THE MOLECULAR BASIS FOR GLUCOCORTICOID-MEDIATED SURVIVAL OF HUMAN NEUTROPHILS

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

MASTER OF SCIENCE

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Dedications

Dedicated to my wife, Anoushe, who was in this with me in every way possible, who stood with me during the hardest times and in the coldest climates! I would not have been able to achieve so much so fast without you by my side.

Mom and dad, whose sacrifices ensured I got education, and who continue to motivate me in my career and life.

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List of Abbreviations

ActD: actinomycin D

ACTH: adrenocorticotropic hormone

ADCC: antibody-mediated cellular cytotoxicity

AP: apoptotic

AP-1: activator protein-1

Apaf-1: apoptotic protease activating factor 1

Bad: Bcl-2-associated death promoter

BAL: bronchoalveolar lavage

Bak: Bcl-2 homologous antagonist/killer

Bax: Bcl-2–associated X protein

BH: Bcl-2 homology

Bid: BH3 interacting domain death agonist

BIR: baculoviral IAP repeat

BLT-1: LTB4 high affinity receptor

BRUCE: BIR repeat-containing ubiquitin-conjugating enzyme

cDNA: complementary DNA

c-FLIP: cellular FLICE inhibitory protein

CHX: cycloheximide

CIAP: cellular IAP

COPD: chronic obstructive pulmonary disease

CpdA: compound A

CR: complement receptor

CRH: corticotropin releasing hormone

$\Delta\Psi_m$: mitochondrial membrane potential

Dex: dexamethasone

DIABLO: direct IAP binding protein with low pI

DISC: death inducing signaling complex

DMSO: dimethyl sulfoxide

DUSP-1: dual specificity phosphatase-1

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-regulated kinase

FACS: fluorescent activated cell sorting

FADD: Fas associated death domain

FasL: Fas ligand

FBS: fetal bovine serum

FcR: fraction crystallizable receptor

FITC: fluorescein isothiocyanate

fMLP: formyl-met-leu-phe

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

G-CSF: granulocyte colony stimulating factor

GM-CSF: granulocyte-macrophage colony stimulating factor

GR: glucocorticoid receptor

GRE: glucocorticoid response element

HBXIP: hepatitis B X-interacting protein

HPA: hypothalamic-pituitary-adrenal

HRP: horse radish peroxidase

HtrA2: high temperature requirement A2

IAP: inhibitor of apoptosis protein

ICAD: inhibitor of caspase activated DNAse

IκB: inhibitor of kappa B

IL: interleukin

IFN: interferon

IP-10: inducible protein 10 kDa

JAK: Janus kinase

JNK: c-Jun NH2-terminal kinase

LPS: lipopolysaccharide

LTB4: leukotriene B4

mAb: monoclonal antibody

MAPK: mitogen activated protein kinase

MAPKK: MAPK kinase

MAPKKK: MAPKK kinase

Mcl-1: myeloid cell leukemia-1

Med: medium

MFI: mean fluorescence intensity

MKP-1: MAPK phosphatase-1

MMP: matrix metalloproease

MnSOD: manganese superoxide dismutase

Mule: Mcl-1 ubiquitin ligase E3

NET: neutrophil extracellular trap

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor kappa B

nGRE: negative GRE

NIAP: neuronal inhibitor of apoptosis protein

NP-40: nonident p-40

p53: protein 53

PAGE: polyacrylamide gel electrophoresis

PAMPs: pathogen associated molecular patterns

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PDK: phosphoinositide-dependent kinases

PDTC: ammonium pyrrolidinedithiocarbamate

PE: phycoerythrin

PEST: proline-, glutamate-, serine- and threonine-rich

PHD: pleckstrin homology-domain

PI: propidium iodide

PI3K: phosphatidylinositol 3 kinase

PKB: protein kinase B

PVDF: polyvinylidene difluoride

RING: really interesting new gene

RT-PCR: reverse transcription polymerase chain reaction

RU: RU-486

RU-486: 11β-aminophenyl-substituted 19-nonsteroid RU 38486

SB: SB203580

SDS: sodium dodecyl sulfate

SGK-1: serum and glucocorticoid activated kinase-1

siRNA: small interfering RNA

SMAC: second mitochondria-derived activator of caspases

SP: SP600125

STAT: signal transducers and activators of transcription

tBid: truncated Bid

TBST: TBS/0.1% Tween 20

TLR: toll-like receptor

TNF-α: tumor necrosis factor-α

TRAIL: TNF-related apoptosis-inducing ligand

Ts-IAP: testicular IAP

Wort: wortmannin

XIAP: X-linked IAP

ABSTRACT

Glucocorticoids have been shown to inhibit neutrophil apoptosis, with implications that this might accentuate neutrophilic inflammation. The aim of this study was to investigate the molecular mechanisms involved in glucocorticoid-mediated inhibition of human neutrophil apoptosis.

Primary human neutrophils were isolated from peripheral blood of healthy volunteers and cultured *in-vitro* with dexamethasone. Here we confirm that dexamethasone, a classical glucocorticoid, significantly inhibited apoptosis of neutrophils. This inhibition was not dependent on transrepression of pro-apoptotic molecules but was associated with induction of anti-apoptotic protein Mcl-1. Remarkably, dexamethasone mediated enhancement of Mcl-1 and survival were significantly suppressed by pharmacological inhibitors of p38 MAPK or PI3K. Inhibition of the above kinases also blocked dexamethasone-induced maintenance of mitochondrial transmembrane potential and suppression of caspases.

In conclusion, human neutrophils mount a robust anti-apoptotic response to dexamethasone that relies on signaling through PI3K and p38 MAPK. These results warrant further caution in treatment of neutrophil-dominated disease with steroids.

1. INTRODUCTION

1.1 Chapter 1: Neutrophils

Neutrophils are polymorphonuclear leukocytes that are essential components of the innate immune system. They form the first line of defense and play a very important role by combating bacterial and fungal infections. Neutrophils are terminally differentiated cells and are produced in the bone marrow from myeloid stem cells by a process called `granulopoiesis'. These cells are then released into the circulation in large numbers and constitute 50-70% of white blood cells. Upon initiation of inflammation, local inflammatory signals such as chemokines, cytokines and adhesion molecules initiate an orchestrated process of actively recruiting neutrophils into the tissue.

Because neutrophils are members of the innate immune system, they do not possess numerous clonal receptors specific for everything foreign. Rather, they act in a quick manner by recognizing pathogen associated molecular patterns (PAMPs) through their germline encoded toll like receptors (TLRs). Human neutrophils are known to express the majority of TLRs.[1] Furthermore, they act in concert with other components of immunity, by expressing receptors for antibody (fraction crystallizable receptors: FcRs) and complement (complement receptors: CRs). Activation of neutrophil receptors such as TLRs, CRs and FcRs generally leads to an aggressive phenotype with multiple pro-inflammatory consequences. These include phagocytosis, antibody-mediated cellular cytotoxicity (ADCC), release of reactive oxygen species, toxic granule content release and cytokine/chemokine synthesis. Moreover, these processes are usually associated with delayed apoptosis of these cells.

Neutrophils in human blood are partitioned between two systems: a) the circulating pool of neutrophils is found in large blood vessels; b) the "marginating" pool of neutrophils is transiently arrested in narrow capillaries mainly in the lung.[2] This marginating pool equals or may even exceed circulating neutrophils.[3] These two populations constantly exchange with each other (at a rate of 1.5±0.5% per second),[4] and have different kinetics.[5] Unlike the systemic circulation, large numbers of intracapillary neutrophils are retained in the pulmonary circulation without impeding regional alveolar blood flow, thus accommodating the marginated pool.[6]

In inflamed organs, an active process of adhesion occurs that involves a selectin- and integrin- dependent sequestration of neutrophils in capillaries and post-capillary venules. Neutrophils will go through the process of adhesion which involves multiple steps of rolling and tethering, firm adhesion and spreading, and finally diapedesis. Graded concentrations of multiple chemoattractants, for which neutrophils have many receptors, actively guide these cells in the above process towards the site of inflammation.

Bacterial killing is among other functions of neutrophils. This protective mechanism usually occurs through phagocytosis, or up-take of individual pathogens. Phagocytosis of neutrophils involves two different receptor classes, FcRs and CRs. Functional neutrophil phagocytosis receptors are FcγRII and CR3, while CR1 and FcγRIIIB mostly act as co-receptors.[7] Cross-talk among these receptors is often necessary for full activation of the phagocytosis process.[8] Another mechanism of bacterial killing utilized by neutrophils is degranulation, a process in which neutrophils release their pre-stored granule contents to the outside environment in an effort to kill target cells. Neutrophils contain different granule types with various ingredients. "Azurophilic" granules emerge first during

neutrophil differentiation and contain true microbicidal agents such as myeloperoxidase, serine proteases and antibiotic proteins. "Secondary" granules have lactoferrin and collagenase, whereas "tertiary" granules contain gelatinase. "Secretory vesicles" or the fourth type of granules appear only in mature neutrophils and contain plasma proteins such as albumin.[7] The above granules are released following ionophore stimulation in the reverse order.[9] Neutrophils also have the capacity to generate massive amounts of oxygen and nitrogen intermediates through "respiratory burst" and other mechanisms. These intermediates result from the assembly and activation of the NADPH oxidase, a transmembrane electron transport chain that reduces oxygen to superoxide anion. Subsequent reactions lead to the formation of other toxic species, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical (OH), and singlet oxygen ($^1O_2$). These intermediates have free radicals and function alone or in combination with other molecules such as chloride to destroy phagocytosed microbes.[10]

A novel anti-microbial mechanism of neutrophils has recently been characterized.[11] Neutrophil extracellular traps (NETs) are extracellular proteins mainly composed of chromatin, with specific granular components of neutrophils attached to them.[12] The backbone of these structures is formed by threads of unfolded nucleosomes and can be degraded by DNAse. These threads form cables and three-dimensional structures resembling webs.[11] Nets are formed in response to activation of various receptors such cytokine and FcRs as well as TLRs.[12, 13] It should be noted that NET formation by neutrophils is also a distinct form of cell death, as neutrophils undergoing NET formation gradually dissolve their nuclear membranes and granules, allowing them to mix.[14] Such NETosis[15] is in contrast with apoptosis where chromatin is condensed and divided into

several membrane bound apoptotic bodies. NETosis does not require caspases and is not accompanied by DNA fragmentation.[14] NETs have been shown to combat Gram- positive and negative bacteria as well as fungi.[11]

Cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) and chemokines such IL-8 have profound effects upon neutrophils. They amplify several responses of these cells such as adhesion and respiratory burst.[7] However, a great body of evidence has accumulated in the past two decades, indicating that neutrophils are not only the target of, but also a source of various cytokines and chemokines.[16] Neutrophils were long considered to be devoid of transcriptional activity. New evidence suggests that neutrophils constitutively or inducibly synthesize and release these mediators.[17] Even though neutrophil capacity to produce such agents is much lower than that of monocytes,[16] *in vivo* this may be irrelevant considering that: a) neutrophils are at least 20 times more numerous than monocytes; and b) neutrophils arrive at the sight of inflammation hours before monocytes.[7] The production of cytokines by neutrophils shows diversity and is regulated in distinct manner in each case. For instance, IL-12 synthesis requires at least two stimuli, interferon (IFN)-γ + lipopolysaccharide (LPS).[18]

The major role of neutrophils in the immune system is to rapidly respond to invading microorganisms. However, neutrophils cannot efficiently differentiate between foreign and self antigens without the help of soluble immune molecules such as antibodies, complement and cytokines. This non-specific response combined with the powerful weapons of neutrophils in some cases may lead to damage to normal tissue. When

regulatory processes that control recruitment, activation and apoptosis are impaired, neutrophils may become the predominant contributor to tissue injury.[7]

1.2 Chapter 2: Apoptosis

Apoptosis is the programmed process of cell death that requires energy. It is central to the development and homeostasis of all mammals, and its dysregulation has grave consequences for the body.[19] In the immune system, apoptosis plays an important role by preventing over-activation of immunity, thus avoiding self-inflicted pathology. As an example, leukemia is a process in which immune cells or their precursors replicate excessively and do not undergo apoptosis, thus giving rise to cancer. In another example, autoimmune diseases are often caused by a subset of immune cells that rather than undergo apoptosis after encountering self antigen, become activated and proliferate. These expanded clones are then capable of destroying normal self cells and causing pathology. On the other hand, an excess of apoptosis in the immune system will predispose the host to bacterial/viral infections by destroying clones that are reactive to pathogens, thus providing an escape mechanism for the invaders, and leading to immune deficiencies.

Apoptosis is an evolutionarily conserved process. Since the concept of apoptosis was established in 1972,[20] many genes controlling the process have been discovered. It is known that all nucleated cells contain the proteins necessary for induction of apoptosis. The morphological characteristics of apoptotic cells are also conserved and include membrane blebbing, chromatic condensation and formation of apoptotic bodies. Multiple proteins are responsible for these characteristic features, the most important of which are caspases.

1.2.1 Caspases

Caspases are cysteine proteases that carry out the final stages of apoptosis by cleaving more than 200 substrate proteins at specific consensus sites.[21] These substrate proteins are involved in various cell functions, and cleavage of some of them such as inhibitor of caspase activated DNAse (ICAD) is responsible for the apoptotic morphology. Caspases are crucial to apoptosis and mammalian development. Each caspase is present constitutively in a precursor zymogen form that must be proteolytically cleaved in order to be activated. This cleavage will result in an enzymatically active protease that cleaves after aspartic acid residues.

More than a dozen caspases have been identified to date, and 11 of them have been identified in humans.[22] Apoptosis is not the only function of caspases,[23] however the seven caspases involved in apoptosis can generally be divided in two categories:

a) Initiator caspases: these proteins do not possess direct effector function in apoptosis. They include caspases-2, 8, 9 and 10, which in turn activate the final effector caspases.

b) Executioner (effector) caspases: proteases that perform the point of no return in apoptosis by irreversibly cleaving many targets. These include caspases-3, 6 and 7.

The initiator caspases are characterized by an extended N-terminal region, which comprises one or more adapter domains. Effector or executioner caspases usually contain 20-30 residues in their prodomain sequence, and exist constitutively as homodimers even after activation.[22]

1.2.2 An overview of pathway leading to caspase activation

Caspases involved in apoptosis are generally activated in two separate pathways of apoptosis. These are termed "intrinsic" and "extrinsic" pathways of apoptosis, and are activated by distinct pro-apoptotic signals of separate origins. The intrinsic pathway of apoptosis is triggered in response to a wide range of death stimuli that are generated from within the cell, such as oncogene activation and DNA damage. This pathway is characteristically inactive in cancer cells.[24] The intrinsic pathway of apoptosis is mediated by mitochondria. The key event is for mitochondria to release proteins that initiate the cascade. Examples include cytochrome c and second mitochondria-derived activator of caspases (Smac) aka direct IAP binding protein with low pI (Diablo). The molecules responsible for mitochondrial membrane integrity control constitute the major checkpoint of the intrinsic pathway, and are called the Bcl-2 family. Once this checkpoint is cleared by pro-apoptotic signals, cytochrome c is released from within mitochondria, and binds a cytoplasmic protein called apoptotic protease activating factor 1 (Apaf-1). This binding induces a conformational change that allows Apaf-1 to bind ATP/dATP along with caspase-9 and form a complex called "apoptosome". Apoptosome activates initiator caspase-9, and this leads to effector caspase-3 activation.[25]

The extrinsic pathway of apoptosis is initiated by binding of death ligands to their receptors on cell surface. An example is binding of Fas ligand (FasL) to Fas receptor on cell surface. Death ligands constitutively exist as homotrimers and induce trimerization of their receptors. The apoptosis signal is transduced to the intracellular environment by recruitment of initiator caspase-8 to FADD (Fas associated death domain) of Fas. This

complex is called DISC (death inducing signaling complex) and serves a similar function as apoptosome by activating executioner caspase-3.[25]

1.2.3 Bcl-2 family of proteins

Bcl-2 family of proteins is a group of closely related molecules that control the fate of every cell by inducing or inhibiting apoptosis. The Bcl-2 family of proteins plays a pivotal role in the regulation of apoptosis by controlling mitochondrial integrity and response to apoptotic signals.[26] The twenty or so members of Bcl-2 family proteins contain up to four evolutionarily conserved domains called Bcl-2 homology (BH) domains 1 to 4. These domains are shared to varying extent and similarity by both pro-apoptotic and anti-apoptotic members of the family.[27] Members of the family are found in various compartments of the cell. Some are found bound to membranes of endoplasmic reticulum, mitochondria or nucleus; whereas others are in a soluble state in the cytoplasm.[26]

In general, this family of molecules is divided in two groups: pro-apoptotic and anti-apoptotic. There are currently five members of the anti-apoptotic Bcl-2 family molecules: Bcl-2, Bcl-x_L, Bcl-w, myeloid cell leukemia-1 (Mcl-1) and A1. Among the anti-apoptotic members, Bcl-2 and Bcl-x_L share all the conserved BH domains (BH1–4), whereas Mcl-1 has BH1-3. The pro-apoptotic members of the Bcl-2 protein family are divided into the BH1–3 multidomain and BH3-only subgroups. The multidomain BH1-3 family members are Bcl-2–associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak). The BH3 only group consists of many molecules: BH3 interacting domain death agonist

(Bid), Bik, Bcl-2-associated death promoter (Bad), Bim, Bfk, Noxa, Puma and Hrk.[28] Bax and Bak are essential for induction of apoptosis. For instance, while Bak or Bax deficient mice develop rather normally, cells deficient in both Bak and Bax are extremely resistant to induction of apoptosis, indicating some level of redundancy between the two molecules.[29] Bax and Bak are thought to induce apoptosis through mediating outer mitochondrial membrane permeabilization, loss of mitochondrial membrane integrity as well mitochondrial membrane potential ($\Delta\Psi_m$), leading to release of pro-apoptotic molecules normally held at bay in the mitochondrial intermembrane space.[26] There are two potential mechanisms for this effect of Bax and Bak. The first hypothesis is based on similarity of Bax/Bak to bacterial pore-forming toxins, and proposes a direct hole-forming action of aggregated Bax/Bak on outer mitochondrial membrane.[30] In the second hypothesis, activated Bax/Bak opens mitochondrial membrane pores by interacting with the molecules controlling permeability.[28] Activation of Bax/Bak consists of a change in their conformation, as well as formation of oligomeric complexes. In the case of Bax, there is also a translocation from cytosol to mitochondria; In contrast, Bak is constitutively bound to outer mitochondrial membrane.[28]

BH3 only members such as Bad and Bid act as rheostats of apoptosis by allowing activation of Bax and Bak. In other words, they act as sensors of apoptosis signals and trigger the induction of apoptotic processes. Each member of this group might be activated in response to a particular set of stimuli and through a distinct mechanism.[31] These molecules on their own are incapable of inducing full-scale apoptosis.[32] There are at least two potential mechanisms through which BH3 only molecules are presumed to activate pro-apoptotic Bax/Bak. In the latest model, a few select BH3 only molecules, i.e.

truncated Bid (tBid), Bim and Puma have the capacity to "directly" activate Bax and Bak and are hence termed "activators".[33] In the second proposed mechanism, all BH3 only molecules are capable of binding to and neutralizing the anti-apoptotic Bcl-2 family members, thus allowing an "indirect" activation of Bax and Bak, hence the term "sensitizers".[33] A good example is the activation of Bad, which results in its coupling to Bcl-x_L. Once Bcl-x_L is coupled with Bad, Bax can now dissociate and freely insert into the mitochondria thereby inducing apoptosis.[34]

Active BH3 only molecules are generated via transcriptional and post-transcriptional mechanisms. For instance, Noxa[35] and Puma[36] are transcribed by activated protein 53 (p53); Bid is activated by caspase-mediated cleavage into tBid,[37] and Bad phosphorylation by Akt inactivates it.[34] Binding of active BH3 molecules to their cognate partners then occurs through the interaction between the hydrophobic face formed by amphipathic α-helical BH3 domain and the hydrophobic groove of the target.[27]

The balance between anti-apoptotic and pro-apoptotic members is probably the crucial factor in the control of mitochondrial integrity by Bcl-2 family members. These proteins interact with each other so as to block each other's function. The function of anti-apoptotic members of the Bcl-2 family is generally considered to be neutralization and sequestration of the pro-apoptotic BH3 members.[38] There is however evidence that Bcl-2 and Bcl-x_L can also block Bax/Bak oligomerization without interfering with BH3 members.[39] With the existence of strong apoptosis signals and in the absence of significant opposition from anti-apoptotic Bcl-2 members, BH3 molecules will activate Bax/Bak triggering their aggregation and insertion into the mitochondria. Through complex mechanisms, this event is thought to facilitate loss of mitochondrial

transmembrane potential as well as integrity. Via the pores created in mitochondria, various mitochondrial proteins such as cytochrome c and SMAC/Diablo are released which then activate caspases.[26]

1.2.4 Mcl-1

Mcl-1 was originally identified in 1993 in differentiating myeloid cells as a protein with sequence homology to Bcl-2.[40] Since then, Mcl-1 has been shown to be expressed in multiple cell types and has emerged as a key member of the Bcl-2 family.[41] Mcl-1 is essential for embryogenesis[42] and for the development and maintenance of both B and T lymphocytes in mice.[43] Overexpression of Mcl-1 in transgenic mice results in a high incidence of lymphomas.[44]

The prototypical Mcl-1 protein is comprised of 350 amino acids and has BH1-3 domains but lacks the BH4 domain of Bcl-2 and Bcl-x_L. The C-terminal section of the protein contains a membrane anchoring domain that is often associated with the outer mitochondrial membrane.[41] Most importantly, this protein contains 2 proline-, glutamate-, serine- and threonine-rich (PEST) domains rich in proline. These domains serve to target Mcl-1 to the proteasome, in response to constitutive ubiquitination by Mcl-1 ubiquitin ligase E3 (Mule).[45] Mcl-1 is thus a protein with a very short half-life.[46] The rapid induction and degradation of Mcl-1 suggests its role in controlling cell fate in response to rapidly changing environmental stimuli.

