Regulation of Toll-like Receptor 4 Signaling and Expression by Endogenous Heat Shock Protein 70 in the Newborn Intestinal Epithelium

by

Amin Afrazi

BSc. Molecular Biology, University of Pittsburgh 2006

Submitted to the Graduate Faculty of

Cellular and Molecular Pathology in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This thesis dissertation was presented

by

Amin Afrazi

It was defended on

August 31, 2012

and approved by

Youhua Liu, PhD, Professor and Committee Chair, Department of Pathology

Timothy R. Billiar, MD, Professor, Departments of Surgery and Pathology

Jeffrey Brodsky, PhD, Professor, Department of Biological Sciences

Jon D. Piganelli, PhD, Associate Professor, Departments of Immunology and Pathology

David Binion, MD, Professor, Department of Medicine, Division of Gastroenterology

Dissertation Advisor: David J. Hackam, MD, PhD, Professor, Departments of Surgery and Pathology

REGULATION OF TOLL-LIKE RECEPTOR 4 SIGNALING AND EXPRESSION BY ENDOGENOUS HEAT SHOCK PROTEIN 70 IN THE NEWBORN INTESTINAL EPITHELIUM

Amin Afrazi, PhD

University of Pittsburgh, 2012

Necrotizing enterocolitis (NEC) is the leading cause of gastrointestinal-related mortality in premature infants and we have previously shown it develops, in part, under conditions of exaggerated Toll-Like Receptor 4 (TLR4) signaling and expression in the preterm newborn intestinal epithelium. Because NEC does not develop spontaneously, despite the presence of seemingly tonic stimulation of intestinal TLR4 by commensal flora in the newborn, we hypothesized that mechanisms must exist to constrain TLR4 signaling that become diminished during NEC pathogenesis. Microarray analysis of murine ileal tissue from animals subjected to a model of NEC demonstrated a sharp decrease of expression of the intracellular stress response protein and chaperone Heat Shock Protein-70 (HSP70). We demonstrate that the induction of intracellular HSP70 in enterocytes dramatically reduced TLR4 signaling, as assessed by LPS-induced NF-κB translocation, cytokine expression, and apoptosis. These findings were confirmed *in vivo*, using mice that either globally lacked HSP70 or overexpressed HSP70 specifically within the intestinal epithelium. TLR4 activation itself significantly increased HSP70 expression in enterocytes, which provided a mechanism of autoinhibition of TLR4 signaling in

enterocytes. In seeking to define the mechanisms involved, intracellular HSP70-mediated inhibition of TLR4 signaling required both its C-terminal co-chaperone-binding EEVD domain and association with its co-chaperone CHIP, an E3 ligase responsible for the targeted ubiquitination and proteasomal degradation of TLR4. The expression of HSP70 in the intestinal epithelium was significantly decreased in murine and human NEC compared with healthy controls, suggesting that loss of HSP70 protection from TLR4 could lead to NEC. In support of this, intestinal HSP70 overexpression in mice and pharmacologic up-regulation of HSP70, via Celastrol, reversed TLR4-induced cytokines, TLR4 expression, enterocyte apoptosis, as well as prevented and treated experimental NEC in an HSP70-dependent manner. Thus, these data shed light upon a novel TLR4 regulatory pathway within the newborn intestinal epithelium involving HSP70 that may be pharmacologically activated to prevent and/or limit NEC severity.

Keywords:

Intestine, Lipopolysaccharide, Toll-Like Receptors, Innate Immunity, Heat Shock Response, Chaperone, Heat Shock Proteins, ER stress, Unfolded Protein Response, Necrotizing Enterocolitis, Neonate, Inflammation, Apoptosis, Ubiquitination, CHIP, Caspase

TABLE OF CONTENTS

LIST OF TABLES

List of figures

PREFACE

I would like to sincerely thank members of the Hackam lab who I have worked closely with during my thesis research for their numerous insights, technical advice and friendship in particular Chhinder Sodhi, Richard Siggers, Charlotte Egan, Hongpeng Jia and Ibrahim Yazji. I would particularly like to thank the members of my thesis committee for their invaluable assistance and patience throughout my thesis work and training. I am indebted to their intellectual support that has consistently served as a true example to follow throughout my training and will continue to during my career as a scientist. It has been through them that I have come to realize what defines a successful career is not the acquisition of accolades but learning to think as a scientist, always asking and chasing the critical question. I would also like to thank my friends and family for their unwavering support for which I am and will remain truly grateful. Finally, I would like to thank my Thesis Advisor, Dr. David J. Hackam, for all of his personal support and mentorship over the past few years.

The children of Adam are limbs of each other
Having been created of one essence.
When the calamity of time afflicts one limb
The other limbs cannot remain at rest.
If you have no sympathy for the troubles of others
You are not worthy to be called by the name of "man".

-Saadi

The truth may be puzzling. It may take some work to grapple with. It may be counterintuitive. It may contradict deeply held prejudices. It may not be consonant with what we desperately want to be true. But our preferences do not determine what's true. We have a method, and that method helps us to reach not absolute truth, only asymptotic approaches to the truth — never there, just closer and closer, always finding vast new oceans of undiscovered possibilities. Cleverly designed experiments are the key.

-Carl Sagan

If you take a look at science in its everyday function, of course you find that scientists run the gamut of human emotions and personalities and character and so on. But there's one thing that is really striking to the outsider, and that is the gauntlet of criticism that is considered acceptable or even desirable. The poor graduate student at his or her Ph.D. oral exam is subjected to a withering crossfire of questions that sometimes seem hostile or contemptuous; this from the professors who have the candidate's future in their grasp. The students naturally are nervous; who wouldn't be? True, they've prepared for it for years. But they understand that at that critical moment they really have to be able to answer questions. So in preparing to defend their theses, they must anticipate questions; they have to think, "Where in my thesis is there a weakness that someone else might find—because I sure better find it before they do, because if they find it and I'm not prepared, I'm in deep trouble."

-Carl Sagan

1.0 INTRODUCTION

1.1 INNATE IMMUNITY

The immune system is composed of a series of biological mediators both, cellular and humoral, primarily aimed at combating invading pathogens in order to not only return the organism to homeostasis but to maintain it as well (1). The immune system is divided into two major arms: the innate and adaptive. The adaptive or acquired arm consists of a host of specialized cells arising from the lymphoid cell lineage in hematopoiesis. Comprising this cellular group are the CD4+ and CD8+ T cells along with B cells chiefly responsible for antibody production (2,3). These members of the adaptive immune system possess a large and unique repertoire of antigen receptors that maintain the impressive ability to recognize a wide array of specific antigens and induce the clonal expansion of those lymphocytes possessing the critical antigen receptor specific to an invading pathogen (1). The ability of the immune system to mount a specific and directed clonal expansion and honed immune response to a particular invading pathogen bears the significant cost of time. An effective adaptive immune response may require 3 to 5 days, which would leave the organism dangerously vulnerable if it were not for the “first-line” of host defense: the innate immune system. The innate immune system is in fact the evolutionarily older arm of the immune system from which the adaptive arm sprouted and exists in nearly all multicellular organisms (1). The innate immune system, like the adaptive

arm, consists of both cellular and innate humoral factors, which are capable of responding to and attenuating a wide variety of invading pathogens **(Figure 1).** The ability of the host to recognize invading pathogens is a fundamental event that must take place rapidly if clearance of the invader and a return to homeostasis is to occur. This rapid recognition and response to invading microbes occurs primarily via a group of microbial recognition receptors known as Pattern Recognition Receptors (PRRs) which maintain the ability to recognize evolutionarily conserved microbial motifs termed Pathogen-Associated Molecular Patterns (PAMPs) (4). The PRRs serve as sentinel receptors upon the cells of the innate immune system comprising a majority of their ability to recognize microbial invaders and initiate an effective immune response. Following PAMP-PRR recognition a beautifully well coordinated signaling and response cascade is initiated culminating in the generation of antimicrobial agents such as reactive oxygen and nitrogen species alongside the generation and release of cytokines and chemokines aimed at the activation and recruitment of neighboring immune cells. Furthermore, released cytokines and chemokines result in up-regulation of adhesion molecules on both the endothelium and immune cells to facilitate immune cell recruitment. PRR signaling also gives rise to induction of acute phase protein production, which comprises a family of proteins with varying physiological functions for the immune system including antimicrobial agents (e.g. C-Reactive Protein and Complement factors), pro-coagulant factors, as well as negative regulators to ensure attenuation of the immune response following clearance of the inciting pathogen (5). Additionally, the two arms do not exist separately from one another and are in fact heavily integrated. As elegantly demonstrated by Medizhtov and Janeway(6), PRR stimulation leads to a significant up-regulation of co-stimulatory molecules critical to T cell activation and initiating crosstalk between the innate and adaptive immune system.

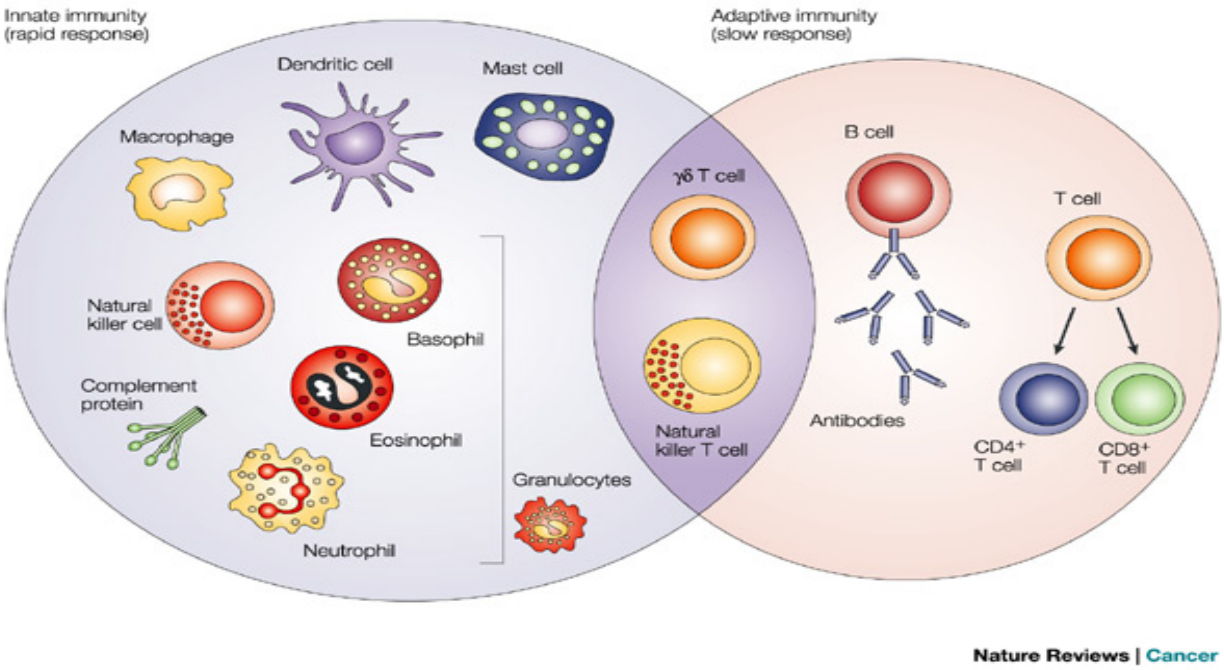


Figure 1.Cellular components of both the innate and adaptive arms of the immune system. The innate immune system is comprised macrophages, dendritic cells, mast cells, neutrophils, eosinophils, basophils and NK cells which initiate and mediate the rapid response to invading pathogens. The innate immune system comprises the front line of defense allowing the cells of the adaptive immune system, CD4 and CD8 T cells and B cells, to mount a slower but specific and directed immune response characterized by clonal expansion of lymphocytes and immune antigenic memory generation and maintenance. *Figure reprinted by permission of Macmillian Publishers Ltd: Nature Reviews Cancer. Article: (7). Copyright 2004*

1.1.1 Toll-like Receptors

The first group of PRRs to be discovered and subsequently the best characterized were the Toll Receptor family. Initially, Toll Receptors were identified and classified in Drosophila as playing a fundamental role in mounting antifugal immune responses in adults and in the signaling cascade regulating dorsoventral polarity in the fruit fly embryo (8). Shortly after this characterization, the human homologue of the Drosophila Toll receptors, named the Toll-like Receptor (TLR) were identified as playing an important role in crosstalk between the innate and adaptive immune system (6). To date a total of 10 and 12 TLRs have been identified in humans and mice respectively (4). The first PRR-PAMP identification was mapped to TLR4 as bacterial endotoxin lipopolysaccharide (LPS) derived from the cell wall of Gram-negative bacteria (6,9).

Subsequent identification of distinct PAMP recognition has been delineated and extended to various PAMPs derived from viruses, bacteria, mycobacteria, fungi and parasites for all TLRs except for human TLR10 and mouse TLR12 and 13 (4). These include lipoproteins (TLRs1, 2, 6), double-stranded RNA (TLR3), Flagellin (TLR5), single-stranded RNA (TLR7, 8) and DNA (TLR9) (10) (**Figure 2**). The TLRs are a group of type I transmembrane proteins that bear a horseshoe-like solenoid shaped ectodomain containing 16-28 tandem leucine-rich repeats, which are important for ligand recognition, (11-14). The ectodomain sequences of the TLRs are strikingly similar despite the vast diversity of ligands they are capable of recognizing. One manner by which this diversity manifests is via amino acid variations within the leucine-rich modalities (LxxLxLxxN) (4). TLRs also bear a single transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain (15), so named as the TIR domain consists of roughly 150 amino acids which display homology with the IL-1 receptor, which is important for the actual adaptor protein recruitment that are fundamental to the signal propagation of the TLRs. TLRs occur as dimers (16). A number of TLRs have been shown to form heterodimers (TLR1-TLR2, TLR2-TLR6, TLR8-TLR7 or TLR9) while others (TLR3, TLR4 and TLR9) have been shown to homodimerize (17,18). TLR dimers are believed to exist pre-assembled in a low-affinity complex prior to ligand binding. Upon ligand binding to symmetrically dimerized ectodomains, a conformational change is believed to occur, positioning the two TIR domains in closer proximity and yielding a suitable platform for the formation of an adaptor protein signaling complex that subsequently leads to activation of specific transcription factors (17). Most TLRs exists as surface bound receptors, however a small number (TLR3, TLR7 and TLR9) are localized intracellularly within endosomes as illustrated in **Figure 2.**

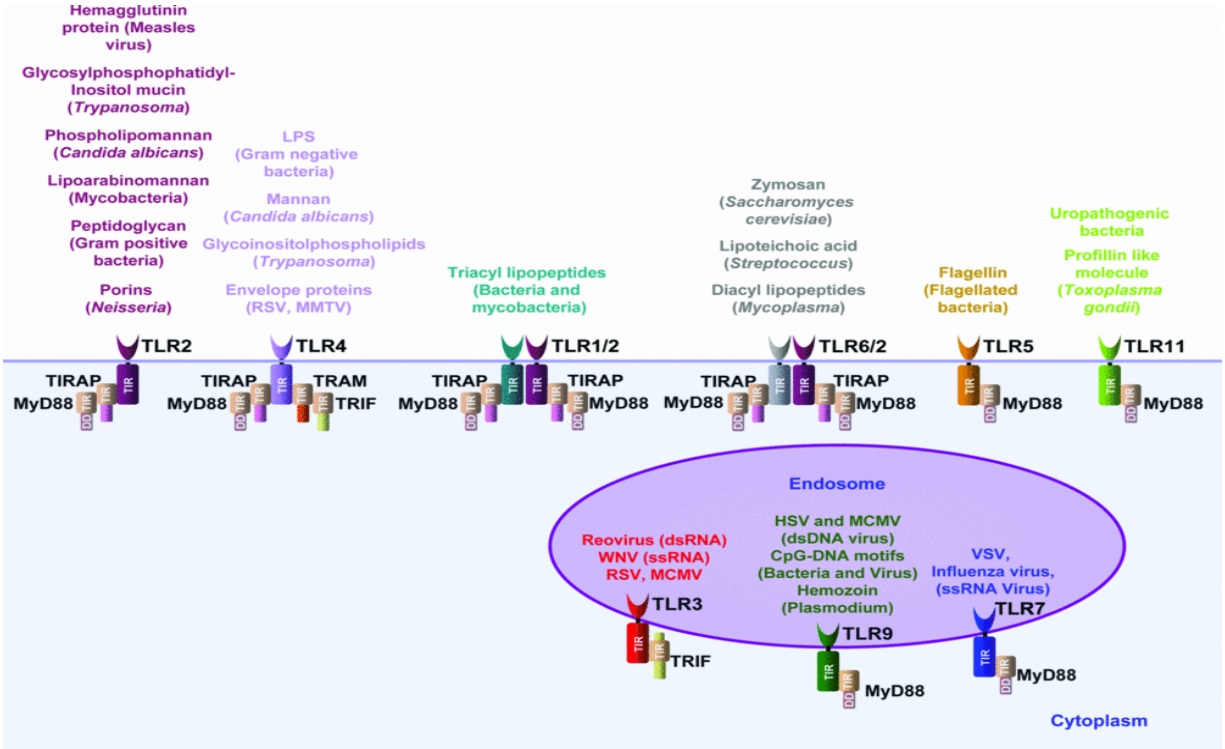


Figure 2.Classical TLR localization and PAMP recognition. Plasma membrane localized TLRs include TLR2, TLR4, TLR1/2, TLR2/6, TLR5 and TLR11. Endosomal TLRs include TLR3, TLR9 and TLR7. All TLRs signaling through the adaptor MyD88 except for TLR3 (TRIF only) and TLR4 (both MyD88 and TRIF). *Figure reproduced with permission of Biochem J. Article: (10). Copyright: the Biochemical Society.*

In addition to the TLRs, additional members of the PRR family include Nucleotide-binding oligomerization domain-like Receptors (NLRs) which detect DAP-type tripeptide motifs (NOD1) and muramyl-dipeptide motifs (NOD2) of peptidoglycan and cytosolic helicases Rig-I-Like receptors (RLRs) which sense the presence of RNA(19). Both NOD1 and NOD2 have been demonstrated to cooperatively protect the colonic epithelium during bacterial infection (20,21) and specific deficiencies in NOD2 expression has been demonstrated to lead to Paneth cell dysfunction (discussed later) and significant intestinal injury (22,23). Additionally, a second group of NLRs exists which, instead of leading to immune transcriptional responses, leads to the activation of the inflammasome, a process integral to the activation of two specific cytokines that are critical supporters of host mucosal defense: pro-IL-1B and pro-IL-18 (19). It is noteworthy to

recognize not only the diversity but the impressive redundancy among the PRRs in terms of both PAMP/DAMP recognition and signaling cascades.

1.1.2 Adaptor Proteins and TLR signaling Cascades

Of the various adaptor proteins containing TIR domains, two fundamental members include Myeloid Differentiation primary response gene 88 (MyD88) and TIR-containing adaptor-inducing interferon-b (TRIF) (10,17). Adaptor proteins are also implicated in adding to the complexity and diversity of TLR signaling in how they associate with the TIR domains of the various TLRs in response to ligand binding (4). MyD88 is generally viewed as the universal adaptor for the TLRs (excluding TLR3) as studies with MyD88 deficient mice demonstrated a significantly attenuated response to ligands of TLR2, TLR4, TLR5, TLR7 and TLR9 (24,25) and a member of the IL-1 Receptor subfamily (26,27). MyD88 recruitment upon TLR ligand binding subsequently results in recruitment of members of the IL-1R-associated kinase (IRAK) family immediately downstream, specifically IRAK4 and IRAK1, which initiate a complex signaling cascade involving the specific polyubiquitination of key signaling molecules. A critical downstream target of the IRAK kinases is the E3-ligase, Tumor necrosis factor-Receptor-Associated Factor 6 (TRAF6) which following activation is recruited and undergoes K63 autoubiquitination. Polyubiquitinated TRAF6 serves as a foundation for the recruitment of transforming-growth-factor-b-activated kinase (TAK1) via the ubiquitin-binding adaptors TAK1-binding protein 1and 2 (TAB) (28) as well as IKK kinases through binding of the IKKγ subunit. Activated TAK1 subsequently leads to activation of the Nuclear Factor-kB kinase (IKK) complex and thus NF-kB (described further below) (29-31) as well as the upstream kinases

responsible for p38 and JNK activation and specific interferon-regulatory factors (IRFs) leading to cytokine and type I interferon production (32).

The adapter protein TRIF, like other adapter proteins was identified based upon screening for TIR-domain-containing proteins (33). TRIF was found to play an important role in both TLR3 and TLR4 signaling resulting in IFNB and cytokine production (33-35) and is proposed to be the only signaling pathway responsible for induction of apoptosis (36). The importance of TRIF is underscored by an abolishment of NF-kB activation or gene up-regulation in response to LPS (37) and a loss of cytokine responses to TLR3 ligands with increased sensitivity to viral infections (38) in its absence. TRIF serves as the sole adaptor for TLR3 and comprises the MyD88-independent signaling arm of TLR4. TRIF signaling cascades utilize many of the same downstream effectors as the MyD88 arm to carry out signal propagation. Important exceptions to note are the importance of both TRAF3 and RIP1 as being critical to TRIF-mediated NF-kB activation in response to TLR3 ligands (39). These signaling cascades are illustrated below in **Figure 3** for TLR4, which utilizes both MyD88 and TRIF.

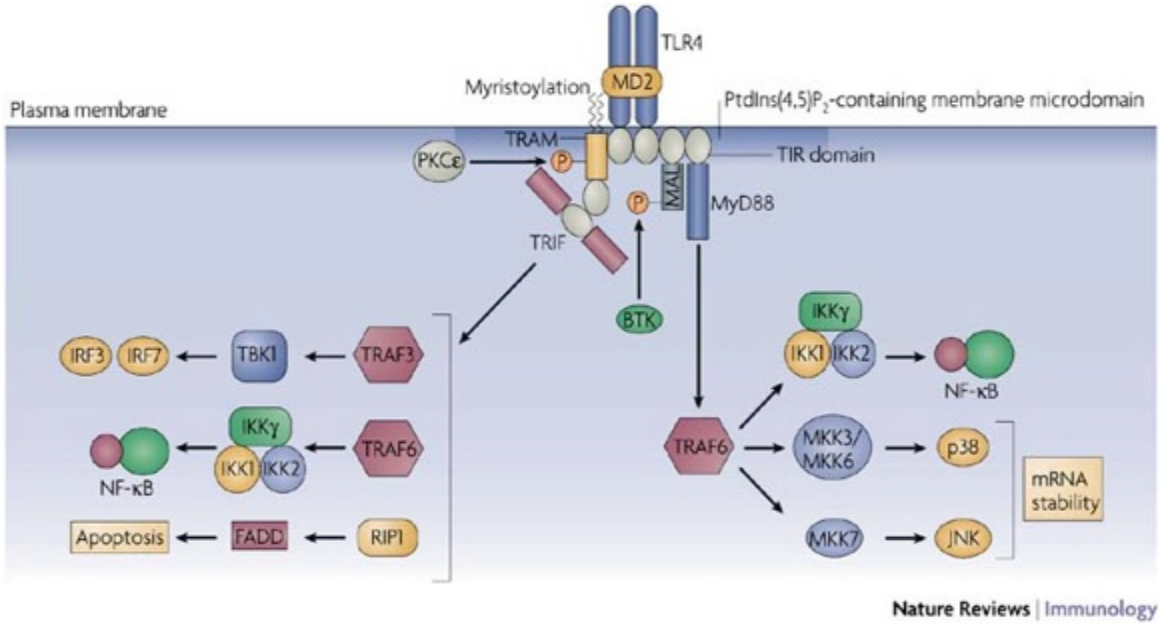


Figure 3.Adaptor molecule signaling by TLR4. TLR4 is the most complex TLR receptor in that it recruits and signals via both MyD88 and TRIF via recruitment of bridging adaptors Mal and TRAM respectively. Both TRIF and MyD88 pathways give rise to IKK and subsequently NF-kB activation via TRAF6 activity. TRIF, via activation of RIP1 is believed to also lead to pro-apoptotic signaling. TRIF mediated signals also activate IRF3 and IRF7 leading to Type I IFN production. MyD88 signaling also leads to activation of MAPK and JNK. FADD (Fas-associated death domain), IRF (IFN-regulatory factor), TNF-associated factor (TRAF), TBK (TAK1) *Figure reprinted by permission of Macmillian Publishers Ltd: Nature Reviews Immunology. Article: (4). Copyright 2012.*

A key downstream target of inflammatory signaling, e.g. PRR-PAMP signaling, is NF-kB as touched upon briefly above. Expression and activity of NF-kB is present in a large number of cell types (40) and serves as an important regulator of a vast number of cellular processes including immune function, inflammatory gene expression as well as expression of both pro- and anti-apoptotic signals (5,41,42). As a regulator and integrator of such a large number of cellular processes, NF-kB activation can occur following an equally diverse set of stimuli not limited to microbial antigens or inflammatory cytokines but physical and chemical stresses as well (5). The NF-kB transcription factor family is comprised of several members: NF-kB1 (or p50), NF-kB2 (or p52), RelA (or p65), c-Rel and RelB where Rel designates these members as bearing a Rel homology domain at their N terminus. The transcriptionally active NF-kB typically consists of p65 and p50 subunits. While homo and heterodimers of the family members can form, typically

those not bearing a Rel subunit are not transcriptionally active. NF-kB at resting conditions exists as a cytoplasmic complex with IkB proteins, which are inhibitors of NF-kB activity via masking of nuclear localization sequences present on the Rel subunits (40). Following activation of upstream receptors, e.g. PRRs, IkB specific kinases are activated and are termed IkB kinases (IKK). The IKK kinases exist as trimmers consisting of the catalytically active IKKa and IKKb, as well as IKKg bearing a regulatory domain (43,44). IKKa catalyzes the phosphorylation IkBa, while in complex with NF-kB, which results in targeted polyubiquitination of IkBa and its shuttling to the proteasome for degradation (45). NF-kB, now free from the inhibitory complex, translocates to the nucleus and initiates transcription of target genes (46) **(Figure 4).**

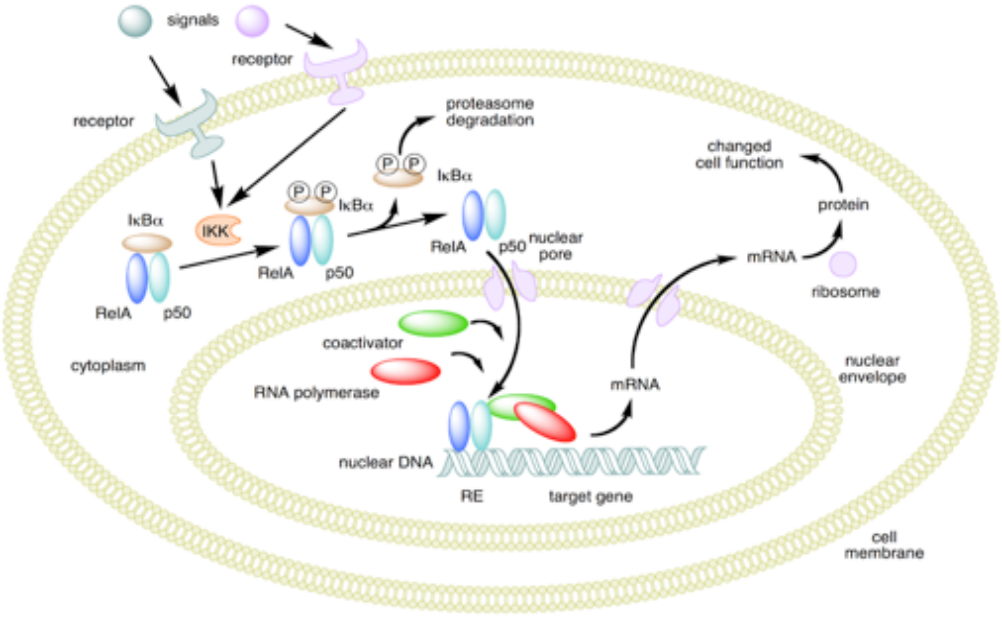


Figure 4.Downstream signals, such as from TLR4, lead to activation of the IKK complex. The catalytically active subunits IKKa/IKKb lead to the phosphorylation of the NF-kB inhibitor complex subunit IkBa, subsequently tagging it for proteosomal degradation via its ubiquitination and shuttling to the proteasome following its dissociation from NF-kB. NF-kB, a heterodimer of RelA-p50, is now free to translocate into the nucleus and binds DNA at specific response elements (RE). Specific proteins are subsequently recruited like co-activators and RNA Polymerase leading to transcription of target response genes. *Figure taken from (47)*

1.1.3 Toll-like Receptor 4

As described previously, TLR4 serves as an important sentinel receptor recognizing a number of PAMPs, primarily bacterial endotoxin LPS. TLR4 as well as other TLRs have been also demonstrated to recognize specific endogenous ligands released from injured or dying host cells. These ligands have been designated as Damage-Associated Molecular Pattern molecules (DAMPs) and include host DNA, hyaluronic acid, Heat Shock Proteins (HSPs), and High Mobility Group protein B-1 (HMGB1) (**Table 1**). Signaling in response to DAMPs allows TLRs to serve not only as sentinel receptors for invading microbes but as sensors of tissue and cellular injury and death; i.e. the presence of "danger signals"(48).

PAMP	DAMP	TLR	Adaptor protein	Transcription factor
Diacylated lipopeptides	β-defensin-3	TLR1/2	MyD88, MAL	NFκB
Triacylated lipopeptides		TLR2/6	MyD88, MAL	NFκB
	Serum amyloid A, neutrophil elastase, HSP60, HSP70, GP96, surfactant A and D, eosinophil-derived neurotoxin, biglycan, versican, hyaluronic acid, HMBG1, anti-phospholipid antibodies	TLR2		
Double-stranded RNA	mRNA	TLR3	TRIF	IRFs, NFκB
LPS	Biclycan, heparan sulphate, hyaluronic acid, neutrophil elastase, serum amyloid A, oxidized LDL, fibronectin EDA, fibrinogen, tenascin-C, lactoferrin, β-defensin-2, saturated fatty acids, HMGB1, HSP22, HSP60, HSP70, HSP72, GP96, lactoferrin	TLR4	MyD88, MAL, TRIF, TRAM	NFκB, IRFs
Flagellin		TLR5	MyD88	NFκB
Guanosine- and uridine-rich single-stranded RNA	Anti-phospholipid antibodies	TLR7/8	MyD88	IRFs, NFκB
Unmethylated CpG dinuceotides, hemozoin	IgG-chromatin complexes	TLR9	MyD88	IRFs, NFκB

CpG: cytosine-guanine; DAMPs: danger-associated molecular patterns; GP: glycoprotein; HMGB1: high-mobility group protein-B1; HSP: heat-shock protein; IgG: immunoglobulin G; IRFs: interferon regulatory factors; MAL: MyD88-adapter-like; MyD88: myeloid differentiation primary response protein-88; NFκB: nuclear factor-κB; LDL: low-density lipoprotein; LPS: lipopolysaccharide; PAMPs: pathogen-associated molecular patterns; TRAM: TIR-domain-containing adapter molecule; TRIF: TIR-domain-containing adapter inducing interferon-β; TLR: toll-like receptor.

Table 1.PAMP/DAMP TLR ligands. In addition to recognition of conserved microbial motifs, TLRs also recognize molecular motifs of endogenous molecules released following cellular/tissue stress and injury so called Damage-Associated Molecular Patterns (DAMPs). Nearly all TLRs have been demonstrated to recognize DAMPs. Both TLR2 and TLR4 recognize a wide array of DAMPs including Heat Shock Proteins, heparin sulfate, hyaluronic acid and HMGB1. *Table reproduced with permission of Remedica. Article: (49). Copyright 2011.*

In describing the various ligands TLR4 is able to recognize, it is also important to touch upon the key accessory proteins that play an important role in TLR4's ability to bind them. An important event in TLR4 signaling in response to LPS is LPS delivery to the receptor itself via LPS binding Protein (LBP) (**Figure5A**). LBP is a acute phase protein with a very high affinity for binding LPS (50). LBP-LPS interactions lead to disaggregation of LPS for delivery to CD14, an additional accessory molecule important for TLR4 responsiveness. LBPs interactions are not limited to LPS or TLR4 as it plays a role in ligand delivery for TLR1, TLR2 and TLR6 (51-53).

CD14 is a glycoprotein bearing leucine-rich repeats and exists in two forms: a soluble form within the blood or a glycosylphosphatidylinositol-anchored membrane protein present upon myeloid cells (4). CD14 like LBP plays an important role in signal transduction of a number of TLRs derived from its high affinity for a variety of microbial ligands (51,54-57). CD14 similar to TLRs exists as a dimer with the ectodomains together forming a structure reminiscent of the horse-shoe like solenoid structure of the TLRs with a hydrophobic pocket key for ligand (LPS) binding (58). Interestingly in response to LPS, CD14 is required for TRIF-dependent signaling and at MyD88-dependent signaling at low doses of LPS (59,60). CD14 has also been implicated in the chaperoning of LPS from LBP to TLR4 at the cell surface. Recent evidence also suggests CD14 plays a key role in the LPS induced endocytosis of TLR4 into early endosomes where the signaling complex can engage TRIF-dependent signaling (60-63) (**Figure 5B)**.

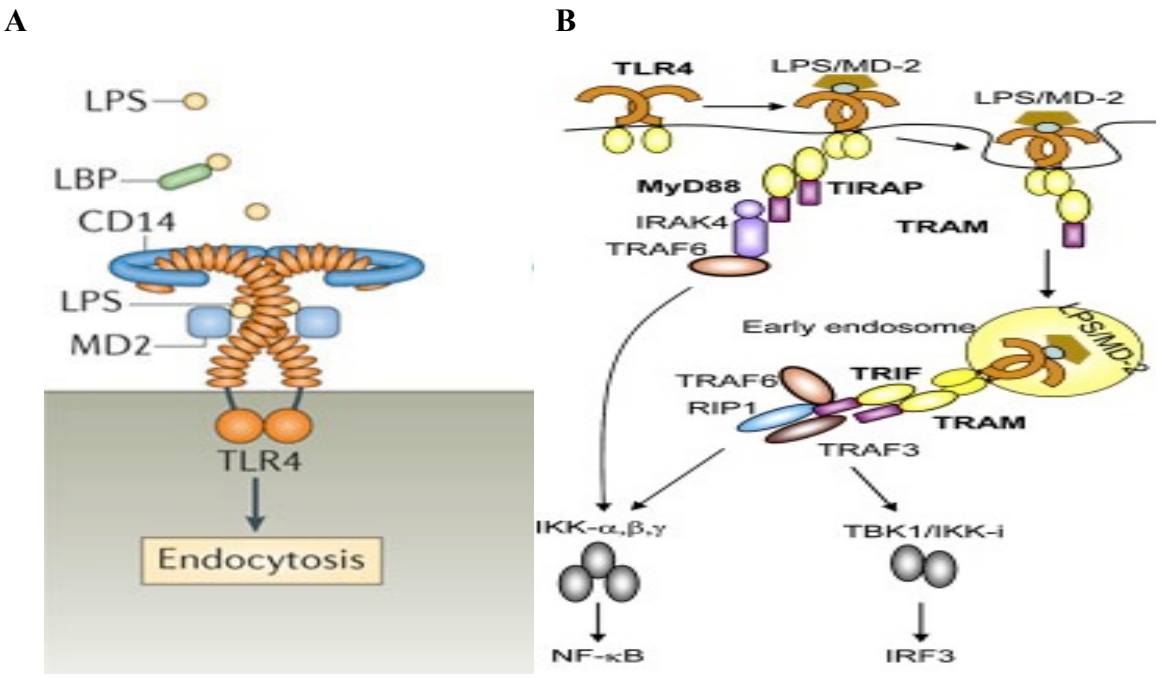


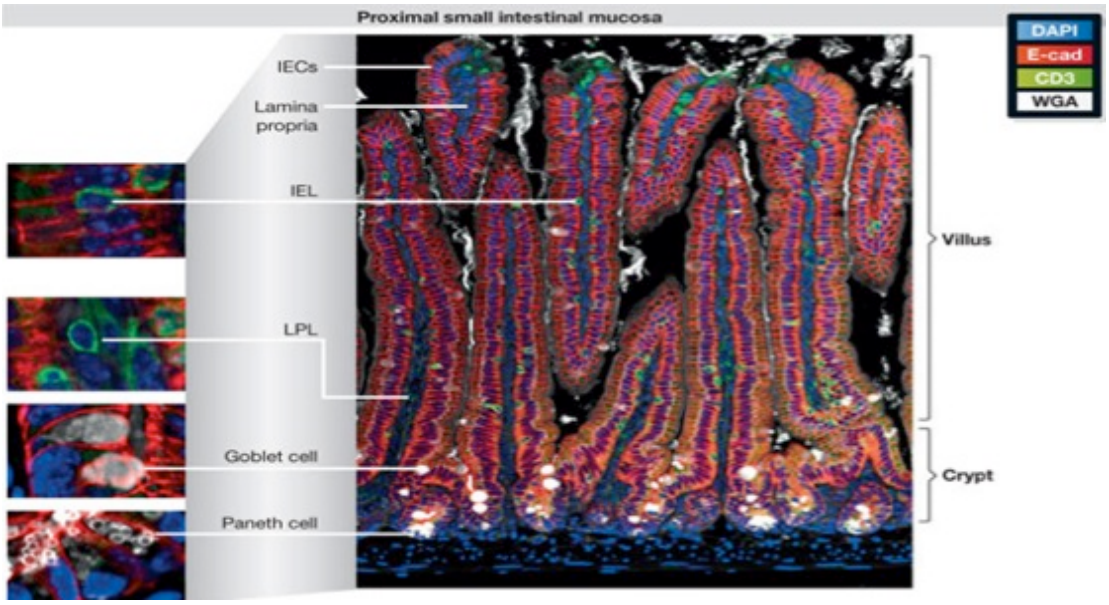
Figure 5.TLR4-LPS binding complex and TLR4 endocytosis. **A.** Lipopolysaccharide (LPS) recognition by TLR4 is heavily dependent upon several upstream accessory proteins. The acute phase protein LPS-binding protein (LBP) efficiently binds LPS within the extracellular space and delivers it to CD14. CD14 is anchored to the plasma membrane via GPI links. MD2 is critical for the homodimerization of TLR4 and creates a LPS binding pocket critical for the binding and recognition of LPS by TLR4. **B.** Upon LPS binding the CD14-MD2-TLR4 complex a host of adaptor molecules are recruited to the TIR-domain of TLR4. MAL(TIRAP) serves as a bridging adaptor recruiting MyD88 giving rise to TRAF6 and IRAK4 activation and subsequently NF-kB activation. The accessory protein TRAM functions to sort TLR4 to the early endosome followed by its subsequent recruitment of TRIF to the TIR-domain. TRIF-mediated NF-kB activation occurs via TRAF6 and RIP1 dependent activation of the IKK complex. IRF3 activity occurs via TRAF3 mediated signal transduction. *Figure reprinted with permission of Macmillian Publishers Ltd:Nature Reviews Immunology. Article:(4). Copyright 2012. Figure reprinted with permission of Elsevier. Article (64)*

Another important accessory molecule is MD2, a glycolsylated soluble protein that associates with the extracellular domain of TLR4 that is fundamental to TLR4 expression on the cell surface (65,66). Recent crystal structures of the TLR4-MD2 complex demonstrate that LPS inserts 5-6 of its lipid chains within the hydrophobic pocket of MD2 (67). Interestingly, of the TLR-Ligand crystal structures that have been resolved, TLR4 is the only one requiring accessory molecules for ligand binding (68). Presumably this is due to the little direct interaction the two TLR4 molecules make within the dimer itself making MD2 required not only for ligand binding but TLR4 dimerization (67).

1.1.4 Innate Immunity in the Small Intestine

The small intestine is the organ critically responsible for the absorption by the host of essential nutrients. While performing this fundamental function the small bowel must also maintain a strict barrier and mount effective and contained immune responses to defend against pathogenic microbes and maintain homeostasis with commensal flora. The gut represents a dynamic immunological system where the presence of commensal flora and exposure to external antigens from ingested sources. The gut immune system must be robustly activated but tightly and delicately controlled. Disruptions in the activation or the regulatory mechanisms governing gut immune homeostasis unsurprisingly can give way to a host of gastrointestinal pathologies. The immune system within the small bowel can be divided into two distinct sections: the outer epithelial layer and the inner lamina propria **(Figure 6)**. The principle component of the outer section is a single layer of Intestinal Epithelial Cells (IECs) which differentiate into four distinct cell types: absorptive enterocytes, enteroendocrine cells, mucus-secreting goblet cells and antimicrobial-secreting Paneth cells; all derived from a common stem cell progenitor located within the crypts of each villus critically dependent upon Notch-mediated epithelial differentiation (69,70). Interspersed between the IECs are Intraepithelial Lymphocytes (IELs) along with dendritic cell extensions. DC extensions, IELs and M cells present in this layer play an important role in first-line defense as partaking in luminal antigen sampling. The lamina propria contains the body of the DCs, neutrophils, macrophages, IgA-producing plasma cells, NK, T and T-reg cells. This inner section of the intestinal immune system is known collectively as the gut-associated lymphoid tissue (GALT), which includes Peyer patches, isolated lymphoid follicles, and mesenteric lymph nodes (19,71) (**Figure 6**).

A



B

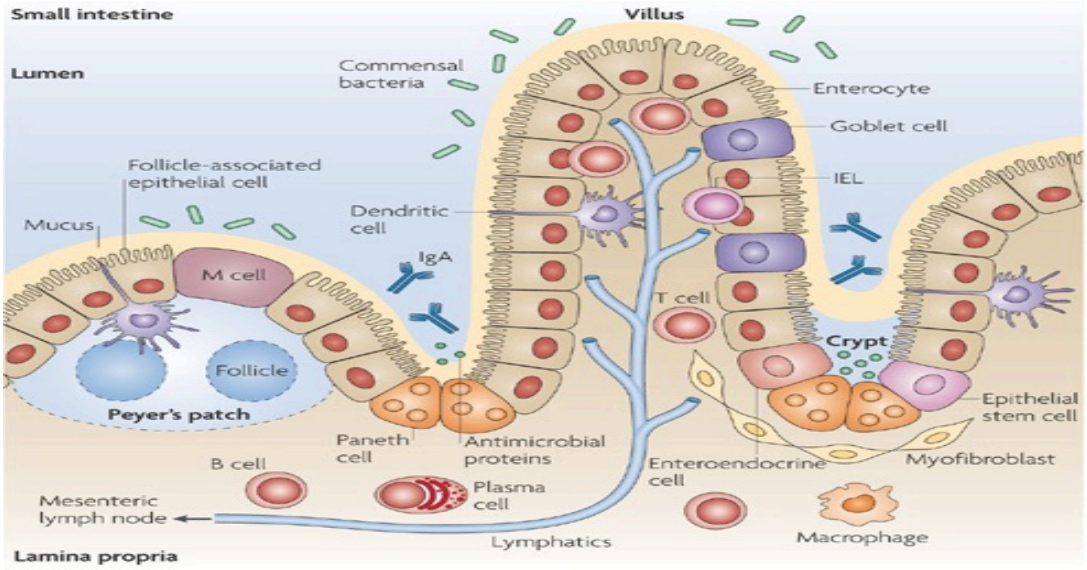


Figure 6.Structural and cellular features of the intestinal mucosa. A. Fixed section of the small bowel from a C57bl/6 mouse stained for various structural and cell-specific proteins. Ecadherin (red) and Dapi stains delineate villus structure and cell nuclei respectively. CD3 staining (green) detects Tcells within the epithelial monolayer, Intraepithelial lymphocytes (IELs) or within the lamina propria (LPL). Highly secretory cells within the epithelium: the Paneth and Goblet cells are depicted via wheat germ agglutinin stain (WGA). Paneth cells exist within the crypts intercalated between stem cells (not depicted). Goblet cells exist within the epithelial monolayer higher up on the crypt-villus axis. **B.** A cartoon representation of **A** clearly illustrating the intestinal milieu and localization of epithelial cell types as well as intestinal immune cells including macrophages, T cells and IgA producing B cells. Intestinal APCs, dendritic cells with luminal sampling extensions and M cells are also depicted. Together these cells and their secreted factors including mucus and anti-microbial peptides, maintain gut-immune homeostasis in the face of the ever-present commensal flora. *Figure reprinted by permission of Macmillian Publishers Ltd: EMBO Reports. Article: (19). Copyright 2012 and Nature Reviews Immunology. Article: (71). Copyright 2012*

A predominant secreted product within the intestine is IgA from the plasma cells, which is important for the prevention of microbes from traversing the intestinal barrier as well as their clearance and activation of effector immune cells (72,73). An additional highly produced and secreted product within the intestine is mucus. Mucins are large glycoproteins produced by goblet cells that are secreted to provide a physical barrier and can facilitate removal of bacteria and decreasing bacterial loads via binding and clearance via peristalsis (74). A host of mucin genes lead to the production of proteins that are anchored to the cell membrane while the most abundant produced form, derived from the MUC2 gene, is packaged into storage granules and secreted into the lumen (75). MUC2-encoded mucus secretion occurs at baseline but has also been demonstrated to increase upon inflammatory stimuli to aid in microbial clearance (76,77). Mucus also maintains the ability to capture and hold signaling molecules and can play a key role in signaling processes promoting wound healing, decreasing apoptosis and regulating inflammatory responses (78-80). Recent work also has implicated goblet cells as contributing to the delivery of luminal antigens to antigen-presenting cells residing in the lamina propria (81).

