# Investigation of the Effects of Moxifloxacin on Human Neutrophils and Mononuclear Leucocytes *in vitro*

by

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Submitted in fulfillment for the degree of Master of Science (MSc)

in

The Department of Immunology
Faculty of Health Sciences
University of Pretoria

March 2007

## DECLARATION

I declare that the work contained in this dissertation is my original work and has not been presented for a degree in any other institution. It is being submitted in fulfilment for the MSc degree at the University of Pretoria.

Signed: _____ Date: _____

## ACKNOWLEDGEMENTS

First and foremost I want to thank the Almighty for the strength, courage and wisdom He has given me till this point of my academic life.

This dissertation is by far most significant scientific accomplishment in my life and would be impossible without the people who supported and believed in me.

My supervisor: Professor Ronald Anderson, Head, Department of Immunology, University of Pretoria, who gave me the opportunity to do this research and generously gave of his expertise and time.

My co-supervisor: Professor Annette Theron, Department of Immunology, University of Pretoria, for her invaluable guidance, encouragement and support.

Last, but not least, I want to express appreciation for the assistance received from the following with guidance and advice in respect of the laboratory work and preparation of this manuscript: Dr Helen Steel, Dr Riana Cockeran and Mrs Joyce Oommen.

Not to forget my beloved family for their unconditional love and trust they always had in me.

# SUMMARY

Moxifloxacin is considered to be a broad-spectrum fluoroquinolone due to its activity against both gram positive and gram negative bacteria. Importantly this agent is currently being evaluated in ongoing clinical trials in South Africa and South America as a treatment for pulmonary tuberculosis, with the specific objective of decreasing the duration of chemotherapy. However, relatively little is known about the effects of moxifloxacin on host defenses, particularly innate protective mechanisms, involving neutrophils.

The primary theme of the laboratory research presented in this dissertation was to investigate the role of moxifloxacin in modulating the host immune system, specifically neutrophil protective functions, as well as lymphocyte proliferation and cytokine production (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL13, IL-17, IFN-γ, GM-CSF, G-CSF, TNF-α, and MCP-1).

The generation of reactive oxidants and elastase release by neutrophils activated with the chemoattractant, fMLP, or the phorbol ester, PMA, were assayed using luminol- and lucigenin-enhanced chemiluminescence (LECL) and colorimetric procedures, while alterations in cytosolic $Ca^{2+}$ concentrations were monitored by radiometric ($^{45}Ca^{2+}$) procedures. Moxifloxacin (1-20 μg/ml) was found to have no significant priming or inhibitory effects on oxidant generation by human neutrophils activated with fMLP or PMA, while elastase release was increased at the highest concentrations of the antibiotic. The magnitude of efflux or store-operated $Ca^{2+}$ influx was unaffected following activation of neutrophils with fMLP.

Moxifloxacin at all concentrations tested, did not affect either lymphocyte proliferation or CD25 expression by PHA-activated mononuclear leukocytes (MNLs). Similarly, none of the cytokines measured were significantly affected by moxifloxacin, either in the absence or presence of PHA, compatible with a lack of effect of this agent on Th1 and Th2 lymphocytes.

In conclusion, this study suggests that moxifloxacin, at therapeutic doses, does not affect the protective functions of human neutrophils and lymphocytes.

# SAMEVATTING

Moksifloksasin word beskou as ‘n breë spektrum fluoroquinoloon met aktiwiteit teen beide gram positiewe en gram negatiewe bakterieë. Dit is noemenswaardig dat hierdie agent tans in kliniese proewe in Suid Afrika en Suid Amerika getoets word as behandeling vir pulmonêre tuberkulose, met die spesifieke doel om die duur van chemoterapie te verminder. Daar is egter relatief min bekend oor die uitwerking van moksifloksasin op gasheerverdediging, veral intrinsieke beskermende meganismes soos neutrofiele.

Die hooftema van die laboratorium navorsing wat in hierdie verhandeling aangebied word, is om die rol van moksifloksasin in die modulering van die gasheer immuunsisteem te ondersoek veral met betrekking tot neutrofiel beskermende funksies, sowel as limfosiet proliferasie en sitokien produksie (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, IFN-γ, GM-CSF, G-CSF,TNF-α and MCP-1).

Die produksie van reaktiewe oksidante en vrystelling van elastase deur neutrofiele, geaktiveer deur die leukolokmiddel, fMLP, of die forbol ester, PMA, is getoets deur gebruik te maak van luminol- en lusigenin-verhoogde chemiluminessensie en kolorimetriese prosedures. Veranderinge in sitosoliese $Ca^{2+}$ konsentrasies is gemeet met behulp van radiometriese ($^{45}Ca^{2+}$) prosedures. Moksifloksasin (1-20μg/ml) het nie ‘n betekenisvolle sensitiserende of inhiberende uitwerking op oksidant generasie van mens neutrofiele geaktiveer met fMLP of PMA gehad nie terwyl elastase vrystelling verhoog is by die hoogste konsentrasies van die antibiotika. Moksifloksasin het ook nie die effluks of stoor-operatiewe $Ca^{2+}$ influks in neutrofiele geaktiveer met fMLP, geaffekteer nie.

Moksifloksasin het by alle konsentrasies getoets, nie limfosiet proliferasie of CD25 uitdrukking deur PHA-geaktiveerde mononukleêre leukosiete,geaffekteer nie. Eweneens is geen van die sitokiene gemeet, betekenisvol geaffekteer deur moksifloksasin in die afwesigheid of teenwoordigheid van PHA nie. Hierdie resultaat toon dat die antibiotika nie ‘n effek op Th1 en Th2 limfosiete het nie.

Ten slotte, dui die studie aan dat mokifloksasin by terapeutiese dosisse geen uitwerking op die produktiewe funksies van mens neutrofiele en T-limfosiete het nie.

# TABLE OF CONTENTS


| | Page |
|---|---|
| **Acknowledgements** | **ii** |
| **Summary** | **iii** |
| **Table of contents** | **vii** |
| **List of figures** | **xi** |
| **List of tables** | **xiii** |
| **List of abbreviations** | **xiv** |
| **CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW** | |
| 1.1 INTRODUCTION | 1 |
| 1.2 LITERATURE REVIEW | 3 |
| 1.2.1 History of Quinolones | 3 |
| 1.2.2 Moxifloxacin structure and functions | 4 |
| 1.3 Phamacology | 5 |
| 1.3.1 Metabolism | 5 |
| 1.3.2 Mechanism of action | 6 |
| 1.3.3 Resistance | 7 |
| 1.4 Inflammation | 9 |
| 1.4.1 Leukocyte entry into sites of inflammation | 9 |
| 1.5 Neutrophils | 12 |
| 1.5.1 Cytoplasmic granules | 13 |
| 1.5.1.1 Primary granules | 13 |
| 1.5.1.2 Secondary granules | 14 |
| 1.5.1.3 Tertiary and secretory granules | 14 |
| 1.5.2 Neutrophil elastase | 15 |

1.6 Antimicrobial mechanisms of neutrophils 16
1.6.1 NADPH oxidase 17
1.6.2 NADPH oxidase activation 17

1.7 Respiratory bust of human neutrophils 19
1.8 Nitric oxide synthase 21

1.9 Calcium and neutrophil activation 21
1.9.1 Calcium fluxes and restoration of Ca2+ homeostasis in Activated neutrophils 21
1.9.2 Ca2+ release from stores 22
1.9.3 Restoration of Ca2+ homeostasis 23

1.10 Interaction of pore forming pneumolysin with neutrophils 25

1.11 Lymphocyte development and heterogeneity 25

1.12 CD4 and CD8 T-cell functions 27

1.13 Helper T-lymphocytes 27

1.14 Antigen processing and presentation to T-cells 28

1.15 Antigen recognition by T-cells 31

1.16 T-cell activation 32

1.17 T lymphocyte proliferation 33

1.18 IL-2 receptor structure and composition 36
1.19 Properties and functions of cytokines 37

1.19.1 Anti-inflammatory cytokines 38

1.20 Chemokines 40

**CHAPTER 2: HYPOTHESIS AND OBJECTIVES**

2.1 Hypothesis 42

2.2 Objectives 42

**CHAPTER 3: EFFECTS OF MOXIFLOXACIN ON HUMAN NEUTROPHIL FUNCTIONS**

3.1 INTRODUCTION 44

3.2 MATERIALS AND METHODS 44
- 3.2.1 Reagents 44
- 3.2.2 Neutrophil isolation 44
- 3.2.3 Oxidant generation 45
- 3.2.4 Elastase release 45
- 3.2.5 Radiometric assessment of Ca2+ fluxes 46
  - 3.2.5.1 Influx of 45 Ca2+ into moxifloxacin-treated neutrophils 46
  - 3.2.5.2 Efflux of 45 Ca2+ from moxifloxacin-treated neutrophils 47
- 3.2.6 Cellular ATP levels 47
- 3.2.7 Statistical analysis 48

3.3 RESULTS 48
- 3.3.1 Effects of moxifloxacin on luminol- and lucigenin-enhanced chemiluminescence responses of fMLP- and PMA-activated neutrophils 48
- 3.3.2 Effects of moxifloxacin on elastase release by fMLP/CB-activated neutropfils 49

3.3.3 Effects of moxifloxacin on ATP levels as an index of viability 49
3.3.4 Effects of moxifloxacin on Ca2+ fluxes 49
3.3.4.1 Effects on influx of 45 Ca2+ 49
3.3.4.2 Effects on efflux of 45 Ca2+ 50

3.4 DISCUSSION 56

**CHAPTER 4: EFFECTS OF MOXIFLOXACIN ON PHA-ACTIVATED HUMAN MONONUCLEAR LEUKOCYTES**

4.1 INTRODUCTION 60

4.2 MATERIALS AND METHODS 60
4.2.1 Reagents 60
4.2.2 Isolation of Mononuclear leukocytes 60
4.2.3 Lymphocyte proliferation assay 61
4.2.4 CD25 expression by PHA-activated T lymphocytes 61
4.2.5 Cytokine production by PHA-activated mononuclear leukocytes 62
4.2.6 Statistical analysis 63

4.3 RESULTS 63
4.3.1 Effects of moxifloxacin on lymphocyte proliferation and expression of CD25 63
4.3.2 Effects of moxifloxacin on cytokine production by PHA-activated mononuclear leukocytes 64

4.4 DISCUSSION 72

**CHAPTER 5: GENERAL CONCLUSION** 75

**CHAPTER 6: REFERENCES** 76

# LIST OF FIGURES


| | | Page |
|---|---|---|
| **Figure 1.1:** | Chemical structure of moxifloxacin | 5 |
| **Figure 1.2:** | Fluoroquinolone mechanism of action | 8 |
| **Figure 1.3:** | Leukocyte recruitment to the site of inflammation | 11 |
| **Figure 1.4:** | Activation of NADPH oxidase | 19 |
| **Figure 1.5:** | Release of $Ca^{2+}$ from intracellular stores and clearance of cytosolic $Ca^{2+}$ in fMLP- activated human neutrophils | 24 |
| **Figure 1.6:** | Antigen processing and presentation to T lymphocytes | 31 |
| **Figure 1.7:** | T-cell proliferation | 35 |
| **Figure 3.1:** | Effects of moxifloxacin on the release of elastase by fMLP/CB-activated neutrophils | 55 |
| **Figure 4.1:** | Effects of moxifloxacin on PHA-activated lymphocyte proliferation | 65 |
| **Figure 4.2:** | Effects of moxifloxacin on CD25 expression by PHA-activated mononuclear leukocytes | 66 |
| **Figure 4.3:** | Effects of moxifloxacin on IL-6 production by PHA-activated mononuclear leukocytes | 68 |

**Figure 4.4:** Effects of moxifloxacin on IL-12 production by PHA-activated mononuclear leukocytes 69

# LIST OF TABLES


| | | Page |
|---|---|---|
| **Table 1.1:** | Major human cytokines | 39 |
| **Table 1.2:** | Common chemokines | 41 |
| **Table 3.1a:** | Effects of moxifloxacin on lucigenin-enhanced chemiluminescence superoxide production by fMLP-activated neutrophils | 51 |
| **Table 3.1b:** | Effects of moxifloxacin on lucigenin-enhanced chemiluminescence superoxide production by PMA-activated neutrophils | 52 |
| **Table 3.2a:** | Effects of moxifloxacin on luminol-enhanced chemiluminescence oxidant production by fMLP-activated neutrophils | 53 |
| **Table 3.2b:** | Effects of moxifloxacin on luminol-enhanced chemiluminescence oxidant production by PMA-activated neutrophils | 54 |
| **Table 4.1:** | Effects of moxifloxacin on IL-6 and IL-12 production by PHA-activated mononuclear cells | 67 |
| **Table 4.2:** | Effects of moxifloxacin on cytokine production by PHA-activated mononuclear cells | 70 |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| ANOVA | Analysis of variance |
| APCs | Antigen presenting cells |
| ATP | Adenosine 3', 5-triphosphate |
| $Ca^{2+}$ | Calcium ion |
| $[Ca^{2+}]i$ | Concentration of intracellular calcium |
| $^{45}Ca^{2+}$ | Calcium-45 chloride |
| $Ca^{2+}$-ATPase | Calcium-adenosine 3', 5'-triphosphatase |
| $CaCl_2$ | Calcium chloride |
| CB | Cytochalasin B |
| CD | Cluster of differentiation |
| CG | Cathepsin |
| CGD | Chronic granulomatous disease |
| $Cl^-$ | Chloride ion |
| CSF | Colony stimulating factor |
| CTL | Cytotoxic T lymphocyte |
| DMSO | Dimethyl sulphoxide |
| EGTA | Ethylene glycol-bis (beta-amino-ethyl-ether)-N, N, N', N'-tetraacetic acid |
| ER | Endoplasmic reticulum |
| FCS | Fetal calf serum |
| $Fe^{2+}$ | Ferrous ion |
| $Fe^{3+}$ | Ferric ion |
| FITC | Fluorescein isothiocyanate |
| FMLP | N-formyl-L-methionyl-L-leucyl-L-phenylalanine |
| GM-CSF | Granulocyte/macrophage colony stimulating factor |
| GTP | Guanosine triphosphate |
| $H^+$ | Proton |
| $^3H$ | Thymidine (tritiated) |
| HBSS | Hanks' balanced salt solution |
| HLA | Human histocompatibility leukocyte antigen |

| | |
|---|---|
| $H_2O_2$ | Hydrogen peroxide |
| HOCL | Hypochlorous acid |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| iNOS | Induced nitric oxide synthase |
| $IP_3$ | Inositol1, 4,5-triphosphate |
| $IP_3$-ICR | $IP_3$-induced $Ca^{2+}$ release |
| $iPLA_2$ | $Ca^{2+}$-intended phospholipase $A_2$ |
| $IP_3ROC$ | $IP_3$ receptor-operated channel |
| KDa | kiloDalton |
| Licigenin | bis-N-methylacridinium nitrate |
| Luminol | 5-amino-2,5-dihydro-1,4-phthalazinedione |
| LECL | Lucigenin-enhanced chemiluminescence |
| LPA | Lymphocyte proliferation assay |
| LPS | Lipopolysaccharide |
| mAb | Monoclonal antibody |
| MCP-1 | Monocyte chemotactic protein-1 |
| MHC | Major histocompatibility complex |
| MPO | Myeloperoxidase |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate (oxidized form) |
| NaOH | Sodium hydroxide |
| NE | Neutrophil elastase |
| NF-$_k$B | Nuclear transcription factor-kappa B |
| $NH_4Cl$ | Ammonium chloride |
| NO | Nitric oxide |
| NRS | Nucleotide releasing substrate |
| $O_2$ | Oxygen |
| $O_2^-$ | Superoxide anion |
| $^1O_2$ | Singlet oxygen |
| .OH/$HO^-$ | Hydroxyl radical |
| p22 $^{phox}$ | Protein/polypeptide phagocyte oxidase, 22kDa molecular weight |

| | |
|---|---|
| PBS | Phosphate-buffer saline |
| PHA | Phytohaemagglutinin |
| PMA | Phorbol-12-myristate 13-acetate |
| PMNL | Polymorphonuclear leukocyte |
| Rho-GDI | Guanosine nucleotide dissociation inhibitor |
| RIA | Radioimmunoassay |
| ROCC | Receptor-operated $Ca^{2+}$ channel |
| ROI | Reactive oxygen intermediate |
| ROS | Reactive oxygen species |
| SEM | Standard error of the mean |
| SER | Sarco-endoplasmic reticulum |
| SERCA | Sarco-endoplasmic reticulum $Ca^{2+}$-ATPase |
| SNF | Supernatant fluid |
| SOC | Store operated channel |
| SOCC | Store-operated calcium channels |
| SOCE | Store-operated $Ca^{2+}$ entry |
| SOD | Superoxide dismutase |
| TCR | T-cell receptor |
| Th | T helper cell |
| TNF-α | Tumor necrosis factor alpha |
| TRPC | Transient receptor potential channel |
| VGCC | Voltage-gated $Ca^{2+}$ channels CIF - $Ca^{2+}$ influx factor |

# *Chapter 1: Introduction and Literature Review*

## 1.1 INTRODUCTION

Moxifloxacin is an antibiotic widely used as a bacteriostatic or bactericidal drug for the therapy of bacterial infections. Notwithstanding the direct interactions between antibiotics and bacteria, many antibiotics also interact, albeit indirectly, with the immune system. The immunomodulatory effects of antibiotics include alteration of phagocytosis, chemotaxis, endotoxin release, cytokine production, and hematopoietic recovery after immunosuppression. Moreover, some antibiotics can affect the life-span of immune and inflammatory cells through the induction or inhibition of apoptosis (Choi *et al.*, 2003). Such properties may have clinical significance for the modulation of the immune response of patients, especially those who are immunodeficient, and those with microbial infections that have harmful inflammatory effects (e.g. septic shock). Thus, antibiotics may play a dual role in infections, by having both direct antimicrobial effects, as well as indirect effects, which can be either beneficial or detrimental to the host response (Araujo *et al.,* 2002).

The increasing evidence for the involvement of immune system-derived cytokines and reactive oxygen species (ROS) in the severity of, or even death due to, infection indicates the importance of defining the immunomodulatory activity of antibiotics. It has become apparent that in the infected host, excessive or unrestricted activity of the immune response can be detrimental. Potentially harmful effects of T-cells and other leukocytes can be prevented by down-regulation of inflammatory responses. Lack of such control can result in tissue injury and death of the host. Among the various classes of antibiotics, fluoroquinolones exert immunomodulatory effects. These agents are widely used in clinical practice, and newer fluoroquinolones with enhanced potencies against microbial pathogens are continuously being developed (Ono *et al.,* 2000). Although moxifloxacin is considered to be a broad-spectrum fluoroquinolone due to its enhanced activity against both gram-positive and gram-negative bacteria (Weiss *et al,* 2004), relatively little is known about the effect of moxifloxacin on host defences, particularly innate protective mechanisms involving neutrophils.

This study has been designed to investigate the *in vitro* immunomodulatory effects of moxifloxacin, a synthetic methoxyfluoroquinolone with a broader antibacterial

spectrum than first, second and third generation fluoroquinolones, on the functions of human neutrophils and mononuclear leukocytes (MNL).

## 1.2 LITERATURE REVIEW

### 1.2.1 History of quinolones

Quinolones are entirely synthetic antibacterial drugs, with the first representative (nalidixic acid) having been synthesized in 1962. Their structures are based on the 4-oxo-1,4-dihydroquinolone skeleton (Levine *et al.*, 1998; Labro, 2000). They have evolved from agents used solely for the treatment of urinary tract infections to molecules with potent activity against a wide spectrum of significant bacterial pathogens, with resultant broad clinical utility. This evolutionary pattern has arisen through the development of new core and side-chain structures, with associated improvements in activity, pharmacokinetics and tolerability, and through the selection of molecules that remain useful and well tolerated (Ball, 2000).

All quinolones with antibacterial activity have a 4-quinolone nucleus with a nitrogen atom at position 1, a carboxyl group at position 3, and a ketone at position 4. The structure of the quinolones has developed along two parallel pathways: the naphthyridones (with the original naphthyridine core of nalidixic acid) and the fluoroquinolones, in which a carbon atom is substituted for nitrogen at position 8 of the naphthyridine nucleus. As mentioned above, the first member of the quinolone class of antibiotics was nalidixic acid, which has limited antibacterial activity against gram-positive bacteria (Owens *et al.,* 2000). Since then, thousands of compounds have been synthesized, of which the 6-fluorinated molecules (fluoroquinolones) represent a breakthrough in 4-quinolone research (Labro, 2000).

Recently, interest in the quinolone antibiotics has intensified following the publication of clinical and pre-clinical data confirming their potential for use in treatment of tuberculosis (TB) (Duncan and Barry, 2004). Data from quinolone studies suggest that these drugs may be used to shorten the duration of chemotherapy. In the development of fluoroquinolone containing “third-line” regimens, moxifloxacin was found to be superior to ofloxacin or levofloxacin, with sterilization being achieved in nine months (Veziris *et al.,* 2003; Duncan and Barry, 2004).

All fluoroquinolones have a fluorine substitution at the 6-position, which confers greater antibacterial potency and a broader spectrum of activity (Owens *et al.,* 2000;

Shalit *et al.,* 2002) than that of nalidixic acid and other nonfluorinated quinolones (e.g. cinoxacin, oxilinic acid). Addition of the fluorine and piperazine moiety at positions 6 and 7 respectively, substitution of carbon for nitrogen at position 8, and modification of the side chain at position 1 yielded the second generation agents ciprofloxacin and ofloxacin.

