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MECHANISMS REGULATING GRANULOCYTE APOPTOSIS

Carol Ward

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DECLARATION

The work presented in this thesis was carried out solely by the author, unless otherwise stated, under the supervision of Dr Adriano Rossi, Professor Edwin Chilvers, Rayne Laboratory, University of Edinburgh, and Dr Stuart Farrow, Glaxo-Wellcome, Stevenage, Herts.

Carol Ward

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ABSTRACT

Inflammation is a physiological, innate, normally beneficial response to tissue injury and infection. If the inflammatory response occurs in an uncontrolled manner or fails to resolve, pathological consequences may include excessive tissue injury, chronic inflammation and scarring. Neutrophilic and eosinophilic granulocytes are key effector cells of the inflammatory response which contain proteases and have the ability to generate inflammatory mediators. If these potentially histotoxic contents are released indiscriminately excessive tissue damage may result. Apoptosis in granulocytes is now thought to represent a key process in the normal control and resolution of inflammation. It leads to shut-down of granulocyte secretory processes and removal of the intact senescent cell by macrophages which utilise a novel phagocytic recognition mechanism that fails to trigger macrophage mediator release.

Few factors present at inflammatory foci have been demonstrated to accelerate granulocyte apoptosis; most inhibit the process, adding to the longevity of these cells. The purpose of these studies was to examine the role of different classes of inflammatory mediators in the induction of granulocyte apoptosis and investigate the possible mechanisms involved.

We have shown that the majority of pro-inflammatory cytokines inhibit the process of apoptosis in granulocytes. The anti-inflammatory cytokine IL-10 had no effect on granulocyte apoptosis, but in neutrophils, it inhibited the survival effects of LPS. TGF-β, inhibited granulocyte apoptosis but potentiated the pro-apoptotic effects of TNF-α, at early timepoints in neutrophils. Eicosanoids also exert differential effects on granulocyte apoptosis, however prostaglandins of the J series proved to be potent inducers of apoptosis in both cell types. The use of receptor agonists demonstrated that these pro-apoptotic effects may be mediated via a class of intracellular peroxisome proliferator activated receptors (PPARs), for which these prostanoids are ligands.

Both TGF-β and PPARs can inhibit the transcription factor nuclear factor-kappa B (NF-κB). Further examination of the role of this transcription factor in granulocyte apoptosis illustrated that it exerts potent survival effects in both neutrophils and eosinophils, and that inhibition of its activation synergistically enhanced the pro-apoptotic response of TNF-α in neutrophils, and induced eosinophils to respond to the pro-apoptotic effects of this cytokine.

The data presented strongly support the hypothesis that NF-κB activation is necessary for the survival of granulocytes, and that certain physiological mediators, such as TNF-α, prostaglandins of the J series and the cytokine TGF-β may be involved in the physiological resolution of inflammation, by inhibiting the activity of this transcription factor.

ABBREVIATIONS

AA	Arachidonic acid
ARDS	Adult respiratory distress syndrome
AP-1	Activator protein 1
ASK-1	Apoptosis signal-regulating kinase-1
ATF2	Activating transcription factor 2
BAL	Bronchoalveolar lavage fluid
BPI	Bacterial/permeability-inducing protein
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CCR	Chemokine receptor
CD	Cluster differentiation antigen
COX	Cyclooxygenase
db-cAMP	Dibutyral cyclic adenosine monophosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxy-ribonucleic acid
DPC4	Deleted in pancreatic cancer 4
EMSA	Electromobility shift assay
EPC	Eosinophil cationic protein
ERK	Extracellular signal-related kinase
FADD	Fas-associated protein with death domain
FAN	Factor associated with N-Smase activation
FITC	Fluorescein isothiocynate
FLICE	FADD-like interleukin-1β converting enzyme
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GSH	Glutathione
GSNO	S-nitrosoglutathione
GSSG	Oxidised glutathione
HPETE	Hydroperoxyeicosantetraenoic acid

ICAM-1	Intercellular adhesion molecule-1
ICE	Interleukin-1β converting enzyme
IFN-γ	Interferon-γ
IκB	NF-κB inhibitory protein
IL-1	Interleukin 1
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-8	Interleukin 8
IL-10	Interleukin 110
INOS	Inducible nitric oxide synthase
Iscove's MDM	Iscove's Modified Dulbecco's medium
JAK	Janus kinase
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
MPB	Major basic protein
$MgCl_2$	Magnesium chloride
NF-κB	Nuclear factor-κB
NIK	NF-κB-inducing kinase
NO	Nitric oxide
PAF	Platelet-activating factor
PAK	p21-activated protein kinase
PBS	Phosphate-buffered saline
PDC	Programmed cell death
PDTC	Pyrrolidine dithiocarbamate
PGA_2	Prostaglandin A_2
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$

PGI_2	Prostaglandin I_2
PGHS	Prostaglandin H_2 synthase
PGJ_2	9-deoxy-Δ^9-PGD_2
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
PTP	Protein tyrosine phosphatase
RAIDD	RIP-associated ICH-I/CED homologous protein with a death domain
RANTES	Regulated and normal T-cell expressed and secreted
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SMAD	Small 'mothers against decapentapleigic
SNP	Sodium nitroprusside
STAT	Signal transducers and activators of transcription
TAB1	Tak1 binding protein
TAK1	TGF-β activated protein
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis Factor-α
TNFR	Tumour necrosis factor receptor
TNFR1	55 kD TNF receptor
TNFR2	75 kD TNF receptor
TPCK	Tosyl-Phe-chloromethylketone
TRADD	TNFR55-associated death domain protein
TRAF	TNFR associated factor
Tris	Tris[hydroxymethyl]-aminomethane
TXA_2	Thromboxane A_2
5-HETE	5-hydroxy-6,8,11,14-eicosantetraenoic acid
5-oxo-ETE	5-oxo-6,8,11,14-eicosantetraenoic acid
$15dPGJ_2$	15-deoxy-Δ^{12},Δ^{14}-PGJ_2

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CHAPTER 1

INTRODUCTION

1.1 The Inflammatory Response

The inflammatory response occurs as a consequence of tissue injury, trauma or infection, and consists of a series of reactions to limit ongoing tissue damage, isolate and destroy the infective organism and activate the repair processes needed for healing and gain of normal function (Baumann & Gauldie, 1994). The body reacts to inflammatory stimuli by increasing blood flow and capillary permeability in the affected site to help amplify the migration of cells into the area. The cell types seen in different inflammatory loci vary according to the stimulus, its persistence and the type of immune reaction it elicits; for example, neutrophils are common in sites of acute inflammation, while eosinophils accumulate in allergic reactions. An inflammatory response can be either beneficial or detrimental to the host. If the inflammatory stimulus is removed, resolution of the damage allows healing to begin, but if the stimulus persists or the inflammatory response goes awry, chronic inflammation may result.

1.2 Neutrophils

Neutrophils develop from the myeloid line and have a limited life span in the circulation with a half-life of 4 – 6 hours (Bicknell et al., 1994). They have a mean diameter of 7 μm, a multi-lobed nucleus and a large number of granules. Neutrophils are peripheral blood cells, and must migrate to inflammatory foci during the acute immune response. During the first stage, cells respond to chemotaxins and adhere to endothelial cells. Initial adhesion is dependent on the interaction of neutrophil and endothelial cell selectins with their ligands. Selectin-mediated adhesion is relatively weak, allowing neutrophils to roll on the endothelium. Firmer adhesion (margination) is achieved through the engagement of neutrophil CD11/CD18 β_2 integrins with endothelial cell ligands such as intercellular adhesion molecule-1

(ICAM-1) (Carlos & Harlan, 1994, Springer, 1994). Margination allows a large number of cells to be available when needed to increase the circulating neutrophil pool. An important site of neutrophil margination is the lung, because of the large lung capillary surface area (approximately 60 m^2) available for endothelial cell contact, and because the relative diameter of the lung capillaries (5 μM), means that deformation of the neutrophil must occur for the cell to pass through the capillary (Hogg, 1987). Neutrophils accumulate at the site of tissue injury or infection by responding to chemotaxins released from the inflammatory locus. This involves diapedisis of neutrophils between the cells of the endothelium and migration along a chemotactic gradient.

The major role of these cells is the phagocytosis and killing of bacterial and fungal pathogens, in which granule contents play a part. Human neutrophils contain a large number of granules which are morphologically, biochemically and functionally distinct and contain more than 20 enzymes (for a recent review see Borregaard & Cowland, 1997). The two main types are azurophil and specific granules. Azurophil granules are peroxidase positive, containing myeloperoxidase, which is involved in the generation of toxic reactive oxygen species in the phagolysosome. These granules also contain cationic proteins involved in antimicrobial activity such as bacterial/permeability-inducing protein (BP1), which is active intracellularly and defensins which are released extracellularly when neutrophils degranulate. Other granule constituents with antibacterial activity include cathepsin G, and lysozyme. Neutrophil specific granules contain glycoproteins, lysozyme, lactoferrin and vitamin B_{12}. However, the physiological functions of many granule constituents are poorly understood (Borregaard & Cowland, 1997).

Activated neutrophils also have the capacity to release a large variety of other products at inflammatory sites; these include oxygen radicals and lipid mediators (Malech & Gallin, 1987). Arachidonic acid (AA) is cleaved from plasma membrane phospholipids and immediately metabolised to generate one or more biologically active eicosanoids (Section 1.9) (Samuelsson, 1983). Thromboxanes and prostaglandins are primarily generated by platelets, while leukotrienes are the

products of neutrophil arachidonic acid metabolism. Phospholipase A_2 can release arachidonate from the *sn*-2 position of phospholipids, which can then be enzymatically converted via acetyltransferase to platelet-activating factor (PAF), an an important mediator with diverse biological activity (Hanahan, 1986). The oxidative burst and degranulation in human neutrophils are stimulated by a variety of agonists. Activation of neutrophils can produce a release of superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) within seconds (Lehrer et al., 1988). Further reduction of these radicals produces more toxic oxygen radicals such as the hydroxyl radical ($^{\cdot}OH$), through a reaction involving the reduction of ferrous to ferric iron, and hypochlorous acid (HOCl), through the catalytic effect of myeloperoxidase (Lehrer et al., 1988). Cytokines produced by other inflammatory cells can act synergistically with agonists to produce a potentiated oxidative burst (referred to as priming).

In the course of eliminating the invading pathogen, neutrophils can directly injure healthy tissue. In adult respiratory distress syndrome (ARDS), neutrophils may damage tissue through H_2O_2 generation (McGuire et al., 1982). Neutrophil infiltration, in myocardial infarction in humans and animals (Lucchesi & Mullane, 1986) can exacerbate ischemia-induced myocardial damage, increasing the area of myocardial necrosis (Simpson et al., 1990). This is also evident in reperfusion-induced injury of other tissues including the central nervous system and gastrointestinal tract (Simpson et al., 1990). Neutrophil oxidants are also implicated in glomerulonephritis (Johnson et al., 1988), while proteases released during degranulation of neutrophils including elastase, collagenase, and gelatinase, can cause tissue injury in ARDS, rheumatoid arthritis, cystic fibrosis, and emphysema (Wong & Travis, 1980, Lee et al., 1981, Janoff, 1985, Bruce et al., 1992).

1.3 Eosinophils

Eosinophils, like neutrophils, are derived from the myeloid line and are important in immunological defence against parasites (Butterworth et al., 1975, Gleich & Adolphson, 1986). They are predominantly tissue cells and large numbers can be found in the epithelial lining of the gastrointestinal and respiratory tracts even when blood eosinophil counts are low, suggesting that eosinophils may have a prolonged life span in the tissues (Weller, 1991). In the blood, the half-life of human eosinophils is 18 ± 2.1 h (Steinbach et al., 1979); healthy non-allergic subjects have eosinophil counts of less than 0.4 x 10^9/l of blood. The cells circulate for an estimated 3 to 8 h after which they diapedese and migrate into the tissues. The process of extravasion appears to be random, and bears no relation to the time the cells have already spent in the circulation (Spry, 1993). Eosinophils, like neutrophils, marginate and diapedese into tissues similarly through post-capillary venules where slow blood flow allows adhesion to the endothelium. Eosinophil adherence is partially mediated through VLA-4 and VCAM-1 (Weller et al., 1994). After attachment the cells migrate between endothelial cells into the connective tissue surrounding the vessels, where they are most prominent in disease (Weller, 1994). Survival time in the tissues is unknown, but eosinophil survival may be extended *in vitro* by adding growth factors such as GM-CSF, IL-3 and IL-5 (Spry, 1993).

Eosinophils have a diameter of 8 μm, with a bi-lobed nucleus and characteristic granules (Sokol et al., 1987, 1991). The granules of mature eosinophils are ovoid, cytoplasmic membrane-bound organelles, 0.15 to 1.5 μm in length, which contain major basic protein (MBP) and eosinophil cationic protein (EPC) (Gleich et al., 1976, Egesten et al., 1986, Peters et al., 1986, Gleich 1990) as well as peroxidase, aryl sulphatase, and hydrolytic lysosomal enzymes (Bainton & Farquhar, 1970). The peroxidase of eosinophilic granules differs from the myeloperoxidase of neutrophils in substrate specificity and sensitivity to a variety of inhibitors (Bolscher et al., 1991). In addition to the specific granules, mature eosinophils possess some smaller

granules (0.1 to 0.5 μm) which contain aryl sulphatase and acid phosphatase (Parmley & Spicer, 1974). Eosinophils also generate oxidative products, hypohalous acids, lipid mediators (for example, LTC_4 and PAF), ribonucleases, cytokines and chemokines (Spry 1993, Bruijnzeel 1994, Thomas 1995). Several cytokines have been shown to be transcribed, or released by eosinophils including TGF-α (Wong et al., 1990), IL-1α, (Weller et al., 1993), IL-3, (Kita et al., 1991, Fujisawa et al., 1994), IL-4 (Moqbel et al., 1995, Nonaka et al., 1995), IL-5 (Broide et al., 1992, Dubucquoi et al., 1994), IL-6 (Hamid et al., 1992, Melani et al., 1993), IL-8 (Braun et al., 1993, Kita et al., 1995), GM-CSF (Weller et al., 1993), TNF-α (Costa et al., 1993), TGF-β, (Wong et al., 1991, Ohno et al., 1992) and RANTES (Lim et al., 1995).

Although these products play a part in host defence, they may also cause damage to the host (particularly granule proteases) in chronic inflammatory diseases such as asthma, causing increased airway reactivity, epithelial damage and inflammation of the airway tissue (Gleich & Adolphson, 1986). MBP is toxic to many mammalian cells, including human lung epithelium, where it produces pathologic changes similar to those observed in asthma (Gleich et al., 1979, Motojima et al., 1989, Sedgwick 1995).

Asthma is a bronchial inflammatory disease characterised by a leukocyte mucosal infiltration which is predominantly eosinophilic (Bousquet et al., 1990, Djukanovic et al., 1990). The cells are recruited from the circulation following inhalation of asthmogens such as allergen in sensitised subjects. Cytokines and chemotactic factors are released by resident cells, such as mast cells and lymphocytes (Barnes, 1989a, b). These mediators increase chemotaxis, the expression of adhesion molecules on bronchial endothelial cells, and lead to increased expression of their ligands on eosinophils (Silberstein & David, 1987, Resnick & Weller, 1993). In the bronchial mucosa, eosinophils are activated and release mediators and cytokines, amplifying the inflammatory process and increasing bronchial responsiveness to cause asthma symptoms (Gleich, 1990, Smith 1992). Repeated provocation results in chronic inflammation in the airways and a marked hyperresponsiveness to

constrictor mediators which correlates with the numbers of eosinophils present in the lung (Bousquet et al., 1990, Bradley et al., 1991).

Eosinophils can also be involved in a range of pathologies ranging from allergic (for example, rhinitis) and inflammatory (such as inflammatory bowel disease and eosinophilic gastroenteritis) to malignant disorders such as Hodgkins's diseases and certain leukaemias (Seminario & Gleich, 1994). In such conditions, increased levels of eosinophils are often found in the circulation and tissues.

In inflamed tissues there is a close spatial and temporal association between eosinophils and recirculating T lymphocytes (Spry, 1971a, b). Increasing evidence suggests that the late phase reaction, including eosinophilia and IgE production, are orchestrated by T-lymphocytes, particularly T helper cells of the Th2 subset. Following specific recognition of an antigen, Th2 cells elaborate cytokines, such as IL-4, which favour the synthesis of IgE by B-lymphocytes, or IL-5 (Basten & Beeson, 1970, Sanderson et al., 1985, Campbell et al., 1988), which is an important differentiation, growth and survival factor for eosinophils (Sanderson, 1992). Eosinophils have receptors for IgE, which play a central role in anti-parasitic defence (Capron et al., 1984, Gounni et al., 1994), but which also cause sensitisation to allergens, the first stage in allergic reactions. Human eosinophil IgE receptors are increased in atopic conditions such as allergen-induced atopic asthma (Barata et al., 1997, Sihra et al., 1997, Rajakulasingam et al., 1998, Ying et al., 1998). Activated Th lymphocytes are present in bronchoalveolar lavage fluid (BAL), bronchial biopsies and peripheral blood from atopic subjects, their numbers correlating with that of circulating eosinophils (Azzawi et al., 1990). Cytokines produced by Th lymphocytes in response to antigen stimulation include IL-3 and GM-CSF, which support the proliferation of granulocyte and macrophage precursors, and increase eosinophil longevity (Alam et al., 1994a). Studies have correlated the concentration of IL-5 in biological fluids with the number of T-cells and eosinophils in BAL and serum from patients with allergic rhinitis (Sedgwick et al., 1991) and bronchial asthma (Corrigan et al., 1993).

1.4 Clearance of Inflammatory Cells

As part of the process of resolution, excess inflammatory cells must be removed. Neutrophils and eosinophils do not re-enter the circulation after extravasion (Spry, 1993). Since tissue damage does not normally result from resolution, it follows that these cells must be removed from an inflammatory foci without discharging inflammatory mediators or intracellular contents, which would cause recruitment of more cells to the site (Spry 1993). Therefore the fate of ageing granulocytes in the tissues has been the focus of many relatively recent studies.

The inflammatory role of granulocytes is terminated by apoptosis, which is associated with attenuation of cytotoxic responses and recognition and phagocytosis by macrophages (Savill et al., 1989a, Savill 1992, Whyte et al., 1993a). This process limits tissue injury and allows for removal of these cells without the liberation of their cytotoxic contents. *In vitro* studies have shown that there is a direct relationship between the amount of apoptosis in samples of aged neutrophils and their recognition and phagocytosis by macrophages and that freshly isolated peripheral blood neutrophils, are not recognised by monocyte-derived macrophages (Savill et al., 1989a). However, during an inflammatory response *in vivo*, the process of apoptosis may be delayed in granulocytes which have been exposed to various inflammatory mediators (Squier et al., 1995, Chilvers et al., 1998).

The ingestion of apoptotic cells by macrophages causes no significant increase in granule enzyme release or the secretion of thromboxane B_2, an important inflammatory mediator (Meagher et al., 1992). Therefore this mode of clearance may be crucial in resolving the inflammatory process (Haslett et al., 1989), and has been proposed as an important mechanism for the removal of granulocytes from sites of inflammation (Savill et al., 1989a, 1990a, Haslett et al., 1994). This is supported by *in vivo* studies demonstrating the removal of apoptotic neutrophils in endotoxin-induced experimental lung injury (Cox et al., 1995), the neonatal respiratory distress syndrome (Grigg et al., 1991) and experimental glomerulonephritis (Savill et al., 1992a).

Why ingestion of apoptotic cells, in contrast to that of bacteria or opsonised particles, does not produce activation of phagocytic cells is currently unknown. Several mechanisms appear to play a role in recognition. For example, the vitronectin receptor $\alpha_v\beta_3$ integrin (Savill et al., 1990b), CD36 (Savill et al., 1992b), the class A scavenger receptor (Platt et al., 1996) the ATP-binding cassette transporter ABC-1 (Luciani & Chimini, 1996), the phosphatidyl serine receptor (Fadok et al., 1992), and the CD14 receptor (Devitt et al., 1998) have all been demonstrated to be involved in this process. Interestingly, CD14 is the receptor, which also binds LPS to trigger inflammatory responses; it is not clear why one ligand (LPS) should cause activation and another (an apoptotic cell), should not.

1.5 Apoptosis

Cell death occurs in two different forms (Wyllie & Morris 1982). Necrosis refers to processes occurring when cells die from severe injury such as physical or chemical trauma and apoptosis or programmed cell death (PCD) which is a physiological process. In necrosis there are early changes in mitochondrial shape and function, and the cell rapidly becomes unable to maintain homeostasis. If the plasma membrane is damaged, the cell loses its ability to regulate osmotic pressure and the cell swells and ruptures. Intracellular contents spill into the surrounding tissue space and provoke an inflammatory response (Cohen 1993).

Apoptosis refers to a series of morphological changes that differ from those seen in necrosis. They occur in response to physiological stimuli such as growth factor withdrawal and are similar in all cell types (Wyllie & Morris 1982). The plasma membrane becomes ruffled and blebbed, the cell shrinks through fluid loss, but the organelles appear normal, apart from the nucleus in which chromatin condenses. This is accompanied by the fragmentation of DNA first into large (50 to 300 kb) and subsequently into small oligonucleosomal fragments (Wyllie et al., 1980, Arends et al., 1990, Roy et al., 1992). The cell membrane does not become permeable to vital dyes at this stage. The cell may ultimately break up into apoptotic bodies, which are

sealed and maintain their osmotic gradients, avoiding the release of intracellular contents (Arends & Wyllie 1991, Cohen 1993). Granulocytes do not normally form apoptotic bodies, but remain as an intact apoptotic cell until ingested by macrophages (Savill et al., 1989a, Rossi & Haslett, 1997)

Apoptosis occurs in different cell types in response to different stimuli (Wyllie et al., 1981). During cell culture, eosinophils and neutrophils undergo spontaneous apoptosis in contrast to thymocytes that require treatment with UV radiation or glucocorticoids (Wyllie & Morris 1982). This process can be detected by various methods, such as nuclear changes (chromatin condensation or DNA laddering), changes in dye binding to nuclear material (acridine orange or PI staining) (Nicoletti et al., 1991), or by measuring surface changes, for example, using 3G8 antibody to measure CD16 shedding (Dransfield et al., 1994), or Annexin V binding to exposed phosphatidyl serine as the cells become apoptotic (Homburg et al., 1994).

1.6 Control of Apoptosis

The study of apoptosis and its control is complicated by the fact that diverse stimuli are responsible for inducing PCD in different cell types. A stimulus that produces apoptosis in a specific cell type may have differing or negligible effects on other cells. For example, glucocorticoids will inhibit apoptosis in neutrophils but induce apoptosis in thymocytes and eosinophils (Wyllie & Morris 1982, Cox 1995, Meagher et al., 1996). *In vivo*, cells will interact with other cell types, cell products such as cytokines, and with environmental factors which will influence signalling systems and ultimately affect the fate of a given cell. However, because granulocytes are known to undergo apoptosis *in situ* at an inflammatory focus to allow inflammation to resolve, it is possible that inflammatory mediators, such as eicosanoids or cytokines can induce programmed cell death in these cells. However, little is known of the physiological processes that drive apoptosis enabling the process of resolution to begin. Most of the inflammatory mediators which have been investigated prolong the longevity of inflammatory cells (Colotta et al., 1992, Lee et al., 1993, Alam et al., 1994a).

Early studies indicated that the transcription and translation of specific genes were required to induce apoptosis. This was based on the effects of cycloheximide and actinomycin D in preventing thymocyte apoptosis triggered by irradiation, glucocorticoid hormones and calcium ionophore (Cohen and Duke, 1984, Wyllie et al, 1984, Kizaki et al, 1989). However, the protein synthesis inhibitor cycloheximide, can either block the induction of apoptosis, promote it, or have no effect, depending on the cell type studied (Cohen et al, 1992). When protein synthesis inhibitors trigger apoptosis, it is possible that programmed cell death is held in check by survival proteins that are not produced when protein synthesis is blocked. This would also suggest that apoptotic effector molecules are constitutively present in most mammalian cells (Raff et al., 1993). Cells without a nucleus can undergo the characteristic cytoplasmic changes of apoptosis, while in contrast, isolated nuclei can exhibit chromatin condensation and DNA fragmentation in a cell-free system (Lazebnik et al., 1993, Newmeyer et al., 1994), implying that different cellular compartments have considerable autonomy in undergoing structural changes during apoptosis.

To try to understand the physiological factors that control apoptosis and resolution *in vivo*, we examined different classes of mediators involved in the inflammatory response and their effects on the constitutive rates of apoptosis in both neutrophils and eosinophils. These included glucocorticoids, which have an anti-inflammatory action, chemokines, various classes of eicosanoids (leukotrienes, 5-oxo-ETE and prostaglandins) and pro- and anti-inflammatory cytokines.

1.7 Glucocorticoids

Glucocorticoids are steroid hormones with anti-inflammatory capabilities. Clinically, treatment of hypereosinophilia in a range of diseases is by administration of glucocorticoids, yet the mechanism(s) by which they exert their effects *in vivo* are poorly understood (Barnes 1989b, 1990, Barnes & Adcock, 1993, Goulding & Guyre, 1993). Glucocorticoids reduce both the numbers of eosinophils and the

number of hypodense (activated) eosinophils in the circulation of asthmatics (Baigelman et al., 1983, O'Connor et al., 1991), and reduce the secretion of IL-3, IL-5 and GM-CSF from CD4 T cells (Corrigan et al., 1995). They bind directly to cytosolic glucocorticoid receptors (GRs), which, when activated, exhibit DNA-binding ability and interact with a wide range of nuclear targets. GRs are present within the cell both in the cytoplasm and nucleus and undergo a conformational change after ligand-binding to yield an active form of the receptor (Beato, 1989). The activated receptor can recognise and bind to specific DNA sequences and bring about activation or repression of transcription initiation of a nearby target gene. Therefore glucocorticoids can both increase or decrease the expression of specific proteins (Maroder et al., 1993).

1.8 Chemokines

Cell extravasion involves selectins, integrins and chemokines (chemoattractant cytokines) and their receptors (Schall & Bacon, 1994, Ben-Baruch et al., 1995). Chemokines are a family of secreted 8 – 10 kDa proteins (Baggiolini et al., 1994), which induce cell migration and activation (Murphy 1994). They are usually divided into three subgroups: C, C-X-C (where X is any amino acid) and C-C, according to variation in a shared cysteine motif (Schall & Bacon, 1994). Most C-X-C chemokines are chemoattractants for neutrophils, whereas C-C chemokines usually act on eosinophils (Miller & Krangel, 1992, Schall & Bacon, 1994, Premack & Schall, 1996). For example, IL-8, a member of the C-X-C family of chemokines (Murphy et al., 1994), is a neutrophil chemoattractant which is produced by monocytes, alveolar macrophages, endothelial cells, fibroblasts and epithelial cells after stimulation with IL-1, TNF-α or LPS (Baggiolini et al., 1989, Oppenheim et al., 1989).

Eotaxin is a specific C-C eosinophil chemokine secreted by eosinophils (Griffiths-Johnson et al, 1993, Ponath et al, 1996), which has no activity on mononuclear cells or neutrophils (Lim et al., 1995, Garcia-Zepeda et al., 1996). This chemokine was isolated from an animal model of asthma with increased expression in the lungs

within three hours of antigen challenge (Jose et al., 1994a, b, Rothenberg et al., 1994). Eotaxin may be constitutively produced (Bartels et al., 1996), but it is markedly upregulated in some human inflammatory diseases. The pro-inflammatory cytokines TNF-α, and IL-1 α induce eotaxin mRNA expression in endothelial and epithelial cells (Garcia-Zepeda et al., 1996). It is also produced by fibroblasts (Bartels et al., 1996), macrophages, ciliated and non-ciliated bronchial epithelial cells (Ponath et al., 1996, Li et al., 1997), and chondrocytes (Li et al., 1997).

RANTES (Regulated and Normal T-cell Expressed and Secreted) is another C-C chemokine which acts on eosinophils both *in vitro* and *in vivo* (Kameyoshi et al., 1992, Rot et al, 1992, Meurer et al., 1993, Ebisawa et al., 1994, Schröder et al, 1994). This chemokine induces mild activation of eosinophils *in vitro* (as measured by eosinophil cationic protein release, superoxide anion production, and induction of hypodensity) (Kameyoshi et al., 1992, Rot et 1992, Alam et al., 1993, al., Schweizer et al., 1994). Bronchial epithelial cells produce RANTES *in vitro* (Stellato et al., 1995), and expression of this chemokine is enhanced in the bronchial epithelium, alveolar macrophages and smooth muscle cells of asthmatic patients (Alam et al., 1994b). In bronchial epithelial cells chemokine production can be modulated by glucocorticoids (Stellato et al., 1995).

Some chemokines can bind to more than one receptor subtype. For example, RANTES can bind to CCR1, CCR3, CCR4 and CCR5, while eotaxin binds to CCR3 (Premack & Schall, 1996). The MIP-1α/RANTES receptor can bind several of the C-C chemokines (Neote et al, 1993). This means that chemokines may have differential effects on cells depending on the type and number of receptor or concentration of chemokine present. For example, RANTES stimulates chemotaxis at low concentrations in T cells, but at higher concentrations it produces a spectrum of responses, including cytokine production and proliferation, which are normally associated with T-cell activation (Bacon et al., 1995). Chemokine receptors are functionally linked to phospholipases via heterotrimeric G proteins, with downstream generation of inositol triphosphate, resulting in intracellular calcium release, calcium channel opening and protein kinase C activation (Murphy, 1994, Bokoch 1995).

Various chemokines have been shown to influence inflammatory cell activation. However few studies have considered the effects of classical chemokines on cell survival. Therefore, we examined whether such mediators could inhibit or increase the constitutive rate of neutrophil and eosinophil apoptosis.

1.9 Eicosanoids

Eicosanoids are a group of substances formed from the C_{20} polyunsaturated fatty acid, arachidonic acid (5,8,11,14-eicosatetraenoic acid) (AA). AA is liberated from membrane phopholipids through activation of phospholipases (Scott et al., 1980). There are two main pathways of arachidonate metabolism, the linear pathway which leads to the formation of leukotrienes and hydroperoxyeicosantetraenoic acids (HPETEs) and the cyclic pathway which leads to the formation of prostaglandins, prostacyclins and thromboxanes (Scott et al., 1980). Such metabolites are important modulators of inflammatory responses (Davis et al., 1984). The specific products produced by this pathway depend on the tissue involved. The formation of metabolites of A A is shown in Figure 1.1.

1.9.1 The 5-lipoxygenase Products

One major pathway for AA metabolism in human neutrophils is initiated by the action of 5-lipoxygenase, which is possibly regulated by 5-lipoxygenase activating protein (FLAP) (Borgeat et al., 1976, Borgeat & Samuelsson 1979a, Ford-Hutchinson, 1994). The initial product of this reaction is 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid which is either converted to LTA_4 (Borgeat & Samuelsson 1979b) by the same enzyme (Rouzer et al., 1986) or reduced to the hydroxy compound, 5-hydroxy-6, 8,11,14-eicosatetraenoic acid (5-HETE), by a peroxidase (Borgeat et al., 1976). 5-HETE can also be oxidified by a highly specific microsomal dehydrogenase in neutrophils to 5-oxo-6, 8,11,14-eicosatetraenoic acid (5-oxo-ETE) (Powell et al., 1992), which can also be produced by eosinophils (Powell et al, 1995);

this eicosanoid is a potent stimulator of both cell types (O'Flaherty et al., 1993, Powell et al., 1993, 1995, Schwenk & Schröder, 1995). 5-oxo-ETE is up to 100-fold more active than 5-HETE in causing PMN calcium transients and chemotaxis and in promoting the degranulation responses of PMN challenged with PAF or TNF- (O'Flaherty et al., 1993, Powell et al., 1993).

LTA_4 is quite unstable and is rapidly converted by LTA_4 hydrolase to the proinflammatory compound LTB_4; a potent activator of neutrophils causing chemotaxis, homotypic and heterotypic adhesion, diapedisis, and reactive oxygen generation (Ford-Hutchinson et al., 1980, Samuelsson et al., 1987, Chatila et al., 1989, Borgeat & Naccache, 1990, Lewis et al., 1990, O'Flaherty et al., 1991, 1994, Henderson, 1994). LTB_4 is known to inhibit neutrophil apoptosis (Hébert et al, 1996). However, eosinophils are relatively unresponsive to LTB_4 (Richards et al, 1991, Powell et al, 1995).

1.9.2 Prostaglandins

Prostaglandins are a group of C_{20} carboxylic acids containing a cyclopentane ring. They have wide-ranging effects in the body and are important mediators of inflammation. As shown in Figure 1.1 cyclooxygenase (COX) plays a key role in prostaglandin synthesis. This enzyme occurs in both constitutive (COX-1) and inducible (COX-2) isoforms (Kujubu et al., 1991, Xie et al., 1991). COX-1 is postulated to provide cytoprotective effects (Mitchell et al., 1993), whereas COX-2 is the major isoform in inflammatory cells (DeWitt & Meade, 1993). The main mechanism of action of most non-steroidal anti-inflammatory drugs such as aspirin (acetylsalicylic acid), is the inhibition of cyclooxygenases also known as prostaglandin H_2 synthases (PGHS) (Vane & Botting, 1987, Loll et al., 1995).

Cyclooxygenase oxygenates arachidonate converting it to PGG_2; PGG_2 is then released from the cyclooxygenase active site and travels to the peroxidase site of the enzyme where PGG_2 is reduced to PGH_2 (Eling et al., 1991). PGH_2 is converted via distinct synthases to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 or thromboxane A_2 (Pierce et al.,

1995, Smith & DeWitt, 1996). PGD_2 can be further metabolised to form 9-deoxy Δ^9-PGD_2 (PGJ_2) (Fukushima et al., 1982a), Δ^{12}-PGJ_2 (Kikawa et al., 1984) and 15-deoxy-Δ^{12},Δ^{14}-PGJ_2. PGE_2 can be also be metabolised to form PGA_2 (Ohno et al., 1986). Prostaglandins formed in the endoplasmic reticulum through the action of COX-1 exit cells and function through cell surface receptors. COX-2 is normally absent from cells but is expressed transiently in response to growth factors and cytokines such as IL-1 and TNF-α (Lee et al., 1992, O'Sullivan et al., 1992, Riese, et al., 1994). COX-2 expression can be inhibited by anti-inflammatory glucocorticoids such as dexamethasone (Lee et al., 1992, O'Bannion et al., 1992, Herschman 1996).

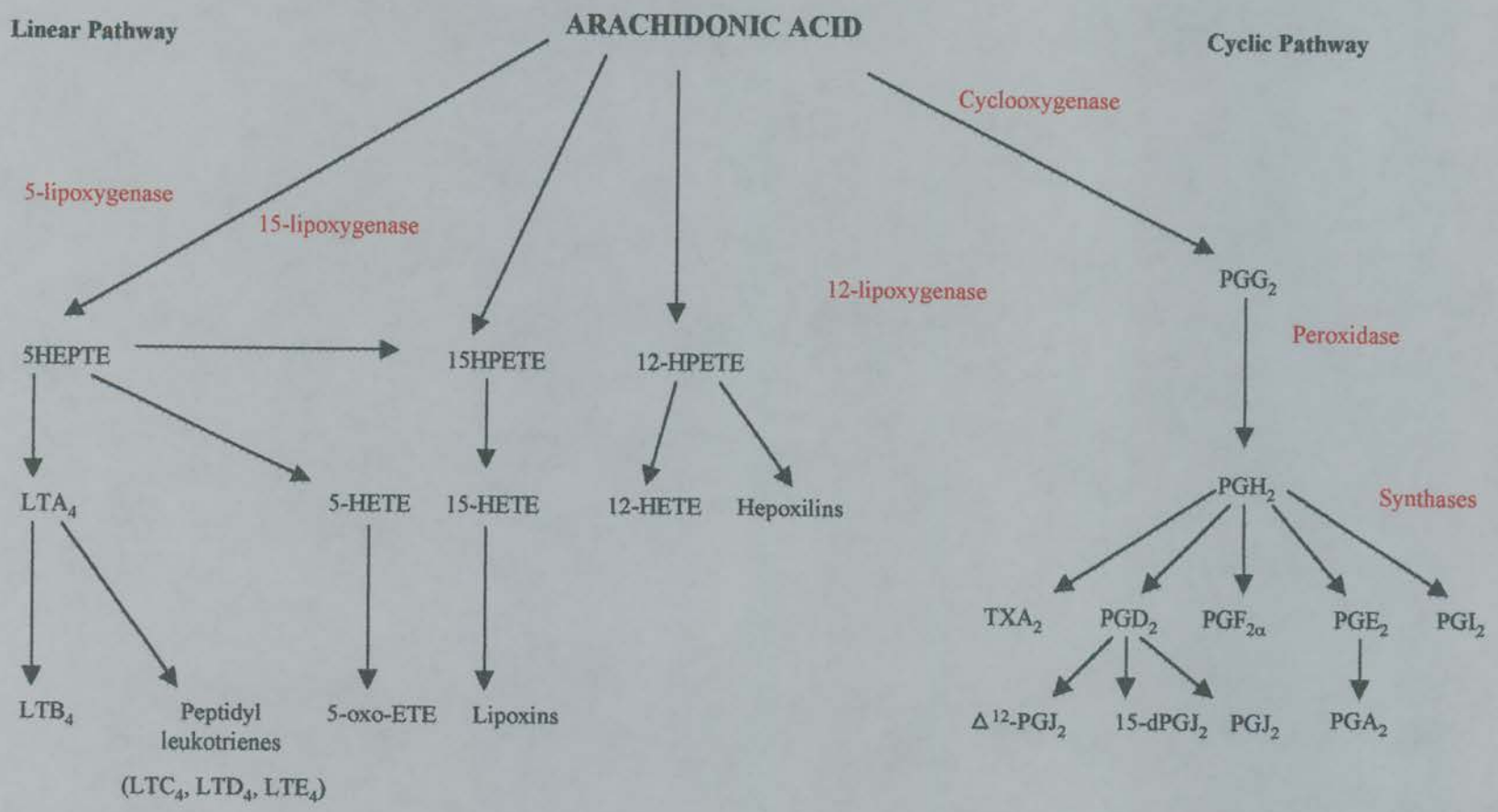


Figure 1.1 Simplified Scheme of Arachidonic Metabolism Pathways

1.9.3 Prostanoid Receptors

The classification of prostanoid receptor subtypes was based upon the activities of natural and synthetic agonists (Kennedy et al., 1982, Coleman et al., 1984). This classification has been verified by cloning data and the use of specific receptor antagonists (Hirata et al., 1991, Abramovitz et al., 1994, Boie et al., 1994, Coleman et al., 1994, Katsuyama et al., 1994, Lake et al., 1994, Nakagawa et al., 1994, Pierce et al., 1995a). The receptors have been categorised into 5 groups, DP, EP, IP, FP and TP, based on the binding characteristics of the five main prostanoids, PGD_2, PGE_2, PGI_2, PGF_2 and thromboxane A_2 respectively (Coleman et al., 1994). Four subtypes of EP receptors, $EP_1 - _4$ have also been cloned (An et al., 1993, Funk et al., 1993, Adam et al., 1994, Bastien et al., 1994, Regan et al., 1994a, b).

DP receptors are expressed on eosinophils (Butchers & Vardey, 1990), but there is no evidence for the presence of IP, FP or TP receptors on these cells (Giembycz et al., 1990). There are both EP_2 and DP receptors on human neutrophils (Rossi & O'Flaherty, 1989, Ney & Schrör, 1991, Wheeldon & Varday, 1993, Darius et al., 1994). Activation of the TP receptor in immature thymocytes has been shown to induce apoptosis (Ushikubi et al., 1993). Occupation of EP_2, or DP prostanoid receptors inhibits certain neutrophil functions, including the respiratory burst, homotypic aggregation and secretion (Rossi & O'Flaherty, 1989, Wheeldon & Vardey, 1993, Wise & Jones, 1994, Talpain et al., 1995). These receptors appear to inhibit neutrophil function via elevation of cAMP (Coleman et al., 1994). Activation of other receptor types has been shown to affect Ca^{2+} levels (Table 1.1). Interestingly, increases in Ca^{2+} and cAMP have been demonstrated to modulate the rate of constitutive apoptosis in neutrophils and eosinophils (Rossi et al., 1995, Cousin et al., 1997). The receptor types, ligands and effects on the two main cellular signalling pathways, cAMP and calcium are shown in Table 1.1.

Receptor	Ligands	Effect on cAMP and Ca^{2+}
DP	PGD_2, PGJ_2	↑ cAMP (1,2) ↑ Ca^{2+} (14)
EP_1	PGE_2,PGE_1, $PGF_{2\alpha}$, PGD_2	↑ Ca^{2+}(3, 4, 5, 6) cAMP no effect (5)
EP_2	PGE_2	↑ cAMP (7)
EP_3	PGE_1	↓ cAMP (8) ↑ Ca^{2+} (8)
EP_4	PGE_2,PGE_1	↑cAMP (12,13)
FP	$PGF_{2\alpha}$, PGD_2, PGE_2	↑ Ca^{2+} (9, 10)
IP	PGI_2, PGE_1, PGE_2, PGD_2, $PGF_{2\alpha}$	↑ cAMP (11)
TP	TXA_2, PGD_2, PGF_2	↑ Ca^{2+} (10)

TABLE 1.1 Cell Surface Prostaglandin Receptors, Ligands and Intracellular Effects

Table based on Coleman et al., 1994 and references as follows – 1. Ito et al., 1990, 2. Bundy et al., 1983, 3. Creese & Denborough, 1981, 4. Coleman & Kennedy, 1985, 5. Watabe et al., 1993, 6. Funk et al., 1993, 7. Jumblatt & Peterson, 1991, 8. Reeves et al., 1988, 9. Behrman et al., 1985, 10. Nakano et al., 1993, 11. Ito et al., 1992, 12. Nishigaki et al., 1998, 13. Kiriyama et al., 1997, 14. Raible et al., 1992.

As shown in Table 1.1 prostaglandin receptors are promiscuous and the effects of prostaglandins on inflammatory cells could be mediated through several receptor types. The prostaglandin metabolites PGA_2, Δ^{12}-PGJ_2, and $15dPGJ_2$ have been shown to bind to intracellular, rather than cell surface receptors (Narumiya et al., 1987). One such receptor family demonstrated to bind some of these metabolites is the peroxisome proliferator-activated receptors (PPARs). PPARs are transducer proteins belonging to the steroid/thyroid/retinoid receptor superfamily (Lemberger et al, 1996). These are nuclear hormone receptors that regulate gene transcription in response to peroxisome proliferators and fatty acids and are predominantly expressed

in adipose tissue, adrenal gland and spleen (Kliewer et al., 1994, Tontonoz et al., 1994, Lemberger et al., 1996). There are multiple subtypes of PPAR called α, δ or γ in mammals and these subtypes are differentially activated by various agents (Dreyer et al., 1992, Schmidt et al., 1992, Chen et al., 1993, Zhu et al., 1993, Kliewer et al., 1994). PPAR-γ is expressed in the immune system, by human monocytes, several myeloid cell lines and bone-marrow precursors (Greene et al., 1995).

There has been much research on the influence of eicosanoids on the activation and functional behaviour of inflammatory cells. Because pro-inflammatory cytokines such as TNF-α can upregulate COX-2 activity, increased expression of eicosanoids would be expected at an inflammatory site. However, the effects of only a few of these mediators on granulocyte apoptosis have been examined formerly, and therefore, several eicosanoids were chosen for study in this work.

1.10 Cytokines

Cytokines are involved in co-ordinating the immune system, where they control the amplitude and duration of the response by regulating the growth, differentiation and functional activities of immune and inflammatory cells (Karnitz & Abraham 1995). They generally act in an autocrine or paracrine manner by binding to high affinity cell receptors on target cells, which may be specific for each cytokine or cytokines of a related group (Balkwill 1989).

Cytokines often regulate cell growth and some exhibit growth inhibitory or cytotoxic actions. They have multiple, overlapping and sometimes contradictory functions depending on local concentration, cell type and other environmental influences to which the cell is exposed. Cytokines form part of a complex network where production of one cytokine influences the production of, or the response to other cytokines. For example, IL-3 or IL-5 stimulates eosinophils to express GM-CSF (Moqbel et al., 1991). Cytokine-binding and culture conditions may change the pattern of cellular behaviour by altering DNA, RNA and protein synthesis in cells (Baumann & Gauldie 1994); for instance, freshly isolated eosinophils constitutively

express mRNAs for IL-4, TGF-β1 and RANTES; in culture, the cells also produce mRNA for IL-8 and GM-CSF. If cultured with IL-5 the cells produce IL-10, but culture with TNF-α causes the production of IL-3 and TNF-α (Nakajima et al. 1996).

The effect of many pro-inflammatory cytokines on granulocyte apoptosis has been examined. However, in an *in vivo* situation, cells in an inflammatory foci will be affected by a plethora of cytokines, both pro- and anti-inflammatory, as well as chemokines and eicosanoids. Cytokines may have antagonistic or synergistic actions depending on the presence of other factors. Therefore this thesis examines the effects of pro- and anti-inflammatory cytokines and their interactions with other factors likely to be present during an inflammatory response, on granulocyte apoptosis.

1.10.1 Pro-inflammatory Cytokines

Pro-inflammatory cytokines are those which activate or prime immune effector cells which are associated with inflammatory conditions. Many cytokines can be considered as pro-inflammatory although this definition is loosely defined since cytokine action will depend on concentration and cell type studied. We elected to study two main cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α). GM-CSF was chosen for study because of the prior demonstration of its actions in inhibiting apoptosis in neutrophils and eosinophils (Lee et al., 1993, Alam et al., 1994a). TNF-α has been demonstrated to have both pro-and anti-apoptotic effects on neutrophils (Murray et al., 1997), but its effect on eosinophil apoptosis is unknown.

1.10.2 Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

Human GM-CSF is a 22 kDa glycoprotein named for its ability to stimulate bone marrow progenitors to proliferate and differentiate into granulocytes and macrophages (Clark & Kamen, 1987). Recombinant human GM-CSF affects the activity of both neutrophils and eosinophils, for example increasing both antibody-

dependent cytotoxicity and phagocytosis. It is known to activate the neutrophil respiratory burst and enhance the action of other activators such as phorbol-myristate acetate (Dispersio et al., 1986). Recombinant GM-CSF is chemotactic for neutrophils, but does not promote adhesion to endothelial cells. There is a single class of high affinity receptors expressed at low levels on human neutrophils (200 – 800 per cell) (Dipersio et al., 1988). GM-CSF signalling shares some signalling components with IL-3 and IL-5, which also inhibit apoptosis in neutrophils and/or eosinophils. The receptors for these three cytokines consist of 2 subunits: an α subunit that is specific to each cytokine, but the β subunit is identical (Dower, 1993). Dimerisation/oligomerisation of the β subunit is essential for signal transduction. Although none of these receptor subunits has intrinsic kinase activity, these cytokines induce tyrosine phosphorylation (Yousefi et al., 1994), activation of Ras, Raf-1 and mitogen activated protein (MAP) kinase and transcriptionally activate nuclear protooncogenes such as *c-myc*, *c-fos* and *c-jun* (Dower, 1993) (Section 1.14.2). The β subunit is also associated with a novel family of receptor-coupled protein tyrosine kinases, termed JAK kinases (Janus kinases or the 'just another kinase' family) and a set of downstream signalling pathways which appear to be highly conserved (Briscoe et al., 1994, Karnitz & Abraham 1995). When JAKs are phosphorylated on tyrosine residues their enzymatic activity increases (Briscoe et al., 1994). Signal transduction is thought to be dependent on association of the receptor's cytoplasmic domains with specific JAKs, although this interaction is not fully understood (Ihle, et al., 1994, Karnitz & Abraham 1995). For example, the JAK2 kinase constitutively associates with the IL-3/IL-5/GM-CSF receptor β subunit in eosinophils and is activated after stimulation with these cytokines (Quelle et al., 1994).

JAK phosphorylation causes activation of a family of transcription factors termed STATs (signal transducers and activators of transcription) which are present as latent monomers in unstimulated cells, but can be activated by phosphorylation of a conserved tyrosine (Briscoe et al., 1994, Ihle et al., 1994, Karnitz & Abraham 1995). This causes the formation of STAT protein dimers that translocate to the nucleus where they function as sequence-specific activators of gene transcription. The array

of STAT factors, which are phosphorylated and activated by each cytokine, is determined by the availability of various STAT proteins in the target cell, and by the affinities of these proteins for the stimulated receptor-JAK complex (Karnitz & Abraham 1995). For example, IL-5 induces STAT1 activation in eosinophils (Pazdrak et al., 1995). Other signal transduction pathways that are activated in the cell will also influence the expression of genes that are induced (Pawson 1995).

1.10.3 Tumour Necrosis Factor-α (TNF-α)

TNF-α plays a central role in immunity and inflammation and regulates the functions of many cell types. It was first named and isolated in 1975 (Carswell et al., 1975) and cloned in 1984 (Gray et al., 1984) and is a primary mediator of endotoxin-induced septic shock, cachexia, and inflammation (Beutler & Cerami 1988, 1989). TNF-α is expressed on a wide range of cells as a 26 kDa integral transmembrane precursor protein from which a 17 kDa homotrimeric soluble form is released after proteolytic cleavage (Kriegler et al., 1988). This cell surface form can kill target cells by cell-cell contact and induce apoptosis (Perez et al., 1990, Monastra et al., 1996). Mature TNF-α in solution is a compact trimer of 52 kDa (Wingfield et al., 1987), which is composed of the secreted 17 kDa protein and the 26 kDa pre-peptide which spontaneously trimerise to form the mature protein (Smith & Baglioni, 1987).

The mammalian TNFR gene family consists of at least 12 different members that interact with a corresponding TNF-related ligand family (Smith et al., 1994). TNF/NGF receptor family consists of TNFR1, TNFR2, TNFRIII, Fas, CD40, CD30, CD27, p75 NGF, OX-40, 4-1BB and DR3. Most are type I transmembrane proteins with a characteristic cysteine-rich pseudorepeat in the extracellular region. This family is involved in a wide range of biological effects including acute phase responses, cell growth and apoptosis and lymphocyte activation (Smith et al., 1994). Ligand-dependent activation of the TNFR superfamily depends on receptor trimerisation (Banner et al., 1993, Bigda et al., 1994); each trimeric complex contains three receptors bound to one ligand trimer.

TNF-α interacts with cells through two types of receptor; the p55 receptor (TNFR1) and the p75 receptor (TNFR2) (Hohmann et al., 1989, Brockhaus et al., 1990, Loetscher et al., 1990, Schall et al., 1990). It binds to both receptors with high affinity and independently. The membrane-bound form of TNF is biologically active on both receptor subtypes (Perez et al., 1990, Decoster et al., 1995). TNFRs occur on almost all nucleated cell types except unstimulated lymphocytes (Vandenabeele et al., 1995). The number of receptors varies from about 200 to 10,000 per cell, but there is no correlation between the number of receptors and the magnitude of response (Fiers et al., 1987, Brockhaus et al., 1990). After binding the ligand-receptor complexes are internalised by clathrin-coated pits (Brockhaus et al., 1990, Porteu & Hieblot, 1994); the internalised receptor is subsequently degraded in lysosomes (Mosselmans et al., 1988). Unlike TNFR1, TNFR2 contains no tyrosine residues in its intracellular domain and therefore, lacks a consensus sequence for rapid cellular internalisation through coated pits (Collawn et al., 1990).

Soluble TNF-binding proteins have been discovered (Seckinger et al., 1988, Engelmann et al., 1989, Gatanaga et al., 1990). These are truncated forms of the extracellular domain of TNFR1 and TNFR2 and appear to arise by proteolytic cleavage from surface-bound TNF receptors (Englemann et al., 1990, Lantz et al., 1990). A major source of these binding proteins may be neutrophils, which reportedly shed 50 % of TNF receptors within two minutes in response to many physiological and pharmacological agents (Porteu & Nathan, 1990). These soluble proteins modulate the availability of biologically active TNF.

The cytoplasmic domains of the TNFR superfamily members are relatively short and contain no known catalytic motif. They also lack significant sequence homology among themselves except for a domain of approximately 80 amino acids long referred to as the death domain (DD), which is involved in TNF-mediated cell killing and is found in both TNFRI and Fas (Itoh & Nagata, 1993, Tartaglia et al., 1993). The use of yeast-two hybrid screens led to identification of the receptor associated molecules, TRADD, FADD/MORT1, TRAF2, 1-TRAF, RIP, FAN, TRAP1, 55.11, FLICE/MACH, and sentrin which associate with TNFR1 and TRAF1, TRAF2, c-

IAP-1, and c-IAP-2, which associate with TNFR2 (Figure 1.2- Section 1.12) (Darnay & Aggarwal, 1997).

Many TNF signals are mediated via TNFR1, but signalling through TNFR2 is controversial. For example, TNFR2 lacks a death domain, but it is implicated in TNF-dependent cell killing (Heller et al., 1992, Higuchi & Aggarwal, 1993, Murray et al., 1997). The development of TNF muteins which allow binding to either TNFR1 or TNFR2, were used to determine the specific effects of each receptor (Loetscher et al., 1993, Van Ostade et al., 1993, Barbara et al., 1994). These muteins demonstrated that both NF-κB activation and cytotoxicity is mediated through TNFR1 in some cells (Chainy et al., 1996). Other reports show activation of NF-κB and apoptosis mediated via TNFR2 (Tartaglia et al., 1993, Laegreid et al., 1994, Rothe et al., 1994, Zheng et al., 1995). In other cells each receptor is able to induce cytotoxicity and apoptosis on its own, and additive effects are observed when both receptors are triggered together (Grell et al., 1993). The analysis of TNF actions on various tumour cell lines revealed that TNF-induced cytotoxicity occurred in cells expressing TNFR1 regardless of expression of TNFR2, while apoptosis was induced by TNF in cells which expressed both receptors (Higuchi & Aggarwal, 1994). A TNFR2-associated kinase that phosphorylates both receptors has been found, and may provide a link between signals from both receptors (Darnay et al., 1994). Cell death induction by both TNF and Fas receptors involves a critical function of protease(s) related to ICE (Enari et al., 1995, Los et al., 1995, Tewari & Dixit, 1995) (Section 1.14.1), and both can be suppressed in a cell-type manner by Bcl-2 (Hennet et al., 1993, Itoh et al., 1993).

1.10.4 Anti-inflammatory Cytokines

Although many cytokines have been shown to be involved in cell activation, few have been demonstrated to downregulate the production of pro-inflammatory cytokines or inflammatory mediators from immune cells. Of these few, we chose interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) for study. Disruption of IL-10 or TGF-β expression in mice is correlated with a non-resolving

inflammatory state (Schull et al., 1992, Khun et al, 1993,) which suggests that these cytokines may play a role in inflammatory resolution.

1.10.5 Interleukin 10 (IL-10)

Human IL-10, a member of the four α-helix family of cytokines (Vieira et al., 1991), is an 18.5 kDa protein of 160 amino acids, containing two intramolecular disulphide bonds (Moore et al, 1993, Windsor et al, 1993). It is acid labile and appears in soluble form as a homodimer of 39 kDa (de Waal Malefyt, 1992). The gene encoding IL-10 is located on chromosome 1 in both mouse and man and includes possible binding sites for the transcription factors NF-κB and AP-1 (Kim et al., 1992). IL-10 is produced by subsets of T cells as well as by monocytes, macrophages and B cells (Fiorentino et al., 1989, Moore et al., 1990, de Waal Malefyt et al., 1991, Vieira et al., 1991).

The IL-10 receptor is a transmembrane glycoprotein approximately 111 kDa in size which closely resembles that for INF-γ (Ho et al., 1993, Lui et al., 1994). The receptor is a member of the type II cytokine receptor family (Bazan, 1990), and is expressed in low numbers on responsive cells, however, cytokine-receptor binding is of high affinity with a K_d of 200 pM (Ho et al., 1993, Lui et al., 1994).

