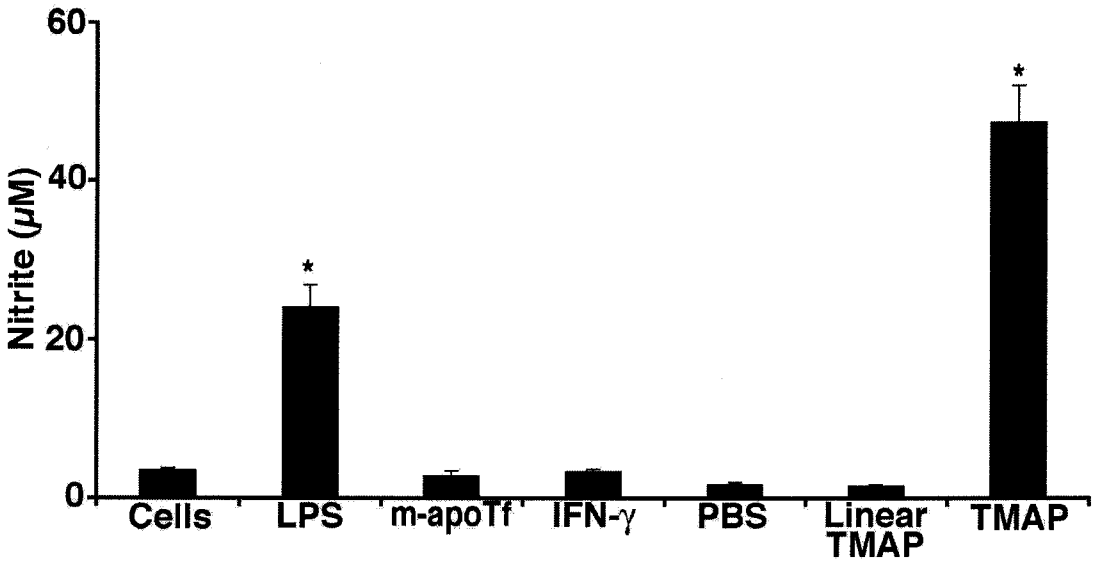

Figure 5.4: Nitric oxide response of bone marrow-derived (BMDM) macrophages treated with the synthetic transferrin macrophage-activating peptide (TMAP). Nitrite production was measured by the Griess reaction and nitrite standard curve. The cells were either left alone or treated with one of the following: 1 μg/mL LPS, 50 μg/mL m-apoTf, 100 U/mL mrIFN-γ, 25 μL PBSA, 50 μg/mL linear TMAP, or 50 μg/mL TMAP. Each point on the graph represents the mean ± SEM of triplicate cultures of two independent experiments that were performed (n=6). * $P < 0.05$ vs. cells alone.

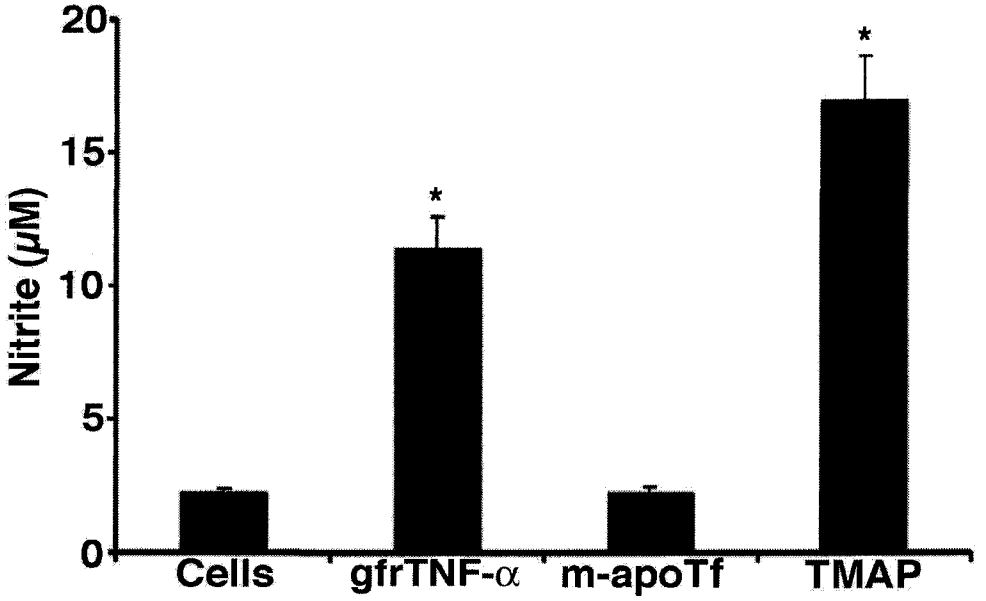

Figure 5.5: The nitric oxide response of primary *in vitro*-derived goldfish macrophage cultures treated with the synthetic transferrin macrophage-activating peptide (TMAP). The nitrite production was measured by the Griess reaction and nitrite standard curve. The cells were either left alone or treated with one of the following: 200 ng/mL recombinant goldfish TNF-α, 50 μg/mL m-apoTf, or 50 μg/mL TMAP. Each point on the graph represents the mean ± SEM of triplicate cultures of seven goldfish macrophage cultures (n=7) established from individual fish. * $P < 0.05$ vs. cells alone.

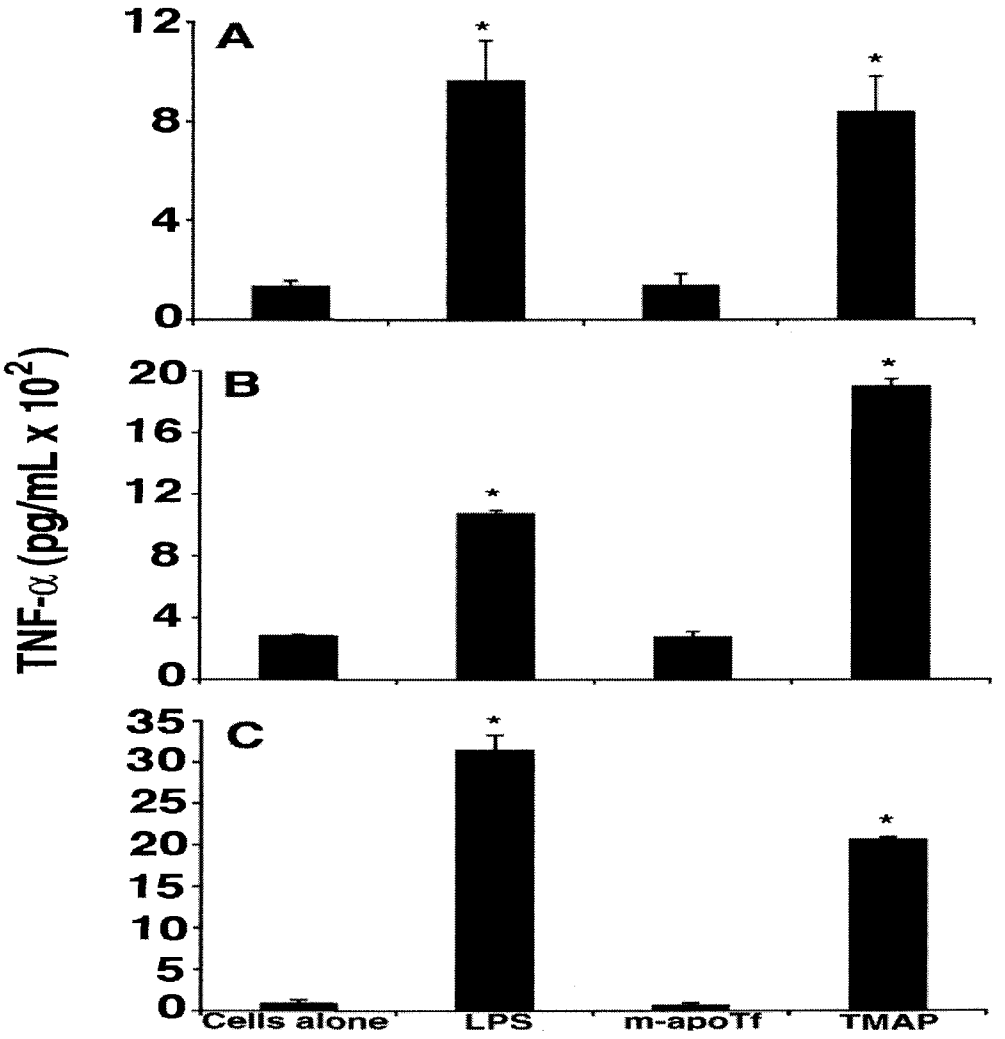

Figure 5.6: Production of TNF-α by different macrophage populations treated with synthetic transferrin macrophage-activating peptide (TMAP). A = $P388D_1$ macrophages; B = murine peritoneal macrophages; C = murine bone marrow-derived macrophages. The cells were either left alone or treated with one of the following: 1 μg/mL LPS, 50 μg/mL m-apoTf, or 50 μg/mL TMAP. TNF-α was quantified using ELISA. Each point on the graph represents the mean ± SEM of duplicate cultures of two independent experiments that were performed (n=4).
* $P < 0.05$ Vs cells alone.