Other modifications to the fluoroquinolone structure yielded third generation agents (e.g. levofloxacin, sparfloxacin, grepafloxacin) with an improved antibacterial spectrum of activity, greater potency and an extended half-life. Recently a fourth generation of quinolones (e.g. gatifloxacin, moxifloxacin, trovafloxacin) with expanded coverage against anaerobes has been developed (Ball, 2000; Owens *et al.,* 2000). The methoxy group at position 8 of moxifloxacin and gatifloxavin theoretically may confer enhanced activity against resistant gram-positive bacteria and reduce development of resistance (Owens *et al.,* 2000).

### 1.2.2 Moxifloxacin structure and function

Moxifloxacin (1-cyclopropyl-7-(2, 8-diazabicyclo [4.3.0] nonane)-6-fluoro-8-methoxy-1,4-di-hydro-4-oxo-3-quiline carboxylic acid hydrochloride), is an 8-methoxy-1,quinolone (figure 1)and is considered a broad spectrum fluoroquinolone due to its enhanced activity against gram-positive and aerobic bacteria (Dalhoff *et al.,* 1998; Araujo *et al.,* 2002; Weiss *et al.,* 2004).

Moxifloxacin consists of a bicyclic aromatic core with a fluorine atom at the C-6 position, a methoxy group at position 8, an N-1 cyclopropyl group and an azobicycline group at the C-7-position. The C-8 methoxy group is thought to contribute to enhanced activity against gram-positive organisms, decreased development of resistance, (Kishii *et al.,* 2003), and to limit the potential for phototoxicity (Caeiro and Lannini, 2003). The bulky diazobicycline group at C-7 also contributes to its spectrum of activity and makes moxifloxacin a poor substrate for the active bacterial efflux pump (Owens *et al.,* 2000; Caeiro and Lannini, 2003). It has also been suggested that moxifloxacin has both inhibitory and stimulatory effects on the immune system, primarily affecting the production of several cytokines by both human and murine leukocytes (Weiss *et al.,* 2004).

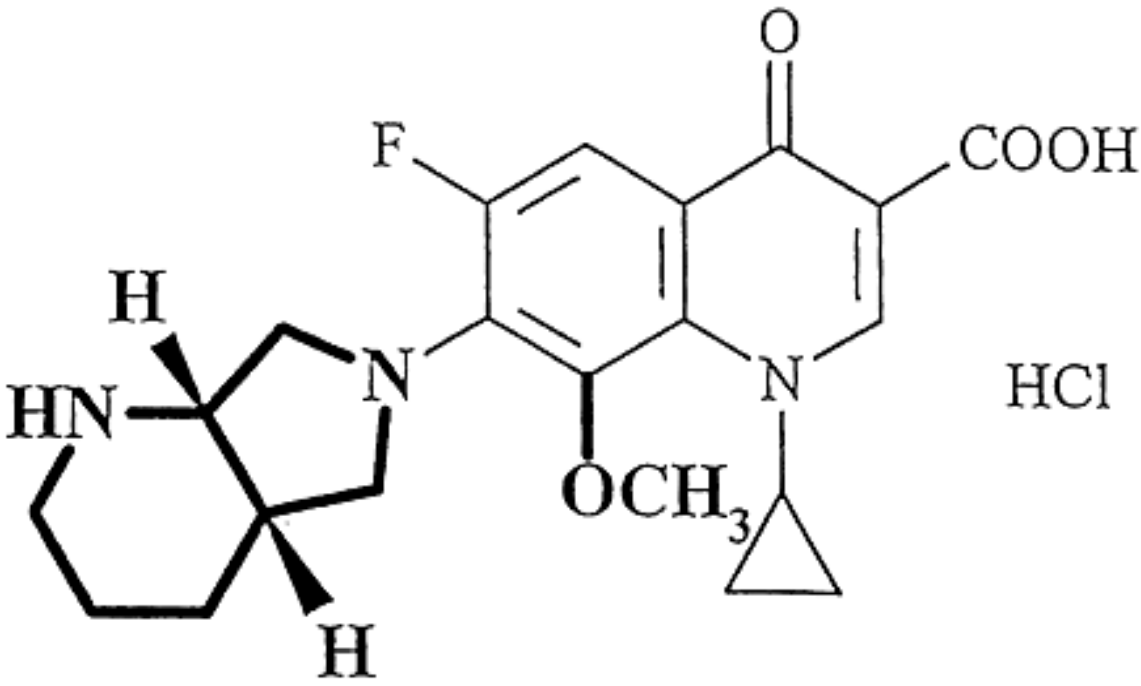



**Fig. 1:** Chemical structure of moxifloxacin (Source: Stass, *et al.*, 1999).

## 1.3 PHARMACOLOGY

Moxifloxacin is a broad-spectrum fluoroquinolone antibiotic. It is approved for the treatment of acute bacterial exacerbations of chronic bronchitis, acute bacterial sinusitis, and mild-to-moderate community-acquired pneumonia. It is usually administered by the oral route and is well absorbed from the gastrointestinal tract, although intravenous administration is also an option. Its absolute bioavailability is 90%. Approximately 50% of moxifloxacin is bound to serum proteins, independent of drug concentration. The volume of distribution of moxifloxacin ranges from 1.7 to 2.7 L/kg. It is widely distributed throughout the body, with tissue concentrations often exceeding plasma concentrations (Ball, 2000).

### 1.3.1 Metabolism

Approximately 52% of an oral or intravenous dose of moxifloxacin is metabolized via glucuronide and sulfate conjugation. Cytochrome P450 is not involved in moxifloxacin

metabolism and its activity is not affected by moxifloxacin (Caeiro and Lannini, 2003). The sulfates conjugate (M1) accounts for approximately 38% of the dose, and are eliminated primarily in the feces. Approximately 14% of an oral or intravenous dose is converted to a glucuronide conjugate (M2), which is excreted exclusively in urine. Peak plasma concentrations of M2 are approximately 40% of that of the parent drug, while plasma concentrations of M1 are generally less than 10% of that of moxifloxacin (Owens *et al.,* 2000).

### 1.3.2 Mechanism of action

Although the mechanism of action of the quinolones, including moxifloxacin, is not fully known, it differs from that of aminoglycosides, β-lactam antibiotics, macrolides and tetracyclines; therefore, microorganisms resistant to these classes of drugs may be susceptible to moxifloxacin and other quinolones (Owens *et al.,* 2000). Bacterial chromosomes require topoisomerase enzymes to maintain the function and configuration (topology) of the intricate DNA molecule (Owens *et al.,* 2000; Caeiro and Lannini, 2003). Type II topoisomerases (topoisomerase IV and DNA gyrase) characteristically alter DNA topology by introducing a transient double strand break in DNA, passing another duplex segment of DNA through the break (Stroman *et al.,* 2005), and relegating the broken ends. In general, type II enzymes are dyadic molecules that, in an ATP-dependent manner, catalyze the relaxation of supercoiled DNA, catenation and decatenation of DNA-rings, and knotting and unknotting of duplex DNA. These enzymes are found in all organisms comprising a family, which is structurally and evolutionarily conserved (Levine *et al.,* 1998).

Similar to other fluoroquinolone agents, moxifloxacin exhibits antimicrobial activity against susceptible bacteria through inhibition of the DNA gyrase (topoisomerase II) activity (Galley *et al.,* 2000; Bearden and Danziger, 2001), an essential bacterial enzyme required for DNA replication, transcription, repair and recombination (Pestova *et al.,* 2000). The drug is bactericidal during the stationary growth phase, as well as the logarithmic growth phase of certain bacteria. Moxifloxacin also inhibits topoisomerase IV, an enzyme structurally similar to DNA gyrase and essential for bacterial DNA replication. Topoisomerase IV may be the primary target of many quinolones in gram-positive bacteria (DNA gyrase appears to be the main target in gram-negative bacteria) (Owens *et al*., 2000; Stroman *et al.,* 2005). The enzymes are tetramers consisting of two

subunits, *GyrA* and *GyrB* in DNA gyrase, and *ParC* and *ParE* in topoisomerase IV (Caeiro and Lannini, 2003)

Levine and colleagues (1998) suggested that quinolones block DNA replication, not by depriving the cell of gyrase, but by converting gyrase to a poison of DNA replication (illustrated in figure 2). Topoisomerase poisons act by affecting the cleavage-relegation equilibrium (Marians and Hiasa, 1997; Caeiro and Lannini, 2003), effectively trapping the enzyme in a drug-DNA-enzyme ternary complex during the topoisomerization reaction in which the DNA gate is open. Ultimate denaturation of the enzyme therefore results in the generation of double strand breaks in the DNA (Levine *et al.,* 1998). Fluoroquinolone cytotoxicity is correlated with the appearance of double strand breaks (Drlica, 1999). These bactericidal drugs, which inhibit DNA gyrase, are highly active against *Mycobacterium tuberculosis*, including strains resistant to first line drugs (Duncan, 2003; Gosling *et al.,* 2003; Yoshimatsu *et al.,* 2002). Moxifloxacin has been shown to inhibit the growth of the main species of mycobacterium infecting humans (Ji *et al.,* 1998; Miyazaki *et al.,* 1999).

Although the formation of a drug-quinolone-topoisomerase ternary complex is critical for antimicrobial activity, these complexes are completely reversible, and the broken DNA strands can be relegated (Caeiro and Lannini, 2003). It has been proposed that the lesion must be fixed in some manner which will result in disruption of the complex, in order to generate the ultimate cytotoxic agent, the double strand break (Levine *et al.,* 1998; Drlica, 1999). A general scheme for intracellular quinolone action is sketched in Figure 1.2, page 10.

### 1.3.3 Resistance

Although quinolones are well tolerated and relatively safe, certain adverse effects are common to all agents in this antibiotic class. Quinolone resistance develops through four main mechanisms: 1) mutations in the target enzymes, which reduce the affinities of the fluoroquinolones for DNA gyrase or topoisomerase IV enzymes; 2) production of gyrase protection protein; 3) alterations in bacterial cell permeability; 4) drug efflux, preventing lethal levels of fluoroquinolones via decreased accumulation in the cytoplasm. The action of the efflux pump is dependent on the ability of the

fluoroquinolone to bind to the bacterial efflux protein, which expels it from the cell (Hooper, 1999).

Some fluoroquinolones, particularly moxifloxacin, are less affected by bacterial efflux mechanisms due to their bulky side chain moiety at position 7, which hinders export out of the cell (Caeiro and Lannini, 2003; Stroman *et al.,* 2005). Fluoroquinolone resistance usually develops in a step-wise fashion with initial mutation of the *ParC* subunit followed by mutation of the *GyrA* subunit (Wang, 1996; Fournier and Hooper, 1998; Caeiro and Lannini, 2003). Activity against single-step mutants may be an important characteristic of moxifloxacin, with the potential to limit higher levels of resistance development.

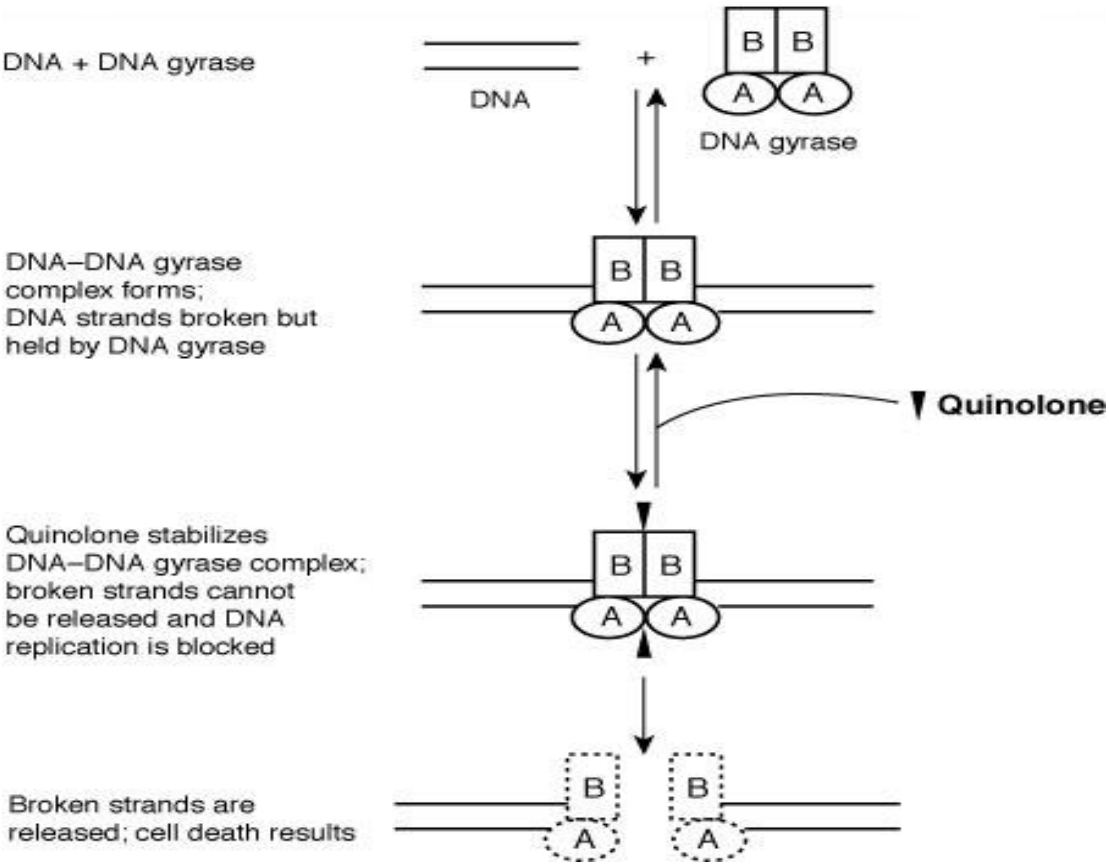



**Fig. 1.2:** Fluoroquinolones inhibit DNA synthesis by attaching to a complex of DNA gyrase or topoisomerase IV, blocking the attachment site to prevent replication, causing cell death (Adapted from: Drlica, 1999).

## 1.4 INFLAMMATION

Inflammation is a basic pathological mechanism that underlies a variety of diseases. The inflammatory reaction involves the complex interaction between inflammatory cells (neutrophils, lymphocytes, and monocytes/macrophages) and vascular cells (endothelial cells and smooth muscle cells). Multiple cytokines and growth factors are present at the site of inflammation, and each of these can potentially influence the nature of the inflammatory response. Endothelial cells and smooth muscle cells must integrate the signals generated by these multiple factors to effectively regulate the immunoinflammatory response through expression of adhesion molecules, cytokines, chemokines, matrix metalloproteinases, and growth factors (Tedgui and Mallat, 2001).

### 1.4.1 Leukocyte entry into sites of inflammation

During an infectious process, the recruitment and migration of leukocytes towards and inside a target tissue is crucial for resolving the infection and for re-establishment of homeostasis (Anderson, 1995; Cassatella, 1999). Both recruitment and migration are multi-step processes that depend on the nature and state of activation of the leukocyte generation of pro-inflammatory cytokines, expression of adhesion molecules, and extracellular matrix (ECM) components, and co-ordination of these events by a mosaic of chemoattractant molecules named chemokines (Shimizu and Shaw, 1991; Nathan and Sporn, 1991; Del Pozo *et al.,* 1995; Gilat *et al.,* 1996; Sallusto *et al.,* 2000; Gerard and Rollins, 2001).

At least four steps, with multiple signalling events at each step, control leukocyte emigration and contribute to its selectivity (figure 3). The attachment or tethering of circulating leukocytes to the vessel wall through labile adhesion permits leukocytes to roll in the direction of flow (step 1) and brings them into proximity with activating signals displayed on the endothelium. These signals (step 2) activate a second class of adhesion receptors, integrins, which firmly bind to immunoglobulin superfamily members inducibly expressed on the inflamed blood vessel (step 3). This results in an arrest of the rolling leukocyte. The arrested leukocyte is then sensitized by chemoattractant gradients, which originate in the nearby extravascular tissue, resulting in activation of integrins which is required for crossing the endothelial lining of the blood vessel and migration into tissue (step 4).

Because most integrins are unable to tether a circulating cell in shear flow, the initial tethering steps are obligatory for the recruitment of leukocytes at sites of extravasation. Binding of endothelial selectins (P-or E-selectin) or of the leukocyte selectin, L-selectin, a three member family of lectin adhesion molecules, to carbohydrate ligands expressed on leukocyte subsets or on specific endothelial surfaces, respectively, in shear flow, are the fastest cell-cell recognition events known in nature. Recently a unique leukocyte integrin member of the beta-1 integrin family, VLA-4, has been identified which is capable of supporting both tethering rolling and arrest on its endothelial ligand, VCAM-1, but is unable to interact in shear flow with its second ligand, the extracellular matrix protein, fibronectin. The ability of an integrin to participate in both labile rolling and firm adhesion depends on its state of activation, which is regulated by multiple cellular factors (Alon *et al.,* 2003; Steeber *et al.,* 1999).

Leukocyte interactions with vascular endothelium during inflammation depend on cascades of adhesion molecule engagement (Zen and Parkos, 2003), particularly during selectin-mediated leukocyte rolling. Leukocyte rolling is also facilitated by members of the integrin and immunoglobulin (Ig) super families. Specifically, leukocyte rolling velocities during inflammation are significantly increased in ICAM-1-deficient mice, with ICAM-1 expression required for optimal P- and L-selectin-mediated rolling. (Steeber *et al.,* 1999; Witko-Sarsat *et al.,* 2000).

In many cases, the loss of both L-selectin and ICAM-1 expression dramatically reduced leukocyte migration into sites of inflammation beyond that which was observed with loss of either receptor alone. In fact, the loss of both L-selectin and ICAM-1 effectively eliminated multiple chronic inflammatory responses in L-selectin/ICAM-$1^{-/-}$ mice. In contrast, the combined loss of L-selectin and ICAM-1 expression had minimal effects on the generation of Ag-specific T cell responses or humoral immunity. Thus, members of the selectin and Ig families function synergistically to mediate optimal leukocyte rolling and entry into tissues, which is essential for the generation of effective inflammatory responses *in vivo* (Steeber *et al.,* 1999).

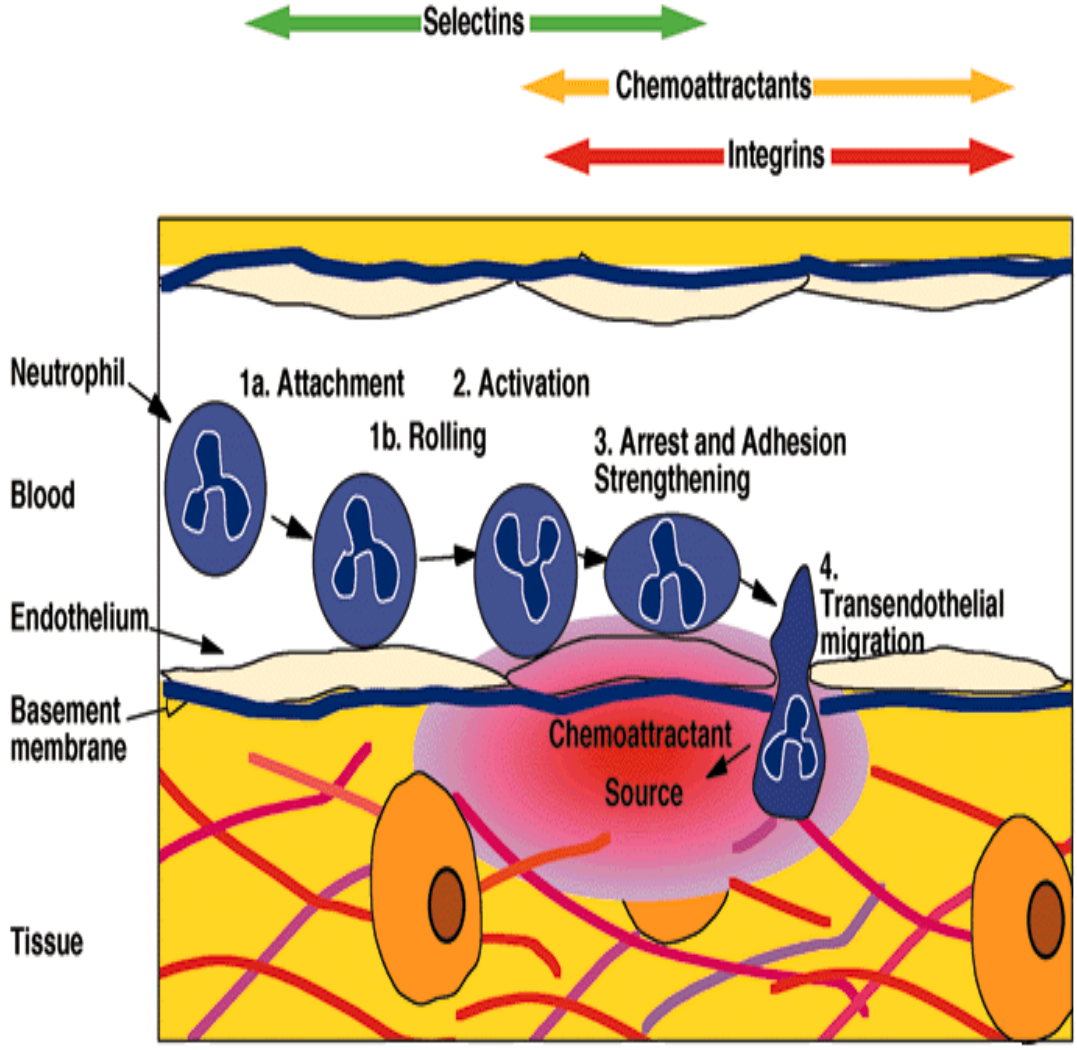



**Fig. 3:** The four-step model for leukocyte recruitment at sites of inflammation. Selectins, chemoattractants (or other activating signals) and integrins act sequentially with some overlap. Selectins mediate both leukocyte tethering and rolling (steps 1a and 1b). Alpha-4 integrins, can support the early steps of rolling, but still require further activation for their participation in firm leukocyte adhesion to endothelium together with other integrins (Source: Alon *et al.*, 2003).

