

**MACROPHAGE PHAGOCYTOSIS OF
APOPTOTIC NEUTROPHILS IS
CRITICALLY REGULATED BY THE
OPPOSING ACTIONS OF
PRO-INFLAMMATORY AND
ANTI-INFLAMMATORY AGENTS:
KEY ROLE FOR TNF- α**

SYLWIA MICHLEWSKA

Presented for the degree of Doctor of Philosophy
The University of Edinburgh
June 2010

DECLARATION

I HEREBY DECLARE THAT THE DATA PUBLISHED IN THIS THESIS ARE THE RESULT OF MY OWN WORK CARRIED OUT UNDER SUPERVISION OF PROFESSORS ADRIANO G ROSSI (PRIMARY SUPERVISOR), IAN DRANSFIELD AND IAN L MEGSON AT THE UNIVERSITY OF EDINBURGH. THIS THESIS HAS BEEN COMPLETED ENTIRELY BY MYSELF AND HAS NOT PREVIOUSLY BEEN SUBMITTED FOR ANY OTHER DEGREE OR QUALIFICATION.

SYLWIA MICHLEWSKA

ABSTRACT

Development of chronic inflammation or autoimmunity may be related to deregulated mechanisms orchestrating successful resolution of inflammation, especially apoptosis of inflammatory cells and their subsequent clearance by macrophages (M ϕ). Chronically inflamed sites are characterised by an excess of the key pro-inflammatory cytokine tumor necrosis factor- α (TNF- α) and importantly, TNF- α inhibitors, widely used in the clinical setting for the treatment of rheumatoid arthritis (RA), inflammatory bowel disease and psoriasis, significantly delay disease progression. TNF- α therefore may affect processes implicated in resolution of inflammation. Although TNF- α and pro-inflammatory bacterial products such as lipopolysaccharide (LPS) influence rates of inflammatory cell apoptosis, little is known about their effects on M ϕ phagocytosis of apoptotic cells (efferocytosis). In this PhD thesis, the effects of several pro-inflammatory agents (i.e., LPS, lipoteichoic acid (LTA), peptidoglycan (PGN) and TNF- α) on efferocytosis by human blood monocyte-derived M ϕ (MDM ϕ) have been investigated. LPS, LTA and PGN all inhibited MDM ϕ efferocytosis in a concentration- and time-dependent manner; however, LPS did not inhibit the uptake of immunoglobulin-G (IgG)-opsonized erythrocytes. Moreover, although TNF- α did inhibit efferocytosis, phagocytosis of IgG-opsonized erythrocytes was not inhibited. Furthermore, the LPS effect was attenuated by dimeric soluble human recombinant TNF receptor-1 (sTNF-R1/Fc), indicating a critical role of TNF- α . Concomitant treatments with monomeric soluble human recombinant TNF receptor-1 (sTNF-R1) or the TNF- α Converting Enzyme (TACE) inhibitor, TOPI-0, only partially reversed the inhibitory effect of LPS.

Even though TNF- α release takes place within the first few hours following LPS stimulation, the LPS-induced inhibitory effect occurred only if treatment was performed for 96 hours or longer. Analysis of supernatants obtained from LPS-treated MDM ϕ revealed that there appears to be interplay between concentrations of TNF- α and interleukin-10 (IL-10) and that these cytokines exert opposing actions on efferocytosis. IL-10 *per se* increased MDM ϕ efferocytosis and addition of exogenous IL-10 to LPS-treated samples rescued

phagocytosis. The latter effect was associated with the IL-10-induced, concentration-dependent inhibition of TNF- α release. Interestingly, when IL-10 was added to TNF- α -treated MDM ϕ , only slight augmentation of phagocytosis was observed. Furthermore, when IL-10-mediated effects were blocked by concomitant treatment with anti-human IL-10 receptor 1 antibody (anti-IL-10-R1Ab), the LPS inhibitory effect on phagocytosis was much greater and occurred at 24 hours after treatment. The role of IL-10 on efferocytosis was also investigated using IL-10 deficient murine bone marrow-derived M ϕ (BMDM ϕ). IL-10 deficient BMDM ϕ , when compared to wild-type, were characterised by a much lower ability to phagocytose apoptotic neutrophils and this effect was independent of culture conditions (control samples and LPS or TNF- α treatments). Finally, effects of the synthetic steroid (dexamethasone) and non-steroidal anti-inflammatory drugs (NSAID) on MDM ϕ phagocytosis were examined. Dexamethasone, like IL-10, augmented MDM ϕ efferocytosis, reversed the inhibitory effects of both LPS and TNF- α , and suppressed LPS-induced production of TNF- α . In contrast NSAID did not increase MDM ϕ efferocytosis *per se*. However, preliminary data suggest that aspirin blocks the inhibitory effect of TNF- α on phagocytosis.

In summary, it has been determined that prolonged treatment with pro-inflammatory agents such as LPS, LTA and PGN inhibits MDM ϕ efferocytosis which may potentially postpone the resolution of inflammation *in vivo*. I have shown that TNF- α is a key mediator in this process and that IL-10 exerts an important regulatory effect on TNF- α production and consequently on efferocytosis. Furthermore, several approaches have been unveiled to successfully reverse LPS-mediated inhibition of efferocytosis by decreasing either TNF- α production or its inhibitory effect with sTNF-R1/Fc, exogenous IL-10 or dexamethasone. These findings indicate that TNF- α and other agents which influence efferocytosis may have significance in the resolution phase of inflammation. In addition, presented findings provide important mechanistic information into the potential mode of action of anti-TNF- α agents and steroids and may help to explain their clinical success in the treatment of chronic inflammatory diseases.

ACKNOWLEDGMENTS

Firstly I would like to express my gratitude towards the Wellcome Trust for funding my PhD study. Without its generous support this PhD thesis would not have been completed.

I would also like to thank all of the volunteers who have donated blood for my research and the many people who also prepare blood samples that shared/exchanged human blood cells with me.

I would also like to express appreciation for all of the people that helped me in so many ways during the course of this project. Thank you all for helpful suggestions and important advice on all the technical matters during the course of this work. Special thanks goes to Aisleen, Fiona, Nikki, Jillian, Lynsey, Michela, Olga, Paula, Sandra, Sarah, Sonia, Stelios and Tara and many others. Most importantly, my keen appreciation goes to Shonna for great patience and endless flow cytometry problem solving and to Ying, Irini and Marta for showing to me how to make perfect western blots ... mine have never been that great!. Many thanks to Sarah, Roger and Paula for proof-reading the final version of this thesis and their useful comments.

Thanks to everyone that I had the pleasure to work with and shared great times with during my years in the CIR!

Finally, I owe my deepest gratitude to my supervisors, Professors Adriano G Rossi, Ian Dransfield and Ian L Megson for their enthusiasm, inspiration and encouragement when they were most required. Especially, many thanks to Professor Adriano G Rossi who was the supervisor I could have only wished for, giving me enough freedom to explore on my own but at the same time always being there for me and supporting me with his guidance, advice and constructive criticism.

And last but not least to my family, who has supported me, has believed in me and have been there for me.....

TABLE OF CONTENTS

DECLARATION	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
ABBREVIATIONS	x
1. INTRODUCTION	2
1.1 INFLAMMATION	2
1.2. INFLAMMATORY MEDIATORS	4
1.2.1. TUMOR NECROSIS FACTOR – ALPHA	5
1.2.2 TNF- α PRODUCTION	7
1.2.2.1 TLR AGONISTS ARE POSITIVE REGULATORS OF TNF- α PRODUCTION	8
1.2.2.2 IL-10 IS A NEGATIVE REGULATOR OF TNF- α PRODUCTION	11
1.2.3 TNF- α SIGNALLING	14
1.2.3.1 NF- κ B ACTIVATION <i>via</i> RIP SIGNALLING	14
1.2.3.1.1 THE CANONICAL PATHWAY OF NF- κ B ACTIVATION	16
1.2.3.1.2 THE NON-CANONICAL PATHWAY OF NF- κ B ACTIVATION	17
1.2.3.2 THE ANTI-APOPTOTIC ACTION AND MAPKKK ACTIVATION <i>via</i> TRAF2 SIGNALLING	17
1.2.3.3 THE PRO-APOPTOTIC PATHWAY <i>via</i> FADD SIGNALLING	18
1.3 RESOLUTION OF INFLAMMATION	21
1.3.1 APOPTOSIS OF INFLAMMATORY CELLS	22
1.3.2 RECOGNITION OF APOPTOTIC CELLS BY PHAGOCYTES	24
1.3.2.1 ATTRACTION OF PHAGOCYTES BY APOPTOTIC CELLS	24
1.3.2.2 PHAGOCYTE RECOGNITION OF APOPTOTIC CELLS	25
1.3.2.2.1 “EAT ME” and “DON’T EAT ME” SIGNALS	27
1.3.2.2.2 INTERACTIONS OF APOPTOTIC CELLS WITH PHAGOCYTES <i>via</i> BRIDGE MOLECULES	29
1.3.2.2.3 PHAGOCYtic RECEPTORS	30
1.3.2.2.4 DIFFERENCES IN RECOGNITION OF APOPTOTIC AND NECROTIC CELLS	33
1.3.3 ENGULFMENT OF APOPTOTIC CELLS BY M Φ	34
1.3.4 IMMUNOLOGICAL CONSEQUENCES OF APOPTOTIC CELL CLEARANCE	37
1.4 CHRONIC INFLAMMATORY CONDITIONS	40
1.4.1 THE ROLE OF CYTOKINES IN CHRONIC INFLAMMATION	40
1.4.2 CLEARANCE DEFICIENCY AND AUTOIMMUNITY	43
1.5 ANTI-INFLAMMATORY THERAPEUTICS AND RESOLUTION OF INFLAMMATION	48
1.5.1 GLUCOCORTICOIDS	48
1.5.2 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS	53
1.5.3 CYTOKINE INHIBITORS	56
2 MATERIALS AND METHODS	62
2.1 REAGENTS	62
2.1.1 TISSUE CULTURE SOLUTIONS	62
2.1.2 PLASTIC WARE	62

2.1.3 REAGENTS FOR ISOLATION OF HUMAN BLOOD CELLS	63
2.1.4 BACTERIAL WALL COMPONENTS	63
2.1.5 RECOMBINANT PROTEINS	63
2.1.6 CELL TRACKER™ PROBES	64
2.1.7 CYTOKINE MEASUREMENT	64
2.1.8 ANTIBODIES	64
2.1.9 OTHER REAGENTS	65
2.2 CELL ISOLATION AND CULTURE	66
2.2.1 HUMAN PERIPHERAL BLOOD CELLS	66
2.2.1.1 CELL ISOLATION FROM HUMAN PERIPHERAL BLOOD	66
2.2.1.2 CULTURE OF HUMAN MONOCYTE-DERIVED MΦ (MDMΦ)	70
2.2.1.3 CULTURE OF HUMAN NEUTROPHILS	70
2.2.2 MURINE BONE MARROW DERIVED MΦ (BMDMΦ)	71
2.3 ASSESSMENT OF CELL VIABILITY	74
2.3.1 CELL MORPHOLOGY	74
2.3.2 FLOW CYTOMETRY	76
2.3.3 ASSESSMENT OF CELL MEMBRANE INTEGRITY	78
2.4 ASSESSMENT OF Mφ EFFEROCYTOSIS	79
2.4 ASSESSMENT OF Mφ EFFEROCYTOSIS	79
2.4.1 HUMAN MONOCYTE-DERIVED MΦ (MDMφ)	79
2.4.1.1 PHAGOCYtic TARGETS	79
2.4.1.2 PHAGOCYtOSIS ASSAY BY FLOW CYTOMETRY	79
2.4.1.3 PHAGOCYtOSIS OF ERYTHROCYTES OPSONISED WITH HUMAN IgG ANTIBODIES	80
2.4.1.4 PHAGOCYtOSIS ASSAY BY MICROSCOPY	80
2.4.2 MURINE BONE MARROW DERIVED MΦ (BMDMφ)	83
2.5 MEASUREMENT OF CYTOKINE CONCENTRATIONS	84
2.5.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)	84
2.5.1.1 PRINCIPLES OF ELISA	84
2.5.1.2 EXPERIMENTAL PROTOCOLS	85
2.5.2 CYTOMETRIC BEAD ARRAY (CBA)	87
2.5.2.1 PRINCIPLES OF CBA	87
2.5.2.2 EXPERIMENTAL PROCEDURES	87
2.6 sTNF-R1/Fc BINDING ASSAY	89
2.7 WESTERN BLOTTING	90
2.7.1 PRINCIPLES OF WESTERN BLOTTING	90
2.7.2 EXPERIMENTAL PROTOCOLS	92
2.7.2.1 PREPARATION OF MDMφ LYSATES	92
2.7.2.2 MEASUREMENT OF PROTEIN CONCENTRATION	93
2.7.2.3 SOLUTIONS AND REAGENTS	94
2.7.2.4 PROTEIN ELECTROPHORESIS	95
2.7.2.5 MEMBRANE TRANSFER OF PROTEINS	95
2.7.2.6 ANTIBODIES, INCUBATION CONDITIONS AND DETECTION	96
2.8 STATISTICAL ANALYSIS	97
3 THE EFFECTS OF PRO-INFLAMMATORY STIMULI ON MΦ EFFEROCYTOSIS	99
3.1 INTRODUCTION	99

3.2 METHODS	102
3.2.1 ISOLATION AND CULTURE OF HUMAN BLOOD CELLS	102
3.2.2 ASSESSMENT OF APOPTOSIS AND NECROSIS	102
3.2.3 ASSESSMENT OF PHAGOCYTOSIS BY FLOW CYTOMETRY	103
3.2.4 ASSESSMENT OF PHAGOCYTOSIS BY MICROSCOPY	103
3.3 RESULTS	104
3.3.1 LPS INHIBITS MDM Φ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER	104
3.3.2 LTA INHIBITS MDM Φ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER	108
3.3.3 PGN INHIBITS MDM Φ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER	110
3.3.4 TNF- α INHIBITS MDM ϕ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER	112
3.3.5 LPS - AND TNF- α - INDUCED INHIBITION OF EFFEROCYTOSIS APPEARS TO BE SPECIFIC FOR APOPTOTIC CELLS	116
3.3.6 IL-1 β INHIBITS MDM ϕ CLEARANCE OF APOPTOTIC NEUTROPHILS	118
3.3.7 SUMMARY OF THE RESULTS	119
3.4 DISCUSSION	120
4 LPS INHIBITS PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS VIA A TNF-α DEPENDENT MECHANISM	129
4.1 INTRODUCTION	129
4.2 METHODS	131
4.2.1 ISOLATION AND CULTURE OF HUMAN BLOOD CELLS	131
4.2.2 ASSESSMENT OF APOPTOSIS AND NECROSIS	131
4.2.3 ASSESSMENT OF PHAGOCYTOSIS BY FLOW CYTOMETRY	132
4.2.4 BINDING OF sTNF-R1/Fc AND IC TO THE SURFACE OF HUMAN APOPTOTIC NEUTROPHILS	132
4.2.5 MEASUREMENT OF CYTOKINE LEVELS AFTER LPS STIMULATION	132
4.3 RESULTS	133
4.3.1 THE ABILITY OF MDM Φ TO PHAGOCYTOSE APOPTOTIC NEUTROPHILS IS CORRELATED WITH THE LEVELS OF TNF- α IN CULTURE MEDIUM	133
4.3.2 DEPLETION OF TNF- α IN CULTURE MEDIUM REVERSES THE INHIBITORY EFFECT OF LPS ON MDM Φ EFFEROCYTOSIS OF APOPTOTIC NEUTROPHILS	138
4.3.3 sTNF-R1/Fc DOES NOT INDUCE Fc-DIRECTED PHAGOCYTOSIS AND PRIMARILY DEPLETES TNF- α IN CULTURE MEDIUM	140
4.3.4 THE EFFECT OF MONOMERIC sTNF-R1 ON LPS- OR TNF- α -INDUCED INHIBITION OF MDM ϕ EFFEROCYTOSIS	143
4.3.5 THE EFFECT OF TNF- α CONVERTING ENZYME INHIBITOR ON LPS- OR TNF- α -INDUCED INHIBITION OF MDM Φ EFFEROCYTOSIS	145
4.3.6 SUMMARY OF THE RESULTS	147
4.4 DISCUSSION	148
5 ANTI- AND PRO-INFLAMMATORY BIOLOGICS EXERT OPPOSING ACTIONS ON MΦ CLEARANCE OF APOPTOTIC NEUTROPHILS	155
5.1 INTRODUCTION	155
5.2 METHODS	157

5.2.1 ISOLATION AND CULTURE OF HUMAN BLOOD CELLS	157
5.2.2 ISOLATION AND CULTURE OF MOUSE BONE MARROW CELLS	158
5.2.3 ASSESSMENT OF APOPTOSIS AND NECROSIS	158
5.2.4 ASSESSMENT OF PHAGOCYTOSIS BY FLOW CYTOMETRY	158
5.2.4.1 HUMAN MDM Φ	158
5.2.4.2 MURINE BMDM Φ	159
5.2.5 MEASUREMENT OF TNF- α AND IL-10 CONCENTRATIONS IN CULTURE SUPERNATANTS	159
5.3 RESULTS	160
5.3.1 CHARACTERISATION OF TNF- α AND IL-10 PRODUCTION BY MDM Φ STIMULATED WITH LPS	160
5.3.2 CHARACTERISATION OF TNF- α AND IL-10 PRODUCTION BY MDM Φ STIMULATED WITH LTA OR PGN	163
5.3.3 PRESENCE OF EXOGENOUS IL-10 INHIBITS TNF- α PRODUCTION AND RESCUES INHIBITION OF MDM Φ EFFEROCYTOSIS INDUCED BY LPS	166
5.3.4 PRESENCE OF MONOCLONAL ANTI-HUMAN IL-10-R1 ANTIBODY AUGMENTS THE INHIBITORY EFFECT OF LPS	170
5.3.5 BMDM Φ FROM IL-10 $-/-$ MICE ARE CHARACTERISED BY LOWER CAPACITY FOR PHAGOCYTOSIS IN COMPARISON TO BMDM Φ FROM WILD TYPE MICE	172
5.3.6 DEXAMETHASONE RESCUES THE ABILITY OF LPS AND TNF- α TREATED MDM Φ TO PHAGOCYTOSE APOPTOTIC NEUTROPHILS	174
5.3.7 DEXAMETHASONE RESCUES THE ABILITY OF IL-1 β TREATED MDM Φ TO PHAGOCYTOSE APOPTOTIC NEUTROPHILS	177
5.3.8 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS DO NOT AUGMENT MDM Φ PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS	179
5.3.9 ASPIRIN INTERFERES WITH THE INHIBITORY EFFECT OF TNF- α ON MDM Φ EFFEROCYTOSIS OF APOPTOTIC NEUTROPHILS	184
5.3.10 SUMMARY OF THE RESULTS	187
5.4 DISCUSSION	188
6. GENERAL DISCUSSION AND FUTURE DIRECTIONS	203
6.1 INTRODUCTION	203
6.2 SUMMARY	206
6.3 CLINICAL IMPLICATION	212
6.4 FUTURE DIRECTIONS	216
6.4.1 THE EFFECTS OF TNF- α ON CLEARANCE OF APOPTOTIC INFLAMMATORY CELLS <i>IN VIVO</i>	216
6.4.2 THE ROLE OF IL-10 IN PREVENTION OF CHRONIC INFLAMMATION (ALTERNATIVE ACTIVATION OF M Φ)	217
6.4.3 THE MECHANISM UNDERLYING TNF- α -INDUCED INHIBITION OF PHAGOCYTOSIS	218
6.4.4 THE MECHANISM UNDERLYING IL-10- AND DEXAMETHASONE-INDUCED AUGMENTATION OF PHAGOCYTOSIS	219
<u>REFERENCE</u>	222
<u>APPENDIX ONE</u>	257

ABBREVIATIONS

ABBREVIATION	DEFINITIONS
AA	arachidonic acid
anti-IL-10-R1Ab	anti-human IL-10 receptor 1 antibody
AP-1	activator protein 1
Anx 1	annexin 1
AREs	AU rich elements
BMDMϕ	bone marrow-derived
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBA	cytometric bead array
CD	Crohn's disease
COPD	chronic obstructive pulmonary disease
COX1(2)	cyclooxygenase 1(2)
DC	dendritic cell
DISC	death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DX	Dexamethasone
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FITC	fluorescein isothiocyanate
GILZ	glucocorticoid-inducible leucine zipper
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HBSS	Hank's Buffered Salt Solution
IBD	inflammatory bowel disease
IC	immune-complex
IFN	Interferon
IL-1-R	interleukin-1 receptor
IL-1β(4,10)	interleukin 1 β (4,10)
IL-10-R	IL-10 receptor
IκBα	inhibitor kappa B- α

IKK	I- κ B kinase complex
IMDM	Iscove's modified Dulbecco's medium
IRAK	IL-1-R associated kinase
LBP	LPS binding protein
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotrienes
LTA	lipoteichoic acid
LX	Lipoxins
MAPK	mitogen-activated protein (MAP) kinases (e.g., p38 and JNK (c-Jun NH ₂ -terminal kinase), ERK1/2 (extracellular signal-regulated kinases))
MC	medium control
MD-2	myeloid differentiation 2 receptor
MM	molecular weight size marker
Mϕ	Macrophages
MDMϕ	monocyte-derived macrophages
MMP	matrix metalloproteinases
MS	multiple sclerosis
MyD88	myeloid differentiation primary-response gene 88
MTX	Methotrexate
Nϕ	Neutrophils
NAC	N-acetylcysteine
NFκB	nuclear factor kappa B
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drugs
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PG	Prostaglandins
PGN	Peptidoglycan
PI	propidium iodide
RIP1	receptor interacting protein 1
ROS	reactive oxygen species
SLE	systemic lupus erythematosus SLE
SOCS	suppressor of cytokine signalling

sTNF-R1	monomeric human recombinant soluble TNF receptor 1
sTNF-R1/Fc	dimeric human recombinant soluble TNF receptor 1/Fc chimera
TACE	TNF- α converting enzyme
TB	Tuberculosis
TGF-β	transforming growth factor β
TLR	Toll-like receptor family
TNF-α/sTNF-α/mTNF-α	tumor necrosis factor α / soluble (s) tumor necrosis factor α / transmembrane (m) tumor necrosis factor α
TNF-R1(2)/sTNF-R	TNF receptor 1(2)/soluble TNF receptor
TIRAP/MAL	myeloid differentiation primary-response gene 88 adaptor like protein
TRADD	TNF receptor-associated death domain
TRAF2(6)	TNF-R-associated factor 2(6)
TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing interferon β
TX	Thromboxane

CHAPTER ONE
INTRODUCTION

1. INTRODUCTION

1.1 INFLAMMATION

Inflammation is a defensive response of an organism that is induced in response to any trauma, i.e., infectious, post-ischaemic, toxic or autoimmune injury. An inflammatory response is characterised by highly complex interactions between the inflammatory cells (leukocytes) and other cells as well as soluble pro- and anti-inflammatory mediators. As long as it is controlled, inflammation is beneficial and leads to recovery from an injury and to healing. For instance, it has been shown that genetic deficiencies in principal components of the inflammatory process, e.g., type I, II or III leukocyte adhesion deficiency are associated with increased risk of grave infections (Etzioni, 2007). Furthermore, people unable to produce the complement components properdin and factors D, C5, C6, C7, C8 or C9 are predisposed to meningococcal infection (Biesma *et al.*, 2001). However, if inflammation does not progress to its resolving phase and occurs in an exaggerated manner, it leads to development of various pathological states, such as granulomas (aggregates of M ϕ and often other inflammatory cells), fibrosis (development of a tissue with excess of collagen) or carcinogenesis (DNA oxidation) (Nathan, 2002).

Inflamed tissues are usually characterised by redness (erythema), swelling, heat, pain and often loss of function, resulting from concerted action of a number of processes. For instance, augmented blood flow to the inflamed tissues leads to their redness and increased warmth, whereas changes into vascular permeability are associated with swelling or oedema. At the basic level, inflammatory response involves the recruitment of inflammatory cells (leukocytes) from blood vessel to the site of infection or injury (extravasation) (Medzhitov, 2008). A number of chemoattractants (platelet-activating factor (PAF), leukotriene B₄, various chemokines) is involved which play a crucial role in inducing leukocyte adherence on endothelial cells and stimulating leukocyte chemotaxis (Witko-Sarsat *et al.*, 2000; Kim, 2004). Recruitment of leukocytes involves several steps. First of all, inflammatory cells begin to adhere loosely (tethering and rolling) to the postcapillary endothelial cells. This process is primarily mediated by selectins (E-, L- and P-

selectins) and their respective ligands (Phillips *et al.*, 1995). Subsequently, inflammatory cells flatten and undergo firm adhesion to the endothelial cells due to the adhesive functions of the integrins (i.e., β_1 and β_2 integrins) and the immunoglobulin superfamily (i.e., ICAM-1, ICAM-2, VCAM-1) (Witko-Sarsat *et al.*, 2000). Eventually, inflammatory cells traverse the blood vessel wall and reach the affected area. The type of recruited cells is determined by a number of factors including the inflammatory stimuli present, structure and physiology of the vasculature, expression of various chemokines (e.g., IL-8 for neutrophils or eotaxin for eosinophils) or adhesion molecules (e.g., VLA-4 [$\alpha_4\beta_1$] is highly expressed on eosinophils and lymphocytes but not on neutrophils) (Yang & Haggmann, 2003). Once leukocytes reach the site of inflammation, they are activated by either direct contact with pathogens or through the actions of mediators secreted by tissue-resident cells. Induction of the inflammatory response by pathogens and mediators regulating inflammatory processes are briefly reviewed in chapter 1.2.

Activated leukocytes e.g., neutrophils, at the infected or injured tissue, exert multiple functions including phagocytosis, antigen presentation or production of immune, potentially toxic mediators such as reactive oxygen species (ROS), reactive nitrogen species, antimicrobial peptides (e.g., defensins), proteases (e.g., protease 3, cathepsin G, elastase) and matrix metalloproteinases (MMP) (Witko-Sarsat *et al.*, 2000). Since some of these mediators do not discriminate between microbial and host cells, their release is usually associated with the increased risk of host tissue breakdown. Consequently, the acute inflammatory response is successful only if it is followed by resolution and repair phases (Medzhitov, 2008). The mechanisms involved in the resolution phase of inflammation are described in more detail in chapter 1.3.

1.2. INFLAMMATORY MEDIATORS

The inflammatory response is regulated by a number of mediators, including many cytokines (a major subject of this thesis). The term ‘cytokine’, derived from *cyto* meaning ‘cell’ and *kinin* meaning ‘hormones’ was first proposed by Stanley Cohen in 1974 (Cohen, 2004). Today, it comprises of a family of proteins such as lymphokines, monokines, interleukins, colony stimulating factors (CSFs), tumor necrosis factor alpha (TNF- α) and chemokines (Tayal & Kalra, 2008), which are produced in response to inducing stimuli mainly by helper T-cells or M ϕ but also other cell types. All cytokines share some common properties:

- Pleiotropy of action – cytokines may influence growth and differentiation of many different cell types or exert opposing actions within different immune and inflammatory pathways.
- Redundancy – different cytokines may exert the same effect on cells.
- Multifunctionality – the same cytokine may regulate multiple immune functions.

Cytokines are a fundamental part of the immune system. They generate and control its responses and play an important role in communication between cells or cells and their environment. Thus, abnormalities in cytokine production, their receptors as well as signalling pathways that they initiate are involved in a wide variety of diseases. The role of cytokines in pathogenesis of inflammatory diseases is described in section 1.4.1.

1.2.1. TUMOR NECROSIS FACTOR – ALPHA

Tumor necrosis factor alpha (TNF- α) is one of the most rapidly released cytokines after exposure to pro-inflammatory stimuli such as pathogens or injury. It was first discovered about 30 years ago as a cytokine that is produced upon activation by the immune system and able to cause necrosis in many tumor cell lines *in vitro* and in certain animal models (Sugarman *et al.*, 1985; Old, 1988). Initially, TNF- α was believed to directly induce apoptosis of tumor cells and therefore to be a powerful tumoricidal agent. However, further studies revealed that this was not the case and that the anti-tumoral activity of TNF- α is influenced by a functional immune response (Hock *et al.*, 1993). In addition, systemic administration of TNF- α in humans results in serious side effects, ranging from influenza-like symptoms to the development of shock (Mannel & Echtenacher, 2000). Yet, in conditions that prevent systemic administration, such as isolated limb perfusion, TNF- α in combination with melphalan (chemotherapeutic) has been shown effective in treatment of soft-tissue sarcoma (Eggermont & ten Hagen, 2001). The underlying mechanism involves degeneration of the tumor vasculature leading to death of tumor cells (Ruegg *et al.*, 1998).

TNF- α is the prototypical member of the TNF superfamily of cytokines able to induce signalling pathways for inflammation, cell proliferation, differentiation, cell survival and cell death. Interestingly, TNF- α may exert opposing effects such as stimulation of processes of regeneration or destruction, depending on many factors such as type of tissue, type of TNF- α receptors (TNF-R) or timing and duration of TNF- α action *in vivo* (Wajant *et al.*, 2003). One example here is the role of TNF- α in neurodegeneration. Although in transgenic mice, upregulation of TNF- α in the CNS results in demyelination, TNF- α expression is also induced in response to brain injury, thus, potentially exerting protective functions (Probert *et al.*, 1995). Furthermore, in a murine model of retinal ischaemia, the net effect of TNF- α action was dependent upon the type of TNF-R expressed in a tissue. In the presence of TNF-R1, TNF- α potentiated tissue destruction while in the presence of TNF-R2 it had a tissue-protective function (Fontaine *et al.*, 2002). Abnormalities in TNF- α production or sustained activation of TNF- α signalling has been associated with numerous

pathological conditions including sepsis, cerebral malaria, diabetes, cancer, osteoporosis, allograft rejection and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease (IBD) (Chen & Goeddel, 2002; Feldmann *et al.*, 2005).

To exert its biological functions TNF- α interacts with its cognate membrane receptors – TNF-R superfamily, whose members include TNF-R1 (CD120a, p55/60), TNF-R2 (CD120b, p75/80), Fas antigen, CD27, CD30, CD40 and several other receptors (Smith *et al.*, 1994). All of them are characterised by some sequence similarities in their extracellular domain (one to six cysteine-rich repeats) and usually no similarities in their intracellular domain (Naismith & Sprang, 1998). However, both TNF-R1 and Fas can induce apoptosis and share 28% identity of their intracellular ‘death domains’ (Tartaglia *et al.*, 1993). Two receptors TNF-R1 and TNF-R2 were found to interact with TNF- α . The membrane-bound TNF- α (mTNF- α) activates both receptors and its soluble form (sTNF- α) mainly stimulates TNF-R1. TNF-R1 is constitutively expressed in most tissues, while expression of TNF-R2 is highly regulated and takes place only in immune cells. The proteolytical cleavage of extracellular domains of both receptors results in formation of soluble receptors fragments. Such soluble receptors (sTNF-R) have been demonstrated to be able to neutralise the activity of TNF- α (Wallach *et al.*, 1991). For instance, sTNF-R1 has been reported to play a crucial role in the regulation of TNF- α functions *in vivo* and cleavage-resistant TNF-R1 mutations are linked with dominantly inherited autoinflammatory syndromes (TNF-R1 associated periodic syndromes; TRAPS) (McDermott *et al.*, 1999).

Furthermore, binding of TNF- α to TNF-R1 triggers activation of two important transcription factors: nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1). These transcription factors regulate expression of genes important for the majority of biological functions of TNF- α (Wajant *et al.*, 2003). In addition, TNF- α has the potential to induce apoptosis *via* the cytoplasmic death domain of TNF-R1. The latter phenomenon has been observed in a variety of cell lines and *in vivo* models but, interestingly, this effect of TNF- α is masked by simultaneous activation of NF- κ B pathway (Chen & Goeddel, 2002).

1.2.2 TNF- α PRODUCTION

TNF- α is primarily produced by monocytes and M ϕ as a transmembrane protein (mTNF- α) that forms stable homotrimers (51 kDa). The TNF- α protomers (17 kDa; 157 amino acid) are built up of two antiparallel β -pleated sheets with antiparallel β -strands forming a β -structure named 'jelly roll' (Bazan, 1993). As shown in Figure 1.1, sTNF- α is released after proteolytic cleavage by TNF- α converting enzyme (TACE) (Black *et al.*, 1997). Pro-inflammatory mediators stimulate production of sTNF- α whereas anti-inflammatory mediators usually exert opposite effects (see below).

FIGURE 1.1 Schematic representation of mTNF- α processing by TACE. A soluble form of TNF- α (sTNF- α) is produced from its transmembrane precursor (mTNF- α) by proteolytic cleavage catalysed by transmembrane TNF- α converting enzyme (TACE). sTNF- α exerts its biological responses *via* two transmembrane TNF- α receptors, namely TNF-R1 and TNF-R2 or alternatively is deactivated after binding to their soluble forms (e.g. sTNF-R1).

1.2.2.1 TLR AGONISTS ARE POSITIVE REGULATORS OF TNF- α PRODUCTION

The human innate immune system provides a first line of defence against pathogens. Bacteria stimulate an inflammatory response *via* highly conserved pathogen-associated molecular patterns (PAMP) interacting with Toll-like receptor family (TLR) and scavenger receptors (Kopp & Medzhitov, 2003). PAMP motifs are carried by bacterial lipopolysaccharide (LPS), lipopeptides, DNA, dsRNA (double-stranded ribonucleic acid) or ssRNA (single-stranded ribonucleic acid). Ten members of the TLR family have been identified so far in humans (Means *et al.*, 2000). Initially, TLR receptors have been demonstrated to be implicated in control of embryonic development (Hashimoto *et al.*, 1988). However, further studies revealed that TLR intracellular carboxyl terminal domain (Toll/IL-1-R (TIR)) shares great similarity with that of the vertebrate interleukin-1 receptor (IL-1-R). Activation of IL-1-R is correlated with acute phase response to bacterial or fungal infections (Hashimoto *et al.*, 1988).

LPS is a core element of the outer membrane of Gram-negative bacteria cells. It is composed of three distinct domains, namely lipid A, a short core of oligosaccharide and O-antigen polysaccharide. Lipid A domain carries PAMP motifs which are recognised by TLR4 (Chow *et al.*, 1999). However, before LPS is able to interact with TLR4, LPS aggregates have to be disrupted by binding to LPS-binding protein (LBP) (O'Neill & Bowie, 2007). LPS-LBP complex is then presented to TLR4 by another adaptor protein-a monocyte/M ϕ receptor molecule (CD14) (Wright *et al.*, 1990) resulting in TLR4 homodimerisation and binding of myeloid differentiation 2 receptor (MD-2) (Shimazu *et al.*, 1999). Finally, an intracellular TIR domain is assembled into the complex, which as demonstrated in Figure 1.2, recruits several adaptor proteins that trigger a signalling cascade regulating gene expression (Jerala, 2007). Several adaptor proteins have been so far identified including myeloid differentiation primary-response gene 88 adaptor like protein (TIRAP/MAL) which acts as a bridging adaptor to recruit the myeloid differentiation primary-response gene 88 (MyD88). The recruitment of MyD88 induces activation of IL-1-R associated kinase 4 (IRAK4) and IRAK1 and subsequently TNF-R-associated factor

6 (TRAF6). The latter results in activating of transcription factors NF- κ B and AP-1 as well as p38 and JNK/MAPK kinases (Doyle & O'Neill, 2006). In the second signalling pathway, triggered by the TLR complex, TRIF related adaptor molecule (TRAM) recruits TIR-domain-containing adaptor protein inducing interferon β (TRIF), which, in turn, activates several signalling pathways leading from either TBK1 to IRF3 or TRAF6 to NF- κ B as well as from receptor interacting protein 1 (RIP1) to apoptosis (Jiang *et al.*, 2005).

The process of TNF- α production is highly controlled by multiple and complex mechanisms. TNF- α gene transcription involves a number of transcription factors, namely CREB, LITAF, ATF-2, c-jun, Egr-1, NFAT, Ets, Elk-1, Sp1 or NF- κ B (Skinner *et al.*, 2008). Some of them, e.g., NF- κ B are activated upon stimulation of cells with LPS. Furthermore, LPS has been demonstrated to upregulate TNF- α production on post-transcriptional levels by increasing the stability and translation of TNF- α mRNA. LPS stimulation activates several MAP Kinases (MAPK), which were originally described as LPS-activated kinases (Han *et al.*, 1994). Their relevance to TNF- α production has been established with the use of MAPK inhibitors. For instance, blockade of p38 MAPK activation with pyridinyl imidazole-based inhibitor SB203580 substantially reduced translation of TNF- α mRNA (Campbell *et al.*, 2004). Similarly, mice that do not express MAPK-activated protein kinase-2 (MK2), show a profound defect in translation of TNF- α mRNA. MK2 is an immediate substrate of p38 MAPK. It has been proposed that p38 MAPK may control stability of TNF- α mRNA or activity of NF- κ B (Kotlyarov *et al.*, 1999). Moreover, Tpl2/ERK signalling pathway has been reported to be implicated in nuclear export of TNF- α mRNA to the cytoplasm. In resting cells Tpl2 is bound to NF- κ B p105. However, when cells are stimulated with either TLR ligands (e.g., LPS) or pro-inflammatory cytokines, Tpl2 separates and activates ERK pathway which is critical for nuclear transport of TNF- α (Dumitru *et al.*, 2000). Post-transcriptional regulation of TNF- α was demonstrated to operate mainly through AU rich elements (ARE) in the 3'-UTR of TNF- α mRNA. In unstimulated cells, ARE may exert a destabilising effect on TNF- α mRNA and contribute significantly to the permanent silencing of TNF- α mRNA. Yet, in activated cells, ARE increase both stability and translation of TNF- α mRNA by playing an important role in its nuclear transport.

ARE serve as binding sites for certain proteins (i.e. HuR) and their functional state is regulated by JNK/SAPK and p38 MAPK-mediated signals, which may be induced by LPS stimulation (Kontoyiannis *et al.*, 1999). Consequently, ARE are critical for restoration of homeostasis in TNF- α biosynthesis by mediating destabilization of TNF- α mRNA and translational silencing after the induction phase of the inflammatory response.

Figure 1.2 Schematic representation of TLR-4 signal transduction pathway.

Following TLR-4 homodimerisation several adaptor proteins are assembled into TLR-4 complex. Two of them, MyD88 and TRIF, activate downstream kinases e.g., IRAK1/4 and TBK1 and subsequently a few transcription factors i.e., NF- κ B, AP1 and IRF. In addition, assembly of RIP leads to execution of apoptosis. Key adaptor proteins and enzymes involved in TLR-4 mediated signalling are described in more depth in chapter 1.2.2.1.

1.2.2.2 IL-10 IS A NEGATIVE REGULATOR OF TNF- α PRODUCTION

IL-10 is an anti-inflammatory cytokine, first described as a soluble factor that is able to inhibit production of cytokines e.g., IL-2 and interferon- γ (IFN- γ) by Th1-type T cells (Fiorentino *et al.*, 1989). This phenomenon is a consequence of the inhibitory effect of IL-10 on antigen-presenting cells (APC), namely monocytes, M ϕ and dendritic cells (Kaur *et al.*, 2008). IL-10 is encoded by a single gene that is highly homologous to an open reading frame of Epstein-Barr virus genome (Moore *et al.*, 1990). In its bioactive form, IL-10 is a homodimer composed of two 18 kDa subunits that interacts with a single class of cell surface receptors (IL-10-R). These receptors are mostly expressed by B cells, T cells, NK cells, monocytes and M ϕ . The functional IL-10-R is a dimer of heterodimers of IL-10-R1 and IL-10-R2. IL-10-R1 serves as a ligand-binding subunit and recruits JAK1 kinase into IL-10-R. In contrast, IL-10-R2 recruits Tyk2 and plays a role in signal transduction (Mosser & Zhang, 2008). Presence of IL-10 induces bridging of two IL-10-R1 molecules and their paired association with two IL-10-R2 molecules. Formation of IL-10-R activates Tyk2 kinase which subsequently *trans*-phosphorylates IL-10-R1 and JAK1. The latter is essential for activation and nuclear translocation of a transcription factor STAT3, which drives transcription of various IL-10-responsive genes (Donnelly *et al.*, 1999). One is a suppressor of cytokine signalling-3 (SOCS-3) that has been demonstrated to feedback inhibit a JAK/STAT pathway - a common signalling pathway for cytokines (see Figure 1.3). Importantly, IL-10-R does not have SOCS-3 binding sites and therefore is not regulated by this factor *per se* (Murray, 2007). The mechanism by which SOCS proteins inhibit the JAK/STAT signalling is thought to involve binding of the appropriate substrate by a SOCS Src homology 2 (SH2) domain followed by formation an E3 ubiquitin ligase by a second domain (SOCS box). The formation of the E3 ubiquitin ligase involves interactions of SOCS box with several proteins i.e., elongins B and C, a cullin and Rbx2 and mediates proteasome degradation of the substrate (O'Shea & Murray, 2008).

IL-10 is produced by a number of cell types (T cells, B cells, monocytes, M ϕ , dendritic cells) in response to a number of stimuli, e.g., Gram-positive or Gram-

negative bacteria, purified endotoxin, bacterial exotoxin or certain viruses. Following LPS stimulation, production of IL-10 is delayed in comparison to other cytokines (e.g., TNF- α , IL-1, IL-6, IL-8, IL-12) (de Waal *et al.*, 1991). Furthermore, its appearance is associated with downregulation of other cytokines such as TNF- α . Thus, it has been suggested that IL-10 is a negative regulator of continued cytokine production (Donnelly *et al.*, 1995). This hypothesis has been further supported by the observation that in LPS-stimulated monocytes, neutralisation of IL-10 activity with anti-IL-10 antibodies prolongs TNF- α expression and increases 'net' TNF- α production (Berg *et al.*, 1995). However, the exact molecular mechanism underlying IL-10 inhibition of LPS-inducible gene expression is yet to be identified. Initially, it has been suggested that IL-10 blocks activation of NF- κ B or MAPK by LPS but no consensus has been reached with this regard (Donnelly *et al.*, 1999). Interestingly, it has been demonstrated in monocytes that IL-10 induces *de novo* synthesis of SOCS-3 and that this correlates with inhibited expression of LPS - inducible cytokines, such as TNF- α or IL-1 (Donnelly *et al.*, 1999). Moreover, phenotypic analysis of JAK1 deficient mice (Rodig *et al.*, 1998) and targeted deletion of STAT3 in neutrophils and M ϕ (Takeda *et al.*, 1999) revealed that both JAK1 and STAT3 are critical for IL-10 inhibition of LPS - induced cytokine production. Therefore it is possible that IL-10-induced SOCS-3 not only interferes with cytokine signalling but also inhibits their production.

FIGURE 1.3 Schematic representation of the IL-10 signal transduction pathway.

Transduction of IL-10 signalling is initiated by formation of the IL-10-R complex that is composed of IL-10 homodimer and two IL-10-R1/IL-10-R2 heterodimers. The formation of IL-10-R complex activates tyrosine kinases Jak1 and Tyk2 and subsequent phosphorylation of intracellular domains of IL-10-R1. The latter results in activation and homodimerisation of a transcription factor STAT3, which translocates to the nucleus and drives transcription of IL-10-induced genes, such as SOCS-3. IL-10 signal transduction pathway and key proteins are described in more depth in section 1.2.2.2.

1.2.3 TNF- α SIGNALLING

TNF- α signalling is initiated by binding of a TNF- α trimer to the extracellular domain of TNF-R1. This event causes a release of the inhibitory protein named silencer of death domain (SODD) from the intracellular domain TNF-R1 (Jiang *et al.*, 1999). Following SODD liberation, intracellular TNF-R1 domain is recognised by an adaptor protein called TNF receptor-associated death domain (TRADD). As indicated in Figure 1.4, TRADD serves as an assembly platform for recruitment of additional adaptor proteins: receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2) and Fas-associated death domain (FADD) (Hsu *et al.*, 1996), which initiate distinct signalling pathways (Wajant *et al.*, 2003).

1.2.3.1 NF- κ B ACTIVATION via RIP SIGNALLING

Following TNF- α stimulation, the signalling pathway for NF- κ B activation is initiated by association of TRADD and subsequent assembly of TRAF2 and RIP into the TNF-R1 complex (Chen & Goeddel, 2002). Both TRAF2 and RIP play an important role in the degradation of inhibitor κ B (I- κ B) *via* activating a multicomponent I- κ B kinase (IKK) complex (Ghosh & Karin, 2002). It has been shown that TRAF2 is responsible for recruitment and RIP for activation (possibly by interacting with mitogen-activated kinase kinase kinase (MEKK3)) of IKK kinases within TNF-R1 signalling complex (Devin *et al.*, 2000).

The NF- κ B/Rel family of transcription factors consists of 5 members (dimers) in mammalian cells: p105 (constitutively processed to NF- κ B1/p50), p100 (processed to NF- κ B2/p52 in certain circumstances), c-Rel, RelA/p65 and RelB (Verma *et al.*, 1995). These factors, in response to some cytokines (i.e., TNF- α and IL-1), bacterial products (i.e., LPS) or physical stress (e.g., UV radiation or ROS) activate transcription of numerous inflammatory-related genes (Baud & Karin, 2001). Two different signalling pathways are involved in activation of NF- κ B (Simmonds & Foxwell, 2008). One of them, namely 'classical' or 'canonical', involves phosphorylation-dependent ubiquitination and degradation of I κ B proteins. I κ B proteins are normally responsible for retention of NF- κ B within the cytoplasm of

unstimulated cells. In the ‘alternative’ or ‘non-canonical’ pathway, the p100 itself (not I- κ B) inhibits and retains RelB in the cytosol. Detailed description of both pathways is presented below.

FIGURE 1.4 Schematic representation of TNF- α signal transduction pathway.

Engagement of TNF- α with TNF-R1 results in assembly of several adaptor proteins, namely TRADD, FADD, TRAF2 and RIP, followed by recruitment of additional key enzymes (e.g. caspases-8, IKK2) that initiate downstream processes leading to apoptosis, cell survival or activation of transcription factors such as NF- κ B or AP1. Detailed description of TNF- α signal transduction pathway and key adaptor proteins and enzymes is presented in section 1.2.3.

1.2.3.1.1 THE CANONICAL PATHWAY OF NF- κ B ACTIVATION

All NF- κ B/Rel proteins possess a highly conserved Rel homology domain (RHD), which facilitates dimerisation, DNA binding, nuclear localization and interaction with the members of inhibitory I- κ B protein family. However, only three NF- κ B/Rel proteins (p65, RelB, c-Rel) contain transactivation domains (TADs) enabling their interaction with general transcription factors and consequently induction of gene expression. The presence of TADs determines whether specific NF- κ B/Rel protein acts as an activator or a repressor of transcription. For instance, common heterodimer p50/p65 acts as an activator of gene transcription due to the presence of a TAD in p65 subunit. On the contrary, the p50 subunit does not have the TAD domain and therefore acts as a repressor of transcription by competing for p50/p65 binding to the NF- κ B consensus sequence (Simmonds & Foxwell, 2008).

In unstimulated cells, the activity of NF- κ B is blocked by I- κ B proteins i.e., I- κ B α , I- κ B β , I- κ B γ , I- κ B ϵ , Bcl-3 or p105 and p100 (the precursors of NF- κ B1 and NF- κ B2 respectively). I- κ B proteins mask the nuclear localization sequences in NF- κ B dimers and therefore they stay in the cytoplasm (Wajant *et al.*, 2003). Upon cell activation, e.g., with LPS, TNF- α or IL-1, I- κ B is degraded by the proteasome and NF- κ B is liberated. Further modifications (phosphorylation or acetylation of subunits) result in NF- κ B translocation into nucleus (Perkins, 2000). Several kinases have been reported to take part in this secondary process i.e. mitogen activated protein kinases (MAPK) and protein kinase C (PKC) (Wajant *et al.*, 2003).

It has been demonstrated that I- κ B degradation is facilitated by a multicomponent protein kinase complex, I- κ B kinase complex (IKK) (Simmonds & Foxwell, 2008). Various cellular stimuli, e.g., LPS or TNF- α induce signalling pathways that converge in the formation of IKK. This complex is composed of a number of proteins including two related I- κ B kinases, IKK1 and IKK2 (IKK α and IKK β), regulatory protein NEMO (Fip3, IKK γ , IKKAP), a heat shock protein – 90 (Hsp90) and Hsp90 – associated cdc37 protein (Chen & Goeddel, 2002). The activated IKK facilitates phosphorylation of the regulatory domain of I- κ B giving a signal for its recognition by an SKP1-Cullin-Fbox-type E3 ubiquitin-protein ligase complex (Wajant *et al.*, 2003).

One interesting feature of the canonical NF- κ B pathway is its rapid but short activation. It prevents a persistent response that can potentially result in pathological changes in affected cells. Indeed, it has been demonstrated that a promoter of the I- κ B gene is highly responsive to NF- κ B activation and contains 11 consensus sequences recognisable by this transcription factor. Consequently, whenever NF- κ B triggers gene expression, I- κ B is also produced and, in turn, binds NF- κ B dimers reducing their activity (Covert *et al.*, 2005).

1.2.3.1.2 THE NON-CANONICAL PATHWAY OF NF- κ B ACTIVATION

The non-canonical (alternative) pathway of NF- κ B activation is specific for B cells. In unstimulated cells, p100 acts as an inhibitory factor responsible for retention of RelB in the cytosol. However, when B cells are stimulated, p100 is processed by IKK1 complexed with NF- κ B inducing kinase NIK and then proteolytically cleaved by the proteasome. This leads to liberation of the p52 subunit and formation of transcriptionally active p52/RelB heterodimers (Xiao *et al.*, 2006). It is thought now that p100 is a new member of the I- κ B family, designated I- κ B δ (Basak *et al.*, 2007).

1.2.3.2 THE ANTI-APOPTOTIC ACTION AND MAPKKK ACTIVATION *via* TRAF2 SIGNALLING

Following TNF- α stimulation, anti-apoptotic signalling begins with recruitment of an adaptor protein TRAF2 into TNF-R1 signalling complex (see Figure 1.4). It has been demonstrated that TRAF2 depletion sensitises cells for the pro-apoptotic action of TNF- α (Li *et al.*, 2002; Fotin-Mleczek *et al.*, 2002; Brown *et al.*, 2002a). This observation can be explained by the fact that TRAF2 mediates recruitment of the inhibitor of apoptosis proteins (IAP), namely cIAP1 and cIAP2 into the TNF-R1 complex (Shu *et al.*, 1996). Both cIAP1 and cIAP2 inhibit activity of caspase-3 and caspase-7 *via* binding to their amino-terminal BIR (baculovirus IAP repeat) domains (Roy *et al.*, 1997). Furthermore, the carboxy-terminal RING domain of cIAP1 and cIAP2 proteins act as E3 ubiquitin ligase involved in proteosomal degradation of caspase-3 and -7 (Yang *et al.*, 2000). A more detailed description of the IAP family of proteins and their role in regulation of apoptosis is presented in

chapter 1.3.1. On the contrary, stimulation of TNF-R2 and assembly of TRAF2 into the TNF-R2 complex augments TNF-R1-induced cell death possibly due to competition of TNF-R1 and TNF-R2 for binding of TRAF2 and associated cIAP1 and cIAP2 proteins (Fotin-Mleczek *et al.*, 2002).

TRAF2 is also thought to activate a mitogen-activated protein kinase kinase kinase (MAPKKK) such as extracellular signal-regulated kinase kinase kinase 1 (MEKK1) or apoptosis-stimulated kinase 1 (ASK1), which, in turn, activate a cascade of kinases regulating activation of c-Jun NH₂-terminal kinase (JNK). The latter kinase phosphorylates c-Jun enhancing its transcriptional activity (Chen & Goeddel, 2002). c-Jun belongs to a family of AP-1 transcription factors, which play an important role in a variety of cellular processes such as proliferation, differentiation or prevention of apoptosis.

1.2.3.3 THE PRO-APOPTOTIC PATHWAY *via* FADD SIGNALLING

Following TNF- α stimulation, signalling for apoptosis begins with association of an adaptor molecule FADD to TNF-R1 signalling complex *via* the bridging molecule TRADD. As indicated in Figures 1.4 and 1.5, subsequent interaction of receptor-bound FADD with inactive caspase-8/10 *via* their death effector domains leads to their autoproteolytic cleavage and initiation of the apoptosis process (Chen & Goeddel, 2002). After activation of caspase-8, downstream signalling for apoptosis occurs in principle through the same pathways for all death receptors, i.e., Fas, TRAIL-R1/2 or TNF-R1 and depends on the type of cell involved. As shown in Figure 1.5, in type 1 cells, activated caspase-8 *per se* is sufficient to process robustly pro-caspase-3 into caspase-3 and induce the execution phase of apoptosis. The activated caspase-3 cleaves inhibitors of caspase-activated DNase resulting in degradation of DNA in the nucleus. In type 2 cells, processing of pro-caspase-3 by caspase-8 alone is not sufficient for triggering apoptosis. Thus, efficient activation of pro-caspase-3 in this model depends on release of cytochrome c from mitochondria and formation of the caspase-3-inducing apoptosome complex. Activated caspase-3

is also able to further activate pro-caspase-8 creating a positive feedback loop (Li *et al.*, 1998).

Interestingly, as shown in Figure 1.4, the pro-apoptotic effect of TNF- α is masked *in vivo* by simultaneous activation of NF- κ B (TNF-R1 is mainly an NF- κ B inducing receptor). In contrast, the other death receptors such as Fas or TRAIL-R1/2 show significant pro-apoptotic functions *in vivo* and Fas- or TRAIL-R1/2 – induced NF- κ B activation occurs only when the pro-apoptotic pathway is blocked (Wajant *et al.*, 2000). The above phenomenon could be explained, at least in part, by various abilities of death receptors to assemble a death-inducing signalling complex (DISC). While formation of DISC has been proven for Fas and TRAIL receptors, demonstration of a TNF-R1–DISC complex has not been successful (Wajant *et al.*, 2003). Possibly, in the case of TNF-R1, DISC complexes are characterised by much lower stability or additional regulatory mechanisms exist that selectively affect formation or activity of DISC. For example, several research groups have observed that TRAF2 depletion (adaptor protein specific for TNF-R1 but not Fas or TRAIL signalling complex) sensitises cells for TNF- α –induced apoptosis (Li *et al.*, 2002). The anti-apoptotic role of TRAF2 signalling is described in more detail in section 1.2.2.2.