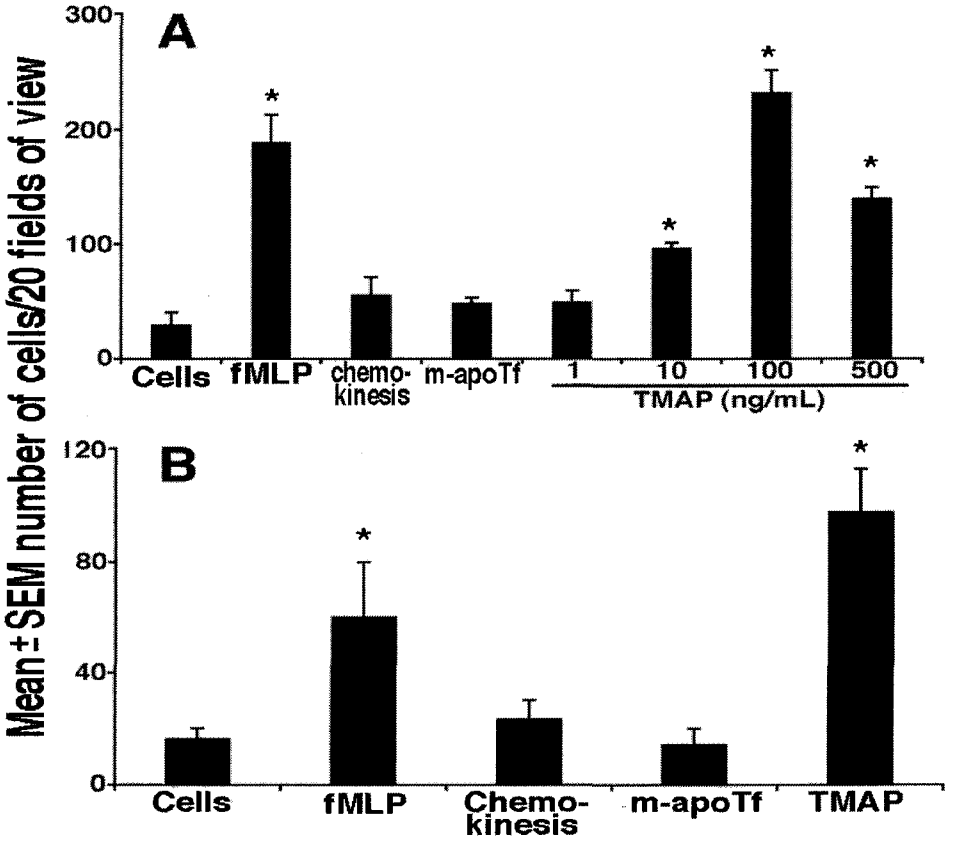

Figure 5.7: The chemotactic response of P388D$_1$ macrophages, and bone marrow-derived macrophages induced by synthetic transferrin macrophage activating peptide (TMAP) determined using blind-well chemotaxis chambers. A = Chemotaxis response in P388D$_1$ macrophages. The treatments were as follows: non-treated cells, fMLP = f-Met-Leu-Phe (positive control, 10 ng/mL), Chemokinesis (100 ng/mL TMAP added to top and bottom chambers), m-apoTf (100 ng/mL), or various concentrations of TMAP. B = Chemotaxis response in bone marrow-derived macrophages. The cells were non-treated or treated with reagents described in (A) except that only one concentration of TMAP was used, 100 ng/mL. Each point on the graph represents the mean ± SEM number of cells/20 randomly selected fields of duplicate filters from three independent experiments that were performed (n=3).
* $P<0.05$ vs. cells alone.

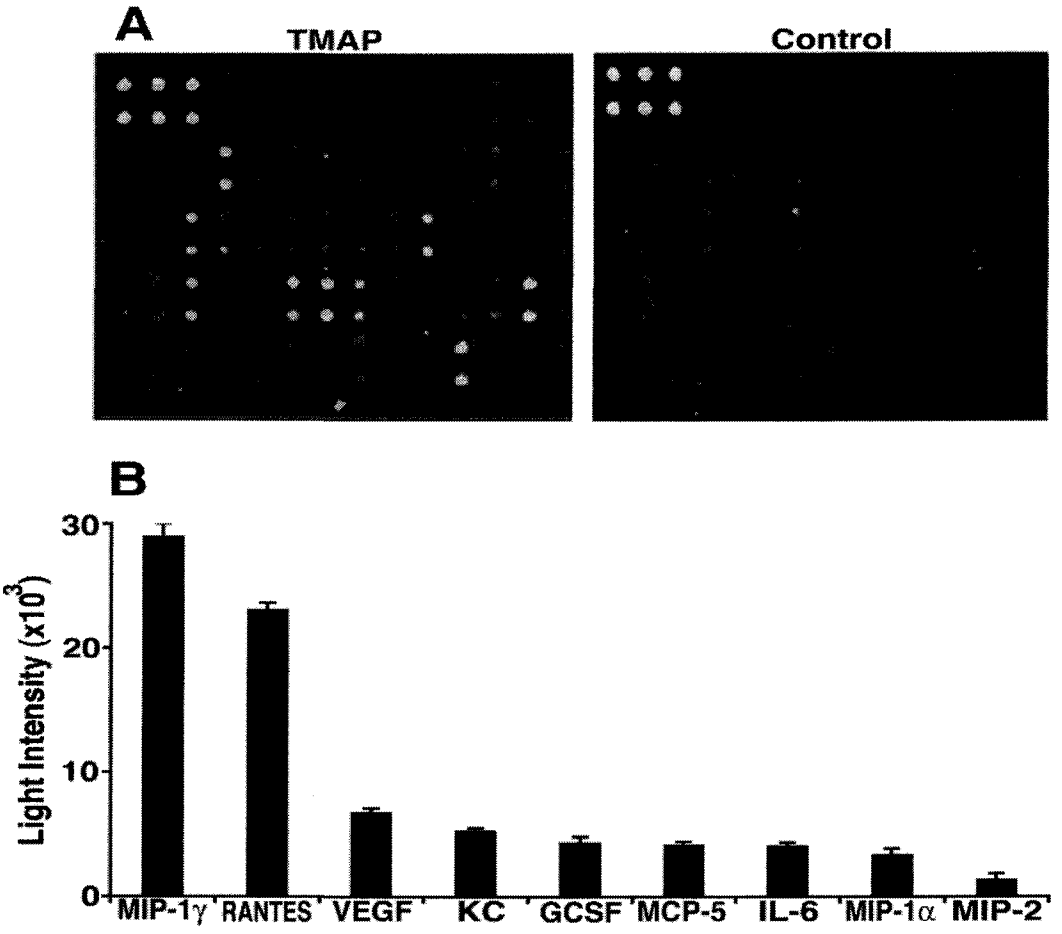

Figure 5.8: The production of pro-inflammatory cytokines/chemokines by bone marrow-derived macrophages treated with 50 μg/mL of transferrin macrophage-activating peptide (TMAP). Each cytokine/chemokine measured is represented by two spots on the array (see Table 5.1 for cytokine/chemokine map). A = the top six spots on the left-hand corner of each array represent the positive controls and the bottom six spots of the right-hand corner represent the negative controls. B = The light intensity of the array spots were analyzed using Gene Pix 6.0 and Raybiotech GIII cytokine array series software. The light intensity results are mean ± SEM of duplicate determinations from two independent cytokine array experiments. The background fluorescence and the light intensity of the control arrays were subtracted from the light intensity of the experimental arrays.

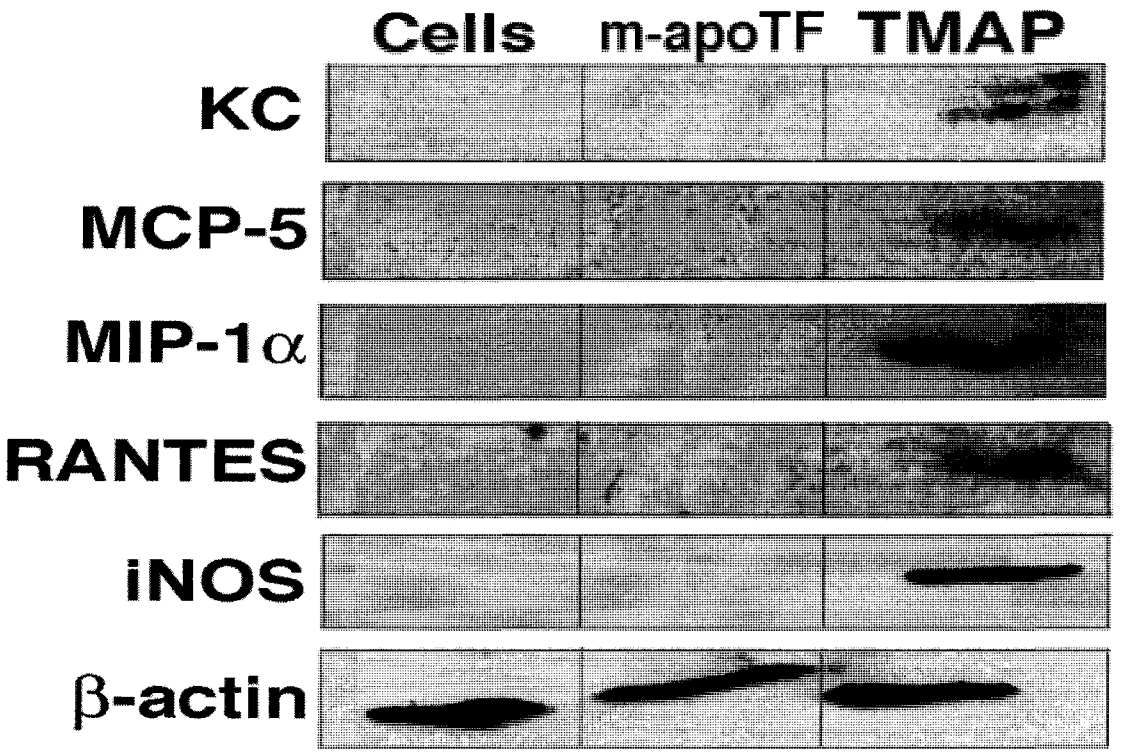

Figure 5.9: Western blot analysis of select chemokines present in supernatants of bone-marrow-derived macrophage (BMDM) cultures following treatment with 50 μg/mL of synthetic transferrin macrophage activating peptide (TMAP). Inducible nitric oxide synthase (iNOS) was measured in cell lysates of TMAP-treated BMDM. The results are from a representative experiment of two independent experiments that were performed. Cells = non-treated cells. m-apoTf = mouse apo-transferrin.