Alternate splicing of Mcl-1 has been reported to occur through skipping of the 2nd exon, giving rise to a short isoform that lacks BH1-2 and transmembrane domains. The

structure thus mimics those of pro-apoptotic BH3 only Bcl-2 family members, and its overexpression has been shown to cause apoptosis.[47] Other variants of the protein have also been identified, including caspase cleavage products. Caspase-3 specially seems to cleave Mcl-1 within a PEST domain at aspartic acid 127, producing a pro-apoptotic C-terminal fragment.[41, 48]

There have been multiple mechanisms proposed for induction or repression of Mcl-1 at the promoter level. In fact, the promoter of Mcl-1 includes binding sites for a variety of transcription factors including ELK-1, Sp1, SRF[49] and PU.1.[50] It appears that the mode in which Mcl-1 transcription is regulated by such transcription factors is highly dependent on the type of stimulus and cells that are stimulated. For example, in multiple myeloma cells, IL-6 has been found to activate the JAK/STAT pathway, resulting in a STAT-3 dependent up-regulation of Mcl-1.[51] STAT-3 is also important in enhancing Mcl-1 and survival in large leukemic granular lymphocytes.[52] In neutrophils stimulated with GM-CSF, the JAK/STAT pathways as well as PI3K are important for induction of Mcl-1.[53] In a separate study, GM-CSF and IL-3 were found to up-regulate Mcl-1 via the PI3K/Akt pathway, leading to a complex containing the transcription factor CREB.[54] Moreover, IL-3 in murine pro-B cells brings about PU.1 mediated transcription of Mcl-1 through activation of p38 MAPK.[50] In contrast, E2F1 is a transcription factor that represses the Mcl-1 promoter, sensitizing cells to apoptosis.[55]

Pro-survival signals such as cytokines and growth factors have been reported to induce Mcl-1 expression, whereas its levels are down-regulated during apoptosis.[28, 56] Direct phosphorylation of Mcl-1 may also play a role in controlling its expression/function.[57] The exact mode of function of Mcl-1 is not clear but it is thought to prevent loss of

mitochondrial integrity and cytochrome c release. This may occur though heterodimerization with pro-apoptotic Bcl-2 family molecules such as Bim or Bak.[43] In the case of Bak for example, it has been shown that Mcl-1 and Bcl-x_L are its only anti-apoptotic partners.[58] As such, in cells such as neutrophils that lack Bcl-x_L,[53, 59, 60] Mcl-1 may be the only restraint on Bak activation.

1.2.5 Inhibitor of apoptosis proteins (IAPs)

The IAPs are a family of proteins that function as intrinsic regulators of the caspase cascade. These proteins are characterized by one or more 70–80 amino-acid baculoviral IAP repeat (BIR) domains. Family members include: X-linked Inhibitor of Apoptosis Protein (XIAP), Cellular Inhibitor of Apoptosis Protein (cIAP) 1 and 2, Livin, Neuronal Inhibitor of Apoptosis Protein (NIAP), survivin, BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE) and Testicular Inhibitor of Apoptosis Protein (TsIAP).[61]

IAPs function in a variety of ways. First, IAPs are the only known endogenous proteins that regulate the activity of both initiator and effector caspases.[62] This has been shown to be due to the interaction of the IAP BIR domains with caspases which inhibits caspase activity.[62] While low nanomolar concentrations of XIAP can inhibit caspases, cIAP1 and -2 are estimated to be 1000-fold less potent and might not exist in cells at high enough concentrations to directly inhibit caspases.[21] XIAP inhibits both initiator (caspase-9) and effector caspases (-3 and -7) using two different domains.[63] Other mammalian IAPs may not be caspase inhibitors considering their domain structure,[64] although it has been

reported that survivin together with hepatitis B X-interacting protein (HBXIP) may prevent caspase-9 activation at the apoptosome.[65]

Second, cIAP1, cIAP2 and XIAP have ubiquitinating properties through their "really interesting new gene" (RING) domains, which possess E3 ligase ubiquitinating activity. The E3 ligase activity of IAPs is important in regulating their own level and apoptosis-inhibiting activity. In addition, XIAP, cIAP1, and cIAP2 not only regulate their own levels in cells, but may also modulate the amount of their (putative) substrates, caspases.[61] These functions are achieved through targeting of the ubiquitinated molecule to the proteasome for degradation. In the case of caspases, this may be considered anti-apoptotic; however, autoubiquitination of IAPs presumably should aid the process of apoptosis.

At least two proteins are released from the mitochondria that antagonize IAPs by binding their BIR domains, thus allowing caspase activity to pursue. These are Smac/DIABLO and Omi aka high temperature requirement A2 (HtrA2), which are released only after mitochondrial membrane integrity has been lost. Smac for example will bind two different domains of XIAP, thus releasing caspases. This step may in fact be necessary for full activation of caspases in the process of apoptosis.[61]

It should be mentioned that although IAPs are best known for their apoptosis inhibiting properties, not all IAPs inhibit apoptosis or caspases. Furthermore, IAPs have many functions that are quite distinct from their anti-apoptotic effects. For example, cIAP1 and -2 participate in TNF receptor signaling,[66] and survivin is implicated in the regulation of

mitosis.[67] Interestingly, overexpression of XIAP has been shown to induce cell cycle arrest.[68]

Although inhibition of caspases is usually regarded as a function of IAPs, a structurally unrelated anti-apoptotic protein called "cellular FLICE inhibitory protein" (c-FLIP) was discovered shortly after identification of caspase-8.[69] c-FLIP was cloned based on structural similarity to caspase-8 and in its full length has the capacity to inhibit this caspase. c-FLIP is identical in length to caspase-8 but has a modified caspase domain and thus functions as a dominant negative inhibitor of caspase-8.[69] Inhibition of the extrinsic apoptosis pathway by c-FLIP has been confirmed in c-FLIP$^{-/-}$ mouse embryonic fibroblasts, which demonstrate increased sensitivity to death receptor mediated apoptosis.[70]

1.2.6 PI3 kinase

The phosphatidylinositol 3-kinases (PI3Ks) constitute a unique and conserved family of intracellular lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides. Many intracellular signaling pathways are activated as a result, and these regulate functions as diverse as cell metabolism, survival, polarity, and vesicle trafficking. A variety of intracellular proteins have the ability to bind to the lipid products of PI3Ks and therefore become activated by PI3K signaling. The conserved role for PI3Ks is probably to mark specific cellular membranes for trafficking events.[71]

PI3Ks are grouped into three classes (I–III) according to their substrate preference and sequence homology. Different classes of PI3K as well as the different isoforms within each class have distinct roles in cellular signal transduction.[72] Class I PI3Ks consist of a catalytic and a regulatory subunit. There are four isoforms of the catalytic subunit: p110α, p110β, p110γ and p110δ. In granulocytes, Class I PI3Ks are considered to be the isoforms responsible for changes in phosphatidylinositol lipid levels.[73]

There are several molecular mechanisms by which PI3K activity can be regulated by extracellular stimuli. Activation generally involves re-location of the PI3K isoform from an inactive, cytoplasmic pool, to an active membrane-associated pool. After translocation, active PI3K phosphorylates phosphatidylinositol lipid substrates, generating the second messengers PI(3,4)P_2 and PI(3,4,5)P_3. Subsequently, there is translocation of a variety of pleckstrin homology-domain (PHD) containing proteins to the plasma membrane. In the context of cellular survival, the serine/threonine kinases Akt aka protein kinase B (PKB), and the constitutively active PDKs (phosphoinositide-dependent kinases), on which Akt depends for its activation, seem to be most important.[74]

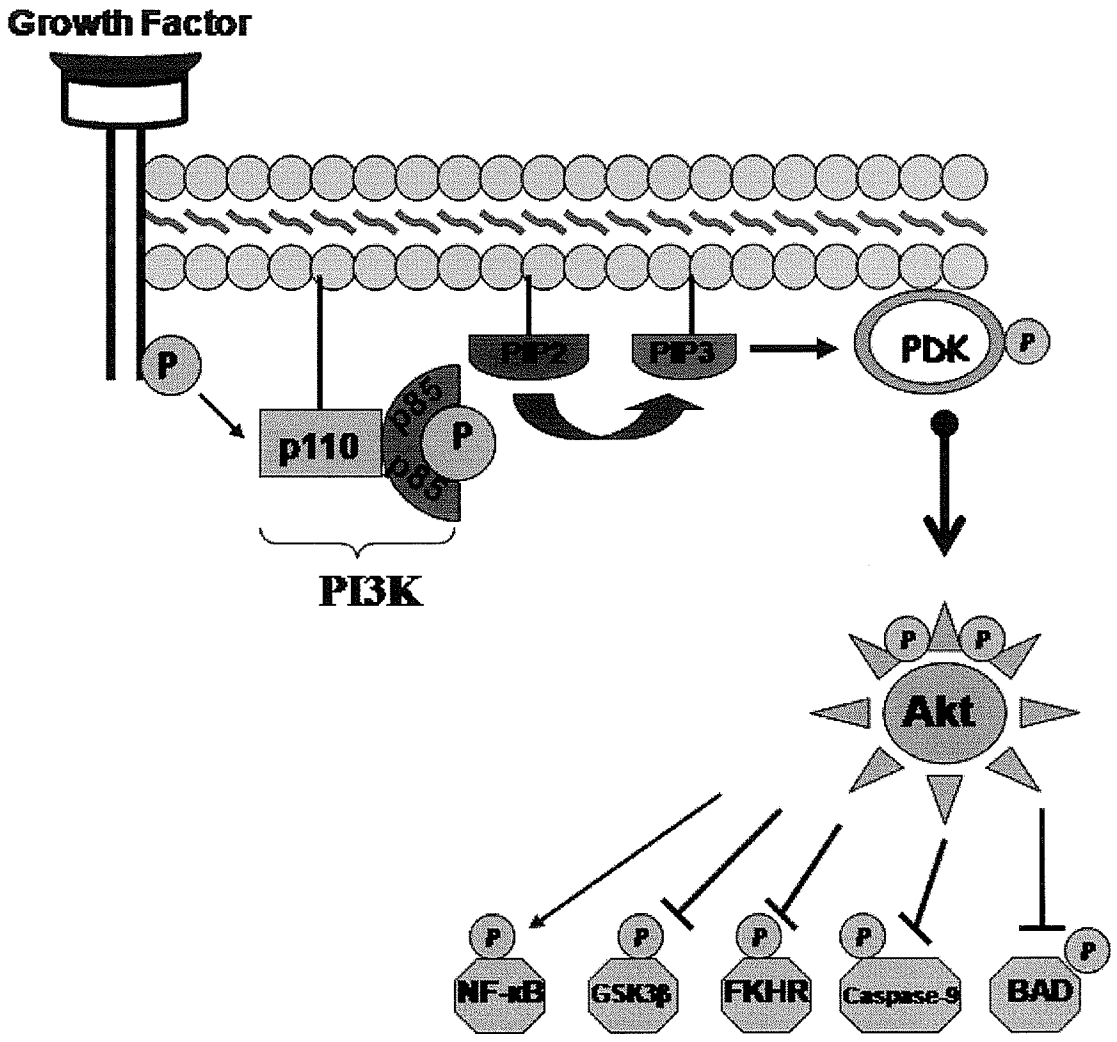

Diagram 1. Outline of PI3K signal transduction in mammalian cells. The PI3K signal transduction pathway is often activated by a growth factor receptor that recruits an adaptor protein to relay activation to the membrane bound lipid signaling PI3K. The net result of PI3K activation is the generation of lipid messenger PIP3, responsible for recruiting several signaling mediators to the membrane to be targeted by various kinases.

Overexpression of Akt has an anti-apoptotic effect in many cell types.[73] As an example, Akt phosphorylates BAD, a pro-apoptotic BH3 only Bcl-2 family member. Phosphorylated BAD is sequestered to 14-3-3 protein in the cytoplasm, and loses it apoptosis inducing effect.[34] Another example is the phosphorylation of FOXO transcription factors by Akt, which results in diminished transcription of pro-apoptotic molecules such as Bim and Fas.[75] In neutrophils, GM-CSF-mediated survival depends on Src-induced activation of PI3K.[76] GM-CSF also activates Akt in a PI3K dependent manner in these cells.[77] Other inducers of PI3K activation in neutrophils include IFN-β,[78] formyl-met-leu-phe (fMLP) and insulin.[79]

The PI3K inhibitor wortmannin was initially discovered as a potent immunosuppressor *in vivo*, long before its discovery as a PI3K inhibitor.[80] Wortmannin is a derivative of a fungal metabolite from *Penicillium Wortmanii*, and is used as a major pharmacological tool to study PI3Ks.

1.2.7 Mitogen Activated Protein Kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are a family of conserved protein kinases that phosphorylate specific serine and threonine residues of target protein substrates and regulate a number of cellular activities including gene expression, mitosis, cell movement, metabolism, cell survival and apoptosis. MAPKs are one part of a three-tiered cascade composed of a MAPK kinase (MAPKK, MKK, or MEK) and a MAPKK kinase (MAPKKK or MEKK). Phosphorylation of the MAPK results in a conformational change

and a >1000-fold increase in specific activity so that, in effect, MAPKs are inactive unless phosphorylated by their respective upstream kinases.[81]

Conventional MAPKs consist of three family members: the extracellular signal-regulated kinase (ERK); the c-Jun NH2-terminal kinase (JNK); and the p38-MAPK, and each family member has its own subfamilies: ERKs (ERK1 and ERK2), JNKs (JNK1, JNK2, and JNK3), and p38-MAPKs (p38-MAPKα, p38-MAPKβ, p38-MAPKγ, and p38-MAPKδ). ERK3, ERK5 and ERK7 are other MAPKs that have distinct regulation and functions

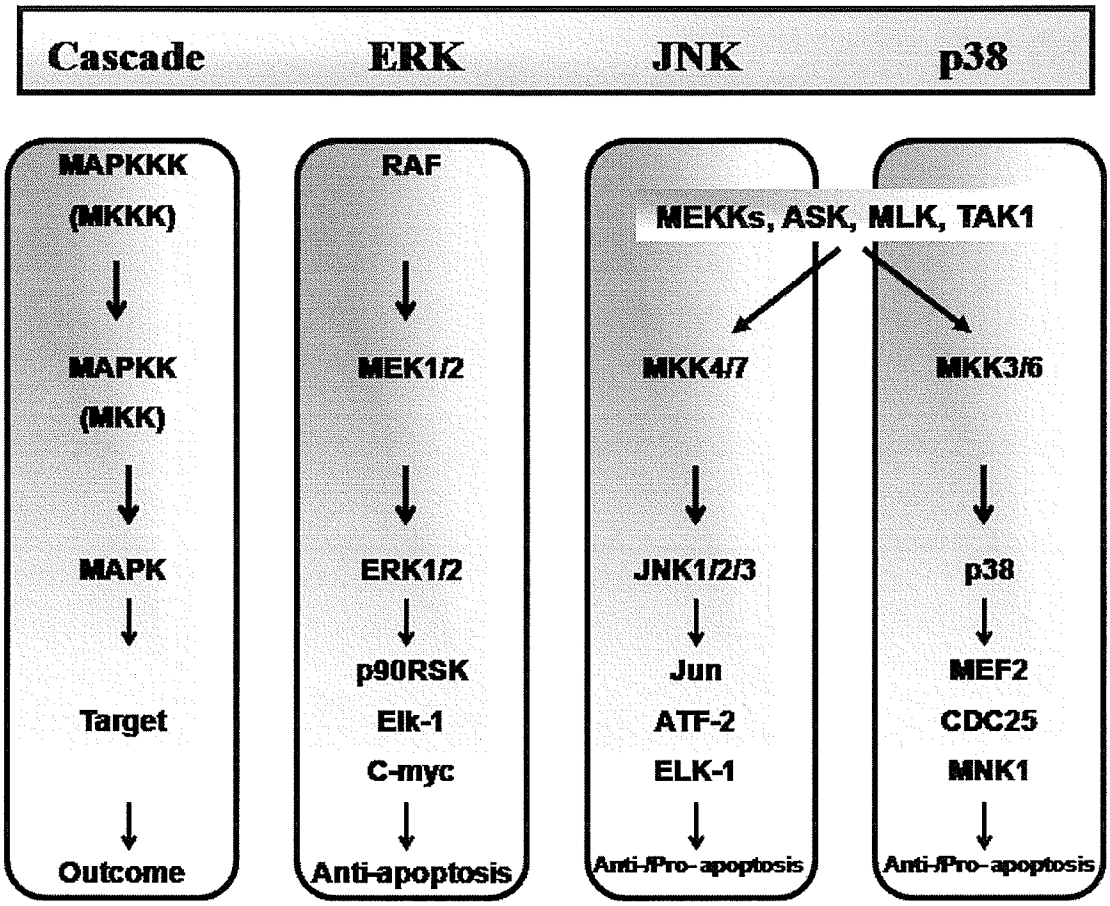

Diagram 2. An overview of MAPK signaling pathways. The above diagram depicts a simplified version of the three main MAPK signaling modules, namely ERK, JNK and p38. The signaling cascade begins with phosphorylation of an upstream kinase called MAPKKK, which then leads to sequential activation of MAPKK, MAPK and various MAPK substrates. Depending on the cell type and experiment settings, the result of such chain phosphorylation may have critical consequences for cell fate.

ERK. ERK was the first identified of all the MAPK family, and is made up of two isoforms 1/2. These are 43 and 41 kDa, share 83% identity, and are ubiquitously expressed. ERK1/2 are distributed throughout the cell. They are associated with plasma membrane receptors and transporters, as well as lipid rafts and caveolae.[82] MEK1 and -2 are the upstream ERK1/2 kinases that phosphorylate tyrosine and threonine residues in the ERK1/2 activation loops.[82] Inhibition of ERK activation prevents animal caps from differentiating into mesodermal tissues, and is thus embryonically lethal.[83] The MEK1/2-ERK1/2 pathway appears to be important for anti-apoptotic signals, although the regulations of apoptosis by the ERK pathway appears to be less complicated than for JNK and p38-MAPKs (please see below for descriptions of dual pro-/anti-apoptotic roles of JNK and p38).[84]

JNK. Three distinct genes encode JNK: JNK1, JNK2 and JNK3. These proteins are greater than 85% identical and exist as 10 or more spliced forms with molecular weights ranging from 46 to 55 kDa. MEK4/MEK7 phosphorylate and activate JNK on the TPY motif within the activation loop.[84] JNKs are activated in response to inflammatory cytokines; environmental stresses, such as heat shock, ionizing radiation, oxidant stress and DNA damage; DNA and protein synthesis inhibition; and growth factors. JNKs phosphorylate transcription factors c-Jun, ATF-2, p53, Elk-1, and nuclear factor of activated T cells (NFAT).[84] Knockout mice deficient in any of the individual JNK isoforms can develop normally; however, compound mutations of JNK1 and JNK2 result in embryonic lethality.[83]

Many lines of evidence support a pro-apoptotic role for JNK. JNK activation triggers apoptosis in response to many types of stress, including ultraviolet and γ-irradiation,

protein synthesis inhibitors, hyperosmolarity, toxins, ischemia/reperfusion injury in heart attacks, heat shock, anticancer drugs, ceramide, T-cell receptor stimulation, peroxide, or inflammatory cytokines such as TNF-α. The overexpression of dominant-negative MKK4, inactive c-Jun or dominant-negative MEKK1 has been shown to inhibit the induction of cell death by various stimuli.[84] Furthermore, embryonic fibroblasts deficient in both JNK1 and JNK2 are resistant to stress-induced apoptosis.[85] Active JNK causes the release of apoptogenic factors, such as cytochrome *c* and Smac/Diablo from isolated mitochondria in a cell-free system.[84]

There is also evidence that the role of JNK in regulation of cell survival is complex. Whereas the above studies all support pro-apoptotic activity of JNK, $CD8^+$ $CD44^{hi}$ T cells in LCMV infected $JNK1^{-/-}$ mice undergo more apoptosis than their wild type counterparts,[86] implying JNK1-induced survival of activated T cells. Furthermore, JNK activity is increased in response to growth factors and co-stimulatory molecules, leading to IL-2 production and proliferation of T cells.[87, 88] In hepatocytes, genetic manipulation of JNK signaling ablates a proliferative response to growth factors and causes embryonic lethality due to liver formation defects.[89, 90]

p38. There are four p38 family members (α, β, γ and δ). p38 MAPKs respond to a wide range of extracellular cues particularly cellular stressors such as UV radiation, osmotic shock, hypoxia, pro-inflammatory cytokines and less often growth factors. p38 is activated via dual phosphorylation within its activation loop by its upstream protein kinases MEK3 and -6. p38s share about 40% sequence identity with other MAPKs, but they share only about 60% identity among themselves, suggesting highly diverse functions.[82] Of the four isoforms, p38α is the best characterized and is expressed in most

cell types. p38 regulates expression of many cytokines and has been identified as the molecular target of anti-inflammatory compounds. p38α-deficient mice are embryonic lethal mainly due to a defect in placental angiogenesis.[83]

The involvement of p38-MAPK in apoptosis is diverse. It has been shown that p38-MAPK signaling promotes cell death,[91] whereas it has also been shown that p38-MAPK cascades enhance survival,[92] cell growth,[93] and differentiation.[94] While these studies may seem controversial at best, it should be noted that such paradoxical results may be due to differences in the specific cell types used as well experiment settings. For example, engagement of $CD4^+$ T cell CD3 using anti-CD3 monoclonal antibody (mAb) has been shown to induce apoptosis both independently of [95] and via [96] p38. Interestingly, $CD4^+$ T cells from $MEK3^{-/-}$ but not $MEK6^{-/-}$ mice are resistant to activation-induced cell death,[97] suggesting differential regulation of p38 mediated T cell apoptosis by its upstream kinases. In neurons and cardiomyocytes, p38 and its downstream partner MEF2 stimulate survival,[98, 99] whereas in T cells they control apoptosis by regulating Nur77 expression.[100]

1.3 Chapter 3: Neutrophil apoptosis

1.3.1 Mcl-1 in neutrophil apoptosis

The expression of anti-apoptotic Bcl-2 family members in neutrophils has been a subject of much debate in the past decade. Although an initial report claimed Bcl-2 expression in neutrophils,[101] the literature now has a consensus about the lack of Bcl-2 itself in human neutrophils.[59, 60, 102-105] The expression of Bcl-x_L and A1 has also been a matter of controversy, with the majority of studies reporting an absence of Bcl-x_L protein in neutrophils.[53, 59, 60, 106] Since the discovery of Mcl-1 and its specific expression in the myeloid lineage, the focus has shifted towards this molecule in neutrophils and the mechanisms through which its expression and function is regulated.

