EVALUATING THE ROLE OF NF-κB SUPPRESSION IN AMELIORATING MAMMALIAN DISEASE: AN EXAMINATION OF INFLAMMATORY BOWEL DISEASE AND DISEASES ASSOCIATED WITH AGING

by

Jeremy Tilstra

Bachelor of Science in Biochemistry, University of Maryland, 2003

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This thesis was presented

By

Jeremy Tilstra

It was defended on

August 11, 2009

and approved by

Olivera Finn, Distinguished Professor and Chair, Dept of Immunology

Lawrence Kane, Assistant Professor, Dept of Immunology

Prabir Ray, Associate Professor, Dept of Immunology

Co Dissertation Advisor: Laura Niedernhofer, Assistant Professor, Dept. of MMG

Dissertation Advisor: Paul Robbins, Professor , Dept of MMG

EVALUATING THE ROLE OF NF-κB SUPPRESSION IN AMELIORATING MAMMALIAN DISEASE: AN EXAMINATION OF INFLAMMATORY BOWEL DISEASE AND DISEASES ASSOCIATED WITH AGING

Jeremy Tilstra PhD

NF-κB is a family of transcription factors that play a pivotal role in inflammation, cell proliferation, cell survival, and apoptosis in response to endogenous and exogenous stress stimuli. NF-κB is implicated in numerous chronic inflammatory and degenerative diseases. In this thesis, the consequences of NF-κB suppression on pathologies associated with inflammatory bowel disease (IBD) and age-associated degeneration are explored. To test the hypothesis that NF-κB plays a causal role in driving the degenerative changes associated with the diseases we evaluated the efficacy of a pharmacologic peptide IKK/NF-κB activation inhibitor in the *IL-10*$^{-/-}$ model of colitis and genetic depletion and pharmacologic inhibition of NF-κB in a mouse model of accelerated aging (*Ercc1*$^{-/\Delta}$ mice).

Pathologic and immunologic markers of IBD were reduced in the presence of systemic pharmacologic NF-κB suppression. Furthermore, this study provides evidence of the efficacy of the NBD inhibitory peptide *in vitro* and *in vivo* experiments, and importantly provided possible therapeutic avenues for the treatment of IBD. Like naturally aged mice, NF-κB is activated in *Ercc1*$^{-/\Delta}$ mice compared to wild type littermates. *Ercc1*$^{-/\Delta}$ mice haploinsufficient for the p65/RelA subunit of NF-κB had a modest delay in the onset of age-related symptoms. This was recapitulated in mice chronically treated with the peptide inhibitor of NF-κB, which exhibited a significant delay in overall aging score and improved histopathological alterations. These

implicate NF-κB as a major driver of degenerative changes associated with aging and set a precedent for therapeutic intervention.

As activated macrophages are mediators of inflammatory and age-associated degenerative changes, we futher evaluated the role of NF-κB suppression in this cell type. Macropahges and monocyte derived DC cells underwent programmed cell death (PCD) in the presence of pharmacologic NF-κB inhibition. Unlike previous studies which implicated TNFα signaling in this pathway, the mechanisms behind this PCD is induction is induction of ROS formation. This observed macrophage NF-κB induced PCD may be one of the mechanisms by which inflammatory and age-associated disease pathologies are reduced in response to NF-κB suppression.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to take the opportunity to thank all of the people who were instrumental in helping me become who I am today and allowed me to complete my dissertation research. First and foremost, I would like to thank my wife and best friend, Sarah. She was always there to support me in the hard times, and was there to share in the joy of the good times. Of all my accomplishments my most amazing feet of my time as a Ph.D. student was convincing her to marry me. I know that without her my research would have probably not differed all that much, but I am sure that my time spent doing it would not have been as fulfilling.

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1.0 INTRODUCTION

1.1 NF-KB/IKK SIGNALING

1.1.1 Introduction to NF-κB

The NF-κB transcription factor (TF) was discovered nearly 23 years ago as proinflammatory regulatory element which signals downstream of LPS[1, 2]. Since its discovery, vast amounts of research has been undertaken to evaluated NF-κB signaling, function, and relevance resulting in nearly 35,000 publications. NF-κB has traditionally been viewed as an immunologic TF, involved in the activation of inflammatory cells, and transcription of cytokines and chemokines. Over time scientists realized the vast implications of dysregulated NF-κB signaling because of the plethora of pathologic conditions associated with this[3]. Furthermore, the number of biologic processes known to be controlled by NF-κB continues to increase[4]. Thus, the understanding of dysregulated NF-κB signaling is critically important to the treatment of many pathologic disease states.

1.1.2 NF-κB Subunits

The NF-κB TF is a dimer which can be formed by combinations of five distinct family members: p65(relA), relB, c-rel, p105/p50, and p100/p52 (for the latter NF-κB subunits the

larger form is the non-cleaved component). These NF-κB subunits act either as homo- or heterodimers and are defined by a short rel-homology domain (RHD), which is responsible for dimerization as well as DNA binding. Three of the subunits, p65, relB, and c-rel, also contain transcription activation domains (TAD), which are regions that promote the transcription of DNA into RNA. The other two subunits do not have TADs and thus act as transcriptional suppressors (Figure 1)[5]. For example, the p65/p50 heterodimer is the traditional inflammatory NF-κB dimer and activates the transcription of a variety of genes, while the p50/p50 homodimer can bind also bind to the NF-κB consensus sequence (GGGRNNYYCC) and has a higher affinity than a p65 homodimer[6], but results in blocked transcription.

1.1.3 NF-κB stimulatory molecules

As stated previously, there are a wide variety of NF-κB activators (Figure 2), which include numerous inflammatory stimulating factors. The toll like receptor (TLR) ligands, which are part of a larger group of pattern recognition receptors (which recognize conserved attributes found in bacterial, viral and parasitic pathogens), are potent NF-κB activators. TLR ligands signal through several PRR exemplified by: lipopolysaccaride (LPS) via TLR4[7], CPG via TLR9[8], Flagellin via TLR2[9], and muramyl dipeptides (MDP) via NOD2[10]. Additionally, tumor necrosis factors (TNF) and interleukin-1 (IL-1), two major proinflammatory cytokines, are both activators as well as transcriptional targets of NF-κB. Other NF-κB activators also include the antigen receptors found on the adaptive immune cells, specifically the T-cell receptor and B-cell receptor (TCR and BCR), as well as varying receptors found on antigen presenting cells including the already mentioned TLRs and CD40R. Additionally growth factors such as: hepatocyte growth factor (HGF), follicle stimulating hormone (FSH), granulocyte macrophage-

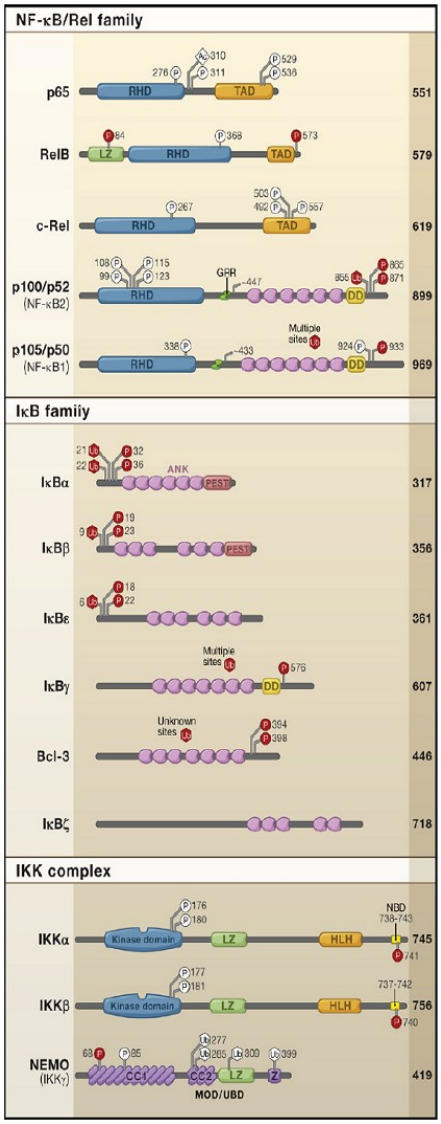


Figure 1: NF-κB, IκB, and IKK family members. Postranslational modifications are indicated with P, U, Ac, for phosphorylation, ubiquitination, and acetylation respectively. These modifications in white indicate activators while those in red describe either inhibitory or proteosmal degredation mediators. Amino acid in human proteins are shown at the right, and NF-κB1 and NF-κB2 are shown in their uncleaved (inhibitory) form. Other abbreviations include; GRR, glycine-rich region; HLH, helix-loop-helix domain; Z, zinc fingerdomain; CC1/2, coiled-coil domains; NBD, NEMO-binding domain; MOD/UBD, minimal oligomerization domain and ubiquitin-binding domain; and DD, death domain. (Reprinted from Cell press, Vol 132, Hayden MS and Ghosh S., Shared Principles in NF-κB Signaling, 344-362, 2008[5])

colony stimulating factor (GM-CSF), and nerve growth factor (NGF) can also activate NF-κB vai their associated receptors[4]

Interestingly, the NF-κB signaling pathway can be activated secondary to DNA damage via the DNA response protein ataxia telangiectasia mutated (ATM), which acts directly at the level of the Inhibitor of Kappa B Kinase (IKK) complex[11, 12]. There is also abundant amounts of data which suggest that ROS can activate and/or suppress NF-κB as reviewed in[13], and these variations may depend on cell types and specific conditions. Thus, the NF-κB response is a ubiquitous signaling pathway which is activated in response to stimuli, the majority of which are mediators or markers of stress.

There are hundreds of genes defined as NF-κB transcriptionally regulated genes; however, they are preferentially activated in response to certain stimuli but not others likely due to the role of other signaling pathways and transcription factors activated by NF-κB activating receptors. Therefore, it is likely that other pathways activated in concert with NF-κB are integral in determining the exact cellular response to any one stimuli. Exploration of these differential responses is beyond the scope of this manuscript, but is important to take into account when evaluating the effects of specific NF-κB suppression in models of disease.

1.1.4 Activation of NF-κB through the IKK complex

The canonical or activated NF-κB signaling cascade is initiated by numerous membrane and intracellular receptors as described above[4]. Of the numerous signaling cascade which activate NF-κB, many share a small number of signaling molecules upstream of the IKK complex. For example LPS activates the Tollip which activates MyD88 and IRAK, these components the signaling through TRAF6, which leads to subsequent activation of the IKK

complex. Other common signaling proteins involved in TLR, IL-1, TNF, and TCR signaling pathways include MyD88, RIP, NIK and TRAF proteins which act upstream of the IKK complex[5]. The IKK complex lies at the confluence of the many different NF-κB signaling cascades, whether activated by TLRs, TCR, or ATM. It is composed of two catalytic subunits, IKKα and IKKβ, as well as a regulatory subunit IKKγ also known as the NF-κB essential modulator (NEMO)[14].

IKKγ interacts with the upstream RIP, NIK, or TRAF proteins which results in the oligomerization of NEMO. IKKγ then induces phosphorylation or possible auto-phosphorylation of the IKKα and β subunits. After phosphorylation, the catalytic subunits are released from the IKKγ; the free catalytic subunits can then act to phosphorylate IκB at Ser32 and Ser36. After phosphorylation, IκBα is then poly-ubiquitinated at Lys22 and degraded by the 26S proteosome. This degradation leads to the loss of the nuclear export signal provided by IκB as well as the revealing of a nuclear localization signal on the NF-κB subunits. This cascade results in the migration of NF-κB to the nucleas[15]. Once in the nucleus NF-κB acts to promote gene expression or suppress various pro-inflammatory, cell growth, and other regulated genes (Figure 2).

