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DISRUPTION OF APOPTOTIC SIGNALING PATHWAYS DURING GLUCOCORTICOID-INDUCED SURVIVAL OF HUMAN NEUTROPHILS

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Major Professor's Signature

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Date

DISRUPTION OF APOPTOTIC SIGNALING PATHWAYS DURING GLUCOCORTICOID-INDUCED SURVIVAL OF HUMAN NEUTROPHILS

By

Joseph W. Frentzel

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ABSTRACT

DISRUPTION OF APOPTOTIC SIGNALING PATHWAYS DURING GLUCOCORTICOID-INDUCED SURVIVAL OF HUMAN NEUTROPHILS

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The polymorphic neutrophil is an abundantly produced immune cell charged with protecting the host organism from potentially harmful prokaryotes and fungi. Neutrophils utilize an array of defenses to neutralize these microorganisms including reactive oxygen production and proteolytic enzymes which degrade an assortment of microbial proteins. Of particular importance to the effective clearance of bacteria is the enhanced survival of the normally short-lived neutrophil. The half-life of the neutrophil, which undergoes apoptosis rapidly both *in vitro* and *in vivo*, can be extended through treatment with several microbial metabolites including lipopolysaccharide. The stress hormone glucocorticoids (GC)s also prolong the life of the neutrophil which is especially surprising given the detrimental effects of these compounds on other immune cell types (e.g., lymphocytes). The induction of the stress response and a corresponding increase in neutrophil production at the expense of other immune cells likely represents a shift in strategy for dealing with microorganisms during periods of stress and is the subject of investigation presented herein.

We observed a rapid translocation of the Glucocorticoid Receptor (GR) to the

nucleus of neutrophils within 30 minutes of GC exposure. Like other members of this family of proteins, ligand-activated GR migrates to the nucleus where it affects gene expression through direct DNA binding and indirect protein-protein interactions. Of the perhaps hundreds of genes induced by GCs, induction of glucocorticoid-induced leucine zipper (GILZ) has shown particular promise in playing a role in GC-mediated neutrophil survival as this protein specifically promotes the survival of T-lineage cells in response to GCs. Similarly, induction of both GILZ mRNA and protein was observed in GC-treated neutrophils, also lending to the suggestion that this protein participates in the survival of these cells. Since several genes related to apoptosis are likely to be altered by GCs, apoptosis-centric microarray analysis was performed on neutrophils treated with GCs. Using this broad approach, 25 genes were identified whose expression changed at least 2.0-fold in response to GCs. Among these gene candidates, a 2.0-fold decrease in Bid mRNA was detected in response to GCs. Further analysis of Bid revealed that GC treatment resulted in loss of both Bid mRNA as well as the apoptogenic t-Bid protein. Moreover, both upstream (FasL) and downstream (caspase 8) molecules involved in death receptor apoptosis signaling were similarly downregulated by GCs. Finally, treatment of neutrophils with a BH3-only cell-permeable peptide representing the apoptosis-inducing domain of Bid resulted in a significant increase in apoptosis of these cells. Thus, the loss of Bid likely represents an important mechanism through which GCs act to promote neutrophil survival.

DEDICATION PAGE

This body of work is dedicated to my wife, Dr. Tonya Duguid, who never balked at the thought of seeing this degree to completion even when I was overwhelmed with doubt. Although the conferring of this degree will do little to change my identity, the sacrifices through which it was obtained will forever leave me humbled. I love you, Tonya.

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I would also like to recognize the contribution that each committee member has made to the development of this project. The receptor signaling component of this project would not have been so quickly resolved had it not been for Dr. Donald Jump's careful critique during the early stages. Dr. John LaPres was absolutely instrumental in teaching me gene expression analysis and related tools, more specifically: real-time PCR. I would also like to acknowledge Dr. Richard Schwartz who was an invaluable resource concerning myeloid topics as well as being knowledgeable on the execution of many signaling pathways. Lastly, I would like to recognize Dr. John Wang who not only used specific and thought-provoking questions to improve the quality of this project, but also never lost sight of the big picture and allowed me to borrow from that vision to bring this story to a conclusion.

Finally, I would like to recognize a host of others who helped make this project move forward, even when I was unsure as to where to go next. This would include Dr. Louis King who always offered his invaluable expertise and excellent cytometry skills. Moreover, his fervor for science and his genuine integrity, unbeknownst to him, have always served as inspiration to me. I would also like to acknowledge Dr. James Mann who graciously took me under his wing as a young graduate student and with whom I have developed a sincere friendship. I would especially like to thank Dr. Mark Trottier who offered so much encouragement and support in the twilight of my graduate career, all of which was especially helpful in propelling me to finish this degree. I also want to recognize the outstanding RTSF support facilities we have here at Michigan State which enabled me to accomplish this degree. Some of the highly proficient personnel included: Joe Leykam, David DeWitt and Colleen Curry. There are several undergraduate students and/or researchers with whom I worked that I would also like to thank including: Heather Gibson, Matt Ankney, Matt Newsted and Nick Hoover. And, in conclusion, I would like to thank the entire Michigan State University community all of whom make East Lansing such a wonderful place to live and work.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin-releasing hormone
AP-1	activator protein 1
APAF1	Apoptosis protease activating factor 1
BH3	Bcl homology 3
BIR	baculovirus IAP repeat
BPI	bactericidal/permeability increasing
CAD	caspase-activated DNase
CARD	caspase recruitment domain
CASP3	caspase 3
CASP9	caspase 9
CD	cluster of differentiation antigen
COPD	chronic obstructive pulmonary disease
CREB	cyclic AMP-response element binding protein
CRF	corticotropin releasing factor
CRH	corticotropin releasing hormone
CRHBP	CRH-binding protein
CytC	cytochrome c
DBD	DNA binding domain
DEX	dexamethasone
DISC	Death-inducing signaling complex
DNA	deoxyribonucleic acid
DR	Death receptor
FADD	Fas-associated protein with death domain
FKBP	FK506 binding protein
G-CSF	granulocyte colony stimulating factor
GC	glucocorticoid
GILZ	glucocorticoid-induced leucine zipper protein
GM-CSF	granulocyte macrophage colony stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamus-pituitary-adrenal
HSD11B2	11 beta hydroxysteroid dehydrogenase 2
HSP	heat shock protein
IAP	inhibitor of apoptosis
IFN	Interferon
Ig	immunoglobulin

IL	Interleukin
kDa	Kilodalton
LBD	ligand binding domain
LPS	lipopolysaccharide
LZ	leucine zipper
MAPK	mitogen activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
MEKK	mitogen-activated protein/ERK kinase kinases
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
MR	mineralcorticoid receptor
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NLS	nuclear localization signal
NPC	nuclear pore complex
NuMa	nuclear matrix associated gene
OMM	Outer mitochondrial membrane
PAR	poly-proline rich domain
PARP	Poly(ADP-ribose) Polymerase
PCASP8	procaspase 8
PEPCK	phosphoenolpyruvate carboxykinase
PKA	protein kinase A
POMC	proopiomelanocortin
PPIase	Peptidylprolyl isomerase
PVN	periventricular nucleus
Ser	Serine
sFasL	soluble FasL
TCR	T-cell receptor
TM	transmembrane
TNF	tumor necrosis factor
TRADD	TNFRSF1A-associated via death domain
TSC	tumor secreting clone

INTRODUCTION

Neutrophils:

Peripheral blood neutrophils are one of the most abundantly produced cells in the human body. Indeed, 100 billion neutrophils are manufactured daily in the average human with the strict purpose to serve in host defense (Edwards, 2005). Since a substantial portion of these cells may never be called to action, most neutrophils will be destroyed through programmed cell death or apoptosis after spending only 6-10hrs in circulation (Davis et al., 1991). Apoptotic neutrophils are then scavenged and ingested by macrophages and are thus effectively cleared from the body. This highly organized method for senescent neutrophil elimination ensures that very few of the potentially damaging anti-microbial proteins/molecules are incidentally released from aging neutrophils. Indeed, various inflammatory disorders have demonstrated the dire consequences of premature release of a neutrophil's lysosomal contents (Holt et al., 2006). Moreover, the rigid apoptotic programming of the neutrophil may also limit the oncogenic potential of this cell type. Despite many advances in the elucidation of the molecular details of neutrophil apoptosis, large gaps in the understanding of this process still exist.

Polymorphonuclear neutrophils, by definition, have neutral-staining patterns (pink using Paul Ehrlich's triacid solution) and possess a uniquely shaped lobular nuclear structure with each lobe connected by thin strands of chromatin. During neutrophil differentiation, the genomic structure of the neutrophil condenses into

tightly compacted heterochromatin, forming 3-4 lobular structures tethered together by thin filaments of DNA (**FIGURE I.1**). While each individual lobe comprises different chromosomes, the DNA filaments which connects these lobes are remarkably constant in composition (Karni et al., 2001) thus indicating that the formation of these filaments are likely to be regulated by the cell. The multi-lobular structure of neutrophil chromatin was once thought to represent an intermediate stage of apoptosis, but this idea has since been rebuffed (Payne et al., 1994). It has been shown during neutrophil apoptosis that neutrophil chromatin condenses even *further* while the nuclear envelope completely disintegrates. Interestingly, it was recently demonstrated that neutrophil chromatin - released after death - can also be used in the construction of Neutrophil Extracellular Traps (NETs) which function to ensnare microbes in the extracelluar environment (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007). During construction of these NETs, excreted neutrophil chromatin is released in tandem with granule proteins to create a high local concentration of anti-microbial compounds which limits the spread of bacterial infection. Thus, even during death neutrophils possess the unique ability to destroy potentially pathogenic microorganisms. The immunological value of this cell type is only fully appreciated, however, when patients who possess few neutrophils are examined.

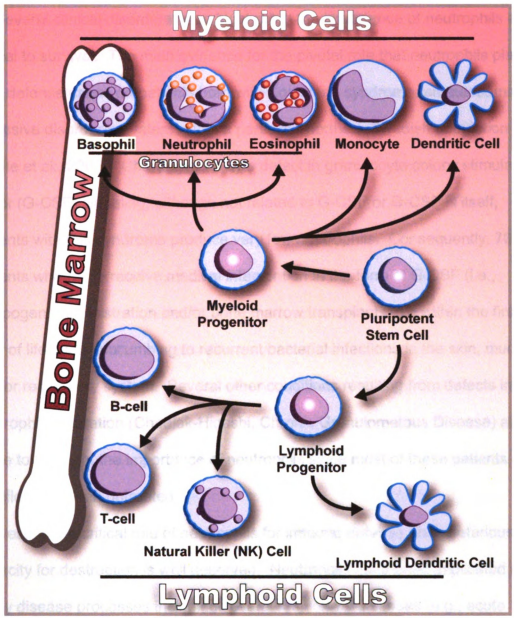

Figure I.1. The Cellular Composition of the Immune System. The immune system can be principally divided into two components shown here: (1) myeloid lineage cells; and (2) lymphoid. Both lineages derive from a common self-renewing, pluripotent stem cell. Each respective branch of the immune system, however, descends from a common lineage-specific progenitor cell. Granulocytic myeloid cells can be further subdivided into basophils, eosinophils and, the focus of this work, neutrophils.

Neutrophils in Medicine:

Several clinical disorders have highlighted the importance of neutrophils as critical to survival. The main evidence for the pivotal role that neutrophils play in host defense draws on patients born with Kostmann syndrome, an autosomal recessive disorder prevalent within a particular northern Swedish population (Welte et al., 2006). Characterized by a defect in granulocyte colony stimulating factor (G-CSF) signaling, although not related to G-CSF or G-CSF R itself, patients with this syndrome produce very few neutrophils. Consequently, 70% of patients who fail to receive medical intervention in the form of G-CSF (i.e., Neupogen) administration and/or bone marrow transplants, die within the first year of life often succumbing to recurrent bacterial infections in the skin, mucosa and/or respiratory system. Several other conditions resulting from defects in neutrophil maturation (Chediak-Higashi, Chronic Granulomatous Disease) all serve to highlight the importance of neutrophils since most of these patients die in childhood, if left untreated.

Despite the critical role of neutrophils for immune defense, their nefarious capacity for destruction is well deserved. Neutrophils have been implicated in many disease processes including several respiratory diseases (e.g., acute respiratory distress syndrome (Ware and Matthay, 2000), chronic obstructive pulmonary disease (COPD) (Stockley, 2002)) and autoimmune disorders such as ulcerative colitis (Hanauer, 2006) and rheumatoid arthritis (Edwards and Hallett, 1997). The damaging capacity of neutrophils is further compounded by their ability to secrete several pro-inflammatory cytokines/chemokines (e.g., IL-1, IL-6,

TNF-a and IL-8) which serves to recruit and incite additional pro-inflammatory cells to the inflammatory foci (Gabrilovich, 2004). The production of these chemotactic factors by neutrophils is usually turned off at the resolution of ordinary inflammation, but their production remains turned on during certain inflammatory disorders. Indeed, granulocyte depletion in particular arthritis mouse models has demonstrated the importance of neutrophils in the maintenance of inflammatory states (Brown et al., 2004; Tanaka et al., 2006). Therefore, understanding the dual and sometimes contrasting capacities of neutrophils as both protectors and destroyers will be critical to advancing clinical medicine in the areas of infection as well as autoimmunity. Owing to the enormous amount of production of neutrophils by the human body, molecular control of their survival may be a very critical aspect of their biology with respect to therapeutic treatments. Due to the substantial hematopoietic and nutritional energy invested in neutrophil production, minor perturbations in the creation and/or elimination of these cells could manifest as major shifts in inflammatory outcomes. Therefore, a full understanding of neutrophil apoptosis as well as agents that might delay this process will also be critical to the overall understanding of inflammation.

Neutrophil Function – Extravasation:

As the first line of defense, neutrophils are entrusted with the critical responsibility of capturing and destroying microbes, especially bacteria and fungi, throughout the host organism. Neutrophils fall within a distinct class of immune defenders categorized by their ability to ingest microorganisms and are thus

termed phagocytes. Phagocytic cells are lured to sites of tissue injury or infection by chemoattractants that have been released by cells within the inflammatory foci. Neutrophils quickly leave the vasculature via an extravasation process which begins with endothelial cells upregulating stored adhesion receptors, selectins, which serve to slow down circulating neutrophils through a "rolling" event. Neutrophils then penetrate adjacent endothelial cells as well as the basement membrane (diapedesis) and travel up a concentration gradient of the chemotactic agent through a process termed chemotaxis.

Neutrophil Function – Phagocytosis:

The neutrophil can then engage the offending microrganism(s) by receptor-mediated interaction with various opsonins (IgG or C3b, C3bi) which have attached to the intruder(s). Neutrophils possess opsonin receptors (CD11b-FcG, CD16b, CD32, CD35, CD64) that recognize both the Fc region(s) of several classes of antibodies as well as activated complement (C3b, C3bi) deposited on surface of bacteria and/or fungi. This ligation reaction initiates a series of molecular events that ultimately results in the ingestion or phagocytosis and ultimate destruction of the offending microbe. In a recent publication by Hashimoto et al, neutrophils were shown to phagocytose Influenza-infected lung cells (Hashimoto et al., 2007). This report was somewhat surprising since neutrophils usually exhibit "frustrated phagocytosis" or extracellular degranulation when encountering particles greater than a few microns in diameter. Nevertheless, neutrophils could conceivably function in this capacity during conditions in which large amounts of apoptotic bodies are available for

consumption (e.g., infection, stress, etc.), provided that these cells are relatively small.

Neutrophil Function – Degranulation:

The receptor-engaged microorganism initiates an invagination of the neutrophil lipid bilayer resulting in the formation of the phagosome vacuole which completely surrounds the microorganism. The phagosome may then fuse with intracellular vacuoles, termed lysosomes, within the neutrophil resulting in the formation of the phagolysosome (aka degranulation or mobilization). These lysosomes and their contents are ultimately responsible for the destruction of the bacteria/fungi via either (1) oxygen-dependent or (2) oxygen-independent destruction processes (Segal, 2005). Lysosomes can also fuse with the plasma membrane resulting in the extracellular release of lysosomal contents including reactive oxygen species (oxygen burst). The term oxygen-dependent toxicity derives principally from the fact that oxygen consumption increases ~100-fold during ingestion of the opsonized particle. Oxygen-dependent toxicity, however, is insensitive to mitochondrial respiratory chain disruptors thus implicating the involvement of another enzymatic oxygen-production process. Indeed, the production of superoxide anion, one of several important anti-microbial compounds produced by the neutrophil, occurs via NADPH oxidase - a membrane-bound multimer protein complex. Superoxide anion can be further converted to the even more potent HOCl- via the catalytic actions of myeloperoxidase (MPO). In fact, it is estimated that up to 5% of neutrophil dry

weight consists of MPO enzyme, perhaps indicating the importance of this protein (Edwards, 2005).

Additionally, the destruction of microbes can occur via oxygen-independent toxicity which capitalizes on the fact that lysosomes contain several preformed cytotoxic proteins. Similar to MPO, it is estimated that defensins comprise a substantial portion of neutrophil proteins, estimated up to 25-30% of azurophilic protein content (Chertov et al., 1996). Defensins are small cationic peptides that possess a broad range of cytotoxic activity against bacteria, fungi and even some enveloped viruses, but also kills bystander eukaryotic cells (Lehrer, 2007). Consequently, the indiscriminate release of defensins is prevented by the fact that azurophilic granules seldom fuse with the plasma membrane, instead preferentially forming phagolysosomes during phagocytosis. The lethal effects of defensins are mediated by their insertion into the cellular membrane and through the formation of voltage-gated channels. Interestingly, murine neutrophils were largely thought to be devoid of defensins altogether. Recent studies, however, demonstrated that murine neutrophils do in fact express a defensin-like protein, bactericidal/permeability increasing (BPI) protein, but at much lower levels relative to their human counterparts (Mestas and Hughes, 2004). BPI binds strongly to lipopolysaccharides (LPS) found in the bacterial membrane leading to both the destruction of the bacterium as well as neutralization of the pyrogenic LPS. The different levels of BPI found in mice and human neutrophils as well as the absence of defensins altogether in mice, may indicate that each organism has different strategies for dealing with microorganisms.

Neutrophil Apoptosis

Review:

The fate of an individual cell can have critical impact on the survival of the host as the death of a rare cell might mean loss of function or, in the case of neutrophils, the increased survival of a well represented population might translate into a burgeoning reactive oxygen load. The overpopulation of the immune system by neutrophils is tightly controlled by the programmed cell death process, apoptosis. Although many exceptions exist, apoptosis occurs via two primary pathways: (1) Intrinsic; and or (2) Extrinsic (**Figure I.2**) (Taylor et al., 2008). In the case of the former pathway, the decision to commit to cell death is regulated at the mitochondria by a group of membrane proteins – the Bcl-2 family. The extrinsic pathway, however, is initiated by external stimuli which engages and stimulates the apoptosis signal via a death receptor (DR). As described below, several other proteins and protein families participate in the execution of apoptosis signaling and many of these pathways – be it intrinsic or extrinsic – overlap to a great degree.

Bcl-2 family proteins are divided into two categories: (1) pro-apoptotic; and (2) anti-apoptotic (**Figure I.3**). Almost all pro-apoptotic Bcl-2 members possess a BH3 (Bcl Homology 3) domain (except Bad) which has been shown to be critical for their function. Anti-apoptotic proteins, on the other hand, possess both BH1 and BH2 domains (among others). Many members of the entire Bcl-2 family contain transmembrane (TM) domains which can insert into the outer mitochondrial membrane (OMM). The insertion into the OMM and

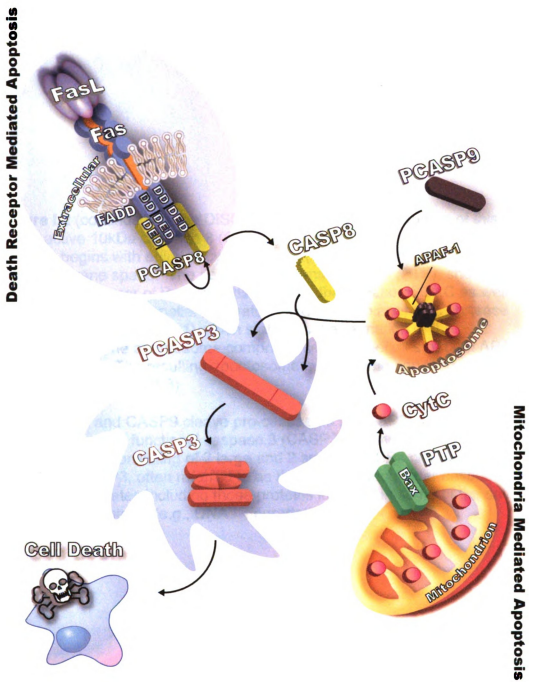

Figure I.2. Death-receptor (Extrinsic) and Mitochondria-mediated (Intrinsic) Modes of Apoptosis. In the example shown here for extrinsic apoptosis, FasL binds to and promotes the oligomerization of Fas (CD95) molecules. The adapter protein FADD associates with the cluster of Fas proteins via Death Domain (DD) motifs. Similarly, pro-caspase 8 (PCASP8) proteins are recruited to the Death-Inducing Signaling Complex (DISC) by FADD via Death Effector Domains (DEDs). The oligomerization of PCASP8

Figure I.2 (continued) at the DISC facilitates the autoproteolysis of this molecule to the active 10kDa caspase 8 (CASP8). Intrinsic apoptosis, on the other hand, typically begins with efflux of cytochrome c (CytC) from the mitochondrial intermembrane space. The permeability transition pore (PTP), shown here to include an oligomer of Bax proteins, enables the release of CytC as well as other proteins from this organelle. Liberated CytC binds to apoptotic protease activating factor 1 (APAF-1) which stabilizes the formation of the apoptosome. The apoptosome comprises seven molecules of both APAF-1 and pro-caspase 9. The resulting apoptosome holoenzyme can then converge on pro-caspase 3 (PCASP3).

Both CASP8 and CASP9 cleave pro-caspase 3 (PCASP3) at specific aspartate residues to reveal functional caspase 3 (CASP3). Active CASP3 is a heterotetramer consisting of 2 large and 2 small PCASP3 cleavage products. CASP3, often referred to as the central executioner caspase, cleaves over 100 substrates including those proteins involved in some of the salient features of apoptosis (e.g., DNA degradation).

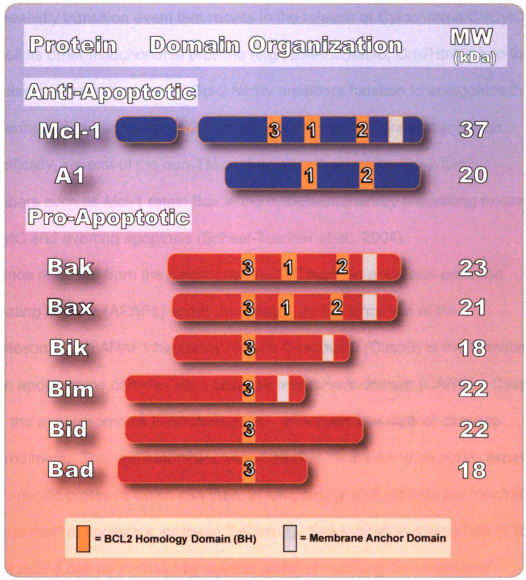

Figure I.3. Bcl-2 Family Members Expressed in Neutrophils. The Bcl-2 family of proteins can be divided into two subclasses shown here: (1) Anti-apoptotic; and (2) Pro-apoptotic. The latter grouping of proteins uniformly possess a third amphipathic α-helical (BH) domain which is essential for their apoptotic activities (shown in orange). The BH3 domain enables the oligomerization of BH3-containing proteins and execution of the permeability transition event. Anti-apoptotic proteins characteristically possess BH1 and BH2 domains, but with some members (not expressed in granulocytes) containing a fourth BH domain. Like the BH3 domain, BH1 and BH2 domains function to promote dimerization of Bcl-2 family proteins. In addition to BH domains, several of the Bcl-2 family proteins feature a C-terminal membrane anchor domain which facilitates localization of the protein to the mitochondria (shown in grey).

oligomerization of pro-apoptotic Bcl-2 family members such as Bax initiates a permeability transition event that results in the release of Cytochrome C (CytC) as well as other mitochondrial proteins (e.g., SMAC/Diablo, Omi/HtrA2) into the cytoplasm. The anti-apoptotic Bcl-2 family members function to antagonize this clustering of pro-apoptotic Bcl-2 family members through direct association. Specifically, several of the non-TM containing anti-apoptotic Bcl-2 family members such as Mcl-1 retain Bax in the cytoplasm thereby preventing release of CytC and averting apoptosis (Scheel-Toellner et al., 2004).

Once released from the mitochondria, CytC binds to apoptosis protease activating factor 1 (APAF1) which then stabilizes the formation of the apoptosome. An APAF1 heptamer recruits Caspase 9 (Casp9) in the formation of the apoptosome complex via a caspase recruitment domain (CARD). Casp9 joins the apoptosome as heterotetramers – effectively two distinct caspase heterodimers. Through autoproteolysis, Casp9 is cleaved into an active aspartic acid-specific protease which can then, in turn, cleave and activate the "central executioner" of apoptosis, caspase 3 (Casp3). The formation and activity of the apoptosome can be inhibited by several proteins including the Inhibitor of Apoptosis (IAP) family. Members of this family possess at least one Zn-binding Baculovirus IAP Repeat (BIR) domain(s) which has been shown to be necessary for binding and inhibiting caspase activity. Although two members of this family possess CARD domains, cIAP1 and cIAP2, these motifs have been shown to be dispensable for caspase inhibition (**Figure I.4**) (Roy et al., 1997).

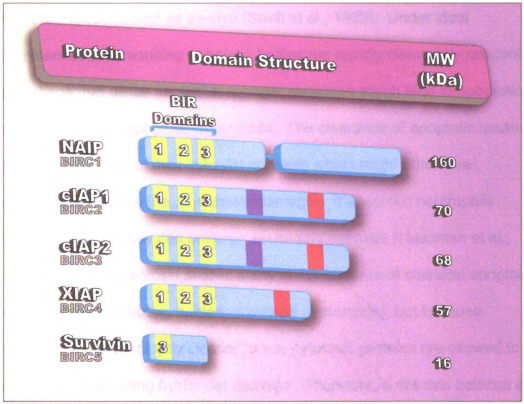

Figure I.4. IAP (BIRC) Family Proteins Expressed in Neutrophils. The inhibitor of apoptosis protein (IAP) family functions largely as antagonists to molecular apoptotic processes. Three members of this family, including cIAP1, cIAP2 and XIAP, bind to and inhibit effector caspases. As shown in this figure, neutrophils express five members of this family of proteins (BIRC1-5). IAP proteins characteristically feature at least one N-terminal 70 amino acid zinc-binding domain termed a baculoviral IAP repeat (BIR) domain. Several members of the IAP family, including cIAP-1 and -2, also possess centrally located caspase recruitment domains (CARD; shown here in purple) which consists of a cluster of 6-7 anti-parallel α-helices. Additionally, cIAP1/2 and XIAP contain a C-terminal really interesting new gene (RING) motif (shown in orange) which facilitates the ubiquitination and degradation of IAPs and other RING-containing proteins.

Peripheral blood neutrophils undergo spontaneous apoptosis both in-vivo (Cox et al., 1995) as well as ex-vivo (Savill et al., 1989). Under ideal circumstances, the resulting apoptotic bodies are rapidly cleared by resident macrophage that utilize several scavenger receptors which bind to apoptotic markers located on apoptotic neutrophils. The clearance of apoptotic neutrophils prevents the release of their cytolytic contents which might otherwise damage nearby tissue (i.e., bystander damage). If apoptotic neutrophils languish, however, they can undergo secondary necrosis (Heasman et al., 2003). Secondary necrosis shares many of the features of classical apoptosis (e.g., membrane blebbing, nuclear envelope destruction), but because neutrophils are not promptly cleared, some cytotoxic proteins are allowed to escape thereby causing bystander damage. Therefore, a delicate balance exists between the elimination of neutrophils via apoptosis and the clearance of these apoptotic bodies by resident macrophages, fibroblasts and mesangial cells. A slight shift in either the abilities of phagocytes to eliminate apoptotic cells or the numbers of apoptotic neutrophils at the site of tissue injury is likely to impact the successful resolution of inflammation.

Intrinsic Apoptosis (Bcl-2 Family Regulated):

Neutrophil apoptosis remains an incompletely understood phenomenon. Spontaneous apoptosis involves components of both the extrinsic (death receptor mediated) as well as intrinsic (mitochondrial-mediated) pathways (**Figure I.5**). The latter mode of apoptotic death was difficult to explain until recently, however, since neutrophils were thought to possess few if any

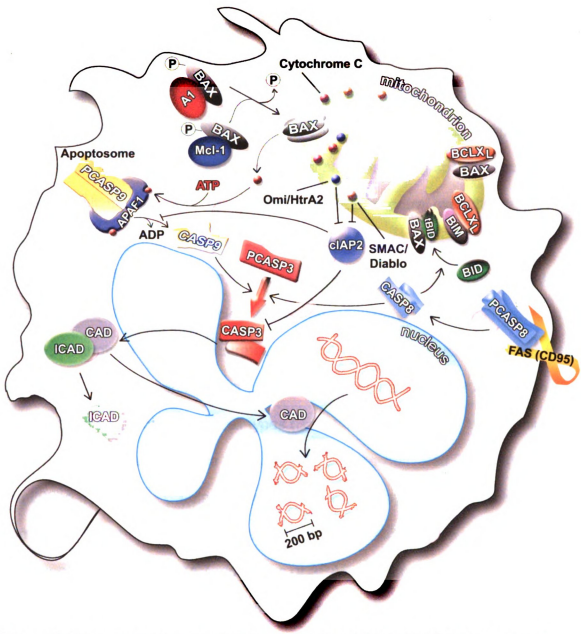

Figure 5. Known Mediators and Antagonists of Neutrophil Spontaneous Apoptosis. Shown in this figure are proteins identified to participate in the execution of apoptotic processes during neutrophil apoptosis. Neutrophil spontaneous apoptosis occurs via the extrinsic (death receptor-mediated) and intrinsic (Bcl-2-family regulated) pathways. Both caspase-8 and -9 have been shown to play critical roles in neutrophil apoptosis. These caspases converge to activate the central executioner, caspase , which results in the cleavage of several nuclear substrates including ICAD as well as many other unidentified proteins. The degradation of ICAD relieves the inhibition of the DNase CAD which is then permitted to degrade nucleosomal DNA into 200bp fragments.

functional mitochondria (Clark et al., 1980; Edwards, 2005). Contrary evidence collected using Mito Tracker probes, though, suggests that neutrophils have a rather elaborate network of mitochondria which are critical to both mobility and respiratory burst functions (Fossati et al., 2003; Maianski et al., 2002). Both caspase-8 and -9 have been shown to play critical roles in neutrophil apoptosis as evidenced by their characteristic cleavage patterns during spontaneous death. These two initiator caspases converge to activate the central executioner, caspase 3, through their aspartate-specific protease action. Caspase 3 activation typically results in the cleavage of several nuclear substrates including PARP, NuMA, U1 small ribonucleoprotein and many other unidentified proteins. Although Sanghavi et al demonstrated the absence of these nuclear proteins in neutrophils, these researchers did detect both Fodrin and Lamin B at levels in neutrophils equivalent to other cell-types (Sanghavi et al., 1998). Neutrophil DNA is ultimately degraded via the action of Caspase-Activated DNase (CAD) into 200bp fragments. Caspase 9 activity is regulated by cytochrome c release from the mitochondria which binds to APAF-1 thus stabilizing the apoptosome. The apoptosome comprises 7 molecules of APAF-1 and cytochrome c and an unknown number of caspase-9 dimers. The apoptosome formation is a necessary event for caspase 9-dependent cleavage of procaspase 3. In addition to cytochrome c, the serine protease Omi/HtrA2 and SMAC/Diablo are both released from the mitochondria during neutrophil apoptosis. These proteins can bind BIRC family proteins (e.g., XIAP, cIAP2, etc.) thereby inhibiting their ability to block caspase 9 activation and caspase 3 activity. Caspase 8 is normally

activated via the clustering of death receptors and their respective death domain adaptor proteins (e.g., FADD, TRADD). It is unknown what molecular events are occurring in the neutrophil during spontaneous apoptosis that result in the activation of caspase 8.

Several Bcl-2 family members have been shown to be important for granulocyte survival pathways. It is generally conceived that even in response to anti-apoptotic stimuli such as LPS or GM-CSF, neutrophils maintain constant levels of pro-apoptotic Bcl-2 family members such as Bax, Bad and Bak (Moulding et al., 2001). During spontaneous neutrophil apoptosis, proapoptotic Bax is dephosphorylated at Ser^{184} which promotes its translocation from the cytoplasm to the mitochondria (Gardai et al., 2004). In healthy neutrophils, Bax is maintained in the cytoplasm by physical association with anti-apoptotic Bcl-2 family members such as A1 and/or Mcl-1. Upon activation, the N-terminus of Bax inserts into the mitochondria and oligomerizes with other Bax molecules thereby initiating the mitochondrial permeability transition event. This culminates in a disturbance of the mitochondrial membrane integrity and the release of mitochondrial intermembrane space proteins Smac/DIABLO, Omi/HtrA2 and, most importantly, Cytochrome c (among others) into the cytoplasm. Smac/DIABLO functions as a dimer and antagonizes the activity of inhibitor of apoptosis proteins (IAPs; e.g., cIAP1, cIAP2, XIAP). Omi/HtrA2 is a serine protease that also inhibits IAP activity and is necessary for caspase-independent cell death. Cytochrome c stabilizes the apoptosome via binding to APAF1 thereby initiating the activation of Caspase 9. Bax translocation in neutrophils

can be prevented with the addition of granulocyte-colony stimulating factor (G-CSF), a pro-neutrophil cytokine (Maianski et al., 2002). As for other pro-apoptotic Bcl-2 family members, Andreas Strasser's lab has shown a 200% increase in survival of bone marrow-derived granulocytes from $bim^{-/-}$ mice when compared to controls (Villunger et al., 2003). Granulocytes from $bax^{-/-}$ mice, on the other hand, did not exhibit improved survival in culture, further demonstrating an important role for bim in the regulation of *murine* granulocyte apoptosis. Furthermore, it has been demonstrated by Lagasse and Weissman that human Bcl-2 overexpression in murine neutrophils results in the decrease of neutrophil spontaneous apoptosis (Lagasse and Weissman, 1994). The physiological significance of such a finding is rather vague since it has been reported that neutrophils do not express Bcl-2 protein. Several myeloid-specific anti-apoptotic bcl-2 members exist, however, which can be quite variable in expression. Both A1 (Bfl1) and Mcl-1 were found to be inducible in response to various stimuli (Chuang et al., 1998; Epling-Burnette et al., 2001).

A1 is a 20kDa anti-apoptotic Bcl-2 family member that is constitutively expressed by cells of hematopoietic lineage. A1 mRNA was found to be upregulated in neutrophils treated with G-CSF, GM-CSF or LPS after only 3 hours (Chuang et al., 1998). In addition to these stimuli, Edwards et al also found that A1 mRNA was increased in response to TNF-α and IFN-γ - both cytokines which also delay neutrophil apoptosis (Moulding et al., 2001). Further substantiating a role for A1 in neutrophil survival, peripheral blood neutrophils purified from $A1^{-/-}$ mice exhibited increased spontaneous apoptosis during routine

culture (Hamasaki et al., 1998). In two separate studies, an increase in A1 protein levels was observed in neutrophils from mice injected with endotoxin (Kotani et al., 2003; Kupfner et al., 2001). In addition to LPS, several exogenously supplied TLR agonists also have been shown to upregulate A1 expression in neutrophils derived from mice (Francois et al., 2005). Finally, the synthetic glucocorticoid (GC), dexamethasone (DEX), was shown to upregulate both A1 mRNA as well as protein in cultured bovine neutrophils (Madsen-Bouterse et al., 2006). Collectively, these findings all suggest a role for A1 as a critical regulator for neutrophil survival.

Several survival cues have been shown to induce Mcl-1 expression in neutrophils, including: GM-CSF, butyrate, IL-1b and LPS (Epling-Burnette et al., 2001; Moulding et al., 1998). Treatment with Mcl-1 anti-sense increased the levels of spontaneous apoptosis for cultured neutrophils, indicating a role for this protein in cell survival (Leuenroth et al., 2000). Even more compelling evidence comes from Mcl-$1^{-/-}$ mice in which granulocyte numbers were reduced by 80% in the peripheral blood (Dzhagalov et al., 2007). In a separate study, the Mcl-1 anti-sense approach also revealed the importance of Mcl-1L (the long isoform) to glucocorticoid- (GC) induced survival of granulocytes. When neutrophils were pretreated with Mcl-1 antisense oligos, the ability of DEX to protect neutrophils was nearly cut in half (Sivertson et al., 2007). These data indicated that Mcl-1L expression may be a major component of GC-mediated protection of neutrophils, especially at the late stage of survival signaling. The overall body of data on Mcl-

1 expression in neutrophils supports the idea that this protein is critical to neutrophil viability in many pro-survival situations.

Extrinsic Apopotosis (Death Receptor Regulated):

Fas-induced cell death is a classical example of death-receptor mediated apoptosis. In this system, membrane-bound FasL binds to CD95 resulting in the formation of the death-inducing signaling complex (DISC) comprising Fas, FADD (an adaptor molecule) and Procaspase-8 (Pcasp8). Oligomerization of Pcasp8 proteins within the DISC causes autoproteolysis of Pcasp-8 proteins which are then converted to the active Casp8. Casp8 can then act at other downstream apoptosis checkpoints such as the cleavages of Bid to t-Bid (i.e., a truncated form of Bid) and Procaspase-3 to the active Caspase-3, the central executioner of apoptosis.

The role of Fas in neutrophil apoptosis has been somewhat controversial. Much of this controversy stems from the observation that soluble FasL (sFasL) fails to induce apoptosis in neutrophils – it actually proved to be an effective chemoattractant (Dupont and Warrens, 2007). Other labs, however, have successfully induced apoptosis in neutrophils using traditional FasL which is typically found on the cell surface as a Type II membrane protein (Chang et al., 2004). Increasing the ambiguity of the role of Fas/FasL in neutrophil survival, *gld* mice which carry a point mutation in FasL, as well as *lpr* mice which carry a defect in the Fas receptor, both have normal granulocyte and granulopoiesis parameters (Fecho et al., 1998). In an unpublished report, however, Holroyd et al described the results of crossing *lpr* mice with Lyn knockout mice (mice that

have lost Lyn kinase activity). Much to their surprise, these double knockout mice had a "vast" increase in granulocytes and granulocyte precursors (Holroyd S, 2004). Lyn kinase has been shown to be important for granulocyte survival in response to GM-CSF stimulation (Wei et al., 1996). In fact, Lyn was shown to have a physical association with the GM-CSF receptor and anti-sense to Lyn reversed any survival advantage in response to GM-CSF exposure. Along the death-receptor mediated axis, a distinct possibility exists in which Fas/FasL engagement as well as the activation of a second signal (e.g., Lyn) results in the spontaneous apoptosis of neutrophils. The contradictory role of Lyn kinase as both a survival protein (e.g., GM-CSF stimulation) and as a death-mediator (e.g., *lpr*/Lyn double knockouts) still needs to be resolved in an effort to determine the absolute role of Fas in the control of neutrophil fate.

