

Investigation of the neutrophil-directed antiinflammatory properties of the cysteinyl leukotriene receptor antagonist, montelukast.

by

Cornelia Magdalena Lodder

(Gravett)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

In

The Department of Immunology

Faculty of Health Sciences

University of Pretoria

October 2011



Publications

Part of this thesis have been published in the following papers:

- Theron AJ, Gravett CM, Steel HC, Tintinger GR, Feldman C, Anderson R. Leukotriene C₄ and D₄ sensitize human neutrophils for hyperreactivity to chemoattractants. *Inflamm Res.* 2009; 57: 1-6.
- Anderson R, Theron AJ, Gravett CM, Steel HC, Tintinger GR, Feldman C. Montelukast inhibits neutrophil pro-inflammatory activity by a cyclic AMP-dependent mechanism. *Br J Pharmacol.* 2009; 156: 105-115.
- Gravett CM, Theron AJ, Steel HC, Tintinger GR, Cockeran R, Feldman C, Anderson R. Interactive inhibitory effects of formoterol and montelukast on activated human neutrophils. *Eur Respir J.* 2010; 36: 1417-1424.
- Gravett CM, Tintinger G, Theron A, Anderson R, Feldman C, Green R. Montelukast sodium: Administration to children to control intermittent asthma. *Clin Med Rev Therap*. 2010; 2: 1-10.



Acknowledgements

I want to sincerely thank the following people for their assistance in my research and accomplishing this thesis:

My supervisor, Prof. R. Anderson for giving me the opportunity to work in the department of Immunology and his guidance in the practical research and in writing these scientific research papers.

My co-supervisor, Prof. A Theron for constant motivation and guidance in the

laboratory work and in helping with pre-reading and preparation of this thesis.

Prof. G Tintinger for helping with laboratory work and scientific research.

Dr. H Steel and Dr R Cockeran for assistance in laboratory work and support.

Prof. C Feldman for believing in giving me a chance to do a PhD.

My husband for his unselfish support.

My mother for her prayers.

My partners, Drs V Diab and A Nortje for the time out of practice to do my research at the University.

Ms M Madgwick in preparing this manuscript.



Abstract

Montelukast (ML) is primarily an antagonist of type 1 cysteinyl leukotriene receptors (CysLT₁R), an activity which underpins its therapeutic efficacy in bronchial asthma. However, ML has also been reported to be useful in the treatment of acute and chronic inflammatory disorders of both infective and noninfective origin in which CysLTs are unlikely to be the predominant mediators of harmful inflammatory responses. These include conditions such as chronic obstructive pulmonary disease and cystic fibrosis in which the neutrophil is believed to be the primary offender, suggesting that ML may possess neutrophiltargeted, CysLT₁R-independent mechanisms of anti-inflammatory activity. Accordingly, the laboratory research presented in this thesis was designed with the primary objectives of characterizing possible CysLT₁R-dependent and independent neutrophil-targeted anti-inflammatory activities of ML in vitro, and consisted of 3 phases. These were investigation of: i) the effects of the CvsLTs, LTC₄ and LTD₄ (in the absence and presence of ML) on mobilization of intracellular Ca²⁺ stores, generation of reactive oxygen species (ROS) and release of primary and secondary granule proteinases; ii) the effects of ML on a series of proinflammatory activities of neutrophils following activation of the cells with the chemoattractants FMLP and platelet-activating factor (PAF); and iii) the interactive, anti-inflammatory effects on neutrophils of ML in combination with the long-acting beta-2 agonist, formoterol. In addition to the aforementioned activities, measurement of the production and expression of CR3, as well as generation of inositol triphosphate (IP3), cyclic AMP, and activities of the enzymes cAMP- and



cGMP-phosphodiesterases (PDEs) in isolated neutrophil cytosol and membrane fractions, were also included.

The following assays were used: i) chemiluminescence procedures for the detection of ROS; ii) a colourimetric procedure for the detection of elastase; iii) ELISA procedures for the detection of the matrix metalloproteinases (MMPs) 8- and -9, LTB₄, and cyclic AMP; iv) fura-2-based spectrofluorimetry and a radiometric procedure for monitoring cytosolic Ca²⁺ fluxes; v) flow cytometry for CR3; and vi) radioassays for IP3 and activity of cAMP- and cGMP-PDEs.

Exposure of neutrophils to LTD₄, but not LTC₄, activated a very modest and transient increase in cytosolic Ca²⁺, but failed to initiate the generation of ROS or release of elastase or MMP-8. However, brief pre-treatment with either LTC₄ or LTD₄ sensitized the cells for increased production of ROS and release of granule proteinases following activation with FMLP, which was partially attenuated by inclusion of ML. In the second part of the study, pre-treatment of neutrophils with ML, at therapeutically relevant concentrations, resulted in dose-related inhibition of the FMLP- or PAF-activated generation of ROS and LTB₄, as well as the release of elastase, with the former being unaffected by an inhibitor of 5-lipoxygenase (MK886), compatible with a CysLT₁R-independent mechanism of anti-inflammatory activity. From a mechanistic perspective, these interactions of ML with neutrophils were associated with accelerated clearance of Ca²⁺ from the cytosol of the cells which could not be attributed to inhibition of production of IP3, but was, however,



specific inhibition of cyclic nucleotide phosphodiesterases. In the third part of the study, combining ML with formoterol caused (in most cases) additive inhibitory effects on the generation of ROS and LTB_4 , release of granule proteinases, as well as expression of CR3, which again were associated with elevations in cAMP and interference with Ca²⁺ mobilization.

In conclusion, ML appears to attenuate neutrophil activation by CysLT1Rdependent and –independent mechanisms. In the case of the former by interfering with the modest sensitizing (priming) interactions of LTC₄ and LTD₄ with neutrophils, and in the latter by inhibition of PDEs, leading a to sustained elevation in cAMP, resulting in rapid clearance of Ca²⁺ from the cytosol and decreased uptake of the cation from the extracellular milieu.



Abstrak:

Montelukast (ML) is primêr 'n antagonis van tipe 1 sisteniel leukotriene reseptore (CysLT₁R), 'n aktiwiteit wat die terapeutiese effektiwiteit daarvan in brongiale asma ondersteun. ML is egter ook beskryf om effektief te wees in die behandeling van akute en chroniese inflammatoriese toestande van beide infektiewe en nieinfektiewe oorsprong waar die CysLTs onwaarskynlik die predominante skadelike inflammatoriese bemiddelaars is. Hierdie toestande sluit chroniese obstruktiewe lugwegsiekte en sistiese fibrose in, waar die neutrofiel waarskynlik die primêre skadelike agent is, wat dan impliseer dat ML moontlik neutrofiel-gerigte, CysLT₁Ronafhanklike meganismes van anti-inflammatoriese aktiwiteite besit. Die laboratoratorium navorsing wat in hierdie verhandeling uiteengesit word, is ontwerp met die primêre doel om die moontlike CysLTR-afhanlike en-onafhanlike neutrofielgerigte anti-inflammatoriese aktiwiteite van ML in vitro, te karakteriseer, en bestaan uit 3 fases. Dit sluit in: i) die uitwerking van die CysLT, LTC₄ en LTD₄ (met of sonder ML) op die mobilisasie van intrasellulêre Ca²⁺ store, generasie van reaktiewe suurstof spesies (ROS) en die vrystelling van primêre en sekondêre granule proteïenases; ii) die uitwerking van ML op 'n reeks pro-inflammatoriese aktiwiteite van neutrofiele na aktivering van die selle met die leukolokmiddels FMLP en plaatjie-aktiveringsfaktor (PAF); en iii) die interaktiewe, antiinflammatoriese uitwerking op neutrofiele van ML in kombinasie met die langwerkende beta-2 agonis, formoterol. Bo en behalwe die bogenoemde aktiwiteite, is die bepaling van die produksie en uitdrukking van CR3, sowel as die

vii



generasie van inositol trifosfaat (IP3), sikliese AMP (cAMP), en aktiwiteite van die ensieme cAMP en cGMP-fosfodiesterases in geïsoleerde neutrofiel sitosol en membraanfraksies, ook ingesluit.

Die volgende toetse is gebruik: i) chemiluminessensie prosedures vir die meting van ROS; ii) 'n kleurbepaling prosedure vir die waarneming van elastase; iii) ELISA prosedures vir waarneming van matriksmetalloproteïenases (MMPs) 8 en -9, LTB₄ en sikliese AMP; iv) fura-2-gebaseerde spektrofluorometrie en 'n radiometriese prosedure vir monitering van sitosoliese Ca²⁺ flukse; v) vloeisitometrie vir CR3; en vi) radiometriese toetse vir IP3 en die aktiwiteit van cAMP-en cGMP-PDEs.

Blootstelling van neutrofiele aan LTD₄, maar nie LTC₄ nie, het 'n matige en verbygaande verhoging van sitosoliese Ca²⁺ geaktiveer maar het nie die generasie van ROS of die vrystelling van elastase en MMP-8, geinisieer nie. Kort voorafgaande blootstelling aan of LTC₄ of LTD₄ het egter die selle gesensitiseer vir verhoogde produksie van ROS en vrystelling van granule proteïenases na aktivering met FMLP wat gedeeltelik deur ML verminder is. In die tweede gedeelte van die studie het voorafbehandeling van neutrofiele met ML, teen terapeuties relevante konsentrasies, gelei tot dosis verwante inhibisie van FMLP- of PAF-geaktiveerde generasie van ROS en LTB₄ asook die vrystelling van elastase, met die eersgenoemde wat ongeaffekteerd was deur 'n 5-lipoksigenase inhibibeerder (MK886), wat verenigbaar is met 'n CysLT₁R-onafhanklike meganisme van anti-inflammatoriese aktiwiteit. Vanuit 'n meganistiese perspektief, was hierdie interaksies van ML met neutrofiele geassosieer met versnelde opruiming van Ca²⁺ van die sitosol van die selle wat nie toegeskryf kan word aan inhibisie van IP3



produksie nie, maar egter geassosieer was met verhoogde vlakke van cAMP, oënskynlik as gevolg van nie-spesifieke inhibisie van sikliese nukleotiedfosfodiesterases. In die derde deel van die studie het die kombinasie van ML en formoterol (in meeste gevalle) additiewe inhibitoriese effekte op die generasie van ROS en LTB₄, die vrystelling van granule proteïenases asook die uitdrukking van CR3 gehad wat ook geassosieer was met verhoging van cAMP en inmenging met Ca²⁺ mobilisasie.

Samevattend, blyk ML om neutrofiel aktivering te verminder deur CysLT-afhanklike en –onafhanklike meganismes. Eersgenoemde as gevolg van matige sensitiseringinteraksies van LTC₄ en LTD₄ met neutrofiele en laasgenoemde deur inhibisie van PDEs wat volgehoue verhoging van cAMP tot gevolg het en aanleiding gee tot versnelde opruiming van Ca²⁺ van die sitosol en 'n vermindering in opname van die katioon van die ekstasellulêre milieu.



Table of Contents

Publications	ii
Acknowledgements	iii
Abstract	iv
Abstrak	vii
Table of Contents	x
List of Figures	civ
List of Tables	xvii
Abbreviationsx	viii

Chapter 1: Introduction and Literature Review

1.1	Introc	luction		2
1.2	Litera	ture Rev	iew	2
	1.2.1	Resting	Neutrophils	2
	1.2.2	Migratio	n of Neutrophils	3
		1.2.2.1	Margination	3
		1.2.2.2	Transmigration	4
		1.2.2.3	Transendothelial migration in the Lung	6
	1.2.3	Chemota	actic Mediators	7
		1.2.3.1	Chemokines	7
		1.2.3.2	Lipid Mediators	7
	1.2.4	Neutrop	hil Priming and Activation	8
		1.2.4.1	Priming	8
		1.2.4.2	Calcium Homeostasis	10
	1.2.5	Activate	d Neutrophils	15
		1.2.5.1	Proteases	15
			1.2.5.1.1 Serine Proteases	17



			1.2.5.1.2 Matrix Metalloproteinases	19
		1.2.5.2	Oxygen Radicals	21
		1.2.5.3	Leukotriene B₄	26
	1.2.6	Neutrop	nil Clearance	30
	1.2.7	Asthma.		31
		1.2.7.1	Neutrophils in Asthma	35
		1.2.7.2	Cysteinyl Leukotrienes	37
		1.2.7.3	Treatment of Asthma	41
		1.2.7.4	New Targets for Treatment	43
			1.2.7.4.1 Phosphodiesterase Inhibitors	44
			1.2.7.4.2 Montelukast	46
1.3	Нуро	thesis		51

Chapter 2: Leukotrienes C₄ and D₄ Sensitize Human Neutrophils for Hyperreactivity to Chemoattractants

2.1	Introd	luction	54
2.2	Mater	ials and Methods	55
	2.2.1	Leukotrienes	55
	2.2.2	Preparation of neutrophils	55
	2.2.3	Spectrofluorimetric Measurement of Cytosolic Ca ²⁺	56
	2.2.4	Measurement of Superoxide Production	57
	2.2.5	Elastase and MMP-8 Release	58
	2.2.6	Expression and Statistical Analysis of Results	59
2.3	Resul	ts	59
	2.3.1	Fura-2 Fluorescence Response	59
	2.3.2	Superoxide Production	60
	2.3.3	Elastase and MMP-8 release	61
2.4	Discu	ssion	61



Chapter 3: Montelukast Inhibits Neutrophil Pro-Inflammatory Activity by a Cyclic AMP-Dependent Mechanism

3.1	Introd	luction	73
3.2	Mater	ials and Methods	74
	3.2.1	Chemicals and Reagents	74
	3.2.2	Neutrophils	75
	3.2.3	Measurement of Reactive Oxygen Species	75
	3.2.4	NADPH Oxidase in Isolated Neutrophil Membranes	76
	3.2.5	Oxygen Consumption	77
	3.2.6	Elastase Release	77
	3.2.7	Spectrofluorimetric Measurement of Cytosolic Ca ²⁺	78
	3.2.8	Radiometric Assessment of Ca ²⁺ Influx	79
	3.2.9	Measurement of LTB ₄ and Cyclic cAMP	80
	3.2.10	PDE Activity	81
	3.2.11	Inositol Triphosphate	82
	3.2.11	Cellular ATP Levels	83
	3.2.12	2 Statistical Analysis	83
3.3	Resu	lts	84
	3.3.1	Production of ROS	84
	3.3.2	Elastase Release	86
	3.3.3	Leukotriene B₄	86
	3.3.4	Fura-2 Fluorescence Responses of Activated Neutrophils	86
	3.3.5	Ca ²⁺ Influx	88
	3.3.6	Cyclic cAMP	88
	3.3.7	PDE	88
	3.3.8	Inositol Triphosphate	91
	3.3.9	ATP Levels	92
3.4	Discu	ssion and Conclusions	93



Chapter 4: Ineractive Inhibitory Effects of Formoterol and Montelukast on Activated Human Neutrophils

4.1	Introc	luction	109
4.2	Mater	ials and Methods	111
	4.2.1	Chemicals and Reagents	111
	4.2.2	Preparation of Neutrophils	112
	4.2.3	Superoxide Generation	112
	4.2.4	Elastase Release	113
	4.2.5	CR3 Expression	114
	4.2.6	Spectrofuorimetric Measurement of Cytosolic Ca ²⁺	114
	4.2.7	Measurement of Leukotriene B_4 (LTB ₄) and Cyclic AMP (cAMP).	115
	4.2.8	Statistical Analysis	117
4.3	Resu	lts	117
	4.3.1	Superoxide Production, Elastase Release and Leukotriene B_{4}	117
	4.3.2	CR3 Expression	118
	4.3.3	Cyclic AMP	119
	4.3.4	Cytosolic Calcium	119
4.4	Discu	ssion	121
Cha	oter 5:	Final Conclusion	132

Chapter 6: References	13	3	8	3
-----------------------	----	---	---	---



List of Figures

Figure 1.1: Calcium-mobilizing stimuli interact with membrane G-protein	
coupled receptors (GPCR) to activate phospholipase C (PLC) generating	
inositol triphosphate (IP3) which interacts with IP3 receptors (IP3R)	
releasing Ca ²⁺ from storage vesicles	
Figure 1.2: A schematic diagram of NOX/NADPH and NOX/DUOX	5
Figure 1.3: The 5-LO pathway eicosanoids and inhibitors. Arachidonic acid	
metabolism initiated by 5-lipoxygenase leads to the bioactive eicosanoids	
shown here along with their receptors27	,
Figure 2.1: Fura-2 fluorescence responses of neutrophils activated with	
FMLP, LTC4 or LTD465	
Figure 2.2: Fura-2 fluorescence responses of neutrophils activated with	
FMLP or LTD ₄ individually or in combination66	
Figure 2.3: LTD ₄ -activated fura-2 fluorescence responses of control and	
montelukast-treated neutrophils	
Figure 2.4: Effects of LTC ₄ or LTD ₄ on superoxide production by	
unstimulated cells and cells activated with FMLP68	
Figure 2.5: The effects of montelukast on superoxide production	
by neutrophils activated with a combination of LTC ₄ or LTD ₄ and FMLP69	
Figure 2.6: Effects of varying concentrations of LTC ₄ or LTD ₄ on elastase	
release by neutrophils activated with FMLP/CB70	
Figure 3.1: Effects of montelukast on the lucigenin and luminol-enhanced	
chemiluminescence responses of neutrophils activated by N-formyl-L-	



methionyl-L-leucyl-L-phenylalanine (FMLP)101
Figure 3.2: Effects of montelukast on the release of elastase from
neutrophils activated with N-formyl-L-methionyl-L-leucyl-L- phenylalanine
/cytochalasin B102
Figure 3.3: Effects of montelukast on the production of leukotriene B ₄
(LTB ₄₎ by neutrophils activated with platelet activating factor103.
Figure 3.4: Chemoattractant-activated fura-2 fluorescence responses of
control and montelukast -treated neutrophils104
Figure 3.5: Effects of montelukast on the influx of ⁴⁵ Ca ²⁺ into the
neutrophils activated with either N-formyl-L-methionyl-L-leucyl-L-
phenylalanine or platelet-activating factor (PAF) 105
Figure 3.6: Effects of CGS21680, salbutamol, rolipram and montelukast
individually, as well as those of montelukast in combination with the other
agents on neutrophil intracellular cAMP106
Figure 3.7: Effects of montelukast, rolipram and 3-isobutyl-1-
methylxanthine on cAMP and cGMP phosphodiesterase (PDE) activities in
neutrophil cytosol107
Figure 4.1: Effects of formoterol and montelukast individually and
in combination on a) the generation of superoxide, b) the release of
elastase, and c) production of leukotriene B4 following activation of
neutrophils with FMLP in combination with cytochalasin B128
Figure 4.2: Effects of formoterol and montelukast individually and in
combination on neutrophil intracellular cyclic AMP (cAMP) levels



Figure 4.3: N-FormyI-L-methionyI-leucyI-phenylalanine (FMLP)/
cytochalasin B (CB)-activated fura-2 fluorescence responses of control
neutrophils and cells treated with formoterol and montelukast individually
and in combination
Figure 4.4: Effects of salbutamol and montelukast alone and in combination
on platelet factor-activated neutrophils



List of Tables

Table 1.1:	Neutrophil cytoplasmic granules and their major contents	16
Table 1.2:	Proposed relationship between asthma phenotypes and	
endotypes:	asthma phenotypes can be present in more than 1 endotype,	
and endoty	pes can contain more than 1 phenotype	34
Table 2.1:	Effects of LTC ₄ or LTD ₄ on MMP-8 release by cells activated	
with FMLP.		.71
Table 3.1:	Effects of MK886 and montelukast, separately and in	
combination	n, on the lucigenin-enhanced chemiluminescence (LECL)	
responses	of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) –	
activated ne	eutrophils	90
Table 4.1:	Peak cytosolic Ca ²⁺ concentrations, duration of the plateau	
phase of th	e peak cytosolic Ca ²⁺ response, and area under the curve	
measured i	n neutrophils activated with FMLP/CB in the absence and	
presence o	f formoterol and montelukast individually and in combination1	26
Table 4.2 :	Peak cytosolic Ca ²⁺ concentrations, duration of the plateau	
phase of the	e peak cytosolic Ca ²⁺ response, and the magnitude of decrement	
in cytosolic	Ca ²⁺ concentrations in neutrophils activated with PAF in the	
presence o	r absence of salbutamol and montelukast individually and in	
combinatio	٦1	27



List of Abbreviations

(in order of appearance in the text)

Cys LT₁R	Cysteinyl leukotriene receptor-1 antagonist
NADPH	Nicotinamide adenine dinucleotide phosphate
PSGL-1	P-selecin glycoprotein ligand-1
ICAM	Intracellular adhesion molecule
VCAM	Vascular cell adhesion molecule
MADCAM	Mucosal addressin cell adhesion molecule
TEM	Transendothelial migration
EC	Endothelial cell
TNF	Tumor necrosis factor
IL	Interleukin
LTB ₄	Leukotriene B ₄
BM	Basement membrane
LERs	Low expression regions
CR3	Complement receptor 3
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
BLT	Leukotriene B ₄ receptor
PPARα	Peroxisome proliferator-activated receptor alpha
CXC	Cysteine-C-cysteine
C-C	Double cysteine molecule
GM-CSF	Granulocyte-macrophage colony-stimulating factor
PAF	Platelet-activating factor
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PKC	Protein kinase C
PLD	Phospholipase D
PLA	Phospholipase A
IP3	Inositol-1,4,5-triphosphate
ATP	Adenosine triphosphate



AR	Adenosine receptor		
SOCE	Store-operated Ca ²⁺ entry		
TRPL	Transport receptor potential channel		
NET	Neutrophil extracellular traps		
DPPI	Dipeptide peptidase 1		
NSP	Neutrophil serine protease		
NE	Neutrophil elastase		
PR3	Proteinase 3		
CG	Cathepsin G		
PGRN	Progranlin		
ROS	Reactive oxygen species		
MMP	Matrix metalloproteinase		
TRL	Toll-like receptor		
LPS	Liposaccharide		
PECAM	Platelet/endothelial-cell adhesion molecule		
TIMP	Tissue inhibitor of metalloproteinase		
MPO	Myeloperoxidase		
CGD	Chronic granulomatous disease		
NOX	NADPH oxidase isoforms		
Phox	Phagocyte oxidase		
GDP	Guanosine diphosphate		
FAD	Flavin adenine dinucleotide		
LPC	Lysophosphatidylcholine		
VSMC	Vascular smooth muscle cell		
5-LO	5-lipoxygenase		
LTA	Leukotriene A		
FLAP	5-lipoxygenase-activating protein		
FOG	5-Oxo-7-glutathionyl-8,11,14-eicosatrienoic acid		
5-oxo-ETE	5-Oxo-eicosatrienoic acid		
OXE	Oxeicosanoid		
ASA	Aspirin-sensitive asthma		



G-CSF	Granulocyte colony stimulating factor		
NF-κβ	Nuclear factor kappa beta		
McI-1	Myeloid cell leukaemia-1		
AC	Adenylyl cyclase		
cAMP	Cyclic adenosine monophosphate		
cGMP	Cyclic guanosine monophosphate		
PBS	Phosphate-buffered saline		
CL	Chemiluminescence		
HBSS	Hanks' balanced salt solution		
PMSF	Phenylmethanesulfonyl fluoride		
DMSO	Dimethylsulfoxide		
SPA	Scintillation proximity assay		



Chapter 1

Introduction and Literature Review.



1.1 Introduction:

Although primarily an anti-asthma agent, the cysteinyl leukotriene receptor antagonist, montelukast, has been reported to be useful in the treatment of other acute and chronic inflammatory diseases in which the neutrophil is believed to be the primary offender. As neutrophils do not synthesize cysteinyl leukotrienes, it is possible that montelukast possesses neutrophil-directed anti-inflammatory properties that are unrelated to its conventional cysteinyl leukotriene receptor (CysLT₁R)-targeted activities. These putative alternative neutrophil-directed antiinflammatory activities of montelukast represent the focus of the current study.

1.2. Literature Review:

This literature review will focus on neutrophils, including activation of neutrophils and their role in the pathogenesis of disease, with emphasis on asthma and new targets for pharmacotherapy in controlling neutrophilic inflammation, especially in asthma.

1.2.1 Resting Neutrophils:

Neutrophils play a key role in the innate immune system of host defence (Kobayashi *et al*, 2005). Host defence systems consist of a number of cellular and protein components that interact to protect the host environment from outside attack.



Neutrophils are formed in the bone marrow from a pluripotent progenitor cell. Differentiation leads to the formation of monopotent progenitor cells, myeloblasts, promyeloblasts, metamyelocytes to band forms and maturing neutrophils. This maturation process takes 10-15 days to complete. Mature neutrophils are incapable of cell division (Adkinson *et al*, 2003). Mature neutrophils contain a variety of granules containing pre-formed products necessary to participate in the eradication of microbial pathogens, and also have the capacity to synthesise potent antimicrobial agents, once activated (Borregaard, Sørensen & Theilgaard-Mönch, 2007). Among these products are different proteases, oxygen radicals, defensins and certain lipid mediators. Neutrophils are also equipped to express genes to form products such as Fc-receptors, complement components, NADPH oxidase proteins and a number of cytokines and chemokines which play an important role in inflammatory and immune responses (Scapini *et al*, 2000).

1.2.2 Migration of neutrophils:

1.2.2.1 Margination:

Mature neutrophils are released from the bone marrow and migrate through the circulation, reaching tissue sites within 6-8 hours. This process is known as margination and ensures that large numbers of these cells are available when needed in the defence against harmful microbes.



Neutrophil migration from the circulation to the site of infection or inflammation is controlled by vascular endothelial interactions. Loose tethering of the neutrophils on the endothelium is mediated by L-selectin on the neutrophil surface and ligands on the surface of endothelial cells, including E- and P-selectin, and P-selectin glycoprotein ligand-1 (PSGL-1) (Wright *et al*, 2010). Tight adhesion of the neutrophil to the endothelium is mediated though interactions between ligands expressed on the surface of leucocytes with ligands on the endothelium such as intercellular adhesion molecule-1(ICAM-1), ICAM-2 and vascular cell adhesion molecule-1 (VCAM-1), as well as mucosal addressin cell adhesion molecule-1(MADCAM-1) (Wright *et al*, 2010).

A large number of marginated neutrophils are found in the pulmonary capillaries. At any given time, there are 60-100 times more neutrophils in the pulmonary capillaries than in the systemic circulation (Hogg & Doerschuk, 1995).

Migration of the neutrophils through the pulmonary capillaries is slow due to the fact that they have to deform to be able to pass through the narrow spaces in the capillary bed (Doerschuk *et al*, 1993). The slow movement of the neutrophils contributes to the high number in the pulmonary circulation.

1.2.2.2 Transmigration:

Following margination of the neutrophil to the wall of the vessel, tethering, rolling and adhesion to the vascular endothelium, the process of neutrophil transendothelial migration (TEM), across the vessel takes place. Diapedesis



across the vessel structure occurs by movement either through endothelial cell (EC) junctions (paracellular route) or through the body of the EC itself (transcellular route) (Gane & Stockley, 2011).

Paracellular transmigration occurs typically at the intersection of three or more ECs known as the tricellular corner (Burns *et al*, 1997). Neutrophil migration through the EC is enhanced by activation of the endothelial cell, or the neutrophil, or both, and the presence of a chemotactic gradient across the endothelium (Yang *et al*, 2005). Activation of these these cells can be induced by cytokines such as tumor necrosis factor (TNF), interleukin-1 β (IL-1 β) (Kumar *et al*, 2011) or endothelial-bound chemoattractants, including leukotriene B₄ (LTB₄), C5a and interleukin 8 (IL-8), or bacterial endotoxin (Gane & Stockley, 2011). Neutrophils can also change shape and migrate through an EC junction. This process can be mediated by adhesion molecules that relocate to allow the passage of the neutrophil, or it could be mediated by neutrophil proteases as was suggested by some studies (Su, Chen & Jen, 2002).

Diapedesis through the basement membrane (BM) has to be achieved without causing damage to the barrier function of the vessel (Hallman *et al*, 2005). Low expression regions (LERs) within the BM express less key matrix proteins and collagen IV and these regions have been shown to align with the EC junctions (Voisin, Woodfin & Nourshargh, 2009). Neutrophils adhere mostly to the endothelium close to these LERs. Neutrophils have the ability to increase the size of the LER, a process possibly mediated by elastase (Wang *et al*, 2006).



1.2.2.3 Transendothelial migration in the lung:

Integrins are transmembrane-spanning CAMs on the surface of leukocytes. Complement receptor 3 (CR3) was the first integrin identified and has several synonyms: Mac-1, Mo-1, α M β 2, CD11b/CD18. It is a member of the β 2 integrins, which share the common CD18 (β 2) subunit and are expressed exclusively by leukocytes; the other members are LFA-1 (CD11a/CD18), CR4 (CD11c/CD18), and the enigmatic α D β 2 (CD11d/CD18) (Ehlers, 2000).

Neutrophil migration to the lung tissue occurs via two different pathways, either via a β 2-integrin-dependent, or an -independent mechanism (Doerschuk, Tasaka & Wang, 2000). The specific stimulus will determine the pathway chosen, as well as the inflammatory stimulus, whether acute or chronic. Mackarel *et al*, have shown in a study done in COPD, cystic fibrosis or bronchiectasis patients, compared to healthy controls, that a difference could be determined. Neutrophils in healthy adults and the stable patients, transmigrated via a CD18-independent way in response to IL-8 and LTB₄ stimulation, whereas the neutrophils from patients with acute exacerbations migrated via a CD18-independent way. Neutrophils of all the groups migrated via a CD18-dependent mechanism on N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) stimulation, confirming the stimulus specific nature of the process (Mackarel *et al*, 2001). Once neutrophils have passed through the endothelium, migration follows a chemotactic gradient.



1.2.3 Chemotactic Mediators:

Several chemokines, bacterial products, complement split products and lipid mediators act as chemoattractants for neutrophils. Important chemoattractants with respect to the lung include IL-8 and LTB₄.

1.2.3.1 Chemokines:

More than 40 chemokines have been identified in humans and are classified into two major groups: cysteine-X-cysteine (CXC) and the C-C (double-cysteine molecules) subfamilies. Chemokines bind to trans-membrane-spanning receptors signalling through G-protein interactions (Thelen & Didichenko, 1997).

Interleukin-8 (IL-8) is a potent chemoattractant for neutrophils in the lung. IL-8 can act as an activator of neutrophils through coupling of the chemokine to receptors (CXCR1 or CXCR2) on the neutrophils (Murphy, 1997). Sources of IL-8 include macrophages, neutrophils, endothelial cells, epithelial cells, fibroblasts and smooth muscle cells. Neutrophils are thus both a source of IL-8 and a target for the chemokine. This was demonstrated in a study done by Jatakanon *et al.* in severe persistent asthma, which demonstrated high concentrations of both IL-8 and neutrophils in the sputum of these patients (Jatakanon *et al.* 1999).

1.2.3.2 Lipid mediators:

Leukotriene B_4 (LTB₄) is a lipid mediator with potent chemoattractant properties that is rapidly generated from activated innate immune cells such as neutrophils,



macrophages, and mast cells (Ohnishi, Miyahara & Gelfand, 2008). The production of leukotrienes from membrane-derived arachidonic acid will be discussed in detail in a later section.

Three receptors have been identified to bind LTB₄. BLT1 and BLT2 are both G protein-coupled seven transmembrane domain receptors on the cell surface of specific cells. BLT1 is a high affinity receptor expressed predominantly on granulocytes, monocytes/ macrophages, mast cells, dendritic cells, and effector T cells (Tager & Luster, 2003). BLT2 also plays a role in chemotaxis of neutrophils (Yokomizo *et al*, 2001). The third receptor, PPAR α , is a nuclear receptor that binds eicosanoids including LTB₄. Interaction with PPAR α promotes degradation of the lipid mediators (Devchand *et al*, 1996).