In addition to the mucus layer, the epithelial layer itself serves as a physical immune barrier through a system of junctional proteins act as both gatekeepers of transcellular transport and “spot-welds” and fortifications between epithelial cells to invading microbes. These junctional proteins are grouped into tight junctions (TJ), which serve to bind cells together at the apical surface, and adherens junctions (AJ) which bind cells at their lateral surfaces. TJs include Occuldins, Claudins, and junctional Adhesion Molecules (JAMs). Each of these proteins plays an important regulatory role in porosity of the epithelial layer and the macro/micromolecule absorbance (82). Loss of function of any of the TJs can lead to altered cellular polarity and permeability (83). AJs also are important sites of intracellular signaling and loss of these

connections not only can disrupt cellular polarization but can lead to apoptosis (84).

Alongside mucins and IgA, the gut, chiefly via the Paneth cells, also actively secretes a host of anti-microbial peptides aimed at curbing bacterial load and maintaining gut homeostasis (85). Paneth cells, located within the crypt base, maintain and release granules containing a number of AMPs including lysozyme, α-defensins, TNFα, anti-microbial lectins known as RegIIIα (RegIIIγ in mice) (86-88). Typically secreted AMPs remain local within the crypt acting to safe-guard the precious stem cell progenitors, but under inflammatory stimuli have been demonstrated to be secreted to support immune responses and defenses of the epithelial layer as well adding to the antimicrobial activity of epithelial secreted AMPs including β-defensins and RegIIIα/γ (88,89).

An integral part of the intestinal immune system is the presence of previously described PRRs in the intestinal epithelium. In the human intestine epithelium, TLR1 to 5 and TLR9 have been detected and serve as sentinels for PAMPs and DAMPs (90). PRR expression by IECs is fundamental to maintain intestinal homeostasis, discerning between pathogenic and commensal bacteria. PRR signaling within the epithelium is important for stimulating the protective factors mentioned above as well as immune signals including cytokines such as thymic stromal lymphopoietin (TSLP) important for T cell maturation, chemokines and growth factors. The important factors for maintaining microbial homeostasis are summarized in **Figure 7.**

With the presence of commensal bacteria within the intestinal lumen, given what is known of PRR-PAMP signaling, indiscriminate PAMP recognition would be expected to yield a significant inflammatory response. However, this is known to not occur and in fact the epithelium seems to tolerate the presence of these PAMPs and actually depends on the presence of these signals for normal function and sensing when breaches by pathogenic microbes occurs

(71). To underscore the importance of tonic innate immune signals in maintaining gut homeostasis, epithelial-specific deficiencies of IKKγ, IKKα/β, TAK1 and p65 in addition to expression of a dominant-negative form of MyD88 leads to spontaneous barrier dysfunction and subsequent mucosal injury and inflammation (91-94).

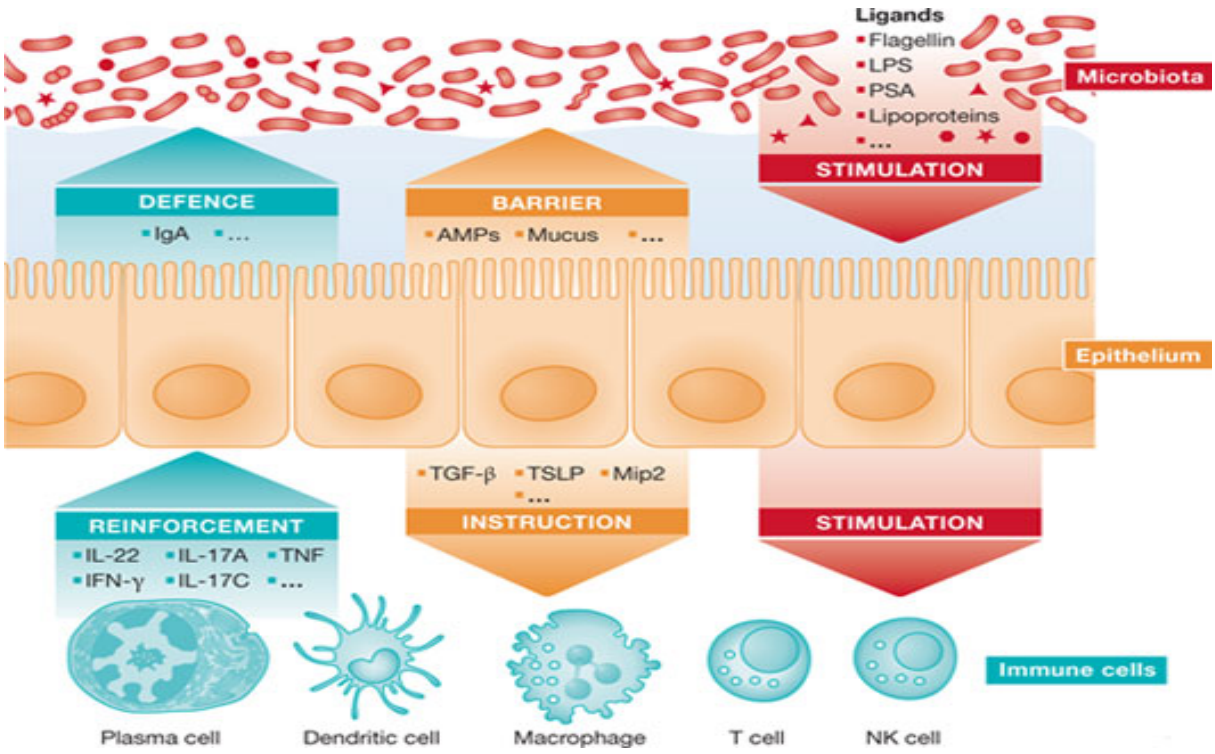


Figure 7.Role of the intestinal epithelium in maintaining gut immune homeostasis. Host immune protective effects are illustrated in blue and include IgA secretion via plasma cells and several cytokines and chemokines released from immune cells residing in both the lamina propria and within the epithelial monolayer. Epithelial effects are demonstrated in orange and include barrier maintenance via mucus production and secretion of antimicrobial peptides from IECs and Paneth cells. IECs also play a key role in communication with the underlying immune cells via release of important immune signaling proteins including macrophage inflammatory protein 2 (Mip2) and thymic stromal lymophoietin(TSLP). Depicted in red are various microbial ligands i.e. PAMPs that provide important homeostatic signals but if unchecked can lead to significant intestinal inflammation and injury. *Figure reprinted by permission of Macmillian Publishers Ltd: EMBO Reports. Article:(19). Copyright 2012*

One contributing factor to ensuring balance between mucosal immunity and commensal flora is receptor compartmentalization. The apical surface is frequently exposed to commensal flora while the basolateral side, in physiologic conditions is relatively protected from such

encounters. Under physiological conditions different PRRs are varyingly expressed both in level and spatially when comparing large versus small bowel which may be driven, in part, by density of microbial flora (19,71). A prime example of how PRR compartmentalization contributes to maintenance of mucosal immune homeostasis and discriminate targeting of pathogenic versus commensal flora is evident in TLR5 and TLR9 localization. IEC TLR5 expression is restricted to the basolateral side of human colonic epithelium and was found to only respond to flagellin in *ex vivo* murine colonic cultures following epithelial barrier disruption (95,96). TLR9, however, is present on both the apical and basolateral membranes (97), but, impressively, initiates different signaling cascades depending in which compartment ligand binding occurs. Apical signals are translated into epithelial-commensal "tolerogenic" and anti-inflammatory signals where interpretation of basolateral signals results in significant pro-inflammatory signals (98). Similarly receptor compartmentalization is also effective at limiting and tailoring responses to released endogenous immune mediators. For example, IEC responses *in vivo* to type III IFNs produced following viral infection are robust while responses to type I IFNs are not, despite expression of both receptors. This is presumably due to type I IFN receptors localized to apical surfaces (99). Of note, comparison of PRR localization between "professional" immune cells and IECs is also suggestive of significant differences in the PRR-PAMP responses between these cells e.g. TLR9 "tolerogenic" signals in IECs vs pro-inflammatory signals in macrophages. PRRs have been demonstrated to undergo significant up-regulation and relocalization following stimulation (71) especially in regards to TLR2 and TLR4 (16,100). In the small intestine, epithelial expression of TLR4 is primarily restricted to an intracellular compartment (101-103). Therefore TLR4 stimulation requires ligand internalization facilitating a sustained epithelial response similar to intracellular-restricted TLR9 signaling and responses in macrophages (104).

Another mechanism ensuring immune responses remain in a steady-state balance despite tonic contact with commensal bacteria and bacterial products arises from a set of proteins specifically charged with limiting PRR signaling. One such protein is Toll-interacting protein (TOLLIP) that inhibits TLR2 and TLR4 signaling. TOLLIP directly associates with both TLR2 and TLR4 and leads to a decrease in IRAK phosphorylation and activity and also serves as a substrate for IRAK activity, with no affect upon IRAK recruitment (105,106). TOLLIP functions as a negative regulator of TLR signaling in IECs through up-regulation following LPS or lipotechoic acid treatment (107). A critical role for TOLLIP has also been identified in patients with inflammatory bowel disease where IECs isolated from these patients demonstrated a significantly reduced ability to up-regulate TOLLIP expression (108). A highly expressed regulator of TLR signaling in IECs, along with IL-1R and IL-33R is single immunoglobulin IL-1R-related molecule (SIGIRR) (109). Impaired up-regulation of SIGIRR expression, like TOLLIP, leads to exacerbation of intestinal inflammation (110,111). SIGIRR is characterized by a single extracellular Ig domain and a TIR domain that are required for its inhibitory activity which includes trapping of TRAF6 and IRAK1 (109). An additional regulator of TLR signaling is a member of the IRAK family, which is specifically comprised of two active kinases (IRAK and IRAK4) and two inactive kinases (IRAK2 and IRAK-M). IRAK-M serves as a negative regulator of TLR signaling, initially believed to be restricted to macrophages (112). However, it is also present and functionally active in IECs (113). IRAK-M is up-regulated following LPS signaling in macrophages and subsequently induces LPS tolerance. IRAK-M prevents formation of IRAK-TRAF6 complex formation indirectly via substantially increasing the affinity of both phosphorylated and unphosphorylated IRAK4 for MyD88 preventing the disassociation of the early TLR signaling complex and TLR signal propagation (112). Key events in TLR signaling

and NF-kB activation cascades involve the polyubiquitination of specific target protein. Therefore, deubiquitination of these targets by deubiquitinases (DUBs) serve as a central regulatory crossroad. One of the most important DUBs regulating NF-kB activation is A20 (114,115). Multiple NF-kB activating stimuli were found to induce A20 expression (116,117) and A20 overexpression inhibited this activation (118,119). A20 functions to catalyze the deubiquitination and inactivation of TRAF6 (120). The importance of A20-mediated negative feedback is underscored by the fact that mice lacking A20 died prematurely due to spontaneous multi-organ inflammation and displayed a significant inability to curb inflammatory responses even when exposed to sub-lethal doses of LPS (121).

The epithelial monolayer exists as a simple sheet composed of numerous IECs that maintain the ability to communicate with its horizontal neighbors via intracellular connections known as Gap junctions (122-125). These connections have proved vital to a variety of tissue processes including electrical coupling and neuronal synapses (126) and serve as important cellular communication relays among IECs in coordinating immune responses. IECs forward immune responses horizontally via the generation and diffusion of messenger molecules, such as Reactive Oxygen Species (ROS), through gap junctions into neighboring cells (127,128). PRR-PAMP/DAMP signals also promote gap junction communication via up-regulation of a group of proteins that compose gap junctions named connexins (129). Aside from gap junctions cytokine secretion functioning in an autocrine manner also serves to foreword inflammatory signals to neighboring epithelia (130,131) **(Figure 8)**.

Thus, the presence and activation of PRRs is not only important for immune responses but for key intestinal processes including IEC proliferation (19,71), TJ maintenance (132), AMP expression (19) and intestinal wound healing (133). Despite the many beneficial functions of

TLR4 signaling within the intestinal epithelium, a number of diseases have been associated with exaggerated TLR4 expression and signaling including Uleratvive Colitis, Crohn's disease, intestinal malignancies and Necrotizing Enterocolitis (71,134).

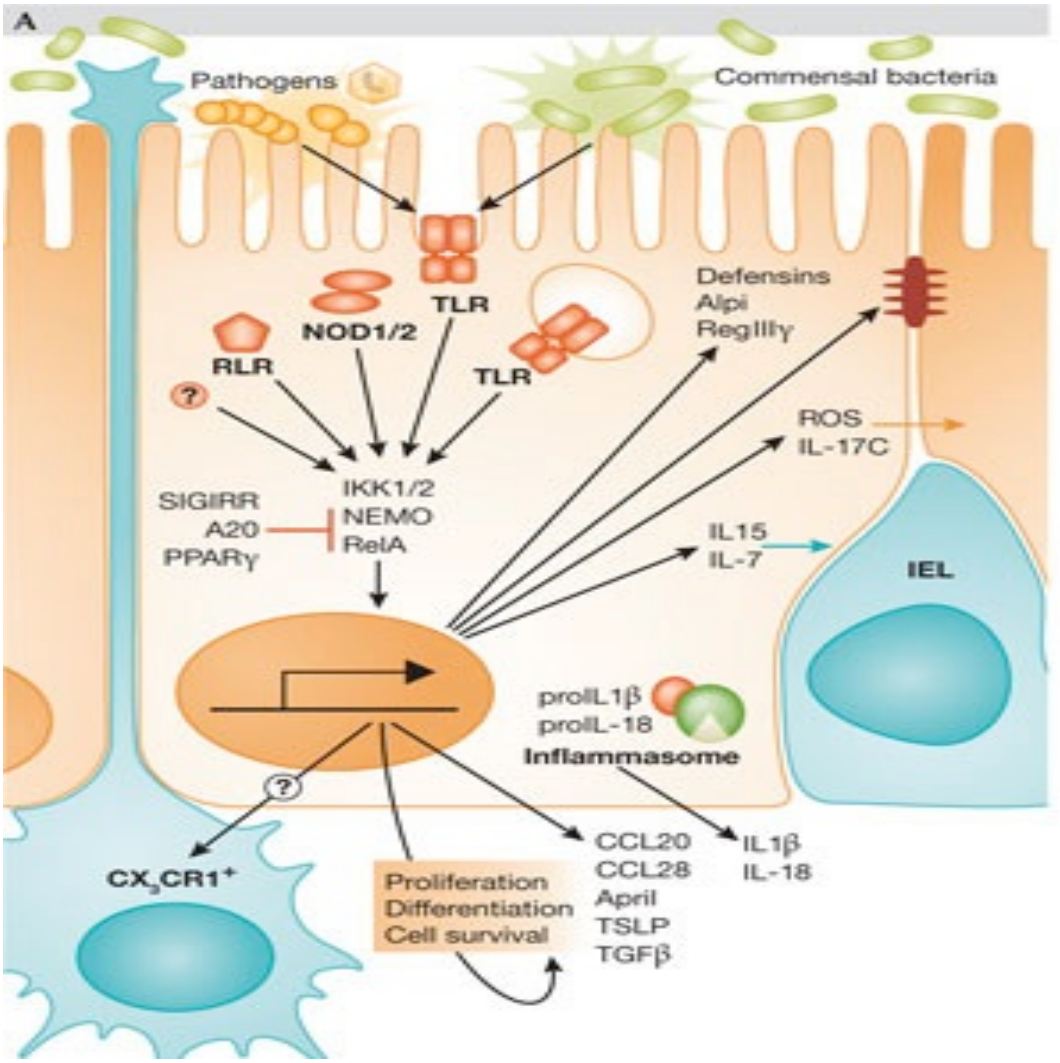


Figure 8.Innate immune signaling of the IEC. PRRs (TLRs, NLRs and RLRs) recognize microbial motifs from both pathogenic and commensal microbes. PRR signals give rise to IKK activation and NF-kB (RelA) activation and subsequent cytokine/chemokine and anti-microbial peptide production. NF-kB responses also lead to activation of proliferative, differentiation and survival signals. Increased Gap junction proteins maintain key intracellular channels for conducting signals (e.g. cytokines, ROS) to neighboring IECs and IELs. PRR signals also lead to inflammasome activation leading to production of active IL-1b and IL-18. Negative regulators of PRR signaling ensure intestinal inflammatory responses do not occur unchecked and remain tightly titrated. SIGIRR, A20 and PPARγ are among the fundamental PRR regulators important to immune homeostasis and attenuation of inflammatory signaling following clearance of invading microbes. *Figure reprinted by permission of Macmillian Publishers Ltd: EMBO Reports. Article: (19). Copyright 2012*

1.2 NEONATES: PRETERM VS TERM

With the increase in the number of induced vaginal deliveries and elective cesarean sections, the number of preterm births, defined as <37 weeks, has significantly increased (42). As medical facilities become more advanced, there has been a significant improvement in the survival rate of these preterm infants. However, this is not without its complications, one of which includes severe gastrointestinal disorders, and Necrotizing Enterocolitis (NEC) is the most significant. The preterm infant demonstrates various signs of organ immaturity resulting in a maladaptation to postnatal life. The gastrointestinal tract is among the most stressed organs in the preterm infant as it is ischemic, developmentally immature, unable to adequately meet the nutrient demand of the infant and rapidly colonized with commensal flora much earlier than anticipated. Compared with the intestinal tract of the term infant, the preterm infant displays deficiencies in structural integrity of the gut barrier, global and gut-specific immune function and dysregulated blood flow predisposing the gut to ischemia. These factors, as well as the onset of enteral feeds in these infants, represent major pathogenic factors for the development of NEC.

1.2.1 Amniotic fluid

The human fetus while in utero is bathed in amniotic fluid (AF) and estimated to swallow 100-200ml per kilogram body weight per day (29), which constitute a significant nutrient and energy source for the fetus (30). AF plays an important role in normal gut development, through the presence of many beneficial factors present such as epidermal growth factor (EGF) and insulin-like growth factor (IGF-1). These factors stimulate tissue and enterocyte growth in utero

and aid in nutrition uptake postnatally (31). In addition, AF plays an important immunomodulatory role via the presence of IL-10 (71) and TGF-β (72). Contained within AF are also anti-microbial peptides such as defensins and lysozymes conferring protection against bacterial infection (73). The preterm infant however is at a significant disadvantage as its exposure to AF is significantly decreased compared with the term infant, predisposing it to gastrointestinal disorders. Indeed, the key protective effects of orally administered AF have been demonstrated to limit NEC development in both mice (117) and pig (135) models of this disease through reduction in both intestinal inflammation and apoptosis. These studies demonstrate that the bioactive properties of AF *in utero*, among them EGF, may positively affect the immediate postnatal development of the intestine in preterm infants as well. There is a considerable functional overlap between the prenatal effects of AF and the postnatal effects of breast milk, which contains very similar biologically active factors to those contained within AF. It appears that AF possibly functions to prepare the gut for the dramatic shift between the *in utero* environment and that immediately following birth.

1.2.2 Breast Milk

There is a growing body of evidence (42) that breast milk is far superior to infant formula in stimulating the maturation of the gut, which is especially important in the preterm infant where digestive and absorptive functions are quite immature. Factors present within the breast milk and absent in formula are believed to mediate the beneficial intestinal effects of breast milk in the neonate. These factors are biologically active within the gut as they are quite resistant to proteolytic degradation. Growth factors present in breast milk such as EGF and IGF-1 have been shown to stimulate gut growth and maturation in terms of increasing intestinal barrier integrity,

villus height and brush-border digestive enzyme expression (75). Along with macronutrients, breast milk also provides important immunoprotective properties during the critical post-natal period underscored by the increased occurrence of infection and gastrointestinal disease in preterm infants fed formula (77,78). Among the protective immunological factors conferred via breast milk are immunoglobulins, lymphocytes and macrophages (79). These factors not only provide direct protection against infection, but also serve as important stimulators of the currently immature newborn immune system. They also provide fundamental tolerance signals critical for the maintenance of gut immune homeostasis in the face of both harmful and harmless antigens. Failure of tolerance signals are believed to be major contributing factors to the development of food allergies, autoimmunity as well as inflammatory bowel disorders (80,82,83). Similar to their significantly attenuated exposure to AF, preterm infants are also significantly deprived of breast milk and fed primarily through total parenteral nutrition and/or infant formula. It remains a critical area of research to identify key factors within breast milk that are "NEC-protective". Recent studies have identified key components of breast milk such as Erythropoietin (136) to significantly protect against models of NEC.

1.2.3 The Immune System of the Newborn Intestine

Bacterial colonization of the intestine is a key step in the development and maturation not only for the gut immune system but for remote organs as well. Critical lines of defense against both pathogenic and commensal flora are the physical and chemical barriers aimed at microbial clearance mentioned in the previous section including: peristalsis, AMP release, barrier integrity, and mucus layers. However, in the preterm gut these critical defense mechanisms are significantly under-developed. Microbes that cross this first-line of defense will activate immune

responses in both the innate and adaptive arms aimed at clearing the invader. The immune system development begins early in the embryonic period but continues to mature up to about 1-2 years of age (137) depicted schematically for both the innate and adaptive arms in **Table 2**.

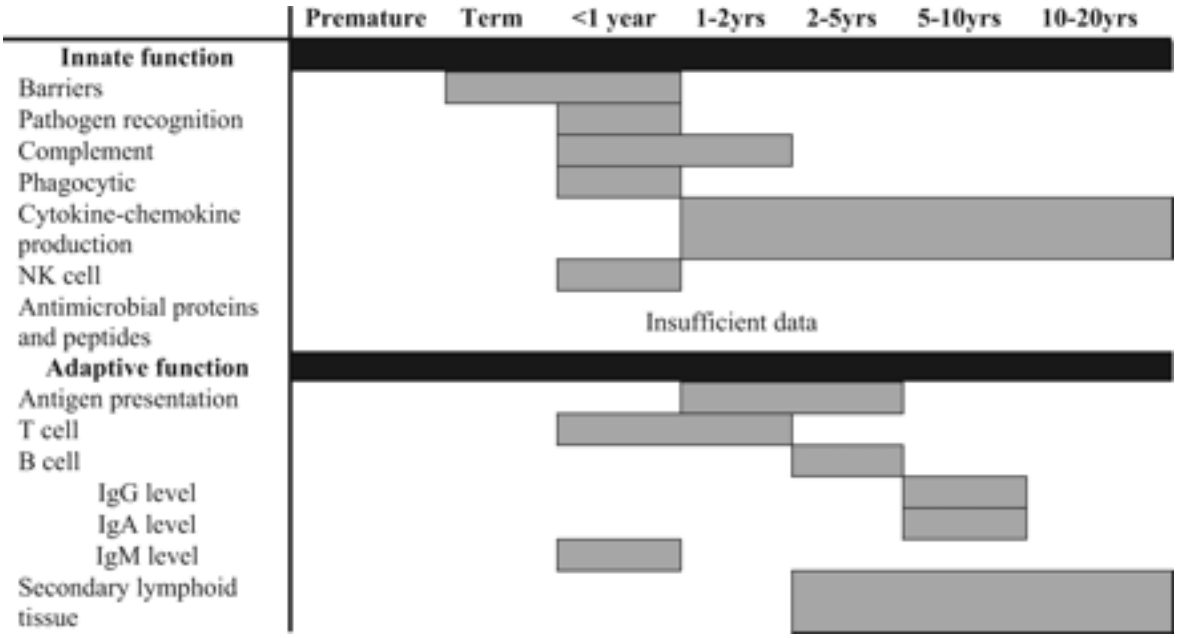


Table 2.Timing of acquisition of mature immune function in the human. Preterm infants, most at risk of developing NEC, are afflicted by disorders of prematurity such as generalized hypoxia due to immature lung development. Not surprisingly preterm infants have a significantly immature innate and adaptive immune system. Intestinal barriers in these infants are also underdeveloped. These factors make preterm infants far more susceptible to acquiring infections and uncontrolled and undirected immune responses following colonization. *Table repreoduced with permission from Pediatrics article (136) by the AAP. Copyright 2010*

This therefore results in an "immunocompromised" neonate at birth, whose proper development is dependent upon both AF prenatally and breast milk postnatally. The immature physical and chemical barriers, limited functions of key immune effector cells and proteins, which characterize the preterm infant, unsurprisingly predispose them to diseases. To highlight a few adaptive arm deficits, the preterm neonate displays: T-cell deficiencies (T-helper 2 skewed cytokine responses, increased T-reg populations and decreased CD8 T-cell cytolytic activity), weak B-cell activity (dampened Ig production especially in response to polysaccharide antigens, poor T-cell-mediated B cell activation) and underdeveloped secondary lymphoid tissues

(138,139). Both adaptive and innate immunological deficits in the preterm infant compared with the term neonate are summarized in **Table 3.**

A summary of some immune system characteristics in preterm neonates

Component	Description in preterm neonates
First line defense	
Mechanical barriers	↓ Stratum corneum function
Intestinal mucosa	↓ IgA, IgM, IgG
	↑ Intestinal permeability
	↓ Antibacterial peptides
Innate immune response	
Antigen presenting cells	↓ MHC receptors
Complement system	↓ Protein level and activity
Neutrophils	↓ Number
	↓ Adhesion
	↓ Oxidative burst
	↓ Deformability
	↓ Phagocytosis
Natural killer cells	↓ Number and function
Adaptive immune response	
Lymphocytes	↓ T and B cell count
	↓ T helper
	↓ Cytotoxic lymphocytes
	↑ T regulatory (inhibitory) cells
	T-helper 2 polarized

Table 3. Specific immunological deficits in both the adaptive and innate arms of the preterm infant compared with the term infant. *Table reprinted with perimission from Elsevier. Article: (42). Copyright 2011.*

In **Figure 9** key differences between the milieu of the preterm and term infant are depicted illustrating that the preterm infant is significantly at risk for development of gastrointestinal diseases such as NEC. The preterm gut therefore represents a "perfect storm" of maladaption to the outside environment with deficits of physiologic digestive/absorptive gut functions as well as incoordination of immune signals and responses.

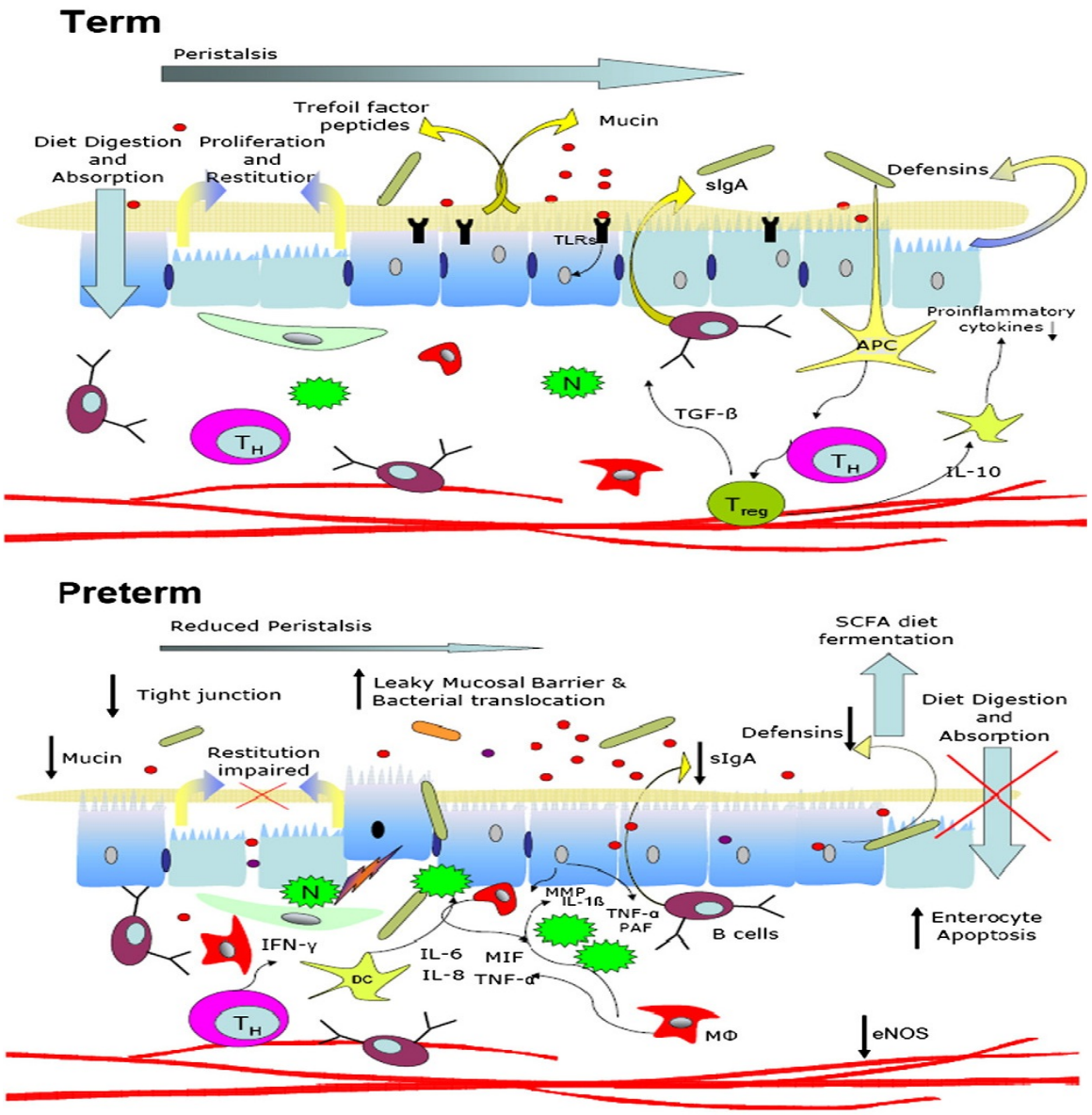


Figure 9. Intestinal milieu: Term vs. Preterm. Compared to the term neonate (top), preterm infants (bottom) demonstrate significantly reduced digestive and absorptive capacity, impaired barrier integrity, blood flow and dysfunctional mucosal immunity. The preterm gut barrier is compromised due to increased IEC apoptosis, decreased AMP and IgA production as well as decreased TJ and mucus expression. A dysregulation of gut immunity gives rise to exaggerated immune responses to both translocating pathogenic and commensal flora. IEC injury and controlled inflammation further facilitate bacterial translocation yielding a vicious cycle typically culminating in the development of NEC. *Figure reprinted with permission from Elsevier. Article: (42). Copyright 2011.*

1.3 NECROTIZING ENTEROCOLITIS

Necrotizing Enterocolitis (NEC) is the leading cause of death for gastrointestinal disease in neonates, and remains a major cause of morbidity in those who survive this devastating disease (140,141). NEC occurs in approximately 1 to 3 per 1000 live births and accounts for nearly up to 10 percent of all neonatal intensive care unit admissions (142). The excessive and extensive intestinal inflammation and injury characteristic of NEC leads to mucosal ulceration and frank necrosis **(Figure 10)** of intestinal tissue along with disruption of the intestinal barrier. This subsequently can extend systemically leading to multisystem organ failure as well as significant brain injury and can lead to significant developmental delay in those infants who survive. Despite the marked improvements in overall neonatal care in general, the management approach to the infant with NEC has not changed in the past 30 years, and the outcome is generally as poor today as it was 3 decades ago (143). Based on these sobering statistics, it is clear that new therapeutic approaches to NEC are required and that such approaches will demand a greater understanding of the molecular mechanisms that contribute to the development of this disease. As was recently summarized by the 2006 NICHD workshop on NEC research, "NEC can be thought to arise from an uncontrolled exuberant inflammatory response to bacterial colonization that characterizes the intestine of premature infant" (144). In the management of this disease there is no specific treatment that exists –primary support is focused upon maintenance of hemodynamic parameters and administration of broad-spectrum antibiotics. In severe cases surgical resection of afflicted bowel is required leading in many cases that are associated with morbidity, such as short gut syndrome. This is underscored by the inability to acquire adequate nutrition with overall surgical survival remaining at 50% (145). Due to the fact that since its initial description in 1965 (143) little progress has been made in improving the

overall survival, there remains a profound interest from individuals involved in the care of preterm infants and research community to develop a cure or adequate preventive strategy for NEC. Several investigators have now examined the mechanisms that mediate the signaling response of the newborn intestine to bacteria and have detailed the consequences of this signaling response to the pathogenesis of NEC. In particular, these studies have uncovered an essential role for a class of bacterial receptors named Toll-like receptors (TLRs) in the pathogenesis of NEC and have provided compelling evidence to suggest that blunting the ability of TLRs to signal within the intestinal epithelium of the newborn infant may either prevent or treat NEC. These findings place the spotlight on the molecular basis that underlies the interaction between the intestinal epithelium and the commensal microbial flora. These studies also suggest that the ability of TLRs to respond to bacteria within the newborn intestinal epithelium may, in part, explain the particular susceptibility of the premature infant to the development of NEC. The following subsections will delve deeper into the role of TLR4 in the pathogenesis of NEC as well as recent advances in the elucidation of the pathogenesis and therapeutic targets for this disorder.

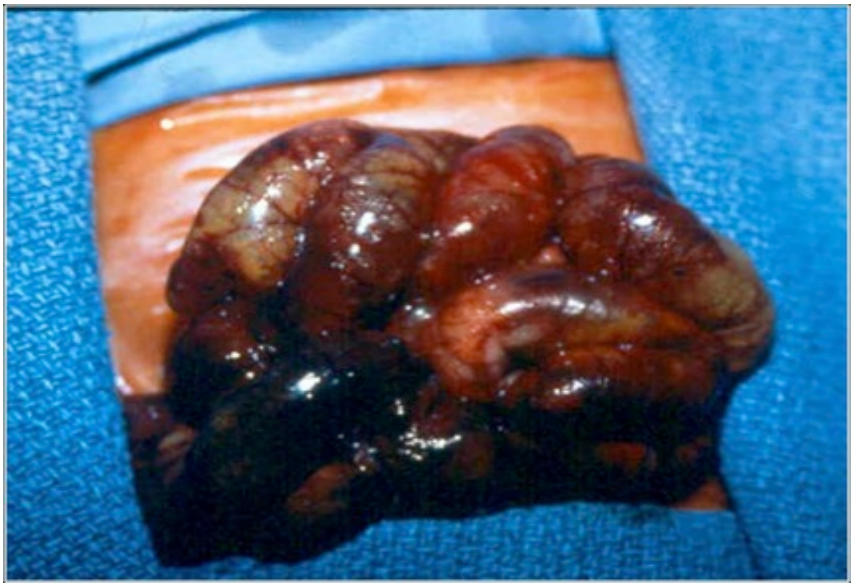


Figure 10.Gross image of frank necrotic bowel at time of bowel resection in a preterm infant afflicted by NEC. *Figure taken from(146)*

1.3.1 An Essential Role for TLR4 in the Pathogenesis of Necrotizing Enterocolitis

A role for LPS—and by extension for TLR4—in the pathogenesis of NEC is highlighted by the findings that LPS administration in association with systemic stress induces NEC in animals (147-149). Based on the observation that NEC is known to develop after the intestine has been colonized with Gram-negative bacteria, serum levels of LPS are increased in patients with both NEC and inflammatory bowel disease, a disorder that shares histopathological features with NEC (150-153), and levels of LPS are significantly increased in the plasma and stools of infants and mice that develop NEC compared with healthy counterparts (154,155), several laboratories have sought to establish whether TLR4 may play a role in the pathogenesis of this disease. The Caplan laboratory showed in 2006 that mice with mutations in TLR4 are protected from the development of NEC (156). The Hackam laboratory in 2007 confirmed that mice lacking TLR4 do not develop NEC and extended these observations by demonstrating that TLR4 signaling regulates the balance between injury and repair in the newborn intestine (155). Several laboratories have shown that TLR4 is increased in the intestinal mucosa of mice, rats, and humans with NEC compared with controls (155,157) and that activation of enterocyte TLR4 leads to an increase in death of the cells that line the intestine through the process of apoptosis (158). Along with the increase in epithelial death that accompanies TLR4 activation in the newborn small intestine, TLR4 activation within the intestine was also found to reduce the capacity of mucosal healing to occur because of a reduction in enterocyte proliferation (159) and reduced enterocyte migration (160). Taken together, these findings demonstrate that TLR4 activation in the newborn small intestine leads to the development of NEC through profound and deleterious effects on promoting intestinal injury and reducing the capacity for mucosal repair.

Other authors have shed light on additional roles played by TLR4 in the pathogenesis of NEC. Luk and coworkers (157) have shown that the activity of the TLR4 signaling pathway was up-regulated in intestinal tissues from premature neonates and rats with NEC compared with controls. This concept is supported by the work of Liu *et al.* (161) who reported that TLR4 expression and signaling is increased in the intestine of rats with NEC when compared with control rats, and that such increases precede histological evidence of mucosal injury in this disease. Further evidence of the physiological relevance of these findings is demonstrated by Lu *et al*. (162), who showed that while TLR4 expression is increased in the intestinal mucosa of rats with NEC, agents known to reduce NEC may protect from the development of NEC through a reduction in TLR4 expression. Wolfs *et al.*(163) have shown that the absence of the key TLR4 regulatory molecule MD-2 in the immature infant bowel may lead to impaired bacterial sensing and thus predispose to NEC on microbial colonization of the premature intestine via disruptions of gut immune homeostasis.

1.3.2 TLR4 Modulation of Intestinal Barrier Integrity and Bacterial Translocation

The integrity of the intestinal barrier is paramount to preventing frank translocation of bacteria as well as PAMPs into the systemic circulation. Increases in the concentration of circulating LPS and bacterial species characterizes infants with NEC and greatly increases the likelihood for these patients to progress to systemic sepsis and organ failure. Increases in the concentration of circulating LPS and TLR4 expression as seen in NEC exert significant deleterious effects upon the integrity of the intestinal barrier. Increased NO production due to increased activity of iNOS has been demonstrated in settings of gut inflammatory states(164)including in response to LPS (165). Through the utilization of iNOS mutant animals,

LPS-induced intestinal injury, barrier dysfunction and bacterial translocation were found to be, in part (166), iNOS dependent (167-170). LPS-induced barrier disruption via NO generation by the enterocytes themselves (171). NO donors have been demonstrated to lead to increased bacterial translocation via enterocyte damage directly as well as tight junction dilatation (172) and inducing bacterial internalization (173). Exaggerated LPS-TLR4 signaling has also been demonstrated to lead to TJ and AJ disruption and down-regulation directly (174,175). Enterocyte TLR4 was also shown, by the Hackam laboratory, to lead directly to translocation of bacteria via TLR4-mediated internalization of Gram-negative bacteria into membrane-bound phagosomes. Furthermore, TLR4 signaling was found to be critical and sufficient for enterocyte phagocytosis as LPS-coated latex particles were preferentially internalized compared with uncoated controls. Additionally, HEK-293 cells, which do not express TLR4, acquired the ability to internalize E. coli once transfected with TLR4-MD2 compared with untransfected controls. Internalization of Gram-negative bacteria across the epithelium to mesenteric lymph nodes was demonstrated to be significantly decreased in TLR4-mutant animals compared with wild-type counterparts (103).

1.3.3 TLR4 Inhibition of Intestinal Restitution

The intestinal epithelial monolayer is subjected to a variety of physiologic stressors and subsequently undergoes complete epithelial turnover every 3-5 days (176). In order for this to occur with little to no lag in intestinal function and barrier integrity, the epithelial monolayer must possess robust regenerative capacity. Following epithelial injury or physiological sloughing, IECs within the epithelial monolayer will be lost, posing a potentially critical problem of gut barrier maintenance. Gaps within the monolayer are filled through two coordinated processes: Epithelial Restitution and Proliferation. Restitution involves migration of surrounding

IECs to close the gap rapidly. Subsequent generation of new IECs from both intestinal stem cells and surrounding epithelium completely restores and closes the monolayer defect (41). In seeking to explore the mechanisms by which TLR4 adversely affects mucosal repair, TLR4 activation was observed to increase the adhesiveness that enterocytes exert on their underlying matrix, profoundly restricting their ability to move along the basement membrane via increased TLR4-mediated activity of Rho-GTPase and focal adhesion kinase and focal adhesion generation. These adhesions culminated in a significantly reduced migration rate of cultured IECs following wound generation of a confluent epithelial monolayer as well as *in vivo* as demonstrated via decreased migration of BrdU labeled IECs following intraperitoneal injection of LPS and subjection to NEC model (105,155,177). The activation of Rho-GTPases culminating in decreased IEC migration was demonstrated to occur upon NO generation via TLR4 stimulation (178).

In addition to significant reductions in enterocyte restitution TLR4 signaling concurrently inhibits IEC proliferation, in the small bowel, upon exposure to LPS injection and NEC models (155). TLR4 activation inhibits enterocyte proliferation through direct dysregulation of the wnt-β-catenin signaling pathway, which is the predominant signaling cascade that regulates cell division within the intestine (159). It is also important to emphasize that TLR4 activation in enterocytes —as opposed to other TLR4-expressing cells such as macrophages—was found to be important in the pathogenesis of experimental NEC, as the delivery of viral particles expressing a dominant negative form of TLR4 to the intestinal epithelium of mice reduced NEC severity (159) as well as through the generation and use of mice lacking TLR4 expression specifically in the intestinal epithelium via CRE-LOXP technology (70).

1.3.4 TLR4 Induction of Enterocyte Apoptosis

One of the most critical aspects of the pathogenesis of NEC remains IEC apoptosis. Apoptosis refers to a type of cellular death that is programmed and well coordinated with a set of standard morphological features culminating in the removal of dying cells (179) (**Figure 11**). Unlike necrosis, which is a form of cell death that incorporates rapid breakdown of cellular membranes and release of pro-inflammatory cellular contents, such as DAMPs, into the extracellular space (180) apoptosis is typically non-inflammatory. Triggers of enterocyte necrosis in the gut include trauma (ischemia-reperfusion) and bacterial toxins (181). Necrosis and apoptosis differ also in regards to their respective etiologies where necrosis is due to pathogenic means and apoptosis can be physiologic or pathologic. Indeed, enterocyte apoptosis is quite necessary to allow for epithelial turnover and maintenance of monolayer integrity (41).