1.1.5 Non-canonical NF-κB signaling

The non-canonical NF-κB signaling pathway is largely involved in lymphoid organ development, which is required for B and T-cell development via activation of the p52/RelB

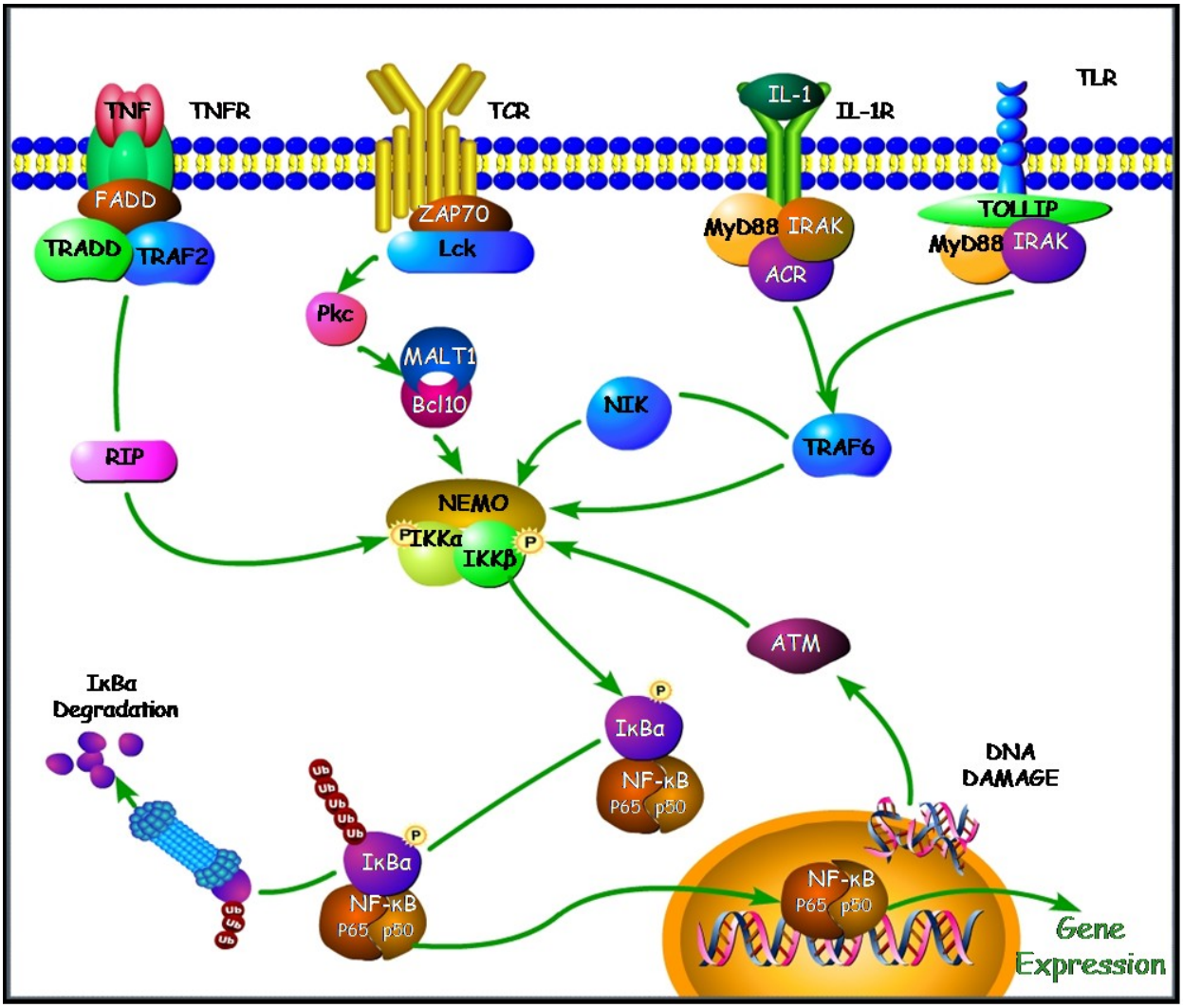


Figure 2: Signaling via the IKK/NF-κB Classical Pathway. The IKK complex (NEMO, IKK1 (IKKα), and IKK2 (IKKβ) can be activated by numerous stimuli and via shared signaling components. Extracellular receptors bind to their ligands and signaling via TRAF/RIP/NIK molecules leading to phosphorylation of IKK subunits, which subsequently phosphorylate IκBα and lead to its ubiquitination and proteosomal degredation. This then releases NF-κB into the nucleus where it acts as a transcription factor. In addition, ATM responds to DNA damage and can also activate the IKK complex. (Figure adapted from [5, 16, 17])

homodimer. This signaling pathway is activated by a limited number of receptors (Lymphotoxin B, B-cell activating factor, and CD40), which subsequently stimulate an IKKα homodimer. This complex then phosphorylates p100 and leads to processing of p100 to p52, allowing for translocation of the p52/RelB complex to the nucleus where it acts as a transcription factor[18]. In addition to lymphoid organ development, studies evaluating IKKα$^{-/-}$ mice suggest a role of the non-canonical pathway in epidermal and skeletal development[19]. While the role of the non-canonical pathway seems most important during development, it is the canonical pathway that seems to be the most relevant in the treatments of mammalian disease described in this manuscript.

1.1.6 IKK and IκB signaling components

The IκB and IKK proteins are the central regulators of activated NF-κB signaling. It is thought that the IKK complex is solely a trimerization of IKKα/β/γ and in fact *in vivo* may be found as a higher order complex with numerous IKK trimers interacting. However, some studies suggest that IKKα is less important than IKKβ with regards to canonical signaling; and within certain cell types the IKK complex may be composed of IKKβ/β/γ trimer rather than the traditional IKKα/β/γ[20]. The IKK kinases α and β share a 52% sequence homology[21] with a greater homology in their catalytic or kinase domains, however, genetic knockout (ko) studies suggest differential and non-redundant roles for these two proteins. While IKKα is more important in non-canonical signaling, IKKβ ko mice show a high level of similarity to *p65*$^{-/-}$ cells. Interestingly there is some evidence that IKKα contributes to canonical signaling[22]. As the IKKβ$^{-/-}$ mouse does not have all of the defects seen in the IKKγ$^{-/-}$ mouse[23], there is likely some differential roles for α and β in the canonical signaling pathways.

The major ligands of the IKK proteins are the IκB cytoplasmic inhibitors. The common component of the IκB-like proteins is the ankyrin repeats which are found on IκBα,β,γ,δ,ε, bcl-3 and the uncleaved p50 and p52 subunits p100 and p105 proteins. These ankyrin repeats act to bind to the Rel portion of NF-κB blocking the NLS. Of the typical IκBs (α/β/ε), the most studied IκB protein is IκBα, the main inhibitor of the canonically activated p65/p50 NF-κB heterodimer, which act as described above. The non-typical IκBs, bcl-3 and IκBδ are thought to inhibit NF-κB transcription by binding to NF-κB in the nucleus. For instance, Bcl-3 stabilizes p50 homodimers on DNA, blocking the NF-κB promoter regions from other NF-κB subunits and preventing transactivation domain (TAD) positive NF-κB subunits from binding the consensus sequence[24].

1.1.7 Genes under NF-κB transcriptional control

Since the discovery of NF-κB[1], hundreds of genes have been shown to be directly transcriptionally regulated by NF-κB[4]. These genes fall under the following categories: cytokines and chemokines, immunoreceptors, antigen presentation, cell adhesion, acute phase response, stress response, cell surface receptors, growth factors and ligands, early response genes, and other transcription factors as defined by Gilmore (nf-kb.org)[4] and summarized below and in Figure 3.

It is clear that the vast numbers of these categories transcriptionally controlled by NF-κB are involved in immune signaling and inflammatory responses. NF-κB transcriptional control of

cytokines is likely one of the most important factors when evaluating the role of NF-κB in

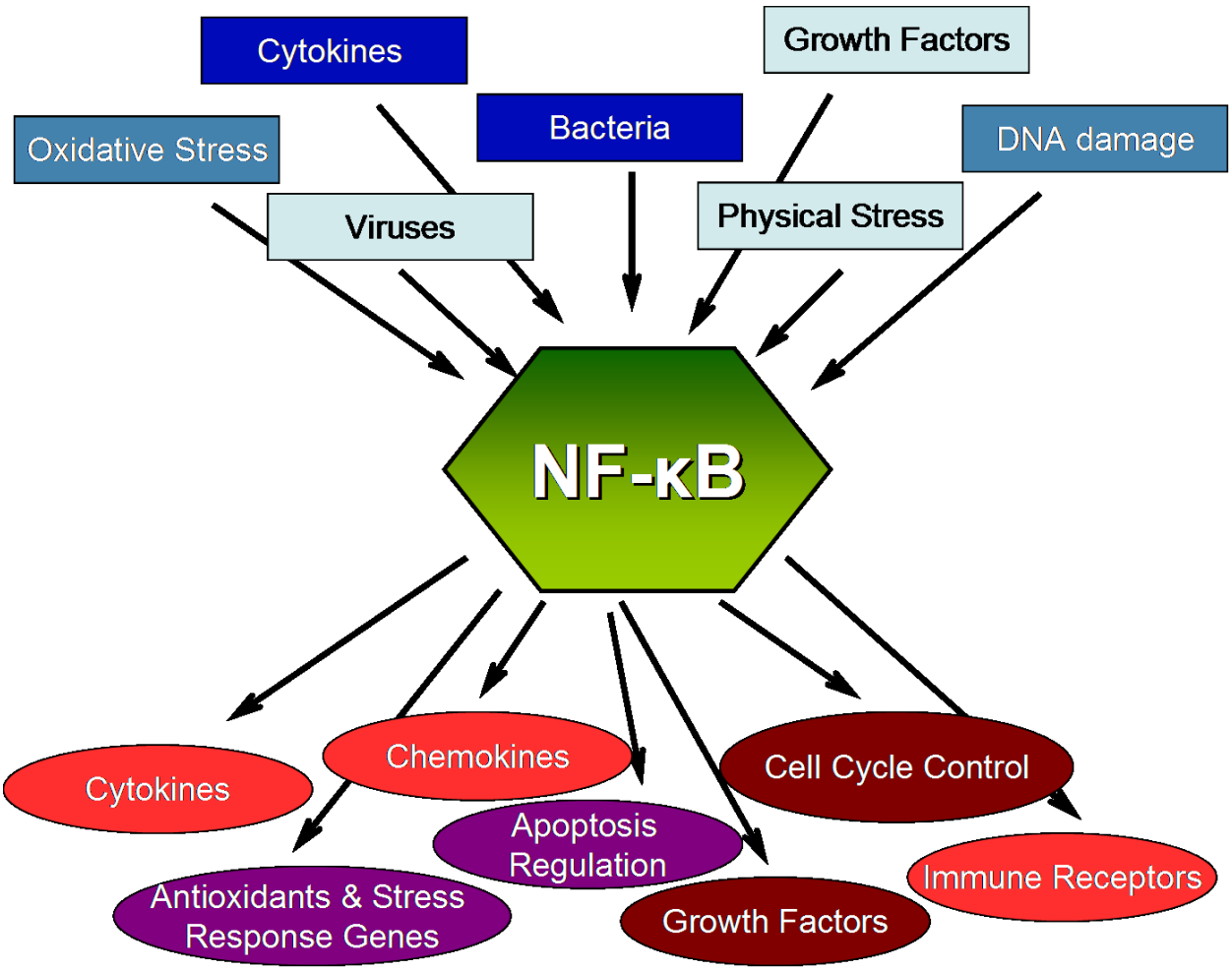


Figure 3: NF-κB is a central regulator in stress response. The NF-κB signaling pathway can be activated by numerous stimuli as listed in the blue boxes. In response to these different stimuli NF-κB transcriptionally regulate hundereds of genes which are listed in the red and purple circles. Of particular importance to chapters 2 are the cytokine and bacterial activators which alter cytokine, chemokine and immune receptor reponse. In chapter 3 of importance are again the cytokine and bacterial activators, but NF-κB regulation of apoptosis and antioxidant production is of note. While in Chapter 4 cell stressors such as DNA damage, oxidative stress, and physical stress are the important inducers of NF-κB while cell cycle, growth factor and immune gene regulation is of interest.

pathologic states. Some of these cytokines include but are not limited to TNFα, IL-1α/β, IL-2,3,6,12, GM-CSF, M-CSF, and G-CSF. NF-κB also regulates chemokines (MCP-1, KC, MIP-1 and several CCLs) and adhesion molecules (ICAM-1, E-selectin, and VCAM-1) which allow for the recruitment and attachment of immune cells to sites of inflammation. In addition to recruitment and activation of immune cells NF-κB also upregulates receptors (CD80/81, IL-2Rα chain, TLR-2) and antigen presenting architecture (MHC class I and β2 microglobulin) on immune cells to allow for the proper innate and adaptive immune responses.

