Investigating the Anti-inflammatory Activity of Honey

A thesis

submitted in fulfilment

of the requirements for the Degree

of

Doctor of Philosophy

in

Biological Sciences

at

The University of Waikato

by

AMANDA BEAN

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2012

Abstract

Honey has various bioactivities with health-benefiting properties. It has been registered for use in wound dressings but before it can be registered with regulatory authorities for sale with claims for therapeutic actions the components responsible for these bioactivities must be identified to allow standardisation. This thesis focuses on the anti-inflammatory activity of honey. Emphasis was put on identifying the mode of action of the anti-inflammatory component, creating an *in vitro* assay to measure the anti-inflammatory activity of individual honeys and attempting to identify and characterise the component(s) responsible for the activity.

A cell line (THP-1) was selected for this study based on its macrophage phenotype upon activation and its role as an inflammatory regulator. Initially this cell line was characterised to better understand the treatments that activate its phagocytosis ability, mobility, phenotype and gene expression profile. The cell line was then monitored for the change in expression of its pro-inflammatory and anti-inflammatory cytokines and growth factors in response to common cell-activating treatments (LPS, PMA and Vit-D3) and Manuka honey. It was found that Manuka honey increased the expression of pro-inflammatory cytokines TNF-α and anti-inflammatory cytokines IL-10, IL-1ra and the growth factors PDGF and TGF-β. This indicated that Manuka honey may allow inflammation to proceed at a modulated level, allowing healing to occur.

The cell line THP-1 was further investigated as a model of a phagocyte when activated. A phagocytosis assay using latex particles was utilised to study the

effect of honey on the ability of cells to phagocytose. It was found that honey reduced phagocytosis, and that Manuka honey had a superior ability to other honey types to do this. The activity was isolated to the high molecular weight compounds in honey. As phagocytosis produces large amounts of reactive oxygen species (ROS) and ROS are pro-inflammatory, the ROS produced by phagocytising THP-1 cells was investigated using a dichlorofluorescein assay. It was found that ROS produced by phagocytising THP-1 cells decrease in the presence of Manuka honey but not artificial honey or Rewarewa honey which has high anti-oxidant activity. This indicated that the effect of Manuka in decreasing ROS was not solely due to its osmotic effects or anti-oxidant capabilities. As phagocytosis starts the inflammatory cascade, it was hypothesised that the phagocytosis-inhibiting component resulted in honey having anti-inflammatory activity.

Next, attempts were made to identify the anti-inflammatory component. Manuka honey was separated using chromatography and the fractions obtained assayed for their inhibitory activity in the phagocytosis assay. The active fractions were found to contain MRJP-1 and to a small extent MRJP-3, which were identified by MALDI-TOF mass spectrometry. It was confirmed that these were glycoproteins with a high mannose content by using a ConA lectin column.

Next a mode of action for the glycoproteins was investigated. It was demonstrated by using mannan that honey was acting on a mannose-binding phagocytic receptor on the cell surface of macrophages, most likely the mannose receptor. It was hypothesised that with the glycoproteins bound to the receptor, the cell was unable to have phagocytosis triggered thus ROS production and expression of inflammatory cytokines in response to ROS produced were prevented.

A wide range of honey varieties was assayed and the anti-inflammatory activity was found in high levels only in Manuka honey and to a lesser extent in Kanuka and Rewarewa. To identify why this activity was much greater in Manuka honey,

as all honey contains the glycoproteins MRJP-1 and MRJP-3, the effect of the recently discovered high levels of MGO in Manuka honey was investigated. It was found that Manuka honey had a high fluorescence that was not seen in other honey types to the same extent and that this fluorescence was due to MGO modifying the proteins in honey. It was found that this fluorescence could be used as an indicator of anti-inflammatory activity for Manuka honey and that this fluorescence, and anti-inflammatory activity increased over time upon incubation of honey with high MGO levels.

The anti-inflammatory activity seen in the phagocytosis assay was compared with that seen in a commonly used *in vivo* assay of anti-inflammatory activity, the HET-CAM assay. The HET-CAM assay depends on the experimental production of inflammation and the reduction of this inflammation with an anti-inflammatory agent on the chorionic membrane of a fertilised hen's egg. It was concluded that highly fluorescent Manuka honey at a concentration of 5% had and anti-inflammatory effect equivalent to that of 5 mg/ml hydrocortisone, a commonly prescribed anti-inflammatory drug.

The anti-inflammatory activity seen in the honeys assayed in the *in vivo* HET-CAM assay concurred with the results for the same honeys assayed in the phagocytosis assay which indicated that the phagocytosis assay was a reliable indicator for assaying the anti-inflammatory activity of honey *in vitro*.

Acknowledgements

There are many people I need to acknowledge for all the assistance I have received during this research. Firstly I would like to thank Prof. Peter Molan, and Dr. Ray Cursons. Their dedication to and knowledge of wound healing and cell biology is second to none.

Secondly, I will always be grateful for Dr. Ray Cursons and Dr. Dick Wilkins for providing me a desk in the most enjoyable lab on campus. They are constantly striving to provide the best education for their students. Their superior intelligence on virtually every subject continues to amaze me and their science knowledge is not bad either.

I have been very fortunate to have great friends in the lab, Olivia, Bruce, Greg, Ali and Emma, to name but a few, and the lucky souls that managed to escape unscathed, Shona, Catherine, and Vicki. They were always around for a coffee and many important scientific discussions. Olivia, you are also a brilliant technician!

To Raewyn Towers and Dave Musgrave, thank you for making the lab a sophisticated place to work, I envy your travels as much as your abilities in the lab. Thanks for all the help over the years.

A big thank you to the many intelligent people who helped (or did for me!) technical things on technical equipment, Dr. Barry O'Brien, Kerry Allen,

Johnathon Puddick, Dr. Chris Adams and Stacey Meyer. I know how lucky I was to have your expertise, here's hoping some of it rubbed off on me.

Thanks also to the Bright Future Scheme and Watson and Son for jointly providing me an Enterprise Scholarship which gave me so many opportunities. I couldn't have done it without this support.

Last but not least, thank you to my wonderful husband and kids. Charles never complained about the weekend visits to the lab, conference attendance or late nights spent reading and writing. He was always willing to discuss things I'm sure were not all that interesting to him or have even understood most of the time.

For my darling children, Max, Jemma, Alyssa and Samantha, the lovely art you created for my work surely made mine the most decorated desk in the lab. To Max and Jemma, I only realised how much of an impact my research had on you when the kindy teachers worried about you drawing blood cells and discussing microbes over lunch. To Alyssa and Sammi, you provided me with a deadline to finish by and plenty of distraction to stray from it! I love you all and hope you each get the opportunity to write your own book one day. This one is dedicated to you!

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Abbreviations

3-DG	3-deoxyglucosone
AGE	advanced glycation end-product
APS	ammonium persulfate
AU	arbitrary units
β_2M	beta-2 microglobulin
BCG	Bacille Calmetle Guerin – attenuated tuberculosis
BSA	bovine serum albumin
ACN	acetonitrile
CCL2	chemokine (C-C motif) ligand 2
CCL5	chemokine (C-C motif) ligand 5
CCD	charge-coupled device
CD14	cluster of differentiation 14
cDNA	complimentary DNA
CHCA	d-Cyano-4-hydroxycinnamic acid
ConA	Concanavilin A
COX	cyclo-oxygenase
CPM	carboxypeptidase M
CR	cystein-rich domain (of MR)
CRD	carbohydrate recognition domains
Da	daltons
DCFH	dichlorofluorescein
DCFH-DA	2'3'dichlorofluorescein diacetate
DDT	dithiothreitol

DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
dntps	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fc	receptor fragment crystallizable
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
RJ	royal jelly
FPLC	fast protein liquid chromatography
GFP	green fluorescent protein
GM CSF	granulocyte-macrophage colony-stimulating factor
GOI	gene of interest
HCgp-39	human cartilage group protein 39
HCL	hydrochloric acid
HEPES	buffering agent (4-(2-hydroethyl)-1-piperazineethanesulfonic acid)
HET-CAM	hen egg test-chorioallantoic membrane
IL-1	interleukin 1
IL-10	interleukin 10
IL-1ra	interleukin 1 receptor antagonist
IL-6	interleukin 6
IκB	inhibitor of kapa B
IκK	IκB kinase
LinReg	linear regression programme
LPS	lipopolysaccharide
M1	type 1 macrophage
M2	type 2 macrophage
MALDI	matrix assisted laser desorption ionisation
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MDC	myeloid dendritic cell
MGO	methylglyoxal

MR	mannose receptor
MRJP	major royal jelly protein
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
MW	molecular weight
MyPo	myeloperoxidase
N°	starting concentration
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOSAIDS	nonsteroidal anti-inflammatory drugs
OD	optical density
P13K	phosphatidylinositol 3-kinases
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood monocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PLCγ	phospholipase C-gamma
PMA	phorbol-12-myristate-13-acetate
PPR	pattern recognition receptor
Q-RT-PCR	quantitative real time PCR
RAGE	receptor for AGE
RANTES	regulated upon activation, normal T-cell expressed, and secreted
RNA	ribose□nucleic acid
ROS	reactive oxygen species
RPMI	Royal Park Memorial Institute
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SOD	superoxide-dismutase
SR	scavenger receptor
TAE	tris-acetate EDTA
Taq	enzyme isolated from *Thermus aquaticus*
TCA	trichloroacetic Acid

TE	tris EDTA
TEMED	N,N,N',N'-Tetramethyl-etylenediamine
THP-1	human acute monocytic cell line
TLR4	toll-like receptor 4
Tm	melting temperature
TNF-α	tumor necrosis factor alpha
TOF	time of flight
t-PA	tissue-type plasminogen activator
UV	ultraviolet
V/V	volume/volume
Vit-D3	1,25-dihydroxyvitamin D-3
W/V	weight/volume

Preamble

This thesis has been divided into separate chapters each containing an introduction, methods, results and discussion section. The references are found at the end of the thesis. A brief introduction (Chapter 1) and final discussion (Chapter 10) are presented but the main topics are introduced and discussed in each chapter.

Chapter 2 is focussed on characterising the THP-1 cell line used in the research requiring monocytes and macrophages.

Chapter 3 looks at pro- and anti-inflammatory cytokines expressed by THP-1 after stimulation by LPS, honey or a combination of these.

Chapter 4 identifies Manuka honey as having an anti-inflammatory effect in an *in vitro* assay measuring phagocytosis of latex particles by THP-1 cells.

Chapter 5 studies the effect of honey on ROS generated by THP-1 cells.

Chapter 6 identifies an anti-inflammatory component of Manuka honey.

Chapter 7 attempts to identify the receptor on THP-1 cells that the anti-inflammatory component of Manuka honey works on.

Chapter 8 looks at the fluorescence of Manuka honey and the effect of MGO on protein in honey.

Chapter 9 compares the phagocytosis assay with an *in vivo* HET-CAM assay for inflammation.

Chapter 1

Introduction and Literature review

1.1 Introduction

Honey has been used for thousands of years as a medicinal agent and dietary supplement. It has been prescribed for a wide range of ailments, most successfully in recent times, for wound healing (Zumla and Lulat 1989). Being considered a "natural cure" means that honey is not always accepted as a therapeutic product. What for some are seemingly outlandish claims have been made for honey, with talk of anti-cancer, anti-viral, anti-bacterial, anti-inflammatory and anti-fungal properties to name a few. Honey has been said to cure athlete's foot, jock itch, ringworm and nail fungus, bacterial contamination and skin infection in wounds and burns, including Methicillin-resistant *Staphylococcus aureus*, acne, stomach aches, stomach ulcers, sore throats, strep throat, gastritis, acid reflux disease, irritable bowel syndrome, heartburn, indigestion, cold and flu symptoms, cold sores, arthritis, gout, smallpox and even baldness. At least, these are the claims made by companies marketing honey products. All bee products, it seems, have important medicinal properties. Propolis, thought to be one of the earliest bee products used by man, provides relief of various conditions, including inflammation, viral diseases, ulcers, superficial burns or scalds, tumorous growth to name but only a few. Royal Jelly is another product with health-benefiting properties. While it is

accepted that it provides a good health supplement due to its free amino acids, sugars, minerals and vitamins, there is possibly no health complaint that Royal jelly has not been claimed to fix. Bee venom is also said to have important health benefits for the treatment of rheumatism and joint diseases, due to its anti-coagulant and anti-inflammatory properties.

Honey is registered for use in wound dressings, but for honey to be registered with regulatory authorities for sale in the pharmaceutical market as a therapeutic agent, components responsible for the various bioactivities must be identified to allow quality assurance and standardisation of products. This will also be needed to carry out the clinical trials necessary for therapeutic claims to be made when honey products are registered as medicines. Without standardised honey (*i.e.* honey with a known amount of the bioactive substance present) being used, a trial would only prove that the particular batch of honey being used had the observed effect. Ideally, the bioactive components of honey need to be chemically identified and assayed as known substances, but there needs to be at least a reliable bioassay available for each activity to be able to standardise honey for use in clinical trials.

Progress is slow in proving many of these declarations so most claims for honey remain unsubstantiated by modern science, but one of the best established is honey's anti-bacterial properties (Molan 2009). All honey appears to have, to some extent, anti-bacterial properties. Claims that Manuka honey from New Zealand in particular is exceptional for its wound healing abilities are commonly known and these claims have some acceptance in the medical community based on *in-vitro* studies (Allen, Molan *et al.* 1991). Derived from particular floral sources in Australia and New Zealand (*Leptospermum spp*) the actual anti-bacterial component of Manuka honey was, until recently, unidentified. The new discovery of methylglyoxal (MGO) in Manuka honey, however, has brought this "natural cure" back into the limelight (Adams, Boult *et al.* 2008; Mavric,

Wittmann *et al.* 2008). MGO has been shown to originate from dihydroxyacetone, which is present in the nectar of Manuka flowers in varying amounts (Adams, Manley-Harris *et al.* 2009). MGO is highly anti-bacterial. It has been demonstrated that the bioactivity of MGO, at the levels at which it is present in the honey, correlates to the non-peroxide activity (Adams, Boult *et al.* 2008; Mavric, Wittmann *et al.* 2008). No longer unidentified, Manuka honey's main anti-bacterial component can be measured and precisely listed on the jar, enabling better prescription for different ailments and perhaps putting to rest the disputed anti-bacterial property of Manuka honey. Further anti-bacterial agents may contribute to the total anti-bacterial activity of honey but have yet to be identified.

The purpose of the research in this thesis was to focus on another therapeutic aspect of honey, its anti-inflammatory properties. These have been observed in wound healing associated with deep and superficial burns and full thickness wounds (Molan 2002) and in other ailments such as inflammatory bowel conditions (Prakash, Medhi *et al*. 2008). Section 1.6.7 discusses the anti-inflammatory activity in more detail.

The investigations for this thesis are based upon the effect honey has on the inflammatory stage of tissue repair. Therefore this chapter reviews the active components of the immune system involved in wound healing and the therapeutic effects of honey.

1.2 The Immune system

The immune system plays an integral role in successful wound healing after injury (Park and Barbul, 2004). Park and Barbul (2004) state that the immune system is designed to defend the body against foreign or dangerous substances that invade it or to recognise damaged tissue due to wounds or burns. This is achieved with controlled inflammation. Inflammation (from the Latin, *inflammare*, to set on fire) is a complex biological response to harmful stimuli or irritants or to necrotic tissue. Inflammation is a protective attempt by the organism to remove the injurious stimuli or debris and to initiate the healing process.

The wound healing process can be subdivided into three consecutive and overlapping stages: inflammation, new tissue formation and remodelling (Gurtner, Werner *et al.* 2008). The transition through the stages is dependent on the maturation and differentiation of the main cell populations involved. Of interest to the study in this thesis is the inflammation stage. Substances which induce inflammation include micro-organisms (bacteria, viruses and fungi), parasites (such as worms), cancer cells and even transplanted organs and tissues.

The first line of defence against invaders is mechanical or physical barriers - the skin, conjunctiva of the eye cornea and membranes lining the respiratory, digestive, urinary and reproductive tracts. If a barrier is broken, for example open wounds or burns on skin, the risk of infection is increased. The first event after injury is the formation of a blood clot. To prevent blood loss, blood vessels constrict within seconds after wounding, platelets aggregate and clotting and complement cascades are activated (Midwood, Williams *et al.* 2004). The clot serves as a temporary protection of the wound. Platelets are one of the earliest sources of cytokines which mediate macrophage activation and chemotaxis to the wound site. TGF-β and PDGF are two of the cytokines released by platelets in the clot to act as chemo-attractants. Platelets also release thrombin which

stimulates the release of pro-inflammatory cytokines like IL-6 and IL-8 from endothelial cells which induce monocyte chemotaxis (Marin, Montero-Julian *et al.* 2001).

The next line of defence is initiated with the newly recruited white blood cells that travel through the bloodstream and into tissues searching for and immobilising micro-organisms. This defence has two parts: specific and non-specific immunity. These two parts interact, influencing each other directly or through substances that attract or activate other cells of the immune system, part of the mobilisation step of defence. There is a predictable sequence of immune cell migration into the wound which is vital to the regulation of the wound-healing process through the secretion of signalling molecules (Park and Barbul 2004) called cytokines, chemokines, and lipid derived mediators such as prostaglandins, leukotrienes and platelet-activating-factor amongst others. Such molecules are released from the cell sending the signal, cross the gap between cells by diffusion, and interact with specific receptors in another cell, triggering a response in that cell by activating a series of enzyme-controlled reactions which lead to changes inside the cell, rather than from physico-chemical effects on the plasma membrane or other membranes of the cell.

The term professional phagocytes can be used to describe both macrophages and neutrophils, as these are considered to have phagocytosis as their primary function. Professional phagocytes are essentially the main responding cells to the signalling molecules.

1.2.1 Neutrophils

Neutrophil granulocytes, generally referred to as neutrophils, are the most abundant type of white blood cell (~70% of all white blood cells) and form an integral part of the immune system. They are normally found in the blood

stream. However, during the acute phase of inflammation, particularly as a result of infection, neutrophils leave the vasculature and migrate to the site of infection by chemotaxis, becoming highly motile, and act within an hour of the insult (Gelderman *et al.* 1998). During an inflammatory response neutrophils are the first cells to infiltrate the wound. Their role is primarily phagocytosis and wound debridement. Within 24 hours neutrophils become the dominant leukocyte in the wound.

Unlike the longer-living macrophages, non-activated neutrophils in the circulation live for 4-10 hours and 1-2 days in the tissue upon activation from pathogenic stimuli (Rico, Ripamonti *et al.* 2002). They only execute one phagocytic event, expending all their glucose content in an extremely vigorous "respiratory burst". The respiratory burst involves the activation of an NADPH oxidase enzyme, which produces large quantities of superoxide, a reactive oxygen species. Superoxide is toxic to pathogens but can also lead to tissue damage, prolonging the inflammatory event, and can also be converted to hydrogen peroxide (H_2O_2) which gives rise to very reactive hydroxyl radicals (•OH) (Cathcart 2004). Neutrophils communicate the inflammatory status to other immune cells using ROS, cytokines and chemokines, recruiting them to the site. Although they contribute to decreasing the likelihood of infection in the wound, neutrophils are not paramount to the overall process of wound healing (Butterfield, Best *et al.* 2006).

1.2.2 Monocytes and macrophages

Macrophages develop from a type of white blood cell called monocytes, after monocytes are signalled to leave the bloodstream to enter tissues (Silva, de Assis *et al.* 2002). These transformed monocytes, now termed macrophages, migrate into the wound 48 to 96 hours after injury and become the predominant cell population before fibroblast migration and replication (Park and Barbul 2004).

Over a period of approximately eight hours, monocytes greatly enlarge and develop into macrophages. Macrophages stay in the tissues and ingest foreign and damaged cells, and are important in participating in and concluding the inflammatory and debridement process. Impaired wound debridement and fibroplasia occur in guinea pigs treated with anti-macrophage serum and steroids to fully deplete circulating monocytes (Leibovich and Ross 1975).

Macrophages are phagocytic and secretory cells, vital to the regulation of immune responses and the development of inflammation. After digesting a pathogen, a macrophage will present the antigen of the pathogen to a corresponding helper T cell. The presentation is done by integrating it into the cell membrane and displaying it to other T cells. Eventually the antigen presentation to the T cells results in the production of antibodies by the B cells that attach to the antigens of other circulating identical pathogens, making it easier for macrophages to adhere to and phagocytose the pathogen. This is a process termed opsonisation. In some cases, pathogens are very resistant to adhesion by the macrophages. Some receptors on the macrophages are believed to function in innate immunity by recognizing unopsonised microorganisms bearing terminal mannose, fucose, *N*-acetylglucosamine, or glucose residues on surface oligosaccharides (Celli and Finlay 2002). Most mammalian cells and serum glycoproteins do not express terminal mannose-bearing carbohydrate. Macrophages have evolved receptors that recognize and directly bind these terminal residues. Engagement of these receptors is termed opsonin-independent phagocytosis (Celli and Finlay 2002). Relevant to the research in this thesis is the stimulation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria which induces the release of critical pro-inflammatory cytokines that are necessary to activate potent immune responses (Lu, Yeh *et al.* 2008). TLR4 plays a pivotal role in the induction of inflammatory responses. TLR4 activation by LPS is achieved by the coordinate and sequential action of three other proteins, LPS binding protein,

CD14 and MD-2 receptors, that bind LPS and present it to TLR4 by forming the activated (TLR4-MD-2-LPS)(2) complex (Peri and Piazza 2011).

A major contribution of macrophages to wound healing is the secretion of cytokines, complement proteins and growth factors. These substances activate and recruit other cells and regulate fibroblast chemotaxis, proliferation and collagen synthesis (Wahl 1985). Macrophages also express inducible nitric oxide synthase (iNOS) and are a source of nitric oxide production in the early phase of wound healing (Schaffer, Tantry *et al.* 1997; Reichner, Meszaros *et al.* 1999; Lee, Efron *et al.* 2001). The importance of nitric oxide in this process surpasses its antimicrobial functions (Efron, Most *et al.* 2000). In murine models, inhibition of iNOS significantly impairs re-epithelialisation (Stallmeyer, Kampfer *et al.* 1999), reduces the rate of closure of full thickness wounds (Yamasaki, Edington *et al.* 1998), and decreases collagen deposition (Schaffer, Tantry *et al.* 1999).

Occasionally, inflammation is not controlled and macrophages especially are responsible for chronic inflammation developing. Macrophages produce large quantities of reactive oxygen species (ROS) upon activation, due to the increased energy requirements for membrane reorganization required for actin polymerisation during phagocytosis (Castellano, Chavrier *et al.* 2001; Novo and Parola 2008) and produce it for directly killing pathogens (Slauch 2011). Many signalling pathways that have a ROS signal component can be overloaded, with excess ROS upsetting the balance and control of feedback loops, self-amplifying the inflammatory response by oxidative activation of nuclear transcription factor NF-κB, which then promotes the production of pro-inflammatory cytokines from leukocytes (Iles and Forman 2002).

1.2.3 Involvement of macrophages in symptoms of diseases

Due to their role in phagocytosis, macrophages are involved in many diseases of the immune system. Two cell populations of macrophages perform multiple functions at various times in the inflammatory process. The pro-inflammatory classically activated macrophages (M1) are most prominent within necrotic tissue as early as 1 day after neutrophil invasion and are activated by the pro-inflammatory cytokines, such as TNF-α and IL-1β. This cell signalling serves to magnify the macrophage response in the tissue but may also recruit additional neutrophils, resulting in a positive pro-inflammatory feedback loop (Mantovani, Sica *et al.* 2004).

Once pro-inflammatory macrophages are activated, they contribute to the exacerbation of inflammation by producing and releasing more than 100 substances, including pro-inflammatory cytokines such as IL-1β and prostaglandins (Scott, Khan *et al.* 2004).

The alternately activated M2 macrophages appear during the latter stages of inflammation and can be divided into three sub-populations: M2a macrophages which promote a Th2 type of inflammation resulting in increased IgE as observed in allergy and parasite immunity; M2b macrophages, that promote Th2 inflammation and bear some immune-regulation properties; and the deactivated macrophages, M2c which are able to control the inflammation and are implicated in tissue remodelling (Mantovani, Sica *et al.* 2004). The primary role for M2 macrophages is tissue repair through cell signalling and cytokine production, releasing a series of growth factors such as fibroblast growth factor, insulin-like growth factor, and transforming growth factor-β1, essential for tissue repair and regeneration (Butterfield, Best *et al.* 2006).

Pro-inflammatory macrophages participate in the formation of granulomas, inflammatory lesions that may be associated with and/or reflect chronic inflammation (Levine, Smith *et al.* 2005). Macrophages are the predominant

cells involved in creating the progressive plaque lesions of atherosclerosis (Tabas 2009).

1.3 Immunity

To be able to eradicate invaders of the body, the immune system must first recognise them. Distinguishing self from non-self is important here. The immune system can make this distinction as all cells have identification molecules on their surface (antigens). Antigens may be contained within or on bacteria, viruses, other micro-organisms or cancer cells. Antigens may also exist on their own, for example, as pollen or food molecules. A normal immune response consists of recognising a foreign antigen or damaged tissue, mobilising forces to defend against it and attacking it. The immune system can make a distinction between foreign invading antigens and the body's own familiar antigens. When a foreign antigen is found, the immune system attacks that cell. B and T lymphocytes need help from other cells in the immune system to recognise invaders. Two immune categories are at work here, specific and non-specific.

1.3.1 Specific immunity

Specific immunity is often sub-divided into two major types depending on how the immunity was introduced. Naturally acquired immunity which occurs through contact with a disease causing agent, when the contact was not deliberate and artificially acquired immunity which develops only through deliberate actions such as vaccination (Pulendran and Ahmed 2006). Both naturally and artificially acquired immunity can be further subdivided depending on whether immunity is induced in the host or passively transferred from an immune host. Passive immunity is acquired through transfer of antibodies or activated T-cells from an immune host, and is short lived, usually lasting only a few months, for example the transfer of maternal antibodies across the placenta

to the fetus (Keller and Stiehm 2000). Active immunity is induced in the host itself by antigen, and lasts much longer, sometimes life-long (Pulendran and Ahmed 2006). The specific (or adaptive) immune system acts as a second line of defence to the innate immune system and affords protection against re-exposure to the same pathogen. Specific immunity takes time to develop after lymphocytes are exposed to a new antigen. However, because a memory is formed, subsequent responses to a previously encountered antigen are more rapid than those generated by an un-encountered antigen. Lymphocytes enable the body to remember antigens and to distinguish self from non-self. This lymphocyte memory can continue for years or even decades (Schmidlin, Diehl *et al.* 2009) preventing certain diseases from recurring. Lymphocytes include B lymphocytes, T lymphocytes and natural killer cells (involved in non-specific immunity).

1.3.1.1 B-Lymphocytes

The abbreviation "B", in B cell, comes from the 'bursa of fabricius' in birds, where they mature. In mammals, immature B cells are formed in the bone marrow, which is used as a backronym for the cells' name. After reaching the IgM+ immature stage in the bone marrow, these immature B cells migrate to the spleen, where they are called transitional B cells, and some of these cells differentiate into mature B lymphocytes (Allman, Srivastava *et al.* 2004). B-lymphocytes have receptors for specific antigens recognition. When an antigen attaches to a receptor on a B-lymphocyte, the B-lymphocyte is stimulated to turn into a plasma cell which, in turn, produces antibodies. These antibodies are specific to the antigen that stimulated their production. Based solely on histological studies the role of these cells is unclear and, given the absence of activating factors, B-lymphocytes are unlikely to play a significant role in the regulation of wound healing (Park and Barbul 2004).

1.3.1.2 T-Lymphocytes

T-Lymphocytes are white blood cells that are made in the bone marrow but matured in the thymus gland. They circulate in the blood stream and can attack particular or abnormal cells. They can be distinguished from other lymphocyte types by the presence of a special receptor on their cell surface called T cell receptors (TCR). The T cell receptor exists as a complex of several proteins. The actual T cell receptor is composed of two separate peptide chains, which are produced from the independent genes for T cell receptor alpha and beta (TCRα and TCRβ). The other proteins in the complex are the four CD3 proteins; CD3γ, CD3δ and two CD3ε which associate with the TCR. T-lymphocytes migrate into a wound after inflammatory cells and macrophages, on the fifth day following injury and peak at day seven (Fishel, Barbul *et al.* 1987). Several different subsets of T cells have been discovered, each with a distinct function:

1. Cytotoxic T cells (T_C cells) which recognise and destroy foreign cells by injecting enzymes into the abnormal cell. They are also known as $CD8^+$ T cells since they express the CD8 glycoprotein at their surface (Walsh, Gleeson *et al.* 2011).

2. T-helper cells (T_H cells) which help B-lymphocytes recognise and produce antibodies against foreign cells. T_H cells assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and B memory cells, and activation of cytotoxic T cells and macrophages, among other functions. These cells can differentiate into one of several subtypes, including T_H1, T_H2, T_H3, T_H17 or T_{FH} which secrete different cytokines to facilitate a different type of immune response (Walsh, Gleeson *et al.* 2011).

3. Regulatory T cells (T_{reg} cells), are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus (Walsh, Gleeson *et al.* 2011).

4. Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has been resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise two subtypes: central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells) (Walsh, Gleeson *et al.* 2011).

T-lymphocytes that develop without the ability to distinguish self from non-self are responsible for auto-immune disorders in which the body's own tissues are attacked.

1.3.2 Non specific immunity

Non-specific (innate) immunity is present at birth. Non-specific immunity components treat all the foreign substances they encounter in much the same way. The white blood cells involved in non-specific immunity are monocytes, neutrophils, eosinophils, basophils and natural killer cells. Each type has a slightly different function. The complement system and cytokines also participate in non-specific immunity.

1.3.2.1 Eosinophils

Eosinophils can ingest bacteria and other foreign cells. However they are less active against bacteria than are neutrophils and macrophages. Their main

function may be to attach to and immobilize invaders, allowing the macrophages and neutrophils to destroy them. Eosinophils, like neutrophils, freshly isolated from blood, are capable of responding vigorously to chemokines (Ponath, Qin *et al.* 1996). Following activation the effector functions of eosinphils include production of growth factors, ROS and cytokines. Eosinophils also produce RNases, contained within their granules which combat virus infections and also play a role in fibrin removal during inflammation (Rothenberg and Hogan 2006).

1.3.2.2 Basophil granulocytes

Basophil granulocytes, sometimes referred to as basophils, are the least common of the granulocytes, representing about 0.01% to 0.3% of circulating white blood cells. Basophils do not ingest foreign cells. They contain granules that release the vasodilator histamine which promotes blood flow to tissues and produce substances that attract neutrophils and eosinophils to the wound site. Basophils also contain the anticoagulant heparin, which prevents blood from clotting too quickly. Basophils have been shown to constitute a significant source of cytokines (IL-4 and IL-13) which play a major role in the pathogenesis of allergic disease, and may modulate T-helper 2-type inflammation at the level of T cell/dendritic cell interactions (Schroeder and Frederick 2009).

1.3.2.3 Natural killer cells

Natural killer cells are lymphocytes that attach to receptors on foreign cells and release enzymes and other substances that damage the outer cell membrane of the foreign cell. They are often the first type of cell to destroy invading cells as they are ready to kill as soon as they are formed. Natural killer cells also produce cytokines that regulate some of the functions of T lymphocytes, B lymphocytes and macrophages (Park and Barbul 2004).

1.3.2.4 Mast cells

Mast cells express critical effector functions in classic IgE-associated allergic disorders, but also play important roles in host defence against parasites and bacteria. Mast cells can contribute to host defence in the context of either acquired or innate immune responses through the release of a myriad of pro-inflammatory and immunoregulatory molecules and the expression of a wide spectrum of surface receptors for cytokines and chemokines. Mast cells also exert distinct non-immunological functions, playing a relevant role in tissue homeostasis, remodeling and fibrosis as well as in the processes of tissue angiogenesis (Park and Barbul 2004).

1.3.2.5 The complement system

The complement system is one of the major mechanisms by which pathogen recognition is converted into an effective host defence against initial infection. Complement is a system of more than 30 plasma proteins that can be activated directly by pathogens or indirectly by pathogen-bound antibody, leading to a cascade of reactions that occurs on the surface of pathogens and generates active components with various effector functions. Complement proteins can work as chemo-attractants, kill bacteria directly (cell lysis) or help destroy bacteria by attaching to them making them easier to detect for macrophages and neutrophils (opsonisation). They can also cause bacteria to clump together, and neutralise viruses by altering the molecular structure of the virus (Mastellos, Morikis *et al.* 2003). Three biochemical pathways activate the complement system: the classical complement pathway, the alternative complement pathway, and the mannose-binding lectin pathway.

The classical pathway is triggered by antigen-bound antibody molecules, IgG or IgM. The initial enzyme, C1, is a complex formed through a calcium-dependent association between two reversibly interacting subunits, C1q and C1 (C1qr2s2) (Kishore and Reid 2000). It is the binding of a specific part of the antibody

molecule to the C1 component that initiates this pathway (Mastellos, Morikis *et al.* 2003).

The alternative pathway is triggered by spontaneous hydrolysis of the protein complement-component-3 (C3) to form protein fragments C3a and C3b. It does not rely on pathogen-binding antibodies like the other pathways. Macrophages and neutrophils possess receptors for C3b, so cells coated with C3b are targeted for phagocytosis (opsonization) (Janeway, Travers *et al.* 2001).

The lectin pathway is homologous to the classical pathway, but with the opsonin, mannose-binding lectin (MBL), and ficolins, instead of C1q. This pathway is activated by the binding of mannose-binding lectin to mannose residues on the pathogen surface, which activates the MBL-associated serine proteases, MASP-1, and MASP-2 (very similar to C1r and C1s, respectively), which can then split C4 into C4a and C4b and C2 into C2a and C2b. C4b and C2b then bind together to form the C3-convertase, as in the classical pathway (Janeway, Travers *et al.* 2001).

1.3.3 Dendritic cells

The main function of dendritic cells is antigen presentation to other cells of the immune system bridging innate and adaptive immunity. Two main types of dendritic cells have been identified, myeloid or plasmacytoid. The myeloid dendritic cell is similar to a monocyte and is made up of at least two subsets: (1) the more common myeloid dendritic cell-1, which is a major stimulator of T cells and (2) the extremely rare myeloid dendritic cell-2, which may have a function in fighting wound infection (Schuurhuis, Fu *et al.* 2006). Once activated, they migrate to the lymph nodes where they interact with T cells and B cells to initiate the adaptive immune response. At certain development stages they grow branched projections, the dendrites, that give the cell its name (δένδρον or déndron being Greek for "tree". Dendritic cells produce cytokine IL-12, amongst others, a signal that helps send naive CD4 T cells towards a Th1 phenotype

activating the immune system against the antigens which the dendritic cell presents on its surface (Schuurhuis, Fu *et al.* 2006).

1.4 Cytokines

Cytokines are messengers of the immune system. White blood cells and certain other cells of the immune system produce cytokines when an antigen is detected. There are many types which affect different parts of the immune system. Cytokines may stimulate or inactivate activity of the immune system, attract cells to damaged tissues (chemokines), or help end the immune response. Chemokines (chemotactic cytokines) are classified according to shared structural characteristics such as small size (they are all approximately 8-10 kilodaltons in size), and the presence of four cysteine residues in conserved locations that are key to forming their 3-dimensional shape. Some chemokines are considered pro-inflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection, while others are considered homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development (Laing and Secombes 2004).

1.4.1 Interleukins

Interleukins are a group of cytokines that were first seen to be expressed by white blood cells (leukocytes, hence the *leukin*) as a means of communication (*inter*-). It has since been found that a wide variety cells produce interleukins. The function of the immune system depends, in a large part, on interleukins, and rare deficiencies of a number of them have been described, all featuring auto-immune diseases or immune deficiency. Of interest to this study are the interleukins IL-1β and IL-6 secreted by macrophages, and IL-8 involved in neutrophil chemotaxis and IL-10 which inhibits cytokine production in Th1 cells. It is important to note that some cytokines have both pro- and anti-inflammatory effects. The net effect of an inflammatory response is determined by the

balance between pro-inflammatory cytokines and anti-inflammatory cytokines. The cytokine TNF-α has both pro- and anti-inflammatory properties. It has been shown that TNF-α induces the release of the type II IL-1, which represents a unique pathway of negative regulation of the IL-1 system resulting in a net anti-inflammatory effect (Orlando, Matteucci *et al.* 1997).

1.4.2 Anti-chemokine therapy

CCL5 and CCL2 (small cytokines belonging to the CC chemokine family) are mediators of acute inflammatory responses (Castellani, De Lutiis *et al.* 2010). CCL5 is an 8kDa protein classified as a chemokine also known as RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted). CCL5 is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites in conjunction with particular cytokines (*i.e.*, IL-2 and IFN-γ) that are released by T cells. CCL5 also induces the proliferation and activation of certain natural-killer (NK) cells to form CHAK (CC-Chemokine-activated killer) cells. CCL5 is strongly induced by viral and bacterial infections and plays a role in allergic diseases, in exacerbation of asthma, in interstitial pneumonia, in allograft rejection and in cancers. CCL2 is also known as monocyte chemotactic protein-1 (MCP-1) and small inducible cytokine A2. CCL2 recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury, infection, and inflammation (Castellani, De Lutiis *et al.* 2010).

These chemokines have become targets for diagnostic procedures and therapeutic intervention, for example, administration of anti-CCL2 antibodies in a model of glomerulonephritis have shown reduced macrophage and T cell infiltration, as well as reduced scarring and renal impairment (Castellani, Bhattacharya *et al.* 2007). Treatment with the anti-CCL5 antibodies results in reduced circulating levels of chemokines and a reduction of neutrophil and macrophage infiltration in models of atherosclerosis (Montecucco, Braunersreuther *et al.* 2011).

1.5 Inflammation

Without inflammation, wounds and infections would never heal. Similarly, progressive destruction of the tissue would compromise the survival of the organism. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the affected area. A cascade of biochemical events propagates the inflammatory response involving the local vascular system, the immune system, and various cells within the injured tissue. Early on in this cascade, phagocytic cells such as macrophages and neutrophils, recognise and remove the inflammatory stimuli. Once the stimulus is removed, the inflammation subsides.

However, excessive and prolonged (chronic) inflammation in wounds, when the stimulus is not removed, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. ROS released by phagocytic cells, attempting to remove the initial inflammatory stimuli, play a major role in chronic inflammation by generating harmful feedback loops preventing the resolution of inflammation. Chronic inflammation can cause pain, ulceration, scarring and fibrosis and result in non-healing wounds (Singer and Clark 1999; Menke, Ward *et al.* 2007). It is for that reason that inflammation is normally closely regulated by the body. Anti-inflammatory agents therefore are of great importance to medicine.

1.5.1 Anti-inflammatory agents

Anti-inflammatory refers to the property of a substance or treatment that reduces inflammation, remedying pain, as opposed to opioids, which affect the central nervous system. Several main types of anti-inflammatory agents used to control inflammation with are briefly discussed here. As mentioned previously,

wounds must progress through three distinct phases of healing: acute, proliferative, and remodelling, yet these wound healing processes can be vulnerable to inhibition by drug therapy and therefore new anti-inflammatory agents that do not inhibit these processes are being sought.

1.5.1.1 Corticosteriods

Corticosteroids reduce inflammation or swelling by binding to glucocorticoid receptors. Corticosteroids can inhibit fibroplasia, vascular proliferation, and wound contraction in soft tissue healing (Karukonda, Flynn *et al.* 2000).

1.5.1.2 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDS) are agents/analgesics such as aspirin and ibuprofen. These medications relieve pain by blocking the production of pain-signaling molecules. One of the steps in this pathway involves certain types of cyclo-oxygenase (COX) enzymes. By blocking the COX-2 type, NSAIDs relieve pain felt in joints, muscles, and other soft tissues (McDaid, Maund *et al.* 2010). NSAIDS and COX-2-selective inhibitors may impair the acute phase of healing and compromise the normal process of wound healing (Karukonda, Flynn *et al.* 2000).

1.5.1.3 Herbs

Certain herbs have been investigated extensively for their anti-inflammatory qualities based on age old remedies, including Harpagophytum, hyssop, ginger, turmeric (Aggarwal 2010), Arnica montana (Zhao, Lee *et al.*), and willow bark, which contains salicylic acid, the active ingredient in aspirin (Jones 2011).

1.5.1.4 Immune Selective Anti-Inflammatory Derivatives

The immune selective anti-inflammatory derivatives (ImSAIDs) represent a new category of anti-inflammatory and are unrelated to steroid hormones or non-steroidal anti-inflammatory agents. ImSAIDs have diverse biological properties, including anti-inflammatory properties. ImSAIDs work by altering the activation and migration of leukocytes responsible for amplifying the inflammatory response (Bao, John *et al.* 2006).

1.6 Honey

Honey is a commercial product of the bee hive, along with propolis, royal jelly and bee venom. Made from a wide range of nectar gathered and processed by the honeybee (*Apis mellifera)* its content varies greatly but generally consists of sugars, vitamins, minerals and bee proteins along with phytochemicals that act as antioxidants (Gheldof, Wang *et al.* 2002). Compelling research has been presented on the healing properties of honey with its antibacterial and anti-inflammatory activities.

1.6.1 Anti-bacterial activity of honey

Infection of wounds by micro-organisms have been successfully cleared by honey, demonstrating its anti-bacterial activities (Molan 2009). Even antibiotic resistant strains of bacteria have been successfully cleared from wounds (Vardi, Barzilay *et al.* 1998; Dunford, Cooper *et al.* 2000a). Briefly the known antibacterial properties of honey are as follows:

1.6.1.1 Osmotic effects

The high sugar content of honey inhibits microbial growth. The low water content (15-21%) deprives bacteria of the water needed for cells to function

(Willix, Molan *et al.*, 1992; Chirife, Scarmato *et al.*, 1982), and can draw water out of bacterial cells by osmosis (Manjo, 1975). This has been demonstrated by using artificial honey solutions containing the same proportions of sugars as honey, and measuring bacterial inhibition (Postmes, van den Bogaard *et al.*, 1993).

1.6.1.2 Hydrogen peroxide

Hydrogen peroxide is a well-known antibacterial molecule and a key component in honey (Weston, 2002; Molan, 1992; Bang, Buntting *et al.*, 2003). Honey contains the enzyme glucose oxidase which is secreted into the nectar by bees when honey is made. It is this enzyme that produces hydrogen peroxide from glucose and oxygen (White, 1975).

1.6.1.3 Acidity

During the ripening of nectar into honey, the action of glucose oxidase also produces gluconolactone/gluconic acid which gives honey its characteristically low pH of 3.2-4.5 (White, 1975). This means honey has a lower pH than the tolerated values for survival by many wound-infecting bacteria (*e.g. Pseudomonas aeruginosa*, pH 4.4, *Escherichia coli*, 4.3, *Salmonella sp.* 4.0) (Molan 1992 a).

1.6.1.4 Non-peroxide anti-bacterial components

The non-peroxide antibacterial activity (NPA) found in Manuka honey has proven to be a very effective antibacterial component and the major part of this activity has been attributed to the methyglyoxal content (MGO) (Adams, Boult *et al.* 2008; Mavric, Wittmann *et al.* 2008). Manuka honey also has very high amounts of MGO, ranging from 38 to 828 mg/kg, which is up to 100-fold higher compared with non-Manuka honeys (Adams, Boult *et al.* 2008). Other components have been isolated from honey with anti-bacterial properties (*e.g.* chrysin,

pinobanksin and pinocembrin (Marcucci, Ferreres *et al.* 2001)) but are thought to have ineffective low concentrations in honey.

1.6.2 Variation between honey types

There is a demonstrated variation in antibacterial activity between honeys obtained from different floral origins and geographical sources that confirm different honey types will have different antibacterial properties (Allen, Molan *et al.*, 1991; Farouk, Hassan *et al.*, 1988; Molan, 1992b). For example, Manuka honey is high in NPA but low in hydrogen peroxide activity whereas Pasture honey has the opposite profile (Willix, 1992).

Variation also exists in the antioxidant content of honey. Antioxidants, which are abundant in natural honey, are free-radical scavengers that either reduce the formation of or neutralize free radicals and determined to be due to the phenolic compounds in honey (Kishore, Halim *et al*. 2011; Tonks, Cooper *et al*. 2001).

Honeys immuno-stimulatory activity has also been determined to be variable, most notably by increasing inflammatory cytokine release in monocytes (Tonks, Cooper *et al.* 2003).

1.6.3 Honey promotes tissue repair as a cell growth stimulant

There is existing evidence for honey as a cell growth stimulant (Lusby *et al.*, 2002; Wood *et al.*, 1997). Though the mechanisms have been speculated, they have not been identified. Recent relevant work showed that 1% Manuka honey concentrations stimulated epithelial cell division in a respiratory epithelial cell line model (Harcourt, 2005). Increasing concentrations beyond this had no effect. Adding to this, tumor necrosis factor α (TNF-α), IL-1β and IL-6 cytokine

release is increased following stimulation with honey and these cytokines have been determined to promote the healing process in wounds (Tonks, Cooper *et al.* 2003).

Honey has also been demonstrated to: promote the development of connective tissue around regenerating blood vessels (Subrahmanyam, 1998; Efem, 1993; Efem, 1988), stimulate formation of epithelial cover (Misirlioglu, Eroglu *et al.*, 2003; Topham, 2002), stimulate collagen synthesis and stimulate new development of blood vessels to increase oxygenation (Kumar, Sharma *et al.*, 1993). Nutrient supply may also be increased along with the osmotic outflow of lymph (Lusby, Coombes *et al.*, 2002). Compared with sugar alone, it appears that honey has a superior ability to promote re-epithelialisation (Bose, 1982).

1.6.4 Honey as an immune stimulant

There is evidence that honey may reduce infection by stimulating the immune system. Studies by Arbuharfeil *et al.* (1999) have shown that honey stimulates the proliferation of B and T lymphocytes and activates neutrophils. Al-Waili (2003) showed that oral ingestion of honey increased the number of circulating monocytes and lymphocytes in blood. Honey has been found to stimulate monocytes *in vitro* to release TNF-α (Tonks, Cooper *et al.* 2001). This was determined to be due to a 5.8kD component in honey which acts via the TLR-4 receptor (Tonks, Dudley *et al.* 2007). Honey has also been found to stimulate the release of IL-1, IL-6 (Tonks, Cooper *et al.* 2003) activating the immune response.

1.6.5 Debriding action

It has been documented that honey provides a rapid debriding action (removal of attached pus and dead cells) on wounds (Molan 2009). It has been suggested that this is due to the flushing effect that occurs as lymph is drawn out by the osmotic action of sugars (Topham, 2002; Molan 2009), although it has also been

suggested that it may be due to the stimulation of proteolysis (Molan 2009). This osmotic activity also lifts dead tissue from the wound bed, eliminating the need for surgical debridement. Debridement of the wound is crucial as a contaminated wound cannot heal due to the growth of bacteria in the dead tissue (McInerney, 1990; Cavanagh, Beazley *et al.*, 1970).

1.6.6 Reduction of scar formation

Honey also provides a moist environment essential for the healing of wounds as protein-digesting enzymes function more effectively (Molan, 2001; Bradley, Cullum *et al.*, 1999; Archer, Barnett *et al.*, 1990). In these moist conditions fibroblast proliferation is also optimised as is keratinocyte migration across the wound bed to restore epithelial cover. This reduces the likelihood of a scar forming (Misirlioglu, Eroglu *et al.*, 2003; Niessen, Spauwen *et al.*, 1999). It has been observed (Topham, 2002) that honey is associated with scar-free healing.

1.6.7 Anti-inflammatory evidence

An anti-inflammatory effect of honey has been observed clinically in numerous reports where it has been applied to wounds. Localised swelling, redness, pain and heat associated with inflammation were reduced (Molan, 1999). In clinical studies there have been direct evidence of anti-inflammatory activity obtained in the form of decreased levels of malondialdehyde (Subrahmanyam, Sahapure *et al.* 2001) and histological observation of reduced numbers of inflammatory cells present in biopsy samples (Subrahmanyam 1998) in clinical trials where burns were dressed with honey compared with silver sulfadiazine. Microscopic examination confirmed that the application of honey to tissues significantly reduced the leukocyte count (Postmes, Bosch *et al.*, 1997). In studies by Postmes and Bosch *et al.* (1997); Kumar and Sharma *et al.* (1993) and Oryan and Zaker *et al.* (1998) reduction of inflammation in wounds where honey was applied was taken as evidence that honey had a direct anti-inflammatory effect.

As infection was not present, the observed anti-inflammatory effect could not have been due to any removal of bacteria promoting inflammation.

Though the process by which honey acts as an anti-inflammatory agent has yet to be demonstrated, processes have been speculated such as the possibility that honey inhibits the prostaglandin synthesis which is often responsible for the observed characteristic heat, itchiness and pain associated with inflammation (Kassim, Achoui *et al.* 2010).

Hydrogen peroxide has been proven to stimulate inflammation in separate studies by Reth (2002) where it diffused through cell membranes to enter the cell nucleus. Honey has been shown to reduce the ROS levels (Tonks, Cooper *et al.* 2001) promoting wound healing. The antioxidant content of honey may be partially responsible for the observed anti-inflammatory effects. The antioxidants in honey were hypothesised to be responsible for inhibition of ROS production by activated human PMNs, (van den Berg, van den Worm *et al.* 2008).

1.7 Research aims of this thesis

The aim for this thesis was to create an *in vitro* assay to measure the anti-inflammatory effect of honey and to characterise this activity. Initially cytokine release was studied as it has been reported that at a 1% concentration, honey is immuno-stimulatory increasing the levels of TNF-α, IL-1β and IL-6 (Tonks, Cooper *et al.* 2001, 2003). A wider range of cytokines and growth factors were investigated including the ones used in previous studies: IL-1β, IL-6, IL-10, IL-1ra, TGF-β, PDGF, and TNF-α. Standard PCR and real time quantitative PCR was used with LPS stimulated THP-1 cells.

The ROS production from activated monocytes was also studied. Honey was found to reduce ROS levels (Harcourt 2005; Tonks, Dudley *et al.* 2007) and this

was determined to be due to the antioxidants in honey. To discover if this was the only reason for ROS reduction, an assay was created to measure the effect of honey on phagocytosis of fluorescent latex beads using LPS stimulated immunocompetent THP-1 cells. As phagocytosis produces ROS, this assay was used in conjunction with a method of measuring the ROS release from actively phagocytising macrophages. Observations were made microscopically to determine bead uptake.

To identify whether the active component in honey was widespread amongst all honey varieties, a honey survey was undertaken, assaying different honey varieties using the phagocytosis assay in conjunction with the NPA and other relevant qualities. Correlation was sought between the variables for each honey variety to determine if there was an indicator for the anti-inflammatory activity in honey.

Next, the component in honey responsible for the observed activity on phagocytic cells was isolated and identified using chromatography and matrix-assisted laser desorption/ionization (MALDI) methods. A mechanism of action of honey was also sought, focusing on the receptor that the active component in honey binds to.

An *in vivo* assay was considered important to the anti-inflammatory research of honey. Therefore the anti-inflammatory properties of honey were further investigated using the *in vivo* assay the hen egg chorioallantoic membrane test (HET-CAM) which is a commonly used assay for measuring the anti-inflammatory properties of an agent against irritants such as SDS.

Chapter 2

Characterising THP-1

2.1 Summary

This chapter covers preliminary investigations of the cell line and treatments used throughout the research contributing to the thesis. Several methods were utilised to characterise the cell line.

The monocyte cell line THP-1 and three activating cell treatments were investigated. Phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS) and 1,25-dihydroxyvitamin D-3 (Vit-D3) were all used to activate the monocyte cells to generate active macrophages. These three activators were compared for their effect on phenotype according to adherence, loss of proliferation, cell shape, phagocytosis of latex particles and expression of two macrophage markers, human cartilage group protein-39 (HCgp-39) and carboxypeptidase M (CPM), so that the most effective and efficient treatment could be selected for further research.

LPS was selected for the main activator in further research due to its quick activation time, low mortality rate and the ability to produce a highly active and phagocytic macrophage.

In addition it has been proposed that a previously unknown state of activation can be achieved with THP-1. Parts of this research were presented at the 2008 QMB conference in the form of a poster.

2.2 Introduction

The THP-1 human monocyte/macrophage cell line, commonly used by researchers to study the response to LPS at various stages in the monocyte/macrophage differentiation process, was chosen for the study. THP-1 cells have been used in numerous studies investigating cytokine expression and phagocytosis.

The THP-1 cell line was cultured from the blood of a one-year-old-boy with acute monocytic leukemia (Tsuchiya, Yamabe *et al.* 1980). The monocytic nature of the cell line was characterized by the presence of alpha-naphthyl butyrate esterase activities which could be inhibited by sodium fluoride, lysozyme production, the phagocytosis of latex particles and sensitized sheep erythrocytes and the ability to restore T-lymphocyte response to Concanavalin A (Con A). This indicated that THP-1 was a leukemia cell line with distinct monocytic features. During culture, THP-1 maintained these monocytic characteristics for over 14 months, meeting the requirements for banking a new cell line, and subsequently the cell line was submitted to cell banks (ATCC, TIB-202) for future research.

THP-1 cells have previously been shown to be an appropriate model for studying LPS-induced changes in gene expression. Though THP-1 cells are transformed and immortalised, their LPS-induced gene expression signature remains very similar to primary monocytes (Sharif, Bolshakov *et al.* 2007). Sharif *et al.* used a focussed microarray strategy and real-time PCR to characterise and compare the response to LPS in THP-1 cell lines and human PBMC derived macrophages. They found a close correlation between THP-1 cells and PBMC-derived macrophages, suggesting they provide a good model system.

Research since then has shown THP-1 can differentiate along the monocytic lineage following exposure to a range of activators, most commonly LPS (Megyeri, Issekutz *et al.* 1990), PMA, and Vit-D3 (Schwende, Fitzke *et al.* 1996) forming macrophage-like cells inducing inflammatory responses such as release of cytokines, ROS and promoting cell chemotaxis towards them.

As mentioned previously, macrophages are central to inflammation and wound healing. They can encourage chronic inflammation by producing inflammatory cytokines and reactive oxygen intermediates that cause tissue damage and create feedback loops amplifying the inflammation. Macrophages are responsible for the generation of bioactive substances, orchestrating the complex processes of cellular proliferation and functional tissue regeneration within wounds. Specific proteins produced by macrophages have a variety of functions most importantly, 1) chemo-attractants that recruit and activate additional macrophages at the site of injury, 2) growth factors that promote cellular proliferation and protein synthesis, 3) proteases and extra-cellular matrix molecules, and 4) factors that may restrain tissue growth once repair is completed (DiPietro 1995).

Release of cytokines by monocytes and macrophages stimulates the synthesis and secretion of a variety of other cytokines including IL-1 and TNF-α. This initiates further pro-inflammatory cytokine release, causes fever by stimulating the release of prostaglandins (Dinarello 2000) and activates NF-κB. Additionally activated monocytes and macrophages release chemokines which induce directed chemotaxis in nearby responsive cells escalating the inflammation (Arai, Nishida *et al.* 1990).

The literature states that monocyte activation can be determined by observing 1) loss of proliferation, 2) increased adherence, 3) development of a different gene expression profile, 4) the ability to phagocytose latex particles, 5) adoption of an irregular cell shape with obvious pseudopodia (Verreck, de Boer *et al.* 2006; Sharif, Bolshakov *et al.* 2007).

Expanding on the observation of morphology changes, Verreck *et al.* showed that type 1 macrophages (M1) typically appear as adherent cells with a classical “fried egg morphology” whereas type 2 macrophages (M2) appear as adherent and stretched, "spindle-like" cells. Verreck *et al.*, observed that LPS-activated THP-1 cells have a very similar gene expression signature to that of primary macrophages, even though they are a transformed and immortalised cell, making them an ideal cell line for LPS-activated gene expression studies. Other studies have found two macrophage phenotypes M1 being classically activated and M2 being alternatively activated (Van Ginderachter, Movahedi *et al.* 2006, Martinez, Sica *et al.* 2008).

Tissue macrophages have many important roles but essentially they are involved in phagocytosis of pathogens, the elimination of dying cells and the secretion of immune system regulators which influence the physiological functions and differentiation of neighbouring cells (Auwerx 1991).

During the monocyte activation process at the site of inflammation the monocyte rolls along the blood vessel wall, slows down and binds to the surface of endothelial cells, then moves into sites of inflammation by extravasation of capillary walls. This contact is possible due to adhesion molecules (selectins and integrins), expressed on the surface of phagocytising and endothelial cells of blood vessels upon endothelial activation (Alom-Ruiz 2008). The phagocytes move up the chemo-tactic gradient to accumulate at the site of inflammation to initiate the clearance of pathogens.

There are several types of receptors involved in the recognition of target pathogens. These are localized on the surface of the phagocytising cells. Examples of these receptors are lectins (proteins binding carbohydrate structures), mannose and galactose receptors, receptors for lipopolysaccharide, scavenger receptors, Fc receptors for antibodies (binding Fc fragment of immunoglobulin) and receptors for complement fragments C3b and iC3b

(Newman, Mikus *et al.* 1991; Allen and Aderem 1996). After adherence of the pathogen to the surface of the phagocytising cell the ingestion phase is initiated.

The ingestion phase is enabled by activating an actin - myosin contractile system which extends pseudopodia around the particle. The plasma membrane is pulled around the pathogen until the pathogen is completely enclosed in a vacuole (phagosome). Cytoplasmic granules fuse with the phagosome and the pathogen is then destroyed by microbicidal mechanisms. This may consist of reactive oxygen species (ROS), nitric oxide or oxygen- independent mechanisms such as, lysozyme, lactoferrin, cathepsin G or other proteolytic enzymes (Aderem and Underhill 1999). It is the product of these microbicidal mechanisms that left unregulated, cause the tissue damaging component of inflammation.

Macrophages control the balance between pro- and anti-inflammatory responses, depending on the activating stimuli. During an infection, inflammatory processes are critical to pathogen removal. However, prolonged inflammation is associated with deleterious effects in the tissue and must be controlled and/or repressed to allow for healing and homeostasis of the tissue. Macrophages control the state of inflammation by responding to their environment accordingly. To achieve this, monocytes can be activated in one of two subsets to generate a pro- or anti-inflammatory collection of cells. M1 cells are pro-inflammatory effectors defined by their ability to produce pro-inflammatory cytokines (IL-1α and β, IL-6, IL-8, TNF-α) and M2 exhibit anti-inflammatory properties as they produce cytokines antagonistic to M1 cytokines (IL-4, IL-10, TGF-β). M2 macrophages are mostly observed during the healing phase of acute inflammation and wound healing (Porcheray, Viaud *et al.* 2005; Verreck, de Boer *et al.* 2006). These activation states have been characterised by their cytokine expression and the presence of macrophage-specific markers. However, Porcheray *et al.* (2005) found that macrophages stimulated towards a specific phenotype (M1 or M2) have the ability to return to a quiescent state

after signal arrest, or to switch their activation phenotype rapidly upon counter-stimulation.

The acquisition of a mature macrophage phenotype is clearly distinct from that of monocytes and requires expression of a new set of genes (Verreck, de Boer *et al.* 2006). This characteristic can be used to determine the extent of activation by different treatments. Most "macrophage-specific" marker genes that have been documented are already expressed in peripheral blood monocytes and do not show up-regulation which would confirm the macrophage phenotype. Several genes were selected to find one that would offer the best indication of monocytes/macrophage populations. Krause *et al.*, (1996) identified several genes that can be used for this purpose with one in particular, HC-gp39, that is expressed only in late-stage macrophage differentiation.

HC-gp39, also known as YKL-40, is a mammalian member of glycosyl hydrolase family. *In vivo*, HC-gp39 levels are increased in serum, synovial fluid, and cartilage of rheumatoid arthritis patients (Johansen, Jensen *et al.* 1993), in various inflammatory disorders (Vos, Steenbakkers *et al.* 2000), in the liver of patients with alcoholic cirrhosis (Johansen, Møller *et al.* 1997), in serum of patients with recurrent breast cancer (Johansen, Cintin *et al.* 1995), colorectal cancer (Cintin, Johansen *et al.* 1999) and in macrophages in the atherosclerotic plaque (Boot, van Achterberg *et al.* 1999). The described expression pattern suggests a predictable role in inflammation, tissue remodelling, and tumour metastasis.

The expression of HC-gp39 is restricted to a small number of cell types such as chondrocytes, synovial cells, neutrophils, and macrophages (Krause, Rehli *et al.* 1996) and shows a strict correlation with late macrophage differentiation *in vitro*; expression is undetectable in monocytes and only marginally detectable in monocyte-derived dendritic cells (Krause, Rehli *et al.* 1996).

During differentiation of human blood monocytes the expression of cell surface antigens changes. Two monoclonal antibodies Max.1 and Max.11 recognize cell surface antigens that are almost undetectable on monocytes but highly expressed on differentiated macrophages (Rehli, Krause *et al.* 1995). Rehli *et al.* found that Caboxypeptidase is identical to the antigens recognised by Max.1 and Max.11 and is produced by activated THP-1 cells. Specific primers for Carboxypeptidase M mRNA can be used to determine the macrophage population and extent of activation after exposure to certain agents.

To confirm that the activating treatments (LPS, PMA, Vit-D3) used on THP-1 cells were in fact generating macrophages, two markers HC-gp39 and Carboxypeptidase M, were screened for after treatment.

Macrophages are not proliferative cells. Their main goal is to remove pathogens and foreign debris or apoptotic cells. Monocyte precursors replenish the monocyte/macrophage population in response to growth factors (Sluiter, van Waarde *et al.* 1982; Nicola and Metcalf 1985). A monocyte cell suspension will differentiate into one expressing functional properties and differentiation markers of macrophages in response to an activating treatment such as PMA (Traore, Trush *et al.* 2005), LPS (Suzuki, Hashimoto *et al.* 2000) or Vit-D3 (Hmama, Nandan *et al.* 1999). However this leads to a decrease in cell proliferation (Traore, Trush *et al.* 2005). This drop in cell proliferation can be used as an indicator for successful cell activation, providing that cell viability does not decrease.

During the change-over from the monocyte to macrophage, the ability to phagocytose increases. This can be easily monitored in THP-1 cells due to their non-specific ability to phagocytose particles such as polystyrene latex particles upon activation using latex particles labelled with fluorescein isothiocyanate (FITC). Phagocytosis can be measured by visually counting the number of fluorescent particles inside live cells.

To assess the amount of expression of macrophage marker genes, a housekeeping gene was selected as a reference. A housekeeping gene should be expressed in stable amounts between treatments as gene of interest (GOI) expression levels may be affected by culture, stimulation or disease severity (Bustin 2000). Beta-2-microglobulin (β_2M) was chosen for this study as β_2M is expressed with low variability between cell treatments (Ishii, Wallace *et al.* 2006). This allows for the direct comparison of samples as long as the amounts of β_2M remain stable between treatments. It is assumed that the appearance of the macrophage markers after stimulation with activating treatments would be gradual until a peak level indicates that monocytes have changed over to macrophages.

2.2.1 Outline of experiments

Several assays were employed in this research to confirm these properties in LPS-, PMA-, and Vit-D3-stimulated THP-1 cells after suitable activation times. The morphology of the resulting cells was investigated using microscopy techniques (Section 2.3.9). Macrophage markers were screened for in the activated THP-1 cells (Section 2.4.3). A phagocytosis assay was used to determine the ability of macrophages to phagocytose latex particles after an activating treatment with LPS, PMA or Vit-D3 (Section 2.4.4). The proliferation rate of the cells activated by each of the treatments was determined (Section 2.4.5).

2.3 Materials and Methods

2.3.1 Cell culture

THP-1 cells (ATCC TIB-202, acquisition date 2006) were maintained at 37°C, 5% CO_2, and 95% relative humidity (RH) in growth medium and used between passage 40 and 50. Cells were cultured in 25 ml Falcon culture flasks (Sigma-

Aldrich) in a 5 ml suspension and split as required to maintain densities between 5 x 10^5 and 1 x 10^6 cells/ml. Cells were activated in a 24 well plates (Greiner, Sigma-Aldrich) in 1 ml samples.

2.3.2 THP-1 growth medium

The growth medium contained an endotoxin-free RPMI 1640 (Sigma) supplemented with 10% volume by volume (V/V) heat-inactivated foetal bovine serum (GIBCO) and 25 mmol/l HEPES with 50 U/ml penicillin, 50 µg/ml streptomycin, 40 µg/ml vancomycin and 50 µg/ml gentomycin. The medium was sterilised by filtering. The medium was then stored at 4°C.

2.3.3 Tetrazolium salt (MTT) assay

MTT was purchased from Sigma-Aldrich (Cat. No.# M-0283). This test is a quantitative colorimetric method to determine cell proliferation or viability for THP-1 cells (Auwerx 1991). It utilizes the yellow tetrazolium salt [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] which is metabolized by NAD(P)H oxidoreductases in the cytosol of proliferating/live cells to yield a purple formazan reaction product. A solubilization solution, diluted hydrochloric acid, is added to dissolve the insoluble purple formazan product into a coloured solution. The resulting purple solution is spectrophotometrically measured.

Briefly, cells were treated and grown in a 96-well plate for 1 – 7 days. Prior to assay suspended cells were removed, and adherent cells and wells were washed twice in PBS and incubated in the presence of 50 µl of MTT solution (5 mg/ml) for 2 h at 37°C. For the non-adherent control cells or cells where the treatment did not promote adhesion, the cells were removed from the test well, transfered to an Eppendorf tube and briefly spun down to pellet cells. The medium was removed and the cells were gently washed in 500 µl PBS. The test well was also washed briefly in PBS. The cells were spun again to pellet them and 50 µl MTT added to the Eppendorf tube to re-suspend the cells. The cells were then

returned to the test well and incubated in the same way as the other test wells. After incubation 0·3 ml of extraction buffer (8% HCl in isopropanol) is added and optical density measured at 540–630 nm. The OD of blank wells (lacking cells, but manipulated in the same way) is subtracted from the values obtained for the test wells.

2.3.4 Trypan Blue exclusion

Trypan blue was purchased from Gibco in a ready-to-use 0.4% solution. The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. Dead cells are therefore blue and live cells are not. The cell suspension was mixed 1:1 with 0.4% trypan and cells counted on a haemocytometer to give a percentage of viable THP-1 cells (Auwerx 1991).