IL-10 was first discovered as a novel factor produced by murine Th2 helper cells which inhibits INF-γ synthesis by murine Th1 cells (Fiorentino et al., 1989, 1991). Mouse Th clones can be divided into two types based on the pattern of cytokines secreted in response to antigen or lectin stimulation. Th1 clones secrete IL-2, INF-γ and lymphotoxin, whereas Th2 express IL-4, IL-5, IL-6 and IL-10 (Fiorentino et al., 1989). Both types of clone secrete IL-3, GM-CSF and TNF-α. Human IL-10 is also produced by Th cell clones: however production is not restricted to Th2 cells, since most Th0 and Th1 clones are also able to produce considerable levels of IL-10 (de Waal Malefyt et al., 1992).

Because macrophages and T cells both produce and respond to IL-10, it has been proposed that IL-10 may provide a mechanism whereby these cells limit their own participation and that of other immune-reactive cells in antigen-driven reactions (de Waal Malefyt et al., 1991, Rennick et al., 1995). IL-10 is expressed later than other cytokines released by activation of T cells and macrophages, which may reflect its role in inhibiting the activation of these cells (de Waal Malefyt et al., 1991, Florentino et al., 1991). Human monocytes activated by LPS produce high levels of IL-10, but production is relatively late when compared with other cytokines with maximal production at 24 – 28 h, while early cytokines such as TNF-α and GM-CSF are produced by 4 h (de Waal Malefyt et al., 1991).

In humans, IL-10 inhibits IL-5 synthesis by resting $CD4^+$ T cells and by differentiated Th0 or Th2 clones (Del Prete et al., 1993, Schandené et al., 1994). In the context of allergic inflammation, inhibition of IL-5 generation may limit eosinophil recruitment (Section 1.3). Mast cells can regulate a variety of eosinophil functions via the production of cytokines that can be down regulated by IL-10 (Arock et al., 1996).

The inhibitory action of IL-10 on most cell-mediated immune responses *in vitro* suggested that recombinant IL-10 might prove useful for the prevention and treatment of certain inflammatory processes *in vivo*. Phase III trials to investigate the efficacy of intravenous IL-10 administration in humans with inflammatory bowel disease have been undertaken (Van Deventer et al., 1997). In a randomised, controlled trial of IL-10 in humans there were no adverse signs after a single intravenous injection of IL-10, which had inhibitory effects on T cells and suppressed the production of TNF-α and IL-1β by whole blood stimulated *ex vivo* with LPS (Chernoff et al., 1995, Huhn et al., 1996). Because of its ability to inhibit production of these cytokines, which are involved in acute and chronic inflammatory processes, IL-10 may be useful in a number of conditions such as sepsis, chronic arthritis, and inflammatory bowel disease. Humans with inflammatory bowel disease have been shown to have low levels of circulating IL-10 (Ishida 1993). In a recent

study, 50% of patients with steroid-refractory Crohn's disease, achieved remission in symptoms with IL-10 treatment (Van Deventer et al., 1997).

Since eosinophils and neutrophils are pro-inflammatory cells, it is possible that IL-10 could be involved in the control of apoptosis and hence clearance from inflammatory foci. IL-10 could act directly on these cells or indirectly by influencing the secretion of cytokines or inflammatory mediators from other immune cells, alternatively it may act in concert with other modulators of apoptosis.

1.10.6 Transforming Growth Factor-β (TGF-β)

TGF-β is a member of the TGF-β superfamily. These proteins are dimeric secreted signalling molecules and include TGF-βs, activins, Mullerian inhibitory substance and bone morphogenetic proteins (Kingsley, 1994, Massagué, et al., 1994) which regulate proliferation, differentiation, migration and apoptosis of many cell types (Ten Dijke, et al., 1996). These cytokines are also involved in immunomodulation, inflammation, and bone and cartilage formation. Mammalian species express three distinct isoforms of TGF-β, 1, 2 and 3 which are encoded by specific genes and are expressed in unique patterns *in vivo* (Sporn et al., 1987, Derynck et al., 1988, Ten Duke et al., 1988, Kondaiah et al., 1989, Millan et al., 1991). The expression of both TGF-β2 and 3 is developmentally and hormonally regulated (Roberts & Sporn, 1992). Two other isoforms have been cloned; β4 has only been detected in chick embryo chondrocytes (Jakowlew, et al., 1988), and β5 in *Xenopus* embryos and adult tissues (Kondaiah et al., 1990). *In vitro* studies have demonstrated that leukocytes principally express the type I isoform which exerts profound suppressive effects on immune cell function (Wahl, 1994), therefore type I was chosen for these studies.

Mature TGF-β1 is a 25 kDa disulphide-linked dimer of 112 amino acids which is produced by platelets, lymphocytes, macrophages and various other cells (Assoian et al., 1983, 1987, Kehrl et al., 1986). It has bifunctional actions in many cells, stimulating or inhibiting growth depending on culture conditions and the presence of other growth factors (Tucker et al., 1984, Roberts et al., 1985, Anzano et al., 1986).

TGF-β1 controls many functions in cells such as cell cycle progression, differentiation, adhesion, migration and extracellular matrix production (Lyons & Moses, 1990, Massaguè, 1990). It is mainly a growth inhibitor (Roberts et al., 1986, Moses et al., 1990), but also affects the expression of a number of genes for structural and metabolic proteins (Keski-Oja et al., 1987, Massagué, 1987). TGF-β enhances normal wound healing via stimulation of chemotaxis, angiogenesis, extracellular matrix accumulation and control of the production of cytokines and other inflammatory mediators (Kehrl et al., 1986, Roberts et al., 1986, Tsunawaki et al., 1988, Wahl et al., 1987, Gold et al., 1990). It stimulates the synthesis of individual matrix components including fibronectin, tenascin, collagens and proteoglycans (Ignotz & Massagué, 1986, Roberts et al., 1986, Balza et al., 1988, Bassols & Massagué, 1988). It also blocks matrix degradation by decreasing the synthesis of proteases and increasing the levels of protease inhibitors (Edwards et al., 1987, Laiho et al., 1987). TGF-β1 is the only isoform of TGF-β found in α-granules of platelets, and its release from degranulating platelets may be critical to the initiation of repair responses (Assoian et al., 1983). Expression of TGF-β1 is induced by its own ligand which serves to enhance and extend its expression, throughout the course of the healing process (van Obberghen-Schilling et al., 1988, Kim et al., 1990). However, this may also cause scarring and fibrosis that can develop into chronic, progressive conditions that may adversely affect tissue structure; this cytokine has been implicated in acute glomerulonephritis (Border et al., 1990, Okuda et al., 1990, Yamaguchi et al., 1990), diabetic nephropathy (Mauer et al., 1984, Velasquez et al., 1990), idiopathic fibrosis of the lung (Brockelmann et al., 1991), pulmonary fibrosis (Raghow et al., 1989), liver cirrhosis (Castilla et al., 1991) and postoperative adhesions (Williams et al., 1992).

The most widely distributed TGF-β receptors are type I (53 kDa) and type II (70 kDa) (Cheifetz et al., 1987, 1990, Segarini et al., 1989). Two other receptors are known; the type III receptor (betaglycan), and endoglin. Betaglycan is a membrane proteoglycan that binds TGF-β through a 100 kDa core protein, but has no obvious signalling motif (Cheifetz et al., 1987, Segarini & Seyedin, 1988, Andres et al., 1989,

Lopez-Casillas et al., 1991, Wang et al., 1991). Endoglin is a disulphide-linked protein homodimer, structurally related to betaglycan and present at high levels in vascular endothelial cells (Gougos & Letarte, 1988, Cheifetz et al., 1992). Type I, II and betaglycan bind all three TGF-β isoforms: endoglin does not bind β2 (Cheifetz et al., 1987, 1992, Bellón et al., 1993). Betaglycan and endoglin may function to capture the soluble ligand and then present it to signalling receptors (Massagué et al., 1994), since the expression of betaglycan correlates with elevated binding of TGF-β to receptor II. However, betaglycan is expressed at low levels on haematopoetic cells (Wang et al., 1991) and among immune cells, only activated monocytes and tissue macrophages express endoglin (Lastres et al., 1992, O'Connel et al., 1992).

TGF-β transduces signals via two different types of serine/threonine kinases, type I (Attisano et al., 1993, Ebner et al., 1993, Bassing et al., 1994, Ten Dijke et al., 1994) and type II receptors (Lin et al., 1992, Moustakas et al, 1993). The structures of these receptors are similar and consist of small cysteine-rich extracellular regions, a transmembrane region, and intracellular parts containing serine/threonine kinase domains (Massagué, et al., 1994, Kingsley, 1994). Type II receptors, have carboxyl-terminal extensions of variable length that are rich in threonine and serine residues, while type I receptors have, in their intracellular juxtamembrane region, a stretch of residues rich in serine and glycine (Kingsley, 1994, Massagué, et al., 1994). These receptors are the only signalling receptors identified for TGF-β. TGF-β induces the formation of a hetero-oligomeric complex, thought to be a heterotetramer consisting of two molecules each of type I and II (Yamashita et al., 1994, 1995, Ten Dijke et al., 1996, Weiss-Garcia & Massagué, 1996). The type II receptor is a constitutively active kinase, the autophosphorylation of which is not upregulated upon ligand binding (Wrana et al., 1994). After binding of TGF-β to type II, type I is recruited into a stable complex and is phosphorylated, by type II on its serine and threonine residues (Wrana et al., 1994). Phosphorylation of type I is important for signal transduction, (Wrana et al., 1992, Cárcamo et al., 1994, Wieser et al., 1995). Activated type I does not autophosphorylate or phosphorylate type II (Wrana et al., 1994, Wieser et al., 1995). Thus, type I functions as a substrate for type II; its phosphorylation and activation by type II is essential and sufficient for most TGF-β

mediated signalling. However, both receptors are required for signalling; whereas type II receptors can bind ligand by themselves, type I receptors cannot (Wrana et al., 1992).

An early effect of TGF-β treatment is the transcription of *jun B* and *c-jun* protooncogene mRNA and the mRNA for IκB-α, an inhibitor of the transcription factor NF-κB and a downregulation of c-Myc (Pertovaara et al., 1989, Pientenpol et al., 1990, Arsura et al., 1996, Iavarone and Massagué, 1997). However, the intracellular signalling pathways of this cytokine are still unclear, but recent research has uncovered various proteins that seem to be involved in signal transduction.

TRIP-1 (TGF-β-receptor interacting protein-1) specifically associates with the type II receptor in a kinase-dependent way and is phosphorylated on serine and threonine by the receptor kinase, suggesting that it plays a role in receptor signalling (Chen et al., 1995a). TRIP-1 may be a substrate of the Type II receptor *in vivo* (Chen et al., 1995a). Other elements in the pathway appear to be TAK1, a TGF-β activated MAPKKK (Section 1.12.2)(Yamaguchi et al., 1995).

Inactivation of the type II receptor has been detected in some tumour types, suggesting that this receptor may function as a tumour suppressor (Garrigue-Antar et al., 1995, Markowitz et al., 1995, Parsons et al., 1995, Pierce et al., 1995b). Studies on this role of the cytokine uncovered more signalling molecules that may be involved in signal transduction. The retinoblastoma gene products (Rb) and $p15^{ink4B}$ have been identified as tumour suppressor genes and are implicated in TGF-β signalling (Alexandrow & Moses, 1995). TGF-β inhibits expression and activation of cyclins (Howe et al., 1991, Geng & Weinberg, 1993) and cyclin-dependent kinases (Ewen et al., 1993) and induces cyclin-dependent kinase inhibitors including p15 (Hannon & Beach, 1994), p21 (Datto et al., 1995) and p27 (Polyak et al., 1994). It inhibits phosphorylation of Rb and the induction of c-*myc* (Moses et al., 1990, Alexandrow & Moses, 1995).

A novel suppressor gene, DPC4, (deleted in pancreatic cancer), (Hahn et al., 1996), is also involved in TGF-β signalling. DPC4 or Smad4 belongs to the SMAD family of proteins (Derynck et al., 1996, Massagué, 1996), which are phosphorylated by activated TGF-β receptors, and propagate the signal through homo- and hetero-oligomeric interactions (Eppert et al., 1996, Lagna et al., 1996, Zhang et al., 1996, Attisano & Wrana, 1998). Smad4/DPC4 plays a central role as it is the shared hetero-oligomerization partner of the other specific SMADs that can be activated by other TGF-β family members.

TGF-β receptor activation induces phosphorylation of Smad2 and Smad3 (Eppert et al., 1996, Lagna et al., 1996, Macías-Silva et al., 1996, Zhang et al., 1996, Nakao et al., 1997a). These, in turn, form hetero-oligomeric complexes with Smad4/DPC4 (Lagna et al., 1996, Kretzschmar et al., 1997, Shi et al., 1997, Wu et al., 1997), and translocate to the nucleus (Eppert et al., 1996, Macías-Silva et al., 1996, Nakao et al., 1997a, Zhang et al., 1997), where they regulate transcriptional responses (Chen et al., 1996a).

SMAD proteins are also involved in curtailing TGF-β signals. Smad7, is a TGF-β-inducible antagonist of TGF-β signalling (Nakao et al., 1997b). Smad 7 associates stably with the TGF-β receptor complex, but is not phosphorylated upon TGF-β stimulation (Nakao et al., 1997b); it appears to inhibit TGF-β-mediated phosphorylation of Smad2 and Smad3 (Hayashi et al., 1997, Nakao et al., 1997b). TGF-β rapidly induces expression of Smad7 mRNA suggesting that Smad7 may participate in a negative feedback loop to control TGF-β-mediated responses (Nakao et al., 1997b). Smad6 forms stable associations with the type I receptor and interferes with the phosphorylation of Smad 2 and the subsequent heteromerisation with Smad4 signalling (Imamura et al., 1997). Smad6 is therefore likely to be another inhibitor of TGF-β . However other pathways have been implicated in TGF-β signalling (Alevizopoulos & Mermod, 1997) therefore inhibitory SMADs may only target this specific pathway.

1.11 Interactions Between Cytokines and Other Inflammatory Mediators

There are many obvious overlaps between the biological processes evoked by cytokine and lipid mediator networks. Cytokines influence the production and actions of lipid mediators at several broad levels. They can stimulate *de novo* synthesis of lipid mediators, induce the enzymes that regulate lipid mediator biosynthesis, cause degradation of or upregulation of receptor expression, and act synergistically with a given lipid mediator to exert biological effects. Likewise, certain lipid mediators can affect the biosynthesis and actions of cytokines.

AA can be liberated directly from phopholipids by the action of phospholipase A_2 (PLA_2) (Peplow, 1996). Cytokines such as IL-1 and TNF-α induce increased expression of group II secretory PLA_2 in a variety of cells (Coyne et al., 1992). Group II $sPLA_2$ plays a role in mediating arachidonic acid release and prostaglandin production (Barbour et al, 1993). The 85 kDa cytosolic PLA_2 ($cPLA_2$) was the first arachidonic acid-selective PLA_2 identified, and is now recognised as an important enzyme for mediating agonist-induced arachidonic acid release (Clark et al, 1995). $cPLA_2$ is regulated by both transcriptional and post-translational mechanisms. IL-1 and TNF-α each induce an increase in the synthesis of PLA_2 and COX-2, whereas TGF-β has inhibitory effects (Clark et al., 1995, Pruzanski et al., 1998). Therefore the signals and effects which can be produced by a specific cytokine or eicosanoid are complex and may, depending on the timepoint chosen for study, reflect synergistic, additive or antagonistic effects of other mediators activated or produced by the original stimulus.

1.12 TNF Signalling Pathways

Cytokines can have differential effects on specific cells because of variation in receptor expression and through interactions with other factors. However, signalling can become complex because cytokines can trigger more than one intracellular pathway and recent work has shown that many of these pathways can interact. This complexity can be illustrated by means of the three main TNF-α signalling

pathways; caspase, mitogen-activated protein (MAP) kinase and NF-κB activation. (Figure 1.2). Since this cytokine has both anti- and pro-apoptotic effects it therefore influences both survival and death pathways. Why some cells are susceptible to the pro-apoptotic effects of TNF-α while others are resistant is currently unknown. However, study of the intracellular inducers of the biological effects of TNF-α are likely to help elucidate mechanisms involved in driving both survival and programmed cell death.

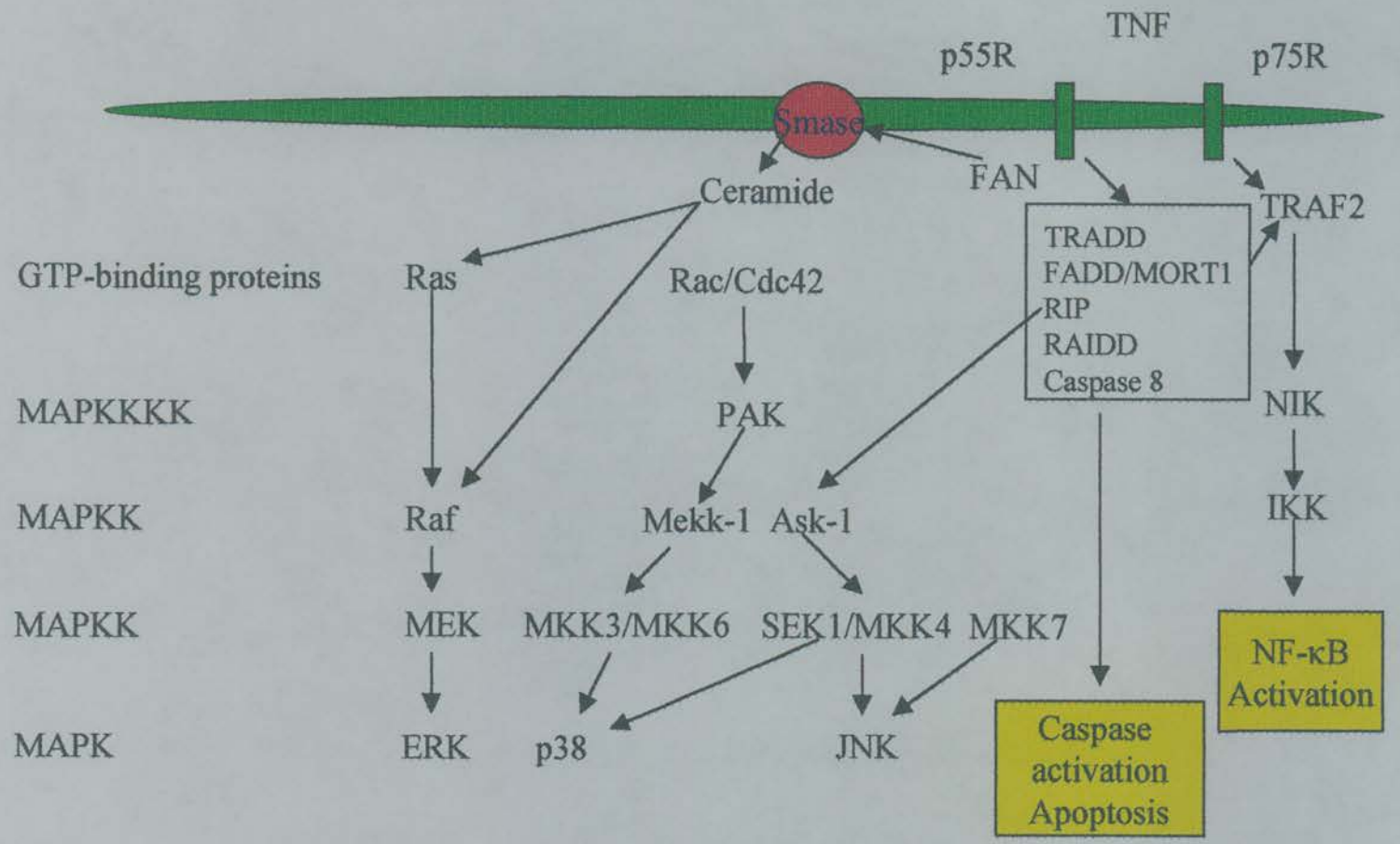


Figure 1.2 TNF-α Signalling Pathways

1.12.1 The Caspase-Cascade Pathway

Apoptosis is regulated by a series of biochemical events; a common feature of which is the activation of caspases (cytosolic aspartate-specific cysteine proteases) (Alnemri et al., 1996). Caspases are inactive until a signal initiates activation of one, which in turn will proteolytically activate the next protease in the pathway. The activation of this cascade and the subsequent proteolytic cleavage of specific substrates is thought to lead to the structural changes of apoptosis and the activation of other effectors critical for death (Martin & Green, 1995).

Caspase substrates are rapidly being identified but the general assumption is that caspases recognise a limited set of cellular proteins (Nicholson & Thornberry, 1997). In human cells, several substrates for these proteases have been defined, including enzymes functioning in DNA repair [e. g. PARP and DNA-PK_{cs} (Casciola-Rosen et al., 1995)], and structural proteins such as lamins A and B (Lazebnik et al., 1995, Neamati et al., 1995, Orth et al., 1996) and fodrin (Martin et al., 1995). Mature neutrophils have been shown to contain precursors of caspases 1, 3 and 7, but not PARP or DNA-PK_{cs} (Sanghavi et al., 1998).

Much of the current knowledge of proteases involved in the caspase-cascade was derived from genetic studies in the nematode *Caenorhabditis elegans* (Ellis et al., 1991, Hengartner & Horvitz, 1994). The *C. elegans* gene *ced-3* encodes a protein that is similar to the family of cysteine proteases, which includes interleukin-1-β-converting enzyme (ICE) (Cerreti et al., 1992, Thornberry et al., 1992, Yuan et al., 1993) and the 32 kDa putative cysteine protease (CPP32) (Fernandes-Alnemri et al., 1994). At least 5 proteases in the ICE family are expressed in mammalian cells, and the activity of some of these enzymes appears to be required for apoptosis to occur (Martin & Green 1995). Inappropriate expression of intracellular proteases including ICE has been shown to drive cells into the apoptotic programme. Conversely, specific ICE family protease inhibitors such as the viral protein crmA (Miura et al., 1993) can prevent normal apoptosis.

TNF-α activates the caspase pathway when TNF-induced trimerisation of TNFR1 triggers an association with TNFR1-associated death domain protein (TRADD), which recruits Fas-associated death domain protein (FADD) also known as MORT1 (Chinnaiyan et al., 1995, Hsu et al., 1996). This interaction with TRADD and FADD/MORT1 allows activation of the caspase cascade (Boldin et al., 1996, Muzio et al., 1996), when FADD/MORT1 recruits pro-caspase 8 to the receptor complex (Darney & Aggarwal, 1997). When pro-caspase 8 is activated, the resulting cascade of caspase activation causes cleavage of cytosolic, cytoskeletal, and nuclear proteins leads to apoptosis (Patel et al., 1996). As shown in Figure 1.2, the TNF receptor complex also interacts via TRADD with RIP (Receptor Interacting Protein) and TRAF2 (TNFR associated factor) proteins which are involved in the activation of stress-activated protein kinases (SAPK), c-Jun N-terminal kinases (JNK) and NF-κB (Hsu et al., 1996, Lui et al., 1996). Another protein RAIDD, can associate with RIP and can cause the recruitment and activation of caspase 2 (ICH-1) (Duan & Dixit, 1997).

1.12.2 The MAP Kinase Pathway

As shown in Figure 1.2, TNF-α also activates the MAP kinase pathway. MAP kinase cascades are ubiquitous components of various signal transduction pathways (Robinson & Cobb, 1997). They generally consist of a MAP kinase kinase kinase kinase (MAPKKKK) which activates through phosphorylation, a MAP kinase kinase kinase (MAPKKK), which phosphorylates and thereby activates a MAP kinase kinase, (MAPKK), which in turn phosphorylates and activates the corresponding MAP kinase (MAPK) (Illustrated in Figure 1.2). There are three different types of MAP kinases: the p42 and p44 extracellular signal-regulated kinases (ERKs), (Avruch et al., 1994), the JNKs/SAPKs (Derijard et al., 1994, Kyriakis et al., 1994) and p38 kinases (Han et al., 1994).

ERKs are most efficiently stimulated by growth factors and phorbol esters (Davis, 1994, Kyriakis et al., 1994, Minden et al., 1994, Cobb & Goldsmith, 1995), The Raf-

Mek-Erk cascade can be activated through FAN, a protein that binds to TNFR1 and activates neutral sphingomyelinase (Adam-Klages et al., 1996) (Figure 1.2).

JNK/SAPKs and p38 play a crucial role in the responses stimulated by pro-inflammatory cytokines, environmental stresses and apoptotic agents (Derijard et al., 1994, Han et al., 1994, Kyriakis et al., 1994, Kyriakis & Avruch, 1996a, b). These kinases can be activated by small GTP-binding proteins that include Cdc42 and Rac1 (Coso et al., 1995, Minden et al., 1995). JNK and p38 can be activated by phosphorylation of Tyr and Thr residues by the upstream kinase MKK7 (Tournier et al., 1997) or by MKK4/SEK-1 (Lange-Carter et al., 1993, Sanchez et al., 1994, Derijard et al., 1995, Lin et al., 1995a), which is phosphorylated and activated by MEKK1 (Han et al., 1994, Sanchez et al., 1994). MEKK1, can in turn, be activated by p21-activated protein kinase (PAK) (Bagrodia et al., 1995). Similarly, p38 kinases are activated by MKK3 and MKK6/SAPKK3 (Cuenda et al., 1996, Raingeaud et al., 1996).

As shown in Figure 1.2 TRAF2 mediates activation of NF-κB, and JNK and p38 kinases in response to TNF-α. Recent studies (Lui et al., 1996, Natoli et al., 1997, Reinhard et al., 1997) have shown that TRAF2-mediated activation of JNK, but not NF-κB can be inhibited by an inactive mutant of MEKK1, indicating that JNK and NF-κB signalling diverge downstream of TRAF2. MEKK1 has been implicated in TNF-induced JNK/SAPK activation as has ASK1, a kinase which is TNF-inducible, and also activates p38 (Ichijo et al., 1997). MKK7 has also been found to be activated in response to TNF-α (Foltz et al., 1998).

Stress stimuli can induce apoptosis in a variety of cell types; therefore JNK/SAPK and p38 have been implicated as part of the apoptotic mechanism in response to these stimuli (Xia et al., 1995, Verheij et al., 1996, Zanke et al., 1996). For example, in Jurkat cells, JNK and p38 MAP kinase activation have been coupled to Fas-induced apoptosis, and it appears that this coupling requires the activation of caspases (Juo et al., 1997), while ASK1 activation, can also cause apoptosis (Ichijo et al., 1997, Malinin et al., 1997). Because TNF-α is known to activate both JNK and

p38, these kinases may play a role in either TNF-α-induced survival, or cell death. However the role of the stress-activated kinases during apoptosis induced by various stimuli is controversial. Inhibition of early TNF-induced JNK and p38 kinase by expression of dominant negative mutants of MKK4, MKK6 or the p38 inhibitor SB203580 increased TNF-induced apoptosis, whereas expression of wild type MKK4 and MKK6 enhanced cell survival in the presence of TNF (Roulston et al., 1998), suggesting that JNK and p38 activation may mediate cell survival signals in response to TNF-α. Recently JNKs/SAPK and the p38 kinases, have also been shown to be activated by hematopoietic growth factors such as IL-3, GM-CSF and G-CSF (Foltz et al., 1998), which are known to enhance survival in granulocytes (Lee et al., 1993, Alam et al., 1994a).

1.12.3 Activation of NF-κB

The third major pathway in TNF-α signalling causes activation of NF-κB. NF-κB is an inducible transcription factor that is usually found in the cytoplasm of unstimulated cells, bound to an inhibitor subunit known as Iκ-B. This factor is activated when Iκ-B is phosphorylated, ubiquitinated, and degraded via the proteasome, thus exposing the nuclear localisation sequence (NLS) which allows activated NF-κB to enter the nucleus and bind to DNA (Baeuerle & Henkel, 1994).(Section 1.15)

As shown in Figure 1.2, TRAF2 binds directly to TNFR2 (Darney and Aggarwal, 1997). The NF-κB-inducing kinase (NIK) is a MAPKKK-related kinase that binds TRAF2 and activates NF-κB when overexpressed (Malinin et al., 1997). TRAF2 appears to be the bifurcation point of two kinase cascades leading to activation of NF-κB and JNK, respectively (Song et al., 1997). Therefore, stimuli that induce NF-κB, such as TNF-α, also activate the JNK cascade, and the two pathways may be interlinked. Transfection of MEKK1 can induce IκB degradation and activate NF-κB reporter genes (Hirano et al., 1996, Meyer et al., 1996). This activation of NF-κB occurs through site-specific phosphorylation of IκB at Ser-32 and 36 (Lee et al.,

1997a). MEKK-1 can also induce the site-specific phosphorylation of IκB *in vivo* and activates the IκB kinase *in vitro* (Lee et al., 1997a, Mercurio et al. 1997). MEKK1 is therefore a critical component of both the c-Jun and NF-κB stress response pathways.

1.12.4 Other Signalling Pathways Involved in TNF-α Signalling

TNF-α signalling also involves activation of a phosphatidylcholine-specific phospholipase C, which generates 1,2-diacylglycerol (DAG), in response to ligand. DAG, as well as directly activating protein kinase C, may in turn initiate a signalling cascade that involves the activation of acidic sphingomyelinases, the generation of ceramide and ceramide-dependent activation of serine-threonine kinases (Siebenlist et al., 1994).

A role for ceramide produced by the sphingomyelin cycle was proposed as a second messenger of TNF-α mediated cellular responses including the induction of apoptosis (Obeid et al., 1993, Jarvis et al., 1994), and the activation of NF-κB (Schutze et al., 1992). In contrast there are also several reports that demonstrate that ceramides are not involved in the signal transduction of TNF-α (Dbaido et al., 1993, Betts et al., 1994). Neutral sphingomyelinase hydrolyses sphingomyelin in the outer leaflet of the plasma membrane, while an acidic sphingomyelinase produces ceramide in the endosomal/lysosomal compartments (Hannun et al., 1993). However, ceramides do not mimic TNF-α-induced apoptosis (Murray, 1998), although inhibition of acidic sphingomyelinase inhibits TNF-induced apoptosis, but does not inhibit NF-κB activation (Higuchi et al., 1996). Therefore although TNF-α activates NF-κB, ceramide does not (Higuchi et al., 1996).

1.13 Transcription Factors

The primary control of eukaryotic gene expression occurs at the level of transcription where genes may be regulated in response to a specific signal or in a particular tissue type (Darnell, 1982). Transcriptional control of gene expression involves the binding of regulatory proteins or transcription factors to short, *cis*-acting DNA sequence elements located within and near the promoter of a gene. There are several types of DNA sequences to which transcription factors will bind. These include promoter, regulatory and enhancer/repressor elements. The transcription factors that bind to regulatory and enhancer/repressor sites will increase or decrease the rate of transcription but are not essential for basal activity (Wildeman et al., 1986, Zenk et al., 1986).

Two major signal transduction systems utilise cAMP and diacylglycerol (DAG) as secondary messengers (Nishizuka, 1986). Each of these pathways is also characterised by the mobilisation of a specific protein kinase, protein kinase A and protein kinase C, respectively. Intracellular levels of cAMP are regulated primarily by adenylate cyclase. cAMP, in turn, binds co-operatively to two sites on the regulatory subunit of protein kinase A (PKA), releasing the active catalytic subunit (Roesler et al, 1988, Lalli & Sassone-Corsi, 1994). The activated catalytic subunit is released from cytoplasmic and Golgi complex anchoring sites and used to phosphorylate a number of cytoplasmic and nuclear proteins on serines in the context X-Arg-Arg-X-Ser-X (where X is any amino acid) (McKnight et al, 1988, Roesler et al, 1988). In the nucleus, PKA-mediated phosphorylation ultimately influences the transcriptional regulation of various genes through distinct, cAMP-inducible promoter responsive sites (Ziff, 1990, Borrelli et al, 1992).

Exposure of cells to TNF-α results in activation of at least two transcription factors, activator protein-1 (AP-1) (Brenner et al., 1989) and NF-κB (Osborn et al., 1989). These transcription factors mediate induction of other cytokine and immunoregulatory genes.

1.14 AP-1

AP-1 is a sequence-specific transcriptional activator composed of members of the Jun and Fos families which associate to form a variety of homo-and heterodimers that bind to a common site (Angel & Karin, 1991). AP-1 activity can be induced by an increase in AP-1 components or by phosphorylation at specific sites which can enhance the transactivating potential of several AP-1 proteins, including c-Jun and c-Fos (Smeal, et al., 1991, Suzuki et al., 1991, Deng & Karin, 1994). Most of the genes that encode AP-1 components behave as "immediate-early" genes, i.e. genes whose transcription is rapidly induced independently of *de novo* protein synthesis, following cell stimulation. Among these, the regulation of *c-fos* and *c-jun* transcription is best understood.

All three MAPK families contribute to induction of AP-1 activity in response to a diverse array of extracellular stimuli; each affecting AP-1 through phosphorylation of a different substrate. The most relevant MAPKs that contribute to AP-1 activation appear to be the JNK and p38 kinases (Karin, 1995, Karin et al., 1997) which activate various transcription factors. For example, both c-Jun (Devary et al., 1992), activating transcription factor 2 (ATF2) (Gupta et al., 1995), induce the *c-jun* and *c-fos* genes. Another part of the increase in AP-1 activity is due to c-Jun phosphorylation. Phosphorylation of c-Jun potentiates its ability to activate transcription as either a homodimer (Pulverer et al., 1991, Smeal et al., 1991) or as a heterodimer with c-Fos (Deng & Karin, 1994). AP-1 is typically upregulated in proliferative processes and can be activated by phorbol esters via the protein kinase C signal transduction pathway.

1.15 NF-κB

NF-κB was first identified as a nuclear factor that binds the decameric DNA sequence 5`-GGGACTTTCC-3` within the intronic immunoglobulin kappa light chain enhancer in mature B cells (Sen & Baltimore, 1986). Many inflammatory mediators regulate gene expression in target cells through transcription factors such

as NF-κB. NF-κB is activated rapidly in response to many pathogenic signals, participates in cytoplasmic/nuclear signalling, and potently activates transcription of a variety of genes encoding cytokines such as IL-1, IL-2, IL-6, IL-8, TNF, and GM-CSF (Baeuerle & Henkel, 1994).

NF-κB is formed from members of the Rel family of proteins that comprises two groups classified on the basis of their structure, function and mode of synthesis. Group 1 includes p50 and p52, which are synthesised as precursor proteins of 105 (p105) and 100 (p100) kDa respectively (Ghosh et al., 1990, Kieran et al., 1990, Schmid et al., 1991). These precursors are able to dimerise with other family members (Mercurio et al., 1993, Naumann et al., 1993, Rice et al., 1993, Scheinman et al., 1993). In such heterodimers, the long C-terminal portion of the precursor blocks the NLS of each subunit, resulting in cytoplasmic localisation (Blank et al., 1992, Henkel et al., 1992, Matthews et al., 1993). However, both precursors can be processed to provide active DNA-binding NF-κB dimers (Fan & Maniatis, 1991, Mercurio et al., 1993). Group II comprises p65/RelA, c-Rel, RelB and the *Drosophila* proteins Dorsal and Dif (Sen & Packer, 1996). Rel proteins are characterised by the presence of a Rel homology domain, which is about 300 amino acids in length and includes a DNA binding domain and an NLS. Rel/NF-κB proteins can form homo- or heterodimers (Baeuerle & Henkel, 1994, Thanos & Maniatis, 1995).

The most common form of NF-κB is a heterodimer of p50 and RelA/p65 proteins (Ghosh et al., 1990, Kieran et al., 1990, Nolan et al., 1991, Ruben et al., 1991). Rel B readily associates with p50 and p52, forming potent transcriptional activators, but no RelB homodimers or heterodimers with RelA (p65) and c-Rel have been observed *in vitro* or *in vivo* (Bours et al., 1990, Ryseck et al., 1992, Dobrzanski et al., 1993). Combinations of p50 with p65 or c-Rel are transcriptionally active as are p65 homodimers and whereas p52 and p50 homodimers are transcriptionally inactive and can repress κB-dependent transcription (Ballard et al., 1992, Lernbecher et al., 1993, Brown et al., 1994).

NF-κB dimers are sequestered in the cytoplasm by physical association with inhibitor proteins referred to as IκB (Henkel et al., 1993). Treatment of cells with various inducers leads to the dissolution of this cytoplasmic complex and the translocation of free NF-κB to the nucleus (Baeuerle et al., 1988).

Currently the IκB family consists of IκB-α, IκB-β, IκB-γ, IκB-ε and Bcl-3 (Ohno et al., 1990, Bhatia et al., 1991, Haskill et al., 1991, Davis et al., 1991, Franzoso et al., 1992, Hatada et al., 1992, Inoue et al., 1992a, b, Liou et al., 1992, Tewari et al., 1992), the most predominant being the α and β subunits. These inhibitors have 5 - 7 conserved domains known as ankyrin repeats (Schmitz et al., 1991, Blank et al., 1992, Nolan & Baltimore, 1992), which interact with NF-κB subunits thus masking the nuclear localisation signals and preventing activation of NF-κB and its transport to the nucleus (Siebenlist et al., 1994, Baldwin, 1996). They also contain a carboxy-terminal protein destabilising sequence rich in proline, glutamate/aspartate and serine/threonine, also known as the PEST sequence, which facilitates proteolytic degradation of IκB proteins (Baeuerle & Henkel, 1994, Thanos & Maniatis, 1995).

IκBα is capable of binding to most dimers, thereby blocking their nuclear localisation signals (Zabel & Baeuerle, 1990, Beg et al., 1992, Ganchi et al., 1992, Kumar & Gelinas, 1993, Zabel et al., 1993,). Like IκBα, IκBβ also binds to many dimers and undergoes phosphorylation and degradation upon stimulation (Thompson et al., 1995). IκBε is induced by phorbol esters and acts as a potent inhibitor of κB dependent gene expression (Baeuerle & Baltimore, 1996). An alternately spliced form of p105, IκBγ, which consists solely of the long C-terminal ankyrin repeat-containing domain, has been seen in some cells. IκBγ is able to bind various dimers and retain them in the cytoplasm (Inoue et al., 1992c).

With the exception of Bcl-3, the IκBs inhibit NF-κB activity (Baeuerle & Baltimore, 1988a,b, Inoue et al., 1992b, Zabel et al., 1993). Bcl-3 appears to interact specifically with only p50 and p52 homodimers and can form a nuclear complex. Bcl-3 has been reported to both inhibit these homodimers and cause transcriptional

activation (Franzoso et al., 1992, Inoue et al., 1993, Zhang et al., 1994). Bcl-3 may prevent inactive p50 homodimers from binding to DNA thus allowing the binding of a transcriptionally active NF-κB complex to the same site (Franzoso et al., 1993). Different isoforms of IκB appear to target different combinations of Rel proteins, for example IκBα and IκB-β associate predominantly with p50/RelA and p50/c-Rel heterodimers (Davis et al, 1991, Haskill et al., 1991, Kerr et al., 1991), IκB-ε binds to p65 and c-Rel homodimers (Whiteside et al., 1997) and Bcl-3 interacts with p50 and p52 homodimers (Franzoso et al. 1992, Nolan et al., 1993). The precursor proteins p105 and p100, can also be considered IκB proteins because they bind p50, c-Rel and RelA and inhibit their DNA binding and nuclear translocation (Mercurio et al., 1993, Naumann et al., 1993, Rice et al., 1993).

The activation of NF-κB results in the rapid degradation of IκB causing release of NF-κB from its cytosolic inhibitor (Beg et al., 1993, Beg & Baldwin 1993, Brown et al., 1993, Henkel et al., 1993, Scott et al 1993, Sun et al., 1993). Upon stimulation, specific kinases phosphorylate IκB leading to its ubiqitination (DiDonato et al., 1996), and degradation by the proteasome complex (Chen et al., 1995b). All known inducers of NF-κB cause degradation of IκB-α. By contrast, IκB-β is only affected by a subset of inducers (e.g. LPS and IL-1), which cause a persistent activation of NF-κB that varies in magnitude in a cell-type-specific manner (McKinsey et al., 1996, Meyer et al., 1996).

Stimuli that activate NF-κB act by means of proteins kinases that phosphorylate IκB. A specific NF-κB-inducing kinase (NIK) has been characterised which activates an IκB-α kinase previously known as CHUK (Regnier et al., 1997), while a cytokine-responsive IκB kinase (IKK) has been described which specifically phosphorylates IκB-α, resulting in activation of NF-κB in response to pro-inflammatory cytokines (DiDonato et al., 1997). Casein kinase II also phosphorylates IκB-α (Janosch et al., 1996), while a multi-subunit complex in HeLa cell cytoplasmic extracts that can phosphorylate IκB-α at Ser-32 and 36 has been isolated (Chen et al., 1996b). A novel property of this kinase is that it can be activated *in vitro* by ubiquitination.

NF-κB activation is regulated at various levels. The IκBα gene has a κB recognition sequence in its promoter region and its constitutive expression is strongly enhanced by NF-κB activating stimuli (Haskill et al., 1991, Brown et al., 1993, Sun et al., 1993). Free IκB-α can enter the nucleus (Morin & Gilmore, 1992, Zabel et al., 1993), and can dissociate the NF-κB-DNA complex (Zabel & Baeuerle, 1990). This causes passage of NF-κB to the cytoplasm, thereby terminating gene activation (Arenza-Seisdedos et al., 1995). This type of feedback loop causes transient expression of NF-κB responsive genes.

The genes encoding p105, p100, c-Rel and Bcl-3 also contain κB binding sites in their promoter regions, and stimulation of cells leads to increased synthesis of these proteins (Bours et al., 1990, Hannick & Temin, 1990, Ohno et al., 1990, Ten et al., 1992, Cogswell et al., 1993, Brown et al., 1993, Cheng et al., 1994, Chiao et al., 1994, Liptay et al., 1994, Nolan et al., 1993).

While ubiquitination of IκBα leads to its complete degradation, ubiquitination of p105 results in an increase in the rate at which p105 is processed to p50 (Beg et al., 1993). P50 homodimers bind to κB sites, but inhibit rather than trigger transcription (Franzoso et al., 1993, Baeuerle & Henkel, 1994, Baldwin, 1996). The RelA gene, is ubiquitously expressed at very low levels (Ruben et al., 1991, Costello et al., 1993), but synthesis of p65/RelA is not upregulated by Rel/NF-kB dimers (Ueberla et al., 1993). All the genes encoding negative regulators of RelA activity seem to be under positive transcriptional regulation by RelA-containing complexes. This imposes a tight negative control on RelA, the strongest transactivating subunit of the NF-κB system. Therefore, after activation, there may be a shift in the composition of NF-κB. Upregulation of p50 homodimers could inactivate transcription of some genes while the change in dimers complexes could activate different gene profiles, since dimer composition may determine the DNA-binding specificity of a given NF-κB complex (Urban et al., 1991, Urban & Baeuerle, 1991, Kunsch et al., 1992).

1.16 Interactions Between Transcription Factors

Accumulating evidence indicates that a relatively small number of transcription factors have a critical role in controlling the complex gene expression involved in the immune response. These include the NF-κB, NFIL-6, CREB/ATF, AP-1, STAT and NF-AT families of transcription factors (Akira & Kishimoto, 1997).

NF-κB has been shown to synergise with a number of different transcriptional activator proteins, including Ets, ATF-2/c-Jun and NF-IL6. This synergy is a consequence of direct interactions between NF-κB and these proteins and co-operative binding to adjacent binding sites. The interacting transcription factors can be present constitutively or co-induced with NF-κB. Several inducers of NF-κB activate JNK, which in turn phosphorylates ATF-2 and c-Jun both of which synergise with NF-κB (Gupta et al., 1995). NF-kB also to interacts with AP-1 (Stein et al., 1993a), SP1 (Perkins et al., 1992), and C/EBPβ (Stein et al., 1993b). Interaction of NF-κB and AP-1 results in the synergistic activation of both κB and AP-1 response elements, while interaction of NF-κB and the transcription factor C/EBPβ results in the enhancement of C/EBPβ and the repression of κB response element function.

The interplay between transcription factors means that the end result of a stimulus will be dependent on the end products of a variety of signalling pathways. This situation is further complicated by links between these pathways such as already discussed for MEKK-1. Therefore the dissection of cause and effect with regard to a specific stimulus is difficult and complex.

1.17 Aims of Thesis

The aims of this thesis were to investigate the regulation of granulocyte apoptosis by physiological, pro- and anti-inflammatory mediators. In view of the role of apoptosis in inflammatory resolution, we were most interested those which would drive the process of apoptosis in granulocytes, and in particular, any mediators which appeared to have specific effects on either neutrophil or eosinophil apoptosis. Therefore, the main body of this work involved an investigation into the effects of various classes of mediators including cytokines, chemokines, prostaglandins, and leukotrienes on the constitutive rates of both neutrophil and eosinophil apoptosis. Further to such results, we wished to examine the mechanisms involved in enhancing both granulocyte survival and programmed cell death and to investigate the signalling pathways controlling these processes.

CHAPTER 2

METHODS AND MATERIALS

2.1 Methods

2.1.1 Isolation of Human Peripheral Blood Granulocytes

Human neutrophils and eosinophils were purified by previously described methods (Haslett et al., 1985, Savill et al., 1989a, Stern et al., 1992). In the case of eosinophil donors, those with a history of atopy were asymptomatic at the time of the study and none were undergoing glucocorticoid treatment. Cell isolation was performed under sterile conditions using LPS-free reagents and plastic ware (Flacon, Oxford, UK).

Venous blood (36 ml) was collected into 50 ml polypropylene tubes, each containing 4 ml of sterile 3.8% sodium citrate solution to prevent coagulation. The citrate and blood were mixed by gentle invertion of the tubes several times to minimise cell activation, before centrifugation at 370g for 20 min at room temperature. This gives two layers, an upper layer containing plasma and platelets, and lower layer containing a leukocyte/erythrocyte pellet.

The platelet-rich plasma (PRP) was aspirated without disturbing the lower pellet. This erythrocyte/leukocyte layer was sedimented by the addition of 5 ml of 6% dextran (T500) (2.5 ml dextran per 10 ml cell pellet), and the volume made up to 50 ml with sterile 0.9% saline. Both these reagents had been warmed to 37 ^{o}C in a water bath for 30 min, since the efficiency of dextran sedimentation depends upon temperature. The cells were allowed to sediment at room temperature for 30 min, resulting in two clearly-defined layers, an upper leukocyte-rich layer and sedimented erythrocytes.

2.1.2 Neutrophil Isolation

Neutrophil isolation was performed at room temperature and under sterile conditions. The leukocyte-rich layer was aspirated from the sedimented red cells, centrifuged (235g, 6 min) and resuspended in 2.5 ml of 55% isotonic Percoll (9:1 v/v Percoll:10 x PBS) in 1 x PBS without Ca^{2+}/Mg^{2+}. Discontinous Percoll gradients were prepared by overlaying 2.5 ml of 70% iostonic Percoll onto 2.5 ml of 81% isotonic Percoll in a 15 ml Falcon tube, and the resuspened cell fraction overlayed to form the final layer of the gradient.

These gradients were centrifuged at 720g for 20 min at room temperature. Neutrophils were harvested from the 70%/81% interface (monocytes can be harvested from the 70%/55% interface). Cells were sequentially washed twice in PBS without divalent cations and PBS with divalent cations (235g, 6 min) with yield assessed before the final wash. Although, density gradient centrifugation does not separate neutrophils from eosinophils or basophils, neutrophils were generally > 98% pure, with basophils rarely seen and preparations containing > 5% eosinophils discarded or purified for eosinophil studies. Typical neutrophil yield was approx 100 x 10^6/40 mls of whole blood.

2.1.3 Eosinophil Isolation

Eosinophils isolation was performed using a two stage procedure based on a modification of a previously described method involving a combination of dextran sedimentation and centrifugation through six isotonic discontinuous plasma-Percoll gradients to produce cell fractions which partially separate neutrophils and eosinophils (Haslett et al., 1985), followed by a negative selection step (Bach et al., 1990) using immunomagnetic separation to purify eosinophils from contaminating neutrophils. This stage was performed using the murine anti-neutrophil ab 3G8 (anti-CD16)-coated sheep anti-mouse IgG-Dynabeads.

Eosinophils were prepared as described for neutrophils (Section 2.1.2). The leukocyte-rich layer was aspirated from the sedimented red cells, centrifuged (530g, 6 min) and resuspended in 2 ml autologous platelet-poor plasma (PPP) (Section 2.1.7). Percoll-plasma gradients (60 -77% Percoll) were prepared by diluting stock Percoll (9:1 v/v Percoll: 10 X PBS) in 1 x PBS without divalent cations and overlaying 2 ml of each gradient in a 15 ml Falcon tube, with the leukocyte suspension as the final layer. The gradients were centrifuged (570g, 40 min) at room temperature, with the brake and accelerator off. Eosinophilic and neutrophilic granulocytes were harvested from the 74/77% interface and washed sequentially in PPP (370g, 5 min), followed by PBS without divalent cations (260g, 5 min) before suspension in PBS without divalent cations containing 0.1% gelatin. The cell yield was assessed and the percentage of neutrophil contamination in each fraction was quantified either by flow cytometry analysis involving antibody detection of cells expressing surface CD16 or morphologically by examination of cytocentrifuge preparations. CD16-Dynabeads were prepared under sterile conditions by combining 3G8 cell supernatants with dynal M450 sheep anti-mouse dynabeads (10 ml supt:700 µl dynabeads), in a 15 ml falcon tube. The antibody/bead mixture was rotated at 4 ^{o}C overnight to allow maximal coating of beads. Coated beads were washed three times in HBSS without divalent cations containing 0.1% gelatin and the beads retrieved by stationary contact (5 min) using a magnet (Dynal Magnetic Particle Concentrator, MPC-1). Harvested granulocytes were incubated with washed 3G8-Dynabeads at a bead:neutrophil ratio of 3:2 on a rotary mixer at 4 ^{o}C for 20 min, and the beads with attached neutrophils were magnetically separated by stationary contact (3 min) with a magnet. This procedure was repeated once. Purified eosinophils (>99% pure with neutrophils as the major contaminant) were washed twice in 8 ml of PBS without divalent cations containing 0.1% gelatin by centrifugation (235g, 5 min) and final yield of purified cells was assessed. Cell yield was typically 30 x 10^{6} cells per 240 ml of blood, but this varied with donor.

Alternatively, eosinophils were isolated using the neutrophil separation method followed by extraction of the contaminating neutrophils using the murine anti-neutrophil ab 3G8 (anti-CD16)-coated sheep anti-mouse IgG-Dynabeads as already

described. This method allowed higher yields of eosinophil (approximately 10 –20 %) without affecting cell purity or viability.

2.1.4 Cell Culture of Neutrophils

Purified neutrophils were routinely suspended at a density of 5 x 10^6 in Iscoves's DMEM supplemented with 10 % autologous serum and 50 U/ml penicillin and 50 U/ml streptomycin. Cells were cultured in flat-bottomed 96 well Falcon flexiwell plates (Becton-Dickenson, UK), in a final volume of 150 µl, at 37 °C in a humidified 5% CO_2 atmosphere for the time periods indicated. Reagents to be examined in this assay system were diluted to 10 x the final concentration required in Iscove's DMEM, before the addition of 15 µl of each reagent to be investigated.

2.1.5 Cell Culture of Eosinophils

Purified eosinophils were suspended at a density of 2.0 x 10^6/ml in Iscove's DMEM supplemented with 10 % autologous serum and 50 U/ml penicillin and 50 U/ml streptomycin. Eosinophils were cultured in flat-bottomed 96 well Falcon flexiwell plates, in a final volume of 150 µl, at 37 °C in a humidified 5% CO_2 atmosphere for the time periods indicated. Reagents to be examined in this assay system were diluted to 10 x the final concentration required in Iscove's DMEM, before the addition of 15 µl of each reagent to be investigated.

2.1.6 Preparation of Autologous Serum

PRP (15 ml) was added to a sterile glass tube and 330 µl of $CaCl_2$ at a final concentration of 20 µM. This was incubated for approximately 45 min at 37 °C to allow clotting of plasma components, forming autologous serum.

2.1.7 Preparation of Platlet Poor Plasma (PPP)

PRP was placed in a 15 ml Falcon tube and centrifuged at 2400g for 20 min causing platelets to pellet. The PPP was decanted off and the pelleted platelets were discarded.

2.1.8 Assessment of Apoptosis

2.1.8.1 Morphological Criteria

Cells were removed from culture at various times, as indicated in the Results for assessment of morphology according to the method of Savill et al (1989a). Cytocentrifuge preparations of cell samples in each experiment were prepared in a Shandon Cytospin II (Shandon, UK), air dried, fixed in methanol, and stained with Diff-Quik™. Cells were examined under oil immersion light microscopy (x 1250 magnification), and apoptotic cells were defined as those containing one or more darkly stained pyknotic nuclei (See Chapter 5, Figure 5.4 and 5.5). For each condition examined, slides were prepared from duplicate or triplicate incubations and a total of at least 500 cells were counted over five fields of view, with the observer blinded to the assay conditions.

2.1.8.2 Annexin V Binding

Assessment of apoptosis was also performed by flow cytometry using FITC-labelled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells. Stock annexin V was diluted 1:200 with binding buffer and then added (25 µl) to 75 µl of the recovered cell samples. Following a 10 min incubation at 4 ^{o}C, these samples were fixed by the addition of 100 µl of 3% paraformaldehyde before analysis using an EPICS Profile II (Coulter Electronics, Luton, U.K.).

2.1.8.3 Chromatin fragmentation assay

DNA was extracted as described previously by the method of Dignam et al., (1983). Neutrophils (2 x 10^6) were taken after the indicated treatment and lysed in 500 µl of lysis buffer (6 M guanidine hydrochloride, 50 mM Tris-CL, pH 8.0, and 0.1 % N-lauroyl sarcosine) at 4 ° C and the nucleic acids extracted by the addition of an equal volume of 10 mM Tris-Cl pH 8.0 saturated phenol:chloroform mixture (50:50 v/v). The resulting emulsion was centrifuged at 1,200 g for 10 min at room temperature and the aqueous phase removed and precipitated with 0.6 volumes isopropanol at room temperature. The precipitated nucleic acids were then pelleted by centrifugation at 1,000 g for 5 min and re-dissolved in 50 µl TE buffer (10 mM Trizma base, 10 mM boric acid and 1 mM EDTA, pH 8.0) containing 50 µg/ml Rnase A. The fragmented DNA was separated by agarose gel electrophoresis on a 1.4 % (w/v) agarose, 0.5 x TBE (10 mM Trizma base, 10 mM boric acid and 1 mM EDTA, pH 8.3) gel. The gel was run for 2 h at 75 V and stained using ethidium bromide (0.5 µg/ml).

2.1.9 Cell viability and recovery

Cell viability was determined as the ability to exclude trypan blue dye (10 µl of cells:40 µl of trypan blue) using microscopic examination of treated cells. Cell recovery was determined by counting the number of granulocytes occupying 25 squares of the haemocytometer. From this value the percentage of cells retrieved after the experiment, compared with the number of cells added to each well initially was estimated. Thus, at an initial cell concentration of 2 x 10^6/ml, cell recovery was calculated using the following formula: Recovery = No of cell in 25 squares/200.

2.1.10 Assessment of Cell Membrane Integrity

Samples of treated cells (150 ml cells at 2 x 10^6/ml) were centrifuged and resuspended in 150 µl of propidium iodide solution (33 µg/ml propidium iodide in PBS containing 1.67 µg/ml Rnase). Cells were incubated for 5 min before analysis using an EPICS Profile II (Coulter Electronics, Luton, U.K.). Heat-treated (necrotic cells) from the same samples were used as controls (See Chapter 5, Figure 5.8).