## 1.5 NEUTROPHILS

Polymorphonuclear leukocytes (PMNLs) constitute the largest population of circulating leukocytes and the first line of cellular defense of mammalian organisms against invading microbes. They are not a homogeneous cell population since subpopulations exist in various stages from dormant to primed, to fully activated (Labro *et al*., 1993). Neutrophils evolve from pluripotent stem cells under the influence of cytokines and colony stimulating factors. Approximately 8 – 14 days are required for a cell to move through the sequence of 4 – 6 cell divisions and complete maturation. During this time the maturing cells can be released from the bone marrow into the blood under conditions of sufficient stress (Bainton, 1999).

Specific signals, including IL-1, IL-3, TNF-α, G-CSF, complement factors C3e, C5a and chemokines mobilize neutrophils from the bone marrow, which circulate in the unstimulated state. Under normal conditions, 90% of the neutrophil pool is in the bone marrow, 2 – 3% in circulation and the rest in the tissue. Up-regulation of the production of these signals during inflammatory stress increases the production and release of neutrophils from the bone marrow (Cooper, 1999).

The myeloblast is the first recognizable precursor cell and is followed by the promyelocyte, which is characterized by the appearance of lysosomal granules, known as primary or azurophil granules. The promyelocyte divides and differentiates into the myelocyte, which in addition to the azurophil, also contains secondary or specific granules. Following this stage, further division occurs and during the final stages of maturation, the cell passes through the metamyelocyte and the band phases. On maturation of the band form, the nucleus becomes lobulated, consisting of up to four segments.

The life-span of neutrophils is estimated to be between 24 – 48 hours, after which they undergo apoptosis (programmed cell death) and removal by the mononuclear phagocyte system, a process which is dependent on the interactions between phosphatidylserine translocated from the inner to the outer plasma membrane of the apoptotic neutrophil and the phosphatidylserine receptors on monocyte/macrophages. Prolongation of their life span occurs following migration of these cells out of circulation to sites of

inflammation and exposure to anti-apoptotic cytokines such as granulocyte/macrophage colony stimulating factor (GM-CSF) (Watson *et al.,* 1999).

### 1.5.1 Cytoplasmic granules

The most notable structural features of neutrophils are the abundant, heterogeneous cytoplasmic granules and the highly dynamic plasma membrane; these make this cell ideally suited to the performance of its primary function, which include, adherence to locally activated vascular endothelium, extravasation, migration to the site of infection and engulfment and intracellular destruction of invasive microbial pathogens (Anderson, 1995; Mayer-Scholl *et al.,* 2004).

Neutrophil granules consist of four different groups distinguished on the basis of protein content, size and density. These are primary (azurophil), secondary (specific) and tertiary (gelatinase) granules and secretory vesicles (Witko-Sarsat *et al.,* 2000). Primary and secondary granules are formed during the promyelocyte and myelocyte/metamyelocyte stages respectively, while tertiary granules develop at the metamyelocyte/band cell stage (Le Cabec *et al.,* 1996). Secretory vesicles are the last to appear (becoming evident) in band and segmented cells (Borregaard and Cowland, 1997). These different granule sub-types vary with respect to efficiency of mobilization during neutrophil activation.

In addition to functioning as a mobilisable reservoir of membrane constituents, these various neutrophil granules contain an array of proteases and antimicrobial peptides and polypeptides, which participate in the migratory and antimicrobial activity of neutrophils. The fact that several granule polypeptides are shared by different granules (e.g. lysozyme and protease 3), is indicative of overlap between these granules, which may represent a continuum, as opposed to clearly demarcated granule types (Witko-Sarsat *et al.,* 2000).

#### 1.5.1.1 Primary granules

The major constituents of the primary granules are myeloperoxidase (MPO), an abundant component comprising about 5% of the total cellular protein, the antimicrobial enzyme, lysozyme, and at least 3 neutral serine proteinases, elastase, cathepsin G and proteinase 3 (Theilgaard-Mönch *et al.,* 2006). Unlike other granule

sub-types, azurophil granules do not function as reservoirs of membrane receptor and polypeptides, although CD63 and CD68 are present on their membranes, but the functional significance of this remains to be established (Cham *et al*., 1994). Myeloperoxidase and the defensins, also known as human neutrophil peptides (HNP-1 to HNP4), small cationic, broad-spectrum antimicrobial peptides that contain six cysteines in disulphide linkage, and are present in extremely high concentrations in primary granules (Witko-Sarta *et al.,* 2000).

Although azurophil granules have not been considered to act as reservoirs of membrane components involved in the migratory, phagocytic and oxidant-generating activities of neutrophils, the translocation of Sialyl Lewis-X from their membrane to the plasma membrane during activation of the cells with chemoattractants, suggests that this may not be the case (Suzuki *et al.,* 2000).

#### 1.5.1.2 Secondary granules

Secondary granules outnumber primary granules by about 2:1. They are rapidly mobilized during cell migration, and, in addition to intragranule constituents, their membranes act as a reservoir for adhesion molecules, leukoattractant receptors and cytochrome $b_{558}$, thereby sustaining neutrophil activation and antimicrobial activities of the cell. Approximately 90% of the total cellular lysosome, vitamin B12- binding protein, the bacteriostatic iron binding protein, lactoferrin as well as the latent metallo-enzymes, collagenase and gelatinase are located in the secondary granules.

#### 1.5.1.3 Tertiary and secretory granules

These granules act as a reservoir for the membrane polypeptides involved in neutrophil activation and function. Their major contribution to neutrophil function is thought to be achieved through extracellular release of gelatinase, which cleaves type IV (basement membrane) and V (interstitial tissues) collagen, thereby facilitating movement of the cells through basement membranes and into underlying tissues (Witko-Sarsat *et al.,* 2000). Secretory vesicles are thought to be endocytic in origin because they contain plasma proteins such as albumin. Interestingly, proteinase 3, a serine proteinase present in azurophil is also localized in the membrane of secretory granules, which are the most mobilizable granules of neutrophils.

### 1.5.2 Neutrophil elastase

Reactive oxygen species (ROS) and proteases are neutrophil-derived toxic molecules that have long been considered important in the pathophysiology of acute and chronic inflammation. Neutrophil elastase (NE) is a member of the chymotrypsin superfamily of serine proteases, being a 33-kDa enzyme with several isoforms that differ in their extent of glycosylation (Ohlsson and Olsson, 1974). NE is capable of degrading almost all extracellular matrix proteins, as well as a variety of key plasma proteins (Witko-Sarsat *et al.,* 2000). Under physiological conditions, NE is a powerful component of host defense and its activity is tightly regulated by endogenous protease inhibitors (Kawabata *et al.,* 2002). Upon activation, NE is rapidly released from the granules into the extracellular space with some portion remaining bound to the neutrophil plasma membrane (Owen *et al.,* 1997; Kawabata *et al.,* 2002).

### 1.5.3 Neutrophil elastase target molecules

The main intracellular physiological function of NE is the degradation of foreign organic molecules phagocytosed by neutrophils, whereas the main target for extracellular elastase is elastin (Kawabata *et al.,* 2002). Although NE has been defined as a protease that can degrade the elastin fibre, other proteases including protease 3, cathepsins, G, L and S, macrophage elastase, and gelatinase are able to degrade elastin as well (Lee and Downey, 2001; Kawabata *et al.,* 2002). NE is unique and recognizable as one of the most destructive enzymes because of its ability to degrade almost all extracellular matrix and key plasma proteins. In addition to elastin, NE is known to degrade the extracellular matrix proteins, collagen types I – IV, proteoglycan, fibronectin, platelet IIb/ IIIa receptor, complement receptor, thrombomodulin, and cadherins (Kawabata *et al.,* 2002).

With respect to plasma proteins, NE is able to cleave coagulation and complement factors, and immunoglobulin, as well as several proteases and protease inhibitors, leading to their activation or loss of function. Interestingly, NE-degraded fragments such as those derived from fibrin and laminin are known to be chemotactic for neutrophils. However, as opposed to the classical notion that the NE is a pro-inflammatory factor, recent studies suggest that NE is capable of degrading various pro-inflammatory cytokines such as IL-1, TNF (Owen *et al.,* 1997), IL-2 and IL-6 (Bank *et al.,* 1999). Kawabata and colleagues (2002) suggested that NE-induced release of

transforming growth factor-β, an anti-inflammatory cytokine, may be important in the remodelling of inflammation.

## 1.6 ANTIMICROBIAL MECHANISMS OF NEUTROPHILS

Among the elements of the human host defence armamentarium against microbial pathogens, neutrophils have a prominent role (Seguchi and Kobayashi, 2002). They are secretory cells that, on interaction with a wide array of stimuli, release microbicidal and pro-inflammatory agents into the extracellular milieu. In general, they utilize oxygen ($O_2$)-dependent and -independent mechanisms to eradicate microbial pathogens.

The oxygen-independent mechanisms encompass the contents of the three neutrophil granules: azurophil, specific and gelatinase granules, which contain characteristic proteases, antimicrobial proteins and peptides, and enzymes. Antimicrobial proteins such as defensins, bactericidal/permeability-increasing protein and the enzyme lysozyme, predominantly function by disrupting anionic bacterial surfaces, probably rendering bacteria more permeable. Proteases such as neutrophil elastase (NE) and cathepsin G (CG), degrade bacterial proteins, including virulence factors (Mayer-Scholl *et al.,* 2004). Roos and colleagues (2003) suggested that degranulation could be induced by a wide variety of stimuli that interact with distinct surface receptors and are able to elicit other neutrophil responses. Stimuli inducing degranulation include chemotactic factors (fMLP), chemokines (IL-8), FcγR ligands, and cytokines (G-CSF, GM-CSF, and TNF). The importance of the oxygen-independent defence mechanism is made clear in two rare inherited diseases, the Chediak-Higashi syndrome (neutrophils contain giant granules resulting from specific and azurophil-granule fusion) and specific granule deficiency.

Although neutrophils produce and release a variety of toxic agents directed toward microbial killing, those systems that depend on reactive products of oxygen metabolism are especially potent (Roos *et al.,* 2003). These agents are produced as a consequence of the respiratory burst, a series of events triggered by phagocytosis on exposure to certain inflammatory mediators, and featuring a dramatic increase in oxidative metabolism, with direct conversion of molecular oxygen to its univalent reduction product, the superoxide anion ($O_2^-$). Subsequent reactions lead to the formation of other

toxic species, including hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$).

### 1.6.1 NADPH oxidase

The phagocyte NADPH oxidase (respiratory burst oxidase) is a membrane-associated enzyme complex that generates superoxide during the respiratory burst by catalyzing the transfer of electrons from NADPH to molecular oxygen (Kim and Dinauer, 2001; Roos *et al.,* 2003). The $O_2^-$ generated by this enzyme serves as the starting material for the production of a vast array of reactive oxidants. These oxidants are used by phagocytes to kill invading microorganisms, but they also cause "collateral damage" to nearby tissues, such that their production has to be tightly regulated to ensure that they are only generated when and where required (Babior, 1999).

The active NADPH oxidase is made up of two membrane-bound components: gp91$^{phox}$ and p22$^{phox}$, that together form the oxidase flavocytochrome *b* (cytochrome $b_{558}$) and at least three cytoplasmic subunits p40 $^{phox}$, p47 $^{phox}$, and p67 $^{phox}$ that translocate to the membrane upon cellular activation (figure 4). In addition to these five *phox* components, two small GTPases, Rap1A, which is located in membranes, as well as Rac, which is located in the cytoplasm in a dimeric complex with Rho-GDI (Guanine nucleotide Dissociation Inhibitor), both have been implicated in the regulation of the NADPH oxidase complex (Werner, 2004). Cytochrome $b_{558}$ and p47 $^{phox}$ and p67 $^{phox}$ subunits are required for superoxide production, since a deficiency in either of these components results in chronic granulomatous disease (CGD), an inherited disorder characterized by absent phagocyte NADPH oxidase activity and recurrent bacterial and fungal infections (Kim and Dinauer, 2001; Kuribayashi *et al.,* 2002). Cytochrome $b_{558}$ is the redox center of the enzyme and appears to be activated upon binding of the p47 $^{phox}$ and p67 $^{phox}$ subunits (Kim and Dinauer, 2001).

### 1.6.2 NADPH oxidase activation

Activation of NADPH oxidase through receptor-mediated signaling by chemoattractants involves modification of the GDP-binding state of G-proteins (DeLeo and Quinn, 1996), thus, the activating receptor catalyses exchange of GDP for GTP by both the G-protein α subunit and low molecular weight G-proteins of Ras, Rho and

ARF (ADP-ribosylation factor) families (Kuribayashi *et al.,* 2002). This process leads to serial activation of phospholipases (PL) C and D, and the generation of lipid second messengers. Several converging pathways (protein tyrosine kinases/phosphatidylinositol 3-kinase; Ras/Rho; PLD) activate the serine kinase Raf, which together with the serine/threonine kinase, protein kinase C (activated by PLC/PLD-derived diacylglycerol), activates MAP kinases, which in turn cause the phosphorylative activation of cytosolic $PLA_2$ (Alonso *et al.,* 1998).

With respect to activation of NADPH oxidase in neutrophils and other phagocytes, the key events are activation of kinases, particularly protein kinase C, and cytosolic $PLA_2$ (Shiose and Sumimoto, 2000). Phosphorylation of p47 $^{phox}$ at several serine residues in the SH3-containing C-terminal region alters the conformation of the polypeptide. This in turn leads to unmasking of SH3 domains enabling weak interaction of p47 $^{phox}$ with p22 $^{phox}$ (Segal *et al.,* 2000; Kuribayashi *et al.,* 2002), by binding to the proline-rich region (PRR) of p22$^{phox}$. However, this event alone is insufficient for efficient activation of NADPH oxidase, a second complementary/synergistic mechanism being required. This is provided by low concentrations of arachidonic acid, generated during cleavage of membrane phosphatidylcholine by cytosolic phospholipase$A_2$ ($PLA_2$). Arachidonic acid maximizes the interactions of phosphorylated p47 $^{phox}$ with p22 $^{phox}$ resulting in complete activation of oxidase (Shiose and Sumimoto, 2000). However, a recent report has suggested that cytosolic $PLA_2$ is not involved in the activation of NADPH oxidase.

The component p47 $^{phox}$ in combination with p67 $^{phox}$/ p40 $^{phox}$ interacts with p22$^{phox}$ and initiates the electron-transporting activity of the oxidase; p47 $^{phox}$ and p67 $^{phox}$, both which are essential for NADPH oxidase activation, have distinct roles in the regulation of electron flow in cytochrome $b_{558}$; p67 $^{phox}$ facilitates electron flow from NADPH to the flavin center resulting in the reduction of flavin adenine dinucleotide (FAD), while p47 $^{phox}$ is required for electron flow to proceed beyond the flavin center to the heme groups in cytochrome $b_{558}$ and then to molecular oxygen. These events are summarized in figure 1.4. The final step in the electron transport chain occurs when oxygen accepts an electron and is converted to the superoxide radical:

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

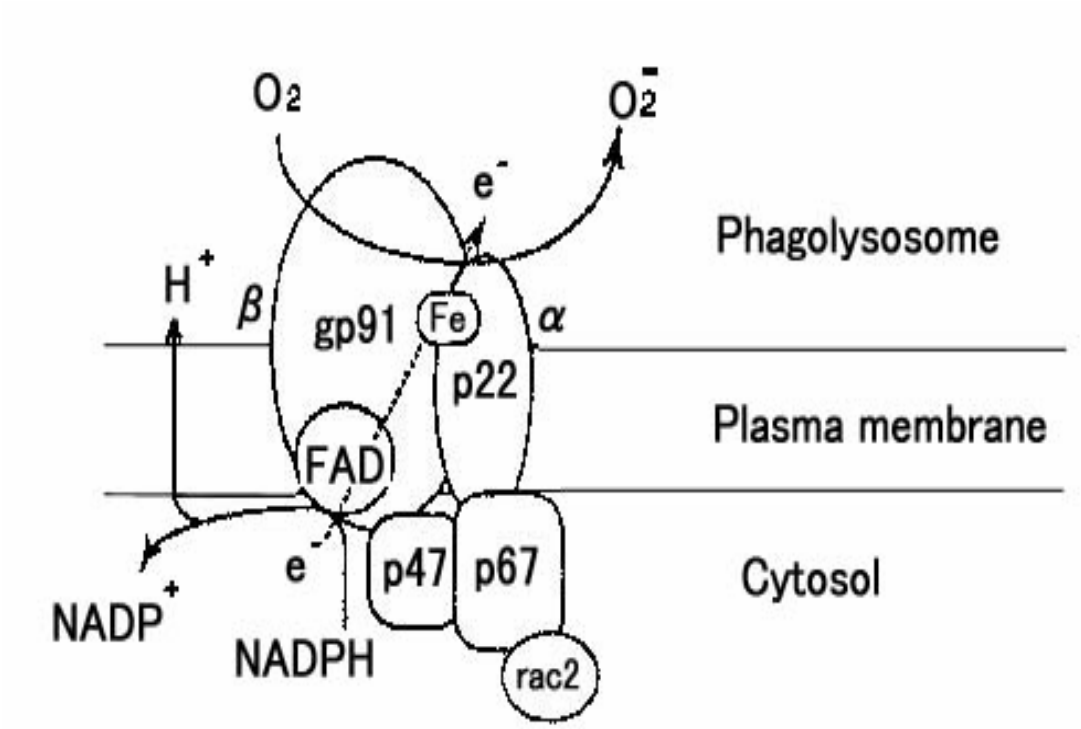



**Fig. 1.4:** Following stimulation by variety of stimuli, the cytosolic components of NADPH oxidase in the neutrophil become heavily phosphorylated and migrate to the membrane, where they associate with cytochrome $b_{558}$ to assemble the active oxidase, which transfers electrons from the substrate to oxygen by means of its electron-carrying prosthetic group with resultant generation of $2O_2^{\cdot -}$.

### 1.7 Respiratory burst of human neutrophils

During the phagocytosis of microbial intruders, professional phagocytes of the innate immune system increase their oxygen consumption through the activation of NADPH-oxidase, resulting in the generation of superoxide anion $2O_2^{\cdot -}$. This oxygen-derived radical is a weak and unstable antimicrobial oxidant, but functions as precursor of a series of more potent microbial oxidants (Leto, 1999).

Superoxide is rapidly converted to the stable oxidant hydrogen peroxide ($H_2O_2$), either by spontaneous dismutation or by enzymatic transformation by superoxide dismutase (Dröge, 2002):

$$O_2^{\cdot -} : O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The antimicrobial potential of $H_2O_2$ is dramatically potentiated by the granule enzyme, myeloperoxidase (MPO), which utilizes this oxidant to oxidize chloride to the extremely potent oxidizing agent hypochlorous acid (HOCl) (Roos *et al.,* 2003):

$$H_2O_2 + Cl \xrightarrow{MPO} H_2O + HOCl$$

Neutrophils transform $H_2O_2$/ $O_2^{\cdot -}$ to hydroxyl radical ($^{\cdot}OH$), the most potent oxidant known in biological systems by the iron-catalyzed Haber-Weiss reaction:

$$H_2O_2 + O_2^{\cdot -} \xrightarrow{Fe^{2+}/\ Fe^{3+}} {}^{\cdot}OH + OH^- + O_2$$

However, production of $^{\cdot}OH$ by neutrophils via this pathway has only been demonstrated *in vitro* in the presence of added iron. A transition metal-independent pathway of $^{\cdot}OH$ generation has been demonstrated in neutrophils which involves the interaction of $O_2^{\cdot -}$ and HOCl (Ramos *et al.,* 1992):

$$HOCl + O_2^{\cdot -} \longrightarrow {}^{\cdot}OH + O_2 + Cl^-$$

Neutrophils have also been reported to generate a significant amount of singlet oxygen [$O_2$ ($^1\Delta g$)], a highly reactive, diffusible and long-lived electronically excited state of molecular $O_2$. They do this by transforming up to 20% of $O_2$ consumed by NADPH Oxidase to [$O_2$ ($^1\Delta g$)] by a MPO-dependent pathway involving interaction of $H_2O_2$ and HOCl (Steinbeck *et al.,* 1992; Mayer-Scholl *et al.,* 2004):

$$H_2O_2 + OCl^- \longrightarrow O_2(^1\Delta g) + H_2O + Cl^-$$

These phagocyte-derived oxidants, acting directly, or through more stable intermediates, are powerful antimicrobial agents. They are indiscriminate, and if released extracellularly during hyperacute or chronic activation of phagocytes, they pose the potential threat of oxygen toxicity to bystander host cells and tissues in the vicinity of inflammatory reactions. In this regard they are cytotoxic for eukaryotic cells,

as well as being potential carcinogens, pro-proteolytic, pro-adhesive and immunosuppressive (Mayer-Scholl *et al.,* 2004).

## 1.8 NITRIC OXIDE SYNTHASE

Production of nitric oxide (NO) within phagocytes is an important component of host defence against microbial infections. Although NO *per se* is only weakly antimicrobial, its microbicidal activity is considerably enhanced by reaction with $O_2^-$ to yield the highly reactive anion, peroxynitrite (Koppenol, 1998).

## 1.9 CALCIUM AND NEUTROPHIL ACTIVATION

Unraveling of the mechanisms by which activated neutrophils handle calcium ($Ca^{2+}$) has resulted in identification of strategies utilized by microbial pathogens to promote dysregulation of $Ca^{2+}$ homeostasis, which in turn may contribute to excessive inflammatory responses, as well as to microbial virulence and persistence (Anderson *et al.,* 2002).