In addition to immune response genes, which are integral to the control of pathogen induced inflammatory response and likely a major contributor to pathology in human diseases there are several other processes which are controlled by NF-κB. Interestingly, NF-κB transcriptionally regulates both pro-apoptotic (Bim, Bax, Fas and Fas-ligand, and caspase 11) and anti-apoptotic genes (XIAP, bcl-2, A1/bfl-1, and c-Flip). It has been shown that NF-κB blocks apoptosis in a number of inflammatory cells including macrophages, dendritic cells, T-cells, B-cells, and neutrophils, as well as being a pro-survival factor in several types of malignancies especially lymphomas (as described below). On the other hand, the inflammatory response often induces apoptotic cell death in infected cells. This inflammatory cell death response is initiated by the production of cell death receptors ie. Fas/FasL and intracellular apoptosis inducing proteins. This apoptotic death is further assisted by activated immune cells which secrete granzyme, perforirin and nitric oxide, all apoptosis inducing factors.

The other major NF-κB transcriptionally regulated genes include growth factors such as: nerve growth factor (NGF), vascular endothelial growth factor (VEGF), insulin-like growth factor binding protein (IGFBP), bone morphogenic protein (BMP), fibroblast growth factor (FGF) and numerous others. Many of these receptors are involved in the expansion and

maturation of immune cells. Thus, the majority NF-κB controlled genes are considered cell stress responders to create inflammation, block apoptosis, and increase cellular growth. Unfortunately many of these processes can be co-opted by cancerous cells to promote their survival and growth.

1.1.8 Other substrates of IKK complex:

While it is likely that the actions of the IKK inhibitors used in these studies exert the majority of their effects due to suppression of the NF-κB canonical signaling pathway, there are also a number of other IKK substrates and interacting proteins separate from this pathway which may also be altered by inhibiting the IKK complex. Proteins known to be phosphorylated by IKK include Bcl-10, β-catenin, cyclinD1, FOXO3a , and ERα. Interestingly, these genes are known to control cell growth and proliferation and many of these have been implicated in cancers[25-27]. NEMO has also been shown to interact with HIF1α and HIF2α to promote transcriptional activity of these two factors, which are known to play a role in anti-oxidant function[25, 28]. Thus, while the majority of the effects observed after IKK inhibition are likely mediated by NF-κB suppression, there may be other pathways affected by IKK suppression, specifically those regarding cell growth and proliferation.

1.1.9 NF-κB in human disease

Dysregulation of NF-κB is implicated in a vast number of human diseases, through inherited genetic mutations, somatic mutations, indirect mutations, or non-epigenetic dysregulation of NF-κB signaling (described below). Only a few diseases, however, result from

genetic mutations in specific NF-κB signaling components, including Incontinentia pigmenti (ID), anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID), osteopetrosis and lymphedema (OL)-EDA-ID, Immunodeficiency without EDA, EDA-ID(with impaired T-cell proliferation) most of which are due to mutations in NEMO or IκBα[29]. Each of these diseases is defined by severe skin inflammation, but this can progress with ocular and hair defects and in severe cases even central nervous system disfunction[29].

REL, or human c-rel, amplification is seen mainly in cancers and is more common than inherited mutations of NF-κB components. Interestingly, REL amplification is likely due to the pro-survival (growth factor, proliferation induction, and anti-apoptotic regulation) transcriptional activity of NF-κB. Rel amplification has been reported in Hodgkins lymphoma, and a variety of non-Hodgkin's lymphoma including diffuse large B-cell lymphoma, follicular, primary mediastinal, natural killer T-cell and others. In these lymphomas REL amplification was present in varying percentages of patients: specifically amplification of REL was observed in upwards of 54% in Hodgkin's, 75% in Cutaneous CD30+ anaplastic large cell, but only 5% in aggressive B-cell lymphoma. Additionally there is a retroviral oncoprotein homologue to REL, v-Rel of the avian Rev-T retrovirus. Recent studies demonstrate efficacy of treating cancers with chemotherapeutics containing adjunct NF-κB inhibitory compounds[30-32], suggesting that the over-active NF-κB is not a marker but rather a contributing factor to tumorgenesis and/or tumor cell survival[29, 33].

The vast majority of diseases in which NF-κB has been implicated are those with single nucleotide polymorphisms (SNPs), alterations in enhancer or promoter regions of NF-κB regulated genes or mutations in genes implicated in the NF-κB signaling pathways. These mutations and associated diseases are described in detail by Courtois et al.[29] Not suprisingly,

many of the diseases associated with increased NF-κB signaling are inflammatory diseases including familial mediterranean fever, Blau's disease, inflammatory bowel disease, cold induced autoinflammatory syndrome, or conditions wich incresase susceptibility to asthma and sepsis.

In addition to the diseases with known genetic alteration dysregulating NF-κB signaling, a vast number of diseases states exhibit upregulated NF-κB signaling despite having no mutations in the NF-κB sigaling pathway. Suggesting that NF-κB may be upregulated in response to altered proteins or stress responses generated by these disease states. This list of indirect NF-κB implicated diseases includes an ever growing number of diseases from Parkinson's to Padget's, glaucoma, cystic fibrosis, systemic lupus erytematous, COPD, pain disorders, osteoporosis, and metobolic disorders[4]. Thus, the implications of NF-κB dysregulation in human disease are vast and a greater understanding of its physiologic role and the effects of altering this signaling pathway is of criticial importance in understanding and treating human pathology. The use of animal models is an important tool to allow for us to complete these goals.

1.1.10 NF-κB inhibition/Nemo Binding Domain (NBD) Peptide:

Many methods to inhibit NF-κB signaling *in vitro* and *in vivo* have been evaluated. Some mechanisms previously used in inflammatory diseases include NF-κB decoy oligoneuclodides[34], proteosome or ubiquitin inhibitors which block the degradation of IκBα, or systemic anti-inflammatory agents which block NF-κB non-specifically (PTDC[35], dexamethasone[36], aspirin[37] and others). However, the problems associated with these therapeutics results from their lack of specificity, or in the case of the NF-κB decoy peptides or p65 translocation inhibitors, they block

both activated and basal NF-κB signaling, thereby increases potential side effects. A more appropriate therapeutically target to suppress NF-κB activation is the IKK complex. While many of the major pharmaceutical companies and various laboratories are generating compounds which have great *in vitro* potential, many of these compounds have less than optimal efficacy *in vivo*. One exception to this low *in vivo* bioavailability is the NEMO binding domain (NBD) peptide. NBD is an 11 amino acid sequence within IKKß that binds to IKKγ[14]. The addition of exogenous NBD peptide blocks the interaction of both IKKα and β with the regulatory subunit IKKγ. While there are numerous small molecule inhibitors of NF-κB, there are some distinct advantages of the NBD peptide. The site of action is highly defined, and due to this specific IKK targeting, only activated, but not basal levels of NF-κB is inhibited. Also, because of the high specificity of the NBD peptide sequence it is unlikely to affect other essential kinases, which is not the case for other more general NF-κB inhibitors[38]. Furthermore, the *in vivo* bioavailability of peptides linked to protein transduction domains is very high[39].

1.1.11 Protein Transduction as method of delivering therapeutics

Protein transduction domains (PTDs) are small peptides which are able to transport much larger molecules such as oligonucleotides, peptides, full-length proteins, 40nm iron nanoparticles, bacteriophages, and even 200 nm liposomes across cellular membranes[40-44]. They have proven useful in delivering biologically active cargoes *in vivo*, and, remarkably, they have the ability to transduce nearly all tissues, including the brain, following intraperitoneal administration of fusion proteins[14, 45, 46].

There are at least three classes of PTDs, which include positively charged (cationic), protein leader sequence derived domains (hydrophobic) and peptides identified by phage display

able to transduce cells in a cell type specific manner (tissue specific) (Table 1). I will focus on the positively charged, cationic transduction domains since they are the most efficient and the best characterized and will be used in the studies outlined in this manuscript.

In 1988 and 1994 respectively, two proteins were observed to cross biologic membranes, TAT (derived from HIV, RQIKIWFQNRRMKWKK)[47, 48] and Antp (derived from within the *Drosophila* antenepedia protein, YGRKKRRQRRR)[49]. Both Antp and TAT transduction domains are comprised of numerous cationic amino acids, or basic residues, which play a crucial role in their transduction properties. These positively charged residues interact with the negatively charged cell membrane which mediates uptake. Studies have suggested that the transduction potential of these peptides is due to their amphipathic helical 3 dimensional structure[50]. However, the fact that peptides containing a minimum number of arginine or lysine residues works as efficiently as TAT PTD suggests that the charge of the peptide is the most important feature of transduction domains.

Initial binding of the cationic PTDs to cells is mediated through glycosaminoglycans.[51]. It is generally accepted that PTDs bind glycosaminoglycans, specifically heparin sulfate, and then transduce cells through a mechanism of receptor-independent, but possibly energy dependent endocytosis. Several mechanisms have been suggested to explain the second phase of transduction of PTDs, and it is still unknown whether all peptides transduce cells via the same mechanism. The fact that transduction can occur in an energy independent manner, albeit at a reduced level suggests that there may be at least two independent mechanisms.

Studies evaluating TAT protein transduction suggest a mechanism of receptor independent macropinocytosis associated with lipid rafts[52]. However, other studies suggest a caveolae-dependent mechanism of PTD uptake since two caveolae endocytosis inhibitors

Table 1: Protein Transduction domains or Cell Penetrating Peptides

Cationic Peptides		
Name	Peptide Sequence	Reference
Antp	RQIKIWFQNRRMKW	[49]
TAT	YGRKKRRQRRR	[47, 48]
8K	KKKKKKKK	[53]
6R	RRRRRR	[53]
PTD-5	RRQRRTSKLMKR	[54]
Amphipathic Peptides		
Name	Peptide Sequence	Reference
MAP	KLALKLALKALKAALKLA	[55]
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	[56]
ppTG20	GLFRALLRLLRSLWRLLLRA	[57]
Trimer	(VRLPPP)3	[58]
P1	MGLGLHLLVLAAALQGAWSQPKKKRKV	[59]
K-FGF	AAVALLPAVLLALLAP	[60]
Cell Targeting Peptides:		
Peptide Sequence	Target Tissue	Cellular Target	Reference
TSPLNIHNGQKL	Human head and neck solid tumors	Unknown	[61]
CGKRK	Tumour neovasculature	Heparan sulfate	[62]
RGD	Integrin receptor	αVβ3	[63]
VHSPNKK	Endothelial VCAM-1 expressing cells	VCAM-1	[64]
LTVSPWY	Breast carcinoma	erbB2	[65]
ATWLPPR	Tumour neovasculature	VEGF receptor	[55]

Modified from Vivés et al.[66], Tilstra et al.[67], and Deshayes et al.[68]

nystatin, and fillipin reduced transduction[69]. Thus, the exact mechanism of uptake may not only be dependent on the PTD itself but also its cargo[70].

1.1.12 Characterization of Transduction Efficiency

Previosuly, our lab characterized a panel of positively charged PTDs for ability to transduce different cell types in culture. Peptides containing 6-8 arginines, lysines or ornithines are highly effective for delivery of fluorescent markers or a ß-gal fusion protein through a receptor independent mechanism[53]. Lysine (8K) was the most effective for transduction of fibroblasts and epithelial cell lines in culture. However, these studies as well as the majority of other studies evaluating PTD sequences examine cellular uptake, bio-distribution, and endosomal escape have used PTDs carrying fluorescent cargos. While this approach offers insight into the mechanism of PTD action and allows for examination of transduction efficiency, it provides very little information about the potential for therapeutic application of this technology. Therefore, we recently chose to evaluate various PTD sequences linked to a biologically relevant peptide cargo which, when present intracellularly, can inhibit the NF-κB signaling pathway (Figure 4).