Furthermore, it has been demonstrated that exclusive activation of TNF-R2 with agonistic TNF-R2–specific antibodies also induces cell apoptosis. The underlying mechanism involves induction of endogenous mTNF- α by activated TNF-R2. Subsequently, mTNF- α triggers TNF-R1 signalling (Grell *et al.*, 1999). Furthermore, the co-existing stimulation of TNF-R2 greatly augments the pro-apoptotic signalling from TNF-R1 by recruitment of TRAF2 and TRAF2–associated anti-apoptotic factors into TNF-R2 signalling complex. Similarly, apoptosis may be induced by other members of the TNF receptor family which do not have intracellular death domains (Eliopoulos *et al.*, 2000).

FIGURE 1.5 Schematic representation of death signalling pathways in granulocytes. Binding of TNF- α to its receptor (TNF-R1) activates procaspase-8 *via* death receptor associated proteins (TRADD, FAD). Caspase-8-directed pathways lead to apoptosis through caspase cascade or mitochondrial pathway associated with cleavage of Bid, release of cytochrome c from mitochondria and formation of the apoptosome. Both pathways finally activate pro-caspase-3 resulting in apoptosis. The death signalling pathways in granulocytes are described in more detail in section 1.2.3.3.

1.3 RESOLUTION OF INFLAMMATION

Currently, two key mechanisms are postulated to play a crucial role in successful resolution of the inflammatory response, namely apoptosis of inflammatory cells and their clearance by phagocytes. Apoptosis is a mode of cell death that, in contrast to necrosis (cell disintegration), does not seem to trigger pro-inflammatory responses and plays an essential role in almost all physiological processes (Haslett, 1999;Wyllie *et al.*, 1980). Apoptotic cells are characterised by several common features such as rapid shrinkage of the cytoplasm, nuclear coalescence, membrane budding and finally formation of one or more apoptotic bodies. More importantly, during apoptosis membrane integrity is maintained and potentially toxic intracellular contents are not liberated into surrounding tissue. This, in turn, prevents tissue damage and consequential propagation and exaggeration of inflammatory processes. Yet, effective apoptosis of activated granulocytes is not the only mechanism determining successful resolution of inflammatory responses. Apoptotic cells must be subsequently removed by neighbour cells or in higher organisms by professional phagocytes to avoid cell disintegration (secondary necrosis) and the release of histotoxic intracellular material leading to harmful chronic inflammatory effects. In mammals, apoptotic granulocytes are thought to undergo non-inflammatory phagocytosis by M ϕ or dendritic cells but in certain circumstances other cell types such as endothelial cells (Dini *et al.*, 1995), vascular smooth muscle cells (Bennett *et al.*, 1995) and fibroblasts may also remove apoptotic cells (Hall *et al.*, 1994). Moreover, it has been shown that M ϕ clearance of apoptotic cells (efferocytosis) induces anti-inflammatory responses in phagocytes such as secretion of anti-inflammatory cytokines, e.g., TGF- β , IL-10 and IL-13 (Huynh *et al.*, 2002;Hoffmann *et al.*, 2005). In contrast, uptake of necrotic cells including secondarily necrotic cells derived from nonengulfed apoptotic cells, stimulates release of pro-inflammatory mediators i.e. TNF- α , IL-1 β , NO (Fadok *et al.*, 1998;Voll *et al.*, 1997;Fadok *et al.*, 2001). It is now accepted that failure of sufficient clearance of apoptotic cells and subsequent secondary necrosis induces pro-inflammatory responses and may lead to the development of autoimmune diseases such as systemic lupus erythematosus (SLE) (Ren *et al.*, 2003), cystic fibrosis

(Vandivier *et al.*, 2002a) or chronic obstructive pulmonary disease (COPD) (Hodge *et al.*, 2003).

1.3.1 APOPTOSIS OF INFLAMMATORY CELLS

Apoptosis is a highly conserved process that engages similar pathways and enzymes in almost all living organisms (Hengartner, 2000). One of the examples here is a family of caspases (proteases dependent on a cysteine nucleophile), which cleave motifs possessing aspartic acid. Caspases are produced as inactive pro-enzymes, composed of a large and a small subunit preceded by an N-terminal pro-domain. When pro-caspase undergoes activation, two Asp sites are cleaved sequentially and the large and the small subunits associate to provide the active site of the enzyme. In fact, the active caspase is a tetramer of two heterodimers and contains two active sites. Upstream caspases (initiators) can activate themselves in an autocatalytic way while downstream caspases (effectors) require initiator caspases for their activation *via* transprocessing. The examples of effector caspases implicated in an execution phase of apoptosis are caspase-3 and caspase-7 (Danial & Korsmeyer, 2004). Activation of caspases leads to morphological changes associated with apoptosis, namely DNA degradation, chromatin condensation and membrane blebbing (Fulda & Debatin, 2006). In order to protect cells from unwanted execution of apoptosis a family of proteins (IAP) has evolved. IAP were first identified as baculovirus proteins that block apoptosis of infected cells *via* direct or indirect inhibition of caspases (Clem *et al.*, 1991). These proteins are characterised by one to three zinc-binding motifs whose role is to bind activated caspases. These motifs are termed baculovirus IAP repeat (BIR) (Salvesen & Duckett, 2002). Some of the IAP contain also an additional, highly conserved, carboxy-terminal RING domain, which plays an important role in targeted degradation of proteins by ubiquitylation (Roy *et al.*, 1997). In humans, there are eight distinct IAP able to block the cytokine cascade and promote cell survival, namely cIAP-1, cIAP-2, XIAP, ILP-2, NAIP, ML-IAP, apollon and survivin (Danial & Korsmeyer, 2004). Their activity is, in turn, controlled by a number of other proteins, e.g., SMAC, which possess IAP binding motifs (IBM). IBM recognise and bind IAP *via* their BIR domains and consequently, active

caspases which execute the apoptosis process (Chai *et al.*, 2000). However, some IAP, e.g., survivin, interact directly with SMAC and inhibit its activity (Song *et al.*, 2003). Interestingly, survivin has been demonstrated to be expressed mainly by transformed cell lines and malignancies but not in normal adult tissues. Furthermore, cells expressing survivin, such as cancer cells, are resistant to various pro-apoptotic stimuli including a wide range of anti-cancer agents (Zangemeister-Wittke & Simon, 2004). In addition, survivin forms complexes with hepatitis BX-interacting protein (HBXIP) or XIAP and interferes with activation of caspase 9 in this way (Marusawa *et al.*, 2003). The role of cIAP-1 and cIAP-2 in TNF- α signalling is described in section 1.2.3.2.

Three different pathways are involved in the activation of effector caspases and subsequent execution of the apoptosis process: the extrinsic death receptor pathway (type I cells), the intrinsic (mitochondrial) pathway (type II cells) and the endoplasmic reticulum (ER) or stress-induced pathway (Fulda & Debatin, 2006). The extrinsic death receptor pathway, induced by ligands of TNF superfamily membrane receptors and associated with formation of DISC has been briefly described in chapter 1.2.3.3. The ER/stress induced pathway has not been characterised well yet but involves activation of caspase 12 by Ca^{2+} and oxidant stress (Kadowaki *et al.*, 2004). The mitochondrial pathway can be initiated by the absence of growth factors, e.g., IL-2, IL-4 or granulocyte M ϕ -colony stimulating factor (GM-CSF) as well as a presence of numerous mediators such as IL-1, IL-6, steroids, ROS, peroxynitrite and NO which, in turn, activate pro- or anti-apoptotic members of BCL-2 family (Fulda & Debatin, 2006). The BCL-2 family of proteins can be divided into three subclasses based on the sequence similarities within four BCL-2 homology (BH) 1-4 domains. BAK, BAX and BID are the pro-apoptotic members of the BCL-2 family and possess either three BH1-3 domains (BAK or BAX) or one BH3 domain (BID). In contrast, anti-apoptotic members of the BCL-2 family (BCL-2, BCL-X_L, MCL-1, A1 and BCL-W) are highly homologous within all four BH1-4 domains. The balance between anti- and pro-apoptotic BCL-2 mediators decides if cells enter the intrinsic pathway of apoptosis (Danial & Korsmeyer, 2004).

In viable cells BAK and BAX exist as monomers. However, when cells receive pro-apoptotic stimulation, BAK and BAX form oligomers that cause

permeabilisation of the mitochondrial outer membrane (MOM) and liberation of intermembrane space (IMS) proteins such as cytochrome c, SMAC/DIABLO and apoptotic protease activating factor 1 (Apaf-1) (Danial & Korsmeyer, 2004). The ‘BH-3 only’ members of BCL-2 family, e.g., BID, are important proteins regulating BAK and BAX activation. It has been also shown that BID requires BAK and BAX for execution of cell apoptosis (Desagher *et al.*, 1999). In contrast, the anti-apoptotic members of the BCL-2 family bind and sequester ‘BH-3 only’ molecules and interfere with BAK and BAX activation in this way (Cheng *et al.*, 2001). Following cytochrome c liberation from IMS of the mitochondrion, it forms a complex (apoptosome) with Apaf-1, which subsequently, in the presence of ATP/dATP, recruits pro-caspase-9 into the complex. These events enable self-processing of pro-caspase 9 into an initiator caspases 9, followed by activation of caspases 3 and initiation of apoptosis (Danial & Korsmeyer, 2004).

1.3.2 RECOGNITION OF APOPTOTIC CELLS BY PHAGOCYTES

Removal of apoptotic cells by professional phagocytes (efferocytosis) in a non-phlogistic manner, according to the recent concept, consists of three central elements: 1) attraction of phagocytes *via* soluble “find me” signals; 2) recognition and engulfment *via* displayed “eat me” and lacking “don’t eat me” signals; and 3) production of anti-inflammatory cytokines such as IL-10 or TGF- β by phagocytes that have ingested apoptotic cells (Lauber *et al.*, 2004). All of these elements are discussed in more detail in the following sections.

1.3.2.1 ATTRACTION OF PHAGOCYTES BY APOPTOTIC CELLS

In higher organisms apoptotic cells are not usually located in close proximity to phagocytic populations therefore secretion of specialised chemotactic signals by apoptotic prey appears to be an important factor determining their sufficient clearance by phagocytes. Although “find me” signals are so far poorly characterised, recent studies demonstrated that apoptotic bodies secrete the phospholipid

lysophosphatidylcholine (LPC), which attracts phagocytic cells. LPC is generated by the calcium-independent phospholipase-A₂ which is activated in presence of caspase-3 during apoptosis (Lauber *et al.*, 2003). LPC is not the only chemotactic signal reported so far. Other examples include S19 (ribosomal protein dimmer) (Horino *et al.*, 1998), split human tyrosyl-tRNA synthetase (Wakasugi & Schimmel, 1999) and thrombospondin-1 (Moodley *et al.*, 2003).

M ϕ receptors recognising soluble ‘find me’ signals and the mechanisms by which ‘find me’ signals phagocyte chemotaxis are yet poorly identified. Quite recently, Peter *et al* have demonstrated (using RNA interference and expression studies) that the G-protein coupled receptor (G2A) on monocytic cells is able to bind LPC and induce migration of monocytes towards apoptotic cell culture supernatants (Peter *et al.*, 2008). Another study by Truman and colleagues has demonstrated that lymphocytes undergoing apoptosis release a chemokine and intracellular adhesion molecule named CX3CL1/fractalkine, which is then recognised by fractalkine receptor, CX3CR1, on the surface of M ϕ and stimulates their chemotaxis towards apoptotic cells (Truman *et al.*, 2008).

Another intriguing hypothesis that explains migration of phagocytes towards apoptotic cells is the generation of electric fields by cells undergoing apoptosis. Apoptotic cells lose membrane asymmetry and, consequently, negative charge appears on their surfaces which can potentially attract phagocytes (Erwig & Henson, 2008). Electric fields have been demonstrated to stimulate migration of endothelial cells and neutrophils in the direction of a wound centre (Zhao *et al.*, 2006) as well as budding of endothelial cell toward the dying cells (Weihua *et al.*, 2005).

1.3.2.2 PHAGOCYTE RECOGNITION OF APOPTOTIC CELLS

Recognition and engulfment of apoptotic particles is a very complex process involving multiple ligand-receptor interactions. The contact point between apoptotic prey and phagocyte, especially in a view of its resemblances to the neuronal synapse, has been termed an engulfment “synapse”. Numerous molecules are involved in the formation of the engulfment “synapse”, namely “eat me” signals (molecules enabling recognition of apoptotic cells by phagocytes), bridge molecules and phagocytic

receptors. All of them are presented in Table 1.1 and briefly reviewed in separate sections below.

TABLE 1.1 Efficient phagocytosis of apoptotic cells is dependent upon expression of numerous ‘eat me’ signals that interact with phagocytic receptors in a direct or an indirect (via bridging molecules) manner.

<i>‘Eat me’ signals</i>	<i>Bridging molecules</i>	<i>Phagocytic receptors</i>
<ul style="list-style-type: none"> • <i>Phosphatidylserine (PS)</i> • <i>Calreticulin (CRT)</i> • <i>TSP binding sites</i> • <i>Collectin binding sites</i> • <i>Lectin binding sites</i> • <i>C1q or C3b binding sites</i> • <i>Sites resembling oxidised lipoproteins</i> 	<ul style="list-style-type: none"> • <i>Annexin 1 (Anx1)</i> • <i>Milk fat-globule-EGF-factor 8 (MFG-E8)</i> • <i>Growth-arrest-specific 6 (GAS6)</i> • <i>β2-glycoprotein-I (β2-GPI)</i> • <i>Protein S</i> • <i>Thrombospondin-1 (TSP-1)</i> • <i>Mannose binding lectin (MBL)</i> • <i>Lung surfactant proteins A and D (SP-A, SP-D)</i> • <i>First component of the classical complement cascade (C1q)</i> 	<ul style="list-style-type: none"> • <i>T-cell immunoglobulin</i> • <i>Brain specific angiogenesis inhibitor (BAI1)</i> • <i>Stabilin2</i> • <i>B2-GPI receptor</i> • <i>Receptor-tyrosine kinase MER</i> • <i>CD91 (LRP1 or α2 macroglobulin)</i> • <i>Vitronectin receptor ($\alpha$$\gamma$$\beta$3-integrin)</i> • <i>Receptor-tyrosine kinase MER</i> • <i>Class A Mϕ scavenger receptor (SR-A)</i> • <i>Lectin-like oxLDL-receptor 1 (LOX-1)</i> • <i>CD68 and CD36</i> • <i>Formylpeptide receptor (FPR2/ALX) for lipoxinA4 and annexin-derived peptide (Ac2-26)</i>

1.3.2.2.1 “EAT ME” and “DON’T EAT ME” SIGNALS

The translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane during apoptosis is one of the most extensively studied “eat me” signals (Fadok *et al.*, 1992). Although detail of the mechanism underlying exposure of PS remains unclear, a few processes are thought to be engaged. One of them is the inhibition of the flippase that normally transfers PS to the inner leaflet of the cell membrane. The second one is the activation of a bidirectional non-specific phospholipid scramblase (Williamson & Schlegel, 2002). Finally, the potential role of the ATP binding cassette transporter (ABC1) in the redistribution of phospholipids has also been postulated (Luciani & Chimini, 1996). Even though externalisation of PS to the outer leaflet of the cell membrane is clearly associated with apoptosis; its role in apoptotic cell recognition and engulfment by phagocytes remains to be established. It has been demonstrated that the presence of annexin V that masks external PS on apoptotic cell surface, blocks phagocytosis (Krahling *et al.*, 1999). In contrast, extensive studies show that some living cells expose PS on the outer leaflet of the cell membrane, e.g., activated B cells (Dillon *et al.*, 2001), neutrophils in Barth syndrome (Kuijpers *et al.*, 2004) or T lymphocytes characterised by low levels of the transmembrane tyrosine phosphatase CD45RB (Elliott *et al.*, 2005). Interestingly, these cells are not ingested by phagocytes.

During apoptosis, in addition to PS, other molecules, which are normally present on the intracellular side of the membrane, appear on the cell surface. These include, colocalising with PS, annexin I and the endoplasmic reticulum protein calreticulin (CRT) (Erwig & Henson, 2008). CRT is expressed by most cell types and its surface levels increases during apoptosis and in response to any cellular stress (Heal & McGivan, 1998). Several lines of evidence indicate that CRT plays a significant role in clearance of apoptotic cells. First of all, cells deficient in this protein undergo apoptosis but their removal by M ϕ is impaired (Mesaeli *et al.*, 1999). Furthermore, addition of soluble CRT to apoptotic cells not expressing their own CRT rescue their clearance by phagocytes (Gardai *et al.*, 2005). In addition, interaction of CRT with CD91 has been shown to be essential for efficient removal of apoptotic cells. CD91 is also known as a low-density lipoprotein (LDL) receptor-related protein (LRP1) or α 2 macroglobulin receptor and has been shown to be a

highly effective internalisation receptor on phagocytes (Orr *et al.*, 2003). Since CRT is expressed on the surface of either apoptotic cells or phagocytes, it may activate CD91 in either *trans* or *cis* fashion. In *cis* mode, apoptotic cells activate CRT localised on the surface of phagocytes whereas in *trans* mode CRT is attached to the apoptotic cell surface and directly interacts with CD91 on phagocytes (Gardai *et al.*, 2006).

During apoptosis, surface proteins undergo various, yet, so far poorly described, modifications, e.g., oxidation (i.e. phospholipids), alteration of sugar chains or surface charge. Consequently, the surface of apoptotic cells is characterised by the presence of specific sites, i.e., thrombospondin (TSP) binding sites, various collectin-binding sites, sites capable of binding lectins or the complement proteins C1q and C3b as well as sites that resemble oxidised lipoprotein particles (Erwig & Henson, 2008). They can interact directly with the receptors on the M ϕ surface or bind serum proteins that serve as links (bridge molecules) between the phagocytes and their apoptotic meal. The bridge molecules are described in more detail in the next section.

Several studies have demonstrated that “eat me” signals like PS or CRT may be expressed by viable cells (Gardai *et al.*, 2006). These findings suggest that living cells may express on their surface some kind of inhibitory (“don’t eat me”) signals that block phagocytosis of viable “self” cells. Similar mechanisms regulate elimination of target cells by NK cells. NK cells express on their surfaces activating receptors, which bind certain ligands presented by other cells. This interaction enables NK cell activation. However, NK cells also express inhibitory receptors (killer cell Ig-like receptor (KIR)) which bind ‘self’ major histocompatibility complex (MHC) class 1 molecules. This interaction suppresses activation of NK cells. It has been postulated that recruitment of src-homology 2-containing protein tyrosine phosphatase-1(SHP-1) and SHP-2 to two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) may be involved (Yusa *et al.*, 2004). It is believed that phagocytosis of viable cells may be prevented in a similar manner by presentation of markers of “self” on the surface of viable cells. These markers would recognise M ϕ inhibitory receptors such as signal regulatory protein α (SIRP α) (Kharitonov *et al.*, 1997).

Interestingly, some “don’t eat me” signals have been identified and shown to inhibit uptake of viable cells. One of them, platelet–endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31, has been demonstrated to facilitate both detachment of viable cells and attachment of apoptotic cells to either human recombinant CD31 coated non-adhesive coverslips or M ϕ (Brown *et al.*, 2002b). Therefore, CD31 seems to be essential for prevention of engulfment of viable “self” cells but also for sufficient uptake of cells undergoing apoptosis. Preincubation of CD31 positive apoptotic cells with blocking antibody reduced their clearance by M ϕ . CD47, also known as integrin-associated protein, is another surface molecule which is believed to suppress removal of “self” viable cells following its interaction with extracellular domain of SIRP α on M ϕ (Oldenberg *et al.*, 2001). For instance, erythrocytes express CD47 on their surface and thus are not eaten by phagocytes (Oldenberg *et al.*, 2000). However, this molecule is also highly expressed on the surface of some cancer cells (ovarian cancer cells) and by viruses such as smallpox and vaccinia viruses. That impairs their clearance by phagocytes and it is not beneficial for host organism (Campbell *et al.*, 1992).

1.3.2.2.2 INTERACTIONS OF APOPTOTIC CELLS WITH PHAGOCYTES via BRIDGE MOLECULES

Bridge molecules (opsonins) enable indirect recognition of the apoptotic cells by phagocytes and therefore enable more efficient phagocytosis. The examples of bridge molecules for PS include annexin I (Anx I) (Arur *et al.*, 2003), milk-fat-globule-EGF-factor 8 (MFG-E8), growth-arrest-specific factor 6 (Gas 6), β 2-glycoprotein-I (β 2-GPI) (Lauber *et al.*, 2004) and serum protein S (Anderson *et al.*, 2003).

Other bridge molecules recognise altered sugars and/or lipids on the apoptotic cell surface and include thrombospondin-1 (TSP-1) (Stern *et al.*, 1996) and the members of the collectin family of molecules: mannose binding lectin (MBL), lung surfactant proteins A and D (SP-A, SP-D) (Vandivier *et al.*, 2002b) and the collectin-like first component of the classical complement cascade C1q (Takizawa *et al.*, 1996).

1.3.2.2.3 PHAGOCYTTIC RECEPTORS

Given the prominence of PS in the alterations of membrane composition during apoptosis and the ability of exogenous PS or PS-binding proteins to block phagocytosis it seems to be likely that PS receptors (PSR) on the phagocyte surface exist and play an important role in phagocytosis. However, adequate identification of this receptor took a lot of time and effort of many research groups. Their effort is briefly summarised below.

One approach aiming at identification of a phagocyte PSR receptor involved the phage display technique. In this approach a range of antibodies was generated against human M ϕ and one of them, monoclonal antibody 217 (mAb 217), able to recognise PS liposomes and was then selected for further studies. Interestingly, addition of mAb217 to M ϕ inhibited engulfment of apoptotic cells. Subsequently, the antigen recognised by mAb217 was cloned and identified in the GenBank database as a KIAA 0585 protein (47-48 kDa) of unknown function. Interestingly, transfection of two cell lines (class II negative B cell line M12.C3 and Jurkat T cells), which did not have the ability to express PSR and phagocytose apoptotic targets, with psr gene resulted in proper binding and engulfment of apoptotic cells. Therefore, authors drew a final conclusion that the antigen for mAb217 is a PSR (Fadok *et al.*, 2000). Further studies have showed that interaction of PS and this putative PSR promotes engulfment but not binding of apoptotic targets by phagocytes. Addition of mAb217 to M ϕ induced uptake of previously bound cells but if added prior to apoptotic cells it inhibited their uptake (Hoffmann *et al.*, 2001). Furthermore, several studies have been performed with PSR deficient animals, which showed tendency to accumulate apoptotic cells. However, these studies did not compare expression of the antigen recognised by mAb217 in wild-type and knockout animals (Williamson & Schlegel, 2004). In contrast, a study published by Bose and colleagues with PSR knockout mice has showed that the ablation of psr function resulted in the abnormal development of mouse embryos but M ϕ maintained normal ability to remove apoptotic cells. Interestingly, in this study the expression of the antigen recognisable by mAb217 in knockout mice was comparable with that of wild-type animals. However, when a commercially-generated antibody against PSR has been used, authors showed that this protein did disappear from knockout mice (Bose *et al.*,

2004). Furthermore, some data demonstrated that the protein cloned by Fadok *et al* is indeed a nuclear protein carrying five nuclear localisation signals (NLS) (Cui *et al.*, 2004) and that this protein did not contain a consensus sequence for PS-binding motif (FxFxLKxxxKxR), which is common in such enzymes as PKC, PLC or PS decarboxylase. Consequently, the antibody mAb217 appeared to be the only experimental link between PSR and presumptive psr gene, which is now thought to encode a nuclear protein playing a role in the development and differentiation and perhaps belonging to iron-oxidase family (Cikala *et al.*, 2004).

Quite recently, three new PSR have been found simultaneously by three separate research teams. One of them is T-cell immunoglobulin and mucin-domain-containing molecule (Tim4 or Timd4), which was identified with the use of a library of hamster monoclonal antibodies recognising mouse peritoneal M ϕ (Miyanishi *et al.*, 2007). Tim4 interacted with PS *via* its immunoglobulin domain and mAb Kat5-18 against Tim4 blocked PS-dependent phagocytosis of apoptotic cells. Furthermore, when Tim4 was expressed in fibroblasts, these cells gained ability to phagocytose apoptotic cells. Interestingly, Tim4 has been proposed to be a tethering receptor for PS since its augmentation of apoptotic cell clearance does not seem to involve the two known signalling pathways for phagocytosis (Park *et al.*, 2009). Signalling pathways for phagocytosis are described in more detail in chapter 1.3.2.3. From other members of Tim family proteins, Tim1, but not Tim2 and Tim3, seems to have similar ability to stimulate phagocytosis in a PS-dependent manner (Miyanishi *et al.*, 2007).

The second PSR has been identified by Park and colleagues by means of the yeast two-hybride system (Park *et al.*, 2007). This protein is a brain specific angiogenesis inhibitor 1 (BAI1) that belongs to the adhesion-type-G-protein coupled receptor (GPCR) family. Significantly, it contains thrombospondin type 1 repeats (TSR) similar to that of thrombospondin 1, which was earlier characterised as a bridge molecule mediating interaction between PS and phagocytic receptors (Stern *et al.*, 1996). Importantly, BAI1 is also an upstream mediator for ELMO/Dock180/Rac and its activation directly triggers signalling pathways for phagocytosis. Similarly to Tim4, BAI1 expression in fibroblast results in these cells gaining ability to ingest

apoptotic cells. On the contrary, abolished expression or function of BAI1 blocked uptake of apoptotic particles in both *in vitro* and *in vivo* settings.

Finally, the third recently characterised PSR is stabilin-2, previously described as hepatic hyaluronan (HA)-binding protein, responsible for the removal of excess HA from blood (McCourt *et al.*, 1999). Interestingly, in a recent study by Park *et al.*, it has been shown that stabilin-2 recognises and binds PS exposed on the surface of aging erythrocytes or apoptotic cells and that this interaction is pivotal for their subsequent clearance by M ϕ (Park *et al.*, 2008). Furthermore, interference with stabilin-2 expression or function significantly abolished both binding and engulfment of both phagocytic preys by M ϕ .

There are also a number of phagocyte receptors that recognise PS *via* bridge molecules. These receptors include vitronectin receptor ($\alpha_v\beta_3$ -integrin), the receptor-tyrosine kinase Mer, and the β_2 -glycoprotein-I (β_2 -GPI) receptor (Lauber *et al.*, 2004). Several phagocyte receptors interact also with oxidized low-density lipoproteins (oxLDL), namely SR-A (class A M ϕ scavenger receptor) (Platt *et al.*, 1996), LOX-1 (lectin-like oxLDL-receptor1) (Oka *et al.*, 1998), CD-68 (Erdoesova *et al.*, 2002) and CD36 (Ren *et al.*, 1995). Furthermore, TSP binding sites bind to a receptor complex on the phagocyte that comprises of the integrin $\alpha_v\beta_3$ and the scavenger receptor CD36. In addition, formylpeptide receptor FPR2/ALX have been demonstrated to play an important role in lipoxin A4 and annexin-derived peptide (Ac2-26) – stimulated phagocytosis (Maderna *et al.*, 2010).

Recognition and engulfment of apoptotic cells by phagocytes is a complex process characterised by multiple interactions between surface molecules presented by both phagocytes and apoptotic cells. However, some of the phagocyte surface proteins (i.e. CD44) do not directly recognise apoptotic corpses but rather exert indirect effects. CD44 is a well known cell surface receptor for HA, fibronectin, collagen and fibrin. It plays an important role in a number of processes, e.g., adhesion and proliferation of lymphocytes, killing of target cells by NK cells and tumor metastasis (Henke *et al.*, 1996). Interestingly, our group has shown that binding of bivalent monoclonal anti-CD44 antibody (CD44mAb) to human monocyte-derived M ϕ *in vitro* enhances uptake of apoptotic neutrophils. Consequently, in *in vivo* settings CD44 may also induce clearance of apoptotic neutrophils and mediate

resolution of inflammation (Hart *et al.*, 1997). The latter suggestion has been supported by studies on CD44 deficient mice characterised by bleomycin-induced inflammation of alveolar interstitium (Teder *et al.*, 2002). These animals accumulated apoptotic material in lungs to a much greater extent than wild-type controls. Impaired phagocytosis was also associated with prolonged inflammation and development of lung injury.

In addition, phagocytosis can be regulated pharmacologically by numerous compounds, e.g., glucocorticoids, lipoxins, prostaglandins or statins, which are briefly characterised in separate sections of chapter 1.5.

1.3.2.2.4 DIFFERENCES IN RECOGNITION OF APOPTOTIC AND NECROTIC CELLS

The clearance of apoptotic and necrotic cells including secondary necrotic cells is thought to elicit different anti-inflammatory or pro-inflammatory responses from phagocytes. Thus, these processes seem to engage specific receptors or adaptor molecules. For instance, uptake of apoptotic cells triggers strong anti-inflammatory responses from both monocytes and M ϕ , such as decreased production of TNF- α , IL-1 β , IL-12 and up-regulation of IL-10 or TGF- β (Voll *et al.*, 1997; Fadok *et al.*, 1998). Furthermore, complement components C1q, C3 and C4 interact selectively with primary or secondary necrotic cells and serve as an opsonin for apoptotic cells, which were not phagocytosed by regular scavenger mechanisms (Gaipl *et al.*, 2001). C1q, together with serum DNase 1, is implicated in the degradation of chromatin from necrotic cells and subsequent uptake of chromatin fragments by monocyte-derived phagocytes (Gaipl *et al.*, 2004). Similarly, C-reactive protein (CRP) does not recognise early apoptotic neutrophils but binds intracellularly to late apoptotic, membrane-permeable cells (Hart *et al.*, 2005). On the contrary several receptors systems such as thrombospondin-CD36- $\alpha_v\beta_3$ complex, CD14 and surface receptor recognised by mAB217G8E9 are involved in uptake of both apoptotic and necrotic cells (Bottcher *et al.*, 2006). The role of PS and its receptors in phagocytosis remains controversial, however, PS is exposed on the outer leaflet of apoptotic as well as necrotic cells and therefore may play a role in both processes (Brouckaert *et al.*,

2004). Some data indicate, however, that the presence of PS on the apoptotic cell surface may induce their engulfment by phagocytes and subsequent production of anti-inflammatory mediators, e.g., TGF- β (Park *et al.*, 2008). The role of TGF- β in resolution of inflammation is described in more detail in chapter 1.3.2.4.

1.3.3 ENGULFMENT OF APOPTOTIC CELLS BY M Φ

Engulfment is a very complex process and depends on numerous factors such as the type of ingested particles (apoptotic or necrotic) or the type of receptors involved. However, a few common principles have been established with regard to this process. Following recognition of a target particle by phagocytes, actin polymerisation occurs at the site of uptake and the particle is ingested *via* an actin-dependent mechanism. After internalisation, the phagosome undergoes complex changes leading to the creation of a mature phagolysosome. In the mature phagolysosome ingested particles are trafficked into a series of increasingly acidified membrane-bound structures, where they subsequently undergo degradation (Erwig & Henson, 2008). The process of internalisation *per se* seems to depend on the type of receptors involved. For instance, phagocytosis mediated by Fc γ receptors relies on their sequential interactions with IgG-opsonised particles. Following recognition by Fc γ receptors, IgG-opsonised particles are phagocytosed by tight pseudopod extensions, which are formed on the surface of phagocytes ('zipper' mechanism) (Griffin, Jr. *et al.*, 1976). In contrast, the uptake of apoptotic cells involves formation of the spacious phagosomes, which are accompanied by ruffling of the cell membrane and uptake of the surrounding fluid (deCathelineau & Henson, 2003).

Furthermore, elegant studies in such model organisms as nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster* and mammalian cells revealed that the engulfment machinery involves very conserved signalling pathways (Reddien & Horvitz, 2000; Gumienny & Hengartner, 2001). Two signalling pathways have been identified so far, which regulate activity of Rho family GTPases (Gardai *et al.*, 2006). Rho family GTPases includes such enzymes as RhoA and Rac-1, which exert opposite effects on phagocytosis of apoptotic cells - RhoA inhibits this process while Rac-1 is essential for proper engulfment (Morimoto *et al.*, 2006). Therefore, the

'net' capacity of phagocytes for uptake of target particles depends on the activation of RhoA and Rac-1 and the RhoA/Rac-1 balance on the surface of phagocytes. As demonstrated in Figure 1.6, signalling pathways for phagocytosis converge in a trimolecular complex ELMO/CrkII/DOCK180 in mammals (known respectively as CED-12, CED-2 and CED-5 in the *C.elegans*) (Henson, 2005). It has been shown that assembly of this complex activates the guanine nucleotide exchange activity of DOCK180 for Rac-1 (CED-10 in the *C.elegans*), which in turn promotes reorganisation of cytoskeletal elements and enables uptake of target particles (Krysko *et al.*, 2006). The important role of Rho-GTPases in engulfment of apoptotic cells is further supported by a number of studies. For instance, the study by Gumienny and colleagues demonstrated that Rac activation was critical for phagocytes to ingest apoptotic cells *in vitro*, whereas activation of RhoA inhibited this process (Gumienny *et al.*, 2001). Interestingly, activation of Rac was quite rapidly followed by activation of RhoA. This observation may explain, at least in part, the fact that uptake of apoptotic cells by M ϕ reduces their ability to engulf a second apoptotic meal (Erwig *et al.*, 1999).

As mentioned above, the activation of signalling pathways for phagocytosis leads to assembly of a trimolecular complex ELMO/CrkII/DOCK180 in mammals or analogical CED-12/CED-2/CED-5 in the *C.elegans*. It is known that one signalling pathway is composed of recently identified in both, mammalian cells and worms, RhoG/MIG-2 and its activator TRIO/UNC-73 (guanine exchange factor (GEF) for RhoG) (deBakker *et al.*, 2004). However, the cell surface receptor for this pathway is still unknown. A second signalling pathway in mammalian cells is formed of the two cell surface receptors ABC1 (CED-7 in *C.elegans*) and CD-91/LRP (CED1 in *C.elegans*) and its adaptor protein GULP (CED-6 in *C.elegans*). ABC1 downstream ligands have not been identified yet (Zhou *et al.*, 2001). Quite recently, another cell surface receptor, named BAI1, has been postulated to signal through ELMO/Dock180/Rac-1. This molecule is a surface receptor for PS and its role in the phagocytosis is described in section 1.3.2.2.

FIGURE 1.6 Schematic representation of the signal transduction pathway for M ϕ engulfment of apoptotic cells. Phosphatidylserine (PS) and possibly other ‘eat me’ signals on the surface of apoptotic cells are recognised by phagocytic receptors such as BAI1 and possibly other unidentified yet receptors resulting in formation of CRKII/ELMO/DOCK180 complex. The latter enables activation of Rac-1, which in turn promotes reorganisation of cytoskeletal elements with subsequent uptake of target particles.

1.3.4 IMMUNOLOGICAL CONSEQUENCES OF APOPTOTIC CELL CLEARANCE

Clearance of apoptotic cells, in contrast to the uptake of necrotic cells, is assumed not to induce pro-inflammatory responses in M ϕ (Meagher *et al.*, 1992). Importantly, it has been demonstrated that engulfment of apoptotic cells stimulates M ϕ production of anti-inflammatory mediators such as transforming growth factor β (TGF- β), prostaglandin E₂ or platelet-activating factor (PAF) (Fadok *et al.*, 1998;Huynh *et al.*, 2002). TGF- β is implicated in inhibition of pro-inflammatory cytokine production *via* inhibition of NF- κ B activation and phosphorylation of p38 MAPK kinase (Xiao *et al.*, 2002). However, the production of TGF- β by M ϕ as well as its anti-inflammatory effects appear only after a longer (> 18 hours) interaction of M ϕ with apoptotic targets (Fadok *et al.*, 1998;Huynh *et al.*, 2002). Currently, it is believed that apoptotic cells exert their anti-inflammatory effect much earlier, just upon binding to the M ϕ . The contact of activated M ϕ with apoptotic cells has been reported to change their functional state from pro- to anti-inflammatory (Cvetanovic & Ucker, 2004) and inhibit IL-12 production by activated M ϕ (Kim *et al.*, 2004). In addition, apoptotic cells *per se* are able to release anti-inflammatory mediators, e.g., IL-10 or TGF- β and manipulate the M ϕ activation in this way (Gao *et al.*, 1998;Chen *et al.*, 2001). The role of IL-10 in regulation of pro-inflammatory cytokine production and signalling is discussed in more detail in chapter 1.2.2.2.

Even though M ϕ uptake of apoptotic cells is usually seen as favourable in terms of the resolution of inflammation, there are a few scenarios in which this process triggers pro-inflammatory consequences. For example, it has been reported that bacterially-triggered apoptotic neutrophils upregulate the production of the pro-inflammatory cytokine TNF- α in human M ϕ (Zheng *et al.*, 2004). Furthermore, apoptotic cells, opsonised with IgG antibody, are consequently recognised *via* Fc receptors and trigger pro-inflammatory response in phagocytes (Hart *et al.*, 2004a;Gregory & Devitt, 2004).

The immunological consequences of phagocytosis are even more complicated when late apoptotic cells (also called secondary necrotic cells) are considered. Secondary necrotic cells, similarly to primary necrotic cells, do not maintain

membrane integrity and thus, the intracellular contents escape into surrounding tissue. For a long time it has been assumed that these cells induce pro-inflammatory responses in M ϕ . However, it has been demonstrated that clearance of secondary necrotic neutrophils by M ϕ did not induce production of pro-inflammatory mediators, i.e., IL-8 or TNF- α (Ren *et al.*, 2001). Furthermore, uptake of secondary necrotic cells by LPS-activated J774A.1 M ϕ blocked production of pro-inflammatory cytokines, i.e., TNF- α and IL-6 in these cells (Cocco & Ucker, 2001). In contrast, it is widely appreciated that uptake of primary necrotic (lysed) neutrophils usually induce production of macrophage - inflammatory protein 2 (MIP-2), IL-8, TNF- α and IL-10 (Fadok *et al.*, 2001; Li *et al.*, 2009). These observations led to a conclusion that the onset of secondary necrosis does not always trigger inflammatory consequences and that the secondary necrotic cells can not be simply compared with the primary necrotic cells. First of all, during apoptosis the contents of secondary necrotic cells is exposed to the activated caspases, which take apart crucial cellular components, processes or enzymes. In contrast, lysed (primary necrotic) neutrophils release numerous proteases and other mediators into the tissue, which further exaggerate the inflammatory response. One of the examples here is a high mobility group box 1 protein (HMGB-1). This protein leaks from primary necrotic cells and activates M ϕ , similarly to LPS, *via* interaction with TLR2 and TLR4. However, HMGB-1 remains bound in secondary necrotic cells (Scaffidi *et al.*, 2002). Similarly, heat-shock proteins and uric acid are released from primary necrotic cells but remain within the secondary necrotic cells (Krysko *et al.*, 2006). Significantly, it has been reported that primary necrotic cells are characterised by activated transcription factor NF- κ B, phosphorylated p38 MAPK and are capable of producing certain pro-inflammatory proteins (e.g., IL-6) (Vanden *et al.*, 2006).

Taken together, the immunological consequences of phagocytosis of apoptotic, secondary necrotic or primary necrotic cells seem to be quite complex. Furthermore, functional responses of M ϕ to phagocytosis are also influenced by multiple factors such as the activation state of M ϕ , source of target cells, stimuli that induce cell death or receptors involved in uptake.

FIGURE 1.7 Immunological consequences of phagocytosis. A. In general the M ϕ uptake of apoptotic cells is believed to be anti-inflammatory. Apoptotic cells produce anti-inflammatory mediators such as IL-10 or TGF- β and following phagocytosis induce their release by phagocytes. In addition, the uptake of apoptotic cells by phagocytes inhibits the release of pro-inflammatory mediators (i.e., TNF- α). **B.** In general, the M ϕ uptake of primary necrotic cells is believed to be pro-inflammatory. Primary necrotic cells release pro-inflammatory mediators i.e., cytokines (TNF- α , IL-6) or proteins such as HMGB-1. HMGB-1, similarly to bacterial products, induces pro-inflammatory activation of phagocytes *via* TLR2 or TLR4. Following phagocytosis, primary necrotic cells induce the release of pro-inflammatory mediators such as TNF- α , MIP2 or IL-8 from phagocytes. As described in chapter 1.3.4 many additional factors may change immunological consequences of phagocytosis and therefore presented diagram is not always applicable.

1.4 CHRONIC INFLAMMATORY CONDITIONS

1.4.1 THE ROLE OF CYTOKINES IN CHRONIC INFLAMMATION

Cytokines are a large family of low-molecular weight proteins that enable communication between cells and coordinate almost all biological processes, e.g., cell proliferation, migration, inflammation, immunity, fibrosis, repair and angiogenesis. Consequently, abnormalities in cytokine levels or their receptors and signalling pathways play an essential role in the pathogenesis of chronic inflammatory conditions. The first observations supporting this hypothesis came from various parallel studies of thyroiditis, diabetes and rheumatoid arthritis (RA) in the early 1980s and were then followed by studies performed by Feldmann's group showing that synovial cells chronically express IL-1 (Feldmann & Maini, 2003). The same research group further demonstrated that TNF- α is a 'master regulator' of production of other equally important pro-inflammatory cytokines and that TNF- α blockade reduces production of IL-1, GM-CSF, IL-6 or IL-8, introducing the concept of a TNF- α -dependent cytokine cascade (Brennan *et al.*, 1989; Feldmann, 1996). The latter finding led to the development of anti-TNF- α therapy, first licensed for treatment of RA. Therefore, for historical reasons, RA has been chosen as an example of chronic inflammatory conditions, which are associated with abnormalities in cytokine levels or functions and as such is briefly described below.

The affected joints in RA are characterised by several common features, namely pain, stiffness and inflamed synovial membranes (synovitis). Chronic inflammation of synovium leads to joint destruction, limited functionality and significant comorbidity in functional systems of the body, e.g., cardiovascular, neurologic and metabolic systems. Another characteristic of RA is a huge number of immune cells recruited into the synovium, namely activated neutrophils, M ϕ , B and T cells, mast cells or plasma cells. In addition, other cells such as activated synovial fibroblasts, chondrocytes and osteoclasts contribute substantially to the processes of

cartilage and bone destruction and further promote inflammatory responses (Brennan & McInnes, 2008).

A number of studies have demonstrated an essential role for cytokines in orchestrating the inflammatory processes in RA. First of all, synovial fluid has been shown to contain numerous pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-18), chemokines (IL-8, IP-10, MCP-1, MIP-1 and RANTES), MMPs (MMP-1, -3, -9, -13) or enzymes (COX-1, COX-2 and iNOS) (Simmonds & Foxwell, 2008). The complex interactions between them prolong and exaggerate pro-inflammatory signals resulting in a chronic and persistent inflammation. As it has been mentioned above, the production of pro-inflammatory mediators seems to be highly regulated by TNF- α and TNF- α blockade reduces synthesis of all the other pro-inflammatory cytokines in joints (Feldmann *et al.*, 2005). Moreover, TNF- α *per se* is able to induce degradation of cartilage (Dayer *et al.*, 1985) and bone (Bertolini *et al.*, 1986) *in vitro*. It has been appreciated that TNF- α mediates a number of processes relevant to the pathogenesis of RA (Brennan & McInnes, 2008) including:

- the release of pro-inflammatory cytokines including IL-1, IL-6, IL-23, GM-CSF,
- the release of hepcidin, an endogenous antimicrobial peptide, that induces the acute phase response,
- the release of chemokines including IL-8, MCP-1, RANTES and SDF-1 leading to leukocyte accumulation,
- the production of PGE₂,
- the activation of osteoclasts resulting in bone resorption
- the activation of chondrocytes resulting in cartilage destruction
- the activation of endothelial cells and up-regulation of E-selectin and VCAM-1 resulting in leukocyte accumulation,
- the processes of angiogenesis.

In addition to TNF- α , other members of the TNF superfamily, namely lymphotoxin- β , B lymphocyte regulator (BLyS), proliferation-inducing ligand (APRIL), are also implicated in development of RA. They regulate maturation, differentiation and activation of B cells, formation of higher lymphoid organisations,

as well as production of autoantibodies (i.e., rheumatoid factor) (Brennan & McInnes, 2008).

Furthermore, several members of the IL-1 superfamily play important roles in the pathogenesis of RA. The synovial membrane in arthritic joints has been demonstrated to be characterised by high levels of IL-1 α and IL-1 β as well as the natural IL-1 receptor antagonist (IL-1ra). The latter limits the pro-inflammatory stimulation of cells by IL-1 α and IL-1 β (Dayer, 2003). Interestingly, IL-1ra-deficient mice developed spontaneous arthritis, in which Th-17 cells seemed to mediate most of the observed pro-inflammatory effects (Nakae *et al.*, 2003). Furthermore, IL-1 α and IL-1 β have been shown to be directly implicated in several pathological processes in RA, such as production of pro-inflammatory cytokines by synovial mononuclear cells or chondrocytes, the release of MMPs and prostanoids by fibroblasts, and osteoclast-mediated bone erosion (Dayer, 2003).

Another important cytokine implicated in the pathogenesis of RA is IL-6. It controls maturation and activation of a number of cells that mediate pathological changes in RA, e.g., B and T cells, M ϕ , osteoclasts, chondrocytes and endothelial cells. It also exerts a broad effect on haematopoiesis in the bone marrow (Kishimoto, 2005). IL-6 is also likely to trigger the hepatic acute phase response in RA and induce synthesis of hepcidin that is directly implicated in the development of anaemia of chronic disease (anaemia caused by chronic infection, inflammation, renal failure or cancer) (Brennan & McInnes, 2008). Described in more detail TNF- α , IL-1 and IL-6 are only a few of the cytokines, which are expressed in inflamed synovium. The other examples include IL-23, IL-12, IL-17, IL-22, IL-15 or IL-7 and their role in RA is still under investigation.

Summing up, abnormal levels of cytokines may significantly contribute to the development of chronic inflammatory and autoimmune disorders, e.g., RA, ankylosing spondylitis, psoriasis, asthma, chronic obstructive pulmonary disease (COPD), IBD, multiple sclerosis (MS) or Alzheimer's disease (AD). The inflamed synovium in RA or similarly sputum in individuals with asthma or COPD (Barnes, 2008) and many other chronically inflamed tissues are characterised by overexpression of these mediators. Consequently, there is a growing interest in cytokines as therapeutic targets and a number of cytokine inhibitors (anti-cytokines)

have been developed and evaluated during clinical trials for their inflammation resolving properties. Some of them are currently licensed for treatment of RA, psoriasis or IBD, as described in more detail in chapter 1.5.3.

1.4.2 CLEARANCE DEFICIENCY AND AUTOIMMUNITY

Clearance of apoptotic cells is a fundamental mechanism of resolution of inflammation. It is believed that if this process is impaired, apoptotic cells undergo secondary necrosis, exacerbating inflammatory processes and leading to tissue injury. In addition, it has been demonstrated that uptake of apoptotic targets actively triggers production of anti-inflammatory cytokines (e.g., IL-10 or TGF- β) by phagocytes helping to maintain an anti-inflammatory milieu (Voll *et al.*, 1997; Fadok *et al.*, 1998). Furthermore elegant studies by Xu and colleagues have shown that the increase in M ϕ production of IL-10 is positively correlated with augmentation of M ϕ uptake of early apoptotic cells (Xu *et al.*, 2006). Mounting evidence implicates defects in clearance of apoptotic cells in pathogenesis of human autoimmune diseases such as systemic lupus erythematosus (SLE), COPD or cystic fibrosis (Krysko *et al.*, 2006). It has been reported that in SLE patients M ϕ uptake of various phagocytic targets, e.g., yeast and bacteria, is impaired (Hurst *et al.*, 1984; Salmon *et al.*, 1984). In addition, Herrmann and colleagues demonstrated that M ϕ isolated from SLE patients are characterised by reduced ability to ingest apoptotic cells. Similarly, in SLE patients phagocytosis by tingible body M ϕ that usually phagocytose apoptotic cells in lymph nodes is impaired (Baumann *et al.*, 2002; Herrmann *et al.*, 1998).

It is believed, that there is a strong link between cell apoptosis and autoimmunity. As mentioned above, deficient clearance of apoptotic cells results in these cells undergoing secondary necrosis, which are a source of harmful, cytotoxic and pro-inflammatory agents. In addition, secondary necrotic cells contain potentially immunogenic autoantigens. As demonstrated in Figure 1.8, in higher organisms formation of autoantigens may have detrimental consequences as their innate immune system presents autoantigens to the adaptive immune system and triggers production of autoantibodies (Mahoney & Rosen, 2005). The most typical class of autoantigens produced in autoimmune disorders are nucleosomal proteins, which are generated by

internucleosomal cleavage of DNA during apoptosis (Baumann *et al.*, 2002). Consequently, the rise in antibodies against nucleosomes is a common characteristic of SLE and other autoimmune diseases. Additionally, if uptake of apoptotic cells is delayed, specific enzymes such as caspases generate neoantigens that induce the loss of tolerance by B cells and formation of immune complexes (Casiano *et al.*, 1996). Importantly, autoantibodies have the ability to opsonise apoptotic particles and influence phagocytosis in this manner. For instance, anti-phospholipid antibodies recognise externalised PS on apoptotic cell surfaces and change the outcome of the uptake of apoptotic cells by M ϕ . The opsonised cells are recognised by M ϕ Fc receptors and, consequently, their uptake by M ϕ triggers pro-inflammatory responses (Price *et al.*, 1996).

In addition to M ϕ , apoptotic and necrotic cells may be phagocytosed by dendritic cells (DCs), also known as antigen presenting cells. It is believed that phagocytosis by tissue or follicular DCs takes place whenever removal of apoptotic cells by M ϕ is impaired. Phagocytosis by DCs may induce various immunoresponses (i.e. tolerance or immune activation), which are determined by the levels of DCs maturation. Only mature DCs are able to cross-present self or foreign antigens to CD8⁺ cytotoxic T lymphocytes (CTL) and stimulate production of antibodies by B cells (Savill *et al.*, 2002). DCs maturation is, in turn, regulated in a positive or a negative manner by multiple environmental factors. For instance, uptake of necrotic cells and the presence of pro-inflammatory signals (e.g., LPS, TNF- α , IL-1 β or IFN- α , viral or bacterial proteins or high antigen load) are very powerful inducers of DCs maturation and activate immune responses (Manderson *et al.*, 2004). In contrast, uptake of apoptotic cells and the presence of anti-inflammatory cytokines such as TGF- β or IL-10 strongly inhibit maturation of DCs and promote peripheral tolerance (Manderson *et al.*, 2004). It has been shown that engulfment of apoptotic material by DCs may inhibit even strong pro-maturation signals such as LPS, indirectly reducing capacity of DCs to stimulate T cells (Chen *et al.*, 2001). Furthermore, uptake of purely apoptotic cells by DCs does not induce antigen presentation and some authors hypothesise that this effect is related to interaction of PS, present on the surface of apoptotic cells, with PSR and stimulation of anti-inflammatory cytokine production. On the contrary, uptake of necrotic cells induce opposite effects promoting DC

maturation as well as a break in peripheral tolerance (Sato *et al.*, 2002; Sauter *et al.*, 2000).

Little is known about the exact processes reducing M ϕ capacity for clearance of apoptotic cells. It has been postulated that downregulation of certain “find me” or “eat me” signals as well as phagocytic receptors may contribute to defects in removal of apoptotic cells and development of autoimmunity. Mounting evidence suggests that homozygous deficiency of any of the early components of classical complement activation pathways (C1q, C1r, C1s, C4 and C2) may be associated with the development of SLE (Manderson *et al.*, 2004). This is a direct consequence of accumulation of apoptotic material and upregulation of self-antigens and immune complexes which induce tissue inflammation, DC maturation and production of autoantibodies. Reduced levels of complement fragments are also associated with decreased threshold activation of T and B cells due to lack of regulatory signals that maintain peripheral tolerance to self-antigens (Manderson *et al.*, 2004; Manderson *et al.*, 2004; Munoz *et al.*, 2005). In addition, it has been postulated that impaired clearance of apoptotic cells significantly contributes to pathogenesis of cystic fibrosis (CF). This autoimmune condition is characterised by massive recruitment of inflammatory cells and release of intracellular proteases in lungs. Apoptotic cells are accumulated in the airways of CF patients and it has been suggested initially that their reduced uptake is caused by elastase-mediated cleavage of PSR from surface of phagocytes (Vandivier *et al.*, 2002a). However, as described in a section 1.3.2.2.3 the identification of a putative PSR has been quite troublesome and three new PSRs have been recently identified. Thus, further work is required in order to fully determine the role of PSR in CF.

In summary, based on current knowledge, it is widely appreciated that deficient clearance of apoptotic cells is crucial for development of autoimmunity and is strongly related to production of autoantigens and creation of a pro-inflammatory environment. However, the exact mechanisms underlying the actual defects in phagocytosis are still poorly understood and likely to be disease-specific.

FIGURE 1.8 Diagram illustrating the cascade of events resulting from efficient (A) or impaired (B) clearance of apoptotic cells (next pages). Efficient clearance of apoptotic cells prevents cell necrosis and stimulates production of anti-inflammatory mediators by phagocytes. In an anti-inflammatory environment maturation of antigen presenting cells (i.e. dendritic cells) and immune responses are inhibited. On the contrary, when clearance of apoptotic cells is affected, accumulated apoptotic cells undergo secondary necrosis resulting in production of autoantigens and pro-inflammatory mediators. In the presence of pro-inflammatory mediators maturation of dendritic cells is induced. Mature dendritic cells, after gaining access to autoantigens, present them to T helper cells followed by subsequent B cell production of autoantibodies and development of autoimmunity. **M ϕ** – macrophages, **DC** - dendritic cells.