2.3.5 Lipopolysaccharide

1 mg of *E.coli* LPS (*E. coli* serotype 0127:B8; Sigma-Aldrich; Cat. No.# L4516-1 mg) was purchased. The LPS was diluted with 1 ml of RPMI 1640 medium and stored at -20°C. LPS was used at a final assay concentration of 100 ng/ml unless otherwise stated.

2.3.6 Phorbol 12-myristate 13-acetate (PMA)

PMA was purchased from Sigma-Aldrich (Cat. No.# P1585-1 mg) and diluted in 1 ml DMSO, and further diluted to a working concentration of 10^{-6} mol/l in RPMI 1640 medium and stored at -20°C. PMA was used at a final concentration of 10 nmol/l unless otherwise stated.

2.3.7 1α,25-dihydroxyvitamin D(3) (Vitamin-D3)

Vit-D3 was purchased from Sigma-Aldrich (Cat. No.# D1530-10 µg) and diluted in 1 ml ETOH and stored at -20°C. Vit-D3 was used at assay concentrations of 100 nmol/l in RPMI medium unless otherwise stated.

2.3.8. Latex particles

Yellow/green fluorescent latex-coated polystyrene particles with carboxylate modifications were purchased from Sigma-Aldrich (1.0 µm, Cat. No.# L5405) and used at densities of approximately 25 particles per cell. The particles were sterilized by pasteurization for 24 hours at 70°C as recommended by the supplier. Detection wavelengths used were excitation 470 nm and emission 505 nm.

2.3.9 Morphology Assessment

The cell morphology, cell viability and phagocytosis activity was assessed using a Axostar plus Zeiss inverted microscope and 40x lens.

The images of macrophages phagocytising latex particles were recorded with a Nikon PCM2000 laser scanning confocal microscope equipped with a 100 mW argon laser. The 488 nm laser line was selected with a band-pass interference filter. The sample was visualized with a 60x oil-immersion objective lens (numerical aperture, 1.4) with a 20 µm confocal pinhole. GFP fluorescence was selected with a 505 nm dichroic mirror and an interference band-pass filter transmitting light from about 500 to 527 nm. Images were recorded by scanning the laser over a field of view that was typically 30.8 by 30.8 µm. Images were 512 by 512 pixels, with a scan speed of 3 µs per pixel. Images were averaged from 10 successive scans. The *xy* resolution of the microscope was approximately 250 nm. The resolution was dependent on the confocal pinhole used. With the 20 µm confocal pinhole, it was about 0.75 µm (half-width at half-maximum).

The images are taken from a time lapse movie generated using a confocal microscope one frame per second in conjunction with Dr Barry O'Brien from the University of Waikato.

2.3.10 Agarose gel electrophoresis

2.3.10.1 Tris-acetate-EDTA (TAE) buffer

To make 1 l of 50 x TAE buffer: 242 g Tris, 100 ml of 0.5 mol/l EDTA pH 8.0, 57.1 ml glacial acetic acid. Add enough MilliQ H_2O to dissolve solids, then bring up to final volume of 1000 ml.

2.3.10.2 Tris-EDTA (TE) buffer

To make 100 ml of TE buffer; Add 1 ml of 1 mol/l tris HCL (pH 8.0) and 0.2 ml EDTA (0.5 mol/l) and make up with MilliQ H_2O to 100 ml.

2.3.11 Preparation of a 2% agarose gel

Agarose (2 g) was added to 100 ml 1 x TAE buffer and weighed. The agarose was dissolved by microwaving on high for approximately 2 minutes and then re-weighed and topped up to the original weight with purified water. The agarose was cooled by swirling the flask in a cold water bath, and 2 µl of ethidium bromide was added before being poured (about 30 ml) into a gel caster (Horizon® 11.14, GibcoRBL) with 12 well combs. The gel was left to set at room temperature.

2.3.12 Oligodeoxynucleotide Primers

Olgodeoxynucleotide primer pairs for the macrophage markers HC-gp39 and CPM, as well as the house keeping genes β-actin and β_2M, were either selected based on previous publications or constructed using a primer designer programme (Genamics Primer Designer). All primers were purchased from

Sigma-Aldrich. Accession numbers for genes and primer sequences are listed in Appendix 2. Original individual primer concentrations varied depending on olgodeoxynucleotide length, and so were subsequently diluted with TE (10 mM Tris, 1 mM EDTA pH 8) buffer to give a stock solution of 200 ng/ml. Working primer solutions were prepared for each cytokine by adding 80 µl of TE buffer to 10 µl each of forward and reverse primer stock, and stored at 4°C.

2.4 Experiments

2.4.1 Activating treatment concentrations

The use of varying concentrations of LPS, PMA and Vit-D3 have been described in the literature and it was important to find concentrations that were tolerable to the cells, yet effective in initiating macrophage differentiation for THP-1 cells. A range of LPS, PMA and Vit-D3 concentrations were used and the mortality recorded to find the highest tolerable concentration for the cell line that would still produce a macrophage, determined by morphological changes. Mortality was assessed using the trypan blue viability assay (Section 2.3.4). The literature was used as a guideline for starting concentrations in the range and were as follows: LPS; 100 ng/ml – 1 µg/ml for 24 hours (Suzuki, Hashimoto *et al.* 2000), PMA; 1 nmol/l – 1 µmol/l from 24 hours (Traore, Trush *et al.* 2005)., Vit-D3; 10 nmol/l –1 µmol/l from 24 hours (Schwende, Fitzke *et al.* 1996; Hmama, Nandan *et al.* 1999).

To grade treatments of THP-1 with LPS, PMA or Vit-D3, cells were assessed using an inverted microscope to observe morphology changes which indicate a successful transformation. Control THP-1 cells (no activating treatment) maintained a round shape and did not clump or adhere to the culture plate surface whereas activated THP-1 cells became flat and amoeboid with obvious pseudopodia, and adhered to the culture plate surface with the exception of the

Vit-D3 treatment which resulted in a non-adherent suspension. The time to achieve this morphology change differed between treatments, as did the actual morphology.

The treatments were assessed over 7 days and optimum conditions for activation were found by assessing the time required to achieve morphology change, and the viability of the cells, to arrive at a concentration of activating agent and the time of treatment best to use for assays in subsequent research.

2.4.2 Viability assay

Cells stimulated with LPS, PMA and Vit-D3 were assayed for viability using the tetrazolium salt (MTT) assay and trypan blue exclusion assay. A period of two days of activation was utilized as any cell death relating to the treatment concentrations would be expected in the typical life span of the cell (26 hours). MTT results were representative of the number of viable cells, confirmed by counting the number of cells in five microscope fields per well by trypan blue exclusion. The results obtained with trypan blue were similar to those obtained with the MTT assay (data not shown) but due to the Vit-D3-treated cells and the untreated-control cells being non-adherent, the trypan blue assay was used in subsequent experiments requiring testing for each treatment and incubation time in triplicate wells.

2.4.3 Macrophage marker gene expression assay

THP-1 cells were incubated (37°C, 95% air, 5% $CO_{2)}$ in growth medium, with LPS, PMA or Vit-D3 for up to 7 days to assess macrophage marker mRNA expression. Cells were grown in 3 ml aliquots in individual tissue culture flasks. At time intervals of 1, 3 and 7 days, medium was removed and RNA extracted. At these time intervals optimal transformation from monocyte to macrophage is expected for all three treatments. After 3 days and 6 days, treated medium was removed and replaced to ensure optimum growing conditions. As a control, the

housekeeping gene β_2M was also amplified from monocytes and macrophages to normalising gene expression between samples.

2.4.3.1 Lysis of cells and RNA extraction

Flasks were placed upright for 20 minutes to allow suspended cells to settle. The medium was pipetted off carefully to leave cells behind. The Trizol reagent (1 ml of ProGenz) was added to the flask and the flask slowly agitated for 2 minutes on its side to remove adherent cells. The flask was then vortexed for 10 seconds to lyse cells and then incubated at room temperature for a further 3 minutes. This way adherent cells and suspended cells could be lysed together.

The flask was then briefly centrifuged to pool lysed cells and these were then pipetted out into a 1.6 ml Ependorf tube. After incubation 200 µl chloroform was added and the tube vortexed for 10 seconds. The tubes were incubated on ice for 10 minutes then centrifuged for 15 minutes at 12 000 *x g*. The upper aqueous layer was removed and placed in a 1.5 ml Eppendorf tube containing an equal amount (~ 500 µl) of isopropanol and the tube incubated at -20° for 30 minutes. The tubes were then centrifuged for 10 minutes at 12 000 *x g* and the supernatant discarded. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged for 5 minutes at 12 000 *x g*. The ethanol was removed by pipette and the pellet was allowed to air dry at room temperature for 5 minutes. The RNA samples were then dissolved in 20 µl of 10 mM Tris (pH 7.8) with 0.66 mol/l $MnCl_2$ by vortex. Residual DNA was removed by treating with 1 µl (1 unit) Promega DNAse and incubated for 30 minutes at 37°C. One unit (1 µl) of Promega DNAse Stop solution was added to each tube and then incubated at 65°C for 10 minutes. To check the quality of the RNA in the extract 2.5 µl was run on a 2% agarose gel in TAE buffer (for agarose gel methods see Section 2.3.11).

The samples were electrophoretically separated at 55 volts for 45 minutes. RNA integrity was indicated by the sharp, clear 28S and 18S rRNA bands. RNA bands

were visualised using a GIBCO BRL ultra-violet trans-illuminator (Life Technologies) at 312 nm, photographed using a COHU High Performance CCD camera and digitally stored. All RNA-band base-pair lengths were checked by comparison with a 1 kb DNA ladder (Invitrogen) unless otherwise stated. The RNA was then stored at -70°C until required for cDNA synthesis.

2.4.3.2 First strand cDNA synthesis

To obtain an estimate of concentration of RNA each RNA sample was measured on a nano-drop spectrophotometer. Readings were done in duplicate to ensure accuracy. Reagents used to produce cDNA were as described by the protocol outlined by SUPERSCRIPT III (GibcoBRL). An appropriate amount of RNA in water was used to ensure cDNA samples were uniform. Briefly, for each sample approximately 5 µl of RNA (50 ng measured by nanodrop spectrophotometer) and 1 µl of oligo dT (GibcoBRL) were used and the balance made up to 11 µl with MilliQ water in a 200 µl Axygen PCR tube. The tubes were incubated for 10 minutes at 65°C to separate secondary structures and then placed on ice for 10 minutes to allow the oligo dT primers to anneal to single stranded RNA.

In a separate tube, 4 µl MilliQ water, 5 µl buffer, 1 µl Dithiothreitol (MDTT), 1 µl of deoxynucleotide triphosphates (dNTPs) and 1 µl of SUPERSCRIPT III reverse transcriptase was added and this mixture was incubated on ice until being added (9 µl) to the tubes containing RNA. The tubes were then incubated for 1 hour at 50°C and then the enzyme inactivated at 65°C for 10 minutes.

2.4.3.3 Conventional RT-PCR analysis of cDNA

Conventional RT-PCR analysis was used to confirm cDNA synthesis from RNA using the primer set for β-actin. Briefly, the PCR process involves multiple cycles of template denaturisation, primer annealing, and primer elongation to amplify DNA sequences. HotStar® *Taq* DNA Polymerase (Qiagen, Valencia, NV) was used for PCR amplifications using the manufacturer's instructions.

A PTC-100 Thermocycler (MJ Research, Reno, NV) was used for thermo cycling. After pre-incubation for 2 minutes at 94°C, to activate Taq polymerase (Qiagen), the target DNA was amplified with 40 cycles, each cycle consisting of a denaturisation step at 94°C (20 seconds melting), annealing for 20 seconds at 55°C (optimised for specific genes, as presented in Appendix 2) and extension at 68°C for 35 seconds.

2.4.3.4 Electrophoresis of cDNA

A 2% gel was run to confirm that cDNA had been successfully amplified. A DNA ladder (10 µl of 100 bp, Invitrogen) was run alongside 10 µl of sample. Gels were prepared as described in Section 2.3.11. The cDNA samples were premixed with a loading dye (40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol). Electrophoresis was carried out in Horizon tanks (Invitrogen) containing TAE buffer, at 100 volts until separation of cDNA bands was achieved. Bands were visualised using a GIBCO BRL ultra-violet trans-illuminator (Life Technologies) at 312 nm, photographed using a COHU High Performance CCD camera and digitally stored. All product band base-pair lengths were checked by comparison with a 100 bp DNA ladder (Invitrogen) unless otherwise stated. A single cDNA band confirms no contamination or miss-priming is present. The primers used for β-actin (described in Appendix 2) amplify a product 100 bp in size (see Figure 2.5). Once the presence of β-actin was confirmed the samples were then quantified for expression of macrophage specific markers CPM and HC-gp39 and the housekeeping gene β_2M, using RT-PCR primer sequences that are listed in Appendix 2.

2.4.4 Phagocytosis assay

In order to transform the monocytes to macrophages THP-1 cells were maintained as described in Section 2.3.2. Cells were sub-cultured with fresh growth medium prior to the assay and assessed for viability as described in Sections 2.3.1 and 2.3.4.

LPS, PMA and Vit-D3 were each added to freshly sub-cultured cells to a final concentration of 100 ng/ml LPS, 10 nmol/l PMA and 100 nmol/l Vit-D3. THP-1 cells had a viability of 95% or higher. Cells were plated out at 1 ml per well (approximately 5 x 10^5 cells) in a 24 well plate (Cellstar, Greiner bio-one) and the plate partially sealed with tape to reduce evaporation but allow CO_2 passage. Plates were incubated for 24 hours (LPS), 3 days (PMA) or 7 days (Vit-D3) at 37°C with 5% CO_2, and 95% relative humidity to allow activation (according to previous findings, Section 2.4.1 indicating time to macrophage differentiation).

Prior to the phagocytosis assay, each well was checked on an inverted microscope to observe morphological changes which indicate a successful transformation. Control THP-1 cells (no treatment) maintained a round shape and did not clump or adhere to the culture plate surface whereas LPS- and PMA-treated THP-1 cells adopted macrophage characteristics as described in Section 2.4.1. Vit-D3 activated cells were notably less adherent than the LPS or PMA activated cells.

Each well had sterile 1.0 µm latex-coated polystyrene particles (approx 25 particles per cell) added to the wells, then the plates were resealed with tape and re-incubated. For each treatment there where at least three replicates per experiment.

After four hours of incubation, any suspended cells were removed by pipette and phagocytosis halted by washing the monolayer with 1 ml of ice-cold sterile phosphate-buffered saline (PBS) three times (adherent LPS and PMA treated cells) or plates chilled to 4°C (suspended control cells and Vit-D3 treated cells) and cells removed by pipette and gently spun down by centrifuge (500 *x g*) in 1.5 ml Eppendorf tubes. The medium was then pipetted off and replaced with 1 ml of ice-cold PBS. This was repeated two more times to remove any dead cells or cell debris. Adherent cells were loosened from the plate by gentle pipetting of 100 µl PBS up and down on the well. At least 200 cells from each sample were counted and examined on a haemocytometer using an Axostar Plus Zeiss

fluorescent microscope phase II 40x lens. Cells that contained at least three particles were considered positive for phagocytosis. Non-activated control THP-1 cells (monocytes) had a phagocytosis rate less than 5% as observed in preliminary assays. Viability was checked to ensure treatments had not induced apoptosis.

The ability of THP-1 cells to phagocytose latex particles was quantified as a percentage of cells undergoing phagocytosis. The phagocytosis rate was obtained by dividing the number of phagocytic cells by the number of cells counted on a haemocytometer.

For the phagocytosis assay with PMA transformed cells, counting on a haemocytometer was hampered by the adhesion of the transformed cells. These cells could not be loosened from the plate without damaging them. PMA treated cells were examined adhered to the plate under the microscope. To give a percentage of phagocytosis, 200 cells from randomly selected fields of view were counted.

2.4.5 Proliferation

Due to the nature of macrophages described earlier, a drop in proliferation, yet not viability, indicates a transformation from a proliferative monocyte to a non-proliferative macrophage. The doubling time for THP-1 cells is approximately 26 hours. Therefore, a cell count of viable cells (determined using trypan blue exclusion) was undertaken at 48 hours post-treatment.

2.5 Results

2.5.1 Cell viability after activation treatment

The viability of the cells assessed over a period of incubation with a range of concentrations of activating treatments is shown in Figures 2.1-2.3. The optimum incubation time required for successful morphology change was found to be: LPS 24 hours, PMA 1-3 days, Vit-D3 7 days. The concentrations of activating agents selected for subsequent research were as follows: LPS; 100 ng/ml, PMA; 10 nmol/l and Vit-D3; 100 nmol/l. At these concentrations there was no significant loss of viability (viability remained 95% or higher). In the case of Vit-D3, which was diluted in ethanol, a control assay was carried out with ethanol only at identical concentrations and it was found that ethanol did not have an effect (results not shown). resist

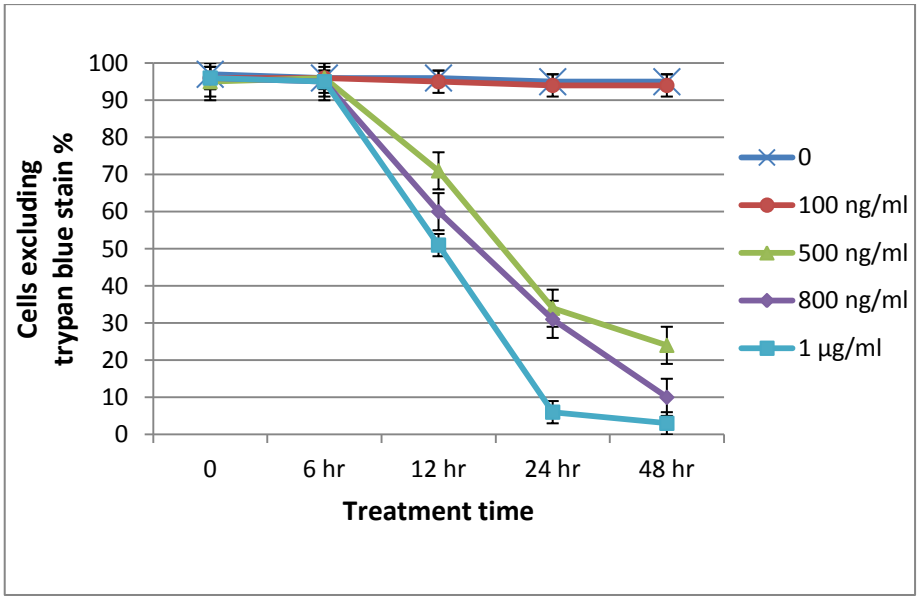

Figure 2.1. Viability of THP-1 cells after treatment with various concentrations of LPS for varying periods of time, as determined by trypan blue staining. Error bars show ± 1 SD of the mean from three experiments.

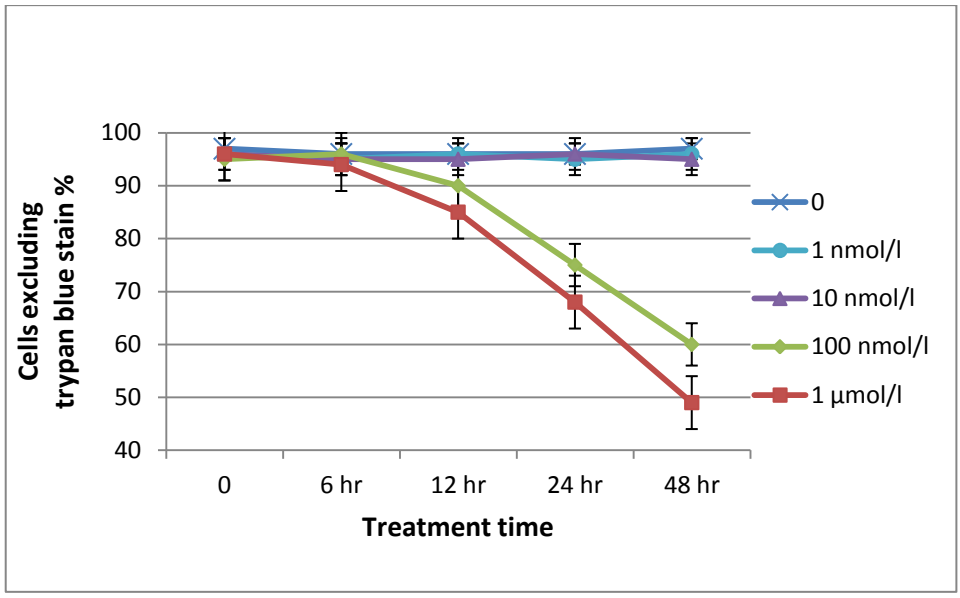

Figure 2.2. Viability of THP-1 cells after treatment with various concentrations of PMA for various periods of time, as determined by trypan blue staining. Error bars show ± 1 SD of the mean from three experiments.

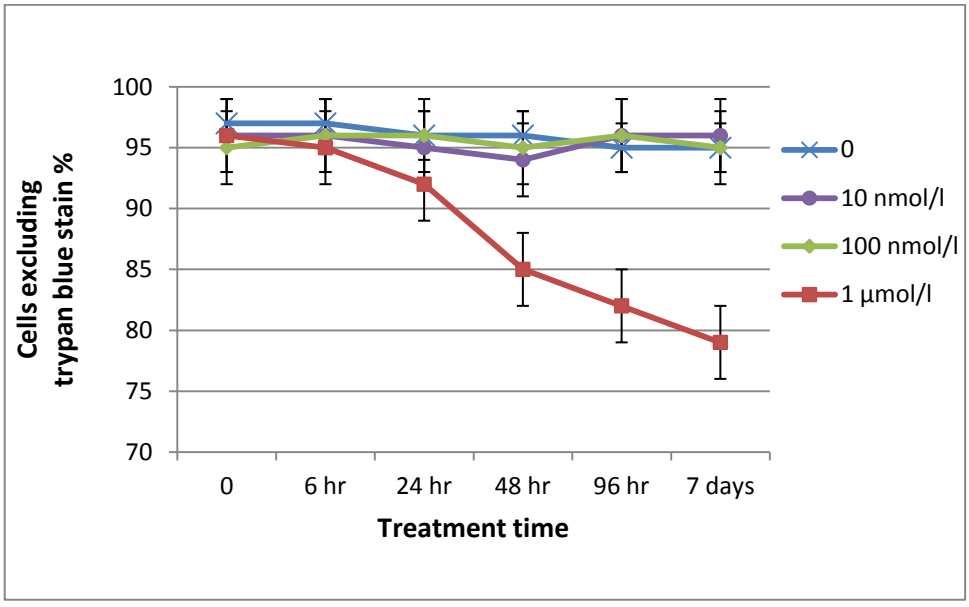

Figure 2.3. Viability of THP-1 cells after treatment with various concentrations of Vit-D3 for various periods of time, as determined by trypan blue staining. Error bars show ± 1 SD of the mean from three experiments.

2.5.2 Macrophage markers and morphology treatment

The RNA extracted from the LPS transformed cells that was run on electrophoresis to verify the integrity of the RNA is shown in Figure 2.4. (PMA and Vit-D3 transformed cells are not shown). The bands are clearly visible in the gel indicating intact 18s and 28s. To confirm that cDNA could be produced from the extracted RNA, the primer set for β-actin was used to amplify cDNA generating a 90bp amplicon. Representative results are presented in Figure 2.5. The cDNA from LPS, PMA and Vit-D3 activated cells were amplified with primers for HCgp-39 and CPM, with β_2M used as the housekeeping control. Figures 2.6 and 2.7 show the images of electrophoresis gels for PCR reactions for HCgp-39 and CPM primer sets respectively. A strong band indicates successful amplification from cDNA. These PCR results demonstrate that the macrophage marker HC-gp39 is expressed in THP-1 monocytes just as strongly as in transformed THP-1 cells. There was no visible expression of CPM in THP-1 monocytes and only a weak expression for Vit-D3 transformed THP-1 cells (24 h stimulation) represented by a weak band (primer-dimer also visible in lane 6 of Figure 2.7). The LPS and PMA transformed cells (24 h stimulation) produced a strong band for CPM indicating it is strongly expressed. After 72 h all three treatments induce strong expression of the CPM gene. After 7 days stimulation the expression of CPM was identical to the 72 hour expression rate for each treatment (results not shown). This indicates that Vit-D3 treatment is slower to transform the monocytes than the other treatments. These results combined suggest that CPM is a superior macrophage marker to HC-gp39 in THP-1 cells.

At the time intervals indicated, the transformation from monocyte to macrophage was expected to have been completed as indicated by the morphology observations for each of the treatments. Representative images of the cells' morphology before and after treatment are shown in Figures 2.8 – 2.11. To assess the morphological change in cell shape from the typical spherical monocyte phenotype to the amoeboid, adherent macrophage phenotype, cells

were microscopically examined daily until their shape was no longer considered spherical. The cells shown in Figure 2.9 – 2.11 were at this stage.

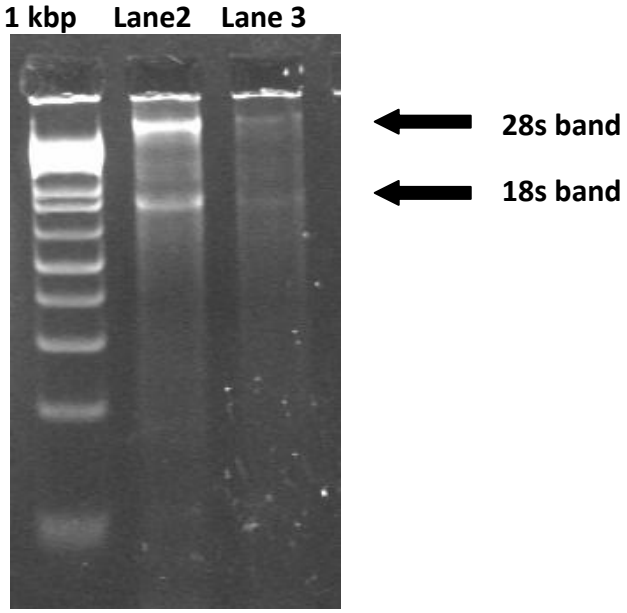

Figure 2.4. THP-1 RNA electrophoresed on 2% gel showing the 28s and 18s bands. A 1 kbp ladder is shown. Lane 2 had a higher concentration of RNA loaded than lane 3.

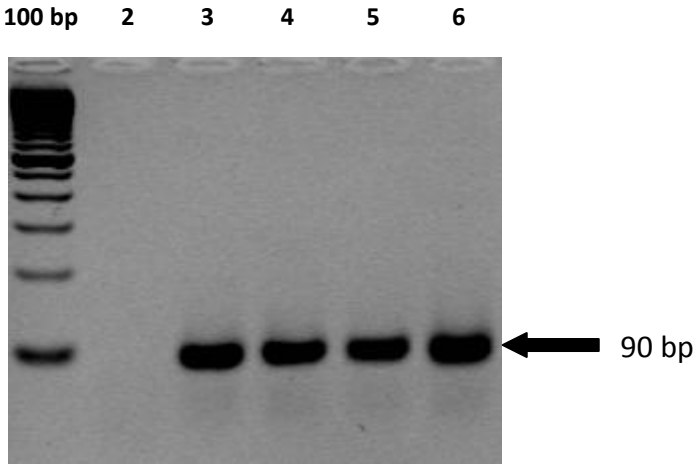

Figure 2.5. A 2% agarose gel showing the PCR products (90 bp) amplified using the β-actin primer set. Lane 1, 100 bp ladder; Lane 2, negative control; Lane 3, monocyte cDNA; lanes 4-6, cells activated for 48 h with LPS, PMA, Vit-D3 respectively. The strong band indicates successful amplification of β-actin cDNA from both monocytes and macrophages.

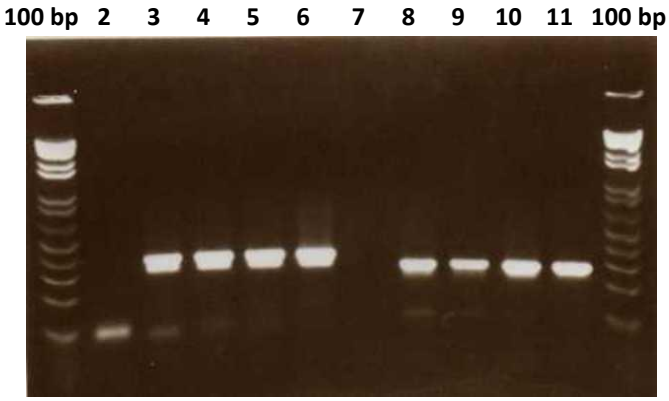

Figure 2.6. A 2% agarose gel showing the PCR products amplified using the β_2M (295 bp) and HCgp-39 (236 bp primer sets. Lane 1, 100 bp ladder; Lane 2, negative (primer dimer band visible); Lanes 3-6, β_2M from control Monocytes and LPS-, PMA-, Vit-D3- activated macrophages after 1, 3 or 7 days treatment respectively; lane 7, blank; Lanes 8-11, HCgp-39 from Monocytes and LPS-, PMA-, Vit-D3-activated macrophages respectively after 1, 3 or 7 days treatment respectively .

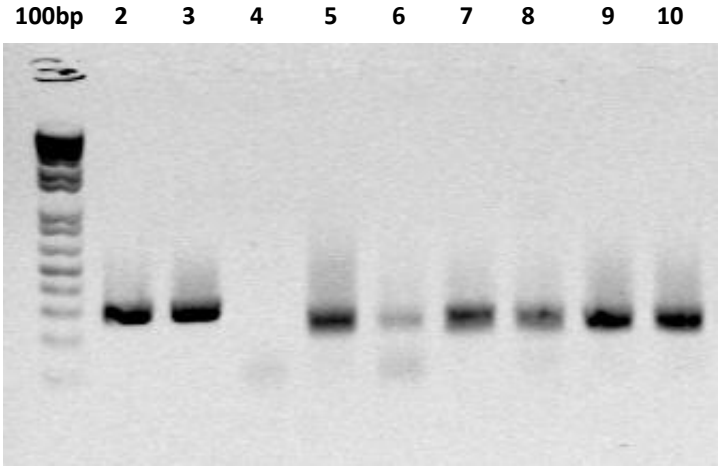

Figure 2.7. A 2% agarose gel showing the PCR products amplified using the β_2M and CPM primer set. Lane 1: 100 bp ladder, lanes 2 and 3: β_2M (295 bp) expression for monocyte and LPS-activated macrophage cells respectively. Lane 4: CPM (233 bp) expression for THP-1 monocytes. Lanes 5-7: THP-1 CPM expression for PMA, Vit-D, LPS, 24 h treatment respectively Lanes 8-10: CPM expression for PMA, Vit-D3, LPS 72 h stimulated THP-1 cells respectively.

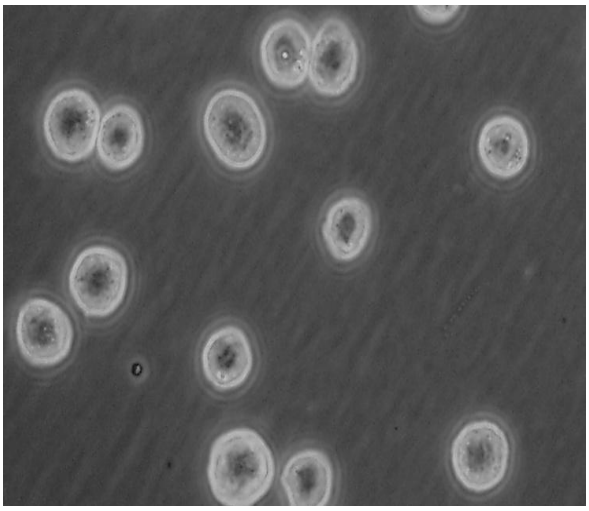

Figure 2.8. THP-1 monocytes showing the non-activated phenotype (400 x magnification).

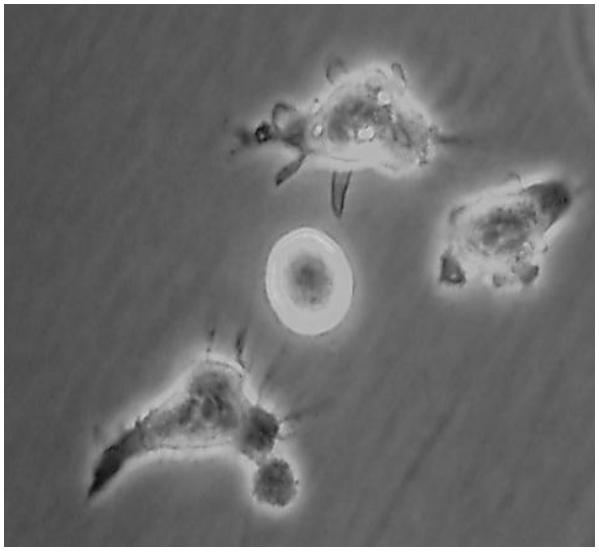

Figure 2.9. LPS-activated THP-1 cells after 24 hours activation with 100 ng/ml LPS. A non-activated spherical THP-1 cell is visible in the centre (400 x magnification).

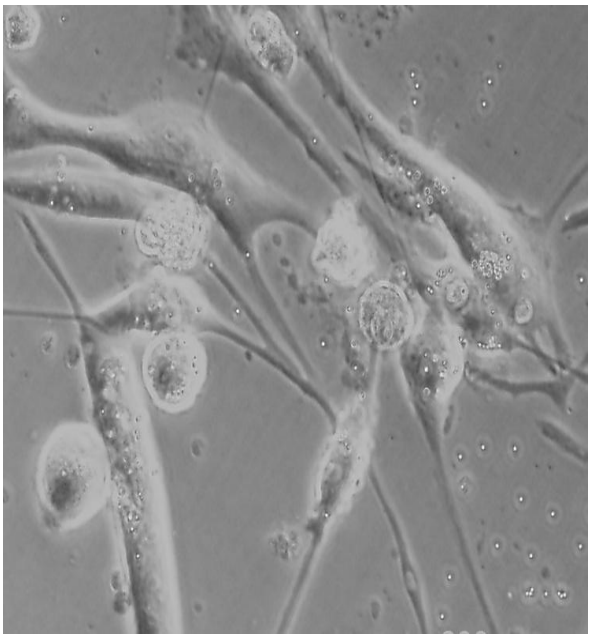

Figure 2.10. PMA-activated THP-1 cells after 3 days activation with 10 nmol/l PMA (400 x magnification).

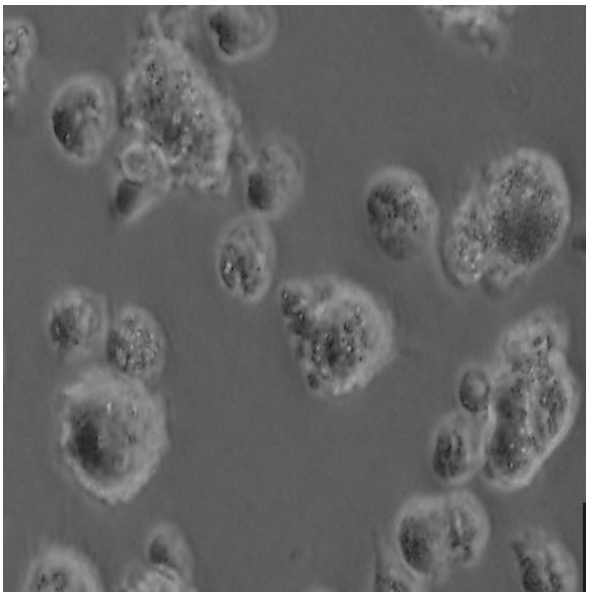

Figure 2.11. Vit-D3-activated THP-1 cells after 7 days activation with 100 nmol/l Vit-D3 (400 x magnification).

The morphological changes between the cell activation treatments varied greatly. The PMA treatment caused the cells to enlarge, become flat and amoeboid, with long pseudopodia extensions and become comparably slow moving. The PMA-treated cells seemed to increase 2–3 times in volume (visually assessed). They resembled dendritic cells in their appearance. PMA-treated cells became the most adherent population, fixing to the cell culture flasks and becoming removable only by trypsin treatment.

LPS-treated cells were moderately adherent, possibly due to the fact that they were very mobile and therefore not fixed to the culture flask like PMA-treated cells. They could be removed from the flask with rigorous tapping, or direct pipetting of fluid on to the cells to dislodge them. Pseudopodia were much shorter but more numerous than with PMA-activated cells, radiating from the cell in all directions. LPS-treated cells had approximately twice the volume of untreated THP-1 cells (visual assessment).

The Vit-D3-treated cells were less adherent than those from the other treatments, only needing a slight tap of the flask to dislodge them. Their appearance remained generally spherical like the untreated THP-1 cell, but they developed a ruffled membrane. This was possibly due to pseudopodia extensions but in a much reduced form compared with that of PMA- and LPS-treated cells.

2.5.3 Phagocytosis assay

Figure 2.12 shows time-lapse photography of an LPS-activated macrophage phagocytising a fluorescent particle. The FTIC-labelled latex particles were easily visualised within the cell. The pseudopodia are clearly visible binding the particle and bringing it closer to be phagocytosed. This is available to view as a movie on the DVD rom supplied in Appendix 1. The results from the quantitative phagocytosis assay are displayed in Figure 2.13.

It can be seen that all three treatments gave a significant increase in phagocytosis with the treatments giving significantly different degrees of activation of phagocytosis from each other. The effect of PMA was approximately twice that of Vit-D3 and of LPS was a further three times greater.

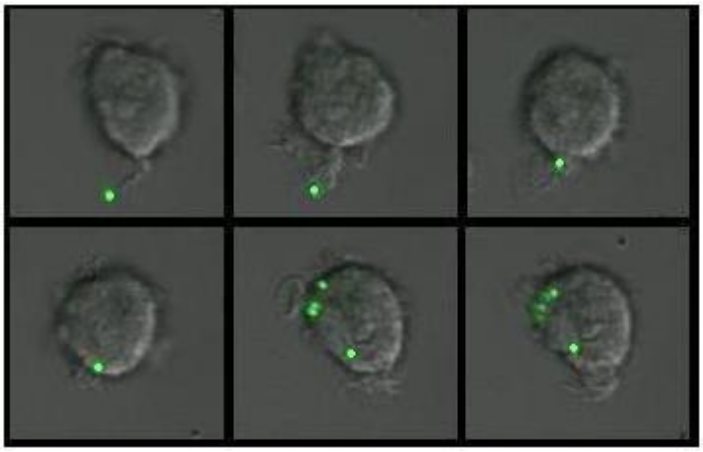

Figure 2.12. Time lapse photographs under a fluorescent microscope of an LPS-activated macrophage (100 ng/ml LPS for 1 day) phagocytising a FITC-labelled latex particle. The whole event took approximately 25 seconds. As the cell moves, other particles already phagocytosed become visible (400 x magnification).

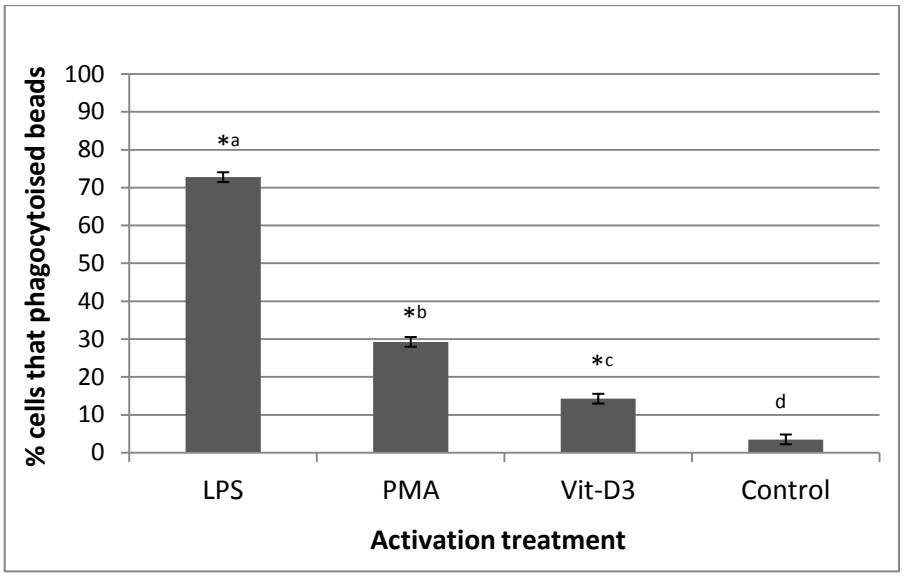

*Figure 2.13. Results from phagocytosis assays for the three THP-1 activating treatments, 100 ng/ml LPS, 10 nmol/l PMA, 100 nmol/l Vit-D3 after 1, 3 or 7 days activation respectively (these times optimal for each specific treatment as indicated in Section 2.5.1). The control received no activating treatment. Error bars show ± 1 SD of the mean of five experiments. *Results are significantly different (*$p<0.001$*) analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the non-activated control. Results not showing the same letter are significantly different to each other.*

2.5.4 Proliferation

Cell density was checked after 48 hours of treatment to indicate the extent of proliferation after exposure to the treatment. Macrophages are not proliferative cells unlike the monocyte precursor. A reduction in proliferation after receiving the activating treatment is an indicator for a change in phenotype as long as the viability remains greater than 95%. Cells that received treatment with the activating substance were compared with the non-treated control cells (monocytes). Any treatment that affects cell proliferation will be noticeable after 48 hours due to the cell cycle of THP-1 being 26 hours. Viability remained above 95% for the duration of the activation treatment determined by the

Trypan Blue assay (Section 2.4.2 results not shown). The proliferation results are displayed in Figure 2.14. PMA-activation generated a significantly less proliferative cell population than Vit-D3- and LPS-activated cells, though all three treatments reduced the proliferation rate significantly from that of the control monocyte population.

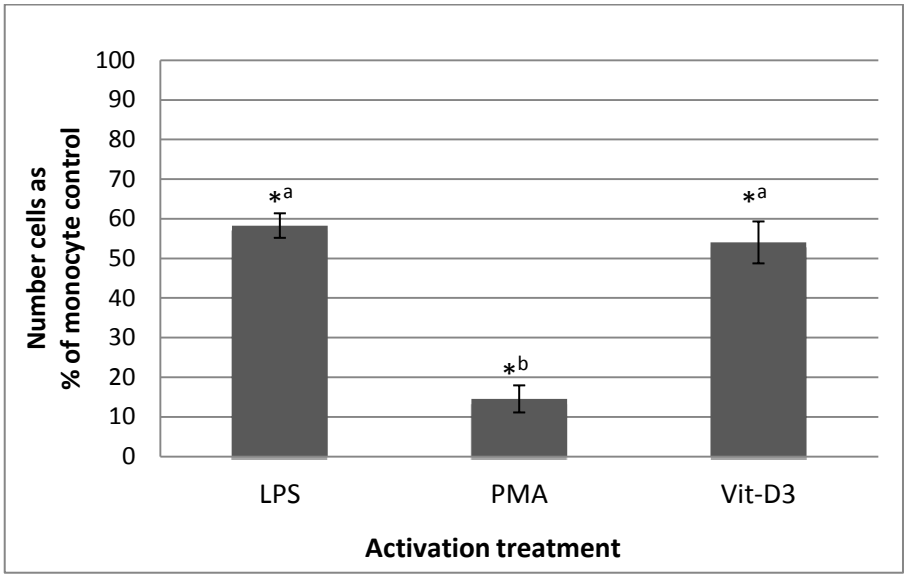

*Figure 2.14 Results from the proliferation assays for the three THP-1 activating treatments; 100 ng/ml LPS, 10^{-8} mol/l PMA, 10^{-7}mol/l Vit-D3 after 48 hours activation compared with the final count of the control cells (un-activated monocytes which displayed a normal increase in cell numbers over the treatment time. Error bars show ± 1 SD of the mean of five experiments. *Results are significantly different (p<0.001) analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the non-activated control. Results not showing the same letter are significantly different to each other.*

2.6 Discussion

A non-cytotoxic concentration of each activating treatment was determined. At this level the cells maintained a high viability yet displayed characteristics which would indicate successful transformation to a macrophage phenotype. The PMA and Vit-D3 concentrations were similar to those found in most of the literature. The optimum LPS concentration, however, was a lot lower than commonly reported (up to 1 μg/ml). It is not known, however, if in the reported studies the viability of the cells was checked after the assay was completed. In the present study at 6 hours post-treatment, all three treatments had an identical viability rate to that of the monocyte control. However, this viability dropped off suddenly over the next 18 hours for high concentrations, indicating that the cell line was not surviving the treatment. This would indicate that for the duration of the first 6 hours, cells may be starting to undergo necrosis. Any results (for example cytokine expression profiles) obtained from an assay during this time may not accurately show the effect of prolonged treatments being assayed. It was for this reason that the highest possible concentration of activating treatment that did not alter viability rate was chosen. These concentrations were used for the remainder of the research in this chapter and following chapters unless otherwise stated. Final concentrations were as follows: LPS, 100 ng/ml; PMA, 10 nmol/l; Vit-D3, 100 nmol/l. At these concentrations proliferation remained positive. PMA-activated macrophages however had a significantly lower proliferation rate that LPS- and Vit-D3-activated macrophages (p>0.001). Recently it has been found that tissue macrophages undergo rapid *in situ* proliferation in order to increase population density (Jenkins, Ruckerl *et al.* 2011). This inflammatory mechanism occurs during TH2-related pathologies (corresponding to M2 macrophages with wound healing or tissue-remodelling functions) under the control of the TH2 cytokine IL-4. This is a fundamental component of TH2 inflammation because exogenous IL-4 causes accumulation of tissue macrophages through self-renewal (Jenkins, Ruckerl *et al.* 2011). This information may indicate that the expansion of innate cells necessary for

pathogen control or wound repair can occur without recruitment of potentially tissue-destructive inflammatory cells. It may be useful in future studies to determine if PMA-activated macrophages are producing higher amounts of chemokines for the purpose of cell recruitment than LPS- or Vit-D3-activated macrophages do, and whether honey may have an effect on this process.

Macrophage marker gene expression was assayed using conventional RT-PCR to identify if the treatments initiated the change-over to a macrophage phenotype. CPM was found to be more definitive for this than HC-gp39 which gave a uniform expression between monocytes and macrophages. This suggests that the THP-1 cell line is already expressing HC-gp39 unlike fresh peripheral blood mononuclear cells as reported in the literature**.** CPM expression was initiated after treatment with the activating agents, indicating that the cell was responding to the treatment by modifying its expression of this gene along with its morphology. All three treatments were found to activate the THP-1 cell line to express the CPM macrophage marker with maximum expression achieved after 72 hours for all three treatments. The LPS treatment and PMA treatment showed a greater increase in the first 24 hours of CPM gene expression compared with Vit-D3. However after 72 hours all three treatments gave the same expression. As previously established, Vit-D3 treatment takes 7 days to achieve a macrophage phenotype, so CPM is useful in indicating the transformation is taking place before this time. It may be speculated from these combined results that Vit-D3 treatment transforms cells at a much slower rate than LPS and PMA.

To assess which was the best activating treatment to provide a phagocytising cell, the three treatments were compared at these above-mentioned concentrations in a phagocytic assay which assayed the amount of FITC-labelled latex particles phagocytosed in a set amount of time (4 hours).

In this time the LPS-activated THP-1 cells provided the cells with the most phagocytic potential, with rates reaching over 70% of the cells phagocytising at least three particles. This was achieved after only 24 hours of treatment with

LPS. At this time point PMA- and Vit-D3-treated cells had a very low phagocytosis rate yet all three treatments produced phagocytic cells that underwent significantly more phagocytosis events than the non-treated control monocytes. This may indicate that the PMA and Vit-D3 treatments activate a macrophage for a purpose other than phagocytosis, as the macrophage marker genes were still expressed. When the PMA- and Vit-D3-activated cells were additionally assayed for phagocytosis after longer treatment times, the phagocytosis rates did not alter (results not shown).

LPS was selected for ongoing research as it provided the most phagocytic cell with macrophage characteristics. The shorter treatment time using LPS also provides a more practical time period for the experiment.

Phagocytic activity, adherence, proliferation rate and mobility between the treatments were found to differ greatly, suggesting that not all of the three treatments produce similarly activated cells. All three treatments produced cells with very different morphologies, proliferation rate, size and phagocytic ability. This research demonstrates how results can be misinterpreted in a study where gene expression is exclusively relied upon to indicate a successful transformation from monocyte to macrophage.

It was observed that the three different treatments, all thought to activate the cell to the macrophage phenotype for phagocytosis assays, did not give uniform results. Upon visualisation it was apparent the cells had different morphologies and functions. Cells treated with LPS became highly phagocytic whereas those treated with Vit-D3 did not. PMA activated cells to a very different phenotype and one could be forgiven for mistaking it for another cell entirely. All cell treatments, however, produced a positive result in the RT-PCR for CPM, confirming that macrophage-specific genes were up-regulated by the treatment.

Since this study was undertaken, other reports have been published supporting this hypothesis. Mosser and Edwards (2008) suggest three basic macrophage populations, host defence, wound healing and immune regulation, which can be

assigned categories much like primary colours, which may “blend into various other 'shades' of activation” depending on their location, state of activation *etc*. This challenges the proposal of two activation states (M1, M2) discussed previously, yet possibly explains why three different cell-activating treatments resulted in three different morphologies in this research. The term “alternatively activated” should perhaps apply to macrophages other than type one, of which there may be several types. Mosser and Edwards (2008) suggest that macrophages populations that switch phenotypes may be responsible for many diseases such as atherosclerosis which is considered an inflammatory disease due to the high levels of pro-inflammatory cytokines released from macrophage plaques.

It is recommended for future studies that macrophages from chronic wounds and acute wounds be compared by assaying for macrophage type markers. A switch in macrophage phenotype may take place prior to the onset of complications in wound healing, causing the release of high amounts of inflammatory cytokines and ROS damaging surrounding tissue and preventing healing taking place. It would be of interest to see if the anti-inflammatory effect of honey is targeting one phenotype of macrophage over other phenotypes.

Chapter 3

The effect of honey on cytokine expression in THP-1 cells

3.1 Summary

It was hypothesised that the induction of the expression of anti-inflammatory cytokines, or suppression of the expression of inflammatory cytokines, may be the mechanism(s) by which honey works in its anti-inflammatory activity.

The messenger RNA expression of inflammatory and anti-inflammatory cytokines induced by honey was investigated. To test the modulatory effects of honey on gene expression, THP-1 cells were exposed to LPS (100 ng/ml), Manuka honey (0.25%) or LPS and Manuka honey simultaneously. Artificial honey (0.25% osmotic control) or medium alone were used as controls.

RNA was removed from cells using Trizol then cDNA was made from the cells' mRNA. Conventional reverse transcriptase-PCR was used to amplify cDNA using the primer set for the β-actin gene to quantify the cDNA. Quantitative real-time RT-PCR was then used to quantify the abundance of cytokine mRNA transcripts expressed from human THP-1 cell lines at a range of times (0, 6, 8 or 24 hours post-treatment). Seven genes were selected for the study; IL-1β, IL-6, IL-10, IL-1ra, TGF-β, PDGF, and TNF-α. β_2M was used as the house-keeping gene for RT-PCR.

The results showed that honey increased expression of TNF-α, IL-1β, IL-10, IL-1ra and TGF-β but reduced expression of PDGF in monocytes. In conjunction with LPS, honey increased expression of all cytokines and growth factors assayed in THP-1 cells. There were no conclusive results for IL-6. These results indicate, that while gene expression for inflammatory cytokines is increased by honey, so is the gene expression for anti-inflammatory cytokines and growth factors, which may allow inflammation to proceed but at a controlled and modulated level.

3.2 Introduction

Cytokines are messengers of the immune system which help regulate an immune response. They are soluble glycoproteins released by white blood cells and certain other cells of the host when an antigen is detected. There are many types which affect different parts of the immune system. Cytokines may stimulate or inactivate the immune system, recruit cells, or help end the immune response.

Cytokines possess typical activities such as:

1. They are released by certain cell types that react specifically with other cells, (target cell types) and regulate specific vital functions that are controlled by feedback mechanisms;
2. They generally act at short range in a paracrine manner or autocrine manner. Some can act in an endocrine manner depending on their ability to enter the circulation and their half-life;
3. They interact with high-affinity cell surface receptors and through this regulate the transcription of a number of cellular genes which results in changes in cell behaviour.

Chemokines are a class of cytokines that have chemo-attractant properties, inducing cells with the appropriate receptors to migrate towards the source of the cytokine. Chemokines function mainly as chemo-attractants for leukocytes,

recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection (Rottman 1999). Because they were first identified in cytokine assays they were initially named as interleukins. Since then members of the chemokine family are divided into four groups depending on the spacing of their first two cysteine residues; CC, CXC, C, CX_3C. The current nomenclature for chemokines is, e.g.: CCL1 for the ligand 1 of the CC-family of chemokines, and CCR1 for its respective receptor (Fernandez and Lolis 2002).

Because of the potent effects of cytokines their activities are tightly regulated, most notably at the levels of secretion and receptor expression. Cytokines can be grouped into two categories, pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. Anti-inflammatory cytokines are T-cell-derived cytokines and are involved in the down-regulation of inflammatory reactions. The functional definition of an anti-inflammatory cytokine is one which inhibits the function of pro-inflammatory cytokines (Opal, *et al.* 2006). The influence of honey on the secretion of the pro-inflammatory cytokines; IL-1β, IL-5, IL-12, IL-18, TNF-α and INF-γ has been previously studied by Dr. Nichola Harcourt at the University of Waikato. Harcourt reported that honey up-regulated the expression of TNF-α, IL-1β, and TGF-β in bovine blood cells. It has also been reported that honey at a concentration of 1% significantly increased the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in a human monocyte cell line MonoMac-6 (MM6) (Tonks, Cooper *et al.* 2003), and that honey stimulated the production of TNF-α in un-activated monocytes but not if activated by LPS (Tonks, Cooper *et al.* 2001). To broaden the reported findings on the bioactivity of honey, this chapter focuses on the anti-inflammatory cytokines, growth factors and pro-inflammatory cytokine, IL-6.

To choose a set of cytokines for the present study, consideration was given to other studies on cytokine mRNA expression and factors that stimulate or suppress their release. Several studies on the effects of anti-inflammatory

substances on cytokine expression have been undertaken. The effects of *Melaleuca alternifolia* essential oil on human peripheral blood monocytes (PBMC) were studied and it was found that the essential oil reduced pro-inflammatory IL-2 secretion, and increased anti-inflammatory IL-4 and IL-10 secretion, as determined by ELISA following incubation of PBMCs with essential oil for 24 hours (Caldefie-Chezet, Fusillier *et al.* 2006). The essential oil also exerted an antioxidant effect by decreasing ROS production. It was concluded that essential oil may work as an anti-inflammatory mediator through its antioxidant activity, and may also efficiently protect the organism by reducing the proliferation of inflammatory cells, without affecting their capacity to secrete anti-inflammatory cytokines.

The possible therapeutic effect of garlic as an anti-inflammatory agent was also investigated by looking at cytokine expression in the treatment of patients with irritable bowel disease, an inflammatory condition (Hodge, Hodge *et al.* 2002). Garlic (*Allium sativum*) has several compounds known to modulate leukocyte cell proliferation and cytokine production. *In vitro* results showed that production of pro-inflammatory IL-12 by monocytes was inhibited significantly in the presence of low concentrations of garlic extract. Production of anti-inflammatory IL-10 significantly increased. At slightly higher concentrations of garlic extract the production of pro-inflammatory TNF-α, IL-1β, IL-2, IL-6, IL-8 and IFN-γ decreased significantly. Methylprednisolone is an anti-inflammatory glucocorticosteriod commonly used for irritable bowel disease. It was stated that the inhibitory activity of methylprednisolone combined with garlic extract on leukocyte cytokine production was additive.

A separate study found that methylprednisolone may act to inhibit the release of pro-inflammatory cytokines IL-1α and β, IL-2, IFN-γ, and TNF-α and up-regulate the anti-inflammatory cytokine IL-10 (Hodge, Hodge *et al.* 1999). IL-10 decreases pro-inflammatory cytokines in circulation so has an over-all anti-inflammatory effect (Opal and DePalo 2000).

Two recent studies used ELISA and RT-PCR to show a dose-dependent inhibition of TNF-α, IL-1 and IL-6 by the immunosuppressive drug dexamethasone. Production of cytokines IL-2, IL-4, IL-6, IL-10, INF-γ and TNF-α indicate that dexamethasone is a broad-range immunosuppressant at the level of both the secreted product and mRNA (Bruin, Hommes *et al.* 1995; Rowland, McHugh *et al.* 1998).

The functional definition of an anti-inflammatory cytokine is the ability of the cytokine to inhibit the synthesis of IL-1, tumour necrosis factor (TNF), and other major pro-inflammatory cytokines. The principal anti-inflammatory cytokines are IL-1ra (Interleukin-1 receptor antagonist), IL-4, IL-10, IL-11, IL-13 and TGF-β and in some cases TNF-α as mentioned previously. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response (Opal and DePalo 2000). IL-1ra blocks the action of IL-1α and IL-1β functional ligands by competitive inhibition at the IL-1 receptor level. The anti-inflammatory cytokines IL-4, IL-6, IL-10, and IL-13 inhibit the synthesis of IL-1β, and stimulate the synthesis of IL-1ra (Dinarello 1997).

3.2.1 Tumor necrosis factor-α

TNF-α most commonly acts as an inflammatory cytokine that binds the TNF receptor (TNFR). Contact with the ligand causes a conformational change to occur in the receptor, leading to the dissociation of the inhibitory protein SODD from the intracellular domain. This dissociation enables the adaptor protein TRADD to bind to the domain, serving as a platform for subsequent protein binding. Following TRADD binding, three pathways can be induced (Locksley 2001). Of key importance to inflammation is the NF-κB pathway. To get activation of NF-κB, TRADD recruits TRAF2 and RIP. TRAF2 in turn recruits the multi-component protein kinase IκB, enabling the serine-threonine kinase RIP to activate it. An inhibitory protein, IκBα, that normally binds to NF-κB and inhibits its translocation, is phosphorylated by IκB and subsequently degraded, releasing NF-κB. NF-κB is a heterodimeric transcription factor that translocates to the

nucleus and mediates the transcription of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors (Stegmaier, Kirchhoff *et al.* 2008).

When TNF-α acts as an anti-inflammatory cytokine it negatively regulates the IL-1 system by inducing the release of IL-1 type II decoy receptor. This results in an overall reduction of IL-1 stimulus to the cells resulting in an anti-inflammatory effect through a multifunctional aminopeptidase (Orlando, Matteucci *et al.* 1997).

3.2.2 Interleukin-1

One of the earliest cytokines described, IL-1 was found to induce fever and control lymphocytes. There are two forms of IL-1, IL-1α and IL-1β, which are both pro-inflammatory. The IL-1 receptor antagonist (IL-1ra) is a molecule that competes with IL-1α and IL-1β for receptor binding to the IL-1 receptor (IL-1r), but does not induce any intracellular response, thus acting as an anti-inflammatory agent (Gabay 1997). IL-1α, IL-1β and IL-1ra are produced by both macrophages and monocytes during an inflammatory response (Dinarello 2000). IL-1ra is produced by monocytes and macrophages and is released into the systemic circulation in greater than 100-fold excess over either IL-1α or IL-1β after stimulation by LPS. The synthesis of IL-1ra and IL-1β are differentially regulated at their own promoter sites. Although bacterial LPS stimulates the synthesis of both IL-1β and IL-1ra, other stimuli cause differential release of IL-1ra and IL-1β, but these anti-inflammatory stimuli must be present in far greater concentrations than those of pro-inflammatory IL-1α and IL-1β cytokines to inhibit their actions (Gabay 1997; Arend, Malyak *et al.* 1998). This explains why acute inflammation can develop into chronic inflammation. IL-1ra has been proposed to be expressed in the skin as a protective measure to ensure that the antagonist is immediately available to counteract the potent effects of IL-1 as a result of injuries. The expression of IL-1ra in the skin suggests that it also could serve to attenuate effects of IL-1 in damaged tissue (Arend, Malyak *et al.* 1998).

3.2.3 Interleukin-6

In wound repair, expression of IL-1α, IL-1β, TNF-α and IL-6 was shown to be strongly up-regulated during the inflammatory phase of healing (Gallucci, Simeonova *et al.* 2000). For this reason these cytokines are labelled pro-inflammatory cytokines. Wounds in IL-6 knock-out animals were found to take up to three times longer to heal than those inflicted in wild-type controls and their subsequent granulation tissue formation was impaired (Gallucci, Simeonova *et al.* 2000). These abnormalities were completely rescued by administration of recombinant murine IL-6 protein 1 hour before wounding. Researchers concluded that IL-6 is crucial for kick-starting the healing response, both via its mitogenic effects on wound edge keratinocytes and via its chemoattractive effect on neutrophils (Gallucci, Simeonova *et al.* 2000).

3.2.4 Interleukin-10

IL-10 is an anti-inflammatory cytokine thought to play a major role in the limitation and cessation of inflammatory responses. IL-10 decreases production of monocyte/macrophage-derived pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-8 and IL-12 (Opal and DePalo 2000). Produced primarily from monocytes, it regulates growth and/or differentiation of various immune cells, as well as keratinocytes and endothelial cells during the process of wound healing (Gallucci, Simeonova *et al.* 2000). Wounded IL-10 null mice were characterized by a significantly higher inflammatory cell infiltration and collagen deposition compared with control mice, showing that IL-10 inhibits the infiltration of neutrophils and macrophages toward the site of injury as well as inhibiting the expression of several chemokines and pro-inflammatory cytokines (Liechty, Kim *et al.* 1999).

3.2.5 Growth factors

Also of interest to this research are growth factors. A growth factor is a naturally occurring substance capable of stimulating cellular growth, proliferation and cellular differentiation. Growth factors are important for regulating a variety of cellular processes, including wound healing and inflammation. Growth factors typically act as signalling molecules between cells, with actions mediated by specific receptors on the surface of their target cells (Pierce, Mustoe *et al.* 1991). They often promote cell differentiation and maturation, the extent of which varies between growth factors. For example, bone morphogenic proteins stimulate bone cell differentiation, inducing the formation of bone and cartilage (Vukicevic, Stavljenic *et al.* 1995), while fibroblast growth factors and vascular endothelial growth factors stimulate blood vessel differentiation (angiogenesis) (Risaua 1990).

3.2.5.1 Platelet derived growth factor

A key player in wound healing is platelet-derived growth factor (PDGF). PDGF is one of the numerous growth factors, or proteins, that regulate cell growth and division. In particular, it plays a significant role in angiogenesis. Migration of human dermal fibroblasts is critical for skin wound healing. The platelet-derived growth factor (PDGF) is the major pro-motility factor in human serum for fibroblast motility on type I collagen (Guan, Fan *et al.* 2009). Fibroblasts play essential roles in cutaneous wound repair and remodelling. They proliferate to expand, migrate into the wound bed, synthesize new extra cellular matrix, and express thick actin bundles as myo-fibroblasts to bring about contraction of the wound (Singer 1999). A number of growth factors/cytokines have been reported to affect, directly or indirectly, fibroblast motility. They include basic and acidic fibroblast growth factors, transforming growth factor-β1 and β2, vascular endothelial growth factor and PDGF (Singer 1999; Imanishi 2000).

PDGF-BB elicits its biological responses via binding to its specific cell surface receptors, PDGFR-α and PDGFR-β. These two receptor isoforms dimerise upon binding the PDGF dimer, leading to three possible receptor combinations, namely -αα, -ββ and -αβ. PDGF binding activates the PDGFR protein tyrosine kinase within seconds, leading to auto-phosphorylation of the receptor creating binding sites for downstream signal transduction molecules, which in many cases also are substrates for the kinase. Examples of the different signaling cascades induced by PDGFR tyrosine kinase receptors are the mitogen-activated protein kinase (MAPK) pathway, the phosphatidyl-inositol-3 kinase (PI3K) pathway and the phospholipase-γ (PLCγ) pathway (Heldin 1999).

MAPK family members have been found to regulate various biological functions by phosphorylation of particular target molecules (such as transcription factors, other kinases *etc*.) located in cell membrane, the cytoplasm and the nucleus, and thus contribute to the regulation of different cellular processes such as cell proliferation, differentiation, apoptosis and immune responses.

PI3K is activated by the majority of tyrosine kinase receptors. Similarly to other SH2 domain-containing proteins, PI3K forms a complex with PY sites on activated receptors. The main function of PI3K activation is the generation of PIP3, which functions as a second messenger to activate downstream tyrosine kinases Btk and Itk, and the Ser/Thr kinases PDK1 and Akt. The major biological functions of Akt activation can be classified into three categories – cell survival, cell proliferation and cell growth (Schabbauer, Tencati *et al.* 2004), all of these being important in inflammation.

In vivo, direct application of PDGF-BB to skin wounds induces increased formation of granulation tissue (Grotendorst 1985; Sprugel 1987) and increased wound breaking strength, thereby increasing the rate of healing (Pierce 1994). Honey has been shown to have almost identical actions on wounds to those of PDGF-BB (Subrahmanyam 1991; Subrahmanyam 1998; Misirlioglu, Eroglu *et al.*

2003) so it was of interest to include this growth factor in the cytokine analysis to determine if honey had an effect on the transcription of PDGF.

3.2.5.2 Transforming growth factor-β

Transforming growth factor beta (TGF-β) is a secreted protein that exists in three isoforms; TGF-β1, TGF-β2 and TGF-β3. TGF-β was the original name for TGF-β1 which was the first factor described. TGF-β1 is a multifunctional growth factor. It plays a pivotal role in wound healing and has been shown to accelerate impaired wound healing. However, high systemic levels of TGF-β1 have generally been associated with fibrotic disease processes such as myelofibrosis and pulmonary fibrosis (Hakvoort 2000). It is always present in platelets, but it is also produced by several cell types present in wounds, including activated macrophages, granulocytes, fibroblasts and keratinocytes (Martin 1992). TGF-β receptors are widely distributed and found on essentially all cell types (Slavin 1996). Immediately after wounding, TGF-β1 is released in large amounts from platelets. This initial kick-start of active TGF-β1 from platelets serves as a chemo-attractant for neutrophils, macrophages, and fibroblasts, and these cell types further enhance TGF-β1 levels. TGF-β is released as an inactive peptide, and requires activation either by proteolysis or as a result of the acidic environment within a wound (Werner and Grose 2003).