2.1.11 Elecromobility Shift Assay

EMSAs were carried out as described by the manufacturer (Promega Corp, Southampton). Nuclear extracts were prepared from 5 x 10^6 cells using a modification of the method of Dignam et al., (1983). Pelleted cells were resuspended in 200 µl hypotonic buffer (buffer A; 10 mM Tris-HCl, pH 7.8, 1.5 mM NaEDTA, 10 mM KCl, 0.5 mM dithiothreitol, 1 µg/ml aprotinin, leupeptin, and pepstatin A, 1 µM 4-(2-aminoethyl)benzenesulfonyl fluroride, 1 mM sodium orthovanadate, 0.5 mM benzamidine and 2 mM levamisole) and placed on ice for 10 min. Following the addition of 0.1 volumes of 10 % NP-40 (v/v) the cells were vortexed briefly and centrifuged at 12,000 g for 2 min at 4 °C. The supernatant was discarded and the pellet washed in 100 µl buffer A minus NP-40 and recentrifuged. The pelleted nuclei were then resuspended in 50 µl hypertonic buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 50 mM KCl, 1.5 mM EDTA, 5 mM dithiothreitol, 1 µg/ml aprotinin, leupeptin and pepstatin A, 1 mM 4-(2-aminoethyl(benzenesulfonyl fluoride, 1 mM sodium orthovanadate, 0.5 mM benzamidine and 2 mM levamisole) and stored at -80 °C until use

Nuclear extracts (approximately 2 µg protein, 7 x 10^5 cell equivalent in 7 µl) were incubated in binding buffer (4 % glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, with 50 µg/ml poly(dI.dC)(dI.dC) with 0.017 pM of γ^{32}P-labelled double stranded oligonucleotide containing the decameric κB binding site at room temperature for 20 min prior to

addition of 5 µl loading buffer (0.01 % bromophenol blue, 20 % Ficoll 400 (w/v) and 1 mM EDTA). Samples were loaded onto an 8 % native acrylamide gel in 0.5 x TBE buffer and run at 250 V for 2 h. The gel was then dried using 3M Whatman paper and exposed to BioMax MS-1 X-ray film. Processed films were analysed using GrabIt and GelPlate software.

2.1.12 Statistical Analysis

All data are presented as mean +/- SEM of the indicated number of experiments. Data were analysed by the student's t-test and where appropriate by one way analysis of variance followed by Newman-Keuls procedure. P values less than 0.05 were considered significant.

2.2 Materials

Reagents were obtained from the following suppliers:-

Affinity, (Exeter, UK); $\Delta^{12}PGJ_2$ (dissolved in DMSO at 10 mM and stored at –20 °C), 15-deoxy$^{12}PGJ_2$ (dissolved in DMSO at 10 mM and stored at –20 °C), SN50 (dissolved in H_2O at 10 mg/ml and stored at –20 °C), and SN50M (dissolved in H_2O at 10 mg/ml and stored at –20 °C).

Alexis Corporation, (Nottingham UK); GEA 3162 (dissolved in H_2O at 30 mM and stored at 4 °C).

Amersham, (Buckinghamshire, UK); [γ-^{32}P]ATP (3,000 Ci/mM) and Igepal-C630.

Bachem, (Saffron Walden, UK); ZVAD-fmk (dissolved in DMSO at 100 mM and stored at –20 °C).

Baxter Healthcare Ltd, (Ballieston, Glasgow, Scotland, UK); Diff-Quik™ stain Solution I (Eosin G in phosphate buffer, pH 6.0), Solution II (Thiazine blue in phosphate buffer, pH 6.0), saline solution, 0.9% (sterile).

Bender MedSystems, (Vienna, Austria); Fluorescein isothianate (FITC)-labelled recombinant human Annexin V.

Calbiochem-Novabiochem Ltd (Beeston, Nottingham, UK); Ro31-8220 was dissolved in DMSO at 1 mM and stored at 4 °C

Cascade Biochem Ltd (Reading, Berkshire, UK); prostaglandin D_2 (PGD_2) (dissolved in ethanol at 2.84 mM and stored at –20 °C), 11-deoxy prostaglandin E_1 (PGE_1) (dissolved in ethanol at 2.95 mM and stored at –20 °C), prostaglandin $F_{2\alpha}$

(PG $F_{2\alpha}$) (dissolved in ethanol at 2.83 mM and stored at –20 °C) and leukotriene B_4 (dissolved in ethanol at 3 mM and stored at –20 °C).

Dako Corporation, (Buckinghamshire, UK); FITC-conjugated F(ab`)2 fragments of rabbit anti-mouse immunoglobulin (Ig).

David Ball Laboratories, (Warwick, UK); dexamethasone (stored at 4 °C at a stock concentration of 8.3 mM.

Dynal (UK) Ltd, (Wirral, UK); dynabeads M-450 sheep anti-mouse IgG (supplied as 4 x 10^8 beads/ml in PBS pH 7.4 with 0.1% human serum albumin and 0.2 % sodium azide.

Genzyme Diagnostics, (Kent, UK); recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (dissolved in PBS without divalent cations at 1000 U/ml and stored at –70 °C).

Gibco Life Technologies, (Paisley, Scotland, UK); Hanks balanced salt solutions (HBSS), pH 7.4, HBSS with 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+}, pH 7.4 (HBSS with divalent cations), Iscove's Dulbecco's modified Eagles medium without supplement with L-glutamine (Iscove's DMEM) and culture supplements, penicillin (50 U/ml), and streptomycin (50 U/ml), and phenol.

Marindale Pharmaceuticals, (Romford, UK); calcium chloride.

Peprotech, Inc (Rocky Hill, N. J., USA); eotaxin (stored at 0.1 mg/ml at –80 °C).

Pharmacia Fine Chemicals, (Uppsala, Sweden); percoll, and poly(dI.dc)(dI. dc).

Phoenix Pharmaceuticals, (Gloucestershire, UK); sterile 3.8% sodium citrate

R & D Systems Europe Ltd, (Oxon, UK); TNF-α (dissolved in PBS at 10 μg/ml and stored at – 80 °C), IL-10 (dissolved in PBS at 1 μg/ml and stored at – 80 °C) and RANTES (dissolved in PBS at 10 μg/ml and stored at –80 °C).

Roche Products Ltd, (Welwyn Garden City, Herts, UK); Ro-31-8220 (dissolved in DMSO at 30 mM and stored at 4 °C).

Schering Health Care, (West Sussex, UK); ZK 118.182 (dissolved in DMSO at 10 mM and stored at –20 °C).

Sigma Chemical Company, (Poole, Dorset, UK); Dulbecco's phosphate buffered saline (PBS) sterile, endotoxin-free, pH 7.4, PBS with 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+}, trypan blue, propidium iodide, cycloheximide (dissolved in DMSO at 10 mM and stored at 4 °C), dextran-500 (dissolved in sterile 0.9% saline (6% w/v) and stored at 4 °C), dimethyl sulphoxide (DMSO), N6, 2`-O-dibutyrladensine 3`:5`-cyclic monophosphate (dibutyryl-cAMP) (dissolved in Iscove's DMEM at 20 mM and stored at –20 °C), lipopolysaccharide (LPS; E Coli 0127:B8) (dissolved in PBS at 1 mg/ml, sonicated (ultrawave sonic bath, Belmont Instruments, Glasgow, UK) and stored at –20 °C), thapsigargin (dissolved in DMSO at 10 mM and stored at – 20 °C), gelatin, paraformaldehyde, guanidine hydrochloride, N-lauroyl sarcosine, chloroform, TRIZMA base, boric acid, ethylenediaminetetraacetic acid (EDTA), RNase A, ethidium bromide, dithiothreitol (dissolved in H_2O at 50 mM and stored at 4 °C), aprotinin (dissolved in H_2O at 100 μg/ml and stored at –20 °C), leupeptin pepstatin A (dissolved in methanol at 100 μg/ml and stored at –20 °C), 4-(2-amonoethyl)-benzenesufonyl fluoride (dissolved in H_2O at 50 mM and stored at –20 °C), sodium orthovanadate (dissolved in H_2O at 100 mM and stored at –20 °C), benzamidine (dissolved in H_2O at 50 mM and stored at –20 °C), levamisole (dissolved in H_2O at 200 mM and stored at – 20 °C), bromophenol blue, prostaglandin E_2 (dissolved in ethanol at 3 mM and stored at – 20 °C), prostaglandin A_2 (dissolved in ethanol at 2.99 mM and stored at – 20 °C), prostaglandin J_2 (dissolved in ethanol at 2.99 mM and stored at –20 °C), gliotoxin (dissolved in DMSO at 2 mg/ml), methylthiogliotoxin (dissolved in DMSO at 2 mg/ml),

pyrrolidine dithiocarbamate (PDCT) (dissolved in PBS at 300 mM and stored at – 20 °C), and Tosyl-Phe-chloromethylketone (TPCK) (dissolved in ethanol at 50 mM and stored at - 20°C).

The hybridoma clone 3G8 (CD16) was a generous gift from Dr. J. Unkeless, Mount Sinai Medical School, NY.; 5-oxo-ETE was synthesised from arachidonate by Dr. J. O'Flaherty and Dr. A. G. Rossi as previously described (O'Flaherty & Rossi, 1993, O'Flaherty et al., 1993).

All other chemicals were of molecular, reagent or cell culture grade and were obtained from **BDH, (Leicestershire, UK).**

CHAPTER 3

THE MODULATION OF GRANULOCYTE APOPTOSIS BY INFLAMMATORY MEDIATORS

3.1 Introduction

Apoptosis is thought to play a major role in the processes of inflammatory resolution by allowing the removal of effete cells without the release of intracellular contents, which, by itself constitutes an inflammatory stimulus (Section 1.4, 1.5 and 1.6). However, the physiological mediators that drive this process in granulocytes remain ill defined. Most inflammatory mediators investigated increase the longevity of inflammatory cells (Lee et al., 1993). Cytokines released by inflammatory cells and likely to be present at an inflammatory site, such as TNF-α, GM-CSF and IL-5, are known to prolong granulocyte life span (Colotta, et al., 1992, Alam et al, 1994a, Murray et al., 1997) but others are likely to be important. Few studies have considered the role of other factors, such as eicosanoids and chemokines in controlling the longevity of neutrophils and eosinophils. We therefore examined the effects of other inflammatory mediators on the rate of constitutive apoptosis of these cells *in vitro*.

The inhibition of apoptosis by cytokines and other mediators may be due to the synthesis of survival proteins. Cycloheximide, an inhibitor of protein synthesis, can either block the induction of apoptosis, promote it, or have no effect, depending on the cell type studied (Cohen et al., 1992). For example, in thymocytes treated with glucocorticoids or irradiation, cell death is delayed by protein synthesis inhibitors, suggesting that the cell-death pathway is protein synthesis dependent (Cohen & Duke, 1984, Wyllie et al., 1984, Kizaki et al., 1989). In granulocytes, protein synthesis inhibitors induce apoptosis, and therefore cell death may be held in check by survival proteins (Whyte et al., 1997).

Glucocorticoids are the most potent and effective agents for controlling chronic inflammatory diseases (Goulding & Guyre, 1993); little is known of the mechanisms involved, although they are known to modulate gene transcription (Beato, 1989). Dexamethasone, a synthetic glucocorticoid, inhibits apoptosis in neutrophils, while inducing cell death in eosinophils (Cox, 1995, Meagher et al., 1996, Cox & Austin, 1997). While this would explain the therapeutic efficiency of steroids in eosinophilic inflammation, it does not account for their efficacy in other inflammatory diseases. Other inflammatory mediators such as lipopolysaccharide (LPS) inhibit apoptosis in both neutrophils and eosinophils (Colotta et al., 1992, Takanaski et al., 1994). Neutrophil responses to LPS include actin assembly, chemotaxis, adherence and the ability to prime for enhanced secretory responses to other stimuli such as platelet activating factor (PAF) (Kitchen et al., 1996, Nick et al., 1996).

During extravasion from the peripheral blood to the tissues, granulocytes will come into contact with other factors that may activate, or affect the lifespan of these cells. RANTES and eotaxin are chemokines that act as direct eosinophil chemoattractants (Kameyoshi et al., 1992, Rot et al., 1992, Meurer et al., 1993, Ebisawa et al., 1994, Jose et al., 1994a, b, Schröder et al., 1994, Garcia-Zepeda et al., 1996). RANTES induces mild activation of eosinophils *in vitro*; it increases cationic protein release, O_2^- production, and induction of a hypodense phenotype (Rot et al., 1992, Alam et al., 1993, Schweizer et al., 1994); it is also synthesised and released by human eosinophils (Lim et al., 1995).

Eotaxin was first isolated as an eosinophil chemoattractant in a guinea pig model of asthma and cloned in 1996 (Griffiths-Johnson et al., 1993, Jose et al., 1994a, b, Rothenberg et al., 1994, Ponath et al., 1996). It is constitutively expressed at high levels in normal human small bowel and colon while detectable levels are also found in the lungs; however it is upregulated in certain human inflammatory diseases (Garcia-Zepeda et al., 1996).

Eotaxin and human RANTES elevate $[Ca^{2+}]_i$ in human eosinophils. Both chemokines bind to the CCR3 chemokine receptor and therefore may have similar effects on

human eosinophils *in vitro* (Jose et al., 1994a, Rothenberg et al., 1995, Ponath et al., 1996, Premack & Schall, 1996).

Granulocytes themselves synthesise and release chemotactic inflammatory mediators, many of which are metabolites of arachidonic acid (AA). There are various pathways by which AA can be metabolised to products with biological activity. For example, leukotriene B_4 (LTB_4), a product of the 5-lipoxygenase pathway, is among the most potent neutrophil chemotactic factors and is produced by neutrophils, eosinophils, macrophages, mast cells, and endothelial and other cell types (Chatila et al., 1989, Borgeat & Naccache, 1990, Nolan et al., 1990). LTB_4 rapidly activates neutrophils and is a potent stimulus for chemotaxis, homotypic and heterotypic adhesion, diapedesis, O_2^- generation, and degranulation (Ford-Hutchison et al., 1980, Samuelsson et al., 1987, Rossi et al., 1988, Tonnesen et al., 1989, Lewis et al., 1990, O'Flaherty et al., 1991, Henderson, 1994). A rapid, transient rise in $[Ca^{2+}]_i$ is seen after activation with LTB_4 (Naccache et al., 1985, Rossi et al., 1988, O'Flaherty et al., 1991). LTB_4 primes neutrophils and like other priming agents has been found to inhibit neutrophil apoptosis (Gay et al., 1984, Stewart et al., 1991, Hébert et al., 1996). High affinity binding sites for LTB_4 have been identified in human neutrophils (Kriesle & Parker, 1983, Goldman & Goetzl, 1984, O'Flaherty al., 1994). While LTB_4 has potent activity on human neutrophils, it is only weakly active on human eosinophils (Morita et al., 1989, Richards et al., 1991, Powell et al., 1995). In these cells, LTB_4 stimulates chemotaxis and the oxidative burst, but not degranulation (Palmblad et al., 1984).

Another product of the 5-lipoxygenase pathway, 5-oxo-6, 8,11,14-eicosatetraenoic acid (5-oxo-ETE), stimulates $[Ca^{2+}]_i$ mobilisation, chemotaxis and aggregation in neutrophils and has similar effects on eosinophils (O'Flaherty et al., 1993, 1994, 1996, O'Flaherty & Rossi, 1993, Powell et al., 1993, 1995, Schwenk & Schröder, 1995). It is synthesised by both granulocytes (Powell et al., 1992, 1995). 5-oxo-ETE stimulates the expression of adhesion molecules on neutrophils and may therefore influence the infiltration of neutrophils into inflammatory sites (Powell et al., 1997). 5-oxo-ETE does not cause neutrophils to produce O_2^-, but it primes PAF-induced O_2^-

release and GM-CSF or TNF-α degranulation responses (O'Flaherty & Rossi, 1993, O'Flaherty et al., 1994). Little is known about the specificity of the cellular recognition mechanism for 5-oxo-ETE and its precursor 5 (S)-HETE. Although they are less active than LTB_4 on neutrophils, studies using cross-desensitisation and an LTB_4 antagonist showed that 5 (S)-HETE and 5-oxo-ETE do not act through the LTB_4 receptor (O'Flaherty & Rossi, 1993, Powell et al., 1993, O'Flaherty et al., 1994).

The synthesis of other metabolites such as prostaglandins is detailed in Figure 1.1. There are five primary active PG metabolites, prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2), prostaglandin D_2 (PGD_2) and thromboxane A_2 (TXA_2) (Pierce et al., 1995a). Both neutrophils and eosinophils synthesise, to varying degrees, some of these prostaglandins (Hubscher, 1975, Goldstein et al., 1977).

PGEs inhibit the production of O_2^-, chemotaxis, aggregation, and lysosomal enzyme release in neutrophils (Zurier et al., 1974, Marone et al., 1980, Babior et al., 1981, Fantone & Kinnes, 1983, Ham et al., 1983, Sedgwick et al., 1985, Ney & Schrör, 1991,). PGE_2 inhibits both opsonised zymosan-induced LTB_4 release and FMLP-induced O_2^- release in neutrophils and degranulation responses to LTB_4 and PAF (Rossi & O'Flaherty, 1989, Wheeldon & Vardey, 1993). In eosinophils, PGE_2 weakly inhibits FMLP-induced eosinophil cationic protein (ECP) release and also stimulates migration, but it does not raise $[Ca^{2+}]_i$ (Butchers & Vardey, 1990, Raible et al., 1992).

PGE_2 is thought to suppress neutrophil function and inhibit neutrophil apoptosis through elevation of cAMP levels (Ottonello et al., 1995, Rossi et al., 1995, Walker et al., 1997). It also delays apoptosis of U937 cells by the same mechanism, but it induces programmed cell death in thymocytes (McConkey et al., 1990, Heidenreich et al., 1996).

PGE_2 can be further metabolised to prostaglandin A_2 (PGA_2) which is produced by the enzymatic dehydration of PGE_2, or from non-enzymatic dehydration in aqueous solutions (Ohno et al., 1986, Fukushima, 1990, 1992, Noyori & Suzuki, 1993). Cyclopentenone prostaglandins such as PGA_2 have antiproliferative effects on various cultured tumour cells (Narumiya & Toda, 1985, Ohno et al., 1986). Although PGE_2 increased cAMP during studies of its effects on growth inhibition, PGA_2 did not, suggesting that cAMP was not involved in PGA_2 activity (Willey et al., 1983, Narumiya & Toda, 1985, Ohno et al., 1986). PGA_2 is actively transported into cells by a specific carrier and accumulates in the nucleus by a temperature-sensitive process (Narumiya et al., 1986, 1987, Narumiya & Fukushima, 1986). The uptake and accumulation correlates closely with growth inhibition. Therefore, these effects may be due to influences on gene activation or transcription via nuclear receptors. PGA_2 has been demonstrated to cause apoptosis in murine leukaemia L1210 cells, but there are no reports on its effects on granulocyte lifespan (Kim et al., 1993).

PGD_2 is formed in virtually all mammalian tissues and has diverse biological effects (Ito et al., 1989). It is the major product of arachidonic acid metabolism in human mast cells in which it is selectively produced and released after immunological challenge (Lewis et al., 1982, Hardy et al., 1984, Peters et al., 1984). Both neutrophils and eosinophils produce PGD_2 (McGuire & Sun, 1980, Kroegel & Matthys, 1993). This PG is considered to be an important mediator in allergic disorders such as allergic rhinitis and is found in the airways of patients with allergic asthma after antigen challenge (Murray et al., 1986, Johnson et al., 1993). However, the biological effects elicited by PGD_2 show considerable species variability (Giles & Leff, 1988).

PGD_2 inhibits O_2^- release in neutrophils stimulated by FMLP, but has no stimulatory effect on its own (Johnson et al., 1985, Simpkins et al., 1990). PGD_2 has no effect on the initial increase in $[Ca^{2+}]_i$ in FMLP-stimulated neutrophils, but causes a more rapid decrease in elevated $[Ca^{2+}]_i$. However, dbcAMP, a non-hydrolysable, cell-permeable cAMP analogue, produced similar results to PGD_2 leading to the suggestion that PGD_2 increases cAMP levels in neutrophils (Simpkins et al., 1990).

PGD_2 inhibits opsonised zymosan-induced LTB_4 release, FMLP-induced O_2^- release, lysosomal enzyme release, and LTB_4 and PAF-induced degranulation in neutrophils (Zurier et al., 1974, Ham et al., 1983, Rossi & O'Flaherty, 1989, Ney & Schrör, 1991, Wheeldon & Vardey, 1993). This prostanoid also has biological activity on eosinophils, in which it stimulates Ca^{2+} mobilisation, and primes eosinophils for enhanced release of LTC_4 in response to the calcium ionophore A23187 (Raible et al., 1992). In addition, it reduces the number of circulating blood eosinophils and is chemotactic for eosinophils in a dog trachea animal model (Marsden et al., 1984, Emery et al., 1989).

The metabolic fate of PGD_2 has been extensively investigated *in vivo* in primates including humans (Ellis et al., 1979, Liston & Roberts, 1985a, b, Roberts & Sweetman, 1985). Products of PGD_2 metabolism include other cytopentenone PGs with biological activity such as 9-deoxy-Δ^9-PGD_2 (PGJ_2), Δ^{12}-PGJ_2 and 15-deoxy-Δ^{12},Δ^{14}-PGJ_2 ($15dPGJ_2$) (Fitzpatrick & Wynalda, 1983, Kikawa et al., 1984, Ito et al., 1988). Early work suggested that PGD_2 could inhibit the growth of rodent and human tumour cells (Fitzpatrick & Stringfellow, 1979, Fukushima et al., 1982a, b, Simmet & Jaffe, 1983, Sakai et al., 1984). However, further studies demonstrated that PGD_2 *per se* was inactive in these studies, and that the cyclopentenone metabolites of PGD_2 were the active forms (Fukushima et al., 1982a, Narumiya & Fukushima 1985).

A large problem in defining the effects of PGD_2 is the biological activity of some of these possible metabolites. A metabolically stable prostaglandin D_2 mimetic, (5Z,13E)-(9R,11R,15S)-9-chloro-15-hydroxy-16,17,18,19,20-pentano-3-oxa-5,13-prostadienoic acid, (ZK 118.182) has been synthesised (Schulz et al., 1990). This compound caused a concentration-dependent activation of adenylate cyclase, comparable to PGD_2, and it also concentration-dependently reduced FMLP- or PAF-induced O_2^- release and degranulation responses in human neutrophils (Darius et al., 1994). ZK118.182 might therefore help to distinguish between cellular responses to PGD_2 and its metabolites.

PGJ_2 is one such PGD_2 metabolite formed by albumin transformation of PGD_2. It has known biological activity and has been demonstrated to inhibit platelet aggregation and produce vasodilation (Coleman et al., 1990). PGJ_2 has anti-tumour effects *in vivo*, and stimulates TNF–α release from LPS-activated monocytes (Vedin et al., 1996).

Another metabolite, Δ^{12}-PGJ_2, has been reported to have potent growth inhibitory activities in some tumour cells, but unlike PGD_2, it does not elicit an increase in cAMP levels when used in growth inhibition studies (Mahmud et al., 1984, Narumiya & Fukushima, 1985, Ohno et al., 1986). Δ^{12}-PGJ_2 is also actively transported into cells by a specific carrier and accumulates in the nucleus (Narumiya & Fukushima, 1986, Narumiya et al., 1986). Binding of this molecule to nuclear proteins appears to regulate the expression of specific genes that are responsible for growth inhibition since, as is the case for PGA_2, nuclear accumulation correlates with growth inhibition (Narumiya et al, 1987). Δ^{12}-PGJ_2 induces apoptosis in human hepatocarcinoma SK-HEP-1 cells and in murine leukaemia L1210 cells (Kim et al., 1993, Lee et al., 1995a). The molecular mechanisms by which Δ^{12}-PGJ_2 causes cell death have not been determined.

15-$dPGJ_2$, another PGD_2 metabolite, like PGJ_2, stimulates TNF-α release from LPS-activated monocytes (Vedin et al., 1996). This metabolite is formed via prostaglandin D_2 synthase, which is highly expressed in macrophages and specialised antigen-presenting cells (Urade et al., 1989). 15-$dPGJ_2$ also binds to a nuclear receptor and appears to stimulate or repress transcription of target genes especially, those involved in lipid metabolism (Forman et al., 1995, Kliewer et al., 1995, 1997, Krey et al., 1997).

$PGF_{2\alpha}$ is identical to PGD_2 except that $PGF_{2\alpha}$ has a hydroxyl group instead of a carbonyl group at the C11 position (Simpkins et al., 1990). $PGF_{2\alpha}$ is reported to have inhibitory effects on neutrophils. It impedes O_2^- production, opsonised zymosan-induced LTB_4 release and FMLP-induced O_2^- release (Gryglewski et al., 1987, Wheeldon & Vardey, 1993). $PGF_{2\alpha}$ reportedly elevates $[Ca^{2+}]_i$ levels in neutrophils

and is known to stimulate eosinophil migration, but there are no reports of its effects on granulocyte survival (Ito et al., 1990).

Almost all the inflammatory mediators discussed above have been demonstrated to affect either intracellular cAMP or $[Ca^{2+}]_i$ levels. Cyclic AMP can regulate certain eosinophil functions, such as inhibition of degranulation, thromboxane and LTC_4 generation, O_2^- production and apoptosis (Dent et al., 1991, Kita et al., 1991, Munoz et al., 1994, Souness et al., 1994, Hallsworth et al., 1996, Cousin et al., 1997). It inhibits apoptosis in cultured rat sympathetic neurones (Edwards et al., 1991, Nobes & Tolkovsky, 1995) and rat phaetochromocytoma PC12 cells (Michel et al., 1995), and human neutrophils (Rossi et al., 1995), but induces apoptosis in rat primary granulosa cells (Keren-Tal et al., 1995) and in resting human B lymphocytes (Lomo et al., 1995).

Studies using lymphoid cells implicate increases in $[Ca^{2+}]_i$ in the triggering of apoptosis. For example, elevation of $[Ca^{2+}]_i$ using Ca^{2+} ionophores induces apoptosis in thymocytes (Cohen & Duke, 1984, Durant et al., 1980, McConkey et al., 1989, Wyllie et al., 1984). However, neutrophil apoptosis is delayed by inflammatory mediators associated with $[Ca^{2+}]_i$ mobilisation (Lew et al., 1987, Smith et al., 1987, Korchak et al., 1988). The Ca^{2+} ionophore A23187 inhibits neutrophil apoptosis while chelation of $[Ca^{2+}]_i$ promotes it (Whyte et al., 1993b). For eosinophils, the converse is true; agents that elevate $[Ca^{2+}]_i$ promote eosinophil apoptosis (Cousin et al., 1997).

Although many researchers have investigated the role of pro-inflammatory cytokines in granulocyte survival, few have considered the effects of chemokines and AA metabolites on the fate of these cells. Many chemotaxins act to down-regulate granulocyte function when present in high concentrations; prostaglandins and other eicosanoids have various effects as detailed above. This study therefore investigated the role of these mediators on neutrophil and eosinophil apoptosis with the specific aim of dissecting possible underlying regulatory mechanisms involved in the apoptotic programme of these closely related granulocytes.

3.2 Results

3.2.1 The Effect of Cycloheximide, Dexamethasone and LPS on Granulocyte Apoptosis

The onset of apoptosis in granulocytes can be recognised by morphological changes such as chromatin condensation and cytoplasmic shrinkage, using oil immersion microscopy. Examples of apoptotic and normal morphology of neutrophils and eosinophils are shown in Figures 5.4 and 5.5 (See Chapter 5). Time courses for constitutive apoptosis in both cell types are shown in Figure 3.1. Eosinophils are known to survive for longer periods than neutrophils in the tissues; Figure 3.1 illustrates that this is also true of cultured cells. For example neutrophils cultured for 20 h exhibit 69.7 ± 6.8 % apoptosis whereas eosinophils exhibited only 13.4 ± 1.8 % at the same time point.

Cycloheximide accelerates the apoptotic process in neutrophils (Figure 3.2 A), where apoptosis was increased from 63.6 ± 7.5 % to 80.8 ± 5.3 % in cycloheximide-treated cells at 20 h. Eosinophils were also susceptible to the effects of protein synthesis inhibition with apoptosis increased from 11.8 ± 3.0 % to 88.5 ± 7.7 % at 20 h (Figure 3.2 B). These data suggest that the survival of granulocytes may depend on synthesis of one or more proteins that suppress the process of programmed cell death, in striking contrast to the case in T cells, where protein synthesis inhibitors protect against apoptotic stimuli.

As shown in Figure 3.3 A and B, dexamethasone, a synthetic glucocorticoid, significantly inhibits neutrophil apoptosis at 20 h, (65.4 ± 3.0 % control; 42.5 ± 3.3 % with dexamethasone treatment) while significantly increasing programmed cell death in eosinophils at both 20 and 40 h. For example, in eosinophils, control apoptosis at 40 h was 28.2 ± 6.9 % compared with 63.7 ± 11.8 % in dexamethasone treated cells. These data suggest that apoptosis in neutrophils and eosinophils is differentially regulated, notwithstanding their ontogenic and functional similarities

and that inflammatory mediators may have dissimilar effects on the rate of apoptosis in specific inflammatory cells.

LPS dramatically inhibits neutrophil apoptosis by 20 h with constitutive apoptosis reduced from 53.6 ± 5.5 % to 20.6 ± 1.9 % with LPS treatment, an inhibition of greater than 50% (Figure 3.4 A). In eosinophils (Figure 3.4 B) there was no significant inhibition at the early 20 h timepoint, but by 40 h a significant decrease in programmed cell death had occurred indicating that LPS, a potent stimulus for initiating the innate inflammatory response, is a survival factor in both types of granulocytes.

3.2.2 The Effect of the Chemokines RANTES and Eotaxin on Granulocyte Apoptosis

Figures 3.5 and 3.6 illustrate the effects of RANTES and eotaxin respectively on granulocyte apoptosis. Because both of these chemokines are considered to be chemotactic for eosinophils but not for neutrophils, it was expected that only eosinophils would show responses to these factors. However, as indicated in Figures 3.5 B and 3.6 B neither RANTES nor eotaxin significantly affected the constitutive rate of eosinophil apoptosis. However, while the constitutive rate of neutrophil apoptosis was unaltered by eotaxin (Figure 3.6 A), the higher concentrations of RANTES (10 and 100 nM), (Figure 3.5 A) showed a small but significant inhibition of programmed cell death in these cells.

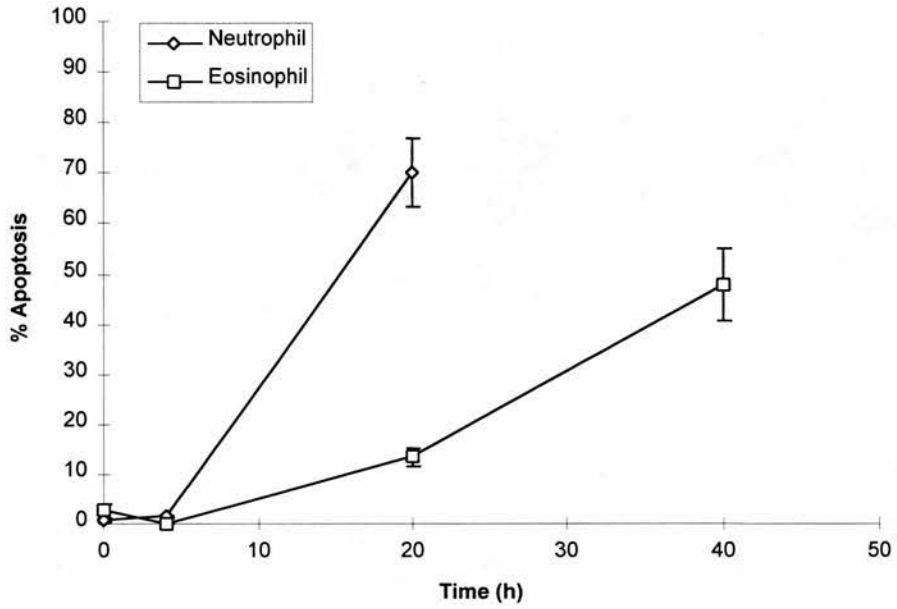


Figure 3.1 Constitutive Apoptosis in Granulocytes

Neutrophils (5 x 10^6/ml) and eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with 10% autologous serum and harvested at various timepoints as shown above. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 7 separate experiments, each performed in triplicate.

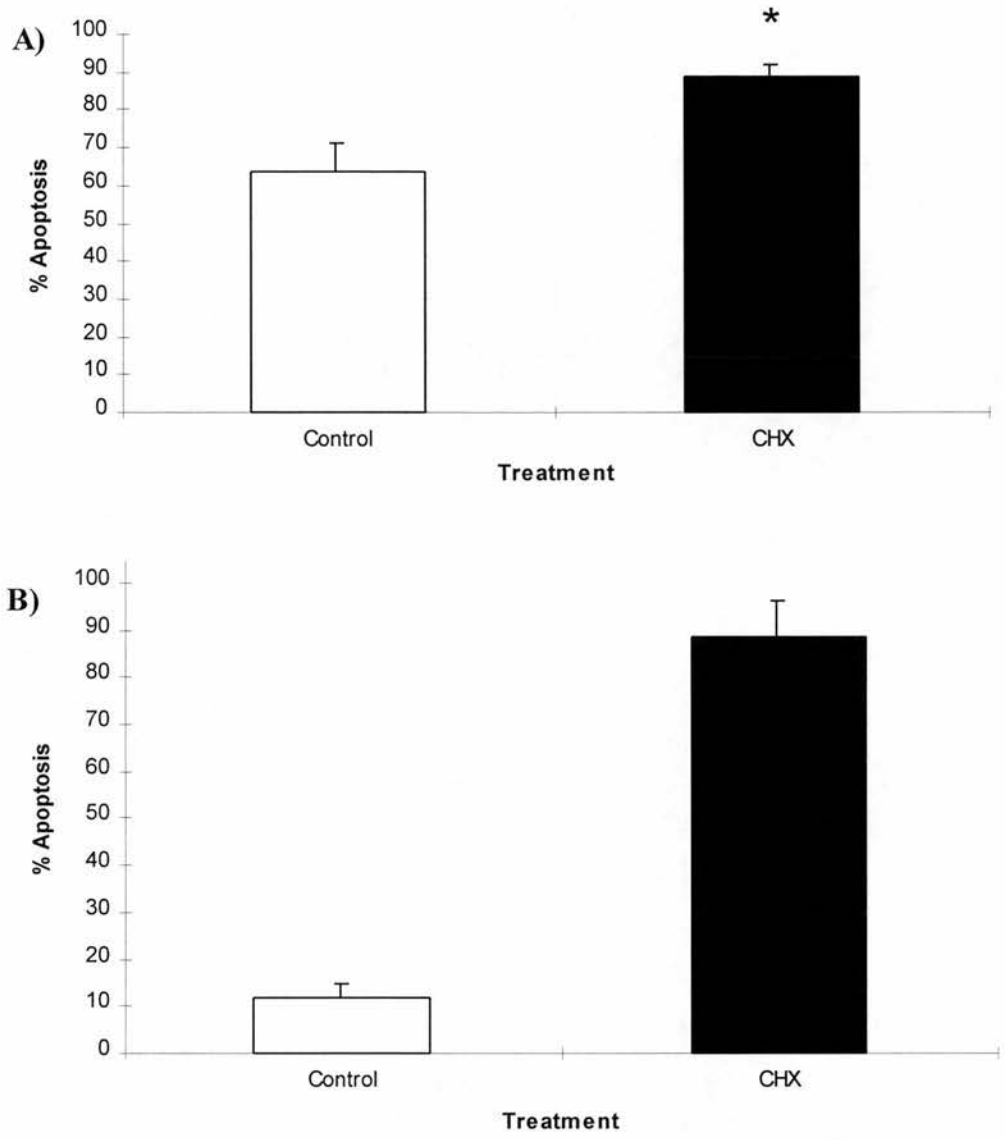


Figure 3.2 The Effect of Cycloheximide on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or in the presence of cycloheximide (50 μM) and harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 7 separate experiments for neutrophils, and 4 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

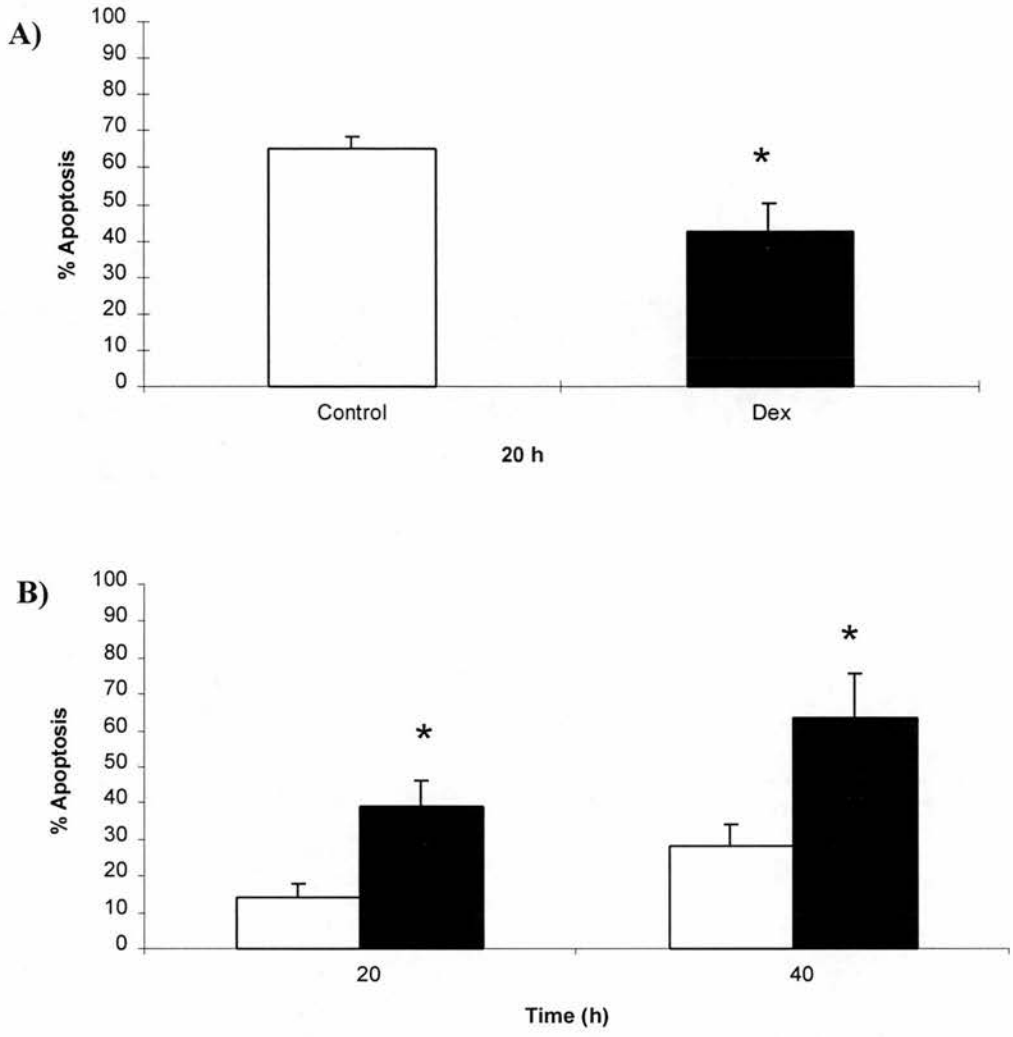


Figure 3.3 The Effect of Dexamethasone on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with dexamethasone (1 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 7 separate experiments for neutrophils, and 4 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

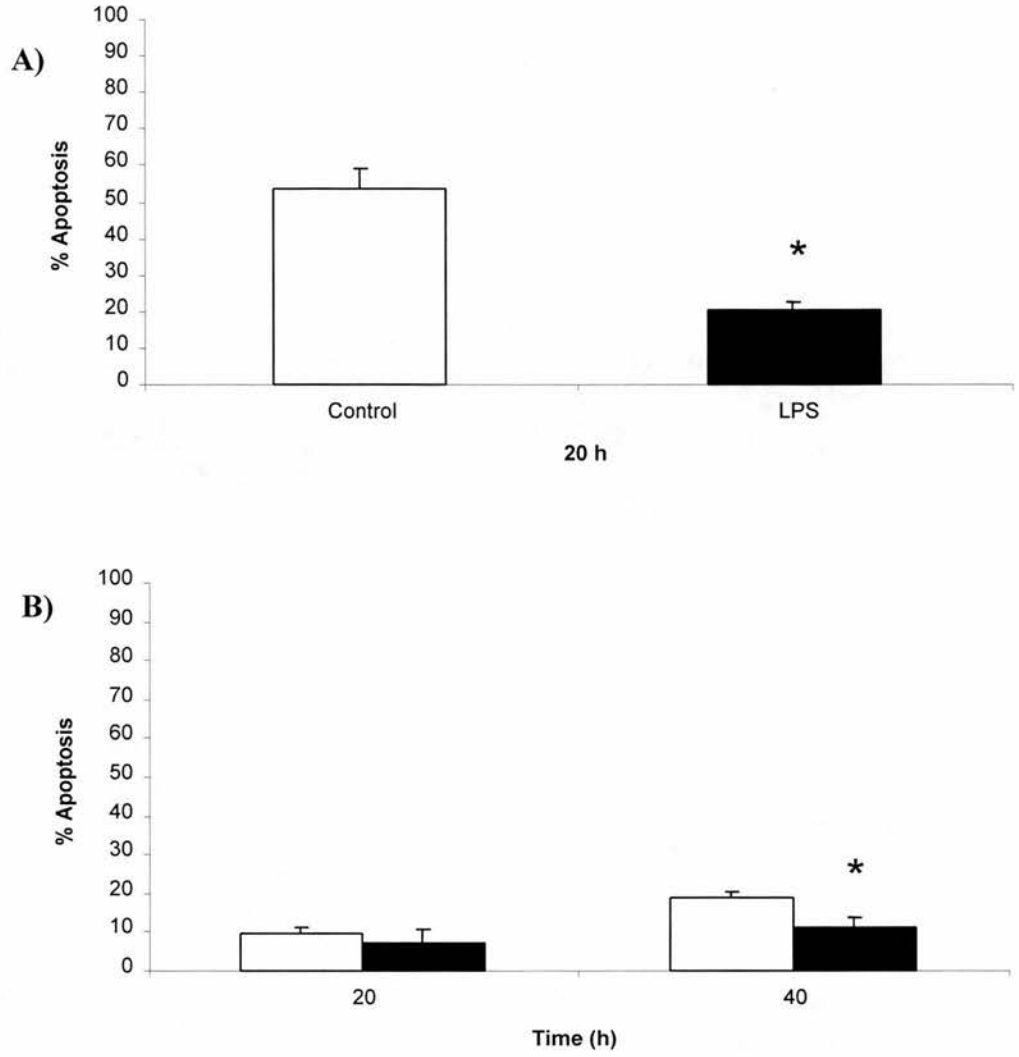


Figure 3.4 The Effect of Lipopolysaccharide on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with LPS (1 μg/ml) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 4 separate experiments for neutrophils, and 4 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

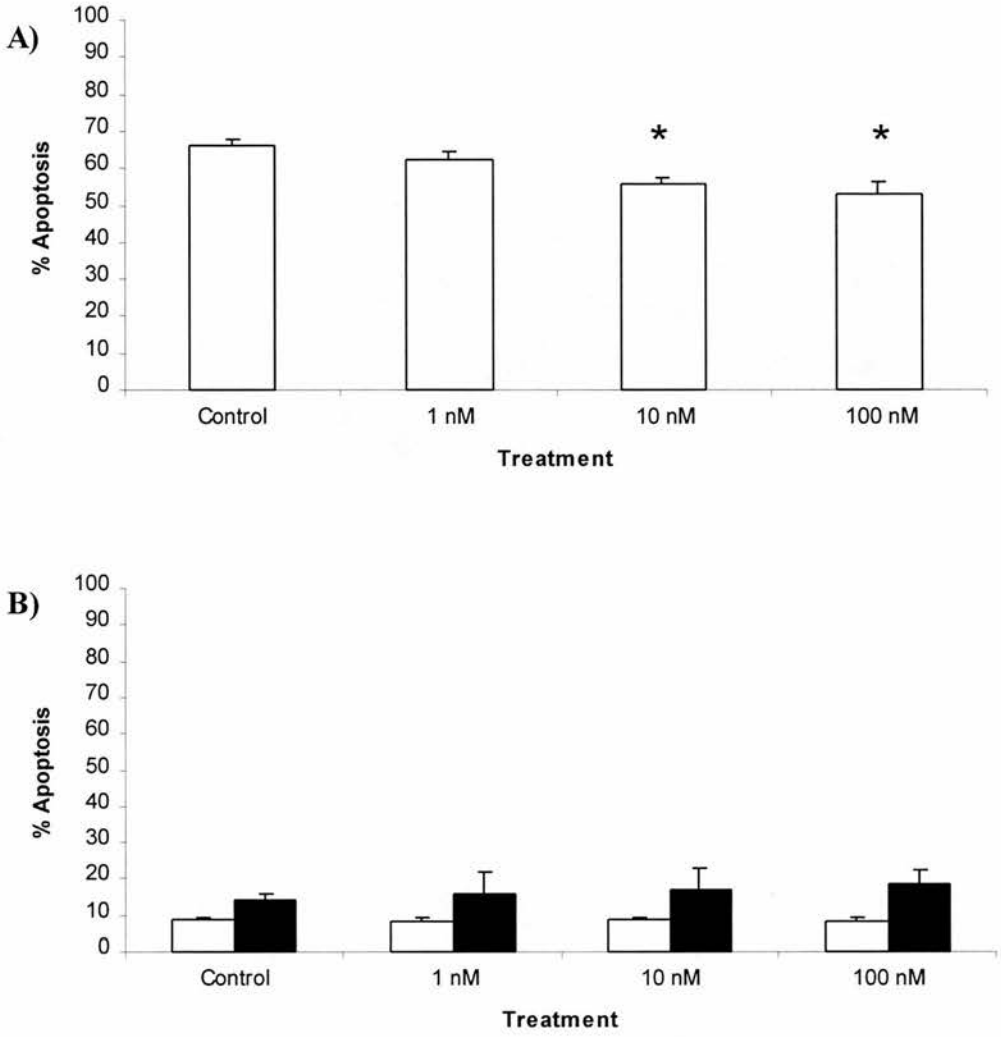


Figure 3.5 The Effect of RANTES on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with RANTES (1 – 100 nM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Open bars indicate 20 h timepoint, filled bars are results for 40 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 4 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

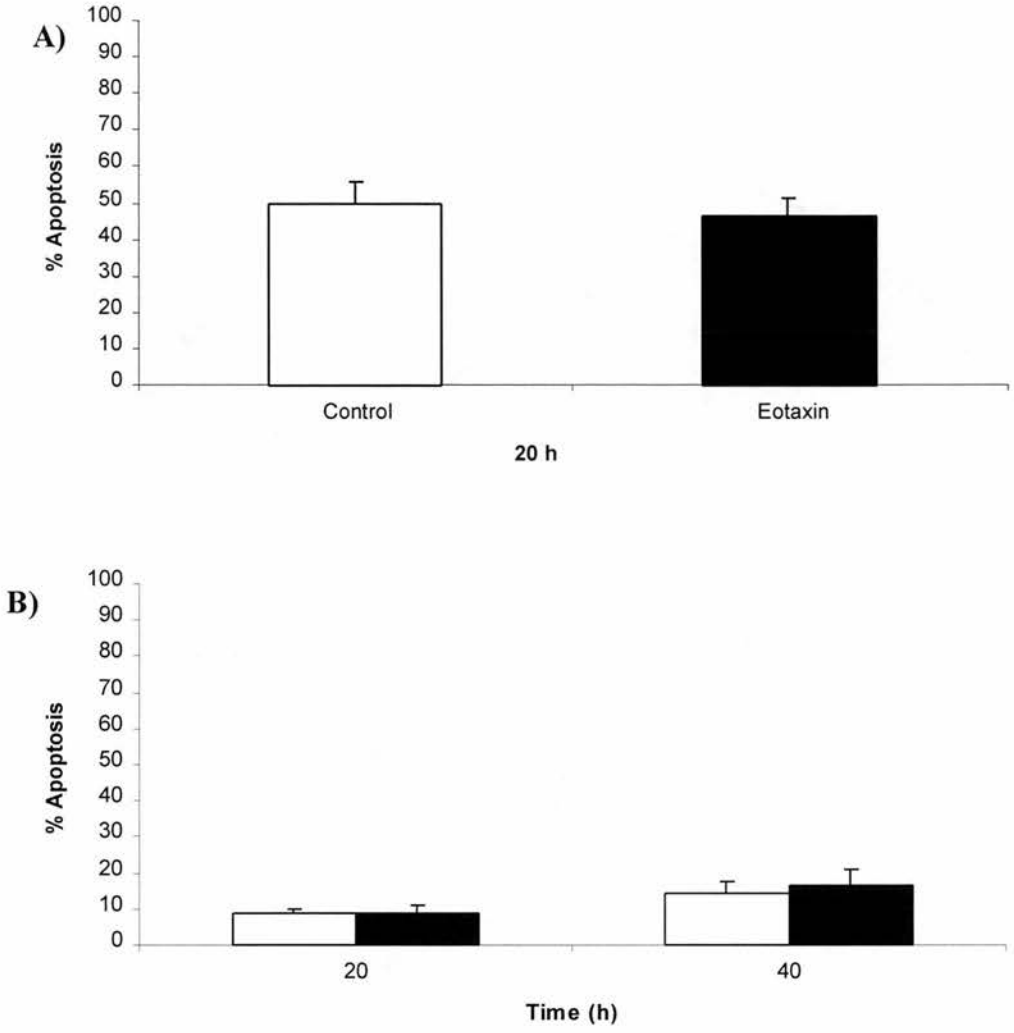


Figure 3.6 The Effect of Eotaxin on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with eotaxin (100 ng/ml) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 5 separate experiments for eosinophils. All experiments were performed in triplicate.

3.2.3 The Effect of the 5-lipoxygenase products, LTB_4 and 5-oxo-ETE on Granulocyte Apoptosis

As shown in Figure 3.7 A, LTB_4 significantly decreased the rate of neutrophil apoptosis from 61.2 ± 6.3 % (control) to 43.1 ± 4.4 %. However this mediator had no significant effect on eosinophil apoptosis at either the 20 or 40 h timepoints (Figure 3.7 B). Similarly, 5-oxo-ETE produced a small but significant reduction in neutrophil apoptosis at 20 h (Figure 3.8 A) but had no effect on eosinophil apoptosis at either 20 or 40 h (Figure 3.8 B).

3.2.4 The Effect of PGE_2, PGA_2 and the EP_2 agonist 11-deoxy PGE_1 on Granulocyte Apoptosis

The prostaglandin PGE_2 displayed similar effects to the 5-lipoxygenase products. As illustrated in Figure 3.9 A and B, PGE_2 inhibited neutrophil apoptosis at 20 h with a reduction from a control value of 65.9 ± 6.6 % to 45.8 ± 6.8 % in PGE_2 treated cells, but had no effect on eosinophil survival. The enhanced survival of neutrophils was mediated by PGE_2 and not by its metabolite PGA_2; Figure 3.10 A and B demonstrate that PGA_2 had no significant effect on apoptosis in either neutrophils or eosinophils. PGE_2 can bind to several subtypes of the EP receptor (discussed in Section 1.9.3) which have been linked to different intracellular signalling pathways. We therefore used 11-deoxy PGE_1, which is an EP_2 receptor agonist. This agonist produced similar results to PGE_2, inhibiting neutrophil apoptosis from 65.3 ± 6.9 % in control cells to 50.3 ± 7.0 % in treated cells, but having no significant effect on eosinophil survival (Fig 3.11 A and B).