### 1.9.1 Calcium fluxes and restoration of $Ca^{2+}$ homeostasis in activated neutrophils

During evolution cells have developed many sophisticated and uniquely tailored systems to effect efficient and fast spatio-temporal changes in cytosolic $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$). Thus, both the outer cell plasma membrane and that of intracellular organelles are equipped with highly specialized proteins, which regulate $[Ca^{2+}]_i$ through influx from the extracellular space and by active extrusion in the case of plasma membrane, and those which allow mobilization from, and uptake into intracellular stores such as the sarco-endoplasmic reticulum, mitochondria, Golgi apparatus, nucleus and acidic granules (Arredouani, 2004).

The increase in $[Ca^{2+}]_i$ is a powerful stimulus to cell activation. Transient elevations in cytosolic free $Ca^{2+}$, precede and are a prerequisite for the receptor-mediated activation of many neutrophil functions, including activation of $\beta_2$-integrin and adhesion to vascular endothelium, superoxide production through NADPH oxidase (Lucas *et al.,* 2003), degranulation, and activation of pro-inflammatory cytosolic nuclear transcription factors, including NF-kB (Dolmetsch *et al.,* 1997; Tintinger, *et al.,* 2005).

### 1.9.2 $Ca^{2+}$ release from Stores

Calcium mobilization from stores during cellular responses to chemoattractants appears to be an essential mechanism for cellular activation (Bolotina, 2004; Oommen *et al.,* 2004). Intracellular $Ca^{2+}$ in the neutrophils is reportedly stored in specialized storage vesicles known as calciosomes (Favre *et al.,* 1996; Corbett and Michalak, 2000; Balsinde and Balboa, 2005). However, since there are two distinct cellular locations of $Ca^{2+}$ stores in neutrophils, these may have differential involvement in the activation of proinflammatory functions that utilize different molecular/biochemical mechanisms of $Ca^{2+}$ mobilization (Pettit and Hallet, 1996; Steel and Anderson, 2002). One site is located peripherally under the plasma membrane and the other (probably calciosomes) is localized in the juxtanuclear space and is mobilized by the chemoatractant, N-formyl-L-leucyl-L-phenylalanine (fMLP) (Pettit and Hallet, 1996).

Occupation of neutrophil membrane receptors for the chemotactic tripeptide, fMLP, results in receptor-G-protein coupling with consequent activation of phospholipase C (PLC) and generation of inositol 1,4,5 triphosphate ($IP_3$) by hydrolysis of phosphatidylinositol 4,5-biphosphate (Alonso *et al.,* 1998; Patti and Banting, 2004). Once generated, inositol 1,4,5 triphosphate binds to an intracellular $IP_3$ receptor located on the surface of intracellular $Ca^{2+}$ stores (endoplasmic reticulum), resulting in a rise in $[Ca^{2+}]_i$ (Machaca and Hartzell, 1999; Linn and Gafka, 2001). The phenomenon of calcium release via the $IP_3$ receptor is commonly termed $IP_3$-induced $Ca^{2+}$ release ($IP_3$-ICR). These events are extremely rapid, occurring within less than 1 second after the ligand-receptor interaction (Arredouani, 2004). In the case of neutrophils the abrupt increase in cytosolic $Ca^{2+}$ following exposure to fMLP, results exclusively from release of the cation from intracellular stores with little or no contribution at this early stage (within the first 30 – 60 sec.) from extracellular $Ca^{2+}$ and results in an increase in the basal $[Ca^{2+}]_i$ from around 100 nM to ± 1 µM (Anderson and Goolam Mahomed, 1997; Geiszt *et al.,* 1997).

Extracellular $Ca^{2+}$ influx is delayed, being detectable one minute after addition of fMLP and terminating around 5 minutes. This type of influx is a characteristic of store-operated $Ca^{2+}$ influx (i.e. primarily involved in refilling of stores like ER, as opposed to contributing to activation of neutrophils) and is operative in a large variety of non-excitable cells, including neutrophils. Thus, the empty $Ca^{2+}$ stores activate store-

operated $Ca^{2+}$ channels (SOCCs) in the plasma membrane, which then allow $Ca^{2+}$ ions to enter the cell. $Ca^{2+}$ entry through this pathway is termed store-operated $Ca^{2+}$ entry (SOCE), formerly known as capacitative $Ca^{2+}$ entry (Machaca and Hartzell, 1999). When the $Ca^{2+}$ stores are replete, the store-operated channels are closed, but once the stores discharge their contents, the store-operated channels open and $Ca^{2+}$ ions enter the cell (Berridge *et al.,* 2000). The mechanism for coupling the stores to activation and deactivation of the store-operated channels are unknown (García-Sancho, 2000).

### 1.9.3 Restoration of $Ca^{2+}$ homeostasis

Restoration of $Ca^{2+}$ homeostasis in neutrophils is essential to prevent $Ca^{2+}$ overload and hyperactivity of the cells (Corbett and Michalak, 2000; Anderson *et al.,* 2002). This is achieved by rapid clearance of cytosolic $Ca^{2+}$ by efflux and is accomplished primarily through the action of the plasma membrane and endo-membrane $Ca^{2+}$-ATPases which are regulated by calmodulin and adenosine 3´, 5´ cyclic monophosphate (cyclic AMP)-dependent protein kinase (Steel and Anderson, 2002) respectively, while extracellular cation is excluded from the cells through the membrane depolarizing activity of NADPH oxidase.

Efficient $Ca^{2+}$ clearance by these systems is greatly facilitated by the membrane depolarizing action of NADPH oxidase, which limits influx of extracellular $Ca^{2+}$ (Rada *et al.*, 2003; Oommen *et al.,* 2004). The superoxide-generating NADPH oxidase undergoes $Ca^{2+}$-dependent activation during exposure of cells to chemoattractants, cytokines and opsonised agents. When activated, the oxidase causes the membrane potential to rise between +30 and +50 mV, reaching positive values (Schrenzel *et al.,* 1998; Jankowski and Grinstein, 1999). In the absence of any electrical effect, $Ca^{2+}$ influx would occur when $Ca^{2+}$ channels are open in the phagosomal and plasma membranes (Lundqvist-Gustafsson *et al.,* 2000).

However, when the oxidase is activated and the membrane potential reverses, the cell becomes more positive inside relative to the outside, so that the influx of positively charged ions, like $Ca^{2+}$, is impeded (Hallett, 2003). In other words, despite the $Ca^{2+}$ channels being open, there would be no further net flux of $Ca^{2+}$ into the cells. This has been shown with neutrophils from patients CGD, which are unable to activate NADPH oxidase with consequent failure of membrane depolarization. In CGD neutrophils, the

$Ca^{2+}$ influx is exaggerated (Rada, *et al.*, 2003). Such cells are easily activated, hyper-responsive and prone to degranulate, all conditions that may lead to inappropriate activation and inflammatory disease. Thus, the activity of the neutrophil oxidase may be an important checkpoint for inflammatory conditions by restraining excessive $Ca^{2+}$ influx and controlling neutrophil aggression. These events are summarized in Figure 1.5.

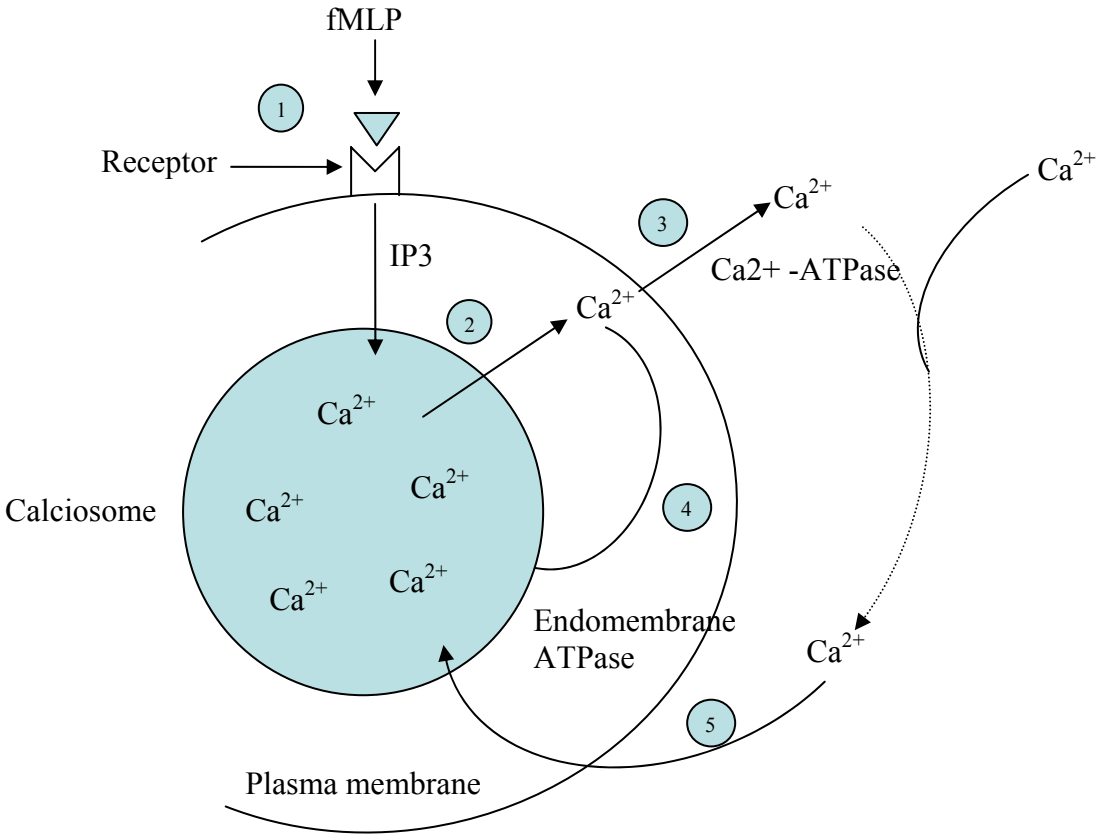



**Fig. 1.5:** Mechanisms of release of $Ca^{2+}$ from intracellular stores, and clearance of cytosolic $Ca^{2+}$ in fMLP-activated human neutrophils: 1) fMLP binds to its receptor and generates inositol triphosphate (IP3); 2) immediate release of $Ca^{2+}$ from calciosomes into the cytosol 3) concomitant efflux of $Ca^{2+}$ across the plasma membrane ($Ca^{2+}$-ATPase mediated); 4) early re-uptake of $Ca^{2+}$ into calciosomes mediated by the endomembrane $Ca^{2+}$-ATPase; and 5) delayed (60 sec – 5 min) store-operated influx of extracellular $Ca^{2+}$ to refill intracellular stores (Redrawn from: Tintinger et *al.*, 2005).

## 1.10 INTERACTIONS OF PORE-FORMING PNEUMOLYSIN WITH NEUTROPHILS

Pneumolysin, a membrane-damaging, pore-forming toxin produced by *Streptococcus pneumoniae* is considered to be intimately involved in the pathogenesis of infections caused by this microbial pathogens (Cockeran *et al.,* 2001). It is released during autolysis of bacteria, and has also been reported to disrupt $Ca^{2+}$ homeostasis in neutrophils (Anderson *et al.,* 2002) by a non-cytolytic pore-forming mechanism, which results in influx of extracellular $Ca^{2+}$ and flooding of the cytosol with the cation (Cockeran *et al.,* 2001).

In addition to being cytotoxic for eukaryotic cells, pneumolysin has been demonstrated to potentiate the pro-inflammatory activities of neutrophils and macrophages (Wellmer *et al*., 2002), and has also been reported to activate $Ca^{2+}$-dependent, cytosolic nuclear transcription factor in monocytes/macrophages, resulting in activation of the genes encoding IL-1, IL-6, TNF-α and inducible nitric oxide synthase (Anderson *et al.,* 2002). The pro-inflammatory potential of pneumolysin is supported by *in vitro* studies, in which treatment of phagocytes with this microbial toxin resulted in increased release of granule enzymes and proinflammatory cytokines (Cockeran *et al.,* 2002; 2003). Some of the pro-inflammatory effects of pneumolysin may also be due to cytolysis of various host cell types, including ciliated respiratory epithelium, and endothelial cells, as well as to activation of PL $A_2$ in endothelial cells.

## 1.11 LYMPHOCYTE DEVELOPMENT AND HETEROGENEITY

Lymphocytes under normal conditions make up about 20% to 35% of all white blood cells, but proliferate rapidly in the face of infection. There are two basic types of lymphocytes: the B lymphocytes and the T lymphocytes. B lymphocytes develop into plasma cells, which in turn produce highly specific antibodies against foreign antigens. Other B lymphocytes act as memory cells, ready for subsequent activation by the same organism. Some T lymphocytes kill invading cells directly, while others interact with different immune system cells, regulating the immune response (Abbas *et al.,* 1997).

In the initial stages of their development, lymphocytes do not produce surface receptors for antigens and are, therefore, unresponsive to antigens. As they mature, they begin to

express antigen receptors, become responsive to antigenic stimulation, and develop into different functional classes (Hale and Haynes, 1999). Lymphocytes consist of distinct subsets that are quite different in respect of their functions and protein products, even though they all appear morphologically similar. In humans, B lymphocytes, are so called because in birds they were first shown to mature in the Bursa of Fabricius. In humans, B lymphocytes mature in the bone marrow, and are the only cells capable of producing antibodies. The antigen receptors of B-cells are membrane-bound forms of antibodies. Interaction of antigen with these membrane antibody molecules initiates the sequence of B-cell activation, which culminates in the development of effector cells that actively secrete antibody molecules.

A second major class consists of T lymphocytes, whose precursors also arise in the bone marrow and then migrate to and mature in the thymus (thymus-derived). T lymphocytes are further subdivided into functionally distinct populations, the helper/inducer T-cells and the cytolytic/suppressor T-cells. The principal functions of the T lymphocytes are to regulate all immune responses to protein antigens and to serve as effector cells for the elimination of intracellular microbes. T-cells do not produce antibodies, they have specificity for antigens; they recognize only peptide antigens attached to proteins that are encoded by genes in the major histocompatibility complex (MHC) and expressed on the surface of other cells, and as a result, they recognize and respond to cell surface-associated but not soluble antigens (Katagiri *et al.,* 2002). Two major classes of T-cells are distinguished one from the other by the expression of surface markers CD4 and CD8. Helper cells are $CD4^+$ T-cells; cytotoxic cells are $CD8^+$ T-cells. CD4 and CD8 are surface glycoproteins, which function as adhesion molecules and T-cell "co-receptors" for antigen.

In addition to T and B lymphocytes up to 10% of the circulating lymphocyte population is composed of large granular lymphocytes known as natural killer (NK) cells (Hale and Haynes, 1999). NK cells were named for their ability to kill tumours in a non-MHC-restricted fashion without the need for prior activation by tumour antigens. NK cells arise in the bone marrow and share a common precursor with T lymphocytes. Although NK cells can develop within and traffic through the thymus, they do not rearrange or productively express a T-cell receptor (TCR), and they do not require the thymic microenvironment for maturation.

## 1.12 CD4 AND CD8 T-CELL FUNCTIONS

Although the CD4 and CD8 glycoproteins show no close sequence or structural similarities, they are nevertheless functionally homologous. CD4 and CD8 are expressed on two mutually exclusive subsets of mature T-cells. The CD4 molecule is expressed by cells (mainly helper T-cells [Th], but also a small subset of cytotoxic T-cells [Tc]) that interact with MHC class II antigen, whereas CD8 exhibits a reciprocal expression on cytotoxic T-cells that recognize class I antigens (Dofman and Germain, 2002).

CD4 and CD8 increase the intensity of cell-to-cell interactions by binding to monomorphic determinants on the appropriate MHC molecules of the target cell. They also have a regulatory function; their antibodies can synergize with anti-CD3 antibodies in T-cell activation because of their association with the T-cell specific tyrosine kinase $p56^{lck}$, which might bring $p56^{lck}$ into close proximity with the TCR complex, particularly its potential substrate the CD3ζ chain, during transient interactions of CD4 and CD8 with TCR-antigen/MHC complex (Hale and Hyners, 1999; Sewell *et al.,* 1999).

## 1.13 Helper T lymphocytes

$CD4^+$ T-cells can be subdivided into several populations using various operational and phenotypic parameters (Mosmann and Sad, 1996). At least two general classes of Th cells can be defined, based on their cytokine secretion profiles. In very broad terms, Th1 T-cells are considered to be responsible for cell-mediated effector mechanisms; they are characterized by production of interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-β (TNF-β), whereas Th2 T-cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, cytokines that play a greater role in the regulation of antibody production. Th1 and Th2 cells appear to differ quite markedly in their requirements for activation and growth. Th1 cells use IL-2 as their autocrine growth factor and respond weakly or not at all, to IL-4. Th2 cells produce and respond to IL-4, but will also proliferate strongly in response to IL-2. An important feature of Th1 and Th2 cells is the ability of one subset to regulate the activities of the other (Prabhakar *et al.,* 2004).

Th1 cells down-regulate antibody responses driven by Th2 cells. In addition, products of one subset can antagonize the activation of the other; IFN-γ inhibits the proliferative response of Th2 cells (driven by either IL-2 or IL-4), but has no effect on that of Th1 cells. Similarly, IL-10, originally called cytokine synthesis inhibitory factor (CSIF), inhibits the production of cytokines, particularly IFN-γ , by Th1 cells and inhibits their autocrine growth. The occurrence of such mutually inhibitory interactions of Th1 and Th2 cells helps to explain how the immune system can selectively trigger various effector mechanisms.

However, the divisions are not absolute and there is considerable overlap or redundancy in function between the Th cells that are assigned to the different subsets. Indeed, some researchers feel that the division of helper T cells into Th1 and Th2 subsets has been overplayed, and that in reality the situation is not so polarised, with many cells falling into the Th0 (intermediate) category (Hickling, 1998). Another subset of T-cells with immunosuppressive bystander effects, secretes T-cell growth factor-β as the dominant cytokine and has been designated Th3.

### 1.14 ANTIGEN PROCESSING AND PRESENTATION TO T-CELLS

Antigen processing and recognition is a key feature of the immune response to intracellular microorganisms and viruses (Kaufmann and Schaible, 2005). It has long been known that B-cells can recognize soluble antigens directly, but T-cells require the presence of an accessory cell population in order to be activated by antigens (Bachman and Kopf, 2002). The first evidence that an active processing step was required before antigen could be recognized by T-cells came from the studies by Ziegler and Unanué (1981) on macrophage presentation of *Listeria monocytogenes* antigens to polyclonal class II-restricted T-cells. They found that there was a lag period between the binding of antigen to the macrophage and detection of antigen recognition by T-cells. Macrophages rendered metabolically inactive by fixation with paraformaldehyde immediately after pulsing with antigen were not recognized by the T-cells, but were able to present *L. monocytogenes* antigens to specific T-cells if fixed after a lag period of 45 – 60 minutes. Similar results were subsequently obtained by other investigators using soluble protein rather than particulate bacterial antigens.

There are two major pathways of antigen processing (Figure 1.6) within the antigen-presenting cell (APC) and target cell (Hudrisier and Bongrand, 2002; Katagiri *et al.,* 2002). In the exogenous pathway, soluble proteins are taken up from the extracellular environment, generally by specialised or 'professional' APCs such as macrophages, B-cells or dendritic cells (DC). During processing, antigens are partially degraded and resulting peptide fragments are bound to MHC class II molecules. Peptide-MHC-class II complexes are brought to the cell surface of the APC for recognition by the TCRs of the $CD4^+$ T-cells (Hale and Haynes, 1999; Hudrisier and Bongrand, 2002).

As with CD8, the CD4 molecule functions as a co-receptor, increasing the strength of the interaction between the T-cell and the APC (Hickling, 1998). $CD4^+$ helper T-cells that are activated by the peptide-class II MHC complexes secrete cytokines such as IL-2 (IL-2, T-cell growth factor), which ultimately activate and promote the proliferation of T-cells (Blattman *et al.,* 2003; Driver, 2004), as well as other cells, including B-cells and macrophages to participate in antigen-specific immune responses.

Alternatively, antigens may be released within the cell as the result of infection by virus or other obligate intracellular pathogens or from alterations in normal cellular proteins generated by the tumour cells (Hale and Haynes, 1999). The endogenous pathway processes proteins that have been synthesized within the APC. In this pathway, proteins in the cytoplasm are cleaved by proteosomes (a proteolytic organelle) into peptide fragments of ~ 20 amino acids in length. These fragments are then transported into the lumen of the endoplasmic reticulum (ER) via the transporters associated with antigen processing (TAP) complex, where they encounter newly formed heavy-chain molecules of MHC class I and their associated $b_2$ microglobulin ($b_2$mg) light chains (Hudrisier and Bongrand, 2002; Al-Daccak *et al.,* 2004). The heavy chain, light chain and peptide form a trimeric complex, which is then transported to and expressed on the cell surface.

T-cells that express the CD8 cell-surface marker recognise antigens that are presented by MHC class I molecules, while those which express the CD4 cell-surface marker recognise antigens that are presented by MHC class II molecules (Hickling, 1998; Hale and Haynes, 1999; Dustin and Cooper, 2000; Kaufmann and Schaible, 2005). Cytolytic T-cells lyse or kill host cells that produce foreign antigens, such as cells infected by viruses and other intracellular microbes. Suppressor T-cells down-regulate B and T-

cells and thus modulate humoral and cell mediated immune responses (Dorfman and Germain, 2002).

