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Investigating The Effects of *Haemophilus influenzae* on Neutrophils in Chronic Obstructive Pulmonary Disease

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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I declare that this thesis entitled:

'Investigating The Effects of *Haemophilus influenzae* on Neutrophils in Chronic Obstructive Pulmonary Disease'

is entirely my own work, except where collaboration is indicated in the text.

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Publications

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Abstract

Chronic Obstructive Pulmonary Disease (COPD) is an inflammatory lung disease that is characterized by a slowly progressive and irreversible deterioration in lung function due to chronic bronchitis or emphysema, resulting in an increased shortness of breath. It is the 12th most prevalent disease world wide and cigarette smoking is the main risk factor for developing the disease - over 90% of patients are cigarette smokers. Exacerbations of COPD can occur due to infection of the lungs with bacteria.

Neutrophils are the most abundant cells of the immune system, constituting the primary line of defence against bacterial infection. A process called the respiratory burst generates reactive oxygen species (ROS) which are used along with proteases to kill bacteria. These products are toxic in high concentrations and may cause damage to surrounding lung tissue when released. Infected lungs have an increased number of neutrophils, which have been recruited into the lungs to kill the bacteria. Despite this, large numbers of bacteria are still present, and increased damage to the lungs is evident. Therefore, the neutrophils may not be working efficiently to kill the bacteria in the COPD lung.

A common bacterium found in the lungs of patients with COPD is *Haemophilus influenzae*. Previous research has shown that *H. influenzae* is not killed by neutrophils. The neutrophils engulf the bacteria, but eventually die by necrosis allowing toxic ROS and proteases to be released into the surrounding tissue, resulting in further damage to the lung. The engulfed *H. influenzae* are released at the same time causing more neutrophils to be recruited to the site of infection. The increased number of neutrophils may therefore be responsible for the tissue damage that can be observed. This may explain the paradox in COPD: large numbers of *H. influenzae*, large numbers of non-functional neutrophils and high levels of tissue damage. Cultured *H. influenzae* secretes a factor(s) that mimics the effect of intact bacteria and induces neutrophil necrosis.

The aims of this project were to identify the necrotic factor(s) released by *H. influenzae*, to elucidate the mechanism by which necrosis is induced and to determine if these processes occur in the COPD lung. I have shown that *H. influenzae* releases at least 2 factors that induce neutrophil necrosis. The use of intracellular signalling inhibitors showed that the factors worked through the protein kinase C and p38-MAPK pathways to induce neutrophil necrosis. Further research indicated that the necrotic factors work by binding to the Toll-like receptors TLR2 and TLR4 and at least one of factors released by *H. influenzae* may be a bacterial lipoprotein. Analysis of neutrophils isolated from the sputum of COPD patients with *H. influenzae* infection have indicated that the results observed *in vitro* also occur *in vivo* inside the COPD lung.

Identification of the necrotic factors and further elucidation of the mechanism by which necrosis is induced may be beneficial in finding a therapeutic target for the treatment of *H. influenzae* exacerbations of COPD.

Abbreviations

AP-1	Transcription Factor Activator Protein-1
ATP	Adenosine Triphosphate
BAL	Bronchoalveolar Lavage
Bcl	B-Cell Lymphocytic-Leukaemia Proto-Oncogene
BH Domain	Bcl-2 Homology Domain
BPI	Bacterial/Permeability-Inducing Protein
CAM	Cell Adhesion Molecule
CB	Cytochalasin B
CBF	Ciliary Beat Frequency
CD16	FcγRIIIb Receptor
CGD	Chronic Granulomatous Disease
CKRA	Chemokine Receptor Agonist
CL	Chemiluminescence
CpG ODN	Unmethylated CpG dinucleotide
COPD	Chronic Obstructive Pulmonary Disease
CR1	Complement Receptor 1
CR3	Complement Receptor 3
CSF	Colony-Stimulating Factor
DISC	Death-Inducing Signalling Complex
DMF	Dimethylformamide
DMSO	Dimethyl Sulphoxide

ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetra-acetate
EGF	Epidermal Growth Factor
ELR	Glutamate-Leucine-Arginine Motif
EM	Electron Microscopy
ERK	Extracellular Signal Regulated Kinase
ESL-1	E-selectin Ligand-1
FAD	Flavin Adenine Dinucleotide
FADD	Fas-Activated Death Domain
FCS	Foetal Calf Serum
FEV_1	Forced Expiratory Volume in 1 s
FITC	Fluorescein Isothiocyanate
fMLP	N-Formylmethionyl-Leucyl-Phenylalanine (N-formyl Met-Leu-Phe)
FVC	FEV_1/Forced Vital Capacity Ratio
G-CSF	Granulocyte Colony-Stimulating Factor
GDI	GDP-Dissociation Inhibitor factor
GDP	Guanosine Diphosphate
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GRO	Growth-Related Oncogene
GTP	Guanosine Triphosphate
H. influenzae	*Haemophilus influenzae*
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balanced Salt Solution

HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulphonic Acid
HMP	Hexose Monophosphate
HOCl	Hypochlorous Acid
HRP	Horseradish Peroxidase
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
I-Latex	IgG-Opsonised Latex
IP-10	Interferon-γ-Inducible Protein
IRAK	Interleukin-1 Receptor Associated Kinase
ITAM	Immunoreceptor Tyrosine-based Activation Motif
JAM	Junctional Adhesion Molecule
JNK	c-Jun NH2-terminal Kinases
LPS	Lipopolysaccharide
MALP-2	Macrophage-activating Lipopeptide 2
MAPK	Mitogen Activated Protein Kinase
MCL-1	Myeloid Cell Leukemia 1
MCP	Monocyte Chemotactic Protein
M-CSF	Macrophage Colony-Stimulating Factor
MEK	MAP Kinase Kinase
MIG	Monokine Induced by IFN-γ
MIP	Macrophage Inflammatory Protein

MPO	Myeloperoxidase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAP-2	Neutrophil-Activating Peptide-2
NF-κB	Nuclear Factor-kappa B
O_2-	Superoxide Anion
p38 MAPK	p38 MAP Kinase
PAF	Platelet Activating Factor
PAK	p21 Activated Kinase
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
Phox	Phagocytic Oxidase
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PMN	Polymorphonuclear Leukocyte
Poly(I:C)	Polyinosinic:Polycytidylic Acid
Poly (U)	Polyuridylic Acid
PSGL-1	P-selectin Glycoprotein Ligand-1

PX	*Phox*-homology Domain
RA	Rheumatoid Arthritis
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute 1640
S. aureus	*Staphylococcus aureus*
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SH3	*Src*-homology 3 Domain
S-Latex	Serum-Opsonised Latex
SOD	Superoxide Dismutase
TLR	Toll-Like Receptor
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor-α
TNFR1	Tumour Necrosis Factor Receptor
TPR	Tetratricopeptide Repeat
U-Latex	Unopsonised Latex

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Chapter 1: Introduction

1.1 Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is the 12th most prevalent disease worldwide and is expected to rise to the 5th leading cause of death by 2012 (*1, 2*). COPD is an inflammatory lung disease that is defined by chronic irreversible airflow limitation associated with an abnormal inflammatory response of the lungs to noxious gases and particles (*3, 4*). The disease is characterized by a slowly progressive and irreversible deterioration in lung function due to chronic bronchitis and emphysema (*5, 6*). COPD patients show destruction of the lung parenchyma and inflammation of the peripheral and central airways (*4, 7*). The obstruction of small airways, chronic cough and sputum production are related to chronic bronchitis and the enlargement of airspaces and destruction of the lung parenchyma is a result of emphysema (*8-10*). Figure 1.1 shows the appearance of a COPD lung compared to a healthy lung. Patients have a combination of these two conditions but the proportions differ between individuals (*8*).

Symptoms of COPD typically begin when the patient is 35-45 years of age, starting with breathlessness when exercising (*11*). This then progresses to breathlessness at rest, a persistent cough and an increase in purulent sputum production. Patients with severe COPD may develop cyanosis, a bluish colour in the lips and fingers, resulting from a lack of oxygen (*4, 8, 12*). There is no single definitive test for COPD and so diagnosis

is usually suggested by symptoms and patient history. Spirometric measurements of the ratio of Forced Expiratory Volume over 1 second to Forced Vital Capacity (FEV_1:FVC) are used to help with diagnosis and to determine the progression of the disease. FEV_1 levels of < 50 % indicate a decreased risk of survival; patients with very severe COPD can have FEV_1 values of approx 30 % (*3, 4, 13*).

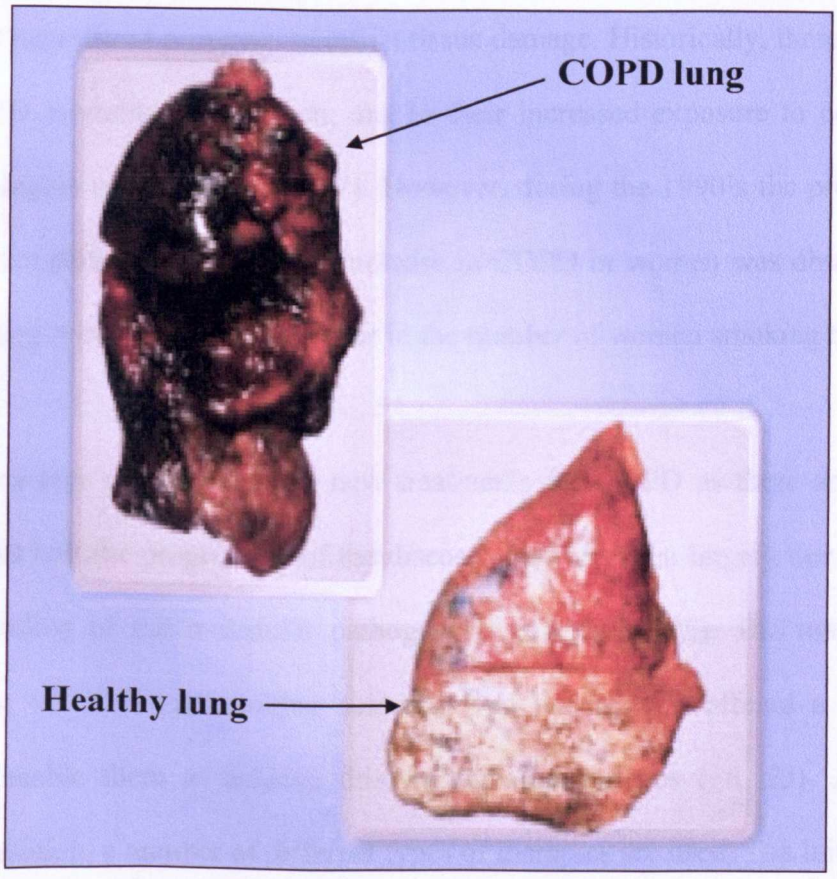


Figure 1.1: The morphological appearance of a healthy lung compared to the lung of a COPD patient. The COPD lung is smaller in size and has a blackened appearance resulting from tissue damage and inflammation.
(www.swen.uwaterloo.ca/~as3/images/lung.gif)

The main risk factors for developing COPD are tobacco smoke (over 90 % patients are cigarette smokers) (*14*), environmental exposure to air pollutants and occupational dust/chemicals e.g. from mining or construction industries (*15*). The development of the disease is usually due to complex interactions of these main risk factors (*2, 16, 17*). There is also a genetic link to COPD with a mutation leading to a deficiency in α_1-antitryspin (*18, 19*). This protease inhibitor normally protects the lungs from damage via neutrophil proteases e.g. elastase (*20*). Therefore, a mutation to this gene will increase the capacity of proteases to inflict tissue damage. Historically, there has been a higher COPD mortality rate in men, due to their increased exposure to occupational risks and a higher rate of smoking (*21*). However, during the 1990's the prevalence of COPD in men plateaued and a large increase in COPD in women was observed. This has been thought to be due to an increase in the number of women smoking tobacco (*1*).

There is a greater need to develop new treatments for COPD as there are no drugs available that halt the progression of the disease. This has been largely due to the lack of understanding of the molecular pathogenesis of COPD (*22*). The main form of management of COPD is smoking cessation and patients are offered a number of options to enable them to achieve this e.g. nicotine patches (*16, 23*). As well as smoking cessation, a number of different types of therapies are used. The initial form of treatment of COPD is with short-acting bronchodilators e.g. salbutamol. These are used in the early stages of COPD and offer quick relief to symptoms such as dyspnea, wheezing and coughing (*24*). Long-acting bronchodilators e.g. tiotropium bromide are used as the disease progresses, to manage moderate to severe COPD. Patients are

usually given a combination of inhaled anticholinergics and β2 adrenoceptor agonists as these drugs are thought to have an additive effect in the treatment of the disease (*25, 26*). The use of inhaled corticosteroids as a therapy is controversial since they do not prevent the progression of the disease as initially thought, and side effects are common with high doses. However, patients are usually given a short course of systemic corticosteroids during exacerbations of the disease (*27-29*). When patients have severe COPD and chronic hypoxia, long term oxygen therapy is also considered in their treatment (*30, 31*).

A number of inflammatory cells have been implicated in the pathogenesis of COPD e.g. monocytes and T-lymphocytes (*32, 33*). The cell type of particular interest in this disease is the neutrophil. Sputum and bronchoalveolar lavage (BAL) fluid from COPD patients have an increased number of neutrophils present compared to healthy controls (*9, 34*). Bronchial biopsies have also shown an increase in the number of neutrophils present in the lungs of patients with severe COPD, correlating with inflammation, indicating that neutrophils are important in the pathogenesis of COPD (*35-37*).

1.2 Neutrophils

Neutrophilic polymorphonuclear leukocytes (neutrophils) are the most abundant cells of the immune system, comprising approximately 45-60 % of white blood cells and are found at concentrations of 3-5 x 10^6 cells per ml of blood. Mature neutrophils are

approximately 7 μm in diameter, have a multilobed nucleus and a cytoplasm with a dense granular appearance (*38*). Neutrophils are the predominate infiltrating cell type in acute inflammation and act as the body's primary line of defence against invading pathogens; the number of blood neutrophils dramatically increases upon infection *(39)*. Neutrophils are now recognised as an important part of both the innate and adaptive immune system. They develop from blast cells in the bone marrow over a period of 15 days after which they are released into the circulation *(38, 40)*. Neutrophils contain many toxic products which can cause damage to tissues if the neutrophil is inappropriately activated, resulting in inflammation. Neutrophils thus have a short half-life of between 6-18 h and constitutively undergo apoptosis to allow their safe removal from the bloodstream by macrophages or fibroblasts (*41*). Neutrophils may also die by necrosis, releasing their toxic contents into the surrounding environment. The necrotic cells may be engulfed by macrophages which enhances the inflammatory response. Due to their short half-life, at least 5×10^{10} neutrophils must be released from the bone marrow each day, in order to protect the body against infections (*41, 42*). This process can be impossible for immunosuppressed patients e.g. patients undergoing chemotherapy, who are then unable to fight infections (*38*).

Neutrophils are rescued from constitutive apoptosis by the recruitment of the cells into tissues. This prolonged survival enables the neutrophil to carry out its functions in order to fight the infection (*41*). These functions are the phagocytosis and killing of pathogens and the recruitment of more neutrophils to the site of infection (*43*). This extended life-span of the cells, though beneficial in infections, can lead to persistent

inflammation and tissue damage as the neutrophils have a greater capacity to secrete cytotoxic molecules such as reactive oxygen species (ROS) and proteases *(41)*.

Neutrophils in the bloodstream are in a non-activated state to prevent host tissue damage but can be primed by a number of chemical stimuli such as cytokines e.g. GM-CSF and TNFα and by the attachment of microorganisms to plasma membrane receptors *(44, 45)*. Priming of neutrophils increases their ability to produce a response to factors such as pathogens. A number of events take place in a primed neutrophil including polarization and change in the cellular shape, change in levels of activity of receptors expressed on the plasma membrane and the increase in ROS production *(46)*.

1.2.1 Adhesion and Diapedesis

Neutrophils present in the circulation are in a non-activated state. They are able to "roll" along the surface of endothelial cells mediated by the expression of selectins. Selectins are type 1 membrane glycoproteins with a NH_2-terminal C-type lectin domain and an EGF-like domain. L-selectin, present on the surface of neutrophils, allows interactions with endothelial cells and other neutrophils via the P-selectin glycoprotein ligand, PSGL-1 (*47, 48*). These interactions allow neutrophils to respond to the presence of chemoattractants, such as IL-8 and fMLP, which are released from the site of infection and activate the circulating neutrophils (*39, 49*).

Activation of neutrophils results in shedding of L-selectin which, in turn, enables neutrophils to adhere to the endothelial cells (*50*). This adhesion is mediated by the β_2 integrin family which consist of a variable α-subunit and a common β-subunit. The α-subunit can be either CD11a, -b or -c and the β-subunit is CD18, the two main β_2 integrins in neutrophils being CD11a/CD18 and CD11b/CD18 (*51, 52*). Activation of the neutrophil results in an increased affinity of the β2 integrins to bind to their ligands, enabling the neutrophils to adhere to the endothelial cells (*39*).

After adhesion, neutrophils migrate between the endothelial cells by a process called diapedesis. During diapedesis, platelet endothelial cell adhesion molecule-1 (PECAM-1), present on the neutrophil surface and on endothelial cells, allows neutrophil extravasation by the formation of PECAM-1/PECAM-1 interactions (*38, 53*).

Chemoattractants are generated at the site of infection by pathogens or by endothelial and other white blood cells e.g. IL-8, leukotriene B_4. IL-8 in particular is a major neutrophil chemoattractant (*54*). Once the neutrophils have been recruited into the tissues they are able to migrate towards the site of infection by their ability to move along a concentration gradient of chemoattractants - a process called chemotaxis (*55, 56*). Once at the site of infection, neutrophils bind the chemoattractants via specific GTP-binding protein (G-protein) coupled receptors and a cascade of events occurs inside the neutrophils leading to activation. This enables the neutrophil to phagocytose and kill the bacteria (*39*).

1.2.2 Phagocytosis

Neutrophils migrate towards the site of infection by chemotaxis. Once at this site bacteria are recognised by receptors on the plasma membrane of the neutrophils (*38, 39*). Bacteria are likely to be opsonised by serum proteins e.g. antibodies such as IgG, enhancing the ability of the neutrophil to recognise the pathogen. There are three different types of receptor classes involved in phagocytosis – Fcγ receptors, the Complement receptors and the Toll-like receptors; these will be discussed in more detail in *1.3 Receptors* (*57, 58*). Once the bacteria have been recognised, the plasma membrane of the neutrophil forms pseudopodia which begin to surround the bacterium, which eventually becomes enclosed within a phagocytic vesicle. This phagocytic vesicle is initially very small and excludes extracellular fluid so that a new optimum environment can be formed within it (see figure 1.2) (*39, 49, 59, 60*). A phagolysosome (also referred to as a phagosome) forms, by the fusion of the cytoplasmic granules with the vesicle, into which the contents of the granules are quickly released. This process is called degranulation (*61*). There are 4 different types of granules which contain a mixture of enzymes, proteases and other proteins. These granule contents are important in enabling the neutrophil to kill the bacteria and will be described in more detail in *1.2.3 Granule Proteins* (*62*).

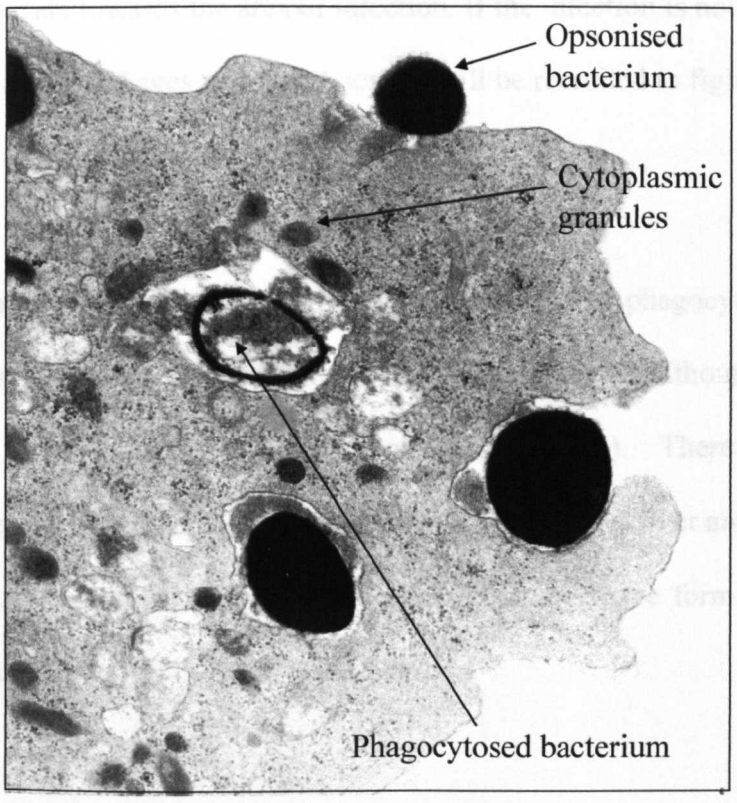


Figure 1.2: Electronmicrograph of a neutrophil phagocytosing and killing *Staphylococcus aureus*. Pseudopodia forming around the bacterium can be observed along with the presence of the cytoplasmic granules. The granules fuse with the vacoule enabling the subsequent death of the pathogen (*25*).

After degranulation, ROS are generated by a process called the respiratory burst (non-mitochondrial O_2 consumption). These ROS are toxic to the bacteria when produced in high concentrations and, along with the change in pH of the phagolysome, play an important part in killing bacteria. This will be discussed in detail in *1.2.4 The Respiratory Burst* (*59, 63*).

Some neutrophil products will activate an inflammatory response enabling more neutrophils to migrate towards the area of infection. If the infection is not cleared, other white blood cells, macrophages and lymphocytes, will be recruited to fight the infection enhancing the inflammatory response (*64*).

When a pathogen is too large to be fully enclosed within the phagocytic vesicle the neutrophil releases its toxic products directly onto the pathogen without phagocytosis occurring. This process is called frustrated phagocytosis (*65*). There is also some evidence to suggest that neutrophils may be able to take up and deliver antibiotics to the site of infection. These agents may then be released in an active form at the site of infection, and can help to kill the bacteria (*38, 66*).

1.2.3 Granule Proteins

The fusion of cytoplasmic granules with the phagocytic vesicle results in the release of granule contents into the vesicle. There are 2 main types of cytoplasmic granules involved in pathogen killing: the peroxidase-positive and peroxidase-negative granules which contain a mixture of proteases, enzymes and other proteins (*62, 67*). The first granules to develop at the promyelocyte stage are the azurophilic granules. These are the peroxidase-positive granules and contain proteins required for bacterial killing and digestion; serine proteases, antibiotic proteins and myeloperoxidase (MPO), an enzyme of particular importance that will be discussed later in more detail (*68*). These granules

also contain defensins which target bacterial cell membranes, increasing their permeability (*69*).

The peroxidase negative granules are further classed into the specific, tertiary and secretory granules. The specific granules contain another enzyme of particular interest, lactoferrin, as well as proteins such as collagenase and cathelicidin (*70*). Tertiary granules are also present which contain gelatinase and the 4th type of granules that exists are known as the secretory granules and contain a number of plasma proteins, such as albumin. Upon phagocytosis of the pathogen, degranulation occurs where the granule contents are quickly discharged into the phagosomes. This enables the proteases and other granule contents to work together to kill the pathogen (*62, 68*).

MPO is a myeloid cell specific enzyme present in the azurophilic granules. It is present in high concentrations in neutrophils, accounting for 1-5 % of the dry weight of the cell (*71*). The enzyme has an intense green colour that is responsible for the colour of pus. During MPO synthesis, an initial 80 kDa peptide is modified in the endoplasmic reticulum to produce the inactive apopro MPO form which is 90 kDa. Heme is inserted into this peptide to form pro MPO which is later cleaved to form the active MPO. Active MPO is a tetramer of 2 identical subunits, a large 59 kDa α subunit and a smaller 13.5 kDa β subunit, with a molecular mass of 150 kDa. It is a strongly basic peptide with an isoelectric point of > 10 (*69, 71-73*).

During the respiratory burst, MPO can react with hydrogen peroxide (H_2O_2), amplifying its toxic potential, to produce hypohalous acids. In physiological conditions, chloride is the most abundant halogen and so hypochlorous acid (HOCl) is the main product formed. HOCl is an extremely potent bactericidal agent and is very important in the destruction of bacteria (*63*). Generation of the hypohalous acids by MPO is via a 2 step process. Ferric MPO (MP^{3+}) initially reacts with H_2O_2 to form compound I, a redox intermediate compound. Compound I is a powerful oxidant and oxidizes the halides (X) to produce the hypohalous acids (HOX) (*61*).

1. $MP^{3+} + H_2O_2 \longrightarrow$ Compound I $+ H_2O$

2. Compound I $+ X^- + H^+ \longrightarrow MP^{3+} + HOX$

As well as being important in host defence MPO plays a role in inflammation. If neutrophils lyse or die by necrosis, MPO will be released into the surrounding environment. Here, MPO will be able to react with H_2O_2 and superoxide (O_2^-) present to produce hypochlorous acid. Instead of killing bacteria, these ROS will cause tissue damage resulting in prolonged inflammation (*69, 71*).

Human deficiencies in MPO may occur and these can be either congenital or acquired. Genetic deficiencies are due to germline mutations that lead to detection and expression in the neutrophils and monocytes of family members. Acquired deficiencies may be a result of disease e.g. leukaemia, iron deficiency and severe infectious disease and are

also common in pregnant women. These acquired deficiencies are usually transient, easily treated and are only present in some neutrophils (*74-76*).

Lactoferrin is another widely-studied granule enzyme. It is present in the specific granules and is an 80 kDa non-heme iron-binding glycoprotein generated by limited proteolysis of a larger protein. It has an antimicrobial amino terminal domain (lactoferricin) which is liberated by pepsin cleavage. Lactoferrin binds and sequesters iron and copper and has many antimicrobial properties including direct bacteriolysis and bacteriostasis (*77, 78*).

1.2.4 The Respiratory Burst

A process called the respiratory burst converts non-mitochondrial O_2 into ROS in order to aid in the killing of phagocytosed bacteria. The NADPH oxidase, present on the plasma membrane of the phagosome, converts O_2 into O_2^- using NADPH generated via the hexose monophosphate shunt, the activity of which increases upon phagocytosis (*38, 61*).

NADPH oxidase is an enzymatic complex comprising at least 6 components. In non-activated neutrophils separate cytosolic and membrane components. $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ form a complex in the cytoplasm of the cell (phox: phagocytic oxidase) (*79*). The components, $p22^{phox}$ and $gp91^{phox}$, form a heterodimeric flavohaemoprotein in

the plasma membrane and the membranes of the secretory, tertiary and specific granules. This complex is the catalytic core of the oxidase, termed cytochrome b_{558}. The assembly of NADPH oxidase also requires the presence of 2 low molecular weight guanine nucleotide-binding proteins, cytosolic Rac2 and the membrane protein Rap1A (*80-82*).

Upon activation of the neutrophil, $p47^{phox}$ is phosphorylated by protein kinase C (PKC) and associates with $p67^{phox}$, causing a conformational change that enables it to travel to the plasma membrane and associate with cytochrome b_{558} to form the active oxidase (*83*). Active NADPH oxidase is able to transfer electrons from cytoplasmic NADPH to molecular O_2 generating O_2^-. It has been shown that $p40^{phox}$ is not essential for *in vitro* function of NADPH oxidase and it is therefore thought, that this component may have a regulatory function (*81, 82*).

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

O_2^- produced in this reaction is a mild oxidant that is able to inactivate bacterial iron/sulphur proteins but has limited membrane permeability (*84*). O_2^- rapidly dismutates, either spontaneously or via superoxide dismutase, to form H_2O_2. The hydroxyl free radical ($^{\cdot}OH$) is formed as a by product of this reaction and is the most reactive oxidative species known. It has a short range of action but causes DNA modification, enzyme inactivation and lipid peroxidation. However, due to its high reactivity it is extremely unstable. H_2O_2 is a membrane permeable oxidising agent and

is highly microbicidal at high concentrations. It reacts with haem proteins and peroxidases to produce reactive free radicals that may cause lipid peroxidation. The granule protein MPO is able to catalyse the conversion of H_2O_2 into HOCl, another microbicidal agent. The oxidants produced via the respiratory burst are shown in figure 1.3 (*61, 63, 85*).

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCl + H_2O$$

HOCl is a strong, non-radical oxidant, able to kill pathogens directly by interacting with iron/sulphur proteins, membrane transport proteins and DNA synthesis. HOCl is able to react further and produce chloramines. These are also directly microbicidal oxidants but are less reactive than HOCl and so are longer lived. The toxicity of these chloramines varies depending upon the precise type of molecule formed (*61, 63*).

$$HOCl + R\text{-}NH_2 \longrightarrow R\text{-}NHCl + H_2O$$

The pH of the phagolysosome rises from 6 up to 7.8 - 8 after phagocytosis due to NADPH oxidase activity, despite the acidic contents of the cytoplasmic granules. This rise in pH is mainly due to the entry of K^+ ions into the vacuole. ROS production is electrogenic and causes a negative charge inside the vacuole. Therefore, K^+ ions are pumped into the vacuole, via Ca^{2+} activated K^+ channels, to balance out the negative charge. The azurophilic granule proteins are highly cationic proteins and are strongly bound to highly-negatively charged proteoglycans e.g. heparin and chondroitin, making

them inactive. These proteins are activated by the increase in pH of the vacuole due to the influx of K^+ ions. These activated proteases are then able to kill the bacterium inside the vacuole (*63, 86*).

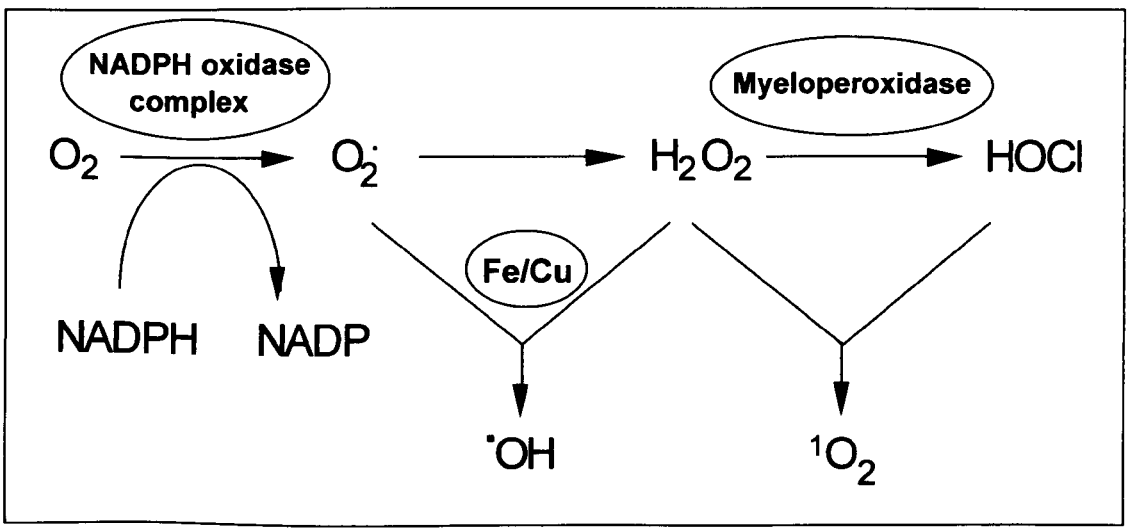


Figure 1.3: The Respiratory Burst. NADPH oxidase catalyses the conversion of O_2 into $O_2^{\cdot}$ which then rapidly dismutates into H_2O_2 which is converted into HOCl via MPO. All the ROS produced in this way play an important role in killing microorganisms that invade the body. However, in inflammatory diseases they MAY also contribute to tissue damage. Evidence for the generation of $^{\cdot}OH$ by phagocytosing neutrophils is not conclusive.

Neutrophils generate large quantities of ROS, some of which can have half lives of up to 18 h (*63*). If neutrophils lyse or die by necrosis their contents will be released into the surrounding area, resulting in tissue damage and inflammation. In particular, HOCl is a potent ROS which oxidises molecules such as amino acids, nucleotides and

hemoproteins. It also causes damage to host tissue due to its ability to inactivate α-1 antiproteinase, a major serine proteinase inhibitor, and by activating pro-collagenase and pro-gelatinase (*87*). This will enable the activation of the granule proteins resulting in tissue damage (*63*). The release of granule enzymes, in particular MPO, will increase the amount of HOCl present in the tissue causing further damage. Some anti-inflammatory medicines are able to prevent this damage e.g. indomethacin stops the production of HOCl by inhibiting the chlorinating activity of MPO (*69*).

The importance of NADPH oxidase in fighting infection was confirmed in patients with Chronic Granulomatous Disease (CGD) (*88*). This disease is a genetic defect and the most common form results from an X-linked mutation. A mutation can occur in any of the 4 phox subunit genes resulting in a defect in NADPH oxidase (*89*). CGD patients are able to phagocytose bacteria but are unable to produce a respiratory burst response to generate the ROS required for bacterial killing. Patients have recurrent, life-threatening bacterial and fungal infections. However, CGD patients are able to kill some bacteria which can produce their own H_2O_2 supply e.g. *Pneumococci*. The H_2O_2 generated by the bacteria may be converted to HOCl by the neutrophils MPO resulting in the production of ROS and the optimum environment required for bacterial killing inside the vacuole (*63, 81, 90*).

1.2.5 Neutrophils and Cytokines

Cytokines are small proteins/peptides that act as extracellular signalling molecules. They are usually small, water soluble proteins between 8-30 kDa in size and play important roles in the innate and adaptive immune responses (*38*). Monocytes produce large quantities of cytokines in comparison to neutrophils. However, the vast numbers of neutrophils attracted to sites of infection results in significant levels of cytokines being released by neutrophils in the acute inflammatory response (*91*). There are a number of different types of cytokines generated by neutrophils; pro-inflammatory cytokines e.g. TNFα, IL-12, anti-inflammatory cytokines e.g. IL-1 receptor antagonist (IL-1Ra), chemokines e.g. IL-8 and other molecules such as growth factors e.g. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (*92-94*).

Neutrophils are both responsive to, and a source of, a number of pro-inflammatory cytokines and chemokines, and in this way are able to auto-regulate the inflammatory pathway. As well as being activators and attractants of neutrophils, many cytokines are also targets for neutrophil proteases, providing a further mechanism of regulation of the response (*92*). The production of cytokines is usually triggered by stimulating agents such as other cytokines and bacterial endotoxins e.g. LPS. Cytokines largely act via G-protein coupled receptors to prime neutrophils and amplify a number of functions e.g. adherence to endothelial cells and ROS production (*92, 95*).

Chemokines are chemotactic cytokines, that specifically recruit cells to the site of inflammation. There are more than 40 different human cytokines, indicating their importance in controlling the immune response. They are 8-14 kDa in size and have between 20-70 % homology (*96*). Chemokines are classified into 2 groups, C-X-C or C-C, depending on the position of the first two cysteine residues of the molecule. The C-X-C group itself is then sub-divided into two classes depending on the presence of a glutamate-leucine-arginine (ELR) motif before the cysteine residues. The presence of an ELR motif usually indicates that the molecule is chemotactic for neutrophils (*96-98*). IL-8 is a member of this group and is of particular importance due to its role in the pathology of COPD (*99, 100*).

IL-8 is a chemokine expressed in response to factors such as IL-15, LPS, PMA and TNFα (*101*). It is secreted by T-lymphocytes, epithelial cells, keratinocytes, fibroblasts, endothelial cells and neutrophils, and so is an abundantly secreted cytokine. The primary cellular target of IL-8 is the neutrophil which has two IL-8 receptors; CXCR1 and CXCR2 (*55, 99, 100, 102*). IL-8 production at the site of infection initiates the migration of neutrophils along a chemotactic gradient. Once at the site of infection, recruited neutrophils will release further IL-8 promoting further neutrophil recruitment. Neutrophils undergoing phagocytosis also have the ability to synthesize and release large amounts of IL-8 (*103*).

IL-8 is of particular importance in COPD due to its interactions with nicotine and certain air pollutants (*100*). Nicotine, inhaled by smokers in cigarette smoke, is

chemotactic for neutrophils and so will stimulate the migration of neutrophils towards the lungs. Nicotine also stimulates neutrophils to release IL-8 (*36, 104*). Therefore, neutrophils recruited to the lungs by nicotine will then be stimulated to release IL-8, promoting the migration of more neutrophils to the lungs (*104*). Nicotine also induces the production of ROS in the cells, which could exacerbate tissue damage in COPD (*105*). The release of these ROS into the lungs will cause oxidative stress, again resulting in IL-8 release and the recruitment of more neutrophils to the lungs. The plasma levels of IL-8 in smokers was measured to be significantly higher that in non-smokers, further confirming its importance in this disease (*104*).

Air pollutant exposure also results in an influx of neutrophils into the lungs. One particular pollutant of interest is sodium sulphite (Na_2SO_3) which primes neutrophils and directly induces ROS production by the activation of NADPH oxidase via PKC activation. This mechanism could also result in neutrophil-derived ROS-mediated tissue damage. Na_2SO_3 has also been shown to induce the release of IL-8, therefore continuing the vicious cycle (*106*).

1.3 Neutrophil Receptors

There are 3 main types of receptors present on the surface of neutrophils that are involved in killing pathogens. These are the Fcγ receptors, the Complement receptors

and Toll-Like receptors. When activated, these receptors initiate a variety of neutrophil functions that lead to phagocytosis and killing of bacteria (*38*).

1.3.1 Fcγ Receptors

The Fcγ receptors play a key role in host defence by providing a link between the humoral immune system and cell mediated effector systems. They are expressed on granulocytes, monocytes, macrophages and natural killer cells, and are able to bind pathogens opsonised with IgG to facilitate the uptake of immune complexes (*107*). IgG molecules are the most predominant type of immunoglobulin found in the blood, and are also present in the lymph, cerebrospinal and peritoneal fluids. They have 2 parts to their structure: two fragment antigen binding (Fab) domains and a fragment crystallisable (Fc) domain. The Fab fragments bind antigens present on the surface of pathogens and are different in antibodies secreted by different B cell clones, whereas the Fc regions are homologous in structure and it is this region that is recognised and bound by the Fcγ receptors. Fcγ receptors are divided into 3 classes; FcγRI, FcγRII and FcγRIII, which differ in their affinity for IgG or IgG-containing immune complexes. These 3 subclasses also have multiple isoforms (*108, 109*).

FcγRI (CD64) has 3 immunoglobulin-like domains in the extracellular ligand binding domain which may account for its high affinity for binding monomeric IgG. It is a heavily glycosylated protein of 72 kDa, is anchored into the plasma membrane receptor

by a single transmembrane domain and is present in three different isoforms; FcγRIa, FcγRIb and FcγRIc. FcγRI is a characteristic marker of mononuclear phagocytes but is also expressed on activated neutrophils (*108*).

FcγRII (CD32) is a 40 kDa integral membrane glycoprotein which initiates a signalling cascade inside the cell. It is expressed on granulocytes, macrophages, monocytes and platelets and has a lower affinity for binding monomeric IgG than FcγRI due to the presence of only two immunoglobulin-like domains in the extracellular ligand binding domain. This receptor binds IgG aggregates and immune complexes and is again present in three different isoforms; a, b and c, which are differentially expressed in immune cells. FcγRIIa and FcγRIIc are expressed in monocytes, macrophages and neutrophils, where they are involved in phagocytosis, whereas FcγRIIb receptors are expressed in monocytes, macrophages and lymphocytes but not in neutrophils, NK cells or T cells (*108-110*).

FcγRIII (CD16) is a heavily glycosylated receptor between 50-70 kDa. It is present on the surface of neutrophils, eosinophils, macrophages and natural killer cells. This receptor binds complexed IgG molecules, has a very low affinity for monomeric IgG and is present in two isoforms; a and b. Fcγ RIIIb is the isoform constitutively expressed on the surface of neutrophils, and approximately 100,000-200,000 receptors are present, as a phosphoinositol linked receptor (GPI) which is easily cleaved enabling its removal from the cell surface (*108-110*).

During phagocytosis it is thought that FcγRIIa acts as a functional phagocytic receptor and FcγRIIIb acts as a co-receptor. FcγRII receptors are cross-linked by IgG opsonised pathogens leading to the aggregation and recruitment of the FcγRIII receptors (*58, 109*). This leads to the phosphorylation of cytoplasmic immunoreceptor tyrosine based activation motifs (ITAMS) and phospholipase C (PLC) resulting in the production of diacylglycerol (DAG), a known activator of PKC. Phosphatidylinositol-3 kinase (PI3K) and the mitogen activated protein kinases (MAPK) are also activated. As previously described PKC is required for activation of the NADPH oxidase by phosphorylation of the $p47^{phox}$ subunit (*80*).

1.3.2 Complement Receptors

There are 20 plasma proteins present in the blood and other bodily fluids that comprise the complement system, which clears pathogens from the body. These proteins are usually present in an inactive form but can be cleaved to form activated proteins by two major pathways; the classical pathway and the alternative pathway. The classical pathway requires activation of the C1 complex, the first of nine major complement proteins, by antibody binding. After activation of the initial protein, each product is an enzyme that is able to catalyse the next step of the reaction resulting in a cascade of events (*111, 112*). This cascade allows amplification of the response and stops non-specific activation of the pathway. The alternative pathway does not require activation via antibodies, but instead is activated by bacterial endotoxin and zymosan (*38*).

Neutrophils possess receptors for activated complement protein binding, with 100,000-300,000 receptors present on the neutrophil surface (*38*). These receptors are 42-48 kDa in size and activate a signalling cascade via interactions with G-proteins. The receptors required for phagocytosis are CR3 (CD11b/CD18) which acts as a functional phagocytic receptor and CR1 (CD35) which acts as a co-receptor (*111, 113*). CR1 and CR3 cross-linking is insufficient for phagocytosis and neutrophils first need to be activated by agents, such as PMA. Once activated these receptors initiate a number of responses inside the cell, such as adherence of neutrophils to endothelial cells, phagocytosis, degranulation and ROS production (*114*). These processes will enable the neutrophil to kill the pathogen, as previously described. The affinity of the receptors to bind to their ligands is increased upon priming (*115*).

1.3.3 Toll-like Receptors

Toll-like receptors play an important role in the recognition of microbial pathogens and the activation of the innate immune response. Toll is a type 1 transmembrane receptor first isolated and characterized in *Drosophila.* The extracellular domain contains leucine-rich repeats (LRR) whereas the cytoplasmic domain is 200 amino acids and has a large homology with the IL-receptors. It is commonly referred to as the Toll/IL-1R (TIR) domain (see figure 1.4). TLRs recognize conserved pathogen associated molecular patterns (PAMPs) rather than specific microbial structures, and binding of these PAMPs initiates a signal transduction cascade resulting in cellular activation. Toll

receptors are evolutionary-conserved and homologs are found in insects, plants and mammals. To date, ten TLRs have been identified in humans and these are essential for microbial recognition (*116-118*).

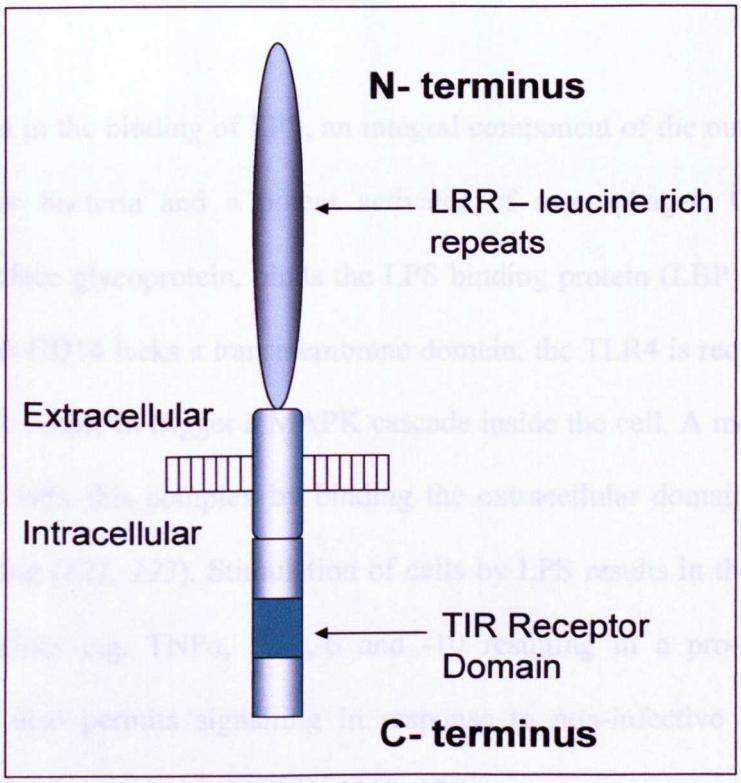


Figure 1.4: Toll-like receptors are transmembrane receptors that play an important role in microbial detection. They contain an extracellular leucine rich repeat domain and a cytoplasmic domain of 200 amino acids with a large homology to IL-receptors (figure adapted from *119*).

TLRs 1-10 are expressed in humans and bind a range of different PAMPs. TLR2 forms heterodimers with TLR1 and TLR6 to bind a variety of bacterial products. TLR2/TLR1 heterodimers bind triacylated bacterial lipoproteins and TLR2/TLR6 heterodimers bind diacylated bacterial lipoproteins as well as peptidoglycan components of Gram positive bacterial cell walls. TLR2 is also thought to be involved in LPS signalling by binding atypical types of LPS i.e. LPS with a cylindrical lipid A component as opposed to the more usual conical form of lipid A (*119-122*).

TLR4 is involved in the binding of LPS, an integral component of the outer membrane of gram negative bacteria and a potent activator of macrophages. CD14, a GPI anchored cell surface glycoprotein, binds the LPS binding protein (LBP) bound to the LPS molecule. As CD14 lacks a transmembrane domain, the TLR4 is required as a co-receptor, where it is able to trigger a MAPK cascade inside the cell. A molecule called MD-2 associates with this complex by binding the extracellular domain of TLR4 to enable LPS binding (*121, 123*). Stimulation of cells by LPS results in the release of a number of cytokines e.g. TNFα, IL-1,-6 and -10 resulting in a pro-inflammatory response. TLR4 also permits signalling in response to non-infective inflammatory stimuli e.g. heat shock protein 60 (HSP60) (*119, 120*).

TLR3 is involved in the binding of double stranded RNA (poly (I:C) is a TLR3 ligand) and is thought to be involved in viral recognition. Unlike the rest of the TLRs, TLR3 is not expressed in human neutrophils. TLR5 binds flagellin, a 55 kDa monomer obtained from bacterial flagella, which is a potent pro-inflammatory factor (*124*).

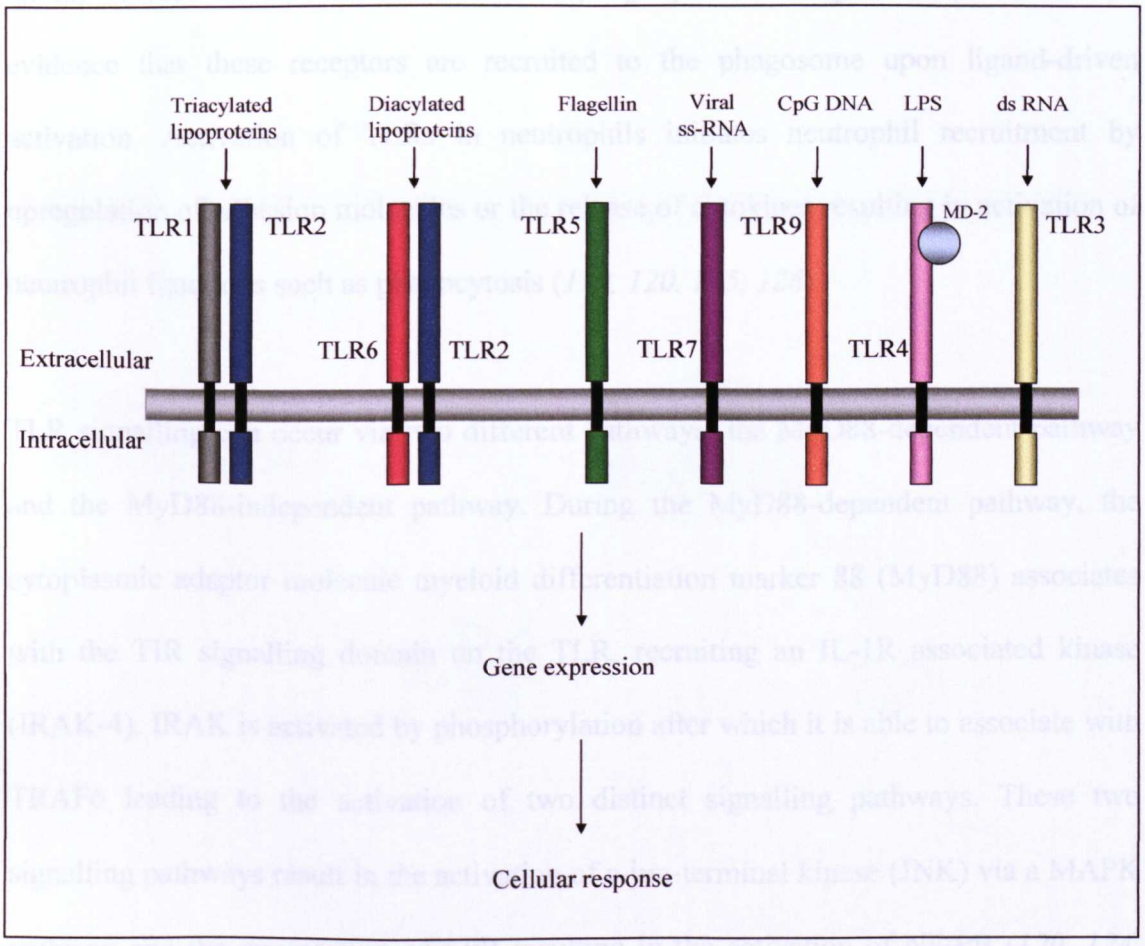


Figure 1.5: Schematic diagram of TLRs and their ligands. PAMPs bind to TLRs initiating a signalling cascade inside the cell. This results in the expression of genes and production of a cellular response (figure adapted from *119*).

TLR7 and TLR8 both bind single stranded viral RNA and, like TLR3, are therefore involved in viral recognition. TLR9 is involved in the detection of bacterial DNA. Unlike TLR3, -7, -8 and -9 which are intra-cellular, TLR1, -2, -4, -5 and -6 are found on the surface on the cell membrane. In phagocytic cells e.g. neutrophils, there is evidence that these receptors are recruited to the phagosome upon ligand-driven activation. Activation of TLRs in neutrophils initiates neutrophil recruitment by upregulation of adhesion molecules or the release of cytokines resulting in activation of neutrophil functions such as phagocytosis (*119, 120, 125, 126*).

TLR signalling can occur via two different pathways; the MyD88-dependent pathway and the MyD88-independent pathway. During the MyD88-dependent pathway, the cytoplasmic adaptor molecule myeloid differentiation marker 88 (MyD88) associates with the TIR signalling domain on the TLR, recruiting an IL-1R associated kinase (IRAK-4). IRAK is activated by phosphorylation after which it is able to associate with TRAF6 leading to the activation of two distinct signalling pathways. These two signalling pathways result in the activation of c-jun-terminal kinase (JNK) via a MAPK pathway and the degradation of I-κB resulting in the activation of NF-κB (*120, 124, 126, 127*).

The MyD88-independent signalling pathway results in activation of the transcription factor IRF-3 which induces IFN-β enabling it to activate Stat-1. This results in the induction of several IFN inducible genes (*120, 124, 126*).

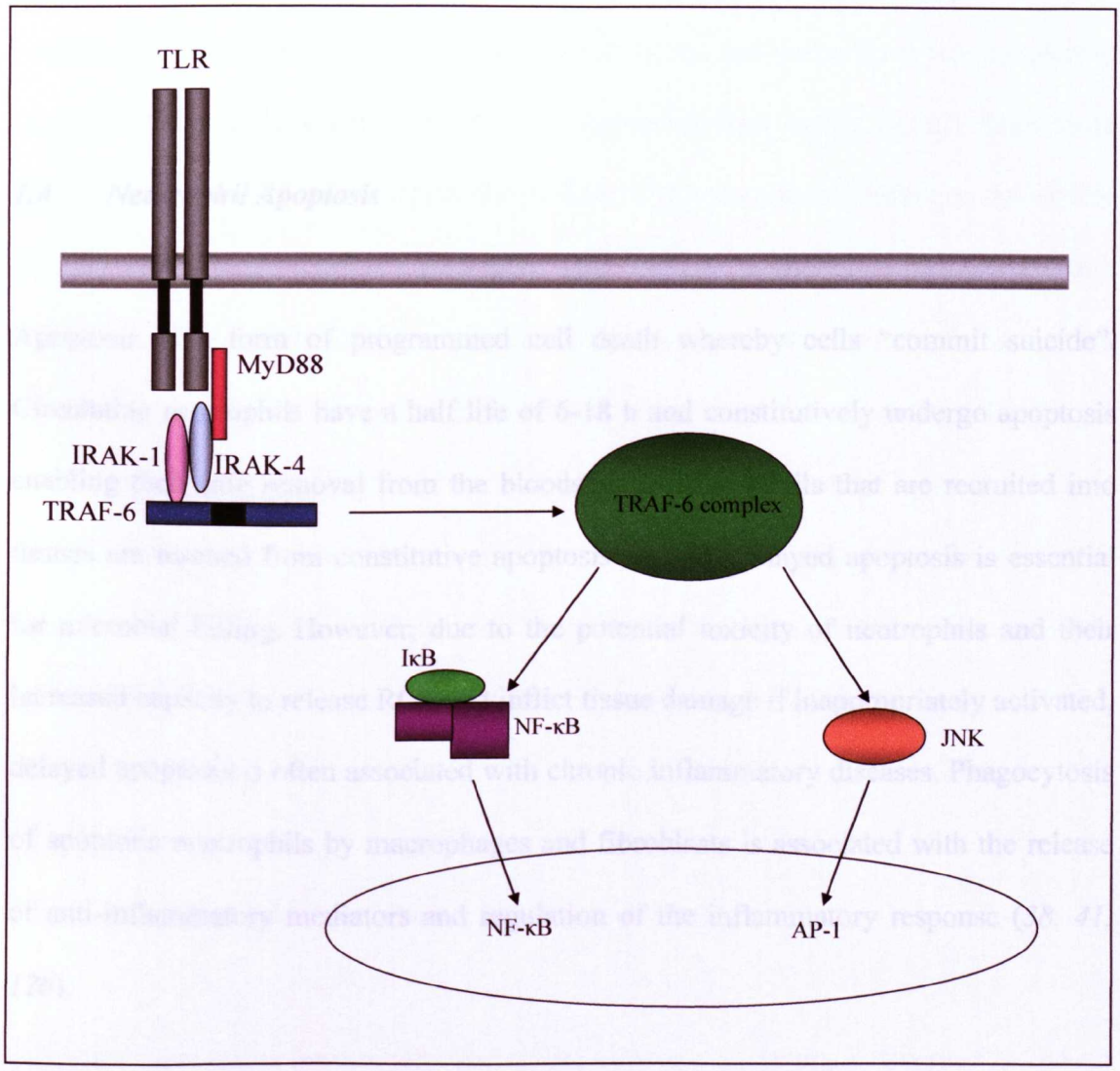


Figure 1.6: Schematic diagram of the TLR MyD88 dependent signalling pathway MyD88 associates with IRAK-4 which is then able to associate with TRAF-6 leading to the activation of the NF-κB pathway and a MAPK pathway (figure adapted from *124*).

Activation of neutrophils by these two pathways results in the release of cytokines, shedding of L-selectin, increase in phagocytosis and ROS production, degranulation and a number of other functions such as enabling the body pathogen killing *(122)*.

1.4 Neutrophil Apoptosis

Apoptosis is a form of programmed cell death whereby cells "commit suicide". Circulating neutrophils have a half life of 6-18 h and constitutively undergo apoptosis enabling their safe removal from the bloodstream. Neutrophils that are recruited into tissues are rescued from constitutive apoptosis and this delayed apoptosis is essential for microbial killing. However, due to the potential toxicity of neutrophils and their increased capacity to release ROS and inflict tissue damage if inappropriately activated, delayed apoptosis is often associated with chronic inflammatory diseases. Phagocytosis of apoptotic neutrophils by macrophages and fibroblasts is associated with the release of anti-inflammatory mediators and regulation of the inflammatory response (*38, 41, 128*).

A number of factors can regulate neutrophil survival in either a pro- or anti-apoptotic way. Pro-inflammatory cytokines e.g. IL-1β, IL-2, G-CSF and GM-CSF and some glucocorticoids can delay neutrophil apoptosis, whereas, anti-inflammatory cytokines e.g. IL-10 initiate apoptosis which in turn inhibits the inflammatory response (*92, 129, 130*). Hypoxia has also been shown to delay apoptosis in human neutrophils. Inflamed

tissues have low O_2 tensions and promote the survival of neutrophils that have migrated from the bloodstream. However, when ROS are released into the tissue the O_2 tension is increased and cells become apoptotic (*41, 131*). This shows an extremely fine balance in the regulation of neutrophil survival. Some antibiotics have been shown to have an anti-apoptotic effect on neutrophils increasing their survival (*132*). This could enable the neutrophil to transport the antibiotic to the site of infection to enable bacterial killing and will also increase the ability of the neutrophil to phagocytose and kill bacteria (*132*). Nicotine has also been shown to suppress neutrophil apoptosis leading to an increase in neutrophil survival. This is mediated via noncholinergic receptors present on the neutrophil surface and the effect is preserved in the presence of sputum from patients who are usually cigarette smokers (*133*). This effect may be important in COPD as an extended life span of the neutrophils will increase the cells ability to inflict tissue damage in the lungs of the patient.

1.4.1 Apoptotic Neutrophil Properties

Apoptotic neutrophils have a very distinct morphology as shown in figure 1.7. Instead of the multilobed nucleus and granular cytoplasm seen in mature neutrophils, apoptotic cells are round and compact with a condensed and rounded nucleus (*41*). This loss of a multilobed nucleus is a result of condensation of chromatin followed by fragmentation into a ladder of nucleosome length fragments. Apoptotic cells lose the expression of a number of surface receptors, in particular CD16, resulting in the cells inability to

respond to cellular signals, mount a response and carry out their functions (*134*). In apoptotic neutrophils phosphatidylserine residues appear on the cell surface. These residues are normally present on the inner plasma membrane leaflet of normal cells and "flip" to the outer membrane in apoptotic cells. This aids recognition of the cells for clearance by macrophages and fibroblasts (*135, 136*). The plasma membrane of apoptotic neutrophils remains intact so that the cytotoxic cellular components are retained, and inflammation can be resolved (*41, 128*).

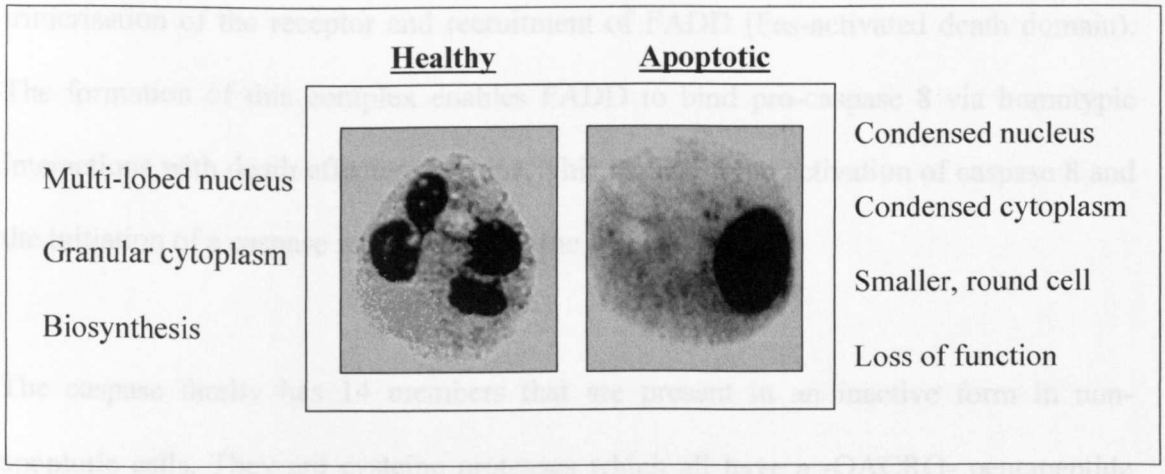


Figure 1.7: Morphological analysis of a healthy neutrophil (left) and an apoptotic neutrophil (right). Healthy neutrophils have a multi-lobed nucleus and a granular cytoplasm. An apoptotic neutrophil is small and the nucleus and cytoplasm condense resulting in an inability of the cell to carry out functions (*28*).

1..4.2 Molecular Control of Apoptosis

There are three main ways in which apoptosis is controlled at a molecular level: 1) through the activation of death receptors, ii) mitochondrial release of cytochrome c and iii) the expression of pro and anti-apoptotic proteins; namely the Bcl-2 family members (*41, 128*).

Cellular stress is detected via death receptors on the plasma membrane of the cell surface by binding of extrinsic factors such as Fas and TNFα. These factors can activate a pro-apoptotic pathway. The Fas ligand binds the Fas receptor resulting in trimerisation of the receptor and recruitment of FADD (Fas-activated death domain). The formation of this complex enables FADD to bind pro-caspase 8 via homotypic interactions with death effector domains. This results in the activation of caspase 8 and the initiation of a caspase response inside the cell (*43, 137*).

The caspase family has 14 members that are present in an inactive form in non-apoptotic cells. They are cysteine proteases which all have a -QACRG- pentapeptide sequence forming the active site of the enzyme (*41*). This gives the proteins the ability to cleave their substrates on the carboxyl side of aspartate residues. These precursor molecules have an N-terminal pro-domain, a large subunit (17-22 kDa in size) and a small subunit (10-12 kDa in size). Upon activation, the pro-domain is removed allowing the monomers to dimerise forming active molecules with protease activity (*138*). Caspases are activated by other caspases and so fall into two groups; the

regulatory caspases and the effector caspases. The regulatory caspases, caspase 2, 8, 9 and 10, have long pro-domains and also contain recognizable homotypic interaction motifs (death effector domain, DED, and caspase activation and recruitment domain, CARD). The effector caspases (caspase 3, 6 and 7) have short pro-domains and lack intrinsic enzyme activity. However, after cleavage between the large and small subunits they are able to cleave most known apoptotic substrates (*137, 139, 140*).

After activation of caspase 8 by the death receptor activation, a cascade of reactions occurs inside the cell. This cascade is necessary to amplify the initial response but also guard against inappropriate activation of the end points of the pathway. The activation of this response results in the disablement of neutrophil functions and the formation of the apoptotic phenotype. Caspases can target molecules such as actin, disruption of which regulates cell shrinkage and blebbing, and can cleave poly (ADP ribose) polymerase, which prevents the repair of fragmented DNA (*41, 138, 139*).

Under some circumstances, the activation of caspase 8 from procaspase 8 is insufficient to initiate apoptosis. In this way, apoptosis occurs via the release of cytochrome c from the mitochondria. After the activation of caspase 8 via Fas binding to the Fas receptor a pro-apoptotic protein, e.g. Bid, is activated. The protein is able to form pores in the membrane of the mitochondria resulting in the release of cytochrome c (*141*). This released cytochrome c interacts with apoptotic protease activating factor 1 (Apaf-1) using dATP. This results in a conformation change and oligomerization of Apaf-1. Pro-capsase 9 is able to bind to Apaf-1 oligomers via CARD resulting in the formation of

the high molecular weigh apoptosome. This apoptosome is then able to activate caspases 3, 6 and 7 triggering the initiation of the pro-apoptotic pathway inside the cell (*43, 142*).

Apoptosis is also controlled by a family of proteins called the B-cell lymphocytic leukaemia proto-oncogene 2 (Bcl-2) proteins. These proteins play a central role in regulating a cell's life/death cycle and can be either pro-apoptotic e.g. Bax, Bak, Bid and Bad or anti-apoptotic e.g. Bcl-2 and Bcl-X_L. The first of these proteins to be identified was Bcl-2 as it was found to be overexpressed in B-cell lymphoma. The other family members have since been identified by possession of conserved BH domains in the proteins (*41, 143*).

All of the Bcl-2 family members are able to form hetero- or homo-dimers due to the presence of these BH domains, as the α helical structures enable them to form protein:protein interactions. For example, Bcl-X_L and Bax are able to form heterodimers. The BH1, 2 and 3 domains of Bcl-X_L form a hydrophobic pocket into which the BH3 domain of Bax can fit. The complex prevents Bax from triggering a death pathway. The relative abundance of pro- and anti-apoptotic proteins and the formation of hetero- and homo-dimers is thought to control cell survival (*144, 145*).

Bcl-2 family members regulate the release of cytochrome c from the mitochondria. Anti-apoptotic proteins are localised to the outer mitochondrial membrane whereas pro-apoptotic proteins are normally cytoplasmic but are able to translocate to the

mitochondria after dephosphorylation. Once at the mitochondria they insert into the membrane and destabilise it, resulting in pore formation and the release of cytochrome c (*41, 146*).

Neutrophils express high levels of the pro-apoptotic proteins Bax, Bak, Bid, Bad and Bik. The proteins have a long half life explaining the constitutive apoptosis and short half life of neutrophils. However, neutrophils do not express the anti-apoptotic proteins Bcl-2 and Bcl-X_L. Instead of these two proteins, neutrophils express two other anti-apoptotic Bcl-2 family members; Mcl-1 and A1 (Bfl-1). These two proteins, unlike the other anti-apoptotic family members, do not contain a BH4 domain indicating that they may interact with different proteins to Bcl-2 and Bcl-X_L(*41, 42, 143*).

Mcl-1 is 40-42 kDa in size and is expressed in a number of tissues and cells as well as neutrophils. Along with the 1-3 BH domains it contains a PEST sequence and several motifs indicating that the protein has a high turnover rate that is between 1-5 h. It is also rapidly induced via transcription and translation (*42, 147*) Full length Mcl-1 protein is derived from transcripts containing 3 exons, but alternative splicing generates a transcript that contains only exons 1 and 3 resulting in the expression of a BH3 only protein. Mcl-1 has several phosphorylation sites, e.g. Ser^{121} and Thr^{163}, and it has been shown they activate a JNK dependent pathway in response to oxidative stress, which results in the inactivation of Mcl-1 via phosphorylation. This hyperphosphorylation of Mcl-1 targets the protein for turnover via the proteasome (*148, 149*).

A number of pro-apoptotic agents have been shown to decrease Mcl-1 protein levels indicating that Mcl-1 expression is cytokine regulated. The pro-apoptotic proteins present in neutrophils have a long half life and hence it has been proposed that the levels of Mcl-1 expression and its variable rate of degradation is the main mechanism controlling neutrophil apoptosis (*42, 150*). When Mcl-1 levels decrease, due to degradation, the pro-apoptotic proteins predominate and neutrophil apoptosis occurs. However, in response to inflammatory signals, Mcl-1 expression is induced and levels of the protein increase via enhanced stability (*151, 152*). These mechanisms enhance levels of Mcl-1 thereby prolonging cell survival enabling the cells to carry out their functions. This continues until external factors result in a decrease in Mcl-1 levels or its natural rate of turnover decreases cellular levels (*147, 148, 153*).

Bfl-1 is another anti-apoptotic molecule present in neutrophils and its predicted molecular weight is 20.1 kDa. However, due to the lack of an Bfl-1 antibody the study of this protein is limited and expression at the mRNA level can only be measured (*41*).

1.4.3 Neutrophil Necrosis

The physiological form of cell death is apoptosis however, cells may also die pathologically and this process if referred to as necrosis. Necrosis may occur under a number of different conditions e.g. cancer, injury, inflammation and infection (*41*). When cells die by necrosis they rapidly swell, the mitochondria dilate and the

organelles begin to disrupt. The plasma membrane of the cells also ruptures releasing the cytoplasmic contents into the surrounding area. The necrotic cell debris is cleared by phagocytic cells, but induces an inflammatory response (*142, 154*). When neutrophils die by necrosis, the released cytoplasmic contents such as ROS, proteases and other granule enzymes may be cytotoxic if they overwhelm tissue defence systems e.g. anti-oxidants and anti-proteases. These toxic products will cause damage to the surrounding tissue. Secondary necrosis of apoptotic cells can occur if the cells are not efficiently cleared by macrophages or fibroblasts (*38, 155*).

1.4.4 Neutrophil Netosis

Recent research has identified a third form of neutrophil cell death, termed netosis. It has been shown that neutrophils are able to release extracellular traps (NETs), which are extracellular fibres that are able to bind Gram positive and Gram negative bacteria. NETs enable neutrophils to enhance killing of extracellular pathogens whilst minimizing damage to the surrounding environment (*156*).

In vitro, NETs have been shown to contain granule proteins and chromatin to form an extracellular fibril matrix after stimulation with agents such as PMA, IL-8 and LPS. This process is dependent on ROS production. By providing a localised environment with high concentration of antimicrobial proteins, NETs enable neutrophils to kill bacteria independently of phagocytosis. It is also thought that NETs provide a physical

barrier that prevents the spread of pathogens. During the formation of NETs, the nuclear morphology of the neutrophil is lost and the internal membranes begin to disappear allowing the NET components to interact. NETs emerge as the cell membrane ruptures, resulting in the subsequent death of the cell; a process termed netosis (*156, 157*).

1.4.5 Neutrophil Survival and Bacterial Infections

After phagocytosis of most types of bacteria, neutrophils will die by apoptosis allowing the resolution of inflammation. However, some bacteria e.g. *Streptococcus pneumoniae* and *Anaplasma phagocytophilum* will delay neutrophil apoptosis to enable their survival (*158, 159*). Some bacteria may block neutrophil apoptosis, enabling intracellular survival of the pathogen or else to induce cell lysis to eliminate the neutrophils (*158, 160, 161*).

1.5 Neutrophil Involvement in Pathogenesis of COPD

COPD is an inflammatory lung disease that results in irreversible deterioration in lung function (*30*). Neutrophils have been implicated in the pathogenesis of this disease due to their increased presence in bronchial biopsies of COPD patients compared to healthy

controls. There is also an increase in the number of neutrophils in the BAL fluid and sputum of patients with COPD compared to control fluids (*2, 32*).

The recruitment of neutrophils into healthy lungs is essential in order to protect against infection. During a 24 h period, a person will usually inhale at least 10,000 micro-organisms into their lungs (*12*). However, the response of neutrophils to these infectious bacteria is usually sufficient to prevent disease.

Cigarette smoke contains nicotine and 10^{14} oxidant molecules per puff (*32, 133*). As described in 1.2.5, nicotine acts as a chemotactic factor for neutrophils. Inhaling nicotine will stimulate neutrophils in the circulation to migrate towards the lungs along a chemotactic gradient. Nicotine also stimulates the release of IL-8 from neutrophils; increased IL-8 levels have been measured in the sputum and bronchial washings of smokers (*32, 104, 133, 162*). After migration, neutrophils will infiltrate the lungs via selectin mediated cell rolling and adherence. Adherence is dependent upon the presence of the β2 integrin CD11b/CD18, the expression of which has been shown to be increased on the surface of neutrophils in patients with COPD, due to mobilization by increased levels of IL-8 (*2, 7, 35, 163*). Once cells have migrated into the lungs, a number of factors have been shown to be important in the neutrophil's ability to inflict damage.

Inflammation in the lung can occur due to a combination of many processes. Neutrophil degranulation occurs, releasing proteases and other enzymes into the tissue. The excess

proteolytic activity of the granule enzyme elastase is a potent mediator of lung damage as it stimulates excessive generation of mucus resulting in impaired lung clearance. An imbalance of proteinases and anti-proteinases in the lung contributes to the pathogenesis of the disease (*5, 34*).

Oxidative stress in the lung, that is implicated in COPD pathology, results from an imbalance of anti-oxidant and oxidant molecules with the shift being towards the oxidants and is thought to contribute to the pathogenesis of COPD (*164-166*). Lungs are exposed to oxidants either endogenously from activated phagocytes, which can release molecules such as O_2- and H_2O_2, or exogenously via air pollutants and cigarette smoke (*133, 167*). The highly cytotoxic oxidant molecules previously described, e.g. H_2O_2, HOCl and ·OH, may cause tissue damage due to their high reactivity, ability to inhibit enzymes and induce mucus hypersecretion (*63*). ROS inhibits anti-elastase, thereby enhancing the damaging effects of elastase. The presence of H_2O_2 in breath condensate is a marker of oxidative stress in the lungs and levels of this have been shown to be higher in patients with COPD than in healthy controls (*168*).

As previously described, apoptosis is required for the resolution of inflammation. However, in COPD increased levels of cytokines, such as GM-CSF, are present and these all have a stabilising effect on a neutrophils life span, decreasing the rate of apoptosis (*4, 169*). Inflamed lung tissue will have lower O_2 tensions than healthy tissue, and hypoxia has also been shown to delay neutrophil apoptosis (*170*). The extended life span of the neutrophils will increase their ability to release cytotoxic products into the

lung, resulting in tissue damage. Cigarette smoke has also been shown to impair the ingestion of apoptotic neutrophils by macrophages, reducing the rate of clearance of these cells from the lungs (*171*). This, along with the decrease in mucociliary clearance of cells from COPD lungs, results in the occurrence of secondary necrosis, which increases the cells capacity to release cytotoxic products and inflict tissue damage.

1.6 Bacterial Infection in COPD

Bacterial infections are implicated in the pathogenesis of COPD in a number of ways. Adults with a history of lower respiratory tract infections, such as bronchitis, pneumonia or whooping cough, when they were children have below average FEV_1 and lower forced vital capacity. This indicates that the respiratory diseases suffered as a child have led to the development of smaller lung capacity and impaired lung function (*172*). This in itself is not a cause of COPD, but it can make a person more susceptible when associated with the other risk factors of the disease. Bacterial pathogens are able to invade and persist in the respiratory tissue where they are able to alter the host response to tobacco, and then induce an inflammatory response (*12, 172*). Bacterial antigens are able to enhance hypersensitivity in the lung, resulting in eosinophil influx and the induction of eosinophilic inflammation. Bacteria are also able to chronically colonise the lower respiratory tract of COPD patients which can result in exacerbations of the disease (*173*). These latter two processes will be discussed in more detail.

Certain strains of bacteria can chronically colonize the lower respiratory tract of COPD patients, with 25-50 % of patients affected (*35, 174*). The main risk factors for developing such a bacterial colonization are the degree of airway obstruction in the lung and whether or not the patient continues to smoke (*175, 176*). The three most common colonizing bacteria are non-encapsulated *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis*, three micro-organisms that normally present in the nasopharynx of healthy adults and children (*177, 178*). Bacterial colonization in the lungs of stable patients, indicate that a breach of host defence by the pathogen may have occurred because the lungs can contain, but not completely eliminate the bacteria. The increased inflammation is often proportional to the bacterial load (*179*). The incidence of bacterial colonization increases with disease severity and patients with colonizations tend to have longer and more severe exacerbations (*174*).

Exacerbations in COPD can occur and are usually associated with increase in bacterial number, change in the airway location of the bacterium and virulent changes in the strain of colonizing bacteria, rather than just the presence of bacteria (*180-182*). Patients usually have 1-3 exacerbations per year and these are associated with enhanced mortality (*183*). Symptoms of exacerbations are an increase in dyspnea, increase in coughing and wheezing, and an increase in sputum production and purulence (*183*). Bacterial exacerbations result in inflammation and neutrophil recruitment to the lungs, leading to further tissue damage due to the release of ROS and proteases (*184*). Exacerbating bacteria can be identified in BAL fluid and again the three most common

forms are non-encapsulated *H. influenzae, S. pneumoniae and M. catarrhalis* (*176, 181*).

However, only 50 % of COPD exacerbations are directly attributed to bacteria, others being be due to viruses; most commonly rhinovirus (*173, 185*). This virus is able to induce cytokines and chemokines e.g. IL-8 and therefore, induce the inflammatory response (*176, 185*). Exacerbations contribute to the vicious cycle theory of the progression of the disease: an increase in bacteria leads to neutrophils being recruited to the lungs which in turn may lead to an increase in lung tissue destruction (see figure 1.8) (*186*).

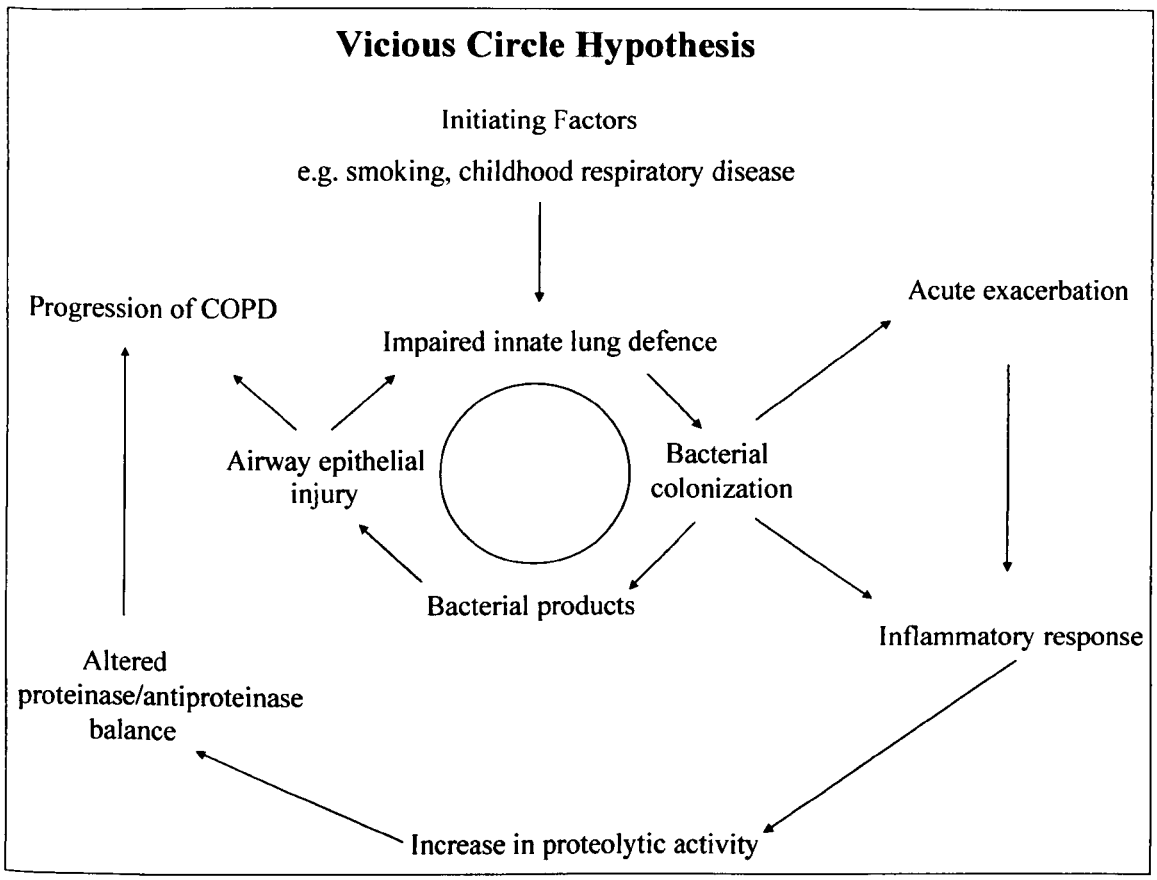


Figure 1.8: The Vicious Circle Hypothesis of COPD. This hypothesis shows factors that contribute to the development and progression of COPD. Initiating factors increase the risk of developing COPD due to a decrease in lung function and impaired lung defense to pathogens. Bacterial colonization can occur resulting in the recruitment of cells to fight the infection and the induction of an inflammatory response. This can lead to an increase in tissue damage resulting in decreased lung function, allowing more bacteria to colonize the lungs (*172, 187*).

1.7 Haemophilus influenzae

Haemophilus influenzae are small, non-motile, gram negative bacteria as shown in figure 1.9 (*188*). They are coccoid bacilli, are usually between 0.4-2 μm in size, sometimes occurring in pairs or short chains (*172*). *Haemophilus* is derived from "heme loving" and the bacteria require this factor for growth. It is usually grown on chocolate agar plates or in media supplemented with Factor X (heme) and Factor V (NAD). The genome of *H. influenzae* was sequenced in 1995, with the completion of 2-D map of the proteome following in 2000 (*189, 190*).

Capsulated *H. influenzae* has 6 serotype strains which are identified by immunologically distinct capsular polysaccharide antigens (*172*). Strains of *H. influenzae* serotype b (Hib) cause diseases such as meningitis in children, cellulitis and pneumonia. *H. influenzae* is also present in non-encapsulated forms and is present in the nasopharynx of approximately 75 % healthy adults and children (*191*). Non-encapsulated *H. influenzae* is also referred to as non-typeable due to the lack of detectable capsular polysaccharides. Non-encapsulated *H. influenzae* is pleomorphic and often exists as long threads and filaments (*192*). Non-encapsulated *H. influenzae* causes chronic bronchitis, sinusitis, conjunctivitis and otitis media in children and is the most common clinical isolate found in COPD where it chronically colonizes the lower respiratory tract of patients (*193, 194*). During colonization, *H. influenzae* contributes to inflammation in the lung by inducing the release of potent inflammatory molecules (*195, 196*).