Leukotriene B_4 is not only a chemoattractant for neutrophils, but also an activator of these cells. Complement 5a also acts as a chemotactic factor for neutrophils (Czermak *et al*, 1998).

1.2.4 Neutrophil Priming and Activation:

1.2.4.1 *Priming:*

Neutrophil priming refers to a process whereby exposure of these cells to various inflammatory mediators such as lipopolysaccharide (LPS), TNF, or granulocyte macrophage colony stimulating factor (GM-CSF) greatly enhances subsequent agonist-induced respiratory burst activity and degranulation responses



(Cadwallader *et al,* 2002). Priming of neutrophils has an effect on neutrophil survival and integrin/selectin expression (Condliffe *et al,* 1998). This process leads to enhanced killing of microbes, but will lead to tissue damage if uncontrolled.

Neutrophils are primed by binding of agonists to a number of different membrane receptors. These receptors include: (1) G-protein-linked seven-transmembrane-domain receptors which are single-transmembrane-domain receptors that require crosslinking for activation such as Fc-receptors; and (2) single-transmembrane-domain receptors for growth-regulating cytokines including tumor necrosis factor (TNF) and GM-CSF. The G-protein-linked seven-transmembrane-domain receptors bind platelet-activating factor (PAF), complement component 5a (C5a), substance P and IL-8. Activation of any of these receptors will prime the oxidase of neutrophils for subsequent activation. Activation of the seven-transmembrane-domain receptors, excluding IL-8, only leads to priming (Hallet & Lloyds, 1995), while cross-linking of the receptors will prime the neutrophil oxidase at low concentrations and will activate it at high concentrations. The growth-regulating cytokine receptors will only lead to priming (Guichard *et al*, 2007).

Intracellular signalling pathways involved in the regulation of the neutrophil oxidase include those driven by phosphoinositide 3-kinase (PI3K), phospholipase C (PLC)/Ca²⁺-dependent protein kinase C (PKC), phospholipase D (PLD), phospholipase A2 (PLA2), and p38/Erk (Condliffe *et al*, 2005).



Activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase results from an increase in the cytosolic free Ca²⁺ in neutrophils (Guichard *et al*, 2007). Once the membrane receptors of the neutrophils interact with the chemoattractants and adhesion molecules, cytosolic Ca²⁺ is abruptly and transiently increased, which is a prerequisite for the initiation of the crucial proinflammatory actions of neutrophils, including generation of superoxide by the membrane-associated electron transporting NADPH oxidase, adhesion to vascular endothelium, degranulation, activation of cytosolic phospholipase A2 and 5lipoxygenase, as well as synthesis of IL-8 (Tintinger *et al*, 2008).

1.2.4.2 Calcium Homeostasis:

Uncontrolled release of the mediators of activated neutrophils can lead to tissue damage and needs strict control. This is neatly done by the control mechanisms of Ca²⁺ homeostasis in neutrophils.

Mobilisation and restoration of Ca^{2+} homeostasis will be explained according to the diagram in Figure 1.1 (page 11). (Reprinted from Drug Design, development and Therapy. 2008;2:95-104) (Tintinger *et al*, 2008).





Figure 1.1: Calcium-mobilizing stimuli interact with membrane G-protein coupled receptors (GPCR) to activate phospholipase C (PLC) generating inositol triphosphate (IP3) which interacts with IP3 receptors (IP3R) releasing Ca^{2+} from storage vesicles. Cytosolic phospholipase A2 (cPLA2) mobilizes arachidonic acid (AA) for the 5-lipoxygenase (5-LO) pathway. The AA metabolite leukotriene B_4 (LTB₄) is actively transported to the cell exterior where it binds to its receptor to activate PLC, completing a positive feedback autocrine loop. Ca^{2+} released into the cytosol is rapidly extruded from the cell by the plasma membrane Ca^{2+} ATPase and re-sequestered into storage vesicles by the protein kinase A (PKA)-sensitive endomembrane Ca^{2+} ATPase. Protein kinase C (PKC) activated by Ca^{2+} and diacylglycerol (DAG) facilitates assembly and activation of NADPH oxidase on the outer membrane which generates reactive oxygen species (ROS) with concomitant membrane depolarization. The depolarized membrane potential delays Ca^{2+} entry through store operated channels (SOCCs) until the Ca^{2+} -activatible Na+/Ca²⁺ exchanger, operating in reverse mode, mediates recovery of the membrane potential promoting Ca^{2+} reuptake via SOCCs. PKC down-regulates PLC as part of a negative feedback loop to terminate IP3 production (Pharmacological control of neutrophil-mediated inflammation: Strategies targeting calcium handling by activated polymorphonuclear leucocytes. Drug design, Develop and Therapy. 2008;2:95-104)



Following receptor-mediated activation by chemoattractants including C5a, IL-8, FMLP, PAF and LTB₄, Ca²⁺ is released from intracellular stores of the neutrophil to reach a concentration of free cytosolic Ca²⁺, 5-10 fold higher than basal value. Binding to the 7-transmembrane, G-protein-coupled receptors mentioned earlier, is controlled by various G α and B $\beta\gamma$ subunits and leads to activation of β isomers of phospholipase C (PLC). PLC mediates production of inositol-1,4,5-triphosphate (IP₃) by hydrolysis of phosphatidylinositol-4,5,-biphosphate. Interaction between IP₃ and Ca²⁺-mobilising receptors on intracellular storage vesicles results in discharge of stored Ca²⁺ into the cytosol (Tintinger *et al*, 2008). The presence of two distinct intracellular Ca²⁺ stores in human neutrophils has been demonstrated, one immediately below the plasma membrane, and the other at the centre of the cell, near the nuclear lobes (Pettit & Hallett, 1998).

The duration of the peak increase in the cytosolic Ca^{2+} is usually brief and is followed by a progressive decline, returning to basal levels. The duration of the peak cytosolic concentration of Ca^{2+} and the rate of return to basal level is determined by several different, albeit coordinated, mechanisms. At least four mechanisms have been identified: (1) shuttling of Ca^{2+} between the stores and the cytosol (Anderson, Steel & Tintinger, 2005); (2) activation of a secondary wave of Ca^{2+} influx stimulated by endogenously-generated LTB₄ by activated neutrophils (Steel *et al*, 2007); (3) the efficiency of mechanisms promoting clearance of Ca^{2+} from the cytosol (Steel & Anderson, 2002); and (4) the regulatory mechanisms controlling the time of onset, rate and magnitude of influx of extracellular Ca^{2+} (Tintinger *et al*, 2008).



The removal of Ca^{2+} from the cytosol of activated neutrophils is essential to avoid hyperactivity of these cells that can cause tissue damage. This is achieved by two separate adenosine triphosphate (ATP)-driven pumps. These are the plasma membrane Ca^{2+} -ATPase, mediating Ca^{2+} efflux, and the endomembrane ATPase, which is responsible for resequestration. The contributions of these pumps in removing the cation from the cytosol are equal (Pettit & Hallett, 1998).

Activation of neutrophils by chemoattractants like FMLP leads to activation of PLC and release of stored Ca^{2+} , but also transient activation of adenylate cyclase, resulting from the interaction of adenosine with G-protein/adenylyl cyclase-coupled adenosine receptors (AR) of the A2_A subtype on the neutrophil membrane (lannone, Wolberg & Zimmerman 1989; Theron *et al*, 2002), which leads to activation of adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase A (PKA). Phospholamban on the endomembrane Ca^{2+} -ATPase undergoes PKA-mediated phosphorylation which leads to up-regulation of the Ca^{2+} sequestrating/ resequestrating activity of the pump (Chu *et al*, 2000).

The membrane depolarisation action of NADPH oxidase facilitates the effective $Ca^{2+}clearance$ actions of the two $Ca^{2+}ATPase$ pumps. Depolarisation restricts the influx of extracellular Ca^{2+} (Tintinger *et al*, 2008). The electrogenic properties of the oxidase lead to an abrupt and steep decrease in membrane potential (Steel & Anderson, 2002). The driving force for Ca^{2+} entry is abolished when depolarisation of the cells occurs, due to the reduction of the electrical component of the



electrochemical gradient that promotes Ca^{2+} entry. "Consequently, NADPH oxidase-mediated membrane depolarization enables the plasma membrane and endomembrane Ca^{2+} -ATPases to mediate clearance of Ca^{2+} from the cytosol of activated neutrophils, unhindered by influx of extracellular Ca^{2+} " (Tintinger *et al*, 2001).

Following the depletion of the intracellular Ca^{2+} stores, refilling occurs through a process known as store-operated Ca^{2+} influx. The duration of NADPH oxidase activity, as well as the intensity of the activity will determine the onset and rate of store-operated Ca^{2+} influx in neutrophils. Inefficient activators of the oxidase, such as PAF, cause rapid influx of Ca^{2+} which overwhelms the Ca^{2+} ATPases, resulting in prolonged elevations in peak cytosolic Ca^{2+} concentrations (Tintinger *et al*, 2001). On the other hand, extracellular Ca^{2+} uptake into neutrophils activated by FMLP is delayed due to the activities of the oxidase, with the influx only being detectable at ± 1 min after activation by the chemoattractant. When NADPH oxidase activity declines, influx proceeds gradually over 5 min. This rate of Ca^{2+} influx is superimposable on that of membrane repolarisation (Tintinger, Steel & Anderson, 2005).

From the above, it is clear that calcium signalling in neutrophils is triggered by the activity of PLC, leading to the opening of IP3-gated channels in the membranes of intracellular calcium stores. During receptor stimulated Ca^{2+} release, the stores are depleted of Ca^{2+} , leading to store-operated Ca^{2+} entry (SOCE) (Schaff *et al*, 2010).



The main components of the SOCE pathway are the sensor of calcium store depletion, STIM 1 (Collins & Meyer, 2011), on the endoplasmic reticulum, and the plasma membrane component Orial 1 (Feske *et al*, 2006). Orial 1 mediates Ca²⁺ entry in neutrophils, in cooperation with transient receptor potential channels (TRPCs). TRPC 6 is the SOCE channel, regulating Ca²⁺ influx in neutrophils activated by E-selectin and G-protein coupled receptors (Bréchard, 2008).

SOCE play a role in neutrophil recruitment. A rise in Ca^{2+} causes an increase in the affinity of the β_2 -integrin, MAC-1, which was mentioned before to play a role in the migration of neutrophils.

1.2.5 Activated Neutrophils:

Activated neutrophils can respond via two separate mechanisms. Either with rapid release of pre-formed serine proteinases stored in primary (azurophilic) granules and release of oxygen radicals, or by activating transcription factors that trigger *de novo* expression of molecules (e.g. receptors and cytokines) (Kasama *et al*, 2005).

1.2.5.1 Proteases:

Neutrophils take part in antibacterial activities via three routes: phagocytosis, degranulation and formation of neutrophil extracellular traps (NETs) (Urban *et al*, 2009). Neutrophil granules are formed during the maturation process and are released into the extracellular space from activated neutrophils by degranulation. Three types of granules have been identified: Azurophil granules (primary),



specific granules (secondary) and small storage granules (tertiary) (Wright *et al,* 2010). The contents of these granules are shown in Table 1.1.

Table 1.1: Neutrophil cytoplasmic granules and their major contents.

Azurophil Granules	Specific Granules	Small Storage Granules
 a) Antimicrobial Substances Myeloperoxidase Defensins Lysozyme BPI (bacterial/permeability-increasing protein) 	a) Specific GranulesLactoferrinLysozyme	a) ProteinasesGelatinase B (MMP-9)
b) Serine ProteasesElastaseCathepsin GProteinase 3	 b) Proteinases Collagenase (MMP- 8) Gelatinase (MMP-9) Complement activator 	<i>b) Membrane Receptors</i>FMLP receptorsMAC-1 receptor
<i>c) Acid Hydrolases</i>Cathepsin BCathepsin D	 <i>c)</i> Membrane receptors FMLP-receptor MAC-1 Laminin receptor 	<i>c) Acid Hydrolases</i>Cathepsin DDAG lipase
 <i>d) Phospholipases</i> Secretory phospholipase A₂ 	 <i>d) Other</i> Histaminase Cytochrome b₅₅₈ 	<i>d) Other</i>B2-microglobulin

Neutrophil proteases include matrix metalloproteinases (MMPs) and neutrophil serine proteases (NSP).


1.2.5.1.1 Serine Proteases

Neutrophil serine proteases (NSP) contain the amino acid, serine, as the key residue of their enzymatic centre, which initiates the cleavage of the protein substrates (Kessenbrock, Dau & Jenne, 2011).

The serine proteases include, neutrophil cathepsin G (CG), neutrophil elastase (NE) and proteinase 3 (PR3), which are synthesized as inactive zymogens. The inactive molecules require two N-terminal proteolytic modifications to become active. After the signal peptide removal, the proenzyme is further processed by the lysosomal cysteine protease dipeptidyl peptidase I (DPPI, also known as cathepsin C) en route to the granules where they are stored as active enzymes (Pham, 2008).

They play an important role in bacterial defence, but can also alter the immune response by altering, either enhancing or abolishing the function of cytokines and chemokines by cleaving these proteins (Shapiro, 2002).

N-terminal proteolytic modification of IL-8 by the NSPs can alter cell function in different ways. PR3 converts it to a more potent neutrophil activating stimulant, whereas NE and CG will inactivate IL-8 (Padrines *et al,* 1994).

Progranulin (PGRN) also known as granulin-epithelin precursor, has been described to suppress the adhesion-dependent oxidative burst and protease



release by neutrophils in the presence of TNF. However, PRN loses its antiinflammatory potential once cleaved by NE (Kessenbrock *et al*, 2008).

Once the NSPs are released into the extracellular space, they bind to surface receptors. The presence of CG on the surface of cells triggers integrin clustering on neutrophils favouring interaction with immobilised immune complexes, leading to cytoskeletal rearrangements, increased reactive oxygen species (ROS) production and the secretion of chemokines (Raptis *et al*, 2005).

Integrins may also provide binding sites for NSP on the cell surface. Neutrophil elastase binds directly to the integrin CD11b/CD18 (CR3, Mac-1), regulating integrin-mediated cellular attachment and detachment (Cai & Wright, 1996). Release of IL-8, cathepsin B and matrix metalloproteinase- 2 (MMP-2), can be induced by NE through a MyD88/IRAK/TNF-receptor-associated factor 6 (TRAF-6)-dependent pathway which also involves Toll-like receptor 4 (TLR4) (Geraghty *et al*, 2007). The Toll-like receptors are intra- and extracellular membrane-anchored molecules. These receptors sense inflammatory stimuli such as bacterial DNA or lipopolysaccharide (LPS) and, via stimulation of the nuclear κ B pathway, mediate cellular activation (Xu *et al*, 2010).

As mentioned before, it was shown that NE plays a role in neutrophil transmigration though the vascular wall into the tissue (Wang *et al*, 2006). The mechanisms involved include: (1) cooperation with platelet/endothelial-cell adhesion molecule 1 (PECAM-1) and alpha 6 integrins (Wang *et al*, 2005); (2)



cleavage of adhesion molecules by NE and CG. The process of cleavage, especially at EC cell junctions, might cause gaps through which the neutrophils transmigrate (Robledo *et al*, 2003). These adhesion molecules include vascular endothelial cadherins (E-cadherins) (Mayerle *et al*, 2005), ICAM-1 (Robledo *et al*, 2003) and VCAM-1 ((Xu *et al*, 2005).

Uncontrolled proteolysis would be harmful for the host and tight control is necessary. Serine protease inhibitors are produced by the liver to neutralise the NSPs after their release (Kessenbrock, Dau & Jenne, 2011). Imbalance between proteases and their inhibitors can lead to certain autoimmune diseases (Heutinck *et al*, 2010), while emphysema can develop in patients with a genetic deficiency of α 1-antitrypsin (Fregonese & Stolk, 2008).

There are ways that the NSPs maintain their activity and protect themselves: (1) NSPs are present in high concentrations which overwhelm the inhibitors (Owen & Campbell, 1995); (2) NSPs localise close to the vascular cell surface to shield themselves from their inhibitors (Campbell, Campbell & Owen, 2000); and (3) Neutrophil-released proteases such as MMP-9, can degrade inhibitors of NSPs (Liu *et al,* 2000).

1.2.5.1.2 Matrix Metalloproteinase:

Matrix metalloproteinases (MMPs) are a family of enzymes responsible for digestion of structural components of the extracellular matrix, including membranes collagens, elastin, laminin and fibronectin (Kessenbrock, Plaks & Werb, 2010). The



MMPs can also cleave other proteins such as receptors, growth factors, cytokines, chemokines and other proteases (McCawley & Matrisian, 2001).

In humans, more than 27 different MMPs have been described. They are grouped into five categories: Collagenases, gelatinases, stromelysins, matrilysins and membrane-associated types, and others. Activities of the MMPs are regulated by tissue inhibitors of metalloproteinases (TIMP) (Tandon & Sinha (2011).

Neutrophils produce high levels of MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B). These MMPs remodel the extracellular matrix, facilitating leucocyte trafficking through the endothelial barriers into solid organs. In a study conducted by Jung *et al* (2009), it was demonstrated that neutrophils are key mediators of recruitment of both Th1 and Th2 cells to the airways. The ratio between MMP-8, MMP-9 and TIMP-1 is crucial in the recruitment process. The study suggested that both neutrophils and MMPs could be targets for new anti-inflammatory asthma treatment (Jung *et al*, 2009).

Uncontrolled activity of the MMPs, has been implicated in chronic lung disorders, including asthma (Cauwe & Opdenakker, 2010) cystic fibrosis (Flifiel *et al*, 2006), and chronic obstructive pulmonary disease (Köhrmann *et al*, 2009). MMP-2,-8 and -9 were also found in acute-onset pulmonary diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (Kong *et al*, 2009).



Increased expression of MMP-8 in bronchial biopsies correlated well with disease severity and was a good predictor of COPD development in smokers (Ilumets *et al*, 2007). In a study by Prikk *et al*, a correlation was shown between the level of MMP-8 and -9, neutrophils and markers of neutrophil activation such as myeloperoxidase (MPO), a marker of oxidant generation (Prikk *et al*, 2002).

1.2.5.2 Oxygen Radicals:

Already in 1933, it was observed that neutrophils demonstrated an increase in oxygen consumption during phagocytosis, a process known as the respiratory burst (Baldridge & Gerard, 1933). The respiratory burst is essential for effective bacterial killing as is clearly evident from chronic granulomatous disease (CGD) in which the absence of this process leads to overwhelming infection (Holmes, Page & Good, 1967). This oxygen-dependent form of bacterial killing leads to consumption of molecular oxygen due to the formation of superoxide and other reactive oxygen species (ROS).

Once activated, neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multicomponent enzyme, transfers electrons from cytoplasmic NADPH to molecular oxygen to generate superoxide:

$\mathsf{NADPH} + \mathsf{O}_2 \ \rightarrow \ \mathsf{NADPH}^{+} + \mathsf{O}_2^{-} + \mathsf{H}^{+}$

 O_2^- is converted to H_2O_2 , either spontaneously or by superoxide dismutase. H_2O_2 has antimicrobial potential in its own right, but is converted to the more potent



ROS, HOCI (reaction 1), by the primary granule enzyme myeloperoxidase (MPO). The reaction of H_2O_2 + HOCI leads to formation of singlet oxygen (reaction 2, 1O_2) (Babior, Lambeth & Nauseef, 2002).

- 1. $H_2O_2 + CI \rightarrow HOCI + H_2O$
- 2. $H_2O_2 + OCI^- \rightarrow {}^1O_2 + H_2O + CI^-$

The components of NADPH oxidase are the membrane-bound cytochrome b_{558} , comprising gp91^{*phox*} (NOX2) and p22^{*phox*} (phox stands for phagocyte oxidase), and four cytosolic components, p47^{*phox*}, p67^{*phox*}, p40^{*phox*} and guanosine diphospate (GDP)-bound GTP-binding protein Rac1/2 (Chessa *et al*, 2010). NOX2 acts as the catalytic subunit of the enzyme.

Cytochrome b_{558} is a membrane-bound flavohemoprotein which contains NOX2 and p22^{phox}, but also a flavin adenine dinucleotide (FAD), which serves as a NADPH-binding site, and two hemo-prosthetic groups, one of which binds gp91^{phox} only, and the other binds both gp91^{phox} and p22^{phox}. In resting neutrophils, 15% of these subunits of the cytochrome b_{558} are plasma membrane bound and 85% within membranes of specific granules and secretory vesicles which translocate to the plasma membrane on oxidase activation (Sheppard *et al*, 2005).

The different units of NADPH oxidase are influenced by neutrophil priming agents. However, priming does not cause activation of the enzyme. Priming of the NADPH oxidase is defined operationally as augmentation of superoxide generation in



response to a second, activating stimulus (Sheppard *et al*, 2005). The time it takes to reach maximal augmentation in this priming process, differs for different priming agents binding to the G-protein-coupled receptor of the neutrophil and can be a few minutes or up to 2h.

When primed by a long-acting agent such as lysophosphatidylcholine (LPC) or GM-CSF, $p47^{phox}$ translocates to the plasma membrane (Mansfield *et al*, 2002). Phosphorylation is a prerequisite for translocation and the degree of phosphorylation correlates with the potency of the priming agent. For instance, in the case of TNF, only partial phosphorylation of $p47^{phox}$ takes place and the molecule is not translocated (Dewas *et al*, 2003). However, the rapid primer PAF, causes phosphorylation of $p67^{phox}$, $p40^{phox}$, and Rac2, but not $p47^{phox}$ (Gay, 1990).

Lipid mediators such as LPC, arachidonic acid and LTB₄ can also increase the release of O_2^- in response to a subsequent stimulus (Palmblad *et al*, 1984). LTB₄ participates in translocation of Rac2 (Abdel-Latif *et al*, 2004). Lipid mediators may activate PKC and phosphoinositide-3 kinase (PI-3K) (Brown *et al*, 2003). A key component of neutrophil priming is Ca²⁺, many of the aforementioned priming activities being Ca²⁺ dependent, such as activation of PKC (Heyworth & Badwey, 1990). Many signalling cascades essential for priming and assembly of the NADPH oxidase components, are initiated by a rise in the cytosolic Ca²⁺ concentration (Silliman *et al*, 2003). Priming of the neutrophil will lead to fusion of the cytoplasmic granules with the plasma membrane which will allow gp91^{phox} and p22^{phox} to interact with the membrane (Borregaard, 1988).



In the activated neutrophil, additional phosphorylation of $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ and translocation to the plasma membrane is necessary to complete assembly of the oxidase and is the final step in activation of the enzyme.

These final steps include: "guanine-5'-triphosphate loading of Rac, allowing its translocation to the membrane and its interaction with the tetratricopeptide repeat domain of p67^{phox} (Lapouge et al, 2000); the interaction of p67^{phox} with gp91^{phox}; and the phosphorylation of p47^{phox} (Chessa et al, 2010)." Phosphorylation of p47^{phox} leads to the exposure of the SH3 domain allowing interaction with p22^{phox}. The C-terminus of p47^{phox} binds to the C-terminal SH3 domain of p67^{phox}. The phox homology (PX) domain of p47^{phox} interacts with phosphatidylinositol 3,4biphosphate (PtdIns $(3,4)P_2$) and phosphatidic acid. This binding may play a role in the efficiency of the assembly and activation of the enzyme (Karathanassis et al, 2002). The subunit p40^{phox} binds to p67^{phox}. It also contains a SH3 domain as well as a threonine (T) 154 -, and serine (S) 315 conserved phosphorylation site (Bouin et al, 1998). Phosphorylation of both these sites is triggered by PKC, an enzyme shown to be important in activation of the oxidase. PKC isoforms also cause phosphorylation of p22^{phox}, enhancing the activity of NADPH and regulating the p22^{phox}-p47^{phox} interaction in the membrane (Lewis et al. 2010). A schematic diagram of NOX/NADPH and NOX/DUOX reproduced from an article in the Journal of Pharmacological Science, 2010, by M Katsuyama is shown in Figure 1.2 (page 25) (used with permission)





Figure 1.2: Schematic diagrams of NOX/NADPH oxidases. A membrane association of NOX and other components. Requirement of other components depends on the NOX isoforms. B. Schematic diagrams of NOX/DUOX. Closed boxes, transmembrane regions. Open diamonds, EF-hands.



Production of O_2^- in an NADPH-dependent manner has been reported in nonphagocytic cells including vascular smooth muscle cells (VSMC) (Katsuyama, 2010) and endothelial cells (Frey, Ushio-Fukai & Malik, 2009). NOX2 is not formed in these cells, but non-phagocyte NOX2 homologs have been identified. Five NOX isoforms (NOX1-5) and two related enzymes (DUOX1/2) have been reported (Katsuyama, 2010).

1.2.5.3. Leukotriene B₄:

The lipid mediator, Leukotriene B_4 (LTB₄), is rapidly generated by activated neutrophils and acts as a potent chemoattractant. LTB₄ is also produced by macrophages and mast cells (Monteiro *et al*, 2011). The cysteinyl leukotrienes together with LTB₄ form the family of leukotrienes.

The leukotrienes are formed from arachidonic acid, which is released from cellular membranes by cytosolic phospholipase A2 (cPLA2), and further converted by the 5-lipoxygenase (5-LO) pathway (Peters-Golden & Henderson, 2007). The pathway will be explained via a diagram (Figure 1.3, page 27) from an article by Paul Rubin and Karl W. Mollison which was published in "Prostaglandins and other Lipid Mediators" in 2007 (Rubin & Mollison, 2007).

In activated cells, arachidonic acid is released from the nuclear membrane and is metabolised by 5-LO in conjunction with the 5-lipoxygenase-activating protein (FLAP) to form 5-hydroperoxyeicosatetraenoic acid (5-HETE) and subsequently, Leukotriene A (LTA₄). LTA₄ can be conjugated with glutathione by LTC₄ synthase,



leading to the production of the cysteinyl leukotrienes, or it can be metabolised by LTA₄ hydrolase to form LTB₄ (Singh *et al*, 2010). Studies have identified two new branches of the 5-LO pathway, producing 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) which shares the OXE receptor with 5-HETE (Brink *et al*, 2004), and can be transformed to 5-oxo-7-glutathionyl-8,11,14-eicosatetraenoic acid (FOG7) which has a distinct receptor (Bowers *et al*, 2000).



Figure 1.3: The 5-LO pathway eicosanoids and inhibitors. Arachidonic acid metabolism initiated by 5-lipoxygenase leads to the bioactive eicosanoids shown here along with their receptors. Points of intervention are indicated for currently approved therapeutic agents. (Paul Rubin P, Mollison KW. Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. Prostaglandins Other Lipid Mediat. 2007 May;83(3):188-97)



Two LTB₄ receptors have been identified, BLT_1 and BLT_2 (Tager & Luster, 2003). Chemotaxis of neutrophils can be mediated by OXE (Brink *et al*, 2004), FOG7 (Bowers *et al*, 2000) or BLT1/2.

The majority of evidence suggests that the chemotactic action of LTB₄ is mediated through binding to the high affinity BLT1 receptor on target cells (Yokomizo *et al*, 2001). Neutrophil influx is a known feature in severe asthma and high levels of LTB₄ (Jatakanon *et al*, 1999) are found in the lungs of these patients, which may be due to the involvement of more than one receptor (Rubin & Mollison, 2007). Both BLT₁ and BLT₂ are G protein-coupled seven transmembrane domain receptors. Binding of LTB₄ to either of these receptors activates a range of intracellular activities including intracellular Ca²⁺ mobilisation (Salmon & Ahluwalia, 2010), activation of extracellular signal-regulated kinase 1 / 2, phosphoinositide-3 kinase, as well as degranulation (Lundeen *et al*, 2006).

Another receptor that plays a role in the metabolism of LTB_4 is the PPAR α that binds eicosanoids, promoting degradation of the lipid mediators. They play an important role in clearance of LTB_4 , controlling the inflammatory process (Narala *et al*, 2010).

Levels of LTB_4 are elevated in the sputum and plasma in asthmatic patients during an acute asthma attack and not in normal individuals (Sampson *et al*, 1995). LTB_4 was also shown to be elevated during nocturnal asthma and correlates with nocturnal fall in forced expiratory volume in one second (FEV₁) (Wenzel *et al*,



1994). In patients with aspirin-sensitive asthma (ASA), a condition associated with severe asthma, aspirin sensitivity and severe rhinosinusitis with recurrent nasal polyposis, high levels of LTB₄, as well as CysLTs in the airways, have been reported (Sousa *et al*, 2002).

LTB₄ has been detected in higher than normal levels in other allergic diseases such as allergic rhinitis (Ohnishi, Miyahara & Gelfand, 2008), atopic dermatitis (Reilly *et al*, 2000) and allergic conjunctivitis (Akman, Irkeç & Orhan, 1998).

A significant correlation was found between the annual fall in post-bronchodilator FEV₁ and numbers of peri-bronchial CD8⁺ T cells, a correlation that was not evident with eosinophils, CD4⁺ T cells, or mast cells (Van Rensen *et al*, 2005). In a study by Gelfand *et al*, CD8⁺/BLT1⁺/IL-13⁺ T cells were found in broncho-alveolar lavage (BAL) fluid of asthmatics, but not normal subjects (Gelfand & Dakhama, 2006). BLT1-expressing effector memory CD8⁺ T cells are more resistant to corticosteroids than CD4⁺ T cells, and corticosteroids can even enhance the activation and effector function of these CD8⁺ T cells due to up-regulation of BLT1 expression. Corticosteroid-mediated up-regulation of BLT1 on effector memory CD8⁺ T cells may contribute to the ability of these agents to enhance the development of allergic airway inflammation (Ohnishi *et al*, 2008).



1.2.6 Neutrophil Clearance

The resolution of the inflammatory process relies on the effective 'switching off' of the neutrophil, the promotion of apoptosis and the successful clearance of these cells (Fox *et al*, 2010).

Apoptosis is a physiological process of programmed cell death, and, in the case of inflammatory cells, is necessary to control tissue damage. Many neutrophils will undergo apoptosis even before leaving the bone marrow to maintain cell numbers. Certain mediators play a role in delaying apoptosis for instance, GM-CSF and granulocyte colony-stimulating factor (G-CSF), are associated with suppression of neutrophil apoptosis (Fox *et al*, 2010). Activation of nuclear factor- κ B (NF- κ B) has been shown to be of importance in the regulation of human granulocyte apoptosis, possibly via the regulation of the production proteins, which protect the neutrophil from cytotoxic effects of cytokines such as TNF (Ward *et al*, 1999).

Two pathways of apoptosis have been identified. The key protein in the intrinsic pathway, regulating constitutive neutrophil apoptosis, is the anti-apoptotic protein, myeloid cell leukaemia-1 (Mcl-1). This protein is rapidly expressed and has a short half-life of 2-3h. Once the survival signal is lost, it is degraded and the cellular level of the protein correlates with apoptosis (Leuenroth *et al*, 2000). Mcl-1 is a member of the anti-apoptotic members of the Bcl-2 family; the others being Bcl-X_L and A1. The pro-apoptotic members include Bax, Bad, Bcl-Xs, Bak and Bid (Andina *et al*, 2009). Death receptors, including Fas, TNF-related apoptosis-inducing ligand



(TRAIL) receptors-1 and -2, and TNF receptors-1 and -2 regulate the extrinsic pathway of apoptosis. Binding of the death receptors with their ligands induces apoptosis via caspase-8 activation. Mcl-1 is also a target of caspase-8 (Akgul & Edwards, 2003).