The decision for a specific cell to begin the programmed cell death cascade is one that represents a very delicate and intricate balance of pro- and anti- apoptotic signals. In mammalian cells, the decision to initiate apoptosis signaling cascades is heavily influenced by the activation and activity of a family of cysteine proteases named Caspases. The activation of these proteases, which exist within cells as inactive precursors, can occur via two distinct pathways: The intrinsic and extrinsic pathways (183) (**Figure 12**). Classical activators of the extrinsic pathway includes Tumor Necrosis Factor-a (TNFα), Fas Ligand and a host of TLR ligands including LPS (180,184-187). Binding to their respective receptors leads to a pro-apoptotic signal that yields the recruitment of Fas-Associated Death Domain (FADD) or MyD88, in the case of TLR4, to the death domain of the receptor thereby leading to formation of the Death-Inducing Signaling

Complex (DISC) upon complex formation with and activation, via proteolytic cleavage, of pro-caspase 8 (188). Once Caspase 8 has been released from the DISC in its active form, it will next

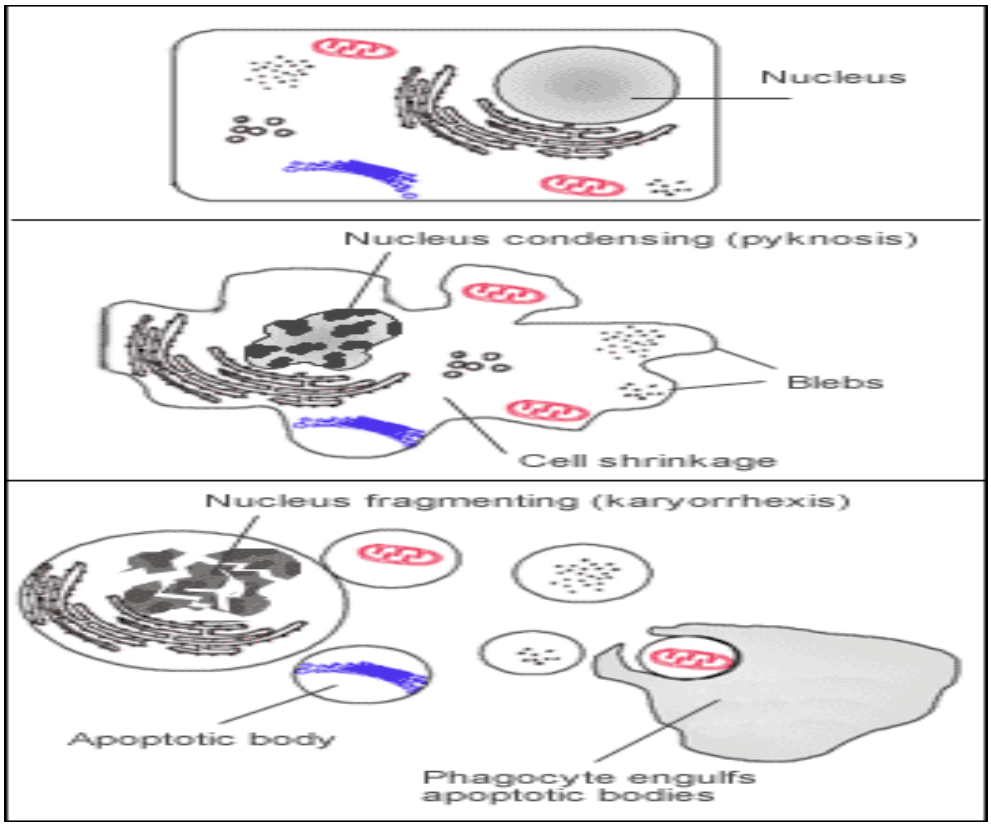


Figure 11.Basic cellular morphology and processes of apoptosis. Following initiation of apoptosis via a variety of stresses, biochemical events are put into play that lead to characteristic morphological changes and cell death. Changes include rapid chromatin/nuclear condensation into compact patches alongside the nuclear envelope (pyknosis) and chromatin/nuclear fragmentation (karyorrhexia). The nuclear envelope becomes discontinuous. Cells undergo significant shrinkage and rounding with generalized blebbing of the plasma membrane. Cells subsequently break apart into several vesicles termed apoptotic bodies containing condensed cytoplasm and tightly packed organelles which are quickly cleared via surrounding phagocytic cells. The rapid packaging and clearance of apoptotic bodies ensures, unlike in necrotic death, dying cells do not elicit an inflammatory response. *Figure taken from(182)*

continue on to activate downstream caspases including caspase 3 and 7 in addition to BH3-interacting domain death agonist (BID). This culminates in an increase in mitochondrial outermembrane permeability and cell death (189). Increased permeability is achieved via pro-apoptotic proteins including Bcl-2 associated X (BAX) and Bcl-2 antagonist/killer (BAK) which are mitochondrial membrane bound and lead to pore formation, the mitochondrial apoptosis-

channel, upon their activation (190). This thereby causes the release of both Cytochrome c as well as the Second mitochondrial activator of caspases (Smac) (190,191). Upon its release, Cytochrome c is free, along with apoptotic protease-activating factor (APAF1) and procaspase 9, form the Apoptosome. The apoptosome then functions to activate procaspase 9 releasing caspase 9, which in turn cleaves and activates procaspase 3 (180,192). Release of Smac serves to prevent inhibitors-of-apoptosis proteins (IAP) from blocking the function of the apoptosome (189,190). Once activated, caspase 3 proceeds to target specific proteins which include the inhibitor of caspase-activated DNase (ICAD), which thus frees CAD to enter the nucleus and cause DNA fragmentation characteristic of apoptosis (193).

Activation of the intrinsic pathway typically stems from toxic stressors such as DNA damage or pH alterations (181,191,194-196). Initiation of the intrinsic pathway via these means activates a group of pro-apoptotic proteins that are members of the B cell lymphoma-2 (Bcl-2) family and known as BH-3-only proteins. BH-3-only protein activation leads to increased activity of BAK and BAX either directly (197-200) or through antagonizing anti-apoptotic proteins including Bcl-2 and Bcl-2-like protein 1 (194,197,198,201).

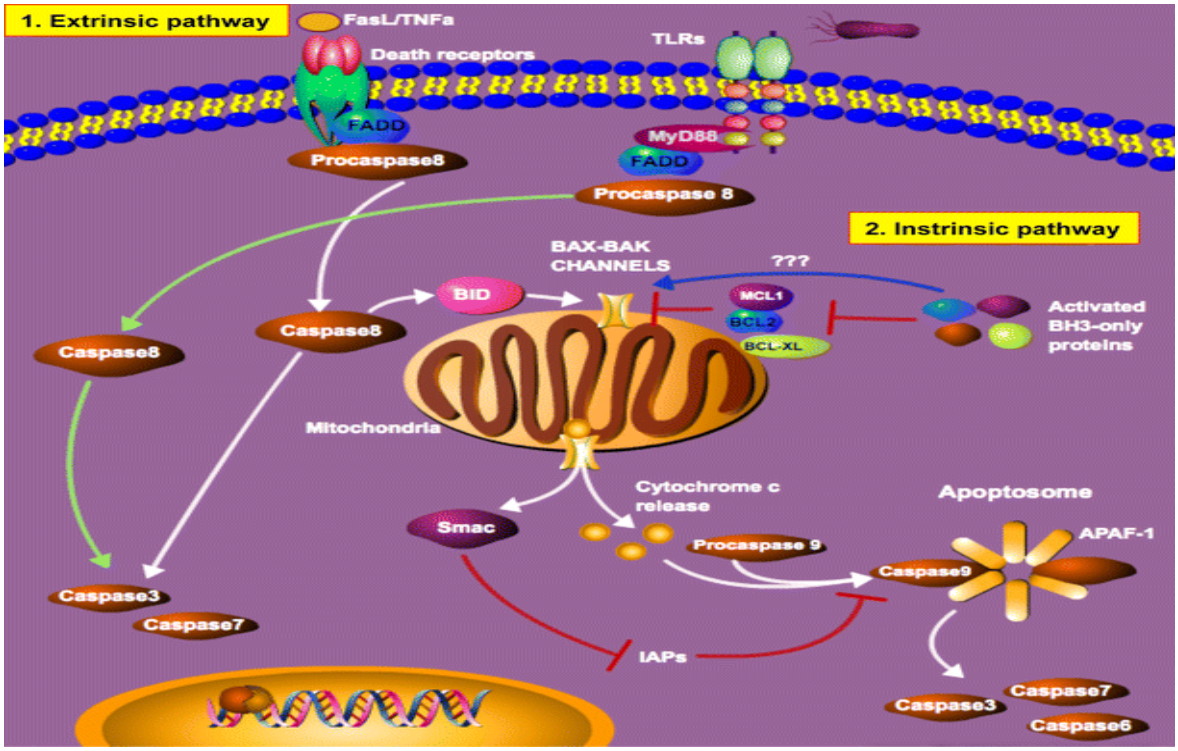


Figure 12. Molecular Signaling cascades governing the intrinsic and extrinsic pathways of apoptosis in mammalian cells. As described in the text, extrinsic pathway initiation occurs classically via FasL, TNFa, or PAMP/DAMP binding to their respective receptors activating downstream caspases. Intrinsic pathway activation can occur in response to toxic stress or DNA damage giving rise to BH3-only protein activation, which antagonize anti-apoptotic proteins Bcl-2, Bcl-XL or MCL1 or directly activate pro-apoptotic proteins BAK and BAX. procaspase 9, APAF1 and cytochrome c, following its release from the mitochondria, form the apoptosome which activates the effector caspase 3. *Figure reprinted with permission from Springer Science and Business Media. Article: (41). Copyright 2011.*

Although enterocyte apoptosis is a key physiologic process in gut homeostasis and function of the epithelial barrier via the removal of senescent, malfunctioning or harmful cells (184) the rate at which apoptosis occurs is a key factor in determining whether the reparative and regenerative mechanisms in place will be sufficient to ensure barrier integrity. Loss of integrity and ensuing microbe or microbial product translocation will lead to activation of the intestinal immune system initiating a dangerous positive feedback loop of bacterial translocation, inflammation, inflammation-induced apoptosis and further barrier disruption. The role of TLR4 in disrupting both enterocyte proliferation and migration were discussed above. Now we

consider the direct role of TLR4 and NF-kB induction of enterocyte apoptosis. As mentioned previously, NF-kB is critical for a number of cellular process including pro-survival and pro-apoptotic signals. Studies of mice deficient in NF-kB responses consistently demonstrated increased intestinal apoptosis and severe intestinal inflammation compared with wild-type mice (202) and increased intestinal injury in response to ischemia-reperfusion injury (203). Also mice lacking NF-kB signaling in IECs of the distal small bowel and colon led to increased susceptibility to intestinal injury and inflammation in a dextran sodium sulphate-induced colitis model partly due to increased rates of epithelial apoptosis (94). These studies are in contrast to the induction of apoptosis via NF-kB activation in the newborn small intestine not seen in the TLR4-deficient or adult mouse. This significant increase in enterocyte apoptosis serves as a key pathogenic factor in the development of NEC (158). TLR4-mediated injury within the newborn small intestine is also in stark contrast to previous work in the adult colon in which TLR4 signaling results in optimal proliferation and protection of colonic epithelia from exaggerated apoptosis (204). Differences in injury model (NEC vs Colitis), location of injury (small vs large bowel) and age (newborn vs adult) are likely contributing factors to the observed differences. This is supported by the demonstration that TLR4 signaling within the colon and adult small bowel did not result in apoptosis (158). One possible explanation accounting for such differences between the newborn and adult small bowel as well as the adult colon is TLR4 ontogeny. TLR4 expression within the developing intestine *in utero* was found to significantly elevated throughout the gut during development, then fall shortly after birth, to rise, then fall once more immediately after weaning. TLR4 was found to be responsive at each of these time-points, including *in utero* where TLR4 was hyper-responsive to stimulation by LPS following in utero injections (113). With the transition from a sterile to a non-sterile environment, as occurs

following birth, the gut is rapidly colonized with what will become commensal flora. It is not surprising therefore that with premature birth and associated high levels of TLR4 expression, the newborn small bowel is significantly predisposed to exaggerated inflammation and NEC. The gut inflammation and injury due to higher baseline TLR4 expression in the premature gut is also exacerbated by conditions of prematurity including hypoxia and remote infections, both of which lead to increased and sustained TLR4 expression and signaling (41,155). However, in the adult colon, where TLR4 expression is relatively low, one would expect minimal signaling to occur with TLR4 playing more of a homeostatic role than pathologic (41).

Contributing aspects of TLR4 signaling leading to induction of enterocyte apoptosis in the pathogenesis of NEC involves the generation of pro-inflammatory mediators downstream of NF-kB activation. iNOS activity remains one of the key downstream effectors up-regulated in this setting (205,206). Among the three isoforms of the nitric oxide synthases, which catalyze the formation of NO from Arginine and O_2, iNOS plays a key role in host immunity and is expressed not under normal conditions but during inflammatory states (207). NO generated by iNOS, unlike that generated by other NOS isoforms, is not rapidly scavenged by red blood cells into nontoxic nitrates. iNOS generated NO is thus free to interact with ROS such as superoxide anions yielding the highly toxic intermediate peroxynitrite ($ONOO^-$) which is significantly more reactive than either NO and superoxide (208). Peroxynitrite generation is considered a hallmark for the inflammatory response and host defense given its highly effective ability to kill microbial targets via its ability to directly oxidize target protein motifs including thiols, iron/sulfur centers and zinc fingers significantly disrupting target protein function (209). Peroxynitrite, while not a free radical itself, can also lead to damage through its breakdown into highly reactive free radicals hydroxide and nitrogen dioxide (210). Peroxynitrite generation can also lead to the

nitration of tyrosine residues of proteins. As nitration can serve as an alternative to tyrosine phosphorylation, this greatly affects the target protein function leading to disruption of intracellular signaling processes (211). As discussed above, exaggerated TLR4 signaling and expression are key factors to the pathogenesis of NEC. With TLR4 stimulation by LPS in IECs, iNOS expression is significantly up-regulated (170,212) from its low expression at baseline in distal ileal tissue (213). Increased and sustained iNOS activity can lead to intestinal barrier failure not only through direct inhibition of IEC migration, proliferation and induction of bacterial internalization but through the induction of enterocyte apoptosis (210). The increased enterocyte apoptosis as seen in NEC is strongly correlated to increased peroxynitrite activity where apoptotic cells also co-localize with increased 3-nitrotyrosine and iNOS staining (206,214). Upon exposure of enterocytes to peroxynitrite, apoptosis occurs via caspase-dependent pathways discussed above (215) as well as necrosis via inhibition of mitochondrial respiration (210). Following TLR4-mediated NF-kB activation in IECs there is a significant increase in pro-inflammatory mediators such as TNFα, IL-1, IL-6, platelet activating factor (216) which have all been demonstrated to play a role in murine and human NEC pathogenesis (210,214,217-219).

Figure 13 summarizes the key pathogenic features of TLR4 signaling that serve as critical factors in the etiology of NEC. The importance to understand the molecular mechanisms leading to TLR4-mediated apoptosis in the setting of NEC stems from the fact that it is a programmable, reversible, early event in NEC and as such a key therapeutic target for novel treatments.

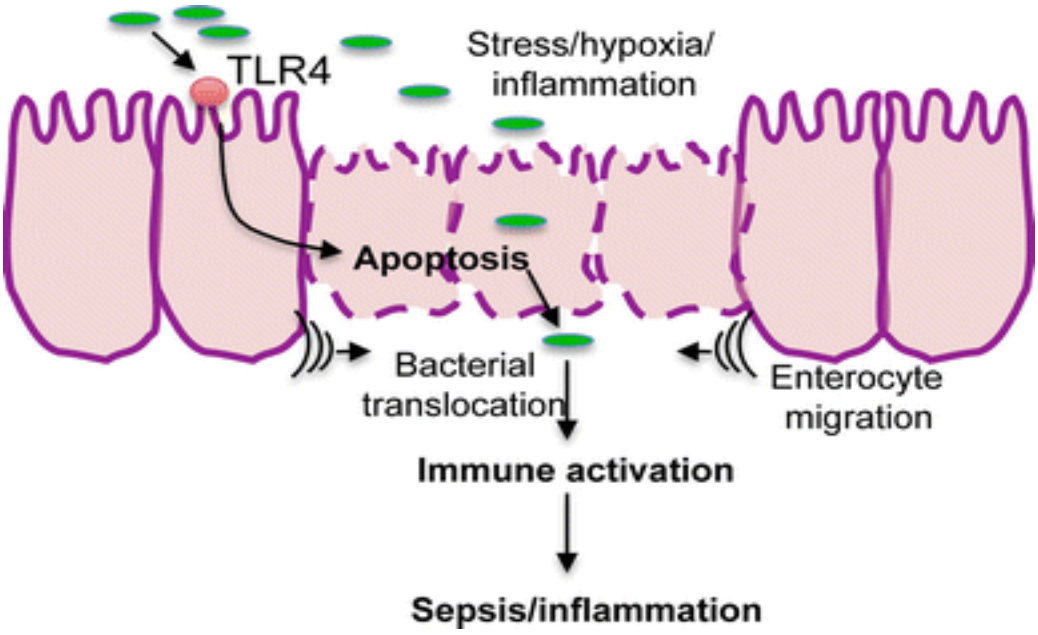


Figure 13.Detrimental effects of exaggerated TLR4 activation. Increased TLR4 signaling in conditions conducive to increased TLR4 expression (prematurity, hypoxia, inflammation), plays a key role in mediating gut inflammation and injury via inhibition of intestinal repair mechanisms: Restitution and Proliferation. IECs lost to apoptosis in the epithelium generate gaps within the monolayer potentially compromising barrier integrity. Typically, these gaps are quickly filled by migration and proliferation of surrounding IECs. TLR4 activation, however, not only leads to exaggerated IEC apoptosis that quickly outpaces the regenerative capacity, but also directly inhibits migration and proliferation of surrounding IECs. Therefore, bacteria are more likely to translocate the discontinuous barrier leading to immune activation and subsequent sepsis and systemic inflammation. *Figure reprinted with permission from Springer Science and Business Media. Article: (41). Copyright 2011.*

1.3.5 Modulation of Enterocyte TLR4 Signaling

TLR4 signaling in the intestinal epithelium in response to commensal enteric flora can lead to NEC, but this is kept in check by the PRR inhibitory mechanisms discussed previously. As an additional example of this method by which TLR4 signaling can be reduced in enterocytes, Lotz *et al.* (80) have shown that the enterocytes of vaginally born—but not C-section delivered mice—are relatively resistant to TLR4 activation *in vitro* because of a posttranscriptional down-regulation of IRAK-1. Wang *et al.* (220) have shown that expression of MAPK phosphatase 1 leads to a reduction in the extent of signaling through TLR4— and other

TLRs—in enterocytes, as a negative feedback loop requiring NF-kB. This phosphatase leads to the dephosphorylation of MAP kinases, which are important effectors of TLR4 signaling. Recent evidence also suggests that cross-talk between two other innate immune receptors—namely TLR9 and NOD2—can prevent exaggerated TLR4 signaling. TLR9 is the receptor in enterocytes for bacterial DNA, which is rich in CpG groups and significantly hypomethylated, in contrast to mammalian DNA. The Hackam laboratory demonstrated that activation of TLR9 with CpG-DNA in enterocytes both *in vitro* and in the newborn intestine led to reduced TLR4 signaling as manifest by reduced cytokine production and decreased apoptosis through a mechanism that involved the up-regulation of the cytoplasmic inhibitor IRAK-M (113). The reciprocal expression of TLR9 and TLR4 was found to influence the extent of TLR4 signaling, and the development of NEC was accompanied by a relative increase in TLR4 with a concomitant reduction in the protective TLR9 (113). Indeed, these findings may explain the relative protective value observed from probiotic administration to infants with NEC (221), as these probiotic preparations are rich in bacterial DNA, through which activation of TLR9 on the host would be expected to limit TLR4 signaling and reduce NEC severity. In recent experiments, the Hackam laboratory has also shown that the cytoplasmic bacterial sensor NOD2—which recognizes the bacterial motif MDP and which has risen to recent prominence because mutations in NOD2 are linked to the development of inflammatory bowel disease in humans (222)— limits TLR4 signaling through posttranslational effects on TLR4 and through the up-regulation of the pro-apoptotic protein SMAC (158). From a therapeutic point of view, activation of TLR9 by the administration of CpG-DNA or activation of NOD2 with administration of MDP resulted in a marked reduction in TLR4-signaling (**figure 14)** and in the severity of experimental NEC in mice.

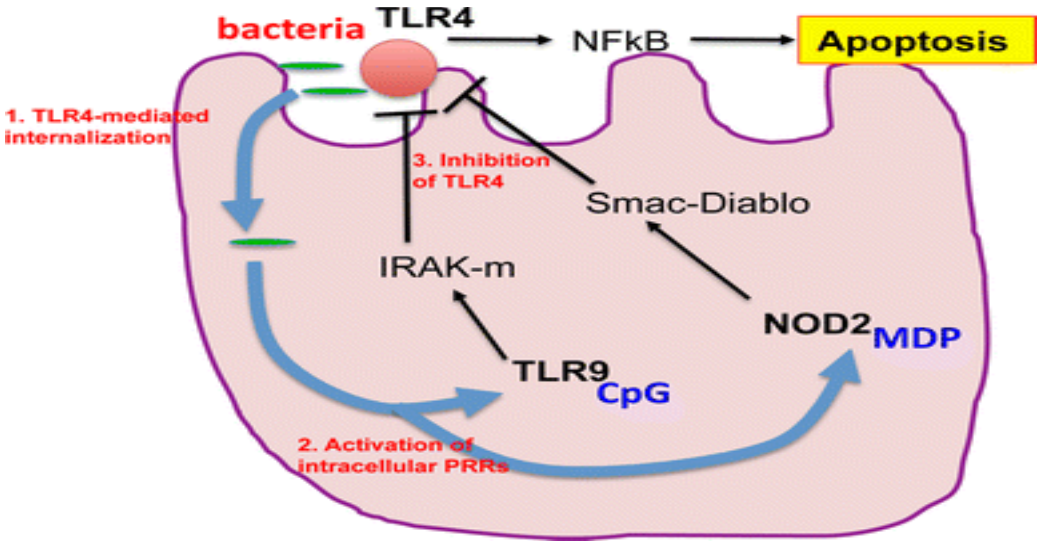


Figure 14.Crosstalk between PRRs as regulators of TLR4 signaling in IECs. The Hackam lab has previously demonstrated that NOD2 (158) andTLR9 (113) activation can limit TLR4-mediated apoptosis in IECs and NEC via inhibition of Smac and activation of IRAK-M respectively. The putative sequence of events for this regulation includes the TLR4-mediated internalization of luminal bacteria, activation of the intracellular PRRs via the PAMPs MDP (NOD2) and CpG (TLR9). *Figure reprinted with permission from Springer Science and Business Media. Article: (41). Copyright 2011.*

These findings not only highlight the mechanisms that maintain levels of TLR4 signaling under physiological conditions but also suggest novel therapeutic approaches to this devastating disease. These studies raise the possibility that the stimulation of these and other, yet to be defined, TLR4 regulatory pathways may provide therapeutic benefit to infants with NEC, or alternatively, may serve as a preventive strategy when administered to premature infants who are at risk. In order to uncover additional TLR4 regulatory pathways important to the pathogenesis of NEC, microarray analysis was performed comparing murine distal ileal tissue of wild-type animals subjected to a NEC model to those who were not. A selected table of the microarray results is presented in **Table 4**. Among a host of proteins down-regulated in the setting of NEC, the molecular chaperone molecule, Heat Shock Protein 70 (HSP70) was most significant.

Gene Name	Fold Down- Regulation
Hspa1a (HSP70)	-4.79
Ly96	-3.70
Caspase 8	-3.50
Cd14	-3.17
IL1b	-2.46
IL6ra	-2.30
Cd80	-2.23
Ptgs2 (Cox-2)	-2.02

Table 4. This table lists the most significantly down-regulated genes that were found in intestinal tissue taken from mice subjected to a NEC model compared with healthy controls

1.4 MOLECULAR CHAPERONES

Along with the discovery that the linear polypeptide chain contained all necessary information for a specific protein's primary, secondary and tertiary structure (223), protein folding was believed to occur spontaneously. However this notion is very unlikely due to the Levinthal paradox which refutes that peptide chains undergo an exploration of possible conformations as this would require a physiologically unrealistic time to "find" the correct conformation (224). Repulsive and attractive forces contained in the chain restrict the possibilities and direct folding through the generation of various folding intermediates (225,226). While some proteins do not require assistance in assuming their final proper (i.e. functional) structure, for a significant number of others, assistance is fundamental to not only generate native proteins but prevent detrimental partially or misfolded aggregate formation. Aggregate formation can occur among these non-native protein intermediates due to exposed hydrophobic residues contained within the polypeptide chain and are highly toxic in eurkaryotes (224,227). Given the propensity of newly translated peptide chains to undergo aggregate formation most chaperones interact with these chains co-translationally alongside the ribosome (224). Subsequently a molecular chaperone is

broadly defined as any protein that interacts, stabilizes, or aids a non-native protein or peptide chain acquire its native structure but not present within that final native structure (228,229). Chaperones are involved in a vast number of cellular processes including nascent peptide chain folding, refolding of misfolded or denatured proteins and proteolytic degradation of proteins alongside Ubiquitin and the proteasome**.** Chaperones, like the Heat Shock Proteins recognize and bind non-native hydrophobic residues and promote, via ATP-dependent binding and release steps (224).

1.5 HEAT SHOCK PROTEINS

Heat Shock Proteins (HSPs) encompass a family of highly conserved across virtually all living organisms cytoprotective molecular chaperones integral to proteostasis and protection against a variety of cellular stresses (230). HSPs, so named from their initial description, by Ritossa in the 1960s, as being strongly up-regulated upon heat exposure (231) are more accurately thought of as stress response proteins. They are potently induced by a variety of stresses, both physiologic and pathologic. These stresses lead to protein misfolding, aggregation or disruption of regulatory protein complexes and HSP induction in an attempt to restore cellular balance(232). Additionally, HSPs are both conditionally expressed or inducible and behave as molecular chaperone molecules for other cellular proteins. Their chaperone function, as discussed above, is critically important to the proper folding and refolding of unfolded or misfolded nascent peptide chains, respectively. As also important for proper proteostasis, HSPs, along with ubiquitin and the proteasome, are central to the degradation of damaged or incorrectly folded proteins, again as a result of both physiologic or pathologic processes (233,234).

Mammalian HSPs have been stratified into two different groups: High and low molecular weight (**Table 5).**

Family	Name	Other names	location	Normal	Stress
hsp 100	hsp 110		Nucleus/nucleolus	+	++
	hsp 104		Cytosol	+	+++
	grp 100		ER/Golgi	+	++*
hsp 90	hsp 90	hsp 82, HtpG	Cytosol/nucleus	++	+++
	grp 94	ERp90	ER	+	++*
hsp 70	hsp 70	hsp 72, DnaK	Cytosol/nucleus	–	+++
	hsc 70	hsp 73	Cytosol/nucleus	++	?
	grp 78	BIP, Kar2p	ER	++	+++*
	mtp 70	Ssc1p, grp 75	Mitochondria	+	++
hsp 60	hsp 60	GroEL, cpn60	Cytosol	+	+
	hsp 58	HuCha 60	Mitochondria	+	+
hsp 40	hsp 40	DnaJ, hdj-1	Cytosol/nucleus	+	++
hsp 30	hsp 32	heme oxygenase	Cytosol	+	++
	hsp 35	G3PDH	Cytosol	+	++
small hsp	hsp 27	α-crystallin	Cytosol/nucleus	+	++
	hsp 42p			+	?
	hsp 10	GroES, cpn-10	Mitochondria	+	++

Table 5.List of major HSP families and family members. The table depicts the subcellular localization of each of the major HSP family members and their respective expression level both at baseline and during settings of stress. *Table reprinted with permission from Wolters Kluwer Health. Article: (230). Copyright 1999.*

The small HSP family is a group of ATP-independent chaperone HSPs varying between ~15-30KDa (235). Small HSPs include the prominent member HSP27 which is important to protect against protein aggregation (236). HSP27 is able to form significant oligomers in its dephosphorylated state (237), but during stress HSP27 phosphorylation is catalyzed by MAPKs. Phosphorylation following MAPK activation occurs by a variety of signals including mitogens, cytokines such as IL-1b and TNFa as well as ROS (232). HSP27 induction occurs relatively late in response to ROS stress, serum starvation or radiation and in a variety of cells and tissue types (232).

Four major members of the high molecular weight subfamily include HSP90, HSP70, HSP60 and HSP40. These subfamilies and their individual members can be localized to various subcellular compartments and vary in their expression i.e. constitutive versus inducible. Each of

the members of this group, aside from HSP40, are ATP-dependent chaperone molecules requiring co-chaperone proteins to modulate both their conformation as well as ATP-binding (232). Mammalian HSP60 is primarily localized to the mitochondrial matrix but homologues have also been detected in extra-mitochondrial sites including the cytosol (238,239). HSP60 family members are typically constitutively expressed but do demonstrate a moderate induction upon stressors, most potently in response to heat (240). Given its localization, HSP60 primarily plays a role in the folding as well as degradation of misfolded or denatured mitochondrial proteins in an ATP-dependent manner. The ATPase activity of HSP60 is regulated by the small co-chaperone HSP10 which, via direct binding of HSP60, subsequently regulates its substrate binding (232). A loss of HSP10 significantly affects newly imported mitochondrial proteins (241). Prominent members of the HSP90 family include HSP90a and HSP90b (242). HSP90 family members are quite essential for the viability of eukaryotic cells and as such are very highly expressed, constituting nearly 1-2% of all cytosolic proteins with additional capacity for induction upon exposure to cellular stresses (232). HSP90 maintains a role in ensuring the stability of a number of target proteins through direct and indirect associations. These client proteins include ligand-dependent and independent receptors (243,244), including a variety of tyrosine and serine/threonine kinases (245,246). HSP90 inhibition leads to the targeting of these client proteins for proteosomal degradation. The HSP70 family represents one of the most well-conserved and well studied chaperones and encoded by a host, roughly 11, of genes in humans (232). HSP70 family members vary in terms of subcellular location, with some members located within the cytosol like the highly inducible HSP70 or the constitutively expressed, Heat Shock Cognate 70 (HSC70) or localized to mitochondria (mtHSP70). A key HSP70 family member resides in the Endoplasmic Reticulum (ER) known as BiP. BiP is a fundamental player in the

maintenance of ER homeostasis via resident chaperone functions and a key activator of the Unfolded Protein Response (UPR) in settings of ER stress (247). Eukaryotic HSP70s possess two functional domains: a N-terminal ATPase domain, like HSP90, critical to its chaperone function and a peptide/substrate-binding domain where HSP70-substrate interactions occur via a cleft composed of several hydrophobic residues. They also possess a C-terminal a-helical domain that functions like a lid of the substrate-binding domain opening and closing under the control of the ATPase domain. Contained in the C-terminus is also an EEVD motif important for co-chaperone associations (**Figure 15**). HSP70 interacts with a wide-variety of co-chaperone molecules that are important supporters of its role in proteostasis such as HSP40, HSP-organizing protein (HOP), HSP-interacting protein (HIP), BAG-1, BAG-3 and the C-terminus of HSP70 interacting protein (CHIP)(232). Co-chaperones are able to interact with either the N or C-terminal domains of HSP70 and thereby significantly modify its function. These functions include in addition to nascent peptide chain folding, the assembly of multiprotein complexes and protein trafficking (248). The ability of HSP70 to protect a variety of cell types from a wide variety of apoptosis-inducing stresses is well characterized (235).

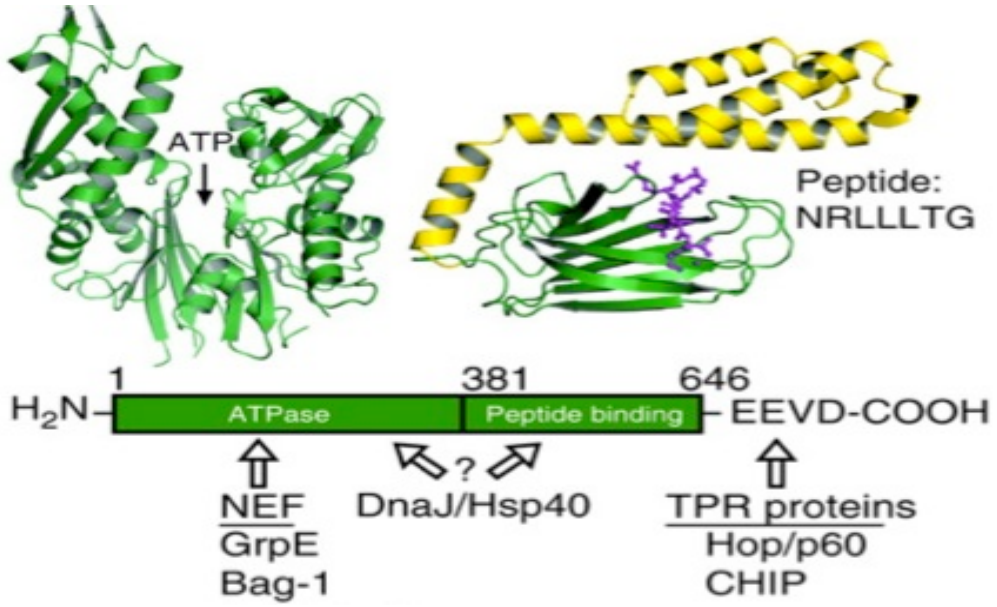


Figure 15.Structure of the key functional domains of the HSP70 family. HSP70 bears a N-terminal ATPase domain critical to inducing conformational changes allowing HSP70 to "catch and release" client proteins in its chaperoning cycle depicted in **Figure 16** and a peptide-binding domain consisting of a hydrophobic cleft. The C-terminal "lid" domain of HSP70 additionally contains an EEVD motif critical to HSP70 ability to interact with TPR containing co-chaperone proteins such as Hop and the E3 ligase CHIP. The N-terminal domain also binds co-chaperone proteins including nucleotide exchange factors, GrpE and BAG-1. HSP70 also directly interacts with the HSP40 chaperone family critical to nascent peptide chain chaperoning, see **Figure 16.** *Figure reprinted with permission from Macmillan Publishers Ltd: Nature Structural & molecular biology. Article: (229). Copyright 2009.*

1.5.1 HSP70 Family-mediated Protein Folding

Chaperones such as HSP70 function by holding nascent and newly synthesized peptide chains in a state to allow for proper folding upon their release into the cytosol. This is in contrast to the large cylindrical chaperones known as chaperonins (e.g. HSP60 family members) found in the eukaryotic cytosol and organelles like mitochondria, that offer physically confined compartments for the entire protein or particular domains to fold sequestered away from the cytosol (249). The majority of small peptide chains may fold upon release from the ribosome without requiring chaperone interaction. A significant amount of nascent chains reach their native state via HSP70-HSP40 assistance with a few number of these requiring additional assistance from HSP90 for folding including eukaryotic kinases and other signal-transduction

proteins (250,251). Although the importance of the barrel shaped chaperonins has been long appreciated, defining an essential role for nascent chain-binding chaperones in protein folding initially proved difficult due to the significant redundancy among individual components (249). A number of the members these chaperones, such as specialized HSP70 proteins such as Ssb1 in yeast species and nascent chain-associated complex (NAC), bind directly to the ribosome near the peptide chain exit site and are perfectly positioned to associate with newly synthesized chains and dissociate upon chain release from the ribosome (228,252). Longer polypeptide chains interact with a second class of nascent chain-binding chaperones, including classical HSP70s, which do no directly associate with ribosomes (253-255). These chaperones not only serve to stabilize peptide chains but aid in the co- or posttranslational folding or mediate transfer to downstream chaperones (253,254,256).

The classic i.e. non-ribosome binding HSP70 family members localized to various subcellular compartments require members of the HSP40 family in aiding with protein folding in an ATP-dependent manner (249). As previously mentioned and depicted in **Figure 15** above, HSP70 is portioned into a N-terminal ATPase domain and a C-terminal peptide binding domain which is further divided into a beta-sandwich subdomain with a peptide-binding cleft and an a-helical latch-like segment that functions similar to a lid for the substrate binding domain (257). Target peptides are roughly seven, typically hydrophobic, residues with a preference for leucine and isoleucine (228,258). Peptides are HSP70 bound, not only through hydrophobic side-chain interactions, but through hydrogen bonds with the peptide backbone itself (257). Rapid binding of peptides by HSP70s occurs in the ATP-bound state in which the a-helical “latch” over the peptide-binding cleft is in the open position. Maintaining a stable hold upon bound peptides requires closure of the latch, which is achieved via a conformational change upon ATP

hydrolysis. Cycling between ATP-bound and unbound states is regulated by HSP40 family members as well as Nucleotide Exchange Factors (NEF) such as the co-chaperones BAG-1 and BAG-2. The C-terminus of HSP40 family members functions as a chaperone in recognizing hydrophobic residues and thereby can facilitate recruitment/shuttling of nascent chains to HSP70s (259-261).NEFs induce the release of ADP from HSP70 family members and upon rebinding ATP the HSP70-Peptide complex dissociates ending the chaperone cycle (**Figure 16**).

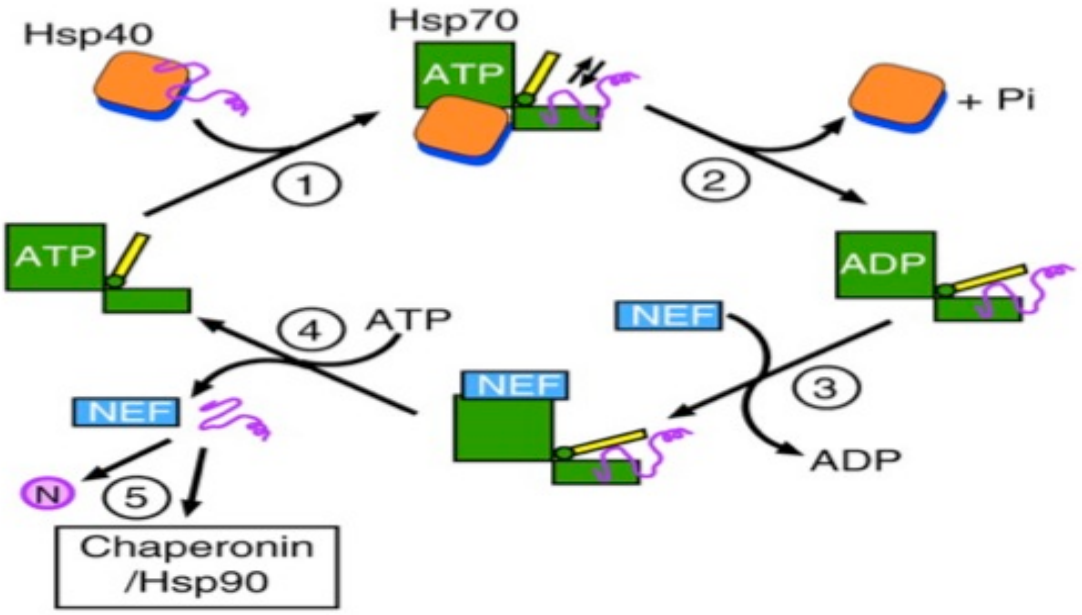


Figure 16. HSP70-mediated nascent peptide chain chaperone cycle. Nascent peptide chains are bound to HSP40 following translation by the ribosome. (1) HSP40 shuttles the nascent chains and passes them to the C-terminal protein-binding domain ofHSP70 while in direct contact with the chaperone. The peptide-binding domain of HSP70 is further divided into a beta-sandwich subdomain with a peptide-binding cleft and an a-helical latch-like segment. Rapid peptide binding to HSP70 occurs in its ATP-bound state. (2) Subsequent hydrolysis of ATP results in a conformational change and "clamping down" of the latch-like a-helical motif ensuring tight binding. (3) NEFs such as BAG-1 efficiently exchange ADP for ATP subsequently allowing HSP70 to release previously bound peptides, (4) freeing it to bind new chains. (5) released peptides are successfully repaired/folded or shuttled to chaperonins/HSP90 via HSP70-Hop for additional chaperoning. *Figure reprinted with permission from Macmillan Publishers Ltd: Nature Structural & molecular biology. Article: (229). Copyright 2009.*

Other HSP70 homologs such as BIP in the ER cooperate in protein folding with co-chaperones that lack separate affinity for hydrophobic residues. These HSP70 family members may be able to bind longer peptide chains and independent of hydrophobic sequences, which is

in contrast to the classical cytosolic HSP70s like HSC70 (262). Although all HSP70s cooperate with HSP40 family co-chaperones, most eukaryotic HSP70s may be NEF-independent for their general function. The dispensability of these factors stems from the fact that the rate-limiting step in the ATPase cycle is the hydrolysis of bound ATP and not the disassociation of the resulting ADP (249).The NEF, BAG-1 has been shown to regulate the chaperone activity of HSC70 via accelerated release of ADP and competing for ATPase domain binding with HiP which stabilizes the ADP bound state. This regulatory activity of BAG-1 has been implicated in aiding in the anti-apoptotic function of HSC70 (263). With release of peptides from HSP70s they are free to conform to their native state. Slow-folding intermediates can rebind to HSP70s to prevent detrimental aggregation. Long peptide chains with multiple domains can be involved in folding cycles facilitated by multiple HSP70s aiding the folding of each individual domain by preventing/reversing intramolecular misfolding. The discovery of HSP70 isomerase activity supports this function as evident by spontaneous refolding of chemically denatured firefly luciferase *in vitro* (264,265). An additional example of chaperone coupling in the eukaryotic cytosol is that between HSC70 and HSP90 in the folding of signal transduction proteins (251). Substrate transfer between HSC70 and HSP90 is accomplished via the co-chaperone HOP acting as a physical linker between the two chaperones. HOP possesses two tetratricopeptide repeat (TPR) domains, which bind C-terminal sequences in HSC70 and HSP90 (266). Similar peptide transfer mechanisms are in place regulating the transfer of misfolded or non-native peptides from these chaperones to the proteasome machinery to facilitate degradation. The co-chaperone CHIP, via a C-terminal U-box E3-ligase domain (268,269), associates with the C-terminal domain of HSP70 as well as that of HSP90 via its N-terminal TPR domain and targets proteins for degradation. Among these proteins is HSP70 itself as a regulatory mechanism of HSP expression

(267). CHIP cooperates with BAG-1, which binds to HSC70/HSP70 and to the proteasome (270).

1.5.2 Ubiquitin: Modulator of Degradation and Signal Transduction

Ubiquitin (Ub) is a protein that is covalently bound to lysine residues that can either trigger proteosomal degradation or protein trafficking of tagged proteins, signal transduction or DNA damage responses (114). The process of ubiquitination involves a three-step enzymatic reaction catalyzed by three classes of proteins: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (271). E1 enzymes, using ATP, activate ubiquitin forming a thioester bond between the catalytic cysteine of the E1 and the C terminal glycine residue of ubiquitin. The activated ubiquitin is subsequently transferred to a specific E2 ubiquitin-conjugating enzyme via another thioester bond. Finally, the E3 ubiquitin ligase catalyzes the conjugation of the activated ubiquitin to a lysine residue on specific target proteins (114). In humans, over 600 E3 ligases have been identified and classified into different subgroups in regards to mechanism of ubiquitin transfer, e.g. direct transfer (HECT E3 ligases) and in coordination with E2s via E3 scaffolding (RING E3 ligases) (272). E3 ligases directly contact all protein substrates and therefore are believed to confer specificity for targeted ubiquitination (114). With the linkage of several Ub molecules upon a specific protein, the target protein is shuttled to and bound by the proteasome via these Ub tags to facilitate degradation. The proteasomal degradation process yields peptides 7-8 residues long that can be broken-down further and recycled into new proteins. Proteosomes are cylindrical complexes containing a core of four stacked rings around a central pore. Within the inner surfaces of the two inner rings exist three to seven protease active sites bearing chymotrypsin-like, trypsin-like and peptidylglutamyl-

peptide hydrolyzing activity (273,274). The outer rings function more as gates regulating ATP-dependent entry of tagged proteins via the recognition of poly-Ub tags by the inner subunits of the outer rings (273). The most common proteasome is the 26S containing a 20S core structure and two 19S caps. With the critical role of the proteasome in proteostasis, it is not surprising that it plays a central role in a number of cellular processes such as cell cycle control (275), apoptosis (276-278), viral clearance (279) and antigen presentation (280,281). Following degradation, Ub molecules are simultaneously removed and recycled by deubiquitinating enzymes (**Figure 17**).