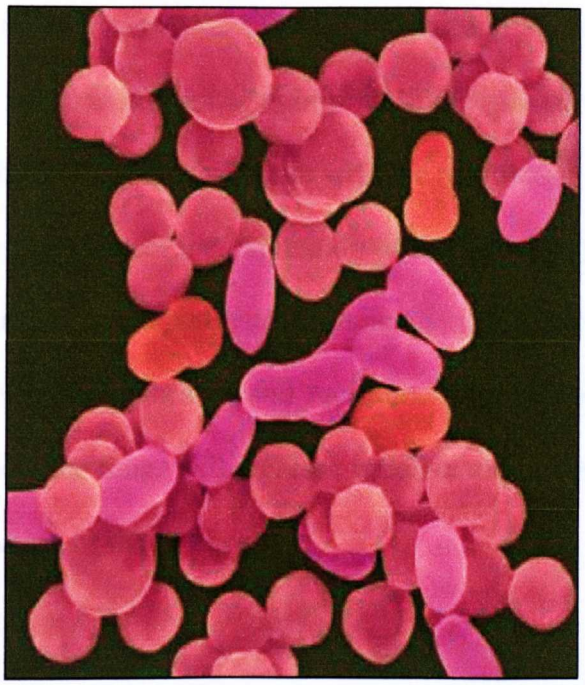


Figure 1.9: *Haemophilus influenzae* are gram-negative coccoid bacillus bacteria. They are able to chronically colonize the lower respiratory tract of COPD patients and contribute to exacerbations of the disease.
(www.prokariotae.tripod.com/Haemophilus_influenzae)

As well as chronically colonising the lower respiratory tract of COPD patients, *H. influenzae* is the main clinical isolate found in exacerbations of the disease (*197*). Due to the strain specific immune response of the body, individuals are susceptible to COPD exacerbations by acquiring new strains of the bacteria that arise from mutations to bacterial genes (*198*). Non-encapsulated *H. influenzae* express multiple adhesin molecules. Complex interactions between bacterial adhesins and host molecules can occur enabling the bacteria to adhere to the respiratory epithelial cells, reducing

clearance of bacteria from the lungs (*199*). Non-encapsulated *H. influenzae* is also capable of intracellular and intercellular invasion resulting in intracellular infection (*200*). In this way the bacteria invades sites beyond the respiratory epithelium, thereby amplifying the exacerbation. By direct contact with the respiratory epithelial cells, non-encapsulated *H. influenzae* induces the expression of the adhesive glycoprotein ICAM-1 on the epithelial surface in response to inflammation. ICAM-1 interacts with β_2-integrins to enable neutrophil adhesion and recruitment to the lung (*201, 202*). This influx of neutrophils into the lungs will increase inflammation as previously discussed, increasing the exacerbation of the disease.

1.8 H. influenzae and Neutrophil Interactions in COPD

The importance of non-encapsulated *H. influenzae* in exacerbations of COPD has been discussed above. Exacerbations of the disease in this way have been shown to cause an increase in neutrophil influx into the lungs in order to fight the infection, resulting in an increase in inflammation (*203*). Previous research into the relationship between neutrophils and non-encapsulated *H. influenzae* in COPD showed some unusual interactions (164). This showed that neutrophils are able to phagocytose non-encapsulated *H. influenzae* but are unable to kill the bacteria. Phagocytosis increased the survival of the bacteria and stimulated the release of chemotactic IL-8 from neutrophils. Morphological analysis of the neutrophils by electron microscopy showed an abnormal morphology (see figure 1.10). Neutrophils were unable to form functional

phagosomes around the bacteria and instead, large vacuoles were observed with many bacteria present inside. Some bacteria were also shown to be present in the cytoplasm of the cell. The plasma membrane of the neutrophils was not intact and it was shown that the cells eventually died by necrosis (*204*).

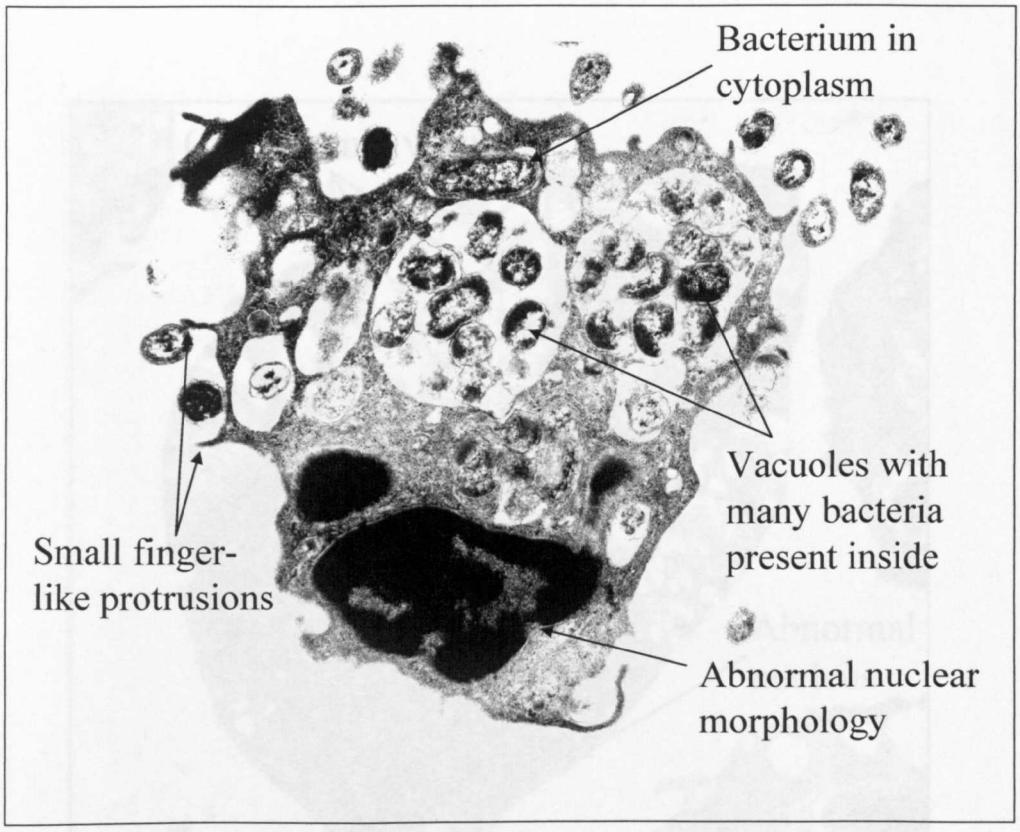


Figure 1.10: Electron microscopy analysis of a neutrophil incubated with *H. influenzae*. Neutrophils had large vacuoles with many bacteria present inside, bacteria present in the cytoplasm, an abnormal nuclear morphology and a loss of plasma membrane integrity (*204*).

Non-encapsulated *H. influenzae* was cultured and the culture filtrate was shown to contain a factor(s) that induced neutrophil necrosis, indicating that *H. influenzae* releases a factor that induces neutrophil necrosis. The morphological effects of the supernatant on neutrophils were analysed via electron microscopy and similar abnormal features were observed (see figure 1.11). The plasma membranes of the neutrophils did not appear to be intact and large vacuoles were present in the cytoplasm of the cells (*205*).

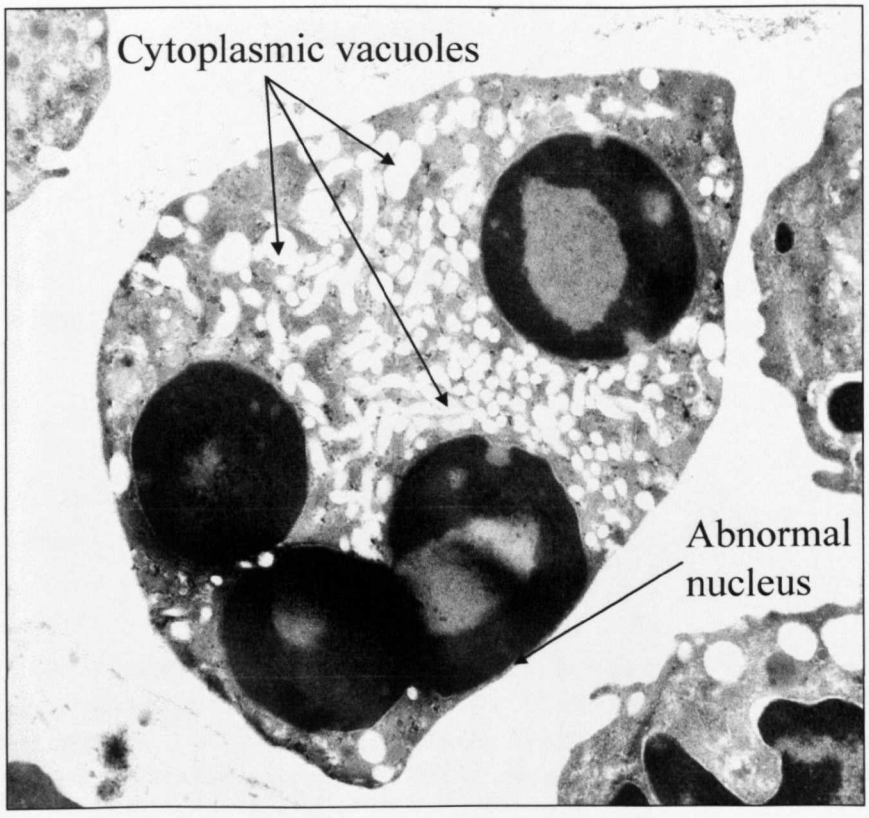


Figure 1.11: Electron microscopy analysis of a neutrophil incubated with *H. influenzae* culture filtrate. Neutrophils have cytoplasmic vacuoles, abnormal nuclear morphology and a loss in the plasma membrane integrity (*205*).

If this phenomenon occurred *in vivo*, whereby neutrophils die by necrosis upon contact with the bacteria, then their cytotoxic products and live bacteria would be released into the tissue. This would result in an increase in the inflammatory response as a result of tissue damage and an increase in bacterial infection resulting in the recruitment of more neutrophils to the site of infection and for the process to start again. This may explain the paradox in COPD: large numbers of *H. influenzae*, large numbers of non-functional neutrophils, high levels of tissue damage (*206*) (figure 1.12)

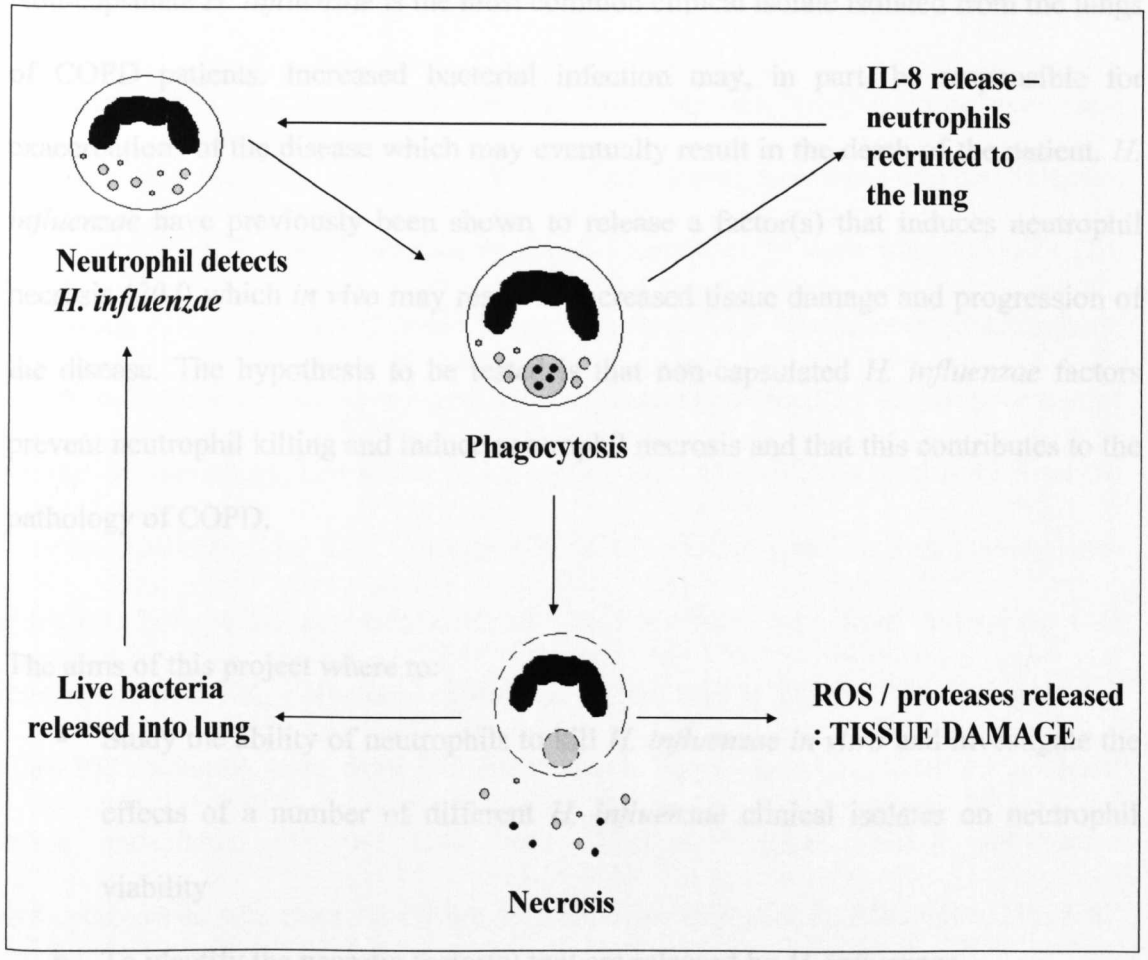


Figure 1.12: The suggested phenomenon occurring inside the COPD lung. Neutrophils phagocytose *H. influenzae* which stimulates the release of IL-8 further recruiting neutrophils to the lungs. However, neutrophils die by necrosis upon phagocytosis of *H. influenzae*, releasing live bacteria and cytotoxic products into the lungs resulting in induction of the inflammatory response and an increase in tissue damage.

Aims of this Project

Non-capsulate *H. influenzae* is the most common clinical isolate isolated from the lungs of COPD patients. Increased bacterial infection may, in part, be responsible for exacerbations of the disease which may eventually result in the death of the patient. *H. influenzae* have previously been shown to release a factor(s) that induces neutrophil necrosis (*204*) which *in vivo* may result in increased tissue damage and progression of the disease. The hypothesis to be tested is that non-capsulated *H. influenzae* factors prevent neutrophil killing and induce neutrophil necrosis and that this contributes to the pathology of COPD.

The aims of this project where to:

- Study the ability of neutrophils to kill *H. influenzae in vitro* and investigate the effects of a number of different *H. influenzae* clinical isolates on neutrophil viability
- To identify the necrotic factor(s) that are released by *H. influenzae*
- To elucidate the mechanism by which necrosis is induced in neutrophils using cell signalling inhibitors and antibodies that detect cell signalling pathways
- To analyse neutrophils isolated from the blood and sputum of patients with COPD and *H. influenzae* infections to determine whether the effects observed *in vitro* also occur *in vivo*.

Chapter 2: Materials and Methods

2.1 Materials

Polymorphprep™ was from AXIS-SHIELD, Oslo, Norway. Rapid Romanowsky Stain was from HD Supplies, Aylesbury, Foetal Calf Serum was from Harlan Seralabs, Loughborough, UK. UK. Columbia agar and CampyGen™ were from OXOID Ltd, Hampshire, UK. Defibrinated horse blood was from TCS Biosciences, Buckingham, UK. *H. influenzae* and *S. aureus* were clinical isolates provided by The Department of Medical Microbiology, University of Liverpool, UK. All cell lines used were from the European Collection of Cell Cultures (ECACC). ECL Hyperfilm and donkey anti-rabbit IgG horseradish peroxidase linked whole antibody were from Amersham Life Sciences, Bucks, UK. Polyclonal rabbit anti-human Mcl-1, TLR2-FITC conjugate and TLR4-PE conjugate were from BD Biosciences, San Diego, CA, USA. Monoclonal mouse anti-human GAPDH was from Nivelles, Belgium. Sheep anti-human myeloperoxidase was from Biodesign International, Kennebunk, ME, USA. GM-CSF was from Roche. PD98059, SB202190, LY294002, Bis-1, JNK inhibitor, SYK inhibitor, caspase inhibitor VI and H89 were from Calbiochem, Nottingham, UK. Phospho Akt, phospho Erk, phospho p38 MAPK, phospho PKC, pan Akt, pan ERK and pan p38 MAPK were from Cell Signalling, Danvers, MA, USA. All other reagents were from Sigma-Aldrich Co. Ltd, Dorset, UK, BDH Laboratory Supplies, Poole, UK, Fisher Scientific, Loughborough, UK or GIBCO, Paisley, UK.

2.2 *Neutrophil Isolation and Culture*

Studies of control, human neutrophils were approved by the University of Liverpool CORE panel. Neutrophils were isolated from heparinized blood by a one-step centrifugation with Polymorphprep^{TM}. Fresh blood was layered onto Polymorphprep^{TM} in a 2:1 ratio. The tubes were centrifuged at 1000 g for 30 min. The neutrophil layer was removed and washed with RPMI 1640 by centrifugation at 1600 g for 5 min. Contaminating erythrocytes were removed by lysing the cells with ammonium chloride lysis soultion (13.4 mM $KHCO_3$, 155 mM NH_4Cl, 96.7 μM EDTA) in a 9:1 ratio with RPMI 1640. The cells were then resuspended in. RMPI 1640 + 25 mM HEPES + 2 mM L-glutamine supplemented with 10 % (v/v) FCS. Neutrophils were counted using a Coulter counter and the cell viability was measured using trypan blue exclusion and was routinely > 98 % for freshly isolated cells. Cell purity was determined using Rapid Romanowsky Staining and was > 95 %. Neutrophils were cultured at concentrations of 5 x 10^6 cells/ml in RMPI 1640 + 25 mM HEPES + 2 mM L-glutamine supplemented with 10 % (v/v) FCS at 37 °C with gentle agitation.

2.2.1 *Neutrophil Priming*

Neutrophils at a concentration of 5 x 10^6 cells/ml were primed with GM-CSF (50 U/ml) for 40 min at 37 °C with gentle agitation.

2.2.2 *Inhibitors*

Cell cultures were treated with a range of inhibitors (Table 2.1) for 20 min before stimulation. Cells were incubated at 37 °C with gentle agitation.

Name	Solvent	Final Concentration	Target
PD98059	DMSO	50 μM	MEK
SB202190	DMSO	1 μM	p38 MAPK
LY294002	DMSO	10 μM	PI3K
Bisindolymalemide-1	DMSO	5 μg/ml	PKC
JNK inhibitor	DMSO	20 μM	JNK
SYK inhibitor	DMSO	10 μM	SYK
Caspase Inhibitor VI	DMSO	50 μM	Pan-caspase
H89	DMSO	10 μM	PKA
DPI	DMSO	10 μM	NADPH-Oxidase

Table 2.1: The concentrations and targets of cell signalling inhibitors used in experiments.

2.3 *Cell Culture*

Cell lines (listed in table 2.2) were cultured in polystyrene tissue culture flasks at 37 °C, with 5 % CO_2 in a humidified incubator. When adherent cells had formed a confluent

monolayer, the cells were passaged and harvested by trypsinisation with 1 x Trypsin-EDTA and were then counted using a Coulter counter. When suspension cells had become confluent, i.e. the media had become acidic; they were passaged, harvested and again counted using a Coulter counter. A known number of cells were added to the wells of polystyrene tissue culture 12-, 24- or 48-well plates and incubated overnight at 37 °C.

Name	Cell Type	Media	Growth
A549	Human Lung Carcinoma	DMEM + 10 % FCS	Adherent
Cos-1	Monkey African Green Kidney	DMEM + 10 % FCS	Adherent
HEK 293 WT	Human Embryonic Kidney Cells	DMEM + 10 % FCS	Adherent
HEK 293 CD14	Human Embryonic Kidney Cells transfected with CD14	DMEM + 10 % FCS + Pen/Strep + Puromycin	Adherent
HEK 293 TLR2	Human Embryonic Kidney Cells transfected with CD14 + TLR2	DMEM + 10 % FCS + Pen/Strep + Puromycin	Adherent
HEK 293 TLR4	Human Embryonic Kidney Cells transfected with CD14 + TLR4	DMEM + 10 % FCS + Pen/Strep + Puromycin	Adherent
HeLa	Human Cervical Carcinoma	MEM + 10 % FCS + 1 x Non-essential Amino Acids	Adherent
HL-60	Human Promyelocytic Leukaemia	RPMI + 10 % FCS + 2 mM L-Glutamine	Suspension
HT-29	Human Colon Adenocarcinoma	DMEM + 10 % FCS	Adherent
SK-N-AS	Human Neuroblastoma	RPMI + 10 % FCS + 2 mM L-Glutamine + 1 x Non-essential Amino Acids	Adherent
U937	Human Histiocytic Lymphoma	RMPI + 10 % FCS + 2 mM L-Glutamine	Suspension

Table 2.2: Cell lines and culture conditions used in experiments

2.4 Bacterial Culture

2.4.1 Haemophilus influenzae

11 different non-capsulated *H. influenzae* clinical isolates, from patients with exacerbations of COPD, were tested for effects on neutrophil viability, and were obtained from Prof C.A. Hart, Department of Medical Microbiology. *H. influenzae* G682 was the strain routinely used in all experiments unless otherwise stated. Chocolate agar plates were made by autoclaving Columbia agar and aseptically adding 10 % (v/v) defibrinated horse blood once the agar had cooled to 70-75 °C. The agar was then placed in a 70 °C water bath and mixed until the blood turned a chocolate brown colour, indicating the lysis of the red blood cells. 25 ml plates were poured and allowed to dry overnight at 37 °C. *H. influenzae* was grown on chocolate agar plates at 37 °C with 5 % (v/v) CO_2 or in a candle jar containing a sachet of CampyGen™. Bacteria were removed from the plates using sterile swabs and transferred to PBS so that the OD_{540} could be measured and the number of bacteria/ml determined (from a previously obtained calibration curve).

2.4.2 H. influenzae Culture Filtrate

H. influenzae lawns were grown on chocolate agar plates overnight. The bacteria were removed and resuspended in 5 ml of PBS. RPMI 1640 + HEPES was supplemented

with 5 % (v/v) glycerol, 30 mg/l hemin (10 mg/ml stock in 1.4 M ammonium hydroxide) and 10 mg/ml β-NAD. *H. influenzae* require hemin, Factor X, and β-NAD, Factor V, for growth. The media was inoculated with ~2 x10^6 bacteria/ml and incubated for 4 d at 37 °C with CO_2 and shaking (100 rpm).

After 4 d incubation, 5 ml of the culture was removed and serial dilutions prepared so that a viable count of the bacteria could be carried out. The remaining culture was centrifuged at 1000 g for 15 min. The supernatant was removed and this step was repeated. The supernatant was filtered through a 0.2 μm filter and stored at -20 °C in aliquots until required.

2.4.3 Staphylcoccus aureus

S. aureus (Oxford strain) was grown on LB agar plates at 37 °C overnight. A single colony was used to inoculate 20 ml of LB broth that was incubated overnight at 37 °C with shaking at 100 rpm. 200 μl of overnight culture was aseptically plated onto LB agar plates and incubated at 37 °C overnight. Bacterial lawns were removed with a sterile swab and transferred to PBS. Serial dilutions of these suspensions were made and aliquots plated onto LB agar plates in order to estimate viable counts. The concentration of *S. aureus* was adjusted to 1 x 10^{10}/ml, heat-killed by incubating at 60 °C for 30 min and then stored at –20 °C.

2.4.4 Bacterial Opsonisation

1 x 10^9 bacteria were pelleted by centrifugation at 500g for 5 min. The supernatant was removed and the bacteria resuspended in 30 % (v/v) heat-inactivated human pooled AB serum and incubated at 37 °C with shaking for 1 h. After incubation, bacteria were washed three times and resuspended in PBS to give a known concentration of bacteria/ml. Unopsonised samples were prepared by incubating bacteria in PBS at 37 °C for 1 h.

2.4.5 Size Fractionation of H. influenzae Culture Filtrate

H. influenzae culture filtrate was initially filtered through a 0.2 μm filter to remove any contaminating bacterial products. The culture filtrate was then filtered through a 50 kDa Macrosep filter by centrifuging at 1000 g for 90 min. Macrosep filters contain a low protein-binding Omega™ membrane made from polyethersulphone which significantly reduces non-specific adsorption to enable high recoveries. Pretreating the membrane with 10 % glycerol can further reduce non-specific binding. After centrifugation, the filter was washed with PBS to remove the > 50 kDa molecules, which were resuspended at the original volume with PBS and stored at - 20 °C. 2 ml of the < 50 kDa eluate was removed and stored at -20 °C until required. The remaining < 50 kDa eluate was then filtered through 30 kDa, 10 kDa, 5 kDa, 3 kDa and 1 kDa filters by

centrifuging at 1000 g for 90 min. The following fractions obtained were analysed for their effects on cell viability:

10 kDa	< 10 kDa
10-5 kDa	< 5 kDa
5-3 kDa	< 3 kDa
3-1 kDa	< 1 kDa

2.5 Isolation of Neutrophils from Sputum

Studies on samples obtained from patients (COPD and asthmatic) were approved by Sefton Local Research Ethics Committee.

Sputum was collected by expectoration into a 50 ml sterile container. Specimens were processed within 2 h of collection. Sputum plugs were collected and separated from saliva using forceps. The sputum was transferred to a pre-weighed Falcon tube and the weight of the sputum was determined. To each g of sputum, 10 ml PBS was added. The mixture was vigorously shaken to disperse the sputum and then incubated at 37 °C for 30 min with shaking. After incubation, this step was repeated with fresh PBS. The mixture was diluted to 50 ml with PBS and the cells were collected by centrifugation and washed twice with PBS. The cells were then resuspended in 10 ml PBS.

The solution was layered onto Ficoll Paque solution and centrifuged at 1000 g for 30 min. The supernatant was removed and pelleted cells (neutrophils) were resuspended in RPMI 1640 + 25 mM HEPES + 2 mM L-Glutamine supplemented with 10 % (v/v) FCS.

2.6 ROS Production by Luminol Amplified Chemiluminescence

Assays were performed at 37 °C using a LKB 1251 luminometer. 10 μM luminol was added to a volume of 1 ml RPMI 1640 containing 1 x 10^6 neutrophils. The respiratory burst was triggered using either 0.1 μg/ml PMA or 0.1 μg/ml fMLP.

2.7 Bacterial Killing

Live *H. influenzae* were prepared as described in section *2.4.1.* Neutrophils resuspended in RPMI 1640 + HEPES + 2 mM L-Glutamine supplemented with 10 % FCS at a concentration of 5 x 10^6/ml. Cultures with bacteria and neutrophils at a ratio of 20:1 were prepared and incubated at 37 °C, with gentle agitation for 3 h. A control culture containing only bacteria was also prepared in the same medium so that the number of viable bacteria at the start of the experiment could be determined. This control culture measured the ability of the bacteria to survive and replicate in the medium.

After incubation, the neutrophils were lysed by a 1:2000 dilution with dH_2O so that phagocytosed bacteria were released. Further dilutions were carried out in PBS so that an aliquot of 100 µl would give approximately 300 colonies after incubation on a chocolate agar plate. The plates were incubated at 37 °C overnight with 5 % (v/v) CO_2. The number of colonies on each plate was counted and the number of viable bacteria/ml calculated for each culture.

2.8 Flow Cytometry

1 x 10^6 cells were removed from culture and centrifuged at 500 g for 5 min. The supernatant was discarded and the cells resuspended in 100 µl of HBSS. The required amount of antibody was added, as indicated by the manufacturer (and subsequently checked experimentally), and the cells incubated for 30 min at 4 °C. When staining with Annexin-V FITC conjugate and PI, cells were resuspended in Annexin-V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 1 µl of Annexin-V-FITC. The samples were incubated for 15 min in the dark at 4 °C. After incubation, the cells were pelleted at 500 g and resuspended in 1 ml HBSS before adding PI (1 µg/ml) and then incubated for a further 15 min in the dark at room temperature. The cells were analysed using a Coulter-Epics Altra Flow Cytometer where 20,000 cells/sample were analysed.

2.9 *Western Blotting*

Neutrophils were cultured in RPMI 1640 medium containing 25 mM HEPES and 2 mM L-Glutamine supplemented with 10 % (v/v) FCS at concentrations of 5 x 10^6 cells/ml or 1 x 10^7 cell/ml for the preparation of protein lysates from cell pellets or cell supernatants respectively. Protein lysates were prepared by centrifuging neutrophils at 500 g for 3 min and then adding boiling 5 x SDS-PAGE sample buffer (50 % glycerol, 5 % β-mercaptoethanol, 3 % SDS, 0.005 % bromophenol blue, 12.5 % 1 M Tris pH 6.8) to cell supernatants and 1 x SDS-PAGE sample buffer (10 % glycerol, 5 % β-mercaptoethanol, 3 % SDS, 0.001 % bromophenol blue, 12.5 % 1 M Tris pH 6.8) to cell pellets. Samples were boiled for 5 min and stored at -20 °C until required. When preparing protein lysates for the detection of phosphorylated proteins, cells were cultured at 5 x 10^6 cells/ml in RMPI 1640 containing 25 mM HEPES and 2 mM L-glutamine. After stimulation the cells were put on ice to stop the reaction and pelleted by centrifugation at 500 g for 5 min at 4 °C. Cell pellets were then resuspended in 1 x SDS-PAGE sample buffer (10 % glycerol, 5 % β-mercaptoethanol, 3 % SDS, 0.001 % bromophenol blue, 12.5 % 1 M Tris pH 6.8) containing protease inhibitors (Roche Cat. No. 11697498001) and phosphatase inhibitors (Calbiochem Cat. No. 524625).

Proteins were separated by SDS-PAGE using 8-12 % polyacrylamide gels depending on the size of the protein being investigated. 0.5 x 10^6 cells or the equivalent volume of cell supernatants were loaded per lane. After electrophoresis, proteins were transferred to a PVDF membrane using a BioRad Mini Protean II Transfer apparatus. The

membrane was then blocked for 1 h at room temperature in blocking buffer (TBS (10mM Tris, 150mM NaCl, pH 8.0), 0.5 % (w/v) Marvel, 0.075 % (v/v) Tween 20) with gentle agitation. Membranes were washed for 2 x 30 s in wash buffer (TBS, 0.075 % (v/v) Tween 20) and then incubated with the primary antibody in antibody buffer (TBS, 0.5 % (w/v) Marvel, 0.075 % (v/v) Tween 20) at 4 °C overnight with gentle agitation. Membranes were washed for 2 x 30 s, 1 x 5 min and 1 x 15 min with wash buffer before being incubated with a HRP-linked secondary antibody for at least 1h at room temperature. Membranes were again washed in wash buffer for 2 x 30 s, 1 x 5 min and 1 x 15 min. Bound secondary antibody was detected using ECL detection reagents and hyperfilm. After detection, the membrane was stained with Ponceau S to ensure that protein loading was equal.

Protein	Primary Antibody Dilution	Secondary Antibody Dilution
Calgranulin	Mouse anti-human calgranulin (1:2000)	Donkey anti-mouse IgG HRP conjugate (1:20,000)
Mcl-1	Rabbit anti-human Mcl-1 (1:20,000)	Donkey anti-rabbit IgG HRP conjugate (1:20,000)
Lactoferrin	Rabbit anti-human lactoferrin (1:20,000)	Donkey anti-rabbit IgG HRP conjugate (1:20,000)
MPO	Sheep anti-human MPO (1:20,000)	Donkey anti-sheep IgG HRP conjugate (1:20,000)
GAPDH	Mouse anti-human GAPDH (1:20,000)	Donkey anti-mouse IgG HRP conjugate (1:20,000)
Phospho Akt	Rabbit anti-human phospho Akt (1:2000)	Donkey anti-rabbit IgG HRP conjugate (1:2000)
Phospho Erk	Rabbit anti-human phospho Ekt (1:2000)	Donkey anti-rabbit IgG HRP conjugate (1:2000)
Phospho p38	Mouse anti-human phospho p38 (1:2000)	Donkey anti-mouse IgG HRP conjugate (1:2000)
Phospho PKC	Rabbit anti-human phospho PKC (1:2000)	Donkey anti-rabbit IgG HRP conjugate (1:2000)

Table 2.3: Dilutions of primary and secondary antibodies used for protein detection during Western Blotting.

2.10 Confocal Microscopy

Neutrophils were cultured in RPMI 1640 medium + HEPES + 2 mM L-glutamine supplemented with 10 % (v/v) FCS and cell lines were cultured as previously described in *2.2 Neutrophil Isolation and Culture* and *2.3 Cell Culture.* 1 µl of Annexin-V FITC conjugate and 1 µg/ml PI were added to the cultures. The cells were observed on a Zeiss LSM 510 confocal microscope using a 20 x magnification. Annexin-V-FITC was excited with 488 nm and the emission at 530 nm was recorded with a 505-550 nm band

pass filter. PI excitation was at 543 nm and emission was recorded at 585 nm with a 585 nm long pass filter. Cells were imaged every 30 min for 18 h to observe apoptosis and necrosis. Images were analysed using LSM 510 computer software.

2.11 Transmission Electron Microscopy

Neutrophils were pelleted at 500 g for 5 min and resuspended in EM fixative (2.5 % (v/v) glutaraldehyde) for 1 h. Cells were pelleted, washed twice in dH_20 and resuspended in 1 ml of 50 % ethanol. Samples were then stable at room temperature and were sent for analysis by transmission electron microscopy in the Department of Medical Microbiology, University of Liverpool.

2.12 Statistical Analysis

Data sets were analysed using the ANOVA test with a Bonferroni correction and the unpaired Student's T-test.

Chapter 3: Effects of *H. influenzae* and *H. influenzae* Culture Filtrate on Neutrophil Viability

Introduction

Neutrophils are an essential part of the immune system due to their ability to recognise, phagocytose and kill invading pathogens *(39)*. During phagocytosis, a small phagosome will form around a bacterium providing the optimum environment for the generation of ROS and the release and activation of granule enzymes. This process enables the neutrophils to kill the bacteria resulting in eradication of the infection *(39, 49)*. The inflammatory response initiated as a result of the infection can then be resolved by the ability of neutrophils to undergo apoptosis. If neutrophils die by necrosis rather than apoptosis, or apoptotic cells are not efficiently cleared and undergo secondary necrosis, the cytotoxic products of the cell will be released into the surrounding environment. This can cause an increase in tissue damage and a prolonged inflammatory response *(41)*.

The lungs of COPD patients have been shown to contain large numbers of bacteria, large numbers of neutrophils and a high level of tissue damage. This clinical picture suggests that the neutrophils may not be working efficiently to clear the bacteria inside the COPD lung and their cytotoxic products may be contributing to the increase in tissue damage and an increase in inflammation *(186)*.

Previous research has shown that *H. influenzae*, the most common clinical isolate found in COPD lungs, has unusual interactions with neutrophils. Morphological analysis by electron microscopy of neutrophils incubated with *H. influenzae* has shown that the cells are able to phagocytose but not kill the bacteria (*204*). Phagocytosing neutrophils have a number of large vacuoles detectable inside the cell, with many bacteria inside and bacteria were also found in the cytoplasm of the cell. Neutrophils had an abnormal nuclear morphology, a loss of plasma membrane integrity and appeared to die by necrosis. *H. influenzae* was shown to induce the release of IL-8 by neutrophils. This neutrophil chemoattractant will initiate the migration of more neutrophils to the site of infection where the neutrophils will continue to phagocytose but not kill the bacteria and then die by necrosis *(204)*.

Further studies indicated that *H. influenzae* releases a factor(s) that induces neutrophil necrosis. Neutrophils were incubated with *H. influenzae* culture filtrate and similar morphological features to those occurring when incubated with bacteria were observed. Neutrophils had a number of large vacuoles in the cytoplasm, even though there were no bacteria present, an abnormal nuclear morphology and again appeared to die by necrosis *(205)*.

The aims of this chapter were to:

- Examine the ability of neutrophils to phagocytose and kill *H. influenzae.*
- Investigate the effects of increasing ratios of *H. influenzae* on neutrophil viability.
- Analyse the effects of 11 different clinical isolates of *H. influenzae*, isolated from the lungs of patients with COPD, on neutrophil viability.
- Investigate the extracellular release of granule enzymes from neutrophils incubated with *H. influenzae* and *S. aureus*.
- Examine the effects of *H. influenzae* culture filtrate on neutrophil ROS production, cell viability and the extracellular release of granule proteins.
- Determine the approximate size of the necrotic factor(s) present in *H. influenzae* culture filtrate.
- Analyse the cell specificity of *H. influenzae* culture filtrate.

3.1 Neutrophil killing of H. influenzae

It is essential for neutrophils to phagocytose and kill bacteria to stop the spread of infection around the body. The efficiency of neutrophils to phagocytose and kill *H. influenzae* was investigated in this section.

Figure 3.1 shows there was no decrease in the number of viable *H. influenzae* after incubation with neutrophils compared to the (no neutrophil) control. In fact, the number of viable *H. influenzae* actually increased following incubation with neutrophils. This indicates that not only are neutrophils unable to kill the bacteria, *H. influenzae* may be able to survive and replicate inside the cell, indicating that the environment inside the neutrophils may be more favourable for bacterial growth than the external environment.

When *H. influenzae* were incubated with neutrophils that had been primed with either GM-CSF or LPS the number of viable bacteria increased further. This increase was not due to the priming agents, as there was no increase in bacterial number when *H. influenzae* were treated with either GM-CSF or LPS alone. This large increase may be due to the increased capacity of primed neutrophils to phagocytose bacteria; more bacteria will be phagocytosed and thus be able to survive and replicate in the favourable environment of the neutrophil, increasing

the number of bacteria released after neutrophil lysis. Alternatively, it may be that the intracellular environment of the primed neutrophils is a more favourable environment for *H. influenzae* growth. Therefore, it can be deduced that neutrophils phagocytose but are unable to kill *H. influenzae* resulting in the survival and replication of the bacteria.

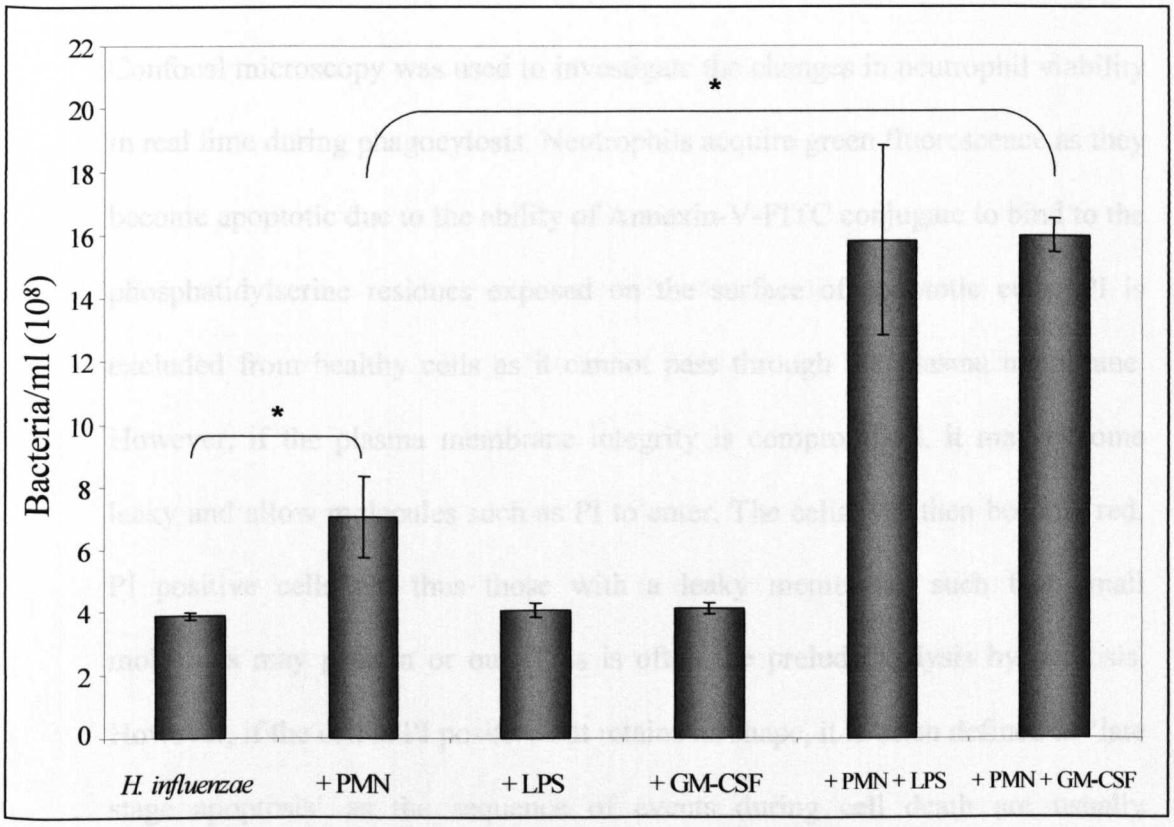


Figure 3.1: Survival of *H. influenzae* after incubation with neutrophils under various conditions. *H. influenzae* were incubated with neutrophils (PMN), LPS (100 ng/ml), GM-CSF (50 U/ml) and a combination of PMN + LPS or GM-CSF for 3 h. The number of viable bacteria in the culture was determined by incubation on chocolate agar plates in CO_2 at 37 °C overnight. For this experiment n = 3, $\pm$ S.D. * $p < 0.05$.

3.2 *Effect of Increasing Ratios of H. influenzae on Neutrophil Viability*

Morphological analysis of neutrophils incubated with *H. influenzae* indicated that the neutrophils died by necrosis.

Confocal microscopy was used to investigate the changes in neutrophil viability in real time during phagocytosis. Neutrophils acquire green fluorescence as they become apoptotic due to the ability of Annexin-V-FITC conjugate to bind to the phosphatidylserine residues exposed on the surface of apoptotic cells. PI is excluded from healthy cells as it cannot pass through the plasma membrane. However, if the plasma membrane integrity is compromised, it may become leaky and allow molecules such as PI to enter. The cells will then become red. PI positive cells are thus those with a leaky membrane, such that small molecules may pass in or out. This is often the prelude to lysis by necrosis. However, if the cell is PI positive but retains its shape, it is often defined as 'late stage apoptosis' as the sequence of events during cell death are usually Annexin-V positive followed by PI positive. However, in the experiments described later in this thesis, cells become PI positive without apparently previously showing signs of Annexin-V positivity. They are therefore termed 'necrotic' although clearly the plasma membrane has not completely disrupted.

Figure 3.2.1 shows the effects of increasing ratios of *H. influenzae* on neutrophil apoptosis. The results show that the lowest ratio of bacteria to neutrophils, 10:1,

induced a similar rate of apoptosis to that of untreated neutrophils. Neutrophil apoptosis decreased as the ratio of *H. influenzae* increased; a 1000:1 ratio of bacteria to neutrophils resulting in the lowest amount of neutrophil apoptosis. The effect of increasing ratios of bacteria on neutrophil necrosis is shown in figure 3.2.2. All ratios of bacteria to neutrophils tested resulted in an increase in neutrophil necrosis compared to untreated cells and this effect increased as the ratio of bacteria present increased i.e. a ratio of 1000:1 resulting in the greatest level of neutrophil necrosis. The initial rate of necrosis increased with higher ratios of bacteria, with a higher number of necrotic cells detected as early as 3 h when cells were incubated with 500:1 or 1000:1 bacteria to neutrophils.

Unopsonised bacteria, at bacteria to neutrophil ratio of 250:1, were also shown to induce necrosis. However, the effect was not a great as that observed in neutrophils incubated with the same ratio of serum-opsonised bacteria indicating that opsonised bacteria are more readily phagocytosed by the neutrophils inducing necrosis more quickly. Figure 3.2.3 shows images taken during the experiment at 8 h. At 8 h, nearly all neutrophils are necrotic when incubated with bacteria to neutrophils at a ratio of 250:1 and upwards. 8 h control cells have a significant number of apoptotic cells which are decreased by the addition of *H. influenzae*.

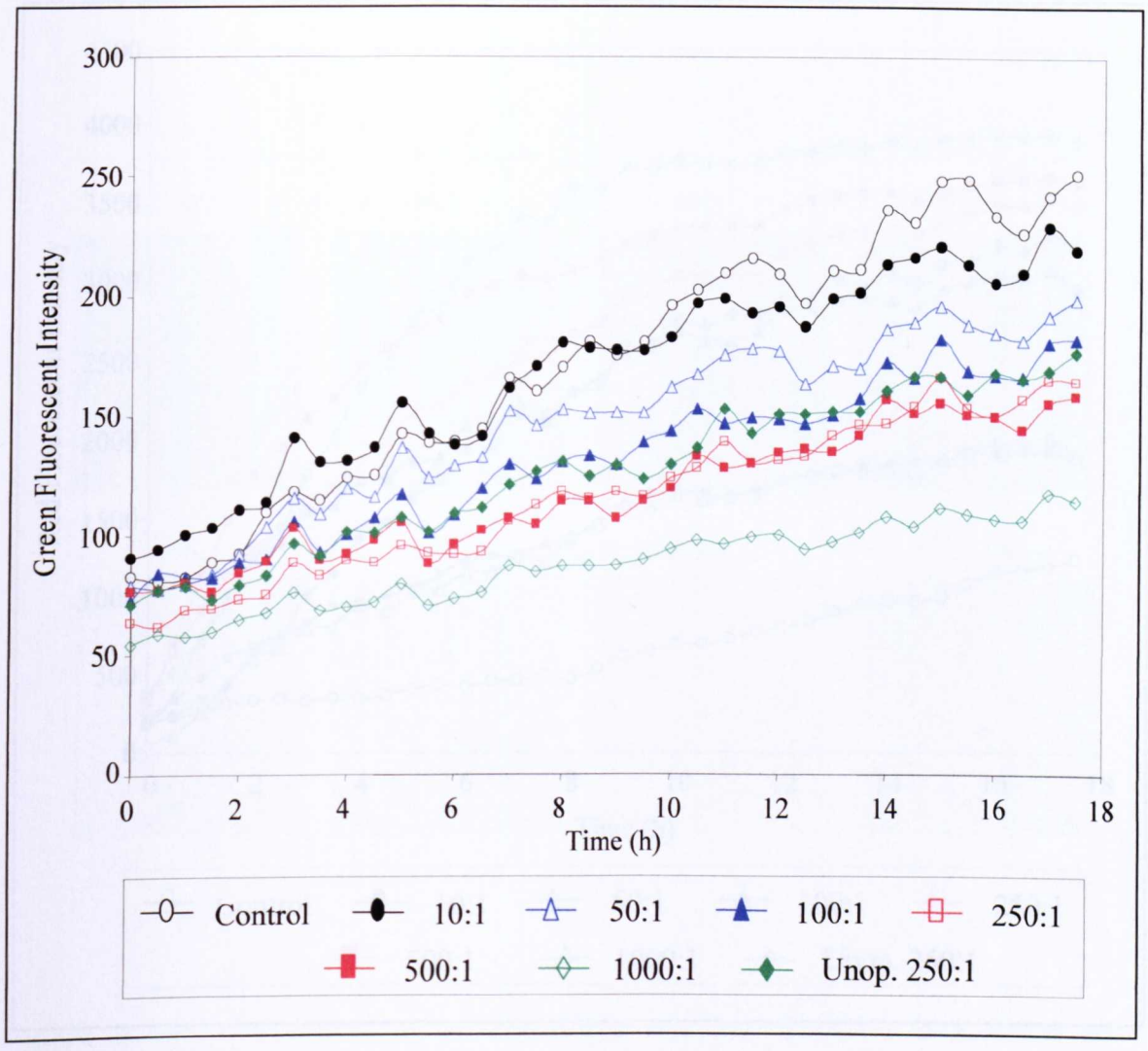


Figure 3.2.1: Effect of increasing *H. influenzae*/neutrophil ratio on neutrophil apoptosis. Green fluorescence intensity (apoptosis) from confocal microscopy of neutrophils incubated with increasing ratios of serum-opsonised *H. influenzae* is shown. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) and propidium iodide (1 µg/ml, red fluorescence). Images were taken every 3 min for 18 h at x 20 magnification. Graph is a representative of 3 experiments.

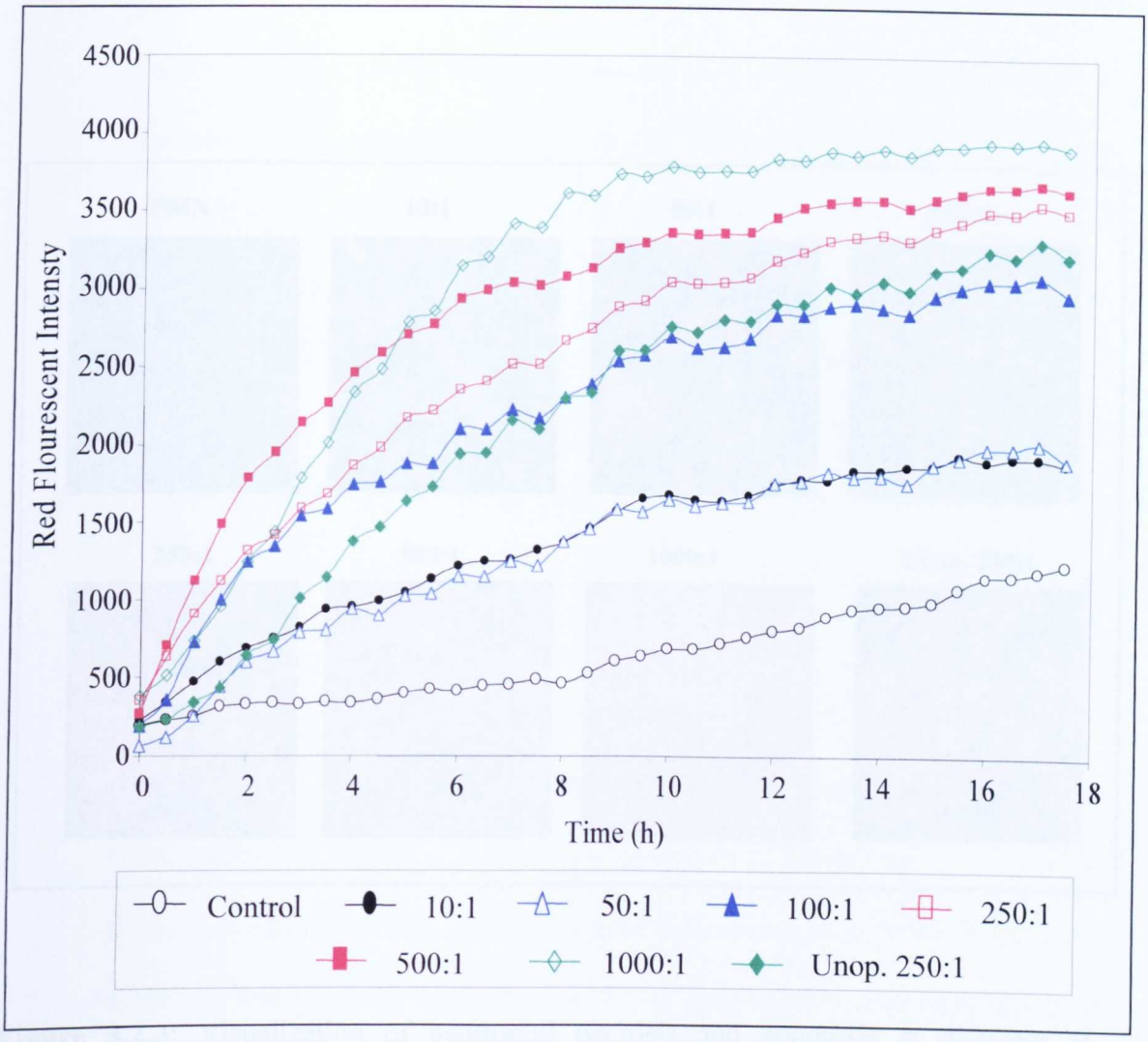


Figure 3.2.2: Effect of increasing *H. influenzae*/neutrophil ratio on neutrophil necrosis. Red fluorescence intensity (necrosis) from confocal microscopy of neutrophils incubated with increasing ratios of serum-opsonised *H. influenzae* is shown. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) and propidium iodide (1 µg/ml, red fluorescence). Images were taken every 3 min for 18 h at x 20 magnification. Graph is a representative of 3 experiments.

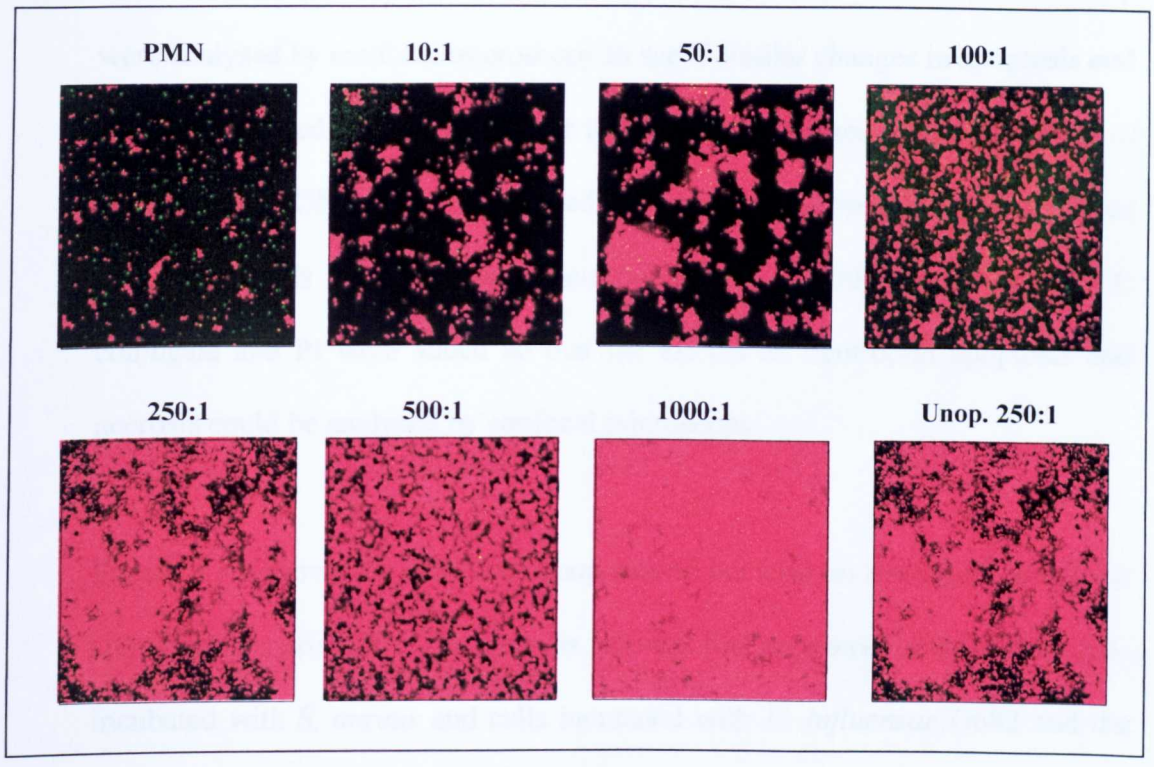


Figure 3.2.3: Visualization of neutrophil necrosis and apoptosis at different *H. influenzae*/neutrophil ratios. 8 h images from confocal microscopy of neutrophils incubated with increasing ratios of serum opsonised *H. influenzae* are shown. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) and propidium iodide (1 µg/ml, red fluorescence). Images were taken every 3 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

3.3 *Effects of Different H. influenzae Clinical Isolates on Neutrophil Viability*

The previous experiments used a clinical isolate that induced neutrophil necrosis, *H. influenzae* G682. The effects of 10 different non-capsulate *H. influenzae* clinical isolates and *S. aureus* (Oxford strain) on neutrophil viability were analysed by confocal microscopy to see if similar changes in apoptosis and necrosis occurred. The *H. influenzae* isolates were obtained from the sputum of patients with COPD. Serum-opsonised and unopsonised bacteria were incubated with neutrophils at a bacteria to neutrophil ratio of 100:1. Annexin-V-FITC conjugate and PI were added so that the effects on neutrophil apoptosis and necrosis could be analysed by confocal microscopy.

Figure 3.3.1 shows the effects of unopsonised bacteria on neutrophil apoptosis (A) and necrosis (B). The data is shown for untreated neutrophils, cells incubated with *S. aureus* and cells incubated with *H. influenzae* G682 and the strain of *H. influenzae* that caused the greatest increase in necrosis (H525) and the lowest increase in necrosis (H530). All *H. influenzae* strains were shown to decrease neutrophil apoptosis compared to untreated cells and cells treated with *S. aureus*. Necrosis induced by H530 was similar to that observed in cells treated with *S. aureus,* all other isolates were shown to increase necrosis. Necrosis induced by H525 started to occur at ~ 6 h however, the other unopsonised isolates were not shown to have an effect until ~ 12 h. Figure 3.3.2 shows the level of neutrophil necrosis at 8 h.

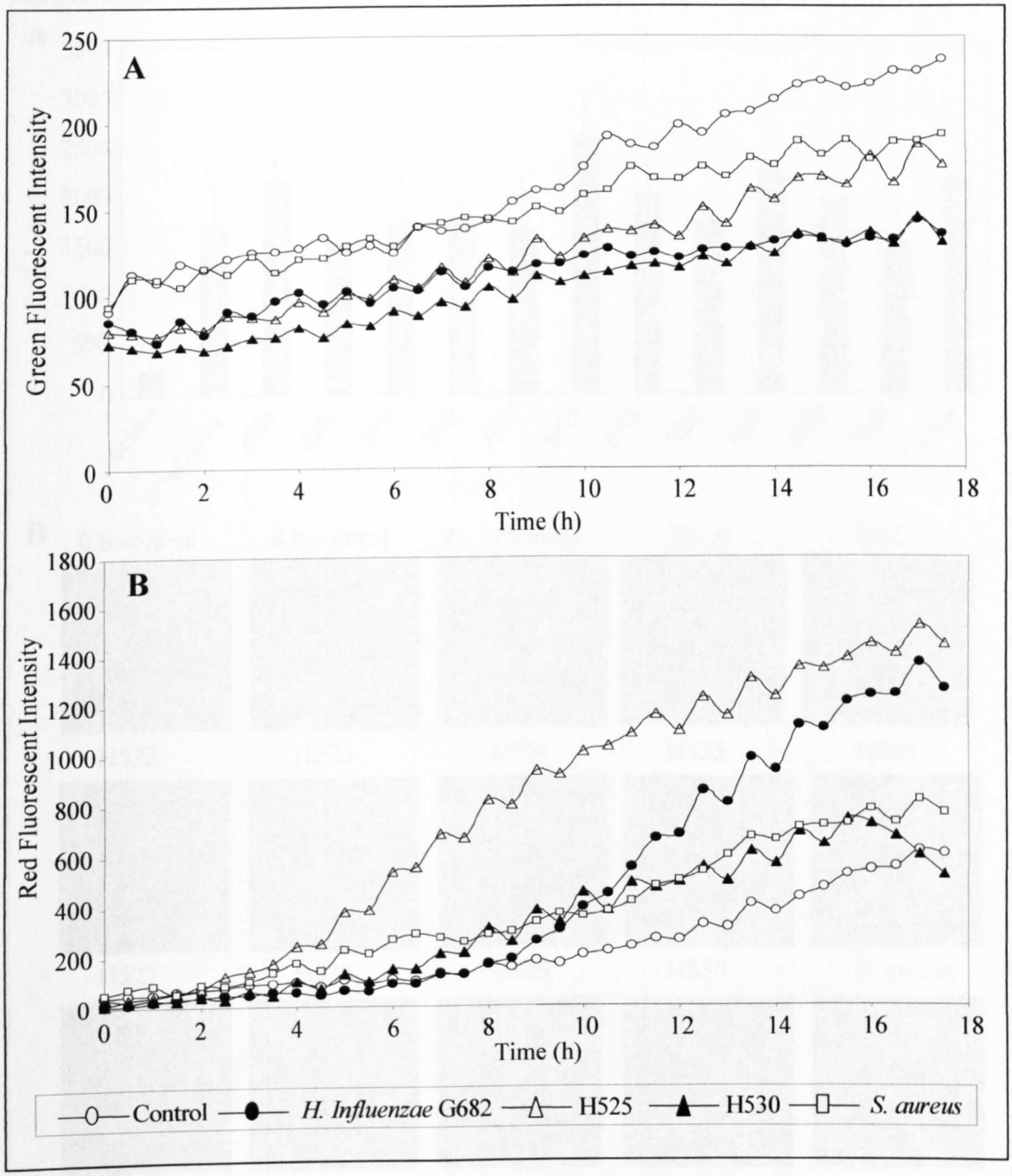


Figure 3.3.1: Effect of *H. influenzae* unopsonised clinical isolates on neutrophil apoptosis and necrosis. Fluorescence changes of neutrophils incubated in a 100:1 bacteria to neutrophil ratio with *H. influenzae* clinical isolates. Neutrophils were stained with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) to measure the effects on neutrophil apoptosis (A) and necrosis (B). Data was obtained for 10 different clinical isolates of *H. influenzae* and those shown are for untreated neutrophils, *S. aureus*, *H. influenzae* G682, and the isolate which caused the greatest (H525) and the lowest (H530) increase in necrosis above controls. Graphs are representative of 3 experiments.

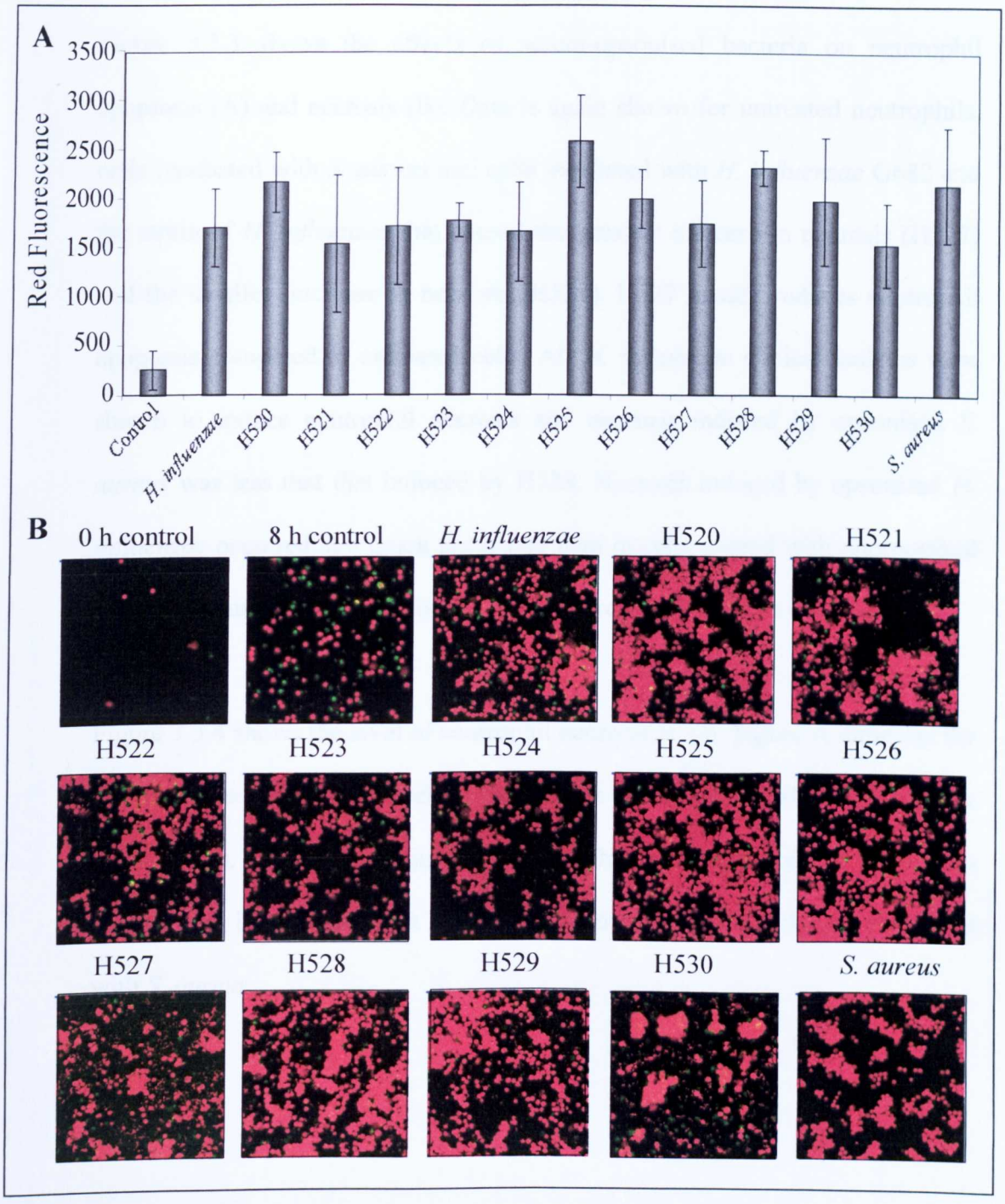


Figure 3.3.2: Effect of *H. influenzae* clinical isolates on neutrophil necrosis at 8 h. Fluorescence intensities and images from confocal microscopy of neutrophils incubated in a 100:1 bacteria to neutrophil ratio with 10 different unopsonised non-capsulate *H. influenzae* clinical isolates. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml). Figure A shows the red fluorescent intensity (necrosis) of neutrophils after 8 h incubation. All isolates were shown to increase necrosis. Figure B shows the images taken at 8 h. Fluorescent intensities are a mean of 3 experiments, ± S.D.

Figure 3.3.3 shows the effects of serum-opsonised bacteria on neutrophil apoptosis (A) and necrosis (B). Data is again shown for untreated neutrophils, cells incubated with *S. aureus* and cells incubated with *H. influenzae* G682 and the strain of *H. influenzae* that caused the greatest increase in necrosis (H527) and the smallest increase in necrosis (H528). H527 greatly reduces neutrophil apoptosis compared to untreated cells. All *H. influenzae* clinical isolates were shown to induce neutrophil necrosis and necrosis induced by opsonised *S. aureus* was less that that induced by H528. Necrosis induced by opsonised *H. influenzae* occurred at a much faster rate than in cells treated with unopsonised bacteria (figure 3.3.1), with high levels of necrosis observed as early as 2 h.

Figure 3.3.4 shows the level of neutrophil necrosis at 8 h. Figure A indicates the red fluorescent intensity reflecting the necrotic cells. Figure B shows the images taken at 8 h. At 8 h the majority of neutrophils incubated with *H. influenzae* isolates had become necrotic compared to untreated cells and cells incubated with *S. aureus*.

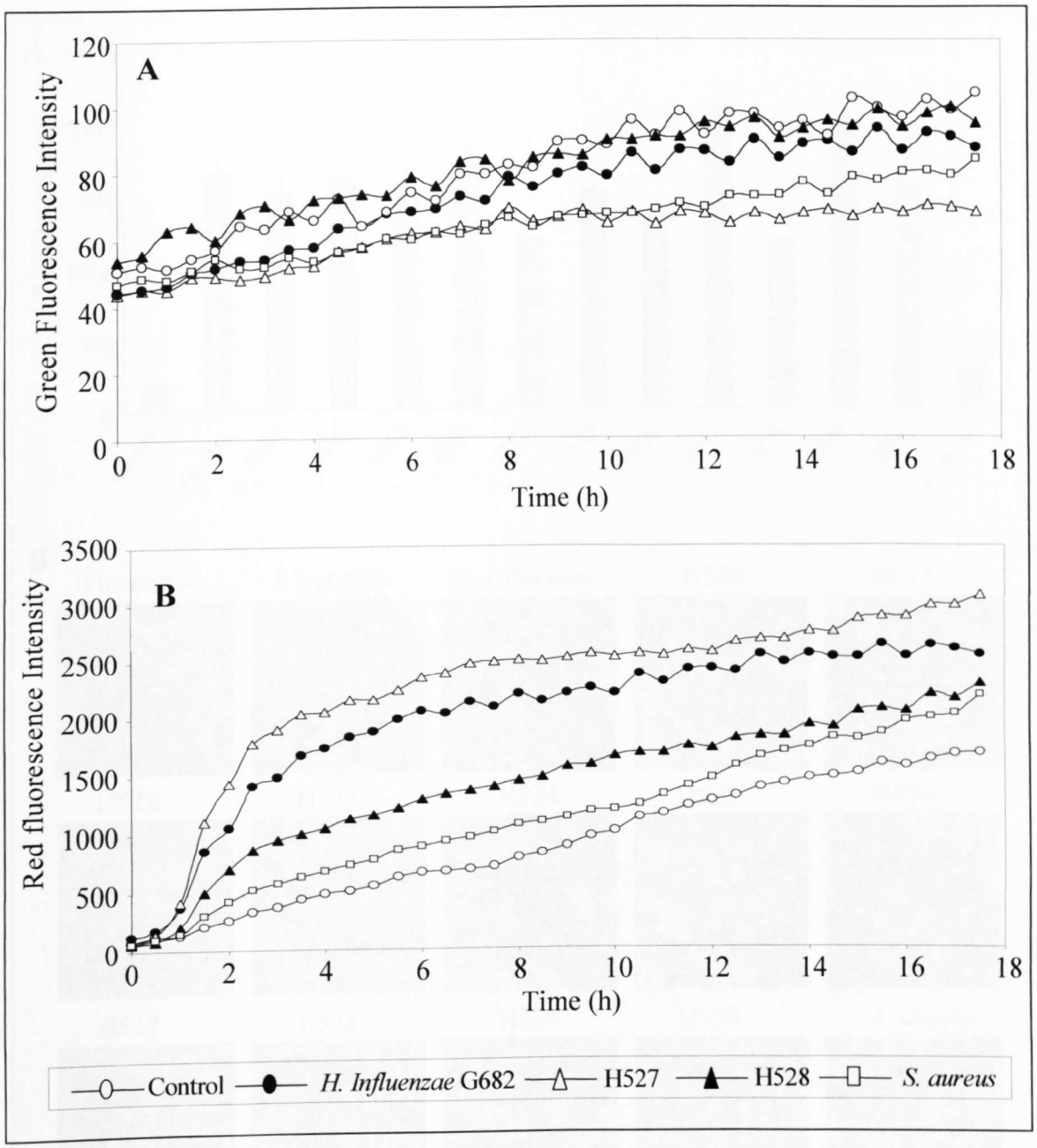


Figure 3.3.3: Effect of *H. influenzae* opsonised clinical isolates on neutrophil apoptosis and necrosis. Fluorescence intensity from confocal microscopy of neutrophils incubated in a 100:1 bacteria to neutrophil ratio *H. influenzae* clinical isolates. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) to measure the effects on neutrophil apoptosis (A) and necrosis (B) were. Data was obtained for 10 different clinical isolated of *H. influenzae* and those shown are for untreated neutrophils, *S. aureus*, *H. influenzae* G682, and the isolate which caused the greatest (H527) and the lowest (H528) increase in necrosis. Graphs are representative of 3 experiments.

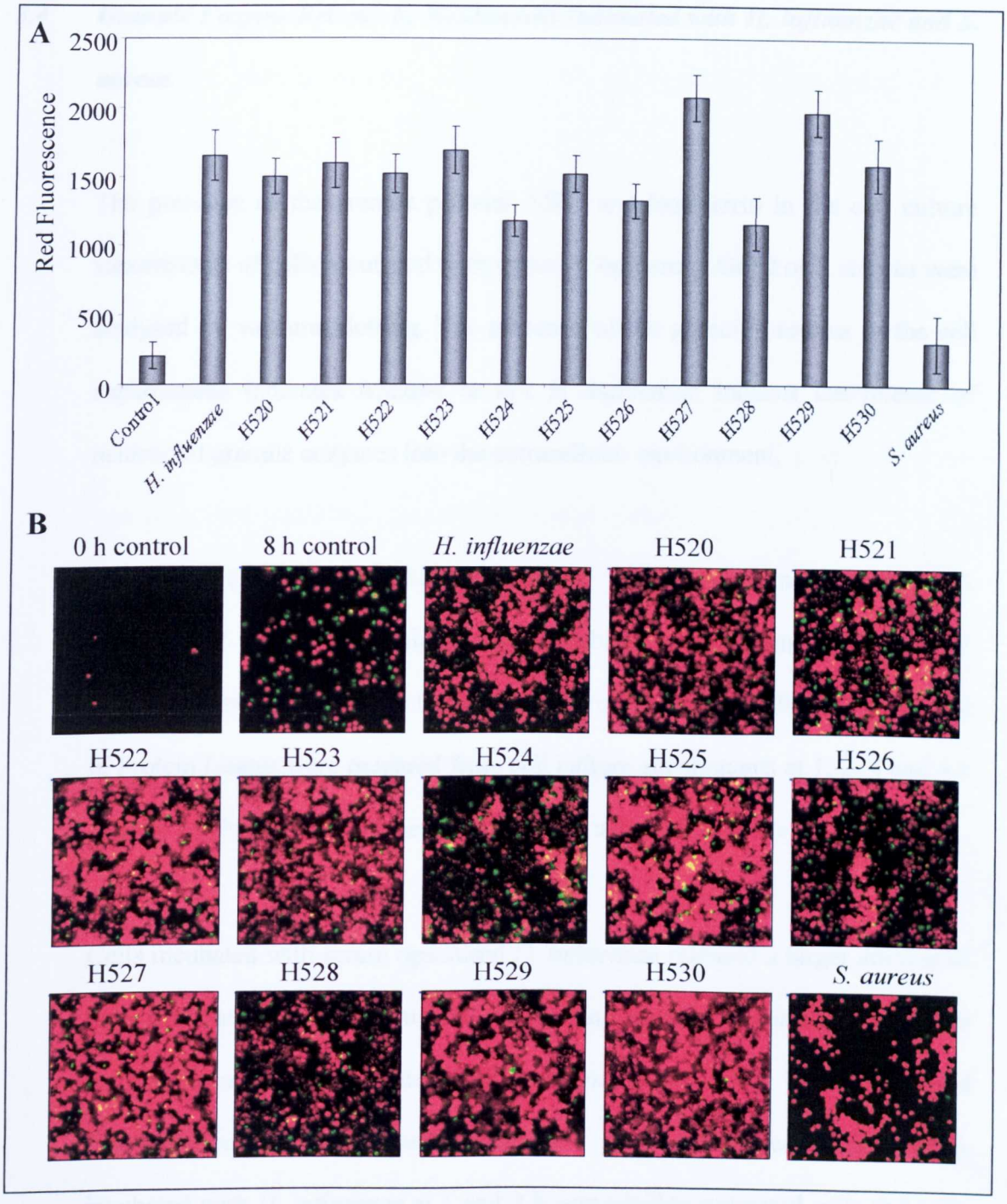


Figure 3.3.4: Effect of *H. influenzae* clinical isolates on neutrophil necrosis at 8 h. Fluorescence intensities and images from confocal microscopy of neutrophils incubated in a 100:1 bacteria to neutrophil ratio with 10 different opsonised non-capsulate *H. influenzae* clinical isolates. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml). Figure A shows the red fluorescent intensity (necrosis) of neutrophils after 8 h incubation. Figure B shows the images taken at 8 h. Fluorescent intensities are a mean of 3 experiments, ± S.D.

3.4 *Granule Enzyme Release by Neutrophils Incubated with H. influenzae and S. aureus*

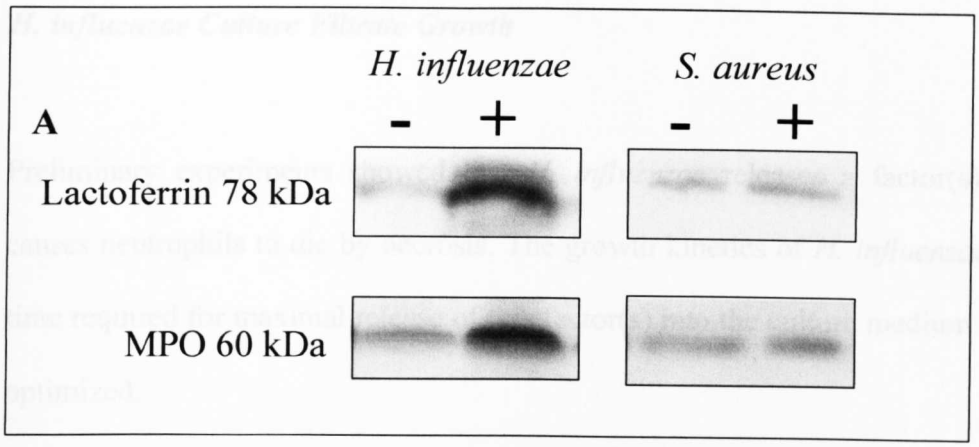
The presence of the granule proteins MPO and lactoferrin in the cell culture supernatants of cells incubated with either *H. influenzae* G682 or *S. aureus* were analysed by western blotting. The presence of the granule proteins in the cell supernatants indicates whether or not *H. influenzae* induces the release of neutrophil granule enzymes into the extracellular environment.

Neutrophils (1 x 10^7 cells/ml in RPMI 1640 + HEPES supplemented with 10 % FCS and 2 mM L-glutamine) were incubated with serum opsonised *H. influenzae* and *S. aureus* at a bacteria to neutrophil ratio of 250:1 at 37 °C for 6 h. Protein lysates were prepared from cell culture supernatants at 1, 2, 4 and 6 h and the levels of MPO and lactoferrin present detected by western blotting.

Cells incubated with serum opsonised *H. influenzae* released a larger amount of both MPO and lactoferrin into the culture supernatants compared to untreated neutrophils and cells incubated with *S. aureus* (figure 3.4). The presence of lactoferrin in cell culture supernatants was greatly increased in neutrophils incubated with *H. influenzae* at 1 and 2 h compared to untreated cells and cells incubated with *S. aureus*. This release was sustained over 6 h (data not shown). *S. aureus* and *H. influenzae* both slightly increased the levels of MPO present in cell culture supernatants at 1 h compared to untreated cells. However, the level

of MPO present in supernatants increased over time in cells incubated with *H. influenzae*, and this increase was not seen when cells were incubated with *S. aureus*.

These results indicate that when neutrophils are incubated with *H. influenzae* granule enzymes are released into the surrounding environment to a much greater extent than controls or when neutrophils were incubated with *S. aureus*, as they undergo necrosis. If this occurs *in vivo* then released granule enzymes and other toxic products could result in tissue damage.



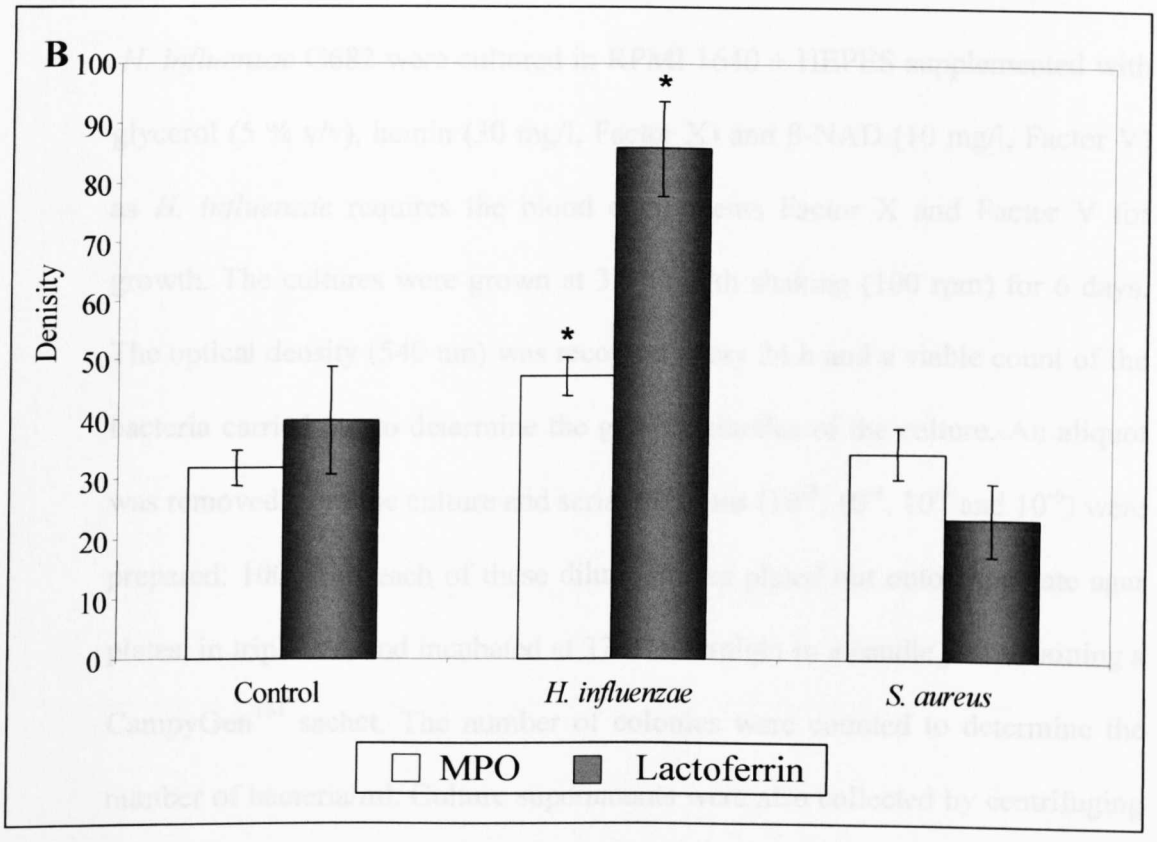


Figure 3.4: Analysis of granule enzyme release from neutrophils incubated with *H. influenzae* and *S. aureus*. Serum opsonised *H. influenzae* or *S. aureus* were incubated with neutrophils in a 250:1 ratio for up to 6 h at 37 °C. Neutrophils were incubated with (+) and without (-) bacteria. Protein lysates were prepared from culture supernatants and the extracellular release of MPO and lactoferrin were detected by western blotting. Figure A shows MPO and lactoferrin release at 2 h. Figure B indicates the MPO and lactoferrin release at 2 h, respectively. Western blots shown are a mean of 3 experiments, $\pm$ SD * $p < 0.05$ compared to control neutrophils.

3.5 *H. influenzae Culture Filtrate Growth*

Preliminary experiments showed that *H. influenzae* releases a factor(s) that causes neutrophils to die by necrosis. The growth kinetics of *H. influenzae* and time required for maximal release of this factor(s) into the culture medium were optimized.

H. influenzae G682 were cultured in RPMI 1640 + HEPES supplemented with glycerol (5 % v/v), hemin (30 mg/l, Factor X) and β-NAD (10 mg/l, Factor V) as *H. influenzae* requires the blood components Factor X and Factor V for growth. The cultures were grown at 37 °C with shaking (100 rpm) for 6 days. The optical density (540 nm) was recorded every 24 h and a viable count of the bacteria carried out to determine the growth kinetics of the culture. An aliquot was removed from the culture and serial dilutions (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were prepared. 100 µl of each of these dilutions was plated out onto chocolate agar plates, in triplicate, and incubated at 37 °C overnight in a candle jar containing a CampyGen™ sachet. The number of colonies were counted to determine the number of bacteria/ml. Culture supernatants were also collected by centrifuging bacterial cultures (twice) at 1600 g for 30 min. The culture filtrate was filtered through a 0.2 µm filter to remove any bacterial debris and the remaining culture filtrate stored at -20 °C until required.