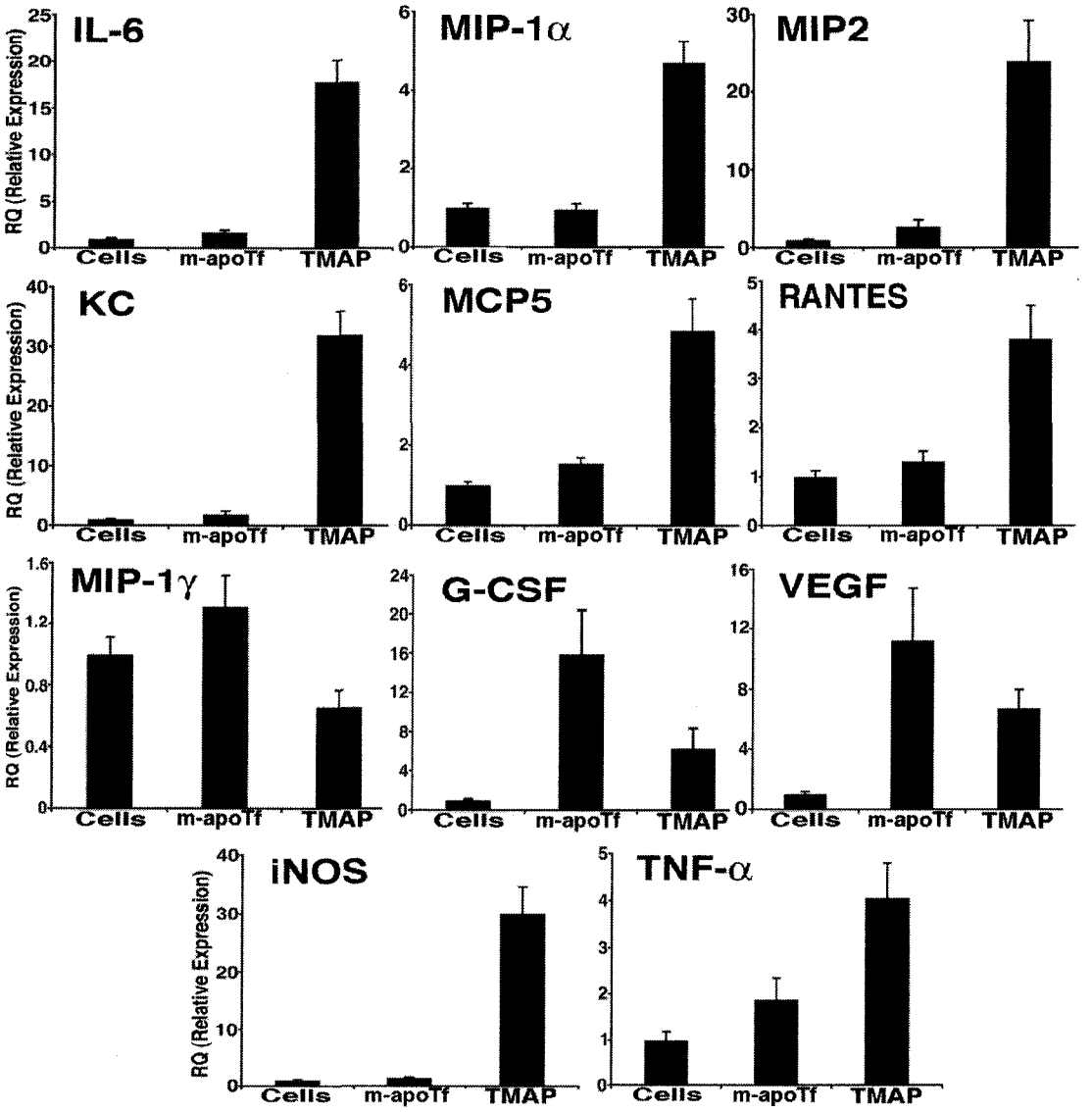

Figure 5.10: Quantitative PCR of select cytokines/chemokine genes induced by treatment of bone marrow-derived macrophages (BMDM) with 50 μg/mL of synthetic transferrin macrophage activating peptide (TMAP). Three μg of total RNA, were isolated from either BMDM treated with TMAP, murine apo-transferrin (mapoTf), or non-treated cells. Each point on the graph is a representative experiment of two independent experiments performed, showing the mean ± SEM of triplicate determinations.

CHAPTER VI

GENERAL DISCUSSION

6.0 Overview of findings

Innate immunity is the first line of defense aimed at protecting the host from invading pathogens by recognizing conserved motifs released by dead or damaged pathogens. However, in the event of tissue injury and in the absence of pathogens the immune system must have means of recognizing any tissue damage in order to mount the appropriate responses eventually leading to tissue repair. The major premise of the "danger model" of immunity proposed by Matzinger (1994) is that the immune system is more concerned with tissue damage than with foreignness, and that the primary mode of immune system activation is through the alarm signals released by damaged tissues. Despite the fact that the "danger model" does not fully address issues such as why transplants between identical twins are not rejected, and why is there an immune response to viruses that cause cell death by apoptosis. Nevertheless, this model of immunity has paved the way for an emerging field of immune system activation by modified endogenous molecules.

Previous studies in our laboratory by Neumann and Stafford [13, 171] revealed that transferrin fragments were present in mitogen-stimulated goldfish kidney supernatants, and that the affinity purified transferrin fragments induced a strong nitric oxide response in goldfish as well as mammalian macrophages.

However, these studies did not identify the enzyme(s) that cleaved goldfish transferrin present in the mitogen-stimulated goldfish kidney supernatants.

My thesis work focused on identifying the potential enzyme found at inflammatory that can cleave transferrin from different species into immunomodulatory fragments. In addition, I examined the pro-inflammatory responses of different macrophage populations after stimulation with transferrin-derived synthetic peptide, TMAP.

It is not surprising that the end result of elastase digestion of transferrin were fragments that had immunomodulating activities. Previously, Doring *et al.* (1988) and Britigan *et al.* (1993) showed that transferrin could be cleaved by *Pseudomonas* elastase and human neutrophil elastase, and that transferrin fragments were present in the BAL of *Pseudomonas areuginosa* infected CF patients. However, Britigan *et al.* (1993) did not hypothesize that these transferrin peptides might enhance the inflammatory response in the lungs of CF patients. Instead, they suggested that transferrin peptides contributed to the pathogenicity of CF lung disease by increasing iron availability for *P. areuginosa* growth and metabolism. They also suggested that free iron that was liberated as a result of enzymatic cleavage of transferrin, formed complexes capable of catalyzing the production of highly cytotoxic hydroxyl radical (•OH) from neutrophil-derived superoxide ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) via the Haber-Weiss reaction [171].

I indicated earlier that neutrophils and macrophages release a plethora of proteolytic enzymes at inflammatory sites and that the major enzyme produced in copious amounts is elastase. I hypothesized that elastase might be one of the

enzymes found at the inflammatory site capable of cleaving transferrin into immunomodulatory fragments. To test this hypothesis, I digested transferrin from different species (human, murine, bovine, and goldfish) and tested liquid chromatography resolved transferrin digest for nitric oxide-inducing activity using $P388D_1$ macrophages. The nitric oxide-inducing activity of elastase digested goldfish transferrin was examined using *in vitro*-derived goldfish macrophages.

That the elastase-digested transferrin fragments induced nitric oxide response in different macrophage populations, suggested that this process could be a feature of the inflammatory microenvironments. Interestingly, elastase-digested murine transferrin also induced a nitric oxide response in *in vitro*-derived goldfish macrophages suggesting that this pathway of macrophage activation may be highly conserved through evolution.

I reported that the enzymatic modification of transferrin did not only result in the generation of fragments with nitric oxide inducing activity but also of fragments with nitric oxide suppressing activity. To my knowledge, there are no reports in the literature that described modified host proteins with both pro- and anti-inflammatory properties. It has been shown that hyaluronan, fibronectin and lactoferrin are proteins similar to transferrin in that when they are cleaved due to an infection or tissue injury their peptides induce an inflammatory response [41, 45,77]. The only endogenous molecule with both pro- and anti-inflammatory properties is the high-mobility group box 1 (HMGB1). In addition to its role as an alarmin molecule as described in chapter 1, HMGB1 appears to be involved in tissue repair, by recruiting stem cells to the site of injury and promoting their proliferation [173]. However, the HMBG1

mediates both pro- and anti-inflammatory functions as an *intact* molecule [51, 55], whereas transferrin must be enzymatically *modified* in order to exert its pro- and anti-inflammatory effects.

The major contribution of my thesis work is the identification and the functional characterization of the transferrin macrophage-activating peptide that I named TMAP. Although the mass spectrometry analysis indicated that elastase-cleaved transferrin did not result in a consistent pool of transferrin peptides, one peptide (TMAP) was consistently present in the digests. The size and cysteine residue pattern of TMAP resembled a defensin-like molecule [174], as well as the antimicrobial domain found in the N-Lobe of ovo-transferrin [175].

TMAP is much smaller (3 kDa) compared to the other alarmin peptides that were generated by enzymatic modification of host proteins. For example, active hyaluronan fragment is ~135 kDa [41], fibronectin fragment is ~110 to 120 kDa [46], and lactoferrin fragments are ~32, 23, 22, and 19 kDa [77]. It is not known whether the entire fragments of hyaluronan, fibronectin, or lactoferrin are required for their biological activity. The functional analysis of TMAP has provided evidence that modified host proteins may possess small hidden domains susceptible to proteolytic enzymes that are commonly produced during inflammation.

In summary, my thesis work has advanced our understanding of the immunomodulatory properties of the enzyme-modified transferrin. Furthermore, it has identified and functionally characterized a transferrin-derived peptide that induced significant and highly conserved pro-inflammatory responses of macrophages.

6.1 Future studies

The study of innate immunity is attracting more attention in light of the discovery of Toll-like receptors and immune cells activation by modified endogenous proteins. However, more research is required in this area to increase our understanding of the mechanisms of innate immunity. In the course of this thesis, I performed experiments to advance our knowledge regarding what was previously known about modified transferrin and its role in the innate immunity of the host. Based on my results, I believe there are two main areas of future research that could be pursued for better understanding of the mechanisms by which transferrin fragments exert their immunomodulatory activities. They are: (1) the examination of mechanisms mediating transferrin's induction and inhibition of nitrite production in macrophages; and (2) the identification and characterization of receptor(s) for both nitric oxide transferrin-inducing and transferrin-inhibiting fragments, and their downstream signaling pathways.

(1) The examination of the mechanisms of induction and/or inhibition of nitric oxide response in mammalian and fish macrophages by transferrin peptides:

I have identified and functionally characterized TMAP, however elastase digestion of transferrin generated an enormous database of transferrin peptides. It is important to also examine the functional properties of additional transferrin peptides and determine their pro- or anti-inflammatory properties. I hypothesize that transferrin molecule may have other macrophage-activating domains similar to TMAP. To test this hypothesis, different recombinant proteins may be generated and expressed and their immunomodulating activities examined. In addition, when

TMAP is folded, it likely forms a homotrimer; therefore, it would be of interest to examine the role of the six cysteine residues in conferring the functionality of TMAP. This can be accomplished by mutating one cysteine residue at a time using site-directed mutagenesis, or by generating different synthetic peptides. The generation of antibody to TMAP would further enhance the functional assessment of this peptide. This antibody could potentially be used to block the activity of the peptide, and for detection of TMAP in inflammatory sites *in vivo*. The antibody may also be required for the identification of the TMAP receptor using co-immunoprecipitation.