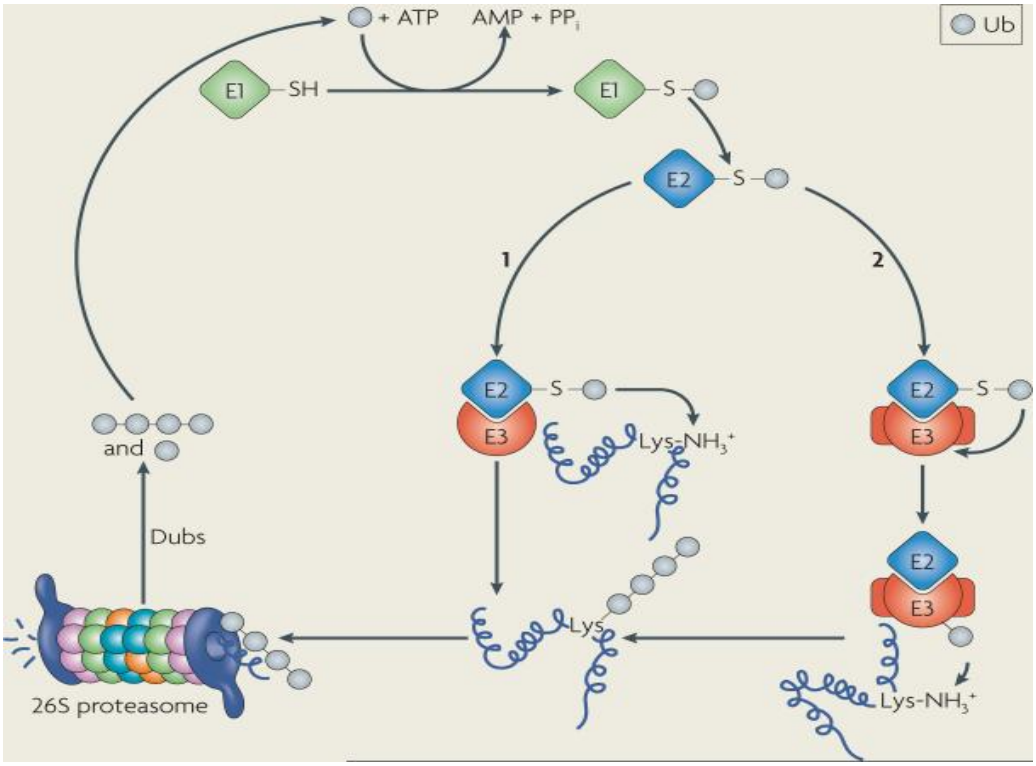


Figure 17**.**Fundamental steps of client protein ubiquitination and proteosomal degradation. Ubiquitin molecules are initially activated via an ATP-dependent coupling to E1 ubiquitin-activating enzymes via thioester linkages. Activated ubiquitin species are subsequently transferred to specific E2 ubiquitin conjugating enzymes via another thioester bond. E2 enzymes subsequently directly interact with specific E3 ubiquitin-ligase enzymes leading to the conjugation of activated ubiquitin molecules to target proteins. The mechanism of ligation differs among two main families of E3 ligases, the RING and HECT families. (1) RING E3 ligases facilitate the transfer of ubiquitin molecules onto target proteins from the E2 enzyme. (2) HECT E3 ligases facilitate the direct transfer ubiquitin molecules onto target proteins from the E3 ligases directly. All E3 ligases make direct contact with target substrates and are viewed as conferring specificity for target protein identification. Once poly-ubiquitinated client protiens are shuttled and bound to the 26S proteasome, via ubiquitin receptors on the proteosomal 19S caps, for degradation by core proteases possessing trypsin-like, chymotrypsin-like and post-glutamylpeptide hydrolyzing activity. Ubiquitin tags may be removed prior or during degradation for recycling. *Figure reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology. Article: (282). Copyright 2008.*

Polyubiquitination requires the linkage of several Ub molecules via connection of several internal lysine residues to form an oligameric Ub chain. Seven lysine residues exist as potential linkage sites including the predominant and most well-characterized targets K48 and K63 (283). Staggered K48 links typically trigger proteosomal binding and degradation of tagged proteins (e.g. IkBα ubiquitination) while K63 links serve as nonproteolytic tags for such processes as signal transduction (e.g. TRAF6 ubiquitination), receptor trafficking, kinase activation and the DNA damage response (271,283). In addition to K48 and K63, linear linkages can be formed between carboxy and amino termini of two Ub molecules (284). Linear polyubiquitination has recently arisen as an important player in NF-kB activation, where the regulatory subunit of IKK complex, IKKγ, is targeted. The role of linear ubiquitination while important for NF-kB activation is still relatively poorly understood (285). Further evidence supports a key role of linear poly-Ub of IKKγ in regulating inflammatory and apoptotic NF-kB responses, where animals lacking key components of the linear ubiquitin chain assembly complex leads to multi-organ inflammation and exaggerated NF-kB pro-apoptotic signaling pathways (286,287).

1.6 THE HEAT SHOCK RESPONSE

The Heat Shock Response (HSR) is the term given to the ancient and highly conserved response to stresses, both physiologic or pathologic, that may disrupt proteostasis that functions, alongside the Unfolded Protein Response (UPR), to return the cell back to homeostasis. The HSR and UPR serve as key stress sensors within the cell, regulating expression of members of the "proteostasis network" including molecular chaperones, ubiquitin-proteosome machinery as well as autophagic activities to clear or repair damaged proteins (233). **Figure 18** gives a non-

exhaustive list of a variety of the stresses that can lead to HSR and UPR activation, including heat shock, ROS, noxious chemicals, growth and developmental cues such as growth factors, viral/cellular oncogene activation, tissue injury, inflammation and a number of protein conformational disorders.

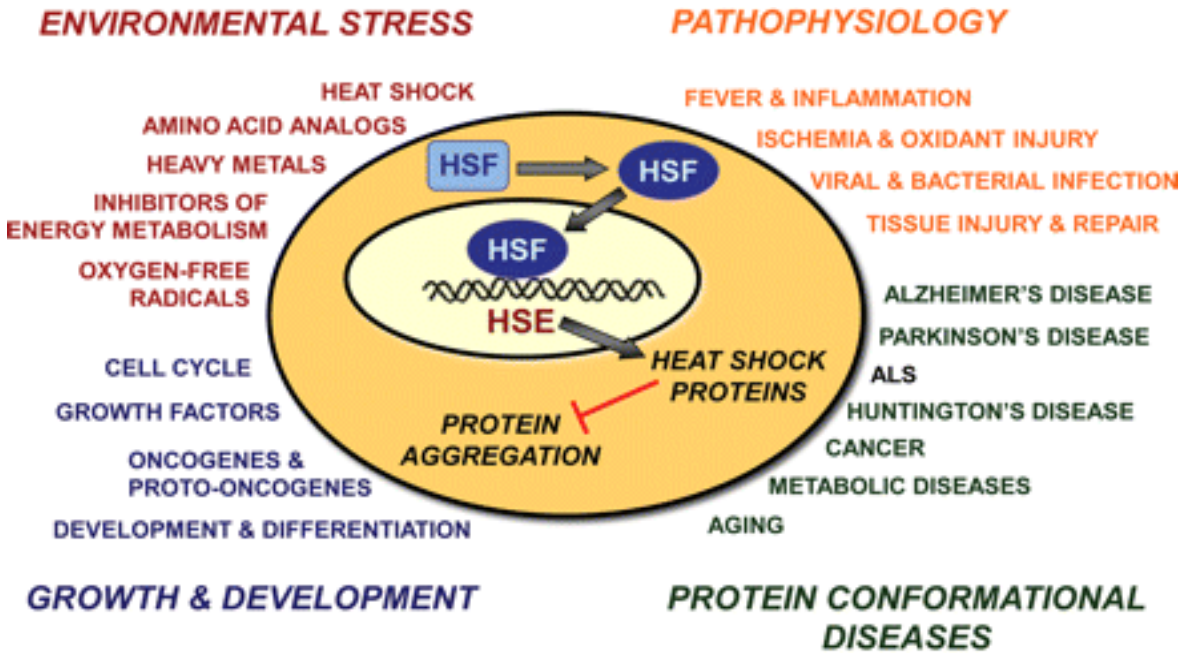


Figure 18.Initiators of the Heat Shock Response: Pathologic and Physiologic. The HSF1-mediated expression of heat shock elements (HSEs) during the HSR occurs following a variety of signals and factors to maintain proteostasis. The major inducers of the HSR are classified into four groups including Environmental stress, Pathophysiology, Growth/development and Protein conformational diseases. Associated with each major subdivision are a non-exhaustive list of representative HSR-inducing conditions within that class. *Figure taken from (234)*

These stress responses, especially the UPR, also function to ease protein folding burdens and loads within the ER in the settings of cell stresses via activation of pathways leading but not limited to the regulation of translation, ER-protein folding machinery expression and ER-associated degradation (234,288,289). The HSR (and UPR) thus maintains diverse cytoprotective capabilities and demonstrates the ability to protect not only against subsequent repeat exposure to the initial HSR-inducing stressor but to confer tolerance to other stresses (e.g. sub-lethal Heat Shock protecting against lethal heat shock or ROS exposure) (234,289). The cell's ability to

activate chaperone machinery to respond to significant stresses that cannot be predicted raises questions regarding its initial activation. One view holds that chaperones possess a significant and excess folding capacity and a fraction of the total chaperone molecules exist in a reserve capacity should emergencies arise. The counter and more accurate view to this holds that cells maintain little excess chaperone capacity and that their expression is titrated fairly closely, if not precisely, allowing the HSR to regulate the proteostasis network with great precision.

The induction of the HSR depends heavily upon a family of transcription factors known as Heat Shock Factors (HSF), first identified in Drosophila (290,291), and their transcription of Heat Shock Elements (HSE) and induce HSP gene expression. The mammalian HSF family consists of four members: HSF1-HSF4. Each HSF possess unique and overlapping functions and can exhibit tissue-specific expression patterns and interacting protein partners that extend beyond solely heat shock response genes (289). HSF1 was originally recognized as the primary stress-response regulator but recent evidence also supports its role in several developmental processes alongside HSF2 and HSF4, whereas HSF4 is the only HSF to date with no ascribed role in the HSR (289). Work utilizing Hsf1 knockout murine and cell models have demonstrated that HSF1 is a requirement for transactivation of HSP genes and thus maintaining cellular tolerances to stress including thermotolerance (292-295). HSF1 is constitutively expressed in most tissues and cell types but in an inactive state during a lack of stress stimuli (296). HSF1 DNA-binding and activation capacity are regulated via multiple post-translational mechanisms, protein-protein interactions and subcellular localization. Mammalian HSF1 has intrinsic stress-sensing capability as it can be homotrimerized from its monomeric form in response to thermal or ROS stress (297-299). HSF1 activation relies on formation of DNA-binding trimerization as the initial step in HSF1 activation in all eukaryotes (300). Extensive evidence exists demonstrating HSF1 interacts

with a number of HSPs at different phases of the activation cycle. Monomeric HSF1 exists in a weak complex with HSP90 and HSP70, preventing HSF1 trimerization in the absence of stress. Following stress stimuli, HSP90 and HSP70 disassociate from HSF1 in order to perform chaperone functions directed at proteins damaged or misfolded due to the inciting stress, thereby freeing HSF1 for trimerization(301-303)(**Figure 19**). HSF1 trimer formation is mediated by arrays of hydrophobic heptad repeats (HR-A and HR-B) that form a coiled-coil (304,305). An additional hydrophobic array HR-C functions to curb spontaneous trimer formation (306-308). Trimers can also be kept inactive when its regulatory domain remains bound to a multi-chaperone complex including HSP90 and the co-chaperone p23 (302,309-312). Activated HSF1 trimers can interact with HSP70 as well as HSP40 but does not suppress DNA-binding activity or post-translationally modify HSF1 but inhibits transactivation capacity via direct interaction with the transactivation domain of HSF1 repressing HSE transcription (303). Thus providing a key negative feedback mechanism to regulate HSP expression during stress and post-stress resolution. The degree of HSR activation is assumed correlative to levels of chaperones and nascent/misfolded peptides (289).

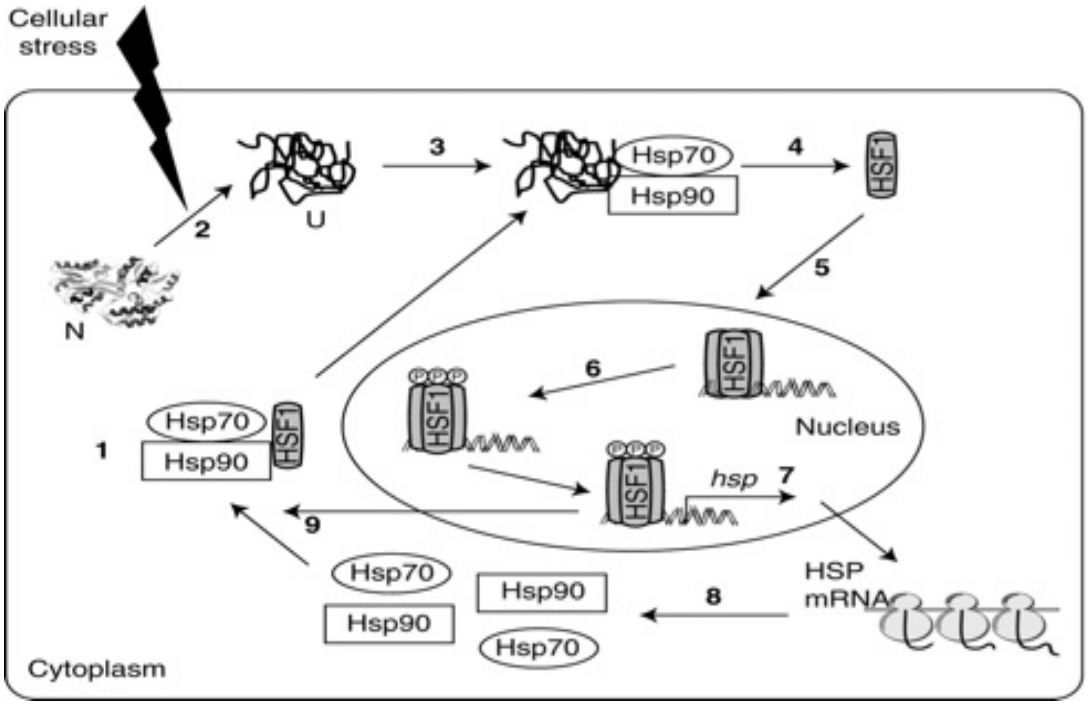


Figure 19.Activation and attenuation of HSF1-mediated HSE transcription. (1) Inactive HSF1 monomers exist in complex with HSP70 and HSP90 preventing its trimerization and phosphorylation. (2) With stress, proteostasis is disturbed yielding a rise in misfolded/damaged proteins. (3) HSP70 and HSP90 disassociate from HSF1 to perform critical cytoprotective HSR functions. (4) HSF1 is free from inhibition and (5) rapidly trimerizes and translocates to the nucleus (6) where it is subsequently phosphorylated and bound to HSEs. (7) Activated HSF1 transcribes heat shock genes leading to (8) heat shock mRNA translation. Newly synthesized chaperones continue to aid in HSR responses to the inciting stress. (9) Following stress resolution induced chaperones function to again form inactivating complexes with HSF1 as well as the targeted degradation of the induced chaperones themselves, returning chaperone expression to baseline. *Figure taken from (224)*

HSF1 is subjected to extensive post-translational modification including acetylation, phosphorylation and sumoylation. HSF1 contains several stress-inducible phosphorylation sites contributing to its transactivation activity (313,314). Additionally, phosphorylation of other sites of HSF1 can lead to conjugation of small ubiquitin-related modifier (SUMO) to lysine residues (315). Phosphorylation-dependent sumolaytion of HSF1 inversely correlates to the severity of heat stress, where SUMO conjugation is absent when maximal HSF1 is required (316). Also, given that transactivation capacity is significantly decreased upon sumolaytion, this serves as an additional regulatory mechanism of the HSR (289). Acetylation of HSF1 occurs on a much greater time scale and coincides more with the attenuation phase of the HSF1 activation cycle,

leading to transcriptional repression affecting DNA binding to HSEs. This can be reversed by SIRT1 activity thereby maintaining HSF1 in a competent state for DNA-binding. Together with HSP expression level, SIRT1 plays an important role in HSF1 activity (289).

Given the critical role of HSPs in a number of cellular processes, pharmacologic modulators of HSFs has garnered some interest in several fields. Hyperactivation of HSFs and increased HSP expression have been implicated in tumorigenesis leading to HSF1 inhibitors as potential therapeutics (317). Several HSF1 inducers have been proposed including the HSP90 inhibitors 17-AAG (318) and Geldanamycin (319) as well as Celastrol (320). As an example, Celastrol, which possesses anti-oxidant activity and is a natural compound isolated from the Celastreace family of plants, activates HSF1 and induces HSP expression profiles with nearly identical kinetics to that of heat shock. Inducers of the HSR and subsequent up-regulation of HSPs may serve as a possible therapeutic for several disorders (320,321).

1.7 HEAT SHOCK PROTEINS AS MODULATORS OF APOPTOSIS

Induction of the HSR leads to HSP induction, which have been shown to intersect and inhibit apoptosis via interfering with caspase activation. Overexpression of HSP27, HSP60, HSP70 and HSP90 inhibit apoptosis via blocking caspase activation in a variety of cell stresses such as accumulation of misfolded proteins, ROS or DNA damage (322-325). Strategies aimed at knocking down expression of these HSPs subsequently renders the host cell significantly more sensitive to various apoptotic stimuli (326-329). Selective depletion of HSP70 is sufficient to lead to apoptosis in certain cells even in the absence of stimuli such as seen in malignancies (330,331). HSPs play a role in inhibiting apoptosis initiated from either the intrinsic or extrinsic

pathways with multiple interceding points along each individual pathway (232) (**Figures 20 and 21**).

1.7.1 TARGET: Upstream Signaling

Upon growth factor binding a number of survival signaling pathways can be activated. One such pathway includes the phosphatidylinositol 3-kinase (PI3-K) pathway. PI3-K functions to phosphorylate inositol lipids in the plasma membrane leading to recruitment and activation of AKT. AKT itself can target a number of apoptotic proteins (332,333), including Bad (334) and caspase 9 (335). HSP27, as well as HSP70, have been demonstrated to promote anti-apoptotic signaling via interactions with the kinase AKT, an association that has proved critical to AKT activation in stressed cells (336,337). HSP27 also plays a role in cytoskeleton stabilization during bouts of cellular stress, which includes effects upon mitochondria membrane stabilization, preventing the critical apoptotic event of cytochrome c release (338). HSP27 has also been implicated in preventing TNFα-induced apoptotic signals, in cancer cells, via the increased ubiquitination of IkBα and subsequent NF-kB transcription of anti-apoptotic genes including Bcl-2, Bcl-xL and IAPs (232,339). Targeted degradation of the cell cycle protein $p27^{kip1}$ by HSP27 under conditions of stress leads to an increase in cell proliferation following stress resolution (340).

HSP70 has also been shown to inhibit stress-induced kinases like Apoptosis Signaling-regulating Kinase (ASK1). HSP70 down-regulation increases ROS-mediated ASK1 activation and apoptosis (341). HSP70 binds to and inhibits c-Jun N-terminal Kinase (JNK) for which its ATPase domain is dispensable (324,342,343) and loss of HSP70 expression leads to exaggerated apoptosis via exaggerated JNK activation in response to hyperosmolarity (344). HSP70

additionally was demonstrated to also affect p38 kinase activity (345). HSP70 has also been shown to interact with transcription factors involved in expression of Bcl-2 proteins. Bcl-2 and Bax are gene targets of p53, where p53 represses and induces transcription of Bcl-2 and Bax respectively. HSP70/HSC70 may play a role in masking nuclear localization sequences of p53, thereby impeding its nuclear import preventing apoptosis in response to DNA damage, a pathway at play in many tumor cells (346,347). The role of HSP70 regulating NF-kB remains fairly controversial. Evidence supports a role of cytoplasmic HSP70 inhibiting NF-kB activation while membrane bound HSP70 leading to its activation (348,349). Elevation of HSP70 to significant levels can actually lead to increased sensitivity to TNFα-induced apoptosis via inhibition of NF-kB survival pathways. This effect however could be the result of HSP70 promoting elimination of cells with significant DNA damage where tumor suppressor genes induce expression of HSP70s in response to significant DNA-damage potentiating TNFα activity (84). HSP70-mediated inhibition of NF-kB activation is perceived to be due to inhibition of both IKK activation and ubiquitination of IkBα especially important in the regulation of inflammatory signaling cascades (350,351).

HSP90, like the HSPs mentioned above, regulates activity and stability of various transcription factors and kinases including the aforementioned NF-kB, p53 (352), AKT, and JNK (353). HSP90 significantly affects NF-kB survival pathway activity via interaction with the IKK complex and is in fact an additional component of the IKK complex in addition to Cdc37. HSP90 and Cdc37 exist bound to one another as well as IKKα/IKKβ. Inhibition of HSP90 disrupts this complex leading to impediment of TNF-induced IKK recruitment and NF-kB activation (354). HSP90 mediates survival also through stabilization of phosphorylated AKT molecules. Activated AKT promotes the phosphorylation, i.e. inhibition of Bcl-2 family member

Bad and caspase 9, and prevents their pro-apoptotic activity (335). Activated AKT also leads to activation of NF-kB survival pathway signals (333).

1.7.2 TARGET: Mitochondria

Touched upon briefly above, HSP27 plays an important role in the prevention of cytoskeletal disruption in response to stress, but also attenuates Bid relocalization to the mitochondria preventing cytochrome c release (335) as well as release of Smac (355). Similarly, HSP70 prevents the translocation of Bax and subsequent outer membrane permeability facilitating cytochrome c and Apoptosis-Inducing Factor (AIF) release, which can trigger caspase-independent apoptosis via direct translocation to the nucleus (356). HSP60 and Bax complex formation is also a likely route of apoptosis prevention where targeted knockdown of HSP60 yields an increase in Bax and decreased Bcl-2 culminating in increased apoptosis in cardiomyocytes (357,358). More generally HSP60 also plays an important function in ensuring proper functioning of the complexes of the ETC safeguarding ATP generation and decreased cytochrome c release (359). Interestingly, HSP90 expression within mitochondria in tumor cells, not typically seen in normal tissues, regulates mitochondrial permeability and cytochrome c release (360). Also evidence exists of HSP90β forming complexes with Bcl-2 leading to apoptosis prevention in macrophages and dendritic cells (361).

1.7.3 TARGET: Post-Mitochondria

HSP27-mediated apoptosis prevention may also occur post-cytochrome c release via its sequestration, preventing capsase activation (362,363) or association with and prevention of activation of caspase 3 directly (364). HSP27 can also increase oxidant tolerance via both a

reduction in ROS levels (365) and neutralization of oxidized proteins directly (366). The ability of HSP27 to stabilize cytoskeleton also confers an ability to regulate membrane bleb formation (367,368). HSP70's and HSP90's role in apoptosis prevention has also been extended to direct interactions with APAF-1 preventing recruitment of caspase 9 into the apoptosome and caspase 3 activation (369-371). The ATPase domain of HSP70 was subsequently found to be necessary for this activity (372). HSP70's role in prevention of apoptosis extends further downstream of caspase 3 inhibition as it has been shown to prevent apoptosis in the face of activated caspase 3 via inhibition of phospholipase A2 and characteristic nuclear changes (247). Interestingly, CAD, downstream of caspase 3 activation, requires HSP70 and HSP40 in addition of ICAD for proper folding and enzymatic activity, thereby highlighting it as an additional target for HSP70-mediated cytoprotection. ICAD-mediated CAD inhibition occurs via recognition and binding of a CAD folding intermediate in association with HSP70-HSP40 (373). HSP90 has additionally been implicated in modulating affects downstream of caspase activation including intermediate filament stabilization, a key target of activated caspases leading to nuclear condensation and fragmentation (374). It is important to note that evidence has implicated both HSP60 and HSP10 as maintaining pro-apoptotic roles following their release from mitochondria via direct association with procaspase 3 and enhancing its activation by cytochrome c via the apoptosome (375,376).

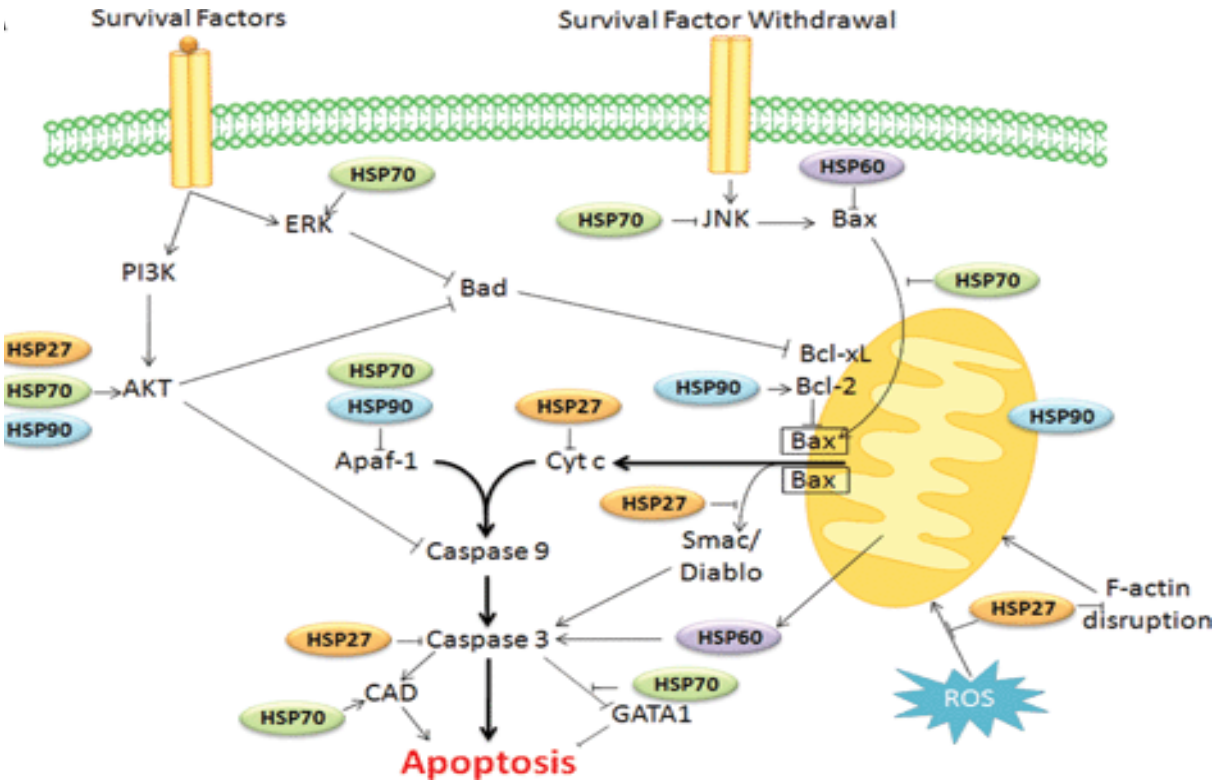


Figure 20. Schematic depicting regulation of the intrinsic apoptosis pathway via HSPs. HSPs can block mitochondrial apoptotic signals such as induced via ROS, through interaction upstream from the mitochondria through modulation of stress kinase (AKT, JNK, ERK) activity. HSPs can intercede at the mitochondrial level via limiting cytochrome c release (HSP27 membrane stabilization, HSP70 and HSP60 inhibition of Bax, HSP90 interaction with Bcl-2), blocking APAF1-cytochrome c interactions. HSPs also can intercede at the post-mitochondrial level via direct interaction with APAF1 (HSP70/HSP90), cytochrome c (HSP27), or caspase 3 (HSP27). These intersection points lead to a decrease in effector caspase activation and/or activity preventing initiation of apoptosis by limiting Caspase-activated DNase (CAD) activity among other mechanisms. *Figure reprinted with permission from John Wiley & sons Inc. Article: (232). Copyright 2008.*

1.7.4 TARGET: Extrinsic and Caspase-Independent Pathway

With specific respect to the extrinsic apoptotic pathway, HSP27 and HSP70 have been demonstrated to inhibit Fas-induced apoptosis (377). HSP27 and HSP70/HSP90 may inhibit TNFα-induced cell death via blockade of Bid translocation and Bid cleavage by activated caspase 8 (378). In addition to preventing Bax translocation and AIF release, HSP70 can also directly bind AIF and prevent its translocation to the nucleus (379-381). Stress stimuli have been

demonstrated to lead to translocation of lysosomal proteases from the lysosome to the cytosol serving as an important death pathway (382). These inciting stresses include TNFα (383,384), Fas (385), p53 activation (386), ROS (385,387) and growth factor withdrawal (385). Cathepsins are the best characterized of the released lysosomal proteases and demonstrated to lead to increased mitochondrial outer membrane permeability (383,386,388-390). HSP70 has been demonstrated to be expressed within endolysosomal membranes where it can inhibit release of cathepsins into the cytosol (391,392).

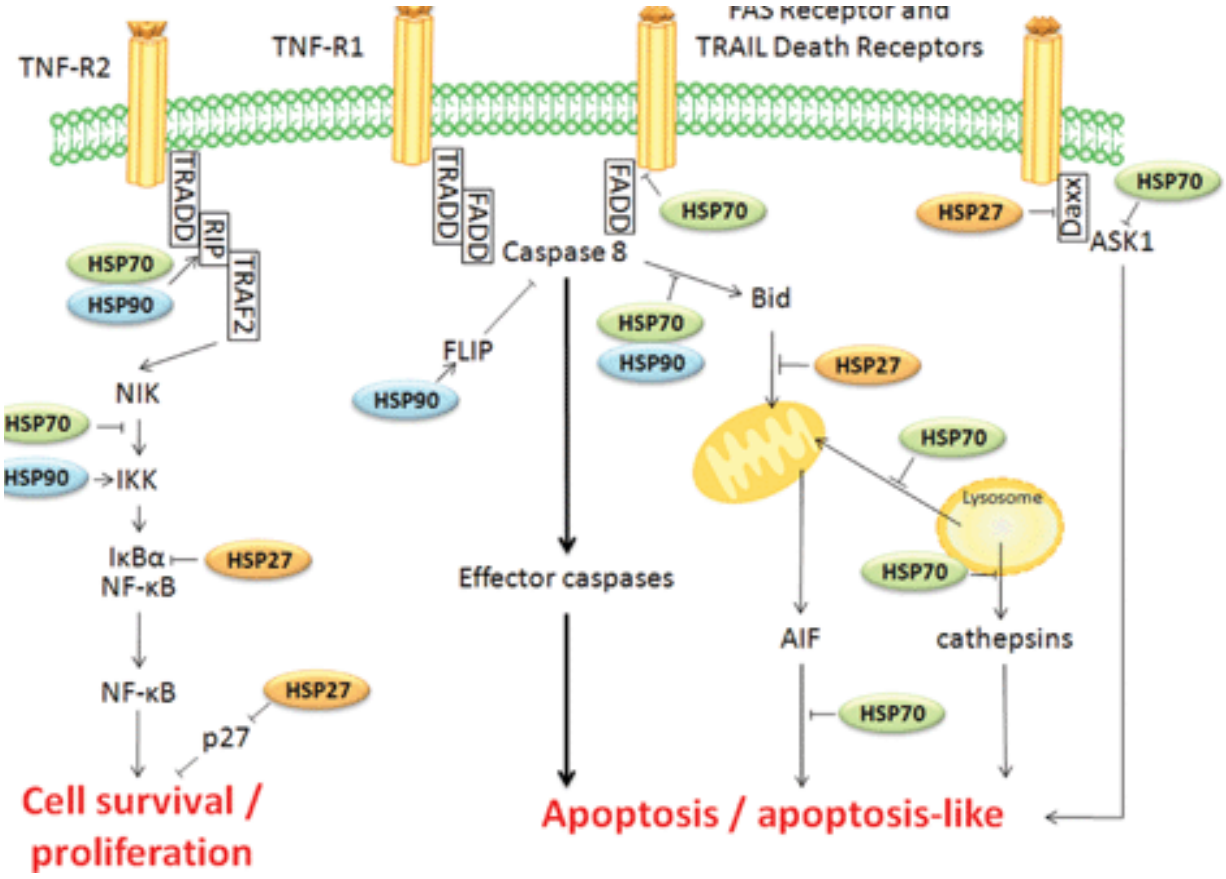


Figure 21.Schematic depicting regulation of the extrinsic and caspase-independent apoptosis and apoptosis-like pathways via HSPs. Both HSP70 and HSP90 can interact with RIP1 at the death receptor level blocking signal transduction and promoting survival. HSP70 and HSP90 can also block caspase 8 activation via recruitment of TRAIL/FADD and FLIP respectively.HSP70 can directly inhibit AIF activity and prevent release of lysosomal proteases (such as cathepsins) into the cytosol. HSP70 and HSP90 can also limit Bid cleavage while HSP27 can affect Bid translocation to the mitochondria prevent mitochondrial membrane disruption. HSP70 can also limit stress kinase ASK1 activity limiting death receptor signaling. *Figure reprinted with permission from John Wiley & sons Inc. Article: (232). Copyright 2008.*

1.8 CURRENT STUDY RATIONALE

As previously mentioned, a significant focus of NEC research is aimed at the specific targeting and modulation of TLR4 signaling. Such therapies have been demonstrated to possess great potential in limiting the severity of NEC. Our lab specifically has identified a number of key regulators of TLR4 signaling that have not only furthered our understanding of NEC pathology but represent promising avenues for pursuing and developing directed NEC therapies. Among these are TLR9 (113), NOD2 (158) and most recently EGFR (117). As shown in **table 4**, microarray analysis of ileal tissue obtained from wild-type mice subjected to our murine model of NEC demonstrated a significant decrease in HSP70 expression when compared with healthy controls. Given our previous findings indicating that TLR4 is significantly up-regulated during and reciprocally expressed in comparison to TLR9 and NOD2 in murine and human NEC, we hypothesized HSP70, like TLR9 and NOD2, also negatively regulates TLR4 in NEC. We next sought to test whether HSP70 may represent a previously unrecognized inhibitor of TLR4 signaling in the enterocyte and, by extension, a key pathogenic factor of NEC. Specifically, the aims of the current work are summarized below:

1.To assess the protective effects of HSP70 induction on TLR4-mediated intestinal injury and repair.

Given the role of HSP70 in mediating both apoptosis and inflammation we specifically sought to determine whether induction of HSP70 could decrease TLR4-mediated NF-kB activation, pro-inflammatory cytokine production and apoptosis. We additionally sought to determine the mechanism of action by which HSP70 could limit TLR4 signaling. Given HSP70's role in proteostasis as a molecular chaperone and reciprocal expression with TLR4 we tested

whether HSP70 and TLR4 could form inhibitory complexes leading to ubiquitination and degradation curbing exaggerated TLR4 expression

2. To determine the role of HSP70 in the prevention and treatment of Necrotizing Enterocolitis via TLR4 inhibition.

Through the use of genetically modified mice either over-expressing HSP70 specifically within the intestinal epithelium or globally HSP70-deficient we tested the role and relevance of HSP70-mediated modulation of TLR4 in the setting of NEC. In order to test whether HSP70's protective role could be manipulated for clinical gain we utilized a known pharmacologic inducer of HSP70 to determine whether its introduction could lead to prevention and/or treatment of NEC.

2.0 INTRACELLULAR HEAT SHOCK PROTEIN-70 NEGATIVELY REGULATES TOLL-LIKE RECEPTOR 4 SIGNALING IN THE NEWBORN INTESTINAL EPITHELIUM

2.1 ABSTRACT

Necrotizing enterocolitis (NEC) is the leading cause of gastrointestinal-related mortality in premature infants, and it develops under conditions of exaggerated TLR4 signaling in the newborn intestinal epithelium. Because NEC does not develop spontaneously, despite the presence of seemingly tonic stimulation of intestinal TLR4, we hypothesized that mechanisms must exist to constrain TLR4 signaling that become diminished during NEC pathogenesis and focused on the intracellular stress response protein and chaperone heat shock protein-70 (HSP70). We demonstrate that the induction of intracellular HSP70 in enterocytes dramatically reduced TLR4 signaling, as assessed by LPS-induced NF-kB translocation, cytokine expression, and apoptosis. These findings were confirmed *in vivo*, using mice that either globally lacked HSP70 or overexpressed HSP70 within the intestinal epithelium. TLR4 activation itself significantly increased HSP70 expression in enterocytes, which provided a mechanism of auto-inhibition of TLR4 signaling in enterocytes. In seeking to define the mechanisms involved, intracellular HSP70-mediated inhibition of TLR4 signaling required both its cochaperone-binding EEVD domain and association with the co-chaperone CHIP, resulting in ubiquitination and proteasomal degradation of TLR4. The expression of HSP70 in the intestinal epithelium was

significantly decreased in murine and human NEC compared with healthy controls, suggesting that loss of HSP70 protection from TLR4 could lead to NEC. In support of this, intestinal HSP70 overexpression in mice and pharmacologic up-regulation of HSP70 reversed TLR4-induced cytokines and enterocyte apoptosis, as well as prevented and treated experimental NEC. Thus, a novel TLR4 regulatory pathway exists within the newborn gut involving HSP70 that may be pharmacologically activated to limit NEC severity.

2.2 INTRODUCTION

Necrotizing enterocolitis (NEC) is the leading cause of death from gastrointestinal disease in premature infants, and it is characterized by the sudden onset of feeding intolerance that rapidly progresses to abdominal distention, systemic sepsis, and death due to acute necrosis of the intestine (393). The intestinal epithelium in infants with NEC displays exaggerated enterocyte apoptosis and mucosal disruption, which is widely thought to lead to the trans-luminal passage of indigenous microbes and an unbridled activation of the host immune system (219). In seeking to determine the inciting molecular mechanisms leading to the development of this cascade, we (155,159) and other investigators (156,163) determined that activation of the innate immune receptor TLR4 within the intestinal epithelium plays an important role in NEC pathogenesis. Specifically, TLR4 signaling in enterocytes leads to increased enterocyte apoptosis *in vitro* and *in vivo*, whereas inhibition of TLR4 signaling in the newborn intestinal epithelium prevents NEC development (155,158,159). Although these studies have clearly placed the spotlight on the role of TLR4 in the pathogenesis of NEC, the observation that most premature infants do not develop NEC, despite seemingly tonic activation of TLR4 within the gut, raises

the possibility that TLR4 signaling must somehow be curtailed within the newborn intestinal epithelium to limit disease development. Importantly, however, the presence of negative-regulatory strategies for TLR4 within the newborn intestinal epithelium and the degree to which such strategies may participate in the pathogenesis of NEC remain largely unexplored.

In the current studies, we test the hypothesis that the intracellular chaperone Heat Shock Protein-70 (HSP70) could negatively regulate TLR4 signaling within enterocytes and, by extension, that a loss of HSP70 could lead to NEC development through unbridled TLR4 activation. The heat shock proteins, of which HSP70 is a predominant member, represent a family of intracellular proteins that is activated by a variety of stressors and that can assist in the delivery of target proteins to the ubiquitin–proteasome system for degradation through co-chaperone molecules, such as CHIP (394). An intracellular role for HSP70 has not been linked to the pathogenesis of NEC nor to regulation of TLR4 within enterocytes, although HSP70 was shown to play an important role in themodulation of apoptosis after various forms of stress (395-398). Through its combined roles of both clearing proteins and modulating cell death, the net effect of HSP70 induction within cells is to restore the host to a non-stressed environment(399-401). Although cytoplasmic HSP70 has not been linked to the regulation of TLR4 signaling inside the enterocyte, HSP70 was shown to serve a protective role in the intestine, as demonstrated by Chang and colleagues (402,403), although upstream regulatory pathways within the gut were not identified. Taken together, these findings in the literature now raise the exciting possibility that intracellular HSP70 could represent a novel regulator of TLR4 signaling at baseline and in the development of NEC.

In support of this hypothesis, using enterocytes that either lack or are induced to express HSP70, as well as by examining mice that either lack HSP70 or that overexpress HSP70 within

the intestinal epithelium, we now determine that intracellular HSP70 limits TLR4 signaling in enterocytes and, moreover, that HSP70 plays a central role in the pathogenesis of NEC. The mechanism by which HSP70 limits TLR4 signaling in the gut involves an increase in CHIP-mediated ubiquitination and degradation of TLR4 via the ubiquitin–proteasomal pathway. Importantly, pharmacologic up-regulation of HSP70 within the intestinal mucosa led to a reduction in TLR4 signaling and a decrease in enterocyte apoptosis, leading to attenuation of NEC severity. Taken together, these findings illustrate a novel pathway linking the regulation of HSP70 with the negative control of TLR4 signaling within the gut and provide evidence that the development of NEC results, in part, from exaggerated TLR4-induced enterocyte apoptosis that is due, in part, to reduced HSP70 activity. Moreover, these results suggest that pharmacologic up-regulation of HSP70 could provide a novel approach to the prevention and/or treatment of NEC through the inhibition of TLR4 signaling in the newborn intestine.

2.3 MATERIALS AND METHODS

2.3.1 *Cell culture and reagents*

IEC-6 enterocytes were obtained from the American Type Culture Collection (Manassas, VA). LPS (Escherichia coli 0111:B4 purified by gel-filtration chromatography, >99% pure) was from Sigma-Aldrich. The TLR5 ligand flagellin was obtained from InvivoGen. Abs were as follows: p65 subunit of NF-kB (Santa Cruz Biotechnology); TLR4 (IMGENEX, Santa Cruz Biotechnology [L14]); cleaved-caspase 3 (Cell Signaling); the inducible isoform of HSP70 (StressGen SPA-810 and Santa Cruz Biotechnology [inducible, K20]); the constitutive (control) isoform of HSP70, i.e., HSC70 (StressGen SPA-815); Ubiquitin (Millipore); V5 (GenScript).

Where indicated, cells were pretreated with the proteasome inhibitor MG-132 (10μM; Calbiochem) 2h prior to the indicated experimental condition. IEC-6 enterocytes were treated with LPS at concentrations that we have shown to be present in mice and humans with NEC (i.e. 50 μg/ml)(155).

2.3.2 *Preparation of lentiviruses and cell transfection*

Lentiviruses expressing V5-tagged HSP70-dominant–negative C-terminal deletion mutant (ΔEEVD), wild-type HSP70, small interfering RNA (siRNA) to HSP70, CHIP HSP70-docking N-terminal mutants (K30A), CHIP C-terminal U-Box (H260Q) mutants, and LacZ were generated using a combination of ViraPower HiPerform Lentiviral and Lentiviral pLenti6.3/V5-DEST Gateway expression system (Invitrogen). In brief, the recombinant V5-tagged pLenti6.3/V5-DEST expression plasmids expressing wild-type and dominant-negative C-terminal deletion mutant (ΔEEVD) HSP70, CHIP HSP70-docking mutants (K30A), CHIP U-Box (H260Q) mutants, and LacZ were first generated using Gateway directional TOPO cloning systems (Invitrogen). V5-tagged pLenti6.3/V5-DEST expression plasmids are under the control of immediate early promoter human CMV and contain WPRE and cPPT elements, which yields cell-specific, high- performance expression of recombinant proteins. Recombinant V5-tagged pLenti6.3/V5-DEST expression plasmids were end sequenced to verify the correct directional cloning. High-expression lentiviral particles were next generated by co-transfection of recombinant V5-tagged pLenti6.3/V5-DEST plasmids and ViraPower Packaging Mix in receptive 293FT cells and used for transduction in destination IEC-6 cells for expression of recombinant proteins. mRNA and protein expression of recombinant proteins were verified by quantitative RT-PCR, Western blot, and immunofluorescence staining. Cells were transduced

with virus particles 48h prior to additional treatment.