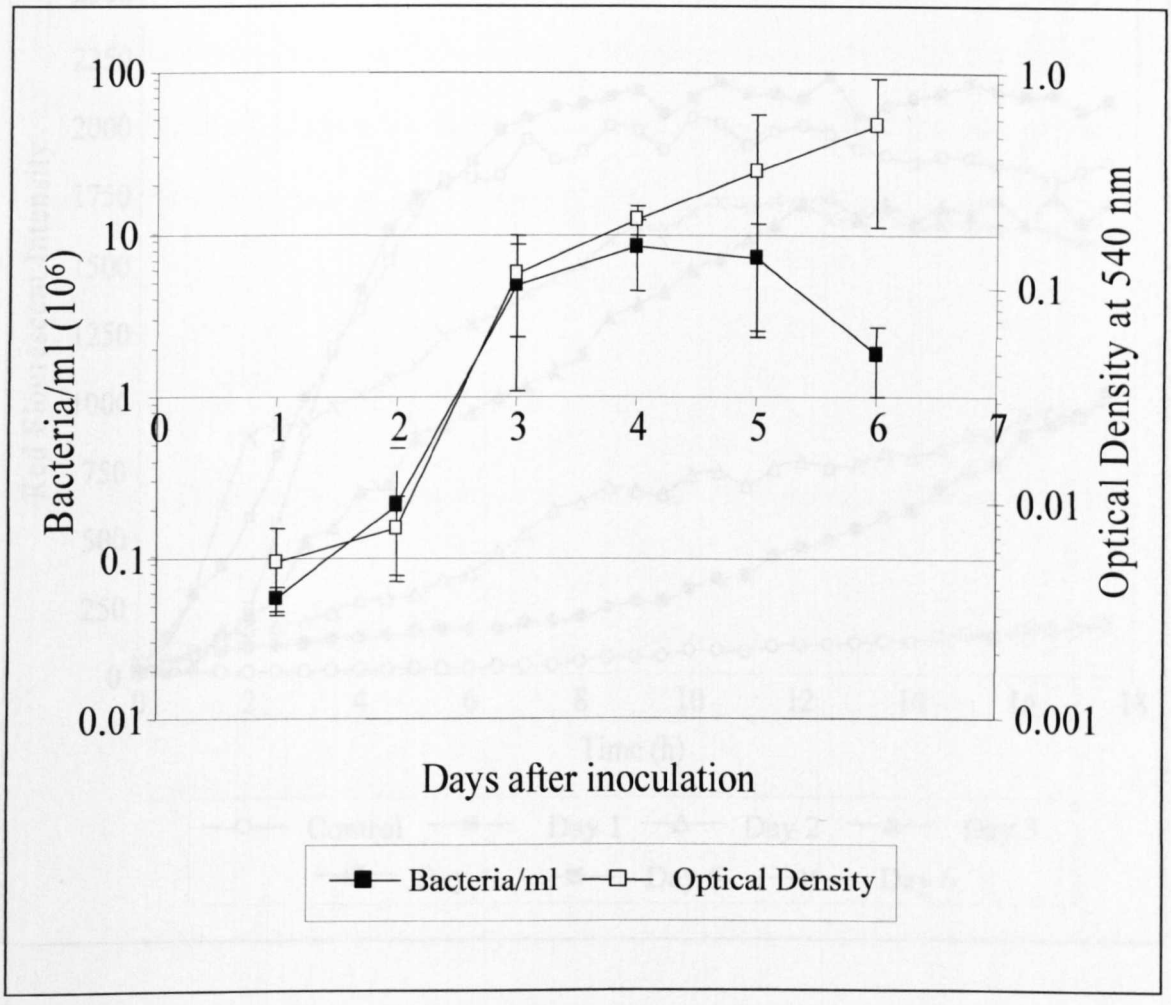


Figure 3.5.1: *H. influenzae* growth curve over 6 days. The culture was incubated at 37 °C with agitation and growth was measured by optical density (OD_{540}) and viable counts of bacteria. Data shown is a mean of 4 experiments, ± S.D.

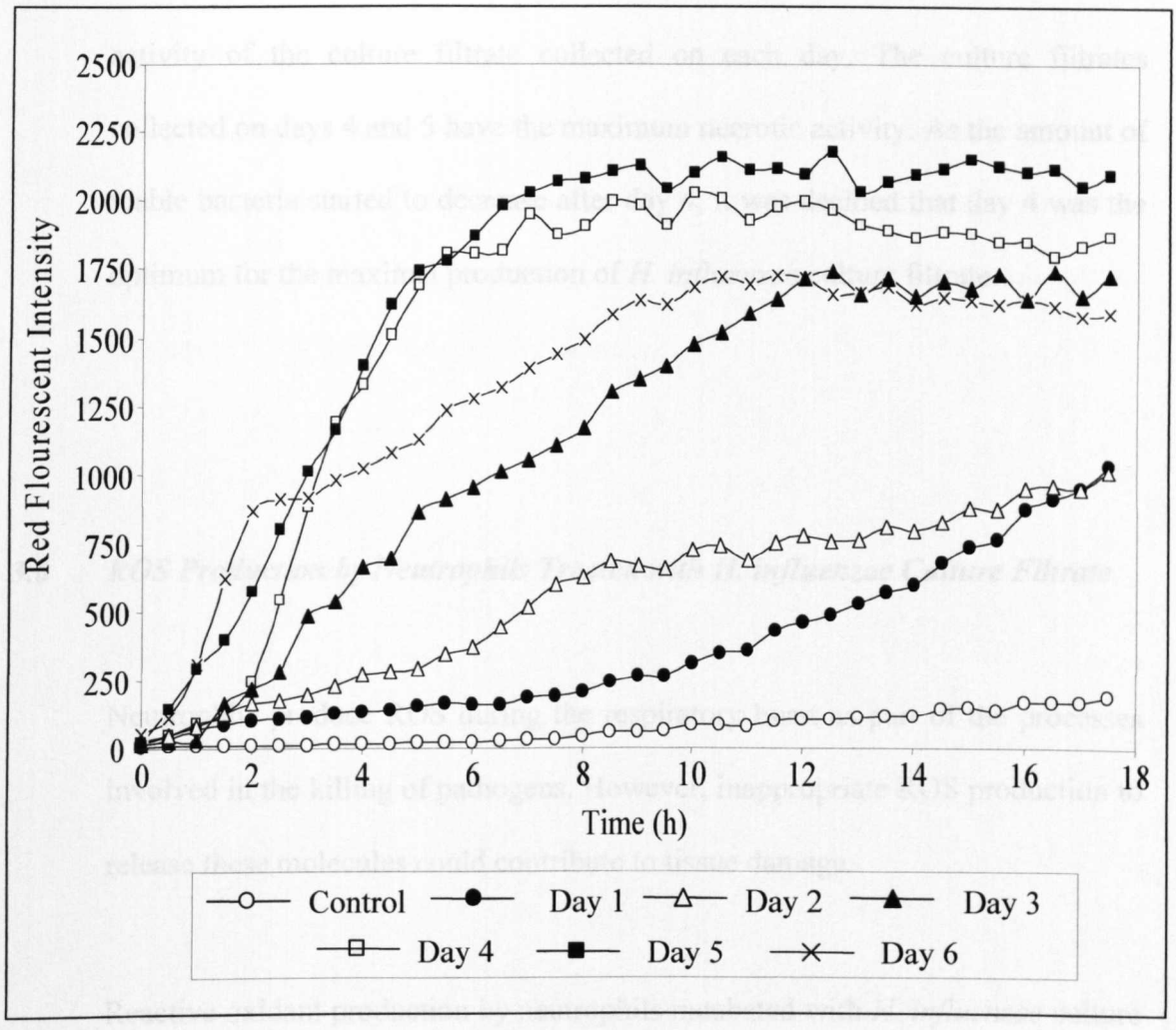


Figure 3.5.2: Effect of *H. influenzae* culture filtrate collected over 6 days on neutrophil viability. Red fluorescence intensity (necrosis) from confocal microscopy of *H. influenzae* culture filtrate activity over 6 days. Neutrophils were incubated with 200 µl/ml of each fraction and incubated with PI (1 µg/ml) to determine the effects on neutrophil necrosis. Graphs are representative of 4 experiments.

Figure 3.5.1 shows that the OD_{540} increased up to day 6 but the viable number of bacteria/ml started to decrease after day 4. Figure 3.5.2 shows the necrotic activity of the culture filtrate collected on each day. The culture filtrates collected on days 4 and 5 have the maximum necrotic activity. As the amount of viable bacteria started to decrease after day 4, it was decided that day 4 was the optimum for the maximal production of *H. influenzae* culture filtrate.

3.6 *ROS Production by Neutrophils Treated with H. influenzae Culture Filtrate*

Neutrophils produce ROS during the respiratory burst as part of the processes involved in the killing of pathogens. However, inappropriate ROS production to release these molecules could contribute to tissue damage.

Reactive oxidant production by neutrophils incubated with *H. influenzae* culture filtrate in RPMI 1640 + HEPES was measured using luminol-amplified chemiluminescence. Neutrophils were primed for 40 min with GM-CSF. 10 μM luminol was added to 1 x 10^5 neutrophils after stimulation with different volumes of culture filtrate or PMA (0.1 μg/ml) and chemiluminescence was measured at 37 °C over 45 minutes.

Figure 3.6 shows that primed neutrophils treated with *H. influenzae* culture filtrate have increased levels of ROS production compared to primed or unprimed cells that have not been stimulated. Cells stimulated with PMA produced the greatest amount of ROS overall. However, neutrophils stimulated with 400 µl of *H. influenzae* culture filtrate produced ROS levels similar to those obtained with PMA. As the volume of culture filtrate decreased the amount of ROS produced also decreased.

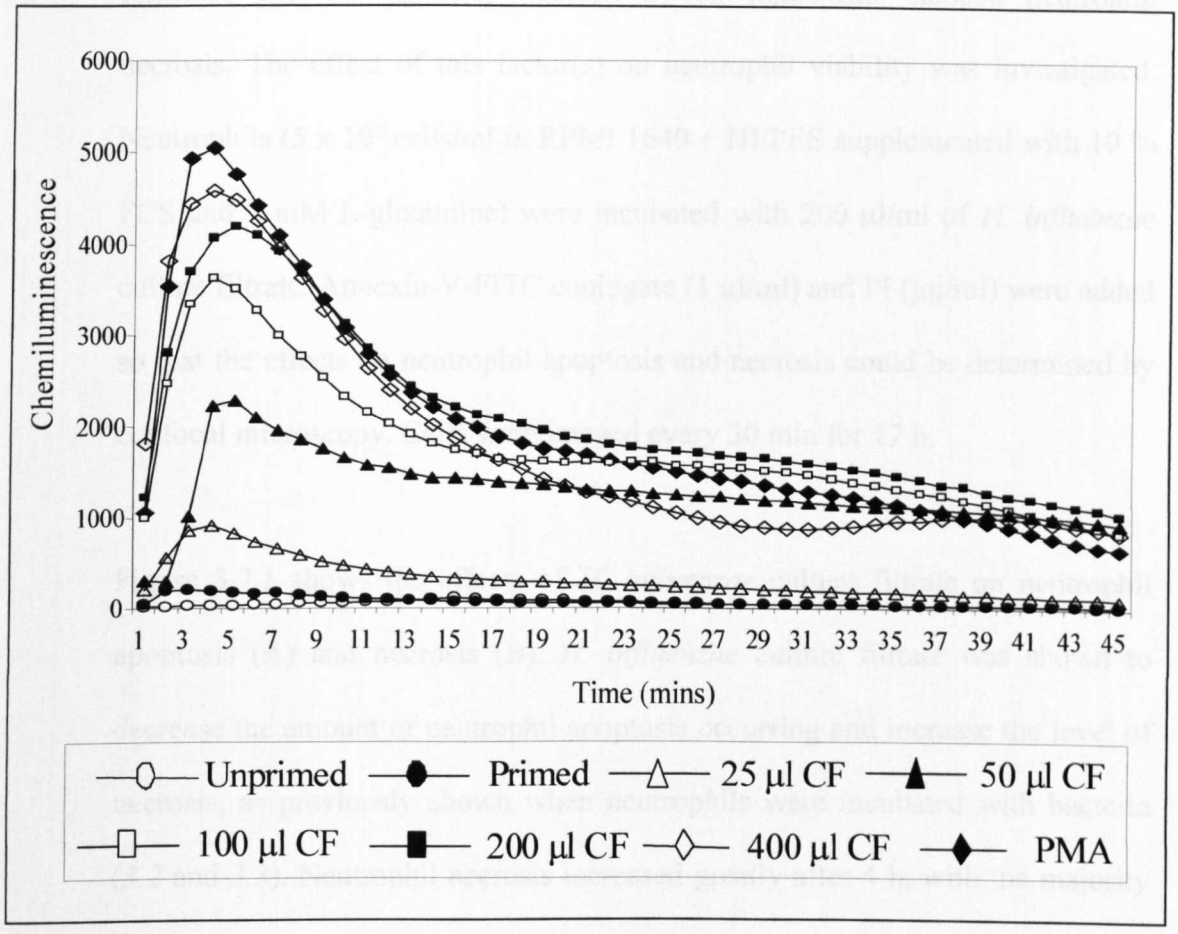


Figure 3.6: Effect *of H. influenzae* culture filtrate on neutrophil ROS release. Neutrophils were primed for 40 min with GM-CSF (50 U/ml). 10 µM luminol was used to measure the ROS production induced by PMA, or different volumes of *H. influenzae* culture filtrate (CF) by chemiluminescence. Cells were measured every 15 sec for 45 min. Data is representative of 3 or more experiments.

3.7 Effects of H. influenzae Culture Filtrate on Neutrophil Viability

EM analysis of neutrophils incubated with *H. influenzae* culture filtrate indicates that the factor(s) released by *H. influenzae* induced neutrophil necrosis. The effect of this factor(s) on neutrophil viability was investigated. Neutrophils (5 x 10^6 cells/ml in RPMI 1640 + HEPES supplemented with 10 % FCS and 2 mM L-glutamine) were incubated with 200 µl/ml of *H. influenzae* culture filtrate. Annexin-V-FITC conjugate (1 µl/ml) and PI (µg/ml) were added so that the effects on neutrophil apoptosis and necrosis could be determined by confocal microscopy. Cells were imaged every 30 min for 17 h.

Figure 3.7.1 shows the effects of *H. influenzae* culture filtrate on neutrophil apoptosis (A) and necrosis (B). *H. influenzae* culture filtrate was shown to decrease the amount of neutrophil apoptosis occurring and increase the level of necrosis, as previously shown when neutrophils were incubated with bacteria (*3.2* and *3.3*). Neutrophil necrosis increased greatly after 4 h, with the majority of cells becoming necrotic by 8 h. Figure 3.7.2 shows the images taken at 0, 4, 8 and 18 h. Green apoptotic cells can be clearly seen in the images of the untreated control cells. The images of cells treated with culture filtrate do not show any green apoptotic cells but show large numbers of red necrotic cells.

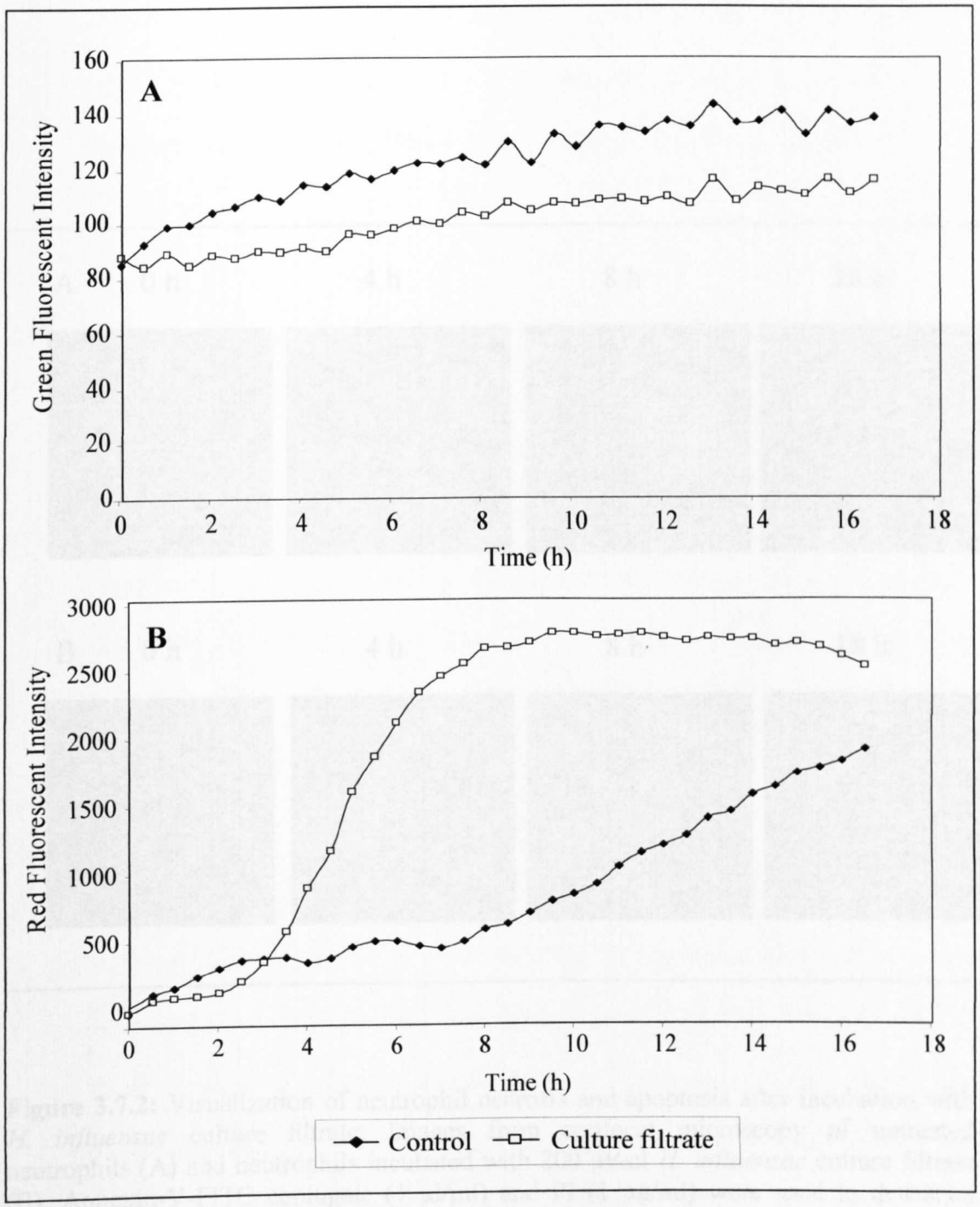


Figure 3.7.1: Effect of *H. influenzae* culture filtrate on neutrophil apoptosis and necrosis. Fluorescent intensity from confocal microscopy of neutrophils incubated with 200 µl/ml *H. influenzae* culture filtrate. Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) were used to detect an increase in apoptosis (A) and necrosis (B) by increases in green and red fluorescence respectively. Images were taken every 30 min for 17 h. Graphs are representative of 3 or more experiments.

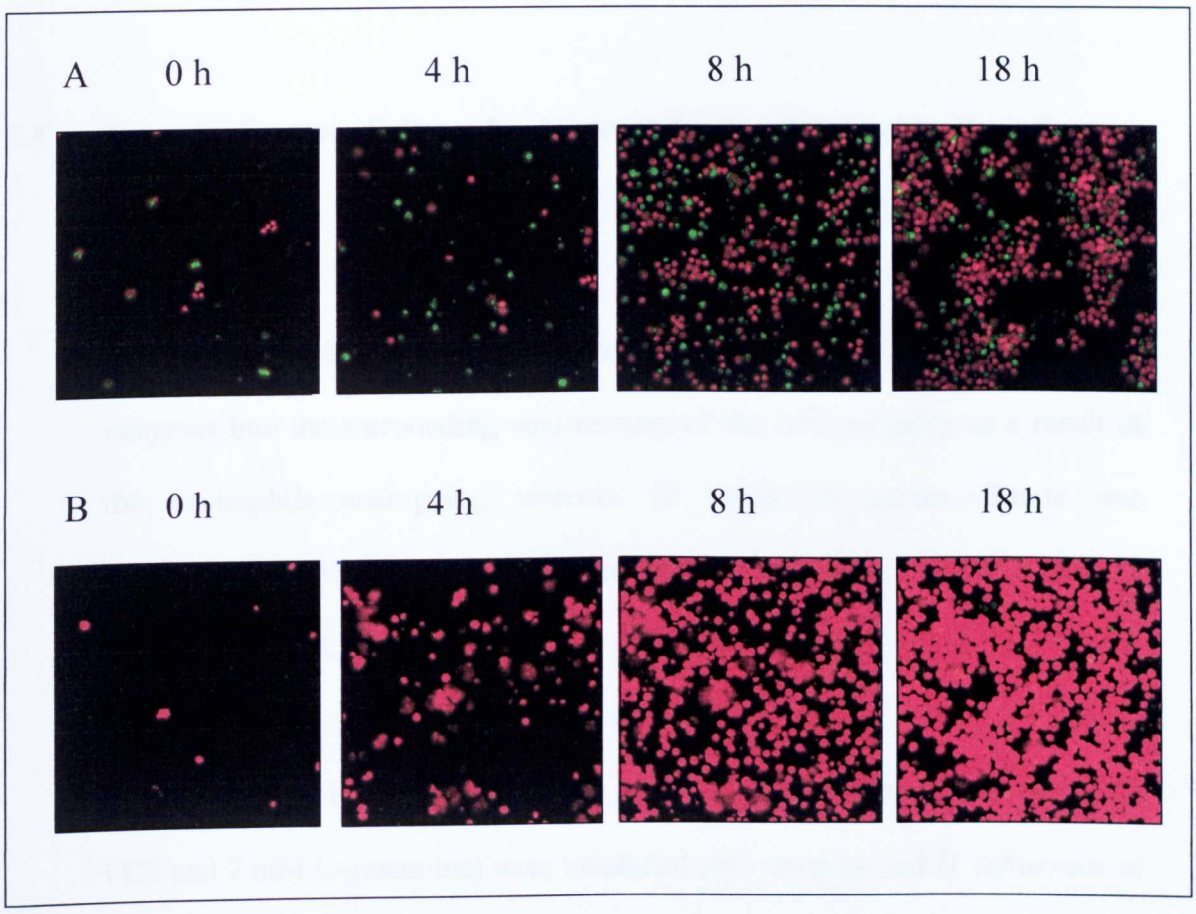


Figure 3.7.2: Visualization of neutrophil necrosis and apoptosis after incubation with *H. influenzae* culture filtrate. Images from confocal microscopy of untreated neutrophils (A) and neutrophils incubated with 200 µl/ml *H. influenzae* culture filtrate (B). Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) were used to detect an increase in apoptosis and necrosis by an increase in green and red fluorescence, respectively. Images were taken every 30 min for 17 h. Images are representative of 3 or more experiments.

It is unclear whether or not the factor(s) actually inhibit apoptosis or if apoptosis does not occur due to the induction of necrosis. These results show that *H. influenzae* culture filtrate, like bacteria, induces neutrophil necrosis.

3.8 *Granule Enzyme Release by Neutrophils Incubated with H. influenzae Culture Filtrate*

H. influenzae G682 have been shown to induce the release of neutrophil granule enzymes into the surrounding environment of the cell, probably as a result of the neutrophils undergoing necrosis. *H. influenzae* culture filtrate was investigated to determine whether or not the factor(s) present within it resulted in a similar effect.

Neutrophils (1 x 10^7 cells/ml in RPMI 1640 + HEPES supplemented with 10 % FCS and 2 mM L-glutamine) were incubated with unopsonised *H. influenzae* or serum-opsonised *H. influenzae* in a bacteria to neutrophil ratio of 250:1 or 200 µl/ml *H. influenzae* culture filtrate. Protein lysates were prepared from cell culture supernatants at 1, 2, 4 and 6 h and the levels of the granule enzymes MPO and lactoferrin, present in the supernatants were analysed by western blotting.

Figure 3.8 shows that the presence of both MPO and lactoferrin is greater in the cell culture supernatants from cells incubated with unopsonised and opsonised *H. influenzae* and culture filtrate, compared to untreated neutrophils. Levels of both enzymes present in cell culture supernatants from neutrophils incubated with unopsonised bacteria are lower compared to that from cells incubated with either opsonised *H. influenzae* or culture filtrate. The levels of MPO detected in the cell culture supernatants were slightly higher when cells have been incubated with opsonised *H. influenzae* compared to culture filtrate, but the levels of MPO detected when incubated with culture filtrate were still increased compared to control cells. The levels of lactoferrin detected were greater in culture supernatants from cells incubated with culture filtrate compared to those incubated with opsonised *H. influenzae*. These results indicate that *H. influenzae* culture filtrate also induces the release of neutrophil granule enzymes into the surrounding environment, again suggesting that the cells are dying by necrosis.

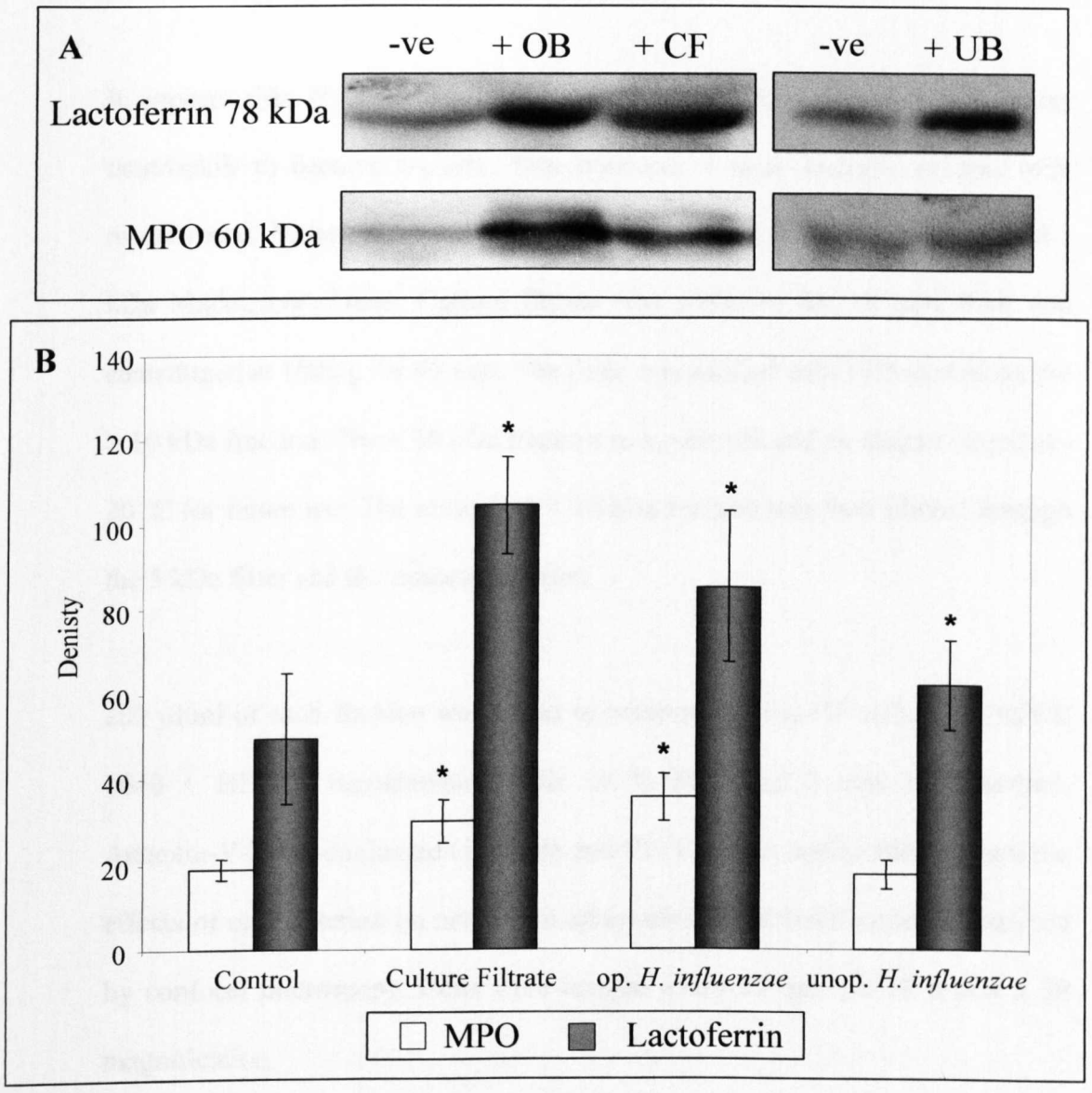


Figure 3.8: Analysis of neutrophil granule enzyme release from neutrophils incubated with *H. influenzae* and *H. influenzae* culture filtrate. Neutrophils were incubated in RPMI 1640 alone (-ve), with serum-opsonised of *H. influenzae* (OB) 250:1 ratio, unopsonised *H. influenzae* (UB) 250:1 ration or 200 µl/ml *H. influenzae* culture filtrate (CF) for up to 6 h at 37 °C. After incubation protein lysates were prepared from culture supernatants and the extracellular release of MPO and lactoferrin detected via western blotting. Figure A shows MPO and lactoferrin release at 2 h. Figure B indicates the MPO and lactoferrin release at 2 h. Results are a mean of 3 experiments, $\pm$ S.D. * $p < 0.05$ compared to control neutrophils.

3.9 *Size Fractionation of H. influenzae Culture Filtrate*

It appears that *H. influenzae* culture filtrate contains a factor(s) that causes neutrophils to become necrotic. Size fractions of these factor(s) present were investigated. *H. influenzae* culture filtrate was filtered through 10-, 5-, 3- and 1 kDa Macrosep® filters. Culture filtrate was added to the 10 kDa filter and centrifuged at 1000 g for 90 min. The filter was washed with PBS to remove the > 10 kDa fraction. The < 10 kDa fraction was removed and an aliquot stored at -20 °C for future use. The remaining < 10 kDa fraction was then filtered through the 5 kDa filter and the process repeated.

200 µl/ml of each fraction was added to neutrophils (5 x 10^6 cells/ml in RPMI 1640 + HEPES supplemented with 10 % FCS and 2 mM L-glutamine). Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) were added so that the effects of each fraction on neutrophil apoptosis and necrosis could be analysed by confocal microscopy. Cells were imaged every 30 min for 18 h at a x 20 magnification.

The results shown in figure 3.9.1 indicate that there may be at least 2 factors present that induce neutrophil necrosis: a factor > 10 kDa in size and a factor < 5 kDa. The > 10 kDa fraction appeared to induce rapid neutrophil necrosis, as a large increase in necrosis was observed as early as 2 h. However, the neutrophil necrosis induced by this fraction did not reach the maximum levels observed by

the unfractionated culture filtrate. In contrast, the < 5 kDa fraction did not have an initial effect on neutrophil necrosis, as there was no increase observed between 0-6 h. The effects of this fraction occur after 6 h and appear to peak at ~ 14 h. The necrosis induced by this fraction eventually reached the maximum levels of that from unfractionated culture filtrate. Figure 3.9.2 shows that both the unfractionated culture filtrate and the > 10 kDa fraction decreased neutrophil apoptosis. However, the < 5 kDa fraction had a slight increase in neutrophil apoptosis compared to untreated cells. This suggests that the > 10 kDa factor may inhibit neutrophil apoptosis as well as inducing initial necrosis.

Neutrophils were treated with 200 µl/ml culture filtrate, > 10 kDa fraction, < 5 kDa fraction and 200 µl/ml of > 10 kDa and < 5 kDa fractions combined to see if the necrotic effect of the intact culture filtrate could be recovered. Figure 3.9.3 shows that when incubated with both the > 10 kDa and < 5 kDa fractions recombined the necrotic effect of the unfiltered culture filtrate was fully recovered. Apoptosis was inhibited and necrosis was induced at a faster rate than with the unfractionated culture filtrate but this was probably due to different concentrations of the factor(s) present in the combined fractions. This further confirms that there are at least two factors present in the culture filtrate that work together to induce neutrophil necrosis.

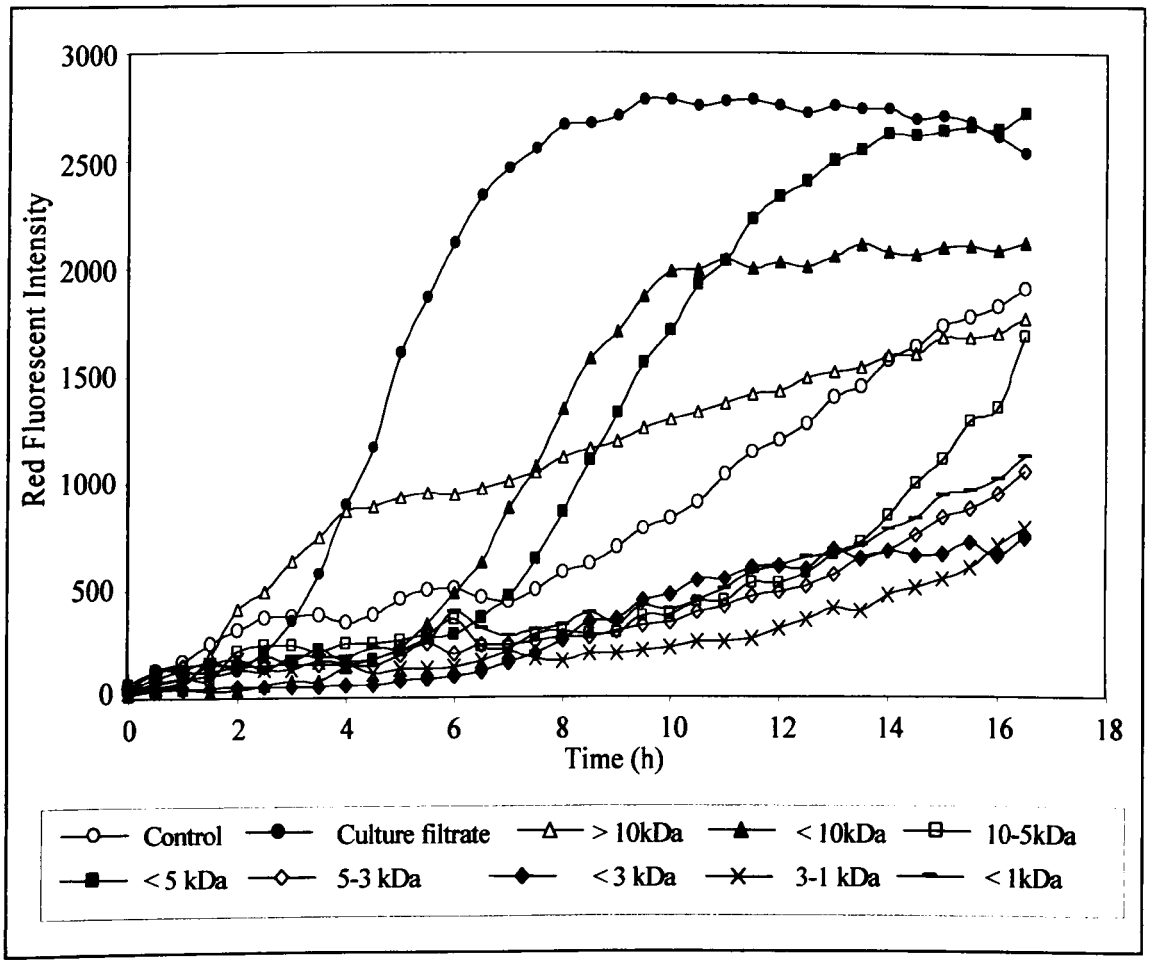


Figure 3.9.1: Effect of size fractionated *H. influenzae* culture filtrate on neutrophil necrosis. Red fluorescence intensity (necrosis) from confocal microscopy of *H. influenzae* culture filtrate fractions. Culture filtrate was filtered through 10 kDa, 5 kDa, 3 kDa and 1 kDa Macrosep filters. Neutrophils were incubated with 200 µl/ml of each fraction and incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) to determine the effects on neutrophil apoptosis and necrosis. Images were taken every 30 min for 17 h. Graphs are representative of 3 experiments.

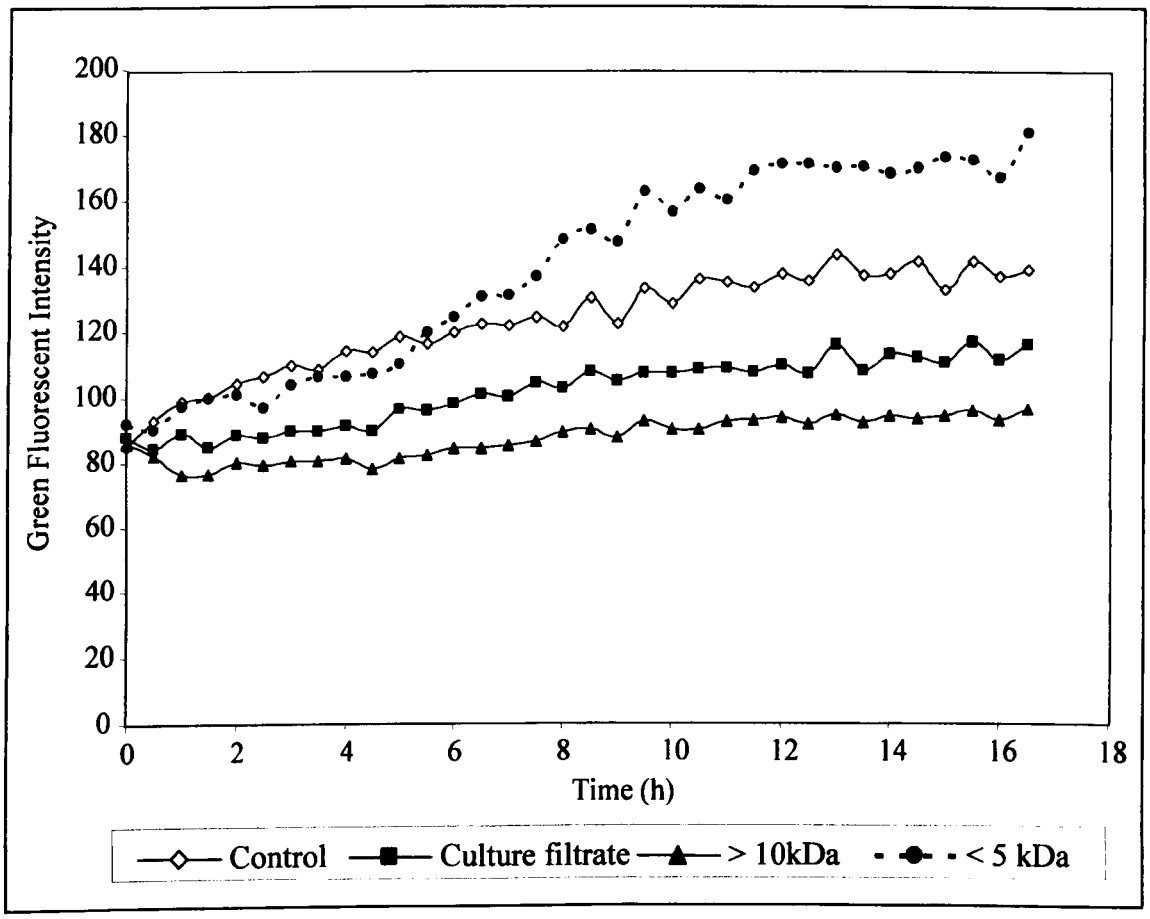


Figure 3.9.2: Effect of size fractionated *H. influenzae* culture filtrate on neutrophil apoptosis. Green fluorescence intensity (apoptosis) from confocal microscopy of *H. influenzae* culture filtrate fractions. Culture filtrate was filtered through 10 kDa, 5 kDa, 3 kDa and 1 kDa Macrosep filters. Neutrophils were incubated with 200 µl/ml of each fraction and incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) to determine the effects on neutrophil apoptosis and necrosis. Images were taken every 30 min for 17 h. Graphs are representative of 3 experiments.

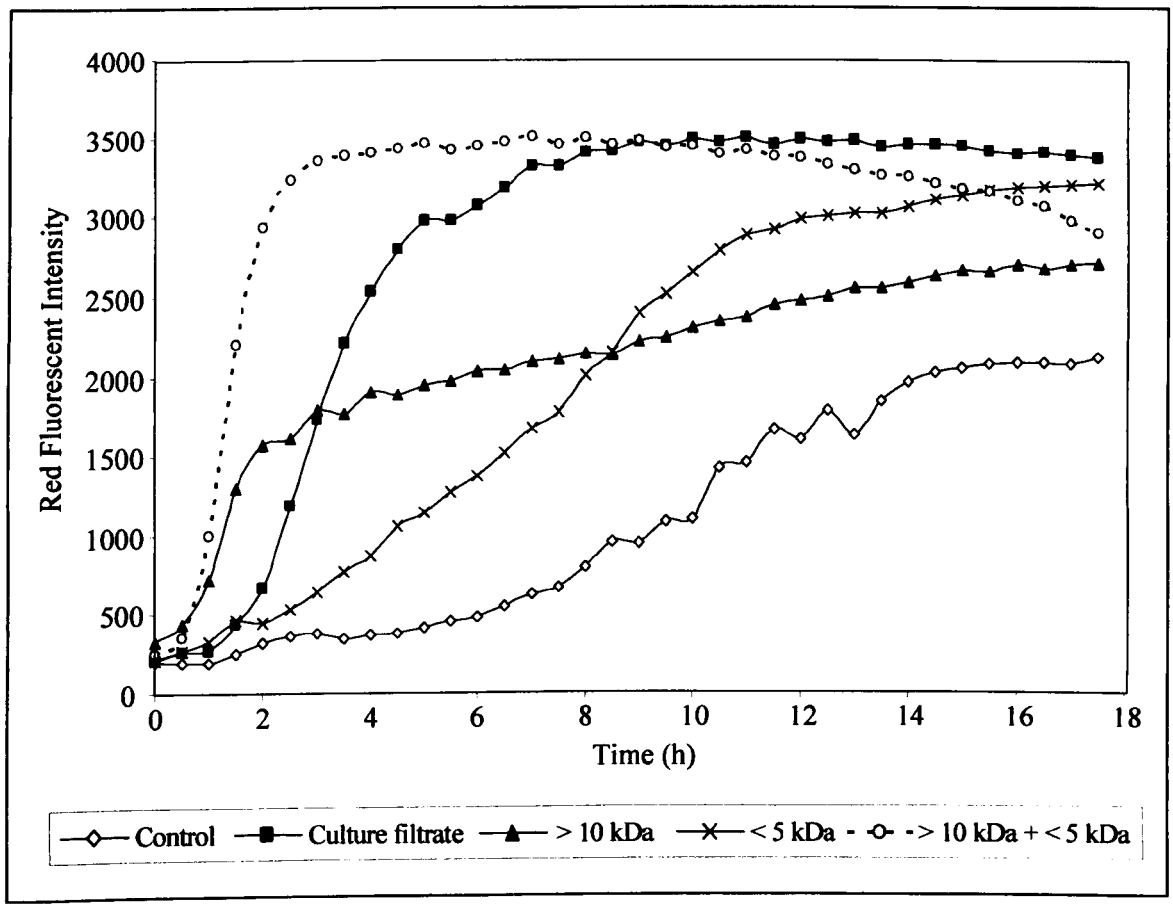


Figure 3.9.3: Effect of reconstituted size fractionated *H. influenzae* culture filtrate on neutrophil necrosis Red fluorescence intensity (necrosis) from confocal microscopy of *H. influenzae* culture filtrate fractions. *H. influenzae* culture filtrate was filtered through 10 kDa, 5 kDa, 3 kDa and 1 kDa Macrosep filters. The > 10 kDa fraction and the < 5 kDa fraction were reconstituted to investigate whether the full necrotic activity of the culture filtrate could be recovered. Neutrophils were incubated with 200 µl/ml of each fraction and incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) to determine the effects on neutrophil apoptosis and necrosis respectively. Graphs are representative of 3 experiments.

Attempts to gain an insight into the size of the > 10 kDa fraction using this approach (i.e. using 50-, 30-, and 10 kDa Macrosep filters) proved unsuccessful. It must be stressed that these filters only give a very crude estimate of size as the shape of the molecule and its ability to bind the membrane influence its ability to pass through the filters.

3.10 Cell Specificity of H. influenzae Culture Filtrate

To determine whether the necrotic effect of *H. influenzae* supernatant was specific to neutrophils the effects of the culture filtrate on a range of different cells lines were analysed. Cell lines were cultured in their optimal growth media at 37 °C until almost confluent (*Materials and Methods 2.3*). The cells lines were treated with 200 µl/ml of *H. influenzae* culture filtrate and Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) were added so that the effects on apoptosis and necrosis could be analysed by confocal microscopy. Fluorescence was detected on a Zeiss LSM 510 confocal microscope, fitted with a temperature control incubator maintained at 37 °C. Cells were imaged every 30 min for 18 h at a x 20 magnification.

Figure 3.10 shows images of untreated cells and cells treated with culture filtrate after 18 h incubation. Necrosis was induced in neutrophils (PMN), peripheral blood monouclear cells (PBMC), HL-60 and U937 cells, all of which

are myeloid cells. A549, Cos-1, HeLa, HT-29 and SK-N-AS cells were not affected by the supernatant. This suggests that the factor may only affect cells of the immune system. A549 cells are a lung epithelial cell line and are not affected by the culture filtrate.

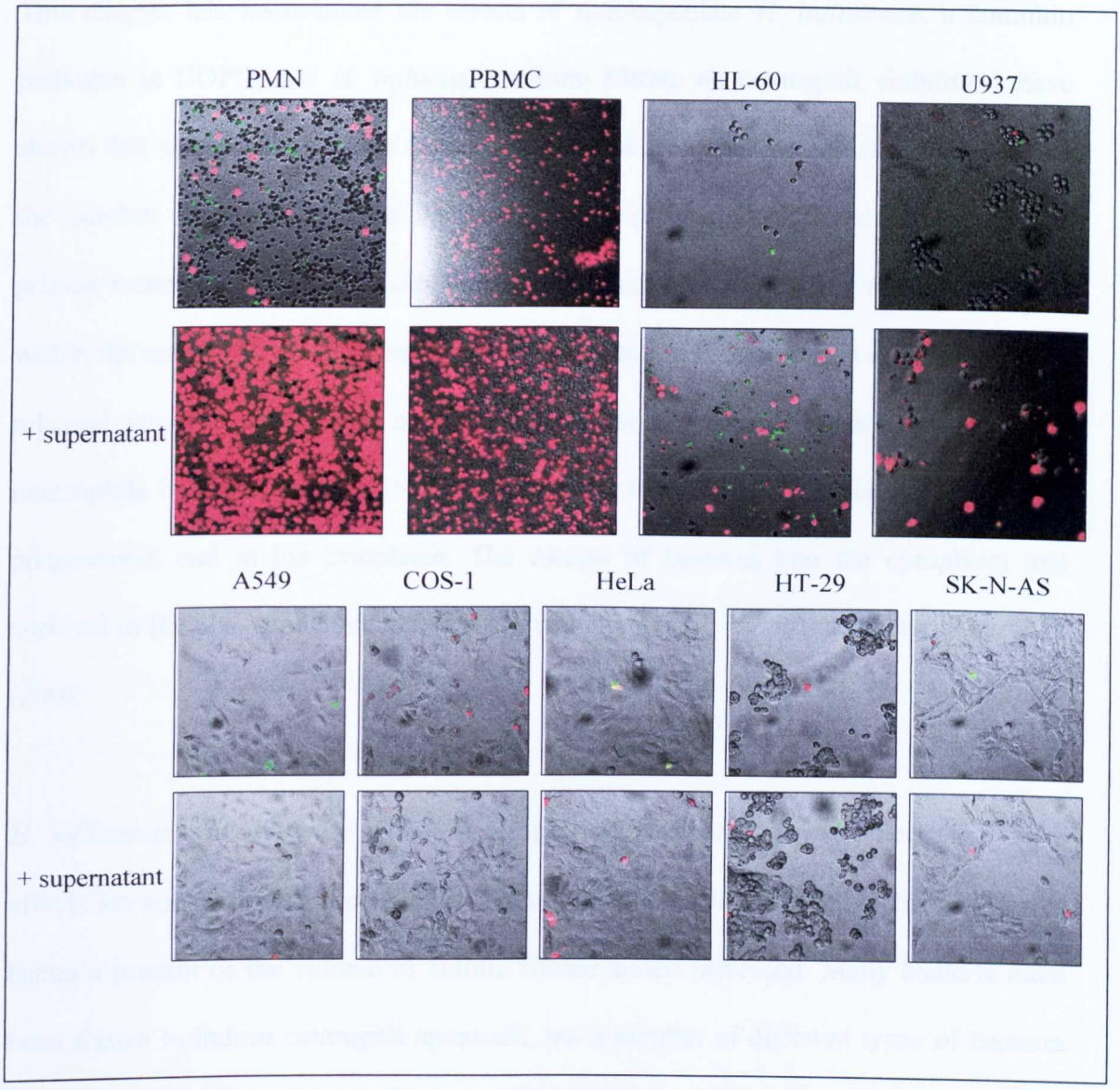


Figure 3.10: Visualization of the effects of *H. influenzae* culture filtrate on different cell lines. 18 h images from confocal microscopy of different cell lines incubated with 200 µl/ml of *H. influenzae* culture filtrate. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) so that the effects of the culture filtrate on apoptosis and necrosis could be detected by an increase in green and red fluorescence respectively. Images were taken every 30 min for 18 h and are representative of 3 separate experiments.

Discussion

This chapter has investigated the effects of non-capsulate *H. influenzae*, a common pathogen in COPD, and *H. influenzae* culture filtrate on neutrophil viability. I have shown that neutrophils are able to phagocytose but are unable to kill *H. influenzae* and the number of viable bacteria increases during phagocytosis, increasing further in primed neutrophils. This indicates that *H. influenzae* are able to survive and multiply within the neutrophil phagosome exacerbating infection if the bacteria are subsequently released into the surrounding environment *in vivo*. Electron microscopy analysis of neutrophils incubated with *H. influenzae* showed the presence of bacteria both in the phagosomes and in the cytoplasm. The escape of bacteria into the cytoplasm and survival in the phagosome are well known strategies of *H. influenzae* bacterial survival (*200*).

H. influenzae was shown to reduce neutrophil apoptosis and increase necrosis. These effects are concentration-dependent and neutrophil necrosis increased as the number of bacteria present or the volume of culture filtrate added increased. Many bacteria have been shown to induce neutrophil apoptosis, but a number of different types of bacteria have been shown to have different effects on neutrophil apoptosis and necrosis. *Anaplasma phagocytophilum*, *Chlamydia pneumoniae* and *Mycobacterium bovis* have all been shown to decrease apoptosis whereas *Pseudomonas aeruginosa (158, 159)*, a common pathogen in cystic fibrosis, *Shigella flexneri* and *Streptococcus pneumoniae* have all been shown to induce necrosis or lysis (*207, 208*).

If numbers of *H. influenzae* increase after phagocytosis via replication within neutrophils followed by neutrophil necrosis *in vivo* in the lung, this could contribute to exacerbations of COPD. This process could enhance bacterial numbers via intracellular replication and then if the cell then dies by necrosis the cellular contents i.e. viable bacteria and cytotoxic products will be released into the surrounding environment. This would enhance bacterial load and enhance tissue damage due to the release of ROS and cytotoxic proteins. More neutrophils will then be recruited to the lungs in response to this infection via IL-8 production and the process will cycle.

A number of different *H. influenzae* clinical isolates, obtained from the lungs of patients with COPD, were studied to determine their effects on neutrophil viability. All of the clinical isolates induced neutrophil necrosis, although levels of activity observed varied between isolates. Unopsonised isolates induced necrosis at a slower rate than opsonised bacteria, which occurred as early as 2 h after incubation. The effects of opsonised *H. influenzae* are more clinically relevant, as bacteria in the body will be opsonised by proteins and antibodies. No difference in necrotic activity or phagocytosis was observed when bacteria were opsonised with serum, IgG or IgA (data not shown).

To determine whether the release of granule enzymes occurred following phagocytosis two granule proteins present in different neutrophil granules, MPO and lactoferrin, were measured in culture supernatants after incubation of neutrophils with *H. influenzae*. It was shown that over a 6 h incubation there was an increase in the levels of both MPO and lactoferrin in cell supernatant of neutrophils incubated with *H.*

influenzae compared to cells incubated with *S. aureus* or untreated cells. This suggests that *H. influenzae* causes neutrophils to release their granules into the surrounding environment either in an attempt to kill the bacteria or as a result of the cell lysing or dying by necrosis. If the release is a result of neutrophil necrosis, other toxic neutrophil products will also be released e.g. proteases, ROS that may contribute to tissue damage *(62)*.

I have also shown that *H. influenzae* releases a factor(s) into the culture filtrate that induces neutrophil necrosis. The released factor(s) did not increase ROS production in unprimed neutrophils, but did stimulate production in cells that had been previously primed with GM-CSF. Neutrophils migrating to the lung may be primed by a number of factors such as cytokines or bacterial products *(44, 45)*. During an infection of *H. influenzae* these neutrophils will enter the lungs and be further stimulated to produce ROS by released *H. influenzae* factor(s). As previously discussed, the neutrophils die by necrosis after interaction with the factor(s) and are predicted to release ROS and granule enzymes into the lung, resulting in an increase in tissue damage and a prolonged inflammatory response.

The effects of *H. influenzae* culture filtrate on neutrophil viability were investigated and it was shown that necrosis was induced by a factor(s) present in the culture filtrate. As well as an increase in necrosis, a decrease in neutrophil apoptosis was also observed. This decrease in apoptosis may be due to the high rate of necrosis occurring or the factor(s) may be able to inhibit neutrophil apoptosis, preventing the safe removal of

neutrophils from the site of infection and the resolution of inflammation. Many studies have demonstrated that some bacteria and their products are able to control neutrophil apoptosis either by inhibiting or inducing it *(159)*. For example *P. aeruginosa* has been shown to release a cytotoxic protein, pyocyanin, that is capable of inducing neutrophil apoptosis (*209, 210*).

H. influenzae induces the release of neutrophil granule proteins into the external environment when neutrophils die by necrosis. *H. influenzae* culture filtrate was investigated to see if the released factor(s) had similar effects. Similar levels of MPO and lactoferrin were detected in the supernatants of cells incubated with *H. influenzae* culture filtrate as in the supernatants of cells incubated with opsonised *H. influenzae*. This indicates that the factor(s) causes the release of neutrophil granules in a similar way to the bacteria, probably as a result of neutrophil necrosis. Release of these enzymes and other neutrophil products into the environment will enhance tissue damage *(62)*.

Crude size-fractionation by ultrafiltration was performed and it was found that there were at least two factors present in the culture filtrate, one > 10 kDa in size and one < 5 kDa in size. Both factors induced neutrophil necrosis but, the high molecular mass fraction induced necrosis very rapidly and the effect of the smaller fraction only occurred after 8 h. The two factors appear to have different effects on apoptosis. No apoptosis was observed in cells treated with the > 10 kDa factor whereas some apoptosis is observed in cells treated with the < 5 kDa factor. Thus, the > 10 kDa factor

may be able to inhibit neutrophil apoptosis. When both factors are reconstituted, the full necrotic activity of the supernatant was recovered further confirming that two necrotic factor are released by *H. influenzae.*

Several bacteria release factors that can induce neutrophil necrosis. *S. pneumoniae* has been shown to release a 53 kDa pore forming toxin, pneumolysin, that inhibits ROS production and induces neutrophil necrosis *(208)*. *Escherichia coli* and *Burkholderia cepacia* have both been shown to release hemolysins which are capable of inducing apoptosis and necrosis *(211, 212)*. The 2D map of the *H. influenzae* proteome has been characterized and *H. influenzae* does not express any hemolysins or pneumolysins *(189, 190)*. However, *H. influenzae* do express porins which increase the permeability of the outer membrane of cells to low molecular weight compounds. *H. influenzae* porins are 35-42 kDa in size and it may be possible that the > 10 kDa factor released by *H. influenzae* is a porin capable of inducing necrosis and sensitizing the cell to the < 5 kDa factor *(213)*.

The culture filtrate was shown to only affect myeloid cells. Myeloid cells include both monocytes and granulocytes and are essential in resolving infection. The specificity of the factor(s) to induce necrosis of myeloid cells would enable *H. influenzae* to invade the immune system resulting in prolonged infection due to impaired host defence. This may be an explanation for the recurrent infections found in COPD patients *(183)*.

This chapter has investigated the effects of *H. influenzae* and *H. influenzae* culture filtrate on neutrophil viability. It has been shown that *H. influenzae* releases at least two factors that work together to inhibit neutrophil apoptosis and induce necrosis. The factors also increase ROS production in primed neutrophils, therefore increasing the capacity of the cells to inflict damage when they die by necrosis. The factors have been shown to be specific to myeloid cells, cell of the immune system, enabling *H. influenzae* to invade the immune system resulting in an inability of the body to fight the infection, increased bacterial infection and an increase in tissue damage to the lungs.

Chapter 4: The Effects of *H. influenzae* Culture Filtrate on Cellular Signalling Pathways

Introduction

Chapter 3 described the ability of non-capsulate *H. influenzae* and *H. influenzae* culture filtrate to induce neutrophil necrosis. *H. influenzae* culture filtrate was shown to contain at least 2 factors that work together to induce necrosis. This effect may be specific to myeloid cells and therefore, only affects cells of the immune system. In this chapter the ability of the *H. influenzae* factors to induce necrosis was investigated by analysing the intracellular signalling pathways involved in this process.

External stimuli are detected by cells either by cell-surface or intracellular receptors that are coupled to signalling networks to control cell fate *(38)*. The observation that the released necrotic factor(s) does not affect all cells, argues against it working as a non-specific membrane-permeablising agent, and is probably acting via a receptor. Many signalling pathways have been identified in neutrophils to control cell function and they often involve kinase phosphorylation cascades (*214, 215*). Many of the intracellular processes that regulate apoptosis (but not necrosis) have been defined. Inhibitors for many of these pathways have been characterised and more specific assays (e.g. using activation-specific antibodies) are available.

The aims of this chapter were to:

- Investigate the effects of a number of cell signalling inhibitors on neutrophil apoptosis and necrosis.
- Determine whether or not the inhibitors have an effect on neutrophil necrosis induced by *H. influenzae* culture filtrate and the < 5 kDa fraction.
- Investigate the effects of cell signalling inhibitors on *H. influenzae* culture filtrate induced necrosis in a cell line that is sensitive to the factors (U937).
- Analyze the phosphorylation states of a number of important cell signalling proteins in neutrophils after stimulation of the cells with the unfractionated culture filtrate and the < 5 kDa fraction.

4.1 Effects of Cell Signalling Inhibitors on Neutrophil Viability

Before I could determine the effects of cell signalling inhibitors on neutrophil necrosis induced by *H. influenzae* culture filtrate and the < 5 kDa fraction, it was first necessary to examine the effects of these inhibitors on apoptosis and necrosis of unstimulated neutrophils (see page 56 for list of inhibitors).

Figure 4.1.1 shows the effects of the inhibitors on unprimed neutrophils and neutrophils primed with GM-CSF (50 U/ml). Some of the inhibitors were shown to slightly increase neutrophil necrosis in unprimed cells, in particular LY294002 (PI3K inhibitor) and Bis-1 (PKC inhibitor), but these effects were not seen in cells that had been primed with GM-CSF. In fact, cells primed with GM-CSF and then treated with SB202190, LY294002, Bis-1, SYK inhibitor, H89 and DPI all had significantly reduced necrosis, compared to untreated unprimed neutrophils (figure 4.1.3). SYK inhibitor was shown to greatly increase neutrophil apoptosis but again this effect was not observed in the cells that had been primed with GM-CSF, as shown in figure 4.1.2. None of the inhibitors caused a significant increase in neutrophil necrosis compared to untreated cells. Therefore, any effects of the inhibitors on neutrophil necrosis in cells treated with *H. influenzae* culture filtrate will be a result of inhibition of the signalling pathway which the necrotic factors activate.

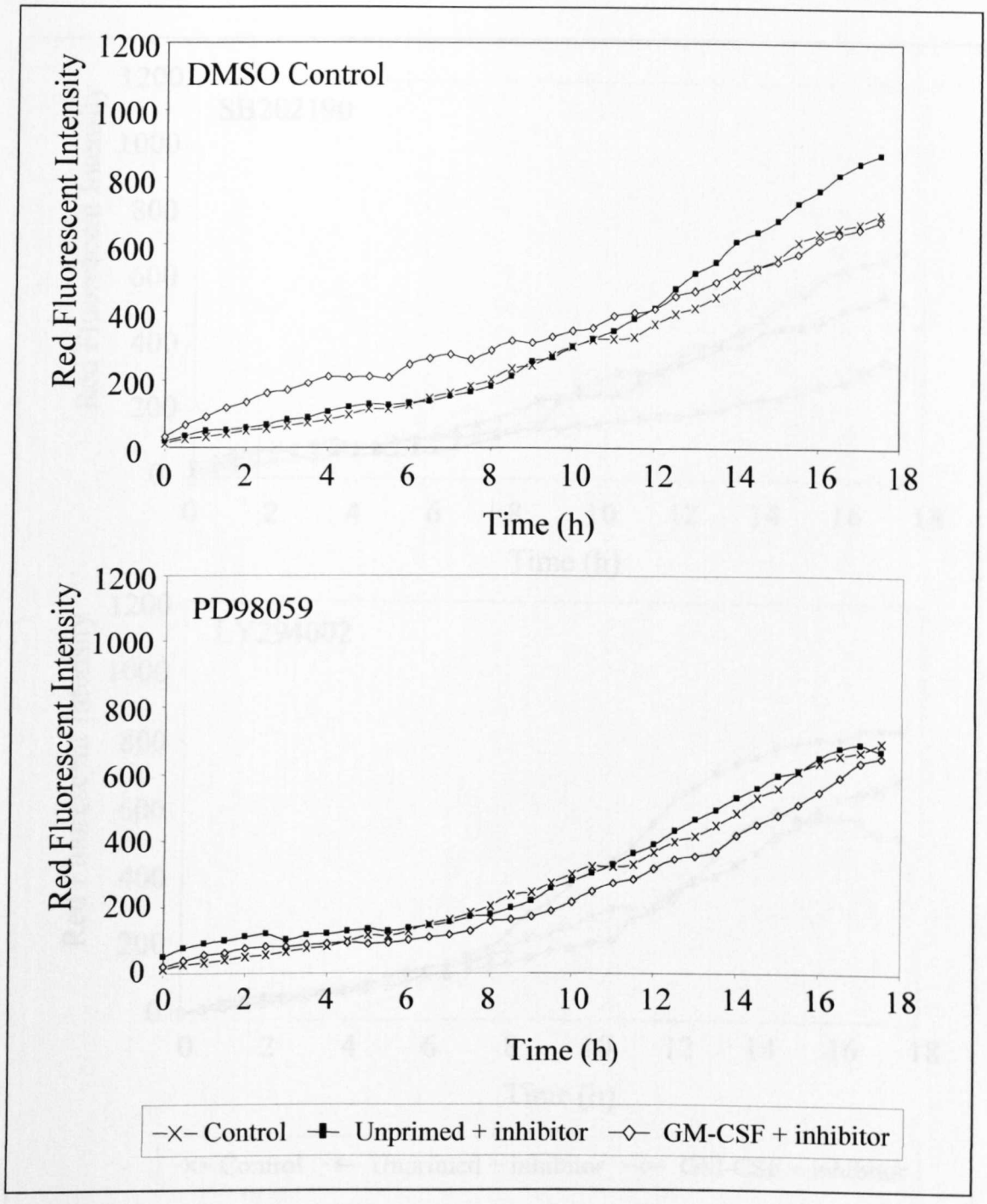


Figure 4.1.1: Fluorescence intensity from confocal microscopy of neutrophils incubated with cell signaling inhibitors (or vehicle control, DMSO) in the presence of GM-CSF (50 U/ml). Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Graphs are representative of 3 experiments.

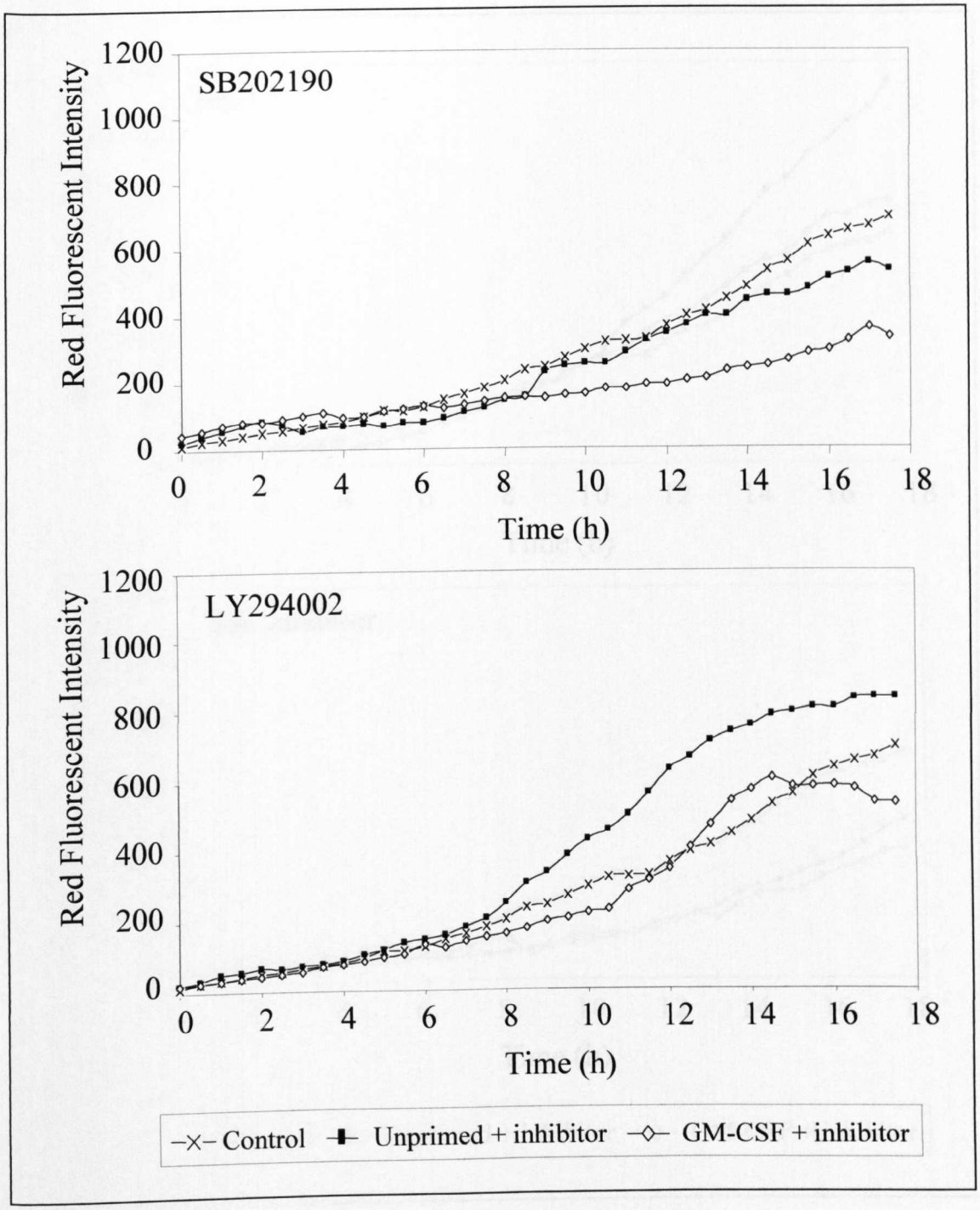
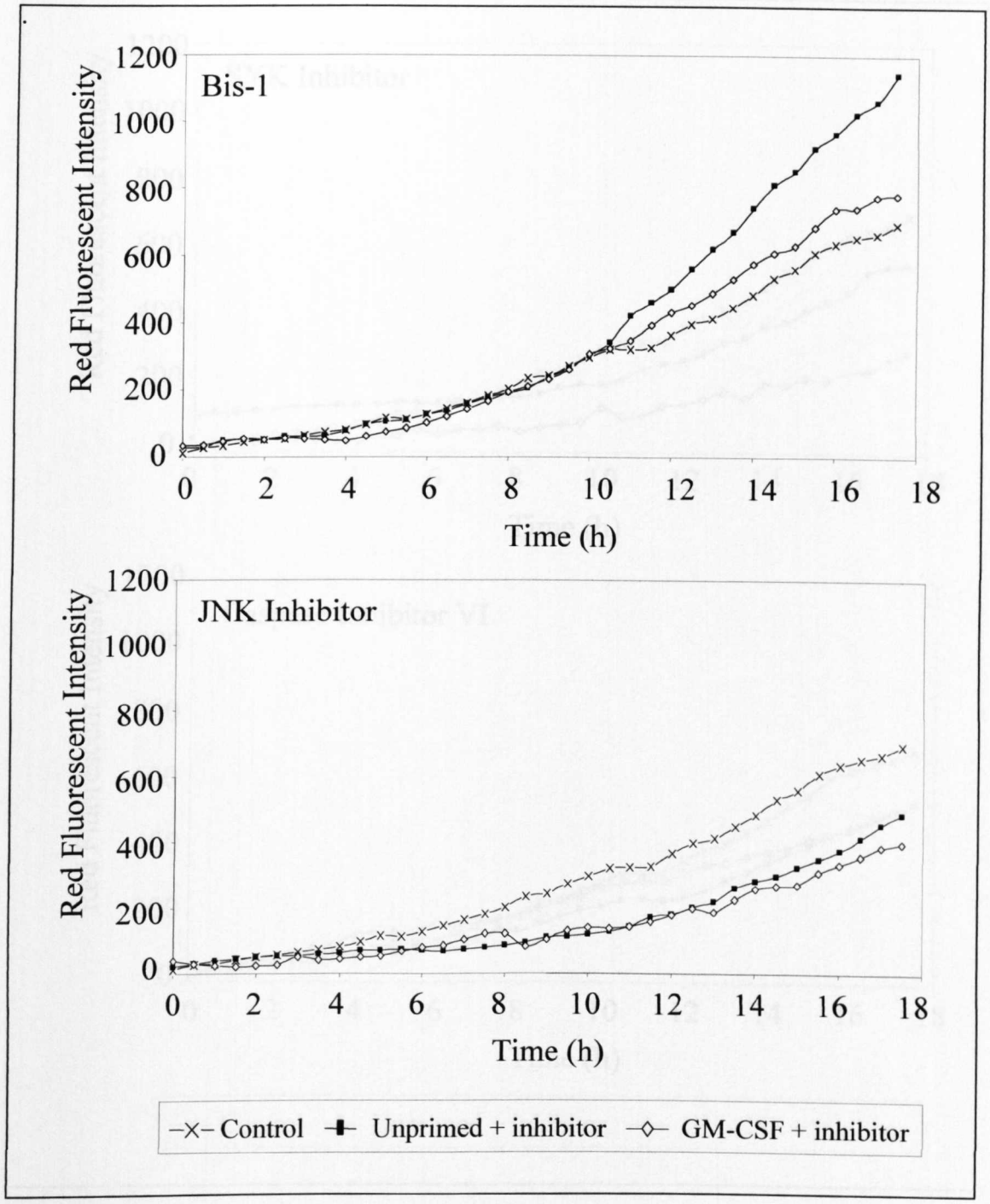


Figure 4.1.1 *(contd.)*



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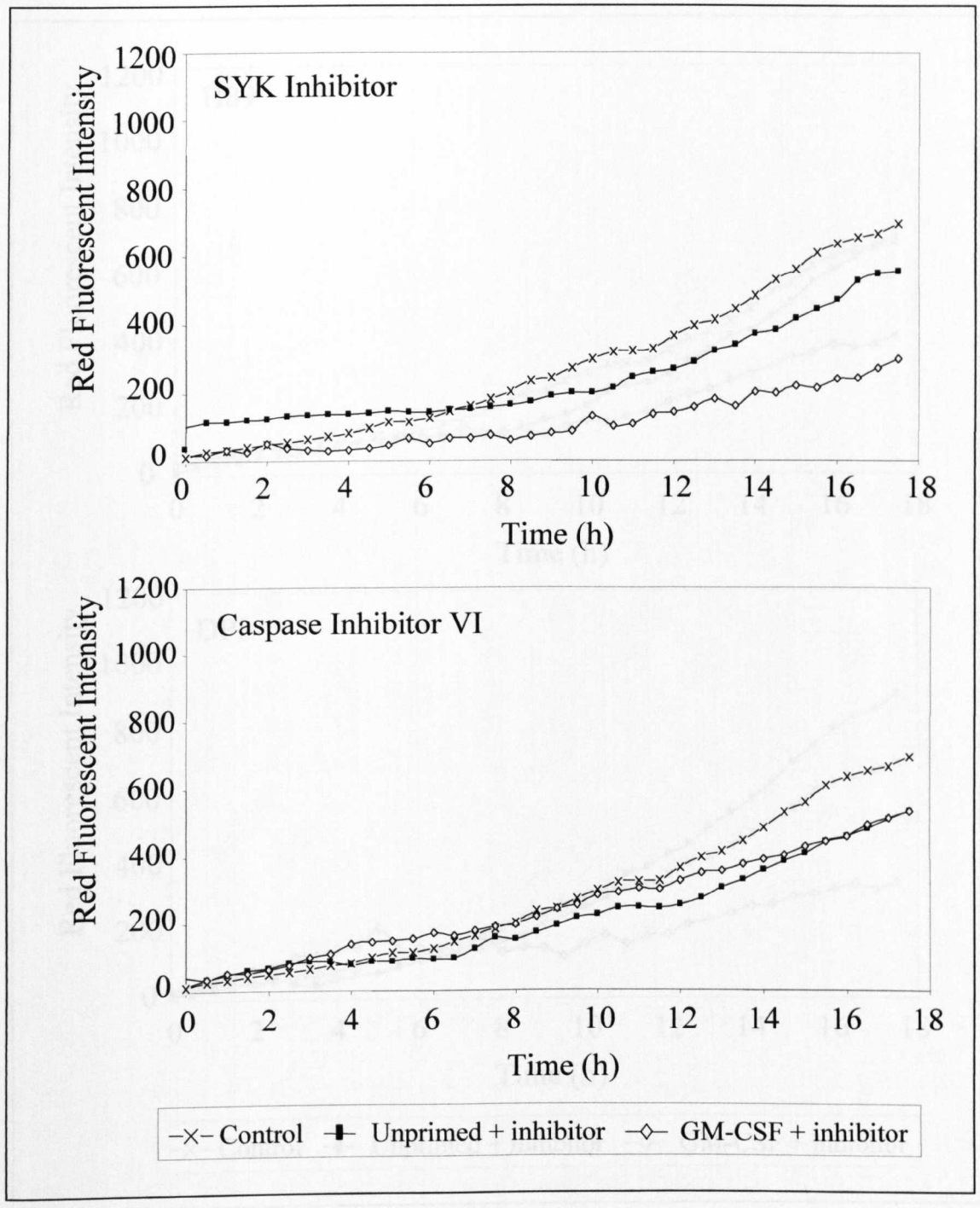

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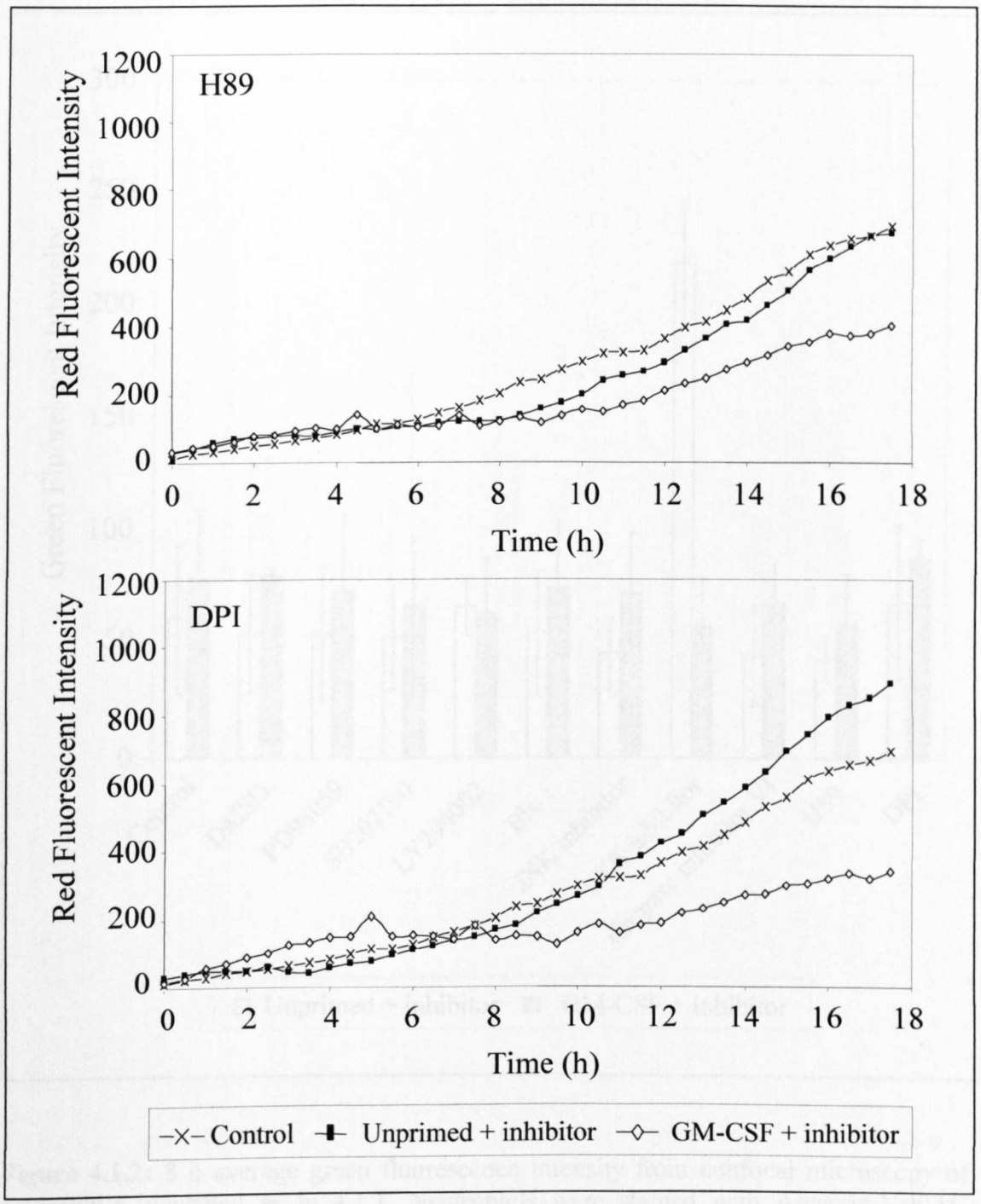


Figure 4.1.1 *(contd.)*

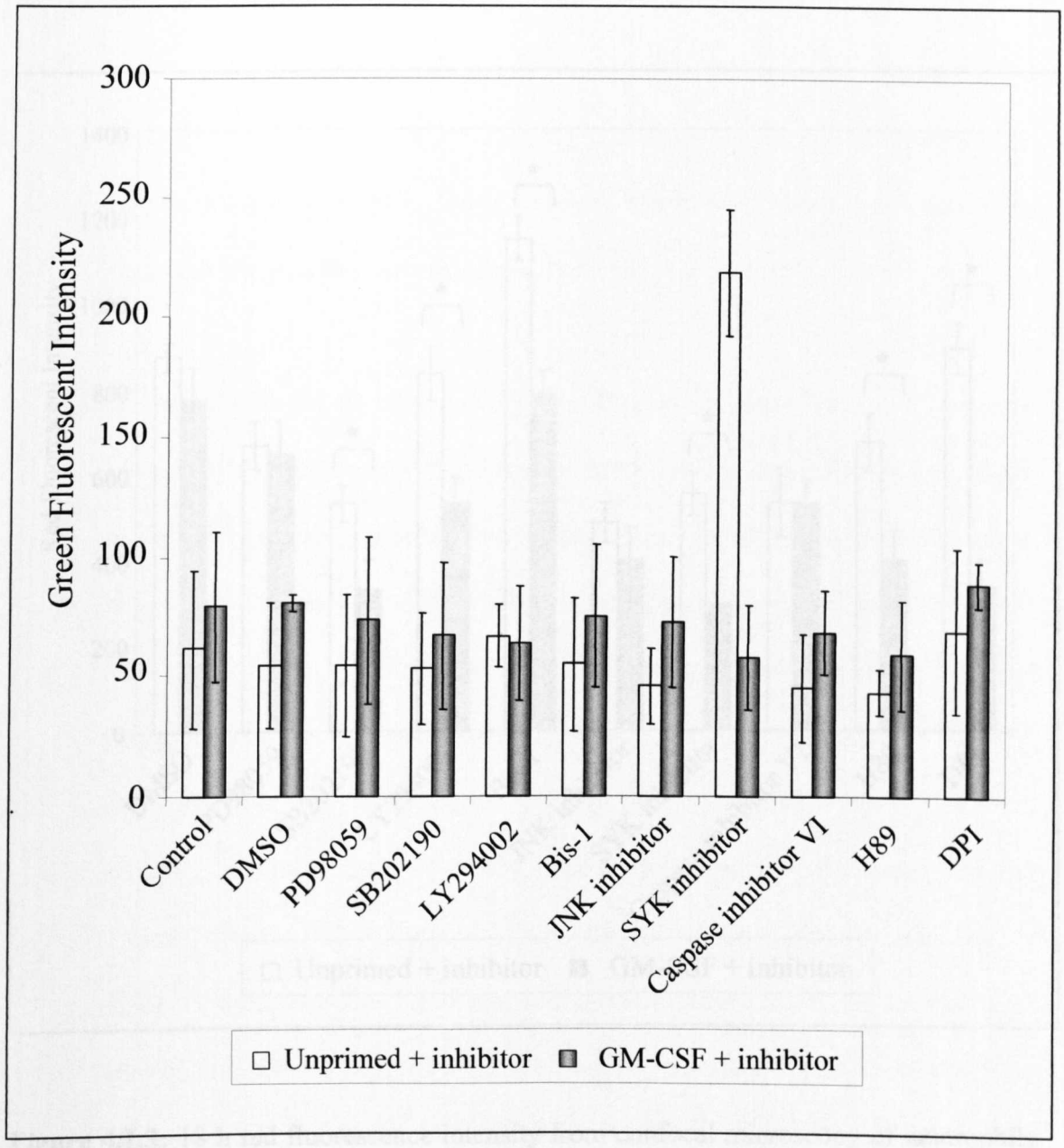


Figure 4.1.2: 8 h average green fluorescence intensity from confocal microscopy of neutrophils incubated as in 4.1.1. Neutrophils were stained with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Results are means of 3 experiments (±SD).