In a study reported by Petrin *et al* (2006), it was illustrated that LTB₄ inhibition of neutrophil apoptosis involves upregulation of McI-1 and a decrease in Bax proteins. LTB₄ activates phosphatidylinositol 3-kinase (PI3-K) via its BLT1 receptor. Neutrophil exposure to GM-CSF or IL-8 delays apoptosis by activating PI3-K and ERK-dependent pathways (Pétrin *et al*, 2006).

Glucocorticosteroids also prolong neutrophil survival due to anti-apoptotic effects. These include high levels of the glucocorticoid receptor β (GR β) in comparison to the GR α (Marwick, Adcock & Chung, 2010). Strickland *et al.*, have shown that neutrophil survival was prolonged if exposed to dexamethasone and that the ratio of GR β to GR α was increased. This effect was enhanced if the neutrophils were pre-incubated with IL-8 (Strickland *et al.*, 2001). The fact that neutrophil apoptosis is inhibited by glucocorticoids, is compatible with a role for this mechanism in steroid-resistant asthma.

1.2.7 Asthma

Asthma is a complex clinical syndrome characterised by variable symptoms of airway obstruction and bronchial hyperresponsiveness due to chronic airway



inflammation (National Heart, Lung and Blood Institute, 2007). Although it is a chronic disease, acute exacerbations and symptoms can be induced by a number of triggers, including amongst others, respiratory viral infections, allergen exposure and exercise (Gravett *et al*, 2010). Inflammatory cell infiltration in the airways during acute episodes may involve eosinophils, which are traditionally associated with asthmatic inflammation, as well as neutrophils and lymphocytes (Cosmi *et al*, 2011). The chronic inflammation leads to hypertrophy of the airway smooth muscle, thickening of the basement membrane, and mucus production. This process can lead to airway remodeling and fixed airway obstruction if poorly treated (Durrani, Viswanathan & Busse, 2011).

Over the years, asthma has proven to be a complex disease that includes different clinical presentations, inflammatory processes and diverse genetic profiles. These groups respond differently to available treatment regimens, and present in a range of age groups with varying grades of severity irrespective of the length of time that the individual has been diagnosed with asthma. In an attempt to better classify the groups and optimum therapies more accurately; different phenotypes have been described in the literature. According to The Encarta World Dictionary, the definition of "Phenotype" is: "the visible characteristics of an organism resulting from the interaction between its genetic makeup and the environment". In a review in the Lancet of August/September 2006, Wenzel proposes the following potential phenotype categories: "Some patients will have asthma that can be classified under more than one phenotype and this division still will not give a clear indication of the underlying disease process or response to treatment" (Wenzel, 2006).



Clinical or physiological phenotypes

Severity-defined

Exacerbation-prone

Defined by chronic restriction

Treatment-resistant

Defined by age at onset

Phenotypes related to the following triggers

Aspirin or non-steroidal anti-inflammatory drugs

Environmental allergens

Occupational allergens or irritants

Menses

Exercise

Inflammatory phenotypes

Eosinophilic

Neutrophilic

Pauci-granulocytic

Lötvall *et al*, therefore suggest a classification of "endotypes" where endotypes are defined as: "a subtype of a condition, which is defined by a distinct functional or pathophysiological mechanism" (Lötvall *et al*, 2011). The possible relationship between asthma phenotypes and endotypes is shown below in Table 1.2 (page 34).



Table 1.2: Proposed relationship between asthma phenotypes and endotypes: asthma phenotypes can be present in more than 1 endotype, and endotypes can contain more than 1 phenotype.

Phenotype:	Eosinophilic asthma
	Endotypes: allergic asthma (adult), aspirin-sensitive asthma, severe late- onset hypereosinophilic asthma, ABPM (allergic broncho-pulmonary mycosis)
Phenotype:	Exacerbation-prone asthma
	Endotypes: allergic asthma (adult),aspirin-sensitive asthma, late-onset hypereosinophilic asthma, API-positive (asthma-predictive indices) preschool wheezer, ABPM, viral-exacerbated asthma, premenstrual asthma
Phenotype:	Obesity-related asthma
	Endotypes: airflow obstruction caused by obesity, severe steroid-dependent asthma, severe late-onset hypereosinophilic asthma
Phenotype:	Exercise-induced asthma
	Endotypes: cross-country skiers' asthma, other forms of elite-athlete asthma, allergic asthma, API-positive preschool wheezer
Phenotype:	Adult-onset asthma
	Endotypes: aspirin-sensitive asthma, infection-induced asthma, severe late- onset hypereosinophilic asthma
Phenotype:	Fixed airflow limitation
	Endotypes: noneosinophilic (neutrophilic) asthma
Phenotype:	Poorly steroid-responsive asthma
	Endotypes: noneosinophilic (neutrophilic) asthma, steroid-insensitive eosinophilic asthma, airflow obstruction caused by obesity

This table is reproduced from: Journal of Allergy and Clinical Immunology Volume 127, Issue 2, February 2011, Pages 355-360



From the above discussion, it is clear that more questions than answers exist with respect to the understanding of asthma and predicting outcomes with treatment.

Treatment aims in chronic inflammatory diseases such as rheumatoid arthritis and ulcerative colitis, are usually to induce remission, but in all the guidelines of asthma treatment, control is the target. In a recent article by Upham & James on remission in asthma, it is again acknowledged that the heterogeneity of asthma and the natural history of the disease have to be taken into consideration when defining remission (Upham & James, 2011). Early treatment of asthma in children did not prevent disease progression. Eosinophils are generally considered to be the key inflammatory cell in asthmatic inflammation, but blocking Interleukin-5 (IL-5), a known growth factor for eosinophils, did not lead to asthma control. This may again be due to the fact that other inflammatory cells including neutrophils may play an important role in the chronic inflammation and that reducing the eosinophil count is not enough (Upham & James, 2011).

1.2.7.1 Neutrophils in Asthma:

Neutrophils are present in the airways of some asthmatic patients, especially those with acute exacerbations (Ito *et al*, 2008), and fatal asthma that may occur within hours of the exacerbation (Lamblin *et al*, 1998), has been described in nocturnal asthma (Nadif *et al*, 2009) and severe-steroid resistant asthma (Wenzel *et al*, 1997).



Neutrophils are present in bronchoalveolar lavage fluid in some patients following allergen challenge in both the acute phase, as well as the late phase of asthma, which might be due to the fact that neutrophils express high-affinity receptors for IgE (FcɛRI). Activation of these receptors leads to IL-8 release (Gounni *et al*, 2001). Interleukin-9 (IL-9) can also activate neutrophils through surface receptor stimulation causing an increase in IL-8 release. IL-9 receptor expression is increased in asthma, being induced by the Th2 cytokine, IL-4, as well as GM-CSF (Abdelilah *et al*, 2001).

In acute asthma, neutrophils will release oxygen radicals and proteases that contribute to tissue damage and increased mucus gland hyperplasia, mucus secretion, and epithelial damage. Elastase exposure increases fibroblast migration that may lead to airway remodeling (Vignola, Kips & Bousquet, 2000).

In sudden-onset fatal asthma, neutrophils have been found to be the predominant inflammatory cells involved (Carroll *et al*, 1996).

Chronic severe asthma is another type of asthma in which neutrophils are found in high concentrations in sputum, correlating with increased levels of IL-8 and myeloperoxidase (MPO). This group of severe asthmatics is poorly responsive to corticosteroid treatment. It is debatable whether the neutrophil infiltration in these severe cases is a consequence or cause of treatment with high doses of corticosteroids (Jatakanon *et al,* 1999).



Asthmatics that smoke also represent a group with high levels of neutrophils in the airways (Cowan *et al*, 2009).

1.2.7.2 Cysteinyl Leukotrienes (CysLTs):

The synthesis of leukotrienes was discussed earlier in this chapter, with emphasis on LTB₄. However, the CysLTs will be discussed in more detail because of the important role they play in the pathogenesis of asthma, and the fact that they are the primary target of treatment with montelukast. Of necessity this section will repeat some of the detail mentioned in the preceding section on leukotriene synthesis.

Feldberg and Kellaway identified a material they named "slow reaction smooth muscle-stimulating substance (SRS)" 60 years ago (Feldberg & Kellaway, 1938). This substance was observed to be released by antigen challenge in human lungs and was renamed, "slow-reacting substance of anaphylaxis (SRS-A) (Brocklehurst, 1960). The discovery of the precise biochemical nature of SRS-A was anticipated by the isolation of 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE) in polymorphonuclear leucocytes (Borgeat & Samuelsson, 1979a; Borgeat & Samuelsson 1979b; Borgeat & Sameulsson, 1979c) and finally identified as a cysteine-containing derivative of 5-hydroxy-7,9,14-eicosatetraenoic acid (Murphy, Hammaström & Sameulsson, 1979. These compounds were named leukotrienes as they were found in leucocytes and were characterised by the presence of three conjugated double-bonds (Samuelsson, 1983).



The synthesis of leukotrienes from phospholipase A_2 was depicted earlier in this literature review (Figure 1.3, page 27). The cysteinyl leukotrienes are synthesised from LTA₄ (Peters-Golden & Henderson, 2007). This intermediate is converted by either of the two enzymes, LTA₄ hydrolase or LTC₄ synthase, to form LTB₄ or LTC₄, respectively. In order for 5-lipoxygenase to function effectively in cells, 5-lipoxygenase-activating protein (FLAP) needs to be present. FLAP does not have any enzymatic activity, but rather enhances the activity of 5-lipoxygenase (Peters-Golden & Brock, 2003). The tripepetide side chain of LTC₄ may be cleaved in two successive steps to generate LTD₄ and LTE₄, which together with the parent compound, comprise the cysteinyl-leukotrienes (cys-LTs) (Duroudier, Tulah & Sayers, 2009).

The capacity to generate large amounts of leukotrienes from arachidonate is largely confined to leukocytes; however, the amounts of LTB₄ and cys-LTs that various types of leukocytes produce depend on the distal enzymes LTA₄ hydrolase and LTC₄ synthase, respectively. Although non-leukocyte cells generally do not have sufficient 5-lipoxygenase and FLAP to synthesize appreciable amounts of leukotrienes from arachidonate, such cells expressing distal LTA₄ –metabolizing enzymes can take up leukocyte-derived LTA₄ and metabolize it into bioactive leukotrienes, a process that is termed "transcellular bio-synthesis (Folko & Murphy, 2006).



The output of the leukotriene synthetic pathway is regulated by: (1) the amount of free arachidonate that phospholipase A₂ releases from cell-membrane phospholipids (Uozumi *et al*, 1997; Henderson *et al*, 2007); (2) the level of each of the proteins in the 5-lipoxygenase pathway; (3) the catalytic activity per enzyme molecule (e.g., modulated by protein kinase-directed phosphorylation); and (4) the availability of small molecules (e.g., ATP, nitric oxide (Coffey, Phare & Peters-Golden, 2000), and reactive oxygen intermediates) that modulate 5-lipoxygenase activity.

Leukotrienes act by binding to specific heptahelical receptors of the rhodosin class that are located on the outer plasma membrane of structural and inflammatory cells (Kanaoka & Boyce, 2004; Salmon & Ahluwalia, 2010). Once ligated by the leukotriene, the receptor interacts with G-proteins in the cytoplasm, thereby eliciting increases in intracellular calcium and reductions in intracellular 3'-5'-cyclic adenosine monophosphate (cAMP).

CysLTs in humans appear to function through at least two receptors (CysLT₁ and CysLT₂) (Capra, 2004). CysLT₁Rs have a relatively restricted occurrence, being expressed on the plasma membrane of epithelial cells, fibroblasts/myoblasts, smooth muscle cells and endothelial cells in the structural group of cells, while the inflammatory group includes neutrophils, monocytes/macrophages, basophils, mast cells, dendritic cells, B lymphoctes and CD4⁺ T cells (Yoshisue *et al*, 2007). Interaction of the CysLTs with the CysLT₁ receptor on the structural cells, leads to mucus production and collagen synthesis and release with implications for airway



remodelling, contractility, proliferation of smooth muscle, vascular permeability, and oedema (Hui & Funk, 2002).

Although receptors are found on the aforementioned inflammatory cells, only mast cells, basophils, eosinophils and, to a lesser extent, monocytes/macrophages, possess the necessary enzymes to convert LTA₄ to CysLTs (Peters-Golden & Henderson, 2007). Interaction of the CysLTs with CysLT₁Rs on the inflammatory cells: (1) recruits and activates T_H2 cells and eosinophils; (2) prolongs eosinophil survival; (3) and may be able to act as co-factor for the enhanced production of eosinophils from the bone marrow in combination with GM-CSF; (4) increases the production of ROS by neutrophils, eosinophils and monocytes/macrophages, these oxidants being mediators of vascular permeability and bronchial hyper-reactivity; and (5) induce the release of proteolytic enzymes such as elastase and matrix metalloproteinases from phagocytic cells, which promote airway re-modelling (Holgate *et al*, 2003).

Certain reported actions of Cys-LTs are not readily explained by their interactions with either CysLT₁ or CysLT₂, raising the possibility of the presence of CysLT₁-CysLT₂ heterodimers or additional receptors (Daniele *et al*, 2011). One candidate is G protein-coupled receptor 17 (GPR17), a dual-uracil nucleotide-cysteinyl leukotriene receptor (Ciana *et al*, 2006).



1.2.7.3 Treatment of Asthma:

The aim of treatment of chronic asthma is to gain control (Bateman *et al*, 2008). In patients with intermittent asthma, short acting β 2 stimulants are used as reliever medication. When this is needed more than three times per week, the asthma is classified as persistent asthma, and according to the GINA guidelines, persistent asthma should be treated with a controller (anti-inflammatory) medication with short-acting β 2-stimulants only being used as reliever treatment if symptoms are experienced (Bateman *et al*, 2008).

Inhaled corticosteroids are the first line anti-inflammatory agents with the addition of a long-acting β 2-stimulant (LABA) if the patient remains symptomatic. A leukotriene antagonist can be used as an additional controller agent in adults being added either to an inhaled steroid or to the combination of an inhaled steroid and LABA (Sears, 2011). Outcomes with combination therapy with inhaled steroids and long-acting beta 2-receptor agonists (LABAs), either in separate devices, or combined in single inhalers, are well documented, and show improved control (Maneechotesuwan *et al*, 2005). The guidelines in children provide for options to use leukotriene antagonists as first-line treatment (Bacharier *et al*, 2008). Montelukast is the only drug in this group that has registration for use in children below the age of twelve.

Phenotypes of asthma with predominantly neutrophilic inflammation will generally respond poorly to inhaled steroids due to mechanisms mentioned previously (Strickland *et al*, 2001; Marwick, Adcock & Chung, 2010). Clinical studies in



asthmatics that smoke, show reduced efficacy of corticosteroids (Lazarus *et al*, 2007) and emphasise the need for new targets for controlling inflammation in asthma. A few trials with combination anti-inflammatory drugs, have underscored the need to target neutrophils as well as eosinophils (Hanania, 2008).

There is evidence that salmeterol reduces the number of neutrophils in the airway mucosa, as well as in broncho-alveolar lavage fluid of patients with mild asthma, whereas the inhaled corticosteroid, fluticasone, was ineffective. Treatment with salmeterol resulted in more symptom-free days than treatment with fluticasone or placebo (Jeffery *et al*, 2002). Formoterol inhalation reduced sputum neutrophils after four weeks of treatment, without any effect on eosinophil counts, while budesonide had the reverse effect with a reduction in eosinophils and no effect on neutrophils (Maneechotesuwan *et al*, 2005). In fact, corticosteroids prolong neutrophil survival by inhibiting their apoptosis, as mentioned previously (Strickland *et al*, 2001; Marwick, Adcock & Chung, 2010). These findings may explain why the combination of inhaled steroids and LABA's is more effective in the treatment of asthma than inhaled steroids alone. Treatment with a LABA without concurrent use of an inhaled steroid is contraindicated due to the potential increase in severe and life-threatening asthma exacerbations (Salpeter *et al*, 2006).

Acute exacerbations of asthma are associated with an increase of both eosinophils and neutrophils. The combination product of formoterol and budesonide, known as Symbicord, has efficacy as reliever therapy in acute asthma. This may be due to the complementary action of the agents and the fact that formoterol has a quick



onset of action (Bousquet *et al,* 2007). The combination of salmeterol and fluticasone is not suitable as reliever therapy because salmeterol has a slow onset of action.

Addition of either LABAs or montelukast to inhaled steroid treatment has also been studied, and montelukast was proven to be as effective as addition of salmeterol to fluticasone (llowite *et al,* 2004).

A pilot study conducted by Dupont *et al*, suggested that the addition of montelukast to a fixed-association inhaled steroid and LABA, may result in significant improvement in asthma control (Dupont *et al*, 2005). Beneficial additive effects of salmeterol and montelukast in asthma control were also reported in two other studies (Dempsey *et al*, 2000; Deykin *et al*, 2007).

Theophylline has been used in the treatment of asthma and COPD for many years. It is known that theophylline inhibits all phosphodiesterase (PDE) isozymes non-selectively. This might contribute to the therapeutic effects in asthma but the non-selective inhibition of all PDEs, leads to side effects that limit its usefulness as does its antagonist actions on type $A2_A$ adenosine receptors (Fukuda *et al*, 2011).

1.2.7.4 New Targets for Treatment:

Total control of asthma in all the different groups, remains a challenge and new targets for pharmacotherapy are constantly being investigated. In a review by Walsh published in *Discovery Medicine* in April 2011, the limitations of new



cytokine-directed therapies of asthma are discussed. Omalizumab, a humanised monoclonal antibody directed at the FccRI binding domain of human IgE, is the only biologic drug that has proven efficacy in the treatment of asthma. It is mainly used in severe allergic asthmatics. Additionally, anti-IL-13 monoclonal antibodies have been reported to be therapeutically useful in a subset of patients with bronchial asthma, specifically those patients with high circulating levels of periostin (Corren *et al.*, 2011). Revisiting the potential of existing drugs is also a target for investigation (Walsh 2011).

1.2.7.4.1 Phosphodiesterase Inhibitors:

Cyclic AMP (3'-5'-cyclic adenosine monophosphate cAMP), the "original second messenger," is generated when a first messenger, including chemokines, lipid mediators, hormones or drugs, bind to a seven-transmembrane-spanning G protein-coupled receptor (GPCR), which in turn is coupled to a stimulatory G protein α subunit (G α s) (Serezani *et al*, 2008). This leads to the exchange of GDP for GTP on the G α s protein and dissociation of the $\beta\gamma$ subunit complex. The enzyme adenylyl cyclase (AC) is then activated to catalyse the cyclisation of ATP to generate cAMP and pyrophosphate. Ligands which activate G α i subunits on the other hand inhibit AC and the production of cAMP, and include LTB₄, LTC₄ and LTD₄. Intracellular levels of cAMP are controlled by AC and the enzyme cAMP phosphodiesterase (PDE). To date, eleven distinct cyclic nucleotide PDE gene families are recognised (Torphy, 1998).



Inactivation of cyclic nucleotides by PDEs is mediated via hydrolytic cleavage of the 3'-phosphoester bond to an inactive 5'-nucleotide monophosphate. The affinities of the various PDEs differ for cAMP and cGMP. PDE4 and PDE7 are selective for cAMP. PDE4 is also the predominant PDE expressed in neutrophils (Torphy, 1998).

Roflumilast, an orally administered selective PDE4 inhibitor for the treatment of COPD, was first identified in 1993 (Rabe, 2011). *In vitro*, inhibition of PDE4 will lead to a reduction in apoptosis and release of inflammatory mediators from neutrophils (Hatzelmann *et al*, 2010). *In vivo*, this reduction will lead to inhibition of cell movement and cytokine and chemokine release from inflammatory cells such as neutrophils, eosinophils, macrophages and T-cells (Sanz, Cortijo & Morcillo, 2005). Roflumilast has been approved for use in COPD in the USA and Europe (Page & Spina, 2011). The gastro-intestinal side-effects such as nausea and vomiting might, however, limit its use in practise (Field, 2008). The molecule has been studied in asthmatics with effects comparable to those of inhaled corticosteroids (Rabe, 2011).

In addition, GlaxoSmithKline plc is developing 256066, an inhaled formulation of a PDE4 inhibitor that has demonstrated efficacy in trials in asthma (Higgs, 2010).

Although the effects of montelukast on PDEs are unknown, it is noteworthy that the early, first generation experimental CysLT₁R antagonists were found to possess PDE inhibitory activity (Fleish, Rinkema & Marshall, 1984).



1.2.7.4.2 Montelukast:

Montelukast sodium, 2-[1[[1-[3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-2[1hydroxy-1-methyl-ethyl)phenyl]-propyl]sulphanyl-methyl]-propyl]cyclopropyl] ethanoic acid, is a selective, pharmacological antagonist of type 1 cysteinyl leukotriene receptors (CysLT₁Rs) (Al Omari *et al*, 2007).

The primary mode of action of montelukast is via antagonism of the CysLT₁Rs, blocking the pro-asthmatic/ pro-inflammatory effects of the CysTLs. This mode of action forms the basis of the use of this agent in the treatment of the inflammation in asthma (Bateman *et al,* 2008). Montelukast is marketed by Merck (Merck Research Laboratories, Rahway, NJ, USA) as Singulair.

The varied clinical responses in different individuals after oral intake of montelukast might be due to genetic variations of the efflux and uptake transport proteins (Lima, 2007). It is rapidly absorbed from the intestine after intake of the different oral formulations. The mean peak plasma concentration is achieved at 3-4 hours after intake of the 10mg tablet in fasted adults and at 2-2.5 hours after intake of the 5 mg chewable tablet in fasted adults. In fasted 2 to 5 year old children, the mean peak plasma concentration after intake of the 4 mg chewable tablet was reached at 2 hours while the 4 mg granule formulation is bioequivalent to the 4 mg chewable tablet after oral intake by fasted adults.

The mean oral bioavailability in those taking the 10 mg tablet was 64% while those of the 4 mg and 5 mg chewable tablets were 63% and 73% respectively (Merck



Research Laboratories). Maximum plasma concentrations (Cmax) after oral administration of a 10 mg tablet ranged between 350-385 ng/ml in healthy adults (Cheng *et al,* 1996). In 2-5 year old children, the Cmax value after intake of 4 mg chewable tablets was 471 ng/ml, while in 6-14 month old children the Cmax was 514 ng/ml after administration of 4 mg oral granules (Knorr *et al,* 2001).

Montelukast is considered to be safe in children from 6 months of age and is the only CysLT antagonist that has registration in children under the age of 12 years as mentioned above (Gravett *et al*, 2010).

Evidence of successful treatment of patients with several different phenotypes of asthma with montelukast is well documented, including, atopic asthma (Riccioni *et al*, 2007), aspirin-sensitive asthma (Currie & McLaughlin, 2006), acute exacerbations due to viral infections (Bisgaard, 2003), asthma in children (Bacharier *et al*, 2008), exercise-induced asthma (Currie & McLaughlin, 2006), and asthma in patients that smoke (Rabinovich et al, 2008). The efficacy of montelukast in these patient groups is primarily due to the blockade of CysTLRs, but may also involve other mechanisms. In this context montelukast has also been shown to play a role in reducing airway remodelling via reducing fibronectin-induced migration of human lung fibroblasts that stimulate smooth muscle proliferation (Tokuriki *et al*, 2007), while studies have shown secondary anti-inflammatory effects of montelukast, unrelated to the antagonism of the CysLT₁Rs, such as:



- Inhibition of 5-lipoxygenase in activated neutrophils as well as monocytes/ macrophages, which leads to a decrease in the synthesis of CysLTs as well as in LTB₄. The mechanisms have not been fully described, but are not via CysLT₁R blockade. The concentrations of montelukast needed for this action, are slightly higher than those needed for antagonism of the CysLT₁Rs (Ramires *et al*, 2004). The blocking of LTB₄ production is potentially an added benefit in treating neutrophil-mediated inflammation in asthma, which was mentioned to be corticosteroid-resistant.
- Inhibition of adherence of eosinophils to vascular endothelium by interfering with the interaction of the eosinophil adherence molecule, α₄β1, with vascular cell adhesion molecule-1, its counter receptor (Robinson *et al*, 2008). This inhibition was unaffected by the inclusion of the 5-lipoxygenase-activating protein inhibitor, MK886, confirming the CysLT₁R-independent mechanism. Montelukast also decreased eosinophil migration activated by of the chemoattractant, 5-oxo-6,8,11,14-eicosatetraenoic acid, which was also associated with a decreased expression of the urokinase plasminogen receptor and a decrease in secretion of MMP-9, which in turn protects against tissue extracellular matrix digestion (Lanlois *et al*, 2006).
- Montelukast was reported, together with pranlukast and zafirlukast, to antagonise the effects of nucleotides acting at P2Y receptors on both a monocyte/macrophage cell line and primary human monocytes, effects that were characterised by inhibition of phospholipase C. This inhibition leads to the



failure to produce inositol triphosphate and Ca^{2+} mobilisation from intracellular stores with decreased production of IL-8 (Mamedova *et al*, 2005).

 In studies on some of the earlier CysLT₁R antagonists such as FLP55712 (Fleish, Rinkema & Marshall, 1984) and LY171883 (Hay *et al*, 1987) and the novel agent CR3465 (Ferrari *et al*, 2004), secondary, non-specific PDE inhibition was described. CR3465 like montelukast possesses a quinoline moiety which may underpin the inhibition of the PDEs.

Another agent, ibudilast, also known as KC-404, AV-411 and MN-166, was developed in Japan and has been marketed there for the treatment of asthma and cerebrovascular disorders. This molecule combines CysLT₁R antagonism and PDE inhibitory properties (Barkhof *et al*, 2010).

The successful use of montelukast in the treatment of diseases other than asthma is well documented. In patients with moderate-to-severe COPD, montelukast therapy leads to clinical improvement (Drakatos *et al*, 2009), while in another study, montelukast added to existing treatment regimes, resulted in decreases in serum levels of LTB₄, IL-8 and TNF (Gueli *et al*, 2011). Several studies done in patients with cystic fibrosis have shown efficacy of montelukast treatment (Stelmach *et al*, 2005), while in children with reactive airway disease following respiratory syncytial virus infection, montelukast led to reduction of symptoms and a delay in acute exacerbations (Bisgaard, 2003). In a recent study done on patients with bronchiolitis obliterans syndrome after lung transplantation, montelukast



added to azithromycin resulted in a decrease in FEV₁ decline (Verleden *et al*, 2011).

Besides lung diseases, montelukast was also reported to be of benefit in sepsisinduced hepatic and ileal injury in a rat model (Sener *et al*, 2005), and in another study also in rats, it led to improved wound healing in burn injury (Turtay *et al*, 2010). In a model of gouty arthritis, montelukast led to a decrease in the total inflammatory cell count, with a predominant effect on polymorphonuclear cells (Ponce *et al*, 2011). Data from a study in atherosclerotic rabbits showed that montelukast inhibited neointimal hyperplasia which was associated with decreased expression of MMP-2 and MMP-9 independent of plasma lipid levels (Liu *et al*, 2009). In a recent study by Wang *et al* in experimented autoimmune encephalomyelitis, montelukast and zafirlukast, both targeting CysTLR1, effectively blocked central nervous system infiltration by inflammatory cells, indicative of potential use in the treatment of multiple sclerosis (Wang *et al*, 2011).

In conclusion, this literature review suggests that neutrophilic inflammation in asthma should be targeted in treatment regimens and that new properties of existing drugs should be explored. It also emphasises the potential of montelukast in controlling inflammation.



1.3 Hypothesis

The hypothesis which forms the basis of this study is that the anti-asthma agent, montelukast, possesses secondary neutrophil-directed anti-inflammatory properties which are distinct from conventional CysLT₁R antagonism. Alternatively, the pro-inflammatory function of these cells is not affected by montelukast.

Objectives:

The primary objectives of the laboratory research described in this thesis were to investigate the effects of:

- The cysteinyl leukotrienes (CysLTs) C₄ and D₄ on isolated human neutrophils, which may result in hyperreactivity of the cells on subsequent exposure to chemoattractants.
- 2) Montelukast, at therapeutically relevant concentrations, on the mobilization of stored and extracellular Ca²⁺, as well as on several Ca²⁺-dependent, proinflammatory activities of neutrophils, in relation to alterations in intracellular cAMP levels, and activities of cAMP and cGMP PDEs following exposure of isolated human neutrophils to chemoattractants.
- 3) Formoterol and montelukast, individually and in combination, on the generation of ROS and LTB₄, as well as release of the matrix metalloproteinases, MMP-8 and MMP-9, and expression of the neutrophil adhesion molecule CR3 in



relation to alterations in the intracellular concentrations of cAMP and cytosolic

Ca²⁺.


Chapter 2

Leukotrienes C₄ and D₄ Sensitize Human Neutrophils for Hyperreactivity to Chemoattractants



2.1 Introduction:

Although primarily involved in the immunopathogenesis of bronchial asthma and other atopic disorders, cysteinyl leukotrienes (CysLTs) are being increasingly implicated in the aetiology of acute and chronic inflammatory diseases of nonallergic origin, including cardiovascular diseases, autoimmune diseases, and certain malignancies (Peters-Golden & Henderson, 2007). The spectrum of proinflammatory activities of CysLTs may therefore extend beyond eosinophils, monocytes/macrophages, type 2 helper T (Th2) lymphocytes, and airway smooth muscle cells, to other types of inflammatory cells such as neutrophils. Human neutrophils do not possess the enzyme LTC₄ synthase and are therefore unable to generate CysLTs (Peters-Golden & Henderson, 2007). Interestingly, however, these cells do possess G protein/ phospholipase C-coupled membrane receptors for LTC₄ and LTD₄ (Akgul & Edwards, 2003; Andina et al, 2009; Heimbürger & Palmblad, 1996), with ligand/receptor binding resulting in modest activation of the cells, specifically Ca²⁺ mobilization and generation of nitric oxide (Heimbürger & Palmblad, 1996; Andina et al, 2009). On the other hand, relatively little is known about the potential of CysLTs to sensitize/"prime" neutrophils for enhanced reactivity to conventional activators, such as chemoattractants, as well as the potential of antagonists of type 1 CysLT receptors (CysLT₁ Rs) such as montelukast to attenuate these interactions.

54



In the current study, the effects of pretreatment of human neutrophils with the CysLTs, C_4 and D_4 , on production of superoxide and release of elastase on subsequent activation of the cells with the chemoattractant, FMLP, as well as the effects of montelukast on superoxide generation, have been investigated.

2.2 Materials and methods

2.2.1 Leukotrienes

Leukotrienes C₄ and D₄ (50 μ g.ml⁻¹ and 55 μ g.ml⁻¹ respectively, in 70% aqueous methanol) were purchased from Sigma-Aldrich, St Louis, MO, USA, and used at a final concentration range of 50–300 nmol.L⁻¹. Montelukast sodium,2-[1[[1-[3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-2[1-hydroxy-1-methyl-ethyl)phenyl]-propyl] sulfanyl-methyl]propyl] cyclopropyl] ethanoic acid, was kindly provided by Merck Research Laboratories, Rahway, NJ, USA, dissolved in dimethyl sulphoxide (DMSO) to a stock concentration of 10 mmol.L⁻¹, and used at a fixed, final concentration of 100 nmol.L⁻¹, which is a therapeutically relevant concentration of this agent as measured in blood after oral administration.(Cheng *et al*, 1996; Knorr *et al*, 2001). Unless indicated, all other chemicals and reagents were purchased from Sigma-Aldrich. Appropriate solvent controls were used in all of the assays described below.