This classical segregation of $CD4^+$ and $CD8^+$ T-cells is critical for control of bacterial and viral infections respectively, and was derived from the notion that intracellular bacteria typically survive inside the phagosomal compartments with access to the MHC II molecules, which are responsible for peptide presentation to $CD4^+$ T-cells. By contrast, viruses are newly generated by the protein-synthesis machinery within various host cells; which is the cytoplasm and the ER. Hence, viral peptides can be readily loaded onto MHC I molecules, which present them to $CD8^+$ T-cells. Accordingly, the intracellular habitat of the pathogen dictates the type of T-cell population responsible for protection. It is well known that not only $CD4^+$ T-cells, but also $CD8^+$ T-cells participate in acquired immunity to numerous intracellular bacteria. In fact, recent studies suggest that antigen from phagosome-processed bacteria might be presented to $CD8^+$ T-cells by a cross presentation pathway (Kaufmann and Schaible, 2005). These events are summarized in Figure 1.6.

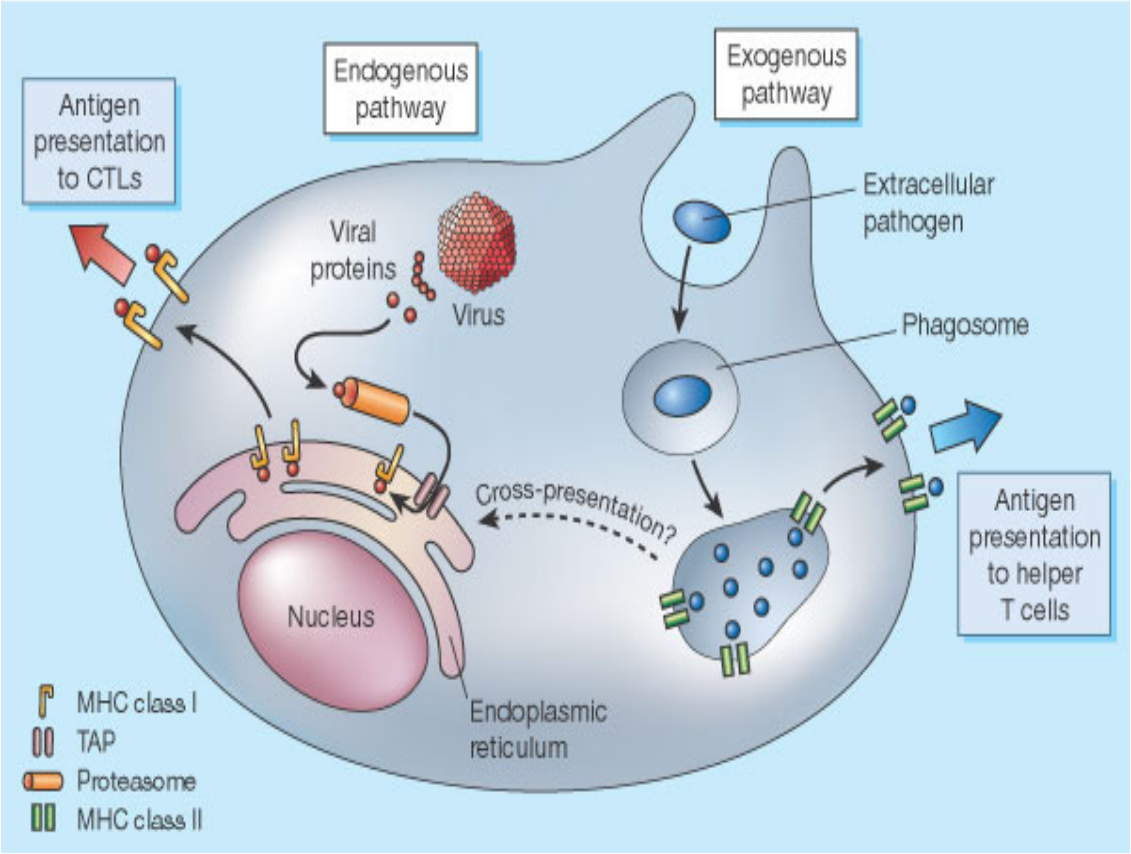



**Fig. 1.6:** Two major pathways of antigen processing and presentation exist. These are the endogenous MHC class I, and exogenous MHC class II pathways. Until recently the way in which antigen from phagosomal bacteria enter the MHC I pathway was unknown. These pathways are not mutually exclusive. Phagosome-processed bacteria are introduced to MHC I molecules by cross-presentation. (Source: http://www.vetmed.wsu.edu/research_vmp/itp/).

### 1.15 Antigen recognition by T-cells

Sensitivity, specificity, and context discrimination are three key properties of T-cell antigen recognition. T-cells recognize protein antigens in the form of peptide fragments that are presented at the cell surface by major histocompatibility complex (MHC) class I or MHC class II molecules (Lanzavecchia *et al.*, 1999; Pecht and Gakamsky, 2005). When the antigen-specific T-cell receptor (TCR) on the T-cell surface (specifically the α/β chains of the CD3 complex) interacts with the appropriate peptide–MHC complex, it triggers phosphorylation of the intracellular domains of the CD3 z (zeta) chains. Subsequently, the zeta-associated protein 70 (ZAP-70) binds to the phosphorylated zeta

chains, and is activated. Simultaneous co-ligation of CD4 (or CD8) with the MHC class II (or class I) molecule results in the phosphorylation of the *lck* kinases.

These events stimulate the activation of at least three intracellular signalling cascades. T-cell activation also requires a second co-stimulatory signal (such as the interaction between the cell markers CD28 on the T-cell, and CD80/CD86 on the antigen-presenting cell). This interaction also triggers several intracellular signalling pathways. Activation of T-cells can lead to cell division, cytokine secretion by the T-cell as well as expression of markers associated with the activated state. Alternatively, in the case of cytotoxic T lymphocytes (CTLs), interaction with antigen via the specific TCR leads to destruction of target cells (Hickling, 1998; Pecht and Gakamsky, 2005).

**1.16 T-cell activation**

Critical to the adaptive immune system is T-cell activation, which depends on the interaction of TCR with antigenic peptides bound to the MHC displayed on the surface of APC (Pahlavani, 1998; Hashemi *et al.,* 1999; Rachmilewitz and Lanzavecchia, 2002). T-cell activation presents a paradigm for both signal transduction and the orchestration of extracellular interactions that lead to the incredible sensitivity and specificity of antigen recognition (Shaw and Dustin, 1997; Lanzavecchia *et al.,* 1999). A characteristic feature of T-cell activation is that sustained TCR signalling is required for cytokine secretion and proliferation.

When finally activated, the TCR receptor complex transmits further signals via a cascade of signalling pathways involving phospholipase C-γ, calcium mobilization, protein kinase C (Trautmann and Valituti, 2003), calcium-dependent kinases and phosphatases, and mitogen-activated protein (MAP) kinase. These signals ultimately result in the activation of the nuclear factor of activated T lymphocytes (NFAT) family and other transcription factors, and the surface expression of activation markers on the plasma membrane.

The earliest surface marker is the CD69 molecule, which is expressed within a few hours of activation (Reddy *et al.,* 2004) and does not initially require new RNA or protein synthesis (Hashemi *et al.,* 1999). This is then followed by the expression of

CD71. The CD71 is the serum iron-transport protein that non-covalently associates with the TCRζ chain in the T-cells where it may play a role in signal transduction (Reddy *et al.,* 2004). Upon full activation of pathways necessary for gene expression, the receptor for IL-2 (CD25) is expressed on the plasma membrane. Surface expression of CD25 requires gene transcription beginning within 2 hrs after TCR stimulation (Hashemi *et al.,* 1999; Reddy *et al.,* 2004).

Although signalling responses occur within seconds of TCR triggering, the biochemical changes associated with TCR activation occur over a period of hours and may be subject to modification by other cellular regulation pathways. The approximate time course of TCR activation pathways is as follows: secretion of cytokines, 2 hours; initiation of DNA replication, 24 hours; cell division, 48 hours; and differentiation into effector cell, days. In the periphery during antigen-specific T-cell responses, most of the T-cells participating in the immune response are eliminated, but a subset of these cells survive and differentiate into long-lived memory cells.

## 1.17 T LYMPHOCYTE PROLIFERATION

Lymphocyte proliferation upon antigenic challenge plays an essential role in mounting an effective immune response. The large diversity of lymphocyte receptors means that there will be at least a few that can bind to any given foreign antigen. Because each lymphocyte has a different antigen receptor, the number of antigen-responsive cells is very small. To generate sufficient antigen-specific effector lymphocytes to fight an infection, a lymphocyte with appropriate receptor specificity must be activated to proliferate before its progeny finally differentiate into effector cells (Hunt *et al.,* 1999). Lymphocyte proliferation is initiated in the draining lymphoid tissues, where naïve lymphocytes and activated antigen presenting cells come together. On recognition of its specific antigen, a small lymphocyte stops migrating and becomes activated. One of the most rapid consequences of T lymphocyte activation through its antigen receptor is the *de novo* synthesis of IL-2. This is quickly followed by expression of high affinity IL-2 receptor, thus permitting rapid and selective expansion of effector T-cell populations activated by antigens (Abbas *et al.,* 1997; Ellery and Nicholls, 2002; Gaffen and Liu, 2004).

IL-2 is the principal mitogenic factor for activated T-cells, and delivers a proliferative signal through ligation of the IL-2 receptor. This proliferative signal is critically dependent upon cytoplasmic tyrosines on the β-chain of this receptor (IL-2Rβ) becoming phosphorylated in response to ligand (Lord *et al.,* 1998; Blattman *et al.,* 2003). IL-2 exerts its cellular effects through binding to specific cell surface receptors (Hunt *et al.,* 1999). The high affinity IL-2 receptor is a heterotrimeric complex consisting of α-, β-, and γ-subunits, the γc subunit being shared with the receptors of other T-cell mitogens, IL-4, IL-7, IL-9, and IL-15. The α-subunit is responsible for conferring high affinity cytokine binding, while the β- and γ-subunits recruit cytoplasmic molecules, thereby transducing the proliferative signal (Hunt *et al*., 1999; Driver, 2004).

Although other cytokines appear to be partially redundant with IL-2 in this regard, this cytokine is vital for determining the magnitude and duration of primary and memory immune responses. IL-2 also plays a central role in down regulating immune responses (Ellery and Nicholls, 2002). Its absence results in severe autoimmunity due to a failure to eliminate activated T-cells (Gaffen and Liu, 2004). Therefore, IL-2 is described as a growth and survival factor capable of inducing T-cell proliferation. This is supported by the *in vivo* studies of Chen and colleagues (2002), which show that elimination of IL-2 from proliferating T-cells can lead to cytokine withdrawal mediating cell death (Manjunath *et al*., 2001).

Following binding of IL-2 to IL-2R, the lymphoblast now begins to divide, normally duplicating two to four times every 24 hours for 3 - 5 days, so that one naive lymphocyte gives rise to a clone of around 1000 daughter cells of identical specificity, which differentiate into effector cells. After a naïve T lymphocyte has been activated, it takes 4 - 5 days before clonal expansion is completed and lymphocytes have differentiated into effector cells, and adaptive immune responses occur only after a delay of several days. Thus, specific recognition by clonally distributed receptors evolved as a late addition to existing innate effector mechanisms to produce an adaptive immune response. Induced lymphocyte proliferation is represented in Figure 1.7.

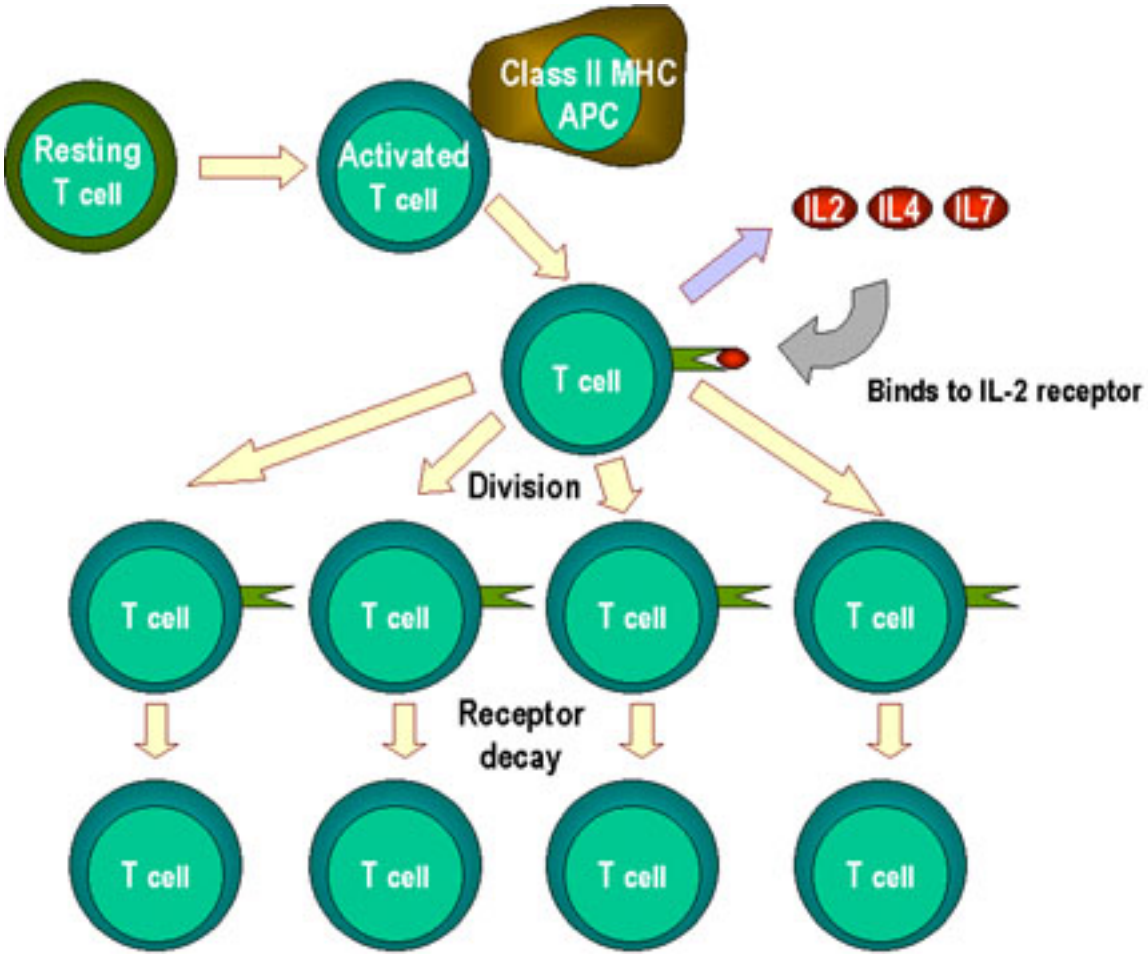



**Fig 1.7:** When T-cells are resting, they do not produce cytokines such as interleukins 2, 4 or 7, nor do they express large amounts of their receptors. Activation of T cells results in the formation of high affinity IL-2 receptors and induction of the synthesis and secretion of IL-2 and IL-4. These bind to their receptors and the T-cells proliferate into effector T-cells.

During an immune response, antigen-specific T-cells proliferate enormously and develop into effector T-cells capable of immediate effector functions, such as cytotoxicity and cytokine production (Butz and Bevan, 1998). Following a successful immune response, activated effector T-cells undergo large-scale apoptosis, presumably to maintain homeostasis in T-cell numbers (Manjunath *et al.,* 2001). However, some persist after the antigen has been eliminated and are known as memory cells, which ensure more rapid and effective responses on the second encounter with pathogen, thereby providing lasting protective immunity.

The measurement of the proliferation of lymphocytes that occurs following various stimuli (such as exposure to mitogenic agents, polyclonal stimuli or specific antigens) is a fundamental technique for assaying T-cell responses. However, simple enumeration of T-cells before and after such stimulation is laborious, and in most cases is not possible, because the cells that are responding represent only a small percentage of the total cell population at the start of the assay (Hickling, 1998).

## 1.18 IL-2 receptor structure and composition

As mentioned in section 1.17 page 33, the IL-2R complex is a heterotrimer composed of three distinct subunits, termed IL-2Rα (also known as CD25 or Tac), and is homologous to a similar affinity-modulating subunit in the IL-15 receptor complex (IL-15Rα), IL-2Rβ (p75, CD122; human chromosome 22) and IL-2Rγc (IL-2Rγ, p64, CD132; X chromosome), and these subunits work in concert to coordinate and drive specific signals (Nelson *et al.,* 1996; Chen *et al.,* 2002; Gaffen and Liu, 2004). The IL-2Rβ chain is shared with the IL-15 receptor, and γc is shared with the IL-4, IL-7, IL-9, and IL-15 receptors, all of which also deliver proliferative signals (Chen *et al.,* 2002). Alone neither IL-2Rβ nor γc bind IL-2 detectably, but the IL-2Rβ/γc complex comprises the intermediate affinity IL-2 receptor complex, and is capable of mediating the full spectrum of IL-2-dependent activities if exposed to IL-2 in sufficient quantities (Hunt *et al.,* 1999; Gaffen and Liu, 2004). IL-2Rβ and γc are members of the type I cytokine receptor superfamily, and activate a variety of signalling pathways common to this family (Nelson *et al.,* 1996).

Despite the inability of IL-2Rα to deliver intracellular signals (Ellery and Nicholls, 2002), its physiological significance should not be underrated. Mice with targeted deletions in IL-2Rα and humans with genetic deficiencies in this chain have clearly demonstrated that absence of IL-2α is functionally equivalent to absence of IL-2 (Sharfe *et al.,* 1997; Gaffen, 2001). When IL-2Rα is expressed on one cell, it can augment IL-2 signalling on an adjacent cell that expresses IL-2Rβ and γc but not IL-2Rα. For that reason, IL-2Rα plays a critical role in regulating responses to IL-2 by controlling the affinity of the IL-2R for ligand, even though this chain does not contribute directly to signal transduction due to the extremely short cytoplasmic tail. Resting T-cells express IL-2R in the form of β and γc chains, which bind with moderate

affinity, allowing T-cells to respond to very high concentrations of IL-2. Association of the α chain with the β and γ chains creates a receptor with much higher affinity for IL-2, allowing the cells to respond to very low concentrations of IL-2 (Janeway *et al.,* 2001; Ludányi *et al.,* 2004).

## 1.19 PROPERTIES AND FUNCTIONS OF CYTOKINES

The development of an effective immune response involves lymphoid cells, inflammatory cells, and haematopoietic cells. The complex interactions among these cells are mediated by a group of secreted, low-molecular-weight proteins that are collectively designated cytokines to denote their role in cell-to-cell communication (Prabhakar, *et al.,* 2004). They assist in regulating the development of immune effector cells, and some cytokines possess direct effector functions of their own. Just as hormones serve as messengers of the endocrine system, so cytokines serve as messengers for the immune system; however, unlike endocrine hormones, which exert their effects over large distances, cytokines generally act locally (Prabhakar *et al.,* 2004).

Unlike hormones, cytokines are not stored in glands as preformed molecules, but are rapidly synthesized and secreted by different cells, mostly after stimulation. Cytokines are pleiotropic in their biological activities and play pivotal roles in a variety of responses, including the immune response, haematopoiesis, neurogenesis, embryogenesis, and oncogenesis. They frequently affect the action of other cytokines in an additive, synergistic, or antagonistic manner. Cytokines have been classified on the basis of their biological response properties into pro-inflammatory (Th1 type) cytokines, for example, IL-1 (α and β), TNF-α, IL-8, IL-11, and IL-6 (Feghali and Wright, 1997; Nikolous *et al*., 1998), or anti-inflammatory (Th2 type) cytokines; IL-10, IL-4 and IL-13 (which is a series of immunoregulatory molecules that control the pro-inflammatory cytokine response) (Gimenes *et al.,* 2005) major properties of different human cytokines are listed in Table 1.1.

The net effect of any cytokine is dependent on the timing of cytokine release, the local milieu in which it acts, the presence of competing or synergistic elements, cytokine receptor density, and tissue responsiveness to each cytokine (Opal and Depalo, 2000).

Cytokines play an important role in the communication between cells of multicellular organisms. Besides their pleiotropic effects, which are often redundant, they exert their actions, which can be auto-, para- or endocrine, via specific cell-surface receptors on their target cells. They are key players in the regulation of the immune response, particularly during infections (Abbas *et al.,* 1997; Gouwy *et al.,* 2005).

### 1.19.1 Anti-inflammatory cytokines

The immune system is fighting a constant war against pathogens in its territory. This requires not only a potent arsenal for efficient control of pathogens, but also tight regulatory mechanisms in order to avoid excessive collateral damage (Bachmann and Kopf, 2002). Maintaining equilibrium is the daily challenge of the immune system. In order to counterbalance overshooting immune responses, T-cells and APCs secrete anti-inflammatory cytokines that are critical for maintaining a healthy balance between protection and immunopathology. Consequently, downregulation of inflammation is equally important in the host's inflammatory response as initiation inflammation. The failure to control inflammatory responses can lead to extensive tissue damage, thus, defective regulation of inflammation may contribute to the pathogenesis of many autoimmune diseases (Ben-Baruch, 2006). Mechanisms which downregulate inflammatory responses include apoptosis of inflammatory cells, production of inhibitors of activated complement components and production of cytokine receptor antagonists. The cytokines, IL-4, IL-10 and IL-13 are produced predominantly by T-cells and have intrinsic anti-inflammatory activities.