Glucocorticoid Signaling

Molecular Events During the Induction of the HPA Stress Axis:

The release of stress hormones, specifically glucocorticoids, into the blood is the final step in executing the stress response. This forward tilt in the hypothalamus-pituitary-adrenal (HPA) stress axis, so named for the involvement of the indicated organs, ultimately results in an increase in serum GCs which can cause a host of effects on diverse cell types (**Figure I.6**). These effects range from alterations in metabolism to influencing cell fate decisions, specifically apoptosis. Many of these cellular changes serve to ensure the organism's survival through a preservation and/or utilization of energy resources and proper execution of the flight or fight response. In one classical example, GCs cause

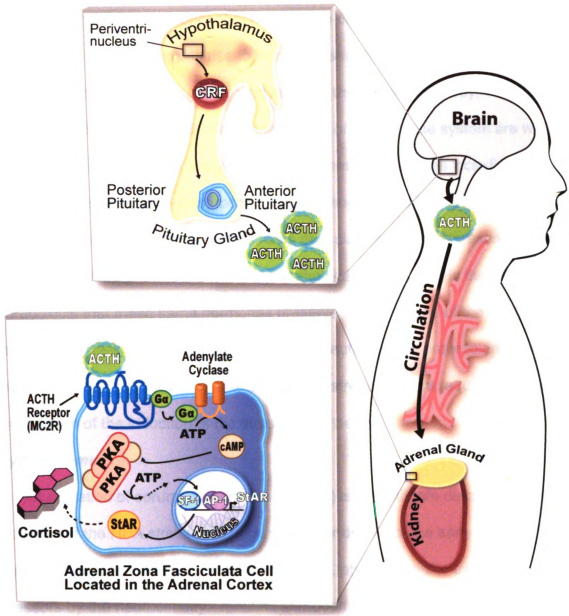

Figure I.6. Induction of the Hypothalamus-Pituitary-Adrenal (HPA) Stress Axis. The stress axis is activated when a stressor is integrated by the periventrinuclear region of the hypothalamus. This stimulates the increased production and secretion of corticotropin releasing factor (CRF). CRF then causes the proximal anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH) into circulation. The adrenal zona fasciculata cells are among the various the targets of ACTH which react with ACTH-specific G-protein coupled receptors resulting in the upregulation steroidogenesis proteins including steroidogeneic autoregulatory (StAR) protein. The net result of this pathway is the production and secretion of glucocorticoids into circulation by cortical adrenal cells.

the upregulation of several enzymes in the liver, particularly those involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK). The upregulation of PEPCK gives rise to increased serum glucose levels which would provide a rapid energy source for many tissues throughout the body. Additionally, the effects of GCs on various cells of the immune system are well documented. GCs induce apoptosis in many immune cell types including thymocytes, immature B-cells, monocytes, eosinophils and basophils. Due to their potent anti-inflammatory effects, GCs as a class of drugs represents 3 of the top 50 prescribed pharmaceutical drugs in the United States. It is therefore paradoxical that these same hormones preserve the life of one of the most potent inflammatory cells of the immune system, the neutrophil. In order to answer why these particular cells would be spared during a stressful event requires a fuller explanation of the induction of the stress response.

Hypothalamus:

An elevation of serum glucocorticoids (GCs) is the ultimate outcome of induction of the HPA stress axis. The molecular details of the early steps in this signaling cascade - the integration of emotional, physical and/or traumatic stressors by the neurocircuity - remains shrouded in mystery. Nevertheless, the HPA stress axis induction begins with the stimulation of periventricular nuclear bodies to secrete corticotropin releasing hormone (CRH) into the hypophyseal duct. CRH is a 41 amino acid neuropeptide that is derived from a 196 amino acid precursor, prepro-CRH. Transcriptional regulation of CRF expression appears to be heavily dependent on protein kinase A (PKA) activation of cyclic

AMP-Response Element Binding (CREB) protein. Indeed, rapid phosphorylation of CREB and a near simultaneous increase in CRH mRNA levels is observed in the PVN as result of stress (Arzt and Holsboer, 2006). CRH is then released by PVN nerve terminals into the hypothalamohypophysial portal system where the neuropeptide descends to its site of action, the anterior pituitary gland.

Pituitary Gland:

At the pituitary, CRF reacts with both high affinity (CRF1) and low-affinity CRH receptors (CRF2) located on the cell surface of pituitary corticotrophs. These G protein-coupled CRH receptors stimulate an increase in cAMP levels which then results in the secretion of adrenocorticotropin-releasing hormone (ACTH) by pituitary cells into the blood. The CRH/CRF signaling system is regulated, at least in part, by alterations in CRF protein numbers expressed by pituitary cells. To this extent, adrenalectomized mice have been shown to express higher levels of CRF whereas mice treated with GCs possess lower amounts of the CRF protein thus demonstrating this feedback mechanism can turn in either direction (Sawchenko, 1987). CRH-binding protein (CRHBP) also functions as another layer of CRH-mediated ACTH secretion regulation. Produced by the hypothalamus and several other cell lineages, CRHBP abrogates ACTH secretion through its physical interactions with CRH consequently diminishing its signaling capacity. These layers of regulation, as well as the direct effects of GCs on ACTH production and secretion, serve to negatively impact ACTH production by pituitary corticotrophs.

Adrenal Glands:

ACTH is one of several proteolytic products derived from the pro-opiomelanocortin (POMC) protein, the others being ß-endorphin and melanocyte stimulating hormones. POMC processing, including several proteolysis steps and ß-amidation, occurs in the trans-Golgi of pituitary cells. The secreted ACTH then travels to adrenal gland where it binds to melanocortin 2 receptors (MC2R) found on the surface of adrenal cells within the zona fasciculata of the adrenals. These ACTH-receptors are also G-protein coupled receptors which when activated result in increases in intracellular cAMP. The net result is an increase in genes involved in steroidgenesis in addition to steroid transport genes. The induction of the HPA stress axis then concludes with the secretion of GCs into circulation by these adrenal cells. Glucocorticoid levels are regulated in several manners, including metabolism by some cells using 11 beta hydroxysteroid dehydrogenase 2 (HSD11B2) which converts cortisol to inactive cortisone and/or binding to the serum protein, corticosteroid binding protein (CBP). In fact, during homeostasis, it is estimated that up to 90% of GCs are bound up by CBP leaving only a limited amount of biologically active GC available. HSD11B2, however, appears to have restricted expression - particularly in lung and kidney cells – due to its role in maintaining cellular sensitivity to mineralcorticoids (Sandeep and Walker, 2001). HSD11B2 activity results in oxidation of cortisol to the inactive glucocorticoid cortisone. Since cortisol can bind to and activate mineralcorticoid receptors (MRs), HSD11B2 would limit the type of steroid that could potentially bind MR. Therefore, this enzyme is expressed preferentially in cells that regulate

Na+/K+ transport and wherein must maintain exclusive sensitivity to mineralcorticoid receptor (MR) activity which can be illicitly activated by certain GCs.

Glucocorticoids in Medicine:

The anti-inflammatory activity of GCs has been exploited by physicians in the treatment of several immune disorders including asthma, arthritis and specific autoimmune diseases. Henry Mason isolated several hormones from the adrenal cortex including one he termed Compound E which would later be named cortisol. Philip Hench was one of the first to use these extracts to treat patients with debilitating arthritis for which he, along with two chemists, received the Nobel Prize in 1950. Since then, GC-based medicine has been advanced through the invention of synthetic GCs which are more slowly metabolized by the liver and have longer receptor-associated half-lives and therefore exert more potent activity on target cells. Unfortunately, most cells express GR, meaning that nearly all cells in the body are affected by GC treatment. This issue manifests in the form of various unwanted side-effects including immunosuppression, hyperglycemia, osteoporosis and muscle dissolution (among others).

Long term treatment with GCs, while in some cases necessary, often results in physicians reckoning with the cost-benefit ratio and in many cases, lowering the dosage and/or duration of GCs in treatment. The side-effects of GCs are divided into 3 categories: (1) immediate; (2) gradual; and (3) idiosyncratic. The immediate effects of GCs treatment typically refer to patient complaints of

blurriness of vision, mood changes, insomnia, weight gain and immune modulation. The more gradual effects, on the other hand, reference changes in gluconeogenesis, osteoporosis, central obesity and adrenal suppression. Finally, the idiosyncratic side-effects include avascular necrosis, cataract formation and psychosis (Trence, 2003). Improvements in delivery of GCs, especially in instances of allergic rhinitis or asthma or reactive airways, has translated into GCs being delivered almost directly to the affected tissues with very limited systemic bioavailability to consider. This has been especially important for the treatment of asthma in children for which GCs are often contraindicated due to their abilities to alter bone formation and stunt growth. Although the anti-inflammatory effects by GCs on several immune cell types, particular lymphocytes, has been given a great deal of attention, it seems as though little consideration has been given to the increase in neutrophil survival caused by these drugs.

Several lung conditions are advocated for treatment with GCs, including chronic obstructive pulmonary disease (COPD) (Barnes, 2007). COPD is the world's 5th deadliest disease, but is estimated to move to the 4th position by year 2030 (WHO). In addition to bronchodilators (e.g., beta-agonists), inhaled GCs are often used as the principal anti-inflammatory treatment option. Data from the laboratory of Philip J. Barnes has caused researchers to reconsider this treatment option since GC treatment did not improve the condition of COPD patients in at least one study (Culpitt et al., 1999). Moreover, Barnes et al determined that neutrophils are present in high numbers in COPD patients and

therefore would be less prone to clearance by normal inflammatory resolution mechanisms due to their increased survival. In a more recent study, Barnes et al withdrew GC treatment of asthmatic patients in an effort to determine the role that neutrophils play in disease progression (Maneechotesuwan et al., 2007). Interestingly, eight out of ten patients that had stopped GC treatment lost control of their disease whereas only 1 out of ten patients that maintained GC treatment had similar outcomes. These data demonstrate that both researchers and clinicians still have much to consider with respect to GCs and their effects on neutrophils. Moreover, the role that neutrophils play in various disease processes is still underdeveloped and will require further advancement in order to balance GC treatment with increases in neutrophil survival.

Glucocorticoid Receptor (GR):

The primary signaling apparatus for the glucocorticoid is the glucocorticoid receptor (GR), a 94kDa member of the nuclear hormone receptor family. The GR which regulates lung maturation (among many other processes) is essential for survival. The ligand-receptive form consists of one GR molecule, two hsp90s and one each of hsp70 and hsp56. This heterocomplex, especially hsp90, is necessary to maintain the cytosolic GR in a high-affinity ligand binding state. GCs are hydrophobic molecules that readily diffuse across the plasma membrane which can bind GR in the cytoplasm. Similar to its receptor, the GC ligand is also critical for survival. Upon ligand binding, the GR dissociates from the receptor-associated chaperone proteins and migrates to the nucleus via the importin nuclear transport system. The involvement of other chaperone proteins

as well as immunophilins ensures that the receptor reaches its appropriate configuration as a nascent protein.

GR Domain Structure:

Like all nuclear hormone receptors, the GR comprises an N-terminal transactivation domain (t1); a centrally located "zinc-finger" containing DNA-binding domain (DBD); and a transactivation domain (t2), ligand binding domain (LBD) and nuclear localization sequences (NLS1 and NLS2) at the carboxy-terminus (**Figure I.7**). Upon ligand binding, the receptor undergoes a conformational change during which the cofactors dissociate and a nuclear localization sequence (NLS) is revealed. There are two types of GC signaling: type one and type two responses. A type one response is the characteristic binding of a GR dimer to a glucocorticoid response element (GRE) of some gene(s) thereby altering gene expression. The type two response is the interaction of GR with various transcription factors which can result in either enhancement of function (e.g., STAT5 and STAT6) or diminished capacity for transactivation (e.g., AP-1 and NF-kB).

Chaperones

Hsp90:

Hsp90 is estimated to comprise 1-2% of all cytoplasmic proteins in unstressed cells and up to 6% in stressed cells (Csermely et al., 1998). These large amounts of Hsp90 are likely required to cover its diverse roles in signaling as well as the proper folding of at least 100 proteins. Hsp90 homodimers play an essential role in preserving the ligand binding pocket of the GR in a high affinity

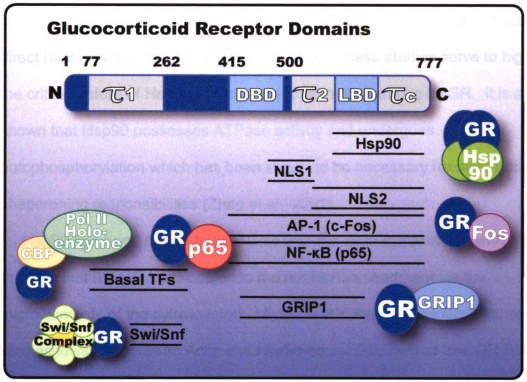

Figure I.7. Domain Structure of the Glucocorticoid Receptor (GR). Characteristic of nuclear hormone receptors, the GR features both a DNA-binding domain (DBD) and a ligand binding domain (LBD). The 777 amino acid protein also possesses three transactivation domains including τ1/2 and τc. Additionally, the various partner proteins which associate with the GR and the general sites for these interactions are shown. The approximate position of nuclear localization signals (NLS1/2) which facilitate the localization of the GR to the nucleus are also presented above.

state thus maintaining the ligand receptivity of receptor. To this end, studies using purified GR and Hsp90 have shown that when Hsp90 association with the receptor is blocked, a loss of hormone responsiveness occurs. Fusion protein studies have also demonstrated that by simply constructing an unrelated protein containing the LBD, and expressing Hsp90, a protein was created that was under direct hormonal control (Scherrer et al., 1993). These studies serve to highlight the critical nature of Hsp90 with respect to hormone binding by GR. It is also known that Hsp90 possesses ATPase activity and undergoes autophosphorylation which has been shown to be necessary for relief from its chaperoning responsibilities (Zhao et al., 2001).

Hsp90 has been shown to associate with the actin cytoskeleton which lent to the idea that the GR translocated to the nuclear compartment via the microfilaments of the cytoskeleton. Miyata and Yahara in fact identified an in-vitro association between Actin and Hsp90-bound GR, but not free GR (Yahara et al., 1998). More recent studies, however, present evidence to the contrary. Oren et al described their studies in which they treated cells with an Actin depolymerization agent, latrunculin A, which did not impact either GR levels nor its translocation to the nucleus (Oren et al., 1999). Moreover, confocal microscopy studies from various sources have consistently reported a mottled appearance from cells stained with fluorescently labeled antibodies to GR (Martins et al., 1991). The preponderance of evidence, then, seems to contradict the idea that GC signaling requires the cytoskeleton. One alternative explanation is that the Hsp90-associated receptors are tethered to actin filaments during

various phases of protein folding/unfolding and are therefore never consistently associated with the cytoskeleton.

Hsp70:

An additional chaperone protein, Hsp70, also assists in the proper folding of the GR, even though it is not recovered as a significant component of native GR heterocomplexes. Hsp70 which associates with the LBD of GR has been shown to be essential for association between Hsp90 and the GR. Hsp70 was also shown to associate with nuclear import sequences (NLSs) which are required for nuclear localization via nuclear pore complexes (NPCs). Interestingly, DeFranco et al determined that Hsp70 was not necessary for GR nuclear translocation thus complicating any role Hsp70 might play in nuclear translocation (Yang and DeFranco, 1994). One explanation for this result is that the exact folding stage of a given protein (e.g., GR) has to precisely coincide with the timing of the presentation of the NLS to the NPC. Although Hsp70 does not make physical contact with Hps90, it does indeed bind the LBD and has been shown to be essential for the high-affinity ligand binding state of GR.

Immunophilins:

In addition to both Hsp90 and Hsp70, the proper folding of GR and maintenance of the ligand-binding state requires the assistance of at least one more protein, FKBP52 (formerly referred to as Hsp56). FKBP52 is an immunophilin which characteristically binds to immunosuppressive agents such as cyclosporin, FK506 or rapamycin all of which can inhibit its intrinsic peptidylprolyl isomerase (PPIase) activity. Despite being the exclusive

enzymatic activity of this class of proteins, PPIase inhibition appears to have no effect on GR signaling (Denny et al., 2005). As its primary function in GR signaling, FKBP52 associates and binds to Hsp90 via its tetratricopeptide repeat (TPR) domains in a reversible equilibrium. Indeed, immunopurified GR protein complexes have been shown to bind [3H]FK506 thus confirming the presence of immunophilins in the heterocomplex. Most importantly, FKBP52 has been shown to potentiate GR signaling in *S. cerevisiae* which is not known to express any member of the FKBP family (Riggs et al., 2003). In mammalian cells, FKBP52 must displace FKBP51 which would otherwise impair the steroid binding capacity of GR. Strengthening the argument for the involvement of the cytoskeleton in GR shuttling to the nucleus is the finding that GR and FKBP52 copurifies with dynein, a motor protein associated with cytoskeleton microtubules. Studies using deletion mutants have determined that Domains I and III of FKBP52 are necessary to copurify GR and dynein. It is therefore possible that the switching of FKBP51 with FKBP52 would temporally permit the GR heterocomplex to associate with the cytoskeleton and thereby facilitate translocation of the receptor to the nucleus. Since it is unclear why actin depolymerization drugs fail to impact GR shuttling, a more extensive inquiry into the role that the cytoskeleton plays in GR signaling may be required.

GR Signal Transduction

Classical Type I GR Signaling:

In the nuclear compartment at the site of transcription, the GR, sans chaperone proteins, homodimerizes via Zn-finger D-boxes and binds to a

palindromic glucocorticoid response element (GRE) (**Figure I.8**). The receptor can function to positively regulate genes as is the case with PEPCK and TAT or turn off gene expression through binding to negative GREs (nGREs) in the promoters of certain genes (e.g., IL-1β, prolactin). Additionally, the GR can inhibit the transactivation of NF-κB and AP-1 via direct interaction, commonly referred to as a Type II response.

Type II GR Signaling:

The physiological importance of these two mechanisms of GR signaling was further elucidated by the laboratory of Guenther Schuetz. GR knockout mice die shortly following birth due to a defect in lung maturation (Cole et al., 1995). It was unclear, however, if this was due to loss of either Type I or Type II GR signaling (or both). In a rather elegant experiment, the Schuetz lab substituted a single alanine with a threonine within the D-loop of the GR (Reichardt et al., 1998). This would effectively prevent dimerization of GR, but still allow for type II interactions with other transcription factors such as NF-kB or AP-1. Surprisingly, these mice – termed GR dim – survived presumably as the result of intact type II signaling. It was later discovered that other GC-dependent mechanisms which were unrelated to survival such as induction of thymocyte apoptosis were in fact altered by loss of GR dimerization. The major finding that binding of DNA by GR is not required for survival represents a major shift in nuclear hormone receptor dogma. Protein-protein interaction among the two major GR isoforms, GRα and GRβ, was also determined to be an important regulatory feature of GR signaling.

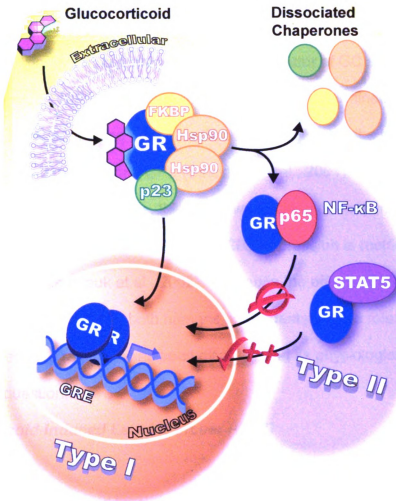

Figure I.8. Two Modalities of Glucocorticoid Receptor (GR) Signal Transduction. GCs freely pass through the plasma membrane lipid bilayer to react with the GR. The GR then dissociates from the chaperone proteins (Hsp90, FKBP, p23) that maintain this protein in a ligand-receptive state. As shown in the figure above, the GR can affect gene expression through at least two mechanisms. In a Type I response, the GR localizes to the nucleus where it binds to a glucocorticoid response element (GRE) as a homodimer and either upregulates (cGRE) or downregulates (nGRE) gene expression. Alternatively, the GR can also directly interact with specific proteins thereby affecting their transactivation activities. In this Type II mechanism, the GR inhibits gene expression (NF-κB, AP-1) or enhances gene expression (STAT5) via protein-protein interactions.

The human GR gene consists of 9 exons, the last of which can be alternatively spliced to give rise to two isoforms: GRα and GRβ. GRα is the predominant isoform found in nearly all cell types studied with some notable exceptions such as HEK293 and Hep3B cell lines. It has been suggested that the β-isoform may act as a dominant-negative regulator of GC signaling (Bamberger et al., 1995). To this end, Strickland et al determined that human neutrophils have, depending on the method used to quantify the two isoforms, 1.4 - 2.6 times more GRβ than GRα (Strickland et al., 2001). Additionally, using immunoprecipitation techniques, this group was able to co-immunoprecipitate GRβ using GRα antibodies. Interestingly, GRβ expression is restricted to human cell lines and tissue (Hauk et al., 2002). Because the effects of GCs on hematopoiesis are similar in both humans and rodents, GRβ's role as a dominant-negative regulator has been disputed and its physiological importance remains in question.

Glucocorticoid-Induced Leucine Zipper (GILZ)

Review:

Glucocorticoid-induced leucine zipper (GILZ) is a 137 amino acid (aa) protein so named for the presence of a characteristic leucine zipper (LZ) at aa 76-97 (**Figure I.9**). Leucine residues at positions 76, 83, 90, 96 and 97 help form an α-helix in the d-position which can then interact with like domains in a coiled-coil fashion. Unlike typical LZ proteins (e.g., AP-1, etc.), GILZ does not possess a conserved DNA binding element which would enable it to act directly as a transcription factor. GILZ has been shown, however, to form homodimers using

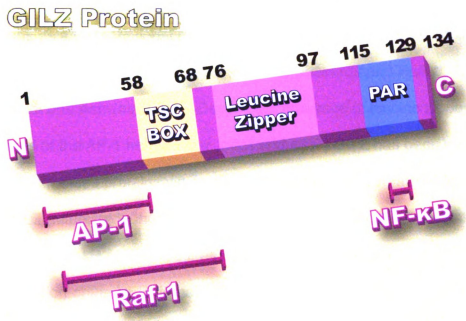

Figure I.9. Glucocorticoid-Induced Leucine Zipper (GILZ) protein domains. GILZ protein consists of three domains: (1) transforming growth factor β-secreting clone 22 (TSC) box; (2) leucine zipper; and (3) a proline- and acid-rich region (PAR). The TSC box is a 10 amino acid stretch that is conserved among other members of the TSC family of leucine zipper proteins. Amino acids 1-60 are required for GILZ-AP-1 interaction. Amino acids 9-73 are required for GILZ-Raf-1 interactions. Interactions with NF-κB requires both a GILZ homodimer mediated by the central leucine zipper as well as region 121-123.

its own zipper domains. The LZ domain of GILZ is flanked at the N-terminus by a transformation-growth factor β secreting clone 22 (TSC) box and a C-terminal poly-proline rich (PAR) domain (98-137aa). The TSC box is a 10 amino acid region that is conserved among members of this type of leucine zipper. GILZ functions as a transcriptional modulator that diminishes the activities of transcription factors such as AP-1 and NF-κB (Edwards and Hallett, 1997; Mittelstadt and Ashwell, 2001). Despite the presence of leucine zippers in both members of the AP-1 heterodimer, c-fos and c-jun, GILZ has been shown to interact with these proteins via a unique N-terminal domain (aa 1-60), but not with its LZ (Mittelstadt and Ashwell, 2001). Interestingly, GILZ-mediated inhibition of NF-kB activity requires both the homodimerization of GILZ and a unique region of the PER domain (Di Marco et al., 2007).

GILZ was first identified by Riccardi et al through subtractive hybridization of a cDNA library using thymocytes treated with GCs (D'Adamio et al., 1997). Additionally, a 9000 gene microarray identified a 5-fold increase in GILZ expression in GC-treated human peripheral blood monocytes (Ehrchen et al., 2007). Yamamoto et al, using chromatin IP, demonstrated the presence of several GREs contained within the GILZ promoter (Wang et al., 2004). Many cell types including monocytes, dendritic cells, mast cells, T-cells, several cell lines and neutrophils, which we will demonstrate, have been shown to have GC-inducible GILZ expression (Berrebi et al., 2003; Cohen et al., 2006; D'Adamio et al., 1997; Godot et al., 2006).

GCs have been shown to block anti-CD3 induced apoptosis and upregulate GILZ mRNA and protein expression in T-cells (D'Adamio et al., 1997). Furthermore, GILZ overexpression in T-cells has been shown to protect against anti-CD3 antibody cell death thereby mimicking the effects of GCs on these cells. In monocytes, GILZ has been shown to diminish the production of pro-inflammatory chemokines such as RANTES and MIP-1a in INF-γ stimulated monocytes (Berrebi et al., 2003).

GILZ Inhibition of NF-kB:

The impact of GCs on NF-kB transactivation is well documented in the literature (De Bosscher et al., 2003). This loss of NF-kB activity usually occurs by three mechanisms: (1) an increase in IkBα expression - an endogenous inhibitor of NF-kB activity; (2) competition for coactivators such as CBP/p300 (squelching); and/or (3) physical association of GR with NF-kB subunits. In addition to these three modes of NF-kB inhibition, GILZ has also been shown to directly associate with NF-kB and negatively modulate its activity. Unlike AP-1, which simply requires a distinct region of GILZ for interactions, NF-kB requires both a discrete domain (aa 121-123 of the PER domain) as well as GILZ homodimerization. Indeed, when the polar amino acids within the LZ domain of GILZ were substituted with non-polar residues effectively preventing GILZ dimerization, a loss of GILZ-NF-kB interaction occurred (Di Marco et al., 2007). One explanation for this two-pronged interaction scheme is that inhibition of NF-kB by GILZ would be regulated so that these interactions only occur when GILZ is in abundance and can sufficiently homodimerize.

Although GILZ was initially described as having nuclear localization in GILZ-transfected 3DO T-cell lymphomas, data from both our lab and others have provided evidence to the contrary (D'Adamio et al., 1997). As will be described later, we have detected predominantly cytoplasmic localization of GILZ protein in several immune cell types assayed, including neutrophils. In a separate study which examined GILZ localization in mast cells, Godot et al detected almost exclusive cytoplasmic GILZ distribution using immunocytochemistry (Godot et al., 2006). Additional evidence for cytoplasmic GILZ localization comes from another group of researchers who performed intracellular staining of GILZ using permeabilization reagents that do not effectively permeabilize the nucleus (personal correspondence with author) (Cohen et al., 2006). Since these scientists detected GC-induced increases in GILZ expression using flow cytometry, it is very likely that this signal derived from the cytoplasmic pool of GILZ protein. Other proteins, including several Bcl-2 family members, show atypical localization when overexpressed. More recently, the Riccardi laboratory indirectly revisited the GILZ localization question by overexpressing a myc-tagged GILZ construct in COS cells (Ayroldi et al., 2007). While examining the protein-protein interactions between GILZ and Ras, this group identified a "juxta-" membrane colocalization of GILZ and Ras proteins. Although these authors presented conflicting evidence as to the localization of GILZ in two separate publications, the varied localization of GILZ was never explained. The finding that GILZ is likely cytoplasmic may complicate the physiological relevance of GILZ-AP-1 interactions owing to the nuclear localization of both c-Jun and c-Fos.

Other GILZ interaction partners such as Raf-1 and NF-kB are localized to the cytoplasm and are therefore more likely to be relevant to in-vivo signaling processes.

GILZ Inhibition of Raf-1:

Raf-1 is a 73kDa proto-oncogene with serine-threonine kinase activity which functions in the Mitogen-activated Protein Kinase (MAPK) cascade to phosphorylate MEK or ERK, both MAPK kinases (Dhillon et al., 2007; Leicht et al., 2007). During its activation, Raf-1 dissociates from 14-3-3 in the cytoplasm and associates with Ras, a small G protein, at the plasma membrane. Raf-1 activation results in ERK1 phosphorylation which, in turn, can phosphorylate c-jun at serines 63 and 73 resulting in its activation. c-jun can then dimerize with ERK-1 activated c-fos via the respective LZ domains and bind to consensus DNA sequences located in the gene promoter. Two groups of researchers have independently demonstrated the inhibitory effects of GCs on Raf-1 activity (Cissel and Beaven, 2000; Rider et al., 1996). Several mechanisms have been postulated by these laboratories as to how GCs inhibit Raf-1 including: (1) direct inhibition by GR-Raf-1 interactions; (2) loss of Hsp90 from the cytosolic Raf-1 complex; (3) an increase in MAPK phosphatase 1; and (4) GR directly associates with AP-1.

In addition to these Raf-1 inhibitory pathways, Riccardi et al has also demonstrated that GILZ can directly interfere with Raf-1 signaling by directly displacing Ras (Ayroldi et al., 2002). Several deletion mutants were constructed in order to show that the N-terminal domain of GILZ was required for effective

Raf-1-GILZ interaction. Moreover, the inhibition of Raf-1 by GILZ blocked the phosphorylation of several downstream MAPK components including MEK and ERK, but not JNK. The inhibition of Raf-1 by GILZ appears to be the result of either steric hindrance which effectively displaces Ras from binding to Raf-1. Inhibition of Raf-1 by GILZ resulted in loss of AP-1 activation. Even though GILZ can directly associate with and inhibit components of AP-1 (c-fos and c-jun), it appears as though GILZ-mediated inhibition of AP-1 is accomplished via inhibition of Raf-1.

GILZ Regulation of FasL:

During thymopoiesis, a negative selection process is required to deplete autoreactive T-cells. This selection step is accomplished through the engagement of the TCR/CD3 receptor complex which induces apoptosis in absence of costimulation (e.g., CD28 and/or CTLA4). CD3 stimulation triggers induction of apoptosis in thymocytes as well as mature T-cells and T-cell hybridomas via the Fas apoptosis pathway (Ju et al., 1995). Specifically, anti-CD3 treatment of T-cell hybridomas resulted in an increase in both FasL as well as CD95 (FasR). Interestingly, GCs, which ordinarily induce apoptosis in several T-cell lineages, protect T-cells from activation-induced death (AID) by preventing the upregulation of both Fas signaling components (Yang et al., 1995). Monocytes, on the other hand, have been shown to undergo Fas/FasL-dependent apoptosis in response to GCs (Schmidt et al., 1999). Schmidt et al showed that monocytes undergoing GC-induced apoptosis upregulate and shed FasL into the supernatant (Schmidt et al., 2001), likely resulting in the

autocrinic/paracrinic death of these cells. Co-treatment of these cells with both GCs and anti-FasL neutralizing antibody prevented GC-induced monocytic cell death. Collectively, these studies indicated that GC-mediated control of FasL is quite complex and may be cell-context specific in some cases. It noteworthy to mention that this seems true for many cellular processes involving GC signaling as GCs induce apoptosis in some cell types, but still spare others.

The role that GILZ plays on FasL regulation is not completely understood. FasL expression is transcriptionally regulated by several transcription factors including NFAT, NF-κB, Egr-3, Sp-1 and AP-1 (Kavurma and Khachigian, 2003). Of these 5 transcription factors, AP-1 appears to be the most critical for regulation of FasL expression during cell stress. Faris et al demonstrated that either PMA/ionomycin or environmental stress (UVR or γ-irradiation) both induced FasL expression in Jurkat T-cells (Faris et al., 1998a). Using dominant-active (DA) MEKK1 Jurkat cells along with deletion mapping, this group identified a MEKK1-dependent site at position -335 of the FasL promoter (Faris et al., 1998b). MEKK1 phosphorylates MKK4/7 which in turn phosphorylates Jun N-terminal Kinase (JNK) leading to the activation of the AP-1 transcription factor complex. When c-Jun activity was disrupted through mutations, FasL promoter activity was reduced by 75% in DA MEKK1 Jurkats indicating the importance of c-jun in FasL expression. In addition to cell activation (PMA/Ionomycin) and environmental stress, GCs have also been shown to play a major role in the regulation of FasL expression.

Mittelstadt and Ashwell first reported that GCs inhibit the upregulation of FasL in response to T-cell activation (Mittelstadt and Ashwell, 2001). Since then, at least two mechanisms have been proposed to explain how GCs impair FasL expression including: (1) GR-dependent loss of NF-κB activity and (2) binding of GR to nGREs within the FasL promoter. Several reports have suggested that GR blocks FasL upregulation by inhibition of NF-κB activity (Lin et al., 1999; Novac et al., 2006). This is plausible since the FasL promoter has two NF-κB response elements (-128, -429, -1076) and, in the case of etoposide-induced apoptosis of T-cells, FasL has been shown to be under the control of NF-κB (Kasibhatla et al., 1998; Kasibhatla et al., 1999; Kavurma and Khachigian, 2003).

The ability of the GR to associate with NF-κB and negatively impact its transactivation potential is well documented (De Bosscher et al., 2003). Using HepG2 cells transfected with wild type GR or GR-dim (a dimerization-deficient mutant), Novac et al demonstrated that GCs not only mediate repression of an hFasL-linked reporter, but that this repression is also dependent on GR dimerization (Novac et al., 2006). Moreover, through deletion mapping analysis, it was further determined that an nGRE overlapped with a NF-kB binding site. Indeed, chromatin immunoprecipitation (ChIP) confirmed GR binding at the overlapping nGRE/NF-κB sites and a corresponding displacement of NF-kB in Jurkat T-cells. Although NF-κB binding to the overlapping elements was reduced (3-fold), it was not fully displaced indicating that this mechanism may not be completely sufficient to explain GC-mediated reduction of FasL expression. Rifampicin, an antibiotic used to treat tubercolosis, is a known GR agonist in

certain cell types including T-cells (Calleja et al., 1998). Similar to GCs, rifampicin has been shown to reduce both FasL mRNA and protein expression in Jurkat T-cells. When Jurkats are retrovirally transduced with dominant-negative (DN) IkBa, these cells become insensitive to rifampicin treatment (Yerramasetti et al., 2002). These data would implicate the NF-κB pathway as a molecular target for GR-mediated inhibition. An additional mechanism, such as GILZ-mediated interference of FasL-dependent transcription factors, may be part of a more comprehensive explanation of how GCs influence FasL expression.

In their efforts to further dissect AID T-cell death, D'Adamio et al explored whether GILZ impacted FasL expression (D'Adamio et al., 1997). To this end, 3DO T-cell hybridomas were transfected with GILZ and then stimulated with anti-CD3 antibody. GILZ clones had significantly less apoptosis compared to controls indicating that GILZ was responsible for the GC-mediated survival of T-cells undergoing AID. The improvement in the survival of these cells was attributed to a reduction in FasL expression in GILZ-transfected 3DO cells. After 10hrs of treatment with anti-CD3 antibody, flow cytometric analysis indicated 81.1% FasL positivity of 3DO cells transfected with a control plasmid. When transfected with GILZ, however, FasL levels were reduced to 1.4%. To further explain how GILZ blocks induction of FasL, Mittelstadt and Ashwell fused the hFasL promoter to a reporter which was coexpressed in Jurkat T-cells with GILZ (Mittelstadt and Ashwell, 2001). Indeed, increasing concentrations of GILZ plasmid resulted in a marked reduction in reporter activity confirming the role that GILZ plays in altering transcription of FasL. When GILZ was coexpressed with a reporter

fused to an AP-1 response element (TRE), a loss of reporter activity was observed indicating that the ablation of FasL transcription may be due to a loss of AP-1 activity. Using several deletion constructs, this group was able to demonstrate that aa 1-60 of GILZ interacts with both c-jun and c-fos of AP-1. Therefore, AP-1 may also play a major role in FasL expression, especially during periods of stress.

Major Hypothesis and Experimental Plan

Treatment of neutrophils with GCs suppressed apoptosis levels through an as of yet to be identified mechanism. Since GCs potently alter gene expression in nearly all cell types, it stands to reason that these compounds also induce a pro-survival regimen of GC-responsive genes in neutrophils which express the prerequisite GR. GC-induced upregulation of GILZ, a diminutive inhibitor of the activities of several transcription factors, represents one possible mechanism by which neutrophils evade spontaneous apoptosis in response to GCs (**Figure I.10**). Therefore, the hypothesis that GILZ is a critical component of GC-induced neutrophil survival will be tested through gene and protein expression analysis to assess the extent to which this protein is involved in survival. Moreover, antisense targeting of GILZ will be used to ascertain the functional significance of this protein in neutrophils. As will be presented herein, GCs induced GILZ expression in neutrophils thus implicating this protein in cell survival decisions. Given the reported role for GILZ in the survival of T-lineage cells, this molecule presents itself as a particularly attractive candidate as a key mediator of GC-induced survival in neutrophils.

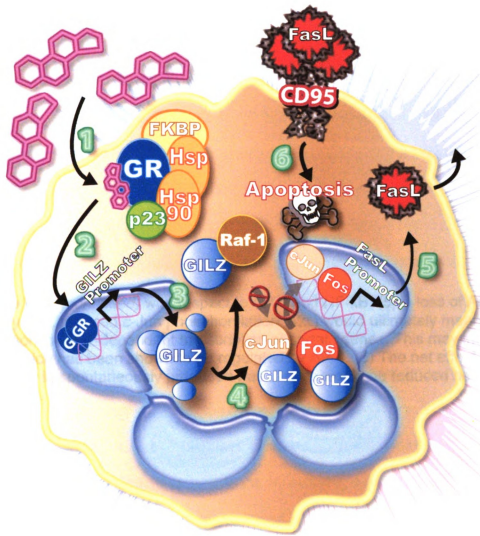

Figure I.10. Summary of a Proposed Pathway in GC-Mediated Neutrophil Survival. (1) Glucocorticoids freely traverse the lipid bilayer to bind to the glucocorticoid receptor (GR) complex which is held in a ligand receptive state by several chaperone proteins. (2) The GR then dissociates from these chaperones and is imported into the nucleus where it can bind to glucocorticoid response elements (GREs) located in the promoter of certain GC-responsive genes (e.g., GILZ). (3) GILZ is the prototypical GC-responsive gene since the promoter of this gene contains several GREs. (4) GILZ has been identified as a transcriptional modulator of several transcription factors and kinases including AP-1, NF-κB and Raf-1. GILZ functions as a transcriptional inhibitor

Figure I.10. *(continued)* with respect to AP-1 and NF-κB. (5) A loss of AP-1 activity (i.e., reduced c-Jun phosphorylation, etc.) would ultimately mean a reduction in AP-1-dependent transcription of certain genes. This may include FasL which has been shown to be regulated by AP-1. (6) The net effect of a reduction in spontaneously produced FasL levels would be reduced neutrophil apoptosis.