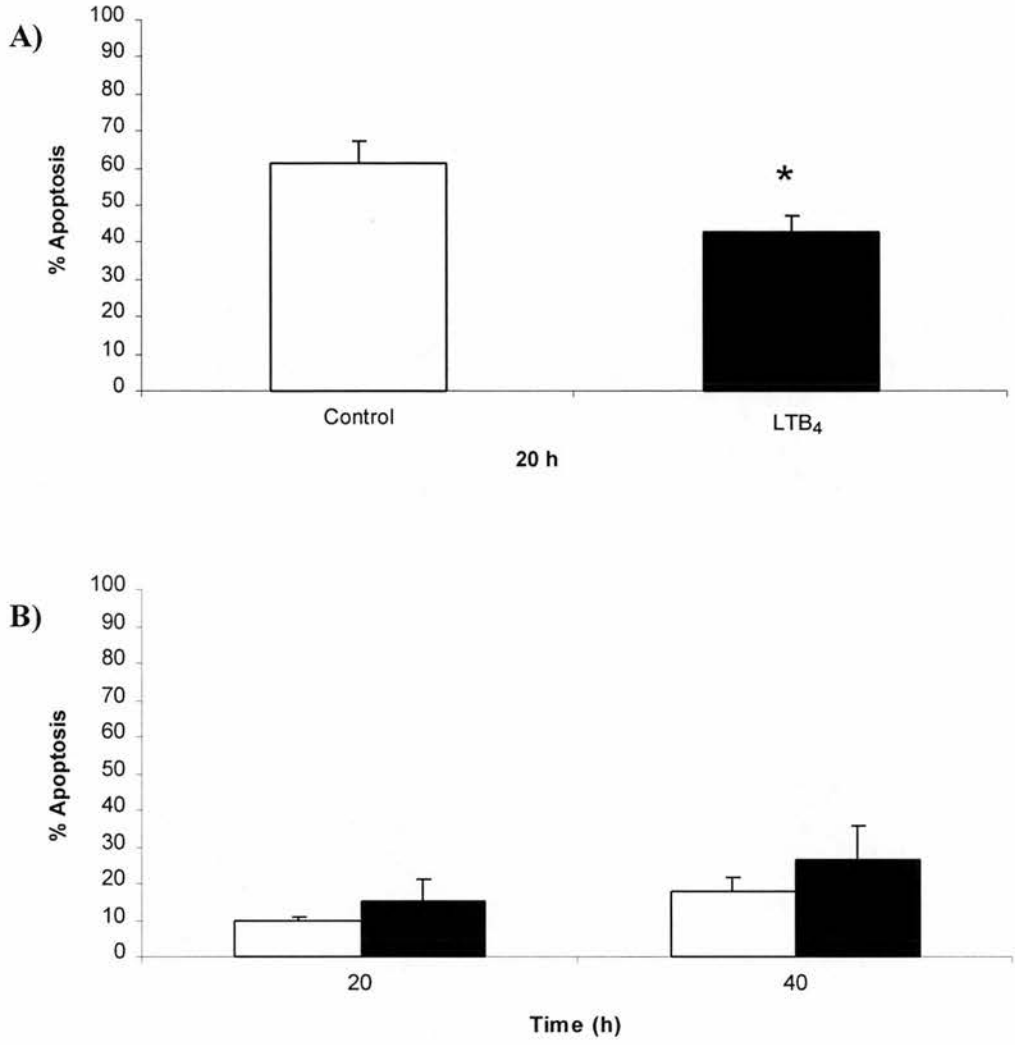


Figure 3.7 The Effect of LTB_4 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with LTB_4 (300 nM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

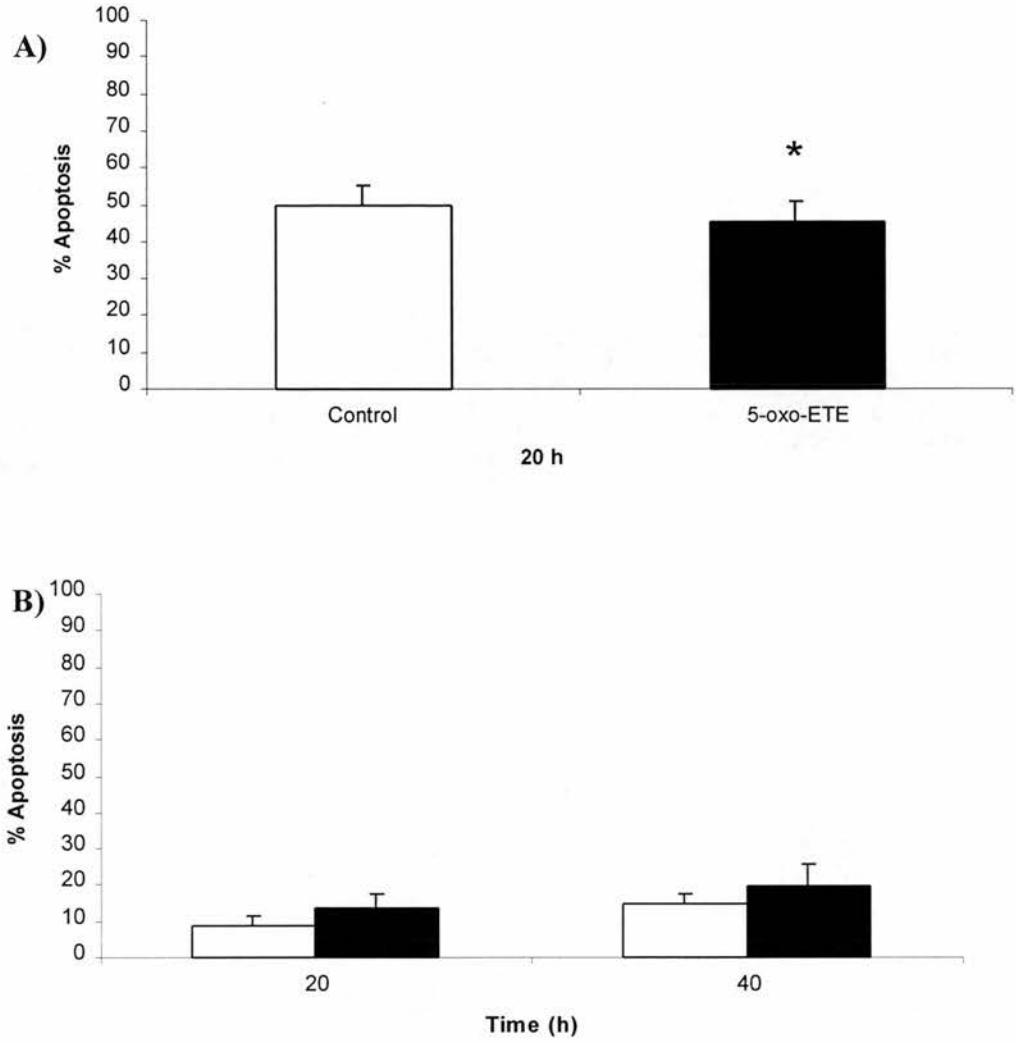


Figure 3.8 The Effect of 5-oxo-ETE on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with 5-oxo-ETE (300 nM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 5 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

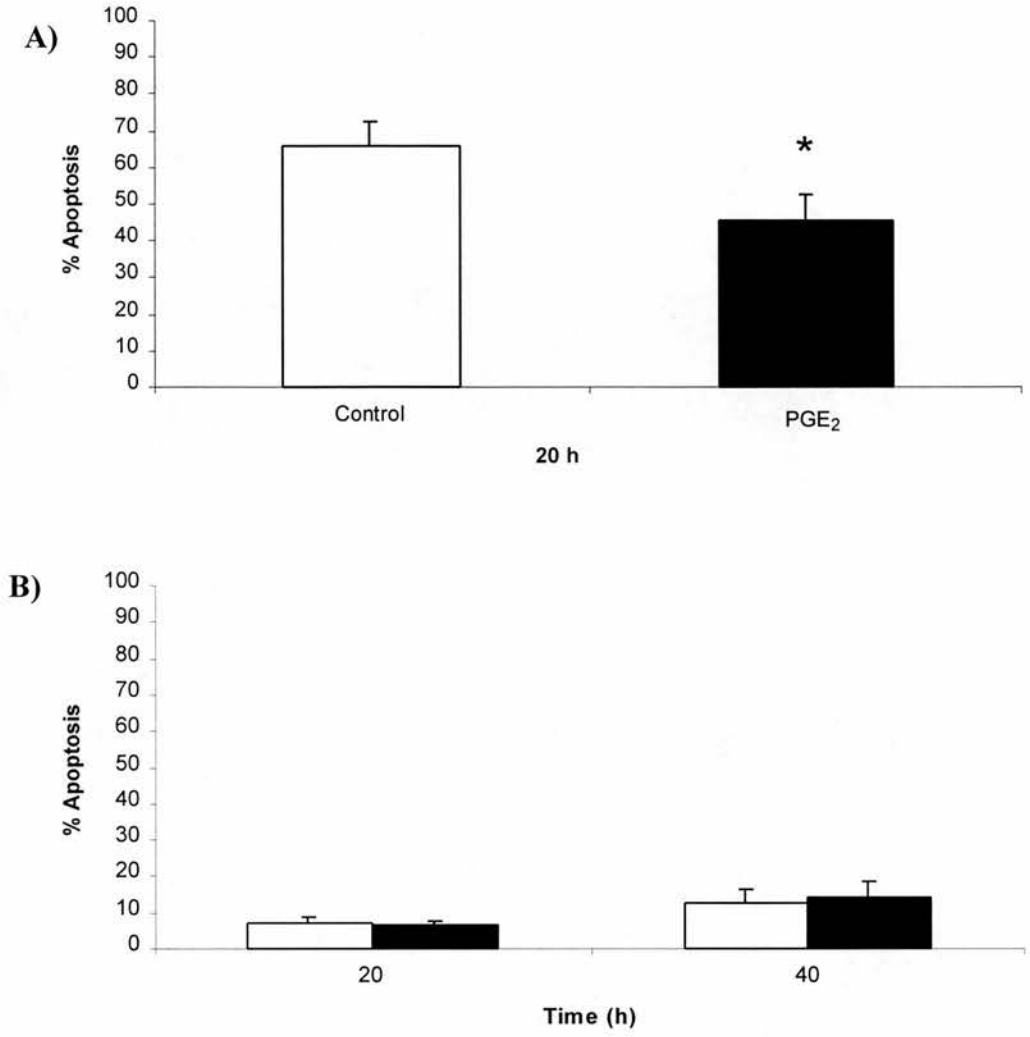


Figure 3.9 The Effect of PGE_2 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with PGE_2 (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 4 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

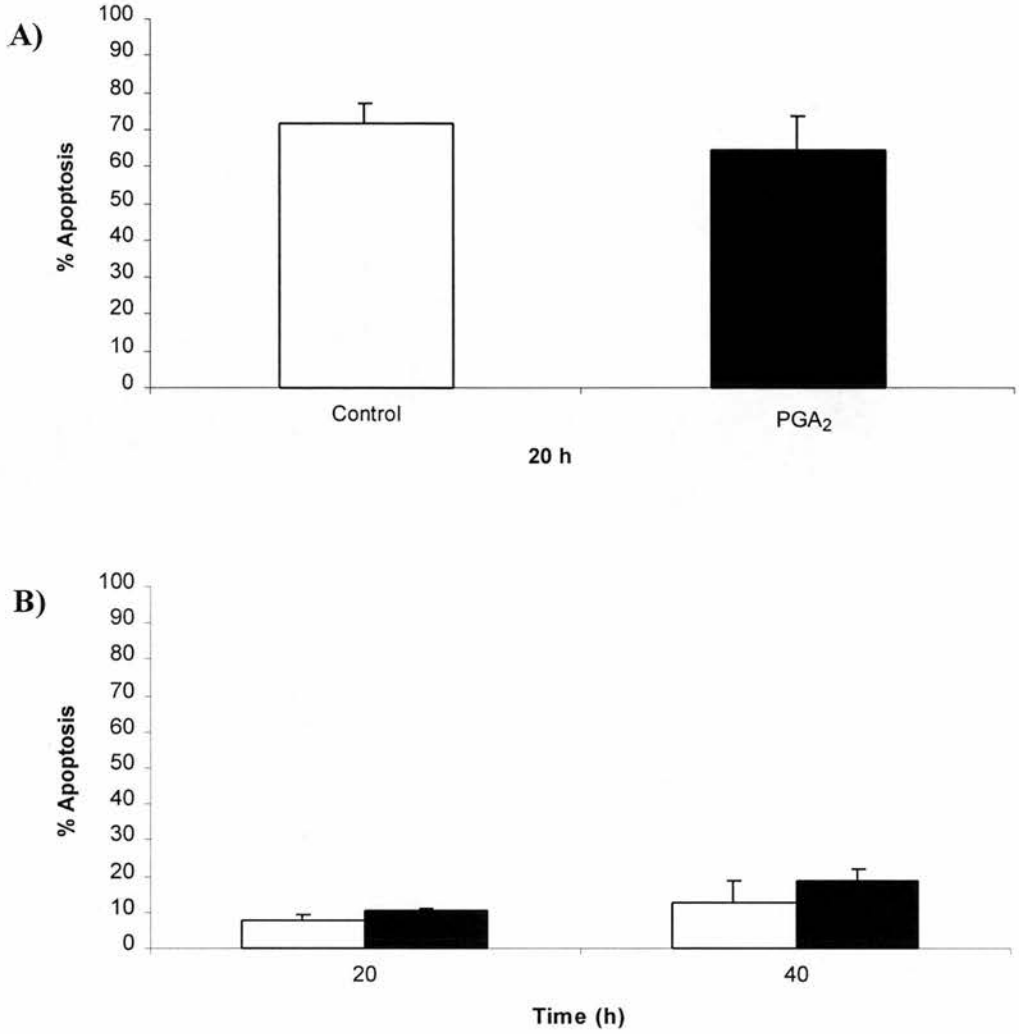


Figure 3.10 The Effect of PGA_2 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with PGA_2 (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate.

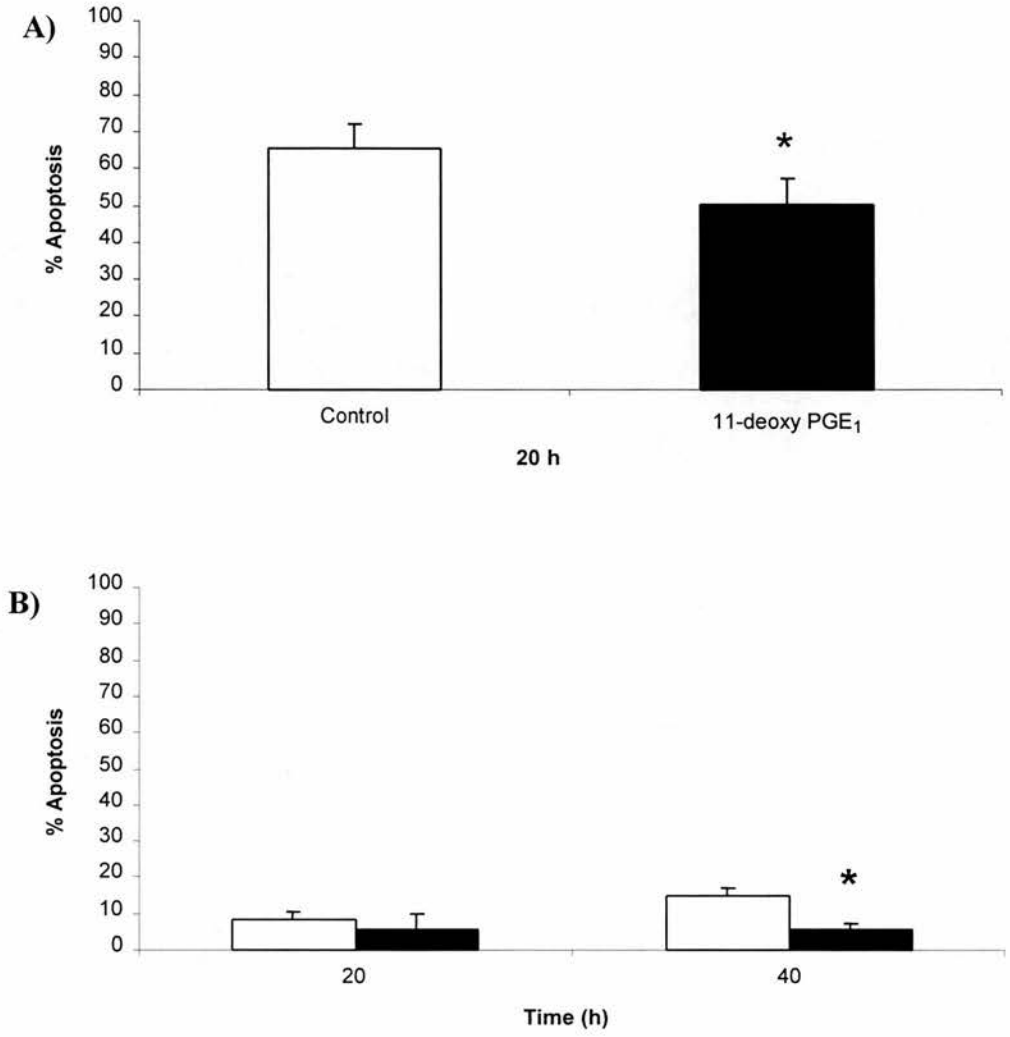


Figure 3.11 The Effect of 11-deoxy PGE_1 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with 11 deoxy-PGE_1 (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 4 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

3.2.5 The Effect of PGD_2, the PGD_2 Mimetic ZK118.182, $PGF_{2\alpha}$ and PGJ_2 on Granulocyte Apoptosis

As shown in Figure 3.12 A, PGD_2 had no effect on neutrophil apoptosis, though it significantly and dramatically increased the rate of eosinophil programmed cell death at 20 and 40 h. For example, at 40 h PGD_2 increased control apoptosis from 19.9 ± 5.0 % to 83.7 ± 5.5 % in eosinophils. To directly demonstrate the efficacy of the PGD_2 effect, experiments were performed with PGD_2 and dexamethasone on the same day with the same donors. Figure 3.13 compares eosinophil apoptosis driven by dexamethasone, an inhibitor of eosinophil survival (See Figure 3.3 B), with PGD_2. At 20 h, control apoptosis was 13.7 ± 5.6 %, apoptosis induced by dexamethasone 21.7 ± 8.8 %, and PGD_2 induced a rate of apoptosis of 49.3 ± 8.1 %. This result shows for the first time that PGD_2 is a powerful inducer of programmed cell death in eosinophils.

However, as already discussed, PGD_2, like PGE_2, can be metabolised to various biologically active breakdown products. Therefore to further characterise this result, the effect on granuloctye apoptosis of ZK118.182, a stable PGD_2 mimetic, was examined. This compound would allow investigation of the effects of activating the DP receptor without the production of active PGD_2 metabolites. Figure 3.14 A and B show that ZK118.182 produced increased eosinophil apoptosis (40 h control, 14.9 ± 4.5 %; ZK118.182, 23.8 ± 6.1 %) but in contrast to PGD_2, it significantly inhibited apoptosis in neutrophils from 50.0 ± 6.1 % to 19.1 ± 3.3 % in treated cells. This suggests that activation of the DP receptor by PGD_2 should cause elevated levels of apoptosis in eosinophils, but decrease neutrophil programmed cell death.

PGD_2 has been shown to bind to other prostanoid receptors such as the FP receptor, which also binds $PGF_{2\alpha}$. $PGF_{2\alpha}$ inhibits several functional activities in human neutrophils but little is known of its effects on eosinophils. As shown in Fig 3.15 A and B, there was a small, but significant increase in neutrophil survival, while at 40 h, eosinophil apoptosis was almost 50% of control values with a decrease from 12.8 ± 1.0 % to 6.9 ± 0.7 % in treated cells.

PGJ_2 is an active PGD_2 metabolite that is a potent and highly selective DP agonist (Bundy et al., 1983). As demonstrated in Fig 3.16 A, PGJ_2 produced similar results to PGD_2 in neutrophils, causing no significant change in the constitutive rate of apoptosis at 20 h. Like PGD_2, it significantly increased eosinophil apoptosis at both 20 h (7.7 ± 1.4 % control, to 15.7 ± 1.7 % with PGJ_2 treatment) and 40 h (12.8 ± 1.0 % control, to 29.8 ± 4.2 % with PGJ_2 treatment) (Fig 3.16 B). This increase is less than that induced by the parent compound, PGD_2. As shown in Figure 3.17, after 40 h of culture apoptosis in control eosinophils was 12.8 ± 1.0 %, PGJ_2 produced a rate of apoptosis of 29.8 ± 4.2 %, while PGD_2 increased apoptosis to 87.9 ± 3.8 %.

These results suggest that activation of the DP receptor causes elevated levels of apoptosis in eosinophils. However, although the stable mimetic ZK118.182 increased eosinophil apoptosis, it decreased neutrophil programmed cell death. The DP agonists PGD_2 and PGJ_2 did not increase neutrophil survival. Neither ZK118.182 nor PGJ_2 achieved the levels of apoptosis in eosinophils caused by PGD_2. It is therefore possible that PGD_2 is metabolised by both eosinophils and neutrophils. In the case of neutrophils, the metabolite may be inactive with regard to apoptosis, while in eosinophils, a metabolite with more powerful pro-apoptotic effects may be produced. Such metabolites may bind to receptors other than the DP receptor.

3.2.6 The Effect of the PGD_2 Metabolites $\Delta^{12}PGJ_2$ and 15-deoxy PGJ_2 on Granulocyte Apoptosis

It has been previously demonstrated that $\Delta^{12}PGJ_2$ can induce apoptosis in other cell types, however, there have been no prior reports of 15d-PGJ_2 being associated with programmed cell death. Neutrophils and eosinophils were therefore cultured with these metabolites. The results for $\Delta^{12}PGJ_2$ and 15d-PGJ_2 are shown in Figure 3.18 A and B and Figure 19 A and B respectively. These graphs demonstrate that both metabolites are potent inducers of programmed cell death in both granulocytes and at all timepoints shown. $\Delta^{12}PGJ_2$ induced neutrophil apoptosis from 66.0 ± 6.7 % to

91.1 ± 3.4 %, while 15d-PGJ_2 increased the control rate to 89.5 ± 4.1 %. In eosinophils at 40 h of culture, control apoptosis (32.0 ± 10.5 %) was increased dramatically to 97.4 ± 2.3 % by $\Delta^{12}PGJ_2$ and to 99.0 ± 0.6 % by 15d-PGJ_2. Therefore both $\Delta^{12}PGJ_2$ and 15d-PGJ_2 caused increases in eosinophil apoptosis which were similar to those achieved by PGD_2, however they also had a potent stimulatory effect on neutrophil apoptosis. This suggests that neutrophils and eosinophils may produce different metabolites.

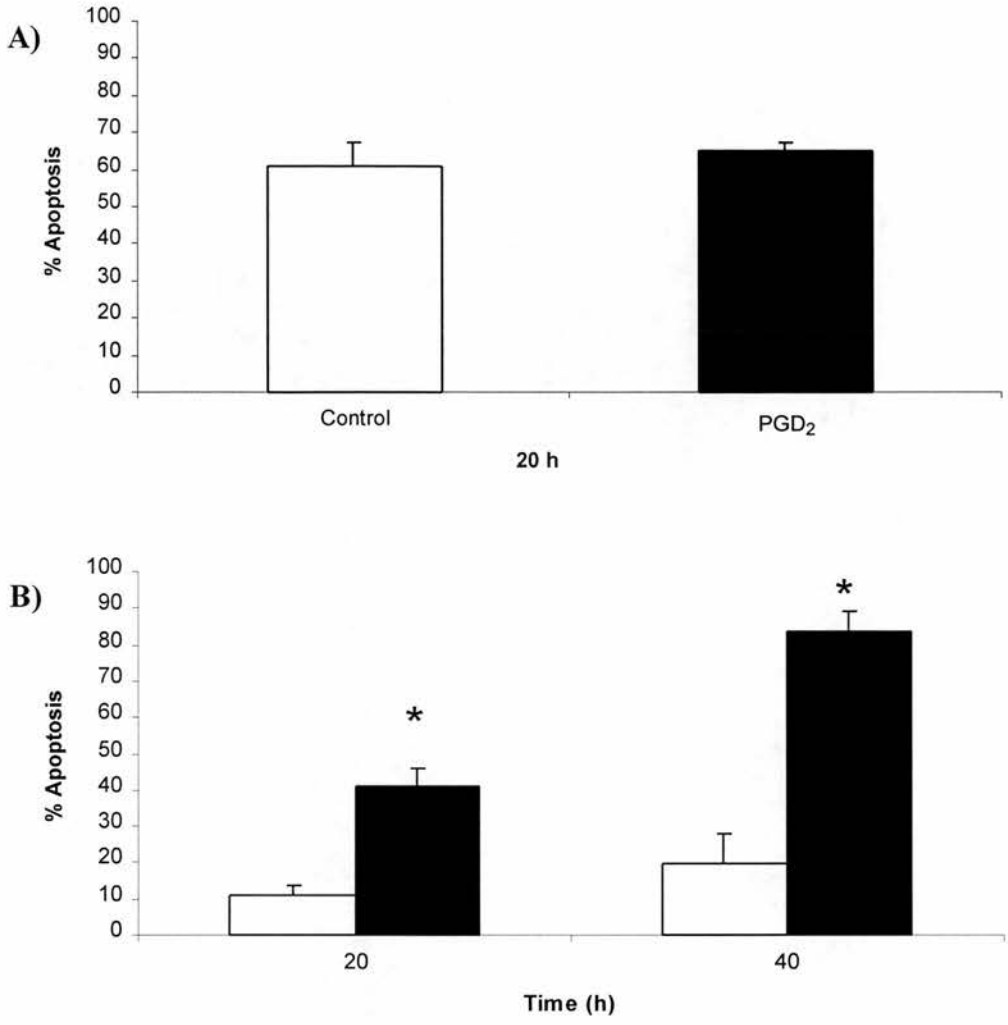


Figure 3.12 The Effect of PGD_2 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with PGD_2 (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 7 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values.

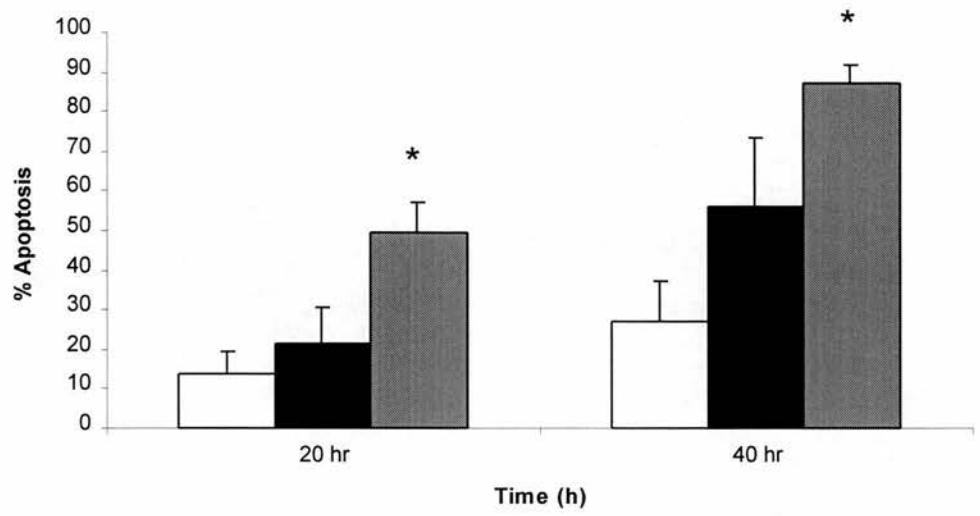


Figure 3.13 A Comparison of the Effects of PGD_2 and Dexamethasone on Constitutive Eosinophil Apoptosis

Eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) (open bars), dexamethasone (1 μM) (solid bars), or with PGD_2 (10 μM) (hatched grey bars) and harvested at 20 and 40 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

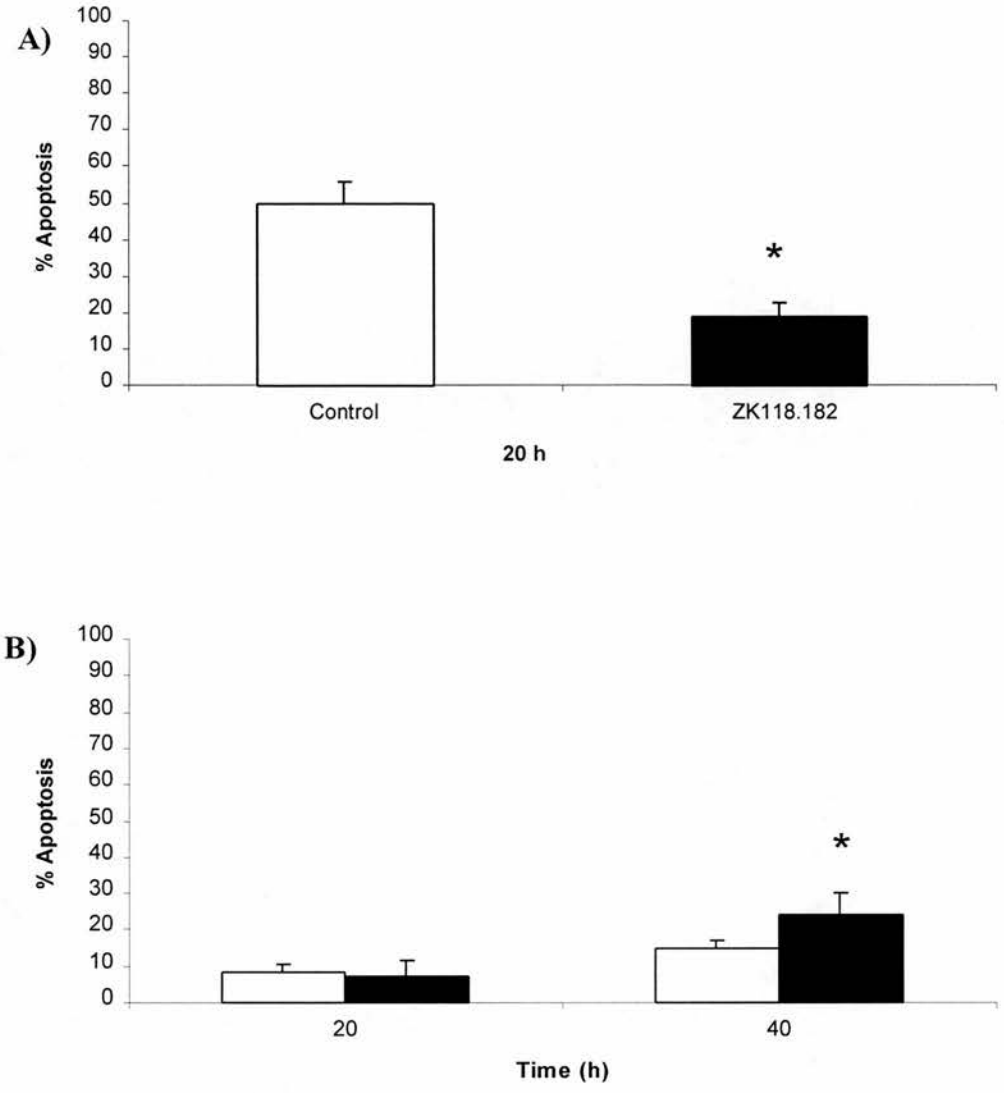


Figure 3.14 The Effect of the PGD_2 Mimetic ZK 118.182 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with ZK 118.182 (30 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars indicate treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

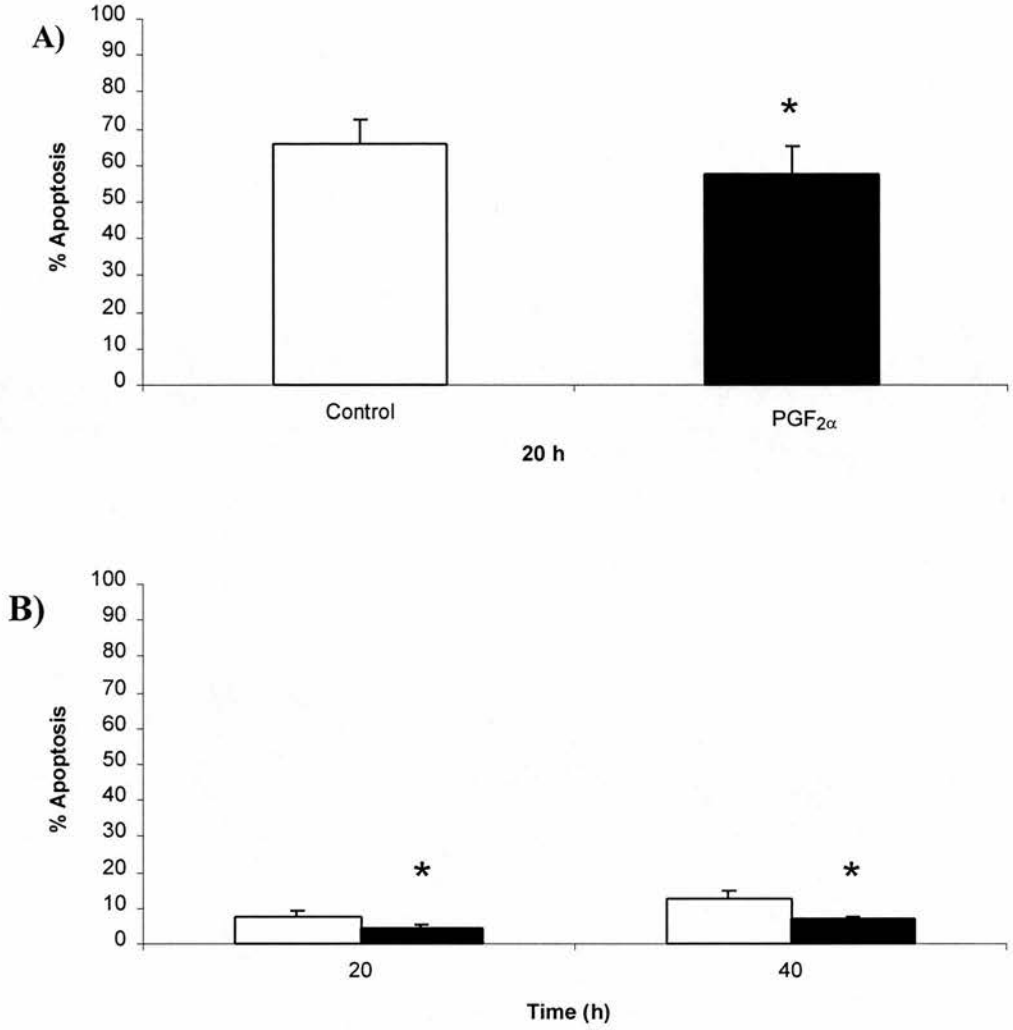


Figure 3.15 The Effect of $PGF_{2\alpha}$ on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with $PGF_{2\alpha}$ (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 4 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

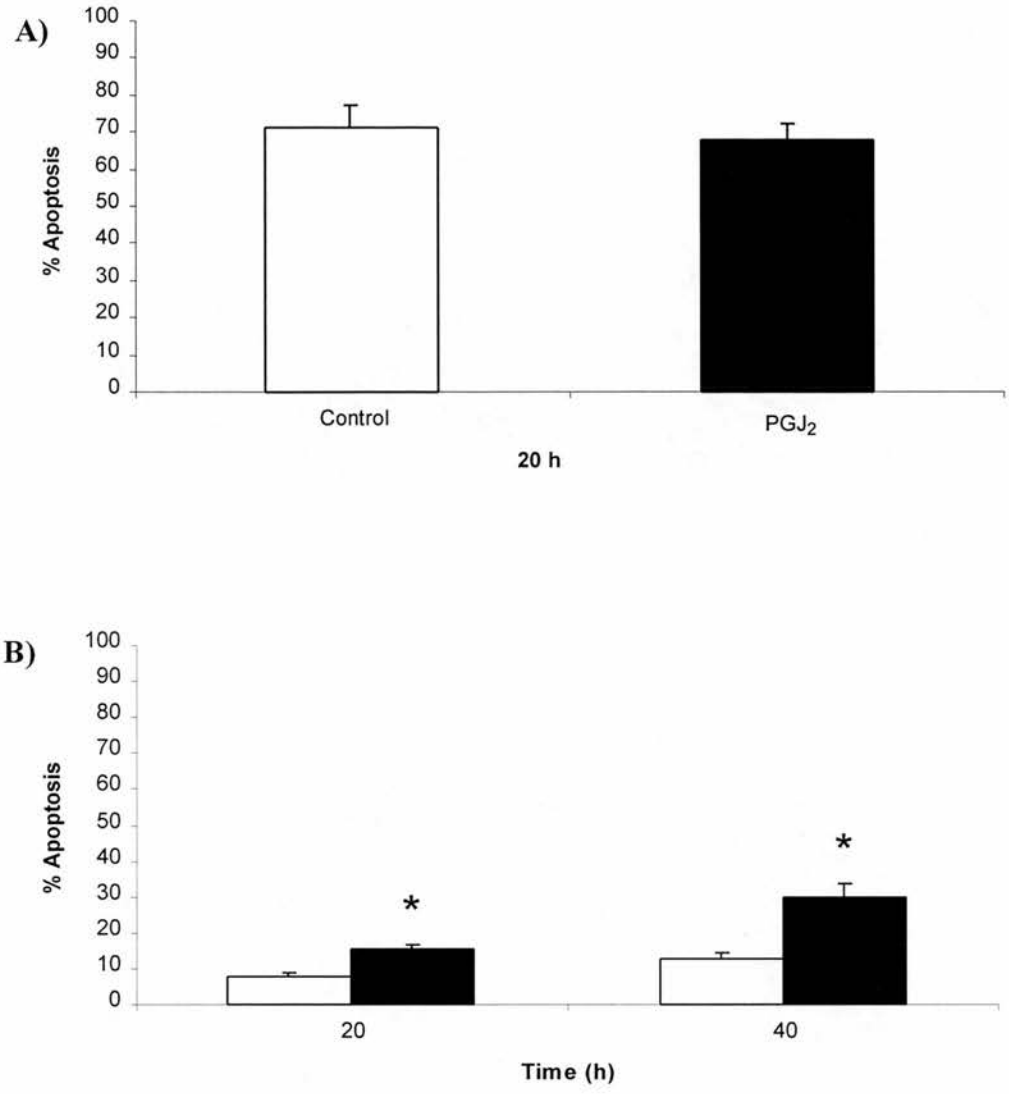


Figure 3.16 The Effects of PGJ_2 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with PGJ_2 (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars indicate treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.5$ compared with control values).

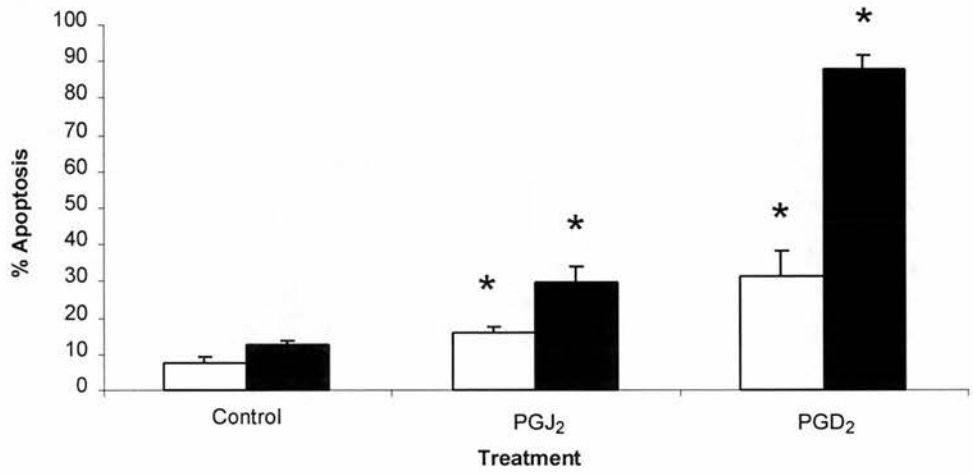


Figure 3.17 A Comparison of the Effects of PGJ_2 and PGD_2 on Constitutive Eosinophil Apoptosis

Eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with PGJ_2 (10 μM) or PGD_2 (10 μM) and harvested at and 20 and 40 h. Open bars indicate 20 h timepoint, filled bars are results for 40 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

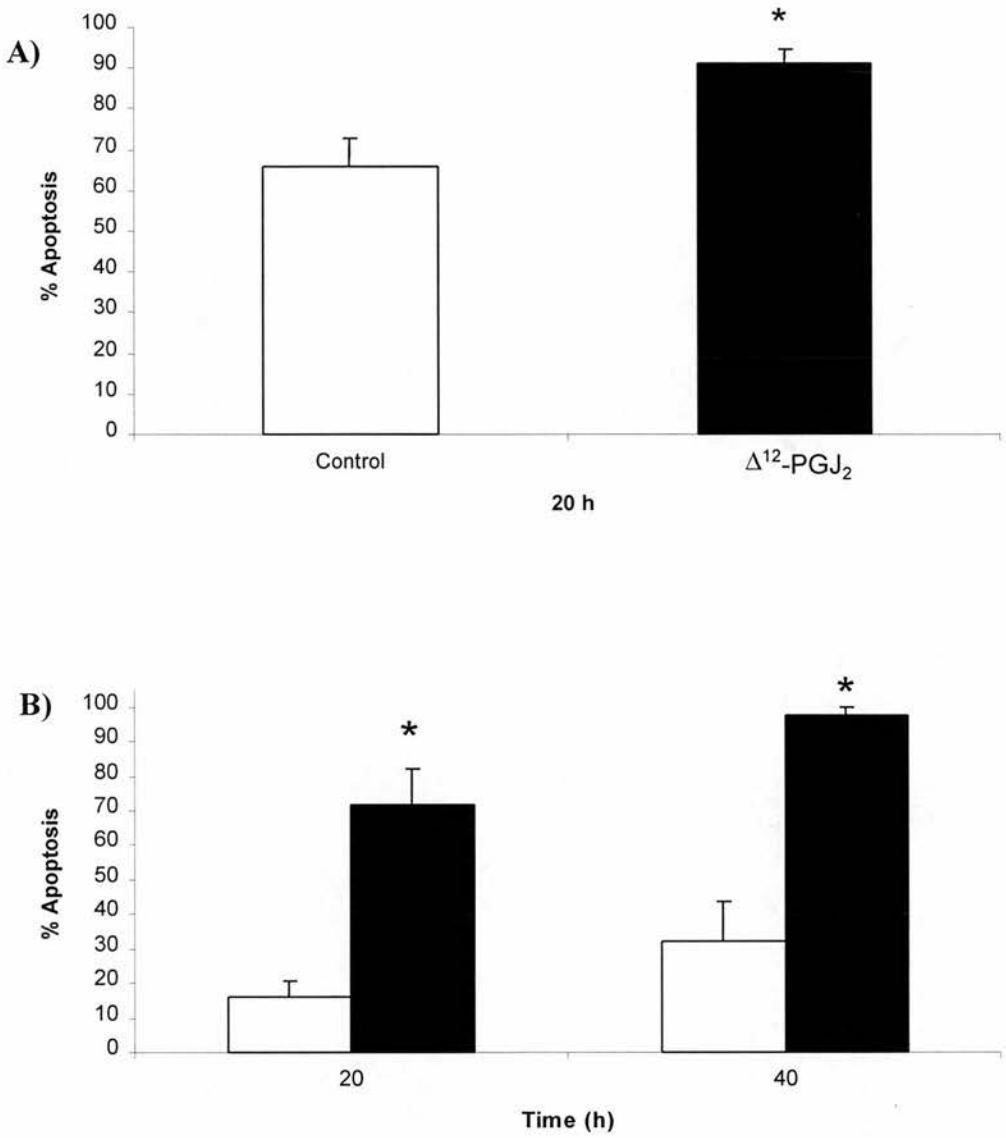


Figure 3.18 The Effect of Δ^{12}-PGJ_2 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with Δ^{12}-PGJ_2 (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 4 separate experiments for neutrophils, and 4 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

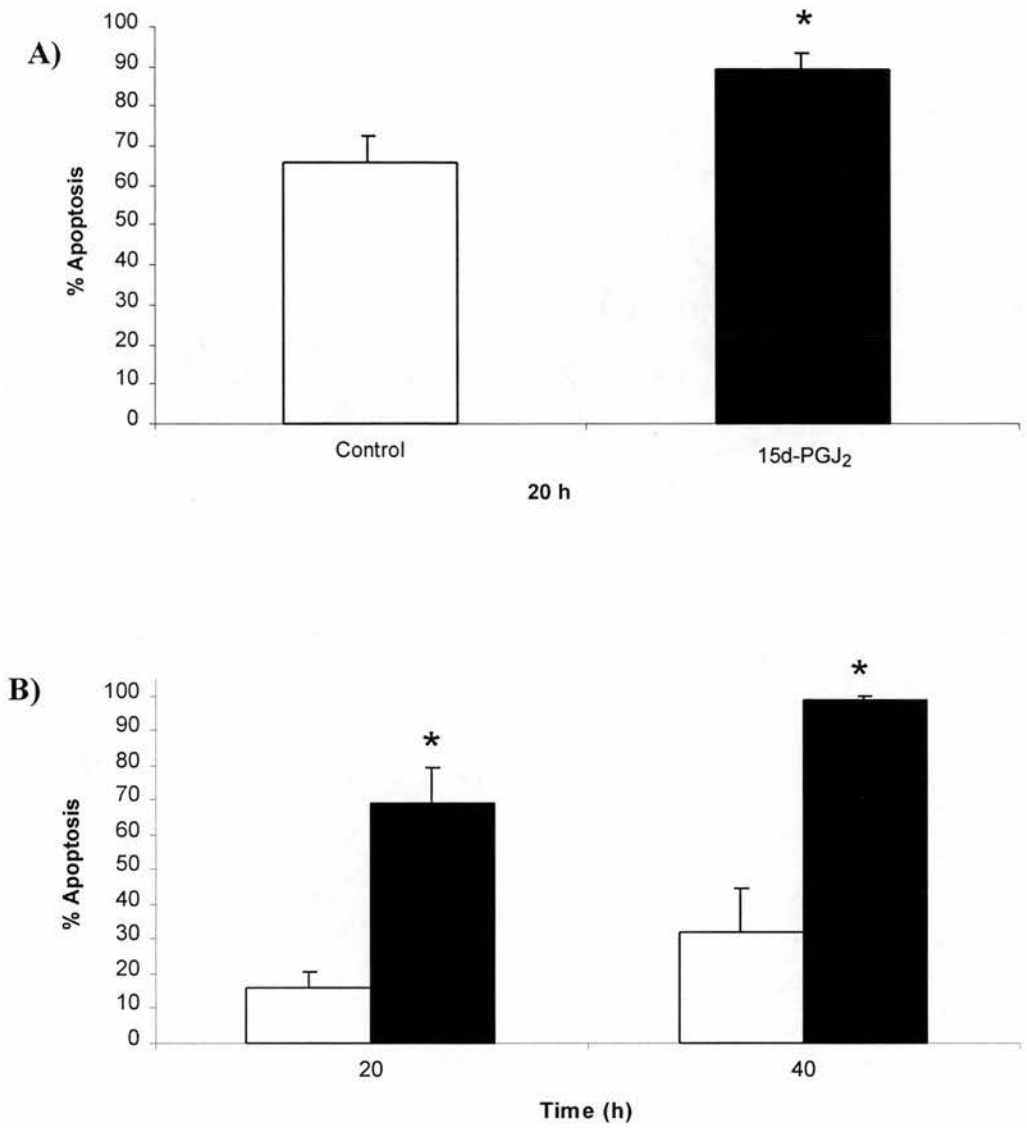


Figure 3.19 The Effect of 15 deoxy-PGJ_2 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with 15 deoxy-PGJ_2 (10 μM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 4 separate experiments for neutrophils, and 4 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

3.2.7 The Effect of Increased Levels of Intracellular cAMP and on Granulocyte Apoptosis

DP receptors have been reported to activate adenylate cyclase activity causing increases in intracellular levels of cAMP (Chapter 1, Table 1.1). In this work, the stable, cell-permeable analogue of cAMP, dbcAMP, was used to mimic elevation of the second messenger cAMP in both eosinophils and neutrophils. As shown in Fig 20 A and B, both granulocytes show inhibition of programmed cell death in response to increased intracellular cAMP levels. For example, in these experiments, neutrophil apoptosis was reduced from a control value of 58.5 ± 3.7 % to 27.4 ± 6.9 % in treated cells at 20 h, while eosinophil apoptosis was decreased from 21.8 ± 3.6 % to 4.5 ± 1.1 % at 40 h with dbcAMP treatment. This indicates that PGD_2 and the active metabolites tested do not increase apoptosis via increases in intracellular cAMP.

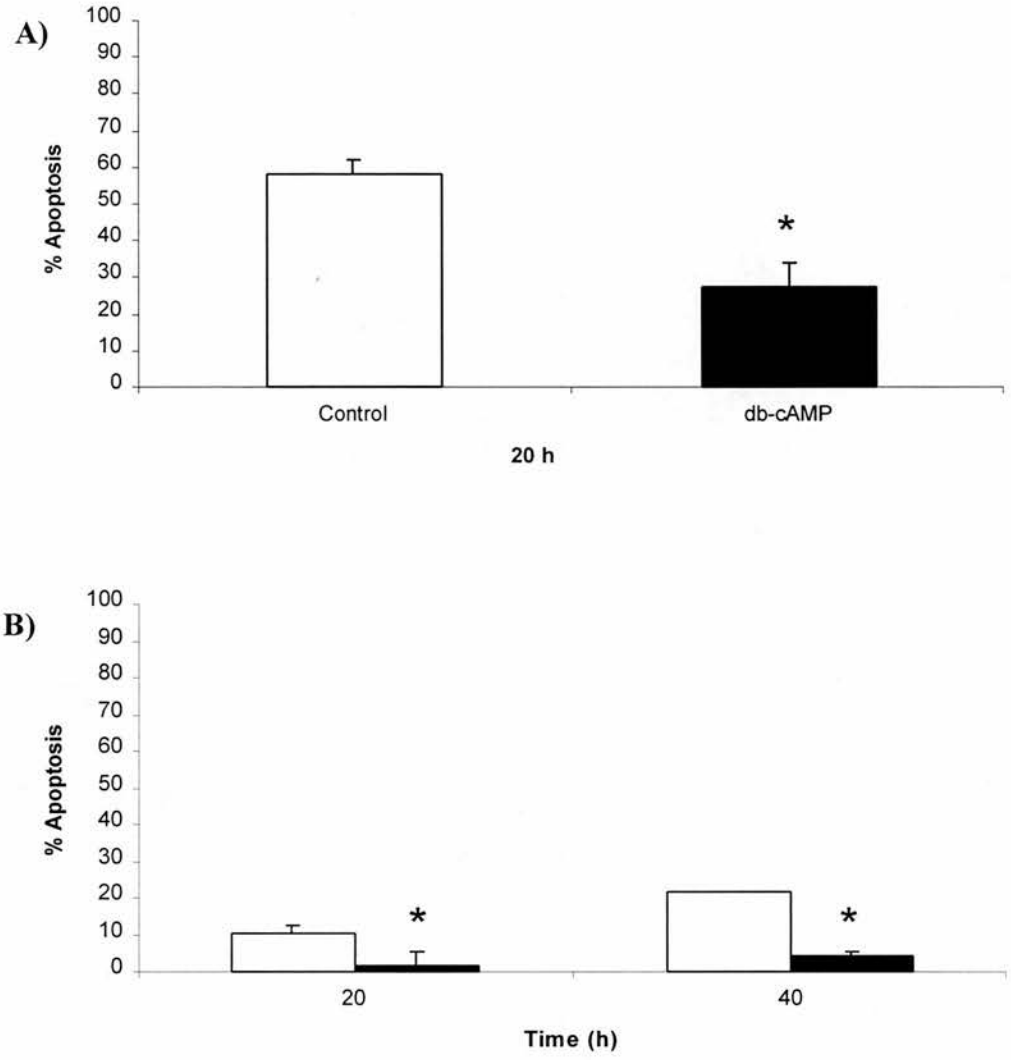


Figure 3.20 The Effect of Increased Intracellular cAMP on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with dbcAMP (0.2 mM) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils, and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

3.3 Discussion

The results show that both neutrophils and eosinophils undergo constitutive apoptosis *in vitro*, and that this process can be modulated by a variety of agents. Granulocyte survival appears to be dependent on *de novo* protein synthesis since both cells types show enhanced rates of apoptosis when cultured with cycloheximide, a protein synthesis inhibitor. However, this could be an indirect effect on cell processes not directly involved in the cell death pathway.

Interestingly, the synthetic glucocorticoid, dexamethasone has differential effects on granulocyte apoptosis. Dexamethasone inhibits neutrophil apoptosis while driving programmed cell death in eosinophils; these results are in accordance with Meagher et al., (1996) and others. Dexamethasone does not prime or activate neutrophils, but still increases the survival of these cells (Cox, 1995). This enhanced survival is prevented by co-treatment with protein synthesis inhibitors suggesting that dexamethasone enhances neutrophil survival by the induction of a protein or proteins (Cox & Austin, 1997).

Why glucocorticoids should have differential effects on the survival of these cells is currently unknown. Glucocorticoid receptors are expressed in almost every type of cell although the density of glucocorticoid receptors may differ from cell to cell, giving different responses (Burnstein & Cidlowski, 1992). However, binding characteristics have been shown to be similar in both neutrophils and eosinophils (Peterson et al., 1981). The pro- and anti-apoptotic effect of dexamethasone has been demonstrated to occur via GR receptors as the effect is not mimicked by mineralocorticoids or steroid sex hormones and is blocked by the glucocorticoid receptor antagonist RU486 (Meagher et al., 1996)

Transcriptional repression of AP-1 response elements involving the physical interaction of the GR and the transcription factor AP-1 has been described (Diamond et al., 1990, Jonat et al., 1990, Schüle et al., 1990, Yang et al., 1990, Heck et al., 1994). AP-1 is formed from dimers of the Jun and Fos family, usually c-Jun and c-Fos; but other family members of AP-1 interact differently with the GR, being positively regulated (Miner & Yamamoto, 1991, Teurich & Angel, 1995). Since different cell types contain one or more of these AP-1-related proteins (Jun B, Jun D, Fos B, Fra-1 and Fra-2), the AP-1/GR interaction can cause positive or negative regulation of genes in a specific cell (Teurich & Angel, 1995). The role of AP-1 in the control of apoptosis is controversial, but it is possible that neutrophils and eosinophils have different profiles of AP-1 family proteins and that this could account for differences in their responses to glucocorticoids.

Dexamethasone can enhance the expression of IκBα, the inhibitory subunit of NF-κB (Auphan et al., 1995, Scheinman et al., 1995a, b). However, the role of NF-κB in cell survival is unclear since activation of NF-κB correlates with apoptosis in some systems and enhanced survival in others (Grimm et al., 1996, Wu et al., 1996). However, in granulocytes, many pro-inflammatory stimuli cause NF-κB activation. This transcription factor regulates genes for cytokines that are associated with enhanced survival in granulocytes such as GM-CSF (Stein et al., 1993a, b), and it is therefore unlikely that this would lead to differential effects on granulocyte apoptosis. However, in T cells, which also undergo apoptosis in response to treatment with glucocorticoids, activation of the GR causes the abrupt downregulation of c-*myc* RNA, and this gene is under the influence of NF-κB (Yuh & Thompson, 1989).

NF-κB is also known to interact with AP-1 (Stein et al., 1993a, b), causing synergistic activation of both κB and AP-1 response elements. However, while both AP-1 and NF-κB can be inhibited by activated GR, synergistic NF-κB/AP-1 activity is largely unaffected (Scheinman et al., 1995b).

Therefore the end result of GR activation by glucocorticoids may be dependent on the spectrum of AP-1 family proteins in a specific cell, and the interactions of these isoforms with NF-κB. If these differ between neutrophils and eosinophils, this may account for the dissimilar effects of dexamethasone on apoptosis in these cells. However, in dexamethasone-induced apoptosis of rat thymocytes, both AP-1 and NF-κB were shown to be activated rather than inhibited at early timepoints, but whether this activation was synergistic or not is unknown (Sikora et al., 1993).

LPS primes and activates both neutrophils and eosinophils, and like most priming agents, it enhances the longevity of both cell types (Lee et al., 1993, Nick et al., 1996). LPS, a powerful survival factor for these cells, is known to strongly activate NF-κB in granulocytes (See Chapter 5, Figure 5.14) (McDonald et al., 1997a) which again suggests that the dexamethasone-mediated survival of neutrophils is unlikely to be due to NF-κB suppression.

RANTES and eotaxin are both eosinophil chemoattractants (Griffiths-Johnson et al., 1993, Rot et al., 1992), but had no effect on the rate of eosinophil apoptosis. Although, it was expected that neutrophils would not respond to these chemokines, RANTES at higher concentrations did inhibit programmed cell death in these cells. Chemokines have been traditionally divided into two main groups, C-C and C-X-C. Generally, neutrophils are affected by C-X-C chemokines, and eosinophils primarily by C-C chemokines (Premack & Schall, 1996). However, this division is not complete; IL-8, a neutrophilic C-X-C chemotaxin, has been shown to be a potent chemoattractant for activated or primed eosinophils (Sehmi et al., 1993, Villar et al., 1993). Therefore it is possible that RANTES may bind to chemokine receptors on neutrophils, causing a small but significant decrease in apoptosis. Another explanation is that RANTES may cause contaminating eosinophils to release a neutrophil survival factor, while eotaxin may not or may cause the generation of different mediators.

LTB_4 activates neutrophils and eosinophils by binding to a cell-surface receptor (Bomalaski & Mong, 1987, Goetzel et al., 1995, Ng et al., 1991, O'Flaherty et al., 1991, Alexander & Peters 1997). However, while it significantly enhanced the survival of neutrophils, it had no effect on eosinophil apoptosis. This result for neutrophils is in agreement with Hébert et al., (1996). Since LTB_4 is weakly active on human eosinophils (Powell et al., 1995), the differential apoptotic response in granulocytes to LTB_4 may reflect this.

LTB_4-binding is associated with increased $[Ca^{2+}]_i$ in neutrophils, eosinophils and other cells, as well as an inhibition of adenylate cyclase activity (Raible et al., 1992, Powell et al., 1996, Yokomizo et al., 1997). However, it has recently been shown that LTB_4 can react with both cell-surface and nuclear receptors (Fiore et al., 1992, Devchand, 1996, Yokomizo et al., 1997). Whether each type of receptor is differentially expressed in specific cells is unknown.

5-oxo-ETE is synthesised by both eosinophils and neutrophils (Powell et al., 1992, 1995). Although it does not act through the LTB_4 receptor (Powell et al., 1993, O'Flaherty et al., 1994), it produced similar results to LTB_4 in these studies, inhibiting neutrophil apoptosis but having no effect on eosinophil programmed cell death. 5-oxo-ETE is known to increase $[Ca^{2+}]_i$ in both types of granulocytes (Powell et al., 1995, 1996), but there are no reports of its effects on adenylate cyclase activity in these cells.

The prostaglandins used in these studies produced diverse results. PGE_2 is a prostanoid with suppressive effects on neutrophil function (Rivkin et al., 1975, Ottonello et al., 1995, Rossi et al., 1995). However, this mediator significantly enhanced neutrophil survival, but had no effect on eosinophil apoptosis. Rossi et al., (1995) and Walker et al., (1997), reported that this PGE_2-induced enhancement of survival was mediated by elevation of intracellular cAMP concentration. Eosinophils showed no significant change in rates of apoptosis in response to this prostanoid.

PGE_2 can bind to several different EP receptor subtypes that utilise different intracellular signalling pathways (Table 1.1). It is possible that there are differences in types of EP receptor on neutrophils and eosinophils and that this would account for the contrasting responses observed. However, since PGE_2 may be metabolised in culture, the results could be due to breakdown products rather than the actions of PGE_2 *per se*. However, the metabolite PGA_2 had no effect on programmed cell death in either cell type. The EP_2 receptor agonist, 11-deoxy PGE_1, produced similar results to PGE_2, indicating that EP2 is the probable receptor involved in inhibition of neutrophil apoptosis. This receptor is known to be present on human neutrophils (Wheeldon & Vardey, 1993), and its activation has been demonstrated to cause increases in intracellular cAMP (Jumblatt & Peterson, 1991).