We initially tested a panel of PTD-NBD fusion peptides for their ability to inhibit IL-1ß induced NF-κB transcriptional activity, measured using an NF-κB luciferase reporter in HELA cells. In culture, delivery of NBD with TAT, Antp and PTD-5 resulted in the most effective inhibition of IL-1ß induced NF-κB activity. This result is in contrast to *in vivo* analysis in a murine footpad delayed-type hypersensitivity (DTH) model where 8K, and six arginine (6R), worked far more efficiently in blocking footpad swelling following local injection. In the DTH model, injection of TAT-NBD and Antp-NBD were significantly less effective in

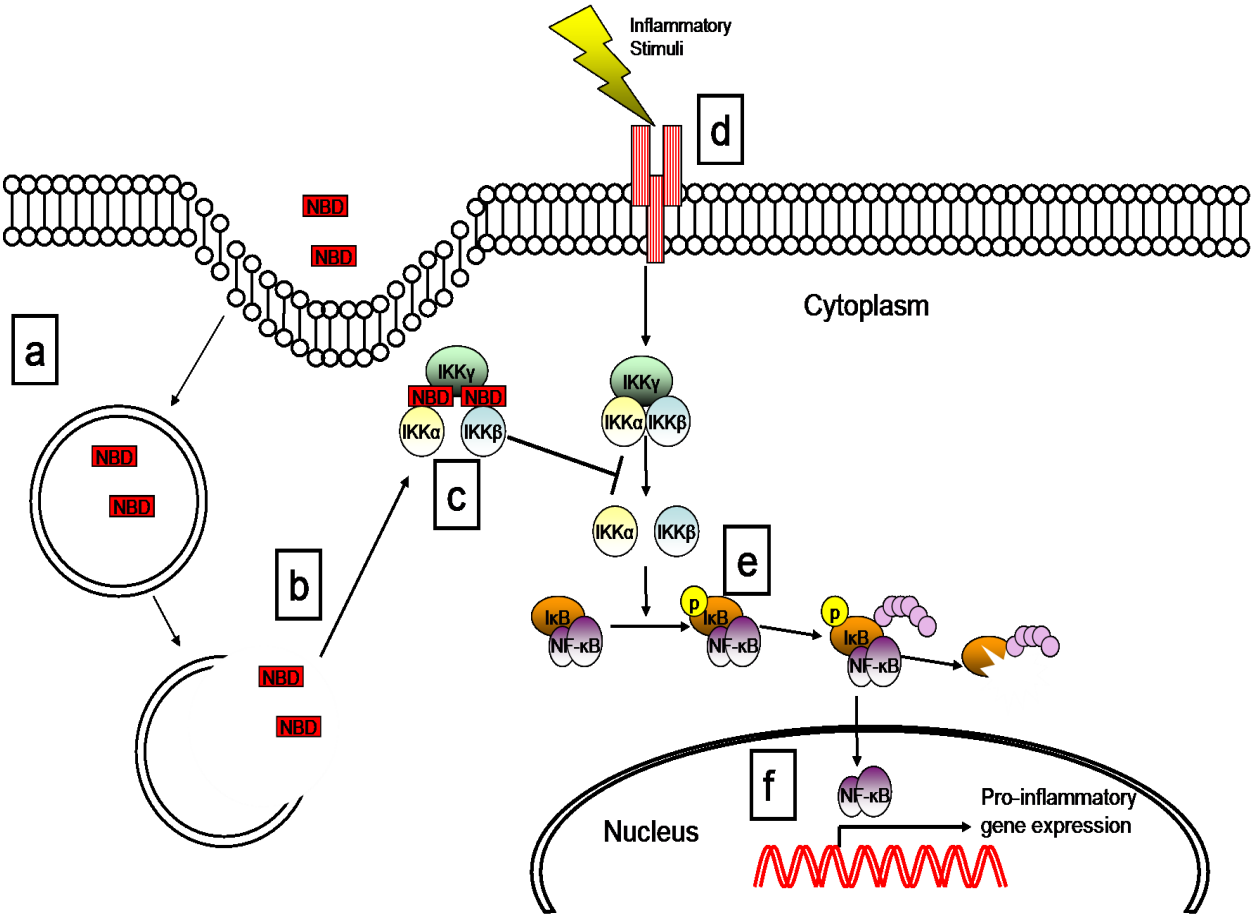


Figure 4: NBD inhibits NF-κB (A) PTD-NBD enters the cell due to its positive charge interacting with the negative charge on the cell surface mediating receptor independent endocytosis (B) NBD escapes the endosome via retrograde transport or Golgi/ER (endoplasmic reticulum) endosome fusion. (C) NBD interacts by binding to IKK*γ* and preventing the association with the *α* and *β* subunits. (D) This prevents the activation of NF-*κ*B stimulated by the pro-inflammatory signals (E) which leads to the phosphorylation of I*κ*B and its subsequent degradation. (F) Finally NF-*κ*B dimer p65p50 is released and then translocates to the nucleus whereby it transcribes pro-inflammatory genes. Reproduced with permission, J. Tilstra, K.K. Rehman, T. Hennon, S.E. Plevy, P. Clemens, and P.D. Robbins, 2007, Biochemical Society Transactions, Vol 35, 811-815(http://www.biochemsoctrans.org/)

inhibiting footpad swelling (paper in preparation). Although the reasons for the difference in activity observed in cell culture and *in vivo* is unclear, we speculate it is due to the ability of 8K and 6R PTDs to persist *in vivo* or to efficiently transduce the appropriate target cells, most likely antigen presenting cells *in vivo*.

1.1.13 Treatment of murine disease models with 8K-NBD

Prior to and during the preparation of this dissertation numerous groups have evaluated the use of the NBD peptide. Chronic administration of PTD-NBD is efficacious in the treatment of numerous murine models of degenerative disease including arthritis[71], diabetes[67], Parkinson's disease[72], muscular dystrophy (MD)[73], pancreatitis[74], and multiple sclerosis[75] (Table 2). Many groups have used either the TAT or Antp PTDs linked to NEMO while we have consistently used 8K-NBD. However, in each case the peptides have had efficacious results in ameliorating disease states. The syndromes evaluated by our laboratory or in collaboration with others include type I diabetes, collagen-induced arthritis (CIA)[67], and Duchene muscular dystrophy (DMD) [67, 73].

1.1.14 Use of PTD-NBD in this manuscript:

As NBD has proven to have efficacy in numerous models of inflammatory disease, as well as, improved bioavailability over other NF-κB inhibitors, this peptide was used to evaluate the effects of NF-κB suppression in two murine models of disease in this manuscript. Not only will NBD have the potential to be a possible therapeutic, but it is also an appropriate tool to evaluate contribution of NF-κB to disease pathogenesis. Here I will assess the effects of NF-κB

Table 2: Use of NBD peptide in mammalian models of human disease

Disease	PTD	Length of Trt	Result of NBD treatment	Pub.
Acute Inflammation (PMA ear edema, Zymosan -induced peritonitis)	Antp	100μM Single dose	Reduced ear thickness, and decreased exudate	[14]
Multiple Sclerosis (EAE model)	Antp	1.0mg/kg q24 hrs for 31 days	Reduced clinical score, Th2 phenotype skewing, Decreased inflammatory cytokines	[75]
Inflammatory Arthritis (arthritogenic serum addition)	TAT	3.0 & 6.0 mg/kg at d0 and d3 of 7 day experiment	Reduced osteoclast formation and bone destruction	[71]
Arthritis (Collagen induced arthritis)	Antp	Daily injections for 30 day	Reduced inflammation and bone destruction, reduced inflammatory cytokine,	[76]
Colitis (DSS and TNBS)	Antp	DSS 0.1mg/kg for 10 day, 1.0mg/kg 3 doses over 48 hrs	Improved histology and decreased cytokine production	[77]
Parkinson's (MPTP)	Antp	0.75 mg/kg given at 1d prior to or 2d after MPTP daily until 7d after MPTP injection	Increased dopaminergic neurons, reduced CD11b infiltration	[72]
Duchene Muscular Dystrophy (MDX mice)	?	Prevention: daily injection 10mg/kg from d6 to d22 Treatment: 200μg q3d from d23 to d50	Decreased macrophage and inflammatory infiltrate, improved muscle regeneration and function	[73]
Acute Pancreatitis (cerulein-induced)	? (Antp)	600μg injection prior to cerulein injection	Reduced inflammation, and MPO activation	[74]
Colitis (IL-10-/- mice)	8K	2 and 10mg/kg 10 of 14 days	Reduced macroscopic and microscopic histologic inflammatory changes, reduced cytokine secretion	[78]
Pancreatitis (Sodium taurocholate (5%) (RAT)	TAT	13mg/kg	Significant reduction in tissue necrosis and improved histology	[79]
Acute respiratory distress syndrome (surfactant induced) (PIG)	Antp	1.25 mg per piglet	improved gas exchange, lung function and reduced pulmonary inflammation	[80]

signaling in both a model of inflammatory bowel disease (IBD) and a model of accelerated aging.

1.2 INFLAMMATORY BOWEL DISEASE (IBD)

Inflammatory Bowel Disease (IBD) affects nearly 1 million people in the United States and includes two diseases, ulcerative colitis and Crohn's disease. The patho-physiology of ulcerative colitis and Crohn's disease are similar in that both are inflammatory in nature and involve dysregulation of cytokines and inflammatory cell infiltrates. Genetic factors, immunological factors, and environmental triggers all play a role in the pathogenesis[81]. There is continuing research on understanding of the pathogenesis of IBD and evaluating new treatment possibilities. Unfortunately, current therapies often do not control symptoms and are associated with significant side effects. A recent study evaluated IBD disease outcome comparing 1998-1999 with 2004-2005[82]. From 2004-2005, one in every 12.5 patients with Crohn's disease and one in every 20 patients with ulcerative colitis were hospitalized secondary to complications from their disease. In addition, 1 in 28.5 patients with Crohn's disease required surgery a ration not significantly decreased from 1998-1999[82]. A much lower number of patients with ulcerative colitis underwent surgery[82] however, often times these surgeries are more invasive and result in colonic resection (colectomy). Nearly 8% of patients with ulcerative colitis will undergo colectomy within five years of diagnosis and 28% by 10 years.[83] The most successful current treatment for Crohn's disease is infliximab, an artificial/chimeric antibody that blocks TNFα. Infliximab is successful in reducing disease severity and fistula formation, but approximately one third of patients do not respond to this therapy[84]. In addition to nonresponders, some patients

develop anti-chimeric antibodies which block the infliximab[85]. Furthermore, infliximab treatment has been linked with increased susceptibility to infections, most importantly tuberculosis, as well as a small, but increased risk of lymphoma. Thus, there is still a great need for improved understanding of IBD physiology and other targeted therapeutics which can reduce symptoms associated with disease.

1.2.1 Pathology of IBD

The two IBD variants are differentiated and distinguished by their localization in the bowel as well as their pathophysiology. Crohn's disease is usually defined as a transmural disease affecting all the layers of the colon including epithelium, mucosa, submucosa, muscle, and serosal layers. Crohn's disease is also defined by skip lesions, small regions of inflammation affecting various areas of the intestine from the esophagus to the anus; however, is most common focused at the ileo-colic junction (Figure 5). Microscopically Crohn's disease is defined by crypt abscesses, villus atrophy, mononuclear infiltrate, which contributes to mucosal thickening and non-caseating granuloma formation. Ulceration, fissures, and thickening of all layers of the intestine (Figure 6) are macroscopic components of Crohn's disease, as well as secondary formation of fistulas and abscesses.

In contrast, in ulcerative colitis occurs only in the colon and is limited to the epithelial, mucosal and occasionally submuscosal layers. Inflammation in ulcerative colitis leads to a loss of the mucosal layer and formation of pseudopolys (intact tissue amongst loss of mucosal tissue) (Figure 5). Microscopically, ulcerative colitis is defined by numerous inflammatory cellular infiltrates associated with abnormal crypt and villus formation, ulceration, and mucosal thickening (Figure 6)[86-88].