2.2.2 Preparation of neutrophils



The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, and prior informed consent was obtained from all blood donors. Neutrophils were isolated from heparinized venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Health assessments were done by qualified nursing sisters and the donors were medication free as determined by questionnaires. Neutrophils were separated from centrifugation on Histopaque-1077 mononuclear leucocytes by (Sigma Diagnostics) cushions at 400 x q for 25 min at room temperature. The resultant pellets were suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. Following centrifugation (280 x g at 4°C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), determined by flow cytometric procedures, were re-suspended to 1×10^7 ml⁻¹ in PBS and held on ice until used. Contaminating cells were not regularly analized but were predominatly mononuclear cells (<5%).

2.2.3 Spectrofluorimetric measurement of cytosolic Ca²⁺

Fura-2/AM was used as the fluorescent, Ca^{2+} -sensitive indicator for these experiments (Grynkiewicz, Poenie & Tsien, 1985). Neutrophils (1 × 10⁷.ml⁻¹) were incubated with fura-2/AM (0.5 µmol.L⁻¹) for 30 min at 37°C in PBS, washed and resuspended in indicator-free Hanks balanced salt solution (HBSS, pH 7.4), containing 1.25 mmol.L⁻¹ CaCl₂. The fura-2-loaded cells (2 × 10⁶.ml⁻¹) were then



pre-incubated for 8 min at 37°C with montelukast (100 nmol.L⁻¹) or an equivalent volume of DMSO in control systems, after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Perkin Elmer LS45 luminescence spectrometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (\pm 1 min), the neutrophils were activated by the addition of either LTC₄ or LTD₄ (100 nmol.L⁻¹), or the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µmol.L⁻¹, final), and alterations in fluorescence intensity monitored over a 1–3 min time course. The following were investigated in additional experiments: i) the effects of pre-treatment of neutrophils with montelukast (100 nmol.L⁻¹) on cytosolic Ca²⁺ fluxes in neutrophils activated with the CysLTs; and ii) the effects of pre-treatment of the cells with either LTC₄ or LTD₄ (both at 100 nmol.L⁻¹) on the FMLP-activated alterations in cytosolic Ca²⁺ (the CysLTs were added to the cells 10 sec before FMLP).

2.2.4 Measurement of superoxide production

This was measured using a lucigenin (bis-*N*-methyl-acridinium nitrate)-based chemiluminescence procedure which detects superoxide (Minkenberg & Ferber, 1984). Briefly, neutrophils (1×10^6) were preincubated for 10 min at 37°C without and with montelukast (100 nmol.L⁻¹) in 900 µl of HBSS containing lucigenin (0.2 mmol.L⁻¹), followed by addition of either LTC₄ or LTD₄ (100 nmol.L⁻¹) and 10 sec later by HBSS (unstimulated control systems) or FMLP (1 µmol.L-1) and chemiluminescence responses recorded using a Lumac Biocounter (Model 2010,

57



Lumac Systems). The final volume in each vial was 1 ml, and the results, which are expressed in relative light units (rlu), are the peak values for FMLP-activated systems which were reached 40-50 sec after addition of the stimulant. FMLP was used at 1 μ mol.L⁻¹ because this is the concentration which is optimal in our hands for NADPH oxidase activation and superoxide production.

2.2.5 Elastase and MMP-8 release

Neutrophil degranulation was measured according to the extent of release of elastase and MMP-8 from the primary and specific granules, respectively. In the case of elastase release, neutrophils were incubated at a concentration of 2×10^{6} .ml⁻¹ in HBSS for 10 min at 37°C followed by successive addition at 10 sec intervals of either LTC₄ or LTD₄ (50–300 nmol.L⁻¹) and FMLP (1 µmol.L⁻¹) in combination with a submaximal concentration of cytochalasin B (CB; 0.5 µmol.L⁻¹, final) and the cells incubated for 15 min at 37°C. Neutrophil–free supernatants were assayed for elastase using a micromodification of a standard colourimetric procedure (Beatty et al, 1982). Briefly, 125 ml of supernatant were added to the elastase substrate, N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide [3 mmol•L-1 in dimethylsulphoxide (DMSO)] in 0.05 M Tris-HCI (pH 8.0), and elastase activity was monitored spectrophoto-metrically at a wavelength of 405 nm.

In the case of MMP-8 release, the experimental design was essentially similar, the only exceptions being that FMLP (1 μ mol.L⁻¹) was used without added CB and the



CysLTs were used at a fixed, final concentration of 100 nmol.L⁻¹. MMP-8 in the supernatants of the cells was assayed using an ELISA procedure (Quantikine, R&D Systems, Inc. Minneapolis) and the results expressed as ng.ml⁻¹.

2.2.6 Expression and statistical analysis of results

With the exception of the results of the fura-2 fluorescence experiments, some of which are presented as the traces from representative experiments, the results of each series of experiments are presented as the mean values ± the standard error of the mean (SEM). Levels of statistical significance were determined by the Mann-Whitney U-test where appropriate by comparing LT-treated systems with the corresponding control system.

2.3 Results

2.3.1 Fura-2 fluorescence response

The results shown in Figure 2.1 (page 65) are representative traces from one experiment (6 in the series all of which showed similar effects) which depict the fura-2 fluorescence responses of neutrophils following exposure to 100 nmol.L⁻¹ LTD₄ or LTC₄. Addition of these CysLTs to neutrophils was accompanied by an abrupt, albeit moderate, increase in fura-2 fluorescence, compatible with an elevation in the cytosolic concentration of Ca²⁺, with LTD₄ being more potent than LTC₄. FMLP (1 μ mol.L⁻¹) was included for comparison, and was found to be a considerably more potent activator of Ca²⁺ mobilization than either LTC₄ or LTD₄



(Figure 2.1). Pre-treatment of neutrophils with LTD₄ (100 nmol.L⁻¹) did not affect the FMLP-activated alterations in cytosolic Ca²⁺ (Figure 2.2, page 66), compatible with lack of effect of LTD₄ on either phospholipase C activation and store-operated Ca²⁺ influx in FMLP-activated neutrophils. These responses of FMLP-activated cells were also unaffected by exposure to LTC₄ (results not shown). Comparable results were obtained when FMLP was used at a final concentration of 100 nmol.L⁻¹ (not shown). As shown in Figure 2.3 (page 67) pre-treatment of neutrophils with 100 nmol.L⁻¹ montelukast attenuated the increase in cytosolic Ca²⁺ following exposure of the cells to LTD₄.

2.3.2 Superoxide production

The effects of addition of LTC₄ or LTD₄ individually on superoxide production by neutrophils, as well as their sensitizing effects on oxidant generation by FMLP-activated neutrophils, are shown in Figure 2.4 (page 68). Addition of either LTC₄ or LTD₄ at a concentration of 100 nmol.L⁻¹ to neutrophils had no significant effects on superoxide generation. However, addition of either of the CysLTs to the cells 10 sec prior to FMLP (1 μ mol.L⁻¹) resulted in significant augmentation of the chemoattractant-activated responses. These observations demonstrate that LTC₄ and LTD₄ sensitize/"prime" neutrophils for enhanced oxidant production following activation with FMLP. The "priming" of the FMLP-activated superoxide responses of neutrophils are shown in Figure 2.5 (page 69). Pre-treatment of neutrophils with montelukast resulted in partial, but statistically significant (P < 0.05) antagonism of the sensitizing effects of the CysLTs on FMLP-activated superoxide generation.



Importantly, montelukast at the concentration used (100 nmol.L⁻¹) did not affect superoxide production by cells activated with FMLP in the absence of the CysLTs (data not shown).

2.3.3 Elastase and MMP-8 release

Exposure of neutrophils to either LTC_4 or LTD_4 did not result in release of either elastase or MMP-8 from neutrophils (results not shown). However, as shown in Figure 2.6 (page 70), addition of LTC_4 or LTD_4 (50–300 nmol.L⁻¹) to neutrophils prior to FMLP/CB, resulted in augmentation of elastase release in comparison with the responses of cells exposed to FMLP/CB only. As shown in Table 2.1 (page 71), similar effects were observed with respect to release of MMP-8 from cells activated with FMLP (without CB) in the presence of the CysLTs at a fixed concentration of 100 nmol.L⁻¹.

2.4 Discussion

CysLTs exert their effects through cysteinyl leukotriene receptors. These receptors are seven transmembrane-spanning receptors that couple to G proteins and activate intracellular signaling pathways in response to agonist binding (Capra, 2004; Peters-Golden & Henderson, 2007). Neutrophils have been shown to possess G protein/phospholipase C (PLC)-coupled membrane receptors for LTC₄ and LTD₄ (Heimbürger & Palmblad, 1996; Lärfars *et al*, 1999; Zhu *et al*, 2005), although they are unable to produce these leukotrienes (Peters-Golden &



Henderson, 2007). Two CysLT receptors, CysLT₁ and CysLT₂ have been identified, cloned, and characterized (Kanaoka & Boyce, 2004; Peters-Golden & Henderson, 2007). The CysLT1R has nanomolar affinity for LTD₄ and couples to the heterotrimeric G protein Gq to promote calcium mobilization. LTC₄ is also a full agonist of the CysLT₁R, but is 10 times less potent (Naik *et al*, 2005). The rank order of potency of agonist activation for the CysLT1R is LTD₄ > LTC₄ > LTE₄, while for the CysLT₂R it is LTC₄ = LTD₄ > LTE₄ (Capra, 2004).

In the current study, addition of either LTC₄ or LTD₄, at a fixed concentration of 100 nmol.L⁻¹ to human neutrophils, cause rapid, transient elevations in cytosolic Ca²⁺, which were of lesser magnitude than those observed following activation of the cells with FMLP (1 μ mol.L⁻¹). LTD₄ was found to be a more potent activator of Ca²⁺ mobilization than LTC₄. In another study, Heimbürger and Palmblad reported that exposure of neutrophils to LTD₄ at 100 nmol.L⁻¹, but not LTC₄, caused transient elevations in cytosolic Ca²⁺, which were of lesser magnitude than those observed following activation of the cells with FMLP (100 nmol.L-1) (Heimbürger & Palmblad, 1996). As observed in the present study with montelukast, these authors observed that pretreatment of neutrophils with the CysLT₁R antagonist, SKF104353, abrogated the LTD₄-mediated elevation in cytosolic Ca²⁺. In apparent contrast, Bautz et al. reported that LTD₄-activated Ca²⁺ fluxes in neutrophils were not affected by pretreatment of the cells with MK571, a selective leukotriene D₄ receptor antagonist, suggesting the involvement of other types of receptors, such as CysLT2 (Bautz et al, 2001). Mobilization of both stored and extracellular Ca²⁺ normally precedes, and is a prerequisite, for membrane receptor- mediated



activation of the proinflammatory activities of human neutrophils, including generation of superoxide, degranulation, and adhesion of the cells to vascular endothelium. Previous studies have reported that exposure of neutrophils to either LTC₄ or LTD₄ results in activation of nitric oxide synthase and mobilization of secondary granules, while, in keeping with the current study, NADPH oxidase and primary granule release are unaffected (Lärfars *et al*, 1999; Lew *et al*, 1987).

However, CysLT-mediated sensitization of neutrophil NADPH oxidase activity and mobilization of primary granules on exposure of the cells to a second, more potent stimulus, although potentially important, has not to our knowledge been addressed in previous studies. Importantly, we observed that brief, pretreatment of neutrophils with either of the CysLTs, sensitized the cells for enhanced generation of superoxide and release of elastase and MMP-8 on subsequent exposure to the chemoattractant, FMLP. Interestingly, the CysLTs were equipotent with respect to their priming interactions with neutrophils, which were attenuated in part by montelukast, compatible with involvement of CysLT₁Rs. Because of concerns about alternative mechanisms of montelukast-mediated anti-inflammatory activity, such as inhibition of 5-lipoxygenase (Ramires *et al*, 2004), which are unrelated to CysLT₁R antagonism, reservations were evident about using the drug at concentrations in excess of 100 nmol.L⁻¹.

Although the precise mechanisms involved in the priming process are poorly understood, two lines of evidence appear to exclude the involvement of Ca²⁺ in CysLT-mediated priming of superoxide production by FMLP-activated neutrophils,

63



as well as elastase release. These are: i) LTD_4 is more effective than LTC_4 with respect to Ca^{2+} mobilization, however, the priming potencies of the two CysLTs are comparable; and ii) the fura-2 responses of FMLP-activated neutrophils are unaffected by pretreatment of the cells with either of the CysLTs. Alternative possibilities include alterations in the activities of various intracellular kinases involve in signal transduction, including tyrosine kinase, PI3K and MAPK family members (Hallett & Lloyds, 1995; Dang *et al*, 2006).

Irrespective of the molecular/biochemical mechanisms of CysLT-mediated priming of neutrophils, the observations reported in the current study clearly identify a previously unrecognized proinflammatory interaction of CysLTs with human neutrophils, which results in the exaggerated production of superoxide and release of the granule proteases, elastase and MMP-8. All of these have been implicated in the pathogenesis of airway hyperresponsiveness, damage and remodelling (Suzuki *et al*, 1996; Prikk *et al*, 2002; Hiraguchi *et al*, 2008; Sugiura & Ichinose, 2008;). If operative in vivo, this mechanism may explain the therapeutic activity of CysLT₁R antagonists, such as montelukast, in diseases such as COPD (Rubinstein, Kumar & Schriever, 2004; Celik *et al*, 2005) and certain types of bronchial asthma, in which the neutrophil is thought to be the primary offender (Barnes, 2007).





Figure 2.1: Fura-2 fluorescence responses of neutrophils activated with FMLP (1 μ mol.L⁻¹), LTC₄ or LTD₄ (100 nmol.L⁻¹). FMLP and the CysLTs were added as indicated (\downarrow) when a stable baseline was obtained. These are typical traces of 6 different experiments.





Figure 2.2: Fura-2 fluorescence responses of neutrophils activated with FMLP (1 μ mol.L⁻¹) or LTD4 (100 nmol.L⁻¹) individually or in combination. LTD₄ was added 10 sec prior to FMLP. FMLP and LTD₄ were added as indicated (\downarrow) when a stable base-line was obtained. These are typical traces of 3 experiments.





Figure 2.3: LTD₄-activated fura-2 fluorescence responses of control and montelukast (100 nmol.L⁻¹)-treated neutrophils. LTD₄ was added as indicated (\downarrow) when a stable base-line was obtained. These are typical traces of 3 experiments.





Figure 2.4: Effects of LTC₄ or LTD₄ (100 nmol.L⁻¹) on superoxide production by unstimulated cells and cells activated with FMLP (1 μ mol.L⁻¹). The leukotrienes were added 10 sec prior to the FMLP. The results of 6 different experiments are presented as the mean peak lucigenin enhanced chemiluminescence values ± S.E.M. * P < 0.05 for comparison with neutrophils activated with FMLP only.





Figure 2.5: The effects of montelukast (100 nmol.L⁻¹) on superoxide production by neutrophils activated with a combination of LTC_4 or LTD_4 (100 nmol.L⁻¹) and FMLP (1 µmol.L⁻¹). The leukotrienes were added 10 sec prior to FMLP. The results of 5 different experiments are presented as the mean peak lucigenin-enhanced chemiluminescence values ± S.E.M.

* P < 0.05 for comparison with neutrophils activated by a combination of LTC_4/LTD_4 and FMLP in the absence of the drug.





Figure 2.6: Effects of varying concentrations of LTC_4 or LTD_4 (50-300 nmol.L⁻¹) on elastase release by neutrophils activated with FMLP/CB. The leukotrienes were added 10 sec prior to FMLP. The results of 3 different experiments are presented as the mean values \pm S.E.M for release of elastase.

* P < 0.05 for comparison with neutrophils activated by FMLP/CB only.



Table 2.1: Effects of LTC_4 or LTD_4 (100 nmol.L⁻¹) on MMP-8 release by cells activated with FMLP (1 µmol.L⁻¹).

System	MMP-8 Level (ng.ml ⁻¹)	P-value	
FMLP (1 µmol.L ⁻¹)	86 ± 4		
LTC ₄ + FMLP	108 ± 6	0.001*	
LTD ₄ + FMLP	116 ± 10	0.001*	

The results of 4 experiments are expressed as the mean values \pm S.E.M.

* P-value with respect to FMLP only control system. MMP-8 was undetectable in unstimulated cells.



Chapter 3

Montelukast Inhibits Neutrophil Pro-Inflammatory Activity by a Cyclic AMP-Dependent Mechanism



3.1 Introduction:

Montelukast, a highly selective antagonist of cysteinyl leukotriene (CysLT) receptors, is widely used in the treatment of bronchial asthma, primarily as an adjunct to corticosteroids (Anonymous, 2004; Currie et al, 2005; Diamant & van der Molen, 2005; Riccioni et al. 2007). In this setting, the therapeutic activity of montelukast is achieved through antagonism CysLT-mediated of bronchoconstriction, increased vascular permeability and mucus secretion, following release of these mediators, mainly from monocytes/macrophages, eosinophils, mast cells and basophils, as well as by anti-inflammatory actions targeting type 2 helper CD4⁺ T-lymphocytes (Peters-Golden & Henderson, 2007). Unlike corticosteroids, montelukast has been reported to modulate airway remodelling in patients with chronic asthma, compatible with an extended spectrum of anti-inflammatory activity (Henderson et al, 2006; Muz et al, 2006). Montelukast has also been reported to possess therapeutic activity in other diseases such as chronic obstructive pulmonary disease, a disorder that is believed to be of neutrophilic aetiology (Celik et al, 2005; Rubinstein, Kumar & Schriver, 2004). Although they do not produce CysLTs, neutrophils do possess receptors for LTC₄ and LTD₄, activation of which triggers relatively modest pro-inflammatory responses in these cells (Lärfars et al, 1999; Zhu et al, 2005). Interference with neutrophil activation by CysLTs released from other cell types, such as monocytes/macrophages, mast cells or eosinophils, may therefore underlie the neutrophil-directed therapeutic efficacy of montelukast. Alternatively, montelukast

73



may possess secondary anti-inflammatory properties that are distinct from conventional antagonism of CysLT receptors. These include interference with activation of the transcription factor, nuclear factor kappa B in immune and inflammatory cells, promotion of sustained production of interleukin-10 in inflamed airways or by inhibition of signalling pathways triggered by P2Y receptors (Mamedova *et al*, 2005; Wu, Zhou & Li, 2006). However, the contribution of these mechanisms to the possible neutrophil-targeted anti-inflammatory activity of montelukast is unclear.

In the current study, the effects of montelukast, at therapeutically relevant concentrations, on the mobilization of stored and extracellular Ca^{2+} by chemoattractant-activated human neutrophils, as well as on several Ca^{2+} -dependent, pro-inflammatory activities of the cells have been investigated. The results demonstrate that montelukast antagonizes the pro-inflammatory activities of neutrophils by a mechanism involving inhibition of cyclic nucleotide phosphodiesterases (PDE), favouring cAMP-mediated attenuation of Ca^{2+} influx.

3.2 Materials and Methods:

3.2.1 Chemicals and reagents

Montelukast sodium, 2-[1[[1-[3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-2[1hydroxy-1-methyl-ethyl)phenyl]-propyl]sulphanyl-methyl]-propyl]cyclopropyl] ethanoic acid, was kindly provided by Merck Research Laboratories, Rahway, NJ,

74



USA, and dissolved in DMSO to a stock concentration of 10 mmol·L⁻¹. Unless indicated, all other chemicals and reagents were purchased from Sigma-Aldrich, St Louis, MO, USA. In the various assay systems described below, montelukast was used at final concentrations of 0.1, 0.25, 0.5, 1 and 2 μ mol·L⁻¹. Peak serum concentrations of 0.5–1 μ mol·L⁻¹ are attainable during oral administration of montelukast (Cheng *et al,* 1996; Knorr *et al,* 2001). The final DMSO concentration was 0.05%, and DMSO control systems were included in each assay.

3.2.2 Neutrophils

The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, and prior informed consent was obtained from all blood donors. Neutrophils were isolated according to methods described in Chapter 2, 2.2.1.

3.2.3 Measurement of reactive oxygen species

These were measured using lucigenin (bis-*N*-methylacridinium nitrate)- and luminol (5-amino-2,3-dihydro-1,4-phthalazine dione) -enhanced chemiluminescence (CL) procedures that predominantly detect superoxide and reactive oxygen species (ROS) generated by the myeloperoxidase/H₂O₂/halide system respectively (Minkenberg & Ferber, 1984). Briefly, neutrophils (10^6 cells) were pre-incubated for 10 min at 37°C, without and with montelukast (0.1-2 mmol·L⁻¹) in 900 ml of Hanks' balanced salt solution (HBSS) containing either lucigenin (0.2 mmol·L⁻¹) or luminol (0.1 mmol·L⁻¹), followed by addition of either 100 µl of HBSS (unstimulated control



systems) or the chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µmol·L⁻¹) and CL responses recorded using a Lumac Biocounter (Model 2010, Lumac Systems Inc., Titusville, FL, USA). The final volume in each vial was 1 ml, and the results, which are expressed in relative light units (rlu), are the peak values for FMLP-activated systems that were reached 40–50 s after addition of the stimulant. MK886, an inhibitor of 5-lipoxygenase-activating protein, was used to investigate the possible contribution of LTs generated by neutrophils, as well as by contaminating cells in the neutrophil suspensions, to superoxide generation by FMLP activated cells, especially the involvement of LTC₄ and LTD₄. Neutrophils were pre-incubated with MK886 (0.5 µmol·L⁻¹, final) for 5 min at 37°C followed by addition of montelukast (0.5 μ mol·L⁻¹) and a further pre-incubation of 5 min followed by addition of FMLP (1 μ mol·L⁻¹) and measurement of lucigenin-enhanced CL. Control systems included neutrophils only, as well as cells treated with either MK886 or montelukast only. The efficacy of MK886 as an inhibitor of 5lipoxygenase in FMLP-activated neutrophils was measured according to the magnitude of inhibition of production of LTB₄ by the cells using the method described below. The superoxide-scavenging potential of montelukast (2 μ mol·L⁻¹) was measured using a cell-free xanthine (1 mmol·L⁻¹)/xanthine oxidase (130 mU ml⁻¹) lucigenin dependent CL procedure.

3.2.4 NADPH oxidase from isolated neutrophil membranes

Neutrophils (1 x 10^6 ml⁻¹) were pre-incubated for 10 min at 37°C without or with montelukast at a fixed concentration of 2 mmol·L⁻¹, followed by addition of FMLP (1



 μ mol·L⁻¹). After 3 min of incubation at 37°C, the reactions were terminated by addition of a large volume of ice-cold HBSS and the tubes transferred to an ice bath. The cells were then pelleted by centrifugation at 4°C and the pellets pooled and re-suspended to 5 x10⁶ ml⁻¹ in 0.34 M sucrose supplemented with 0.5 mmol·L⁻¹ phenylmethylsulphonyl fluoride (PMSF, Calbiochem Corp., La Jolla, CA, USA) and disrupted by sonication. Cellular debris was removed by centrifugation and the membrane fractions in the supernatants were harvested after centrifugation at 70000 x *g* for 30 min. The membrane pellets were dispersed in 1 ml of sucrose and assayed for NADPH oxidase activity using lucigenin-enhanced CL. Reaction mixtures (1 ml) contained lucigenin, membrane fractions (200 µl) and NADPH (2 mmol·L⁻¹), which was added last to initiate superoxide generation.

3.2.5 Oxygen consumption

This was measured using a three-channel oxygen electrode (Model DW1, Hansatech Ltd, King's Lynn, Norfolk, UK). Neutrophils (2 x 10^6 ml⁻¹) were preincubated for 10 min at 37°C in HBSS without or with montelukast at a fixed concentration of 1 µmol·L⁻¹ followed by addition of FMLP (1 µmol·L⁻¹) and measurement of PO₂ over a 5 min time course.

3.2.6 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule enzyme, elastase. Neutrophils were incubated at a concentration of 2×10^6 ml⁻¹ in HBSS with and without montelukast (0.1–2 µmol·L⁻¹) for 10 min at



37°C. FMLP (1 μ mol·L⁻¹) in combination with a submaximal concentration of cytochalasin B (0.5 μ mol·L⁻¹, final) was then added to the cells that were incubated for 15 min at 37°C. The tubes were then transferred to an ice bath, followed by centrifugation at 400 x *g* for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase according to the method described in chapter 2,2.2.5.

3.2.7 Spectrofluorimetric measurement of cytosolic Ca²⁺

Fura-2/AM was used as the fluorescent, Ca^{2+} -sensitive indicator for these experiments (Grynkiewicz, Poenie & Tsien, 1985). Neutrophils (1 x 10⁷ ml⁻¹) were incubated with fura-2/AM (2 µmol·L⁻¹) for 30 min at 37°C in PBS, washed and resuspended in indicator-free HBSS (pH 7.4), containing 1.25 mmol·L⁻¹ CaCl₂. The fura-2-loaded cells (2 x 10⁶ ml⁻¹) were then pre-incubated for 5 min at 37°C with montelukast (0.25–2 µmol·L⁻¹) or an equivalent volume of DMSO in control systems, after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm respectively. After a stable baseline was obtained (+/-1 min), the neutrophils were activated by the addition of the chemoattractants FMLP (1 µmol·L⁻¹, final), or platelet-activating factor (PAF, 200 nmol·L⁻¹, final) and alterations in fluorescence intensity monitored over a 5–10 min time course. Cytosolic calcium concentrations were calculated as described previously (Grynkiewicz, Poenie & Tsien, 1985).



3.2.8 Radiometric assessment of Ca²⁺ influx

A radiometric procedure was also used to measure the net influx of ${}^{45}Ca^{2+}$ into FMLP (1 µmol·L⁻¹)- or PAF (200 nmol·L⁻¹)- activated neutrophils uncomplicated by concomitant efflux of the radiolabelled cation. The cells were pre-incubated for 10 min at 37°C in Ca²⁺-replete (1.25 mmol·L⁻¹) HBSS to ensure that intracellular Ca²⁺ stores were full to minimize spontaneous uptake of ${}^{45}Ca^{2+}$ (unrelated to activation with FMLP or PAF) in the influx assay. The cells were then pelleted by centrifugation and re-suspended to a concentration of 1 x 10⁷ ml⁻¹ in HBSS containing 25 µmol·L⁻¹ cold, carrier CaCl₂.

The Ca²⁺-loaded neutrophils (2 x 10⁶ ml⁻¹) were then incubated for 5 min at 37°C in HBSS containing 25 µmol·L⁻¹ CaCl₂ in the absence or presence of montelukast (0.25 µmol·L⁻¹) followed by simultaneous addition of FMLP or PAF and 2 µCi ml⁻¹ ⁴⁵Ca²⁺ (as ⁴⁵[Ca]Cl₂, specific activity 24.3 mCi mg⁻¹, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA), or ⁴⁵Ca²⁺ only to control, unstimulated systems. The cells, in a final volume of 5 ml, were then incubated for 5 min at 37°C, after which chemoattractant-activated, store-operated uptake of Ca²⁺ is complete (Steel & Anderson, 2002), and the reactions stopped by the addition of 10 ml of ice-cold, Ca²⁺-replete HBSS to the tubes, which were transferred immediately to an ice bath. The cells were then pelleted by centrifugation at 400 x *g* for 5 min followed by washing with 15 ml of ice-cold, Ca²⁺-replete HBSS and the cell pellets dissolved in 0.5 ml 0.1% Triton X-100/0.1M NaOH and the radioactivity measured in a liquid scintillation spectrometer. The results are presented as the amount of cell-associated radioactivity (pmol ⁴⁵Ca²⁺.10⁷ cells⁻¹).



3.2.9 Measurement of LTB₄ and cyclic AMP

Competitive binding enzyme immunoassay procedures (Correlate-EIA[™], Assay Designs Inc., Ann Arbor, MI, USA) were used to measure LTB₄ in the supernatants of neutrophils activated with PAF (200 nmol·L⁻¹), while cAMP was measured in the extracts of unstimulated neutrophils, in the absence and presence of montelukast $(0.25-2 \text{ }\mu\text{mol}\cdot\text{L}^{-1})$. In the case of LTB₄, neutrophils (2 x 10⁶ ml⁻¹, final) in HBSS were preincubated for 10 min at 37°C with montelukast after which PAF was added to the cells and the reactions stopped after 3 min incubation at 37°C (predetermined in preliminary time course experiments) by the addition of an equal volume of ice-cold HBSS to the tubes, which were then held in an ice bath prior to pelletting the cells by centrifugation. The cell free supernatants were then assaved for LTB₄ using the enzyme immunoassay procedure. Supernatants from cells activated with PAF were diluted 1:4 prior to assay. These results are expressed as pg 10^7 cells⁻¹. In the case of cAMP, neutrophils (2 x 10^6 ml⁻¹, final) were preincubated for 10 min at 37°C followed by the addition of montelukast (0.25-2 μ mol·L⁻¹) after which the cells were incubated for a further period of 5 min at 37°C and the reactions were stopped by the addition of an equal volume of ice-cold HBSS to the tubes, which were then held on ice prior to pelletting the cells by centrifugation. Following centrifugation, the supernatants were discarded and cAMP extracted from the cell pellets by addition of 1 ml of 0.1 M HCl for 10-15 min followed by centrifugation to remove cell debris and the supernatants decanted and assayed for cAMP. These results are expressed as pmol cAMP 10⁷ cells⁻¹.



In an additional series of experiments, the cells were exposed to montelukast (2 μ mol·L⁻¹) or vehicle (0.05% DMSO) for 5 min at 37°C followed by the addition of salbutamol (β 2-adrenoreceptor agonist, 5 μ mol·L⁻¹), CGS21680 (adenosine A_{2A} receptor agonist, 1 μ mol·L⁻¹) or rolipram (type 4 PDE inhibitor, 0.1 μ mol·L⁻¹) for 3–5 min at 37°C after which cAMP was assayed in the cell extracts.

3.2.10 PDE activity

To prepare neutrophil cytosol, the cells (5 x 10^6 ml⁻¹) in PBS were pelleted by centrifugation, then re-suspended in 0.34 M sucrose and 0.5 mmol·L⁻¹ PMSF. The cells were then disrupted by sonication and cellular debris removed by centrifugation. The sonicates were then fractionated by ultracentrifugation at 70000 x q for 30 min and the supernatants harvested for assessment of PDE activity using a scintillation proximity assay (SPA, Amersham Biosciences, UK). Briefly, assays were performed at 30°C for 10 min in buffer containing 50 mmol·L⁻¹ Tris-HCI (pH 7.5), 8.3 mmol·L⁻¹ MgCl₂, 17 mmol·L⁻¹ EGTA and 0.3 mg ml⁻¹ bovine serum albumin. Each assay was performed in a reaction volume of 200 µl containing neutrophil cytosol (20 µl) as a source of PDE and approximately 0.05 mCi [³H]cAMP or [³H]cGMP in the absence and presence of montelukast (0.25–2 μ mol·L⁻¹), as well as rolipram (20 μ mol·L⁻¹), or the non-specific PDE inhibitor, 3isobutyl-1-methylxanthine (50 μ mol·L⁻¹) in control systems. Reactions were terminated by the addition of 75 µl of PDE SPA beads suspended in 18 mmol·L⁻¹ zinc sulphate and PDE-mediated hydrolysis of [³H]cAMP or [³H]cGMP determined by liquid scintillation spectrometry. The effects of montelukast on the activity of



PDE in a preparation isolated from bovine heart (Sigma Chemical Co.) were also investigated, using the enzyme preparation at a fixed, final concentration of 1 mU ml⁻¹.