3.2.6 Real-time PCR

Real-time PCR is a rapid, highly sensitive and high-throughput molecular technique to quantify nucleic acids by amplifying particular regions of DNA between two predetermined positions termed the amplicon. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared with standard PCR, where the product of the reaction is

detected at the end of the reaction. For mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase, and the cDNA is amplified in order to robustly detect and quantify gene expression from small amounts of RNA.

PCR has three phases, exponential phase, linear phase and plateau phase. The exponential phase is the earliest phase in the PCR, in which product increases exponentially since the reagents are not limited. During the exponential phase the PCR product (cDNA) will ideally double during each cycle if efficiency is perfect, *i.e*. 100% (maximum efficiency is therefore calculated as 2) if the PCR conditions, primer characteristics, template purity, and amplicon lengths are optimal. The linear phase is characterized by a linear increase in product as PCR reagents become limited. The PCR will eventually reach the plateau phase during later cycles, and the amount of product will not change, because some reagents become depleted. Real-time PCR exploits the fact that the quantity of PCR products in the exponential phase is in proportion to the quantity of initial template under ideal conditions. This allows calculations to be made estimating the initial copy number of the expressed gene that was present, comparing this with the initial copy numbers of a reference gene and other sample genes quantified in the same run (Yuan, Reed *et al.* 2006). A "house-keeping gene" (one expressed in constant amounts in cells) is used as the reference gene.

Detection is fluorescence-dependent at the end of each PCR cycle. A fluorescent marker is hybridised to the cDNA between two PCR primers for the gene of interest. As the PCR proceeds, the 5' nuclease activity of the *Taq* polymerase cleaves the probe, releasing the tag such that the fluorescence is proportional to the amount of cDNA present. Several methods are available for analysis of results. The method chosen for this study is fully described in Section 3.4.8.

3.3 Aims

The aim of this study was to determine the effect of honey on the expression of cytokine genes in THP-1 cells. The growth factors PDGF and TGF-β and the following cytokines were selected for this study: IL-1ra, IL-1β, IL-6, IL-10 and TNF-α. Their expression was assayed using real time-PCR. Specific incubation times were chosen according to current research on cytokine and growth factor expression in THP-1, to achieve maximal expression or change in expression of the individual gene. Expression of TNF-α was assayed after 6 hours incubation, IL-1β and IL-6 after 24 hours (Segura 2001), TGF-β and PDGF after 8 hours (Shiffman, Mikita *et al.* 2000) and IL-10 after 6 hours (Lee 1996).

3.4 Methods

3.4.1 Reagents

3.4.1.1 Honey solutions

The required concentrations of Manuka honey and artificial honey were achieved by dilution of the honey with sterile RPMI 1640 complete medium. Manuka honey (M144) was obtained from the Honey Research Unit, University of Waikato, NZ and had a non-peroxide antibacterial activity equivalent to 12% w/v phenol (Allen, Molan *et al.* 1991). Honey was diluted by weighing out 1.37 g (1 ml) of honey and diluting this with 19 ml of sterile RPMI 1640 (to achieve a V/V concentration of 5%) immediately prior to use and filtered (50 µm, 8 µm and 3 µm, Minisart Sartorius filters, Millipore corporation) to remove pollen, bee tissues *etc.* Undiluted honey was kept at 4°C in dark coloured containers to prevent enzyme denaturation and degradation. Artificial honey was used to provide a control for the osmotic effect of the natural sugars found in honey. The composition of this simulated the sugar composition of honey, as published

(White 1975). Throughout the thesis honey concentrations are shown as % V/V. Endotoxin-free flasks, disposable equipment and reagents were used throughout this research.

3.4.1.2 LPS solutions

A 1 mg/ml stock solution of LPS (*E. coli* serotype 0127:B8; Sigma-Aldrich UK; Cat. No.# L4516) was diluted to working concentrations of 100 µg/ml diluted with endotoxin-free RPMI 1640 medium. A final assay concentration of 100 ng/ml was achieved after adding to the cells according to viability testing in Section 2.3.3.

3.4.1.3 THP-1 cell lines

THP-1 cells were cultured in suspension and passaged as required to maintain densities between 5 x 10^5 and 1 x 10^6 cells/ml as described in Section 2.3.1. Cells were grown in RPMI 1640 complete medium with 10% foetal calf serum, at 37°C (95% air, 5% CO_2) as described in Section 2.3.2.

3.4.2 Osmotic tolerance

Preliminary work on osmotic tolerance ranges for THP-1 cell lines was undertaken and an acceptable honey concentration of 0.25% was chosen for the study. This concentration was also used by Dr Nichola Harcourt as mentioned previously. Briefly cells were exposed to varying concentrations of honey (0.05%, 0.15%, 0.25%, 0.5%, 1%, 2%). The mortality of the cells was assessed after 24 hours and 48 hours using the MTT assay (Section 2.3.3.). Viability remained over 95% for concentrations of honey up to 1%, results not shown.

3.4.3 Assay design

THP-1 cells were incubated with Manuka honey and/or LPS to investigate the effect of honey on the expression of cytokine mRNA. THP-1 cells with medium alone served as controls for constitutive cytokine expression.

Cells were cultured for 24 hours in endotoxin free tissue culture flasks (Nunc) with six treatments; (1) RPMI growth medium alone (negative control), (2) Manuka honey (to measure stimulatory effects), (3) artificial honey (osmotic control), (4) LPS (positive control), (5) Manuka honey and LPS and (6) artificial honey and LPS (to measure modulatory effects). A 7 ml sample of freshly sub-cultured cells was treated in each flask. To achieve the final 0.25% concentration of honey, 50 µl of 5% diluted honey was added per ml of cells treated (350 µl per flask) or the equivalent in medium for the negative control. To achieve a final concentration of 100 ng/ml LPS for cell treatment, 1 µl of 100 µg/ml working solution LPS was added per ml of cells (7 µl per flask).

At specific time intervals (0, 6, 8 or 24 hours) flasks were inverted carefully to re-suspend cells and 1 ml samples were removed and placed on ice. The RNA was then removed. In the case of adherent LPS-treated cells the flasks were tapped to loosen adhered cells prior to sample removal or attached cells were lysed in the flask with Trizol.

3.4.4 Lysis of cells and RNA extraction

The protocol from Section 2.4.3.1 was followed for RNA extraction and storage.

3.4.5 First strand cDNA synthesis

The procedure described in Section 2.4.3.2 was followed for synthesis of cDNA.

3.4.6 Conventional RT-PCR

3.4.6.1 Oligodeoxynucleotide primers

All oligo-deoxynucleotide primers were purchased from Sigma-Aldrich. Oligo-deoxynucleotide primer pair sequences used in this Chapter are listed in Table 1 of Appendix 2 and prepared according to the method described in Section 2.3.12. Working oligo-deoxynucleotide primer solutions were prepared for each

cytokine by adding 80 µl of TE buffer (described in Section 2.3.10.2.) to 10 µl each of forward and reverse primer stock, and stored at 4°C.

3.4.6.2 Conventional RT-PCR analysis

The procedure in Section 2.4.3.3 was followed for conventional RT-PCR analysis to confirm cDNA synthesis from RNA, using the primer set for the housekeeping gene β-actin.

3.4.6.3 Electrophoresis of DNA

A 2% gel was run to confirm that cDNA had been successfully amplified using the primer pairs for β-actin. Samples (10 µl) were run along-side 10 µl of 100 bp DNA ladder (Invitrogen). Gels were prepared and electrophoresed as described in Section 2.4.3.4. DNA was illuminated using UV and photographed using the GEL-DOC system (Bio-Rad). Once the presence of β-actin was confirmed the samples were then used to further quantify the cytokine levels in quantitative real-time RT-PCR (qRT-PCR).

3.4.7 Quantitative Real-Time PCR

The method qRT-PCR was used to provide a quantitative measure of the transcription of mRNA of target genes for the proteins of interest. SYBER Green® (Roche) was used as a fluorescent marker, allowing any double-stranded DNA generated during PCR to be selected. Specificity of the PCR products was confirmed by dissociation curve analysis and electrophoresis on a 2% agarose gel.

Purified RNA was prepared for qRT-PCR according to a published method (Konnai, Usui *et al.* 2003) adapted by Dr Ray Cursons (University of Waikato) as follows.

All steps were carried out in a dedicated PCR cabinet. Quantitative RT-PCR was performed using a Rotor-Gene™ 6000 instrument (Corbett Research) to perform the PCR reaction, including fluorescent emission and detection of the signals. The qRT-PCR reactions were carried out in a total volume of 20 µl in 100 µl thin-walled tubes (Corbett Research).

3.4.7.1 qRT-PCR reaction master mix

A reaction master mix was prepared consisting of: Master Mix (10x) Thermostart® Reaction Buffer (AB Ltd), 1/20,000 dilution of SYBR® Green I dye (Invitrogen), 250 µmol/l deoxynucleotide triphosphates (Invitrogen), 5 mmol/l $MgCl_2$ (pH 8.5) (Invitrogen), 0.5 U of ABGene Thermostart® DNA polymerase (AB Ltd), 5 pmol of specific primers, and 200 ng of cDNA (measured using a Nanodrop Spectrophotometer).

A frequent cause of concern among investigators performing qRT-PCR is false positives caused by genomic DNA contamination from the sample itself or the environment it was prepared in. For this reason a negative control was used to check there was no genomic DNA contamination of the reagents by substituting cDNA with sterile water for every reaction set.

3.4.7.2 RT-PCR thermo-cycling program

Touchdown thermo-cycling steps were as follows:

1. Initial denaturation at 95°C for 10 minutes
2. 94°C for 20 seconds
3. Annealing temperature started at 70°C for 20 seconds and then decreased at 1°C/cycle for 20 seconds for the first 15 cycles
4. Extension at 68°C for 40 seconds
5. Fluorescence acquisition step at 80°C for 15 seconds (excitation at 470 nm, detection at 510 nm)
6. Steps 2-5 repeated 39 times

3.4.7.3 SYBR® Green I melting curve analysis

A melting curve analysis followed touchdown thermo-cycling to ensure products were specifically amplified. PCR products were heated from 75°C to 99°C in 0.5°C increments every 5 seconds. The denaturing conditions dissociated the double stranded DNA into single strands as the melting temperature (Tm) was reached for each specific PCR product. As a consequence of denaturing, the SYBR® Green I dye is released and fluorescence intensity drops. This indicates the correct Tm for the gene.

Seven genes were selected for study and each sample was analysed for both the target gene and an endogenous control gene β2-microglobµlin (β_2M), a housekeeping gene with expression that remains fairly consistent regardless of treatment (Ishii, Wallace *et al.* 2006). The expression levels of the target genes were reported relative to the expression levels of β_2M.

Each primer pair has a specific product melting point above 80°C influenced by product length and GC%. Examples are shown in Figures 3.1 and 3.2.

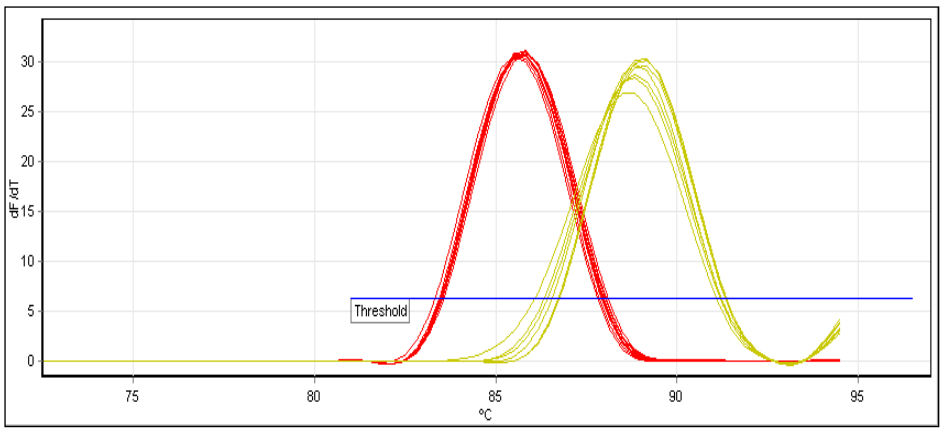

Figure 3.1: CYBR® Green I melting curves for β_2M (85.6 °C) and IL-1β (88.9 °C).

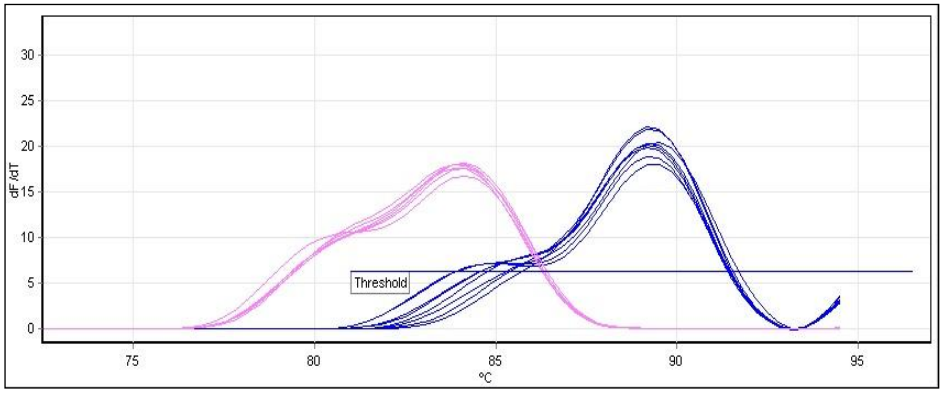

Figure 3.2: CYBR® Green I melting curves for IL-10 (85.6 °C) and TGF-β (88.9 °C).

Products with melting points different from those expected were considered to be miss-primed or non-specific, and therefore the mRNA not present in large enough copy numbers to be detected. A result of zero was given to these samples for the purposes of comparing amplification for gene of interest (GOI) to the housekeeping gene, β_2M. All primers were selected to span intron sequences so that genomic DNA amplification was not possible.

End products for real-time PCR were further analysed by gel electrophoresis (Figure 3.3). This verifies the size of the amplified product by comparison with the standard ladder, and shows up contamination in the form of multiple bands. The agarose gel was prepared as described in Section 2.4.3.4 omitting the ethidium bromide because SYBER Green® dye was present. Product bands were visualised using a GIBCO BRL ultra-violet trans-illuminator (Life Technologies) at 312 nm, photographed using a COHU High Performance CCD camera and digitally stored. All product band base-pair lengths were checked by comparison with a 100 bp DNA ladder (Invitrogen) unless otherwise stated.

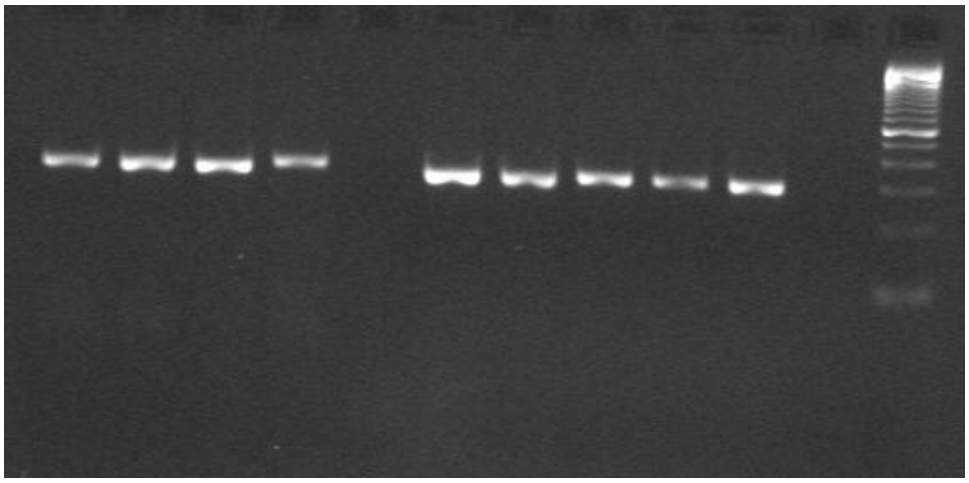

Figure 3.3: Quantitative real-time RT-PCR products run on a 2% gel. IL-1β (315 bp) and β_2M (295 bp) are shown alongside a 100 bp ladder.

3.4.8 Analysis of real-time quantitative RT-PCR results

A linear regression program (LinReg) used exported fluorescence level data from each cycle during the real-time quantitative PCR runs. Regression lines for each sample were calculated to fit the log-linear phase of the data points. A minimum of four data points were included in the data analysis for each sample, and the slope was chosen for each data set by taking in combination the slope closest to the maximum and the highest r^2 value.

Only samples with PCR efficiencies above 1.5 were selected. The initial copy number (ICN - the starting concentration (N^o) of the cDNA template of each sample was calculated from the intercept extrapolated from the straight line that fitted best to include the data points which were expressed in terms of fluorescence intensity.

All values were expressed as the expression relative to the control sample (time 0) and corrected for β_2M gene expression (endogenous control gene). To normalise the level of fluorescence of each gene of interest the following equation was used:

Step 1

$$\text{Relative expression at time 0} = \frac{\text{Concentration N}^{o}\text{ value for GOI a time 0}}{\text{Concentration N}^{o}\text{ for }\beta_2\text{M at time 0}}$$

Step 2

For each time point *(x*) the relative expression of the GOI was calculated by normalising the levels of fluorescence to that obtained for β_2M at the same time point:

$$\text{Relative expression at time } x = \frac{\text{Concentration N}^{o}\text{ value for GOI at time } x}{\text{Concentration N}^{o}\text{ value for }\beta_2\text{M at time } x}$$

Step 3

The relative expression of the GOI at time *x* was then normalised for the relative expression of the same gene at time 0, by dividing the value obtained from Step 2 by the value obtained from Step 1.

3.5 Results

Results from RT-PCR are displayed graphically (Figures 3.4 – 3.9) by the calculated individual initial copy number (ICN) of the GOI cDNA templates, relative to the reference gene (housekeeping β_2M). This provides a normalised expression so that the results can be compared with each other directly. Results were analysed by ANOVA and Tukey's multiple pairwise comparisons compared

with the non-activated control (monocytes) and or the LPS-activated control (macrophages). A confidence level of $p<0.001$ was selected for the analysis.

Figure 3.4 shows LPS had a significant effect ($p<0.001$) of increasing the expression of IL-1β (from 0.009 to 0.7) compared with non-treated control monocytes, whereas honey does not. The expression of IL-1β in LPS-activated macrophages was not significantly affected by the addition of honey.

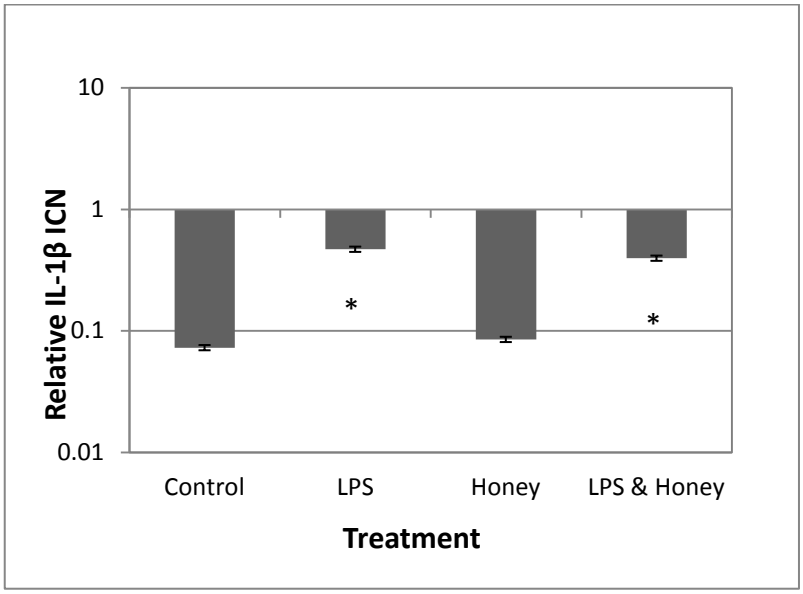

*Figure 3.4. The effect of honey (0.25%) and LPS (100 ng/ml), separately and in combination, on IL-1β gene expression after 24 h treatment. Results were calculated as individual initial copy number (ICN) of the GOI cDNA templates, relative to the reference gene (housekeeping β_2M) from the same cell population. Error bars show ± 1 SD of the mean of five experiments. *p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with un-treated monocytes.*

Figure 3.5 shows that LPS and honey each significantly ($p<0.001$) increased the expression of the IL-1ra (from 0.6 to 1.5 and 1.1 respectively), from the expression in non-treated control monocytes. The effect of honey on the expression of the IL-1ra in macrophages activated by LPS was to give a significant ($p<0.001$) further increase (about four times the increase that resulted from LPS alone, from 1.5 to 6).

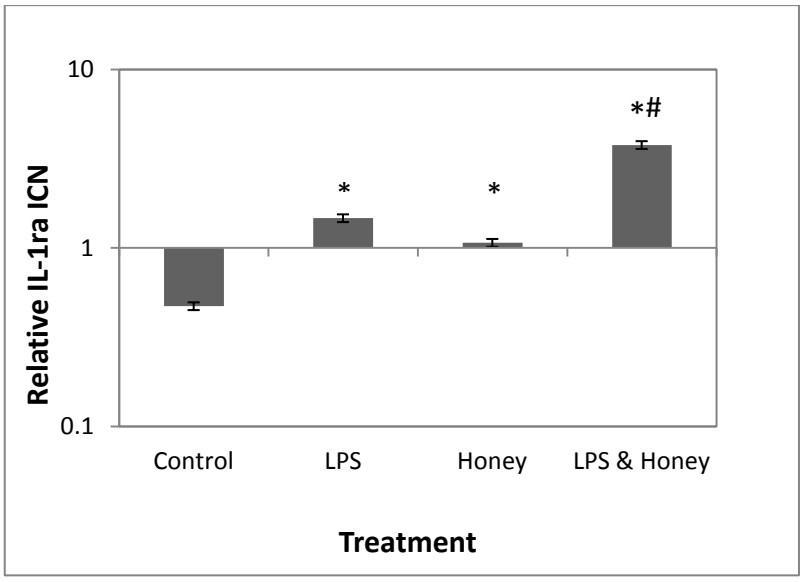

*Figure 3.5. The effect of honey (0.25%) and LPS (100 ng/ml), separately and in combination, on IL-1ra gene expression after 24 h treatment. Results were calculated as individual initial copy number (ICN) of the GOI cDNA templates, relative to the reference gene (housekeeping β_2M) from the same cell population. Error bars show ± 1 SD of the mean of five experiments. *p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the un-activated monocytes. #p<0.001 analysed by ANOVA compared with the LPS-activated control.*

Figure 3.6 shows that LPS and honey each significantly (p<0.001) increased the expression of TNF-α compared to non-treated control monocytes, but the increase with honey was less than that with LPS. When honey was added to LPS-activated macrophages this resulted in a significant (p<0.001) reduction of TNF-α expression (decreased from 6 to 1), yet the expression level was still higher than with honey alone (0.1).

Figure 3.7 shows that LPS and honey each increased the expression of IL-10 significantly (p<0.001) (0.065 and 0.05 respectively) compared with the expression of non-treated control monocytes (0.006). In the LPS-activated macrophages honey had a significant (p<0.001) effect of increasing the expression of IL-10 beyond that caused by LPS (from 0.065 to 0.6).

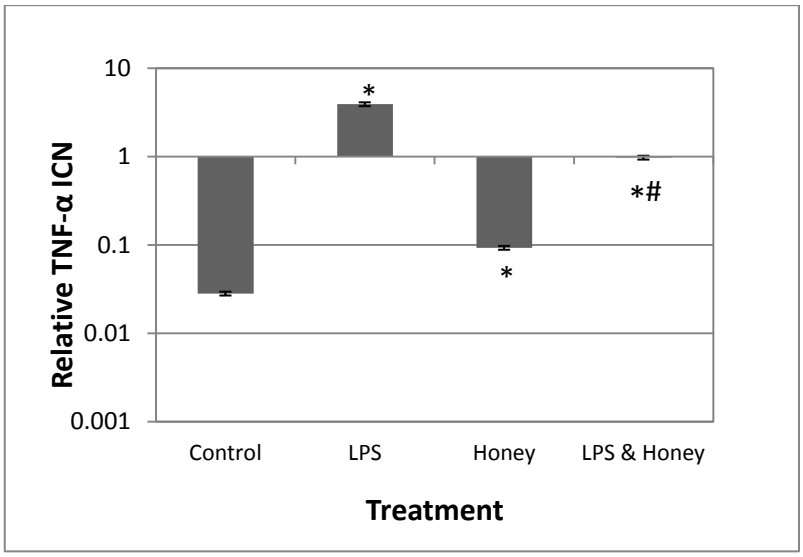

*Figure 3.6. The effect honey (0.25%) and LPS (100 ng/ml), separately and in combination, on TNF-α gene expression after 6 h treatment. Results were calculated as individual initial copy number (ICN) of the GOI cDNA templates, relative to the reference gene (housekeeping β_2M) from the same cell population. Error bars show ± 1 SD of the mean of five experiments. *p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the un-activated monocytes. #p<0.001 analysed by ANOVA compared with the LPS-activated control.*

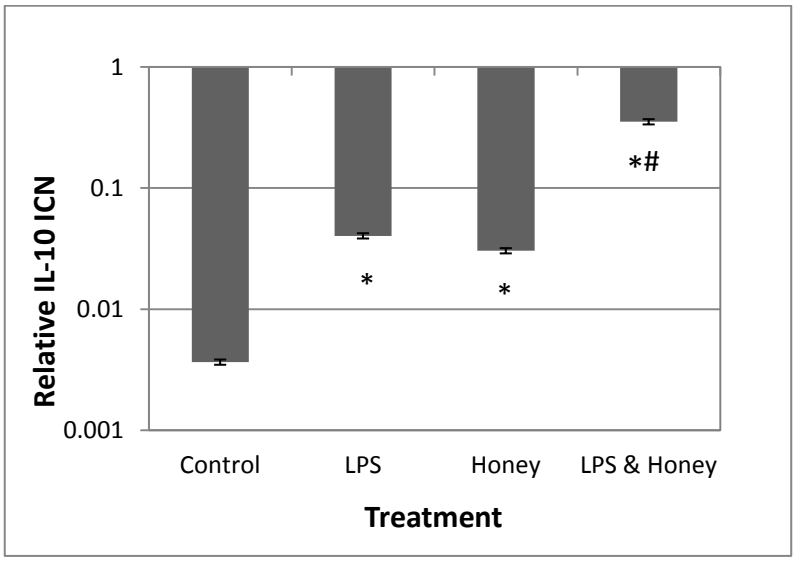

*Figure 3.7. The effect of honey (0.25%) and LPS (100 ng/ml), separately and in combination, on IL-10 gene expression after 6 h treatment. Results were calculated as individual initial copy number (ICN) of the GOI cDNA templates, relative to the reference gene (housekeeping β_2M) from the same cell population. Error bars show ± 1 SD of the mean of five experiments. *p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the un-activated monocytes. #p<0.001 analysed by ANOVA compared with the LPS-activated control.*

Figure 3.8 shows that LPS and honey each significantly (p<0.001) increased the expression of TGF-β compared to the expression in non-treated control monocytes (from 0.5 to 1 and 7.5 respectively). In LPS-activated macrophages honey significantly (p<0.001) reduced the expression of TGF-β compared with that caused by LPS (from 1 to 0.75) yet this expression level was still significantly (p<0.001) higher than in non-activated monocytes (0.5).

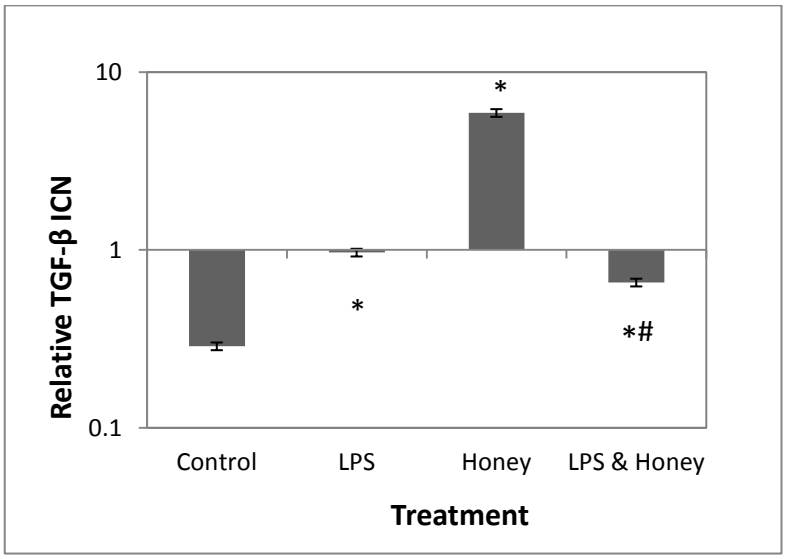

*Figure 3.8. The effect of honey (0.25%) and LPS (100 ng/ml), separately and in combination, on TGF-β gene expression after 8 h treatment. Results were calculated as individual initial copy number (ICN) of the GOI cDNA templates, relative to the reference gene (housekeeping β_2M) from the same cell population. Error bars show ± 1 SD of the mean of five experiments. *p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the un-treated monocytes. #p<0.001 analysed by ANOVA compared with the LPS-activated control.*

Figure 3.9 shows that LPS and honey each significantly (p<0.001) reduced the expression of PDGF compared with the expression in non-treated control monocytes (from 1 to 0.001 and 0.085 respectively). In LPS-activated macrophages honey significantly (p<0.001) increased the expression of PDGF compared with that caused by LPS (from 0.01 to 4). The expression level with

honey combined with LPS was significantly (p<0.001) higher (4) than in non-activated monocytes (1).

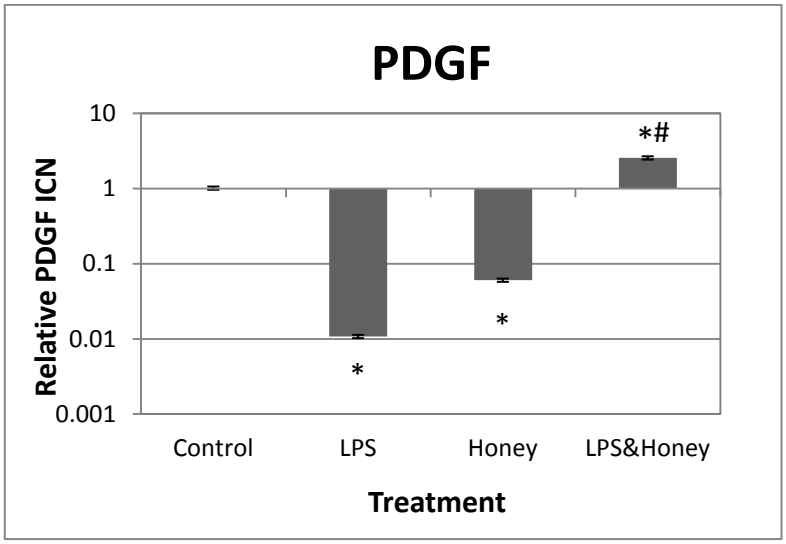

*Figure 3.9. The effect honey (0.25%) and LPS (100 ng/ml) on PDGF gene expression after 8 h treatment. Results were calculated as individual initial copy number (ICN) of the GOI cDNA templates, relative to the reference gene (housekeeping β_2M) from the same cell population. Error bars show ± 1 SD of the mean of five experiments. *p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the un-treated monocytes. #p<0.001 analysed by ANOVA compared with the LPS-activated control.*

Table 3.1. Summary of cytokine gene expression in response to honey (0.25%), in un-activated THP-1 cells (monocytes) and LPS activated THP-1 cells (macrophages). A significant increase or decrease in relative ICN gene expression is cited compared to the non-honey treated controls. Data taken from Figures 3.4-3.9.

Cytokine/growth factor	Un-activated monocytes	LPS activated macrophages
IL1-β	No change	No change
IL1ra	Increase 0.3 - 1	Increase 1.5 - 6
TNF-α	Increase 0.04 - 0.1	Decrease 6 - 1
IL-10	Increase 0.0065 - 0.05	Increase 0.065 - 0.6
TGFβ	Increase 0.5 - 7.5	Decrease 1 - 0.75
PDGF	Decrease 1 - 0.85	Increase 0.01 - 4

3.6 Discussion

Artificial honey had no effect on cytokine expression in monocytes or LPS-activated macrophages which is to be expected due to the low dilution rate of 0.25% (results not shown). This low concentration adds only a very small amount of extra sugars to what was already available to the cells in the medium. A similar finding has been reported by other researchers as well (Tonks, Cooper *et al.* 2003).

Shown in Figures 3.4 – 3.9 are the effects of LPS, honey or a combination on the expression of cytokines in THP-1 cells. Honey induced TNF-α expression significantly (p<0.001), but the increase was not to the magnitude expected in THP-1 cells compared with research reported elsewhere (Tonks, Cooper *et al.* 2003). This may be due to the different cell lines or honey varieties being used in the different studies. It can be concluded that honey has a modulating effect, reducing the expression of TNF-α stimulated by adding LPS to the cells, and this is in line with published findings (Tonks, Cooper *et al.* 2003). This may not be a direct effect of honey on TNF-α expression, but as a result of increasing one or more of the anti-inflammatory cytokines, which, in turn, cause a decrease in TNF-α expression through various feedback loops *etc.*

As mentioned previously, the significance of increasing the cytokine expression of TNF-α may lie in one of two possibilities (or both): 1) TNF-α can acts as an anti-inflammatory cytokine by negatively regulating the IL-1 system by inducing the release of IL-1 type II decoy receptor, 2) TNF-α can act in a pro-inflammatory manner by initiating the NF-κB pathway important in the onset of inflammation. The research presented in this chapter potentially explains one of the mechanisms at least, for the therapeutic properties of honey of reducing inflammation. A topical application of honey to an uninfected wound (*ie*: no bacterial LPS present) would be expected to initiate inflammation allowing healing to begin or allow inflammation to conclude in a controlled way by slightly increasing the expression of TNF-α and initiating the NF-κB pathway. Whereas a

topical application of honey to an infected wound where LPS is present and therefore the tissue is inflamed, would reduce the expression of TNF-α preventing a chronic state of inflammation by allowing the inflammation to resolve.

IL-1β gene expression was significantly (p<0.001) increased by LPS activation in monocytes. Honey did not significantly affect the gene expression of IL-1β either with or without LPS treatment. Interestingly, there was an increase in IL-1ra expression by honey. Honey and LPS separately had a significant (p<0.001) effect by increasing the expression of IL-1ra from that in non-activated monocytes, and honey had a significant (p<0.001) additive effect by increasing the expression from that in LPS-activated macrophages (from 1.5 – 6). So while the inflammatory IL-1β was not changed in response to honey as previously published, the anti-inflammatory competitor (for receptor sites) was increased. This may allow the monocytes/macrophages to recruit more immune cells to the affected site, yet dampen down the inflammatory response of these recruited cells so inflammation is not uncontrolled and thus healing can take place.

The anti-inflammatory cytokine IL-10 was also significantly (p<0.001) increased in honey-treated THP-1 cells. Honey had approximately the same effect on IL-10 expression levels as LPS did on non-activated THP-1 cells, but when used in conjunction with LPS had a significant (p<0.001) additive effect (from 0.065 – 0.6). As mentioned previously, research has shown that increasing levels of IL-10 inhibit macrophage differentiation from monocytes by 20%, resulting in an anti-inflammatory effect (Gredmark 2004). IL-10 works as an anti-inflammatory cytokine to down-regulate the switch from monocyte to macrophage and is primarily produced by monocytes. Increased levels of IL-10 at the wound site will reduce the number of macrophages being activated. Fewer active macrophages at a wound site will mean fewer recruited inflammatory cells. While this percentage is not large it may be all that is required to switch the trend towards chronic inflammation back to controlled acute inflammation. Gredmark (2004) noticed that increased levels of IL-10 also resulted in

macrophages with a lower ability for attachment to a Transwell filter system designed for migration assays, resulting in a more monocyte phenotype. Monocytes are much more spherical and uniform in appearance and this was definitely observed in honey-treated cells. While their activity increased from that of untreated monocytes, they were more spherical in phenotype than the LPS-activated THP-1 cells (see Appendix 1 for comparison of phenotype and activity between treatments).

The expression of the growth factors, PDGF and TGF-β, was examined and conflicting results were produced. In honey-treated cells PDGF expression was significantly decreased. A reduction in PDGF will result in reduced cell migration to the site, which results in an anti-inflammatory effect (Guan, Fan *et al.* 2009). So in controlled inflammation, this would be of benefit to the tissue. LPS-treated cells also had a significant ($p<0.001$) reduction in PDGF expression but to a greater extent. When honey and LPS were used in conjunction, the expression of PDGF significantly increased to that above the normal control level. Increased levels of PDGF induce increased formation of granulation tissue and increased wound-breaking strength, hastening the rate of healing as mentioned previously. This would be of great benefit to an inflamed wound, especially a chronic wound where healing is delayed. Indeed, these therapeutic properties have already been identified in honey-treated wounds, as mentioned previously and this increase in PDGF may be responsible for these findings. Perhaps the reason for reduced healing in chronic wounds is explained by the decrease in PDGF in LPS-treated monocytes. A badly infected wound that will not heal obviously benefits from honey application, and it may be due to the PDGF levels increasing from the reduced rate observed here.

Expression of TGF-β was observed to significantly increase when either honey or LPS was present. The honey-induced expression in the cells was significantly higher than that induced by LPS. When honey was used in conjunction with LPS there was significantly less expression than with either alone but expression remained at a significantly higher level than in the untreated control suggesting

that honey partially abrogates the effect of LPS. As mentioned previously, TGF-β has been shown to slow wound healing, yet excessive levels are associated with fibrotic disease (Hakvoort 2000; Zhou, Li *et al.* 2004).

The ability of honey to increase the expression of TGF-β will be of great benefit to the wound as the healing rate will be increased. In the absence of LPS, honey increased TGF- β expression 15-fold, while in the presence of LPS only a partial (50%) reversal was seen. To speculate, the reduced expression of TGF-β when LPS is present may prevent any fibrotic impairment. Future work on the effect of honey on fibroblasts will be necessary to confirm this. Based on these results it can be theorised that honey will reduce the time a wound takes to heal and prevent excessive scar tissue being formed. These therapeutic properties have already been reported for honey (Al-Waili and Saloom 1999, Al-Waili, 2011) so these findings may provide the mechanism of action.

When these results are considered together it seems highly likely that honey may be having an anti-inflammatory effect on monocytes, and that these effects could also be taking place *in vivo* on monocytes through its effects on expression of genes for cytokines, and potentially other immune cells such as neutrophils and fibroblasts. It must be appreciated though, that the gene expression of a gene does not mirror the translation and production of the protein. Certainly it has been shown that honey hastens wound healing, reduces redness and pain associated with inflammation and reduces scaring (Molan 1999). Achieving a therapeutic balance between limiting inflammation and stimulating repair is important, and honey may be doing this by modulating expression of growth factors as well as cytokines. Further work on protein translation for the cytokines gene expression mentioned here will be important.

As honey increased and decreased the expression of different cytokines, it was of interest to see how the THP-1 cellular phenotype changed during these treatments. The honey-treated monocytes were observed to have increased

pseudopodia activity and it was postulated that these cells would have an increased phagocytosis rate. This became the focus of study for Chapter 4.

Future studies should aim to investigate the effect of honey on neutrophil and monocyte attachment to endothelial cell monolayers. Published work indicates that anti-inflammatory drugs decrease attachment (Diaz-Gonzalez, Gonzalez-Alvaro *et al.* 1995). While honey-treated cells seemed to clump, with increased attachment to each other, the increased IL-10 levels may result in less attachment to other cell types, thus reducing cell migration and damping down the inflammatory feedback loops. Investigating the mechanism of the clumping observed in honey-treated cells should be undertaken. This would be relevant to the current work on inflammation.

These results confirmed previous findings that honey increases the expression of TNF-α (Tonks, Cooper *et al.* 2001; Tonks, Cooper *et al.* 2003), though their finding that is also increased the expression of IL-1β and IL-6 was not confirmed in the present study. This was thought to be due to low expression of the IL-6 gene. An ELISA assay may prove to be more sensitive.

Chapter 4

The effect of honey on the phagocytic activity in THP-1 macrophages

4.1 Summary

In the work in Chapter 3 it was observed that treatment of monocytes with LPS and Manuka honey increased pseudopodia activity, resulting in clumping of cells and increased mobility. As mentioned earlier (Section 2.2) monocytes do not phagocytose yet can be transformed into phagocytic macrophages after exposure to activators such as LPS. It was hypothesized that the honey treatment may be acting as an activator by causing differentiation of monocytes into macrophages, and therefore may be causing an increase in phagocytosis. A phagocytosis assay was used to quantify the effect of Manuka honey on the phagocytosis of LPS-activated THP-1 macrophages and non-activated THP-monocytes. Manuka honey had the unexpected effect of reducing the rate of phagocytosis in THP-1 cells.

Manuka honey (up to 1%) prevented up to 80% of the phagocytosis of latex particles, *E.coli* and BCG, and zymosan particles. To characterize the component in Manuka honey contributing to the change in the phagocytosis rate, honey was

dialysed to separate the sugars from the honey and the dialysate and retentate were assayed in the phagocytosis assay with latex particles.

It was established that the active component in Manuka honey was a high molecular weight compound, inhibiting phagocytosis.

4.2 Introduction

Phagocytosis is a specific form of endocytosis involving the actin-based vesicular internalization of solid particles, such as bacteria, a major mechanism used to remove pathogens and cell debris. Phagocytosis is distinct from other forms of endocytosis such as pinocytosis which is the vesicular internalization of various liquids (Doherty and McMahon 2009).

Phagocytosis can be viewed as either an opportunity or an obstacle for microbial organisms. Some pathogens have evolved strategies to enter and survive within phagocytic cells and others have developed ways to prevent their phagocytosis as part of their pathogenic profile (Celli and Finlay 2002). Of interest to this research are the receptors for phagocytosis. To discriminate between pathogens and self, macrophages have evolved a number of phagocytic receptors, such as the mannose receptor, that recognize conserved motifs on pathogens. When a pathogen binds to a receptor the innate immune system is initiated, which, in turn, coordinates the adaptive response where the immune cells are primed to recognize non-self antigens and form an immunological memory. Pathogens and foreign objects are also phagocytosed by complement receptors after relatively nonspecific opsonization with complement, and by Fc receptors after specific opsonization with antibodies. All these receptors induce rearrangements in the actin cytoskeleton leading to internalization of the particle and subsequent degradation (Allen and Aderem 1996; Aderem and Underhill 1999; Celli and Finlay 2002).

The best characterized phagocytic receptors on macrophages are the complement receptor 3 (CR3), Fc gamma receptor (FcγR), mannose receptor (MR) and scavenger receptor (SR). The CR3 and FcγR are involved in the uptake of opsonized micro-organisms during infection. Opsonization involves the binding of an opsonin (antibody), to an receptor (antigen) on the pathogen's cell membrane. After opsonin binds to the membrane, phagocytes are attracted to the pathogen and phagocytose it. CR3 binds C3bi on complement-opsonized targets, whereas FcγR bind to immunoglobulin G (IgG)-coated targets. Mannose receptors and scavenger receptors are involved in phagocytosis of un-opsonised targets. Phagocytosis by both types of receptors is driven by the reorganization of filamentous actin (f-actin), but to different extents and the mechanisms of uptake are different (Newman, Mikus *et al.* 1991; Allen and Aderem 1996).

CR3- and FcR-mediated phagocytosis is a relatively passive process where complement-opsonized particles appear to sink into the cell with elaboration of small, if any, pseudopodia. During FcR-mediated phagocytosis, the membrane rises above the cell surface and tightly surrounds the particle before drawing it into the body of the macrophage. Unopsonised targets are bound by the MR and SR and are dependent on actin rearrangement in a similar manner to FcγR (Kaplan 1977; Castellano, Chavrier *et al.* 2001; May and Machesky 2001; Tricker and Cheng). FcR, MR and SR actin rearrangement is accompanied by the activation of the respiratory burst (producing ROS) and by the production of arachidonic acid metabolites, a key inflammatory intermediate, and inflammatory cytokines, such as TNF-α (Celli and Finlay 2002). CR3-dependent uptake can occur in the absence of any of these pro-inflammatory signals (Caron and Hall 1998).

A substance or process which impedes or prevents the action of phagocytes is termed anti-phagocytic. Cytochalasin B is a cell-permeable mycotoxin. It shortens actin filaments by blocking monomer addition at the fast-growing end of polymers. At high concentrations it inhibits motility, adhesion and macrophage fusion, but at lower concentrations F-actin-dependent phagocytosis

and macrophage fusion is inhibited (DeFife, Jenney *et al.* 1999). It is therefore an anti-phagocytic agent and routinely used to identify which phagocytic receptors are being used in an assay for phagocytosis (DeFife, Jenney *et al.* 1999; May and Machesky 2001).

Yeast mannans have been shown to inhibit binding and phagocytosis of zymosan (yeast cell wall particle) by macrophages (Sung, Nelson *et al.* 1983). β-glucan also binds Dectin-1 preventing phagocytosis. (Brown, Taylor *et al.* 2002; Esteban, Rodriguez *et al.* 2004; Taylor, Brown *et al.* 2004).

Phagocytosis can be enhanced by opsonisation agents. Mannan-binding lectin (MBL) constitutes an important part of the human innate immune defence system. It has been shown to mediate the activation of complement upon binding to specific microbial carbohydrate motifs, to directly opsonise organisms, and to enhance the phagocytosis of targets (Arora, Munoz *et al.* 2001).

During cell preparation for RT-PCR assays (Chapter 2) it was observed that after activation with LPS, THP-1 cells adhered to the flask. Microscopy work showed that the cells were changing morphology and that different activation treatments (PMA, LPS, Vit-D3) gave different cell morphologies (Section 2.4). Time-lapse photography of these cells showed that with activation the cells became adherent, motile and possessed pseudopodia. Honey treatment in conjunction with LPS treatment caused the cells to clump and form large mobile aggregations. It was assumed that honey was increasing their ability to adhere or perhaps increase their scavenging ability. It was postulated that honey increased phagocytosis, based on these observations during time-lapse movies (presented in Appendix 1).

The phagocytosis assay was modified to test the effect of the addition of honey on phagocytosis of fluorescent latex particles, two strains of bacteria, *E. coli* and BCG (Bacille Calmette Guerin), and zymosan. Versions of *E.coli* and BCG expressing green fluorescent protein and FITC-labeled zymosan particles, were obtained so that the phagocytosis of the bacteria/particles could be viewed and

counted in the same way as the fluorescent latex particles. The green fluorescent protein (GFP) is a 27 kDa protein originally isolated from the jellyfish *Aequorea victoria*/*Aequorea aequorea*/*Aequorea forskalea* that fluoresces green when exposed to blue light (Prendergast 1978). GFP has many characteristics that make it useful for localization studies in bacteria, primarily its ability to fluoresce when fused to target polypeptides without the addition of exogenously added substrates (Tsien 1998).

Latex particles, *E. coli* and zymosan are all commonly used in phagocytosis assays using THP-1 cells (Akiko, Ikuko *et al.* 2008). *Mycobacterium tuberculosis* is a facultative intracellular pathogen, which can reside within the macrophages of the host. The major immune-pathology associated with tuberculosis results from interactions between the live tubercle bacillus and the host's immune response. Mycobacteria have the ability to enter a number of different cell types, but the primary cell type that they are thought to replicate within during human disease is macrophages (El-Etr and Cirillo 2001). It gains entry to the host cell by taking advantage of the phagocytic cells' ability to phagocytose as a result of binding the mannose receptor (Kang 1998; Astarie-Dequeker, N'Diaye *et al.* 1999; Diaz-Silvestre, Espinosa-Cueto *et al.* 2005). BCG is an attenuated vaccine strain, yet it retains its ability to survive within the macrophage (Monahan, Betts *et al.* 2001).

4.2.1 Outline of experiments

The phagocytosis assay utilizing fluorescent latex particles (Section 2.4.4) was modified to include the addition of honey (0.25% - 2%) to measure its effect on the phagocytosis rate of THP-1 macrophages (Section 4.3.1 and Section 4.3.2). Further assays used *E.coli* (Section 4.3.3), BCG (Section 4.3.4) and zymosan (Section 4.3.5) as the phagocytic particle. To determine whether honey was directly affecting the cytokine expression a medium change was done prior to assay (Section 4.3.6). Then honey was fractioned to characterize the component responsible for the inhibition of phagocytosis which honey was found to give. This included dialysis of honey which identified the high molecular weight

component being active (Section 4.3.7). Cytochalasin B was used in a phagocytosis assay to identify whether f-actin rearrangement was taking place in latex particle phagocytosis (Section 4.3.8).

4.3 Methods

4.3.1 Phagocytosis assay with latex particles

The phagocytosis assay procedure was as described in Section 2.4.4 with various final concentrations of honey (0.125% – 2%) added at various time points. Freshly sub-cultured cells (500 µl ~ 1 x 10^6 cells) were transformed with LPS (100 ng/ml) for 24 hours before treatment with honey in the assay wells. A control was included which received only medium. The method used to calculate the reduction of phagocytosis was as described in Section 2.4.4.

4.3.1.1 Honey

The honey solutions used are described in Chapter 3.4.1.1. Manuka (M144) and Pasture (P59), Kanuka (K17) and Clover (CLO42) and artificial honey were selected for individual assessment using this assay. All other honeys referred to can be found in Appendix 3. Honey was added to wells to achieve final concentrations ranging from 0.125% – 2% diluted in culture medium. Control wells had only culture medium added. To achieve the final 0.125% concentration of honey, 25 µl of 5% diluted honey was added per 500 µl of cells, 50 µl for 0.25%, 100 µl for 0.5%, 200 µl for 1%, 400 µl for 2% respectively. The medium was topped up to achieve a final assay volume of 1 ml per well. The honey solution was added to the well after the extra medium.

4.3.2 Phagocytosis assay with GFP *E.coli*

The phagocytosis assay described in Section 4.3.1 was carried out replacing the latex particles with GFP *E.coli*.

4.3.2.1 Luria-Bertani medium

A Luria-Bertani (LB) medium was made by dissolving 40 g LB powder (Invitrogen Cat. No.# 12795-0-84) in 1 l of deionized water. The pH of the solution was adjusted to 7.4 using sodium hydroxide. The solution was then autoclaved at 121°C for 20 minutes to sterilize the medium. The mixture was allowed to cool down to about 50 to 60°C then the antibiotics (50 µg/ml ampicillin) were added. The medium was then stored at 4°C.

4.3.2.2 Luria-Bertani plate

Bacteriological agar (15 g) was added to the LB medium before autoclaving. The solution was mixed and then autoclaved at 121°C for 20 minutes. The solution was allowed to cool down to about 50 to 60°C then the antibiotics (50 µg/ml ampicillin) were added. The solution was poured into petri dishes and kept at room temperature for the medium to solidify. The plates were stored at 4°C in plastic bags.

4.3.2.3 GFP *E.coli* pBAD24

A GFP *E. coli* pBAD24 strain was purchased (Bio-Rad). One loop scraping from an inoculated LB plate was re-suspended in LB medium and grown at 37°C overnight using sterile techniques.

GFP *E.coli* were induced to fluoresce using the arabinose-inducible pBAD24 vector, a plasmid vector containing the PBAD promoter of the araBAD (arabinose) operon. In the presence of arabinose, low levels of the GFP protein rapidly saturate the cell and when observed under blue light glow green.

GFP *E. coli* pBAD24 were induced with arabinose (0.1% final concentration) 2 hours before use. Cells were grown for a further 2 to 3 h, harvested by centrifugation, and washed and re-suspended in LB medium. The fluorescent *E. coli* cells were counted prior to assay on a haemocytometer.

4.3.2.4 Phagocytosis assay

The assay with GFP *E. coli* was carried out the same way as for the latex particle assay (Section 4.3.1) with the following modifications:

Cultures of THP-1 macrophages were inoculated with approximately 25 *E.coli* cells per THP-1 cell, with or without 0.5% honey present and left to incubate at 37°C for 2 hours for phagocytosis to take place.

Cells were checked for viability with trypan blue prior to counting to ensure cells had not been overwhelmed by the bacterial inoculation. Pasteurisation of *E.coli* was trialled to prevent adverse effects on THP-1 cells but this weakened the GFP fluorescence, preventing distinguishable phagocytosis. In the shortened two-hour incubation time viability was found to be comparable to un-infected cell lines not exposed to the infection, so the effect of phagocytosed bacteria was not considered to be lethal.

Cells were placed on a haemocytometer (20 µl of cell suspension) and THP-1 cells that phagocytosed bacteria counted the same as in the latex particle assay.

4.3.3 Phagocytosis assay with GFP BCG

The phagocytosis assay described in Section 4.3.1. was carried out replacing the latex particles with GFP BCG.

4.3.3.1 GFP BCG

GFP BCG were gifted by Dr Ray Cursons, University of Waikato, grown in Sauton's medium (0.2 g KH_2PO_4, 0.2 g $MgSO_4$, 1.6 g L-asparagine, 0.02 g $FeNH_4$ citrate, 0.96 g citric acid, 24 ml glycerol, made up to 400 ml with purified water. The pH was adjusted to pH 7.2. The solution was microwaved for 2 minutes to dissolve the agar, then autoclaved). Cultures were grown at 37°C.

4.3.3.2 Phagocytosis assay

The phagocytosis assay procedure was the same as the latex particle assay (Section 4.3.1.) but with honey added at a final concentration of 0.5%. BCG cells were counted on a haemocytometer and sub-cultured prior assay and used at a ratio of 25:1 (BCG:THP-1 cells).

Cells were placed on a haemocytometer (20 µl of cell suspension) and THP-1 cells that phagocytosed bacteria counted in the same way as the latex particle assay.

4.3.4 Phagocytosis assay with zymosan particles

The phagocytosis assay described in Section 4.3.1. was carried out replacing the latex particles with FITC zymosan particles.

4.3.4.1 Zymosan particles

Fluorescent labelled (FITC) zymosan particles prepared from the cell wall of the yeast *Saccharomyces cerevisiae* were purchased from Sigma-Aldrich (Cat. No.# Z4250).

4.3.4.2 Phagocytosis assay

The phagocytosis assay procedure was the same as the latex particle assay (Section 4.3.1.) but with honey added at a final concentration of 0.5%. Zymosan

particles were counted on a haemocytometer and diluted prior assay and used at a ratio of 25:1 (zymosan particles: THP-1 cells).

Cells were placed on a haemocytometer (20 µl of cell suspension) and THP-1 cells that phagocytosed zymosan particles counted in the same way as the latex particle assay.

4.3.5 Assay for the effect of cytokines on phagocytosis

It was established in the previous Sections that honey prevents phagocytosis by macrophages. It was hypothesized that the macrophages were responding to honey by releasing cytokines that somehow prevented other cells from undertaking phagocytosis by changing the function of the macrophage. In Chapter 3 it was identified that there was a modification of cytokine expression in response to exposure of the cells to honey. The cytokine expression profile of a monocyte or macrophage determines its function (Chapter 3). Factors that increase or decrease the pro- and/or anti-inflammatory cytokine expression will ultimately decide the function of the transformed macrophage and therefore its phagocytic ability.

To test this theory, activated THP-1 cells were treated with Manuka honey and /or LPS as described in Section 3.4.3. These cells were washed to remove honey and LPS, and placed in fresh growth medium. They were incubated for four hours to express cytokines. This conditioned medium (cell free but containing cytokines released from the THP-1 cells) was then removed and fresh THP-1 cells (not previously exposed to LPS or honey) were placed in the medium to respond to any cytokines released from the honey- or honey- and LPS-treated cells. Latex particles were then added to the cells as described in Section 3.4.3 and the rate of phagocytosis was quantified and compared with control cells that had the same treatments but not exposed to honey.

Artificial honey was used as a control for the sugar content of Manuka honey. The details of the procedure are given below in Section 4.3.5.1. As it has been

previously established (Chapter 2) that un-activated THP-1 cells (no activating treatment) do not phagocytose it was deemed not necessary to include a monocyte control.

4.3.5.1 Phagocytosis assay

THP-1 cells (5 ml) were treated in growth medium with 100 ng/ml LPS and 0.5% honey (flask 1). A control was repeated with artificial honey (flask 2). The flasks were incubated for 24 hours 37°C, 5% CO_2, and 95% relative humidity to allow activation.

After the incubation time the spent medium was removed in the honey treated flask and the control flask. The cells and flasks where washed 3 times in fresh growth medium. After this, 3 ml fresh growth medium was added to the flasks. The flasks were re-incubated for a further 4 hours to allow cells to release cytokines.

After the incubation the medium was removed from the honey treated flask (1) and the control flask (2). This conditioned medium was added to fresh monocytes, to give final volume of 3 ml (the growth medium from the fresh monocytes was removed first).

The flasks then had latex particles added (25 latex particles per cell) and the flasks incubated for 4 hours to allow phagocytosis. The cells that phagocytosed latex particles were then counted on a haemocytometer.

4.3.6 Phagocytosis assay with dialysed honey

In an attempt to characterize the component of honey responsible for the inhibition of phagocytosis, honey was dialysed to a high and low molecular weight fraction. These fractions were assayed for their effect on phagocytosis in the same manner as whole honey in Section 4.3.1.

4.3.6.1 Dialysis of honey

Twenty-five grams (18.2 ml) of Manuka honey (M144,) and Pasture honey (PS9) were each suspended in 25 ml of purified water and dialyzed against 1 l of tap water for 48 h at 4°C with three changes of water during this time. Dialysis tubing with a molecular mass cut off of 3 500 Da (Cellu Sep T1, Membrane Filtration Products, Inc., Seguin, TX; EEUU) was used. The dialysate and retentate was lyophilized and then reconstituted to 5 ml in purified water to concentrate the sample. Both samples were kept at -20°C until required.

4.3.6.2 Phagocytosis assay

The phagocytosis assay procedure was the same as for the latex particle assay (Section 4.3.1.) but with the addition of a 1% final concentration of honey retentate or dialysate.

4.3.7 The effect of Cytochalasin B on phagocytosis

Cytochalasin B was used in a phagocytosis assay to identify whether f-actin rearrangement was taking place during the latex particle phagocytosis.

4.3.7.1 Cytochalasin B

Dihydro-Cytochalasin B was purchased from Sigma-Aldrich (Cat. No.# C6762-1 mg) and dissolved in 1 ml dimethyl sulfoxide to create a stock solution. Cytochalasin B was then used at 12.5 µmol/l, 25 µmol/l and 50 µmol/l concentrations in RPMI 1640 medium.

4.3.7.2 Phagocytosis assay

The phagocytosis assay procedure was the same as for the latex particle assay (Section 4.3.1.) but Cytochalasin B added.

4.4 Results

All phagocytosis assays were performed in triplicate (at least) using the same equipment throughout the research. The latex particles were present for four hours unless otherwise stated. Bacteria and zymosan were present for two hours. After this time some bacteria/zymosan particles were digested and unable to be singularly identified in the macrophage, resulting in a glowing THP-1 cell. This may have resulted in a higher or lower result than for the latex particles.

4.4.1 Phagocytosis assay with latex particles

The results from main assay variations are presented here. The effect of varying concentrations of one sample (M144) of Manuka honey on phagocytosis after four hours of incubation are shown in Figure 4.1, and for 0.5% of the same Manuka honey, Pasture and Artificial honey on phagocytosis over different time periods are shown in Figure 4.2. Four hours after incubation with Manuka honey, all concentrations used (0.125% -2%) showed a significant (p<0.001) reduction in phagocytosis of latex particles compared with the phagocytosis of cells not treated with honey (Figure 4.1). A dose dependent response was observed, with higher concentrations of honey giving a higher inhibition of phagocytosis. Manuka honey gave a significant inhibition of phagocytosis at all the time points, but the degree of inhibition steadily decrease over time. Pasture honey gave a significant (p<0.001) reduction of phagocytosis at 2, 4 and 24 hours after latex bead addition but to a lesser extent than Manuka honey (14% reduction compared with 65% reduction for Manuka honey at 4 hours). Artificial honey had the opposite effect to honey by significantly (p<0.001) increasing the phagocytosis of latex particle at all time-points though this promotion of phagocytosis gradually reduced (32% at 2 hours, to 3% promotion at 24 hours after latex particle addition).

These results show that honey has a bioactivity beyond that of the sugar content alone, and that the bioactivity of Manuka honey is better than that of Clover honey. The bioactivity of both Manuka honey and Pasture honey could still be detected after 24 hours of treatment suggesting a lasting effect.

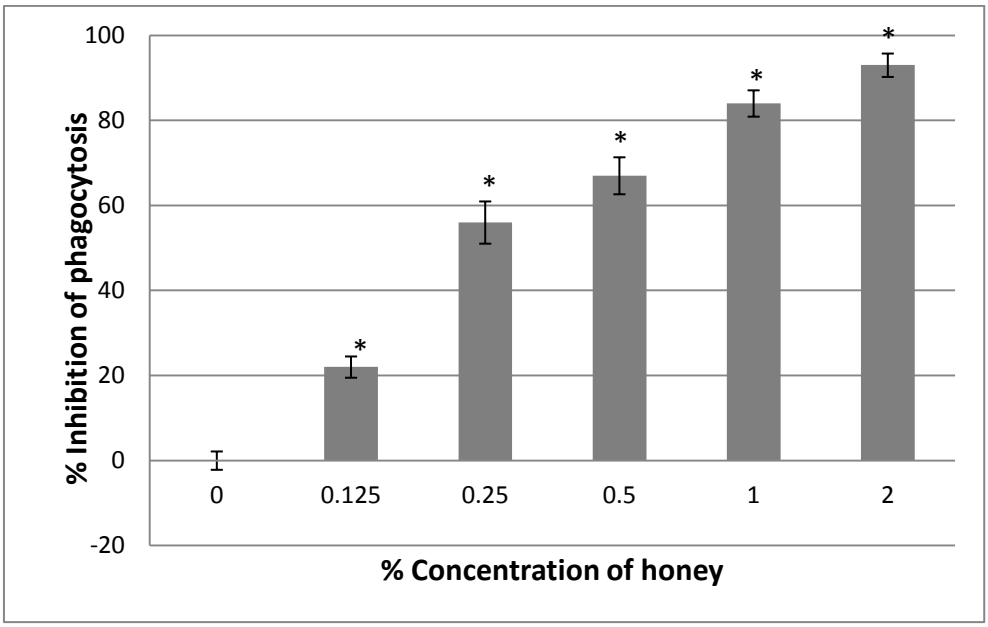

*Figure 4.1: The effect of increasing concentrations of Manuka honey on phagocytosis of latex particles in LPS-activated THP-1 cells. The assay was performed 4 h after latex particle addition. Error bars show ± 1 SD of the mean of 3 experiments. * p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the control (not treated with honey).*

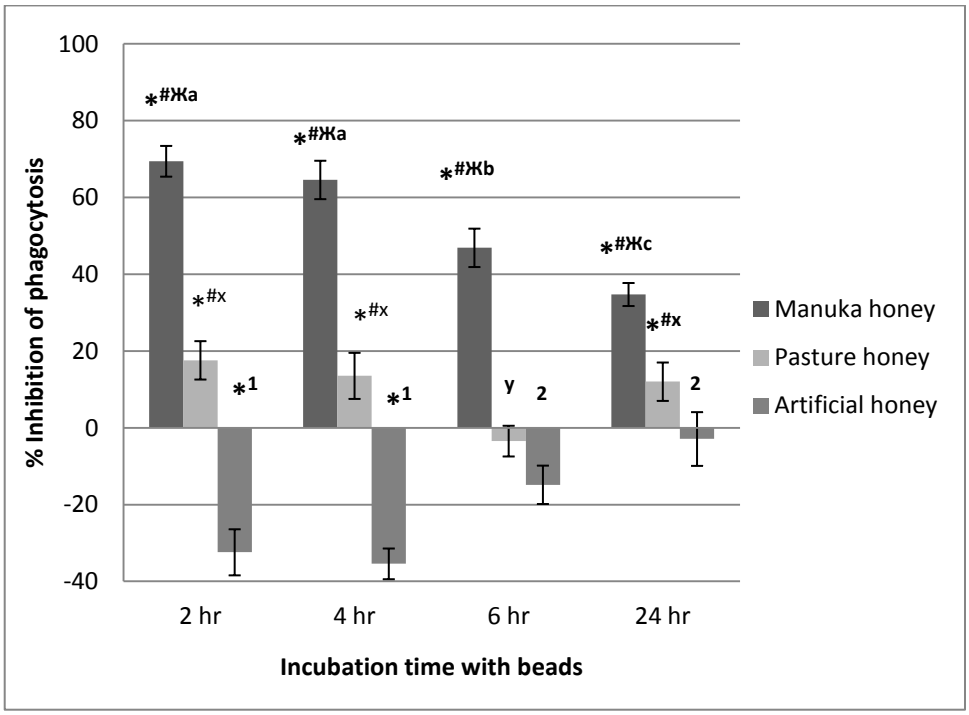

*Figure 4.2: The effect Manuka honey, Pasture honey and artificial honey (all at a concentration of 0.5%) on phagocytosis of latex particles in LPS-activated THP-1 cells at different time points after addition of latex particles compared to the control which received no honey treatment. Error bars show ± 1 SD of the mean of 3 experiments. A negative value indicates a promotion of phagocytosis compared to the control. The phagocytosis inhibition results were analysed by ANOVA and Tukey's multiple pairwise comparisons. * p<0.001 compared with control (not treated with honey) at the same incubation time point. # p<0.001 compared with artificial honey at the same incubation time points. Ж p<0.001 Manuka honey compared with Pasture honey at the same incubation time points. Changes in inhibition of phagocytosis between incubation time points for each type of honey ((a,b,c Manuka),(x,y,z Pasture),(1,2,3 artificial) p<0.001)). Values not showing the same letter are significant to each other.*

The effect of a small range of honey varieties on phagocytosis of latex particles is shown in Figure 4.3. The sample of Manuka honey had a significantly (p<0.001) larger effect on the reduction of phagocytosis compared with the samples of other honey varieties.