1.5 ANTI-INFLAMMATORY THERAPEUTICS AND RESOLUTION OF INFLAMMATION

Our group and others have shown that pharmacological or immunological intervention may drastically modulate M ϕ ability to phagocytose apoptotic cells. In sections below there is a short description of glucocorticoids, NSAID and cytokine inhibitors, which are widely used as anti-inflammatory therapeutics. Some of them have been demonstrated to influence the rate of M ϕ clearance of apoptotic cells and consequently to play a role in the resolution of inflammation. These data are briefly presented below.

1.5.1 GLUCOCORTICOIDS

Glucocorticoids are hormones that play an important role in the control of inflammation. Normally, inflammation triggers a negative feedback loop to the hypothalamic – pituitary – adrenal (HPA) axis, which stimulates release of glucocorticoids (e.g. cortisol) by the adrenal cortex (Johnson & Rn, 2006). Glucocorticoid stimulation triggers a number of signalling pathways and eventually opposes the inflammatory response. A number of synthetic glucocorticoids (e.g., hydrocortisone, fludrocortisone, prednisolone, dexamethasone, betamethasone, triamcinolon) have been introduced into the clinic and are widely used as the most potent and cost-effective anti-inflammatory and immunosuppressive drugs. However, in addition to their beneficial effects, treatment with glucocorticoids is inevitably associated with precipitation of adverse drug reactions (ADR) outweighing, in some cases, the benefit of the treatment. These include: diabetes, osteoporosis, proximal myopathy, Cushing's syndrome (acne, moon face, striae) and many others. Glucocorticoids act through the intracellular glucocorticoid receptor (GR), mainly its isoform alpha (GR α), that belongs to the nuclear receptor superfamily (Gronemeyer *et al.*, 2004). GR, in its inactive state, resides in the cytoplasm, where it forms a complex with a number of proteins i.e. heat shock proteins (hsp90, hsp70) co-chaperones and immunofillins. Following association of a ligand, GR undergoes a series of conformational changes leading to exposure of nuclear localisation signal

and rapid translocation to the nucleus (Defranco, 1999). In the nucleus, GR either activates or suppresses transcription of certain genes *via* interaction with glucocorticoid response elements (GRE) (Yamamoto *et al.*, 1998). Interestingly, some GRE do not directly recognise GR but initially interact with other transcription factors and then recruit GR *via* protein:protein interaction (tethering) (Reichardt *et al.*, 2001). Several transcription factors have been reported to provide tether for GR, namely AP-1, NF- κ B, STAT3 or STAT5 (Chinenov & Rogatsky, 2007).

As demonstrated in Figure 1.9, glucocorticoids exert their anti-inflammatory effects *via* a number of distinct mechanisms. One of them is inhibition of AP-1 and NF- κ B activation, especially important in downregulation of TLR-mediated inflammation. AP-1 and NF- κ B are transcription factors that induce transcription of many pro-inflammatory mediators in immune cells, especially M ϕ , monocytes, neutrophils and dendritic cells as well as in epithelial and endothelial cell types (Oda & Kitano, 2006). Glucocorticoids induce also expression of certain proteins, such as I κ B and glucocorticoid-inducible leucine zipper (GILZ), which interfere with AP-1 and NF- κ B binding of DNA and inhibit their transcriptional activity (Chinenov & Rogatsky, 2007). Interestingly, recent evidence suggests that glucocorticoids may act in a more complex manner and target pathways upstream of, or unrelated to, AP-1 and NF- κ B. For instance, GR inhibit several protein kinases including JNK and p38 without inhibiting their transcription (Caelles *et al.*, 1997;Hirasawa *et al.*, 1998). This observation can be explained, at least in part, by glucocorticoid augmentation of transcription of an inhibitory MAPK phosphatase 1 (MKP1) (Kassel *et al.*, 2001;Furst *et al.*, 2007). In fact, in MKP1^{-/-} mouse M ϕ , glucocorticoid treatment does not inhibit activation of JNK and p38 by LPS and the consequential production of inflammatory mediators such as TNF- α or IL-1 β (Abraham & Clark, 2006). Furthermore, GR interferes with activation of transcription factors belonging to IRF family. They are activated by the majority of TLRs *via* both MyD88-dependent and independent pathways and induce transcription of a number of genes e.g. interferon β , IP10 and RANTES. For instance, it has been demonstrated that activation of IRF3 requires binding of additional protein named GRIP1. Importantly, GRIP1 is also a cofactor of an active GR complex. In the presence of glucocorticoids, activated GR competes with IRF3 for GRIP1 and consequently transcription of IRF3-inducible

genes is decreased (Chinenov & Rogatsky, 2007). Glucocorticoids have been also reported to interfere with pro-inflammatory cytokine signalling *via* upregulation of transcription of SOCS1. Expression of SOCS protein is induced by TLR signalling and provides a negative feedback loop that limits an inflammatory response (the detailed mechanism is described in chapter 1.2.2.2). Indeed, mice deficient in SOCS1 are characterised by upregulated production of cytokines in response to LPS and are more sensitive to septic shock (Nakagawa *et al.*, 2002). Importantly, data from *in vitro* and *in vivo* studies clearly indicate that glucocorticoids have the ability to induce transcription of SOCS1 (Chinenov & Rogatsky, 2007).

The effects of glucocorticoids on apoptosis of inflammatory cells and their subsequent clearance by phagocytes have been evaluated. For instance, it has been demonstrated that glucocorticoids dramatically delay neutrophil but promote eosinophil and lymphocyte apoptosis (Meagher *et al.*, 1996). The latter may be of particular importance for treatment of asthma, which is characterised by an eosinophilic inflammation of airways (Walsh *et al.*, 2003). Treatment of exacerbations of asthma with steroids results in the resolution of the eosinophilic inflammation, possibly by inducing apoptosis in lung eosinophils. Glucocorticoid-induced apoptotic eosinophils are subsequently recognised and phagocytosed by alveolar M ϕ (Woolley *et al.*, 1996). Furthermore, severity of asthma, as assessed by symptom scores and airflow obstruction, has been positively correlated with reduction of eosinophil apoptosis (Duncan *et al.*, 2003). In addition to regulation of cell apoptosis, glucocorticoids significantly augment, in a concentration- and time-dependent manner, the ability of M ϕ to phagocytose apoptotic cells (Liu *et al.*, 1999). Our group has demonstrated that monocytes treated with dexamethasone for the first 24 hours of 5 day culture displayed significantly increased phagocytic capacity. This effect was mediated *via* the GR and blocked by GR antagonist RU38486 (Liu *et al.*, 1999). Furthermore, monocytes treated with dexamethasone for a duration of 5 days and longer were characterised by “reprogrammed” differentiation associated with high phagocytic capability, altered morphology and reorganisation of cytoskeletal elements. This phenotype of M ϕ also displayed high levels of active Rac and, when compared with untreated cells, high cytoskeletal activity (formation of cellular extensions and lamellipodia). The latter observation may significantly contribute to

glucocorticoid-induced increase in clearance of apoptotic neutrophils. Furthermore, these cells were distinctively round, smaller and demonstrated decreased adhesion as a consequence of decreased activation of paxillin and *pyk2* (important proteins involved in adhesion contacts) as well as downregulated expression of p130Cas (mediator of adhesion signalling). In addition, the cell membrane was characterised by increased activity, which was likely to result from both increased cytoskeletal elements remodelling and decreased cell adhesion (Giles *et al.*, 2001). M ϕ are thought to be one of the most important cells involved in removal of apoptotic cells. However, non-professional phagocytes such as DC, hepatocytes, fibroblasts and epithelial cells may also be involved in this process. For instance, small airway epithelial cells are capable of ingesting apoptotic eosinophils and, importantly, this process is significantly augmented by dexamethasone treatment. The latter observation adds another important fact to the role of glucocorticoids in therapy of asthma (Walsh *et al.*, 1999).

FIGURE 1.9 GR exerts its anti-inflammatory effects by interfering with pro-inflammatory signalling at multiple levels and *via* various mechanisms. For illustrative purposes TLR-mediated pro-inflammatory pathways are depicted. GR induces expression of numerous genes e.g. I κ B, GILZ or MKP1 that inhibit activation or action of transcription factors NF- κ B and AP1 (1,2,3). In addition GR inhibits activity of IRF transcription factors. The exact mechanism is not fully understood but it is possible that GR competes with IRF3 for GRIP1 binding (4). More detailed description of GR interference with pro-inflammatory signalling is presented in chapter 1.5.1. TLR-mediated signal transduction and key adaptor proteins and enzymes are described in more depth in section 1.2.2.1. **GR** – glucocorticoid receptor.

1.5.2 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

The term non-steroidal anti-inflammatory drugs (NSAID) covers a group of various therapeutics characterised by anti-inflammatory, anti-analgesic and anti-pyretic properties. The examples include aspirin, ibuprofen, ketoprofen, flurbiprofen, indomethacin, etodolac meloxicam, diclofenac. They all demonstrate ability to inhibit activity of the enzyme called cyclooxygenase (COX). Aspirin is one of the most commonly used NSAID and, as so, it is used in the majority of studies investigating mechanism of action as well as the role of NSAID in resolution of inflammation, inflammatory cell apoptosis and their clearance by M ϕ .

Eicosanoids are a group of signalling molecules, which are generated in the process of oxygenation of twenty-carbon essential fatty acids (EFAs), such as arachidonic acid (AA). Eicosanoids are further divided into several groups depending on the enzymes involved in their biosynthesis. One of them, prostanoids, is composed of the products of AA metabolism by COX, i.e., prostaglandins (PG) prostacyclins (PGI₂) and thromboxanes (TX). The second group includes leukotrienes (LT) – products of AA metabolism by 5-lipoxygenase. There is also a third group of lipoxins (LX), which are also AA metabolites playing a crucial role in resolution of inflammation as further described below. Prostanoids are important mediators of the inflammatory response and control processes of vasodilation (PGI₂), vasoconstriction (TXA₂), platelet aggregation (TXA₂, PGI₂) as well as pain and fever (PGE₂). LT such as LTB₄ stimulate chemotaxis and adhesion of leukocytes as well as release of ROS and enzymes from neutrophils (LTB₄). Furthermore, cysteinyl LT (i.e., LTC₄, LTD₄, and LTE₄) were reported to be the slow reacting substance of anaphylaxis. They are potent inducers of bronchoconstriction, vasodilatation of most vessels and coronary vasoconstriction. They also increase vascular permeability and stimulate mucus secretion. They play a crucial role in development of such pathophysiological conditions as asthma, allergic rhinitis as well as gastrointestinal diseases and atherosclerosis (Capra, 2004). Therefore, cysteinyl LT inhibitors have been introduced to clinic for treatment of asthma and allergic rhinitis. The example is leukotriene receptor antagonists, namely montelukast and zafirlukast.

NSAID exert their therapeutic effects *via* blocking the activity of the above-mentioned COX enzyme. COX metabolises the first step of a conversion of AA into PGG₂ and H₂. PGG₂ and PGH₂ are, in turn, metabolised into individual prostanoids (PGD₂, PGE₂, PGJ₂, PGI₂, PGF_{2α} and TXA₂) (Gilroy, 2005). There are at least two isoforms of COX. COX1 is expressed constitutively whereas COX2 is expressed in response to variety of stimuli and is responsible for PG biosynthesis in inflamed tissue (Amann & Peskar, 2002). However, it has been demonstrated that COX2 is constitutively expressed in some tissues, e.g., in the central nervous system and kidney (Harris *et al.*, 1994; Yamagata *et al.*, 1993). NSAID differ in their potency to block COX1 and COX2 and aspirin is regarded as non-selective COX inhibitor whereas ibuprofen and indomethacin show greater selectivity towards COX2. Blockade of COX by aspirin prevents formation of prostanoids, but, at the same time, allows formation of 15(R)–hydroxyeicosatetraenoic acid (15(R) – HETE), which can be further metabolised by lipoxygenase (LOX) isoenzymes into LX i.e., 15-epi-lipoxin A₄ and 15-epi-lipoxin B₄ (LXA₄ and LXB₄ respectively). Formation of LX is an exclusive characteristic of aspirin, not shared with other NSAID (Claria & Serhan, 1995). LXs exert a number of anti-inflammatory effects, e.g., inhibition of granulocyte recruitment but they stimulate monocyte chemotaxis and adherence. The anti-inflammatory as well as vasorelaxing properties of both LXA₄ and LXB₄ can be explained, at least in part, by their ability to stimulate the synthesis of PGI₂ and nitric oxide (NO) in endothelial cells (Gilroy, 2005). There are several reports in the literature demonstrating the ability of aspirin to inhibit activation of pro-inflammatory transcription factors NF-κB and AP-1. (Amann & Peskar, 2002). However, the extent to which these effects contribute to the anti-inflammatory properties of aspirin remains to be established. Importantly, in mice deficient in p105 (the precursor of the p50 component of NF-κB) aspirin maintained its anti-inflammatory properties, while the anti-inflammatory effect of dexamethasone was abolished (Cronstein *et al.*, 1999).

Several lines of evidence indicate that eicosanoids may play an important role in the resolution of inflammation *via* regulation of Mφ efferocytosis. For instance, PGs were demonstrated to reduce Mφ capacity for clearance of apoptotic neutrophils. This effect was correlated with the increases in cellular cAMP levels. Pretreatment of Mφ with PGE₂, PGD₂ or cAMP stable analogues such as 8-bromo-cAMP and

dibutyryl-cAMP changed M ϕ adhesion, reduced membrane activity and cell locomotion as well as significantly decreased in the proportion of M ϕ that phagocytosed apoptotic cells (Rossi *et al.*, 1998b). In addition, it has been reported that other pro-inflammatory mediators, which stimulate activity of adenylate cyclase and protein kinase A (PKA) are likely to inhibit clearance of apoptotic cells (Oropeza-Rendon *et al.*, 1979). In cytotoxic T cells, PKA is able to phosphorylate Rho, which is a key negative regulator of cytoskeletal organisation. These data further support the pivotal role of cAMP in regulation of the phagocytosis process (Lang *et al.*, 1996). The activation of the Rho family GTPases requires prenylation (covalent attachment of lipid adducts) and subsequent membrane insertion. Interestingly, statins have been demonstrated to reduce cellular levels of prenylation substrates *via* inhibition of HMG-CoA reductase. For instance, lovastatin suppresses prenylation and membrane localization of RhoA to a greater extent than Rac-1 thus altering RhoA–Rac-1 membrane balance towards Rac-1. Since Rac-1 is a positive regulator of phagocytosis, lovastatin is able to increase M ϕ ability to phagocytose apoptotic cells (Morimoto *et al.*, 2006).

In addition, it has been demonstrated that LX promote the resolution phase of inflammation by inhibiting neutrophil chemotaxis and adhesion (Serhan, 1997). For instance, findings presented by Godson and colleagues clearly show that LXA₄ and its stable synthetic analogues augment phagocytosis in a concentration-dependent manner (Godson *et al.*, 2000). Interestingly, stable cAMP analogue 8-bromo-cAMP attenuated while PKA inhibitor–Rp-cAMP mimicked LXA₄ effects on phagocytosis. The effects of Rp-cAMP and LXA₄ were not additive suggesting that the same mechanism is involved, possibly, inhibition of PKA. The latter has been further supported by the fact that upon cell stimulation with PGE₂, LXA₄ did indeed inhibit PKA activity. These findings clearly point out the critical role of PKA in regulation of phagocytosis. One of PKA's cellular substrates is scavenger receptor CD36 (Hatmi *et al.*, 1996) and loss of CD36 phosphorylation in platelets results in increased cytoadhesion (Asch *et al.*, 1993). Monoclonal antibodies against CD36 blocked pro-phagocytic properties of LXA₄ indicating that LXA₄ may induce dephosphorylation of CD36 and promote recognition of apoptotic cells by phagocytes (Godson *et al.*,

2000). A new phagocytic receptor FPR2/ALX has been demonstrated to play an important role in LXA₄–stimulated phagocytosis (MADERNA *et al.*, 2010).

1.5.3 CYTOKINE INHIBITORS

Cytokines are very important inflammatory mediators, which are produced by immune cells and orchestrate immune and inflammatory responses. However, if their levels are abnormal, cytokines significantly contribute to the pathogenesis of chronic inflammatory conditions, as described in more detail in section 1.4. Cytokines and their receptors are either cell surface molecules or found as soluble forms that have been released to the extracellular environment. Consequently, their action can be blocked by protein-based biologics such as monoclonal antibodies and soluble receptor-IgG fusion proteins. Research is progressing rapidly in the field of cytokine inhibitors and they seem to be a very promising, new class of anti-inflammatory therapeutics. Currently, a few of them are licensed for the treatment of certain chronic inflammatory conditions i.e., RA, psoriasis or Crohn's disease.

The first cytokine inhibitors introduced to the clinic were TNF- α inhibitors. This was based on the observation that TNF- α participates in the pathogenesis of numerous diseases such as RA, diabetes, septic shock or congestive heart failure. Some of these conditions have been demonstrated to have autoimmune or chronic inflammatory component (TAYAL & KALRA, 2008). So far, two strategies have been developed to block the action of circulating TNF- α , namely monoclonal anti-TNF- α antibodies (used under generic names infliximab and adalimumab) and the dimeric soluble TNF-R2 Fc fusion protein (used under generic name etanercept). Initially, infliximab was approved to be used with the disease modifying anti-rheumatic drug methotrexate (MTX) for RA patients with an inadequate response to sole MTX therapy. This was then expanded to include psoriasis, ulcerative colitis, psoriatic arthritis, Crohn's disease and ankylosis spondylitis and is further progressing to include such conditions as diabetes, stroke, asthma and malignancy (WILLIAMS *et al.*, 2007).

The TNF- α blocking agents are thought to exert their anti-inflammatory effect by a number of mechanisms (WILLIAMS *et al.*, 2007). They decrease expression of the pro-inflammatory cytokines, reduce recruitment of leukocytes into the joints, reduce

angiogenesis, increase activity of the regulatory T cells, normalise T cell hyporesponsiveness and prevent joint erosion. However, a few concerns are associated with the use of anti - TNF- α agents. First of all, their efficacy is quite variable but can be improved by combined treatment with MTX (Maini *et al.*, 1998). It has been demonstrated that combination of TNF- α inhibitors with MTX augments joint protection (Lipsky *et al.*, 2000) and probably induces joint repair (Klareskog *et al.*, 2004). Another problem is the risk of infection, especially tuberculosis (TB) and sepsis. TNF- α is the main mediator of host defence and its inhibition has been reported to augment the risk of recrudescence of TB (Keane *et al.*, 2001). Therefore, it is recommended that all the patients should be evaluated for TB before starting TNF- α inhibitors.

Interleukin-1 (IL-1) is another cytokine that has been shown to play an important role in the pathogenesis of RA. Similarly to TNF- α inhibitors, several approaches have been used to block interaction between IL-1 and IL-1 type I receptor (IL-1-R1). One of them is a recombinant form of human IL-1 receptor antagonist (used under generic name anakinra), which has been licensed for treatment of RA in combination with MTX, following successful clinical trials (Bresnihan *et al.*, 1998). An alternative approach includes fully human anti-IL-1 mAb and soluble IL-1-R1 Fc fusion protein which are still under investigation (Williams *et al.*, 2007). Interestingly, and in contrast to animal studies, IL-1 blockers are less effective in human RA than TNF- α blockers (Williams *et al.*, 2000). The examples of other cytokines that have been targeted in RA include IL-2 (a humanised antibody against the α -chain of the IL-2 receptor used under generic name daclizumab) and IL-6 (a humanised anti-IL-6 receptor antibody used under generic name tocilizumab) (Williams *et al.*, 2007).

Summing up, if mechanisms implicated in disease pathogenesis are relatively simple and involve abnormalities in cytokine levels or signalling, cytokine therapy seems to be a very promising approach. Therefore, a number of other cytokine (IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-18, IL-21 etc.) inhibitors have been used experimentally in animals and are being tested in patients for various conditions (Tayal & Kalra, 2008). However, given the pleiotropic and redundant functions of cytokines, there is a great chance that a particular cytokine may play opposing roles

in immune and inflammatory pathways. For instance, IFN γ -mediated activation of myeloid cells significantly contributes to inflammation. However, this cytokine also helps to limit autoimmune responses by reducing proliferation of lymphoid cells. Consequently, the 'net' effect of IFN γ blockade is detrimental in some animal models (Williams *et al.*, 1993; Vermeire *et al.*, 1997). Similarly, the effect of TNF- α inhibition is beneficial in a number of diseases. Yet, in patients with MS it was found to worsen the disease, augmenting disease progression (1999). This phenomenon can be explained, at least in part, by neuroprotective effects of TNF- α . Therefore, the successful identification of suitable cytokine targets in chronic inflammatory or autoimmune conditions requires fine characterisation of the underlying biology. Nevertheless, research is continuing in the field of cytokine inhibitors aiming at development of new therapies, improvement of those already in use and increase of clinical benefit by establishing the safest and the most effective dosage levels.

1.6 HYPOTHESES AND AIMS

Inflammation, as long as it is controlled, is a crucial mechanism defending organisms in case of infection or tissue injury. Inflammatory processes are regulated in a very complex manner by numerous mediators, e.g., cytokines. Furthermore, abnormalities in cytokine levels have been demonstrated to play a fundamental role in development of chronic inflammation and autoimmunity. Overexpression of TNF- α seems to be of particular importance and inhibition of TNF- α with TNF- α blocking agents has been shown to be a relatively successful approach for treatment of RA, psoriasis and Crohn's disease.

Currently, it is postulated that at least two key mechanisms are involved in resolution of inflammatory responses, namely apoptosis of inflammatory cells and their subsequent clearance by phagocytes. Accumulation of apoptotic cells, usually associated with impaired clearance, results in secondary necrosis, further tissue damage and exacerbation of inflammation.

Based on the above observations the main hypotheses addressed in this PhD thesis are as follows:

1) **Given that sites of chronic inflammation are characterised by an overexpression of TNF- α and that TNF- α inhibitors enhance resolution of inflammation, agents that induce TNF- α production or TNF- α *per se* interfere with processes involved in the resolution of inflammation.** This hypothesis is further supported by observations by Ward and colleagues that TNF- α , depending on time of treatment, may either induce or inhibit apoptosis of inflammatory cells (Ward *et al.*, 1999). Yet, data regarding TNF- α effects on M ϕ clearance of apoptotic cells are not conclusive. For instance, a report by Savill and colleagues demonstrates that TNF- α may augment clearance of apoptotic cells by immature M ϕ (Ren & Savill, 1995). In contrast, findings by McPhillips and colleagues regarding mature M ϕ demonstrate otherwise (McPhillips *et al.*, 2007).

2) **Anti-inflammatory agents (e.g., IL-10 or glucocorticoids) oppose TNF- α effects on M ϕ clearance of apoptotic cells and consequently promote resolution of inflammation.** IL-10 and glucocorticoids have been previously shown to augment M ϕ clearance of apoptotic cells (Liu *et al.*, 1999; Xu *et al.*, 2006). Yet, there is not

much known about these agents exerting similar effects in pro-inflammatory conditions (e.g., overexpression of TNF- α). It seems to be likely that pro- and anti-inflammatory mediators exert opposite effects on certain processes (e.g., M ϕ phagocytosis of apoptotic neutrophils) and therefore the 'net' effect depends on the balance between them.

Therefore the aims of this PhD thesis were to:

- 1) Characterise the effects of the pro-inflammatory cytokine TNF- α on M ϕ efferocytosis of apoptotic neutrophils. A number of pro-inflammatory mediators, i.e., LPS, lipoteichoic acid (LTA), peptidoglycan (PGN) have been used to induce M ϕ production of TNF- α . Their effects on M ϕ phagocytosis were compared with those of exogenous human recombinant TNF- α .
- 2) Examine, if the LPS, LTA or PGN effects on phagocytosis are mediated by stimulation of M ϕ production of TNF- α . We compared the efficacy of several TNF- α inhibitors, i.e., dimeric soluble human recombinant TNF-R1/Fc chimera (sTNF-R1/Fc), monomeric soluble human recombinant TNF-R1 (sTNF-R1) and TACE inhibitor to reduce bioactivity of TNF- α released into culture medium.
- 3) Evaluate the role of IL-10 in regulation of M ϕ ability to phagocytose apoptotic neutrophils in the absence or presence of pro-inflammatory mediators (LPS or TNF- α). The effect of IL-10 on production of TNF- α by M ϕ stimulated with pro-inflammatory agents has also been investigated.
- 4) Examine the effects of anti-inflammatory drugs such as glucocorticoids and NSAID (i.e., aspirin, ibuprofen, and indomethacin) on M ϕ phagocytosis of apoptotic neutrophils. Selective COX1 and COX2 inhibitors (SC-650 and NS-398) as well as paracetamol (limited effect on COX1 and COX2) have been included as additional controls. Their effects have also been assessed in the presence of pro-inflammatory mediators (LPS or TNF- α).

CHAPTER TWO
MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 REAGENTS

All reagents were obtained from Sigma-Aldrich Co. (Poole, UK) unless otherwise stated.

2.1.1 TISSUE CULTURE SOLUTIONS

Tissue culture Iscove's modified dulbecco's medium (IMDM) was obtained from PAA Laboratories (Pasching, Austria) and Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco Lige Technologies (Paisley, UK) and they were supplied sterile, endotoxin free, at pH 7.

Hank's Buffered Salt Solution (HBSS), phosphate buffered saline (PBS) (1x and 10x) both with or without Ca^{2+} and Mg^{2+} , penicillin/streptomycin (100 U/ml) solution and Trypsin (0.25%), ethylenediaminetetraacetic acid (EDTA) solution (all sterile, endotoxin free) were from PAA Laboratories (Pasching, Austria).

Foetal bovine serum and foetal calf serum were heat inactivated (50°C, 1 h) before addition to media.

All tissue culture solutions were stored at 4°C and pre-warmed to 37°C in a water bath prior to use.

2.1.2 PLASTIC WARE

Tissue culture 50 ml flasks were from Becton Dickinson Labware (Oxford, UK) and 75 ml flasks were from Corning Incorporated (Corning, NY).

Tissue culture plates (48-well, 6-well, 96-well EIA/RIA high binding) were from Corning Incorporated (Corning, NY) and flexible 96-well plates were from Becton Dickinson Labware (Oxford, UK).

Falcon® tubes, 15 ml and 50 ml, were from Becton Dickinson Labware (Oxford, UK).

2.1.3 REAGENTS FOR ISOLATION OF HUMAN BLOOD CELLS

Dextran T500 from Amersham Pharmacia Biotech (Buckingham UK) was prepared as a 6% solution in 0.9% NaCl, aliquoted and stored at 4°C. Sodium chloride solution 0.9% from Baxter (Glasgow, UK) was stored at RT, sodium citrate solution 3.8% and 1M calcium chloride solution from Sigma (St.Louis MO, USA) were aliquoted and stored at 4°C, Percoll™ from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) 100% solution was aliquoted (27 ml) and stored at 4°C.

2.1.4 BACTERIAL WALL COMPONENTS

Lipopolysaccharide (LPS) from *E.coli*, serotype 0127:B8, was purchased from Sigma-Chemicals Co. (St.Louis MO, USA) and stock solutions (1mg/ml in sterile PBS without Ca²⁺ and Mg²⁺) were aliquoted and stored at -20°C.

Peptidoglycan (PGN) and lipoteichoic acid (LTA) from *S.aureus* were purchased from Fluka (Buchs, Switzerland) and stock solutions (10 mg/ml in sterile PBS without Ca²⁺ and Mg²⁺) were aliquoted and stored at -20°C.

2.1.5 RECOMBINANT PROTEINS

Recombinant human (rh) TNF- α , recombinant mouse (rm) TNF- α , rh IL-6, rh IL-10 were from R&D Systems (Minneapolis, LA, USA) and rh IL-1 β was from Merck Chemicals (Nottingham, UK). Stock solutions of the recombinant proteins (10 μ g/ml in sterile PBS without Ca²⁺ and Mg²⁺) were aliquoted and stored at -20°C.

Soluble (s)rh TNF receptor 1/ Fc Chimera (sTNF-R1/Fc) and monomeric srh TNF-R1 (sTNF-R1) were from R&D Systems (Minneapolis, LA, USA) and stock solutions (50 μ g/ml in sterile PBS without Ca²⁺ and Mg²⁺) were aliquoted and stored at -20°C.

Recombinant human Annexin-V fluorescein isothiocyanate (FITC) conjugate was purchased from Roche (Hertfordshire, UK) and used in a 1:500 dilution, whereas

Annexin-V APC conjugate was purchased from Invitrogen (Paisley, UK) and used in a 1:50 dilution.

2.1.6 CELL TRACKER™ PROBES

Cell Tracker™ Green CMFDA and Cell Tracker™ Far Red DDAO-SE were purchased from Invitrogen (Paisley, UK). Stock solutions (in sterile PBS without Ca^{2+} and Mg^{2+}) were aliquoted and used in a 1:1000 dilution and stored at -20°C .

2.1.7 CYTOKINE MEASUREMENT

BD™ - Cytometric Bead Array (CBA) Human Inflammation Kit was from BD Biosciences Pharmingen (San Diego, CA, USA). DuoSet® ELISA (human/mouse TNF- α , human/mouse IL-10) was from R&D Systems (Minneapolis, LA, USA).

2.1.8 ANTIBODIES

Monoclonal anti-human IL-10 receptor 1 antibody (anti-IL-10-R1Ab) was from R&D Systems (Minneapolis, LA, USA) and stock solution (1 mg/ml in sterile PBS without Ca^{2+} and Mg^{2+}) was further diluted to 1 $\mu\text{g}/\text{ml}$ and stored at -20°C . F(ab')₂ anti-human IgG FITC conjugated antibody from goat was from CALTAG laboratories (Buckingham, UK) and used in 1:50 dilution of a stock solution (1mg/ml).

Antibodies used for western blotting were obtained from Cell Signalling (Herts, UK) and diluted according to manufacturer's instructions. These include:

1) Primary antibodies (from rabbit):

- phospho-p44/42 MAP Kinase (Thr202/Tyr204) and p44/42 Kinase antibodies,
- phospho-SAPK/JNK (Thr183/Tyr185) and SAPK/JNK antibodies,
- PhosphoPlus® p38 MAP Kinase (Thr180/Tyr182) and p38 MAP Kinase antibodies, kits were from Cell Signalling and diluted according to manufacturer's instructions.

- 2) Secondary anti-rabbit, HRP-linked antibody
- 3) anti-biotin, HRP – linked antibody.

2.1.9 OTHER REAGENTS

R-roscovitine was purchased from Merck Chemicals LTD (Nottingham, UK). Dexamethasone was purchased from Organon (Cambridge, UK). SC-650 and NS-398 inhibitors as well as TNF- α Convertase (TACE) Inhibitor (TAPI-1) were from Merck Chemicals (Nottingham, UK). Bio-Rad Dc Protein Assay Reagent A and Reagent B were obtained from Bio-Rad Laboratories (Hercules, CA).

2.2 CELL ISOLATION AND CULTURE

2.2.1 HUMAN PERIPHERAL BLOOD CELLS

2.2.1.1 CELL ISOLATION FROM HUMAN PERIPHERAL BLOOD

Human mononuclear cells, neutrophils and erythrocytes were isolated from peripheral blood of healthy volunteers (Haslett *et al.*, 1985; Rossi *et al.*, 1998a). As demonstrated in Figure 2.1 the main steps of the isolation process are:

- 1) 40 ml of blood was drawn from the antecubial fossa through a 19-gauge (19 G) needle into 50 ml BD Falcon® tubes that contained 4 ml of citrate (0.38 % final concentration) and mixed by gentle inversion of tube,
- 2) platelet rich plasma (PRP) was separated from whole blood by centrifugation (350 g, 20 min),
- 3) PRP was aspirated from the erythrocyte/leukocyte rich layer and was then used to prepare autologous serum by incubating (37°C, 30-60 min) with addition of calcium chloride (220µl of 1M CaCl₂ per 10 ml of PRP),
- 4) erythrocytes were separated from leukocytes by dextran sedimentation: 6 ml of pre-warmed (37°C) 6 % dextran was added into each tube, topped up to 50 ml with pre-warmed (37°C) saline solution (0.9 % NaCl), gently mixed and allowed to sediment for not longer than 30 min at RT,
- 5) leukocyte-rich upper layer was gently aspirated and washed with saline solution, followed by centrifugation (350 g, 6 min) to obtain a leukocyte-rich pellet,
- 6) isotonic 90 % Percoll solution was prepared by adding 3 ml of 10 x PBS without Ca²⁺ and Mg²⁺ into 27 ml of 100 % Percoll™ (9:1 ratio); obtained solution was further diluted into 55 %, 68 % and 81 % isotonic Percoll™ solutions in 1x PBS without Ca²⁺ and Mg²⁺; discontinuous gradients were prepared by layering 3 ml of 68 % Percoll solution (middle layer) onto 3 ml of 81 % Percoll™ solution (lower layer) in 15 ml polypropylene tubes,
- 7) leukocyte pellet was re-suspended in 3 ml of 55 % Percoll solution and layered on the top of 68 % Percoll solution to form the upper layer of the gradient,

8) sub-populations of leukocytes were separated by centrifugation (720 g, 20 min, acceleration 0, break 0) of gradients,

9) mononuclear cells and granulocytes were next harvested (55:68 % and 68:81 % interface respectively) into separate 50 ml polypropylene tubes and washed twice in 1x PBS without Ca^{2+} and Mg^{2+} (220 g, 6 min). Erythrocytes may also be harvested at this point and stored at 4°C.

Yields of isolated cells were assessed with the use of a bright line haemocytometer. For this purpose 10 μl of cell suspension was placed under a cover slip on the haemocytometer, the number of cells in the central 25 squares counted by light microscopy (x10 magnification) and the cell yield calculated according to the following equations:

$$\text{Cell Number in central 25 squares of a hemocytometer} \times 10\,000 = \text{Cell Number in 1 ml of a cell suspension}$$

$$\text{Cell Number in 1 ml of a cell suspension} \times \text{Total volume of a cell suspension} = \text{Total cell yield}$$

After cell number had been assessed the cells were centrifuged (220 g, 6 min) and re-suspended at the appropriate concentration in pre-warmed (37°C) IMDM supplemented with penicillin/streptomycin. The typical yield from 160 ml of whole blood was 100×10^6 for mononuclear cells and 250×10^6 for granulocytes, however, considerable variation between donors was observed. Typical photomicrographs of freshly isolated human blood neutrophils and monocytes are shown in Figure 2.2.

FIGURE 2.1. Schematic diagram demonstrating isolation of cells from human peripheral blood. All steps of the isolation of human blood cells are described in more detail in section 2.2.1.1 and indicated in the above figure.

FIGURE 2.2. Typical photomicrographs of freshly isolated human blood neutrophils (A) and monocytes (B). Human blood cells have been isolated following the protocol described in section 2.2.1.1. Following isolation, 100 μ l of cell suspension have been used to prepare slides, which were then stained using Diff-Quik™ physiological stain. Images were obtained using an Axiovert S100 inverted microscope (Carl Zeiss), RS Photometrix camera and analysed with Open lab software, 3.1.5 version. Magnification 1000x.

2.2.1.2 CULTURE OF HUMAN MONOCYTE-DERIVED MΦ (MDMΦ)

Mononuclear cells isolated by Percoll™ gradient centrifugation were re-suspended in IMDM at the concentration 4×10^6 cells/ml (monocyte/lymphocyte ratio was assessed by flow cytometry), plated at 0.5 ml/well in 48-well plates and incubated for 1 hour (37°C, 5 % CO₂) to allow monocyte adhesion to the plate. Following 1 hour incubation, tissue culture medium was aspirated and non-adherent cells including contaminating lymphocytes were removed by washing twice with HBSS with Ca²⁺ and Mg²⁺. Monocytes were then cultured in IMDM supplemented with penicillin/streptomycin and 10 % (v/v) autologous serum +/- treatment for 6 days at 37°C and 5 % CO₂ atmosphere to allow their maturation into Mφ.

2.2.1.3 CULTURE OF HUMAN NEUTROPHILS

Following isolation by Percoll™ gradient centrifugation neutrophils (on average >98% pure as assessed by morphology or flow cytometry) were cultured at 37°C and 5% CO₂ atmosphere at a concentration of 5×10^6 /ml in IMDM supplemented with penicillin/streptomycin and 10% autologous serum either for 20-24 hours to undergo constitutive apoptosis or alternatively for 8 hours, when apoptosis (>90% on average) was induced with 20 μM R-roscovitine (Rossi *et al.*, 2006).

If apoptotic neutrophils were used as phagocytic targets to assess Mφ phagocytosis by flow cytometry, neutrophils immediately after isolation were re-suspended in IMDM supplemented with penicillin/streptomycin at the concentration of 20×10^6 cells/ml and incubated for 15 minutes (37°C, 5% CO₂) with Cell Tracker™ Green CMFDA at a final concentration of 2 μg/ml (1 μl of 1 mg/ml stock solution per 1×10^6 of neutrophils). Following centrifugation (220 g, 6 minutes) neutrophils were re-suspended 5×10^6 /ml in IMDM supplemented with penicillin/streptomycin and 10% autologous serum and cultured as described above (in majority the of experiments we used neutrophils that had undergone constitutive apoptosis).

Apoptosis of neutrophils was assessed by oil-immersion light comparative microscopy of DiffQuik™ stained cyto-centrifuge preparations or alternatively by an annexin-V binding assay (Rossi *et al.*, 2006), which are described in more detail in section 2.3. Only neutrophil samples with less than 5 % necrosis (assessed by propidium iodide uptake) were used.

2.2.2 MURINE BONE MARROW DERIVED MΦ (BMDMΦ)

Bone marrow was obtained from femurs prepared from IL-10^{-/-} and wild type (wt) mice (Kuhn *et al.*, 1993). To obtain bone marrow, both femurs were removed and placed in HBSS and kept at 4°C. BMDMφ growth/conditioned medium was used throughout for isolating bone marrow cells and during the culture (Dulbecco's modified eagle medium (DMEM) F-12 GlutMAX supplemented with 10 % FBS (v/v), 1 % penicillin/streptomycin cocktail (v/v) and 10 % L929 conditioned medium). To obtain bone marrow the following procedure was applied:

- 1) Femurs were placed on the bottom of Petri dish in a hood and one by one surface sterilized by wiping with bacteriological wipes and dipping into ethanol. From this point onward sterile techniques were adopted.
- 2) Sterilized femurs were placed on the lid of Petri dish containing a small amount of BMDM growth/conditioned medium to prevent bones from drying up (due to ethanol dip).
- 3) Using a 19-gauge (19G) needle 10 ml of BMDMφ medium were aspirated into a 10 ml syringe and the 19G needle was replaced with a 25G needle.
- 4) The scalpel and forceps were sterilized in 70 % ethanol and used to firmly hold and sever the femur at its both ends to expose the cavity of the bone marrow (red circular dot in cross-section).
- 5) The femur was then picked up with sterile forceps, the 25G needle inserted into marrow cavity and bone marrow flushed out into the Falcon® tube. Next, the femur was inverted, the 25G needle inserted into its second end and marrow flushed out again until the cavity looked white.
- 6) The 25G needle was replaced with a 19G needle and the content of the Falcon® tube was aspirated and flushed out to break up any clumps and form a single cell

suspension which was next transferred into a Teflon pot and topped up with 30 ml of BMDM ϕ growth/conditioned medium to a final volume of 40 ml.

7) Teflon pots were placed into an incubator (37°C, 5 % CO₂) and 10 ml of BMDM growth/conditioned medium was replaced every other day.

On day 4 of culture, cells were harvested and yield assessed by use of a bright line haemocytometer (see section 2.2.1.1 for isolation of cells from human peripheral blood). Next, cells were re-suspended at the concentration 2×10^6 cells/ml in BMDM ϕ growth/conditioned medium, plated at 0.5 ml/well on 48-well plates and cultured for 4 days at 37°C and 5 % CO₂. Medium was changed every 48 hours and appropriate treatment was applied. Typical photomicrographs of BMDM ϕ cultured for 4 days in growth/conditioned medium are shown in Figure 2.3.

FIGURE 2.3. Typical photomicrographs showing wt (A) and IL-10^{-/-} (B) BMDMφ cultured for 4 days in BMDMφ growth/conditioned medium. BMDMφ have been isolated and cultured for 4 days following the protocol described in section 2.2.2. On day 4, 100 µl of cell suspension has been used to prepare slides, which were then stained using Diff-Quik™ physiological stain. Images were obtained using an Axiovert S100 inverted microscope (Carl Zeiss), RS Photometrix camera and analysed with Open lab software, 3.1.5 version. Magnification 1000x.

2.3 ASSESSMENT OF CELL VIABILITY

2.3.1 CELL MORPHOLOGY

Morphology of cells was assessed following cyto-centrifugation (300 rpm, 3 min). Obtained slides were fixed in methanol (100 %, 1 minute) and then stained using Diff-Quik™ physiological stain before they were analysed by light microscopy (x 400-1000 magnification) (Rossi *et al.*, 2006). Apoptotic cells were characterised by condensed, pyknotic nucleus or alternatively lack of nucleus (so called ghost cells) while membrane integrity was maintained. In contrast, necrotic cells were defined as those with disrupted membrane. To assess cell morphology at least 500 cells per slide were counted. Typical photomicrographs of apoptotic neutrophils are shown in Figure 2.4.

FIGURE 2.4. Typical photomicrographs of apoptotic neutrophils (next page). Human blood neutrophils have been cultured for either 8 hours in culture medium (**A**) or for 8 hours in the presence of 20 μ M R-roscovitine (**B**) or for 24 hours in culture medium (**C**). Following culture, 100 μ l of cell suspension has been used to prepare slides, which were then stained using Diff-Quik™ physiological stain. Apoptotic cells are characterised by distinct, condensed, pyknotic nucleus or alternatively lack of nucleus, whereas non-apoptotic neutrophils have typical multi-lobed nucleus. Images were obtained using an Axiovert S100 inverted microscope (Carl Zeiss), RS Photometrix camera and analysed with Open lab software, 3.1.5 version. Magnification 1000x. **aN ϕ** – apoptotic neutrophil, **naN ϕ** – non apoptotic neutrophil.

2.3.2 FLOW CYTOMETRY

In order to assess apoptosis of neutrophils prior to the phagocytosis assay, flow cytometric analysis was used (Rossi *et al.*, 2006). This method allows characterisation of individual cells suspended in a focused stream of fluid, which passes through different laser beams. Particular cells vary in their ability to fluoresce or scatter the laser light and these fluctuations are then recorded by specific detectors. The light scatter provides information about the relative size (forward scatter; FS) and the relative granularity (side scatter; SS) of a cell. For instance, M ϕ are bigger (have higher FS) and less granular (have lower SS) than neutrophils.

In addition, the flow cytometry technique can be used to detect the presence of certain proteins on the cell surface by means of fluorescent-conjugated antibodies or reagents. The latter is of particular importance for assessment of cell apoptosis. During apoptosis some of the cellular markers disappear from the cell surface whereas some are translocated to the outer leaflet of the plasma membrane. PS is an example of a marker that externalises during apoptosis (as described in section 1.3.2). Importantly, another protein, Annexin-V shows high affinity for PS binding and therefore FITC-conjugated Annexin-V (detected by FL-1 channel of the flow cytometer) is extensively used to quantify percentage of apoptotic cells within cell populations (Vermees *et al.*, 1995). In addition, necrotic or secondary necrotic (late apoptotic) cells are characterised by lack of cell membrane integrity. Consequently, dyes such as propidium iodide (PI) can freely enter cells and these cells are positive for PI staining (detected by the FL-2 channel of the flow cytometer). As demonstrated in Figure 2.5, healthy, viable cells are Annexin-V and PI negative (FL-1⁻ and FL-2⁻), early apoptotic cells are Annexin-V positive but PI negative (FL-1⁺ and FL-2⁻), whereas necrotic cells and late apoptotic/secondary necrotic cells are positive for both Annexin-V and PI (FL-1⁺ and FL-2⁺).

To assess neutrophil viability by the above-described approach Annexin-V was diluted (1:500) in binding buffer (500 ml HBSS supplemented with 2.5 ml of 1M CaCl₂) and aliquoted 280 μ l per FACS tube. Next 20 μ l aliquots of neutrophil suspension (4×10^6 /ml) were added to FACS tubes and incubated for 15 minutes on

ice. Finally 1 μ l of PI (1 mg/ml) was added to FACS tubes approximately 2 minutes before flow cytometric analysis was carried out.

FIGURE 2.5 Representative flow cytometric profiles of freshly isolated (left panels) and cultured for 24 hours (right panels) neutrophils. The % of apoptotic cells within the cell population can be estimated on the basis of Annexin-V binding (higher FL-1 signal), whereas the % of necrotic cells is demonstrated by internalisation of PI (higher FL-2 signal). The data can be plotted as two dimensional dot plots diagrams (A) or histogram plots of cells detected by FL-1 (B) or alternatively FL-2 channels (C). N ϕ – neutrophils.

2.3.3 ASSESSMENT OF CELL MEMBRANE INTEGRITY

Staining of cells with Trypan blue, a derivative of toluidine, allows distinguishing between live/apoptotic and necrotic cells. Live/apoptotic cells maintain membrane integrity and are selective towards compounds that can enter the intracellular compartments. In contrast, necrotic cells have disrupted membranes and the above-mentioned selectivity is lost. Thus, upon staining, Trypan blue can freely traverse the membrane and stain necrotic cells. Cells are then examined under the light microscope, counted and the percentage of necrotic cells can be evaluated.

In all experiments, where the viability of M ϕ was assessed by this method, cells were first trypsinised and harvested to Eppendorf™ tubes. Subsequently, an equal volume of 0.4% Trypan blue was added to 150 μ l of cell suspension and incubated in room temperature for 5–15 minutes. Following incubation, 10 μ l of cell suspension was placed on the haemocytometer and the number of both stained and unstained cells was assessed.

2.4 ASSESSMENT OF M ϕ PHAGOCYTOSIS

In this thesis phagocytosis has been assessed for human MDM ϕ (see section 2.4.1) and mouse BMDM ϕ (see section 2.4.2). Information presented in this chapter characterise phagocytic targets and methods used for assessment of phagocytosis for these two types of cells.

2.4.1 HUMAN MONOCYTE-DERIVED M ϕ (MDM ϕ)

MDM ϕ were cultured for the duration of 6 days in IMDM supplemented with penicillin/streptomycin and 10 % autologous serum, following which the appropriate treatment was applied. For ELISA or CBA assays, culture supernatants were removed and stored at -80°C. For the phagocytosis assay, adherent MDM ϕ were washed with HBSS with Ca²⁺ and Mg²⁺ and incubated with apoptotic neutrophils (re-suspended in serum-free IMDM supplemented with penicillin/streptomycin at 5x10⁶/ml) for 30-60 minutes at 37°C, providing a phagocyte-to-target ratio of approximately 1:10 (0.5 ml of neutrophil suspension was added to each well).

2.4.1.1 PHAGOCYTOTIC TARGETS

In the majority of experiments, apoptotic human neutrophils were used as phagocytic targets. These cells were isolated from human blood cells (see section 2.2.1.1 for isolation protocol) and cultured for 20 hours to obtain cells undergoing constitutive apoptosis (see section 2.2.1.3 for culture conditions). Apoptosis was also induced by R-roscovitine (see section 2.2.1.3 for culture conditions). Human erythrocytes opsonised with anti-human erythrocyte Ab were used as another phagocytic target (see section 2.2.1.1 for isolation protocol and 2.4.1.3 for labelling and opsonisation protocols).

2.4.1.2 PHAGOCYTOTIS ASSAY BY FLOW CYTOMETRY

For the flow cytometric phagocytosis assay (Jersmann *et al.*, 2003), neutrophils were labelled with Cell Tracker™ Green CMFDA (see section 2.2.1.3 for

labelling protocol). After incubation of M ϕ with apoptotic neutrophils (30-60 minutes), neutrophils and medium were removed from wells. Remaining cells were incubated with trypsin/EDTA for 15 minutes at 37°C followed by 15 minutes on ice and transferred to FACS tubes. In order to ensure that all the M ϕ were removed from wells, plates were examined by light microscopy. Harvested cells were analysed by flow cytometry in a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, UK). Neutrophil and M ϕ populations were separated by forward and side scatter profiles and a minimum of 6000 events within the M ϕ gate were acquired (see Figure 2.6). The number of FL1⁺ events in the M ϕ gate was divided by the total number of M ϕ to obtain the % of M ϕ that had internalised neutrophils.

2.4.1.3 PHAGOCYTOSIS OF ERYTHROCYTES OPSONISED WITH HUMAN IgG ANTIBODIES

Following discontinuous Percoll™ density gradient centrifugation, erythrocytes were harvested, washed and resuspended at 10⁷/ml in Iscove's DMEM, stained with Cell Tracker™ Green CMFDA for 3 hours and then incubated at 4°C with rabbit polyclonal anti-human erythrocyte Ab (1:1000) for 30 minutes. Opsonized erythrocytes were then washed and resuspended at 4x10⁶/ml and 2x10⁶ erythrocytes were added to each well with adherent M ϕ and incubated at 37°C for 30 minutes (Hart *et al.*, 1997). The % phagocytosis was assessed by flow cytometry following the same protocol as for phagocytosis of apoptotic neutrophils.

2.4.1.4 PHAGOCYTOSIS ASSAY BY MICROSCOPY

In order to assess phagocytosis by microscopy (Rossi *et al.*, 1998b), M ϕ were incubated with unlabelled apoptotic neutrophils as described above (see section 2.4.1.2). Following the phagocytosis assay, medium was removed from each well and wells were washed approximately 4 times with ice-cold PBS without Ca²⁺ and Mg²⁺ to remove all non-engulfed neutrophils. Next, cells were fixed with 2.5 % glutaraldehyde in PBS without Ca²⁺ and Mg²⁺ for 10 minutes and stained for myeloperoxidase using 0.1 mg/ml dimethoxybenzidine and 0.03 % (v/v) hydrogen

peroxide in PBS without Ca^{2+} and Mg^{2+} (solution was prepared just prior to staining). To achieve appropriate staining cells were incubated at 37°C , in 5 % CO_2 for approximately 10-20 minutes. Finally, uptake of apoptotic neutrophils by $\text{M}\phi$ was assessed by inverted light microscopy. Typical photomicrograph representing $\text{MDM}\phi$ uptake of apoptotic neutrophils are shown in Figure 2.7.

FIGURE 2.6. Representative flow cytometric dot-plots demonstrating two $\text{MDM}\phi$ subpopulations: ingesting and non-ingesting apoptotic neutrophils. Some treatments may influence levels of phagocytosis and consequently change the size of $\text{MDM}\phi$ subpopulation that performs efferocytosis, e.g., treatment with dexamethasone augments efferocytosis but treatment with LPS reduces this process. **DX** – dexamethasone.

FIGURE 2.7. Typical photomicrograph representing MDM ϕ uptake of apoptotic neutrophils. Unlabelled apoptotic neutrophils (cultured for 20 hours) were incubated with mature MDM ϕ (cultured for 6 days in culture medium) for 30-60 minutes and then cells were fixed and stained as described in section 2.4.1.4. Images were obtained using an Axiovert S100 inverted microscope (Carl Zeiss), RS Photometrix camera and analysed with Open lab software, 3.1.5 version. Magnification 320x. **aN ϕ** – apoptotic (20 hours culture) neutrophils.

2.4.2 MURINE BONE MARROW-DERIVED MΦ (BMDMφ)

On the day of the phagocytosis assay BMDMφ culture supernatants were removed and Mφ were washed with HBSS with Ca²⁺ and Mg²⁺ and then labelled with Cell Tracker™ Far Red DDAO-SE at the final concentration of 1 µg/ml in HBSS with Ca²⁺ and Mg²⁺ (150 µl added to each well in 48-well plate) for 30 minutes at 37°C, 5 % CO₂. Subsequently 100 µl of FBS was added to each well to bind excess of Cell Tracker™ Far Red DDAO-SE and incubated for 15 minutes at 37°C, 5 % CO₂. Finally, cells were washed twice with HBSS with Ca²⁺ and Mg²⁺ and apoptotic neutrophils, labelled with Cell Tracker™ Green CMFDA, added, following the same protocol as for phagocytosis by human Mφ.

Since populations of murine BMDMφ and human neutrophils overlap with human neutrophils when plotted SS against FS two fluorescent labels were used: Cell Tracker™ Far Red DDAO-SE (for labelling of BMDMφ, FL-4⁺) and Cell Tracker™ Green CMFDA (for labelling of human neutrophils, FL-1⁺) allowing gating BMDMφ as a FL-4⁺ positive cells and then assessing percentage of FL-1⁺ subpopulation within it. All the BMDMφ cells were FL-4⁺ but only those that had phagocytosed apoptotic neutrophils were both FL-4⁺ and FL-1⁺.

2.5 MEASUREMENT OF CYTOKINE CONCENTRATIONS

2.5.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

2.5.1.1 PRINCIPLES OF ELISA

ELISA is a technique that belongs to the family of enzyme-linked immunoassays, developed to measure microamounts of substances in test samples (Lequin, 2005). All these techniques are based on interaction between an antigen (usually a substance of interest) and a specific antibody and consist of several common steps (van Weemen & Schuurs, 1974; Engvall & Perlmann, 1971), namely:

- 1) immobilization of antigen on the surface (often a U-bottomed 96 well),
- 2) blocking of the surface with non-interacting protein i.e., bovine serum albumin (BSA) to avoid non-specific binding of detection antibody to the surface,
- 3) interaction between antigen and specific detection antibody which is linked to an enzyme,
- 4) addition of a substance that is converted by an enzyme to a detectable signal.