As shown in Table 1.1, acting alone or in concert, IL-4 and IL-10 decrease the production of the pro-inflammatory cytokines, IL-1, IL-6, IL-8, IL-12 and TNF-α (Opal and Depalo, 2000). IL-13 inhibits the production by lipopolysaccharide (LPS)-stimulated monocytes of IL-1, IL-6, IL-8, IL-10, IL-12, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein-1α (MIP-1α) and TNF-α, but upregulates IL-1Rα expression. IL-13 does not globally inhibit macrophage functions, but instead selectively inhibits cytotoxic and pro-inflammatory macrophage activities, since it increases the ability of macrophages to present antigens, resulting in an increased T-cell proliferative response.

| CYTOKINE | CELL SOURCE | PRINCIPAL EFFECTS |
|---|---|---|
| IL-1 α and β | Macrophages and other APCs | Costimulation of APCs and T-cells, Phagocyte activation |
| IL-2 | Activated Th1 cells, Tc cells, NK cells | Proliferation of activated T-cells, NK and Tc cell functions |
| IL-4 | Th2 cells, mast cells | Class II MHC expression, Th2 and Tc-cell proliferation and functions, inhibition of monocyte functions |
| IL-6 | Activated Th2 cells, APCs | Synergistic effects with IL-1 Or TNF to costimulate T-cells |
| IL-8 | Macrophage and other somatic cells | Chemoattractant for neutrophils and T-cells |
| IL-10 | Activated Th2, CD8 T-cells B-cells and macrophages | Inhibition of cytokine production by Th1 cells, promotion of B-cell proliferation, suppression of cellular immunity |
| IL12 | B-cells and macrophages | Proliferation and function of activated Tc and NK cells, Th1 induction, promotion of cell-mediated immunity |
| TNF-α and -β | Activated macrophage and other stromatic cells | IL-1 like effects |
| INF-α and -β | Macrophages, neutrophils | Antiviral effects, up-regulation of class I MHC expression |
| INF-γ | Activated Th1 and NK cells | Promotion of cell-mediated immunity, induction of MHC class I and class II |
| G-CSF | Fibroblasts, monocytes | Promotion of granulocyte growth |
| GM-CSF | T lymphocytes, fibroblasts, endothelial cells, monocytes | Promotion of macrophage growth |
| MCP-1 | Monocytes, basophils, mast cells | Macrophage activation, basophil degranulation (histamine release) |
| MIP-1α abd -β | T-cells, monocytes | Chemoattractant for monocytes and T lymphocytes, integrin expression by T-cells |
| TGF-β | Activated T-cells, platelets, macrophages | Suppression of cytokine production, antiproliferative for macrophages and lymphocytes |

**Table 1.1** Major properties of human interleukins and other immunoregulatory cytokines (Source: Janeway, *et al.,* 2001).

### 1.19.2 Chemokines

The recruitment of inflammatory and immune effector cells to sites of injury or infection is a hallmark of inflammation and an early event leading to the development of an immune response. This process is tightly regulated, in part by a network of locally released mediators, including chemokines, the largest family of cytokines, which form a complex system for the chemotactic activation of all types of leukocytes. These mediators control leukocyte trafficking during homeostatic migration, as well as during inflammation, and provide a linkage between innate and adaptive immunity (Rot and von Andrian, 2004). Together with adhesion molecules, such as integrins and selectins, chemokines and their receptors act primarily as part of a complex molecular network that facilitates the selective movement of specific cell types into, and out of tissues (Coelho *et al.*, 2005)

Production of chemokines is induced directly by exogenous irritants (bacterial and viral products), as well as by endogenous mediators (cytokines; such as IL-1, TNF-α and IFN-γ). Because they bind to specific cell surface receptors, chemokines can be considered second-order cytokines (Thelen, 2001). They appear to be less pleiotropic than first-order pro-inflammatory cytokines because they are not potent inducers of other cytokines and exhibit more specialized functions in inflammation and repair. In response to infection, cytokines and chemokines are produced simultaneously and interact in a complex network as either activators or inhibitors of inflammation (Gouwy *et al.*, 2005).

Receptor expression and generation of different chemokines form the basis for the selective recruitment of leukocytes and determine the composition of the inflammatory infiltrate. Traditionally, chemokines have four conserved cysteines that form two disulfide bonds (Cys1-Cys3 and Cys2-Cys4) C-X-C (where X is any amino acid) and C-C chemokines or α and β chemokines, are distinguished according to the position of the first two cysteines that are adjacent (CC) or separated by one amino acid (CXC) (Laing and Secombes., 2004). Most of the CXC chemokines are chemoattractants for neutrophils (and to some extent lymphocytes) but not monocytes, whereas CC chemokines appear to attract monocytes, granulocytes (except neutrophils) and

lymphocytes. Recently, the third C (γ) branch of these molecules has been discovered. The main chemokines are shown in Table 1.2 below.

**Table 1.2:** Common human chemokines

| Family | Official name | Common name |
|---|---|---|
| CxC (α) | CxCL8 | IL-8 |
| | CxCL4 | PF-4 |
| | CxCL7 | NAP-2 |
| CC (β) | CCL2 | MCP-1 |
| | CCL3 | MIP-1α |
| | CCL4 | MIP-1β |
| | CCL5 | RANTES |
| | CCL7 | MCP-3 |
| | CCL8 | MCP-2 |
| | CCL9 | MIP-1γ |
| | CCL12 | MCP-5 |
| | CCL13 | MCP-4 |
| C (γ) | CL1 | Lymphotactin-α |
| | CL2 | Lymphotactin-β |
| $Cx_3C$ (δ) | $Cx_3CL1$ | Neurotactin/fractalkine |

Source: Laing and Secombers, 2004.

# *Chapter 2: Hypothesis and Objectives*

## 2.1 HYPOTHESIS

The hypotheses to be tested in this study are that moxifloxacin

1) possesses anti-inflammatory properties in addition to its conventional antimicrobial activity, and
2) neutralizes the pro-inflammatory activities of the pneumococcal pore-forming toxin, pneumolysin.

## 2.2 GENERAL OBJECTIVES

This study has been designed to investigate the *in vitro* immunomodulatory effects of moxifloxacin, on stimulated and unstimulated human blood neutrophils and T lymphocytes.

### 2.2.1 SPECIFIC OBJECTIVES

- To evaluate the effects of moxifloxacin on oxidant production by PMA and fMLP-activated neutrophils (using luminol- and lucigenin-dependent chemiluminescence), and the release of elastase (colorimetric procedure) by fMLP/CB-activated neutrophils.

- To measure store-operated $Ca^{2+}$ influx into fMLP-stimulated neutrophils using a radiometric procedure.

- To evaluate the potential of moxifloxacin to antagonize the injurious and pro-inflammatory interactions of the pore-forming cytotoxic and pro-inflammatory microbial toxin, pneumolysin, with neutrophils.

- To investigate the effect of moxifloxacin on mitogen (phytohaemagglutinin; PHA)-activated proliferative responses of human mononuclear leukocytes (MNL) using a conventional assay based on the uptake of radiolabelled thymidine.

- Flow cytometric assessment of the effects of moxifloxacin on the expression of CD25 (interleukin-2 receptor) on MNL activated with PHA.

- Measurement of the cytokine profiles (both pro-inflammatory and anti-inflammatory cytokines) of PHA-activated MNL using suspension bead protein array technology (Bio-Plex™).

# *Chapter 3: Effects of Moxifloxacin on Human Neutrophil Functions*

## 3.1 INTRODUCTION

Neutrophils are an essential component of the inflammatory response and for the resolution of microbial infection. On encountering microorganisms, neutrophils engulf these microbes into a phagosome, which fuses with intracellular granules to form a phagolysosome, in which the organisms are killed after exposure to antimicrobial enzymes and peptides, and reactive oxygen species (ROS). The laboratory research described in this chapter was undertaken with the primary objective of determining the effects of moxifloxacin on human neutrophil activities, with emphasis on superoxide production, elastase release and calcium fluxes.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Reagents

Moxifloxacin was kindly supplied by Bayer Healthcare AG, Leverkusen, Germany. Moxifloxacin was dissolved in sterile distilled water and used in the assays described below at final concentrations of 0.5 – 20 µg/ml. Recombinant pneumolysin was kindly supplied by Professor T.J. Mitchell, Division of Infection and Immunity, University of Glasgow, UK. Unless indicated all other reagents were purchased from Sigma Diagnostics (St. Louis, Mo, USA).

### 3.2.2 Neutrophil isolation

Purified human neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult volunteers and separated from mononuclear leukocytes by centrifugation on Histopaque®-1077 cushions at 400 g for 25 minutes at room temperature. The granulocyte/erythrocyte fractions were sedimented with 3% gelatin for 15 minutes at 37 ºC to remove most of the red blood cells (RBC). After centrifugation, residual RBCs were removed by selective lysis with 0.84% ammonium chloride at 4 ºC for 10 minutes. The neutrophils, which were routinely of high purity and viability (>90%), were resuspended to 1 x $10^7$ cells/ml in phosphate-buffered saline (PBS; 0.15M, pH 7.4) and held on ice until use. Purity of isolated neutrophils was assessed microscopically and assessment of viability was done by dye-exclusion using 0.1% methylene blue.

### 3.2.3 Oxidant generation

The generation of superoxide and oxidants derived from the MPO/$H_2O_2$/halide system, were measured using lucigenin (bis-N-methylacridinium nitrate) and luminol (5-amino-2, 5-dihydro-1, 4-phthalazinedione)-enhanced chemiluminescence (LECL) methods respectively (Minkenberg and Ferber, 1984). Neutrophils (1 x $10^6$ final) were preincubated for 15 minutes in 900 µl indicator-free Hanks balanced salt solution (HBSS, pH 7.4, Highveld Biological, Johannesburg, RSA) in combination with 0.2 mM lucigenin or 0.1 mM luminol in the presence and absence of moxifloxacin (1-20 µg/ml, final), prior to activation with phorbol 12-myristate 13-acetate (PMA, 25 ng/ml final). Spontaneous and PMA-activated LECL responses were recorded using an LKB Wallac 1251 chemiluminometer (Turku, Finland) and the readings were recorded as mV/sec. Additional experiments were performed, in the same manner, to investigate the effect of moxifloxacin on the LECL response of neutrophils activated with the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1 µM, final) using a Lumac Biocounter® M2010 (Lumac Systems, Schaumberg, The Netherlands). LECL readings were integrated for 10-second intervals and recorded as relative light units (r.l.u).

### 3.2.4 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils (1 x $10^6$ cells/ml final) suspended in HBSS in the presence and absence of moxifloxacin (1, 2.5, 5, 10 and 20 µg/ml) was incubated for 10 minutes at 37 ºC. The stimulant fMLP (1 µM final) in combination with cytochalasin B (CB, 1µM) was then added to the cells, which were incubated for 15 min at 37˚C, after which the tubes were transferred to an ice bath, followed by centrifugation at 250 g for 10 minutes to pellet the cells. The neutrophil-free supernatants were assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al.,* 1982). Briefly, 125 µl of supernatant fluid (SNF) was added to 125 µl of the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide, 3 mM in 0.05 M Tris-HCl (pH 8.0). Elastase activity was monitored at the wavelength of 405 nm using a Power $Wave_x$ plate spectrophotometer (Bio-Tec instruments, Inc.,) and the results expressed as the mean

percentages of the total cellular enzyme content released during activation by the corresponding fMLP/CB-activated, drug-free control systems.

### 3.2.5 Radiometric assessment of $Ca^{2+}$ fluxes

Calcium-45 chloride ($^{45}Ca^{2+}$, specific activity 18.53 mCi/mg, Du Pont NEN Research Products, Boston, MA, USA) was used as tracer to label the intracellular $Ca^{2+}$ pool and to monitor $Ca^{2+}$ influx in resting and moxifloxacin-treated neutrophils, as well as efflux of the cation. In the assays of $Ca^{2+}$ influx and efflux described below, the radiolabelled cation was used at a fixed, final concentration of 2 µCi/ml, containing 50 µM cold carrier $Ca^{2+}$ (as $CaCl_2$) and the final assay volumes were 5 ml containing a total of 1 x $10^7$ neutrophils. The standardization of the procedure used to load the cells with $^{45}Ca^{2+}$ has been described previously (Anderson and Goolam Mahomed, 1997).

#### 3.2.5.1 Influx of $^{45}Ca^{2+}$ into moxifloxacin-treated neutrophils

This procedure was used to measure the magnitude of $Ca^{2+}$ influx following activation of neutrophils with the chemotactic tripeptide fMLP, as well as the effect of moxifloxacin on $Ca^{2+}$ influx. To measure the net influx of $^{45}Ca^{2+}$ into fMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were preincubated for 15 min at 37 ºC in $Ca^{2+}$-replete HBSS, then pelleted by centrifugation and resuspended to 1 x $10^7$/ml in HBSS containing 250 µM cold $Ca^{2+}$. Pre-loading of neutrophils with cold $Ca^{2+}$ was undertaken to ensure that intracellular $Ca^{2+}$ stores were replete, thereby minimizing spontaneous uptake of $^{45}Ca^{2+}$ in the influx assay. The $Ca^{2+}$-loaded neutrophils (2 x $10^6$/ml) were then preincubated for 10 min at 37 ºC in HBSS containing a final concentration of 50 µM cold, carrier $Ca^{2+}$ in the presence and absence of moxifloxacin (10 µg/ml final). This was followed by the simultaneous addition of fMLP (1 µM) and $^{45}Ca^{2+}$ (2 µCi/ml), or $^{45}Ca^{2+}$ only to control, unstimulated systems. The influx of $^{45}Ca^{2+}$ into fMLP-activated neutrophils was determined 5 min later when influx is complete, and the values compared with the uptake of the radiolabelled cation by identically processed unstimulated cells using liquid scintillation spectrometry. Briefly, the cells were washed twice in ice-cold HBSS, followed by lysis of the cell pellets with 0.5 ml of Triton X-100/ NaOH

(0.1%: 0.05 M), addition of scintillation cocktail and detection of the amount of cell-associated radioactivity (counts per minute) using a Tri-Carb – 2100TR (Packard) liquid scintillation spectrometer. Additional experiments were performed in the same manner to measure the influx of $^{45}Ca^{2+}$ into pneumolysin (8.37 ng/ml)-activated neutrophils.

### 3.2.5.2 Efflux of $^{45}Ca^{2+}$ from moxifloxacin-treated neutrophils

To measure net efflux of $^{45}Ca^{2+}$ from neutrophils, uncomplicated by concomitant influx of the radiolabelled cation, the cells ($10^7$/ml) were loaded with $^{45}Ca^{2+}$ (2 µCi/ml) for 15 min at 37 ºC in HBSS. The neutrophils were then pelleted by centrifugation, washed once with, and resuspended in $Ca^{2+}$-replete HBSS. The $^{45}Ca^{2+}$-loaded neutrophils (2 x $10^6$/ml) were then pre-incubated for 10 min at 37 ºC followed by addition of moxifloxacin (10 µg/ml final), activation with fMLP (1 µM) and measurement of the net efflux of $^{45}Ca^{2+}$ after a fixed time period of 60 sec. Reactions were stopped by adding 10 ml ice-cold $Ca^{2+}$-replete HBSS to the tubes, which were then transferred immediately to an ice-bath. The cells were pelleted by centrifugation at 400 g for 5 min followed by washing with ice-cold $Ca^{2+}$-replete HBSS and the cell pellets finally dissolved in 0.5 ml of Triton X-100/ NaOH (0.1%: 0.05 M), and radioactivity assayed in a scintillation spectrometer.

### 3.2.6 Cellular ATP levels

Measurement of cellular ATP levels was performed to investigate the cytotoxic potential of moxifloxacin for neutrophils. Neutrophils (1 x $10^6$ cells/ml) were incubated in the presence and absence of moxifloxacin (2.5, 5, 10 and 20 µg/ml) for 10 min in a 37ºC waterbath. Following incubation, 20 µl of cell suspension were added into pre-prepared chemiluminometer cuvettes containing 100 µl of nucleotide releasing agent (NRS), which causes release of ATP from the cells, and 30 µl of ATP assay mix dilution buffer (FL-AAM). After vortexing, 20 µl of ATP assay mix was added to the mixture, and chemiluminescence measured using the Lumac Biocounter® 2010M and the results (r.l.u.) converted to nmoles/$10^6$ cells using a standard curve.

### 3.2.7 STATISTICAL ANALYSIS

The results of each series of experiments are expressed as the mean values ± the standard error of the mean (SEM). Levels of statistical significance were calculated by paired Student's *t*-test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. P values of $\leq 0.05$ were considered significant.

## 3.3 RESULTS

### 3.3.1 Effects of moxifloxacin on luminol- and lucigenin-enhanced chemiluminescence responses of neutrophils activated with fMLP or PMA

Neutrophils (1 x $10^6$ cells/ml) were pre-incubated in the presence of 0.1mM luminol (5-amino-2,5-dihydro-1,4-phthalazinedione) or 0.2 mM lucigenin (10-10'-dimethyl-bis-9,9'-biacridinium nitrate) with moxifloxacin at final concentrations of 1, 2.5, 5, 10 and 20 µg/ml for 15 minutes at 37 °C. The cells were then exposed to the activators of oxidant production, fMLP (1 µM) or PMA (25 ng/ml). The results of these experiments with fMLP and PMA are shown in Tables 2.1a, b and 2.2a, b respectively. In both luminol- and lucigenin-chemiluminescence systems, moxifloxacin did not affect oxidant (superoxide and oxidants derived from the MPO/$H_2O_2$/halide system) generation by either fMLP- or PMA-stimulated neutrophils.

### 3.3.2 Effect of moxifloxacin on elastase release by fMLP/CB-activated neutrophils

Moxifloxacin at concentrations of 1, 2.5, and 5 µg/ml did not affect the release of elastase by neutrophils measured 30 min after the addition of fMLP/CB, while at concentrations of 10 and 20 µg/ml release of elastase was significantly increased. These results are presented in Figure 2.1. Moxifloxacin alone did not affect the release of elastase from neutrophils. The values for the untreated control were 100 ± 9, and for moxifloxacin (1, 2.5, 5, 10 and 20 µg/ml)-treated cells were 112 ± 9, 110 ± 6, 122 ± 3, 117 ± 3 and 110 ± 6 percentage of control/$10^6$ cells respectively.

### 3.3.3 Effect of moxifloxacin on neutrophil ATP levels as an index of viability

The ATP content of the neutrophils was measured using the luciferin-luciferase firefly luminescence method (Jabs *et al.,* 1997). These experiments were performed to investigate the cytotoxic potential of moxifloxacin for neutrophils. Neutrophils were exposed to moxifloxacin (2.5 - 20 µg/ml) for 10 min at 37 °C. The values for untreated cells were 65.2 ± 5.2 nmoles/$10^6$ cells while the values for those treated with moxifloxacin (2.5, 5, 10 and 20 µg/ml) were 64.4 ± 5.6, 67.4 ± 7.9, 70.8 ± 5.7, and 64.9 ± 5.7 nmoles/$10^6$ cells respectively. These results indicate that treatment of neutrophils with moxifloxacin at concentrations of up to 20 µg/ml does not affect cell viability.

### 3.3.4 Effects of moxifloxacin on $^{45}Ca^{2+}$ fluxes

#### 3.3.4.1 Effects on influx of $^{45}Ca^{2+}$

For these experiments, neutrophils were preloaded with cold $Ca^{2+}$ (to minimize the spontaneous uptake of $^{45}Ca^{2+}$ in the influx assay), transferred to $Ca^{2+}$-free HBSS, and incubated with moxifloxacin for 10 min at 37 °C prior to the simultaneous addition of fMLP (1 µM) and $^{45}Ca^{2+}$ (2 µCi/ml). Activation of control, drug-free neutrophils with fMLP resulted in a delayed influx of $^{45}Ca^{2+}$, which occurred after a lag phase of 30 – 60 sec. The influx of $^{45}Ca^{2+}$ appeared to be a true consequence of the activation of neutrophils with fMLP, as the unstimulated control did not show a marked increase in intracellular $^{45}Ca^{2+}$ levels. The mean uptakes of $^{45}Ca^{2+}$ by fMLP-activated control and moxifloxacin-treated neutrophils were 526 ± 35 and 570 ± 32 pmol$^{45}Ca^{2+}$ /$10^7$ cells respectively, measured 5 min after addition of fMLP when influx is completed (Anderson and Goolam Mahomed, 1997).

Measurement of the effects of moxifloxacin on pneumolysin-mediated influx of $^{45}Ca^{2+}$ into human neutrophils revealed that moxifloxacin (10 µg/ml) did not affect the pore-forming interactions of pneumolysin (8.37 ng/ml) with neutrophils. The mean uptakes of $^{45}Ca^{2+}$ were 151 ± 7 for the pneumolysin-free control system, and 2538 ± 60 and 2494 ± 113 pmol$^{45}Ca^{2+}$ /$10^7$ cells for the pneumolysin-treated systems in the absence and presence of moxifloxacin respectively. To confirm that moxifloxacin does not affect calcium fluxes, additional experiments were performed

to determine the effects of varying concentrations of moxifloxacin (2.5 – 20 µg/ml) on the spontaneous influx of $^{45}Ca^{2+}$ into unstimulated neutrophils. At all concentrations tested, moxifloxacin did not affect spontaneous $Ca^{2+}$ uptake. The mean peak values were 667 ± 22 for resting cells and 684 ± 17, 667 ± 21, 648 ± 26 and 711 ± 53 pmol/$10^7$ cells for moxifloxacin (2.5, 5, 10 and 20 µg/ml)-treated neutrophils.