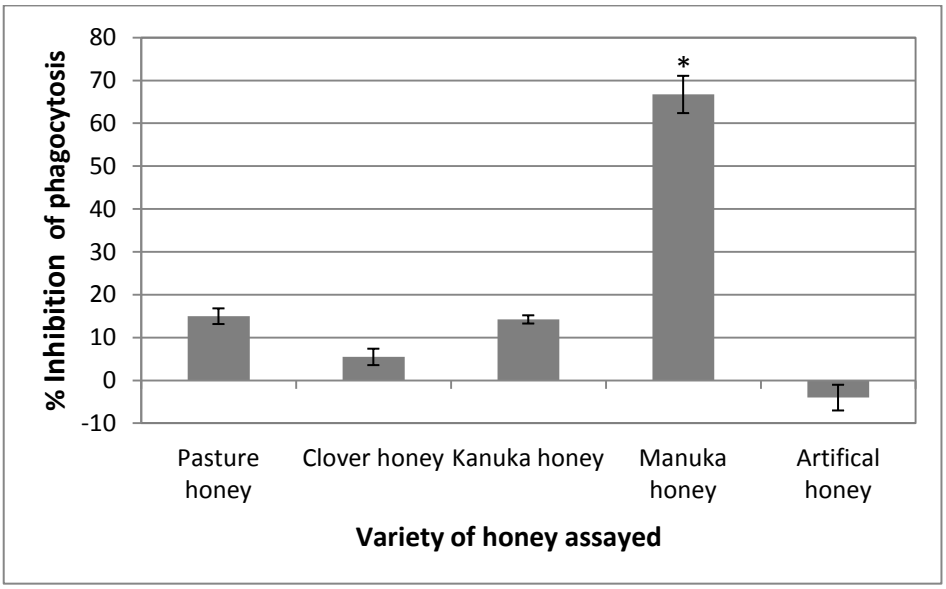

*Figure 4.3: The effect of different types of honey (at a concentration of 0.5%) on phagocytosis of latex particles in LPS-activated THP-1 cells. The assay was performed 4 h after latex particle addition. Error bars show ± 1 SD of the mean of 3 experiments. ** p*<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared with the non-Manuka samples.*

Figure 4.4 shows the results from comparing a larger number of samples of honey of different floral varieties excluding Manuka honey (compiled from honeys presented in Appendix 3). Again there was a difference in phagocytosis inhibition between honey varieties. Only Rewarewa and Kanuka honey had a phagocytosis inhibition of over 10% (a figure that Manuka honey with no detectable NPA scored higher than in Figure 4.5).

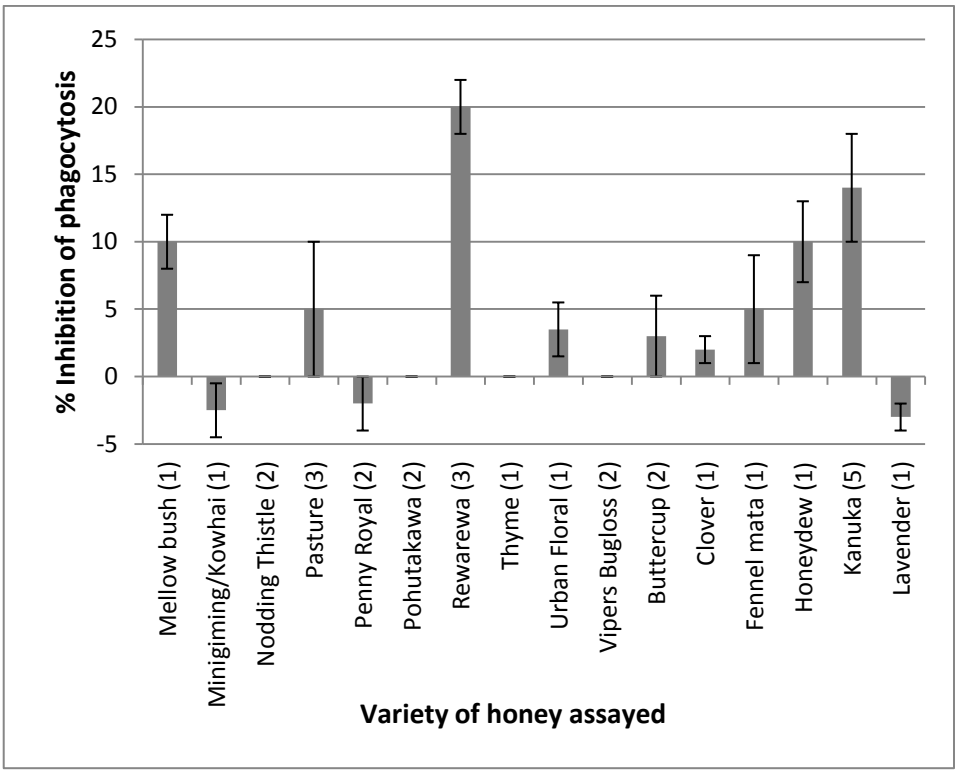

Figure 4.4: The effect of different types of honey excluding Manuka at a concentration of 0.5%, multiple samples of some of the types of honey tested (The number of samples of each type of honey is shown in brackets) on phagocytosis of latex particles in LPS-activated THP-1 cells. The assay was performed 4 h after latex particle addition. Error bars show ± 1 SD of the mean of 3 experiments for individual samples of honey. Compiled from honeys presented in Appendix 3.

There was a large variability between different Manuka honey samples. Figure 4.5 shows the effect on the phagocytosis of latex particles of a 0.5% concentration of a small selection of Manuka honeys with various levels of NPA. There appeared to be no relationship between the degree of inhibition of phagocytosis and the level of NPA of the sample. Manuka honey samples were examined individually in Chapter 8 to examine this variability. In Appendix 3 the phagocytosis rate of THP-1 after exposure to all individual honey samples is presented.

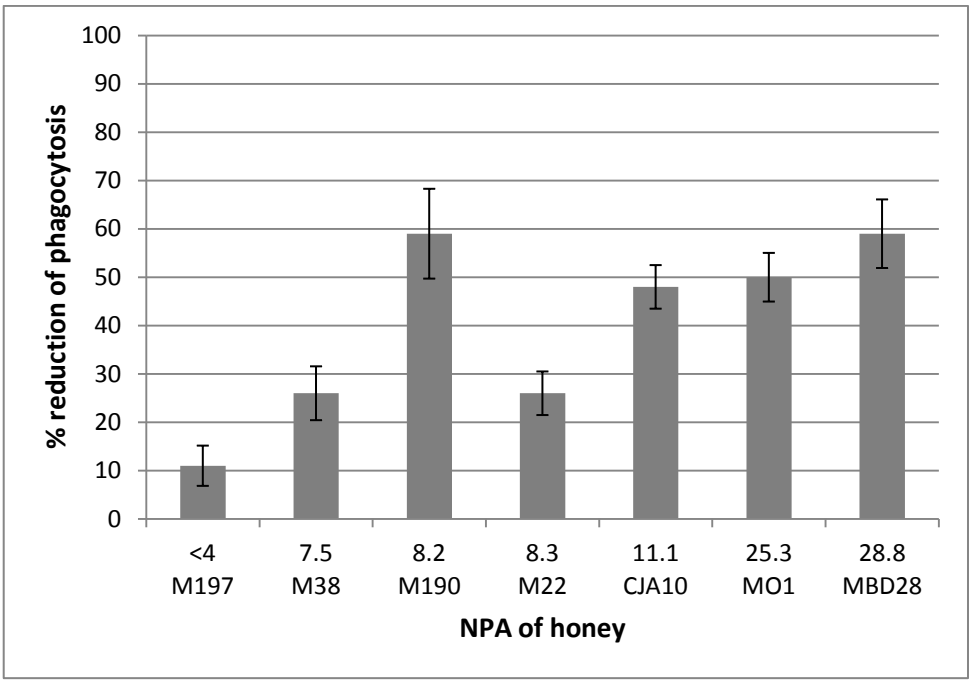

Figure 4.5: The effect of different types of Manuka honey (at a concentration of 0.5%) of varying NPA on phagocytosis of latex particles in LPS-activated THP-1 cells. The assay was performed 4 h after latex particle addition. Error bars show ± 1 SD of the mean of 3 experiments.

4.4.2 Phagocytosis assay with GFP *E.coli*

E.coli was used in place of latex particles in the phagocytosis assay to determine how extensive the effect of honey in inhibiting phagocytosis was with a biological particle in place of a synthetic material. The results are shown in Figure 4.6. Manuka honey gave a significant (p<0.001) reduction of phagocytosis of *E.coli*, though this was less than the inhibition observed with latex particles. Pasture honey also reduced the phagocytosis of *E.coli* when compared to artificial honey but to a lesser degree.

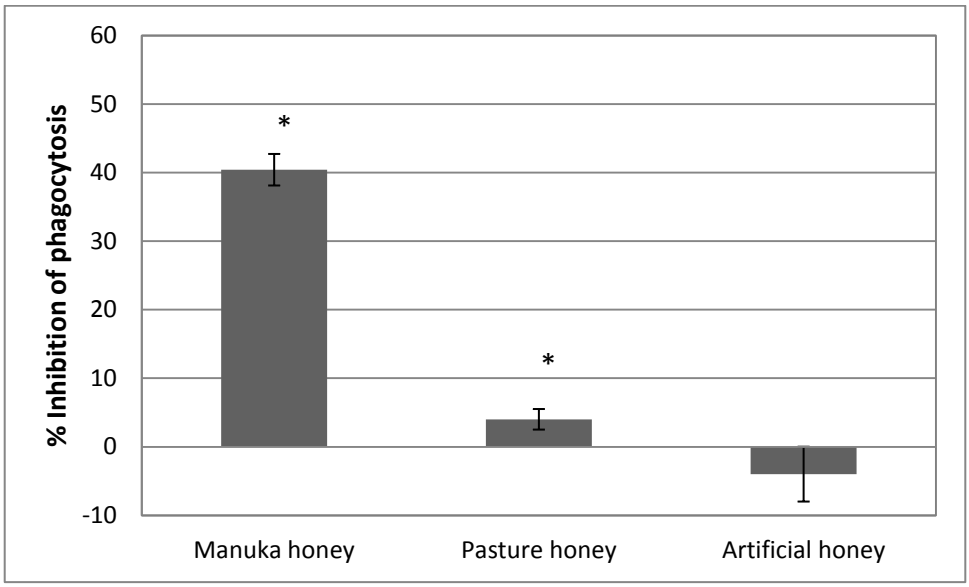

Figure 4.6: The effect of Manuka honey (at a conc. of 0.5%) on phagocytosis of E. coli *in LPS-treated THP-1 cells. The assay was performed 2 h after the addition of* E. coli*. Error bars show ± 1 SD of the mean of 3 experiments. ** p*<0.001 analysed by ANOVA compared with the non honey-treated control.*

4.4.3 Phagocytosis assay with GFP BCG

BCG was used in place of latex particles in the phagocytosis assay to further determine how extensive the effect of honey was at inhibiting phagocytosis on different types of biological particles. The results are shown in Figure 4.7. Manuka honey gave a significant (p<0.001) reduction of phagocytosis of BCG, though this was less than the inhibition observed with latex particles. Pasture honey also reduced the phagocytosis of BCG when compared to artificial honey but to a lesser degree.

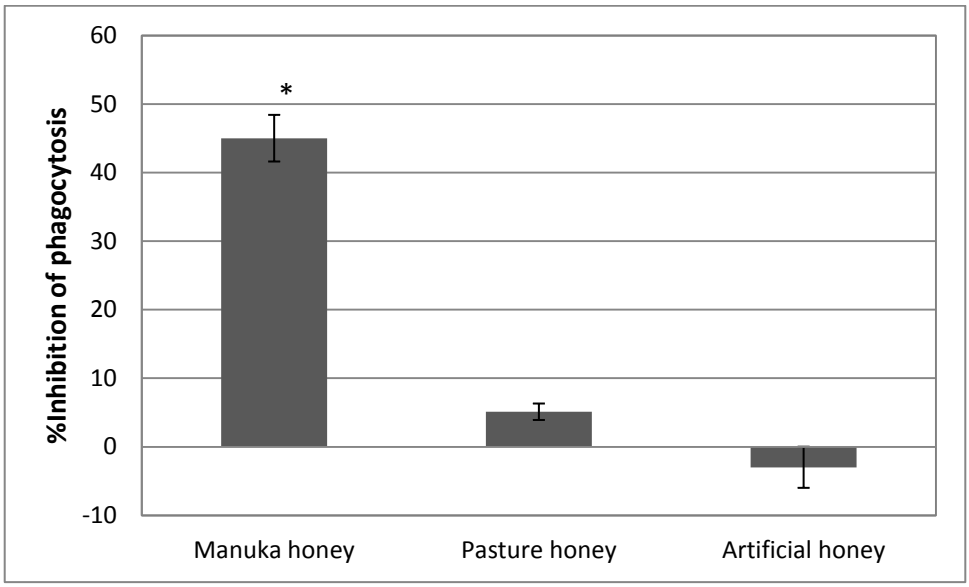

*Figure 4.7: The effect of Manuka honey (at a concentration of 0.5%) on the phagocytosis of BCG in LPS-activated THP-1 cells. The assay was performed 2 h after the addition of BCG. Error bars are ± 1 SD of the mean of 3 experiments. * p<0.001 analysed by ANOVA compared with the non honey-treated control.*

4.4.4 Phagocytosis assay with zymosan

Zymosan was used in place of latex particles in the phagocytosis assay to further determine how extensive honey was at inhibiting phagocytosis on different types of biological particles. The results are shown in Figure 4.8. All Manuka honey concentrations had a significant (p<0.001) reduction of phagocytosis of zymosan in a somewhat dose-dependent manner. However this dose dependent inhibition is lost after the concentration of honey reached 0.5%, unlike the inhibition seen with latex particles (Figure 4.1). This possibly indicates that zymosan is only bound by one receptor that honey blocks, whereas latex particles bind more than one.

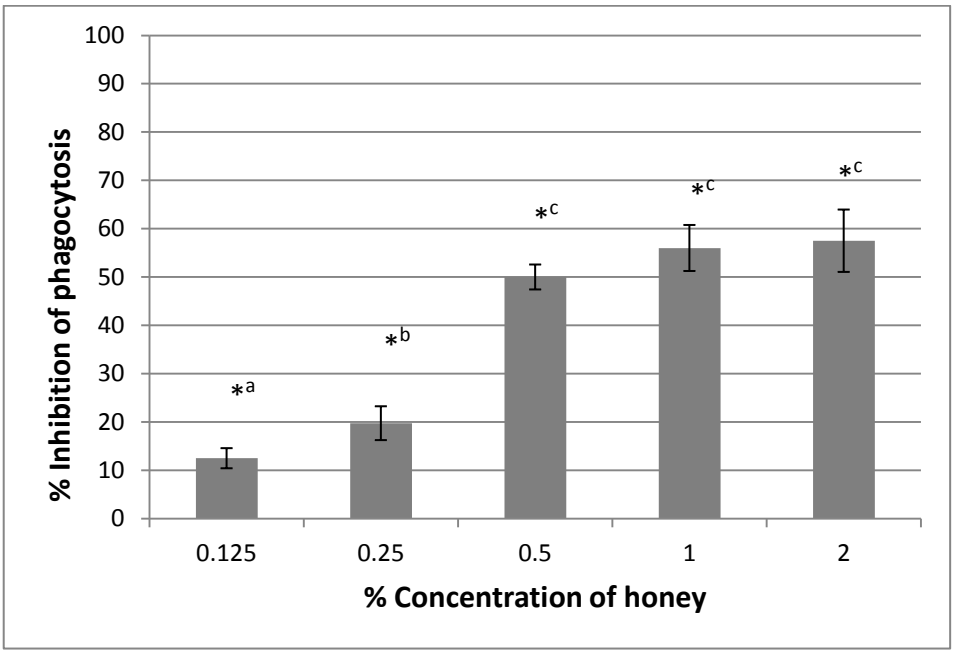

*Figure 4.8: The effect of a range of concentrations of Manuka honey on phagocytosis of zymosan particles in LPS-activated THP-1 cells. The assay was performed 4 h after zymosan addition. Error bars show ± 1 SD of the mean of 3 experiments. *p<0.001 analysed by ANOVA compared with the non honey-treated control. [(a,b,c)]p<0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared to other honey concentrations. Values not showing the same letter are significant to each other.*

4.4.5 Assay for the effect of cytokines on phagocytosis

It was previously shown that honey modifies the cytokine expression in THP-1 cells (Chapter 2). To determine if the effect of honeys on reducing phagocytosis in LPS-activated THP-1 cells was due to the cytokine profile changing in response to honey, the phagocytosis assay was modified to allow the effect of cytokines to be measured as described in Section 4.3.5. The results are presented in Figure 4.9. There were no significant difference between treatments and LPS alone

suggesting that an influence on cytokine production is not playing a role in the phagocytosis-inhibiting effect of honey.

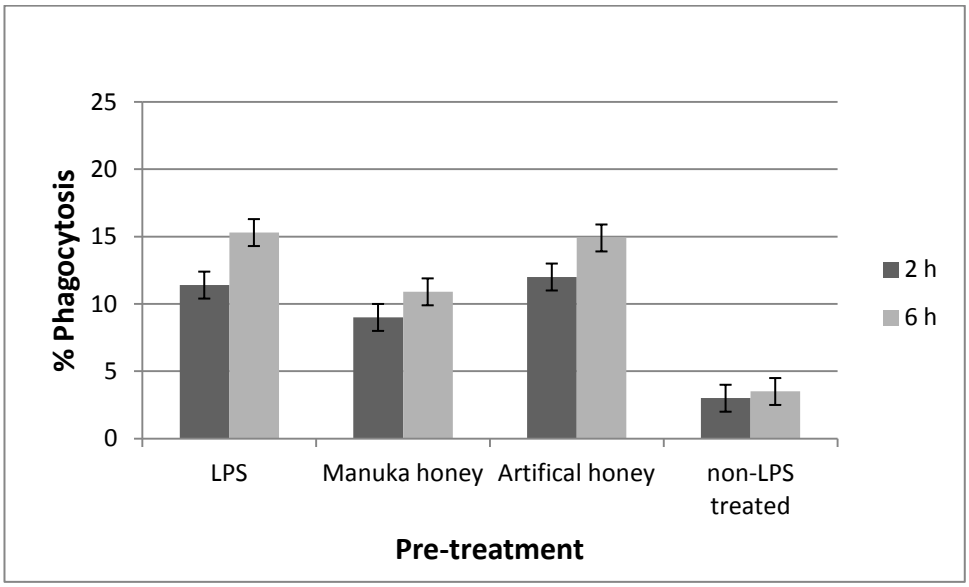

Figure 4.9: The effect on phagocytosis of THP-1 cells treated with the conditioned medium from cells pre-treated with LPS, Manuka honey or artificial honey or from control cells not treated with LPS. The assay was counted at 2 and 6 h after the addition of the latex particles. Error bars show ± 1 SD of the mean of three measurements. There was no significant difference between treatments analysed by ANOVA compared with the LPS-treated control.

4.4.6 Phagocytosis assay with dialysed honey

To begin characterizing the components in honey responsible for inhibiting phagocytosis in THP-1 cells, honey was dialysed to separate the high and low molecular weight compounds. These fractions where then assayed in the phagocytosis assay. The results are shown in Figure 4.10. The high molecular weight component of honey had a significantly *(p*<0.001) greater effect of

reducing phagocytosis of latex particles compared with the low molecular weight components, acting in a dose-dependent manner.

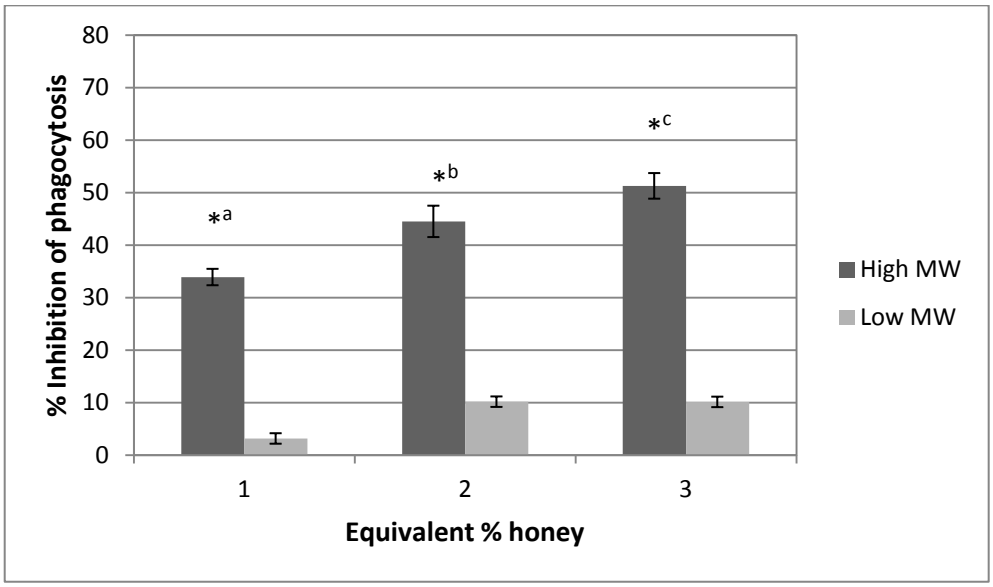

*Figure 4.10: Effect of high and low molecular weight (MW) components of Manuka honey on phagocytosis of latex particles in LPS-activated THP-1 cells. The assay was performed 4 h after addition of the latex particles. Error bars show ± 1 SD of the mean of three measurements. * p<0.001 analysed by ANOVA comparing the high and low MW components for each honey concentration. $^{(a,b,c)}$p <0.001 analysed by ANOVA and Tukey's multiple pairwise comparisons compared to other honey concentrations. Values not showing the same letter are significant to each other.*

4.4.7 Phagocytosis assay of latex particles with Cytochalasin B

Cytochalasin B (12.5 and 25 µmol/l) had a 100% inhibitory effect on the phagocytosis of latex particles (results not shown). Cytochalasin B inhibits F-actin-dependent phagocytosis. This indicates that for latex particle phagocytosis, f-actin rearrangement is occurring. At 12.5 µmol/l and 25 µmol/l Cytochalasin B had no effect on the viability of the THP-1 cells but at higher concentrations

viability dropped off slightly suggesting the cells could not tolerate a high level of blocking of f-actin polymerization.

4.5 Discussion

Certain samples of Manuka honey at low concentrations (0.5%) significantly (p<0.001) reduced phagocytosis of latex particles, *E.coli*, BCG and zymosan particles in the macrophage cell line THP-1. A range of concentrations of these Manuka honeys showed that honey gave a dose-dependent effect. Other honeys tested had a lower inhibitory effect than these Manuka honeys. Pasture honey and Kanuka honey at the same concentration had only 20% of the inhibitory effect of Manuka honey yet this was still a significant reduction. There is a probability that there was Manuka nectar present in these samples which was responsible for the inhibition observed.

The variability of the effect of Manuka honey with varying NPA was explored briefly and even a Manuka honey with no anti-bacterial activity (NPA 0) had a higher phagocytosis inhibiting action than all but two of the honey varieties assayed. The two honey varieties that had higher activity were Kanuka (14 %) and Rewarewa (20 %). As mentioned previously there is a probability that there was Manuka nectar present in these samples which was responsible for the inhibition observed. This variability between all honey samples is explored in Chapter 8.

The component responsible being in the high molecular weight fraction of honey suggested a protein or protein complex. BCG (Kang 1998; Diaz-Silvestre, Espinosa-Cueto *et al.* 2005) and *E.coli* (Akiko, Ikuko *et al.* 2008) are known to be phagocytosed by interaction with the mannose receptor. It was hypothesized that the high molecular weight component of Manuka honey exerted its effect on phagocytosis by interacting with the mannose receptor, possibly blocking/immobilizing receptors or preventing their recycling back to the

membrane (Stahl, Schlesinger *et al.* 1980). Indeed there have been reports of proteins in honey that have high-mannose type carbohydrate glycoproteins (Kimura, Miyagi *et al.* 2000) that may able to bind the mannose receptor. This hypothesis may be backed up by the observed prevention of phagocytosis of latex particles by addition of cytochalasin B which indicated that for phagocytosis of these the rearrangement of f-actin is essential, ruling out CR3 type phagocytosis. This suggests that the mannose or scavenger receptors are responsible for latex particle phagocytosis.

This finding is important regarding the inflammatory aspects of wound healing. Chronic wounds are overpopulated with active macrophages causing large amounts of pro-inflammatory cytokines and ROS to be released, aggravating the situation and creating feedback loops that worsen the condition. By reducing the phagocytosis of active macrophages these cytokines and ROS would be reduced. It has been reported that Manuka honey reduces ROS production in macrophages (Tonks, Cooper *et al.* 2001) but it was not reported by what mechanism. The possibility exists that the antioxidants in honey may have scavenged the ROS free radicals that Tonks *et al.* were measuring.

It is important to be aware of the different states achievable by activated macrophages. Currently there are two widely accepted activation states, type 1 and type 2. Type 1 is the classical inflammatory state where upon stimulation by LPS or similar factors, the cell releases pro-inflammatory cytokines such as TNF-α and IL1-β. Type 2 is termed alternative activation where an anti-inflammatory cytokine profile is produced. Both states have membrane-bound mannose receptor though type 1 has less than type 2, which could mean type 2 Macrophages are more able to respond to the active compound in honey.

The THP-1 cell line used in this study could be activated by LPS, PMA or Vit-D3 to achieve a macrophage phenotype (Chapter 2). With all of these it was confirmed that the monocytes had achieved a macrophage phenotype according to expression of macrophage-specific gene HCgp-39 detected by RT-PCR. The LPS-

stimulated cells were found to perform best in the phagocytosis assay (higher rates of phagocytosis in controls) suggesting different states of activation between the treatments (Chapter 2). Manuka honey may have an effect on only one state ensuring adequate protection/clearance of foreign particles in the wound with the other macrophage state/s and immune cells. This should be investigated in future work.

The effect of honey on phagocytosis was lost gradually over time, with a reduction in the inhibition of approximately 50% after 24 hours of incubation with the latex particles. At two hours after latex particle addition, Manuka honey had a 70% inhibitory effect on latex particle phagocytosis compared with 35% inhibitory effect after 24 hours. This may indicate that the inhibitory component in honey is either being depleted or the cells are able to increase the number of receptors available for phagocytosis or manufacture a different receptor. If the cell is making more of the receptor that binds the honey component then the rate of uptake of the active component will be faster, therefore depleting it. If the cell is able to make a different type of receptor that does not bind the active component in honey then honey will have less effect on the cell. Both these scenarios could explain why the inhibitory action of the honey component lost its effect over time.

A further assay was carried out to test whether the observed phagocytosis reduction is caused by a compound in honey (*e.g.* a protein found in honey), or a cell response to honey (*e.g.* a cytokine released that stimulates other cells). The assay took active macrophages and placed them in fresh medium with no treatment. After a period of time where the cells could theoretically release agents, these cells were removed and resting monocytes were added. These cells were left to be activated by the medium containing the conditioned medium. Latex particles were added to these cells and phagocytosis was measured, (Figure 4.9). The activation of the cells (ability to phagocytose) was significantly (p<0.001) reduced to 12% and 15% at the two hour and six hour

time points respectively, compared with the usual direct stimulation with LPS giving activation rates up to 70-90% (Chapter 2).

These results suggest that LPS needs to stay in contact with the cells for activation to proceed. The activation rate was low, and a slightly lower amount of phagocytosis was observed in the honey-treated cells, but this result was not significant. This indicates that the factor(s) in the medium produced as a result of stimulation of the monocytes by LPS gave a low level of activation of fresh monocytes, but that the factor(s) was not as potent as direct LPS exposure. The agent(s) responsible for the activation in the absence of LPS may be cytokine(s), the mRNA for which have been observed to change after treatment with honey or LPS, as demonstrated by RT-PCR analysis (Chapter 3).

Treatment with Manuka honey (but not artificial honey) gave a slightly lower activation indicating a slight suppressive effect on production of the factor(s) after removal of the Manuka honey but this result was not significant. The overall conclusion is that honey is not acting via an effect on cytokine production (or other factors) when it suppresses phagocytosis.

These findings have numerous possible implications involving the mannose receptor or other phagocytic receptors with regards to honeys' anti-inflammatory effects. One important consideration is that myeloperoxidase released by neutrophils during immune activity is bound through the mannose receptor on macrophages (Shepherd and Hoidal 1990). Myeloperoxidase is a strong signalling factor for macrophages to initiate and amplify inflammation (Rodrigues, Rodriguez *et al.* 2002). If honey does block the mannose receptor then this signal would be downgraded, preventing an over-production of inflammatory cytokines at the site.

Another consideration is the possible effect on tissue-type plasminogen activator (t-PA), a serine protease that activates fibrinolysis by converting plasminogen into plasmin, which cleaves fibrin into soluble degradation products. Recent research has shown that fibrin plays a key role in the inflammatory response and

is also involved in forming scar tissue (Bouma and Mosnier 2006). Thus t-PA is beneficial to healing wounds. It has been shown that t-PA is cleared from plasma through the mannose receptor probably through its high mannose-type oligosaccharide (Biessen, van Teijlingen *et al.* 1997; Noorman, Barrett-Bergshoeff *et al.* 1997).

Because of its rapid clearance in humans, t-PA is often administered in large doses to achieve efficient thrombolysis. Mannosides can inhibit t-PA binding to the mannose receptor by immobilizing the receptor, effectively inhibiting t-PA clearance. The active component in Manuka honey may also inhibit t-PA clearance by blocking the mannose receptor. One of the benefits of using Manuka honey in wounds is the reduction of scarring (Topham 2002). This action of honey may be through allowing t-PA to stay in circulation for longer. Future research should be directed to exploring this possibility.

The suppression of phagocytosis in macrophages by honey on the face of it seems non-beneficial as this would delay pathogen clearance. But when consideration is given to the nature of phagocytosis, an anti-inflammatory benefit can be clearly seen.

Phagocytosis produces large amounts of inflammatory ROS, arachidonic acid metabolites and inflammatory cytokines. As it is excessive inflammation that prevents wound healing, reducing phagocytosis may reduce the amount of these pro-inflammatory mediators allowing chronic wounds to heal, and acute wounds to heal with-out entering a chronic state.

This action may also protect the organism by reducing the proliferation of inflammatory cells without affecting their capacity to secrete anti-inflammatory cytokines. Phagocytosis of pathogens and other debris can then be carried out at a controlled level and by the other phagocytic cells already present in the tissue.

This theory agrees with recent research (Harcourt 2005) which found that honey increases the rate of phagocytosis in neutrophils. Future research should look at

the effects of honey on other immune cells that phagocytose such as dendritic cells and mast cells.

Chapter 5

The effect of honey on the release of reactive oxygen species in THP-1 macrophages

5.1 Summary

This chapter expands on work completed in Chapter 4 where it was found that Manuka honey reduced phagocytosis in activated THP-1 macrophages. It was necessary to see if this reduction in phagocytosis also resulted in a reduction of the level of reactive oxygen species (ROS), a by-product of phagocytising cells that when accumulated in high amounts can cause tissue damage and inflammation.

A dichlorofluorescein (DCFH) assay was used to quantitate ROS release by actively phagocytising THP-1 cells in the presence of 0.5% honey.

Manuka honey was found to decrease ROS release from macrophages but not monocytes. Artificial honey increased the release of ROS and Rewarewa honey had no effect on ROS release.

5.2 Introduction

ROS, also known as reactive oxygen intermediates (ROI), are ions or very small molecules that include oxygen and hydroxyl free radicals and peroxides, both inorganic and organic. ROS are highly reactive due to the presence of unpaired valence shell electrons able to participate in bonding of other atoms. Peroxides are termed ROS because from such species ROS form as a natural by-product of their normal reactions, or directly from their metabolism for destruction of phagocytosed microbes and cell debris. Microbial phagocytosis triggers production of pro-inflammatory cytokines, and generation of ROS. ROS function in antigen processing and presentation, as well as suppression of genes encoding molecules involved with bacterial recognition and internalization (Reth 2002).

Hydrogen peroxide ($H_2O_{2)}$ and superoxide ($O_2^{\bullet-}$, which forms hydrogen peroxide) can cause or contribute to various kinds of oxidative stress and they can react directly with tissue components themselves. However since these molecules are comparatively non-toxic, cells have well-known means of dealing with them, and they are even used in cellular signalling under normal conditions. $O_2^{\bullet-}$ is a relatively un-reactive intermediate on its own. In living tissue $O_2^{\bullet-}$ can be converted into H_2O_2 enzymatically by superoxide dismutase (SOD) isoforms, or non-enzymatically. H_2O_2 is a major ROS contributor to oxidative stress which is formed from the superoxide that leaks from the mitochondria. Catalase minimises the damaging effects of hydrogen peroxide by converting it to oxygen and water but this conversion is not 100% efficient as residual peroxides remain. Normal cellular functioning produces minimal amounts which can be tolerated, yet excessive amounts can cause deleterious effects (Horton 2003).

ROS levels may increase during certain conditions, stressing the surrounding cells and causing significant damage called oxidative stress to cell structures. Oxidative stress leads to considerable DNA damage (Evans 2004) and to the polymerisation and denaturation of proteins (Davies 2005). The most common damaging species, the hydroxyl radical, is created by the reaction of peroxide

and superoxide with un-liganded or incompletely liganded iron ions. The hydroxyl radical is exceptionally damaging, and a major cause of chronic inflammation (Kell 2009). The hydroxyl radical ($^{\bullet}OH$) has a very short half-life (10^{-9} s) and high reactivity. In biological systems it does not diffuse from the site of generation and can rapidly damage any surrounding carbohydrates and macromolecules, including proteins, potentially leading to their inactivation/denaturation (Novo and Parola 2008).

Phagocytic cells such as neutrophils, macrophages and eosinophils generate ROS via NADPH oxidase upon activation, due to the increased energy requirements for membrane reorganization required for actin polymerisation during phagocytosis (Castellano, Chavrier *et al.* 2001; Novo and Parola 2008) and produce it for directly killing pathogens. This may cause an increased localised ROS load which, under normal conditions, can be successfully controlled by cellular control feedback loops via cell signalling pathways, which, in turn, activate various effector functions that make up the initial immune response (Gwinn 2006).

Inducible nitric oxide synthase (iNOS), which was first identified in macrophages and then in other cells, including hepatocytes, is known to be up-regulated by pro-inflammatory cytokines and/or bacterial lipopolysaccharide, and is able to generate low levels of nitric oxide. Nitric oxide exerts physiological effects by controlling vascular tone, cell adhesion, vascular permeability and platelet adhesion. It also exerts several potentially toxic effects. Nitric oxide is not particularly toxic *in vivo* because of efficient systems able to minimize its accumulation. Nitric oxide is removed as a consequence of its rapid diffusion through tissues. Under pro-inflammatory conditions, simultaneous production of $O_2^{\bullet-}$ and nitric oxide can be strongly activated and significant amounts of peroxynitrite ($ONOO^-$) are generated, which may cause significant injury to different cellular structures (Pacher, Beckman *et al.* 2006).

ROS causing oxidative stress can activate transcription factors, most importantly nuclear factor κB (NF-κB). NF-κB is a protein complex that acts as a transcription factor involved in cellular responses shown to respond to stimuli such as stress, cytokines (such as IL-1 and TNF-α), inflammation, control of cell growth and the balance between ultimate cell survival and apoptosis (Chapter 3.2.1). NF-κB plays a key role in regulating the immune response to infection by initiating transcription of a range of inflammatory cytokines (Baldwin 1996). It is activated by H_2O_2 produced by the respiratory burst, and controls the inducible expression of genes whose products are part of the inflammatory response. In this way, H_2O_2 increases the inflammatory response. This is a critical link between the respiratory burst and other inflammatory responses (Iles and Forman 2002). Incorrect regulation of NF-κB has been linked to cancer, inflammatory and auto-immune diseases, septic shock, viral infection, and improper immune development (Perkins 2007). NF-κB recognises DNA sequences called κB sites and is involved in maintaining mitochondrial integrity and in regulating antioxidant activity.

In resting cells, the NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Inhibitor of κB). Activation of NF-κB is initiated by the signal-induced degradation of IκB proteins. This occurs primarily via activation of a kinase called the IκB kinase (IκK). With the degradation of the IκB inhibitor, the NF-κB complex is then freed to enter the nucleus where it can 'turn on' the expression of specific genes that have NF-κB binding sites within promoter regions. Many of these NF-κB response genes are pro-inflammatory or immune related. NF-κB turns on expression of its own repressor, IκBα. The newly synthesized IκBα then re-inhibits NF-κB and, thus, forms an auto feedback loop, which results in oscillating levels of NF-κB activity. ROS, produced intra-cellularly as a part of the response induced by inflammatory cytokines, contribute to reinforce the signal to activate NF-κB by oxidising and activating IκK causing IκBα degradation (Nelson, Ihekwaba *et al.* 2004).

Many signalling pathways that have a ROS signal component can be overloaded with excess ROS. ROS can bypass the initial ligand binding step, entering the pathway midway creating a false positive activation, upsetting balance and the controlling of feedback loops.

Agents that control ROS levels or inhibit ROS production can therefore have therapeutic properties in certain diseases. Antioxidants are commonly used to control excess ROS in tissue. Antioxidative agents include small-molecule ROS scavengers, inhibitors of ROS-generating enzymes, and antioxidative enzymes (Fang, Seki *et al.* 2009). Antioxidants are very successful in assisting wound healing such as burns (Horton 2003). Antioxidants prevent the oxidation of other molecules by removing ROS, thus inhibiting oxidation reactions by being oxidized themselves. In wounds where chronic inflammation is present or likely to occur, controlling excessive ROS will be beneficial. This may be achieved by either preventing/decreasing the release of ROS, or removing excess ROS by way of antioxidants. Honey has been shown to quench free radicals in a cell-free system (Gheldof, Wang *et al.* 2002; Henriques, Jackson *et al.* 2006; van den Berg, van den Worm *et al.* 2008), but it is not known if honey can reduce the production of ROS in cells actually phagocytising pathogens or particles.

ROS scavengers have been shown to inhibit production of IL-8 in LPS-stimulated whole blood, thus reducing inflammation, while addition of hydrogen peroxide caused a dose-dependent stimulation of IL-8 production, thus increasing inflammation (DeForge, Fantone *et al.* 1992; Stegmaier, Kirchhoff *et al.* 2008). IL-8 is a cytokine produced by monocytes, endothelial cells and fibroblasts in response to inflammatory stimuli such as LPS and pro-inflammatory cytokines such as TNF-α and IL-1β. IL-8 transcription can be activated by NF-κB when the nuclear factor binds the gene after translocation into the nucleus (Baldwin 1996; Stegmaier, Kirchhoff *et al.* 2008). IL-8 activates neutrophils causing their migration and accumulation at the site of its release and causing consequent inflammation due to active phagocytosis releasing bioactive lipids, enzymes and

excessive ROS, mediating extensive tissue damage (Baggiolini, Walz *et al.* 1989). This provides evidence that excessive levels of ROS play an important role in cytokine expression, resulting in increased inflammation.

Macrophage activation has been found to generate high levels of myeloperoxidase (MyPo) (Rodrigues, Rodriguez *et al.* 2002). The increase in MyPo content is accompanied by an increased capability of macrophages to generate hypochlorous acid and increased peroxidase activity. Lowering the amount of MyPo generated and received by macrophages could greatly reduce inflammation. In an experimental model of inflammatory bowel disease, intra-rectal honey administration was as effective as prednisolone treatment, a steroid drug commonly used to treat the condition. Its activity was attributed to the reduction of MyPo levels (Bilsel, Bugra *et al.* 2002).

It was found in the present study (Chapter 4) that Manuka honey decreased phagocytosis. One possible explanation for this is that honey reduces the activation rate of macrophages, thus reducing the release of MyPo. Therefore it was hypothesised that this resulted in less actin polymerisation in the cells for cytoplasmic movement of phagosome. As actin polymerisation produces large amount of ROS, it was also hypothesised that less actin polymerisation would result in less ROS production for microbial killing. This would reduce ROS levels, consequently reducing cellular damage and feedback loops, exerting an anti-inflammatory effect in wounds. To test this hypothesis a commonly used assay for measuring cell-generated ROS was used.

Cell-generated ROS levels can be measured using the Dichlorofluorescein (DCFH) assay. When applied to intact cells, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall ROS released from phagocytising cells (LeBel, Ischiropoulos *et al.* 1992).

This chapter investigates ROS formation in actively phagocytising THP-1 cells and its decrease due to honey. To find out if the honey was actually preventing ROS release, or simply working as an anti-oxidant mopping up ROS as it was formed by the cell, a honey with high antioxidant properties was used. Rewarewa honey has been shown to have high anti-oxidant activity (personal communication with Professor Peter Molan), so was compared with Manuka honey. Latex beads were used to induce phagocytosis

LPS-activated cells were previously identified as producing the most actively phagocytosis cells (Chapter 2) so LPS was initially used for the DCFH assay. PMA was used as an alternative cell treatment to activate monocytes. PMA was found in earlier research to activate monocytes to macrophages but these cells showed less phagocytic activity. It was expected that PMA-activated cells would have less ROS release corresponding with the lower level of phagocytosis observed.

A control replicated the experiment using resting monocytes. Monocytes have little or no phagocytic activity and so would not produce the same levels of ROS as active macrophages. Any fluorescence developed from ROS reactions with DCFH would be generated by normal cell ROS levels and not by ROS produced when there is actin polymerisation or microbial killing. It was hypothesised that honey would have no effect on release of ROS by monocytes.

5.3 Methods

5.3.1 Reagents

5.3.1.1 THP-1 Cells

THP-1 cells were maintained at 37°C, 5% CO_2, and 95% relative humidity in growth medium (as described in Section 2.3.2). Cells were split with fresh

growth medium prior to the assay and assessed for viability (as described in Sections 2.3.1 and 2.3.4). The cells were activated with LPS or PMA as described in Section 2.4.1. Phagocytosis of latex beads was carried out as described in Section 2.4.4.

5.3.1.2 Dichlorofluorescin

6-Carboxy-2′,7′-dichlorofluorescin diacetate (DCFH-DA) was purchased from Sigma-Aldrich (Cat. No.# 35845-1 G) and dissolved in DMSO to create a 0.5 mg/ml stock solution. DCFH-DA was mixed with growth medium to a final concentration of 100 µmol/l when added to the cells.

5.3.1.3 Honey

Stock solutions of Manuka honey (M144) and Rewarewa honey (Re37-807) were as described in Section 3.4.1.1. Honey solutions (0.5% final concentrations) were added to wells diluted in growth culture medium. Control wells had only the medium added. Artificial honey (as described in Section 3.4.1.1) was used to provide a control for the osmotic effect of the natural sugars found in honey.

5.3.2 DCFH assay procedure

THP-1 cells were split in fresh growth medium, counted, and checked for viability by trypan blue exclusion (as described in Chapter 2.3.4). Viable cells were treated with 100 ng/ml LPS or 10 nmol/l PMA or left untreated to have resting monocytes for comparison with the macrophages. Then 200 µl cell suspensions (10^6 cells/well) were plated into 96-well sterile black plates for fluorescence detection (Nunc) and incubated (5% CO_2/95% air at 37°C) for 24 hours prior to experiments to allow differentiation to the macrophage phenotype. Two hours prior to adding DCFH-DA, Manuka honey, Rewarewa or artificial honey was added to each well to give a final concentration of 0.5%. Control wells had

equivalent amounts of RPMI medium added. Latex beads (25/cell) were added to the wells to initiate phagocytosis, and plates were further incubated.

After 2 hours incubation, DCFH-DA in 50 µl assay medium was added to each well to give a final concentration of 100 µmol/l DCFH-DA. The assay was performed at 4℃ to slow the reaction down, which allows for higher accuracy of readings across the plate. Fluorescence was measured on a LAS -1000 imaging system (Fuji Film life Sciences) using the fluorescence setting (λex 473 nm/λem 520 nm) and analyzed using the provided software. Preliminary experiments indicated that a 30 minutes incubation time gave the highest fluorescence readings before dropping which indicates the end of the reaction. Figure 5.1 shows an example of the intensity of the fluorescence of the LPS-activated THP-1 cells after a 30 minute DCFH assay.

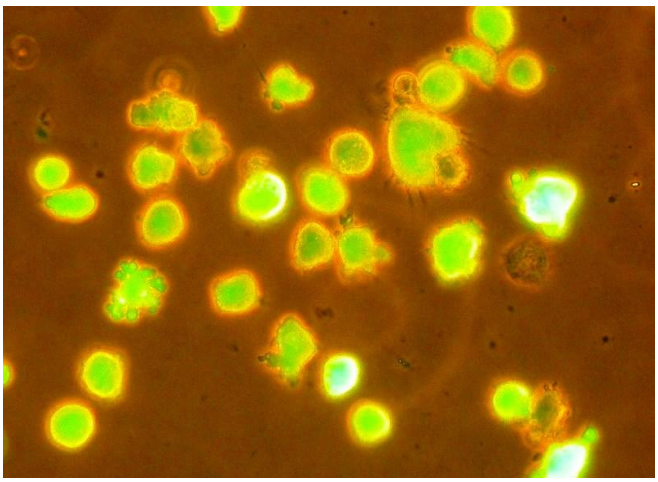

Figure 5.1. LPS activated THP-1 cells after 30 minutes incubation with DCFH-DA. Cells are adherent to culture plate. Pictures taken on an Axostar plus Zeiss fluorescent microscope, phase II, 40x lens with a canon digital camera, λex 470 nm, λem 505 nm.

5.3.3 Fluorescence readings and calculation

An initial fluorescence reading was taken immediately after adding DCFH-DA and a subsequent reading 30 minutes later. Fluorescence measurements are shown in arbitrary units (AU) to illustrate the differences between maximal fluorescence generation using LPS-, PMA- or un-activated THP-1 cells. To compare honey types the percentage increase in fluorescence per well was calculated by the formula; $[(Ft_{30} - Ft_0)/Ft_0 * 100]$, were Ft_{30} = fluorescence at time 30 minutes and Ft_0 = fluorescence at time 0 minutes. This method of analysis has advantages over analyzing just the net change in fluorescence in that not only did the calculated data directly reflect the percentage changes of fluorescence over time from the cells in the same well, they also effectively control for any variability among wells (Wang and Joseph 1999) and variability in latex bead fluorescence.

Background fluorescence was monitored using a control with cells and latex beads only, as the latex beads had their own fluorescence. The fluorescence generated from the latex beads was minimal (~ 500 AU) compared to the fluorescence due to oxidation of DCFH in control cells (~ 13 000 AU). These measurements remained unchanged for the duration of the assay. Three replicates were used.

5.4 Results

Manuka honey had a significant (p<0.005) effect by decreasing ROS release in phagocytosing macrophages activated with LPS (Figure 5.2) or PMA (Figure 5.3) whereas artificial honey did not. This shows that the decrease in production of ROS was not due to an osmotic effect of the sugar content. Figure 5.4 shows the effect of honey on ROS release in resting monocytes which shows that resting monocytes produce less than half the ROS than actively phagocytosing activated macrophages do, and this was not affected by honey. Neither Manuka honey

nor artificial honey had a significant effect on the release of ROS (p=0.527 and p=0.722 respectively). Figure 5.5 shows a comparison of the effect of Manuka honey, Rewarewa honey and artificial honey on the release of ROS from LPS-activated macrophages with the measurements reported in % increase in fluorescence. Only Manuka honey gave a significant decrease in ROS release (p=0.003 for Manuka honey, p=0.655 for Rewarewa honey and p=0.824 for artificial honey).

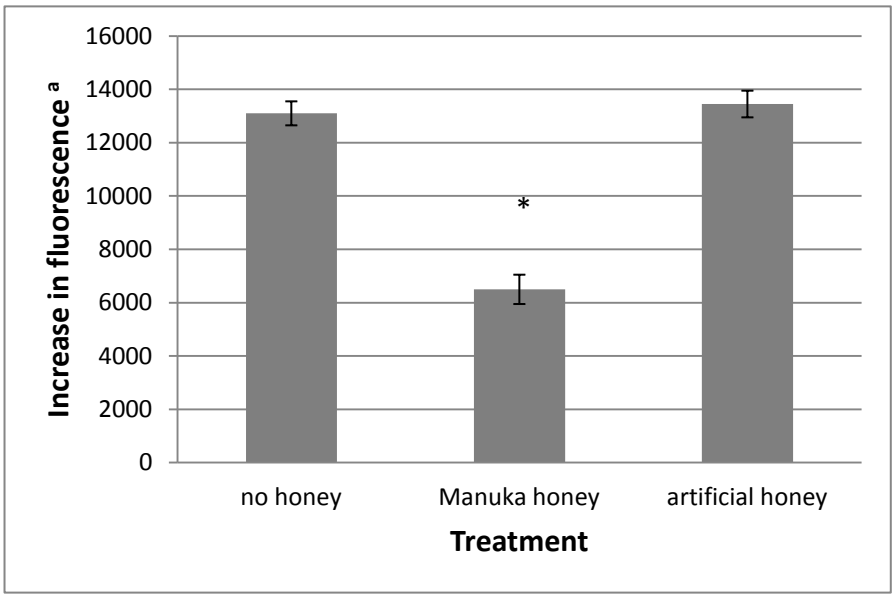

Figure 5.2: The effect of honey on release of ROS in phagocytosing LPS-activated THP-1 cells. [a] *Fluorescence measurements are in arbitrary units (AU) and show the increase in fluorescence after 30 minutes exposure to DCFH-DA, compared with the fluorescence measured at the start of the experiment (Ft_0). Error bars show the mean ± 1 SD of three experiments.* * p*<0.001 analysed by ANOVA comparing honey treated cells with control cells not treated with honey.*

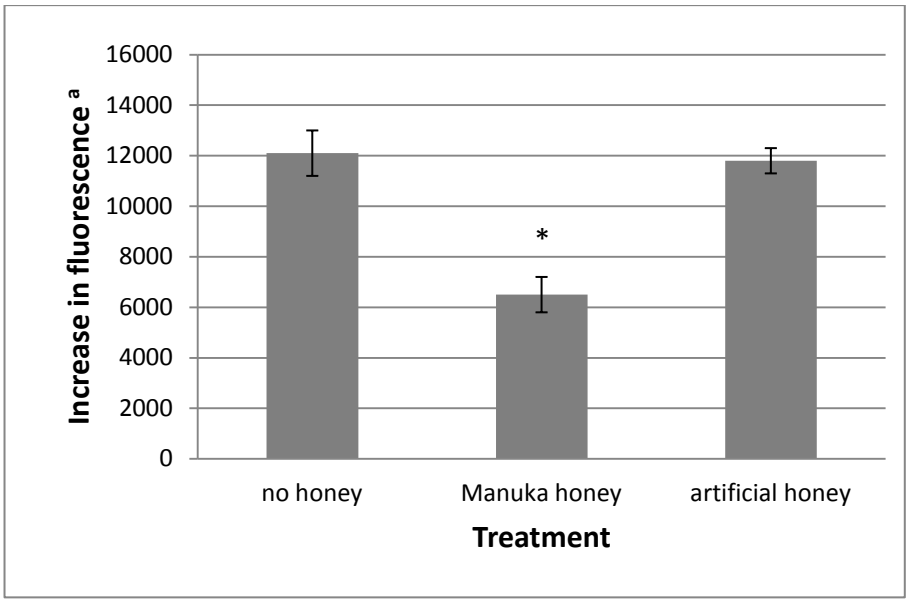

Figure 5.3: The effect of honey on release of ROS in phagocytosing PMA-activated THP-1 cells. [a] *Fluorescence measurements are in arbitrary units (AU) and show the increase in fluorescence after 30 minutes exposure to DCFH-DA, compared with the fluorescence measured at the start of the experiment (Ft_0). Error bars show the mean ± 1 SD of three experiments.* * $p<0.001$ *analysed by ANOVA comparing honey treated cells with control cells not treated with honey.*

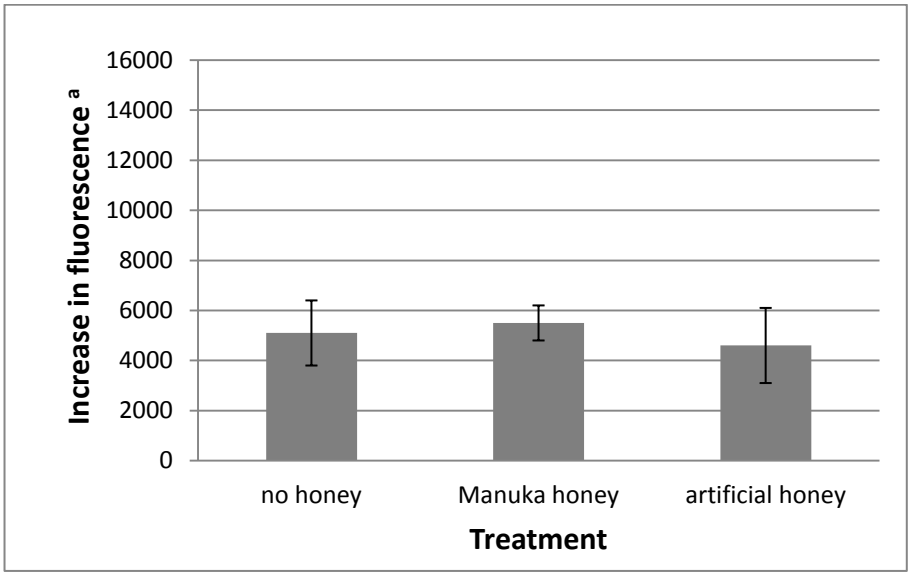

Figure 5.4: The effect of honey on release of ROS in resting THP-1 cells (monocytes). [a] *Fluorescence measurements are in arbitrary units (AU) and show the increase in fluorescence after 30 minutes exposure to DCFH-DA, compared with the fluorescence measured at the start of the experiment (Ft_0). Error bars show the mean ± 1 SD of three experiments. Latex particles were used as the phagocytic stimulus.*

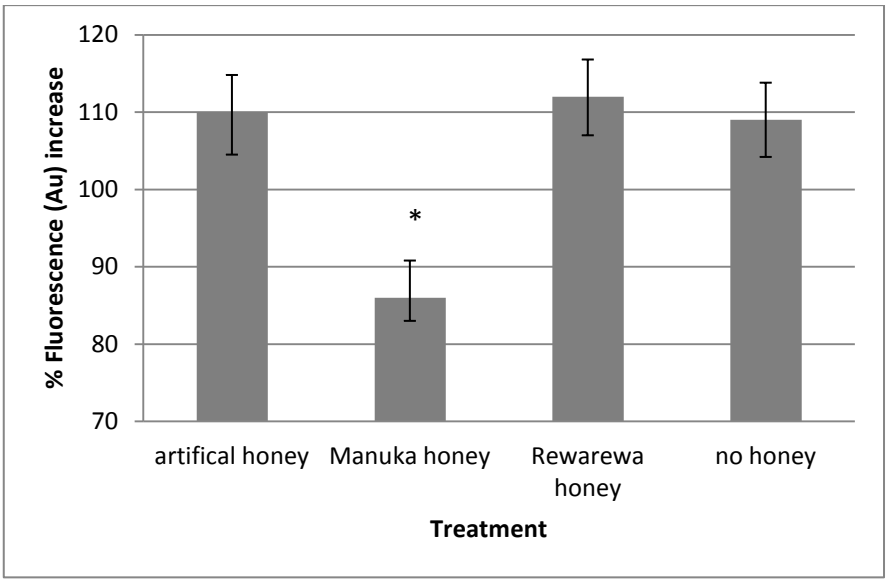

*Figure 5.5: Comparison of the effect of Manuka honey, Rewarewa honey and artificial honey on the release of ROS in LPS-activated THP-1 cells compared with control cells which received no honey treatment. Fluorescence measurements are in arbitrary units and show the increase in fluorescence after 30 minutes exposure to DCFH-DA compared to the fluorescence at the start of the experiment (Ft_0). Error bars show the mean ± 1 SD of three experiments. * p<0.005 analysed by ANOVA comparing honey treated cells with control cells not treated with honey*.

5.5 Discussion

The monocyte control provides an indication of how much ROS is produced by resting monocytes during normal cell replication and maintenance. After providing latex particles to stimulate phagocytosis, the fluorescence did not increase beyond 6 000 AU (compared to the fluorescence measured at the start of the assay Ft_0= ~ 500 AU) for each honey treatment after the assay time of 30 minutes. When this assay was repeated using monocytes activated with LPS or PMA (to form macrophages), this fluorescence increased to 13 000 AU and 12 000 AU respectively.

In LPS-activated phagocytosing cells Manuka honey had a large, significant (p<0.001) effect in decreasing ROS production, preventing fluorescence from increasing much beyond the baseline achieved by the monocyte control. This effect was not seen with Rewarewa honey which is known for its high antioxidant levels. This suggests that Rewarewa honey does not scavenge ROS from actively phagocytising macrophages, and perhaps the antioxidant properties are limited to scavenging ROS free in solution. Manuka honey may be preventing the formation of ROS, not neutralising already released ROS. However it may be that the antioxidants in Rewarewa honey do not cross all membranes and those in Manuka honey do.

The results were similar in PMA-activated THP-1 cells. Unexpectedly the ROS production was similar in LPS- and PMA-activated cells. As mentioned previously, activation with PMA produced a much less mobile cell and it was expected that the ROS production would also be less. Manuka honey still had an inhibitory effect on ROS production in these macrophages.

Interestingly, artificial honey slightly increased the ROS release in macrophages, though this increase was not significant. It was noted in Chapter 4 that artificial honey also increased the phagocytosis of latex beads. This may be due to the increased energy supplement that sugars provide. None the less it can be said that increasing the rate of phagocytosis also increased the ROS release from macrophages. The sugars in the artificial honey are also present in Manuka honey and if taken into account would give the inhibitory factor in Manuka honey a greater inhibition of ROS release. The results from these assays show that the osmotic effects of the sugars in honey cannot be simply inhibiting cell activity/vitality with regards to the phagocytosis rate.

Research has been published on the effect of honey on ROS release, which is in agreement with these results. It was reported that ROS production was significantly (p<0.001) decreased by Pasture honey and Manuka honey (Tonks, Cooper *et al.* 2001) and more recently that honey appears to modify the

oxidative burst process by inhibiting phagocytic MyPo activity causing an inhibition of ROS production including H_2O_2, •OH and hypochlorous acid (Mesaik, Azim *et al.* 2008). This is because MyPo produces hypochlorous acid and •OH from H_2O_2 during the respiratory burst (Rodrigues, Rodriguez *et al.* 2002).

Future work should attempt to identify the actual mode of action honey has on reducing ROS. Currently it would seem that honey may be working to decrease MyPo activity which will ultimately reduce the production of ROS (Mesaik, Azim *et al.* 2008) and research should look in this direction. Evidence also suggests that MyPo released from macrophages is involved in, and is possibly responsible for, atherosclerosis (Gelderman 1998).

Research also indicated that MyPo derived from neutrophils is cleared by the macrophage mannose receptor (Shepherd and Hoidal 1990) suggesting the macrophage mannose receptor plays a potentially physiological role in regulating extracellular MyPo levels. The receptor-mediated uptake may either support the macrophage to contribute to oxidant-mediated tissue damage or may function to clear extracellular MyPo during the resolution phase of the inflammatory process.

Future work should focus on the role of the mannose receptor, MyPo and honey. A wide range of honeys should also be tested using the DCFH assay.

Chapter 6

Identifying the anti-inflammatory agent in honey

6.1 Summary

This Chapter covers the work done to isolate and identify the component of honey that in previous Chapters that was found to have anti-inflammatory properties by way of inhibiting phagocytosis and ROS release. Fractions of Manuka honey were subjected to a series of different treatments to determine the likely properties of the phagocytosis-inhibiting component of Manuka honey. Trypsin treatment and heat treatment resulted in a loss of the inhibitory activity in a phagocytosis assay, indicating that the active component was a protein.

The active protein fraction in Manuka honey was identified by separating the proteins out, using gel permeation chromatography on a Sephadex column and subsequently assaying these fractions in the phagocytosis assay. The active fraction was isolated as an early peak on the elution trace, suggesting a larger protein or protein complex. It was determined that the high molecular weight protein fraction was active in the phagocytosis assay. This fraction was further separated into two peaks by FPLC (fast protein liquid chromatography) on a Superose 12 FPLC gel permeation column and one of these peaks was identified as having the inhibitory activity. This fraction was then run on a reverse phase

FPLC column to further separate out the proteins in the fraction but the products were not able to be identified by MALDI mass spectrometry due to low concentrations. Instead the whole fraction was subjected to analysis by MALDI mass spectrometry.

This fraction was identified as having the protein Apalbumin-1 (MRJP-1) by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, it being found to have a molecular weight of 48.8 kDa. Apalbumins are proteins secreted by the bee into the gathered nectar during honey maturation. Apalbumin-1 is the most abundant protein in honey, and known to be a glycoprotein. The protein bound to a ConA lectin column and showed loss of activity after mannosidase treatment, indicating mannose modifications on the protein conferred at least some of the activity. A second protein in the fraction was identified by MALDI mass spectrometry as being Apalbumin-3 (MRJP-3). Apalbumin-3 is a slightly larger 61.6 kDa glycoprotein.

6.2 Introduction

In Chapter 4 it was found that honey reduced the phagocytosis of latex particles and a range of other biological materials. This was hypothesised to have an anti-inflammatory effect by reducing the amount of ROS produced which is a pro-inflammatory agent. This hypothesis was backed up by the observations made in Chapter 5 which showed that honey did reduce the release of ROS which results in an anti-inflammatory effect. Further to that it was found that honey changed the expression of cytokines to have a net anti-inflammatory effect on THP-1 cells. It was for these reasons that an attempt was made to isolate and identify the active component in honey. In Chapter 4 the high molecular weight component of honey was found to contain the active component of honey. This Chapter focuses on further isolating this component and identifying it.

6.2.1 Royal jelly proteins

The honey bee (*Apis mellifera)* caste determination occurs when young worker bees in the hive (nurse bees) produce, secrete, and feed a substance called royal Jelly to developing larvae. Royal jelly is a natural source of essential amino acids, lipids, vitamins, acetylcholine, and other nutrients (Schmitzová, Klaudiny *et al.* 1998; Lerrer, Zinger-Yosovich *et al.* 2007). In the initial 3 days of development, all larvae are fed royal jelly, but thereafter only larvae designated by workers to become queens receive royal jelly. In its place, a mixture of honey, pollen, and water is fed to larvae selected to become workers.

Royal jelly proteins make up 50% (dry mass) of royal jelly (Schönleben, Sickmann *et al.* 2007). Of these proteins more than 80% are from the major royal jelly protein family (MRJP) also called the yellow protein family. Multiple yellow-like proteins have been found in all insect genomes including that of the honeybee. MRJPs are restricted to honeybees. Nine MRJP proteins, termed MRJP I-9, have been characterized so far (Albert and Klaudiny 2004) with MRJP 1-5 being the most prominent. These are named apalbumins. They range in size from 49 kDa – 87 kDa. Size polymorphisms exist, with MRJP-3 having at least five different isoforms (Albert, Klaudiny *et al.* 1999). MRJP-1 is reported to constitute 48% of the royal jelly proteins (Scarselli, Donadio *et al.* 2005). The genes of these proteins have been identified and the proteins are suggested to be multifunctional, performing diverse nutritional, physiological and developmental roles affecting various tissues (Schmitzová, Klaudiny *et al.* 1998).

Royal jelly products have been used for various therapeutic purposes including cholesterol-lowering (Guo, Saiga *et al.* 2007), anti-inflammatory (Kohno, Okamoto *et al.* 2004), antioxidant, anti-tumour (Viuda-Martos, Ruiz-Navajas *et al.* 2008), or wound-healing (Calli, Tugyan *et al.* 2008), and for their anti-bacterial effects (Fujiwara, Imai *et al.* 1990). MRJPs have also been identified as having immuno-stimulatory properties (Simuth, Bilikova *et al.* 2004). MRJP-1 is described as the major honey protein. It is a glycoprotein with various biological

properties, and is able to stimulate macrophages to release TNF-α (Majtan, Kovacova *et al.* 2006). Honey contains around 1% protein (Lerrer, Zinger-Yosovich *et al.* 2007) with MRJP-1 being the major honey protein.

6.2.2 Importance of structural integrity of proteins

Many proteins play important roles in cell signalling, often due to their specific folding properties, allowing them to correctly fit into receptor sites of membranes, initiating signalling cascades. For many proteins the correct three-dimensional structure is essential to function (Anfinsen 1972). Failure to fold into the intended shape usually produces inactive proteins with different properties, unable to bind receptors as their interactive sites are lost.

Denaturation of proteins involves the disruption and possible destruction of both the secondary and tertiary structures. Since denaturation reactions are not strong enough to break the peptide bonds, the primary structure (sequence of amino acids) remains the same after a denaturation process. Denaturation disrupts the normal alpha helix and beta sheets in a protein and uncoils it into a random shape.

In tertiary structure there are four types of bonding interactions between "side chains" including: hydrogen bonding, salt bridges, disulfide bonds, and non-polar hydrophobic interactions which may be disrupted. Therefore, a variety of reagents and conditions can cause denaturation. The most common observation in the denaturation process is the precipitation or coagulation of the protein. High temperatures disrupt hydrogen bonds and non-polar hydrophobic interactions (Shortle 1996). This occurs because heat increases the kinetic energy and causes the molecules to vibrate so rapidly and violently that the bonds are disrupted, causing loss of ordered folding and thus loss of activity.

Protease treatment also causes a loss of activity by cleaving the peptide chain. Trypsin is a serine protease found in the digestive system of many vertebrates, where it breaks down proteins (Walsh, Gertrude *et al.* 1970). Trypsin

predominantly cleaves peptide chains at the carboxyl side of the amino acids lysine and arginine. The conventional source for trypsin is porcine or bovine pancreas. It is commonly used in proteomics for protein digestion prior to mass spectrometry and other protein assay work.

The previous work in Chapter 4 showed that the phagocytosis-inhibiting activity in Manuka honey was of high molecular weight and therefore possibly a protein, so it was important to see if the activity could be lost by denaturing or digesting the proteins, while keeping all the honey components in solution, to find out if the phagocytosis-inhibiting component is a protein. Then, is it modified in any way with sugars.