In an interesting early report in 1998, Moulding et al. compared the expression of various Bcl-2 family members in human neutrophils. They reported a lack of Bcl-2 and Bcl-x_L but an abundance of Mcl-1 protein. The levels of Mcl-1 protein declined with time in cultured neutrophils and constantly correlated with the viable fraction of the population. Furthermore, the pro-survival GM-CSF cytokine greatly diminished the loss of Mcl-1 in neutrophils.[60]

In a later report in 2001, the same research group reported on Mcl-1 transcript levels in neutrophils and found an increase in Mcl-1 mRNA with GM-CSF or LPS stimulation but not TNF-α or IFN-γ treatment. They also verified the GM-CSF induced increase in Mcl-1. Interestingly, and in contrast to Mcl-1, the levels of pro-apoptotic Bcl-2 family members Bax, Bak, Bad and Bid did not change during neutrophils culture, and seemed stable even after inhibition of translation.[59]

In a third paper published in 2004, Moulding et al. studied the regulation of Mcl-1 protein in human neutrophils. It was found that similar to GM-CSF, inhibition of the proteasome by MG-132 not only up-regulates Mcl-1 but also delays neutrophils apoptosis and prevents caspase activation. Furthermore, by blocking new protein translation, they discovered a protein translation independent up-regulation of Mcl-1 by GM-CSF. This up-regulation occurred through ERK and PI3K phosphorylation and led to increased stability of Mcl-1 protein.[107]

Regulation of Mcl-1 in human neutrophils was the subject of an additional study by Epling-Burnette et al. in 2001 who verified increases in Mcl-1 mRNA and protein in GM-CSF-stimulated cells. The increase in Mcl-1 was sensitive to inhibitors of PI3K and Janus kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) signaling pathways, especially when they were used in combination. Moreover, they found Mcl-1 to co-immunoprecipitate with Bax in GM-CSF treated neutrophils.[53] Sakamoto et al. in 2002 found 3-fold increases of Mcl-1 mRNA by real-time reverse transcription polymerase chain reaction (RT-PCR) at 1h of culture with G-CSF or GM-CSF that gradually decreased to baseline levels in a span of less than 4h. They also reported Mcl-1 protein increases that were blocked by inhibiting the JAK/STAT pathway.[108]

Human neutrophils have an extremely short life span and are notoriously non-transfectable. As such, the use of small interfering RNA (siRNA) technology to prove direct causal effects of Mcl-1 on neutrophils survival has remained unfeasible. Nevertheless, anti-sense oligonucleotides have been reported to effectively reduce Mcl-1 protein in human neutrophils. This study by Leuenroth et al. found that silencing Mcl-1 through anti-sense technology significantly increased neutrophils apoptosis after only 8h

of culture. Interestingly, hypoxia-induced survival of neutrophils but not adhesion-induced survival was blocked by the specific anti-sense against Mcl-1, indicating signal specific regulation of this protein.[109]

Although the expression of Mcl-1 in neutrophils and its correlation with survival was shown a long time ago, the most convincing data regarding the critical role of Mcl-1 in neutrophil survival was only recently published. Using a specific knock-down of Mcl-1 in myeloid cell lineage, Dzhagalov et al. demonstrated Mcl-1 to be crucial for neutrophil but not macrophage survival.[110] The granulocyte compartment in the blood, spleen, and bone marrow of Mcl-1 conditional knockout mice exhibited an approximately 2- to 3-fold higher apoptotic rate than control cells. These mice also demonstrated 80-86% reduction in granulocyte numbers in spleen, bone marrow and blood. The remaining neutrophils in these mice are those that miraculously escaped the Cre deletion mechanism.

1.3.2 Apoptosis of Neutrophils: what's special?

With regard to the classical pathways of apoptosis, there are a few facts to mention regarding apoptosis of neutrophils in particular:

a) Neutrophils undergo spontaneous apoptosis and do not require external stimuli to signal death. However, this spontaneous process can be accelerated in response to pro-apoptotic factors such as FasL.

b) The intrinsic pathway of apoptosis rather than the extrinsic pathway is thought to be associated with constitutive apoptosis of neutrophils.[111, 112]

c) Cytochrome c has been reported as either non-detectable[113] or barely detectable[114] in neutrophils.

d) Unlike most other types of cells, the requirement for cytochrome c release from mitochondria to activate caspase-9 is not strict in neutrophils.[113, 114]

e) Neutrophils lack the expression of classical anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-x_L.[53, 59, 60]

f) A1 (Bfl-1) is another Bcl-2 family member, whose expression has been reported with conflicting results in neutrophils. Whereas knock-out of A1 in murine neutrophils accelerated their apoptosis,[115] and A1 was apparently identified in human neutrophil lysates as well,[1, 116, 117] other reports reject the idea of A1 expression at the protein level in human neutrophils.[53, 59]

g) Multiple cytokines and other soluble factors have been shown to alter the rate of neutrophil spontaneous apoptosis. In general, pro-inflammatory mediators prolong neutrophil survival, whereas inhibitory cytokines seem to suppress such survival signals. Agents that augment neutrophil survival include: GM-CSF,[77] G-CSF,[118] LPS and other TLR ligands,[1] type I interferons,[78] IFN-γ, TNF-α, IL-1β,[119] IL-6,[120] IL-8,[121] IL-15,[122] leptin.[123] When combined with pro-survival signals, IL-10[124] and IL-17[125] seem to attenuate their survival effect.

h) PI3 kinase seems to function as a signaling molecule that is ubiquitously needed for survival of neutrophils. Almost every anti-apoptotic agent reported so far in the neutrophil literature requires the PI3K signal. These agents include: GM-CSF,[53, 77] LPS,[1, 124, 126] leptin,[123] type I IFNs,[78] leukotriene B4 (LTB4)[127], TNF-α,[128] thermal injury[129] and IL-8.[77]

1.3.3 Apoptosis of Neutrophil: why bother?

Inflammation, and chronic persistence of granulocytes in tissue, is now recognized as a central process in the pathogenesis of diseases such as chronic obstructive pulmonary disease (COPD) and bronchial asthma.[130] Neutrophils are the first cells to arrive at inflamed tissues, and subsequent steps of inflammation such as monocyte migration and edema depend on this initial response.[130] These leukocytes are terminally differentiated cells with extremely short half lives (8-20h).[131] Aged neutrophils undergo spontaneous apoptosis in the absence of survival signals such as cytokines and pro-inflammatory agents. Intact apoptotic neutrophils are removed via phagocytosis by macrophages. This silent process prevents dead neutrophils from releasing their cytotoxic content into the extracellular milieu that would occur if the cells died by necrosis. In inflammation, neutrophil numbers within tissues can rise because of influx from the circulation. In addition, neutrophil apoptosis is delayed in inflamed tissue and the surroundings by inflammatory mediators. As such, the potential for neutrophils to cause tissue damage via the release of toxic reactive oxygen species and granule enzymes such as proteases is very high. A special concern is the overloading of tissue macrophages by an excessive frequency of apoptotic neutrophils in inflamed tissue. In these cases as has been reported in the airways, secondary necrosis of apoptotic neutrophils leads to release of cytotoxic granules, causing harm to resident structural cells.[132]

Death by apoptosis and safe removal by phagocytic cells thus helps to limit tissue damage during the resolution of inflammation. Importantly, phagocytic clearance of apoptotic granulocytes does not lead to release of pro-inflammatory mediators from

macrophages; on the contrary, up- take of post-apoptotic secondary necrotic bodies by macrophages has the potential of switching on macrophage inflammatory properties.[133]

Apoptosis is the major mechanism controlling the functional longevity of neutrophils at inflamed sites.[130] In treatment of asthma, clinical improvement is associated with granulocyte apoptosis and appearance of apoptotic bodies within airway macrophages.[134] As such, understanding the processes that regulate constitutive neutrophil apoptosis and cytokine-mediated delay of cell death will lead to a better understanding of the molecular pathology of inflammatory diseases in which neutrophil apoptosis may be perturbed and could also identify new therapeutic targets.[131]

1.4 Chapter 4: Glucocorticoids

Glucocorticoids are part of the steroid family of hormones. The endogenous glucocorticoid released from the cortex of the adrenal gland in humans is cortisol. This endogenous hormone exhibits both glucocorticoid and mineralcorticoid activity that are necessary in the regular function of the body. Glucocorticoids in particular are important in the response to stress and in regulating immune responses, although they influence almost every tissue in the body.[135] It has been shown that glucocorticoid receptor deficient mice have severe abnormalities and die shortly after birth.[136] On the contrary, overexpression of GR renders mice resistant to stress and endotoxic shock, apparently due to a decrease in inflammatory responses.[137]

The release of endogenous glucocorticoids from the adrenal gland is controlled by the hypothalamic-pituitary-adrenal (HPA) axis. In this feedback control mechanism, the hypothalamus releases corticotropin releasing hormone (CRH) that then acts on the pituitary gland to cause secretion of adrenocorticotropic hormone (ACTH). ACTH then is transferred via the circulation to the adrenal cortex where it stimulates cells to synthesize and release cortisol. The elevation of cortisol in the blood later results in down-regulation of CRH in the hypothalamus thus providing a feedback response.

The medicinal properties of glucocorticoids have vastly been recognized and generously taken advantage of in the past few decades. Pharmacologic compounds with glucocorticoid activity are now available that demonstrate much higher affinity for the glucocorticoid receptor (GR), and avoid the mineralcorticoid side effects of cortisol. A

classical example of these drugs is Dexamethasone (Dex) which has long-acting glucocorticoid effects with fewer side effects than cortisol.

Perhaps the most important pharmacologic property of glucocorticoids is their immunosuppressive effects, and as such they have been extensively used to avoid immune and inflammatory responses. These characteristics are attributed to multiple effects of glucocorticoids on immune cells, inducing apoptosis of thymocytes, T cells and eosinophils as well as general prevention of pro-inflammatory molecule expression including chemokines, cytokines, lipid mediators and adhesion molecules.[135] Recently, it has become evident that glucocorticoids also induce the expression of various anti-inflammatory molecules.[138]

1.4.1 Glucocorticoid receptor mode of function

Glucocorticoids mediate their effect on target cells by directly binding to their cytosolic receptor known as the glucocorticoid receptor (GR). The unliganded GR is normally found in the cytoplasm in a complex with multiple other proteins. Binding of the ligand then induces release of the receptor from its protein complex, dimerization and translocation to the nucleus, where the GR can regulate the expression of genes in different ways. The nuclear GR can bind special sequences of nucleic acids called glucocorticoid response elements (GRE) in the promoter region of responsive genes, and induce "transactivation". In a few genes, a new consensus site has been recognized as the negative GRE (nGRE), which upon binding the GR will repress expression of the related transcript. Alternatively, the GR can directly bind to and suppress other transcription

factors, in a process called "transrepression". Recently, it has become clear that the GR can also influence cell function by modulating various intra-cellular signaling pathways in a rapid "non-genomic" or "non-transcriptional" manner.[139, 140]

1.4.2 RU-486: a specific antagonist of the GR

11β-aminophenyl-substituted 19-nonsteroid RU 38486 (RU-486) was discovered in the early 1980s[141] after a long search for a specific GR antagonist to pharmacologically modulate the effects of glucocorticoids in the human body. The anti-glucocorticoid activity of RU-486 has been very helpful in elucidating the molecular events leading to GR activation,[142] as well as in the treatment of patients with hypercortisolism (Cushing's syndrome).[143]

RU-486 strongly binds to the GR, with an affinity 3-4 times higher than Dex, and 18 times higher than cortisol,[144, 145] although these all have the same binding site in the GR.[146] The binding of the antagonist causes a different conformation of the GR than that induced by agonists.[147] RU-486 is often used in *in-vitro* studies to prove that a presumed glucocorticoid effect is indeed mediated by the GR.

RU-486 uses both active and passive antagonism to block glucocorticoid-induced GR activity. Passive antagonism is simply to prevent the agonist (such as Dex) from binding to GR. Active antagonism is more complex and involves bulky side chains of RU-486, which alter GR phosphorylation status and its subcellular localization. This inhibits coactivator binding to GR, with subsequent recruitment of corepressors.[148, 149]

1.4.3 Glucocorticoids and cell signaling

The interaction between the GR and cellular signaling has attracted increasing attention in recent years. This has mostly been due to technological advances in the study of cell signaling as well as discovery of the non-genomic aspect of GR function. The cellular signaling changes induced by glucocorticoids can be divided into various categories:

a) Interaction of the GR with transcription factors: the interaction of GR with various pro-inflammatory transcription factors has been known for a number of years, and was discovered after realization that most pro-inflammatory genes suppressed by glucocorticoids did not include a GR binding site in their promoter. It is now well-known that activated GR can interact with and suppress transcription factors such as Nuclear Factor kappa B (NF-κB), Activator Protein-1 (AP-1) and STATs.[150] In most cases, this results in "trans-repression" of pro-inflammatory genes, although in some cases cooperative action of GR with such transcription factors has been demonstrated as well.[135, 151]

b) Induction of cell signaling molecules: it has been shown recently that a few targets of GR have signaling capabilities and do indeed exert changes in cell signaling environment. For instance, Inhibitor of kappa B (IκB), an inhibitor of NF-κB signaling, has been shown to contain a GRE in its promoter, and is a target of the GR. MAPK phosphatase-1 (MKP-1), also known as Dual Specificity Phosphatase-1 (DUSP-1), is a transcriptional target of the GR,[152] which can actively dephosphorylate p38 and JNK MAPKs, protecting mice from LPS-induced endotoxic shock.[153] Another example is Serum and Glucocorticoid Activated Kinase (SGK-1), an Akt-like kinase with pro-survival signaling characteristics.[154]

c) Non-genomic activation of cell signaling pathways: in the past few years, there have been multiple examples of activation of various cell signaling pathways by glucocorticoids in an often rapid non-transcriptional manner.[139, 140] For instance, ligand activated GR has been shown to activate Akt downstream of PI3K in a non-transcriptional manner, protecting against ischemic heart damage[155] and stroke.[156] Akt and ERK activation by glucocorticoids protected ovarian follicular cells from apoptosis.[157]

1.4.4 Glucocorticoids and cell survival

Although glucocorticoids are mostly notorious for their apoptosis inducing properties, it has become increasingly clear in the past decade that this is not necessarily the case. In fact, multiple cell types have now been identified in which glucocorticoids inhibit apoptosis and induce survival.[152, 154, 157-170] Just as diverse as the cell types in which glucocorticoids induce survival are the mechanisms by which they appear to do so. The following is a summary of the identified pathways:

a) Up-regulation of anti-apoptotic Bcl-2 family members[158, 159, 162]

b) Stabilization[163] and induction[170] of IAPs

c) Activation of NF-κB[164, 165]

d) Suppression of components of the extrinsic pathway of apoptosis[160, 167]

e) Induction of signaling molecules such as MKP-1 and SGK-1[152, 154]

1.4.5 Glucocorticoids and asthma/COPD

Glucocorticoids are generally considered to be the treatment of choice in many inflammatory diseases, in particular asthma and COPD. In asthma, glucocorticoid therapy frequently results in decreased airway hyperresponsiveness. However, the magnitude of the reduction varies, and airway responsiveness often remains improved but abnormal.[171] If therapy is discontinued, airway hyperresponsiveness will return to pre-treatment levels.[172]

In a fraction of asthma patients,[173, 174] and in a large number of COPD patients,[175] glucocorticoid responsiveness is poor. It is likely that there is a spectrum of steroid responsiveness, and also that various mechanisms account for the lack of response to glucocorticoids in resistant cases.[138] Glucocorticoid resistance in the case of asthma,[176, 177] COPD,[178] and in many other inflammatory diseases including septic shock[179] and idiopathic pulmonary fibrosis,[180] has been associated with neutrophilic inflammation. For instance, one mechanism of neutrophil steroid resistance in rheumatoid arthritis may be down-regulation of GR expression in neutrophils of synovial fluid.[181]

It is known that neutrophilic asthma represents a fairly large proportion of asthma overall, up to 50% by some reports.[182] Neutrophils have frequently been implicated in the pathophysiology of severe asthma.[183, 184] Although glucocorticoids lead to marked reduction of eosinophils, mast cells, T lymphocytes and macrophages in sputum, bronchoalveolar lavage (BAL) and bronchial wall,[185] changes in the neutrophilic component of asthma are often the reverse, with reports of increase in neutrophils after glucocorticoid therapy.[186, 187] For example, some glucocorticoid resistant patients develop

exacerbations that often appear to have a neutrophil-dependent inflammatory mechanism.[176] Neutrophil numbers are significantly increased in human airway submucosa following oral steroid therapy.[188] Moreover, neutrophil numbers as well as their Matrix Metalloprotease-9 (MMP-9) content in BAL are high in severe asthmatics, and poorly controlled by glucocorticoids.[189] Interestingly, an analysis of human bronchial biopsy specimens revealed that a two week treatment with oral prednisone resulted in an increase of neutrophil numbers as well as neutrophil chemoattractant proteins such as Inducible Protein 10 kDa (IP-10) and IL-8.[190] In induced human sputum samples, all cell types were steroid responsive following inhaled glucocorticoid administration, except neutrophils, which did not display nuclear translocation of GR.[191] In a mouse model of airway inflammation, prior treatment with Dex was shown to synergize with LPS in bringing about an accumulation of neutrophils.[192]

It is tempting to speculate from the evidence above, that at least in a fraction of patients receiving glucocorticoids, elevated neutrophil numbers appear that combined with factors in the local milieu may exacerbate the disease status.

1.5 Chapter 5: Glucocorticoids and neutrophils

GR expression in the human body is ubiquitous, and neutrophils are no exception. As such, neutrophils are expected to display biological and functional changes following steroid administration. However, it has been established that among immune cells, neutrophils express the lowest amount of GR.[193] Furthermore, it has been reported that the dominant negative GRβ isoform is expressed at a higher level than GRα in neutrophils.[194] The above points have been proposed as possible explanations for the relative glucocorticoid resistance of these cells. A recent study of global changes in gene expression clearly demonstrates that neutrophils do respond to glucocorticoids by significantly modifying their expression of hundreds of genes.[195]

The effects of glucocorticoids on various functions of granulocytes have often been reported with controversial results.[196] For example, neutrophil chemotactic activity has been claimed to be unaffected,[197] partially inhibited[198] or even enhanced[199] after glucocorticoid treatment. Other examples of such paradoxical findings include the effect of glucocorticoids on various pro-inflammatory properties of neutrophils such as super oxide anion production, L-selectin expression and cytokine/chemokine release.[196]

There is consensus in the literature that glucocorticoids cause a dose-dependent neutrophilia *in vivo* in the circulation,[200-202] similar to endotoxin.[203] This has been attributed to enhanced release from bone marrow, as well as delayed migration from the intravascular space to tissue compartments, resulting in prolonged neutrophilia in the circulation.[204] In fact, this property of glucocorticoids is used in conjunction with neutrophil stimulating cytokine G-CSF to treat neutropenic patients.[205, 206]

1.5.1 Glucocorticoids and neutrophil apoptosis

Three independent research groups simultaneously reported the *in vitro* anti-apoptotic effect of glucocorticoids on human neutrophils.[207-209] There have been a number of papers published since then that studied this phenomenon. These papers together have clarified that:

1. Glucocorticoids including Dex inhibit spontaneous neutrophil apoptosis in a concentration-dependent manner with an optimal response at 10^{-7} to 10^{-6}M.[207-212]
2. Other steroid hormones with no glucocorticoid activity such as aldosterone and progesterone had no effect on neutrophil apoptosis.[207-209, 213]
3. The anti-apoptotic effect of Dex is abolished by transcription/translation inhibitors.[207, 214]
4. The anti-apoptotic effect of Dex was mediated through the GR.[207, 212, 213]
5. Unlike GM-CSF and LPS, the anti-apoptotic effect of Dex was not significantly maintained past 24h.[209, 210]
6. Unlike GM-CSF and LPS, Dex did not lead to activation of neutrophils, as measured by such criteria as IL-8 and superoxide production.[209]
7. Dex augmented GM-CSF but not LPS-induced survival of neutrophils.[209, 210, 212]
8. Glucocorticoid-induced survival of neutrophils did not depend on autocrine release of mediators.[213, 214]

There have been a number of mechanisms proposed so far for the above-described phenomenon of glucocorticoid-mediated neutrophil survival:

1. Strickland et al.[194] in 2001 reported preferential dominant expression of GRβ and not GRα in human neutrophils. Furthermore, they found that murine neutrophils that are normally non-responsive to Dex become Dex responsive after transfection with GRβ.

2. Stankova et al.[215] in 2002 reported that incubation of human neutrophils with Dex caused an up-regulation of LTB4 high affinity receptor (BLT1). As well, they demonstrated an anti-apoptotic effect of LTB4 on human neutrophils. This was proposed as a potential mechanism for Dex-induced neutrophil survival. However, autocrine production of LTB4 itself was in fact found to be lowered in Dex treated neutrophils compared with control treatment.

3. Chang et al.[216] in 2004 found that bovine neutrophils treated with Dex demonstrated a decreased expression of Fas mRNA and protein, which correlated with decreased caspase-8 activity in these cells.

4. Madsen-Bouterse et al.[217] in 2005 observed an increase of anti-apoptotic A1 and a decrease of pro-apoptotic Bak in Dex treated bovine neutrophils. These findings correlated with decreased activity of caspase-9, and were proposed as a possible mechanism of glucocorticoid-mediated survival.