IEC-6 cells were pretreated with either HSP70 siRNA (100nM; Dharmacon) via Lipofectamine LTX (Invitrogen), according to the manufacturer's directions, or a negative control siRNA that is directed at no known target for 48h in antibiotic-free media at 37℃ in a humidified chamber with 5% CO_2. The media were changed, and cells were treated with LPS and assessed for apoptosis, NF-kB translocation, and/or cytokine expression.

The NF-kB promoter GFP-reporter system was generated via the pPACKF1 Lentivector Packaging kit (System Biosciences) using the pSIF1-H1-siLuc-copGFP positive-control expression plasmid and delivered via lentiviral transduction of IEC-6 cells 48h prior to stimulation with LPS and/or heat treatment, as described above.

2.3.3 *HSP70 induction and knockdown*

For the induction of HSP70, IEC-6 cells were placed in an incubator at 42℃ (5% CO2 and 23% O2) for 45min, followed by either a 4h (LPS-mediated apoptosis analysis) or 8h (NF-kB translocation assay) recovery period at 37℃ under otherwise the same ambient conditions, after which they were exposed to the indicated treatment. In parallel, IEC-6 cells were treated with Celastrol (3μM in DMSO) for 30 min at 37℃ and allowed an 8h recovery period at 37℃ prior to treatment with LPS. Targeted knockdown of HSP70 was accomplished via transfection with siRNA to HSP70 or siRNA against no known target. Chemical inhibition of HSP70 was performed via 2h pretreatment with quercetin (20 μM; Sigma) in DMSO.

Induction of HSP70 within cells was accomplished *in vivo* through the administration of Celastrol (1 mg/kg), as described below. To confirm that the effects of this compound on TLR4 signaling occurred through up-regulation of HSP70, all studies were performed in HSP70-

deficient mice after injection of Celastrol for comparison, as described below

2.3.4 *Experimental endotoxemia and NEC*

All experiments were approved by the Children's Hospital of Pittsburgh Animal Care Committee and the Institutional Review Board of the University of Pittsburgh. C57BL-6, Swiss Webster, $HSP70^{-/-}$, and mice overexpressing HSP70 on the villin promoter ($HSP70^{villin}$), as well as their appropriate wild-type controls, were generated as described (402,403) or obtained from The Jackson Laboratory. All animals were age and sex matched prior to use. Experimental NEC was induced in 10d-old mice, as we described (113,158,159), using formula gavage (Similac Advanced infant formula [Ross Pediatrics]/Esbilac canine milk replacer, 2:1) five times/d and hypoxia (5% O_2, 95% N_2) for 10 min in a hypoxic chamber (Billups-Rothenberg) twice daily for 4d. Where indicated, mice were administered Celastrol (1 mg/kg) via i.p. injections 1d prior to the model and on day 3 of the model. Volume-matched DMSO was administered as a vehicle control in all Celastrol-treatment models. The severity of disease was determined on histologic sections of the terminal ileum by a pediatric pathologist, who was blinded to the study condition, according to the work of Radulescu et al. (404), as follows: 0: normal intestine; 1: epithelial lifting or separation; 2: sloughing of epithelial cells to the mid-villous level; and 3: necrosis of the entire villous. This protocol results in the development of patchy necrosis involving the small intestine similar to human NEC, with an increase in circulating cytokines that mimics that seen in human NEC (113).

2.3.5 *Preparation and processing of human tissue from infants with and without NEC*

Intestinal samples were obtained from human neonates undergoing intestinal resection for NEC, for unrelated indications (control), or at the time of stoma closure. All human tissue was obtained and processed as discarded tissue via waiver of consent with approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue-procurement guidelines.

2.3.6 *Immunohistochemistry, immunoprecipitation and SDS-PAGE*

The immunoconfocal microscopy of IEC-6 enterocytes, as well as mouse and human intestine, was performed as previously described (405). In brief, cells were fixed in 4%PFA for 20min, exposed to 0.1% Triton-X for 20min for antigen retrieval. Cells were blocked in 5% BSA for 1h and probed with specific antibodies for 1h. Cells were washed in 1x PBS and probed with fluorescently labeled secondary antibodies for visualization. In parallel, Cryo-Gel (Cancer Diagnostics) frozen sections of terminal ileum were sectioned (6 μm), rehydrated with PBS, and fixed with 2% paraformaldehyde. Nonspecific binding was blocked with 5% BSA. Stained tissues and cells were evaluated using an Olympus FluoView 1000 confocal microscope under oil-immersion objectives. Images were assembled using Adobe Photoshop CS2 software (Adobe Systems).

For immunoprecipitation, IEC-6 lysates were collected, and 500μg total protein was pre-cleared with 20 μl/sample Agarose Protein A/G beads (Santa Cruz Biotechnology) for 30 min at 4℃. Samples were centrifuged, and supernatants were collected and treated with Abs to HSP70

(K20) or ubiquitin, as indicated, or an isotype IgG control Ab and then incubated with Agarose Protein A/G beads overnight at 4℃. Samples were then centrifuged, supernatants were discarded, and beads were washed three times in PBS and resuspended in equal volumes of 2xgel-loading buffer and boiled at 95 ℃ for 3min. Lysates were then subjected to SDS-PAGE (177) and immunoblotted with various Abs (1:1000), as indicated. Blots were developed using ECL reagent (ECL-Super Signal; Pierce) and developed on radiographic film.

2.3.7 *Quantitative real-time PCR*

Quantitative real-time PCR was performed, as previously described, using the Bio-Rad CFX96 Real-Time System (155) and the primers listed in **Table 6**. In brief, total RNA was isolated from the ileal mucosal scrapings of mice that had been breast-fed (control) or induced to develop experimental NEC, as well human control and NEC tissues using the RNeasy kit (Qiagen) and reverse transcribed (1 μg of RNA) using the QuantiTect Reverse Transcription Kit (Qiagen). Gene-specific cDNA was amplified and quantified in a real-time thermal cycler system (SYBER Green I; iCycler iQ Real-Time PCR Detection System;). PCR amplification was then performed in triplicate. In all cases, water was used instead of cDNA to serve as a non-template control. The reaction protocol included pre-incubation at 95°C for 15 min to activate AmpliTaq Gold DNA Polymerase (Applied Biosystems) and amplification for 40 cycles (15 s at 95°C, 30 s at 56°C, and 60 s at 72°C). Where indicated, gene expression was assessed on 2.5% agarose gels using ethidium bromide staining. Images were obtained with a Kodak Gel Logic 100 Imaging System using Kodak Molecular Imaging software. The expression of the following genes by quantitative RT-PCR was measured relative to the housekeeping genes b-actin and GAPDH.

Gene	Species	Forward Sequence	Reverse Sequence	Amplicon Size (bp)
β-actin	Mouse/rat	5′-CCACAGCTGAGAGGGAAATC-3′	5′-TCTCCAGGGAGGAAGAGGAT-3′	108
	Human	5′-TCCCTGGAGAAGAGCTACG-3′	5′-GTAGTTTCGTGGATGCCACA-3′	131
Hsp70	Mouse/human	5′-GCCAACAAGATCACCATCAC-3′	5′-TGTTGAAGGCATAGGATTCG-3′	154
	Rat	5′-TTCAATATGAAGAGCGCCGTGGAG-3′	5′-TCCTCTTTCTCAGCCAGCCAGCGTGTTA-3′	134
GAPDH	Mouse	5′-TGAAGCAGGCATCTGAGGG-3′	5′-CGAAGGTGGAAGAGTGGGAG-3′	102
	Human	5′-TCTCCTCTGACTTCAACAGCGACA-3′	5′-CCCTGTTGCTGTAGCCAAATTCGT-3′	126
IL6	Mouse/rat	5′-CCAATTTCCAATGCTCTCCT-3′	5′-ACCACAGTGAGGAATGTCCA-3′	182
	Human	5′-TCTCCACAAGCGCCTTCG-3′	5′-CTCAGGGCTGAGATGCCG-3′	193
iNOS	Mouse/rat	5′-CTGCTGGTGGTGACAAGCACATTT-3′	5′-ATGTCATGAGCAAAGGCGCAGAAC-3′	167
	Human	5′-AATGAGTCCCCGCAGCCCCT-3′	5′-AGTCATCCCGCTGCCCCAGT-3′	143
TLR4	Mouse	5′-TTTATTCAGAGCCGTTGGTG-3′	5′-CAGAGGATTGTCCTCCCATT-3′	186
	Human	5′-AAGCCGAAAGGTGATTGTTG-3′	5′-CTGAGCAGGGTCTTCTCCAC-3′	153
	Rat	5′-TGCTCAGACATGGCAGTTTC-3′	5′-GCGATACAATTCGACCTGCT-3′	102

Table 6**.** Table of primers used for the detection of specific mRNAs in the current work. *Table reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

2.3.8 *Determination of enterocyte signaling and mucosal injury in response to TLR4 activation*

For the determination of NF-kB translocation, IEC-6 cells were treated with LPS (50 μg/ml, 45 min). The extent of NF-kB translocation was determined as described(113), using an adaptation of the methodology of Ding and Li (406). In brief, a threshold limit was set based upon the emission signal for the nuclear stain DAPI in serial micrographs of cells that were co-stained for the p65 subunit of NF-kB, whereas a corresponding cytoplasmic region of interest was defined by stenciling a circular region, 12 pixels beyond the nucleus, upon each cell. The average integrated pixel intensity pertaining to the corresponding NF-kB emission within the cytoplasmic and nuclear regions was then determined for >200 cells/treatment group, in at least four experiments per group, using Meta-Morph software version 6.1 (Molecular Devices).

The release of IL-6 from IEC-6 cells was determined by an ELISA kit (R&D Systems), according to the manufacturer's instructions.

Enterocyte apoptosis was determined in IEC-6 cells after 16h of treatment with LPS under concentrations that we measured in NEC(155) (50 μg/ml) by immunostaining with Abs to cleaved-caspase 3 and performing confocal immunofluorescent analysis. The number of cleaved-caspase 3-positive cells was identified in a blinded fashion using MetaMorph software (Molecular Devices) and expressed as a percentage of cleaved-caspase 3-positivecells/high-power field, with >100 fields/experiment studied and >100 cells/field.

Enterocyte apoptosis *in vivo* was determined by measuring the percentage of enterocytes positive for cleaved-caspase 3 by confocal microscopy per high-power field. More than 50 fields/sample were evaluated, as we described (158).

2.3.9 *Statistical analysis*

All experiments were repeated at least in triplicate, with >100 cells/high-power field. For mouse experiments of endotoxemia, >4 mice/group were assessed; for experiments of NEC, >10 mouse pups/group were included, and litter-matched controls were included in all cases. Statistical analysis was performed using SPSS 13.0 software. ANOVA was used for comparisons for experiments involving more than two experimental groups. Two-tailed Student t test was used for comparison for experiments consisting of two experimental groups. For analysis of the severity of NEC, x^2 analysis was performed. In all cases, statistical significance was accepted at $p < 0.05$ between groups.

2.4 RESULTS

2.4.1 *Heat Shock Response induction limits TLR4 signaling in enterocytes*

To determine whether the induction of HSP70 could limit TLR4 signaling, we first briefly exposed cultured enterocytes (IEC-6 cells) to conditions known to increase the expression of HSP70 (42 ℃, 45 min **Fig. 22A)** and then treated cells with the TLR4 agonist LPS at concentrations that we previously measured from the stool of humans and mice with NEC (155). The extent of TLR4 signaling was determined by assessing the extent of NF-kB activation, as reflected by the degree of translocation of the p65 subunit of NF-kB from the cytoplasm to the nucleus as described in *Materials and Methods*, the degree of induction of the pro-inflammatory cytokine IL-6, and the degree of enterocyte apoptosis. As shown in **Fig. 22**, LPS treatment caused a marked increase in NF-kB activation (**Fig. 22Di, 22Dii**) that was significantly reduced following heat treatment (**Fig. 22C, 22Diii**). Heat treatment also prevented the increase in IL-6 expression that occurred in LPS-treated IEC-6 cells (**Fig. 22B**) (primer sequences for quantitative RT-PCR are listed in **Table 6**) as well as a reduction in the extent of LPS-mediated apoptosis, both down to levels similar to untreated control cells (**Fig. 22Fi–iii**). We used the expression of cleaved-caspase 3 as an estimate of apoptosis in these studies, because its increased expression was shown to be a terminal event in the apoptosis cascade in enterocytes, and it provides a reliable and reproducible estimate of enterocyte apoptosis in both human and experimental NEC (41,158,407,408). To determine whether a heat-induced increase in HSP70 was required for the observed attenuation in TLR4 signaling, cells were exposed to heat after siRNA-mediated knockdown of HSP70 in IEC-6 cells (**Fig. 22E**), which abrogated the protection in TLR4 signaling previously observed after heat treatment with regard to the

induction of IL-6 expression (**Fig. 22B**), NF-kB translocation (**Fig. 22C**), and enterocyte apoptosis (**Fig. 22F**). Taken together, these findings indicate that HSP70 can inhibit TLR4 signaling in enterocytes. We next sought to investigate the mechanisms mediating this effect and focused initially on determining whether an association between HSP70 and TLR4 was required for the negative effects of HSP70 on TLR4 signaling.

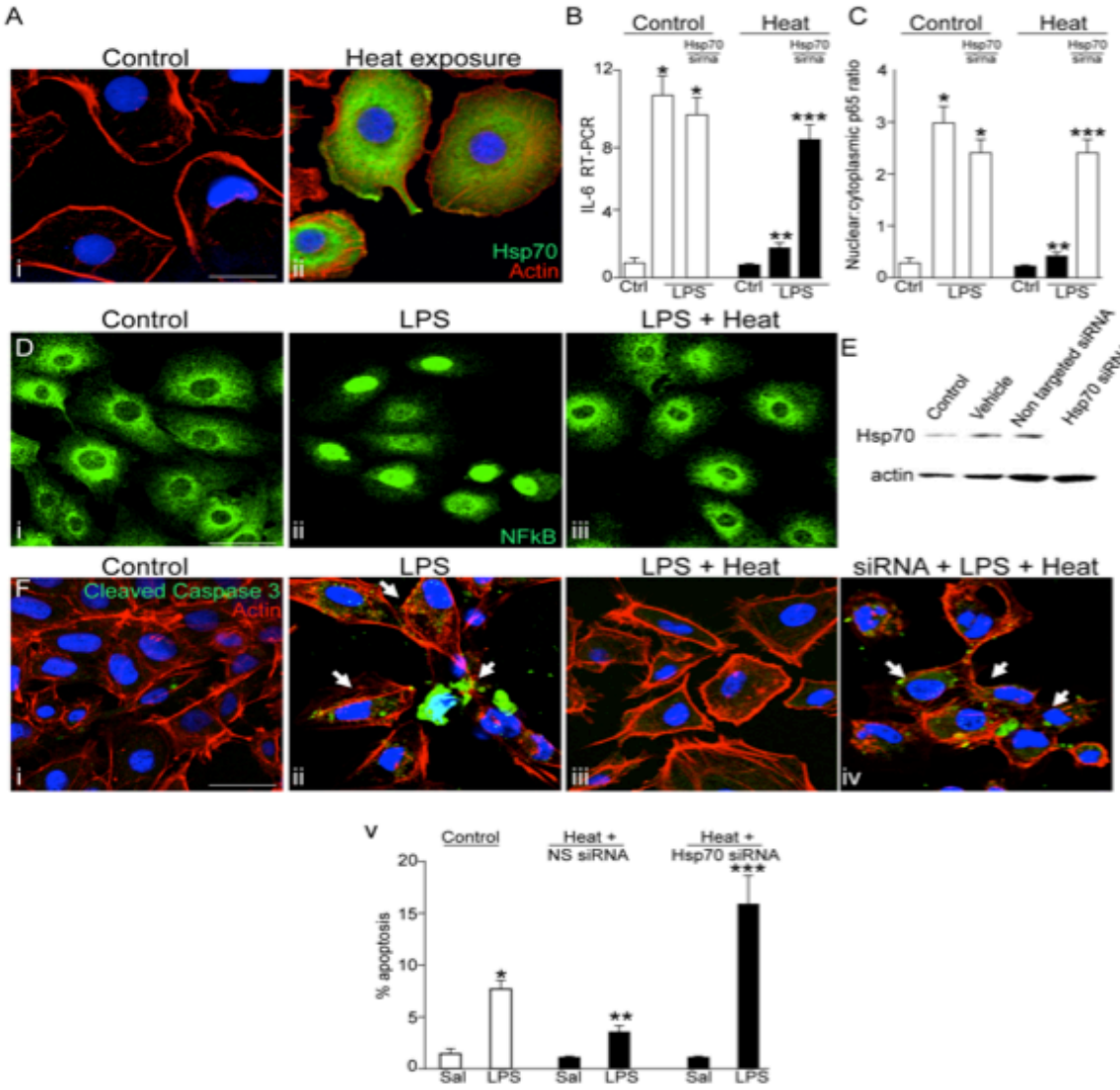


Figure 22.HSP70 induction limits TLR4 signaling in enterocytes. (A) Confocal photomicrographs showing the expression of HSP70 (green), b-actin (red), and DAPI (blue) in IEC-6 enterocytes that were either untreated (i) or exposed to 42 ℃ for 45 min (ii). Quantitative RT-PCR showing the expression of IL-6 (B) or quantification of the extent of NF-kB translocation (C) in IEC-6 cells that were either untreated (white bars) or exposed to heat (black bars) and were either untransfected or were transfected with HSP70 siRNA. Quantification in (C) is based upon >50 cells/field and >50 fields examined in four separate experiments.*p <0.05, versus untreated control, **p <0.01, versus heat control, ***p <0.001, versus control cells transfected with HSP70 siRNA. (D) Confocal photomicrographs of IEC-6 enterocytes that were untreated (i), treated with LPS (50 μg/ml, 45 min) (ii), or treated with LPS after pretreatment with heat (iii). (E) Representative SDS-PAGE showing lysates of IEC-6 that were untreated (control), incubated with PBS alone (vehicle), or transfected with either control siRNA against no known substrate (nontargeted siRNA) or siRNA to HSP70 (HSP70 siRNA). Blot was stripped then and reprobed with Abs to b-actin. (F) Confocal photomicrographs (i–iv) and quantification (v) of IEC-6 enterocytes that were untreated (i), treated with LPS in the absence (ii) or presence of pre-exposure to heat as above (iii), or pretreated 48 h prior with siRNA to HSP70, as in (iv). Representative images are taken of >50 fields examined with >50 cells/field in four separate experiments. Scale bar, 10um. Representative apoptotic cells are indicated by arrows. *p<0.05, versus control, **p <0.01, versus LPS control, ***p <0.001, versus heat+HSP70 siRNA saline. *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

2.4.2 *An EEVD-mediated association between TLR4 and HSP70 is required for the inhibition of TLR4 signaling in enterocytes by HSP70*

The effects of HSP70 on target proteins are largely influenced by interactions with accessory proteins called co-chaperones (409,410). In a variety of cell types, HSP70 regulates the intracellular function and fate of proteins through the formation of direct protein–protein interactions that occur largely through an EEVD-binding domain in its C-terminus (266,411,412). To investigate whether an association between TLR4 and HSP70 was required for the negative effects on TLR4 signaling, IEC-6 cells were stably transduced with lentivirus expressing either LacZ as a vector control or with HSP70 bearing a truncation mutation in the EEVD domain while sparing the N-terminal ATPase domain, as described (413) (**Fig. 23Ai**). After LPS treatment, cells were subjected to immunoprecipitation using Abs to HSP70 in the absence or presence of heat exposure. As shown in **Fig. 23Ai**, TLR4 was detected in immunoprecipitates obtained from IEC-6 lysates that had been transduced with control vector, and this association was increased after heat exposure. By contrast, the stable delivery via lentiviral transduction of a mutant HSP70 lacking the C-terminus EEVD-binding domain markedly reduced the ability to detect TLR4 in the immunoprecipitates after LPS treatment, which did not vary after heat exposure, indicating that the EEVD mutation resulted in a reduction in the extent of association between HSP70 and TLR4 (**Fig. 23Ai**). Although heat treatment of IEC-6 cells that had been transduced with LacZ vector conferred significant protection from both LPS-induced NF-kB translocation (**Fig. 23Bi–iii**) and enterocyte apoptosis (**Fig. 23Ci–iii**), this heat-mediated reduction in LPS signaling was lost in IEC-6 cells that had been transduced with the C-terminus EEVD-binding mutant (**Fig. 23Biv–vii, 23Civ–vii**). As expected, LPS caused a

significantly greater degree of IL-6 expression after transduction of ΔEEVD HSP70 compared with nontransfected cells (**Fig. 23Bxi**), consistent with the reduced effect of HSP70 in inhibiting TLR4 signaling in these cells. To further investigate the link between HSP70 expression and TLR4 signaling, IEC-6 cells were transduced with lentiviruses expressing wild-type HSP70, which resulted in a significant increase in the degree of HSP70 expression compared with nontransfected cells (**Fig. 23Aii**). Importantly, as shown in **Fig. 23Bvii–x**, compared with LacZ-transfected control cells, the addition of LPS led to a minimal degree of NF-kB translocation in IEC-6 cells that overexpress HSP70, consistent with the notion that these cells are less responsive to TLR4 signaling due to the effects of HSP70 on TLR4. Moreover, the degree of LPS-induced NF-kB translocation in HSP70–IEC-6 cells was minimally affected by heat exposure, consistent with the observation that these cells already overexpress HSP70. Taken together, these findings demonstrate that the induction of HSP70 leads to a reduction in TLR4 signaling and that the effects of HSP70 occur through an association between TLR4 and HSP70 dependent upon EEVD domain in its C-terminus. Given the role of HSP70 in regulating the ubiquitin-mediated degradation of target proteins, we, therefore, next sought to investigate whether HSP70 associations with TLR4 could subsequently alter the ubiquitination state of TLR4 in enterocytes.

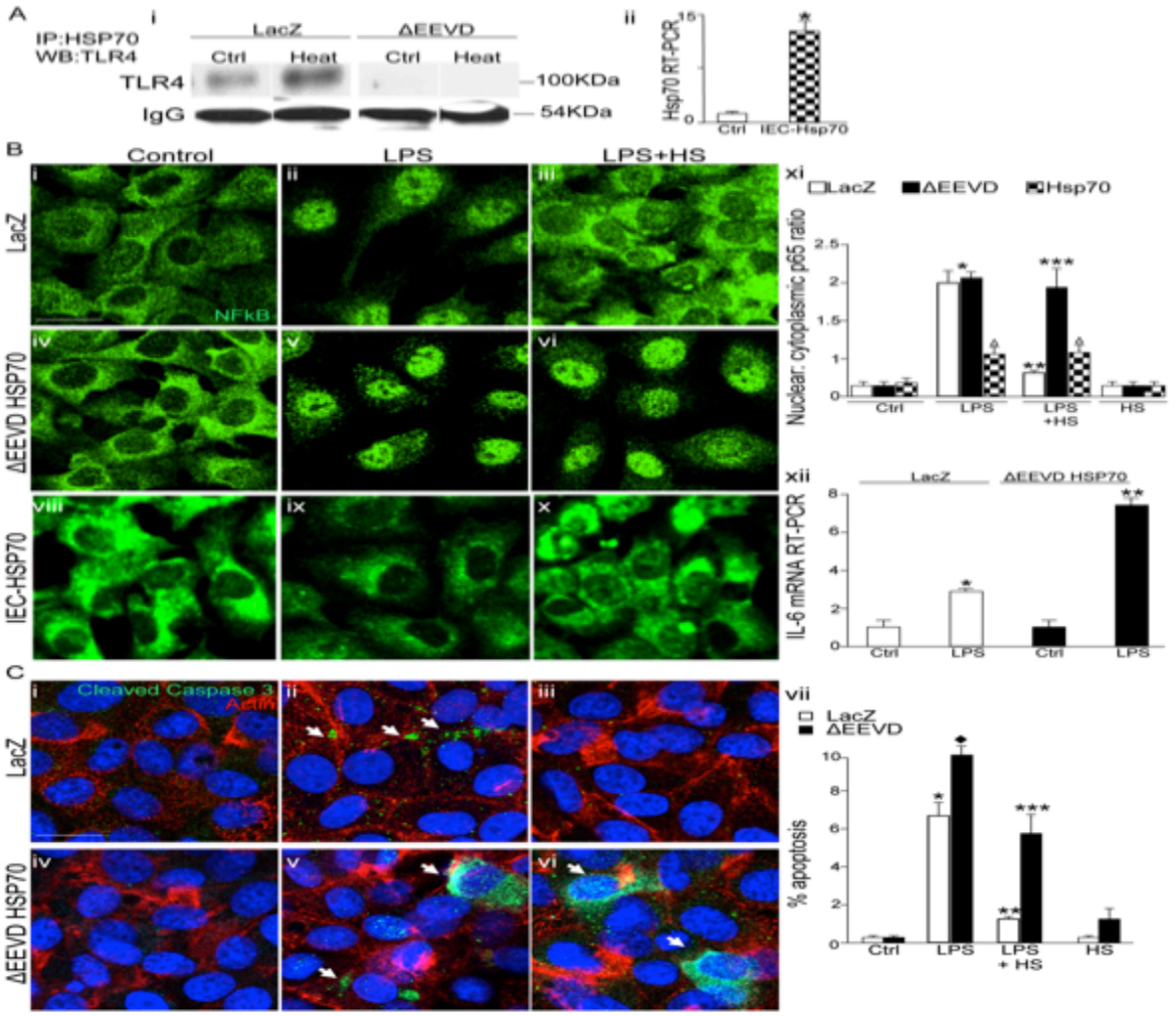


Figure 23. An EEVD-mediated association between TLR4 and HSP70 is required for the inhibition of TLR4 signaling in enterocytes by HSP70. (Ai) Representative SDS-PAGE showing lysates of IEC-6 enterocytes that had been virally transduced with either LacZ or HSP70 lacking the EEVD substrate-binding domain (ΔEEVD), prior to treatment with LPS, and maintained at 37℃ or treated at 42℃ for 45 min (heat) and then immunoprecipitated with Abs to HSP70 and immunoblotted with anti-TLR4 Abs; shown is IgG as a loading control. (Aii) Quantitative RT-PCR showing the expression of HSP70 in nontransfected control IEC-6 cells and IEC-6 cells that were transfected with full-length HSP70. Representative confocal photomicrographs of IEC-6 enterocytes that were either transduced with LacZ (Bi–iii, Ci–iii) or ΔEEVD-HSP70 (Biv–vi, Civ–vi) and then left untreated (Bi, Biv, Ci, Civ), treated with LPS (Bii, Bv, Cii, Cv), or treated with LPS plus pretreatment with heat (Biii, Bvi, Ciii, Cvi). Cells were then stained for NF-kB (green in B), or cleaved-caspase 3 (green), b-actin (red), and DAPI (blue) in (C). Scale bar, 10 mm. NF-kB translocation (Bx) and percentage of apoptosis (Cvii) based upon >50 fields with >50 cells/field. *$p<0.05$, LPS open and solid bars versus control open and solid bar, **$p<0.01$, LPS+HS versus LPS open bar, ***$p<0.001$, control black bar versus LPS+HS black bar, ♦$p<0.05$, LPS black bar versus LPS open bar. Δ, no significant difference between untreated, LPS-treated, or heat-exposed LPS-treated HSP70–IEC-6 cells. Summary of four separate experiments. (Bvii–ix) Confocal photomicrographs of IEC-6 cells that were transfected with HSP70 and treated as indicated. (Bxi) Quantitative RT-PCR showing IL-6 expression in IEC-6 cells that were transfected with LacZ or ΔEEVD and then treated with LPS as in Materials and Methods (6 h, 50 mg/ml). Representative apoptotic cells are indicated by arrows. *$p<0.05$, versus Ctrl, **$p<0.01$ versus LPS in LacZ-transfected cells. Representative of three separate experiments. *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

2.4.3 *The induction of HSP70 leads to ubiquitination and degradation of TLR4 via the co-chaperone CHIP*

In various cell types, HSP70 regulates the fate of target proteins, in part, by critically influencing their ubiquitination state and subsequent degradation through the proteasomal system (32). The degree of ubiquitination affected by HSP70 is directly influenced by the activity of a co-chaperone molecule CHIP, an E3-ligase bearing a ubiquitin ligase U-box domain and a HSP70-docking domain (33, 34). We now postulate that HSP70 induction limits TLR4 signaling by promoting its ubiquitination state via CHIP. To determine whether HSP70 induction affected TLR4 expression via effects on the extent of ubiquitination of TLR4, we first immunoprecipitated lysates of IEC-6 cells using anti-ubiquitin Abs in the absence or presence of heat and then performed SDS-PAGE using antibodies to TLR4. As shown in **Fig. 24Ai**, heat exposure increased the degree of TLR4 ubiquitination, which was associated with a reduction in TLR4 expression (**Fig. 24Aii**). Further evidence of the importance of HSP70 in regulating TLR4 expression after heat treatment is shown in **Fig. 24Aiii.** After the administration of siRNA for HSP70, the expression of TLR4 was significantly greater, as detected by SDS-PAGE, than in wild-type cells that express HSP70 (see **Fig. 22E** for expression of HSP70 in the siRNA-treated cells, confirming knockdown of HSP70 by the siRNA approach). Importantly, heat treatment did not cause a reduction in TLR4 in IEC-6 cells after knockdown of HSP70, consistent with the important role of heat-induced HSP70 in mediating the regulation of TLR4 (**Fig. 24Aiii**), which is lost in these cells after HSP70 knockdown. Furthermore, the importance of ubiquitination in mediating the effects of HSP70 on TLR4 expression and signaling is demonstrated, because the treatment of IEC-6 cells with the proteasomal inhibitor MG-132 prevented the loss of TLR4

expression in response to heat while reversing the protective effects of heat shock on TLR4-induced enterocyte apoptosis (**Fig. 24B, 24C**). To explore whether CHIP/HSP70-mediated interactions were required for the protective effects of HSP70 on TLR4 signaling and function via CHIP-mediated docking and ubiquitination of TLR4, we next introduced, via lentiviral-mediated transduction, two dominant negative forms of CHIP into IEC-6 cells: H260Q, the E3-ligase U-box mutant, which impairs the ability of HSP70/CHIP to polyubiquitinate target proteins, and K30A, a docking-domain mutant that impairs the ability of CHIP to interact with HSP70 and target proteins (267,414). We then assessed effects on protein stability, ubiquitination state, and TLR4 signaling. Introduction of the H260Q ubiquitin ligase mutant prevented the increase in the ubiquitination of TLR4 in response to heat exposure compared with IEC-6 cells that were infected with empty vector (**LacZ; Fig. 24Ai**) and significantly reduced the protective effects of heat exposure on LPS-mediated enterocyte apoptosis (**Fig. 24B, 24D, 24E**). Furthermore, transduction of IEC-6 cells with the K30A docking mutant also reversed the protection in LPS-induced enterocyte apoptosis that was previously provided from heat exposure (**Fig. 24B, 24F**). Taken together, these findings demonstrate that HSP70 limits TLR4 activation through ubiquitin-mediated protein degradation via CHIP. We next sought to determine whether TLR4 could limit its own signaling, in part through up-regulation of HSP70. Transduction controls are shown in **Appendix Figure 31** where expression of the V5 tag is demonstrated in transduced cells

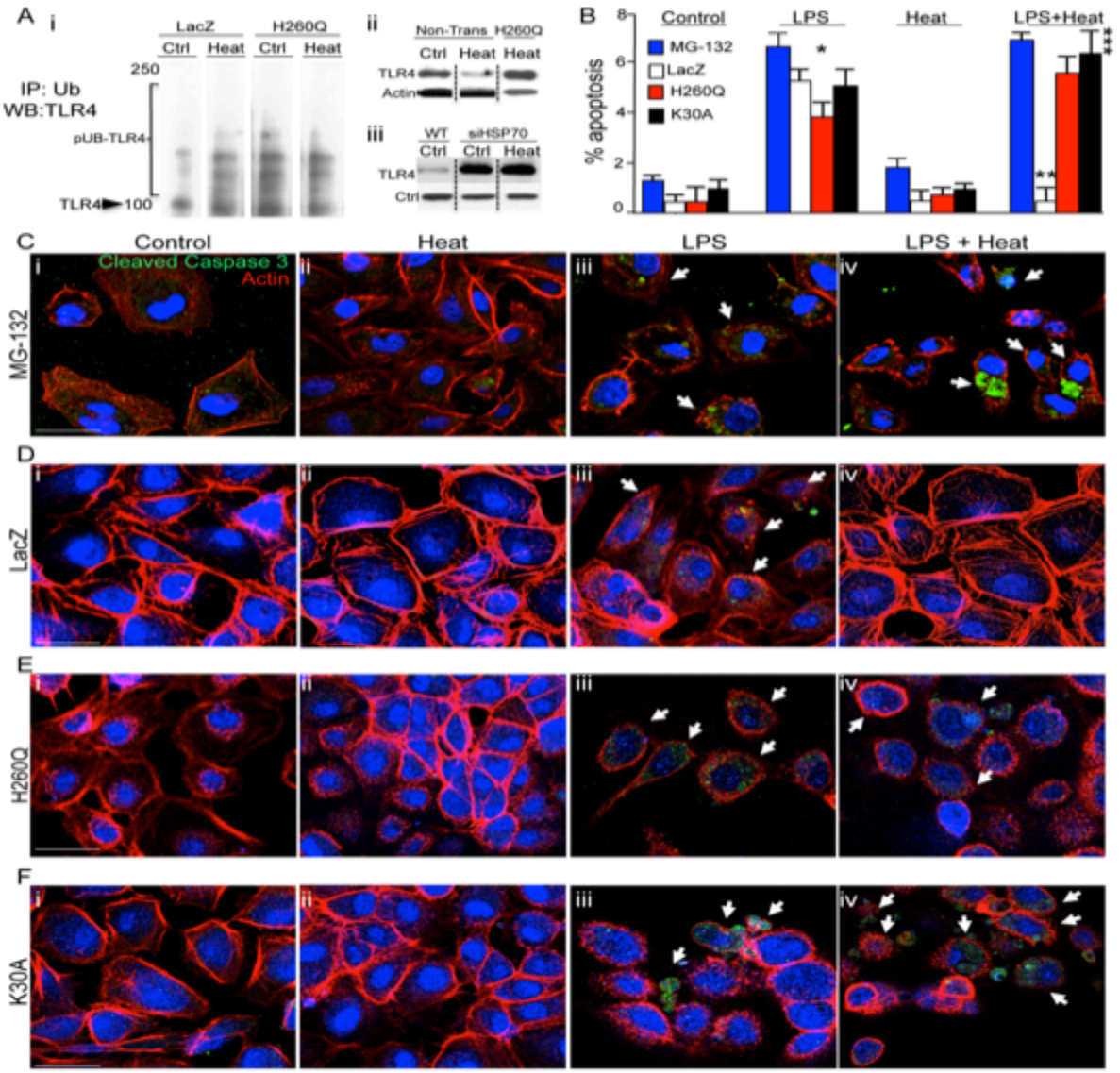


Figure 24.The induction of HSP70 leads to the ubiquitination and degradation of TLR4 via the co-chaperone CHIP. (Ai) Representative immunoblots in which LacZ- and H260Q-transfected IEC-6 enterocytes were exposed to heat or were maintained at 37℃ and then immunoprecipitated with anti-ubiquitin Abs and immunoblotted with anti-TLR4 Abs, displaying polyubiquitinated species (pUB-TLR4). The location of TLR4 on the gel is shown. (Aii) Representative SDS-PAGE of IEC-6 cells probed with anti-TLR4 Abs that were either nontransfected or transfected with H260Q and then maintained at 37℃ or exposed to heat, in which heat exposure leads to a reduction in TLR4 expression that is not seen in H260Q-transfected cells. (Aiii) SDS-PAGE showing expression of TLR4 and loading protein control in either wild-type (WT) IEC-6 cells or IEC-6 cells treated with siRNA to HSP70 (siHSP70) that were either untreated (Ctrl) or treated with heat as in Materials and Methods (Heat). (B) Percentage apoptosis per high-power field >50 fields with >50 cells/field. Representative confocal photomicrographs of IEC-6 enterocytes treated with MG-132 (C), or transduced with LacZ (D), H260Q-CHIP (E), or K30A-CHIP (F), treated as indicated, and immunostained with cleaved-caspase 3 (green), b-actin (red), and DAPI (blue). Arrows point to apoptotic cells. Scale bar, 10 mm. *p <0.05, control (all bars) versus LPS (all bars), **p <0.01, LPS (open bar) versus LPS+Heat (open bar) versus LPS open bar, ***p <0.001, Control versus LPS + Heat (black, red, and blue bars) in three separate experiments. *Table reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

2.4.4 *TLR4 induces HSP70 expression, which then negatively affects TLR4 signaling*

During the inflammatory response, the activation of TLR4 in response to LPS must be carefully controlled; the failure to negatively regulate a TLR4 response would necessarily result in a pro-inflammatory cytokine storm each time a TLR4 signal was initiated. Having shown that HSP70 induction can inhibit TLR4 signaling in enterocytes, we next considered the possibility that TLR4 activation itself could also lead to an induction of HSP70, which could then serve to negatively regulate (and therefore to self-limit) TLR4 responsiveness. To do so, we first sought to evaluate the time dependency of the signaling response of TLR4 in IEC-6 cells using two separate techniques. IEC-6 cells were transiently transduced with an NF-kB–promoter driven GFP-reporter construct as a readout of TLR4 activation, treated with LPS, and assessed for the expression of GFP by confocal microscopy over time. Cells were also stained for HSP70 to assess its induction in response to LPS. Second, the induction of the pro-inflammatory molecule inducible NO synthase (iNOS) was assessed by RT-PCR over time after treatment with LPS. As shown in **Fig. 25A** (red staining) and **Fig. 25B** (solid bars), LPS caused a time-dependent signaling response in IEC-6 cells, which reached a maximum at 4–6 and then decreased by 16h. LPS also caused a time-dependent increase in the expression of HSP70 in IEC-6 cells (**Fig. 25A–C**), which peaked at the time at which TLR4 signaling decreased (**Fig. 25B, 25C**). As a positive control for the effects of HSP70 on the confocal-based assay, heat exposure resulted in a marked inhibition of TLR4 signaling, as revealed by reduced GFP expression and an increase in HSP70 expression (**Fig. 25Aiv–vi**). Of note, the effects of LPS and heat on the intracellular induction of HSP70 in IEC-6 cells is also shown in **Fig. 25D**.Several lines of evidence indicate that the intracellular increase in HSP70 expression that was observed to occur in response to LPS could limit TLR4 signaling. First, treatment of IEC-6 with the HSP70 inhibitor Quercetin resulted in an

increase in the extent of TLR4-induced IL-6 expression (**Fig. 25Fi**). Second, the increased expression of HSP70 that was noted in response to LPS resulted in an increase in the degree to which TLR4 could be detected in lysates of IEC-6 cells that had been immunoprecipitated with Abs to HSP70 (**Fig. 25E**), indicating that the increased HSP70 expression in response to TLR4 activation also resulted in increased association of HSP70 with TLR4, consistent with the mechanism of action of HSP70 in reducing TLR4 signaling shown in **Figs. 23 and 24**. Third, inhibition of HSP70 using siRNA resulted in a marked exaggeration in the extent of TLR4 signaling, as measured by an increase in both LPS-induced iNOS expression (**Fig. 25Fii**) and LPS-induced enterocyte apoptosis (**Fig. 25Fiii**) compared with control cells that had been transfected with control siRNA or no siRNA. Importantly, heat exposure did not prevent NF-kB translocation in IEC-6 cells that were treated with the TLR5 ligand flagellin, and these findings were not affected by the presence of Quercetin, suggesting that the effect of heat-induced TLR4 suppression is specific for TLR4 signaling (**Appendix fig 32**). Taken together, these findings reveal that TLR4 can induce the expression of HSP70, which inhibits LPS signaling in enterocytes, and by extension, that HSP70 can exert a physiological role in constraining the effect of TLR4 signaling that occurs. Therefore, we next sought to investigate the role of HSP70 on LPS-induced TLR4 signaling *in vivo*.

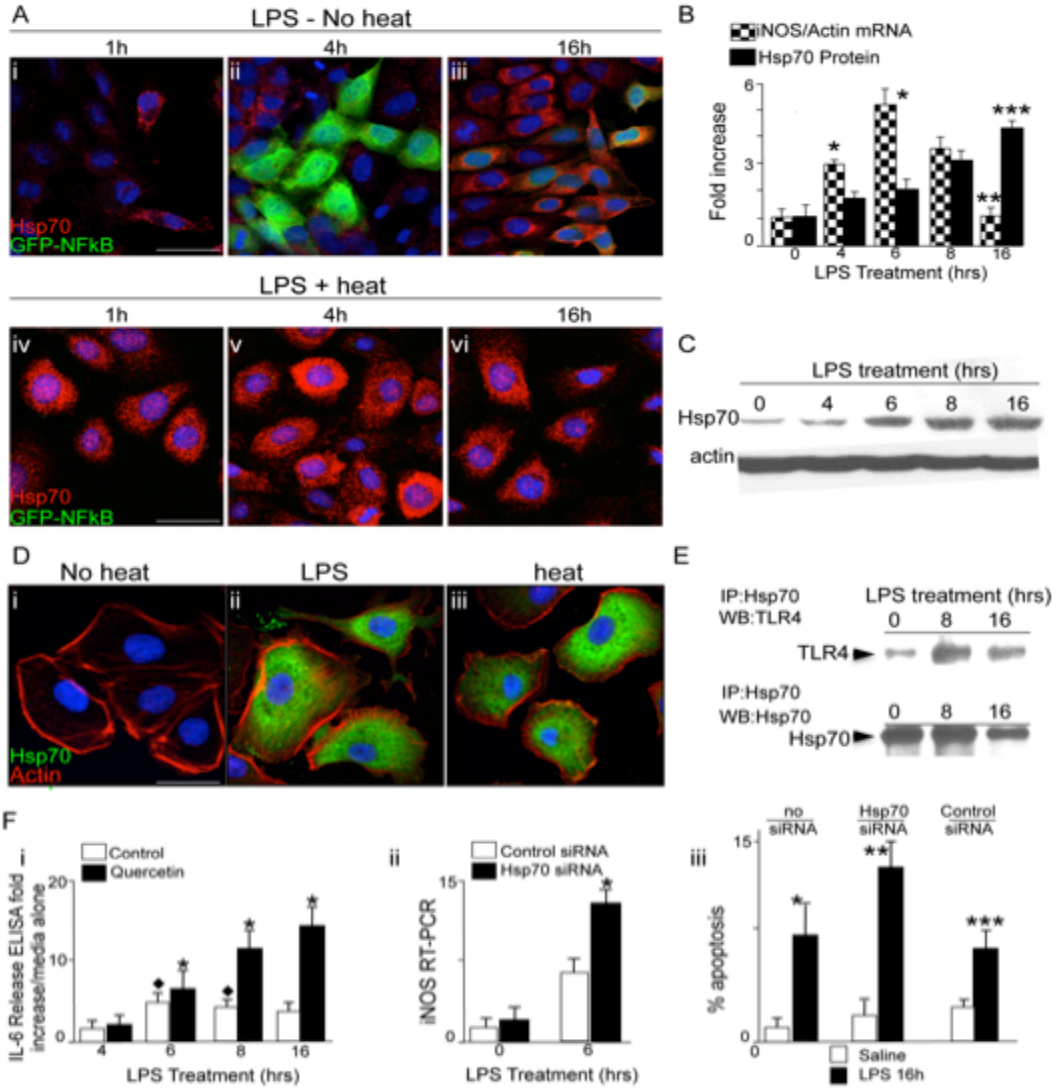


Figure 25. TLR4 induces HSP70 expression, which then negatively effects TLR4 signaling. (A) Representative confocal photomicrographs of IEC-6 enterocytes transduced with GFP–NF-kB and then treated with LPS (t = 0) in the absence of heat (i–iii) or LPS after pretreatment with heat (iv–vi); cells were stained for HSP70 (red) and assessed for GFP (green) at the indicated time point. Scale bar, 10 mm. (B) Quantification of iNOS mRNA by RT-PCR and HSP70 protein relative to b-actin. *p<0.05, versus t = 0 checkered bars, **p <0.01, 16h versus 8, 6 and 4h, checkered bars, ***p <0.001, t = 16 h versus other time points, solid bars. Data are representative of four separate experiments. (C) Representative SDS-PAGE showing HSP70 in IEC-6 cells treated with LPS. (D) Representative confocal photomicrographs of IEC-6 enterocytes under the indicated conditions and stained for HSP70 (green), b-actin (red), and DAPI (blue). Scale bar, 10 um. (E) Representative SDS-PAGE of IEC-6 lysates treated with LPS, immunoprecipitated with anti-HSP70 Abs, and immunoblotted with TLR4 (upper bands) and HSP70. (Fi) Fold increase of IL-6 release by ELISA over media alone in IEC-6 cells treated as indicated. *p<0.05, solid bars versus open bars indicated point, ♦p <0.05, open bars versus 4-h time point. Representative of three separate experiments. (Fii) iNOS RT- PCR in IEC-6 cells treated with LPS, as indicated. Representative of four separate experiments. (Fiii) Apoptosis in IEC-6 cells treated as indicated. Based upon four separate experiments with >50 fields/experiment and >50 cells/field. *p<0.05, no siRNA solid versus open bars, **p <0.01, HSP70 siRNA versus no siRNA solid bars, ***p <0.001, versus HSP70 siRNA versus control siRNA, solid bars. *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

2.4.5 *HSP70 negatively regulates TLR4 signaling in the intestinal epithelium*

The systemic administration of LPS to newborn mice is known to cause a significant inflammatory response in the small intestine that includes an increase in the expression of iNOS and an induction in enterocyte apoptosis (178,415,416). Based upon the above findings, in which HSP70 was found to inhibit enterocyte TLR4 *in vitro*, we next sought to evaluate the effects of HSP70 on enterocyte TLR4 signaling *in vivo*. To do so, we injected saline or LPS into wild-type mice or into two additional mouse strains: mice that were globally deficient in HSP70 ($HSP70^{-/-}$) and mice that selectively overexpress HSP70 within the intestinal epithelium ($HSP70^{villin}$). As shown in **Fig. 26A**, the injection of LPS into wild-type mice caused a time-dependent increase in the mucosal expression of iNOS, as well as a significant induction in enterocyte apoptosis (**Fig. 26Bi, 26Bii, 26C**). In contrast, injection of LPS into mice that selectively overexpress HSP70 within the intestinal epithelium resulted in a marked reduction in LPS-mediated iNOS expression within the intestinal epithelium compared with wild-type mice (**Fig. 26A**) and a reduction in enterocyte apoptosis (**Fig. 26Biii, 26Biv, 26C**), demonstrating that HSP70 could negatively regulate TLR4 signaling *in vivo* and supporting the *in vitro* data shown in **Fig. 25**. Moreover, the injection of LPS into $HSP70^{-/-}$ mice resulted in a significantly increased degree of apoptosis compared with LPS-injected wild-type mice (**Fig. 26Bv, 26Bvi, 26H**), providing further evidence that HSP70 negatively regulates TLR4 signaling *in vivo*. Therefore, we next sought to determine the physiological relevance of these findings in the pathogenesis of NEC.