Two antibodies may be used for detection of an antigen; a primary antibody specific for antigen and a secondary antibody that is linked to the enzyme that enables detection. Secondary antibody is specific for an Fc region of a primary antibody and therefore its use is not limited to one particular primary antibody.

Performance of ELISA may vary and currently the most important methods are (van Weemen, 2005):

- 1) 'indirect' ELISA – where antigen is immobilised on the surface just by non-specific adsorption and this is a main limitation of this method.
- 2) sandwich ELISA – where surface is covered with a specific, capture antibody which in turn binds a specific antigen. The main advantage of this method is selective binding of antigen of interest to the surface what allows detection even in crude or

impure samples. The main principles of sandwich ELISA are demonstrated in Figure 2.8.

3) competitive ELISA – where an antibody is added to a sample where it binds an antigen present in the sample. Samples are then washed over the surface, previously coated with an antigen which recognises an unbound antibody from the sample. Hence the antigen from the sample and from the surface competes for the antibody. The antibody bound to the surface is next detected by a secondary antibody linked to the enzyme. In competitive ELISA the higher the original concentration of an antigen in the sample the weaker the final signal.

2.5.1.2 EXPERIMENTAL PROTOCOLS

In this PhD thesis the sandwich ELISA has been used to assess concentrations of TNF- α and IL-10 in MDM ϕ culture supernatants. DuoSet ELISA Development kit has been used according to the manufacturer's instruction. Culture supernatants were collected on day 6 of the MDM ϕ culture, centrifuged (600g; 6 minutes) and stored in aliquots at -80°C until subjected to ELISA analysis.

FIGURE 2.8. Schematic diagram demonstrating the principles of ‘sandwich’ ELISA. The entire procedure is described in more detail in section 2.5.1 and important steps indicated in the above figure.

2.5.2 CYTOMETRIC BEAD ARRAY (CBA)

2.5.2.1 PRINCIPLES OF CBA

Similar to the ‘sandwich’ ELISA, the CBA is based on antigen–antibody interactions, where capture antibody is used to immobilise antigen and detection antibody is linked to an enzyme that produces detectable signal. The main difference to an ELISA technique is the surface that carries captures antibodies. In ELISA, as described in section 2.5.1.1, capture antibodies coat a well of typically 96-well plate. In contrast, in CBA, capture antibodies coat a surface of a bead. Interaction between an antigen and antibodies and formation of a ‘sandwich’ in CBE approach is schematically demonstrated in Figure 2.9. Beads may differ in size, colour or intensity of the colour and thus can be analysed separately by different channels of the flow cytometer. Consequently, CBA has a number of advantages over ELISA, namely:

- multiple substances are analysed simultaneously
- volume of a sample is reduced
- wide dynamic range of fluorescence is used
- time of an assay is reduced (approximate 4 hours).

2.5.2.2 EXPERIMENTAL PROCEDURES

In this PhD thesis, CBA (Human Inflammation Kit) has been used that measures concentrations of IL-8, IL-1 β , IL-6, IL-10, TNF- α and IL-12p70. Cytokine concentrations in the MDM ϕ culture supernatants were determined according to the manufacturer’s instructions. Culture supernatants were collected on day 6 of the MDM ϕ culture, centrifuged (600g; 6 minutes) and stored in aliquots at -80°C until subjected to CBA analysis.

FIGURE 2.9 Formation of a ‘sandwich’ in CBA assay. The above diagram demonstrates complex interaction between an antigen and antibodies in CBA approach to measure the antigen levels. The principles of this technique are described in more detail in section 2.5.2.

2.6 sTNF-R1/Fc BINDING ASSAY

Neutrophils, isolated from human blood (as described in section 2.2.1), were cultured at 37°C and 5 % CO₂ atmosphere at a concentration of 4x10⁶/ml in IMDM supplemented with penicillin/streptomycin and 10% FBS (human IgG free) for 20 hours, then washed twice (300g, 5 minutes, 4°C) in PBS without Mg²⁺/Ca²⁺, re-suspended at the concentration of 0.8x10⁶/ml in flow buffer (PBS without Mg²⁺/Ca²⁺ with 2% FBS) and plated at 100 µl/well in 96-well FlexiPlate. Cells were incubated on ice for 20 minutes either in flow buffer alone or in the presence of 500 ng/ml sTNF-R1/Fc or 100 µg/ml heat aggregated human recombinant IgG. Next, cells were washed twice (300g, 5 minutes, 4°C) in PBS without Mg²⁺/Ca²⁺, incubated for 20 minutes on ice with goat F(ab')₂ anti-human IgG, FITC conjugated antibody (1:50, detected by FL-1 channel on flow cytometer) washed twice (300g, 5 minutes, 4°C) in PBS without Mg²⁺/Ca²⁺, and re-suspended in Annexin-V (1:40, APC conjugate, detected as FL-3 on flow cytometer) binding buffer. Assessment of binding was performed by flow cytometry analysis (Hart *et al.*, 2004a). The % necrotic cells was assessed flow cytometrically by PI uptake (FL-2 channel on flow cytometer).

2.7 WESTERN BLOTTING

2.7.1 PRINCIPLES OF WESTERN BLOTTING

Western blotting is an analytical technique, related to CBA and ELISA which are described in section 2.5. It is used for detection of a specific protein in a given sample i.e., cell extract. W. Neal Burnette named this method by analogy to Southern blotting, which is a DNA detection method, developed earlier by Edwin Southern (Burnette, 1981). Detection of RNA is termed northern blotting. Western blotting combines several steps which are indicated in Figure 2.10 and reviewed in detail by Towbin *et al.* (Towbin *et al.*, 1979). These steps include:

- 1) tissue homogenation and cell lysis,
- 2) gel electrophoresis to separate native or denatured proteins by the length of the polypeptides (denaturing conditions) or by their 3-D structures (native / non-denaturing conditions),
- 3) transfer of proteins to the membrane (typically nitrocellulose or PVDF),
- 4) blocking of the membrane in a dilute solution of protein, typically BSA, to prevent interaction between membrane and antibodies used for detection of a target protein,
- 5) probing with primary antibody specific for protein of interest,
- 6) probing with secondary antibody that allows easy detection,
- 7) appropriate detection method

FIGURE 2.10 Schematic diagram demonstrating the main steps of protein detection by western blotting. The entire procedure is described in more details in section 2.7 and important steps are indicated in the above figure.

2.7.2 EXPERIMENTAL PROTOCOLS

2.7.2.1 PREPARATION OF MDM ϕ LYSATES

Mononuclear cells isolated by Percoll™ gradient centrifugation (see section 2.2) were re-suspended in IMDM at the concentration 4×10^6 cells/ml, plated at 3 ml/well in 6-well plates (1 hour, 37°C, 5 % CO₂). Then non-adherent cells were removed by washing twice with HBSS with Ca²⁺ and Mg²⁺ and monocytes were cultured in IMDM supplemented with penicillin/streptomycin and 10 % (v/v) autologous serum +/- treatment (6 days, 37°C, 5 % CO₂) to allow their maturation into MDM ϕ . On day 6 MDM ϕ were washed twice in ice-cold PBS without Ca²⁺ and Mg²⁺ supplemented with 100 μ M Na₃VO₄ and lysed with 150 μ l RIPA lysis buffer (30 minutes, 4°C). The composition of RIPA lysis buffer is presented in Table 2.1. In order to minimise protein degradation, proteinase inhibitor cocktail has been added to RIPA lysis buffer just before the lysis (10 μ l for every 1 ml of the buffer). Following lysis, cells were scraped, lysates were harvested into Eppendorf™ tubes and cytocentrifuged (15 minutes, 13000 rpm, 4 °C). Supernatants were aliquoted and stored at either -30 or -80°C depending on when intended to use.

TABLE 2.1 Composition of RIPA lysis buffer

<i>RIPA lysis buffer</i>	<i>Stock solutions</i>	<i>Volume / 50 ml</i>
<i>100 μM Na₃VO₄</i>	<i>1 mM Na₃VO₄</i>	<i>5 ml</i>
<i>50 mM NaCl</i>	<i>1 M NaCl</i>	<i>2.5 ml</i>
<i>50 mM TRIS pH 7.4</i>	<i>1 M TRIS pH 7.4</i>	<i>2.5 ml</i>
<i>0.5 % deoxycholate</i>	<i>10 % deoxycholate</i>	<i>2.5 ml</i>
<i>0.1 % SDS</i>	<i>10 % SDS</i>	<i>0.5 ml</i>
<i>1% NP40</i>	<i>100 % NP40</i>	<i>0.5 ml</i>
<i>1 mM EGTA</i>	<i>0.5 M EGTA</i>	<i>100 μl</i>
<i>1 mM NaF</i>	<i>0.5 M NaF</i>	<i>100 μl</i>
<i>1 mM PMSF</i>	<i>1 M PMSF</i>	<i>50 μl</i>

RIPA lysis buffer was made out of stock solutions on the day of lysis and kept on ice. Just prior to lysis proteinase inhibitors were added. Stock solutions may be prepared in advance and stored in the laboratory.

2.7.2.2 MEASUREMENT OF PROTEIN CONCENTRATION

The concentration of proteins in obtained lysates was measured by Bio-Rad Dc Protein Assay following the manufacturer's instruction. This assay is based on the colorimetric method of Lowry in which copper (II) ion in alkaline solution reacts with amino bonds of the protein and is reduced to copper (I) (Lowry *et al.*, 1951). Next copper (I) reduces Folin reagent and it turns blue. Intensity of the colour (proportional to protein concentration) is measured as a maximum absorbance (A_{\max}) at 750 nm with a spectrophotometer or microplate reader. Finally, by a comparison to a standard curve, relative concentration of the protein in samples of interest can be measured. The standard curve was created by preparing series of dilutions of 1.35 mg/ml BSA solution.

2.7.2.3 SOLUTIONS AND REAGENTS

- **Running Buffer** - NuPAGE[®] 20X MES SDS Running Buffer (Invitrogen[™], Carlsbad, CA), reconstituted in purified water.
- **Sample Buffer** – NuPAGE[®] 4X LDL Sample Buffer (Invitrogen[™], Carlsbad, CA).
- **Sample Reducing Agent** - NuPAGE[®] 10X Sample Reducing Agent (Invitrogen[™], Carlsbad, CA).
- **Molecular weight size marker** – biotinylated protein ladder (Cell Signalling, Herts, UK)
- **Gels for electrophoresis** - NuPAGE[®] Novex 4-12 % Bis-Tris precast gels (Invitrogen[™], Carlsbad, CA).
- **Transfer buffer** - NuPAGE[®] 20X Transfer Buffer (Invitrogen[™], Carlsbad, CA), in 10 % solution of methanol in purified water (i.e., 50 ml of NuPAGE[®] 20X Transfer Buffer, 100 ml of methanol and 850 ml of purified water have been used for total volume of 1000 ml).
- **Blocking Buffer** – 1X TBS, 0.1 % Tween-20, 5 % w/v nonfat dry milk in purified water.
- **10 X TBS (Tris-buffered saline)** – for total volume of 1000 ml: 24.2 g Tris base, 80 g NaCl in purified water, pH adjusted to 7.6 with HCl.
- **Wash Buffer TBS/T** – 1X TBS, 0.1 % Tween-20 in purified water.
- **Primary Antibody Dilution Buffer** – 1X TBS, 0.1 % Tween-20 with 5 % BSA in purified water.
- **Detection Reagents** - 20X LumiGLO[®] chemiluminescent reagent and 20X peroxide used along with horseradish peroxidase (HRP) tagged detection antibodies (Cell Signalling, Herts, UK). In the presence of hydrogen peroxide, HRP converts luminol to an excited intermediate dianion. This dianion emits light on return to its ground state which is captured by x-ray film.

2.7.2.4 PROTEIN ELECTROPHORESIS

In general, gel electrophoresis is a method of separation of charged molecules, i.e., DNA or proteins, based on their ability to migrate in a gel toward opposite charge. In this PhD thesis polyacrylamide (PAGE) gel electrophoresis has been used to separate proteins in analysed samples. PAGE gels may be composed of various concentrations of acrylamide and cross-linker producing gels that differ in the size of pores. The choice of suitable PAGE gel depends on the size of analysed proteins. The higher the molecular mass of proteins the lower percentage of PAGE gel is usually used. Proteins are separated due to their differences in mass – low molecular mass proteins migrate slower than those of higher molecular mass. In this project, the gradient 4-12 % PAGE gels have been used. Prior to electrophoresis, proteins were resuspended in a sample buffer and treated with sample reducing agent containing sodium dodecyl sulphate (SDS), which denatures secondary and non-disulfide-linked tertiary structures. In addition, incubation with SDS results in each protein having a negative charge proportional to its mass and independent of its primary structure. Consequently, migration of proteins in the gel towards the positive pole depends mainly on its mass (charge). Finally, the mass of analysed proteins is established by a comparison to specific molecular weight size markers. Gels and all the necessary reagents and equipment to run electrophoresis were purchased from Invitrogen™ (Carlsbad, CA) and are described in section 2.7.2.3. The manufacturer's instructions have been followed.

2.7.2.5 MEMBRANE TRANSFER OF PROTEINS

Following gel electrophoresis, proteins have been transferred from PAGE gel on nitrocellulose blotting membrane (Gottingen, Germany). This step allows further detection of proteins with the use of specific antibodies. In the presented study, proteins were transferred to the membrane in transfer buffer (described in section 2.7.2.3) by application of electric current. For this purpose, a sheet of nitrocellulose membrane has been placed on the top of PAGE gel, covered with a stack of filter papers (Whatmann 3mm) and sponges, all soaked in the transfer buffer, and placed into transfer apparatus, where proteins move in the direction of anode. The binding of proteins to the surface of the membrane is based upon hydrophobic and charged

interactions between the membrane and a protein. The manufacturer's instructions have been followed throughout the entire procedure.

2.7.2.6 ANTIBODIES, INCUBATION CONDITIONS AND DETECTION

After proteins had been transferred to the membrane, the following steps were undertaken in order to detect proteins of interest:

- 1) Wash with 25 ml 1 X TBS (5 minutes, room temperature (RT), gentle agitation).
- 2) Incubation in 25 ml of Blocking Buffer (1 hour, RT, gentle agitation).
- 3) Wash 3 times with 15 ml of Wash Buffer (5 minutes each wash, RT, gentle agitation).
- 4) Incubation in 10 ml of Primary Antibody Dilution Buffer. Primary antibodies used in this study are listed in section 2.1.8 and 1:1000 dilution was used (overnight, 4°C, gentle agitation).
- 5) Wash 3 times with 15 ml of Wash Buffer (5 minutes each wash, RT, gentle agitation).
- 6) Incubation in 10 ml of Blocking Buffer containing HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers (1 hour, RT, gentle agitation). These antibodies are listed in section 2.1.8.
- 7) Wash 3 times with 15 ml of Wash Buffer (5 minutes each wash, RT, gentle agitation).
- 8) Incubation with 10 ml LumiGLO[®] (0.5 ml 20 X LumiGLO[®], 0.5 ml 20 X Peroxide and 9.0 ml Milli – Q water) (1 minute, RT, gentle agitation).
- 9) The membrane was drained of excess of developing solution, wrapped in plastic wrap and exposed to x-ray film (Kodak BioMax light film) to read results.
- 10) X-ray films have been developed in Medical Film Processor (Konica SRX-101A).

2.8 STATISTICAL ANALYSIS

Results are expressed as the mean \pm SEM of n=number of independent experiments using M ϕ from different donors. Statistical significance (defined as $p < 0.05$) was evaluated by ANOVA (InStat3) with comparisons between groups using the Student-Newman-Keuls procedure when $n \geq 5$ or the Dunn's test when $n = 4$.

CHAPTER THREE

THE EFFECTS OF

PRO-INFLAMMATORY STIMULI

ON MΦ EFFEROCYTOSIS

3 THE EFFECTS OF PRO-INFLAMMATORY STIMULI ON M ϕ EFFEROCYTOSIS

3.1 INTRODUCTION

The inflammatory response is a physiological way of defending a host organism, subjected to tissue injury, infection or other forms of trauma. However, inflammation must be appropriately controlled and resolved in order to prevent its harmful consequences including progressive tissue damage, which is associated with pathological conditions (e.g., RA, Crohn's disease, asthma etc.) (Savill *et al.*, 1989). Sites of chronic inflammation are characterised by overexpression of numerous pro-inflammatory mediators including TNF- α (Feldmann *et al.*, 1994). Yet, few studies describe the effects of pro-inflammatory cytokines on two important mechanisms underlying resolution of inflammation: apoptosis of inflammatory cells and their subsequent clearance by M ϕ . For instance, it has been previously shown that TNF- α , depending on duration of treatment, may exert variable effects on apoptosis of inflammatory cells (Ward *et al.*, 1999). Acute treatment with TNF- α induces neutrophil apoptosis while prolonged exposure results in cell survival. Furthermore, the findings regarding the effects of TNF- α on M ϕ clearance of apoptotic cells are contradictory. In the study published by Savill and colleagues, TNF- α augmented clearance of apoptotic cells by immature M ϕ (Ren & Savill, 1995). In contrast, some studies have recently demonstrated that TNF- α inhibits clearance of apoptotic cells by mature M ϕ (McPhillips *et al.*, 2007; Borges *et al.*, 2009).

One of the objectives of this PhD thesis was a thorough evaluation of the effects of TNF- α on MDM ϕ clearance of apoptotic neutrophils. The production of TNF- α in MDM ϕ has been induced by several agents, i.e., LPS, LTA and PGN. These are constituents of bacterial cell walls that are recognised by the TLR family of receptors (Draing *et al.*, 2008). LPS is a component of Gram-negative bacterial cell walls, which is recognised by TLR4, located on the surface of host cells. Due to its powerful pro-inflammatory properties, LPS is commonly used by scientists to induce

inflammation in various model systems (Marshall, 2005). LPS, its recognition by host cells and pro-inflammatory signalling pathways are described in more detail in section 1.2.2.1.

LTA and PGN are also components of Gram-positive bacterial cell walls and are recognised by the cell surface receptor TLR2. Even though administration of LTA *in vivo* exerts similar effect to LPS (systemic shock), it has been reported that signalling pathways triggered by these agents are not identical (Draing *et al.*, 2008). For instance, no final consensus has been reached regarding the requirement of CD14 and LBP in LTA-induced cellular responses (see chapter 1.2.2.1 for more information on CD14 and LBP). Furthermore, TLR4 recruits two types of adaptor proteins TRIF/TRAM and MyD88/Mal while TLR2 engages only the latter. TLR2 has been also demonstrated to act in a complex with other receptors such as TLR1, TLR6 or CD36 (Deininger *et al.*, 2008). Activation of either TLR4 or TLR2 leads to subsequent activation of pro-inflammatory transcription factors (e.g., NF- κ B or AP1) and production of certain pro-inflammatory mediators such as TNF- α , as described in section 1.2.2.1.

In this PhD thesis, in order to establish the effects of TNF- α on phagocytosis, monocytes isolated from human blood were differentiated into MDM ϕ during 6 days culture. MDM ϕ production of TNF- α was induced with LPS, LTA and PGN (increasing concentrations and durations of treatment were used). Alternatively, MDM ϕ were cultured for various durations of time with increasing concentrations of exogenous human recombinant TNF- α . On day 6, M ϕ ability to phagocytose apoptotic neutrophils (constitutive or R-roscovitine-induced apoptosis) has been assessed by either flow cytometry (majority of experiments) or microscopy, as described in section 2.4.1. Furthermore, the effects of TNF- α on various phagocytic targets, i.e., apoptotic neutrophils and IgG-opsonised human erythrocytes, have been studied. It is recognised that mechanisms involved in the uptake of apoptotic cells are different from that involved in engulfment of opsonised particles (Rossi *et al.*, 1998b). During apoptosis, the cell membrane undergoes thorough remodelling and some of these changes may provide binding sites for opsonins such as antibodies and complement proteins. Opsonins, in turn, may mediate M ϕ phagocytosis by classical phagocytic receptors e.g., Fc receptors (Hart *et al.*, 2004b). The latter process is very

efficient and may provide an additional mechanism preventing secondary necrosis and consequential tissue damage in tissues overload with inflammatory cells during inflammation. Thus, in order to investigate the TNF- α regulation of M ϕ phagocytosis further, human erythrocytes were opsonised with human IgG antibodies and their uptake by MDM ϕ was assessed by flow cytometry (protocol described in section 2.4.1.3).

3.2 METHODS

3.2.1 ISOLATION AND CULTURE OF HUMAN BLOOD CELLS

Human blood cells were isolated by Percoll™ gradient centrifugation, following a protocol described in section 2.2.1.1. Subsequently, human monocytes were cultured for 6 days in culture medium (see section 2.2.1.3 for culture conditions) undergoing differentiation into mature human MDM ϕ . During this time MDM ϕ were treated with a number of pro- and anti-inflammatory agents. Typical treatments were as follows: 1 μ M dexamethasone (96-144 hours), 0.1–100 ng/ml TNF- α or LPS (6, 24, 48, 96 or 144 hours), 0.01–10 ng/ml IL-1 β (120 hours), 0.1 – 100 μ g/ml LTA or PGN (6, 16, 32, 48, 72, 96 hours).

On day 6, MDM ϕ were assessed for their ability to phagocytose different targets. In the majority of experiments these targets were human neutrophils that had undergone constitutive apoptosis for 20 hours of culture (see section 2.2.1.3 for culture conditions). Apoptosis of human neutrophils was also induced by 20 μ M R-roscovitine over 8 hours of culture (see chapter 2.2.1.3 for culture conditions). Other phagocytic targets used in this study were opsonised human erythrocytes (see section 2.2.1.1 for isolation protocol and 2.4.1.3 for labelling and opsonisation protocols).

3.2.2 ASSESSMENT OF APOPTOSIS AND NECROSIS

Prior to performing the phagocytosis assay, the percentage of apoptotic cells within neutrophil populations was determined by morphology or alternatively by Annexin-V binding assessed by flow cytometry (see sections 2.3.1 and 2.3.2 for experimental protocol). The percentage of necrotic cells was determined by PI staining assessed by flow cytometry. Only neutrophils with less than 5 % of necrosis were used in the phagocytosis assay.

3.2.3 ASSESSMENT OF PHAGOCYTOSIS BY FLOW CYTOMETRY

In the majority of experiments described in this chapter efferocytosis was assessed by flow cytometric analysis that employs labelling of phagocytic targets with FL1⁺ Cell Tracker™ Green (CMFDA) prior to the phagocytosis assay. Following the phagocytosis assay, the % of FL1⁺ MDM ϕ (MDM ϕ that had engulfed phagocytic targets) within the entire MDM ϕ population was assessed by flow cytometry as described in section 2.4.1.1.

3.2.4 ASSESSMENT OF PHAGOCYTOSIS BY MICROSCOPY

To obtain photomicrographs of MDM ϕ that have been cultured in different conditions and have or have not engulfed apoptotic neutrophils, unlabelled apoptotic neutrophils were incubated with mature MDM ϕ and then cells were fixed, stained and observed under light microscope. Photomicrographs were taken as described in section 2.4.1.2.

3.3 RESULTS

3.3.1 LPS INHIBITS MDM ϕ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER

Human blood monocytes were differentiated *in vitro* for 6 days following a well-established protocol for generating M ϕ (Rossi *et al.*, 1998b). In order to examine effects of LPS on MDM ϕ efferocytosis of apoptotic cells, monocytes were treated with LPS (10 ng/ml) for the duration of 96–144 hours and then co-cultured with apoptotic neutrophils to determine efferocytosis. The % of MDM ϕ ingesting apoptotic neutrophils was assessed by flow cytometric analysis. Efferocytosis was decreased by LPS treatment from control levels of 39.4 ± 3.4 % to 20.5 ± 3.0 % ($p < 0.001$; $n = 19$; Figure 3.1A). MDM ϕ cultured in the presence of dexamethasone (1 μ M, 96–144 hours), previously shown to augment efferocytosis of apoptotic neutrophils, were characterised by increased uptake of apoptotic neutrophils up to 64.6 ± 3.4 % ($p < 0.001$; $n = 19$; Figure 3.1A). Representative flow cytometric dot-plots demonstrating differences in MDM ϕ uptake of apoptotic neutrophils upon treatments with dexamethasone or LPS are presented in Figure 3.1B. Their confirmation by microscopy is depicted in Figure 3.6.

FIGURE 3.1 Effects of dexamethasone and LPS on MDM ϕ efferocytosis of neutrophils. **A.** Human MDM ϕ cultured in medium (control) for 6 days and treated on average for 96-144 hours with either 1 μ M dexamethasone or 10 ng/ml LPS were assessed for their ability to phagocytose apoptotic neutrophils. The % efferocytosis assessed on day 6 by flow cytometry is expressed as mean \pm SEM for n=19 experiments. Significant difference for comparison to medium control is represented by ***p<0.001. **B.** Representative flow cytometric dot-plot profiles demonstrating changes in efferocytosis for treatments with either 1 μ M dexamethasone or 10 ng/ml LPS for 120 hours in comparison to medium control. **aN ϕ** –apoptotic (20 hours culture) neutrophils, **DX** – dexamethasone.

Having found that LPS treatment inhibits efferocytosis, I next sought to investigate if this effect was concentration- and time-dependent. For this purpose human blood monocytes were cultured for 6 days and treated with increasing concentrations of LPS for 120 hours or with 10 ng/ml LPS for 6, 48, 96 and 144 hours. Efferocytosis was assessed on day 6 by flow cytometry. As demonstrated in Figure 3.2A, the LPS inhibitory effect was dependent on its concentration. The first significant decrease from 42.9 ± 6.2 % (control levels) to 29.3 ± 3.6 % was observed upon treatment with 0.1 ng/ml LPS ($p < 0.05$; $n = 4$) and this effect further increased in parallel with increase in LPS concentration. Efferocytosis levels were 17.7 ± 4.6 % for treatment with 1 ng/ml LPS ($p < 0.01$, $n = 4$), 14.0 ± 4.3 % for treatment with 10 ng/ml LPS ($p < 0.001$; $n = 4$) and 13.9 ± 3.9 % for treatment with 100 ng/ml LPS ($p < 0.001$; $n = 4$). The approximate IC_{50} value for LPS was 0.6 ng/ml. The inhibitory effect of LPS was also dependent upon length of treatment. Interestingly, LPS required at least 96 hours to induce significant reduction in efferocytosis levels from 38.7 ± 4.8 % (control) to 21.8 ± 3.3 % ($p < 0.05$; $n = 4$). Inhibition was even greater after 144 hours of LPS treatment- 8.4 ± 2.3 % ($p < 0.001$; $n = 4$).

FIGURE 3.2 Concentration- and time-dependent effects of LPS on MDM ϕ efferocytosis of neutrophils. Human MDM ϕ were cultured with either increasing concentrations of LPS for 120 hours (**A**) or with 10 ng/ml LPS for increasing durations of time (**B**) and then assessed for their ability to ingest apoptotic neutrophils. The % efferocytosis assessed on day 6 by flow cytometry is expressed as mean \pm SEM of n=4 separate experiments. Significant differences for comparisons to medium control are represented by *p<0.05, **p<0.01 and ***p<0.001.

3.3.2 LTA INHIBITS MDM ϕ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER

Having found that the TLR-4 agonist, LPS, reduced MDM ϕ efferocytosis I next sought to investigate whether the TLR-2 agonist, lipoteichoic acid (LTA), would exert a similar effect. For this purpose, human blood monocytes were differentiated *in vitro* for 6 days and treated with increasing concentrations of LTA for various durations of time. Efferocytosis was measured on day 6 by flow cytometry. As shown in Figure 3.3A, after 32 hours of treatment, LTA, at a concentration of 100 $\mu\text{g/ml}$, significantly reduced efferocytosis to $21.3 \pm 2.6 \%$ in comparison to control levels $34.3 \pm 3.6 \%$ ($p < 0.05$; $n = 5$). Next, I examined the time-dependence of the LTA effect. Human blood monocytes were differentiated for 6 days into MDM ϕ and treated for increasing durations of time: 6, 16, 32, 48, 72 and 96 hours with 100 $\mu\text{g/ml}$ LTA. On day 6, MDM ϕ phagocytosis was assessed by flow cytometry. Interestingly, I was able to detect significant inhibition at early time points. At the 16 hour time point, efferocytosis was reduced from $34.9 \pm 4.5 \%$ (control) to $24.3 \pm 3.9 \%$ ($p < 0.05$; $n = 4$). At 32 hours after treatment the LTA inhibitory effect was even greater ($18.5 \pm 3.2 \%$, $p < 0.01$, $n = 4$ in comparison to control) and was sustained till the 96 hour time point (Figure 3.3B).

FIGURE 3.3 Concentration- and time-dependent effect of LTA on MDM ϕ efferocytosis of neutrophils. Human MDM ϕ were cultured for 6 days and treated as indicated with either increasing concentrations of LTA for 32 hours (**A**) or with 100 μ g/ml LTA for increasing durations of time (**B**). On day 6, their ability to ingest apoptotic neutrophils was assessed by flow cytometry. Data are expressed as mean \pm SEM of n=5 (**A**) or n=4 (**B**) separate experiments. Significant differences for comparisons to medium control are represented by *p<0.05, **p<0.01*** and p<0.001.

3.3.3 PGN INHIBITS MDM ϕ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER

Since I demonstrated that LTA can block MDM ϕ uptake of apoptotic neutrophils, I then decided to use another TLR-2 agonist, PGN, to examine its effect upon efferocytosis. For this purpose human blood monocytes were differentiated *in vitro* for 6 days and cultured for 16 hours with increasing concentrations (0.1, 1, 10, 100 $\mu\text{g/ml}$) of PGN. Efferocytosis was measured on day 6 by flow cytometry. As shown in Figure 3.4A PGN at concentrations 10 and 100 $\mu\text{g/ml}$ significantly reduced efferocytosis from control levels $29.0 \pm 5.8\%$ to $14.6 \pm 2.5\%$ ($p < 0.05$, $n = 4$) and $7.0 \pm 0.9\%$ ($p < 0.01$, $n = 4$) respectively. Next, I examined the time-dependence of the PGN effect. Human blood monocytes were differentiated for 6 days into MDM ϕ and treated for increasing durations of time: 6, 16, 32, 48, 72 and 96 hours with 10 $\mu\text{g/ml}$ PGN. Phagocytosis was measured on day 6 of the culture by flow cytometry. The first significant inhibition of efferocytosis was observed at 16 hours; $18.7 \pm 1.9\%$ in comparison to control $35.3 \pm 4.5\%$ ($p < 0.01$, $n = 4$) but this effect disappeared at 32 and 48 hours after treatment. Intriguingly, at later time points: 72 and 96 hours, MDM ϕ again showed reduced ability for phagocytosis; $21.2 \pm 8.3\%$ ($p < 0.05$, $n = 4$) and $18.4 \pm 6.7\%$ ($p < 0.01$, $n = 4$) respectively (Figure 3.4B).

FIGURE 3.4 Concentration- and time-dependent effect of PGN on MDM ϕ efferocytosis of neutrophils. Human MDM ϕ were cultured for 6 days and treated as indicated with either increasing concentrations of PGN for 16 hours (**A**) or with 10 μ g/ml PGN for increasing durations of time (**B**). On day 6 their ability to ingest apoptotic neutrophils was assessed by flow cytometry. Data are expressed as mean \pm SEM of n=4 separate experiments. Significant differences for comparisons to medium control are represented by *p<0.05 and **p<0.01.

3.3.4 TNF- α INHIBITS MDM ϕ EFFEROCYTOSIS IN A CONCENTRATION- AND TIME-DEPENDENT MANNER

Since LPS, LTA and PGN have been shown to induce M ϕ production of TNF- α (Fan *et al.*, 2003; Su *et al.*, 2006), I investigated whether M ϕ treated with TNF- α would have decreased ability to phagocytose apoptotic neutrophils. Thus, human blood monocytes were differentiated for 6 days and treated first with increasing concentrations (0.1, 1, 10 and 100 ng/ml) of TNF- α for 120 hours. As shown in Figure 3.5A, TNF- α inhibited efferocytosis in a concentration-dependent fashion. Significant inhibition from control levels 31.9 ± 3.4 % to 24.5 ± 4.4 % ($p < 0.01$; $n = 13$) was observed upon treatment with 1 ng/ml TNF- α . The further increase in TNF- α concentration up to 10 ng/ml resulted in augmentation of its inhibitory effect and the levels of efferocytosis were 13.4 ± 2.0 % ($p < 0.001$; $n = 13$). Similar levels of efferocytosis (13.5 ± 2.3 %; $p < 0.001$; $n = 13$) were obtained upon treatment with 100 ng/ml TNF- α . The approximate IC_{50} for TNF- α was 8.3 ng/ml. Next, I investigated the time dependence of the TNF- α effect and found that TNF- α (10 ng/ml) requires a shorter time than LPS to induce significant inhibition of efferocytosis. As indicated in Figure 3.5B, after 6 hours of TNF- α treatment, the levels of phagocytosis were reduced from 42.4 ± 5.3 % to 28.0 ± 2.5 % ($p < 0.05$; $n = 6$). Longer TNF- α treatments, 48, 96 and 144 hours, further decreased efferocytosis to similar levels: 15.3 ± 2.1 %, 14.8 ± 2.6 % and 13.3 ± 6.3 % respectively ($p < 0.001$, $n = 6$). Figure 3.5C compares typical flow cytometric profiles of efferocytosis by control and TNF- α treated MDM ϕ and Figure 3.6 depicts their confirmation by microscopy.

FIGURE 3.5 (previous page). Concentration- and time-dependent effect of TNF- α on MDM ϕ efferocytosis of neutrophils. A. Human MDM ϕ for concentration-response experiments were cultured for 6 days and treated for 120 hours with increasing concentrations of TNF- α (**A**) whereas for time-course experiments MDM ϕ were treated for increasing durations of time with 10 ng/ml TNF- α (**B**). On day 6, MDM ϕ efferocytosis of neutrophils was assessed by flow cytometry. The % efferocytosis is expressed as the mean \pm SEM of n=13 (**A**) or n=6 (**B**) experiments. Significant differences for comparisons to medium control are represented by *p<0.05, **p<0.01 and ***p<0.001. C. Representative flow cytometric dot-plot profiles demonstrating changes in efferocytosis of neutrophils resulting from 96 hours treatment with 10 ng/ml TNF- α in comparison to medium control. **aN ϕ** – apoptotic (20 hours culture) neutrophils

FIGURE 3.6 (next page). Representative photomicrographs showing MDM ϕ efferocytosis of apoptotic neutrophils. Human MDM ϕ were cultured for 6 days in culture medium (control) or treated for 120 hours with either 1 μ M dexamethasone, 10 ng/ml LPS or 10 ng/ml TNF- α . On day 6, MDM ϕ efferocytosis of apoptotic neutrophils was assessed by microscopy. Images were obtained using an Axiovert S100 inverted microscope (Carl Zeiss), RS Photometrix camera and analysed with Open lab software, 3.1.5 version. Magnification 320x. **aN ϕ** – apoptotic (20 hours culture) neutrophils, **DX** - dexamethasone.

3.3.5 LPS - AND TNF- α - INDUCED INHIBITION OF EFFEROCYTOSIS APPEARS TO BE SPECIFIC FOR APOPTOTIC CELLS

I next investigated whether the effects of TNF- α and LPS on efferocytosis were specific only for neutrophils undergoing constitutive apoptosis. For this purpose I induced apoptosis in neutrophils with the cyclin-dependent kinase inhibitor, R-roscovitine (8 hours treatment), previously shown by our group to augment neutrophil apoptosis (Rossi *et al.*, 2006). As shown in Figure 3.7A TNF- α (10 ng/ml) and LPS (10 ng/ml) markedly inhibited uptake of R-roscovitine-induced apoptotic neutrophils from 40.6 ± 5.2 % to 25.0 ± 5.2 % and 23.5 ± 7.9 % ($p < 0.05$, $n = 4$) respectively whereas dexamethasone increased the uptake up to 70.1 ± 5.5 % ($p < 0.001$, $n = 4$). On the contrary, MDM ϕ phagocytosis of Ig-G opsonised erythrocytes was not inhibited by 10 ng/ml TNF- α (59.2 ± 16.3 %) and 10 ng/ml LPS (50.5 ± 10.8 %) in comparison to control (40.5 ± 7.6 %) but here again dexamethasone (1 μ M) significantly increased phagocytosis up to 62.8 ± 8.0 % ($p < 0.05$, $n = 4$ Figure 3.7B).

FIGURE 3.7 LPS and TNF- α inhibit MDM ϕ uptake of R-roscovitine-induced apoptotic neutrophils but not opsonised erythrocytes. MDM ϕ were cultured for 6 days and treated for 120 hours with either 1 μ M dexamethasone, 10 ng/ml TNF- α or 10 ng/ml LPS. On day 6, MDM ϕ ability to ingest either apoptotic neutrophils (apoptosis was induced by 20 μ M R-roscovitine, 8 hours treatment) (**A**) or human IgG opsonized erythrocytes (**B**) was assessed by laser flow cytometric analysis. Data are expressed as mean phagocytosis \pm SEM for n=4 experiments. Significant differences for comparisons to medium control are represented by *p<0.05 and ***p<0.001.

3.3.6 IL-1 β INHIBITS MDM ϕ CLEARANCE OF APOPTOTIC NEUTROPHILS

Since TNF- α triggers production of other pro-inflammatory cytokines by M ϕ (Feldmann *et al.*, 1995) I next investigated whether MDM ϕ treated with another cytokine, i.e., IL-1 β , would also display impaired efferocytosis. For this purpose human blood monocytes were cultured for 6 days in medium (medium control) or treated for 96 hours with either 10 ng/ml TNF- α (TNF- α control) or increasing concentrations (0.01, 0.1, 1, 10 ng/ml) of IL-1 β . As shown in Figure 3.8, TNF- α significantly decreased efferocytosis and the mean levels were 8.6 ± 2.9 % ($p < 0.001$, $n = 6$) in comparison to medium control levels 29.4 ± 5.9 %. Interestingly, MDM ϕ treated with 1 and 10 ng/ml IL-1 β were also characterised by decreased ability for efferocytosis, 14.0 ± 5.2 % ($p < 0.01$, $n = 6$) and 16.0 ± 6.8 %, ($p < 0.05$, $n = 6$) respectively. Similar experiment has also been conducted with IL-6 and obtained data suggest that IL-6 did not exert any effect on efferocytosis (data not shown).

FIGURE 3.8 Effects of IL-1 β on MDM ϕ efferocytosis of apoptotic neutrophils.

Human MDM ϕ were cultured in medium (control) for 6 days and treated as indicated for 96 hours with either 10 ng/ml TNF- α or increasing concentrations of IL-1 β . On day 6, their ability to ingest apoptotic neutrophils was assessed by flow cytometry. The % efferocytosis is expressed as mean \pm SEM for $n = 6$ experiments. Significant differences for comparisons to medium control are represented by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.3.7 SUMMARY OF THE RESULTS

- Efferocytosis of apoptotic neutrophils is inhibited by bacterial products, i.e., LPS, LTA and PGN. These effects are concentration-dependent and the timing of response differs between analysed compounds.
- TNF- α inhibits efferocytosis of apoptotic neutrophils in a concentration- and time-dependent manner. My preliminary data also suggest that TNF- α -inducible cytokine, IL-1 β , may exert similar inhibitory effects.
- TNF- α and LPS inhibit the uptake of R-roscovitine-induced apoptotic cells but not human IgG-opsonised erythrocytes. On the contrary, dexamethasone augments the uptake of both apoptotic neutrophils and human IgG-opsonised erythrocytes.

3.4 DISCUSSION

The interrelationship between over expression of pro-inflammatory cytokines such as TNF- α at inflamed tissues and development of chronic inflammation is not fully understood. Since chronic inflammation has been shown to be associated with defects in resolution of inflammation (Gilroy *et al.*, 2004; Serhan *et al.*, 2007), I hypothesised that the presence of pro-inflammatory mediators may delay the resolving phase of inflammation. There are at least two main mechanisms involved in resolution of inflammation: inflammatory cell apoptosis and subsequent clearance by M ϕ (Serhan *et al.*, 2007; Serhan & Savill, 2005; Gilroy *et al.*, 2004). It has been previously shown that key pro-inflammatory mediators such as LPS and TNF- α can influence the rate of inflammatory cell apoptosis (Lee *et al.*, 1993; Murray *et al.*, 1997; Ward *et al.*, 1999). The experiments presented in chapter 3 focus on the effects of several pro-inflammatory agents, i.e., bacterial products LPS, LTA, PGN (which are well-known inducers of TNF- α production) as well as human recombinant TNF- α on M ϕ clearance of apoptotic neutrophils.

Data presented in chapter 3 show for the first time that prolonged treatment with LPS decreases the ability of human monocyte derived M ϕ to ingest apoptotic neutrophils in a concentration- and time-dependent manner. I was particularly interested in the effects of prolonged pro-inflammatory challenge that would more likely mimic chronic inflammatory conditions. Therefore, I initially, treated human monocytes during their differentiation into mature M ϕ with LPS for a duration of 96–144 hours. The inhibition of phagocytosis was observed with concentration of LPS as low as 0.1 ng/ml and the effect was positively associated with the increase in LPS concentration. Interestingly, when the time-dependence of LPS (10 ng/ml) effects was examined, I found that at least 96 hours incubation was required to achieve significant reduction in the phagocytosis. This is partially in agreement with the observation by McPhillips and colleagues, who demonstrated that treatment of the murine M ϕ cell line, J774, with 10 ng/ml LPS for 24 hours did not result in decreased uptake of apoptotic Jurkat cells (McPhillips *et al.*, 2007). The latter study did not examine the effects of LPS treatment for durations longer than 24 hours.

I also used two TLR2 agonists, LTA and PGN, to evaluate their effects on M ϕ phagocytosis of apoptotic neutrophils. Interestingly, both agents were able to exert inhibitory effects, yet, the kinetics of observed inhibition was distinct from that induced by LPS. First of all, much higher concentrations of LTA (100 μ g/ml) and PGN (10 μ g/ml) were required to induce significant decreases in phagocytosis levels. The minimal time of incubation, which was necessary to observe the inhibitory effect, varied between analysed agents. Upon LTA and PGN treatments the inhibition of phagocytosis was observed after 16 hours incubation whereas upon LPS treatment inhibitory effect occurred only after 96 hours incubation. Interestingly, upon PGN treatment, inhibitory effect occurred after 16 hours, then disappeared and occurred again after 96 hours.

The above-mentioned differences between the inhibitory effects of LPS, LTA and PGN may be explained, at least in part, by the fact that LPS is a much more powerful activator of immune cells and has a greater efficacy to induce cytokine production in M ϕ . Several possible mechanisms underlie the lower ability of LTA to activate immune cells in experimental settings. First of all, LTA is a major immunostimulatory constituent of the Gram-positive bacteria cell wall, which is anchored in the bacterial membrane and protrudes through the murein sacculus (PGN) (Gutberlet *et al.*, 1997). It has been shown that adhesion of LTA to e.g., a polystyrene surface (imitating the physiological situation) drastically increases its immunostimulatory potency in comparison to soluble LTA (Deininger *et al.*, 2008). The surface presentation of LTA has been associated with 2- to 10-fold enhancement of the release of pro-inflammatory cytokines e.g., TNF- α . It has been also reported that soluble LTA does not activate JNK, which is crucial for pro-inflammatory cytokine production (Draing *et al.*, 2008). In this PhD thesis, the soluble form of LTA has been used and this could potentially explain the much lower effect of LTA in comparison to LPS. Importantly, the surface presentation of LPS does not further increase its immunostimulatory properties (in nature LPS is a constituent of the outer bacterial membrane). It has been also suggested that LTA is a major immunostimulatory constituent of PGN and, consequently, highly pure (LTA-free) PGN does not activate TLR-2 (Travassos *et al.*, 2004). Small amounts of LTA

presented in the PGN framework were demonstrated to induce cytokine production, probably by reproducing natural conditions (Travassos *et al.*, 2004).

There is contradiction in the literature with regard to the role of LBP in LTA-induced cell activation. While some studies suggest that LBP enhances or has no effect on LTA-induced signalling, other studies demonstrate that LBP may actually block this process (Schroder *et al.*, 2003; Mueller *et al.*, 2006). This discrepancy may, at least in part, result from contamination of commercially available LTA preparations with LPS (LBP plays an important role in induction of LPS signalling as described in section 1.2.2.1). Importantly, one recent study has demonstrated that the activity of pure LTA (no TLR4 activity) is blocked in the presence of soluble LBP (Mueller *et al.*, 2006). This is in contrast to LPS, which requires soluble LBP in order to be presented to the cell surface receptor TLR4 and exert its pro-inflammatory effects. Since in this PhD thesis experiments with LPS, LTA and PGN were performed in the serum-supplemented medium (soluble LBP is a serum constituent), it is possible that presence of soluble LBP interfered with pro-inflammatory activity of LTA in my system.

Finally, LPS, LTA and PGN may exert various effects on phagocytosis because they stimulate distinct host cell surface receptors (TLR-4 or TLR-2 respectively). The 'net' outcome of TLR-4 and TLR-2 stimulation differ in the pattern of released cytokines, e.g., LTA, in contrast to LPS, triggers strong chemokine production and almost no IL-12 and IFN γ release (Draing *et al.*, 2008). Thus, it is likely that overall differences in the released mediators are responsible for the observed various effects of LPS and LTA on phagocytosis. Nevertheless, since LPS, LTA and PGN are well-known inducers of TNF- α production I wanted to establish the effects of exogenous TNF- α on phagocytosis. Interestingly, in my system, TNF- α treatment of monocyte/M ϕ resulted in significant reduction of the M ϕ clearance of apoptotic neutrophils. I also obtained preliminary data demonstrating that IL-1 β but not IL-6 (TNF- α -inducible cytokines) exerts a similar effect. The TNF- α -induced inhibitory effects were observed as early as 6 hours after TNF- α treatment and were positively associated with the duration of treatment (the maximal inhibition was observed after 144 hours treatment). These effects were also concentration-dependent and 1 ng/ml concentration was sufficient to induce significant inhibition of

phagocytosis. Further increase in the TNF- α concentration resulted in additional reduction of phagocytosis. This observation is partially consistent with the study by McPhillips and colleagues, which demonstrated that short-term treatment (up to 4 hours) of murine J774 M ϕ with TNF- α (1-10 ng/ml) significantly reduced their ability to phagocytose apoptotic Jurkat cells (McPhillips *et al.*, 2007). However, in the latter study the TNF- α effect disappeared if treatment was performed for longer than 4 hours. It is likely that different experimental conditions (possibly the fact that I used human cells) contributed, at least partially, to the above-described discrepancy. In this PhD thesis, human primary cells have been used (human monocytes and neutrophils isolated from peripheral blood), while in the study by McPhillips *et al* experiments were performed with murine J774 M ϕ and apoptotic Jurkat cells. Furthermore, in this PhD thesis, M ϕ were stimulated with human recombinant TNF- α and in the study by McPhillips *et al* with mouse recombinant TNF- α , which could potentially affect the kinetics of the response.

The study published by McPhillips *et al* also suggests the possible mechanism underlying TNF- α inhibition of phagocytosis. The authors demonstrated that TNF- α treatment induced generation of ROS, followed by activation of phospholipase cPLA₂ and upregulation of the active Rho levels. Indeed, the latter effect significantly reduces M ϕ phagocytosis as described in more detail in section 1.3.3. My data complement the observation by McPhillips *et al* and indicate that TNF- α impairs phagocytosis of apoptotic neutrophils. If this is the case *in vivo*, the presented data add more insight into how over expression of TNF- α in inflamed tissue contributes to development of chronic inflammatory conditions. Indeed, one recent *in vivo* study in mice has demonstrated that TNF- α inhibits clearance of apoptotic cells by alveolar M ϕ . This effect was associated with increased lung recruitment of inflammatory leukocytes and elevated release of pro-inflammatory cytokines, i.e., IL-6, KC and MCP-1 while levels of anti-inflammatory IL-10 and TGF- β remained unaffected (Borges *et al.*, 2009).

It is also noteworthy that the effect of TNF- α on phagocytosis is suggested to be influenced by the degree of M ϕ maturation. One study demonstrates that TNF- α significantly augments phagocytosis by immature (4 day differentiated) human monocyte derived M ϕ (Ren & Savill, 1995). This observation has been confirmed by

McPhillips *et al* who demonstrated that TNF- α augmented phagocytosis of apoptotic cells by immature M ϕ (4 day differentiated) and inhibited phagocytosis by mature M ϕ (7 day differentiated). Importantly, TNF- α treatment increased the levels of active Rho only in mature M ϕ while the levels of active Rho in immature M ϕ remained unaltered. It is therefore possible that, at inflammatory sites, TNF- α can inhibit clearance of apoptotic cells by mature M ϕ but would not affect phagocytic functions of incoming and maturing M ϕ .

It is intriguing that LPS did not exert inhibitory effects on phagocytosis during the first 24 hours of M ϕ treatment, particularly since M ϕ produce TNF- α as early as 2 hours after LPS treatment (de Waal *et al.*, 1991). Furthermore, as shown in Figure 3.5B, TNF- α inhibited M ϕ phagocytosis of apoptotic neutrophils after 6 hours incubation (shorter durations of time have not been here studied) and according to McPhillips *et al* M ϕ treated with TNF- α for 20 minutes had reduced ability to phagocytose apoptotic cells. In this PhD study, after 6 hours treatment with LPS, minor decreases in the phagocytosis levels have been observed for each individual experiment. Yet, statistical analysis demonstrates that these results, when plotted together, are not significantly different from controls (Figure 3.2B). The lack of the significant inhibitory effect of LPS during the first 24 hours of treatment could be explained, at least in part, by findings by Ding and colleagues (Ding *et al.*, 1989). The authors demonstrated that during endotoxaemia M ϕ and some other host cells may be unresponsive to TNF- α because such cells internalise their TNF-Rs in response to LPS prior to TNF- α production. The latter effect was dependent on both the concentration of LPS and duration of treatment (1 hour treatment with 10 ng/ml LPS resulted in a complete loss of cell surface TNF- α binding sites). Furthermore, authors of this study provided several lines of evidence indicating that this effect is mediated by LPS *per se* and that TNF- α , which is released from M ϕ in response to LPS, is responsible for only a minor portion of TNF-Rs internalisation. Interestingly, in this PhD thesis high concentrations of TNF- α were detected at M ϕ culture supernatants at 6 hours after treatment with 10 ng/ml LPS (data presented in Figure 5.1, chapter 5). Therefore, one may speculate that M ϕ treated with LPS are unresponsive to TNF- α at early time points or endogenously produced TNF- α does not mediate inhibitory effects of LPS. I performed a number of experiments in order to shed more light into

significant delay of LPS-inhibitory effects. They included depletion of endogenously produced TNF- α from culture medium, treatment with anti-inflammatory cytokine IL-10 or assessment of phagocytosis by IL-10 deficient BMDM ϕ . The results are presented and discussed in the following chapters.

It is believed that apoptotic cells are cleared by mechanisms different from those that are implicated in the phagocytosis of necrotic or opsonised cells (Hart *et al.*, 2004b). Thus, control experiments have been performed, in which I tried to establish specificity of TNF- α or LPS effects on phagocytosis. For this purpose, in order to obtain negligible levels of necrosis, human neutrophils have been synchronised in apoptosis with the use of R-roscovitine (8 hours treatment). The M ϕ uptake of R-roscovitine-induced apoptotic neutrophils (Rossi *et al.*, 2006) was inhibited by both TNF- α and LPS further supporting my findings. Next, I examined the effects of TNF- α and LPS on the M ϕ uptake of human IgG-opsonised erythrocytes. Interestingly, this process was not affected by TNF- α and LPS treatments indicating that pro-inflammatory stimuli do not block all phagocytic pathways in M ϕ . This observation is in agreement with previously published data (Rossi *et al.*, 1998b) demonstrating that M ϕ phagocytosis of apoptotic neutrophils is regulated by mechanisms distinct from those modulating phagocytosis of cells coated with IgG and complement (Michlewska *et al.*, 2007; Ravichandran & Lorenz, 2007). Interestingly, opsonins such as antibodies or complement have been shown to bind to apoptotic cells and enable their clearance *via* classical phagocytic receptors, such as Fc receptors. For instance, patients with antiphospholipid syndrome (APLS) are characterised by elevated levels of ‘antiphospholipid antibodies’ including β 2-glycoprotein I (β 2-GPI) antibodies, which bind to the surface of apoptotic cells and augment their clearance by immature dendritic cells (Rovere *et al.*, 1998). The latter observation suggests an important role in antigen presentation and exacerbation of autoimmune disease. In addition, C1q deficiency in SLE is associated with reduced phagocytosis of apoptotic cells (Michlewska *et al.*, 2007). As discussed in section 1.3.2.4 the outcome of phagocytosis may differ depending on the type of removed particles. In general, phagocytosis of apoptotic cells is thought to be anti-inflammatory and to actively inhibit release of pro-inflammatory mediators such as TNF- α , IL-1 β or IL-8 and promote the secretion of anti-inflammatory cytokines

including TGF- β , PGE₂ or IL-10 (Fadok *et al.*, 1998; Voll *et al.*, 1997). Furthermore, *in vivo* studies showed that in a murine model of LPS-induced endotoxic shock administration of apoptotic cells protect mice from LPS-induced death (Ren *et al.*, 2008). The latter effect was associated with decreased levels of circulating pro-inflammatory cytokines such as TNF- α , inhibition of neutrophil recruitment and reduction in LPS serum levels. On the contrary, phagocytosis of opsonised particles is considered to be pro-inflammatory and stimulate release of pro-inflammatory cytokines e.g., TNF- α (Fadok *et al.*, 2001). For instance, in Wegener's granulomatosis, opsonisation of apoptotic neutrophils with anti-neutrophil cytoplasmic antibodies (ANCA) has been demonstrated to augment their clearance by M ϕ (Moosig *et al.*, 2000). This effect was associated with increased production of TNF- α . TNF- α may then stimulate recruitment of further neutrophils, which are cleared in a similar way stimulating further release of TNF- α and exacerbation of inflammation. Interestingly, in this study phagocytosis of apoptotic cells opsonised with IgG did not result in induction of TNF- α release but since other studies suggest otherwise no final consensus has been reached with this regard. Data presented in this PhD thesis demonstrate that TNF- α inhibits clearance of apoptotic neutrophils, but not opsonised erythrocytes. These results suggest that even though TNF- α inhibits phagocytosis of apoptotic cells, these cells may undergo opsonisation and be cleared *via* Fc receptors (preventing secondary necrosis and exacerbation of inflammation). Yet, in the recent study by McPhillips *et al.*, mice injected intraperitoneally with TNF- α and apoptotic thymocytes were characterised by decreased phagocytosis in comparison to control animals (McPhillips *et al.*, 2007). These findings indicate that over expression of TNF- α in inflamed tissue may indeed lead to accumulation of apoptotic cells, their secondary necrosis and exacerbation of inflammation. Thus, further *in vivo* studies are required in order to fully establish immunological consequences of the inhibition of apoptotic cell clearance by TNF- α in humans.