In my thesis research, no attempts were made to characterize the nitric oxide response-inhibiting transferrin fragments. Therefore, identifying and characterizing the exact amino acid sequence(s) of the nitric oxide response-inhibiting fragments(s) would be of importance since there are no reports describing that a modified endogenous protein can induce both pro- and anti-inflammatory effects. Preliminary analysis of the transferrin C-lobe sequence using SMART, suggests that the anti-inflammatory fragments may be present in this lobe. I would first analyze using mass spectrometry the fractions that contained fragments that induced anti-inflammatory responses of macrophages. Once the amino acid sequences were identified, I would synthesize different peptides and examine whether they suppress the nitric oxide response of macrophages. The generation of antibodies that recognize fragments that have nitric oxide-suppressing activities would further enhance the functional assessment of these fragments and the identification of their receptor(s).

(2) The identification and characterization of the mammalian and fish macrophage receptor(s) for both nitric oxide transferrin-inducing and transferrin-inhibiting fragments:

The identification of the receptors for the macrophage-activating and -inhibiting fragments is important for understanding the mechanisms by which transferrin fragments exerts their activities. I hypothesize that PRRs, and more specifically TLRs, may be involved since many modified endogenous or "danger" molecules, such as HSP70 [34], hyaluronan fragment [41], and fibronectin EDA fragment [48], have been shown to signal through TLRs. To test this hypothesis, blocking TLR-2 and TLR-4 and then treating macrophages with TMAP could be done to test directly whether these two receptors are involved in TMAP signaling. Other proven approaches for the identification of receptors for known ligands may also be required, such as yeast two-hybrid system or co-immunoprecipitation technique.

The information gathered by studies outlined above would further enhance our understanding about the roles of modified transferrin in inflammation of both higher and lower vertebrates.

CHAPTER VII

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APPENDIX I

Molecular and functional characterization of goldfish (*Carassius auratus* L.) transforming growth factor-beta

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Abstract

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine with important roles in the regulation of cell proliferation, differentiation, survival, migration, activation and de-activation. It is one of the first cytokines released during an immune response and plays a strong immunomodulatory role in the activation and subsequent deactivation of macrophages and other immune cells. TGF-β is a highly conserved molecule, and members of the TGF superfamily can be found in organisms as evolutionary distant as arthropods. In this manuscript we described the identification of a goldfish TGF-β molecule, that was highly expressed in the skin, kidney and spleen of the goldfish and its expression was up-regulated in macrophages treated with LPS or recombinant goldfish TNF-α. Goldfish TGF-β shared a high amino acid identity with, and was phylogenetically related to TGF-β1 of other teleost fish, birds, amphibians and mammals. Recombinant goldfish TGF-β (rTGF-β) induced the proliferation of a goldfish fibroblast cell line (CCL71) in a dose-dependant manner. In addition, rTGF-β down-regulated the nitric oxide response of TNF-α activated macrophages. This is the first report of teleost TGF-β function in an ectothermic vertebrate.

Keywords: transforming growth factor-beta; TGF-β; fish; goldfish; macrophages; deactivation; proliferation; inflammation; innate immunity.

Introduction

Transforming growth factor beta (TGF-β) is an important molecule involved in the regulation of cell proliferation, differentiation, survival, migration and activation and de-activation of immune cell functions [175]. TGF-β is produced primarily by macrophages, fibroblasts and platelets [176] and is initially released from the cell in a latent form called latent TGF-β or the small latent complex (SLC) because of the association of a latency associated protein (LAP) with the mature TGF-β peptide. Latent TGF-β cannot bind to its receptor, but must be separated from the LAP and the latent TGF-β-binding protein (LTBP) before it is functional. This separation can occur by an increase in pH, heat or the addition of several proteases *in vitro* [177], and is thought to occur *in vivo* via a number of mechanisms including proteolytic activation by transglutaminase or by a conformational change in LAP by interaction with thrombospondin [178; 179; 180].

The TGF-β system appears to be very conserved, ligands, receptors and signalling molecules associated with this system can be traced back to arthropods, suggesting that it is over one billion years old [181]. Recently, TGF-β's role in the biology of regulatory T cells has rejuvenated interest in the TGF-β immunomodulatory role in mammals [175], however, TGF-β also plays many important roles in the regulation of innate immunity which may be more applicable to non-mammalian organisms. TGF-β has been shown to regulate the activation states of monocytes and macrophages [182], and upregulate the expression of adhesion molecules, such as LFA-1 and the fibronectin receptor on monocytes [183; 184; 185], making it a potent chemoattractant [186; 187]. Interestingly, TGF-β is an effective inhibitor of activated macrophage function, this likely occurs through down-regulation of MyD88 signaling via the degradation of MyD88 molecules probably induced by activation of Smad3 [188; 189]. TGF-β is also a central cytokine of wound healing in part because of its regulatory role in inducing the expression of connective tissue growth factor (CTGF) which induces the proliferation of fibroblasts at the wound site [190; 191].

Here we report, for the first time, quantitative expression in macrophage subpopulations and functional analyses of a TGF-β molecule from bony fish, the

goldfish (*Carassius auratus* L.). This TGF-β molecule was found to be expressed in all tissues of the goldfish with the highest mRNA levels observed in the skin, kidney and spleen. Monocytes, macrophages and their progenitors also expressed TGF-β with highest expression observed in macrophages which was up-regulated after addition of LPS or recombinant goldfish TNF-α (goldfish rTNF-α). Goldfish TGF-β was phylogenetically related to other fish TGF-β molecules, which were most closely related to mammalian TGF-β1. Recombinant goldfish TGF-β (rTGF-β) induced the proliferation of a goldfish fibroblast cell line CCL71. Goldfish rTGF-β deactivated goldfish macrophages by down-regulating the production of reactive nitrogen intermediates in monocytes/macrophages that have been previously activated with goldfish rTNF-α

Materials and Methods

2.1 Fish

Goldfish (*Carassius auratus* L.) were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 20°C in a flow-through water system and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least three weeks prior to use in experiments. All of the fish used in the experiments detailed in this paper ranged from 10 to 15 cm in length and whenever possible an equal representation of both sexes was used.

2.2 Culturing of goldfish macrophages and CCL71 fibroblasts

The culture medium (NMGFL-15) we used was described previously [192]. Complete NMGFL-15 medium used for the cultivation of goldfish macrophages contained 5% carp serum and 10% newborn calf serum (NCS; Hyclone, Logan, UT). Cultures were grown in the absence of any additional CO_2 at 20°C. CCL71 fibroblast cell line was purchased from ATCC, and grown according to the directions provided. Briefly cells were thawed from a frozen stock and grown at 20°C in DMEM supplemented with 10% FBS and L-glutamine for 5-6 days before passage.

2.3 Isolation and generation of primary kidney macrophage cultures from goldfish

Isolation of goldfish kidney leukocytes and the generation of primary kidney macrophages (PKM) were performed as previously described [192]. Briefly, goldfish macrophage cultures were established by seeding freshly isolated kidney leukocytes (1.8 to $2.0x10^7$ cells/flask from individual fish) into 75 cm^2 tissue culture flasks containing 15 mL of complete medium and 5 mL of cell-conditioned medium (CCM) from previous cultures. PKM cultures were composed of a heterogeneous population of cells, as determined by flow cytometry, morphology, cytochemistry, and function. Three distinct macrophage subpopulations were a feature of PKM cultures which represent macrophage subsets temporally arrested at distinct differentiation junctures in development: the early progenitors, the monocytes and mature macrophages [193].

2.4 DNA sequencing and in silico analysis of goldfish TGF-β

The goldfish TGF-β transcript was identified by homology based PCR using the previously identified carp sequences as references. All primers sequences used for sequencing, expression and cloning of goldfish TGF-β can be found in table 1. From this fragment, RACE PCR (BD Biosciences, Palo Alto, CA) was performed following the manufacturers specifications to obtain the full open reading frame and untranslated 5' and 3' sequence of goldfish TGF-β. Sequencing was performed by first cloning the TGF-β PCR amplicon into the pCR 2.1 TOPO vector (Invitrogen) and then identifying the colonies that contained positive inserts by colony PCR using the vector specific M13 primers (Invitrogen). Positive clones were identified using the QIAquick PCR purification kit (Qiagen), and sequenced using a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia) and a PE Applied Biosystems 377 automated sequencer. Single-pass sequences were analyzed using Vector NTI (Invitrogen) and subsequent gene annotations were conducted using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/).

Conserved TGF-β motifs were identified in the putative amino acid sequence, and subsequent predictions were based on analytical tools provided in the ExPASy proteomics server (http://www.expasy.org). Sequence alignments were performed

using ClustalX v1.83 (http://bips.u-strasbg.fr/fr/Documentation/ClustalX. Phylogenetic analysis was performed using the program MEGA 3 (http://www.megasoftware.net/).

2.5 Real-time PCR analysis of goldfish TGF-β expression in different tissues and sorted macrophage subpopulations

Goldfish skin, kidney, spleen, liver, heart, gill, intestine and brain were isolated and cultured goldfish kidney macrophages were sorted into early progenitor, monocyte and macrophage subpopulations using a FACS Calibur flow cytometer (Becton/Dickinson) according to a protocol we described previously [193]. RNA from tissues and sorted cells was isolated immediately after sorting using Trizol (Invitrogen). First-strand cDNA synthesis was done using the Superscript II cDNA synthesis kit (Invitrogen). Quantitative real-time PCR analyses were carried out using the Applied Biosystems 7500 Fast real-time PCR equipment. The relative expression of goldfish TGF-β in relation to β-actin in the skin, kidney, spleen, liver, heart, gill, intestine, and brain as well as sorted cells was assessed using primers generated with the Primer Express software (Applied Biosystems). Thermocycling parameters were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Analyses of the relative tissue expression data for five fish was carried out using the 7500 Fast software (Applied Biosystems). Statistical significance of the data obtained from the quantitative PCR was analyzed using a 1-way ANOVA, results were deemed to be significant at $P<0.05$.