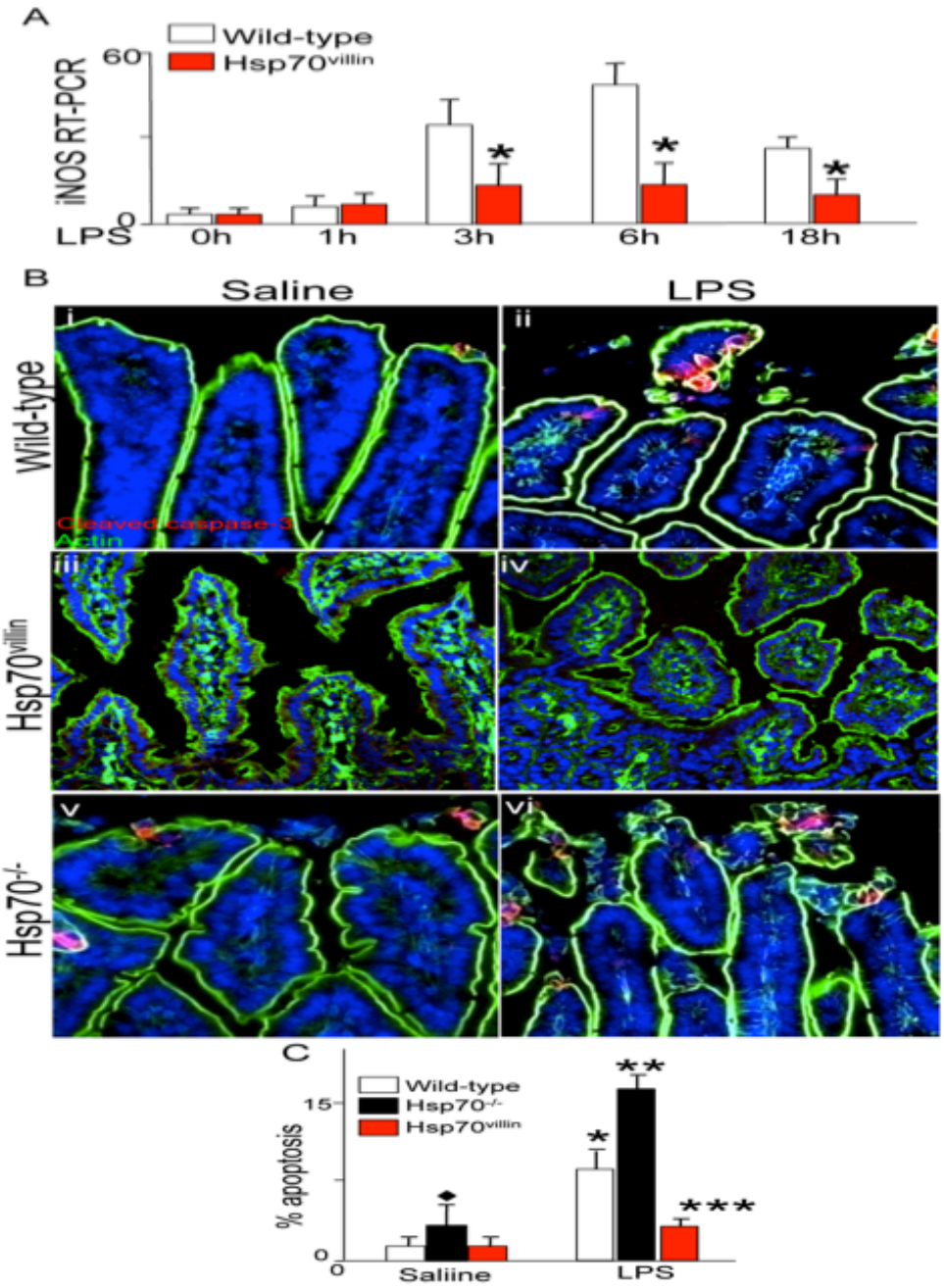


Figure 26.HSP70 negatively regulates TLR4 signaling in the intestinal epithelium. (A) RT-PCR for iNOS in the intestinal epithelium in wild-type or HSP70^{villin} mice treated with LPS for the time points indicated. *p<0.05, red bar versus open bar for each point indicated. (B) Confocal photomicrographs of newborn intestine obtained from the terminal ileum after injection with saline (Bi, Biii, Bv) or LPS (5 mg/kg, 16 h; Bii, Biv, Bvi) in wild-type (Bi, Bii), HSP70villin (Biii, Biv), or HSP70$^{-/-}$(Bv, Bvi) mice. Original magnification 340. (C) Quantification of apoptosis in the small intestine of newborn mice, as in (B), after injection with saline or LPS, as indicated. Based upon four separate experiments with more than four mice/group and >50 fields examined per group. *p<0.05, saline versus LPS for open and black bars, **p <0.01, LPS white bar versus LPS solid bar, ***p <0.001, LPS red bar versus LPS solid bar. *Saline-treated black bar versus open bar or red bar. *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

2.4.6 *HSP70 signaling negatively regulates the development of NEC*

We and other investigators demonstrated that NEC is a disease characterized by TLR4-mediated apoptosis within the newborn small intestine (155,156,407). Having now shown that HSP70 can limit TLR4 signaling in enterocytes both *in vivo* and *in vitro*, we next sought to evaluate whether a lack of HSP70 may lead to an increase in the severity of NEC. As shown in **Fig. 27A**, the expression of HSP70 in both mice (**Fig. 27Ai, 27Aii**) and humans (**Fig. 27Aiii, 27Aiv**) with NEC was significantly decreased compared with control bowel, indicating the possibility that disturbances of HSP70 expression or function may play a role in NEC development. To investigate directly whether HSP70 could regulate the development of this disease, NEC was induced in wild-type, $HSP70^{-/-}$, and $HSP70^{villin}$ mice using a combination of formula gavage and intermittent hypoxia. As shown in **Fig. 27B**, the induction of NEC in wild-type mice resulted in enterocyte apoptosis (**Fig. 27B, 27Bii, 27Ei**), mucosal disruption (**Fig. 27Biii, 27Biv**), and an increase in the expression of iNOS in the intestinal mucosa (**Fig. 27Eii**). Importantly, the induction of NEC in $HSP70^{-/-}$ mice showed a significant increase in the extent of enterocyte apoptosis (**Fig. 27Ci, 26Cii, 26Ei**), mucosal disruption (**Fig. 27Ciii, 27Civ**), and iNOS expression (**Fig. 27Eii**), as well as increased disease severity (**Fig. 27Eiii**), whereas the induction of NEC in the $HSP70^{villin}$ mouse that overexpresses HSP70 in the intestinal epithelium resulted in a marked reduction in each of these measures (**Fig. 27D, 27E).** Taken together, these findings illustrate that HSP70 plays a key role in the regulation of NEC. We next sought to evaluate whether pharmacologic induction of HSP70 could inhibit TLR4 signaling and affect NEC severity.

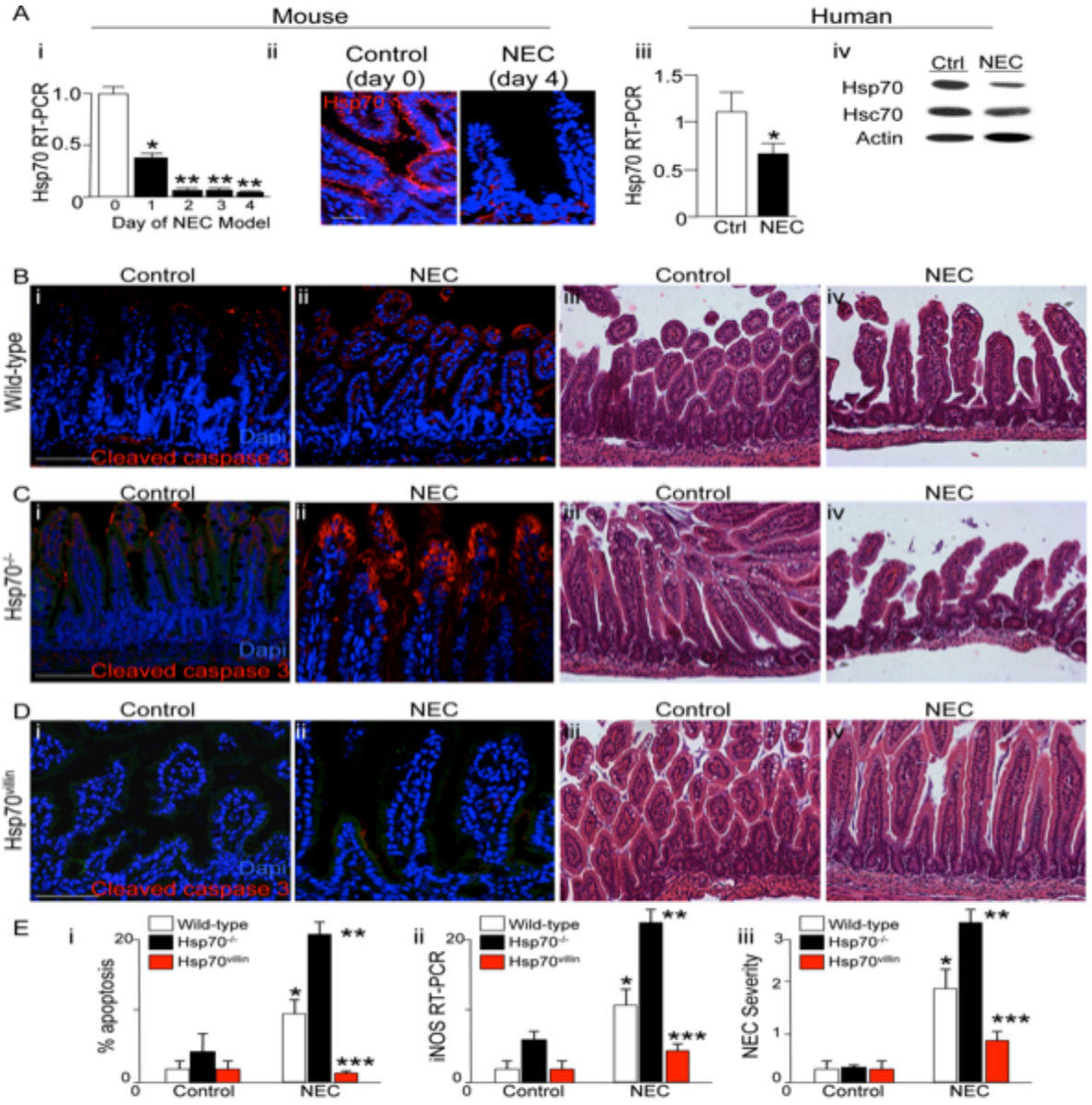


Figure 27.HSP70 signaling negatively regulates the development of NEC. (Ai) RT-PCR showing HSP70 on each day of the 4d NEC model. *p<0.05, versus day 0, *p <0.01, versus day 1. Representative of more than four separate experiments (n = 10 mice/group). (Aii) Representative confocal photomicrographs showing the expression of HSP70 (red) and DAPI (blue) in the terminal ileum of mice without (Control) and with NEC (NEC). (Aiii) PCR showing the expression of HSP70 in intestine from infants without (open bar) and with NEC (solid bar). *p<0.05, solid versus open, based upon nine separate samples/group. (Aiv) Representative SDS- PAGE from infant without (Ctrl) and with NEC blotted with Abs to HSP70 and then probed for Hsc70 and b-actin. Representative confocal and H&E photomicrographs of sections of the terminal ileum from wild-type (B), HSP70$^{-/-}$(C), or HSP70^{villin} (D) newborn mice with or without NEC. In (Bi, Bii), (Ci, Cii), and (Di, Dii), slides were stained for cleaved-caspase 3 (red) and DAPI (blue). Scale bar, 250 μm. (Ei) Apoptosis. (Eii) iNOS RT-PCR in the terminal ileum. (Eiii) NEC severity. Based upon at least four experiments with >15 mice/strain/group. *p<0.05, NEC in wild-type versus control, **p <0.01, NEC in HSP70$^{-/-}$versus wild-type, ***NEC in HSP70^{villin} versus wild-type and HSP70$^{-/-}$. *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

2.4.7 Pharmacologic induction of HSP70 limits TLR4 signaling in enterocytes *in vitro* and *in vivo*, and attenuates the severity of experimental necrotizing enterocolitis

In the final series of studies, we sought to evaluate whether the pharmacologic induction of HSP70 could inhibit TLR4 signaling *in vitro* and *in vivo* and, thus, attenuate the severity of NEC. To do so, we used the small molecule Celastrol (320,321), a novel cell-permeable triterpenoid antioxidant that was shown to induce HSP70 expression and activity in a variety of cells (417,418). As shown in **Fig. 28Ai**, treatment of IEC-6 cells with Celastrol led to a rapid induction of HSP70, as determined by SDS-PAGE. In parallel, the injection of Celastrol into mice on three consecutive days resulted in an increase in the expression of HSP70 within the intestinal mucosa on each day (**Fig. 28Aii**). Importantly, the exposure of IEC-6 cells to Celastrol resulted in a marked increase in cytoplasmic HSP70 expression (**red staining in Fig. 28Bi–iv**), as well as a significant reduction in the extent of TLR4 signaling, as measured by a reduction in the extent of LPS-induced apoptosis (**Fig. 28Biv versus Fig. 28Bii, 28Ei**), a reduction in the extent of LPS-induced NF-kB translocation (**Fig. 28C, 28Eii**), and a significant reduction in the extent of LPS-induced IL-6 expression (**Fig. 28Eiii**). There were no effects of Celastrol treatment alone on enterocyte apoptosis, NF-kB translocation, or IL-6 expression (**Fig. 28Biii, 28Ciii, 28Ei**). Having shown that the injection of Celastrol into mice can induce HSP70 expression within the intestinal mucosa **(Fig. 28Aii**), we next sought to determine whether Celastrol could inhibit TLR4 signaling in the intestinal epithelium via effects on HSP70 induction. To do so, wild-type and HSP70$^{-/-}$ mice were injected with Celastrol 24h prior to LPS and then assessed for the extent of enterocyte apoptosis and iNOS expression in the intestinal mucosa. As shown in **Fig. 28Di-iv and 28Fi**, the administration of Celastrol to wild-type mice led to a significant reduction in the extent of LPS-induced enterocyte apoptosis compared with

the effects of LPS in wild-type mice that did not receive Celastrol, as well as to a significant reduction in the extent of LPS-induced expression of iNOS in the intestinal mucosa compared with wild-type mice (**Fig. 28Fii**). Importantly, there was no protective benefit of Celastrol when it was administered to $HSP70^{-/-}$ mice, confirming that its protective effects required HSP70 induction (**Fig. 28Dv–viii, 28F**). The specificity of Celastrol for TLR4-mediated enterocyte apoptosis was confirmed *in vitro*. Although LPS caused a significant increase in enterocyte apoptosis in HSP70-deficient IEC-6 cells, the addition of Celastrol did not confer protection; in fact, these HSP70-deficient enterocytes were significantly more susceptible to apoptosis than were their nontransfected counterparts (**Appendix Fig. 33**).

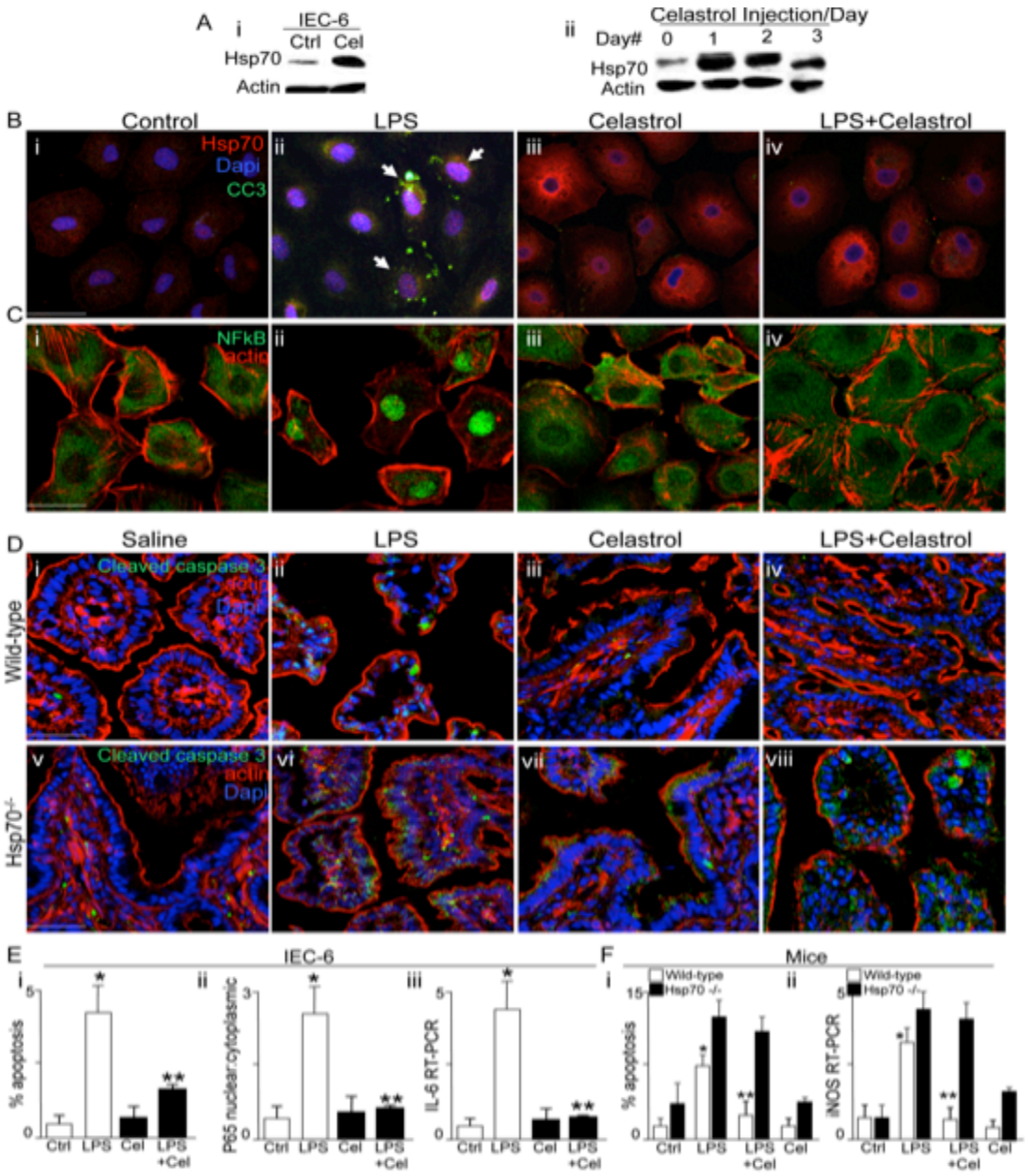


Figure 28.Pharmacologic induction of HSP70 limits TLR4 signaling in IECs *in vitro* and *in vivo*. (Ai) Representative SDS-PAGE of IEC-6 enterocytes treated with Celastrol or DMSO and blotted with HSP70 and then reprobed for b-actin. (Aii) Representative SDS-PAGE of mucosal scrapings from terminal ileum of newborn mice administered Celastrol daily for 3d. Representative confocal photomicrographs of IEC-6 enterocytes treated as indicated and immunostained for HSP70 [red in (B)] and DAPI [blue in (B)] or NF-kB [green in (C)] and b-actin [red in (C)].Representative apoptotic cells are indicated by arrows. Scale bar, 10 um. Representative confocal photomicrographs of terminal ileum in newborn wild-type (Di–iv) or HSP70$^{-/-}$(Dv–viii) mice that were treated, as indicated, and stained for cleaved-caspase 3 (green), DAPI (blue), and b-actin (red). Scale bar, 250 um. (E) Quantification of apoptosis, p65 translocation, and IL-6 mRNA expression in IEC-6 cells. *p<0.05, control versus LPS open bars, **LPS open bars versus LPS+ Celastrol closed bars in four separate experiments. Quantification of apoptosis (Fi) and iNOS expression (Fii) by RT-PCR in the terminal ileum of newborn wild-type or HSP70$^{-/-}$mice. Representative of four separate experiments, with >10 mice/group. *p<0.05, open bars LPS versus control, **p<0.01, LPS+Celastrol versus LPS open bars. *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

Based upon the above findings, we next sought to evaluate whether the induction of HSP70 that occurs with the administration of Celastrol could attenuate the severity of experimental NEC. To test this directly, we first administered either DMSO or Celastrol by i.p. injection to newborn pups on days 0 and 1 of the experimental model and assessed the effects on extent of mucosal disruption, enterocyte apoptosis, induction of iNOS, and disease severity. As shown in **Fig. 29**, the administration of Celastrol markedly reduced the degree of mucosal disruption (**Fig. 29Ai–iii**), enterocyte apoptosis (**Fig. 29Av–vii, 29Ci**), mucosal iNOS expression (**Fig. 29Cii**), and disease severity (**Fig. 29Ciii**) compared with mice that had been administered DMSO. It is noteworthy that the injection of Celastrol resulted in a marked increase in the expression of HSP70 and a reduction in TLR4 compared with mice with NEC that received DMSO alone, consistent with the mechanism of action for Celastrol shown in **Fig. 28**. Having shown that the induction of HSP70 through the administration of Celastrol could prevent the development of NEC when administered prior to the start of the model, we next sought to determine whether Celastrol administration could reduce the severity of NEC once the disease had been established in mice. To do so, we injected mice with Celastrol on the last 2d of the 4d model, at a time in which significant inflammation is already established. Strikingly, as shown in **Fig. 29**, mice with NEC that received Celastrol after disease induction showed restoration of mucosal architecture (**Fig. 29Aiv**), a significant reduction in enterocyte apoptosis (**Fig. 29Aviii**, **29Ci**), and a reduction in NEC severity (**Fig. 29Ciii**), all to levels that were similar to mice without NEC and comparable to levels observed in mice receiving Celastrol as a prevention strategy (checkered vs solid bars in **Fig. 29C**). Taken together, these findings suggest that the pharmaceutical induction of HSP70 may be used as a novel approach to the prevention or treatment of NEC through effects on the inhibition of TLR4 signaling in the newborn small intestine.

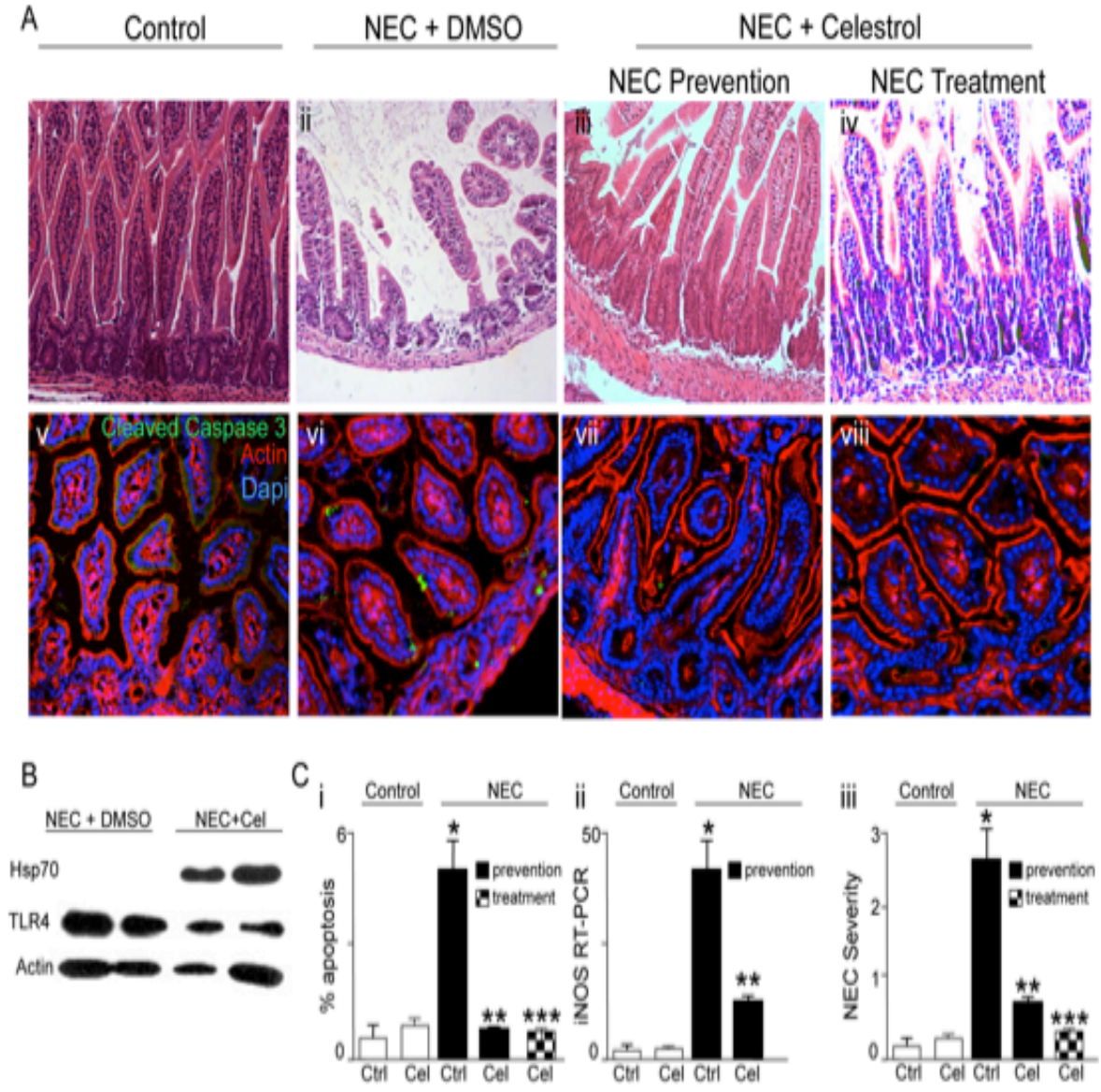


Figure 29. Pharmacologic induction of HSP70 prevents and also treats experimental NEC in mice. Representative H&E (Ai–iv) and confocal images [(Av–viii), cleaved-caspase 3 (green), b-actin (red), DAPI (blue)] of sections from terminal ileum of newborn mice that were either breastfed [Control, (Ai) and (Av)] or induced to develop NEC and administered either DMSO [(Aii) and (Avi)] or 1 mg/kg Celastrol on days 0 and 1 of the 4d model [(Aiii) and (Avii)]. In parallel, mice that had NEC for 2d were administered 1 mg/kg Celastrol for 2d [(Aiv) and (Aviii)]. Scale bar, 250 um. (B) SDS-PAGE of mucosal scrapings from mice subjected to experimental NEC and injected with either DMSO or Celastrol on the first 2d of the model; blots were probed for HSP70 and then stripped and reprobed for TLR4 and b-actin. Quantification of enterocyte apoptosis (Ci), iNOS by RT-PCR in the terminal ileum (Cii), and NEC severity (Ciii).Representative of four separate experiments with >10 mice/group. *p<0.05, NEC Ctrl (solid bar) versus control (open bar), **p <0.01, NEC Celastrol – prevention (solid bar) versus NEC Ctrl – prevention (solid bar), ***p <0.001, NEC Celastrol treatment (checkered bar) versus NEC control (solid bar). *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

3.0 DISCUSSION

We now define a novel mechanism by which TLR4 is regulated in the newborn intestinal epithelium that has important implications in the pathogenesis of NEC, a disease that is characterized by exaggerated TLR4 signaling within the intestinal mucosa (155,156,159). Specifically, we identify that the induction of HSP70 leads to a reduction in TLR4-induced signaling in enterocytes, as measured by a reduction in NF-kB activation, cytokine induction, and apoptosis, and that induction of HSP70 either pharmacologically or genetically leads to a reduction in TLR4 signaling and a marked inhibition in the severity of NEC. The current results identify a novel pathway that links cytoplasmic HSP70 induction with TLR4 regulation and demonstrate that impaired HSP70 expression or function may, in part, underlie the causes of this devastating disease. These findings represent a novel departure from current thinking in the field by revealing that future treatments for NEC may involve nonspecific immunological approaches, such as the elimination of microbial pathogens or the administration of particular feeding regimens (419), as well as the pharmacologic induction of an intracellular chaperone, such as HSP70, to limit disease progression through inhibitory effects on the innate immune receptor TLR4.

An important finding of the current study involves the proposed mechanism of action of HSP70 in limiting TLR4 signaling within enterocytes. As a molecular chaperone, HSP70 can associate with co-chaperone proteins through an EEVD motif in its C-terminus (411,412). As

shown in **Fig. 23**, we now demonstrate that the inhibition of TLR4 signaling in response to HSP70 induction required this EEVD-binding motif (266), because the introduction of a mutant lacking this domain prevented the association between TLR4 and HSP70, presumably via a co-chaperone intermediary, as well as reversed the protection of HSP70 induction on TLR4 signaling (**Fig. 23**). This mechanism of action for HSP70 is in agreement with recent work of Chow *et al*. (420), who showed that HSP70 mutants with a functional EEVD motif but lacking N-terminus ATPase activity were still capable of protecting L929 fibroblasts from apoptosis induced by pro-inflammatory cytokines.

We also determined that the association between HSP70 and TLR4 results in enhanced ubiquitination and degradation of TLR4, a process that we have now determined to require the co-chaperone and E3 ligase CHIP (**Fig. 24**). CHIP has not previously been linked to TLR4 signaling within the intestinal epithelium, and we further reveal that mutations in both its docking (K30A) or ubiquitination U-Box domain (H260Q) prevented the protective effects of HSP70 induction on TLR4. These findings define the mechanism by which CHIP may act to mediate the inhibitory effects of HSP70 on TLR4 signaling and are in agreement with the known function of CHIP in regulating the activity of other HSP70 targets through ubiquitination (421,422), yet to our knowledge, they represent the first direct link of CHIP to an intestinal inflammatory disease. It is noteworthy that in the original description of the CHIP-deficient mouse, attention was drawn to the intestinal phenotype that was observed when mice were subjected to a brief hyperthermic stress, characterized by friability of the small intestine with marked apoptosis of the intestinal epithelium (423), although potential CHIP targets that could mediate this effect on the small intestine during stress were not identified. It is tempting to now speculate that CHIP may play a central role in the maintenance of intestinal homeostasis, in part

by preventing the unbridled activation of immune targets of CHIP, such as TLR4 in such settings as NEC.

It should be noted that the current findings in which cytoplasmic HSP70 serves to curtail the signaling of TLR4 within the intestinal epithelium lie in distinction to a growing and somewhat controversial body of work concerning the extracellular role of HSP70 and other heat shock proteins in activating the innate immune system via TLR4 (348,424-428). In this regard, Retzlaff *et. al.* (429) showed that the exogenous administration of HSP70 could increase IL-1, IL-6, and TNF in cultured macrophages, whereas Wheeler *et. al.* (430) showed that the extracellular exposure of HSP70 to neutrophils from wild-type mice leads to the release of IL-8, yet this effect is not observed in neutrophils from C3H/HeJ mice that have inhibitory mutations in TLR4. Although very exciting, such studies were recently called into question by concerns that the observed effects might actually result not from the heat shock proteins themselves, but rather from contaminants, such as LPS, which could inadvertently be present within the protein preparations or be bound specifically to the heat shock proteins (56). For example, Wallin *et. al.* (431), Bausinger *et al.* (432), and Gao and Tsan (433) showed that the activation of immune cells previously attributed to HSP70 were lost when highly purified recombinant proteins were used, although these results were recently and convincingly rebutted in two review articles on this topic (434,435). In contrast to studies in the field of extracellular HSP70 biology, the novelty and importance of the current findings lie in the newly discovered link between TLR4 and HSP70 within the enterocyte both *in vitro* and *in vivo*, as well as the potential etiological relevance to the development of NEC. And although they represent an extension of the classic role of HSP70 in modulating the fate of cytoplasmic proteins, the relevance, if any, to the body of literature surrounding the fate of HSP70 outside of the cells is unknown.

Other diseases of intestinal inflammation, including ulcerative colitis and Crohn's disease, in which TLR4 signaling may play a lesser, or perhaps even opposite, role make integrating the current work complex. Although we (155,159) and other investigators (156,163) showed that the development of NEC requires TLR4 activation, it was shown that TLR4 plays a protective role in experimental colitis (133,436). Several reasons may account for this apparent discrepancy that have relevance to the current study. TLR4 activation leads to intestinal injury in a well-defined and physiologically relevant context (i.e., the newborn small intestine). In support of this concept, we recently demonstrated that TLR4 activation with LPS leads to increased enterocyte apoptosis in the terminal ileum of newborn mice but not adult mice, as well as in the small intestine but not the newborn colon (158). Further, reports that demonstrate a protective role for TLR4 in models of colitis have typically been based upon the use of global TLR4 knockout mice, in which TLR4 signaling is disrupted in enterocytes as well as T cells and myeloid cells. We recently showed that TLR4 signaling within the enterocyte itself is important for the induction of intestinal injury leading to NEC, using enterally administered adenoviral constructs that bear inhibitory mutations in TLR4 whose expression is largely favored within the small bowel mucosa (113,159) and also in enterocyte specific TLR4 conditional knockout mice (70). Therefore, it is reasonable to conclude that the protective effects attributed to TLR4 signaling in the gut by previous investigators may reflect, in part, the mitigating effects of TLR4 signaling on other cells. In support of this possibility, we note that Fukata *et al*. (437) recently showed, in an elegant study using chimeric mice, that TLR4 signaling in colonic epithelial cells worsened intestinal inflammation. These findings argue that the effects of TLR4 in the development of intestinal inflammation are strongly influenced by a variety of factors, including the effector cells involved, developmental factors, and involved region of the intestine. The precise effects of

HSP70 at these varying stages of development and within these different cell types remain to be explored in further detail, but they are likely to provide important clues to the underlying causes of these diseases.

It is important to note that the current findings do not represent the sole HSP70-dependent mechanism in play to curb IECs injury and inflammation associated with exaggerated TLR4 signaling. Recent work by Chen *et.al*. (438) illustrates HSP70 can also block the ubiquitination and activation of TRAF6 via direct binding in macrophages. Interestingly, this interaction is dependent upon the C-terminus of HSP70 consistent with our work illustrating a dependence upon the C-terminal EEVD motif of HSP70 for the inhibition of NF-kB activation and apoptosis. Additionally, previous work has demonstrated that HSP70 can negatively regulate NF-kB activation. Ran *et. al*. (84) demonstrate that HSP70 directly interacts with the IKKγ subunit of the IKK complex preventing the oligomerization of IKKγ proteins and formation of the IKK complex upon HSR activation. While HSP90 functions to stabilize the IKK complex playing an important role in NF-kB activation (85), the presence of HSP70 in the IKK complex has been shown to disturb its function and IkBα phosphorylation and ubiquitination (89). Other studies have illustrated that HSF1 activation and nuclear translocation can lead to inhibition of NF-kB DNA binding directly (90). Although this mechanism may play a lesser role in the current studies as we have found induction of HSP70 in IEC6 leads to inhibition of NF-kB nuclear translocation.

While not directly addressed in this work, DAMP release may play an important casual role in NEC. Recent work in the Hackam lab has demonstrated that HMGB1 expression is significantly increased in both murine and human NEC. HMGB1 was demonstrated to inhibit enterocyte migration via TLR4 activation both *in vitro* and *in vivo*. Interestingly, this was unique

to enterocytes as inflammatory cell migration was significantly enhanced following exposure to HMGB1 (105). Work by Tang *et. al.* (106,108) has demonstrated that the induction of HSP70 can lead to translocation to the nucleus where stress-induced HMGB1 release by macrophages can be significantly attenuated. It stands to reason, therefore, that over-expression of HSP70 in enterocytes, or gut immune cells, may lead to decreased HMGB1 release in the setting of intestinal inflammation. HSR activation *in vitro* or *in vivo* may directly inhibit HMGB1 or other DAMP release in the setting of NEC further attenuating the severity of this disorder.

The cytoprotective mechanisms of HSP70 extensively studied and summarized above involve multiple intersection points along different apoptotic (intrinsic vs extrinsic and caspase-dependent vs caspase-independent) and apoptotic-like pathways. The current work demonstrates a significant attenuation in TLR4-mediated enterocyte apoptosis via HSP70 up-regulation and correlates this to a significant attenuation in NEC severity. HSP70 activity leads to an inhibition of Caspase 3 activation consistently seen in NEC. While in the current work this is correlated to attenuation in caspase-dependent apoptosis, further studies are necessary to determine the exact mechanism of apoptosis prevention, e.g. inhibition of APAF1-Cytochrome c-Caspase 9 apoptosome formation. These studies also do not rule out the possibility of other mechanisms of HSP70-mediated cytoprotection against caspase-independent apoptosis, e.g. inhibition of AIF activity. The role of apoptosis-like cell death pathways such as pyroptosis or necroptosis in the setting of NEC have yet to be elucidated. Caspase 1 via inflammasome formation and death receptor caspase 8 activity via kinases RIP1 or RIP3 which play key initiating roles in pyroptosis (107) and necroptosis (110), respectively, have been demonstrated to be regulated via HSP activity as described previously or via inhibition of downstream effectors (109) (382). Further work is necessary to not only determine the role of these apoptotic-like cell death pathways in

NEC as well as the role HSP70 induction may play in modulating these pathways in protecting the newborn gut from this disorder.

Based upon the current findings, we now propose a model by which HSP70 limits TLR4 signaling and plays a key role in influencing the development of enterocyte apoptosis and the development of NEC (**Fig. 30**). Under healthy conditions, the relationship between the indigenous flora of the host and the baseline activation of TLR4 exists in homeostatic balance, which we now attribute, in part, to a constitutive role of HSP70 in limiting the extent of TLR4 signaling by controlling its degradation through proteasomal pathways. The interaction between TLR4 and HSP70 may occur within intracellular compartments, such as the Golgi apparatus, where TLR4 signaling was shown to reside primarily and occur within the enterocyte (101). In contrast, under the conditions of stress that favor the development of NEC (increased LPS, hypoxia, and prematurity), the exhaustion of HSP70 signaling, accompanied by the relative increase in TLR4 expression in the preterm gut (113), leads to exaggerated TLR4 activation and the development of the increased enterocyte apoptosis and pro-inflammatory cytokine expression in the newborn intestine that leads to NEC. It is also notable that the pharmacologic induction of HSP70 can curtail TLR4 signaling and both prevent and treat experimental NEC.