In summary, data presented in this chapter demonstrate that the presence of TNF- α during monocyte differentiation into M ϕ significantly affects their ability to phagocytose apoptotic neutrophils but not human IgG-opsonised erythrocytes. Interestingly, the general pro-inflammatory activators of M ϕ , namely LPS, LTA and PGN were shown to exert similar albeit delayed inhibitory properties (the next

chapters give more insight into this phenomenon). One important finding presented in this chapter is a differential effect of TNF- α and LPS on clearance of apoptotic and opsonised targets. If this occurs *in vivo*, it is likely that at the inflammatory site TNF- α blocks clearance of apoptotic neutrophils by M ϕ enabling more efficient removal of foreign organisms or opsonised particles. The latter effect seems to be more beneficial at the onset of inflammation, when phagocytosis of apoptotic immune cells is not necessary. However, if inflammatory processes are associated with excessive or prolonged exposure to TNF- α , removal of apoptotic cells is impaired leading to cell necrosis, subsequent tissue damage and development of many pathological conditions. Alternatively, apoptotic neutrophils may bind numerous opsonins and be cleared *via* other phagocytic receptors. The immunological outcome of the latter process may vary depending on numerous factors (e.g., type of opsonins) and in some circumstances it may be associated with the release of pro-inflammatory mediators and exacerbation of inflammation.

CHAPTER FOUR

LPS INHIBITS PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS *via* A TNF- α DEPENDENT MECHANISM

4 LPS INHIBITS PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS *via* A TNF- α DEPENDENT MECHANISM

4.1 INTRODUCTION

Data presented in chapter 3.3 demonstrate that treatment of MDM ϕ with the pro-inflammatory cytokine TNF- α significantly decreases MDM ϕ ability to phagocytose apoptotic neutrophils. This effect was observable as early as 6 hours after TNF- α treatment. Interestingly, when M ϕ were treated with other M ϕ activators such as LPS, LTA or PGN, inhibitory effects also occurred, yet, after much longer incubation (LPS > 96 hours, LTA > 16 hours, PGN > 16 hours). It is noteworthy that LPS, LTA and PGN are well-known inducers of TNF- α production. Inhibition of phagocytosis was the strongest and, intriguingly, the most delayed when M ϕ were treated with LPS. Thus, I have chosen LPS for further investigation.

A number of studies have demonstrated that LPS-treated cells release TNF- α as early as 2 hours following stimulation and TNF- α production is maximal after 6 hours (de Waal *et al.*, 1991). Interestingly, in this PhD thesis, phagocytosis by LPS-treated MDM ϕ has not been significantly reduced at such early time points. These observations suggest that, in my system, LPS-induced inhibition of phagocytosis is not necessarily mediated by TNF- α production. Alternatively, other mechanisms may interfere with either production or action of TNF- α .

In experiments presented in chapter 4 I attempted to establish whether LPS-induced inhibition of phagocytosis is mediated by induction of TNF- α production. Specifically, I sought to investigate if depletion of biologically active TNF- α in MDM ϕ culture rescued phagocytosis inhibited by LPS treatment. For this purpose several reagents have been used including two soluble TNF R1 (sTNF-R1/Fc and sTNF-R1) and the TACE inhibitor, TOPI-0. The sTNF-Rs bind sTNF- α in culture supernatants and reduce its bioactivity. Their role in treatment of chronic

inflammatory conditions characterised by an excess of TNF- α is well-established and discussed in section 1.5.3. In contrast, TACE inhibitors block processing of 26 kDa, membrane-bound TNF- α into its soluble form and consequently reduce sTNF- α concentration in culture supernatants. All experiments presented in chapter 4 have been performed with human blood monocytes, differentiated into MDM ϕ over 6 day culture and treated with LPS and increasing concentrations of the specific TNF- α inhibitor. On day 6 of the culture phagocytosis of human apoptotic neutrophils were assessed by flow cytometry. The levels of phagocytosis were compared to medium control or LPS treated samples. The ability of TNF- α inhibitors to bind and reverse the effects of TNF- α present in culture medium was evaluated by treatment of MDM ϕ with exogenous TNF- α and increasing concentrations of appropriate inhibitor.

4.2 METHODS

4.2.1 ISOLATION AND CULTURE OF HUMAN BLOOD CELLS

Human blood cells were isolated by Percoll™ gradient centrifugation, following a protocol described in section 2.2.1.1. Subsequently, human monocytes were cultured for 6 days in culture medium (see section 2.2.1.3 for culture conditions) undergoing differentiation into mature human MDM ϕ . During this time MDM ϕ were treated with a number of pro- and anti-inflammatory agents. In experiments described in this chapter human monocytes were treated with TNF- α and LPS at a concentration of 10 ng/ml (96-144 hours), sTNF-R1/Fc using a range of concentrations: 5–500 ng/ml (96-144 hours), sTNF-R1 using a range of concentrations: 62.5-1000 ng/ml (96-144 hours) or TACE inhibitor–TOPI-0 using a range of concentrations: 0.1–10 ng/ml (96-144 hours).

On day 6, MDM ϕ were assessed for their ability to phagocytose human apoptotic neutrophils that had undergone constitutive apoptosis for 20 hours of culture (see section 2.2.1.3 for culture conditions).

4.2.2 ASSESSMENT OF APOPTOSIS AND NECROSIS

Prior to the phagocytosis assay, the % of apoptotic cells within neutrophil populations was assessed by morphology (see section 2.3.1 for experimental protocol) or alternatively by Annexin-V binding assessed by flow cytometry (see section 2.3.2 for experimental protocol). The % of necrotic cells was assessed by PI staining assessed by flow cytometry. Only neutrophils with less than 5 % of necrosis were used in the phagocytosis assay.

4.2.3 ASSESSMENT OF PHAGOCYTOSIS BY FLOW CYTOMETRY

In all the experiments described in this chapter phagocytosis was assessed by flow cytometric analysis that employs labelling of phagocytic targets with FL1⁺ Cell Tracker™ Green (CMFDA) prior to the phagocytosis assay. Following the phagocytosis assay, the % of FL1⁺ MDM ϕ (MDM ϕ that had engulfed phagocytic targets) within the entire MDM ϕ population was assessed by flow cytometry as described in section 2.4.1.1.

4.2.4 BINDING OF sTNF-R1/Fc AND IC TO THE SURFACE OF HUMAN APOPTOTIC NEUTROPHILS

The sTNF-R1/Fc is a chimeric protein containing an Fc region of human IgG₁. Thus, it is potentially able to induce Fc-mediated phagocytosis in similar way to immune-complexes (IC, heat-aggregated IgG) (Hart *et al.*, 2004a). I therefore compared binding of sTNF-R1/Fc and IC to the surface of human apoptotic neutrophils, cultured for 20 hours (see section 2.2.1.3 for culture conditions) prior to the binding assay described in section 2.6.

4.2.5 MEASUREMENT OF CYTOKINE LEVELS AFTER LPS STIMULATION

In experiments described in section 4.3 MDM ϕ were treated for 120 hours with 10 ng/ml LPS. Prior to assessment of MDM ϕ efferocytosis, cytokine levels in MDM ϕ culture supernatants were determined. To quantify cytokine concentrations, a CBA (Human Inflammation Kit) has been used as described in section 2.5.1.

4.3 RESULTS

4.3.1 THE ABILITY OF MDM ϕ TO PHAGOCYTOSE APOPTOTIC NEUTROPHILS IS CORRELATED WITH THE LEVELS OF TNF- α IN CULTURE MEDIUM

Figure 4.1 depicts a scatter diagram where paired data showing % efferocytosis upon treatment with 10 ng/ml LPS (96–144 hours) versus % efferocytosis in control conditions are plotted (n = 40). MDM ϕ were cultured for 6 days and phagocytosis was assessed by flow cytometry. Even though the inhibitory effect of LPS is highly significant ($p < 0.001$), some variation was found in MDM ϕ response to LPS treatment between donors.

FIGURE 4.1 Variability of LPS effects upon efferocytosis. The scatter diagram demonstrates paired data (control efferocytosis versus efferocytosis upon LPS treatment) from 40 experiments.

I hypothesised that the extent of inhibition of efferocytosis induced by LPS was dependent on endogenous TNF- α production by cultured MDM ϕ and consequently on the concentration of TNF- α in culture medium prior to the phagocytosis assay. To test this, monocytes were isolated from 6 selected donors, where I previously observed variability in response to LPS. Monocytes were cultured for 6 days, treated with 10 ng/ml LPS for 120 hours. On day 6 we performed paired experiments (schematic representation is displayed in Figure 4.2) that involved collection of culture supernatants, measurement of cytokines by CBA and, in parallel, assessment of MDM ϕ ability to phagocytose apoptotic neutrophils by flow cytometric analysis. As shown in Figure 4.3 the % of MDM ϕ ingesting apoptotic neutrophils was negatively correlated with the concentration of TNF- α in the supernatants ($R^2 = 0.84$). In addition, I observed an interesting correlation between efferocytosis and concentrations of TNF- α and IL-10. Figure 4.4 depicts levels of TNF- α and IL-10 in MDM ϕ culture supernatants for two separate experiments. In the first experiment (Figure 4.4A), LPS treatment exerted a strong inhibitory effect upon efferocytosis and culture supernatants were characterised by high concentrations of TNF- α (above 15 ng/ml) and low levels of IL-10 (approximately 0.3 ng/ml). In the second experiment (Figure 4.4B), LPS treatment was associated with augmentation of phagocytosis and, interestingly, levels of TNF- α in culture supernatants were undetectable whereas IL-10 concentrations were elevated (approximately 3ng/ml).

FIGURE 4.2 Schematic representation of paired experiments performed to examine correlations between TNF- α concentrations and efferocytosis. MDM ϕ were cultured for 6 days and treated for 120 hours with 10 ng/ml LPS. On day 6, just prior to the efferocytosis assay: 1) culture supernatants were collected and subjected to CBA analysis, 2) apoptotic neutrophils (cultured for 20 hours) were added to adherent MDM ϕ and 3) efferocytosis was measured by flow cytometry. **aN ϕ** – apoptotic neutrophils.

FIGURE 4.3 The effect of LPS on MDM ϕ efferocytosis is associated with the concentrations of TNF- α in the culture medium. Monocytes isolated from blood of 6 donors were cultured for 6 days and treated with 10 ng/ml LPS for 120 hours. On day 6, culture supernatants were collected and TNF- α levels were measured by CBA while efferocytosis was assessed by flow cytometry. Negative correlation ($R^2=0.84$) was identified between efferocytosis levels and TNF- α concentrations. For comparisons, efferocytosis levels were normalised (medium control levels represented by 100%).

FIGURE 4.4 Low TNF- α production upon LPS treatment is associated with elevated IL-10 release. Human MDM ϕ were cultured for 6 days and treated with 10 ng/ml LPS for 120 hours. On day 6, culture supernatants were collected and TNF- α and IL-10 levels were measured by CBA while efferocytosis was assessed by flow cytometry. Concentrations of TNF- α and IL-10 are demonstrated for two separate experiments, where LPS treatment induced **A.** strong inhibitory effect upon efferocytosis and **B.** augmentation of efferocytosis.

4.3.2 DEPLETION OF TNF- α IN CULTURE MEDIUM REVERSES THE INHIBITORY EFFECT OF LPS ON MDM ϕ EFFEROCYTOSIS OF APOPTOTIC NEUTROPHILS

Having found that exogenous TNF- α inhibits MDM ϕ phagocytosis of apoptotic neutrophils (Figure 3.5) and that variability of responses to LPS treatment is correlated with various levels of TNF- α in culture supernatants (Figure 4.3), I hypothesised that LPS-mediated effects were dependent upon production of endogenous TNF- α by MDM ϕ . Thus, I next aimed to rescue MDM ϕ efferocytosis upon LPS treatment by reducing the biological activity of TNF- α produced by MDM ϕ . For this purpose I used sTNF-R1/Fc that binds TNF- α present in the culture medium. Human monocytes were cultured for 6 days in culture medium (control) or co-treated for 120 hours with 10 ng/ml LPS or 10 ng/ml TNF- α +/- increasing concentrations of sTNF-R1/Fc. I showed that addition of 500 ng/ml sTNF-R1/Fc reversed both exogenous TNF- α and LPS-mediated inhibition of efferocytosis back to control levels (30.5 ± 3.5 %). The % of phagocytosis when cells were treated with LPS alone was 11.4 ± 1.1 % and for TNF- α alone was 14.2 ± 2.3 % whereas addition of sTNF-R1/Fc enhanced efferocytosis up to 32.1 ± 4.1 % and 30.1 ± 5.0 % respectively ($p < 0.01$; $n = 4$) (Figure 4.5A). Addition of sTNF-R1/Fc to control MDM ϕ (no LPS or TNF- α treatment) did not augment efferocytosis *per se* (28.9 ± 3.5 %). Representative flow cytometric dot-plot profiles demonstrating reversal of inhibitory effects of LPS by addition of srh TNF-R1/Fc are demonstrated in Figure 4.5B.

A

B

FIGURE 4.5 Neutralization of TNF- α released by LPS-stimulated MDM ϕ rescue efferocytosis. **A.** Human MDM ϕ were cultured for 6 days in culture medium alone or treated on average for the duration of 96-144 hours with 10 ng/ml LPS or 10 ng/ml TNF- α +/- increasing concentrations of sTNF-R1/Fc, as indicated. On day 6 MDM ϕ efferocytosis was assessed by flow cytometric analysis. Data shown as mean \pm SEM for n=4 separate experiments. Significant differences for comparisons to appropriate controls (treatments with LPS or TNF- α alone) are represented by **p<0.01. **B.** Representative flow cytometric dot-plot profiles demonstrating levels of efferocytosis for controls and treatments with either 10 ng/ml LPS alone or 10 ng/ml LPS with 500 ng/ml sTNF-R1/Fc. **aN ϕ** – apoptotic neutrophils.

4.3.3 sTNF-R1/Fc DOES NOT INDUCE Fc-DIRECTED PHAGOCYTOSIS AND PRIMARILY DEPLETES TNF- α IN CULTURE MEDIUM

sTNF-R1/Fc is a chimeric protein containing two subunits of sTNF-R1 and an Fc region of human IgG₁. Dimeric sTNF-R1s were previously shown to be approximately 45-fold more potent inhibitors of TNF- α than the monomeric sTNF-R1s (Aggarwal & Natarajan, 1996). However, the presence of an Fc region in sTNF-R1/Fc raises the possibility that, in my system, reversal of the inhibitory effect of LPS and TNF- α is accomplished not only by a decrease in TNF- α activity but also by induction of Fc mediated phagocytosis, similar to augmentation of phagocytosis by immune-complexes (heat-aggregated IgG) (Hart *et al.*, 2004a). It has been reported that binding of ICs to Fc receptors is a rapid process and 0.5 hour is sufficient to obtain optimal opsonisation (Hart *et al.*, 2004a). Thus, a series of controls were performed comparing effectiveness of sTNF-R1/Fc (500 ng/ml) on reversal of LPS- or TNF- α -induced inhibition of phagocytosis. In all the experiments monocytes were cultured for 6 days in culture medium alone or treated for 96 hours with either TNF- α (10ng/ml) or LPS (10 ng/ml). sTNF-R1/Fc was added either synchronically with LPS (10 ng/ml) or TNF- α (10ng/ml) for 96 hours to monocyte culture or only for the last 30 minutes of the MDM ϕ or neutrophil culture, just prior to the phagocytosis assay. Alternatively, sTNF-R1/Fc was added directly to the phagocytosis assay (1 hour), where both MDM ϕ and apoptotic neutrophils were present. As indicated in Figure 4.6A, the presence of sTNF-R1/Fc in monocyte culture for 96 hours significantly reversed the inhibitory effects of LPS and TNF- α . However, sTNF-R1/Fc added for short durations of time (0.5 and 1 hour), sufficient for binding to the cell surface receptors, but not sufficient to neutralise TNF- α throughout the culture, did not have any effect on efferocytosis in comparison to appropriate controls. Furthermore, I compared binding of sTNF-R1/Fc and heat aggregated IgGs (immune complexes), to the aged (20 hours) neutrophils. Neutrophils, in this experiment, were cultured in IMDM with 10 % FCS (human IgG free). As shown in Figure 4.6B, following 30 min incubation, more than 98% of neutrophils (viable and apoptotic) were opsonised with

ICs whereas more than 98% did not bind sTNF-R1/Fc, excluding possibility of Fc induced augmentation of efferocytosis in my study. Since nonspecific binding of anti-human IgG antibodies to necrotic cells (less than 10 %) were detected (no difference between control, sTNF-R1/Fc and IC samples), these cells were excluded from analysis.

FIGURE 4.6 sTNF-R1/Fc does not facilitate Fc mediated efferocytosis (next page). **A.** Human MDM ϕ were cultured for 6 days in culture medium or treated for the duration of 96 hours with either 10 ng/ml LPS or 10 ng/ml TNF- α alone. sTNF-R1/Fc was added to MDM ϕ or alternatively to apoptotic (20 hours culture) neutrophils at various times or was present in the efferocytosis assay, as indicated. On day 6 MDM ϕ efferocytosis was assessed by flow cytometric analysis. Data shown as mean \pm SEM for n=4 separate experiments. Significant differences for comparisons to appropriate controls (samples treated only with LPS or TNF- α) are represented by *p<0.05 and ***p<0.001. **B.** Apoptotic neutrophils, cultured for 20 hours in Iscove's DMEM with 10% FCS, were incubated for 20 minutes in flow buffer alone (control) or with 500ng/ml sTNF-R1/Fc or 100 μ g/ml heat aggregated IgGs-immune-complexes, washed twice and incubated with anti-human IgG antibody. Percentage of apoptotic neutrophils was assessed by annexin-V binding assay. **N ϕ** –neutrophils, **aN ϕ** –apoptotic neutrophils, **naN ϕ** –non-apoptotic neutrophils, **IC**–immune-complexes.

4.3.4 THE EFFECT OF MONOMERIC sTNF-R1 ON LPS-OR TNF- α -INDUCED INHIBITION OF MDM ϕ EFFEROCYTOSIS

Having successfully reversed the inhibitory effect of LPS and TNF- α on efferocytosis with the use of sTNF-R1/Fc I aimed to investigate if its monomeric form – sTNF-R1 would have a similar effect. Previously published data showed that the monomeric form of sTNF-R1 is a much weaker inhibitor of the biological action of TNF- α in comparison to its chimeric form (Aggarwal & Natarajan, 1996). To examine this, human blood monocytes were differentiated for 6 days and co-treated on average for 96-144 hours with 10 ng/ml LPS or 10 ng/ml TNF- α alone or with addition of increasing concentrations (125, 250, 500 and 1000 ng/ml) of sTNF-R1. As indicated in Figure 4.7 treatment with sTNF-R1 did not significantly rescue efferocytosis that was inhibited with LPS or TNF- α treatment. However, it is noteworthy that in the presence of sTNF-R1, M ϕ in samples treated with either LPS or TNF- α showed a slight tendency for an increase in efferocytosis levels. The effect appears to be concentration-dependent; 1 μ g/ml sTNF-R1 had the most prominent effect and increased efferocytosis upon LPS treatment from 14.1 ± 5.6 % up to 21.8 ± 8.5 % and upon TNF- α treatment from 18.3 ± 3.2 % up to 22.9 ± 4.9 %. Medium control levels were 36.6 ± 2.1 % and interestingly MDM ϕ treated with 1 μ g/ml sTNF-R1 alone showed decreased ability for efferocytosis (30.0 ± 3.9 %).

FIGURE 4.7 Effect of sTNF-R1 on LPS- or TNF- α -induced inhibition of MDM ϕ efferocytosis. A. Human MDM ϕ were cultured for 6 days in culture medium alone or treated on average for the duration of 96-144 hours with 10 ng/ml LPS or 10 ng/ml TNF- α +/- increasing concentrations of sTNF-R1, as indicated. On day 6 MDM ϕ efferocytosis was assessed by flow cytometric analysis. Data shown as mean \pm SEM for n=4 separate experiments

4.3.5 THE EFFECT OF TNF- α CONVERTING ENZYME INHIBITOR ON LPS- OR TNF- α -INDUCED INHIBITION OF MDM ϕ EFFEROCYTOSIS

Another approach has been also applied to rescue efferocytosis upon LPS treatment involving inhibition of the activity of TNF- α Converting Enzyme (TACE). TACE processes membrane bound proTNF- α to its active soluble form (TNF- α) and therefore TACE inhibitors reduce TNF- α production by LPS-stimulated cells (Newton *et al.*, 2001). Thus, I hypothesised that a TACE inhibitor would interfere with the inhibitory effect of LPS but not exogenous TNF- α on MDM ϕ efferocytosis. To verify this, human blood monocytes were differentiated for 6 days and co-treated for 96-144 hours with 10 ng/ml LPS or 10 ng/ml TNF- α alone or with addition of increasing concentrations (0.1, 1 and 10 ng/ml) of TACE inhibitor–TOPI-0. As indicated in Figure 4.8 treatment with TOPI-0 did not significantly reverse LPS- or TNF- α -induced inhibition of efferocytosis. However, I observed a tendency for the elevation of phagocytosis levels when increasing concentrations of TOPI-0 were added into LPS treated samples. The phagocytosis levels were 19.1 ± 2.2 % for LPS treatment and increased up to 27.5 ± 3.5 % for combined LPS and 10 ng/ml TOPI-0 treatments. Interestingly, control treatment with 10 ng/ml TOPI-0 alone reduced efferocytosis from control levels 33.9 ± 1.4 % to 27.9 ± 3.0 %. A similar pattern was identified for samples treated with TNF- α , where in samples treated with TNF- α alone levels of efferocytosis were 19.6 ± 2.8 % and addition of 10 ng/ml TOPI-0 further reduced efferocytosis to 14.7 ± 0.7 %.

FIGURE 4.8 Effect of the TNF- α converting enzyme (TACE) inhibitor, TOPI-0, on inhibition of MDM ϕ efferocytosis by LPS and TNF- α . Human MDM ϕ were cultured for 6 days in culture medium alone or treated on average for the duration of 96-144 hours with 10 ng/ml LPS or 10 ng/ml TNF- α +/- increasing concentrations of TOPI-0, as indicated. On day 6 MDM ϕ efferocytosis was assessed by flow cytometric analysis. Data shown as mean \pm SEM for n=4 separate experiments.

4.3.6 SUMMARY OF THE RESULTS

- The LPS inhibitory effect on phagocytosis is positively correlated with the TNF- α concentration in MDM ϕ culture supernatants. In addition, decreased TNF- α concentrations and a lack of LPS inhibitory effects on phagocytosis are associated with elevated IL-10 concentrations.
- The addition of sTNF RI/Fc to MDM ϕ treated with either LPS or TNF- α rescues MDM ϕ ability to phagocytose apoptotic neutrophils. This effect occurs only if sTNF RI/Fc is present during the entire duration of either LPS or TNF- α treatment. sTNF RI/Fc does not bind to the surface of apoptotic neutrophils and, thus, does not trigger Fc-mediated phagocytosis.
- The addition of monomeric sTNF RI or the TACE inhibitor, TOPI-0, to MDM ϕ treated with either LPS or TNF- α does not rescue MDM ϕ ability to phagocytose apoptotic neutrophils.

4.4 DISCUSSION

TNF- α is one of the most rapidly released cytokines after trauma or infection (e.g., exposure to bacterial derived LPS) and is one of the most abundant mediators in inflamed tissues (Feldmann *et al.*, 1994). The data presented in chapter 3, as well as recent findings by McPhillips and colleagues, clearly demonstrate that treatment of M ϕ with TNF- α greatly reduces their ability to phagocytose apoptotic neutrophils (McPhillips *et al.*, 2007). LPS, LTA or PGN are activators of M ϕ and short-time stimulation is sufficient to induce significant TNF- α release from these cells (de Waal *et al.*, 1991; Wang *et al.*, 2000). However, in this PhD thesis, inhibition of phagocytosis by LPS appeared after at least 96 hours incubation. Therefore, in the experiments presented in chapter 4, I tried to establish whether TNF- α primarily mediates inhibitory effects of LPS on phagocytosis in my system.

One interesting aspect of LPS-induced inhibition was the variability of the responses. As demonstrated in figure 4.1., when M ϕ were treated with 10 ng/ml LPS the levels of phagocytosis varied between donors. Even though LPS exerted its inhibitory effects in the majority of experiments, in some cases I did not observe inhibition. This has been investigated further (paired experiments presented in figure 4.2) and significant positive correlation has been identified between levels of inhibition and concentrations of TNF- α in M ϕ culture supernatants. It is noteworthy, that in samples, in which presence of LPS did not cause inhibition, TNF- α levels were undetectable. This led to the formation of a hypothesis that upon LPS treatment, TNF- α may indeed, at least in part, mediate inhibition of phagocytosis in my system. To investigate this further, several approaches have been used, all of them aiming at reduction of either the concentration or bioactivity of TNF- α released by M ϕ into culture medium upon treatment with LPS.

One regulator of TNF- α action occurring in physiological conditions is a sTNF-R (Tracey *et al.*, 2008). It is believed that soluble cytokine receptors act as cytokine ‘carriers’ and change the biodistribution of the interacting cytokine in natural settings. It has been also demonstrated that *in vivo* administration of recombinant soluble receptors may antagonise immune and inflammatory processes. In this PhD thesis, two different sTNF-R1s were used to reduce biologic activity of

TNF- α , namely monomeric sTNF-R1 and a dimeric sTNF-R1/Fc. The latter is a fusion protein, composed of two subunits of sTNF-R1 and Fc portion of human IgG₁. As indicated in Figure 4.5, the presence of sTNF-R1/Fc at its highest concentration (500 ng/ml) reversed the inhibitory effects of both LPS and exogenous TNF- α in our system, suggesting its ability to bind TNF- α in culture medium. Interestingly, when monomeric sTNF-R1 was used no significant reversal of the inhibitory effects of LPS and TNF- α on phagocytosis was observed (Figure 4.7). A few possible explanations behind this observation are presented in the following paragraphs.

The sTNF-R1/Fc, in contrast to sTNF-R1, contains the Fc portion of human IgG₁. Therefore, one may speculate that the augmentation of phagocytosis observed upon addition of sTNF-R1/Fc to LPS treated M ϕ may result from induction of Fc-mediated phagocytosis. This process occurs when phagocytic targets are opsonised with IgG antibodies, which Fc portions are recognised by Fc receptors on the surface of M ϕ , e.g., phagocytosis of immune-complexes (IC) (Hart *et al.*, 2004a). A couple of control experiments have been performed to investigate if in our study Fc-mediated phagocytosis plays an important role in the reversal of LPS- and TNF- α - induced inhibitory effects. As shown in Figure 4.6, I was able to demonstrate that sTNF-R1/Fc, in contrast to ICs, does not have the ability to opsonise human apoptotic neutrophils. In addition, sTNF-R1/Fc reversed inhibitory effects of LPS providing it was added concurrently with LPS. Even though opsonisation is a very quick process, 30-60 minutes incubation of cells with sTNF-R1/Fc at the end of the culture or during the phagocytosis assay was not sufficient to achieve augmentation of phagocytosis. Taken together, the above findings clearly suggest that sTNF-R1/Fc reduces the biological activity of TNF- α in our system and that this effect is primarily implicated in the reversal of the inhibitory effects of LPS and TNF- α on phagocytosis.

The sTNF-R1/Fc used in this thesis is composed of two subunits of sTNF-R1. This is in contrast to sTNF-R1, which is a monomeric molecule. This difference may contribute, at least in part, to the much greater ability of sTNF-R1/Fc to bind TNF- α and reverse inhibitory effects of LPS on phagocytosis. Naturally occurring TNF-Rs are type I membrane receptors, which are characterised by one to six extracellular cysteine-rich domains (CRDs) (Chan, 2007). These domains and especially the membrane-distal first CRD, termed pre-ligand assembly domain (PLAD), have been

demonstrated to play an important role in the formation of homotypic, ligand-independent receptor complexes. For instance, the unliganded structure of TNF-R1 was demonstrated to be a dimer (Naismith *et al.*, 1996). Therefore, the structure of sTNF-R1/Fc used in this study is similar in its structure to the naturally occurring membrane-bound dimeric receptors. The latter may explain the high affinity of sTNF-R1/Fc to TNF- α . Furthermore, several studies reported that monomeric TNF-Rs, in comparison to its dimeric forms, have severely compromised ability to bind ligands (Scallon *et al.*, 1995b; Mohler *et al.*, 1993). Furthermore, linkage of the Fc portion of IgG to the sTNF-R has been shown to change its pharmacokinetic parameters and significantly prolong its serum half-life. The latter likely contribute to the greater efficacy in neutralising TNF- α (Jacobs *et al.*, 1993).

Another interesting difference between sTNF-R1 and sTNF-R1/Fc used in this PhD thesis is their effects on control M ϕ . As demonstrated in Figure 4.5 and 4.7 greater concentrations of monomeric sTNF-R1 reduced ability of M ϕ to phagocytose apoptotic neutrophils whereas sTNF-R1/Fc did not exert such an effect. Interestingly, the study by Mohler and colleagues reports that monomeric sTNF-R2 or low concentrations of sTNF-R2/Fc have agonistic effects on release of TNF- α (Mohler *et al.*, 1993), which could potentially explain the reduction in phagocytosis levels in our control samples. It is possible that in the case of high concentrations of sTNF-R1 two independent albeit opposing processes take place, induction of TNF- α release and binding of released TNF- α . Since LPS *per se* is a strong inducer of TNF- α production and therefore greatly inhibits phagocytosis, it is likely that in LPS treated samples sTNF-R1 does not further augment TNF- α release and the inhibitory effect of sTNF-R1 on phagocytosis does not occur. However, the concentrations and bioactivity of TNF- α in culture supernatants have not been measured and I was not able to draw any final conclusions with this regard.

In summary, my findings suggest that TNF- α is a primary mediator involved in the inhibition of phagocytosis by LPS. I was able to demonstrate the successful reversal of LPS- or TNF- α -induced inhibition of phagocytosis with the use of sTNF-R1/Fc but not sTNF R1. This is in agreement with previously published data suggesting that dimeric sTNF-Rs are more powerful inhibitors of TNF- α than monomeric sTNF-Rs. TNF- α is not only a ‘master regulator’ of pro-inflammatory

cytokine production (Maini *et al.*, 1995) but also regulates many inflammatory processes such as inflammatory cell activation and recruitment and, thus, is likely a key molecule involved in development of chronic inflammation (Clark, 2007). Consequently, TNF- α inhibitors such as infliximab (mouse-human chimeric antibody), adalimumab (human monoclonal antibody) or etanercept (TNF receptor II-IgG fusion protein) have been shown to have therapeutic benefit in treatment of chronic inflammatory conditions (Feldmann, 1996; Feldmann & Maini, 2003). Etanercept is a bivalent receptor that binds TNF- α with 50-1000 times higher affinity to TNF- α than monomeric sTNF-Rs and linkage of Fc portion of IgG significantly prolongs its half-life (Mazzon & Cuzzocrea, 2008). So far, it has not been fully understood why binding and deactivation of TNF- α at inflamed tissues leads to resolution of inflammation. Here, evidence is given that excess TNF- α in inflamed tissue may result in insufficient clearance of apoptotic neutrophils and potentially other apoptotic cells and that neutralisation of TNF- α by means of TNF- α inhibitors rescues phagocytosis. As shown in Figure 4.5, sTNF-R1/Fc reduces the biological activity of TNF- α and reverses the inhibitory effects of pro-inflammatory stimuli (LPS) on efferocytosis. Moreover, one study demonstrates that binding of mTNF- α on the cell surface induces antibody-dependent and complement-dependent cytotoxicity and results in lysis of cell expressing TNF- α (Scallon *et al.*, 1995a). The latter may provide an additional mechanism contributing to the therapeutic effects of TNF- α inhibitors.

The efficacy of TNF- α inhibitors (infliximab, adalimumab or etanercept), showed that the blockade of TNF- α was a successful approach for the control of certain chronic inflammatory conditions. Yet, their major disadvantage is the intravenous way of administration. There is growing interest in development of selective, orally administered, small molecule inhibitors of TNF- α , namely TACE inhibitors. As described in more detail in section 1.2.2, TNF- α is produced as a membrane-bound precursor, which is then proteolytically cleaved by TACE into soluble TNF- α . Inhibition of TACE would, therefore, reduce the amount of soluble TNF- α released from stimulated cells. In this PhD thesis, a pilot experiment was performed, in which I sought to establish if the TACE inhibitor, TOPI-0, can interfere with LPS-induced inhibition of M ϕ phagocytosis. As shown in Figure 4.8, when

samples were treated with increasing concentrations of TOPI-0 and LPS I could observe a trend towards increased phagocytosis, yet, it was not significant in comparison to controls. Interestingly, when the highest concentrations of TOPI-0 were added to either control or TNF- α treated samples, I observed a small, non-significant decrease in the phagocytosis levels. These findings could be explained, at least in part, by the fact that TOPI-0 is a broad spectrum inhibitor of matrix metalloproteinases (MMP) and blocks the activity of several MMP at the same time. One may therefore speculate that in unstimulated or TNF- α -treated samples TOPI-0 may inhibit phagocytosis by blocking enzymes other than TACE. Upon LPS-treatment, when M ϕ produce TNF- α , TOPI-0 counteracts this process by blocking TACE activity and consequently augments phagocytosis. The latter effect is, however, masked by the concurrent ability of TOPI-0 to reduce phagocytosis.

In addition, several studies provide data that could potentially explain why, in this PhD thesis, TOPI-0 did not significantly reverse inhibitory effects of LPS on phagocytosis. For instance, Williams and colleagues have demonstrated that TACE inhibitor BB-2275 not only inhibits formation of the soluble TNF- α but also shedding of TNF-R1 and TNF-R2 from the cell membrane (Williams *et al.*, 1996). As a consequence, higher levels of membrane-bound TNF- α (mTNF- α) and TNF-Rs (mTNF-R) accumulated on the surface of cells. Interestingly, upon treatment with BB-2275, cells expressed higher surface levels of mTNF-R and were more sensitive to the cytotoxic effect of sTNF- α . Furthermore, mTNF- α was able to signal through mTNF-R and induce production of downstream cytokines, i.e., IL-1, IL-6 and IL-8. The above findings clearly suggest that although the studied TACE inhibitor, BB-2275, is able to reduce the release of sTNF- α from cells, it may not necessarily block TNF- α signalling and therefore exert some unwanted pro-inflammatory effects. This is of particular interest since exposure to LPS has been reported to induce internalisation of mTNF-Rs in several cell types, i.e., the mouse M ϕ -like cell line RAW 264.7 or primary human or murine M ϕ (Ding *et al.*, 1989). It is possible that in this PhD thesis, when M ϕ are treated with LPS and TOPI-0 two opposing processes are taking place; TOPI-0 blocks TNF- α release but also increase the levels of mTNF-Rs that would normally be decreased by LPS treatment alone. On the contrary, Dekkers and colleagues have demonstrated that, in humans, oral administration of

MMP inhibitor, GI5402, followed by a bolus intravenous injection of LPS, reduced the plasma concentrations of sTNF- α and sTNF-Rs but did not increase the levels of mTNF- α and mTNF-Rs in comparison to LPS treatment alone (Dekkers *et al.*, 1999). This observation indicates that the alterations in the release of sTNF-Rs and levels of mTNF-Rs upon MMP inhibitor treatment are not directly correlated phenomena. Furthermore, the 'net' effect of MMP inhibitors on TNF- α and its receptors may vary depending on experimental settings (*in vivo* or *ex vivo*) or differences in modes or spectrum of action of particular agents. Therefore, in order to draw a final conclusion with regard to the effects of TOPI-0 on the LPS-induced inhibition phagocytosis further work should be performed including measurement of concentrations of both soluble (culture supernatants) and M ϕ membrane-bound TNF- α and TNF Rs.

In summary, data presented in chapter 4 show that the inhibitory effect of LPS on phagocytosis is dependent upon TNF- α release and is positively correlated with the concentration of TNF- α in culture medium just prior to the phagocytosis assay. Reduction of TNF- α bioactivity in culture supernatants with sTNF-R1/Fc significantly augmented phagocytosis levels. Further experiments with sTNF-R1 confirmed findings by others demonstrating that sTNF-R1 is a much weaker inhibitor of TNF- α than sTNF-R1/Fc. In addition sTNF-R1 appears to exert some inhibitory effect on phagocytosis itself. Similarly, the TACE inhibitor, TOPI-0, did not significantly reverse LPS-induced inhibition and reduced phagocytosis levels when M ϕ were treated with greater concentrations of TOPI-0 alone. Further work should be performed in order to establish changes in soluble and membrane-bound TNF- α and TNF-Rs upon treatment with sTNF-R1 and TOPI-0 to provide more insight into my observation. Even though an important role for TNF- α in the regulation of M ϕ ability to phagocytose apoptotic neutrophils has been demonstrated, it still remains to be established why this effect occurred after at least 96 hours incubation with LPS. In order to shed more light into this phenomenon I performed the experiments presented in chapter 5.

CHAPTER FIVE

**ANTI- AND PRO-
INFLAMMATORY BIOLOGICS
EXERT OPPOSING ACTIONS ON
M Φ CLEARANCE OF
APOPTOTIC NEUTROPHILS**

5 ANTI- AND PRO-INFLAMMATORY BIOLOGICS EXERT OPPOSING ACTIONS ON M ϕ CLEARANCE OF APOPTOTIC NEUTROPHILS

5.1 INTRODUCTION

Data presented in chapter 4 demonstrate that LPS-induced inhibition of efferocytosis by MDM ϕ is, at least in part, mediated by the release of TNF- α . First of all, the extent to which LPS inhibits phagocytosis was correlated with the levels of TNF- α in culture medium. Second, depletion of biologically-active TNF- α in culture medium with the use of sTNF-R1/Fc rescued phagocytosis. Interestingly, when M ϕ were treated with LPS the inhibitory effect of TNF- α was observed only if treatment was performed for a duration of 96 hours or longer. Since LPS is a general activator of M ϕ we hypothesised that upon LPS treatment, MDM ϕ produce/release mediators that either interfere with TNF- α production or negate TNF- α function.

Numerous reports throughout the literature demonstrate an important role of cytokines with anti-inflammatory properties (e.g., IL-10, IL-4 or TGF- β) as negative regulators of TNF- α production. For instance, the study by Ebert investigated the release of TNF- α by resting T-cells and monocytes in the presence of antibodies neutralising IL-10, IL-4 or TGF- β and demonstrated elevated release of TNF- α in such conditions (Ebert, 2005). Interestingly, the authors of this study reported that when IL-4 or TGF- β were neutralised with appropriate antibodies, elevated levels of IL-10 were produced by monocytes, providing an additional protection against the release of exceptionally high amounts of TNF- α by these cells. The importance of IL-10 as an endogenous regulator of TNF- α is further supported by the findings that, in humans, subjects deficient in serum IL-10 levels are more prone to develop chronic inflammatory diseases, e.g., ulcerative colitis (Tagore *et al.*, 1999; Ebert *et al.*, 2009). IL-10 has been also reported to augment phagocytosis of apoptotic cells by M ϕ

(Ogden *et al.*, 2005; Xu *et al.*, 2006). In experiments presented in chapter 4 I correlated TNF- α and IL-10 concentrations with phagocytosis levels upon LPS treatment. As indicated in Figure 4.4, high levels of phagocytosis were correlated with low concentrations of TNF- α and, interestingly, elevated concentrations of IL-10. We hypothesised that IL-10 might block TNF- α production or release and therefore interfere with inhibitory effect of LPS in our system.

In this chapter, I aimed to establish whether production/release of IL-10 by LPS-activated MDM ϕ delayed the inhibitory effects of LPS on phagocytosis of apoptotic neutrophils. For this purpose concentrations of TNF- α and IL-10 in MDM ϕ culture supernatants were measured during LPS stimulation. Next, I investigated the effects of IL-10 on LPS-induced inhibition of phagocytosis by using either exogenous IL-10, IL-10 receptor blocking antibodies (anti-IL-10-R1Ab) or by comparing phagocytosis by wt and IL-10^{-/-} murine BMDM ϕ . In addition, I compared the effects of IL-10 to those of other anti-inflammatory agents (i.e., glucocorticoids and NSAID).

5.2 METHODS

5.2.1 ISOLATION AND CULTURE OF HUMAN BLOOD CELLS

Human blood cells were isolated by Percoll™ gradient centrifugation, following the protocol described in section 2.2.1.1. Subsequently, human monocytes were cultured for 6 days in culture medium (see section 2.2.1.3 for culture conditions) undergoing differentiation into mature human MDM ϕ . During this time MDM ϕ were treated with a number of pro- and anti- inflammatory agents. In experiments described in this chapter human monocytes were treated with LPS using a range of concentrations: 0.01–100 ng/ml (6, 24, 48, 72, 96, 120 and 144 hours), both LTA (100 μ g/ml) and PG (10 μ g/ml) for 1, 6, 16, 32, 48, 72 and 96 hours, TNF- α using a range of concentrations: 0.1–100 ng/ml (72, 120 hours), anti-IL-10-R1Ab at a concentration 10 μ g/ml (treatment was added every 48 hours), IL-1 β at a concentration of 10 ng/ml (120 hours), IL-10 at a range of concentrations: 0.1–100 ng/ml (72, 120 hours), 1 μ M dexamethasone using a range of concentrations: 0.001–1 μ M (96-144 hours), aspirin, ibuprofen, paracetamol using a range of concentrations: 10–1200 μ M, indomethacin using a range of concentrations: 10–100 μ M, SC-650 and NS-398 using a range of concentrations 2.5–250 μ M for 48-72 hours. On day 6, MDM ϕ were assessed for their ability to phagocytose apoptotic neutrophils that had undergone constitutive apoptosis for 20 hours of culture (see section 2.2.1.3 for culture conditions).

5.2.2 ISOLATION AND CULTURE OF MOUSE BONE MARROW CELLS

In experiments described in section 5.3.4, efferocytosis by wt and IL-10^{-/-} murine BMDMφ have been compared, for control and pro-inflammatory conditions. Bone marrow cells were isolated from mouse femurs, cultured in Teflon pots for 4 days and then transferred to 48-well plates and cultured for the next 4 days as described in section 2.2.2. BMDMφ were treated for 24 or 48 hours with 10 ng/ml LPS or 10 ng/ml mouse recombinant TNF-α. On the last day of culture BMDMφ were assessed for their ability to phagocytose human apoptotic neutrophils that had undergone constitutive apoptosis for 20 hours of culture (see section 2.2.1.3 for culture conditions).

5.2.3 ASSESSMENT OF APOPTOSIS AND NECROSIS

Prior to the phagocytosis assay, the % of apoptotic cells within neutrophil populations was assessed by morphology (see section 2.3.1 for experimental protocol) or alternatively by Annexin-V binding assessed by flow cytometry (see section 2.3.2 for experimental protocol). The % of necrotic cells was assessed by PI staining as described in section 2.3.2. Only neutrophils with less than 5 % of necrosis were used in phagocytosis assay.

5.2.4 ASSESSMENT OF PHAGOCYTOSIS BY FLOW CYTOMETRY

5.2.4.1 HUMAN MDMφ

In all the experiments described in this chapter efferocytosis was assessed by flow cytometric analysis that employs labelling of phagocytic targets with FL1⁺ Cell Tracker™ Green (CMFDA) prior to the phagocytosis assay. Following the phagocytosis assay the % of FL1⁺ MDMφ (MDMφ that had engulfed phagocytic

targets) within the entire MDM ϕ population was assessed by flow cytometry as described in chapter 2.4.1.1.

5.2.4.2 MURINE BMDM ϕ

Efferocytosis by BMDM ϕ was assessed by 2 colour flow cytometric assay as described in chapter 2.4.2. Human apoptotic neutrophils, used as phagocytic targets, overlap with mouse BMDM ϕ when plotted SS against FS. Therefore two fluorescent labels were used: Cell Tracker™ Far Red (for labelling of BMDM, FL-4+) and Cell Tracker™ Green (for labelling of human neutrophils, FL-1+). This allowed the gating of BMDM ϕ as a population of FL-4+ positive cells and then assessing the percentage of the FL-1+ cells within entire FL-4+ population. Only BMDM ϕ that were both FL-4+ and FL-1+ positive were deemed to have engulfed apoptotic neutrophils.

5.2.5 MEASUREMENT OF TNF- α AND IL-10 CONCENTRATIONS IN CULTURE SUPERNATANTS

In experiments described in this chapter I assessed changes in TNF- α and IL-10 levels at different time points after LPS, LTA and PGN stimulation (see sections 5.3.1 and 5.3.2) and examined the effect of exogenous IL-10 (see sections 5.3.3) and dexamethasone (see sections 5.3.6) on TNF- α production by LPS-stimulated MDM ϕ . In all these experiments, culture supernatants were collected and processed as described in section 2.5.2. Cytokine concentrations were measured by specific ELISA following the manufacturer's instruction (see section 2.5.2 for more details).

5.3 RESULTS

5.3.1 CHARACTERISATION OF TNF- α AND IL-10 PRODUCTION BY MDM ϕ STIMULATED WITH LPS

Experiments in chapter 4 show that the % of MDM ϕ ingesting apoptotic neutrophils was negatively correlated with the concentration of TNF- α in the supernatant ($R^2 = 0.84$, Figure 4.3). I also noted that culture supernatants containing low concentrations of TNF- α were characterised by increased levels of IL-10 (Figure 4.4). Interestingly, IL-10 was previously reported to augment uptake of apoptotic cells by M ϕ (Ogden *et al.*, 2005; Xu *et al.*, 2006). In this PhD thesis, one of the most striking characteristic of LPS-induced inhibition of phagocytosis is significant delay in comparison to TNF- α -induced inhibition. Therefore, I sought to investigate whether LPS-stimulated MDM ϕ produce IL-10 and if this cytokine interferes with the LPS inhibitory effect upon efferocytosis. To answer these questions I first aimed to measure the production of TNF- α and IL-10 by MDM ϕ in response to LPS treatment. For this purpose human blood monocytes were cultured for 6 days and LPS (10 ng/ml) was added at a number of time points: 1, 6, 12, 24, 48, 72, 96 and 120 hours before the phagocytosis assay. On day 6, culture supernatants were collected and TNF- α and IL-10 levels measured by ELISA. Figure 5.1 shows changes in concentrations of TNF- α and IL-10 throughout the culture. The first significant increase in TNF- α levels appears at the 6 hour time point (18.1 ± 7.2 ng/ml in comparison to the 1 hour time point 0.4 ± 0.3 ng/ml, $p < 0.01$, $n = 7$), followed by a steady decrease. TNF- α concentration markedly rises again at the 96 hour time point (16.8 ± 7.5 ng/ml in comparison to 1 hour control, $p < 0.05$, $n = 7$). I also found significant elevation in IL-10 levels at the 12 hour time point (15.3 ± 3.1 ng/ml in comparison to 1 hour control 0.01 ± 0.004 ng/ml, $p < 0.01$, $n = 7$) with maximum concentrations at 24 hours (21.4 ± 5.0 ng/ml in comparison to 1 hour control 0.01 ± 0.004 ng/ml, $p < 0.01$, $n = 7$), followed by a steady decline (3.9 ± 1.4 ng/ml at 120 hour time point). As shown in Figure 5.1A, the increases in the IL-10 levels are associated with decreases in TNF- α levels and vice versa. In addition, I measured

concentrations of TNF- α and IL-10 following 96 hours treatment with increasing concentrations of LPS. As indicated in Figure 5.1B TNF- α concentrations are positively correlated with LPS concentrations and rise significantly from 0.09 ± 0.07 ng/ml (medium control) to 5.2 ± 1.5 ng/ml for 10 ng/ml LPS ($p < 0.05$, $n=4$) or to 6.0 ± 2.7 for 100 ng/ml LPS ($p < 0.01$, $n=4$). The increases in TNF- α concentration are also associated with low levels of IL-10. It is noteworthy that in the latter experiment LPS (1-100 ng/ml) significantly inhibited MDM ϕ ability to phagocytose apoptotic neutrophils in comparison to control (data not shown).

FIGURE 5.1 Production of TNF- α and IL-10 by LPS stimulated MDM ϕ (next page). Human MDM ϕ were cultured for 6 days and treated for increasing durations of time with 10 ng/ml LPS (**A**) or treated for 96 hours with increasing concentrations of LPS (**B**). On day 6, culture supernatants were collected and TNF- α and IL-10 concentrations were measured by ELISA. Concentrations of TNF- α and IL-10 are expressed as the mean concentration \pm SEM for $n=7$ (**A**) or $n=4$ (**B**) experiments. Significant differences for comparisons to respective concentrations at 1 hour time-point (**A**) or to samples not treated with LPS (**B**) are represented by * $p < 0.05$ and ** $p < 0.01$.

5.3.2 CHARACTERISATION OF TNF- α AND IL-10 PRODUCTION BY MDM ϕ STIMULATED WITH LTA OR PGN

Experiments described in chapter 3 (sections 3.3.2 and 3.3.3) showed that inhibitory effects of LTA and PGN on efferocytosis depend on the duration of treatment. Upon treatment with either LTA or PGN reduction in efferocytosis levels occurred quicker (16 hours after treatment) than upon LPS treatment. I therefore aimed to establish if the timing of TNF- α or IL-10 production/release by LTA- or PGN-stimulated MDM ϕ is different from that obtained for LPS. For this purpose human blood monocytes were cultured for 6 days and either 100 μ g/ml LTA or 10 μ g/ml PGN were added at several time points: 1, 6, 16, 32, 48, 72, 96 hours before assessing phagocytosis. On day 6, culture supernatants were collected and TNF- α and IL-10 levels were measured by ELISA.

Figure 5.2A shows changes in concentrations of TNF- α and IL-10 throughout the culture upon treatment with 100 μ g/ml LTA. The first significant increase in TNF- α levels appears at the 6 hour time point (6.2 ± 0.6 ng/ml in comparison to 1 hour time point 0.025 ± 0.008 ng/ml; $p < 0.01$, $n = 4$), and further increases at 32 hours up to 7.2 ± 1.6 ng/ml ($p < 0.01$, $n = 4$, in comparison to 1 hour control) and then markedly decreases at the 48 hour time point (0.9 ± 0.1 ng/ml). Levels of TNF- α do not increase again at later time points of the culture. I also found significant and maximal elevation in IL-10 concentrations at 16 hours after LTA treatment (6.5 ± 2.5 ng/ml in comparison to 1 hour control 0.10 ± 0.04 ng/ml, $p < 0.05$, $n = 4$), followed by a steady decline. Similar to TNF- α , levels of IL-10 do not rise again at later time points of the culture.

Figure 5.2B shows changes in concentrations of TNF- α and IL-10 throughout the culture upon treatment with 10 μ g/ml PGN. The first significant increase in TNF- α levels appears at the 6 hour time point (12.7 \pm 1.7 ng/ml in comparison to 1 hour time point 0.035 \pm 0.002 ng/ml; p <0.01, n =4), and further increases at the 16 hour time point up to 13.4 \pm 1.6 ng/ml (p <0.01, n = 4 in comparison to 1 hour control) and then declines and remains low at 32, 48 and 72 hours after PGN treatment. However, levels of TNF- α rise again at the end of the culture at 96 hour time point up to 10.9 \pm 1.9 ng/ml (p <0.01, n =4 in comparison to 1 hour control). Significant elevation in IL-10 concentration was identified at 16 hour time point after PGN treatment (6.4 \pm 0.8 ng/ml in comparison to 1 hour control 0.09 \pm 0.02 ng/ml, p <0.01, n =4), followed by further increase at the 32 hour time point up to 9.1 \pm 1.7 ng/ml (p <0.01, n =4 in comparison to 1 hour control) and decrease at later time points.

Figure 5.2 Production of TNF- α and IL-10 by LTA or PGN stimulated MDM ϕ .

Human MDM ϕ were cultured for 6 days and treated for increasing durations of time with 100 μ g/ml LTA (**A**) or 10 μ g/ml PGN (**B**). On day 6, culture supernatants were collected and TNF- α and IL-10 concentrations were measured by ELISA. Concentrations of TNF- α and IL-10 are expressed as the mean concentration \pm SEM for n=4 experiments. Significant differences for comparisons to respective concentrations at 1 hour time point are represented by *p<0.05 and **p<0.01.