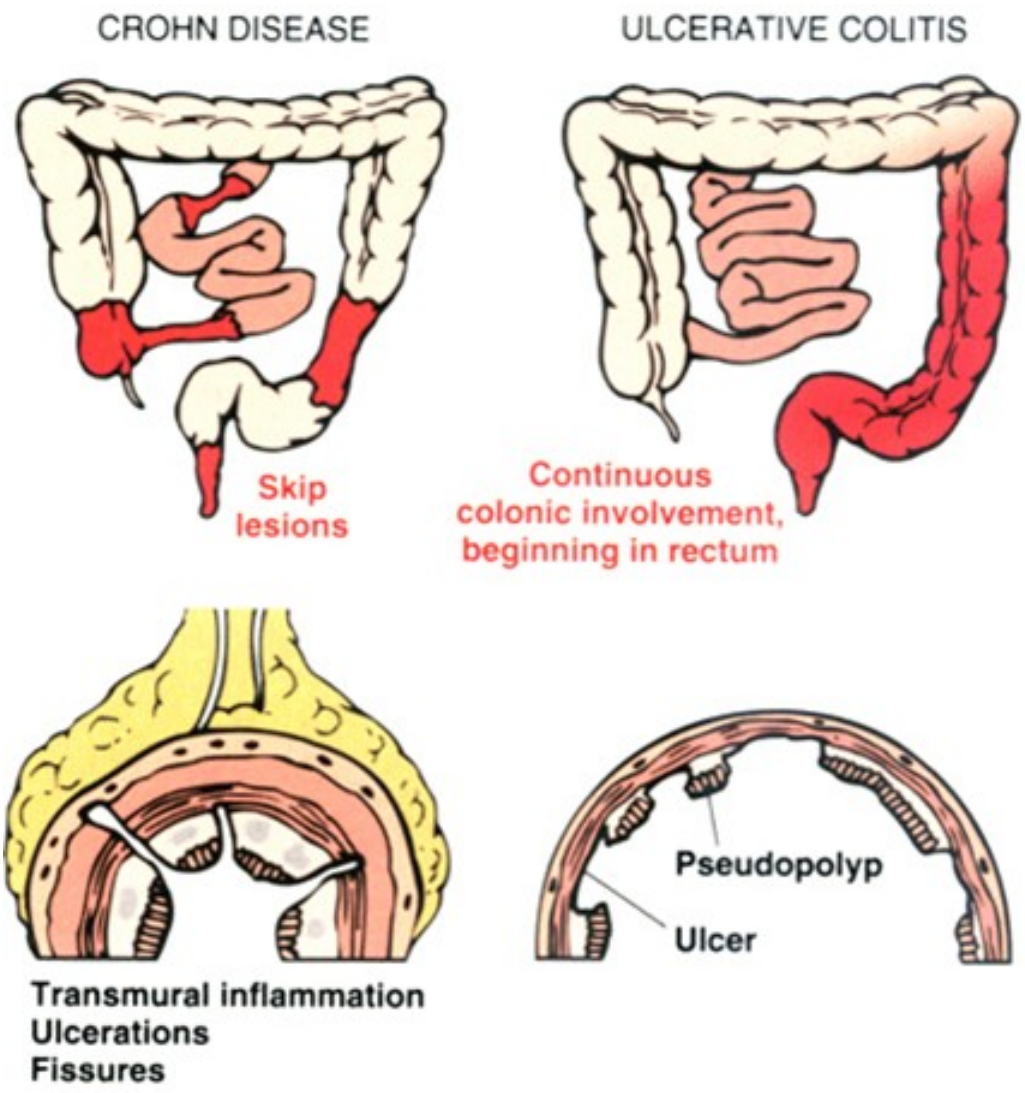


Figure 5: The distribution patterns of the two variants of IBD. The top panels describe the location and generalized pattern of disease within the small intestine and colon of both Crohn's disease and ulcerative colitis. The bottom two panels describe the macropathologic changes in the two variants. (Courtesy of Elsevier originally published in Kumar: Robbins and Cotran: Pathologic Basis of Disease, 7th ed.[89])

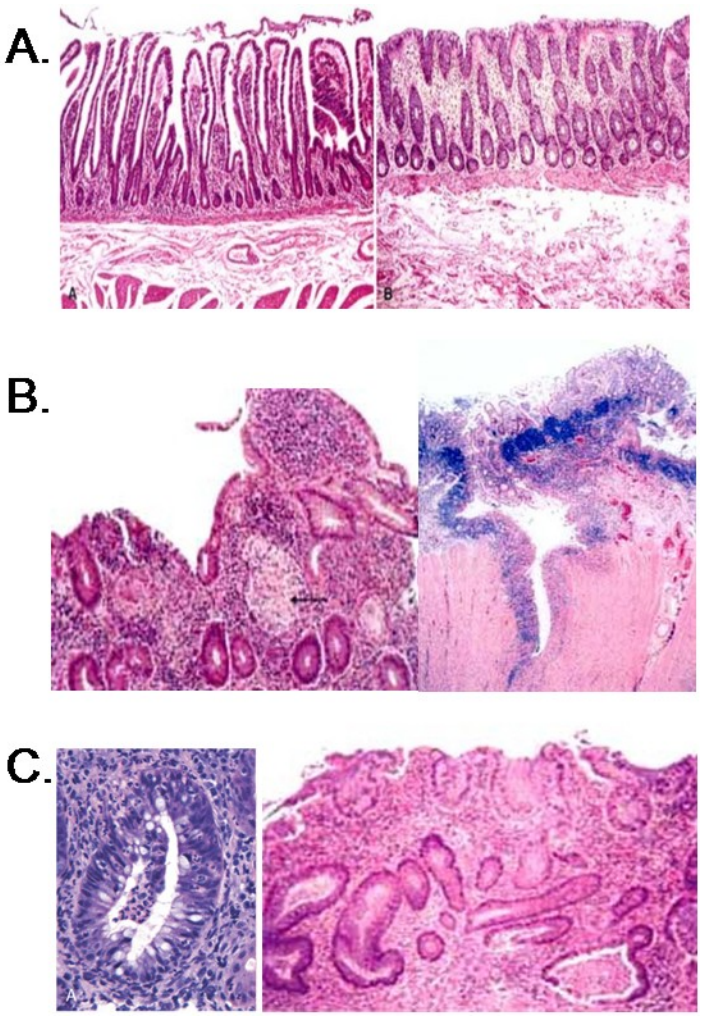


Figure 6: Histopathology of IBD. (A) Normal small intestine/colon with crypt and villus formation. (B) Crohn's disease micrographs, Left image, intestine is exhibiting inflammatory (mononuclear cell) infiltrate with non-caseating granuloma formation and loss of normal villus structure. Right image, transmural fissure (center) which extends through epithelium, submucosa, mucosa and into the muscle layer, with large inflammatory infiltrate exhibited between the mucosa and submucosa (blue patches), and shallow ulcer (right). (C) Ulcerative colitis micrographs. Left image, crypt abcess formation Right image, loss of villus structure inflammatory infiltrate into mucosal layer (Courtesy of Elsevier originally published in Kumar: Robbins and Cotran: Pathologic Basis of Disease, 7th ed.[89])

1.2.2 Extra-intestinal Manifestation of IBD

IBD pathology is normally localized to the intestine, but is in fact a systemic disease which has numerous extraintestinal manifestations that can affect several organ systems as reviewed in [88, 90, 91] and summarized below. Arthritis is the most common extaintestinal manifestation of IBD. Manifestations of arthritis are varied, but three major forms include ankylosing spondilitis, sacroilitis, and migratory monoarthritis. Ankylosing spondilitis is a chronic inflammatory arthritis which effects the spine and sacroilium causing severe back pain and stiffness. The spine eventually fuses and causes rigidity and debilitation. Sacroiliitis is inflammation localized to the sacroiliac joint and causes low back pain. Patients with IBD can also experience migratory monoarthritis which affects large joints usually one at a time and runs a course unrelated to that of the intestinal disease. Other extraintestinal manifestations include: dermatologic changes including erythema nodosum (inflammation of the fatty layer of the skin also seen in sarcoidosis and infectious mononucleosis), pyoderma gangrenosum (a cutaneous ulcerative lesion thought to do with neutrophil dysregulation, and is also seen in seropostive arthritis and less commonly systemic lupus erythema (SLE) and Sjogren's syndrome)[92], and aphthous ulcers (well circumscribed ulcers of the buccal mucosa in the mouth also seen in SLE)[93]. Interestingly, these signs and symptoms are characteristics of other inflammatory disorders as well, underlying the systemic inflammatory nature of IBD.

1.2.3 Inflammation and role in IBD:

It is likely that an exaggerated immune response to enteric bacteria plays a significant role in the development of disease. The intestine is normally considered a suppressive

environment from an immunologic perspective, and dysregulation of this suppressive environment is thought to be a major contributor to the pathogenesis of IBD. In numerous mouse models of IBD, mice do not generate disease under germ free conditions[94], and further both ulcerative colitis and Crohn's disease are localized to the ileal-colic junction and colon, the two areas of intestine with the highest bacteria burden[87].

Features of the intestine closely resemble other lymphoid organs. Macrophages, dendritic cells, monocytes, and lymphocytes reside in the lamina propria and submucosal layers of the gut. In addition, the intestine has intrinsic lymph tissue known as gut-associated-lymphoid tissue (GALT) also described as Peyer's patches (in the small intestine) and follicles (in the colon). GALT contains a high percentage of T and B-cells. In contrast to other lymph organs, GALT has many suppressive elements.

The gut secretes numerous molecules to prevent invasion of bacteria, however these molecules do not activate a gross inflammatory response. For example, the major antibody secreted by the intestine is immunoglobulin A (IgA), which acts as a dimers and is linked together by the J-chain, allowing for its secretion into the intestinal lumen. Importantly, IgA is unable to activate complement or bind Fc receptors. Thus IgA acting as a neutralizing agent binding to bacteria and preventing invasion, but not as an inflammatory activator[95, 96]. The intestinal epithelial layer includes the villus or surface epithelium (intestinal epithelial cells (IECs), goblet cells, neuroendocrine cells, and paneth cells. Each of these cells in the epithelial layer provides microbial protection without overt stimulation of the immune system[87]. Goblet cells secrete mucin and glycoproteins, creating the glycocalyx. which overlays the epithelium and helps prevent bacterial invasion. Paneth cells are responsible for secreting defensins and anti-microbial peptides. Deficiencies in the defensins secreted by the paneth cells can exacerbate

or increase the risk of IBD development[97]. Further, IECs express CD1d which leades to T regulatory cells (T-reg) or suppressive T-cells, and the IECs are one of the few non-professional antigen presenting cells (APC) to express MHC class II molecules[98]. APC from the intestine have suppressive qualities. Mucosal macrophages in the intestine are also more suppressive than there general counterparts. These mucosal macrophages do not express CD14, a co-receptor for TLR4[99], and produce less inflammatory cytokines (TNFα and IL-1β) than other macrophages. There is also a special population of intestinal dendritic cells which sample apoptotic IECs and tolerize naïve T-cells against self antigens[100]. In addition to DC tolerization of T-cells, lamina propria T-cells do not proliferate due to TCR engagement, but seem to activate alternative pathways via CD2 and CD28. These findings exemplify the role of intestinal immune response as a protective yet suppressive environment.

Unfortunately, in IBD the normally suppressive environment in the intestine becomes an activated environment. Immune dysregulation and activation appears to be a common factor in both ulcerative colitis and Crohn's disease although the immune activation differs between them. While ulcerative colitis and Crohn's disease are distinctive diseases, the clinical picture can vary from patient to patient even with the same pathology and often colonic Crohn's disease is confused with ulcerative colitis. It is traditionally thought that ulcerative colitis is a T-helper 2 (Th2) skewed disease marked by increased IL-5, IL-6 and IL-13 secretion whereas Crohn's disease is a Th1 skewed disease which is associated with increased IL-12, Il-23, and tumor necrosis factor alpha (TNFα) secretion[101]. There are numerous reports of an over active NF-κB pathway in patients with Crohn's disease[102]. In ulcerative colitis it is thought that the relative deficiency of interleukin 10 (IL-10), which has anti-inflammatory and NF-κB inhibitory effects, may contribute to persistent inflammatory changes[14, 103].

1.2.4 NF-κB and IBD

Evidence suggests that the pathophysiology of IBD is secondary to intestinal immune dysregulation. It is thought that NF-κB is a master switch for inflammatory gene expression including cytokines, chemokines, adhesion molecules, and cellular defense molecules[22, 104]. Furthermore, activation of NF-κB has been shown to be important in a wide variety of inflammatory and non-inflammatory diseases[15]. The role of NF-κB in IBD disease pathology has been defined by studies of patients with IBD and examination of numerous mouse models of intestinal inflammation. Activated NF-κB is found Interestingly, numerous studies have shown that *IL-10*$^{-/-}$ mice develop colitis only in the presence of bacteria[105]. in inflamed mucosa and macrophages of patients with IBD [102, 106]. RelA/p65 was shown to be overexpressed in the intestines of patients with ulcerative colitis and Crohn's disease. Further analysis of lamina propria macrophages derived from the IBD patients showed increase DNA binding of NF-κB subunits, which were responsible for inflammatory cytokine production, IL-1β, IL-6, and TNF[106].