2.6 Design of expression constructs, PCR amplification, and cloning

Goldfish TGF-β constructs were designed for expression using the pET 151D/TOPO prokaryotic expression system (Invitrogen). Primers were designed to amplify the 336bp portion of the goldfish TGF-β transcript that encoded for the mature peptide portion of the predicted amino acid sequence. PCR amplification of the expression insert was performed as follows: 7 μL of the TGF-β clone template was added to 76 μL ddH2O, dNTPs (0.2 μL of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10x PCR buffer (10 μL of 100 mM Tris-HCl pH8.3, 500 mM KCl, 15

mM $MgCl_2$, 0.01% (w/v) gelatin), expression primers and a 15:1 ratio of Taq:Pfu DNA polymerases (1 μL of 5 U/mL solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient thermal cycler. The amplification program consisted of 30 cycles of: 94°C for 15 sec, 59°C for 15 sec, 72°C for 2 min, and a final elongation step of 72°C for 7 min. Amplification was confirmed by agarose gel electrophoresis.

2.8 Production of recombinant goldfish TGF-β (rTGF-β)

Plasmid DNA containing the TGF-β expression construct was transformed into BL21 Star competent cells (Invitrogen) for recombinant protein expression. 10 ng of plasmid DNA was transfected into the BL21 cells according to the manufacturer's specifications and the cells were grown and tested for presence of recombinant protein before and after induction with IPTG following the manufacturer's specifications. After optimization of the recombinant protein expression procedure, BL21 cells were grown in 1L cultures and collected and tested for recombinant protein via Western blot using an antibody specific for the 6x HIS tag located on the N-terminal end of the recombinant protein.

2.9 Purification and analysis of rTGF-β

BL21 cells expressing the rTGF-β were lysed by freeze-thaw cycles and these lysates were resuspended in 100mM HEPES solution prior to protein purification. Recombinant goldfish TGF-B was purified from cell lysates using MagneHIS beads (Promega) according to the manufacturer's specifications. Purified proteins were eluted and stored for 4 hours at 4°C in a solution containing 100mM HEPES, 500mM, 1M 3-(1-Pyridinio)-1-propanesulfonate (NDSB201) (Sigma) [194; 195] and 500mM imidazole (pH 9.0) which also facilitated the refolding of the protein correctly, and then dialyzed overnight against 1X PBS. LPS removal was achieved using the EndoTrap Red endotoxin removal column (Cambrex) as per the manufacturers directions. Samples were then filter-sterilized in preparation for immunodetection and analysis of biological activity. Total protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce) according to the

manufacturer's protocols. Presence of the rTGF-β in the purified sample was confirmed by mass spectroscopy [196]. Briefly, proteins were separated by SDS-PAGE under reducing conditions using 12.5% polyacrylamide gels, transferred to 0.2 μm nitrocellulose membranes (BioRad), and incubated over night at 4°C in the presence of the anti-6x HIS primary antibody. Membranes were subsequently washed, incubated with an alkaline phosphatase (AP)-conjugated mouse IgG mAb, and developed using BCIP and NBT.

To determine whether rTGF-β formed a homodimer after purification goldfish rTGF-β was biotinylated using the EZ-Link Biotinylation Kit (Pierce) according to the manufacturer's protocol. Effective biotinylation of each protein was confirmed using the EZ-Biotin Quantification kit (Pierce) according to the manufacturer's protocol. 100 μg of rTGF-β was then incubated in de-ionized water (pH 7.5) for 60 min and the protein mixture was cross-linked using bis (sulfosuccinimidyl) suberate (BS^3) for 15 min and then run on a reducing SDS-PAGE gel. The proteins were then transferred to nitrocellulose membrane (Bio-Rad) and probed using Alkaline phosphatase-conjugated avidin (Pierce). The blot was then developed using NBT and BCIP (BioRad) according to the manufacturers protocol.

2.10 Proliferation assay

The effect of rTGF-β on the proliferation of a goldfish fibroblast cell line CCL71 was assessed using the Cell Proliferation ELISA BrdU colorimetric assay (Roche). This technique is based on the incorporation of the pyrimidine analog, 5-bromo-2'-deoxyuridine (BrdU), into the DNA of proliferating cells. CCL71 fibroblasts were grown as directed (ATCC) using DMEM supplemented with 10% FBS and L-glutamine. Upon reaching confluency, cells were counted and seeded at a density of 1x 10^4 cells $well^{-1}$ in 96-well culture plates (Falcon) in 50 μL of DMEM and treated with 50 μL of 200, 100, 50, 10 and 1 ng/mL rTGF-β mixed with DMEM, and incubated with BrdU labelling reagent at a concentration of 15 μM. Cells were incubated with BrdU labelling reagent for 24 hours and then subsequent samples were taken every 2 days from the day 0 time point. The reaction was developed according

to the manufacturer's specifications and optical densities determined at 450 nm using a micro plate spectrophotometer (Biotek Instruments). In control experiments, the colorimetric reaction was found to be directly proportional to the number of proliferating fibroblasts in culture. Recordings from the untreated cells were subtracted from the experimental groups to account for the regular growth of the CCL71 cells. Six replicates of CCL71 cells were seeded at a density of 10000 cells $well^{-1}$ and counted every 48 hours using a haemocytometer to confirm data collected using the BrdU assay.

2.11 Nitric oxide assay

The goldfish PKM cultures were established from six fish and after 8 to 10 days of culture were assayed for a nitric oxide response measured using the Griess reaction. Briefly, $5x10^4$ viable cells/100 µL of medium from 6 individual fish, were seeded into 96-well flat-bottom tissue culture plates. The experimental groups were: untreated cells (medium control), cells treated with either 100 ng rTNF-α or rTGF-β only, or cells treated with either 100 ng rTNF-α or rTGF-β for one hour, and then 100 ng of rTGF-β was added to the cultures pre-treated with rTNF-α, and 100 ng of rTNF-α were added to cultures pre-treated with rTGF-β. To assess whether higher amounts of rTGF-β would further down-regulate the nitric oxide response of rTNF-α-activated macrophages, additional 100 ng rTGF-β were added at each time point (total rTGF-β concentration at 12, 24 and 48 hrs were 200, 300 and 400 ng, respectively) after initial pre-treatment with rTNF-α/rTGF-β. The plates were incubated for 72 hours at 20°C prior to determination of nitrite concentration for each culture (n=4).

2.12 Statistical Analyses

Statistical significance of the data obtained from the quantitative real-time PCR, BrdU proliferation assay and nitric oxide assays were analyzed using a one-way ANOVA. Probability level of $P < 0.05$ was considered to be significant.

Results

3.1 Goldfish TGF-β transcript analysis and phylogenetic analysis of goldfish TGF-β

Goldfish TGF-β (Genbank accession # EU086521) was encoded for by a 1134bp transcript that generates a predicted peptide possessing both the pro-peptide and 112 amino acid mature portion of TGF-β. In the precursor region a characteristic RGD integrin binding site as well as a RRKR cut site were identified suggesting that the mechanism behind goldfish TGF-β regulation after translation was similar to that reported for mammals. In the 112 amino acid mature peptide portion of the molecule the nine conserved cysteine residues that allow for formation of inter-chain and intra-chain disulphide bonds and the C-terminal cysteine knot were present, as well as a conserved proline at position 36 of the mature peptide and a glycine at position 42, both of which are hallmarks of the TGF superfamily (Fig 1).

Goldfish TGF-β appeared to be most closely related to the TGF-β1 molecules of mammals and other fish. It shared closest phylogenetic relationship with carp TGF-β1 (94% amino acid identity), zebrafish TGF-β1 (76% amino acid identity) and rainbow trout TGF-β (67% amino acid identity). These fish TGF-β1 molecules grouped with those of chicken, frog and mammals, and had a more distant relationship to the TGF-β2 and TGF-β3 of fish, chicken, frogs and mammals (Fig. 2). The predicted amino acid sequence of goldfish TGF-β possessed both the pro-peptide (LAP) and the mature peptide. The mature peptide contained all of the essential cysteine residues required for the formation of inter-chain and intra-chain disulphide bonds.

3.2 Expression of goldfish TGF-β in tissues and sorted mononuclear cells

Goldfish TGF-β transcript expression in tissues and mononuclear cells was analyzed by quantitative PCR. Expression of goldfish TGF-β was compared to the expression of the endogenous control transcript, β-actin and then normalized to the heart (Fig 3A) or untreated progenitor cells (Fig 3B). Goldfish TGF-β was observed to be highly expressed in all tissues when compared to β-actin and the highest tissue expression was observed in the skin, a 10-fold higher expression than that in the heart. Similarly, the TGF-β mRNA levels in the kidney and spleen were 5- and 4-

fold higher than in the heart, respectively. mRNA levels in the liver, brain, intestine and gill were similar to that observed for the heart (Fig 3A).

Goldfish TGF-β transcript expression was also analyzed in sorted goldfish monocytes, macrophages and their progenitor cells. Sorted cells were analyzed in resting state as well as after treatment with 1μg/ml LPS and 100 ng/ml of goldfish rTNF-α for 24 hours. Goldfish TGF-β mRNA levels were the highest in mature macrophages and monocytes (3-fold and 2- fold, respectively) compared to progenitor cells. Treatment with 1μg/mL LPS increased the expression of goldfish TGF-β in sorted macrophages to almost 5-fold higher than that observed in progenitor cells. Expression levels in sorted monocytes and progenitor cells were not affected by treatment with LPS. Similarly, treatment with 100 ng/ml goldfish rTNF-α induced a significant increase in TGF-β expression levels in sorted macrophages but had no significant effect on the expression of TGF-β in sorted monocytes or progenitor cells (Fig 3B).