5.3.3 PRESENCE OF EXOGENOUS IL-10 INHIBITS TNF- α PRODUCTION AND RESCUES INHIBITION OF MDM ϕ EFFEROCYTOSIS INDUCED BY LPS

Measurement of TNF- α and IL-10 concentrations in culture supernatants upon MDM ϕ treatment with either LPS, LTA or PGN revealed intriguing interplay between them. As described in sections 5.3.1 and 5.3.2, TNF- α was produced at early time points and reached maximum concentration approximately 6 hours after treatment, whereas the first peak of IL-10 levels occurred later on, approximately 12-16 hours after treatment. The increase in IL-10 levels was accompanied by decrease in TNF- α concentration and either subsequent decrease in IL-10 levels (LPS and PGN treatment) or alternatively reductions in TNF- α and IL-10 levels were synchronised (LTA treatment). Thus, I hypothesised that, in our system, IL-10 is a negative regulator of TNF- α production by MDM ϕ exposed to pro-inflammatory stimuli such as LPS. To investigate this further, human blood monocytes were differentiated for 6 days into MDM ϕ . MDM ϕ were cultured in medium (control) or treated with either 10 ng/ml LPS (LPS control) or 10 ng/ml TNF- α (TNF- α control). Increasing concentrations of IL-10 (0.1-100 ng/ml) were added to appropriate samples, as indicated in Figure 5.3A. Treatment was performed for the duration of 120 hours and efferocytosis was assessed by flow cytometry. IL-10 alone significantly increased MDM ϕ uptake of apoptotic cells from 24.0 ± 3.17 % (medium control) to 37.9 ± 2.9 % at a concentration of 100 ng/ml ($p < 0.05$; $n = 5$). Furthermore, IL-10 rescued efferocytosis in LPS treated MDM ϕ in a concentration-dependent manner but not in TNF- α (10 ng/ml) treated MDM ϕ . Upon LPS treatment, efferocytosis was decreased to 10.7 ± 2.1 % but addition of IL-10 at concentrations 10 ng/ml or 100 ng/ml increased phagocytosis in LPS treated MDM ϕ up to 36.1 ± 4.3 % ($p < 0.001$; $n = 5$) and 36.0 ± 7.4 % ($p < 0.001$; $n = 5$) respectively. It is noteworthy that these levels of efferocytosis are similar to those represented by MDM ϕ treated with 10 ng/ml or 100 ng/ml IL-10 alone. To further verify that IL-10 indeed inhibited production of TNF- α in LPS treated (120 hours) MDM ϕ , I measured TNF- α concentrations in MDM ϕ culture supernatants. As demonstrated in Figure 5.3B, IL-10 reduced production of TNF- α by LPS-treated MDM ϕ in a concentration-dependent manner. IL-10 (0.1

ng/ml and 1 ng/ml) significantly decreased TNF- α production by MDM ϕ from 22.0 \pm 4.8 ng/ml for LPS alone to 11.5 \pm 3.5 ng/ml (p <0.05, n =4) and 3.6 \pm 0.9 ng/ml (p <0.001, n =4) respectively. However, as shown in Figures 3.3A and 5.3A these levels of TNF- α are still capable of inhibiting efferocytosis. Higher concentrations of IL-10, 10 and 100 ng/ml, further decreased TNF- α concentrations to 1.6 \pm 0.5 ng/ml (p <0.001, n =4) and 1.1 \pm 0.2 ng/ml (p <0.001, n =4) respectively and effectively reversed LPS-mediated inhibition of phagocytosis.

Since the concentration response for TNF- α (Figure 3.3A) clearly indicates that TNF- α , at 1 ng/ml, significantly inhibits uptake of apoptotic neutrophils, I examined this phenomenon further. Human monocytes were cultured for 6 days and treated for 72 hours with increasing concentrations of TNF- α alone or with addition of IL-10 (100 ng/ml). As indicated in Figure 5.4, IL-10 at this concentration does not override the effect of higher concentrations of TNF- α (10 and 100 ng/ml), but did significantly reverse the inhibition of efferocytosis caused by 1 ng/ml TNF- α (p <0.01, n = 4). Note that control (no treatment) samples and samples co-treated with 1 ng/ml TNF- α and 100 ng/ml IL-10 are undistinguishable in terms of phagocytosis levels.

FIGURE 5.3 Presence of exogenous IL-10 reverses the inhibition of efferocytosis and production of TNF- α induced by LPS. Human MDM ϕ were cultured for 6 days in culture medium alone or treated for 120 hours with either 10 ng/ml LPS or 10 ng/ml TNF- α alone +/- increasing concentrations of IL-10. **A.** Efferocytosis was assessed on day 6 by flow cytometry. Data shown as mean \pm SEM for n=5 separate experiments (*p<0.05 compared with medium control and ***p<0.001 compared with samples treated only with LPS). **B.** Culture supernatants were collected on day 6 just before efferocytosis assay and TNF- α concentration measured by ELISA. Data shown as mean concentration \pm SEM for n=4 separate experiments (*p<0.05 ***p<0.001 compared with samples treated only with LPS).

FIGURE 5.4 IL-10 masks inhibitory action of low concentrations of TNF- α . Human MDM ϕ were cultured for 6 days and treated for 72 hours with increasing concentrations of TNF- α alone or with addition of 100 ng/ml IL-10. Efferocytosis was assessed on day 6 by flow cytometry. Data shown as mean \pm SEM for n=5 separate experiments. Significant differences for comparisons to medium control are represented by *p<0.05 and **p<0.01.

5.3.4 PRESENCE OF MONOCLONAL ANTI-HUMAN IL-10-R1 ANTIBODY AUGMENTS THE INHIBITORY EFFECT OF LPS

Having found that the presence of exogenous IL-10 rescues efferocytosis in LPS-treated MDM ϕ and that this effect is associated with a reduction in TNF- α concentration, I next sought to investigate if endogenous production of IL-10 upon LPS stimulation is responsible for the observed delay of LPS inhibitory effect. For this purpose MDM ϕ were treated with LPS and the biological activity of endogenously produced IL-10 was blocked with the use of anti-human IL-10-R1 antibody (anti-IL-10-R1Ab). Human blood monocytes were differentiated for 6 days and cultured with 10 ng/ml LPS either alone or in the presence of 10 μ g/ml anti-IL-10-R1Ab for different durations of time: 24, 48, 72 and 96 hours. Efferocytosis was assessed on day 6 by flow cytometry. Interestingly, in the presence of anti-IL-10-R1Ab, LPS treatment causes significant inhibition at the 24 hour time point ($24.2\pm 3.3\%$ in comparison to control level $34.9\pm 4.9\%$, $p < 0.05$, $n=8$, Figure 5.5) and this effect was maintained at 48, 72 and 96 hours after treatment. Concurring with data presented in Figure 3.2B samples treated with LPS alone showed decrease in efferocytosis only after 96 hours of treatment ($24.2\pm 3.3\%$, $p < 0.05$, $n=8$, in comparison to medium control, Figure 5.5). Moreover, at the 96 hour time point, LPS induced much greater inhibition in the presence of anti-IL-10-R1Ab ($14.2\pm 2.5\%$, $p < 0.001$, $n=8$, in comparison to medium control, Figure 5.5) than when used alone.

Figure 5.5 IL-10 produced by LPS stimulated MDM ϕ delays LPS-induced inhibition of efferocytosis. Human MDM ϕ were cultured for 6 days and treated for 24, 48, 72 and 96 hours with 10 ng/ml LPS alone or with addition of 10 μ g/ml anti-IL-10-R1Ab. Efferocytosis was assessed on day 6 by flow cytometry. Data are expressed as mean \pm SEM of n=8 separate experiments. Significant differences for comparisons to medium control are represented by *p<0.05, **p<0.01 and ***p<0.001.

5.3.5 BMDM ϕ FROM IL-10 $-/-$ MICE ARE CHARACTERISED BY LOWER CAPACITY FOR PHAGOCYTOSIS IN COMPARISON TO BMDM ϕ FROM WILD TYPE MICE

Having identified the importance of endogenous IL-10 production on protection of MDM ϕ ability to phagocytose apoptotic neutrophils I investigated this phenomenon further. I compared efferocytosis by wt and IL-10 deficient murine BMDM ϕ in control and pro-inflammatory conditions. Bone marrow cells were isolated from wt and IL-10 $^{-/-}$ mice, differentiated them into BMDM ϕ for 8 days and treated with LPS or TNF- α for 24 or 48 hours prior to phagocytosis assay. Phagocytosis was assessed in a similar way as in the experiments using MDM ϕ and human apoptotic neutrophils were used as phagocytic targets. The obtained data clearly show that IL-10 deficient BMDM ϕ have a much lower ability to phagocytose apoptotic neutrophils in comparison to wt BMDM ϕ . As indicated in Table 5.1 decrease in efferocytosis levels was observed for all experimental conditions and % of inhibition varied from 37.3 \pm 5.9 % for 24 hours treatment with LPS up to 75.7 \pm 9.4 % for 48 hours treatment with TNF- α .

TABLE 5.1 Comparison of efferocytosis by wt and IL-10^{-/-} BMDMφ.

<i>Treatment and genotype</i>	<i>%Phagocytosis¹</i>	<i>% Inhibition²</i>
<i>Control wt</i>	<i>36.3 ± 1.4</i>	
<i>Control IL-10^{-/-}</i>	<i>17.3 ± 3.3</i>	<i>52.6 ± 8.4</i>
<i>LPS 24 wt</i>	<i>24.4 ± 4.3</i>	
<i>LPS24 IL-10^{-/-}</i>	<i>15.0 ± 2.2</i>	<i>37.3 ± 5.9</i>
<i>LPS 48 wt</i>	<i>4.5 ± 2.1</i>	
<i>LPS 48 IL-10^{-/-}</i>	<i>2.1 ± 0.9</i>	<i>50.2 ± 3.0</i>
<i>TNF-α 24 wt</i>	<i>23.3 ± 2.4</i>	
<i>TNF-α 24 IL-10^{-/-}</i>	<i>12.0 ± 0.7</i>	<i>47.4 ± 5.5</i>
<i>TNF-α 48 wt</i>	<i>7.5 ± 3.0</i>	
<i>TNF-α 48 IL-10^{-/-}</i>	<i>1.9 ± 0.5</i>	<i>75.7 ± 9.4</i>

Phagocytosis by BMDMφ was assessed by flow cytometry. Data are expressed as ¹ mean % efferocytosis±SEM, ² mean % inhibition of efferocytosis±SEM, where corresponding wt and IL-10^{-/-} treatments were compared. Three animals / group were used.

5.3.6 DEXAMETHASONE RESCUES THE ABILITY OF LPS AND TNF- α TREATED MDM ϕ TO PHAGOCYTOSE APOPTOTIC NEUTROPHILS

Glucocorticoids, including the synthetic glucocorticoid, dexamethasone, are recognised for their anti-inflammatory properties and ability to inhibit production of pro-inflammatory cytokines such as TNF- α (Barnes, 1998). Furthermore, they are reported to increase non-phlogistic phagocytosis of apoptotic cells (Heasman *et al.*, 2004; Giles *et al.*, 2001; Liu *et al.*, 1999). I therefore aimed to determine, whether dexamethasone was able to exert similar effects as IL-10 and rescue LPS-inhibited efferocytosis through inhibition of TNF- α production or reverse the inhibitory effect of TNF- α . To answer these questions human monocytes were treated for 120 hours with increasing concentrations of dexamethasone alone or in combination with either 10 ng/ml LPS or 10 ng/ml TNF- α . Dexamethasone, as expected, augmented efferocytosis in a concentration-dependent manner with the most prominent effect at 0.1 μ M concentration or higher. When MDM ϕ were pre-treated with LPS and dexamethasone (Figure 5.6A), efferocytosis levels were markedly increased from 22.1 \pm 0.8 % for LPS alone (control level=43.9 \pm 4.1 %) up to 61.8 \pm 3.5 % for combined LPS and 1 μ M dexamethasone (p <0.001; n =5). Levels of phagocytosis for treatment with 1 μ M dexamethasone alone were 72.6 \pm 2.8 %.

As indicated in Figure 5.6A dexamethasone also reversed the inhibitory effect of TNF- α on efferocytosis in a concentration-dependent fashion. With TNF- α treatment phagocytosis was inhibited (18.5 \pm 2.9 %) in comparison to control and was significantly enhanced by the presence of 1 μ M dexamethasone up to 68.7 \pm 2.7 % (p <0.001, n = 4).

Since I was able to demonstrate that the presence of dexamethasone reversed the inhibitory effects of LPS and TNF- α on efferocytosis of apoptotic neutrophils, I next sought to investigate if dexamethasone treatment alters production of TNF- α or IL-10 by MDM ϕ stimulated with LPS. For this purpose human blood monocytes were differentiated for 6 days and cultured in medium (control) or treated with either 1 μ M dexamethasone alone (DX control) or 10 ng/ml LPS alone or with both LPS and dexamethasone for 120 hours. Subsequently, culture supernatants were collected

and cytokine levels measured by CBA. The TNF- α and IL-10 levels in both controls were barely detectable (data not shown). However, as shown in Figure 5.6B, I found that dexamethasone did not change production of IL-10 but partially decreased production of TNF- α in LPS-stimulated MDM ϕ from 44.5 \pm 21.1 ng/ml to 14.5 \pm 10.5 ng/ml (p <0.05; n =5). This effect was accompanied by increases in efferocytosis, even though TNF- α at this concentration greatly inhibits MDM ϕ uptake of apoptotic neutrophils (Figure 3.3A).

FIGURE 5.6 Dexamethasone reverses the inhibitory effects of TNF α and LPS on efferocytosis (next page). **A.** Human MDM ϕ were cultured for 6 days and treated for 120 hours with increasing concentrations of dexamethasone alone or with addition of either 10 ng/ml LPS or 10 ng/ml TNF- α . Efferocytosis was assessed on day 6 by flow cytometry. Data are expressed as mean \pm SEM of n =4-8 separate experiments. Significant differences for comparisons to respectively medium, TNF- α or LPS control are represented by * p <0.05, ** p <0.01 and *** p <0.001. **B.** Human MDM ϕ were cultured for 120 hours in the presence of 10 ng/ml LPS alone or with addition of 1 μ M dexamethasone. After 120 hours supernatants were collected and TNF- α and IL-10 concentrations were measured by CBA. Data shown as mean concentration \pm SEM for n =5 separate experiments. Combined LPS and dexamethasone treatment at 120 hours significantly changed TNF- α . (* p <0.05) but not IL-10 levels as compared to treatment with LPS alone. **DX**-dexamethasone.

5.3.7 DEXAMETHASONE RESCUES THE ABILITY OF IL-1 β TREATED MDM ϕ TO PHAGOCYTOSE APOPTOTIC NEUTROPHILS

As described in chapter 3 (section 3.3.6) treatment of MDM ϕ with exogenous IL-1 β decreased their ability to phagocytose apoptotic neutrophils, similar to LPS or TNF- α treatments. I therefore sought to investigate if the presence of dexamethasone would have any effect on inhibition of efferocytosis by IL-1 β . For this purpose we cultured human blood monocytes for 6 days and treated them for 120 hours with either 10 ng/ml IL-1 β alone or with addition of 1 μ g/ml dexamethasone. Efferocytosis was assessed on day 6 by flow cytometry. Obtained results clearly showed that when MDM ϕ were treated with IL-1 β efferocytosis was reduced from 23.3 \pm 6.2 % (control) to 8.3 \pm 0.7 %. Dexamethasone significantly augmented uptake of apoptotic neutrophils in both groups up to 51.6 \pm 8.6 % for control samples (p <0.01; n =4; in comparison to medium control) and 57.4 \pm 7.4 % for IL-1 β treated samples (p <0.001; n =4; in comparison to IL-1 β treatment). It is noteworthy, that when MDM ϕ were pre-treated with IL-1 β augmentation of efferocytosis was even greater than for control samples (Figures 5.7).

FIGURE 5.7 Dexamethasone reverses the inhibitory effects of IL-1 β on efferocytosis. Human MDM ϕ were cultured for 6 days in culture medium or treated for 120 hours with 10 ng/ml IL-1 β +/- 1 μ M dexamethasone. Efferocytosis was assessed on day 6 by flow cytometry. Data are expressed as mean \pm SEM of n=4 separate experiments. Significant differences for comparisons to medium or IL-1 β control are represented by **p<0.01 and ***p<0.001. **DX**-dexamethasone.

5.3.8 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS DO NOT AUGMENT MDM ϕ PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS

Data described in chapter 5.3.6 demonstrate that dexamethasone, an anti-inflammatory steroid, not only augments MDM ϕ efferocytosis of apoptotic neutrophils *per se* but also reverses inhibitory effects of LPS and TNF- α upon this process. In contrast, this section focuses on non-steroidal anti-inflammatory drugs (NSAID) and their effects on MDM ϕ efferocytosis. These drugs act mainly through inhibition of COX-1 and COX-2 enzymes (i.e., aspirin, ibuprofen or indomethacin), therefore, for comparison, I also analysed effects of the selective COX-1 (SC-650) and the COX-2 (NS-398) inhibitors. Furthermore, I investigated the effect of paracetamol, an analgesic, which arguably blocks the COX-3 enzyme as described in detail in section 1.5.2.

In order to assess the effects of aspirin, ibuprofen, indomethacin, SC-650, NS-398 and paracetamol on MDM ϕ efferocytosis of apoptotic neutrophils we cultured human blood monocytes for 6 days and treated them for 48-72 hours with 1 μ M dexamethasone or increasing concentrations of aspirin (10, 100, 1000 μ M), ibuprofen (10, 100, 1000 μ M), paracetamol (10, 100, 1000 μ M), SC-650 (2.5, 25, 250 μ M) or NS-398 (2.5, 25, 250 μ M). Due to relatively high cell toxicity, indomethacin was used only at low concentrations (10, 50, 100 μ M). Efferocytosis was assessed on day 6 by flow cytometry. The control levels of efferocytosis in all experiments ranged between 27.7 \pm 2.5 % (Figure 5.8F) and 43.9 \pm 4.8 % (Figure 5.8A, B and C). Dexamethasone significantly augmented uptake of apoptotic neutrophils in all experiments and the levels of efferocytosis varied between 56.6 \pm 6.4 % (Figure 5.8E) and 67.6 \pm 5.4 % (Figure 5.8A, B and C) (p <0.001; n =4; in comparison to medium controls). All tested non-steroidal agents at lower concentrations did not exert any major effects on efferocytosis but significantly reduced this process when used at the highest concentrations. The levels of efferocytosis for treatment with 1000 μ M aspirin were 25.1 \pm 6.8 % (p <0.05; n =4; in comparison to medium control, Figure 5.8A), 1000 μ M ibuprofen were 19.1 \pm 5.6 % (p <0.001; n =4; in comparison to medium control, Figure 5.8B), 100 μ M indomethacin were 27.5 \pm 4.1 % (p <0.05; n =4;

in comparison to medium control, Figure 5.8C), 1000 μ M paracetamol were 28.1 ± 7.0 % ($p < 0.05$; $n = 4$; in comparison to medium control, Figure 5.8D), 250 μ M SC-650 were 6.0 ± 2.8 % ($p < 0.001$; $n = 4$; in comparison to medium control, Figure 5.8E) and 250 μ M NS-398 were 6.5 ± 1.4 % ($p < 0.001$; $n = 4$; in comparison to medium control, Figure 5.8F). Viability of MDM ϕ was confirmed just before the phagocytosis assay by staining with the vital dye-Trypan Blue and observation under light microscope. Percentage of distinctive blue cells (dead cells) within each tested population is demonstrated in Table 5.2.

FIGURE 5.8 (next pages) NSAID do not augment MDM ϕ efferocytosis of apoptotic neutrophils. Human MDM ϕ were cultured for 6 days in culture medium or treated for 48-72 hours with 1 μ M dexamethasone, increasing concentrations of NSAID (aspirin (A), ibuprofen (B), indomethacin (C), paracetamol (D)) or selective COX-1 inhibitor SC-650 (E) and selective COX-2 inhibitor NS-398 (F). Efferocytosis was assessed on day 6 by flow cytometry. Data are expressed as mean \pm SEM of $n = 4$ separate experiments. Significant differences for comparisons to medium control are represented by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. **DX-**dexamethasone.

TABLE 5.2 Viability of MDM ϕ confirmed by Trypan Blue staining

<i>Treatment</i>	<i>Trypan blue positive MDMϕ</i>
<i>Control MDMϕ (no treatment)</i>	<i>5.1 \pm 0.5 %</i>
<i>Aspirin 1000 μM</i>	<i>3.2 \pm 0.2 %</i>
<i>Ibuprofen 1000 μM</i>	<i>4.8 \pm 0.4 %</i>
<i>Indomethacin 100 μM</i>	<i>2.1 \pm 0.1 %</i>
<i>Paracetamol 1000 μM</i>	<i>4.5 \pm 0.5 %</i>
<i>SC-650 250 μM</i>	<i>2.9 \pm 0.3 %</i>
<i>NS-398 250 μM</i>	<i>2.4 \pm 0.2 %</i>

Viability of MDM ϕ was confirmed by Trypan Blue staining. At least 500 cells were counted under light microscope. Data are presented as mean % Trypan Blue positive cells \pm SEM of n=3 separate experiments.

5.3.9 ASPIRIN INTERFERES WITH THE INHIBITORY EFFECT OF TNF- α ON MDM ϕ EFFEROCYTOSIS OF APOPTOTIC NEUTROPHILS

Data described in section 5.3.6 demonstrate that the presence of dexamethasone interferes with inhibitory effects of LPS and TNF- α upon MDM ϕ phagocytosis. For comparison, I sought to investigate whether non-steroidal anti-inflammatory drugs would exert similar effects. Results presented in this chapter show the influence of NSAID, namely aspirin, as well as SC-650 and NS-398 on TNF- α induced inhibition of phagocytosis.

In this series of experiments human blood monocytes were cultured for 6 days in culture medium alone or with addition of 10 ng/ml TNF for 72 hours. Increasing concentrations (300, 600, 1200 μ M) of aspirin were added to both control and TNF- α treated samples for 72 hours. Efferocytosis was assessed on day 6 by flow cytometry. As shown in Figure 5.9A, upon TNF- α treatment, efferocytosis was significantly reduced from 40.1 ± 2.2 % (control level) to 22.3 ± 2.8 % ($p < 0.05$, $n = 4$ in comparison to control). Interestingly, the presence of aspirin at concentrations 300 μ M and 600 μ M reversed the inhibitory effect of TNF- α and the levels of phagocytosis were 36.3 ± 2.4 % (combined treatment with 300 μ M aspirin and 10 ng/ml TNF- α) and 37.1 ± 7.9 % (combined treatment with 600 μ M aspirin and 10 ng/ml TNF- α). It is noteworthy that aspirin at the above concentrations does not inhibit phagocytosis by itself. Aspirin at the concentration 1200 μ M significantly inhibited MDM ϕ efferocytosis (18.21 ± 2.7 %, $p < 0.01$, $n = 4$ in comparison to control) and did not augment efferocytosis reduced by the presence of TNF- α (22.6 ± 3.2 %, $p < 0.05$, $n = 4$ in comparison to control).

Following the above observation, I sought to investigate whether the selective COX-1 inhibitor, SC-650, or the selective COX-2 inhibitor, NS-398, would exert similar effects as aspirin and rescue MDM ϕ phagocytosis in the presence of TNF- α . I performed pilot experiment in which human blood monocytes were cultured for 6 days in culture medium alone or with addition of 10 ng/ml TNF for 72 hours. Increasing concentrations (2.5, 25, 250 μ M) of SC-650 (Figure 5.9B) or NS-398

(Figure 5.9C) were added to both control and TNF – α treated samples for 72 hours. Efferocytosis was assessed on day 6 by flow cytometry. Preliminary data demonstrated in Figure 5.9B and C suggest that the presence of either compound, SC-650 or NS-398, does not interfere with inhibition of MDM ϕ efferocytosis by TNF– α and that perhaps mechanisms other than inhibition of COX enzyme underlies the observed ability of aspirin to interfere with TNF– α action.

FIGURE 5.9 Aspirin, at lower concentration, interferes with TNF- α inhibitory effect on MDM ϕ efferocytosis of apoptotic neutrophils. Human MDM ϕ were cultured for 6 days in culture medium or treated for 72 hours with increasing concentrations of aspirin (**A**), SC-650 (**B**) or NS-398 (**C**) +/- 10 ng/ml TNF- α . Efferocytosis was assessed on day 6 by flow cytometry. Data are expressed: **A.** as mean \pm SEM of n=4 separate experiments (*p<0.05, **p<0.01, compared with medium control). **B** and **C.** as mean \pm SD of n=1 experiment (duplicate).

5.3.10 SUMMARY OF THE RESULTS

- During MDM ϕ treatment with pro-inflammatory agents an interesting interplay occurs between the concentrations of TNF- α and IL-10 in culture supernatants (the most prominent for LPS, the least for LTA treatment). Elevated IL-10 concentrations are associated with a decrease in TNF- α levels and minimal inhibition of phagocytosis. On the contrary, at the time points where TNF- α levels are increased, IL-10 concentrations are reduced.
- Exogenous IL-10 rescues the MDM ϕ phagocytosis inhibited by LPS treatment. This effect is associated with inhibition of TNF- α production by MDM ϕ . The effect of exogenous IL-10 on inhibition of phagocytosis by exogenous TNF- α is less prominent.
- IL-10 deficient murine BMDM ϕ are characterised by a decreased ability to phagocytose apoptotic neutrophils in comparison to wt BMDM ϕ , in both control and pro-inflammatory conditions.
- The anti-IL-10-R1Ab potentiates LPS-induced inhibition of MDM ϕ phagocytosis. In addition, upon treatment with anti-IL-10-R1Ab, the LPS inhibitory effect occurs at much earlier time points in comparison to samples treated only with LPS.
- Dexamethasone rescues phagocytosis by MDM ϕ treated with LPS but only partially inhibits TNF- α production. Dexamethasone also rescues phagocytosis by MDM ϕ treated with exogenous TNF- α or IL-1 β .
- In contrast to dexamethasone, NSAID *per se* do not augment MDM ϕ phagocytosis of apoptotic neutrophils. Preliminary data suggest that aspirin reverses the inhibitory effect of TNF- α on phagocytosis by mechanism other than an inhibition of COX1 and/or COX2.

5.4 DISCUSSION

In chapter 3 of this PhD thesis, pro-inflammatory stimuli, namely LPS, LTA and PGN as well as TNF- α , have been shown to inhibit M ϕ phagocytosis of apoptotic neutrophils. As demonstrated in chapter 4, LPS-induced inhibition was mediated by TNF- α release but intriguingly was significantly delayed (96 hours) in comparison to exogenous TNF- α -mediated effects (6 hours in our study). The inhibition of phagocytosis by LTA and PGN occurred at 32 and 16 hours respectively. Previously published data demonstrated that in monocytes maximal production of pro-inflammatory TNF- α , IL-1 α , IL-1 β , IL-6 or IL-8 appears 4-8 hours after pro-inflammatory challenge (i.e., LPS or interferon- γ) and is followed by maximal IL-10 production 24-48 hours after activation (de Waal *et al.*, 1991). Therefore, in chapter 5, I sought to investigate the mechanism underlying the delay of the inhibition of phagocytosis by LPS. LPS has been chosen for the majority of experiments because its inhibitory effect was characterised by the most prominent delay.

As shown in Figure 5.1, when M ϕ were treated with LPS an interesting interplay occurred between TNF- α and IL-10 concentrations in culture supernatants and inhibition of phagocytosis. TNF- α concentration rose twice during the M ϕ culture (it was maximal 6 and 96 hours after treatment), while IL-10 concentrations increased between 12 and 24 hours (the latter effect was associated with decreased TNF- α levels). Interestingly, inhibition of phagocytosis was observed only during the second increase in TNF- α concentration. When M ϕ were treated with PGN I observed two peaks of maximum TNF- α concentration (at 16 and 96 hours after PGN treatment) and both of them were associated with maximal inhibition of phagocytosis. IL-10 concentration was maximal at 32 and 48 hours after PGN treatment and correlated with normal efferocytosis levels. The interplay between TNF- α , IL-10 and phagocytosis was not so clear-cut with LTA treatment (perhaps because LTA was the weakest inductor of TNF- α production in our system). The TNF- α concentration rose only once during the culture (it was maximal 32 hours after LTA treatment) and it was associated with the maximum inhibition of phagocytosis. Although the maximum increase in IL-10 concentration was observed at 16 hours, the TNF- α concentration was decreasing between 32 and 48 hours after LTA treatment.

The observed interplay between TNF- α and IL-10 may be explained, at least in part, by the phenomenon known as ‘endotoxin tolerance’ (Dobrovolskaia & Vogel, 2002). It has been shown that pre-treatment of cells with LPS results in their hyporesponsiveness to LPS during subsequent stimulation (‘endotoxin tolerance’). It is interesting, especially in regard to data presented in this PhD thesis, that endotoxin tolerant and non-tolerant cells, when stimulated with LPS, differ in the pattern of expressed genes. During LPS treatment, M ϕ that have been already exposed to LPS, fail to produce pro-inflammatory cytokines i.e., TNF- α , IL-1 β , IL-6 or IL-12 while production of anti-inflammatory IL-10, IL-1 receptor antagonist or soluble TNF-R2 is normal or elevated. It is suggested that in endotoxin tolerant cells activation of transcription factor NF- κ B is impaired by a number of mechanisms and this is associated with inhibition of TNF- α production (Dobrovolskaia *et al.*, 2003). Furthermore, the phenomenon of tolerance is not specific for LPS but shared with other TLR agonists (Lehner *et al.*, 2001). I therefore speculated that, in our system, cells responded to LPS, PGN and LTA treatments by producing TNF- α , then became hyporesponsive and produced increased amounts of IL-10. The latter was associated with inhibited release of TNF- α . Interestingly, the more powerful agent used (e.g., LPS) the earlier cells became tolerant (between 6-12 hours after LPS treatment). It is possible that observed tolerance is transient and the second spike of TNF- α occurred when cells regain sensitivity towards LPS or PGN. It still remains to be established why the second peak of TNF- α was not observed in case of LTA treatment. It is possible that the fact that this agent was the weakest inducer of TNF- α may be an important factor.

Interestingly, although TNF- α concentration rose twice during LPS treatment only the second peak of TNF- α was correlated with significant decrease of phagocytosis. This is in contrast to PGN-treated samples where both peaks of TNF- α were associated with significant inhibition of phagocytosis. However, it is noteworthy, that upon PGN treatment the initial increase in TNF- α concentration lasted longer (up to 16 hours following PGN treatment) than in case of LPS (up to 6 hours following LPS treatment). The second important observation is that upon PGN treatment, during the first increase of TNF- α , significant reduction of phagocytosis appeared after 16 hours while phagocytosis was inhibited almost instantly during the

second rise in TNF- α concentration for both PGN and LPS treatments. Thus, it is possible that, in my system, some transient processes were taking place that made cells unresponsive to TNF- α during the first few hours of treatment and therefore played an important role in maintaining phagocytosis during the first few hours of LPS treatment. One potential mechanism here is internalisation of TNF-Rs, which has been reported in cells treated with LPS (Ding *et al.*, 1989). If this is a case in my study it would be interesting to investigate it further and establish e.g., the duration of time needed for restoration of TNF-Rs on the cell surface and if this process is specific for LPS or perhaps shared with other TLR agonists such as PGN or LTA.

Since convincing evidence exists indicating that IL-10 can inhibit TNF- α production (Donnelly *et al.*, 1999; Rossato *et al.*, 2007; Raychaudhuri *et al.*, 2000; Kontoyiannis *et al.*, 2001; Rossato *et al.*, 2007) and up-regulate efferocytosis (Ogden *et al.*, 2005; Xu *et al.*, 2006) I hypothesised that in my system IL-10 opposed, at least in part, the LPS inhibitory effect on phagocytosis. I investigated this further and provided several lines of evidence demonstrating that IL-10 plays a crucial role in sustaining the clearance of apoptotic neutrophils by LPS-stimulated M ϕ . First of all, addition of exogenous IL-10 reversed the LPS-induced inhibitory effect and this effect was associated with concentration-dependent inhibition of TNF- α production (see Figure 5.3). Of note, exogenous IL-10 had very limited ability to reverse the inhibition of phagocytosis induced by TNF- α already present in culture medium. Furthermore, presence of IL-10 in control samples augmented M ϕ phagocytosis of apoptotic neutrophils in a concentration-dependent manner. Next, I blocked the activity of endogenous IL-10 produced by LPS-stimulated M ϕ with an anti-IL-10-R1Ab and observed much earlier and greater inhibition of phagocytosis (see Figure 5.5). Finally, I compared the phagocytosis by wt and IL-10^{-/-} BMDM ϕ and found that the latter had much lower ability to phagocytose apoptotic neutrophils in control and pro-inflammatory conditions (see Table 5.1). Thus, I concluded that IL-10 mediates its protective effects over phagocytosis mainly by inhibition of TNF- α production but does not have the ability to completely override the inhibitory effects of TNF- α already present in culture medium. However, since IL-10 *per se* augments phagocytosis of apoptotic neutrophils it can mask the effects of TNF- α to a small extent (see Figure 5.4). The above-described data are consistent with previously

published findings and show, for the first time, that phagocytosis of apoptotic neutrophils is dynamically regulated by the subtle balance between pro- and anti-inflammatory cytokines, specifically TNF- α and IL-10. It is clear that exposure of cells to pro-inflammatory stimuli, such as LPS, induces production of pro-inflammatory cytokines (e.g., TNF- α), followed by anti-inflammatory cytokines (e.g., IL-10). IL-10 then feeds back to inhibit pro-inflammatory responses. Here, I show that these events directly affect M ϕ clearance of apoptotic neutrophils, the process which we believe to be essential for effective resolution of the inflammatory response.

My observation is supported by a recently published study by Fernandez-Boyanapalli and colleagues demonstrating that in a murine model of X-linked chronic granulomatous disease (X-CGD) M ϕ are characterised by increased production of pro-inflammatory cytokines and decreased ability to phagocytose apoptotic cells *in vivo* and *ex vivo* (Fernandez-Boyanapalli *et al.*, 2009). Furthermore, the authors show that pre-treatment of X-CGD M ϕ with anti-inflammatory cytokine IL-4 can change M ϕ activation status and increase their ability to ingest apoptotic cells. When wt M ϕ were cultured with IL-4 neutralising antibodies the phagocytosis was found to be significantly decreased, further supporting the role of IL-4 as a positive regulator of phagocytosis. Quite surprisingly, when the authors pre-treated X-CGD M ϕ with IL-10, phagocytosis did not increase. However, X-CGD M ϕ were characterised by defective IL-4 expression in comparison to wt M ϕ . It is possible that IL-10 acts in concert with IL-4 and, thus, pre-treatment of cells with IL-10 alone did not augment phagocytosis. Furthermore, M ϕ isolated from X-CGD animals were plated for at least a few hours before addition of IL-10. Since these cells produced pro-inflammatory cytokines, e.g., TNF- α , it is likely that IL-10 was added to the M ϕ already exposed to TNF- α . This is in agreement with data presented in this PhD thesis, where IL-10 had a limited ability to interfere with the effects of TNF- α and reversed inhibitory effects of LPS mainly by blocking TNF- α production.

It is now accepted that M ϕ , depending on the type of cytokines that they are exposed to, may undergo either classical (Th1) or alternative (Th2) activation (Classen *et al.*, 2009). The classically activated (pro-inflammatory) M ϕ are obtained by stimulation with IFN- α or LPS, characterised by production of pro-inflammatory

cytokines e.g., TNF- α and, as demonstrated in this PhD thesis, by decreased clearance of apoptotic neutrophils. The classically activated M ϕ produce NO and other intermediates, which destroy microorganisms at inflammatory sites and therefore play an important function at the onset of inflammation. On the contrary, anti-inflammatory mediators such as IL-4, IL-10 or IL-13 are postulated to induce alternative activation of M ϕ . Two subclasses of alternatively activated M ϕ are currently recognised, regulatory and wound-healing M ϕ (Mosser & Edwards, 2008). The wound-healing M ϕ play an important role in tissue repair whereas regulatory M ϕ in suppression of immune responses. Regulatory M ϕ are characterised by decreased production of pro-inflammatory mediators such as TNF- α , increased release of IL-10 and, as demonstrated in this PhD thesis, increased ability to phagocytose apoptotic neutrophils promoting resolution of inflammation. It is possible that in *in vivo* settings impaired production or signalling of IL-10 (or other alternative activators of M ϕ) may lead to overexpression of TNF- α and consequential decreased clearance of apoptotic cells, secondary necrosis and exacerbation of inflammation. Several clinical trials have been conducted in order to establish efficacy of IL-10 in treatment of chronic inflammatory conditions such as Crohn's disease or ulcerative colitis. However, IL-10 (when administered systemically) does not seem to be as a powerful anti-inflammatory agent as TNF- α inhibitors (see chapter 1.5.3 for more information). Based on the observation provided in this PhD thesis one may speculate that, in contrast to TNF- α inhibitors, IL-10 does not counteract the effects of TNF- α already present at inflamed tissue and therefore this cytokine may not be able to rescue phagocytosis of apoptotic cells and resolve inflammation. Yet, further *in vivo* studies should be performed in order to draw any final conclusion with this regard.

The mechanism(s) underlying LPS-stimulation of IL-10 production is not fully understood. It has been suggested that several of the LPS-induced pro-inflammatory cytokines may stimulate IL-10 release, which, in turn, feeds back to inhibit further production of pro-inflammatory cytokines. Indeed, it has been shown that LPS-stimulated BMDM ϕ , which were characterised by impaired production or signalling of type I IFN produce excessive amounts of pro-inflammatory cytokines (Chang *et al.*, 2007). Furthermore, some studies suggest a number of possible mechanisms underlying the opposing actions of IL-10 and LPS on many cellular

processes also including IL-10-induced inhibition of TNF- α production upon LPS activation. For instance, one study demonstrated that IL-10 blocks expression of the MyD88 adaptor protein, crucial for transduction of LPS-induced signalling (LPS signalling pathways are discussed in chapter 1.2.2.1). The authors reported that the levels of MyD88 mRNA in LPS-stimulated RAW 264.7 cells remained the same irrespective of pre-treatment of cells with IL-10. However, cells cultured in the presence of IL-10 were characterised by reduced levels of MyD88 protein resulting in affected LPS-signalling, impaired activation of NF- κ B, p38 and stress-activated protein kinase (SAPK) (Dagvadorj *et al.*, 2008). IL-10 may also exert some direct effects on NF- κ B by blocking I κ B kinase activity and by inhibiting NF- κ B already present in the nucleus. The latter is a direct consequence of IL-10-induced nuclear translocation of repressive p50/p50 homodimers, which bind DNA and block its transcriptional activity (Driessler *et al.*, 2004). Interestingly, it is been suggested that IL-10 induces the latter effect *via* stimulation of Bcl-3 expression. It has been shown that in LPS-activated M ϕ , Bcl-3 interacts with p50 subunit of NF- κ B and promotes its binding to the TNF- α promoter in the nucleus and subsequent inhibition of TNF- α expression (Kuwata *et al.*, 2003). In contrast, some studies demonstrate that IL-10 does not block the LPS-induced NF- κ B activation but rather inhibits LPS signalling through stimulation of suppressor of cytokine signalling SOCS-3 (discussed in more detail in chapter 1.2.2.2) (Qin *et al.*, 2006). It has been also demonstrated that IL-10 counteracts the LPS-induced survival of neutrophils through a mechanism that involves inhibition of ERK activation (Ward *et al.*, 2005). There is contradiction in literature whether IL-10 blocks TNF- α production in LPS stimulated cells *via* inhibition of activation of MAPK/SAPK (i.e., p38, JNK and ERK). While some studies suggest that IL-10 exerts direct inhibitory effects on these kinases (Kontoyiannis *et al.*, 2001) another report demonstrates otherwise (Denys *et al.*, 2002). In this PhD thesis, we investigated MAPK/SAPK activation upon LPS treatment. As demonstrated in Figure 5.10, LPS treatment (10ng/ml) transiently activates p38, JNK and ERK MAPK/SAPK in MDM ϕ . These effects were correlated with an increase in TNF- α production (as confirmed by ELISA, data not shown). I also obtained preliminary data that show that inhibition of p38 phosphorylation with the kinase inhibitor SB202190 is associated with decreased culture medium

concentration of TNF- α (data not shown). This observation is supported by many others indicating the crucial role of p38 kinase in TNF- α expression (see chapter 1.2.2.1 for more details). Yet, when I incubated M ϕ with LPS and IL-10 no difference has been identified in p38 activation in comparison to samples treated only with LPS (see Figure 5.11). Since this was only a pilot experiment this data was not conclusive. Further work is required to fully establish if, in my system, IL-10 blocks production of TNF- α *via* inhibition of p38 activation. For instance various concentration of IL-10 and LPS or durations of incubation should be examined. It is also possible that IL-10 affects other MAPK/SAPK and, thus, future work should include blotting for ERK and JNK.

Figure 5.10 MAPK/SAPK expression and activation in MDM ϕ following LPS stimulation (next page). Western blots were obtained with the use of phosphorylation status-dependent and independent antibodies against p38 (38 kDa, **A**), ERK1/2 (42 and 44 kDa, **B**) and JNK (46 and 54 kDa, **C**) kinases following pre-treatment of MDM ϕ with LPS for 30, 60, 120 and 180 minutes as indicated. **MM** – molecular weight size marker, **MC** – medium control, **(p)** - phosphorylated forms of MAPKs.

Figure 5.11 p38 expression and activation in LPS-stimulated MDM ϕ in the presence of IL-10. Western blots were obtained with the use of phosphorylation status-dependent and independent antibodies against p38 following pre-treatment of MDM ϕ with either LPS or concurrently with LPS and IL-10 for 15 and 30 minutes as indicated. **MM** – molecular weight size marker, **MC** – medium control, **(p)** - phosphorylated form of p38.

Having found that IL-10, at least in part, rescued phagocytosis upon LPS treatment I sought to investigate whether another powerful anti-inflammatory agent, dexamethasone would exert similar effects. This synthetic glucocorticosteroid exerts numerous anti-inflammatory effects and in many ways resembles the anti-inflammatory cytokine IL-10. For instance, it has been demonstrated that both glucocorticoids and IL-10 stimulate expression of glucocorticoid-induced leucine zipper (GILZ), which plays an essential role in the inhibition of transcription factors AP-1 and NF- κ B (Godot *et al.*, 2006). Dexamethasone has been also reported to augment, in a concentration- and time-dependent manner, M ϕ efferocytosis (Giles *et al.*, 2001; Liu *et al.*, 1999) as well as inhibit production of pro-inflammatory cytokines in M ϕ (Barnes, 1998). In this study, I have demonstrated that dexamethasone, like IL-10, reversed the inhibitory effect of LPS on M ϕ phagocytosis. However, in contrast to IL-10, dexamethasone turned out to be a powerful suppressor of TNF- α -induced inhibition of phagocytosis. Since dexamethasone did not completely inhibit the TNF- α production in LPS-treated MDM ϕ (see Figure 5.6) and increased the uptake of IgG-opsonized erythrocytes (see Figure 3.7) it is possible that that its effect is more complex and likely involves modulation of several signalling pathways. For instance, glucocorticoids exert some of their anti-inflammatory effects *via* stimulating Anx 1 production (Parente & Solito, 2004). Anx 1 was originally identified as a phospholipase A₂ (PLA₂) inhibitory protein (PLA₂ plays an important role in the release of AA from plasma membrane phospholipids and subsequent yield of its active metabolites; see chapter 1.5.2 for further information). Now, it appears that Anx 1 also inhibits the expression and/or activity of other inflammatory enzymes such as inducible nitric oxide synthetase (iNOS) or COX2. Some of these effects may be related, at least in part, to Anx 1-induced IL-10 production (Parente & Solito, 2004). Future work would therefore include investigation of the exact mechanism underlying the dexamethasone-induced augmentation of efferocytosis and reversal of LPS and TNF- α inhibitory effects. However, I hypothesise that at least a few processes may be here involved and they are briefly discussed below.

It has been demonstrated that dexamethasone, possibly due to enhanced Anx 1 expression, stimulates production of IL-10 in M ϕ (Mozo *et al.*, 2004; Xia *et al.*, 2005). In this PhD thesis, I have shown that IL-10 augments M ϕ clearance of

apoptotic neutrophils *per se* as well as inhibits production of TNF- α by LPS-stimulated M ϕ . Yet, as shown in Figure 5.6 I did not identify elevated levels of IL-10 in samples treated with dexamethasone just prior to the phagocytosis assay. It is possible that experimental conditions e.g., duration of treatment or type of cells play an important role in IL-10 induction by dexamethasone. Thus, more experiments should be performed in order to fully answer the question whether increased IL-10 production contributes to dexamethasone-induced reversal of inhibitory effects of LPS or TNF- α on phagocytosis. For instance, IL-10 concentrations should be measured at earlier time points after dexamethasone treatment.

Furthermore, Anx 1 has been reported to be directly involved in the phagocytosis process by serving as a bridge molecule between 'eat me' signals on the surface of apoptotic cells and phagocytic receptors on M ϕ (see chapter 1.3.2.2 for more information). There is also evidence that Anx 1 is recognised by a G protein-coupled receptor (ALXR, originally identified as low-affinity N-formyl-methionyl-leucyl-phenylalanine receptor-like 1), which also binds LXA₄ and ATLs (Scannell & Maderna, 2006). Lipoxins are well-known for their pro-phagocytic properties (Godson *et al.*, 2000). It is therefore possible that Anx 1-induced phagocytosis involves the same pathways as lipoxin-induced phagocytosis (Scannell & Maderna, 2006).

Interestingly, several lines of evidence indicate that TNF- α exerts its cellular effects not only by stimulating transcription of NF- κ B-directed genes but also by activating PLA₂ and inducing the AA cascade. AA metabolites, especially leukotrienes, seem to be involved in variety of intracellular TNF- α -induced events including NF- κ B activation. In fact, some studies have demonstrated in TNF- α -treated cells that PLA₂ and LOX5 inhibitors interfere with transcription of genes dependent on NF- κ B (van Puijenbroek *et al.*, 1999; Anthonsen *et al.*, 2001). This observation gives more insight into the potential mechanism underlying dexamethasone's ability to fully reverse the TNF- α inhibitory effect on phagocytosis in our system. In addition, as discussed in chapter 1.5.2, PGs and particularly PGE₂, inhibit M ϕ phagocytosis and therefore the reduction of AA metabolism by dexamethasone could significantly contribute to its pro-phagocytic properties. Yet, in order to prove that in my study dexamethasone overrides TNF- α -induced inhibition

of phagocytosis by blocking AA cascade future work should be performed involving e.g., measurement of PLA₂ activity, release of AA metabolites or NF-κB activation. Nevertheless, my findings provide further insight into the anti-inflammatory properties of glucocorticoids and may explain, at least in part, their efficacy in treatment of chronic inflammatory diseases (see chapter 1.5.1 for further information).

Next, I performed some experiments in order to investigate if another class of anti-inflammatory drugs, NSAID (see chapter 1.5.2 for more details), would exert similar effects to dexamethasone. As demonstrated in Figure 5.8, in contrast to dexamethasone, none of the tested NSAID augmented Mφ phagocytosis of apoptotic cells. In fact, when used at very high concentrations (1000 μM, except indomethacin 100 μM), they inhibited the MDMφ clearance of apoptotic neutrophils. In addition, MDMφ treatment with selective COX1 (SC-560) or COX2 (NS-398) inhibitors (250 μM) also reduced MDMφ capacity for phagocytosis. Interestingly, when viability of cells was assessed (trypan blue), the results suggested that decrease in phagocytosis did not result from NSAID - induced cell toxicity (see Table 5.2). However, this assay does not distinguish between non-apoptotic and apoptotic cells and it is well-established that NSAID can induce cell apoptosis by COX2 dependent and COX2-independent mechanism (Jana, 2008). Thus, in order to give more insight into the exact mechanism underlying the above observation, apoptosis levels should be confirmed by additional methods e.g., flow cytometry or morphology. It is also possible that very high concentrations of NSAID exert some pro-inflammatory effects on cells. In fact, it has been reported that aspirin at 1000 μM may upregulate COX2 activity (Mortaz *et al.*, 2005). If this is the case, it is possible that subsequent production of PGs significantly contribute to the observed decreased phagocytosis (see chapter 1.5.2 for further information regarding the PGs effects on phagocytosis). On the other hand, NSAID have been shown to augment TNF-α release from LPS-stimulated cells (Rouzer *et al.*, 2004; Cho, 2007). The latter effect is associated with the ability of exogenous PGE₂ and prostacyclin to suppress TNF-α release in LPS-treated Mφ (Rouzer *et al.*, 2004). It would be interesting, especially in a context of this study, to investigate, if PGs play important roles in TNF-α regulation in my system. PGE₂ has been demonstrated to reduce phagocytosis *per se* (Rossi *et al.*,

1998b) and therefore its effect may be insignificant in terms of augmentation of phagocytosis upon LPS treatment.

Aspirin, when compared to other NSAID, possesses quite unique anti-inflammatory properties. First of all, aspirin has been shown to interfere with NF- κ B activation *via* a quite specific inhibition of IKK2 (Mazur *et al.*, 2007). Second, aspirin induces production of so-called aspirin-triggered lipoxins that have been shown to augment phagocytosis of apoptotic cells (as further discussed in chapter 1.5.2). Therefore, I sought to investigate if aspirin, similarly to dexamethasone, may reverse the inhibitory effects of TNF- α on phagocytosis. As demonstrated in Figure 5.9 aspirin at 300 μ M rescued phagocytosis inhibited by TNF- α . Yet, when used at a high concentration (1200 μ M) this effect was minimal (possibly due to inhibitory effect on phagocytosis discussed in previous paragraphs). The exact mechanism underlying the reversal of TNF- α inhibition of phagocytosis by aspirin is still to be investigated however it quite likely involves inhibition of COX, NF- κ B or production of aspirin-triggered lipoxins.

Summing up, the above data demonstrate that MDM ϕ efferocytosis is dramatically regulated by their environment. After exposure to pro-inflammatory stimuli, at the onset of inflammation, the balance between cytokines shifts towards pro-inflammatory mediators (e.g., TNF- α) and this effect is associated with decreased uptake of apoptotic cells. However, during the resolution phase the balance of cytokines shifts towards anti-inflammatory mediators (e.g., IL-10) thereby augmenting efferocytosis and facilitating resolution of inflammation. As demonstrated in Figure 5.3, IL-10 is a very powerful inhibitor of pro-inflammatory cytokine production (Moore *et al.*, 1990; Kontoyiannis *et al.*, 2001; Denys *et al.*, 2002) and consequently defects in either IL-10 secretion or function may represent a critical factor in development of inflammatory diseases (Ebert *et al.*, 2009). It is also noteworthy that uptake of various phagocytic targets may alter cytokine production in M ϕ and consequently influence their environment in an autocrine fashion. Phagocytosis of apoptotic cells actively inhibits, whereas phagocytosis of IgG-opsonized cells induces production of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-8 in both control or LPS treated M ϕ (Fadok *et al.*, 1998; Voll *et al.*, 1997). Furthermore, it has been reported that apoptotic cells may have therapeutic potential

in LPS-induced septic shock by enhancing IL-10 production and reducing release of pro-inflammatory cytokines (Ren *et al.*, 2008).

In addition, I was able to show that dexamethasone not only augments clearance of apoptotic cells but also rescue phagocytosis inhibited in the presence of LPS or TNF- α . This observation may explain, at least in part, the powerful anti-inflammatory properties of steroids and their success in treatment of chronic inflammatory conditions. In contrast, NSAID do not increase phagocytosis *per se*. However, I did not evaluate their effects in cells challenged with pro-inflammatory stimuli (e.g., LPS) and upregulated levels of COX2. It is possible that in such circumstances NSAID, and especially aspirin (aspirin reversed TNF- α -induced inhibition of phagocytosis), would exert some protective effects over phagocytosis.

CHAPTER SIX

GENERAL DISCUSSION

&

FUTURE DIRECTIONS

6. GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 INTRODUCTION

Despite decades of research, little is known about the exact signals/mechanisms that trigger chronic inflammation and autoimmunity. Since numerous diseases have been demonstrated to have a chronic inflammatory component, it is necessary to better understand the underlying mechanisms. Currently, it is generally accepted that apoptosis of inflammatory cells and their subsequent non-inflammatory clearance by phagocytes are two key processes underlying the resolution of inflammation. They are controlled by many factors including cytokines, which allow cell communication and coordinate many physiological processes. TNF- α is a major pro-inflammatory cytokine, which is released in response to trauma and infection and plays an important role at the onset of inflammation. Yet, a few observations suggest that excessive or prolonged TNF- α release may be associated with development of chronic inflammation and autoimmunity (Michlewska *et al.*, 2007). Chronically-inflamed sites are characterised by excess TNF- α and, importantly, TNF- α inhibitors, widely used in the clinical setting for treatment of RA, IBD or psoriasis, significantly delay disease progression. One can therefore speculate that the presence of TNF- α may affect processes implicated in resolution of inflammation (i.e., inflammatory cell apoptosis and their clearance by phagocytes) to influence the development of chronic inflammation and its harmful consequences (see Figure 6.1).

FIGURE 6.1 Diagram representing the main hypothesis of this PhD thesis. An inflamed tissue is characterised by increased levels of TNF- α and defective processes implicated in resolution of inflammation. As demonstrated in this PhD thesis, TNF- α inhibits phagocytosis of apoptotic neutrophils; a key process involved in the resolving phase of inflammation.

Previously published data demonstrated that TNF- α may exert variable effects on survival of inflammatory cells, such as neutrophils; inducing apoptosis at early time points (2-6 hours) and promoting survival after longer (12-24 hours) treatment (Ward *et al.*, 1999). Furthermore, a few recent studies including this PhD thesis, showed that TNF- α , at least *in vitro*, inhibited clearance of apoptotic cells by mature M ϕ (McPhillips *et al.*, 2007; Borges *et al.*, 2009; Michlewska *et al.*, 2009). If this is the case *in vivo*, the excess of TNF- α may lead to accumulation of apoptotic material, secondary necrosis, consequential tissue damage, further release of TNF- α and exacerbation of inflammation. Thus, treatment with TNF- α inhibitors and consequential TNF- α depletion are likely to restore phagocytosis and resolve inflammation. However, TNF- α is critical to maintain immune functions and treatment with TNF- α inhibitors is associated with dampened immune responses and higher risk of severe infections, e.g., TB. This observation indicates that although TNF- α inhibition results in resolution of inflammation, this is not an optimal way of treatment. The precise timing of TNF- α release seems to be an important factor regulating onset and resolution of inflammation. Thus, there is a clinical need to better understand mechanisms underlying regulation of TNF- α release and its effects upon resolution of inflammation *per se* in order to develop new therapeutic strategies that would induce resolution of inflammation but at the same time allow maintenance of basic immune functions.