Initial studies focusing on the role of NF-κB in development of IBD was completed in animal models of colitis. The most commonly used genetic model of colisi is the *IL-10*$^{-/-}$ mouse. This mouse developes normally in utero, however, at approximately 4 weeks of age these mice exhibit weight loss and anemia, which coincides with the colonization of their intestines with bacteria, which is necessary for the development of colitis in this model[105]. Interestingly, when either *IL-10*$^{-/-}$ or wild-type mice were exposed to intestinal bacteria, they exhibited increased NF-κB signaling in the enterocytes of the intestine, however, as the *IL-10*$^{-/-}$ mice alone develop intestinal inflammation by seven weeks of age, which is defined by inflammatory infiltrations, villus atrophy, and ulceration. It was origiannly observed that *IL-10*$^{-/-}$ mice exhibit increased

NF-κB signal in the lamina propria as well as inflammatory cell populations including macrophages, dendritic cells, T-cells, and B-cells[107]. Thus, NF-κB inhibitors were evaluated in this model, specifically after it was observed that NF-κB DNA binding was increased in lamina propria macrophages derived from *IL-10*$^{-/-}$ mice[108]. *In vivo* administration of the NF-κB antisense oligonucleotide successfully treated established colitis in both chemical-induced and spontaneous occurring colitis of *IL-10*$^{-/-}$ mice[109], as shown by reduction in macroscopic and histological pathology of colitis [108, 110, 111].

NF-κB is clearly implicated in the pathogenesis of IBD, but NF-κB activation is not entirely detrimental. NF-κB is necessary to block invasion of pathogens; conditional deletion of NEMO or IKKα and β to the intestinal epithelium led to massive intestinal inflammation subsequent a loss of the eptheliums protective function [112] suggesting that NF-κB is protective and can prevent the onset of intestinal inflammation. However, NF-κB activation in inflammatory cells can have a negative role by propagating inflammation once initiation of disease occurs.

In addition to these murine studies, numerous IBD therapeutics used to treat human disease have been shown to inhibit NF-κB signaling. Sulfasalazine, which is frequently used for treatment of Crohn's disease and is currently used for ulcerative colitis. Sulfasalazine inhibits NF-κB by blocking IκBα degradation and inhibiting RelA phosphorylation[113]. The former mainstay of IBD therapy, corticosteroids, have inhibitory effects on NF-κB by suppressing transcription through activation of histone deacetlyases[114]. Even the most notable current IBD therapy, the TNFα antibodies, infliximab and adalimumab, referred to as disease modifying biologic therapies, target the NF-κB pathway by blocking one of its activating factors TNFα[101].

It appears that increased NF-κB signaling, specifically in the immune compartment, myeloid and lymphoid cells, is a major component of the pathophysiology of IBD. Furthermore, the majority of therapeutics used to treat IBD suppress NF-κB signaling. Interestingly, genetic studies (discussed below) evaluating common gene variants associated with IBD, implicate genes involved in inflammatory signaling and specifically with NF-κB. Overall, while NF-κB may have differential roles in disease development depending on cell type it appears that suppression of NF-κB may have beneficial effects in treating IBD.

1.2.5 Genetics of IBD

Crohn's disease has been shown to have a higher concordance of disease in monozygotic twins than ulcerative colitis, suggesting a greater genetic component[115]. Interestingly, in familial studies 80% of patients have the same disease type (Crohn's disease or ulcerative colitis). However, in 20% of cases disease affecting multiple family members is mixed, suggesting that there is a combined genetic susceptibility for all IBD diseases[115]. There are three major gene SNPs which are associated with Crohn's disease including NOD2 (CARD15), TNFSF15, and IL23R.[115] It has been shown that while NOD2 SNPs are associated with Crohn's disease alone, SNPs of IL23R, IL12B, and STAT3 are associated with both ulcerative colitis and Crohn's disease. Importantly, the above genes are all involved in immune regulation suggesting a genetic component contributing to the dysregulation of the immune system. There are also other genetic components associated with IBD that are not specifically inflammatory related genes including ATG16L1, IRGM, NKX2-3. However, each has been shown to play a role in immunologic control of bacterial invasion or inflammatory signaling[87]. These genes that are associated with

immune signaling are not only genetic predisposing factors, but may also contribute to pathology.

NOD2 is a pattern recognition protein which leads to NF-κB activation. A loss of function mutation in NOD2 appears to lead to overactive NF-κB signaling over time[116]. IL-23 and IL-12 are inflammatory cytokines from the same family, both of which lead to the activation of lymphocytes. Using specific genetic models, IL-12 and IL-23 have been shown to contribute to IBD pathogenesis. More recent evidence suggests IL-23 signaling is highly important in IBD, both in its role as a innate immune signaling factor as well as its role in differentiation and activation of the pro-inflammatory Th17 population[117]. Furthermore, Stat3 is a downstream signaling component of the IL-23 pathway thus suggesting another important role for this inflammatory signaling pathway in pathogenesis of Crohn's disease. Interestingly, IL-23/Il-12 production is transcriptionally regulated by NF-κB, specifically RelA, C-rel, and p50[118]. IL-23 signaling can also activate NF-κB transcription via degradation of IκBα and allowing for IL-1 production[119]. Each component of genetic susceptibility, with regards to IBD, implicates the NF-κB signaling pathway, further confirming that the NF-κB pathway is an appropriate target for therapeutic intervention.

1.2.6 Evaluation of NF-κB in IBD

The above data suggests that overactive NF-κB signaling has a causal role in IBD pathology, whether it is through initiation and or progression. Current therapies for IBD are improving with the advent of biologics. Other therapies need to be developed due to the lack of efficacy of current therapies in a large fraction of patients as well their significant side effects. It will be imperative to further address the specific roles of NF-κB signaling in this disease to help

with the development of new therapies. Additionally, the evaluation of NF-κB suppression in IBD will have potential therapeutic and mechanistic consequences to help tailor a more direct, targeted treatment for IBD patients.

1.3 INFLAMMATION AND AGING

Aging is usually defined as the advancement in chronologic age; however, aging is often characterized by the presence of aging-associated diseases, such as kyphosis, cachexia, sarcopenia, frailty and neurodegeneration. Interestingly, the greatest risk factor for a wide variety of diseases is an increase in chronologic age. The risk of cancer increases 10 fold for patients over 65 compared to those under 65[120]. In fact the trend for many age-associated diseases including cardiovascular disease, neurodegeneration, diabetes, arthritis and osteoporosis is similar[121]. Furthermore, there is to be an exponential increase in disease with a linear increase in chronologic age[120] suggesting specific mechanisms are contributing to this change. By determining what these mechanisms are and how they contribute to aging; in the future scientists and clinicians may be able to use systemic therapies to reduce several age-associated pathologies simultaneously. The number of individuals over the age of 65 will double over the next 25 years[122], and the average age of our population is increasing. In fact 27% of the 327 billion dollar Medicare budget in 2008 was spent on care in a patients last year of life[123]. This reveals the tremendous burden that age-related diseases can have on our health care system and our national economy. Therefore, understanding the mechanisms of aging and defining therapies to reduce this burden is of critical importance.

1.3.1 Stochastic Theory of Aging

There are two predominant theories to explain why aging occurs, either through genetic contributions and/or time dependent damage events which lead to altered cellular function. The theory of programmed genetic aging has recently fallen out of favor, but may have potential implications with regards to the aging process. While aging is not likely the result of genetic programming, it may be in part attributed to a evolved trait that leads to a reduction in somatic maintenance after an animal reaches sexual maturation[121].

There may be some evolutionarily based gene and protein functions which contribute to the aging process. For instance, the maximum age of mice bred in captivity is approximately three years of age; while in the wild, mice live an average of one year. Thus an investment in gene expression that would block age-related changes at three years would not be selected for in the wild as compared to a gene that would allow mice to survive a cold harsh winter (the most common cause of death in wild-type mice)[121]. For example, mice likely have evolved gene expression which allows for increased storage of fat deposits to allow them to surivive a cold winter, however, this same evolved fat storage trait may lead to an increase in fat storage which leads to increased cytokine secretion, reduced insulin. Furthmore, if energy is diverted to energy and fat storage, then maintence of other cellular material and organelles such as mitochondira and DNA may suffer, thus leading to increased cellular damage and age-associated changes. In whole this suggests that genes which promote young mouse survival but not aged survival are selected for during the evolutionary process. Therefore it is likely that aging is not an evolved trait but possibly a byproduct the shortened lifespan and the perils of survival for animals in the wild.

The stochastic theory of aging, the foremost accepted theory of aging, states that endogenous and exogenous damage lead to breakdown of macromolecules and organelles, ultimately resulting in cellular and organismal senescence and aging[124]. There are numerous hypotheses regarding the source of damage events which contribute to aging including, somatic mutations, mitochondrial/ROS, telomere shortening, and accumulation of waste, all of which likely contribute to stochastic aging as described by Kirkwood[121], and are discussed in greater detail below.

1.3.1.1 Somatic Theory of Aging

The somatic theory of aging centers on damage to DNA. Several lines of evidence support the hypothesis that DNA damage is the most important type of molecular damage that contributes to aging. First, DNA lesions and genetic mutations caused by DNA damage accumulate in tissues of aged organisms [125, 126]. Second, mice harboring germ-line mutations that confer resistance to genotoxic stress are long-lived[127,128]. Third, the majority of human progerias (or syndromes of accelerated aging) are caused by inherited mutations in genes required for genome maintenance (described in 1.3.5)[129]. For example, deficiency of the DNA repair endonuclease ERCC1-XPF causes progeria[130]. Additionally, members within mammalian species with increased Poly(ADP-Ribose) polymerase levels, an integral member in stress response to DNA damage, have increased lifespans[131]. Comparison of the transcriptome of DNA repair deficient mice and old wt mice revealed a highly significant correlation that was recapitulated in young wt mice exposed to genotoxic stress[130]. Also mice exposed to long term low doses of irradiation develop phenotypes and gene expression profiles similar to that of normative aging animals. These data suggest that accumulation of DNA damage is a major contributing factor to mammalian aging.

1.3.1.2 Mitochondrial Damage/ Reactive Oxygen Species (ROS) contribuation to Aging

Mitochondrial damage and subsequent ROS production is thought to be the other major contributing factor to stochastic aging along with DNA damage. Mitochondria, often defined as the energy center of the cell, are responsible for producing ATP using oxidative phosphorylation[132]. Aged mitochondria in old and postmitotic cells, showed uncoupling demonstrated by a loss in mitochondrial membrane potential and accumulated damage of mitochondrial DNA (mtDNA)[133]. Damage to mitochondria results in increased ROS production. This increased ROS in the cytoplam negatively impacts the cellular environment by causing further damage mitochondria, other organelles, and resulting in DNA damage. Depite numerous sources of ROS within the cell, nearly 90% of these can be traced to the mitochondria[132]. Additionally, transgenic mouse with increased susceptibility to mtDNA damage, developed a shortened lifespan and symptoms of premature aging[134]. In addition to mtDNA damage, loss of mitochondrial function induced aging is in part carried out by increased ROS production. A murine model with reduced capability to degrade of H_2O_2 resuled in disease mimicing age-associated neural degeneration[135]. Furthermore, cells with p21 and Ras mutations resulting in premature senescence, exhibited increased ROS production[136], and mouse embryonic fibroblast (MEF) replicative senesecnce can be induced using 20% oxygen compared to 3% O_2 growth conditions[130, 137]. Thus, damaged mitochondrial due to mtDNA damage and increased oxidative stress can advance the onset cellular senescence, and likely contribute to pathologies associated with aging.

1.3.1.3 Shortening Telomeres Contribution to Aging

Telomeres are evolutionarily conserved repeating DNA sequences meant to protect the ends of linear chromosomes from DNA damage responses[138]. There is a loss of nearly 100-200

base pairs per replication cycle[139, 140], and this shortening may result in recognition of telomere ends as sites of damage by DNA repair machinery. This DNA repair response to the chromosome ends can result in cell death or senescence.[138] Additionally, exogenous expression of the catalytic subunit of telomerase in primary human cells led to their immortalization[141]; suggesting that telomerase plays an integral part in cellular senescence and aging. However, there is no evidence that increased telomere length leads to reduced aging *in vivo*, in fact, nematodes with altered telomere length had no change in lifespans[142]. Additionally, animals with significantly increased telomere length still undergo the aging process. One prime example is mice which have longer telomeres than humans but live only for a maximum of 3 years. Therefore the length of telomers alone does not confer resistance to aging, but perhaps the maintenance is integral to reduce cellular senesense. In support of this theory, recent studies suggest that oxidative and damage events may have a greater role in telomere shortening than replication[121, 143]. Von Zglinicki et al. observed that fibroblasts have significantly increased rates of telomere shortening under conditions of oxidative stress and suggests that alkylation, UV exposure, and irradiation induced damage has the same effects[143]. Thus, shortened telomeres, which may be responsible for replicative senescence, may contribute to aging secondary to stochastic damage.