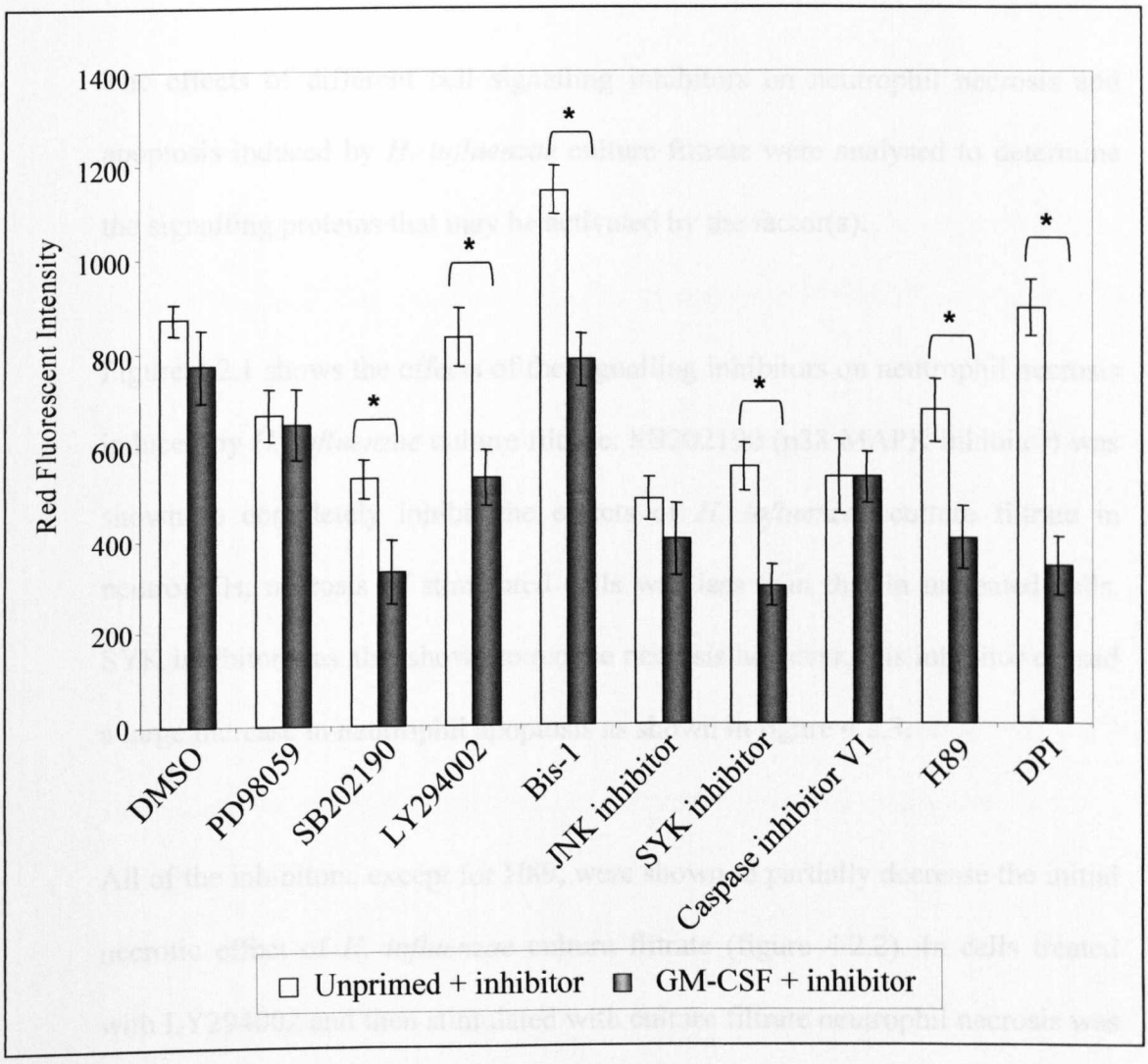


Figure 4.1.3: 18 h red fluorescence intensity from confocal microscopy of neutrophils incubated with cell signaling inhibitors (or vehicle control, DMSO) in the presence of GM-CSF (50 U/ml). Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Results are means of 3 experiments (± SD), * $p > 0.05$.

4.2 *Effects of Signalling Inhibitors on Neutrophils Treated with H. influenzae Culture Filtrate*

The effects of different cell signalling inhibitors on neutrophil necrosis and apoptosis induced by *H. influenzae* culture filtrate were analysed to determine the signalling proteins that may be activated by the factor(s).

Figure 4.2.1 shows the effects of the signalling inhibitors on neutrophil necrosis induced by *H. influenzae* culture filtrate. SB202190 (p38 MAPK inhibitor) was shown to completely inhibit the effects of *H. influenzae* culture filtrate in neutrophils; necrosis of stimulated cells was less than that in untreated cells. SYK inhibitor was also shown to reduce necrosis however, this inhibitor caused a large increase in neutrophil apoptosis as shown in figure 4.2.3.

All of the inhibitors, except for H89, were shown to partially decrease the initial necrotic effect of *H. influenzae* culture filtrate (figure 4.2.2). In cells treated with LY294002 and then stimulated with culture filtrate neutrophil necrosis was delayed compared to cells treated with culture filtrate alone. Between 0 - 4 h levels of necrosis were similar to those observed in control cells. However, after 4 h neutrophil necrosis increased and reached levels of necrosis observed in cells treated with *H. influenzae* culture filtrate at 6 h. Neutrophil necrosis in cells treated with Bis-1 and JNK inhibitor were also shown to be similar to control cells between 0 - 6 h. In cells pretreated with Bis-1, after 6 h neutrophil

necrosis increased and eventually reached the levels seen in cells treated with culture filtrate at 14 h.

H89 and DPI were also shown to slightly inhibit the initial neutrophil necrosis induced by *H. influenzae* culture filtrate but by 18 h the necrosis was similar to controls. Caspase inhibitor VI was shown to inhibit initial necrosis however cells treated with this inhibitor and cells treated with PD98059 were shown to have higher levels of necrosis after 6 h than cells treated with *H. influenzae* culture filtrate alone. Figure 4.2.3 shows images of cells at 8 h, and the changes in neutrophil necrosis are clearly visible by the differences in red fluorescence. Again, it is clear that SB202190, JNK inhibitor and Bis-1 inhibit the effect of *H. influenzae* culture filtrate. Figure 4.2.4 shows the effects of the inhibitors on neutrophil apoptosis and, as previously discussed, only cells treated with SYK inhibitor show a significant increase in apoptosis compared to untreated cells.

These results indicate that the factors present in *H. influenzae* culture filtrate induce neutrophil necrosis through a signalling cascade inside the cell. There are a number of signalling proteins that appear to be involved in this pathway but the ones shown to have the greatest importance appear to involve p38 MAPK, protein kinase C and JNK, as inhibition of these enzymes significantly decreases the necrotic effect.

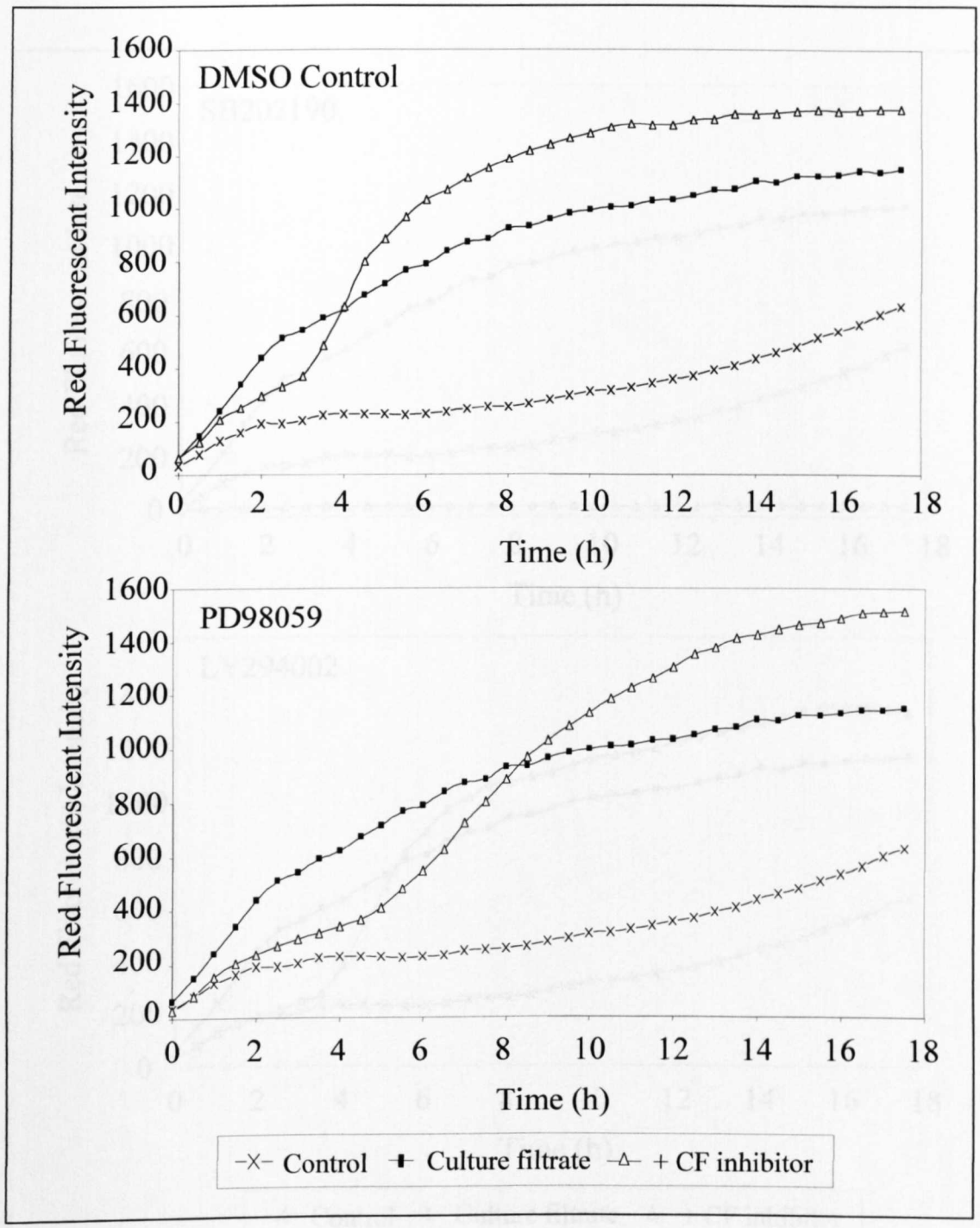


Figure 4.2.1: Fluorescence intensity from confocal microscopy of neutrophils incubated with *H. influenzae* culture filtrate (200 µl/ml) or culture filtrate and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Graphs are representative of 3 experiments.

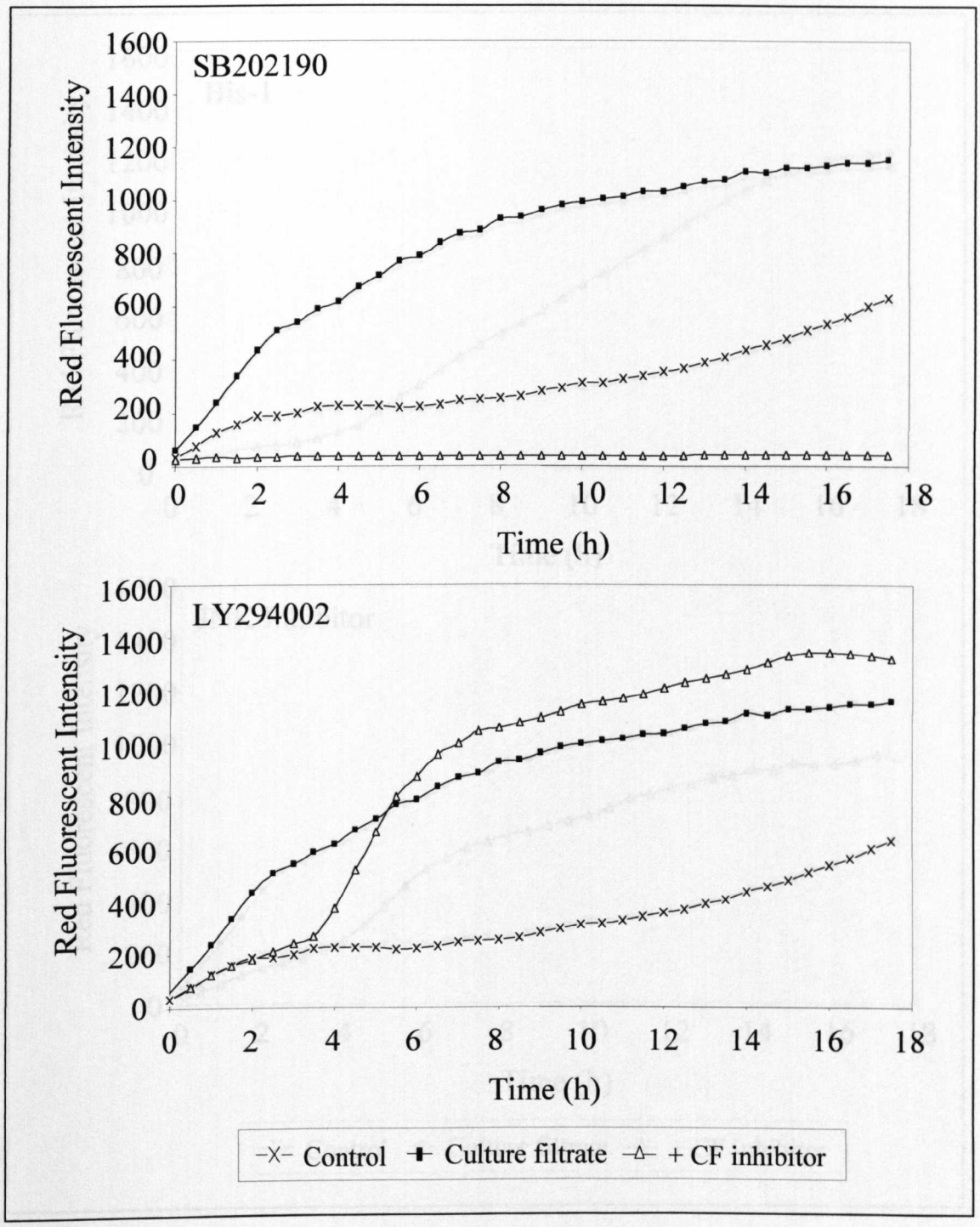
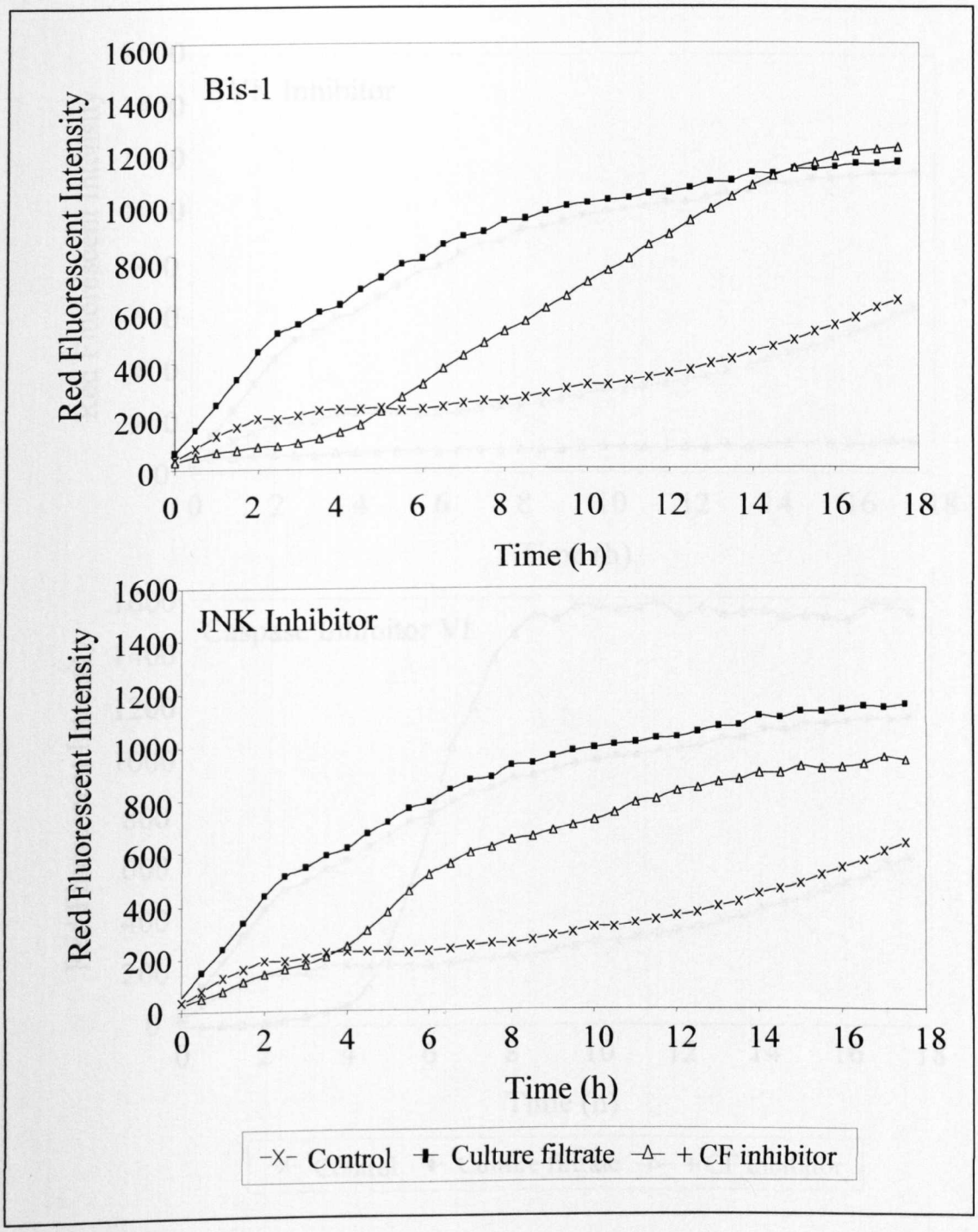


Figure 4.2.1 *(contd.)*



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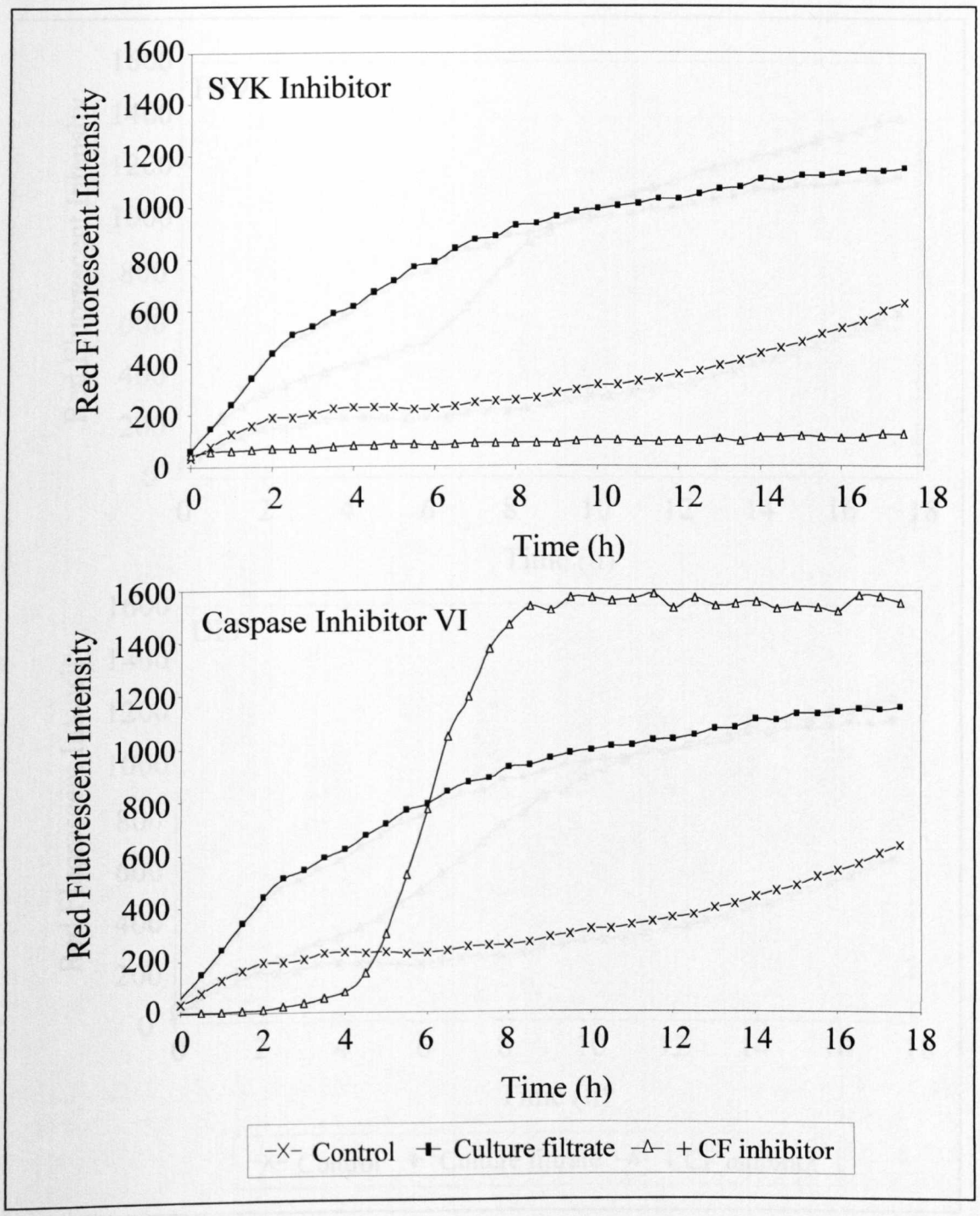


Figure 4.2.1 *(contd.)*

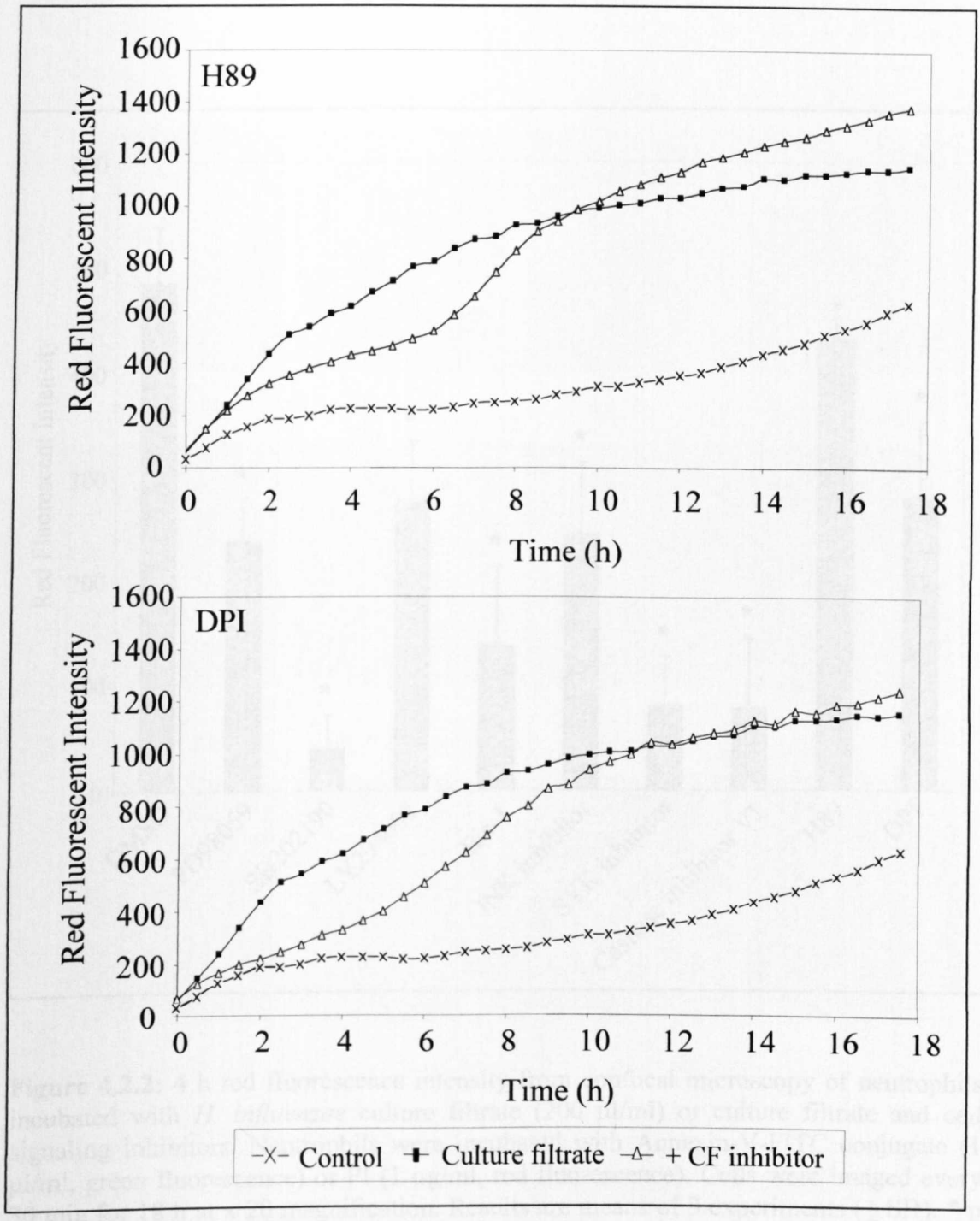


Figure 4.2.1 *(contd.)*

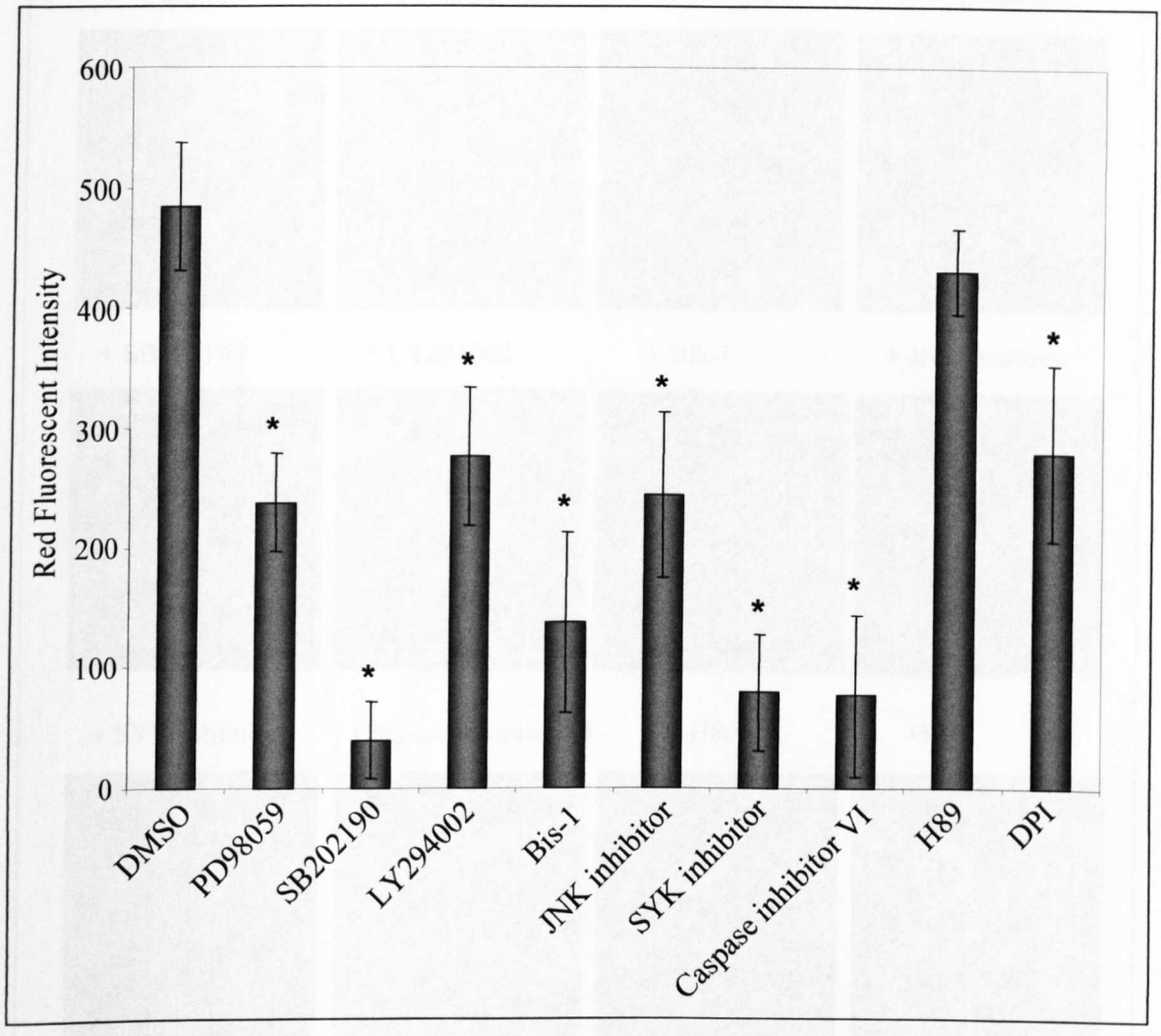


Figure 4.2.2: 4 h red fluorescence intensity from confocal microscopy of neutrophils incubated with *H. influenzae* culture filtrate (200 µl/ml) or culture filtrate and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Results are means of 3 experiments (± SD), * $p > 0.05$ compared to DMSO control.

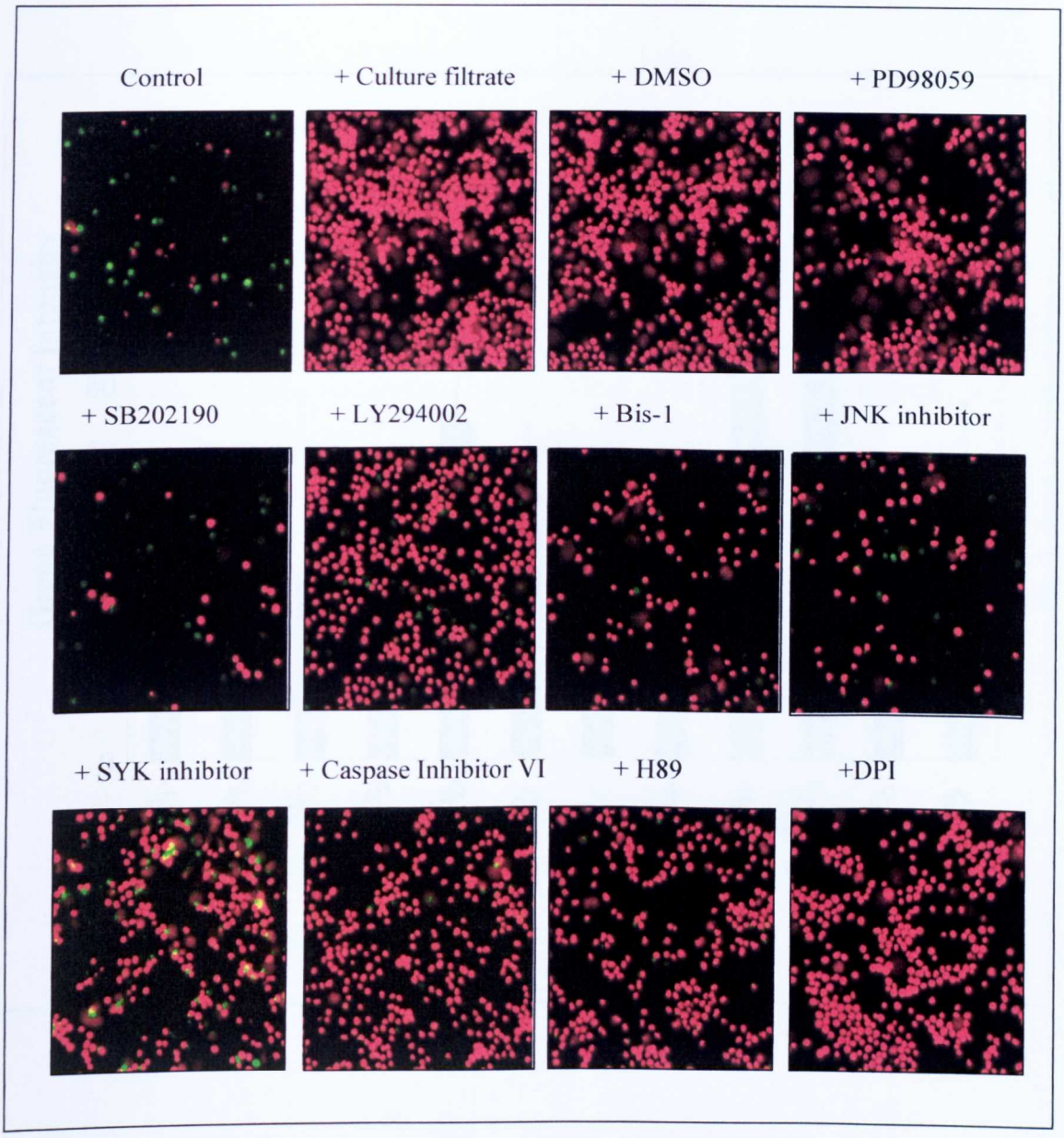


Figure 4.2.3: 8 h images from confocal microscopy of neutrophils incubated with *H. influenzae* culture filtrate (200 µl/ml) or culture filtrate and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Images shown were obtained after 8 h incubation. Images are representative of 3 experiments.

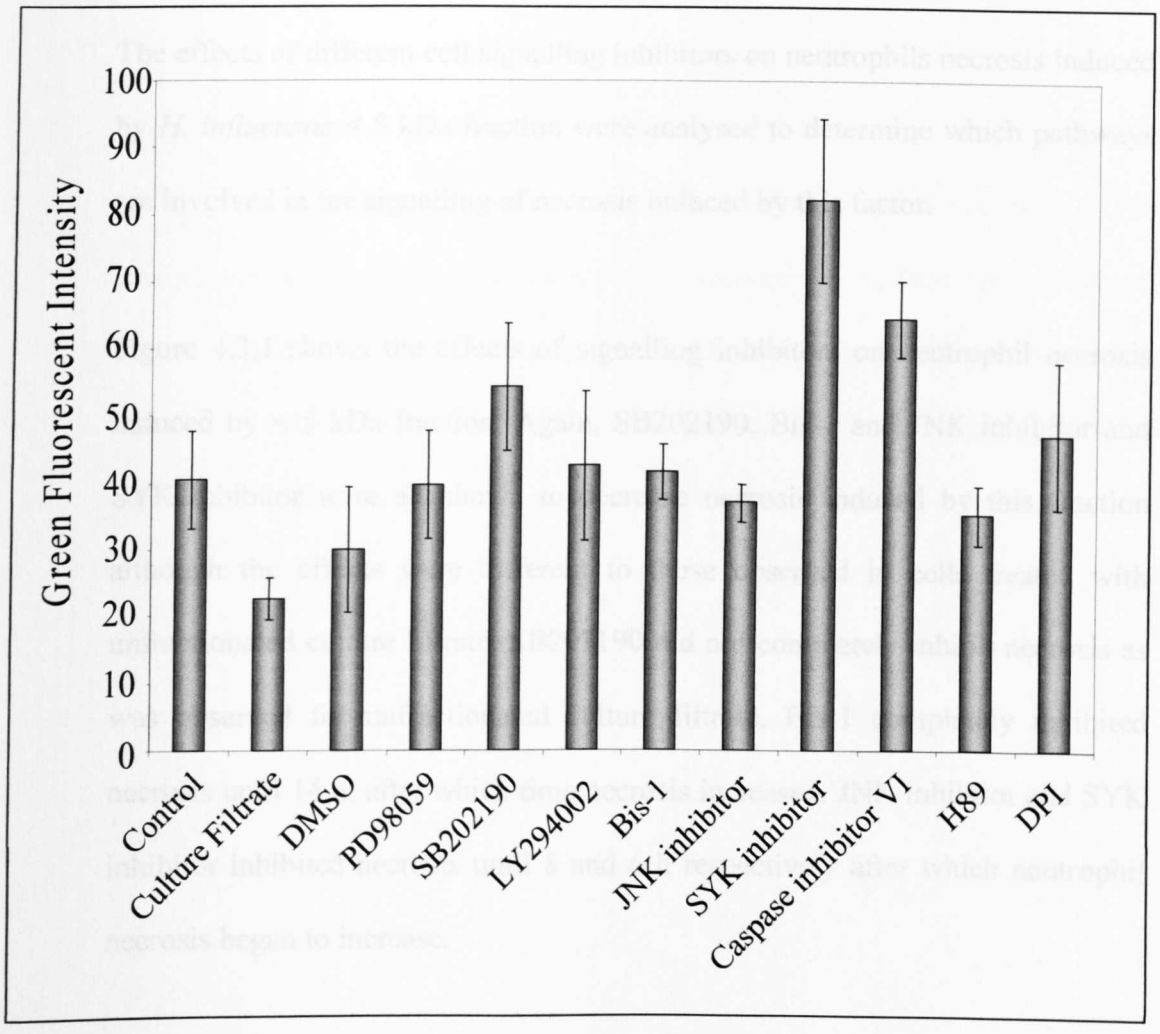


Figure 4.2.4: 8 h green fluorescent intensity from confocal microscopy of neutrophils incubated with *H. influenzae* culture filtrate (200 µl/ml) plus inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Results are means of 3 experiments (±SD), n = 3.

4.3 *Effects of Signalling Inhibitors on Neutrophils Treated with H. influenzae < 5 kDa Fraction*

The effects of different cell signalling inhibitors on neutrophils necrosis induced by *H. influenzae* < 5 kDa fraction were analysed to determine which pathways are involved in the signalling of necrosis induced by this factor.

Figure 4.3.1 shows the effects of signalling inhibitors on neutrophil necrosis induced by < 5 kDa fraction. Again, SB202190, Bis-1 and JNK inhibitor and SYK inhibitor were all shown to decrease necrosis induced by this fraction although the effects were different to those observed in cells treated with unfractionated culture filtrate. SB202190 did not completely inhibit necrosis as was observed for unfractionated culture filtrate. Bis-1 completely inhibited necrosis until 14 h, after which time necrosis increased. JNK inhibitor and SYK inhibitor inhibited necrosis until 8 and 6 h respectively after which neutrophil necrosis began to increase.

PD98059 and LY294002 were shown to slightly decrease necrosis induced by this fraction whereas H89 and DPI were both shown to increase necrosis. Caspase inhibitor VI did not have an effect on neutrophil necrosis induced by < 5 kDa fraction. Images taken at 8 h are shown in figure 4.3.2. Again, the decrease in red fluorescence i.e. necrosis, can be clearly observed in cells treated with SB202190, Bis-1, JNK inhibitor and SYK inhibitor. Figure 4.3.3

shows green fluorescence intensities (apoptosis) in cells treated with the signalling inhibitors. Again, SYK inhibitor was shown to greatly increase neutrophil apoptosis compared to untreated cells.

These results further confirm the importance of p38 MAPK, PKC, JNK and SYK in the signalling pathways induced by *H. influenzae* culture filtrate. The differences in the inhibition of necrosis induced by unfractionated culture filtrate and < 5 kDa fraction further confirm the presence of at least 2 factors in the culture filtrate, > 10 kDa and < 5 kDa, that have different kinetics but work together to induce necrosis.

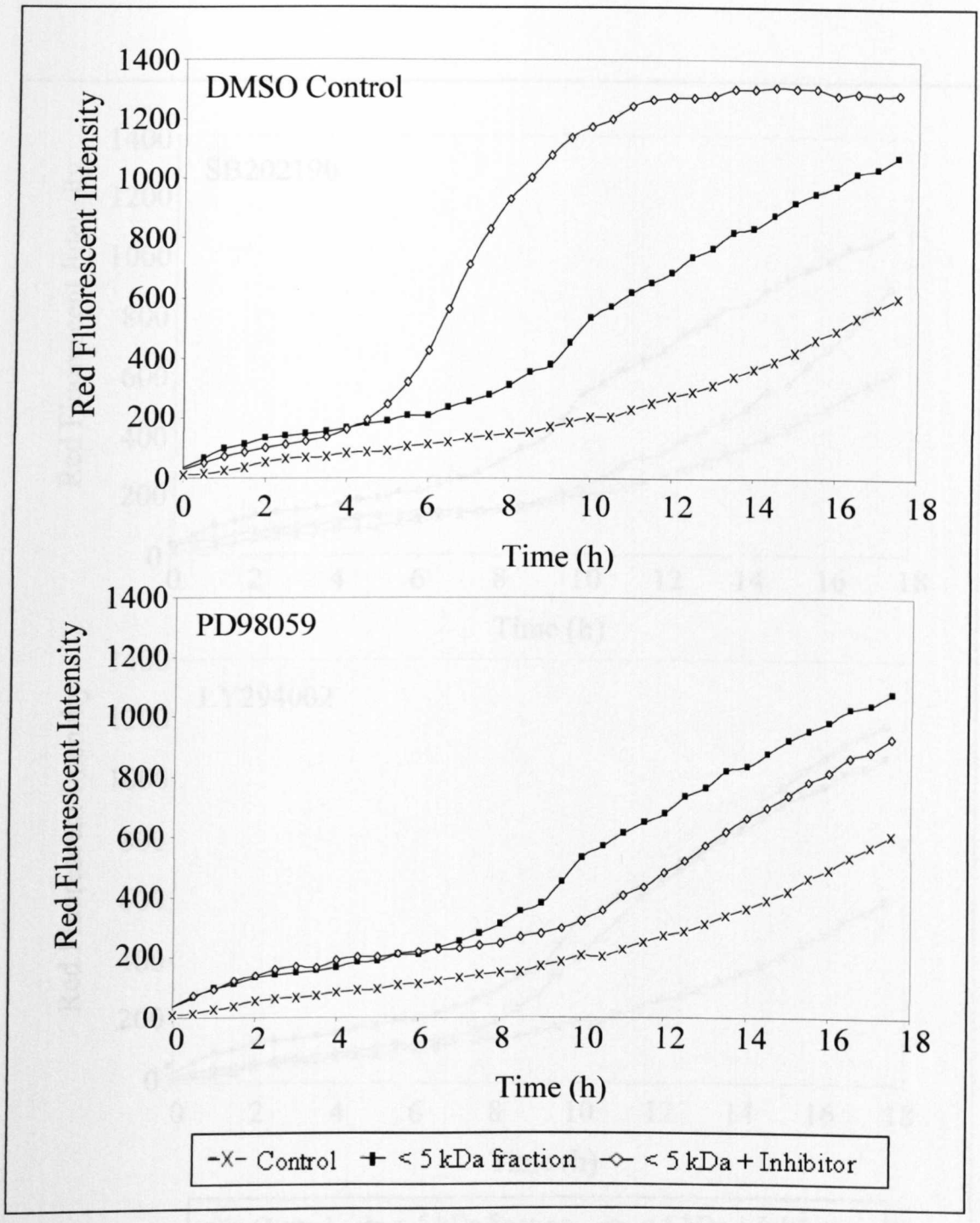


Figure 4.3.1: Fluorescence intensities from confocal microscopy of neutrophils incubated with *H. influenzae* < 5 kDa fraction (200 µl/ml) or < 5 kDa fraction plus inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Graphs are representative of 3 experiments.

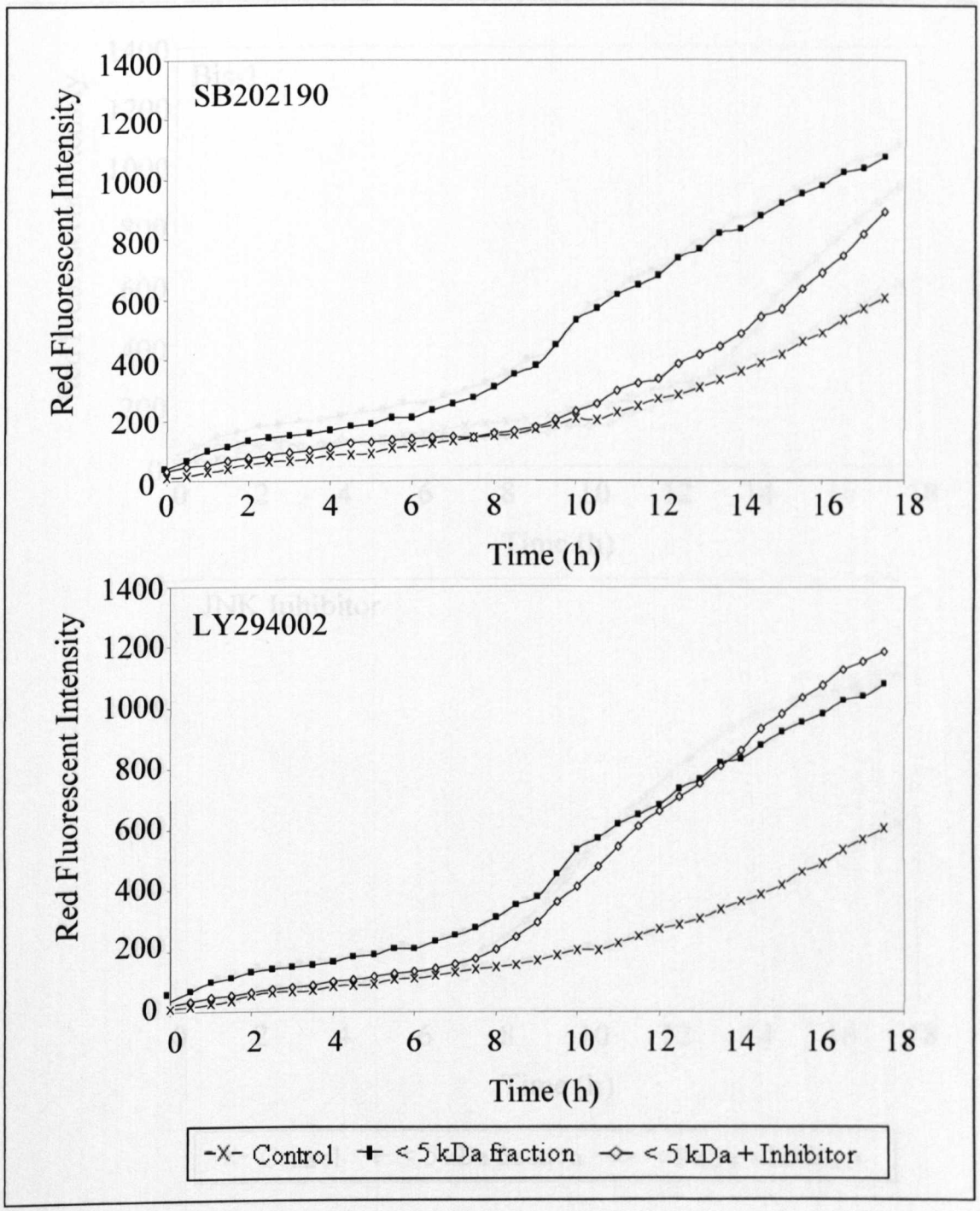


Figure 4.3.1 *(contd.)*

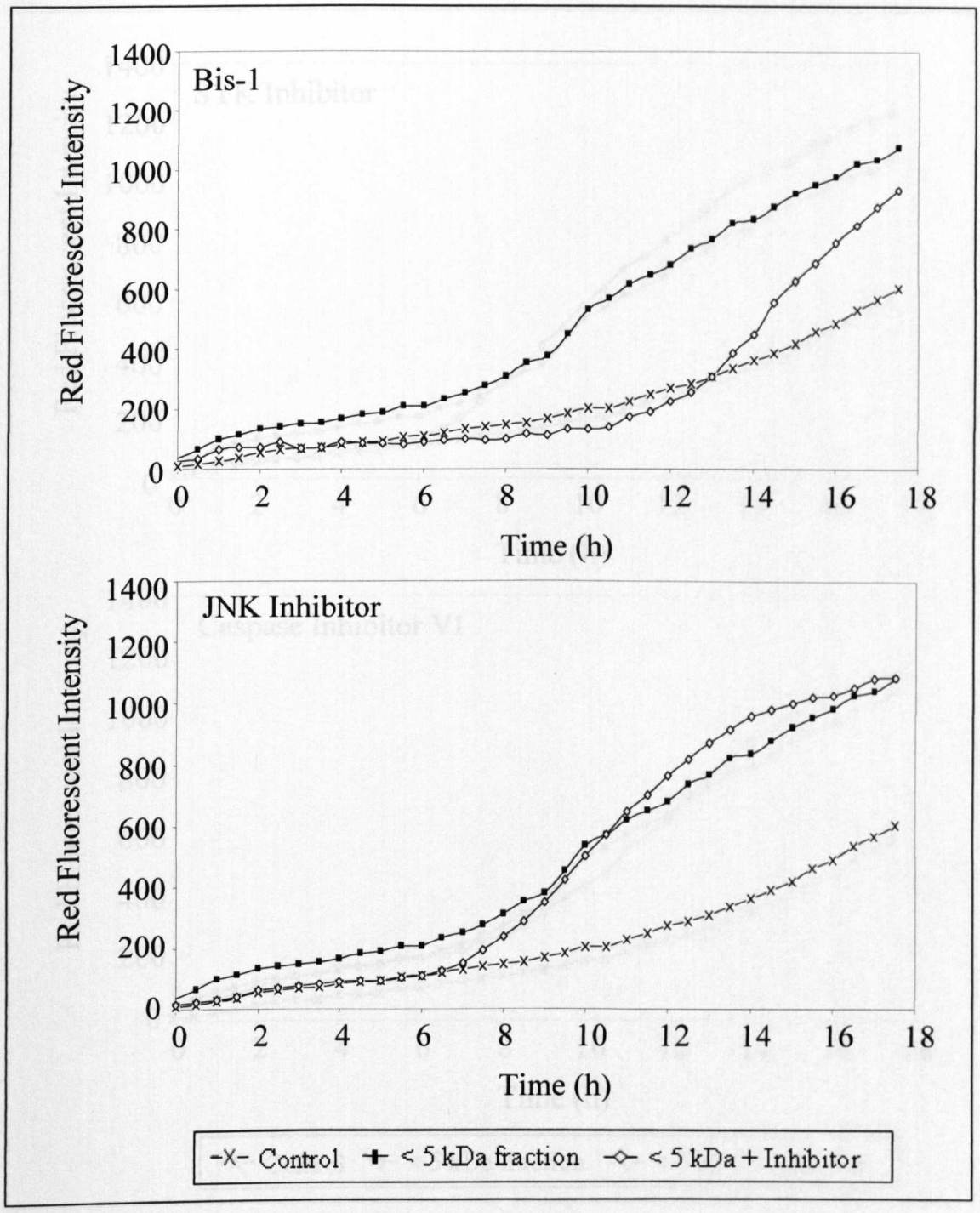
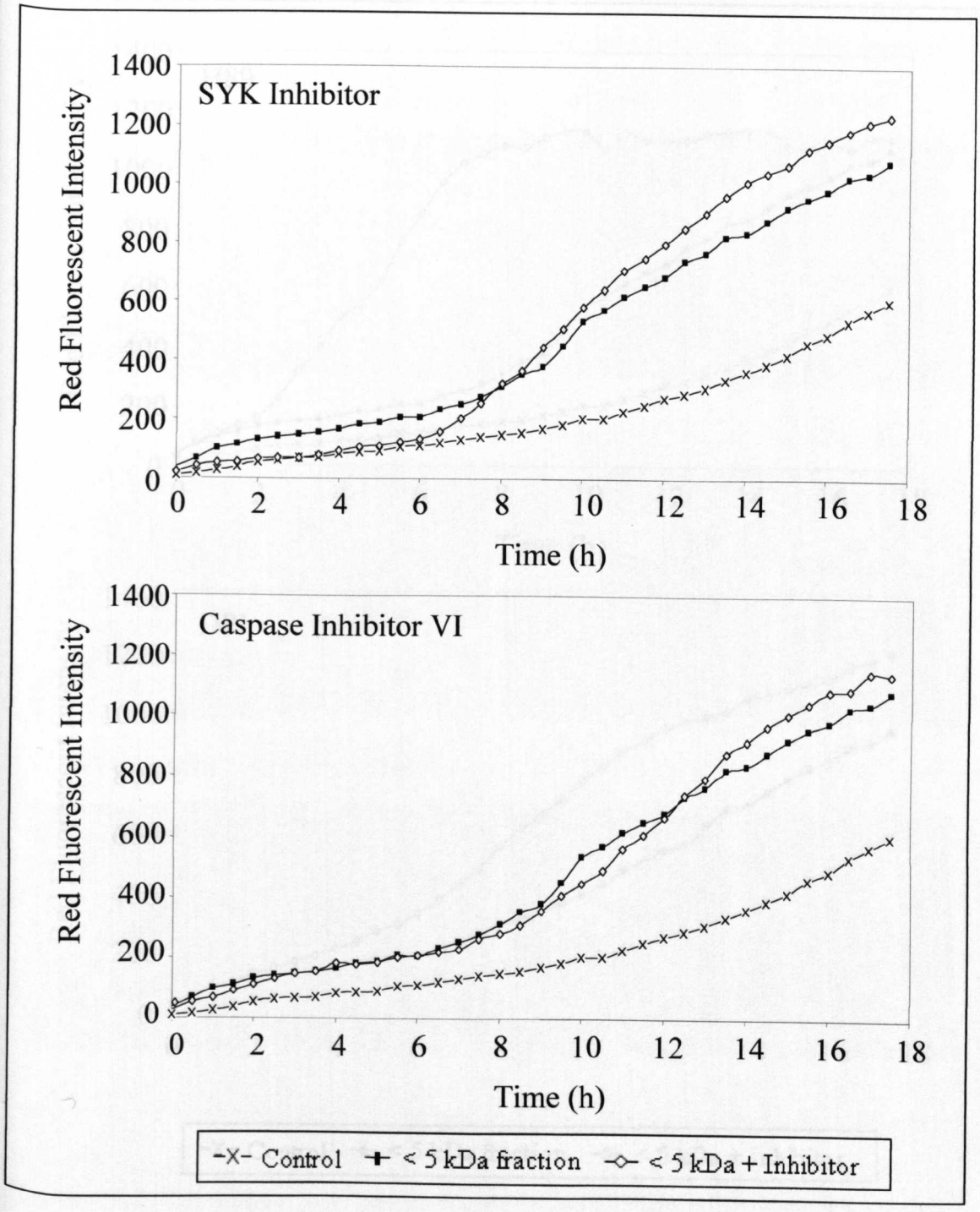


Figure 4.2.1 *(contd.)*



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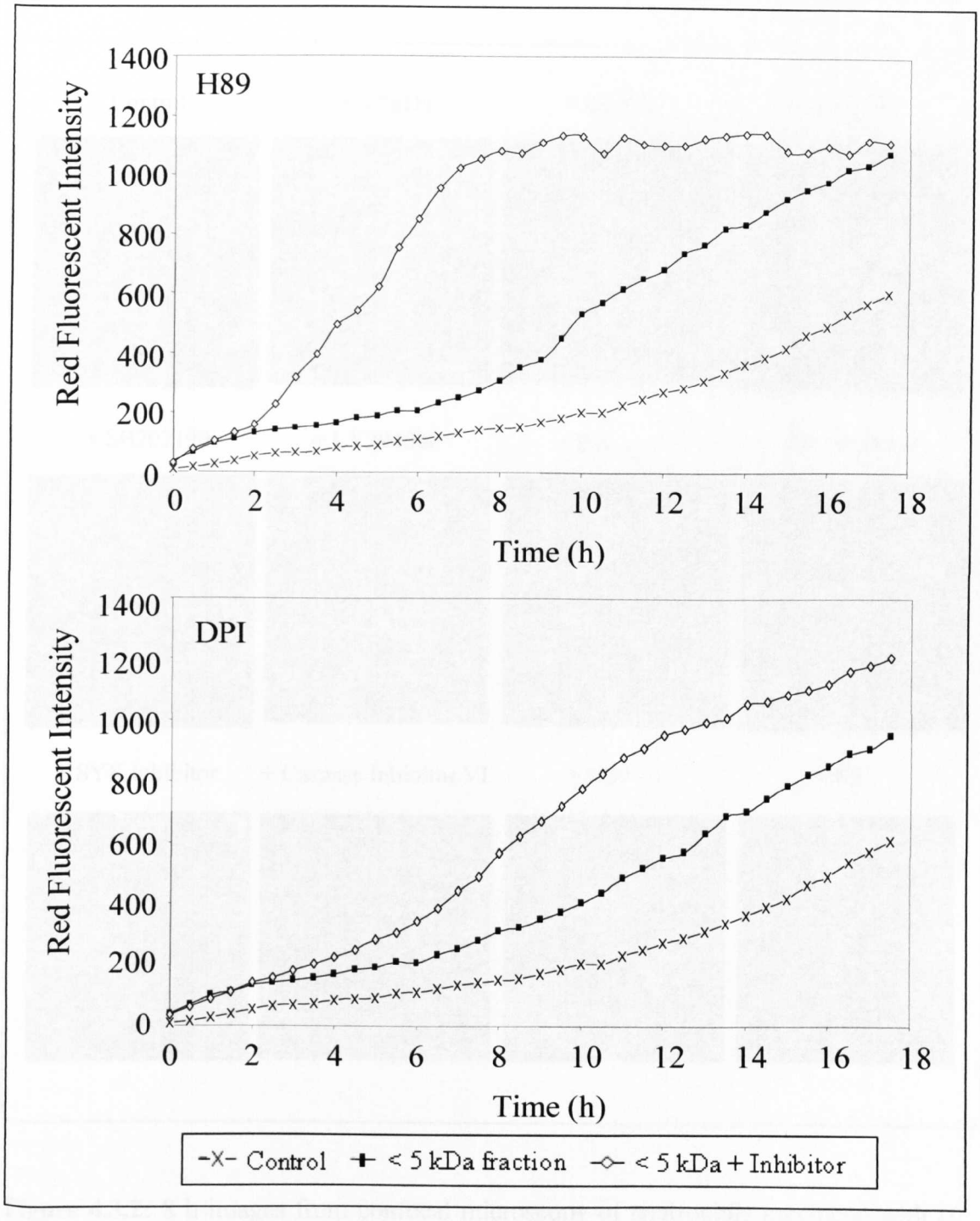

Figure 4.2.1 *(contd.)*

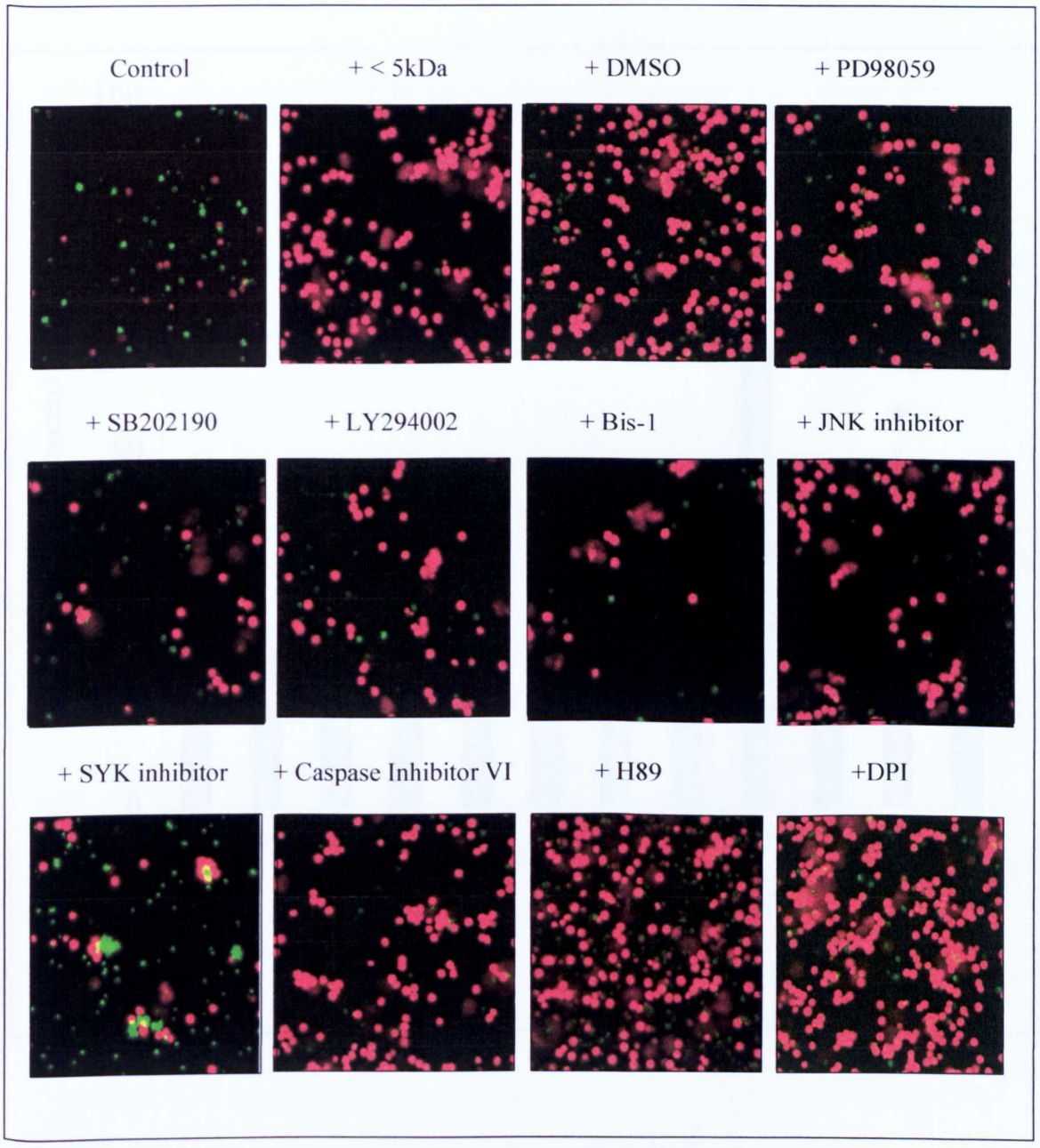

Figure 4.3.2: 8 h images from confocal microscopy of neutrophils incubated with *H. influenzae* culture filtrate (200 µl/ml) or culture filtrate and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Images shown were obtained after 8 h incubation. Images are representative of 3 experiments.

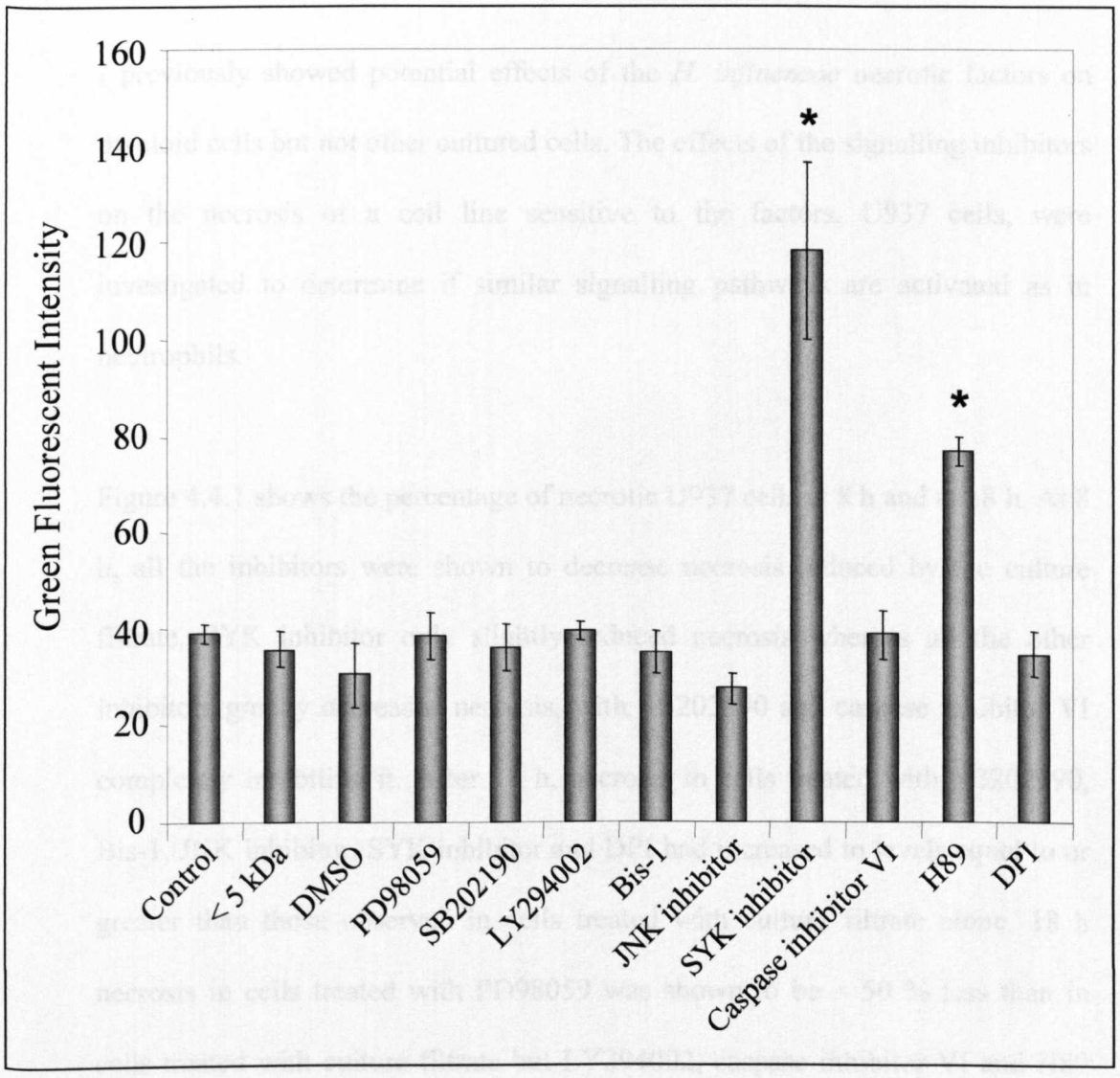

Figure 4.3.3: 8 h green fluorescence intensities from confocal microscopy of neutrophils incubated with *H. influenzae* < 5 kDa (200 µl/ml) or < 5 kDa plus cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Fluorescence was measured after 8 h incubation and mean values (± SD) of 3 separate experiments are shown. * indicates $p < 0.05$ compared to DMSO control.

4.4 *Effects of Signalling Inhibitors on a Cell Line Sensitive to H. influenzae Culture Filtrate*

I previously showed potential effects of the *H. influenzae* necrotic factors on myeloid cells but not other cultured cells. The effects of the signalling inhibitors on the necrosis of a cell line sensitive to the factors, U937 cells, were investigated to determine if similar signalling pathways are activated as in neutrophils.

Figure 4.4.1 shows the percentage of necrotic U937 cells at 8 h and at 18 h. At 8 h, all the inhibitors were shown to decrease necrosis induced by the culture filtrate. SYK inhibitor only slightly reduced necrosis whereas all the other inhibitors greatly decreased necrosis, with SB202190 and caspase inhibitor VI completely inhibiting it. After 18 h, necrosis in cells treated with SB202190, Bis-1, JNK inhibitor, SYK inhibitor and DPI had increased to levels equal to or greater than those observed in cells treated with culture filtrate alone. 18 h necrosis in cells treated with PD98059 was shown to be ~ 50 % less than in cells treated with culture filtrate but LY294002, caspase inhibitor VI and H89 completely inhibited necrosis. These results are different to those observed in neutrophils indicating that different signalling proteins are involved in the pathway in U937 cells. Images taken at 8 h are shown in figure 4.4.2 and again differences in cell viability at this time point can be clearly seen by differences in green and red fluorescence.

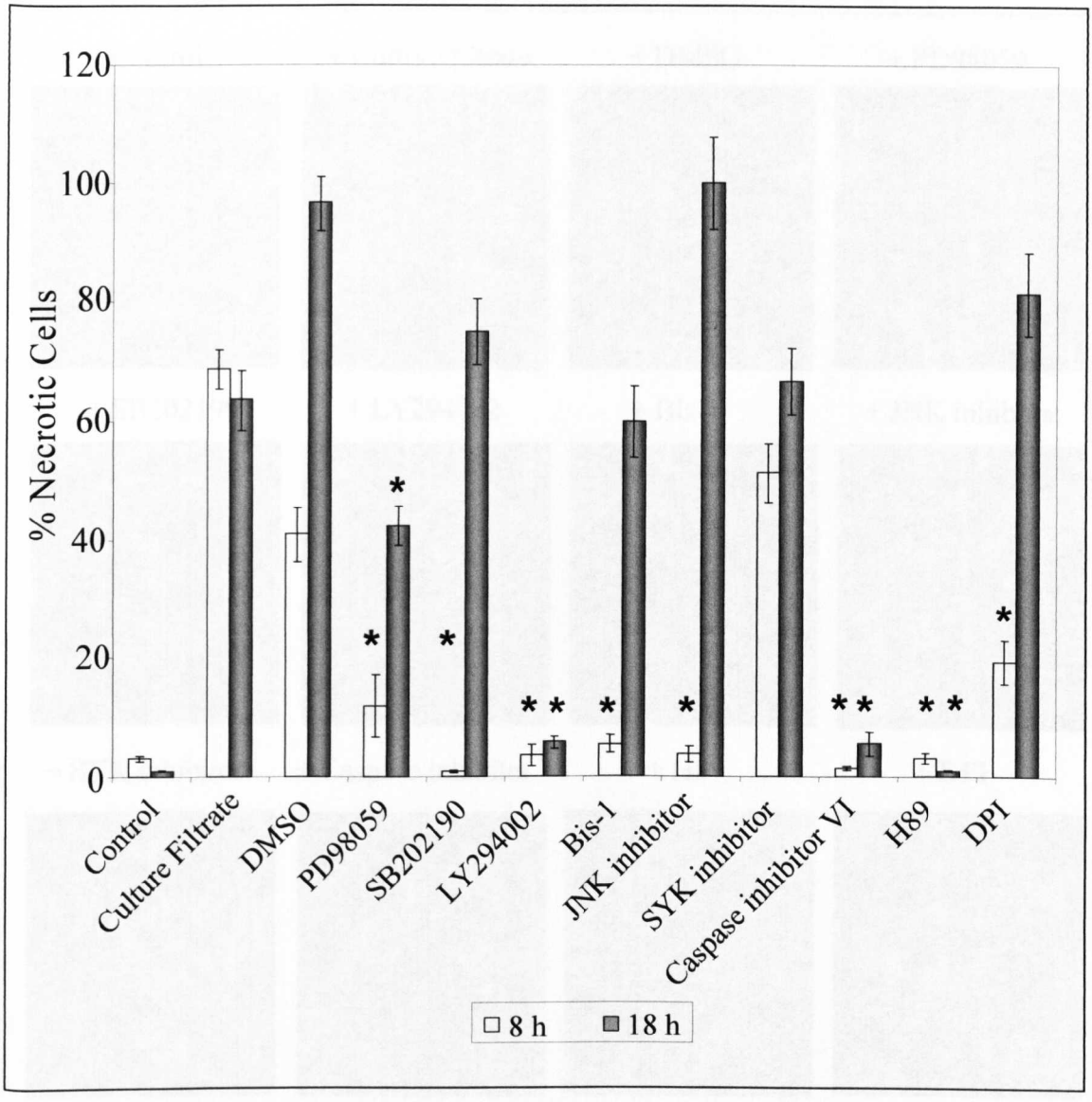

Figure 4.4.1: Percentage of necrotic U937 cells at 8 and 18 h. U937 cells were treated with cell signaling inhibitors and incubated with *H. influenzae* culture filtrate (200 µl/ml). Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence) and the effect on cell viability analysed by confocal microscopy. Data shown are mean values (± SD), n = 3. * $p < 0.05$ compared to DMSO control.

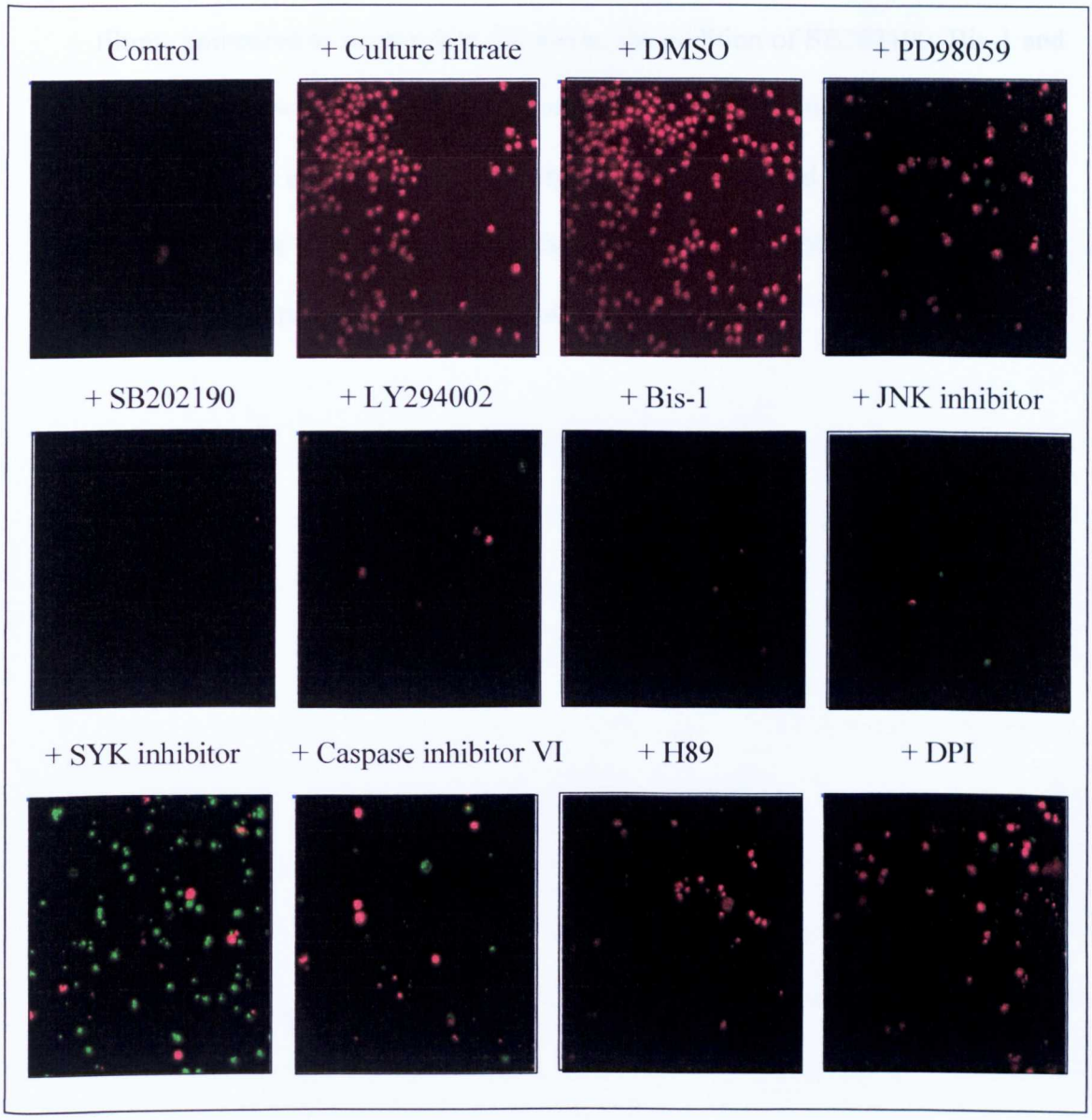

Figure 4.4.2: 8 h images from confocal microscopy of U937 cells incubated with *H. influenzae* culture filtrate (200 µl/ml) or culture filtrate and cell signaling inhibitors. U937 cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification and images shown were obtained after 8 h incubation. Images are representative of 3 experiments.

The addition of LY294002, caspase inhibitor VI and H89 were shown to have the greatest effect on inhibiting U937 necrosis induced by *H. influenzae* culture filtrate compared to neutrophils. However, the addition of SB202190, Bis-1 and JNK inhibitor was shown to inhibit necrosis at 8 h indicating that p38 MAPK, PKC and JNK are activated in the signal cascade induced by the factors. The results confirm that the necrotic factors released by *H. influenzae* induce necrosis via intracellular signalling pathways.

4.5 *Phosphorylation of Signalling Proteins by H. influenzae < 5 kDa Fraction*

The previous experiments indicated that p38 MAPK and PKC were important in the signalling pathways leading to necrosis of neutrophils in response to the < 5 kDa fraction. The kinetics of phosphorylation following the addition of the < 5 kDa fraction of these 2 proteins and also ERK 1/2 and Akt were analysed by western blotting.

Neutrophils (1 x 10^7 cell/ml in RPMI 1640 + HEPES supplemented with 2 mM L-glutamine) were stimulated with *H. influenzae* < 5 kDa fraction and at time intervals, levels of activated kinases were determined.

Figure 4.5.1 shows the levels of phosphorylated p38 MAPK compared to levels of total p38 MAPK before and after stimulation. Levels of phosphorylated p38 MAPK were very low in 0 h control and 18 h control samples and in 18 h stimulated samples. Phosphorylation of the protein was seen after stimulation of the neutrophils with the < 5 kDa fraction and reached a maximum level after 40 sec stimulation. The phosphorylation levels then begin to decrease again until a second increase in phosphorylation was seen at 2 h showing that cycles of phosphorylation and dephosphorylation of the protein occur. The protein appears to be activated early after stimulation and again later, corresponding to the time when necrosis begins to become detected.

Figure 4.5.2 shows the levels of phosphorylation of ERK 1/2. The pattern of phosphorylation of this protein is different to that of p38 MAPK as phosphorylation occurred after 40 sec of stimulation and peaked at 5 min. Phosphorylation then decreased and no second peak was observed. This suggests that ERK 1/2 is activated very soon after addition of the factor to the cells.

Figure 4.5.3 shows the levels of phosphorylated Akt after stimulation of the neutrophils with the < 5 kDa fraction. Again, only one peak in phosphorylation was observed over the time course, occurring after 5 min. The levels of phosphorylated Akt decreased until the phosphorylated protein was no longer detected by 2 h. Akt activation occurs after p38 MAPK and ERK 1/2 activation indicating that Akt may be phosphorylated downstream of these two proteins.

Figure 4.5.4 shows the levels of phosphorylated PKC after stimulation with the < 5 kDa fraction. Confocal microscopy analysis with the PKC inhibitor Bis-1 indicated that PKC is an important protein involved in the signalling pathways activated by < 5 kDa fraction. This is confirmed by the presence of phosphorylated PKC 5 sec after the addition of < 5 kDa fraction. The phosphorylation of the protein was maintained for 5 min and decreased after 15 min. However, increased levels of phosphorylated PKC were detected after 30 min. These results suggest that PKC, like p38 MAPK and ERK1/2, was

phosphorylated both early and late in the sequence of events following addition of the factor.

The levels of phosphorylated p38 MAPK, ERK 1/2, Akt and PKC in untreated neutrophils were analysed by western blotting and the presence of these phosphorylated proteins was shown to be minimal, as shown in 0 h control and 18 h control samples. This confirms that activation of these proteins is a result of neutrophil interaction with the < 5 kDa fraction, likely to be via receptor mediated pathways.

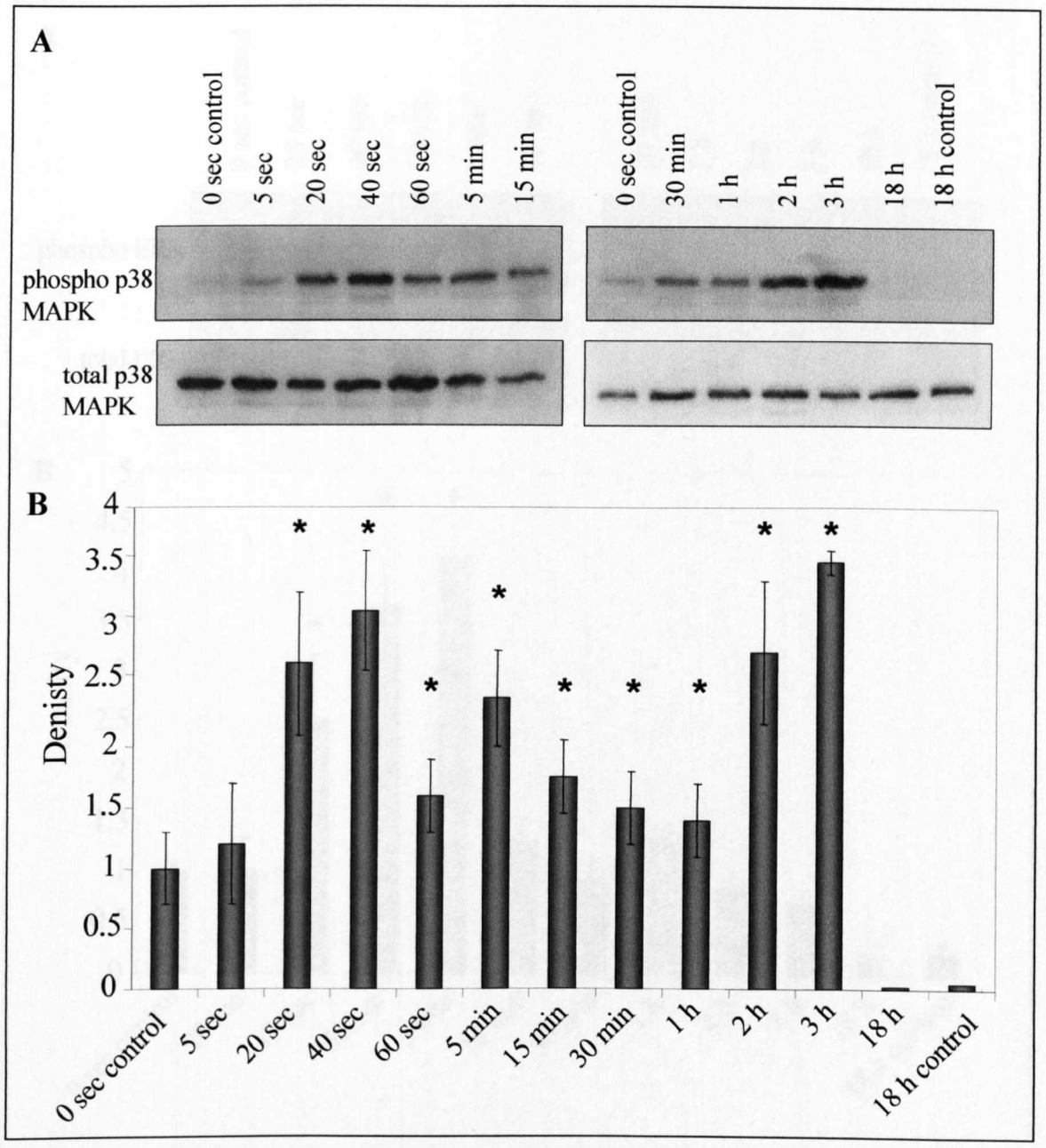

Figure 4.5.1: Neutrophils were stimulated with *H. influenzae* culture filtrate < 5 kDa fraction (200 µl/ml) and incubated at 37 °C. Protein lysates were prepared at regular intervals and the levels of phospho p38 MAPK and total p38 MAPK were detected via western blotting. Images are representative of 3 experiments. B mean values are of 3 separate experiments (± SD). * $p < 0.05$ compared to unstimulated neutrophils.