In this PhD thesis I have investigated the regulation of M ϕ phagocytosis of apoptotic neutrophils by pro- and anti-inflammatory agents. In particular, I was interested in TNF- α effects and used exogenous TNF- α as well as a variety of agents to induce TNF- α production/release in differentiating monocytes/M ϕ . In addition, I investigated the effects of anti-inflammatory agents (i.e., the anti-inflammatory cytokine IL-10, the synthetic steroid dexamethasone and NSAIDs) on M ϕ phagocytosis of apoptotic neutrophils. Their ability to counteract the effects of pro-inflammatory agents has also been examined.

6.2 SUMMARY

In order to investigate the role of TNF- α in the regulation of MDM ϕ phagocytosis of apoptotic neutrophils, MDM ϕ were treated with the bacterial derived cell activators (i.e., LPS, LTA and PGN) to induce MDM ϕ production of TNF- α . For comparison, MDM ϕ were also treated with exogenous TNF- α . As demonstrated in chapter 3, TNF- α treatment of mature MDM ϕ significantly inhibited the uptake of apoptotic neutrophils. This effect was observed at 6 hours after treatment (earlier time points were not examined in this thesis) and was dependent on TNF- α concentration and duration of treatment. These findings complement published studies suggesting that TNF- α inhibits phagocytosis by mature M ϕ but does not exert such an effect on differentiating M ϕ (Ren & Savill, 1995; McPhillips *et al.*, 2007). In this PhD thesis, it has been showed for the first time that LPS treatment inhibits MDM ϕ phagocytosis of apoptotic neutrophils. Interestingly, even though LPS-induced TNF- α release occurred 6 hours after treatment, significant inhibition of phagocytosis was observed only after longer treatment (i.e., 96 hours). This observation complements data by McPhillips *et al.*, who show that M ϕ treated with LPS for 24 hours have unaffected ability to phagocytose apoptotic cells (McPhillips *et al.*, 2007). In comparison to LPS, inhibitory effects of LTA and PGN were less pronounced and occurred at the 16 hour time point while increased concentrations of TNF- α were detectable at 6 hours following both treatments. When MDM ϕ were treated with PGN, initial decrease in phagocytosis occurred at the 16 hour time point, then disappeared at 32 and 48 hour time points and occurred again at 72 and 96 hour time points, partially reflecting the fluctuations in TNF- α concentration in culture medium. The above findings suggested that either the release of endogenous TNF- α is not solely responsible for inhibition of MDM ϕ phagocytosis or additional mechanisms interfered with the TNF- α -induced inhibition and maintained MDM ϕ ability to clear apoptotic cells in the presence of TNF- α (I investigated these hypotheses in more detail in chapters 4 and 5).

Another important finding presented in chapter 3 was that TNF- α and LPS had not blocked all phagocytic pathways in MDM ϕ and while both treatments significantly reduced clearance of apoptotic cells, phagocytosis of human IgG-opsonised erythrocytes was not affected. Since various opsonins may bind to the surface of apoptotic cells, it is difficult to speculate if, in *in vivo* settings, TNF- α -

induced inhibition of clearance of apoptotic cells results in secondary necrosis and exacerbation of inflammation. It is possible that in the presence of TNF- α apoptotic cells coated with opsonins are still cleared by alternative pathways and do not undergo secondary necrosis. Thus, as discussed in chapter 3, further *in vivo* studies should be performed to fully assess immunological consequences of TNF- α inhibitory effects on the phagocytosis of apoptotic neutrophils.

In experiments presented in chapter 4, I sought to investigate whether the LPS inhibitory effect upon phagocytosis was mediated by TNF- α (LPS treatment has been selected for these experiments due to a marked delay in mediating its inhibitory effect). Following 96 hours treatment, the strength of LPS-induced inhibition varied between donors and was positively correlated with the concentration of TNF- α in culture medium. Even though LPS-induced inhibition was significant in the majority of experiments, in a few samples I did not observe such an effect. Interestingly, this was associated with undetectable levels of TNF- α in culture medium prior to the phagocytosis assay.

In order to establish whether TNF- α production/release was critical for LPS to exert its inhibitory effect I tried to reduce TNF- α bioactivity/concentration in culture medium when MDM ϕ were treated with LPS. First of all, I used soluble TNF receptors: sTNF-R1/Fc and sTNF-R1. The sTNF-R1/Fc is composed of two subunits of TNF-R1 linked to the Fc portion of human IgG₁ while sTNF-R1 is a monomeric receptor. Concomitant treatment of MDM ϕ with LPS (10 ng/ml) and sTNF-R1/Fc (500 ng/ml) significantly reversed the inhibitory effect of LPS upon phagocytosis suggesting a crucial role of TNF- α . This was further supported by a few important observations. Concomitant treatment of MDM ϕ with sTNF-R1/Fc and TNF- α blocked TNF- α -induced inhibition of phagocytosis. Furthermore, sTNF-R1/Fc was demonstrated not to induce Fc-mediated phagocytosis in our system. Theoretically, presence of the Fc-portion of human IgG₁ within the sTNF-R1/Fc molecule could induce Fc-mediated phagocytosis and reverse the LPS-induced inhibition in this manner. This would, however, require binding of sTNF-R1/Fc to the cell surface (opsonisation). We demonstrated that the augmentation of phagocytosis by sTNF-R1/Fc required its presence during the entire duration of LPS treatment. The addition of sTNF-R1/Fc into either neutrophil or MDM ϕ culture 30 minutes before the

phagocytosis assay or alternatively directly into the phagocytosis assay did not rescue phagocytosis. This suggested that sTNF-R1/Fc acts rather by neutralising TNF- α produced in response to LPS and not by inducing Fc-mediated phagocytosis (opsonisation is a quick process and 30 minutes would be enough to bind sTNF-R1/Fc to a cell surface). I have also demonstrated that sTNF-R1/Fc, in contrast to ICs, did not bind to the surface of apoptotic neutrophils.

In contrast to sTNF-R1/Fc, treatment with sTNF-R1 did not significantly reverse the inhibitory effect of LPS upon phagocytosis (similar trend towards increased phagocytosis levels was observed). These findings are in agreement with published data (Scallon *et al.*, 1995b; Mohler *et al.*, 1993) demonstrating that dimeric TNF-Rs have much greater capacity for TNF- α binding when compared to monomeric TNF receptors (see section 4.4).

Since there is a growing interest in development of orally available TNF- α inhibitors, I have tried to reduce the concentration of sTNF- α in culture medium by blocking mTNF- α processing with TACE inhibitor TOPI-0. Although I could observe slightly increased phagocytosis when MDM ϕ were co-treated with LPS and TOPI-0, the reversal of LPS effect was not significant. As discussed in section 4.4, this observation may be potentially explained by the fact that TOPI-0 is not a selective TACE inhibitor and blocks activity of other cellular enzymes.

Having established that TNF- α release is necessary for LPS-induced inhibition of phagocytosis, we next sought to investigate mechanisms that delayed this process in our system. In experiments presented in chapter 5, I have partially explained why TNF- α release and inhibition of phagocytosis were not directly correlated phenomena (in our system TNF- α was released at 6 hours and inhibition occurred at 96 hours after LPS treatment). LPS is a general cell activator and induces the release of numerous cytokines, which, in turn, may potentially affect TNF- α release/effects. For instance, elevated IL-10 levels were detected in samples, where LPS treatment (96 hours) did not induce inhibition of phagocytosis and TNF- α concentration was minimal. Then, I measured concentrations of TNF- α and IL-10 at several time points following LPS, PGN and LTA treatments and observed that elevated IL-10 levels were associated with decreased TNF- α concentration. Thus, I hypothesised that presence of IL-10 interferes with TNF- α release/effects in our

system and delays the inhibitory effects of LPS. I supported this hypothesis with a few important findings. First of all, IL-10 *per se* increased MDM ϕ ability to phagocytose apoptotic neutrophils and, second, addition of exogenous IL-10 to LPS treated samples rescued phagocytosis. The latter effect was associated with the IL-10-induced, concentration-dependent inhibition of TNF- α release. Interestingly, when IL-10 was added to TNF- α treated MDM ϕ , only slight augmentation of phagocytosis was observed. This indicated that, in my system, IL-10 delayed inhibitory effects of LPS mainly *via* inhibition of TNF- α release and not interference with the effects of TNF- α already present in the culture medium. Furthermore, when IL-10-mediated effects were blocked by addition of anti-IL-10-R1Ab, the LPS inhibitory effect on phagocytosis was much greater and occurred at 24 hours after treatment. Finally, the important role of IL-10 in maintenance of M ϕ ability to phagocytose apoptotic cells was demonstrated in IL-10 deficient murine BMDM ϕ . IL-10 deficient BMDM ϕ , when compared to wt, were characterised by lower ability to phagocytose apoptotic neutrophils and this effect was independent of culture conditions (control samples, LPS or TNF- α treatments).

As discussed in chapter 5, dexamethasone in many ways resembles IL-10, i.e., augments MDM ϕ phagocytosis of apoptotic neutrophils (Giles *et al.*, 2001; Liu *et al.*, 1999) and inhibits M ϕ production of TNF- α (Barnes, 1998). In this PhD thesis, I demonstrated that dexamethasone rescued MDM ϕ phagocytosis upon treatment with either LPS or TNF- α but, interestingly, did not fully block the production of TNF- α by MDM ϕ treated with LPS. Since dexamethasone increased the uptake of both apoptotic neutrophils and IgG-opsonised erythrocytes, it is possible that dexamethasone-induced modulation of phagocytosis is more complex and involves several signalling pathways. Interestingly, some studies showed that dexamethasone induced M ϕ production of IL-10 (Mozo *et al.*, 2004; Xia *et al.*, 2005). I did not find a significant increase in IL-10 concentration following MDM ϕ treatment with dexamethasone and LPS for 120 hours. Thus, it is possible that in my system dexamethasone effects on phagocytosis were not necessarily associated with increased IL-10 release. However, due to time limitation, IL-10 concentrations were not measured at earlier time points and I was unable to draw any final conclusion in this regard.

In this PhD thesis, I investigated the effects of NSAID on MDM ϕ phagocytosis. In contrast to dexamethasone, NSAID did not augment MDM ϕ phagocytosis of apoptotic neutrophils and, interestingly, when used at greater concentrations (1000 μ M) reduced MDM ϕ phagocytosis. Furthermore, aspirin was demonstrated to reverse the inhibitory effect of TNF- α on phagocytosis when used at lower concentration (300 μ M). The latter finding is potentially associated with the unique ability of aspirin to generate aspirin-triggered lipoxins (Gilroy *et al.*, 2004). Aspirin-triggered lipoxins, as discussed in section 1.5.2, augment MDM ϕ phagocytosis of apoptotic neutrophils.

In summary, it has been determined that prolonged pro-inflammatory challenge may affect resolution of inflammatory responses *via* inhibition of MDM ϕ efferocytosis. I have shown that TNF- α is a key mediator in this process and that IL-10 exerts an important regulatory effect on TNF- α production and consequently on efferocytosis. Furthermore, I found several approaches to successfully decrease either TNF- α production or its inhibitory effect, and consequently reverse LPS-mediated inhibition of efferocytosis. The summary of the results is graphically presented in Figure 6.2. Data presented in this PhD thesis indicate the significance of TNF- α in development of chronic inflammation as well as cytokine regulation in the resolution of inflammation. Furthermore, presented findings provide important mechanistic information into the mode of action of steroids and anti-TNF- α agents and may help to explain their clinical success in treatment of chronic inflammatory diseases.

FIGURE 6.2 Schematic representation of the effects of pro- (TNF- α) and anti-inflammatory (IL-10, dexamethasone, TNF- α inhibitors) agents on clearance of apoptotic cells and resolution of inflammation. Pro-inflammatory situations e.g., trauma or infection (exposure to bacterial products such as LPS) result in release of TNF- α and potentially in reduced clearance of apoptotic neutrophils. An excessive or prolonged TNF- α release is associated with development of chronic inflammation. The latter can be prevented by inhibiting TNF- α expression with IL-10 or steroids (dexamethasone) or alternatively by blocking TNF- α bioactivity with TNF- α inhibitors (e.g., sTNF-R1/Fc).

6.3 CLINICAL IMPLICATION

Apoptosis of inflammatory cells and their subsequent clearance by M ϕ are thought to be key mechanisms underlying resolution of inflammation. The sites of chronic inflammation are characterised by increased release of pro-inflammatory cytokines, e.g., TNF- α , but for a long time the exact TNF- α -induced processes delaying the resolving phase of inflammation have not been fully determined. Recently, a few studies have demonstrated that TNF- α inhibits clearance of apoptotic neutrophils by mature M ϕ (McPhillips *et al.*, 2007; Borges *et al.*, 2009; Michlewska *et al.*, 2009). Thus, potentially, overexpression of TNF- α at inflamed tissue may reduce phagocytosis of apoptotic cells resulting in secondary necrosis, release of cytotoxic cell contents with consequential damage into surrounding tissue, further TNF- α release and exacerbation of inflammation. This may explain, at least in part, the success of TNF- α inhibitors in delaying the progression of chronic inflammatory conditions such as RA, CD or psoriasis (see section 1.5.3). TNF- α inhibitors, by decreasing TNF- α bioactivity, restore clearance of apoptotic cells and prevent harmful consequences of secondary necrosis. Unfortunately, treatment with TNF- α inhibitors is associated with reduced immune responsiveness (as discussed in section 1.2, physiological concentrations of TNF- α are crucial for the onset of inflammation). It is known that patients treated with TNF- α inhibitors are more prone to develop severe infections e.g., TB (Wallis, 2008). This adverse effect could be avoided by development of tissue/site-specific delivery systems that would deliver TNF- α inhibitors to chronically inflamed sites resulting in only local (not systemic) decrease in TNF- α bioactivity. For instance it has been shown that in mice with dextran sulphate sodium (DSS)-induced chronic colitis, administration of *Lactococcus lactis* engineered to secrete monovalent and bivalent murine (m)TNF-neutralising nanobodies resulted in significantly dampened local colon inflammation and did not have a systemic effect (Vandenbroucke *et al.*, 2010).

In contrast to TNF- α , the anti-inflammatory cytokine IL-10 has been reported to augment M ϕ clearance of apoptotic cells (Xu *et al.*, 2006; Ogden *et al.*, 2005). In this PhD thesis, I was able not only to observe the pro-phagocytic effect of IL-10 but also to demonstrate that IL-10 delays the LPS-induced effect upon phagocytosis,

mainly by inhibiting TNF- α production. However, IL-10 has a very limited ability to reverse TNF- α -induced inhibition. Dexamethasone, similar to IL-10, rescued MDM ϕ phagocytosis upon LPS treatment and was shown to override the inhibitory effects of TNF- α . This may explain, at least in part, why synthetic steroids are such powerful anti-inflammatory therapeutics. Furthermore, by evaluating the effects of several pro- and anti-inflammatory agents I established that MDM ϕ phagocytosis of apoptotic neutrophils is actively regulated by a subtle balance between pro- (e.g., TNF- α) and anti-inflammatory (e.g., IL-10) cytokines. These findings are supported by others (Fernandez-Boyanapalli *et al.*, 2009). Thus, as demonstrated in Figure 6.3, any factor that either impairs IL-10 release/signalling or induces an excessive and/or prolonged TNF- α release can potentially trigger exacerbation of inflammation. For instance, IL-10 deficient mice were reported to develop spontaneous chronic IBD (Wei *et al.*, 2008).

Based on the above-mentioned findings, a concept of classically and alternatively activated M ϕ has been recently introduced (Fernandez-Boyanapalli *et al.*, 2009). Accordingly, pro-inflammatory mediators (e.g., TNF- α) are classical activators of M ϕ , they can reduce M ϕ efferocytosis and contribute to development chronic inflammation. On the contrary, anti-inflammatory mediators such as IL-10 and glucocorticoids change M ϕ inflammatory status into anti-inflammatory (alternative activation) (Michlewska *et al.*, 2009; Borges *et al.*, 2009) The latter effect is associated with enhanced clearance of apoptotic cells and resolution of inflammation.

It is possible that, *in vivo*, TNF- α inhibitors deplete TNF- α and rescue phagocytosis of apoptotic cells. Consequently, as discussed in chapter 1.3.2.4, phagocytes start producing anti-inflammatory mediators e.g., IL-10 with associated alteration of M ϕ activation status from pro- into anti-inflammatory. The latter is associated with further release of the anti-inflammatory cytokine IL-10 and resolution of inflammation. Several studies support this hypothesis demonstrating that uptake of apoptotic cells changes the pattern of cytokines released by M ϕ and stimulates IL-10 release (Fadok *et al.*, 1998; Voll *et al.*, 1997). Thus, arguably an ideal therapeutic strategy for treatment of chronic inflammatory conditions should aim at shifting the cytokine balance towards anti-inflammatory IL-10 or IL-4 rather than reducing

bioactivity of TNF- α . As recently demonstrated, apoptotic cells could help to achieve this goal. Administration of apoptotic cells in mice can protect them from LPS-induced septic shock by multiple mechanisms, including suppression of TNF- α and induction of IL-10 release by M ϕ following phagocytosis of apoptotic cells (Ren *et al.*, 2008). It is possible that also in humans administration of apoptotic cells could change the cytokine balance towards an anti-inflammatory scenario without affecting TNF- α bioactivity. Hypothetically, in such settings, cells would be able to produce sufficient concentrations of TNF- α in response to pro-inflammatory stimuli and therefore maintain basic immune functions.

FIGURE 6.3 Schematic representation of fluctuations in a cytokine balance during subsequent phases of an inflammatory response. At the onset of inflammation the cytokine balance is shifted towards pro-inflammatory cytokines (e.g., TNF- α). A resolving phase of inflammation is, in turn, associated with production of anti-inflammatory mediators (e.g., IL-10) and upregulated apoptosis of inflammatory cells and their clearance by phagocytes. If production of anti-inflammatory mediators is impaired or, alternatively, production of pro-inflammatory mediators is excessive, inflammation does not resolve and is associated with prolonged lifespan of inflammatory cells and reduced phagocytosis of apoptotic cells. The latter results in secondary necrosis and exacerbation of inflammation.

6.4 FUTURE DIRECTIONS

In this PhD thesis a few important observations have been made. Firstly, prolonged MDM ϕ treatment with LPS, LTA or PGN resulted in reduced clearance of apoptotic cells. These effects were associated with increased TNF- α release and, in the case of LPS treatment, we showed that TNF- α mediated LPS-induced inhibitory effects. Secondly, IL-10 significantly delayed the LPS inhibitory effect on phagocytosis, mainly by inhibition of TNF- α production. Similar findings were obtained for a synthetic steroid, dexamethasone (see section 6.3 for summary of the results). Interestingly, both IL-10 and glucocorticoids have been demonstrated to play an important role in maintenance of ‘endotoxin tolerance’ (Berg *et al.*, 1995; Rearte *et al.*, 2010). Data presented in this PhD thesis clearly indicate that the balance between pro- and anti-inflammatory mediators defines M ϕ ability to phagocytose apoptotic cells. Yet, in order to fully understand these processes further work should be performed. TNF- α and IL-10 effects on M ϕ clearance of apoptotic cells *in vivo* should be determined as well as elucidating the exact mechanisms underlying TNF- α -induced inhibition or IL-10- and dexamethasone-induced augmentation of phagocytosis.

6.4.1 THE EFFECTS OF TNF- α ON CLEARANCE OF APOPTOTIC INFLAMMATORY CELLS *IN VIVO*

Since all the experiments presented in this thesis were performed *in vitro* I think it would be prudent to investigate the effects of TNF- α on efferocytosis *in vivo*, in animal models of inflammation. TNF- α plays a crucial role in development of arthritis (see section 1.4.1) and, thus, a model of collagen-induced arthritis could be used in further investigations (Earp *et al.*, 2008a; Earp *et al.*, 2008b). It would be interesting to establish whether TNF- α indeed inhibits M ϕ phagocytosis of inflammatory cells *in vivo* in such a model. I demonstrated that while TNF- α inhibited clearance of apoptotic cells it did not exert any significant effects on

clearance of opsonised particles. It is known that in *in vivo* settings apoptotic cells may undergo opsonisation and be cleared by other mechanisms. This would prevent secondary necrosis but not necessarily further TNF- α release. It is generally accepted that phagocytosis of opsonised particles, in contrast to phagocytosis of apoptotic cells, induces TNF- α release by M ϕ (however, some exceptions have been reported and are discussed in section 1.3.2.4). Thus, it would be interesting to investigate whether excess of TNF- α at inflamed tissue results in accumulation of apoptotic material and secondary necrosis or, alternatively, in opsonisation of apoptotic cells and their clearance by distinct mechanisms. If the latter is true, it would be interesting to examine whether further release of TNF- α is induced resulting in exacerbation of inflammation.

6.4.2 THE ROLE OF IL-10 IN PREVENTION OF CHRONIC INFLAMMATION (ALTERNATIVE ACTIVATION OF M ϕ)

This PhD thesis complements data by others and demonstrates that IL-10 increases MDM ϕ phagocytosis and, importantly, upon LPS treatment, inhibits the release of TNF- α and rescues phagocytosis. Therefore, one may speculate that reduced ability to produce IL-10 or defects in IL-10 signal transduction pathways could suffice to account for development of chronic inflammation. I have already demonstrated that MDM ϕ isolated from various donors responded to LPS with various degrees of inhibition of phagocytosis and these effects were associated with variable TNF- α and IL-10 release. Furthermore, in animal models, IL-10 deficiency have been shown to be associated with development of spontaneous IBD (Kuhn *et al.*, 1993). Thus, it would be interesting to investigate if, in humans, development of chronic inflammatory conditions (e.g., RA, Crohn's disease) is associated with defects in IL-10 production or signalling. Some reports have been already published and demonstrated that, in humans, both IL-10 deficiency and disrupted IL-10 signalling were associated with greater risk of developing IBD, particularly ulcerative colitis or Crohn's disease (Grundtner *et al.*, 2009; Ebert *et al.*, 2009). It would be interesting to examine whether IL-10 deficiency or impaired IL-10 signalling *in vivo*

is associated with elevated TNF- α levels, accumulation of apoptotic cells and development of chronic inflammatory conditions. This could be done with the use of animal models of inflammation (e.g., collagen-induced arthritis). Theoretically, animals elicit variable degrees of inflammation depending on the effectiveness of IL-10-R signal transduction pathways or levels of IL-10 expression. One could then identify animals which are more prone to develop inflammation and investigate IL-10 and TNF- α expression, IL-10 signal transduction and clearance of apoptotic immune cells. In addition, it would be interesting to study the effectiveness of IL-10-R signal transduction and IL-10 expression in M ϕ from patients with chronic inflammatory conditions (e.g., RA). If any defects are identified one could investigate whether there is any association with increased plasma levels of TNF- α and accumulation of apoptotic material (reduced phagocytosis) in affected joints.

6.4.3 THE MECHANISM UNDERLYING TNF- α -INDUCED INHIBITION OF PHAGOCYTOSIS

As discussed in section 1.3.2.3 the capacity for phagocytosis ultimately depends on the activation status of Rho family GTPases i.e., Rac-1 and RhoA. Rac-1 is obligatorily required for engulfment while RhoA inhibits this process. The study published by McPhillips and colleagues suggests that TNF- α treatment induces generation of oxidants, which, in turn, activate RhoA resulting in decreased phagocytosis (McPhillips *et al.*, 2007). The mechanism underlying generation of oxidants likely involves activation of phospholipase cPLA₂ and liberation of AA as exogenous AA exerted similar inhibitory effects to TNF- α and TNF- α -induced inhibition was reversed by blocking cPLA₂ activity. TNF- α activates cPLA₂ *via* both TNF-R1 and TNF-R2 signal transduction pathways and activation of ERK and c-Jun or, alternatively, by translocating cPLA₂ to the plasma membrane and consequential calcium efflux (TNF-R2 induced pathway) (Jupp *et al.*, 2003; Schievella *et al.*, 1997). It would be of interest to establish whether, in this PhD thesis, TNF- α inhibited phagocytosis *via* activation of cPLA₂ and generation of oxidants. This could be investigated by blocking cPLA₂ activity with phospholipase inhibitors (e.g., pyrrolidine). Interestingly, a dexamethasone-induced protein, Anx-1, is an inhibitor

of cPLA₂ (see section 5.4 for more details) and, in this PhD thesis, I have already demonstrated that dexamethasone reversed TNF- α -induced inhibition of phagocytosis. In addition, I could block RhoA activity with anti-oxidants such as N-acetylcysteine (NAC) and investigate whether this would reverse TNF- α or LPS-induced inhibition of phagocytosis. It has been recently reported that NAC enhances the resolution of LPS-induced pulmonary inflammation in mice through the inhibition of RhoA activity and the enhancement of apoptotic cell clearance (Moon *et al.*, 2010). Interestingly, in this study, LPS induced a significant increase in active RhoA and subsequent reduction in phagocytosis at 3 days post LPS treatment. These findings complement our data demonstrating that at least 72 hours LPS treatment is necessary for significant reduction of phagocytosis. The authors concluded that critical levels of oxidants are required for RhoA activation, which were only reached after at least 3 days of LPS treatment. It would be interesting to see whether, in our study, the delay of LPS-induced inhibitory effects was due to insufficient oxidants levels at earlier time points.

Furthermore, as described in section 1.3.2.2 expression of numerous cell surface receptors is critical for phagocytosis of apoptotic cells. Since TNF- α exerts its effects on cells by multiple mechanisms, it is possible that TNF- α changes expression of some cell surface receptors. Future work would therefore involve the use of fluorescently labelled antibodies and flow cytometric analysis of changes in expression of surface proteins in response to TNF- α .

6.4.4 THE MECHANISM UNDERLYING IL-10- AND DEXAMETHASONE-INDUCED AUGMENTATION OF PHAGOCYTOSIS

In this PhD thesis, I have shown that both IL-10 and dexamethasone augmented MDM ϕ phagocytosis of apoptotic neutrophils and reversed the LPS-induced inhibitory effect on phagocytosis. Thus, future work would include identification of the exact underlying mechanisms. As described in sections 1.3.2.3 and 6.4.3 the ability of M ϕ for uptake of apoptotic cells is regulated by the balance between active RhoA and Rac-1. McPhillips *et al* have shown that TNF- α inhibits

phagocytosis by activation of cPLA₂, subsequent AA liberation associated with generation of oxidants and RhoA activation. It is possible that, in my study, dexamethasone counteracted the TNF- α inhibitory effect by inhibiting cPLA₂ activation. This hypothesis is further supported by my observation that dexamethasone fully reversed LPS-induced inhibition of phagocytosis without a complete inhibition of TNF- α release (see section 5.3.6 for more details). Theoretically, reduced activity of cPLA₂ would result in an inhibition of AA release and consequently in decreased levels of active RhoA. It is possible that dexamethasone treatment also changes levels of active Rac1 or other small GTPase family members that have been demonstrated to increase phagocytosis (e.g., RhoG and Rab5 (Nakaya *et al.*, 2006)). This could be investigated by western blotting for active forms of these proteins. I could also use pharmacological inhibitors to block activity of small GTPase family members involved in augmentation of phagocytosis (e.g., Rac-1). If Rac-1 activation is implicated, the pharmacological inhibition of Rac-1 will interfere with dexamethasone-induced augmentation and reversal of LPS-induced inhibition of phagocytosis.

Furthermore, data presented in this PhD thesis indicate that IL-10 reverses LPS-induced inhibitory effects on phagocytosis by inhibition of TNF- α production rather than by interference with TNF- α effects (see section 5.3.3 for more details). In the literature, data on mechanisms underlying IL-10-induced inhibition of TNF- α production are not conclusive. Initially, it has been thought that inhibition of NF- κ B or MAPK is implicated but while some reports demonstrate that this is true others show otherwise (see section 1.2.2.2). In this PhD thesis, I obtained preliminary data demonstrating that IL-10 did not interfere with p38 activation upon LPS treatment (see Figures 5.10 and 5.11). It would be interesting to establish an exact mechanism underlying IL-10-induced inhibition of TNF- α production. This could be achieved by western blotting for phosphorylated forms of MAPK in MDM ϕ treated concomitantly with LPS and IL-10 or with the use of pharmacological inhibitors of MAPK. In addition, IL-10 may inhibit activation of transcription factors (e.g., NF- κ B), which could be detected by western blotting. *De novo* synthesis of SOCS-3 (see section 1.2.2.2 for more details) may also play an important role in inhibition of production of LPS-induced cytokines, including TNF- α . If this is true, pharmacological

inhibition of SOCS-3 would block IL-10-induced reversal of the inhibitory effects of LPS on phagocytosis.

Finally, it is possible that MDM ϕ treatment with IL-10 or dexamethasone changes expression of cell surface phagocytic receptors (M ϕ receptors for phagocytosis are discussed in chapter 1.3.2.2). This is relatively easy to investigate with the use of commercially available antibodies labelled with a fluorescent markers and flow cytometric analysis. It would be of interest to examine if TNF- α and IL-10/dexamethasone exert opposing effects on expression of the same receptors. This would potentially allow identification of signalling pathways/important mediators involved in phagocytosis which could be crucial for resolution of the inflammatory response.

Reference List

(1999). TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology* 53, 457-465.

ABRAHAM, S.M. & CLARK, A.R. (2006). Dual-specificity phosphatase 1: a critical regulator of innate immune responses. *Biochem Soc Trans* 34, 1018-1023.

AGGARWAL, B.B. & NATARAJAN, K. (1996). Tumor necrosis factors: developments during the last decade. *Eur Cytokine Netw* 7, 93-124.

AMANN, R. & PESKAR, B.A. (2002). Anti-inflammatory effects of aspirin and sodium salicylate. *Eur J Pharmacol* 447, 1-9.

ANDERSON, H.A., MAYLOCK, C.A., WILLIAMS, J.A., PAWELETZ, C.P., SHU, H., & SHACTER, E. (2003). Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. *Nat Immunol* 4, 87-91.

ANTHONSEN, M.W., SOLHAUG, A., & JOHANSEN, B. (2001). Functional coupling between secretory and cytosolic phospholipase A2 modulates tumor necrosis factor- α - and interleukin-1 β -induced NF- κ B activation. *J Biol Chem* 276, 30527-30536.

ARUR, S., UCHE, U.E., REZAUL, K., FONG, M., SCRANTON, V., COWAN, A.E., MOHLER, W., & HAN, D.K. (2003). Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev Cell* 4, 587-598.

ASCH, A.S., LIU, I., BRICCETTI, F.M., BARNWELL, J.W., KWAKYE-BERKO, F., DOKUN, A., GOLDBERGER, J., & PERNAMBUCO, M. (1993). Analysis of CD36 binding domains: ligand specificity controlled by dephosphorylation of an ectodomain. *Science* 262, 1436-1440.

BARNES, P.J. (2008). The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 118, 3546-3556.

- BARNES, P.J. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)* 94, 557-572.**
- BASAK, S., KIM, H., KEARNS, J.D., TERGAONKAR, V., O'DEA, E., WERNER, S.L., BENEDICT, C.A., WARE, C.F., GHOSH, G., VERMA, I.M., & HOFFMANN, A. (2007). A fourth IkappaB protein within the NF-kappaB signaling module. *Cell* 128, 369-381.**
- BAUD, V. & KARIN, M. (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 11, 372-377.**
- BAUMANN, I., KOLOWOS, W., VOLL, R.E., MANGER, B., GAJPL, U., NEUHUBER, W.L., KIRCHNER, T., KALDEN, J.R., & HERRMANN, M. (2002). Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum* 46, 191-201.**
- BAZAN, J.F. (1993). Emerging families of cytokines and receptors. *Curr Biol* 3, 603-606.**
- BENNETT, M.R., GIBSON, D.F., SCHWARTZ, S.M., & TAIT, J.F. (1995). Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. *Circ Res* 77, 1136-1142.**
- BERG, D.J., KUHN, R., RAJEWSKY, K., MULLER, W., MENON, S., DAVIDSON, N., GRUNIG, G., & RENNICK, D. (1995). Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *J Clin Invest* 96, 2339-2347.**
- BERTOLINI, D.R., NEDWIN, G.E., BRINGMAN, T.S., SMITH, D.D., & MUNDY, G.R. (1986). Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. *Nature* 319, 516-518.**
- BIESMA, D.H., HANNEMA, A.J., VAN VELZEN-BLAD, H., MULDER, L., VAN, Z.R., KLUIJT, I., & ROOS, D. (2001). A family with complement factor D deficiency. *J Clin Invest* 108, 233-240.**
- BLACK, R.A., RAUCH, C.T., KOZLOSKY, C.J., PESCHON, J.J., SLACK, J.L., WOLFSON, M.F., CASTNER, B.J., STOCKING, K.L., REDDY, P., SRINIVASAN, S., NELSON, N., BOIANI, N., SCHOOLEY, K.A., GERHART, M., DAVIS, R., FITZNER, J.N., JOHNSON, R.S., PAXTON,**

R.J., MARCH, C.J., & CERRETTI, D.P. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 385, 729-733.

BORGES, V.M., VANDIVIER, R.W., MCPHILLIPS, K.A., KENCH, J.A., MORIMOTO, K., GROSHONG, S.D., RICHENS, T.R., GRAHAM, B.B., MULDROW, A.M., VAN, H.L., HENSON, P.M., & JANSSEN, W.J. (2009). TNF{alpha} Inhibits Apoptotic Cell Clearance in the Lung Exacerbating Acute Inflammation. *Am J Physiol Lung Cell Mol Physiol*.

BOSE, J., GRUBER, A.D., HELMING, L., SCHIEBE, S., WEGENER, I., HAFNER, M., BEALES, M., KONTGEN, F., & LENGELING, A. (2004). The phosphatidylserine receptor has essential functions during embryogenesis but not in apoptotic cell removal. *J Biol* 3, 15.

BOTTCHER, A., GAIPL, U.S., FURNROHR, B.G., HERRMANN, M., GIRKONTAITE, I., KALDEN, J.R., & VOLL, R.E. (2006). Involvement of phosphatidylserine, alphavbeta3, CD14, CD36, and complement C1q in the phagocytosis of primary necrotic lymphocytes by macrophages. *Arthritis Rheum* 54, 927-938.

BRENNAN, F.M., CHANTRY, D., JACKSON, A., MAINI, R., & FELDMANN, M. (1989). Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet* 2, 244-247.

BRENNAN, F.M. & MCINNES, I.B. (2008). Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest* 118, 3537-3545.

BRESNIHAN, B., VARO-GRACIA, J.M., COBBY, M., DOHERTY, M., DOMLJAN, Z., EMERY, P., NUKI, G., PAVELKA, K., RAU, R., ROZMAN, B., WATT, I., WILLIAMS, B., AITCHISON, R., MCCABE, D., & MUSIKIC, P. (1998). Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum* 41, 2196-2204.

BROUCKAERT, G., KALAI, M., KRYSKO, D.V., SAELENS, X., VERCAMMEN, D., NDLOVU, '., HAEGEMAN, G., D'HERDE, K., & VANDENABEELE, P. (2004). Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. *Mol Biol Cell* 15, 1089-1100.

BROWN, K.D., HOSTAGER, B.S., & BISHOP, G.A. (2002a). Regulation of TRAF2 signaling by self-induced degradation. *J Biol Chem* 277, 19433-19438.

BROWN, S., HEINISCH, I., ROSS, E., SHAW, K., BUCKLEY, C.D., & SAVILL, J. (2002b). Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. *Nature* 418, 200-203.

BURNETTE, W.N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112, 195-203.

CAELLES, C., GONZALEZ-SANCHO, J.M., & MUNOZ, A. (1997). Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev* 11, 3351-3364.

CAMPBELL, I.G., FREEMONT, P.S., FOULKES, W., & TROWSDALE, J. (1992). An ovarian tumor marker with homology to vaccinia virus contains an IgV-like region and multiple transmembrane domains. *Cancer Res* 52, 5416-5420.

CAMPBELL, J., CIESIELSKI, C.J., HUNT, A.E., HORWOOD, N.J., BEECH, J.T., HAYES, L.A., DENYS, A., FELDMANN, M., BRENNAN, F.M., & FOXWELL, B.M. (2004). A novel mechanism for TNF-alpha regulation by p38 MAPK: involvement of NF-kappa B with implications for therapy in rheumatoid arthritis. *J Immunol* 173, 6928-6937.

CAPRA, V. (2004). Molecular and functional aspects of human cysteinyl leukotriene receptors. *Pharmacol Res* 50, 1-11.

CASIANO, C.A., MARTIN, S.J., GREEN, D.R., & TAN, E.M. (1996). Selective cleavage of nuclear autoantigens during CD95 (Fas/APO-1)-mediated T cell apoptosis. *J Exp Med* 184, 765-770.

CHAI, J., DU, C., WU, J.W., KYIN, S., WANG, X., & SHI, Y. (2000). Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 406, 855-862.

CHAN, F.K. (2007). Three is better than one: pre-ligand receptor assembly in the regulation of TNF receptor signaling. *Cytokine* 37, 101-107.

CHANG, E.Y., GUO, B., DOYLE, S.E., & CHENG, G. (2007). Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production. *J Immunol* 178, 6705-6709.

CHEN, G. & GOEDDEL, D.V. (2002). TNF-R1 signaling: a beautiful pathway. *Science* 296, 1634-1635.

CHEN, W., FRANK, M.E., JIN, W., & WAHL, S.M. (2001). TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* 14, 715-725.

CHENG, E.H., WEI, M.C., WEILER, S., FLAVELL, R.A., MAK, T.W., LINDSTEN, T., & KORSMEYER, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 8, 705-711.

CHINENOV, Y. & ROGATSKY, I. (2007). Glucocorticoids and the innate immune system: crosstalk with the toll-like receptor signaling network. *Mol Cell Endocrinol* 275, 30-42.

CHO, J.Y. (2007). Immunomodulatory effect of nonsteroidal anti-inflammatory drugs (NSAIDs) at the clinically available doses. *Arch Pharm Res* 30, 64-74.

CHOW, J.C., YOUNG, D.W., GOLENBOCK, D.T., CHRIST, W.J., & GUSOVSKY, F. (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 274, 10689-10692.

CIKALA, M., ALEXANDROVA, O., DAVID, C.N., PROSCHEL, M., STIENING, B., CRAMER, P., & BOTTGER, A. (2004). The phosphatidylserine receptor from Hydra is a nuclear protein with potential Fe(II) dependent oxygenase activity. *BMC Cell Biol* 5, 26.

CLARIA, J. & SERHAN, C.N. (1995). Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc Natl Acad Sci U S A* 92, 9475-9479.

CLARK, I.A. (2007). How TNF was recognized as a key mechanism of disease. *Cytokine Growth Factor Rev* 18, 335-343.

- CLASSEN, A., LLOBERAS, J., & CELADA, A. (2009).** Macrophage activation: classical versus alternative. *Methods Mol Biol* 531, 29-43.
- CLEM, R.J., FECHHEIMER, M., & MILLER, L.K. (1991).** Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science* 254, 1388-1390.
- COCCO, R.E. & UCKER, D.S. (2001).** Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Mol Biol Cell* 12, 919-930.
- COHEN, S. (2004).** Cytokine: more than a new word, a new concept proposed by Stanley Cohen thirty years ago. *Cytokine* 28, 242-247.
- COVERT, M.W., LEUNG, T.H., GASTON, J.E., & BALTIMORE, D. (2005).** Achieving stability of lipopolysaccharide-induced NF-kappaB activation. *Science* 309, 1854-1857.
- CRONSTEIN, B.N., MONTESINOS, M.C., & WEISSMANN, G. (1999).** Salicylates and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of inhibition of prostaglandin synthesis and p105 of NFkappaB. *Proc Natl Acad Sci U S A* 96, 6377-6381.
- CUI, P., QIN, B., LIU, N., PAN, G., & PEI, D. (2004).** Nuclear localization of the phosphatidylserine receptor protein via multiple nuclear localization signals. *Exp Cell Res* 293, 154-163.
- CVETANOVIC, M. & UCKER, D.S. (2004).** Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol* 172, 880-889.
- DAGVADORJ, J., NAIKI, Y., TUMURKHUU, G., HASSAN, F., ISLAM, S., KOIDE, N., MORI, I., YOSHIDA, T., & YOKOCHI, T. (2008).** Interleukin-10 inhibits tumor necrosis factor-alpha production in lipopolysaccharide-stimulated RAW 264.7 cells through reduced MyD88 expression. *Innate Immun* 14, 109-115.
- DANIAL, N.N. & KORSMEYER, S.J. (2004).** Cell death: critical control points. *Cell* 116, 205-219.

DAYER, J.M. (2003). The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. *Rheumatology (Oxford)* 42 Suppl 2, ii3-10.

DAYER, J.M., BEUTLER, B., & CERAMI, A. (1985). Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. *J Exp Med* 162, 2163-2168.

DE WAAL, M.R., ABRAMS, J., BENNETT, B., FIGDOR, C.G., & DE VRIES, J.E. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 174, 1209-1220.

DEBAKKER, C.D., HANEY, L.B., KINCHEN, J.M., GRIMSLEY, C., LU, M., KLINGELE, D., HSU, P.K., CHOU, B.K., CHENG, L.C., BLANGY, A., SONDEK, J., HENGARTNER, M.O., WU, Y.C., & RAVICHANDRAN, K.S. (2004). Phagocytosis of apoptotic cells is regulated by a UNC-73/TRIO-MIG-2/RhoG signaling module and armadillo repeats of CED-12/ELMO. *Curr Biol* 14, 2208-2216.

DECATHELINÉAU, A.M. & HENSON, P.M. (2003). The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. *Essays Biochem* 39, 105-117.

DEFRANCO, D.B. (1999). Regulation of steroid receptor subcellular trafficking. *Cell Biochem Biophys* 30, 1-24.

DEININGER, S., TRAUB, S., AICHELE, D., RUPP, T., BARIS, T., MOLLER, H.M., HARTUNG, T., & VON, A.S. (2008). Presentation of lipoteichoic acid potentiates its inflammatory activity. *Immunobiology* 213, 519-529.

DEKKERS, P.E., LAUW, F.N., TEN, H.T., TE VELDE, A.A., LUMLEY, P., BECHERER, D., VAN DEVENTER, S.J., & VAN DER, P.T. (1999). The effect of a metalloproteinase inhibitor (GI5402) on tumor necrosis factor-alpha (TNF-alpha) and TNF-alpha receptors during human endotoxemia. *Blood* 94, 2252-2258.

DENYS, A., UDALOVA, I.A., SMITH, C., WILLIAMS, L.M., CIESIELSKI, C.J., CAMPBELL, J., ANDREWS, C., KWAITKOWSKI, D., & FOXWELL, B.M. (2002). Evidence for a dual mechanism for IL-10 suppression of TNF-alpha production that does not involve inhibition of p38 mitogen-activated

protein kinase or NF-kappa B in primary human macrophages. *J Immunol* 168, 4837-4845.

DESAGHER, S., OSEN-SAND, A., NICHOLS, A., ESKES, R., MONTESSUIT, S., LAUPER, S., MAUNDRELL, K., ANTONSSON, B., & MARTINOU, J.C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 144, 891-901.

DEVIN, A., COOK, A., LIN, Y., RODRIGUEZ, Y., KELLIHER, M., & LIU, Z. (2000). The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity* 12, 419-429.

DILLON, S.R., CONSTANTINESCU, A., & SCHLISSEL, M.S. (2001). Annexin V binds to positively selected B cells. *J Immunol* 166, 58-71.

DING, A.H., SANCHEZ, E., SRIMAL, S., & NATHAN, C.F. (1989). Macrophages rapidly internalize their tumor necrosis factor receptors in response to bacterial lipopolysaccharide. *J Biol Chem* 264, 3924-3929.

DINI, L., LENTINI, A., DIEZ, G.D., ROCHA, M., FALASCA, L., SERAFINO, L., & VIDAL-VANACLOCHA, F. (1995). Phagocytosis of apoptotic bodies by liver endothelial cells. *J Cell Sci* 108 (Pt 3), 967-973.

DOBROVOLSKAIA, M.A., MEDVEDEV, A.E., THOMAS, K.E., CUESTA, N., TOSHCHAKOV, V., REN, T., CODY, M.J., MICHALEK, S.M., RICE, N.R., & VOGEL, S.N. (2003). Induction of in vitro reprogramming by Toll-like receptor (TLR)2 and TLR4 agonists in murine macrophages: effects of TLR "homotolerance" versus "heterotolerance" on NF-kappa B signaling pathway components. *J Immunol* 170, 508-519.

DOBROVOLSKAIA, M.A. & VOGEL, S.N. (2002). Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect* 4, 903-914.

DONNELLY, R.P., DICKENSHEETS, H., & FINBLOOM, D.S. (1999). The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J Interferon Cytokine Res* 19, 563-573.

DONNELLY, R.P., FREEMAN, S.L., & HAYES, M.P. (1995). Inhibition of IL-10 expression by IFN-gamma up-regulates transcription of TNF-alpha in human monocytes. *J Immunol* 155, 1420-1427.

DOYLE, S.L. & O'NEILL, L.A. (2006). Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochem Pharmacol* 72, 1102-1113.

DRAING, C., SIGEL, S., DEININGER, S., TRAUB, S., MUNKE, R., MAYER, C., HARENG, L., HARTUNG, T., VON, A.S., & HERMANN, C. (2008). Cytokine induction by Gram-positive bacteria. *Immunobiology* 213, 285-296.

DRIESSLER, F., VENSTROM, K., SABAT, R., ASADULLAH, K., & SCHOTTELIUS, A.J. (2004). Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. *Clin Exp Immunol* 135, 64-73.

DUMITRU, C.D., CECI, J.D., TSATSANIS, C., KONTOYIANNIS, D., STAMATAKIS, K., LIN, J.H., PATRIOTIS, C., JENKINS, N.A., COPELAND, N.G., KOLLIAS, G., & TSICHLIS, P.N. (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103, 1071-1083.

DUNCAN, C.J., LAWRIE, A., BLAYLOCK, M.G., DOUGLAS, J.G., & WALSH, G.M. (2003). Reduced eosinophil apoptosis in induced sputum correlates with asthma severity. *Eur Respir J* 22, 484-490.

EARP, J.C., DUBOIS, D.C., MOLANO, D.S., PYSZCZYNSKI, N.A., ALMON, R.R., & JUSKO, W.J. (2008a). Modeling corticosteroid effects in a rat model of rheumatoid arthritis II: mechanistic pharmacodynamic model for dexamethasone effects in Lewis rats with collagen-induced arthritis. *J Pharmacol Exp Ther* 326, 546-554.

EARP, J.C., DUBOIS, D.C., MOLANO, D.S., PYSZCZYNSKI, N.A., KELLER, C.E., ALMON, R.R., & JUSKO, W.J. (2008b). Modeling corticosteroid effects in a rat model of rheumatoid arthritis I: mechanistic disease progression model for the time course of collagen-induced arthritis in Lewis rats. *J Pharmacol Exp Ther* 326, 532-545.

EBERT, E.C. (2005). Endogenous inhibitory cytokines repress TNFalpha secretion. *Cell Immunol* 237, 106-114.

EBERT, E.C., PANJA, A., DAS, K.M., PRAVEEN, R., GENG, X., REZAC, C., & BAJPAI, M. (2009). Patients with inflammatory bowel disease may have a transforming growth factor-beta-, interleukin (IL)-2- or IL-10-deficient state induced by intrinsic neutralizing antibodies. *Clin Exp Immunol* 155, 65-71.

EGGERMONT, A.M. & TEN HAGEN, T.L. (2001). Isolated limb perfusion for extremity soft-tissue sarcomas, in-transit metastases, and other unresectable tumors: credits, debits, and future perspectives. *Curr Oncol Rep* 3, 359-367.

ELIOPOULOS, A.G., DAVIES, C., KNOX, P.G., GALLAGHER, N.J., AFFORD, S.C., ADAMS, D.H., & YOUNG, L.S. (2000). CD40 induces apoptosis in carcinoma cells through activation of cytotoxic ligands of the tumor necrosis factor superfamily. *Mol Cell Biol* 20, 5503-5515.

ELLIOTT, J.I., SURPRENANT, A., MARELLI-BERG, F.M., COOPER, J.C., CASSADY-CAIN, R.L., WOODING, C., LINTON, K., ALEXANDER, D.R., & HIGGINS, C.F. (2005). Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes. *Nat Cell Biol* 7, 808-816.

ENGVALL, E. & PERLMANN, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 8, 871-874.

ERDOSOVA, B., HLAVKOVA, L., PROCHAZKOVA, J., & LICHNOVSKY, V. (2002). Part of CD68+ macrophages in the clearance of apoptotic bodies in human metanephros. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 146, 41-45.

ERWIG, L.P., GORDON, S., WALSH, G.M., & REES, A.J. (1999). Previous uptake of apoptotic neutrophils or ligation of integrin receptors downmodulates the ability of macrophages to ingest apoptotic neutrophils. *Blood* 93, 1406-1412.

ERWIG, L.P. & HENSON, P.M. (2008). Clearance of apoptotic cells by phagocytes. *Cell Death Differ* 15, 243-250.

ETZIONI, A. (2007). Leukocyte adhesion deficiencies: molecular basis, clinical findings, and therapeutic options. *Adv Exp Med Biol* 601, 51-60.

FADOK, V.A., BRATTON, D.L., GUTHRIE, L., & HENSON, P.M. (2001). Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J Immunol* 166, 6847-6854.

FADOK, V.A., BRATTON, D.L., KONOWAL, A., FREED, P.W., WESTCOTT, J.Y., & HENSON, P.M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 101, 890-898.

FADOK, V.A., BRATTON, D.L., ROSE, D.M., PEARSON, A., EZEKEWITZ, R.A., & HENSON, P.M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 405, 85-90.

FADOK, V.A., VOELKER, D.R., CAMPBELL, P.A., COHEN, J.J., BRATTON, D.L., & HENSON, P.M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148, 2207-2216.

FAN, H., TETI, G., ASHTON, S., GUYTON, K., TEMPEL, G.E., HALUSHKA, P.V., & COOK, J.A. (2003). Involvement of G(i) proteins and Src tyrosine kinase in TNFalpha production induced by lipopolysaccharide, group B Streptococci and Staphylococcus aureus. *Cytokine* 22, 126-133.

FELDMANN, M. (1996). What is the mechanism of action of anti-tumour necrosis factor-alpha antibody in rheumatoid arthritis? *Int Arch Allergy Immunol* 111, 362-365.

FELDMANN, M., BRENNAN, F.M., ELLIOTT, M., KATSIKIS, P., & MAINI, R.N. (1994). TNF alpha as a therapeutic target in rheumatoid arthritis. *Circ Shock* 43, 179-184.

FELDMANN, M., BRENNAN, F.M., FOXWELL, B.M., TAYLOR, P.C., WILLIAMS, R.O., & MAINI, R.N. (2005). Anti-TNF therapy: where have we got to in 2005? *J Autoimmun* 25 Suppl, 26-28.

FELDMANN, M., BRENNAN, F.M., WILLIAMS, R.O., ELLIOTT, M.J., & MAINI, R.N. (1995). Cytokine expression and networks in rheumatoid arthritis: rationale for anti-TNF alpha antibody therapy and its mechanism of action. *J Inflamm* 47, 90-96.

FELDMANN, M. & MAINI, R.N. (2003). Lasker Clinical Medical Research Award. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat Med* 9, 1245-1250.

FERNANDEZ-BOYANAPALLI, R.F., FRASCH, S.C., MCPHILLIPS, K., VANDIVIER, R.W., HARRY, B.L., RICHES, D.W., HENSON, P.M., & BRATTON, D.L. (2009). Impaired apoptotic cell clearance in CGD due to altered macrophage programming is reversed by phosphatidylserine-dependent production of IL-4. *Blood* 113, 2047-2055.

FIorentino, D.F., BOND, M.W., & MOSMANN, T.R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170, 2081-2095.

FONTAINE, V., MOHAND-SAID, S., HANOTEAU, N., FUCHS, C., PFIZENMAIER, K., & EISEL, U. (2002). Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci* 22, RC216.

FOTIN-MLECZEK, M., HENKLER, F., SAMEL, D., REICHWEIN, M., HAUSSER, A., PARMRYD, I., SCHEURICH, P., SCHMID, J.A., & WAJANT, H. (2002). Apoptotic crosstalk of TNF receptors: TNF-R2-induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. *J Cell Sci* 115, 2757-2770.

FULDA, S. & DEBATIN, K.M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* 25, 4798-4811.

FURST, R., SCHROEDER, T., EILKEN, H.M., BUBIK, M.F., KIEMER, A.K., ZAHLER, S., & VOLLMAR, A.M. (2007). MAPK phosphatase-1 represents a novel anti-inflammatory target of glucocorticoids in the human endothelium. *FASEB J* 21, 74-80.

GAIPL, U.S., BEYER, T.D., HEYDER, P., KUENKELE, S., BOTTCHEER, A., VOLL, R.E., KALDEN, J.R., & HERRMANN, M. (2004). Cooperation between C1q and DNase I in the clearance of necrotic cell-derived chromatin. *Arthritis Rheum* 50, 640-649.

GAIPL, U.S., KUENKELE, S., VOLL, R.E., BEYER, T.D., KOLOWOS, W., HEYDER, P., KALDEN, J.R., & HERRMANN, M. (2001). Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death. *Cell Death Differ* 8, 327-334.

GAO, Y., HERNDON, J.M., ZHANG, H., GRIFFITH, T.S., & FERGUSON, T.A. (1998). Antiinflammatory effects of CD95 ligand (FasL)-induced apoptosis. *J Exp Med* 188, 887-896.

GARDAI, S.J., BRATTON, D.L., OGDEN, C.A., & HENSON, P.M. (2006). Recognition ligands on apoptotic cells: a perspective. *J Leukoc Biol* 79, 896-903.

GARDAI, S.J., MCPHILLIPS, K.A., FRASCH, S.C., JANSSEN, W.J., STAREFELDT, A., MURPHY-ULLRICH, J.E., BRATTON, D.L., OLDENBORG, P.A., MICHALAK, M., & HENSON, P.M. (2005). Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* 123, 321-334.