These data present a novel pathway by which HSP70 serves to limit TLR4 signaling in the intestinal epithelium, and moreover, shows that factors that increase HSP70 signaling can attenuate NEC severity through inhibition of TLR4. We believe that such findings offer new insights into the molecular requirements that lead to NEC development, as well as offer novel therapeutic approaches for this devastating disease. It is important to note that while our studies demonstrate a significant protective effect of Celastrol in experimental NEC, this particular drug

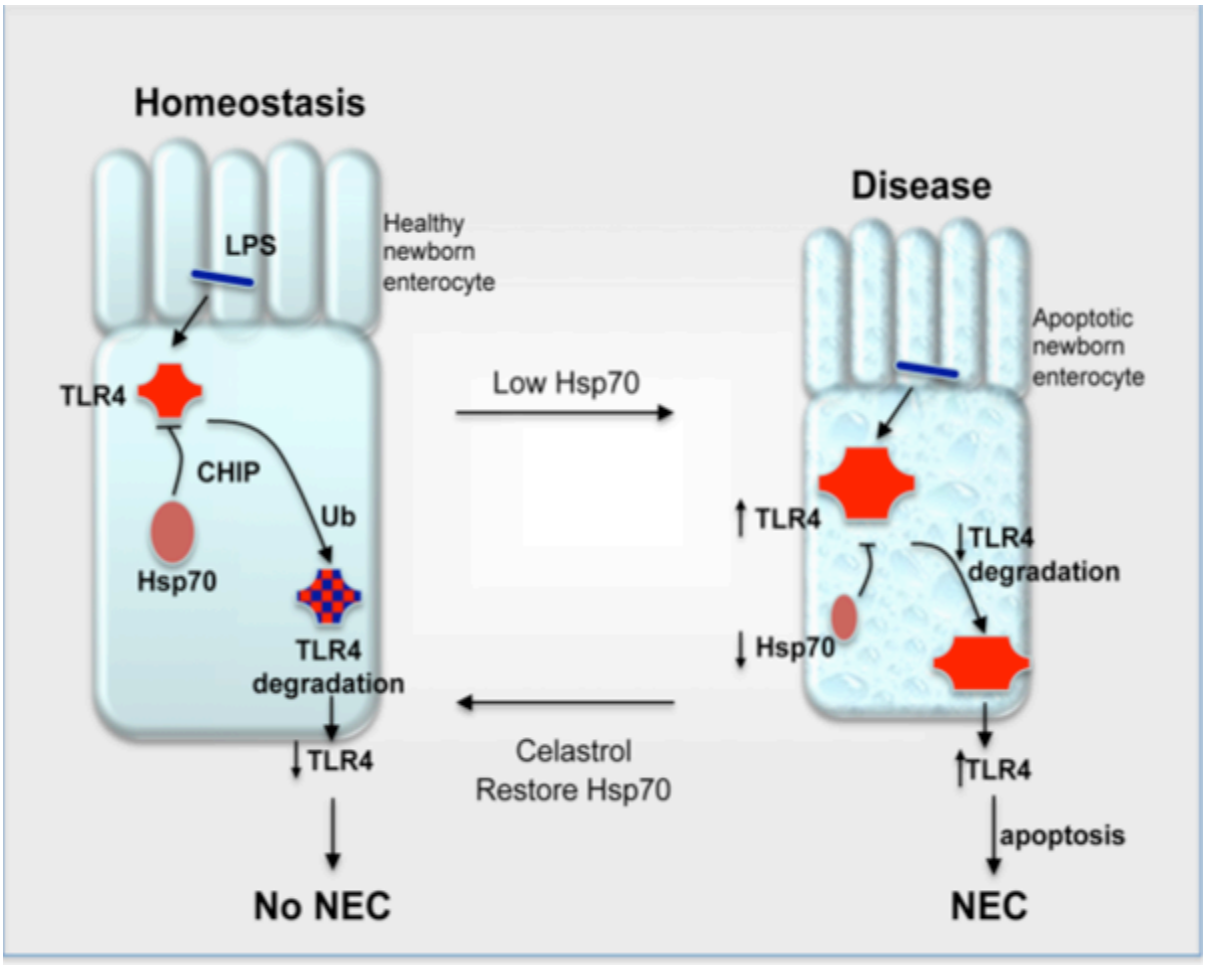


Figure 30**.** Proposed model: HSP70 regulates TLR4 signaling in enterocytes in the pathogenesis of NEC. As described in the text, under healthy conditions. TLR4 is activated by host microbes. The degree of activation is limited by HSP70 through effects on TLR4 degradation through proteasomal pathways via CHIP. In contrast, under the conditions of stress that favor the development of NEC, the reduction in HSP70 expression accompanied by the increase in TLR4 expression leads to exaggerated TLR4 activation and the development of increased enterocyte apoptosis and pro-inflammatory cytokine expression in the newborn intestine. This leads to the development of NEC. Moreover, pharmacologic induction of HSP70 can curtail TLR4 signaling and both prevent and treat experimental NEC. *Figure reprinted with permission from The American Association of Immunologists, Inc. Article: Copyright 2012*

maintains a very narrow therapeutic dose window. Due to the significant toxicity of Celastrol, it is unlikely to translate directly to clinical trials. We have used this specific drug due to its wide availability and as a proof of concept that pharmacologic inducers of HSP70 may provide novel treatments for NEC. However, these treatment options will not be without their side effects. A major concern for the use of HSP70 inducers is the possibility of malignancies arising due to prolonged pharmacologic expression of HSP70 in tissues. These treatments will undoubtedly

require strict monitoring and tailoring for use in diseases like NEC. As NEC does not develop in all preterm infants and rarely manifests in term infants, a major challenge in the field is the elucidation of novel biomarkers or polymorphisms that may shed light on which infants are at risk. The data we present suggest the possibility that certain polymorphisms that may effect HSP70 expression or function could predispose to NEC development. Polymorphisms altering CHIP or HSF expression or activity may also predispose to this disease. Given the critical role of TLR4 in the pathogenesis of NEC, polymorphisms leading to increased TLR4 expression or activity may also predispose infants to NEC. Identifying such markers as well as clinical biomarkers would undoubtedly drastically alter our current approaches to NEC patients and thus patient outcomes.

While the mechanisms at play involving HSP70-mediated cytoprotective effects have been well studied, the mechanisms governing TLR4-mediated apoptosis are less well characterized. The current work demonstrating a novel mechanism of how activation of the HSR and a stress-induced molecular chaperone can curb the injurious effects of an innate immune receptor sheds light upon a possible pathway leading to TLR4-mediated cell death. As touched upon briefly above, the HSR and UPR play key cytoprotective roles in proteostasis. The small bowel is a highly secretory organ as evident by the amount of mucus, AMPs, IgA and hormones produced daily. It is therefore unsurprising that proteostasis and those mechanisms in place which maintain this balance, such as the UPR, are especially critical in the intestine. The intestinal parenchyma is taxed with the mass production and secretion of these products and subsequently maintains a very high demand and reliance upon translational and protein-folding machinery and proteostasis pathways. It is due to this that the intestine remains an organ with a relatively high baseline of ER stress (115).

ER stress refers to a state of cellular stress that culminates in the accumulation of unfolded proteins within the ER, which can be transient and well-controlled or insurmountable. It has been shown that during conditions of cell stress, such as hypoxia as well as during inflammatory conditions leading to increased cytokine synthesis, the protein load in the ER exceeds the capacity for normal folding (112). This accumulation of misfolded/unfolded proteins in the ER leads to the induction of the UPR. Similar to the HSR induction in response to cytoplasmic disturbances in proteostasis, the UPR encompasses signaling pathways aimed at relieving ER protein burdens. If the activation of the UPR is unable to correct this accumulation, prolonged ER stress and by extension prolonged UPR activation will result in apoptosis (112). A key sensor and upstream modulator of the UPR is the resident ER chaperone and HSP70 family member, BiP also referred to as GRP78. BiP plays a key role in the proper folding of nascent peptide chains newly translated and released into the ER from bound ribosomes. Under conditions of ER proteostasis, BiP is found primarily in complex with three resident proteins in the ER: ATF6, PERK and IRE1 (112). These proteins comprise the three arms of the UPR signaling cascade. While in complex with BiP, each arm of the UPR remains in an inhibitory state. Upon accumulation of unfolded nascent peptides, BiP dissociates from its bound state to fold/refold these proteins leaving each of the three arms of the UPR free for activation. ATF6 activation leads to its cleavage and translocation to the nucleus where it transcribes target genes aimed at increasing ER size and protein folding machinery including, among other resident chaperones, BiP (112). PERK activation results in autophosphorylation as well as the phosphorylation of the important translation initiation factor 2α (eIF2α), indirectly inactivating eIF2 and decreasing mRNA translation. This leads to a sharp decrease in translation and leads to the preferential induction of the transcription factor ATF4 and subsequent transcription and

translation of its critical gene target CHOP, a transcription factor controlling expression of pro-apoptotic genes. Activation of the PERK pathway is therefore a significantly protective arm of the UPR at modest levels of activation or detrimental at exaggerated ER stress levels or prolonged amounts of time. IRE1 is both a kinase and endoribonuclease. UPR activation results in the splicing of UPR-specific transcription factor Xbp1 mRNA giving rise to Xbp1(s). Xbp1(s) target genes result in increases in ER capacity, protein folding machinery and ER-associated degradation components. IRE1 activation also decreases ER protein load by directly degrading ER bound mRNA transcripts. The IRE1 and ATF6 arms of the UPR are generally viewed as the adaptive arms primarily activating pathways aimed at relieving ER burden. The key switch between UPR activation as a means to maintain homeostasis vs leading to the cell's demise centers upon its timely resolution. If ER stress is prolonged, sustained PERK activity leads to CHOP-mediated apoptosis of the dysfunctional cell (112).

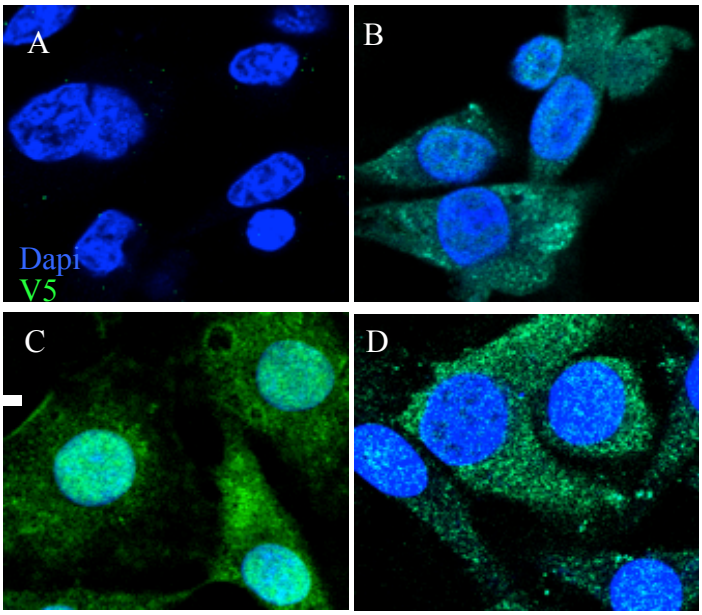
In a seminal paper by Kaser *et. al.* (116) mice lacking Xbp1 expression in the intestinal epithelium demonstrated significant intestinal injury and inflammation as measured by increased susceptibility to experimental colitis. These animals also demonstrated a spontaneous enteritis following Xbp1 removal and subsequently defective UPR. Given the importance of a functional intestinal UPR in modulating intestinal inflammation and injury, it is tempting to hypothesize that these pathways are also critical in the setting of NEC. The current work demonstrates that TLR4, a fundamental player in the pathogenesis of NEC, is inhibited by the molecular chaperone HSP70. With the high physiologic baseline of ER stress within the gut, it may be that the gut is uniquely susceptible to acute increases in ER stress tipping the balance toward injury from homeostasis. Therefore, a potentially fruitful avenue of NEC research may center on the investigation of the role of exaggerated TLR4 signaling in inducing ER stress in the newborn

intestinal epithelium leading to intestinal inflammation, apoptosis and subsequently NEC. This would involve the specific elucidation of which specific UPR pathways are activated in response to TLR4 activation and determining whether manipulation of these pathways can alleviate disease severity. We will seek to determine if enterocyte TLR4 activation induces markers of ER stress both *in vitro* and *in vivo* and in the setting of NEC both murine and human. Generation of cell lines and utilization of animals deficient in the various UPR pathways described above will aid in the delineation of key pathways involved in modulating or inducing TLR4-mediated injury. Utilization of TLR4 mutant animals, which are known to be protected from intestinal injury and NEC development would be expected to also demonstrate a significantly attenuated amount of ER stress markers compared with wild-type animals. We hypothesize that exaggerated TLR4-mediated ER stress will lead to intestinal injury via prolonged PERK/CHOP pathway activation and specific targeting of these pathways, via deficient cells/mice or pharmacologic inhibition via Salubrinal, would prevent or alleviate TLR4-mediated injury. Given the current work, we would also hypothesize that overexpression of HSP70 family members would also demonstrate decreased ER-stress markers in the face of TLR4 activation both *in vitro* and *in vivo*, e.g. in HSP70^{villin} animals exposed to the NEC model. In line with Kaser *et. al.* we would also hypothesize Xbp1 via IRE1 activity plays an important role in modulating TLR4-mediated ER stress and that mice deficient in this pathway would display exaggerated intestinal injury in NEC. We have previously demonstrated that the expression of TLR4 is significantly increased in the preterm murine pup and in the preterm human and that this exaggerated expression predisposes to excessive signaling and NEC (113). Similarly, the preterm infant may display markers of exaggerated ER stress, possibly directly due to TLR4 expression, may predispose to intestinal injury upon colonization and TLR4 activation. Assessment of the ontogeny of ER

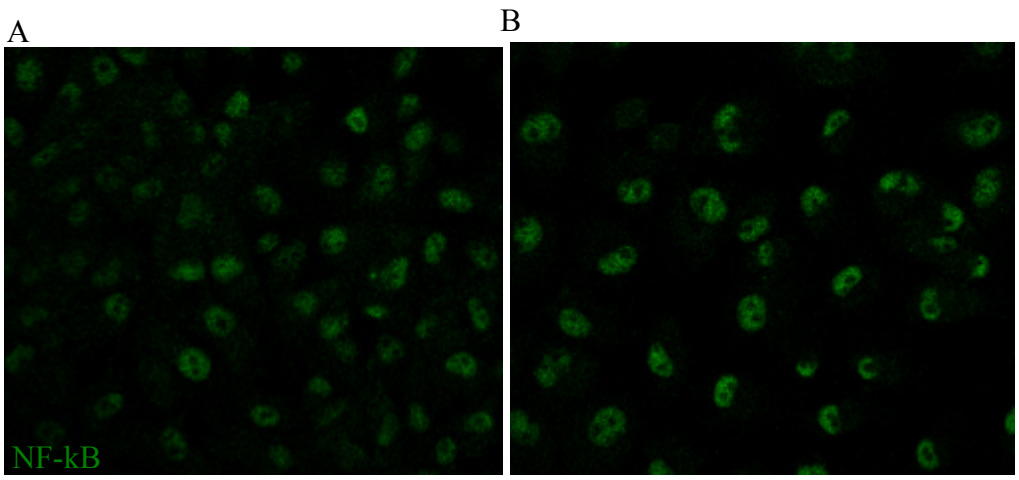
stress markers in both wild-type and TLR4 mutant animals may shed light on this. Our lab has previously utilized *in utero* injection of murine embryos to test intestinal biology (113)(117). Utilizing this technology, specific targeted knockdown of UPR pathways, e.g. PERK, via lentiviral shRNA introduction and transduction, may enable the specific blockade of detrimental ER stress pathways prior to NEC model exposure. Determination of the specific mechanism(s) governing TLR4-mediated ER stress would undoubtedly also serve as therapeutic avenues for novel NEC treatments and insights into potentially novel NEC etiologies. Possible mechanisms include excessive TLR4-mediated protein synthesis and release (e.g. cytokines) that over burden ER capacity. TLR4-mediated ROS generation is also a possible mechanism for induction of enterocyte ER stress. TLR4-mediated ER stress would therefore display sensitivity to ROS scavengers and by extension a subsequent reduction in NEC severity. Another possible mechanism of TLR4-mediated injury via increased ER stress centers upon TLR crosstalk pathways. Recent work has demonstrated the localization and compartmentalization of TLR3, TLR7 and TLR9 into endosomes from the ER is critically dependent upon several proteins including the ER-resident protein UNC93B (118,119). Direct interaction between these TLRs and UNC93B is a critical step in this process. Disruption in UNC93B function has been attributed to defective innate immune responses (120). We have previously identified a significant degree of negative crosstalk between TLR4 and TLR9 in IECs. Both receptors are reciprocally expressed following exposure of their respective ligands as well as during settings of intestinal injury and inflammation (i.e. high TLR4, low TLR9) (113). Therefore, excessive TLR4 signaling leading to this reciprocal TLR9 expression is correlated to increased ER stress in IECs. To date there is, to our knowledge, no evidence, which suggests excessive TLR4 activation affects UNC93B function or expression in IECs or any other cell type or tissue. However, should

excessive activation of TLR4 lead to dysfunction or decreased expression of UNC93B, or other proteins important for ER-endosomal transport, TLR9 nascent peptide chains may accumulate within the ER stimulating the UPR and ER stress. This would serve not only as a mechanism of TLR4-mediated ER stress but a novel target of TLR4 signaling and TLR crosstalk. Future work examining a role for ER stress will elucidate a previously unrecognized role for disturbances of proteostasis in the newborn intestinal epithelium in NEC. Thus suggesting that mechanisms at work to reestablish this homeostasis, the HSR and UPR, play central part in intestinal injury and repair and at the heart of these pathways, the molecular chaperones of the HSP70 family.

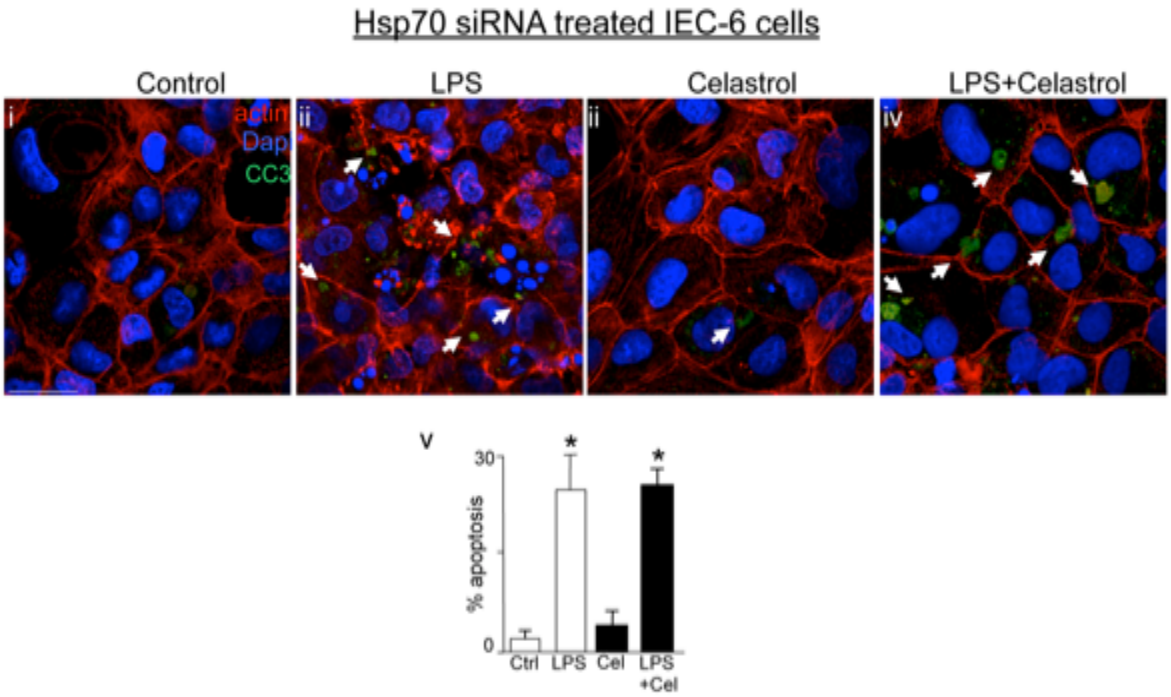
4.0 APPENDIX



Appendix figure 31. Transduced IECs express the V5 marker. (A) Non-transduced IECs. (B) LacZ-V5 construct transduction. (C) K30A CHIP-V5 transduction. (D) H260Q CHIP-V5 transduction.



Appendix Figure 32. HS does not inhibit TLR5-mediated NF-kB induction. *(A)* The TLR5 ligand flagellin leads to NF-kB activation and nuclear translocation in IECs that is (B) not prevented by induction of HSP70 via heat shock, suggesting this inhibition of NF-kB is specific to inhibiting TLR4 derived signals.



Appendix Figure 33. Targeted knockdown of HSP70 via siRNA disrupts Celastrol mediated protection against TLR4-mediated IEC apoptosis. Representative confocal photomicrographs of IEC-6 enterocytes treated as indicated and immunostained for Actin [red] and DAPI [blue] or CC3 [green]. Representative apoptotic cells are indicated by arrows. Scale bar, 10 um. * $p<0.05$

BIBLIOGRAPHY

1. Medzhitov, R., and Janeway, C., Jr. (2000) *The New England journal of medicine* **343**, 338-344
2. Janeway, C. A., Jr. (1999) *Current biology : CB* **9**, R342-345
3. Janeway, C. A., Jr. (1999) *Immunology and cell biology* **77**, 177-179
4. Lee, C. C., Avalos, A. M., and Ploegh, H. L. (2012) *Nature reviews. Immunology* **12**, 168-179
5. Zhang, G., and Ghosh, S. (2001) *The Journal of clinical investigation* **107**, 13-19
6. Medzhitov, R., and Janeway, C. A., Jr. (1998) *Seminars in immunology* **10**, 351-353
7. Dranoff, G. (2004) *Nature reviews. Cancer* **4**, 11-22
8. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996) *Cell* **86**, 973-983
9. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282**, 2085-2088
10. Kumar, H., Kawai, T., and Akira, S. (2009) *The Biochemical journal* **420**, 1-16
11. Matsushima, N., Tanaka, T., Enkhbayar, P., Mikami, T., Taga, M., Yamada, K., and Kuroki, Y. (2007) *BMC genomics* **8**, 124
12. Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O. J., and Lee, J. O. (2007) *Cell* **130**, 906-917
13. Ohto, U., Fukase, K., Miyake, K., and Satow, Y. (2007) *Science* **316**, 1632-1634
14. Jin, M. S., Kim, S. E., Heo, J. Y., Lee, M. E., Kim, H. M., Paik, S. G., Lee, H., and Lee, J. O. (2007) *Cell* **130**, 1071-1082
15. Akira, S., and Takeda, K. (2004) *Nature reviews. Immunology* **4**, 499-511
16. Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000) *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13766-13771

17. O'Neill, L. A., and Bowie, A. G. (2007) *Nature reviews. Immunology* **7**, 353-364
18. Wang, J., Shao, Y., Bennett, T. A., Shankar, R. A., Wightman, P. D., and Reddy, L. G. (2006) *The Journal of biological chemistry* **281**, 37427-37434
19. Pott, J., and Hornef, M. (2012) *EMBO reports* **13**, 684-698
20. Geddes, K., Rubino, S., Streutker, C., Cho, J. H., Magalhaes, J. G., Le Bourhis, L., Selvanantham, T., Girardin, S. E., and Philpott, D. J. (2010) *Infection and immunity* **78**, 5107-5115
21. Geddes, K., Rubino, S. J., Magalhaes, J. G., Streutker, C., Le Bourhis, L., Cho, J. H., Robertson, S. J., Kim, C. J., Kaul, R., Philpott, D. J., and Girardin, S. E. (2011) *Nature medicine* **17**, 837-844
22. Ogura, Y., Lala, S., Xin, W., Smith, E., Dowds, T. A., Chen, F. F., Zimmermann, E., Tretiakova, M., Cho, J. H., Hart, J., Greenson, J. K., Keshav, S., and Nunez, G. (2003) *Gut* **52**, 1591-1597
23. Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R. A. (2005) *Science* **307**, 731-734
24. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11**, 115-122
25. Takeuchi, O., Takeda, K., Hoshino, K., Adachi, O., Ogawa, T., and Akira, S. (2000) *International immunology* **12**, 113-117
26. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. (1998) *Molecular cell* **2**, 253-258
27. Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. (1998) *The Journal of experimental medicine* **187**, 2097-2101
28. Akira, S. (2003) *The Journal of biological chemistry* **278**, 38105-38108
29. Pritchard, J. A. (1965) *Obstetrics and gynecology* **25**, 289-297
30. Mulvihill, S. J., Stone, M. M., Debas, H. T., and Fonkalsrud, E. W. (1985) *Journal of pediatric surgery* **20**, 668-672
31. Michalsky, M. P., Lara-Marquez, M., Chun, L., and Besner, G. E. (2002) *Journal of pediatric surgery* **37**, 1-6
32. Chen, Z. J. (2005) *Nature cell biology* **7**, 758-765
33. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002) *J Immunol* **169**, 6668-6672
34. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003) *Nature immunology* **4**, 161-167

35. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003) *Science* **301**, 640-643
36. Kaiser, W. J., and Offermann, M. K. (2005) *J Immunol* **174**, 4942-4952
37. Hirotani, T., Yamamoto, M., Kumagai, Y., Uematsu, S., Kawase, I., Takeuchi, O., and Akira, S. (2005) *Biochemical and biophysical research communications* **328**, 383-392
38. Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., and Beutler, B. (2003) *Nature* **424**, 743-748
39. Meylan, E., Burns, K., Hofmann, K., Blancheteau, V., Martinon, F., Kelliher, M., and Tschopp, J. (2004) *Nature immunology* **5**, 503-507
40. Karin, M. (1999) *The Journal of biological chemistry* **274**, 27339-27342
41. Siggers, R. H., and Hackam, D. J. (2011) *Cellular and molecular life sciences : CMLS* **68**, 3623-3634
42. Siggers, R. H., Siggers, J., Thymann, T., Boye, M., and Sangild, P. T. (2011) *The Journal of nutritional biochemistry* **22**, 511-521
43. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388**, 548-554
44. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278**, 860-866
45. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87**, 13-20
46. Barnes, P. J., and Karin, M. (1997) *The New England journal of medicine* **336**, 1066-1071
47.
48. Lotze, M. T., Zeh, H. J., Rubartelli, A., Sparvero, L. J., Amoscato, A. A., Washburn, N. R., Devera, M. E., Liang, X., Tor, M., and Billiar, T. (2007) *Immunological reviews* **220**, 60-81
49. Chotirmall, S. H. *CML cystic fibrosis* **1**
50. Ulevitch, R. J., and Tobias, P. S. (1995) *Annual review of immunology* **13**, 437-457
51. Dziarski, R., Tapping, R. I., and Tobias, P. S. (1998) *The Journal of biological chemistry* **273**, 8680-8690
52. Schroder, N. W., Heine, H., Alexander, C., Manukyan, M., Eckert, J., Hamann, L., Gobel, U. B., and Schumann, R. R. (2004) *J Immunol* **173**, 2683-2691
53. Schroder, N. W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zahringer, U., Gobel, U. B., Weber, J. R., and Schumann, R. R. (2003) *The Journal of biological chemistry* **278**, 15587-15594

54. Baumann, C. L., Aspalter, I. M., Sharif, O., Pichlmair, A., Bluml, S., Grebien, F., Bruckner, M., Pasierbek, P., Aumayr, K., Planyavsky, M., Bennett, K. L., Colinge, J., Knapp, S., and Superti-Furga, G. (2010) *The Journal of experimental medicine* **207**, 2689-2701
55. Hailman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M., and Wright, S. D. (1994) *The Journal of experimental medicine* **179**, 269-277
56. Lee, H. K., Dunzendorfer, S., Soldau, K., and Tobias, P. S. (2006) *Immunity* **24**, 153-163
57. Nakata, T., Yasuda, M., Fujita, M., Kataoka, H., Kiura, K., Sano, H., and Shibata, K. (2006) *Cellular microbiology* **8**, 1899-1909
58. Kim, J. I., Lee, C. J., Jin, M. S., Lee, C. H., Paik, S. G., Lee, H., and Lee, J. O. (2005) *The Journal of biological chemistry* **280**, 11347-11351
59. Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., and Beutler, B. (2005) *Nature immunology* **6**, 565-570
60. Zanoni, I., Ostuni, R., Marek, L. R., Barresi, S., Barbalat, R., Barton, G. M., Granucci, F., and Kagan, J. C. (2011) *Cell* **147**, 868-880
61. Akashi-Takamura, S., and Miyake, K. (2008) *Current opinion in immunology* **20**, 420-425
62. da Silva Correia, J., Soldau, K., Christen, U., Tobias, P. S., and Ulevitch, R. J. (2001) *The Journal of biological chemistry* **276**, 21129-21135
63. Gioannini, T. L., Teghanemt, A., Zhang, D., Coussens, N. P., Dockstader, W., Ramaswamy, S., and Weiss, J. P. (2004) *Proceedings of the National Academy of Sciences of the United States of America* **101**, 4186-4191
64. Ishii, K. J., Koyama, S., Nakagawa, A., Coban, C., and Akira, S. (2008) *Cell host & microbe* **3**, 352-363
65. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) *The Journal of experimental medicine* **189**, 1777-1782
66. Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002) *Nature immunology* **3**, 667-672
67. Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., and Lee, J. O. (2009) *Nature* **458**, 1191-1195
68. Kang, J. Y., and Lee, J. O. (2011) *Annual review of biochemistry* **80**, 917-941
69. Guilmeau, S., Flandez, M., Bancroft, L., Sellers, R. S., Tear, B., Stanley, P., and Augenlicht, L. H. (2008) *Gastroenterology* **135**, 849-860, 860 e841-846

70. Sodhi, C. P., Neal, M. D., Siggers, R., Sho, S., Ma, C., Branca, M. F., Prindle, T., Jr., Russo, A. M., Afrazi, A., Good, M., Brower-Sinning, R., Firek, B., Morowitz, M. J., Ozolek, J. A., Gittes, G. K., Billiar, T. R., and Hackam, D. J. (2012) *Gastroenterology*
71. Apuzzio, J., Chan, Y., Al-Khan, A., Illsley, N., Kim, P. L., and Vonhaggen, S. (2004) *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet* **15**, 313-317
72. Sangild, P. T., Mei, J., Fowden, A. L., and Xu, R. J. (2009) *American journal of physiology. Regulatory, integrative and comparative physiology* **296**, R1053-1062
73. Yoshio, H., Tollin, M., Gudmundsson, G. H., Lagercrantz, H., Jornvall, H., Marchini, G., and Agerberth, B. (2003) *Pediatric research* **53**, 211-216
74. Zijlstra, R. T., Odle, J., Hall, W. F., Petschow, B. W., Gelberg, H. B., and Litov, R. E. (1994) *Journal of pediatric gastroenterology and nutrition* **19**, 382-390
75. Petschow, B. W., Carter, D. L., and Hutton, G. D. (1993) *Journal of pediatric gastroenterology and nutrition* **17**, 49-58
76. Hylander, M. A., Strobino, D. M., and Dhanireddy, R. (1998) *Pediatrics* **102**, E38
77. Sangild, P. T., Siggers, R. H., Schmidt, M., Elnif, J., Bjornvad, C. R., Thymann, T., Grondahl, M. L., Hansen, A. K., Jensen, S. K., Boye, M., Moelbak, L., Buddington, R. K., Westrom, B. R., Holst, J. J., and Burrin, D. G. (2006) *Gastroenterology* **130**, 1776-1792
78. Bjornvad, C. R., Schmidt, M., Petersen, Y. M., Jensen, S. K., Offenberg, H., Elnif, J., and Sangild, P. T. (2005) *American journal of physiology. Regulatory, integrative and comparative physiology* **289**, R1212-1222
79. Xanthou, M. (1998) *Biology of the neonate* **74**, 121-133
80. Lotz, M., Gutle, D., Walther, S., Menard, S., Bogdan, C., and Hornef, M. W. (2006) *The Journal of experimental medicine* **203**, 973-984
81. McDole, J. R., Wheeler, L. W., McDonald, K. G., Wang, B., Konjufca, V., Knoop, K. A., Newberry, R. D., and Miller, M. J. (2012) *Nature* **483**, 345-349
82. Strobel, S., and Hourihane, J. O. (2001) *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* **12 Suppl 14**, 43-46
83. Forchielli, M. L., and Walker, W. A. (2005) *The British journal of nutrition* **93 Suppl 1**, S41-48

84. Ran, R., Lu, A., Zhang, L., Tang, Y., Zhu, H., Xu, H., Feng, Y., Han, C., Zhou, G., Rigby, A. C., and Sharp, F. R. (2004) *Genes & development* **18**, 1466-1481
85. Salminen, A., Paimela, T., Suuronen, T., and Kaarniranta, K. (2008) *Immunology letters* **117**, 9-15
86. Bevins, C. L., and Salzman, N. H. (2011) *Nature reviews. Microbiology* **9**, 356-368
87. Vaishnava, S., Behrendt, C. L., Ismail, A. S., Eckmann, L., and Hooper, L. V. (2008) *Proceedings of the National Academy of Sciences of the United States of America* **105**, 20858-20863
88. Vaishnava, S., Behrendt, C. L., and Hooper, L. V. (2008) *Journal of pediatric gastroenterology and nutrition* **46 Suppl 1**, E10-11
89. Weiss, Y. G., Bromberg, Z., Raj, N., Raphael, J., Goloubinoff, P., Ben-Neriah, Y., and Deutschman, C. S. (2007) *Critical care medicine* **35**, 2128-2138
90. Song, M., Pinsky, M. R., and Kellum, J. A. (2008) *Journal of critical care* **23**, 406-415
91. Kajino-Sakamoto, R., Inagaki, M., Lippert, E., Akira, S., Robine, S., Matsumoto, K., Jobin, C., and Ninomiya-Tsuji, J. (2008) *J Immunol* **181**, 1143-1152
92. Gong, J., Xu, J., Zhu, W., Gao, X., Li, N., and Li, J. (2010) *Clin Immunol* **136**, 245-256
93. Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., Huth, M., Nikolaev, A., Neufert, C., Madison, B., Gumucio, D., Neurath, M. F., and Pasparakis, M. (2007) *Nature* **446**, 557-561
94. Steinbrecher, K. A., Harmel-Laws, E., Sitcheran, R., and Baldwin, A. S. (2008) *J Immunol* **180**, 2588-2599
95. Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J., and Madara, J. L. (2001) *J Immunol* **167**, 1882-1885
96. Rhee, S. H., Im, E., Riegler, M., Kokkotou, E., O'Brien, M., and Pothoulakis, C. (2005) *Proceedings of the National Academy of Sciences of the United States of America* **102**, 13610-13615
97. Chabot, S., Wagner, J. S., Farrant, S., and Neutra, M. R. (2006) *J Immunol* **176**, 4275-4283
98. Lee, J., Mo, J. H., Katakura, K., Alkalay, I., Rucker, A. N., Liu, Y. T., Lee, H. K., Shen, C., Cojocaru, G., Shenouda, S., Kagnoff, M., Eckmann, L., Ben-Neriah, Y., and Raz, E. (2006) *Nature cell biology* **8**, 1327-1336
99. Pott, J., Mahlakoiv, T., Mordstein, M., Duerr, C. U., Michiels, T., Stockinger, S., Staeheli, P., and Hornef, M. W. (2011) *Proceedings of the National Academy of Sciences of the United States of America* **108**, 7944-7949
100. Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008) *Nature immunology* **9**, 361-368

101. Hornef, M. W., Normark, B. H., Vandewalle, A., and Normark, S. (2003) *The Journal of experimental medicine* **198**, 1225-1235
102. Ortega-Cava, C. F., Ishihara, S., Rumi, M. A., Kawashima, K., Ishimura, N., Kazumori, H., Udagawa, J., Kadowaki, Y., and Kinoshita, Y. (2003) *J Immunol* **170**, 3977-3985
103. Neal, M. D., Leaphart, C., Levy, R., Prince, J., Billiar, T. R., Watkins, S., Li, J., Cetin, S., Ford, H., Schreiber, A., and Hackam, D. J. (2006) *J Immunol* **176**, 3070-3079
104. Chassin, C., Kocur, M., Pott, J., Duerr, C. U., Gutle, D., Lotz, M., and Hornef, M. W. (2010) *Cell host & microbe* **8**, 358-368
105. Dai, S., Sodhi, C., Cetin, S., Richardson, W., Branca, M., Neal, M. D., Prindle, T., Ma, C., Shapiro, R. A., Li, B., Wang, J. H., and Hackam, D. J. (2010) *The Journal of biological chemistry* **285**, 4995-5002
106. Tang, D., Kang, R., Xiao, W., Wang, H., Calderwood, S. K., and Xiao, X. (2007) *J Immunol* **179**, 1236-1244
107. Fink, S. L., and Cookson, B. T. (2005) *Infection and immunity* **73**, 1907-1916
108. Tang, D., Kang, R., Xiao, W., Jiang, L., Liu, M., Shi, Y., Wang, K., Wang, H., and Xiao, X. (2007) *J Immunol* **178**, 7376-7384
109. Squires, R. C., Muehlbauer, S. M., and Brojatsch, J. (2007) *The Journal of biological chemistry* **282**, 34260-34267
110. Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. (2010) *Nature reviews. Molecular cell biology* **11**, 700-714
111. Levin, T. C., Wickliffe, K. E., Leppla, S. H., and Moayeri, M. (2008) *Cellular microbiology* **10**, 2434-2446
112. Walter, P., and Ron, D. (2011) *Science* **334**, 1081-1086
113. Gribar, S. C., Sodhi, C. P., Richardson, W. M., Anand, R. J., Gittes, G. K., Branca, M. F., Jakub, A., Shi, X. H., Shah, S., Ozolek, J. A., and Hackam, D. J. (2009) *J Immunol* **182**, 636-646
114. Shembade, N., and Harhaj, E. W. (2012) *Cellular & molecular immunology* **9**, 123-130
115. Bogaert, S., De Vos, M., Olievier, K., Peeters, H., Elewaut, D., Lambrecht, B., Pouliot, P., and Laukens, D. (2011) *PloS one* **6**, e25589
116. Kaser, A., Lee, A. H., Franke, A., Glickman, J. N., Zeissig, S., Tilg, H., Nieuwenhuis, E. E., Higgins, D. E., Schreiber, S., Glimcher, L. H., and Blumberg, R. S. (2008) *Cell* **134**, 743-756
117. Good, M., Siggers, R. H., Sodhi, C. P., Afrazi, A., Alkhudari, F., Egan, C. E., Neal, M. D., Yazji, I., Jia, H., Lin, J., Branca, M. F., Ma, C., Prindle, T., Grant, Z., Shah, S., Slagle, D., 2nd, Paredes, J., Ozolek, J., Gittes,

G. K., and Hackam, D. J. (2012) *Proceedings of the National Academy of Sciences of the United States of America* **109**, 11330-11335
118. Tabeta, K., Hoebe, K., Janssen, E. M., Du, X., Georgel, P., Crozat, K., Mudd, S., Mann, N., Sovath, S., Goode, J., Shamel, L., Herskovits, A. A., Portnoy, D. A., Cooke, M., Tarantino, L. M., Wiltshire, T., Steinberg, B. E., Grinstein, S., and Beutler, B. (2006) *Nature immunology* **7**, 156-164
119. Kim, Y. M., Brinkmann, M. M., Paquet, M. E., and Ploegh, H. L. (2008) *Nature* **452**, 234-238
120. Casrouge, A., Zhang, S. Y., Eidenschenk, C., Jouanguy, E., Puel, A., Yang, K., Alcais, A., Picard, C., Mahfoufi, N., Nicolas, N., Lorenzo, L., Plancoulaine, S., Senechal, B., Geissmann, F., Tabeta, K., Hoebe, K., Du, X., Miller, R. L., Heron, B., Mignot, C., de Villemeur, T. B., Lebon, P., Dulac, O., Rozenberg, F., Beutler, B., Tardieu, M., Abel, L., and Casanova, J. L. (2006) *Science* **314**, 308-312
121. Lee, E. G., Boone, D. L., Chai, S., Libby, S. L., Chien, M., Lodolce, J. P., and Ma, A. (2000) *Science* **289**, 2350-2354
122. Revel, J. P., and Karnovsky, M. J. (1967) *The Journal of cell biology* **33**, C7-C12
123. Karnovsky, M. L., Graham, R., Karnovsky, M. J., Saito, K., Shafer, A. W., and Glass, E. (1967) *Protoplasma* **63**, 88-89
124. Young, J. D., Cohn, Z. A., and Gilula, N. B. (1987) *Cell* **48**, 733-743
125. Lampe, P. D., and Lau, A. F. (2004) *The international journal of biochemistry & cell biology* **36**, 1171-1186
126. Todd, K. L., Kristan, W. B., Jr., and French, K. A. (2010) *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**, 15277-15285
127. Dolowschiak, T., Chassin, C., Ben Mkaddem, S., Fuchs, T. M., Weiss, S., Vandewalle, A., and Hornef, M. W. (2010) *PLoS pathogens* **6**, e1001194
128. Kasper, C. A., Sorg, I., Schmutz, C., Tschon, T., Wischnewski, H., Kim, M. L., and Arrieumerlou, C. (2010) *Immunity* **33**, 804-816
129. Ey, B., Eyking, A., Gerken, G., Podolsky, D. K., and Cario, E. (2009) *The Journal of biological chemistry* **284**, 22332-22343
130. Ramirez-Carrozzi, V., Sambandam, A., Luis, E., Lin, Z., Jeet, S., Lesch, J., Hackney, J., Kim, J., Zhou, M., Lai, J., Modrusan, Z., Sai, T., Lee, W., Xu, M., Caplazi, P., Diehl, L., de Voss, J., Balazs, M., Gonzalez, L., Jr., Singh, H., Ouyang, W., and Pappu, R. (2011) *Nature immunology* **12**, 1159-1166

131. Song, X., Zhu, S., Shi, P., Liu, Y., Shi, Y., Levin, S. D., and Qian, Y. (2011) *Nature immunology* **12**, 1151-1158
132. Cario, E., Gerken, G., and Podolsky, D. K. (2004) *Gastroenterology* **127**, 224-238
133. Fukata, M., Michelsen, K. S., Eri, R., Thomas, L. S., Hu, B., Lukasek, K., Nast, C. C., Lechago, J., Xu, R., Naiki, Y., Soliman, A., Arditi, M., and Abreu, M. T. (2005) *American journal of physiology. Gastrointestinal and liver physiology* **288**, G1055-1065
134. Santaolalla, R., and Abreu, M. T. (2012) *Current opinion in gastroenterology* **28**, 124-129
135. Siggers JL, S. R., Skovgaard K, Schmidt M, Moeller HK, Boye M, Sangild PT. (2008) *Gastroenterology* **134**, A259
136. Shiou, S. R., Yu, Y., Chen, S., Ciancio, M. J., Petrof, E. O., Sun, J., and Claud, E. C. (2011) *The Journal of biological chemistry* **286**, 12123-12132
137. Wynn, J., Cornell, T. T., Wong, H. R., Shanley, T. P., and Wheeler, D. S. (2010) *Pediatrics* **125**, 1031-1041
138. Adkins, B., Leclerc, C., and Marshall-Clarke, S. (2004) *Nature reviews. Immunology* **4**, 553-564
139. Levy, O. (2007) *Nature reviews. Immunology* **7**, 379-390
140. Luig, M., and Lui, K. (2005) *Journal of paediatrics and child health* **41**, 169-173
141. Gagliardi, L., Bellu, R., Cardilli, V., and De Curtis, M. (2008) *Journal of pediatric gastroenterology and nutrition* **47**, 206-210
142. Luig, M., and Lui, K. (2005) *Journal of paediatrics and child health* **41**, 174-179
143. Mizrahi, A., Barlow, O., Berdon, W., Blanc, W. A., and Silverman, W. A. (1965) *The Journal of pediatrics* **66**, 697-705
144. Grave, G. D., Nelson, S. A., Walker, W. A., Moss, R. L., Dvorak, B., Hamilton, F. A., Higgins, R., and Raju, T. N. (2007) *Pediatric research* **62**, 510-514
145. Blakely, M. L., Lally, K. P., McDonald, S., Brown, R. L., Barnhart, D. C., Ricketts, R. R., Thompson, W. R., Scherer, L. R., Klein, M. D., Letton, R. W., Chwals, W. J., Touloukian, R. J., Kurkchubasche, A. G., Skinner, M. A., Moss, R. L., and Hilfiker, M. L. (2005) *Annals of surgery* **241**, 984-989; discussion 989-994
146. CHLA.
147. Hotta, T., Yoshida, N., Yoshikawa, T., Sugino, S., and Kondo, M. (1986) *Research in experimental medicine. Zeitschrift fur die gesamte experimentelle Medizin einschliesslich experimenteller Chirurgie* **186**, 61-69
148. Feng, J., El-Assal, O. N., and Besner, G. E. (2005) *Seminars in pediatric surgery* **14**, 167-174