### 3.3.4.2 Effects on efflux of $^{45}Ca^{2+}$

In these experiments, neutrophils, which were pre-loaded with $^{45}Ca^{2+}$ (2 µCi/ml), washed and transferred to $Ca^{2+}$-replete HBSS, were activated with fMLP (1 µM) in the presence and absence of moxifloxacin (10 µg/ml), added 1 min prior to fMLP, followed by measurement of cell-associated $^{45}Ca^{2+}$. Moxifloxacin did not affect the magnitude of efflux of $^{45}Ca^{2+}$ from fMLP-activated neutrophils. The amounts of $^{45}Ca^{2+}$ discharged from unstimulated neutrophils were 567 ± 22 as compared to 1686 ± 34 and 1629 ± 29 pmoles/$10^7$ cells discharged from fMLP-activated control and moxifloxacin-treated neutrophils, respectively.

## 3.1 LUCIGENIN-ENHANCED CHEMILUMINESCENCE

**Table 3.1a: Effects of moxifloxacin (1 - 20 µg/ml) on superoxide production by fMLP-activated neutrophils.**

| System | Superoxide production (r.l.u) |
|---|---|
| Resting cells | 820 ± 58 |
| fMLP control | 3891 ± 563 |
| fMLP + Moxifloxacin 1 µg/ml | 3883 ± 550 |
| fMLP + Moxifloxacin 2.5 µg/ml | 3967 ± 577 |
| fMLP + Moxifloxacin 5 µg/ml | 3577 ± 547 |
| fMLP + Moxifloxacin 10 µg/ml | 3796 ± 472 |
| fMLP + Moxifloxacin 20 µg/ml | 4211 ± 456 |

The results of 10 experiments are presented as the mean absolute peak values ± SEMs measured 1 min after the addition of fMLP.

**Table 3.1b: Effects of moxifloxacin (1 - 20 µg/ml) on superoxide production by PMA-activated neutrophils.**

| System | Superoxide production (mV/s) |
|---|---|
| Resting cells | 485 ± 82 |
| PMA control | 3379 ± 195 |
| PMA + Moxifloxacin 1 µg/ml | 3504 ± 188 |
| PMA + Moxifloxacin 2.5 µg/ml | 3539 ± 228 |
| PMA + Moxifloxacin 5 µg/ml | 3856 ± 340 |
| PMA + Moxifloxacin 10 µg/ml | 3456 ± 221 |
| PMA + Moxifloxacin 20 µg/ml | 3367 ± 194 |

The results of 13 experiments are presented as the mean absolute peak values ± SEMs measured 10 min after the addition of PMA.

## 3.2 LUMINOL-ENHANCED CHEMILUMINESCENCE

**Table 3.2a: Effects of moxifloxacin (1 - 20 µg/ml) on production of oxidants by the MPO/$H_2O_2$/halide system following activation of neutrophils with fMLP.**

| System | Superoxide production (r.l.u) |
|---|---|
| Resting cells | 3142 ± 211 |
| fMLP control | 35898 ± 7256 |
| fMLP + Moxifloxacin1 µg/ml | 33838 ± 6574 |
| fMLP + Moxifloxacin 2.5 µg/ml | 33965 ± 6095 |
| fMLP + Moxifloxacin 5 µg/ml | 34069 ± 6291 |
| fMLP + Moxifloxacin 10 µg/ml | 34691 ± 6325 |
| fMLP + Moxifloxacin 20 µg/ml | 34711 ± 4859 |

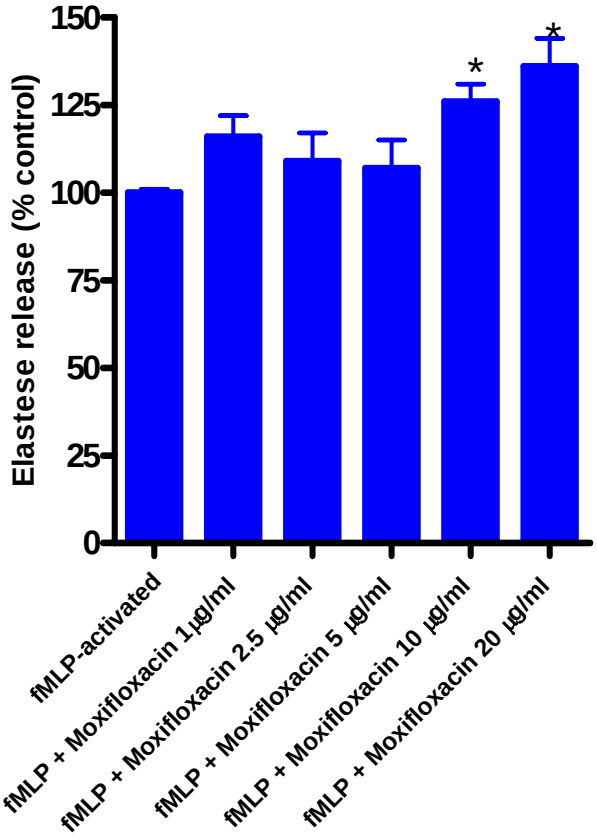
The results of 3 experiments are presented as the mean absolute peak values ± SEMs measured 1 min after the addition of fMLP.

**Table 3.2b: Effects of moxifloxacin (1 - 20 µg/ml) on production of oxidants by the MPO/$H_2O_2$/halide system following the activation of neutrophils with PMA.**

| System | Superoxide production (mV/s) |
|---|---|
| Resting cells | 1804 ± 82 |
| PMA control | 23938 ± 1946 |
| PMA + Moxifloxacin 1 µg/ml | 22108 ± 1770 |
| PMA + Moxifloxacin 2.5 µg/ml | 21217 ± 1656 |
| PMA + Moxifloxacin 5 µg/ml | 20868 ± 1680 |
| PMA + Moxifloxacin 10 µg/ml | 21040 ± 1816 |
| PMA + Moxifloxacin 20 µg/ml | 19273 ± 1521 |

The results of 3 experiments are presented as the mean absolute peak values ± SEMs measured 10 min after the addition of PMA.

**Fig 3.1: Effects of Moxifloxacin on the release of elastase by fMLP/CB-activated neutrophils.**




The results of 4 experiments are presented as the mean values ± SEMs for elastase measured in the supernatants of control and moxifloxacin-treated neutrophils 15 min after the addition of fMLP/CB.

## 3.4 DISCUSSION

Cellular signalling events leading to systemic inflammation are complex. It is generally accepted that recruitment and activation of leukocytes contribute to tissue damage in inflammation (Lucas *et al.,* 2003). Neutrophils migrate to the site of inflammation, where they are activated by different stimuli, resulting in the generation of large amounts of ROS and the release of granular enzymes such as elastase and myeloperoxidase (Choi *et al.,* 2003), which participate in tissue injury. There are a multitude of intracellular processes, which are linked to, or precede the production of inflammatory mediators (Hirayama *et al.,* 2000).

It has been highlighted recently that several fluoroquinolones, including moxifloxacin play an important role in host defence by priming (sensitizing) mature human phagocytes such as neutrophils and mononuclear leukocytes. For example, the ability of fMLP and PMA to stimulate superoxide production is significantly enhanced by pre-incubation of neutrophils with several fluoroquinolones (Niwa *et al.,* 2002; Dalhoff and Shalit, 2003). However, relatively little is known about the effect of moxifloxacin on host defences, particularly innate protective mechanisms involving neutrophils.

Lucigenin and luminol chemiluminescence measurements were used to evaluate the production of superoxide and HOCl respectively during neutrophil activation. Using both lucigenin- and luminol-chemiluminescence systems, moxifloxacin was found to have no significant priming or inhibitory effects on oxidant generation by human neutrophils stimulated with either PMA (which acts via a cascade involving protein kinase C) or fMLP (via a G-protein-coupled receptor) (Liao *et al*., 2005).

Some preliminary studies suggest that neutrophils kill ingested microorganisms by subjecting them to high concentrations of highly toxic ROS and bring about myeloperoxidase (MPO)-catalysed halogenation. Studies performed by Reeves and colleagues (2002) suggest that ROS generation and MPO activity are not themselves sufficient to kill microorganisms, instead ROS and cytoplasmic granules released into the phagocytic vacuole in which the microbes are encapsulated act together to destroy

internalized microbes.

When neutrophils are activated, receptors, adhesion molecules, and enzymes are translocated to the cell surface from intracellular granules, and some of these factors such as elastase (Mayer-Scholl *et al.,* 2004) and MPO (Topham *et al.,* 1998) are secreted. The process of degranulation influences neutrophil functional responses including adhesion to surfaces, aggregation, motility, and microbial killing. In subjects with granule deficiencies or impaired degranulation mechanisms, these events are altered and host defense is compromised (Tophan *et al*., 1998). In this part of my study, neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophil elastase is a neutrophil-derived toxic molecule that has been considered crucial in the pathophysiology of acute inflammatory responses (Kawabata *et al.,* 2002; Korkmaz *et al.,* 2005). Interestingly, moxifloxacin treatment at higher concentrations (10 and 20 μg/ml) showed a significant enhancement of elastase release by human neutrophils following activation with fMLP/CB for 15 min, suggesting that moxifloxacin at higher concentrations enhances neutrophil degranulation. Importantly, elastase, as well as other neutrophil granule serine proteases possesses antimicrobial activity (Reeves *et al.,* 2002), suggesting that moxofloxacin, albeit at relatively high concentrations, has potentiate the bactericidal actions of neutrophils.

Since degranulation by activated neutrophils is a $Ca^{2+}$-dependent process (Nüsse *et al.,* 1997; Ramafi *et al.,* 1999), the biochemical processes which mediate an increase in $[Ca^{2+}]_{i,}$ as well as those which restore $Ca^{2+}$ homeostasis, were identified as possible targets of moxifloxacin. Increases in the concentrations of cytosolic $Ca^{2+}$ are a powerful stimulus to cell activation (Li, 1998; Lucas, *et al.,* 2003). In inflammatory cells such as neutrophils or macrophages, in which $Ca^{2+}$ influx is not mediated by voltage activated channels (Hallett, 2003), receptor mediated and store-operated $Ca^{2+}$ influx pathways appear to be important mechanisms for calcium entry (Patterson and Rossum, 1999).

However, the results of the experiments using the radiometric procedures demonstrated that neither the efflux (an indirect measurement of the abruptly

occurring release of $Ca^{2+}$ from intracellular stores) nor the store-operated influx of $Ca^{2+}$ was affected by moxifloxacin. These results indicate that moxifloxacin alone does not induce movement of $Ca^{2+}$ into or out of neutrophils, neither does it affect the magnitude of efflux or store-operated influx following activation of neutrophils with fMLP. Taken together, these observations clearly demonstrate that moxifloxacin does not affect the fMLP-receptor-G-protein interactions that lead to the activation of phospholipase C which in turn mediate generation of $IP_3$ in fMLP-activated neutrophils.

Notwithstanding complement-activating properties, pore-forming interactions with neutrophils and monocytes, resulting in influx of $Ca^{2+}$, have been implicated in the pro-inflammatory activities of the pneumococcal toxin, pneumolysin (Cockeran *et al.*, 2003). Rather than contributing to the eradication of the infection, however, the resultant, predominantly neutrophil-mediated inflammatory response appears to favour persistence and extrapulmonary dissemination of the pneumococcus (Cockeran *et al.*, 2003). In the current study, moxifloxacin did not affect pneumolysin-mediated influx of $Ca^{2+}$, indicating that moxifloxacin neither antagonizes nor potentiates the pore-forming activities of pneumolysin. Although antagonism of pneumolysin would be a beneficial property of antibiotics used in the treatment of pneumococcal diseases, moxifloxacin appears to be neutral in this respect.

Some preliminary studies reviewed by Dalhoff and Shalit (2003) suggest that fluoroquinolones interact directly with bacteria to inhibit adherence to and colonization of epithelial surfaces, reducing the release of pro-inflammatory bacterial products, while increasing uptake by phagocytosis and intracellular killing. In the case of the quinolones ofloxacin, ciprofloxacin, sparfloxacin and temafloxacin, Aoki *et al.*, (1994) found that these agents differ in their immunomodulating effects. For ofloxacin as well as ciprofloxacin, they found enhancement of the oxidative burst, but in contrast, inhibitory effects on the oxidative burst were observed for sparfloxacin and temafloxacin.

The results of the current study are in agreement with those of Fischer and Adam (2001) who demonstrated that moxifloxacin does not affect oxidative burst activity. I have found that moxifloxacin at therapeutically relevant concentrations and higher does not directly antagonize or enhance phagocytic functions, since it has little or no direct effects on the pro-inflammatory responses of human neutrophils. These observations are compatible with a mechanism whereby moxifloxacin potentiates host defenses indirectly by weakening bacteria, preventing their adherence to host tissues, while increasing susceptibility to phagocytosis and intracellular killing, as opposed to direct immunomodulatory interactions with neutrophils.

# *Chapter 4: Effects of Moxifloxacin on PHA-Activated Human Mononuclear Cells*

## 4.1 INTRODUCTION

Activation of lymphocytes involves a complex, and finely regulated cascade of events that results in the expression of cytokine receptors, production and secretion of cytokines and expression of several surface molecules that eventually lead to divergent immune responses. The laboratory research presented in this chapter was designed to evaluate the effect of moxifloxacin on lymphocyte proliferation, expression of the activation marker CD25 (a hallmark of cellular activation, present on peripheral blood lymphocytes with density increasing *in vitro* upon activation) and cytokine production by PHA-activated lymphocytes.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Reagents

Moxifloxacin was used, as described in 3.2.1 (page 44) of this dissertation. Bio-Plex™ cytokine assay kits were purchased from Bio-Rad, Hercules, CA, USA. Monoclonal antibodies were purchased from Beckman Coulter.

### 4.2.2 Isolation of mononuclear leukocytes

Purified human MNL were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy adult volunteers and separated from granulocytes by centrifugation on Histopaque®-1077 cushions at 400 g for 25 min at room temperature. Due to the various densities of the different types of cells, differential sedimentation velocity resulted in the formation of four cell fractions; plasma containing thrombocytes, mononuclear leukocytes, ficoll and the mixture of erythrocytes and granulocytes. The mononuclear leukocyte (MNL) layer was removed and cells were washed with PBS containing ethylene glycol-bis (beta-amino-ethyl-ether)-N, N, N', N'- tetraacetic acid (EGTA, 1 mM) to prevent aggregation of the cells. After centrifugation at 250 g for 10 minutes, residual erythrocytes were removed by selective lysis with 0.84% $NH_4Cl$ for 10 minutes at 4˚C. The resultant pellet was then washed with PBS/EGTA. The MNLs which were routinely of high purity and viability (>90%), were then resuspended to 1 x $10^7$ cells/ml in RPMI 1640 tissue culture medium and held on ice until use. Purity of isolated lymphocytes was assessed microscopically and assessment of viability was done by dye-exclusion using 0.1% methylene blue.

### 4.2.3 Lymphocyte proliferation assay (LPA)

This procedure measures the ability of lymphocytes to undergo polyclonal/clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. Cells were resuspended at 1 x $10^6$ cells/ml in RPMI 1640 culture medium. Using 96 well flat-bottomed microtitre plates, lymphocytes (50 µl of a 1 x $10^6$ cells/ml MNL suspension i.e. 5 x $10^4$ cells/well) were supplemented with fetal calf serum (FCS; 10% final, 20µl/well) in the presence and absence of moxifloxacin (2.5, 5 and 10 µg/ml; 20 µl/well). The plates were then incubated for 24hrs at 37˚C in a humidified $CO_2$ incubator (5% $CO_2$) before the addition of the mitogen phytohemagglutinin (PHA 2.5 and 5 µg/ml; 20 µl/well). The final volume in each well was 200 µl. The plates were agitated gently on a microplate agitator for 5 seconds and incubated for a further 48 hrs. Proliferation was assessed radiometrically according to the magnitude of uptake of tritiated thymidine ($^3$H, specific activity 0.2 µCi/well, Du Pont-NEN, Research Products, Boston, MA, USA) for 18 hrs, into the newly synthesized DNA of the dividing cells. Cells were then harvested on glass fiber filters using the PHD multi-well cell harvester (Cambridge Technology, USA). The disks were dried using methanol, placed in a glass vials, followed by the addition of 4 ml scintillation fluid (Packard Bioscience, USA). The amount of radioactivity incorporated into DNA in each well was measured using a liquid scintillation counter (TRI-CARB – 2100TR, Packard, Canberra Co, USA) and expressed as counts per minute.

### 4.2.4 Analysis of CD25 expression

The effects of moxifloxacin on the functional response of lymphocytes were also assessed according to the expression of the surface activation marker CD25 (IL-2αR), which is an alternative method to evaluate T-cell proliferation. Lymphocytes (1 x $10^6$ cells/ml) were resuspended in RPMI 1640 in the presence and absence of moxifloxacin (0.625 - 10 µg/ml) and incubated for 24 hrs at 37˚C in a $CO_2$ incubator (5% $CO_2$) before the addition of the mitogen, PHA (2.5 and 5 µg/ml). The tubes, which contained a final volume of 2 ml were incubated for a further 24 hrs, and CD25 was detected flow cytometrically using an anti-CD25 FITC-conjugate. Briefly, 500 µl of cultured lymphocyte suspension were diluted with 500 µl HBSS. The cells were then incubated for 15 min at room temperature in the dark with anti-CD25 FITC monoclonal antibodies (mAb), or anti-IgG FITC conjugate for detection of

nonspecific background staining. The Epics Altra (Beckman Coulter, Miami, FL, USA) equipped with a water-cooled coherent Enterprise laser, was used to detect the CD25 positive cells. Expo 32 software (Beckman Coulter) was used to analyze the results obtained. Both the percentage CD25 positive cells and the density of the activation marker expression (mean fluorescence intensity, MFI) were calculated and represented as the normalized mean fluorescence intensity (NMFI) value, which is the percentage of positive lymphocytes multiplied by the corresponding MFI values (Oxenhandler *et al.*, 1984).

### 4.2.5 Cytokine production by PHA=activated mononuclear leukocytes

#### 4.2.5.1 Suspension array system for cytokine assay

This assay employs a bead-based sandwich enzyme immunoassay technique. A monoclonal antibody specific for the cytokine of interest is coupled onto a designated bead with a known internal fluorescence. Cytokine standards, provided as a lyophilized cocktail, were reconstituted with 500 µl RPMI 1640 to obtain stock concentrations of 50 000 pg/ml of each cytokine. Serial 4 fold dilutions (1.95 - 32 000 pg/ml) for control cytokines were used in all the experiments. The conjugated beads and detection antibodies were diluted 25-fold using assay buffer, while streptavidin-PE was diluted 100-fold using detection diluent.

#### 4.2.5.2 Cytokine detection

Experiments were performed to measure the effects of moxifloxacin on the cytokine profiles (both pro-inflammatory and anti-inflammatory) of PHA-activated MNLs using suspension bead protein array technology (Bio-Plex™). Mononuclear cells (1 x $10^6$ cells/ml) were resuspended in RPMI 1640 in the presence and absence of moxifloxacin (0.625 - 10 µg/ml) and incubated for 24 hrs at 37˚C with 5% $CO_2$ before the addition of the mitogen, PHA (2.5 and 5 µg/ml). The tubes, which contained a final volume of 2 ml, were incubated for a further 24 hrs, and culture supernatants were collected prior to the cytokine assay. Briefly, the assay was performed using 96 well filter plates that were first saturated with 200 µl of assay buffer. The plates were then covered with plastic lids and incubated for 1 hr at room temperature. The assay buffer was removed by using a vacuum manifold apparatus, followed by gentle blotting of the plates on paper. The conjugated beads (50 µl/well) were added, and

deposited on the filter by exposing the plate to a vacuum, after which the plate was washed twice with 100 µl of wash buffer. In each designated well, 50 µl of standard, control, or culture supernatant were added in duplicate. The plates were covered with a sealing tape and aluminium foil, agitated for 30 sec on the plate agitator and for 60 min at 300 rpm on the orbital shaker at room temperature to promote bead-cytokine binding. After incubation, the plates were vacuum filtered as before, followed by the addition of the detection biotinylated antibodies (25 µl/well). The plates were again covered with a sealing tape and aluminium foil, agitated for 30 sec and incubated for 30 min at room temperature on the orbital shaker at 300 rpm. The plates were drained and washed three times with 100 µl/well wash buffer. For the detection and quantification of each captured cytokine, 50 µl of streptavidin-PE (a fluorescently labelled reporter molecule that specifically binds to the analyte), was added to each well. The plates were sealed again, as before, and mixed for 10 min at 300 rpm on the orbital shaker at room temperature, followed by a triplicate wash step, with 100 µl/well wash buffer. After the third wash, assay buffer (125 µl/well) was added into each well, and the plates agitated for 30 sec. The contents of each well were analyzed using Bio-Plex™ plate reader software (version 3.0) and the concentration of each cytokine (pg/ml) calculated from standard curve generated. The following cytokines were assayed: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, and TNF-α.