GHOSH, S. & KARIN, M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell* 109 Suppl, S81-S96.

GILES, K.M., ROSS, K., ROSSI, A.G., HOTCHIN, N.A., HASLETT, C., & DRANSFIELD, I. (2001). Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac. *J Immunol* 167, 976-986.

GILROY, D.W. (2005). The role of aspirin-triggered lipoxins in the mechanism of action of aspirin. *Prostaglandins Leukot Essent Fatty Acids* 73, 203-210.

GILROY, D.W., LAWRENCE, T., PERRETTI, M., & ROSSI, A.G. (2004). Inflammatory resolution: new opportunities for drug discovery. *Nat Rev Drug Discov* 3, 401-416.

GODOT, V., GARCIA, G., CAPEL, F., AROCK, M., DURAND-GASSELIN, I., SSELIN-LABAT, M.L., EMILIE, D., & HUMBERT, M. (2006). Dexamethasone and IL-10 stimulate glucocorticoid-induced leucine zipper synthesis by human mast cells. *Allergy* 61, 886-890.

GODSON, C., MITCHELL, S., HARVEY, K., PETASIS, N.A., HOGG, N., & BRADY, H.R. (2000). Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. *J Immunol* 164, 1663-1667.

GREGORY, C.D. & DEVITT, A. (2004). The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology* 113, 1-14.

GRELL, M., ZIMMERMANN, G., GOTTFRIED, E., CHEN, C.M., GRUNWALD, U., HUANG, D.C., WU LEE, Y.H., DURKOP, H., ENGELMANN, H., SCHEURICH, P., WAJANT, H., & STRASSER, A. (1999). Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. *EMBO J* 18, 3034-3043.

GRIFFIN, F.M., JR., GRIFFIN, J.A., & SILVERSTEIN, S.C. (1976). Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. *J Exp Med* 144, 788-809.

GRONEMEYER, H., GUSTAFSSON, J.A., & LAUDET, V. (2004). Principles for modulation of the nuclear receptor superfamily. *Nat Rev Drug Discov* 3, 950-964.

GRUNDTNER, P., GRUBER, S., MURRAY, S.S., VERMEIRE, S., RUTGEERTS, P., DECKER, T., LAKATOS, P.L., & GASCHÉ, C. (2009). The IL-10R1 S138G loss-of-function allele and ulcerative colitis. *Genes Immun* 10, 84-92.

GUMIENNY, T.L., BRUGNERA, E., TOSELLO-TRAMPONT, A.C., KINCHEN, J.M., HANEY, L.B., NISHIWAKI, K., WALK, S.F., NEMERGUT, M.E., MACARA, I.G., FRANCIS, R., SCHEDL, T., QIN, Y., VAN, A.L., HENGARTNER, M.O., & RAVICHANDRAN, K.S. (2001). CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. *Cell* 107, 27-41.

GUMIENNY, T.L. & HENGARTNER, M.O. (2001). How the worm removes corpses: the nematode *C. elegans* as a model system to study engulfment. *Cell Death Differ* 8, 564-568.

GUTBERLET, T., FRANK, J., BRADACZEK, H., & FISCHER, W. (1997). Effect of lipoteichoic acid on thermotropic membrane properties. *J Bacteriol* 179, 2879-2883.

HALL, S.E., SAVILL, J.S., HENSON, P.M., & HASLETT, C. (1994). Apoptotic neutrophils are phagocytosed by fibroblasts with participation of

the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin. *J Immunol* 153, 3218-3227.

HAN, J., LEE, J.D., BIBBS, L., & ULEVITCH, R.J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265, 808-811.

HARRIS, R.C., MCKANNA, J.A., AKAI, Y., JACOBSON, H.R., DUBOIS, R.N., & BREYER, M.D. (1994). Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J Clin Invest* 94, 2504-2510.

HART, S.P., ALEXANDER, K.M., & DRANSFIELD, I. (2004a). Immune complexes bind preferentially to Fc gamma RIIA (CD32) on apoptotic neutrophils, leading to augmented phagocytosis by macrophages and release of proinflammatory cytokines. *J Immunol* 172, 1882-1887.

HART, S.P., ALEXANDER, K.M., MACCALL, S.M., & DRANSFIELD, I. (2005). C-reactive protein does not opsonize early apoptotic human neutrophils, but binds only membrane-permeable late apoptotic cells and has no effect on their phagocytosis by macrophages. *J Inflamm (Lond)* 2, 5.

HART, S.P., DOUGHERTY, G.J., HASLETT, C., & DRANSFIELD, I. (1997). CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. *J Immunol* 159, 919-925.

HART, S.P., SMITH, J.R., & DRANSFIELD, I. (2004b). Phagocytosis of opsonized apoptotic cells: roles for 'old-fashioned' receptors for antibody and complement. *Clin Exp Immunol* 135, 181-185.

HASHIMOTO, C., HUDSON, K.L., & ANDERSON, K.V. (1988). The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 52, 269-279.

HASLETT, C. (1999). Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 160, S5-11.

HASLETT, C., GUTHRIE, L.A., KOPANIAK, M.M., JOHNSTON, R.B., JR., & HENSON, P.M. (1985). Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol* 119, 101-110.

HATMI, M., GAVARET, J.M., ELALAMY, I., VARGAFTIG, B.B., & JACQUEMIN, C. (1996). Evidence for cAMP-dependent platelet ectoprotein kinase activity that phosphorylates platelet glycoprotein IV (CD36). *J Biol Chem* 271, 24776-24780.

HEAL, R. & MCGIVAN, J. (1998). Induction of calreticulin expression in response to amino acid deprivation in Chinese hamster ovary cells. *Biochem J* 329 (Pt 2), 389-394.

HEASMAN, S.J., GILES, K.M., ROSSI, A.G., ALLEN, J.E., HASLETT, C., & DRANSFIELD, I. (2004). Interferon gamma suppresses glucocorticoid augmentation of macrophage clearance of apoptotic cells. *Eur J Immunol* 34, 1752-1761.

HENGARTNER, M.O. (2000). The biochemistry of apoptosis. *Nature* 407, 770-776.

HENKE, C.A., ROONGTA, U., MICKELSON, D.J., KNUTSON, J.R., & MCCARTHY, J.B. (1996). CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. *J Clin Invest* 97, 2541-2552.

HENSON, P.M. (2005). Engulfment: ingestion and migration with Rac, Rho and TRIO. *Curr Biol* 15, R29-R30.

HERRMANN, M., VOLL, R.E., ZOLLER, O.M., HAGENHOFER, M., PONNER, B.B., & KALDEN, J.R. (1998). Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis Rheum* 41, 1241-1250.

HIRASAWA, N., SATO, Y., FUJITA, Y., MUE, S., & OHUCHI, K. (1998). Inhibition by dexamethasone of antigen-induced c-Jun N-terminal kinase activation in rat basophilic leukemia cells. *J Immunol* 161, 4939-4943.

HOCK, H., DORSCH, M., KUNZENDORF, U., QIN, Z., DIAMANTSTEIN, T., & BLANKENSTEIN, T. (1993). Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon gamma. *Proc Natl Acad Sci U S A* 90, 2774-2778.

HODGE, S., HODGE, G., SCICCHITANO, R., REYNOLDS, P.N., & HOLMES, M. (2003). Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol* 81, 289-296.

HOFFMANN, P.R., DECATHELINER, A.M., OGDEN, C.A., LEVERRIER, Y., BRATTON, D.L., DALEKE, D.L., RIDLEY, A.J., FADOK, V.A., & HENSON, P.M. (2001). Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J Cell Biol* 155, 649-659.

HOFFMANN, P.R., KENCH, J.A., VONDRACEK, A., KRUK, E., DALEKE, D.L., JORDAN, M., MARRACK, P., HENSON, P.M., & FADOK, V.A. (2005). Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo. *J Immunol* 174, 1393-1404.

HORINO, K., NISHIURA, H., OHSAKO, T., SHIBUYA, Y., HIRAOKA, T., KITAMURA, N., & YAMAMOTO, T. (1998). A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells. *Lab Invest* 78, 603-617.

HSU, H., SHU, H.B., PAN, M.G., & GOEDEL, D.V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84, 299-308.

HURST, N.P., NUKI, G., & WALLINGTON, T. (1984). Evidence for intrinsic cellular defects of 'complement' receptor-mediated phagocytosis in patients with systemic lupus erythematosus (SLE). *Clin Exp Immunol* 55, 303-312.

HUYNH, M.L., FADOK, V.A., & HENSON, P.M. (2002). Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* 109, 41-50.

JACOBS, C.A., BECKMANN, M.P., MOHLER, K., MALISZEWSKI, C.R., FANSLOW, W.C., & LYNCH, D.H. (1993). Pharmacokinetic parameters and biodistribution of soluble cytokine receptors. *Int Rev Exp Pathol* 34 Pt B, 123-135.

JANA, N.R. (2008). NSAIDs and apoptosis. *Cell Mol Life Sci* 65, 1295-1301.

- JERALA, R. (2007). Structural biology of the LPS recognition. *Int J Med Microbiol* 297, 353-363.
- JERSMANN, H.P., ROSS, K.A., VIVERS, S., BROWN, S.B., HASLETT, C., & DRANSFIELD, I. (2003). Phagocytosis of apoptotic cells by human macrophages: analysis by multiparameter flow cytometry. *Cytometry A* 51, 7-15.
- JIANG, Y., WORONICZ, J.D., LIU, W., & GOEDDEL, D.V. (1999). Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. *Science* 283, 543-546.
- JIANG, Z., GEORGEL, P., DU, X., SHAMEL, L., SOVATH, S., MUDD, S., HUBER, M., KALIS, C., KECK, S., GALANOS, C., FREUDENBERG, M., & BEUTLER, B. (2005). CD14 is required for MyD88-independent LPS signaling. *Nat Immunol* 6, 565-570.
- JOHNSON, K.L. & RN, C.R. (2006). The hypothalamic-pituitary-adrenal axis in critical illness. *AACN Clin Issues* 17, 39-49.
- JUPP, O.J., VANDENABEELE, P., & MACEWAN, D.J. (2003). Distinct regulation of cytosolic phospholipase A2 phosphorylation, translocation, proteolysis and activation by tumour necrosis factor-receptor subtypes. *Biochem J* 374, 453-461.
- KADOWAKI, H., NISHITOH, H., & ICHIJO, H. (2004). Survival and apoptosis signals in ER stress: the role of protein kinases. *J Chem Neuroanat* 28, 93-100.
- KASSEL, O., SANCONO, A., KRATZSCHMAR, J., KREFT, B., STASSEN, M., & CATO, A.C. (2001). Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *EMBO J* 20, 7108-7116.
- KAUR, K., DHINGRA, S., SLEZAK, J., SHARMA, A.K., BAJAJ, A., & SINGAL, P.K. (2008). Biology of TNFalpha and IL-10, and their imbalance in heart failure. *Heart Fail Rev*.
- KEANE, J., GERSHON, S., WISE, R.P., MIRABILE-LEVENS, E., KASZNICA, J., SCHWIETERMAN, W.D., SIEGEL, J.N., & BRAUN, M.M. (2001). Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 345, 1098-1104.

KHARITONENKOV, A., CHEN, Z., SURES, I., WANG, H., SCHILLING, J., & ULLRICH, A. (1997). A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature* 386, 181-186.

KIM, C.H. (2004). Chemokine-chemokine receptor network in immune cell trafficking. *Curr Drug Targets Immune Endocr Metabol Disord* 4, 343-361.

KIM, S., ELKON, K.B., & MA, X. (2004). Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity* 21, 643-653.

KISHIMOTO, T. (2005). Interleukin-6: from basic science to medicine--40 years in immunology. *Annu Rev Immunol* 23, 1-21.

KLARESKOG, L., VAN DER, H.D., DE JAGER, J.P., GOUGH, A., KALDEN, J., MALAISE, M., MARTIN, M.E., PAVELKA, K., SANY, J., SETTAS, L., WAJDULA, J., PEDERSEN, R., FATENEJAD, S., & SANDA, M. (2004). Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomised controlled trial. *Lancet* 363, 675-681.

KONTOYIANNIS, D., KOTLYAROV, A., CARBALLO, E., ALEXOPOULOU, L., BLACKSHEAR, P.J., GAESTEL, M., DAVIS, R., FLAVELL, R., & KOLLIAS, G. (2001). Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. *EMBO J* 20, 3760-3770.

KONTOYIANNIS, D., PASPARAKIS, M., PIZARRO, T.T., COMINELLI, F., & KOLLIAS, G. (1999). Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 10, 387-398.

KOPP, E. & MEDZHITOV, R. (2003). Recognition of microbial infection by Toll-like receptors. *Curr Opin Immunol* 15, 396-401.

KOTLYAROV, A., NEININGER, A., SCHUBERT, C., ECKERT, R., BIRCHMEIER, C., VOLK, H.D., & GAESTEL, M. (1999). MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nat Cell Biol* 1, 94-97.

KRAHLING, S., CALLAHAN, M.K., WILLIAMSON, P., & SCHLEGEL, R.A. (1999). Exposure of phosphatidylserine is a general feature in the

phagocytosis of apoptotic lymphocytes by macrophages. *Cell Death Differ* 6, 183-189.

KRYSKO, D.V., D'HERDE, K., & VANDENABEELE, P. (2006). Clearance of apoptotic and necrotic cells and its immunological consequences. *Apoptosis* 11, 1709-1726.

KUHN, R., LOHLER, J., RENNICK, D., RAJEWSKY, K., & MULLER, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263-274.

KUIJPERS, T.W., MAIANSKI, N.A., TOOL, A.T., BECKER, K., PLECKO, B., VALIANPOUR, F., WANDERS, R.J., PEREIRA, R., VAN, H.J., VERHOEVEN, A.J., ROOS, D., BAAS, F., & BARTH, P.G. (2004). Neutrophils in Barth syndrome (BTHS) avidly bind annexin-V in the absence of apoptosis. *Blood* 103, 3915-3923.

KUWATA, H., WATANABE, Y., MIYOSHI, H., YAMAMOTO, M., KAISHO, T., TAKEDA, K., & AKIRA, S. (2003). IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-alpha production in macrophages. *Blood* 102, 4123-4129.

LANG, P., GESBERT, F., ESPINE-CARMAGNAT, M., STANCOU, R., POCHELET, M., & BERTOGLIO, J. (1996). Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO J* 15, 510-519.

LAUBER, K., BLUMENTHAL, S.G., WAIBEL, M., & WESSELBORG, S. (2004). Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell* 14, 277-287.

LAUBER, K., BOHN, E., KROBER, S.M., XIAO, Y.J., BLUMENTHAL, S.G., LINDEMANN, R.K., MARINI, P., WIEDIG, C., ZOBYWALSKI, A., BAKSH, S., XU, Y., AUTENRIETH, I.B., SCHULZE-OSTHOFF, K., BELKA, C., STUHLER, G., & WESSELBORG, S. (2003). Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* 113, 717-730.

LEE, A., WHYTE, M.K., & HASLETT, C. (1993). Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukoc Biol* 54, 283-288.

LEHNER, M.D., MORATH, S., MICHELSEN, K.S., SCHUMANN, R.R., & HARTUNG, T. (2001). Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different Toll-like receptors independent of paracrine mediators. *J Immunol* 166, 5161-5167.

LEQUIN, R.M. (2005). Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin Chem* 51, 2415-2418.

LI, H., ZHU, H., XU, C.J., & YUAN, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94, 491-501.

LI, H.N., BARLOW, P.G., BYLUND, J., MACKELLAR, A., BJORSTAD, A., CONLON, J., HIEMSTRA, P.S., HASLETT, C., GRAY, M., SIMPSON, A.J., ROSSI, A.G., & DAVIDSON, D.J. (2009). Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-37 is not proinflammatory to phagocytosing macrophages. *J Leukoc Biol* 86, 891-902.

LI, X., YANG, Y., & ASHWELL, J.D. (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature* 416, 345-347.

LIPSKY, P.E., VAN DER HEIJDE, D.M., ST CLAIR, E.W., FURST, D.E., BREEDVELD, F.C., KALDEN, J.R., SMOLEN, J.S., WEISMAN, M., EMERY, P., FELDMANN, M., HARRIMAN, G.R., & MAINI, R.N. (2000). Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. *N Engl J Med* 343, 1594-1602.

LIU, Y., COUSIN, J.M., HUGHES, J., VAN, D.J., SECKL, J.R., HASLETT, C., DRANSFIELD, I., SAVILL, J., & ROSSI, A.G. (1999). Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. *J Immunol* 162, 3639-3646.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* 193, 265-275.

LUCIANI, M.F. & CHIMINI, G. (1996). The ATP binding cassette transporter ABC1, is required for the engulfment of corpses generated by apoptotic cell death. *EMBO J* 15, 226-235.

MADERNA, P., COTTELL, D.C., TOIVONEN, T., DUFTON, N., DALLI, J., PERRETTI, M., & GODSON, C. (2010). FPR2/ALX receptor expression and internalization are critical for lipoxin A4 and annexin-derived peptide-stimulated phagocytosis. *FASEB J* 24, 4240-4249.

MAHONEY, J.A. & ROSEN, A. (2005). Apoptosis and autoimmunity. *Curr Opin Immunol* 17, 583-588.

MAINI, R.N., BREEDVELD, F.C., KALDEN, J.R., SMOLEN, J.S., DAVIS, D., MACFARLANE, J.D., ANTONI, C., LEEB, B., ELLIOTT, M.J., WOODY, J.N., SCHAIBLE, T.F., & FELDMANN, M. (1998). Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum* 41, 1552-1563.

MAINI, R.N., ELLIOTT, M.J., BRENNAN, F.M., & FELDMANN, M. (1995). Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockade in rheumatoid arthritis (RA). *Clin Exp Immunol* 101, 207-212.

MANDERSON, A.P., BOTTO, M., & WALPORT, M.J. (2004). The role of complement in the development of systemic lupus erythematosus. *Annu Rev Immunol* 22, 431-456.

MANNEL, D.N. & ECHTENACHER, B. (2000). TNF in the inflammatory response. *Chem Immunol* 74, 141-161.

MARSHALL, J.C. (2005). Lipopolysaccharide: an endotoxin or an exogenous hormone? *Clin Infect Dis* 41 Suppl 7, S470-S480.

MARUSAWA, H., MATSUZAWA, S., WELSH, K., ZOU, H., ARMSTRONG, R., TAMM, I., & REED, J.C. (2003). HBXIP functions as a cofactor of survivin in apoptosis suppression. *EMBO J* 22, 2729-2740.

MAZUR, I., WURZER, W.J., EHRHARDT, C., PLESCHKA, S., PUTHAVATHANA, P., SILBERZAHN, T., WOLFF, T., PLANZ, O., & LUDWIG, S. (2007). Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-kappaB-inhibiting activity. *Cell Microbiol* 9, 1683-1694.

MAZZON, E. & CUZZOCREA, S. (2008). Role of TNF- α in ileum tight junction alteration in mouse model of restraint stress. *Am J Physiol Gastrointest Liver Physiol*.

MCCOURT, P.A., SMEDSROD, B.H., MELKKO, J., & JOHANSSON, S. (1999). Characterization of a hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship to scavenger receptors. *Hepatology* 30, 1276-1286.

MCDERMOTT, M.F., AKSENTIJEVICH, I., GALON, J., MCDERMOTT, E.M., OGUNKOLADE, B.W., CENTOLA, M., MANSFIELD, E., GADINA, M., KARENKO, L., PETERSSON, T., MCCARTHY, J., FRUCHT, D.M., ARINGER, M., TOROSYAN, Y., TEPPONEN, A.M., WILSON, M., KARAARSLAN, H.M., WAN, Y., TODD, I., WOOD, G., SCHLIMGEN, R., KUMARAJEEVA, T.R., COOPER, S.M., VELLA, J.P., AMOS, C.I., MULLEY, J., QUANE, K.A., MOLLOY, M.G., RANKI, A., POWELL, R.J., HITMAN, G.A., O'SHEA, J.J., & KASTNER, D.L. (1999). Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. *Cell* 97, 133-144.

MCPHILLIPS, K., JANSSEN, W.J., GHOSH, M., BYRNE, A., GARDAL, S., REMIGIO, L., BRATTON, D.L., KANG, J.L., & HENSON, P. (2007). TNF- α inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase A2 and oxidant-dependent mechanisms. *J Immunol* 178, 8117-8126.

MEAGHER, L.C., COUSIN, J.M., SECKL, J.R., & HASLETT, C. (1996). Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* 156, 4422-4428.

MEAGHER, L.C., SAVILL, J.S., BAKER, A., FULLER, R.W., & HASLETT, C. (1992). Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B2. *J Leukoc Biol* 52, 269-273.

MEANS, T.K., GOLENBOCK, D.T., & FENTON, M.J. (2000). Structure and function of Toll-like receptor proteins. *Life Sci* 68, 241-258.

MEDZHITOV, R. (2008). Origin and physiological roles of inflammation. *Nature* 454, 428-435.

MESAELI, N., NAKAMURA, K., ZVARITCH, E., DICKIE, P., DZIAK, E., KRAUSE, K.H., OPAS, M., MACLENNAN, D.H., & MICHALAK, M. (1999). Calreticulin is essential for cardiac development. *J Cell Biol* 144, 857-868.

MICHLEWSKA, S., DRANSFIELD, I., MEGSON, I.L., & ROSSI, A.G. (2009). Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-alpha. *FASEB J* 23, 844-854.

MICHLEWSKA, S., MCCOLL, A., ROSSI, A.G., MEGSON, I.L., & DRANSFIELD, I. (2007). Clearance of dying cells and autoimmunity. *Autoimmunity* 40, 267-273.

MIYANISHI, M., TADA, K., KOIKE, M., UCHIYAMA, Y., KITAMURA, T., & NAGATA, S. (2007). Identification of Tim4 as a phosphatidylserine receptor. *Nature* 450, 435-439.

MOHLER, K.M., TORRANCE, D.S., SMITH, C.A., GOODWIN, R.G., STREMLER, K.E., FUNG, V.P., MADANI, H., & WIDMER, M.B. (1993). Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. *J Immunol* 151, 1548-1561.

MOODLEY, Y., RIGBY, P., BUNDELL, C., BUNT, S., HAYASHI, H., MISSO, N., MCANULTY, R., LAURENT, G., SCAFFIDI, A., THOMPSON, P., & KNIGHT, D. (2003). Macrophage recognition and phagocytosis of apoptotic fibroblasts is critically dependent on fibroblast-derived thrombospondin 1 and CD36. *Am J Pathol* 162, 771-779.

MOON, C., LEE, Y.J., PARK, H.J., CHONG, Y.H., & KANG, J.L. (2010). N-acetylcysteine inhibits RhoA and promotes apoptotic cell clearance during intense lung inflammation. *Am J Respir Crit Care Med* 181, 374-387.

MOORE, K.W., VIEIRA, P., FIORENTINO, D.F., TROUNSTINE, M.L., KHAN, T.A., & MOSMANN, T.R. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRF1. *Science* 248, 1230-1234.

MOOSIG, F., CSERNOK, E., KUMANOVICS, G., & GROSS, W.L. (2000). Opsonization of apoptotic neutrophils by anti-neutrophil cytoplasmic antibodies (ANCA) leads to enhanced uptake by macrophages and increased release of tumour necrosis factor-alpha (TNF-alpha). *Clin Exp Immunol* 122, 499-503.

MORIMOTO, K., JANSSEN, W.J., FESSLER, M.B., MCPHILLIPS, K.A., BORGES, V.M., BOWLER, R.P., XIAO, Y.Q., KENCH, J.A., HENSON,

- P.M., & VANDIVIER, R.W. (2006). Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. *J Immunol* 176, 7657-7665.
- MORTAZ, E., REDEGELD, F.A., NIJKAMP, F.P., & ENGELS, F. (2005). Dual effects of acetylsalicylic acid on mast cell degranulation, expression of cyclooxygenase-2 and release of pro-inflammatory cytokines. *Biochem Pharmacol* 69, 1049-1057.
- MOSSER, D.M. & EDWARDS, J.P. (2008). Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8, 958-969.
- MOSSER, D.M. & ZHANG, X. (2008). Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* 226, 205-218.
- MOZO, L., SUAREZ, A., & GUTIERREZ, C. (2004). Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes. *Clin Exp Allergy* 34, 406-412.
- MUELLER, M., STAMME, C., DRAING, C., HARTUNG, T., SEYDEL, U., & SCHROMM, A.B. (2006). Cell activation of human macrophages by lipoteichoic acid is strongly attenuated by lipopolysaccharide-binding protein. *J Biol Chem* 281, 31448-31456.
- MUNOZ, L.E., GAJPL, U.S., FRANZ, S., SHERIFF, A., VOLL, R.E., KALDEN, J.R., & HERRMANN, M. (2005). SLE--a disease of clearance deficiency? *Rheumatology (Oxford)* 44, 1101-1107.
- MURRAY, J., BARBARA, J.A., DUNKLEY, S.A., LOPEZ, A.F., VAN, O., X, CONDLIFFE, A.M., DRANSFIELD, I., HASLETT, C., & CHILVERS, E.R. (1997). Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood* 90, 2772-2783.
- MURRAY, P.J. (2007). The JAK-STAT signaling pathway: input and output integration. *J Immunol* 178, 2623-2629.
- NAISMITH, J.H., BRANDHUBER, B.J., DEVINE, T.Q., & SPRANG, S.R. (1996). Seeing double: crystal structures of the type I TNF receptor. *J Mol Recognit* 9, 113-117.

NAISMITH, J.H. & SPRANG, S.R. (1998). Modularity in the TNF-receptor family. *Trends Biochem Sci* 23, 74-79.

NAKAE, S., SAIJO, S., HORAI, R., SUDO, K., MORI, S., & IWAKURA, Y. (2003). IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc Natl Acad Sci U S A* 100, 5986-5990.

NAKAGAWA, R., NAKA, T., TSUTSUI, H., FUJIMOTO, M., KIMURA, A., ABE, T., SEKI, E., SATO, S., TAKEUCHI, O., TAKEDA, K., AKIRA, S., YAMANISHI, K., KAWASE, I., NAKANISHI, K., & KISHIMOTO, T. (2002). SOCS-1 participates in negative regulation of LPS responses. *Immunity* 17, 677-687.

NAKAYA, M., TANAKA, M., OKABE, Y., HANAYAMA, R., & NAGATA, S. (2006). Opposite effects of rho family GTPases on engulfment of apoptotic cells by macrophages. *J Biol Chem* 281, 8836-8842.

NATHAN, C. (2002). Points of control in inflammation. *Nature* 420, 846-852.

NEWTON, R.C., SOLOMON, K.A., COVINGTON, M.B., DECICCO, C.P., HALEY, P.J., FRIEDMAN, S.M., & VADDI, K. (2001). Biology of TACE inhibition. *Ann Rheum Dis* 60 Suppl 3, iii25-iii32.

O'NEILL, L.A. & BOWIE, A.G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 7, 353-364.

O'SHEA, J.J. & MURRAY, P.J. (2008). Cytokine signaling modules in inflammatory responses. *Immunity* 28, 477-487.

ODA, K. & KITANO, H. (2006). A comprehensive map of the toll-like receptor signaling network. *Mol Syst Biol* 2, 2006.

OGDEN, C.A., POUND, J.D., BATH, B.K., OWENS, S., JOHANNESSEN, I., WOOD, K., & GREGORY, C.D. (2005). Enhanced apoptotic cell clearance capacity and B cell survival factor production by IL-10-activated macrophages: implications for Burkitt's lymphoma. *J Immunol* 174, 3015-3023.

OKA, K., SAWAMURA, T., KIKUTA, K., ITOKAWA, S., KUME, N., KITA, T., & MASAKI, T. (1998). Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci U S A* 95, 9535-9540.

OLD, L.J. (1988). Tumor necrosis factor. *Sci Am* 258, 59-75.

OLDENBORG, P.A., GRESHAM, H.D., & LINDBERG, F.P. (2001). CD47-signal regulatory protein alpha (SIRPalpha) regulates Fcgamma and complement receptor-mediated phagocytosis. *J Exp Med* 193, 855-862.

OLDENBORG, P.A., ZHELEZNYAK, A., FANG, Y.F., LAGENAUR, C.F., GRESHAM, H.D., & LINDBERG, F.P. (2000). Role of CD47 as a marker of self on red blood cells. *Science* 288, 2051-2054.

OROPEZA-RENDON, R.L., SPETH, V., HILLER, G., WEBER, K., & FISCHER, H. (1979). Prostaglandin E1 reversibly induces morphological changes in macrophages and inhibits phagocytosis. *Exp Cell Res* 119, 365-371.

ORR, A.W., PEDRAZA, C.E., PALLERO, M.A., ELZIE, C.A., GOICOECHEA, S., STRICKLAND, D.K., & MURPHY-ULLRICH, J.E. (2003). Low density lipoprotein receptor-related protein is a calreticulin coreceptor that signals focal adhesion disassembly. *J Cell Biol* 161, 1179-1189.

PARENTE, L. & SOLITO, E. (2004). Annexin 1: more than an anti-phospholipase protein. *Inflamm Res* 53, 125-132.

PARK, D., HOCHREITER-HUFFORD, A., & RAVICHANDRAN, K.S. (2009). The phosphatidylserine receptor TIM-4 does not mediate direct signaling. *Curr Biol* 19, 346-351.

PARK, D., TOSELLO-TRAMPONT, A.C., ELLIOTT, M.R., LU, M., HANEY, L.B., MA, Z., KLIBANOV, A.L., MANDELL, J.W., & RAVICHANDRAN, K.S. (2007). BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature* 450, 430-434.

PARK, S.Y., JUNG, M.Y., KIM, H.J., LEE, S.J., KIM, S.Y., LEE, B.H., KWON, T.H., PARK, R.W., & KIM, I.S. (2008). Rapid cell corpse clearance

by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death Differ* 15, 192-201.

PERKINS, N.D. (2000). The Rel/NF-kappa B family: friend and foe. *Trends Biochem Sci* 25, 434-440.

PETER, C., WAIBEL, M., RADU, C.G., YANG, L.V., WITTE, O.N., SCHULZE-OSTHOFF, K., WESSELBORG, S., & LAUBER, K. (2008). Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *J Biol Chem* 283, 5296-5305.

PHILLIPS, M.L., SCHWARTZ, B.R., ETZIONI, A., BAYER, R., OCHS, H.D., PAULSON, J.C., & HARLAN, J.M. (1995). Neutrophil adhesion in leukocyte adhesion deficiency syndrome type 2. *J Clin Invest* 96, 2898-2906.

PLATT, N., SUZUKI, H., KURIHARA, Y., KODAMA, T., & GORDON, S. (1996). Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proc Natl Acad Sci U S A* 93, 12456-12460.

PRICE, B.E., RAUCH, J., SHIA, M.A., WALSH, M.T., LIEBERTHAL, W., GILLIGAN, H.M., O'LAUGHLIN, T., KOH, J.S., & LEVINE, J.S. (1996). Anti-phospholipid autoantibodies bind to apoptotic, but not viable, thymocytes in a beta 2-glycoprotein I-dependent manner. *J Immunol* 157, 2201-2208.

PROBERT, L., AKASSOGLU, K., PASPARAKIS, M., KONTOGEORGOS, G., & KOLLIAS, G. (1995). Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor alpha. *Proc Natl Acad Sci U S A* 92, 11294-11298.

QIN, H., WILSON, C.A., ROBERTS, K.L., BAKER, B.J., ZHAO, X., & BENVENISTE, E.N. (2006). IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3. *J Immunol* 177, 7761-7771.

RAVICHANDRAN, K.S. & LORENZ, U. (2007). Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol* 7, 964-974.

RAYCHAUDHURI, B., FISHER, C.J., FARVER, C.F., MALUR, A., DRAZBA, J., KAVURU, M.S., & THOMASSEN, M.J. (2000). Interleukin 10

(IL-10)-mediated inhibition of inflammatory cytokine production by human alveolar macrophages. *Cytokine* 12, 1348-1355.

REARTE, B., LANDONI, V., LABORDE, E., FERNANDEZ, G., & ISTURIZ, M. (2010). Differential effects of glucocorticoids in the establishment and maintenance of endotoxin tolerance. *Clin Exp Immunol* 159, 208-216.

REDDIEN, P.W. & HORVITZ, H.R. (2000). CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*. *Nat Cell Biol* 2, 131-136.

REICHARDT, H.M., TUCKERMANN, J.P., GOTTLICHER, M., VUJIC, M., WEIH, F., ANGEL, P., HERRLICH, P., & SCHUTZ, G. (2001). Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *EMBO J* 20, 7168-7173.

REN, Y. & SAVILL, J. (1995). Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. *J Immunol* 154, 2366-2374.

REN, Y., SILVERSTEIN, R.L., ALLEN, J., & SAVILL, J. (1995). CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis. *J Exp Med* 181, 1857-1862.

REN, Y., STUART, L., LINDBERG, F.P., ROSENKRANZ, A.R., CHEN, Y., MAYADAS, T.N., & SAVILL, J. (2001). Nonphlogistic clearance of late apoptotic neutrophils by macrophages: efficient phagocytosis independent of beta 2 integrins. *J Immunol* 166, 4743-4750.

REN, Y., TANG, J., MOK, M.Y., CHAN, A.W., WU, A., & LAU, C.S. (2003). Increased apoptotic neutrophils and macrophages and impaired macrophage phagocytic clearance of apoptotic neutrophils in systemic lupus erythematosus. *Arthritis Rheum* 48, 2888-2897.

REN, Y., XIE, Y., JIANG, G., FAN, J., YEUNG, J., LI, W., TAM, P.K., & SAVILL, J. (2008). Apoptotic cells protect mice against lipopolysaccharide-induced shock. *J Immunol* 180, 4978-4985.

RODIG, S.J., MERAZ, M.A., WHITE, J.M., LAMPE, P.A., RILEY, J.K., ARTHUR, C.D., KING, K.L., SHEEHAN, K.C., YIN, L., PENNICA, D., JOHNSON, E.M., JR., & SCHREIBER, R.D. (1998). Disruption of the Jak1

gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell* 93, 373-383.

ROSSATO, M., CENCIG, S., GASPERINI, S., CASSATELLA, M.A., & BAZZONI, F. (2007). IL-10 modulates cytokine gene transcription by protein synthesis-independent and dependent mechanisms in lipopolysaccharide-treated neutrophils. *Eur J Immunol* 37, 3176-3189.

ROSSI, A.G., HASLETT, C., HIRANI, N., GREENING, A.P., RAHMAN, I., METZ, C.N., BUCALA, R., & DONNELLY, S.C. (1998a). Human circulating eosinophils secrete macrophage migration inhibitory factor (MIF). Potential role in asthma. *J Clin Invest* 101, 2869-2874.

ROSSI, A.G., MCCUTCHEON, J.C., ROY, N., CHILVERS, E.R., HASLETT, C., & DRANSFIELD, I. (1998b). Regulation of macrophage phagocytosis of apoptotic cells by cAMP. *J Immunol* 160, 3562-3568.

ROSSI, A.G., SAWATZKY, D.A., WALKER, A., WARD, C., SHELDRAKE, T.A., RILEY, N.A., CALDICOTT, A., MARTINEZ-LOSA, M., WALKER, T.R., DUFFIN, R., GRAY, M., CRESCENZI, E., MARTIN, M.C., BRADY, H.J., SAVILL, J.S., DRANSFIELD, I., & HASLETT, C. (2006). Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nat Med* 12, 1056-1064.

ROUZER, C.A., KINGSLEY, P.J., WANG, H., ZHANG, H., MORROW, J.D., DEY, S.K., & MARNETT, L.J. (2004). Cyclooxygenase-1-dependent prostaglandin synthesis modulates tumor necrosis factor-alpha secretion in lipopolysaccharide-challenged murine resident peritoneal macrophages. *J Biol Chem* 279, 34256-34268.

ROVERE, P., MANFREDI, A.A., VALLINOTO, C., ZIMMERMANN, V.S., FASCIO, U., BALESTRIERI, G., RICCIARDI-CASTAGNOLI, P., RUGARLI, C., TINCANI, A., & SABBADINI, M.G. (1998). Dendritic cells preferentially internalize apoptotic cells opsonized by anti-beta2-glycoprotein I antibodies. *J Autoimmun* 11, 403-411.

ROY, N., DEVERAUX, Q.L., TAKAHASHI, R., SALVESEN, G.S., & REED, J.C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J* 16, 6914-6925.

RUEGG, C., YILMAZ, A., BIELER, G., BAMAT, J., CHAUBERT, P., & LEJEUNE, F.J. (1998). Evidence for the involvement of endothelial cell

integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med* 4, 408-414.

SALMON, J.E., KIMBERLY, R.P., GIBOFSKY, A., & FOTINO, M. (1984). Defective mononuclear phagocyte function in systemic lupus erythematosus: dissociation of Fc receptor-ligand binding and internalization. *J Immunol* 133, 2525-2531.

SALVESEN, G.S. & DUCKETT, C.S. (2002). IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* 3, 401-410.

SATO, K., YAMASHITA, N., & MATSUYAMA, T. (2002). Human peripheral blood monocyte-derived interleukin-10-induced semi-mature dendritic cells induce anergic CD4(+) and CD8(+) T cells via presentation of the internalized soluble antigen and cross-presentation of the phagocytosed necrotic cellular fragments. *Cell Immunol* 215, 186-194.

SAUTER, B., ALBERT, M.L., FRANCISCO, L., LARSSON, M., SOMERSAN, S., & BHARDWAJ, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 191, 423-434.

SAVILL, J., DRANSFIELD, I., GREGORY, C., & HASLETT, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol* 2, 965-975.

SAVILL, J.S., WYLLIE, A.H., HENSON, J.E., WALPORT, M.J., HENSON, P.M., & HASLETT, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 83, 865-875.

SCAFFIDI, P., MISTELI, T., & BIANCHI, M.E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418, 191-195.

SCALLON, B.J., MOORE, M.A., TRINH, H., KNIGHT, D.M., & GHAYEB, J. (1995a). Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. *Cytokine* 7, 251-259.

- SCALLON, B.J., TRINH, H., NEDELMAN, M., BRENNAN, F.M., FELDMANN, M., & GHAYEB, J. (1995b). Functional comparisons of different tumour necrosis factor receptor/IgG fusion proteins. *Cytokine* 7, 759-770.
- SCANNELL, M. & MADERNA, P. (2006). Lipoxins and annexin-1: resolution of inflammation and regulation of phagocytosis of apoptotic cells. *ScientificWorldJournal* 6, 1555-1573.
- SCHIEVELLA, A.R., CHEN, J.H., GRAHAM, J.R., & LIN, L.L. (1997). MADD, a novel death domain protein that interacts with the type 1 tumor necrosis factor receptor and activates mitogen-activated protein kinase. *J Biol Chem* 272, 12069-12075.
- SCHRODER, N.W., MORATH, S., ALEXANDER, C., HAMANN, L., HARTUNG, T., ZHRINGER, U., GOBEL, U.B., WEBER, J.R., & SCHUMANN, R.R. (2003). Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J Biol Chem* 278, 15587-15594.
- SERHAN, C.N. (1997). Lipoxins and novel aspirin-triggered 15-epi-lipoxins (ATL): a jungle of cell-cell interactions or a therapeutic opportunity? *Prostaglandins* 53, 107-137.
- SERHAN, C.N., BRAIN, S.D., BUCKLEY, C.D., GILROY, D.W., HASLETT, C., O'NEILL, L.A., PERRETTI, M., ROSSI, A.G., & WALLACE, J.L. (2007). Resolution of inflammation: state of the art, definitions and terms. *FASEB J* 21, 325-332.
- SERHAN, C.N. & SAVILL, J. (2005). Resolution of inflammation: the beginning programs the end. *Nat Immunol* 6, 1191-1197.
- SHIMAZU, R., AKASHI, S., OGATA, H., NAGAI, Y., FUKUDOME, K., MIYAKE, K., & KIMOTO, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 189, 1777-1782.
- SHU, H.B., TAKEUCHI, M., & GOEDDEL, D.V. (1996). The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proc Natl Acad Sci U S A* 93, 13973-13978.

SIMMONDS, R.E. & FOXWELL, B.M. (2008). Signalling, inflammation and arthritis: NF-kappaB and its relevance to arthritis and inflammation. *Rheumatology (Oxford)* 47, 584-590.

SKINNER, S.J., DELEAULT, K.M., FECTEAU, R., & BROOKS, S.A. (2008). Extracellular signal-regulated kinase regulation of tumor necrosis factor-alpha mRNA nucleocytoplasmic transport requires TAP-NxT1 binding and the AU-rich element. *J Biol Chem* 283, 3191-3199.

SMITH, C.A., FARRAH, T., & GOODWIN, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 76, 959-962.

SONG, Z., YAO, X., & WU, M. (2003). Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. *J Biol Chem* 278, 23130-23140.

STERN, M., SAVILL, J., & HASLETT, C. (1996). Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha v beta 3/CD36/thrombospondin recognition mechanism and lack of phlogistic response. *Am J Pathol* 149, 911-921.

SU, S.C., HUA, K.F., LEE, H., CHAO, L.K., TAN, S.K., LEE, H., YANG, S.F., & HSU, H.Y. (2006). LTA and LPS mediated activation of protein kinases in the regulation of inflammatory cytokines expression in macrophages. *Clin Chim Acta* 374, 106-115.

SUGARMAN, B.J., AGGARWAL, B.B., HASS, P.E., FIGARI, I.S., PALLADINO, M.A., JR., & SHEPARD, H.M. (1985). Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. *Science* 230, 943-945.

TAGORE, A., GONSALKORALE, W.M., PRAVICA, V., HAJEER, A.H., MCMAHON, R., WHORWELL, P.J., SINNOTT, P.J., & HUTCHINSON, I.V. (1999). Interleukin-10 (IL-10) genotypes in inflammatory bowel disease. *Tissue Antigens* 54, 386-390.

TAKEDA, K., CLAUSEN, B.E., KAISHO, T., TSUJIMURA, T., TERADA, N., FORSTER, I., & AKIRA, S. (1999). Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10, 39-49.

TAKIZAWA, F., TSUJI, S., & NAGASAWA, S. (1996). Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. *FEBS Lett* 397, 269-272.

TARTAGLIA, L.A., AYRES, T.M., WONG, G.H., & GOEDEL, D.V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell* 74, 845-853.

TAYAL, V. & KALRA, B.S. (2008). Cytokines and anti-cytokines as therapeutics--an update. *Eur J Pharmacol* 579, 1-12.

TEDER, P., VANDIVIER, R.W., JIANG, D., LIANG, J., COHN, L., PURE, E., HENSON, P.M., & NOBLE, P.W. (2002). Resolution of lung inflammation by CD44. *Science* 296, 155-158.

TOWBIN, H., STAEBELIN, T., & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76, 4350-4354.

TRACEY, D., KLARESKOG, L., SASSO, E.H., SALFELD, J.G., & TAK, P.P. (2008). Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther* 117, 244-279.

TRAVASSOS, L.H., GIRARDIN, S.E., PHILPOTT, D.J., BLANOT, D., NAHORI, M.A., WERTS, C., & BONECA, I.G. (2004). Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep* 5, 1000-1006.

TRUMAN, L.A., FORD, C.A., PASIKOWSKA, M., POUND, J.D., WILKINSON, S.J., DUMITRIU, I.E., MELVILLE, L., MELROSE, L.A., OGDEN, C.A., NIBBS, R., GRAHAM, G., COMBADIÈRE, C., & GREGORY, C.D. (2008). CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood* 112, 5026-5036.

VAN PUIJENBROEK, A.A., WISSINK, S., VAN DER SAAG, P.T., & PEPPELENBOSCH, M.P. (1999). Phospholipase A2 inhibitors and leukotriene synthesis inhibitors block TNF-induced NF-kappaB activation. *Cytokine* 11, 104-110.

VAN WEEMEN, B.K. (2005). The rise of EIA/ELISA. *Clin Chem* 51, 2226.

- VAN WEEMEN, B.K. & SCHUURS, A.H. (1974). Immunoassay using antibody--enzyme conjugates. *FEBS Lett* 43, 215-218.
- VANDEN, B.T., KALAI, M., DENECKER, G., MEEUS, A., SAELENS, X., & VANDENABEELE, P. (2006). Necrosis is associated with IL-6 production but apoptosis is not. *Cell Signal* 18, 328-335.
- VANDENBROUCKE, K., DE, H.H., BEIRNAERT, E., DREIER, T., LAUWEREYS, M., HUYCK, L., VAN, H.J., DEMETTER, P., STEIDLER, L., REMAUT, E., CUVELIER, C., & ROTTIERS, P. (2010). Orally administered *L. lactis* secreting an anti-TNF Nanobody demonstrate efficacy in chronic colitis. *Mucosal Immunol* 3, 49-56.
- VANDIVIER, R.W., FADOK, V.A., HOFFMANN, P.R., BRATTON, D.L., PENVARI, C., BROWN, K.K., BRAIN, J.D., ACCURSO, F.J., & HENSON, P.M. (2002a). Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest* 109, 661-670.
- VANDIVIER, R.W., OGDEN, C.A., FADOK, V.A., HOFFMANN, P.R., BROWN, K.K., BOTTO, M., WALPORT, M.J., FISHER, J.H., HENSON, P.M., & GREENE, K.E. (2002b). Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J Immunol* 169, 3978-3986.
- VERMA, I.M., STEVENSON, J.K., SCHWARZ, E.M., VAN, A.D., & MIYAMOTO, S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* 9, 2723-2735.
- VERMEIRE, K., HEREMANS, H., VANDEPUTTE, M., HUANG, S., BILLIAU, A., & MATTHYS, P. (1997). Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol* 158, 5507-5513.
- VERMES, I., HAANEN, C., STEFFENS-NAKKEN, H., & REUTELINGSPERGER, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 184, 39-51.
- VOLL, R.E., HERRMANN, M., ROTH, E.A., STACH, C., KALDEN, J.R., & GIRKONTAITE, I. (1997). Immunosuppressive effects of apoptotic cells. *Nature* 390, 350-351.

WAJANT, H., HAAS, E., SCHWENZER, R., MUHLENBECK, F., KREUZ, S., SCHUBERT, G., GRELL, M., SMITH, C., & SCHEURICH, P. (2000). Inhibition of death receptor-mediated gene induction by a cycloheximide-sensitive factor occurs at the level of or upstream of Fas-associated death domain protein (FADD). *J Biol Chem* 275, 24357-24366.

WAJANT, H., PFIZENMAIER, K., & SCHEURICH, P. (2003). Tumor necrosis factor signaling. *Cell Death Differ* 10, 45-65.

WAKASUGI, K. & SCHIMMEL, P. (1999). Highly differentiated motifs responsible for two cytokine activities of a split human tRNA synthetase. *J Biol Chem* 274, 23155-23159.

WALLACH, D., ENGELMANN, H., NOPHAR, Y., ADERKA, D., KEMPER, O., HORNIK, V., HOLTSMANN, H., & BRAKEBUSCH, C. (1991). Soluble and cell surface receptors for tumor necrosis factor. *Agents Actions Suppl* 35, 51-57.

WALLIS, R.S. (2008). Tumour necrosis factor antagonists: structure, function, and tuberculosis risks. *Lancet Infect Dis* 8, 601-611.

WALSH, G.M., SEXTON, D.W., & BLAYLOCK, M.G. (2003). Corticosteroids, eosinophils and bronchial epithelial cells: new insights into the resolution of inflammation in asthma. *J Endocrinol* 178, 37-43.

WALSH, G.M., SEXTON, D.W., BLAYLOCK, M.G., & CONVERY, C.M. (1999). Resting and cytokine-stimulated human small airway epithelial cells recognize and engulf apoptotic eosinophils. *Blood* 94, 2827-2835.

WANG, J.E., JORGENSEN, P.F., ALMLOF, M., THIEMERMANN, C., FOSTER, S.J., AASEN, A.O., & SOLBERG, R. (2000). Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. *Infect Immun* 68, 3965-3970.

WARD, C., CHILVERS, E.R., LAWSON, M.F., PRYDE, J.G., FUJIHARA, S., FARROW, S.N., HASLETT, C., & ROSSI, A.G. (1999). NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro. *J Biol Chem* 274, 4309-4318.

WARD, C., MURRAY, J., CLUGSTON, A., DRANSFIELD, I., HASLETT, C., & ROSSI, A.G. (2005). Interleukin-10 inhibits lipopolysaccharide-

induced survival and extracellular signal-regulated kinase activation in human neutrophils. *Eur J Immunol* 35, 2728-2737.

WEI, X., GONG, J., ZHU, J., NIU, L., ZHU, W., LI, N., & LI, J. (2008). Therapeutic effects of triptolide on interleukin-10 gene-deficient mice with colitis. *Int Immunopharmacol* 8, 1808-1812.

WEIHUA, Z., TSAN, R., SCHROIT, A.J., & FIDLER, I.J. (2005). Apoptotic cells initiate endothelial cell sprouting via electrostatic signaling. *Cancer Res* 65, 11529-11535.

WILLIAMS, L.M., GIBBONS, D.L., GEARING, A., MAINI, R.N., FELDMANN, M., & BRENNAN, F.M. (1996). Paradoxical effects of a synthetic metalloproteinase inhibitor that blocks both p55 and p75 TNF receptor shedding and TNF alpha processing in RA synovial membrane cell cultures. *J Clin Invest* 97, 2833-2841.

WILLIAMS, R.O., MARINOVA-MUTAFCHIEVA, L., FELDMANN, M., & MAINI, R.N. (2000). Evaluation of TNF-alpha and IL-1 blockade in collagen-induced arthritis and comparison with combined anti-TNF-alpha/anti-CD4 therapy. *J Immunol* 165, 7240-7245.

WILLIAMS, R.O., PALEOLOG, E., & FELDMANN, M. (2007). Cytokine inhibitors in rheumatoid arthritis and other autoimmune diseases. *Curr Opin Pharmacol* 7, 412-417.

WILLIAMS, R.O., WILLIAMS, D.G., FELDMANN, M., & MAINI, R.N. (1993). Increased limb involvement in murine collagen-induced arthritis following treatment with anti-interferon-gamma. *Clin Exp Immunol* 92, 323-327.

WILLIAMSON, P. & SCHLEGEL, R.A. (2004). Hide and seek: the secret identity of the phosphatidylserine receptor. *J Biol* 3, 14.

WILLIAMSON, P. & SCHLEGEL, R.A. (2002). Transbilayer phospholipid movement and the clearance of apoptotic cells. *Biochim Biophys Acta* 1585, 53-63.

WITKO-SARSAT, V., RIEU, P., SCAMPS-LATSCHA, B., LESAVRE, P., & HALBWACHS-MECARELLI, L. (2000). Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 80, 617-653.

WOOLLEY, K.L., GIBSON, P.G., CARTY, K., WILSON, A.J., TWADDELL, S.H., & WOOLLEY, M.J. (1996). Eosinophil apoptosis and the resolution of airway inflammation in asthma. *Am J Respir Crit Care Med* 154, 237-243.

WRIGHT, S.D., RAMOS, R.A., TOBIAS, P.S., ULEVITCH, R.J., & MATHISON, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249, 1431-1433.

WYLLIE, A.H., KERR, J.F., & CURRIE, A.R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* 68, 251-306.

XIA, C.Q., PENG, R., BEATO, F., & CLARE-SALZLER, M.J. (2005). Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity. *Scand J Immunol* 62, 45-54.

XIAO, G., RABSON, A.B., YOUNG, W., QING, G., & QU, Z. (2006). Alternative pathways of NF-kappaB activation: a double-edged sword in health and disease. *Cytokine Growth Factor Rev* 17, 281-293.

XIAO, Y.Q., MALCOLM, K., WORTHEN, G.S., GARDAL, S., SCHIEMANN, W.P., FADOK, V.A., BRATTON, D.L., & HENSON, P.M. (2002). Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. *J Biol Chem* 277, 14884-14893.

XU, W., ROOS, A., SCHLAGWEIN, N., WOLTMAN, A.M., DAHA, M.R., & VAN, K.C. (2006). IL-10-producing macrophages preferentially clear early apoptotic cells. *Blood* 107, 4930-4937.

YAMAGATA, K., ANDREASSON, K.I., KAUFMANN, W.E., BARNES, C.A., & WORLEY, P.F. (1993). Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* 11, 371-386.

YAMAMOTO, K.R., DARIMONT, B.D., WAGNER, R.L., & INIGUEZ-LLUHI, J.A. (1998). Building transcriptional regulatory complexes: signals and surfaces. *Cold Spring Harb Symp Quant Biol* 63, 587-598.

YANG, G.X. & HAGMANN, W.K. (2003). VLA-4 antagonists: potent inhibitors of lymphocyte migration. *Med Res Rev* 23, 369-392.

YANG, Y., FANG, S., JENSEN, J.P., WEISSMAN, A.M., & ASHWELL, J.D. (2000). Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 288, 874-877.

YUSA, S., CATINA, T.L., & CAMPBELL, K.S. (2004). KIR2DL5 can inhibit human NK cell activation via recruitment of Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2). *J Immunol* 172, 7385-7392.

ZANGEMEISTER-WITTKE, U. & SIMON, H.U. (2004). An IAP in action: the multiple roles of survivin in differentiation, immunity and malignancy. *Cell Cycle* 3, 1121-1123.

ZHAO, M., SONG, B., PU, J., WADA, T., REID, B., TAI, G., WANG, F., GUO, A., WALCZYNSKO, P., GU, Y., SASAKI, T., SUZUKI, A., FORRESTER, J.V., BOURNE, H.R., DEVREOTES, P.N., MCCAIG, C.D., & PENNINGER, J.M. (2006). Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. *Nature* 442, 457-460.

ZHENG, L., HE, M., LONG, M., BLOMGRAN, R., & STENDAHL, O. (2004). Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages. *J Immunol* 173, 6319-6326.

ZHOU, Z., HARTWIEG, E., & HORVITZ, H.R. (2001). CED-1 is a transmembrane receptor that mediates cell corpse engulfment in *C. elegans*. *Cell* 104, 43-56.

APPENDIX ONE

PUBLICATIONS