149. Feng, J., and Besner, G. E. (2007) *Journal of pediatric surgery* **42**, 214-220
150. Kruis, W., Schussler, P., Weinzierl, M., Galanos, C., and Eisenburg, J. (1984) *Digestive diseases and sciences* **29**, 502-507
151. Caradonna, L., Amati, L., Lella, P., Jirillo, E., and Caccavo, D. (2000) *The American journal of gastroenterology* **95**, 1495-1502
152. Noerr, B. (2003) *Advances in neonatal care : official journal of the National Association of Neonatal Nurses* **3**, 107-120
153. Sharma, R., Tepas, J. J., 3rd, Hudak, M. L., Mollitt, D. L., Wludyka, P. S., Teng, R. J., and Premachandra, B. R. (2007) *Journal of pediatric surgery* **42**, 454-461
154. Duffy, L. C., Zielezny, M. A., Carrion, V., Griffiths, E., Dryja, D., Hilty, M., Rook, C., and Morin, F., 3rd. (1997) *Digestive diseases and sciences* **42**, 359-365
155. Leaphart, C. L., Cavallo, J., Gribar, S. C., Cetin, S., Li, J., Branca, M. F., Dubowski, T. D., Sodhi, C. P., and Hackam, D. J. (2007) *J Immunol* **179**, 4808-4820
156. Jilling, T., Simon, D., Lu, J., Meng, F. J., Li, D., Schy, R., Thomson, R. B., Soliman, A., Arditi, M., and Caplan, M. S. (2006) *J Immunol* **177**, 3273-3282
157. Chan, K. L., Wong, K. F., and Luk, J. M. (2009) *World journal of gastroenterology : WJG* **15**, 4745-4752
158. Richardson, W. M., Sodhi, C. P., Russo, A., Siggers, R. H., Afrazi, A., Gribar, S. C., Neal, M. D., Dai, S., Prindle, T., Jr., Branca, M., Ma, C., Ozolek, J., and Hackam, D. J. (2010) *Gastroenterology* **139**, 904-917, 917 e901-906
159. Sodhi, C. P., Shi, X. H., Richardson, W. M., Grant, Z. S., Shapiro, R. A., Prindle, T., Jr., Branca, M., Russo, A., Gribar, S. C., Ma, C., and Hackam, D. J. (2010) *Gastroenterology* **138**, 185-196
160. Qureshi, F. G., Leaphart, C., Cetin, S., Li, J., Grishin, A., Watkins, S., Ford, H. R., and Hackam, D. J. (2005) *Gastroenterology* **128**, 1012-1022
161. Liu, Y., Zhu, L., Fatheree, N. Y., Liu, X., Pacheco, S. E., Tatevian, N., and Rhoads, J. M. (2009) *American journal of physiology. Gastrointestinal and liver physiology* **297**, G442-450
162. Lu, J., Jilling, T., Li, D., and Caplan, M. S. (2007) *Pediatric research* **61**, 427-432
163. Wolfs, T. G., Derikx, J. P., Hodin, C. M., Vanderlocht, J., Driessen, A., de Bruine, A. P., Bevins, C. L., Lasitschka, F., Gassler, N., van Gemert, W. G., and Buurman, W. A. (2010) *Inflammatory bowel diseases* **16**, 68-75
164. Szabo, C. (1995) *New Horiz* **3**, 2-32
165. Salzman, A. L. (1995) *New Horiz* **3**, 352-364

166. Lewis, K., Caldwell, J., Phan, V., Prescott, D., Nazli, A., Wang, A., Soderholm, J. D., Perdue, M. H., Sherman, P. M., and McKay, D. M. (2008) *American journal of physiology. Gastrointestinal and liver physiology* **294**, G669-678
167. Mishima, S., Xu, D., and Deitch, E. A. (1999) *Critical care medicine* **27**, 880-886
168. Mishima, S., Xu, D., Lu, Q., and Deitch, E. A. (1998) *The Journal of trauma* **44**, 175-182
169. Mishima, S., Xu, D., Lu, Q., and Deitch, E. A. (1997) *Arch Surg* **132**, 1190-1195
170. Tepperman, B. L., Brown, J. F., and Whittle, B. J. (1993) *The American journal of physiology* **265**, G214-218
171. Forsythe, R. M., Xu, D. Z., Lu, Q., and Deitch, E. A. (2002) *Shock* **17**, 180-184
172. Salzman, A. L., Menconi, M. J., Unno, N., Ezzell, R. M., Casey, D. M., Gonzalez, P. K., and Fink, M. P. (1995) *The American journal of physiology* **268**, G361-373
173. Inaba, T., Alexander, J. W., Ogle, J. D., and Ogle, C. K. (1999) *Shock* **11**, 276-282
174. Sheth, P., Delos Santos, N., Seth, A., LaRusso, N. F., and Rao, R. K. (2007) *American journal of physiology. Gastrointestinal and liver physiology* **293**, G308-318
175. Chin, A. C., Flynn, A. N., Fedwick, J. P., and Buret, A. G. (2006) *Canadian journal of physiology and pharmacology* **84**, 1043-1050
176. Neal, M. D., Richardson, W. M., Sodhi, C. P., Russo, A., and Hackam, D. J. (2011) *The Journal of surgical research* **167**, 1-8
177. Cetin, S., Ford, H. R., Sysko, L. R., Agarwal, C., Wang, J., Neal, M. D., Baty, C., Apodaca, G., and Hackam, D. J. (2004) *The Journal of biological chemistry* **279**, 24592-24600
178. Cetin, S., Leaphart, C. L., Li, J., Ischenko, I., Hayman, M., Upperman, J., Zamora, R., Watkins, S., Ford, H. R., Wang, J., and Hackam, D. J. (2007) *American journal of physiology. Gastrointestinal and liver physiology* **292**, G1347-1358
179. Kepp, O., Galluzzi, L., Lipinski, M., Yuan, J., and Kroemer, G. (2011) *Nature reviews. Drug discovery* **10**, 221-237
180. Taylor, R. C., Cullen, S. P., and Martin, S. J. (2008) *Nature reviews. Molecular cell biology* **9**, 231-241
181. Edelblum, K. L., Yan, F., Yamaoka, T., and Polk, D. B. (2006) *Inflammatory bowel diseases* **12**, 413-424
182. Farmer, E.
183. Barnhart, B. C., Alappat, E. C., and Peter, M. E. (2003) *Seminars in immunology* **15**, 185-193
184. Hausmann, M. (2010) *International journal of inflammation* **2010**, 574568

185. Shen, W. H., Wang, J., Wu, J., Zhurkin, V. B., and Yin, Y. (2006) *Cancer research* **66**, 6033-6039
186. Micheau, O., and Tschopp, J. (2003) *Cell* **114**, 181-190
187. Salaun, B., Romero, P., and Lebecque, S. (2007) *European journal of immunology* **37**, 3311-3318
188. Scaffidi, C., Kirchhoff, S., Krammer, P. H., and Peter, M. E. (1999) *Current opinion in immunology* **11**, 277-285
189. Hengartner, M. O. (2000) *Nature* **407**, 770-776
190. Oh, K. J., Singh, P., Lee, K., Foss, K., Lee, S., Park, M., Lee, S., Aluvila, S., Park, M., Singh, P., Kim, R. S., Symersky, J., and Walters, D. E. (2010) *The Journal of biological chemistry* **285**, 28924-28937
191. Owens, T. W., Valentijn, A. J., Upton, J. P., Keeble, J., Zhang, L., Lindsay, J., Zouq, N. K., and Gilmore, A. P. (2009) *Cell death and differentiation* **16**, 1551-1562
192. Krammer, P. H. (2000) *Nature* **407**, 789-795
193. Lieberman, J. (2003) *Nature reviews. Immunology* **3**, 361-370
194. Youle, R. J., and Strasser, A. (2008) *Nature reviews. Molecular cell biology* **9**, 47-59
195. Khaled, A. R., Reynolds, D. A., Young, H. A., Thompson, C. B., Muegge, K., and Durum, S. K. (2001) *The Journal of biological chemistry* **276**, 6453-6462
196. Pagliari, L. J., Kuwana, T., Bonzon, C., Newmeyer, D. D., Tu, S., Beere, H. M., and Green, D. R. (2005) *Proceedings of the National Academy of Sciences of the United States of America* **102**, 17975-17980
197. Chipuk, J. E., Moldoveanu, T., Llambi, F., Parsons, M. J., and Green, D. R. (2010) *Molecular cell* **37**, 299-310
198. Degterev, A., and Yuan, J. (2008) *Nature reviews. Molecular cell biology* **9**, 378-390
199. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R., and Newmeyer, D. D. (2002) *Cell* **111**, 331-342
200. Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. (2000) *Genes & development* **14**, 2060-2071
201. Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., Colman, P. M., Day, C. L., Adams, J. M., and Huang, D. C. (2005) *Molecular cell* **17**, 393-403
202. Zaph, C., Troy, A. E., Taylor, B. C., Berman-Booty, L. D., Guild, K. J., Du, Y., Yost, E. A., Gruber, A. D., May, M. J., Greten, F. R., Eckmann, L., Karin, M., and Artis, D. (2007) *Nature* **446**, 552-556
203. Chen, L. W., Egan, L., Li, Z. W., Greten, F. R., Kagnoff, M. F., and Karin, M. (2003) *Nature medicine* **9**, 575-581
204. Fukata, M., Chen, A., Klepper, A., Krishnareddy, S., Vamadevan, A. S., Thomas, L. S., Xu, R., Inoue, H., Arditi,

M., Dannenberg, A. J., and Abreu, M. T. (2006) *Gastroenterology* **131**, 862-877
205. Di Lorenzo, M., and Krantis, A. (2001) *Journal of pediatric surgery* **36**, 700-705
206. Nadler, E. P., Dickinson, E., Knisely, A., Zhang, X. R., Boyle, P., Beer-Stolz, D., Watkins, S. C., and Ford, H. R. (2000) *The Journal of surgical research* **92**, 71-77
207. Denicola, A., Souza, J. M., and Radi, R. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 3566-3571
208. Beckman, J. S. (1990) *Nature* **345**, 27-28
209. Beckman, J. S., and Koppenol, W. H. (1996) *The American journal of physiology* **271**, C1424-1437
210. Chokshi, N. K., Guner, Y. S., Hunter, C. J., Upperman, J. S., Grishin, A., and Ford, H. R. (2008) *Seminars in perinatology* **32**, 92-99
211. Schopfer, F. J., Baker, P. R., and Freeman, B. A. (2003) *Trends in biochemical sciences* **28**, 646-654
212. Chen, K., Inoue, M., and Okada, A. (1996) *Biochemical and biophysical research communications* **224**, 703-708
213. Hoffman, R. A., Zhang, G., Nussler, N. C., Gleixner, S. L., Ford, H. R., Simmons, R. L., and Watkins, S. C. (1997) *The American journal of physiology* **272**, G383-392
214. Ford, H., Watkins, S., Reblock, K., and Rowe, M. (1997) *Journal of pediatric surgery* **32**, 275-282
215. Potoka, D. A., Nadler, E. P., Zhou, X., Zhang, X. R., Upperman, J. S., and Ford, H. R. (2000) *Shock* **14**, 366-373
216. Neutra, M. R., Mantis, N. J., and Kraehenbuhl, J. P. (2001) *Nature immunology* **2**, 1004-1009
217. Caplan, M. S., and Hsueh, W. (1990) *The Journal of pediatrics* **117**, S47-51
218. Caplan, M. S., Sun, X. M., Hseuh, W., and Hageman, J. R. (1990) *The Journal of pediatrics* **116**, 960-964
219. Afrazi, A., Sodhi, C. P., Richardson, W., Neal, M., Good, M., Siggers, R., and Hackam, D. J. (2011) *Pediatric research* **69**, 183-188
220. Wang, J., Ouyang, Y., Guner, Y., Ford, H. R., and Grishin, A. V. (2009) *J Immunol* **183**, 1384-1392
221. Lin, H. C., Su, B. H., Chen, A. C., Lin, T. W., Tsai, C. H., Yeh, T. F., and Oh, W. (2005) *Pediatrics* **115**, 1-4
222. Borzutzky, A., Fried, A., Chou, J., Bonilla, F. A., Kim, S., and Dedeoglu, F. (2010) *Clin Immunol* **134**, 251-261
223. Anfinsen, C. B. (1973) *Science* **181**, 223-230
224. Vabulas, R. M., Raychaudhuri, S., Hayer-Hartl, M., and Hartl, F. U. (2010) *Cold Spring Harbor perspectives in biology* **2**, a004390
225. Brockwell, D. J., and Radford, S. E. (2007) *Current opinion in structural biology* **17**, 30-37

226. Bartlett, A. I., and Radford, S. E. (2009) *Nature structural & molecular biology* **16**, 582-588
227. Haass, C., and Selkoe, D. J. (2007) *Nature reviews. Molecular cell biology* **8**, 101-112
228. Hartl, F. U. (1996) *Nature* **381**, 571-579
229. Hartl, F. U., and Hayer-Hartl, M. (2009) *Nature structural & molecular biology* **16**, 574-581
230. De Maio, A. (1999) *Shock* **11**, 1-12
231. Ritossa, F. A. (1962) *Experimentia* **18**, 571-773
232. Lanneau, D., Brunet, M., Frisan, E., Solary, E., Fontenay, M., and Garrido, C. (2008) *Journal of cellular and molecular medicine* **12**, 743-761
233. Morimoto, R. I. (2011) *Cold Spring Harbor symposia on quantitative biology* **76**, 91-99
234. Morimoto, R. I. (2008) *Genes & development* **22**, 1427-1438
235. Schmitt, E., Parcellier, A., Gurbuxani, S., Cande, C., Hammann, A., Morales, M. C., Hunt, C. R., Dix, D. J., Kroemer, R. T., Giordanetto, F., Jaattela, M., Penninger, J. M., Pance, A., Kroemer, G., and Garrido, C. (2003) *Cancer research* **63**, 8233-8240
236. Parcellier, A., Gurbuxani, S., Schmitt, E., Solary, E., and Garrido, C. (2003) *Biochemical and biophysical research communications* **304**, 505-512
237. Garrido, C. (2002) *Cell death and differentiation* **9**, 483-485
238. Ellis, R. J. (1999) *Current biology : CB* **9**, R137-139
239. Khan, I. U., Wallin, R., Gupta, R. S., and Kammer, G. M. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 10425-10430
240. Vargas-Parada, L., Solis, C. F., and Laclette, J. P. (2001) *Parasitology* **122**, 583-588
241. Bukau, B., and Horwich, A. L. (1998) *Cell* **92**, 351-366
242. Sreedhar, A. S., Kalmar, E., Csermely, P., and Shen, Y. F. (2004) *FEBS letters* **562**, 11-15
243. Nathan, D. F., and Lindquist, S. (1995) *Molecular and cellular biology* **15**, 3917-3925
244. Shaknovich, R., Shue, G., and Kohtz, D. S. (1992) *Molecular and cellular biology* **12**, 5059-5068
245. Hartson, S. D., and Matts, R. L. (1994) *Biochemistry* **33**, 8912-8920
246. Wartmann, M., and Davis, R. J. (1994) *The Journal of biological chemistry* **269**, 6695-6701
247. Jaattela, M., Wissing, D., Kokholm, K., Kallunki, T., and Egeblad, M. (1998) *The EMBO journal* **17**, 6124-6134
248. Shi, Y., and Thomas, J. O. (1992) *Molecular and cellular biology* **12**, 2186-2192
249. Hartl, F. U., and Hayer-Hartl, M. (2002) *Science* **295**, 1852-1858

250. Buchner, J. (1999) *Trends in biochemical sciences* **24**, 136-141
251. Young, J. C., Moarefi, I., and Hartl, F. U. (2001) *The Journal of cell biology* **154**, 267-273
252. Beatrix, B., Sakai, H., and Wiedmann, M. (2000) *The Journal of biological chemistry* **275**, 37838-37845
253. Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. (1999) *Nature* **400**, 693-696
254. Teter, S. A., Houry, W. A., Ang, D., Tradler, T., Rockabrand, D., Fischer, G., Blum, P., Georgopoulos, C., and Hartl, F. U. (1999) *Cell* **97**, 755-765
255. Thulasiraman, V., Yang, C. F., and Frydman, J. (1999) *The EMBO journal* **18**, 85-95
256. Siegers, K., Waldmann, T., Leroux, M. R., Grein, K., Shevchenko, A., Schiebel, E., and Hartl, F. U. (1999) *The EMBO journal* **18**, 75-84
257. Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., and Hendrickson, W. A. (1996) *Science* **272**, 1606-1614
258. Rudiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997) *The EMBO journal* **16**, 1501-1507
259. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992) *Nature* **356**, 683-689
260. Rudiger, S., Schneider-Mergener, J., and Bukau, B. (2001) *The EMBO journal* **20**, 1042-1050
261. Sha, B., Lee, S., and Cyr, D. M. (2000) *Structure* **8**, 799-807
262. Misselwitz, B., Staeck, O., and Rapoport, T. A. (1998) *Molecular cell* **2**, 593-603
263. Hohfeld, J., and Jentsch, S. (1997) *The EMBO journal* **16**, 6209-6216
264. Szabo, A., Langer, T., Schroder, H., Flanagan, J., Bukau, B., and Hartl, F. U. (1994) *Proceedings of the National Academy of Sciences of the United States of America* **91**, 10345-10349
265. Herbst, R., Gast, K., and Seckler, R. (1998) *Biochemistry* **37**, 6586-6597
266. Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) *Cell* **101**, 199-210
267. Ballinger, C. A., Connell, P., Wu, Y., Hu, Z., Thompson, L. J., Yin, L. Y., and Patterson, C. (1999) *Molecular and cellular biology* **19**, 4535-4545
268. Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001) *Current biology : CB* **11**, 1569-1577
269. Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Hohfeld, J., and Patterson, C. (2001) *Nature cell biology* **3**, 93-96

270. Luders, J., Demand, J., and Hohfeld, J. (2000) *The Journal of biological chemistry* **275**, 4613-4617
271. Hershko, A., and Ciechanover, A. (1998) *Annual review of biochemistry* **67**, 425-479
272. Bernassola, F., Karin, M., Ciechanover, A., and Melino, G. (2008) *Cancer cell* **14**, 10-21
273. Saeki, Y., and Tanaka, K. (2012) *Methods Mol Biol* **832**, 315-337
274. Wilk, S., and Orlowski, M. (1983) *Journal of neurochemistry* **40**, 842-849
275. Brito, D. A., and Rieder, C. L. (2006) *Current biology : CB* **16**, 1194-1200
276. Haas, A. L., Baboshina, O., Williams, B., and Schwartz, L. M. (1995) *The Journal of biological chemistry* **270**, 9407-9412
277. Schwartz, L. M., Myer, A., Kosz, L., Engelstein, M., and Maier, C. (1990) *Neuron* **5**, 411-419
278. Low, P., Bussell, K., Dawson, S. P., Billett, M. A., Mayer, R. J., and Reynolds, S. E. (1997) *FEBS letters* **400**, 345-349
279. Mallery, D. L., McEwan, W. A., Bidgood, S. R., Towers, G. J., Johnson, C. M., and James, L. C. (2010) *Proceedings of the National Academy of Sciences of the United States of America* **107**, 19985-19990
280. Wang, J., and Maldonado, M. A. (2006) *Cellular & molecular immunology* **3**, 255-261
281. Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y., and Tanaka, K. (2007) *Science* **316**, 1349-1353
282. Vembar, S. S., and Brodsky, J. L. (2008) *Nature reviews. Molecular cell biology* **9**, 944-957
283. Ikeda, F., and Dikic, I. (2008) *EMBO reports* **9**, 536-542
284. Tokunaga, F., Sakata, S., Saeki, Y., Satomi, Y., Kirisako, T., Kamei, K., Nakagawa, T., Kato, M., Murata, S., Yamaoka, S., Yamamoto, M., Akira, S., Takao, T., Tanaka, K., and Iwai, K. (2009) *Nature cell biology* **11**, 123-132
285. Iwai, K., and Tokunaga, F. (2009) *EMBO reports* **10**, 706-713
286. Ikeda, F., Deribe, Y. L., Skanland, S. S., Stieglitz, B., Grabbe, C., Franz-Wachtel, M., van Wijk, S. J., Goswami, P., Nagy, V., Terzic, J., Tokunaga, F., Androulidaki, A., Nakagawa, T., Pasparakis, M., Iwai, K., Sundberg, J. P., Schaefer, L., Rittinger, K., Macek, B., and Dikic, I. (2011) *Nature* **471**, 637-641
287. Seymour, R. E., Hasham, M. G., Cox, G. A., Shultz, L. D., Hogenesch, H., Roopenian, D. C., and Sundberg, J. P. (2007) *Genes and immunity* **8**, 416-421
288. Ron, D., and Walter, P. (2007) *Nature reviews. Molecular cell biology* **8**, 519-529

289. Akerfelt, M., Morimoto, R. I., and Sistonen, L. (2010) *Nature reviews. Molecular cell biology* **11**, 545-555
290. Parker, C. S., and Topol, J. (1984) *Cell* **37**, 273-283
291. Wu, C. (1984) *Nature* **311**, 81-84
292. McMillan, D. R., Xiao, X., Shao, L., Graves, K., and Benjamin, I. J. (1998) *The Journal of biological chemistry* **273**, 7523-7528
293. Xiao, X., Zuo, X., Davis, A. A., McMillan, D. R., Curry, B. B., Richardson, J. A., and Benjamin, I. J. (1999) *The EMBO journal* **18**, 5943-5952
294. Pirkkala, L., Alastalo, T. P., Zuo, X., Benjamin, I. J., and Sistonen, L. (2000) *Molecular and cellular biology* **20**, 2670-2675
295. Zhang, Y., Huang, L., Zhang, J., Moskophidis, D., and Mivechi, N. F. (2002) *Journal of cellular biochemistry* **86**, 376-393
296. Fiorenza, M. T., Farkas, T., Dissing, M., Kolding, D., and Zimarino, V. (1995) *Nucleic acids research* **23**, 467-474
297. Goodson, M. L., and Sarge, K. D. (1995) *The Journal of biological chemistry* **270**, 2447-2450
298. Larson, J. S., Schuetz, T. J., and Kingston, R. E. (1995) *Biochemistry* **34**, 1902-1911
299. Zhong, M., Orosz, A., and Wu, C. (1998) *Molecular cell* **2**, 101-108
300. Baler, R., Dahl, G., and Voellmy, R. (1993) *Molecular and cellular biology* **13**, 2486-2496
301. Ali, A., Bharadwaj, S., O'Carroll, R., and Ovsenek, N. (1998) *Molecular and cellular biology* **18**, 4949-4960
302. Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998) *Cell* **94**, 471-480
303. Shi, Y., Mosser, D. D., and Morimoto, R. I. (1998) *Genes & development* **12**, 654-666
304. Wu, C. (1995) *Annual review of cell and developmental biology* **11**, 441-469
305. Sorger, P. K., and Nelson, H. C. (1989) *Cell* **59**, 807-813
306. Chen, Y., Barlev, N. A., Westergaard, O., and Jakobsen, B. K. (1993) *The EMBO journal* **12**, 5007-5018
307. Rabindran, S. K., Haroun, R. I., Clos, J., Wisniewski, J., and Wu, C. (1993) *Science* **259**, 230-234
308. Nakai, A., Tanabe, M., Kawazoe, Y., Inazawa, J., Morimoto, R. I., and Nagata, K. (1997) *Molecular and cellular biology* **17**, 469-481
309. Pratt, W. B., and Toft, D. O. (1997) *Endocrine reviews* **18**, 306-360
310. Duina, A. A., Kalton, H. M., and Gaber, R. F. (1998) *The Journal of biological chemistry* **273**, 18974-18978
311. Zou, J., Salminen, W. F., Roberts, S. M., and Voellmy, R. (1998) *Cell stress & chaperones* **3**, 130-141

312. Bharadwaj, S., Ali, A., and Ovsenek, N. (1999) *Molecular and cellular biology* **19**, 8033-8041
313. Holmberg, C. I., Hietakangas, V., Mikhailov, A., Rantanen, J. O., Kallio, M., Meinander, A., Hellman, J., Morrice, N., MacKintosh, C., Morimoto, R. I., Eriksson, J. E., and Sistonen, L. (2001) *The EMBO journal* **20**, 3800-3810
314. Guettouche, T., Boellmann, F., Lane, W. S., and Voellmy, R. (2005) *BMC biochemistry* **6**, 4
315. Hietakangas, V., Ahlskog, J. K., Jakobsson, A. M., Hellesuo, M., Sahlberg, N. M., Holmberg, C. I., Mikhailov, A., Palvimo, J. J., Pirkkala, L., and Sistonen, L. (2003) *Molecular and cellular biology* **23**, 2953-2968
316. Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., and Sistonen, L. (2006) *Proceedings of the National Academy of Sciences of the United States of America* **103**, 45-50
317. Whitesell, L., and Lindquist, S. (2009) *Expert opinion on therapeutic targets* **13**, 469-478
318. Bagatell, R., and Whitesell, L. (2004) *Molecular cancer therapeutics* **3**, 1021-1030
319. Kim, H. R., Kang, H. S., and Kim, H. D. (1999) *IUBMB life* **48**, 429-433
320. Westerheide, S. D., Bosman, J. D., Mbadugha, B. N., Kawahara, T. L., Matsumoto, G., Kim, S., Gu, W., Devlin, J. P., Silverman, R. B., and Morimoto, R. I. (2004) *The Journal of biological chemistry* **279**, 56053-56060
321. Trott, A., West, J. D., Klaic, L., Westerheide, S. D., Silverman, R. B., Morimoto, R. I., and Morano, K. A. (2008) *Molecular biology of the cell* **19**, 1104-1112
322. Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., and Kroemer, G. (2006) *Cell Cycle* **5**, 2592-2601
323. Garrido, C., Bruey, J. M., Fromentin, A., Hammann, A., Arrigo, A. P., and Solary, E. (1999) *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13**, 2061-2070
324. Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I., and Massie, B. (2000) *Molecular and cellular biology* **20**, 7146-7159
325. Mosser, D. D., and Morimoto, R. I. (2004) *Oncogene* **23**, 2907-2918
326. Kamada, M., So, A., Muramaki, M., Rocchi, P., Beraldi, E., and Gleave, M. (2007) *Molecular cancer therapeutics* **6**, 299-308
327. Choi, D. H., Ha, J. S., Lee, W. H., Song, J. K., Kim, G. Y., Park, J. H., Cha, H. J., Lee, B. J., and Park, J. W. (2007) *FEBS letters* **581**, 1649-1656

328. Aghdassi, A., Phillips, P., Dudeja, V., Dhaulakhandi, D., Sharif, R., Dawra, R., Lerch, M. M., and Saluja, A. (2007) *Cancer research* **67**, 616-625
329. Compton, S. A., Elmore, L. W., Haydu, K., Jackson-Cook, C. K., and Holt, S. E. (2006) *Molecular and cellular biology* **26**, 1452-1462
330. Nylandsted, J., Rohde, M., Brand, K., Bastholm, L., Elling, F., and Jaattela, M. (2000) *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7871-7876
331. Gurbuxani, S., Bruey, J. M., Fromentin, A., Larmonier, N., Parcellier, A., Jaattela, M., Martin, F., Solary, E., and Garrido, C. (2001) *Oncogene* **20**, 7478-7485
332. Biggs, W. H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W. K., and Arden, K. C. (1999) *Proceedings of the National Academy of Sciences of the United States of America* **96**, 7421-7426
333. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* **401**, 82-85
334. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91**, 231-241
335. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282**, 1318-1321
336. Rane, M. J., Pan, Y., Singh, S., Powell, D. W., Wu, R., Cummins, T., Chen, Q., McLeish, K. R., and Klein, J. B. (2003) *The Journal of biological chemistry* **278**, 27828-27835
337. Gao, T., and Newton, A. C. (2002) *The Journal of biological chemistry* **277**, 31585-31592
338. Paul, C., Manero, F., Gonin, S., Kretz-Remy, C., Virot, S., and Arrigo, A. P. (2002) *Molecular and cellular biology* **22**, 816-834
339. Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantome, A., Plenchette, S., Khochbin, S., Solary, E., and Garrido, C. (2003) *Molecular and cellular biology* **23**, 5790-5802
340. Parcellier, A., Brunet, M., Schmitt, E., Col, E., Didelot, C., Hammann, A., Nakayama, K., Nakayama, K. I., Khochbin, S., Solary, E., and Garrido, C. (2006) *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20**, 1179-1181
341. Park, H. S., Cho, S. G., Kim, C. K., Hwang, H. S., Noh, K. T., Kim, M. S., Huh, S. H., Kim, M. J., Ryoo, K., Kim, E. K., Kang, W. J., Lee, J. S., Seo, J. S., Ko, Y. G., Kim, S., and Choi, E. J. (2002) *Molecular and cellular biology* **22**, 7721-7730
342. Park, H. S., Lee, J. S., Huh, S. H., Seo, J. S., and Choi, E. J. (2001) *The EMBO journal* **20**, 446-456

343. Meriin, A. B., Yaglom, J. A., Gabai, V. L., Zon, L., Ganiatsas, S., Mosser, D. D., Zon, L., and Sherman, M. Y. (1999) *Molecular and cellular biology* **19**, 2547-2555
344. Lee, J. S., Lee, J. J., and Seo, J. S. (2005) *The Journal of biological chemistry* **280**, 6634-6641
345. Gabai, V. L., Yaglom, J. A., Volloch, V., Meriin, A. B., Force, T., Koutroumanis, M., Massie, B., Mosser, D. D., and Sherman, M. Y. (2000) *Molecular and cellular biology* **20**, 6826-6836
346. Akakura, S., Yoshida, M., Yoneda, Y., and Horinouchi, S. (2001) *The Journal of biological chemistry* **276**, 14649-14657
347. Zylicz, M., King, F. W., and Wawrzynow, A. (2001) *The EMBO journal* **20**, 4634-4638
348. Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Bare, O., Auron, P. E., Stevenson, M. A., and Calderwood, S. K. (2002) *The Journal of biological chemistry* **277**, 15028-15034
349. Mijatovic, T., Mathieu, V., Gaussin, J. F., De Neve, N., Ribaucour, F., Van Quaquebeke, E., Dumont, P., Darro, F., and Kiss, R. (2006) *Neoplasia* **8**, 402-412
350. Shanley, T. P., Ryan, M. A., Eaves-Pyles, T., and Wong, H. R. (2000) *Shock* **14**, 447-450
351. Yoo, C. G., Lee, S., Lee, C. T., Kim, Y. W., Han, S. K., and Shim, Y. S. (2000) *J Immunol* **164**, 5416-5423
352. Lin, K., Rockliffe, N., Johnson, G. G., Sherrington, P. D., and Pettitt, A. R. (2008) *Oncogene* **27**, 2445-2455
353. Zhang, H., and Burrows, F. (2004) *J Mol Med (Berl)* **82**, 488-499
354. Chen, G., Cao, P., and Goeddel, D. V. (2002) *Molecular cell* **9**, 401-410
355. Chauhan, D., Li, G., Hideshima, T., Podar, K., Mitsiades, C., Mitsiades, N., Catley, L., Tai, Y. T., Hayashi, T., Shringarpure, R., Burger, R., Munshi, N., Ohtake, Y., Saxena, S., and Anderson, K. C. (2003) *Blood* **102**, 3379-3386
356. Stankiewicz, A. R., Lachapelle, G., Foo, C. P., Radicioni, S. M., and Mosser, D. D. (2005) *The Journal of biological chemistry* **280**, 38729-38739
357. Kirchhoff, S. R., Gupta, S., and Knowlton, A. A. (2002) *Circulation* **105**, 2899-2904
358. Shan, Y. X., Liu, T. J., Su, H. F., Samsamshariat, A., Mestril, R., and Wang, P. H. (2003) *Journal of molecular and cellular cardiology* **35**, 1135-1143
359. Veereshwarayya, V., Kumar, P., Rosen, K. M., Mestril, R., and Querfurth, H. W. (2006) *The Journal of biological chemistry* **281**, 29468-29478
360. Kang, B. H., Plescia, J., Dohi, T., Rosa, J., Doxsey, S. J., and Altieri, D. C. (2007) *Cell* **131**, 257-270
361. Kuo, C. C., Liang, C. M., Lai, C. Y., and Liang, S. M. (2007) *J Immunol* **178**, 6100-6108

362. Bruey, J. M., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S. A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A. P., Kroemer, G., Solary, E., and Garrido, C. (2000) *Nature cell biology* **2**, 645-652
363. Samali, A., Robertson, J. D., Peterson, E., Manero, F., van Zeijl, L., Paul, C., Cotgreave, I. A., Arrigo, A. P., and Orrenius, S. (2001) *Cell stress & chaperones* **6**, 49-58
364. Voss, O. H., Batra, S., Kolattukudy, S. J., Gonzalez-Mejia, M. E., Smith, J. B., and Doseff, A. I. (2007) *The Journal of biological chemistry* **282**, 25088-25099
365. Mehlen, P., Kretz-Remy, C., Preville, X., and Arrigo, A. P. (1996) *The EMBO journal* **15**, 2695-2706
366. Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A. P., Buchner, J., and Gaestel, M. (1999) *The Journal of biological chemistry* **274**, 18947-18956
367. Coleman, M. L., Sahai, E. A., Yeo, M., Bosch, M., Dewar, A., and Olson, M. F. (2001) *Nature cell biology* **3**, 339-345
368. Pivovarova, A. V., Chebotareva, N. A., Chernik, I. S., Gusev, N. B., and Levitsky, D. I. (2007) *The FEBS journal* **274**, 5937-5948
369. Li, C. Y., Lee, J. S., Ko, Y. G., Kim, J. I., and Seo, J. S. (2000) *The Journal of biological chemistry* **275**, 25665-25671
370. Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M., and Green, D. R. (2000) *Nature cell biology* **2**, 469-475
371. Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E. S., Kufe, D., and Kharbanda, S. (2000) *The EMBO journal* **19**, 4310-4322
372. Saleh, A., Srinivasula, S. M., Balkir, L., Robbins, P. D., and Alnemri, E. S. (2000) *Nature cell biology* **2**, 476-483
373. Sakahira, H., and Nagata, S. (2002) *The Journal of biological chemistry* **277**, 3364-3370
374. Zhang, M. H., Lee, J. S., Kim, H. J., Jin, D. I., Kim, J. I., Lee, K. J., and Seo, J. S. (2006) *Molecular and cellular biochemistry* **281**, 111-121
375. Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., and Orrenius, S. (1999) *The EMBO journal* **18**, 2040-2048
376. Xanthoudakis, S., Roy, S., Rasper, D., Hennessey, T., Aubin, Y., Cassady, R., Tawa, P., Ruel, R., Rosen, A., and Nicholson, D. W. (1999) *The EMBO journal* **18**, 2049-2056
377. Mehlen, P., Schulze-Osthoff, K., and Arrigo, A. P. (1996) *The Journal of biological chemistry* **271**, 16510-16514
378. Gabai, V. L., Mabuchi, K., Mosser, D. D., and Sherman, M. Y. (2002) *Molecular and cellular biology* **22**, 3415-3424

379. Ravagnan, L., Gurbuxani, S., Susin, S. A., Maisse, C., Daugas, E., Zamzami, N., Mak, T., Jaattela, M., Penninger, J. M., Garrido, C., and Kroemer, G. (2001) *Nature cell biology* **3**, 839-843
380. Matsumori, Y., Hong, S. M., Aoyama, K., Fan, Y., Kayama, T., Sheldon, R. A., Vexler, Z. S., Ferriero, D. M., Weinstein, P. R., and Liu, J. (2005) *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **25**, 899-910
381. Lui, J. C., and Kong, S. K. (2007) *FEBS letters* **581**, 109-117
382. Jaattela, M., and Tschopp, J. (2003) *Nature immunology* **4**, 416-423
383. Guicciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Svingen, P. A., Peters, C., Kaufmann, S. H., and Gores, G. J. (2000) *The Journal of clinical investigation* **106**, 1127-1137
384. Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M., and Jaattela, M. (2001) *The Journal of cell biology* **153**, 999-1010
385. Brunk, U. T., and Svensson, I. (1999) *Redox report : communications in free radical research* **4**, 3-11
386. Yuan, X. M., Li, W., Dalen, H., Lotem, J., Kama, R., Sachs, L., and Brunk, U. T. (2002) *Proceedings of the National Academy of Sciences of the United States of America* **99**, 6286-6291
387. Brunk, U. T., Dalen, H., Roberg, K., and Hellquist, H. B. (1997) *Free radical biology & medicine* **23**, 616-626
388. Bidere, N., Lorenzo, H. K., Carmona, S., Laforge, M., Harper, F., Dumont, C., and Senik, A. (2003) *The Journal of biological chemistry* **278**, 31401-31411
389. Boya, P., Gonzalez-Polo, R. A., Poncet, D., Andreau, K., Vieira, H. L., Roumier, T., Perfettini, J. L., and Kroemer, G. (2003) *Oncogene* **22**, 3927-3936
390. Roberg, K., Kagedal, K., and Ollinger, K. (2002) *The American journal of pathology* **161**, 89-96
391. Nylandsted, J., Gyrd-Hansen, M., Danielewicz, A., Fehrenbacher, N., Lademann, U., Hoyer-Hansen, M., Weber, E., Multhoff, G., Rohde, M., and Jaattela, M. (2004) *The Journal of experimental medicine* **200**, 425-435
392. Bivik, C., Rosdahl, I., and Ollinger, K. (2007) *Carcinogenesis* **28**, 537-544
393. Neu, J., and Walker, W. A. (2011) *The New England journal of medicine* **364**, 255-264

394. Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I., and Sherman, M. Y. (1997) *The Journal of biological chemistry* **272**, 18033-18037
395. Jiang, B., Liang, P., Deng, G., Tu, Z., Liu, M., and Xiao, X. (2011) *Cell stress & chaperones* **16**, 143-152
396. Tanaka, K., and Mizushima, T. (2009) *International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group* **25**, 668-676
397. Jiang, B., Wang, K., Liang, P., Xiao, W., Wang, H., and Xiao, X. (2009) *The FEBS journal* **276**, 2615-2624
398. Carrizo, L. C., Ruete, C. M., Manucha, W. A., Ciocca, D. R., and Valles, P. G. (2006) *Cell stress & chaperones* **11**, 309-324
399. Didelot, C., Schmitt, E., Brunet, M., Maingret, L., Parcellier, A., and Garrido, C. (2006) *Handbook of experimental pharmacology*, 171-198
400. Beere, H. M., and Green, D. R. (2001) *Trends in cell biology* **11**, 6-10
401. Joly, A. L., Wettstein, G., Mignot, G., Ghiringhelli, F., and Garrido, C. (2010) *Journal of innate immunity* **2**, 238-247
402. Tao, Y., Hart, J., Lichtenstein, L., Joseph, L. J., Ciancio, M. J., Hu, S., Chang, E. B., and Bissonnette, M. (2009) *Carcinogenesis* **30**, 175-182
403. Hu, S., Zhu, X., Triggs, J. R., Tao, Y., Wang, Y., Lichtenstein, L., Bissonnette, M., Musch, M. W., and Chang, E. B. (2009) *American journal of physiology. Gastrointestinal and liver physiology* **296**, G1003-1011
404. Radulescu, A., Zorko, N. A., Yu, X., and Besner, G. E. (2009) *Pediatric research* **65**, 437-442
405. Leaphart, C. L., Qureshi, F., Cetin, S., Li, J., Dubowski, T., Baty, C., Beer-Stolz, D., Guo, F., Murray, S. A., and Hackam, D. J. (2007) *Gastroenterology* **132**, 2395-2411
406. Ding, L. A., and Li, J. S. (2003) *World journal of gastroenterology : WJG* **9**, 1327-1332
407. Khailova, L., Mount Patrick, S. K., Arganbright, K. M., Halpern, M. D., Kinouchi, T., and Dvorak, B. (2010) *American journal of physiology. Gastrointestinal and liver physiology* **299**, G1118-1127
408. Jilling, T., Lu, J., Jackson, M., and Caplan, M. S. (2004) *Pediatric research* **55**, 622-629
409. Houry, W. A. (2001) *Current protein & peptide science* **2**, 227-244
410. Naylor, D. J., and Hartl, F. U. (2001) *Biochemical Society symposium*, 45-68
411. Brinker, A., Scheufler, C., Von Der Mulbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and

Hartl, F. U. (2002) *The Journal of biological chemistry* **277**, 19265-19275
412. Liu, F. H., Wu, S. J., Hu, S. M., Hsiao, C. D., and Wang, C. (1999) *The Journal of biological chemistry* **274**, 34425-34432
413. Freeman, B. C., Myers, M. P., Schumacher, R., and Morimoto, R. I. (1995) *The EMBO journal* **14**, 2281-2292
414. Qian, S. B., McDonough, H., Boellmann, F., Cyr, D. M., and Patterson, C. (2006) *Nature* **440**, 551-555
415. Dickinson, E., Tuncer, R., Nadler, E., Boyle, P., Alber, S., Watkins, S., and Ford, H. (1999) *The American journal of physiology* **277**, G1281-1287
416. Unno, N., Wang, H., Menconi, M. J., Tytgat, S. H., Larkin, V., Smith, M., Morin, M. J., Chavez, A., Hodin, R. A., and Fink, M. P. (1997) *Gastroenterology* **113**, 1246-1257
417. Paimela, T., Hyttinen, J. M., Viiri, J., Ryhanen, T., Karjalainen, R. O., Salminen, A., and Kaarniranta, K. (2011) *Pharmacological research : the official journal of the Italian Pharmacological Society* **64**, 501-508
418. Kalmar, B., and Greensmith, L. (2009) *Cellular & molecular biology letters* **14**, 319-335
419. Morgan, J. A., Young, L., and McGuire, W. (2011) *Current opinion in infectious diseases* **24**, 183-189
420. Chow, A. M., Steel, R., and Anderson, R. L. (2009) *Cell stress & chaperones* **14**, 253-263
421. Jiang, J., Ballinger, C. A., Wu, Y., Dai, Q., Cyr, D. M., Hohfeld, J., and Patterson, C. (2001) *The Journal of biological chemistry* **276**, 42938-42944
422. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001) *Nature cell biology* **3**, 100-105
423. Dai, Q., Zhang, C., Wu, Y., McDonough, H., Whaley, R. A., Godfrey, V., Li, H. H., Madamanchi, N., Xu, W., Neckers, L., Cyr, D., and Patterson, C. (2003) *The EMBO journal* **22**, 5446-5458
424. Vabulas, R. M., Ahmad-Nejad, P., Ghose, S., Kirschning, C. J., Issels, R. D., and Wagner, H. (2002) *The Journal of biological chemistry* **277**, 15107-15112
425. Pockley, A. G., Muthana, M., and Calderwood, S. K. (2008) *Trends in biochemical sciences* **33**, 71-79
426. Chase, M. A., Wheeler, D. S., Lierl, K. M., Hughes, V. S., Wong, H. R., and Page, K. (2007) *J Immunol* **179**, 6318-6324
427. Chen, T., Guo, J., Han, C., Yang, M., and Cao, X. (2009) *J Immunol* **182**, 1449-1459
428. Gong, J., Zhu, B., Murshid, A., Adachi, H., Song, B., Lee, A., Liu, C., and Calderwood, S. K. (2009) *J Immunol* **183**, 3092-3098

429. Retzlaff, C., Yamamoto, Y., Hoffman, P. S., Friedman, H., and Klein, T. W. (1994) *Infection and immunity* **62**, 5689-5693
430. Wheeler, D. S., Chase, M. A., Senft, A. P., Poynter, S. E., Wong, H. R., and Page, K. (2009) *Respiratory research* **10**, 31
431. Wallin, R. P., Lundqvist, A., More, S. H., von Bonin, A., Kiessling, R., and Ljunggren, H. G. (2002) *Trends in immunology* **23**, 130-135
432. Bausinger, H., Lipsker, D., Ziylan, U., Manie, S., Briand, J. P., Cazenave, J. P., Muller, S., Haeuw, J. F., Ravanat, C., de la Salle, H., and Hanau, D. (2002) *European journal of immunology* **32**, 3708-3713
433. Gao, B., and Tsan, M. F. (2003) *The Journal of biological chemistry* **278**, 174-179
434. Henderson, B., Calderwood, S. K., Coates, A. R., Cohen, I., van Eden, W., Lehner, T., and Pockley, A. G. (2010) *Cell stress & chaperones* **15**, 123-141
435. van Wijk, F., and Prakken, B. (2010) *Journal of leukocyte biology* **88**, 431-434
436. Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004) *Cell* **118**, 229-241
437. Fukata, M., Hernandez, Y., Conduah, D., Cohen, J., Chen, A., Breglio, K., Goo, T., Hsu, D., Xu, R., and Abreu, M. T. (2009) *Inflammatory bowel diseases* **15**, 997-1006
438. Chen, H., Wu, Y., Zhang, Y., Jin, L., Luo, L., Xue, B., Lu, C., Zhang, X., and Yin, Z. (2006) *FEBS letters* **580**, 3145-3152