6.2.3 Glycoproteins

Proteins with sugar modifications (glycoproteins) often have additional properties. Glycoproteins play an important role in many biological processes including immune regulation, inflammation, cell-to-cell adhesion and contact inhibition, cell signalling, protection against proteolytic degradation, and several other biological processes. Several papers have reported that proteins in honey have a high glycosylation with mannose (Schmitzová, Klaudiny *et al.* 1998; Simuth, Bilikova *et al.* 2004).

One method for identifying the types of modifications is by using a lectin isolation column. Lectins are proteins that have a selective affinity for carbohydrate moieties. The Concanavalin A (ConA) lectin preferentially recognizes α-linked mannose. These carbohydrates are attached to proteins through asparagine residues (Cummings 1997). Mannosidase can be used to cleave off mannose-linked sugars from proteins, this reducing the effect of the proteins if the sugar additions are responsible for binding the recognition sites.

6.2.4 Characterising and identifying proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to their molecular size. The electrophoretic mobility of a protein without SDS added is a function of length of polypeptide chain or molecular weight as well as higher order protein folding, post-translational modifications and other factors. The addition of SDS causes the protein molecules to unfold, and this massive negative charge (from the SDS molecules which attach to the protein molecules) swamp any effects on modifying from the protein molecules' own charges, thus the protein molecules migrate according to their size. The gel after running SDS-PAGE is stained to visualise the protein bands and these bands are cut from the gel. This technique will be used to isolate active protein(s) from honey in an attempt to identify them from that molecular weight.

Identification of the proteins from honey will be attempted using MALDI mass spectrometry after removing the stained bands from the gel piece. In order to have molecules small enough to be mobilised the protein is digested with trypsin to form peptides.

MALDI is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as proteins, peptides and sugars) and large organic molecules which tend to be fragile and fragment when ionized by more conventional ionization methods. The ionization is triggered by a laser beam (normally a nitrogen laser). A matrix holding the protein is used to protect it from being destroyed by the direct laser beam and to facilitate vaporization and ionization.

The laser is fired at the crystals in the MALDI spot. The matrix absorbs the laser energy and it is thought that primarily the matrix is ionized by this event. The matrix then transfers part of its charge to the analyte molecules (*e.g*. protein), ionizing them while still protecting them from the disruptive energy of the laser.

The type of mass spectrometer most widely used with MALDI is the TOF (time-of-flight mass spectrometer), mainly due to its large mass range. The TOF measurement procedure is also ideally suited to the MALDI ionization process since the pulsed laser takes individual 'shots' rather than working in continuous operation. MALDI-TOF instruments are typically equipped with an "ion mirror", deflecting ions with an electric field, thereby doubling the ion flight path and increasing the resolution

These results are matched with a library of a published protein sequence database of fragments of proteins previously studied by MALDI-TOF mass spectrometry. Preferred libraries are Swiss-Prot and NCBInr.

6.2.5 Aims and experimental approach

The aim for this Chapter was to isolate the active component of honey and identify it. Honey was treated in various ways and assayed for its phagocytosis inhibiting activity to determine if the activity was lost. Diluted honey was treated with heat and trypsin to see if the phagocytosis-inhibiting effect was lost with protein denaturation or enzyme digestion (Sections 6.3.3 and 6.3.4). The results suggested protein was indeed a large part, at least, of honey's phagocytosis-inhibiting activity. The honey proteins were fractioned using gel permeation chromatography, and the individual fractions tested to isolate the activity (Section 6.3.5). These fractions were run on SDS-PAGE and the bands containing the proteins thought to have the activity were then identified by MALDI-TOF mass spectrometry (Section 6.3.7). A ConA glycoprotein affinity column was used to find if the active proteins bound to ConA, which would indicate that the active proteins had terminal modifications with mannose (Section 6.3.8). A mannosidase treatment of whole fractions was used to determine if these sugar modifications of the protein were important in the inhibition of phagocytosis (Section 6.3.9) and these proteins were separated on SDS mini-gels to examine the molecular weight of the proteins before and after mannosidase treatment.

6.3 Methods

6.3.1 Honey dilutions

Manuka honey (M144) was used for assays in this Chapter and the method of dilution is as described in Chapter 3.

6.3.2 THP-1 cell culturing

THP-1 cells for the phagocytic assay were maintained at 37°C, 5% CO_2, and 95% relative humidity in growth medium (as described in Section 2.3.2). Cells were split with fresh growth medium prior to the assay and assessed for viability as described in Sections 2.3.1 and 2.3.4.

6.3.3 Heat treatment of honey

Diluted honey solutions (10% in water) and undiluted honey samples were incubated at different temperatures to denature the proteins present: 65°C for 2 hours and 99°C for 10 minutes. Additional incubations were at 4°C and 37°C, each for 24 hours to test the stability of the proteins. All honey incubations were assayed in the phagocytosis assay as described in Section 4.3.1 but with a 0.5% final concentration of honey (V/V).

6.3.4 Trypsin treatment of honey

Manuka honey proteins were treated with high grade trypsin to see if the phagocytosis-inhibiting activity would be lost with degradation of the protein.

Trypsin was purchased from Sigma-Aldrich (Cat. No#T8128 Porcine pancreas) and dissolved in RPMI 1640 medium (with no FCS) to get a 5% solution. Trypsin was used at a final concentration of 2.5%.

A 5% Manuka honey solution (M144) was treated with a 5% trypsin solution (500 µl of each) at 37°C for 2 hours. A control honey solution was incubated at 37°C for the same amount of time with RPMI 1640 medium added instead of enzyme solution. An additional control included trypsin-treated honey incubated at 4°C. A trypsin inhibitor (Sigma-Aldrich Cat. No#T6522-25 MG) was used to end the reaction. Trypsin inhibitor was used at a final concentration of 2 mg/ml (40 µl of a 50 mg/ml solution). After the trypsin inhibitor was added to the honey/trypsin solution, the solution was incubated at 65°C for 10 minutes.

The phagocytosis assay with latex particles was carried out as described in Section 4.3.1 but using a 0.5% final equivalent concentration of 0.5% trypsin treated honey or control.

6.3.5 Fractioning honey

The fractionation of honey was carried out by Stacey Meyer from the Honey Research Unit, University of Waikato. Honey was initially dialysed to remove the sugars and low molecular weight compounds (Section 6.3.5.1) Then chromatography on Sephadex G-50 (Section 6.3.5.2) and Superose 12 FPLC (Section 6.3.5.3) chromatography columns was carried out. A reverse phase column was used to further separate out the proteins to determine if the fraction was made up of more than one protein (Section 6.3.5.4).

6.3.5.1 Elimination of the low molecular weight components of honey

Twenty-five grams of honey (M141) were suspended in 25 ml of distilled water and dialyzed (Cellu Sep T1 tubing, Membrane Filtration Products, Inc., Seguin, TX; EEUU, molecular mass cut off 3 500 Da) against 1 litre of purified water for 48 h at 4°C with four changes of the 1 litre of water during this time. The dialysis retentate was lyophilized and then stored at −20 °C until used. The lyophilized samples were reconstituted to 2 ml with 0.3 mol/l ammonium acetate solution.

6.3.5.2 Chromatographic separation on Sephadex G-50

The reconstituted retentate from dialysis (2 ml) was then loaded onto a Sephadex G-50 column (180 ml) and the material was eluted in 1 ml fractions (flow rate 0.5 ml/minute monitored at 280 nm). The fractions obtained from the 2 peaks were lyophilized and reconstituted to 100 µl with purified water for preliminary assessment in a SDS gel and tested in the phagocytosis assay. This assay indicated that the inhibitory activity was in the peak that was eluted first, but an assortment of protein sizes were present (37 kD – 250 kD) indicating the possibility of flow-though proteins. Further chromatographic separation was undertaken to generate a larger amount of protein. The chromatography on the Sephadex G-50 column was repeated three times and the fractions in the first peak on the elution trace from each run were pooled all together, rotary evaporated to 200 µl and then this pooled fraction further separated on a Superose 12 column (Section 6.3.5.3).

6.3.5.3 FPLC separation of protein on Superose 12

To further fractionate the first peak on the Sephadex G-50 elution trace, a 100 µl amount of the reconstituted sample was injected on a Superose 12 FPLC column (25 ml) and eluted as 1 ml fractions with phosphate-buffered saline (pH 7.11), flow rate 0.5 ml/minute, 0.5 cm/ml, monitored at 280 nm. These fractions were frozen at -20°C until further use. Two clearly separated major peaks (Fractions 8 and 14 as shown in Figure 6.6) were obtained and assayed for inhibitory activity in the phagocytosis assay (Section 6.3.5.6). These fractions were run on a 10% SDS-PAGE gel and processed for MALDI-TOF mass spectrometry identification (Section 6.3.7).

6.3.5.4 Reverse phase of active fractions

The fraction from chromatography on Superose 12 found to have phagocytosis-inhibiting activity (Fraction 8) was chromatographed on a reverse phase column

to further purify proteins for MALDI mass spectrometry identification. Chromatography was performed using a a FPLC system (Pharmacia-LKB, Uppsala, Sweden). A 500 µl amount of the Fraction 8 sample was injected on the column and eluted with a mobile phase in a gradient from 100% water to 100% of acetonitrile at a flow rate of 1 ml/minute monitored at 260 nm. The fractions comprising the two major peaks obtained (Fractions 23 and 26) were rotary evaporated to 50 µl. The 5 µl of the fractions were loaded onto 10% SDS mini gels to visualise and processed for MALDI-TOF mass spectrometry (see Section 6.3.6 and 6.3.7). The resulting proteins for each fraction obtained returned no results in the phagocytosis assay indicating the concentrations were too low.

6.3.5.5 Estimating protein concentration of fractions

Fractions from the Superose 12 FPLC chromatography column where assessed using a Nanodrop-1000 spectrophotometer to estimate protein concentration (results not shown). The fractions were rotary evaporated to achieve the approximate protein concentrations there would have been in un-fractionated honey.

6.3.5.6 Phagocytosis assay

The phagocytosis assay with latex particles was carried out as described in Section 4.3.1 but in place of honey, the protein fractions obtained from column chromatography (Superose 12, Section 6.3.5.3) were used. Fractions were used at approximately 1% of the concentration they would have been in honey. A 200 µl assay sample size was used due to the smaller amounts of protein available from the fractioning of honey.

6.3.6 Electrophoresis on SDS mini gels

Proteins obtained by fractionation of honey were run on SDS-PAGE gels to give an estimate of their molecular weight. Resolving gels with 10% and 12%

acrylamide were used for separating a different range of proteins. The 10% gel is best for separating 14-205 kDa proteins and 12% gel for 14-66 kDa proteins. The gels were made in an OWL Separation System gel caster (BioLab Scientific LTD) using assembled clean glass plates. The resolution gel was poured first, using a syringe, and left to set covered with a layer of butanol to ensure an even gel surface. When the gel was set the butanol was removed and the stacking gel was poured and a gel comb inserted to make wells for sample loading. The composition of the gels was:

10% resolution gel

6.8 ml 37% Acrylamide

2 ml 1 mol/l Tris pH 9.0

5.9 ml water

150 µl 10% Sodium Dodecyl Sulfate (SDS)

15 µl N,N,N',N'tetramethylenediamine (TEMED)

150 µl 10% ammonium persulfate (APS)

12% resolution gel

8.2 ml 37% Acrylamide

2 ml 1 mol/l Tris pH 9.0

4.5 ml water

150 µl 10% SDS

15 µl TEMED

150 µl 10% APS

5% stacking gel

0.66 ml 37% Acrylamide

1.25 ml 0.5 mol/l Tris pH 6.8

3.0 ml water

50 µl 10% SDS

5 µl TEMED

50 µl 10% APS

When the stacking gel was set, the gel was inserted into the electrophoresis chamber and running buffer (3.0 g Tris, 14.4 g Glycine, 1 g SDS, in 1 l MilliQ water) added in the top and bottom chamber. The comb was removed and the sample wells washed with electrophoresis buffer. The gel was then run for 10 minutes at 10 mA to remove any un-polymerised acrylamide.

Samples to be loaded on the gel were mixed 1:1 with 2X Tricine Sample Buffer (0.1 mol/l Tris pH 6.8, 24% (w/v) glycerol, 8% (w/v) SDS, 0.2 mol/l dithiothreitol, 0.02% (w/v) Coomassie Blue G-250) and heated at 99°C for 5 minutes, then left to cool. Samples were centrifuged (2 000 x *g* for 20 seconds) to pellet any sediment and 10 µl of sample loaded per lane. A BIORAD Precision Plus Dual Colour protein standard ladder (Cat. No.#161-0374) was run alongside the samples to provide an estimated protein size for the samples. The standard contained proteins sized 10-250 kDa: 6 µl was loaded at each end of the gel.

The gel was run at 10 mA until samples had migrated through the stacking gel into the resolution gel. The current was then increased to 20 mA. When the dye in the sample had run off the edge of the gel, the current was switched off and the gel removed and stained.

Two methods were used to visualize bands. Silver staining was used to identify fainter bands as it is more sensitive. A Fast Blue method was used prior to MALDI-TOF mass spectrometry work as the stain is more readily removed than silver. Fast blue is modified Coomassie Brilliant Blue which binds with less affinity to glycoproteins making it ideal for honey which has a large proportion of glycoproteins. This allows for better stain removal prior to protein MALDI mass spectrometry work as remaining stains can interfere with quantification (Deutscher 1990).

All reagents were made fresh 5 minutes before staining took place and kept at 4°C until required. The gel was first fixed for 30 minutes in 50% ethanol/12% acetic acid, then soaked in 30% ethanol for 15 minutes. Then 100 ml of 0.02% sodium thiosulfate was added and left for 1-2 minutes. The gel was soaked in

100 ml 0.1% silver nitrate + 100 µl formaldehyde for 10 minutes. It was then washed in water for 10 seconds and 100 ml developer (3 g sodium carbonate, 100 µl formaldehyde, 40 µl 1% sodium thiosulfate in 100 ml MilliQ water) added. Development was stopped with 10% acetic acid after the bands became visible. Developed gels were stored in MilliQ water until required.

Fast Blue was obtained from Fisher Biotec (Cat. No.#FS-100). Fast Blue was diluted (8 ml of Fast Stain concentrate, 32 ml MilliQ water, 10 ml 45% methanol with 10% acetic acid) before use. Gels were first washed in 40% methanol with 10% acetic acid for 10-20 minutes followed by a rinse in MilliQ water. Then 100 ml of diluted Fast Blue was added and the gel was gently swirled in the solution for 20 minutes. Gels were de-stained with 10% acetic acid for 10 minutes, then stored in MilliQ water until required.

6.3.7 MALDI-TOF mass spectrometry identification of active honey proteins

The MALDI-TOF work was completed in collaboration with Jonathan Puddick, from the Waikato Mass Spectrometry Facility, University of Waikato.

The single protein bands were cut from the 10% SDS mini-gels after electrophoresis of both the Superose 12 Fraction 8 and Fraction 14 (both were 50-65 kDa molecular weight) and prepared for MALDI-TOF identification. The reverse phase fractions were not concentrated enough to visualize a distinct band on the gel, so a band was cut at the expected location of the target protein according to the molecular weight ladder. Unfortunately no results could be obtained from these gel pieces

6.3.7.1 Preparation of proteins

A clean scalpel was used to excise the bands which were then de-stained with 30% ethanol to remove the Fast Blue dye by incubating at 60°C for 15 minutes or until the gel pieces appeared colourless. The bands were washed twice in 30%

ethanol and then shrunk with 100% acetonitrile for 10 minutes. Acetonitrile was removed by aspiration and the gel piece was dried by vacuum in a Speed Vac to remove residual moisture for 30 minutes.

The protein in the gel pieces was cleaved into peptides for analysis by MALDI-TOF mass spectrometry by digesting the protein in the gel piece with trypsin. The method used was adapted from details given by Dr. Jo McKenzie, University of Waikato. To each gel piece 10 µl of 25 mmol/l ammonium bicarbonate was added, followed by 10 µl of sequencing grade trypsin solution (49 µl 25 mmol/l ammonium bicarbonate in 10% acetonitrile, 1 µl sequencing trypsin (1 mg/ml Promega Cat. No.#V5111). Tubes were then left overnight at 37°C. To each tube 14 µl 50 % acetonitrile with 0.3% trifluoroacetic acid was added and the tubes vortexed and sonicated for 10 minutes. The gel piece was discarded and the solutions were refrigerated until use.

6.3.7.2 Preparation of matrix

The method used was obtained from Jonathan Puddick, Waikato Mass Spectrometry Facility, University of Waikato.

Five mg α-Cyano-4-hydroxycinnamic acid (CHCA) was added to 500 µl 65% acetonitrile with 1% trifluoroacetic. The solution was vortexed for 2 minutes, sonicated for 10 minutes, vortexed again for 2 minutes then centrifuged at 12 000 x *g* for 5 minutes. The prepared protein digest solutions and matrix were combined (2:1) and 1 µl was spotted onto the MALDI Anchor chip target plate. The spots were allowed to air dry and then washed by pipetting 5 µl of 1% trifluoroacetic onto and off the dried matrix spot and allowed to dry.

6.3.7.3 Autoflex operating procedure

The external calibration was conducted with the Bruker Daltonics Peptide standard 206 195 prior to analysis. A monoisotopic peptide calibration was used

with a zooming of ± 0.1%. Once a good, clean spectrum had been collected the mass spectrometer automatically recalibrated. The fit result was accepted if the error was no more than ± 10 ppm.

Internal calibration can be conducted if products resulting from the autolysis of the trypsin are present. These products yield peaks at 842 and 2 211 Da. The peaks are assigned as internal calibrants in the flex analysis program.

A Bruker Autoflex II TOF/TOF mass spectrometer was used to analyze peptide digests. An average of 30 shots per sample was taken to build the peptide mass fingerprint spectrum. The mass range selector was set at low range and the detector gain voltage offset was set at 1 400 v. The detection settings were set in the range of 480 – 3 540 Da. The instrument was manually operated using flex control software. Suitable spectra were saved and exported into flex analysis.

6.3.7.4 Analysing MALDI spectra

Using the Biotools software the spectrum collected can be searched against a variety of protein databases. The databases of preferred use are Swiss-Prot and NCBInr. The parameters for the searching of Eukaryota proteins in Swiss-Prot are a peptide tolerance of ± 200 ppm and 1 missed cleavage. A protein score greater than 64 is significant for Swiss-Prot. The parameters for searching of Eukaryota proteins in NCBInr are 1 missed cleavage and a peptide tolerance of ± 200 ppm. The protein score required for a significant hit in NCBInr is 78. These parameters were correct, at least, up until May 2009. Molecular masses can be measured to within an accuracy of 0.01% of the total molecular mass of the molecule/peptide. This is sufficient to allow minor mass changes to be detected, *e.g.* the substitution of one amino acid for another or a post-translational modification.

The main function of the mass analyser is to separate, or resolve, the ions formed in the ionisation source of the mass spectrometer according to their mass-to-charge ratios. The peptides produced by the tryptic digest are separated

by ionisation due to their mass and this mass can be calculated. The detector monitors the ion current, amplifies it and the signal is then transmitted to the data system where it is recorded in the form of a mass spectrum. The mass to charge values of the ions are plotted against their intensities to show the number of components in the sample, the molecular mass of each component, and the relative abundance of the various components in the sample. When using MALDI-TOF to identify a protein, the ions generated must form a clear mass spectrum with intense peaks, indicating that only one protein (in large quantities at least) is present in the band cut from the SDS electrophoresis gel. When more than one protein is present in the sample there are typically no clear peaks correlating to large amounts of the same peptide.

6.3.8 Isolating glycoproteins from the protein fraction

A 1% solution of diluted Manuka honey (in purified water) and the fractions containing the phagocytosis-inhibiting activity (Fraction 8) was assayed using a glycoprotein isolation kit utilising a Concanavalin A (ConA)-Sepharose column to identify any glycosylation with mannose the proteins may have. The honey and Fraction 8 were separated on a ConA column and both the flow-through proteins and the retained proteins were used in the latex particle phagocytosis assay.

6.3.8.1 Procedure for glycoprotein isolation using ConA

A glycoprotein isolation kit was purchased from Thermo Scientific (Cat. No.# 89804 ConA) and for preparation of the sample the manufacturer's directions were followed. Briefly, binding/wash and elution buffers were brought to room temperature and then the samples diluted with 5X binding/wash buffer stock solution (1 to 1.5 mg of total protein/ml mixed 4:1 with 5X Buffer, not exceeding 800 µl total final volume).

The isolation of glycoproteins was as follows; 1X Binding/Wash Buffer was prepared by diluting 460 µl 5X Binding/Wash Buffer with 1 740 µl of ultrapure

water per sample. A fresh column was prepared by adding 200 µl ConA lectin gel, centrifuging for 1 minute at 1 000 × *g* and discarding the storage buffer. The column was placed in a collection tube and 200 µl 1X Binding/Wash Buffer was added to the gel. The top cap was closed and the column centrifuged for 1 minute at 1 000 × *g* and the rinse was discarded. This was repeated twice. The protein sample was added and the column incubated for 10 minutes at room temperature with end-over-end mixing using a rotator. The column was then centrifuged for 1 minute at 1 000 × *g* and the flow-through liquid collected. This step was repeated once more. The column was then rinsed with 400 µl 1X Binding/Wash Buffer and incubated for 5 minutes at room temperature with end-over-end mixing using a rotator. The column was then centrifuged for 1 minute at 1 000 × *g* and the rinse discarded. This step was repeated once more. Then 200 µl of Elution Buffer was added to the column and which was then incubated for 5 minutes at room temperature with end-over-end mixing. The column was then centrifuged for 1 minute at 1 000 × *g* and the eluate was collected. This step was repeated once more. The non-retained and the eluted retained fractions were stored on ice for immediate use or frozen for later analysis.

6.3.8.2 SDS electrophoresis of glycoproteins

The wash-through and the eluted bound proteins from the ConA column from whole honey (1% dilution) and Fraction 8 were subjected to electrophoresis on a 12% SDS gel as described in Section 6.3.6. The ConA binding proteins and the flow through non-binding proteins from Fraction 8 were concentrated by using a Vivaspin column 5 kDa molecular weight cut-off (Viva Science, 1 200 *x g* for 30 minutes at 10°C to 50 µl) and then a Nanodrop-1000 spectrophotometer was used to estimate protein concentration compared with the 1% solution of diluted honey

6.3.8.3 Phagocytosis assay with glycoproteins

Phagocytosis assay conditions were as described in Chapter 4. ConA binding glycoproteins and the flow through (non-ConA binding proteins) were used in the phagocytosis assay. As a control, a sample of Fraction 8 prior to ConA isolation was used. Fractions were used at concentrations that would equal that of 1% diluted Manuka honey. This was achieved by using a Vivaspin column 5 kDa molecular weight cut-off (Viva Science, 1 200 *x g* for 30 minutes at 10°C) to concentrate and a Nanodrop-1000 spectrophotometer was used to estimate protein concentration compared with a 1% solution of diluted honey.

6.3.9 Mannosidase treatment

To determine the importance of the mannose terminal modifications, dialysed honey (1%) and the glycoprotein that bound the ConA column (Section 6.3.8) from Fraction 8 (Section 6.3.8.1) were subjected to mannosidase treatment. A sample of Fraction 8 prior to ConA separation was also subjected to mannosidase treatment for use in the phagocytosis assay. The resulting fractions were subjected to electrophoresis on a 12% SDS gel.

6.3.9.1 Mannosidase

α-Mannosidase from *Canavalia ensiformis* (Jack bean) was purchased from Sigma-Aldrich (Cat. No.#M7257) and used at 1 mg/ml in 1% diluted Manuka honey, ConA fractions (Section 6.3.8) and Fraction 8 (Section 6.3.5.3) and incubated at 37° for 4 hours. As a control, equal amounts of enzyme were added to a 1% dilution of Manuka honey and the solution incubated at 4°C for 4 hours. Proteins separated on ConA (Section 6.3.8) and Fraction 8 (Section 6.3.5.3) after treatment with mannosidase were subjected to electrophoresis on a 12% SDS gel as described in Section 6.3.6.

6.3.9.2 Phagocytosis assay modification

The phagocytosis assay with latex particles was the same as for Chapter 4 using a an equivalent concentration as in 0.5% honey of the mannosidase-treated honey fractions of both ConA separated protein from Fraction 8, and a 1% dilution of Manuka honey separated on a ConA column. The honey equivalent concentration of 0.5% was achieved by using a Vivaspin column 5 kDa molecular weight cut-off (Viva Science, 1 200 *x g* for 30 minutes (approximate) at 10°C) to concentrate. A Nanodrop-1000 spectrophotometer was used to estimate the protein concentration by comparing the protein to the protein content of a 0.5% solution of diluted honey.

6.4 Results

6.4.1 Heat treatment of honey

Manuka honey proteins were subjected to heat treatment (37°C, 65°C and 99°C) with two non-heat treated honey dilutions being freshly diluted honey or diluted honey stored at 4°C for 24 hours prior. All honey dilution treatments were compared with a non honey-treated control. Whole honey incubated at 99°C was unable to be diluted successfully due to caramelisation so was not assayed. The results are shown in Figure 6.1.

All treatments assayed of diluted and whole honey (excluding whole honey incubated at 99°C) had a significant (p<0.001) effect by reducing the phagocytosis-inhibiting effect of honey compared to the control with no honey added. Heat treatments of 65°C and 99°C of diluted honey, and 65°C of whole honey, had a significantly reduced effect on the inhibition of phagocytosis compared to freshly-diluted non-heat-treated honey. This indicated that denaturation had taken place. Whole honey retained all of its activity at 37°C and 4°C, possibly due to protection by the sugar in undiluted honey. Diluted honey

incubated at 37°C for 24 hours had a significantly reduced effect on the inhibition of phagocytosis compared to freshly-diluted non-heat-treated honey.

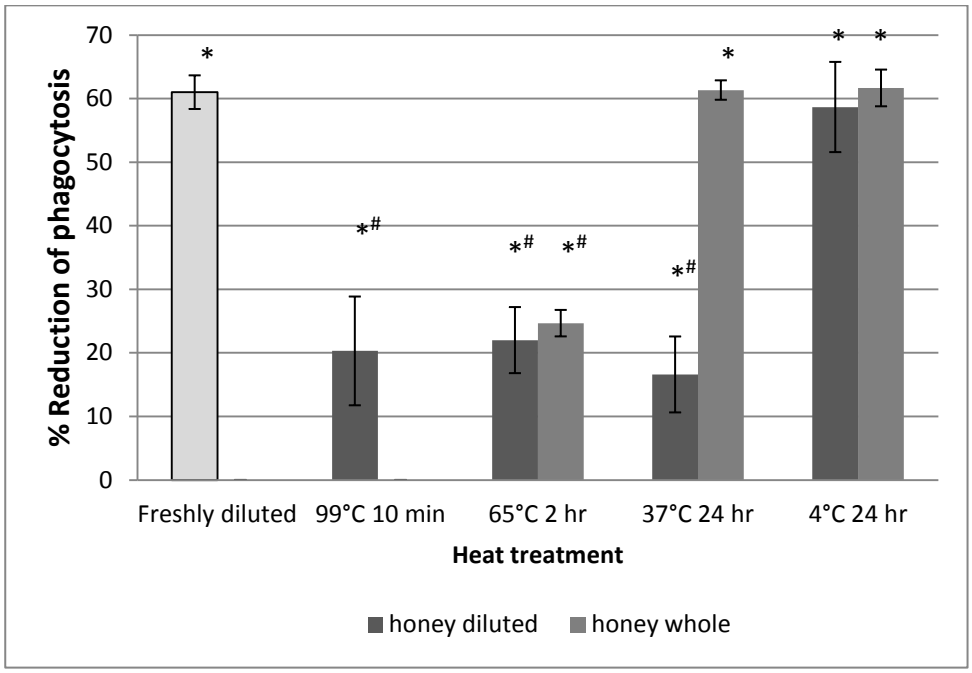

*Figure 6.1. The effect of heat on the phagocytosis-inhibiting activity of Manuka honey in LPS-stimulated THP-1 cells. Manuka honey was exposed to various temperatures for various times prior to an assay for phagocytosis. Results show % reduction of phagocytosis by honey compared with the control with no honey added. The assays were done with a 5% concentration of honey. Whole honey at 99°C was not assayed. Error bars show ±1 SD of the mean from at least three experiments. * p<0.001 analysed by ANOVA compared with the non honey-treated control. # p<0.001 analysed by ANOVA compared with the freshly diluted honey control.*

6.4.2 Trypsin treatment of honey

Manuka honey proteins were subjected to trypsin treatment at 37°C for two hours with a control treatment at 4°C, to see if an intact protein is important in the phagocytosis-inhibiting activity of Manuka honey. The results from the assay of phagocytosis-inhibiting activity after this treatment are shown in Figure 6.2.

Trypsin treatment of honey at 37°C gave a significant (p<0.001) reduction in the phagocytosis-inhibiting effect of honey whereas there was little difference in the honey treated with trypsin at 4°C, indicating that the effect was due to the enzyme activity of trypsin.

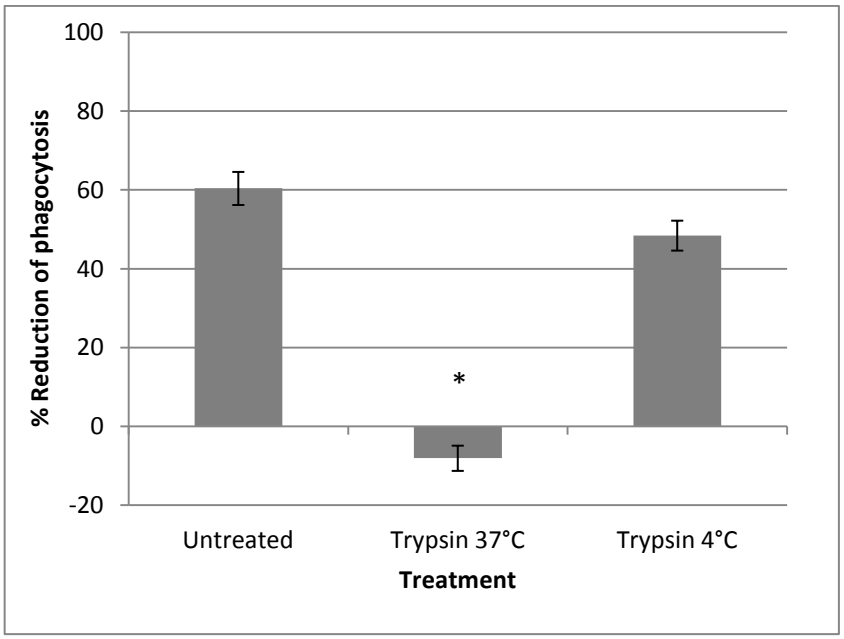

*Figure 6.2. The effect of trypsin on the phagocytosis-inhibiting activity of Manuka honey in LPS-stimulated THP-1 cells. Manuka honey was treated with trypsin (4°C or 37°C) prior to assay for phagocytosis. A 0.5% honey solution was assayed. Results show % reduction of phagocytosis. Error bars show ±1 SD of the mean from three assays. * p<0.001 analysed by ANOVA compared with the untreated control.*

6.4.3 Honey fractions obtained by chromatography

Honey was dialysed (Section 6.3.5.1) and the retentate proteins fractionated on a Sephadex G-50 chromatography column (Section 6.3.5.2). Figure 6.3 shows the elution trace from the Sephadex G-50 chromatography column and Figure 6.4 shows the silver-stained SDS electrophoresis gel image of the fractions obtained from the G-50 chromatography.

The first peak eluted from the column was seen to contain several proteins, with molecular weights up to >200 kDa. The major ones had molecular weights of approximately 37 kDa, 55 kDa and 65 kDa. The second peak eluted from the column was seen to contain little protein, with just a trace of the one with a molecular weight of 65 kDa.

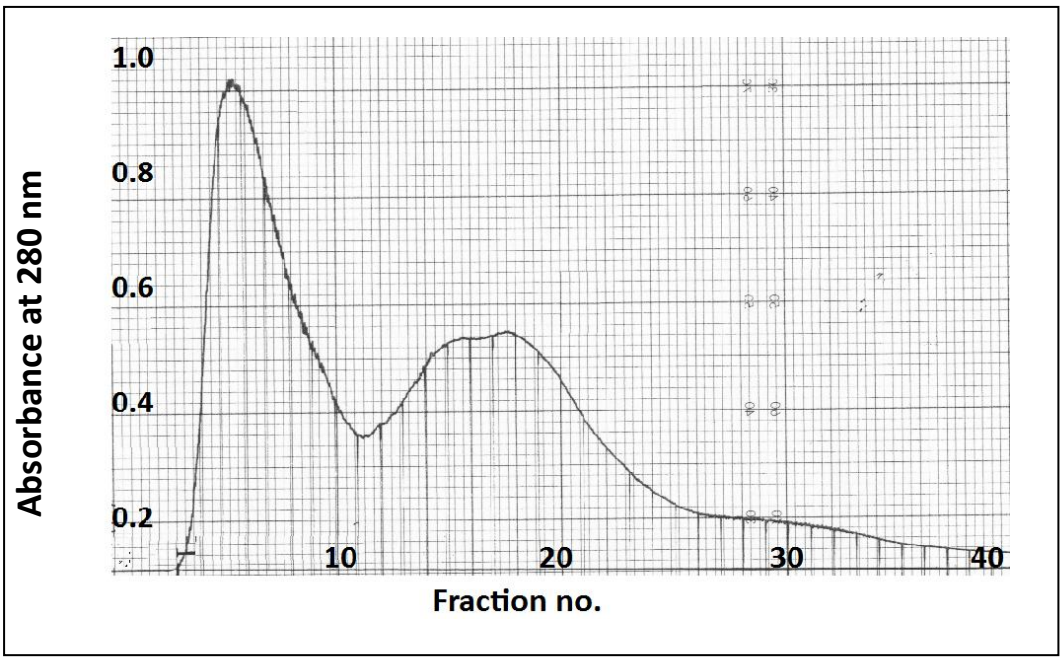

Figure 6.3. Elution trace from chromatography of the dialysis retentate of Manuka honey on a 180 ml G-50 Sephadex column. The fraction size was 1 ml.

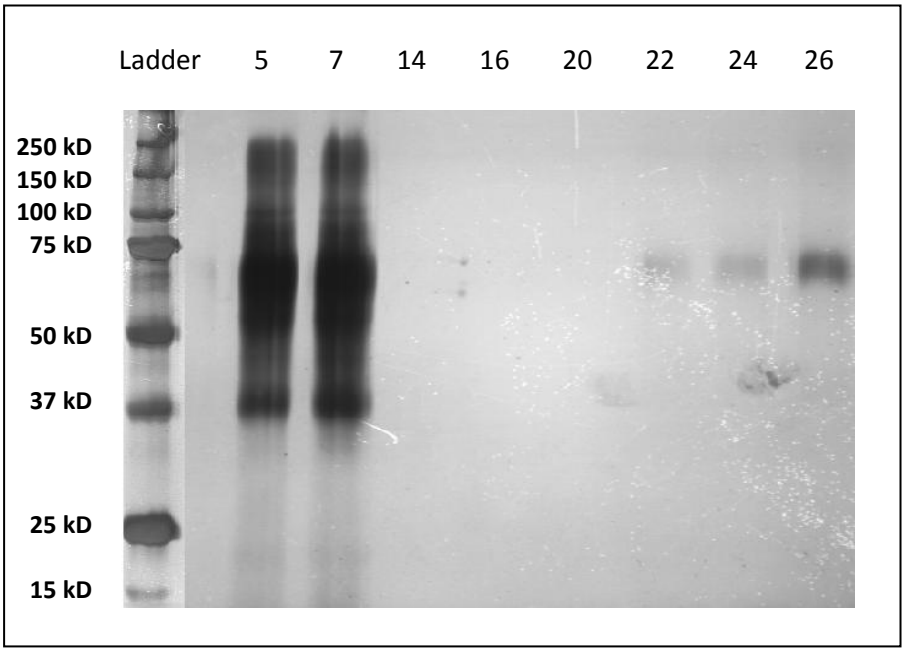

Figure 6.4. Image of a silver-stained SDS electrophoresis gel run with fractions shown in Figure 6.3 from the G-50 chromatography on Sephadex G-50 numbered as in Figure 6.3.

Preliminary phagocytosis assays suggested the bulk of the inhibitory activity was in the first peak (Fractions 4-10) from the chromatography. Figure 6.5 shows the results of the phagocytosis assay. The inhibitory activity was only in the early fractions (Fractions 4-16), with Fraction 6 and 8 having the most. The SDS gel (Figure 6.4) showed no obvious protein in the Fractions 14 and 16 yet these fractions had some inhibitory activity. It is assumed that the protein concentration was too low to be detected by staining in the SDS gel.

Fractions 4-10 from the chromatography on Sephadex G-50 shown in Figure 6.3 were pooled from three repeated runs. The pooled fractions were chromatographed on a Superose 12 FPLC column to further separate the different molecular weights of the proteins, as the SDS gel (Figure 6.4) showed a range of high molecular weight proteins present in the fraction. The results are shown in Figure 6.6.

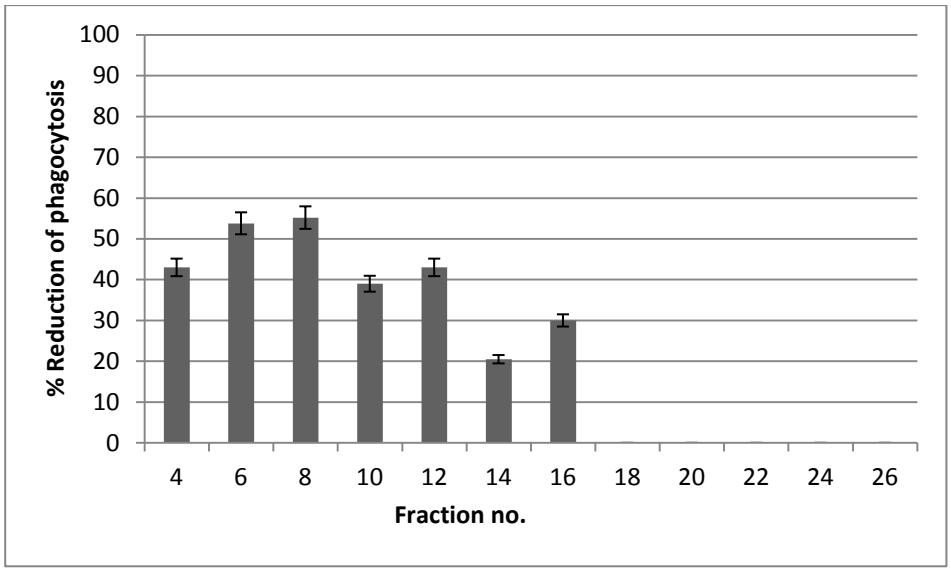

Figure 6.5. Phagocytosis-inhibiting activity of Fractions 4-26 (even numbers only tested) obtained from G-50 Sephadex chromatography shown in Figure 6.3. Results show % reduction of phagocytosis compared with that by the non-treated control in LPS-stimulated THP-1 cells. Error bars show ±1 SD of the mean from three assays.

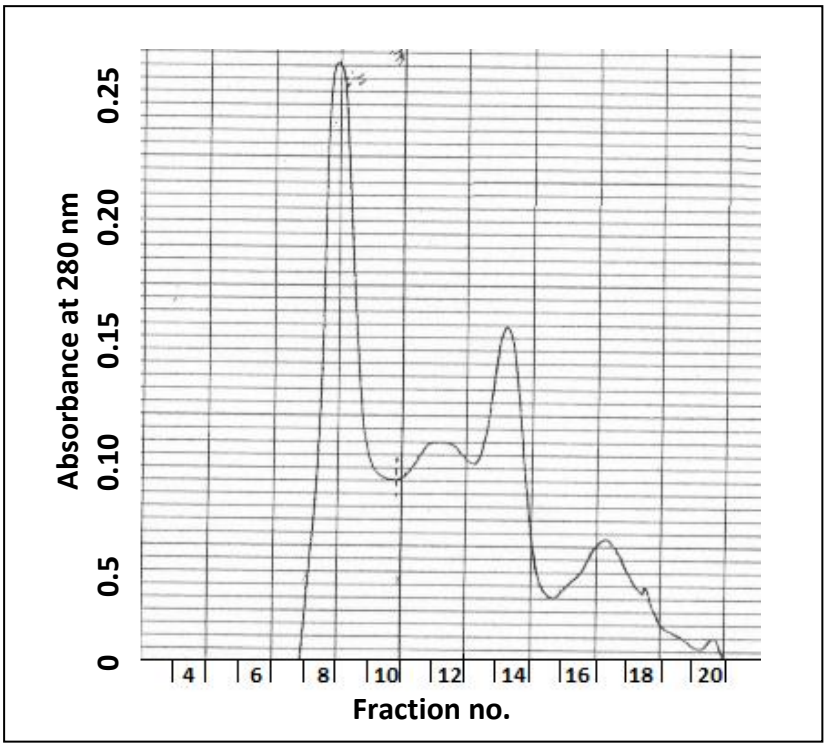

Figure 6.6. Elution profile from chromatography on a 25 ml Superose 12 FPLC column of Fractions 4-10 from chromatography on Sephadex G-50 shown in Figure 6.3. The fraction size was 1 ml.

The two major peaks (Fractions 8 and 14) were run on an SDS PAGE minigel. The results from this are shown in Figure 6.7. Fraction 8 was seen to contain a major band of molecular weight 65 kDa with a trace of protein spread around 200 kDa. Fraction 14 was seen to contain a major band of molecular weight 55 kDa. These fractions were also tested in the phagocytosis assay (Figure 6.8). Fraction 8 was seen to contain the phagocytosis-inhibiting activity whereas Fraction 14 contained no more inhibitory activity than Fraction 23 which was included as a control because it contained little or no protein. The main bands seen in the SDS-PAGE of Fractions 8 and 14 were excised from the gel for analysis by MALDI-TOF as indicated by the arrows in Figure 6.7.

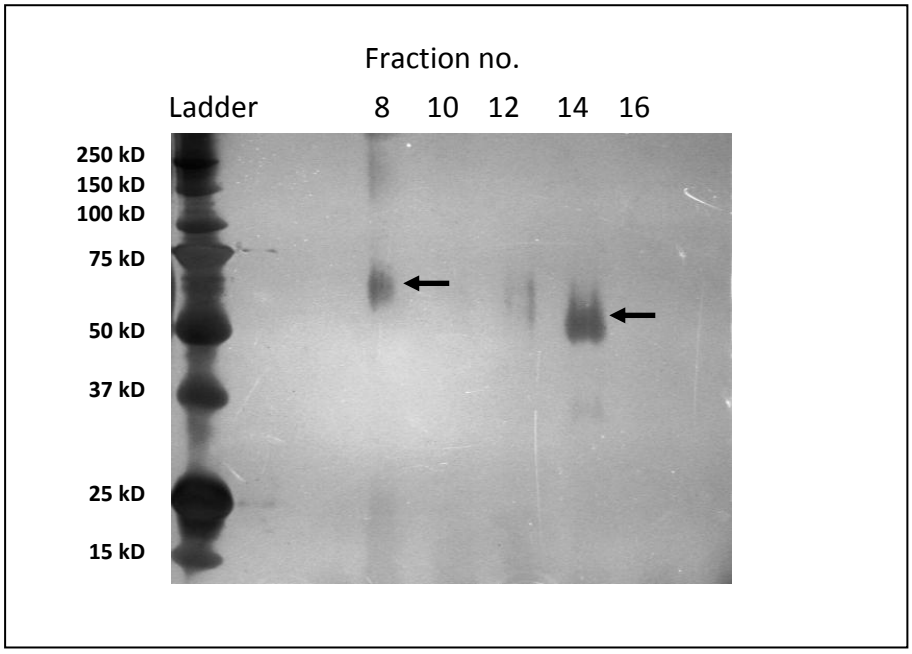

Figure 6.7. Image of a silver-stained SDS electrophoresis gel run with fractions shown in Figure 6.6 from the Superose 12 chromatography column. Arrows indicate bands excised for MALDI-TOF analysis.

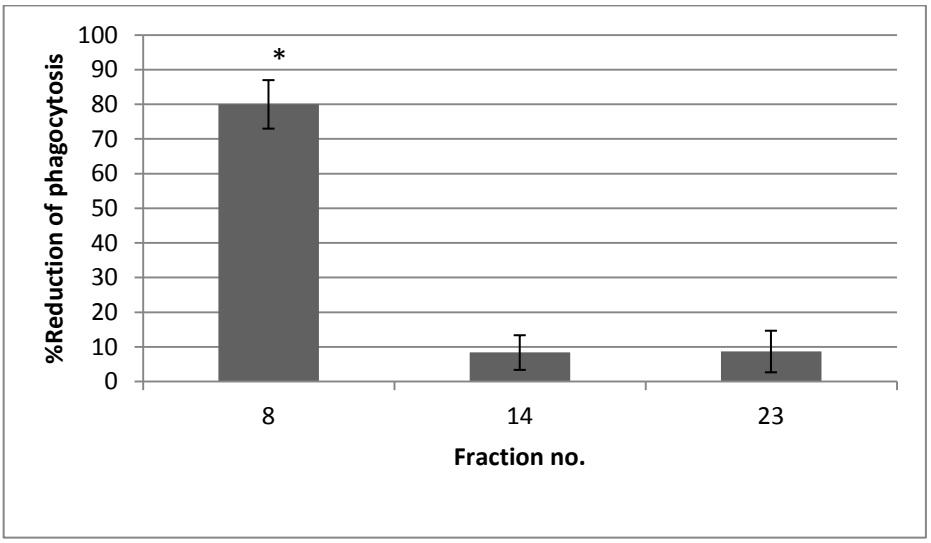

*Figure 6.8. Phagocytosis-inhibiting activity of Fractions 8, 14 and 23 obtained from Superose 12 chromatography in Figure 6.6. Results show % reduction of phagocytosis compared with that by the non-treated control in LPS-stimulated THP-1 cells. Error bars show ±1 SD of the mean from three assays. Fraction 23 was included as a control as it contained little/no protein. * p<0.001 analysed by ANOVA compared with Fractions 14 and 23.*

6.4.4 Reverse phase chromatography of active fraction

The active fraction from the chromatography on Superose 12 (Fraction 8 in Figure 6.6) was chromatographed on a reverse phase column to determine if more than one protein was present in this fraction. Figure 6.9 shows the elution profile for the reverse phase chromatography.

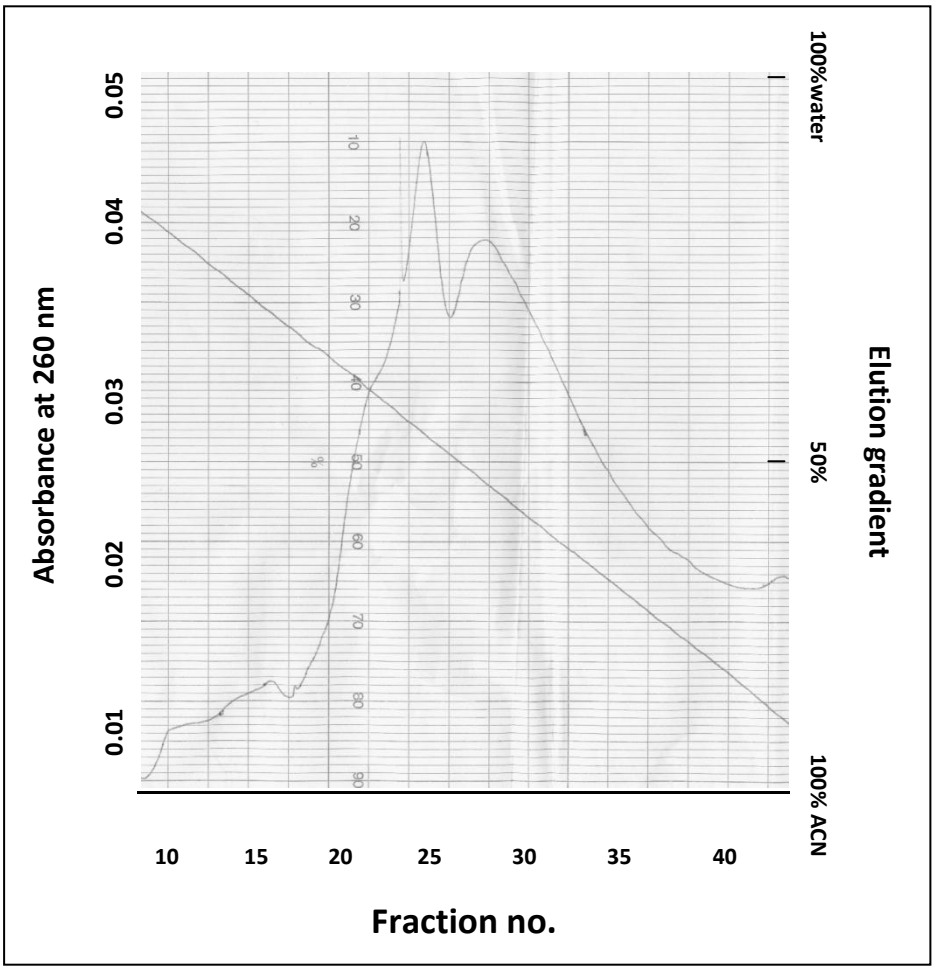

Figure 6.9. Elution profile from chromatography on a reverse phase column of Fraction 8 from chromatography on Superose 12 shown in Figure 6.6. The fraction size was 0.5 ml.

The proteins in Fraction 8 separated by reverse phase were run on a 12% SDS electrophoresis gel but this failed to show any bands possibly, due to low concentrations. Phagocytosis assays on its fractions from the reverse phase chromatography also failed to get a result possibly due to the low protein concentrations.

6.4.5 MALDI-TOF mass spectrometry identification of active proteins

The proteins recovered from the excised bands in the gels from SDS electrophoresis, Fractions 8 and 14 from the Superose 12 chromatographic separation, were subjected to a tryptic digest for MALDI-TOF mass spectrometry analysis for identification (bands excised indicated by arrows in Figure 6.7).

The Autoflex operating procedure described in Section 6.3.7.3 was used to obtain a peptide mass fingerprint spectrum of Fraction 14 isolated from the Superose 12 chromatography column. The mass spectrum generated by MALDI-TOF mass spectrometry is presented in Figure 6.10 a. The molecular weight of each peptide was entered into the Swiss-Prot library and searched for matches. Two hits/matches of the molecular weight of the tryptic peptides to the Swiss-Prot library were obtained. The peptide matches are displayed under the sequence for the most significant matching protein in Figures 6.10 b and 6.10 c. The most significant hit/match was for the protein Apalbumin 3 (MRJP-3). The second hit Apalbumin 1 (MRJP-1), is not a significant match in itself, but because the mass spectrum peaks which correspond to this match are different from the ones which contribute to the match for MRJP-3, it is very likely to be present in the sample.

This was also done for Fraction 8 isolated from the Superose 12 chromatography column. The MALDI mass spectrum of the peptides from Fraction 8 is displayed in Figure 6.11 a. One significant hit was obtained for the mass spectrum, MRJP-1. The sequence coverage for the peptides and the protein match MRJP-1 are displayed in Figure 6.11 b.

The two major peaks (Fractions 23 and 26) from the run of Fraction 8 on reverse phase chromatography (Figure 6.9) were digested for MALDI-TOF mass spectrometry analysis straight from the chromatography but no significant hits were obtained, probably due to due to a low concentration of protein. Attempts were made to concentrate the proteins but still no suitable peptide mass spectrum or significant hits were obtained.

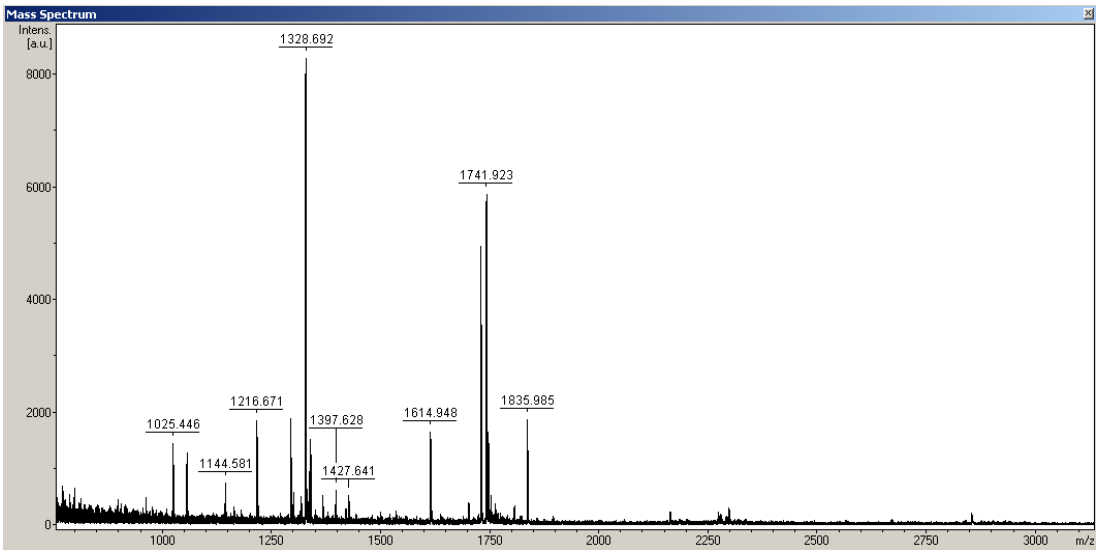

Figure 6.10 a. Mass spectrum of peptides obtained by tryptic digest of Fraction 14 isolated with a Superose 12 chromatography column (Figure 6.6). The single protein band seen on SDS electrophoresis of this fraction (Figure 6.7) was cut from gel and digested with trypsin prior to MALDI-TOF mass spectrometry.

Protein: Major royal jelly protein 3 precursor - Apis mellifera (Honeybee) MRJP3_APIME Peak threshold: 0.0

Intensity coverage: 57.4 % (19035 cnts) Sequence coverage MS: 13.2 % Sequence coverage MS/MS: 0.0 % pI: 6.5 MW (kDa): 61.6

10	20	30	40	50	60	70	80	90
MTKWLLLVVC	LGIACQDVTS	AAVNHQRKSA	NNLAHSMKVI	YEWKHIDFDF	GSDERRDAAI	KSGEFDHTKN	YPFDVDRWRD	KTFVTIERNN
100	110	120	130	140	150	160	170	180
GVPSSLNVVT	NKKGKGGPLL	RPYPDWSFAK	YEDCSGIVSA	FKIAVDKFDR	LWVLDSGLVN	NNQPMCSPKL	LTFDLKTSKL	VKQVEIPHNI
190	200	210	220	230	240	250	260	270
AVNATTGMGE	LVSLAVQAID	RTNTMVYIAD	EKGEGLIMYQ	NSDDSFHRLT	SNTFDYDPRY	TKLTVAGESF	TVKNGIYGIA	LSPVTNNLYY
280	290	300	310	320	330	340	350	360
SPLLSHGLYY	VDTEQFSNPQ	YEENNVQYEG	SQDILNTQSF	GKVVSKNGVL	FLGLVGNSGI	ACVNEHQVLQ	RESFDVVAQN	EETLQMIVSM
370	380	390	400	410	420	430	440	450
KIMENLPQSG	RINDPEGNEY	MLALSNRMQK	IINNDFNFND	VNFRILGANV	DDLMRNTRCG	RYHNQNAGNQ	NADNQNADNQ	NANNQNADNQ
460	470	480	490	500	510	520	530	540
NANKQNGNRQ	NDNRQNDNKQ	NGNRQNDNKQ	NGNRQNDNKQ	NGNRQNGNKQ	NDNKQNGNRQ	NDNKRNGNRQ	NDNQNNQNDN	NRNDNQVHHS
550								
SKLH								

Figure 6.10 b. Sequence coverage of the mass spectrum displayed in Figure 6.10 a. The peptides have a molecular weight that matches that of the peptides generated from tryptic digest of the protein MRJP-3 precursor – Apis mellifera (Honeybee) MRJP3_APIME generating a significant hit (Score of 66). The bars indicate peptides that were observed in the MALDI-TOF data.

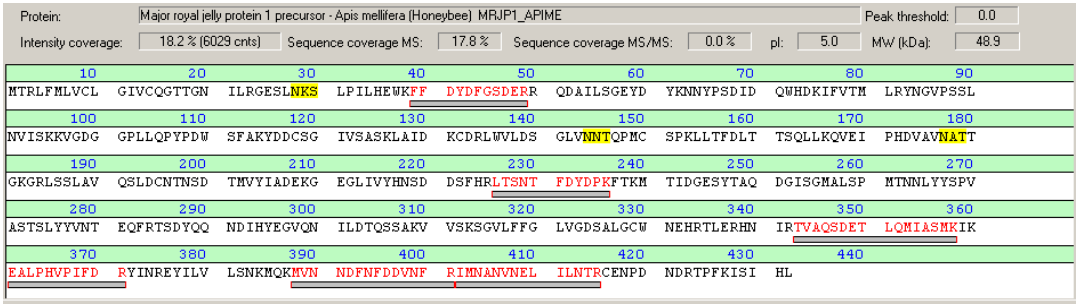

Figure 6.10 c. Sequence coverage of the mass spectrum displayed in Figure 6.10 a. The peptides have a molecular weight that matches that of the peptides generated from tryptic digest of the protein MRJP-1 precursor – Apis mellifera (Honeybee) MRJP1_APIME did not generate a significant hit (Score of 59). The bars indicate peptides that were observed in the MALDI-TOF data.

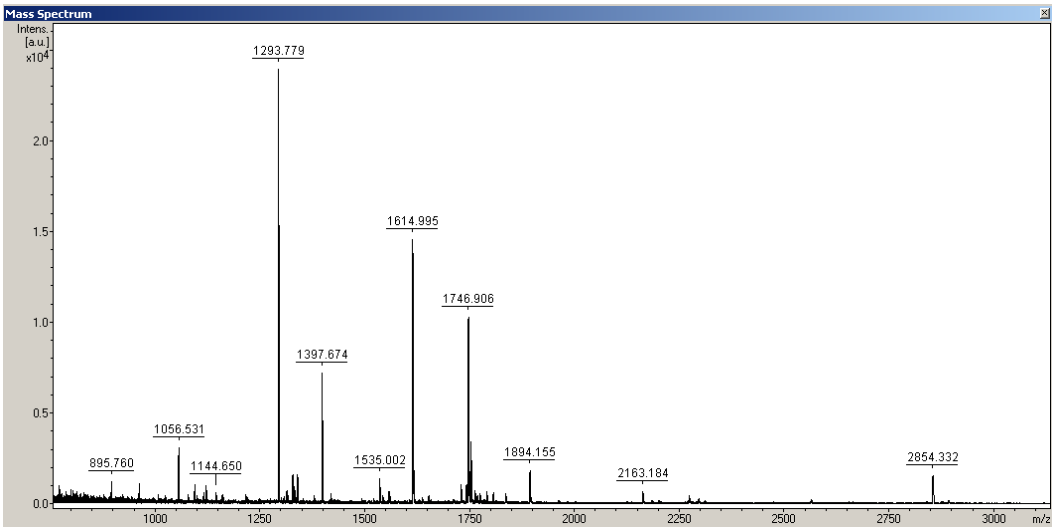

Figure 6.11 a. Mass spectrum of peptides obtained by tryptic digest of Fraction 8 isolated with a Superose 12 chromatography column (Figure 6.6). The single protein band seen on SDS electrophoresis of this fraction (Figure 6.7) was cut from gel and digested with trypsin prior to MALDI-TOF mass spectrometry.

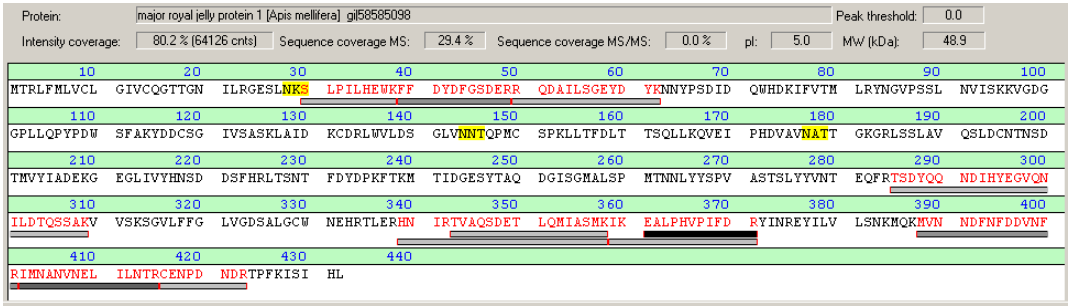

Figure 6.11 b. Sequence coverage of the mass spectrum displayed in Figure 6.11 a. The peptides have a molecular weight that matches that of the peptides generated from tryptic digest of the protein MRJP-1 precursor – Apis mellifera (Honeybee) MRJP1_APIME gi|58585098 generating a significant hit (Score of 117). The bars indicate peptides that were observed in the MALDI-TOF data

6.4.6 ConA glycoprotein isolation column

A ConA column was used to separate mannose-containing glycoproteins from other proteins in fractions identified as having phagocytosis-inhibiting activity (Fractions 4 -10 obtained from Sephadex G-50 chromatography column, Figure 6.3). The separated proteins were subjected to SDS electrophoresis on a 12% gel and an assay of phagocytosis-inhibiting activity. The results of the SDS electrophoresis are shown in Figure 6.12. The results show that the mannose-binding lectin bound proteins of molecular weight of 35 kDa, 50 kDa and 65 kDa.

Results using the glycoproteins isolated on ConA were inconclusive for the phagocytosis assay, possibly due to the high manipulation and low concentrations of protein after glycoprotein isolation. It was necessary to remove the buffer from the glycoproteins as it proved to be toxic to the THP-1 cells in the phagocytosis assay, and this removal resulted in a loss of protein. Protein (brown in colour) was observed to be stuck on the filter membrane after washing steps and could not be fully removed.

6.4.7 Mannosidase treatment

Eluted glycoprotein that was retained and the flow-though from running dialysed honey through a ConA column were treated with mannosidase. The results from the SDS electrophoresis of these treated fractions are displayed in Figure 6.13.

Compared with the bands visible without the mannosidase treatment (approximately 37 kDa, 55 kDa, 65 kDa and 150 kDa), the enzyme-treated protein had two major additional bands. These bands generated from the mannosidase treatment appeared at approximately 43 kDa and 46 kDa and were from the protein that bound to the ConA column. This indicates that these bands must represent the mannose-containing glycoproteins, confirming that the ConA column bound such proteins.

The results from the phagocytosis assay using mannosidase-treated fractions obtained from chromatographic separation or ConA column separations were inconclusive, possibly due to over-manipulation of the protein, or low concentrations, so instead mannosidase-treated 1% dialysed honey was used in the phagocytosis assay. The results are shown in Figure 6.14.

Mannosidase treatment of dialysed honey caused a 21 % reduction of phagocytosis inhibiting activity (from 52 % to 41 %) compared with control dialysed honey that was incubated at 37 °C without the enzyme showing that the presence of the mannose units on the glycoprotein was important for the phagocytosis-inhibiting activity. This was a significant reduction ($p<0.001$).

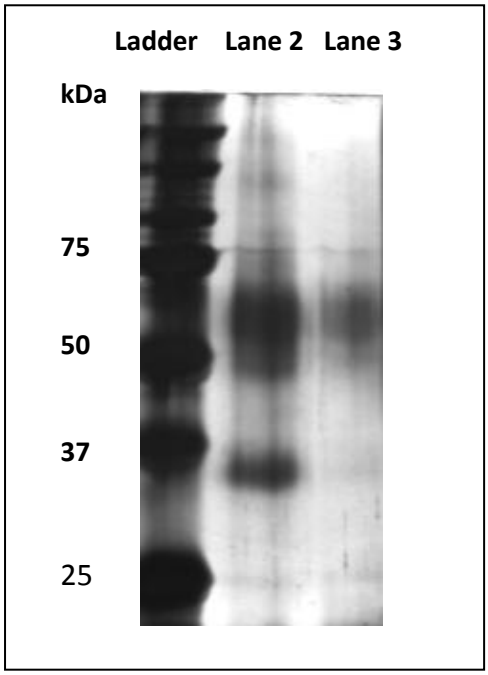

Figure 6.12. SDS electrophoresis gel of active honey protein fraction from Sephadex G50 separated with a ConA column. Lane 2 was the protein that bound the ConA column. Lane three was the protein that did not bind the ConA column.

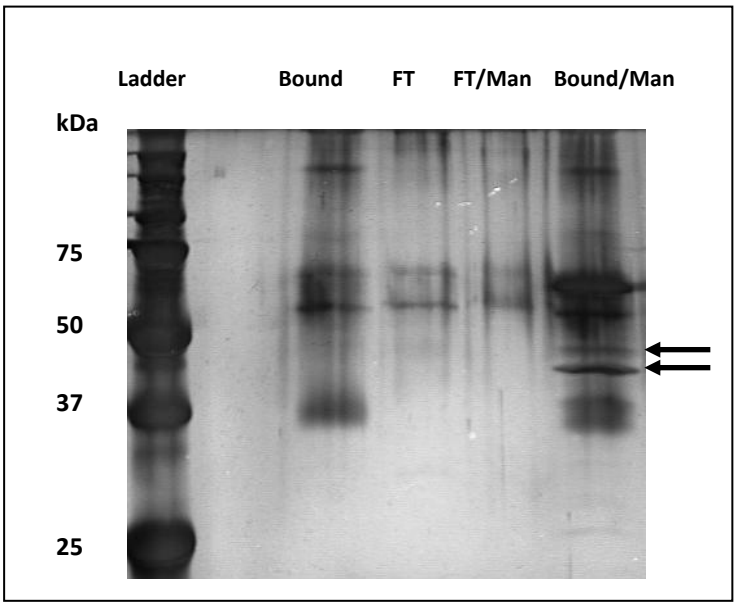

Figure 6.13. SDS electrophoresis gel of honey proteins before and after mannosidase treatment. The proteins had been fractionated with a ConA column, into 'bound' (mannose-containing glycoprotein) and the 'flow-through' protein which did not bind. FT= Flow though, Man= mannosidase treated. Arrows indicate bands generated by mannosidase treatment.