Additionally, induction of specific Bcl-2 family members including Mcl-1 and A1, have been shown to be potent protectors of neutrophils in response to pro-inflammatory ligands. Emerging evidence suggests that GCs also affect expression of select members of this family, further indicating the importance of this class of proteins to neutrophil survival decisions. Therefore, the hypothesis that the protective effects of GCs are the result of an increase in anti-apoptotic members of the Bcl-2 family will be tested through gene and protein expression analysis. Conversely, GCs might also promote the *downregulation* of pro-apoptotic Bcl-2 proteins resulting in a similar outcome in cell fate. To test this alternative hypothesis, a microarray containing ~450 genes related to apoptosis will be employed to comprehensively analyze the effects of GCs on Bcl-2 family member expression as well as other genes which might be involved in this survival pathway. Identification of apoptosis-related proteins besides Bcl-2 family members will also generate additional, previously unconsidered targets for additional analysis. Genes of interest identified by this approach will be further assayed using PCR and, in certain cases, Western analysis. Finally, the functional analysis of candidate genes identified by experiments proposed in this section presents itself as a daunting task since neutrophils stubbornly resist standard gene manipulation techniques. In an effort to overcome these obstacles and identify the significance of changes in the expression of specific genes, several gene dosing techniques which require only brief pretreatment protocols will be considered including antisense strategies and protein transduction. Hence, information generated from the proposed gene

manipulation studies featuring shorter incubation periods might spare data analysis from the confounding effects of increasing apoptosis of the short-lived neutrophil. The approaches presented in this publication serve to achieve the overall goal of determining the mechanism by which GCs promote neutrophil survival. A better comprehension of these molecular processes may offer greater insight into the dynamic interplay between host defense and stress which potentially lends itself to the development of medical treatments for conditions involving inflammatory cells and/or stress and GCs.

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CHAPTER 1

ASSESSMENT OF A ROLE FOR GLUCOCORTICOID-INDUCED LEUCINE ZIPPER (GILZ) IN THE SURVIVAL OF NEUTROPHILS

By

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ABSTRACT

Peripheral blood neutrophils are a short-lived, but critical mediator of the early inflammatory response. Ironically, treatment of neutrophils with glucocorticoids (GCs) – a potent *anti*-inflammatory compound – reduced neutrophil apoptosis by 65% at 12hrs. In this chapter, we analyze several early events that occur in neutrophils during GC signaling including whether the early induction of a glucocorticoid receptor (GR)-regulated gene participates in the survival of these cells. It was determined that GC treatment did not alter GC receptor (GR) mRNA, but did cause the rapid translocation of the GR to the nucleus within 30 minutes of GC treatment. Among a group of genes that increase in response to GCs, a 4.0-fold increase in Glucocorticoid-induced Leucine Zipper (GILZ) protein expression was detected after only 2hrs of GC treatment. GILZ, a 15kDa transcriptional modulator, is an important survival factor for anti-CD3 treated T-cells, but has also been shown to be a pro-death gene *in vivo* in immature T-cells. We further determined the localization of GILZ to be cytoplasmic, even during GC stimulation. Using several types of GC compounds, we observed a direct relationship between GC potency and levels of GILZ protein induction. Moreover, a study of the function of GILZ in neutrophils was attempted using antisense technology. The data presented herein suggests a role for GILZ in neutrophil survival, but more reliable tools for the study of function in neutrophils must first be developed.

Introduction

Neutrophil Survival:

The entry of microorganisms into the body elicits a process of inflammation that is highly coordinated between a variety of both immunological and non-immunological cell types. Of the immune cells, polymorphic neutrophils comprise the first wave of cellular response to these potentially pathogenic microorganisms. Neutrophils function to promptly remove these microbial intruders from inflammation-affected sites in the body through a receptor mediated process of ingestion termed phagocytosis. Moreover, these cells possess the abilities to produce reactive oxygen molecules as well as a host of antimicrobial proteins all of which serve to neutralize these pathogens. Prolonged inflammation has been identified as an underlying cause of a variety of autoimmune dysfunctions such as arthritis and inflammatory bowel disease. Thus, the proper resolution of the inflammatory foci requires the clearance of inflamed neutrophils at the conclusion of microbial engagement. In order to limit the scope of inflammation which is typically accompanied by elevated local concentrations of potent inflammatory proteins, neutrophils must ultimately be eliminated from these affected sites. The highly coordinated death process of apoptosis is required for the successful resolution of these inflammatory processes which would henceforth limit the spread of potentially tissue-damaging enzymes and reactive molecules possessed by the neutrophil (Savill et al., 1989) The survival of the normally short-lived neutrophil, however, is prolonged through stimulation by lipopolysaccharide (LPS) and/or a host of pro-inflammatory

cytokines (e.g., IL-8, leukotrienes, etc) (Colotta et al., 1992; Hebert et al., 1996; Kettritz et al., 1998). Additionally, several laboratories have demonstrated the capability of glucocorticoids (GCs) to increase neutrophil half-life ex-vivo (Cox, 1995; Kato et al., 1995; Liles et al., 1995). This is in stark contrast to other immune cells such as pro-B cells and thymocytes which undergo apoptosis in response to GCs (Lill-Elghanian et al., 2002; Wyllie and Morris, 1982).

Glucocorticoid Impact on Immunity and Disease:

Glucocorticoids (GCs) have long been used medicinally to control inflammatory conditions such as asthma, arthritis and skin disorders (first described in (Carryer et al., 1950), (Hench et al., 1949) and (Rappaport, 1955); later reviewed in ((Barnes, 2002) and (Rhen and Cidlowski, 2005). Clinicians often noted a reduction of lymphocytes in patients undergoing GC therapy, while myeloid cells such as the neutrophil actually increased in numbers (Bioerck et al., 1964). This increase in neutrophils caused by GCs was largely considered a pharmacological anomaly that did not warrant further investigation. In subsequent years, however, it has become apparent that the GC-mediated increase in neutrophil numbers may actually be a conserved biological strategy with significant physiological ramifications (Fraker and King, 2004) .

GCs have been implicated in the progression and scope of a number of diseases and medical maladies (Sapse, 1984). Diverse conditions ranging from surgery (Desborough, 2000) to burns (Dolecek, 1989) to infection (Christeff et al., 1997) and even death (Rothwell and Lawler, 1995) have been associated with elevated GC levels. Indeed, several medical conditions exist such as Crohn's

disease (Straub et al., 1998), rheumatoid arthritis (Catley et al., 2000) and various nutrient deficiencies, including zinc-deficiency (DePasquale-Jardieu and Fraker, 1979), all result in elevated levels of *adrenal* steroids. In Crohn's Disease, an inflammatory disorder of the bowel, serum cortisol levels were found to be elevated; especially during exacerbation of disease symptoms (Straub et al., 1998). It has been estimated that increased serum cortisol can be found in a host of disease pathologies and plays a vast role in such diverse conditions as depression, cancer and AIDS (Sapse, 1997).

This laboratory was one of the first to identify the prominent role that the hypothalamus-adrenal-stress (HPA) axis plays in symptoms associated with nutrient deficiencies. In mice fed a sub-optimal zinc diet, the endogenous GC corticosterone (CS) was found to be elevated in both moderate and severely zinc-deficient animals (Fraker and King, 2004). Moreover, adrenalectomized mice fed a Zn-deficient diet did not undergo thymic atrophy, a salient feature of poor Zn status, thus highlighting the importance of GCs in the progression of this nutritional deficiency (DePasquale-Jardieu and Fraker, 1979). In an analysis of hematopoietic cells of the bone marrow, granulocytes as well as their myeloid progenitors were found to be elevated by 57% and 50%, respectively (King and Fraker, 2002). This increase in bone marrow granulocytes manifested as increased circulating neutrophils in blood samples harvested from Zn-deficient mice (King and Fraker, personal correspondence). Given that several other nutrient deficiencies exist in which increases in GCs and/or neutrophils have been identified, particularly vitamin C- and copper-deficiencies, it is highly

probable that induction of the stress response can be extrapolated to many types of malnutrition (Savino et al., 2007).

Synthetic GCs such as those used by clinicians are favored due to their increased circulatory half-life and greater affinity for cognate receptor. A major drawback to the long term use of GCs in medicine is their lack of specificity. Protracted use of steroids often results in tissue damage including osteoporosis, glaucoma and an increased incidence in opportunistic infections due to the suppression of adaptive immunity. It is not yet clear how the immune system compensates for the loss of adaptive immunity during exposure to GCs. Of particular interest is whether survival of neutrophils during periods of high GCs represents a wholesale adaptation with the net result of increasing the efficiency and fitness of the host defense system (Ueda et al., 2005). A GC-induced increase in neutrophil numbers with a concomitant loss in several lymphoid cell populations conceivably represents a shift in immune strategy away from an adaptive immune response in preference of preservation and even expansion of the innate arm of the immune system. Perhaps just one of the reasons for this dramatic switch in strategies is to limit the seemingly high energy expenditures associated with negative selection events featured in adaptive immunity which can often result in the elimination of up to 99% of immature progenitors. Hence, an increase in neutrophil survival during periods of stress could represent a dual attempt to not only conserve energy resources which may be at a premium, but also to create a broad-reaching yet effective immune barrier to microbes which might take advantage of the stress-associated event.

Glucocorticoid Signaling:

Despite some advances in the understanding of the effects of GCs on neutrophils, there is little known about the primary signaling event that occurs in these cells. In many cell types, GCs initially bind to the 94kDa glucocorticoid receptor (GR) which is held in a ligand-receptive state by a series of chaperones and immunophilins including Hsp90, Hsp70, p23 and FKBP51. Upon GC occupation of the ligand binding domain of GR, the receptor dissociates from the molecular chaperones and enters into the nucleus via two distinct nuclear localization sequences, NLS1 and NLS2 (Ylikomi et al., 1992). The activated GR then homodimerizes and collectively binds to glucocorticoid response elements (GREs) located on GC-responsive genes. This can result in either the upregulation or downregulation of genes depending on the presence of classical GREs or negative GREs, respectively. Alternatively, activated GR can also physically interact with other transcription factors including NF-κB and AP-1 and thus restrict their transactivation potential. Several genes have documented sensitivity to GCs, but until recently there was no clear candidate gene which possessed both GREs and played a role in cell survival.

The Survival Protein GILZ:

Glucocorticoid-Inducible Leucine Zipper (GILZ) was first identified through the subtractive hybridization of normal mRNA versus a DEX-treated pool of cDNA (D'Adamio et al., 1997). GILZ expression has been identified in most cells, except the pancreas and liver (Cannarile et al., 2001). The GILZ induction by GCs derives primarily from the presence of 3 GREs located at positions -1944, -

2439 and -2475bp upstream from the transcriptional start site (Wang et al., 2004). GILZ functions as a 15kDa transcriptional modulator which negatively regulates AP-1, NF-κB and Raf-1 transactivation through protein-protein interactions (Ayroldi et al., 2001; Ayroldi et al., 2002; Mittelstadt and Ashwell, 2001). Despite the presence of an N-terminal leucine zipper, GILZ neither dimerizes nor requires such a motif for interacting with other transcription factors (Mittelstadt and Ashwell, 2001). More importantly, GILZ has been shown to have a pro-survival role in the 3DO T-cell line (D'Adamio et al., 1997). Specifically, GILZ overexpression in anti-CD3-treated T-cell line prevents the induction of apoptosis which would normally occur with the addition of antibody alone. Another important role for GILZ was established for macrophage-derived dendritic cell maturation (Cohen et al., 2006; personal correspondence). It is not known if GC treatment of neutrophils results in GILZ induction. It is conceivable though that GILZ elevation during GC treatment would likely indicate a role for this protein in the observed survival response of neutrophils.

Similar to other laboratories, we have developed a system in which we can detect a reduction in neutrophil apoptosis as a result of GC treatment. Using MC-540, we detected significant reductions in neutrophil apoptosis in response to GCs. In this chapter, we further explore the GC-mediated protection of neutrophils using receptor antagonists to demonstrate that GCs suppress neutrophil apoptosis via the GR. Additionally, for the first time, we show that GR rapidly translocates to the nucleus of human neutrophils during GC signaling. This is in contrast to other studies which suggest that neutrophils downregulate

GR in response to GCs (Chang et al., 2004; Preisler et al., 2000). We also identified increases in both mRNA and protein levels of GILZ in GC-treated neutrophils. Finally, we attempted to ascertain the role of GILZ in neutrophil survival using antisense-mediated knockdown of GILZ expression.

Materials & Methods

Reagents and Antibodies

Becton Dickinson (BD) Vacutainer Tubes containing Acid Citrate Dextrose Solution A and 21G1½ needles were used for drawing blood (BD Vacutainer System, Franklin Lakes, NJ). Dextran T-500, Percoll, PVDF, ECL-plus reagents and HRP-linked anti-mouse and –rabbit antibodies were obtained from GE Biosciences (Piscataway, NJ). Trypsin Inhibitor and Human Neutrophil Elastase Inhibitor (HNEI) were both ordered from EMD Biosciences (San Diego, CA). PMSF, RPMI-1640 Media, Iscove's Media, 100x Pennicillin-Streptomycin, 100x L-Glutamine, Acridine Orange, Propidium Iodide (PI), TRIZOL, Superscript II and III and dNTPs, SYBR Green (1000x) were all obtained from Invitrogen (Carlsbad, CA). Micrococcal Nuclease (MNase) was obtained from USB Corp. (Cleveland, OH). Fetal Bovine Serum was purchased from Hyclone (Logan, UT). All other reagents including Dexamethasone, hydrocortisone and DOTAP were purchased from Sigma (St. Louis, MO). SYBR Green Core Reagent Kits and PCR plates were obtained from Applied Biosystems, Inc. (Foster City, CA). Nuclear extraction reagents and BCA protein determination reagents were purchased from Pierce, Inc. (Rockford, IL). The HL-60 cell line was obtained from the ATCC (Bethesda, MD) and the 3DO cell line was a kind gift from the J. Kappler and P.

Marrack laboratory (National Jewish Medical and Research Center, Denver, Colorado). The antibodies used in these studies were anti-GR (M1 clone, BD Biosciences, San Jose, CA), anti-β-Actin (Sigma, St. Louis, MO), anti-Oct1 (Santa Cruz Biotech., Santa Cruz, CA) and anti-Hsp90 (Stressgen, Ann Arbor, MI). The anti-GILZ antibody was a kind gift from the laboratory of Carlo Riccardi (Univ. of Perugia, IT). All primers were synthesized by MSU RTSF Oligonucleotide Synthesis Facility (E. Lansing, MI). Anti-sense and sense oligonucleotides were purchased from Oligos, Etc (Wilsonville, OR).

Neutrophil Isolation

Peripheral blood neutrophils were harvested from healthy adult donors (ages 18-30) following the submission of donor consent in accordance with Michigan State University Human Use Committee guidelines. Neutrophils were purified as described previously, with minor modifications to the protocol (Savill et al., 1989). Briefly, 34ml of anti-coagulated blood was mixed with 17ml of 3% Dextran in 0.85% NaCl to deplete red blood cells (RBCs). The RBC-depleted cells were then resuspended in 2ml of autologous platelet poor plasma which was obtained from donor serum supernatant following centrifugation at 5000xg. Next, leukocyte subsets were resolved on a discontinuous Percoll gradient comprising two layers of 55% and 64% Percoll in 0.85% NaCl. The granulocytes were then harvested from the 55%-64% interphase and washed in Hanks' Balanced Salts Solution (HBSS; minus Ca^{++} and Mg^{++}). This method routinely yielded preparations with >98% viability as determined with Trypan Blue. Neutrophil

purity was routinely assessed to be 96 – 98% based on nuclear morphology via Acridine Orange staining.

MC-540 Detection of Apoptosis

Apoptosis was measured using a merocyanine-540 (MC-540)-based method that was developed in our lab (Laakko et al., 2002). Neutrophils were cultured in RPMI containing 100 units Penicillin, 100µg Streptomycin, 2mM L-glutamine and 2% heat-inactivated, Charcoal-Dextran treated FBS. Neutrophils were plated on Falcon non-treated 24 well plates to reduce adhesion of healthy cells. Following harvest, cells were stained with 17uM MC-540 for 10 minutes in the dark. PI (1μg/ml) was then added to each tube just prior to FACS analysis in order to detect late stages of apoptosis and exclude necrosis. Cell analysis was done using a FACS-Vantage flow cytometer equipped with 575nm and 660nm filters. Flow data was analyzed and gating was performed with WinList 5.0 (Verity Software) FACS data analysis program.

Real Time PCR Analysis of Gene Expression

Total RNA was extracted from $1x10^7$ neutrophils using TRIZOL RNA extraction reagent. 1μg of anchored oligo d(T_{18}VN) was added to 1μg of total RNA to synthesize cDNA template using Superscipt Reverse Transcriptase. Relative real time PCR was conducted on 0.5µl of cDNA product using ABI Core Reagents in 25µl total volumes. The following primers were used for real time experiments: (1) *β-actin* (F: TGTTGGCGTACAGGTCTTTG; R: TGT TGGCGTACAGGTCTTTG); (2) *GRα* (F: CCATTGTCAAGAGGGAAGGA; R: AAAT GTTTGGAAGCAATAGTTAAGG); and (3) *GILZ* (F:

TGGTGGCCATAGACAACAA G; R: TCTCGGATCTGCTCCTTCA). Primer efficiency for each primer set within the range of analysis was estimated to be >90% as estimated by an amplicon-titrated standard curve. Analysis of real time data was performed using the $-\Delta\Delta C_T$ method of comparison. Data points for the genes of interest were normalized to respective β-actin levels.

Cellular Fractionation and Western Blot Analysis

Cells were fractionated into cytoplasmic and nuclear fractions using the NE-PER nuclear extraction kit (Pierce). Protease inhibitors were used as previously described (Gametchu et al., 1993), but also included: 100μM Phenylarsine Oxide, 2mM NaF, 10mM Na_2MoO_4, 100μM Elastatinal, 100μM Chymostatin and 10μM Human Neutrophil Elastase Inhibitor. Protein concentration was determined using BCA Protein Assay (Pierce). Samples were equally loaded and resolved on a 12% Polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). The separated proteins were then transferred to a PVDF membrane at 25V for 1 hour. Blots were blocked with either 5% dried milk dissolved in Tris-buffered saline with 0.1% Tween-20 (TBS-T) or 2% Bovine serum albumin (BSA) in TBS. The blots were then incubated with 1:500 mouse anti-G or 1:1000 rabbit anti-GILZ diluted in blocking buffer. Following washing, the membranes were treated with HRP-conjugated anti-mouse or -anti-rabbit antibody, developed and exposed to film. To probe for additional antigens, the blots were stripped using 100mM 2-mercaptoethanol and 2% SDS at 55°C for 30 minutes. The membranes were then re-probed using either rabbit anti-Oct1, anti-Hsp90α or anti-β-actin and developed as described above.

Anti-sense Knockdown of Gene Expression

Antisense was designed *in silico* using the antisense generator tool available from the Integrated DNA Technologies (IDT; Coralville, IA) website thus ensuring minimal secondary structure formation. Second generation antisense technology was used to synthesize chimeric oligonucleotides containing mixed backbone linkages and a 5'-RNase H activating region (Oligos, Etc). A 5µg:1µg ratio of DOTAP:oligo was used to enable efficient transfer of oligonucleotides into neutrophils (Sivertson et al., 2007). For gene knockdown experiments, purified neutrophils were pre-incubated with 2.5µM GILZ sense (AGAGGTGGAGGAGAGAGTGT) or antisense (TCTCCACCTCCTCTCTCACA) for 4 hours. Following pre-treatment, GCs were added and cultures were further incubated for an additional 4 hours for Western analysis of GILZ knockdown efficacy; or 12 hours for flow cytometric analysis of neutrophil apoptosis.

Results

Glucocorticoids Promote Neutrophil Survival

In order to establish a model in which GC-regulated mechanisms of neutrophil survival could be studied, an MC-540 based method of apoptosis detection was used to ascertain levels of neutrophil cell death. Treatment of neutrophils with 0.1µM Dexamethasone reduced neutrophil spontaneous apoptosis by 60% at 6hrs and by 65% at 12 hrs (**Figure 1.1**). To assess the degree of protection afforded by more natural stress-like conditions, we also used the endogenously

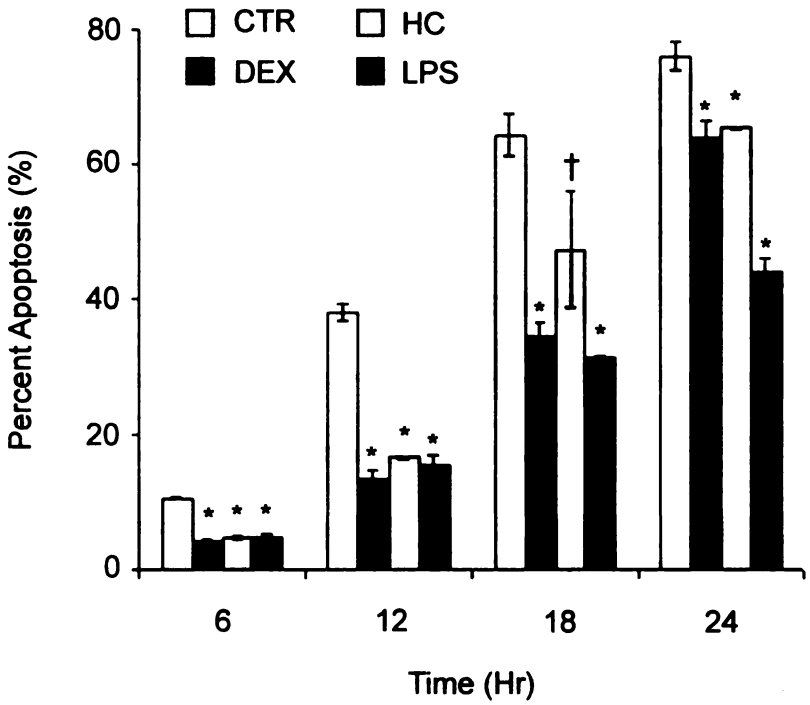

Figure 1.1. Time Course of Glucocorticoid-Induced Neutrophil Survival During Routine Culture. The bar graph shown is the flow cytometric analysis of human neutrophil apoptosis using the merocyanine 540 staining technique. Purified human neutrophils were treated with either 0.1μM dexamethasone (DEX), 1μM hydrocortisone (HC) or 10μg/ml LPS and cultured for 6-24 hours (Hr) to promote apoptosis. Standard error is shown for each data point (*$p<0.01$; †$p<0.05$).

produced steroid hydrocortisone (HC) at concentrations that would be found during periods of stress. Similar to DEX, the addition of the endogenous steroid HC reduced neutrophil apoptosis by 55% and 56% at 6hr and 12hr, respectively. LPS, a potent neutrophil activator and pro-inflammatory component of gram-negative bacteria, was utilized as a positive control during these studies (Colotta et al., 1992). The pro-survival effects of DEX, as well as HC, were observed through 18hrs of GC treatment. It is important to note that neither GCs nor LPS were able to stop apoptosis altogether, rather it delayed apoptosis. These studies effectively demonstrate that MC-540 is a sensitive tool for studying the GC-mediated alterations of neutrophil survival.

GCs Signal Survival via The GR

With the GR being the primary signal messenger by which GCs conduct cell signaling, the role that this receptor plays in neutrophil survival was investigated using selective GR antagonists. Mifepristone (also known as RU38486) as well as the lesser known RU40555 were both utilized to block GR signaling in neutrophils. Mifepristone binds to the GR with an affinity 3-times that of DEX, stabilizing an inactive conformation of the receptor.

Primary neutrophils were pre-treated for 1hr with either vehicle of 1μM of RU40555 or Mifepristone. Following preincubation, neutrophils were then treated for an additional 12hrs with either 0.1μM DEX, 1μM HC or 10μg/ml LPS. Pretreatment of neutrophils with either Mifepristone or RU40555 resulted in apoptosis levels approximately equal to those of control even after the addition of

DEX or HC (**Figure 1.2**). While Mifepristone has been shown previously to inhibit GC-mediated neutrophil survival, this is the first report that RU40555 causes a similar inhibition of GC-enhanced neutrophil survival. The unimpaired ability of LPS to reduce neutrophil apoptosis despite the presence of GR antagonists indicated the pathway exclusivity by which GCs signal survival in neutrophils. This control serves to highlight the one of the critical differences between GC-stimulated neutrophil survival and neutrophil activation by pro-inflammatory compounds such as LPS.

GR mRNA Levels

Homologous regulation of a receptor by its ligand is a prominent feature of GC signaling. Specifically, in many systems GCs possess the capacity to alter the abundance of its cognate receptor, the GR. Homologous down-regulation of the GR by GCs has been described for several cell types with the belief that that this mechanism(s) represents at least one manner by which cells can be desensitized to the effect of ligand. In this vein, we analyzed the expression of GRα in GC-treated neutrophils to gauge any homologous effects exerted by the ligand.

Real-time PCR was used to determine the relative abundances of GRα mRNA in neutrophils treated with GCs. Through 4 hours of GC treatment, no alteration in GRα mRNA reaching the level of significance was detected in GC-treated neutrophil samples (**Figure 1.3A**). At 2 hours of LPS treatment, however, a 40% decrease in GRα mRNA levels occurred thus demonstrating that the PCR tools used in these experiments were sufficiently sensitive to detect changes in transcript levels and further indicating the divergence of the GC and LPS

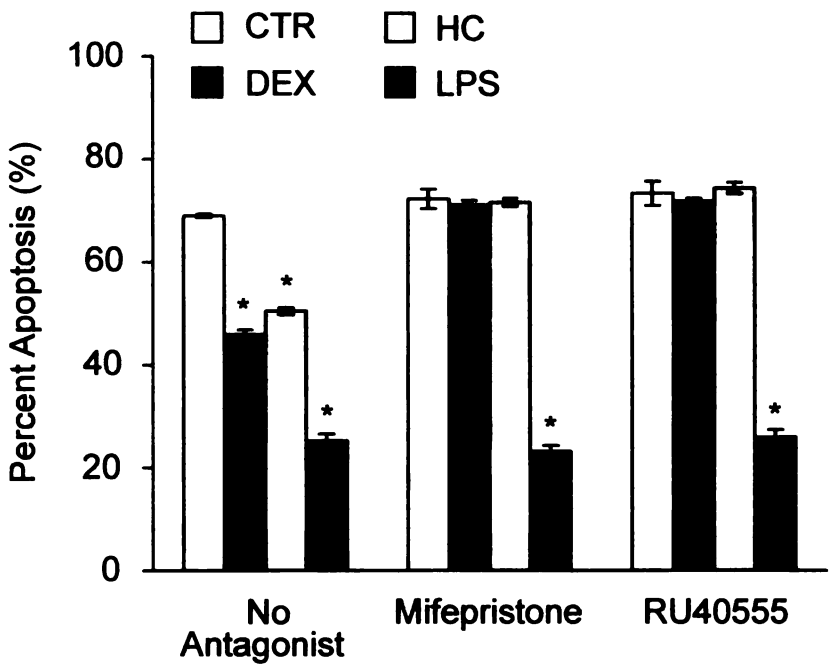

Figure 1.2. Glucocorticoid Receptor Signaling Is Critical to Mediating the GC-Induced Survival Signal In Neutrophils. The bar graph shown is the flow cytometric analysis of neutrophil apoptosis. Purified human neutrophils were pre-treated with either vehicle, 1μM mifepristone or 1μM RU40555 for one hour following which 0.1μM dexamethasone (DEX), 1μM hydrocortisone (HC) or 10μg/ml LPS were added for an additional 12 hours. Standard error is shown for each data point (*$p<0.01$) and this experiment is representative of three independent experiments.

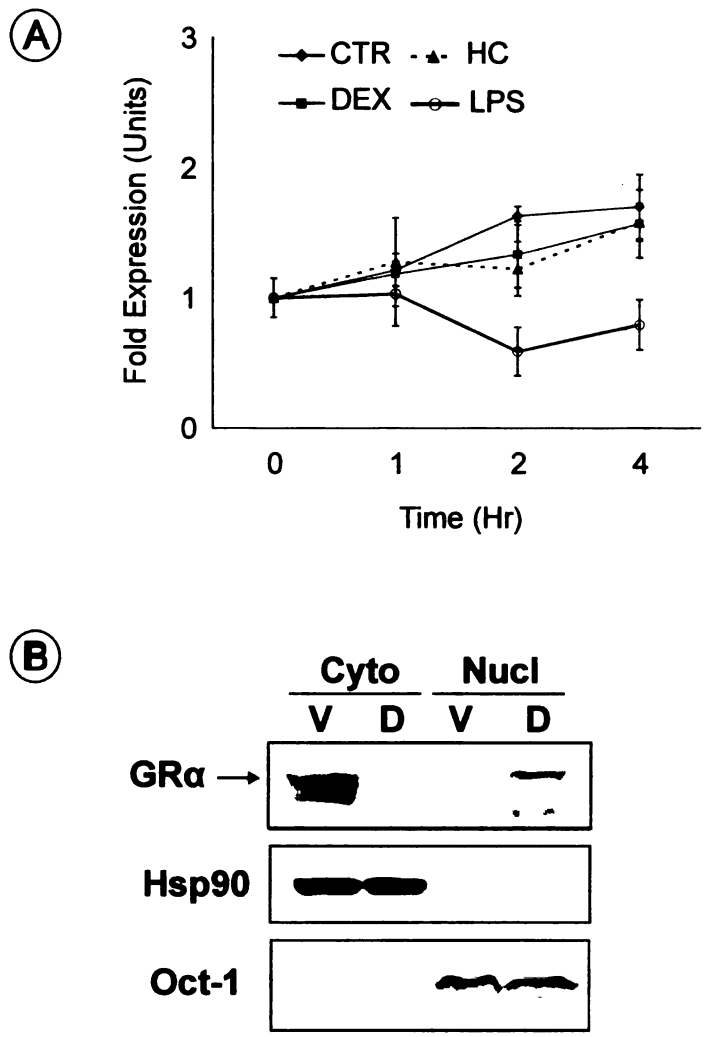

Figure 1.3. Glucocorticoids Promote The Migration of GR to the Nucleus While Leaving GR mRNA Levels Unaffected. (A) The line graph depicted here represents the relative expression of GRα mRNA in neutrophils treated with either vehicle (ethanol; VEH), 0.1μM dexamethasone, 1μM hydrocortisone or 10μg/ml lipolysaccharide (LPS). Each data point was normalized to β-Actin mRNA as well as time zero for that data set. Standard error is shown and this experiment is representative of three independent experiments (*$p<0.01$, †$p<0.05$) (B) Data shown is the Western analysis of glucocorticoid receptor (GRα) expression in fractionated neutrophils including the cytoplasmic (Cyto) and nuclear compartments (Nucl). Neutrophils were treated with either vehicle (ethanol; V) or 0.1μM dexamethasone (D) for 30 minutes. Hsp90 and Oct-1 were used to verify relative cytoplasmic and nuclear compartment purity, respectively. Data shown is representative of two independent experiments.

pathways. The inability of GCs to alter GRα mRNA expression in neutrophils demonstrates the novelty of this system with respect to other models that have been studied. It is interesting to note the negative impact that a pro-inflammatory molecule(s) like LPS has on an anti-inflammatory pathway (i.e., the GC pathway). These experiments indicated that GR mRNA is not down-regulated in response to GCs in neutrophils. Alterations in receptor transcript abundance, however, do not necessarily provide information as to the cellular whereabouts of the corresponding protein. This is especially important for nuclear hormone receptors due to the vast number of nuclear genes regulated by these family members, including the GR. Therefore, it was also important to ascertain the cellular localization of the GR during GC treatment of neutrophils.

GR Movement to the Nucleus

A major component of GC signaling is the translocation of the GR to the nucleus where activated receptors can then either bind GREs as a homodimer or interact with other transcription factors. There are reports that the GR is downregulated in neutrophils during GC-treatment (Chang et al., 2004; Preisler et al., 2000). Indeed, routine Western preparation of GC-treated samples resulted in the loss of GR-associated chromatin when the insoluble fraction is discarded (data not shown). In order to ascertain the cellular localization of GR, GC-treated neutrophils were fractionated into cytoplasmic and nuclear components. Using Western analysis and immunoblotting, a major shift in compartmental distribution of GR within 30 minutes of GC stimulation was identified (**Figure 1.3B**). Kinetic analysis of GR translocation during GC

treatment indicated that this event occurs as early as 15 minutes (data not shown), but reaches its peak at 30 minutes. Despite the preferential cytoplasmic localization of GR in unstimulated neutrophils, there was a remarkable loss of this protein in this compartment during GC treatment. Interestingly, this loss of GR in the cytoplasm closely resembled Western data obtained using *whole cell* lysates from GC-treated neutrophils. This data suggests that early GR translocation to the nucleus in response to homologous ligand may represent the very first step in a signaling cascade which culminates in the improved survival of neutrophils. This finding highlights the significant difference in GR responses to GC treatment between neutrophils and other cell types. Specifically, while GC treatment of neutrophils promotes the translocation of GR to the nucleus; GC treatment of other cell types results in the downregulation of GR.(Oakley and Cidlowski, 1993) These results necessitated the investigation of downstream events, with special emphasis on gene(s) that have been shown to be regulated by GCs and coincidentally affect survival.

Induction of GILZ in Neutrophils

In an effort to identify a GC-responsive gene with a tangible relationship to cell survival, we analyzed GILZ gene expression in response to GCs. As outlined in the introduction, GILZ, which is induced by GCs in a variety of cell types, acts as a pro-survival protein in T-cells. Therefore, relative real-time PCR was used to examine GC-induced expression of GILZ mRNA. A 4.2-fold increase in GILZ expression was observed after 2hrs of treatment with 0.1μM DEX and a 3.2-fold increase in GILZ was observed with 1μM HC (**Figure 1.4**). Similarly, GILZ

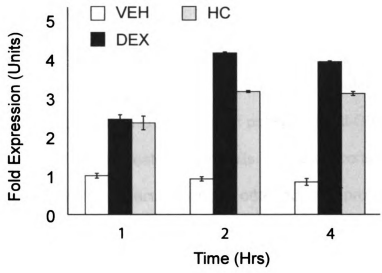

Figure 1.4. Glucocorticoids Upregulate GILZ mRNA Expression in Human Neutrophils. Purified human neutrophils were treated with either vehicle (ethanol; VEH), 0.1μM dexamethasone (DEX) or 1μM hydrocortisone (HC) for 1-4hrs. A cDNA population was generated from total RNA for each sample. The bar graph shown is the relative abundance of GILZ mRNA for each treatment. Data were normalized to β-Actin expression levels for each cDNA sample. Standard error is shown and data is representative of three independent experiments.

remained elevated by 4hrs at 3.9-fold with DEX treatment and 3.1-fold with HC. GILZ gene expression remained significantly elevated throughout 8hr of incubation with GCs (data not shown). The induction of this gene in neutrophils is consistent with the finding that GILZ is one of the most highly GC-inducible genes across a variety of cell types. It is therefore not surprising that this gene would also be identified as a GC target in neutrophils as well. Since there is often a discrepancy between gene and corresponding protein expression, the ability of GCs to alter GILZ protein levels was also investigated.

To ascertain GILZ protein levels, a rabbit polyclonal anti-GILZ antibody was used to detect GILZ in GC-treated neutrophils. Recent reports have identified GILZ protein isoforms of molecular weights other than the prototypical 15kDa molecular weight form. While a higher molecular weight immunoreactive band was frequently seen, particularly in unstimulated neutrophils, these studies focused exclusively on the 15kDa GILZ isoform (also known as GILZ1). Western blot analysis was used to correlate changes in GILZ gene expression with protein levels of GILZ. Indeed, we observed a 4.0-fold increase in GILZ expression in response to 0.1μM DEX relative to the 2hr-matched vehicle control (**Figure 1.5**). By 4hrs, the difference between vehicle and DEX was 3.4-fold. GILZ protein levels continued to remain elevated even through 8hrs of GC treatment.

To further investigate the mode by which GCs induce GILZ expression in neutrophils, GR antagonists were used to block GR activity in these cells. Pre-treatment of neutrophils for 1hr with 1μM of either Mifepristone (M) or RU40555 (R) potently blocked the ability of either DEX or HC to upregulate GILZ

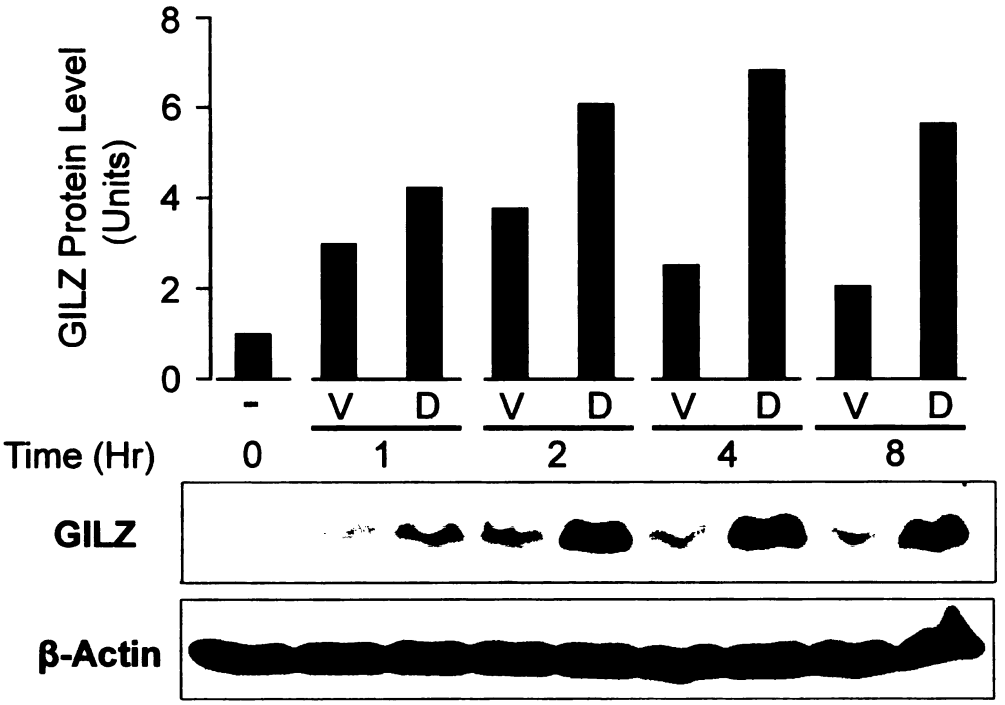

Figure 1.5. Glucocorticoids Induce GILZ Protein Expression in Human Neutrophils. Purified human neutrophils were incubated with either vehicle (V; ethanol) or 0.1μM dexamethasone (D) for the indicated times. Western analysis of GILZ expression for each sample is shown. Densitometric analysis, including normalization to β-Actin, is displayed as a bar graph. Data shown is representative of two independent experiments.

expression (**Figure 1.6**). GILZ levels were reduced to approximately that of untreated controls when cells were pre-treated with the inhibitors. These data indicate that GILZ induction during GC treatment is regulated by the GR. This finding helps in discerning the transcriptional regulation of GILZ in neutrophils; a gene which is not only under the control of GR, but also FoxO3 (Asselin-Labat et al., 2004).