5. In a very recent report, Sivertson et al.[218] studied Mcl-1 modulation by Dex in human neutrophils. Interestingly, they reported an increase in mRNA and protein content of full-length Mcl-1 but not its short pro-apoptotic splice variant in glucocorticoid treated human neutrophils. They also demonstrated that Mcl-1 anti-sense oligonucleotides abolished Dex-induced Mcl-1 expression and survival in human neutrophils.

2. Materials and Methods

2.1 Reagents

Fluorescein Isothiocyanate (FITC)-conjugated monoclonal anti-Fas was purchased from Dako Cytomation. FITC-conjugated mouse IgG1 (clone MOPC-21) isotype control, Dex, RU-486, ready-made Cycloheximide (CHX) solution, Dimethyl Sulfoxide (DMSO), Ammonium Pyrrolidinedithiocarbamate (PDTC) and Propidium Iodide (PI) were obtained from Sigma. Compound A (CpdA), MG-132, actinomycin D, SB203580, U0126, SP600125, SN-50 peptide and wortmannin were purchased from Calbiochem. Recombinant human GM-CSF was from PeproTech. HyQ® RPMI 1640 and Fetal Bovine Serum (FBS) were obtained from Hyclone Laboratories. FITC-conjugated annexin-V, polyclonal rabbit anti- Smac and Bax, and monoclonal anti- Mcl-1, cIAP-2 and Manganese Superoxide Dismutase (MnSOD) were purchased from BD Biosciences. RNaseA was obtained from Qiagen. Wright-Giemsa staining agents were purchased from Fisher Scientific. Polyclonal rabbit anti- cIAP-1, active caspase-3 and survivin, and Phycoerythrin (PE)-monoclonal anti-Fas ligand were from R&D systems. Horse Radish Peroxidase (HRP)-conjugated monoclonal anti-Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH) was obtained from Abcam. Rabbit polyclonal anti-XIAP, Bid and caspase-3 were from Cell Signaling. Anti-Fas IgM (CH11) was purchased from Upstate.

2.2 Subjects

This study was approved by the Ethics Committee of the Faculty of Medicine, University of Manitoba. Adult non-smoker non-allergic healthy subjects volunteered to donate blood. Between 30-80ml of venous blood was taken from each donor, between 9-11am on weekdays only. EDTA coated tubes from BD biosciences were used to store the blood, which was immediately transferred to a cell culture hood for further processing.

2.3 Isolation and purification of human peripheral blood neutrophils

Blood was collected into sterile syringes from the peripheral vein of healthy donors. Every 20ml of blood was then mixed with 10ml of normal saline stored at room temperature. The resulting mix was added in 10ml portions to 3ml of Ficoll (Amersham) in 15ml tubes. These tubes were transferred to a centrifuge and rotated for 20min at 1600RPM at room temperature with the brakes off. The resulting layered tube contained the granulocyte-erythrocyte layer at the bottom. The upper layers of Ficoll, Peripheral Blood Mononuclear Cells (PBMC) and plasma were disposed of. An equal volume of dextran-saline was subsequently added to the tubes and mixed with the bottom layer to allow sedimentation of red blood cells in approximately 30min at room temperature. Following the incubation, the resulting clear layer on top that contained granulocytes was removed and transferred to a 50ml tube, while the bottom deep red layer was disposed of. The granulocyte mix in the 50ml tube still contains residual erythrocytes that will impact purity. As such, further purification by hypotonic lysis is required. The 50ml tube was filled up with room-temperature normal saline and 2% volume of patient's own plasma

kept on ice, followed by centrifugation at 1200RPM for 5min at 4°C. After this step, granulocytes with the residual erythrocytes were left at the bottom of the tube, while the supernatant was gently poured out. Immediately, the cell layer at the bottom was mixed in 10ml of hypotonic ice-cold 0.2% saline for only 50sec. Instantly after the 50sec period, 10ml of hypertonic ice-cold 1.6% saline was added followed by 25ml of 4°C normal saline. The tube was centrifuged at 1200RPM for 5min at 4°C, the supernatant was disposed of, and the cell pellet was visually inspected for the amount of remaining erythrocytes. The above hypotonic shock procedure was repeated if deemed necessary; otherwise, the pellet was resuspended in the appropriate amount of 4°C RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100units/ml penicillin and 100μg/ml streptomycin. This step was usually followed by repeated centrifugation and resuspension in the medium to ensure a clean population of cells. Samples were then taken for manual counting and viability assessment under a light microscope (40×) using trypan blue die according to standard laboratory protocols. Subsequently, neutrophils were resuspended in the same medium but at a fixed density of 5million/ml. Furthermore, cytological examination of stained neutrophils by the Wright-Giemsa method was carried out on a sample of 100,000 neutrophils spread on a glass slide by a cytospin, routinely yielding between 95-98% neutrophil purity.

2.4 Culture of human neutrophils

Neutrophils were either used fresh (time 0) or cultured with Dex (1μM unless stated otherwise), GM-CSF (10ng/ml) or CpdA at 37°C in 5% CO_2 in volumes varying between

200µl to 2ml in 48- or 24-well plates. For some assays, cells were pre-incubated for 15min with RU-486 (10µM), cycloheximide (5µg/ml unless stated otherwise), actinomycin D (5µg/ml), MG-132 (50µM) or left unstimulated (medium). For signaling assays, neutrophils were pre-incubated for 15min with wortmannin (10, 25nM), SB203580 (10, 25µM), U0126 (1, 5µM) or SP600125 (10µM).

2.5 Flow-Cytometric analysis of Fas and Fas ligand expression on human neutrophils

Cell preparations of $4x10^5$ neutrophils in 100µl of PBS+5% FBS were incubated for 30min on ice with primary anti-Fas, -FasL antibody or isotype control antibody (1µg/ml). Cells were subsequently washed twice with 3ml of Phosphate Buffered Saline (PBS), resuspended in 200µl of PBS+5% FBS and analyzed by FACS using CellQuest-Pro software (BD) at a rate of approximately 400 events per second.

2.6 Annexin-V/PI staining

Apoptosis was detected by annexin-V-FITC/PI staining assay to detect early apoptotic cells (membrane intact cells with externalized phosphatidylserine residues), late apoptotic (apoptotic cells with compromised membrane integrity) and non-apoptotic cells at 18h of culture.

Approximately 100,000 cells were washed once in ice cold PBS and resuspended in 100µl of annexin-V binding buffer (140 mM NaCl, 2.5mM $CaCl_2$, 1.5 mM $MgCl_2$, and

10 mM HEPES, pH 7.4) containing 5µl annexin-V-FITC and PI (1µg/ml) and incubated for 15min at room temperature on a shaker in dark. The volume was then increased to 200µl. Analysis was immediately performed by Fluorescent Activated Cell Sorting (FACS) using CellQuest-Pro software at a rate of approximately 400 events per second, and cells negative for both annexin-V and PI were considered viable (survival).

2.7 DNA fragmentation assay

The rate of neutrophil apoptosis was also assessed by the staining characteristics of fixed cells exposed to PI, a DNA binding dye. Between 2-5million neutrophils were washed with ice cold PBS, and fixed in 3ml of ice-cold 70% ethanol in 15ml tubes by adding the cells in small drops while gently shaking the tube on a vortex. Fixed cells in ethanol were kept at 4°C for varying lengths of time. At the appropriate time, cells were washed twice with 3ml PBS with extreme care and re-suspended in 400µl of PBS containing RNase A at 200 U/ml for 30min at 37°C. PI (30µg/ml) was then added to the suspension, and the cells were transferred to FACS tubes through a strainer cap. Neutrophils were analyzed using cell cycle parameters on an EPICS XL-II flow cytometer (Beckman Coulter). Doublets of cells were excluded.

2.8 Mitochondrial transmembrane potential assessment

Mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed by two-color density plot analysis of JC-1 (Alexis Biochemicals) 610nm/527nm fluorescence staining. Neutrophils

were harvested after 18h of culture, resuspended in PBS (1×10^6/200µl) and incubated with JC-1 (7.7µM) at 37°C for 20min. Cells were then washed and resuspended in 1ml of PBS and analyzed immediately by FACS using CellQuest-Pro software (BD).

2.9 Caspase Activity Assay

$15x10^3$ cultured neutrophils at various time points were subjected to 25µl Caspase-Glo (Promega) substrate solution according to the manufacturer's instructions in triplicate in refractive plates suitable for luminometry. Following a 30sec vigorous shake of the plate by an Enzyme-linked Immunosorbent Assay (ELISA) reader, the plate was incubated for 1hr at room temperature in dark. Enzymatic activity was acquired by an EG&G Berthold microplate luminometer.

2.10 Western blots

Neutrophils ($5x10^6$ or more per experimental group) were washed with PBS and subsequently the pellet was lysed with 150µl or more of an Nonident P-40 (NP-40) or Triton-X lysis buffer supplemented with a protease inhibitor cocktail (Roche). Following a 15min lysis on ice, the tube containing lysed cells was centrifuged at 12,000RPM for 10min at 4°C. The supernatant was stored immediately at -60°C, and the DNA containing sticky pellet was disposed of. Protein lysate for each sample was measured for concentration and immediately aliquoted in 10 or 15µl portions in 200µl centrifuge tubes

at the earliest convenient time and restored at -60°C until each aliquot was used for blotting.

Thawed protein sample aliquots were adjusted for differences in concentration and then mixed with the appropriate amount of lysis buffer to reach an equal volume for all samples run in the same gel. The samples were then mixed with a reducing 4× sample buffer containing Sodium Dodecyl Sulfate (SDS) and β-mercaptoethanol. Prepared samples were boiled at 100°C for 5min, shortly centrifuged and then transferred to polyacrylamide gels (SDS-PAGE) under denaturing conditions. The gel was run in an electrophoresis apparatus at range of voltages starting from 80 to 110V until the proteins were completely resolved.

The proteins were then transferred to charged PVDF (polyvinylidene difluoride) membranes either at 100V for 90min (<60kDa) or overnight at 30V (>60kDa). The PVDF membrane was stained with ponceau red after transfer to ensure transfer integrity and equal loading. Subsequently, the membranes were blocked by incubating with 5% non-fat milk/bovine serum albumin in TBS/0.1% Tween 20 (TBST) for 1h at room temperature or overnight at 4°C on a shaker.

Blocked membranes were incubated with primary antibodies diluted in non-fat milk/bovine serum albumin in TBST overnight at 4°C or for 2h at room temperature on a shaker. The membranes were washed three times for 15min each with copious amounts of TBST, and then incubated with the appropriate secondary antibodies for 1h at room temperature, followed by extensive washing at short intervals for 2h with TBST. Bands were visualized by ECL Plus/Advance Western Blotting Detection System (Amersham)

according to manufacturer's instructions using AlphaEase FC software v3.1.2 on a Fluorchem 8800 (Alpha Innotech).

Antibody concentrations used for western blotting were: Mcl-1 (1/250), XIAP (1/1000), cIAP-1 (1/1000), cIAP-2 (1/250), Survivin (1/250), GAPDH (1/10,000), Bax (1/1000), Bad (1/1000), Smac (1/500), MnSOD (1/500), active caspase-3 (1/1000) and total caspase-3 (1/1000).

2.11 Subcellular fractionation

Two different methods were used to isolate subcellular fractions for western blotting. In the first method, cytoplasmic and mitochondrial fractions were separated by differential centrifugation. Briefly, neutrophils were harvested and washed once with PBS after the indicated time points. The cells were re-suspended for 5min on ice in a lysis buffer: 10mM Tris-HCl (pH 7.8), 1% NP-40, 10mM β-mercaptoethanol, 0.5mM PMSF, 1 mg/ml aprotinin and 1mg/ml leupeptin. Cells were then sheared by passing them through a 22-gauge needle. The nuclear fraction was recovered by centrifugation at 600*g* for 5min, and the low-speed supernatant was centrifuged at 10,000*g* for 30min to obtain the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The mitochondrial fraction was further lysed in the buffer: 10mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 5mM EDTA (pH 8.0). The above method worked best to detect translocation and truncation of Bid.

In a second method,[113] cells treated under various conditions were washed in ice-cold PBS and resuspended in the ice-cold cytosol extraction buffer (250mM sucrose, 70mM

KCl, 250μg/ml digitonin, PIM, 2mM DFP in PBS). After a 10–15 min incubation on ice, when >90% cells became trypan blue positive, the preparations were spun at 1000*g* for 5min, and the supernatants were kept as cytosolic fractions. The pellets were resuspended in half the volume (as the cytosol extraction buffer) of the ice-cold mitochondria lysis buffer (100mM NaCl, 10mM $MgCl_2.6H_2O$, 2mM EGTA, 2mM EDTA, 1% NP-40 (v/v), 10% glycerol (v/v), PIM, 2mM DFP in 50mM Tris, pH 7.5) and incubated for 10min on ice, followed by a 10min centrifugation at 10,000*g*. The supernatants were taken as mitochondrial fractions. The second method worked best in detecting translocation and truncation of Bax.

2.12 Densitometry Analysis

The intensity of bands was determined using AlphaEase FC software v3.1.2 on a Fluorchem 8800 (Alpha Innotech) relative to control GAPDH levels. Changes in protein quantity were expressed as fold increase over the value obtained with unstimulated control cells at time 0.

2.13 Quantitative Real Time RT-PCR analysis

RNA was purified from primary human neutrophils using TRIzol (Invitrogen) according to manufacturer protocols. RNA concentration and integrity were evaluated using a spectrophotometer. Reverse transcription was performed with 2μg of total RNA using a high capacity reverse transcription kit (Applied Biosystems) according to manufacturer protocols. Briefly, DNA standards were prepared from PCR using complementary DNA

(cDNA) of neutrophils. The amount of extracted DNA was quantified by a spectrophotometer and expressed as copy numbers and serial dilutions were used to generate the standard curve. RNA and sequence specific Mcl-1 primers (0.25μM) forward 5'-GCC AAG GAC ACA AAG CCA AT-3' and reverse 3'-AAC TCC ACA AAC CCA TCC CA-5', and the house keeping gene GAPDH forward 5'-CCG GAG GGG CCA TCC ACA GTC T-3' reverse 5'-AGC AAT GCC TCC TGC ACC ACC AAC-3' were added to the LightCycler-DNA Master SYBR Green I solution (Roche Molecular Biochemicals). PCR was performed in glass capillaries with an initial denaturation step of 10min at 95°C followed by 1 cycle of denaturation, 35 cycles of PCR, 1 cycle of melting and 1 cooling cycle. Detection of the fluorescent product was obtained at the end of the 72°C extension period. Product specificity was determined by melting curve analysis and by visualization of the PCR products on agarose gel. The amplification of target genes in stimulated cells was calculated by first normalizing to the amplification of GAPDH and then expressing the normalized values as fold increase over the value obtained with unstimulated control cells at time 0.

2.14 Statistics

Data were analyzed by one-way ANOVA first to determine if any significant differences may generally exist among various experimental groups. Non-parametric Mann-Whitney U tests were then performed to detect statistically significant differences in each pair of experimental groups. GraphPad Prism 3.0 was the software of choice and $p<0.05$ was considered statistically significant.

3. Hypothesis and Aims

3.1 Hypothesis

Glucocorticoids alter the balance between pro- and anti-apoptotic molecules in human neutrophils to favor prolonged survival.

3.2 Aims

1. Verify previous *in vitro* findings regarding glucocorticoid-mediated neutrophil survival.

2. Clarify the role of GR-induced transrepression in glucocorticoid-mediated neutrophil survival.

3. Identify pro- and anti-apoptotic Bcl-2 family targets of glucocorticoids in human neutrophils and characterize their modifications as a result of glucocorticoid treatment.

4. Determine changes in anti-apoptotic IAPs in neutrophils following Dex stimulation.

5. Study dependence of glucocorticoids on alteration of caspase activity to inhibit apoptosis in neutrophils.

6. Determine changes in key signaling molecules following Dex treatment of neutrophils.

7. Analyze the mechanisms through which changes in neutrophil signaling relate to modifications in anti-apoptotic Bcl-2/IAP family molecules.

4. Results

4.1 Dex inhibited neutrophil apoptosis

We first confirmed that Dex significantly inhibited spontaneous cell death of neutrophils ($p<0.001$) with an optimal response at 1μM. Using two different methods to quantify apoptosis at two different time points, the percentage of non-apoptotic cells was found to increase by approximately two-fold in cultures with 1μM Dex, to a level similar to that of cultures with 10ng/ml GM-CSF (Fig. 1-2). Using the annexin-V/PI staining method, an average of approximately 37% of human neutrophils cultured for 18h in medium were detected as non-apoptotic, whereas a mean 68% of neutrophils survived without undergoing apoptosis when cultured with the addition of 1μM Dex (Fig. 1A). Furthermore, when a second method of detecting apoptosis (i.e. DNA fragmentation) was utilized at 24h, it was evident that while only 15% of cells cultured in medium did not show signs of chromatin cleavage, approximately 38% of those incubated in the presence of 1μM Dex were identified as non-apoptotic (Fig. 2A).

To verify that Dex exerted its effect through the GR, neutrophils were pre-incubated for 10min with 10μM RU-486 (RU), an antagonist of the GR, before the addition of Dex. The survival effect of Dex was completely abolished by RU. CHX, an inhibitor of protein translation, also resulted in a complete abrogation of the anti-apoptotic effect of Dex, implying that the anti-apoptotic effect of Dex is dependent on *de novo* protein synthesis (Fig. 1-2). Taken together, the above data demonstrate that Dex suppressed human neutrophil apoptosis by stimulating translation of proteins specifically through the GR.

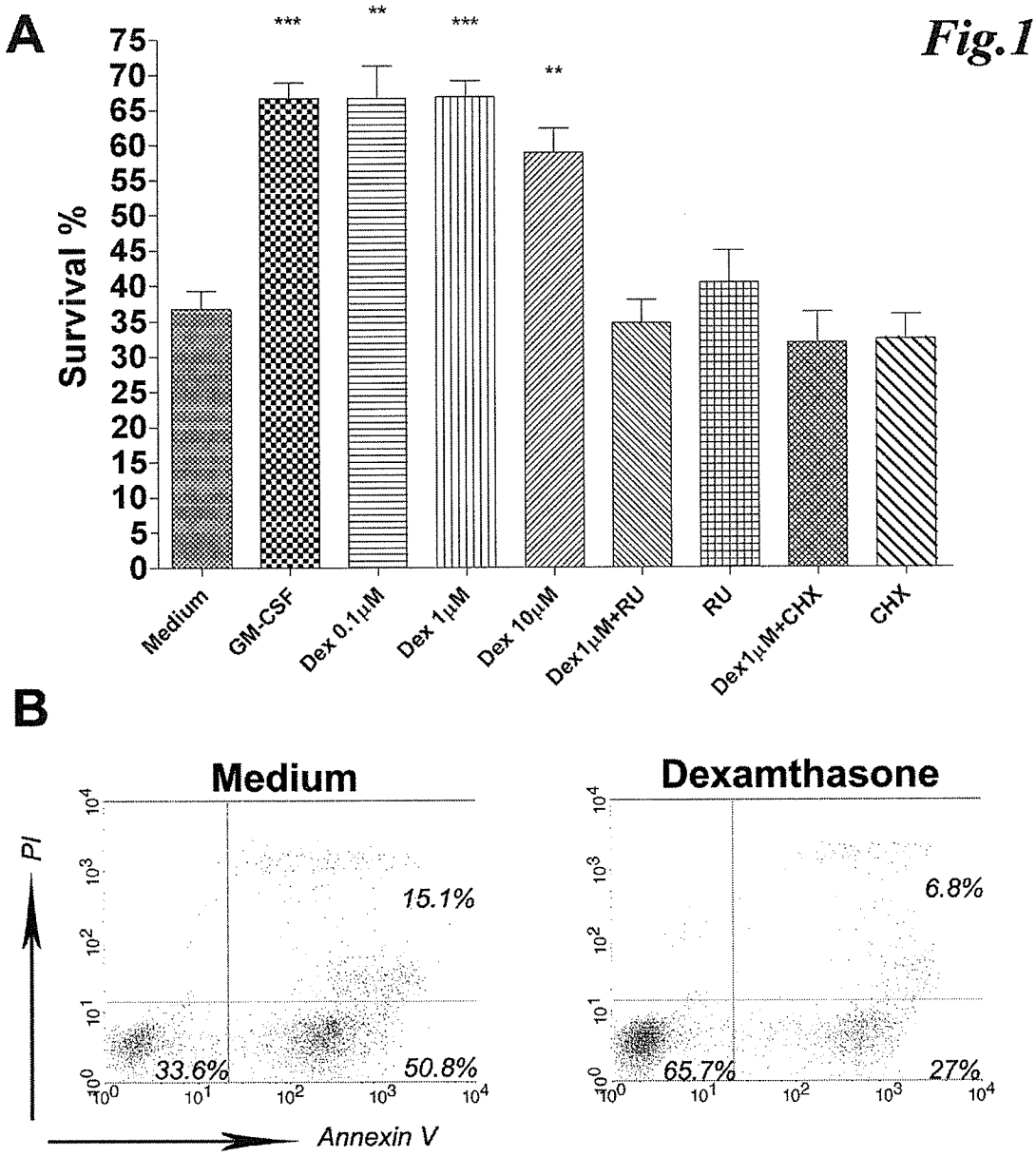

Fig.1

Figure 1. Dex inhibited apoptosis of neutrophils at 18h. (A) Freshly isolated human neutrophils were cultured for 18h in complete medium supplemented with 10% FBS and antibiotics. The following concentrations of reagents were used to stimulate these cells: 10ng/ml GM-CSF, 0.1, 1, and 10μM Dex, 10μM RU-486 and 10ng/ml of CHX. In the case of RU-486 and CHX, these reagents were added 10min before adding Dex. These neutrophils were assessed for apoptosis using annexin-V/PI staining method and the

percentage of cells with negative staining for annexin-V and PI was reported as survival (*** $p<0.0001$, ** $p<0.005$ compared with the Medium; n=8). The above data confirm previous reports that the anti-apoptotic effect of Dex on neutrophils is optimal at the 1μM concentration. Furthermore, RU-486, a specific antagonist of GR, and CHX, a protein translation inhibitor, abrogated the effect of Dex, implying a GR-specific survival effect that relies on protein translation. (B) Representative plots of annexin-V/PI staining patterns of neutrophils cultured for 18h with and without 1μM Dex. Non-apoptotic cells appear low in both annexin-V and PI.