### 4.2.6 Statistical analysis

Statistical analyses were performed as described in 3.2.7 (page 48)

## 4.3 RESULTS

### 4.3.1 Effects of moxifloxacin on lymphocyte proliferation and expression of CD25

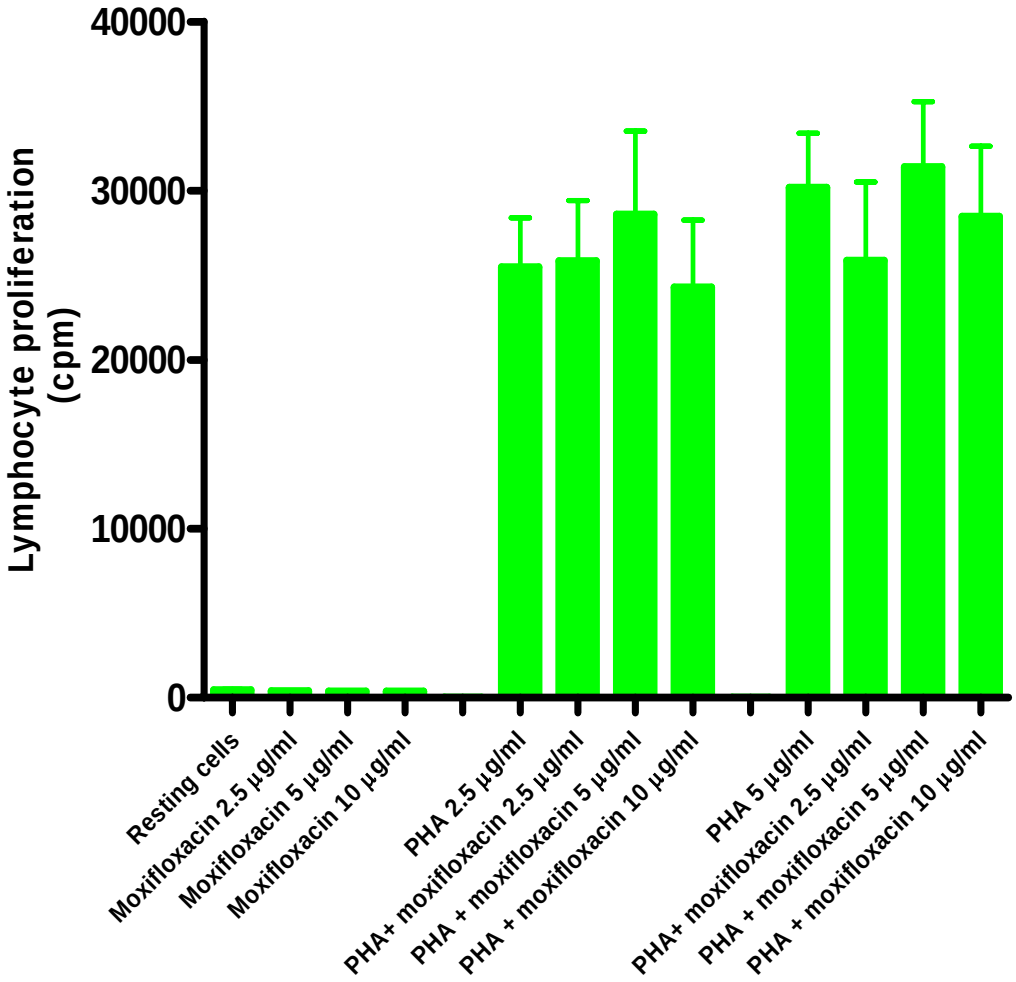
To investigate the effect of moxifloxacin on lymphocyte proliferation, $^{3}$H-thymidine incorporation into newly synthesized DNA of T-cells was measured after treatment of cells with moxifloxacin followed by activation with PHA, while expression of CD25 was measured by flow cytometry. As shown in Figures 4.1 and 4.2 moxifloxacin at

concentrations 0.625, 1.25, 2.5, 5 and 10 μg/ml did not affect either lymphocyte proliferation or CD25 expression.

### 4.3.2 Effects of moxifloxacin on cytokine production by PHA-stimulated mononuclear leukocytes cultures

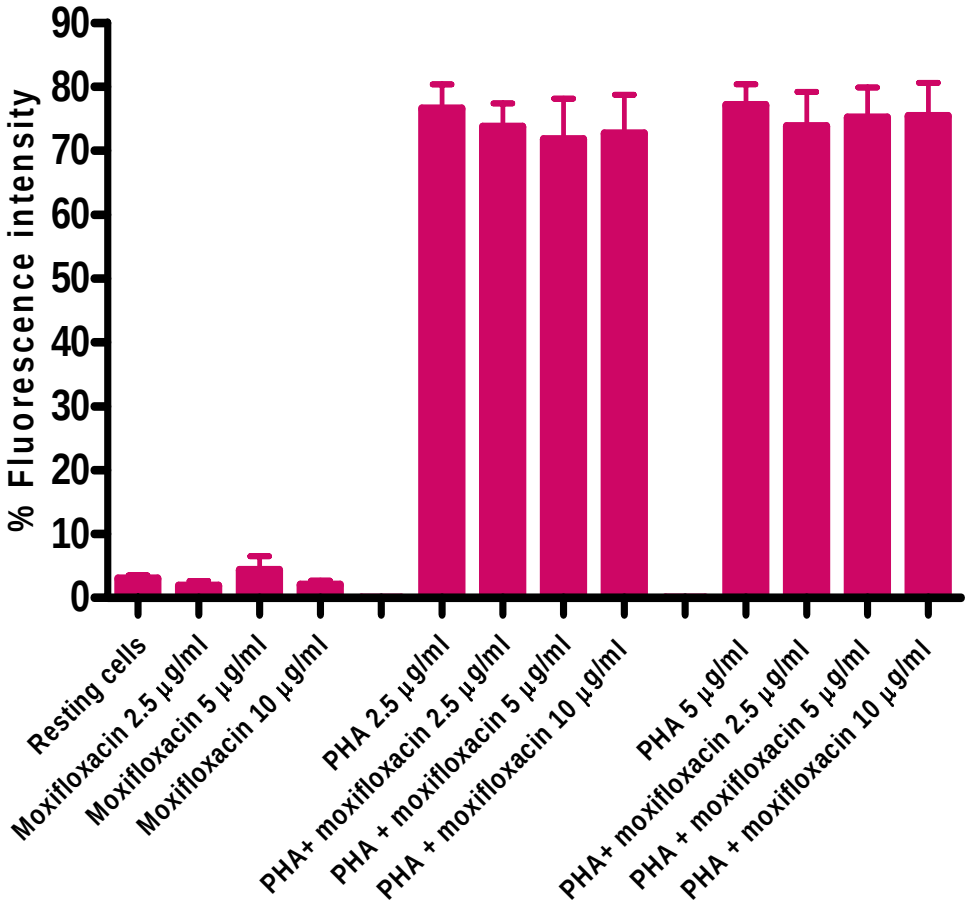
Stimulation of lymphocytes with PHA (2.5 and 5μg/ml) resulted in a significant increase in the production of most of the cytokines in the cell culture supernatants, which was more pronounced with PHA 5μg/ml. As shown in Table 4.1, exposure of PHA-stimulated lymphocytes to moxifloxacin (0.625 - 10 μg/ml) did not have a significant effect on the production of the following cytokines: IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL13, IL-17, IFN-γ, GM-CSF, G-CSF, TNF-α, and MCP-1. Although levels of IL-6 and IL-12 were decreased following exposure of MNL to moxifloxacin, the magnitudes of inhibition did not achieve statistical significance (Table 4.1 and Figures 4.3 and 4.4).

**Fig 4.1 Effects of moxifloxacin on proliferation of PHA-activated MNL**




The results of 15 experiments are presented as the mean values ± SEMs for uptake of radiolabelled thymidine by unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

**Fig 4.2 Effect of moxifloxacin on CD25 expression by PHA-activated MNL**



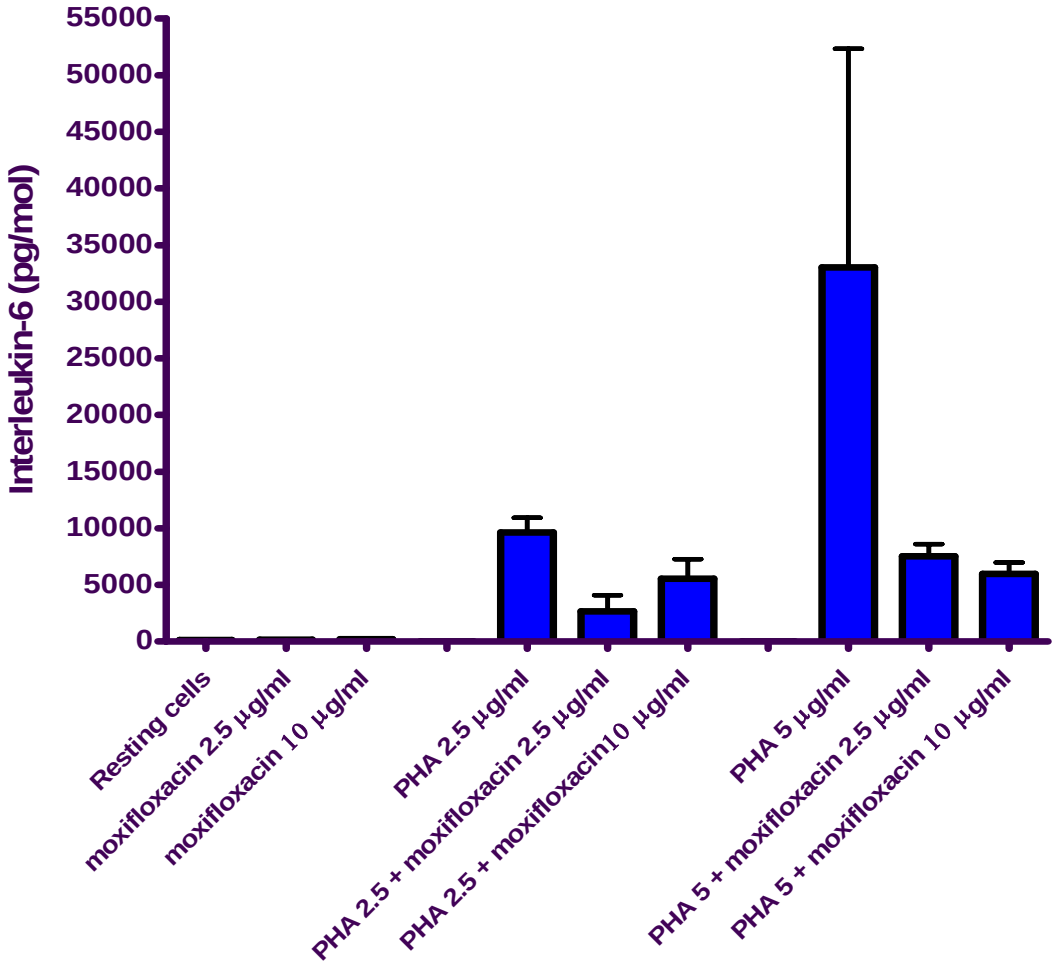

The results of 7 experiments are presented as the mean values ± SEMs for CD25 expression of unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

**Table 4.1 Effects of Moxifloxacin on IL-6 and IL-12 production by PHA-activated MNLs**

| CYTOKINE | IL-6 (pg/ml) | IL-12 (pg/ml) |
|---|---|---|
| Resting cells | 154.5 ± 9.3 | 1.8 ± 0.5 |
| Moxifloxacin (2.5 µg/ml) | 193.3 ± 21.8 | 1.6 ± 0.7 |
| Moxifloxacin (10 µg/ml) | 206.3 ± 38.4 | 1.2 ± 1.1 |
| PHA (2.5 µg/ml) | 9646.0 ± 1278.1 | 15.6 ± 5.4 |
| PHA(2.5 µg/ml) + Moxifloxacin (2.5 µg/ml) | 2672.1 ± 1433.7 | 8.2 ± 2.0 |
| PHA (2.5 µg/ml) + Moxifloxacin (10 µg/ml) | 5565.6 ± 1699.0 | 5.6 ± 1.0 |
| PHA (5 µg/ml) | 33048.3 ± 19325.0 | 36.7 ± 22.6 |
| PHA (5 µg/ml) + Moxifloxacin (2.5 µg/ml) | 7547.5 ± 1069.8 | 11.3 ± 3.8 |
| PHA (5 µg/ml) + Moxifloxacin (10 µg/ml) | 5982.4 ± 997.4 | 4.1 ± 0.4 |

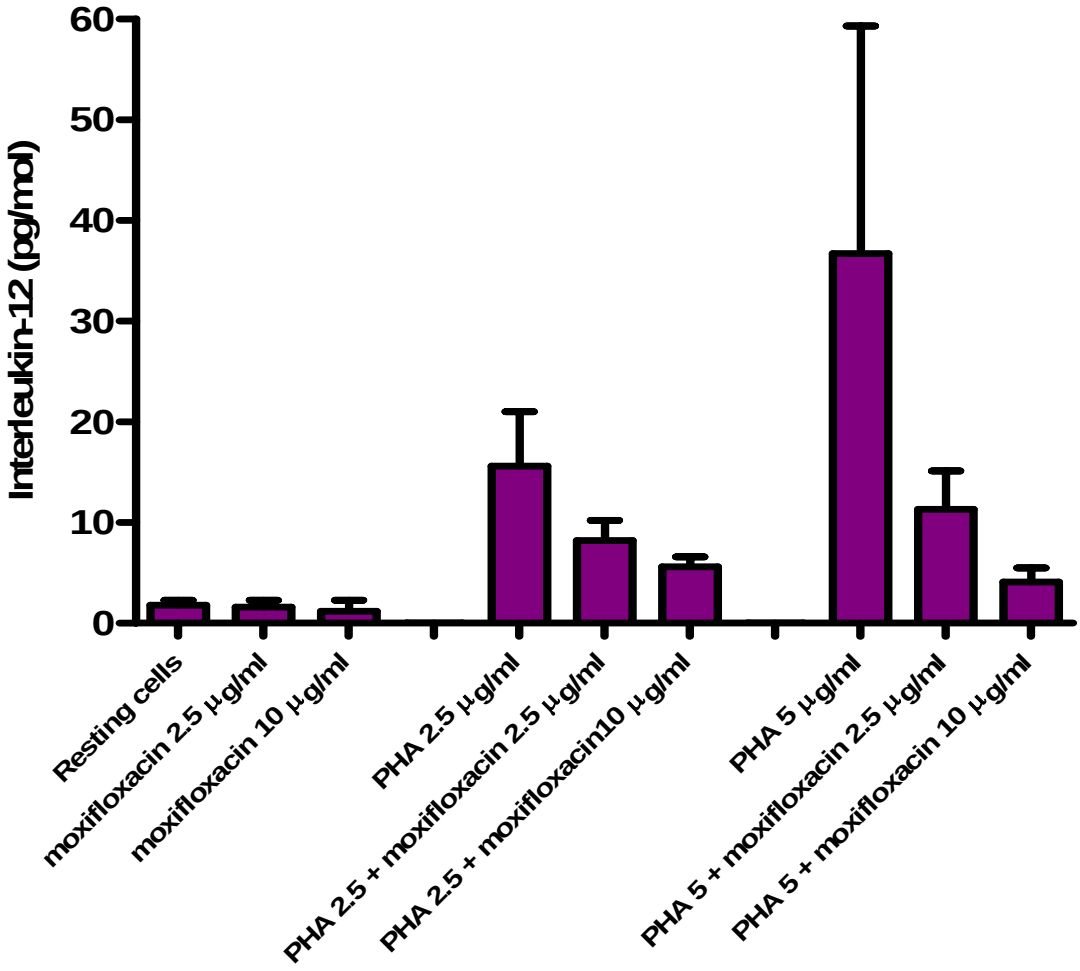
The results of 4 experiments are presented as the mean values ± SEMs as pg/ml for each cytokine.

**Fig 4.3: Effects of moxifloxacin on IL-6 production by PHA-activated MNLs**




The results of 4 experiments are presented as the mean values ± SEMs for IL-6 production by unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

**Fig 4.4: Effects of moxifloxacin on IL-12 production by PHA-activated MNLs**




The results of 4 experiments are presented as the mean values ± SEMs for IL-12 production by unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

**Table 4.2 Effects of Moxifloxacin on cytokine production by PHA-activated MNLs**

| CYTOKINE | IL-1β | IL-2 | IL-4 | IL-5 | IL-7 | IL-10 | IL-13 |
|---|---|---|---|---|---|---|---|
| Resting cells | 10.2 ± 3.5 | 31.1 ± 0.3 | 73.2 ± 22.8 | 1.5 ± 0.1 | 2.0 ± 0.5 | 11.0 ± 5.1 | 4.6 ± 1.3 |
| Moxifloxacin (2.5 μg/ml) | 22.0 ± 8.6 | 47.2 ± 5.0 | 85.3 ± 22.7 | 1.6 ± 0.2 | 2.5 ± 0.6 | 12.1 ± 5.2 | 5.7 ± 2.0 |
| Moxifloxacin (10 μg/ml) | 9.1 ± 3.1 | 33.9 ± 2.8 | 64.2 ± 24.1 | 1.8 ± 0.1 | 1.8 ± 0.6 | 9.1 ± 5.0 | 4.2 ± 2.0 |
| PHA (2.5 μg/ml) | 115.7 ± 68.3 | 139.3 ± 32 | 237.5 ± 23.9 | 26 ± 2.1 | 5.5 ± 1.4 | 165.3 ± 69.0 | 92.5 ± 25.1 |
| PHA (2.5 μg/ml) + Moxifloxacin (2.5 μg/ml) | 108.4 ± 64.7 | 158.5 ± 56.7 | 266.2 ± 48.3 | 22 ± 6.1 | 5.5 ± 1.0 | 190.2 ± 80.4 | 47.2 ± 16.9 |
| PHA (2.5 μg/ml) + Moxifloxacin (10 μg/ml) | 68.4 ± 25.7 | 149.3 ± 17.0 | 214.9 ± 54.7 | 18.5 ± 2.1 | 4.7 ± 1.0 | 133.0 ± 69.3 | 99.5 ± 25.3 |
| PHA (5 μg/ml) | 149.3 ± 69.8 | 113.1 ± 11.8 | 280.7 ± 28 | 37.1 ± 2.7 | 6.9 ± 1.5 | 323.4 ± 163.2 | 117.6 ± 30.0 |
| PHA (5 μg/ml) + Moxifloxacin (2.5 μg/ml) | 119.6 ± 61.2 | 171.3 ± 43.7 | 224.2 ± 45.4 | 38.5 ± 13.9 | 5.1 ± 1.0 | 534.4 ± 251 | 124.8 ± 42.1 |
| PHA (5 μg/ml) + Moxifloxacin (10 μg/ml) | 137.7 ± 61.2 | 128 ± 1.9 | 275.2 ± 60.5 | 38.7 ± 7.89 | 6.2 ± 1.7 | 451.6 ± 214.3 | 251.0 ± 91.4 |

Table continued (P.T.O)

**Table 4.2 Effects of Moxifloxacin on cytokine production by PHA-activated MNLs**

| CYTOKINE | IL-17 | TNF-α | IFN-γ | G-CSF | GM-CSF | MCP-1 |
|---|---|---|---|---|---|---|
| Resting cells | 6.2 ± 1.7 | 23.8 ± 7.3 | 107.3 ± 43.3 | 70.5 ± 26.2 | 56.0 ± 17.4 | 2909.6 ± 1672.7 |
| Moxifloxacin (2.5 μg/ml) | 6.3 ± 3.2 | 44.3 ± 16.4 | 172.0 ± 64.5 | 144 ± 58.3 | 61.4 ± 25.7 | 8209.6 ± 4290.4 |
| Moxifloxacin (10 μg/ml) | 6.9 ± 2.6 | 28.2 ± 9.1 | 114.3 ± 40.3 | 63.7 ± 20.8 | 38.1 ± 14.6 | 2230.1 ± 1298.1 |
| PHA (2.5 μg/ml) | 30.8 ± 8.0 | 456.0 ± 203.0 | 939.6 ± 373.9 | 509.6 ± 112.6 | 250.6 ± 48.9 | 25259.0 ± 11748.0 |
| PHA (2.5 μg/ml) + Moxifloxacin (2.5 μg/ml) | 25.5 ± 6.2 | 293.5 ± 169.3 | 796.8 ± 406.7 | 531.4 ± 148.8 | 233.6 ± 46.9 | 17030.4 ± 4565.1 |
| PHA (2.5 μg/ml) + Moxifloxacin (10 μg/ml) | 29.1 ± 6.6 | 243.6 ± 117.4 | 734.1 ± 334.1 | 466.0 ± 198.9 | 169.4 ± 47.0 | 26127.6 ± 9497.8 |
| PHA (5 μg/ml) | 34.0 ± 4.6 | 455.7 ± 177.5 | 1348.7 ± 346.2 | 842.0 ± 229.9 | 296.7 ± 38.0 | 32046.9 ± 9145.2 |
| PHA (5 μg/ml) + Moxifloxacin (2.5 μg/ml) | 33.3 ± 8.9 | 598.5 ± 274.7 | 1664.4 ± 607.3 | 743.2 ± 228.0 | 297.0 ± 82.8 | 23998.1 ± 8406.3 |
| PHA (5 μg/ml) + Moxifloxacin (10 μg/ml) | 46.7 ± 19.0 | 370.4 ± 245.5 | 1667.2 ± 528.8 | 767.3 ± 223.9 | 315.2 ± 99.9 | 24838.3 ± 1333.2 |

The results of 4 experiments are presented as the mean values ± SEMs as pg/ml for each cytokine.

# *Chapter 5: General Conclusion*

## GENERAL CONCLUSION

The effect of quinolones on the immune system has been mainly studied *in vitro*. Despite some conflicting results due to variation in study methodologies, certain conclusions can be drawn. Clinically relevant concentrations of most of quinolones seem to have no direct effect on isolated immune parameters, such as phagocytic cell functions, lymphocyte proliferation immunoglobulin production, cytokine production and bone marrow progenitor cell proliferation. *In vivo* studies are few, and are generally in agreement with the *in vitro* findings. Only high doses administered to experimental animals caused suppressive effects, while therapeutic doses are usually not associated with measurable alterations in immune functions. Secondary anti-inflammatory properties would be clinically useful for treating acute lung injury and many chronic lung diseases.

Therefore, I conclude that moxifloxacin at therapeutically relevant concentrations does not have any direct effects, either inhibitory or stimulatory, on human leukocytes (neutrophils and lymphocytes) functions *in vitro*, but rather interacts directly with target bacteria rendering them more vulnerable to eradication by leucocytes.

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