3.3 Visualization of rTGF-β homodimerization by protein cross-linking

Recombinant goldfish TGF-β was assessed for its ability to form functional homodimers by biotinylating the protein and then cross-linking it with the protein cross-linking agent bis (sulfosuccinimidyl) suberate (BS^3). rTGF-β molecules that were in association with each other at the time of cross-linking were frozen in this state, and 100 μg of protein was either untreated, treated with β-mercaptoethanol and then BS^3, treated with BS^3 and then β-mercaptoethanol, or just BS^3 and observed for its ability to form homodimers. Protein left untreated was observed to migrate on SDS-PAGE at approximately 12 kDa, the estimated size of monomeric rTGF-β. Protein treated with β-mercaptoethanol and then BS^3 was found to migrate at 12 kDa suggesting that no homodimerization was observed after reducing disulphide bonds. Protein treated with BS^3 and then β-mercaptoethanol exhibited 2 bands migrating at 12 kDa and 25 kDa likely representing the monomeric and dimeric forms of rTGF-β. Protein treated with BS^3 only also migrated at 12 and 25 kDa (Fig. 4)

3.4 Induction of CCL71 goldfish fibroblast proliferation by rTGF-β

Recombinant goldfish TGF-β induces proliferation in the goldfish fibroblast cell line CCL71. A BrdU cell proliferation assay was done to assess the growth characteristics of the CCL71 fibroblasts after treatment with 1, 10, 50, 100 and 200 ng/mL of rTGF-β. The CCL71 cells grew linearly when $1x10^4$ cells/well were used. The growth of CCL71 cells was enhanced when the cultures were treated with 50, 100 or 200ng/mL of rTGF-β. One and 10 ng/mL rTGF-β had no significant effect on the growth of CCL71 cells, whereas significant differences between non treated cells and cells treated with 100 ng/mL rTGF-β were documented in two of three independent experiments, as early as day 4 after treatment (Fig 5A). Cell counts were performed to support the BrdU proliferation data. Untreated CCL71 cells exhibited increases in cell number throughout the 8-day monitoring period at an average doubling rate of approximately 52 hours. Cells treated with rTGF-β also increased in cell number throughout the treatment period and exhibited a doubling time of approximately 50, 48.5 and 45 hours when treated with 10, 50 and 100 ng/mL rTGF-β respectively. Cells treated with 100ng/ml rTGF-β resulted in approximately 2.3-fold more cells then untreated or elution buffer treated cells, and the effect of rTGF-β on the CCL71 cells was dose dependant with approximately 2- and 1.3-fold increases in cell number over untreated and elution buffer controls in the 50 and 10 ng/ml rTGF-β treated cells respectively (Fig. 5B).

3.5 Goldfish rTGF-β inhibits nitric oxide response of macrophages

Macrophages pre-treated with 100 ng of rTGF-β and then treated with rTNF-α exhibited slightly higher nitric oxide response compared to that induced by rTNF-α alone, whereas rTGF-β on its own did not induce a nitric oxide response in macrophages (Fig 6A). On the other hand, the nitric oxide response was significantly reduced in macrophages that were first activated with rTNF-α and then treated with rTGF-β (Fig 6A), indicating that rTGF-β down-regulated the nitric oxide response of activated goldfish macrophages.

The effect of rTGF-β on deactivation of goldfish macrophages was dose-dependent. Macrophages pre-treated with rTGF-β and then treated with rTNF-α or those treated with rTNF-α alone exhibited a gradual increase in nitrite production which was further enhanced by subsequent addition of 100 ng of rTNF-α, whereas in

cells pre-treated with rTNF-α the nitric oxide response gradually decreased following subsequent addition of 100 ng of rTGF-β (Fig. 6B). The nitric oxide response of this experimental group was significantly lower after the 3rd (total amount per culture: 300 ng) and 4th (total amount per culture: 400 ng) additions of rTGF-β.

Discussion

In this manuscript, we described for the first time quantitative expression and functional analyses of a bony fish TGF-β. Goldfish TGF-β was highly expressed in the skin, kidney and spleen and was differentially expressed as monocyte/macrophage progenitor cells developed into functional macrophages. Goldfish TGF-β expression in macrophages increased after activation of macrophages with optimal concentrations of LPS and goldfish rTNF-α, which was similar to that observed for mammalian mononuclear cells [175] and trout macrophages [197]. Trout and sea bream TGF-β molecules exhibited a similar ubiquitous expression pattern in the tissues and high expression in macrophages [198; 199]. Interestingly, the TGF-β mRNA levels were the highest in the skin, supporting TGF-β's role in wound repair and being one of the initial cytokines at the site of wound or infection. Phylogenetically, goldfish TGF-β was closely related to other fish TGF-β1 and mammalian TGF-β1 molecules and was functionally similar to TGF-β1 of mammals.

Addition of goldfish rTGF-β induced a proliferative response of CCL71 cells (goldfish fibroblast cell line). This was similar to observations of Grotendorst [19], who reported that mammalian TGF-β induced the proliferation of fibroblasts. However, recent studies suggest that TGF-β does so indirectly [176]. For example, treatment of fibroblasts with TGF-β induced high connective tissue growth factor (CTGF) expression. CTGF was found to have strong mitogenic effects on fibroblasts and was nearly as effective as TGF-β itself in stimulating extracellular matrix synthesis. These findings suggested that TGF-β may be stimulating fibroblasts to produce CTGF which in turn induced fibroblast proliferation [176; 190; 191]. The CTGF transcript(s) have not been identified in the goldfish, which prevented us from determining whether this induction mechanism was also present in teleosts.

Goldfish rTGF-β was found to be able to form homodimers in solution, suggesting that it is also functional in a dimeric form like the mammalian TGF-β. Not all of the goldfish rTGF-β was found as a dimer, and approximately half of the cross-linked sample was observed as a monomer possibly due to the prokaryotic expression system used to produce the recombinant protein. This is likely the reason that higher then biological levels of rTGF-β were necessary to induce a functional effect in fish cells.

Goldfish rTGF-β inhibited the nitric oxide response in macrophages albeit at a higher concentration than that observed in mammals. The concentration of rTGF-β used in this study was determined empirically and at 100 ng/mL the highest level of inhibition was observed (data not shown). The result obtained using 200 ng/mL of rTGF-β (Fig 6B) added to rTNF-α pre-treated cells was not statistically significant from the experimental groups pre-treated with rTGF-β + rTNF-α or rTNF-α alone, which differed from results reported in Fig. 6A. The observed differences in induction of biological activity by different amounts of rTGF-β in two independent experiments may be due to the variability observed between cultures obtained from individual goldfish.

Goldfish macrophages pre-treated with rTGF-β and then with rTNF-α showed an enhanced nitric oxide response when compared to macrophages treated with rTNF-α alone (Fig. 6A). Moreover, goldfish TGF-β did not activate the macrophages on its own, however, it appears to have possible priming effect on goldfish macrophages. As shown in mammalian systems, TGF-β is one of the first cytokines secreted at the inflammatory site by platelets, and was shown to be responsible for initiation of inflammation by recruiting monocytes and neutrophils to the site of inflammation as well as promoting adhesion and activation of immune cells [200; 201]. Upon arrival at the inflammatory site, immune cells undergo activation and develop antimicrobial armamentarium whose amplitude was reported to be controlled by TGF-β which has been shown to down-regulate antimicrobial functions of activated mammalian neutrophils and macrophages [202].

The immunomodulatory effect of TGF-β described in this study was similar to that observed for mammalian TGF-β where the induction of interleukin-1 (IL-1) and nitric oxide response in murine H4 chondrocyte cell line was down-regulated by 50% and 80%, 12 and 24 hrs after TGF-β treatment, respectively. [203]. TGF-β was reported to down-regulate the nitric oxide response in activated mammalian macrophages where 2 pM of TGF-β1 reduced nitrite concentration by 50% of INF-γ + TNF-α treated mouse peritoneal macrophages. TGF-β suppressed the production of nitric oxide response in macrophages by reducing the translation of iNOS mRNA and by promoting the degradation of the enzyme [204]. Interestingly, exogenous TGF-β was also found to exert its effects across species. For example, bovine TGF-β1 mature peptide, which has 69% amino acid identity to trout TGF-β1, was found to have activating and deactivating properties in trout macrophages. Trout macrophages treated with bovine TGF-β exhibited enhanced respiratory burst response after 24 hrs of cultivation [39]. In the same study, however, the treatment of trout macrophages with bovine TGF-β1 and macrophage activating factor (MAF) was also reported to cause significant decrease of the respiratory burst response [205].

TGF-β effects on neutrophils and monocytes/macrophages contribute to the formation of inflammatory foci due to this cytokine's ability to promote chemotaxis and adhesion activities [206]. TGF-β1 was the most potent chemoattractant described for human peripheral blood neutrophils (PMNs). The maximal chemotactic response was reported at doses of 40 fM for all mammalian TGF-β isoforms, highlighting the critical role for TGF-β in the early stages of the inflammatory response [207]. TGF-β was reported to activate resting blood monocytes by inducing an increase in mRNA expression of pro-inflammatory cytokines such as, TNF-α, IL-1β, and PDGF-BB [208]. In addition, TGF-β increased the expression of integrin receptors, such as LFA-1, VLA-3 (α3β1), and VLA-5 (α5β1) in human monocytes [209; 210]. TGF-β was shown to down-regulate mammalian the inflammatory responses of macrophages by down-modulating the production of INF-γ [211], and by increasing the induction of IL-1 receptor antagonist [212]. The central role for TGF-β in regulation of inflammation was also supported by observations that it facilitated the resolution of

the inflammatory response by promoting phagocytosis of damaged cells by macrophages [206], and accelerated wound healing through multiple pathways that influence cell infiltration, proliferation, angiogenesis, extracellular matrix synthesis and remodelling [213].

TGF-β is a pleiotropic cytokine, and its effects depend on number of factors such as time of secretion, concentration, TGF-β receptor expression, and the state of cellular differentiation and the micromilieu [214; 215; 216]. Our findings represent the first functional characterization of a TGF-β molecule from an ectothermic vertebrate and suggest that the goldfish TGF-β molecule is phylogenetically and functionally similar to mammalian TGF-β1.