PGD_2 is thought to bind to a specific DP receptor that is found on neutrophils and eosinophils (Rossi & O'Flaherty, 1989, Butchers & Vardey, 1990, Ney & Schrör, 1991, Wheeldon & Varday, 1993, Darius et al., 1994). In COS-M6 and HEK 293 cells transfected with the cloned human DP receptor, ligand-binding caused increased cAMP production, and increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Boie et al., 1995). However $[Ca^{2+}]_i$ rises were not observed in neutrophils treated with PGD_2 (Raible et al., 1992). Unlike other stimuli which increase $[Ca^{2+}]_i$ in human eosinophils, such as PAF and LTB_4, PGD_2 induced another secondary rise in $[Ca^{2+}]_i$, seen after the initial increase (Raible et al., 1992).

PGD_2 is known to bind to different prostaglandin receptors; for example it has been demonstrated to bind to the $PGF_{2\alpha}$ and/or thromboxane A_2 receptors (Narumiya & Toda, 1985, Balapure et al., 1989, Hamid-Bloomfield et al., 1990). Therefore it is possible that receptors other than DP are involved in cellular responses to PGD_2. The results shown in Figure 3.15 suggest that if it is binding to the $PGF_{2\alpha}$ receptor, an inhibition of apoptosis should have been noted in both eosinophils and neutrophils.

Although $PGF_{2\alpha}$, at high concentrations, has been shown to inhibit certain neutrophil functions, and is chemotactic for eosinophils (Gryglewski et al., 1987, Wheeldon & Vardey, 1993), this prostaglandin significantly inhibited both neutrophil and eosinophil apoptosis and may therefore have pro-inflammatory properties. Its structure is similar to PGD_2, but PGD_2 proved to be a powerful inducer of eosinophil apoptosis, though it had no effect on neutrophil apoptosis.

The effect of thromboxane A_2 on apoptosis in both cell types should be examined to investigate whether the pro-apoptotic effects of PGD_2 are mediated via the thromboxane receptor, although there is no direct evidence for the presence of IP, FP or TP receptors on granulocytes (Giembycz et al., 1990).

PGD_2 can be rapidly metabolised, and in many studies there is confusion over whether the results are due to this prostanoid or to its breakdown products. Such metabolites could be inactivation products since in a number of instances they are less active than the parent compound. In the specific case of PGD_2, however, some metabolites have been shown to be more potent with regard to several activities. It is possible that this mediator is broken down into different products by neutrophils and eosinophils, hence its differential effects on these cells.

PGJ_2 is a PGD_2 metabolite that is a potent, selective agonist on the DP receptor (Bundy et al., 1983). This prostanoid gave similar results to PGD_2, but the enhancement of eosinophil apoptosis was significantly less than PGD_2. This indicates that activation of the DP receptor does induce eosinophil apoptosis, but does not rule out the possibility that PGD_2 may be metabolised by either cell type; and in the eosinophil, the breakdown product is a more powerful inducer of apoptosis than the parent compound. To address this possibility, the stable PGD_2 mimetic ZK118.182 was used. This produced similar results to PGJ_2 in the eosinophil, but significantly inhibited neutrophil apoptosis, suggesting that PGD_2 can be rapidly metabolised in the neutrophil to a breakdown product with no effect on apoptosis or one which may counteract any PGD_2 mediated inhibition.

While the results in the eosinophil are not conclusive, they are suggestive of a metabolite with more powerful effects on apoptosis than PGJ_2. The results obtained with Δ^{12}-PGJ_2 and 15dPGJ_2 would support this hypothesis. Both these metabolites significantly and efficiently induced apoptosis in neutrophils and eosinophils. Therefore, eosinophils possibly metabolise PGD_2 to breakdown products with pro-apoptotic activity, while neutrophils may not.

How Δ^{12}-PGJ_2 and 15dPGJ_2 induce apoptosis is unknown, but cell surface receptors are not necessarily involved. Accumulating evidence indicates Δ^{12}-PGJ_2 and PGA_2 have their primary targets in the nucleus, where they regulate the expression of specific genes. However, Δ^{12}-PGJ_2 dramatically induced apoptosis while PGA_2 did not, which suggests that either they have different receptors or that there are no nuclear receptors for PGA_2 in these granulocytes. Alternatively, receptor affinity differences could be involved. Studies have shown that PGA_2 nuclear binding is reversible whereas Δ^{12}-PGJ_2 causes an irreversible effect via nuclear protein binding (Narumiya et al., 1987, Ohno et al., 1988).

Possible candidates for these intracellular receptors are peroxisome proliferator-activated receptors (PPARs). PPARs are transducer proteins belonging to the steroid/thyroid/retinoid receptor superfamily which regulate the expression of target genes by binding to DNA sequence elements termed PPAR response elements (Lemberger et al., 1996). Members of one family of these receptors have all been identified in humans; PPAR-γ, PPAR-δ, PPAR-α (Schmidt et al., 1992, Sher et al., 1993, Greene et al., 1995). PPAR-α is primarily expressed in tissues that have high fatty acid catabolism, including the liver and the immune system, while PPAR-γ is expressed in adipose tissue, adrenal gland, spleen and several myeloid cell lines (Kliewer et al., 1994, Tontonoz et al., 1994, Greene et al., 1995, Braissant et al., 1996, Lemberger et al., 1996).

Prostanoids with the most potent anti-tumour and growth inhibitory effects on human cancer cells, are the same subset which have subsequently been shown to bind to PPARs (Bregman et al., 1986, Choi et al., 1992, Fukushima et al., 1992, Holbrook et al., 1992, Kim et al., 1993, Yu et al., 1995). Studies from several investigators have suggested that PPAR subtypes are differentially activated by various agents (Dreyer et al., 1992, Schmidt et al., 1992, Zhu et al., 1993, Kliewer et al., 1994, Yu et al., 1995). Many PPAR activators exert their effects indirectly by either being metabolised in the cell to an active form or by inducing the release or synthesis of endogenous PPAR ligands (Issemann & Green, 1990, Göttlicher et al., 1992).

Prostaglandins from the A, D, and J series have been shown to activate all three PPAR subtypes when applied at concentrations of 10 μM, as used in the experiments described in Section 3.2 (Yu et al, 1995). PPAR-γ was the most responsive subtype with Δ^{12}-PGJ_2 and $15dPGJ_2$ its most powerful activators (Forman et al, 1995, Kliewer et al., 1995). No binding of PGD_2 was detected in these studies, suggesting that PGD_2 activates PPAR-γ through one or more of its metabolites.

PPAR-γ inhibits gene expression by antagonising the transcription factors AP-1, STAT and NF-κB. Therefore activation of PPAR-γ results in inhibition of at least three different classes of transcription factors involved in regulating inflammatory responses in granulocytes and other cell types. This suggests that PPAR-γ and its ligands $15dPGJ_2$ or Δ^{12}-PGJ_2 could be involved in the regulation of inflammatory responses (Ricote et al., 1998). Prostaglandin D_2 synthase, which is required for $15dPGJ_2$ synthesis, is expressed in macrophages and specialised antigen-presenting cells (Urade et al., 1989). Whether this enzyme is also expressed in eosinophils but not in neutrophils is currently unknown.

PPAR-γ is markedly upregulated in activated macrophages and inhibits the expression of inducible nitric oxide synthase in response to 15d-PGJ_2 (Ricote et al., 1998).

In RAW 264.7 cells, which contain little PPAR-γ, PGD_2 only affected these responses in the presence of co-transfected PPAR-γ (Ricote et al., 1998). Since PGD_2 does not bind to PPAR-γ (Forman et al., 1995, Kliewer et al., 1995), this indicates metabolic conversion of PGD_2 to an activating ligand by this macrophage cell line. A similar mechanism may be involved in the pro-apoptotic responses of eosinophils to PGD_2, which raises the exciting possibility that PPAR-γ may regulate inflammatory responses as a result of local production of $15dPGJ_2$ from eosinophils and macrophages.

The antidiabetic group of thiazolidinedoine compounds are known ligands of PPARγ (Lehmann et al., 1995). These drugs could therefore be used to try to mimic the effects of PPAR-γ activation in granulocytes; results would indicate whether this is the mechanism involved in inducing apoptosis in granulocytes via $15dPGJ_2$ or Δ^{12}-PGJ_2.

Another hypothesis which should be considered is that these PGD_2 metabolites may mediate apoptosis by inducing synthesis of proteins which actively drive programmed cell death in granulocytes. If this type of mechanism is involved, a protein synthesis inhibitor such as cycloheximide should block the induction of apoptosis, therefore this hypothesis could be readily investigated.

LTB_4 has also been demonstrated to bind and activate the intranuclear transcription factor PPARα, resulting in the activation of genes that terminate inflammatory processes in macrophages (Devchand et al., 1996). Also of interest is the finding that DHEA-S, an endogenous steroid, mediates gene induction through PPAR-γ (Peters et al, 1996). This allows the possibility that steroids may also utilise these receptors to influence gene transcription.

Many of the inflammatory mediators examined in this work are known to affect intracellular concentrations of Ca^{2+} or cAMP or both. As shown in Figure 3.20, increases in intracellular levels of cAMP inhibited apoptosis in both neutrophils and eosinophils. DbcAMP is a nonhydrolyzable, cell-permeable cAMP analogue (Cronstein et al., 1988) that has been shown to inhibit neutrophil apoptosis (Rossi et al., 1995, Yasui et al., 1997). This suggests that in granulocytes, PGD_2 and its metabolites do not induce apoptosis through increasing intracellular cAMP or adenylate cyclase activity.

However, increases in $[Ca^{2+}]_i$ cause inhibition of neutrophil apoptosis but dramatically increase eosinophil apoptosis (Cousin et al., 1997, Whyte et al., 1993b). This could be due to differential effects on eicosanoid synthesis. After stimulation with A23187, a Ca^{2+} ionophore, the major AA metabolites formed in eosinophils are thromboxane B_2 and LTC_4 with LTB_4 and 5-HETE, the precursor to 5-oxo-ETE, formed in neutrophils (O'Flaherty & Rossi, 1993, Parsons & Roberts, 1988, Sehmi et al., 1992). As already discussed, PGD_2 can bind to the thromboxane receptor and this should be examined as a possible mechanism inducing apoptosis in the eosinophil. Also of note is the fact that increases in $[Ca^{2+}]_i$ cause degranulation in both cell types (O'Flaherty et al., 1991, O'Flaherty & Rossi, 1993, and personal observation). Therefore the differences in responses observed in these cells may be due to the effects of the granule contents of each particular cell type. This possibility also merits investigation.

In signalling responses, intracellular Ca^{2+} and cAMP levels will normally fluctuate transiently, but the use of Ca^{2+} ionophores and stable cAMP analogues may cause larger, more persistent increases, that do not allow natural homeostatic responses to occur, and therefore do not reflect physiological processes. Changing concentrations of $[Ca^{2+}]_i$, have been shown to cause differential effects in specific cells. For example, in B lymphocytes, amplitude and duration of Ca^{2+} signals controls differential activation of the transcription factors NF-κB, JNK and NFAT. NF-κB and JNK are selectively activated by a large transient $[Ca^{2+}]_i$ rise, whereas NFAT is

activated by a low, sustained $[Ca^{2+}]_i$ plateau (Dolmetsch et al., 1997). Therefore, the cellular responses to increased $[Ca^{2+}]_i$ may be more complicated than many results suggest. $[Ca^{2+}]_i$ measurements should be made in eosinophils treated with PGD_2 and its metabolites. However, because increased $[Ca^{2+}]_i$, inhibits neutrophil apoptosis, it is unlikely that Δ^{12}-PGJ_2 and $15dPGJ_2$ are inducing apoptosis through this mechanism.

CHAPTER 4

THE EFFECT OF PRO- AND ANTI-INFLAMMATORY CYTOKINES ON GRANULOCYTE APOPTOSIS

4.1 Introduction

The initation, amplification and resolution of an immune response are regulated in part through the release of cytokines. This chapter investigates the effects of pro-inflammatory (GM-CSF and TNF-α) and anti-inflammatory cytokines (IL-10 and TGF-β) (as defined in Chapter 1, Sections 1.10.1 and 1.10.4 respectively), granulocyte apoptosis *in vitro*.

GM-CSF interacts with a specific receptor that is present on both neutrophils and eosinophils (Park et al., 1986, Dipersio et al., 1988). In neutrophils, GM-CSF causes the release of AA from membrane phospholipids (Sullivan et al., 1987), and increases fMLP receptor expression (Weisbart et al., 1986, Atkinson et al., 1988). GM-CSF can prime neutrophils, causing the cell to become hyperresponsive to a second stimulus such as fMLP or LTB_4 that induces the oxidative burst (Wiesbart et al., 1985, 1987), membrane depolarisation (Fletcher & Gasson, 1988) and phagocytosis (Fleischmann et al., 1988). It also increases tyrosine phosphorylation, activates the Na^+/H^+ antiporter, increases adhesion and guanylate cyclase activity, and reduces adenylate cyclase activity (Begley et al., 1986, Lopez et al., 1986, Weisbart et al., 1986, Coffey et al., 1988, Gomez-Cambronero et al., 1989, Monroy et al, 1990). GM-CSF also activates and primes eosinophils (Monroy et al., 1990). GM-CSF is released by both neutrophils and eosinophils and inhibits constitutive apoptosis of both cell types (Begley et al., 1986, Vancheri et al., 1989, Kita et al., 1991, Moqbel et al., 1991, Colotta et al., 1992, Lee et al., 1993, Hallsworth et al., 1996).

TNF-α exerts multiple effects on different cell types at inflamed tissue sites (Dinarello, 1992). For example, it acts on stromal cells, including fibroblasts and

endothelial cells, causing the release of a secondary wave of cytokines such as IL-8, a neutrophil chemoattractant (Matsushima & Oppenheim, 1989). TNF-α also produces extravascular accumulation of granulocytes through its action on vascular endothelium (Gamble et al., 1985, Cavender et al., 1987, Sayers et al., 1988, Furie & McHugh, 1989, Godding et al., 1995). TNF-α produced by macrophages (Warren et al., 1989) upregulates vascular adhesion molecules, including ICAM-1 and E-selectin (Tosi et al., 1992, Bevilacqa, 1993). This leads to adhesion and neutrophil interaction with endothelial E-selectin and ICAM-1 (Mulligan et al., 1991, 1995), allowing neutrophil extravasion. Eosinophils adhere to vascular endothelium through interactions between CD11a/CD18 and ICAM-1 and between VLA-4 and VCAM-1 (Bochner et al., 1991, Weller et al., 1991). TNF-α increases the adhesion of activated eosinophils to respiratory epithelial cell cultures *in vitro* (Godding et al., 1995) and induces eosinophil accumulation in a rat skin model; this effect is inhibited by a soluble TNFR1 IgG fusion protein that affects α_4 integrin/VCAM-1 adhesion pathways (Hartnell et al., 1997). TNF-α also enhances the cytotoxic capability of eosinophils, which may damage the endothelium (Slungaard et al., 1990), and increase airway hyperreponsiveness in man (Kips et al., 1992).

After entering the tissue, granulocytes themselves become important sources of chemotactic factors (Xing et al., 1993, 1994). For example, activated neutrophils synthesise and secrete IL-8 (Bazzoni et al., 1991a), TNF-α (Bazzoni et al., 1991b, Costa et al., 1993), IL-1β (Cassatella et al., 1993) and MIP-1α (Kasama et al., 1993). Such factors act not only as chemotaxins but as activating stimuli for PMN, T cells, basophils, eosinophils and monocytes (Baggiolini et al., 1989, Larsen et al., 1989, Alam et al., 1992, Rot et al., 1992). The release of these chemokines may be responsible for the influx of other inflammatory cells into the area. Blocking TNF-α activity *in vivo* using a TNF receptor fusion protein inhibits the influx of granulocytes into BAL fluid and skin sites in animal models of lung and dermal inflammation respectively (Gater et al., 1996, Norman et al., 1996, Renzetti et al., 1996); TNF-α expression also correlates closely with mononuclear cell infiltration in such models (Williams et al., 1997).

TNF-α primes for neutrophil phagocytosis (Shalaby et al., 1985, Klebanoff et al., 1986, Seow et al., 1987), antibody-dependent cellular cytotoxicity (Shalaby et al., 1985, Klebanoff et al., 1986, Perussia et al., 1987), intracellular microbicidal activity (Ferrante, 1989), release of oxidants (Berkow et al., 1987), degranulation (Atkinson et al., 1988, Renesto & Chignard, 1991), and responses to secondary stimuli such as fMLP (Berkow et al., 1987). TNF-α also primes and activates eosinophils (Silberstein & David, 1987). Although TNF-α is a powerful priming agonist, it has been shown to induce apoptosis in cells such as T lymphocytes (Zheng et al., 1995), HL-60 cells (Obeid et al., 1993) and neutrophils (Murray et al., 1997). However, some data indicate that TNF-α may also delay apoptosis, for example, in human monocytes, neutrophils, and B104 lymphoma cells (Mangan et al., 1991, Colotta et al., 1992, Genestier et al., 1995, Murray et al., 1997).

IL-10 is synthesised by T cells, monocytes, macrophages, bronchial epithelial cells and activated B cells and is considered anti-inflammatory because it inhibits synthesis of proinflammatory cytokines (de Waal Malefyt et al., 1991, Vieira et al., 1991, Moore et al., 1993, Mosmann, 1994, Bonfield et al., 1995). For example, in monocytes stimulated with LPS, or LPS plus INF-γ, IL-10 reduces synthesis of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF (de Waal Malefyt et al., 1991, Oswald et al, 1992). At the same time, IL-10 enhances production of the IL-1 receptor antagonist (IL-1ra) that has anti-inflammatory properties (Hammum et al., 1990). IL-10 also has suppressive effects on T cell proliferation, macrophage microbicidal activity (nitric oxide-dependent), and production of prostaglandins by macrophages (Bogdan et al., 1991, Ding & Shevack, 1992, Oswald et al., 1992, Taga et al., 1992, Gazzinelli et al, 1992, Mertz et al., 1994, Niiro et al., 1994). IL-10 does not suppress all cytokine release, since it has no effect on the constitutive expression of TGF-β (de Waal Malefyt et al., 1991); therefore its effects are selective.

IL-10 inhibits the expression of cytokines and chemokines from LPS-stimulated neutrophils by shortening the half-live of mRNA for these factors; but it has no influence on O_2^- production in response to fMLP or TNF-α (Cassatella et al., 1994, 1994, Kasama et al., 1994). However, it inhibits the oxidative burst induced by INF-

γ, opsonised zymosan and phorbol esters (Chaves et al., 1996, Capsoni et al., 1997). Eosinophils contribute to the inflammatory process by the release of lipid mediators such as LTB_4, PAF and prostaglandins, and cytokines such as TNF-α, MIP-1α, IL-1, IL-5, IL-8 and GM-CSF (Broide et al., 1992, Braun et al., 1993, Costa et al., 1993). IL-10 inhibits the synthesis of these factors and inhibits LPS-induced eosinophil survival (Takanaski et al., 1994). Human eosinophils express CD40, a member of the TNF receptor superfamily, ligation of which enhances their survival via the autocrine release of GM-CSF. IL-10 decreases CD40 mRNA and suppresses the production of GM-CSF, and might therefore inhibit this enhanced survival (Ohkawara et al., 1996). Cycloheximide abrogates the inhibitory effects of IL-10, suggesting that these effects are mediated through a synthesised repressor protein (Kasama et al., 1994).

An anti-inflammatory role for IL-10 is supported by data from IL-10 gene knockout mice. These mice die prematurely, and suffer from chronic enterocolitis thought to be caused by uncontrolled immune responses to enteric antigens, with chronically activated macrophages causing amplification of the inflammatory process (Khun et al., 1993). Mucosal lesions in the intestines contain abundant inflammatory cells including neutrophils and eosinophils, with high levels of pro-inflammatory cytokines such as TNF-α. TNF neutralising antibodies do not improve the condition, which is however reversed by IL-10 administration. These mice have prolonged immune reactions in the tissues when stimulated with antigen, resulting in excessive inflammation and tissue damage (Rennick et al., 1995). Neutralising anti-TNF-α antibodies prevent this tissue damage, suggesting that one function of IL-10 is to control the local production of pro-inflammatory cytokines such as TNF-α.

Other *in vivo* studies using rodent models support the idea of IL-10 as an anti-inflammatory cytokine. Systemic administration of IL-10 suppresses delayed-type hypersensitivity reactions and T-cell-mediated inflammatory bowel disease (Powrie et al., 1993, Li et al., 1994). In a model of endotoxin shock, neutralisation of IL-10 enhances TNF synthesis and lethality and IgG immune-complex-induced lung injury (Gérard et al., 1993, Howard et al., 1993, Mulligan et al., 1993, Marchant et al.,

1994a, Kasama et al., 1995, Shanley et al., 1995). In this model there was increased lung permeability, increased numbers of neutrophils and higher myeloperoxidase levels in bronchoalveolar lavage. The effects of IL-10 neutralisation were attributed to increased TNF-α levels, resulting in enhanced expression of adhesion molecules promoting neutrophil recruitment and activation (Shanley et al., 1995). IL-10 administration was also of benefit in a collagen-induced model of polyarthritis (Joosten et al, 1997), chronic granulomatous inflammation (Herfarth et al., 1996), and acute pancreatitis (Rongione et al., 1997), and had a protective effect against lung reperfusion injury (Eppinger, 1996).

Animal models demonstrate a protective role for IL-10 in allergic inflammation. IL-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergilliosis (Grünig et al, 1997). Endogenous IL-10 is thought to act via several mechanisms to limit this response (Zuany-Amorim et al., 1995). These include inhibition of TNF-α production which promotes neutrophil and eosinophil accumulation in bronchoalveolar fluid; inhibition of IL-5 production (Schandené et al., 1994), which is a major mediator of eosinophil differentiation, activation and survival; and by direct inhibition of eosinophil effector functions (Takanaski et al., 1994). Although IL-10 production is upregulated by TNF-α, several anti-inflammatory drugs that inhibit TNF-α release increase IL-10 synthesis. For example, PGE_2 and other agents elevating intracellular cAMP suppress TNF-α production and upregulate IL-10 production in monocytes/macrophages (Strassmann et al., 1994, Platzer et al., 1995). Neutrophils constitutively produce small amounts of PGE_2 in culture supernatants, but this is not affected by IL-10 (Niiro et al., 1997). LPS stimulation causes a large increase in PGE_2 levels through COX-2, which IL-10 does inhibit in a concentration-dependent fashion (Niiro et al., 1997).

An anti-inflammatory role for IL-10 is also supported by human studies. In patients with rheumatoid arthritis, neutralisation of IL-10 upregulates TNF-α, while TNF-α increases the production of IL-10 *in vitro* in human monocytes (Wanidworanun &

Strober, 1993, Platzer et al., 1995). These data suggest a negative feedback inhibition of TNF-α production by IL-10 (Katiskis et al., 1994).

TGF-β is a pleiotropic cytokine produced by platelets and inflammatory cells such as macrophages, T cells, eosinophils and neutrophils (Roberts et al., 1985, Grotendorst et al., 1989, Ohno et al., 1992). It acts to limit the growth and activity of inflammatory cells (Wahl, et al., 1994). TGF-β has inhibitory activities on T cells and monocytes and, like IL-10, TGF-β deactivates macrophages, inhibiting production of reactive oxygen species, and cytokines such as TNF-α, (Espevik et al, 1987, Tsunawaki et al., 1988, Massagué, 1990, Nelson et al., 1991, Sporn & Roberts, 1992, Vodovotz et al., 1993); but it enhances IL-10 production in these cells (Maeda et al., 1995).

Both the initiation and resolution of general inflammatory responses seem to involve TGF-β1. TGF-β stimulates leukocyte migration (Wahl, 1992), but it has potent anti-inflammatory effects including inhibition of neutrophil and T-lymphocyte adhesion to endothelium (Gamble & Vadas, 1988, Gamble et al., 1993, Wahl et al., 1993), antagonism of TNF-α function (Espevik et al., 1987, Ranges et al., 1987), inhibition of proliferation of all T-cells subsets, inhibitory effects on proliferation and function of B lymphocytes, down-regulation of natural killer cell activity, and regulation of cytokine production by immune cells (Goey et al., 1989, Gresham et al., 1991, Keller et al., 1991, Palladino et al., 1991, Wahl, 1992). It also affects neutrophil migration by inhibiting the production of IL-8 (Smith et al., 1996). TGF-β does not cause exocytosis, O_2^- production or priming of neutrophils (Brandes et al., 1991, Reibman et al., 1991), but it activates neutrophils adherent to fibrinogen to release H_2O_2 and lactoferrin (Balazovich et al., 1996). TGF-β inhibits activation and survival of eosinophils induced by IL-3, IL-5 and GM-CSF (Alam et al., 1994a).

TGF-β is important for wound healing (Sporn et al., 1983, Roberts et al., 1986). This process, which involves both fibrogenisis and angiogenesis is characterised by an ordered progression of inflammatory cells, beginning with platelets, followed by

neutrophils, macrophages, and then lymphocytes (Wahl, 1987). At femtomolar concentrations, TGF-β is a potent chemoattractant for human blood monocytes and neutrophils *in vivo* and *in vitro* (Mustoe et al., 1987, Wahl et al., 1987, Wiseman et al., 1988, Allen et al., 1990, Reibman et al., 1991). TGF-β bypasses classic signalling mechanisms for chemotaxis (GTPase activity, calcium, and actin polymerisation) and triggers protein synthesis (Reibman et al., 1991). It increases collagen formation and angiogenesis (Roberts et al., 1986, Postlethwaite et al., 1987, Sprugel et al., 1987). In wound healing models, TGF-β increases wound strength (Mustoe et al., 1987) and accelerates wound healing (Mustoe et al., 1990). Oestrogen accelerates cutaneous wound healing by increasing TGF-β1 levels (Ashcroft et al., 1997).

In vivo, TGF-β applied or released locally promotes leukocyte adhesion, infiltration and activation by regulating the synthesis and expression of receptors for integrins such as fibronectin and laminin (Wahl et al., 1993). However, TGF-β administered systemically has the opposite effect, inhibiting the expression of endothelial cell adhesion proteins and decreasing leukocyte recruitment to sites of inflammation (Gamble et al., 1993). *In vitro*, TGF-β promotes neutrophil chemotaxis but blocks neutrophil adhesion to fibrinogen in response to phorbol ester (Gresham et al., 1994). Thus, TGF-β may selectively promote or inhibit PMN functional responses, depending upon specific environmental conditions.

TGF-β enhances macrophage phagocytosis of apoptotic neutrophils (Ren & Savill, 1995). Phosphatidylserine (PS) recognition by macrophages is involved in the removal of apoptotic cells that express PS on their cell membranes. TGF-β is involved in β-glucan induction of PS recognition by macrophages (Rose et al., 1995). Phagocytosis of apoptotic neutrophils inhibits the production of IL-1β, IL-8, IL-10, GM-CSF, TNF-α, LTC_4 and TXB_2 by human monocytic-derived macrophages, while TGF-β1, PGE_2 and PAF are increased (Fadok et al., 1998). Culturing with TGF-β1, PGE_2 or PAF in this system inhibits LPS-stimulated cytokine production

(Fadok et al., 1998). This again suggests a potential role for TGF-β in inflammatory resolution.

Bacterial infection, or direct administration of LPS or TNF-α, can induce isoforms of nitric oxide synthase (iNOS, NOS2), which produce large amounts of NO over a period of hours in animal models (Pittet et al., 1995). This mimics the situation in patients with sepsis (Rixen et al., 1997). TGF-β suppresses expression of NOS2 by regulating the rate of gene transcription, mRNA stability, translation and protein stability (Vodovotz et al., 1993, Perella et al., 1994, Finder et al., 1995). Lack of TGF-β1 in TGF-β null mice is associated with aberrant expression of NOS2 (Vodovotz et al., 1996).

TGF-β1 knock out mice suffer from excessive, multifocal inflammatory responses, with massive infiltration of leukocytes to different organs, beginning during the first week of life and causing severe wasting and death by week four (Schull et al., 1992, Kulkarni et al., 1993). These animals exhibit inflammatory cell infiltration and tissue necrosis in organs such as liver, heart and stomach (Schull et al., 1992). The stomach infiltration consists mainly of neutrophils and some eosinophils, while in liver, granulocytes and lymphocytes are involved (Schull et al., 1992). Elevation of blood neutrophil and monocyte counts are observed (Schull et al., 1992). Thus, TGF-β1 deficiency results in severe pathology and death associated with dysfunction of the immune and inflammatory systems (Schull, et al., 1992).

The phenotype of this mouse suggests that inflammation can be initiated without TGF-β, but the ability to control and promote the resolution of inflammation is lost. This might be the most critical function of this cytokine (Letterio & Roberts, 1996). Chronic systemic exposure to inflammatory cytokines including INF-γ, IL-1, and TNF-α can lead to a progressive wasting syndrome and associated tissue inflammation similar to the phenotype of the TGF-β1 knock-out mouse (Tracey et al., 1988). However, the profound dysregulation of immune cells present in TGF-β1 null mice is not reversed by systemically injected TGF-β1, suggesting that these cells

rely solely on autocrine or possibly even intracrine TGF-β1 (Letterio & Roberts, 1996). This is supported by bone marrow transplantation studies which show the same pathology as the TGF-β1 knock-out mouse, even in the presence of endogenous TGF-β1 produced by other cells in the recipient (Yaswen et al., 1996).

In some epithelial and hematopoietic cells, TGF-β1 has been reported to induce programmed cell death (Rotello et al., 1991, Lotem & Sachs, 1992, Oberhammer et al., 1992, Lomo et al., 1995). It acts synergistically with Ca^{2+} to trigger T and B lymphocyte apoptosis (Andjelic et al., 1997), and has been shown to mediate osteoclast apoptosis induced by oestrogen (Hughes et al., 1996). However, TGF-β has also been shown to inhibit apoptosis in normal human keratinocytes grown in suspension (Sachsenmeier et al., 1996).

The aim of the work presented in this chapter was to examine the capacities of the anti-inflammatory cytokines IL-10 and TGF-β to upregulate granulocyte apoptosis. Both IL-10 and TGF-β1 null mice can mount inflammatory responses, but appear to be unable to resolve the process. Therefore, if these cytokines potentiate granulocyte apoptosis, then given that ingestion of apoptotic cells will cause the release of mediators that downregulate pro-inflammatory cytokines (Fadok et al., 1998), this could be relevant to the mechanism of inflammatory resolution.

4.1 Results

4.2.1 The Effects of GM-CSF and TNF-α on Granulocyte Apoptosis

Figure 4.1 A and B demonstrates that GM-CSF significantly decreased apoptosis in both neutrophils and eosinophils at all timepoints examined. In neutrophils, apoptosis fell from 73.8 ± 2.2 % to 40.9 ± 3.5 % with GM-CSF treatment at 20 h, while control apoptosis in eosinophils fell from 32.0 ± 8.4 % to 21.3 ± 4.6 % at 40h.

As shown in Figure 4.2 B, TNF-α had no significant effect on eosinophil apoptosis at either 20 or 40 h. However, this cytokine significantly increased apoptosis in neutrophils at 6 h (control 3.4 ± 1.3 %, TNF-α, 12.0 ± 2.5 %), but it significantly inhibited apoptosis at 20 h (control 56.1 ± 3.7 %, TNF-α, 47.4 ± 3.3 %) (Figure 4.2 A).

4.2.2 The Effect of IL-10 on Granulocyte Apoptosis

IL-10 had no significant effect on the constitutive rate of neutrophil or eosinophil apoptosis over the range of timepoints shown in Figure 4.3 A and B. To ensure that the IL-10 used in the foregoing and following studies was biologically active, monocytes and monocyte-derived macrophages were incubated with IL-10 after LPS stimulation. Macrophages and monocytes activated by LPS are potent producers of TNF-α (Männel, et al., 1980, Matthews, 1981, Mosmann, 1994). IL-10 has been shown to attenuate such production (Bogdan et al., 1991). As illustrated in Figure 4.4 A and B, the IL-10 used in these experiments significantly inhibited the release of TNF-α from both monocytes and monocyte-derived macrophages. In monocytes treated with LPS, TNF-α production was decreased from 555.1 ± 27.1 pg/ml to 127.3 ± 5.9 pg/ml in cells treated with 100 ng/ml IL-10, while in activated macrophages, it fell from 210.2 ± 4.8 pg/ml to 31.0 ± 2.1 pg/ml. This demonstrates that although IL-10 had no effect on granulocyte apoptosis, this was not due to loss of biological activity.

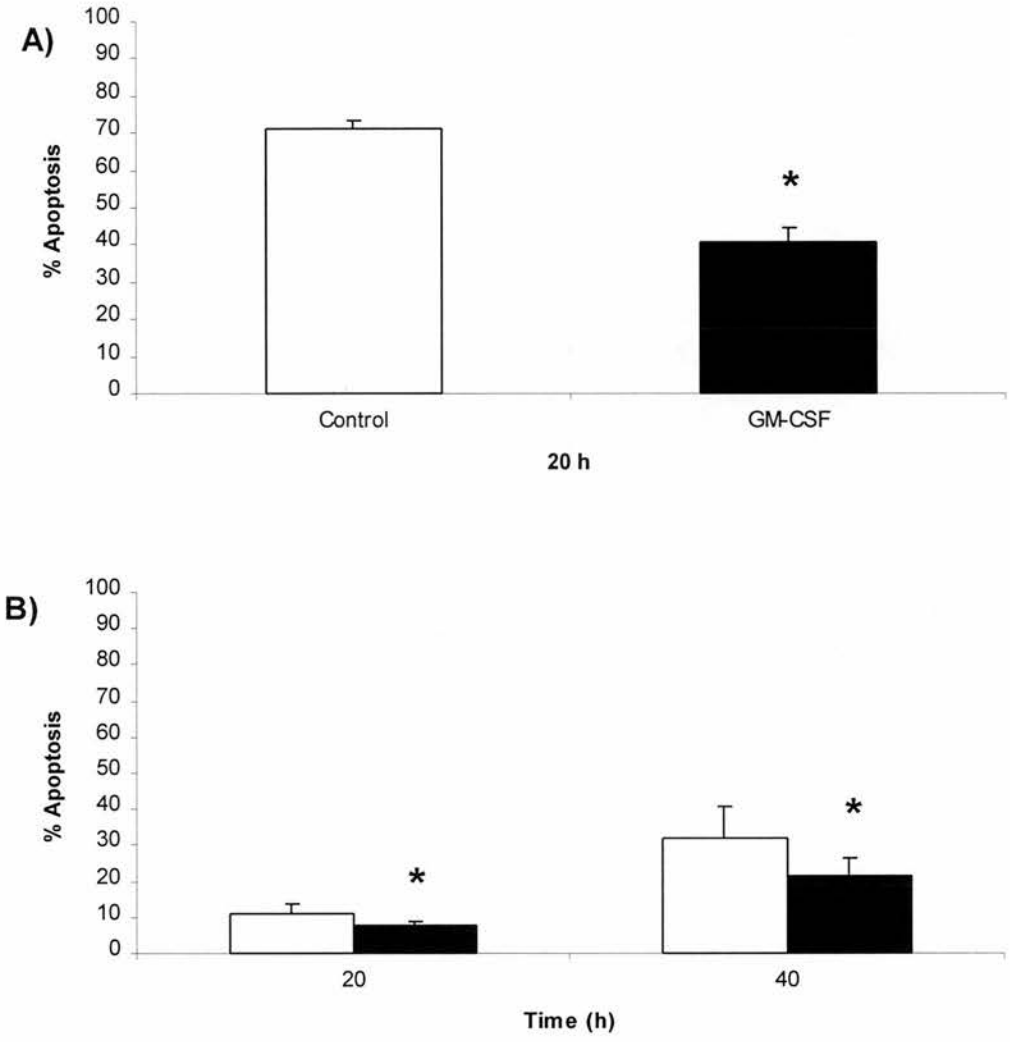


Figure 4.1 The Effect of GM-CSF on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with GM-CSF (50 U/ml) and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils and 5 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

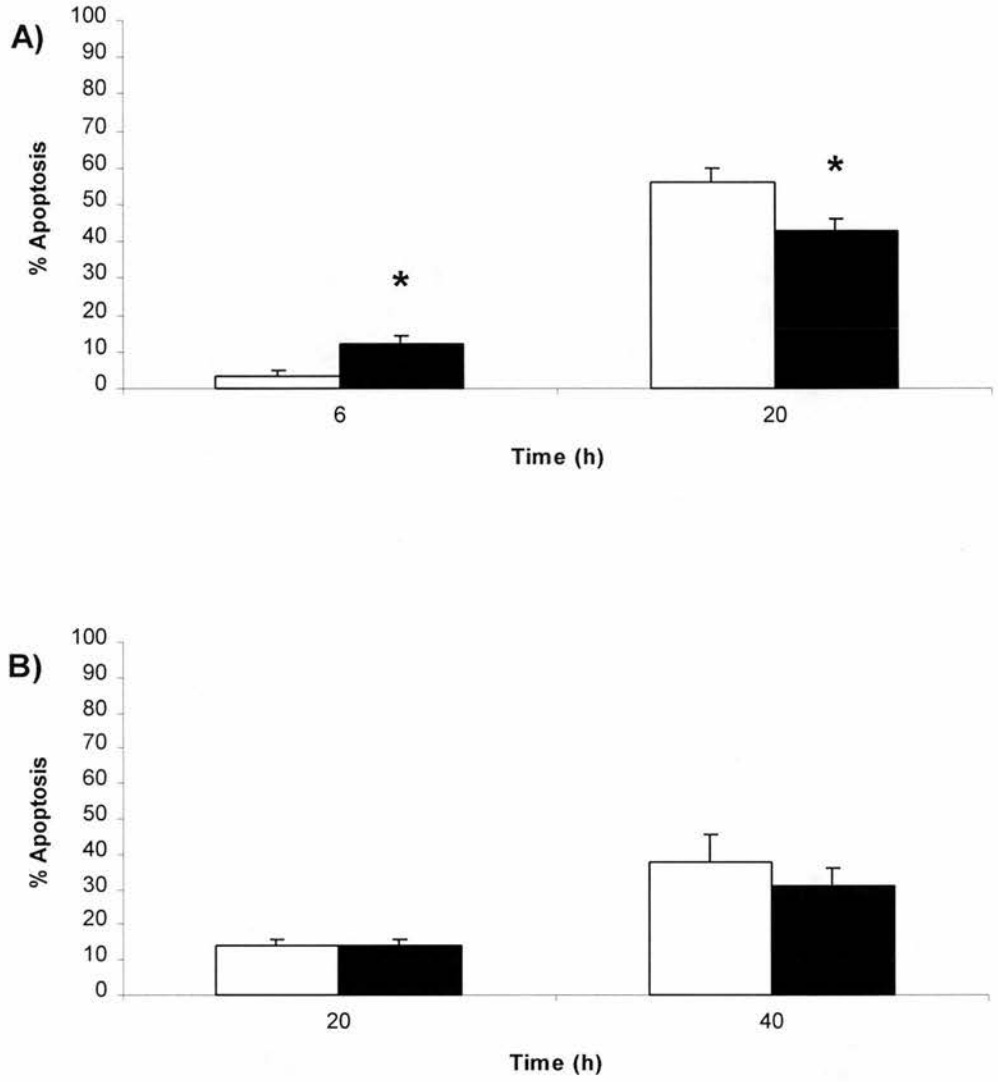


Figure 4.2 The Effect of TNF-α on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with TNF-α (12.5 ng/ml) and harvested at 6 and 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent treated cells. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 7 separate experiments for neutrophils and 8 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

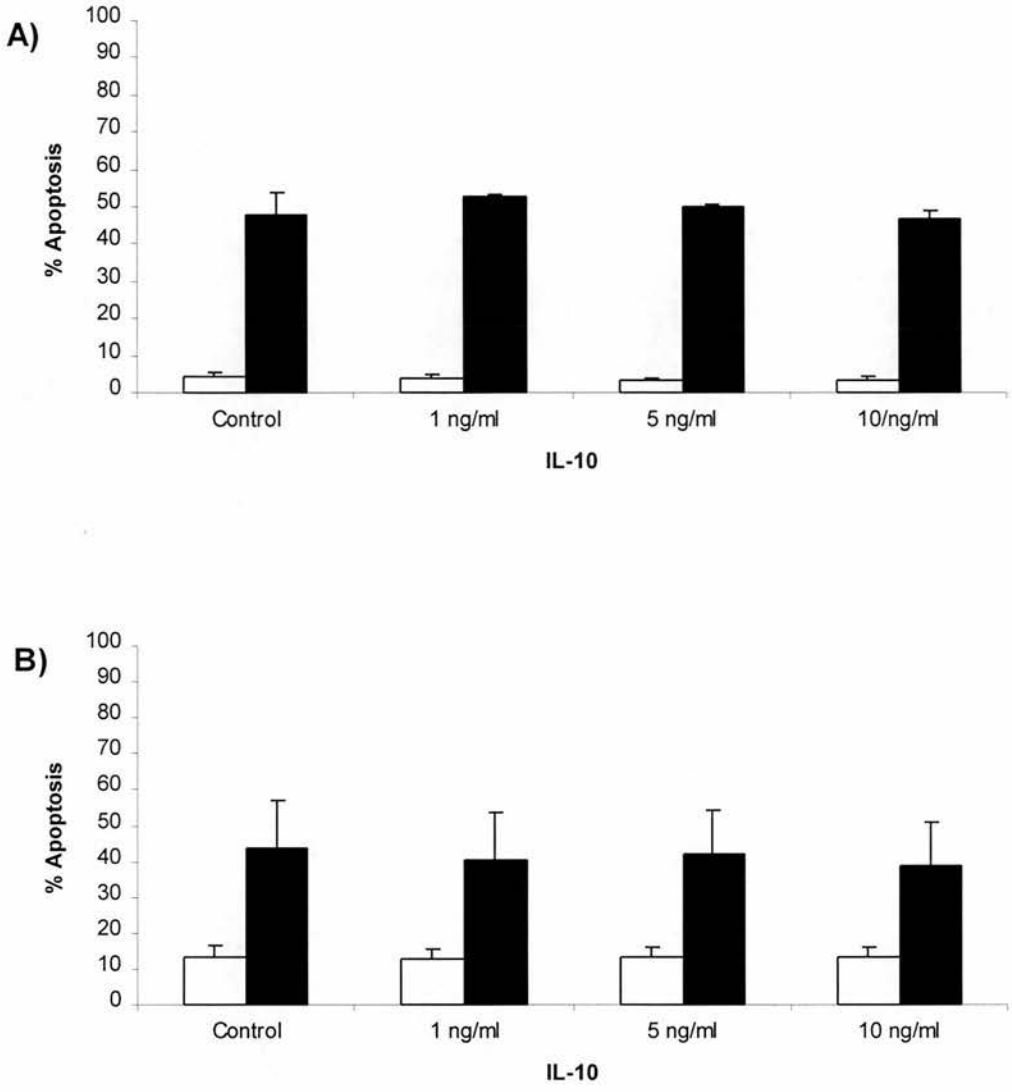


Figure 4.3 The Effect of IL-10 on Constitutive Granulocyte Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with IL-10 (10, 5 or 1 ng/ml) and harvested at 6 and 20 h (neutrophils) and 20 and 40 h (eosinophils). In A, filled bars represent 20 h, in B filled bars represent 40 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils and 4 separate experiments for eosinophils. All experiments were performed in triplicate.

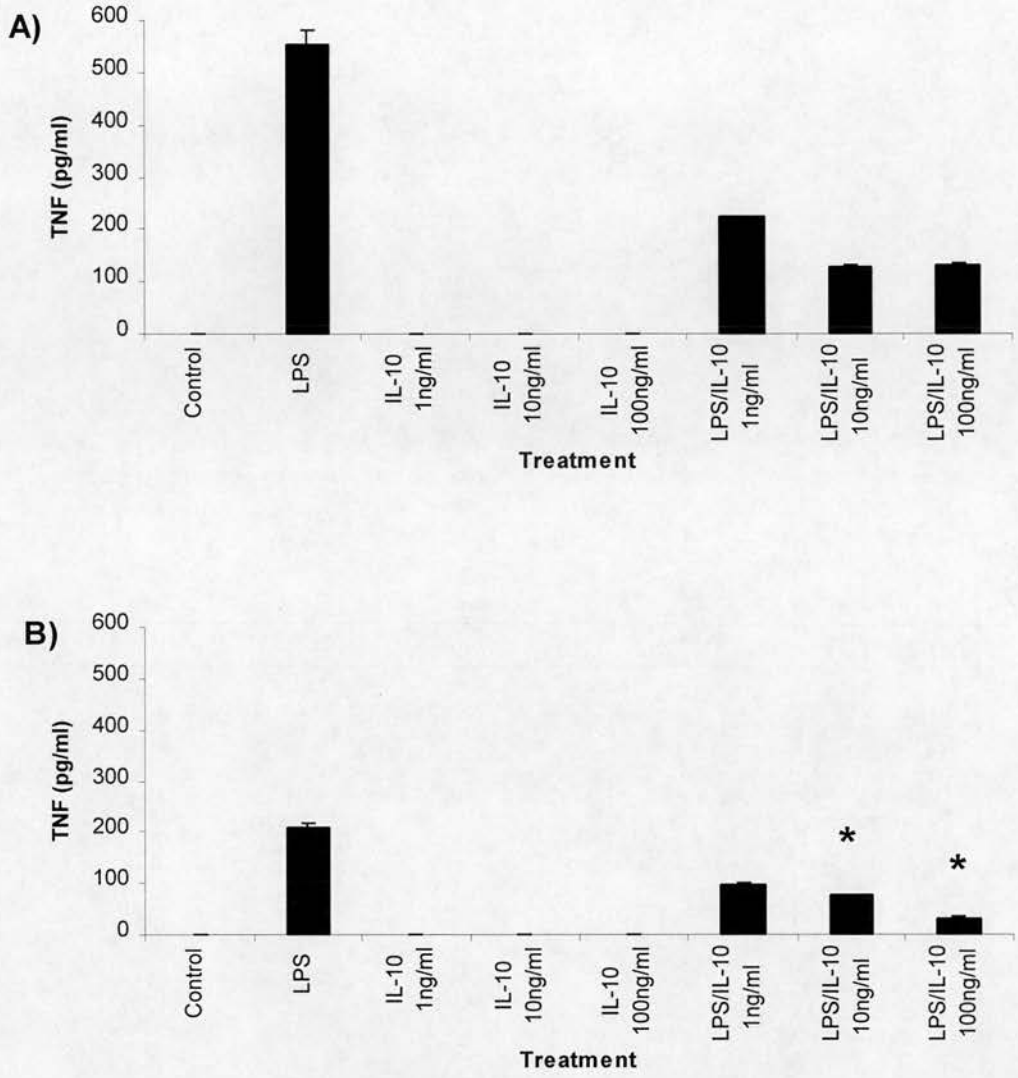


Figure 4.4 The Effect of IL-10 on TNF-α Production by Monocytes and Macrophages

A) Monocytes and B) macrophages (1 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or LPS (1μg/ml) ± IL-10 (100, 10 or 1 ng/ml) for 4 h and supernatants were collected for ELISA determination of TNF-α production (pg/ml). Sensitivity limit was 5pg/ml TNF-α. Data represent the mean ± SEM of 3 separate experiments, performed in duplicate (* $p < 0.05$ in comparison with LPS-stimulated TNF-α production). (Corroborative data provided by L. Bruce, University of Edinburgh).

4.2.3 The Effect of IL-10 on the Inhibition of Granulocyte Apoptosis Mediated by LPS, Dexamethasone, GM-CSF, TNF-α, dbcAMP, 5-oxo-ETE and ZK118.182

Because in an *in vivo* situation, cytokines and inflammatory factors have been shown to work in concert, we examined the effects of IL-10 on other mediators that affect the constitutive rate of apoptosis in neutrophils and/or eosinophils. As shown in Figure 4.5 A, IL-10 (10 ng/ml) significantly inhibited LPS-induced (1 μg/ml) neutrophil survival at 20 h (Control 58.5 ± 3.7 %, LPS 19.8 ± 2.5 %, LPS + IL-10 35.9 ± 4.2 %). This inhibition was however partial rather than total. In eosinophils, IL-10 had no significant effect on apoptosis, and as shown in Figure 3.4 B, LPS enhanced survival at the 40 h timepoint only.

Because IL-10 can inhibit LPS-induced neutrophil survival, we examined whether it had effects on other inhibitors of apoptosis. Dexamethasone inhibits apoptosis in neutrophils but increases eosinophil programmed cell death, as shown in Figure 3.3 A and B (Chapter 3). However, Figure 4.6 demonstrates that IL-10 had no effect on glucocorticoid-mediated granulocyte survival or apoptosis.

Similarly, as illustrated in Figure 4.7 A and B, IL-10 had no significant effect on either neutrophil (at 20 h, control, 58.5 ± 3.7 %, GM-CSF 34.17 ± 6.4 %, GM-CSF + IL-10 33.8 ± 2.8 %) or eosinophil survival induced by GM-CSF (at 40 h, control 18.87 ± 3.8 %, GM-CSF 11.9 ± 1.2 %, GM-CSF + IL-10 12.7 ± 1.2 %.

Figure 4.8 A demonstrates the effect of IL-10 on neutrophils cultured with TNF-α. Because TNF-α has differential effects on neutrophil apoptosis at early and late timepoints (Figure 4.2 A), the effect was examined at 6 and 20 h. As shown in Figure 4.8 A, IL-10 did not affect the pro-apoptotic response (6 h) or the inhibitory response (20 h) induced by TNF-α. In eosinophils (Figure 4.8 B) neither TNF-α nor IL-10 affected the constitutive rate of apoptosis, nor did co-culture with both cytokines.

The effect of IL-10 on dbcAMP-induced granulocyte survival is illustrated in Figure 4.9. IL-10 did not reverse dbcAMP inhibition of neutrophil apoptosis at the timepoint examined, (Figure 4.9 A), nor did it significantly inhibit dbcAMP-induced survival in eosinophils (Figure 4.9 B). Figures 4. 10 and 4.11 demonstrate the co-culture of neutrophils with IL-10 and 5-oxo-ETE and with IL-10 and ZK118.182 respectively. As shown in these figures, culture with 5-oxo-ETE alone or with both 5-oxo-ETE and IL-10 had no effect on the constitutive rate of neutrophil apoptosis; IL-10 also did not significantly alter the inhibition of neutrophil apoptosis induced by ZK118.182 alone.

IL-10 had no effect on constitutive granulocyte apoptosis. Although it partially reversed LPS-mediated neutrophil survival, it did not significantly effect the rates of apoptosis obtained using any of the other factors examined.

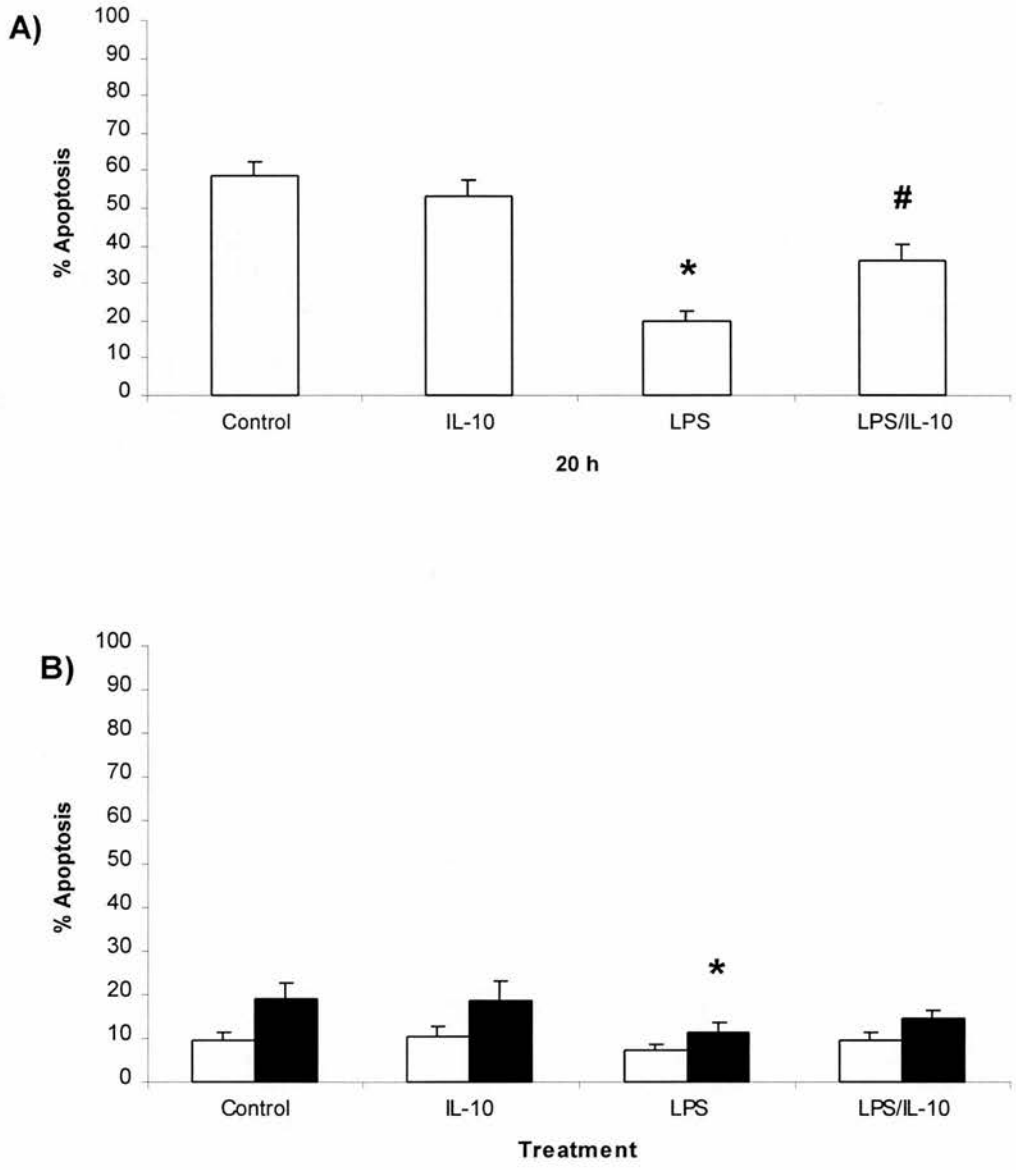


Figure 4.5 The Effect of IL-10 on LPS-induced Granulocyte Survival

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), IL-10 (10 ng/ml), LPS (1 μg/ml) or IL-10 + LPS, and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent 40 h timepoint for eosinophils. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils and 4 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with LPS values).