The upregulation of GILZ protein in response to GCs in neutrophils suggests that this protein may ultimately play a role in the survival of these cells. Using a transfected cell line overexpressing GILZ, D'Adamio et al demonstrated a nuclear localization of GILZ protein. Based on evidence from other overexpression models, such as Bcl-2, proteins of epigenetic origin and produced in great abundance can often have atypical cellular distribution. Therefore, it was necessary to determine the cellular localization of endogenously produced GILZ, especially in neutrophils.

Localization of GILZ

The reported nuclear localization of GILZ fits nicely with its role as an inhibitor of AP-1 since both c-Fos and c-Jun are also localized to this compartment. The role of GILZ in the inhibition of other transcription factors such as NF-κB, however, become more uncertain especially since the normal inhibition of this protein by IκBα usually occurs in the cytoplasm (Baeuerle et al., 1988). In order to identify the localization of endogenously produced GILZ, Western analysis was used to detect GILZ expression in a variety of fractionated cells including the 3DO cell line, primary human neutrophils and peripheral blood mononuclear cells

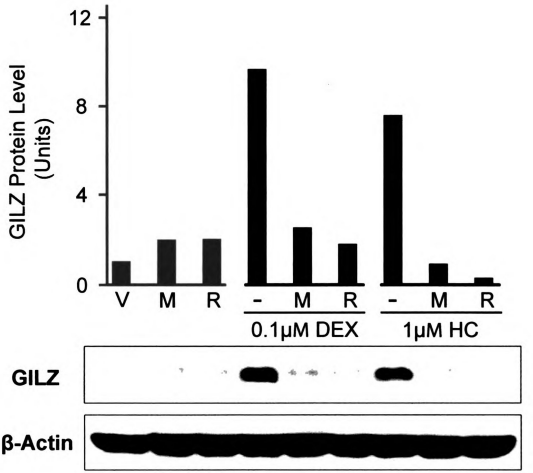

Figure 1.6. Glucocorticoid Receptor (GR) Antagonists Block GILZ Protein Induction In Response to GCs. The importance of the GR in GILZ induction by glucocorticoids was explored by blocking the GR with selective antagonists. Purified human neutrophils were pre-treated with either 1μM mifepristone (M) or 1μM RU40555 (R) for one hour. Following pre-treatment, either 0.1μM dexamethasone (DEX) or 1μM hydrocortisone (HC) was added for an additional 4 hours. Data shown is the Western analysis of GILZ expression for the indicated treatments. The bar graph represents the densitometric values for GILZ expression which were normalized to β-Actin for each sample.

(PBMCs). Contrary the other published findings, endogenously-produced GILZ was localized exclusively in the cytoplasm (**Figure 1.7A**). One noteworthy observation in these experiments was the overabundance of Oct-1 in PBMCs which was used to verify the purity of the nuclear fraction. Lymphocytes, a major component of PBMCs, possess an additional lymphocyte-specific Oct-1 (Oct-1L) which shares a nearly identical sequence with classical Oct-1 save an additional 12 amino acids at the N-terminus (Luchina et al., 2003). The antibody used for these studies recognized the C-terminal end of Oct-1 and therefore would detect both Oct-1 and Oct-1L. This likely explains why such intense staining of Oct-1 was observed for PBMC nuclear lysates.

Since GC treatment of neutrophils alters the localization of other proteins such as the GR, the question of whether GILZ localization can be altered during GC treatment was addressed. Unlike the GR, treatment of peripheral blood neutrophils for 4hrs with 0.1μM DEX only modestly affected GILZ distribution (**Figure 1.7B**). A faint band representing a small increase in nuclear GILZ was observed in samples treated with DEX.

Since neutrophils are terminally differentiated cells with a rather short lifespan in culture, classical molecular approaches such as plasmid transfection or viral transduction are not viable options. Therefore, the inherent differential sensitivity of neutrophils to various GC types (both synthetic and natural) was exploited in an effort to indirectly determine the importance of GILZ to neutrophil survival.

GC Potency Versus GILZ Levels

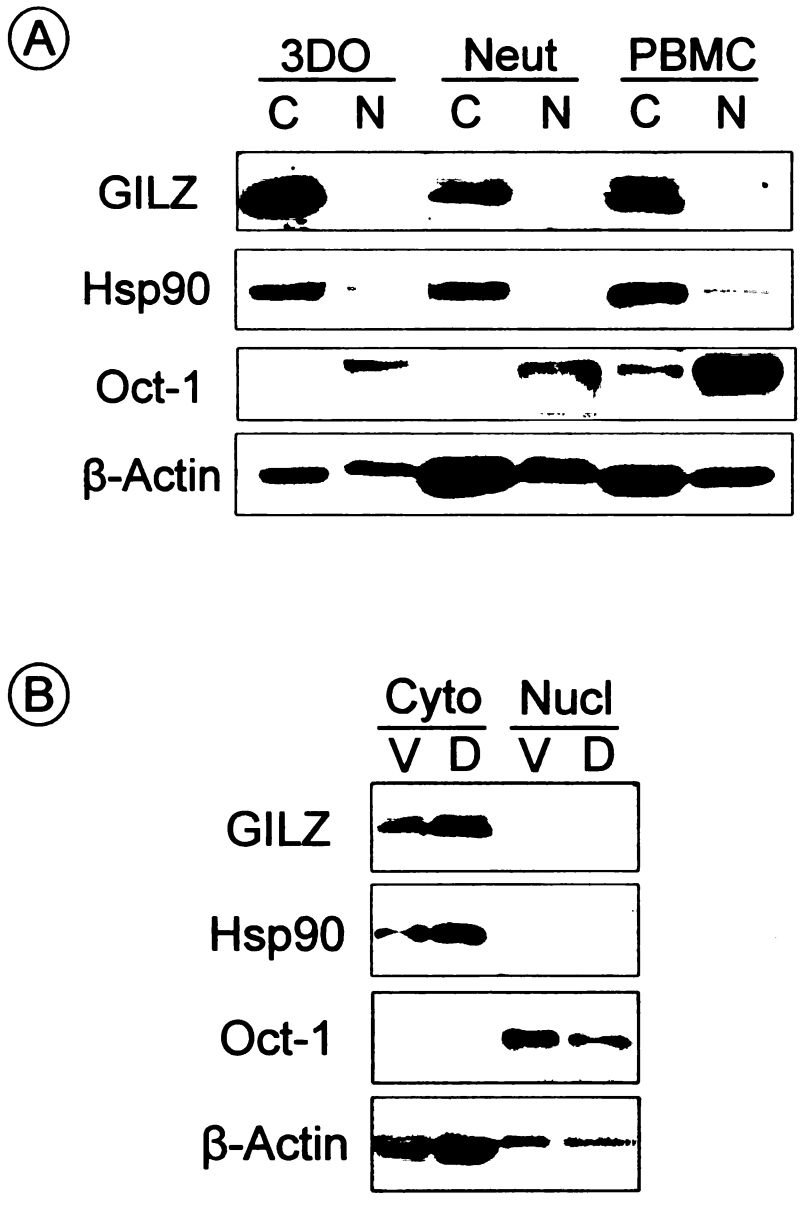

Figure 1.7. GILZ Has Cytoplasmic Distribution in a Variety of Cell Types Including Resting and GC-Treated Neutrophils. (A) Western analysis of GILZ localization in cytoplasmic (C) and nuclear (N) fractions of the 3DO cell line (3DO), purified neutrophils (Neut) and peripheral blood mononuclear cells (PBMC). Hsp90 and Oct-1 expression served as comparment marker controls and β-Actin was used to gauge equal loading. (B) The ability of GCs to modulate GILZ localization was determined by treating purified neutrophils with 0.1μM dexamethasone followed by fractionation and Western analysis of GILZ expression. Controls similar to (A) were employed to verify the relative purity of comparmental fractions.

The different levels of induction of GILZ by either the synthetic GC DEX or the natural HC was duly noted. Based on this, we hypothesized that a correlation between GILZ protein levels and neutrophil survival could offer substantial progress toward demonstrating the critical importance of this protein in protection of neutrophils during GC treatment. Therefore, various GC types ranging in potency from the weakest, Corticosterone to the most potent, Budesonide, were used to construct a GC potency versus neutrophil survival dose curve.

Neutrophils were treated with various GC types, each of which varied by potency, for 4hrs for Western analysis of GILZ expression and 12hrs for apoptosis detection. Prednisone, a prodrug that is converted to an active GC by the liver, was also included as a negative control. After GC concentrations were adjusted for potency (e.g., Budesonide at 0.01μM versus Hydrocortisone at 1μM), densitometric analysis demonstrated similar abilities of each GC type to induce GILZ protein expression (**Figure 1.8A**). Flow cytometric analysis of neutrophil apoptosis, however, was much more sensitive in discerning between GC types. For example, the weakest agonist, Corticosterone (1μM), only reduced neutrophil apoptosis levels by 24% whereas 0.01μM Budesonide reduced apoptosis by 46%. If a correlation between GILZ and neutrophil survival existed, then a plot of GILZ protein expression versus neutrophil survival, if linear, would demonstrate such a relationship.

In order to estimate the degree of correlation between GILZ induction and apoptosis indices, an R-square analysis was used to estimate the type of relationship between the anti-apoptotic potency of GCs and GILZ induction.

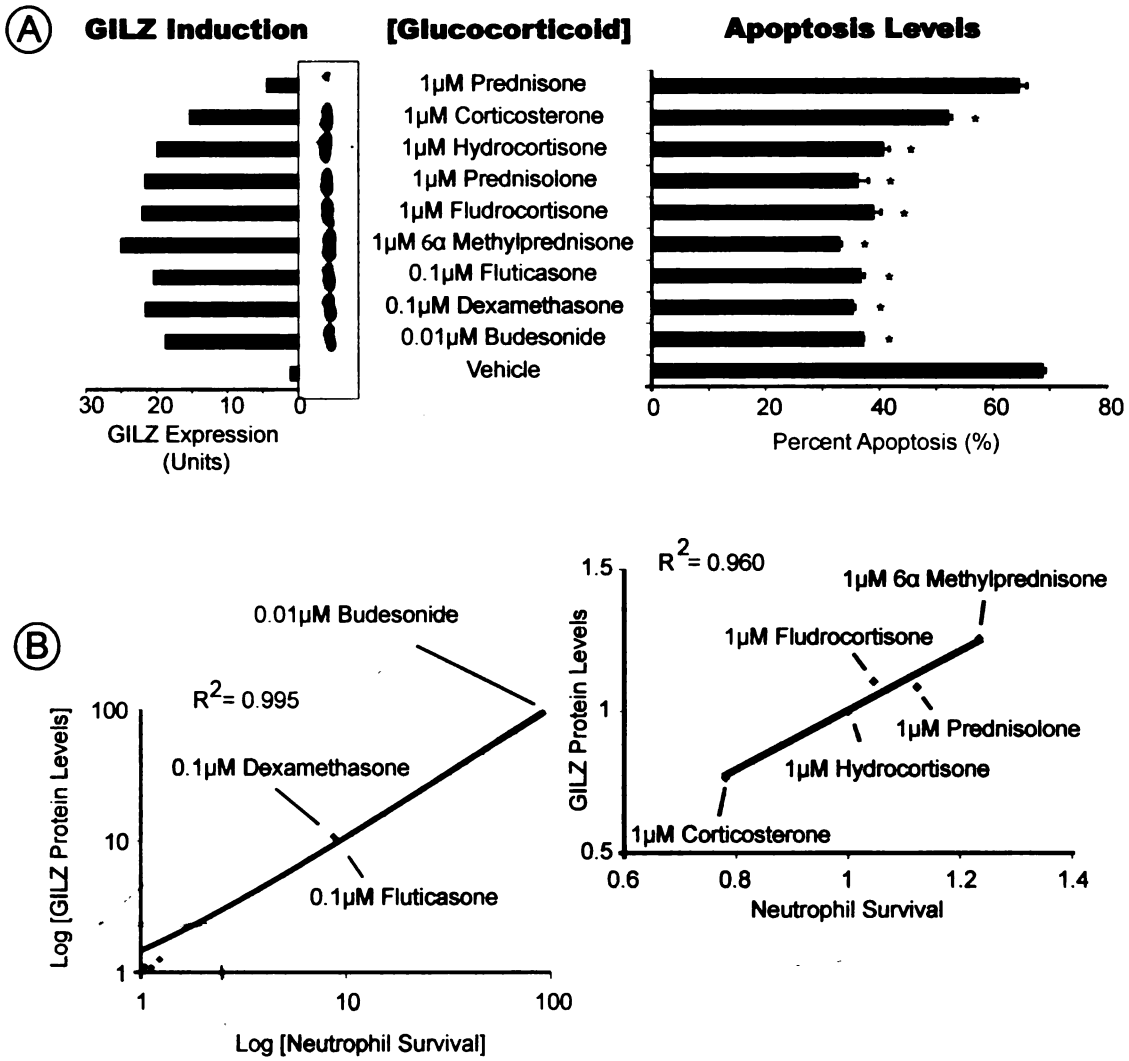

Figure 1.8. A Direct Correlation Between GC-Induced GILZ Protein Levels and Neutrophil Survival. (A) Data shown represents both the level of GILZ protein induction and the amount of apoptosis in human neutrophil cultures treated with various types of GCs. Densitometric data was normalized to both Vehicle and β-Actin (not shown). Apoptosis levels were quantitated using merocyanine 540 and flow cytometric analysis (n=2; $p<0.01$). (B) Data resulting from GILZ protein levels and neutrophil survival in response to various GC types was plotted The weaker GC agonists were plotted as a linear inset graph. R-square values were presented as a correlative measure of linearity.

When all GC types were included in a logarithmic plot, an R-square value of 0.9995 was obtained, indicating a strong linear relationship between GILZ levels and survival (**Figure 1.8B**). Owing to the fact that Corticosterone induced normalized neutrophil apoptosis levels to a value less than one, this data point was plotted separately along with 4 other agonists (**Figure 1.8B inset graph**). This linear analysis of the weaker agonists yielded an R-square value of 0.960, hence further supporting a direct relationship between GILZ levels and neutrophil survival.

Functional Analysis of GILZ Using Antisense

Although a relationship between GILZ expression and neutrophil survival was indicated by the experiments conducted thus far, an alternative role for GILZ as a negative regulator of neutrophil survival could not be ruled out. The possibility existed that not all genes induced by GCs in neutrophils would be related to survival, but rather this other class of genes could be involved with regulating neutrophil sensitivity to GCs and other GC-caused effects. To ascertain a more exact role for GILZ in neutrophil apoptosis, an antisense strategy, one of only two molecular tools available for studying gene effects in neutrophils, was employed.

To define a role for GILZ in neutrophil apoptosis, cells were pre-treated with 2.5μM antisense oligonucleotides (or sense) corresponding to nucleotide positions 204 -223 of GILZ mRNA. Following 4 hours of incubation with oligos, GCs were added and neutrophils were incubated for an additional 4 hours. The effect of these oligos on GILZ protein expression was measured via Western analysis. GILZ levels were unaltered, though, when cells were treated with both

DEX and antisense or sense. These results were particularly surprising since the design of the oligonucleotides specifically targeted the GILZ transcript. Treatment of neutrophils with antisense (AS) alone, however, reduced GILZ protein levels by 27% relative to the vehicle control (VEH) and 15% when compared to sense (S) (**Figure 1.9A**). The inability to knockdown GILZ expression beyond the levels of vehicle-treated samples could be related to the effectiveness of the specific AS sequence selected for use in these studies. The effects of GILZ antisense on neutrophil apoptosis were also measured. Despite the modest effects of antisense on GILZ protein levels, treatment of neutrophils with antisense alone reduced apoptosis by 69% relative to the control and 51% when compared to sense only (**Figure 1.9B**). Remarkably, antisense did not significantly alter apoptosis levels of neutrophils treated with DEX when compared with controls. Compared to control, apoptosis levels of neutrophils treated with DEX and oligonucleotide had reduced levels of apoptosis irregardless of the type of oligo used. These data indicated that DEX in combination with oligo (sense or antisense) caused some non-specific effect on neutrophil apoptosis.

Discussion

Neutrophils are the most abundantly produced immune cell found in circulation. Because of this, subtle increases to neutrophil production and/or survival can result in a major boost to overall immune defense. The immunological value of the neutrophil is easily assessed by examining patients born with a congenital neutrophil deficiency (Carlsson and Fasth, 2001). Prior to

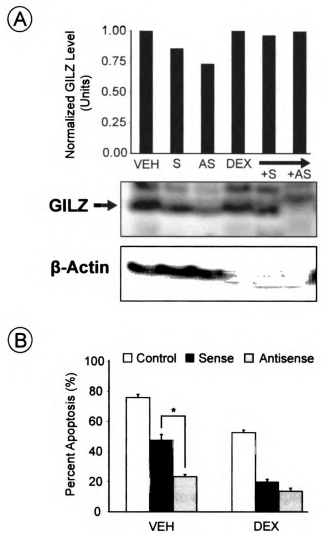

Figure 1.9. GILZ Antisense Inhibits Neutrophil Spontaneous Apoptosis, But Does Not Affect Glucocorticoid-Induced Survival. (A) The ability of GILZ antisense to knockdown GILZ protein expression was monitored using Western blotting. Purified human neutrophils were treated with either vehicle (ethanol; VEH) or 2.5μM GILZ antisense (AS) or sense (S) for 4 hours prior to the addition of 0.1μM dexamethasone (DEX) for an additional 12 hours. The bar graph represents the densitometric ratio of GILZ:β-Actin expression for each sample. Shown values were normalized to VEH which served as a control. (B) The effects of GILZ antisense and sense on neutrophil apoptosis were measured using merocyanine 540 and flow cytometric analysis of viable neutrophils. Results are expressed as the mean for each treatment group (n=3; *$p<0.01$). The replicate shown is representative of three independent experiments.

any therapeutic discovery, these patients had a mean life expectancy of 18mo and often succumbed to lung infections caused by *S. aureus*. It is therefore very clear that these myeloid cells play an irreplaceable role in the daily surveillance against microbes. As demonstrated by our lab and others, GCs promote both granulopoiesis and the survival of neutrophils (Laakko and Fraker, 2002). It is possible, then, that the effects of GCs on neutrophils are the result of an adaptive response by the organism to prepare for stress-accompanying conditions. For example, an increase in neutrophils may be an attempt to compensate for other diminishing defenses such as the compromise of a physical barrier (i.e., epidermis) during a traumatic event. Determining how GCs enable neutrophil survival will be key to understanding the function and adaptation of the immune system during stress conditions.

This paper is the first to show that GCs cause the rapid translocation of GR to the nucleus and the accompanying alteration of a GR-regulated gene in neutrophils. The ability of the GR to alter gene expression either through GREs or interaction with other transcription factors is ligand-dependent. In several studies, GCs were shown to cause the downregulation of GR in neutrophils (Chang et al., 2004; Preisler et al., 2000). While a downregulated GR protein does not necessarily imply complete loss of GR-dependent transcription, it certainly would result in less robust gene expression of GR-sensitive genes. This presents a paradoxical situation since early studies on the effects of GCs on neutrophils indicated that active gene transcription was required for survival (Cox and Austin, 1997). Therefore, the loss of the primary signaling apparatus during

ligand treatment posed an unlikely scenario. Indeed, we found that the GR moves to the nucleus within 15 minutes of GC treatment and peaks at 30 minutes. This experimental design, however, can not rule out that GILZ - given its small molecular weight - may have leaked into the cytoplasmic fractions during cell processing. This seems less likely, however, in light of recent data obtained by Cohen et al who monitored GILZ expression in saponin-permeabilized cells using immunofluorescence (Cohen et al., 2006). Because saponin does not permeabilize the nucleus, any GILZ fluorescent signal would therefore have to come from protein localized in the cytoplasm. Future studies should be conducted to determine if the loss of GR in the cytoplasm is the exclusive result of migration to the nucleus or if a proteasome-dependent degradation process is involved.

Our proposition that an intact GR is required for GC-induced neutrophil survival was bolstered by our discovery of an upregulated GRE-containing gene. GILZ induction in the neutrophil occurs relatively early during GC treatment. Due to the capacity of GILZ to interact with several transcription factors – NF-kB, AP-1, Raf-1 – it is difficult to speculate on what role GILZ may be playing during survival. GCs have been shown to block AICD-induced apoptosis in 3DO T-cells (D'Adamio et al., 1997). It was further discovered that the anti-CD3 treatment of these cells results in substantial production of FasL. Most importantly, overexpression of GILZ in these cells blocks both induction of apoptosis *and* FasL expression. Baumann et al, however, identified the presence of two nGREs in the CD95L promoter thus increasing the complexity by which GCs

regulate FasL expression (Baumann et al., 2005). Similar to other pathways in which GCs interfere (e.g. NF-κB, AP-1), it is likely that GC regulation of FasL occurs via overlapping layers which ensures precise control. Further investigation is required to determine both the significance and mechanism of CD95L down-regulation during GC-mediated survival, especially in neutrophils. Of three types of phagocytes tested, Klebanoff et al determine that neutrophils were the only cell type sensitive to the effects of Fas-stimulated apoptosis (Liles et al., 1996). Moreover, this lab also reported an upregulation of FasL during the progression of neutrophil spontaneous apoptosis. While these data supported a model of paracrinic cell death for neutrophils, when Mifepristone-treated neutrophils were treated with conditioned media from GC-treated neutrophils no further change in neutrophil apoptosis was observed (Cox and Austin, 1997). Since FasL can be expressed as either soluble or membrane-bound forms, the possibility remains that FasL can stimulate apoptosis via cell-to-cell contact. Examining neutrophil apoptosis on a single cell basis would contribute to the better understanding of cell death in these cells. Nevertheless, these data collectively support a role for FasL in the progression of neutrophil apoptosis. It is important to note that Delfino et al reported that GILZ overexpression in thymocytes augments apoptosis (Delfino et al., 2004). This was in contradiction to our hypothesis that the early induction of GILZ in neutrophils represented a first step in the survival signal mediated by GCs. Due to this dichotomy, an effort to test GILZ function was pursued in hopes of clearly defining a role for GILZ in neutrophils.

Several difficulties emerge when attempting to define the role of GILZ in the primary neutrophil. One such barrier which needs to be overcome is that very few molecular tools exist for the study of gene-effects on short-lived cells. Aside from gene deletion or knock-in studies in animals, most gene transfection and transduction techniques require much more time than the short life of the neutrophil will accommodate. Recent papers using the protein transduction domain of HIV TAT linked to a protein under study is one promising approach that requires relatively minimal pre-handling of these cells (Choi et al., 2003). This method has two limitations: (1) it is suitable for small protein domains that can be easily synthesized; and (2) larger stretches of amino acids require the use of prokaryotic expression systems which can result in endotoxin contamination. Indeed, using the same construct that Riccardi et al used, we were unable to depyrogenate a TAT-GILZ peptide preparation sufficiently to be used in our studies. Fortunately, two labs have successfully employed an antisense strategy for the study of Mcl-1 function in neutrophils (Leuenroth et al., 2000; Sivertson et al., 2007). We therefore utilized this method to study the function of GILZ in neutrophils.

Antisense treatment of neutrophils failed to knockdown GILZ protein levels during GC treatment. Similarly, antisense oligos had little effect on the apoptosis levels of DEX-treated neutrophils when compared to sense control. Antisense treatment of unstimulated neutrophils, though, reduced neutrophil apoptosis levels by 50% when compared to sense. The ability of antisense to reduce basal apoptosis levels would ordinarily indicate a pro-apoptotic role for GILZ, but given

the slight reduction in GILZ protein levels in untreated neutrophils, many experimental replicates would be required to assign significance to this finding. The Western results were also confounded both by too few cells used in this protocol and remnant liposomal DOTAP which sedimented alongside cells during centrifugation. It was likely a combination of these factors that resulted in the murky appearance of the developed Western image. Interestingly, DOTAP is generally considered to be a gentle transfection technique, but when neutrophils were incubated with DOTAP alone (i.e., no oligonucleotide), cell viability counts indicated a near complete depletion of cells in these samples. Owing to a host of technical issues, the antisense appeared to be ineffective for the study of GILZ function. This could be the result of incorrect target sequence selection, but all suitable sequences generated by the IDT's "web tool" centered on the sequence used in these studies. Alternatively, antisense could have targeted a different GILZ isoform which shares the same nucleotide sequence.

GILZ is expressed as 4 different splice variants, including 15kDa GILZ1, the focus of the studies presented herein. GILZ3 also shares the same sequence as GILZ1 for which antisense was designed and targeted, but this isoform is 94aa shorter than GILZ1. Consequently, the antisense used in these studies may have targeted the smaller isoform, the effects of which would go unnoticed as the antibody we used principally recognizes the original 15kDa GILZ1 molecule. Therefore, it may be worthwhile in the future to pay careful attention to the array of immunoreactive bands which are developed during probing for GILZ1. Additionally, characterization of the various isoforms expressed by neutrophils

might provide further insight into the function of these molecules. This may be of special importance as the different isoforms of GILZ each possess unique abilities (e.g., GILZ1 simulates Na^+ current, but GILZ3 does not).

An enormous gap remains in our understanding of the role of GILZ in neutrophil survival. Although both GILZ mRNA and protein are induced by GCs via activated GR, we were not able to knockdown expression using antisense specific to GILZ. Other approaches which should be considered such as tethering a domain of GILZ responsible for protein-protein interactions to a cluster of positively-charged amino acids (e.g., aspartate) thus effectively creating a protein transduction construct. One short-coming in this approach is the recent discovery that GILZ interacts with NF-κB at a C-terminal site distinct from other GILZ partner proteins such as AP-1 (Di Marco et al., 2007) (see Introduction Figure 9). Therefore, two separate constructs would have to be synthesized to ascertain the relative importance of each respective domain in a cellular context.

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CHAPTER 2

MICROARRAY ANALYSIS OF APOPTOSIS-RELATED GENES IN GC-TREATED NEUTROPHILS

By

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ABSTRACT

Microarrays are now widely employed to swiftly analyze global expression patterns across a wide variety of cell types and systems. These gene expression tools are being increasingly used for the study of the neutrophil, despite the low abundance of mRNA and static transcriptional status of this cell type. Using a commercial microarray consisting of 350 genes, we sought to establish a pro-survival gene expression profile for neutrophils treated with GCs. Since several Bcl-2 family members including A1 and Mcl1 have been shown to be important for LPS- and cytokine-induced survival of neutrophils, we hypothesized that GCs would cause significant shifts in the expression of these genes. Contrary to our hypothesis, a majority of the genes altered in response to GCs were related to cell signaling, cytokine signaling and neutrophil recruitment. Microarray analysis of neutrophils treated with 0.1μM DEX identified the upregulation of 16 genes, 14 of which were validated using real-time PCR. This included a 24-fold increase in ILR18RAP, a component of the IL-18 receptor, as well as a 9.7-fold increase in Cyclin D3. An additional 9 genes were identified by microarray to be downregulated in response to DEX including a 5-fold reduction in the cytokine IL1β and a 3.3-fold decrease in the chemokine GRO1. Unlike other neutrophil survival pathways, GCs did not cause large shifts in Bcl-2 family members. Moreover, changes in the expression of groups of genes including IκBα and IκBε suggested complex regulation of signaling pathways in response to GCs. The data described herein represents the first application of microarray technology to the study of the effects of GCs on human neutrophil gene expression.

Introduction

Granulocytes are unique among immune cells in that they transit from the bone marrow to the blood supply terminally differentiated replete with a fully mature repertoire of defenses. In contrast, other cell types such as B-cells, T-cells and dendritic cells must further mature at distinct sites beyond the boundaries of the bone marrow. The differentiation status of neutrophils along with lower mRNA levels for this cell type has given rise to the widely held view that neutrophils have limited transcriptional capacity. This conventional wisdom owes not only to the fully differentiated status of this cell, but also to the hypersegmentation of the neutrophil nucleus. Epigenetic regulation - molecular modification of chromatin landscape - has been shown to be a critical determinant of gene expression. Hence, the hypercondensed state of neutrophil DNA was thought to exclude RNA polymerase machinery which would otherwise allow for a robust transcriptional response. The structure of neutrophil chromatin likely represents the major reason for lower levels of mRNA found in this cell type.

Neutrophils engage bacteria and other microorganisms via the receptor-mediated process of phagocytosis. Despite the involvement of several proteins in this process, phagocytosis occurs without the need of additional RNA synthesis. This is probably due to the microgram quantities of preformed antimicrobial proteins such as myeloperoxidase and defensin already present in lysosomal vacuoles. In fact, differentiation stages of granulocytes are often defined by the presence of a particular granule protein (e.g., elastase, gelatinase,

etc.). It seems likely, then, that the majority of gene transcription and protein synthesis occurs in immature neutrophils. This is further evidenced by the relative lack of ribosomes found in these cells. Despite the transcriptional inactivity of mature neutrophils, recent studies have cast new light on the actual capacity of these cells to regulate mRNA expression, especially in response to pathogens. As will be described in the following section, the microarray has been of particular importance in revising the designation of the neutrophil as a transcriptionally stunted cell type.

Microarray Technology Applied to Neutrophils

The microarray is a molecular tool that has been successfully utilized by life scientists to analyze gene expression since the mid-80s. What originally began as cDNA spotted on a glass slide has now morphed into tens of thousands of spots each containing nano-quantities of oligonucleotides (oligos) spotted onto a variety of substrates including nylon and silicon. These tools have enabled researchers to quickly assay most genes expressed by an organism in a short period of time in an effort to measure differential gene expression. One of the largest microarrays, the human genome array, contains oligo spots corresponding to approximately 40,000 genes. Owing to a dearth of mRNA in neutrophils, most researchers scoffed at the idea of utilizing such a large scale gene expression tool for the study of these cells (Newburger et al., 2000).

Departing from conventional wisdom, however, Itoh et al endeavored to construct a cDNA library based on the mRNA present in freshly isolated granulocytes (Itoh et al., 1998). Sequencing of these cloned cDNA revealed that

neutrophils were found to express 748 transcripts with about 10% of these genes comprising cell surface proteins. Genes corresponding to secretory proteins, DNA-binding proteins as well as signal transduction pathways were also moderately expressed in neutrophils. By contrast, genes involved in energy production, lysosomal proteins, protein synthesis and structural proteins (cytoskeleton) were found in least abundance. While these data serve to bolster existing views of neutrophils such as the concept of preformed lysosomal proteins and the minimal role for protein translation machinery; the finding that several classes of genes are active in neutrophils suggests that these cells may be able to utilize a genetic program to respond to the dynamic conditions of host defenses. Of particular interest is the level of activity of signal transduction genes which likely play a prominent role in the neutrophil survival response. While expression libraries are immensely useful open architecture tool for studying moderate to high abundance transcripts, they lack the sensitivity of the microarray to detect low copy number transcripts.

Suzuki et al was one of the first laboratories to apply microarray technology to study gene expression in neutrophils (Suzuki et al., 2002). This group was particularly interested in determining genes responsive to granulocyte colony stimulating factor (G-CSF) treatment. Of 9000 genes assayed by microarray, this group identified the upregulation of 172 transcripts in human neutrophils treated with G-CSF. Interestingly, downregulated genes were not reported in this brief communication. Nevertheless, these data indicated that microarray techniques would be a useful tool for identifying genes of interest for a particular pathway.

The application of microarray to studying neutrophil apoptosis is hampered by the indiscriminate degradation of mRNAs during the execution of the apoptotic program. Indeed, our lab and others have detected decreases in both genes of interest as well as housekeeping genes in apoptotic populations (unpublished). This would therefore complicate any findings regarding modest changes in gene expression since it would be difficult to tease apart effects due to apoptosis versus those caused by a select agent (e.g., G-CSF or GCs). One approach to circumnavigating this difficulty is to analyze early stages of gene induction which would precede any non-specific degradation of mRNAs caused by apoptosis. This method ensures that all transcripts remain mostly intact thus increasing the probability of identifying specific effects due to treatment.

Effects of LPS on Neutrophils:

O'Neill et al published one of the first studies to examine apoptotic pathways in neutrophils using microarrays . Using an Affymatrix Gene Chip containing sequences corresponding to approximately 12,600 genes, O'Neill et al analyzed the impact of LPS on neutrophil gene expression (O'Neill et al., 2004b). Both cIAP1 and cIAP2 were found to be elevated in neutrophils treated with LPS from 1-4hrs. Microarrays performed using neutrophil mRNA derived from patients experiencing sepsis – the presence of bacteria in the blood stream – showed decreases in both of these genes. The discrepancy between *in vivo* and *ex vivo* results is likely caused by the activation of a whole host of inflammatory signaling pathways in the case of sepsis. Nevertheless, the observed decrease in cIAP family members in septic patients suggests that these genes may not play as

large a role as previously thought in LPS-stimulated neutrophil survival. In a separate publication, this same group also reported increases for both Cox-2 as well as NF-κB (O'Neill et al., 2004a). In a separate report, another group of researchers reported that of 4608 genes assayed, 28 were found to be induced in neutrophils after 4hr of treatment with LPS (Malcolm et al., 2003). These researchers also identified increases in NF-kB, as well as several chemokines and cytokines and, most notably, the Bcl-2 family member A1. An increase (2.5-fold) in A1 expression in neutrophils represents at least one pathway through which LPS signals survival in these cells. In addition to LPS, other survival cues have been studied using a microarray approach to detect changes in gene expression.

Effects of GM-CSF on Neutrophils:

Kobayashi et al utilized a 12,500 gene microarray to study the effects of granulocyte-macrophage colony stimulating factor (GM-CSF) on neutrophil gene expression (Kobayashi and DeLeo, 2003). GM-CSF, like many other pro-inflammatory mediators, reduces neutrophil apoptosis during routine culture. These researchers cultured neutrophils for 24hrs with or without GM-CSF. The duration of these cultures, however, represents one of the major criticisms of this study. After 24hrs in culture, most neutrophils are already apoptotic and therefore will exhibit non-specific degradation of mRNAs. Since housekeeping genes such as ß-Actin and GAPDH are also lost as a result of apoptosis, it would be virtually impossible to accurately normalize expression levels for actual genes of interest using these or any other reference

genes. While this experimental flaw would hinder the analysis of modest changes in gene expression, it does not impair the detection or validity of major shifts in transcript levels. Indeed, these researchers identified hundreds of genes that were altered in response to GM-CSF treatment. These data were validated not only using real-time PCR, but also by analyzing the expression of corresponding proteins including several surface markers (CD14, HLA-DR, CD24, CD66) as well as serum- and glucocorticoid-inducible protein kinase (SGK). Moreover, the microarray results obtained in this study helped establish a prominent role for SGK in neutrophils treated with GM-CSF. SGK has been shown to be both regulated by GCs and to control the survival fate of certain cell types including breast epithelial cells (Wu et al., 2004). Given the role that GCs play in SGK regulation, it was surprising to learn that significant changes in SGK expression were not detected using microarrays to study GC-treated neutrophils.

Effects of GCs on Bovine Neutrophils:

As discussed previously, GCs increase the survival of neutrophils both in-vivo ad ex-vivo. In order to assess global changes in gene expression of GC-treated neutrophils, Madsen et al treated bovine neutrophils with either (a) 0.1uM Dexamethasone (DEX); (b) the GR antagonist mifepristone; or (c) both DEX and Mifepristone (Madsen et al., 2002). This experimental design not only enabled these researchers to identify genes regulated by DEX, but it also increased the probability of identifying those genes involved in survival by comparing neutrophils treated with DEX versus those treated with both DEX and MP. Since MP blocks GC-mediated neutrophil survival, genes that are altered in both DEX

and DEX+MP samples are excluded from further analysis owing to a reduced likelihood of playing a major role in survival. Of 18,263 genes assayed, this group identified 502 genes that were differentially regulated by DEX. This list of genes was further reduced to 141 genes when DEX+MP genes were subtracted from DEX alone thus highlighting those genes that are likely involved in GC-mediated survival. Of these 141 genes, the authors chose to validate 14 genes including apoptosis-related genes Caspase 8 and TFAR-19 as well as the tissue remodeling enzyme MMP-9. While these data represent the first global analysis of genes modulated by GCs, it still remained to be determined if these same gene subsets are regulated in human cells as well. Moreover, the fact that only MMP-9 was validated at the protein level highlights one of the major issues concerning the use of microarrays. Indeed, many elaborate pathways are constructed based solely on changes in gene expression. These ideas can be challenged, however, based on the poor correlation between gene and protein expression.

Discordance Between Gene and Protein Levels:

One of the assumptions of many genomic studies is that changes in gene expression infer corresponding changes in protein levels. While such a relationship has been shown to exist for several genes, recent reports have demonstrated that this may in fact be the exception to the rule. Using *S. cerrevisiae* grown in log phase, Gygi et al focused on 150 protein spots resolved by 2-D gel electrophoresis and identified by mass spectrometry (Gygi et al., 1999). These researchers determined that they were unable to predict protein

expression based on SAGE-derived quantitative mRNA levels. For example, some genes had corresponding mRNA levels that remained constant while the protein levels varied by more than 20-fold. On the other hand, certain proteins whose levels remained at steady-state had more than 30-fold changes in corresponding mRNA expression. Other studies have also demonstrated that a disparity between gene and protein expression exists in certain animal cell types as well.

Chen et al analyzed the expression of 98 genes corresponding to 165 protein spots derived from 76 human adenocarcinoma and 9 non-neoplastic lung tissue samples (Chen et al., 2002). Oligonucleotide microarrays were used to analyze the expression of these 98 genes in order to correlate gene expression with protein levels. Of the 165 protein spots assayed, only 28 (17%) exhibited a significant correlation between gene and protein expression. Furthermore, a statistically significant correlation between all mRNA correlation coefficients and protein levels could not be established for these samples. These data demonstrate that with the exception of a small subset of genes, a discrepancy exists between mRNA and protein levels in these human cells. While a preponderance of evidence suggests that mRNA should not be used as a predictive tool for protein changes, a recent study examining gene and protein levels of 4 organelles in 6 different tissues from the mouse suggested otherwise.

In one of the most exhaustive studies concerning the concordance between gene and protein levels, Kiplinger et al quantitated 1700 proteins contained in specific organelles derived from murine brain, heart, kidney, liver, lung and

placenta tissues and then compared these levels with corresponding microarray-quantitated mRNA levels. Interestingly, this group decided to classify correlation levels into three categories: (1) inliers (409 genes; avg. p-score: 0.81; avg. p-value: 0.1); (2) midliers (846 genes; avg. p-score: 0.44; avg. p-value: 0.32); and (3) outliers (503 genes; avg. p-score: 0.00; p-value: 0.43). By suggesting that both inliers as well as midliers met statistical significance, these authors concluded that of the 1758 genes assayed, 1255 (71%) exhibited correlation between gene and protein levels.

It is evident then that in certain cases, gene expression might indeed be reflected by protein expression. The fact that such a correlation does not always exist necessitates the assessment of protein levels for those genes identified by microarray detection. While this extra level of investigation would seem to be contrary to the high throughput potential of the microarray, it is labor-intensive and possibly counterproductive to construct pathways for proteins that may in fact not be altered in expression; that is, if they are expressed at all!