In an additional series of experiments, the effects of pre-treatment of neutrophils with montelukast (2 µmol·L⁻¹) on the activities of cAMP PDE in matched, isolated membranes and cytosol fractions prepared from both unstimulated and FMLP (1 µmol·L⁻¹)-activated cells were investigated. Briefly, neutrophils were pre-incubated for 10 min at 37°C in the absence and presence of montelukast, followed by the addition of FMLP (or an equal volume of HBSS to control cells) and termination of reactions 1 min later by addition of ice-cold HBSS. The cells were then pelleted by centrifugation, re-suspended in 0.34 M sucrose/0.5 mmol·L⁻¹ PMSF, sonicated, and membrane and cytosol fractions prepared as described above and assayed for cAMP PDE activity by SPA. For purposes of comparison, the membrane and cytosol fractions were assayed for protein content and the results expressed as enzyme activity min⁻¹ mg protein⁻¹.

3.2.11 Inositol triphosphate (inositol-1,4,5-triphosphate)

Neutrophils at a concentration of 4 x 10^6 ml⁻¹ were preincubated for 5 min at 37° C in HBSS without or with montelukast (2 µmol·L⁻¹) after which the cells were activated with PAF (200 nmol·L⁻¹) in a final volume of 1 ml. The reactions were terminated and the inositol-1,4,5-triphosphate (IP3) extracted by the addition of 1 ml of 20% perchloric acid at 5 and 10s after the addition of PAF. Following a 20



min incubation on ice, the tubes were centrifuged at 2000 x *g* for 15 min and the supernatants decanted and titrated to pH7.5 with 5 M KOH followed by centrifugation at 2000 x *g* for 15 min to remove precipitated KClO₄. The supernatants were assayed for IP3 using the inositol-1,4,5-triphosphate (³H)radioreceptor assay kit (Perkin Elmer Life and Analytical Sciences), which is a competitive ligand binding assay, and the results expressed as pmol 10^7 cells⁻¹.

3.2.12 Cellular ATP levels

To determine the effects of montelukast (2 μ mol·L⁻¹) on neutrophil viability, intracellular ATP concentrations were measured in cell lysates (1 x 10⁶ cells ml⁻¹) following exposure of the cells to the drug for 15 min at 37°C, using a luciferin/luciferase CL procedure (Holmsen, Storm & Day, 1972). These results are expressed as nmol ATP 10⁷ cells⁻¹.

3.2.13 Statistical analysis

With the exception of the results of the fura-2 fluorescence experiments, some of which are presented as representative traces, the results of each series of experiments are presented as the mean values \pm SEM, either as the absolute values or as mean percentages of the corresponding drug-free control systems where *n* = the number of different donors used in each series of experiments, with the number of replicates for each drug concentration and drug-free control system for each experiment shown in the figure legends and table footnotes. Levels of statistical significance were determined by comparing the absolute values for each



drug-treated system with the corresponding values for the relevant drug-free control systems for each assay using the Friedman repeated measures ANOVA with Dunn's multiple comparisons post-test, or the Wilcoxon matched pairs signed-ranks test where appropriate, while a two-way repeated measures ANOVA with the Bonferroni post-test was used to analyse the data shown in Table3.1 (page 90).

3.3 Results

3.3.1 Production of ROS

The effects of montelukast (0.1–2 μ mol·L⁻¹) on the FMLP-activated generation of ROS using the lucigenin- and luminol-enhanced CL procedures are shown in Figure 3.1 (page 100). Treatment of the cells with montelukast resulted in dose-related inhibition of the generation of ROS, which was evident using both procedures and achieved statistical significance at concentrations of 0.5 μ mol·L⁻¹ (lucigenin, *P* < 0.001) or 1 μ mol·L⁻¹ (luminol, *P* < 0.01). Maximal inhibition was observed at 2 μ mol·L⁻¹ montelukast, resulting in 70% and 60% mean inhibition of the generation of ROS by FMLP-activated neutrophils with the lucigenin- and luminol-enhanced CL procedures respectively, the IC₅₀ for the latter being 1.5 μ mol·L⁻¹ (confidence intervals 1.1–1.9). As shown in Table 3.1 (page 90), pretreatment of neutrophils with MK886 (0.5 μ mol·L⁻¹) did not affect the generation of superoxide by FMLP-activated neutrophils in either the absence or presence of montelukast (0.5 μ mol·L⁻¹). Treatment of neutrophils with MK886 resulted in almost complete inhibition of the FMLP-activated production of LTB₄ by the cells, the



values for unstimulated cells and for FMLP-activated cells in the absence and presence of MK886 being 59 ± 8, 332 ± 23 and 22 ± 3 pg LTB₄ 10^7 cells⁻¹ (n = 5 with a minimum of two replicates for each system).

The activity of NADPH oxidase in isolated membranes prepared from neutrophils activated with FMLP was markedly attenuated by treatment of the cells with montelukast (2 µmol·L⁻¹). The results for membrane fractions prepared from unstimulated neutrophils and those from neutrophils activated with FMLP in the absence and presence of montelukast were 2852 ± 291, 11 543 ±698 and 6518 ± 407 rlu respectively (n = 5 with two replicates for each system in each experiment; P < 0.05 for comparison of FMLP-activated systems without and with montelukast).

At the maximum concentration of montelukast used in these studies (2 μ mol·L⁻¹), the drug did not possess detectable superoxide-scavenging activity, with the lucigenin-enhanced CL values of the xanthine oxidase/xanthine superoxide generating system in the absence and presence of montelukast being 22 939 ± 4850 and 22 271 ± 5413 rlu respectively (data from three separate experiments with three to four replicates for the control and drug-treated systems).

Activation of neutrophils with FMLP (1 μ mol·L⁻¹) resulted in increased oxygen consumption by the cells that was linear over a 1 min period and was significantly attenuated by pre-treatment of the cells with 1 μ mol·L⁻¹ montelukast (65 ±- 11% of control; *n* = 6 with one to three replicates for each system; *P* < 0.05 for comparison of FMLP-activated systems without and with montelukast).



3.3.2 Elastase release

The effects of montelukast on the release of elastase from neutrophils activated with FMLP/cytochalasin B are shown in Figure 3.2 (page 101). Treatment of the cells with montelukast resulted in dose-related inhibition of the release of elastase, which achieved statistical significance (P < 0.001) at concentrations of 0.5 µmol·L⁻¹ and greater, with maximal inhibition (79%) observed at 2 µmol·L⁻¹ of this agent. The IC₅₀ value for montelukast-mediated inhibition of elastase release was 1.2 µmol·L⁻¹ (95% confidence intervals 0.9–1.4).

3.3.3 Leukotriene B₄

The effects of montelukast on the production of LTB₄ by PAF (200 nmol·L⁻¹)activated neutrophils are shown in Figure 3.3 (page 102). Treatment of neutrophils with this agent resulted in dose-related inhibition of the generation of LTB₄, which achieved statistical significance (P < 0.001) at concentrations of 1 µmol·L⁻¹ and greater, with maximal inhibition (89 ± 3%) observed at 2 µmol·L⁻¹ montelukast. The IC₅₀ value for montelukast-mediated inhibition of LTB₄ production was 1.2 µmol·L⁻¹ (95% confidence intervals 0.7–1.6).

3.3.4 Fura-2 fluorescence responses of activated neutrophils

The results shown in Figure 3.4 (page 103), are typical traces of the FMLP and PAF-activated fluorescence responses of neutrophils in the absence and presence of montelukast at 2 μ mol·L⁻¹. Addition of FMLP to neutrophils was accompanied by



the characteristic, abrupt increase in fura-2 fluorescence intensity, which accompanies increased cytosolic concentrations of Ca²⁺, rising from a basal value of 83 ± 8 nmol·L⁻¹ to a peak value of 419 ±60 nmol·L⁻¹. This was followed by a rapid decrease in fluorescence intensity, which slowed after 1-2 min, coincident with influx of Ca²⁺. Although the peak cytosolic Ca²⁺ concentrations were equivalent in control and montelukast treated neutrophils, the rate of decline in fluorescence intensity was faster in montelukast-treated cells. The time taken for fluorescence intensity to decline to half peak values was 1.3 ± 0.1 , 0.9 ± 0.1 and 1.0 ± 0.1 min for FMLP-activated cells in the absence (control system) and presence of 1 and 2 μ mol·L⁻¹ montelukast respectively (n = 8, P < 0.05 to P < 0.001 for comparison of the control system with each drug-treated system). These observations are compatible with increased efficiency of sequestration/ resequestration of cytosolic Ca²⁺ into stores and/or decreased store-operated influx of the cation. In the case of PAF-activated cells, the peak increases in cytosolic Ca²⁺ were sustained for about 1 min (Figure 3.4) as described previously (Steel & Anderson, 2002). Treatment of neutrophils with montelukast (2 µmol·L⁻¹) markedly attenuated the duration of the sustained peak elevation in cytosolic Ca^{2+} , without affecting the magnitude of the peak response. The mean duration of the peak plateau elevation in cytosolic Ca²⁺ for PAF-activated control cells was 1.13 ± 0.1 min, while the corresponding value for systems treated with 2 μ mol·L⁻¹ montelukast was 0.3 ± 0.1 min (n = 12, P < 0.001 for comparison of the control system with the drug-treated system).



3.3.5 Ca²⁺ influx

The effects of varying concentrations of montelukast (0.25– 2 μ mol·L⁻¹) on influx of ⁴⁵Ca²⁺ following activation of the cells with the chemoattractants are shown in Figure 3.5 (page 104). Treatment of the cells with montelukast resulted in a dose-related decrease in the influx of Ca²⁺ activated by both FMLP and PAF, which was statistically significant at concentrations of 1 and 2 μ mol.L⁻¹.

3.3.6 Cyclic AMP

Exposure of neutrophils to montelukast caused a dose-related increase in intracellular cAMP, which achieved statistical significance (P < 0.05) at 1 µmol·L⁻¹, the values for the control system and systems treated with 0.5, 1 and 2 µmol·L⁻¹ montelukast being 4.8 ± 0.3, 6.2 ± 0.2, 7.7 ± 0.3 and 7.3 ± 0.2 pmol cAMP 10⁷ cells⁻¹ respectively. The effects of montelukast alone or in combination with CGS21680, rolipram or salbutamol are shown in Figure 3.6 (page 105). Treatment of neutrophils with montelukast (2 µmol·L⁻¹) in combination with CGS 21680, rolipram or salbutamol resulted in elevations in cAMP, which were significantly (P < 0.05) greater than those observed with the individual agents.

3.3.7 PDE

The effects of montelukast, relative to those of rolipram and 3-isobutyl-1methylxanthine, on cAMP and cGMP PDE activity, when added directly to neutrophil cytosolic extracts, are shown in Figure 3.7 (page 106). Montelukast


caused dose-related inhibition of both cAMP and cGMP PDE activity, which achieved statistical significance (P < 0.05) at concentrations of 1 µmol·L⁻¹ and higher. Although not shown, similar effects of montelukast were observed using the PDE preparation from bovine heart. Using neutrophil cytosol as the source of PDE activity, the IC₅₀ value for montelukast-mediated inhibition of cAMP PDE activity was 3.4 µmol·L⁻¹ (95% confidence intervals 2.9–3.9). The cAMP PDE activities of matched cytosol and membrane fractions prepared from montelukast (2 µmol^{-L}-1)treated and untreated, unstimulated and FMLP-activated neutrophils are shown in Table 3.2 (page 91). Enzyme activity was considerably lower in the membrane fractions, while no redistribution of enzyme activity between the cytosol and membrane compartments was evident following activation of the cells with FMLP. Pretreatment of the cells with montelukast was accompanied by decreased cAMP PDE activity in the cytosol, and especially the membrane fractions. Given that the cell pellets were diluted approximately 20-fold in sucrose/PMSF following exposure to montelukast ± FMLP, it is likely that the inhibitory effects of montelukast on cAMP PDE were underestimated using this experimental design.



Table 3.1: Effects of MK886 and montelukast, separately and in combination, on the lucigenin-enhanced chemiluminescence (LECL) responses of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) - activated neutrophils

System	LECL	P-values
	(relative light units)	
a. FMLP only	6401 ± 496	
b. FMLP + MK886 (0.5 μmol·L ⁻¹)	6448 ± 571	NS ^a
c. FMLP + Montelukast (0.5 µmol·L ⁻¹)	4707 ± 354	<0.01 to <0.001 ^a
d. FMLP + MK886 + Montelukast	4741 ± 349	NS ^b

The results of four experiments (n=4) are expressed as the mean peak LECL values +/- SEM.

^aFor comparison with the FMLP-activated, drug-free control system.

^bFor comparison between systems c and d.



Table 3.2: Cyclic AMP phosphodiesterase (PDE) activities in cytosol and membrane fractions prepared from matched control and montelukast-treated unstimulated and N-formyl-L-methionyl-L-leucyl- L-phenylalanine (FMLP)-activated neutrophils

System	PDE activity	
	(cpm x 10 ⁻² min ⁻¹ mg protein ⁻¹)	
	Membranes	Cytosol
Control, unstimulated cells	108 ± 2	895 ± 56
Unstimulated cells + 2 µmol·L ⁻¹ montelukast	34 ± 10*	718 ± 57*
FMLP-activated control cells	60 ± 10	938 ± 38
FMLP-activated cells + 2 µmol·L ⁻¹ montelukast	21 ± 6*	609 ± 34*

Results are expressed as the mean values \pm SEM (*n* = 4, 2 replicates for each system in each experiment). **P* < 0.05 for comparison with the corresponding drug-free control systems.

3.3.8 Inositol triphosphate

The basal IP3 value for unstimulated cells was $45 \pm 2 \text{ pmol } 10^7 \text{ cells}^{-1}$, increasing to $63 \pm 2 \text{ pmol } 10^7 \text{ cells}^{-1}$ at 10 s following the addition of PAF (200 nmol·L⁻¹) to control neutrophils (P < 0.05 for comparison with the basal value), while the corresponding value for PAF-activated, montelukast (2 µmol·L⁻¹)-treated neutrophils was $60 \pm 3 \text{ pmol } \text{IP3 } 10^7 \text{ cells}^{-1}$, which did not differ significantly from the control system (n = 12, with two to five replicates for each drug concentration and control system in each experiment).



3.3.9 ATP levels

Treatment of neutrophils with montelukast (2 μ mol·L⁻¹) did not affect neutrophil ATP levels; the values for control and drug-treated cells following a 15 min exposure at 37°C were 62 ± 2 and 58 ± 3 pmol ATP 10⁷ cells⁻¹ respectively (*n* = 2, with seven replicates for each system in each experiment).

3.4 Discussion and conclusions

Montelukast, a selective antagonist of CysLT₁ receptors, is used primarily in the treatment of allergic conditions such as bronchial asthma and allergic rhinitis (Fox-Spencer, 2006; Nayak & Langdon, 2007; Peters-Golden & Henderson, 2007). The reported pA₂ value for montelukast antagonism of LTD₄-mediated contraction of quinea pig trachea is 9.3 (Jones et al, 1995). Interestingly, beneficial therapeutic effects of this agent have been reported for diverse diseases in which neutrophils play a pathogenic role, including chronic obstructive pulmonary disease, respiratory bronchiolitis, cystic fibrosis and atherosclerosis (Anonymous, 2004; Rubinstein, Kumar & Schriever, 2004; Celik et al, 2005; Riccioni et al, 2007). The current study was designed to probe potential anti-inflammatory interactions of montelukast with activated human neutrophils in vitro. Montelukast, at concentrations within the therapeutic range (Cheng et al, 1996; Knorr et al, 2001) and above, caused significant dose-related inhibition of superoxide (lucigenin CL) and hypochlorous acid (luminol CL) generation, as well as production of LTB₄ and release of elastase, by activated neutrophils. In the case of superoxide production,



the inhibitory effects of montelukast were found to result from interference with the activation of NADPH oxidase.

This latter contention is based on observations that montelukast, at concentrations of up to 2 µmol·L⁻¹, did not possess superoxide-scavenging activity, while treatment of the cells with this agent resulted in decreased oxygen consumption following activation with FMLP, as well as markedly reduced activity of NADPH oxidase in membrane fractions prepared from these cells. MK886, an inhibitor of 5lipoxygenase activating protein, was used to probe the possible involvement of LTC₄ and LTD₄ generated by contaminating cells in the neutrophil preparations, in the production of superoxide by these cells. The failure of MK886 to affect the production of superoxide by activated control neutrophils demonstrates that LTC₄ and LTD₄ were not present at high enough concentrations in the cell suspensions to affect neutrophil NADPH oxidase activity. More importantly, however, the failure of MK886 to attenuate montelukast-mediated inhibition of superoxide production by FMLP activated neutrophils clearly demonstrates that the observed antiinflammatory effects of montelukast, in this experimental design, are directed primarily at neutrophils and not at contaminating cells in the cell suspension and, further, that the effects were not mediated via antagonism of CysLT₁ receptors.

Given that all the pro-inflammatory activities of neutrophils mentioned above are dependent on elevations in cytosolic Ca^{2+} , the effects of montelukast on Ca^{2+} fluxes in FMLP/PAF-activated neutrophils were also investigated. Peak cytosolic Ca^{2+} concentrations in PAF-activated neutrophils were sustained for 60–90 s,



followed by a gradual subsidence over a time course of several minutes. The prolonged peak cytosolic Ca²⁺ response observed in PAF-activated neutrophils results from the failure of this chemoattractant to activate both NADPH oxidase and adenylyl cyclase (Nick *et al*, 1997; Steel & Anderson, 2002), resulting in early store-operated influx of Ca²⁺ and failure of cAMP-dependent protein kinase (PKA)-mediated restoration of Ca²⁺ homeostasis (as described below) respectively. In the case of FMLP-activated cells, NADPH oxidase-mediated membrane depolarization limits influx of Ca²⁺, while activation of adenylyl cyclase favours rapid clearance of cytosolic Ca²⁺ (lannone, Wolberg & Zimmerman, 1989; Tintinger *et al*, 2001). Consequently, the peak cytosolic Ca²⁺ response observed in FMLP-activated neutrophils is of brief duration, declining rapidly for 1–2 min, followed by a levelling-off, coincident with store-dependent influx of Ca²⁺ (Geiszt *et al*, 1997; Tintinger *et al*, 2001).

Treatment of neutrophils with montelukast did not affect the magnitudes of the immediate peak increase in cytosolic Ca^{2+} in neutrophils activated with either FMLP or PAF. Taken together with the absence of effects of montelukast on IP3 production by PAF-activated neutrophils, these observations demonstrate that neither phospholipase C nor the Ca^{2+} -mobilizing interactions of IP3 with its receptor on intracellular Ca^{2+} stores are affected by this agent. Treatment of neutrophils with montelukast did, however, significantly attenuate the duration of the prolonged peak cytosolic Ca^{2+} response of PAF-activated neutrophils, while hastening the rate of decline in cytosolic Ca^{2+} concentrations in FMLP-activated neutrophils, compatible with decreased store-operated influx of Ca^{2+} in drug-



treated cells. Using procedures that selectively measure the influx of Ca^{2+} into chemoattractant-activated neutrophils, it was observed that montelukast did indeed cause significant, dose-related inhibition of the uptake of Ca^{2+} by cells activated by both FMLP and PAF, with mean values for inhibition of uptake of 50% and 66%, respectively, for cells treated with 2 µmol·L⁻¹ montelukast.

Importantly, Ca²⁺ influx is necessary to sustain the Ca²⁺-dependent proinflammatory activities of neutrophils (Bréchard & Tschirhart, 2008). Treatment of neutrophils with montelukast, at the same concentrations that suppressed the Ca²⁺-dependent proinflammatory activities of the cells, was found to cause a significant increase in basal cAMP. Basal cAMP is probably maintained by the autocrine interactions of secreted adenosine with adenosine A2A receptors (Mundell et al, 2001). From a mechanistic perspective, the elevation in intracellular cAMP observed in montelukast-treated neutrophils represented the most likely explanation for the Ca²⁺ handling-targeted, anti-inflammatory interactions of this agent with activated neutrophils. Interestingly, pre-treatment of neutrophils with montelukast followed by addition of CGS21680, rolipram or salbutamol resulted in elevations in neutrophil cAMP, which were significantly greater than those observed with the individual agents. With respect to CGS21680 and salbutamol, these agents were used at concentrations likely to cause saturation of adenosine A_{2A} and β_2 -adrenoceptors respectively, compatible with lack of agonist interactions of montelukast with either of these G protein/adenylyl cyclase-coupled receptor types. This observation, taken together with the interactive effects of montelukast and rolipram on raising basal cAMP in neutrophils, as well as the findings of a



limited series of experiments that revealed an increase in basal cGMP in montelukast-treated cells (data not included), suggested that the drug possessed non-specific PDE inhibitory activity.

The effects of montelukast on cAMP and cGMP PDE activity were measured using cytosolic fractions from isolated neutrophils, as well as a PDE preparation from bovine heart. Addition of montelukast to either of these resulted in striking, dose-related inhibition of the activities of both cAMP and cGMP PDEs in neutrophil cytosol with an IC₅₀ value of 3.4 µmol·L⁻¹ for the former. The concentrations of montelukast that were found to possess non-specific PDE inhibitory activity therefore closely paralleled those that elevated cAMP and inhibited the Ca²⁺-dependent pro-inflammatory activities of neutrophils, compatible with a causal association between these events. Although the IC₅₀ values for montelukast-mediated inhibition of neutrophil PDEs are somewhat higher than those for inhibition of superoxide and LTB₄ production and elastase release (1, 1.2 and 1.3 µmol·L⁻¹ respectively), this difference may be due to intracellular accumulation of lipophilic montelukast by intact cells, as the drug has an oil : water partition coefficient of Log/KD = 2.3 +/- 0.2 (data on file, Merck Research Laboratories).

The effects of addition of montelukast to intact neutrophils on cAMP PDE activity in cytosol and membrane fractions prepared from unstimulated and FMLP-activated cells were also investigated. Activation of neutrophils with FMLP did not result in either increased activity of cytosolic cAMP PDE, or redistribution of enzyme to the membrane, with activity in the membrane fraction being low relative to the cytosol.



Treatment of intact neutrophils with montelukast resulted in decreased cAMP PDE activity in the cytosolic and especially the membrane fractions of unstimulated and FMLP activated cells. In the case of the cytosol, however, this was of lesser magnitude than that observed following direct addition of the drug to the cytosol, due, presumably, to loss and dilution of the drug during cell processing.

As a consequence of activation of PKA, cAMP promotes restoration of Ca²⁺ homeostasis in neutrophils and other cell types by multiple mechanisms, including phosphorylative inactivation of PLCy (Ali et al, 1998); inactivation of IP3-receptors phosphorylative up-regulation of the Ca²⁺ (Bai & Sanderson, 2006); sequestering/resequestering endomembrane Ca²⁺-ATPase Anderson *et al*, 1998; Anderson *et al.* 2000): inactivation of store-operated Ca²⁺ channels (Binnaz *et al.* 2006); and inhibition of p38 MAP kinase with consequent interference with the activation of 5-lipoxygenase (Flamand et al, 2002), thereby attenuating an autocrine, LTB₄-mediated secondary wave of Ca²⁺ uptake by the cells (Steel et al. 2007). While the first of these mechanisms does not appear to contribute significantly to the effects of montelukast on Ca²⁺ handling by activated neutrophils observed in the current study, all the other mechanisms may be operative. Moreover, cross-activation of PKA and PKG by cAMP and cGMP may also contribute to restoration of Ca²⁺ homeostasis as PKG has also been reported to restrict store-operated uptake of Ca²⁺ (Ruiz-Velasco et al, 1998). Although PDE4 subtype B2 appears to be the predominant PDE in human neutrophils (Wang et al, 1999), it is noteworthy that cilostazol, a PDE3 inhibitor, has been reported to attenuate Ca²⁺ fluxes in activated human neutrophils, as well as superoxide



generation (Yang *et al*, 2006), demonstrating, albeit indirectly, the presence of PDE3 in these cells. Given the ability of PDE3 to hydrolyze cAMP, as well as cross-activation of PKAs A and G by cAMP and cGMP, non-specific PDE inhibitors that target PDEs 3 and 4 neutrophils may be more effective anti-inflammatory agents than those that selectively target PDE4, by preventing compensatory, counteracting increases in the activities of PDE3. The apparent benefits of a combination of PDE3 and PDE4 inhibitors, as opposed to either category of inhibitor alone, have been already described in an animal model of allergen-induced bronchospasm (Underwood *et al*, 1994).

Although the non-specific PDE inhibitory effects of montelukast described here have not, to our knowledge, been reported previously, several of the early, experimental CysLT₁ receptor antagonists such as FPL55712 and LY171883 were documented to possess this property (Fleisch, Rinkema & Marshall, 1984; Ferrari *et al* 2004). More recently, CR3465, a novel CysLT1 receptor antagonist, was reported to possess PDE inhibitory (Barkhof *et al*, 2010). In the case of FPL55712 and LY171883, PDE inhibitory activity appeared to represent a limitation in respect of specificity of pharmacological mode of action and clinical development (Fleisch, Rinkema & Marshall, 1984; Ferrari *et al* 2004;), whereas for CR3465 the combination of CysLT₁ receptor antagonism and PDE inhibitory activity was considered to be beneficial, because the latter property conferred additional protection by targeting spasmogenic and inflammatory mediators other than CysLTs (Barkhof *et al*, 2010). It is noteworthy that PKA also possesses antiinflammatory activities that are distinct from its effects on Ca²⁺ handling by

98



activated immune and inflammatory cells. These include interference with the activation of NADPH oxidase, and inhibition of p38 MAP kinase (as mentioned above) and phosphatidylinositol 3-kinase (Bengis-Garber *et al*, 1996; Flamand *et al*, 2002; Burelout *et al*, 2007). In addition, montelukast has also been reported to inhibit human recombinant 5-lipoxygenase with a relatively high IC₅₀ of 30–50 μ mol·L⁻¹, while synthesis of LTB₄ by activated neutrophils was inhibited at drug concentrations of >1 μ mol·L⁻¹ (Ramires *et al*, 2004). However, effects on Ca²⁺ fluxes and cAMP, which may explain the greater sensitivity of intact cells to the inhibitory effects of montelukast on LTB₄ production, were not investigated in this study. Nevertheless, inhibition of 5-lipoxygenase, either directly or indirectly by the mechanisms described in the current study, together with reported inhibition of signalling via P2Y receptors (Mamedova *et al*, 2005), suggests that montelukast may be particularly effective in attenuating both the generation and action of autocrine inflammatory mediators.

Antagonism of CysLT₁ receptors is clearly the primary mechanism of therapeutic activity of montelukast. However, the PDE-targeted anti-inflammatory activity of this agent described in the current study may contribute to the beneficial effects of this agent, especially when used in combination with inhaled corticosteroids, in some categories of patients with bronchial asthma (Laviolette *et al*, 1999). This strategy may enable control of the corticosteroid-resistant neutrophil (Barnes, 2007), and may also counters bronchospasm via direct cyclic nucleotide mediated effects on airway smooth muscle (Binnaz *et al*, 2006). Impotantly, and as mentioned earlier, the concentrations of montelukast used in this study were based

99



on peak serum concentrations following oral administration of this agent. These are not, however, representative of concentrations in the airways which may differ due to the lipophilicity of this agent, possibly resulting in higher tissue concentrations and an under-estimation of anti-inflammatory potential.





Figure 3.1: Effects of montelukast (0.1–2 µmol·L⁻¹) on the lucigenin and luminol-enhanced chemiluminescence responses of neutrophils activated by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 µmol·L⁻¹). The results are expressed as the mean peak chemiluminescence values in relative light units measured 30–50 s after the addition of FMLP and vertical lines show SEM. In the case of lucigenin-enhanced chemiluminescence (left graph, n = 3 with three to four replicates for each drug concentration and control system in each experiment), the absolute values for unstimulated neutrophils and for cells activated with FMLP in the absence of montelukast were 1086 ± 147 and 4729 ± 325 respectively, while the corresponding values for luminol-enhanced chemiluminescence (right graph, n = 5 with two to three replicates for each drug concentration and control system in each experiment) were 3047 ± 127 and $56 397 \pm 8394$ relative light units. *P < 0.01 to P < 0.001 for comparison with the FMLP-activated, montelukast-free control system.





Figure 3.2: Effects of montelukast $(0.1-2 \ \mu mol \cdot L^{-1})$ on the release of elastase from neutrophils activated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine $(1 \ \mu mol \cdot L^{-1})/$ cytochalasin B (0.5 $\mu mol \cdot L^{-1}$). The results (n = 4 with duplicate data sets for each experiment with 10 replicates for each drug concentration and control system in each experiment) are expressed as the mean values for total extracellular elastase (milliunits 10^7 cells^{-1}) and vertical lines show SEM. The absolute values for the unstimulated control system and for cells activated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine/ cytochalasin B in the absence of montelukast were 221 ± 44 and 892 ± 9.6 milliunits elastase 10^7 cells^{-1} respectively. *P < 0.001 for comparison with the drug-free control systems.





Figure 3.3: Effects of montelukast $(0.25-2 \ \mu mol \cdot L^{-1})$ on the production of leukotriene B4 (LTB4) by neutrophils activated with platelet activating factor (200 nmol \cdot L^{-1}). The results are presented as the mean values for total extracellular LTB₄ (pg 10⁷ cells⁻¹) and vertical lines show SEM (n = 8, with two to three replicates for each drug concentration and control system in each experiment). The absolute values for the unstimulated control system and for cells activated with platelet-activating factor in the absence of montelukast were 16 ± 6 and 993 ± 107 pg LTB₄ 10⁷ cells⁻¹ respectively. *P < 0.001 for comparison with the drug-free control system.





Figure 3.4: Chemoattractant-activated fura-2 fluorescence responses of control and montelukast (2 μ mol·L⁻¹)-treated neutrophils. Platelet activating factor (PAF) (200 nmol·L-1) or N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 μ mol·L⁻¹) were added as indicated (\uparrow) when a stable baseline was obtained (1 min). The traces shown are from three different representative experiments (8 for FMLP and 12 for PAF in the series).







Figure 3.5: Effects of montelukast $(0.25-2 \ \mu \text{mol}\cdot\text{L}^{-1})$ on the influx of ${}^{45}\text{Ca}^{2+}$ into the neutrophils activated with either N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 $\ \mu\text{mol}\cdot\text{L}^{-1}$, upper graph) or platelet-activating factor (PAF) (200 nmol·L⁻¹, lower graph). The results are expressed as the mean percentages of the drug-free control systems and vertical lines show SEM (n = 4–8 with two to four replicates for each drug concentration and control system). The absolute values for uptake of ${}^{45}\text{Ca}^{2+}$ by unstimulated neutrophils and for cells activated with FMLP or PAF were 47 ± 25, 150 ± 34 and 148 ± 14 pmol ${}^{45}\text{Ca}^{2+}$ 10⁷ cells⁻¹ respectively. *P < 0.05 to P < 0.01 for comparison with the corresponding chemoattractant-activated montelukast-free control systems (according to the repeated measures ANOVA, there were significant effects of montelukast at both 1 and 2 $\ \mu\text{mol}\cdot\text{L}^{-1}$).





Figure 3.6: Effects of CGS21680 (CGS; 1 µmol·L⁻¹), salbutamol (Sb; 5 µmol·L⁻¹), rolipram (ROLI; 0.1 µmol·L⁻¹) and montelukast (2 µmol·L⁻¹) individually, as well as those of montelukast in combination with the other agents on neutrophil intracellular cAMP. The results are presented as the mean percentages of the drug-free control system and vertical lines show SEM (n = 6, with two to three replicates for each drug concentration and control system in each experiment). The absolute value for the drug-free control system was 4.3 ± 0.3 pmol cAMP 107 cells⁻¹. *P < 0.05 for comparison with the drug-free control systems.