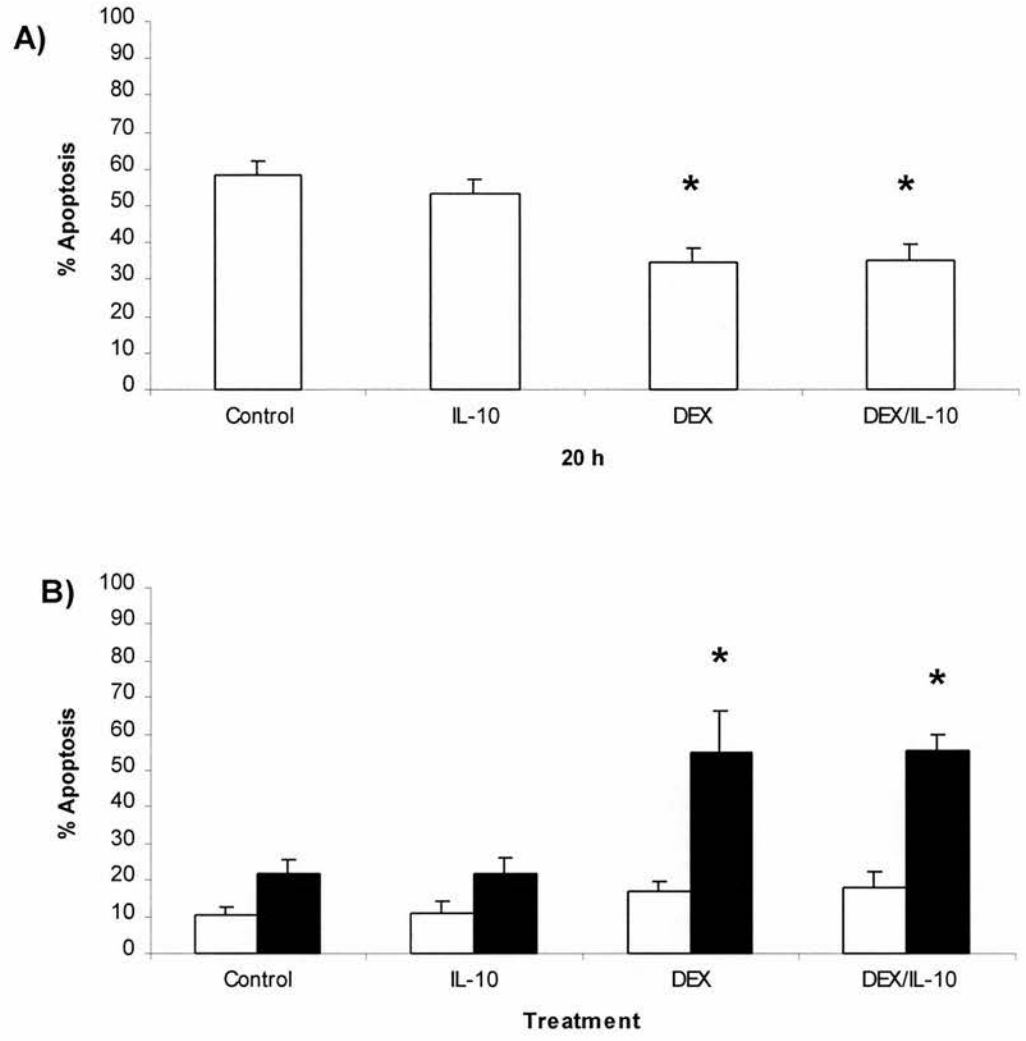


Figure 4.6 The Effect of IL-10 on Dexamethasone-induced Neutrophil Survival and Eosinophil Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), IL-10 (10 ng/ml), dexamethasone (1 μM) or IL-10 + dexamethasone, and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent 40 h timepoint in eosinophils. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

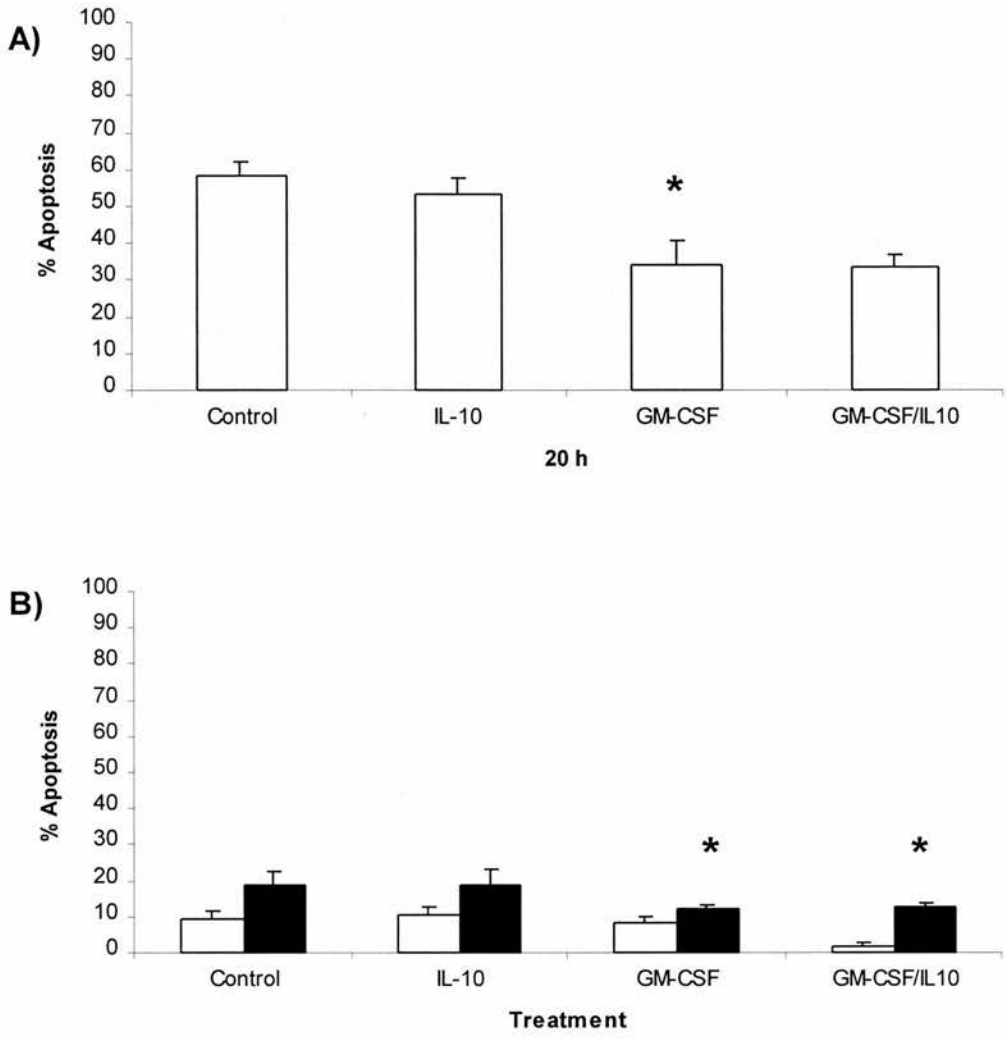


Figure 4.7 The Effect of IL-10 on GM-CSF-induced Granulocyte Survival

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), IL-10 (10 ng/ml), GM-CSF (50 U/ml) or IL-10 plus GM-CSF and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Filled bars represent 40 h of treatment in eosinophils. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

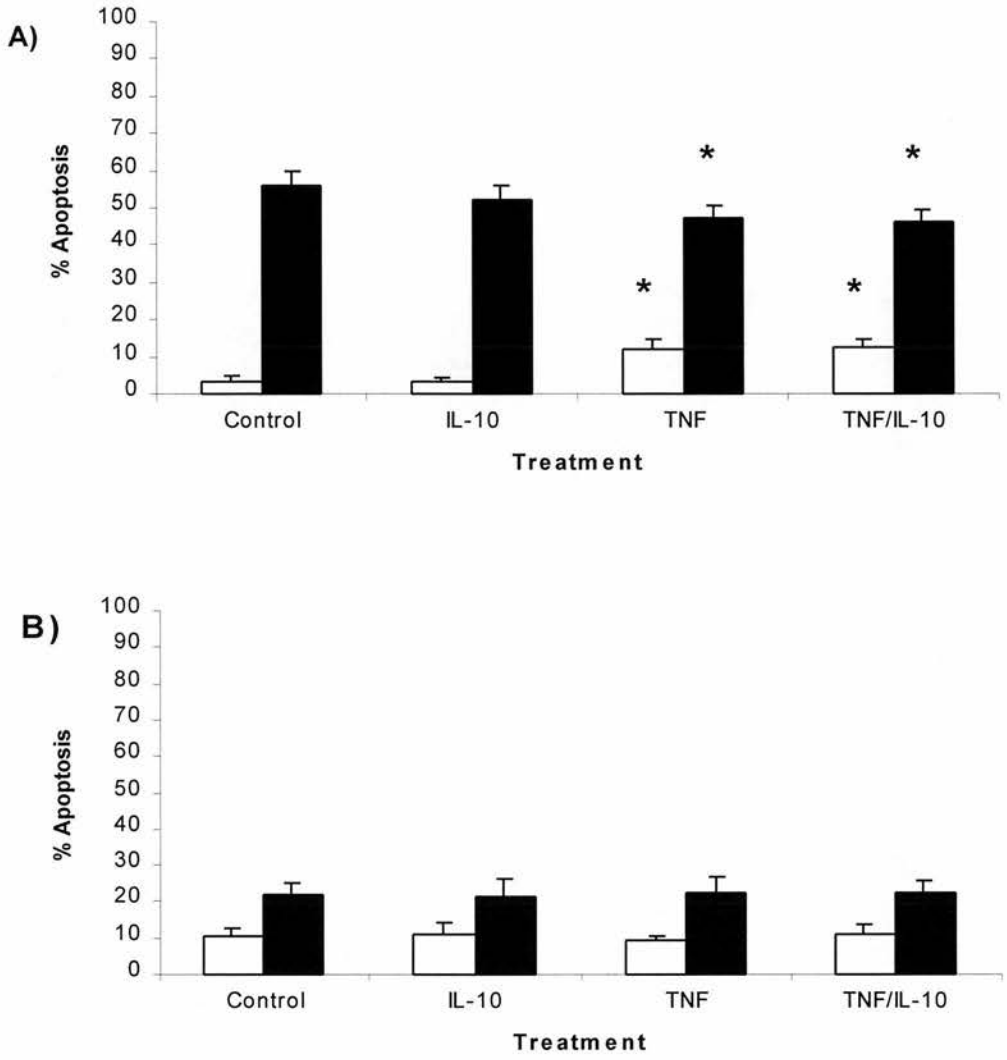


Figure 4.8 The Effect of IL-10 and TNF-α on Granulocyte Survival

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), IL-10 (10 ng/ml), TNF-α (12.5 ng/ml) or IL-10 + TNF-α, and harvested at 6 and 20 h (neutrophils) or 20 and 40 h (eosinophils). Filled bars represent 20 h timepoint (neutrophils) and 40 h (eosinophils). Apoptosis was assessed morphologically. Data represent the mean ± SEM of 7 separate experiments for neutrophils and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

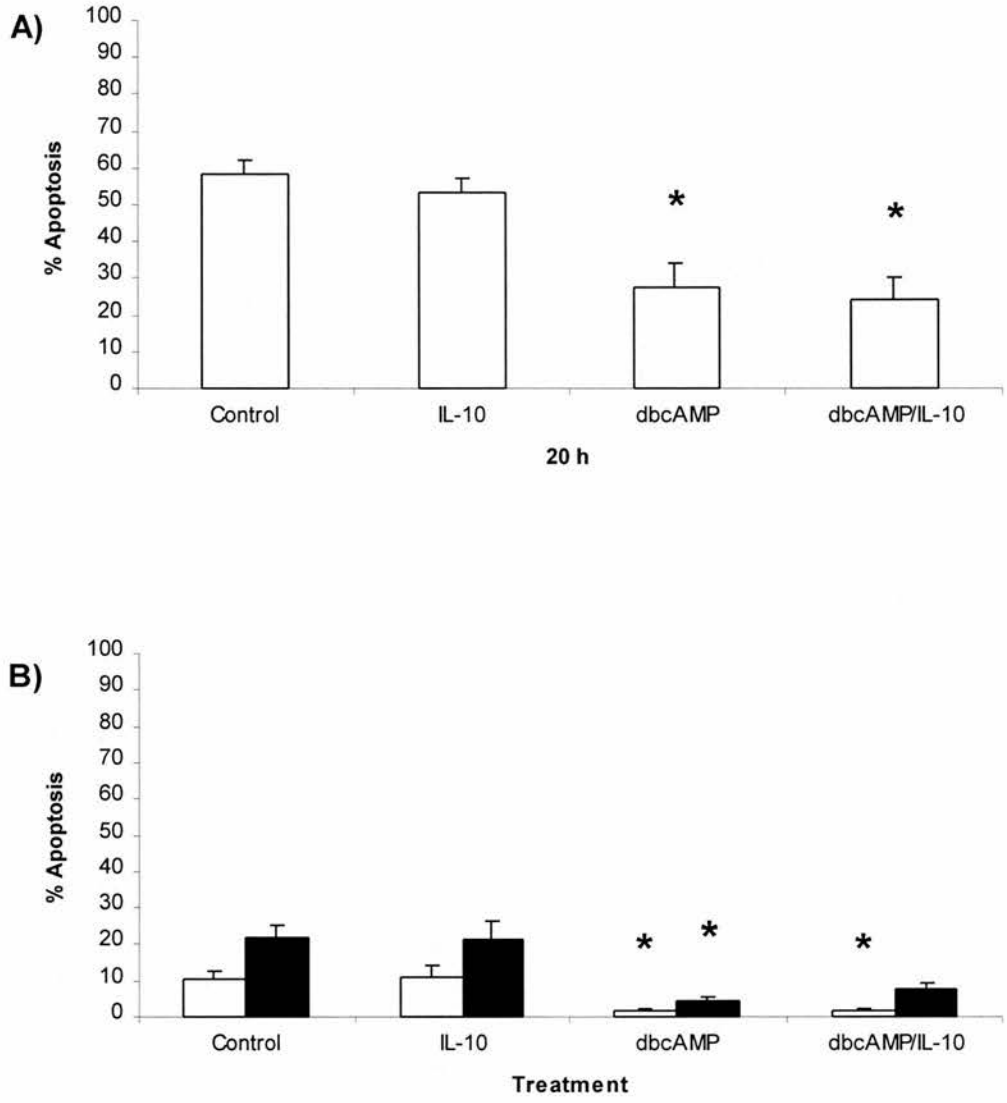


Figure 4.9 The Effect of IL-10 on dbcAMP-induced Granulocyte Survival

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), IL-10 (10 ng/ml), dbcAMP (0.2 mM) or IL-10 + dbcAMP at the same concentrations, and harvested at 20 h (neutrophils) or 20 and 40 h (eosinophils). Filled bars represent 40 h timepoint in eosinophils. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

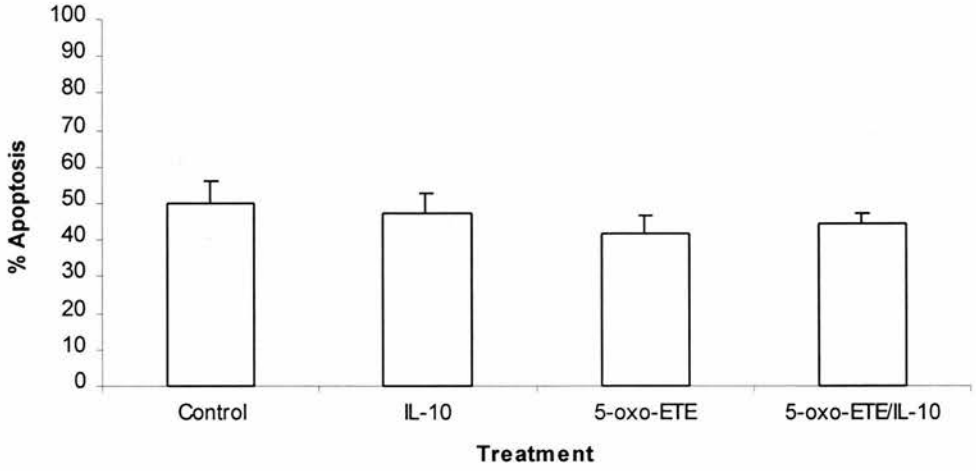


Figure 4.10 The Effect of IL-10 on 5-oxo-ETE-induced Neutrophil Survival

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), IL-10 (10 ng/ml), 5-oxo-ETE (300 nM) or IL-10 + 5-oxo-ETE at the same concentrations above and harvested 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate.

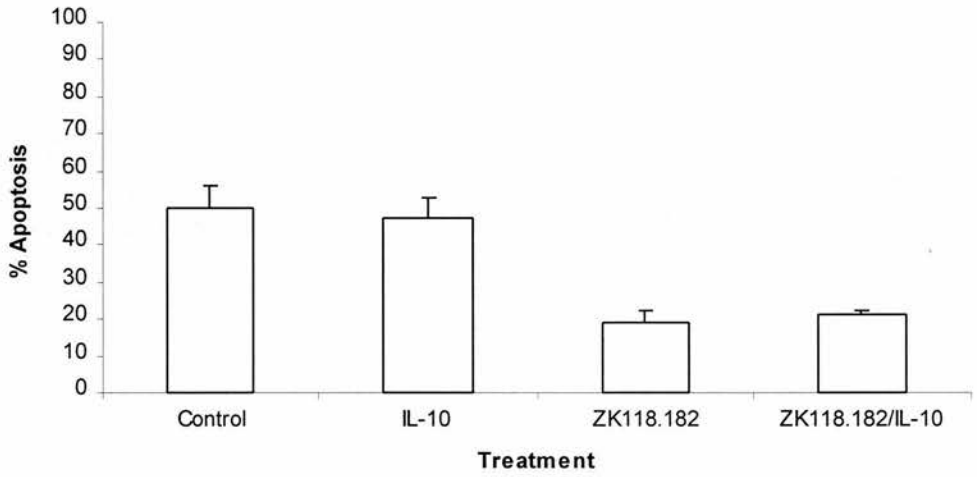


Figure 4.11 The Effect of IL-10 on ZK118.182-induced Neutrophil Survival

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), IL-10 (10 ng/ml), ZK118.182 (30 μM) or IL-10 plus ZK118.182 at the same concentrations, and harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments for neutrophils and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

4.2.4 The Effect of TGF-β on Granulocyte Apoptosis

TGF-β induces apoptosis in some immune cells (Lomo et al., 1995), but as shown in Figure 4.12, it significantly inhibited neutrophil apoptosis at 20 h using concentrations above 300 pM (e. g. Control 61.5 % ± 5.0, TGF-β 300 pM 46.4 ± 3.4 %). A concentration of 400 pM was used in the following neutrophil experiments. Figure 14.3 A demonstrates the significant inhibition of constitutive neutrophil apoptosis using this concentration (control 70.7 ± 2.3 %, TGF-β 400 pM 44.4 ± 4.7 %). Figure 4.13 B illustrates the effect of TGF-β on eosinophil programmed cell death using 1000 pM and 50 pM TGF-β. There was no significant difference at either concentration at 40 h, but at 20 h, 1000 pM TGF-β significantly inhibited apoptosis in these cells (control 23.2 ± 2.0 %, TGF-β 1000 pM 12.2 ± 0.1 %).

4.2.5 The Effect of TGF-β on Dexamethasone and GM-CSF Inhibition of Neutrophil Apoptosis

Figure 4.14 illustrates the effect of TGF-β on dexamethasone-induced neutrophil survival. As shown, both TGF-β and dexamethasone significantly inhibited neutrophil apoptosis. However, the co-culture of neutrophils with TGF-β and dexamethasone potentiated the inhibition induced by both factors alone. Interestingly, the level of inhibition significantly increased in comparison with that mediated by dexamethasone treatment alone (control 69.4 ± 2.1 %, dexamethasone 49.9 ± 0.4 %, TGF-β plus dexamethasone 34.1 ± 3.9 %). Similar results were obtained using GM-CSF and TGF-β (Figure 4.15). Again, TGF-β and GM-CSF both significantly reduced neutrophil apoptosis at 20 h, but co-culture of neutrophils with both cytokines together caused a further significant decrease (TGF-β 46.0 ± 6.1 %, GM-CSF 40.9 ± 3.7 %, TGF-β plus GM-CSF (27.7 ± 1.8 %). These results (Figures 4.14 and 4.15) indicate that TGF-β, rather than inducing neutrophil apoptosis, can enhance the inhibitory effects of both dexamethasone and GM-CSF.

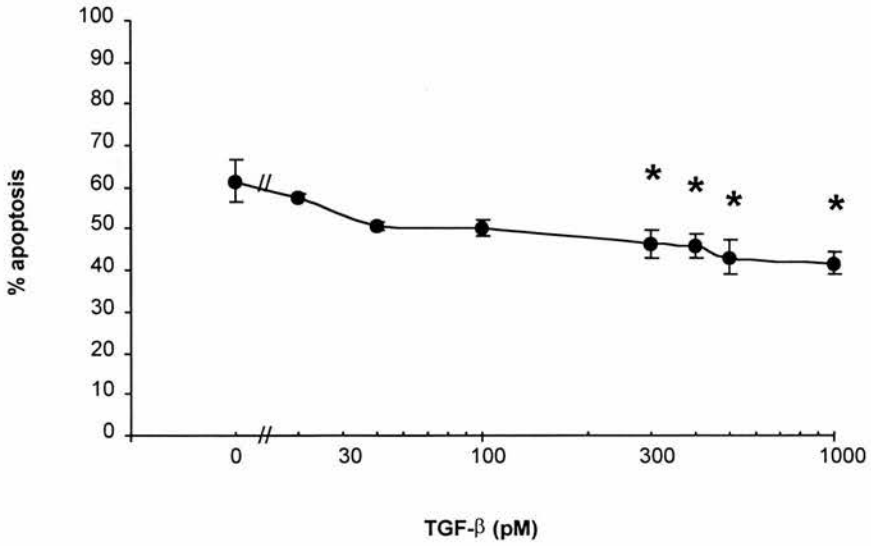


Figure 4.12 The Effect of TGF-β on Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with TGF-β at the concentrations shown and harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 4 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

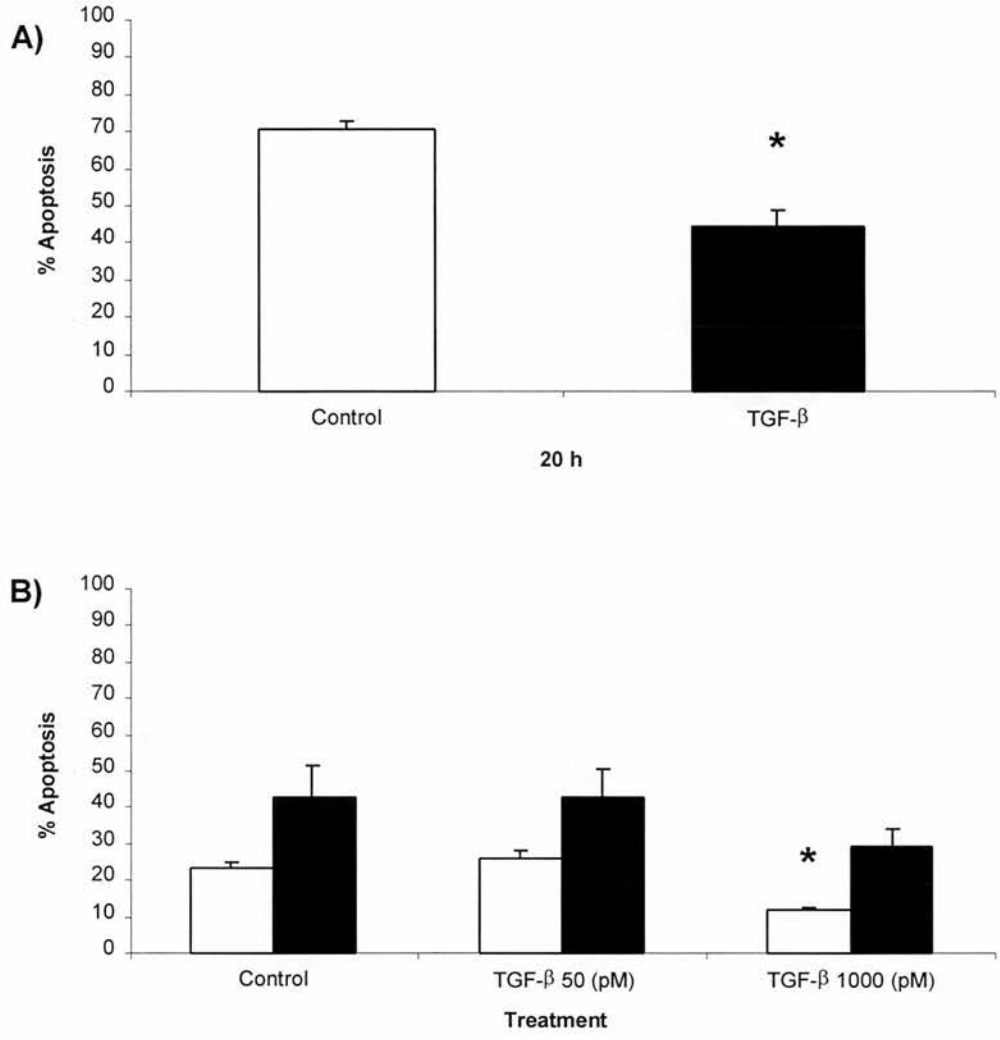


Figure 4.13 The Effect of TGF-β on Neutrophil and Eosinophil Apoptosis

A) Neutrophils (5 x 10^6/ml) and B) eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with TGF-β (400 pM for neutrophils and 50 or 1000 pM for eosinophils) and harvested at 20 h (neutrophils) or 20 (open bars) and 40 h (filled bars) (eosinophils). Apoptosis was assessed morphologically. Data represent the mean ± SEM of 8 separate experiments for neutrophils and 3 separate experiments for eosinophils. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

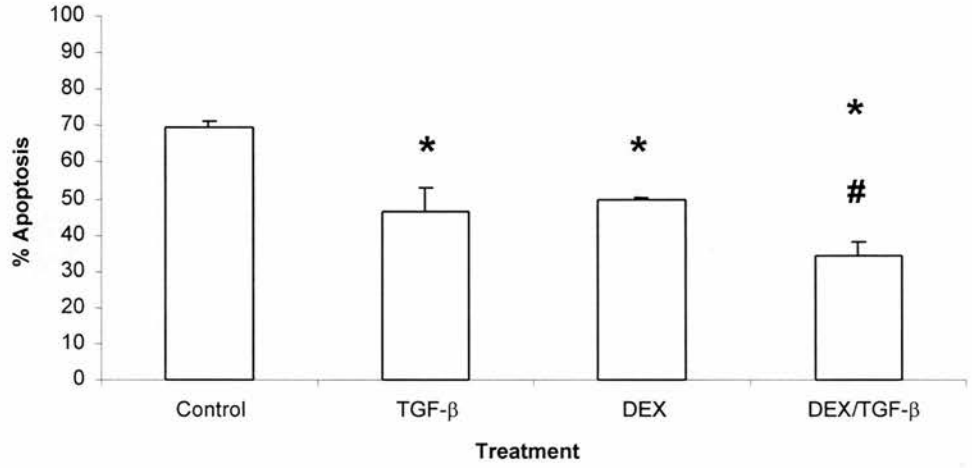


Figure 4.14 The Effect of TGF-β on Dexamethasone Inhibition of Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with TGF-β (400 pM), dexamethasone (1 μM) or both TGF-β and dexamethasone at the same concentrations, and harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with TGF-β or dexamethasone alone).

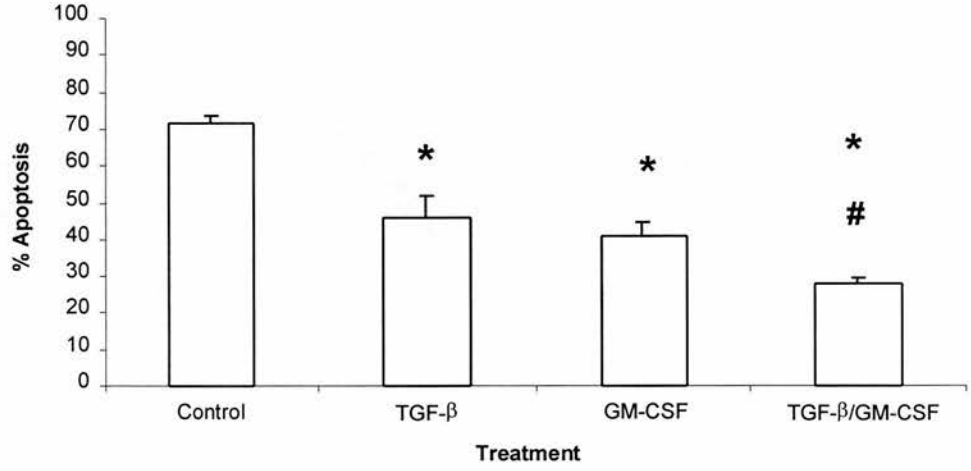


Figure 4.15 The Effect of TGF-β on GM-CSF Inhibition of Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with TGF-β (400 pM), GM-CSF (50 U/ml) or both TGF-β and GM-CSF at the same concentrations, and harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with TGF-β or GM-CSF alone).

4.2.6 The Effect of TGF-β on TNF-α Mediated Apoptosis and Survival of Neutrophils

Neutrophils were cultured for 4 h to examine whether TGF-β could potentiate or inhibit the pro-apoptotic response to TNF-α, and for 20 h to investigate its effect on TNF-α-mediated survival. At 4 h, TGF-β significantly enhanced the pro-apoptotic effect of TNF-α (TNF-α 17.9 ± 2.1 %, TGF-β plus TNF-α 29.9 ± 0.7 %), but TGF-β alone did not inhibit constitutive apoptosis at this timepoint. At 20 h, both TGF-β and TNF-α inhibited neutrophil apoptosis, but co-culture with both cytokines was not significantly different from results with each cytokine alone (Figure 4.16).

To further examine the potentiation of the pro-apoptotic actions of TNF-α, neutrophils were cultured with both cytokines for 2, 4, 6 and 8 h (Figure 4.17). This illustrates significant enhancement of TNF-α-induced apoptosis by TGF-β at early timepoints (2 and 4 h). Although TGF-β produced a slight increase in TNF-α-mediated neutrophil apoptosis in all experiments at 6 and 8 h, the differences at these timepoints between TNF-α and TNF-α plus TGF-β treated cells were not significant.

4.2.7 The Effect of Dexamethasone and GM-CSF on TNF-α-induced Neutrophil Apoptosis

To determine the specificity of the upregulation of the TNF-α effect by TGF-β, neutrophils were incubated with TNF-α plus dexamethasone or TNF-α plus GM-CSF. Both factors enhance neutrophil survival; GM-CSF activates and primes neutrophils but dexamethasone does not (Cox, 1995). As illustrated in Figure 4.18, TGF-β, dexamethasone and GM-CSF significantly decreased neutrophil apoptosis at 6 h. Dexamethasone and GM-CSF also significantly reduced TNF-α induced apoptosis at this timepoint (TNF-α 30.3 ± 2.7 %, dexamethasone plus TNF-α 22.6 ± 2.7 %, GM-CSF plus TNF-α 23.9 ± 2.2 %), but TGF-β did not.

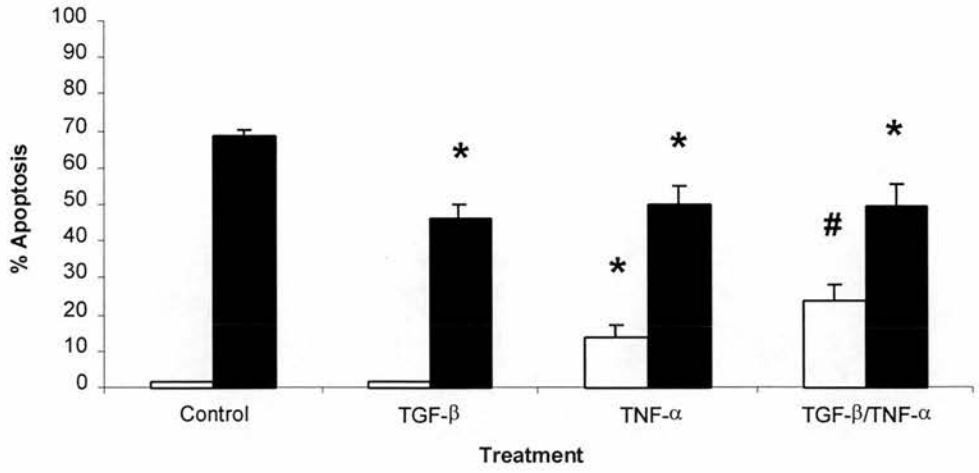


Figure 4.16 The Effect of TGF-β on TNF-α Induction and Inhibition of Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with TGF-β (400 pM), TNF-α (12.5 ng/ml) or both TGF-β and TNF-α at the same concentrations, and harvested at 4 h (open bars) or 20 h (filled bars). Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with TGF-β or TNF-α alone).

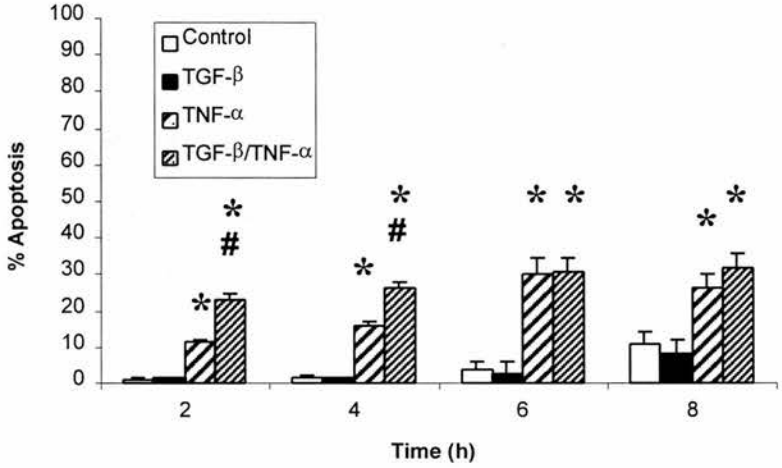


Figure 4.17 The Effect of TGF-β on TNF-α Induced Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with TGF-β (400 pM), TNF-α (12.5 ng/ml) or both TGF-β and TNF-α at the same concentrations, and harvested at 2, 4, 6 and 8 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 6 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with TGF-β or TNF-α alone).

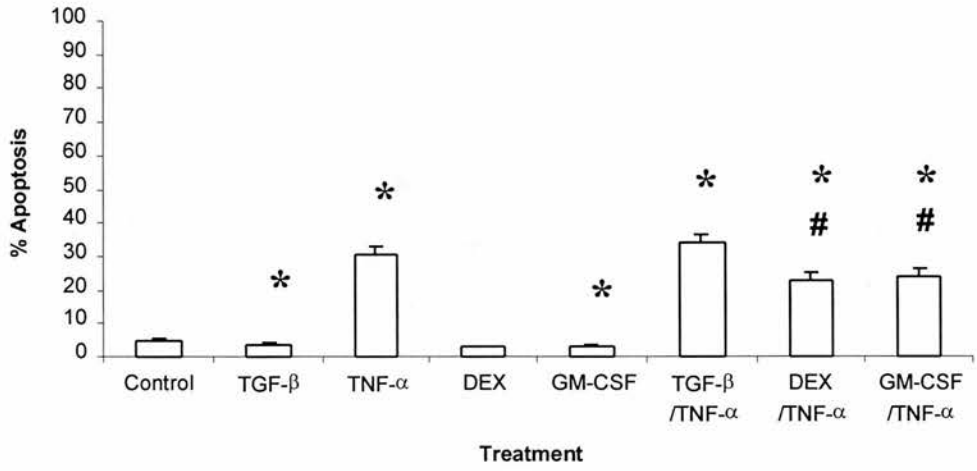


Figure 4.18 The Effect of Dexamethasone, GM-CSF and TGF-β on TNF-α Induced Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), or with TGF-β (400 pM), TNF-α (12.5 ng/ml), dexamethasone (1 μM), GM-CSF (50 U/ml) or both TGF-β and TNF-α, dexamethasone and TNF-α, or GM-CSF and TNF-α at the same concentrations, and harvested at 6 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with values for TNF-α alone).

4.3 Discussion

The pro-inflammatory cytokines and granulocyte priming agents, GM-CSF and TNF-α, enhanced neutrophil survival, but only GM-CSF inhibited eosinophil apoptosis. The results for GM-CSF are in agreement with Wallen et al., (1991), Lee et al., (1993), Kroegel et al., (1994), and Takanaski et al., (1994). TNF-α had no effect on eosinophil programmed cell death, although nerve growth factor, a member of the TNF family, has been shown to enhance the survival of human eosinophils (Hamada et al., 1996). Of great interest is the observation that TNF-α has pro-apoptotic effects at early timepoints in human neutrophils. These results are in agreement with Murray et al., (1997), who demonstrated that the time in culture is crucial for examining the pro-apoptotic effects of this cytokine. In neutrophils, incubation of cells for less than 8 h induces apoptosis, and greater than 12 h causes a decrease in neutrophil apoptosis. Few physiological mediators have been demonstrated to induce programmed cell death in neutrophils; other priming agonists do not have this effect. The early proapoptotic effects of TNF-α appear to be dependent on both TNF receptor subtypes whereas priming, and the later inhibitory effect on neutrophil apoptosis, are mediated via TNFRII (Murray et al., 1997).

Culture of neutrophils and eosinophils with IL-10 produced no differences in rates of apoptosis in comparison with control cells [in agreement with Cox, (1996), and Keel et al., (1997) who found no effect of IL-10 on constitutive neutrophil apoptosis, and Takanaski et al., (1994), who found parallel results in IL-10 treated eosinophils], although IL-10 effectively reduced TNF-α production by LPS-stimulated monocytes and monocyte-derived macrophages.

IL-10 partially reversed LPS-inhibition of apoptosis but did not significantly reduce this inhibition in eosinophils. These results for eosinophils contradict those obtained by Takanaski et al., (1994). However, this group divided eosinophil donors into responders and non-responders to LPS and their published data included only responders. In contrast, all donors in the experiments described in this chapter

showed inhibition of eosinophil apoptosis by LPS. However, of the donors used, results from 2 showed that IL-10 inhibited LPS-induced eosinophil survival, while the other 2 showed no significant difference. It is possible that as a result of selecting donors whose eosinophils show a good response to LPS, the results from Takanaski are skewed, showing a more marked inhibition of LPS-induced survival by IL-10 than those obtained by selecting donors at random as presented here. Alternatively, there may be some donors whose eosinophils do not respond to IL-10 when activated by LPS. All eosinophil donors used in these studies were either non- or mildly atopic and were on no medication at the time of sampling. The atopic eosinophil donors were prone to allergic reactions such as rhinitis or eczema, and therefore it is possible that some were undergoing mild allergic reactions while this study was undertaken. If some of the cells obtained were slightly activated before the experiments began, this might account for differences between donors with regard to the effects of IL-10 on LPS-induced eosinophil survival. The number of donors in this experiment (n = 4) are too small to draw firm conclusions with regard to non-responders to IL-10. It would be interesting to extend the study to include subjects with moderate or severe allergies to examine if there are differences in the response to IL-10 with regard to LPS-activation, and to other functional responses as well as apoptosis. It is unlikely that differences between these results and those obtained by Takanaski et al., (1994) are due to differences in IL-10 concentration, since their experiments were conducted using 5 ng/ml IL-10 and those presented in this chapter used 10 ng/ml. LPS concentrations were also similar.

IL-10 had no effect on the inhibition or stimulation of apoptosis mediated by dexamethasone, GM-CSF, TNF-α, dbcAMP, 5-oxo-ETE or ZK118.182. Takanaski et al., (1994) also found that IL-10 did not inhibit GM-CSF-induced eosinophil survival. However, Keel et al., (1997), found that IL-10 inhibited neutrophil survival induced by LPS, TNF-α and GM-CSF. However, many of these experiments were performed using cells from patients with severe sepsis. This means that the cells they examined could have been exposed to LPS *in vivo*, which would explain the inhibition by IL-10. Such cells might also have been activated by a plethora of pro-inflammatory cytokines in the peripheral blood; for example, as shown by many

studies, TNF-α is markedly upregulated in sepsis (Gérard et al., 1993, Marchant et al., 1994b).

The mechanism of IL-10 inhibition of LPS-induced neutrophil survival is not known. This cytokine may act as a "deactivating factor", as has been demonstrated in macrophages, but inhibition might only occur in activated cells. GM-CSF and TNF-α also prime and activate granulocytes and IL-10 did not affect inhibition of apoptosis induced by these factors. Nevertheless, IL-10 might inhibit other functional activities mediated by TNF-α or GM-CSF in these cells. IL-10 is known to inhibit the synthesis of pro-inflammatory cytokines from both neutrophils and eosinophils (Casatella et al., 1993, Takanaski et al., 1994). GM-CSF neutralising antibodies partially reversed LPS-induced survival of eosinophils (Takanaski et al., 1994), but had no effect on LPS-induced neutrophil survival (Cox, 1996). Therefore there may be differences in the mechanism of IL-10 inhibition between these two cell types.

The production of pro-inflammatory cytokines such as GM-CSF, TNF-α and IL-8, which is inhibited by IL-10, is controlled at least partially by the transcription factor NF-κB, which LPS is known to activate (Wang et al., 1995). IL-10 has been shown to suppress NF-κB activity. For example, in monocytes stimulated with LPS, IL-10 inhibits nuclear localisation of NF-κB in a concentration-dependent manner (Wang et al., 1995), with inhibition detectable at 10 ng/ml IL-10. In a rat model of lung injury, IL-10 was shown to inhibit NF-κB activation, reducing levels of TNF-α (Lentsch et al., 1997). This appeared to be due to upregulation of IκBα, which may have been caused by induction of IκBα gene expression by IL-10 or by the inhibition of IκBα proteolysis (Lentsch et al., 1997). These examples might suggest that inhibition of apoptosis by the cytokines and other factors used in these studies is not mediated solely by NF-κB; alternatively, such factors may activate NF-κB in a more rapid manner. For example, TNF-α can activate NF-κB within two to four minutes after binding to the cell, even when only 20 – 25 % of the receptors are occupied (Hohmann et al., 1990). This possibility could be examined by pre-incubating cells

with IL-10 before the addition of cytokines and other mediators. In the results shown in Figure 4.4, IL-10 inhibited the production of TNF-α from LPS-stimulated monocytes and macrophages, but even at concentrations 10 times greater than those used in the other IL-10 experiments presented in this chapter, the inhibition was partial rather than total. In LPS-treated granulocytes, there was again only a partial reversal of LPS-inhibition. This may indicate that NF-κB is an important mediator of enhanced survival, but that granulocytes may need only a partial activation of this transcription factor to prevent apoptosis from occurring.

Another possible explanation is that GM-CSF, TNF-α and other mediators used in these studies inhibit granulocyte apoptosis using other signalling pathways that cannot be inhibited by IL-10 either instead of or in concert with NF-κB. For example, other transcription factors such as AP-1, GR, CREB, Oct-1 and Sp-1 are not affected by IL-10 (Wang et al., 1995).

LPS upregulates tyrosine phosphorylation (Geng et al., 1994) which is also implicated in the regulation of apoptosis (Yousefi et al., 1994, 1996). IL-10 strongly inhibits tyrosine kinase activity (Geng et al., 1994). The signal transduction pathways triggered by IL-10 remain to be specified (Goldman et al, 1996). IL-10 has been shown to activate Stat1, Stat3 and in certain cells, Stat5 leading to the formation of three or in some cells four distinct DNA binding complexes (Weber-Nordt et al., 1996), but little is known of how this affects gene transcription.

However, because IL-10 blocks LPS enhancement of neutrophil survival, it may play a physiologically relevant role in resolution of bacterial infection and inflammation. In an *in vivo* model of mild acute lung injury using LPS-induced inflammation, IL-10 shortens the period of pulmonary neutrophilia (Cox, 1996). The levels of recruited cells are unchanged, suggesting that clearance of these cells is enhanced by IL-10 (Cox, 1996).

TGF-β enhanced survival of both neutrophils and eosinophils (but only at 20 h in eosinophils). There were no effects on constitutive apoptosis in either cell type at a

concentration of 50 pM, which gives maximal autoinduction of TGF-β (Van Obberghen-Schilling et al., 1988). Rather than inhibiting GM-CSF or dexamethasone induced survival in neutrophils, TGF-β increased this effect to levels that were significantly enhanced compared to those achieved by dexamethasone or GM-CSF alone.

TGF-β did however enhance the pro-apoptotic potential of TNF-α at early timepoints (2 and 4 h), whereas dexamethasone and GM-CSF both significantly inhibited TNF-α-mediated apoptosis at 6 h. The mechanism by which TGF-β upregulates TNF-α-induced apoptosis is unknown, but synergistic and additive effects of TNF-α and TGF-β with co-culture of immune cells have been noted (Kamijo et al., 1990, Hori et al., 1994).

In neutrophils, both TNFRs are required for apoptosis and TNF stimulation results in rapid downregulation of both receptors (Porteu & Hiebolt, 1994, Murray et al., 1997). The number of binding sites and the affinity of TNF-α for its receptors were found to be unchanged by TGF-β, in various cells which respond to co-culture with both cytokines with synergistic effects (Kamijo et al., 1990). This should however, be verified in neutrophils.

TNF cytotoxicity does not require new protein synthesis (Ruff & Gifford, 1981), therefore it is unlikely that TGF-β potentiates the pro-apoptotic effect of TNF-α through this mechanism. TGF-β could upregulate the TNF effect by either inhibiting a survival pathway or by upregulating an apoptotic pathway. Because the induction of apoptosis by TNF-α requires both receptors, TGF-β could interact with signals from either receptor. The signalling pathways known to be activated by TNF-α are illustrated in Figure 1.2.

As shown in Figure 1.2, the transcription factors responsible for many of the TNF-α–mediated effects are NF-κB and AP-1 (Brenner et al., 1989, Israël et al., 1989, Angel & Karin, 1991, Baldwin, 1996). AP-1 is activated through the JNK and p38

kinase (Karin, 1995, Karin et al., 1997). TNF-α induces the simultaneous activation of NF-κB, JNK and p38 kinase.

TRAF2 is part of the signal cascade involved in the activation of both NF-κB and JNKs. However, inhibition of TRAF2 activity prevents the activation of JNK, but not NF-κB, suggesting that TRAF2 has a specific role in the regulation of the JNK pathway. TRAF2 activates anti-apoptotic signals independently of NF-κB genes during TNF-α-mediated apoptosis (Lee et al., 1997b). Examination of TRAF2 deficient cells revealed a severe reduction in TNF-α-mediated JNK/SAPK activation but only a mild effect on NF-κB activation, while thymocytes and other haematopoetic progenitors were found to be highly sensitive to TNF-induced cell death (Yeh et al., 1997). These results suggest that JNK activation mediates a survival effect.

TGF-β also activates AP-1 (Kim et al., 1989, Pertovaara et al., 1989). TGF-β can signal via TAK1 binding protein (TAB1) (Shibuya et al., 1996), which activates a MAPKKK, TGF-β activated kinase (TAK-1), which can activate MEKK1 (Shibuya et al., 1996). This would lead to activation of JNK and p38. From the studies detailed above (Lee et al., 1997, Yeh et al., 1997), this is unlikely to be responsible for the potentiation of the pro-apoptotic effect of TNF-α. It might however, explain the inhibitory effect of TGF-β on granulocyte apoptosis.

TNF-α is known to mediate apoptosis via the caspase cascade pathway that is also utilised by Fas (Figure 1.2). It is not known whether TGF-β can enhance the activity of this pathway, but it would be interesting to examine whether this cytokine can also potentiate the effects of Fas receptor ligation on neutrophil apoptosis.

Other studies in which TNF-α causes apoptosis or growth inhibition correlate this response with a reduction in levels of *c-myc* expression. For example, in HL-60 cells, TNF-α treatment causes growth inhibition and apoptosis with a 50% reduction in c-*myc* mRNA in less than an hour (Krönke et al., 1987, Tobler et al., 1987). c-

Myc protein was rapidly down-regulated in apoptotic HL-60 cells and apoptosis could be induced by *c-myc* antisense oligonucleotides (Kumakura et al., 1996). These results suggest that the down-regulation of c-myc may be closely associated with apoptosis in HL-60 cells treated with TNF-α (Kumakura et al., 1996).

In U937 cells, both TNF-α and TGF-β could marginally inhibit cell proliferation, but together they acted synergistically (Hori et al., 1994). TNF-α concentration-dependently reduced *c-myc* expression in these cells, and again TGF-β behaved synergistically, only marginally reducing c-*myc* mRNA alone. Changes in the half-life of c-Myc were not involved: the effect was mediated via a reduction in transcriptional activity of the gene (Hori et al., 1994). In B cell lymphoma cells, WEH1 231 and CH31 cells, TGF-β mediated apoptosis was preceded by a decline in c-Myc expression (Warner et al., 1992, Fischer et al., 1994, Arsura et al. 1996).

The *c-myc* gene has been strongly implicated in the regulation of cellular death. Overexpression or inappropriate expression of the *c-myc* gene promotes apoptosis in myeloid and fibroblast cells upon removal of growth factors (Askew et al., 1991, Evan et al., 1992). In contrast, in immature B cell models of tolerance, such as WEH1 231 and CH31 and CH33 cells (Kim et al., 1979, Ralph, 1979, Boyd & Schrader, 1981, Monroe & Seyfert, 1988), a drop in c-Myc expression is correlated with induction of apoptosis. An early transient increase, followed by a dramatic decline in levels of c-myc RNA and protein, precedes anti-Ig receptor-mediated induction of apoptosis in WEH1 231 and CH 31 B cells (McCormack et al., 1984, Levine et al., 1986, Ales-Martinez et al., 1988; Benhamou et al., 1990, Hasbold & Klaus, 1990, Maheswaran et al., 1991). When these cells are stably transfected to express IgD, anti IgM and anti IgD treatments cause transient increases of c-Myc expression. However, only IgM caused a decline in c-*myc* RNA levels below baseline, followed by apoptosis. Anti-IgD treatment maintained c-*myc* expression at or above control levels and no induction of apoptosis was noted (Ales-Martinez et al., 1988, Tisch et al., 1988). Similarly, mutants of WEH1 231 that failed to induce apoptosis in response to anti-immunoglobulin displayed sustained c-Myc expression (Hibner et al., 1993).

c-*myc* gene expression is controlled, in part by NF-κB binding (Lee et al., 1995b). Treatment of WEH 231 cells with TGF-β1 decreased NF-κB binding in the nucleus (Arsura et al., 1996), correlating with increased expression of the IκBα gene (Arsura et al., 1996). In turn, the reduction in NF-κB binding correlated with a drop in c-Myc levels (Arsura et al., 1996). It therefore seems likely that inhibition of NF-κB downmodulates c-Myc expression inducing death of these cells (Arsura et al., 1996).

TGF-β treatment rapidly downregulates c-myc mRNA and protein levels in TGF-β sensitive cells (Pientenpol et al., 1990, Munger et al., 1992, Malliri et al., 1996). Neutrophils and eosinophils are both sensitive to the effects of TGF-β (Figure 4.4), Therefore the effects on c-Myc levels of TNF-α and TGF-β both alone and in co-culture in neutrophils should be examined. This could be achieved using in situ hybridisation, northern and western blots.

Enhanced sensitivity to TNF-α in NF-κB deficient cells has been demonstrated (Beg & Baltimore, 1996, Van Antwerp et al., 1996, Wang et al., 1996). Therefore the most likely explanation for the enhancement of the pro-apoptotic effect of TNF-α by TGF-β is that this cytokine upregulates expression of IκBα leading to suppression of NF-κB. This would not cause a permanent suppression but would ensure a faster cycling of the transcription factor from the nucleus back to the cytoplasm. This in turn would cause a fall in levels of c-Myc, a protein with a half-life of only 15 – 30 min (Blackwood & Eisenman, 1991).

Why IL-10, which also inhibits NF-κB, does not potentiate the pro-apoptotic effects of TNF-α is not known. However, Thompson et al., (1995) showed that while some inducers of NF-κB rapidly and transiently activate this transcription factor through IκBα (TNF-α), others such as LPS cause a more persistent activation by affecting IκBα and IκBβ. One possibility is that IL-10 inhibits this more persistent induction through interference with IκBβ activation rather through IκBα. This would explain why IL-10 does not inhibit the effects of TNF-α on neutrophil apoptosis and why

only a partial reversal of LPS-induced activation is achieved. It would therefore be of interest to co-culture neutrophils with TNF-α, TGF-β and IL-10 to examine whether this could further potentiate the pro-apoptotic effect of TNF-α.

In view of the findings discussed here, we concluded that NF-κB might play an important role in granulocyte survival and apoptosis. Therefore we decided to examine the role of this transcription factor in these processes (Chapter 5).

CHAPTER 5

THE ROLE OF THE TRANSCRIPTION FACTOR NF-κB IN GRANULOCYTE APOPTOSIS

5.1 Introduction

The results depicted in Chapter 4, showing an upregulation of TNF-α-induced neutrophil apoptosis by TGF-β, which has been demonstrated to inhibit NF-κB activity by the production of IκB-α (Arsura et al., 1996), prompted an in depth study of the effect of this transcription factor on granulocyte apoptosis. The signalling pathways and activation of NF-κB are discussed in Section 1.14.3.

The role of NF-κB in apoptosis is controversial. Because NF-κB is activated by some apoptotic stimuli, it may be involved in some aspects of programmed cell death (Siebenlist et al., 1994, Verma et al., 1995, Baldwin, 1996). TNF-α, which induces apoptosis in some cells, while also activating NF-κB, is an example of this (Figure 1.2). NF-κB activation in HL60 cells and thymocytes is associated with apoptosis (Marinovich et al., 1996). Pyrrolidine dithiocarbamate (PDTC), a potent non-specific inhibitor of NF-κB activation prevents apoptosis in HL-60 cells and in glucocorticoid-treated thymocytes (Bessho et al., 1994). Several groups have therefore suggested that NF-κB may function pro-apoptotically under some conditions and in certain cell lines (Jung et al., 1995, Lin et al., 1995a, Grimm et al., 1996).

However, since the onset of my PhD studies, inhibition of NF-κB has been shown to induce apoptosis in other cell types, for example, murine B cells (Wu et al., 1996), PC12 cells (Taglialatela et al., 1997), and HT 1080 fibrosarcoma cells (Wang et al., 1996). In HeLa and MCF7 cells, TNF-α-induced NF-κB activation prevents cell death (Lui et al., 1996). The active form of NF-κB is most frequently composed of p50/RelA heterodimers (Blank et al., 1992, Nolan & Baltimore, 1992); mice lacking

the p65/RelA gene die from extensive apoptosis within the liver, suggesting an anti-apoptotic function for NF-κB (Beg et al., 1995). Many cells that respond to TNF-α with strong activation of NF-κB are resistant to programmed cell death (Holtmann et al., 1991). More pertinently to this thesis, disruption of IκB-α in mice results in prolonged activation of NF-κB in response to inflammatory stimuli, with animals dying of widespread inflammation (Klement et al., 1996). Rel B or p50 null mice have defects in both immune and inflammatory responses (Sha et al., 1995, Weih et al., 1995). Several groups have also reported that inactivation of NF-κB increases the cytotoxic effects of TNF-α (Beg & Baltimore, 1996, Van Antwerp et al., 1996, Wang et al., 1996,).

Such studies emphasise the importance of NF-κB in inflammatory responses and cell survival and suggest that NF-κB inhibition might be necessary for resolution to occur, but the role of NF-κB in granuloctye apoptosis is currently unknown. It has recently been reported that LPS, TNF-α and fMLP activate NF-κB in neutrophils (McDonald et al., 1997), but Browning et al., (1997) found no activation in these cells despite obvious NF-κB activation in peripheral blood mononuclear cells. Because granulocytes are short-lived, terminally differentiated cells, NF-κB inhibition by genetic manipulation is not feasible; therefore the studies discussed in this chapter were performed using other methods of inhibition. Most studies use antioxidants or protease inhibitors to block activation of NF-κB.

Antioxidants with this ability include N-acetyl-L-cysteine, 2-mercaptoethanol, dithiocarbamates, α-lipoic acid, vitamin E, and iron and copper chelators (Schreck et al., 1991, 1992a, b, Meyer et al., 1993). These findings suggest that NF-κB activation depends on the cell's redox status which is determined by the concentrations of ROS that occur as intermediates in the reduction of O_2 to H_2O during cellular respiration (Halliwell & Gutteridge, 1989). Important ROS are $O_2^{\cdot -}$, H_2O_2, hydroxyl radicals, and singlet oxygen ($^1O_2$). GSH (glutathione), a major intracellular reducing agent (Hedley & Chow, 1991), reduces and might therefore block NF-κB activation *in vivo*. Well-established roles of GSH are to reduce the

intracellular H_2O_2 load, reduce intracellular free radicals and to maintain protein thiol groups in the reduced state (Ratan et al., 1994). Interestingly, GSH levels fall in neutrophils as the cells undergo ageing (Narayanan et al., 1997).