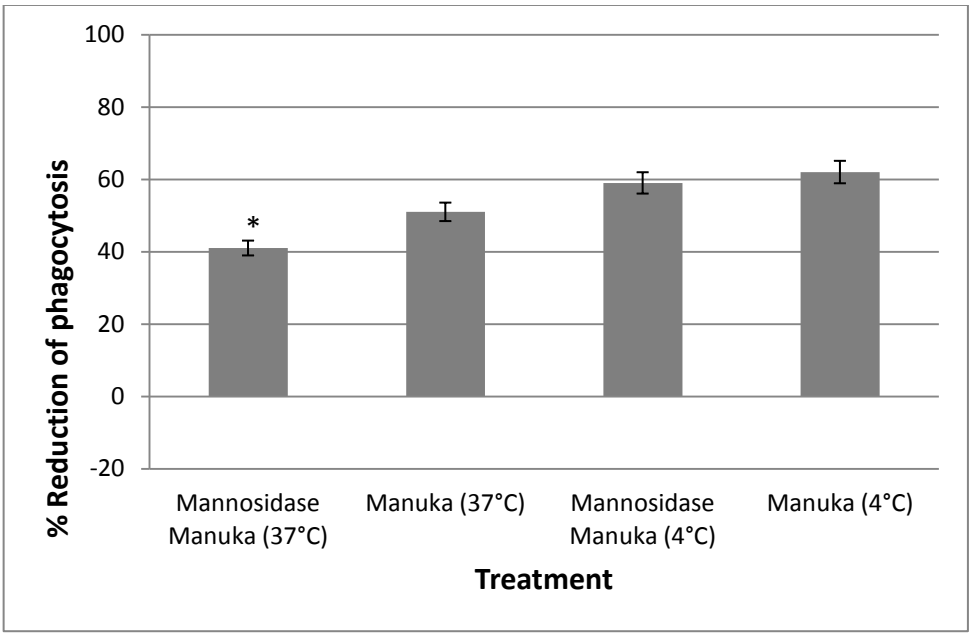

*Figure 6.14. The effect of Mannosidase treatment (4 hr incubation at 37°C or 4°C) of 1% dialysed honey on LPS-stimulated THP-1 cells in the phagocytosis assay. Results show % reduction of phagocytosis compared with that by the control not treated with honey. Error bars show ±1 SD of the mean from at least three assays. * p<0.001 analysed by ANOVA compared with the non enzyme-treated control.*

6.5 Discussion

Heat treatment of Manuka honey showed there was a significant (p<0.001) loss of phagocytosis-inhibiting activity, indicating that most of the activity of honey is due to a native protein. Three heat treatments were assayed with diluted honey (10%): 37°C, 65°C and 99°C and a control treatment at 4°C. Whole honey was also incubated at these temperatures. At 99°C whole honey appeared to caramelise/burn and could not be used in the phagocytosis assay due to the difficulties in diluting and filtering the honey. At 4°C both whole honey and diluted honey had the same effect, indicating that activity is not lost by being in a diluted form for 24 hours at this temperature.

At 37°C for 24 hours, whole/undiluted honey had the same activity after heating as non heat-treated honey did, indicating that proteins in whole honey are stable at this temperature in a non-diluted form. The diluted honey, however, lost a significantly (p<0.001) large proportion of its activity (~ 60%) after 24 hours of incubation at 37°C compared with fresh/non heat-treated honey, possibly because of enzyme activity increased resulting from the increased water activity, or the possibility that there may be hydrolysis of the inhibitory glycoproteins in honey. Although honey contains 17% water, this is bound to the sugars in honey so the water activity is very low due to little free water. The free water is what is measured as the water activity (a_w). Mean a_w values for honey have been reported from 0.562 to 0.62 (Molan 1992 a). With such low a_w values in honey, the glycoproteins may be protected from hydrolysis due to reduced enzyme activity (Molan 1992 a). This finding is important to consider because it indicates that the active proteins are not stable in lower sugar concentrations at 37°C, which would be a consideration in the situation of wound dressings where the honey is diluted by wound exudate and incubated at body temperature. This would imply that fresh honey needs to be applied regularly. It is recommended for future research that this incubation (37°C for 24 hours) be repeated using higher concentrations of honey to find at what concentration these proteins are stable for longer, or rather at what concentrations the enzymes, or hydrolysis, in honey become activated. From the point of wound dressings, honey is applied at a concentration of 100% and then becomes diluted slowly by wound exudate, which may cause it to lose its effectiveness. There needs to be further investigation of the speed of loss of activity with various degrees of dilution.

At 65°C for 2 hours and 99°C for 10 minutes, diluted honey lost a significant (p<0.001) amount of activity (approximately 60%). At 99°C proteins lose their secondary, tertiary and quaternary structure but at lower temperatures some proteins can refold. Of interest is the fact that with heat treatments of 37°C, 65°C and 99°C approximately 40% of the phagocytosis-inhibiting activity was retained. This remaining activity is higher than the activity found in other honey

types not treated with heat suggesting that either denatured proteins in Manuka honey have superior phagocytosis-inhibitory activity to correctly folded proteins in other honey, or that the denatured proteins (at 65°C and 99°C) are able to refold at the assay temperature of 37°C and that their tertiary structure is sufficient to bind the receptors. The activity of refolded or denatured proteins may be due to partial binding of mannose receptors by oligosaccharides released by enzymic or non-enzymic hydrolysis. Another possibility is that the interaction of oligosaccharides still on the protein may still bind with the receptor, but not so well due to altered folding of the protein. Noorman, *et al.* (1997) found that cluster mannoside ligands with longer chains of mannose residues had more affinity for the mannose receptor (Noorman, Barrett-Bergshoeff *et al.* 1997) indicating that the whole protein may not be required to successfully ligate a receptor.

It was important to incubate at 37°C for 24 hours as this is the temperature at which the cells are incubated in the phagocytosis assay, and it is also the body temperature in patients receiving honey treatment. The loss of activity over time in the phagocytosis assay (Chapter 4) may be due to the temperature and dilution causing direct decrease in activity of the honey proteins, rather than receptors on the macrophage cell being renewed or recycled as hypothesised. It may be that the proteins are not being depleted, but, rather are becoming ineffective due to the incubation affecting their structure. Either scenario still requires that honey be applied regularly to a wound to continue effective treatment.

At 99°C the sugars in the diluted and whole honey appeared to caramelise. Whole incubated honey (99°C) was not used in the assay due to dilution/ filtering complications. Diluted honey incubated at 99°C was used in the phagocytosis assay and approximately 60% of the activity was lost due to the heat treatment. While the activity was lost in the diluted honey, other factors may come into play when there is a chemical change occurring in the honey, such as caramelisation.

For future work it is suggested that other heat treatments of temperatures up to 65°C for varying time periods be tested for loss of phagocytosis-inhibiting activity. This would define prime storage conditions in light of the new discovery of methylglyoxal (MGO) which forms in honey at higher rates during storage at higher temperatures (personal communication with Professor Peter Molan). Beekeepers may be encouraged by higher prices to store their honey at higher temperatures, to increase the MGO content in shorter amounts of time. Unfortunately, this may destroy any anti-inflammatory activity. MRJP 1-3 in royal jelly are sensitive to heat when stored for long periods (Li, Feng *et al.* 2008). It is not known, however, if the proteins in honey are affected in the same way. Honey has evolved to be a storable food for bees when nectar is in low demand during winter, or in rainy periods when collection of nectar is not possible whereas royal jelly is a product used freshly made. The proteins may be preserved by the high sugar content or other components in honey.

Trypsin treatment of diluted honey confirmed that denaturing proteins in honey reduced the phagocytosis-inhibiting effect. However, no residual effect was found, unlike heat treatment which left approximately 40% inhibitory activity. This suggests that the heat-denatured honey proteins were better able to bind receptors than trypsinised protein. This may be due to the remaining size of the ligand: as mentioned earlier, larger ligands usually bind the receptor with greater avidity. This also rules out hydrolysis, if there is complete hydrolysis, as an explanation of loss of activity in incubated diluted honey.

The phagocytosis-inhibiting activity was isolated to the protein that eluted first from the chromatography column. Manuka honey was separated by chromatography on Sephadex G-50 and these fractions assayed in the phagocytosis assay to determine where the most of the phagocytosis-inhibiting activity was. This indicated that the activity was due to a large molecule. This area of the elution profile was pooled and re-run on a second chromatography column (Superose 12). Four separate peaks were obtained and the major of the

two (the fractions with the highest protein concentrations), Fraction 8 and 14, were assayed for phagocytosis-inhibitory activity. Fraction 8 had strong phagocytosis inhibiting activity and Fraction 14 had very little activity. Both fractions were identified with significant hits by MALDI-TOF mass spectrometry. The fraction with the majority of the anti-inflammatory activity (Fraction 8) was Apalbumin 1 (MRJP-1). Fraction 14 returned two hits, one being significant which was Apalbumin 3 (MRJP-3). The non-significant hit was Apalbumin 1 (MRJP-1). Apalbumin 1, may have been present also, just at lower concentrations, too low to give a phagocytosis-inhibitory action or definite identification from its peptides.

The degree of confidence for the identification of the active proteins being correct is convincingly high due to the scores being above the accepted margin of error. As mentioned previously, when more than one protein is present in the sample there are typically no clear peaks correlating to large amounts of the same peptide. The mass spectra obtained for this research returned clear peaks which were appropriate for use in the Swiss-Prot database. The two identified proteins belonged to the *Apis mellifera* genome. These proteins have been reported to be in honey (Scarselli, Donadio *et al.* 2005; Schönleben, Sickmann *et al.* 2007). No other significant hits for protein were returned in either fraction indicating that the sample was composed predominantly of the identified proteins. Three peptides returned identical molecular weights between the two different proteins (peaks at 1 144, 1 397 and 1 614 Da on the Mass Spectra in Figure 6.10 and 6.13) indicating that these peptides are homologous to both proteins (or from yet another protein). This may also explain why an insignificant match for Fraction 14 returned MRJP-1. Indeed the MRJPs are reported to be highly homologous (Schönleben, Sickmann *et al.* 2007). The molecular weight returned from the Swiss-Prot database for MRJP-1 and MRJP-3 was 48.9 kDa and 61.6 kDa respectively. These are calculated from the known sequence and will not include glycosylation, which will increase the molecular weight slightly. This is very similar to reported molecular weights in other research, Scarselli and

Donadio *et al.* reported molecular weights for MRJP-1 and MRJP-3 as being 49.3 kDa 61.9 kDa respectively, determined by mass spectrometry. MRJP-3 exists in five isoforms: it was not determined in this study which isoforms was identified. This should be done in future research. The two identified proteins combined may contribute to the majority of the phagocytosis-inhibitory activity. As MRJP-1 is present in much larger amounts it may be responsible for most of the phagocytosis-inhibitory activity.

The most active fraction from chromatography on Superose 12 (Fraction 8) was further assessed using a column specific bearing the lectin ConA, a mannose-specific ligand. While this was unsuccessful, using the whole active fraction (obtained by Sephadex G-50) showed that MRJP-1 was a target for ConA but MRJP-3 was not. This was determined by identifying the proteins by their molecular weight in the SDS electrophoresis gel. MRJP-3 was made up of both ligands and non-ligands. This can be determined by comparing the molecular weight of the bands of the two ConA-separated fractions correlating to the expected position for MRJP-3 on the SDS electrophoresis gel. The protein that bound to the lectin column is the same molecular weight as the protein that did not bind the column. These two bands would appear at the same position on a gel and give a denser band if they had not been separated. This may be due to there being two similar but different sized proteins in the band, one a mannose-terminated glycoprotein and the other not, or one protein may have different degrees of glycosylation. Attempts to identify these two bands by MALDI-TOF mass spectrometry were unsuccessful due to the concentration being too low.

The molecular weight for Fraction 8 (MRJP-1) estimated from the SDS gels is 60 kDa which is 10 kDa larger than would be expected as the Swiss-Prot returned a protein size of only 48.9 kDa. The size disparity could be explained by glycosylation. Three predicted N-linked glycosylation sites have been reported on MRJP-1 (Srisuparbh, Klinbunga *et al.* 2003). Glycosylation of these sites would increase the molecular weight. When the mannosidase treated protein was run

on a SDS electrophoresis gel, extra bands were visualised on the gel, indicating that these proteins were running at a similar band position as a larger protein. As Fraction 8 was in the void volume, (meaning macromolecules that are sufficiently large to be excluded from the column beads elute first from the column (the void volume of the Superose 12 column is 11 ml), this may indicate that MRJP-1 is highly glycosylated and/or cross-linked, as at 48.9 kDa (the molecular weight of MRJP-1 returned by Swiss-Prot) the monomer protein should easily enter the column (Size exclusion limit for Superose 12 is 2 000 kDa). As the protein is boiled in SDS to disrupt protein complexes, the actual size of the protein complex was not established. Oligomers of MRJP-1 have been reported with a molecular weight of 290 kDa, identified using native-PAGE (Tamura, Amano *et al.* 2009). The MRJP-1 oligomer was separated into 55 kDa and 5 kDa spots on 2-D blue native/SDS-PAGE. The 55 kDa protein was identified as MRJP-1 monomer by proteome analysis, whereas the 5 kDa protein was identified as Apisimin by N-terminal amino acid sequencing, and this protein may function as a subunit-joining protein within MRJP-1 oligomers. Another study separated royal jelly of *A. mellifera* by ultracentrifugation and reported the existence of different forms of MRJP-1. These included a monomer (55 kDa) and an oligomer (approximately 420 kDa) (Simuth 2001). It is not known if an oligomer of MRJP-1 exists in honey.

With the current knowledge of the presence of MGO in honey, glycation of MRJPs is to be expected and may give a plausible explanation for the different ConA binding states. MGO is involved in the formation of advanced glycation endproducts (AGEs). In this process, MGO reacts with free amino groups of lysine and arginine and with thiol groups of cysteine forming AGEs. It is an irreversible process and alters the molecular weight of the resulting protein. The modification of proteins by MGO has been reported to be a potent signal for the degradation of proteins by monocytic cells (including THP-1) in which the arginine derivative is the receptor recognition factor (Westwood, Argirov *et al.* 1997). It therefore seems highly likely that MGO modifies MRJP in honey

creating a ligand for THP-1 cells which were the cell type used in the current research.

Mannosidase was used on whole honey and active honey fractions. The gel image of mannosidase-treated honey shows that extra bands were formed on the gel after treatment (at approximately 35 kDa, 43 kDa and 46 kDa) which shows their size was reduced by the treatment. This protein may have been present in the SDS electrophoresis gel as a protein or protein-complex with a higher molecular weight. A possibility is that these were present at 55 -60 kDa as this is the only band that has decreased in proportion to the other bands, if the protein had not been treated with the enzyme. However these extra bands have a molecular weight too low to be MRJP's. The MRJP family have been reported to have molecular weights ranging from 49 kDa - 87 kDa (Schmitzová, Klaudiny *et al.* 1998). If the extra bands came from a larger protein complex that sits higher in the SDS electrophoresis gel, the mannosidase treatment could have disrupted the large protein complex into monomers. As there was a small amount of activity lost it could be assumed that the protein from the extra bands would have run at around 60 kDa, the molecular weight of the MRJP-3 that bound the ConA column and may be responsible in conjunction with MRJP-1 for the phagocytosis-inhibiting activity. If this is the case, the change in size indicates that sugars have been cleaved off a monomerric protein as no low molecular weight protein bands were visualised on the gel that would make up the size difference for a protein complex.

Another possibility for the reduced phagocytosis-inhibitory activity is that Mannosidase-treated whole honey had little hydrolysis. Future research should aim to develop this assay so that more hydrolysis can be realised if this is the case. A suitable control would need to be included to gauge the level of hydrolysis after mannosidase treatment. Secondly, the proteins could be separated by size and then treated with mannosidase. This was attempted in the current research but the concentrations were not high enough to get good

results. Higher concentrations would need to be achieved. The reduced phagocytosis-inhibitory activity suggests that these sugars are important, perhaps by enabling protein complexes to form or by additional properties gained by becoming a glycoprotein. Future work should look at identifying the extent of glycosylation on these proteins and whether an oligomer of MRJP-1 exists in honey.

Chapter 7

Identifying the phagocytic receptor blocked by Manuka honey

7.1 Summary

In the work described in Chapter 6 it was found that the phagocytosis-inhibiting fractions obtained by FPLC column chromatography bound a ConA glycoprotein isolation column. It was thus concluded that the proteins with mannose terminated glycoconjugates were important in the anti-inflammatory action of Manuka honey. To determine if these proteins were acting on the macrophage mannose receptor, which triggers phagocytosis, mannan and β-glucan were used in place of honey in the phagocytosis assay. Results showed that mannan but not β-glucan reduced phagocytosis in the assay, indicating that Manuka honey was likely to be acting on the mannose receptors of macrophages, reducing the phagocytosis capabilities of the cells resulting in an anti-inflammatory action.

7.2 Introduction

It has been determined in work described in previous chapters that Manuka honey in low concentrations decreases phagocytosis of latex particles, *E. coli*,

zymosan and mycobacteria by activated macrophages in a dose-dependent manner by up to 80% which would be expected to give an anti-inflammatory effect.

7.2.1 C-type letins

Several mechanisms are available to the macrophage for engulfment of particles, this Chapter focuses on phagocytosis. Phagocytosis is a complex process that is instrumental in the control of extracellular pathogens mediated by several pattern recognition receptors, including a number of C-type lectins. The C-type lectin family is a large group of proteins that are characterised by the presence of one or more C-type lectin-like domains (CTLDs) (Kerrigan and Brown 2009).

The C-type lectins are divided into 17 groups based on their phylogeny and domain organisation (Drickamer and Fadden 2002). C-type lectins are functionally diverse and have been implicated in various processes including cell adhesion, tissue integration and remodelling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis. The mannose receptor (MR) and β-glucan receptor, called Dectin-1, are C-type lectin receptors that recognize glycoproteins terminated in D-mannose, L-fucose or N-acetyl glucosamine (MR), (Esteban, Rodriguez *et al.* 2004) or β-1,3 and or β-1,6 glucans (Dectin 1) (Brown and Gordon 2001; Taylor, Gordon *et al.* 2005b; Rothfuchs, Bafica *et al.* 2007). These glycoproteins are found in abundance on the cell surface of bacteria and other lower organisms.

Both receptors are expressed in macrophages and a range of other phagocytic cells. Bound β-glucan or mannan ligands activate macrophages, stimulating their phagocytic activity and the production of ROS, inflammatory mediators and cytokines (Janusz, Austen *et al.* 1986; Stahl and Ezekowitz 1998). The precursor monocyte does not express the MR but does express the glucan receptor (Esteban, Rodriguez *et al.* 2004).

The MR is not the only receptor with specificity for mannose. Other receptors sharing similar pattern of ligand binding include SIGNR1 (mouse)/DC-SIGN (human) and Endo-180 (Taylor, Martinez-Pomares *et al.* 2005a), though the mannose receptor is the most studied.

7.2.2 Mannose receptor

The MR is a type-I membrane protein with a single trans-membrane domain and a cytoplasmic domain that mediates receptor internalisation and receptor recycling. It contains three types of domains at its extracellular region, an *N*-terminal cysteine-rich domain capable of Ca^{2+}-independent binding to sulphated sugars terminated in SO_4-3-Gal or SO_4-3/4-GalNAc (Taylor, Gordon *et al.* 2005b), a fibronectin type II domain involved in collagen binding especially collagen types I, II, III, and IV (Martinez-Pomares, Wienke *et al.* 2006) and eight tandemly arranged C-type lectin-like domains responsible for Ca^{2+}-dependent binding (Taylor, Gordon *et al.* 2005b).

The cysteine-rich domain recognises glycoprotein hormones. The C-type lectin-like domain region binds myeloperoxidase (MyPo) and lysosomal hydrolases. The interaction of MR with lysosomal hydrolases and MyPo suggests a crucial role for the MR during the resolution of inflammation, as both initiate the activation of macrophages (Taylor, Gordon *et al.* 2005b). The C-type lectin-like domain region can also bind to ligands of microbial origin, as mannose is frequently found on the surface of many micro-organisms. In this way the MR is considered a pattern recognition receptor.

Studies have shown that MyPo, which is released from neutrophils, is involved in cell killing and induces macrophages to secrete interleukin-1, interferon and tumor necrosis factor. In the neutrophil phagosome, MyPo converts H_2O_2 and chloride into hypochlorous acid, a strong oxidant that acts as a bactericidal agent in phagocytes. MyPo also binds to the MR in macrophages and this interaction between neutrophils and macrophages induces a state of chronic inflammation

(Lefkowitz, Mills *et al.* 1995). Recently D-fructose from honey has been found to have a role in suppressing MyPo-catalyzed ROS production from neutrophils, thus reducing inflammation (Mesaik, Azim *et al.* 2008).

Patterns produced by pathogens and recognised by the MR include those of *Mycobacterium tuberculosis* (Fraser, Koziel *et al.* 1998; Astarie-Dequeker, N'Diaye *et al.* 1999) *Candida albicans* (Marodi, Korchak *et al.* 1991) and *Streptococcus pneumonia* (Zamze, Martinez-Pomares *et al.* 2002) and the common baker's yeast *Saccharomyces cerevisiae* (Fraser, Koziel *et al.* 1998). Due to its pattern regognition feature, the MR has been reported to mediate the uptake of pathogenic and non-pathogenic mycobacteria and bypasses bactericidal responses in macrophages via a 19-kDa antigen (Diaz-Silvestre, Espinosa-Cueto *et al.* 2005). This antigen has been shown to bind the MR in THP-1 cells promoting its phagocytosis (Astarie-Dequeker, N'Diaye *et al.* 1999).

When a mannose-terminated glycoconjugate binds the MR, the cell membrane forms a vesicle enclosing the bound receptor and travels though the cell to fuse with a lysosome were degradation of the foreign particle will occur. An appropriate immune response follows. The receptor is recycled, returning to the cell membrane within 15 minutes. The MR recycles between the plasma membrane and the early endosomal compartment, even in the absence of any ligand. At a steady state 10–30% of the receptor is found at the cell surface and the remaining 70% is localised intracellularly (Gazi and Martinez-Pomares 2009).

Mannan (a yeast cell wall component with high mannose content) inhibits phagocytosis by binding the MR and preventing recycling of the receptor to the membrane (Sung, Nelson *et al.* 1983).

7.2.3 Dectin 1 the β-glucan receptor

Beta-glucans extracted from the common baker's yeast *Saccharomyces cervisiae* are composed of chains of 20-30 glucose units. β-glucan will not bind the MR so can be used as a control in a phagocytosis assay investigating the MR. β-glucan is a chain of sugars, as is mannan, with a similar affinity for its receptor, Dectin-1. *Mycobaterium turberculosis* has also been demonstrated to bind Dectin-1 (Rothfuchs, Bafica *et al.* 2007). β-glucan reduces phagocytosis of *Mycobaterium turberculosis* by macrophages, as does mannan. If β-glucan reduces phagocytosis of latex particles in the previously used assay then it is possible that honey too binds the β-glucan receptor. Thus it was decided to use β-glucan and mannan to determine which receptors may be involved in the phagocytosis of carboxylated-latex particles by THP-1, thus indicating were honey may be working.

7.2.4 Manuka honey

In the work described in Chapter 6 the active proteins in Manuka honey were found to bind to ConA lectin, a lectin specific for mannose-terminated glycoconjugates, indicating the honey proteins contain terminal mannose units. As mannose is the primary lectin for the mannose receptor it was postulated that the active Manuka honey proteins are binding to this receptor to exert their anti-inflammatory effect.

Work done by Noorman *et al.* (1997) showed that longer mannose-containing oligosaccharides inhibited antigen uptake better that shorter oligosaccharides. The MR contains eight carbohydrate recognition domains (CRD) and has a high affinity for mannan. When truncated receptor forms were expressed, it was concluded that CRD 4, 5 and 7 are essential for the high-affinity binding of mannan, as mannan had a much lower affinity for the mutant receptors. Shorter mannosides have fewer CRDs to bind to and therefore have a lower affinity (Noorman, Barrett-Bergshoeff *et al.* 1997). In Chapter 6 MRJP-1 and MRJP-3

where identified as being present in the fraction of honey having phagocytosis-inhibiting activity. These proteins are glycosylated with mannose units (Li, Feng *et al.* 2008). ConA interactions with MRJPs have been characterized and the typical high-mannose-type structure (Man_{9-4} $GlcNAc_2$) accounts for about 72% of them, followed by a biantennary structure ($GlcNAc_2$ Man_3 $GlcNAc_2$) (about 8%), and a hybrid-type structure ($GlcNAc_1Man_4GlcNAc_2$) (about 3%), all of them reacting with ConA (Kimura, Miyagi *et al.* 2000; Kimura, Hama *et al.* 2002).

It was postulated that the MRJPs in Manuka honey, due to their high mannose glycosylation, bind the MR, preventing phagocytosis by blocking the receptor as an antagonist, or like mannan, binds and prevents receptor recycling.

7.2.5 Involvement of methylglyoxal

All honey contains bee proteins with MRJP-1 the major one (Schmitzová, Klaudiny *et al.* 1998), yet Manuka honey has superior phagocytosis-inhibiting activity due to these proteins. The content of bee proteins would be expected to be similar in all honeys, which raises the question why would the activity in Manuka honey be higher of that in other honeys? A unique feature of Manuka honey is the recently discovered content of very high levels of methylglyoxal (MGO). MGO is responsible for the majority of the non-peroxide antibacterial activity (NPA) (Adams, Boult *et al.* 2008; Mavric, Wittmann *et al.* 2008). MGO is a highly reactive 1,2-dicarbonyl compound formed in foods and beverages susceptible to degradation due to processing, cooking and prolonged storage. To date, no honey other than Manuka honey has been found to contain high levels of MGO, which have been up to 828 mg/kg (Adams, Boult *et al.* 2008; Mavric, Wittmann *et al.* 2008).

MGO irreversibly reacts with proteins by modifying the arginine and lysine residues to form advanced glycation end-products (AGEs), which can cause cross-linking of protein molecules (Yim, Yim *et al.* 2001). MGO-modified arginine

residues on human serum albumin have been shown to bind endocytic cell surface receptors and undergo internalisation and degradation in THP-1 cells (Westwood, McLellan *et al.* 1994). Highly modified glucose-derived human serum albumin AGEs were also found to bind specifically to macrophage receptors in this manner, suggesting the irreversible modifications by MGO on albumin are a potent signal for degradation (Westwood, Argirov *et al.* 1997). *Westwood et al.* (1997) did not identify any receptors in their study but it was suggested that scavenger-like receptors may be involved which are highly similar to the MR.

Another receptor important to the present study is the receptor for advanced glycation end-products (RAGE) which reportedly triggers cellular responses implicated in the pathogenesis of diabetes (Yan, Ramasamy *et al.* 2009). When AGEs bind and activate RAGE, vascular cell adhesion molecule-1 (VCAM-1) expression is increased from vascular endothelial cells and TNF-α secretion by mononuclear cells in induced. However, AGE modification and high RAGE binding affinity are not sufficient to generate pro-inflammatory signalling molecules (Valencia, Mone *et al.* 2004).

It is likely the MGO present in Manuka honey is actively modifying the proteins, forming novel ligands for the receptors responsible for phagocytosis which then act in a decoy manner or prevent receptor recycling. Another possibility is that binding of receptors to cross-linked mannose glycoproteins effectively cross-links the receptors preventing them from being internalised by phagocytosis and thus preventing their recycling.

Extensive work has been carried out on MGO-modified human serum albumin and bovine serum albumin. Research shows MGO-modified proteins, such as human serum albumin, are of clinical significance due to their involvement in the development of retinopathy, neuropathy or nephropathy in diabetes mellitus (McLellan, Thornalley *et al.* 1994).

Clearly human cells are able to recognise these modifications if they are involved in disease. It is highly likely that the MGO in Manuka honey is able to modify albumins in honey, as it does with other albumins such as human serum albumin and bovine serum albumin. Possibly Apalbumin-1 (MRJP-1) is modified in this way. Apalbumin-1 was identified as being the protein in Manuka honey with anti-inflammatory activity in Chapter 6. This could explain why Manuka honey has greater effect than other honeys. Other honeys may have less glycation of proteins caused by little or no MGO present. As these MGO-modified proteins have been determined to bind the macrophage receptors, proteins from other honey varieties then, would not bind to the receptors with the same affinity, or even at all.

7.2.6 Aims and experimental approach

To check the hypotheses mentioned above, mannan, and β-glucan as a control, were used with and in place of honey in the latex bead phagocytosis assay to determine if the mannose receptor was likely to be involved in the effect of Manuka honey in inhibiting phagocytosis.

7.3 Methods

7.3.1 Phagocytosis assay with latex particles

The phagocytosis assay using latex particles, was the same as described in Section 4.3.1, but with various concentrations of mannan and β-glucan added. The concentrations of both mannan and β-glucan selected were taken from publications (Esteban, Rodriguez *et al.* 2004; Kang and Schlesinger 1998). Preliminary assays found the concentrations appropriate, but a range of

concentrations was used to determine if inhibition was dose-related as it is with Manuka honey. Mannan was used at concentrations of 1.25 mg/ml – 5 mg/ml. β-glucan was used at concentrations of 50 µg/ml – 200 µg/ml. Manuka honey was used at concentrations ranging from 0.125% - 1%. One objective was to see if either mannan or β-glucan had an additive effect when assayed in conjunction with Manuka honey. To do this 0.125% Manuka honey was used in the phagocytosis assay in conjunction with 1.25 mg/ml mannan or 50 µg/ml β-glucan. Also, in case the cell would use either the MR or Dectin-1 receptor to phagocytose latex particles, mannan and β-glucan were assayed together.

7.3.1.1 Reagents

A solution of Manuka honey (M144) honey was prepared as described in Chapter 3. THP-1 cells for the phagocytic assay were maintained at 37°C, 5% CO_2, and 95% relative humidity in growth medium as described in Section 2.3.1. Cells were split with fresh growth medium prior to the assay and assessed for viability as described in Section 3.2. All conditions of the phagocytosis assay were the same as described in Section 4.3.1 but with mannan or β-glucan either replacing or additional to honey.

7.3.1.2 Mannan

Mannan from baker's yeast *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich (Cat. No.# M7504) and dissolved in MilliQ water to give a stock solution of 100 mg/ml. The stock solution was stored at -20° until required. Mannan stock solution was added at a rate of 15 µl – 50 µl stock solution per ml of cell suspension, to give final concentrations of 1.25 mg/ml – 5 mg/ml. Mannan at a concentration of 4 mg/ml has been reported in the literature to inhibit phagocytosis in macrophages (Kang and Schlesinger 1998).

7.2.1.3 β-glucan

β-glucan from barley was purchased from Sigma-Aldrich (Cat. No.# G6513) and dissolved in MilliQ water to give a stock concentration of 100 mg/ml. A working solution of β-glucan (10 mg/ml) was made by diluting the stock solution 1/10 in RPMI-1640 medium. Both the stock solution and working solution was stored at -20° until required. β-glucan was used at a rate of 5 µl – 20 µl of working solution, to give a final concentration of 50 µg/ml – 200 µg/ml. β-glucan at a concentration of 125 µg/ml has been reported in the literature to inhibit phagocytosis in macrophages (Esteban, Rodriguez *et al.* 2004).

7.4 Results

Results comparing the effect of mannan and β-glucan with that of Manuka honey in the phagocytosis assay are displayed in Figures 7.1-7.3. Results show the percent inhibition of phagocytosis caused by the inhibitor (mannan, β-glucan or Manuka honey) compared with the control with no inhibitor. Mannan was seen to have dose-dependent activity like Manuka honey did. When used in combination with Mannan, Manuka honey significantly (p<0.001) increased this phagocytosis inhibiting activity (Figure 7.1). The activity of the combination was close to the sum of the activity of each alone. β-glucan at 50 µg/ml and 100 µg/ml had no significant effect on reducing phagocytosis. At 200 µg/ml there was a slight reduction of phagocytosis. β-glucan in combination with Manuka honey, (Figure 7.2) or mannan, (Figure 7.3) gave no additional inhibition to either, demonstrating that the mannose receptor is most likely involved as opposed to Dectin-1 in the inhibition of phagocytosis of latex particles in THP-1 cells.

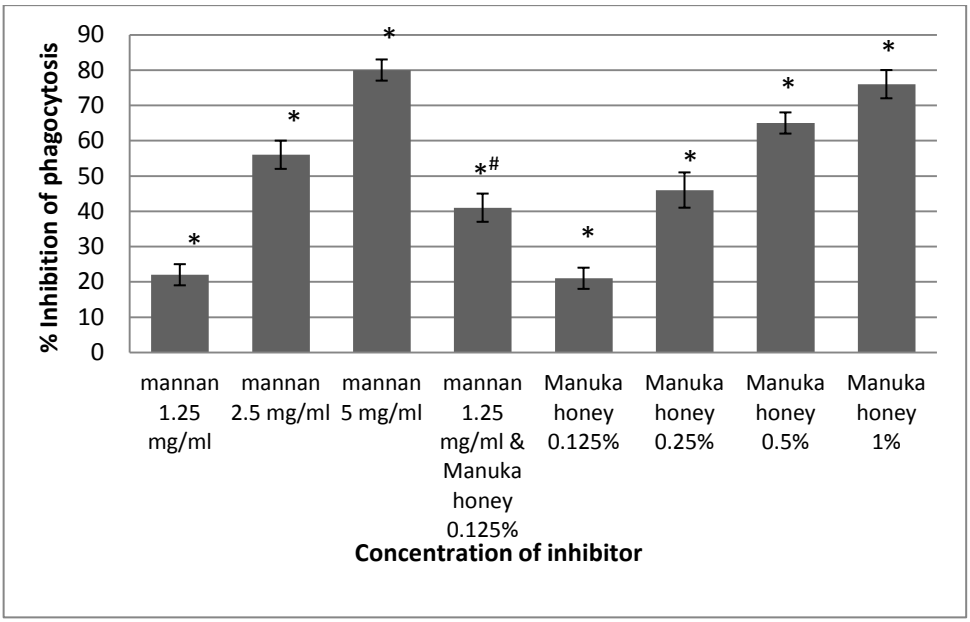

*Figure 7.1. The inhibitory effect of a range of concentrations of mannan, Manuka honey or a combination of these on the phagocytosis of latex particles by LPS-activated THP-1 cells. Error bars show ± 1 SD of the mean from three experiments. *p<0.001 analysed by ANOVA compared with the control not treated with honey or mannan. #p<0.001 analysed by ANOVA compared with 0.125% Manuka honey alone.*

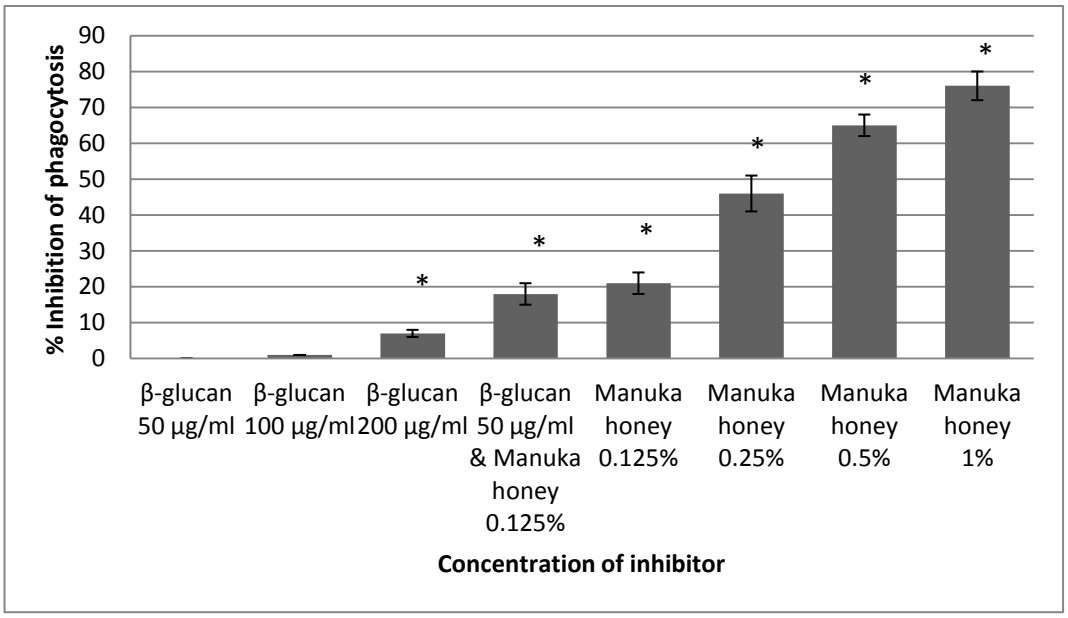

*Figure 7.2. The inhibitory effect of increasing concentrations of β-glucan, Manuka honey or a combination of these on the phagocytosis of latex particles by THP-1 cells. Error bars show ± 1 SD of the mean from three experiments. *p<0.001 analysed by ANOVA compared with the control not treated with honey or β-glucan. No significance was found with 50 µg/ml β-glucan/0.125% Manuka honey and 0.125% Manuka honey alone, analysed by ANOVA.*

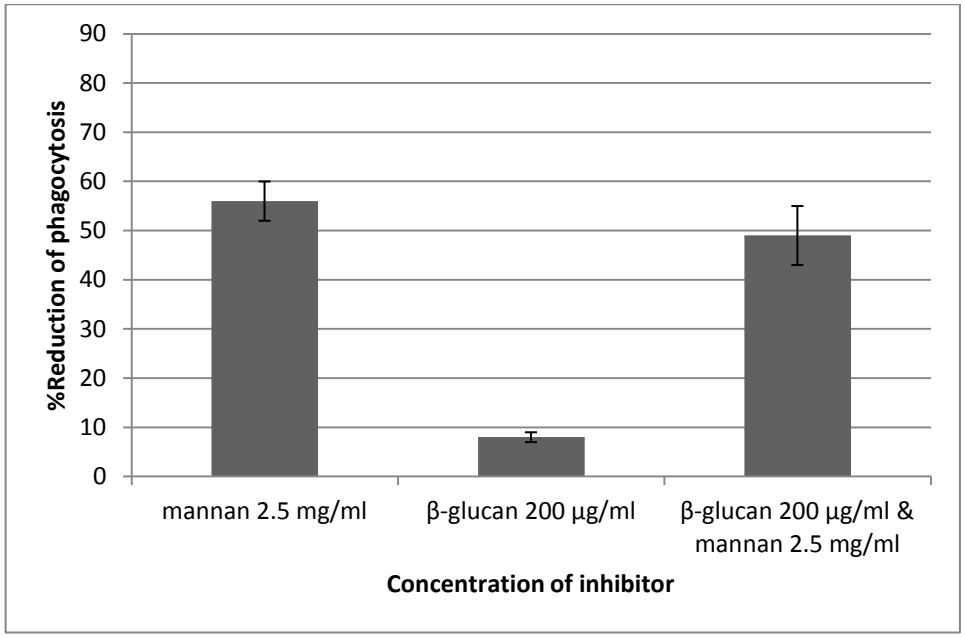

Figure 7.3. The inhibitory effect of β-glucan and mannan, and a combination of these on the phagocytosis of latex particles by THP-1 cells. Error bars show ± 1 SD of the mean from three experiments. 200 µg/ml β-glucan had no significant effect on the inhibition by mannan analysed by ANOVA.

7.5 Discussion

An antigen of *Mycobacterium tuberculosis* has been identified that binds the mannose receptor of THP-1 cells, promoting the phagocytosis of the bacteria (Diaz-Silvestre, Espinosa-Cueto *et al.* 2005). As it was found in the present study that Manuka honey reduces the phagocytosis of Mycobacteria in THP-1 cells, it seems plausible that the active compound of the honey is preventing phagocytosis by blocking the MR. Similar hypotheses can be advanced for zymosan and *E. coli*, both of which have been reported to bind the MR in THP-1 cells (Akiko, Ikuko *et al.* 2008) and were found in the present study to have phagocytosis of them inhibited by Manuka honey.

β-glucan was used as a control for mannan. As mentioned previously, β-glucan will not bind the MR but will bind phagocytic receptors such as Dectin-1. As it

was hypothesised that a component of Manuka honey was binding the MR, it was useful to include β-glucan in the phagocytosis assay. If phagocytosis of the latex particles was being triggered by their being bound by the Dectin-1 receptor then β-glucan may have an inhibitory effect on phagocytosis. Even at the highest dose (200 μg/ml) β-glucan gave very little inhibition of the phagocytosis of latex particles and even then was only 1/10 of that of mannan. This concentration is almost double the reported effective concentration, where up to 50% inhibition of phagocytosis is reported (Esteban, Rodriguez *et al.* 2004). It is likely that a higher concentration may provide more inhibition. This is recommended for future work. To check if it was the case that the cell can use either the MR or Dectin-1 receptor to phagocytose latex particles, β-glucan and mannan were assayed together, thus blocking both receptors. This did not result in additional inhibition to that with mannan alone (p<0.294) In the case of using Manuka honey together with mannan or β-glucan, only mannan caused a significant (p<0.001) additional increase in the inhibition of phagocytosis using published concentrations. It is possible that a higher concentration of β-glucan would increase the inhibition by Manuka honey, though it was noted that the highest concentration used in this study (200 μg/ml) did not increase the inhibition of phagocytosis of latex particles by mannan, suggesting they work on different receptors.

Zymosan is a yeast-derived particle composed primarily of polysaccharides namely mannan and β-glucan that reportedly binds the MR or the Dectin-1 (β-glucan) receptor. Unopsonized zymosan phagocytosis can be inhibited by laminarin, a β-glucan commonly found in brown algae (Brown, Taylor *et al.* 2002) and mannan (Sung, Nelson *et al.* 1983). As Manuka honey stops phagocytosis of zymosan it is likely to be blocking one of these receptors. In the work described in Chapter 4, Manuka honey at a concentration of 0.5% was found to give approximately 50% inhibition of phagocytosis of zymosan.

In this Chapter, mannan was used in place of Manuka honey in the latex particle phagocytosis assay and the effect was like that of Manuka honey. The inhibitory effect of Mannan was also dose-dependent like that of Manuka honey. When used in conjunction with Manuka honey, mannan increased the inhibition of phagocytosis, yet β-glucan did not. Other studies (Sung, Nelson *et al.* 1983; Speert and Silverstein 1985; Brown, Taylor *et al.* 2002) have found up to 50% reduction of phagocytosis of unopsonized zymosan by either β-glucan or mannan, suggesting that each receptor binds zymosan in roughly equal proportions. This suggests that mannan and Manuka honey work on the same receptors, namely the MR, and indicates that the apalbumin glycosylation with mannose terminal sugars is important as it may bind the MR and possibly other receptors that bind this moiety.

It is still possible that a receptor other than the MR is involved. Conclusive evidence of the involvement of the MR is important for future work. Possible experiments may include transfection studies with MR or MR knockdown. If the MR is involved it may be that free mannan has a higher affinity for the MR than latex beads.

Mannan-binding lectin, a serum protein, has been shown to mediate the activation of complement upon binding to specific microbial carbohydrate motifs, to directly opsonize organisms, and to enhance phagocytosis, enhancing FcR-mediated phagocytosis by both monocytes and macrophages *in vitro* (Arora, Munoz *et al.* 2001; Wallis, Lynch *et al.* 2005). The serum mannose-binding protein and the macrophage MR are both pattern recognition molecules that recognise mannose-terminated glycoproteins. When they bind a mannose ligand they initiate an immune response (Fraser, Koziel *et al.* 1998). The active protein in Manuka honey may bind the MR, but somehow prevent phagocytosis or an immune response. This may be due to the cross-linking of mannose glycoproteins blocking the receptors as mentioned previously, or a in a decoy fashion where the large amounts of ligands that honey presents, simply floods

the receptors so that pathogens, or in this case latex particles, are not able to be phagocytosed as freely or as quickly.

Future work should look at testing whether honey, by blocking MR sites on macrophages, prevents the communication of neutrophils and macrophages via released MyPo. This would give a great reduction in chronic inflammation as mentioned earlier.

Another area which should be investigated is the effect Manuka honey may have on the MR binding of tissue-type Plasminogen Activator (t-PA). This activator is a serine protease that activates fibrinolysis by converting plasminogen into plasmin, which cleaves fibrin into soluble degradation products. Recent research has shown that fibrin plays a key role in the inflammatory response and is also involved in forming scar tissue (Bouma and Mosnier 2006). Thus t-PA is beneficial in wound healing. It has been shown that t-PA is cleared from plasma through the MR (Biessen, van Teijlingen *et al.* 1997; Noorman, Barrett-Bergshoeff *et al.* 1997). Mannosides work by immobilizing the receptor, effectively inhibiting t-PA clearance. Manuka honey may exert its anti-inflammatory effects in this manner.

Chapter 8

The effect of methylglyoxal on proteins in Manuka honey

8.1 Summary

Manuka honey has been shown to have high levels of methylglyoxal (MGO) (Adams, Boult *et al.* 2008; Mavric, Wittmann *et al.* 2008). As MGO is a highly reactive compound that is capable of modifying proteins to produce fluorescent derivatives, a wide range of Manuka and non-Manuka honey was screened for fluorescence to see if this reaction occurs in Manuka honey.

Fluorescence was detected using a fluorescence imaging system and determined to be due to modification of proteins in honey by MGO. It was found that the active fractions obtained from chromatography of Manuka honey in Chapter 6 were fluorescent whereas other fractions were not. It was hypothesised that incubating honey would increase the fluorescence and also increase the anti-inflammatory activity determined by the phagocytosis assay. Honey with high MGO but low fluorescence was incubated for three months and monitored throughout this period for an increase in fluorescence and anti-inflammatory activity. Honey with no natural MGO was incubated with high levels of added MGO and monitored in the same way. As a control BSA was also incubated with MGO and monitored for a gain of fluorescence.

A method using thin layer chromatography plates (TLC) and filter paper was developed to visualise the fluorescent protein of Manuka honey, MGO-treated Clover honey and MGO-treated BSA. It was found that by incubating honey or BSA with MGO at 37°C, fluorescence increased. After incubation at 37°C for three months Manuka honey with high MGO levels became more anti-inflammatory, determined by the phagocytosis assay.

8.2 Introduction

8.2.1 Methylglyoxal

During the early part of this research project two independent groups of researchers published the finding stated that the major anti-bacterial component of Manuka honey was MGO (Adams, Boult *et al.* 2008; Mavric, Wittmann *et al.* 2008). They demonstrated that the bioactivity of MGO, at the levels at which it is present in the honey, is equivalent to the NPA (Adams, Boult *et al.* 2008; Atrott and Henle 2009). The levels of MGO and other 1,2-dicarbonyls have been determined in a range of foodstuffs and beverages but this was the first time it had been identified in honey at such high levels. Manuka honey had very high amounts of MGO, ranging from 38 to 828 mg/kg, which is up to 100-fold higher compared with non-Manuka honeys (Mavric, Wittmann *et al.* 2008).

MGO is a highly reactive compound that irreversibly modifies proteins. Until the discovery of there being high levels of MGO present it was unclear why Manuka honey had a high level of anti-inflammatory activity due to bee proteins yet other honeys which had the same amount of protein did not. It was hypothesised in Chapter 6 that at these levels the MGO in Manuka honey could modify the protein in honey over time, either in the hive or during storage by the apiarist, which conferred anti-inflammatory properties to the honey.

One of the most obvious signs of modification of proteins by MGO is the resulting fluorophore, a component of a molecule which causes the molecule to be fluorescent. Proteins highly modified by MGO fluoresce (Westwood, McLellan *et al.* 1994). The high levels of MGO in Manuka honey may modify the proteins resulting in fluorophores, yet to date no other research has investigated this.

It is widely published that carbohydrates in foods and beverages are susceptible to degradation by processing, cooking and prolonged storage, forming reactive 1,2-dicarbonyl compounds, including MGO, glyoxal and 3-deoxyglucosone). The non-enzymatic reactions involving the formation of these compounds are collectively referred to as either caramelisation or, if amino-containing compounds are present, non-enzymatic glycation called Maillard reactions. Often these processes are considered to be desirable as they confer much of the taste and colour upon cooked foods.

8.2.2 Colour of honey and the Maillard reaction

The colour of table honey is one of the factors that determine its price, with the lighter honeys having a more mild and popular taste than dark-coloured honeys. Manuka honey is traditionally a darker honey, with the best NPA Manuka honeys having a typically darker colour. The rate of darkening of honey has been related to the ratio of glucose to fructose, nitrogen content, free amino acids and moisture, alongside temperature, indicating the Maillard reaction is responsible (Gonzales, Burin *et al.* 1999). Dark honeys traditionally have a higher nitrogen content than paler honeys, with a range of 0.04% to 0.1% (White and Landis 1980). MGO is formed endogenously in numerous enzymatic and nonenzymatic reactions (Nemet, Varga-Defterdarovi *et al.* 2006). It is formed by the spontaneous degradation of triosephosphates, oxidative metabolism of ketone bodies and catabolism of threonine (Thornalley 1993). MGO reacts rapidly with

the side chains of arginine, lysine and cysteine residues in proteins to form advanced glycation end products (AGEs) (Thornalley 1993). It is generally believed that the reaction of MGO with proteins produces adverse products, in terms of protein structure and function. However, a few studies have shown beneficial effects, particularly on the chaperone function of human α-crystallin in the eye lens where chemical modification of α-crystallin by MGO enhances its chaperone function which helps in maintaining the transparency of the lens, and by protecting the lens proteins from various stress conditions (Nagaraj, Oya-Ito *et al.* 2003; Kumar, Reddy *et al.* 2004).

8.2.3 MGO-formed fluorophores and AGE formation

The fluorescence of honey has been investigated and found to be a method of successfully determining levels of adulteration with sugar cane syrup or corn syrup to bulk honey, using the natural fluorescence of antioxidant components (Ghosh, Verma *et al.* 2005). However these studies have not tested Manuka honey nor looked for the MGO-formed fluorophores which have a different wavelength with higher intensity. Along with brown colour, fluorescence is one of the qualitative properties used to estimate AGE formation (Ulrich and Cerami 2001), with many of the AGEs detected and isolated based on their fluorescent properties.

Human serum albumin (HSA) and bovine serum albumin (BSA) have been modified with MGO (MG-BSA, MG-HSA) to give fluorescent derivatives, characterised by the arginine-derived fluorphore (Westwood and Thornalley 1995). Further it was shown that MGO binds and irreversibly modifies BSA, producing a protein that binds endocytic receptors initiating uptake of the protein. MGO-modified arginine residues have been identified as the signal for receptor-mediated endocytosis and degradation of proteins by THP-1 cells, indicating there are receptors recognising this modification. These modified proteins are a potent signal for their degradation by monocytic cells, cellular

activation or stimulation (Westwood, Argirov *et al.* 1997). This implies that the immune system is readily capable of detecting and responding to AGEs. AGE-modified proteins have also been implicated in the impairment of the respiratory burst of macrophages in diabetic patients (Ramírez, Bedoya *et al.* 1997) and are thought to be linked to the development of diabetic complications as MGO and AGE levels are higher in diabetic patients. AGEs have also been proposed to play a crucial role in the development of atherosclerosis due to plaques forming of macrophages expressing the receptor for AGE (RAGE) in excessive quantities (Uchida, Khor *et al.* 1997). When there is binding of AGEs to RAGE it initiates inflammatory responses (Beckman, Creager *et al.* 2002; Tekabe, Li *et al.* 2008). The formation of AGEs increases ROS in the tissue, resulting in lipid peroxiation products implicated in Parkinson's disease (Yoritaka, Hattori *et al.* 1996), Alzheimer disease (Mark, Lovell *et al.* 1997) and cancer (Okamoto, Toyokuni *et al.*, 1994). This leads to the assumption that MGO represents one of the primary sources of oxidative damage to proteins in complex biological matrices.

AGE-modified proteins bind to several cellular receptors such as RAGE (Yan, Ramasamy *et al.* 2009), AGE-R1, AGE-R2, AGE-R3 (Thornalley 1998), macrophage scavenger receptor (SR-A and CD36) (Takata, Horiuchi *et al.* 1988; Miyazak, Nakayama *et al.* 2002), and the macrophage receptor (Vlassara, Brownlee *et al.* 1985). AGE-RAGE interaction is thought to activate nuclear factor-κB (NF-κB) which plays a key role in regulating the immune response to infection (Yan, Ramasamy *et al.* 2009). A membrane-associated macrophage receptor has been identified that specifically recognised proteins to which AGEs are bound. This macrophage receptor binds AGEs and induces the synthesis of cytokines such as TNF-α, IL-1β and growth factors PDGF and IGF-1, suggesting that the AGE receptor system plays an important role in normal tissue remodelling (Kirstein, Brett *et al.* 1990; Kirstein, Aston *et al.* 1992). Glycation of arginine residues by MGO is expected to have significant impact on the functional activity of proteins, due to a loss of positive charge and consequent protein miss-folding (Ahmed,

Dobler *et al.* 2005). This may target modified proteins for ligation to receptors on immuno-competent cells otherwise not encountered.

AGE formation *in vivo* is a major source of ROS that leads to oxidative damage, and AGE accumulation in tissues is one of the main causes of diabetic vascular damage (Brownlee 1995). When AGEs bind RAGE receptors in THP-1 cells, a chronic inflammatory cascade is initiated with the release of pro-inflammatory cytokines accelerating damage to the surrounding tissue (Shang-Ming Huang 2006).

8.2.4 AGE inhibitors

Binding of these receptors does not necessarily cause an increase in inflammation (Valencia, Mone *et al.* 2004). AGE inhibitors have been identified that bind the same receptors yet have an anti-inflammatory response on monocytes and macrophages, conferring beneficial effects for the treatment of inflammation. These inhibitors may act as decoys, simply preventing the AGE from binding receptors. The drug LR-90 has been identified as belonging to a new class of AGE inhibitors that inhibits inflammatory responses in THP-1 cells in a dose-dependent fashion (Figarola, Shanmugam *et al.* 2007). Treatment with LR-90 results in reduced expression of RAGE and other pro-inflammatory genes and NF-κB promoter transcriptional activity. LR-90 also prevented oxidative stress in macrophages by reducing intra-cellular superoxide production, preventing other ligands from binding RAGE and inducing ROS formation. It also blocks monocyte adhesion to endothelial cells (Figarola, Scott *et al.* 2003; Figarola, Shanmugam *et al.* 2007). It has been proposed that targeting RAGE may lead to treatments of inflammatory disorders, as ligation to RAGE by AGE in THP-1 monocytes leads to increased expression of inflammatory cytokine expression, growth factors and adhesion molecules as well as initiating chemotaxis (Yan, Ramasamy *et al.* 2009).

8.2.5 Aims and experimental approach

It is hypothesised that the protein in honey may be modified by MGO, resulting in a novel ligand for macrophage receptors that initiates an immune response down-regulating the inflammatory pathways. This may also result in a fluorescent protein product easily identifiable for the screening of anti-inflammatory potency of Manuka honey. The aim of the research in this chapter was to determine if in fact there was MGO-formed fluorphores in honey. Therefore a variety of Manuka and non-Manuka honeys was screened for fluorescence and this was related back to the NPA, MGO content and phagocytosis-inhibiting activity. A range of honeys was selected for assaying fluorescence. Also the colour of honey was rated on a scale of 1-12. A colour scale was developed for use in this study and further detailed in Appendix 4. The brown colour constitutes one of the qualitative properties of AGEs (Ulrich and Cerami 2001).

8.3 Methods

A range of Manuka honeys were obtained from the collection in the Honey Research Unit, University of Waikato, NZ. The fluorescence and phagocytosis-inhibiting activity was measured for each of these honeys. Measurement of the NPA of the honeys had been carried out by Kerry Allen in the Honey Research Unit. MGO levels were determined by Dr. Chris Adams from the Chemistry Department, University of Waikato, which involved treating the honey sample with *o*-phenylenediamine, to react with 1,2-dicarbonyl compounds forming the corresponding quinoxaline derivatives. These derivatives were analysed by HPLC using UV detection (Adams, Boult *et al.* 2008). All the honeys used in this Chapter are outlined in Table 8.1. The fluorescence and colour for all the honeys used for the whole research project are outlined in Appendix 3. Preparation and filtration of solutions of honey prior to assay was as described in Chapter 2. Honeys were assayed at the concentrations mentioned for the procedures

described below. BSA and a honey with naturally low fluorescence were incubated with MGO to determine if fluorophores could be developed.

8.3.1 Measuring fluorescence

The fluorescence of whole honey and honey fractions isolated by chromatography in Chapter 6 was measured.

8.3.1.1 Whole honey

Honey was brought to room temperature and diluted to 10% V/V in purified water. Dilute honey (5 ml) was then filtered through a 0.2 µm Minisart sterile single use syringe filter (Sartoris) to remove pollen and impurities. It was then pipetted (100 µl) into 5 wells of a 96 well black plate (Nunc Cat. No.# 137101). The fluorescent intensity of the honey was measured on a fluorescence imaging system, a Fujifilm LAS-1000 instrument equipped with an intelligent darkbox II (Alphatech). The plate was placed inside the darkbox (level 4) and focused using the Image reader LAS-1000 Plus programme. The blue light fluorescence setting was used with the standard wavelength filters provided used to detect emission at 470 nm. Data was analysed using the provided software, Image Reader LAS-1000 Plus Lite version 1.5 and Image Gauge 4.0. The fluorescence was measured across the well in arbitrary units (AU) and the background intensity subtracted to give a reading. In each plate there was a standard honey with previously measured fluorescence along with water blanks and empty wells for background readings. Measurements from the 5 wells for each sample were averaged to obtain a final reading. So that the honeys could be compared between plates readings were adjusted relative to the standard honey. In some cases the fluorescence intensity was too high for individual honeys and exposure time was reduced to avoid over-exposure. The measurement from the standard honey was used to derive the correct measurement for the honeys in these plates.

Results ranged from 3 000 AU to 260 000 AU, with typical standard deviation of +/- 1 500 AU.

8.3.1.2 Honey fractions

Fractions obtained from Sepharose 12 FPLC chromatography columns (1 ml fractions) in Chapter 6 (Section 6.3.5.3) were screened for fluorescence according to the protocol as described in Section 8.3.1.1.

8.3.2 Phagocytosis assay

The latex bead phagocytosis assay using incubated honey proceeded the same as previously (Section 4.3.1). THP-1 cells and LPS activation were unchanged.

8.3.3 Colour grading

A colour scale was developed by sorting honey into the closest possible match, which resulted in a scale from one to twelve, with one being the palest honey to twelve being the darkest honey. The scale can be found in Appendix 4. The colour of honey was graded by filling a standard size (1.5 cm diameter x 5 cm long) small plastic vial with honey and finding the closest match on the colour scale developed.

8.3.4 Honey incubation

Both Manuka and non-Manuka honeys were incubated. Manuka honey was selected for incubation based on its initial high MGO levels and low fluorescence. Non-Manuka honey was selected based on its low fluorescence and lack of MGO. Honey was incubated for three months, during which time fluorescence readings were tracked. Each honey incubated also had a sample stored at -20° to maintain the original qualities. The incubated honey and the frozen honey were assayed for inhibition of phagocytosis and were subjected to SDS electrophoresis

on a mini-gel to determine any protein size modifications due to the incubation. A Manuka honey with high fluorescence and Manuka honey with low fluorescence was also subjected to SDS electrophoresis for comparison to the incubated and non-incubated honey.

Whole Clover honey and Pasture honey were incubated with MGO or without MGO for three months at 37°C to determine if the presence of MGO would generate fluorescence over time in the non-fluorescent honey and increase the phagocytosis-inhibiting activity. The honey had been assayed previously for phagocytosis-inhibiting activity and had low activity. The honey was electrophoresed on a SDS mini-gel to determine any protein size modifications due to the incubation. The MGO treated honey was assayed in the phagocytosis assay to determine what effect the incubation had on the phagocytosis-inhibiting activity.

8.3.4.1 Methylglyoxal

Methylglyoxal (40%) was purchased from Sigma-Aldrich (Cat. No.#M0252) and used at final concentrations correlating to a NPA activity of 15 (approximately 400 mg/kg honey or 1 ml of 40% MGO per kg honey).

8.3.4.2 Honey incubation

Whole Manuka honey (20 g of MSB20, NPA 20+), Pasture honey (20 g of P59) and Clover honey (20 g of CLO1) were incubated for three months at 37°C. Clover honey, (20 g of CLO1) treated with 400 mg/kg MGO mixed into it, was also incubated for three months at 37°C. Control samples of all the honeys (20 g) were frozen for the duration. The fluorescence of honey immediately after adding the MGO was measured, and at the concentrations added to honey, the fluorescence was found to not increase due to the added MGO. After six weeks

and three months incubation of the honey, the fluorescence was measured as described in Section 8.3.1.1.

8.3.4.3 SDS polyacrylamide gel electrophoresis of incubated honey

Honey (described in Section 8.3.6.1) was run on a 12% SDS mini-gel (prepared and run as described in Section 6.3.6). Also included for comparison was Manuka honey with high fluorescence (M149) and Manuka honey with low fluorescence (M26). Honey was diluted to 10%. Samples to be loaded on the gel were mixed 1:1 with 2X Tricine Sample Buffer and heated at 99°C for 5 minutes, then left to cool prior to loading. Prior to staining, gels were imaged on a UV gel illuminator to observe the fluorescence of the protein band (this fluorescence was not visible after staining). SDS minigels were silver-stained as described in Section 6.3.6.

8.3.5 Bovine serum albumin incubation with methyglyoxal

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Cat. No.# 85040c fraction 5) and dissolved in sterile phosphate buffered solution to achieve a concentration of 10 mg/ml. It was incubated with or without 400 mg/kg MGO (1 µl 40% MGO /g BSA for two days at 37°C. Preliminary results indicated that only short incubation was required to develop fluorescence, accordingly BSA was incubated for two days. Absorbance of the protein was measured at 280 nm before and after modification by MGO was carried out. Unmodified BSA has a molecular weight of 66 kDa. The MGO-modified BSA (from incubation for two days) was electrophoresed on a SDS mini-gel to determine any protein size modifications due to incubation with MGO. Prior to staining, gels were imaged on a Fujifilm imaging system LAS-1000, to observe the fluorescence of the protein band. The SDS mini-gels were then silver stained following the protocol in Chapter 6.3.6.

8.3.6 Visualisation of colour and fluorescent protein on TLC plates

A novel method of visualising the fluorescent protein in solution was developed using aluminium-backed TLC plates. Approximately 50 µl of 10% honey, BSA or MGO-modified BSA was spotted onto a TLC plate (Merck Aluminium sheets, silica gel 60, without fluorescent indicator, Art. 5553) and left to spread by capillary action. This resulted in a ring of protein which did not spread much from the point of application, surrounded by a larger ring where the sugars and other smaller molecules spread further than the protein. The TLC plate was then visualised in the LAS-1000 instrument for comparison of fluorescence intensity before and after incubation, or for comparisons between honey samples. The solutions applied to the plate were: 1 mg/ml BSA with and without 400 µg/g MGO as described in Section 8.3.7, Manuka honey with low fluorescence (M32, M128 and MSB20), and Manuka honey with high fluorescence (MO5, MB102 and M141) and, Clover honey (CLO1 non-incubated, incubated and MGO-treated and incubated as described in Section 8.3.5.2). The TLC plates were also observed before fluorescent imaging for their colour.

In a variation of this experiment Whatman filter paper discs (no. 4) were used, on which the honey sample left a brown ring after the sample had spread by capillary action. This showed the variation in colour between honey samples and gave a greater fluorescence than the TLC plates. The filter paper was then imaged on the LAS-1000 for fluorescence to locate the fluorescent component.

8.3.7 Obtaining a fluorescence emission spectrum for Manuka honey

A 10% Manuka honey solution (M141) was compared with MGO-modified BSA for fluorescence emission wavelengths. An emission spectrum plot was obtained from each using a Confocal microscope imaging system to detect the fluorphore/s present. This work was completed in conjunction with Dr Barry O'Brien from The University of Waikato's Confocal Microscopy Imaging Facility.

8.4 Results

The honeys assayed during this research are listed in Appendix 3. In this Section a range is given of the measurements obtained for each assay. Table 8.1 shows the data for honeys that had the MGO and NPA values tested.

Table 8.1. Results from measurements of various parameters for a range of honeys of different varieties of floral source. Colour was graded on a scale of 1 (lightest colour) to 12 (darkest colour). Fluorescence (Fluor) of the honeys is shown in Arbitrary Units x 10^3. Anti-inflammatory activity (AI) is shown as the mean inhibition (%) of phagocytosis of latex beads. Non-peroxide activity (NPA) is shown as % phenol with equivalent activity. Content of MGO and is shown as µg/kg honey. Data are sorted by AI in ascending order.