1.3.1.4 Altered Protiens and Waste Accumulation Theory of Aging

The final contributer to the stochastic theory of aging is the accumulated waste and altered protein hypothesis. Numerous diseases of aging are associated with accumulation of damaged proteins namely Parkinson's, cataracts, and Alzheimer's. With aging there is a decline in the function of proteosomes and chaperones which alters waste removal and causes a build-up of proteins within cells[121]. Related findings suggest that numerous deficiencies in cellular waste

disposal including proteases, DNA repair enzymes, autophagy, and lysosome activity increase with age[144], likely secondary to damage events. Therefore, as damaged macromolecules accumulate within a cell, a loss of cellular homeostasis can occur. For example, an accumulation of mutated proteins or altered proteins such as amyloid in Alzheimer's can contribute to cellular dysfunction or generating immune response leading to associated pathology. Furthermore, when mitochondria age they become dysfunctional losing their membrane potential and increasing ROS production. Due to decreased autophagy within the cells, these mitochondria persist and contribute to the increased intracellular damage[145], leading to an increased loss of cellular homeostasis.

1.3.1.5 Accumulated Damage and Composite Stochastic Theory of Aging

While evidence supports each of the components of the stochastic theory of aging described above (somatic mutations, mitochondrial damage, telomere shortening, and altered waste disposal), it is likely they are inter-related components in the aging process. Each stochastic event reinforces and contributes to the changes described by each of the damage hypotheses. For example, damage to the mitochondria causes an increase in ROS production. As ROS levels increases inside the cell, this leads to a loss of homeostasis with increased damage to mitochondria other cellular components including DNA as well as contributing to more rapid telomere shortening. Because the aging cell has reduced autophagic response these defective mitochondria cannot be degraded, and continue to produce the ROS. Simultaneously, damaged mitochondria produce less ATP contributing to reduced protein production (possibly less proteosome, autophagic proteins, or DNA repair proteins), which subsequently results in reduced cellular repair and promotes further dysfunction within the cell.

A second example is endogenous DNA damage which passes to the daughter cells following cell division. Dysfunctional proteins are created as a byproduct of this damage leading to an increased need for degradative processing. This results in reduced capacity to repair additional cellular damage resulting in loss of homeostasis. In each case, damage to individual cellular compartments triggers a cascade event contributing to the aging-cell phenotype. The stochastic theory of aging encompasses several theories of aging, each of which likely contributes to the overall aging phenotype. In each case, damage is the root cause of aging seen at the cellular and organismal level. In addition to the damage itself, it appears that the way the cell manages this damage response is integral to the aging process as a whole.

1.3.2 Damage Response and Aging

The most common cellular response to damage is repair, which results in normal cell function. In the event that cellular damage remains unrepaired, cells either apoptose, senescence, or in rare instances transform into tumor cells. The damage responses not including repair all results in alterations which affect cellular function and interaction with the surrounding environment. Recent evidence suggests that the apoptosis and senescence response to damage is a mechanism by which cells reduce tumorigeneity; however these changes then contribute to the aging process[146]. Replicative senescence is defined permanent cell growth arrest which results in altered cellular function. Senescence is often described as aging at the cellular level and is characterized by an altered morphology such as, increased nuclear to cytoplasmic ratio, altered actin structure and a flattened sprawled appearance. Senescent cells are distinguished by increased $p16^{ink4a}$ and SA-β-galactosidase expression[147]. Cellular senescence cab be accelerated by damage events such as DNA damage[130, 148] or oxidative stress[149]. Thus, previous studies on

the pathogenesis of aging have focused on damage and its contribution to the aging process. However, a growing area of interest is now examining not only these damage events but also the damage response and their subsequent effects on aging and age-related diseases.

1.3.3 NF-κB as a component of stress response and aging

Stress response pathways implicated in aging include IGF-1, mTOR, SIRT1, p53, and NF-κB[150]. Of these, NF-κB, is of considerable interest, as it is a transcription factor has been implicated in both oxidative stress and DNA damage response pathways. Additionally, Adler et al. defined NF-κB as the transcription factor most associated with aging[151]. NF-κB is traditionally thought of as a mediator of immune and inflammatory responses, but is also activated in response to many stress stimuli including genotoxic, oxidative, mechanical and other environmental insults that cause cell damage[4]. Specifically, DNA damaging resulting from UV rays, γ-irradiation, topoisomerase poisons and ROS activate NF-κB[152, 153]. While NF-κB signaling is activated in conditions associated with aging, senescence, and age-associated changes, it is not known whether it plays a protectice or degenerative role.

Overexpression of two NF-κB subunits, c-rel and RelA/p65, induced a senescent phenotype in cells[154-156]. In concert with these findings, evaluation of skin-derived human fibroblasts from aged individuals (aged 72-93), and HGPS progeria patients (8-14) showed increased levels of NF-κB activation when compared with cells derived from young individuals (aged 22-33 and 8-14 respectively)[151, 157]. These cellular findings are further supported by studies that observed NF-κB upregulation with age in several tissues including skin, liver, kidney, cerebellum, cardiac muscle and gastric mucosa[158-163]. Adler et al. first observed that genetice depletion of NF-κB in aged mouse skin reversed age-related pathology and gene

expression changes, suggesting a beneficial role for NF-κB inhibition in reversing age-related degeneration[151]. Kawahara et al. then evaluated NF-κB in aging using the *sirt6*$^{-/-}$ mouse, which exhibits degenerative changes. Experiments using haploinsufficient p65 (NF-κB subunit) background resulted in 40% of the *sirt6*$^{-/-}$*p65*$^{+/-}$ mice exhibit improved growth and longer lifespan than their *sirt6*$^{-/-}$ littermates[164]. While this provides some evidence of the role of NF-κB activation in aging, the *sirt6*$^{-/-}$ mouse model exhibits a severe colitis phenotype, suggesting that these mice may have chronic colonic infection leading to NF-κB activation and more of a illness induced degeration rather than a true aging phenotype[165]. Thus, further exploratory studies are necessary to elucidate the role of NF-κB inaging.

One compentent contributing to increased NF-κB activity which occurs with aging is the altered transcriptional phenotype which occurs in senescent cells. A recent article by Coppé et al. defined a specific senescent-associate secretory phenotype (SASP) in senescent cells. SASP is a highly pro-inflammatory phenotype consisting of increased expression of IL-6, IL-8, IL-7, MCP-2, MIP-3, ICAM, Il-1α, and Il-β, and was observed in numerous senescent cell types induced by varying methods. While senescence is initially a tumor-suppressive mechanism[166] there is likely numerous deleterious side effects of this anti-growth, pro-inflammatory senescent phenotype. It is important to note that the vast majority of these SASP profile cytokines and chemokines are transcriptionally regulated by NF-κB[167], again implicating this signaling pathway in aging on a cellular level.

1.3.4 NF-κB in Age-Associated Disease

Aging is widely considered a physiologic condition; however the majority of aging research focuses on age-associated diseases which are treated as pathologic conditions. These

age-associated diseases include Alzheimer's, Parkinson's, type II diabetes, atherosclerosis, sarcopenia, and osteoporosis. Therefore, aging which is defined by these diseases should be considered a treatable condition. One features that these diseases share is an increased inflammatory state; specifically NF-κB signaling and cytokine secretion are upregulated in atherosclerosis[168], osteoarthritis[169], neurodegeneration (Alzheimer's and Parkinson's)[170], osteoporosis[171], and cardiovascular disease[172] as discussed below.

1.3.4.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of age-associated dementia, and is characterized by fibrillary tangles and β-amyloid plaques.[173] AD affects only 0.6% of patients 65-69 years of age, but increases with age to 8.4% of 85+ aged patients. While the causes of AD remain poorly defined one common characteristic is the increased inflammatory state observed in patients suffering from AD. While an overt inflammatory infiltration is not evident in AD, IL-1β and TNFα are present at increased levels in the brains of AD patients, both of which are NF-κB transcriptionally regulated cytokines.[173] Murine studies have begun to confirm the role of cytokines including IL-6 an IL-1β in AD[174], for example mice which underwent brain injections of IL-1α or β exhibited increased AD associated plaque formation[175].

A possible mechanism for this increase in cytokines seen in AD is via Aβ stimulation of NF-κB activity in microglia (brain monocytes)[176]. Suppression of NF-κB in these microglia results in decreased neurotoxicity[177], and evidence suggests that NF-κB regulatory elements lie upstream of the APP protein, which is necessary for plaque formation[178]. The hypothesis that inflammation and NF-κB activation is the underlying cause of AD is further supported by observations that chronic LPS injections accelerate AD progression[179] and patients with systemic infection exhibit increased rates of cognitive decline[180, 181]. Additional studies evaluating links

between inflammation and AD is reviewed in [179] and [182]. On the whole, the evidence suggesting that NF-κB plays a role in plaque formation is strong, but likely more important is the role of NF-κB, inflammation, and cytokine signaling in AD progression.

1.3.4.2 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease that results in a movement disorder that is observed in patients over the age of 55 years[183]. As with neurodegeneration seen in AD patients, there is a similar increase in inflammatory and cytokine signaling in Parkinson's patients. There is a 70 fold increase in p65 activation in dopaminergic neurons, the neurons which are central to disease pathology, from PD patients compared with age-matched controls[184]. TNFα a is increased in both brain tissue and CSF[185] and further IL-1β, IL-2, IL-6 and other cytokines were shown to be increased in the CSF of Parkinson's patients[186]. Furthermore, microglia, the innate immune cells of the brain are highly active in PD and suppression of these cells in mouse models of PD result in reduced disease[183]. Using MPP, a chemical compound known to induce a Parkinson like disease, neurons show upregulation of NF-κB signaling, which was necessary for cell death[187]. The true role of NF-κB in causing neuronal cell death is still unknown, however it is likely that in both PD and AD, the chronic NF-κB activated inflammatory state act through primary or secondary mechanisms to contribute to neuronal cell death and progression of disease state[182, 188].

1.3.4.3 Type II Diabetes

Type II Diabetes (T2D) or non insulin-dependent diabetes results is defined by insulin resistance, and is normally accompanied by numerous sequela or co-morbidities, dyslipidemia, hypertension, atherosclerosis, and central obesity, blindness, end-stage renal disease, and non-

traumatic loss of limb[189]. NF-κB/IKK has been implicated in obesity-induced insulin resistance and glucose metabolism via pharmacologic and genetic suppression[190]. Upregulation of NF-κB signaling in hepatocytes results in a type II diabetes phenotype[191]. It is further thought that innate immune activation and inflammatory response underlie Type II Diabetes (T2D) and its associated features[189]. Macrophages or dendritic cells (DC) that reside in adipose tissue and secrete cytokines can contribute to insulin resistance and mediate disases progression[192]. Novel data suggests that mice deficient in DC have reduced body mass and are not susceptible to Type II diabetes when placed on a high fat diet (private communication). As with AD, systemic inflammation and cytokine secretion likely play a significant role in the onset and progression of T2D. IL-1β can induce β-cell cytotoxicity and inhibit β-cell function[193]. Mice deficient in TNFα signaling have been shown to be resistant to obesity induced insulin resistance[194]. Additionally, IL-6 as well as MCP-1, IL-1β and TNFα levels are found to be increased in T2D patients, however, the role of IL6 remains ill-defined[189]. Thus abherent NF-κB activation in numerous tissues including adipose, pancreas, and liver contribute to disease pathology observed in patients with T2D.

1.3.4.4 Atherosclerosis

Atherosclerosis is a disease of arterial wall thickening and plaque formation associated with increased age and is the leading cause of coronary artery disease. Atherosclerosis is a disease resulting from a combination of endothelial, hematopoietic, T-cell and macrophage dysfunction. Atherosclerotic lesions have increased levels of NF-κB activity, specifically in unstable coronary plaques[195]. In an LPS induced model of atherosclerosis, using *apoE*$^{-/-}$ mice, genetic suppression of NF-κB signaling led to a reduction in the size of atherosclerotic lesions[196]. As with other diseases associated with aging, this NF-κB upregulation is accompanied by an

increase in cytokine release and inflammatory signaling. A comprehensive review on the subject by Kleeman et al characterizes both the pro-atherogenic cytokines, IL-1, IL-12, IL-18, IFN-gamma, TNF-alpha, and M-CSF as well as the anti-atherogenic cytokine, IL-10[197]. TNFα, both an inducer and target of NF-κB, was shown to increase ROS formation, increase apoptosis, and subsequently increase endothelial dysfunction in rat carotid arteries, mimicking changes seen in aging arteries[198]. TNF blockade was further shown to have vasculoprotective effects[198]; thus adding addition support for the role of NF-κB activation as a negative regulator of aging associated atherosclerosis.