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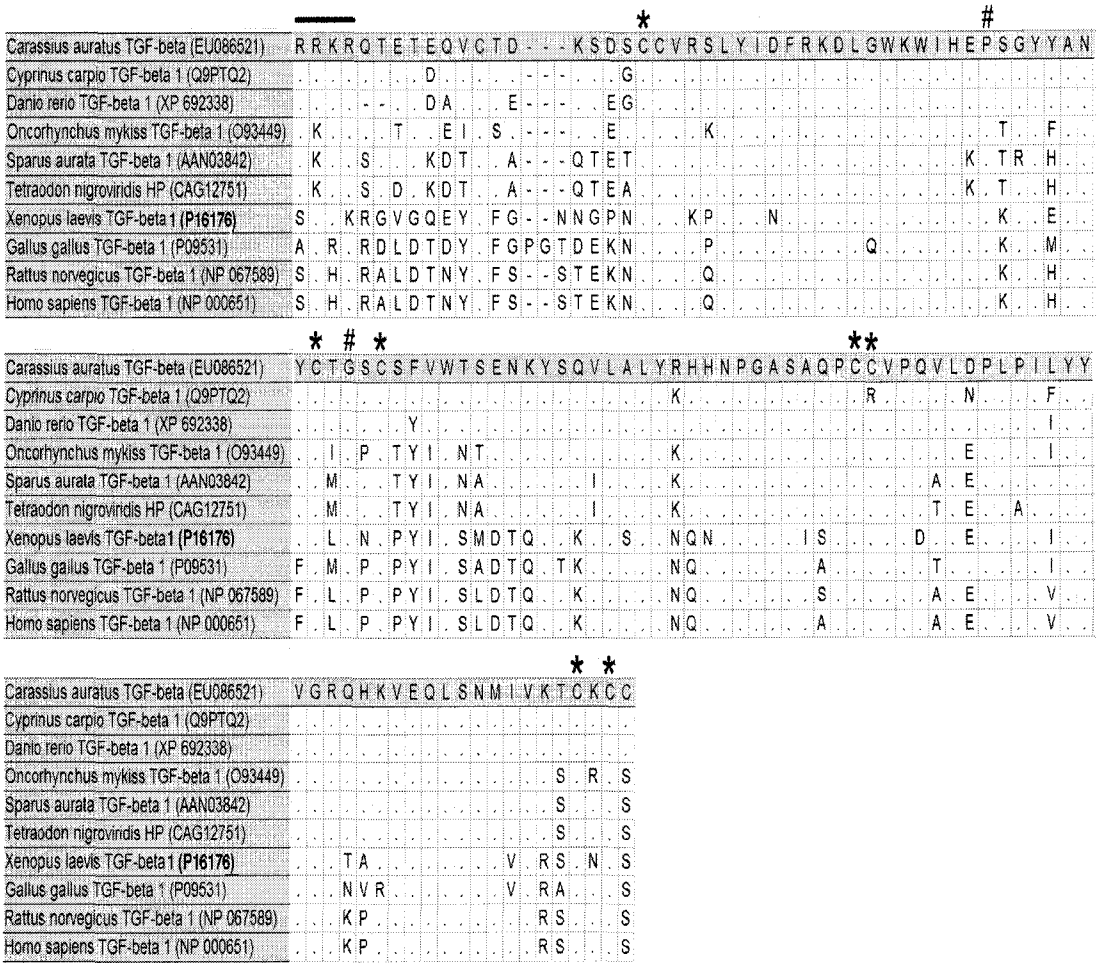

Figure 1: Amino acid alignment of the mature peptide portion of fish, amphibian, bird and mammalian TGF-β molecules. The RRKR (overlined) fragment signifying the beginning of the mature peptide is shown as are the conserved cysteine residues (*) required for disulphide bond formation to create the final functional homodimeric form of TGF-β. In addition, a conserved proline at position 36 (#) of the mature peptide and a glycine at position 42 (#), both of which are hallmarks of the TGF superfamily.

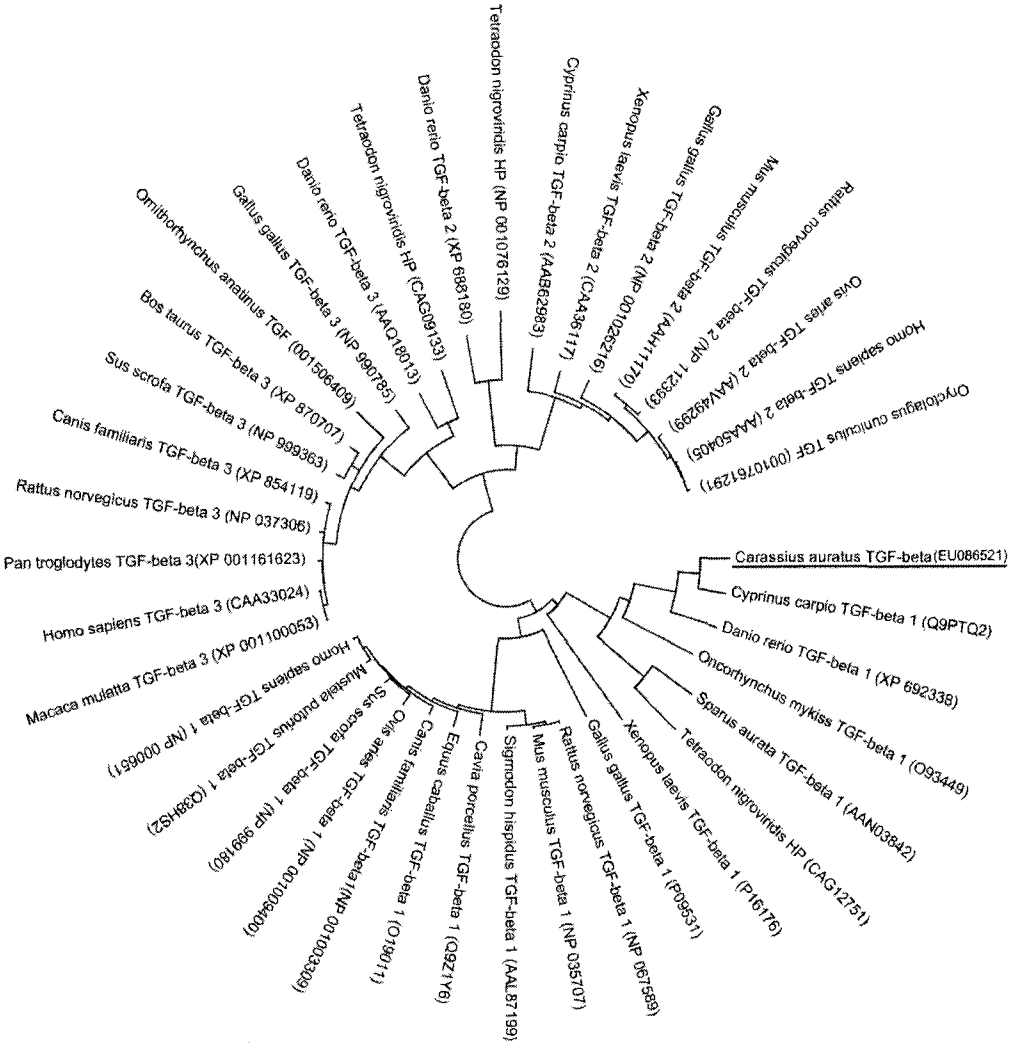

Figure 2: Phylogenetic tree showing the predicted relationship between goldfish TGF-β and other known TGF-β molecules from fish, birds, amphibians and mammals. Goldfish TGF-β groups with other fish TGF-β1 molecules and shares its highest amino acid identity with carp, zebrafish and trout TGF-β1. The phylogenetic tree was generated using the neighbour-joining method and was bootstrapped 10,000 times and no bootstrap values were below 59%. GenBank numbers are in brackets behind each sequence. HP = hypothetical protein.

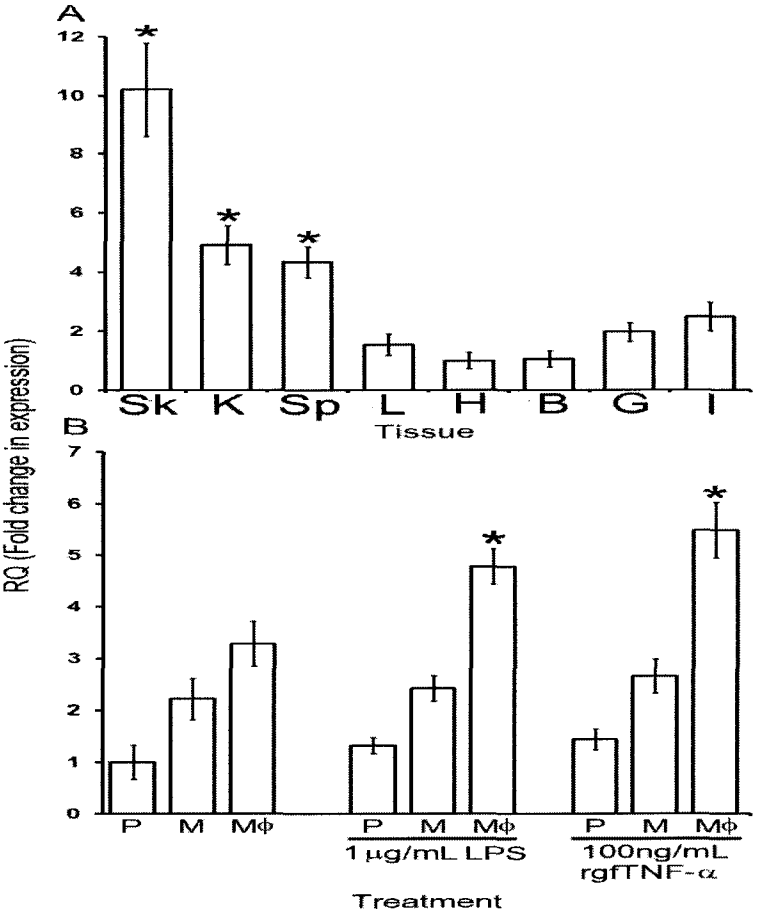

Figure 3: Goldfish TGF-β transcript expression analysis in tissues and sorted kidney-derived mononuclear cell populations using quantitative real-time PCR. TGF-β data were compared relative to a β-actin endogenous control and were normalized to m RNA expression in the heart (A), or non treated progenitor cells (B). Sorted cells (progenitor cells (P), monocytes (M) and macrophages (MΦ)) were not treated, treated with 1µg/mL LPS, or 100ng/mL rTNF-α for 24 hours prior to RNA isolation. Tissue expression was examined in 5 goldfish in the skin (Sk), kidney (K), spleen (Sp), liver (L), heart (H), brain (B), gill (G) and intestine (I), and kidney-derived macrophage cultures were obtained from individual fish (n=5) cultured for 4 days and sorted before use in the assays. The values depicted in the graphs are mean ± SEM. (*) indicates statistical significance ($P < 0.05$; one-way ANOVA).