Code	Variety	Colour	Fluor	AI	NPA	MGO
M128	Manuka	4	20	4	11.8	192
M158	Manuka	4	30	5	13.3	330
M160	Manuka	4	21	5.1	8.3	170
M161	Manuka	4	23	5.6	9.4	223
M165	Manuka	4	28	7	11.8	280
M159	Manuka	4	29	7.2	11.8	280
M197	Manuka	4	20	9	ND	115
M162	Manuka	4	26	9	8.7	360
M181	Manuka	4	72	10	ND	130
M163	Manuka	4	34	10	14	352.8
M24	Manuka	3	10	11	7.9	96.6
M206	Manuka	4	51	12	ND	110
M196	Manuka	4	21	13	ND	130
M28	Manuka	4	19	14	8.6	112
M34	Manuka	7	33	15	4.5	58.6
M173	Manuka	4	50	15	ND	150
P59	Pasture	3	35	15	0	10
M38	Manuka	4	29	16	7.5	89.9
M22	Manuka	3	34	16	8.3	106
M23	Manuka	3	26	16	9.3	141
M13	Manuka	5	25	16	11	167
M188	Manuka	4	66	17	8.7	130
M168	Manuka	4	60	18	ND	140
M169	Manuka	4	68	18	ND	150
M31	Manuka	7	39	20	7.5	107
M209	Manuka	5	81	20	ND	140
M176	Manuka	4	46	20	ND	145

Code	Variety	Colour	AU	AI	NPA	MGO
M170	Manuka	4	45	20	ND	160
M26	Manuka	6	47	21	7.3	67
M30	Manuka	4	30	21	10	103
M21	Manuka	3	35	21	9.2	121
M177	Manuka	4	65	21	ND	150
M32	Manuka	6	40	23	5.9	71
M164	Manuka	4	80	23	ND	118
M198	Manuka	4	77	24	ND	80
M185	Manuka	4	57	24	ND	110
M12	Manuka	5	52	24	14.9	272
M184	Manuka	4	58	25	ND	110
M171	Manuka	4	74	25	ND	150
M167	Manuka	4	61	25	ND	151.2
M207	Manuka	5	80	28	ND	120
M178	Manuka	4	68	28	ND	150
M27	Manuka	6	65	29	7.3	107
M07	Manuka	8	58	29	13.9	205
M201	Manuka	4	73	29	12.4	280
M174	Manuka	4	90	30	ND	85
M175	Manuka	4	102	30	ND	85
M172	Manuka	4	89	30	ND	160
M205	Manuka	5	96	30	8.9	170
M10	Manuka	6	54	30	13.9	219
M36	Manuka	3	53	31	5.4	25.4
M179	Manuka	4	89	31	ND	120
M166	Manuka	4	92	31	ND	145.9
M15	Manuka	7	63	31	15.3	267
M182	Manuka	4	116	32	ND	110
M202	Manuka	4	96	32	ND	150
M16	Manuka	7	68	32	14.4	272
M187	Manuka	4	94	33	8.1	110
M183	Manuka	4	107	34	ND	115
M200	Manuka	4	85	34	ND	140
M11	Manuka	6	74	34	14.8	249
M09	Manuka	8	62	34	17.7	329
M204	Manuka	5	120	35	ND	160
M186	Manuka	4	87	35	ND	95
102	Manuka	9	153	35	0	100
M29	Manuka	5	73	35	8	114
M03	Manuka	7	75	37	23.6	599
M180	Manuka	4	97	38	ND	150
M199	Manuka	4	93	38	ND	160

Code	Variety	Colour	AU	AI	NPA	MGO
CJA10+	Manuka	5	92	38	11.1	189
M17	Manuka	4	97	39	14.2	243
M01	Manuka	8	95	40	25.3	650
M203	Manuka	5	156	41	ND	150
CJA30+	Manuka	7	147	41	27	679
M05	Manuka	9	162	43	17.5	312
M192	Manuka	5	175	44	8.1	130
M193	Manuka	5	214	49	ND	100
M190	Manuka	5	195	49	8.2	105
MBD28.8	Manuka	9	167	49	27.3	708
M191	Manuka	5	216	51	ND	110
M189	Manuka	5	204	55	ND	85
P59	Pasture	3	35	10	0	10
VB6468	Vipers Bugloss	1	3	0	0	0

NPA values ND=non-detectable in the standard assay which has a minimum level for measurements of 8.2. For NPA values listed lower than 8.2, these were measurements made with honey solutions double the usual concentration and corrected by dividing the NPA by 2.

8.4.1 Fluorescence of honey

The range in fluorescence measurements was 3 000 AU – 227 000 AU (± 1 500 AU), with the lowest reading recorded for Vipers Bugloss honey (VB6468) and the highest for an aged Manuka honey (MB102). The lowest fluorescence recorded for a Manuka honey was 10 000 AU (M24). Fluorescence measurements for all samples tested are listed in Appendix 3.

A highly fluorescent aged Manuka honey (MB102) was electrophoresed on an SDS gel and its fluorescence visualised using the LAS-1000 instrument. Figure 8.1 shows the position of the fluorescent protein after electrophoresis (but before staining) and the same gel lane after silver staining.

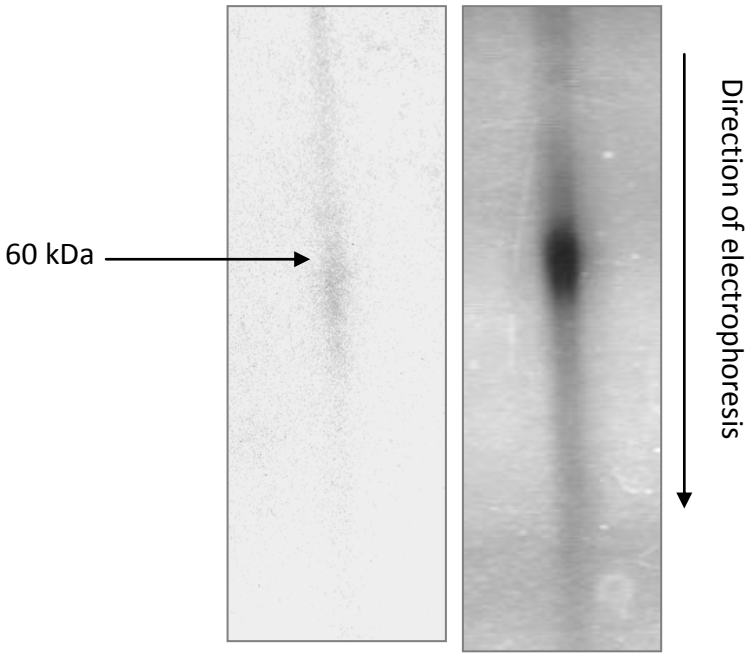

Figure 8.1. SDS electrophoresis gel (12%) with whole Manuka honey run on it. The image to the left shows the visible fluorescent band of Manuka honey protein taken before silver-staining. The image to the right shows the same lane after silver staining.

8.4.2 Colour of honey

The colour grading of each honey in the range of samples studied is shown in Table 8.1. The range was 1-12, with the lowest grade recorded for Vipers Bugloss honey (VB6468) and the highest for an aged Manuka honey (MB102). The lowest grade recorded for a Manuka honey was 3 (M24). The colour scale used is in Appendix 4. The colour score for all the honeys tested is shown in Appendix 3.

8.4.3 Fluorescence of fractionated honey

The fluorescence measurements for the fractions obtained from column chromatography on Sephadex G-50 (described in Chapter 6) are shown in Table 8.2 alongside their phagocytosis-inhibiting activity (data are taken from Section

6.4.3). The fractions with the highest fluorescence were also the fractions that had the most anti-inflammatory activity.

Table 8.2. Fluorescence of fractions isolated from G-50 Sephadex chromatography compared with their phagocytosis-inhibiting activity (data from Chapter 6).

Fraction no.	Fluorescence (AU x 10^3)	Phagocytosis-inhibiting activity (% inhibition)
4	22	42
6	26	53
8	26	55
10	22	39
12	24	43
14	20	20
16	22	30
18	6	0
20	0	0
22	0	0
24	0	0
26	0	0

8.4.4 Finding correlation in assay results

Correlation between parameters in the data presented in Table 8.1 was sought in the graphs shown in Figures 8.2 – 8.9. Only honeys with a full data set have been included. For the comparison it was assumed that there was a linear relationship between the parameters, and the correlation coefficient is given as R^2. Only anti-inflammatory activity plotted against fluorescence (Figure 8.5) returned a significant result for the regression (p<0.0001). The lack of correlation between

the other variables suggests that a complex reaction is occurring. One possibly very relevant variable that is missing is the age of the honey, as the age of the honey samples taken from storage is unknown. The age was known for some fresh honeys from the new season (Codes M159-M209) and some old honeys which were known to be over 10 years old (Codes MB101-MB102 and Spbay old).

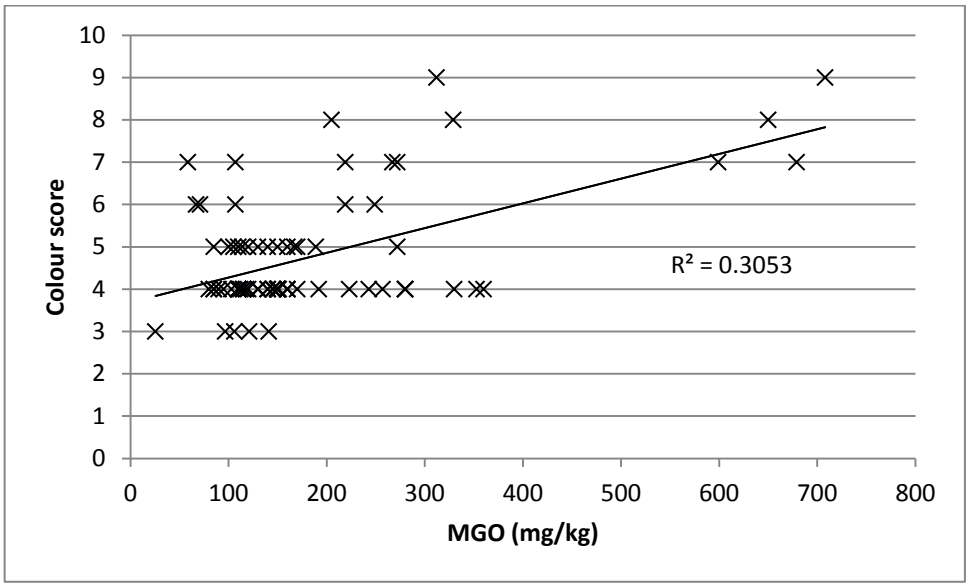

Figure 8.2. Colour plotted against MGO content for the range of Manuka honeys studied. Colour was graded on a scale of 1 (lightest) to 12 (darkest).

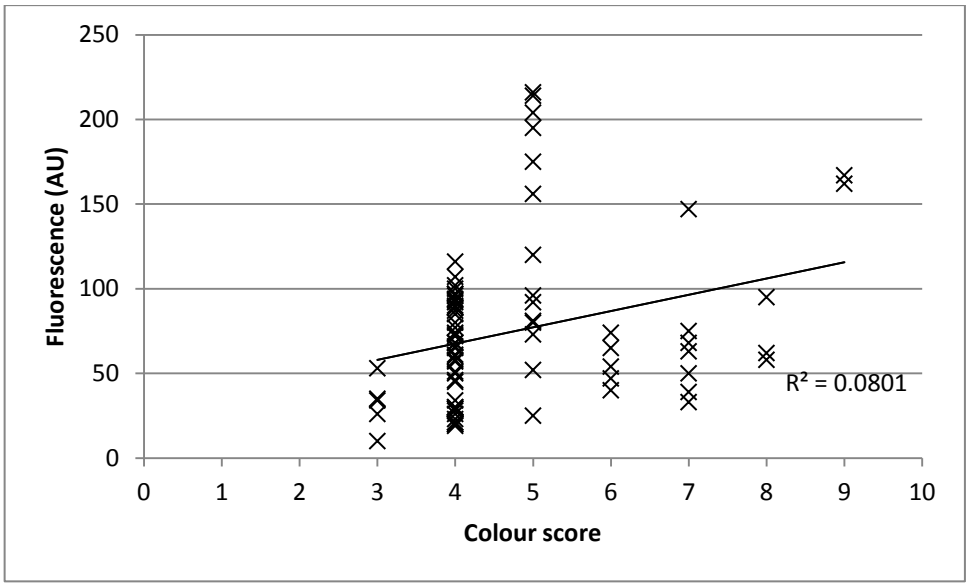

Figure 8.3. Fluorescence plotted against Colour for the range of Manuka honeys studied. Colour was graded on a scale of 1 (lightest) to 12 (darkest). Fluorescence is shown in Arbitrary Units x 10^3.

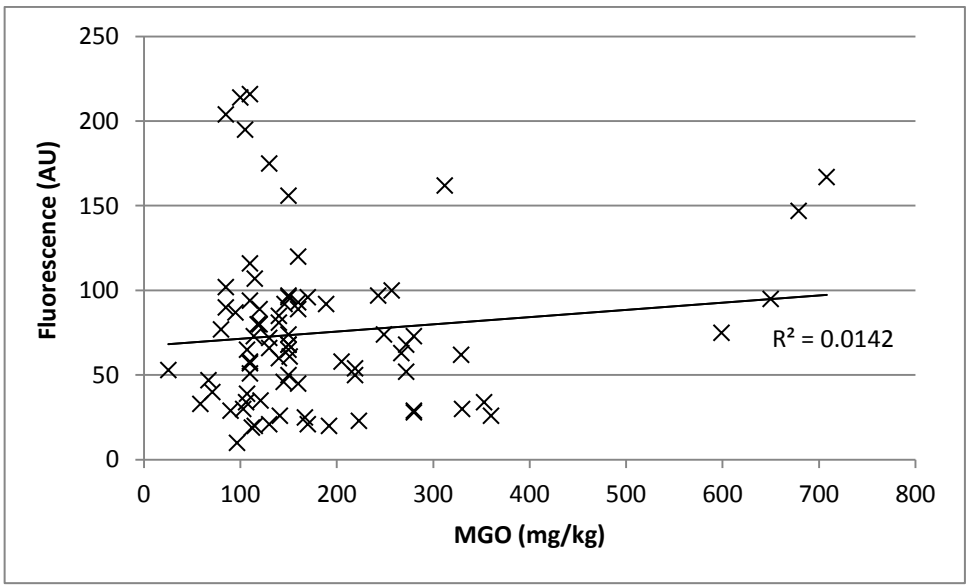

Figure 8.4. Fluorescence plotted against MGO content for the range of Manuka honeys studied. Fluorescence of the honeys is shown in Arbitrary Units x 10^3.

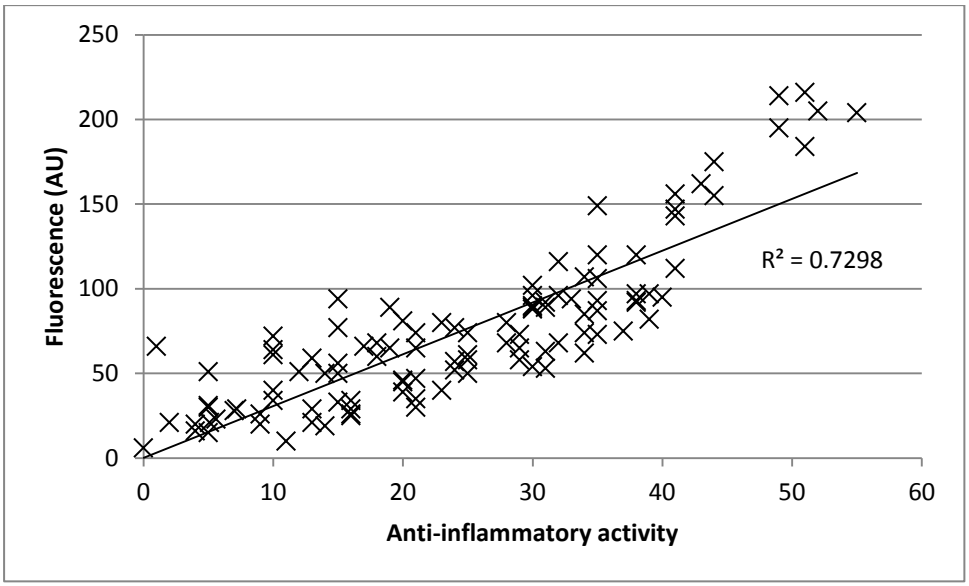

*Figure 8.5. Anti-inflammatory activity plotted against fluorescence for the range of Manuka honeys studied. Fluorescence of the honeys is shown in Arbitrary Units (AU) x 10^3. Anti-inflammatory activity is shown as the mean inhibition (%) of phagocytosis of latex particles (data are sourced from Chapter 6). The regression is highly significant (*p*=0.000 analysed by ANOVA) anti-inflammatory activity = 9.184 + 0.2236 fluorescence (AU).*

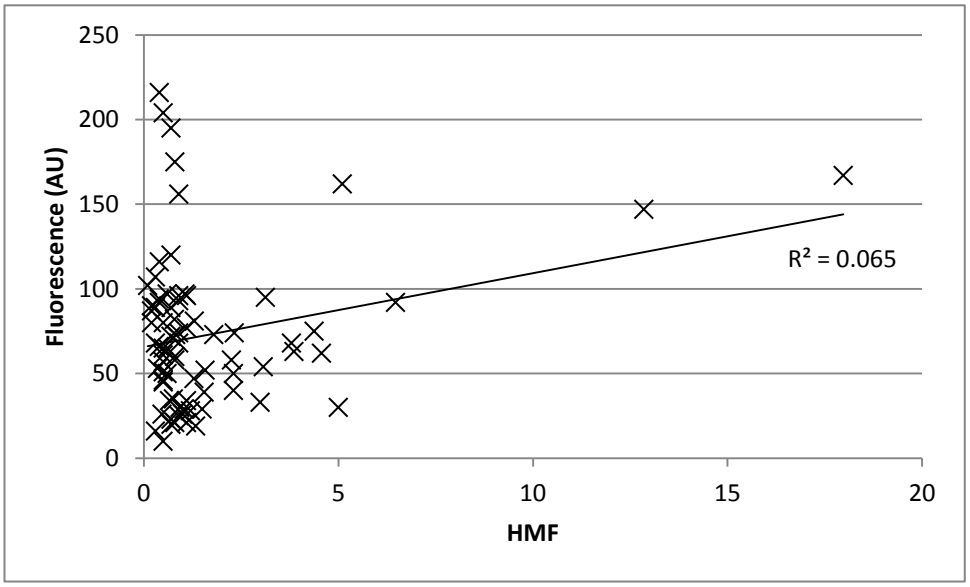

Figure 8.6. Hydroxymethylfurfuraldhyde (HMF) plotted against fluorescence for the range of Manuka honeys studied. Content of HMF is shown as mg/kg. Fluorescence of the honeys is shown in Arbitrary Units x 10^3.

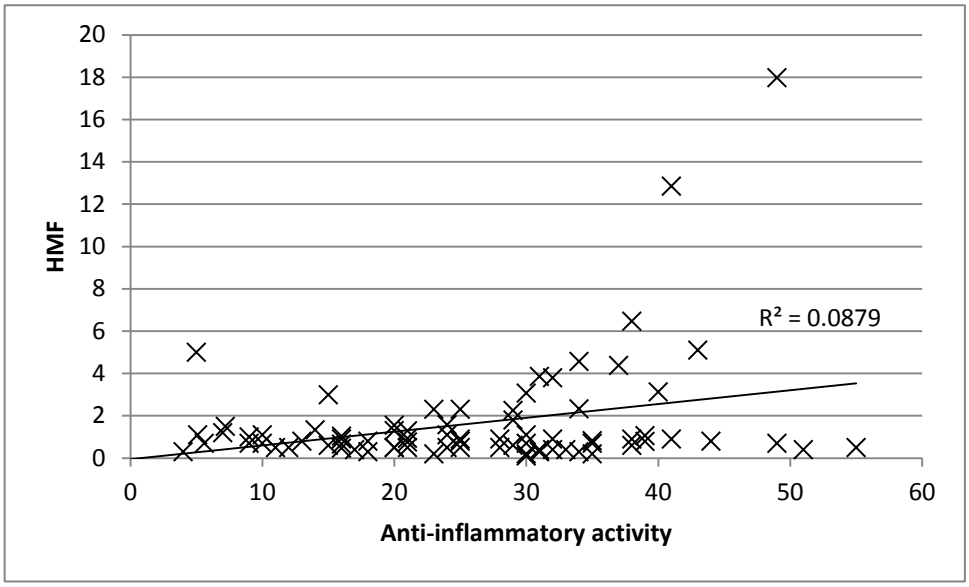

Figure 8.7. Hydroxymethylfurfuraldhyde (HMF) content plotted against anti-inflammatory activity for the range of Manuka honeys studied. Content of HMF is shown as mg/kg. Anti-inflammatory activity is shown as the mean inhibition (%) of phagocytosis of latex particles (data are sourced from Chapter 6).

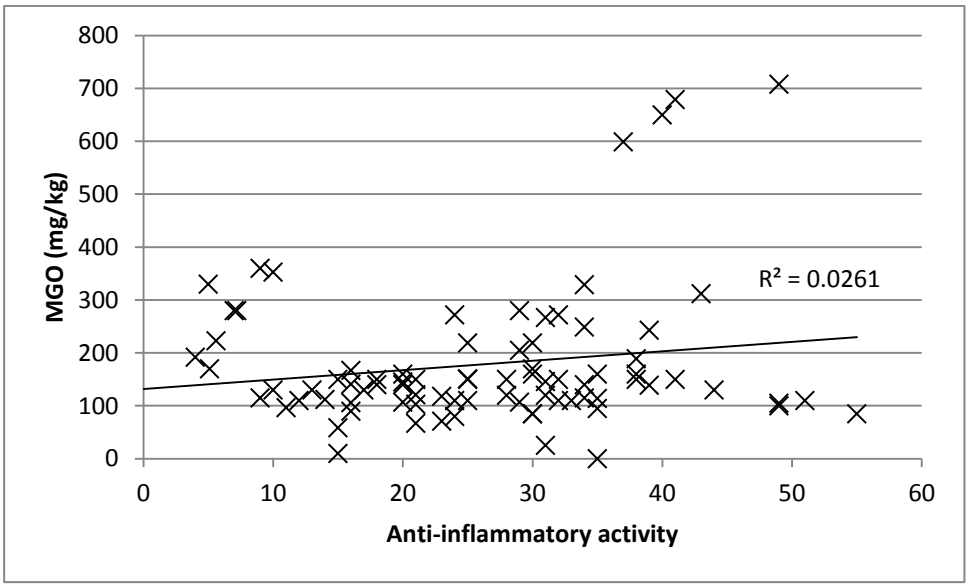

Figure 8.8. MGO content plotted against anti-inflammatory activity for the range of Manuka honeys studied. Anti-inflammatory activity is shown as the mean inhibition (%) of phagocytosis of latex beads, (data are sourced from Chapter 6).

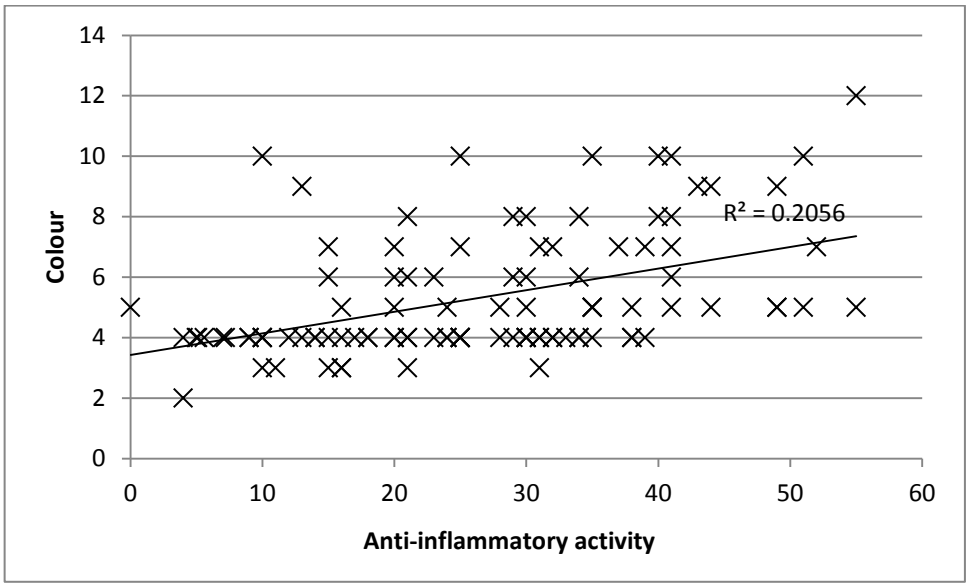

Figure 8.9. Colour plotted against anti-inflammatory activity for the range of Manuka honeys studied. Anti-inflammatory activity is shown as the mean inhibition (%) of phagocytosis of latex beads, (data are sourced from Chapter 6). Colour was graded on a scale of 1 (lightest) to 12 (darkest).

8.4.5 Incubating honey

Table 8.3 and 8.4 shows the fluorescence and colour measurements for selected honeys before, during and after incubation for three months at 37°C with natural MGO levels (Manuka) or 400 mg/kg MGO added (Pasture and Clover). In the controls which were Manuka honey (with a high natural MGO content) kept for three months at 4°C, and Pasture and Clover honey with 400 mg/kg MGO or no added MGO kept for three months at 4°C, there was no increase in fluorescence (results not shown). Table 8.3 and 8.4 also shows the results from BSA also being incubated with or without MGO. After incubation with MGO, BSA turned a pale yellow colour, whereas incubated BSA with no added MGO remained colourless. A further control found that MGO in water at the quantities used had no fluorescence (results not shown).

Table 8.3. Fluorescence measurements (in Arbitrary Units x 10^3) before incubation, after 6 weeks incubation, and after 3 months incubation at 37°C, with the level of natural or added MGO shown for honey and BSA. Manuka honey had natural MGO levels whereas Pasture and Clover had MGO added.

Honey code	MGO mg/kg	Fluorescence before incubation	Fluorescence after 6 weeks incubation	Fluorescence after 3 months incubation
Manuka MSB20	576	88	149	170
Pasture P59	10	35	47	57
Pasture P59	410	35	64	89
Clover CL01	0	41	52	60
Clover CL01	400	41	66	82
BSA	0	2	2	2
BSA	400	2	245	250

Table 8.4. Colour score before incubation, after 6 weeks incubation, and after 3 months incubation at 37°C, with the level of natural or added MGO shown for honey and BSA. Manuka honey had natural MGO levels whereas Pasture and Clover had MGO added.

Honey code	MGO mg/kg	Colour score before incubation	Colour score after 6 weeks incubation	Colour score after 3 months incubation
Manuka MSB20	576	8	9	9.5
Pasture P59	10	3	5	5
Pasture P59	410	3	6	6
Clover CL01	0	2	4	4
Clover CL01	400	2	4	5
BSA	0	colourless	colourless	colourless
BSA	400	colourless	yellow	yellow

The resulting effects on phagocytosis-inhibiting activity of incubating Manuka honey with natural MGO and Clover honey with MGO added (400 mg/kg) are shown in Figure 8.10. Also assayed for phagocytosis-inhibiting activity was BSA before and after modification by incubating with 400 mg/kg MGO. Neither of these gave any inhibitory activity (results not shown). Activity significantly increased by incubation with added/natural MGO in both Clover honey and Manuka honey. The controls showed no significant effect of incubating Clover honey (with no added MGO) or of the presence of MGO (Clover honey with MGO not incubated).

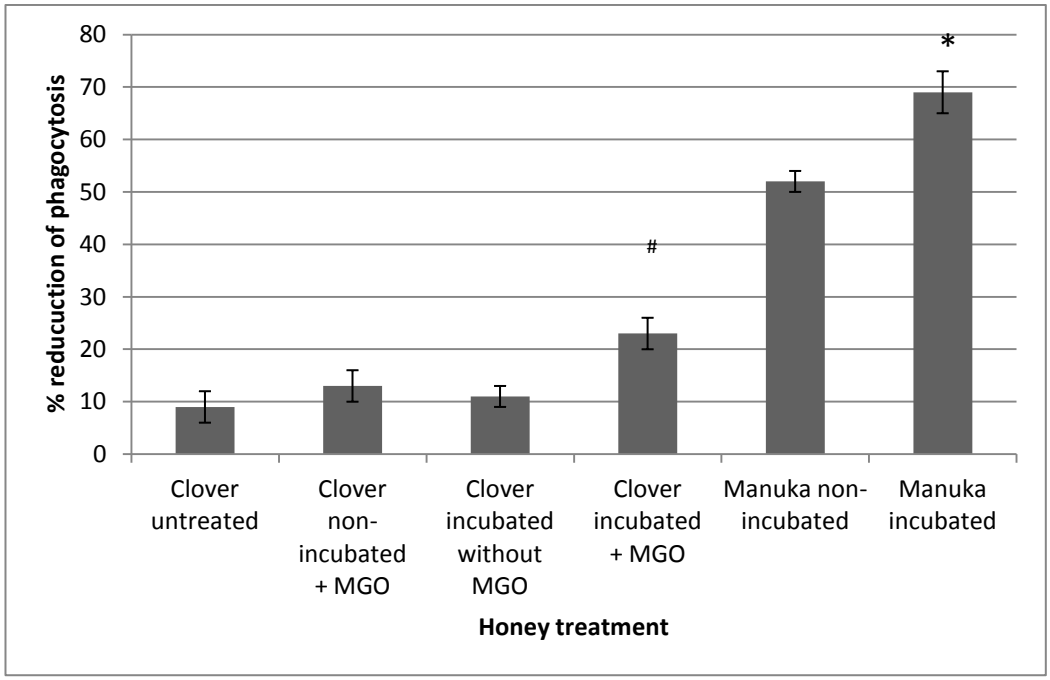

*Figure 8.10. Inhibition of phagocytosis with Manuka honey (MSB20) and Clover honey (CL01), untreated and incubated for 3 months at 37°C. The Clover honey was incubated with and without 400 mg/kg MGO added. As a control, non-incubated Clover honey had 400 mg/kg MGO added immediately before the phagocytosis assay. *p<0.001 analysed by ANOVA compared to the non-incubated Manuka honey control. #p<0.001 analysed by ANOVA compared to the non-incubated Clover honey (with MGO added) control.*

8.4.6 SDS electrophoresis gel images of incubated honeys

The results of electrophoresis of honeys before and after incubation with MGO are shown in Figures 8.11 and 8.12. In Figure 8.11 it can be clearly seen that there was a difference in molecular weight of the major protein between Clover honey (58 kDa) and Manuka honey (63 kDa). Figure 8.12 shows that incubating honey with MGO increased the molecular weight of the major protein (by about 5 kDa) in both honey types. Manuka honey had proportionally more of the higher molecular weight spread (110 kDa and above) than Clover honey. This indicates cross-linking.

Figure 8.13 compares the molecular weight of the major protein band for a range of honeys. It can be clearly seen that Manuka honeys (M149, M26, MSB20) have a larger molecular weight (~ 63 kDa) than Pasture honey (P59) and Clover honey (~58 kDa). Incubated MSB20 had a slightly larger again molecular weight (~65 kDa) of the major honey protein compared with the non-incubated MSB20.

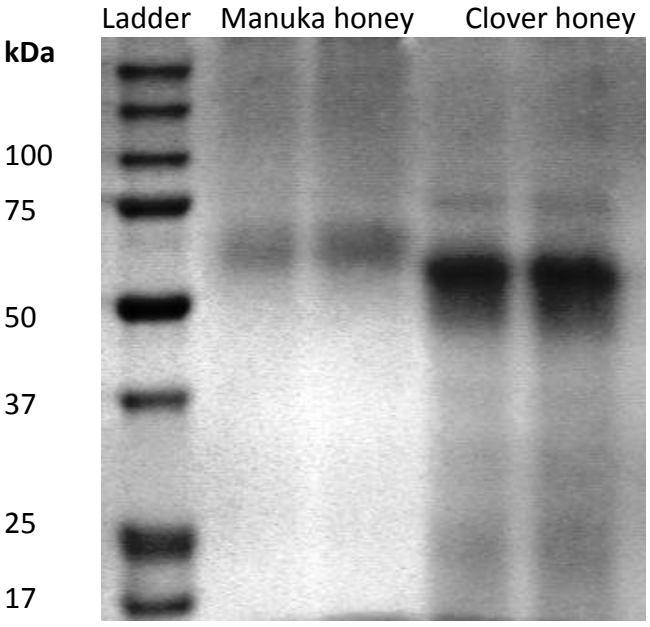

Figure 8.11. Silver-stained gel from SDS electrophoresis of untreated Manuka honey and Clover honey before incubation (each in duplicate).

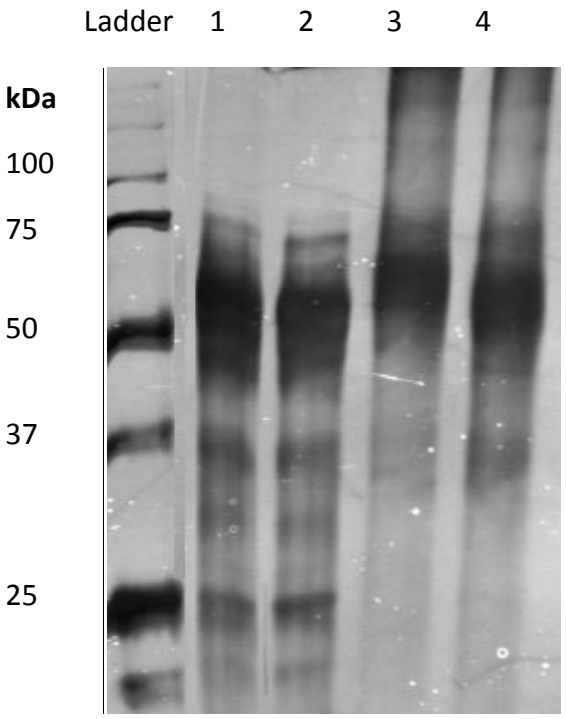

Figure 8.12. Silver-stained gel from SDS electrophoresis of Pasture honey after 3 months incubation (Lane 1) and before incubation (Lane 2) and Manuka honey after 3 months incubation (Lane 3) and before incubation (Lane 4).

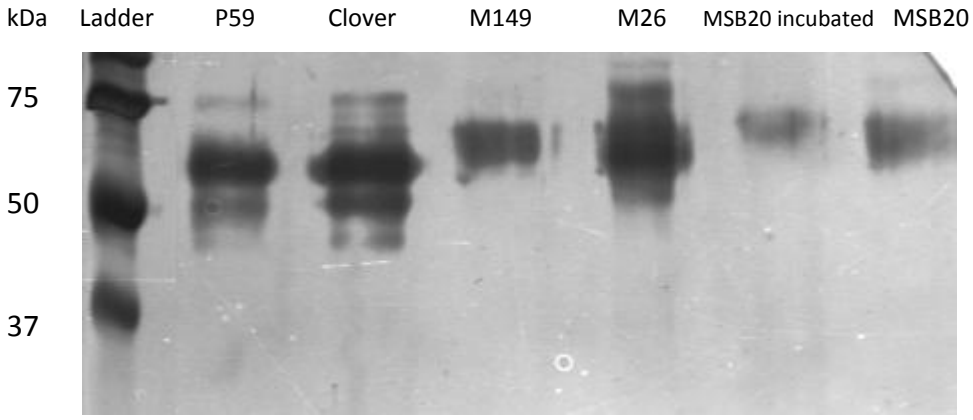

Figure 8.13. Silver-stained gel from SDS electrophoresis of incubated and non-incubated Manuka honey (MSB20), compared with Manuka honey with high fluorescence (M149) and Manuka honey with low fluorescence (M26) and Clover and Pasture honey.

8.4.7 SDS electrophoresis of MGO-modified BSA

The results from electrophoresis of BSA and MGO-modified BSA are shown in Figure 8.14 photographed before staining, using UV illumination to highlight the fluorescence generated by modifying BSA with MGO, and a different gel in Figure 8.15 after staining. A large proportion of MGO-modified BSA only just entered the gel due to the increased protein size or cross-linking of the protein (visible on the gel at approximately 300 kDa). MGO-modified BSA that migrated in the gel formed two major bands (~ 270 kDa and 66 kDa). The untreated BSA was not as visible in the gel, but a slight shadow is visible at 66 kDa.

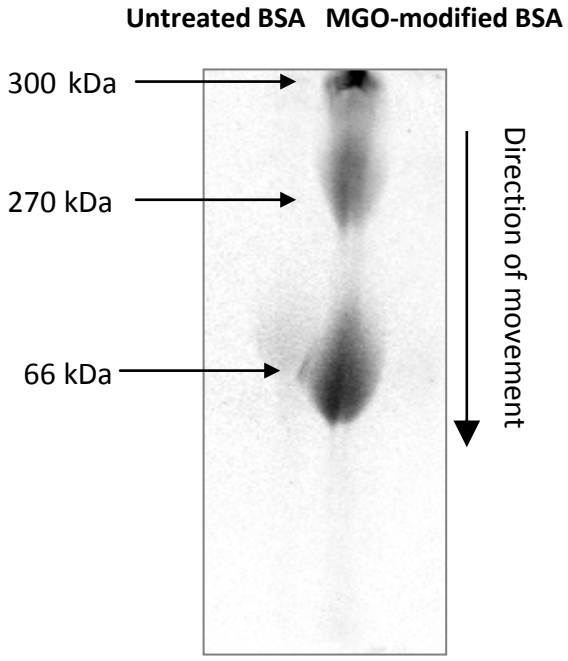

Figure 8.14. Gel from electrophoresis of BSA and MGO-modified BSA, prior to silver-staining. Gel imaged on a LAS-1000 instrument. The fluorescence of the MGO-modified BSA shows in the image of the gel as a dark colour. The molecular weight shown was estimated from the molecular weight ladder run on the gel.

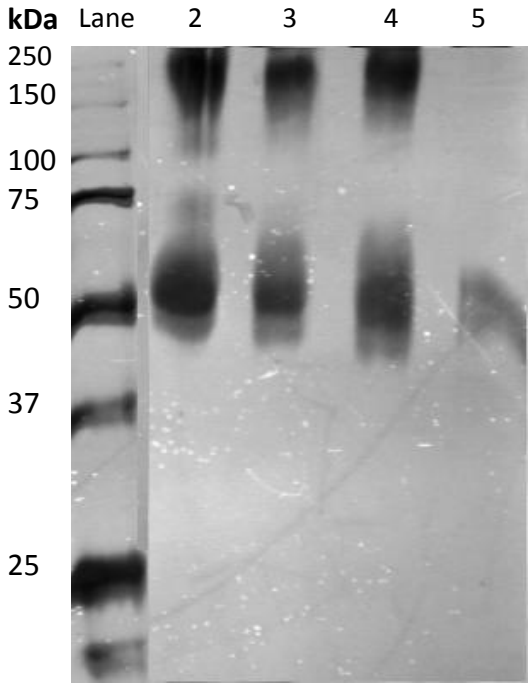

Figure 8.15. Silver-stained gel from SDS electrophoresis of MGO-modified BSA (incubated with 400 µg/kg MGO) (Lanes 2-4) and untreated BSA (Lane 5)

8.4.8 Spot chromatography

8.4.8.1 Fluorescence of spots of MGO-modified BSA and untreated BSA

MGO-modified BSA and untreated BSA were aliquoted on to filter paper and chromatography TLC plates and left to spread by capillary action. The papers were then imaged on the LAS-1000 instrument to highlight the development of fluorphores after incubation with MGO. The results are shown in Figure 8.16 and 8.18. It was concluded that the protein was left at the meniscus of the initial aliquot. On the filter paper the MGO-modified protein was very fluorescent (Figure 8.16). A pale yellow inner ring was visible to the naked eye on the TLC plates. Upon illumination from the LAS-1000, a clear fluorescent ring in the MGO-modified BSA but not the untreated BSA was present (Figure 8.18). The outermost ring of the sample is fluorescent but not a property of the sample. This was concluded by running a water blank (Figure 8.17). This was true for

both the filter paper and the TLC plates. There is a surfactant that enables the samples to run evenly. This runs at the front of the liquid moving out from the point of application of the sample and it may be this surfactant ring that is fluorescing.

The TLC plate allowed for better separation of the fluorescent component but the filter paper showed the greatest overall fluorescence.

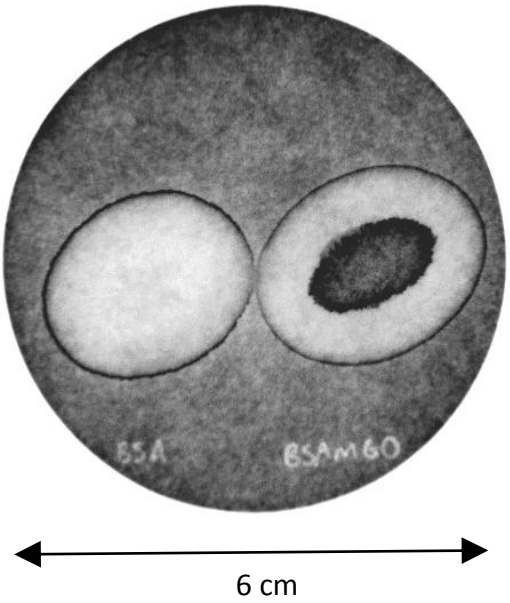

Figure 8.16. Image from the LAS-1000 instrument of untreated BSA (left) and MGO-modified BSA (right) on chromatography paper. The dark colour shows fluorescence. The outer ring of fluorescence may be the surfactant that runs at the front of the liquid moving out from the point of application.

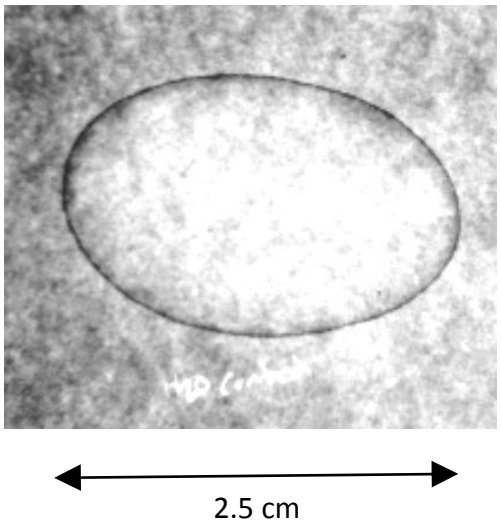

Figure 8.17. Image from the LAS-1000 instrument of water on chromatography paper. The outer ring of fluorescence (shown as a dark colour) may be the surfactant that runs at the front of the liquid moving out from the point of application.

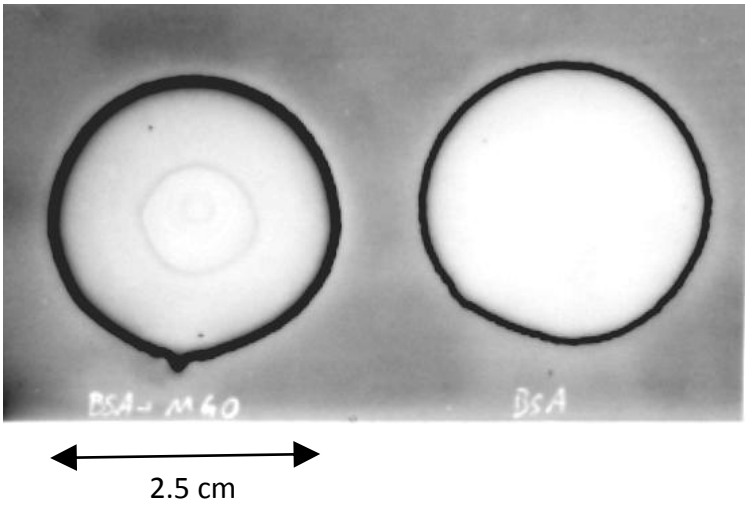

Figure 8.18. Image from the LAS-1000 instrument of MGO-modified BSA (left) and untreated BSA (right) on TLC plates. The dark colour shows fluorescence. The outer ring of fluorescence may be the surfactant that runs at the front of the liquid moving out from the point of application.

8.4.8.2 Fluorescence and colour of spots of honey

Manuka honey (10%) was separated out on filter paper resulting in a brown circle which may be MGO-modified protein adsorbed to the paper as the liquid phase was drawn away from the protein due to capillary action. This enabled the comparison of the brown colour of the protein between honeys. The brown colour variation of the honeys is shown in Figure 8.19. Note, there is only one ring and its colour and thickness is dependent on the colour of the sample of honey, as mentioned previously. Honey with high fluorescence typically had a darker colour. Honeys with high fluorescence (top) and low fluorescence (bottom) are pictured.

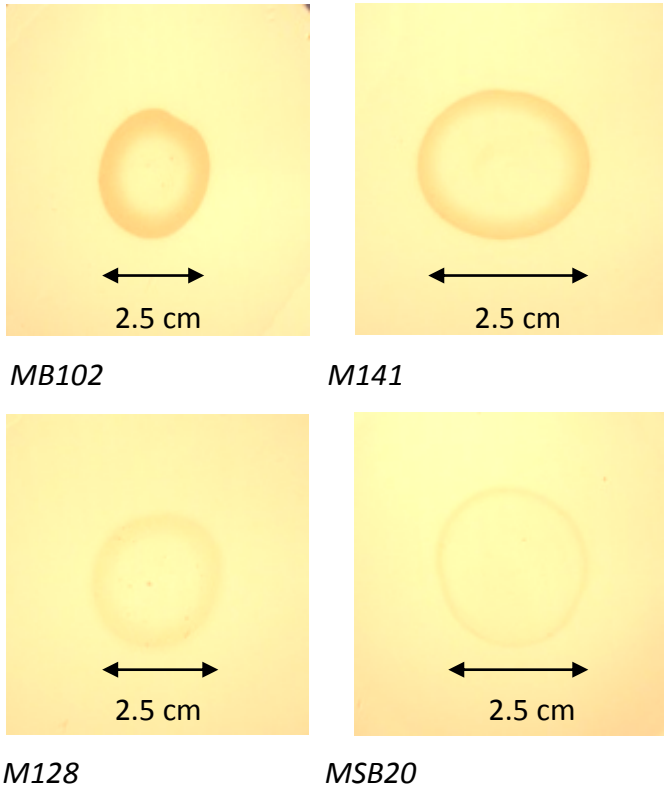

Figure 8.19. The colour of rings of honey protein on filter paper. Manuka honey with high fluorescence (top) and low fluorescence (bottom) spotted on filter paper to show the coloured protein ring. Honey with high fluorescence has darker coloured protein, as shown by the darker ring.

Images from the LAS-1000 instrument were then taken to show the variation in the fluorescence of the rings of the honey spots on TLC plates. The TLC plates enabled better separation of the honey and consequently the liquid moved past the ring of adsorbed protein, leaving a centre ring of brown colour. Two honey samples with different levels of fluorescence are shown in Figure 8.20. There appeared to be two types of fluorescence. The centre ring from honey with high fluorescence had a much more intense fluorescence than the ring from the honey with low fluorescence. Surrounding the fluorescent ring was a halo-like fluorescence that was not present in honey with low fluorescence. This halo-like fluorescence can be seen in Figure 8.20 as grey shading either side of the inner ring. The fluorescence of Clover honey incubated with or without MGO is shown in Figure 8.21. Incubation of Clover honey with MGO gave rise to increased fluorescence in the centre ring. The background fluorescence was present but not as much as in Manuka honey with high fluorescence. Incubation of Clover honey without MGO gave a small increase in the fluorescence of the centre ring but not to the same intensity as when incubated with MGO. The centre rings in Clover honey are in the same position as the fluorescent rings in the Manuka honey.

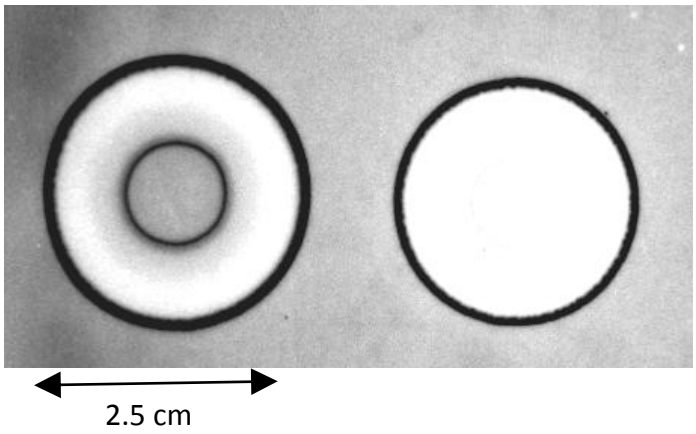

Figure 8.20. Image from the LAS-1000 instrument of two Manuka honeys with high fluorescence (M05 left) and low fluorescence (M32 right) on TLC plates. The outer ring of fluorescence may be the surfactant that runs at the front of the liquid moving out from the point of application.

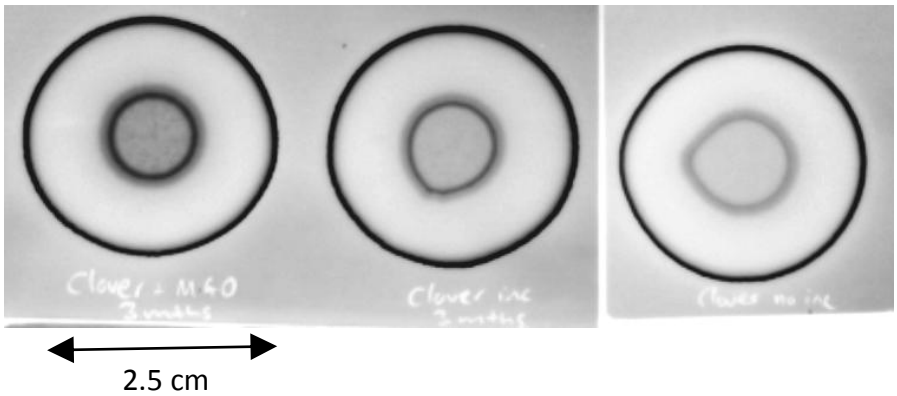

Figure 8.21. Image from the LAS-1000 instrument of incubated Clover honey with 400 mg/kg MGO (left), without MGO (center), or non-incubated clover honey (right) on TLC plates. The outer ring of fluorescence may be the surfactant that runs at the front of the liquid moving out from the point of application.

8.4.9 Fluorescence emission spectrum of BSA and Manuka honey.

Figure 8.22 and 8.23 show the emission spectrum obtained for Manuka honey (10%) and MGO-modified BSA, respectively, using a confocal microscope system to measure the fluorescence wavelength of the fluorphores present in the samples. The analysis was three or four times illustrated by the multiple lines on the emission spectrum. Both 10% Manuka honey and MGO-modified BSA had a maximum emission wavelength of 480-500 nm, and both had emission ≥ half-maximal over the range 445-555 nm.

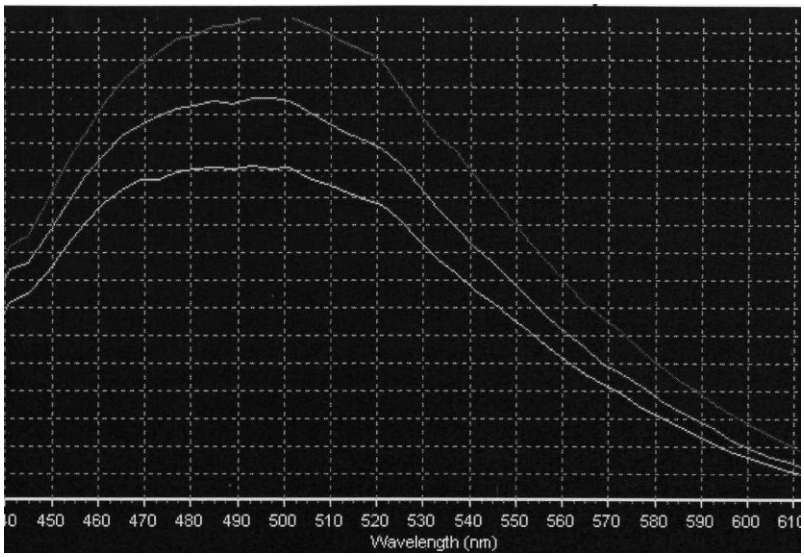

Figure 8.22. Fluorescence emission spectrum of 10% Manuka honey (M144) with naturally high fluorescence. The multiple lines are from three repeats.

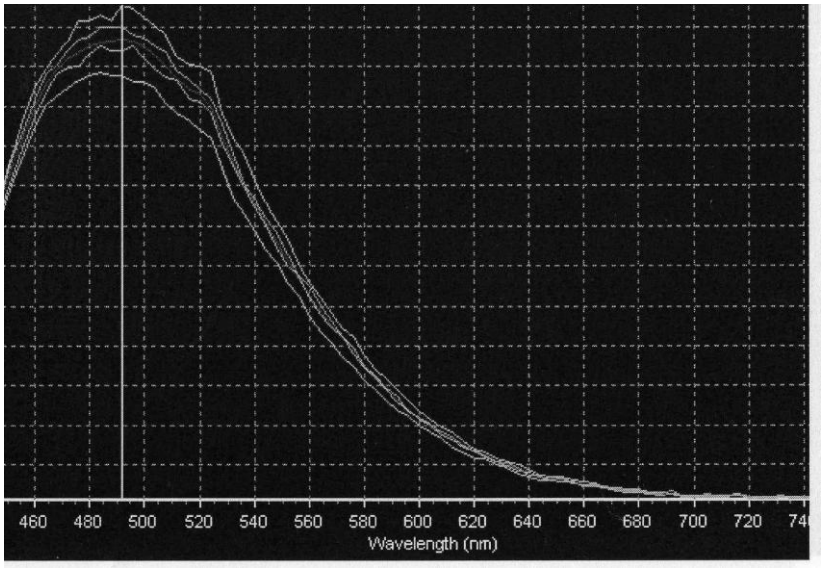

Figure 8.23. Fluorescence emission spectrum of BSA modified by incubating it at 10 mg/ml with MGO 400 mg/kg MGO for two days. The multiple lines are from four repeats.

8.5 Discussion

It was found that Manuka honey is strongly fluorescent whereas non-Manuka honey had little fluorescence. While this fluorescence is not significantly dependent on MGO levels, honeys with no MGO had low fluorescence indicating that the MGO may be responsible for the fluorescence of honey. The fluorescence of honey and the anti-inflammatory activity of honey, however, was highly significant ($p<0.0001$). The majority of the fluorescence is produced from the protein in honey. This is concluded from observation of honey fractions of high molecular weight having fluorescence, seen both in the fractions themselves after chromatography and from electrophoresis gels where the fractionated honey had a fluorescence that could be visualised in the non-stained gel. It was also established (Chapter 6) that it was the protein in honey that had the anti-inflammatory activity. Correlation between MGO content and fluorescence was weak, but there was very little fluorescence in non-Manuka honeys which had no MGO in them. An alternative explanation could be that there is a fluorescent phytochemical specifically from Manuka nectar not present in other types of honey. It has been reported that there are fluorescent phytochemicals in honey (Ghosh, Verma *et al.* 2005) but these are not comparable in fluorescence intensity.

A possible explanation for the intense fluorescence of Manuka honey is the formation of pentosidine. Pentosidine is a fluorescent crosslink with visible wave length fluorescence, making it easy to detect. This is one of the end-stage products of the Maillard reaction known as AGEs (Fagugli, Vanholder *et al.* 2001). Pentosidine levels can be determined by measuring the total fluorescence at a wavelength characteristic for these substances: Excitation 335 nm/Emission 385 nm. The fluorescence emission spectrum of Manuka honey had a peak emission of 480 nm - 500 nm. The fluorescence of Manuka honey must be due primarily to something other than pentosidine-crosslinks in the proteins as this emission range is too high. The intensity of Manuka honey's fluorescence ranged, in this study, up to 227 000 AU, whereas other floral types ranged up to 89 000 AU It is

not known, however, if these other floral varieties had honey with Manuka nectar in them, which is very likely. However, the fluorescence emission wavelength of Manuka honey was the same as MGO-modified BSA. Upon incubation with MGO, BSA became highly fluorescent in as little as one day. This reaction may be taking place in Manuka honey with naturally high levels of MGO.

It has been reported that the fluorescence attributed to MGO-incubated amino acids, has a maximum emission at 520–530 nm and this emission was only present for MGO incubated with arginine and not with lysine (Schmitt *et al.* 2005). Adams *et al*. (2009) found that adding arginine but not lysine to honey reduced the MGO content suggesting that MGO was modifying only the arginine. Therefore the fluorescence observed in Manuka honey is most likely derived from arginine modifications.

As mentioned previously, one of the properties of proteins that have been modified by MGO is browning. The colour of honey varied greatly in the sample used in this study. This colour variation could be seen by separating diluted honey on filter paper. The mobile phase tended to be yellow in colour, leaving a ring of colour which absorbed to the paper at the point of application of the sample. It was concluded that this colour was due to the protein in honey, or at least modified protein, which varied depending on the honey type. Earlier work in Chapter 6 attempted to remove low molecular weight components from fractionated honey using a spin column. This was not successful as the protein in the sample stuck to the ultra filter membrane and was difficult to remove. This protein was dark brown in colour as were the fractions that corresponded with the large peaks on the chromatography trace, indicating there was the most protein in those fractions. By assessing the colour of protein using the TLC plates and filter paper, it was concluded that the majority of brown colour in Manuka honey comes from the protein component. When assessed for fluorescence, these rings on the TLC plates had high fluorescence in Manuka honey but very little or none in other floral varieties of honey. When MGO-modified BSA was visualised on filter paper, it left a yellow accumulation of what could only be

protein (as there was only protein in the sample) after the mobile phase moved out past the point of application. This yellow accumulation was highly fluorescent. It is not known if the non-modified protein adsorbed at this point also as it was not visible.

No strong correlation could be found with the colour of honey and the MGO content or fluorescence, however. The lack of correlation between colour and fluorescence could be because colour could develop from other Maillard reactions which don't give the fluorescent structure. It is postulated that as the MGO modifies the protein in honey over time, the MGO content is therefore reduced. Thus, old, dark Manuka honeys would have a high fluorescence and a low MGO content. Indeed this was true for the older honeys, one in particular (MB102) which had the highest fluorescence found but had no measurable non-peroxide anti-bacterial activity (and therefore no MGO). This honey was almost black and had the darkest colour score of 12. This honey also had the highest anti-inflammatory activity measured by the phagocytosis assay.

The fraction with the most anti-inflammatory activity obtained when Manuka honey was fractioned by FPLC (Chapter 6) was determined to have fluorescence. This fraction contained the MRJP-1 and MRJP-3 (determined in Chapter 6). These proteins show that the anti-inflammatory activity of Manuka honey in the phagocytosis assay was due to fluorescent MRJP. The fluorescent form of these proteins were not found in other honey types in such high quantities, and the quantity of the fluorescent protein varied according to MGO content, storage conditions and possibly age. The age was not known for most of the honey samples taken from storage, but it was observed that the oldest honeys (over 10 years old) were much darker (colour score of 10-12) than honey that came in from the current season which received a colour score of 4. Protein concentration is fairly uniform across all honey varieties though darker honeys have slightly more (White and Landis 1980; Azeredo, Azeredo *et al.* 2003) and so protein concentration alone could not account for the variation in fluorescence or colour due to the protein.

Not all Manuka honey with high MGO levels had high anti-inflammatory activity. The ones with low activity were young honeys which may develop anti-inflammatory activity over time, during which the MGO modification process may occur. Antioxidant content may come into play here also. Honey with high antioxidant levels may retard the MGO modification process preventing or reducing fluorescence development. The antioxidants act as AGE inhibitors, presumably through metal-ion chelation and sequestration of free-radical species (Prakash Reddy and Beyaz, 2006). As MGO modification of proteins is inhibited by antioxidants it is hypothesised that Manuka honeys with high antioxidant levels and MGO levels will develop low fluorescence and therefore low anti-inflammatory activity, which explains some variation in the results for Manuka honey with high MGO levels. The variation in antioxidant levels in Manuka honey may account for why some develop high anti-inflammatory activity and others do not. Time limits prevented measurement of the anti-oxidant in honey but this work should be done in the future.

Clover honey with very low fluorescence could be made to develop fluorescence by incubating with MGO at levels similar to those found naturally in a Manuka honey with a NPA rating of 15. The anti-inflammatory activity, determined by the assay of inhibition of phagocytosis, could also be made to increase upon incubation of Clover honey with added MGO and Manuka honey containing natural MGO, the fluorescence and molecular weight of the protein also increasing (5 kDa) as a result of this incubation. The difference in size between the major protein (approximately 58 kDa and 63 kDa respectively) of both Clover and Manuka honey (Figure 8.11) is possibly due to the increased glycation of the Manuka honey proteins as the size difference is not high enough to be cross-linking. However, there is proportionately more diffuse protein >110 kDa in Manuka honey that could be due to cross-linking. This was especially noticeable in the honeys incubated with MGO (Figure 8.12) were there is almost no trace of protein >75 kDa in Clover honey and a large amount of diffuse protein in the Manuka sample. Also of interest is the lack of protein with low molecular weight

(<37 kDa) in the Manuka honey sample, though there are clear bands in the Pasture honey sample at 15 kDa and 25 kDa. These proteins may be cross-linked or highly glycosylated and found in the >100 kDa molecular weight range for the Manuka honey sample.

Colour varied widely between honey varieties but more interestingly between Manuka honeys. A selection of Manuka honeys from around New Zealand was obtained from the same harvest season (Codes M159-M209). All of these samples had a similar colour but ranged in the fluorescence reading from low to high. Fluorescence may start to develop in the hive which is kept at a fairly constant temperature of about 35°C. Older honeys showed great colour variation. Three honeys were obtained that were at least 10 years old and had not been refrigerated. These honeys had the darkest colour grade and also the brightest fluorescence. The causes of darkening in honey have been attributed to Maillard reaction, fructose caramelization and oxidation reactions of polyphenols (Gonzales, Burin *et al.* 1999), with the majority of these reactions occurring at relatively high ambient temperatures (37°C).

An assay is available to measure the extent of heating that honey has undergone. HMF (Hydroxy-methyl furfuraldehyde), formed in honey by the action of acidity on fructose, increases with heating and is used as an indicator for storage conditions. This assay provides useful information as high temperatures during storage can degrade proteins, most importantly to this study, MRJPs (Kamakura, Fukuda *et al.* 2001). Kamakura *et al.* found that in royal jelly, the MRJPs degraded at room temperature over the period of one month as a result of acceleration of the Maillard reaction. It is likely that the same degradation of MRJPs occurs in honey which is stored at room temperature or above but more slowly. Fresh natural honey can have varying levels of HMF. Normally this is below 1 mg/kg but levels soon start to rise with ambient temperatures above 20°C (Castro-Vazquez, Daaz-Maroto *et al.* 2008; LeBlanc, Eggleston *et al.* 2009). As mentioned previously, as storage temperature increases, honey darkens because of the Maillard reaction and MGO levels increase. HMF levels were

known for some of the honey samples in the current research and these can be found in Appendix 3. Generally, the honeys assayed during this research had very low levels suggesting very little exposure to heat has occurred.

One of the most obvious factors that may explain the lack of correlation of anti-inflammatory activity or fluorescence with MGO, is the fact that all honey can contain nectar from a variety of floral sources. Bees tend to take the most productive option for them when collecting nectar and cannot be easily directed to a particular source. Apiarists may label their honey for what they believe is its floral source. Manuka honey is of high value because of its health benefits so it is likely that much of the honey labelled Manuka actually contains very little Manuka nectar. One of the major issues with Manuka honey is the similarity it has with Kanuka. Both grow in the same location and often flower at the same time. Kanuka does not contain dihydroxyacetone so MGO does not form in Kanuka honey (Adams, Manley-Harris *et al.* 2009).

To date, there is no definitive way to distinguish the two varieties of honey. Most honey varieties can be identified by the morphologies of the pollen they contain, but Manuka and Kanuka have pollen identical in appearance. The Kanuka honey in this study as well as the Rewarewa had more fluorescence and anti-inflammatory activity than the other floral types of honey (excluding Manuka). As mentioned previously these honeys may contain higher amounts of Manuka nectar than the other floral types due to them often being in close proximity to Manuka shrub and flowering at the same time. Until a reliable test is available, much of the industry can opt to avoid accurate labelling. It is suggested that future research is directed to developing a test to distinguish Manuka and Kanuka honey. The research presented here, strongly suggests that a fluorescence based assay may accurately predict honeys with high anti-inflammatory activity, and therefore most likely to be Manuka honey.

Further research is needed to determine if MGO formation takes time and whether or not the MGO-modification of proteins depends mainly on time or on

other factors as well. This may explain the variation in fluorescence for the honeys. It is also likely that as protein modification takes place the pool of MGO is reduced as it is an irreversible reaction and the rate of formation of MGO decreases with time (Adams *et al.* 2009). Possibly old honeys with high fluorescence will have low MGO, whereas the level of MGO may have been higher previously.

Chapter 9

Anti-inflammatory effect of honey in the hen's egg chorioallantoic membrane test

9.1 Summary

The anti-inflammatory action of Manuka honey seen in the phagocytosis assay was compared with that seen in a commonly used *in vivo* assay of anti-inflammatory activity. Previous work identified that Manuka honey with high fluorescence had the most inhibitory phagocytosis activity resulting in an anti-inflammatory action by reducing inflammatory cytokine expression and ROS production in monocytes and macrophages. Also it was found that Manuka honey had higher activity than other honeys in these assays. It was therefore of interest to compare the effects of Manuka honey with high and low fluorescence, and a Pasture honey, in an assay that shows anti-inflammatory activity *in vivo.* In the present chapter, the anti-inflammatory properties of Manuka honey were assessed against two irritants that cause inflammation, sodium dodecyl sulphate (SDS) and LPS, in a commonly used *in vivo* assay of anti-inflammatory activity, the hen's egg chorioallantoic membrane test (HET-CAM).

In the SDS assays, it was concluded that highly fluorescent Manuka honey at a concentration of 5% had an anti-inflammatory effect equivalent to that of 5 mg/ml hydrocortisone, observed by a complete lack of the typical inflammatory

response such as granulomas and a star-like pattern of vasculation observed with SDS alone. Manuka honey with low fluorescence had much less of an inhibitory effect. Artificial honey and Pasture honey had little or no anti-inflammatory action. Use of LPS to cause inflammation in this assay has not been documented in the literature but was used in place of SDS for further assays as LPS was used as the irritant in previous cell work where Manuka honey had an anti-inflammatory effect. Resulting inflammation from LPS stimulation was swelling of the tissue accompanied by redness, due to influx of blood to the tissue. Manuka honey and hydrocortisone both decreased these signs of inflammation, with highly fluorescent Manuka honey having a greater effect than Manuka honey with low fluorescence, and Pasture and artificial honey showed little or no anti-inflammatory effect. The inflammatory response to the two irritants was different suggesting that Manuka honey may have two modes of action in its anti-inflammatory effect.

9.2 Introduction

The HET-CAM assay uses the chorioallantoic membrane, which is a vascular foetal membrane, composed of the fused chorion and allantois surrounding a developing chick embryo. It is a complete tissue lacking sensory innervations that responds to injury with classical inflammatory reactions without the sense of pain (Leighton, Nassauer *et al.* 1985). The HET-CAM assay was chosen as it is a commonly used method for testing substances for anti-inflammatory activity *in vivo* and it is readily accessible for experimentation. The chorioallantoic membrane has been proposed as a model for a living membrane with good correlation between results obtained by the HET-CAM test and those of other widely accepted irritant/inflammatory models such as the Draize rabbit eye test and croton oil ear test which use adult animals (D'Arcy and Howard 1967;

Leighton, Nassauer *et al.* 1985; Brantner, Quehenberger *et al.* 2002; Tavaszi and Budai 2007).