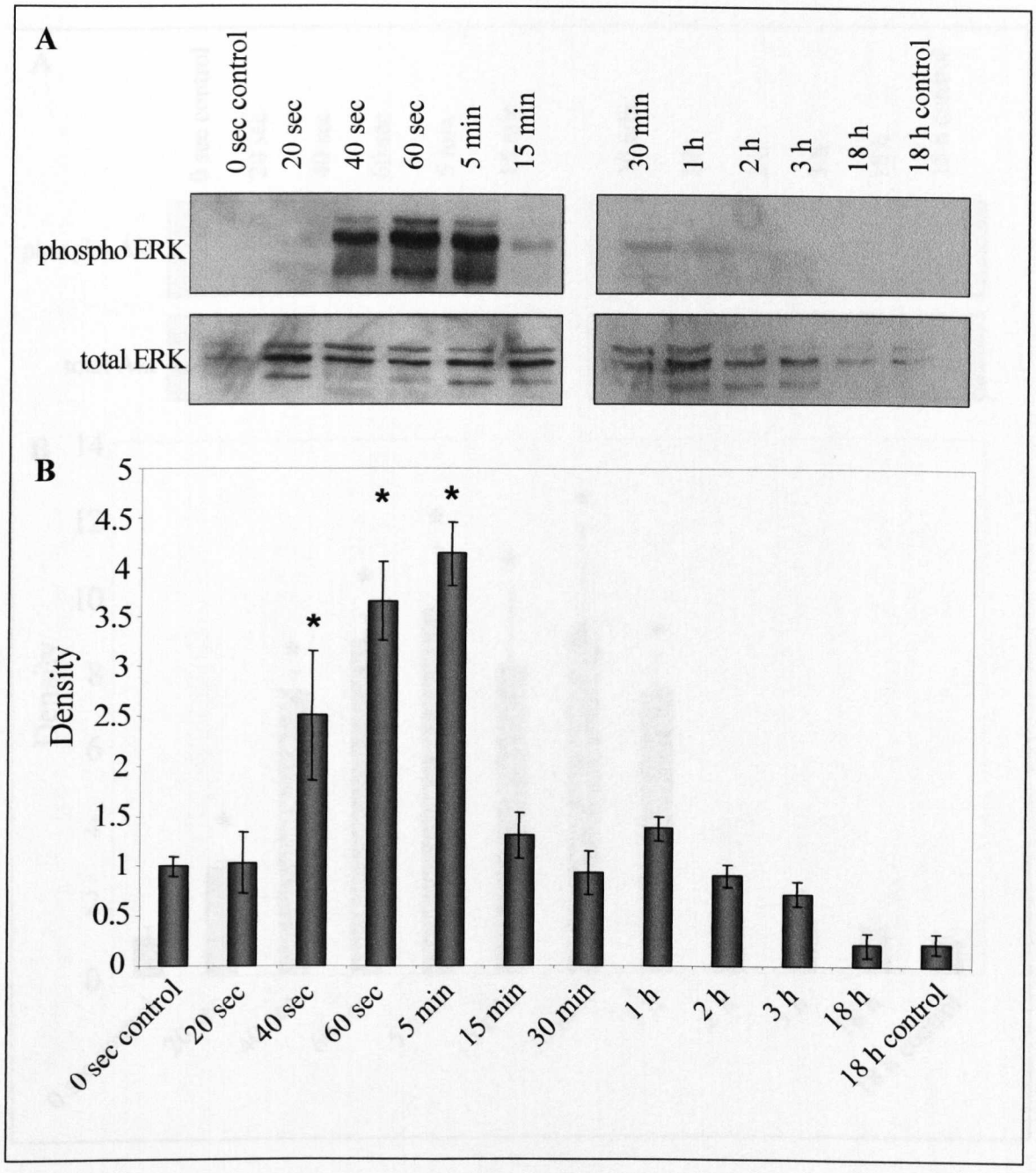

Figure 4.5.2: Neutrophils were stimulated with *H. influenzae* culture filtrate < 5 kDa fraction (200 µl/ml) and incubated at 37 °C. Protein lysates were prepared at regular intervals and the levels of phospho ERK and total ERK were detected via western blotting. Images are representative of 3 experiments. B mean values are of 3 separate experiments (± SD). * $p < 0.05$ compared to unstimulated neutrophils.

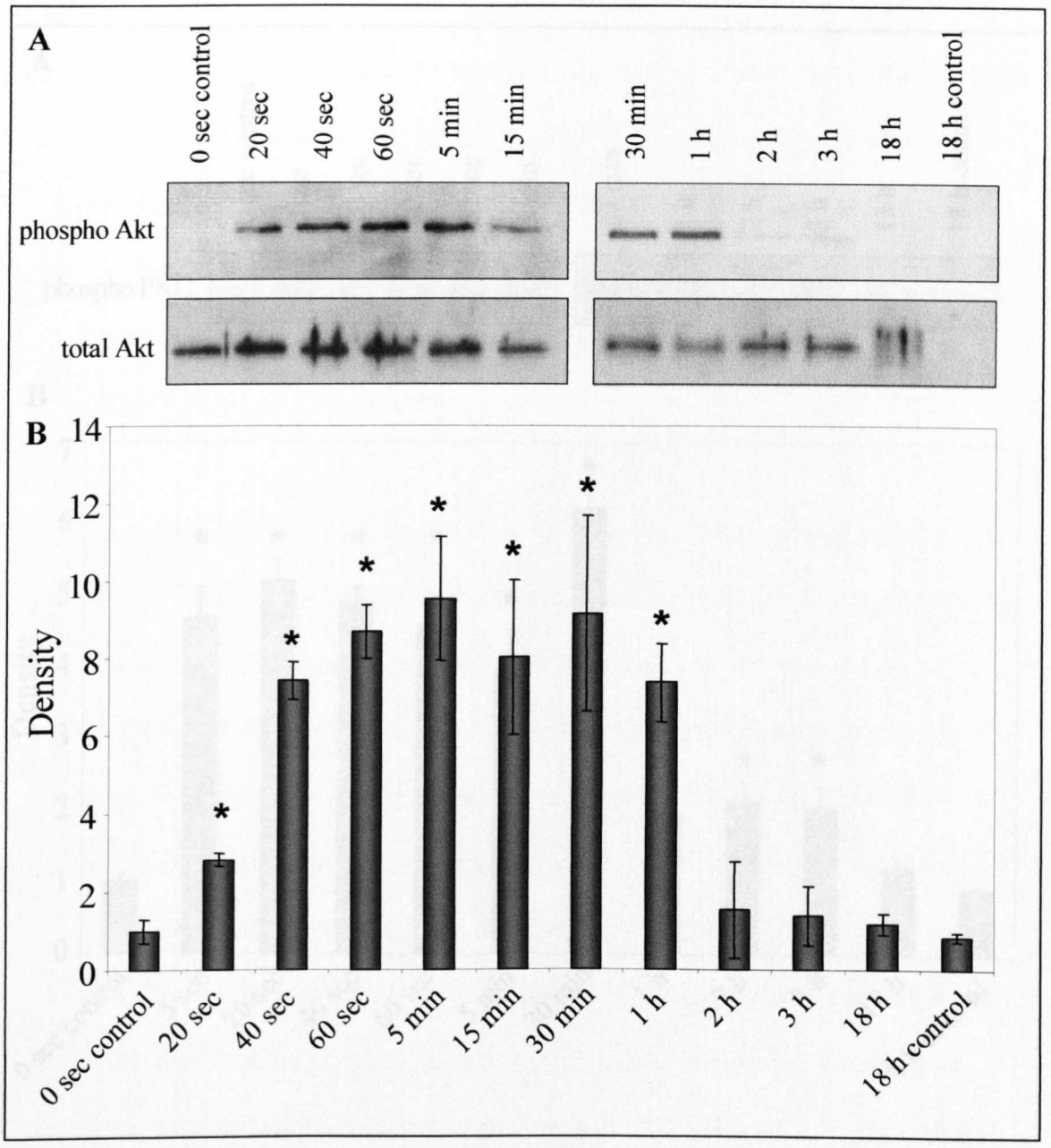

Figure 4.5.3: Neutrophils were stimulated with *H. influenzae* culture filtrate < 5 kDa fraction (200 µl/ml) and incubated at 37 °C. Protein lysates were prepared at regular intervals and the levels of phospho Akt and total Akt were detected via western blotting. Images are representative of 3 experiments. B mean values are of 3 separate experiments (± SD). * $p < 0.05$ compared to unstimulated neutrophils.

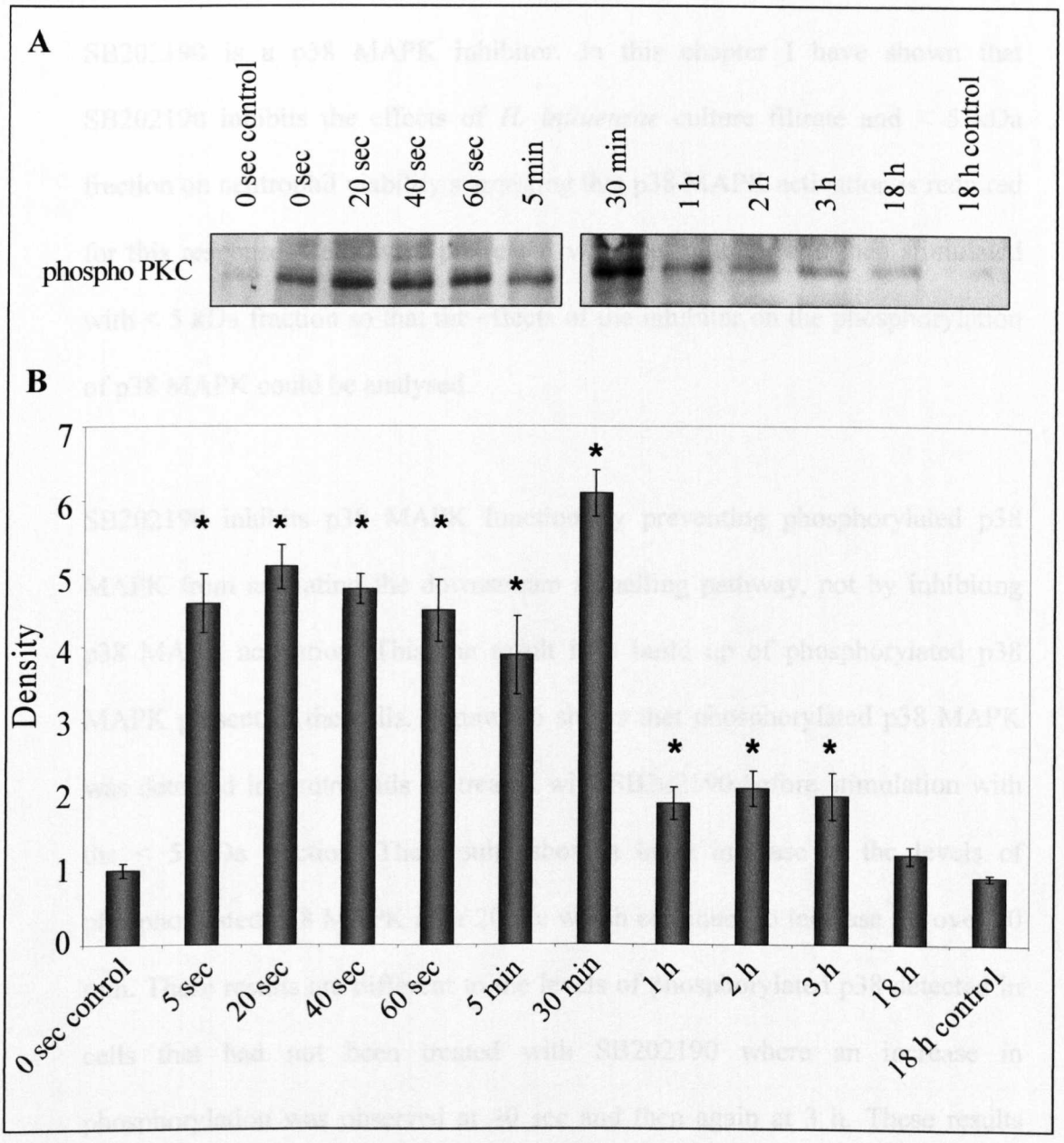

Figure 4.5.4: Neutrophils were stimulated with *H. influenzae* culture filtrate < 5 kDa fraction (200 µl/ml) and incubated at 37 °C. Protein lysates were prepared at regular intervals and the levels of phospho PKC was detected via western blotting. Images are representative of 3 experiments. B mean values are of 3 separate experiments (± SD). * $p < 0.05$ compared to unstimulated neutrophils.

4.6 *Phosphorylation of p38 MAPK in Neutrophils Treated with SB202190*

SB202190 is a p38 MAPK inhibitor. In this chapter I have shown that SB202190 inhibits the effects of *H. influenzae* culture filtrate and < 5 kDa fraction on neutrophil viability suggesting that p38 MAPK activation is required for this response. Cells were pretreated with the inhibitor and then stimulated with < 5 kDa fraction so that the effects of the inhibitor on the phosphorylation of p38 MAPK could be analysed.

SB202190 inhibits p38 MAPK function by preventing phosphorylated p38 MAPK from activating the downstream signalling pathway, not by inhibiting p38 MAPK activation. This can result in a build up of phosphorylated p38 MAPK present in the cells. Figure 4.6 shows that phosphorylated p38 MAPK was detected in neutrophils pretreated with SB202190 before stimulation with the < 5 kDa fraction. The results show a large increase in the levels of phosphorylated p38 MAPK after 20 sec which continues to increase for over 30 min. These results are different to the levels of phosphorylated p38 detected in cells that had not been treated with SB202190 where an increase in phosphorylation was observed at 40 sec and then again at 3 h. These results further confirm that p38 MAPK is activated by the *H. influenzae* < 5 kDa fraction resulting in neutrophil necrosis.

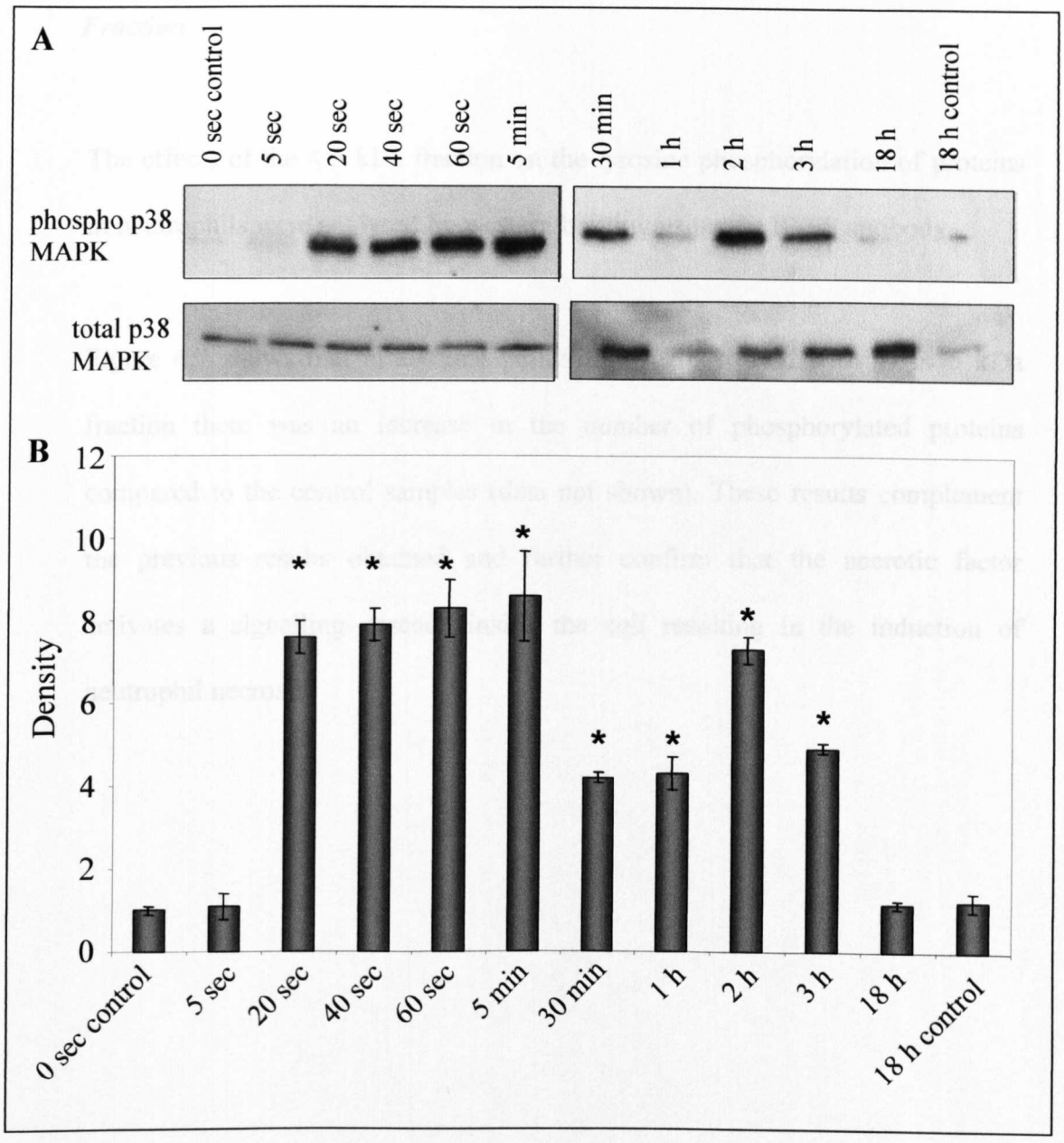

Figure 4.6: Neutrophils were treated with the cell signaling inhibitor SB202190 (1 μM) for 20 min prior to stimulation with *H. influenzae* culture filtrate < 5 kDa fraction (200 μl/ml) and incubated at 37 °C. Protein lysates were prepared at regular intervals and the levels of phospho p38 MAPK detected via western blotting. Images are representative of 3 experiments. * $p < 0.05$ compared to unstimulated neutrophils.

4.7 Tyrosine Phosphorylation of Proteins in Neutrophils Stimulated with < 5 kDa Fraction

The effects of the < 5 kDa fraction on the tyrosine phosphorylation of proteins in neutrophils were analysed by western blotting using the PY99 antibody.

Figure 4.7 shows that in all time points from cells treated with the < 5 kDa fraction there was an increase in the number of phosphorylated proteins compared to the control samples (data not shown). These results complement the previous results obtained and further confirm that the necrotic factor activates a signalling cascade inside the cell resulting in the induction of neutrophil necrosis.

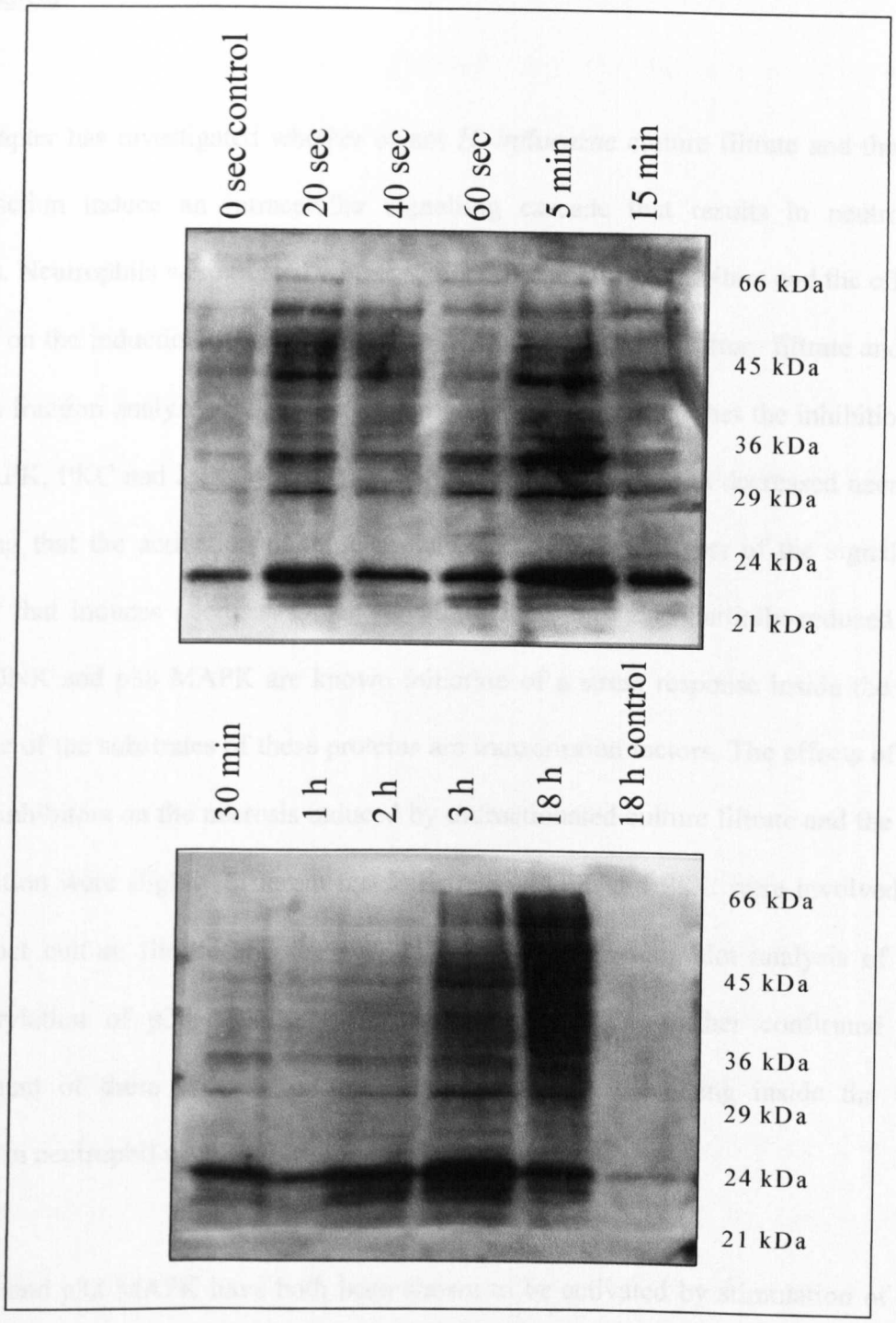

Figure 4.7: Neutrophils were treated stimulated with < 5 kDa fraction (200 µl/ml) and incubated at 37 °C. Protein lysates were prepared at regular intervals and the levels of global tyrosine phosphorylation detected via western blotting using PY99 antibody. Images are representative of 3 experiments.

Discussion

This chapter has investigated whether or not *H. influenzae* culture filtrate and the < 5 kDa fraction induce an intracelullar signalling cascade that results in neutrophil necrosis. Neutrophils were treated with a number of signalling inhibitors and the effects of these on the induction of neutrophil necrosis by *H. influenzae* culture filtrate and the < 5 kDa fraction analysed by confocal microscopy. It was shown that the inhibition of p38 MAPK, PKC and JNK by their signalling inhibitors resulted in decreased necrosis indicating that the activation of these proteins is an important part of the signalling pathway that induces necrosis. Other signalling inhibitors also partially reduced the effects. JNK and p38 MAPK are known initiation of a stress response inside the cell and some of the substrates of these proteins are transcription factors. The effects of the specific inhibitors on the necrosis induced by unfractionated culture filtrate and the < 5 kDa fraction were slightly different but JNK, p38 MAPK and PKC were involved by both intact culture filtrate and the < 5 kDa fraction. Western blot analysis of the phosphorylation of p38 MAPK, ERK 1/2, Akt and PKC further confirmed the involvement of these proteins in the signalling cascade occurring inside the cell resulting in neutrophil necrosis.

ERK 1/2 and p38 MAPK have both been shown to be activated by stimulation of the cells with *H. influenzae* culture filtrate and < 5 kDa fraction. ERK 1/2 and p38 MAPK phosphorylation occurs during activation of the respiratory burst, chemotaxis, IL-8 production and stress induced apoptosis and are involved in initiating a pro-

inflammatory response (*215, 216*). Therefore, activation of these two signalling proteins by the necrotic factors will increase inflammation in the lung by the increased production of ROS, release of pro-inflammatory cytokines and migration of neutrophils to the lungs.

p38 MAPK and ERK are known to activate MAPK-activated protein kinase-2 (MK2) and Akt is a substrate of this kinase (*214, 216*). Western blot analysis of the phosphorylated proteins showed that phosphorylation of Akt occurred later than that of p38 MAPK and ERK 1/2 indicating that Akt may be phosphorylated downstream by MK2. This suggests that the factors work through a signalling pathway where p38 MAPK and ERK 1/2 are activated early in the pathway resulting in the activation of Akt.

Inhibition of neutrophil necrosis by Bis-1 and detection of phosphorylated PKC in neutrophils stimulated with the < 5 kDa fraction has shown that PKC is also activated by the necrotic factors. PKC is an important signalling protein in neutrophils and its activation is required for phagocytosis and ROS production. PKC activation is Ca^{2+} and diacylglycerol dependent and enables the assembly and activation of the NADPH oxidase complex to occur *(83, 217)*. It has been shown that *H. influenzae* is phagocytosed by neutrophils and ROS production increased after stimulation with *H. influenzae* and *H. influenzae* culture filtrate, events requiring activation of PKC.

These results show that necrosis is induced in myeloid cells by the ability of the factors to initiate an intracellular signalling cascade. This suggests that the factors may be binding to plasma membrane receptors in order to initiate this response. If this can be confirmed and the signalling pathway further elucidated then it may be possible to design new therapies to treat exacerbations of COPD by *H. influenzae* infections.

Chapter 5: Does *H. influenzae* Culture Filtrate Induces Necrosis Through Toll-like Receptors?

Introduction

Chapter 4 investigated the ability of the unfractionated culture filtrate and the < 5 kDa fraction to induce neutrophil necrosis by activating an intracellular signalling cascade. Using cell signalling inhibitors to block particular pathways and antibodies that recognise activated kinases it was found that p38 MAPK, ERK 1/2, Akt, PKC and JNK are all activated when neutrophils are incubated with the necrosis-inducing factors. These results would indicate that the factors are inducing necrosis by first binding to plasma membrane receptors present on the surface of the neutrophil. This chapter investigates the involvement of the Toll-like receptors in inducing neutrophil necrosis.

TLRs play important roles in the recognition of microbial pathogens and activation of the immune response. Ten TLRs have been identified in humans and these recognize and bind conserved pathogen associated molecular patterns (PAMPs) rather than specific microbial structures (*119*), as described in *1.3.3 Toll-like Receptors*. The ten different TLRs thus bind a wide range of different PAMPs ranging from bacterial RNA and DNA, to flagellin.

Two TLRs that have received a great deal of interest are TLR2 and TLR4, as these receptors are of particular importance in the host response to bacterial infections *(121)*.

TLR2 forms heterodimers with TLR1 and TLR6 to enable recognition of bacterial lipoproteins. TLR2/TLR1 heterodimers bind triacylated bacterial lipoproteins whereas TLR2/TLR6 heterodimers are able to recognize diacylated lipoproteins. TLR2 is also thought to be involved in the recognition of atypical forms of LPS. TLR4 is involved in binding LPS, an integral component of the outer membrane of gram negative bacteria, by acting as a co-receptor for CD14. Binding of LPS will induce a pro-inflammatory response *(119, 120)*.

Many immune cells express a range of TLRs but definition of the function of individual or combinations of TLRs has been greatly facilitated by the development of cell lines that express functional receptors. The HEK cell line is a good experimental tool for such purposes.

The aims of this chapter were to:

- Determine whether or not *H. influenzae* culture filtrate has any effect on cells that have been transfected with TLR2 and TLR4.
- Investigate the effects of cell signalling inhibitors necrosis of cells transfected with TLR2 and TLR4.
- Analyse the effects of known TLR ligands on neutrophil viability.
- Analyse the expression of TLR2 and TLR4 on cell lines that are either sensitive or resistant to *H. influenzae* culture filtrate.

5.1 *Effects of H. influenzae Culture Filtrate and < 5 kDa Fraction on IL-8 Production in HEK Cells Transfected with CD14, CD14/TLR2 and CD14/TLR4*

The effects of *H. influenzae* culture filtrate and the < 5 kDa fraction on IL-8 production in HEK cells transfected with two TLRs, TLR2 and TLR4, was investigated. HEK cells were transfected with CD14 alone or CD14 was co-transfected with TLR2 or TLR4 as it is required as a co-receptor along with fTLR4 for LPS binding. Production of IL-8 by the cells will indicate whether or not the factors present in the culture filtrate are activating cells via these receptors. Note that these cell lines endogenously express TLR-6.

Transfected HEK cells were cultured as described in *Materials and Methods 2.3* and plated into 96-well plates. MD-2 (5 μg/ml) was added to the cells as this forms a complex with the TLR4 and CD14 receptors that is required for LPS binding. Cells were stimulated with undiluted *H. influenzae* culture filtrate and < 5 kDa fraction as well as 1:1 and 1:4 dilutions of the filtrate. Cells were also stimulated with uninoculated *H. influenzae* culture medium for use as a control. The amount of IL-8 production (pg/ml) by the stimulated cells was measured using an ELISA.

Figure 5.1.1 shows the amount of IL-8 production in HEK cells transfected with CD14, CD14/TLR2 and CD14/TLR4 stimulated with *H. influenzae* culture filtrate. The results indicate that the factors present in the culture filtrate were able to activate the cells via TLR2 and CD14 as there was an increase in IL-8 production compared to cells treated with *H. influenzae* culture media. There was no enhancement of IL-8 production in HEK cells transfected with CD14/TLR4 compared to HEK cells transfected with CD14 alone indicating that the IL-8 production in this cell line was a result of CD14 activation. This suggests that there are possibly 2 factors present in the culture filtrate that are capable of binding and activating CD14 and TLR2. Alternatively, it is also possible that the factors present in the culture filtrate are capable of binding to more than one receptor.

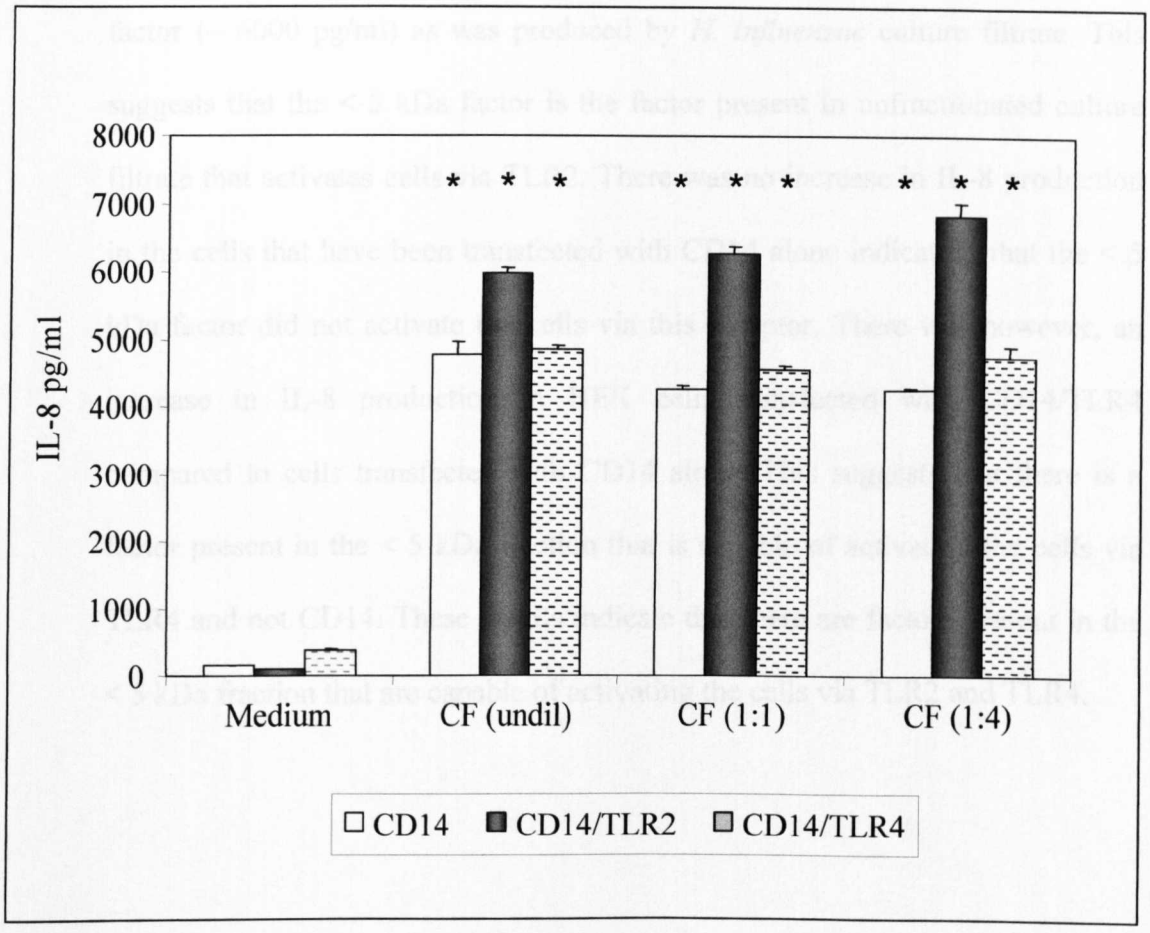

Figure 5.1.1: Amount of IL-8 (pg/ml) produced by HEK cells transfected with CD14, CD14/TLR2 or CD14/TLR4 after stimulation with *H. influenzae* culture filtrate (CF) was measured using an ELISA. The cells were stimulated with undiluted culture filtrate, a 1:1 dilution of CF and a 1:4 dilution of CF and incubated for 18 h. RMPI 1640 + HEPES supplemented with 5 % (v/v) glycerol, 30 mg/l hemin and 10 mg/ml β-NAD was used as a negative control in this experiment (medium). Results are means of 3 experiments (± SD). * $p < 0.05$ compared to unstimulated cells.

Figure 5.1.2 shows the amount of IL-8 production (pg/ml) in transfected HEK cells that were stimulated with *H. influenzae* < 5 kDa factor. The same amount of IL-8 was produced in HEK cells transfected with CD14/TLR2 by the < 5 kDa factor (~ 6000 pg/ml) as was produced by *H. influenzae* culture filtrate. This suggests that the < 5 kDa factor is the factor present in unfractionated culture filtrate that activates cells via TLR2. There was no increase in IL-8 production in the cells that have been transfected with CD14 alone indicating that the < 5 kDa factor did not activate the cells via this receptor. There was however, an increase in IL-8 production in HEK cells transfected with CD14/TLR4 compared to cells transfected with CD14 alone. This suggests that there is a factor present in the < 5 kDa fraction that is capable of activating the cells via TLR4 and not CD14. These results indicate that there are factors present in the < 5 kDa fraction that are capable of activating the cells via TLR2 and TLR4.

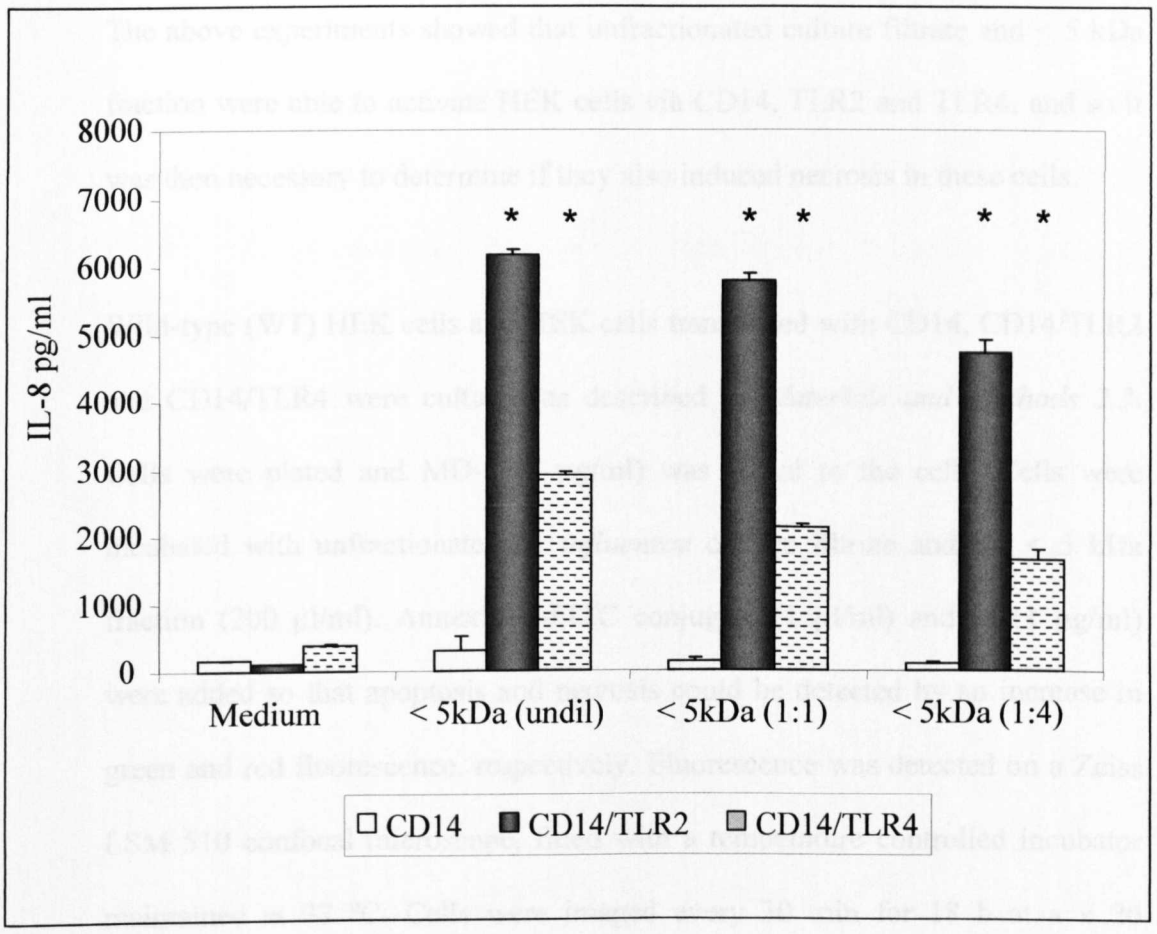

Figure 5.1.2: Amount of IL-8 (pg/ml) produced by HEK cells transfected with CD14, CD14/TLR2 or CD14/TLR4 after stimulation with *H. influenzae* < 5 kDa fraction was measured using an ELISA. The cells were stimulated with undiluted culture filtrate, a 1:1 dilution and a 1:4 dilution of the < 5 kDa fraction and incubated for 18 h. RMPI 1640 + HEPES supplemented with 5 % (v/v) glycerol, 30 mg/l hemin and 10 mg/ml β-NAD was used as a negative control in this experiment (medium). Results are means of 3 experiments (± SD). * $p < 0.05$ compared to unstimulated cells.

5.2 *Effects of H. influenzae Culture Filtrate on the Viability of HEK Cells Transfected with CD14, CD14/TLR2 and CD14/TLR4*

The above experiments showed that unfractionated culture filtrate and < 5 kDa fraction were able to activate HEK cells via CD14, TLR2 and TLR4, and so it was then necessary to determine if they also induced necrosis in these cells.

Wild-type (WT) HEK cells and HEK cells transfected with CD14, CD14/TLR2 and CD14/TLR4 were cultured as described in *Materials and Methods 2.3.* Cells were plated and MD-2 (5 μg/ml) was added to the cells. Cells were incubated with unfractionated *H. influenzae* culture filtrate and the < 5 kDa fraction (200 μl/ml). Annexin-V-FITC conjugate (1 μl/ml) and PI (1 μg/ml) were added so that apoptosis and necrosis could be detected by an increase in green and red fluorescence, respectively. Fluorescence was detected on a Zeiss LSM 510 confocal microscope, fitted with a temperature controlled incubator maintained at 37 °C. Cells were imaged every 30 min for 18 h at a x 20 magnification.

Figure 5.2.1 shows the red fluorescence intensities (necrosis) of the 4 cell lines after addition of *H. influenzae* culture filtrate and the < 5 kDa fraction. In WT HEK cells and CD14 HEK cells there was no increase in the red fluorescence intensity of the cells treated with either culture filtrate or the < 5 kDa fraction compared to unstimulated cells. This indicates that neither the endogenously

expressed receptors nor CD14 responded to the factors to trigger necrosis. Figure 5.2.2 shows 18 h images of HEK cells stimulated with *H. influenzae* culture filtrate and the < 5 kDa factor.

In cells that had been transfected with CD14/TLR2, there was a large increase in red fluorescence after incubation of the cells with *H. influenzae* culture filtrate, indicating that the factors present in the supernatant induced necrosis in these cells. There was also an increase in necrosis in cells treated with the < 5 kDa fraction after ~ 10 h incubation, although necrosis did not reach the levels seen in cells stimulated with culture filtrate. In HEK cells transfected with CD14/TLR4 again there was an increase in red fluorescence in cells treated with both *H. influenzae* culture filtrate and the < 5 kDa fraction. The effects were lower than for CD14/TLR2 HEK cells but greater than untreated control cells. Necrosis of CD14/TLR2 and CD14/TLR4 HEK cells induced by both *H. influenzae* culture filtrate and < 5 kDa fraction was less than that observed in neutrophils and occurs at a slower rate but this is probably due to the different signalling mechanisms of the cells. There was no effect on HEK cell apoptosis after incubation with H. influenzae culture filtrate and the < 5 kDa factor. The results indicate that the necrotic factors released by *H. influenzae* are able to induce necrosis in cells via TLR2 and TLR4.

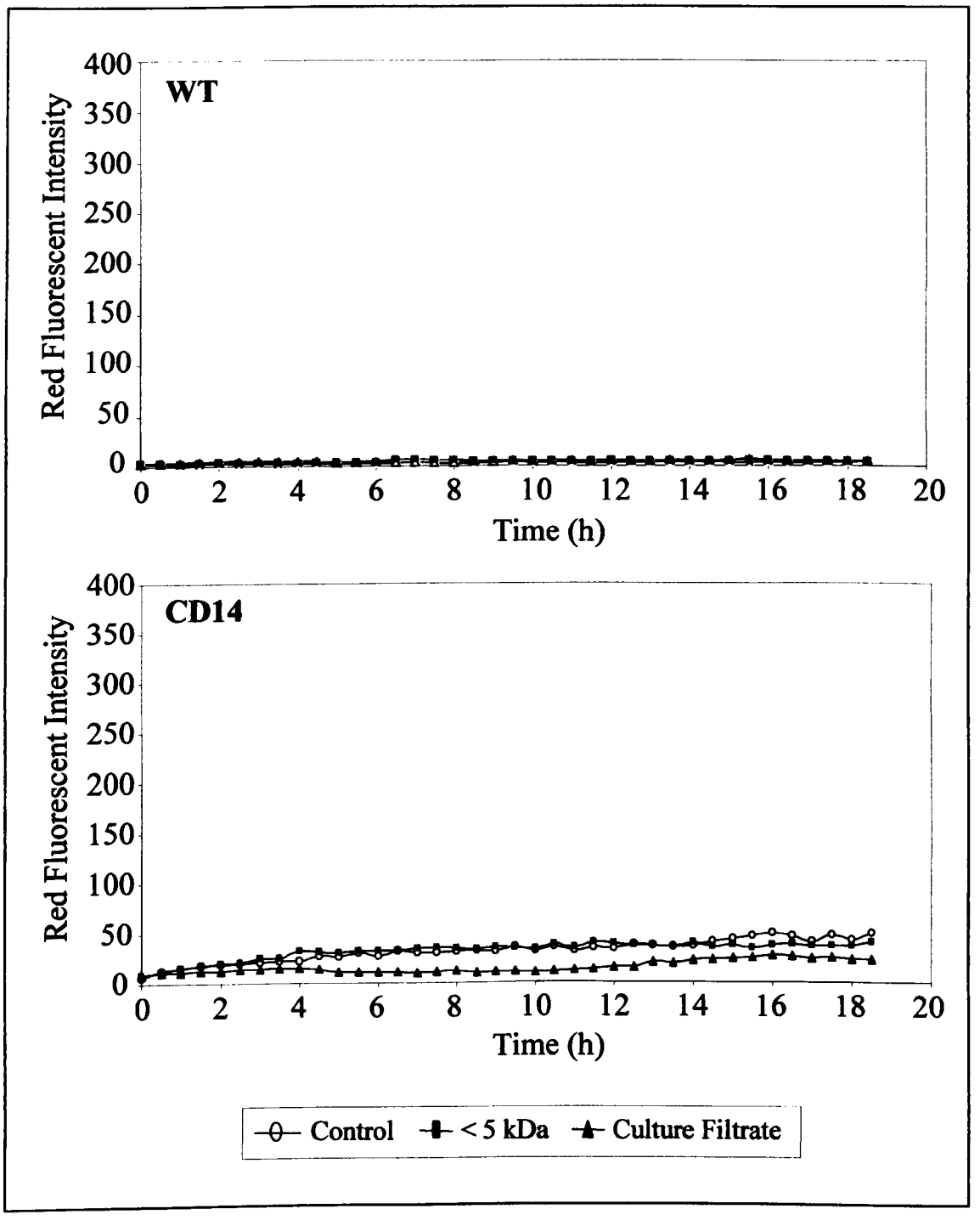

Figure 5.2.1: Red fluorescence intensity (necrosis) from confocal microscopy of wild type HEK cells and HEK cells transfected with CD14, CD14/TLR2 or CD14/TLR4 incubated with *H. influenzae* culture filtrate (200 µl/ml) or < 5 kDa fraction (200 µl/ml). Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) and propidium iodide (1 µg/ml, red fluorescence). Images were taken every 30 min for 18 h at x 20 magnification. Graph is a representative of 3 experiments.

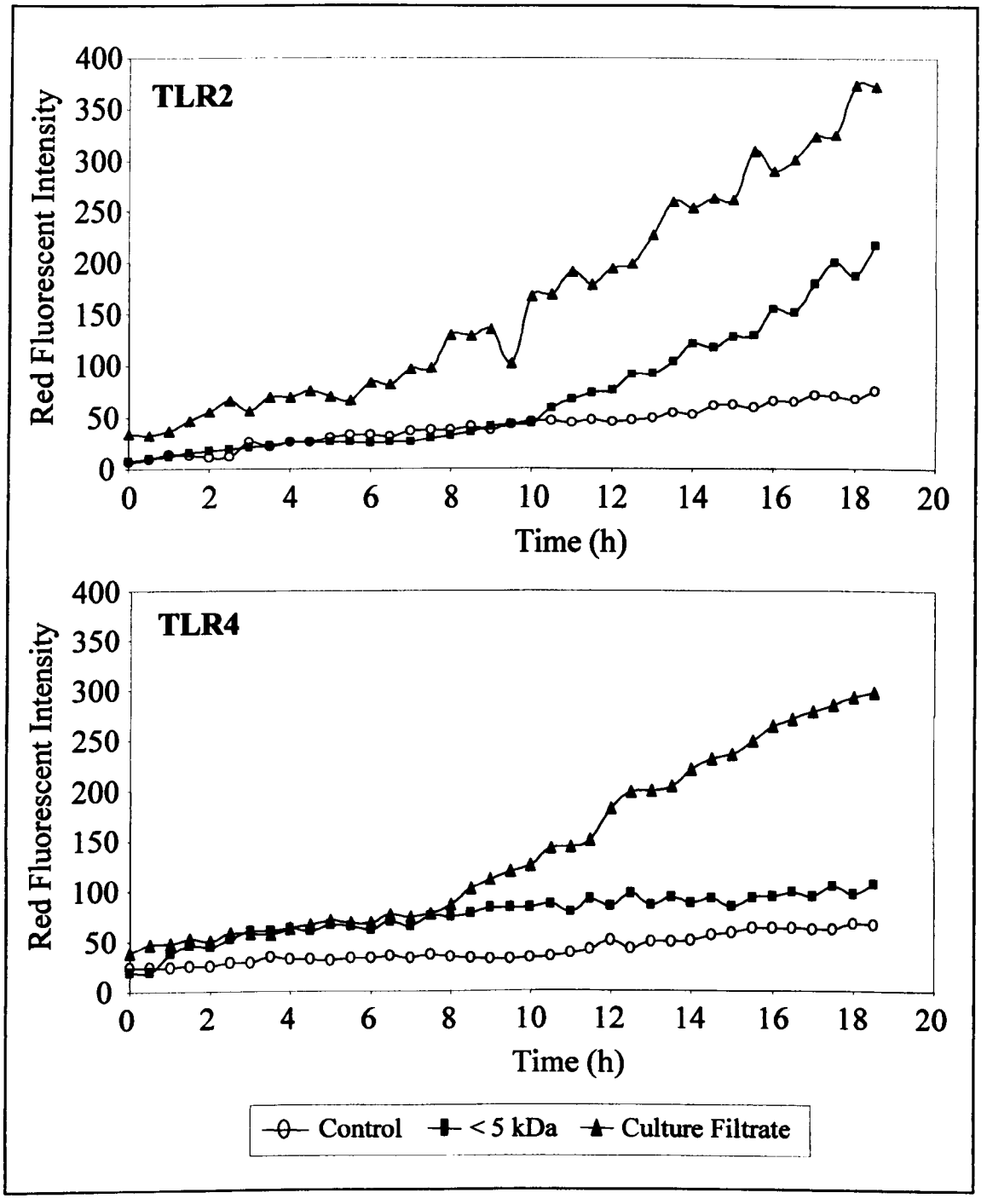

Figure 5.2.1: Red fluorescence intensity (necrosis) from confocal microscopy of wild type HEK cells and HEK cells transfected with CD14, CD14/TLR2 or CD14/TLR4 incubated with *H. influenzae* culture filtrate (200 µl/ml) or < 5 kDa fraction (200 µl/ml). Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) and propidium iodide (1 µg/ml, red fluorescence). Images were taken every 30 min for 18 h at x 20 magnification. Graph is a representative of 3 experiments.

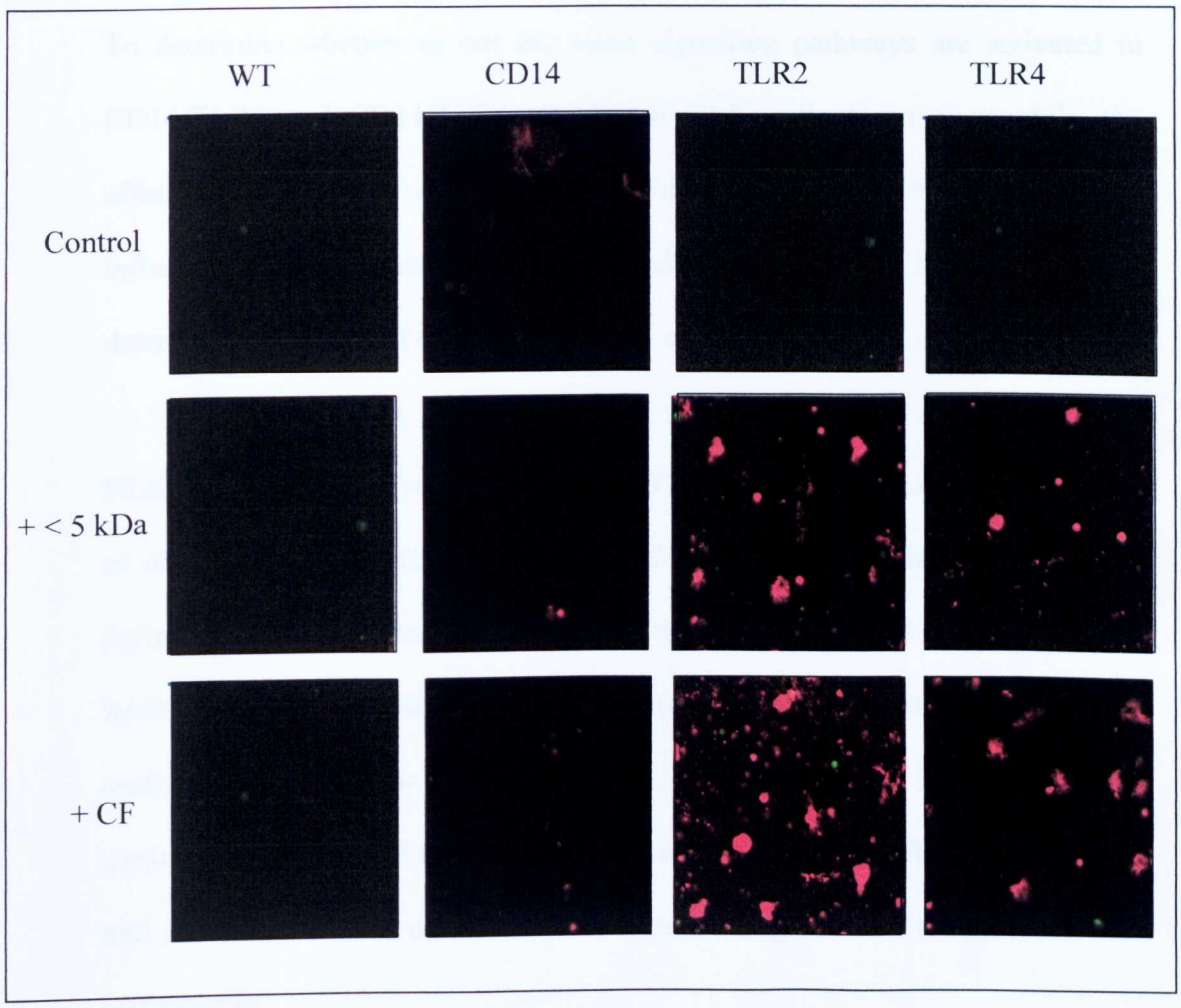

Figure 5.2.2: 18 h images from confocal microscopy of HEK cells transfected with CD14, CD14/TLR2 or CD14/TLR4 treated with *H. influenzae* culture filtrate, CF, (200 µl/ml) or the < 5 kDa fraction (200 µl/ml). Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) and propidium iodide (1 µg/ml, red fluorescence). Images were taken every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

5.3 *Effects of Cell Signalling Inhibitors on the Viability of HEK Cells Transfected with CD14, CD14/TLR2 and CD14/TLR4*

To determine whether or not the same signalling pathways are activated in CD14/TLR2 and CD14/TLR4 transfected HEK cells as in neutrophils, the effects of a range of cell signalling inhibitors on necrosis induced by *H. influenzae* culture filtrate were investigated. It was however, first necessary to determine the effects of these inhibitors on untreated cells.

HEK cells transfected with CD14, CD14/TLR2 and CD14/TLR4 were cultured as described in *Materials and Methods 2.3.* Cells were plated and MD-2 (5 μg/ml) was added to the cells. Cells were treated with a panel of cell signalling inhibitors and the inhibitor solvent DMSO (concentrations as stated in *Materials and Methods 2.2.2*) for 20 min at 37 °C. After incubation, Annexin-V-FITC conjugate (1 μl/ml) and PI (1 μg/ml) were added so that the effects of apoptosis and necrosis could be detected by an increase in green and red fluorescence, respectively.

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Figures 5.3.1, 5.3.3 and 5.3.5 show the 40 min images of HEK cells transfected with CD14, CD14/TLR2 and CD14/TLR4. At 40 min LY294002 resulted in a large increase in the red fluorescence of CD14 HEK and CD14/TLR4 HEK cells, and increased the green and red fluorescence of CD14/TLR2 HEK cells. Bis-1 also induced a large increase in the red fluorescence of all three cell lines.

These increases in red fluorescence after 8 h are also shown in figures 5.3.2, 5.3.4 and 5.3.6. This indicates that PI3-K and PKC play a major part in controlling endogenous cell survival in HEK cells as the inhibition of these two kinases results in cell death. The SYK inhibitor resulted in a large increase in the green fluorescence intensity of the three cells lines, indicating an increase in apoptosis. Caspase inhibitor VI and H89 have also been shown to slightly increase the necrosis of the cells after 18 h. Any necrosis occurring in cells treated with LY294002 and Bis-1 will be a result of inhibition of the signalling pathway involved in controlling endogenous cell survival and therefore not necessarily due to *H. influenzae* culture filtrate. In the light of the effects of inhibitors alone care must be taken in interpreting the results of effects of bacterial filtrate and inhibitors. Figure 5.3.7 shows the effects of the signalling inhibitors on apoptosis. The majority of the inhibitors were not shown to have an effect on apoptosis in any of the 3 cell lines. However, LY294002 and caspase inhibitor VI were shown to increase apoptosis in CD14 and CD14/TLR2 HEK cells, SYK inhibitor was shown to increase apoptosis in all 3 cell lines and H89 increased apoptosis in CD14/TLR2 HEK cells.

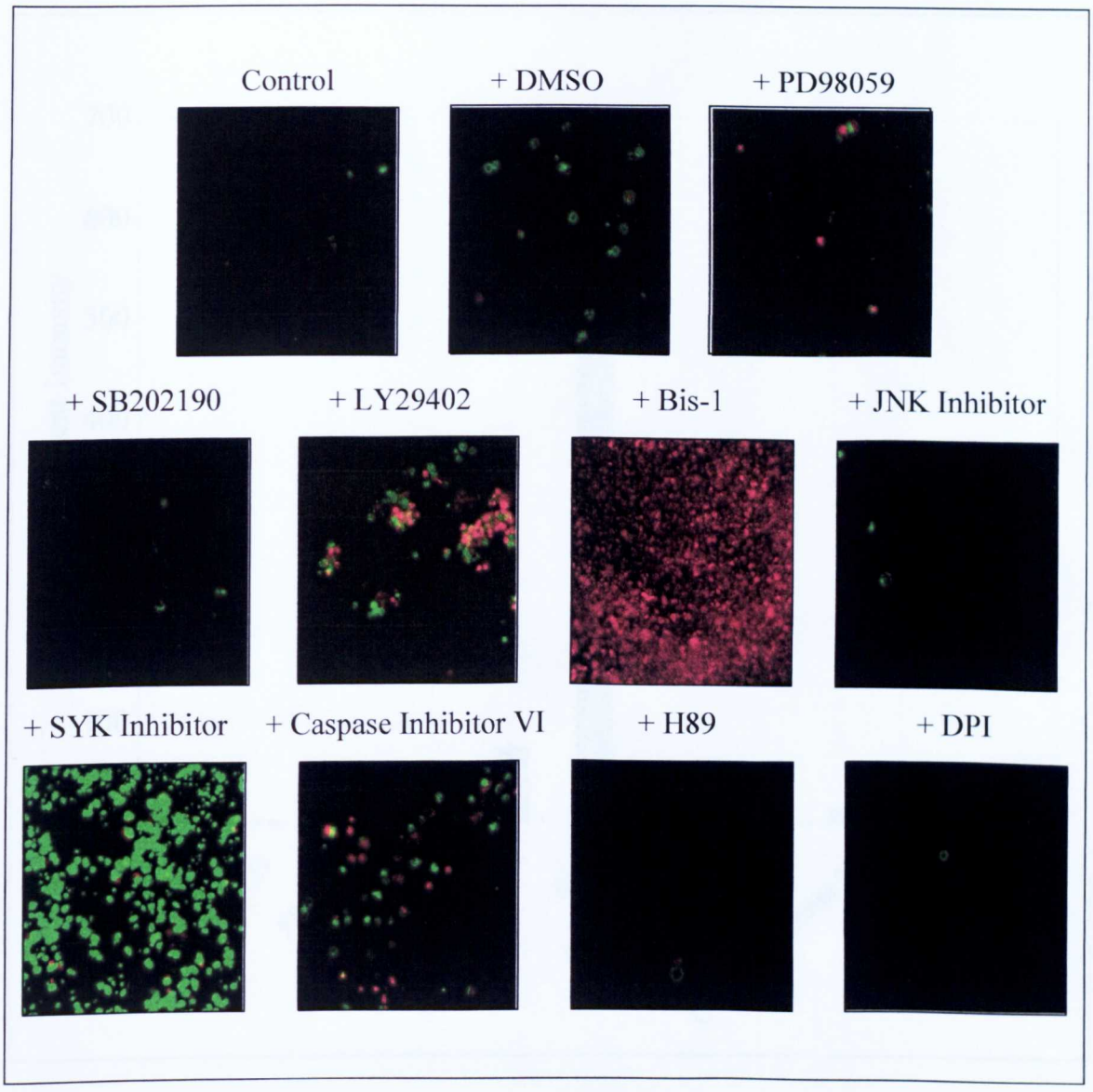


Figure 5.3.1: 1 h images from confocal microscopy of HEK cells transfected with CD14 incubated with cell signaling inhibitors. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

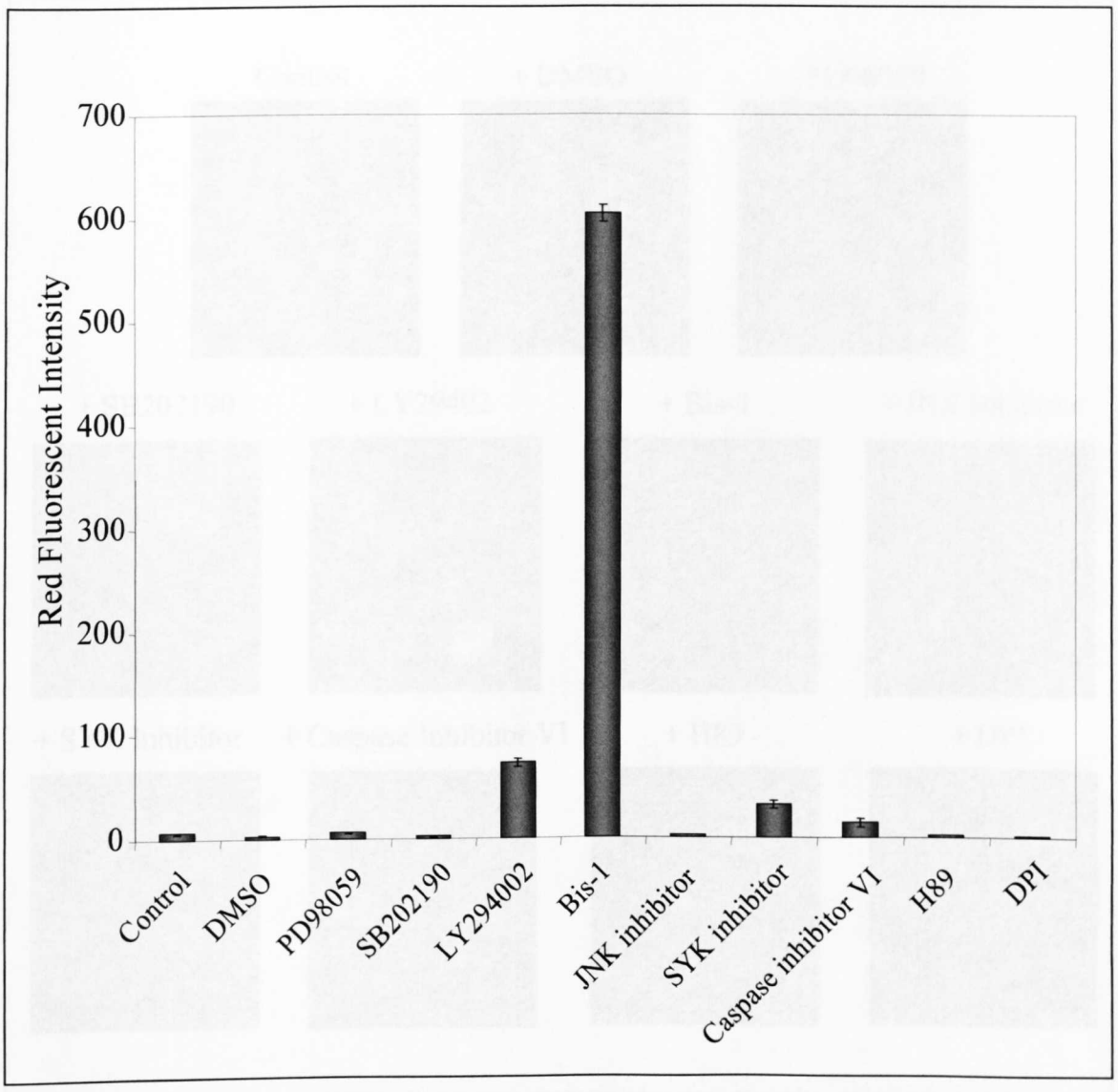


Figure 5.3.2: 8 h red fluorescence intensity (necrosis) from confocal microscopy of HEK cells transfected with CD14 incubated with cell signaling inhibitors. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Data is a mean of 3 experiments ± SD.

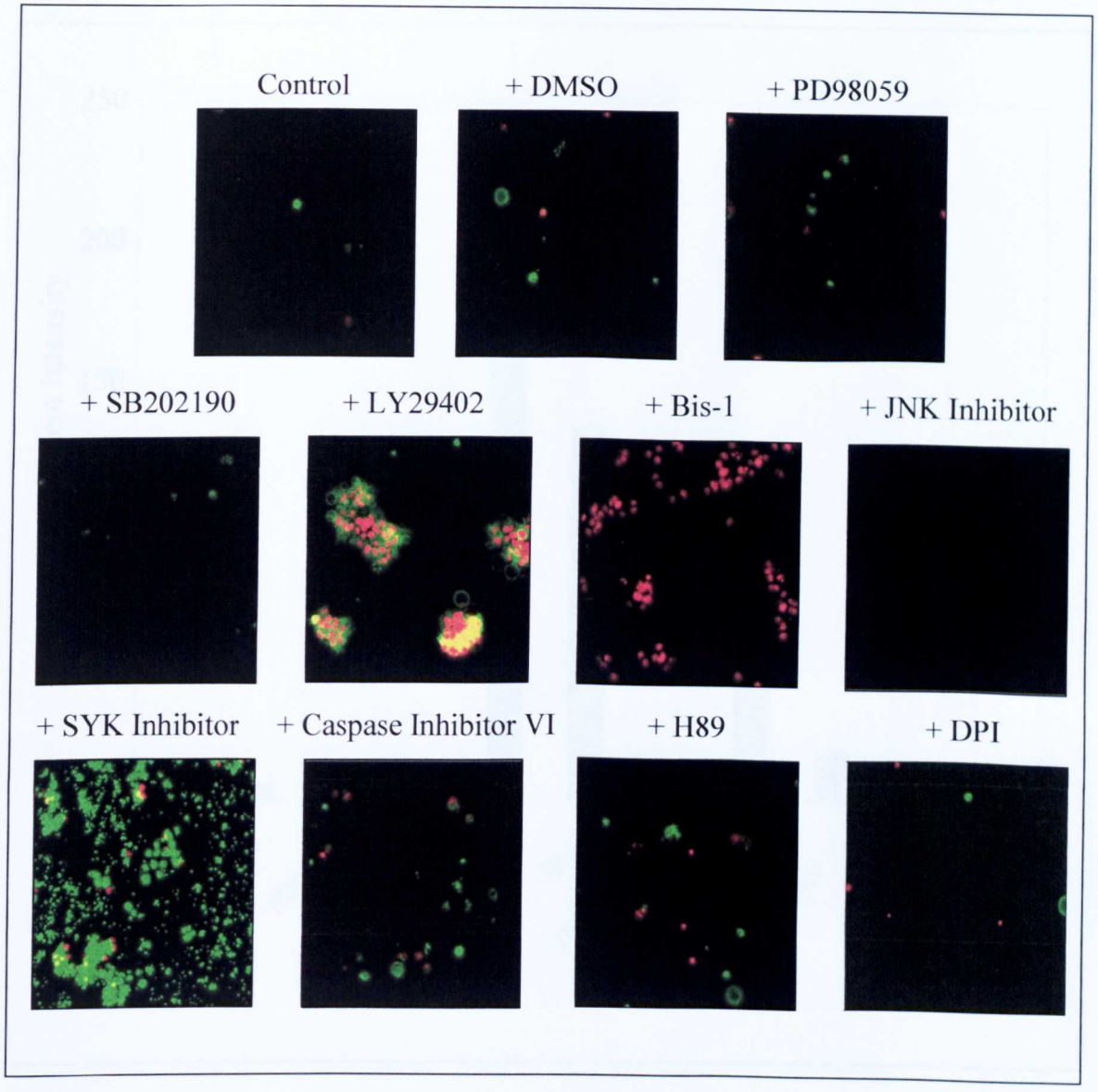


Figure 5.3.3: 1 h images from confocal microscopy of HEK cells transfected with CD14/TLR2 incubated with cell signaling inhibitors. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

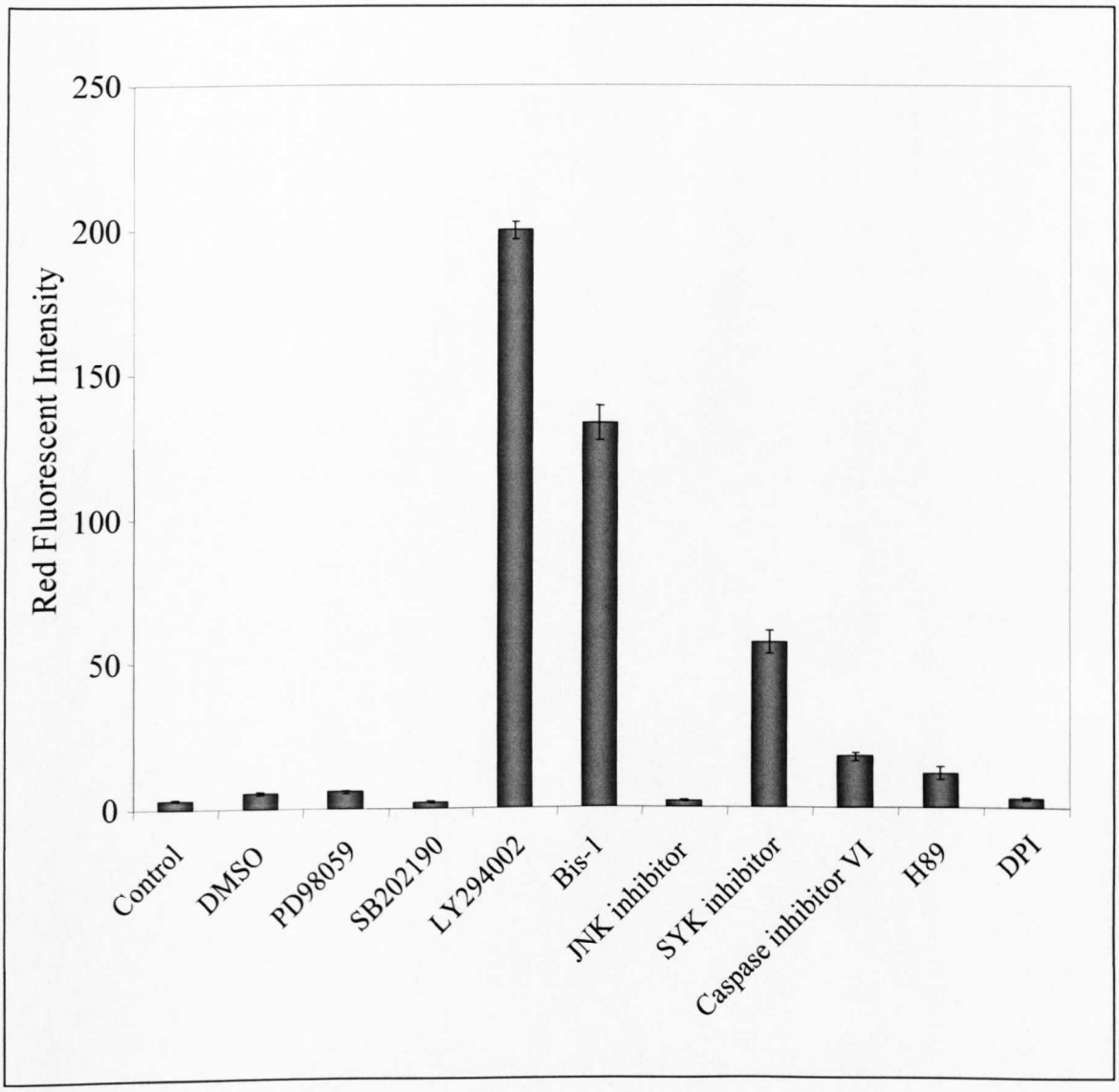


Figure 5.3.4: 8 h red fluorescence intensity (necrosis) from confocal microscopy of HEK cells transfected with CD14/TLR2 incubated with cell signaling inhibitors. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Data is a mean of 3 experiments ± SD.

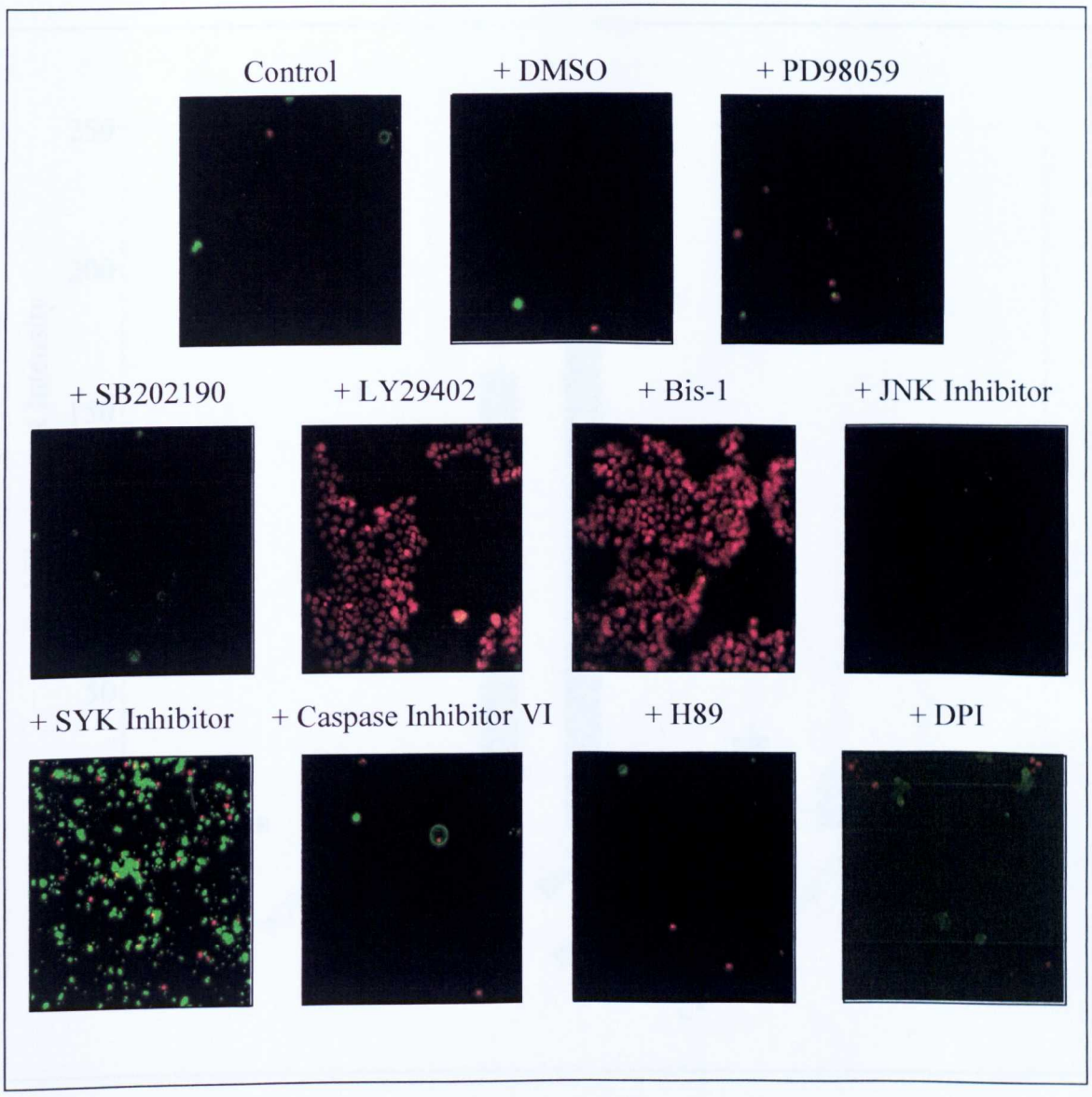


Figure 5.3.5: 1 h images from confocal microscopy of HEK cells transfected with CD14/TLR4 incubated with cell signaling inhibitors. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

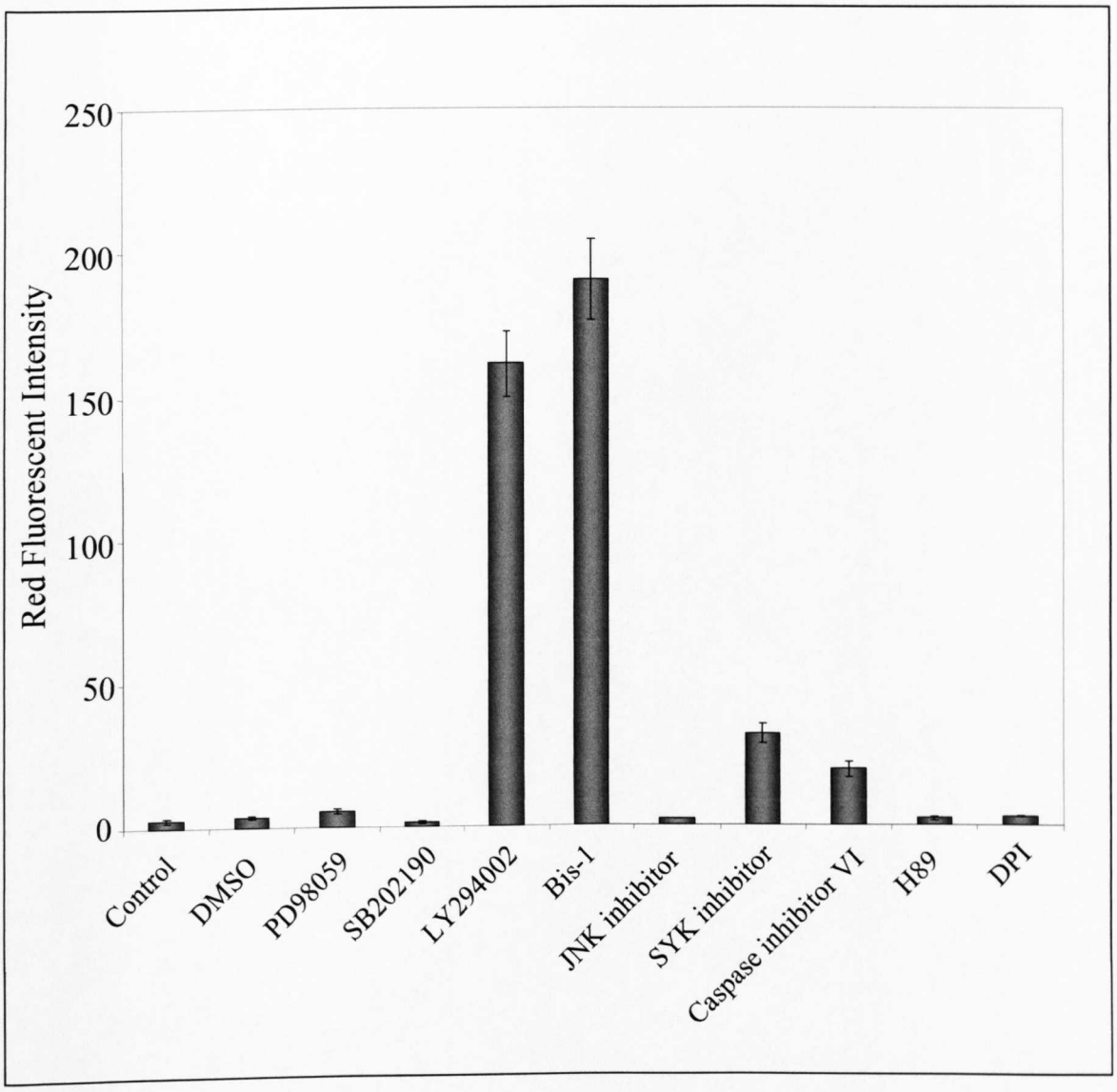


Figure 5.3.6: 8 h red fluorescence intensity (necrosis) from confocal microscopy of HEK cells transfected with TLR4 incubated with cell signaling inhibitors. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Data is a mean of 3 experiments ± SD.