Figure 3.7: Effects of montelukast (0.5–20 µmol·L-1), rolipram (20 µmol·L-1) and 3isobutyl-1-methylxanthine (50 µmol·L-1) on cAMP (upper graph) and cGMP (lower graph) phosphodiesterase (PDE) activities in neutrophil cytosol. The results of four to eight and two to four experiments for cAMP and cGMP PDE activity respectively are presented as the mean percentages of the drug-free control systems and vertical lines show SEM. In the case of the cAMP/PDE experiments, the absolute values for the cytosol-free background system and for the cytosol-containing systems in the absence of the drugs were 1213 ± 53 and 14, 525 ± 232 counts per minute respectively. The corresponding values for the cGMP/PDE experiments were 2031 ± 206 and 21, 381 ± 501 counts per minute. *P < 0.05 for comparison with the corresponding drug-free control system.



Chapter 4

Interactive Inhibitory Effects of Formoterol and Montelukast on Activated Human Neutrophils.



4.1 INTRODUCTION

Anti-inflammatory chemotherapy, most commonly inhaled corticosteroids, is the cornerstone of the pharmacotherapy of bronchial asthma (McFadden, 1998). In patients not controlled on corticosteroids alone, the guideline recommendation is that additional controller therapy should be added, either a long acting beta(2)-adrenoreceptor agonist (LABA) or a cysteinyl leukotriene receptor (cysLTR) antagonist (Van Weel *et al*, 2008). In patients who are still not adequately controlled, a combination of controller medications is added to the inhaled corticosteroids (Van Weel *et al*, 2008). With the possible exception of effects of corticosteroids on beta(2)- receptor expression and function (Johnson, 2002; Johnson, 2006; Lovén *et al*, 2007), the clinical benefit of this triad of agents is thought to result from the collective effects of their distinct mechanisms of therapeutic activity, as opposed to meaningful, beneficial interactions between them.

Corticosteroids possess broad spectrum anti-inflammatory activity, while LABAs and montelukast function primarily as bronchodilators and through antagonism of cysLTRs respectively (Capra *et al*, 2006; Barnes, 2007). There is, however, evidence that LABAs and montelukast, in addition to their conventional therapeutic activities, possess anti-inflammatory properties (Tintinger *et al*, 2000; Johnson, 2002; Anderson *et al*, 2009; Theron *et al*, 2009). If operative *in vivo*, these may complement the anti-inflammatory actions of corticosteroids in controlling severe

109



asthma, especially those types of asthma in which the seemingly corticosteroidresistant neutrophil (Barnes, 2007) is the dominant type of inflammatory cell (MacDowell & Peters, 2007).

The pro-inflammatory activities of neutrophils, as well as other types of immune and inflammatory cells, are suppressed by pharmacological agents which elevate intracellular cyclic AMP (Moore & Willoughby, 1995). Because these cells possess beta(2)-adrenoreceptors, they are amenable to the anti-inflammatory actions of beta(2)-agonists, including the selective, long-acting agent, formoterol (Tintinger *et al*, 2000; Johnson, 2002). As mentioned in the previous chapter, montelukast increases intracellular cyclic AMP by a mechanism distinct from antagonism of cysLTRs, which apparently involves non-specific inhibition of cyclic nucleotide phosphodiesterases [PDEs], resulting in cyclic AMP-dependent inhibition of neutrophil pro-inflammatory activity. Although untested, it is plausible that the combination of formoterol and montelukast may exert augmentative, cyclic AMPdependent anti-inflammatory activity through the respective interactions of these agents with beta(2)-adrenoreceptors and PDEs, especially PDE4 (Wang *et al*, 1999).

In the current study, the effects of formoterol and montelukast, individually and in combination, on several pro-inflammatory activities of activated neutrophils in relation to alterations in cyclic AMP and Ca²⁺ handling by the cells, have been investigated. In a limited series of experiments dexamethasone and salbutamol were included for comparison.



4.2 MATERIALS AND METHODS

4.2.1 Chemicals and reagents

Formoterol fumarate dehydrate and dexamethasone were purchased from Sigma-Aldrich (Pty)Ltd, Johannesburg, South Africa, and montelukast provided by Merck Research Laboratories, Rahway, NJ, USA, while salbutamol and rolipram were provided by GlaxoSmithKline plc, Stockley Park, West London, UK. With the exception of salbutamol (water soluble) all agents were dissolved to a stock concentration of 10 mmol.L⁻¹ in dimethyl sulphoxide (DMSO) and used at final concentrations of 1 and 10 nmol.L⁻¹ (formoterol), 2 μ mol.L⁻¹ (montelukast), 100 nmol.L⁻¹ (salbutamol), 1 μ mol.L⁻¹ (dexamethasone), and 1 μ mol.L⁻¹ (rolipram) in the various assays of neutrophil function described below. The final concentrations of DMSO in each assay ranged from 0.1-0.2% and appropriate solvent controls were included with each experimental system.

The concentrations of montelukast used in the current study (1-2 μ mol.L⁻¹), closely approximate peak serum levels of about 1 μ mol.L⁻¹ attained during chemotherapy with this agent (Knorr *et al*, 2001). The concentration of formoterol in the airways is, however, more difficult to ascertain. Assuming a maximum single dose of 24 μ g and lung deposition of 18.6%, the total amount of formoterol reaching the airways would be approximately 4.5 μ g (Farr *et al*, 1995). Given a tidal volume of 500 mL, this would equate to a local concentration of 9 ng·mL⁻¹, which is equivalent to 11 nmol.L⁻¹, close to the highest concentration of 10 nmol.L⁻¹ used in the current



study. Unless indicated, all other chemicals and reagents were purchased from Sigma-Aldrich.

The chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μ mol.L⁻¹ final), in combination with cytochalasin B (CB, 3 mmol.L⁻¹ final) was used to activate the neutrophils. This relatively strong activator (FMLP/CB) was used because of the anti-inflammatory potency of formoterol and montelukast. An additional chemoattractant, platelet-activating factor (PAF, 200 nmol.L⁻¹) was used in an additional, limited series of experiments.

4.2.2 Preparation of neutrophils

The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, and prior informed consent was obtained from all blood donors. These cells were isolated according to the method described in Chapter 2, 2.2.1.

4.2.3 Superoxide generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) procedure. Briefly, neutrophils (10^6 cells) were preincubated without and with montelukast (2 µmol.L⁻¹) in 900 µL Hanks' balanced salt solution (HBSS, pH 7.4, indicator-free; Highveld Biological (Pty) Ltd, Johannesburg, South Africa) containing 0.2 mmol.L⁻¹ lucigenin. Following preincubation, formoterol (1 or 10 µmol.L⁻¹) was added to the cells (or an equal



volume of solvent to control systems) followed 20 sec later by FMLP/CB, and LECL responses recorded using a Lumac Biocounter (model 2010, Lumac Systems Inc, Titusville, FL,USA). The final volume in each vial was 1 mL, and the results, which are expressed in relative light units (rlu), are the peak values for FMLP/CB– activated systems that were reached 40-50 sec after the addition of the activator. There were 4 systems in each experiment: i) neutrophils only (control); ii) neutrophils + formoterol; iii) neutrophils + montelukast; and iv) neutrophils + montelukast + formoterol.

4.2.4 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule enzyme, elastase. Neutrophils were incubated at a concentration of 2 x 10^{6} ·mL⁻¹ in HBSS without and with montelukast (2 µmol.L⁻¹) for 10 min at 37°C followed by addition of formoterol (1 or 10 nmol.L⁻¹) or solvent control to the relevant systems and 20 sec later by FMLP/CB. The tubes were incubated and the elastase assays performed according to methods described in in chapter 2, 2.2.5. The effects of the corticosteroid, dexamethasone (1 µmol.L⁻¹), on elastase release by FMLP/CB-activated neutrophils were also investigated in a more limited series of experiments. The corticosteroid was present with the cells during the 10 min preincubation period.



4.2.5 CR3 expression

Expression of CR3 on resting and FMLP/CB-activated neutrophils in the absence or presence of formoterol (10 nmol.L⁻¹) and montelukast (2 μ mol.L⁻¹), individually and in combination, was measured flow cytometrically. Neutrophils (1 x 10⁶·mL⁻¹, final) were incubated in HBSS with and without montelukast for 10 min at 37°C followed by the addition of formoterol or solvent control to the relevant systems and 20 sec later by FMLP/CB. The tubes were incubated for 1 min and the reactions stopped by dilution of the cells in ice-cold medium. Phycoerythrin-labelled monoclonal antibody to CD11b (Beckman Coulter, Miami, FL, USA) was added in a 10 μ L volume to 1 mL of cell suspension and CR3 expression analysed using an Epics Altra Flow Cytometer equipped with a water-cooled Enterprise Laser (Beckman Coulter) and the results expressed as mean fluorescence intensity.

4.2.6 Spectrofluorimetric measurement of cytosolic Ca²⁺

Fura-2/AM was used as the fluorescent, Ca^{2+} -sensitive indicator for these experiments (Grynkiewicz, Poenie & Tsien, 1985). Neutrophils $(1x10^{7} \cdot mL^{-1})$ were incubated with fura-2/AM (2 µmol.L⁻¹) for 30 min at 37°C in PBS, washed and resuspended in HBSS. The fura-2-loaded cells (2 x $10^{6} \cdot mL^{-1}$) were then preincubated for 5 min at 37°C without and with montelukast (2 µmol.L⁻¹) after which they were transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500nm respectively. After a stable baseline



was obtained (± 1 min), formoterol (10 nmol.L⁻¹ only) or solvent control was added to the relevant systems followed 20 sec later by FMLP/CB, and alterations in fluorescence intensity monitored over a 5-10min time course. Cytosolic Ca²⁺ concentrations were calculated as described previously (Grynkiewicz, Poenie & Tsien, 1985).

In a more limited series of experiments, the following were also investigated: i) the effects of the short-acting beta(2)-receptor agonist, salbutamol (100 nmol.L⁻¹, final, predetermined in preliminary experiments) without and with montelukast (1 μ mol.L⁻¹) on PAF (200 nmol.L⁻¹)-activated increases in cytosolic Ca²⁺ concentrations in neutrophils; ii) the effects of formoterol (10 nmol.L⁻¹) alone or in combination with the prototype PDE₄ inhibitor, rolipram (fixed, final concentration of 1 μ mol.L⁻¹); and iii) the effects of dexamethasone (1 μ mol.L⁻¹) on FMLP/CB-activated Ca²⁺ fluxes in neutrophils. Montelukast, rolipram and dexamethasone were present with the cells during the 10 min preincubation period, while salbutamol or formoterol were added 20 sec prior to the activation of the cells.

4.2.7 Measurement of leukotriene B₄ (LTB₄) and cyclic AMP (cAMP)

Competitive binding immunoassay procedures (Correlate-EIATM, Assay Designs Inc., Ann Arbor, MI, USA) were used to measure LTB₄ in the supernatants of FMLP/CB-activated neutrophils, while cAMP was assayed in the extracts of unstimulated cells in the absence and presence of formoterol and montelukast individually and in combination. In the case of LTB₄, neutrophils (2 x 10^{6} ·mL⁻¹)



were preincubated for 10 min at 37°C in the absence and presence of montelukast (2 μ mol.L⁻¹) after which formoterol (1 or 10 nmol.L⁻¹) was added to the relevant systems, followed 20 sec later by FMLP/CB. Incubation was terminated 3 min later by the addition of an equal volume of ice-cold HBSS to the tubes which were then held in an ice bath prior to pelletting the cells by centrifugation. The cell-free supernatants were then diluted (1:8) and assayed for LTB₄, with the results expressed as pg LTB₄ ·10⁷ cells⁻¹. As with the other assays of neutrophil pro-inflammatory activity, each experiment consisted of 4 systems: i) neutrophils only (control); ii) neutrophils + formoterol; iii) neutrophils + montelukast; and iv) neutrophils + formoterol + montelukast. In a more limited series of experiments, the following were also investigated: i) the effects of salbutamol (100 nmol.L⁻¹) without and with montelukast (1 μ M) on the production of LTB₄ by PAF (200 nmol.L⁻¹)-activated neutrophils; and ii) the effects of dexamethasone (1 μ mol.L⁻¹)

In the case of cAMP, neutrophils (2 x $10^{6} \cdot mL^{-1}$) were preincubated for 10 min at 37°C without or with montelukast (2 µmol.L⁻¹), after which formoterol (10 nmol.L⁻¹ only) was added to the relevant systems and the reactions terminated 20 sec later by the addition of an equal volume of ice-cold HBSS to the tubes. Following centrifugation, the supernatants were discarded and cAMP extracted from the cell pellets by addition of 0.1M HCl for 15 min followed by centrifugation to remove the cell debris and the supernatants decanted and assayed for cAMP. These results are expressed as pmol cAMP·10⁷ cells⁻¹.



4.2.8 Statistical analysis

With the exception of the results of the fura-2 fluorescence experiments, some of which are presented as representative traces, the results of each series of experiments are presented as the mean values \pm SEM, where n = the number of different donors used in each series of experiments, with the number of replicates for each drug concentration and drug-free control system for each experiment shown in the figure legends. Levels of statistical significance were determined by comparing the absolute values for each drug-treated system with the corresponding values for the relevant drug-free control systems for each assay using repeated measures ANOVA with an appropriate multiple comparisons posttest.

4.3 RESULTS

4.3.1 Superoxide production, elastase release, and leukotriene B₄

These results for neutrophils activated with FMLP/CB in the absence and presence of formoterol (1 and 10 nmol.L⁻¹) and montelukast (2 μ mol.L⁻¹) individually and in combination are shown in Figure 4.1 (page 127). Treatment of neutrophils with formoterol or montelukast significantly (*P*<0.05) attenuated the production of superoxide and LTB₄ in particular, as well as the release of elastase, with the combination, especially in the case of 1 nmol.L⁻¹ formoterol, being significantly (*P*<0.05) more effective in most cases, albeit additive rather than synergistic, than either of the individual agents. The exception was superoxide production for which



the effects of the combination differed only modestly from those of montelukast alone. The magnitude of inhibition of elastase release observed in the presence of montelukast, although statistically significant, was less than that reported in Chapter 3, probably because of the higher concentration of cytochalasin B used in the current study.

Treatment of neutrophils with dexamethasone had minimal effects on the production of LTB₄ and release of elastase from FMLP/CB-activated neutrophils. In the case of elastase the values for cells activated with FMLP/CB in the absence and presence of dexamethasone were 950 ± 15, and 932 ± 15 munits elastase $\cdot 10^7$ cells⁻¹ respectively. The corresponding values for LTB₄ release were 7213 ± 110 and 7242 ± 150 pg LTB₄ $\cdot 10^7$ cells⁻¹ (data from 3 separate experiments). The effects of salbutamol (100 nmol.L⁻¹) and montelukast (2 µmol.L⁻¹) individually and in combination on the release of LTB₄ by PAF-activated neutrophils were also investigated. The results for unstimulated cells and for PAF-activated control cells and cells treated with salbutamol alone, montelukast alone, or the combination of these agents were 24 ± 2, 681 ± 29, 335 ± 55, 132 ± 38, and 65 ± 17pg LTB₄ $\cdot 10^7$ cells⁻¹ respectively (*P*< 0.05 for comparison of each drug-treated system with the control system and for the combination with the individual agents).

4.3.2 CR3 expression

The level of CR3 expression for resting neutrophils was 27 ± 2 mean fluorescence intensity. The corresponding levels of expression for control, FMLP/CB-activated



cells and for the corresponding systems treated with formoterol (10 nmol.L⁻¹) only, montelukast (2 μ mol.L⁻¹) only, or the combination of these two agents were 92 ± 2, 71 ± 3, 81 ± 2 and 60 ± 3 mean fluorescence intensity respectively. The levels of CR3 expression were significantly less (*P*<0.05) than those of the control system for all 3 drug-treated systems, while the levels of CR3 expression in the system treated with both formoterol and montelukast was significantly less (*P*<0.05) than that of the systems treated with the individual drugs.

4.3.3 Cyclic AMP

These results are shown in Figure 4.2 (page 128). Treatment of neutrophils with either formoterol (10 nmol.L⁻¹) for 20 sec, or montelukast (2 μ mol.L⁻¹) resulted in significant (*P* <0.05) elevations in intracellular cAMP. Although higher than that observed with the individual agents, the increase in cAMP which resulted from treatment of the cells with the combination of montelukast and formoterol was not significantly different from that observed with the individual agents (Figure 4.2, page 128).

4.3.4 Cytosolic calcium

The results shown in Figure 4.3 (page 129) are traces from a representative experiment which depicts the alterations in cytosolic Ca^{2+} (fura-2 fluorescence) following activation of the cells with FMLP/CB in the absence and presence of formoterol (10 nmol.L⁻¹) and montelukast (2 μ mol.L⁻¹) individually and in combination. Activation of the cells with FMLP/CB resulted in an abrupt increase in



cytosolic Ca²⁺, with the peak response being sustained for 1-2 min, followed by a gradual subsidence which levelled off within 1-2 min reaching a sustained plateau at a level considerably higher than the pre-activation, basal value for cytosolic Ca²⁺. Treatment of the cells with either montelukast (2 μ mol.L⁻¹) for 5 min or formoterol (10 nmol.L⁻¹) for 20 sec prior to the addition of FMLP/CB caused partial, but significant (*P*<0.05) attenuation of the peak, plateau cytosolic Ca²⁺ response, while accelerating the rate of decline, and decreasing the magnitude of the second, sustained plateau response. Again, the combination of montelukast and formoterol was found to be significantly (*P*<0.05) more effective than the individual agents in promoting clearance of Ca²⁺ from the cytosol of FMLP/CB-activated neutrophils. These results are shown in Table 4.1 (page 125). Similar results (not shown) were obtained with the non-selective PDE inhibitor, rolipram (1 µmol.L⁻¹), in the presence or absence of formoterol (10 nmol.L⁻¹).

Addition of PAF (200 nmol.L⁻¹) to neutrophils resulted in an abrupt increase in cytosolic Ca²⁺, which was followed by a sustained plateau phase and subsequent gradual decline towards basal levels. Pretreatment of neutrophils with salbutamol (100 nmol.L⁻¹) 20 sec prior to addition of the stimulant did not alter the magnitude of the initial peak response, or the duration of the sustained plateau phase. However, the rate of decline in cytosolic Ca²⁺ concentrations was accelerated. In the presence of montelukast (1 μ mol.L⁻¹), the initial plateau phase was markedly attenuated and the rate of decline in cytosolic Ca²⁺ concentrations increased significantly. The combination of salbutamol and montelukast was more effective



than either agent alone. These results are shown in Figure 4.4 (page 130) and Table 4.2 (page 126). Dexamethasone did not detectably alter Ca²⁺ fluxes in FMLP/CB-activated neutrophils (results not shown).

4.4 **DISCUSSION**

Add-on therapy with montelukast has been reported to confer benefit on patients whose asthma is poorly controlled with inhaled corticosteroid monotherapy, or with the combination of a LABA and an inhaled steroid (Dempsey *et al*, 2000; Currie *et al*, 2003; Dupont *et al*, 2005; Dal Negro *et al*, 2009; Keith *et al*, 2009; Korn *et al*, 2009). In this setting, it is believed that montelukast targets inflammatory mechanisms, specifically those orchestrated by cysteinyl leukotrienes and their receptors, thereby complementing the therapeutic actions of inhaled corticosteroids and beta(2)-agonists. However, this may not be the only mechanism by which add-on therapy with montelukast confers benefits on patients with poorly controlled asthma. The effects of montelukast on PDEs (described in the previous chapter) raise the possibility that this agent may act in concert with the other anti-asthma agents, particularly beta(2)-agonists, to augment cAMP-mediated anti-inflammatory activity, and possibly bronchodilatory activity.

To test this hypothesis, we investigated the effects of formoterol (1 nmol.L⁻¹ and 10 nmol.L⁻¹) and montelukast (1-2 μ mol.L⁻¹), individually and in combination on the pro-inflammatory activities of human neutrophils. Treatment of neutrophils with

121



either agent resulted in significant inhibition of the generation of superoxide in particular, as well as expression of CR3 and release of elastase following activation of the cells with FMLP/CB, with the combination being more effective than the individual agents, most notably in the case of CR3 expression and elastase release, the effects being additive as opposed to synergistic. In asthma, phagocyte-derived reactive oxygen species and elastase have been identified as possible mediators of bronchial hyperactivity and obstruction, and airway remodelling respectively (Lee *et al*, 2006; Mak & Chan-Yeung, 2006), while interference with CR3 expression will restrict the migration of neutrophils into the airways.

In the case of production of LTB₄ by activated neutrophils, the individual agents were found to be extremely potent inhibitors of production of this eicosanoid, with small, but nevertheless significant augmentative effects observed with the combination of formoterol and montelukast. Notwithstanding the well-recognised involvement of cysteinyl leukotrienes in asthma, it is noteworthy that LTB₄ also appears to play an important role in the pathogenesis of severe persistent asthma, as well as aspirin- and exercise-induced asthma, allergic rhinitis and atopic dermatitis (Ohnishi, Miyahara & Gelfand, 2008). Moreover, corticosteroids, which generally do not affect LTB₄ production by immune and inflammatory cells, have been reported to upregulate the expression of the BLT1 receptor on corticosteroid-resistant cells such as neutrophils, monocytes and effector memory CD8⁺ T cells (Obinata *et al*, 2003; Lee *et al*, 2006; Ohnishi *et al*, 2008; Ohnishi, Miyahara & Gelfand, 2008). In the current study, dexamethasone, an agent which does not



elevate intracellular cAMP concentrations, had no detectable effects on either elastase or LTB₄ release by activated neutrophils.

Although of limited relevance in the treatment of chronic asthma, or asthma in which inflammation is well-controlled, the effects of the short-acting beta(2)-agonist, salbutamol, on LTB₄ production by neutrophils activated with PAF, a less potent stimulus than FMLP/CB, were also measured. Salbutamol also inhibited the production of LTB₄ by PAF-activated neutrophils which was augmented by inclusion of montelukast, probably by interactive cAMP-dependent mechanisms as described in the previous chapter.

To probe the mechanism of formoterol/montelukast-mediated inhibition of the proinflammatory activity of neutrophils, the effects of these agents on alterations in cAMP and cytosolic Ca²⁺ were investigated. Exposure of neutrophils to either formoterol or montelukast was accompanied by substantial increases in cAMP. In the case of montelukast, these were somewhat higher than reported in the previous chapter. This is most likely attributable to the longer exposure of the cells to the drug (10 min in the current study compared to 5 min in the previous study). It is possible that longer exposure times may have revealed significant effects at lower concentrations of montelukast; however, these experiments were not performed. Although the magnitude of the increase in cAMP observed with the combination was slightly higher than that observed with the individual agents, the difference did not achieve statistical significance. This may be due to the fact that a relatively brief exposure time (20 sec) was used at which the effects of formoterol



were maximal; longer exposure times may have revealed significant effects of the combination.

In neutrophils, the anti-inflammatory actions of agents which elevate intracellular cAMP are achieved primarily by activation of cAMP-dependent protein kinase (PKA). PKA in turn accelerates restoration of Ca^{2+} homeostasis and down-regulation of pro-inflammatory activities following exposure of the cells to receptor-linked, Ca^{2+} mobilizing stimuli by several mechanisms including: phosphorylative inactivation of phospholipase C (Ali *et al*, 1998); inactivation of inositol triphosphate (IP₃) receptors on intracellular Ca^{2+} stores (Bai & Sanderson, 2006); up-regulation of the Ca^{2+} sequestering/resequestering endo-membrane Ca^{2+} -ATPase (Anderson *et al*, 2000); and inhibition of p38 MAP kinase, resulting in interference with the activation of 5-lipoxygenase (Flamand *et al*, 2002) and attenuation of an autocrine, LTB₄-mediated secondary wave of Ca^{2+} uptake by the cells (Steel *et al*, 2007).

In the current study, formoterol, as reported previously (Tintinger *et al*, 2000), and montelukast, facilitated the clearance of Ca^{2+} from the cytosol of neutrophils activated with FMLP/CB without affecting mobilization of the cation from intracellular stores. The two agents were most effective when used in combination. These observations are compatible with a mechanism whereby formoterol and montelukast, by targeting beta(2)-adrenoreceptors and PDEs respectively, exert their individual and combined anti-inflammatory activities via cAMP-dependent, accelerated restoration of Ca^{2+} homeostasis. This contention is supported by the following observations: i) exposure of FMLP/CB-activated neutrophils to the type 4


phosphodiesterase inhibitor, rolipram in combination with formoterol resulted in decreases in cytosolic Ca^{2+} concentrations similar to those observed with montelukast and formoterol; and ii) montelukast potentiated the effects of salbutamol on clearance of Ca^{2+} from the cytosol of PAF-activated neutrophils. In keeping with its lack of effects on release of elastase and LTB₄, dexamethasone did not affect Ca^{2+} fluxes in fMLP/CB-activated neutrophils.

In conclusion, the results of the current study identify an interactive, cAMPdependent mechanism by which formoterol and montelukast may support the antiinflammatory actions of inhaled corticosteroids by targeting the intransigient neutrophil in patients with poorly controlled asthma.



Table 4.1: Peak cytosolic Ca^{2+} concentrations, duration of the plateau phase of the peak cytosolic Ca^{2+} response, and area under the curve measured in neutrophils activated with FMLP/CB in the absence and presence of formoterol (10 nmol.L⁻¹) and montelukast (2 µmol.L⁻¹) individually and in combination.

System	Peak cytosolic Ca ²⁺ concentration (nM)	Duration of peak plateau phase (min)	Area under the curve (cm ²) ⁺
FMLP/CB only (Control)	420 ± 11	1.3 ± 0.1	7.6 ± 0.34
FMLP/CB + formoterol	435 ± 7	0.9 ± 0.1*	$6.3 \pm 0.4^*$
FMLP/CB + montelukast	420 ± 15	0.9 ± 0.1*	$6.7 \pm 0.4^{*}$
FMLP + formoterol + montelukast	415 ± 13	0.4 ± 0.1**	5.4 ± 0.4**

The results of 4 separate experiments using cells from 4 different donors are presented as the mean values \pm SEM.

- ⁺ Measured 2 min after the addition of FMLP/CB
- * *P*<0.05 for comparison with the control system
- ** *P*<0.05 for comparison of the combination with the individual agents



Table 4.2: Peak cytosolic Ca²⁺ concentrations, duration of the plateau phase of the peak cytosolic Ca²⁺ response, and the magnitude of decrement in cytosolic Ca²⁺ concentrations in neutrophils activated with PAF (200 nmol.L⁻¹) in the presence or absence of salbutamol (100 μ mol.L⁻¹) and montelukast (1 μ mol.L⁻¹) individually and in combination.

System	Peak cytosolic Ca ²⁺ concentration (nM)	Duration of peak plateau phase (min)
PAF only (Control)	455 ± 12	1.4 ± 0.4
PAF + salbutamol	440 ± 14	1.2 ± 0.4
PAF + montelukast	416 ± 22	0.17 ± 0.02*
PAF + salbutamol + montelukast	416 ± 16	0.14 ± 0.02**

The results of 5-16 separate experiments using cells from 4-6 different donors are presented as the mean values \pm SEM.

*P<0.05 for comparison with the control system,

**P<0.05 for comparison of the combination with the individual agents.





Figure 4.1: Effects of formoterol (1 and 10nmol.L⁻¹) and montelukast (2µmol.L⁻¹) individually and in combination on a) the generation of superoxide, b) the release of elastase, and c) production of leukotriene B4 following activation of neutrophils with FMLP in combination with cytochalasin B. The results are expressed as a % of control of a minimum of four different experiments using cells from four different donors. +P<0.05 for comparison of formoterol and montelukast combination with the system treated with montelukast alone; *P<0.05 for comparison of the formoterol and montelukast combination with the system treated with formoterol alone. The absolute values for the responses of control (drug-free), unstimulated neutrophils for superoxide generation, release of elastase and production of LTB4 were 3.782 ± 475 rlu, 34 ± 2 milli-units enzyme per 10^7 cells, and 37 ± 5 pg LTB₄ per 10^7 cells, respectively; the corresponding values for the control systems activated with FMLP/CB were 54.954 ± 1.9 rlu, 980 ± 16 milli-units enzyme per 10^7 cells and 7.926 ± 1.17 pg LTB₄ per 10^7 cells.





Figure 4.2: Effects of formoterol (10nmol.L⁻¹) and montelukast (2µmol.L⁻¹) individually and in combination on neutrophil intracellular cyclic AMP (cAMP) levels. The results are expressed as the mean \pm SEM values (data from 5 different experiments using cells from five different donors). Exposure of the cells to either formoterol or montelukast was accompanied by significant (p<0.05) elevations in cAMP, while the effects of the combination of agents, although greater, did not differ significantly from those of the individual agents. *P<0.05.





Figure 4.3: N-Formyl-L-methionyl-L-leucyl-phenylalanine (FMLP).cytochalasin B (CB)activated fura-2 fluorescence responses of control neutrophils and cells treated with formoterol (10nmol.L⁻¹) and montelukast (2µmol.L⁻¹) individually and in combination. FMLP/CB was added as indicated by arrows when a stable baseline was obtained (\pm 1 min). The traces are from an individual representative experiment (four in a series using cells from four different donors).





Figure 4.4: Effects of salbutamol (100nmol.L⁻¹; _ _) and montelukast (1 μ mol.L⁻¹;.....) alone and in combination (**x x x**) on platelet factor-activated (arrow) neutrophils. ____; controls.



Chapter 5

Final Conclusion



The fact that neutrophilic inflammation is not controlled by glucocortico-steroids was acknowledged in the first chapter and remains an unmet need in the treatment of inflammatory conditions.

The research presented in this thesis was focused primarily on the effects of montelukast (ML) a selective cysteinyl leukotriene receptor-1 (CysLTR1) antagonist, on human neutrophils. CysLTR1-dependent and –independent activities were investigated.

The sensitising effects of the cysteinyl leukotrienes, C₄ and D₄ on human neutrophils and the antagonistic effects of ML demonstrated in Chapter two have not been documented previously. These pro-inflammatory interactions of CysLTs with neutrophils may play an important role in acute and chronic inflammatory conditions in both atopic and non-atopic individuals. This interaction between the CysLTs and human neutrophils may prove to be an important therapeutic target for control of neutrophilic inflammation in conditions where CysLTs have not been thought to play an important role. ML was also found to promote rapid clearance of Ca²⁺ from the cytosol of chemoattractant-activated neutrophils, which was associated with an increase in basal levels of cAMP and marked attenuation of the Ca²⁺-dependent pro-inflammatory activities of the cells. At the same concentrations, ML was shown to possess non-specific PDE inhibitory activities, which appear to underpin the CysLTR1-independent inhibitory effects of this agent on neutrophil functions. When tested in combination, the anti-inflammatory effects of ML and formoterol were generally greater than those of the individual agents,



which may support the rationale of using both in combination with inhaled glucocortico-steroids in the treatment of asthma.

There are, however, several limitations to the current study, as well as potential future extensions. Firstly, the effects of ML on other types of inflammatory cells, including monocytes, macrophages and lymphocytes were not studied. This would be an important area of investigation given the fact that cAMP possesses broad spectrum anti-inflammatory activity encompassing cells of both the innate and adaptive immune systems (Moore & Willoughby, 1995; Serezani et al, 2008). Secondly, the effects of ML on other neutrophil-derived mediators of inflammation such as the chemokine IL-8 and the cytokine TNF were not studied. TNF levels have been reported to be decreased by ML in a rat model of experimental inflammation (Coskun et al. 2011), while it is well known that agents which elevate cAMP are potent inhibitors of the synthesis of TNF (Moore & Willoughby, 1995; Serezani et al., 2008). Thirdly, the experiments described in the current study were only done in vitro and did not include an ex vivo component using blood samples of healthy volunteers or patients with bronchial asthma given montelukast at therapeutic doses.