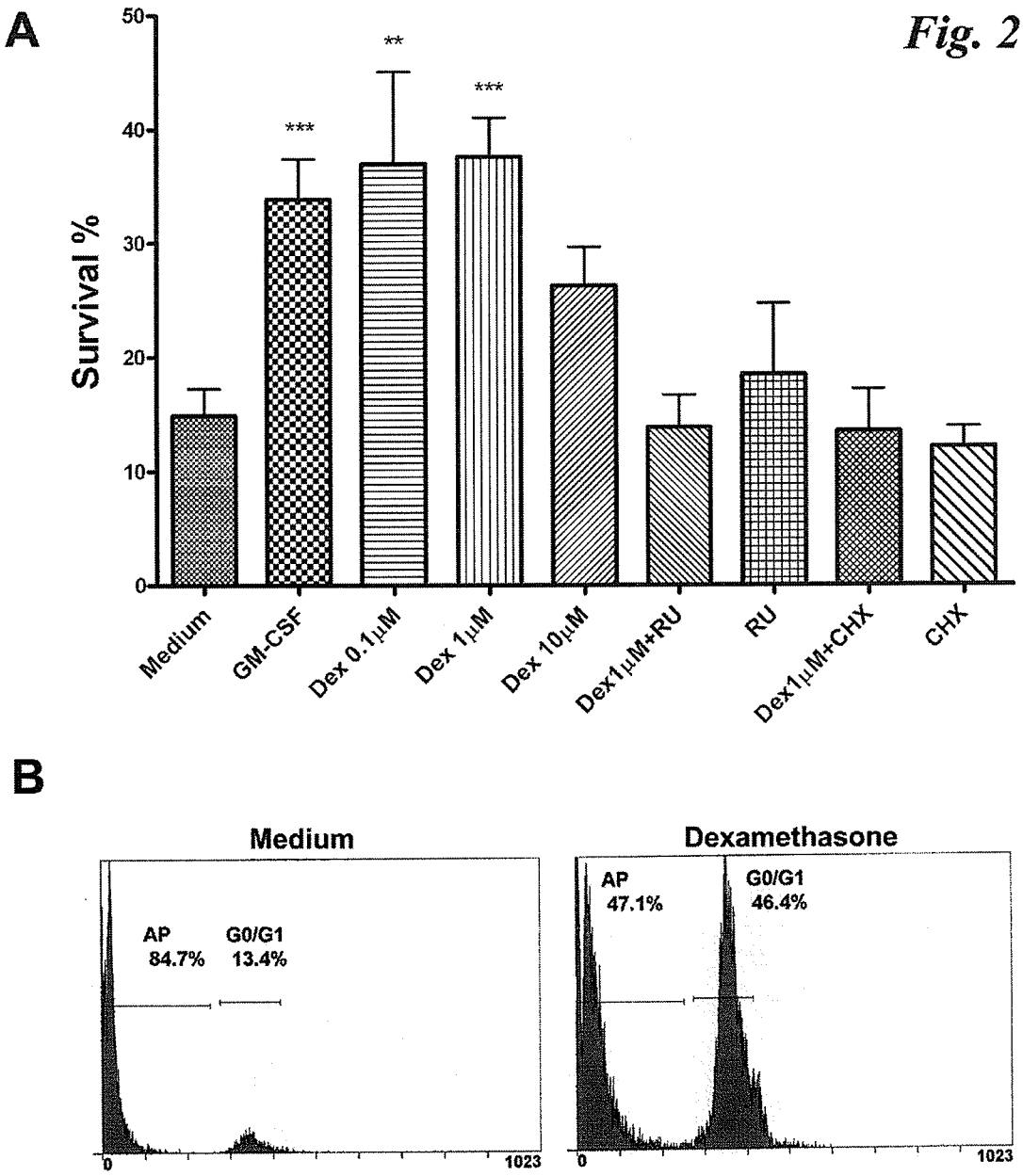

Figure 2. Dex inhibited apoptosis of neutrophils at 24h. (A) Freshly isolated human neutrophils were cultured for 24h in complete medium supplemented with 10% FBS and antibiotics. The following concentrations of reagents were used to stimulate these cells: 10ng/ml GM-CSF, 0.1, 1, and 10μM Dex, 10μM RU-486 and 10ng/ml of CHX. In the case of RU-486 and CHX, these reagents were added 10min before adding Dex. These

neutrophils were assessed for apoptosis by DNA fragmentation assay. Percentage of cells with intact PI stained DNA content was reported as survival (** $p<0.0005$, * $p<0.01$ compared with the Medium; n=5). The above data confirm previous reports that the anti-apoptotic effect of Dex on neutrophils is optimal at the 1μM concentration. Furthermore, RU-486, a specific antagonist of GR, and CHX, a protein translation inhibitor, abrogated the effect of Dex, implying a GR-specific survival effect that relies on protein translation. (B) Representative plots of cell cycle distribution in neutrophils cultured for 24h with and without 1μM Dex (AP: apoptotic). Apoptotic cells display low inclusion of PI due to DNA cleavage.

4.2 Dex-mediated survival occurred independently of transrepression

A hypothesis justifying the inhibition of neutrophil apoptosis by glucocorticoids was that they caused transrepression of pro-apoptotic molecules in neutrophils, and this in turn led to a delay in apoptosis. To test this hypothesis, two sets of experiments were performed: First, the effect of a specific inducer of GR-mediated transrepression of genes on neutrophil apoptosis was examined. CpdA is a recently developed GR modulator that binds the GR with higher affinity than Dex and selectively induces only gene transrepression *in vivo* and *in vitro*.[150] Therefore, we cultured neutrophils with various doses of CpdA and assessed for apoptosis at 18 and 24h by annexin-V/PI staining and DNA fragmentation, respectively. Unlike Dex, CpdA did not inhibit apoptosis of neutrophils at any of the dosages or time points examined (Fig. 3A-B). Using annexin-V/PI staining method to assess apoptosis at 18h of neutrophil culture, neutrophils treated

with 10nM, 100nM or 1μM CpdA demonstrated cell death rates similar to the control group (Fig. 3A). When these cells were further evaluated for apoptosis at 24h of culture by DNA fragmentation analysis, similar results were obtained (Fig. 3B), indicating CpdA did not influence neutrophil programmed cell death. These data argue against a role for GR-mediated transrepression in enhanced neutrophil survival.

Fig. 3

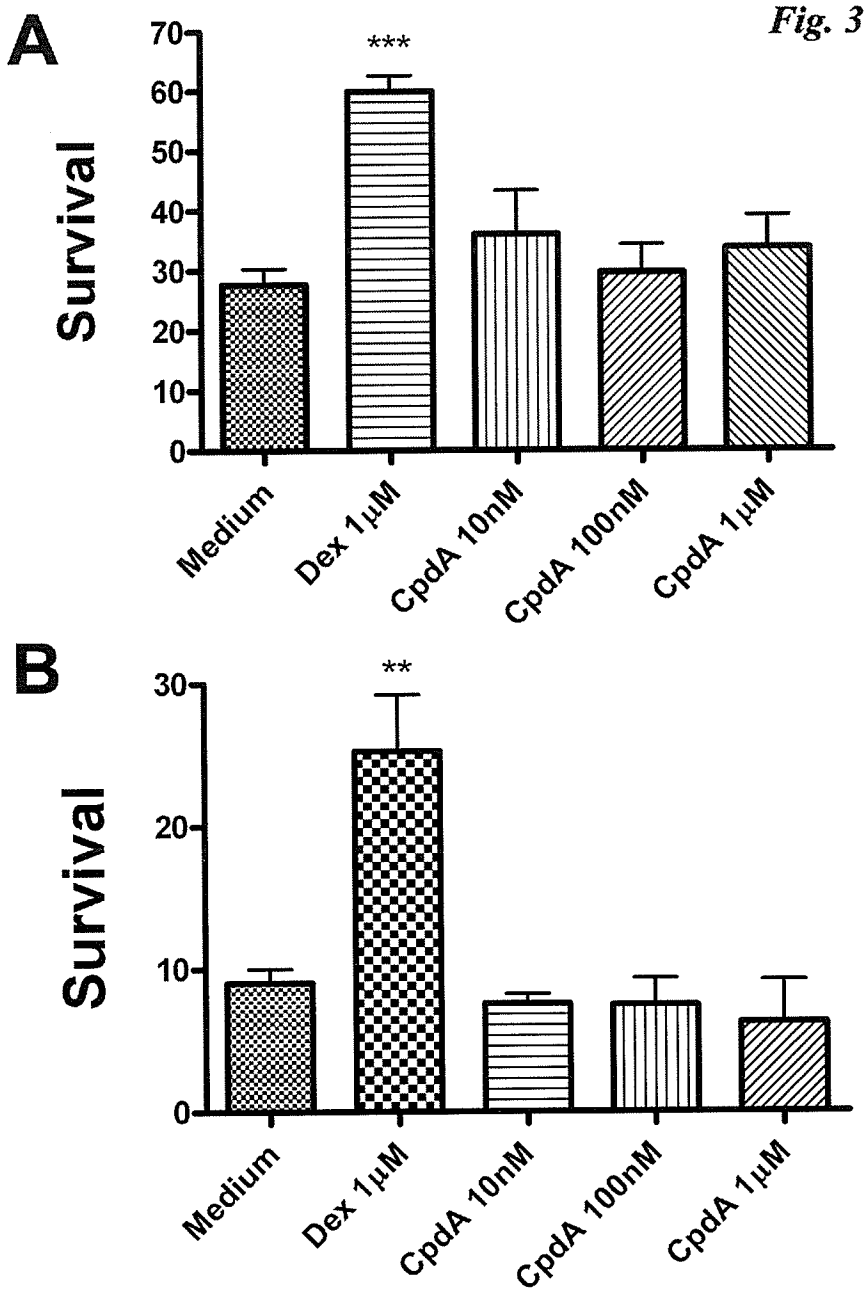

Figure 3. The glucocorticoid receptor modulator CpdA failed to inhibit neutrophil apoptosis. CpdA is a newly found selective and specific modulator of GR activity that has been claimed to induce only the transrepressive effects of liganded GR. However, no survival effects were observed following culture of human neutrophils with three different doses of CpdA, suggesting that GR-mediated transrepression does not lead to

enhanced neutrophil survival. Freshly isolated human neutrophils were cultured in complete medium supplemented with 10% FBS and antibiotics. Various concentrations of CpdA (10nM, 100nM and 1μM) as well as 1μM Dex were used to stimulate these cells. (A) These neutrophils were assessed for apoptosis using annexin-V/PI staining (18h culture; *** $p<0.0001$ compared with Medium; n=4) and the percentage of cells with negative staining for annexin-V and PI was reported as survival. (B) Neutrophils were also assessed for apoptosis by DNA fragmentation assay (24h culture; ** $p<0.005$ compared with Medium; n=3) and the percentage of cells with intact PI stained DNA content was reported as survival.

Next, we analyzed levels of two pro-apoptotic molecules of the extrinsic pathway of neutrophil apoptosis, Fas/Fas ligand.[219] Neutrophils were treated for 18h with Dex, GM-CSF or were left untreated and then analyzed for surface expression of Fas/FasL by FACS. No significant differences were found in the mean fluorescence intensity of Fas (Fig. 4) or FasL (not shown).

Fig. 4

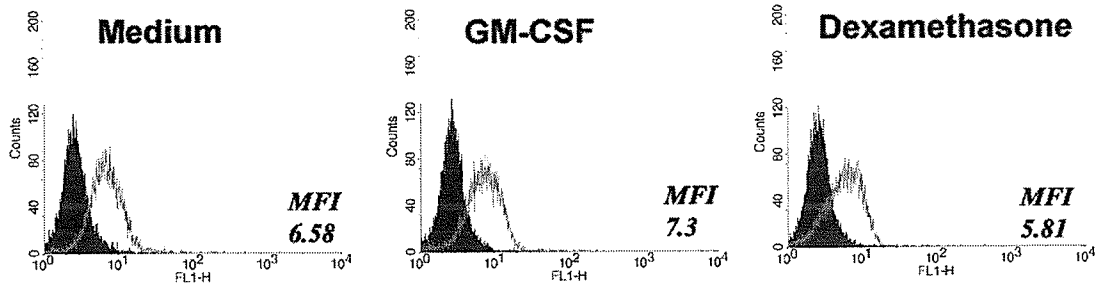

Figure 4. Surface Fas levels of neutrophils after culture with Dex. Fas is a cell surface death receptor that potently induces apoptosis upon engagement by its ligand, FasL. Down- or up-regulation of Fas is often used by various cell types to control susceptibility to pro-apoptotic stimuli. We cultured human neutrophils in complete medium, with the addition of 10ng/ml GM-CSF or 1μM Dex. After 18h, these cells were stained with FITC-conjugated 1μg/ml anti-Fas or an isotype control antibody. Expression of Fas was analyzed by FACS, revealing no significant changes in the Mean Fluorescence Intensity (MFI) of this surface antigen upon treatment with Dex, ruling out Fas down-regulation as a potential mechanism for Dex-mediated survival. The closed line represents the isotype control, whereas the green line represents Fas. The MFI average as shown was calculated from three independent experiments.

Furtheromre, we analyzed levels of two pro-apoptotic members of the intrinsic pathway of apoptosis. Fresh neutrophils or those treated for 6 and 12h with Dex, Dex+RU, GM-CSF or medium alone were analyzed for total cell expression of Bid and Bax by immunoblotting. No significant reductions in Bid/Bax expression were detected at any of the time points examined in Dex treated cells (Fig. 5). These data suggest that GR-

induced gene transrepression is not involved in Dex-enhanced survival of human neutrophils.

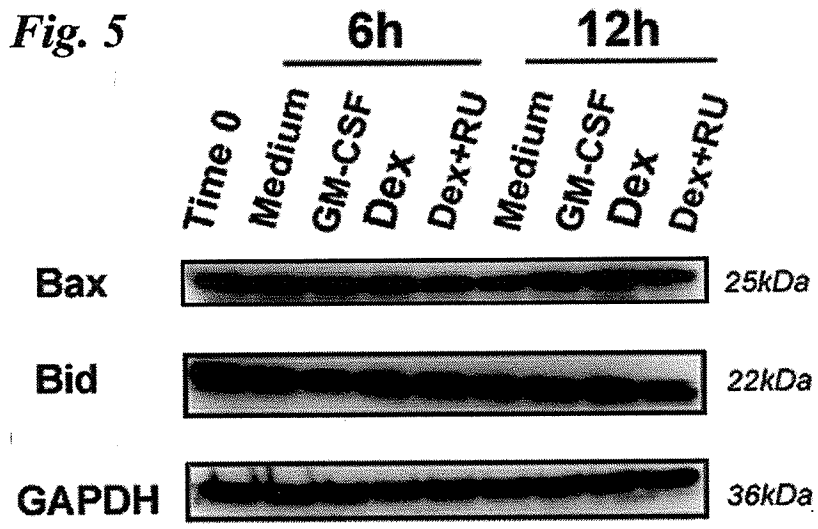

Figure 5. Bax and Bid protein levels were not suppressed by Dex treatment of human neutrophils. Bid and Bax are two representative members of the pro-apoptotic wing of Bcl-2 family members. These molecules are activated upon apoptosis signaling, and their expression level is regulated in various cells to control susceptibility to pro-apoptotic signals. We treated freshly isolated neutrophils (time 0) with 10ng/ml GM-CSF, 1μM Dex or 10μM RU-486 followed by 1μM Dex in complete medium. Cells were incubated for 6 and 12h, after which they were lysed and analyzed for total levels of Bax and Bid by immunoblotting. No decreases in Bax/Bid were observed in Dex treated cells compared with control medium cultures. These results suggest decreases of Bax/Bid protein are not a means by which Dex enhances neutrophil survival. GAPDH was used as an indicator of loaded protein quantity. Figure represents three separate experiments.

4.3 Dex up-regulated Mcl-1

Since de novo protein synthesis was necessary for the induction of neutrophil survival by Dex (Fig. 1-2), we postulated that Dex resulted in translation of new anti-apoptotic protein(s). The first candidate protein in neutrophils was Mcl-1, an anti-apoptotic protein from the Bcl-2 family of proteins whose expression level has been correlated with regulation of neutrophil survival.[220] Therefore, we performed multiple experiments in which neutrophils were incubated with culture medium, Dex, GM-CSF or RU-486. Mcl-1 protein and mRNA expression were analyzed by western blotting and real time RT-PCR at various time points.

Western blotting analyses revealed a significant two-fold increase in Mcl-1 levels after 6h of culture with Dex and this persisted up to 12h ($p<0.05$). This up-regulation was not detected in cells cultured in medium alone or those treated with RU before adding Dex, confirming the specificity of Mcl-1 up-regulation by the GR (Fig. 6A). To determine whether Mcl-1 up-regulation by Dex was due to increased transcriptional activity or increased protein/mRNA stability, neutrophils were pre-incubated with inhibitors of mRNA transcription, protein translation, and proteasome, and then stimulated with Dex for 6h. In agreement with previous experiments, western blots on cell lysates obtained from the above treatments confirmed an up-regulation of Mcl-1 specifically in Dex treated cells. This up-regulation was abrogated by inhibition of transcription/translation. Furthermore, while inhibition of the proteasome alone caused an enhancement of Mcl-1, its combination with Dex had an additive effect on up-regulation of Mcl-1 (Fig. 6B).

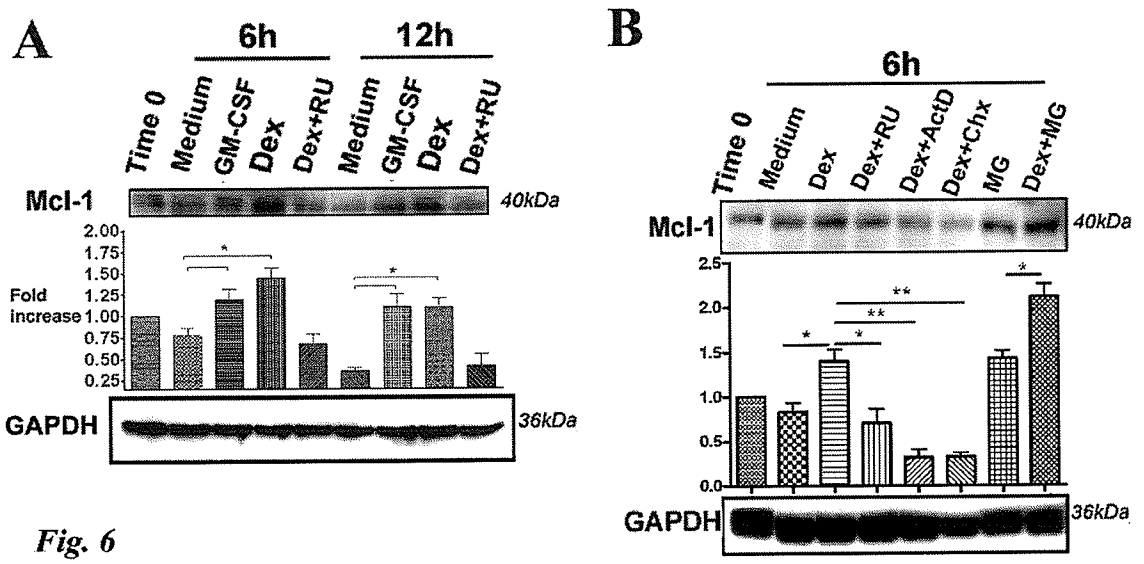

Fig. 6

Figure 6. Dex up-regulated anti-apoptotic Mcl-1 protein. Mcl-1 is the main anti-apoptotic Bcl-2 family member expressed in human neutrophils, whose protein level often correlates with survival rates. We treated freshly isolated neutrophils (time 0) with 10ng/ml GM-CSF, 1μM Dex, 10μM RU-486 followed by 1μM Dex, 5μg/ml CHX followed by 1μM Dex, 5μg/ml actinomycin D followed by 1μM Dex, 50μM MG-132, or 50μM MG-132 followed by 1μM Dex in complete medium. These cells were incubated for 6 or 12h, after which they were lysed and analyzed for total levels of Mcl-1 by immunoblotting and densitometry. Densitometry results are reported as fold increase over average value for Time 0. All analyses indicated up-regulation of Mcl-1 by Dex at both time points. This increase was abrogated by RU-486 mediated blocking of GR as well as CHX and ActD induced inhibition of translation and transcription, implying dependence on GR-mediated transcription/translation. Furthermore, inhibition of the proteasome by MG-132 on its own enhanced Mcl-1 protein, confirming proteasomal degradation of Mcl-1, which synergized with Dex-induced Mcl-1 up-regulation to result

in highest levels of Mcl-1 protein. GAPDH was used as an indicator of loaded protein quantity (* $p<0.05$; ** $p<0.01$; n=3).

These data were further confirmed by quantitative real-time RT-PCR analysis of Mcl-1 transcript levels. Remarkably, Dex-stimulated neutrophils exhibited an increase of approximately 1.5 fold in Mcl-1 mRNA at 3h ($p<0.05$ compared with time 0; Fig. 7). On the contrary, the level of Mcl-1 mRNA in neutrophils cultured with medium only demonstrated a steady decrease that was evident as early as after 90min of culture, in agreement with the spontaneous process of apoptosis in neutrophils. Collectively, the above data suggest that Mcl-1 is indeed specifically induced by Dex, and that Mcl-1 up-regulation by Dex occurs at least at the transcriptional level.