Given that several pro-survival cues for neutrophils alter the expression of Bcl-2 family members preferentially expressed in myeloid cells (e.g., A1, Mcl1, etc), we hypothesized that GCs also affect the expression level(s) of these genes. This would then manifest in microarray analysis of gene expression as a positive shift in the ratio of anti-apoptotic:pro-apoptotic Bcl-2 family expression. We therefore employed a pathway-specific microarray containing approximately 350 genes related to apoptosis in an effort test our hypothesis. Furthermore, we

used relative real-time PCR (sqPCR) to further assess those genes that were positively identified by microarray analysis.

Methods

Reagents

Becton Dickinson (BD) Vacutainer Tubes containing Acid Citrate Dextrose Solution A and 21G1½ needles were used for drawing blood (BD Vacutainer System, Franklin Lakes, NJ). Dextran T-500 and Percoll, were obtained from GE Biosciences (Piscataway, NJ). RPMI-1640 Media, Iscove's Media, 100x Penicillin-Streptomycin, 100x L-Glutamine, Acridine Orange, Propidium Iodide (PI), TRIZOL, Superscript II or III and dNTPs, SYBR Green (1000x) were all obtained from Invitrogen (Carlsbad, CA). Fetal Bovine Serum was purchased from Hyclone (Logan, UT). All other reagents including Dexamethasone and hydrocortisone were from Sigma-Aldrich (St. Louis, MO). SYBR Green Core Reagent Kits and PCR plates were obtained from Applied Biosystems, Inc. (Foster City, CA). All primers were synthesized by MSU RTSF Oligonucleotide Synthesis Facility (E. Lansing, MI).

Neutrophil Isolation

Peripheral blood neutrophils were harvested from healthy adult donors (ages 18-30) following the submission of donor consent in accordance with Michigan State University Human Use Committee guidelines. Neutrophils were purified as described previously, with minor modifications to the protocol (Haslett et al., 1985). Briefly, 34ml of anti-coagulated blood was mixed with 17ml of 3% Dextran in 0.85% NaCl to deplete red blood cells (RBCs). The RBC-depleted cells were

then resuspended in 2ml of autologous platelet poor plasma which was obtained from donor serum supernatant following centrifugation at 5000xg. Next, leukocyte subsets were resolved on a discontinuous Percoll gradient comprising two layers of 55% and 64% Percoll in 0.85% NaCl. The granulocytes were then harvested from the 55%-64% interphase and washed in Hanks' Balanced Salts Solution (HBSS; minus Ca^{++} and Mg^{++}). This method routinely yielded preparations with >98% viability as determined with Trypan Blue. Neutrophil purity was routinely assessed to be 96 – 98% based on nuclear morphology via Acridine Orange staining.

Microarray Analysis

For microarray analysis of gene expression, purified peripheral blood neutrophils were cultured with either vehicle (Ethanol) or Dexamethasone (0.1μM DEX) in RPMI containing 2% heat-inactivated Charcoal-Dextran-depleted FBS serum for 3 hours. Samples were then centrifuged, washed once in ice-cold PBS and finally resuspended in an appropriate amount of TRIZOL (Invitrogen). The TRIZOL was then flash-frozen and the samples were shipped to the Miltenyi corporation (Auburn, CA) which performed the microarray analysis. Prior to sending the samples, we performed relative real time PCR (sqPCR) on both GILZ and IκBα in order to verify GC-treatment and to monitor the sensitivity of the array since IκBα is located on the apoptosis microarray and should be induced at the 3 hour time point. At Miltenyi, fluorescent cDNA was synthesized from purified RNA of both the vehicle (Cy3) and DEX (Cy5) samples. The samples were then hybridized to

the microarray for approximately 6 hours following which the glass slide was washed and scanned using a microarray scanner.

Real Time PCR

Total RNA was extracted from $1x10^7$ neutrophils using TRIZOL extraction reagent. 1μg of total RNA was combined with 1ug of anchored oligo $d(T)_{18}VN$ to synthesize cDNA template using Superscipt Reverse Transcriptase. Relative real time PCR was conducted on 0.5µl of cDNA product using ABI Core Reagents in 25µl total volumes. A list of the primers used in these studies is printed in **Table 2.1**. Primer efficiency for each primer set within the range of analysis was estimated to be >90% as estimated by an amplicon-titrated standard curve. Analysis of real time data was performed using the $-\Delta\Delta C_T$ method of comparison. Data points for the genes of interest were normalized to respective β-actin levels.

Validation of a given gene was determined by the correlation of real-time PCR results with the microarray hits. Significant genes as identified by microarray were defined as 2.0-fold or higher. Similarly, genes were determined to be validated if real time results at any point during a 1-8hr time course also exceeded the 2.0-fold threshold in the correct direction for a given gene.

Results and Gene Descriptions

RNA Quality and Microarray Analysis of GC-Treated Neutrophils:

Figure 2.1A is the digitized 28S:18S analysis of RNA quality for the samples used in these studies. The quality of the purified RNA was analyzed with an Agilent Bioanalyzer prior to microarray analysis. Digital analysis of the resulting

GENE	FORWARD PRIMER	REVERSE PRIMER
ILR18RAP	ATTCCGCATCACATAAGCAA	TCCACAGAGAGGAGTTTTCCA
CCND3	ATTTCCTGGCCTTCATTCTG	CGGGTACATGGCAAAGGT
IL1R2	GCGCTTGTACGTGTTGGTAA	CTGAACTCCCGCTTGTAATG
TLR2	TGATGCTGCCATTCTCATTC	GCCACTCCAGGTAGGTCTTG
CUGBP2	TGCAGATGTTCATGCCTTTT	AGCTTGGATAGCAGCTTGTG
CD30L	TCATGGGCCTACCTCCAA	GATCACCAGATTCCCATCCT
STK17B	TGATCCCATTACCACAGCAA	TTTCTTGATTATCTTCTCCCACAA
MCL1	GCATCGAACCATTAGCAGAA	CATGGAAGAACTCCACAAACC
IKBA	TACGAGCAGATGGTCAAGGA	TCATGGATGATGGCCAAGT
FOS1	CTGGCGTTGTGAAGACCAT	ATTCCTTTCCCTTCGGATTC
IL18R1	CACTGGTCAACAGCACATCA	GGAAATGCACGCAGGAGTA
IL1B	CTCCAGGGACAGGATATGGA	ACACGCAGGACAGGTACAGA
GRO1	CATCCAAAGTGTGAACGTGAA	GATGCAGGATTGAGGCAAG
CD14	GCTGGAACAGGTGCCTAAAG	CCCGTCCAGTGTCAGGTTAT
P53	CAAGCAGTCACAGCACATGA	CCAAATACTCCACACGCAAA
BID	TGGGAGGGCTACGATGAG	CCGGATGATGTCTTCTTGAC
IKBE	ATGGGCATCTCATCCACTCT	ATCAAAGGGCAAAAGGACAA
TNFC	GAGGAGGAGCCAGAAACAGA	GTCCCGCTCGTCAGAAAC

Table 2.1. List of Primers Used in Real-Time Validation Studies

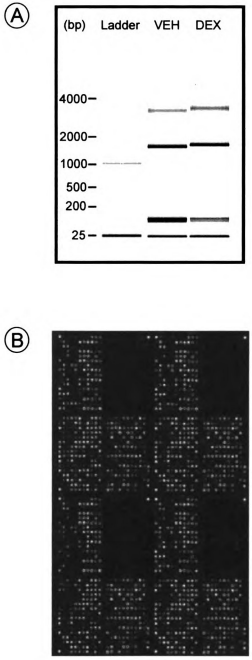

Figure 2.1. Microarray Analysis of GC-treated Neutrophils. (A) Digitized electrophoretogram showing the distribution of RNA in neutrophils treated with either vehicle (VEH) or 0.1μM Dexamethasone (DEX). **(B)** The false color depiction of the scanned microarray comparing the expression profile of neutrophils treated with Vehicle (Cy3; green) or 0.1μM DEX (Cy5; red) for 3hrs.

electrophoretogram indicated RNA Integrity Number (RIN) values for these samples ranged between 7.9 and 8.6. Since the RIN values for both treatment groups was >6, these samples were of sufficient quality to be labeled and used for microarray analysis of gene expression. The microarray experiment is seen in **Figure 2.1B** which represents the false-color scan of the microarray containing Cy5-labeled DEX cDNA and Cy3-labeled VEH cDNA. OD intensities for each treatment group – VEH and DEX – were captured and images were overlayed to reveal ratios for individual spots located on the array.

(A) Upregulated Genes Related to Survival (Table 2.2)

For this section, a brief description of each gene and its relationship to GC signaling is provided along with the results. Relationships between genes are inferred later in the discussion.

ILR18RAP

Of the 16 genes identified by the microarray to be upregulated in response to DEX, Interleukin-18 Receptor Accessory Protein-like (ILR18RAP or IL18Rb) showed the strongest induction at 23.9-fold (**Table 2.2**). sqPCR analysis of ILR18RAP expression confirmed that DEX increases levels of this gene by 3.5-fold at 2hrs and up to 32-fold by 8hrs (**Figure 2.2**). Similarly, HC caused ILR18RAP levels to increase approximately 3-fold by 2hrs and up to 14-fold by 8hrs.

ILR18RAP, along with IL18Ra and CD48, form the GPI-anchored signaling complex responsive to IL18 ligand (Fukushima et al., 2005). IL18 is a proinflammatory cytokine that induces IFNγ production in T-cells and

GENE	qPCR Validation (Hrs) 2	4	8	Microarray Results (3Hrs)
ILR18RAP	3.5	15	32	24
CCND3	3.5	9.3	19	9.7
IL1R2	6.6	7.4	5.1	7.5
TLR2	2.6	2.8	3.3	4.1
CUGBP2	2.9	3.3	3.6	3.4
CD30L	7.0	15	24	3.3
STK17B	3.4	3.8	3.1	3.1
MCL1	1.4	2.0	1.8	2.5
IKBA	2.0	0.6	0.4	2.3
FOS1	2.4	3.7	4.3	2.3
IL18R1	6.0	34	92	2.0

TABLE 2.2. Comparison of Microarray Data and Real-Time Data for Genes Upregulated in Neutrophils by Dexamethasone

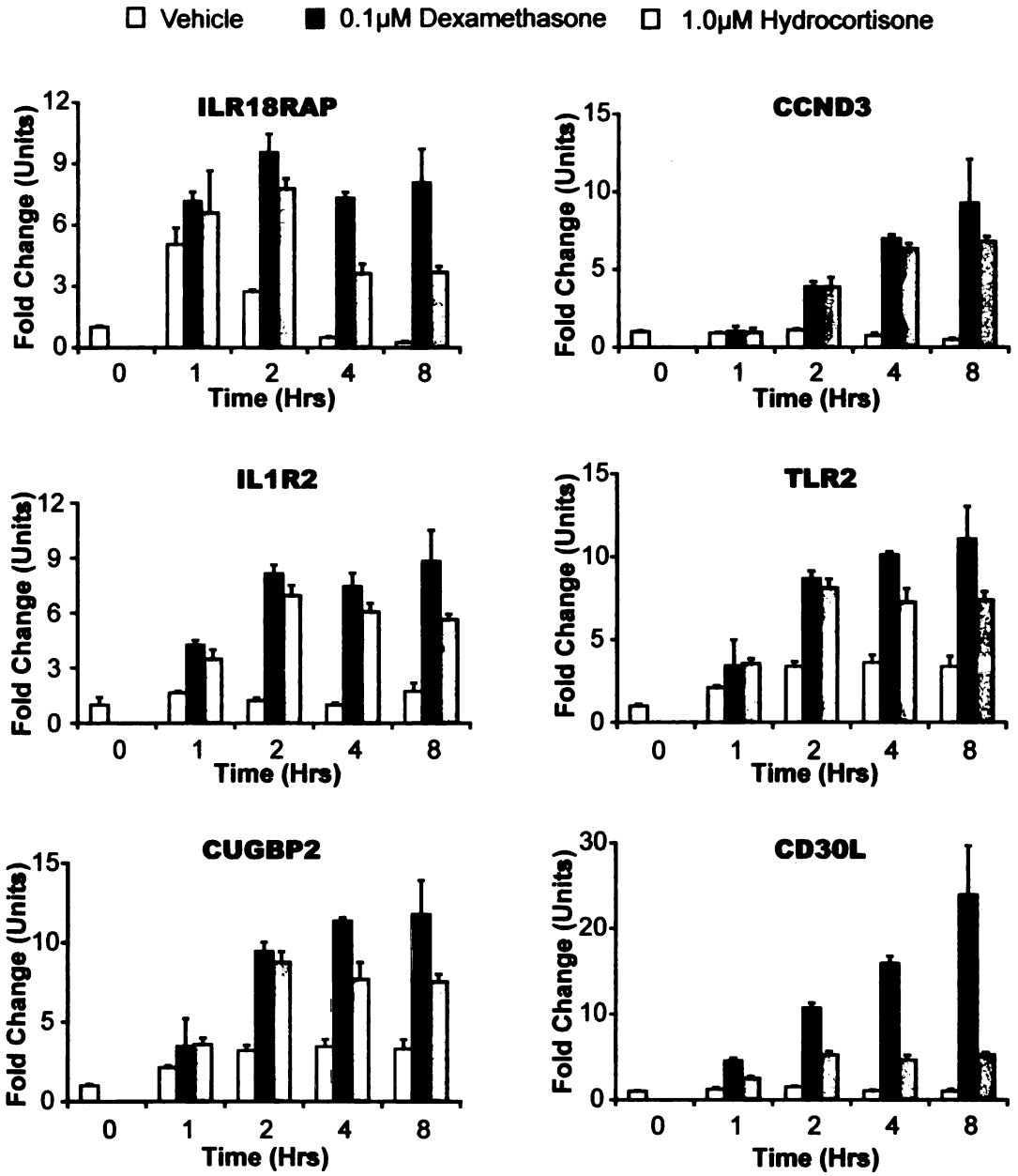

Figure 2.2. Validation of Genes Identified by Microarray Analysis to be Upregulated by GCs. Genes shown in this figure are those that were classified as signficant (>2.0-fold) and were successfully validated using sqPCR. The bar graphs depicted here represent the gene expression levels of those eleven genes positively identified to increase in response to GCs. Each cDNA sample was normalized to β-Actin to correct for loading and/or treatment discrepancies. Standard error is also shown for individual data points (n=3).

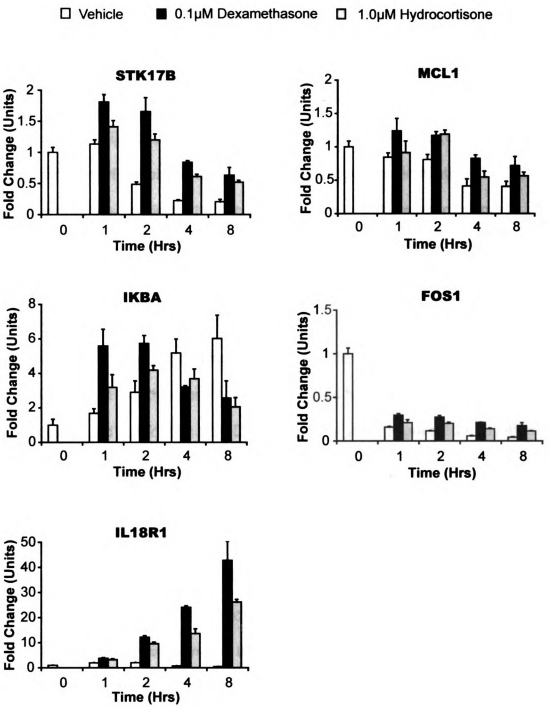

Figure 2.2 *(continued)*. **Validation of Genes Identified by Microarray Analysis to be Upregulated by GCs.**

has been implicated as a fundamental feature of a variety of autoimmune diseases including rheumatoid arthritis (Boraschi and Dinarello, 2006). Although a component of the IL18 receptor complex, this protein does not physically interact with or bind IL18. Leung et al determined that neutrophils treated with IL18 produced significant quantities of the IL8 chemokine. Interestingly, treatment with DEX ablated IL18-induced IL8 production, indicating that GCs interfere with the abilities of IL18 to transduce a pro-inflammatory signal in neutrophils.

CCND3

Microarray analysis indicated that Cyclin D3 (CCND3) was the second highest induced genes of those identified (9.7-fold) (**Table 2.2**). PCR validation of these results indicated that this gene is indeed induced by GCs in these cells. CCND3 was upregulated 3.5-fold by both DEX and HC at 2hrs (**Figure 2.2**). By 4hrs, this gene was increased 9.3-fold and 8.4-fold with DEX and HC, respectively. CCND3 levels continued to rise by 8hrs in DEX- (~19-fold) and HC-treated (~14-fold) samples. These data confirm that CCND3 is indeed upregulated in neutrophils treated with GCs.

CCND3 forms a complex with cyclin dependent kinases 4 or 6 and is required for cell cycle progression through the G2 phase (Gabrielli et al., 1999). Of particular relevance to these studies was the discovery that loss of CCND3 expression resulted in a collapse of the granulocytic compartment of the bone marrow and reduced circulating neutrophil numbers in the blood (Sicinska et al., 2006). This study in particular demonstrates an essential role for Cyclin D3 in

promoting granulopoiesis *in vivo*. Moreover, an increase in the expression of CCND3 caused by GCs likely represents at least one mechanism by which these compounds increase granulopoiesis *in vivo*. The preferential expression of CCND3 protein in immature cell types has also been observed in lymphoid and proliferating epithelial cells, further indicating a role for this protein during various maturation processes including granulopoiesis (Norris et al., 2005). Further supporting a role for this protein in cell survival, increased Cyclin D3 immunoreactivity has been shown to be a prognosticator for both laryngeal squamous cell carcinoma mortality as well as colorectal cancer metastasis (Pruneri et al., 2005) (Tanami et al., 2005). Consistent with a tumor-promoting role, Cyclin $D3^{-/-}$ mice which exhibit dramatically reduced T-cell development are especially resistant to tumor formation caused by various oncogenic stimuli (Sicinska et al., 2003). Of particular relevance to the studies described herein, nuclear Cyclin D3 protein levels in gastrointestinal tumor cells were shown to be independent of CCND3 mRNA thus highlighting a disconnect between protein and mRNA levels for this particular gene (Pruneri et al., 2003). While the preponderance of evidence suggests a pro-survival role for CCND3, especially in the case of tumor cells, the relationship between CCND3 gene induction and corresponding shifts in protein levels appears to be inconsistent. In contrast to neutrophils, a myeloid cell type, GCs have been shown to reduce CCND3 mRNA levels in lymphoid cells.

CCND3 is especially important in the development of pre-B cells as loss of this gene resulted in a 66% reduction in this developmental stage (Cooper et al.,

2006). Given that GCs have divergent activities on various immune cell types - i.e., GCs cause survival in neutrophils, but death in developing lymphoid cells - it is therefore not all that surprising that GCs would also have differing effects on gene expression in these respective cell types. It remains to be determined, however, whether CCND3 plays a critical role in the GC-mediated survival of mature neutrophils. One approach to resolving this matter is to utilize bone marrow-derived neutrophils purified from CCND3$^{-/-}$ mice. A relationship between Cyclin D3 and neutrophil survival could be resolved by observing the protective effects of GCs on neutrophils derived from these knockout animals. If indeed an upregulation in CCND3 expression participates in the survival response, then samples using neutrophils from knockout animals should display a reduced protective effect caused by GCs.

IL1R2

Interleukin-1 Receptor 2 (IL1R2) was induced 7.5-fold as assessed using microarray analysis of neutrophils treated with DEX for 3hrs (**Table 2.2**). sqPCR analysis, however, revealed even higher levels of induction of this gene in cells treated with either DEX or HC. DEX induced a 6.6-fold increase in this gene at 2hrs whereas HC induced a 5.7-fold elevation at the same time point (**Figure 2.2**). Induction of this gene using either DEX or HC was maximal at 4hrs (7.4 and 6.1, respectively). By 8hrs, however, inductions levels had waned to 5.1-fold with DEX and 3.3-fold with HC.

IL1R2 is decoy receptor that binds the cognate proinflammatory cytokines IL1α and IL1β as well as the IL1 receptor, IL1R1 (Colotta et al., 1993). While

IL1R2 has been shown to mediate the formation of the ligand/receptor signaling complex, it does not participate in signaling in this pathway (Malinowsky et al., 1998). Bourke et al demonstrated that neutrophils in particular scavenged IL1b using IL1R2 thereby effectively neutralizing inflammation caused by this cytokine (Bourke et al., 2003). While GCs had not been shown to directly induce IL1R2 expression, elevated levels of this protein *were* associated with septic patients treated with GCs (Ehrchen et al., 2007).

TLR2

Despite the robust 4.1-fold increase in TLR2 expression observed using the microarray, sqPCR analysis of this gene revealed more modest changes (**Table 2.2**). For example, at 2hr DEX and HC caused only 2.6- and 2.4-fold increases in TLR2 expression (**Figure 2.2**). Expression of this gene increased to 2.8-fold in DEX-treated samples by 4hrs and actually decreased to 2.0-fold in the HC samples. By 8hrs, expression remained statistically unchanged from that of the 4hrs time point.

TLR2 belongs to the Toll-like Receptor family whose members possess domains which recognize pathogen-associated molecular patterns (PAMPs). TLR2, in particular, recognizes a number of lipoproteins including lipotechoic acid derived from gram (+) bacteria. Additionally, TLR2 has been shown to bind to extracellular Hsp70 (a chaperokine) which is especially elevated in response to stressful stimuli (Asea et al., 2002). Treatment of neutrophils with the TLR2 agonist peptidoglycan was shown to be nearly equal to LPS in anti-apoptotic potency (Francois et al., 2005). Moreover, ROS-stimulated

NF-κB activation in cardiac myocytes was demonstrated to require TLR2 using anti-TLR2 antibodies (Frantz et al., 2001). A prominent role for TLR2 in mediating signals caused by reactive oxygen species was also described for neutrophils. Using $TLR2^{-/-}$ mice, Williams et al showed a reduction in IL-6 chemoattractant expression in the lung as well as a delay in neutrophil recruitment to this tissue (Lin et al., 1999). Further supporting a role for TLR2 as a stress-sensor was a 47% reduction in GC production observed in $TLR2^{-/-}$ mice treated with a TLR2-specific agonist (Bornstein et al., 2004). GCs themselves have been shown to induce TLR2 mRNA expression in HeLa cells (Shuto et al., 2002). DEX alone was shown to cause a modest 2-fold increase in the expression of this gene whereas treatment with a *Hemophilus influenzae* extract resulted in a 5-fold increase in TLR2 expression. Co-treatment with both DEX *and* the bacteria extract, however, resulted in a sizeable 15-fold increase in TLR2 expression thus demonstrating a synergistic effect.

A cooperative effect on TLR2 expression was also observed using DEX and TNFα (Hermoso et al., 2004). While Hermoso et al identified a GRE-like sequence that was required for the induction of TLR2 by both DEX and TNF, this sequence was not sufficient for induction of this gene with DEX alone. GM-CSF, another survival cue for neutrophils, has also been shown to induce TLR2 expression in these myeloid cells (Kurt-Jones et al., 2002). Unlike the TLR4 agonist LPS, selective TLR2 agonists are not nearly as efficacious in delaying neutrophil apoptosis (Sabroe et al., 2003).

<u>CUGBP2</u>

Microarray determination of CUG triplet repeat, RNA binding protein 2 (CUGBP2) levels revealed a 3.4-fold induction of this gene in DEX-treated neutrophils (**Table 2.2**). Validation results were similar for DEX-treated samples with a 3.3-fold induction by 4hrs and a 3.6-fold induction by 8hrs (**Figure 2.2**). Levels of induction for samples treated with the natural GC were less robust, however, as HC-treated samples demonstrated inductions levels that were 40% of the DEX-treated samples. HC caused a 2.6-fold increase in CUGBP2 levels by 2hrs, but inductions levels declined to 1.3-fold at 4hrs and 1.5-fold at 8hrs.

CUGBP2 is a member of the CELF/BRUNOL family of RNA-binding proteins. This protein comprises one member of a RNA editing complex that causes site-specific C -> U deamination of apoplipoprotein B mRNA (Anant et al., 2001). Also, CUGBP2 has been shown to stabilize COX2 mRNA following irradiation, but ironically this protein also impaired the translation of COX2 mRNA to protein (Mukhopadhyay et al., 2003). The expression of CUGBP2 has also been shown to be inhibited by prostaglandins which are formed from arachadonic acid using COX2. This likely represents a negative feedback mechanism in which levels of COX2 are regulated by the actions of this protein. Using microarrays, CUGBP2 was identified to be upregulated in preadipocytes (Bujalska et al., 2006). Given the well-documented effect of GCs on COX2 protein expression, it is likely that GC-mediated induction of CUGBP2 mRNA represents one mechanism through which loss of COX2 protein is accomplished.

TNFSF8

TNFSF8 (CD30L or CD153) was upregulated 3.3-fold as measured using microanalysis of DEX-treated neutrophils (**Table 2.2**). Kinetic analysis of the expression of this gene, however, revealed a more robust response as DEX caused a 7.0-fold induction as early as 2hrs whereas HC induced more a modest 3.5-fold increase in TNFSF8 expression (**Figure 2.2**). By 4hrs, DEX and HC had induced 15-fold and 4.4-fold increases in the expression of TNFSF8. A 24-fold increase in this gene was observed in response to DEX at 8hrs which represented the third largest shift in gene expression in these studies (the other two being ILR18RAP and CCND3). HC, by comparison rose maximally to 5.2-fold by 8hrs.

TNFSF8 (CD30L) has been shown to induce apoptosis in a variety of cell types including a $CD30^+$ tumor cells (Younes et al., 1997) as well as the close relative of the neutrophil, the eosinophil (Berro et al., 2004). The cognate receptor for this ligand, CD30, is expressed mainly on lymphocytes and plays an important role in the negative selection of autoreactive T-cells (Agrawal et al., 1996). CD30L is among the growing list of proteins that are secreted by neutrophils in response to various stimuli (Gabrilovich, 2004). In neutrophils, CD30 stimulation via crosslinking of CD30L using mAbs resulted in an increase in both IL8 and reactive oxygen production even though CD30 itself was undetectable in these cells using flow cytometry (Wiley et al., 1996). Although direct evidence is sorely lacking, it is important to note that most agents which promote chemokine/cytokine secretion and/or ROS production typically induce a survival response in neutrophils. Additionally, the inability to detect CD30 on the

surface of neutrophils is consistent with the expression of other surface proteins including the LPS-receptor (CD14) which is expressed on both secretory and specific granules, but not on the cell surface. Therefore, the absence of CD30 as detected by FACS should be confirmed using other techniques such as Westerns or intracellular labeling. If indeed CD30L confers a protective effect on neutrophils, as proposed by this scenario, then enhanced production of this gene would confer protection to a population of neutrophils via paracrinic action. Moreover, production of CD30L along with its deleterious effects on cell types other than neutrophils might also help explain the selectivity by which these agents act in promoting neutrophil survival at the expense of immature lymphocytes and eosinophils. Despite causing pleiotropic effects on different cells of the immune system, $CD30L^{-/-}$ knockouts showed no alterations in the immune system (Falini et al., 1995).

STK17B

STK17B (DRAK2; DAP kinase-related apoptosis inducing protein kinase 2) was upregulated 3.1-fold by DEX as identified using microarray analysis (**Table 2.2**). This expression level was closely mirrored in sqPCR analysis as induction levels for this gene were 3.4-fold and 3.8-fold by DEX at 2- and 4hrs, respectively (**Figure 2.2**). Induction of STK17B expression by HC was only slightly diminished with respect to DEX as levels for this gene were 2.5-fold at 2hrs and 2.8-fold at 4hrs. By 8hrs, however, induction of this gene began to wane for both DEX and HC as levels for this gene dipped to 3.1-fold and 2.5-fold, respectively.

STK17B is a serine-threonine kinase whose overexpression induces apoptosis in NRK, NIH3T3 and Caco-2 cell lines, but does not affect the survival of still other cell types including ACL-15, HeLa and WI-38 cells (Kuwahara et al., 2006). In these studies, the chief difference between STK17B-sensitive and -insensitive cell types was the accumulation of this protein in the nuclear compartment of the apoptosis-susceptible cell types. It was further shown that in NRK and NIH3T3 cell lines, which are sensitive to STK17B-induced apoptosis, STK17B accumulated in the nucleus of these cells. By comparison, this protein remained in the cytoplasm of STK17B-insensitive cell types (ACL-15 and HeLa cells) in which no apoptosis was observed. While it is not clear what role this protein plays in neutrophils, the localization of STK17B has been shown to be an important predictor in the determination of cellular fate.

MCL1

Microarray analysis indicated a 2.5-fold induction of Mcl1 in neutrophils treated with DEX for 3hrs (**Table 2.2**). Real-time PCR analysis, however, revealed only slight changes in the expression of this gene with 1.4-fold and 2.0-fold inductions in response to DEX at 2- and 4hrs, respectively (**Figure 2.2**). Expression levels for this gene even began to decline to 1.8-fold by 8hrs. Treatment with the natural GC showed even more subtle changes in Mcl1 expression with 1.5-fold at 2hrs and 1.3-fold at 4hrs. Expression of this gene remained only slightly elevated in response to HC by 8hrs (1.4-fold). It is also important to note that expression of Mcl1 in VEH-treated samples decreased by almost 60% by 4hrs and remained decreased through 8hrs of culture.

Mcl1 is an important Bcl-2 family member involved in several survival pathways in neutrophils. This gene is described in more detail in the introduction chapter of this publication. It is important to note that the levels of induction for this gene were similar to those we have observed in the past when we originally suspected Mcl1 of being involved in GC-mediated neutrophil survival (data not shown).

IKBA

Microarray analysis of DEX-treated neutrophils identified a 2.3-fold upregulation in the expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) (**Table 2.2**). Validation by sqPCR identified a similar 2.0-fold upregulation in this gene with DEX by 2hrs (**Figure 2.2**). A more modest 1.4-fold increase was observed with HC at the same time point. By 4- and 8hrs, however, expression of this gene actually *decreased* by 1.4- and 2.3-fold, respectively, when compared with time-matched VEH-treated samples. In samples treated with HC, IκBα expression was reduced to 1.4-fold by 4hrs and further declined to 2.9-fold by 8hrs. Of the 23 genes assayed by sqPCR, this was the only transcript which exhibited a cyclical pattern in expression.

Induction of IκBα by GCs has been demonstrated for several cell types including lymphocytes and a variety of tissue culture cells. While the exact mechanism(s) for GC-induced expression of IκBα is unclear, the bulk of evidence suggests the involvement of a non-canonical GRE (Deroo and Archer, 2001). IκBα associates with NFκB in the cytoplasm of resting neutrophils thereby

repressing the transactivation potential of this transcription factor. Upon stimulus, IκBα is phosphorylated through a series of IkK kinases which then targets this protein for degradation.

FOS1

FOS1 levels were induced 2.3-fold with DEX at 3hrs as assessed using the microarray (**Table 2.2**). This result paralleled FOS1 expression data obtained using sqPCR which identified 2.4-fold and 3.7-fold increases in FOS1 expression with DEX at 2- and 4hrs, respectively (**Figure 2.2**). By comparison, HC induced FOS1 expression by 1.8-fold and 2.5-fold at similar time points. FOS1 was maximally expressed at 4.3-fold with DEX and 2.7-fold with HC at 8hrs.

FOS1 (cFos) along with cJun collectively form the transcription factor AP-1 which is important in the regulation of several genes including p67phox, a NADPH oxidase cofactor (Gauss et al., 2002). AP-1 transactivation is inhibited by the GR which associates directly with either FOS1 or cJun. Restraint stress, a classical physiological method of inducing elevated GCs in laboratory animals, induced a 5-fold increase in FOS1 mRNA within 30 minutes of the stressor (Imaki et al., 1995). Peripheral blood neutrophils have been shown to express high levels of FOS1 compared to peripheral blood lymphocytes and several myeloid cell lines (Colotta et al., 1987). Expression levels of this protein were further increased in neutrophils treated with GM-CSF or TNF. Most importantly, a 14-fold induction of this gene was observed in bovine neutrophils treated with DEX (Weber et al., 2006). These data, in conjunction with our results, demonstrate

a pattern of FOS1 mRNA induction in a variety of neutrophil survival pathways.

IL18R1

IL18R1 was the last on a list of upregulated genes to achieve the 2.0-fold cutoff for significance in these studies (**Table 2.2**). Despite its low standing, sqPCR analysis revealed an early 6-fold induction of this gene with DEX at 2hrs (**Figure 2.2**). At 4hrs, expression levels of IL18R1 had outperformed every other gene in this study by achieving a 34-fold increase in expression. Expression of this gene increased even higher to 92-fold by 8hrs. Similarly, HC induced a 4.8-fold increase in IL18R1 expression by 2hrs. Expression levels further increased to 19-fold and 56-fold by 4- and 8hrs, respectively.

The IL18 receptor exists as a heterodimer comprising a low-affinity α-chain (IL18R1) and a β-chain (IL18R2). IL18R1 is expressed on lymphoid and myeloid cells including monocytes and neutrophils (Liew et al., 2003). IL18 has been shown to induce IFNγ production which is important in anti-viral host defense. IL18 production has also been shown to be a prominent feature of several immune dysfunctions including inflammatory bowel disease (IBD) (Pizarro et al., 1999), psoriasis (Naik et al., 1999), sarcoidosis (Greene et al., 2000) and rheumatoid arthritis (Gracie et al., 1999). Thus, IL18 itself likely functions as a pro-inflammatory mediator in addition to its anti-viral capacities.

IL18 was established as a neutrophil chemoattractant since IL18 depletion by mAbs resulted in the reduced accumulation of neutrophils in the lungs and liver of mice injected with a lethal dose of LPS (Netea et al., 2000). Moreover, IL18

was shown to be important in neutrophil recruitment to collagen-induced arthritic inflammatory foci in mice (Canetti et al., 2003). Using TNFRp55$^{-/-}$ mice, these authors demonstrated that recruitment of neutrophils by IL18 required TNF. Interestingly, the accumulation of IL18-elicited neutrophils to the peritoneum was blocked in animals injected with both DEX and IL18. IL18 can also impact GC signaling directly by increasing GRβ levels and thus altering the GRα:GRβ isoform ratio in certain cell types (e.g., CEM cells) (Orii et al., 2002). This alteration in GR isoforms has been linked to the GC-resistant phenotype found in approximately 20% of IBD patients. Despite the link between IL18 and GCs, a relationship between IL18R1 and GCs has yet to be established in the literature. The induction of a component of a pro-inflammatory signaling complex by an anti-inflammatory compound (i.e., GCs) represents a rather intriguing finding.

(B) Downregulated Genes Related to Survival (TABLE 2.3)

IL1B

Microarray analysis of IL1β expression in DEX-treated neutrophils revealed a 5-fold reduction in the expression of this gene (**Table 2.3**). Validation results for this gene were similar in that DEX treatment resulted in a 5.7-fold decrease in expression at 2hrs (**Figure 2.3**). Expression levels for IL1β were further reduced to 17-fold and 14-fold at 4- and 8hrs, respectively. Treatment of neutrophils with HC also reduced IL1β expression levels. At 2hrs, a 5.1-fold reduction in this gene was observed in response to HC. An 11-fold reduction in IL1β expression was detected at 4hrs which was further reduced to 16-fold by 8hrs.

GENE	qPCR Validation (Hrs) 2	4	8	Microarray Results (3Hrs)
IL1B	-5.7	-16	-14	-4.3
GRO1	-3.6	-4.7	-2.1	-3.8
CD14	-2.3	-11	-18	-2.6
P53	-1	-2.1	-2.3	-2.5
BID	-1.3	-2.8	-2.4	-2.4
IKBE	-1.8	-8.7	-20	-2.1
TNFC	-2.1	-5.8	-3.9	-2.1

TABLE 2.3. Comparison of Microarray Data and Real-Time Data for Genes Downregulated in Neutrophils by Dexamethasone

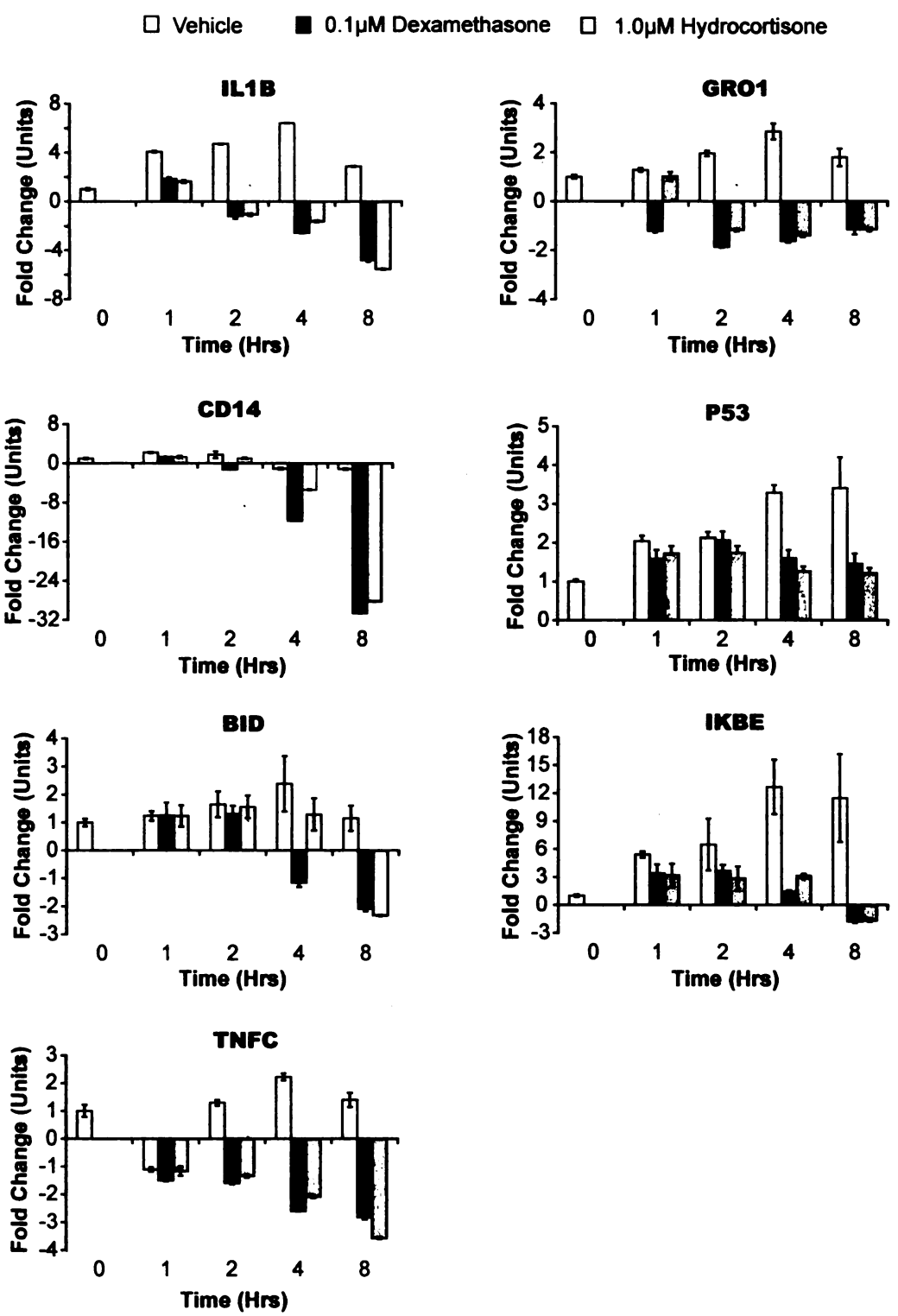

Figure 2.3. Validation of Genes Identified by Microarray Analysis to be Downregulated by GCs.