Other aspects that require further investigation include:

 Investigation of the effects of montelukast on neutrophils from patients with chronic inflammatory disorders of the airways, especially bronchial asthma and chronic obstructive lung disease.

134



- Effects of montelukast on the pro-inflammatory activities of neutrophils activated with different activators such as IL-8, LTB₄ and TNF. The latter would be particularly interesting as it is not a Ca²⁺-mobilizing activator and may not be responsive to montelukast.
- Effects of montelukast on the various purified isoforms of cAMP PDEs to establish their relative sensitivities to the agent
- Because of its anti-oxidative properties the effects of montelukast on neutrophil chemotaxis and phagocytosis are difficult to predict. Interference with NADPH oxidase activity could potentially predispose to bacterial infection and this aspect requires further investigation. However, there are no reports of increase susceptibility to bacterial or viral infections in patients receiving long-term therapy with montelukast.

In addition to these, future studies should include comparison of the antiinflammatory effects of ML on neutrophils with those the other two commercially available CysLT1R antagonists, pranlukast and zafirlukast. Most importantly, however, assessment of the inflammatory potential of ML in genetically engineered mice with selective knockout of the gene encoding the CysLTR1 would be particularly revealing to probe the involvement of CysLTR1-independent antiinflammatory activity in the therapeutic action of this agent.

Future clinical studies should also focus on the anti-inflammatory potential of other agents which combine CysLTR1 antagonism and PDE inhibitory activities. One

135



such agent is CR3465 which combines CysLTR1 antagonism with PDE3 and PDE4 inhibitory effects. A comparison was done with ML, demonstrating reversal of LTD4-induced bronchoconstriction, but not, however, comparing effects on PDE activity (Ferrari *et al*, 2004). Further comparative studies are necessary.

Likewise, the existence of other mechanisms of ML-mediated anti-inflammatory activity, distinct from those described in the current study cannot be discounted. For example, crosstalk between CysLTR1 and P2Y receptors has been described in a study in which the effects of the different CysLTR1 antagonists on human bronchial epithelial cells were investigated (Lau *et al*, 2011).

Importantly, the anti-inflammatory potential of ML in combination with other antiinflammatory agents which target neutrophils by alternative mechanisms, especially the macrolides, also needs investigation. In this respect it is of interest that macrolides have the potential to modify the natural history of chronic inflammatory lung diseases including Post-transplant Bronchiolitis Obliterans Syndrome (BOS) (Friedlander *et al*, 2010). A pilot study done on patients with BOS already on a macrolide (azithromycin) to which ML was added, showed a significant arrest in FEV₁ decline relative to the group treated with the macrolide alone (Verleden *et al*, 2011). Currently, a multi-institutional phase II study of montelukast for the treatment of BOS is underway to which children older than 6 years of age and adults up to 80 years have been recruited. The outcome is awaited. (Martin, 2011)

136



Finally, the results of the current study have demonstrated that ML targets the proinflammatory activities of neutrophils by both CysLTR1-dependent and – independent mechanisms. However, the contribution of the latter to therapeutic activity, if any, remain to be conclusively established.



Chapter 6

References



Abdelilah S, Latifa K, Esra N, Cameron L, Bouchaib L, Nicolaides N, Levitt R, Hamid Q. Functional expression of IL-9 receptor by human neutrophils from asthmatic donors: role in IL-8 release. *J Immunol*. 2001; 166: 2768-2774.
Abdel-Latif D, Steward M, Macdonald DL, Francis GA, Dinauer MC, Lacy P. Rac2 is critical for neutrophil primary granule exocytosis. *Blood*. 2004; 104: 832-839.
Adkinson AF Jr, Yunginger JW, Busse WW, Bochner BS, Holgate S, Simons FE.
Biology of Neutrophils. *Middelton's Allergy Principles & Practice*. 2003; 291;
Akgul C, Edwards SW. Regulation of neutrophil apoptosis via death receptors. *Cell Mol Life Sci*. 2003; 60: 2402-2408.

Akman A, Irkeç M, Orhan M. Effects of Iodoxamide, disodium cromoglycate and fluorometholone on tear leukotriene levels in vernal keratoconjunctivitis. *Eye* (Lond). 1998; 12: 291-295.

Al Omari MM, Zoubi RM, Hasan EI, Khader TZ, Badwan AA. Effect of light and heat on the stability of montelukast in solution and in its solid state. *J Pharm Biomed Anal*. 2007; 45: 465-471.

Ali H, Sozzani S, Fisher I, Barr AJ, Richardson RM, Haribabu B, Snyderman R. Differential regulation of formyl peptide and plateletactivating factor receptors: role of phospholipase Cb3 phosphorylation by protein kinase A. *J Biol Chem.* 1998; 273: 11012–11016.

Anderson R, Goolam Mahomed A, Theron AJ, Ramafi G, Feldman C. Effects of rolipram and dibutyryl cyclic AMP on resequestration of cytosolic calcium in FMLP-activated human neutrophils. *Br J Pharmacol.* 1998; 124: 547–555.

Anderson R, Steel HC, Tintinger GR. Inositol 1,4,5-triphosphate-mediated shuttling between intracellular stores and the cytosol contributes to the sustained elevation in



cytosolic calcium in FMLP-activated human neutrophils. *Biochem Pharmacol*. 2005; 69: 1567-1575.

Anderson R, Theron AJ, Gravett CM, et al. Montelukast inhibits neutrophil proinflammatory activity by a cyclic AMP-dependent mechanism. *Br J Pharmacol.* 2009; 156: 105–115.

Anderson R, Visser SS, Ramafi G, Theron AJ. Accelerated resequestration of cytosolic calcium and suppression of the proinflammatory activities of human neutrophils by CGS 21680. *Br J Pharmacol.* 2000; 130: 717–724.

Andina N, Conus S, Schneider EM, Fey MF, Simon HU. Induction of Bim limits cytokine-mediated prolonged survival of neutrophils. *Cell Death Differ*. 2009; 16: 1248-1255.

Anonymous. Drug Review. Asthma. Montelukast, drugs in context. Part E. *Respir Med.* 2004; Infect 1: 1–40.

Bacharier LB, Boner A, Carlsen KH, et al. Diagnosis and treatment of asthma in childhood: a PRACTALL consensus report. *Allergy*. 2008; 63: 5-34.

Bai Y, Sanderson MJ. Airway smooth muscle relaxation results from a reduction in the frequency of Ca²⁺ oscillations induced by a cAMP-mediated inhibition of the IP3 receptor. *Respir Res.* 2006; 7: 34.

Bai Y, Sanderson MJ. Airway smooth muscle relaxation results from a reduction in the frequency of Ca²⁺ oscillations induced by a cAMP-mediated inhibition of the IP3 receptor. *Respir Res.* 2006; 7: 34

Baldridge, C. W., Gerard, R. W. The extra respiration of phagocytosis. *Am. J. Physiol*. 1933; 103: 235-236



Babior BM, Lambeth JD, Nauseef W. The Neutrophil NADPH Oxidase. Review. *Arch Biochem Biophys*. 2002; 397: 342-344.

Barkhof F, Hulst HE, Drulovic J, Uitdehaag BM, Matsuda K, Landin R. Ibudilast in relapsing-remitting multiple sclerosis: a neuroprotectant? *Neurology*. 2010; 74: 1033-1040.

Barnes PJ. New molecular targets for the treatment of neutrophilic diseases. \boldsymbol{J}

Allergy Clin Immunol. 2007; 119: 1055–1062.

Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, Gibson P,

Ohta K, O'Byrne P, Pedersen SE, Pizzichini E, Sullivan SD, Wenzel SE, Zar HJ.

Global strategy for asthma management and prevention: GINA executive summary.

Eur Respir J. 2008; 31: 143-178

Bautz F, Denzlinger C, Kanz L, Möhle R. Chemotaxis and transendothelial migration of CD34+ hematopoietic progenitor cells induced by the inflammatory mediator leukotriene D4 are mediated by the 7-transmembrane receptor CysLT1. *Blood*. 2001; 97: 3433–3440.

Beatty K, Robertie P, Senior RM, Travis J. Determination of oxidized alpha-1proteinase inhibitor in serum. *J Lab Clin Med.* 1982; 100: 186–192.

Bengis-Garber C, Gruener N. Protein kinase A downregulates the phosphorylation of p47 phox in human neutrophils: a possible pathway for inhibition of the respiratory burst. *Cell Signal*.1996; 8: 291–296.

Binnaz A, Iyanoye A, Sieck G, Prakash YS, Pabelick CM. Cyclic nucleotide regulation of store-operated Ca2+ influx in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol*. 2006; 290: L278–L283.



Bisgaard H. A randomized trial of montelukast in respiratory syncytial virus
postbronchiolitis. *Am J Respir Crit Care Med.* 2003; 167: 379–383.
Borgeat P, Samuealsson B. Arachidonic acid metabolism in polymorphonuclear
leukocytes: unstable intermediate in formation of dihydroxy acid. *Proc Natl Acad Sci USA*. 1979(a); 79: 3213-3217.

Borgeat P, Samuelsson B. Metabolism of aracidonic acid in polymorphonuclear leukocytes. Structural analysis of novel hydroxylated compounds. *J Biol Chem.* 1979(b); 254: 7865-7869.

Borgeat P, Samuelsson B. Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. Formation of a novel dihyrdoxyeichosatetraenoic acid. *J Biol Chem*. 1979(c); 254: 2643-2646.

Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol*. 2007; 28: 340-345.

Borregaard N. Subcellular localization and dynamics of components of the respiratory burst oxidase. *J Bioenerg Biomembr*. 1988; 20: 637-651.

Bouin AP, Grandvaux N, Vignais PV, Fuchs A. p40(phox) is phosphorylated on threonine 154 and serine 315 during activation of the phagocyte NADPH oxidase. Implication of a protein kinase c-type kinase in the phosphorylation process. *J Biol Chem*. 1998; 273: 30097-30103.

Bousquet J, Boulet LP, Peters MJ, Magnussen H, Quiralte J, Martinez-Aguilar NE, Carlsheimer A. Budesonide/formoterol for maintenance and relief in uncontrolled asthma vs. high-dose salmeterol/fluticasone. *Respir Med*. 2007; 101: 2437- 2446.



Bowers RC, Hevko J, Henson PM, Murphy RC. A novel glutathione containing eicosanoid (FOG7) chemotactic for human granulocytes. *J Biol Chem*. 2000; 275: 29931-29934.

Bréchard S, Melchior C, Plançon S, Schenten V, Tschirhart EJ. Store-operated Ca²⁺ channels formed by TRPC1, TRPC6 and Orai1 and non-store-operated channels formed by TRPC3 are involved in the regulation of NADPH oxidase in HL-60 granulocytes. *Cell Calcium*. 2008; 44: 492-506.

Bréchard S, Tschirhart EJ. Regulation of superoxide production in neutrophils: role of calcium influx. *J Leuk Biol*. 2008; 84: 1–15.

Brink C, Dahlén SE, Drazen J, Evans JF, Hay DW, Rovati GE, Serhan CN, Shimizu

T, Yokomizo T. International Union of Pharmacology XLIV. Nomenclature for the oxoeicosanoid receptor. *Pharmacol Rev*. 2004; 56: 149-157.

Brocklehurts WE. The release of histamine and formation of slow reacting substance (SRS-A) during anaphylactic shock. *J Physiol*. (London). 1960; 151: 416-435

Brown GE, Stewart MQ, Liu H, Ha VL, Yaffe MB. A novel assay system implicates PtdIns(3,4)P(2), PtdIns(3)P, and PKC delta in intracellular production of reactive oxygen species by the NADPH oxidase. *Mol Cell*. 2003; 11: 35-47.

Burelout C, Thibault N, Harbour D, Naccache PH, Bourgoin SG. The PGE2-induced inhibition of the PLD activation pathway stimulated by fMLP in human neutrophils is mediated by PKA at the PI3-Kgamma level. *Biochem Pharmacol*. 2007; 74: 730–741.

Burns AR, Walker DC, Brown ES, Thurmon LT, Bowden RA, Keese CR, Simon SI, Entman ML, Smith CW. Neutrophil transendothelial migration is independent of tight



junctions and occurs preferentially at tricellular corners. *J Immunol*. 1997; 159: 2893-2903.

Cadwallader KA, Condliffe AM, McGregor A, et al. Regulation of phosphatidylinositol 3-kinase activity and phosphatidylinositol 3,4,5-trisphosphate accumulation by neutrophil priming agents. *J Immunol*. 2002; 169: 3336-3344.

Cai TQ, Wright SD. Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, alpha M beta 2) and modulates

polymorphonuclear leukocyte adhesion. *J Exp Med*. 1996; 184: 1213-1223.

Campbell EJ, Campbell MA, Owen CA. Bioactive proteinase 3 on the cell surface of human neutrophils: quantification, catalytic activity, and susceptibility to inhibition. *J Immunol*. 2000; 165: 3366-3374.

Capra V, Ambrosio M, Riccioni G, et al. Cysteinyl-leukotriene receptor antagonists:

present situation and future opportunities. Curr Med Chem. 2006; 13: 3213–3216.

Capra V. Molecular and functional aspects of human cysteinyl leukotriene receptors.

Pharmacol Res. 2004; 50: 1-11.

Carroll N, Carello S, Cooke C, James A. Airway structure and inflammatory cells in fatal attacks of asthma. *Eur Respir J*. 1996; 9: 709-715.

Cauwe B, Opdenakker G. Intracellular substrate cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases. *Crit Rev Biochem Mol Biol*. 2010; 45 :351-423.

Celik P, Sakar A, Havlucu Y, Yuksel H, Turkdogan P, Yorganioglu A. Short-term effects of montelukast in stable patients with moderate to severe COPD. *Respir Med.* 2005; 99: 444–450.



Cheng H, Leff JA, Amin R, Gertz BJ, De Smet M, Noonan N, Rogers JD, Malbecq W, Meisner D, Somers G. Pharmacokinetics, bioavailability, and safety of montelukast sodium (MK-0476) in healthy males and females. *Pharm Res*. 1996; 13: 445–448.

Cheng H, Leff JA, Amin R, Gertz BJ, De Smet M, Noonan N, Rogers JD, Malbecq W, Meisner D, Somers G. Pharmacokinetics, bioavailability, and safety of montelukast sodium (MK-0476) in healthy males and females. *Pharm Res.* 1996; 13: 445-448. Chessa TA, Anderson KE, Hu Y, Xu Q, Rausch O, Stephens LR, Hawkins PT. Phosphorylation of threonine 154 in p40phox is an important physiological signal for activation of the neutrophil NADPH oxidase. *Blood*. 2010; 116: 6027-6036. Chu G, Lester JW, Young KB, Luo W, Zhai J, Kranias EG.A single site (Ser16) phosphorylation in phospholamban is sufficient in mediating its maximal cardiac responses to beta-agonists. *J Biol Chem*. 2000; 275: 38938–38943. Ciana P, Fumagalli M, Trincavelli ML, et al. The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. *EMBO J.* 2006; 25: 4615-1627.

Coffey MJ, Phare SM, Peters-Golden M. Prolonged exposure to lipopolysacchride inhibits macrophage 5-lipoxygenase metabolism via induction of nitric oxide synthesis. *J Immunol.* 2000; 165: 3592-3598.

Collins SR, Meyer T. Evolutionary origins of STIM1 and STIM2 within ancient Ca²⁺ signalling systems. *Trends Cell Biol*. 2011; 21: 202-211.

Condliffe A. M, P T. Hawkins L R. Stephens C. Haslett E R. Chilvers. Priming of human neutrophil superoxide generation by tumour necrosis factor- α is signalled by



enhanced phosphatidylinositol 3,4,5-trisphosphate but not inositol 1,4,5trisphosphate accumulation. *FEBS Lett*. 1998; 439: 147.

Condliffe AM, Davidson K, Anderson KE, Ellson CD, Crabbe T, Okkenhaug K, Vanhaesebroeck B, Turner M, Webb L, Wymann MP, Hirsch E, Ruckle T, Camps M, Rommel C, Jackson SP, Chilvers ER, Stephens LR, Hawkins PT. Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils**. Blood**. 2005; 106: 1432-1440.

Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, Su Z, Mosesova S, Eisner MD, Bohen SP, Matthews JG. Lebrikizumab treatment in adults with asthma. *N Engl J Med*. 2011;365: 1088-1098.

Coskun AK, Yigiter M, Oral A, Odabasoglu F, Halici Z, Mentes O, Cadirci E, Atalay F, Suleyman H. The effects of montelukast on antioxidant enzymes and proinflammatory cytokines on the heart, liver, lungs, and kidneys in a rat model of cecal ligation and puncture-induced sepsis. *ScientificWorldJournal*. 2011; 11: 1341-56.

Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. Th17 cells: new players in asthma pathogenesis. *Allergy*. 2011; 66: 989-998.

Cowan DC, Cowan JO, Palmay R, Williamson A, Taylor DR. Heterogeneity of asthma according to blood inflammatory patterns. *Thorax*. 2009; 64: 374-380. Currie GP, Lee DKC, Dempsey OJ, Fowler SJ, Cowan LM, Lipworth BJ. A proof of concept study to evaluate putative benefits of montelukast in moderate persistent asthmatics. *Br J Clin Pharmacol.* 2003; 55: 609–615.



Currie GP, McLaughlin K. The expanding role of leukotriene receptor antagonists in chronic asthma. *Ann Allergy Asthma Immunol*. 2006; 97: 731-741.

Currie GP, Srivastava P, Dempsey OJ, Lee DKC. Therapeutic modulation of allergic airways disease with leukotriene receptor antagonists. *Q J Med.* 2005; 98: 171–182. Czermak BJ, Lentsch AB, Bless NM, Schmal H, Friedl HP, Ward PA. Role of complement in in vitro and in vivo lung inflammatory reactions. *J Leukoc Biol*. 1998; 64: 40-48.

Dal Negro RW, Borderias L, Zhang Q, Fan T, Sazonov V, Guilera M, Taylor SD. Rates of asthma attacks in patients with previously inadequately controlled mild asthma treated in clinical practice with combination drug therapy: an exploratory post-hoc analysis. *BMC Pulm Med.* 2009; 9:10.

Dang PM, Stensballe A, Boussetta T, Dewas H, Kroviarski Y, Hayem G, Jensen ON, Gougerot-Pocidalo MA, El-Benna J. A specific P47phox serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. *J Clin Invest.* 2006; 116: 2033–2043.

Daniele S, Trincavelli ML, Gabelloni P, Lecca D, Rosa P, Abbracchio MP, Martini C. Agonist-Induced Desensitization/Resensitization of Human G Protein-Coupled Receptor 17: A Functional Cross-Talk between Purinergic and Cysteinyl-Leukotriene Ligands. *J Pharmacol Exp Ther*. 2011; 338: 559-567.

Dempsey OJ, Wilson Am, Sims Ej, Mistry C, Lipworth BJ. Additive bronchoprotective and bronchodilator effects with doses of salmeterol and montelukast in asthmatic patients receiving inhaled corticosteroids. *Chest*. 2000; 117: 950-953.



Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W. The PPARalpha-leukotriene B4 pathway to inflammation control. *Nature.* 1996; 384: 39-43.

Dewas C, Dang PM, Gougerot-Pocidalo MA, El-Benna J. TNF-alpha induces phosphorylation of p47(phox) in human neutrophils: partial phosphorylation of p47phox is a common event of priming of human neutrophils by TNF-alpha and granulocyte-macrophage colony-stimulating factor. *J Immunol*. 2003; 171: 4392-4398.

Deykin A, Wechsler ME, Boushey HA, Chinchilli VM, Kunselman SJ, Craig TJ,

DiMango E, Fahy JV, Kraft M, Leone F, Lazarus SC, Lemanske RF Jr, Martin RJ,

Pesola GR, Peters SP, Sorkness CA, Szefler SJ, Israel E. Combination therapy with

a long-acting beta-agonist and a leukotriene antagonist in moderate asthma. Am J

Respir Crit Care Med. 2007; 175: 228-234.

Diamant Z, van der Molen T. Treating asthma: is there a place for leukotriene receptor antagonists? *Respir Med.* 2005; 99: 655–662.

Doerschuk CM, Beyers N, Coxson HO, Wiggs B, Hogg JC. Comparison of neutrophil and capillary diameters and their relation to neutrophil sequestration in the lung. *J*

Appl Physiol. 1993; 74: 3040-3045.

Doerschuk CM, Tasaka S, Wang CD11/CD18-dependent and -independent neutrophil emigration in the lungs: how do neutrophils know which route to take? *Am*

J Respir Cell Mol Biol. 2000; 23: 133-136.

Drakatos P, Lykouras D, Sampsonas F, Karkoulias K, Spiropoulos K. Targeting leukotrienes for the treatment of COPD? *Inflamm Allergy Drug Targets*. 2009; 8: 297-306.



Dupont L, Potvin E, Korn A, Lachman M, Gusman J, Pechè R. Improving asthma in patients suboptimally controlled on inhaled steroids and long-acting beta2-agonists: addition of montelukast in an open-label pilot study. *Curr Med Res Opin*. 2005; 21: 863-869.

Duroudier NP, Tulah AS, Sayers I. Leukotriene pathway genetics and pharmacogenetics in allergy. *Allergy*. 2009; 64: 823-839.

Durrani SR, Viswanathan RK, Busse WW. What effect does asthma treatment have on airway remodeling? Current perspectives. *J Allergy Clin Immunol*. 2011; 128: 439-448.

Ehlers MR. CR3: a general purpose adhesion-recognition receptor essential for innate immunity. *Microbes Infect*. 2000; 2: 289-294.

Farr SJ, Rowe AM, Rubsamen R, et al. Aerosol deposition in the human lung following administration from a microprocessor controlled pressurized metered dose inhaler. *Thorax.* 1995; 50: 639–644.

FeldbergW and Kellaway CW, Liberation of histamine and formation of lysocithin-like substance by cobra venom. *J Physiol*. (London). 1938; 94: 187-226.

Ferrari F, Mennuni L, Caselli G, Zanelli T, Makovec F. Pharmacological profile of CR3465, a new leukotriene CysLT1 receptor antagonist with broad anti-inflammatory activity. *Eur J Pharmacol*. 2004; 504: 223-233.

Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 2006; 441: 179-185.



Field SK. Roflumilast: an oral, once-daily selective PDE-4 inhibitor for the management of COPD and asthma. *Expert Opin Investig Drugs*. 2008; 17: 811-818.

Flamand N, Surette ME, Picard S, Bourgoin S, Borgeat P. Cyclic AMP-mediated inhibition of 5-lipoxygenase translocation and leukotriene biosynthesis in human neutrophils. *Mol Pharmacol.* 2002; 62: 250–256.

Fleisch JH, Rinkema LE, Marshall WS. Pharmacologic receptors for the leukotrienes. *Biochem Pharmacol*. 1984; 33: 3919-3922.

Fligiel SE, Standiford T, Fligiel HM, Tashkin D, Strieter RM, Warner RL, Johnson KJ, Varani J. Matrix metalloproteinases and matrix metalloproteinase inhibitors in acute lung injury. *Hum Pathol*. 2006; 37: 422-430.

Folko G, Murphy RC. Eicosanoid transcellular biosynthesis: from cell-cell interaction to in vivo tissue responses. *Pharmacol Rev.* 2006; 58: 375-388

Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *J Innate Immun*. 2010; 2: 216-227.

Fox-Spencer R. Drug review. Allergic rhinitis and asthma. Montelukast. *Drugs*

Context. 2006; 2: 413–456.

Fregonese L, Stolk J. Hereditary alpha-1-antitrypsin deficiency and its clinical consequences. *Orphanet J Rare Dis.* 2008; 3: 16.

Frey RS, Ushio-Fukai M, Malik AB. NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. *Antioxid Redox Signal*. 2009; 11: 791-810.



Friedlander AL, Albert RK. Chronic macrolide therapy in inflammatory airways diseases. *Chest.* 2010; 138: 1202-12.

Fukuda M, Suzuki Y, Hino H, Morimoto T, Ishii E. Activation of central adenosine (2A) receptors lowers the seizure threshold of hyperthermia-induced seizure in childhood rats. *Seizure*. 2011; 20: 156-159.

Gane J, Stockley R. Mechanisms of neutrophil transmigration across the vascular endothelium in COPD. *Thorax*. 2011; 10: 1136.

Gay JC. Priming of neutrophil oxidative responses by platelet-activating factor. J

Lipid Mediat. 1990; 2 Suppl: S161-S175.

Geiszt M, Kapus A, Nemet K, Farkas L, Ligeti F. Regulation of capacitative calcium influx in neutrophil granulocytes: alterations in chronic granulomatous disease. *J Biol Chem*. 1997; 272: 26471–26478.

Gelfand EW, Dakhama A. CD8+ T lymphocytes and leukotriene B4: novel interactions in the persistence and progression of asthma. *J Allergy Clin Immunol*.

2006; 117: 577-582.

Geraghty P, Rogan MP, Greene CM, Boxio RM, Poiriert T, O'Mahony M, Belaaouaj A, O'Neill SJ, Taggart CC, McElvaney NG. Neutrophil elastase up-regulates cathepsin B and matrix metalloprotease-2 expression. *J Immunol*. 2007 ;178: 5871-5878.

Gounni AS, Lamkhioued B, Koussih L, Ra C, Renzi PM, Hamid Q. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. *FASEB J*. 2001; 15: 940-949.



Gravett CM, Tintinger G, Theron A, Anderson R, Feldman C, Green R. Montelukast sodium: Administration to children to control intermittent asthma. *Clin Med Rev Therap.* 2010; 2: 1-10.

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescent properties. *J Biol Chem*. 1985; 260: 3440–3450.

Gueli N, Verrusio W, Linguanti A, De Santis W, Canitano N, Ippoliti F, Marigliano V, Cacciafesta M. Montelukast therapy and psychological distress in chronic obstructive pulmonary disease (COPD): a preliminary report. *Arch Gerontol Geriatr*. 2011; 52: e36-39.

Guichard C, Pedruzzi E, Dewas C, Fay M, Pouzet C, Bens M, Vandewalle A, Ogier-Denis E, Gougerot-Pocidalo MA, Elbim C.Interleukin-8-induced priming of neutrophil oxidative burst requires sequential recruitment of NADPH oxidase components into lipid rafts. *J Biol Chem*. 2007; 280: 37021–37032.

Hallett MB, Lloyds D. Neutrophil priming: the cellular signals that say 'amber' but not 'green'. *Immunol Today*. 1995; 16: 264-268.

Hallmann R, Horn N, Selg M, Wendler O, Pausch F, Sorokin LM. Expression and function of laminins in the embryonic and mature vasculature. *Physiol Rev*. 2005; 85: 979-1000.

Hanania NA. Targeting airway inflammation in asthma: current and future therapies. *Chest*. 2008; 133: 989-998.

Hatzelmann A, Morcillo EJ, Lungarella G, Adnot S, Sanjar S, Beume R, Schudt C, Tenor H. The preclinical pharmacology of roflumilast--a selective, oral phosphodiesterase 4 inhibitor in development for chronic obstructive pulmonary disease. *Pulm Pharmacol Ther*. 2010; 23: 235-256.



Hay DW, Muccitelli RM, Tucker SS, Vickery-Clark LM, Wilson KA, Gleason JG, Hall RF, Wasserman MA, Torphy TJ. Pharmacologic profile of SK&F 104353: a novel, potent and selective peptidoleukotriene receptor antagonist in guinea pig and human airways. *J Pharmacol Exp Ther*. 1987; 243: 474-4781.

Heimbürger M, Palmblad J. Effects of leukotriene C4 and D4, histamine and bradykinin on cytosolic calcium concentrations and adhesiveness of endothelial cells and neutrophils. *Clin Exp Immunol*. 1996; 103: 454–460.

Henderson WR Jr, Chi EY, Bollinger JG, et al. Importance of group X-secreted phospholipase A2 in allergen-induced airway inflammation and remodelling in a mouse asthma model. *J Exp Med*. 2007; 204: 865-877.

Henderson WR Jr, Chiang GK, Tien YT, Chi EY. Reversal of allergen-induced airway remodeling by CysLT1 receptor blockade. *Am J Respir Crit Care Med.* 2006; 173: 718–728.

Heutinck KM, ten Berge IJ, Hack CE, Hamann J, Rowshani AT. Serine proteases of the human immune system in health and disease. *Mol Immunol*. 2010; 47: 1943-1955.

Heyworth PG, Badwey JA. Protein phosphorylation associated with the stimulation of neutrophils. Modulation of superoxide production by protein kinase C and calcium. J

Bioenerg Biomembr. 1990; 22: 1-26.

Higgs G. Is PDE4 too difficult a drug target? *Curr Opin Investig Drugs*. 2010; 11: 495-498.

Hiraguchi Y, Nagao M, Hosoki K, Tokuda R, Fujisawa T. Neutrophil proteases activate eosinophil function in vitro. *Int Arch Allergy Immunol*. 2008; 146: 16–21.



Hogg JC, Doerschuk CM. Leukocyte traffic in the lung. *Ann Rev Physiol*. 1995; 57: 97-114.

Holgate ST, Peters-Golden M, Panettieri RA, Henderson WR Jr. Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. *J*

Allergy Clin Immunol. 2003; 111: S18-34; discussion S34-36

Holmes, B., Page, A. R., Good, R. A. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. *J Clin Invest*. 1967; 46: 1422-1432.

Holmsen H, Storm E, Day HJ. Determination of ATP and ADP in blood platelets: a modification of the firefly luciferase assay for plasma. *Anal Biochem*. 1972; 46: 489–501.

Hui Y, Funk CD. Cysteinyl leukotriene receptors. *Biochem Pharmacol*. 2002; 64: 1549-1557.

Iannone MA, Wolberg G, Zimmerman TP. Chemotactic peptide induces cAMP
elevation in human neutrophils by amplification of the adenylate cyclase response to
endogenously produced adenosine. *J Biol Chem*. 1989; 264: 20177– 20180.
Ilowite J, Webb R, Friedman B, Kerwin E, Bird SR, Hustad CM, Edelman JM.
Addition of montelukast or salmeterol to fluticasone for protection against asthma
attacks: a randomized, double-blind, multicenter study. *Ann Allergy Asthma Immunol*. 2004; 92: 641-648.

Ilumets H, Rytilä P, Demedts I, Brusselle GG, Sovijärvi A, Myllärniemi M, Sorsa T, Kinnula VL. Matrix metalloproteinases -8, -9 and -12 in smokers and patients with stage 0 COPD. *Int J Chron Obstruct Pulmon Dis*. 2007; 2: 369-379.



Ito K, Herbert C, Siegle JS, Vuppusetty C, Hansbro N, Thomas PS, Foster PS, Barnes PJ, Kumar RK. Steroid-resistant neutrophilic inflammation in a mouse model of an acute exacerbation of asthma. *Am J Respir Cell Mol Biol*. 2008; 39: 543-550. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med*. 1999; 160: 1532-1539.

Jeffery PK, Venge P, Gizycki MJ, Egerod I, Dahl R, Faurschou P. Effects of salmeterol on mucosal inflammation in asthma: a placebo-controlled study. *Eur Respir J*. 2002; 20: 1378-1385.

Johnson M. Effects of β2-agonists on resident and infiltrating inflammatory cells. *J Allergy Clin Immunol*. 2002; 110: S282–S290.

Johnson M. Molecular mechanisms of β2-adrenergic receptor function, response and regulation. *J Allergy Clin Immunol*. 2006; 117: 18–24.