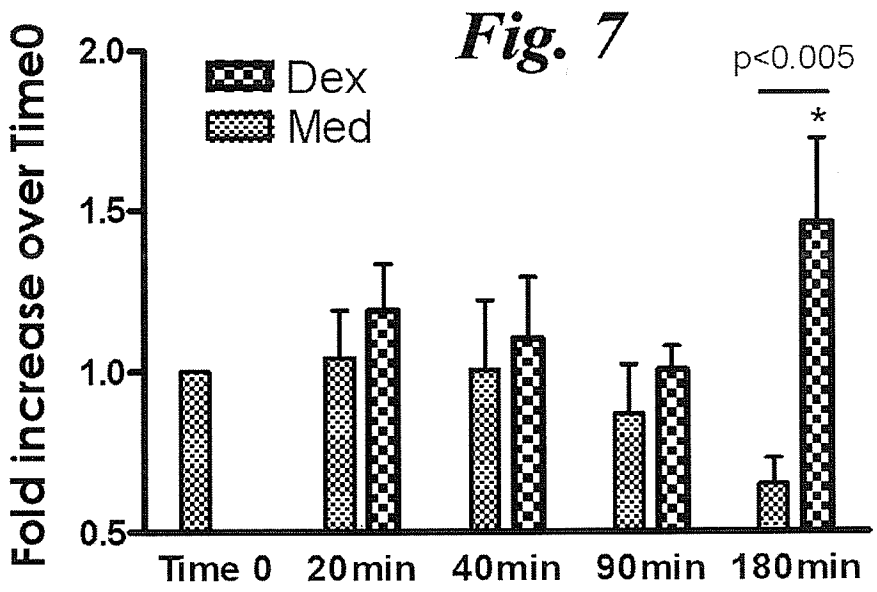

Figure 7. Dex induced Mcl-1 mRNA in human neutrophils. To verify that Dex up-regulated Mcl-1 at the transcriptional level, we analyzed Mcl-1 transcript levels in human neutrophils treated with Dex by quantitative real-time RT-PCR. Total RNA was separated from freshly isolated neutrophils (time 0) and those cultured for various time points with 1μM Dex in complete medium. Quantification of Mcl-1 transcript numbers clearly demonstrated an increase of Mcl-1 in Dex-treated neutrophils at all time points that reached statistical significance at 3h, while the mRNA levels steadily declined in cells maintained in medium alone. RNA isolation using TRIzol reagent and RT-PCR were carried out as described in Materials and Methods. Results are reported as fold increase of Mcl-1 transcript numbers over average value for Time 0 (* $p<0.05$ compared with Time 0; n=6). GAPDH was used as the house keeping gene.

4.4 Anti-apoptotic Mcl-1 up-regulation by Dex was associated with suppression of apoptosis at the level of mitochondria

Mcl-1 is a pro-survival molecule that exerts its anti-apoptotic effect at the level of mitochondria by inhibiting the function of pro-apoptotic Bcl-2 family members. Due to technical limitations in working with human neutrophils, a specific genetic shut-down of Mcl-1 was not possible. As such, we sought to determine whether the observed increase in Mcl-1 correlated with any possible changes in the mitochondrial pathway of apoptosis. Two sets of experiments were performed to analyze the mitochondrial checkpoint of apoptosis in neutrophils treated with Dex. In the first set of investigations, the mitochondrial fraction of neutrophils was separated from the cytosolic fraction and subjected to immunoblotting. Subcellular fractionation of neutrophils revealed that in freshly isolated cells, pro-apoptotic Bid and Bax molecules were mostly localized in the cytosol without any truncation. In control cells cultured for 18h in medium alone or with RU added before Dex, the majority of Bid and Bax molecules were observed in the mitochondrial fraction. However, in cells cultured with Dex or GM-CSF, substantial portions of Bid and Bax remained in the cytosol (Fig. 8A). In addition, cleaved Bid and Bax fragments were only detected in the mitochondrial fraction of cells undergoing extensive apoptosis (untreated and Dex+RU) (Fig. 8A), confirming suppressed activation of Bid and Bax in Dex treated samples.

Smac (second mitochondrial activator of caspases) is released following Bax insertion into mitochondria, allowing caspase activation to proceed.[113] As shown in Fig. 8A, in fresh neutrophils, Smac was detected in the mitochondrial fraction only. This pattern was

generally maintained after 18h of culture with Dex or GM-CSF, but not Dex+RU or medium alone. In the latter, Smac was detectable mostly in the cytoplasmic fraction.

In the second set of experiments, integrity of mitochondrial membrane was analyzed. This was done using fluorescent staining with JC-1, a dye specific for mitochondria. JC-1 binds mitochondria and displays differential fluorescent wave emission in cells with maintained or lost mitochondrial transmembrane potential. The loss of mitochondrial transmembrane potential occurs as a result of Bax/Bak insertion into mitochondria and is associated with opening of mitochondrial pores and release of Smac/Diablo.[26] In this experiment, neutrophils were cultured for 18h and then stained with JC-1 followed by FACS analysis. As can be observed in Fig. 8B and in accord with previous assays of apoptosis (Fig. 1), only 22% of neutrophils cultured in medium alone had maintained their mitochondrial integrity; in contrast, more than 50% of neutrophils cultured with Dex demonstrated maintenance of mitochondrial transmembrane potential ($p<0.05$). This effect was indeed specific to the GR, as RU-486 completely reversed the phenomenon. Taken together, these results indicate that Dex treatment functionally suppressed the process of apoptosis at least at the mitochondrial level.

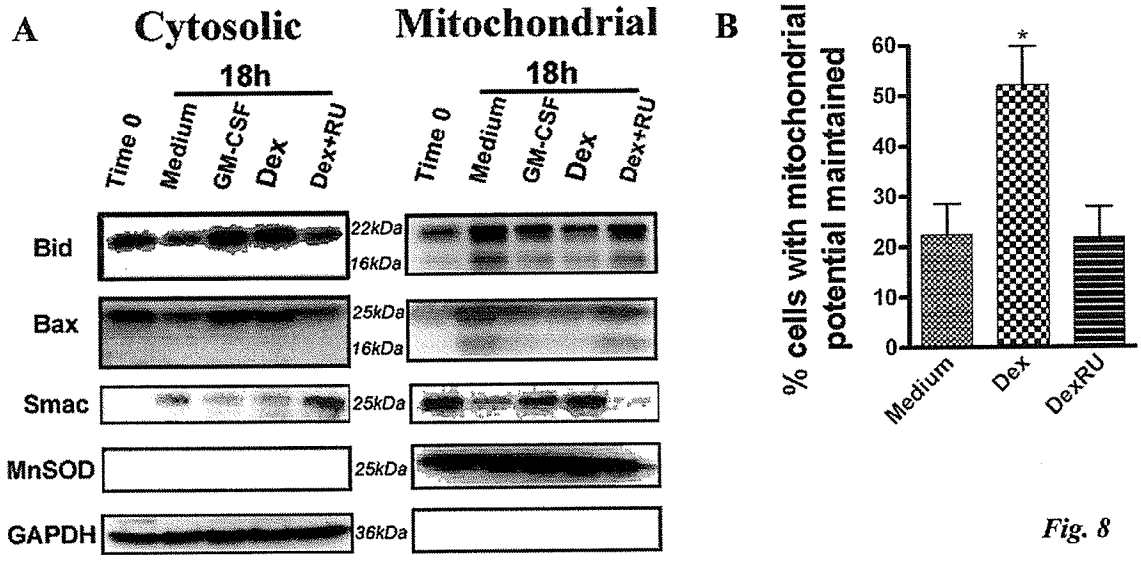

Figure 8. Dex suppressed pro-apoptotic activity at the level of mitochondria. Mitochondria are critical regulators of apoptosis that release a number of key pro-death molecules such as Smac as they lose the integrity of their membranes. Upon receiving the appropriate signal in the form of aggregated Bax and cleaved Bid insertion, mitochondrial membrane potential is lost. To investigate mitochondria as a key stage in Dex-induced survival, (A) subcellular fractions were obtained from freshly isolated neutrophils (Time 0) or those cultured for 18h with 10ng/ml GM-CSF, 1μM Dex or 10μM RU-486 followed by 1μM Dex in complete medium. These lysates were immunoblotted for Bid and Bax, both of which demonstrated cleavage and insertion into mitochondria. In contrast, western blots for Smac revealed its release from mitochondria. The above process was clearly inhibited by Dex, indicating induction of survival at or upstream of mitochondria. MnSOD and GAPDH served as mitochondrial and cytosolic content control, respectively. Panel represents 3 separate experiments. Moreover, cells stimulated with Dex maintained their mitochondrial membrane potential (B), suggesting preserved mitochondrial integrity. These data were obtained from FACS analysis of the

above cells stained with JC-1 die, whose fluorescence properties change upon loss of mitochondrial transmembrane potential (*$p<0.05$; n=6).

4.5 Dex up-regulated XIAP protein

Although the Bcl-2 family is traditionally considered the critical step in safeguarding cell survival, there is clear evidence that a second checkpoint also exists to counter apoptosis. This second checkpoint consists of the IAP family that prevents apoptosis by binding caspases and Smac/Diablo. We assessed total levels of candidate anti-apoptotic IAPs in Dex treated neutrophils by immunoblotting. It was observed that a 12h treatment with Dex specifically stabilized XIAP, and partially maintained survivin (Fig. 9; $p<0.05$). These effects were specific to Dex and were not observed in either the culture medium control, GR blocked control or in cells cultured with GM-CSF. In the case of cIAP-1 and cIAP-2 however, there were no significant changes in any of the groups (Fig. 9). The above results demonstrate an enhancement of anti-apoptotic proteins at yet another level in the regulation of neutrophil survival by Dex.

Fig. 9

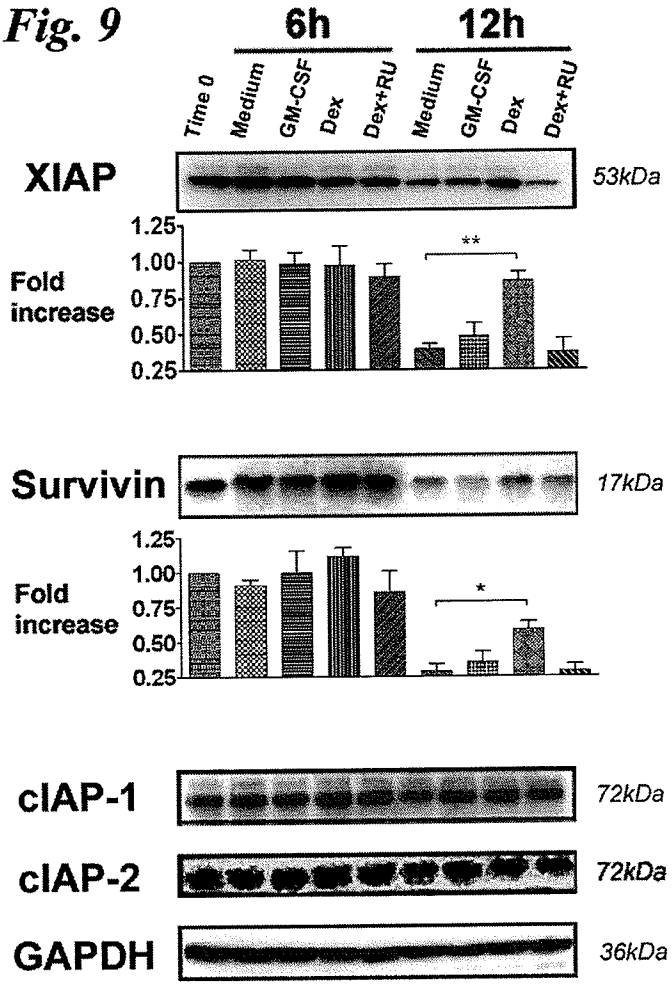

Figure 9. Dex up-regulated XIAP protein in neutrophils. IAPs were detected in total lysates of fresh or neutrophils cultured for 6 and 12h by western blotting and quantified by densitometry. Densitometry results are reported as fold increase over average value for Time 0. Figure represents three independent experiments (* $p<0.05$, ** $p<0.01$).

4.6 Dex blocked activation of caspases

Since caspases perform their pro-apoptotic function downstream of mitochondrial and IAP checkpoints, we sought to determine whether the above findings correlated with downstream caspase activity. To perform a thorough analysis of both initiator and executioner caspases, enzymatic activity of caspases -8, -9 and -3 was measured in neutrophils cultured for various time periods. At 6h, neutrophils treated with Dex and GM-CSF showed decreases of approximately 25% in initiator caspase-8 and 9 enzymatic activities compared with untreated cells (Fig. 10A; $p<0.05$); no significant differences with either Dex or GM-CSF were detected at later time points up to 24h (data not shown) in caspase -8/9 activity. In the case of executioner caspase-3, enzymatic activity measurement at 6h revealed a 4-fold decrease in neutrophils treated with Dex compared with untreated cells (Fig. 10B; $p<0.001$). Significant reductions in caspase-3 protease activity were maintained up to 24h in Dex treated cultures.

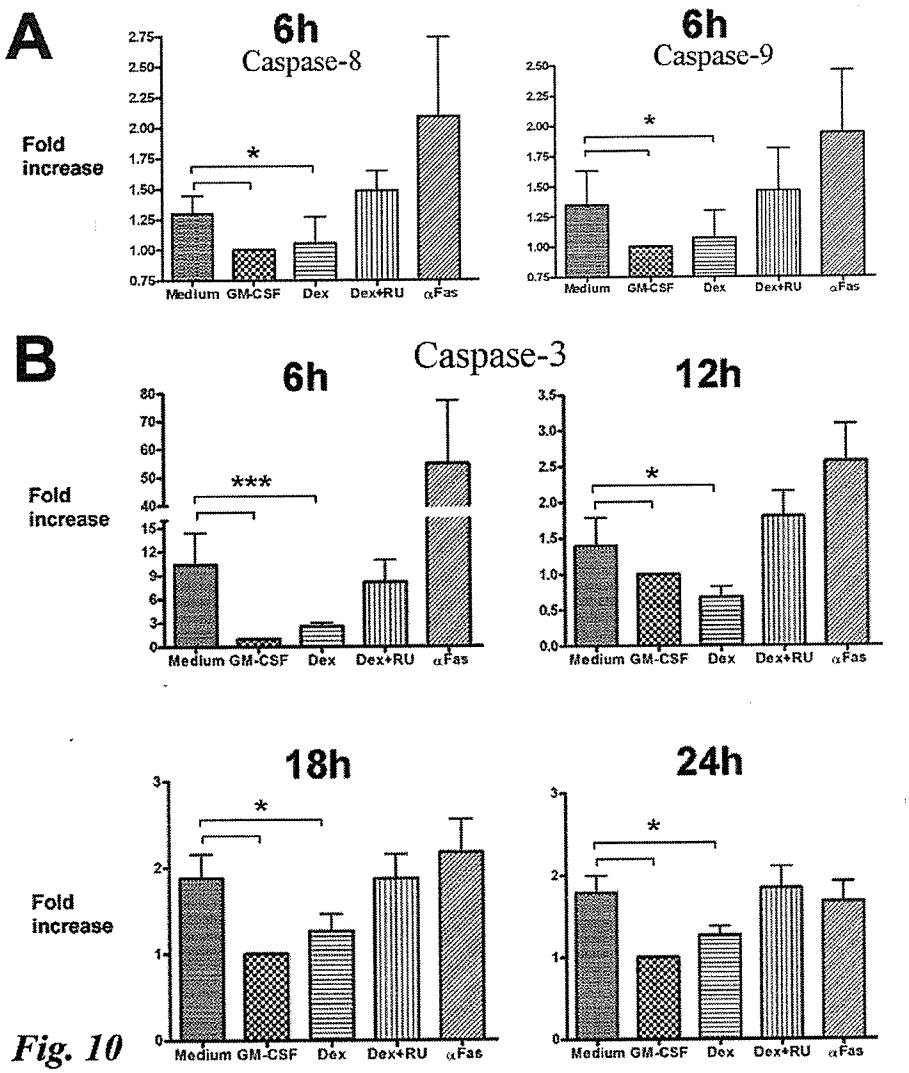

Fig. 10

Figure 10. Caspase activity in neutrophils was suppressed by Dex. Caspase enzymatic activity is a good indicator of downstream pro-apoptotic activity in all cell types. The activity was measured by adding <15,000 cells in culture medium with equal volumes of a specific reagent that lyses the cells to free the caspases inside. Caspases will then cleave and release a fluorogenic caspase substrate, resulting in enzymatic activity as measured by a luminometer. In these sets of experiments, neutrophils were cultured for 6, 12 and 18h in complete medium alone or with the addition of 10ng/ml GM-CSF, 1μM Dex, 10μM RU-486 followed by 1μM Dex or 250ng/ml anti-Fas monoclonal antibody.

Anti-Fas monoclonal antibody served as a positive control that potently induced apoptosis in neutrophils. At the specified time points, approximately 15,000 cells were taken out of culture and assessed for caspase activity as mentioned above. Enzymatic activity of caspases-8, -9 (A) and -3 (B) are reported as fold increase over average value for GM-CSF. Cells treated with Dex or GM-CSF demonstrated a general reduction in caspase enzymatic activity, confirming reduced apoptosis in these cells was in fact dependent on decreases of active caspases. The figure includes data from at least five separate experiments done in quadruplicate (* $p<0.05$, *** $p<0.001$).

To verify the data from enzymatic activity assays, western blots were performed using two different specific antibodies against either the caspase-3 zymogen or its active fragment. In fresh cells, only the zymogen was apparent. By 12 and 18h however, the amount of zymogen had decreased and the active fragment was observed in control cultures, but not in cells treated with Dex or GM-CSF (Fig. 11). Taken together, these data demonstrate that the enhanced levels of anti-apoptotic Mcl-1 and XIAP molecules in Dex-treated neutrophils are indeed associated with downstream inhibition of apoptosis pathways.

Fig. 11

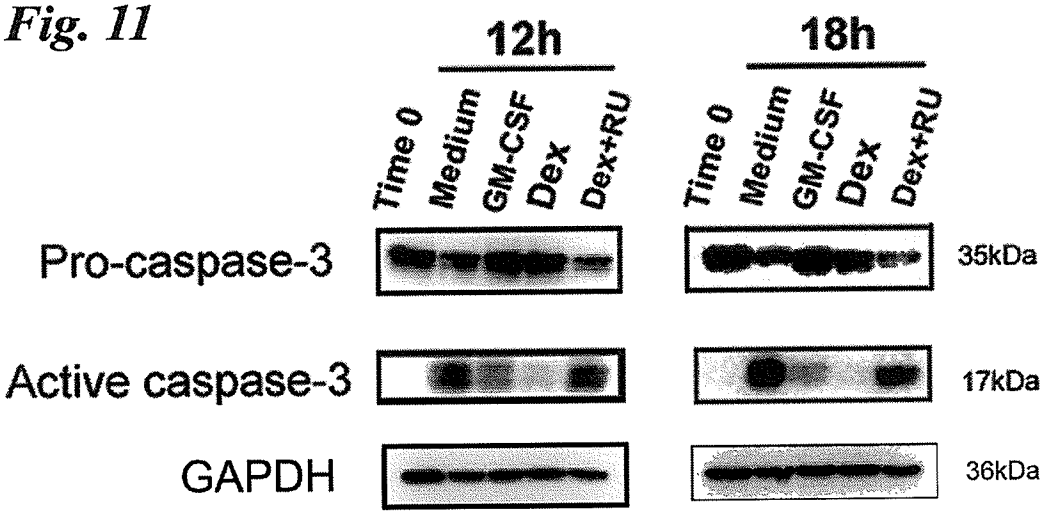

Figure 11. Caspase-3 zymogen was maintained in Dex-treated neutrophils. Caspase cleavage is a sound indicator of overall pro-apoptotic activity in various cell types. To become enzymatically active, caspases have to be cleaved from a zymogen pro-caspase protein into a short active enzyme. The zymogen as well as the active caspase can be simultaneously or separately detected in western blots at specific molecular weights. Such data often correlate with and confirm results from caspase enzymatic activity measurements. In the above experiments, freshly isolated neutrophils (time 0) were cultured in complete medium alone or together with 10ng/ml GM-CSF, 1μM Dex or 10μM RU-486 followed by 1μM Dex for 12 and 18h. At the specified time points, the cells were taken out of culture, washed with PBS, and lysed. Western blots were performed separately for active caspase-3 and its zymogen (pro-caspase-3). GAPDH served as assurance of equal protein loading. It is obvious from the figure that in GM-CSF and Dex treated cultures, neutrophils maintained a great portion of their caspase-3 zymogen, with little cleavage occurring in comparison with cells cultured in medium alone. Panel is representative of three separate experiments.

4.7 PI3K signaling was required for the inhibition of apoptosis by Dex

Induction of survival in human neutrophils by various anti-apoptotic factors such as GM-CSF, [53, 124] IL-8,[77] leptin,[123] IFN-β,[78] LTB_4[127] and LPS [1, 124] has been reported to occur through the PI3K pathway. To determine whether Dex also utilized this pathway to inhibit apoptosis, neutrophils were pre-incubated with wortmannin, a specific inhibitor of PI3K before treatment with Dex, and were examined for apoptosis at 18h.

Interestingly, inhibition of PI3K equally and significantly decreased the survival induced by both Dex and GM-CSF ($p<0.0005$; Fig. 12A). The impact of wortmannin was clearly observed in the form of a shift from annexin-V negative to positive cells in FACS analyses of neutrophil cultures, ruling out necrosis (Fig. 12B). These results indicate a critical role for PI3K in Dex-induced neutrophil survival.