Pro-IL1β is cleaved by caspase 1 to form a potent inflammatory cytokine which exerts actions on a variety of cell types. Both GM-CSF and TNFα have been shown to induce IL1β expression in neutrophils (Roberge et al., 1994). Moreover, GCs have been shown to reduce IL1β expression in LPS-stimulated human monocytes (Amano et al., 1993). C/EBPβ along with a novel STAT-like transcription factor are essential for the expression of this gene. Treatment with GCs resulted in a reduction in the DNA-binding of these transcription factors in THP-1 nuclear extracts thus suggesting a transrepression mode of inhibition by the GR (Waterman et al., 2006). While loss of IL1β expression in response to GCs is not surprising, the concomitant increase in ILR18R1 suggests that these cells are adapting to stress through alterations in cytokine/chemokine modifications. Whether these adaptive changes represent a survival advantage to a stressed host remains as of yet unknown.

GRO1

Microarray analysis of GRO1 expression in DEX-treated neutrophils revealed a 3.3-fold reduction in expression of this gene (**Table 2.3**). These results were also reflected in the sqPCR results as DEX treatment of neutrophils resulted in a 3.6-fold decrease in this gene at 2hrs and 4.7-fold decrease at 4hrs (**Figure 2.3**). Induction of this gene in response to DEX waned, however, as GRO1 levels were reduced only 2.1-fold by 8hrs. Treatment of neutrophils with the naturally occurring HC resulted in induction levels of GRO1 similar to that seen with DEX. Levels of this gene were reduced by 2.3-fold at 2hrs and 4.0-fold by 8hrs.

Expression levels of GRO1 in response to HC were identical to DEX-treated samples at 8hrs (2.1-fold reduction).

GRO1 is a proinflammatory chemokine involved in the recruitment of neutrophils and amplification of the inflammatory foci. GRO1 expression is induced in epithelial cells by a variety of stimuli including IL1β (a gene identified to be upregulated in these studies), as well as LPS and TNF (Ohtsuka et al., 1996). In addition to the elicitation of neutrophils, GRO1 has also been shown to increase tumor formation in a murine model of melanoma (Luan et al., 1997) as well as promote lung angiogenesis (Mohsenin et al., 2007). This gene was originally identified to be repressed by GC activity in a rat kidney epithelial cell line induced to express GRO1 using IL1B (Ohtsuka et al., 1996). It was further determined that loss of GRO1 expression was the result of impairment of NF-kB activity by the activated GR.

GRO1 has also been shown to be important in the pathogenesis of ulcerative colitis which is caused in part by the accumulation of neutrophils at sites of inflammation. Egesten et al determined that treatment with topical prednisolone resulted in a marked decrease in GRO1 perfusate levels in the patient cohort responsive to corticosteroid therapy (Egesten et al., 2007). In contrast to the results reported here, a 12-fold induction of GRO1 was observed in the microarray analysis of bovine neutrophils treated with GCs (Weber et al., 2006). The results reported by this group, however, appear to contradict the large body of literature regarding the effects of GCs on GRO1 expression and may better

reflect either a species difference or, more likely, the inadvertent inclusion of a GRO1-inducing contaminant (e.g., LPS).

CD14

CD14 expression levels as assessed using microarrays were reduced 2.5-fold in neutrophils treated with DEX for 3hrs (**Table 2.3**). Interestingly, expression of this gene increased spontaneously by 2-fold at 1hr, but then steadily declined back to levels comparable to fresh isolates by 4hrs and beyond (**Figure 2.3**). At 2hrs, both DEX and HC reduced CD14 expression by 2-fold, but by 4hrs expression was reduced by 11-fold and 4.9-fold, respectively. Then by 8hrs, expression of this gene plummeted by 18-fold with DEX and 24-fold in HC-treated samples.

CD14 is the cell surface receptor for the Gram (-) bacteria cell wall component, LPS, which initiates the transduction of a signaling cascade that crosses over to the TLR4 pathway. CD14 is also involved in the selective recognition and phagocytosis of apoptotic cells by macrophages (Devitt et al., 1998). This idea was further substantiated using $CD14^{-/-}$ mice which accumulated large numbers of apoptotic bodies in several tissues, but most notably in the thymus (Devitt et al., 2004).

LPS has long been shown to reduce neutrophil apoptosis both *in vivo* and *ex vivo* (Colotta et al., 1992). Unlike other cell types, neutrophils primarily express this protein on intracellular azurophilic granules and, at much reduced levels, on the cell surface (Rodeberg et al., 1997). The GC methylprednisolone has been found to block neutrophil expression of CD14 induced by a variety of agents

including fMLP, LPS and GM-CSF (Brandt et al., 1993). In monocytes, *in vitro* treatment with prednisolone resulted in the downregulation of both membrane CD14 and sCD14 expression (Nockher and Scherberich, 1997). Probably owing to the low expression levels of this protein on the surface of neutrophils, no study to date has examined the effect(s) of GCs on CD14 protein expression in this cell type.

P53

Microarray analysis of p53 expression identified a 2.5-fold decrease in the expression of this gene following treatment with DEX for 3hrs (**Table 2.3**). Validation of this result using sqPCR, however, revealed even more modest changes as DEX did not alter expression of this gene by 2hrs (**Figure 2.3**). Moreover, DEX reduced expression of p53 only by 2-fold at 4hrs and 2.3-fold at 8hrs. HC, on the other hand, initiated a 1.2-fold decrease in p53 at 2hrs, but further decreased expression by 2.6-fold and 2.8-fold at 4- and 8hrs, respectively. As a cautionary note, it appears that this reduction in p53 expression at the later time points may be the mathematical result of a progressive increase in the expression of this gene. Indeed, by 4hrs, expression of p53 had risen to greater than 3-fold and remained at this expression level through 8hrs.

P53 is the classic tumor suppressor protein which functions as a critical regulator of cell cycle control (Braithwaite and Prives, 2006). During cellular quiescence, p53 associates with Cyclin A thus impeding progression of cell cycle. Phosphorylation at Ser315 of p53 causes this protein to dissociate from

Cyclin A and reassociate with E2F1-3 which permits p53 to become transcriptionally active. As levels of cell-cycle proteins rise, p53 will eventually dissociate from E2F1-3 and once again block progression of the cycle.

Repression of p53 expression by GCs has been described for a number of cell type including: glomerular podocytes (Wada et al., 2005), osteocytes (Tsuji et al., 2006) and RAW264.7 monocytic cells (Zhang et al., 2008). Moreover, p53 has been shown to associate directly with the GR in which case the transactivation capacities of both proteins is significantly diminished.

BID

A 2.5-fold decrease in Bid expression was observed with microarray analysis of DEX-treated neutrophils (**Table 2.3**). sqPCR validation of the microarray results revealed a similar pattern of expression as DEX induced 1.3-fold and 2.8-fold decreases in Bid expression at 2- and 4hrs, respectively (**Figure 2.3**). HC did not alter Bid expression at 2hrs, but by 4hrs the natural steroid had reduced expression of this gene to 1.8-fold. By 8hrs, DEX and HC had reduced Bid levels by 2.4-fold and 2.7-fold, respectively.

The BH3-only Bcl-2 family member Bid will be described in greater detail in Chapter 3. It is important to note that Bid downregulation has also been shown for xenograft tumors treated with DEX for 6hrs (1.5-fold decrease) (Pang et al., 2006). Also, cleavage of Bid to the apoptotic t-Bid appears to be constitutively active in neutrophils, but this event can be prevented by the anti-apoptotic G-CSF (Maianski et al., 2002).

IKBE

IκBε expression as measured by microarray analysis was reduced by 2.0-fold in response to DEX (**Table 2.3**). Validation of this gene revealed a similar pattern of expression as IκBε levels were downregulated by 1.8-fold at 2hrs and 8.7-fold at 4hrs (**Figure 2.3**). By 8hrs, however, levels for IκBε had dramatically decreased to a 20-fold reduction in expression relative to the control. HC did not decrease expression of IκBε at 2hrs, but did reduce expression by 1.9-fold at 4hrs. HC further decreased the expression of this gene by 5.5-fold by 8hrs.

IκBε functions similarly to IκBα in sequestering NF-kB in the cytoplasm thereby restricting the nuclear migration and transactivation potential of this protein. It has been proposed that IκBε functions in a delayed manner relative to IκBα such that IκBε would be responsible for diminishing prolonged NF-kB activation.

IκBε was also shown to be upregulated in neutrophils treated with the gram (-) pathogen *Anaplasma sp.* (Zhao et al., 2001) or LPS (O'Neill et al., 2004b). To date though, there are no publications describing an effect by GCs on IκBε expression.

TNFC

Microarray analysis of TNFC expression identified a 2.0-fold reduction in the expression of this gene resulting from DEX treatment for 3hrs (**Table 2.3**). sqPCR validation of this gene revealed a similar degree of gene repression as DEX decreased TNFC expression by 2.1-fold at 2hrs and 5.8-fold at 4hrs (**Figure 2.3**). Expression levels for this gene in response to DEX remained decreased by 3.9-fold by 8hrs. HC, on the other hand, caused only a modest reduction in

expression of TNFC. Expression of this gene was reduced by 1.4-fold at 2hrs and 1.9-fold at 4hrs. HC treatment resulted in a 1.4-fold decrease in TNFC expression by 8hrs.

TNFC (lymphotoxin beta; LTB) is essential for the formation of lymph nodes and Peyer's patches (reviewed by (Tumanov et al., 2003). This gene was also upregulated in infiltrating leukocytes elicited with carrageenin (Lawrence et al., 2001) and may play a role in the recruitment of immune cells and consequent amplification of the inflammatory foci. A relationship between this gene and GCs has yet to be established in the literature.

Discussion

GCs promote the survival neutrophils through a mechanism that has yet to be elucidated. Therefore, we utilized a microarray to assess the expression levels of 350 genes related to apoptosis in neutrophils treated with 0.1μM DEX for 3hrs. We hypothesized that GCs would cause the upregulation of anti-apoptotic Bcl-2 family members (e.g., A1, Mcl1, etc) while pro-apoptotic Bcl-2 proteins either remained unchanged or concomitantly decreased in expression. In employing this strategy, we identified 25 genes (7%) that were altered in response to GCs. Surprisingly, just two Bcl-2 family members exhibited only modest changes in expression: Mcl-1 was upregulated 2.5-fold and Bid was downregulated 2.5-fold. The remainder of the genes identified to be altered in response to GCs fell into other categories with far less obvious connections to apoptosis or apoptosis-related pathways.

<u>Cytokines and Cell Receptors</u>

A large proportion (40%) of the genes identified by the microarray to be altered by DEX treatment were related to cytokine/chemokine signaling including: ILR18RAP (24-fold increase), 1L1R2 (7.5-fold increase), TLR2 (4.1-fold increase), CD30L (3.3-fold increase), IL18R1 (2.0-fold increase), IL1B (5.0-fold decrease), GRO1 (3.3-fold decrease), CD14 (2.5-fold decrease), CD40 (2.0-fold decrease; see supplemental section) and TNFC (2.0-fold decrease). Given that the survival advantage afforded by GCs is non-transferable from the culture media (e.g., neutrophil-secreted cytokines/chemokines) (Cox and Austin, 1997), it seems more likely that alterations of this grouping of genes might explain the anti-inflammatory properties of GCs rather than the execution of a pro-survival pathway in neutrophils. Thus, the decrease in IL1β, GRO1, CD14, CD40 and TNFC may partially explain how GCs extinguish the inflammatory foci by limiting the cytokine/chemokine-mediated recruitment of immune cells. Membrane-bound ligands, which would not transfer into the culture media, might also play a role mediating anti-apoptotic signals in neutrophils. Changes in the expression of these ligands and/or their receptors could conceivably influence neutrophil survival decisions. Both IL1β as well as the ligand for CD40, CD40L, are expressed as soluble and membrane forms. While early reports indicated that IL-1β suppressed neutrophil apoptosis to a degree similar to that seen with other pro-inflammatory mediators, highly purified neutrophils – free of monocytes and other contaminants – were not nearly as responsive to the anti-apoptotic effects of this agent. These data indicated that IL-1β likely mediated its anti-apoptotic effects on neutrophils via an intermediary bystander cell which in turn influenced

neutrophil survival decisions. Based on these findings, the observed *loss* of IL-1β expression in response to GCs could not account for the reduction in neutrophil apoptosis observed during treatment with these compounds.

Using the apoptosis-centric microarray, we also detected a 2-fold decrease in CD40 (see supplemental section). Only recently has the expression of CD40 been reported for neutrophils (Khan et al., 2006). CD40 presents itself as a particularly attractive target for our studies since activation of this pathway suppresses apoptosis in B-cells through the upregulation of the Bcl-2 family member, Bcl-X_L (Zhang et al., 1996). CD40 stimulation by CD40L has also been shown to prime the respiratory burst generated by neutrophils (Vanichakarn et al., 2008). Since agents that prime neutrophils typically activate the anti-apoptotic response, the possibility exists that an increase in CD40 receptors stimulation may cause a suppression of apoptosis in these cells. Activation of this pathway, however, would require the expression of ligand, specifically CD154 (CD40L).

The plausibility of the CD40 pathway activation resulting in delayed neutrophil apoptosis is made less likely, though, by the reported absence of CD154 expression by these cells (Daoussis et al., 2004). This finding was independently verified in our studies by absence of signal from the microarray spot representing CD40L (data not shown). While neutrophils are unable to produce CD40L, the possibility exists that an additional cell type (e.g., monocyte contaminants) could act as a source for this ligand. The small number of monocytes in the culture system described herein, however, would severely limit the number of

neutrophils allowed to make molecular contact with this cell surface ligand. Alternatively, GC-induced survival via this pathway might occur through the secretion of the soluble form of CD40L, sCD40L. Both platelets and monocytes have been shown to secrete the 18kDa soluble form of CD40L. In fact, neutrophils can enhance the secretion of sCD40L by platelets which in turn increases the respiratory burst activities of neutrophils (Vanichakarn et al., 2008). Since priming of neutrophils invariably leads to suppression of apoptosis, the strong possibility exists for a role for CD40 in neutrophil survival. Presumably an upregulation in CD40 would increase cellular sensitivity to this pathway, but because we observed a decrease in expression of this protein using microarray it appears unlikely that this protein plays a major role in the GC-induced survival of neutrophils. Moreover, a greater understanding of the role of CD40L in our neutrophil culture system will be necessary to fully assess a role for CD40 in the survival of these cells. For example, detection of an increase in CD40L and/or sCD40L production in the culture protocol presented herein might provide cause for further study of CD40 signaling in cultured neutrophils.

Bcl-2 Family and Apoptosis-related Genes

Microarray analysis of the complete set of Bcl-2 family members revealed a surprising lack of change in these genes in GC-treated neutrophils. Of the 21 Bcl-2 family members assayed by the microarray, only two genes from this family exhibited significant changes in expression in response to GC treatment including: Mcl-1 (↑ 2.5-fold) and Bid (↓ 2.0-fold). The 2.5-fold increase in Mcl-1 expression did not validate as well as some other genes in these studies since

induction levels for this gene, measured using sqPCR, reached a maximum 2.0-fold at 4hrs. Basal expression of Mcl-1 has been shown to be especially important to the survival of neutrophils as antisense-mediated loss of this gene resulted in an increase in the apoptosis of these cells.

Mcl-1 expression appears to be regulated by several different transcription factors, the participation of which varies by the particular cellular system under study. Given the tremendous role that NF-κB plays in both neutrophil activation and several survival pathways, it is surprising to learn that this particular transcription factor does not appear to regulate Mcl-1 induction in neutrophils (Edwards et al., 2003). Several transcription factor have been shown to be important for Mcl-1 upregulation including: (1) SRF and Elk-1 (myelomonocytic cells) (Townsend et al., 1999); (2) PU.1 (pro-B cell line) (Wang et al., 2003); and (3) STAT3 (neutrophils) (Epling-Burnette et al., 2001). STAT3 is particularly interesting since not only was this transcription factor identified to be important for Mcl-1 expression in neutrophils, but also because activated GR in cooperation with STAT3 can synergistically enhance the expression specific genes. Therefore, one possible mechanism for neutrophil survival exists in which activated GR cooperates with another transcription factor such as STAT3 to promote the upregulation of Mcl-1 in GC-treated neutrophils. Indeed, recent evidence strongly implicates Mcl-1 in the GC-mediated survival of neutrophils.

Given their terminal differentiation state and short half-life in culture, neutrophils are not particularly amenable to most contemporary gene manipulation techniques. Techniques involving the introduction of small

molecules such as oligonucleotide antisense, though, have proven to be the exception. This is precisely the approach utilized by Sivertson et al in their attempt to assess the function of Mcl-1 in GC-treated neutrophils (Sivertson et al., 2007). Using antisense to knock down Mcl-1 expression, this group was able to correlate a loss of GC-mediated protection with a loss of Mcl-1 expression. Despite presenting compelling results demonstrating a survival role for Mcl-1 in GC-treated neutrophils, this report appears to have omitted some critical controls. In particular, Western data showing loss of basal Mcl-1 expression was presented, but samples treated with both oligonucleotide and DEX were conspicuously absent. Thus the results shown by Sivertson et al while providing evidence for loss of basal Mcl-1 expression, fail to provide adequate evidence for antisense-mediated loss of inducible Mcl-1 expression. Since the strongest evidence for a role for Mcl-1 derives from the apoptosis levels of samples treated with DEX and either sense or antisense oligonucleotides, corresponding Western data should have been presented for identical treatment groups. The inducibility of Mcl-1 by GCs also appears to be an issue as levels of Mcl-1, even in the presence of GCs, never exceed that of control (time=0) samples. Therefore, it would appear that Mcl-1 protein levels are merely maintained in DEX-treated samples, albeit at slightly lower levels relative to time zero, but still at higher levels compared to vehicle-treated controls. Collectively, these results call into question the assertion that Mcl-1 is the critical regulator of GC-mediated neutrophil survival and highlight the difficulty and limitations of studying molecular mechanisms using neutrophils.

Loss of the pro-apoptotic Bcl-2 family member Bid represents a contrasting mechanism by which GCs could exert their survival effects on neutrophils. In this instance, GC-induced loss of the pro-apoptotic Bid protein could confer a protective effect on neutrophils. Bid is cleaved into a truncated formed termed t-Bid through the proteolytic actions of Caspase 8 as well as other classes of proteases including cathepsins. t-Bid inserts into the mitochondrial membrane where it promotes the permeability transition even which permits the release of Cytochrome C as well as other pro-apoptotic mitochondrial proteins. Mice deficient in Bid protein exhibited absolute neutrophil counts 8-times that of wild-type animals (Zinkel et al., 2003). Moreover, monocytes derived from $Bid^{-/-}$ mice exhibited reduced sensitivity to death-receptor pathways such as TNFα-induced apoptosis. DEX has been shown to inhibit the expression of Bid mRNA in carcinoma cells induced to undergo apoptosis with chemotherapeutic cisplatin (Herr et al., 2003). Thus, one possibility is that GCs can suppress the execution of apoptosis in neutrophils through the reduction of pro-apoptotic Bid. The molecular events purportedly involved in this proposed mechanism are described in the succeeding paragraphs.

Cell Signaling and Transcription Factors

Owing to the well published effects of GCs on a variety of signaling pathways, it is not surprising to learn that steroids alter the expression of several cell signaling genes in neutrophils. The changes observed in the expression of CCND3 (↑ 9.7-fold), STK17B (↑ 3.1-fold), IκBα (↑ 2.3-fold), FOS1 (↑ 2.3-fold), P53 (↓ 2.5-fold) and IκBε (↓ 2-fold) in response to GCs are indicative of the wide-

ranging effects of these compounds on a variety of signaling pathways. In addition to prolonging the survival of neutrophils, GCs have also been shown to positively regulate the expansion of myeloid progenitors in the bone marrow as well as the differentiation of myeloid cell lines. The nearly 10-fold increase in Cyclin D3 may be partly responsible for GC-induced increases in myeloid lineage cells and/or neutrophil survival.

Lending further intrigue to the possibility of Bid participation in neutrophil apoptosis was the fact that Bid expression has been shown by other labs to be regulated by p53 (Sax et al., 2002). In fact, loss of p53 expression resulted in an increased resistance to apoptosis by myeloid cells cultured with very low levels of growth factor (Lotem and Sachs, 1993). It has been reported, however, that neutrophils do not express p53 (nor Rb) as demonstrated using Western analysis of protein expression (Wei et al., 1996). Careful analysis of this claim revealed a technical problem that is common when dealing with nuclear proteins expressed in this cell type. Wei et al used a gentle NP-40-based method of protein extraction which is suitable for cytoplasmic proteins yet all too often fails to liberate proteins from the nuclear compartment which is eventually discarded as the particulate fraction. Indeed, when the expression of p53 was analyzed on a whole-cell scale using FACS analysis of saponin-permeabilized neutrophils, nearly 95% of these cells were positive for low levels of expression of this protein (Hsieh et al., 1997). Microarray analysis of gene expression in neutrophils and progenitors revealed that p53 expression peaks in immature cells (promyelocytes) and subsequently declines in later stages as these cells mature

(Theilgaard-Monch et al., 2005). It remains to be determined if loss of p53 expression in GC-treated neutrophils plays a major role in the survival of these cells.

E2F has also been shown to regulate Bid transcription (Cao et al., 2004). Interestingly, E2F binds with high affinity to the cyclin D3 (CCND3) promoter, a gene which we identified with a 9.7-fold increase in expression (Ma et al., 2003). Moreover, protein interaction studies revealed that CCND3 can bind to and inhibit E2F protein thereby preventing E2F-dependent transcription (Janicke et al., 1996). Bolstering a role for E2F in normal apoptotic processes is that ectopic expression of E2F in both an immature myeloid cell line (32D) and lung carcinoma cells (H1299) leads to induction of apoptosis (Boonen et al., 1999; Kato and Sherr, 1993). Therefore, one possible scenario emerges in which CCND3 upregulation results in inhibition of E2F transactivation capabilities either through squelching and/or direct inhibition which could then ultimately impact Bid expression levels. These findings would serve to further substantiate a role for Cyclin D3 in a survival pathway and that at least one consequence of increased CCND3 expression may be a reduction in Bid transcription.

Furthering our interest in this protein was the fact that Cyclin D3-null mice were found to be defective in granulocyte maturation, containing fewer mature granulocytes in both the blood and bone marrow (Sicinska et al., 2006). This loss in neutrophils was attributed to a short-circuiting of the G-CSF signaling pathway which when activated results in an increase in Cyclin D3 in wild-type animals. In contrast to our results using GC-treated neutrophils, CCND3 has been shown to

be downregulated in response to GCs in murine lymphoma cells (P1798) (Reisman and Thompson, 1995). Interestingly, these same investigators demonstrated that when both cyclin D3 and c-myc were overexpressed in P1798 cells, cell viability during GC-treatment increased from 40 – 45% (with either CCND3 or c-myc alone) to greater than 90%.

NF-κB activation is typically associated with anti-apoptotic cellular programs (Foo and Nolan, 1999). This is best evidenced by IκBα $^{-/-}$ mice which die within 8d of birth after presenting with robust granulopoiesis, runting and abnormal skin formation (Klement et al., 1996). Of four neutrophil survival cues studied (TNF, fMLP, GM-CSF and IL-8), only TNF was found to induce IκBα degradation in these cells, indicating the existence of specific roles for this protein in promoting survival (Kettritz et al., 2004). NF-kB has also been shown to be an important component in signaling the survival response in neutrophils as NF-kB inhibitors cause dramatic increases in neutrophil apoptosis (Ward et al., 2004). It is therefore somewhat conflicting to determine a role for IκBα in neutrophil survival as increased expression of this protein would diminish the pro-survival effects of NF-κB. Since IκBα preferentially targets p50/p65 heterodimers, the possibility remains that GCs function to funnel NF-kB signaling through a p65 homodimer modality. Loss of IκBε, a gene identified to be downregulated by the studies presented herein, would therefore further support the existence of such a mechanism as a reduction in this protein would facilitate signaling via p65 homodimers.

Of the identified genes of interest, IκBε exhibited the most dramatic decrease in gene expression (↓ 20-fold at 8hrs). Unlike the α isoform which can migrate to the nucleus to inhibit NF-κB activity, IκBε remains localized in the cytoplasm where it functions to restrict the activity of p65 homodimers (i.e., NF-κB) rather than p50/p65 heterodimers (Simeonidis et al., 1997). This molecular selectivity of IκBε results in the suppression of genes whose expression is dependent on homodimers such as IL-8. Although little has been published regarding a potential role for IκBε in the regulation of apoptosis, LPS-induced survival of B-cells has been shown to be associated with the preferential degradation of IκBε (c.f., IκBα) (Souvannavong et al., 2007). It is likely then that perturbations in IκBα:IκBε ratios represent one manner in which NF-kB activity is regulated. Determining the extent to which this result influences neutrophil survival should be the subject of future investigations.

The decision to use a smaller apoptosis-centric array as opposed to a GeneChip (Affymatrix) containing 40,000+ genes was largely based on our ability to manage large volumes of data generated by microarrays. For example, if we identified a similar proportion (7%) of changes in the expression of 40,000 genes, we would potentially have to process and validate 2700 genes. This large volume of gene targets would be difficult to manage, especially for laboratories that do not perform these assays routinely. Of course, a major limitation of the smaller, focused arrays is that the gene(s) that is responsible for GC-mediated protection of neutrophils may not be represented. Given that our interest is apoptosis and agents that delay this process, an array focused on apoptosis

seems to reduce some of the risk associated with fewer genes.

It is important to note that while this is the first microarray to be employed in the study of GC-induced survival of *human* neutrophils, it is certainly not the first study of its kind. Weber et al used a cDNA-based microarray to analyze DEX-modulated gene expression in bovine neutrophils (Weber et al., 2006). This group identified the differential expression of 1,100 genes, but surprisingly, only two of the 25 genes identified by the study described herein were contained in their list of genes. They identified a 14-fold increase in FOS1 expression and a 12-fold increase in GRO1. In contrast, we observed a 2.3-fold increase in FOS1 expression and a 3.3-fold *decrease* in GRO1. Since multiple reports exist concerning the GC-mediated downregulation of GRO1, we feel that GRO1 expression levels should in fact be reduced in response to GCs. The discrepancy between these two studies owes to either a difference in species (bovine versus human) or in neutrophil purification and/or handling. It is likely to be a combination of the two reasons since 23 other genes that we identified to be altered by GCs are not found in this group's list of >1000.

Future investigations should focus on the 21 genes that were validated in these studies. Careful consideration should be given to assaying corresponding protein levels and/or localization since several reports exist describing a discordance between mRNA and protein. Additionally, the localization of proteins is especially important in the case of Bcl-2 family members, some of which exert function by translocating to and inserting into the mitochondria

membrane (e.g., t-Bid). An effort should be made to prioritize these genes based on their demonstrated role(s) in apoptosis and cell-survival decision-making.

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CHAPTER 3

GLUCOCORTICOIDS PROMOTE NEUTROPHIL SURVIVAL THROUGH DOWNREGULATION OF THE DEATH-RECEPTOR PATHWAY

By

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ABSTRACT

Peripheral blood neutrophils undergo spontaneous apoptosis during routine culture, but this death can be prevented by exposure to several types of pro-inflammatory molecules. Surprisingly, glucocorticoids (GCs), an *anti-*inflammatory family of molecules, also decrease neutrophil apoptosis. Several members of the Bcl-2 family of proteins have been identified as important for specific neutrophil survival pathways. Real-time PCR was used to quickly assess GC-mediated alterations of Bcl-2 family members specific to neutrophils. Remarkably, we observed alterations in only two of the genes assayed: a 2.0-fold increase in Mcl-1 and a 2.0-fold decrease in Bid. Kinetic analysis further demonstrated an inhibitory effect by GCs on Bid expression in neutrophils. Though decreases in Bid protein were not observed, cleavage of Bid to apoptogenic t-Bid was in fact prevented by GC treatment. To further test the purported role of Bid, neutrophils were transduced with a cell-permeable BH3-only Bid peptide. Transduction of neutrophils with 30μM Bid peptide resulted in an 82% increase in apoptosis of these cells after 5hrs thus demonstrating a surprising role for Bid in apoptosis of neutrophils. Peptide-induced apoptosis was not reduced with GC treatment, indicating that GCs act upstream of Bid in the survival pathway. Analysis of upstream components of death-receptor mediated apoptosis revealed GC-mediated downregulation of FasL, a pro-apoptotic membrane protein expressed on the surface of several cell types including neutrophils. These data serve to identify both FasL and Bid as important molecular targets for GCs in the preservation of neutrophils.

Introduction

Peripheral blood neutrophils naturally undergo apoptosis without prompting during routine culture and *in vivo*. This spontaneous progression of death can be delayed by a variety of pro-inflammatory molecules including LPS, Interleukins (ILs) and various growth factors (CSFs). The physiological explanation for this extension of neutrophil survival owes, in part, to the important role that these cells play in responding to microbial pathogens. Specifically, amplification of the inflammatory foci requires the secretion of IL-1 to effectively recruit viable neutrophils to sites of infection. These inflammatory compounds therefore have the dual role of not only promoting neutrophil survival, but also eliciting the active recruitment of these cells. In contrast, the stress hormone glucocorticoids (GCs) have been shown to prevent the recruitment of neutrophils to sites of infection through the downregulation of certain adhesion molecules required for extravasation. Despite these anti-inflammatory properties, GCs can *also* delay neutrophil apoptosis to a degree similar to that achieved by *pro*-inflammatory molecules. The immunological significance of these observations remains unresolved, but it has been speculated to be an immunological protective mechanism generated during periods of stress.

GCs, a clinically important anti-inflammatory medicine, have been shown to promote both neutrophil survival and granulopoiesis. The mechanism by which these compounds exert their action(s) has yet to be fully explained. Several anti-apoptotic proteins, including Bcl-2 family members A1, and Mcl-1L as well as Fas, have been implicated in the GC-mediated survival of neutrophils. A direct

link between gene induction by GCs and consequent survival remains unresolved as a definitive GC response element has yet to be identified for these candidate genes. The difficulty of study of apoptotic systems is further compounded by the steady loss of both mRNA transcripts and protein observed during the progression of apoptosis. Indeed, studies in which gene levels are compared between apoptotic and healthy populations, modest differences in levels of these molecules may in fact reflect a loss of transcript in apoptotic cells rather than an increase in response to a stimulus. It is crucial that the analysis of gene and protein alterations in GC-treated neutrophils be further scrutinized in an effort to tease apart gene effects due to GC treatment and those inherent to apoptotic processes.

Mitochondrial Pathways in GC-Treated Neutrophils

As reviewed in the introduction, the Bcl-2 family of proteins are important regulators of apoptosis for most cell types including neutrophils. Despite the notable absence of the namesake member of this family, Bcl-2, several other pro-survival members found in neutrophils including A1 and Mcl-1 have been shown to be induced by a variety of stimuli. Bcl-2 family proteins feature BH domains which facilitate the oligomerization of these molecules with self at the mitochondrial membrane. Bcl-2 oligomers act in concert with: (1) VDAC (ion channel protein which closes during apoptosis); (2) ANT (exports ATP to the cytoplasm); and (3) cyclophilin D (interacts with ANT) – to collectively form the permeability transition pore (PTP) (Mohamad et al., 2005). Matrix swelling and outer membrane rupture permit the release of several pro-apoptotic

mitochondrial proteins including cytochrome c, SMAC/Diablo, AIF and endonuclease G which promote the progression of apoptosis through the activation of caspases and inhibition of anti-apoptoic processes. The static expression of *pro*-apoptotic Bcl-2 members including Bax and Bad is likely a major contributor to the spontaneous apoptosis of these cells. Due to low rates of respiration and other evidence based on microscopy, neutrophils were thought to contain only a small number of dysfunctional mitochondria (Fossati et al., 2003). Recent studies, however, revealed that while few in number, neutrophil mitochondria still play a critical role in mediating the apoptosis of these cells. While GCs have been shown to only modestly affect Bcl-2 family expression in a few cell types, recent reports suggest that these compounds can alter select members of this family in neutrophils.

Of the pro-apoptotic Bcl-2 proteins involved in neutrophil apoptosis, Bax and its insertion into the mitochondria is probably the best understood. Prior to induction of apoptosis, Bax is retained in the cytoplasm through its association with Mcl-1. This protein-protein interaction becomes interrupted, however, when Bax is phosphorylated by the Akt kinase at serine 184 (Gardai et al., 2004). The dissociation of these proteins permits the localization, insertion and oligomerization of Bax at the mitochondrial membrane thereby initiating the permeability transition event. Thus, the equilibrium between Bax and Mcl-1 can be weighted in favor of survival, through the induction of Mcl-1 expression.

Several survival cues induce Mcl-1 expression in neutrophils including GM-CSF, IL-1β and LPS (Moulding et al., 1998; Saffar et al., 2008). Moreover,

neutrophils derived from mice deficient in this protein succumb to apoptosis more quickly in culture thus highlighting the essential role that this protein plays in the normal survival of these cells (Dzhagalov et al., 2007). Given the importance of this protein in other survival pathways, Sivertson et al analyzed Mcl-1L expression in neutrophils treated with GCs. This group observed a ~50% increase in Mcl-1L expression in neutrophils treated with GCs for 12hrs, but their data also suggested that there was a >50% difference in apoptosis between GC-treated and non-treated populations (Sivertson et al., 2007). Therefore, the preservation of Mcl-1L protein levels observed by these authors in DEX-treated neutrophils may in fact owe to a greater proportion of healthy cells in GC-treated samples rather than actual induction by GCs. Conversely, as we will show in the chapter, apoptotic populations of neutrophils exhibit increased degradation of transcripts which would likely include Mcl-1L. Sivertson et al also tackled the daunting task of functional analysis of Mcl-1L activity in neutrophils.

Owing to their differentiation status and short life-span, neutrophils are not amenable to standard laboratory transfection approaches. Notable exceptions have included protein transduction and antisense strategies which simulate overexpression and loss of function, respectively. Sivertson et al utilized the latter technique to further analyze the role of Mcl-1L in neutrophil apoptosis. Despite showing that antisense reduction of Mcl-1L levels resulted in reduced protection of neutrophils by GCs, these authors also observed a reduction in apoptosis in samples treated with sense oligonucleotide. The vehicle used to transfer the oligonucleotides into the cells, in this case DOTAP, may cause a

secondary effect on apoptotic pathways. Therefore, the observed inhibition of GC-mediated survival in samples treated with Mcl-1L antisense may be the result of an interplay between the expected pro-apoptotic effect caused by Mcl-1L antisense and the non-specific anti-apoptotic effect of the transfection technique. Given our analysis of data generated by Sivertson et al, as well as the dearth of publications regarding GC regulation of Mcl-1 expression, the role of Mcl-1 in GC-mediated neutrophil survival remains suspect.

Effects of GCs on Fas/FasL System:

Several apoptosis-related proteins have been shown to be altered in response to GCs in neutrophils including Fas (CD95) (Chang et al., 2004). Fas, a death receptor (DR) protein that is activated through occupation by FasL, is highly expressed on human neutrophils in comparison with other phagocytes (Liles et al., 1996). While a reduction in Fas mRNA and protein has been observed for DEX-treated bovine neutrophils, it is unclear if this finding is significant with respect to the survival of these cells (Chang et al., 2004). Unlike Fas which is expressed in a wide assortment of tissues, the expression of its ligand, FasL, is much more restricted (French et al., 1996).

Among the cell types which express this protein are ocular cells which use the pro-apoptotic FasL to maintain immune privilege by inducing apoptosis in infiltrating leukocytes (reviewed in (Ferguson and Griffith, 2007)). In relation to this project, increases in sFasL expression were identified in neutrophils after 24hrs of culture which persisted through 48hrs (Liles et al., 1996). These results, however, must be tempered against the peculiarly low levels of apoptosis

observed by these researchers which were likely the result of using a Ficoll-based media for neutrophil purification. Ficoll, unlike other cell separation medias such as Percoll, has been shown to alter the appearance and function of neutrophils (Haslett et al., 1985).

As will be shown in this chapter, we did not note a decrease in Fas expression in human cells treated with GCs. On the other hand, a decrease in FasL expression was observed in GC-treated neutrophils, consistent with other reports regarding the negative impact of GCs on the expression of this gene. It seems unlikely that GCs would cause similar outcomes for neutrophil survival by species-specific alterations of separate genes within the same pathway. Furthermore, FasL regulation by GCs has already been described thus validating our observation and increasing the likelihood that this protein plays a prominent role in GC-mediated neutrophil survival (reviewed in (Riccardi et al., 2000).

As already discussed in a previous chapter, overexpression of GILZ resulted in the inhibition of FasL expression. In this model, GILZ, a highly GC-inducible gene, inhibits AP-1 activity which is required for the expression of two critical FasL transcription factors, Egr-2 and -3 (Mittelstadt and Ashwell, 2001). Additionally, FasL expression has been recently shown to be regulated through the dynamic competition between the GR and NF-kB (Novac et al., 2006). Due to overlapping response elements, binding by the GR to the FasL promoter mutually excludes NF-kB binding thereby resulting in a reduction in FasL expression. Thus, the regulation of FasL expression by GCs appears to be complex, requiring an inducible intermediate in the form of GILZ as well as direct

inhibition through the binding of GR to a nGRE within the FasL promoter. An explanation of the molecular events involved in Fas-induced apoptosis will help elucidate downstream targets which can be analyzed for GC-induced alterations.

FasL binding to Fas results in the formation of the death-inducing signaling complex (DISC) comprising Fas, FADD (an adaptor molecule) and Procaspase-8 (PCASP8). Specifically, this receptor-ligand interaction results in the tethering of external Fas molecules by the ligand which consequently initiates internal clustering of corresponding intracellular death domains. The domains selectively recruit adapter molecules including TNF-R associated death domain (TRADD). TRADD mediates the recruitment of procaspase 8 (ProCASP8) proteases via an interaction with an additional adapter molecule, Fas-Associated Death Domain (FADD). FADD has been previously shown to be essential for Fas- and TNF-induced apoptosis, but dispensable for still other types of extrinsic apoptosis such as oncogene-mediated or virus-induced (Yeh et al., 1998). The oligomerization of ProCASP8 facilitates the autoproteolytic activation of these proteins to functional caspases. Activated caspase 8 (CASP8) proteins then cleave a variety of substrates, some of which participate directly in the progression of apoptosis including the "central executioner" caspase 3. Additionally, caspase 8 cleaves the Bcl-2 family member Bid to the truncated form, t-Bid.