Jones TR, Labelle M, Belley M, Champion E, Charette L, Ford-Hutchinson AW, *et al.* Pharmacology of montelukast sodium (Singulair), a potent and selective leukotriene D₄ receptor antagonist. *Can J Physiol Pharmacol*. 1995; 73: 191–201.

Jung YW, Zindi C, Lai j, Weaver CT, Chaplin DD. Matrix Metalloproteinases Induced by Gr-1+ Cells are Crucial for recruitment of Th Cells into the Airways. *Eur J Immunol*. 2009; 39: 2281-2292.

Kanaoka Y, Boyce JA. Cysteinyl leukotrienes and their receptors:cellular distribution and function in immune and inflammatory responses. *J Immunol*. 2004; 173: 1503-1510.

Karathanassis D, Stahelin RV, Bravo J, Perisic O, Pacold CM, Cho W, Williams RL. Binding of the PX domain of p47^{phox} to phosphatidylinositol 3,4-bisphosphate and



phosphatidic acid is masked by an intramolecular interaction. *EMBO J*. 2002; 21: 5057-5068.

Kasama T, Miwa Y, Isozaki T, Odai T, Adachi M, Kunkel SL. Neutrophil-derived cytokines: potential therapeutic targets in inflammation. *Curr Drug Targets Inflamm Allergy*. 2005; 4 :273-279.

Katsuyama M. NOX/NADPH oxidase, the superoxide-generating enzyme: its transcriptional regulation and physiological roles. *J Pharmacol Sci*. 2010; 114: 134-146.

Keith PK, Koch C, Djandji M, et al. Montelukast as add-on therapy with inhaled corticosteroids and long-acting β2-agonists in the management of patients diagnosed with asthma and concurrent allergic rhinitis (the RADAR trial). *Can Respir J*. 2009; 16: 17–24.

Kessenbrock K, Dau T, Jenne DE. Tailor-made inflammation: how neutrophil serine proteases modulate the inflammatory response. *Mol Med*. 2011; 89: 23-28.

Kessenbrock K, Fröhlich L, Sixt M, Lämmermann T, Pfister H, Bateman A, Belaaouaj A, Ring J, Ollert M, Fässler R, Jenne DE. Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. *J Clin Invest*. 2008; 118: 2438-2447.

Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*. 2010; 141: 52-67.

Knorr B, Nguyen HH, Kearns GL, Villaran C, Boza ML, Reiss TF, Rogers JD, Zhang J, Larson P, Spielberg S. Montelukast dose selection in children ages 2 to 5 years: comparison of population pharmacokinetics between children and adults. *J Clin*

Pharmacol. 2001; 41: 612-619.



Kobayashi SD, Voyich JM, Burlak C, DeLeo FR. Neutrophils in the innate immune response. *Arch Immunol Ther Exp (Warsz).* 2005; 53: 505-517.
Köhrmann A, Kammerer U, Kapp M, Dietl J, Anacker J. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. *BMC Cancer*. 2009; 9: 188.
Kong MYF, Gaggar A, Li Y, Winkler M, Blalock E, Clancy JP. Matrix Metalloproteinase Activity in Pediatric Acute Lung Injury.Int *J Med Sci*. 2009; 6: 9-17.

Korn D, Van den Brande P, Potvin E, et al. Efficiency of add-on montelukast in patients with non-controlled asthma: a Belgian open-label study. *Curr Med Res Opin*. 2009; 25: 489–497.

Kumar SD, Krishnamurthy K, Manikandan J, Pakeerappa PN, Pushparaj PN. Deciphering the key molecular and cellular events in neutrophil transmigration during

acute inflammation. *Bioinformation*. 2011; 6: 111-114.

Lamblin C, Gosset P, Tillie-Leblond I, Saulnier F, Marquette CH, Wallaert B, Tonnel

AB. Bronchial neutrophilia in patients with noninfectious status asthmaticus. Am J

Respir Crit Care Med. 1998; 157: 394-402.

Langlois A, Ferland C, Tremblay GM, Laviolette M. Montelukast regulates eosinophil protease activity through a leukotriene-independent mechanism. *J Allergy Clin Immunol*. 2006; 118: 113-119.

Lapouge K, Smith SJ, Walker PA, Gamblin SJ, Smerdon SJ, Rittinger K. Structure of the TPR domain of p67phox in complex with Rac.GTP. *Mol Cell*. 2000; 6: 899-907.



Lärfars G, Lantoine F, Devynck MA, Palmblad J, Gyllenhammar H. Activation of nitric oxide release and oxidative metabolism by leukotrienes B4, C4 and D4 in human polymorphonuclear leukocytes. *Blood*. 1999; 93: 1399–1405.

Laviolette M, Malmstrom K, Lu S, Chervinsky P, Pujet JC, Peszek I, Zhang J, Reiss TF. Montelukast added to inhaled beclamethasone in treatment of asthma. *Am J Respir Crit Care Med*. 1999; 160: 1862–1868.

Lau WK, Chow AW, Au SC, Ko WH. Differential inhibitory effects of CysLT(1) receptor antagonists on P2Y(6) receptor-mediated signaling and ion transport in human bronchial epithelia. PLoS One. 2011; 6: e22363.

Lazarus SC, Chinchilli VM, Rollings NJ, Boushey HA, Cherniack R, Craig TJ, Deykin A, DiMango E, Fish JE, Ford JG, Israel E, Kiley J, Kraft M, Lemanske RF Jr, Leone FT, Martin RJ, Pesola GR, Peters SP, Sorkness CA, Szefler SJ, Wechsler ME, Fahy JV; National Heart Lung and Blood Institute's Asthma Clinical Research Network. Smoking affects response to inhaled corticosteroids or leukotriene receptor antagonists in asthma. *Am J Respir Crit Care Med*. 2007; 175: 783-790.

Lee K-Y, Ho S-C, Lin H-C, Lin SM, Liu CY, Huang CD, Wang CH, Chung KF, Kuo HP. Neutrophil-derived elastase induces TGF-β1 secretion in human airway smooth muscle. *Am J Respir Cell Mol Biol*. 2006; 35: 407–414.

Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH. The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis. *J Leukoc Biol*. 2000; 68: 158-166.

Lew PD, Monod A, Waldvogen FA, Pozzan T. Role of cytosolic free calcium and phospholipase C in leukotriene-B4-stimulated secretion in human neutrophils.



Comparison with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine. *Eur J Biochem*. 1987; 162: 161–168.

Lewis EM, Sergeant S, Ledford B, Stull N, Dinauer MC, McPhail LC. Phosphorylation of p22^{phox} on threonine 147 enhances NADPH oxidase activity by promoting p47^{phox} binding. *J Biol Chem*. 2010; 285: 2959-2967.

Lima JJ. Treatment heterogeneity in asthma: genetics of response to leukotriene modifiers. *Mol Diagn Ther*. 2007; 11 :97-104.

Liu D, Ge S, Zhou G, Xu G, Zhang R, Zhu W, Liu Z, Cheng S, Liu X. Montelukast inhibits matrix metalloproteinases expression in atherosclerotic rabbits. *Cardiovasc*

Drugs Ther. 2009; 23: 431-437.

Liu Z, Zhou X, Shapiro SD, Shipley JM, Twining SS, Diaz LA, Senior RM, Werb Z. The serpin alpha1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo. *Cell*. 2000; 102: 647-655.

Lötvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, Lemanske RF Jr, Wardlaw AJ, Wenzel SE, Greenberger PA. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome**. J Allergy Clin** *Immunol*. 2011; 127: 355-360.

Lovén J, Svitacheva N, Jerre A, et al. Anti-inflammatory activity of β2-agonists in primary lung epithelial cells is independent of glucocorticoid receptor. *Eur Respir J*. 2007; 30: 848–856.

Lundeen KA, Sun B, Karlsson L, Fourie AM. Leukotriene B4 receptors BLT1 and BLT2: expression and function in human and murine mast cells. *J Immunol.* 2006; 177: 3439-3447.



MacDowell AL, Peters SP. Neutrophils in asthma. *Curr Allergy Asthma Rep*. 2007; 7: 464–468.

Mackarel AJ, Russell KJ, Ryan CM, Hislip SJ, Rendall JC, FitzGerald MX, O'Connor CM. CD18 dependency of transendothelial neutrophil migration differs during acute pulmonary inflammation. *J Immunol*. 2001; 167: 2839-2846.

Mak JC, Chan-Yeung MM. Reactive oxidant species in asthma. *Curr Opin Pulm Med*. 2006; 12: 7–11.

Mamedova L, Capra V, Accomazzo MR, Gao ZG, Ferrario S, Fumagalli M, Abbracchio MP, Rovati GE, Jacobson KA. CysLT1 leukotriene receptor antagonists inhibit the effects of nucleotides acting at P2Y receptors. *Biochem Pharmacol*. 2005; 71: 115-125.

Maneechotesuwan K, Essilfie-Quaye S, Meah S, Kelly C, Kharitonov SA, Adcock IM, Barnes PJ. Formoterol attenuates neutrophilic airway inflammation in asthma. *Chest*. 2005; 128: 1936-1942.

Mansfield PJ, Hinkovska-Galcheva V, Shayman JA, Boxer LA. Granulocyte colonystimulating factor primes NADPH oxidase in neutrophils through translocation of cytochrome b(558) by gelatinase-granule release. *J Lab Clin Med*. 2002; 140: 9-16. Martin P. Multi-institutional phase II study of montelukast for the treatment of bronchiolitis obliterans following allogeneic stem cell transplantation in children and adults. http://www.seattlecca.org/clinical-trials/transplant-NCT00656058.cfm. Marwick JA, Adcock IM, Chung KF. Overcoming reduced glucocorticoid sensitivity in airway disease: molecular mechanisms and therapeutic approaches. *Drugs*. 2010; 70: 929-948.


Mayerle J, Schnekenburger J, Krüger B, Kellermann J, Ruthenbürger M, Weiss FU, Nalli A, Domschke W, Lerch MM. Extracellular cleavage of E-cadherin by leukocyte elastase during acute experimental pancreatitis in rats. *Gastroenterology*. 2005; 129: 1251-1267.

McCawley LJ, Matrisian LM. Matrix metalloproteinases: They're not just for matrix anymore! *Curr Opin Cell Biol*. 2001; 13: 534-540.

McFadden ER. Inhaled glucocorticoids and acute asthma: therapeutic breakthrough

or non-specific effect? Am J Respir Crit Care Med. 1998; 157: 677–678.

Merck research Laboratory: Data on file.http://www.merck.com/product/usa/pi

_circulars/s/singulair/singulair_pi.pdf.

Minkenberg I, Ferber E . Lucigenin-dependent chemiluminescence as a new assay for NADPH-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. *J Immunol Methods*. 1984; 71: 61–67.

Monteiro AP, Pinheiro CS, Luna-Gomes T, Alves LR, Maya-Monteiro CM, Porto BN,

Barja-Fidalgo C, Benjamim CF, Peters-Golden M, Bandeira-Melo C, Bozza MT,

Canetti C. Leukotriene B4 mediates neutrophil migration induced by heme. J

Immunol. 2011; 86: 6562-6567.

Moore AR, Willoughby DA. The role of cAMP regulation in controlling inflammation.

Clin Exp Immunol. 1995; 101: 387–389.

Mundell SJ, Olah ME, Panettieri RA, Benovic JL, Penn RB. Regulation of G-proteincoupled receptor-adenyl cyclase responsiveness in human airway smooth muscle by exogenous and autocrine adenosine. *Am J Respir Cell Mol Biol*. 2001; 24: 155– 163.



Murphy PM. Neutrophil receptors for interleukin-8 and related CXC chemokines. *Semin Hematol.* 1997; 34: 311-318.

Murphy RC, Hammaström S, Samuelsson B. and C Leukotriene, slow –reacting substance from murine mastocytoma cells. *Proc Natl Acad USA*. 1979; 76: 4275-4279.

Muz MH, Deveci F, Bulut Y, Illhan N, Yekeler H, Turgut T. The effects of low dose leukotriene receptor antagonist therapy on airway remodeling and cysteinyl leukotriene expression in a mouse asthma model. *Exp Mol Med*. 2006; 38: 109–118. Nadif R, Siroux V, Oryszczyn MP, Ravault C, Pison C, Pin I, Kauffmann F; Epidemiological study on the Genetics and Environment of Asthma (EGEA). Heterogeneity of asthma according to blood inflammatory patterns. *Thorax*. 2009; 64: 374-380.

Naik S, Billington CK, Pascual RM, Deshpande D, Stefano F, Kohout T, Eckman DM, Benovic JL, Penn RB. Regulation of cysteinyl leukotriene type 1 receptor internalization and signalling. *J Biol Chem*. 2005; 280: 8722–8732.

Narala VR, Adapala RK, Suresh MV, Brock TG, Peters-Golden M, Reddy RC. Leukotriene B4 is a physiologically relevant endogenous peroxisome proliferatoractivated receptor-alpha agonist. *J Biol Chem*. 2010; 285: 22067-22074. National Heart, Lung and Blood Institute, National Institutes of Health, US Department of Health and Human Services. Expert panel report 3: guidelines for the diagnosis and management of asthma. 2007; Section 2, p.1. Nayak A, Langdon RB. Montelukast in the treatment of allergic rhinitis. An evidence-

based review. *Drugs*. 2007; 67: 887–901.

162



Nick JA, Avdi NJ, Young SK, Knall C, Gerwins P, Johnson GL , Worthen GS. Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J Clin Invest*. 1997; 99: 975–986. Obinata H, Yokomizo T, Shimizu T, et al. Glucocorticoids up-regulate leukotriene B4 receptor-1 expression during neutrophilic differentiation of HL-60 cells. *Biochem Biophys Res Comm*. 2003; 309: 114–119.

Ohnishi H, Miyahara N, Dakhama A, Takeda K, Mathis S, Haribabu B, Gelfand EW. Corticosteroids enhance CD8+ T cell-mediated airway hyperresponsiveness and allergic inflammation by upregulating leukotriene B4 receptor 1. *J Allergy Clin Immunol*. 2008; 121: 864-871.e4.

Ohnishi H, Miyahara N, Gelfand EW. The role of leukotriene B₄ in allergic diseases. *Allergol Int*. 2008; 57: 291-298.

Owen CA and Campbell EJ. Neutrophil proteinases and matrix degradation. The cell biology of pericellular proteolysis. *Semin Cell Biology*. 1995; 6: 367–376.

Padrines M, Wolf M, Walza A, Baggiolini M. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Letters*. 1994; 352: 231-235

Page CP, Spina D. Phosphodiesterase inhibitors in the treatment of inflammatory

diseases. Handb Exp Pharmacol. 2011; 204: 391-414.

Palmblad J, Gyllenhammar H, Lindgren JA, Malmsten CL. Effects of leukotrienes and f-Met-Leu-Phe on oxidative metabolism of neutrophils and eosinophils. *J*

Immunol. 1984; 132: 3041-3045.

Peters-Golden M and WR Henderson, Mechanisms of disease: Leukotrienes. *New Engl J Med*. 2007; 357: 1841-1854.



Peters-Golden M, Brock TG. 5-lipoxygenase and FLAP. *Prostaglandins Leukot Essent Fatty Acids*. 2003; 69: 99-109.

Pétrin D, Turcotte S, Gilbert AK, Rola-Pleszczynski M, Stankova J. The antiapoptotic effect of leukotriene B4 in neutrophils: a role for phosphatidylinositol 3kinase, extracellular signal-regulated kinase and Mcl-1. *Cell Signal*. 2006; 18: 479-487.

Pettit EJ, Hallett MB. Two distinct Ca²⁺ storage and release sites in human neutrophils**.** *Leukoc Biol*. 1998; 63: 225-232.

Pham CTN. Neutrophil serine proteases fine-tune the inflammatory response. *Int J Biochem Cell Biol*. 2008; 40: 1317–1333.

Ponce L, Arjona M, Blanco G, Alvarez S, Arcila E, Ortega A, Nuñez D, Verzura J, Tovar R, Bethencourt S, Riera R, Mora-Orta S, Corado J. The effect of montelukast in a model of gouty arthritis induced by sodium monourate crystals. The effect of montelukast in a model of gouty arthritis induced by sodium monourate crystals. *Invest Clin*. 2011; 52: 15-22.

Prikk K, Maisi P, Pirilä E, Reintam MA, Salo T, Sorsa T, Sepper R. Airway obstruction correlates with collagenase-2 (MMP-8) expression and activation in bronchial asthma. *Lab Invest*. 2002; 82: 1535-1545.

Rabe KF. Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br J Pharmacol*. 2011; 163: 53-67.

Rabinovitch N, Strand M, Stuhlman K, Gelfand EW. Exposure to tobacco smoke increases leukotriene E4-related albuterol usage and response to montelukast. *J Allergy Clin Immunol*. 2008; 121: 1365-1371.



Ramires R, Caiaffa MF, Tursi A, Haeggström JZ, Macchia L. Novel inhibitory effect on 5-lipoxygenase activity by the anti-asthma drug montelukast. *Biochem Biophys Res Commun*. 2004; 324: 815-821.

Raptis SZ, Shapiro SD, Simmons PM, Cheng AM, Pham CT. Serine protease cathepsin G regulates adhesion-dependent neutrophil effector functions by modulating integrin clustering. *Immunity*. 2005; 22: 679-691.

Reilly DM, Parslew R, Sharpe GR, Powell S, Green MR. Inflammatory mediators in normal, sensitive and diseased skin types. *Acta Derm Venereol*. 2000; 80: 171-174. Riccioni G, Bucciarelli T, Mancini B, Di Ilio C, D'Orazio N. Antileukotriene drugs: clinical application, effectiveness and safety. *Curr Med Chem*. 2007; 14: 1966-1977. Robinson AJ, Kashanin D, O'Dowd F, Williams V, Walsh GM. Montelukast inhibition of resting and GM-CSF-stimulated eosinophil adhesion to VCAM-1 under flow conditions appears independent of cysLT(1)R antagonism. *J Leukoc Biol*. 2008; 83: 1522-1529.

Robledo, A. Papaioannou, B. Ochietti, C. Beauchemin, D. Legault and A. Cantin et al., ICAM-1 isoforms: Specific activity and sensitivity to cleavage by leukocyte elastase and cathepsin G. *Eur J Immun*. 2003; 33: 1351–1360.

Rubin P, Mollison KW. Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. *Prostaglandins Other Lipid Mediat*. 2007; 83: 188-197. Rubinstein I, Kumar B, Schriever C. Long-term montelukast therapy in moderate to severe COPD- a preliminary observation. *Respir Med*. 2004; 98: 134–138. Ruiz-Velasco V, Zhong J, Hume JR, Keef KD. Modulation of Ca²⁺ channels by cyclic nucleotide cross activation of opposing protein kinases in rabbit portal vein. *Circ Res*. 1998; 82: 557–565.



Salmon MD, Ahluwalia J. Discrimination between receptor- and store-operated Ca²⁺influx in human neutrophils. *Cell Immunol*. 2010; 265: 1-5.

Salpeter SR, Buckley NS, Ormiston TM, Salpeter EE. Meta-analysis: effect of longacting beta-agonists on severe asthma exacerbations and asthma-related deaths.

Ann Intern Med. 2006; 144: 904-912.

Sampson AP, Castling DP, Green CP, Price JF. Persistent increase in plasma and urinary leukotrienes after acute asthma. *Arch Dis Child*. 1995; 73: 221-225.

Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science*. 1983; 220: 568-575.

Sanz MJ, Cortijo J, Morcillo EJ. PDE4 inhibitors as new anti-inflammatory drugs: effects on cell trafficking and cell adhesion molecules expression. *Pharmacol Ther*. 2005; 106: 269-297.

Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA. The neutrophil as a cellular source of chemokines. *Immunol Rev*. 2000; 177: 195-203. Schaff UY, Dixit N, Procyk E, Yamayoshi I, Tse T, Somin SI. Orai1 regulates intracellular calcium, arrest, and shape polarization during neutrophil recruitment in shear flow. *Blood*. 2010; 115: 657-666.

Sears MR. The addition of long-acting beta-agonists to inhaled corticosteroids in asthma. *Curr Opin Pulm Med*. 201; 17: 23-28.

Sener G, Sehirli O, Cetinel S, Ercan F, Yüksel M, Gedik N, Yeğen BC. Amelioration of sepsis-induced hepatic and ileal injury in rats by the leukotriene receptor blocker montelukast. *Prostaglandins Leukot Essent Fatty Acids*. 2005; 73: 453-462.



Serezani CH, Ballinger MN, Aronoff DM, Peters-Golden M. Cyclic AMP: master regulator of innate immune cell function. *Am J Respir Cell Mol Biol*. 2008; 39: 127-132.

Shapiro SD. Neutrophil elastase: path clearer, pathogen killer, or just pathologic? *Am J Respir Cell Mol Biol*. 2002; 26: 266-268.

Sheppard FS, Kelher MR, Moore EE, McLaughlin NJD, Banerjee A, Silliman CC.Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *J Leukoc Biol*. 2005; 78: 1025-1042. Silliman CC, Elzi DJ, Ambruso DR, Musters RJ, Hamiel C, Harbeck RJ, Paterson AJ, Bjornsen AJ, Wyman TH, Kelher M, England KM, McLaughlin-Malaxecheberria N, Barnett CC, Aiboshi J, Bannerjee A. Lysophosphatidylcholines prime the NADPH oxidase and stimulate multiple neutrophil functions through changes in cytosolic calcium. *J Leukoc Biol*. 2003; 73: 511-524.

Singh RK, Gupta S, Dastidar S, Ray A. Cysteinyl leukotrienes and their receptors: molecular and functional characteristics. *Pharmacol*. 2010; 85: 336-349.

Sousa AR, Parikh A, Scadding G, Corrigan CJ, Lee TH. Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. *N Engl J Med*. 2002; 347: 1493-1499.

Steel HC, Anderson R. Dissociation of the PAF-receptor from NADPH oxidase and adenylate cyclase in human neutrophils results in accelerated influx and delayed clearance of cytosolic calcium. *Br J Pharmacol*. 2002; 136: 81–89.

Steel HC, Tintinger GR, Theron AJ, Anderson R. Itraconazole-mediated inhibition of calcium entry into platelet-activating factor-stimulated human neutrophils is due to



interference with production of leukotriene B4. *Clin Exp Immunol*. 2007; 150: 144–150.

Stelmach I, Korzeniewska A, Stelmach W, Majak P, Grzelewski T, Jerzynska J.
Effects of montelukast treatment on clinical and inflammatory variables in patients with cystic fibrosis. *Ann Allergy Asthma Immunol*. 2005; 95: 372-380.
Strickland I, Kisich K, Hauk PJ, Vottero A, Chrousos GP, Klemm DJ, Leung DY. High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. *J Exp Med*. 2001; 193: 585-593.

Su WH, Chen HI, Jen CJ. Differential movements of VE-cadherin and PECAM-1 during transmigration of polymorphonuclear leukocytes through human umbilical vein endothelium. *Blood*. 2002; 100: 3597-3603.

Sugiura H, Ichinose M. Oxidative and nitrative stress in bronchial asthma. *Antioxid Redox Signal*. 2008; 10: 785–797.

Suzuki T, Wang W, Lin JT, Shirato K, Mitsuhashi H, Inoue H. Aerosolized neutrophil elastase induces airway constriction and hyperresponsiveness with protection by intravenous pretreatment with half-length secretory leukoprotease inhibitor. *Am J*

Respir Crit Care Med. 1996; 153: 1405–1411.

Tager AM, Luster AD. BLT1 and BLT2: the leukotriene B(4) receptors.

Prostaglandins Leukot Essent Fatty Acids. 2003; 69: 123-34.

Tandon A, Sinha S. Structural insights into the binding of MMP-9 inhibitors. *Bioinformation*. 2011; 5: 310-314.

Thelen M, Didichenko SA. G-protein coupled receptor-mediated activation of PI 3kinase in neutrophils. *Ann N Y Acad Sci*. 1997; 832: 368-382.



Theron AJ, Gravett CM, Steel HC, Tintinger GR, Feldman C, Anderson R. Leukotrienes C₄ and D₄ sensitize human neutrophils for hyperreactivity to chemoattractants. *Inflamm Res*. 2009; 58: 263–268.

Theron AJ, Steel HC, Tintinger GR, Anderson R. Endogenous adenosine regulates neutrophil pro-inflammatory activities by cyclic AMP-dependent accelerated clearance of cytosolic calcium. *Inflamm Res.* 2002; 51: 594-602.

Tintinger G, Steel HC, Anderson R. Taming the neutrophil: calcium clearance and influx mechanisms as novel targets for pharmacological control. *Clin Exp Immunol*. 2005; 141: 191-200.

Tintinger G, Steel HC, Theron AJ, Anderson R. Pharmacological control of neutrophil-mediated inflammation: Strategies targeting calcium handling by activated polymorphonuclear leucocytes. *Drug Design Develop Therapy*. 2008; 2: 95-104. Tintinger GR, Anderson R, Theron AJ, Ramafi G, Ker JA. Comparison of the effects of selective and non-selective β -adrenergic agonists on the pro-inflammatory activities of human neutrophils in vitro. *Inflammation*. 2000; 24: 239–249. Tintinger GR, Theron AJ, Steel HC, Anderson R. Accelerated calcium influx and hyperactivation of neutrophils in chronic granulomatous disease. *Clin Exp Immunol.* 2001; 123: 254–263.

Tokuriki S, Ohshima Y, Yamada A, Ohta N, Tsukahara H, Mayumi M. Leukotriene D4 enhances the function of endothelin-1-primed fibroblasts*. Clin Immunol*. 2007; 125: 88-94.

Torphy TJ. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med*. 1998; 157: 351-370.



Turtay MG, Firat C, Samdanci E, Oguzturk H, Erbatur S, Colak C. Effects of montelukast on burn wound healing in a rat model. *Clin Invest Med*. 2010; 33: E413-421.

Underwood DC, Kotzer CJ, Bochnowicz S, Osborn RR, Luttman MA, Hay DW, Torphy TJ. Comparison of phosphodiesterase III, IV and dual III/IV inhibitors on bronchospasm and pulmonary eosinophils influx in guinea pigs**. J Pharmacol Exp** *Ther*. 1994; 270: 250–259.

Uozumi N, Kune K, Nagase T, et al. Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature*. 1997; 390: 618-622.

Upham JW, James AL. Remission of asthma: The next therapeutic frontier?

Pharmacol Ther. 2011; 130: 38-45.

Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, Brinkmann V, Jungblut PR, Zychlinsky A. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog*. 2009; 5(10): e1000639.

Van Rensen EL, Sont JK, Evertse CE, Willems LN, Mauad T, Hiemstra PS, Sterk PJ; AMPUL Study Group. Bronchial CD8 cell infiltrate and lung function decline in asthma. *Am J Respir Crit Care Med*. 2005; 172: 837-841.

Van Weel C, Bateman ED, Bousquet J, et al. Asthma management pocket reference 2008. *Allergy*. 2008; 63: 997–1004.

Verleden GM, Verleden SE, Vos R, De Vleeschauwer SI, Dupont LJ, Van Raemdonck DE, Vanaudenaerde BM. Montelukast for bronchiolitis obliterans syndrome after lung transplantation: a pilot study. *Transpl Int*. 2011; 24: 651-656.



Vignola AM, Kips J, Bousquet J. Tissue remodeling as a feature of persistent asthma. *J Allergy Clin Immunol*. 2000; 105: 1041-1053.

Voisin MB, Woodfin A, Nourshargh S. Monocytes and neutrophils exhibit both distinct and common mechanisms in penetrating the vascular basement membrane in vivo. *Arterioscler Thromb Vasc Biol*. 2009; 29: 1193-1199.

Walsh GM. Novel cytokine-directed therapies for asthma. *Discov Med*. 2011; 11: 283-291.

Wang L, Du C, Lv J, Wei W, Cui Y, Xie X. Antiasthmatic Drugs Targeting the Cysteinyl Leukotriene Receptor 1 Alleviate Central Nervous System Inflammatory Cell Infiltration and Pathogenesis of Experimental Autoimmune Encephalomyelitis. *J Immunol*. 2011; 187: 2336-2345.

Wang P, Wu P, Ohleth KM, Egan RW, Billah MM. Phosphodiesterase 4B2 is the predominant phosphodiesterase species and undergoes differential regulation of gene expression in human monocytes and neutrophils**.** *Mol Pharmacol.* 1999; 56: 170–174.

Wang S, Voisin MB, Larbi KY, Dangerfield J, Scheiermann C, Tran M, Maxwell PH, Sorokin L, Nourshargh S. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. *J Exp Med*. 2006; 203: 1519-1532.

Wang, J.P. Dangerfield, R.E. Young, S. Nourshargh. PECAM-1, alpha6 integrins and neutrophil elastase cooperate in mediating neutrophil transmigration, *J Cell Sci*. 2005; 118: 2067–2076.

171



Ward C, Chilvers ER, Lawson MF, Pryde JG, Fujihara S, Farrow SN, Haslett C, Rossi AG. NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro. *J Biol Chem*. 1999; 274: 4309-4318.

Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med*. 1997; 156: 737-743.

Wenzel SE, Trudeau JB, Westcott JY, Beam WR, Martin RJ. Single oral dose of prednisone decreases leukotriene B4 production by alveolar macrophages from patients with nocturnal asthma but not control subjects: relationship to changes in cellular influx and FEV1. *J Allergy Clin Immunol*. 1994; 94: 870-881.

Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet*. 2006; 368: 804-813.

Wright HL, Moots RJ, Bucknall RC, Edwards SW. Review. Neutrophil function in inflammation and inflammatory diseases. *Rheumatol* (Oxford). 2010; 49: 1618-1631.
Wu Y, Zhou C, Tao J, Li S. Montelukast prevents the decrease of interleukin-10 and inhibits NF-kappa B activation in inflammatory airway of asthmatic guinea pigs. *Can J Physiol Pharmacol*. 2006; 84: 531–537.

Xu et al., 2005 M. Xu, E. Bruno, J. Chao, S. Huang, G. Finazzi and S.M. Fruchtman et al., Constitutive mobilization of CD34+ cells into the peripheral blood in idiopathic myelofibrosis may be due to the action of a number of proteases. *Blood*. 2005; 105: 4508–4515.

Xu Y, Zhao Y, Huang H, Chen G, Wu X, Wang Y, Chang W, Zhu Z, Feng Y, Wu D. Expression and function of toll-like receptors in multiple myeloma patients: toll-like



receptor ligands promote multiple myeloma cell growth and survival via activation of nuclear factor-kappaB. *Br J Haematol*. 2010; 150: 543-553.

Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood*. 2005; 106: 584-592.

Yang Y, Luo J, Kazumura K, Takeuchi K, Inui N, Hayashi H, Ohashi K, Watanabe H. Cilostazol suppresses adhesion of human neutrophils to HUVECs stimulated by FMLP and its mechanisms. *Life Sci.* 2006; 79: 629–636.

Yokomizo T, Kato K, Hagiya H, Izumi T, Shimizu T. Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. *J Biol Chem*. 2001; 276: 12454-12459.

Yoshisue H, Kirkham-BrownJ, Healy E, Holgate ST, Sampson AP, Davies DE.

Cysteinyl leukotrienes synergize with growth factors to induce proliferation of human bronchial fibroblasts. *J Allergy Clin Immunol*. 2007; 119: 132-140.

Zhu J, Qiu YS, Figueroa DJ, Bandi V, Galczenski H, Hamada K, Guntupalli KK, Evans JF, Jeffery PK. Localization and upregulation of cysteinyl leukotriene-1 receptor in asthmatic bronchial mucosa. *Am J Respir Cell Mol Biol*. 2005; 33: 531– 540.