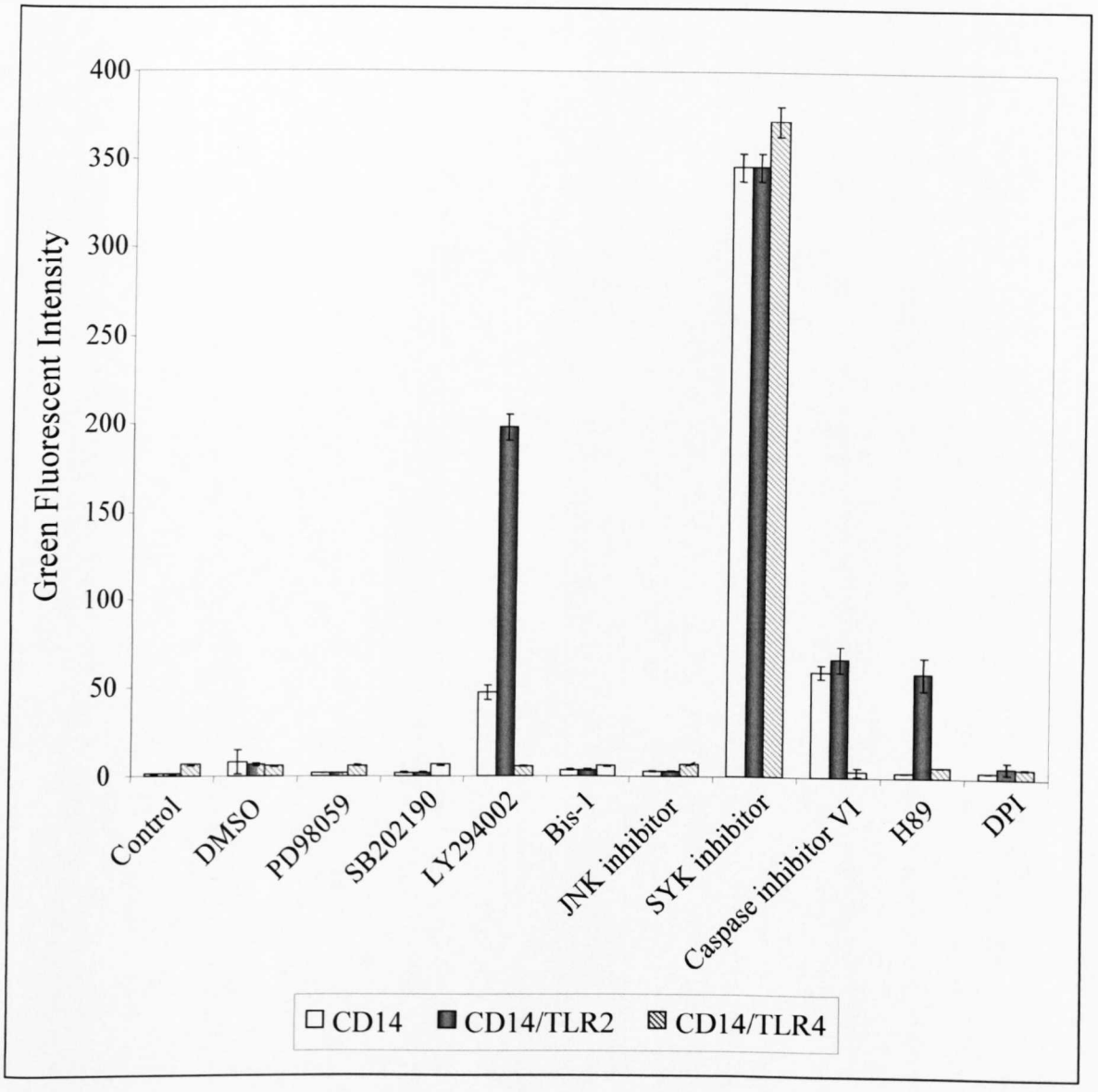


Figure 5.3.7: 8 h green fluorescence intensity (apoptosis) from confocal microscopy of HEK cells transfected with CD14, CD14/TLR2 and CD14/TLR4 incubated with cell signaling inhibitors. Cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Data is a mean of 3 experiments ± SD.

5.4 *Effects of Cell Signalling Inhibitors on the Viability of HEK Cells Transfected with CD14, CD14/TLR2 and CD14/TLR4 after Stimulation with H. influenzae Culture Filtrate*

The effects of the above cell signalling inhibitors on necrosis induced in HEK cells transfected with CD14, CD14/TLR2 and CD14/TLR4 by *H. influenzae* culture filtrate were investigated to determine whether similar signalling pathways activated in neutrophils by *H. influenzae* culture filtrate are also activated in HEK cells.

As bacterial culture filtrate did not induce necrosis in WT or CD14 expressing cells no further experiments were performed on those cells. Figure 5.4.1 shows the increase in the necrosis of CD14/TLR2 HEK cells treated with *H. influenzae* culture filtrate and culture filtrate plus signalling inhibitors. Cells treated with Bis-1 had a greater amount of necrosis compared to control cells and also cells stimulated with *H. influenzae* culture filtrate. This was a result of Bis-1 inducing constitutive necrosis (*figures 5.3.1 and 5.3.2*). Cells treated with LY294002 and then stimulated with *H. influenzae* culture filtrate had an increased amount of necrosis after 40 min compared to cells treated only with *H. influenzae* culture filtrate but this did not increase further over 17 h. JNK inhibitor completely inhibited necrosis induced by *H. influenzae* culture filtrate, an effect which also occurs in neutrophils. PD98059, SB202190, caspase inhibitor VI and H89 all showed a reduction in necrosis induced by *H. influenzae* culture filtrate.

However, DPI was not shown to have an effect on reducing necrosis. Figure 5.4.2 shows an increase in apoptosis in cells that had been pretreated with SYK inhibitor compared to all other cells.

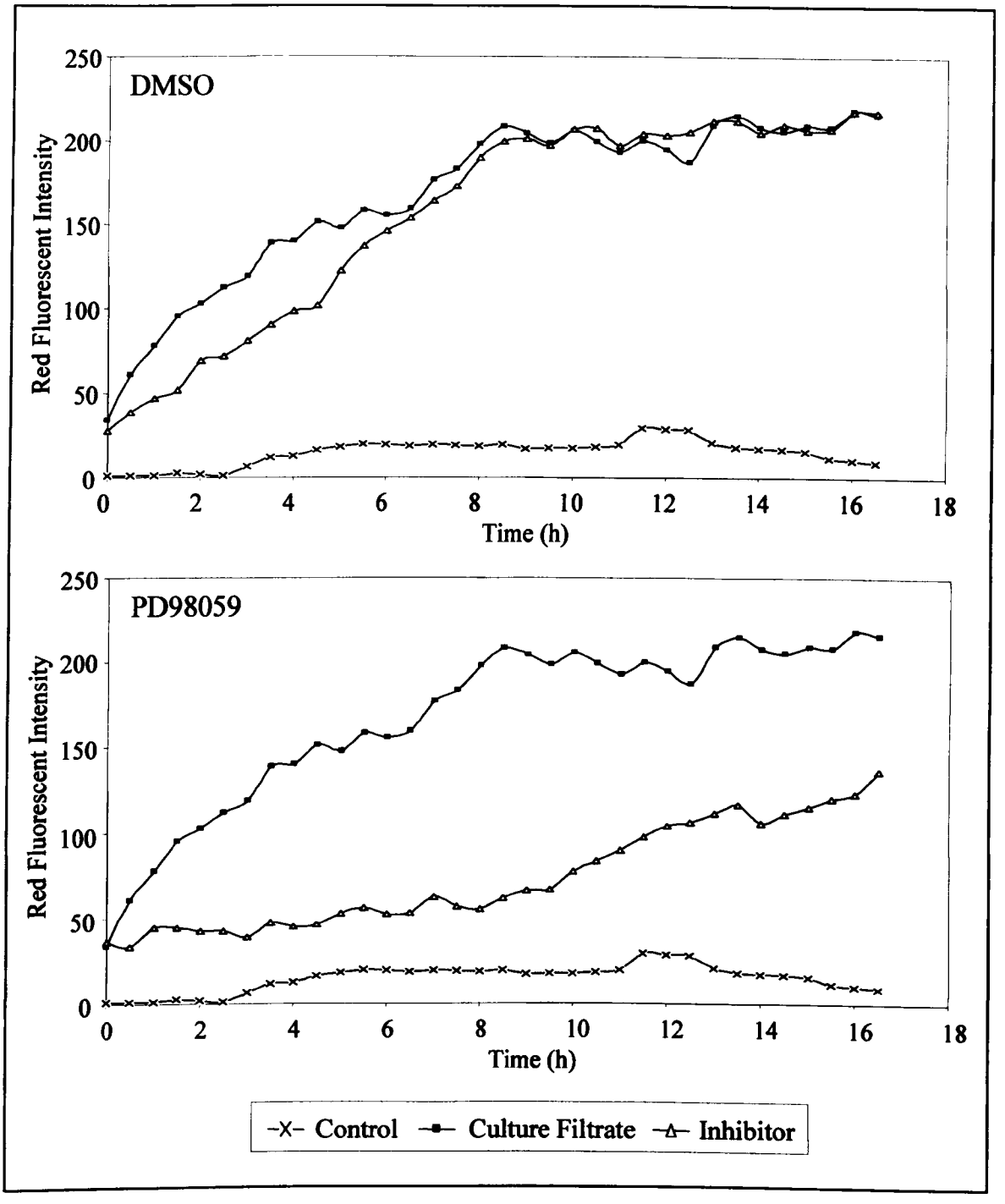


Figure 5.4.1: Red fluorescence intensity from confocal microscopy of HEK cells transfected with CD14/TLR2 incubated with *H. influenzae* culture filtrate (200 µl/ml) and cell signaling inhibitors. HEK cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 17 h at x 20 magnification. Graphs are representative of 3 experiments.

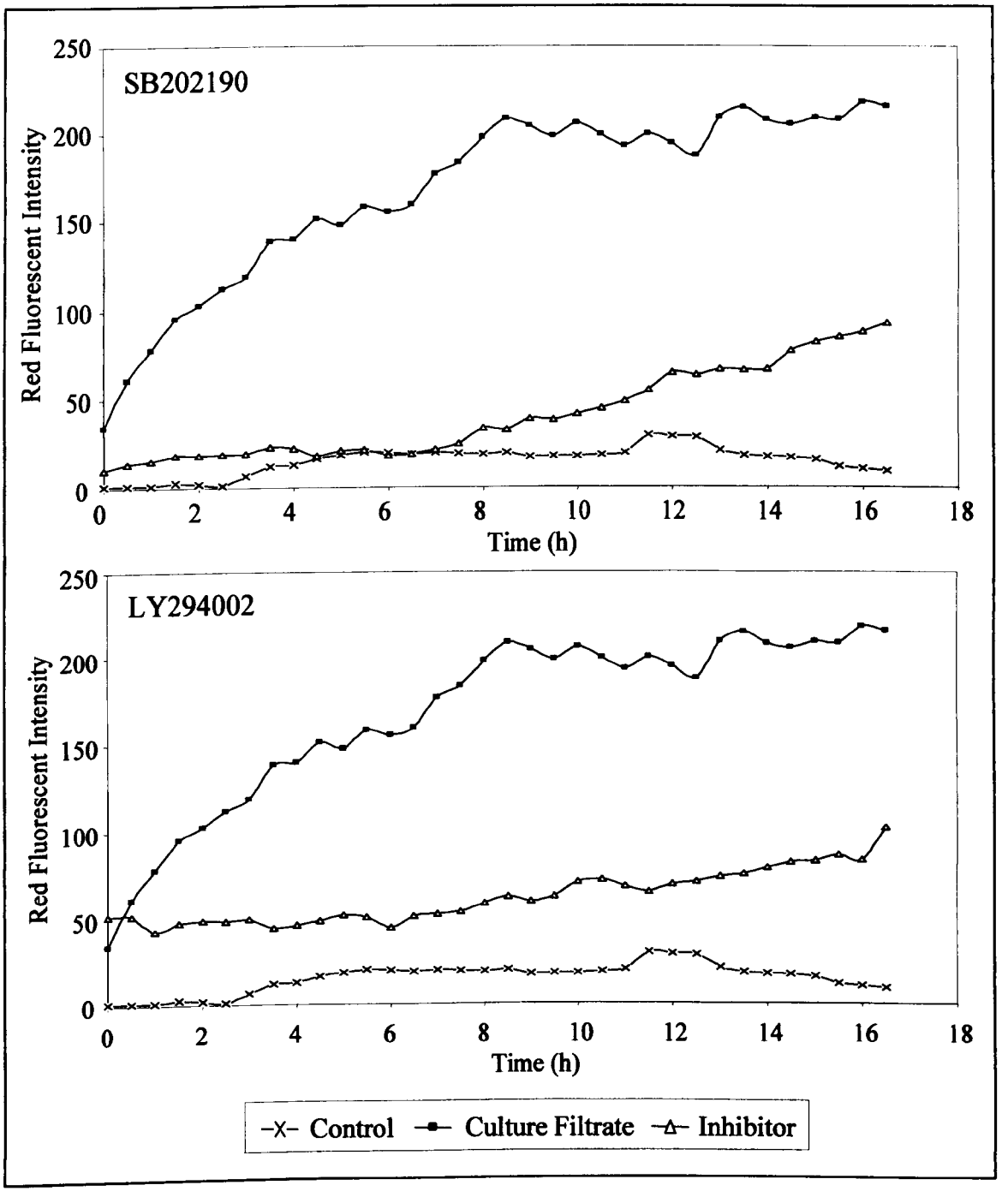
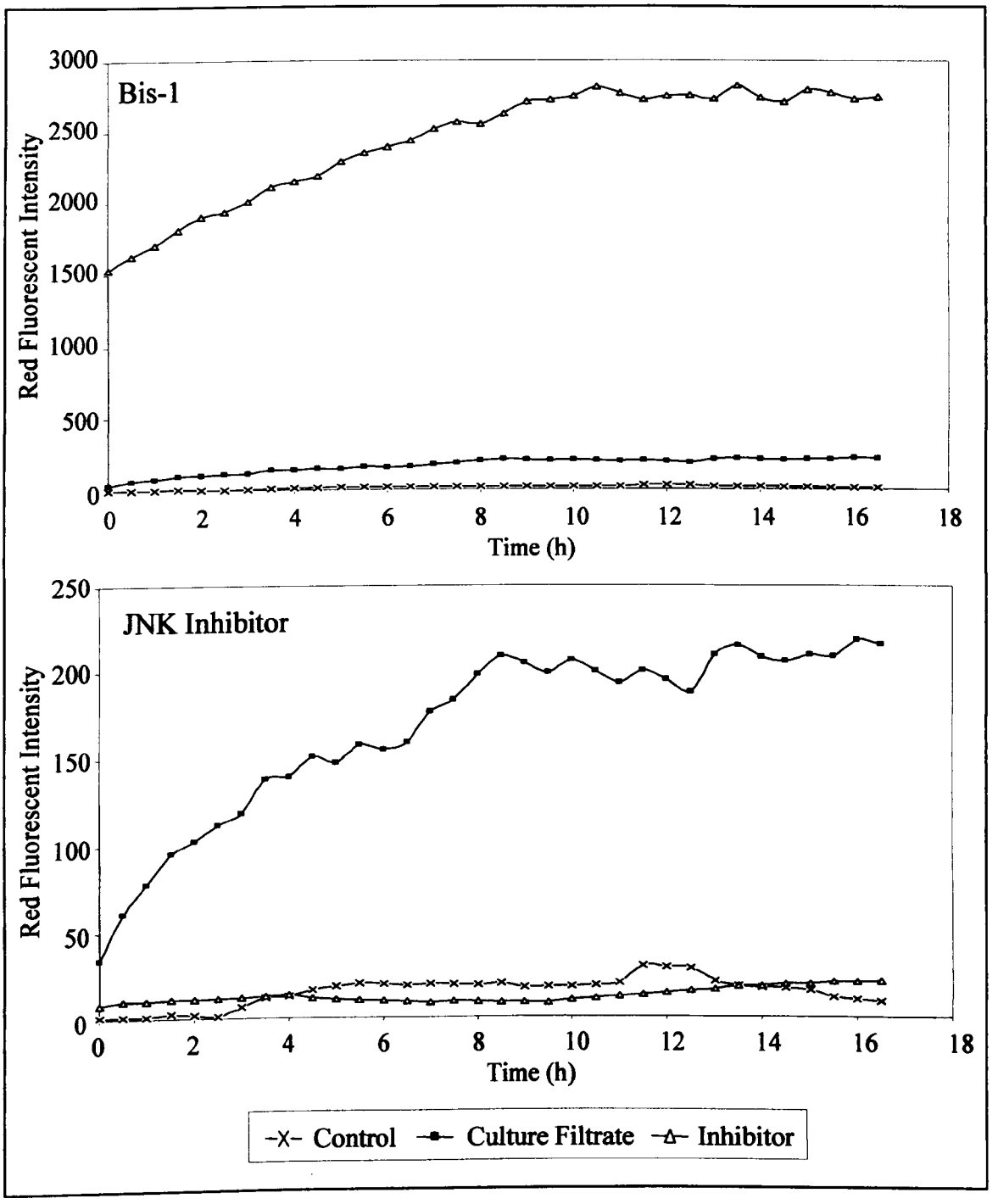


Figure 5.4.1 *(contd.)*



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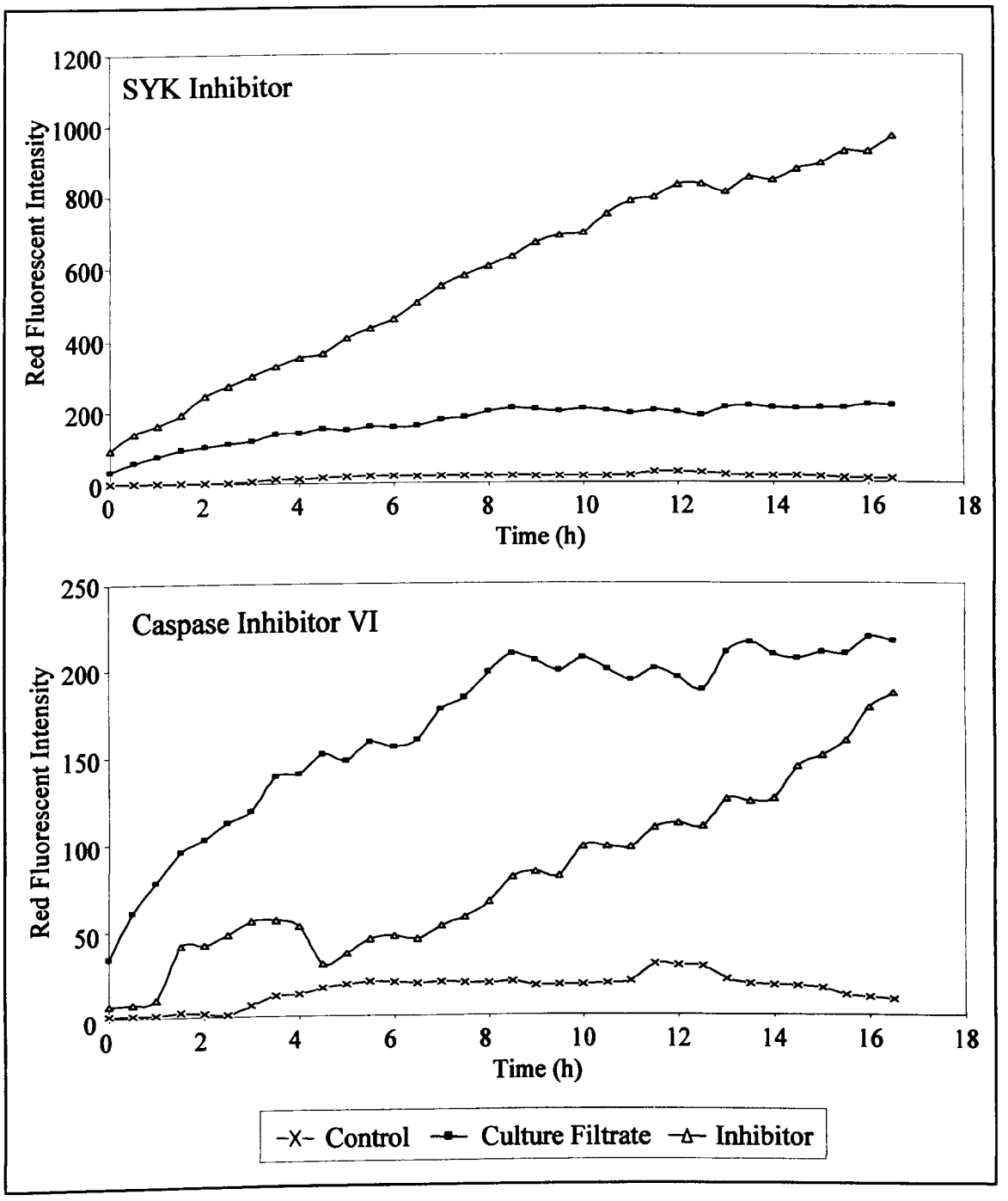

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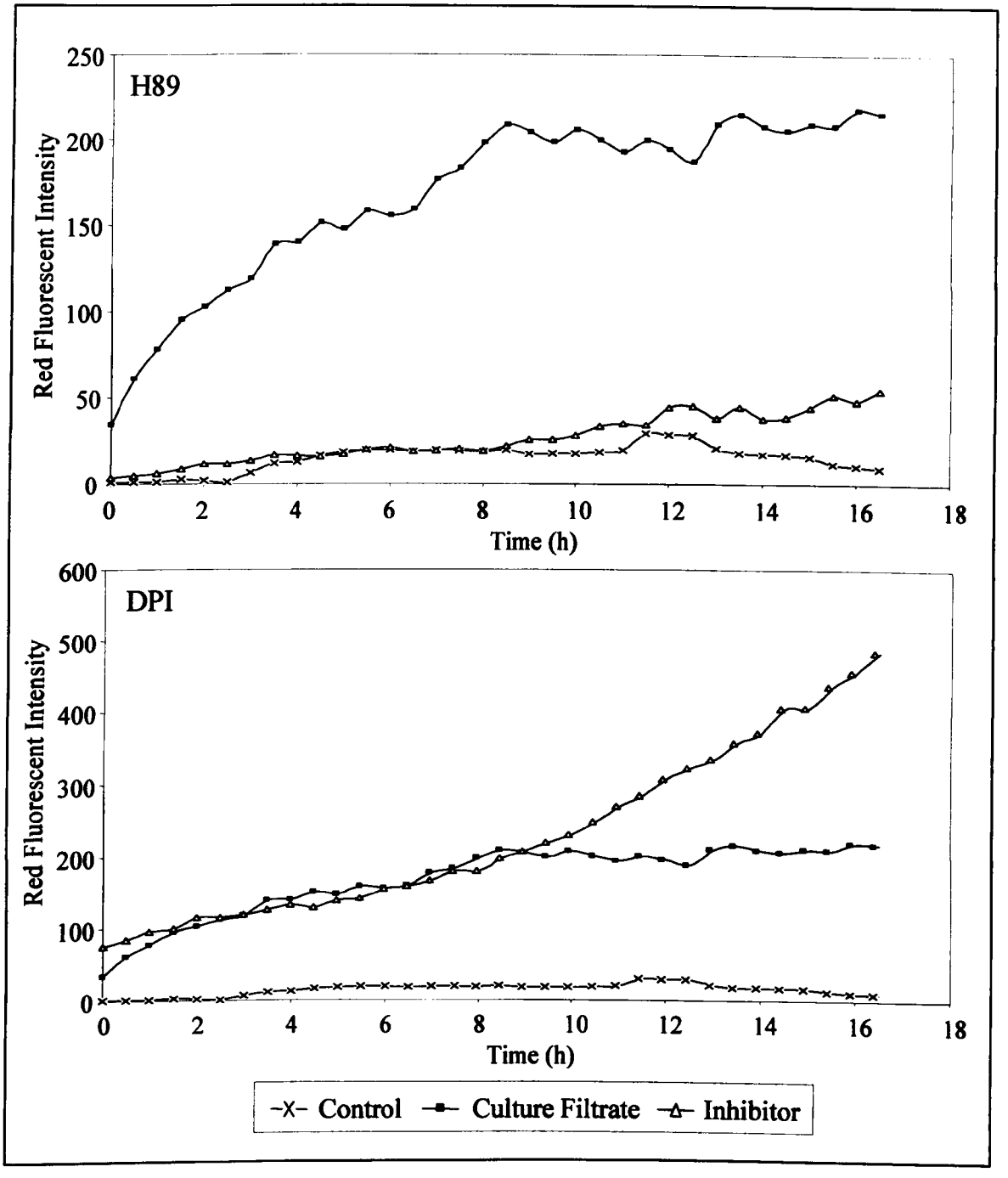


Figure 5.4.1 *(contd.)*

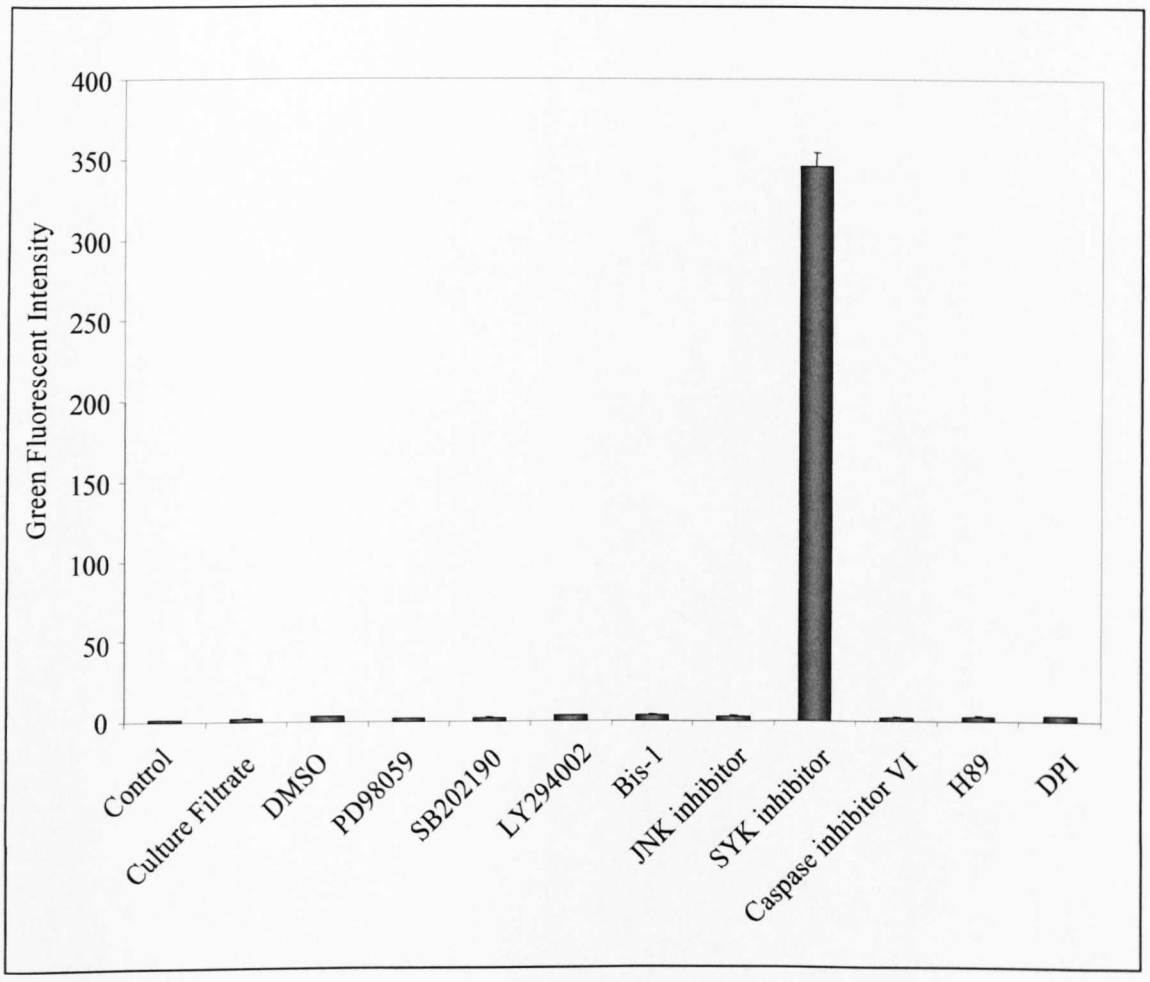


Figure 5.4.2: Green fluorescence intensity from confocal microscopy of HEK cells transfected with CD14/TLR2 incubated with *H. influenzae* culture filtrate (200 µl/ml) and cell signaling inhibitors. HEK cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 17 h at x 20 magnification. Data are a mean of 3 experiments (± SD).

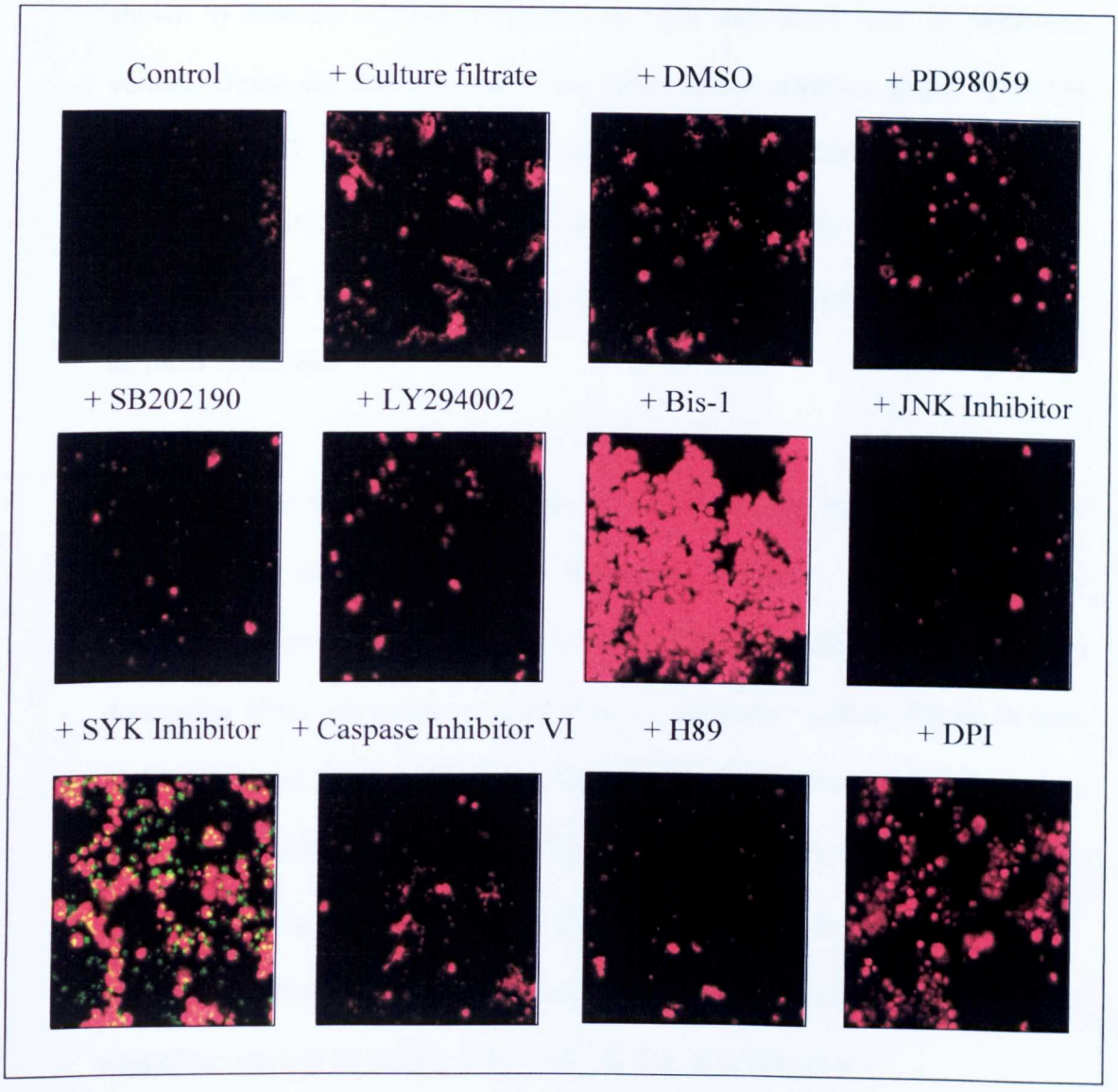


Figure 5.4.3: 17 h images from confocal microscopy of HEK cells transfected with CD14/TLR2 incubated with *H. influenzae* culture filtrate (200 µl/ml) and cell signaling inhibitors. HEK cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

Figure 5.4.4 shows the effects of signalling inhibitors on necrosis induced by *H. influenzae* culture filtrate in TLR4 HEK cells. Again, LY294002 and Bis-1 were shown to increase necrosis compared to cells stimulated with *H. influenzae* culture filtrate and this was due to the effect of the inhibitor alone. PD98059, SB202190, JNK inhibitor, caspase inhibitor VI, H89 and DPI were all shown to decrease necrosis induced by *H. influenzae* culture filtrate. Figure 5.4.5 shows that again, SYK inhibitor resulted in a large increase in apoptosis compared to all other inhibitors.

It is difficult to assess the importance of LY294002 and Bis-1 on inhibition of *H. influenzae* induced necrosis as these two inhibitors resulted in a large increase in necrosis. However, all the other inhibitors used had an effect on decreasing HEK cell necrosis induced by *H. influenzae* culture filtrate in both TLR2 HEK and TLR4 HEK cells. JNK inhibitor completely inhibited necrosis in the TLR2 HEK cells, further confirming the importance of the JNK pathway in the signalling of necrosis. From these results it can be concluded that *H. influenzae* culture filtrate induced necrosis via TLR2 and TLR4 and initiates a signalling cascade inside the cell similar to that in neutrophils.

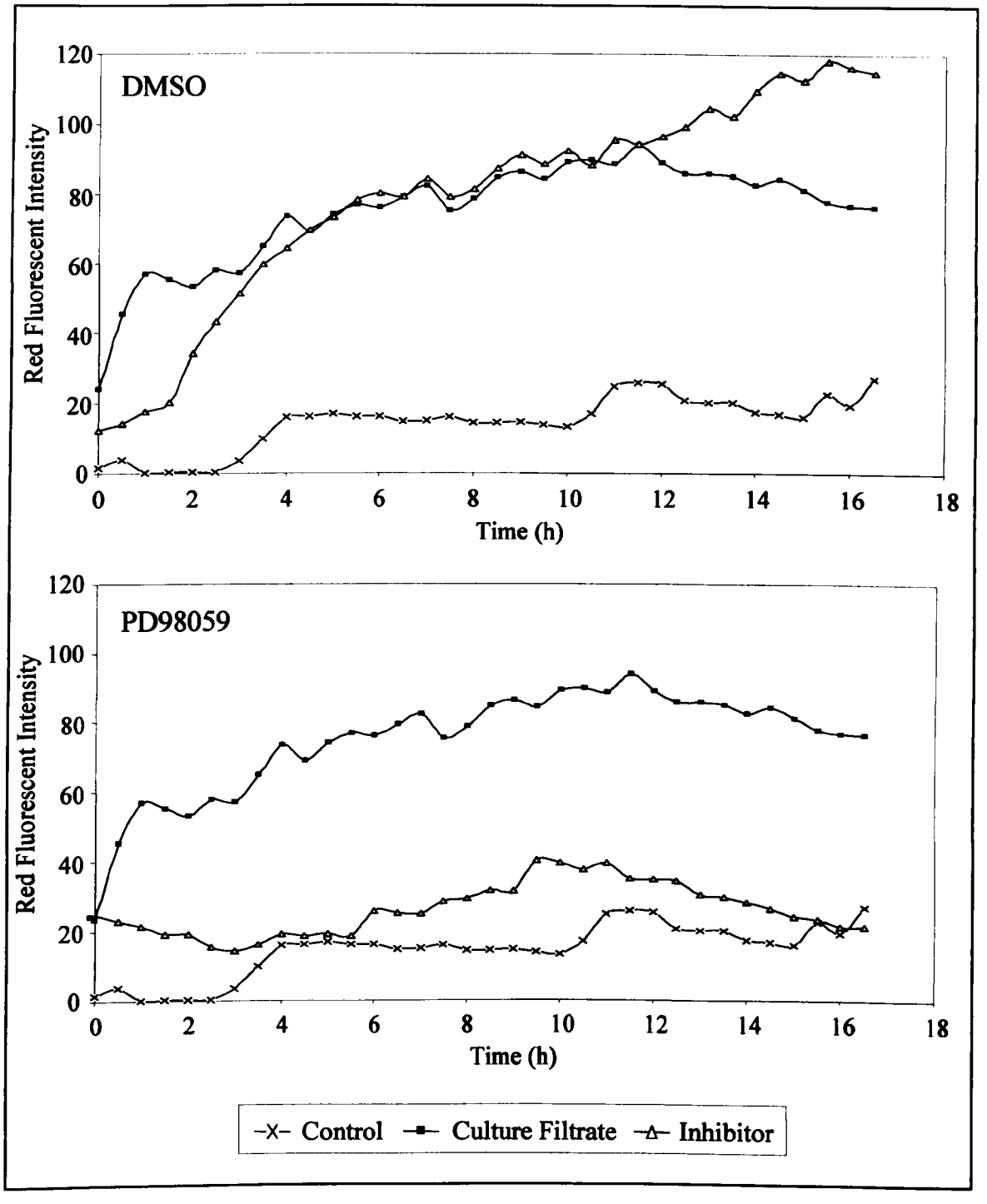


Figure 5.4.4: Red fluorescence intensity from confocal microscopy of HEK cells transfected with CD14/TLR4 incubated with *H. influenzae* culture filtrate (200 µl/ml) and cell signaling inhibitors. HEK cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 17 h at x 20 magnification. Graphs are representative of 3 experiments.

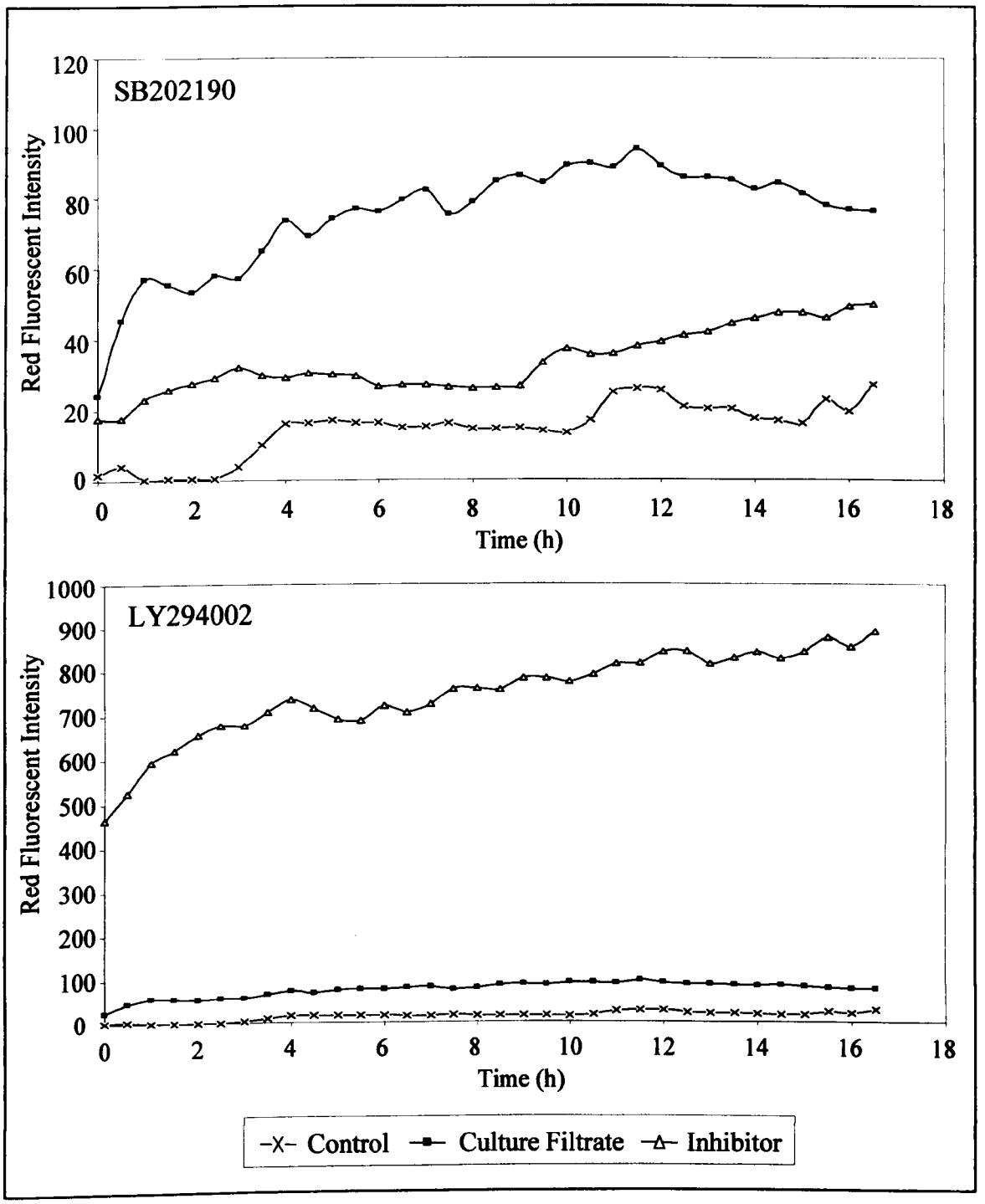


Figure 5.4.4 *(contd.)*

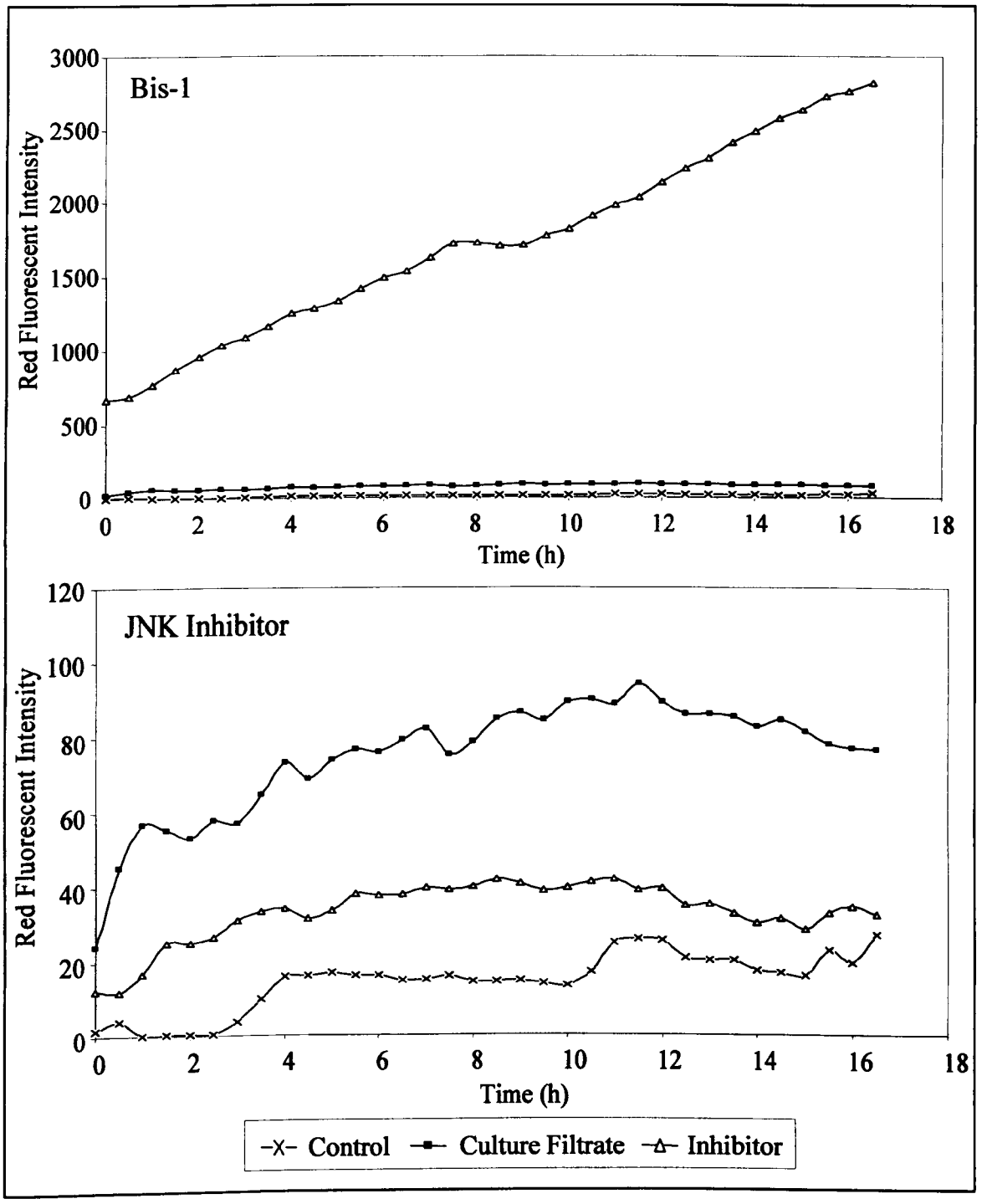
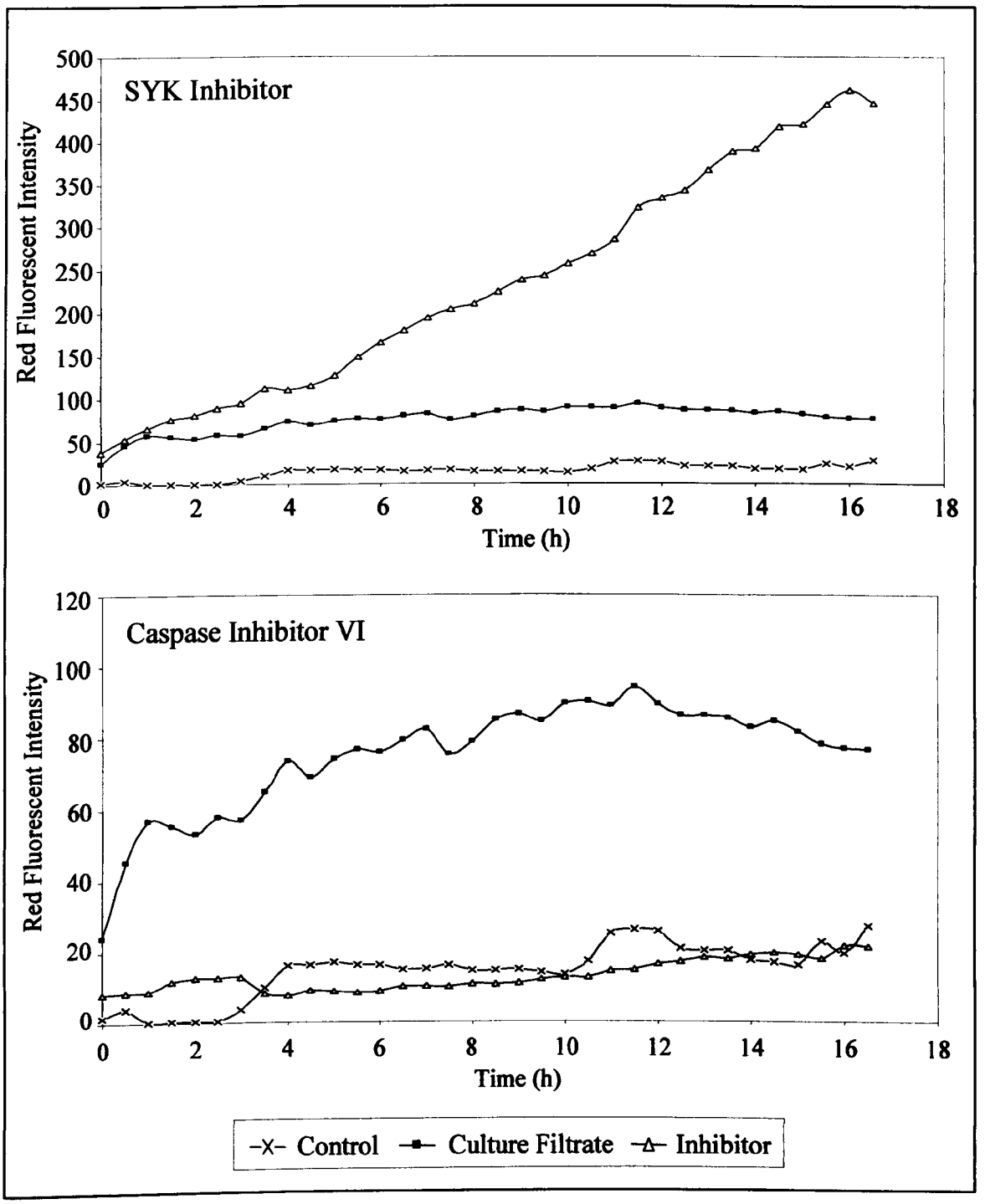


Figure 5.4.4 *(contd.)*



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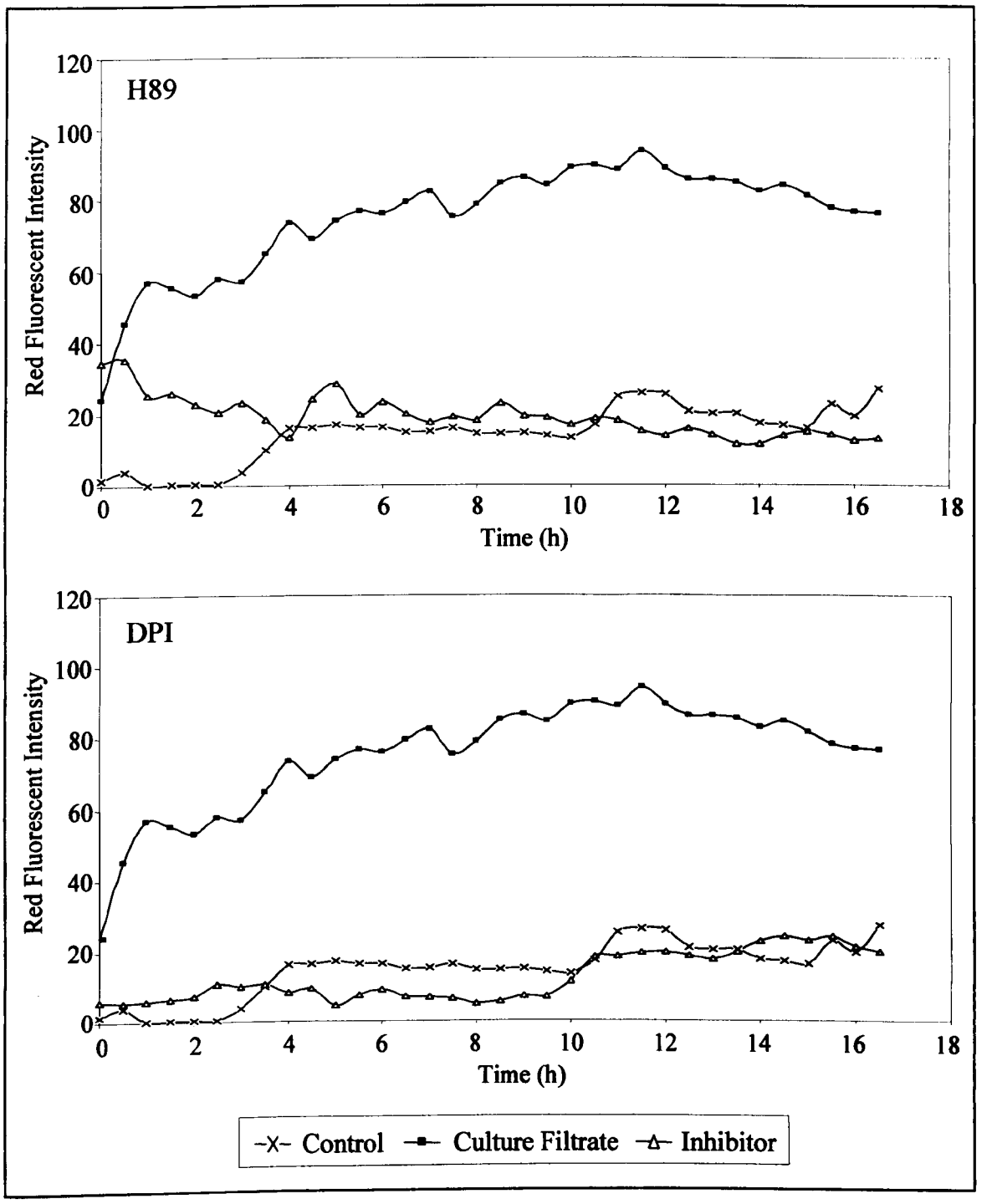

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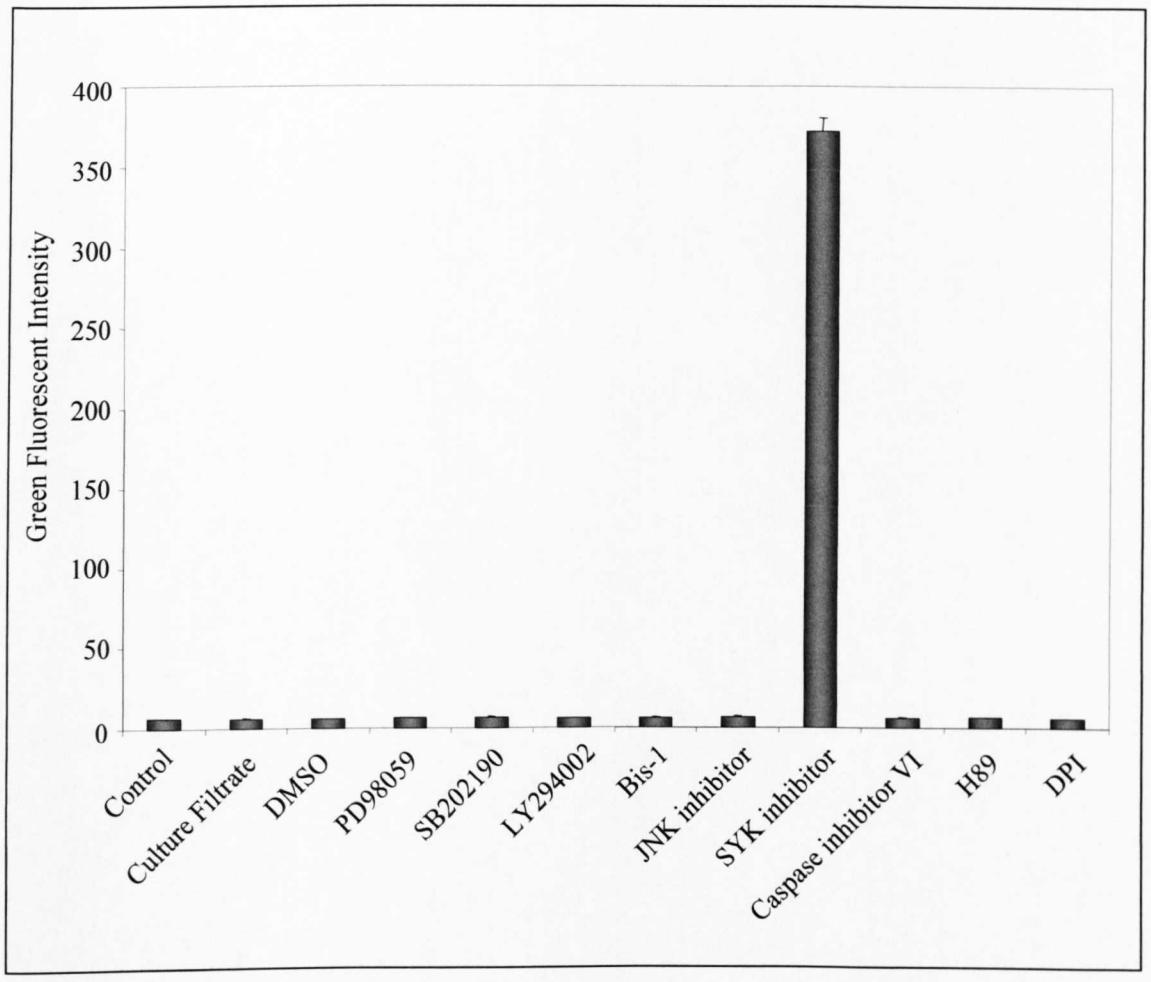


Figure 5.4.5: Green fluorescence intensity from confocal microscopy of HEK cells transfected with CD14/TLR4 incubated with *H. influenzae* culture filtrate (200 µl/ml) and cell signaling inhibitors. HEK cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 17 h at x 20 magnification. Data are a mean of 3 experiments (± SD).

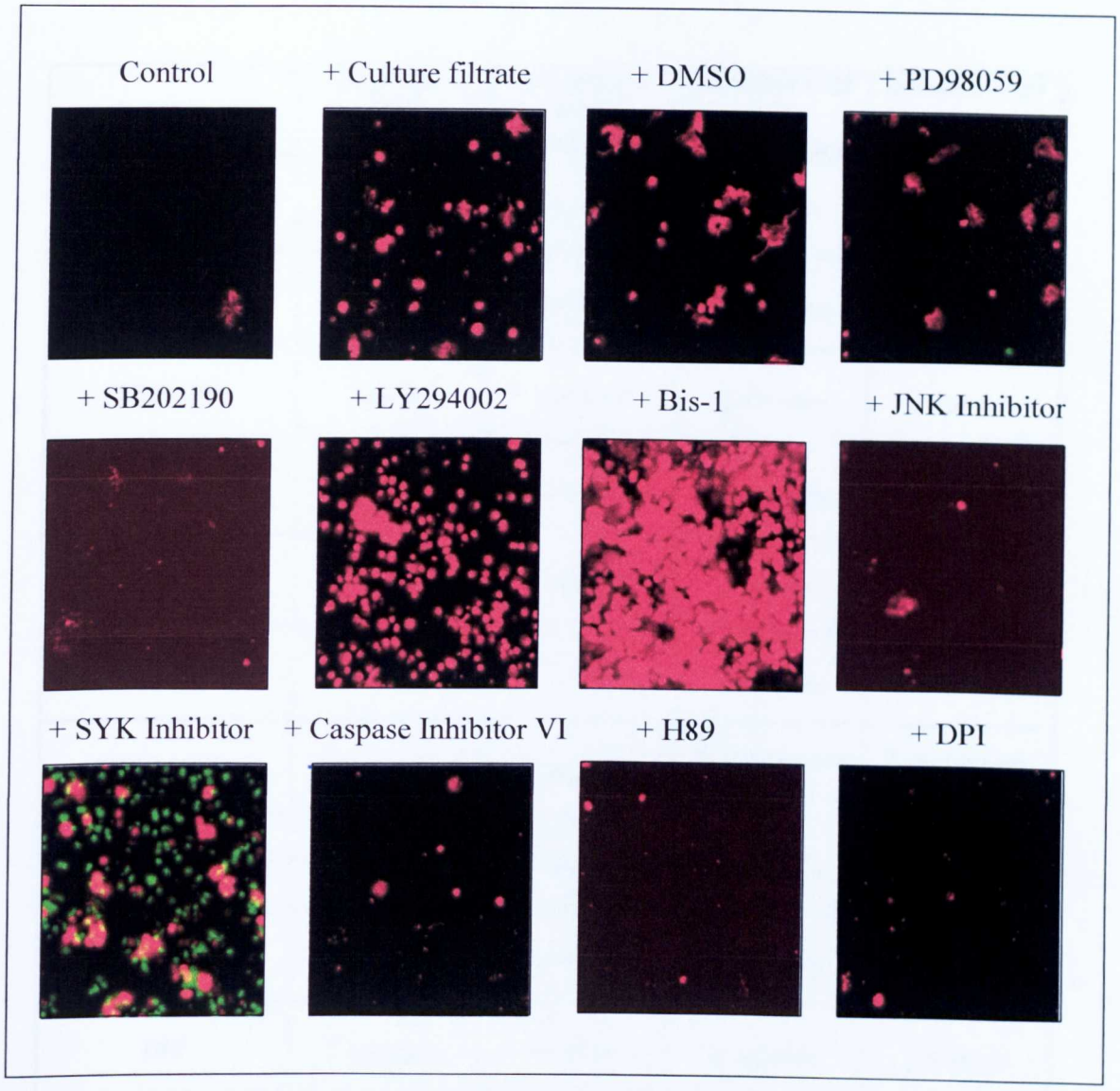


Figure 5.4.6: 17 h images from confocal microscopy of HEK cells transfected with CD14/TLR4 incubated with *H. influenzae* culture filtrate (200 µl/ml) and cell signaling inhibitors. HEK cells were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

Inhibitor	Inhibitor + HEK-CD14/TLR2	Inhibitor + HEK-CD14/TLR4	Inhibitor + CF + HEK-CD14/TLR2	Inhibitor + CF HEK-CD14/TLR4
DMSO	no effect	no effect	no effect	no effect
PD98059	no effect	no effect	↓ necrosis	↓ necrosis
SB202190	no effect	no effect	↓ necrosis	↓ necrosis
LY294002	↑apoptosis and necrosis	↑ necrosis	↓ necrosis	↑ necrosis
Bis-1	↑ necrosis	↑ necrosis	↑ necrosis	↑ necrosis
JNK inhibitor	no effect	no effect	↓ necrosis	↓ necrosis
SYK inhibitor	↑ apoptosis	↑ apoptosis	↑ necrosis and apoptosis	↑ necrosis and apoptosis
Caspase inhibitor VI	↑ apoptosis	↑ necrosis	↓ necrosis	↓ necrosis
H89	↑ apoptosis	no effect	↓ necrosis	↓ necrosis
DPI	↑ apoptosis	no effect	↓ necrosis	↓ necrosis

Table 5.1: Summary of the effects of cell signaling inhibitors on culture filtrate induced necrosis of HEK cells transfected with TLR2 and TLR4.

5.5 *The Effects of TLR Ligands on Neutrophil Viability*

Results obtained in this chapter suggest that *H. influenzae* culture filtrate and the < 5 kDa fraction are able to induce necrosis in myeloid cells via TLR2 and TLR4. The effects of a range of known TLR ligands on neutrophil apoptosis and necrosis were analysed to determine if any ligands, particularly the TLR2 and TLR4 ligands, induced a similar response.

Neutrophils were stimulated with a range of different TLR ligands (*see table 5.2 for ligands and concentrations*). Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) were added so that the effects of the inhibitors on neutrophil apoptosis and necrosis could be analysed by an increase in green and red fluorescence.

Ligand	Compound	TLR	Concentration
Pam_3CSK_4	bacterial lipoprotein	TLR1:TLR2	100 ng/ml
Poly (I:C)	ds RNA	TLR3	100 µg/ml
LPS	lipopolysaccharide	TLR4	1 µg/ml
Flagellin	bacterial flagella	TLR5	100 ng/ml
MALP-2	bacterial lipoprotein	TLR2:TLR6	100 ng/ml
FSL	bacterial lipoprotein	TLR2:TLR6	10 ng/ml
Poly (U)	viral DNA	TLR7/TLR8	10 µg/ml
CpG ODN	bacterial DNA	TLR9	10 µg/ml

Table 5.2 The TLR ligands used in experiments and their targets and concentrations.

Figure 5.5.1 shows increases in neutrophil necrosis in cells stimulated with the TLR ligands. CpG ODN, the TLR9 ligand, caused the greatest increase in neutrophil necrosis and this was concentration dependent (data not shown). LPS, the TLR4 ligand, was also shown to slightly increase necrosis. However, the two ligands that were shown to have similar kinetics as the < 5 kDa fraction were MALP-2 and FSL. These are both TLR2:TLR6 ligands and are synthetic diacylated bacterial lipoproteins. Pam_3CSK_4 is a TLR2:TLR1 ligand and did not cause an increase in necrosis. This suggests that the < 5 kDa factor may be a diacylated lipoprotein working via TLR2:TLR6 heterodimers. None of the other TLR ligands had a significant effect on neutrophil necrosis.

Figure 5.5.2 shows changes in neutrophil apoptosis in cells stimulated with the TLR ligands. Only CpG ODN caused an increase in apoptosis compared to control cells. Pam_3CSK_4, Flagellin and Poly(U) all decreased apoptosis compared to control cells but none of the ligands completely inhibited apoptosis in a similar way to *H. influenzae* culture filtrate.

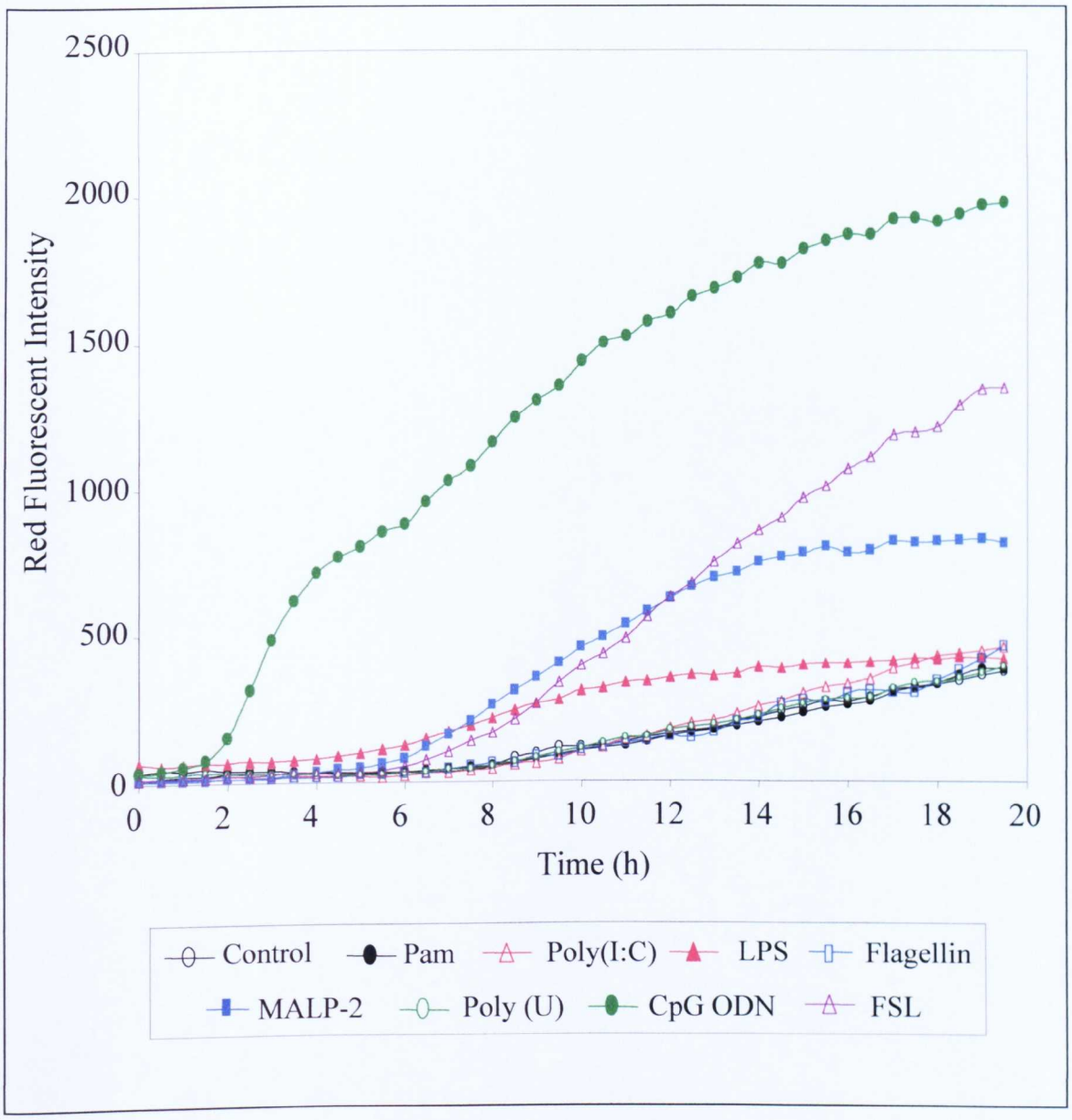


Figure 5.5.1: Red fluorescence intensity (necrosis) from confocal microscopy of neutrophils incubated with a range of TLR ligands. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Graphs are representative of 3 experiments.

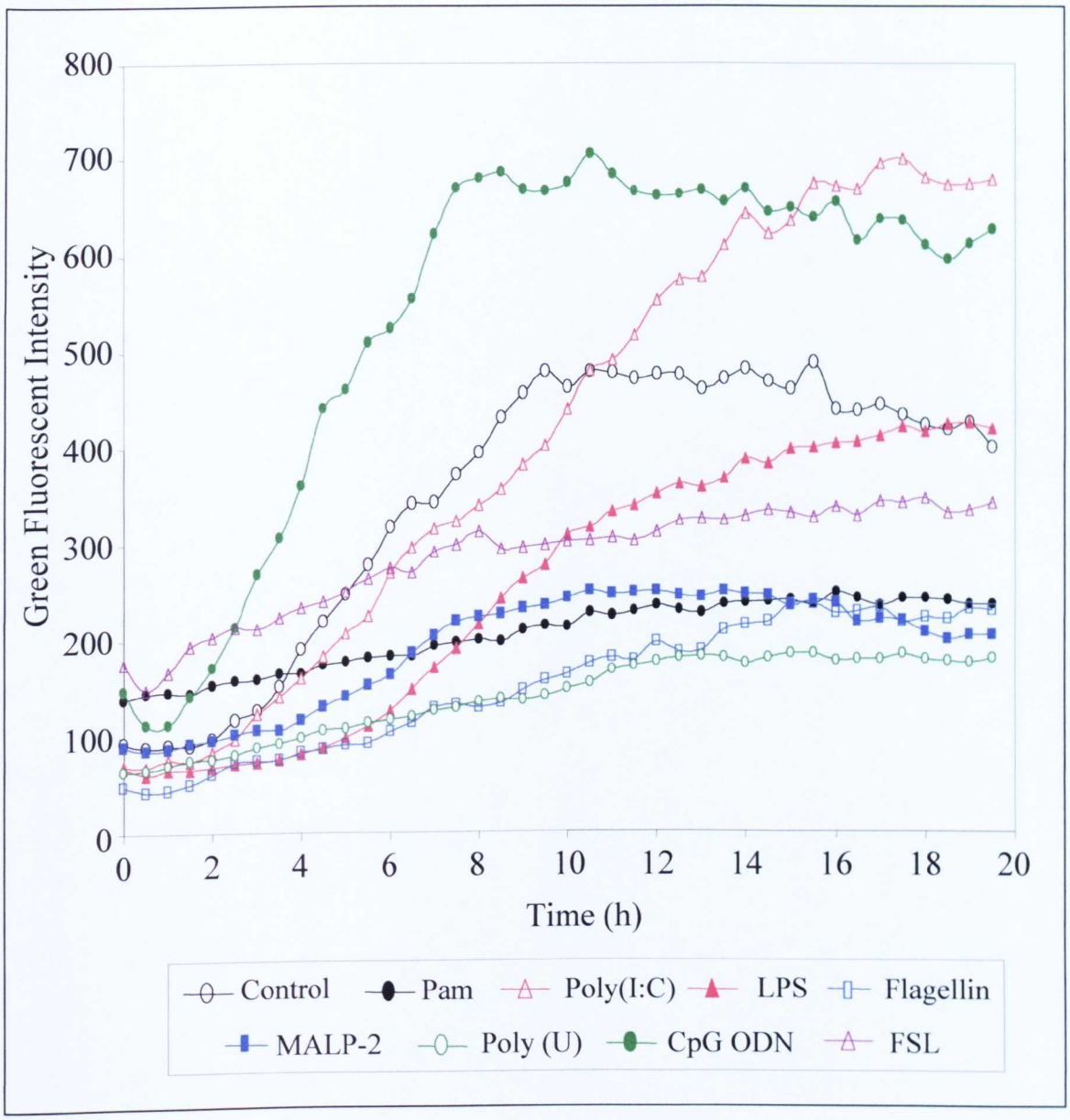


Figure 5.5.2: Green fluorescence intensity (apoptosis) from confocal microscopy of neutrophils incubated with a range of TLR ligands. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Graphs are representative of 3 experiments.

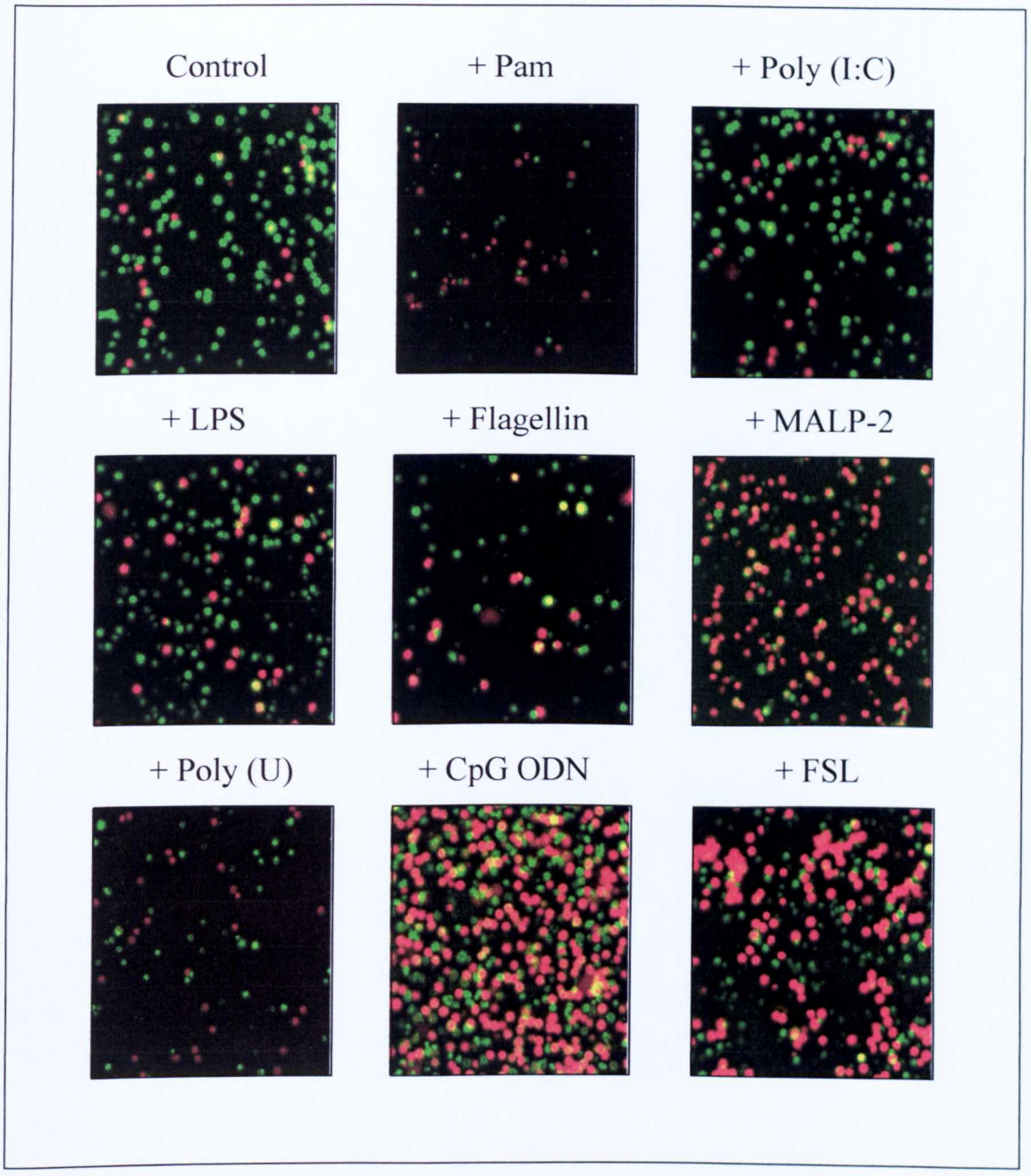


Figure 5.5.3: 8 h images from confocal microscopy of neutrophils incubated with a range of TLR ligands. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

5.6 *Effects of Signalling Inhibitors on Neutrophils Treated with MALP-2 and FSL*

It has been shown that two TLR2:TLR6 ligands, MALP-2 and FSL, induce necrosis in neutrophils. The effects of cell signalling inhibitors on the necrosis induced by these ligands was investigated to determine whether or not the same intracellular signalling pathways were activated as those activated by *H. influenzae* culture filtrate and the < 5 kDa fraction.

Neutrophils were treated with 9 signalling inhibitors and the inhibitor solvent DMSO (concentrations stated in *Materials and Methods 2.2.2*). After incubation, neutrophils were stimulated with either MALP-2 or FSL as described in section *5.5*.

Figure 5.6.1 shows the effects on the red fluorescence intensity i.e. necrosis of the neutrophils stimulated with MALP-2. All of the inhibitors used were shown to inhibit necrosis induced by the ligand. In particular, SB202190, Bis-1 and JNK inhibitor again completely inhibited necrosis. This is similar to the pattern of inhibition in neutrophils treated with *H. influenzae* culture filtrate and the < 5 kDa fraction. Figure 5.6.2 shows the effects of the inhibitors on apoptosis induced by MALP-2. As well as increasing necrosis, MALP-2 also increased apoptosis in neutrophils. None of the inhibitors affected apoptosis compared to cells treated only with MALP-2.

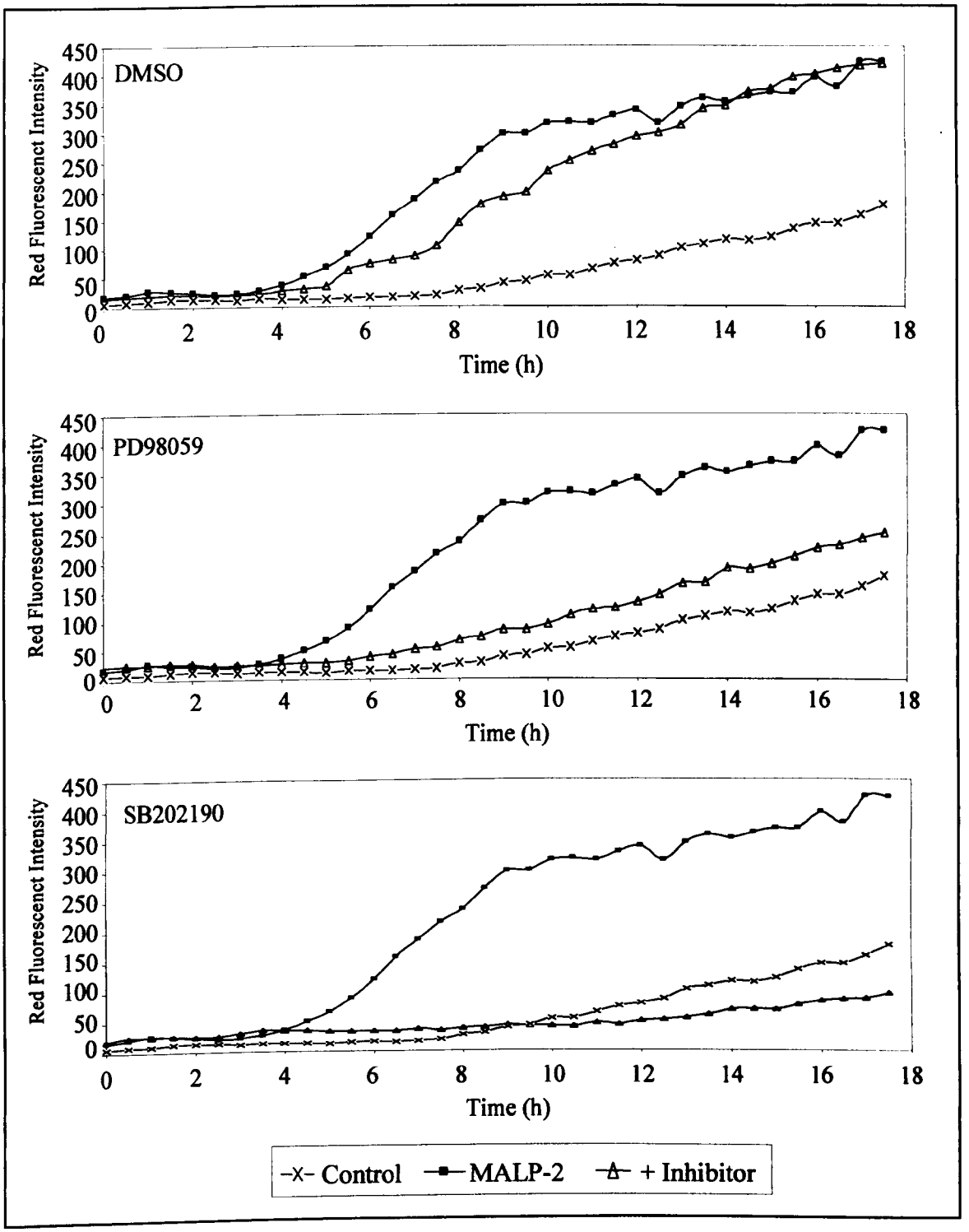


Figure 5.6.1: Red fluorescence intensity from confocal microscopy of neutrophils incubated with the TLR2 ligand MALP-2 (100 ng/ml) and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Graphs are representative of 3 experiments.