Topical application of various substances onto the chorioallantoic membrane results in local injury and reactions that differ in severity from one substance to another. For any single substance, tested as a sequence of dilutions on a series of membranes, the chorioallantoic membrane responds with decreasing intensity that parallels the decreasing dosage (Leighton, Nassauer *et al.* 1985). It is assumed that acute effects induced by a test substance on the small blood vessels and proteins of the membrane are similar to effects induced by the same test substance on the eye of a treated rabbit (Leighton, Nassauer *et al.* 1985) or the ear of a mouse (Brantner, Quehenberger *et al.* 2002). These methods are commonly used to assay anti-inflammatory agents but are not ideal as they use live adult animals, increasing costs and requiring ethics approval. Research on chorioallantoic membranes has concluded that HET-CAM is acceptable as the results are comparable with studies using adult animals, and as the assay uses an embryo at less than half of its gestation meaning ethics approval is not required.

Inflammation and angiogenesis are co-dependent, with many of the mechanisms involved in inflammation also involved with angiogenesis (Ribatti, Vacca *et al.* 1996; Jackson, Seed *et al.* 1997). The chorioallantoic membrane may be evaluated for the development of irritant or inflammatory end points such as hyperaemia, hemorrhage, formation of granulomas and coagulation and angiogenesis. Relevant to the research presented in this thesis, is the finding that pro-inflammatory macrophages participate in the formation of granulomas, which reflects chronic inflammation (Levine, Smith *et al.* 2005). In Chapter 4 it was found that honey had an inhibitory effect on phagocytosis in THP-1 macrophages, resulting in an anti-inflammatory effect *in vitro.* Therefore it is relevant to assay honey for its anti-inflammatory effect *in vivo,* focusing on the formation of granulomas.

The HET-CAM assay depends on the experimental production of inflammation on the chorioallantoic membrane and the reduction of this inflammation with an anti-inflammatory substance. If an irritant is applied in conjunction with an anti-inflammatory substance, complete normalisation of the membrane irritation is observed (Bürgermeister, Paper *et al.* 2002). SDS is commonly used as the irritant in the HET-CAM assay (Marchesan, Paper *et al.* 1998; Bürgermeister, Paper *et al.* 2002; Dietrich, Karall *et al.* 2005). SDS is an anionic surfactant that causes disruption of cell membranes, which causes an inflammatory effect. Test substances can be applied in a diluted liquid form directly to the chorioallantoic membrane or dissolved on a filter disc and placed on the chorioallantoic membrane (Leighton, Nassauer *et al.* 1985; Marchesan, Paper *et al.* 1998; Chakraborty and Brantner 2001). The assay was modified by Dietrich *et al.* (2005) to measure the anti-inflammatory properties of test substances dissolved in agarose pellets. Hydrocortisone, a commonly prescribed anti-inflammatory drug used for the treatment of allergic rashes and other inflammatory skin conditions, is commonly used as an anti-inflammatory control compound in the HET-CAM assay (Marchesan, Paper *et al.* 1998; Bürgermeister, Paper *et al.* 2002; Dietrich, Karall *et al.* 2005). The egg is then incubated for 24 hours to allow the inflammation to develop and the chorioallantoic membrane is then harvested from the egg and observed under magnification for the appearance of membrane irritation, typically in the form of a granuloma and a star-like blood vessel pattern surrounding the granuloma.

Ethics approval was not required for this experiment as the embryo used is at less than half its gestation period (The Animal Welfare Act 1999, Section 6, 2.1).

9.3 Materials and Methods

9.3.1 Materials

9.3.1.1 Agarose bead

A 2.5% solution of agarose (Ultra Pure™ Agarose, Invitrogen) was made in PBS and sterilized by boiling for 5 minutes. When cooled, but still above its setting temperature, test substances were added and pellets formed by placing 10 µl amounts on parafilm to gel. The pellets were 3 – 4 mm in diameter. They were made freshly before each placement on a chorioallantoic membrane.

9.3.1.2 Sodium dodecyl sulphate (SDS) concentrations

The SDS concentration used was taken from current literature (Marchesan, Paper *et al.* 1998; Dietrich, Karall *et al.* 2005). SDS was purchased from Roche and diluted in purified water to a working stock concentration of 0.1 g/ml. A final concentration of 5 mg/ml (50 µg/agarose pellet) was achieved by diluting the stock SDS solution with agarose. Results from preliminary assays at this concentration concurred with the published results.

9.3.1.3 Lipopolysaccharide

LPS from *E.coli* (*E. coli* serotype 0127:B8; Cat No.# L4516-1 mg) was purchased from Sigma-Aldrich, UK. The LPS was dissolved in sterilised purified water to a working concentration of 1 mg/ml and used at a final concentration in agarose of 50 µg/ml (500 ng per agarose pellet), based on preliminary testing on chorioallantoic membranes. At this concentration a noticeable irritation occurred yet the embryo remained viable.

9.3.1.4 Hydrocortisone

The hydrocortisone concentration used was taken from current literature (Marchesan, Paper *et al.* 1998; Dietrich, Karall *et al.* 2005). Hydrocortisone was purchased from Sigma-Aldrich (Cat No.# HO888-1 G) and diluted to 500 mg/ml in ethanol for a stock solution. The stock solution was diluted to a final concentration of 5 mg/ml in agarose (50 µg per agarose pellet). Results from preliminary assays at this concentration concurred with the published results.

9.3.1.5. Honey concentrations

Honey was obtained from the Honey Research Unit, University of Waikato, NZ, and was tested for its non-peroxide antibacterial activity (NPA) (Allen, Molan *et al.* 1991). Two Manuka honeys were selected based on their fluorescence, phagocytosis-inhibiting activity and their NPA. Two Manuka honeys with similar high NPA ratings were chosen for the study, one with high fluorescence and the other with low fluorescence (as determined in Chapter 8). Selecting honeys with similar high NPA ensures that each honey was a mono-floral Manuka honey, not a blend with other floral types. These were selected from the honey survey presented in Appendix 3, and detailed in Table 9.1.

The required concentrations of Manuka and artificial honey were achieved by dilution of the honey with sterile purified water as described in Section 3.4.1.1.

Artificial honey was used to provide a control for the osmotic effect of the natural sugars found in honey. It was prepared using a published recipe (White 1975). Preliminary assays with diluted honey indicated that a 5% concentration of Manuka honey would be sufficient in neutralising the effect of SDS on a chorioallantoic membrane. Consequently concentrations of 2% and 5% honey were used for all the honeys tested.

9.3.2 HET-CAM Methods

9.3.2.1 Egg preparation for HET-CAM

Fresh fertilised eggs were collected on the day of laying from a poultry farm (Alstra Poultry Lodge, Ngaruawahia). Preferred eggs were white, 55 g and unsoiled. Prior to incubation eggs were candled to check for cracks or fractures in the shell, and any damaged eggs were discarded. One day old eggs were preferred for the assay. Eggs not used on the day of collecting were stored at 18°C for no more than two days. Eggs were incubated in a horizontal position for 72 hours at 37°C and 80% relative humidity. The eggs were rotated in an automated incubator several times a day to stop the developing membrane from attaching to the shell.

After 72 hours of incubation the eggs were candled to observe embryo development. Eggs that had no embryo were discarded. Eggs were then swabbed with 70% ethanol at both ends. A small patch (5 mm) of shell was ground away using a rotary tool (a cordless Dremal hobby tool was used) with a sterile cutting/grinding disk, on the pointed end of the egg leaving the outer membrane intact. The egg was then held pointed side down. A hypodermic syringe with a 19 gauge needle was inserted through the hole ground in the shell, piercing the outer membrane and 10 ml of albumin was aspirated out slowly, ensuring the yolk was not disturbed. The egg was then inverted and the hole was sealed with molten candle wax. The egg was then placed pointed end down in an egg tray. A 3 cm diameter circle was then ground into the egg shell using the Dremal hobby tool from the top of the egg leaving the outer membrane untouched. The outer shell membrane was then cut using a sterile scalpel blade, removing the shell and attached shell membrane with tweezers, exposing the chorioallantoic membrane on the yolk. The prior removal of the albumin causes the chorioallantoic membrane to sit low in the egg leaving approximately a 4 cm diameter chorioallantoic membrane to do testing on. The yolk floats in the albumin, orientating the embryo and chorioallantoic membrane

to the top of the egg enabling good viewing. The embryo was checked for viability by noting the presence of a beating heart, and for haemorrhaging, which would indicate imminent death. The egg was covered with parafilm and set back in the incubator for a further 72 hours. The eggs were left stationary during this period. Figures 9.1 to 9.5 show the process of preparing the egg for the HET-CAM work.

Figure 9.1. Removing a small patch of shell using a rotary hobby tool. The outer membrane still intact.

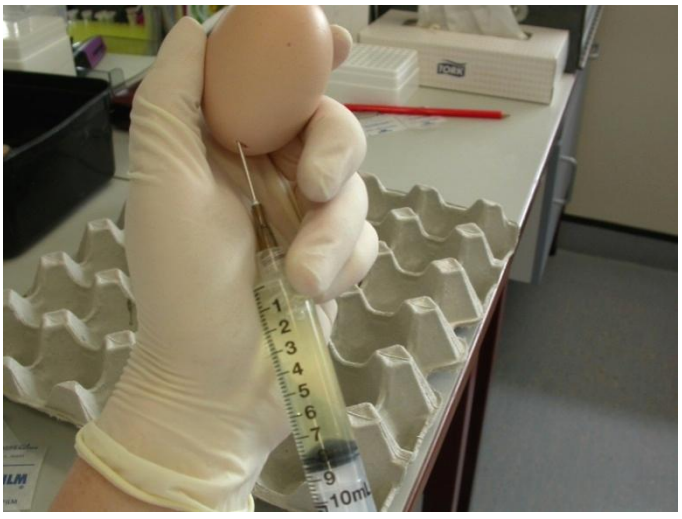

Figure 9.2. Aspirating 10 ml albumin through the exposed outer shell membrane.

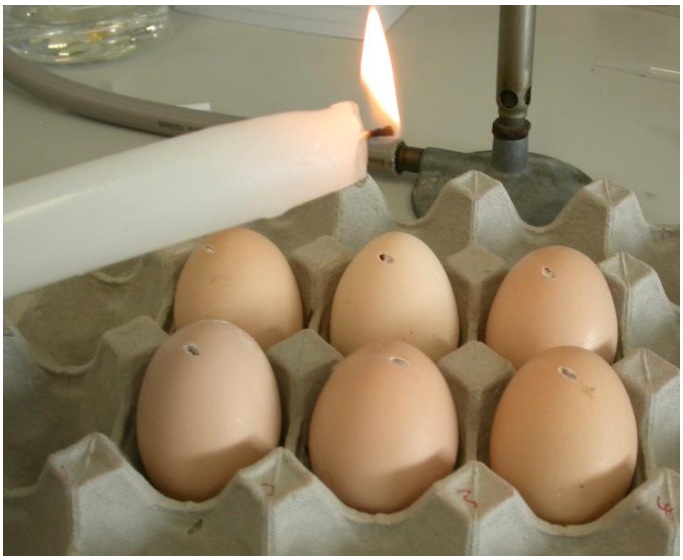

Figure 9.3. Sealing the egg using molten candle wax after aspirating albumin.

Figure 9.4. Preparing the egg for removal of the shell to expose the chorioallantoic membrane. The shell is cut through without cutting the outer shell membrane.

Figure 9.5. The exposed chorioallantoic membrane covered with parafilm.

9.3.2.2 HET-CAM assay using SDS as an irritant

SDS in an agarose pellet was used to cause inflammation on chorioallantoic membranes. As a positive control for detecting anti-inflammatory activity, the SDS was combined with hydrocortisone. The negative controls included agarose-only pellets, hydrocortisone-only, and honey-only pellets at the same concentrations used in the assay.

9.3.2.3 Modified HET-CAM assay using LPS as an irritant.

In the experiments in previous Chapters, LPS was used to cause an inflammatory response in THP-1 cells in cell culture. It was therefore of interest to use a similarly induced inflammatory response with the HET-CAM assay. LPS use in this assay has not been documented in the literature. A range of concentrations of LPS was used in control chorioallantoic membranes to find a suitable concentration to create inflammation. A final concentration of 50 µg/ml in the agarose pellet was selected for further assays. Conditions of the HET-CAM assay remained the same as those for the SDS treatments.

The chorioallantoic membranes responded differently to the LPS compared with when SDS was used. No granuloma was identified in any of the chorioallantoic membranes, and the capillary net remained virtually unchanged. The most obvious reaction to LPS was an intense redness due to the influx of blood in to the tissue surrounding the agarose pellet. Honeys were used at the same concentrations as previously mentioned.

9.3.3 Agarose pellet compound combinations

Agarose pellets used in the HET-CAM assays contained the following combinations;

Test: Honey and SDS, honey and LPS

Positive controls: SDS only, SDS and hydrocortisone, LPS only, LPS and hydrocortisone.

Negative controls: Hydrocortisone only, honey only and nothing added to the agarose.

The details of the honey used in the section are given in Table 9.1.

Table 9.1: Details of the honeys used in the HET-CAM assays.

Code	Type	NPA	Fluorescence. (AU)	Anti-inflam. property
M144	Manuka	12	184	High
M128	Manuka	11.8	20	Low
PS9	Pasture	0	35	Low
Artificial	Artificial	0	0	Low

Abbreviations: NPA = non-peroxide antibacterial activity; AU = Arbitrary units; Inflam = inflammation.

9.3.4 Agarose pellet placement

Pellets were placed on each membrane in duplicate (two per egg) away from any major blood vessels and 1 cm from the shell. This allowed the pellet to be found easily and prevented the pellet from being dislodged by the active embryo. The test substances were each placed on at least four eggs, thus giving a total of eight tests for each of the honey concentrations. At least three eggs were used for each control, each having the control in duplicate. In some experiments, the embryo died during the incubation and these eggs were not included in the final count but the death was noted. Death of the embryo was not specific to any one treatment and was minimal over the entire test period. The length of the assay was 7 days from start of incubation to harvesting of the chorioallantoic membrane.

9.3.5 Harvesting the chorioallantoic membrane

After 24 hours of incubation with the test pellet, the chorioallantoic membrane was harvested from the egg with sterile scissors and tweezers. The agarose pellet was removed from the membrane with tweezers. The membrane was then placed in chilled phosphate-buffered saline and viewed under a stereo

microscope. After observations, a digital picture was taken and the membrane was placed into 100% ethanol to preserve.

9.3.6 Evaluation of results obtained

The degree of irritation of the membrane was observed and graded using a scale of 0 – 2 with increments of 0.5 for SDS and a scale of 0 – 3 with increments of 1 for the LPS assays. These scores were then used to calculate a proportional inhibition of inflammation. Table 9.2 and 9.3 details the score systems used for SDS and LPS respectively. A positive effect, corresponding to anti-inflammatory activity, exists if the irritation of the membrane by SDS or LPS is reduced or appears normal and the blood-vessel net appears normal.

Table 9.2: Score used for the evaluation of the anti-inflammatory effect of test substances in SDS-irritated chorioallantoic membranes.

Score		Effects Observed
0	no effect/strongly irritated	Large granuloma, strongly vascularised, large capillary net surrounding granuloma.
0.5	weak effect/weakly irritated	Large granuloma, reduced capillary net surrounding granuloma.
1.0	weak-medium effect/weakly normalised	Granuloma smaller than score 0 or 0.5, significantly reduced capillary net.
1.5	medium-strong effect/almost normalised	No granuloma, slight increase in capillary growth at site of agarose bead.
2.0	strong effect/completely normalised	No granuloma or obvious increase in capillary growth at site of agarose bead.

Table 9.3: Score values for the evaluation of the anti-inflammatory effect of test substances in LPS-irritated chorioallantoic membranes in the HET-CAM assay.

Score		Effects observed
0	no effect	Membrane extremely red surrounding area of pellet
1	weak effect	Membrane red surrounding pellet but less than in score 0
2	medium effect	Membrane has slight redness visible but less than in score 0 & 1
3	Strong effect	Membrane normalised, no redness

All results are expressed as mean values ± 1 SD. Corresponding anti-inflammatory effects were based on the degree of inhibition as in the publication of Bürgermeister, *et al.* 2002:

Inhibition	**Effect**
<40%:	no anti-inflammatory effect
40-55%:	uncertain anti-inflammatory effect
55-70%:	weak anti-inflammatory effect
70-85%:	good anti-inflammatory effect
>85%:	strong anti-inflammatory effect

9.4 Results

Membranes from the HET-CAM assay where observed under a stereo microscope and scored. Not all membranes were successfully photographed due to breakage however, all viable membranes were scored. The images of some membranes are presented in Section 9.4.1 and the resulting scores are presented in Section 9.4.2.

9.4.1 Observations of inflammation

Figure 9.6 shows a normal membrane (control) after agarose bead placement with no LPS, SDS, hydrocortisone or honey. The perimeter of the pellet is obvious as it leaves a slight indentation in the membrane were it was placed (Score 2).

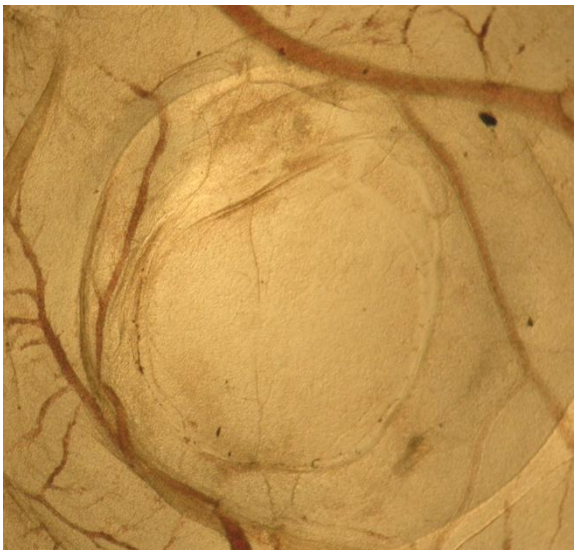

Figure 9.6: Agarose control pellet. No SDS, LPS, hydrocortisone or honey added. The perimeter of the pellet is obvious as it leaves a slight indentation in the membrane were it was placed. Score 2.

Figure 9.7 shows the typical membrane irritation/inflammation by 50 µg SDS (Score 0). In this inflammatory state there is an obvious formation of a granuloma and the number of blood vessels directed to and from the granuloma is high resulting in a star-like formation around the granuloma due to increased angiogenesis (Marchesan, Paper *et al.* 1998).

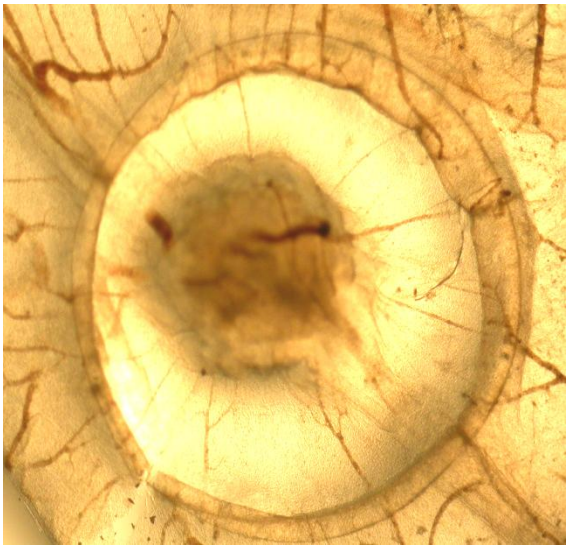

Figure 9.7: Irritation of the membrane caused by SDS (50 µg/pellet). Note capillary growth is directed to and from the granuloma in centre. Score 0.

Figure 9.8 shows the typical membrane irritation/inflammation by 500 ng/pellet of LPS (Score 0). The capillary net growth remains normal and no granuloma is formed, however, capillaries appear dilated.

Figure 9.9 shows the reduction of irritation/inflammation by SDS resulting from the anti-inflammatory effect of 50 µg hydrocortisone. This was a complete reduction of inflammation (Score 2). The perimeter of the pellet is obvious as it leaves a slight indentation in the membrane were it was placed.

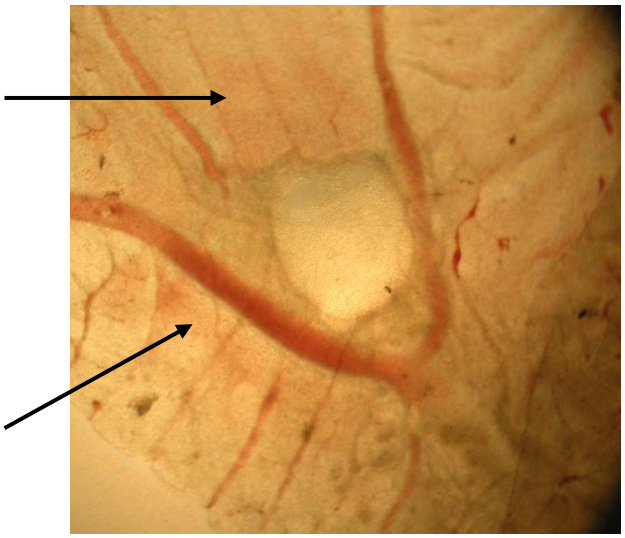

Figure 9.8: The redness/inflammation resulted from 500 ng/pellet LPS agarose pellet (arrows mark the redness). Score 0.

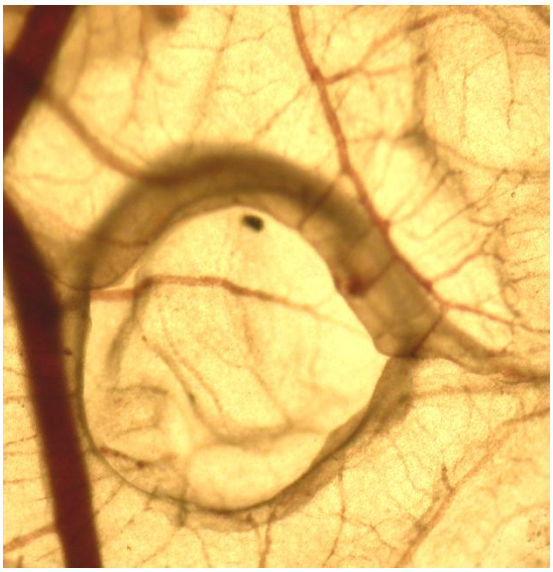

Figure 9.9: Inhibition by hydrocortisone (50 µg/pellet) of membrane irritation by SDS. Score 2.

Figure 9.10 shows the reduction of irritation/inflammation by LPS resulting from the anti-inflammatory effect of 50 µg hydrocortisone. This was a complete reduction of inflammation (Score 3). The perimeter of the pellet is obvious as it leaves a slight indentation in the membrane were it was placed.

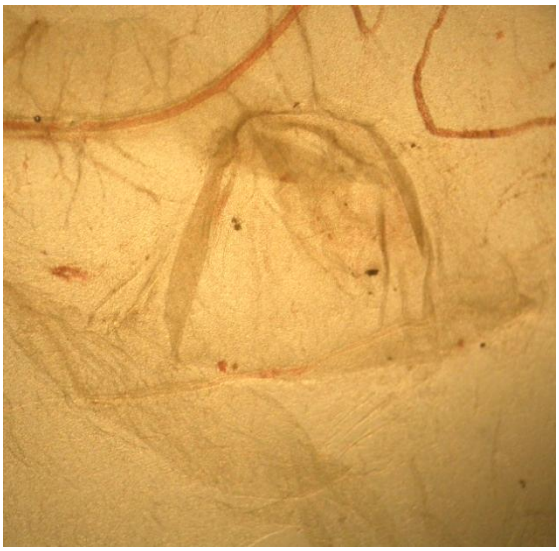

Figure 9.10: Inhibition by 50 µg/pellet hydrocortisone irritation of the membrane by LPS. Score 3.

Figure 9.11 shows the reduction of irritation/inflammation by SDS resulting from the anti-inflammatory effect of 5% highly fluorescent Manuka honey. This also was a complete reduction of inflammation (Score 2).

Figure 9.12 shows the reduction of irritation/inflammation by LPS resulting from the anti-inflammatory effect of 5% highly fluorescent Manuka honey. This also was a complete reduction of inflammation (Score 3).

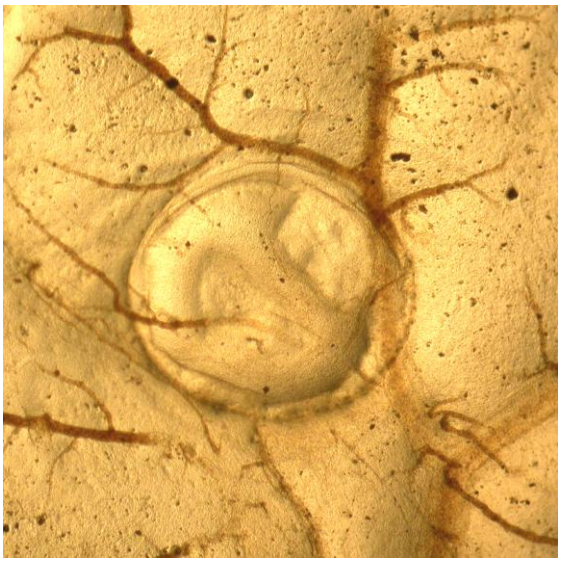

Figure 9.11: Inhibition by 5% highly fluorescent Manuka honey of irritation of the membrane by SDS. Score 2.

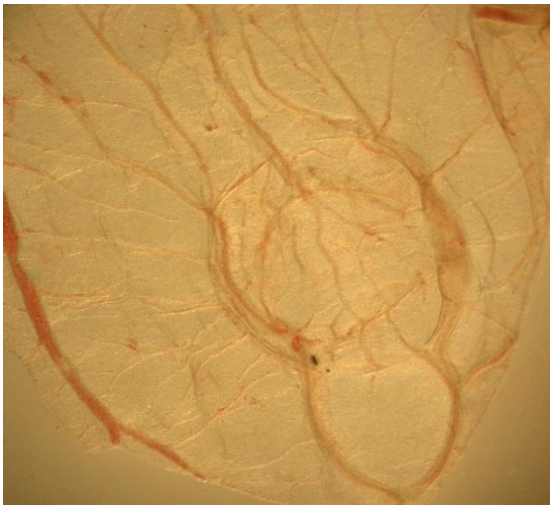

Figure 9.12: Inhibition by 5% highly fluorescent Manuka honey of irritation of the membrane by LPS. Score 3.

Figure 9.13 shows the reduction of irritation/inflammation by SDS resulting from the anti-inflammatory effect of 2% highly fluorescent Manuka honey. The granuloma size and number of blood vessels are reduced, but not completely (Score 1).

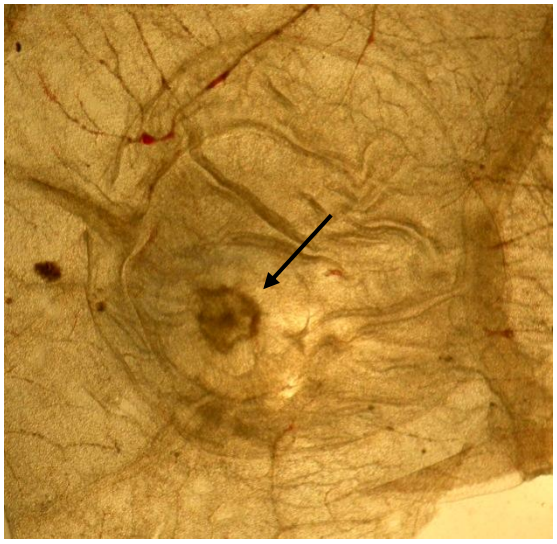

Figure 9.13: Some inhibition by 2% highly fluorescent Manuka honey of irritation of the membrane by SDS. A small granuloma is present (marked with an arrow), and there is no obvious capillary net. Score 1.

Figure 9.14 shows the reduction of irritation/inflammation by LPS resulting from the anti-inflammatory effect of 2% highly fluorescent Manuka honey. The granuloma size and number of blood vessels are reduced, but not completely (Score 1.5).

Figure 9.15 shows no reduction of irritation/inflammation by LPS resulting from the application of 5% Pasture honey (Score 0).

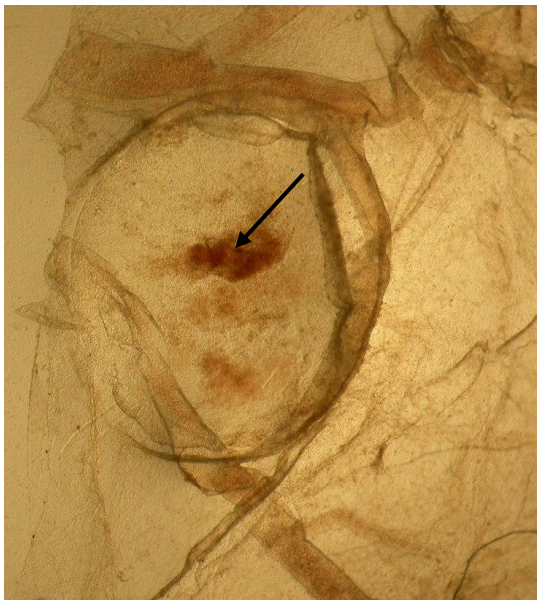

Figure 9.14: Some inhibition by 2% highly fluorescent Manuka honey of irritation of the membrane by LPS. A small granuloma is present (marked with an arrow), and there is no obvious capillary net. Score 1.5.

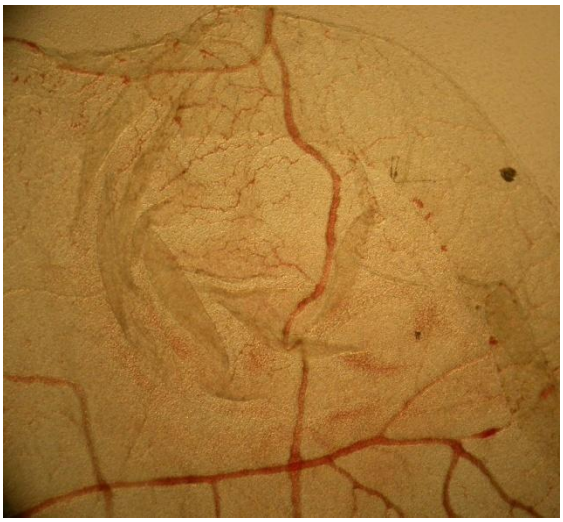

Figure 9.15: No inhibition by 5% Pasture of irritation of the membrane by LPS. Score 0.

9.4.2 Results of HET-CAM assay

The resulting scores assigned to each test membrane after harvesting from the egg are presented here. Section 9.4.2.1 has the results from SDS irritated membranes and Section 9.4.2.1 has the results from the PLS irritated membranes.

9.4.2.1 HET-CAM assay with SDS

Each membrane was assessed and given a score out of 2 (0 = no anti-inflammatory effect, membrane strongly irritated; 2 = strong anti-inflammatory effect/completely normalised membrane). From these scores a percentage reduction in inflammation could be calculated for each substance for all the membranes assayed. This percentage is a measure of the anti-inflammatory activity of the substance tested. Table 9.4 summarises the results of the SDS HET-CAM experiments and Figure 9.16 shows the reduction of inflammation, the score for anti-inflammatory activity being shown as a percentage of the maximum score of 2 which is assigned when a membrane has a completely normal appearance. All Manuka honey treatments significantly ($p<0.001$) reduced the inflammation caused by SDS with the formation of a granuloma being absent and the capillary net appearing normal. Manuka honey with high fluorescence had the greatest anti-inflammatory effect. The 5% Manuka honey with low fluorescence was comparable in effect with the 2% concentration of Manuka honey with high fluorescence. Artificial honey had no anti-inflammatory effect and Pasture honey had a slight effect but this was not significant compared with the artificial honey control.

Table 9.4: Results from HET-CAM assay. The mean anti-inflammatory activity score is shown for various substances in comparison on SDS-irritated membranes. A score of 2 indicates a normal membrane was observed at the end of the assay. A score of 0 indicates inflammation was present in the form of a granuloma and increased capillary growth.

Treatment per pellet	No. of eggs	No. of dead embryos	No. of CAMs harvested	Average score (out of 2)
SDS 50 µg alone	4	1	6	0
Hydrocortisone 50 µg no SDS (control)	3	0	6	2
Manuka honey high AU 5% no SDS (control)	3	0	6	2
Hydrocortisone 50 µg + SDS 50 µg	4	0	8	1.88
Manuka honey high AU 2% + SDS 50 µg	4	1	6	0.92
Manuka honey high AU 5% + SDS 50 µg	4	0	8	1.81
Manuka honey low AU 5% + SDS 50 µg	3	1	6	0.92
Pasture honey 5% + SDS 50 µg	3	0	6	0.16
Artificial honey 5% + SDS 50 µg	3	0	6	0
Agarose only (control)	4	0	8	2

Abbreviations; SDS=Sodium dodecyl sulphate; AU=Arbitrary units measuring fluorescence; CAM=chorioallantoic membrane.

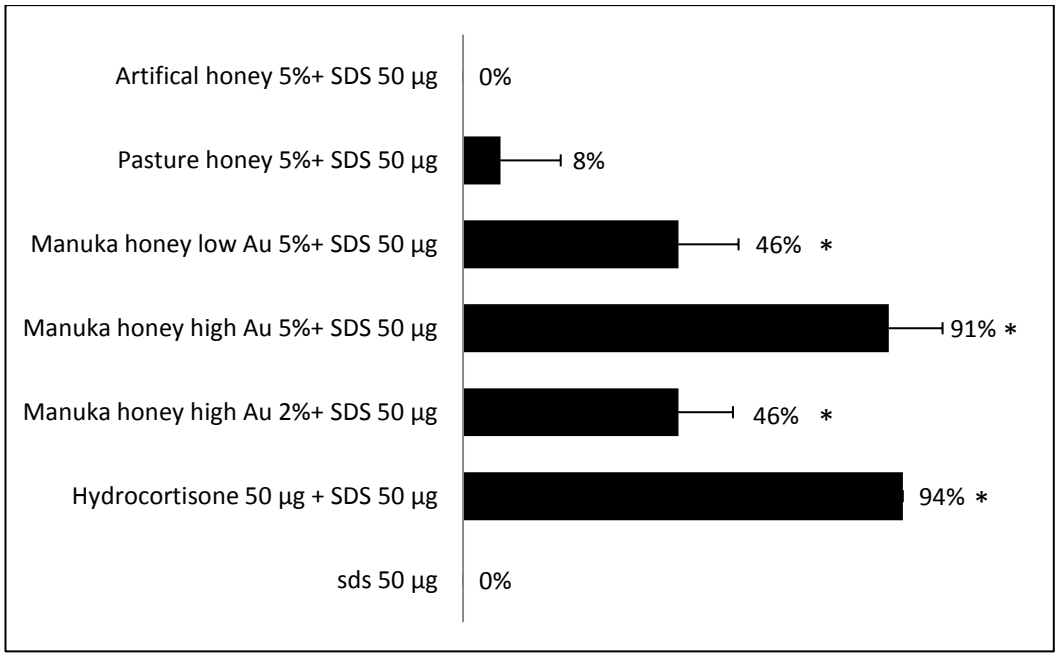

*Figure 9.16: Data from Table 9.4 for the mean anti-inflammatory activity score for SDS irritated chorioallantoic membranes, shown as a percentage of the maximum score of 2 which is assigned to a membrane showing no signs of inflammation. Error bars show ± 1 SD of the mean from at least three experiments. ** p<*0.001 analysed by ANOVA compared with the control membrane treated with artificial honey.*

9.4.2.2 HET-CAM assay with LPS

Each membrane was assessed and given a score out of 3 (0 = no anti-inflammatory effect, membrane strongly irritated; 3 = strong anti-inflammatory effect/completely normalised membrane). From these scores a percentage could be calculated for each substance for all the membranes assayed. This percentage is a measure of the anti-inflammatory activity of the substance tested. Table 9.5 summarises the results of the LPS HET-CAM experiments and Figure 9.17 shows the reduction of inflammation, the score for anti-inflammatory activity being shown as a percentage of the maximum score of 3 which is assigned when a membrane has a completely normal appearance.

Table 9.5: Results from HET-CAM assay. The mean anti-inflammatory activity score is shown for various substances in comparison on LPS-irritated membranes. A score of 3 indicates a normal membrane was observed at the end of the assay. A score of 0 indicates inflammation was present in the in the form of redness.

Treatment Per pellet	No. of eggs	No. of dead embryos	No. of CAMs harvested	Average score (out of 3)
LPS 500 ng alone	3	0	6	0
Hydrocortisone 50 µg no LPS (control)	3	0	6	2.88
Manuka honey high AU 5% no LPS (control)	3	1	6	2.83
Hydrocortisone 50 µg + LPS 500 ng	4	0	8	2.63
Manuka honey high AU 2%+ LPS 500 ng	4	1	6	1.50
Manuka honey high AU 5%+ LPS 500 ng	4	0	8	2.50
Manuka honey low AU 5%+ LPS 500 ng	4	0	8	1.38
Pasture honey 5%+ LPS 500 ng	3	0	6	0.25
Artificial honey 5%+ LPS 500 ng	3	1	4	0.25
Agarose only (control)	4	1	6	3

Abbreviations; LPS, lipopolysaccharide; AU, Arbitrary units measuring fluorescence.

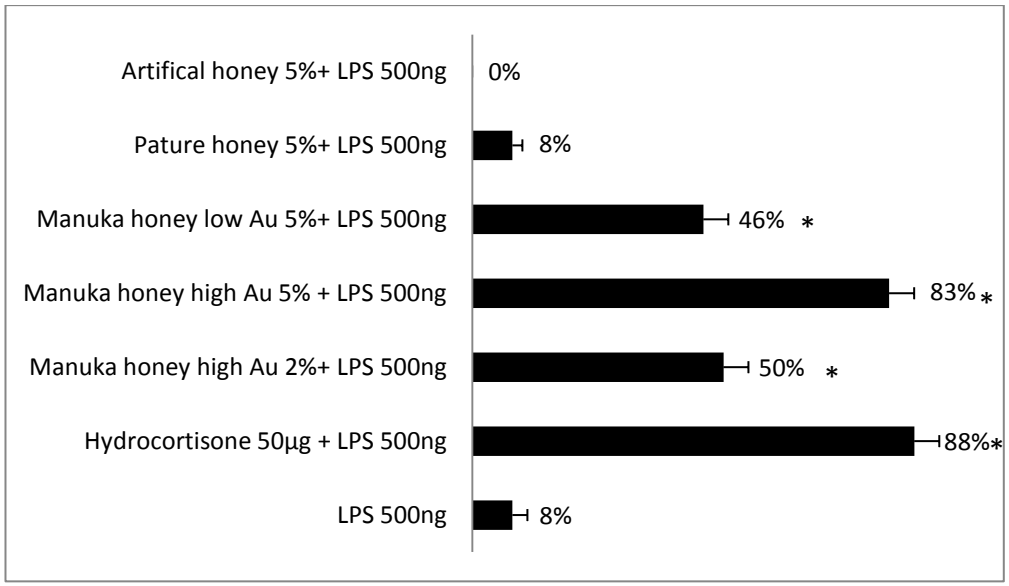

*Figure 9.17: Data from Table 9.5 for the mean anti-inflammatory activity score for LPS irritated chorioallantoic membranes, shown as a percentage of the maximum score of 3 which is assigned to a membrane showing no signs of inflammation. Error bars show ± 1 SD of the mean from at least three experiments. ** p*<0.001 analysed by ANOVA compared with the control membrane treated with artificial honey.*

All Manuka honey treatments significantly (p<0.001) reduced inflammation of the chorioallantoic membrane irritated by LPS. The 5% concentration of highly fluorescent Manuka honey showed the greatest inhibition of chorioallantoic membrane irritation with all the membranes tested. This was apparent by the reduction/lack of redness associated with LPS irritation. At 5% the highly fluorescent Manuka honey was comparable in effect to hydrocortisone at a concentration of 5 mg/ml for LPS irritated chorioallantoic membranes. At 2% Manuka honey that was highly fluorescent was comparable with 5% Manuka honey with low fluorescence. Artificial honey had no effect on inflammation. Pasture honey had a slight effect in reducing inflammation but this was not significant compared with the artificial honey control.

9.5 Discussion

The typical chorioallantoic membrane inflammation due to SDS has been comprehensively examined by researchers and the characteristic scarring, granulomas and capillary nets are easily identifiable (Marchesan, Paper *et al.* 1998; Bürgermeister, Paper *et al.* 2002). By combining anti-inflammatory agents with the irritant a reduction of inflammation can be measured. This assay is commonly used to test the anti-inflammatory properties of new compounds so it was employed for the testing of the anti-inflammatory effect of Manuka honey. SDS was seen to stimulate typical membrane irritation. In this inflammatory state the number of blood vessels is high and there is formation of a large granuloma. In all cases the blood vessels have a star form around the granuloma and the granuloma is raised above the capillary net.

LPS was also used to irritate the membrane as this had been used in previous work to activate macrophages for phagocytosis assay. Preliminary work ascertained a concentration of LPS that did not kill the embryo but still had a visible inflammatory effect. Redness and swelling were the visible symptoms of the resulting inflammation observed from LPS application. This inflammation was able to be prevented by the addition of hydrocortisone proving its anti-inflammatory action against LPS irritation.

Previous work identified that the Manuka honey with high fluorescence had more inhibitory activity on phagocytosis than Manuka honey with low fluorescence did. This activity was presumed to result in an anti-inflammatory action as in conjunction with reduced phagocytosis, inflammatory cytokine expression and ROS production in monocytes and macrophages was reduced.

Manuka honeys with high and low fluorescence, were tested at a 5% concentration. Manuka honey with high fluorescence was also tested at 2% after preliminary work identified the lowest limit of activity and the maximum amount of honey required to neutralise the effects of SDS at commonly used concentrations of SDS. Pasture honey and artificial honey were tested at the

highest concentration of 5%. The same honey concentrations were used in the LPS-irritated chorioallantoic membranes as the SDS-irritated chorioallantoic membranes.

The 5% concentration of highly fluorescent Manuka honey showed the greatest inhibition of chorioallantoic membrane irritation with both SDS- and LPS-irritated membranes. At 5% the highly fluorescent Manuka honey was comparable in effect to hydrocortisone at a concentration of 5 mg/ml for both the SDS and LPS irritated chorioallantoic membranes. At a concentration of 2% Manuka honey that was highly fluorescent had the same effect as 5% Manuka honey with low fluorescence confirming the greater anti-inflammatory properties of Manuka honey with high fluorescence. The results from these initial HET-CAM assays suggest that the anti-inflammatory activity of honey may be attributed to the fluorescent component. Further assays need to be completed with a wider range of honey samples, both Manuka and non-Manuka, with varying fluorescence, to show statistically that there is a correlation between high fluorescence and anti-inflammatory activity.

These results agree with those found *in vitro* with the phagocytosis assay, indicating that the phagocytosis assay gives a reliable indication of anti-inflammatory activity *in vivo*. Artificial honey had no effect in reducing the inflammation caused by LPS or SDS irritation and while Pasture honey had a slight affect this was not significant. As mentioned in earlier Chapters, it is highly likely that some Manuka nectar was present in the Pasture honey giving this activity.

It was not proven that the granuloma formed in response to SDS irritation was predominately consisting of macrophages as most granulomas are deemed to be. Future work may look at determining this to show that the active component in honey is working on macrophages *in vivo* as it was determined to *in vitro* by the phagocytosis assay.

Future work using the HET-CAM assay should include comparison of the anti-inflammatory activity of Manuka honey with that of other commonly used anti-inflammatory agents such as phenylbutazone and sodium diclofenac. More honeys should be tested in the HET-CAM such as RewaRewa and Kanuka which have similar properties to Manuka honey yet lack the fluorescence and content of MGO. Further work should use the HET-CAM assay to test more Manuka honeys with a range of phagocytosis-inhibiting activity to determine the correlation coefficient. HET-CAM assays should also be completed using fractionated Manuka honey as was done in Chapter 6 when isolating the active protein using the phagocytosis assay. This would confirm that it is the phagocytosis-inhibiting glycated MRJP-1 that is responsible for the anti-inflammatory affect seen *in vivo*, and not some other component in honey acting through a different mechanism.

Chapter 10

Summary and discussion of thesis

10.1 Summary

To summarise, it was found during this research that honey reduced phagocytosis in activated THP-1 cells and that certain Manuka honeys had a superior ability to other types of honey to do this. MRJP-1, the major protein in honey, and MRJP-3 was identified as being present in the fraction having this activity in Manuka honey. This finding is important to the anti-inflammatory aspects of wound healing due to the large amounts of ROS released from phagocytising macrophages. Chronic wounds are overpopulated with active macrophages and the high levels of ROS released, creates feedback loops that worsen the situation.

An *in vitro* assay for inflammation utilizing the chorioallantoic membrane of a fertilized hen's egg, (HET-CAM) showed that the phagocytosis-inhibiting activity seen in the phagocytosis assay was a reliable indicator for measuring anti-inflammatory activity of honey *in vivo*, though it was not proven that the honey was working *via* the same means.

The protein in the active fraction was found to bind a ConA lectin chromatography column indicating it had mannose-terminating glycosylation. The phagocytosis inhibiting activity could be significantly reduced by treating

honey with mannosidase suggesting these mannose components are important in the protein's phagocytosis-inhibiting activity.

Attempts to identify the receptor narrowed it down to a mannose-binding receptor by using mannan and β-glucan as inhibitors of phagocytosis in the phagocytosis assay. It was hypothesised that the receptor was the mannose receptor.

MRJP-1, as is most likely to occur with all proteins in honey, was found to be modified by the MGO present in Manuka honey. It appears that this modification occurs gradually over time upon storage of the honey. This modification resulted in Manuka honey developing fluorescence at a wavelength characteristic of that of AGEs formed by reaction of MGO with arginine residues, and this fluorescence can be used to identify which honeys have a high level of anti-inflammatory activity as determined by the phagocytosis assay and the HET-CAM assay.

10.2 Discussion

There may be several anti-inflammatory agents in honey, one of them at least was identified in this research as MRJP-1. It is likely that this protein has several modes of action, as discussed in Chapter 9. The LPS- and SDS-irritated membranes each had a different inflammatory reaction to the treatment, yet Manuka honey normalised both types of inflammation of the membranes. It was not proven that the anti-inflammatory activity seen in the HET-CAM assay was due to MRJP-1, though it was present in the fraction of honey that had this activity. However, it was noted that honey with high anti-inflammatory activity in the phagocytosis assay also had had a high score in the HET-CAM assay (indicating high anti-inflammatory activity also). These honeys had a high fluorescence, which was shown to be due to MGO modifying the protein in

honey. This indicated that MGO-modified MRJP-1 was present. To a lesser extent MRJP-3 may have some anti-inflammatory activity also as it was present in the active fraction. Manuka honey with low fluorescence had a low level of anti-inflammatory activity in both assays. MGO presence alone was not able to inhibit phagocytosis, so the correlation for MGO and fluorescence was low. However, only honey that had MGO developed fluorescence. There is most likely another variable influencing the development of the fluorescence, such as antioxidants retarding the modification of the protein, age of the honey or temperature the honey was stored at. This will need to be investigated further.

THP-1 cells activated by three different agents, LPS, PMA and Vit-D3, all generated macrophages (Chapter 3) which responded to Manuka honey by having reduced phagocytosis ability (Chapter 4). This indicates that the macrophage activation state is diverse as discussed in Chapter 4 which is to be expected due to the different mechanisms of activation. It may mean that in the HET-CAM assay different states of activation of macrophages may occur. The HET-CAM assay could be tested with PMA and other irritants to determine further modes of anti-inflammatory action for Manuka honey. As mentioned previously in Chapter 9, it will be of great importance to confirm which cells are being targeted in the HET-CAM assay. While macrophages are considered to be the main cellular component in granulomas, it needs to be confirmed in this instance before assumptions can be made about the anti-inflammatory component of honey. The activity may be active *via* the same mechanisms in the phagocytosis assay and the HET-CAM assay yet this remains to be proven.

The anti-inflammatory effect could have been due to the stabilization of cytokine expression, anti-oxidants preventing ROS formation activating further inflammation, MGO-modified MRJP-1 blocking mannose receptors, reduction of ROS production or a combination of these. As mentioned previously, adding arginine but not lysine to honey reduced the MGO content of honey, suggesting that MGO was modifying only the arginine on the proteins in honey (Adams, *et*

al. 2009). In addition to that it has been reported that MGO-modified arginine residues are a signal for receptor mediated endocytosis by THP-1 cells (Westwood, *et al*. 1997). Thus it is highly likely that the natural MGO content of Manuka honey is modifying the arginine of the protein in honey, the majority of being MRJP-1, which then become ligands for THP-1 macrophage receptors. In light of the current research, this is most likely the mechanism that honey works in its anti-inflammatory activity.

Anti-inflammatory activity had a highly significant correlation with the fluorescence of honey. Honey with high fluorescence had high anti-inflammatory activity. This finding proves fluorescence is a good indicator for the anti-inflammatory effect of honey. The assay for fluorescence is a much more economical assay than the one for phagocytosis or the HET-CAM assay, both in terms of actual cost and time. It may be possible that future screening of honey for its anti-inflammatory activity be done using only the fluorescence of honey.

Attempts to find correlation between anti-inflammatory activity and other factors such as MGO content, or NPA were unsuccessful, suggesting a complex process is occurring. The mode of action for modulating the cytokine expression was not determined for Manuka honey, but it is likely that it is due to another component entirely. It was shown that decreasing phagocytosis decreased ROS release *in vitro* but it was only hypothesised that this would contribute to the reduction of inflammation *in vivo*.

Research should be directed towards discovering the bioavailability of glycated MRJP-1. Does the MRJP-1 cross membranes? Can it be absorbed from the gut and work systemically? Can it diffuse through intact skin, or does it work only on exposed phagocytes where the mannose receptor is present to bind it? If honey cannot do this, then this would indicate that honey may be restricted to treating inflamed wounds with exposed phagocytes such as skin trauma wounds, stomach ulcers, mouth ulcers, and gingivitis. Investigations into the ability of

the active component of honey to survive the highly acidic condition of the stomach and dilution through the intestines will be important in learning if honey can treat inflammation caused by stomach ulcers, irritable bowel syndrome or Crohn's disease. The HET-CAM work showed that the active component in honey can cross membranes as the chorioallantoic membrane was intact for this assay. But as mentioned previously it is only hypothesised that the anti-inflammatory activity seen in the HET-CAM assay is due to the MGO-modified MRJP-1 and not another component in honey with high fluorescence. It is important to know the bio-availability of the active component in honey, as other areas that might benefit from anti-inflammatory action of honey do not have exposed phagocytes, for example, sunburn and arthritis. Research would also benefit by assaying a wider range of honeys in the HET-CAM.

Obtaining high concentrations of MRJP-1 was a limiting factor for this research. Recombinant MRJP-1 has been prepared by heterologous expression in *E. coli*, retaining the immuno-stimulatory effect of native MRJP-1, by increasing TNF-α expression (Majtan, Kovacova *et al.* 2006). It is highly recommended that in the future MRJP-1 be manufactured by genetic engineering of insects to create larger quantities of pure glycosylated MRJP-1, and this protein be modified with MGO after isolation. An insect cell line would give the correct terminal mannose residues needed to act on the mannose receptor of macrophages. This protein could then be used in the HET-CAM assay and the phagocytosis assay, eliminating the question of whether anti-oxidants or other components have the anti-inflammatory activity.

The presence of cross-links in the active protein needs to be investigated as SDS gel electrophoresis of MGO-modified protein in honey and bovine serum albumin indicated MGO-modified protein became larger, possibly by forming cross-links. The main Manuka honey protein estimated to be around 63 kDa on the SDS electrophoresis gel), was 5 kDa larger than the same protein band in Clover honey (estimated to be 58 kDa). The reported molecular weight of MRJP-

1 in honey is 55 kDa (Tamura, Kono *et al.* 2009). The size differences are expected to be due to differences in glycosylation between the samples of honey. Manuka honey and incubated Manuka honey with naturally high MGO content, both had a diffuse protein band in the higher molecular weight range (150 - 250 kDa) that was not visible in the Clover honey. This is possibly due to glycation and/or cross-linking. The bovine serum albumin had protein that increased in weight to around 240 - 280 kDa from 66 kDa after incubation with MGO. Such a large increase in weight suggests cross-linking. As mentioned in Chapter 5, cross-linked protein binds receptors with more affinity, which could partially explain the anti-inflammatory effect of Manuka honey if it is working on receptors as hypothesised.

One aspect not investigated during this research was the cell-to-cell communication that occurs in inflammatory cascades and feedback loops. It would be of great importance to investigate whether the anti-inflammatory activity of honey affects cell-to cell communication, for example; by way of MyPo as discussed earlier. Though the MyPo pathway is only one method of cell-to-cell communication, it is possible that this pathway is targeted by the active component of honey. Macrophage activation has been found to generate high levels of MyPo and the increase in MyPo content is accompanied by an increased production of inflammatory mediators which recruit further inflammatory cells, both macrophages and other phagocytes, and MyPo derived from neutrophils is cleared by the mannose receptor of macrophages.

Future work should investigate the contribution that anti-oxidants in the honey may make to the anti-inflammatory effect. As mentioned earlier, antioxidants retard the reaction that produces MGO-modified protein. This needs to be investigated in Manuka honey where the natural anti-oxidant content may be influencing the development of anti-inflammatory activity. The antioxidant content may explain why there was a lack of correlation with MGO content and anti-inflammatory activity of honey. With this knowledge it will be possible for

apiarists to select blends of honey so that honey can have an optimal anti-inflammatory activity. Having a method for quantifying the amount of anti-inflammatory activity in individual honey will assist in the marketing of Manuka honey as an anti-inflammatory agent. Depending on the circumstances a honey could be selected for greatest anti-bacterial or anti-inflammatory effect to speed up the wound healing process as not all wounds are infected or inflamed.

It may be that antioxidants are anti-inflammatory but are superfluous if production of ROS is inhibited by the protein. Decreasing ROS release from macrophages hypothetically will suppress inflammation by preventing the ongoing feedback loops in the inflammatory cascade generated by phagocytosis. Inflammatory pathways such as the NF-κB pathway, (NF-κB being a major transcription factor that regulates genes responsible for both the innate and adaptive immune response), won't be activated, and cytokine expression may be diverted to anti-inflammatory production.

These findings have obvious implications for honey and its use as a wound dressing with respect to its anti-inflammatory properties. It would be beneficial for future work to re-run the HET-CAM assays with just the protein from Manuka honey, to see how the results compare with the whole honey. Further to this, a wider range of honey, both Manuka and other floral varieties should be assayed to determine if any other honey types have this bioactivity.

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Appendix 1 – DVD

The DVD can be found attached to the inside cover of this thesis at the back. The DVD can be played on a standard DVD player, or computer with DVD drive. The following is a description of the files on the disc.

Time-lapse movie of untreated-monocyte THP-1 cells, 1 frame every 20 seconds.

Time-lapse movie of honey treated THP-1 cells, 1 frame every 20 seconds, 0.25% Manuka honey.

Time-lapse movie of LPS-treated (100 ng/ml) THP-1 cells, 1 frame every 15 seconds.

Time-lapse movie of honey- and LPS-treated (100 ng/ml) THP-1 cells, 1 frame every 20 seconds, 0.25% Manuka honey.

Time-lapse movie of LPS-treated (100 ng/ml) THP-1 cells 1 frame every second.

Movie made of THP-1 cells for the Queenstown molecular biology conference.

Appendix 2 – Primer sequences

Gene	Primer sequence (forward and reverse, 5'–3')	length (bp)	Product length (bp)	Accn. no.	Reference	Melt temp
IL-1β	F-TACGAATCTCCGACCACCA R-GGGACAGGATATGGAGCAAC	19 20	315	M15330		87
IL-6	F-CTGAGGGCTCTTCGGCAAA R-CTTTCCAAGAAATGATCTGGC	19 21	192	M18403		84.7
IL-10	F-GCTGTTTTCCCTGACCTCC R-CATCTCCTGGGTTCAAGCA	19 19	400	M57627		89
IL-12	F-CACCAAGCAAAGAGGGAGAG R-TTTTGCCTGTGTCTCGTCTG	20 20	186	M65290		89.5
TGFβ	F-CAGCAACAATTCCTGGCGATA R-AAGGCGAAAGCCCTCAATTT	21 20	155	X02812		89.5
PDGF	F-CCGTAGGGAGTGAGGATTCT R-GCTGCTTTAGGTGGGTTTTA	21 20	487	U41745	(Utsugi *et al.*, 2002)	87
IL-1ra	F-GGCCTCCGCAGTCACCTAATCACTCT R-TACTACTCGTCCTCCTGGAAGTAGAA	26 26	500	M55646	(Gabay, 1997)	91
HCgp-39	F-TCAAGAACAGGAACCCCAAC R-AAATTCGGCCTTCATTTCCT	20 20	236	Y08374		
CPM	F-GAAAGCAGCAGTCAGGCAC R-CCTCTGCCTTCTGGGTTCA	19 19	233	AF368463	(Rehli *et al.* 1995)	88.3
β_2M	F-ATGTCTCGCTCCGTGGCCTTA R-ATCTTGGGCTGTGACAAAGTC	21 21	295	AB021288		86
β-actin	F-GGATGCAGAAGGAGATCACTG R-CGATCCACACGGAGTACTTG	21 20	90	NM_001101		N/A

Appendix 3 – Sample data

Code No.	Description	NPA	MGO	Colour	Fluor	AI	HMF
bu496	Buttercup			10	51	5	
bu796	Buttercup			10	66	1	
clo42	Clover			2	21	2	
f1	Fennel mata			8	31	5	
hd1	Honey Dew			8	40	10	
k17	Kanuka			5	59	13	
k24	Kanuka			9	89	19	
k27	Kanuka			8	64	10	
k29	Kanuka			6	56	15	
ko1	Kanuka			8	77	15	
La6	Lavender			2	6	0	
CJA10+	Manuka	11.1	189	5	92	38	6.47
CJA30+	Manuka	27	679	7	147	41	12.85
M01	Manuka	25.3	650	8	95	40	3.13
M03	Manuka	23.6	599	7	75	37	4.38
M05	Manuka	17.5	312	9	162	43	5.10
M07	Manuka	13.9	205	8	58	29	2.25
M08	Manuka	14.7	219	7	50	25	2.31
M09	Manuka	17.7	329	8	62	34	4.57
M10	Manuka	13.9	219	6	54	30	3.07
M11	Manuka	14.8	249	6	74	34	2.33
M118	Manuka				65	19	
M119	Manuka				120	38	
M12	Manuka	14.9	272	5	52	24	1.58
M120	Manuka	19.3		8	74	21	
M122	Manuka			4	50	14	
M124	Manuka	18		8	143	41	
M125	Manuka	16		10	120		
M126	Manuka			6	94	15	
M127	Manuka	19		8	141		
M128	Manuka	11.8	192	4	20	4	
M129	Manuka	14		9	29	13	
M13	Manuka	11	167	5	25	16	0.91
M130	Manuka	15		5	93	35	
M140	Manuka	22		10	106	35	
M142	Manuka	25+		9	155	44	
M144	Manuka	12+		10	184	51	
M145	Manuka	10+		6	112	41	

Code No.	Description	NPA	MGO	Colour	Fluor	AI	HMF
M146	Manuka	20+		8	88	30	
M149	Manuka	5+		7	205	52	
M15	Manuka	15.3	267	7	63	31	3.86
M150	Manuka			3	61	10	
M151	Manuka	ND		2	16	4	0.30
M152	Manuka	6.9			15	5	
M158	Manuka	13.3	330	4	30	5	5.00
M159	Manuka	11.8	280	4	29	7.2	1.50
M16	Manuka	14.4	272	7	68	32	3.80
M160	Manuka	8.3	170	4	21	5.1	1.10
M161	Manuka	9.4	223	4	23	5.6	0.70
M162	Manuka	8.7	360	4	26	9	1.00
M163	Manuka	14	352.8	4	34	10	1.10
M164	Manuka	ND	118	4	80	23	0.20
M165	Manuka	11.8	280	4	28	7	1.20
M166	Manuka	ND	145.9	4	92	31	0.40
M167	Manuka	ND	151.2	4	61	25	0.50
M168	Manuka	ND	140	4	60	18	0.80
M169	Manuka	ND	150	4	68	18	0.30
M17	Manuka	14.2	243	4	97	39	1.06
M170	Manuka	1/4 PI	160	4	45	20	0.50
M171	Manuka	ND	150	4	74	25	0.90
M172	Manuka	ND	160	4	89	30	0.70
M173	Manuka	1/4 PI	150	4	50	15	0.60
M174	Manuka	ND	85	4	90	30	0.20
M175	Manuka	ND	85	4	102	30	0.10
M176	Manuka	ND	145	4	46	20	0.50
M177	Manuka	ND	150	4	65	21	0.50
M178	Manuka	ND	150	4	68	28	0.90
M179	Manuka	ND	120	4	89	31	0.30
M18	Manuka	14.2	257	4			0.82
M180	Manuka	ND	150	4	97	38	0.60
M181	Manuka	1/4 PI	130	4	72	10	0.70
M182	Manuka	ND	110	4	116	32	0.40
M183	Manuka	ND	115	4	107	34	0.30
M184	Manuka	ND	110	4	58	25	0.80
M185	Manuka	ND	110	4	57	24	0.50
M186	Manuka	ND	95	4	87	35	0.20
M187	Manuka	8.1 PI 3/4	110	4	94	33	0.40
M188	Manuka	8.7 1/4 PI	130	4	66	17	0.40
M189	Manuka	ND	85	5	204	55	0.50
M19	Manuka	11.9	186				0.84
M190	Manuka	8.2 PI 2/4	105	5	195	49	0.70
M191	Manuka	ND	110	5	216	51	0.40

Code No.	Description	NPA	MGO	Colour	Fluor	AI	HMF
M192	Manuka	8.1 PI 1/4	130	5	175	44	0.80
M193	Manuka	ND	100	5	214	49	
M196	Manuka	ND	130	4	21	13	0.80
M197	Manuka	ND	115	4	20	9	0.70
M198	Manuka	ND	80	4	77	24	1.10
M199	Manuka	PI 2/4	160	4	93	38	0.90
M20	Manuka		139	7	82	39	0.79
M200	Manuka	PI 1/4	140	4	85	34	
M201	Manuka	12.4	280	4	73	29	1.80
M202	Manuka	ND	150	4	96	32	0.90
M203	Manuka	ND	150	5	156	41	0.90
M204	Manuka	ND	160	5	120	35	0.70
M205	Manuka	8.9 PI 3/4	170	5	96	30	1.10
M206	Manuka	ND	110	4	51	12	0.50
M207	Manuka	ND	120	5	80	28	0.50
M209	Manuka	ND	140	5	81	20	1.30
M21	Manuka	9.2	121	3	35	21	0.76
M217	Manuka		550+		149	35	
M22	Manuka	8.3	106	3	34	16	0.66
M23	Manuka	9.3	141	3	26	16	0.47
M24	Manuka	7.9	96.6	3	10	11	0.50
M26	Manuka	7.3	67	6	47	21	1.29
M27	Manuka	7.3	107	6	65	29	0.61
M28	Manuka	8.6	112	4	19	14	1.33
M29	Manuka	8	114	5	73	35	0.82
M30	Manuka	10	103	4	30	21	0.93
M31	Manuka	7.5	107	7	39	20	1.55
M32	Manuka	5.9	71	6	40	23	2.31
M34	Manuka	4.5	58.6	7	33	15	2.99
M36	Manuka	5.4	25.4	3	53	31	0.35
M38	Manuka	7.5	89.9	4	29	16	1.04
m999	Manuka			4	32	10	
MB02	Manuka	16.8		10	159	40	
MBD28.8	Manuka	27.3	708	9	167	49	17.98
Mt1	Manuka			10	53	10	
Mt2	Manuka			5	65	0	
mwh	Manuka			6	106	20	
sp bay 1	Manuka	20+		4	72	25	
MB101	Manuka > 10 yr old	15.3		10	141	41	
MB102	Manuka > 10 yr old		0		12	227	55
Spbay old	Manuka > 10yr old		10	206			
Mt3	Manuka comb			10	114	25	

Code No.	Description	NPA	MGO	Colour	Fluor	AI	HMF
mb1	Mellow bush			7	89	10	
mk1	Mingimingi/Kowhai			3	21	0	
N13	Nodding Thistle			2	5	0	
N14	Nodding Thistle			5	11	0	
p132	Pasture			5	24	5	
P141	Pasture			4	20	10	
P59	Pasture	0	10	3	35	15	
bw50	Penny Royal			7	14	0	
bw49	Penny Royal			7	44	0	
PO10	Pohutakawa			5	13	0	
PO9	Pohutakawa			2	5	0	
r138	Rewarewa			7	33	20	
r139	Rewarewa			4	34	20	
r140	Rewarewa			5	34	20	
T18	Thyme			8	20	0	
Mf1	Urban floral			5	20	0	
VB4	Vipers Bugloss			3	5	0	
VB6468	Vipers Bugloss	0	0	1	3	0	20.00

NB: Missing data in spreadsheet indicates test was not completed. **Abbreviations**; NPA=non-peroxide antibacterial activity, MGO=methylglyoxal, Fluo.=fluorescence , AI.=Anti-inflammatory level, HMF=HydroxyMethylFurfuraldehyde.

Appendix 4 - Colour scale

Appendix 5

Assesment of LPS as an irritant for the HET-CAM assay.

Preliminary assays were conducted for the HET-CAM assay using varing concentrations of LPS to find a suitable concentration that would not kill the embryo but provide an observable inflammation as a direct result of the LPS application to the membrane. Two embryos were assayed using the same protocol as listed in Chapter 9, Section 9.3.2.1 and 9.3.2.3. A 50 µg/ml concentration of LPS was selected for further assays.

Concentration of LPS	**Embryos alive** **(OUT OF 2)**	**Granuloma present**	**Cappilary net normal**	**Redness observed**
5 µg/ml	2	NO	YES	NO
50 µg/ml	2	NO	YES	YES
200 µg/ml	1	NO	hemorrhage	YES
500 µg/ml	0	NO	N/A	N/A