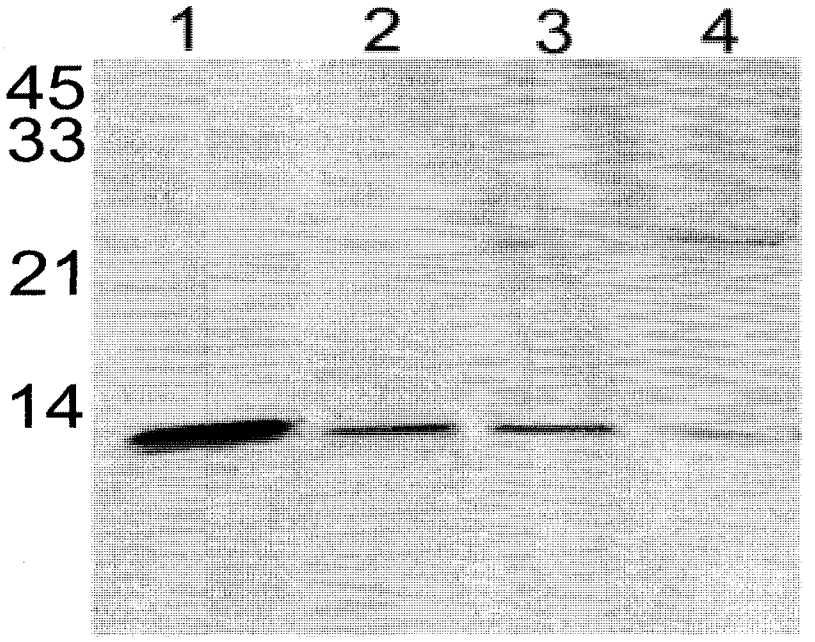

Figure 4: Western blot showing the assessment of recombinant goldfish TGF-β for its ability to form functional homodimers. 100 μg of recombinant protein was biotinylated and then cross-linked with bis (sulfosuccinimidyl) suberate (BS^3). Protein samples were (1) untreated, (2) treated with β-mercaptoethanol and then BS^3, (3) treated with BS^3 and then β-mercaptoethanol, or just (4) BS^3. Size in kilodaltons indicated on left side of the blot.

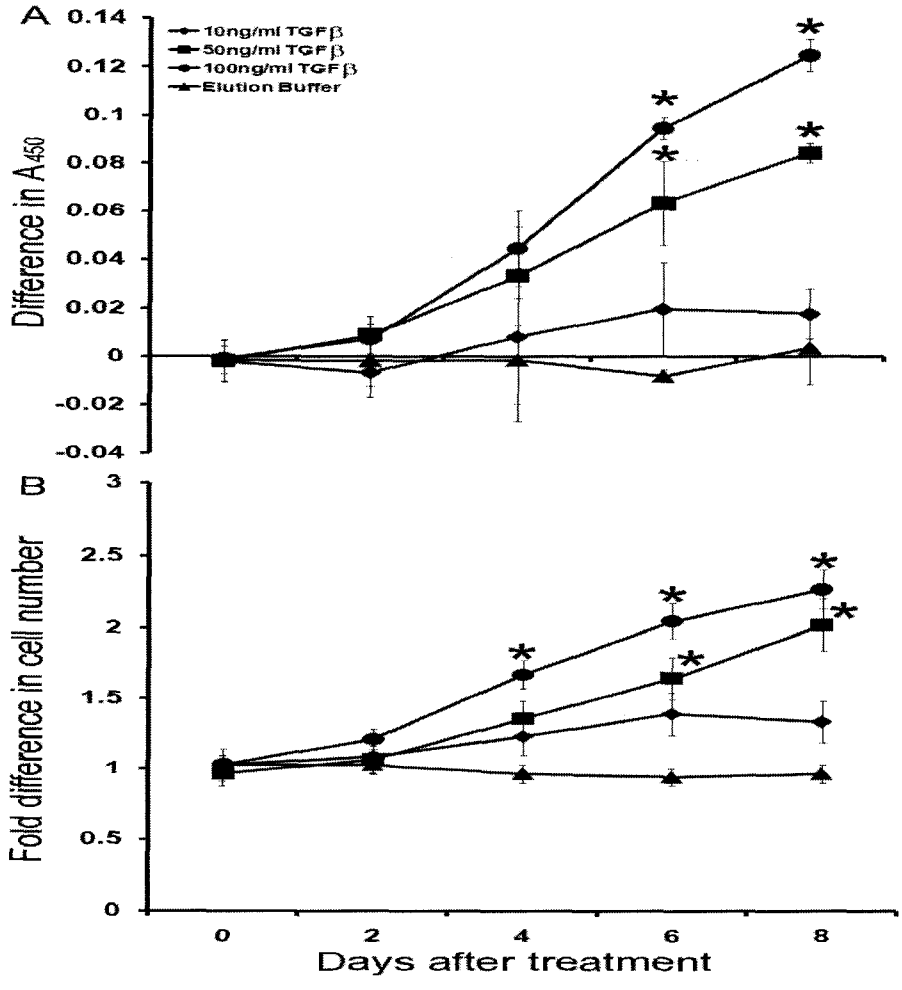

Figure 5: BrdU proliferation analysis of goldfish CCL71 fibroblast cell line with or without rTGF-β treatment. Non-treated CCL71 proliferation data have been subtracted from rTGF-β treated sample data to account for the normal (spontaneous) proliferation by this cell line (A). Fold difference in CCL71 cell number in relation to untreated cells (B). Both experiments are results from 10000 cells well^{-1} original cell density and represent the data from 3 (BrdU) or 6 (cell counts) independent experiments. The results are presented as mean ± SEM. (*) denotes significant difference ($P < 0.05$, one-way ANOVA).

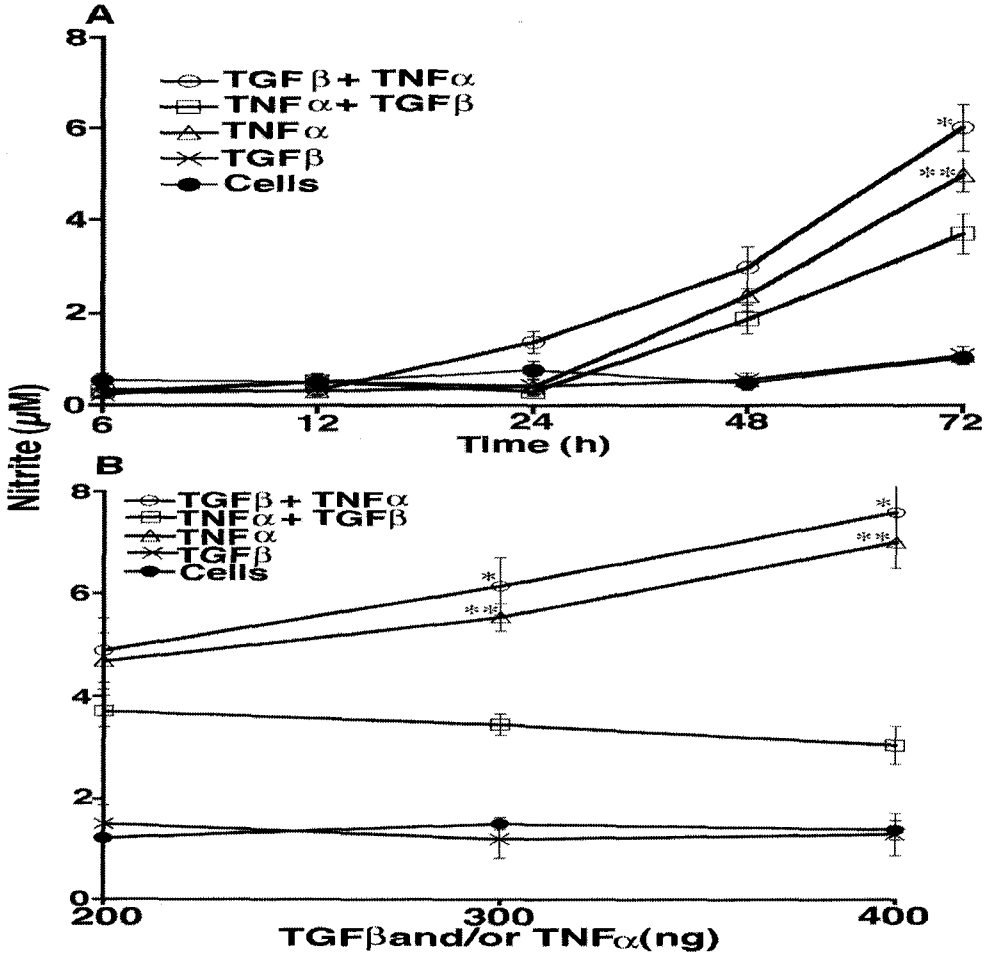

Figure 6: The down-regulation of the nitric oxide response of activated macrophages by goldfish rTGF-β. Nitric oxide response was determined by measuring the nitrite concentration using the Griess reaction. (A) The experimental groups were: untreated cells (medium control), cells treated with either 100 ng rTNF-α or rTGF-β only, or cells treated with either 100 ng rTNF-α or rTGF-β for one hour, and then 100 ng of rTGF-β was added to the cultures pre-treated with rTNF-α, and 100 ng of rTNF-α were added to cultures pre-treated with rTGF-β. Each point on the graph represent the mean ± SEM of six fish and data are from a representative experiment of two independent experiments performed. (*) significantly different ($P < 0.05$), rTGF-β + rTNF-α vs. rTNF-α + rTGF-β; (**) significantly different ($P < 0.05$), rTNF-α vs. rTNF-α + rTGF-β. (B) To assess whether higher amounts of rTGF-β would further down-regulate the nitric oxide response of rTNF-α-activated macrophages, additional 100 ng rTGF-β were added at each time point (total rTGF-α concentration at 12, 24 and 48 hrs were 200, 300 and 400 ng, respectively) after initial pre-treatment with rTNF-α/rTGF-β. Each point on the graph represents the mean ± SEM of four fish and data are from a representative experiment of two independent experiments performed. (*) significantly different ($P < 0.05$) rTGF-β + rTNF-α vs. rTNF-α + rTGF-β. (**) significantly different ($P < 0.05$), rTNF-α vs. rTNF-α + rTGF-β.

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APPENDIX II

GOLDFISH Smad4 OPEN READING FRAME

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CAGTCTGATGTGTCACAGACAGGGCGGCGAGAGCGAGACCTTCGCCAAACGGG
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