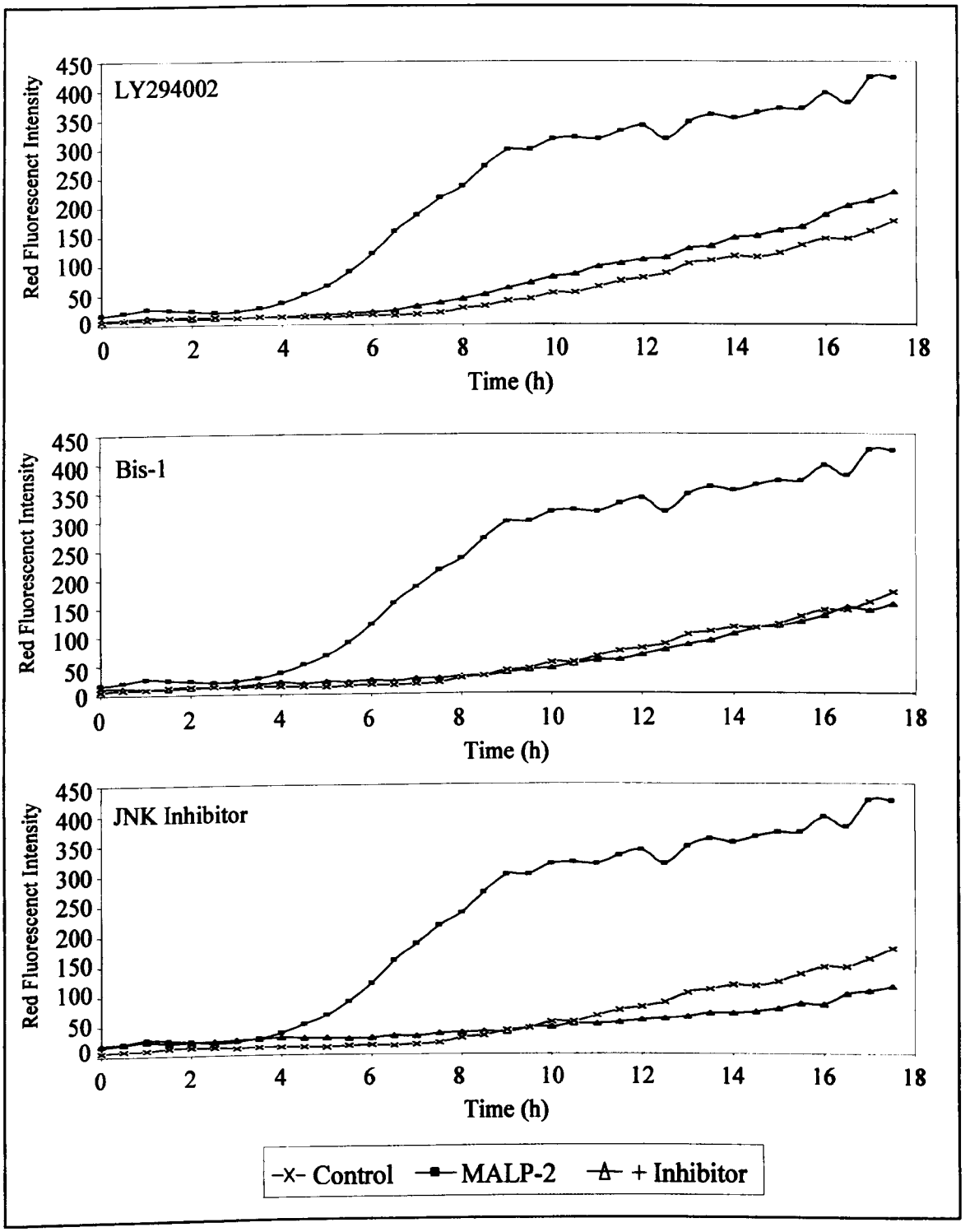
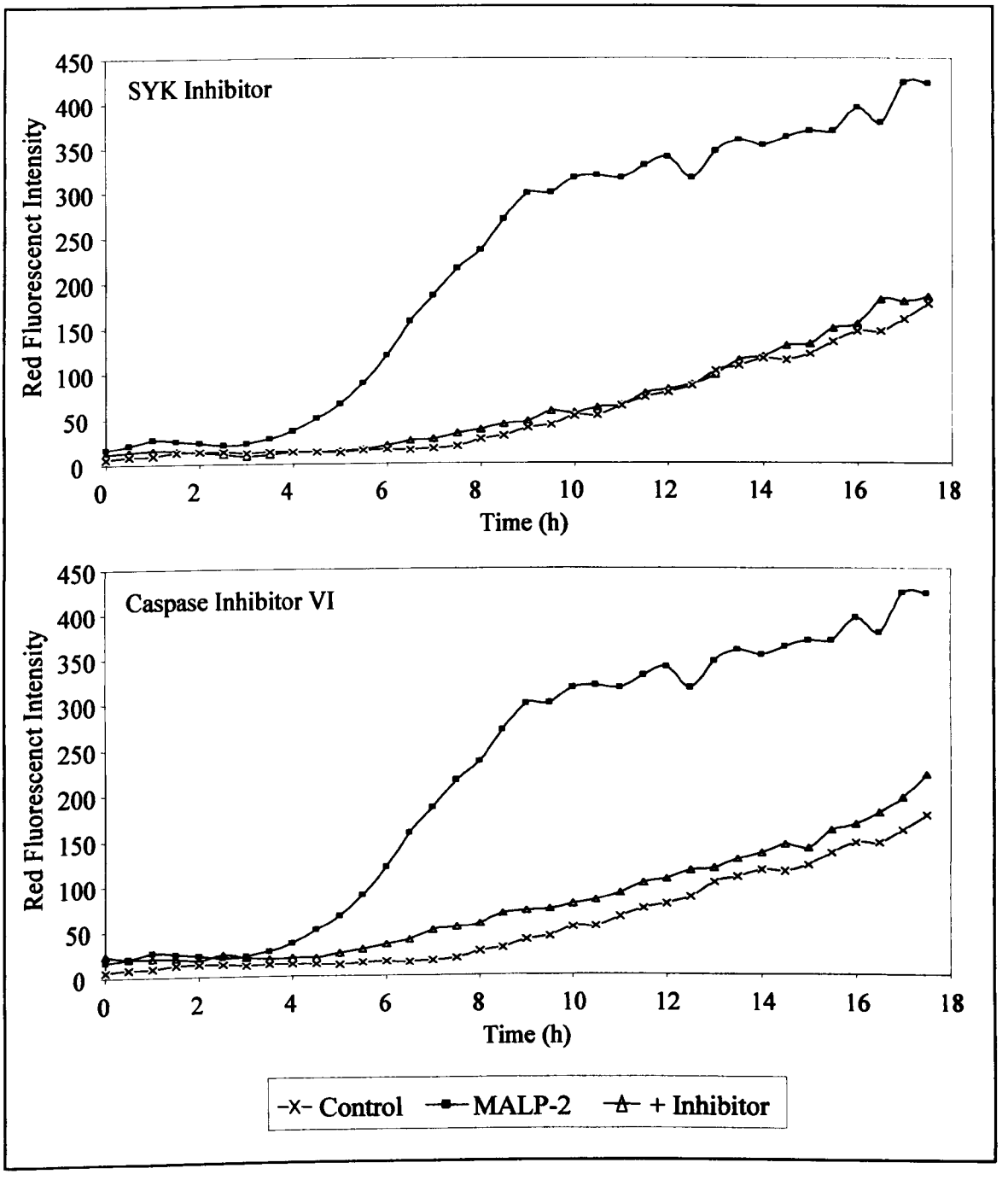


Figure 5.6.1 *(contd.)*



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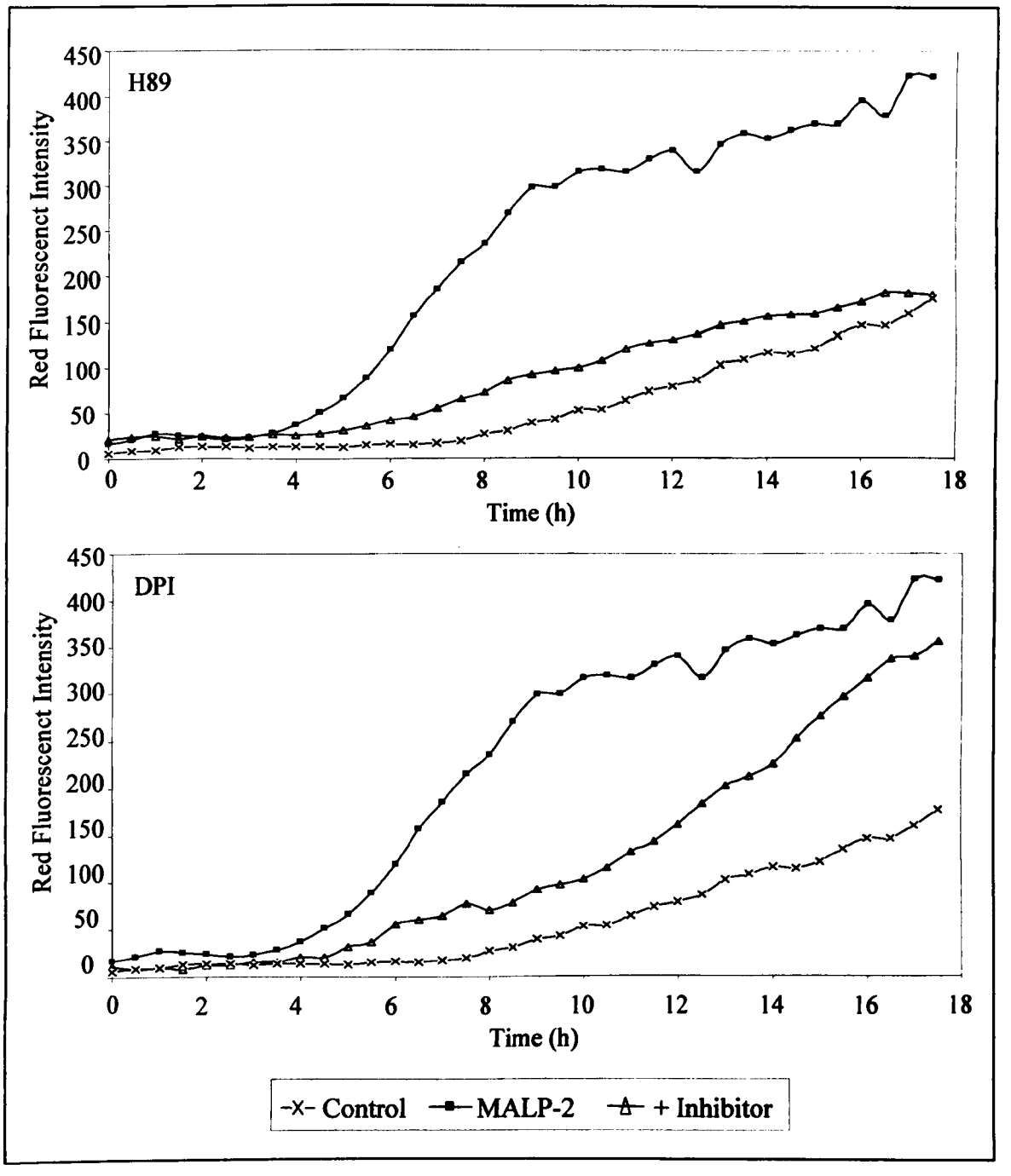


Figure 5.6.1 *(contd.)*

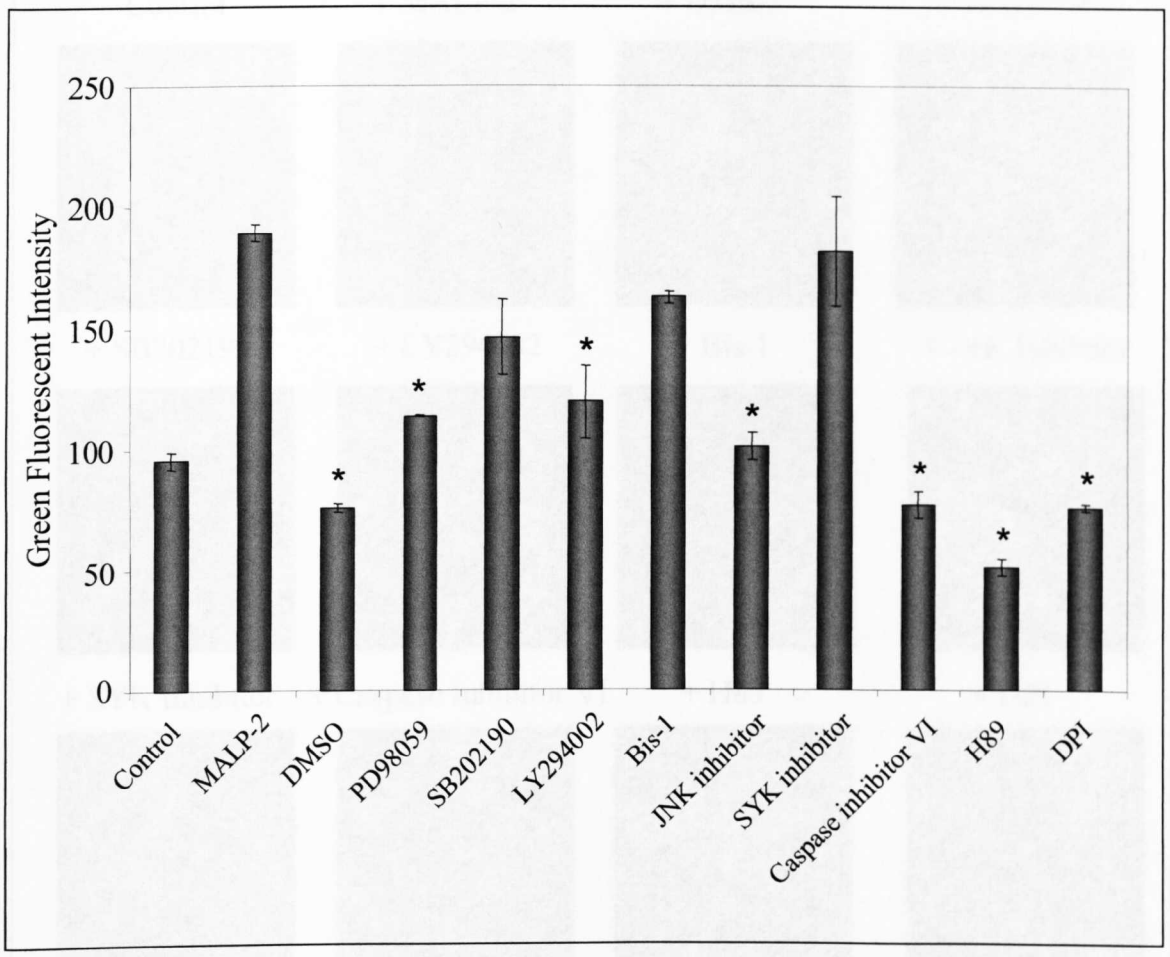


Figure 5.6.2: 8 h green fluorescence intensity from confocal microscopy of neutrophils incubated with the TLR2 ligand MALP-2 (100 ng/ml) and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Data are a mean of 3 experiments (± SD) * $p > 0.02$.

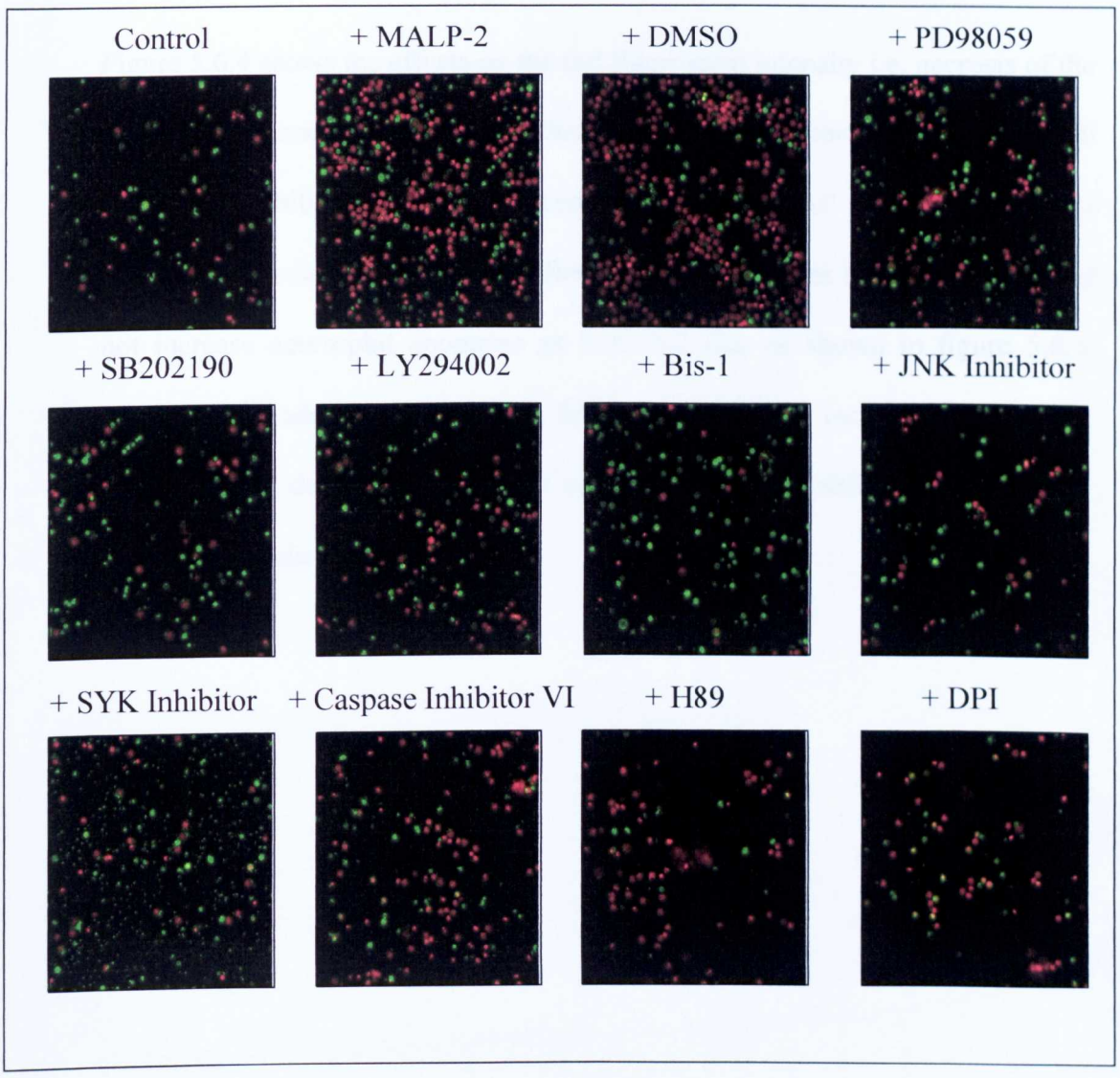


Figure 5.6.3: 8 h images from confocal microscopy of neutrophils incubated with the TLR2 ligand MALP-2 (100 ng/ml) and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

However, PD98059, LY294002, JNK inhibitor, caspase inhibitor VI, H89 and DPI had a significant decrease in MALP-2 induced apoptosis.

Figure 5.6.4 shows the effects on the red fluorescent intensity i.e. necrosis of the neutrophils stimulated with the other TLR2:TLR6 agonist, FSL. SB202190 completely inhibited necrosis induced by this ligand. All the other inhibitors inhibited necrosis from 0 - ~10 h, after this necrosis began to increase. FSL did not increase neutrophil apoptosis as MALP-2 did, as shown in figure 5.6.5. However, the addition of all of the inhibitors caused an increase in apoptosis. This could be due to the inhibition of necrosis by the inhibitors enabling the neutrophils to die by apoptosis.

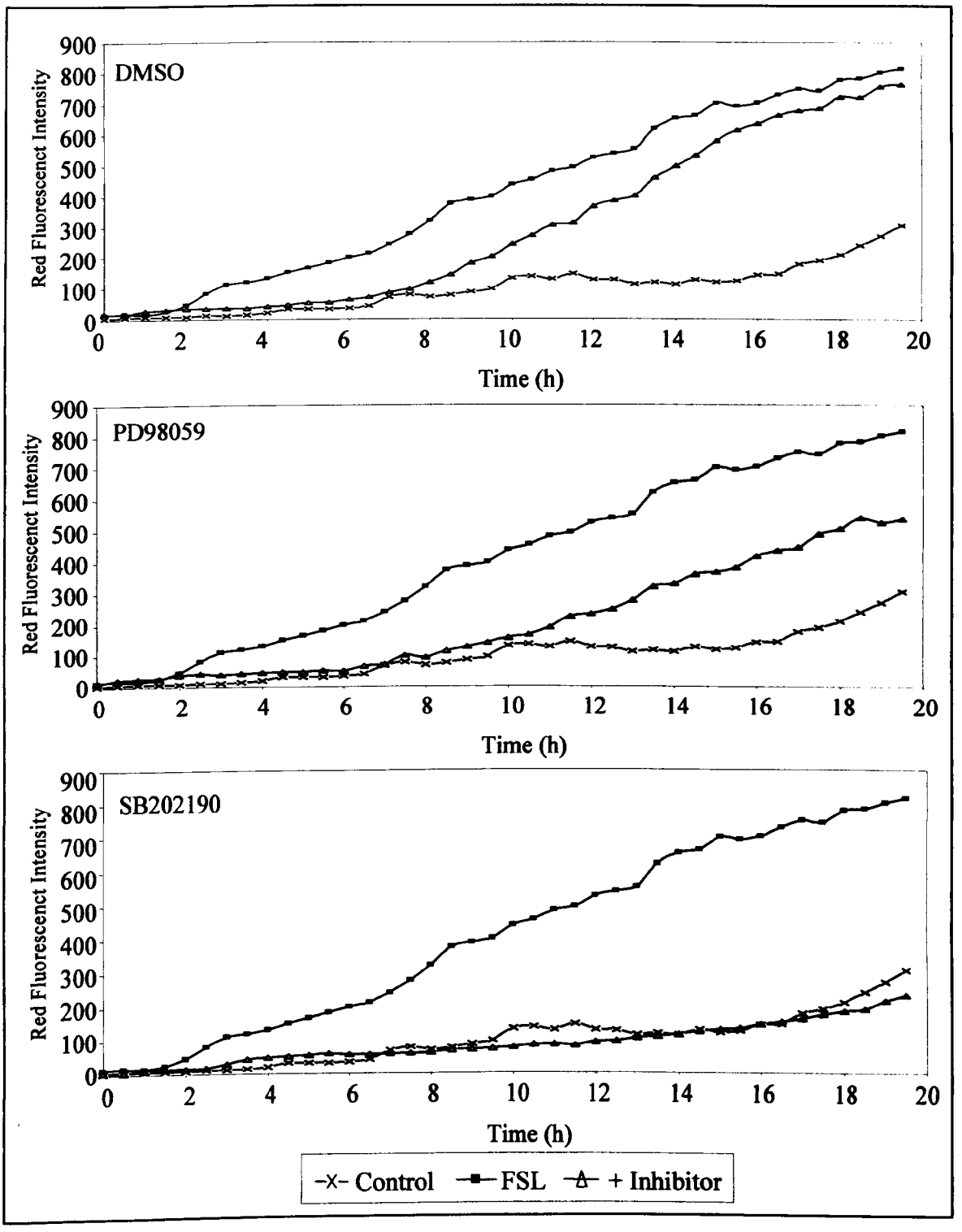
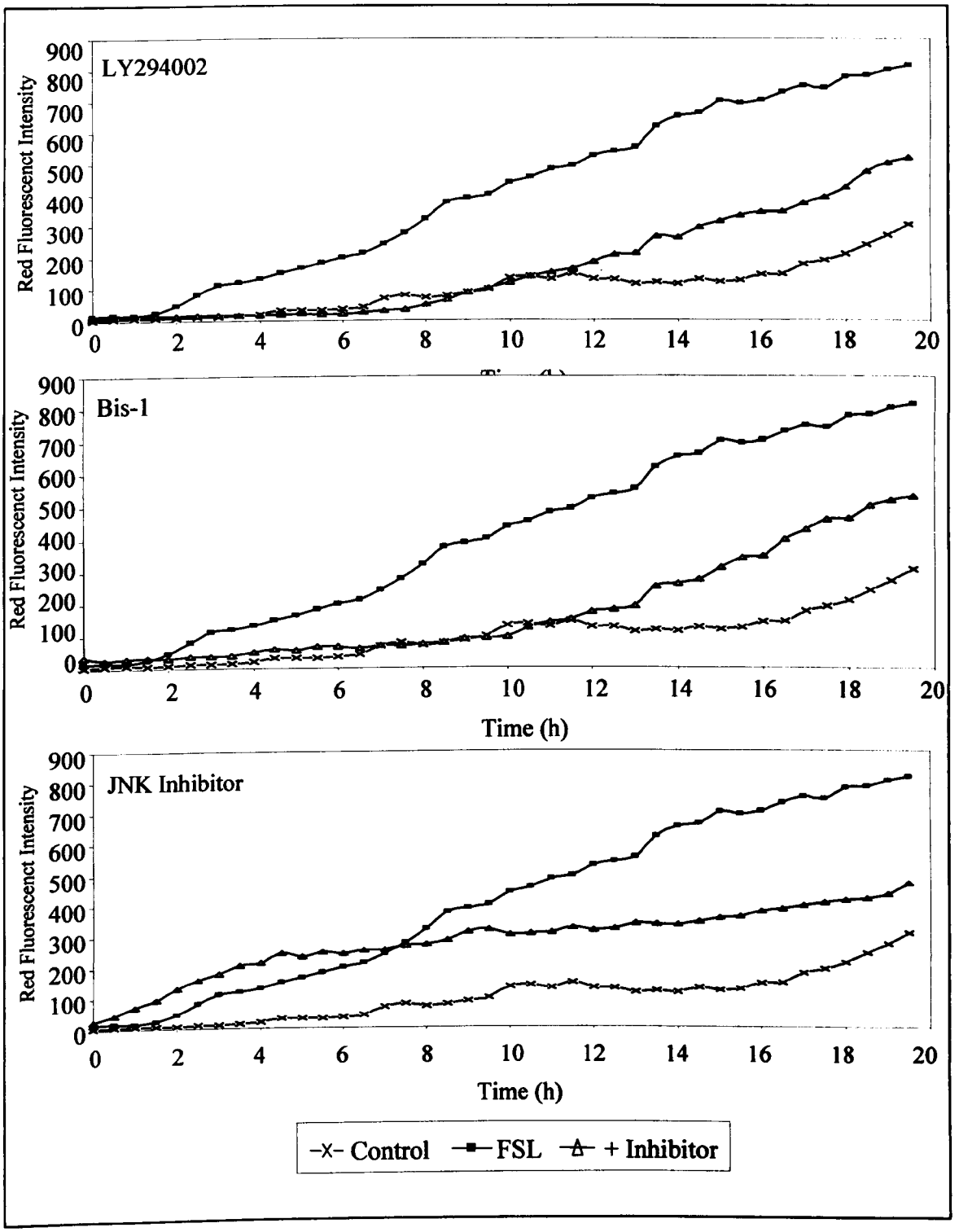


Figure 5.6.4: Red fluorescence intensity from confocal microscopy of neutrophils incubated with the TLR2 ligand FSL (10 ng/ml) and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Graphs are representative of 3 experiments.



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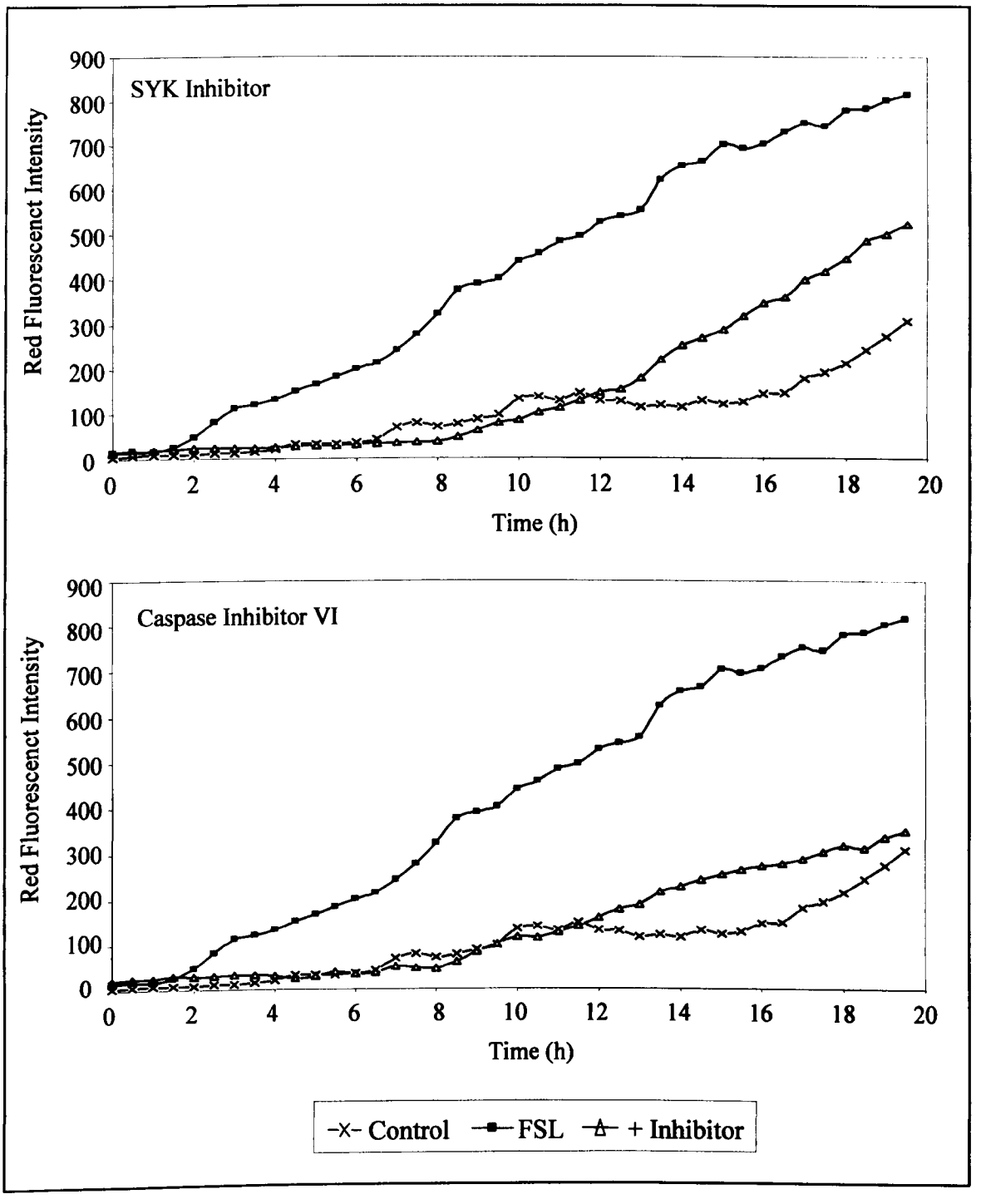

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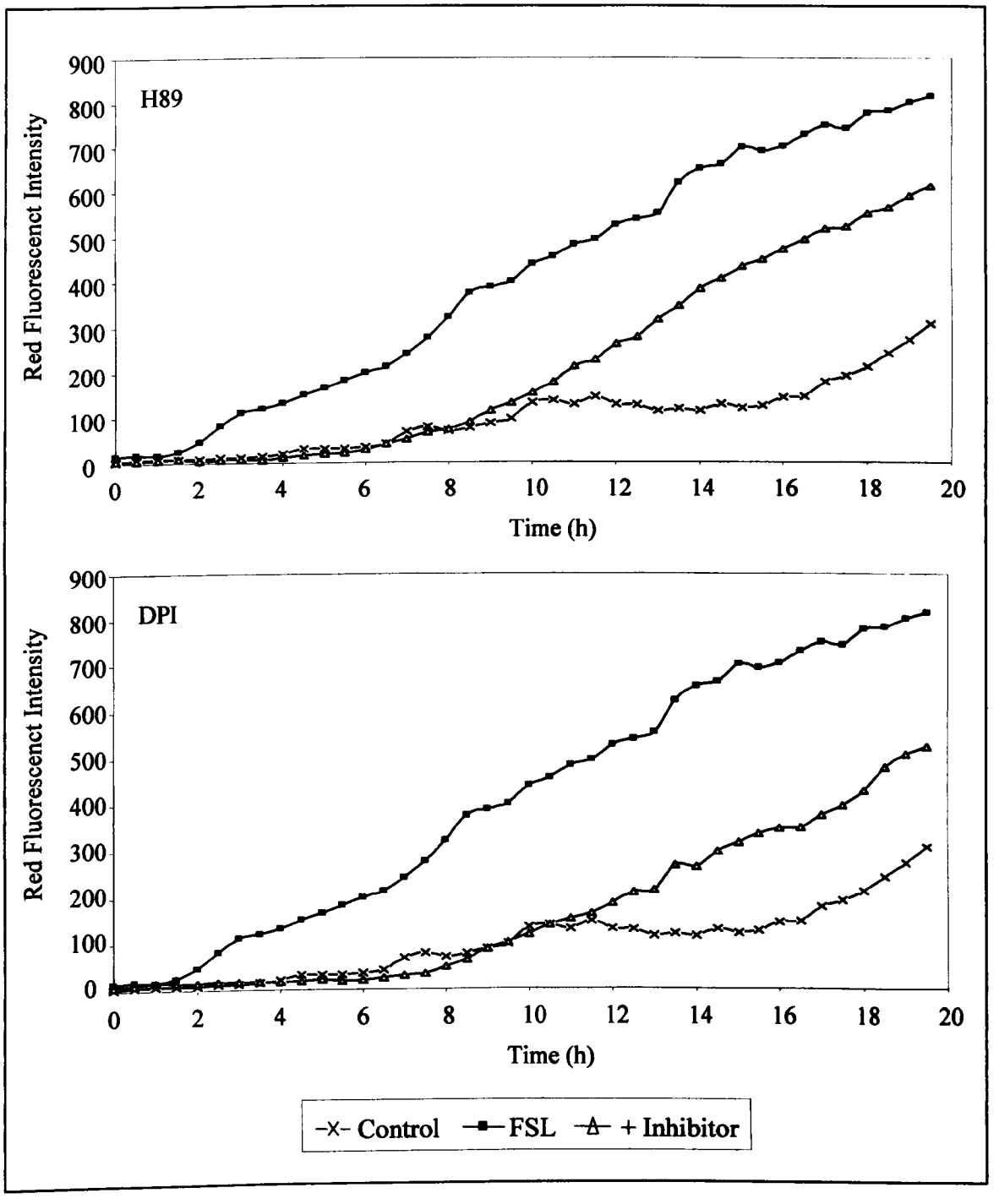

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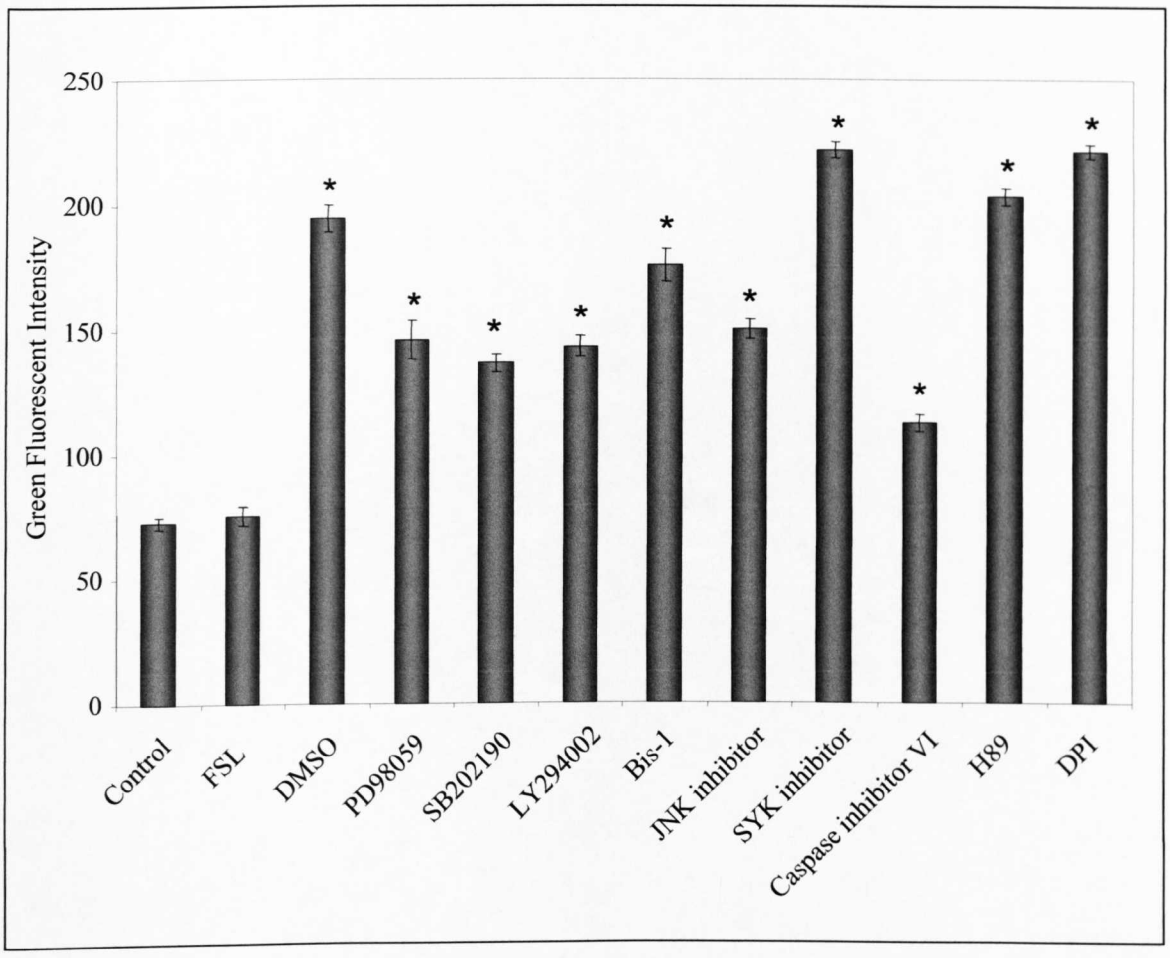

Figure 5.6.5: 8 h green fluorescence intensity from confocal microscopy of neutrophils incubated with the TLR2 ligand FSL (10 ng/ml) and cell signaling inhibitors. Neutrophils were stained with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Data are mean of 3 experiments (± SD). * $p > 0.05$.

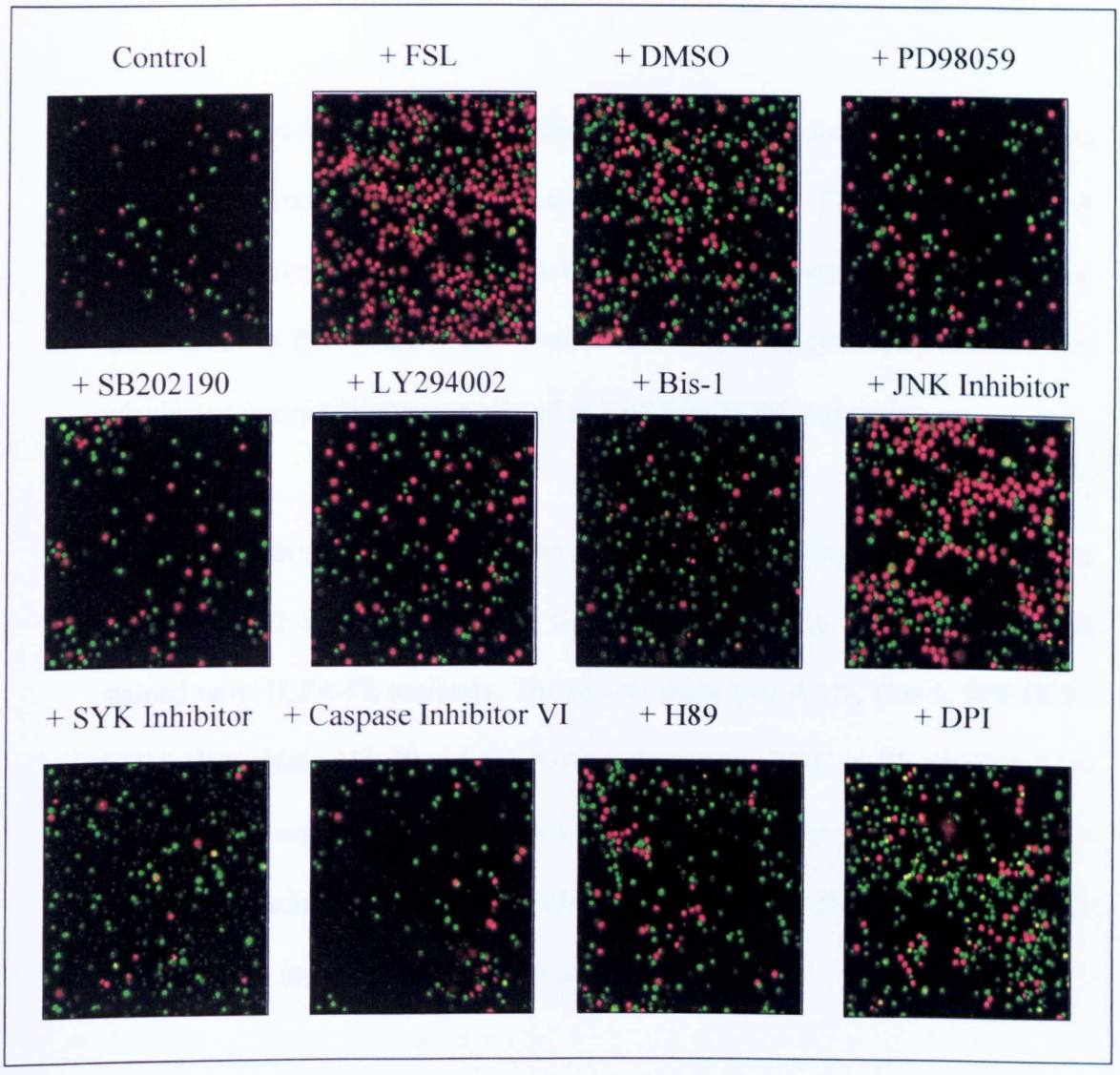

Figure 5.6.6: 8 h images from confocal microscopy of neutrophils incubated with the TLR2 ligand FSL (10 ng/ml) and cell signaling inhibitors. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml, green fluorescence) or PI (1 µg/ml, red fluorescence). Cells were imaged every 30 min for 18 h at x 20 magnification. Images are representative of 3 experiments.

5.7 *Expression of TLR2 and TLR4 in Cell Lines Sensitive and Resistant to H. influenzae Culture Filtrate*

Results obtained in this chapter indicate that *H. influenzae* culture filtrate and the < 5 kDa fraction may induce neutrophil necrosis via TLR2 and TLR4. Cell lines that are sensitive to the culture filtrate i.e. myeloid cells and cell lines that are resistant to the culture filtrate were analysed by flow cytometry to determine whether or not sensitivity was related to TLR2 or TLR4 expression.

Figure 5.7.1 shows the traces for the different cell lines that have been stained with the TLR2-FITC antibody and figure 5.7.2 shows the traces for cell lines stained with TLR4-PE antibody. The results show that A549, Cos-1, WT HEK, CD14 HEK, Hela, HT-29 and SK-N-AS cells are not FITC or PE positive. This indicates that none of these cell lines have the fluorescent antibodies bound to them and therefore do not express either TLR2 or TLR4. These cell lines are all resistant to *H. influenzae* culture filtrate.

Figure 5.7.1 shows an increase in the number of TLR2-FITC positive cells in the TLR2 HEK, HL-60, PBMC, neutrophils and U937 cell. Figure 5.7.2 also shows an increase in the number of TLR4-PE positive cells in the TLR4 HEK, HL60, PBMC, neutrophils and U937 cells. TLR2 and TLR4 transfected HEK cells only express the receptor that has been transfected, as expected. However, all the other cell lines express both TLR2 and TLR4. These cells are all

sensitive to *H. influenzae* culture filtrate and therefore further indicate that the necrotic factors are working via TLR2 and TLR4.

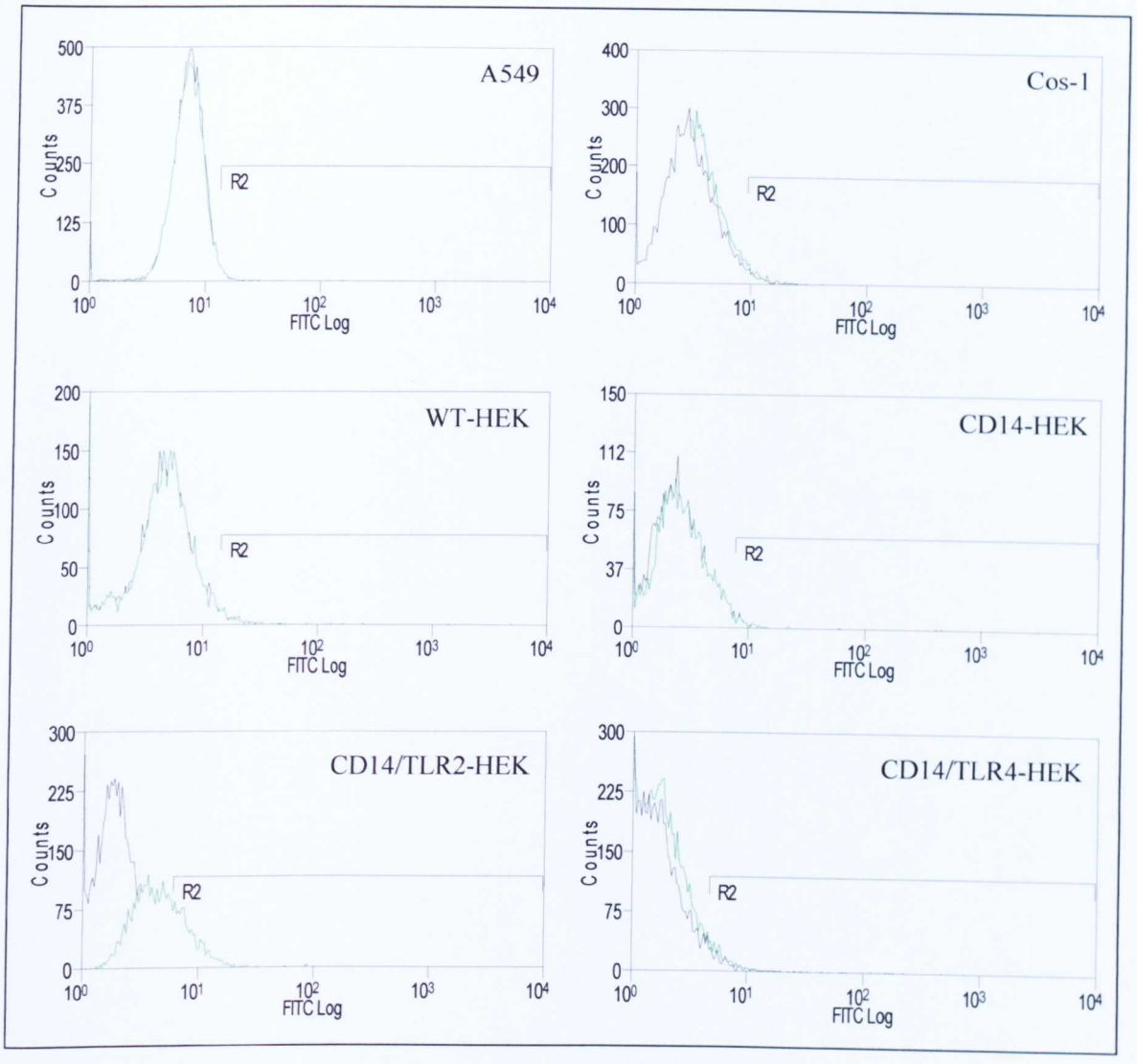

Figure 5.7.1: % of FITC labelled cells of a range of cell types to determine expression levels of TLR2. Cells were stained with TLR2-FITC conjugate (1 µl/ 0.5 x 10^6 cells). 20,000 cells were analysed using a Coulter-Epics Altra Flow Cytometer.

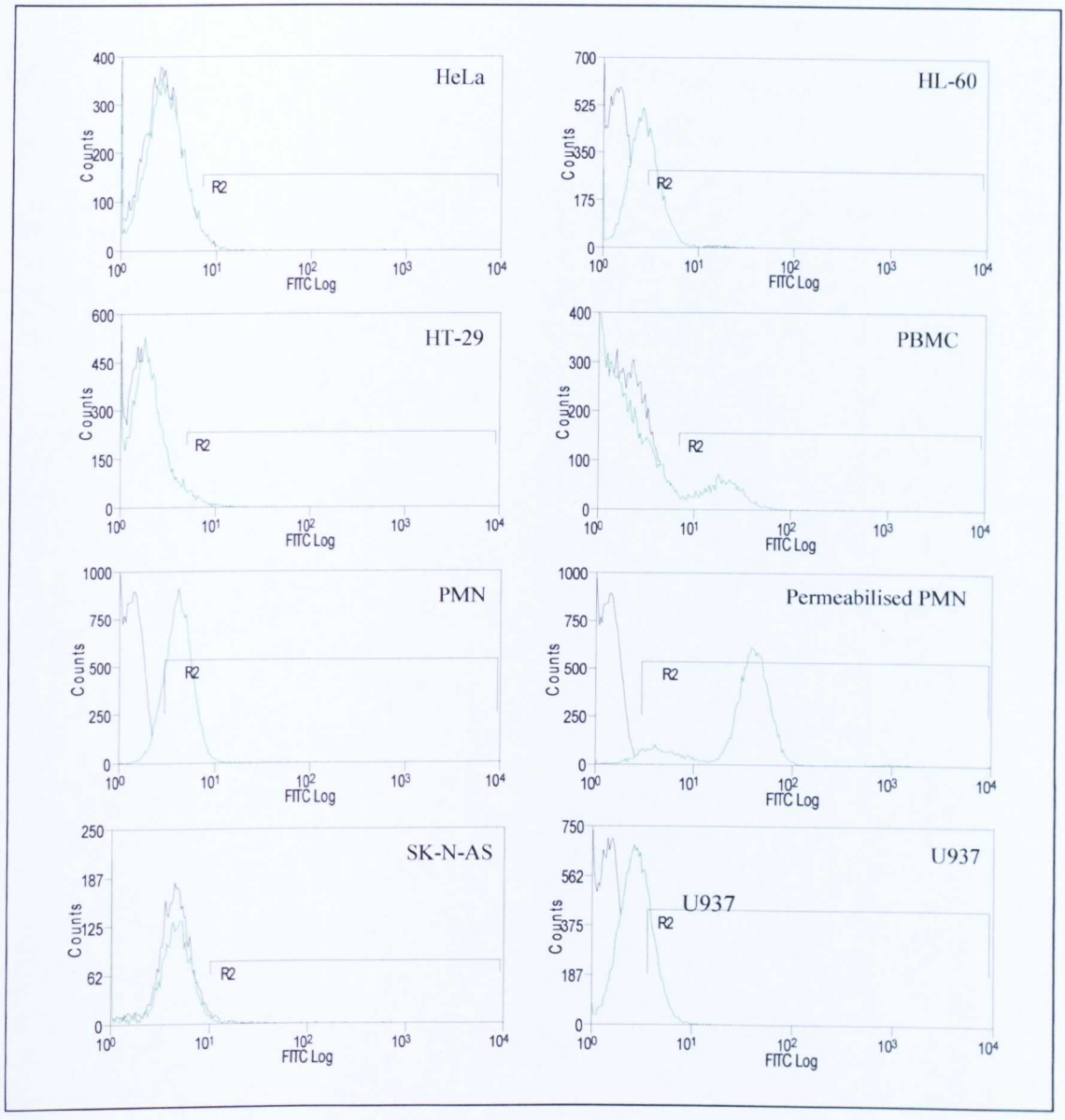

Figure 5.7.1: % of FITC labelled cells of a range of cell types to determine expression levels of TLR2. Cells were stained with TLR2-FITC conjugate (1 µl/ 0.5 x 10^6 cells). 20,000 cells were analysed using a Coulter-Epics Altra Flow Cytometer.

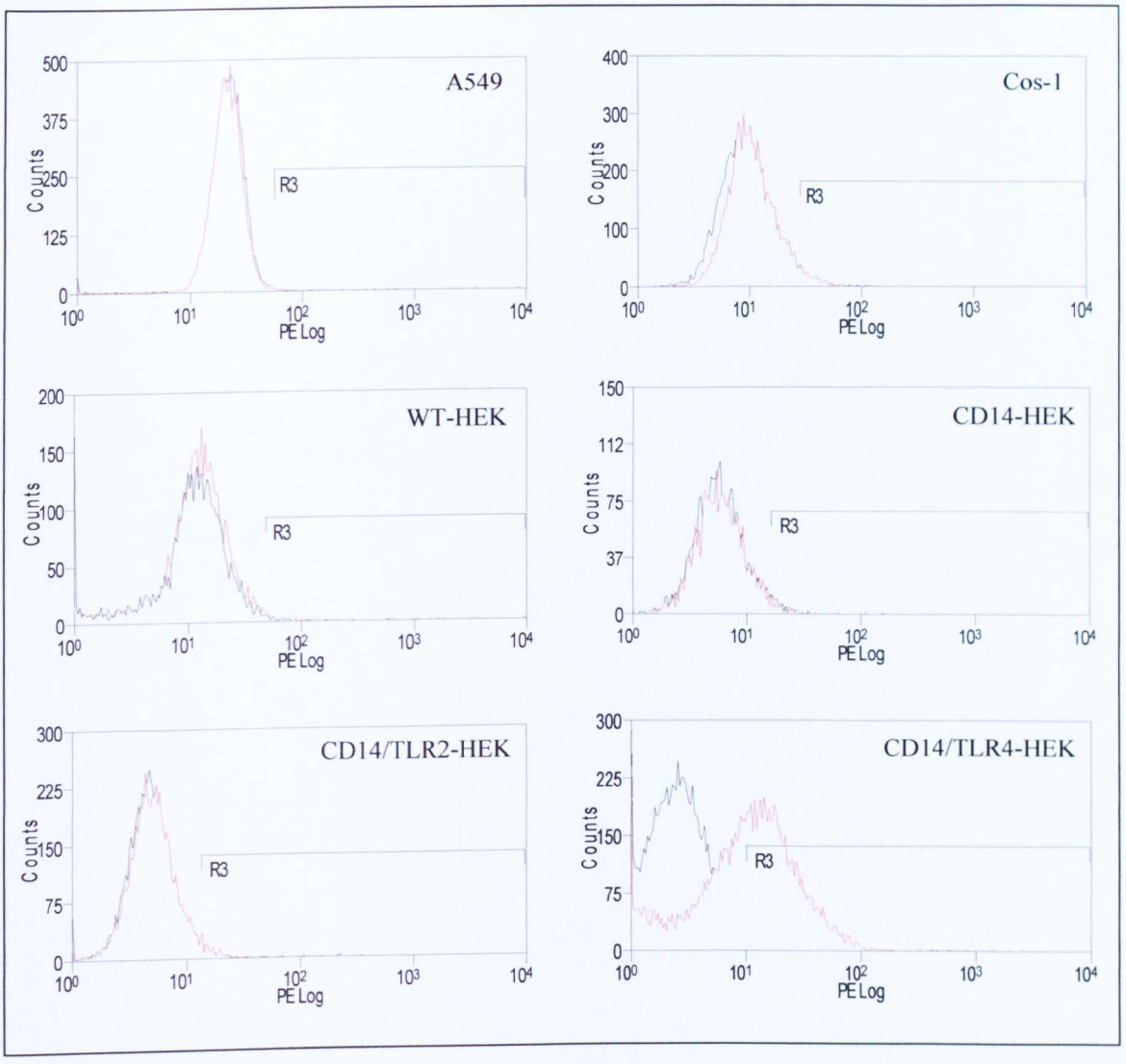

Figure 5.7.2: % of PE labelled cells of a range of cell types to determine expression levels of TLR4. Cells were stained with TLR4-PE conjugate (1 µl/ 0.5 x 10^6 cells). 20,000 cells were analysed using a Coulter-Epics Altra Flow Cytometer.

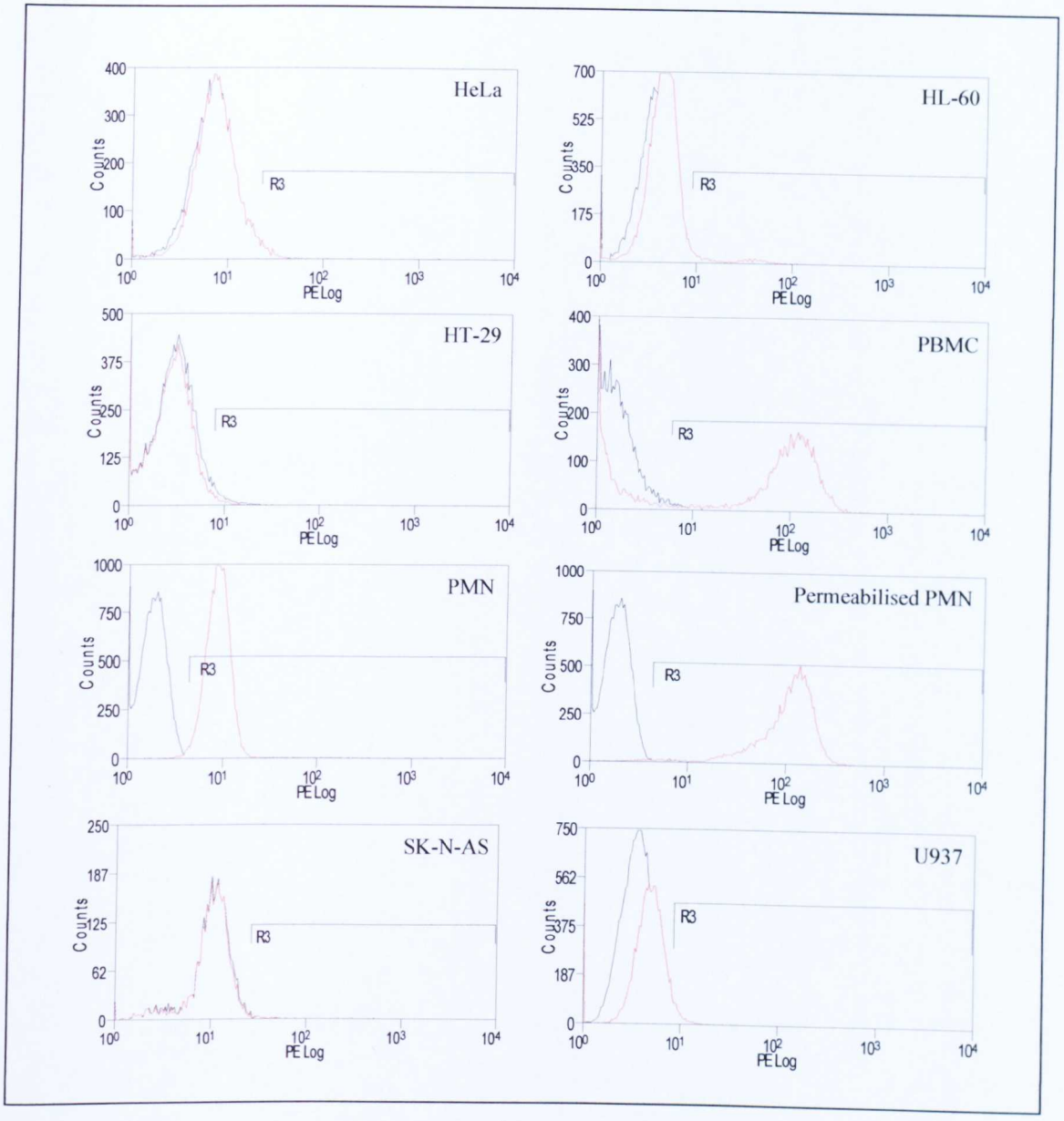

Figure 5.7.2: % of PE labelled cells of a range of cell types to determine expression levels of TLR4. Cells were stained with TLR4-PE conjugate (1 µl/ 0.5 x 10^6 cells). 20,000 cells were analysed using a Coulter-Epics Altra Flow Cytometer.

Discussion

This chapter has investigated the ability of factors present in *H. influenzae* culture filtrate to induce necrosis in myeloid cells and has investigated the roles of TLR2 and TLR4 in this process. ELISA data from the study of HEK cells transfected with CD14, CD14/TLR2 and CD14/TLR4 showed that there are a number of factors present in unfractionated culture filtrate that are capable of activating the cells via CD14, TLR2 and TLR4, as measured by an increase in IL-8 production. The < 5 kDa fraction was shown to contain a factor that activates cells via TLR2 and also TLR4. This fraction could possibly contain 2 factors, one which binds TLR2 and one which binds TLR4, or one factor which is capable of binding both TLR2 and TLR4. Analysis of the ability of *H. influenzae* culture filtrate and the < 5 kDa fraction to induce necrosis in transfected HEK cells showed that both the unfractionated culture filtrate and the < 5 kDa fraction are capable of inducing necrosis to varying degrees in the CD14/TLR2 and CD14/TLR4 HEK cells, but not in CD14 HEK cells. This suggests that although there is a factor present in unfiltered culture filtrate that is capable of activating the cells via CD14 to generate IL-8, this factor does not induce necrosis. These results strongly suggest that the necrotic factors work via TLR2 and TLR4.

The effects of different signaling inhibitors on necrosis in transfected HEK cells induced by *H. influenzae* culture filtrate were also investigated. PI3-K and PKC have both been shown to be activated in neutrophils by *H. influenzae* culture filtrate. However, it was not possible to determine the involvement of these two kinases in

HEK cell necrosis, as the inhibitors caused a large increased in cell death in the absence of culture filtrate. Most of the inhibitors had some effect on decreasing the necrosis induced in transfected HEK cells indicating that their target signaling proteins are activated intracellularly. In particular, SB202190 and JNK inhibitor both resulted in significant inhibition of necrosis in TLR2 and TLR4 HEK cells. The targets of inhibition (p38 MAPK and JNK) were shown to be important in the signaling pathways activated by the necrotic factors in neutrophils and U937 cells. This further confirms that induction of necrosis in the cells via TLR2 and TLR4 in the transfected HEK cells activates a similar MAPK signaling cascade to that of myeloid cells.

The effects of a number of different known TLR ligands on neutrophil viability were investigated and some were shown to increase necrosis. CpG ODN, bacterial DNA that interacts with TLR9, resulted in a large increase in necrosis. LPS, which works via TLR4, slightly increased neutrophil necrosis *(119)*. However, treating *H. influenzae* culture filtrate with polymixin had little effect on necrosis of neutrophils casting doubt on the idea of LPS being the factor which induces necrosis in *H. influenzae* culture filtrate (data not shown). Two TLR2 ligands, MALP-2 and FSL, induced neutrophil necrosis and the kinetics were similar to those observed when cells were stimulated with the < 5 kDa fraction. TLR2 acts as a heterodimer with either TLR1 or TLR6. MALP-2 and FSL are synthetic diacylated lipoproteins and have been shown to work via TLR2:TLR6 heterodimers *(121)*. This suggests that the TLR2 necrotic factor present in the < 5 kDa fraction may act via TLR2:TLR6 and may possibly be a diacylated lipoprotein. This idea is further supported by the inability of Pam_3CSK_4 to

induce neutrophil necrosis. Pam_3CSK_4 is triacylated lipoprotein which activates cells by binding to TLR2:TLR1 heterodimers (*218*).

The effects of the signaling inhibitors on necrosis induced by MALP-2 and FSL were also investigated and again SB202190, Bis-1 and JNK inhibitor were shown to result the greatest inhibition of necrosis. This confirms the importance of p38 MAPK, PKC and JNK activation in *H. influenzae* induced necrosis and also strengthen the idea that the < 5 kDa factor may be a diacylated lipoprotein activating cells via TLR2:TLR6.

TLR signaling occurs via two different pathways; the MyD88 dependent pathway and the MyD88 independent pathway. TLR2 and TLR4 signaling can occur via the MyD88 pathway. MyD88 associates with the TIR domain of the TLR and after stimulation by the TLR ligand, IRAK is recruited to the complex. IRAK is then phosphorylated after which it associates with TRAF6. TRAF6 then activates 2 different pathways; the NFκB pathway and a MAPK pathway. JNK, p38 MAPK and ERK have all been shown to be activated in this TLR MAPK signaling pathway resulting in the activation of the transcription factor AP-1. These results indicate that the factors released by *H. influenzae* are inducing necrosis in the cell via a TLR MyD88 dependent pathway *(117,124, 219).*

Results obtained in Chapter 3 indicated that the ability of the factors to induce necrosis may be specific to myeloid cells as these cells are sensitive to the effects of culture filtrate. A range of sensitive cell lines and resistant cell lines were analysed to

determine the levels of expression of both TLR2 and TLR4. None of the resistant cell lines were shown to express either TLR2 or TLR4. However, all of the sensitive cell lines were shown to express both of the receptors. This further confirms that the factors induce necrosis in myeloid cells by binding to TLR2 and TLR4 and initiate a MAPK signaling cascade inside the cell where p38 MAPK, PKC and JNK are activated.

TLR4 signaling in neutrophils, induced by bacterial LPS, has been shown to induce the expression of TLR2 in endothelial cells in a time dependent manner *(121, 122)*. TLR2 upregulation in endothelial cells is also enhanced by the presence of neutrophil NADPH-oxidase derived oxidants which may be increased in the lungs as a result of *H. influenzae* induced neutrophil necrosis (*220*). If this occurs it may increase the ability of *H. influenzae* to cause tissue damage, as well as causing necrosis of myeloid cells, expression of TLR2 by endothelial cells may enable the < 5 kDa factor to induce necrosis in these cells. Confirmation of the identity of the receptors through which *H. influenzae* necrotic factors induce necrosis may enable therapeutic targets to be developed so that exacerbations of COPD by *H. influenzae* can be treated.

Chapter 6: Does *H. influenzae* Induce Neutrophil Necrosis *in vivo*?

Introduction

COPD is an inflammatory lung disease, characterised by a slowly progressive and irreversible deterioration in lung function. Research since 1983 has shown the importance of neutrophils in contributing to exacerbations of COPD by the increased presence of these cells in the BAL fluid and sputum of patients with COPD compared to control samples. Patients with existing airway colonizations of bacteria are more at risk of developing exacerbations. Non-typeable *H. influenzae* is the most common pathogen isolated from the lungs of patients with COPD and may be responsible for exacerbations, although viral pathogens may also play a role (*176, 202*).

Chapters 3-5 in this thesis have shown that non-capsulated *H. influenzae* and culture filtrate from this bacterium can induce necrosis in neutrophils isolated from healthy individuals. Clinical isolates of the organism obtained from the lungs of patients with COPD have been studied and all of the 12 isolates caused neutrophils to die by necrosis. Results from Chapter 3 indicate the presence of at least 2 factors present in *H. influenzae* culture filtrate capable of inducing neutrophil necrosis, and the data in Chapter 5 indicates that these factors may be activating via TLR2 and TLR4. These receptors may act intracellularly via signalling through MyD88 and MAPK (*124*). These pathways may also be important in the generation of an inflammatory response as they also regulate expression of molecules such as IL-8. My data suggests a vicious

cycle of neutrophil recruitment, activation and lysis but it is very important to determine if such events occur *in vivo* in COPD.

The aims of this chapter were to:

- Analyse by cytospins and electron microscopy, the morphology of neutrophils in the sputum of asthmatic controls and COPD patients with and without infections by non-typeable *H. influenzae*.
- Determine, by chemiluminescence, whether or not neutrophils isolated from the sputum of COPD patients with a *H. influenzae* infection are activated *in vivo*.
- Examine the expression levels of a number of neutrophil proteins in sputum neutrophils compared to blood neutrophils from COPD patients to determine if COPD neutrophils have been activated to secrete granule enzymes *in vivo*.
- Analyse the levels of MPO released into the sputum of COPD patients with a *H. influenzae* infection.

6.1 *Morphological Analysis by Cytospin of Neutrophils Isolated from Patient Blood and Sputum Samples*

To determine whether or not the results observed when *H. influenzae* are incubated with neutrophils *in vitro* also occur *in vivo*, it was necessary to analyse neutrophils isolated from patient blood and sputum. Neutrophils were isolated from the blood and sputum of healthy controls, asthmatic patients, COPD patients with no detectable *H. influenzae* infection and COPD patients with a current *H. influenzae* infection. Analysis of cytospins indicated neutrophil number, purity and viability.

Figure 6.1.1 shows images of cells isolated from the blood and sputum of a healthy control (A) and an asthmatic control (B). Blood neutrophils isolated from the healthy control appear normal, with only < 5 % of the cells showing any signs of apoptosis. Neutrophils isolated from the blood of the asthmatic patient had signs of activation at a high magnification i.e. vacuoles present in the cytoplasm but had a normal morphology. In the cytopsins of cells isolated from the sputum of the healthy control and asthmatic patients there were a number of large cells present, possibly squamous cells, but no neutrophils were detected. This was expected as, in the absence of an infection, neutrophils will not be recruited to the lungs. There would be expected to be a large number of

eosinophils present in the sputum of the asthmatic patient, however this was not observed in this particular patient.

Figure 6.1.2 shows images taken of neutrophils isolated from the blood and sputum of two COPD patients that did not have clinical evidence of current *H. influenzae* infection at the time of sample collection. Again, the blood neutrophils appeared to have a normal morphology. The cells isolated from the sputum of these patients had an increased number of neutrophils present compared to healthy controls and asthmatic controls. These neutrophils were clumped together and appeared to be activated due to the presence of large vacuoles present in the cells which was observed at 40 x magnification (images not shown). The presence of neutrophils in the sputum of these patients could be due to colonization of bacteria in the respiratory tract or infection of bacteria other than *H. influenzae.* Again, a number of other cells are present in the sputum and these could be squamous cells from the mouth.

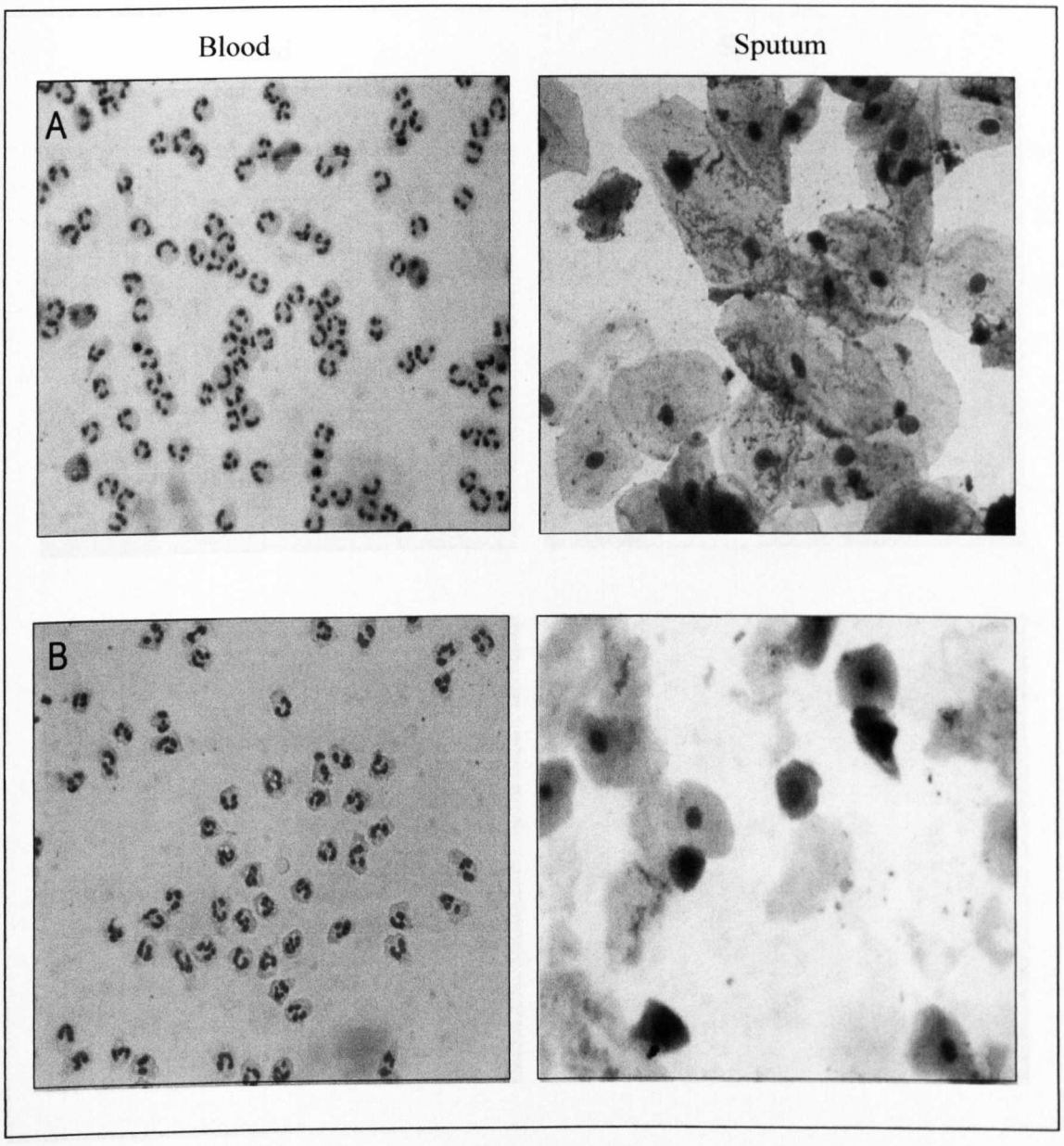

Figure 6.1.1: Cytospins of cells isolated from the blood and sputum of healthy controls (A) and asthmatic controls (B). 1 x 10^5 cells were centrifuged at 500 g for 5 min and stained using Rapid Romanowsky Staining to analyse the morphology of the cells. Images are 10 x magnification. A and B show neutrophils purified from blood, but no neutrophils were detected in the sputum.

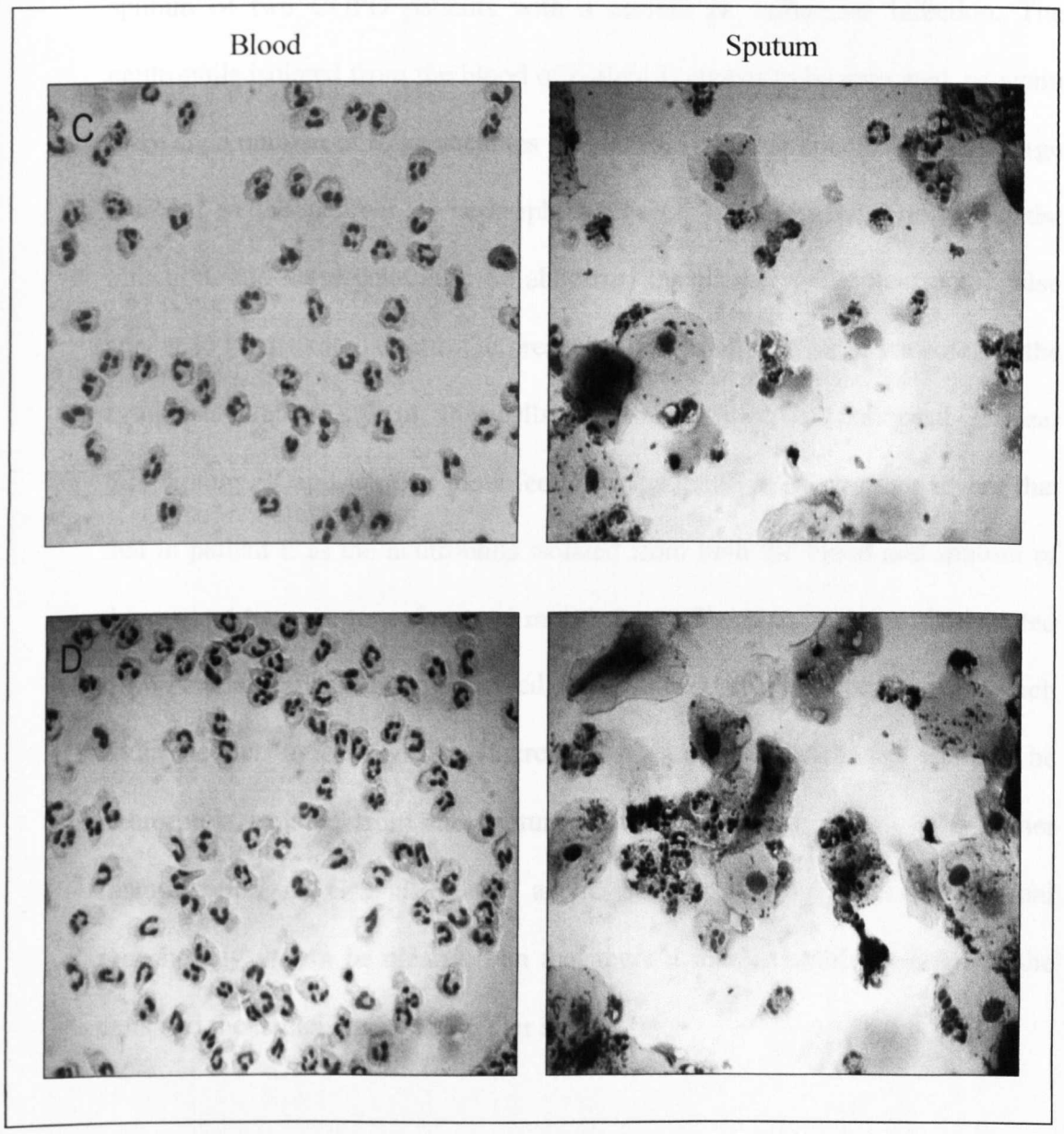

Figure 6.1.2: Cytospins of cells isolated from the blood and sputum of 2 COPD patients without a current *H. influenzae* infection (C & D). 1 x 10^5 cells were centrifuged at 500 g for 5 min and stained using Rapid Romanowsky Staining to analyse the morphology of the cells. Images are a 10 x magnification. Neutrophils were shown to be present in both the blood and sputum of patients.

Figure 6.1.3 shows images taken of neutrophils isolated from the blood and sputum of two COPD patients with a current *H. influenzae* infection. The neutrophils isolated from the blood of patient E appear to be activated, as again there are a number of large vacuoles present in the cytoplasm. There was a large increase in the number of neutrophils present in the sputum, reflecting the infection, and these cells have an abnormal morphology. The neutrophils also appear to be activated due to the presence of a number of large vacuoles in the cytoplasm and many of the cells appear to have an abnormal nuclear morphology. It appears that the infection in patient F is much more severe that that in patient E as the neutrophils isolated from both the blood and sputum of this patient have a more abnormal morphology. The blood neutrophils isolated from patient F appear to be activated. There is a decrease in cell number which could be due to cell lysis as there are smears of stain on the slides. The neutrophils isolated from the sputum of this patient are shown at a higher magnification in G and H and again. The cells have a very abnormal morphology. It can be clearly seen that there a many vacuoles present in the cells and the nuclei have an irregular shape.

Cytospin analysis indicates that neutrophils isolated from the sputum of patients with a *H. influenzae* infection have an abnormal morphology. It is therefore necessary to analyse these cells in more detail at a higher magnification.

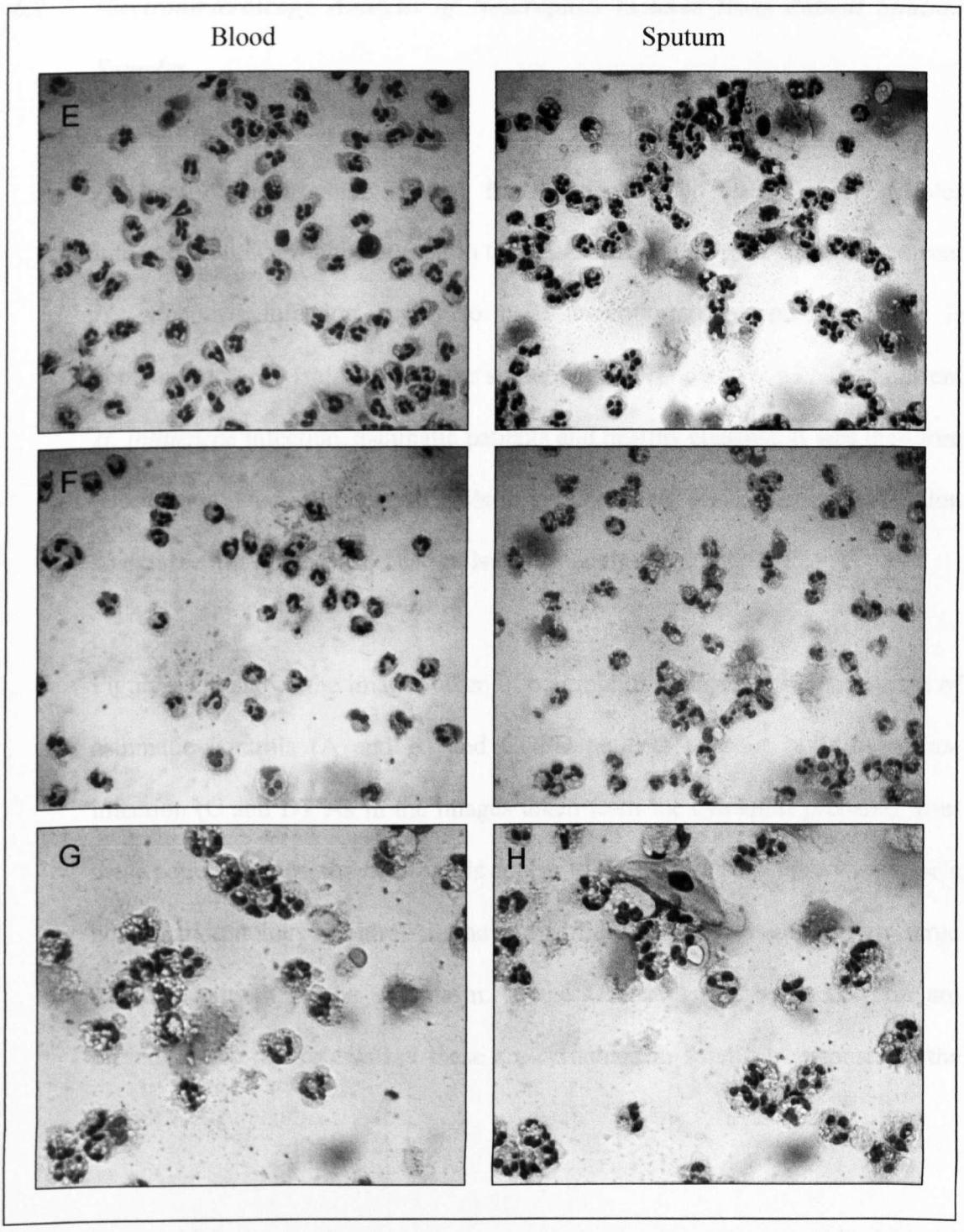

Figure 6.1.3: Cytospins of cells isolated from the blood and sputum of 2 COPD patients with a current *H. influenzae* infection (E & F) at a 10 x magnification. Cytospins G and H show sputum neutrophils from patient F at 25 x magnification. 1 x 10^5 cells were centrifuged at 500 g for 5 min and stained using Rapid Romanowsky Staining to analyse the morphology of the cells.

6.2 *Electronmicroscopy Analysis of Neutrophils Isolated from Patient Sputum Samples*

Analysis of neutrophils isolated from patient blood and sputum samples indicates that neutrophils present in the sputum of COPD patients with a current *H. influenzae* infection appear to have an abnormal morphology. This is compared to cells isolated from the sputum of COPD patients without a current *H. influenzae* infection, asthmatic patients and healthy controls. It was therefore necessary to study these neutrophils in greater detail at a higher magnification so that the morphology of these cells can be analysed.

Figure 6.2.1 shows the images taken of neutrophils isolated from the sputum of asthmatic controls (A and B) and COPD patients without a *H. influenzae* infection (C and D). As in the images taken from the cytospins prepared from these patient groups, the neutrophils appear to be activated but otherwise have a normal morphology. Neutrophils have a ruffled cytoplasm and there are large vacuoles present in the cytoplasm. Image D shows that some bacteria are present in the neutrophils but these are contained in small phagosomes in the cell.

Figure 6.2.2 shows electron microscopy images taken of neutrophils isolated from COPD patients with a current *H. influenzae* infection. These cells have an extremely abnormal morphology. There are many extremely large vacuoles

present in the cytoplasm of the cells and bacteria can also be seen in the cytoplasm of the cells (as shown by arrows in images J and K). Phagosomes are present in the neutrophils but, rather than being small and compact, they are large and have many bacteria present inside (indicated by arrows in image F and K). The plasma membranes of the cells also have small finger-like protrusions forming (as shown by arrows in images E and I) and the nuclei of the cells appear to have a be grossly abnormal (as shown by arrows in images G and I). The morphology of these cells indicate that the cells show signs of necrosis, as shown by the abnormal nuclear morphology and the loss of plasma membrane integrity observed under higher magnifications.