H_2O_2 activates NF-κB in Jurkat T cells and HeLa cells and this is inhibitable by antioxidants (Schreck et al., 1991). It is notable that in many cell lines, $O_2^{\cdot -}$, $^1O_2$ and nitric oxide do not cause rapid activation of NF-κB, suggesting that NF-κB might be specifically influenced by H_2O_2 and other peroxides rather than other ROS (Schreck et al., 1992a, Meyer et al., 1993). However, since in some cell types H_2O_2 does not induce transcription from κB-reporter constructs in unless PMA or TNF-α are added (Israel et al., 1992, Ziegler-Heitbrook et al., 1993), H_2O_2 might act not directly but by establishing a redox state that facilitates activation of NF-κB by other stimuli. In intact cells oxidant treatment induces rather than inhibits NF-κB activity (Schreck et al., 1991). Nevertheless antioxidants, for example reduced thiols such as dithiothreitol, cysteine, dihydrolipoate, and reduced thioredoxin, can enhance the DNA binding of activated NF-κB (Matthews et al., 1992, Okamoto et al., 1992, Hayashi et al., 1993, Galter et al., 1994, Suzuki et al., 1995a, b). Overall, these results suggest the possibility of redox facilitation of both NF-κB activation and DNA binding of the transcription factor.

Redox regulation of DNA binding has also been proposed for AP-1 (Abate et al., 1990), the role of which in cell survival is also controversial. AP-1-related effects might therefore complicate studies of NF-κB inhibition by antioxidants. TNF-α-induced ROS production stimulates AP-1 expression (Lo & Cruz, 1995), and mitogen-induced AP-1 activity is inhibited by antioxidants, suggesting that it is ROS mediated (Goldstone et al., 1995). N-acetyl-choline, (NAC) inhibits AP-1 activation and prevents the induction of c-*fos* and c-*jun* mRNAs (Datta et al., 1992, Collart et al., 1995, Janssen et al., 1995). However, contradictory data have been obtained concerning the mode of AP-1 regulation by reduced thiols; for instance, NAC enhanced DNA binding activity and transactivation (Meyer et al., 1993, Schenk et al., 1994). Treatment with antioxidants alone also strongly activated DNA binding and transactivation of AP-1, suggesting that AP-1 behaves as an antioxidant-

responsive transcription factor (Choi & Moore, 1993, Meyer et al., 1993). Thioredoxin, a powerful thiol reducer and major protein oxidoreductase inside the cell (Holmgren 1985), increased PMA-induced AP-1 transcriptional activity in a concentration-dependent manner in HeLa and L929 cells. This effect was shown to be specific to the antioxidant property of thioredoxin (Meyer et al., 1993, Schenk et al., 1994). These studies also demonstrated that other thiol reducing agents (PDTC, 2-mercaptoenthanol, butylated hydroxyanisole and NAC) enhance DNA binding activity and transactivation of AP-1 (Schenk et al., 1994). Conversely, elevation of intracellular levels of GSSG before stimulation increased AP-1 transactivation (Galter et al., 1994).

PDCT has been shown to inhibit NF-κB in many studies (Beg et al., 1993, Henkel et al., 1993, Sun et al., 1993), but again no simple generalisations can be made. PDTC (3 - 30 μM) increased GSSG levels in Molt-4 (lymphocyte cell line) cells and inhibited NF-κB activity, but a higher concentration (300 μM) reconstituted the NF-κB activity (Galter et al., 1994). Protein tyrosine phosphatase (PTP) inhibitors strongly activate NF-κB, but PDCT did not prevent this activation in lymphocytes, and conversely pre-treatment of these cells with PDCT enhanced rather than inhibited NF-κB activity (Krejsa et al., 1997). Therefore PTP inhibitors can activate NF-κB by a mechanism that is independent of intracellular redox status (Krejsa et al., 1997).

Currently, the interaction between NF-κB activation through IκB phosphorylation and degradation, oxidative stress, and ceramide is not well understood (Akira & Kishimoto, 1997). Therefore, using antioxidants to inhibit NF-κB in this work might be complicated by any effects on the activity of AP-1, since the role of this transcription factor on cell survival is also controversial.

Serine protease inhibitors are powerful inhibitors of NF-kB activation in intact cells, but they are not specific (Henkel et al., 1993). For example, TPCK (Tosyl-Phe-chloromethylketone) inhibited AP-1 binding in TNF-α treated HeLa cells (Finco et al., 1994). Also, TPCK did not strongly interfere with NF-κB activity when added

after stimulation with PMA, IL-1 or LPS (Henkel et al., 1993). Other NF-κB inhibitors with more specific effects are now available. Gliotoxin, a member of the epipolythiodioxoperazine family (Braithwaite et al., 1987), synthesised as a secondary metabolite by *Aspergillus fumigatus* and other pathogenic fungi (Cole & Cox, 1981, Jordan & Cordiner, 1987, Shah & Larsen, 1991), is reported to be a specific inhibitor of the RelA/p50 NF-κB isoform (Pahl et al., 1996). Gliotoxin exhibits immunosuppressive activity both *in vivo* and *in vitro*. For example, it inhibits mitogen-induced proliferation of both T and B cells, induces macrophage and osteoclast apoptosis *in vitro* (Waring et al., 1988, 1990, Ozaki et al., 1997), and causes thymocyte and spleen cell apoptosis *in vivo* (Sutton et al. 1994). The known biological properties of gliotoxin include the ability to undergo redox cycling and generate oxygen free radicals that cause oxidative damage to isolated DNA *in vitro* (Eichner et al., 1988).

The pleiotropic effects of gliotoxin described in various studies may be attributed to the use of different concentrations of the compound. As an epipolythiodioxopiperazine derivative, gliotoxin has the potential to react covalently at high concentrations with sulphydryl residues in polypeptides. However, at relatively low concentrations, gliotoxin was found to selectively inhibit the activation of NF-κB in a number of cell lines, with no detectable effect on the DNA-binding activity of CREB, Oct-1, NFAT, or Stat1, or any inhibition of the tyrosine kinase activities of Lck and Fyn in intact cells (Pahl et al., 1996). Gliotoxin did not directly modify and inactivate NF-κB in intact cells; this only occurred at high concentration (millimolar) *in vitro*, suggesting that the redox-sensitive cysteine residues in the DNA-binding domains of NF-κB proteins remained intact under cell culture conditions, and *ipso facto* that the fungal immunosuppressant very selectively interfered with NF-κB activation (Pahl et al., 1996).

As IκBα and β subunits are degraded, the NLS of NF-kB is exposed and allows nuclear translocation (Cordle et al., 1993, Donald et al., 1995). Synthetic peptides containing a cell-permeable motif and the nuclear localisation sequence (NLS) of p50 have been developed. These peptides inhibit movement of NF-κB complexes

from the cytoplasm to the nucleus by blocking the intracellular recognition mechanism for the NLS present on p50 heterodimers (Lin et al., 1995b). Therefore they should also block translocation of p50/RelA heterodimers.

Several physiological mediators such as protein kinase C (PKC) and nitric oxide (NO) may also be involved in the regulation of NF-κB activity. PKC isoforms are categorised in three groups: conventional PKCs (cPKCs α, β1, β2 and γ), new PKCs (nPKCs δ, ε, and μ) and atypical PKCs (aPKCs ζ and λ) (Nishizuka, 1992). PKC ζ activation via ceramide may link the TNF-α receptor to NF-κB since TNF-α or ceramide increase phosphorylation of this isoform (Lozano et al., 1994, Muller et al., 1995, Spiegel et al., 1996). PKC ζ, has been suggested to phosphorylate IκB-α and has been demonstrated to cause dissociation of the NF-κB-IκB-α complex *in vitro* (Shirikawa & Mizel, 1989) and is necessary for NF-κB activation in *Xenopus* oocytes and mammalian cells (Diaz-Meco et al., 1994); it might act by phosphorylating IκB-α. In apoptotic U937 cells, the expression of PKC ζ is reduced while that of PKC β1 is increased (Pongracz et al., 1995). If PKC ζ is linked to the induction of active NF-κB and NF-κB is a survival factor, this would suggest a role in programmed cell death for both these PKC isoforms. A dominant negative PKC ζ protein inhibits NF-κB activation by TNF-α, while constitutively active PKC ζ activates NF-κB in NIH3T3 cells (Diaz-Meco et al., 1993); though it has been reported that some inhibitors of PKC, which block PMA-induced activation of NF-κB, fail to block TNF-α-induced activation (Meichle et al., 1990). Other studies support a role for PKC in NF-κB activation. Full activation in T cells requires the combined action of a Ca^{2+}-mobilizing and a protein kinase C-activating signal (Emmel et al., 1989, Tong-Starksen et al., 1989). NF-κB is activated in many cell types by PMA, which is a direct stimulator of PKC, and this activation can be efficiently blocked by antioxidants which are known inhibitors of NF-κB (Schreck et al., 1992a, Meyer et al., 1993). H8, a membrane-permeable inhibitor of cyclic nucleotide-dependent protein kinases and PKC, inhibits NF-κB activation by IL-1 and LPS (Shirikawa & Mizel, 1989). A range of antineoplastic agents which activate both PKC and NF-κB in A549 cells were blocked by the PKC inhibitors calphostin C

and GF109203X again suggesting a role for PKC in activation (Das & White, 1997). Pharmacological data argue against a direct phosphorylation of IκB by PKC in intact cells, but PKC may be indirectly involved, for example in a kinase cascade which leads to ultimate phosphorylation and activation of the actual kinase responsible.

Nitric oxide (NO) plays an important role in a number of physiological and pathological processes, displaying pro-inflammatory or anti-inflammatory effects depending on its effective concentration at the inflammatory site (Barnes & Belvisi, 1993, Brady & Poole-Wilson, 1993, Kubes, 1995, Moilanen & Vapaatalo, 1995, Barnes, 1996). It can modulate certain neutrophil responses (Kaplan et al., 1989, Kubes, 1991, Clancey et al., 1992, Moilanen et al., 1993) and induce apoptosis in several other cell types (Albina et al., 1993, Blanco et al., 1995, Kuo et al., 1996). Treatment of cells with nitric oxide (NO)-generating compounds such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine and S-nitrosoglutathione (GSNO) influences activation of NF-κB (Siebenlist et al., 1994, Matthews & Hay, 1995); in human vascular endothelial cells, activation of NF-κB was inhibited by treatment with SNP and GSNO by a mechanism thought to involve both stabilization of IκBα and increased transcription of the IκBα gene (Peng et al., 1995a, b). However, NO has also been found to inhibit p50 and p65 DNA binding activity (Matthews et al., 1996). NO modulates the biological activity of proteins by direct interactions with their iron centres or by S-nitrosylation of cysteines to form S-nitrosothiols, and this could affect the activity of NF-κB (Matthews et al., 1996). Inhibition of NF-κB by physiological mediators such as NO may represent an important mechanism for resolution of inflammation especially since this transcription factor is linked to the survival of inflammatory cells. The controversies surrounding the role of NF-κB in apoptosis and cell survival may be due to characteristic cell differences in responses to inhibitors and activators, or to other non-specific effects of the inhibitors used in blocking studies. Therefore the work presented in this chapter examines the role of NF-κB, using a broad range of reported NF-κB inhibitors on granulocyte survival and on the pro-apoptotic effects of TNF-α, to avoid the uncertainty of inferences from results obtained with single inhibitor types.

5.2 Results

5.2.1 The Effect of Gliotoxin on Constitutive and TNF-α Mediated Granulocyte Apoptosis

Figure 5.1 A illustrates the rapid induction of neutrophil apoptosis *in vitro* by gliotoxin. This induction was both concentration (e.g. at 6 h EC_{50} = 76.1 ± 22.1 ng/ml) and time-dependent. Using a concentration of 1 μg/ml, apoptosis was readily apparent within 2 h and reached 100 % by 6 h. At 20 h, when the rate of constitutive neutrophil apoptosis was 58.7 ± 2.9 %, gliotoxin increased this to 100% at all concentrations greater than 0.1 μg/ml. The inactive analogue of gliotoxin, methylthiogliotoxin (30 μg/ml), did not affect the constitutive rate of neutrophil apoptosis at any of the time-points studied (2, 6 and 20 h) (Figure 5.1 B). Neither gliotoxin nor methylthiogliotoxin caused cell necrosis since less than 1% of the treated cells were permeable to the vital dye trypan blue.

Figure 5.2 A illustrates the effect of gliotoxin on TNF-α-induced neutrophil apoptosis. These experiments were performed at 2 h when the independent pro-apoptotic effects of TNF-α (12.5 ng/ml) and gliotoxin (0.1 μg/ml) (Figure 5.1 A) are only just apparent. As shown in Figure 5.2 A, gliotoxin synergistically increased TNF-α-mediated neutrophil apoptosis even at gliotoxin concentrations as low as 3 ng/ml. A concentration of gliotoxin of 0.1 μg/ml in combination with TNF-α (12.5 ng/ml) caused almost 100 % apoptosis at 2 h. With gliotoxin alone, only 6.2 ± 2.2 % apoptosis was noted at 2 h with 65.9 ± 9.6 % at 6 h (Figure 5.1 A). Interestingly, the synergy between the two agents decreased as the concentration of gliotoxin was increased above 0.1 μg/ml. Again, methylthiogliotoxin (30 μg/ml) had no effect on constitutive or TNF-α-induced apoptosis at 2 h (Figure 5.2 B) and despite the rapid induction of apoptosis, the levels of necrosis were less than 1 % as assessed by trypan blue exclusion.

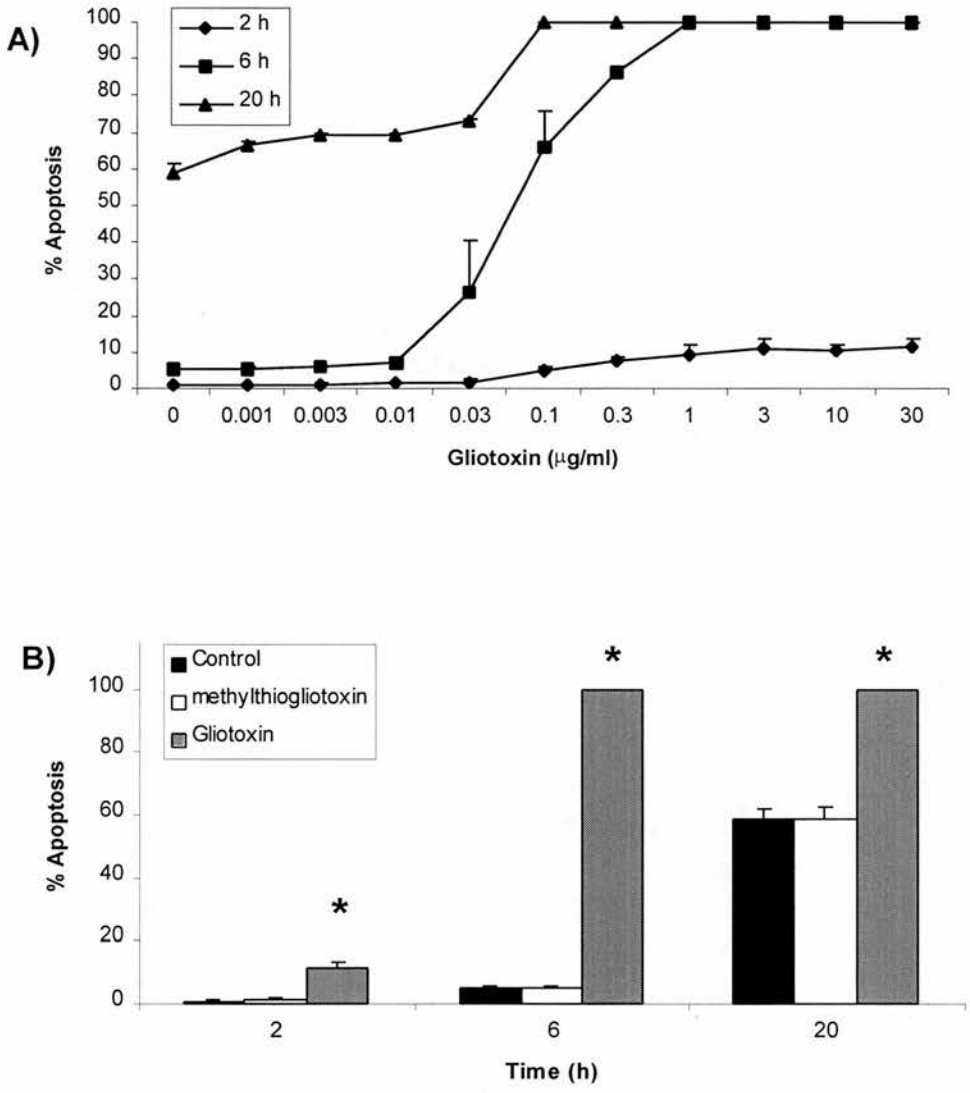


Figure 5.1 The Effect of Gliotoxin on Constitutive Neutrophil Apoptosis

A) Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with gliotoxin (0.001 – 30 μg/ml) and harvested at 2, 6 or 20 h. B) Neutrophils were incubated as above with gliotoxin (30 μg/ml) or methylthiogliotoxin (30 μg/ml) and harvested at 2, 6 or 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values).

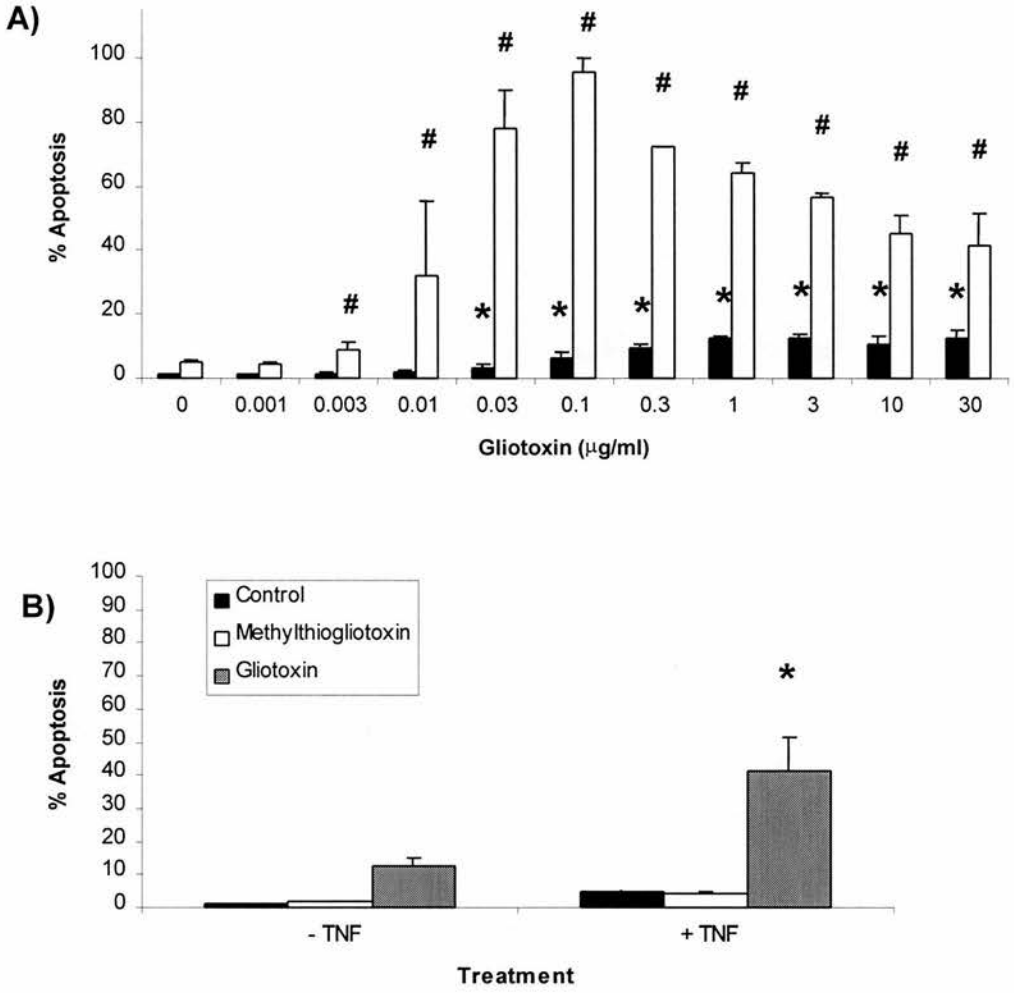


Figure 5.2 The Effect of Gliotoxin on Constitutive Neutrophil Apoptosis

A) Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with gliotoxin (0.001 – 30 μg/ml) ± TNF-α (12.5 ng/ml) and harvested at 2 h. Filled bars indicate gliotoxin alone; open bars indicate TNF-α + gliotoxin. B) Neutrophils were incubated as above with gliotoxin (30 μg/ml) or methylthiogliotoxin (30 μg/ml) ± TNF-α and harvested at 2 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with control or TNF-α alone).

5.2.2 The Effect of Gliotoxin on Constitutive Eosinophil Apoptosis and Co-culture with TNF-α

Eosinophils do not undergo apoptosis in response to TNF-α (Figure 4.8 B). Since NF-κB inhibition has been shown to increases the susceptibility of cells to the cytotoxic effects of TNF-α (Beg & Baltimore, 1996, Van Antwerp et al., 1996, Wang et al., 1996), we investigated the effect of gliotoxin on eosinophil apoptosis at 4 h in the presence and absence of this cytokine. The results shown in Figure 5.3 A demonstrated that gliotoxin induced apoptosis in eosinophils (EC_{50} = 0.37 ± 0.22 μg/ml) as it does in neutrophils, and caused a synergistic increase in the rate of apoptosis when the cells were co-cultured with TNF-α. Almost 100% apoptosis was observed using a gliotoxin concentration of 0.1 μg/ml plus TNF-α at a time-point of 4 h. In comparison, eosinophils cultured in the absence of gliotoxin would normally show only 40% apoptosis at a 40 h time-point (Figure 3.1) (Stern et al., 1992, Ward et al., 1997). Again the synergistic action of TNF-α plus gliotoxin was greatest using a concentration of gliotoxin of 0.1 μg/ml. Necrosis in these cells was less than 2% and the inactive analogue of gliotoxin had no effect on either the constitutive rate of apoptosis alone or in conjunction with TNF-α (Figure 5.3 B).

5.2.3 The Mode of Cell Death Induced by Gliotoxin and TNF-α is Apoptotic Not Necrotic

Figures 5.4 and 5.5 demonstrate the classic apoptotic morphology of neutrophils and eosinophils when treated TNF-α (12.5 ng/ml) and gliotoxin (0.1 μg/ml). The changes from normal cell morphology to apoptotic morphology are clearly seen in Figure 5.4 A, where non-apoptotic neutrophils contain a multi-lobed nucleus, and Figure 5.4 D, where the apoptotic cells have a shrunken appearance with pyknotic nuclei. Figure 5.5 A and D demonstrate a similar effect in eosinophils.

To ensure that this rapid induction of cell death was genuine apoptosis, the effect of gliotoxin and its synergy with TNF-α were also assessed by annexin V binding and

DNA fragmentation. In Figure 5.6 the annexin V 'low peak' represents non-apoptotic cells and the 'high-peak' represents apoptotic cells. Although control cells at 2 h exhibit low rates of apoptosis, the small increase in annexin V positive cells observed with TNF-α and gliotoxin alone is dramatically augmented when the cells are cultured in the presence of both reagents together. These results are consistent with Figure 5.2. Analysis by DNA fragmentation (Figure 5.7) demonstrates that cells cultured alone or in combination with the above reagents exhibit the classical "ladder" of DNA fragmentation associated with apoptosis.

Initial studies used trypan blue as a marker of plasma membrane integrity. However, given the rapid rate of apoptosis mediated by co-culture of granulocytes with TNF-α and gliotoxin, it was important to validate this by assessing necrosis in an independent manner. Figure 5.8 shows the profile of neutrophils at 4 h (2 h later than the experiments shown in Figure 5.2 A) after treatment with gliotoxin (0.1 μg/ml) and TNF-α (12.5 ng/ml). Despite apoptotic rates of 100% at 2 h, almost all cells, showed low fluorescence using propidium iodide staining detected by flow cytometry, indicating that the cell membrane had remained intact. As a positive control, cells cultured initially with TNF-α and gliotoxin were heat-treated (60 °C for 5 min) to ensure 100% necrosis. This resulted in a uniform and major increase in propidium iodide staining (Fig 5.8). These data coincide completely with the results obtained with trypan blue staining and confirm that the cells had undergone apoptotic cell death and were not necrotic.

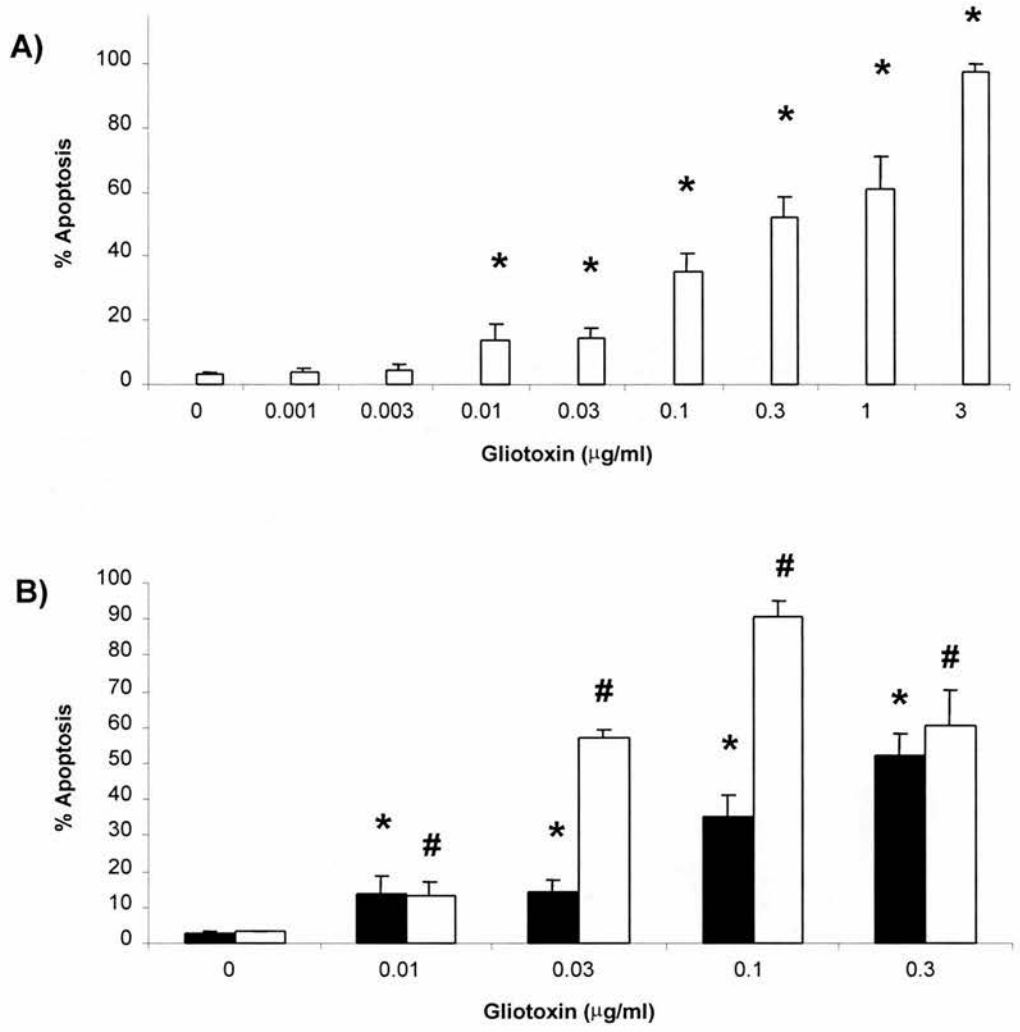


Figure 5.3 The Effect of Gliotoxin on Constitutive Eosinophil Apoptosis

A) Eosinophils (2.0 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with gliotoxin (0.001 – 30 μg/ml) ± and harvested at 4 h. B) Eosinophils were incubated as above with gliotoxin (0.01 - 30 μg/ml) ± TNF-α and harvested at 4 h. (Filled bars, gliotoxin alone, open bars, gliotoxin + TNF-α). Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (* $p < 0.05$ compared with control values; # $p < 0.05$ compared with control or TNF-α alone).

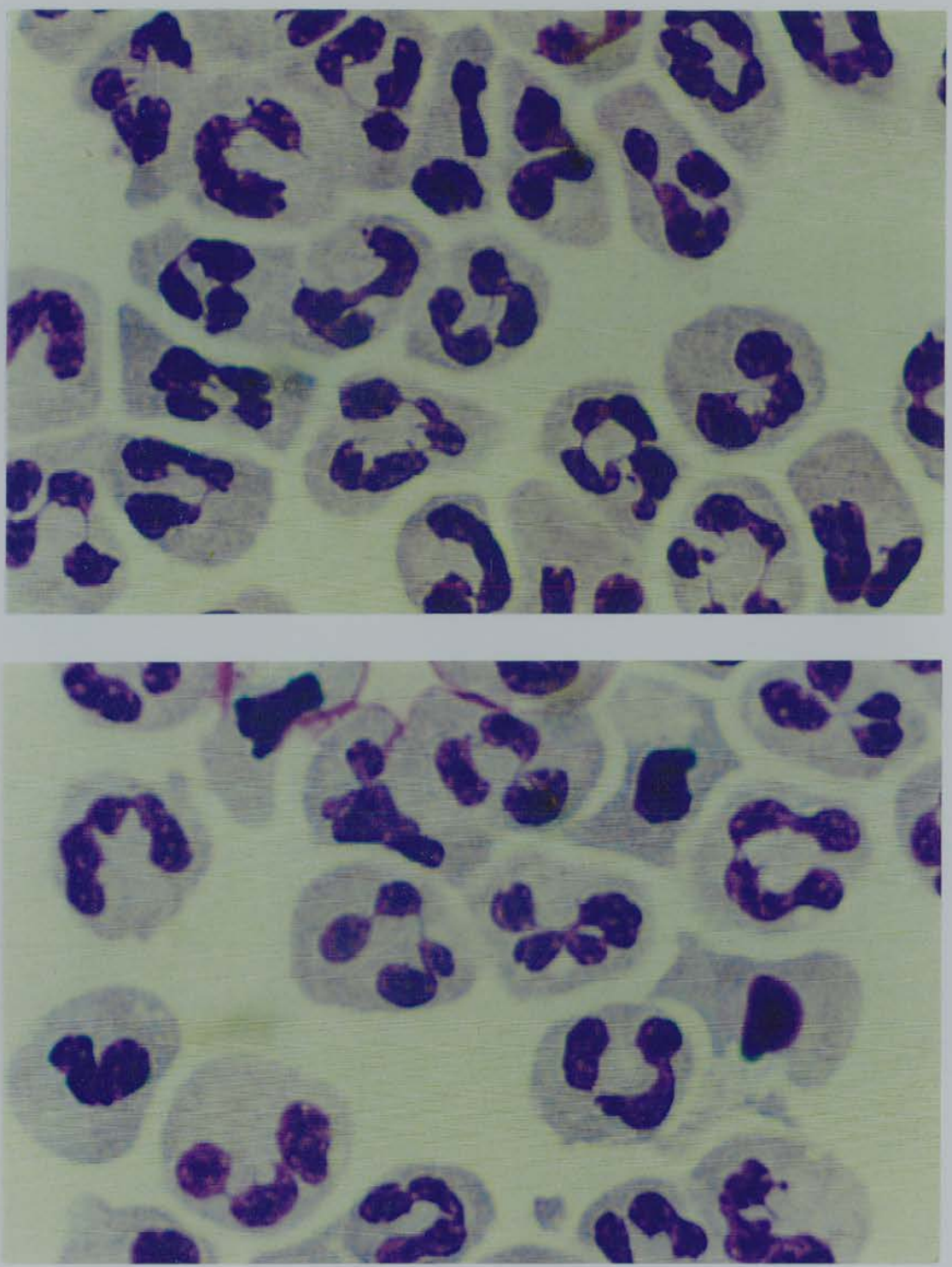
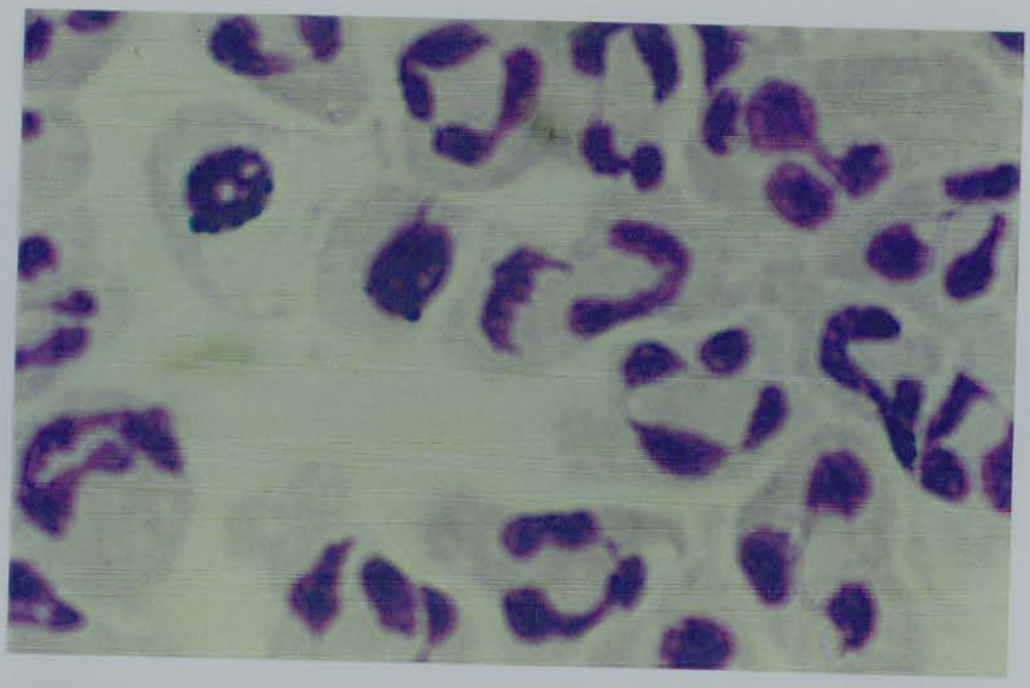


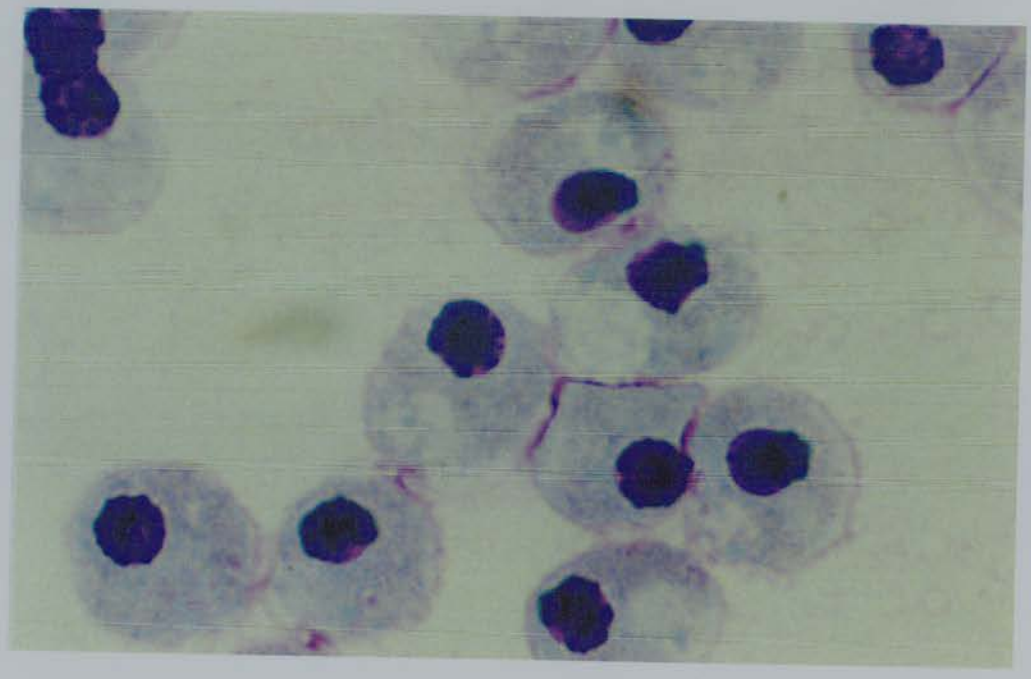
Figure 5.4 Morphology of Neutrophils Treated with Gliotoxin and TNF-α

Photomicrographs (x 1250 magnification) of cytocentrifuge preparations. Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone A) (control), B) with gliotoxin (0.01 μg/ml), C) with TNF-α (12.5 μg/ml) or D) with both gliotoxin and TNF-α. Cells were harvested at 2 h.

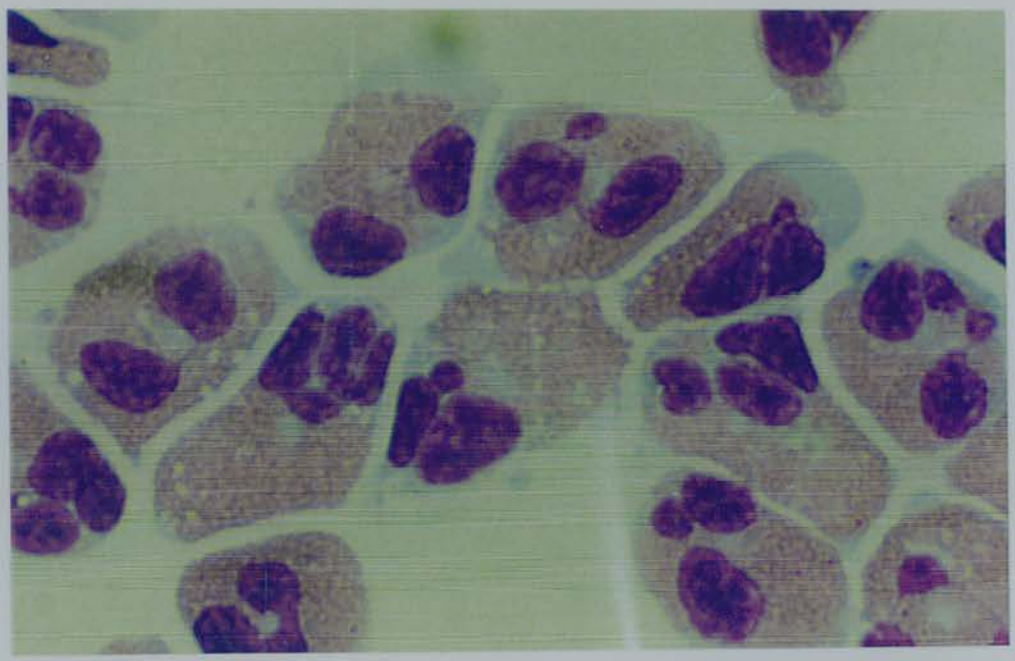
C)



D)



A)



B)

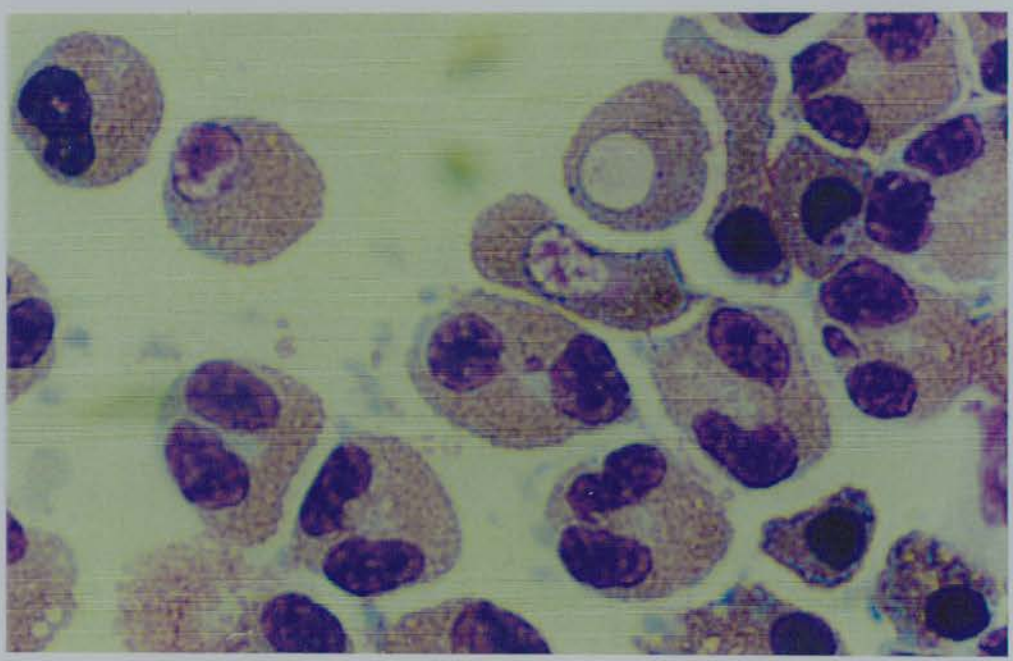
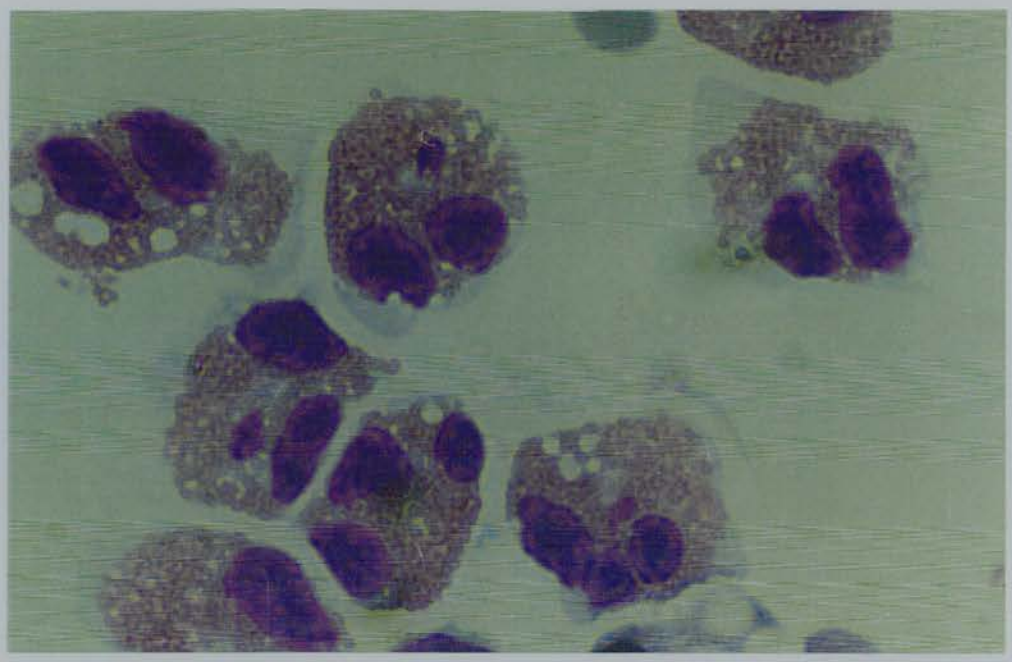


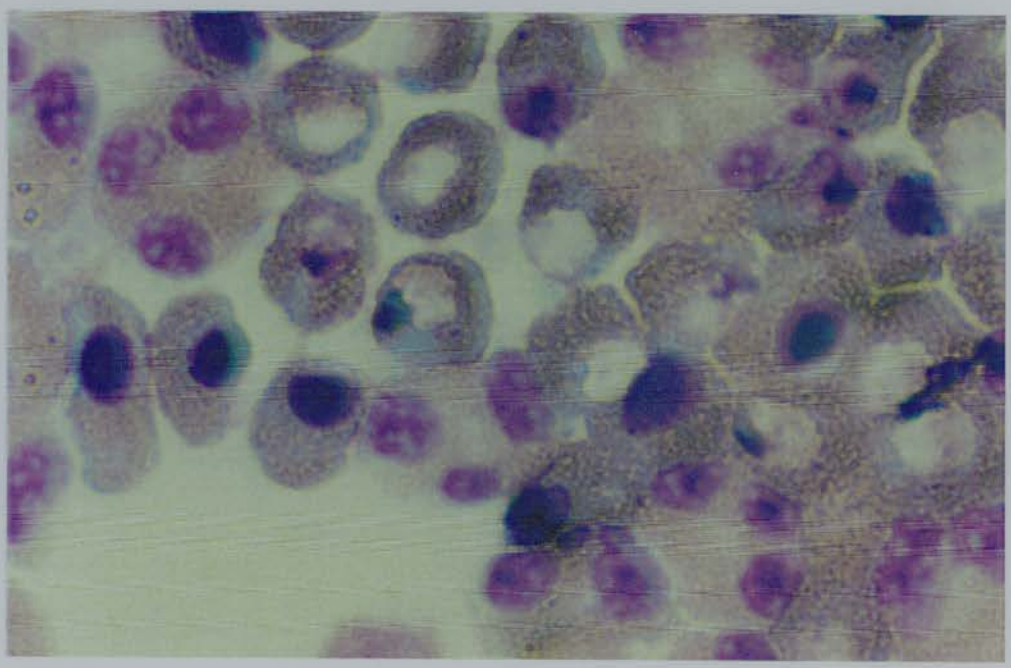
Figure 5.5 Morphology of Eosinophils Treated with Gliotoxin and TNF-α

Photomicrographs (x1250) of cytocentrifuge preparations of cultured eosinophils. Eosinophils (2 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone A) (control), B) with gliotoxin (0.01 μg/ml), C) with TNF-α (12.5 ng/ml) or D) with both gliotoxin and TNF-α. Cells were harvested at 4 h.

C)



D)



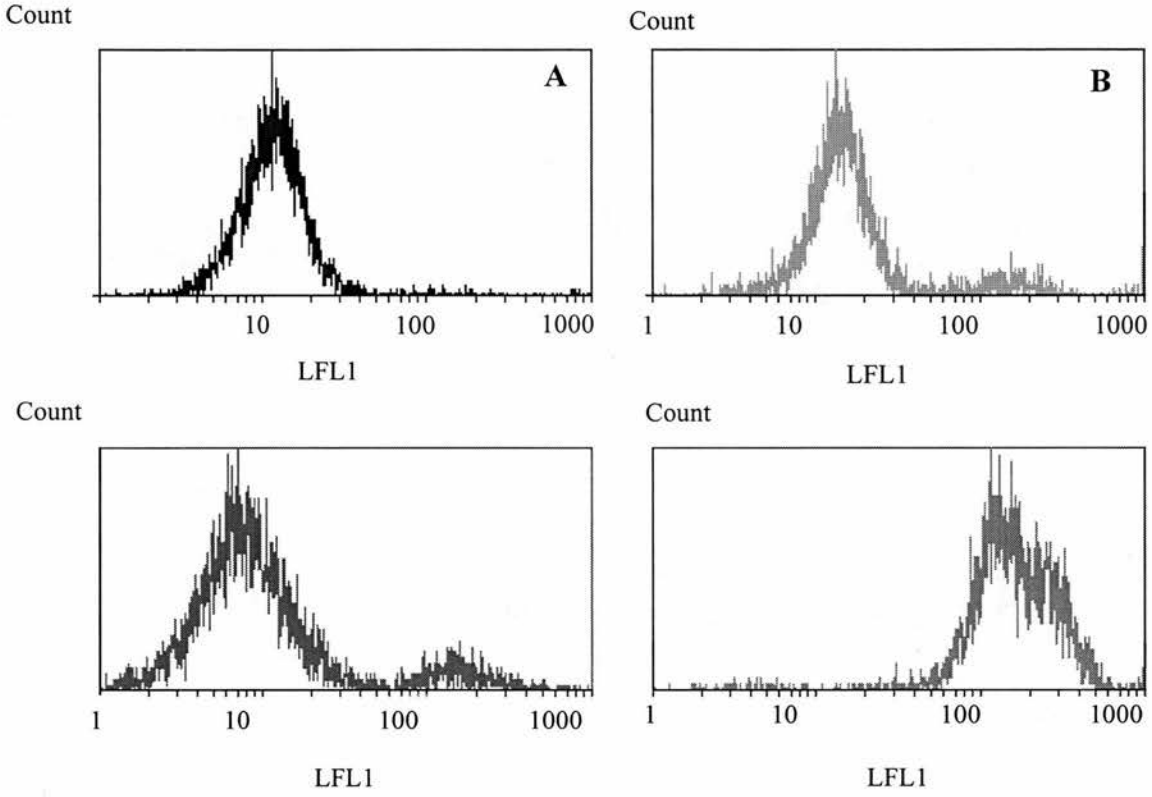


Figure 5.6 Flow Cytometry Profiles of Neutrophils Stained with Annexin V

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone A) (control), B) with gliotoxin (0.01 μg/ml), C) with TNF-α (12.5 ng/ml) or D) with both gliotoxin and TNF-α. Cells were harvested at 2 h and samples from each condition incubated with Annexin 'V'. Data shown are from 1 representative experiment.

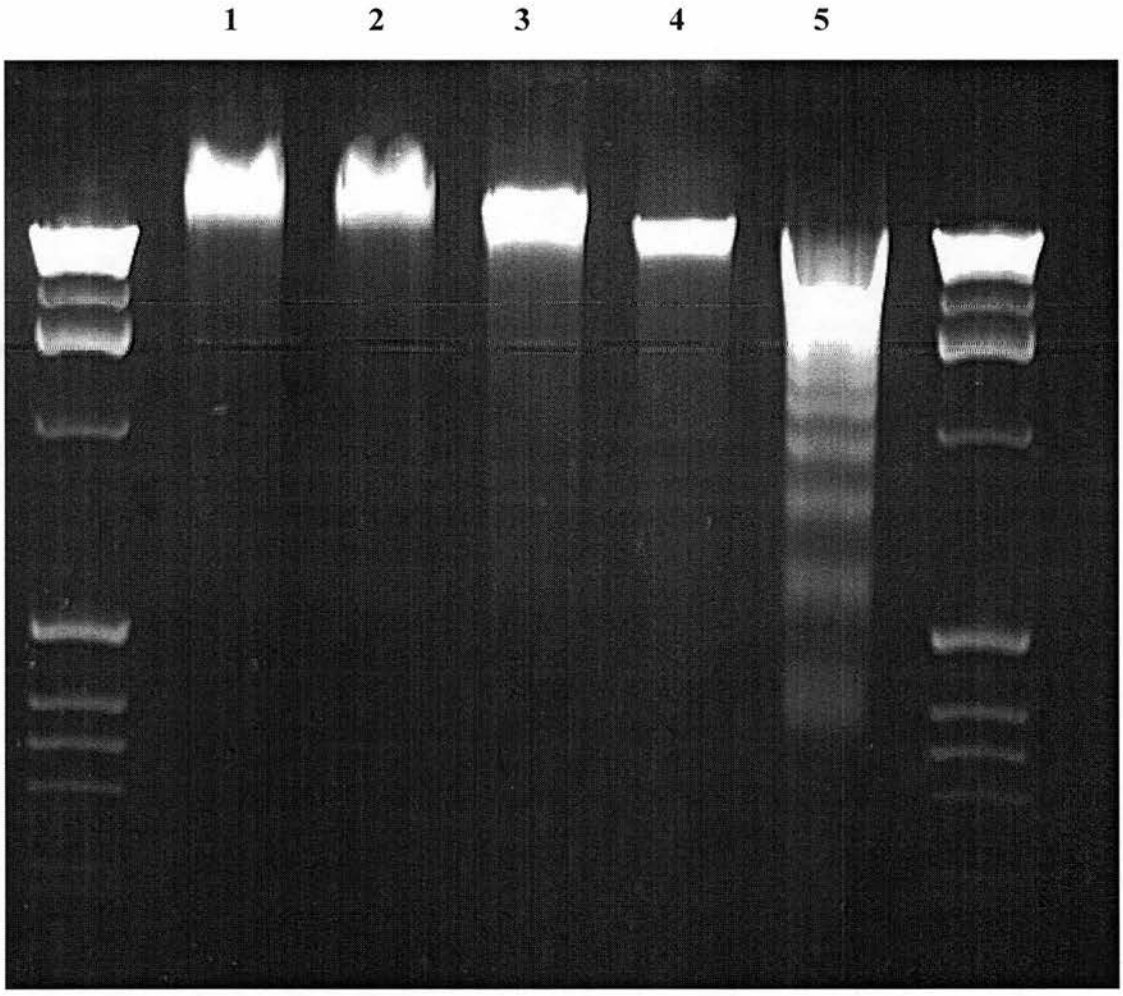


Figure 5.7 DNA Fragmentation

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum. Lane 1) freshly isolated neutrophils, lane 2) control, lane 3) cells cultured with gliotoxin (0.1 μg/ml), lane 4) cells cultured with TNF-α (12.5 ng/ml), lane 5) gliotoxin (0.1 μg/ml), and TNF-α (12.5 ng/ml). Cells were harvested at 2 h and DNA extracted and electrophoresed.

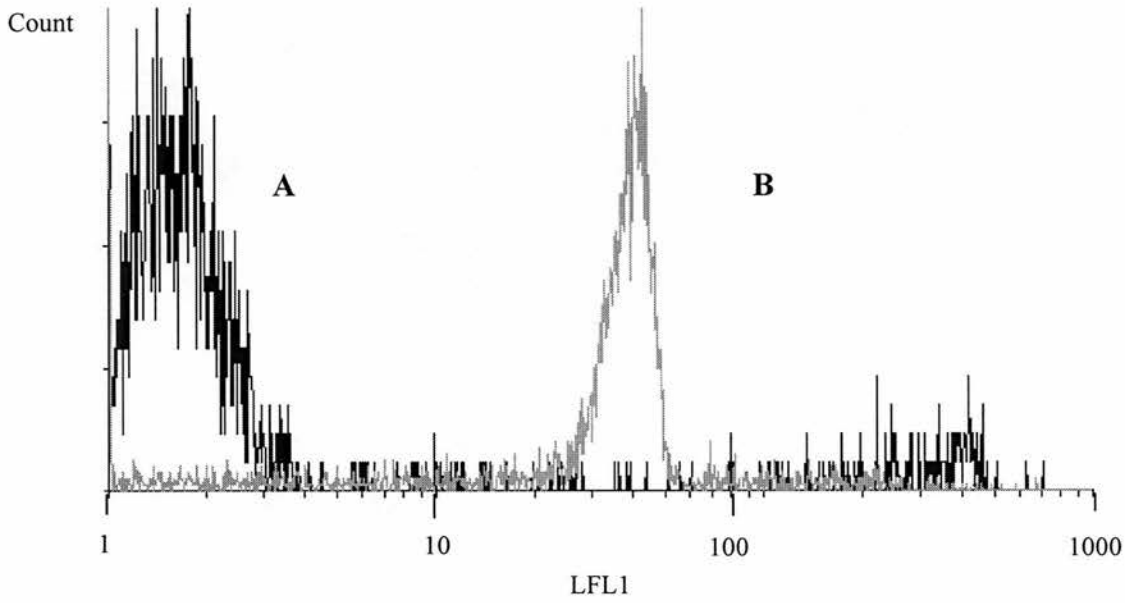


Figure 5.8 Flow Cytometry Profiles of Necrotic and Apoptotic Neutrophils Stained with Propidium Iodide

A) Neutrophils (5 x 10^6/ml) were incubated for 4 h with gliotoxin (0.1 μg/ml) and TNF-α (12.5 ng/ml). B) Cells were treated as above but heated-treated to ensure necrosis. Samples were stained with propidium iodide and analysed by flow cytometry. Data shown are from 1 representative experiment.