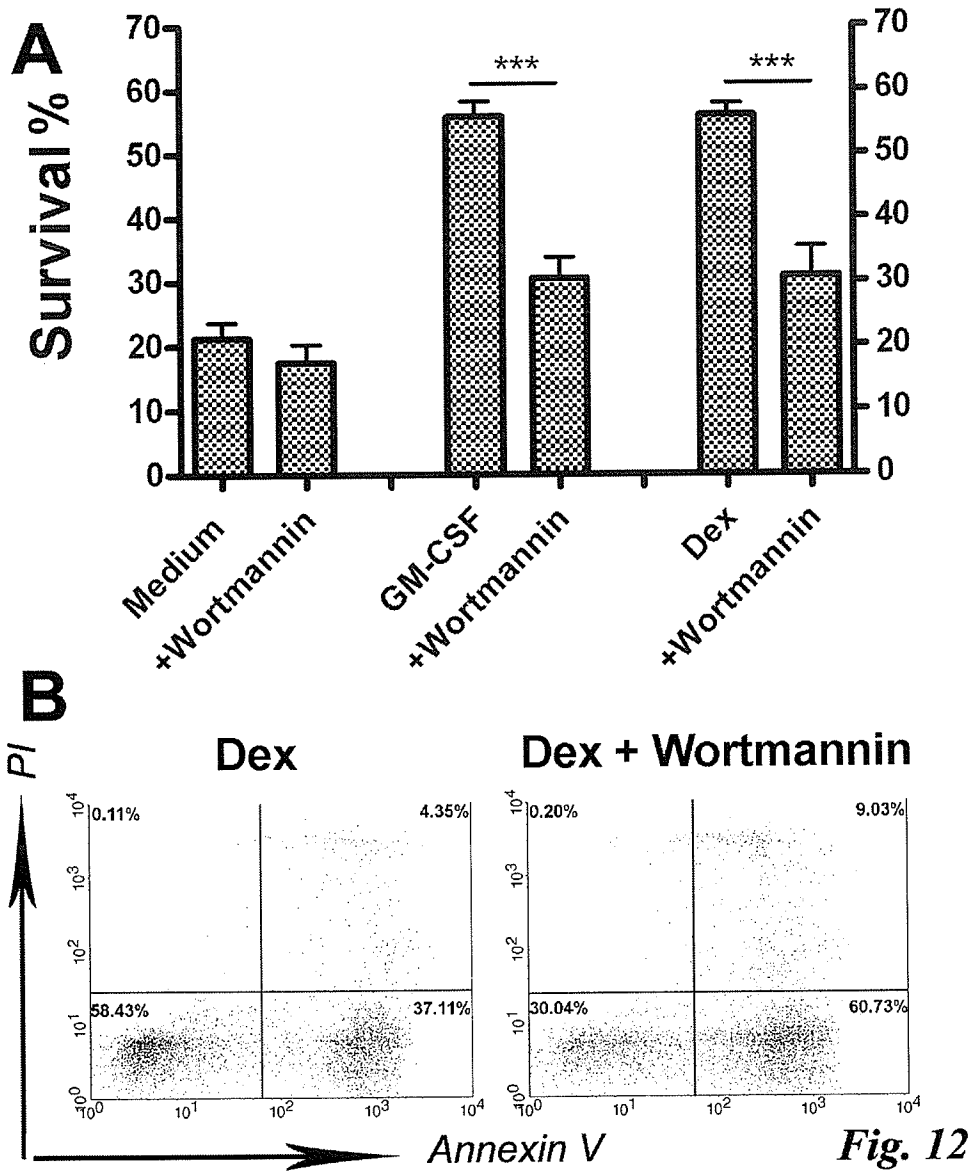

Fig. 12

Figure 12. Inhibition of PI3K suppressed Dex-mediated survival of neutrophils. PI3K is a key mediator of many upstream cellular signaling events, orchestrating complex and diverse phenomena such as cell survival. The necessity of PI3K signaling for downstream effects of any particular agent may be determined by blocking PI3K enzymatic activity. Wortmannin is a cell-permeable specific inhibitor of PI3K. In this experiment, freshly isolated neutrophils were resuspended in complete medium plus DMSO, or 25nM wortmannin. After 15min, 10ng/ml GM-CSF or 1μM Dex was added to the above

cultures, and the cells were incubated for 18h. Apoptosis was then assessed by annexin-V/PI staining (*** $p<0.0001$; n=9). It is evident form panel (A) that by blocking PI3K, the survival effect of both GM-CSF and Dex was nearly abrogated. Panel (B) depicts a representative FACS plot of annexin-V/PI stained cells. The main consequence of wortmannin addition was a shift of cells from the annexin-V negative population to the annexin-V positive group on the lower right, ruling out necrosis as a potential effect of PI3K blockade.

4.8 Dex required p38 but not ERK or JNK MAPK signaling to inhibit apoptosis

MAPKs especially p38 [221, 222] and ERK [77, 124, 127] have been found to play important roles in inhibition of neutrophil apoptosis. We used specific inhibitors of the three MAPKs, namely U0126 for MEK1/2 (preventing phosphorylation of downstream ERK1/2), SB203580 for p38 and SP600125 for JNK prior to stimulation of neutrophils with Dex or GM-CSF. These neutrophil cultures were subsequently assessed for apoptosis following 18h of incubation. In accord with previous reports,[77, 124] we found ERK inhibition significantly suppressed the survival inducing effects of GM-CSF ($p<0.0005$); however, ERK inhibition had only minimal effects on Dex-induced survival (Fig. 13A). Similarly, inhibition of JNK caused a significant decrease in the survival mediated by GM-CSF ($p<0.005$) but not Dex (Fig. 13B). Interestingly, inhibition of p38 equally and significantly suppressed the survival effects of both GM-CSF and Dex ($p<0.005$; Fig. 14), in a manner similar to but smaller than inhibition of PI3K (Fig. 12A).

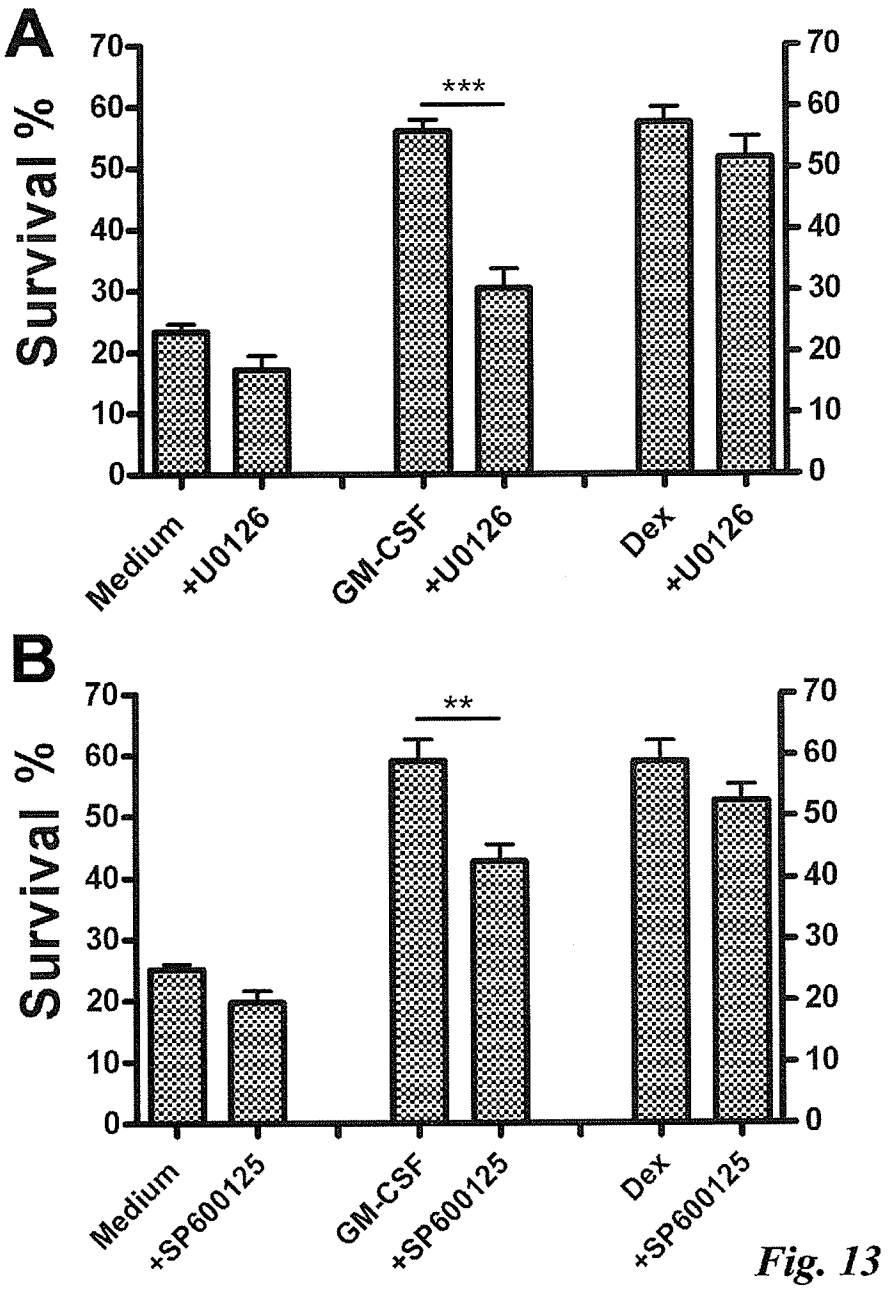

Fig. 13

Figure 13. The effect of ERK and JNK MAPK inhibition on Dex-induced survival of neutrophils. ERK and JNK MAPK are signaling molecules critically required for a variety of cell functions. The necessity of ERK or JNK MAPK signaling for downstream effects of any particular agent may be determined by blocking their enzymatic activity. U0126 and SP600125 are cell-permeable specific inhibitors of ERK and JNK, respectively. In these

experiments, freshly isolated neutrophils were resuspended in complete medium plus DMSO, 5μM U0126 (A) or 10μM SP600125 (B). After 15min, 10ng/ml GM-CSF or 1μM Dex was added to the above cultures, and the cells were incubated for 18h. Apoptosis was then assessed by annexin-V/PI staining (*** $p<0.0005$; ** $p<0.005$; $n=8$). It is evident form panels (A) and (B) that while GM-CSF-induced survival of neutrophils was absolutely dependent on JNK and especially ERK MAPK signaling, Dex-mediated survival was not dependent on neither of those molecules.

Fig. 14

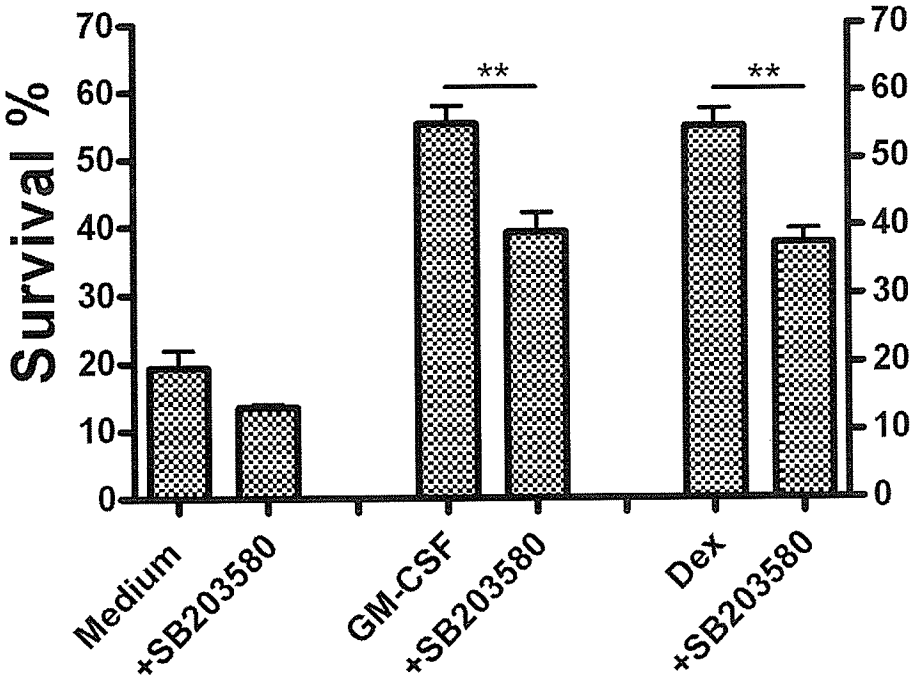

Figure 14. The effect of p38 MAPK inhibition on Dex-induced survival of neutrophils. P38 MAPK is a salient signaling molecule required for a variety of cell functions. The necessity of p38 MAPK signaling for downstream effects of any particular agent may be determined by blocking its enzymatic activity. SB203580 is a cell-permeable specific inhibitor of p38 MAPK. In the above experiments, freshly isolated neutrophils were resuspended in complete medium plus DMSO or 25μM SB203580. After 15min, 10ng/ml GM-CSF or 1μM Dex was added to the above cultures, and the cells were incubated for 18h. Apoptosis was then assessed by annexin-V/PI staining (** $p<0.005$; $n=9$). The above graph shows a grave requirement of p38 MAPK signaling for induction of survival by both GM-CSF and Dex.

These data indicate similarities and differences between pathways utilized by GM-CSF and Dex to inhibit human neutrophil apoptosis. To further examine these differences, we used these inhibitors in combination at suboptimal concentrations. As can be seen in Fig. 15 and in agreement with the above results, combined inhibition of ERK and p38 MAPK nearly abrogated the survival induced by GM-CSF ($p<0.001$) but did not significantly influence Dex-induced survival anymore than inhibition of p38 alone. The above data suggest differential requirements for MAPK signaling in glucocorticoid-induced versus GM-CSF-induced survival. Whereas Dex-mediated survival relies on only one of the three MAPKs (i.e. p38), optimal anti-apoptotic signaling by GM-CSF requires all three MAPKs.

Fig. 15

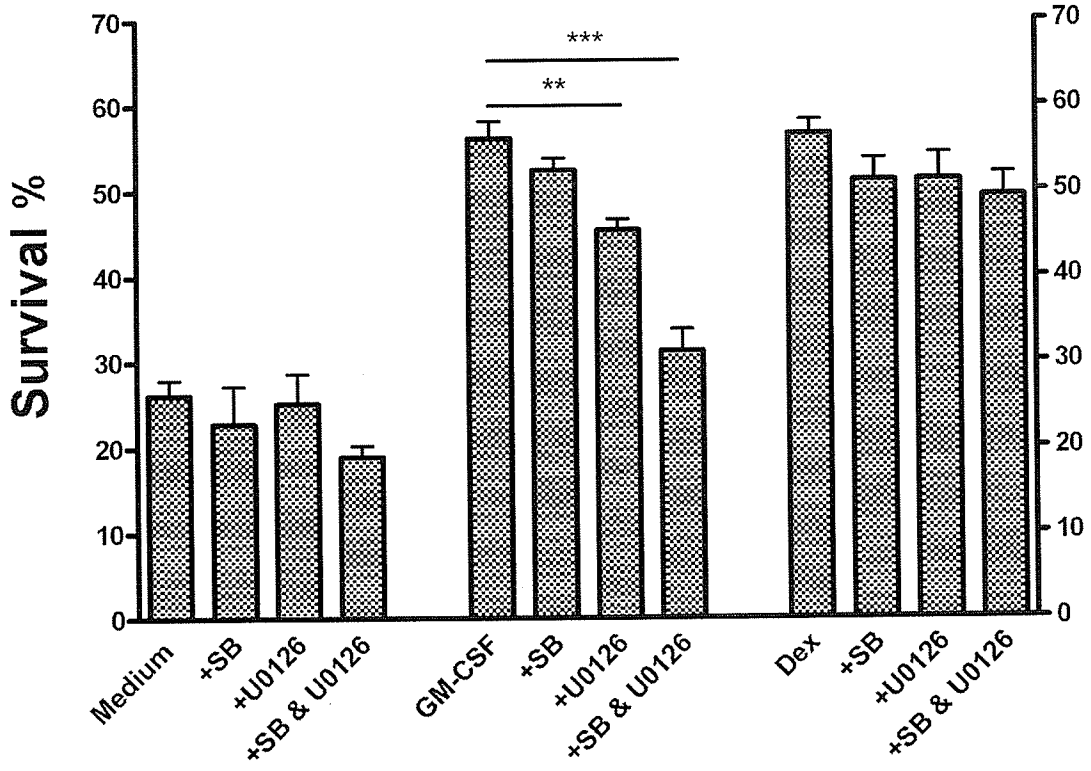

Figure 15. Combination of p38 and ERK MAPK inhibition attenuated GM-CSF- but not Dex-induced survival. Since previous data demonstrated the requirement of both p38 and ERK MAPK signaling for GM-CSF to mediate enhanced survival of neutrophils, we sought to determine whether these molecules functioned in a cooperative manner to inhibit apoptosis. To answer the above question, suboptimal concentrations of SB203580 and U0126 were used alone or in combination, to block p38 or ERK MAPK, respectively. This strategy avoided a complete inhibition of signaling that would abrogate GM-CSF mediated survival. Freshly isolated neutrophils were resuspended in complete medium plus DMSO, 1μM U0126, 10μM SB203580 or the combination. After 15min, 10ng/ml GM-CSF or 1μM Dex was added to the above cultures, and the cells were incubated for 18h. Apoptosis was then assessed by annexin-V/PI staining (***

p<0.001; ** p<0.01; n=6). In agreement with the data above, GM-CSF enhanced neutrophil survival through ERK and p38 MAPK signaling, whereas Dex-mediated neutrophil survival was only minimally affected by the suboptimal concentrations of p38/ERK MAPK inhibition. These data highlight differences in the signaling pathways utilized by GM-CSF versus Dex to delay neutrophil apoptosis.

4.9 Combined inhibition of PI3K and p38 MAPK abrogated Dex-induced survival of neutrophils

The above data indicated that among the pathways examined, PI3K and p38 MAPK were important in Dex-mediated survival of neutrophils, and that the requirement for PI3K was greater than p38 MAPK for the induction of survival by Dex. To determine whether these two molecules played redundant roles in such survival, we used a combination of SB203580 and wortmannin in our cultures at sub-optimal concentrations to avoid their synergistic cytotoxic effect at full dose.

Interestingly, combined inhibition of PI3K and p38 MAPK abrogated Dex-induced but not GM-CSF-mediated survival of neutrophils ($p<0.0005$; Fig. 16). These data indicate total necessity of p38 MAPK and PI3K signaling for Dex but not GM-CSF-induced survival of neutrophils.

Fig. 16

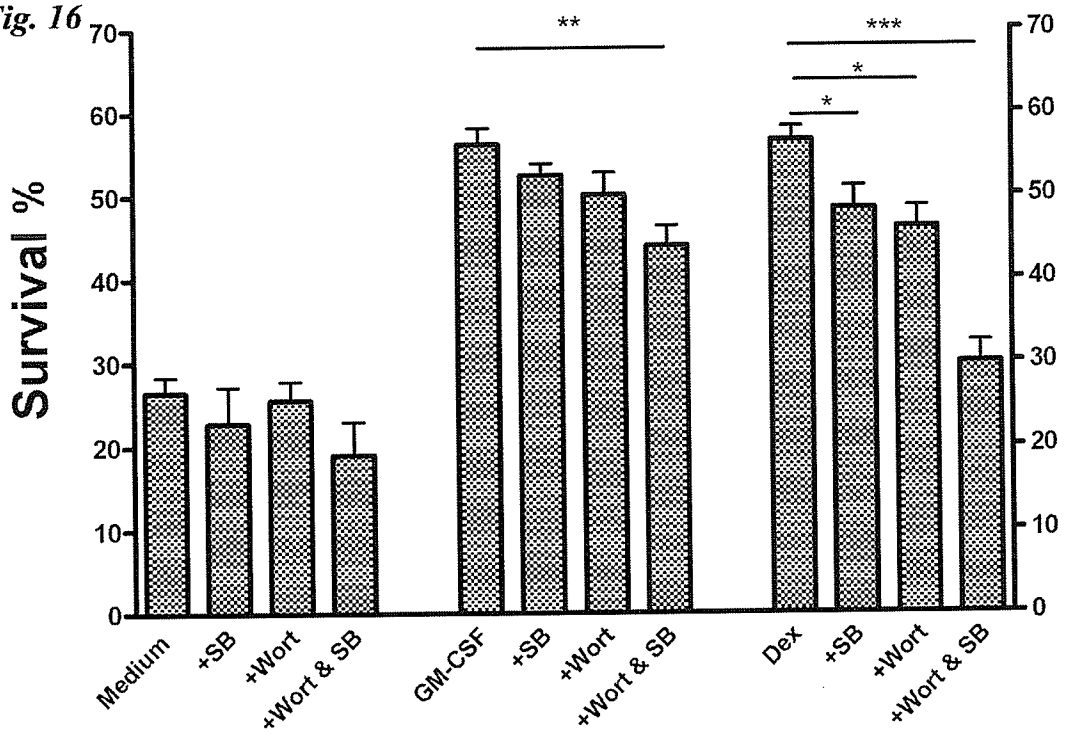

Figure 16. PI3K and p38 MAPK regulated the induction of survival by Dex. The above results indicated the requirement of both p38 MAPK and PI3K signaling for Dex to enhance survival of neutrophils. We then sought to determine whether these molecules functioned in a cooperative manner to inhibit apoptosis. Suboptimal concentrations of SB203580 and wortmannin were used alone or in combination, to inhibit p38 MAPK or PI3K, respectively. These concentrations avoided a complete inhibition of signaling that would abrogate Dex mediated survival. Freshly isolated neutrophils were resuspended in complete medium plus DMSO, 10nM wortmannin, 10μM SB203580 or the combination. After 15min, 10ng/ml GM-CSF or 1μM Dex was added to the above cultures, and the cells were incubated for 18h. Apoptosis was then assessed by annexin-V/PI staining (*** $p<0.001$; ** $p<0.01$; * $p<0.05$; $n=6$). In agreement with previous data, both Dex and GM-CSF enhanced neutrophil survival through PI3K and p38 MAPK

signaling. Remarkably however, the combined inhibition of p38 MAPK and PI3K was more detrimental to the survival induced by Dex than that of GM-CSF.

4.10 Mcl-1 up-regulation by Dex was reversed by inhibitors of PI3K or p38 MAPK

Taken together, the above results indicated two critical phenomena in the pro-survival effect of Dex on human neutrophils:

a) up-regulation of Mcl-1, correlating with maintained mitochondrial integrity

b) signaling through PI3K and p38 MAPK

While both phenomena seemed vitally important, their relevance to each other was not clear. In this step, neutrophils were pre-incubated with inhibitors of p38 MAPK or PI3K and then stimulated with Dex for 6h. Dex-induced Mcl-1 up-regulation was abrogated by inhibiting PI3K or p38 MAPK (Fig. 17A). These results suggest that signaling through p38 MAPK and PI3K are required for up-regulation of Mcl-1 by Dex.