Role of Bcl-2 Member Bid in Apoptosis:

The cleavage of a Bcl-2 family member by Caspase 8 represents at least one example of the cross-talk that exists between the mitochondrial- and DR-

mediated apoptotic pathways. Truncated Bid inserts into the mitochondria where it, along with other Bcl-2 family members, promotes the formation of the permeability transition complex. This event permits the efflux of several pro-apoptotic mitochondrial proteins including SMAC/Diablo and Omi/HtrA2, but most importantly it allows for the release of cytochrome c which effectively results in the convergence of both receptor-mediated apoptosis and mitochondrial apoptosis. In addition to cleavage by Caspase 8, Bid can be digested by several other classes of proteolytic enzymes including calpains, Granzyme B and cathepsins (Chen et al., 2001; Cirman et al., 2004; Sutton et al., 2000). The resulting 11-15kDa Bid fragment t-Bid is short-lived with a half-life of less than 1.5hrs (Breitschopf et al., 2000). Moreover, through the concerted actions of multiple proteases, even shorter t-Bid species have been observed for specific cell types (Imgenex).

Neutrophils in particular have been shown to cleave Bid using cysteine-specific capthepsins (cathepsin B or L) (Blomgran et al., 2007). Additionally, the cleavage products resulting from cathepsin-mediated cleavage of the Bid substrate are the smaller 11kDa and, to a lesser extent, 13kDa fragments of t-Bid. These fragments are slightly shorter than the usually anticipated 15kDa t-Bid that is generated by Caspase 8. Using the cathepsin inhibitor EST*, Blomgran et al not only blocked the progression of ROS-mediated neutrophil apoptosis, but also prevented the formation of truncated Bid (Blomgran et al., 2007). Neutrophils treated with a pan-caspase inhibitor (z-VAD-fmk), however, had reduced levels of apoptosis, but had levels of t-Bid similar to controls. These

results indicated that in at least one apoptotic pathway, neutrophils can utilize non-caspase proteases to activate Bid which contributes to the death of these cells.

Along with several other pro-apoptotic Bcl-2 family members, activation of Bid has been shown to occur during progression of neutrophil spontaneous apoptosis during routine culture (Maianski et al., 2004). As evidence for the importance of this protein in neutrophil apoptosis, Bid-deficient mice develop a myeloproliferative disorder which manifests as an 8-fold increase in circulating granulocytes (Zinkel et al., 2003). This study further showed that myeloid precursor cells deficient in Bid were completely unresponsive to death-receptor mediated apoptotic pathways including TNF and Fas thus indicating that Bid alone was sufficient to execute apoptosis in these cells. Collectively, these data indicated a prominent role for the pro-apoptotic protein Bid with respect to both the survival and DR-induced apoptosis of myeloid cells. The data presented herein reinforces and expands upon the role of Bid in neutrophil apoptosis.

Materials and Methods

Reagents and Antibodies

Becton Dickinson (BD) Vacutainer Tubes containing Acid Citrate Dextrose Solution A and 21G1½ needles were used for drawing blood (BD Vacutainer System, Franklin Lakes, NJ). Dextran T-500, Percoll, PVDF, ECL-plus reagents and HRP-linked anti-mouse and –rabbit antibodies were obtained from GE Biosciences (Piscataway, NJ). Trypsin Inhibitor and Human Neutrophil Elastase Inhibitor (HNEI) were both purchased from EMD Biosciences (San Diego,

CA). PMSF, RPMI-1640 Media, Iscove's Media, 100x Penicillin-Streptomycin, 100x L-Glutamine, Acridine Orange, Propidium Iodide (PI), TRIZOL, Superscript II and III and dNTPs, SYBR Green (1000x) were all obtained from Invitrogen (Carlsbad, CA). Micrococcal Nuclease (MNase) was obtained from USB Corp. (Cleveland, OH). Fetal Bovine Serum was purchased from Hyclone (Logan, UT). All other reagents including Dexamethasone and hydrocortisone were purchased from Sigma (St. Louis, MO). SYBR Green Core Reagent Kits and PCR plates were obtained from Applied Biosystems, Inc. (Foster City, CA). BCA protein determination reagents were purchased from Pierce, Inc. (Rockford, IL). The antibodies used in these studies were rabbit polyclonal anti-Bid (BD Biosciences, San Jose, CA), anti-β-Actin (Sigma, St. Louis, MO), anti-FasL (Santa Cruz Biotech., Santa Cruz, CA) and anti-CASP8 (Cell Signaling Tech., Danvers, MA). All primers were synthesized by MSU RTSF Oligonucleotide Synthesis Facility (E. Lansing, MI).

Neutrophil Isolation

Peripheral blood neutrophils were harvested from healthy adult donors (ages 18-30) following the submission of donor consent in accordance with Michigan State University Human Use Committee guidelines. Neutrophils were purified as described previously, with minor modifications to the protocol (Haslett et al., 1985). Briefly, 34ml of anti-coagulated blood was mixed with 17ml of 3% Dextran in 0.85% NaCl to deplete red blood cells (RBCs). The RBC-depleted cells were then resuspended in 2ml of autologous platelet poor plasma which was obtained

from donor serum supernatant following centrifugation at 5000xg. Next, leukocyte subsets were resolved on a discontinuous Percoll gradient comprising two layers of 55% and 64% Percoll in 0.85% NaCl. The granulocytes were then harvested from the 55%-64% interphase and washed in Hanks' Balanced Salts Solution (HBSS; minus Ca^{++} and Mg^{++}). This method routinely yielded preparations with >98% viability as determined with Trypan Blue. Neutrophil purity was routinely assessed to be 96 – 98% based on nuclear morphology via Acridine Orange staining.

Real Time PCR

Total RNA was extracted from $1x10^7$ neutrophils using TRIZOL RNA extraction reagent. 500ng - 1μg of anchored oligo d(T_{18}VN) was added to 1μg of total RNA to synthesize cDNA template using Superscipt Reverse Transcriptase. Relative real time PCR was conducted on 0.5µl of cDNA product using ABI Core Reagents in 25µl total volumes. The primers used in these studies are identified in **Table 3.1**. Primer efficiency for each primer set within the range of analysis was estimated to be >90% as estimated by an amplicon-titrated standard curve. Analysis of real time data was performed using the $-\Delta\Delta C_T$ method of comparison (ABI User Bulletin #2; ABI, Foster City, CA). Data points for the genes of interest were normalized to respective β-actin levels.

Gene	Forward Primer	Reverse Primer
β-Actin	CTCTTCCAGCCTTCCTTCCT	TGTTGGCGTACAGGTCTTTG
A1	CAGGAGAATGGATAAGGCAAA	CAGGAGAGATAGCATTTCACAGA
Bcl-XL	CAAGGAGATGCAGGTATTGG	GCTGCATTGTTCCCATAGAG
Mcl-1	GCATCGAACCATTAGCAGAA	CATGGAAGAACTCCACAAACC
Bad	GCTCCGGAGGATGAGTGA	CCCACCAGGACTGGAAGA
Bak	GTCACCTTACCTCTGCAACCT	CTGCAACATGGTCTGGAACT
Bax	CAAGAAGCTGAGCGAGTGTCT	GTTGAAGTTGCCGTCAGAAA
Bid	TGGGAGGGCTACGATGAG	CCGGATGATGTCTTCTTGAC
Bik	CTTGGCATGACTGACTCTGAA	CTGAGGCTCACGTCCATCT
Bim	GCAAAGCAACCTTCTGATGTAA	CTTGTGGCTCTGTCTGTAGGG
FasL	GTTCTGGTTGCCTTGGTAG	GCTTCTCCAAAGATGATGCTG

Table 3.1. List of Primers Used in Real-Time PCR Studies

Western Blot Analysis

Cells were fractionated into cytoplasmic and nuclear fractions using the NE-PER nuclear extraction kit (Pierce). Protease inhibitors were used as previously described (Gametchu et al., 1993), but also included: 100μM phenylarsine oxide, 2mM NaF, 10mM Na_2MoO_4, 100μM elastatinal, 100μM chymostatin and 10μM human neutrophil elastase inhibitor. Protein concentration was determined using BCA Protein Assay (Pierce). Samples were equally loaded and resolved on a 12% Polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). The separated proteins were then transferred to a PVDF membrane at 25V for 1 hour. Blots were blocked with either 5% dried milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) or 2% Bovine serum albumin (BSA) in TBS. Blots were then incubated with antibody specific to the antigen under study in 2% BSA in TBS at ambient temperatures for 1hr. Following washing, the membranes were treated with HRP-conjugated anti-mouse or -anti-rabbit antibody, developed and exposed to film. To probe for additional antigens, the blots were stripped using 100mM 2-mercaptoethanol and 2% SDS at 55°C for 30 minutes. The membranes were then stripped, probed with mouse anti-β-actin and redeveloped as described above.

Protein Transduction with Synthetic Peptides

For Bid transduction experiments, two peptides were generated including a control (RRRRRRRRGEDIIRNIARHAAQVGASMDR) and BH3-only peptide (RRRRRRRRGEDIIRNIARHLAQVGDSMDR) (MSU Macromolecular Sequencing

and Synthesis Facility; East Lansing, MI). These peptides were based on sequences published by the Korsmeyer laboratory (Walensky et al., 2004). Per other published reports (Letai et al., 2002), each peptide was dissolved in sufficient DMSO to make 10mM stock solutions. For protein transduction, peptides were added directly to 500μl culture media followed by the addition of $5x10^5$ neutrophils. Following a 5hr incubation, cells were harvested and prepared for analysis of apoptosis levels.

MC-540 Detection of Apoptosis

Apoptosis was measured using a merocyanine-540 (MC-540)-based method that was developed in our lab (Laakko et al., 2002). Neutrophils were cultured in RPMI containing 100 units Penicillin, 100µg Streptomycin, 2mM L-glutamine and 2% heat-inactivated, Charcoal-Dextran treated FBS. Neutrophils were plated on Falcon non-tissue culture treated 24 well plates to reduce adhesion of healthy cells. Following harvest, cells were stained with 17uM MC-540 for 10 minutes in the dark. PI (1μg/ml) was then added to each tube just prior to FACS analysis in order to detect late stages of apoptosis and exclude necrosis. Cell analysis was done using a FACS-Vantage flow cytometer equipped with 575nm and 660nm filters. Flow data was analyzed and gating was performed with WinList 5.0 (Verity Software) FACS data analysis program.

Statistics

The mean is plotted for each data point and standard error of the mean is shown for each plot. For statistical analysis, unless otherwise noted, a two-tailed student t-test with unequal variances was used to ascertain statistical significance (typically, $p<0.01$).

Results

Apoptotic Neutrophils Show Loss of RNA

Normal progression of apoptosis results in the degradation of a variety of cellular substrates via the specific and non-specific actions of activated proteases and nucleases. RNaseL has been shown to be especially important for the proper execution of apoptosis in several cell types including thymocytes and fibroblasts (Zhou et al., 1997). Since gene expression data is particularly susceptible to RNA degradation bias when used in the study of apoptotic systems (Auer et al., 2003), we sought to determine the extent of RNA degradation in apoptotic neutrophils prior to gene analysis.

To assess the status of RNA in cultured neutrophils, FACS technology was utilized to sort these cells into healthy and apoptotic populations. MC-540 brightness which increases with apoptosis was used as a marker to separate these cells into two distinct populations. The purity of these populations was confirmed using Wright-Giemsa staining with subsequent microscopy (**Figure 3.1**). As shown in **Figure 3.2A**, following 12hrs in culture, apoptotic neutrophils demonstrated a 37% reduction in total RNA levels as determined with spectroscopic measurement.

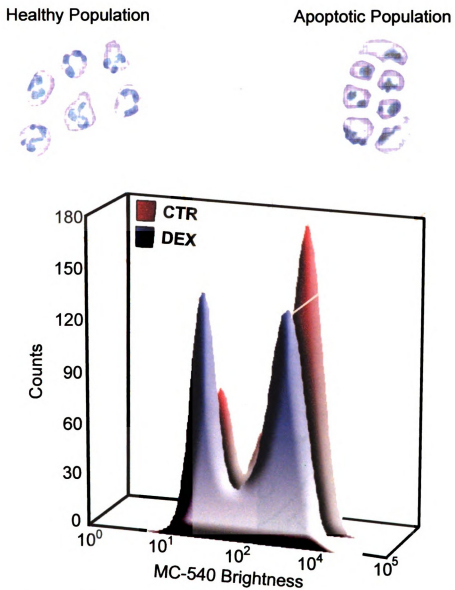

Figure 3.1. Cell Sorting of Neutrophils into Healthy and Apoptotic Populations. Peripheral blood neutrophils were cultured with either media (CTR; control) or 0.1μM Dexamethasone (DEX) for 12hrs. Cells were then stained with MC-540 to quantify levels of apoptosis. The histograms shown above depict MC-540 brightness of each treatment group. Apoptosis levels were measured in tandem as MC-540^{dim} and MC-540^{br} peaks were sorted into separate tubes. Cytospin preparations were prepared for each resulting sample. Slides were stained with Wright-Giemsa staining and images were visualized via a camera-equipped microscope, also shown above. Data shown above is characteristic of 2 independent experiments.

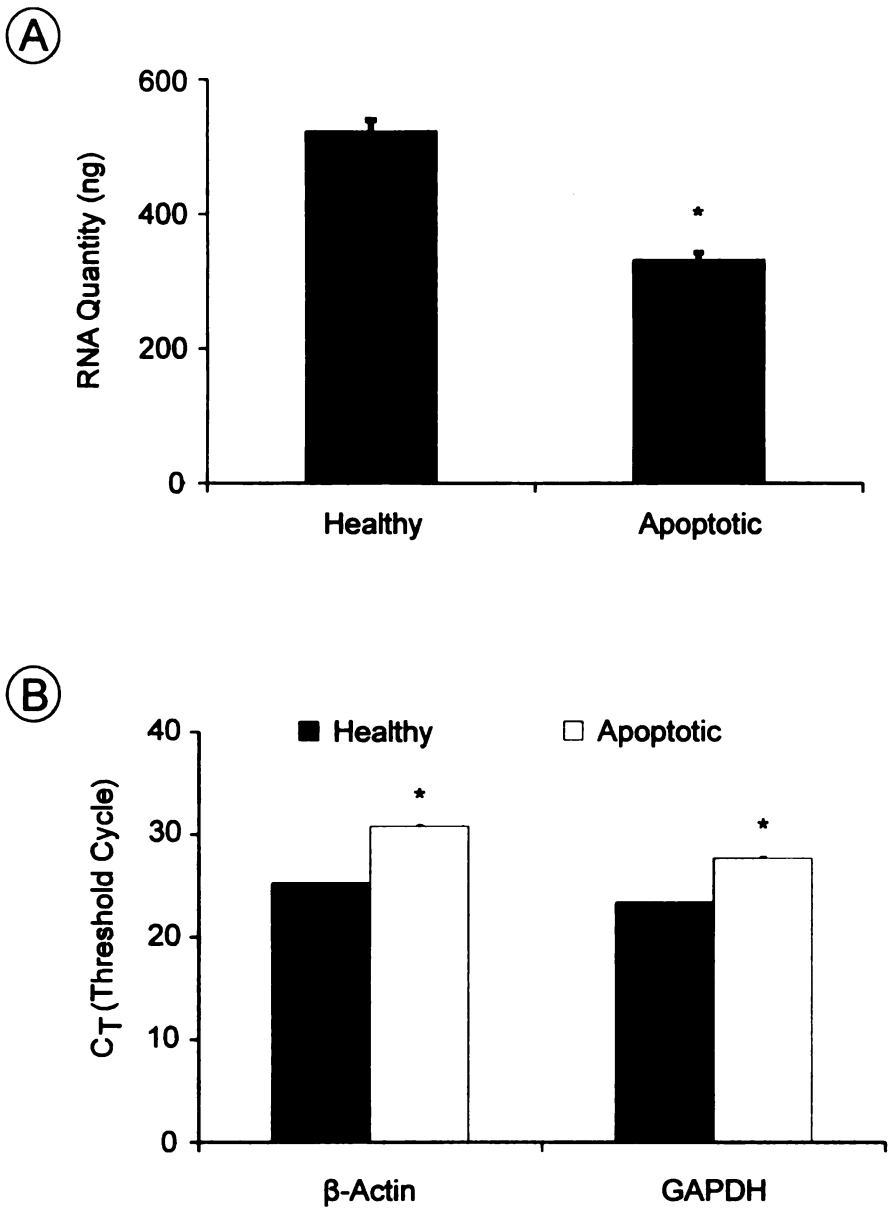

Figure 3.2. Analysis of RNA from Neutrophils Sorted into Healthy and Apoptotic Populations. (A) RNA quantities are shown from neutrophils sorted into healthy and apoptotic populations using FACS analysis. Neutrophils were cultured for 12hrs to induce adequate levels of apoptosis for sorting. This graph represents the average of two independent experiments (*$p<0.01$). **(B)** The average cycle (C_T) at which the PCR signal reached an arbitrary threshold is shown for two housekeeping genes. Each gene was analyzed in healthy (black bar) and apoptotic (white bar) sorted populations. Real-time PCR was used to quantify levels for each respective gene. Standard error is shown for each data point (n=3, *$p<0.01$).

To determine if a wholesale loss of RNA affected gene expression analysis, we analyzed the expression of two housekeeping genes: β-Actin and GAPDH. Despite the loss of RNA owing to degradation, we predicted that experimental normalization of RNA quantities (i.e., increasing sample concentrations) would still permit gene expression analysis. Surprisingly, the C_T values for both β -Actin and GAPDH levels were significantly higher in apoptotic samples (**Figure 3.1B**), indicating a reduction in the levels of these transcripts. Typically, a C_T difference of one accounts for a 3.3-fold change in expression. Therefore, the C_T discrepancy between these two populations could translate to an 18-fold difference in gene expression levels. Thus, for gene expression studies, lengths of culture should be limited to shorter durations in order to control for apoptosis-related mRNA loss. This approach enriches for gene candidates which might be directly influenced by GC treatment.

Bcl-2 Family Expression in Neutrophils Exposed to GCs

Certain members of the Bcl-2 family, including A1, Mcl-1 and Bim, have been shown to be important for the survival of neutrophils. Despite much progress made in understanding the role that this family plays in neutrophil survival, it remains unclear to what extent GCs affect the expression of this family in these cells. To address this question, we utilized real-time PCR in an effort to profile the expression of this family in GC-treated neutrophils.

To avoid a loss of RNA transcripts associated with apoptosis, RNA was harvested from neutrophils cultured for only 4hrs. Since neutrophils demonstrate little appreciable apoptosis at 4hrs (see Chapter 1), this time frame would enable

the reliable measurement of gene levels in these cells. At this time point, neutrophils showed only modest changes in Bcl-2 family members. In particular, Mcl-1 showed 2.0-fold and 2.1-fold increases in expression in response to DEX and HC, respectively. A1 also exhibited a 1.5-fold increase with DEX treatment and a 1.6-fold increase with HC treatment. In contrast, the other anti-apoptotic Bcl-2 family member assayed, Bcl-X_L, remained relatively constant in expression in response to GCs.

Several pro-apoptotic Bcl-2 family members were also assayed in these studies including: Bad, Bak, Bax, Bid, Bik and Bim (**Table 3.2**). Similar to other published reports, Bax maintained constant levels of expression in our studies. Except for Bid, the expression levels of this entire group of genes remained stable in response to GCs. Bid expression, on the other hand, decreased 2-fold and 2.5-fold in response to DEX and HC, respectively. Collectively, these data indicated potential roles for Mcl-1 and Bid in GC-mediated neutrophil survival.

GCs Alter Bid Expression and Cleavage

Despite detection of a 2.0-fold increase in Mcl-1 expression, PCR analysis of this gene in response to GCs revealed even less substantial changes in expression (see **Chapter 2, Figure 2.2**). Contrary to other published reports (Sivertson et al., 2007), we were unable to detect a shift in Mcl-1 protein levels in neutrophils treated with GCs (data not shown). This discrepancy may reflect a difference in antibodies used in each respective study since the antibody generated by Sivertson et al reacted specifically with the long form of Mcl-1 (Mcl-1L). Moreover, since stabilization, rather than an induction, of Mcl-1 during GC

	Gene	DEX	HC	n
Anti-Apoptotic	A1	1.5	1.6	3
	Bcl-XL	0.8	1.2	2
	Mcl-1	2.0	2.1	3
Pro-Apoptotic	Bad	0.8	0.8	3
	Bak	1.1	0.7	2
	Bax	0.7	1.5	3
	Bid	0.5	0.4	2
	Bik	0.8	0.7	1
	Bim	1.1	0.8	2

Table 3.2. Summary of Bcl-2 Family Expression in GC-Treated Neutrophils at 4hrs

treatment of neutrophils has been suggested to be important for the survival of these cells, we instead focused on Bid and Bid-related pathways for the studies presented herein.

A loss in pro-apoptotic Bid expression might represent one pathway by which GCs exert their anti-apoptotic effects. To further assess the degree of downregulation of Bid mRNA caused by GCs, we performed kinetic analysis of the expression of this gene using real-time PCR. Bid mRNA levels were not significantly altered through the first two hours of cultures, but did trend downward in response to GCs by the second hour (**Figure 3.3**). At 4hrs, however DEX and HC caused respective 49% and 68% reductions in Bid expression relative to a vehicle-treated sample. These results for Bid expression in response to GCs validated the single time point results obtained in Table 2 for expression of this gene. The negative effect of GCs on Bid expression persisted through 8hrs of culture as DEX treatment caused a 57% reduction and HC treatment resulted in a 48% reduction in the expression of this gene. These data indicated that the pro-apoptotic Bcl-2 family member Bid was downregulated in response to GC treatment which may help explain how these steroids influence neutrophil survival. Next, the protein levels of Bid were assayed in order to correlate gene expression changes with alterations in protein levels.:

Actuation of death-receptor mediated apoptosis processes typically includes the activation of caspase 8 which in turn, through protease action, cleaves Bid to the 15kDa form t-Bid. Under resting conditions, full-length Bid is a stable molecule with a long half-life; whereas t-Bid is short-lived with a half-life of 1-2hrs

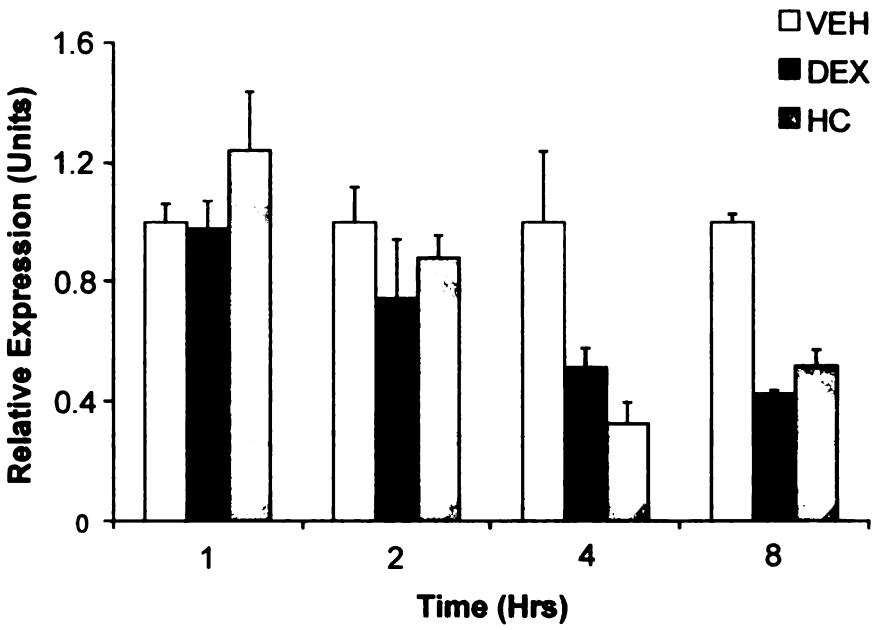

Figure 3.3. GCs Reduce Bid mRNA Expression in Neutrophils. Purified neutrophils were treated with vehicle (VEH), 0.1μM Dexamethasone (DEX) or 1μM hydrocortisone (HC) for 0-8hrs. Data shown are Bid mRNA levels relative to a time-matched vehicle control. Each data point is normalized to β-Actin mRNA levels and standard error is shown for each plot (n=3). This experiment is representative of two independent experiments.

(Breitschopf et al., 2000). While the short lifespan of t-Bid might limit the extent by which this protein can promote apoptosis, it also increases the difficulty of analyzing Bid in a cellular system. Moreover, neutrophils have been shown to cleave Bid to even smaller 11kDa fragments via the action of cathepsins thereby increasing the multiplicity of cleavage products derived from the parent Bid protein (Blomgran et al., 2007).

In order to assess alterations in Bid expression in response to GCs, Western blotting was used to determine levels of this protein and its cleavage products. As shown in **Figure 3.4A**, Bid is stably expressed under basal conditions in cultured neutrophils through at least 8hrs in culture. In contrast to the effects of GCs on Bid mRNA expression, DEX caused a modest increase in the expression of full-length Bid protein (1.9-fold at 4hrs; quantitative data not shown). Similarly, the natural GC, HC, also caused a slight, yet consistent elevation of Bid protein expression (**Figure 3.4B**; 2.3-fold at 12hrs; quantitative data not shown). These data indicated that GCs have contrasting effects on Bid mRNA and protein expression in neutrophils. Despite the divergence of Bid mRNA and full length protein levels, a significant loss in the apoptogenic t-Bid was observed in response to GCs. In fact, the effect of DEX on t-Bid formation was consistent with the effects of GCs on Bid mRNA expression.

Bid cleavage to t-Bid was apparent as early as 8hrs and strongest by 12hrs during the routine culture of neutrophils (**Figure 3.4A**). No 10kDa cleavage products were observed in either experiment at the earlier time point of 4hrs, but this could be due to the sensitivity of Western analysis and/or the proclivity of Bid

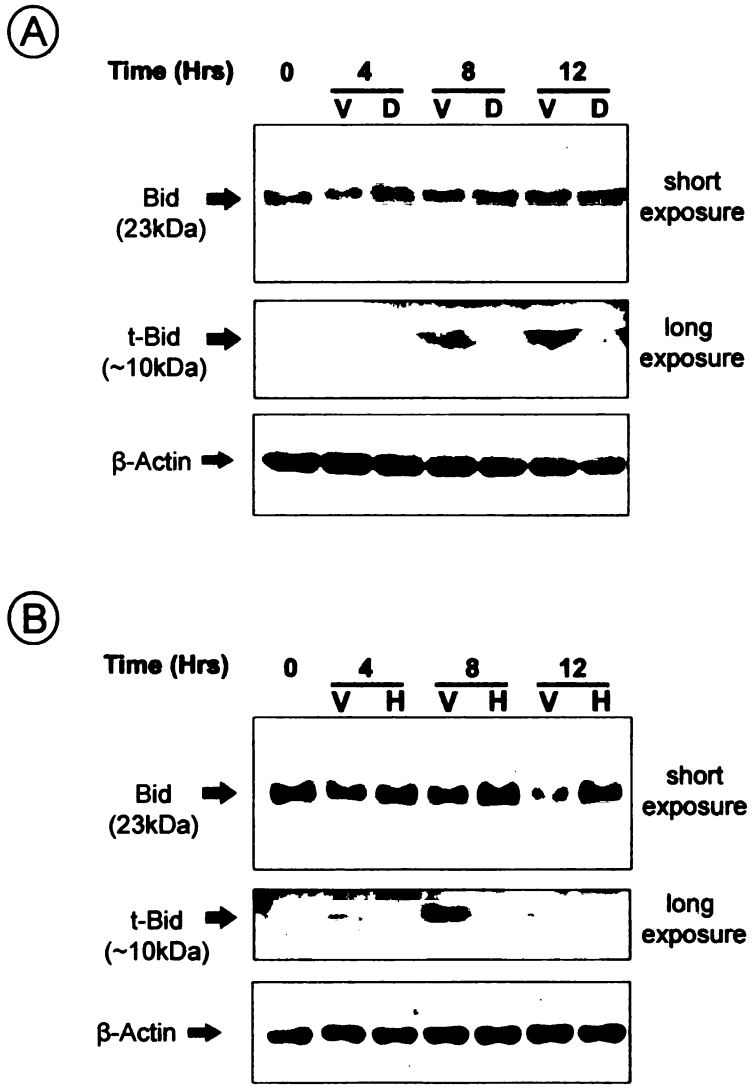

Figure 3.4. Glucocorticoid treatment does not alter full length Bid protein levels, but does affect t-Bid formation. Neutrophils were treated with Vehicle, (A) 0.1μM Dexamethasone (D) or (B) 1μM hydrocortisone (H) for 0-12hrs. Western analysis was used to analyze both full length Bid (23kDa) and the shorter t-Bid cleavage products (<15kDa). The cleavage product t-Bid required longer exposure times relative to the more abundant full length Bid. β-Actin is shown to demonstrate equal loading of the gel.

to degradation. As shown in **Figure 3.4A**, treatment of neutrophils with 0.1μM DEX substantially reduced t-Bid levels at both the 8- and 12hrs time points. HC also caused a comparable reduction in t-Bid formation, but only at the 8hrs time point (**Figure 3.4B**). The discrepancy between DEX and HC samples at this time point could be the result of blood-donor variation since little spontaneous t-Bid formation was observed at this time point. While GCs did not reduce the expression of the inert full-length Bid protein such as that seen with Bid mRNA, these compounds did reduce levels of t-Bid which actively influences apoptosis decisions. Hence, a reduction in Bid activity through loss of t-Bid may explain in part how GCs promote survival in neutrophils. Since CASP8 is important for the activation of Bid to t-Bid, we next analyzed whether CASP8 activity was similarly reduced by treatment with GCs.

GCs Diminish Caspase 8 Activation:

CASP8 activation is a useful predictor of extrinsic apoptosis pathway involvement. This particular caspase has already been shown to play a crucial role in neutrophil spontaneous apoptosis (Maianski et al., 2004), but it is unclear if GCs impact the activation of CASP8 which could in turn affect the execution of apoptosis in these cells. CASP8 activation can be monitored using several techniques including the use of fluorogenic substrates which bind and identify the cleaved form of this protein or by detection of the cleavage event which characteristically results in the formation of a smaller immunoreactive band. We utilized this latter approach to assess whether this pathway was altered in GC-treated neutrophils.

To analyze CASP8 activation, lysates from neutrophils treated with either Vehicle (V) or GCs for 0-12hrs were resolved using Western analysis and probed with an antibody that recognizes both the pro- and cleaved-forms of this protein. The emergence of a ~40kDa immunoreactive band corresponding to activated CASP8 was identified starting at 8hrs (**Figure 3.5A**). In this experiment, the CASP8 activation persisted through 12hrs, although at much reduced levels. Treatment of neutrophils with 0.1μM Dexamethasone (D), however, completely abolished the activation of CASP8 at both 8- and 12hrs. The ability of GCs to impair CASP8 activation was also tested using the natural steroid, HC. As shown in **Figure 3.5B**, neutrophils in this experiment exhibited more robust CASP8 activation during routine culture. In both experiments in **Figure 3.5**, CASP8 activation occurred by 8hr and persisted through 12hrs thus indicating a consistent kinetic pattern. Similar to DEX-treated neutrophils, treatment with HC caused a marked reduction in the 40kDa immunoreactive band at both 8hrs and 12hrs time points. Hence, a loss of CASP8 activity in neutrophils can be attributed indirectly to treatment with physiological stress ligand. Collectively, these results indicated that this class of compounds reduced CASP8 activation in neutrophils. The spontaneous activation of CASP8 required an oligomerization event which is typically orchestrated by the clustering of DRs. While neutrophils are known to express several types of DRs – including TNF-R which actually promotes survival – these cells are especially sensitive to the apoptogenic effects of FasL. Therefore, FasL expression was analyzed to determine if an association between this protein and CASP8 and Bid activation existed.

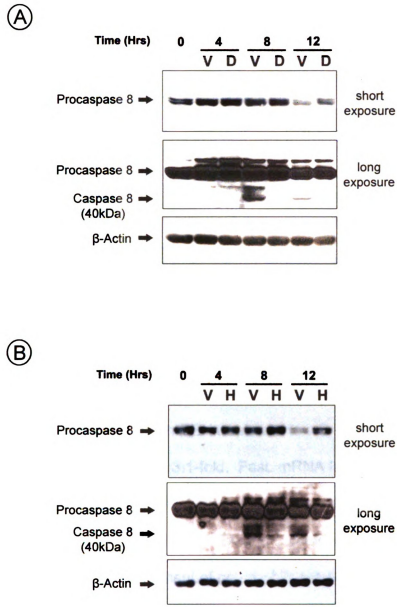

Figure 3.5. GCs Reduce Cleaved Caspase 8 Levels During Neutrophil Apoptosis. Neutrophils were treated with either 0.1μM DEX (A) or 1μM HC (B) and then assayed using Westerns for cleaved Caspase-8. The more abundant Procaspase-8 was detected using a lower exposure time, whereas the products resulting from Caspase-8 cleavage are shown after a prolonged development period. β-Actin served as a loading control for both of these experiments.

Loss of Spontaneous FasL Expression in Response to GCs:

As described in the introduction, GCs have been shown to negatively regulate FasL expression. Since it is also known that neutrophils spontaneously express this death ligand during the normal progression of apoptosis, we wanted to determine if GCs similarly affect this pathway in these myeloid cells. Using relative real-time PCR, kinetic analysis of FasL mRNA expression was performed. Treatment of neutrophils with GCs, either 0.1μM Dexamethasone (DEX) or 1μM hydrocortisone (HC), caused consistent downeregulation of this TNF family member at every time point assayed (**Figure 3.6**). As early as 1hr, DEX and HC caused 3.2-fold and 2.2-fold decreases in FasL mRNA expression, respectively. Expression of this gene decreased even further by 4-fold with DEX and stabilized at a 1.9-fold decrease with HC at 2hrs. At 4hrs, FasL levels began to rebound slightly with DEX treatment to a 2.1-fold decrease whereas HC caused a further decrease to 3.1-fold. FasL mRNA levels, while still depressed relative to control, were at their highest in GC-treated samples by 8hrs as decreases were only 1.4-fold with DEX and 1.5-fold with HC. Collectively, these data indicated that GC treatment of neutrophils resulted in a loss in FasL mRNA expression, but nevertheless began to ramp up expression at late stages of culture.

In order to correlate downregulation of FasL mRNA with FasL protein levels, Western blot analysis was performed on GC-treated neutrophil lysates. Neutrophils treated with 0.1μM DEX were resolved using SDS-PAGE and FasL expression was detected with anti-FasL antibody. As shown in **Figure 3.7A**,

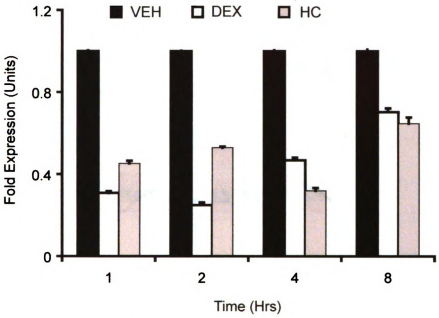

Figure 3.6. GC Treatment Causes a Reduction in FasL mRNA Expression in Neutrophils. Neutrophils were treated with either 0.1μM Dexamethasone (DEX) or 1μM hydrocortisone for 0-8hrs and then analyzed for gene expression using sqPCR. Data shown is the real-time PCR analysis of FasL expression in neutrophils treated with GCs. Standard error is shown and data is representative of two independent experiments.

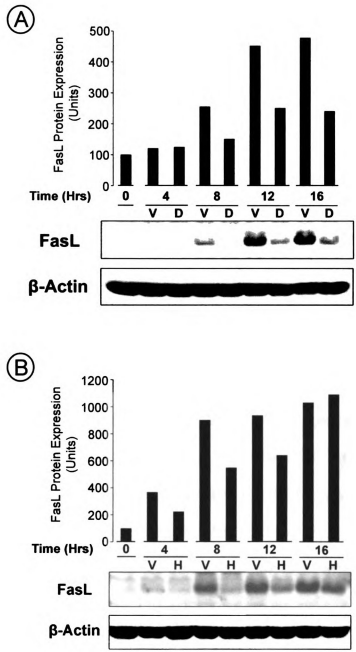

Figure 3.7. GCs Delay Spontaneous FasL Upregulation in Neutrophils. Peripheral blood neutrophils were treated with vehicle (V), **(A)** 0.1μM Dexamethasone (D) or **(B)** 1μM hydrocortisone (H) for 0-16hrs.. Cell lysates were then analyzed via Western analysis for FasL expression. Denistometric data of these immunoblots were normalized using β-Actin (shown here) and the intensity of each band was plotted as arbitrary units in the bar graph depicted above.

neutrophils basally express low levels of FasL even through 4hrs of culture. Spontaneous production of FasL appeared by 8hrs (2.5-fold induction over controls), but was suppressed to basal levels with the addition of DEX. FasL protein expression further increased to 4.5-fold by 12hrs and was maximal at 4.8-fold by 16hrs. In contrast, treatment with DEX reduced FasL levels by 45% at 12hrs and 49% at 16hrs.

Likewise, the natural steroid, HC, caused similar albeit weaker reductions in FasL protein expression (**Figure 3.7B**). In this experiment, 1μM HC suppressed the spontaneous production of FasL which, similar to **Figure 3.7A**, increased with time spent in culture. While HC had a more modest effect on FasL expression at 4hrs, neutrophils treated with this steroid exhibited 49% less apoptosis by 8hrs. Similarly, apoptosis levels continued to be depressed with HC to 32% at 12hrs. At the late time point of 16hrs, however, FasL levels were indistinguishable between vehicle and HC-treated samples. In summary, GCs appeared to inhibit the expression and production of FasL mRNA and protein.

BH3-only Peptide Transduction of Neutrophils

Neutrophils are not amenable to contemporary molecular investigatory techniques such as gene transfection or even some gene interference technologies. Thus the study of a gene or protein in these cells must be accomplished using a very narrow set of tools. Protein transduction in particular has proven to be a valuable technique in introducing a gene product into neutrophils in order to garner more information concerning gene function (Dai et al., 2004). Since sensitivity of neutrophils to Fas-induced apoptosis had already

been established, the downstream target Bid was selected for further study. Moreover, a poly-arginine- (polyR-) linked peptide corresponding to the apoptosis-stimulating BH3 region of Bid had been devised and tested in the laboratory of Stanley Korsmeyer (Letai et al., 2002). This synthetic peptide, which is capable of inducing apoptosis in several cell types, would therefore permit the analysis of Bid function in neutrophils. If indeed neutrophils are sensitive to t-Bid, then introduction of this already apoptogenic form should cause induction of apoptosis in these cells.