5.2.4 The Effect of Gliotoxin on NF-κB Activation

Gliotoxin has been reported to act as a specific inhibitor of NF-κB (Pahl et al., 1996). Experiments were aimed to identify and characterise the expression of NF-κB in human neutrophils and to determine whether gliotoxin could inhibit such activity. Because apoptosis results in the fragmentation of DNA (Figure 5.7), inhibition of NF-κB activity could be due to the loss of binding sites in apoptotic cells. Therefore a preliminary time-course was performed (Figure 5.9 A and B). At 1 h, no synergistic interactions were noted between TNF-α and gliotoxin. Ninety minutes was established as the optimal time to examine basal gliotoxin and TNF-α regulated NF-κB activity in neutrophils, and coincided with the onset of the biologically observable effect of gliotoxin (Figure 5.9 B).

As shown in Figure 5.10, NF-κB EMSAs performed on neutrophil nuclear extracts indicated the presence of 3 discrete bands. To ascertain which of these bands were specifically NF-κB, an excess of unlabelled probe was included in the labelling reaction to displace specific binding; as shown in Figure 5.11, two NF-κB bands were identified and designated A and B. No change in the intensity of band B was observed with any of the treatments undertaken (Fig 5.10). This, together with its strong expression in freshly prepared untreated neutrophils, suggests that this band may represent a form of constitutively active NF-κB. However, band A was markedly upregulated by TNF-α (Fig 5.10), and as shown in Figure 5.10, gliotoxin caused a concentration-dependent inhibition of this NF-κB activity and abolished the TNF-α–stimulated increase in this band. As shown in Figure 5.12, densitometric analysis of these data confirmed that co-treatment of neutrophils with TNF-α and gliotoxin at a lower concentration (0.1 μg/ml) inhibited this band more than treatment with gliotoxin alone. Treating neutrophils with LPS (100 ng/ml for 20 min) was found to cause the appearance of this inducible isoform of NF-κB (Figure 5.13) and this induction could also be inhibited by gliotoxin (0.1 μg/ml).

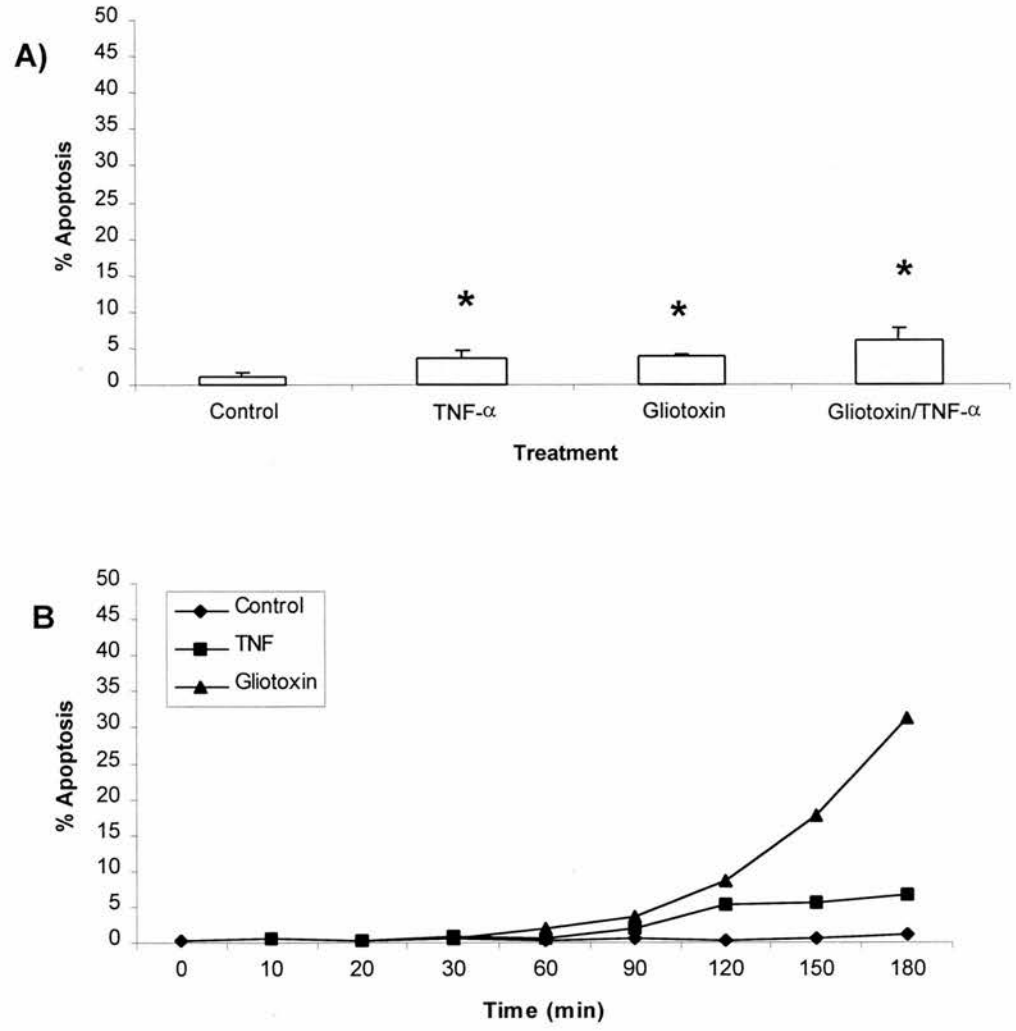


Figure 5.9 The Effect of Gliotoxin on Constitutive Neutrophil Apoptosis

A) Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or with gliotoxin (0.1 μg/ml) ± TNF-α (12.5 ng/ml) and harvested at 1 h. Data represent the mean ± SEM of 3 separate experiments performed in triplicate, (* $p < 0.05$ compared with control values). B) Neutrophils were incubated as above with gliotoxin (0.1 μg/ml) or TNF-α (12.5 ng/ml) and harvested at the timepoints shown. Data shown are from a representative experiment. Apoptosis was assessed morphologically.

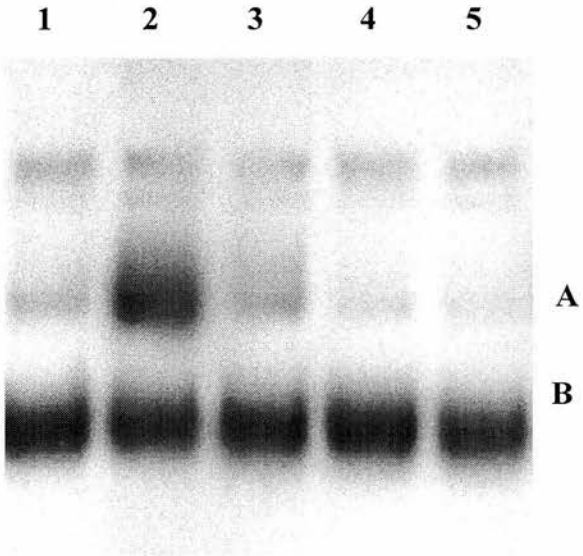


Figure 5.10 EMSA of NK-κB in Neutrophil Nuclear Extracts

Neutrophils (5 x 10^6/ml) were incubated for 90 min. Lane 1) control, lane 2) TNF-α (12.5 ng/ml), lane 3) gliotoxin (1 μg/ml), lane 4) gliotoxin (0.1 μg/ml) and lane 5) gliotoxin (0.1 μg/ml) with TNF-α. Nuclear extracts were prepared as detailed in Section 2.1.11 and samples electrophoresed.

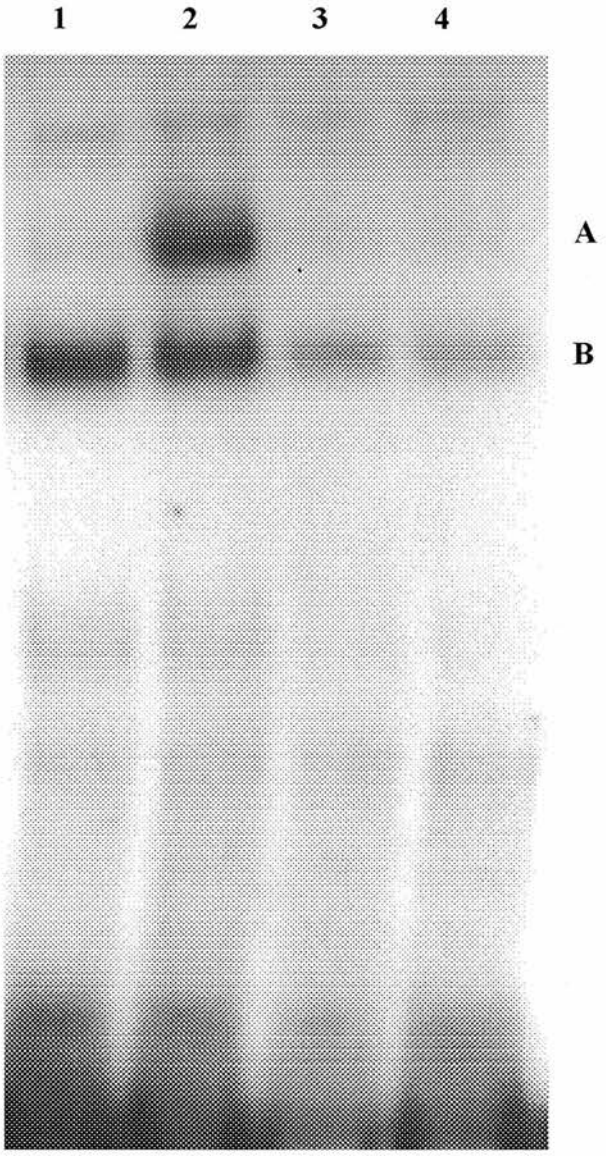


Figure 5.11 Displacement of NK-κB Binding by Cold Probe

Neutrophils (5 x 10^6/ml) were incubated for 90 min with serum alone (control) or TNF-α (12.5 ng/ml) for 90 min. Nuclear extracts were prepared as detailed in Section 2.2.10 Samples were incubated with labelled probe. Lane 1 Control, Lane 2 TNF-α, Lane 3 TNF-α with the addition of 50 x excess cold probe, Lane 4 TNF-α with the addition of 100 x excess cold probe. Excess cold AP-1 (negative control) did not displace binding (M. Lawson – personal communication).

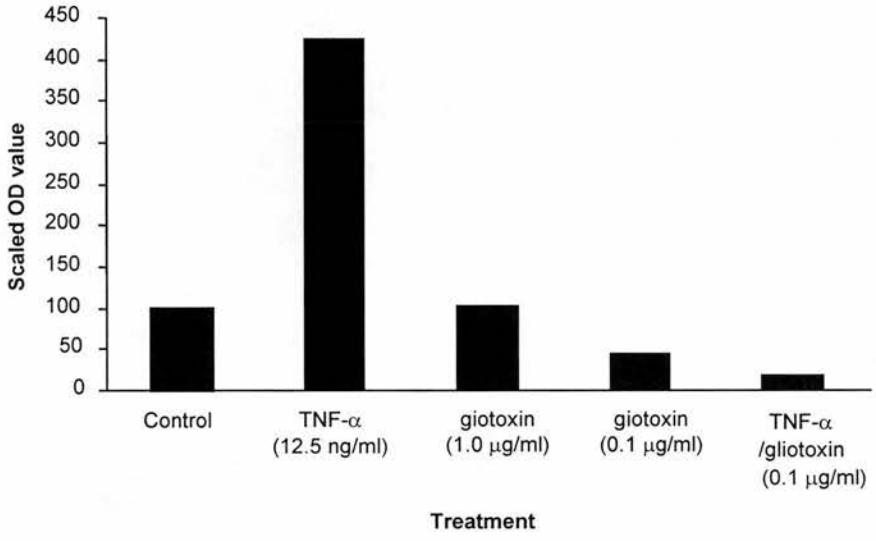


Figure 5.12 Densitometry Scanning of Gel Shown in Figure 5.11

Densitometry scan of gel shown in Figure 5.11. Data shown represent results for the inducible band A.

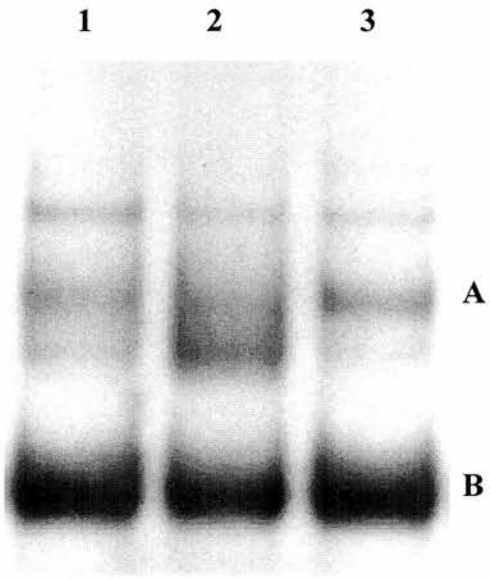


Figure 5.13 EMSA of Gliotoxin and LPS-treated Neutrophils

Neutrophils (5 x 10^6) were incubated in Iscove's DMEM. Lane 1) control, lane 2) LPS (100 ng/ml), and lane 3) LPS plus gliotoxin (0.1 μg/ml). Cells were harvested at 20 min and nuclear extracts prepared as detailed in Section 2.1.11.

5.2.5 The Role of Caspases in TNF-α- and Gliotoxin-Induced Apoptosis

To determine whether the pro-apoptotic effects of gliotoxin and the marked synergism displayed by TNF-α and gliotoxin were mediated via activation of the caspase pathway, neutrophils were co-incubated with TNF-α, gliotoxin and benzyloxycarbonyl-valinyl-alaninyl-aspartyl (O-methyl)-flouromethylketone (zVAD-fmk) (Figure 5.14). At 2 h, zVAD-fmk completely inhibited the increase in apoptosis induced by gliotoxin, TNF-α and by both factors together [TNF-α (12.5 ng/ml) 9.9 ± 3.1 %, TNF-α plus gliotoxin (0.1 μg/ml) 91.7 ± 4.0 %, gliotoxin in the presence of TNF-α plus ZVAD-fmk (10 μM) 2.9 ± 1.0 %]. This demonstrates that apoptosis induced by both factors, alone or together, is dependent on caspase activation.

The results shown in Figure 5.14 suggest that activation of the inducible form of NF-κB may inhibit the pro-apoptotic effects of TNF-α which are mediated by activation of the caspase pathway, possibly by the production of a protective protein or protein(s). As shown in Figure 5.15, the protein synthesis inhibitor CHX used specifically at a concentration (5 μM) that alone had almost no effect on neutrophil apoptosis at 2 h but caused a synergistic increase in the level of apoptosis when cells were co-cultured with TNF-α. [Control 0.5 ± 0.2 %, TNF-α (12.5 ng/ml) 5.9 ± 1.5 %, CHX (5 μM), 1.0 ± 0.2 %, CHX plus TNF-α 75.5 ± 4.9 %, CHX plus TNF-α and ZVAD-fmk (10 μM) 0.9 ± 0.4].

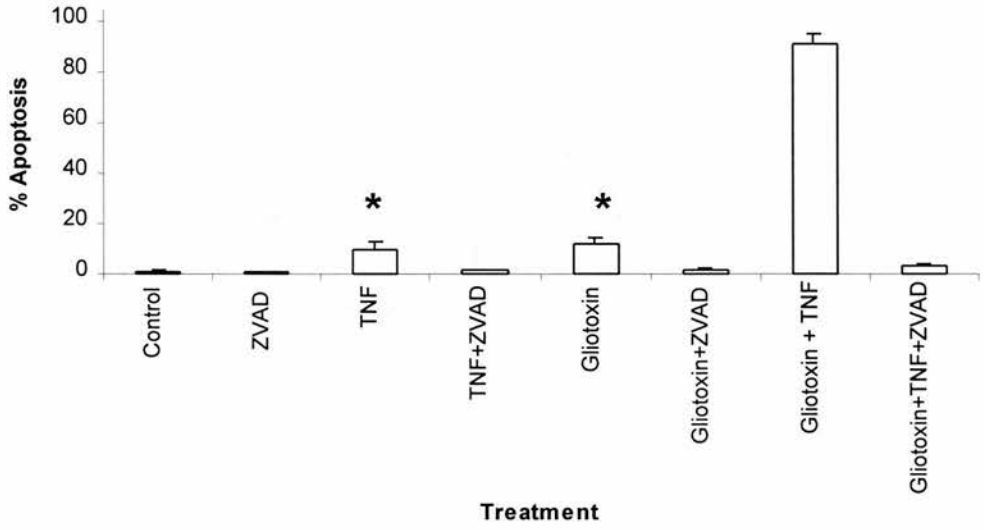


Figure 5.14 The Effect of zVAD-fmk on Gliotoxin and TNF-α-mediated Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), zVAD-fmk (10 mM), TNF-α (12.5 ng/ml) or gliotoxin (0.1 mg/ml), or in combination as shown. Cells were harvested at 2 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of three separate experiments. All experiments were performed in triplicate (*$p < 0.05$ compared with control values).

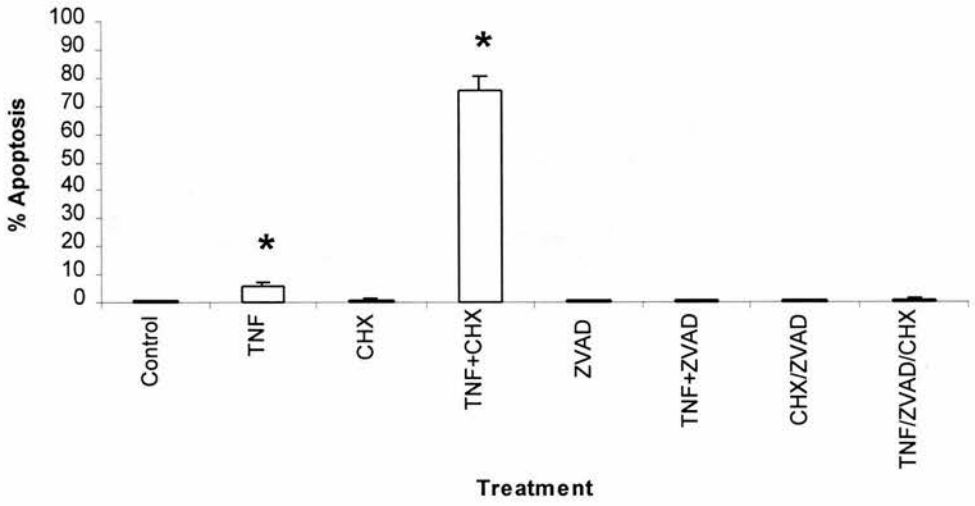


Figure 5.15 The Effect of Cycloheximide on TNF-α-induced Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), TNF-α (12.5 ng/ml), CHX (5 μM) or zVAD-fmk (10 μM), or in combination as shown. Cells were harvested at 2 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (*$p < 0.05$ compared with control values).

5.2.6 Other NF-κB Inhibitors

Cell permeable NF-κB inhibitory peptides (Lin et al., 1995b) also increased the rate of constitutive neutrophil apoptosis (Figure 5.16) despite the fact that less than 5 % of the peptide reportedly enters the cell (Lin et al., 1995b). At 6 h, the SN50 peptide increased neutrophil apoptosis from 4.7 ± 1.2 % to 15.0 ± 3.2 %, whereas the less active peptide SN50M only increased apoptosis from 4.7 ± 1.2 to 5.6 ± 0.8 %. Similar effects on neutrophil apoptosis were seen at 20 h.

Figure 5.17 illustrates the effect of the PKC inhibitor Ro 31,8220 on neutrophil apoptosis at 2h. At this timepoint, concentrations of Ro 31,8220 (0.01 - 10 μM) had no affect on constitutive neutrophil apoptosis alone, but a concentration of 10 μM with TNF-α caused a synergistic increase from 8.0 ± 4.6 % (TNF-α) to 67.0 ± 7.0 %.

NO has also been reported to inhibit NF-κB activity (Matthews et al., 1996). As demonstrated in Figure 5.18, the NO donor GEA 3162 also induced neutrophil apoptosis at 4 h [control 1.4 ± 0.4 %; GEA 3162 (30 μM) 14.8 ± 4.6 %], and more than doubled the TNF-α effect from 11.3 ± 1.9 to 26.3 ± 1.5 % at this timepoint.

The antioxidant PDCT (Figure 5.19) significantly increased neutrophil apoptosis at 20 h [control 59.2 ± 8.0 %; PDCT (300 μM) 85.4 ± 3.3 %]. However there was no significant difference in rates of apoptosis between TPCK (20 μM) treated neutrophils and control at 20 h (Figure 5.20).

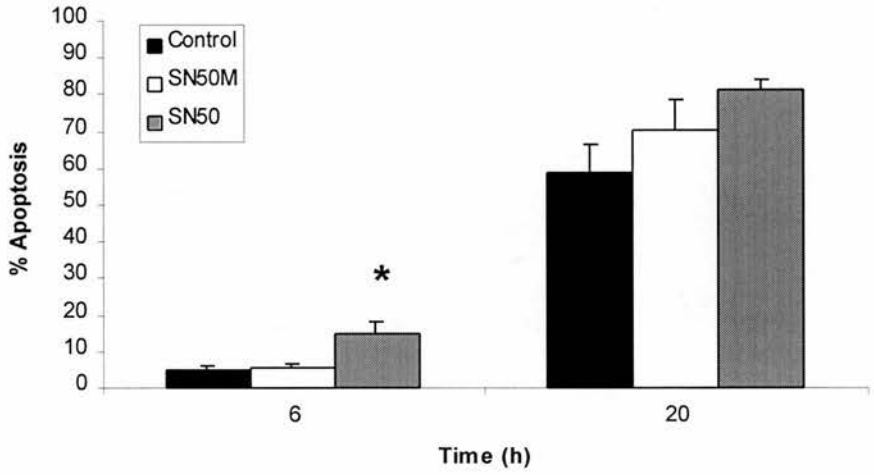


Figure 5.16 The Effect of the NF-κB Inhibitory Peptide SN50 on Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were pre-incubated for 15 min with SN50 or SN50 M (100 μg/ml) in Iscove's DMEM before the addition of 10% autologous serum to all treatments. Cells were harvested at 6 and 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (*$p < 0.05$ compared with control values).

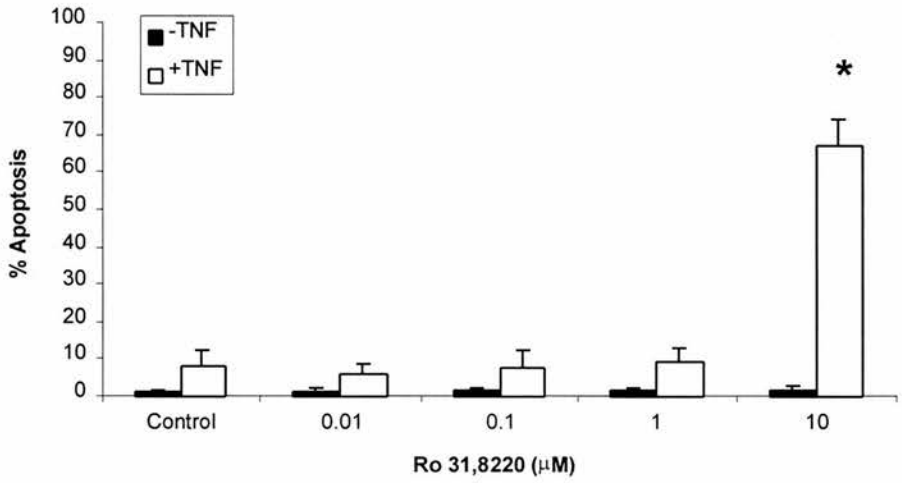


Figure 5.17 The Effect of the PKC inhibitor Ro 31,8220 on Constitutive and TNF-α-induced Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or Ro 31,8220 (0.01 - 10 μM) or TNF-α (12.5 ng/ml) as shown. Cells were harvested at 2 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (*$p < 0.05$ compared with control values).

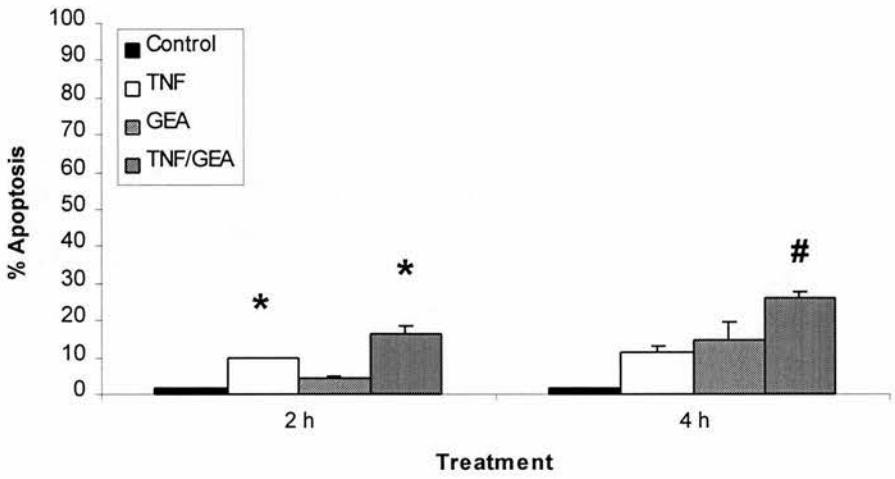


Figure 5. 18 The Effect of the NO Donor GEA 3162 on Constitutive and TNF-α-Induced Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control), TNF-α (12.5 ng/ml) or GEA 3162 (30 μM) as shown. Cells were harvested at 2 and 4 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were preformed in triplicate (*$p < 0.05$ compared with control values, # $p < 0.05$ compared with TNF-α alone).

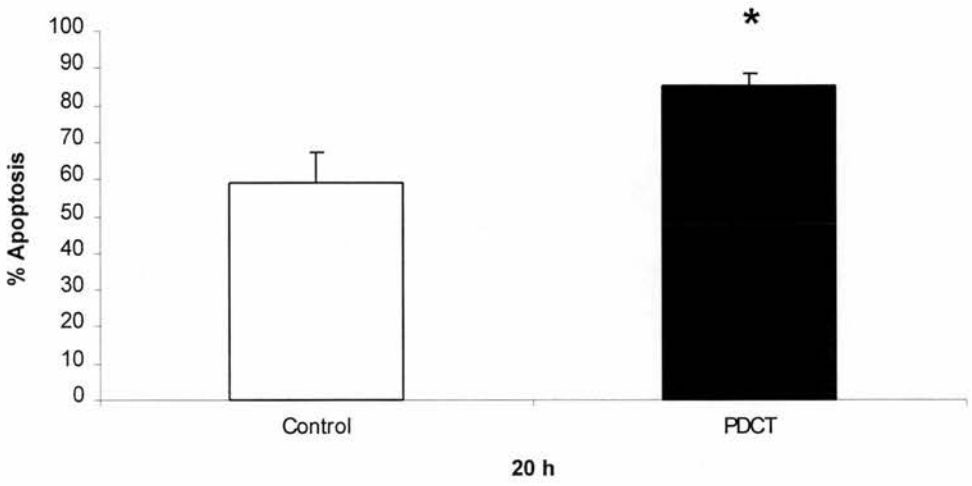


Figure 5.19 The Effect of the Antioxidant PDCT on Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum (control) or PDTC (300 μM). Cells were harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate (*p <0.05 compared with control values).

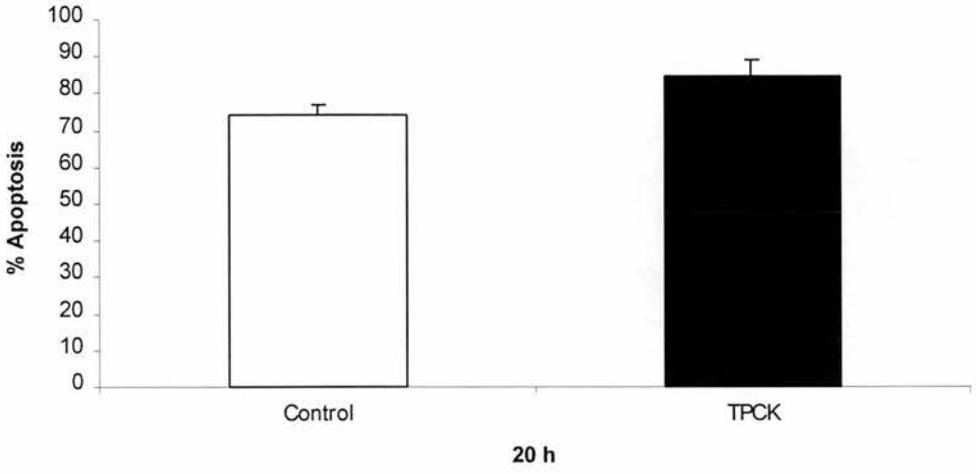


Figure 5.20 The Effect of TPCK on Neutrophil Apoptosis

Neutrophils (5 x 10^6/ml) were incubated in Iscove's DMEM supplemented with serum alone (control) or TPCK (20 μM). Cells were harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SEM of 3 separate experiments. All experiments were performed in triplicate.

5. 3 Discussion

Collectively, the results described in this chapter suggest that the transcription factor NF-κB has a central role in the survival of neutrophils and eosinophils.

Gliotoxin, a reportedly specific inhibitor of the RelA/p50 isoform of NF-κB at the concentrations used in these experiments (Pahl et al., 1996), induced a rapid, time- and concentration-dependent increase in apoptosis in both neutrophils and eosinophils (Figure 5. 1 A and 5.3 A), which was not mimicked by methylthiogliotoxin, an inactive analogue (Pahl et al., 1996). Gliotoxin produced a synergistic increase in TNF-α-mediated apoptosis in neutrophils (Figure 5.2 A), and rendered eosinophils responsive to the pro-apoptotic effects of this cytokine (Figure 5.3). Co-culture of both granulocyte types with gliotoxin and TNF-α produced maximal effects at the same concentration of gliotoxin (0.1 μg/ml), suggesting a common mechanism for the induction of apoptosis in these cells. Although rates of apoptosis fell as the concentration of gliotoxin increased in co-culture experiments, the higher concentrations might have produced non-specific effects, perhaps activating survival pathways. Pahl et al. (1996) found that concentrations of gliotoxin higher than 1.0 μg/ml had inhibitory and stimulatory effects on the activities of several unrelated gene promoters and enhancers. The concentrations that give specific effects may also vary with cell type, therefore the effects of gliotoxin in granulocytes should be examined further by investigating its effects on transcription factors other than NF-κB. However, data (Figures 5.10 - 5.13) clearly show that low concentrations of gliotoxin inhibit NF-κB in neutrophils (Figure 5.10 - 5.13).

EMSAs showed 2 specific bands in neutrophil nuclear extracts. Band B appeared to be constitutively active while Band A showed inducible activity being upregulated by TNF-α and LPS (Figure 5.10 and 5. 14). These results are in accordance with McDonald et al. (1997), who also found constitutive activity in these cells. Band A was markedly diminished by gliotoxin at 1.0 and 0.1 μg/ml. However, the greatest inhibition of this band was induced by gliotoxin at 0.1 μg/ml plus TNF-α. These

concentrations corresponded with those giving the greatest increase in apoptosis in both neutrophils and eosinophils (Figure 5.2 A and 5.3 B). EMSAs distinctly show inhibition of the inducible isoform of this transcription factor (Figure 5.10 to 5.13) before the onset of apoptosis at 90 min (Figure 5.9). Data presented in Figures 5.4 – 5.8 confirm that cell death was apoptotic, and not necrotic, and demonstrate that despite the rapid induction of apoptosis in granulocytes by co-culture with these two agents, no cytotoxic effects were produced. These results suggest a strong correlation between inhibition of this inducible isoform of NF-κB and the onset of apoptosis. This observation is further strengthened by the induction of this band by treatment with LPS (Figure 5. 13), a known inhibitor of granulocyte apoptosis (Lee et al., 1993) and confirmed in Figure 3.4. In control neutrophils incubated for 20 h, where the constitutive rate of apoptosis is approximately 70% (Figure 2.1), the density of the constitutive NF-κB band was unaffected (data not shown), implying that survival effects are mediated totally by the inducible band.

Embryonic fibroblasts from RelA-deficient mice are defective in the TNF-α-mediated induction of mRNA for IκBα and GM-CSF, although basal levels of these transcripts are unaltered. This indicates that RelA controls inducible, but not basal transcription in NF-κB regulated pathways and that Rel A is a key regulator of genes inducible by TNF-α (Beg et al., 1995). These differences in the inducible and constitutive forms of NF-κB are most likely due to differential regulation of activation, for example, by the involvement of different isoforms of the inhibitory IκB subunit, or perhaps to the formation of the constitutively active NF-κB from a different set of dimers from the classical RelA/p50 heterodimer. It has recently been demonstrated that neutrophils contain c-Rel, p50, and p105 (the p50 precursor protein) as well as RelA (Druker et al., 1994, McDonald et al., 1997). The inducible band observed has also been reported to be upregulated by phagocytosis of IgG opsonised yeast particles (McDonald & Cassatella, 1997), and has been shown to consist mainly of RelA/p50 heterodimers and possibly a small amount of c-Rel. In that study, particle phagocytosis did not affect the activity of the constitutive complex.

Several mechanisms, aside from NF-κB inhibition, have been proposed for the pro-apoptotic actions of gliotoxin in other cells. Sutton et al. (1995), have shown that although this fungal metabolite did not affect intracellular Ca^{2+} levels, there was a correlation between increases in cAMP levels and apoptosis in gliotoxin-treated splenocytes. However, agents that elevate intracellular cAMP inhibit apoptosis in both neutrophils and eosinophils (Rossi et al., 1995, Hallsworth et al., 1996 and Figure 3.20). It has also been suggested that PKA-dependent phosphorylation of histone H3 correlates with gliotoxin-induced apoptosis in thymocytes (Waring et al., 1997), but again in neutrophils, activation of PKA inhibits apoptosis (Rossi et al., 1995). Although gliotoxin has been reported to inhibit protein synthesis (Waring, 1990) it is highly unlikely that this mechanism is directly responsible for its pro-apoptotic effects. Firstly, gliotoxin induces apoptosis in thymocytes whereas cycloheximide inhibits it. Secondly, NF-κB activation is involved in the control of multiple genes, many of which encode inflammatory mediators so inactivation of NF-κB would be expected to inhibit protein synthesis in granulocytes. Thirdly, while protein synthesis inhibitors do upregulate the rate of constitutive cell death in granulocytes, the kinetics of this response are very different from those observed with gliotoxin. For example, Whyte et al. (1997), have reported that cycloheximide (50 μg/ml) and actinomycin D (1 μM) induce apoptosis in approximately 30% of neutrophils by 6 h. In the present study, gliotoxin (0.1 μg/ml) induced a rate of almost twice this, whereas 1.0 μg/ml gliotoxin induced 100 % neutrophil apoptosis by 6 h (Figure 5.1 A). Likewise the results with cycloheximide (Figure 5.15) indicate that protein synthesis inhibition alone does not affect the rate of neutrophil apoptosis at 2 h, whereas gliotoxin alone produced almost 15% apoptosis over this period (Figure 5.1 A). This suggests that other mechanisms, such as inhibition of NF-κB, may be involved.

This is supported by our data from other NF-κB inhibitors in neutrophils. The cell-permeable NF-κB inhibitor peptide SN50 (Lin et al., 1995b) significantly increased neutrophil apoptosis at the timepoints examined, although this increase was not as marked as that produced by gliotoxin. However, since less than 5 % of this peptide enters the cell within 30 min (Lin et al., 1995b) the actual intracellular concentration

of this inhibitor in the cell is unknown, but it is likely to be low. The peptide competes with activated NF-κB complexes for translocation to the nucleus, presumably by binding to a receptor/transportation protein, but it is not known whether binding is reversible. Therefore, inhibition could be transient, allowing some activation of NF-κB to occur. It is also possible that other transcription factors/nuclear proteins share the same NLS sequence, so while the peptide inhibits nuclear NF-κB import, the effect may not be specific.

Inhibition of PKC by Ro 31,8220 did not affect constitutive apoptosis in neutrophils after 2 h, but it did synergistically increase the pro-apoptotic effect of TNF-α when used at 10 μM, suggesting that PKC might be involved in NF-κB activation. Ro 31,8220 is a broad spectrum PKC inhibitor, but unfortunately there is no readily available specific PCK ζ inhibitor that would allow investigation of the effect of this isoform, which has been linked to NF-κB activation. These data in Figure 5.17, do suggest a role for PKC in protecting cells against the cytotoxic effects of TNF-α, but further work on activation/inhibition of NF-κB when PKC is manipulated would have to be undertaken before supporting evidence could be obtained.

The NO donor GEA 3162 also induced apoptosis in neutrophils. These results are in agreement with data produced in our laboratory [Wong et al., (1997)]. NO has also been reported to inhibit NF-κB activity (Matthews et al., 1996) and interestingly, it also increased the pro-apoptotic effect of TNF-α. Again, EMSAs should be performed to check the effect of GEA 3162 and other NO donors on NF-κB activity in granulocytes, both alone and in concert with TNF-α.

The antioxidant PDCT and the serine protease inhibitor TPCK are used widely in NF-κB inhibition studies. Interestingly, PDCT significantly induced neutrophil apoptosis, while TPCK had no effect. The results for PDCT are in concert with those obtained by other putative NF-κB inhibitors in these studies, but the negative result for TPCK might be attributed to by non-specific effects of this inhibitor. Increases in ROS have been linked to possible mechanisms for inducing apoptosis, particularly

via TNF-α. However, the results for PDCT treatment (Figure 5.19) would suggest that this is not the mechanism involved in neutrophils, since antioxidants would decrease ROS and therefore should inhibit apoptosis as does hypoxia in these cells (Hannah et al., 1995). However, it is questionable whether reactive oxygen species are widely used by cells to commit suicide. Apoptosis has been observed in the absence of mitochondrial respiration and in cells grown essentially without oxygen, conditions that should greatly reduce the amount of free radicals (Jacobson et al., 1993).

One group has claimed that antioxidants can inhibit spontaneous neutrophil apoptosis (Oishi & Machida, 1997). In this thesis, no neutrophil data are presented on cells incubated for more than 20 h in culture because control cells exhibit rising levels of necrosis after this timepoint. Since Oishi & Machida, (1997) used neutrophils in culture for 60 h, the neutrophils would have been mainly necrotic, and not apoptotic. Other studies, in contrast, have shown that resting neutrophils respond to Fas engagement with accelerated rates of apoptosis, but activated neutrophils, with increased ROI production, are refractory to signals delivered through Fas (Watson et al., 1997). Fas engagement did not alter levels of intracellular ROIs or thiols (Watson et al., 1997), but depletion of endogenous antioxidant thiols induced apoptosis of resting neutrophils (Watson et al., 1997). Pretreatment of neutrophils with butionine sulfoximine (BSO), a specific inhibitor of GSH biosynthesis, did not prevent an LPS-induced reduction in apoptosis (Watson et al., 1997). These studies suggest that ROS production should inhibit neutrophil apoptosis. However, granulocytes use ROS as a major defence against microorganisms and therefore they might have protective mechanisms to prevent self-damage from ROS release. Therefore, granulocytes might respond differently from other cell types to ROS.

Cytotoxic effects of TNF-α have recently been reported to be potentiated by inhibitors of NF-κB (Beg & Baltimore, 1996, Van Antwerp et al., 1996, Wang et al., 1996), supporting the hypothesis that gliotoxin does function to limit NF-κB activation. Embryonic fibroblasts and macrophages from Rel-A deficient mice also showed dramatic loss of viability when treated with TNF-α, leading to the

suggestion that Rel-A regulates a protective mechanism against the cytotoxic effects of TNF-α. Although the experiments presented here indicate that gliotoxin does not inhibit the constitutive form of NF-κB, at least at early time-points, it does inhibit the activation of an inducible isoform of NF-κB, which most likely consists of heterodimers containing the Rel-A/p65 protein and therefore could perform a similar anti-apoptotic function in neutrophils and eosinophils. While TNF-α does not produce significant cytotoxic effects in eosinophils, co-treatment with gliotoxin caused these cells to become highly responsive to this cytokine, producing greatly increased levels of apoptosis. This suggests that both of these inflammatory cell types could be stimulated to undergo apoptosis and hence be cleared rapidly by phagocytes at sites of inflammation if activation of the inducible NF-κB isoforms were inhibited.

The mechanism by which NF-κB inhibition induces granulocyte apoptosis and increases the cytotoxic response to TNF-α is currently unclear. Since gliotoxin and TNF-α driven apoptosis are both inhibited by zVAD-fmk a cell permeable, irreversible tripeptide inhibitor of caspases (Dolle, et al., 1994, Pronk et al., 1996), NF-κB or an NF-κB regulated intermediate might influence granulocyte apoptosis at a stage in the pathway between the TNF-α receptor and caspase activation. However, the fact that a potentiated TNF-α effect can be achieved with the protein synthesis inhibitor CHX (Figure 5.15), which is also blocked by zVAD-fmk, suggests that NF-κB may upregulate the synthesis of a protective protein or proteins, which inhibits activation of the caspase pathway in response to TNF-α.

The simplest conclusion from these observations is that one or more of the NF-κB-dependent genes that are induced by TNF-α protect cells from apoptosis. Such a scenario also helps explain why, in most cell types, TNF-α is not cytotoxic unless the cells are simultaneously exposed to an inhibitor of RNA or protein synthesis, which may block expression of protective NF-κB-dependent genes. The putative anti-apoptotic genes that are activated by NF-κB in response to endogenous TNF-α remain to be identified.

NF-κB induces activation of genes for G-CSF, GM-CSF, TNF-α, NO synthase, and c-*myc* (Baeuerle, 1991, Geller et al., 1993, Muller et al., 1993). Given that both TNF-α and TGF-β (a reputed NF-κB inhibitor) synergistically downregulate the production of c-Myc (Hori et al., 1994, Arsura et al., 1996), this is one protein which may be involved. Hori et al. (1994) found that TNF-α used at concentrations greater than 1 ng/ml reduced c-Myc expression by 30 min with maximal reduction at 1 h. This would correlate with the onset of apoptosis in our studies (Figure 4. 17, 5.2 A, 5.3 B. In HL-60 cells, c-Myc is rapidly downregulated in apoptotic cells when TNF-α is used as the inducing agent; apoptosis could also be induced by c-*myc* antisense (Kumakura et al., 1996). Of interest is the short half-life of this protein (approx. 20 min) (Waters et al., 1991). Therefore the short time between signal and induction of cell death (approx. 90 min in this system) could be mediated via the rapid removal of a protective factor such as c-Myc. This will differ in other systems for example, in activation-induced apoptotic cell death in T cells hybridomas, antisense c-myc prevents apoptosis (Shi et al., 1992). Although the protein is present, there is little expression of c-Myc in peripheral blood neutrophils (Cotter et al., 1994, Hannah, personal communication).

NF-κB has been shown to interact directly with AP-1 subunits and to synergise with a number of different transcriptional activator proteins including Sp1, Ets, ATF-2/c-Jun, and NF-κF6 (Stein et al., 1993a, b). This synergy is a consequence of the direct interactions between NF-κB and these proteins and cooperative binding to adjacent binding sites. The interactive transcription factor can be present constitutively or coinduced with NF-κB. An example of the latter possibility is the recent finding that several inducers of NF-κB activate the c-Jun terminal protein kinase (JNK), which in turn phosphorylates ATF-2 and c-Jun both of which synergise with NF-κB (Gupta et al., 1995, Lee et al., 1997a). C-Jun deficient mice die at mid to late gestation and exhibit impaired hepatogenesis, altered fetal liver erythropoiesis and generalized oedema; in the liver, apoptosis and necrosis of cells are apparent (Hilberg et al., 1993). Interestingly, this pathology is similar to that of the RelA/p65 deficient mouse (Beg et al., 1995). Therefore apoptosis induced by NF-κB inhibition may be

induced by dysregulation of genes which are controlled by other transcription factors due to loss of synergistic and other interactions. The results for the c-Jun-deficient mouse suggest that this protein could also be important. As shown in Figure 1.2, TNF-α will activate both NF-κB and AP-1.

A20 is a novel zinc finger protein originally identified as a TNF-α- and IL-1-inducible gene product in endothelial cells (Dixit et al., 1990). It inhibits TNF-α-induced apoptosis (Opipari et al., 1992). Protection mediated by this protein is specific for TNF-α-induced apoptosis but it also inhibits TNF-α-induced activation of phospholipase A_2 and activation of NF-κB and AP-1 (Jaattela et al., 1996). A20 expression is regulated by NF-κB (Krikos et al., 1992) and interacts with TRAF1 and TRAF 2, inhibiting the activation of NF-κB by TRAF2, TNF, CD40 TRADD and IL-1 (Song et al., 1996). The mechanism by which A20 exerts its protective effect is unknown, but it does bind to several isoforms of the 14-3-3 proteins *in vitro* (Vincenz & Dixit, 1996). Interestingly, 14-3-3 proteins have been shown to function as regulators of PKC (Aitken et al., 1992). Activation of CD40, another TNFR, leads to the induction of A20. The induction of transcripts occurs within 1 h, which renders B cell lines resistant to apoptosis (Sarma et al., 1995). However, A20 does not rescue RelA null fibroblasts from the pro-apoptotic effects of TNF-α (Beg & Baltimore, 1996), indicating that other NF-κB driven mechanisms are involved.

Another protein, c-IAP2 is up regulated following TNF-α treatment and protects cells from apoptosis (Chu et al., 1997). This protein is recruited to the TNF receptor through TRAF proteins (Rothe et al., 1995) and is an NF-κB inducible gene product (Chu et al., 1997). However, exogenous cIAP2 expression could not protect cells from TNF-α-induced apoptosis even with activation of NF-κB (Chu et al., 1997).

Manganese superoxide dismutase (an antioxidant) suppresses TNF-α-induced apoptosis and activation of NF-κB and AP-1 in human breast cancer MCF-7 cells (Manna et al., 1998). This protein provides partial protection against apoptosis when expressed constitutively (Wong et al., 1989). However a role for NF-κB in regulation

of manganese superoxide dismutase gene expression has not yet been established (Baichwal & Baeuerle, 1997).

The role of NF-κB as a promoter or attenuator of cell death may ultimately depend on both the cell type and the nature of the apoptosis-inducing stimulus. In different cell types, NF-κB could perform these opposing functions by activating distinct patterns of genes in conjunction with cell-type-specific transcription factors (Baichwal & Baeuerle, 1997), and its behaviour might vary with different stimuli.

The ability of gliotoxin to enhance the cytotoxic effects of TNF-α and itself produce a rapid onset of apoptosis in inflammatory cells such as neutrophils and eosinophils may suggest NF-κB inhibition as a logical therapeutic target in the treatment of inflammatory conditions. In a rat model of lung inflammation, suppression of NF-κB activity has already been shown to block the development of neutrophil lung inflammation by inhibiting the synthesis of chemotaxins (Blackwell et al., 1996). These results, together with results presented in this Chapter, suggest that NF-κB inhibition may also be of benefit in enhancing the resolution of inflammation by allowing a more rapid clearance of granulocytes.

CHAPTER 6

SUMMARY

The safe removal of aged granulocytes from an inflammatory site by apoptosis is now considered a central part of the mechanism underlying the processes of healing and resolution, but the physiological mediators which control this response are unknown. Many of the inflammatory mediators present at a site of tissue injury *in vivo,* have previously been demonstrated to prolong the life of both neutrophils and eosinophils *in vitro*. If this situation prevailed in an *in vivo* situation, the result would be chronic inflammatory conditions as effete cells became fragile and necrotic and histotoxic cell contents are released. This thesis examined the role of inflammatory mediators of various different classes, in controlling apoptosis in both neutrophils and eosinophils, with particular interest in those which could drive cell death, with the long term aim of finding possible physiological factors that may be involved in the natural process of clearance of these cells.

Generally, culture of these cells with most inflammatory mediators, including chemokines, leukotrienes and prostaglandins either inhibited granulocyte apoptosis or had no significant effect. However, prostaglandins of the J series produced dramatic increases in apoptosis in both neutrophils and eosinophils. PGD_2 is produced in large quantities by mast cells, and is therefore a likely prostanoid to be present at inflammatory sites in conditions such as asthma; it is also produced by both neutrophils and eosinophils. Neutrophil apoptosis however, was not affected by PGD_2, but it produced large increases in the rate of eosinophil apoptosis. The metabolites of PGD_2 were potent inducers of cell death in both cell types, raising the possibility that PGD_2 may be differentially metabolised in neutrophils and eosinophils. The PGD_2 metabolites, Δ^{12}-PGJ_2 and 15-deoxy-Δ^{12},Δ^{14}-PGJ_2 are ligands for peroxisome proliferator activated receptors (PPARs), and therefore these receptors may be involved in the induction of granulocyte apoptosis. PPARs can

antagonise transcription factors such as STATs, AP-1 and NF-κB, all of which are involved in regulating various inflammatory mechanisms. This would therefore be an attractive model to account for granulocyte apoptosis as a part of the processes involved in resolution, since PPARs are known to be upregulated in activated macrophages, and have been shown to inhibit genes controlled by NF-κB. Such genes include many pro-inflammatory cytokines that are known to increases the longevity of both neutrophils and eosinophils.

The pro-apoptotic effects of the cytokine TNF-α are apparent in neutrophils at early timepoints, but there is no induction of cell death in eosinophils in response to this cytokine. IL-10 and TGF-β are cytokines with known anti-inflammatory properties. IL-10, however had no effect on the constitutive rate of apoptosis in either neutrophils or eosinophils, but did significantly inhibit LPS-mediated neutrophil survival. TGF-β significantly decreased constitutive apoptosis in both granulocytes, but interestingly, it enhanced TNF-α-induced apoptosis at early timepoints.

As part of the examination of the mechanism of TGF-β/TNF-α induction of neutrophil apoptosis we examined the role of the transcription factor NF-κB in process of granulocyte cell death. We show that there is both a constitutive and inducible activation of NF-κB in neutrophils, and that the inducible isoform appears to be necessary for granulocyte survival. Treatment of cells with gliotoxin, which specifically inhibited the inducible isoform at the timepoints examined by EMSAs produced a profound increase in apoptosis in both neutrophils and eosinophils. Pro-apoptotic effects were also observed with SN50, a cell-permeable peptide which is known to inhibit NF-κB activation. Co-culture of both granulocytes with TNF-α and gliotoxin caused dramatic increases in cell death, with almost 100% apoptosis reached in 2 h in neutrophils and 4 h in eosinophils.

Interestingly, many of the inflammatory mediators and cytokines known to inhibit granulocyte apoptosis have either been found to activate NF-κB, or are in part, regulated by this transcription factor. One exception to this is glucocorticoids, which we have shown to induce eosinophil apoptosis, while inhibiting neutrophil apoptosis.

Glucocorticoids induce transcription of the IκBα gene, resulting in an increased rate of IκBα protein synthesis, which inhibits NF-κB activation (Akira & Kishimoto, 1997). This mechanisms is similar to that suggested for TGF-β, but dexamethasone significantly inhibited TNF-α-induced apoptosis in neutrophils, suggesting another mechanism is involved in these cells, particularly since many NF-κB inhibitors have been shown to potentiate the cytotoxic effects of TNF-α.

One of the most powerful inhibitors of granulocyte apoptosis was found to be cAMP. The direct effect of cAMP on NF-κB is unlikely to account for this anti-apoptotic effect since direct stimulation of cells with dbcAMP causes only a modest increase in NF-κB activity (Zhong et al., 1997). However, other transcription factors such as cAMP-responsive element binding protein (CREB) and the closely related factor p300 which are activated by increases in cAMP can interact with p65/RelA and potentiate its transactivating ability (Gerritsen et al., 1997, Perkins et al., 1997). CBP/p300 can also form a molecular bridge between DNA-bound p65 and other transcription factors such as ATF-2 or c-Jun and the basal transcriptional apparatus (TFIIB), which may be required for full transcriptional activation. Therefore, it is possible that secondary effects on NF-κB activation could be involved in the inhibitory effects of cAMP on granulocyte apoptosis.

TNF-α, also inhibits neutrophil apoptosis if cultured for 20 h. We show that neutrophils appear to be protected against the cytotoxic effects of this cytokine by a process requiring synthesis of a protein(s), which is induced by the activation of NF-κB. While several proteins have been suggested to fulfil this role, none meets with the requirements needed for total inhibition of TNF-α-induced cell death. The kinetics of the response in neutrophils, in which almost all cells undergo apoptosis within 2 h if cultured with both TNF-α and an inhibitor of NF-κB suggest a protein with a high turnover rate and a short half-life, which are both characteristics of the protein c-Myc. This protein has several attributes that would fit the model: c-Myc is present in small amounts in granulocytes and as granulocyte precursors mature and differentiate, c-Myc levels fall. Decreases in c-Myc concentration have been

correlated with the onset of apoptosis in some cells, and both TGF-β and TNF-α reduce c-Myc levels and can act synergistically to induce this response. NF-κB activation is involved in the regulation of c-Myc levels. In other cells, changes in c-Myc concentrations have been shown to occur well within the timeframe of 90 min as determined for the onset of apoptosis in experiments with gliotoxin and TNF-α.

NF-κB plays a role in other aspects of inflammation. The expression of leukocyte adhesion molecules (ELAM-1, VCAM-1 and ICAM-1) on endothelial cells is induced by TNF-α and other inflammatory cytokines and requires NF-κB (Eck et al., 1993, Siebenlist et al., 1994). Sodium salicylate inhibits activation of NF-κB and inhibits the expression of adhesion molecules and transendothelial migration of neutrophils (Pierce et al., 1997). NF-kB inhibition also blocks the upregulation of CD11b/CD18 on neutrophils (Zhou et al., 1996). Blocking the activation of NF-κB in an *in vivo* rat model of lung inflammation suppresses neutrophil infiltration by blocking the synthesis of neutrophil chemotaxins (Blackwell et al., 1996).

The results presented in this thesis suggest that while NF-κB is a potent stimulator of the innate immune response, any mediator which can inhibit NF-κB is likely to have a beneficial effect on the resolution phase of inflammation by inducing granulocyte apoptosis and inhibiting the influx of inflammatory cells into the site. Logically this could provide a useful target for therapeutic intervention in inflammatory conditions. However, it should be remembered that in almost no case does a transcription factor act alone to regulate its target genes. Promoters or enhancers typically depend on multiple transcription factors for their activity. The particular NF-κB complex that binds a given κB site may be selected from among several by physical interaction with a transcription factor from an adjoining site (Siebenlist et al., 1994). Therefore interactions between NF-κB and other transcription factors may potentiate either the effects of NF-κB or affect activity of the other transcription factor. Therefore by modulating the activities of NF-κB, other genes, which are not normally regarded as being under the control of this transcription factor, may be adversely affected. Very

little is known of the interactions of various transcription factors and NF-κB or the specific combinations of these factors with regard to specific genes.

The controversy over the role of NF-κB in apoptosis means that inhibition of this transcription factor is unlikely to be a specific mechanism that induces cell death in all cells. However, to be therapeutically useful, a degree of specificity would be needed. More *in vivo* models of various inflammatory conditions would be useful to investigate the efficacy of this approach and to examine the effects on other cell types. If TGF-β and J series prostaglandins prove to be physiological inhibitors of NF-κB in granulocytes, further work may elucidate novel strategies to interfere with the activities of this transcription factor in a cell-specific manner. If they do not block activation, and other pathways are involved in their pro-apoptotic activities, further research may lead to the of design specific inducers of apoptosis in these cells which would be of therapeutic benefit.

Publications Arising From This Thesis

Ward C, Hannah S, Chilvers E R, Farrow S, Haslett C, Rossi A G. Transforming Growth Factor -β Increases the Inhibitory Effect of GM-CSF and Dexamethasone on Neutrophil Apoptosis. Biochem. Soc. Trans., 1997, 25: 244S

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Ward C, Cousin, J M, Haslett C, Chilvers E R, Rossi A G. Differential Regulation of Granulocyte Apoptosis by Prostaglandins. Manuscript in preparation.

Ward C, Haslett C, Chilvers E R, Rossi A G. Modulation of TNF-α-induced Granulocyte Apoptosis By An Anti-Inflammatory Cytokine. Manuscript in preparation.

ABSTRACTS

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