4.11 PI3K and p38 MAPK inhibition resulted in loss of mitochondrial integrity and activation of caspase-3

Since Mcl-1 is responsible for maintaining mitochondrial integrity, we sought to confirm that abrogation of Dex-induced Mcl-1 up-regulation through inhibition of PI3K/p38 MAPK had functional consequences in the mitochondrial pathway. Analysis of mitochondrial transmembrane potential using JC-1 staining of neutrophils demonstrated a 2.5 fold increase of cells with maintained mitochondrial integrity among Dex treated neutrophils at 18h of culture. This increase was abolished by pre-incubation with inhibitors of PI3K/p38 MAPK alone or in combination ($p<0.05$; Fig. 17B).

As we showed before in Fig. 10, Dex mediated a significant suppression of caspase-3 activity. In accord with the mitochondrial integrity analyses, Dex-mediated suppression of caspase-3 activity was also significantly impaired following the inhibition of PI3K/p38 MAPK ($p< 0.0001$; Fig. 17C). The above data suggest PI3K and p38 MAPK exert their anti-apoptotic signaling effects at the level or upstream of mitochondria.

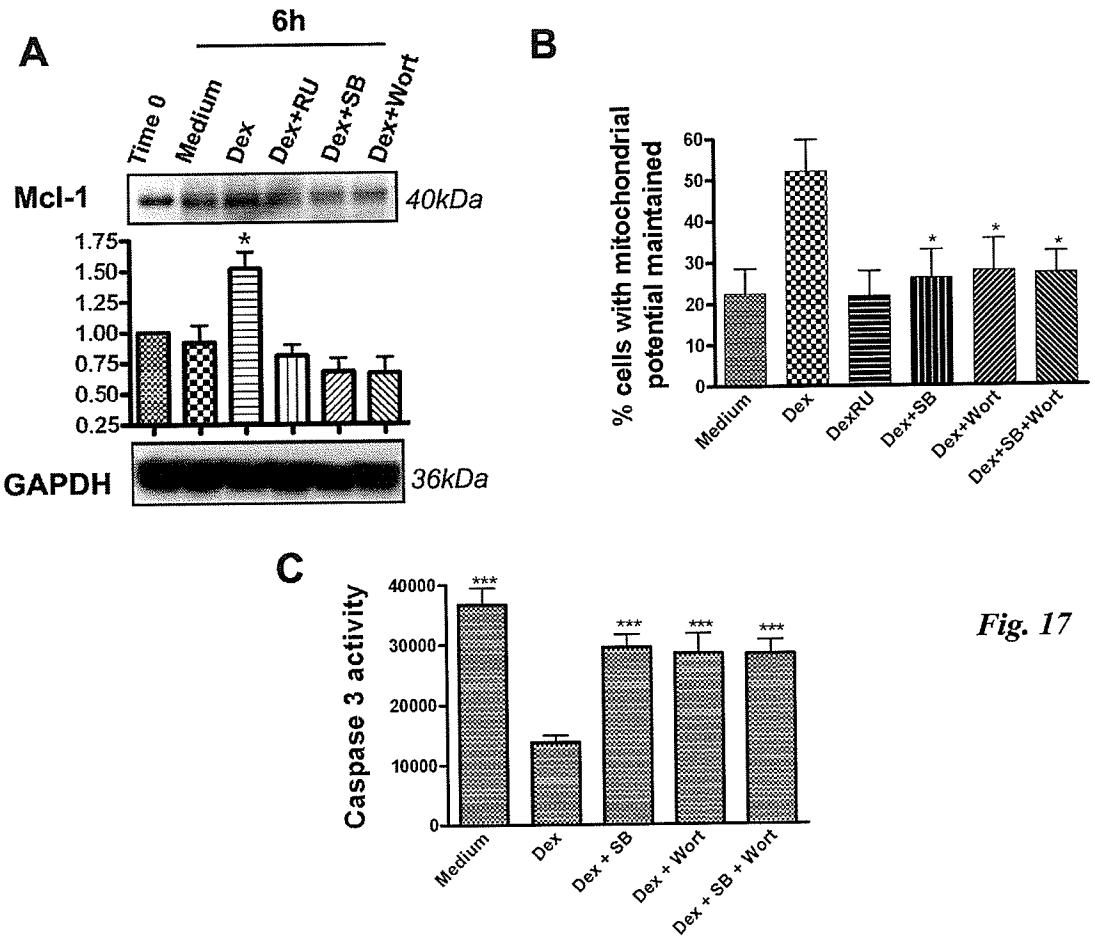

Fig. 17

Figure 17. Inhibition of PI3K and p38 MAPK abrogated the pro-survival effects of Dex. While it was clear from prior experiments that PI3K/p38 MAPK signaling as well as up-regulation of Mcl-1 were important for Dex-mediated neutrophil survival, no mechanisms were evident that linked the above phenomena. Therefore, we sought to determine the apoptosis checkpoint most dramatically affected by blocking PI3K/p38 MAPK signaling. (A) Human neutrophils were either lysed immediately following isolation (time 0) or pre-incubated with: DMSO, RU-486 (10μM), SB203580 (25μM) or wortmannin (25nM) for 15min in complete medium. Neutrophils were then treated with 1μM Dex or left untreated for 6h. Immunoblots were performed for Mcl-1 that

depicted a lack of Mcl-1 protein up-regulation in Dex-stimulated neutrophils pretreated with SB203580/wortmannin. These results suggest PI3K and p38 MAPK are both necessary for Dex to boost Mcl-1 protein in neutrophils. Densitometry results are reported as fold increase over average value for Time 0 (*$p<0.05$ compared with Medium; n=3). (B) Neutrophils were pre-incubated with: DMSO, RU-486, SB203580 (25μM), wortmannin (25nM), or the combination (10μM and 10nM, respectively) for 15min. These cells were then treated with 1μM Dex for 18h. JC-1 staining and mitochondrial membrane potential analysis by FACS were performed (*$p<0.05$ compared with Dex; n=6). Panel B data are in agreement with the results from panel A suggesting PI3K/p38 MAPK signaling inhibit neutrophil apoptosis at or upstream of the mitochondrial checkpoint. (C) Neutrophils were pre-incubated with: DMSO, RU-486, SB203580 (25μM), wortmannin (25nM), or the combination (10μM and 10nM, respectively) for 15min. These cells were then treated with 1μM Dex and incubated for 6h. Caspase-3 enzymatic activity was measured according to Materials and Methods (***$p<0.0001$ compared with Dex; n=6 in quadruplicate). Reversal of Dex-mediated blockade of caspase-3 activity downstream of mitochondria is evident in neutrophils pretreated with PI3K/p38 MAPK inhibitors.

5. Discussion

In this study we provide further insight into the molecular pathways through which glucocorticoids inhibit apoptosis of primary human neutrophils. This inhibition occurs through p38 MAPK and PI3K signaling pathways. These pathways are necessary for the induction of anti-apoptotic Mcl-1 protein in neutrophils by Dex. Mcl-1 enhancement in turn is associated with suppression of downstream pathways of apoptosis (Fig. 18). These mechanisms are of potential clinical value and provide further insight into treatment of neutrophilic inflammation. In asthma for instance, corticosteroids have been shown to increase airway tissue neutrophils.[187, 188] They have also been shown to decrease chemotactic factors for T cells and eosinophils but not neutrophils in asthmatic airway mucosa.[190] Furthermore, glucocorticoids are reportedly inefficient in controlling increased neutrophil MMPs in severe asthma.[189] We speculate that the above-mentioned mechanisms may contribute in such processes, although *in-vivo* pro- and anti-apoptotic signals often coexist in the inflammatory milieu, rendering the pathophysiology much more complex.

From a physiological point of view, the neutrophilic reaction that follows *in vivo* glucocorticoid administration may have developed as a response to stress that boosts the innate immunity. For instance, it has been shown that although increased levels of corticosteroids in mice leads to a decrease in the lymphocyte population and suppression of the adaptive immune response, it increases neutrophil numbers and enhances anti-bacterial immunity.[223] Mice with increased serum glucocorticoids that were exposed to *L.*

monocytogenes and *S. pneumonia* demonstrated enhanced clearance of these bacterial infections.[223]

We first demonstrated that GR engagement by Dex was necessary for induction of neutrophil survival, since treatment of cells with the GR antagonist RU-486 ablated the response. Second, selective induction of the transrepressive function of GR by the GR modulator CpdA did not alter neutrophil apoptosis, although CpdA has been shown to bind GR with high affinity and efficiently repress genes.[150] Third, the inhibitory impact of Dex on neutrophil apoptosis could not be considered non-genomic, since the effect is abrogated by transcription/translation inhibitors.[139, 214]

These findings suggest GR transactivation may be responsible for Dex-mediated survival of neutrophils. In agreement with our hypothesis, we did not detect significant decreases in pro-apoptotic proteins such as Fas, FasL, Bid and Bax following stimulation with Dex. In contrast, enhancement of anti-apoptotic proteins was clearly observed. Reports on neutrophil apoptosis and factors that delay the process generally agree that the level of pro-apoptotic molecules such as Bax is not altered by anti-apoptotic stimuli.[59, 118, 224] Furthermore, findings of a very recent report confirmed our data indicating that the protein levels of Bax in human neutrophils do not significantly change following incubation with Dex.[218] Although Fas/FasL levels did not change significantly in Dex-treated human neutrophils, caspase-8 activity and Bid cleavage were observed in neutrophils undergoing apoptosis. Since "TNF-related apoptosis-inducing ligand" (TRAIL) receptor is an additional death receptor expressed by human neutrophils that can activate caspase-8 leading to Bid truncation,[225] further investigation into potential down-regulation of neutrophil TRAIL or its receptor by Dex is warranted.

The first candidate among pro-survival proteins to be induced by glucocorticoids was the Bcl-2 family. In fact, evidence from the literature suggests glucocorticoids can inhibit apoptosis in a variety of cell types in association with enhancement of anti-apoptotic Bcl-2 family proteins.[158, 159, 162] As mentioned above, we observed an up-regulation of the anti-apoptotic Bcl-2 family member Mcl-1 following stimulation of neutrophils with Dex. Mcl-1 is the sole anti-apoptotic member of Bcl-2 family consistently detected in human neutrophils at the protein level.[131] A1 and Bcl-x_L are two other pro-survival Bcl-2 family molecules that have been variably reported to be expressed at low levels in human neutrophils.[1, 116, 117] However, like others we have not been successful in consistently detecting significant levels of these molecules using commercially available antibodies.[53, 59, 60] Up-regulation of anti-apoptotic Bcl-2 family members by Dex in neutrophils has been recently reported in two other studies as well. In a report on bovine neutrophils, Madsen-Bouterse et al. found an induction of A1 by Dex.[217] Sivertson et al.[218] in agreement with the current study detected increases in Mcl-1 mRNA and protein in Dex treated human neutrophils with similar kinetics. Furthermore, up-regulation of Mcl-1 through PI3K/p38 MAPK signaling pathways is in agreement with previous reports demonstrating the importance of these signaling molecules in neutrophils and other cell types that respond to pro-survival agents by enhancing Mcl-1.[50, 53, 54] These signaling molecules up-regulated Mcl-1 at the transcriptional level by activating transcription factors such as PU.1 and CREB.[50, 54]

The second candidate family of pro-survival molecules was IAPs, which serve as an additional checkpoint for apoptosis in cells. These proteins contain BIR domains in their structure that specifically interact with particular domains in caspases/Smac and prevent

their activity.[62] Neutrophils express a diversity of these proteins, and up-regulate them under certain inflammatory conditions.[226, 227] We demonstrate that the level of XIAP, the prototypic IAP, was specifically maintained by Dex. Similarly, XIAP has been shown to be stabilized by glucocorticoids in two different cancer cell lines.[163, 164] Enhanced levels of XIAP in Dex treated neutrophils may partially account for the decreased activity of caspase-3 observed at all time points in neutrophil cultures.

To study the regulation of the above anti-apoptotic proteins and survival by glucocorticoids, we focused on various intracellular signaling pathways that had previously been delineated in neutrophils. Interestingly, glucocorticoid regulation of apoptosis in other cell types has also been reported to rely on similar signaling molecules.[157, 228, 229] An original finding was the suppression of Dex-induced survival by blocking PI3K and p38 but not JNK or ERK MAPK using previously reported concentrations of wortmannin,[1, 53, 107, 123, 124, 126, 127, 221, 222, 230-232] SB203580,[1, 123, 124, 221, 222] SP600125[230, 232, 233] and U0126,[231, 232] respectively in the neutrophil literature. These inhibitors revealed significant and exclusive properties of glucocorticoid-mediated anti-apoptotic signaling versus GM-CSF in primary human neutrophils. It remains to be established whether glucocorticoids lead to direct activation of PI3K (and downstream Akt) and p38 MAPK in neutrophils as it has been shown for GM-CSF.

GM-CSF-mediated survival of neutrophils and up-regulation of Mcl-1 consist of transcription-dependent and -independent components.[53, 107, 108] Derouet et al.[107] have demonstrated that ERK MAPK and PI3K are responsible for the transcription independent enhancement of Mcl-1 that occurs through protein stabilization. We observed the effect of Dex on both neutrophil survival and Mcl-1 enhancement to be

dependent on protein translation and signaling through PI3K and p38 MAPK. These findings indicate a role for PI3K and p38 MAPK in the translation dependent enhancement of Mcl-1 by Dex; however, these signaling pathways also have alternative translation-independent anti-apoptotic effects that may in fact contribute to glucocorticoid-mediated neutrophil survival. For instance, p38 has been shown to phosphorylate and deactivate caspases- 3 & 8;[221] or PI3K-mediated Akt activity can lead to pro-survival phosphorylation of Bad, Bax and caspase-9.[73, 116]

Our data are in agreement with a recently published research article studying the up-regulation of Mcl-1 in glucocorticoid treated human neutrophils but not eosinophils.[218] Sivertson et al. elegantly demonstrated that the expression of the anti-apoptotic long form of Mcl-1 is enhanced in glucocorticoid stimulated neutrophils at both mRNA and protein level, while expression of the pro-apoptotic short form of Mcl-1 remained stable in these cells.

In 2001, Strickland and colleagues published an article in which they attributed the unusual anti-apoptotic effect of glucocorticoids on human neutrophils to high expression of the beta isoform of GR (GRβ) in these cells.[194] GRβ has been recognized as a splice variant of GR that has no transcriptional activity and a lack of endogenous ligand binding.[234] However, the authors claimed GRβ binding to GRα exerted an inhibitory influence on GR mediated transcription of pro-apoptotic genes in neutrophils, thus enhancing survival. Although the above paper may have demonstrated convincing data indicating GRβ expression in neutrophils, the above hypothesis is in contrast with findings of the current thesis and prior publications. Enhanced survival of neutrophils treated with glucocorticoids has been shown to require translation of new proteins, such

as Mcl-1. Furthermore, current results argue against transrepression of pro-apoptotic genes as a mechanism of glucocorticoid mediated survival. GRβ however, is neither capable of initiating transcription, nor is it associated with preferential suppression of pro- or anti-apoptotic genes.

Another hypothesis put forward to explain glucocorticoid-mediated neutrophil survival came from an article published by Stankova et al. in 2000.[215] Stankova and colleagues showed evidence for up-regulation of the high affinity LTB4 receptor (BLT1) in glucocorticoid treated human neutrophils. Not surprisingly however, LTB4 production itself was suppressed in these cells compared with control culture neutrophils. The authors propose that anti-apoptotic signaling of up-regulated BLT1[127] in glucocorticoid treated human neutrophils mediates enhanced survival. This hypothesis however does not specify how the increase in a cell-surface receptor alone without a concomitant rise in its ligand may lead to anti-apoptotic signaling.

Taken together, our observations provide a model in which Dex-mediated inhibition of primary human neutrophil apoptosis is associated with increased levels of Mcl-1 and XIAP. Up-regulation of these molecules correlates with suppression of various downstream pathways of apoptosis. These mechanisms might be initiated by GR-mediated transactivation of anti-apoptotic genes, independently of or in association with intra-cellular signaling molecules and transcription factors. Collectively, the above results underline mechanisms through which corticosteroids undesirably modulate apoptosis of an inflammatory cell. Development of GR ligands that selectively inhibit neutrophil inflammatory function without inducing survival is thus warranted.

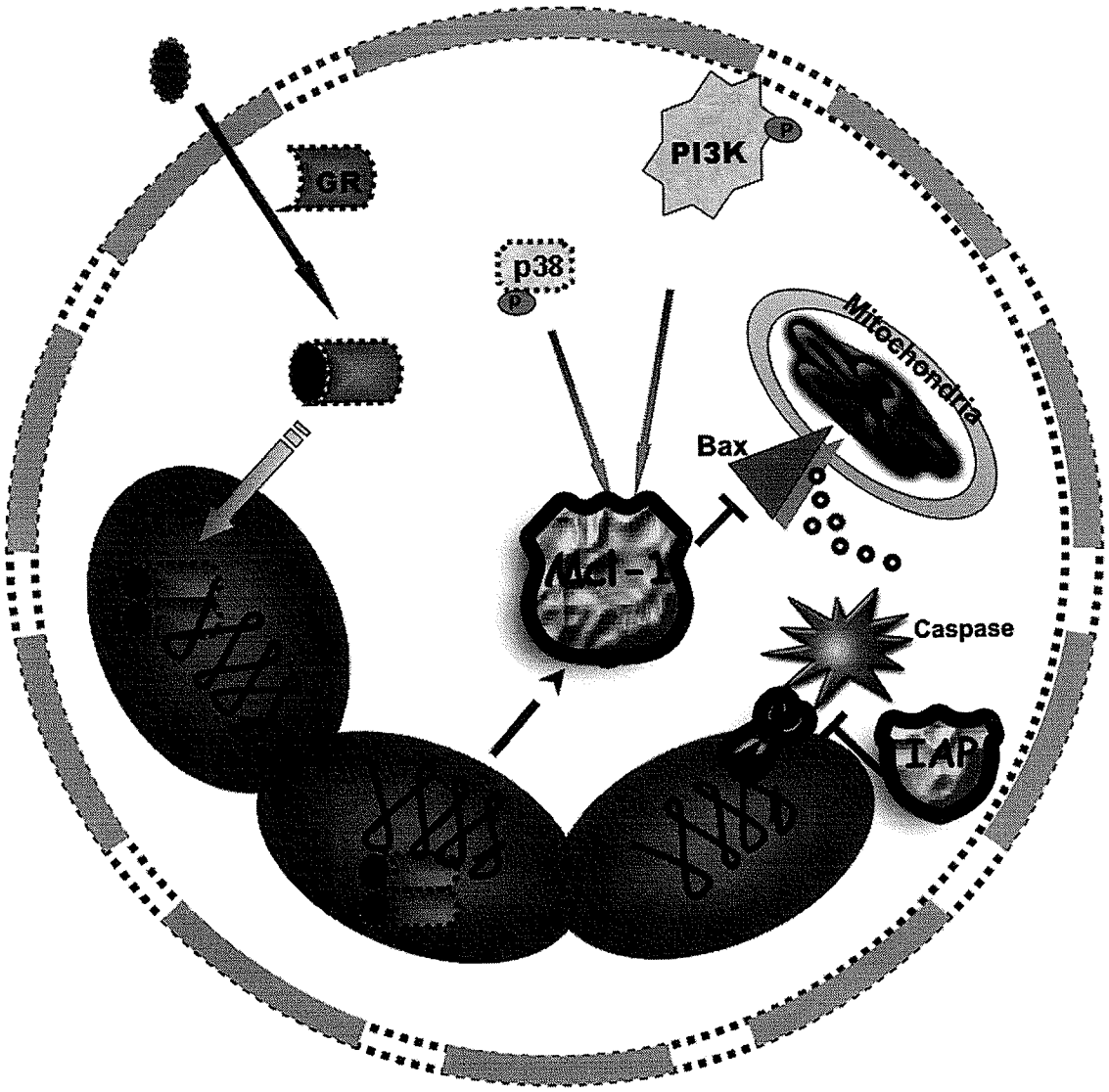

Figure 18. Glucocorticoid-induced survival of human neutrophils: a schematic model. Following treatment of neutrophils with Dex, dimerization and movement of the glucocorticoid receptor to the nucleus is presumed to occur. Within hours, Mcl-1 and IAPs are up-regulated, correlating with maintenance of mitochondrial integrity and suppression of caspases. PI3K and p38 MAPK regulate this process at or upstream of mitochondria.

6. Future Directions

1. Determine the non-genomic effects of glucocorticoids on neutrophils. These effects would generally occur within a timeframe of 30min following glucocorticoid stimulation. Various aspects of cell biology could be studied to detect non-genomic changes. These may include: phosphorylation and dephosphorylation of key cellular proteins such as kinases and phosphatases, changes in mRNA stability, modifications of proteins such as phosphorylation leading to enhanced/impaired function, etc.

2. Identify gene targets of glucocorticoids in neutrophils and how induction/repression of such genes may impact cellular function.

3. Study classical cell signaling pathways following glucocorticoid stimulation to determine pathways downstream and upstream of PI3K/p38 MAPK that are involved in glucocorticoid signaling in neutrophils. Possible targets include the Src and Syk family of kinases, PKC as well as pathways involving calcium release.

4. Study signaling mechanisms of the GR. Specifically determine GR isoforms in neutrophils as well as GR translocation and phosphorylation kinetics in glucocorticoid-stimulated neutrophils. How do such GR properties relate to glucocorticoid resistance in neutrophils of severe asthmatics?

5. Determine which isoforms of PI3K are responsible for glucocorticoid-mediated survival of human neutrophils in comparison with response to GM-CSF.

6. Is there a role for NF-κB in glucocorticoid-induced neutrophil survival?

7. Assess the importance of the above mechanisms in survival mediated by other glucocorticoid drugs available.

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