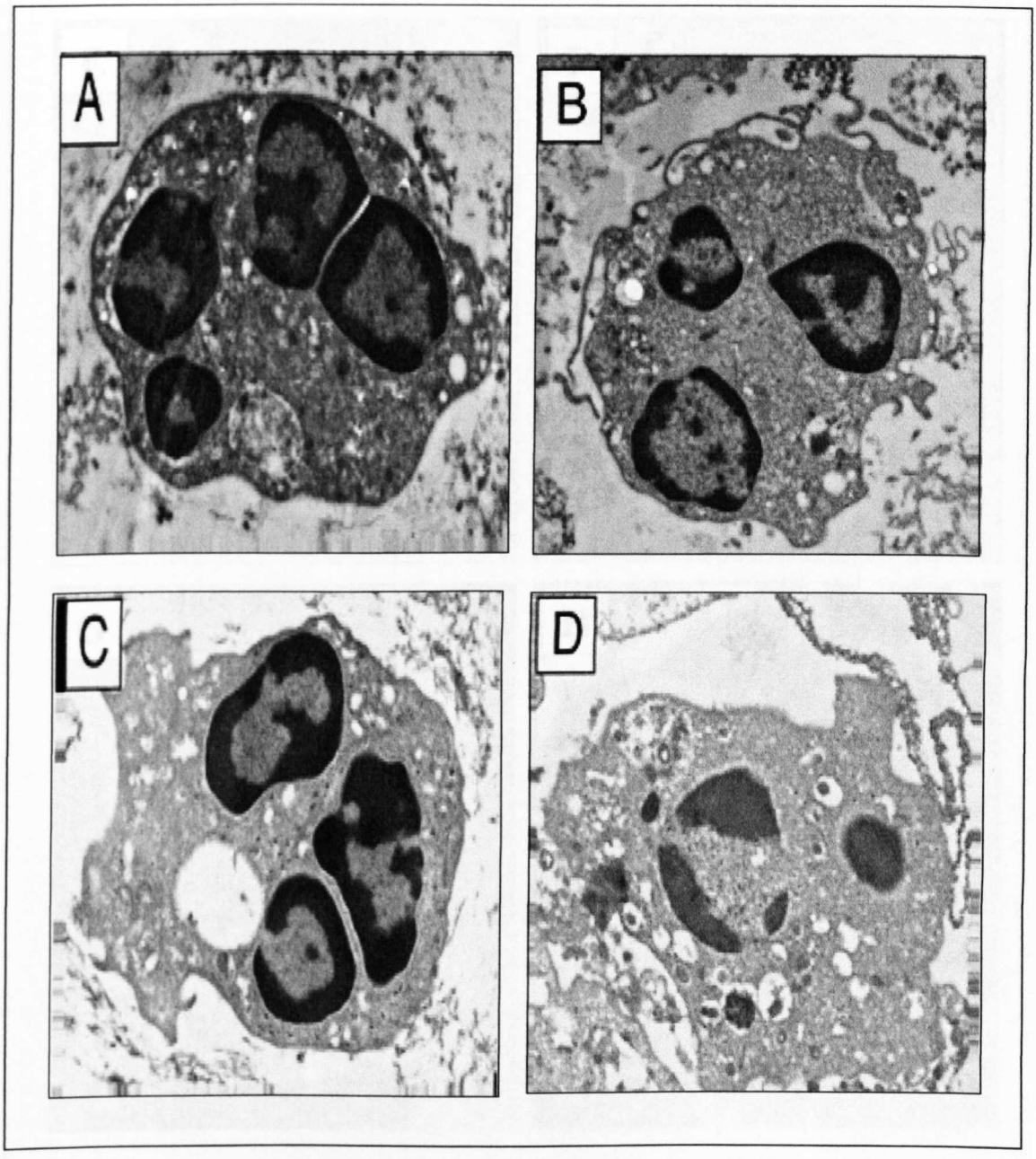

Figure 6.2.1: Neutrophils were isolated from the sputum of asthmatic controls (A & B) and COPD patients without a current *H. influenzae* infection (C & D) and the morphology of the cells analysed by electron microscopy. Images are taken at 75,000 x magnification.

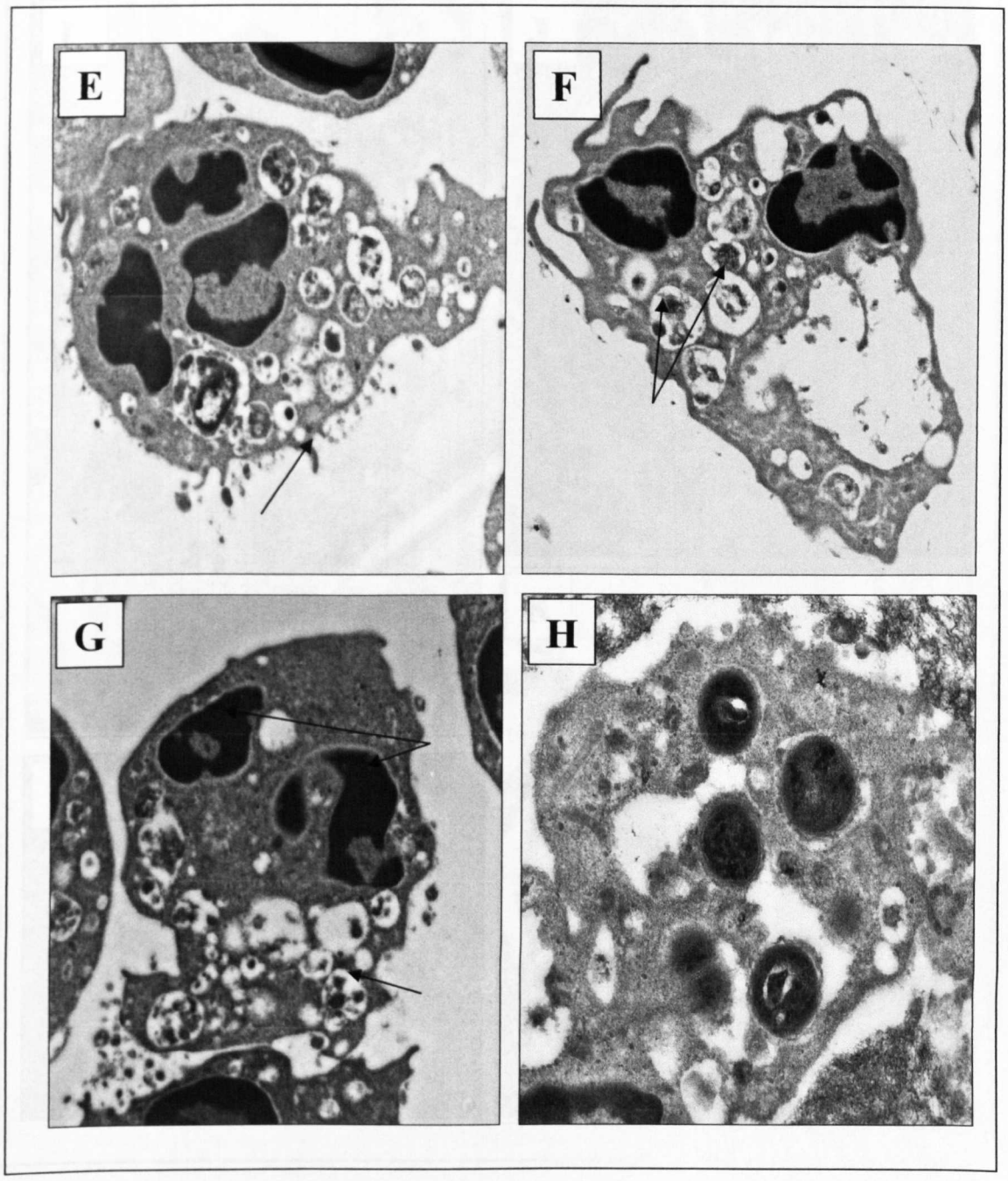

Figure 6.2.2: Neutrophils were isolated from the sputum of COPD patients with a current *H. influenzae* infection and the morphology was analysed by electron microscopy. Images E, F and G were taken at a 66,000 x magnification and H was taken at a 75,000 x magnification.

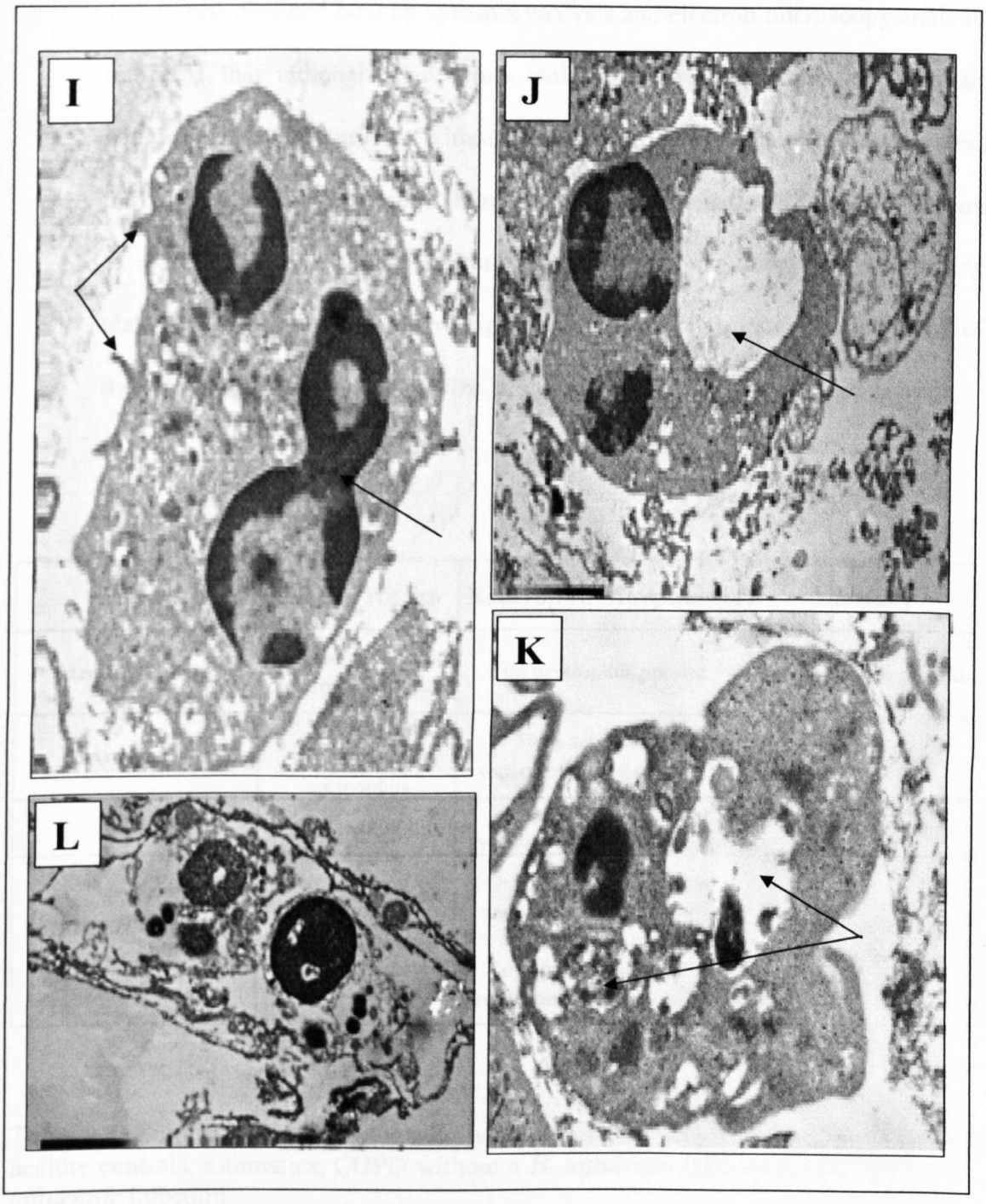

Figure 6.2.2: Neutrophils were isolated from the sputum of COPD patients with a current *H. influenzae* infection and the morphology was analysed by electron microscopy. Image I was taken at a 75,000 x magnification, J and K were taken at a 66,000 x magnification and L was taken at a 33,000 x magnification.

The results obtained both by cytospin analysis and electron microscopy analysis indicated that although neutrophils isolated from the sputum of asthmatic patients and COPD patients without a current infection appear to be activated, they appear otherwise normal. However, neutrophils isolated from the sputum of COPD patients with a current *H. influenzae* infection have a grossly abnormal morphology and show signs of cell death. This indicates that many of the effects of *H. influenzae in vitro*, also occurs *in vivo* inside the COPD lung.

Patient	Cells Present	Neutrophil Morphology	Viability
Healthy Control	Squamous cells, no neutrophils present	no neutrophils present	no neutrophils present
Asthmatic	10 % neutrophils, squamous cell, eosinophils	~ 50 % activated: large vacuoles, ruffled cytoplasm	~ 10 % apoptotic
COPD	~95 % neutrophils	~ 60 % activated: large vacuoles, ruffled cytoplasm	~ 20 % apoptotic
COPD + *H. influenzae* infection	100 % neutrophils	> 90 % abnormal: large vacuoles, many bacteria in vacuoles, bacteria in cytoplasm, finger like protrusions, abnormal nucleus	> 95 % necrotic like appearance

Table 6.1: Summary of morphology and viability of cells isolated from the sputum of healthy controls, asthmatics, COPD without a *H. influenzae* infection and COPD + *H. influenzae* infection.

6.3 *ROS Production by Neutrophils Isolated from Patient Blood and Sputum*

It was necessary to determine whether or not neutrophils isolated from the sputum of COPD patients were activated in the lung. Activated neutrophils will have higher levels of ROS production which will be released if these cells become necrotic.

Figure 6.3.1 shows the chemiluminescence i.e. ROS production of neutrophils isolated from the blood of healthy controls and asthmatic controls. The results show that neutrophils that had been previously primed with GM-CSF (50 U/ml) had an increased amount of ROS production compared to unprimed cells. This indicates that neutrophils present in the blood are in a non-activated resting state but can be primed by the addition of cytokines. As shown in figure 6.1.1, there were little if any neutrophils present in the sputum of these two patients groups therefore ROS production in sputum neutrophils could not be analysed.

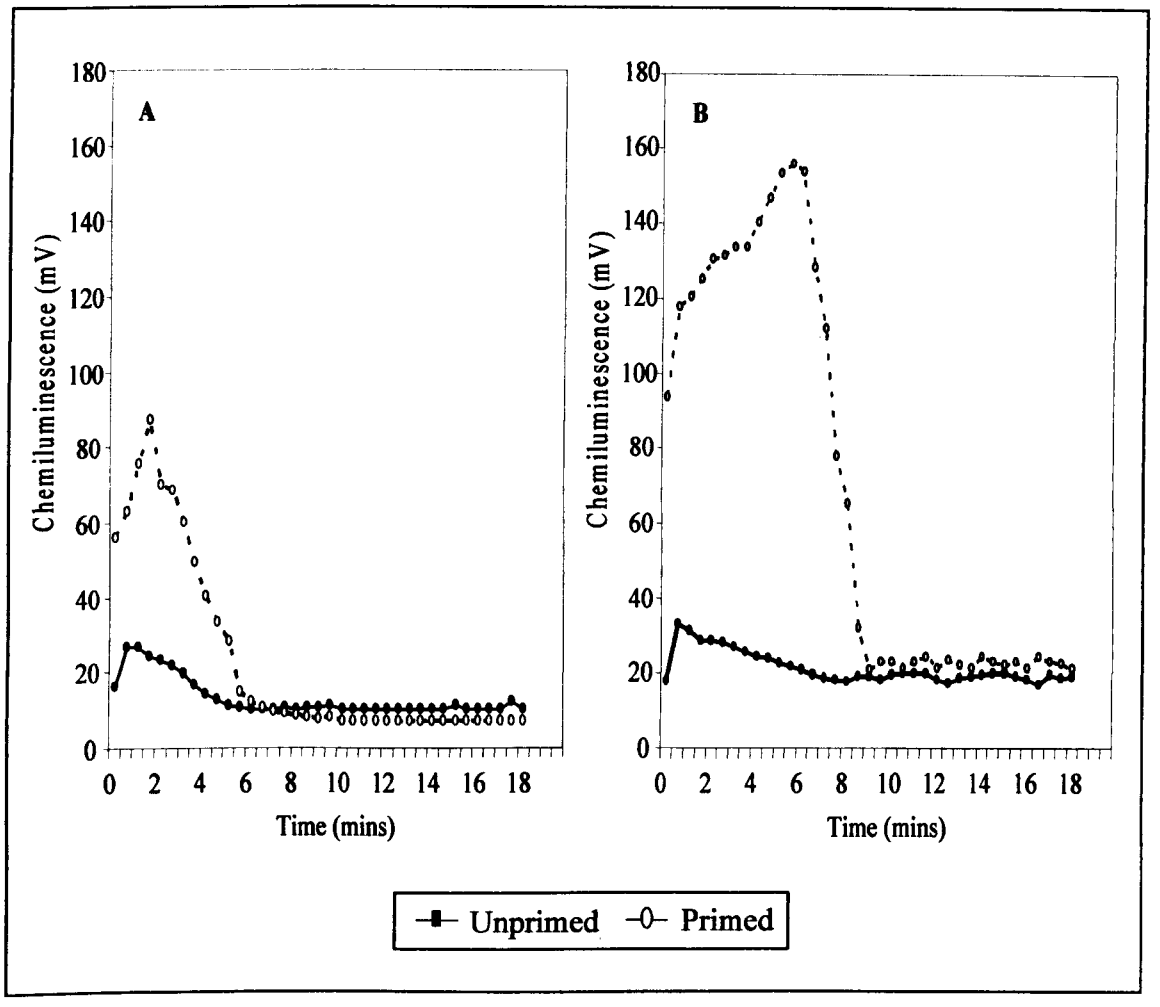

Figure 6.3.1: Neutrophils isolated from the blood of a healthy control (A) and an asthmatic control (B) were primed for 40 min with GM-CSF (50 U/ml). 10 μM luminol was used to measure the intracellular ROS production induced by fMLP in unprimed and primed neutrophils by chemiluminescence. Cells were measured every 15 sec for 20 min. Data is representative of 3 or more experiments.

Figure 6.3.2 shows the chemiluminescence generated from neutrophils isolated from the blood (C) and sputum (D) of COPD patients without a current *H. influenzae* infection after stimulation with fMLP. As in healthy controls and asthmatic patients, unprimed blood neutrophils do not show a marked increase in ROS production after stimulation but this does increase in cells that had been previously primed with GM-CSF. This was also shown in neutrophils isolated from the sputum of these patients. Unprimed cells did not produce a response after stimulation. However, this was greatly increased after priming with GM-CSF. This indicates that neutrophils that are present in the lungs of these patients are not activated and so will not release large amount of cytotoxic ROS and proteases into the lungs.

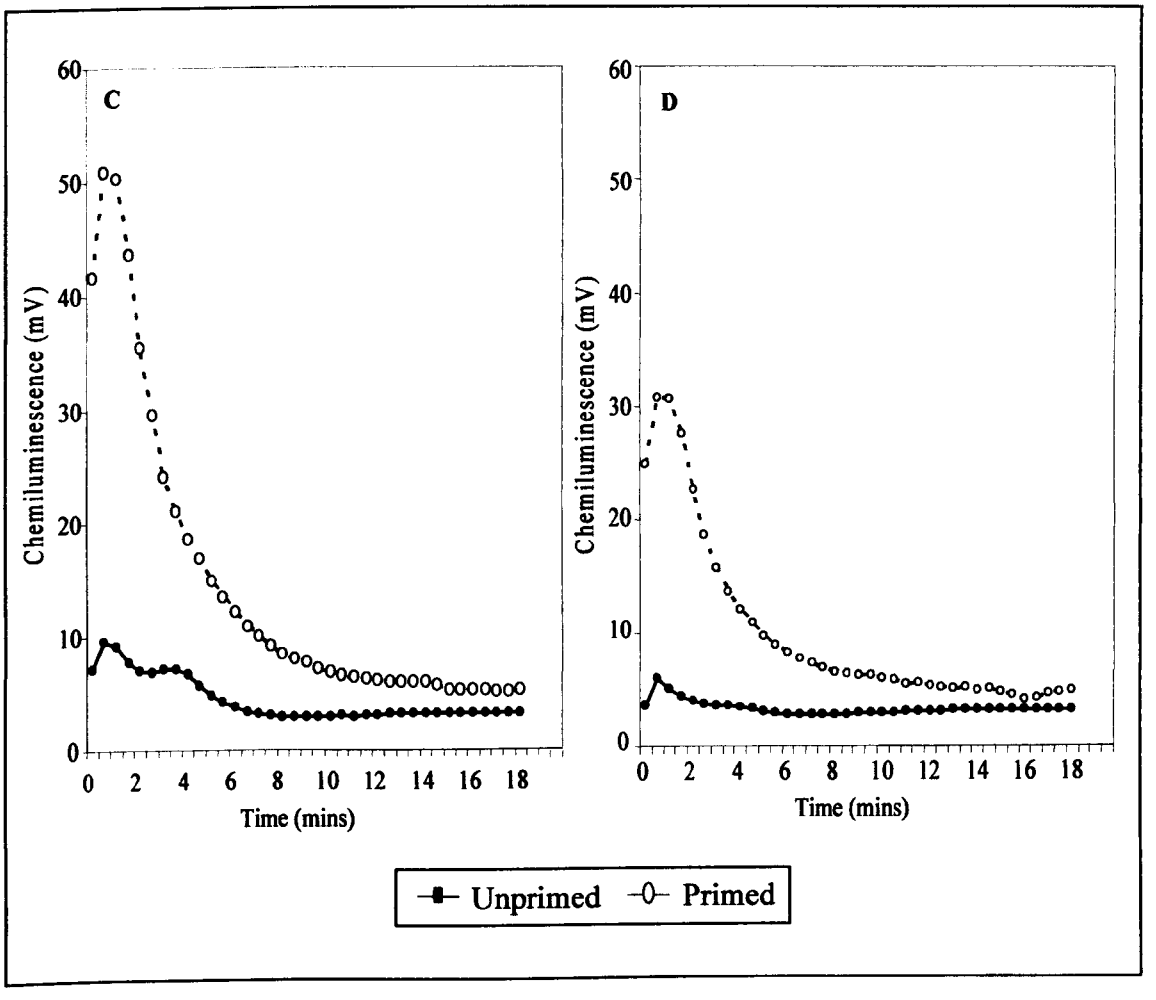

Figure 6.3.2: Neutrophils isolated from the blood (C) and sputum (D) of a COPD patient without a current *H. influenzae* infection were primed for 40 min with GM-CSF (50 U/ml). 10 μM luminol was used to measure the intracellular ROS production induced by fMLP in unprimed and primed neutrophils by chemiluminescence. Cells were measured every 15 sec for 20 min. Data is representative of 3 or more experiments.

Figure 6.3.3 shows the chemiluminescence generated by neutrophils isolated from the blood and sputum of COPD patients with a current *H. influenzae* infection. Figure E shows the chemiluminescence generated from neutrophils isolated from the blood of these patients. As with the other patient groups, neutrophils isolated from the blood are not activated and ROS production increases after the cells have been primed with GM-CSF. However, in cells that have been isolated from the sputum of these patients (F) the chemiluminescence generated was the same in both unprimed and primed cells i.e. ROS production did not increase in cells that had been pretreated with GM-CSF. This indicates that in COPD patients that have a current *H. influenzae* infection neutrophils that are recruited into the lung are primed and cannot be further activated by the addition of cytokines.

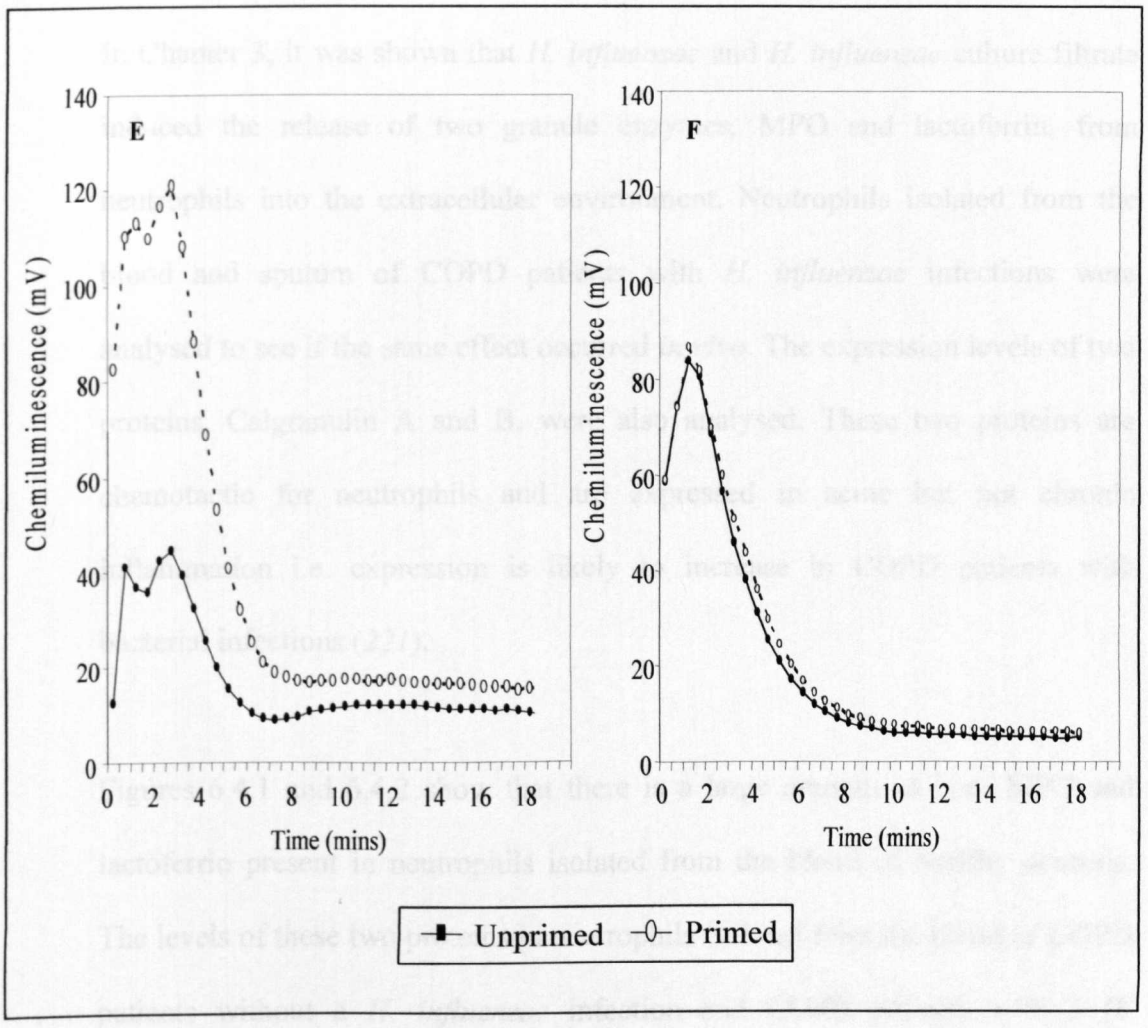

Figure 6.3.3: Neutrophils isolated from the blood (E) and sputum (F) of a COPD patient with a current *H. influenzae* infection were primed for 40 min with GM-CSF (50 U/ml). 10 μM luminol was used to measure the intracellular ROS production induced by fMLP in unprimed and primed neutrophils by chemiluminescence. Cells were measured every 15 sec for 20 min. Data is representative of 3 or more experiments.

6.4 *Analysis of Protein Levels in Neutrophils Isolated from Patient Blood and Sputum*

In Chapter 3, it was shown that *H. influenzae* and *H. influenzae* culture filtrate induced the release of two granule enzymes, MPO and lactoferrin, from neutrophils into the extracellular environment. Neutrophils isolated from the blood and sputum of COPD patients with *H. influenzae* infections were analysed to see if the same effect occurred *in vivo*. The expression levels of two proteins, Calgranulin A and B, were also analysed. These two proteins are chemotactic for neutrophils and are expressed in acute but not chronic inflammation i.e. expression is likely to increase in COPD patients with bacterial infections (*221*).

Figures 6.4.1 and 6.4.2 show that there is a large amount of both MPO and lactoferrin present in neutrophils isolated from the blood of healthy controls. The levels of these two proteins in neutrophils isolated from the blood of COPD patients without a *H. influenzae* infection and COPD patients with a *H. influenzae* infection was decreased slightly compared to healthy control neutrophils. However, there was still a significant amount of each protein present. It was not possible to compare the levels of MPO and lactoferrin present in healthy control sputum neutrophils as neutrophils are not present in these samples. There was a decrease in the levels of both MPO and lactoferrin detected in neutrophils isolated from the sputum of COPD patients without a *H.*

influenzae infection compared to blood neutrophils. However, these results are difficult to interpret because of the varying recovery of neutrophils in the different sputum samples, as reflected by the varying levels of GAPDH (used as a loading control). Nevertheless, the data indicates decreased expression of MPO and lactoferrin in sputum neutrophils of patients with *H. influenzae* infections.

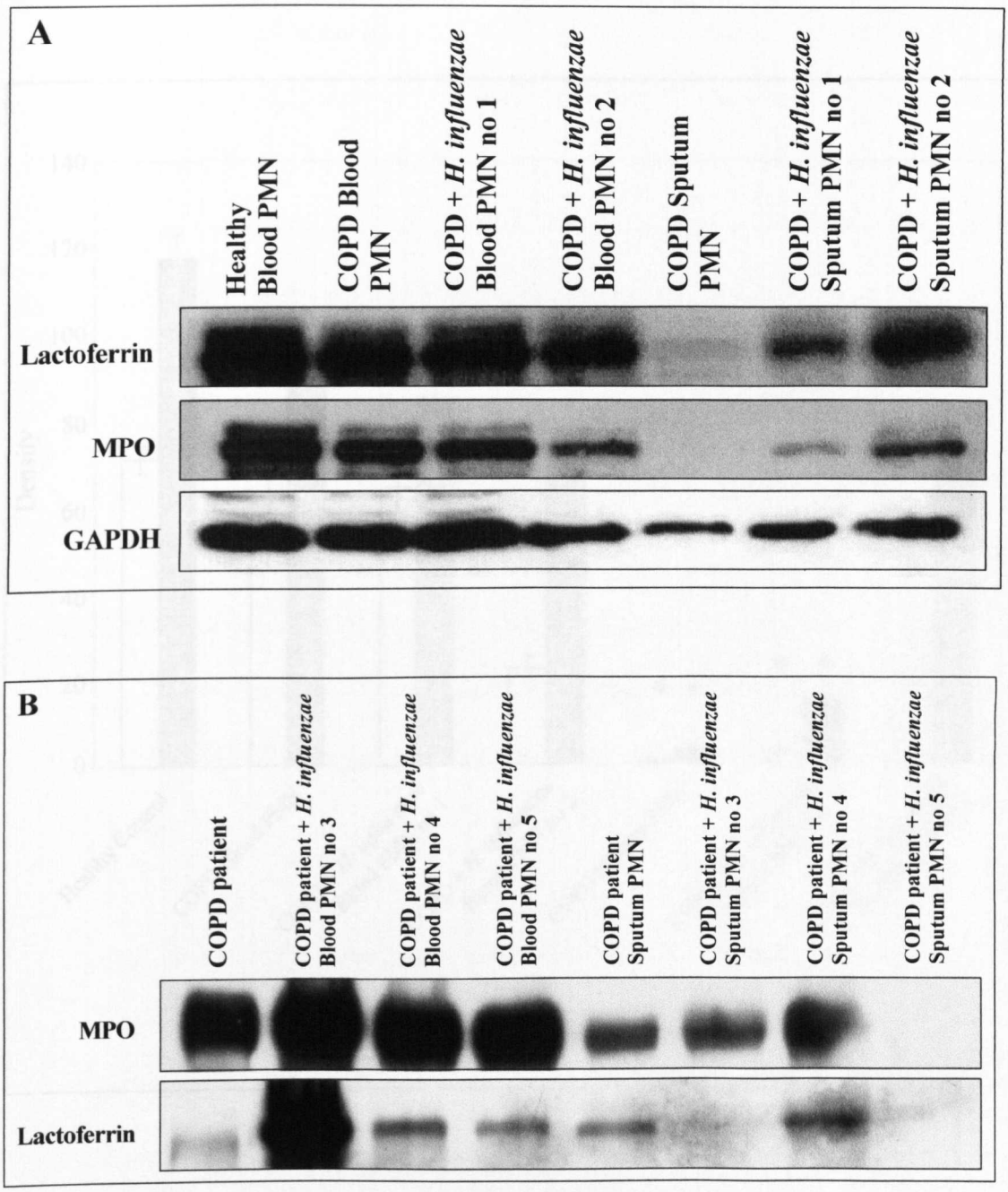

Figure 6.4.1: Neutrophils were isolated from the blood and sputum of healthy controls, COPD patients without a *H. influenzae* infection and COPD patients with a current *H. influenzae* infection. Protein lysates were prepared at 0 h and the levels of Lactoferrin, MPO and GAPDH detected via western blotting. Images are representative of 3 experiments and different patient samples shown in A and B.

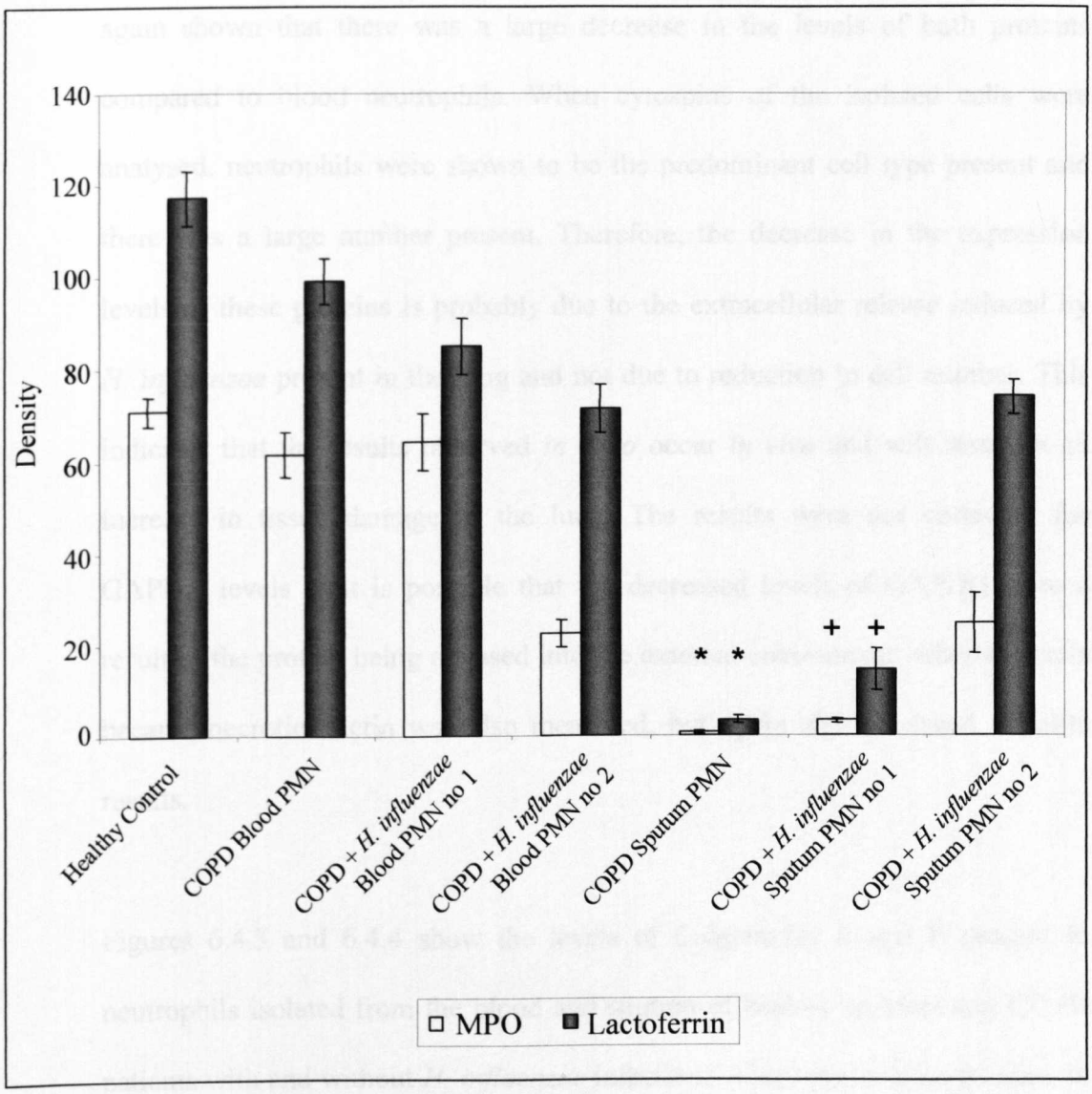

Figure 6.4.2: Neutrophils were isolated from the blood and sputum of healthy controls, COPD patients without a *H. influenzae* infection and COPD patients with a current *H. influenzae* infection. Protein lysates were prepared at 0 h and the levels of Lactoferrin, MPO and GAPDH detected via western blotting. Data are mean of 3 experiments from the images shown in *figure 6.4.1 A* ($\pm$ SD) * and + = $p < 0.05$ compared to the corresponding patient blood neutrophils.

When the levels of MPO and lactoferrin present in sputum neutrophils isolated from COPD patients with a current *H. influenzae* infection were analysed, it was again shown that there was a large decrease in the levels of both proteins compared to blood neutrophils. When cytospins of the isolated cells were analysed, neutrophils were shown to be the predominant cell type present and there was a large number present. Therefore, the decrease in the expression levels of these proteins is probably due to the extracellular release induced by *H. influenzae* present in the lung and not due to reduction in cell number. This indicates that the results observed *in vitro* occur *in vivo* and will result in an increase in tissue damage in the lung. The results were not corrected for GAPDH levels as it is possible that the decreased levels of GAPDH were a result of the protein being released into the external environment when the cells became necrotic. Actin was also measured, but again also produced variable results.

Figures 6.4.3 and 6.4.4 show the levels of Calgranulin A and B present in neutrophils isolated from the blood and sputum of healthy controls and COPD patients with and without *H. influenzae* infections. There was a large increase in the expression levels of calgranulin A in neutrophils isolated from the blood of COPD patients with a *H. influenzae* infection compared to healthy control blood neutrophils. This increased further in neutrophils isolated from the sputum of patients with a *H. influenzae* infection indicating that there is an increase in inflammation in the lung. There was also a significant increase in the expression

of Calgranulin B in neutrophils isolated from the blood of COPD patients with a *H. influenzae* infection compared to healthy controls. However, there was no Calgranulin B present in neutrophils isolated from the sputum of these patients. These results confirm that *H. influenzae* enhances the inflammatory response.

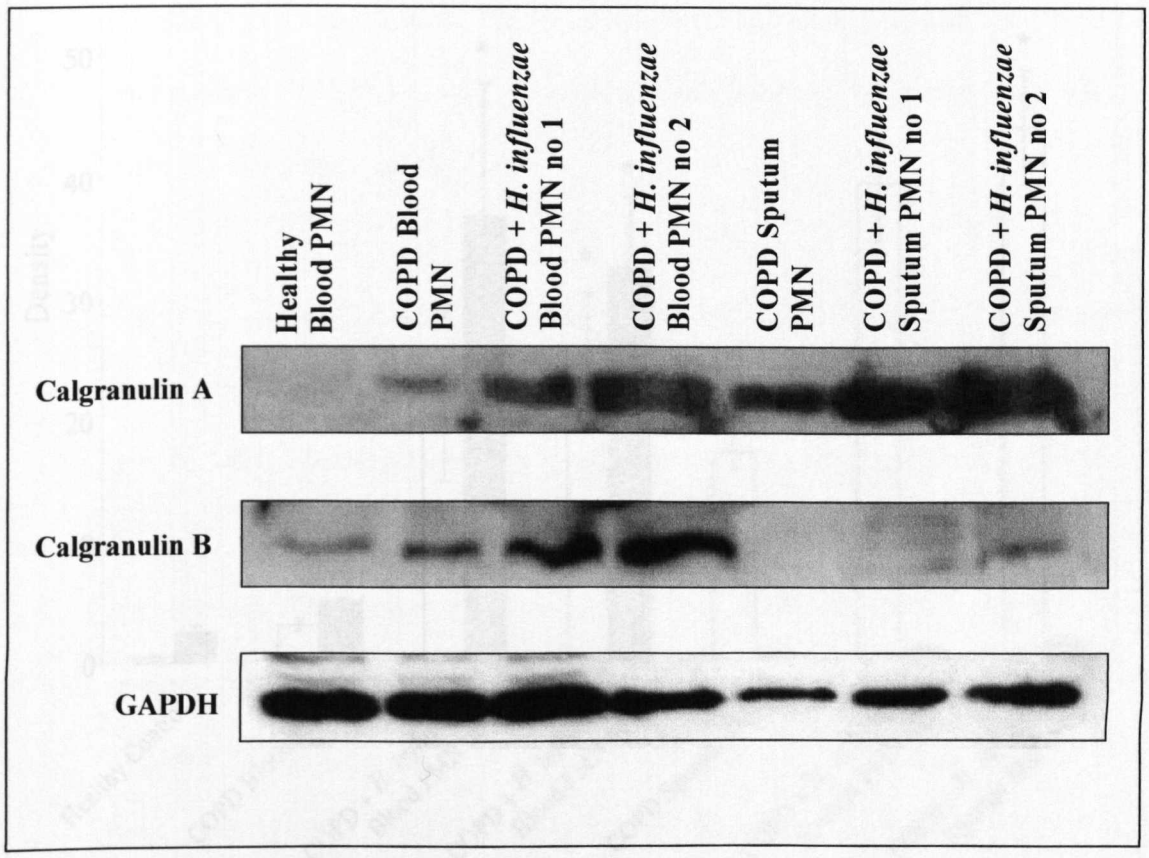

Figure 6.4.3: Neutrophils were isolated from the blood and sputum of healthy controls, COPD patients without a *H. influenzae* infection and COPD patients with a current *H. influenzae* infection. Protein lysates were prepared at 0 h and the levels of Calgranulin A and B and GAPDH detected via western blotting. Images are representative of 3 experiments.

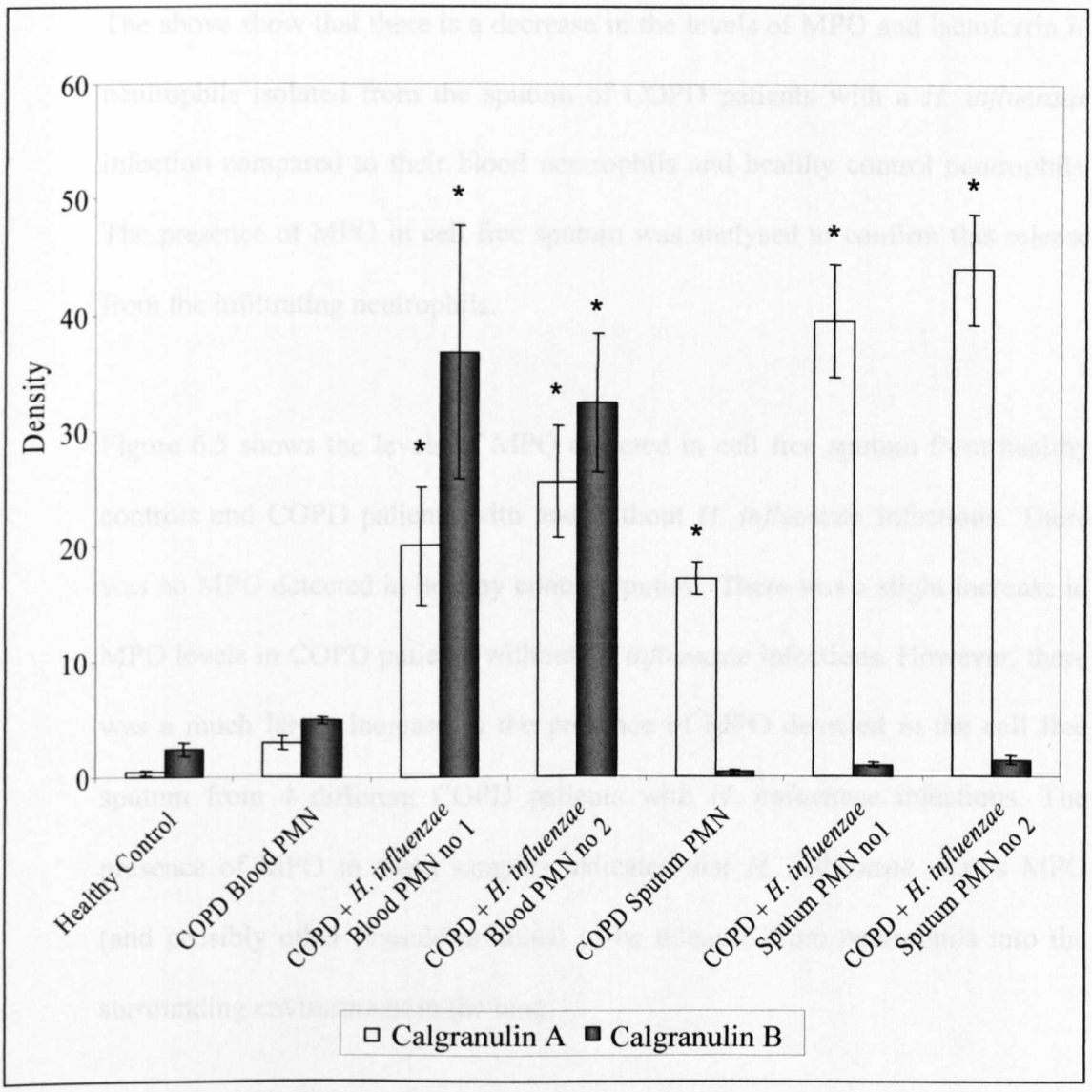

Figure 6.4.4: Neutrophils were isolated from the blood and sputum of healthy controls, COPD patients without a *H. influenzae* infection and COPD patients with a current *H. influenzae* infection. Protein lysates were prepared at 0 h and the levels of Calgranulin A and B detected via western blotting. Data is a mean of 3 experiments (± SD). * $p < 0.05$ compared to healthy control blood neutrophils.

6.5 *Analysis of the Presence of MPO in Cell Free Sputum*

The above show that there is a decrease in the levels of MPO and lactoferrin in neutrophils isolated from the sputum of COPD patients with a *H. influenzae* infection compared to their blood neutrophils and healthy control neutrophils. The presence of MPO in cell free sputum was analysed to confirm this release from the infiltrating neutrophils.

Figure 6.5 shows the levels of MPO detected in cell free sputum from healthy controls and COPD patients with and without *H. influenzae* infections. There was no MPO detected in healthy control sputum. There was a slight increase in MPO levels in COPD patients without *H. influenzae* infections. However, there was a much larger increase in the presence of MPO detected in the cell free sputum from 4 different COPD patients with *H. influenzae* infections. The presence of MPO in these samples indicates that *H. influenzae* causes MPO (and possibly other granule proteins) to be released from neutrophils into the surrounding environment in the lung.

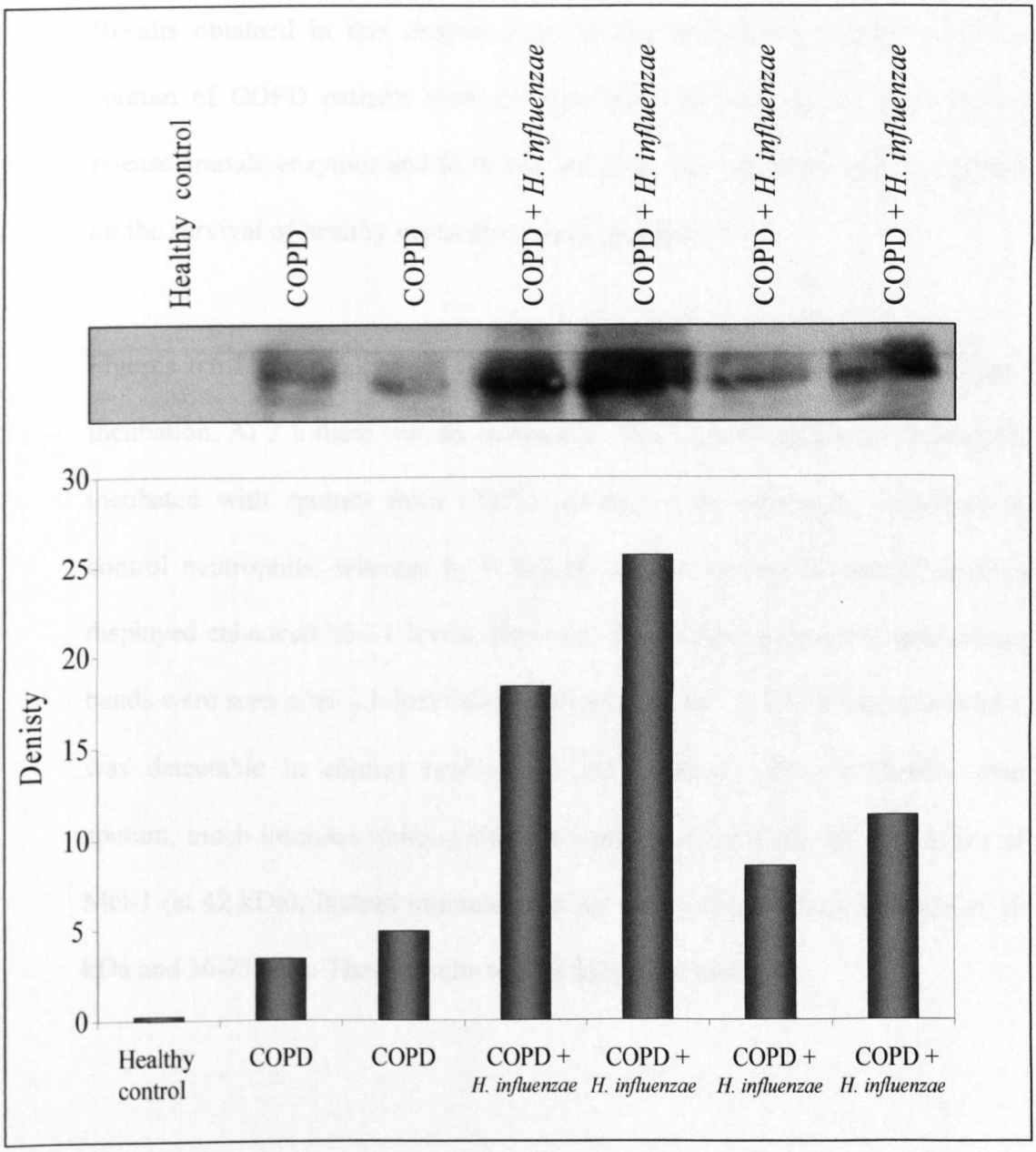

Figure 6.5.1: Cell free sputum was obtained by centrifuging at 600 g for 5 min to pellet the cells. Protein lysates were prepared by the addition of 5 x SDS-PAGE sample buffer to the cell free sputum. Levels of MPO present in the sputum were analysed by western blotting.

6.6 *Effects of Cell Free Sputum on Mcl-1 Levels in Healthy Neutrophils*

Results obtained in this chapter indicate that neutrophils isolated from the sputum of COPD patients with *H. influenzae* infection die by necrosis and release granule enzymes and ROS into the lung. The effects of cell free sputum on the survival of healthy neutrophils were investigated.

Figures 6.6.1 and 6.6.2 show the levels of Mcl-1 present in neutrophils after incubation. At 2 h there was an increase in Mcl-1 levels present in neutrophils incubated with sputum from COPD patients + *H. influenzae* compared to control neutrophils, whereas by 6 h both infected and non- infected sputum displayed enhanced Mcl-1 levels. However, lower and higher molecular weight bands were seen after 6 h incubation with sputum. By 18 h incubation no Mcl-1 was detectable in control neutrophils, but curiously after incubation with sputum, much immuno-staining was detectable, but not at the predicted size of Mcl-1 (at 42 kDa), Instead immuno staining was evident indicating bands at 55 kDa and 36-21 kDa. These results will be discussed later.

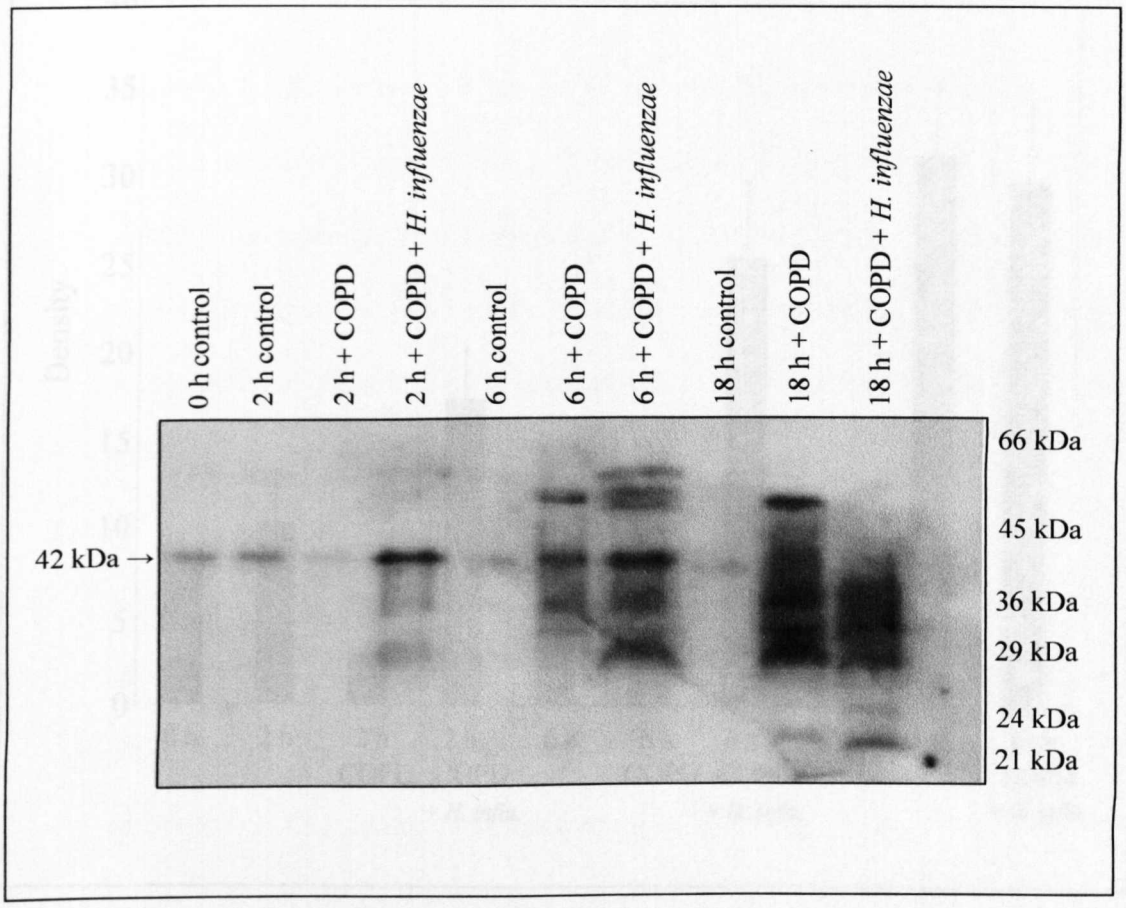

Figure 6.6.1: Neutrophils isolated from a healthy control and incubated with cell free sputum (25 %) from 2 COPD patients with and without *H. influenzae* infections at 37 °C. Protein lysates were prepared at 2, 6 and 18 h and the effects of the sputum on neutrophils Mcl-1 levels analysed by western blotting. Image is representative of 2 experiments.

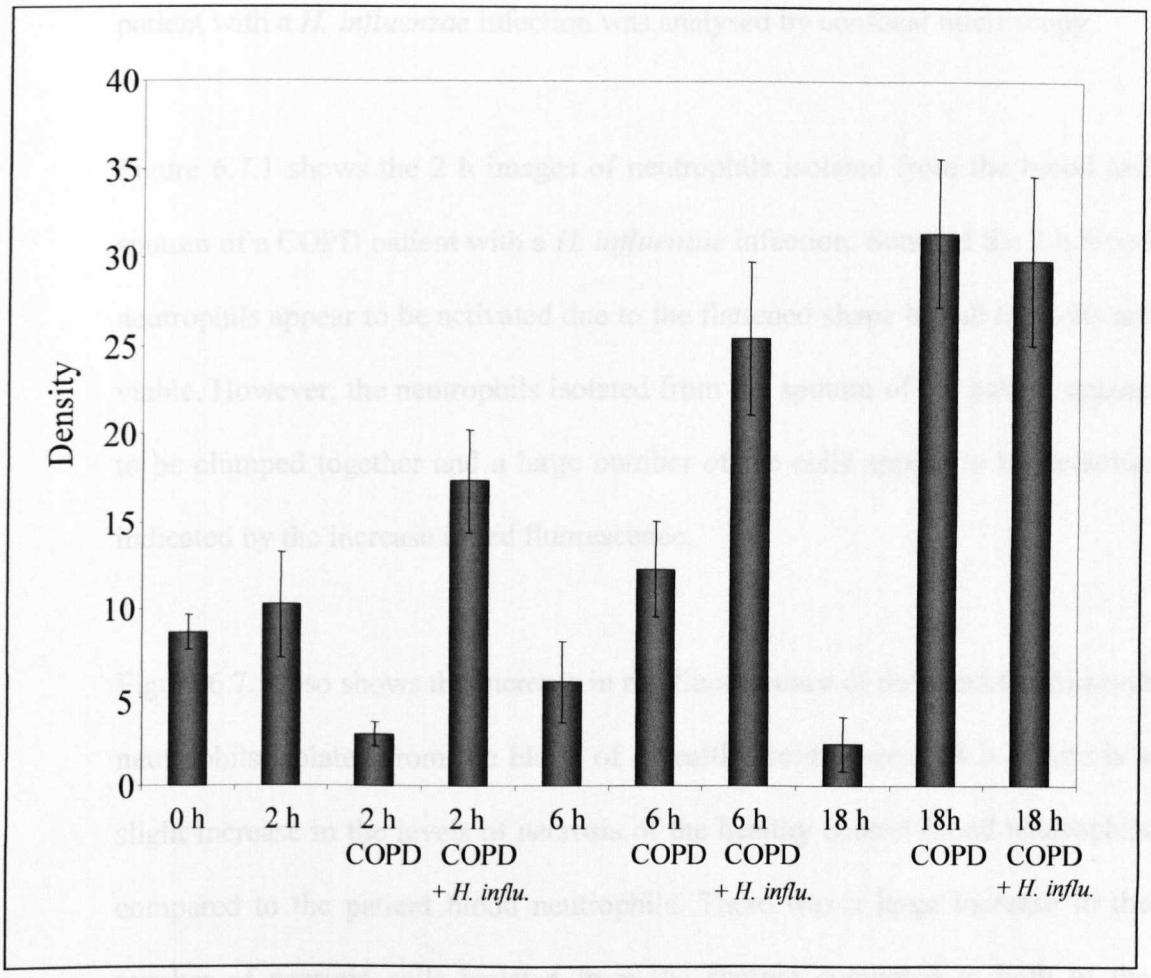

Figure 6.6.2: Neutrophils isolated from a healthy control and incubated with cell free sputum (25 %) from 2 COPD patients with and without *H. influenzae* infections at 37 °C. Protein lysates were prepared at 2, 6 and 18 h and the effects of the sputum on neutrophil Mcl-1 levels analysed by western blotting, densitometry was measured for the 42 kDa band. Graph is representative of 2 experiments.

6.7 *Confocal Analysis of Neutrophils Isolated from Patient Blood and Sputum*

The viability of neutrophils isolated from the blood and sputum of a COPD patient with a *H. influenzae* infection was analysed by confocal microscopy.

Figure 6.7.1 shows the 2 h images of neutrophils isolated from the blood and sputum of a COPD patient with a *H. influenzae* infection. Some of the 2 h blood neutrophils appear to be activated due to the flattened shape but all the cells are viable. However, the neutrophils isolated from the sputum of the patient appear to be clumped together and a large number of the cells appear to be necrotic, indicated by the increase in red fluorescence.

Figure 6.7.1 also shows the increase in red fluorescence of these neutrophils and neutrophils isolated from the blood of a healthy control over 18 h. There is a slight increase in the levels of necrosis of the healthy control blood neutrophils compared to the patient blood neutrophils. There was a large increase in the number of necrotic cells isolated from the sputum compared to both of the blood samples. At ~ 11 h there was a decrease in red fluorescence in cells isolated from the sputum of the COPD patient. It can be seen in figure 6.7.2 that these sputum neutrophils become clumped by 8-12 and that small, red fluorescent particles are detected past 12 h incubation. These are PI-stained bacteria released from the necrotic neutrophils. This confirms the hypothesis that neutrophils phagocytose *H. influenzae* in the lung but then die by necrosis

releasing cytotoxic products and live bacteria into the lung. This results in an increase in infection and tissue damage *in vivo*.

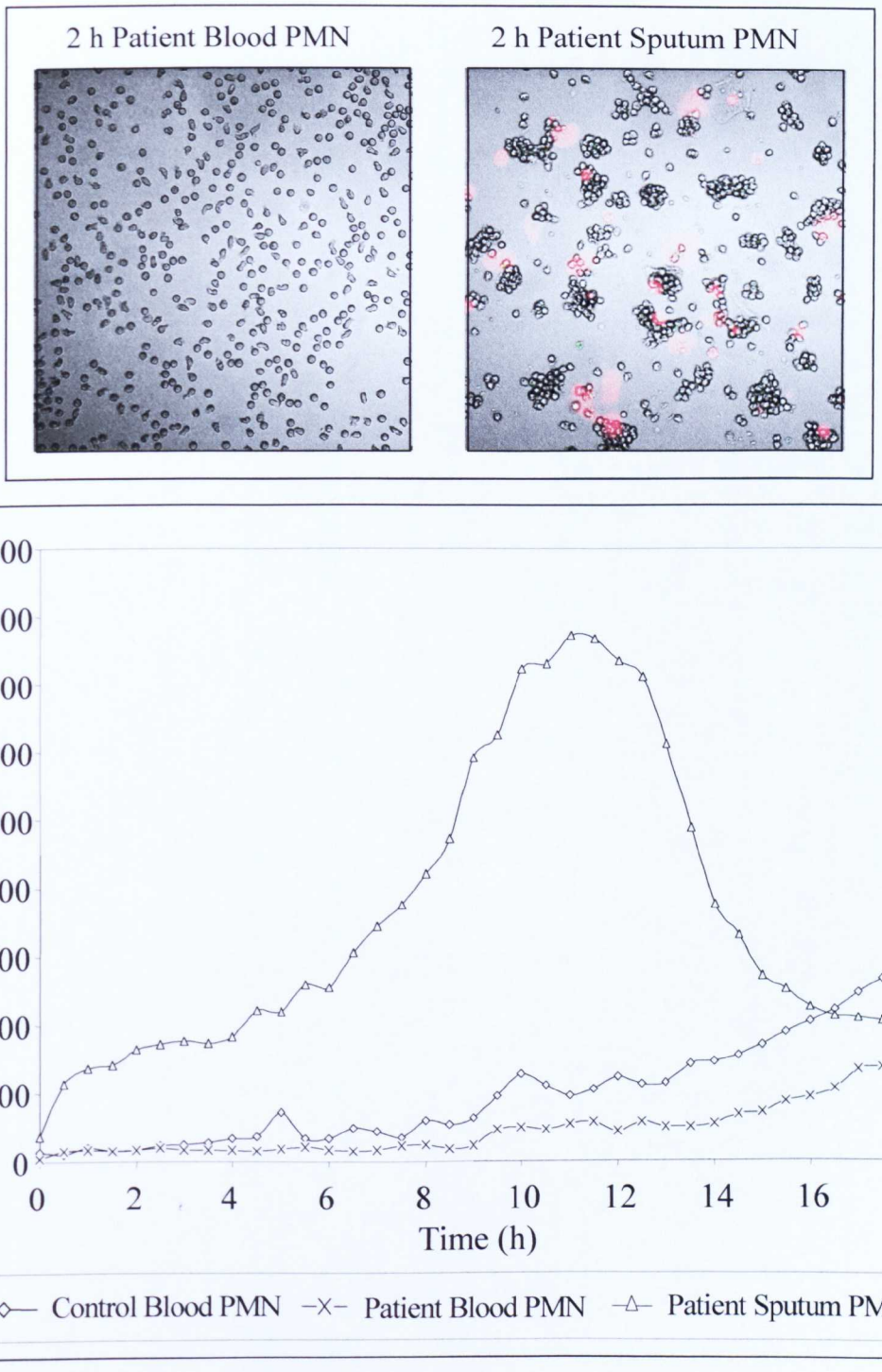

Figure 6.7.1: Confocal microscopy analysis of neutrophils isolated from the blood and sputum of a patient with a *H. influenzae* infection. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) to analyse the viability of the neutrophils. Cells were imaged every 30 min for 18 h at x 20 magnification.

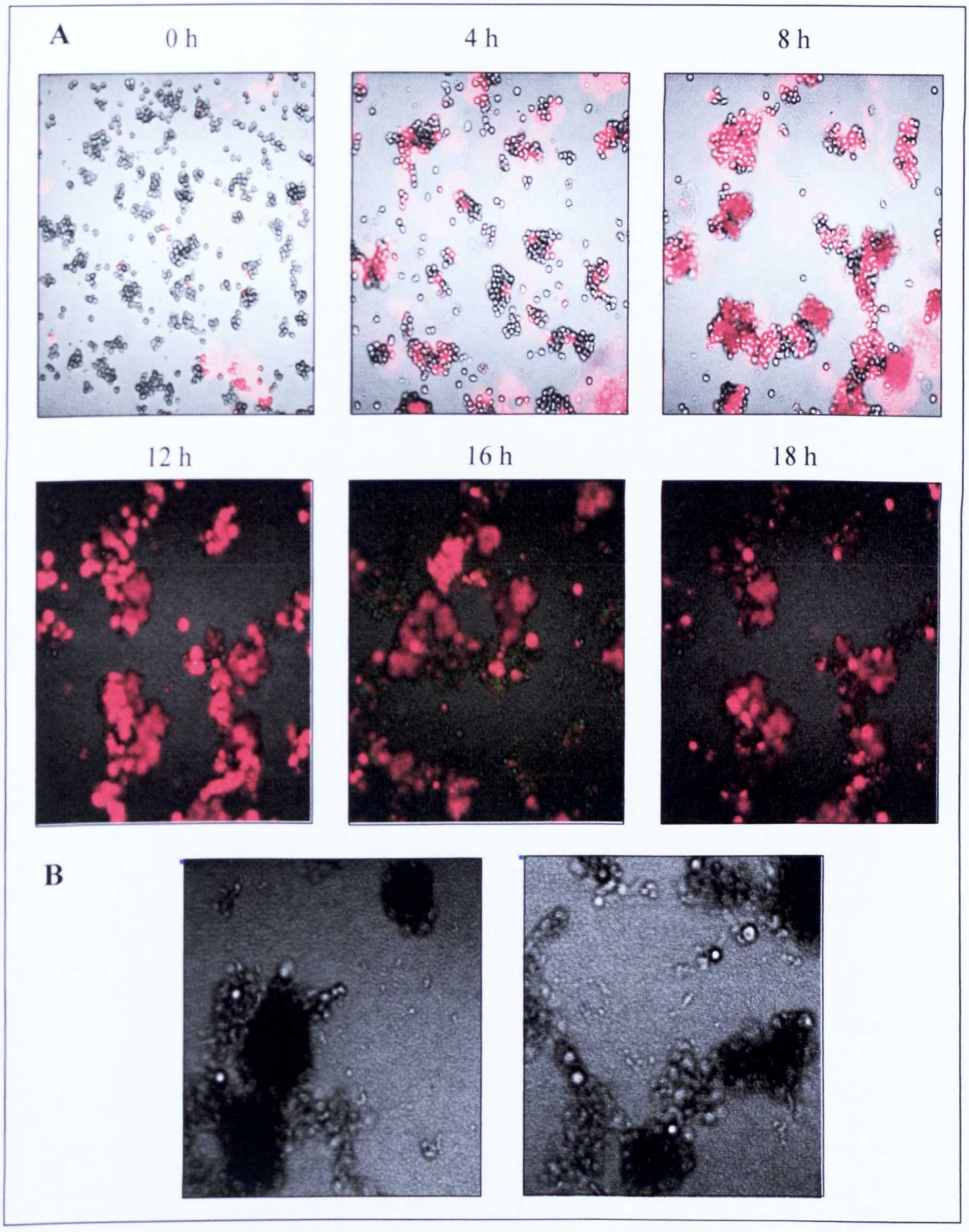

Figure 6.7.2: Confocal microscopy analysis of neutrophils isolated from the sputum of a patient with a *H. influenzae* infection. Neutrophils were incubated with Annexin-V-FITC conjugate (1 µl/ml) and PI (1 µg/ml) to analyse the viability of the neutrophils. Cells were imaged every 30 min for 18 h at x 20 magnification (A). Images shown were taken at 0, 8, 12, 16 and 18 h (A) and 12 h enlarged images (B)

Discussion

This Chapter has studied neutrophils isolated from the blood and sputum of healthy controls, asthmatic patients and COPD patients with and without *H. influenzae* infections. By studying the morphology and the levels of protein expression in these neutrophils it is possible to determine whether the effects of *H. influenzae* observed *in vitro* also occur *in vivo* in the COPD lung contributing to exacerbations of the disease. It must be stressed that due to the rather small sample sizes and variability in the data, these results must be viewed as preliminary. Nevertheless, they are indicative that many of the events observed *in vitro* during incubation of neutrophils with *H. influenzae* also occur *in vivo* during infection of COPD.

The morphology of the isolated neutrophils was analysed by cytospin and electron microscopy. Sputum from healthy controls and asthmatic patients was shown to have very little, if any, neutrophils present. COPD patients had an increase in the number of neutrophils present but other cell types were also observed. In the sputum of COPD patients with a *H. influenzae* infection extremely high numbers of neutrophils were present. When observed under a high magnification, these neutrophils appeared to have an abnormal morphology e.g. an unusual nuclear shape. The neutrophils isolated from the blood of COPD patients with and without infections appeared to be activated. However, when patients had a severe infection these neutrophils also appeared to have an abnormal morphology.

When isolated neutrophils were analysed by electron microscopy it was shown that neutrophils isolated from the sputum of asthmatic patients and COPD patients without a *H. influenzae* infection were activated, cells had a ruffled cytoplasm and vacuoles present, but otherwise had a normal morphology. However, neutrophils isolated from the sputum of COPD patients with a *H. influenzae* infection had a grossly abnormal morphology. Neutrophils contained large phagosomes, with many bacteria inside, rather than 1-2 bacteria observed in small phagosomes, as seen during phagocytosis of organisms such as *S. aureus*. Bacteria were also present in the cytoplasm of the cells. Many large vacuoles were present and the cells also appeared to have small finger like protrusions appearing from the plasma membrane. Nuclear morphology also appeared to be extremely abnormal. The morphology of these isolated neutrophils was similar to that observed in healthy neutrophils that had been incubated with *H. influenzae in vitro*.

The levels of ROS production by the isolated cells was analysed by chemiluminescence. Blood neutrophils isolated from all types of patients were not activated in the bloodstream and could be primed *in vitro* by the addition of GM-CSF. This was also observed in neutrophils isolated from the sputum of COPD patients without a *H. influenzae* infection. However, neutrophils isolated from the sputum of COPD patients with a current *H. influenzae* infection produced the same amount of chemiluminescence as cells that had been previously primed with GM-CSF *in vitro*. This suggests that neutrophils present in the lungs of these patients have been primed or activated *in vivo*.

The levels of MPO and lactoferrin present in neutrophils isolated from the blood and sputum of COPD patients with *H. influenzae* infections were also analysed and it was shown that the levels of both granule proteins in sputum neutrophils were decreased compared to the neutrophils isolated from the patients' blood. This suggests that granule proteins have been released into the extracellular environment i.e. the lung following infection. This was further confirmed by the presence of MPO detected in cell free sputum. The presence of MPO in the cell free sputum is indicative that the neutrophils are dying by necrosis and releasing their cytotoxic products. It was also shown that there are factors in the sputum of COPD patients with a *H. influenzae* infection that can affect degradation of Mcl-1. It is noteworthy that large amounts of immuno staining was detected after incubation with sputum, but not in control cells. Full length Mcl-1 has a relative molecular mass of about 42 kDa, but lower smaller bands may represent Mcl-1 turnover products e.g. as a results of caspase cleavage (*223*). It is possible that the larger bands are ubiquitinated forms of Mcl-1 (*224*). It is also curious to note that in cells that are triggered to undergo necrosis, there is such a large amount of immuno stained Mcl-1, allbeit of altered size and hence likely to be non-functional. This could be a futile attempt by the cells to induce survival mechanisms is response to factors in the sputum.

When the viability of neutrophils isolated from the blood and sputum of a COPD patient with a *H. influenzae* infection was analysed by confocal microscopy, it was shown that sputum neutrophils had increased levels of necrosis compared to blood neutrophils. It was also observed that when these cells died by necrosis live bacteria

were released into the surrounding environment. This further confirms previous results that suggest that *H. influenzae* are able to survive and replicate inside a neutrophil, increasing bacterial infection after neutrophil necrosis (*204*).

The results obtained in this chapter confirm that the phenomenon previously discussed in Chapters 3-5 also occurs *in vivo* inside the COPD lung. Neutrophils die by necrosis upon contact with *H. influenzae* (*186*). Cytotoxic products and live bacteria are then released into the lung, leading to an increase in tissue destruction and bacterial infection. More neutrophils are then recruited to the lung to fight the infection. This results in a prolonged inflammatory response and an increase in tissue damage. The preliminary data described in this Chapter merit a more intensive and detailed study of this phenomenon.

Chapter 7: General Discussion

During exacerbations of COPD, large numbers of neutrophils may be present in the lungs (*2, 9*) often with large numbers of *H. influenzae* present and tissue damage is evident. The large numbers of neutrophils recruited into the lungs should be expected to kill the invading bacteria, but they evidently do not. This suggests that neutrophils are not functioning correctly and are unable to efficiently kill *H. influenzae* inside the COPD lung. The results previously obtained and those described in this thesis may help explain the clinical picture in COPD, via the following series of events (*204, 205*). Neutrophils recruited into the lung phagocytose, but are unable to kill *H. influenzae* and die by necrosis due to the release of necrosis-inducing factors by the bacteria. This results in the release of live bacteria into the surrounding environment as well as released cytotoxic products of the neutrophil i.e. ROS and proteases resulting in lung damage. *H. influenzae* also induces the release of the chemoattractant IL-8 from neutrophils leading to the recruitment of more neutrophils into the lungs to fight the infection and by these processes the vicious circle continues (*204, 205*).

H. influenzae was cultured *in vitro* and it was shown that the culture filtrate obtained also induced neutrophil necrosis with similar kinetics as the bacteria, indicating that these released factor(s) may be the same as those that induce neutrophil necrosis during phagocytosis (*205*). The identity of this factor(s) and the mechanisms by which it can induce neutrophil necrosis could shed new insights into the complex pathology of COPD.

The aims of this thesis were to try and identify the necrotic factor(s) released by non-encapsulated *H. influenzae* and to elucidate the mechanisms by which necrosis is induced. It was also important to determine, by the study of sputum samples, whether the effects of *H. influenzae* observed *in vitro* also occur *in vivo*. I found that:

- 10 different non-encapsulated *H. influenzae* clinical isolates induced neutrophil necrosis, albeit to varying degrees.

- At least 2 factors were present in *H. influenzae* culture filtrate that worked together to inhibit apoptosis and induce necrosis. These factors were shown to be > 10 kDa and < 5 kDa in size (by size exclusion filtration).

- Necrosis was induced by the activation of an intracellular MAPK signalling pathway.

- The factors appear to induce necrosis by binding to TLR2 and TLR4 receptors that are expressed by myeloid cells and this may explain why myeloid cells respond to the factor(s).

- Isolation of neutrophils from the sputum samples of COPD patients with and without *H. influenzae* infections confirms that both the morphological and biochemical effects shown *in vitro* also occur *in vivo*.

In order to obtain insights into the kinetics and mechanisms regulating neutrophil viability, a number of assays were evaluated. Neutrophil viability is commonly studied by morphological analysis as stained cells have a distinctive morphology (as discussed in *1.4 Neutrophil Apoptosis)*. Apoptotic and necrotic cells can also be analysed by flow cytometry by staining the cells with annexin-V and propidium iodide (PI). As cells become apoptotic, phosphatidylserine becomes exposed on the outer leaflet of the plasma membrane and this, in the presence of Ca^{2+} can bind FITC-annexinV to exhibit green fluorescence. As cells progress into apoptosis, the plasma membrane may become leaky (sometimes referred to as late apoptosis or secondary necrosis) and can become leaky to dyes such PI. If PI enters cells it binds nucleic acids and cells become red. Clearly, if cells lyse by necrosis, they are not detected by microscopy or flow cytometry. Thus cells that stain red with PI are often referred to as "necrotic" whereas in reality they have a permeable and dysfunctional plasma membrane. In this thesis, the assay of choice for studying neutrophil cell death was confocal microscopy. This technique was chosen for several reasons. First, staining with annexin and PI requires several centrifugation/washing./resuspension and incubation steps and this can lyse fragile cells prior to analysis by flow cytometry. Second, these essential processing steps for staining for flow cytometry are *time* consuming and hence detailed kinetic data on multiple samples can be difficult to obtain. The assay developed to utilise confocal microscopy overcomes these limitations as cells are never removed from their optimal culture conditions.

This technique, together with electron microscopy has shown that *H. influenzae* induces neutrophil necrosis and the bacteria are also able to survive and replicate inside the neutrophil. Electron microscopy revealed many vacuoles containing numerous bacteria but also bacteria present in the cytoplasm. A number of different microbes have been shown to evade neutrophil killing in similar ways to *H. influenzae* i.e. by surviving inside the phagolysosome and by escaping into the cytoplasm. *Streptococcus pyogenes* is a one such pathogen where streptococcal proteins H and M mediate *S. pyogenes* survival inside the phagolysosome (*225*). *S. pyogenes* also forms a capsule that enables the bacterium to escape into the cytoplasm as shown by electron microscopy (*226*). It is thought that *S. pyogenes* can upregulate expression of Ihk-Irr two-component gene-regulatory. Another possible mechanism of bacterial survival is the ability of bacteria to direct the generation of ROS to the extracellular space as shown by *Helicobacter pylori* (*227*). It has been shown in Chapters 3 and 4 of this thesis that *H. influenzae* culture filtrate increases ROS production from neutrophils and activates PKC, a protein required for the activation of the NADPH oxidase complex indicating that *H. influenzae* is involved in increased ROS production via the respiratory burst.

Further investigations showed that *H. influenzae* release at least 2 factors that have effects on neutrophil apoptosis and necrosis. A factor > 10 kDa induced rapid necrosis and inhibited apoptosis. A number of different bacteria have been shown to have similar effects on neutrophil survival. *S. pyogenes, Clostridium perfrinigens* and *S. aureus* have all been shown to release factors that induce neutrophil lysis (*228, 229,*

230). *S. pneumoniae* releases a 53 kDa pore forming toxin, pneumolysin that inhibits ROS production and induces neutrophil necrosis (*208*) and I originally considered if the factor released by *H. influenzae* could work by a similar mechanism. However, the factors released by *H. influenzae* actually increase ROS production and also the 2-D map of the *H. influenzae* proteome has shown that the bacteria do not express either hemolysins or pneumolysins (*189, 190*).

Another factor < 5 kDa was identified and was shown to slightly increase apoptosis as well as causing an increase in necrosis. Some bacteria act in a similar manner i.e. *Pseudomonas aeruginosa* releases a phenazine called pyocyanin (*231*). Pyocyanin has been shown to increase neutrophil apoptosis thereby suppressing the inflammatory response resulting in increased bacterial survival. *Shigella flexneri* is another example of a pathogen which releases a cytotoxic factor that induces neutrophil apoptosis (*232*). However, the < 5 kDa *H. influenzae* necrotic factor also induces necrosis as well as apoptosis.

Results obtained in Chapter 4 and 5 of this thesis indicate that the necrotic factors are activating intracellular signaling cascades via TLR2 and TLR4. TLR4 is known to bind bacterial LPS and induce an inflammatory response (*120*). Previous research has shown that treatment of the factor with polymixin, an antibiotic that disrupts the structure of many types of LPS, does not have an effect on the ability of the factor to induce necrosis (*205*). However, *H. influenzae* has been shown to produce atypical forms of LPS referred to as lipooligosaccharide (LOS) (*233*). LOS possesses much shorter

oligosaccharide side chains than LPS and may not be affected by polymixin: there is thus a possibility that the > 10 kDa factor may be *H. influenzae* LOS. This can be further investigated by studying the effects of a number of purified *H. influenzae* LOS to determine if similar effects are observed.

Preliminary work has suggested that the < 5 kDa factor may possibly be a diacylated lipopeptide due to its ability to induce necrosis via a TLR2:TLR6 pathway (*121*). Factors that bind via TLR2:TLR6 have been shown to activate an intracellular MyD88 dependent pathway (*234*). This is further confirmed by the ability of the factor to activate an intracellular p38 MAPK pathway. Further work can be carried out using a number of other synthetic diacylated lipopeptides to investigate this further. As previously mentioned, *S. flexneri* releases a cytotoxic factor that is capable of inducing neutrophil apoptosis. Purification approaches have shown that this factor is a triacylated bacterial lipoprotein that works by activating TLR2 (*235*). The factor is also capable of inducing the release of proinflammatory cytokines. This adds strength to my hypothesis that the factors released by *H. influenzae* are bacterial lipoproteins capable of inducing an inflammatory response via TLR2.

While the mechanisms that regulate neutrophil apoptosis are becoming elucidated (*41, 42, 143)* those that control necrosis are relatively understudied. My work in this thesis implicates TLR2/TLR4 and signalling pathways such as p38 MAPK. In view of the potential pathological importance of neutrophil necrosis in COPD, further work into the mechanisms that control this form of death are now required.

COPD is expected to rise to the 5th leading cause of death worldwide by 2012 (*1, 2*). Current therapies for the treatment of this disease are not very efficient and it is therefore necessary to develop new therapies for the treatment of CODP (22). Identifying the necrotic factors that are released by *H. influenzae* and further elucidating the mechanism by which necrosis is induced may lead to potential drug targets which can block the interactions of the necrotic factors with neutrophils and other myeloid cells. This may result in reduced exacerbations of the disease and therefore reduce the cost of treating COPD.

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