In order to examine the effects of Bid on neutrophil apoptosis, we obtained two peptides, one corresponding to the BH3 domain of Bid and the other a mutant form (L90A, D95A) of the same peptide termed control peptide. Each peptide was N-terminally linked to eight D-isomer arginines. This cluster of positively charged, chirally-opposite amino acid residues served to enhance the cellular uptake of the synthetic peptide. For these studies, neutrophils were incubated with 0-30μM of either the BH3-only or control peptide for 6hrs following which samples were harvested and assayed for apoptosis. Additionally, a separate set of samples were incubated with both control/BH3-only peptides and 0.1μM Dexamethasone (DEX) in an effort to gauge pathway interactions.

To ascertain the effects of the BH3-only peptide on neutrophil survival, a dose curve ranging from 0μM peptide (i.e., control) to 30μM peptide was constructed. As shown in **Figure 3.8**, the control peptide caused only modest increases in basal apoptosis levels when compared to the no peptide control (i.e., [0μM]). The mutated, inert peptide caused levels of cell death to rise from 29% of all cells

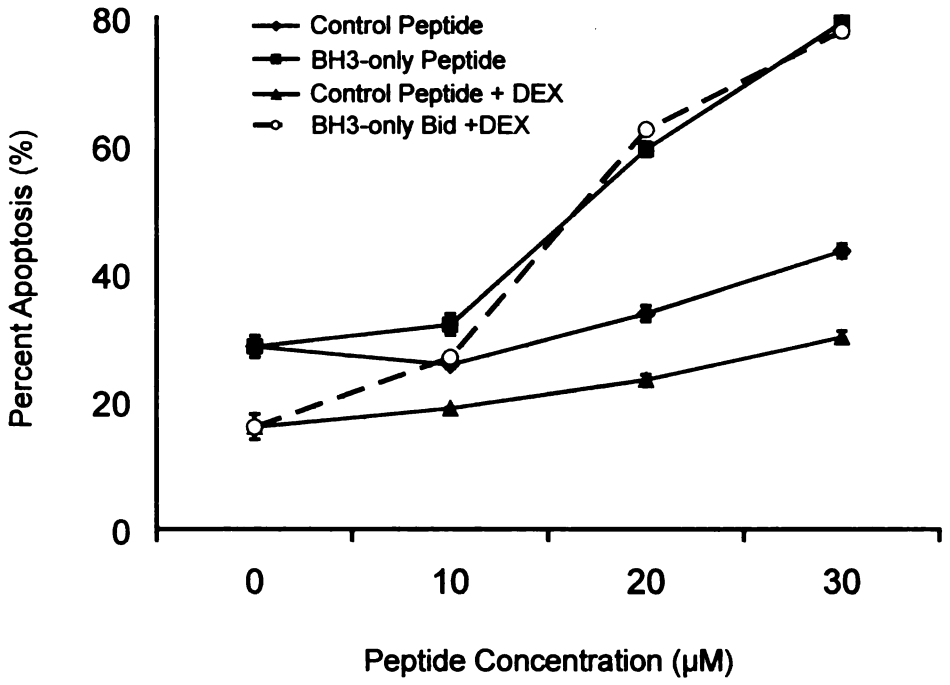

Figure 3.8. Protein Transduction of Neutrophils with the BH3 Domain of Bid Implicates this Pathway in Neutrophil Survival Decisions. Peripheral blood neutrophils were treated with 0-30μM of poly-arginine linked control peptide or the BH3-only domain of the Bid protein for 5hrs. Neutrophils were also treated with 0.1μM Dexamethasone (DEX) in combination with the various peptide treatments. Data shown are the levels of apoptosis that were assessed following treatment using Merocyanine 540 (n=3). This experiment is representative of 3 independent experiments.

in controls to 44% with 30μM of peptide. Compared to the control peptide, 20μM BH3-only Bid caused a 76% increase in apoptosis levels. The effective concentration of BH3-only Bid, though, appeared to be ≥20μM as this peptide did not exert a significant apoptotic effect at the lower 10μM concentration. The highest concentration of peptide tested, 30μM, induced an 82% increase in apoptosis after 5hrs (c.f., 44% for the control peptide). These data indicated that the apoptogenic region of Bid (i.e., the BH3 domain) which would be represented in active t-Bid was indeed capable of inducing apoptosis in neutrophils.

In order to assess pathway interactions and/or redundancies which might exist between GC-mediated pathways and Bid-mediated apoptosis, a separate set of samples were cotreated with both the synthetic peptides and 0.1μM DEX. Treatment of neutrophils with DEX resulted in a 44% reduction of apoptosis after 5hrs of treatment. Cotreatment with both DEX and increasing concentrations of the control peptide resulted in a modest yet steady increase in apoptosis levels, similar to that observed with control peptide alone. By comparison, samples treated with BH3-only Bid and DEX nearly mirrored the results obtained with just BH3-only Bid alone. Treatment of neutrophils with 20μM BH3-only Bid and DEX caused a 167% increase in apoptosis with respect to the control peptide. Samples treated with 30μM of BH3-only Bid and DEX exhibited a 159% increase in apoptosis. Since GCs did not impair BH3-only Bid-induced apoptosis, these data suggested that GCs probably act upstream of the Bid signaling pathway to exert their anti-apoptotic effect(s).

Conclusion

In this chapter we identified the BH3-only Bcl-2 family member Bid as a likely target for GC-mediated suppression of neutrophil apoptosis. We presented evidence that GCs suppress the spontaneous formation of FasL in neutrophils. Moreover, we show that the activities of apoptosis effector proteins CASP8 as well as t-Bid are reduced in response to GCs. Finally, transduction of neutrophils with BH3-only peptide demonstrated the likely involvement of this pathway in the survival of these cells. Collectively, these data indicated that a reduction in both FasL expression and t-Bid formation caused by GCs may explain, in part, the anti-apoptotic effects of these compounds. These findings can be added to the emerging picture of neutrophils as a dynamic and responsive cell type.

The loss of spontaneous FasL expression we observed in GC-treated neutrophils may represent one mechanism by which neutrophils evade cell death. Based on these results, a timeline for the molecular events involved in neutrophil survival can be constructed. The GC-mediated survival of these cells begins with a reduction in FasL mRNA as early as 1hr. Corresponding decreases in FasL protein expression are not observed until 8hrs. The delay between FasL mRNA and protein expression suggests that a separate regulatory mechanism exists to control the timing of FasL protein expression. This may coincide with a cellular commitment to apoptosis which could require an integration of other signaling pathways. Nevertheless, FasL expression preceded a near simultaneous recruitment of other DR-related proteins including CASP8 activation and t-Bid formation which also occurred at 8hrs. We did not include the reduction of Bid mRNA in the construction of this timeline since we

cannot yet link this event with changes in Bid protein expression. Collectively, these results indicated the existence of a FasL threshold which, once reached, resulted in the rapid activation of apoptosis effector proteins including CASP8 and t-Bid. This critical threshold likely represents a level of mFasL required for execution of apoptosis via *inter*cellular ligand occupation of Fas. A threshold theory is supported by the identification of FasL localization to membrane rafts where FasL accumulates during ligand:receptor interactions (Cahuzac et al., 2006). In addition to facilitating the localization of surface proteins, lipid rafts characteristically prevent the internalization of surface receptors thereby amplifying a signaling event. Localization of FasL to lipid rafts, along with the long half-life FasL:Fas complexes, would therefore facilitate the formation of FasL oligomers which are required to produce Fas clustering and consequent apoptosis. Interestingly, an increase in FasL which accumulates during spontaneous apoptosis potentially serves a second purpose of providing a reservoir of chemotactic agent in an *in vivo* setting.

Role of FasL in Neutrophils:

Neutrophils exhibit accelerated apoptosis in response to Fas stimulation by anti-Fas antibodies (Liles et al., 1996). Despite this finding, autocrinic/paracrinic neutrophil apoptosis was largely ruled out as the result of conditioned media transfer experiments (Cox and Austin, 1997). In these studies, Cox et al treated neutrophils with GCs for 24hrs, following which the conditioned supernatant was harvested. Next, a separate set of neutrophils were pre-treated with the GR antagonist mifepristone and then cultured in the conditioned supernatant.

Addition of mifepristone would therefore prevent specific survival effects caused by GCs, yet still permit the activity of other survival agents that might be released into the media in response to these steroids. This group demonstrated that DEX-conditioned media did not alter the survival of Mifepristone-treated neutrophils thereby ruling out an autocrine survival pathway. This approach, however, did not address whether a cell-surface expressed protein might act in a similar manner, but owing to cellular association would therefore not transfer into the supernatant. Coincidentally, this highlights the two cellular states of the proapoptotic protein, Fas which can be expressed as (1) a soluble form (sFasL); or (2) a membrane associated form (mFasL).

FasL is a 32kDa single-pass type II membrane protein expressed on the surface of several cell types, including neutrophils. Membrane associated FasL is cleaved to the smaller (26kDa) soluble form through the proteolytic actions of matrix metalloproteases (MMPs) (Schneider et al., 1998). In comparison to mFasL, the soluble form of this protein appears to play a greater role in cell recruitment through the process of chemotaxis. Indeed, both recombinant sFasL as well as sFasL-containing supernatant failed to induce apoptosis in a number of Fas-sensitive cell types (Oyaizu et al., 1997; Suda et al., 1997), but rather elicited the recruitment of neutrophils (Ottonello et al., 1999). In contrast, the accumulation of the membrane form of this protein through either the use of MMP inhibitors or overexpression increased apoptosis in Fas-sensitive cells (Kang et al., 2000; Oyaizu et al., 1997). Most importantly, the divergent effects of the two forms of FasL on neutrophil survival are in agreement with the existing

body of literature concerning their respective functions. Specifically, we, along with others, propose that neutrophil spontaneous apoptosis is at least partially mediated by mFasL rather than the soluble form of this protein which likely explains why conditioned media failed to alter the survival of these cells.

GCs suppress FasL expression in several cell types (D'Adamio et al., 1997), but this is the first report, to our knowledge, to identify this effect in neutrophils. As described in the introduction, GCs reduce FasL transcription through either (1) inhibition of AP-1 by upregulation of GILZ; or (2) competition of the GR with NF-κB for overlapping response elements. In the latter scenario, activated GR displaces NF-κB thereby diminishing FasL expression. While Fas stimulation is an effective inducer of neutrophil apoptosis, loss of either component of this system - Fas or FasL – did not cause dramatic changes to granulopoiesis (Fecho et al., 1998). Indeed, no significant difference in apoptosis was observed between bone marrow neutrophils derived from control animals and Fas- or FasL-null cells. One explanation for this finding is the overlapping function(s) of several DR-mediated apoptotic pathways which would ensure apoptosis of these cells. Alternatively, a second molecular cue may be required to coordinate multiple pathways to achieve apoptosis.

Evidence for a second molecular event in Fas regulation of neutrophil survival was demonstrated by Holroyd et al through the of crossing *Lyn*$^{-/-}$ and *lpr* mice (Fas null). Animals deficient in these two proteins exhibited a vast increase in granulocytes and their precursors thus indicating important roles for both of these proteins in granulocyte apoptosis (Holroyd S, 2004). This finding demonstrated

that *in vivo* sensitivity of granulocytes to Fas-induced apoptosis required two signals: (1) an intact Fas system; and (2) the tyrosine kinase Lyn. Contrary to these results, however, antisense to Lyn actually blocked increases in neutrophil survival caused by GM-CSF (Wei et al., 1996). Taken together, these findings suggest that Lyn activities in neutrophil survival are complex and may depend on factors associated with particular experimental systems and/or conditions. Future experiments should be careful to examine the activities of various tyrosine kinases found in neutrophils (Lyn, Fyk, Hck, etc), especially during treatment with GCs. Lyn activity, in particular, has been shown to be inactivated by GCs in mast cells (Sancono, 2003). A GC-mediated loss of Lyn in neutrophils, in combination with a reduction in FasL expression, may be sufficient to promote survival in these cells. This would then lead to the activation of various apoptosis effector molecules such as CASP8 and Bid.

Bid-Mediated Signaling:

While cleavage of Bid to apoptogenic t-Bid had been demonstrated for neutrophils during routine culture, it was unclear to what extent this protein promoted apoptosis in these cells. Thus far, very few tools have been developed for researchers wishing to explore gene function in the short-lived neutrophil. To tackle this question, we utilized a protein transduction strategy, originally developed by the Korsmeyer lab, which linked eight D-isomer arginine residues to the BH3-only domain of Bid. Protein transduction is ideal for neutrophil studies since only a short period of time is required for the peptide to be internalized by these cells. Analogous to an overexpressed protein, the positively charged

amino acid residues facilitated the entry of the peptide into the target cell where it accumulates and exerts its function. In these studies, we observed a concentration-dependent increase in the apoptosis of neutrophils treated with the BH3-only Bid peptide. Given the design of our studies, however, we could not deduce that loss of Bid was the sole explanation for GC-mediated neutrophil survival. A critical role for Bid is suggested by the studies presented herein, since addition of GCs did not impact the apoptosis levels of neutrophils treated with BH3-only Bid. This result also indicated that GCs probably act upstream of t-Bid formation which further supports a role for FasL in Bid activation. Alternative approaches, including gene knockdown studies using antisense, could also be helpful in understanding the importance of Bid in neutrophil apoptosis, but these efforts could be mitigated by the stable nature of this protein.

Bcl-2 family member expression in neutrophils is especially responsive to several anti-apoptotic cues including LPS, GM-CSF and ILs. Therefore, we were surprised to observe only modest changes in the expression of this family in response to GCs. Notably, neither A1 nor Mcl-1, Bcl-2 family members typically associated with increases in neutrophil survival, exhibited robust changes in expression. This discrepancy in expression of this family of genes with respect to GC exposure may demonstrate the unique molecular pathway(s) through which GCs signal survival. Several hundred genes have reported GREs, yet none have been associated with any member of the Bcl-2 family. It is curious to note that several pro-inflammatory transcription factors (e.g., NF-κB) are in fact

antagonized by the activated GR, a classical anti-inflammatory protein. This observation further supports the notion that GCs signal non-inflammatory neutrophil survival via a pathway distinct from that of other pro-survival cues. Given the heavy focus on Mcl-1 in GC-mediated survival of neutrophils and due to a lack of changes by other members of this family of protein, Bid was selected for further analysis in these studies.

The inert Bid is cleaved to pro-apoptotic t-Bid through a not as of yet identified mechanism early in neutrophil apoptosis (Maianski et al., 2004; Simon, 2003). The cleavage of Bid is typically accomplished via the action of CASP8, but other proteases including neutrophil cathepsins have demonstrated similar proteolytic capacities. It was demonstrated in this report that both natural and synthetic GCs prevent the formation of t-Bid in cultured neutrophils. To our knowledge, this is the first report that GCs negatively regulate the expression and/or function of a pro-apoptotic Bcl-2-related protein. Given the demonstrated sensitivity of neutrophils to BH3-containing peptides presented herein, a loss in Bid expression in response to GCs conceivably represents one of likely several mechanisms responsible for steroid-mediated survival of these cells. Future efforts should focus on developing a loss of function model for Bid through either antisense or animal knockouts in which the importance of this protein in GC-treated neutrophils can be further assessed.

Similarly, G-CSF - a potent suppressor of neutrophil apoptosis - has also been shown to prevent the formation of Bid in neutrophils. Interestingly, the protein synthesis inhibitor cyclohexamide which abrogates the protective effects

of G-CSF, did not restore t-Bid formation in samples treated with both inhibitor and survival agent (Maianski et al., 2004). It is therefore possible that Bid cleavage occurs via the actions of an unidentified pre-formed protein, although this initially appeared less likely as GC-mediated neutrophil survival has been shown to require intact protein synthesis activities (Cox and Austin, 1997). While we also observed a marked reduction in t-Bid formation, full-length Bid actually exhibited slight increases in expression in response to both types of GCs. The divergent responses of Bid mRNA and protein to GC treatment suggested that loss of Bid mRNA probably did not contribute to reduced t-Bid formation. This finding further suggests a post-translational mechanism for the regulation of Bid activity in neutrophils. The role of Bid in the regulation of myeloid cell survival has also been elucidated through knockout animal models.

Despite appearing normal at birth, Bid null mice develop fatal myeloid hyperplasia, presenting with an 8-fold excess of circulating neutrophils (Zinkel et al., 2003). Moreover, myeloid precursors derived from these $Bid^{-/-}$ mice were unresponsive to DR agonists such as TNF/Actinomycin and anti-Fas. Taken together, these data support a prominent role for Bid in the regulation of neutrophil fate, especially in response to DR agonists. This was therefore cause for our lab to consider what stimuli might initiate the Bid cleavage event. Since neutrophils have demonstrated apoptotic sensitivity to the Fas/FasL system, the possibility that this pathway was a component of GC-mediated neutrophil survival was also assessed.

In this chapter, we showed that GCs caused only modest changes in Bcl-2 family expression. The BH3-only Bcl-2 family member Bid was identified as one of those genes that was altered in response to steroid treatment. Indeed, real-time PCR confirmed that GC treatment caused a 2.0-fold decrease in Bid expression and reduced t-Bid formation. Moreover, several of the components involved in signaling apoptosis through t-Bid, FasL and CASP8 were also downregulated in response to GCs. Finally, we show that protein transduction of neutrophils with a BH3-only construct resulted in the apoptosis of these cells, thus demonstrating the likely involvement of this protein in neutrophil apoptotic processes. GCs appear to target Bid as mRNA for this gene and the active form of Bid, t-Bid, were both decreased. A loss in Bid likely represents one mechanism by which GCs signal survival in neutrophils.

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SUMMARY

A BRIEF OVERVIEW OF: "DISRUPTION OF APOPTOTIC SIGNALING PATHWAYS DURING GLUCOCORTICOID-INDUCED SURVIVAL OF HUMAN NEUTROPHILS"

by

Joseph W. Frentzel

Summary

As described in the introduction section of this thesis, peripheral blood neutrophils are critical mediators of the inflammatory component of the immune system. Indeed, loss of this cell type renders individuals unable to mount an effective defense against pathogens, including routine normal flora such as *S. auerus*. Because neutrophils play such an integral role in immune defense, and because they are produced in very large numbers, minor perturbations in their production and/or survival are an important facet of the resolution of inflammation and host defense. Compounds that result in the increase in neutrophil numbers include several types of bacterial products (e.g., butyrate, LPS), cytokines/chemokines (GM-CSF and G-CSF) and, of interest to the studies described herein, glucocorticoids (GCs).

GCs have been shown to promote the survival of neutrophils both *in vivo* and *in vitro*. While strides have been made toward the determination of the mechanism by which GCs signal survival in these cells, there still exists a dearth of information on the molecules involved in the execution of this survival process. To this end, this body of work describes the efforts made to elucidate new targets involved in GC signaling in neutrophils as well as tests for functionality in mediating the neutrophil survival response. Because neutrophils are a terminally differentiated cell type with low transfection efficiencies, alternative molecular strategies had to be employed to study gene and/or protein function in these cells.

In Chapter 1, GCs were shown to delay neutrophil apoptosis by up to 65% after 12hrs of culture in normal media. This finding was consistent with previously published results which demonstrated the prosurvival effects of GCs. GCs were also shown in this chapter to signal through the GR, but not cause alterations in GR mRNA. Rather, treatment of neutrophils with GCs resulted in the translocation of the GR to the nuclei of these cells. In an effort to identify a GC-responsive gene in neutrophils, the highly GC-sensitive gene GILZ was identified to be upregulated in these cells. GILZ mRNA was found to be upregulated 3.9-fold by 4hrs in DEX-treated neutrophils and GILZ protein was upregulated by 4-fold also at 4hrs. Because GILZ has been shown to be involved in the survival processes of other cell types including B-cells, the role of GILZ in neutrophil survival was further explored in these studies. Similar to the overall survival of neutrophils, GILZ induction was demonstrated to be GR-dependent. Pretreatment of neutrophils with GR antagonists resulted in ablated neutrophil survival responses. Owing to the high induction of GILZ in response to GCs, the possibility of this molecule playing an important role in neutrophil survival was further explored.

Due to the prevalent use of GCs in medicine, several types of GCs, both natural and synthetic, are available for use in neutrophil studies. Each of these GC types possesses different potencies with respect to GR signaling and gene induction. Taking advantage of this feature, it was determined that these GC compounds also induce GILZ to varying levels. Moreover, a correlation of GILZ induction and neutrophil survival in response to the various GCs revealed a

highly linear relationship (R2>0.95) between these two phenomena meaning that neutrophils with the highest levels of GILZ also exhibited the most survival. These data suggested that GILZ was likely involved in promoting the GC-induced survival of neutrophils. To further assess the functional role of this protein, antisense studies were attempted in an effort to deplete GILZ mRNA and/or protein in neutrophils. Due to technical handicaps, including vehicle (DOTAP) disruption of cellular integrity and unaltered GILZ protein levels in antisense-treated samples, these experiments were inconclusive in establishing a role for GILZ. Nevertheless, the data generated thus far by these studies suggested that GILZ plays an important role in neutrophil survival. An improvement in the molecular tools used for the study of neutrophils and/or a better understanding of the domains necessary for GILZ-mediated survival effects will be required to determine the extent by which this protein prolongs neutrophil lifespan.

To further develop a pool of relevant targets for downstream studies, Chapter 2 described how an apoptosis-centric microarray was employed to study the gene effects of GCs on human neutrophils. The array employed in these studies contained 350 genes corresponding to apoptosis-related and other survival proteins. For these experiments, neutrophils were treated with DEX for 3hrs and then assayed for gene expression profiles. Of the 350 genes, 25 (7%) were identified by microarray to be altered in response to GC treatment including cytokine-related genes (40%), Bcl-2-related genes (Mcl-1 and Bid) and cell signaling/transcription-related genes (CCND3, STK17B, IκBα, FOS1, P53 and IκBε).

Of the gene targets identified by these studies, an upregulation in CCND3 (Cyclin D3) and a downregulation in the BH3-only Bcl-2-related molecule Bid appeared to be promising candidates for studying GC-mediated neutrophil survival. These two genes were identified as particularly relevant due the findings that Bid null mice exhibited robust increases in granulocytes whereas Cyclin D3 null mice had defective granulocyte maturation (studies described in Chapter 2). While other genes identified by these studies might also play a significant role in GC-mediated neutrophil survival, the connection with this survival phenomenon is less obvious as gleaned from the literature. Therefore, based on the hypothesis being tested by the studies presented in this thesis which suggested the involvement of Bcl-2 family member(s) in neutrophil survival, it was proposed that the loss of Bid represented at least one mechanism by which neutrophils evade apoptosis during GC treatment.

Using real-time PCR to specifically assay select Bcl-2 family members, Bid was identified to be downregulated 2.0-fold in response to 4hrs of GC treatment as described in Chapter 3. An extended kinetic analysis confirmed the downregulation of Bid mRNA as early as 4hrs which continued through 8hrs of GC treatment. Surprisingly, though, full length Bid protein was unaffected by the downregulation of Bid mRNA. Instead, the apoptogenic truncated form of Bid (tBid) was decreased in samples treated with either DEX or HC. Since tBid executes apoptosis to a much greater extent relative to its inert parent molecule, this finding suggested that a reduction in tBid might be one possible explanation for the reduced apoptosis observed in GC-treated neutrophils. To flesh out the

effects of GCs on Bid-related pathways, the involvement of caspase 8 and FasL was also assessed.

Bid, a relatively inert molecule is cleaved to a truncated form through the action of caspase 8 along with other proteases including neutrophil cathepsins. Prior to this event, caspase 8 activation typically occurs through the clustering of death receptor proteins (e.g., TNF-R and Fas) and subsequent autoproteolysis. To determine the extent of caspase 8 involvement in neutrophil apoptosis/survival, activation of this protein was monitored via the generation of cleavage products which represents the conversion of procaspase 8 to active caspase 8. A 40kDa product representing the cleaved form of caspase 8 was reduced in samples treated with GCs as early as 8hrs. Using real-time PCR, FasL (but not Fas) was also identified to be involved in GC-mediated neutrophil survival as treatment with these compounds caused a 3.2-fold decrease in this gene as early as 1hr. Correspondingly, treatment of neutrophils with GCs also ablated the spontaneous increase of FasL in cultured neutrophils. Finally, a peptide construct possessing the pro-apoptotic BH3-only portion of Bid tethered to a cell permeable amino acid sequence was utilized to study the effects of tBid on neutrophils. Indeed, 20μM of this peptide construct induced a 76% increase in neutrophil apoptosis in comparison to controls. Moreover, the pro-apoptotic effects of these peptide constructs were unaltered by addition of GCs. Collectively, these data suggested a strong apoptotic role for Bid which can be downregulated through the actions of GCs.

This body of work presents several firsts in the field of neutrophils including the first evidence of the involvement of a prototypical GC-responsive gene (GILZ) in these cells. This publication also details the first application of microarray technology to the study of GC-treated *human* neutrophils. This powerful tool has generated ample targets for future study in the determination of neutrophil survival in response to GCs. Finally, this has been the first study to identify the downregulation of a pro-apoptotic protein, Bid, in the regulation of neutrophil survival in response to GCs. Moreover, an alternative molecular approach – protein transduction using polyR constructs - was successfully used in determining the extent by which Bid exerts apoptotic effects in these cells. Collectively, these data will serve to advance the understanding of anti-inflammatory compounds such as GCs on neutrophils and neutrophil survival and to better assist in the generation of more selective therapeutic interventions.

APPENDIX A

MICROARRAY IDENTIFIED GENES EITHER NOT RELATED TO SURVIVAL OR UNABLE TO BE VALIDATED

By

Joseph W. Frentzel

Genes Without Relationship To GCs

NALP6

A 3.4-fold increase in NALP6 expression was identified using microarray analysis (**Table S1**). Similarly, sqPCR identified a 3.6-fold increase in expression of this gene by 2hrs (**Figure S1**). Expression of NALP6 dipped to 3.4-fold by 4hrs in response to DEX, but increased to 8.3-fold by 8hrs. Surprisingly, HC which is generally considered to be a weaker agonist relative to DEX, induced a 4.5-fold increase in NALP6 expression by 2hrs. By 4hrs, expression of this gene dropped to 4.0-fold in response to HC and by 8hrs, reached its nadir at 3.1-fold.

Despite having been discovered nearly 6 years ago, very little information has been published regarding NALP6. Expression of this gene has been shown to be highly restricted to T-cells and granulocytes (Grenier et al., 2002). NALP6, along with an additional protein (ASC), has been shown to activate pro-caspase 1 which functions to process pro-IL1β as well as pro-IL18. While generally considered to be a pro-inflammatory molecule, there is insufficient evidence to conclusively determine a role for this protein.

ZA20D2

DEX caused a 3.3-fold induction of ZA20D2 (ZNF216) expression in neutrophils treated with DEX for 3hrs (**Table S1**). sqPCR validation of these results indicated similar degree of expression in response to DEX as this GC

GENE	qPCR Validation (Hrs) 2	4	8	Microarray Results (3Hrs)
NALP6	3.6	3.4	8.3	3.4
ZA20D2	2.4	3.9	5.3	3.3
SOCS1	DID NOT AMPLIFY			3.0
RALB	1.9	2.7	3.4	2.7
NFAT3	DID NOT AMPLIFY			2.4
NEDD4	-1.0	-1.2	-1.2	-2.2
CD40	1.1	1.3	-1.5	-2.1

TABLE S1. Comparison of Microarray Data and Real-Time Data for Genes not Validated or Related to Glucocorticoid and/or Survival Pathways

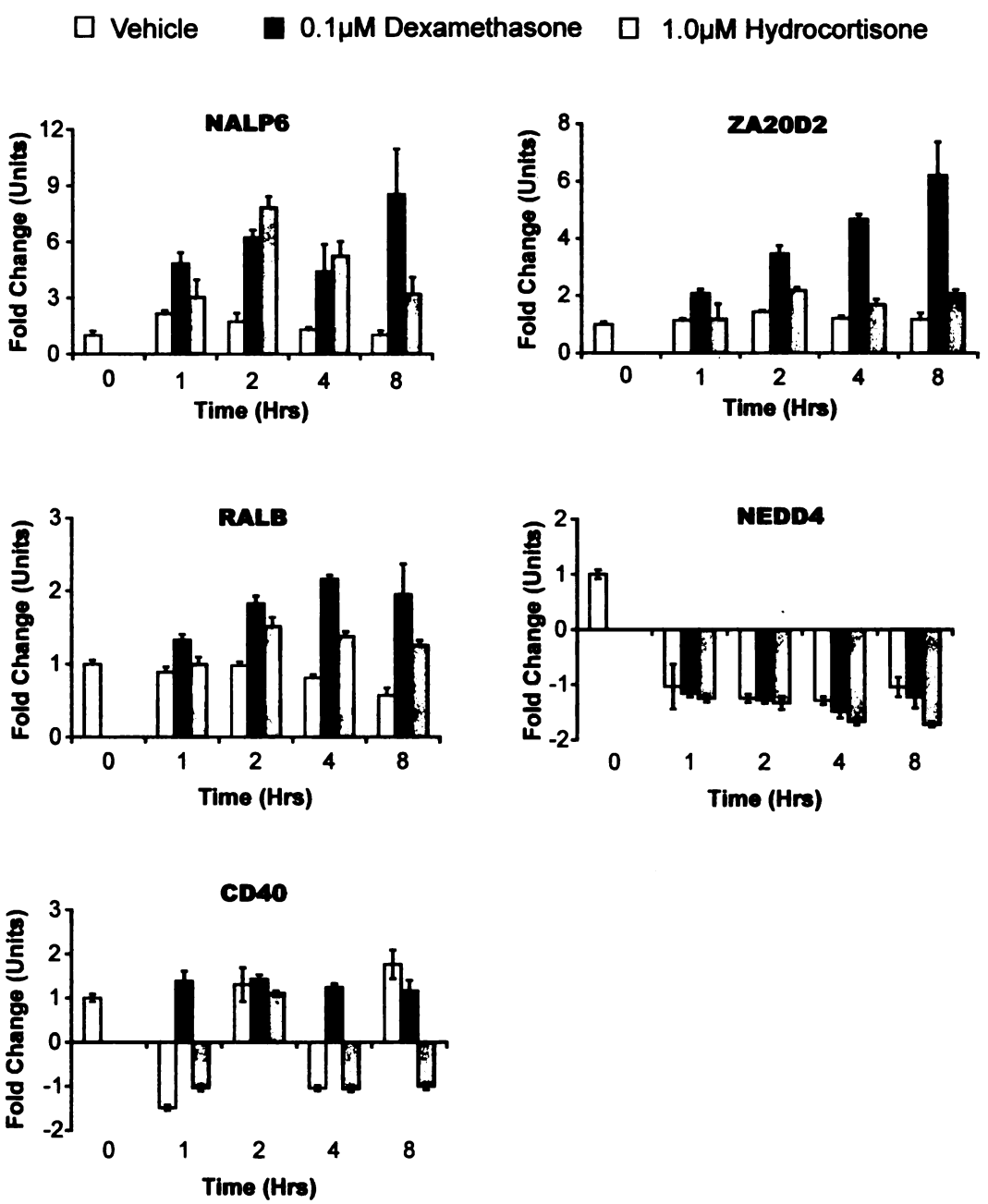

Figure S1. sqPCR Results of Genes not Validated or Related to to Glucocorticoid and/or Survival Pathways. Data shown is the relative real time PCR analysis of either invalid microarray identified genes or genes with insufficient evidence to include as genes of interest. Each data point represents the mean of at least 3 samples and is normalized to β-Actin. Standard error is displayed for each plot.

caused 2.4-fold and 3.9-fold induction levels of this gene by 2- and 4hrs, respectively (**Figure S1**). Expression levels for this gene in response to DEX peaked at 5.3-fold by 8hrs. The natural GC HC, however, induced only modest expression of this gene which peaked at 1.8-fold by 8hrs. The expression levels of ZA20D2 was only 1.5-fold and 1.4-fold at 2- and 4-hrs, respectively. These data especially highlight the potency differences between these two types of GCs, one synthetic; the other naturally occurring.

ZA20D2 (ZNF216) was induced in RAW264.7 monocytic cells using a variety of cytokines (RANKL, TNFα and IL1β) (Hishiya et al., 2005). Expression of the full length form of this protein was able to inhibit osteoclast differentiation thus indicating a role in the maturation of this cell-type. This gene was also shown to be upregulated in both wild type and metallothionein-null fibroblasts treated with 100uM Cu. Metallothionein is a metal-chelating protein that may function to protect cells from excess concentrations of Zn and/or Cu. In an effort to decipher function of this gene, Hishiya et al determined that ZA20D2 binds directly to ubiquitin and, using ZA20D2 knockout mice, they observed the accumulation of ubiquitinylated proteins in the muscle cells of the genetically altered animals (Hishiya et al., 2006). Although these data suggest a role for ZA20D2 in the degradation of cellular proteins, the relationship between this gene and GCs and/or neutrophils remains unclear. The possibility exists that this gene might participate in autophagic processes which would be prevalent during prolonged periods of stress. Autophagy represents an increase in cellular efficiency as the proteins contained in the cell are utilized in an effort to conserve energy. Since

ubiquitination is essential to autophagy (Otto et al., 2003), the accumulation of ubiquitinylated proteins in ZA20D2$^{-/-}$ mice could reflect disruption of autophagic processes in these cells.

RALB

RalB expression was upregulated 2.7-fold in response to DEX as measured using microarray analysis (**Table S1**). sqPCR validation of this gene revealed strikingly similar expression patterns with 1.9-fold and 2.7-fold inductions with DEX at 2- and 4hrs, respectively (**Figure S1**). Expression of this gene continued to rise (3.4-fold) through 8hrs with DEX treatment. HC-treated neutrophils demonstrated a similar pattern in RalB expression with 1.5-fold induction at the 2hr time point and a 1.7-fold induction by 4hrs. Like DEX-treated cells, HC caused a continued rise (up to 2.2-fold) in the expression of this gene through 8hrs.

RalB is a monomeric GTP-binding protein that has been shown to be required for the survival of some tumor cells (e.g., HeLa, MCF7, SW480), but not for non-cancerous cells (human epithelial or prostate cells) (Chien and White, 2003). In a separate publication, Chien et al demonstrated an association between RalB and Sec5, a component of a protein complex (exocyst) involved in tethering vessicles to dynamic plasma membranes (Chien et al., 2006). This complex was further shown to recruit and activate an atypical IκB kinase, TBK1. TBK1 along with IKKε has been shown to be a critical mediator in the production of IFNβ, an important component of the host anti-viral response (Fitzgerald et al., 2003). RalB has also been shown to block neutrophil maturation which may be

important for the GC-mediated expansion neutrophil progenitors in the bone marrow (Omidvar et al, 2006). Moreover, inhibition of RalB expression using siRNA prevented migration of two cancer cell types via the disruption of cytoskeletal Actin (Oxford et al., 2005).

Genes Not Validated

NEDD4

A 2.0-fold reduction in NEDD4 expression was observed with microarray analysis of DEX-treated neutrophils (**Table S1**). These results, however, were not validated by sqPCR as levels of this gene in DEX- and HC-treated samples were similar to controls. These results indicate that the loss in NEDD4 expression observed using the microarray may not be representative of how GCs regulate the expression of this gene.

NEDD4 was identified originally in the murine central nervous system (Harvey and Kumar, 1999). This gene was recently ascribed the function of an E3 ubiquitin ligase which associates with and ubiquitinates RNA pol II following UV-induced damage of DNA (Anindya et al., 2007). Retroviruses also take advantage of this protein to facilitate viral budding as VP40 interacts with NEDD4. Since NEDD4 family members possess a C2 domain which is thought to facilitate interaction with phospholipids, including plasma membranes, the virus takes advantage of this property enabling the passage and dispersal of viral particles from the cell (Ingham et al., 2004). It is unclear if neutrophils express NEDD4 or what role this protein might play in this cell type.

TNFRSF5 (CD40)

Microarray analysis revealed a 2.0-fold increase in TNFRSF5 expression in response to DEX (**Table S1**). Similar to the gene above, NEDD4, this result was not successfully validated using sqPCR. Neutrophils treated with DEX for 8hrs was the only sample that exhibited a decline in expression (1.5-fold reduction). The remaining samples all demonstrated slight increases in expression in response to either DEX or HC.

CD40 belongs to the TNF family and is expressed by a variety of cell types including endothelial cells, epithelial cells, monocytes and, as recently discovered, neutrophils (Khan et al., 2006). The soluble ligand for CD40, sCD40L, is secreted by platelets and elicits a proinflammatory response including the priming of the oxidative response of neutrophils. The synthetic GC budesonide potently inhibited CD40 expression the granulocytic eosinophil (Ohkawara et al., 1996).

SOCS1

Microarray analysis of SOCS1 expression identified a 3.0-fold increase in this gene (**Table S1**). Using primers designed in a manner similar to other genes assayed in this write-up, no amplification of SOCS1 was observed. Since SOCS1 expression has already been described for neutrophils, the amplification issue experienced for this gene is probably due to the primer sequences themselves. A redesign of primers and the addition of a SOCS1 positive sample should be employed in future studies in an effort to validate this gene.

SOCS1 (Suppressor of Cytokine Signaling-1) has been shown to be an important regulator of cell survival decisions especially during serum

starvation. In one study, SOCS1$^{-/-}$ fibroblasts grown in serum-deficient media continued to grow whereas the wild-type cells underwent typical serum starvation-induced apoptosis (Rottapel et al., 2002). Even more interesting is the observation that SOCS1$^{-/-}$ mice die shortly after birth due to a leukemic myeloproliferative disorder. Consistent with the observed role for SOCS1 in fibroblasts, this gene, when overexpressed in an eosinophilic cell line, induced apoptosis. SOCS1 expression was upregulated in both a pre-B-cell line (Yoshida et al., 2002) as well as in lymphoblastic CEM cells (Thompson and Johnson, 2003) induced to undergo apoptosis using GCs. SOCS1 levels were also elevated in neutrophils treated with GM-CSF (Tortorella et al., 2006) and IL8 and fMLP (Stevenson et al., 2004). Since all of these agents – GM-CSF, IL-8, fMLP or GCs - delay neutrophil apoptosis, SOCS1 induction may be a common theme shared by many survival pathways in neutrophils.

NFAT3

Microarray analysis of NFAT3 (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4) expression in DEX-treated neutrophils indicated a 2.4-fold induction in this gene (**Table S1**). Amplification of this gene using the primers described in Table 1, however, was not possible. As in the case of SOCS1, the lack of amplification could be due to the primers or, in the case, the lack of expression of this gene in neutrophils. Since NFAT3 has not been studied in this cell type, a positive control should be employed in future studies to rule out this possibility.

NFAT3 is unique among other NFAT family members in that expression of this is form occurs principally in non-lymphoid tissues (Homey et al, 1995). An interaction between NFAT3 and the AF1 domain of estrogen receptor beta (ERβ) was detected using a yeast-2-hybrid strategy (Zhang et al., 2005). NFAT3 was found to act as a coactivator in ER signaling in breast cancer cells and, conversely, inhibits ER signaling in kidney cells (Zhang et al., 2007). Despite the demonstrated interactions between this gene and a member of the nuclear hormone receptor family, a link between the GR and NFAT3 has yet to be established. Nevertheless, it is apparent that the cellular context of the expression of NFAT3 plays a large role in predicting the function of this gene.

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