1.3.4.5 Sarcopenia

Sarcopenia, defined as the loss of skeletal muscle mass and strength, is highly correlated with advancing age[199]. In hospitalized geriatric patients, those patients with increased inflammation, indicated by elevated CRP and IL-6, had a correlative decreased in grip strength, shoulder extension strength, and exhibited increased muscle fatigue[200]. Furthermore, TNFα and IL-6 are inversely related to muscle mass and strength in elderly patients[201]. While there is little research evaluating NF-κB activation in sarcopenia, numerous studies show a significant causal role for NF-κB activation in muscle atrophy. Mice transgenic for active IKKβ, exhibit a muscle wasting phenotype[202]. Muscle unloading, or loss of muscle innervation, led to an 8-fold increase in NF-κB signaling suggesting an integral role of NF-κB in muscle atrophy[203, 204]. Additionally NF-κB was implicated in the pathology associated with muscular dystrophy (MDX model), a disease of muscle degeneration, Specific suppression of NF-κB activity in macrophages reduced muscle degeneration, and systemic treatment of NBD, an NF-κB inhibitor, reduced pathologies associated with musclar dystrophy[73]. As with T2D and other aging pathologies, NF-κB acts in numerous cell types to contribute to disease pathology.

1.3.4.6 Osteoporosis

Osteoporosis another age-associated pathology is defined by decreased bone density and increased fragility leading to bone breaks[205]. It is known that inflammatory cytokines IL-6, TNFα, and IL-1[205] signaling via NF-κB are activators of osteoclastogenesis and osteoclast function[206]. As ostoclasts are responsible for bone resorbtion, the role of NF-κB in these cells cannot be underestimated. Additionally, IL-6 levels are shown to be a positive predictor for decreased bone density, and TNFα levels were shown to be increased in patients with decreased vertebral bone density. While it unknown whether these cytokines are markers or mediators of osteoporosic changes they implicate NF-κB and inflammation in this disease process[207]. Interestingly, patients with overactive inflammatory and NF-κB signaling exhibit increased risk of developing osteoporosis when suffering from diseases, including HIV infection, hyper-IgE syndrome, rheumatoid arthritis, myeloma, and inflammatory bowel disease[208]. Recent findings also identified an important role for NF-κB in osteoblast function, mice transgenic for a DN-IKK expressed in osetoblasts had reduced bone loss after ovariectomy (12%) compared with wild-type mice which exhibited 40% bone loss. Thus it is likely, that as with many of these age-associated disease, NF-κB acts through numerous mechanisms to promote osteoporotic degeneration[209].

1.3.5 Progeria and evaluating NF-κB in Age-Associated disease

NF-κB is a main mediator or component in the majority of age-associated disease (as discussed above). In each pathology described previously there are likely additional and possibly more important mediators of disease; however, NF-κB is one of the few factors which

have been implicated of the full spectrum of these age-associated diseases. In some pathologies, NF-κB and inflammation may be the initiator of diseases, while in others NF-κB activation may promote disease progression secondary to events such as DNA damage, altered protein production, or oxidative stress. While numerous groups have shown that specific NF-κB inhibition blocks disease pathology, what remains unknown is the effects of NF-κB suppression on cumulative aging seen in mammalian species, which will be explore in this disseration.

While there are many studies which evaluate aging-related disease, aging in its own right is difficult to study. Most of the current research in aging biology focuses on cellular aging by way of primary mouse embryonic fibroblasts (MEFs) or lower organism model systems such as C. elegans or Drosophilla which have lifespans of 2-3 weeks and 4-5 weeks respectively. While these animals have an abundance of genes that have mammalian homologues, the systems have vast differences. When evaluating mammalian models, the shortest lived, the mouse has a maximum lifespan of nearly 3 years, with variation dependent on strain. However, scientifically, this is still a lengthy period to evaluate specific gene involvement or therapeutic intervention associated with aging. Using a natural phenomenon, known as progeria, or diseases of accelerated aging, one can study aging pathology over a compressed period of time.

Progerias are often caused by single gene mutations, some of which will be discussed here, but result in children who appear normal at birth, but in early childhood begin to develop symptoms of accelerated aging. Symptoms associated with progeria, as well as normative aging, include; visual and hearing impairment, liver and kidney dysfunction, impaired hematopoiesis, neurodegenerative changes, osteoporosis, cachexia, and sarcopenia. There are numerous types of progeriod diseases defined in human populations including Hutchinson-Gilford Progeria syndrome (HGPS), Werner's syndrome, Cockayne syndrome, Bloom syndrome, and Rothmund–

Thomson syndrome. All of these diseases have defects in DNA-repair pathways or deficiency in Lamin A/C[210]. Werner's, Bloom, and Rothmund-Thomas syndrome patients all have defects in the RecQ helicases which maintain genome stability[210]. Werner's syndrome, the most studied of these disorders, is developed secondary to a deficiency in *RECQL2,* which is involved in several DNA repair pathways including NER[211] and double strand breaks[212]. Patients with Werner's syndrome have a mean lifespan of 47 years and normally succumb to cancer or heart disease. The only progerias thought to occur due to defects not specifically associated with DNA repair are those induced secondary to defective lamin such as HGPS. Lamin A deficiency leads to abnormalities in the integrity and shape of the nuclear envelope, regulation of transcription, DNA replication, cell-cycle control and cellular differentiation[213], However, recent studies observed that Lamin deficiency induced progeria exhibit a defective DNA damage response secondary to reduced recruitment of DNA-repair proteins[214]. Accordingly, the majority of progerias and progeroid like diseases support the role of somatic damage in contributing to the aging process..

As one would expect, the vast majority of murine models of progeria are based on human disease and thus have defects in DNA repair. Some examples of progeria models include Werner's like mice, HGPS like mice, and CS mice. Interestingly, the *wrn*$^{-/-}$ mice, which recapitulate the human defect, do not have an aging phenotype unless crossed with a deletion in the telomerase gene. These mice then develop numerous features associated with aging including, loss of hair, osteoporosis, diabetes mellitus and cataracts[213]. Several murine models mimics HGPS, most with mutations in the lamin A gene, and thus exhibit several aging-associated features including alopecia, skin defects, growth retardation, and osteoporosis[213].

There are also several progeriod like models which result from direct defects in DNA repair, indcluding the *Ercc1*$^{-/-}$ and *Ercc1*$^{-/\Delta}$ mouse models.

ERCC1 deficient mice have reduced expression of the DNA repair endonuclease, excision repair cross-complementing rodent repair deficiency, complementation group 1 **(**ERCC1), which is the obligate binding partner of xeroderma pigmentosa group F (XPF). Ercc1-XPF is responsible for nucleotide excision repair of helix distorting lesions and is also responsible for double strand break, and interstrand crosslink-repair[215] (Figure 7A). *Ercc1*$^{-/-}$ mice, which have undetectable levels of ERCC1-XPF, have a maximum lifespan of 1 month and pathologic and genome-wide transcriptional changes associated with natural or normative aging[130]. *Ercc1*$^{-/\Delta}$ mice express 10% of the normal level of the ERCC1/XPF endonuclease and live for approximately 7 months. These mice age rapidly as a consequence of their DNA repair defect. Like the previously published *Ercc1*$^{-/-}$ mice[130], *Ercc1*$^{-/\Delta}$ mice prematurely develop numerous signs and symptoms associated with old age, including trembling, ataxia, cerebral atrophy, renal acidosis, decreased liver function, hypoalbuminemia, bone marrow degeneration, osteoporosis, kyphosis, dystonia, muscle wasting, growth retardation, cachexia and loss of vision. However, *Ercc1*$^{-/\Delta}$ mice are asymptomatic until 8 weeks of age, offering a window of opportunity for therapeutic intervention. The phenotype is remarkably homogenous with all mice displaying an identical spectrum of symptoms in a highly predictable order. *Ercc1*$^{-/\Delta}$ mice presenting with progressive signs associated with aging are shown in Figure 7B. The *Ercc1*$^{-/\Delta}$ strain is an accurate and rapid model system for studying mammalian aging and the debilitating symptoms associated with human aging[216] and will be used to evaluate the role of NF-κB transcriptional activity in aging.

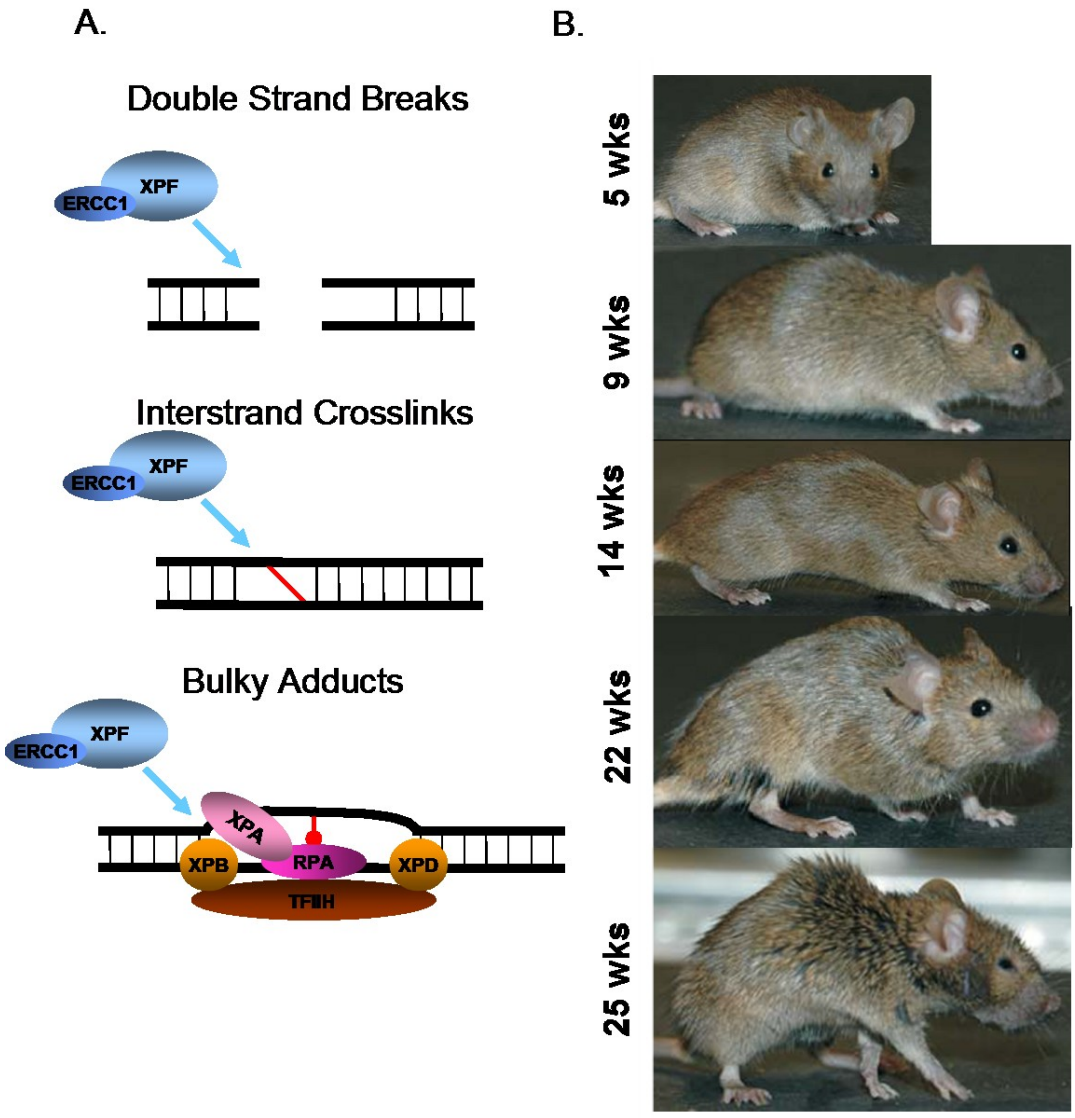


Figure 7: Accelerated aging phenotype of the *Ercc1*$^{-/\Delta}$ over its lifespan and associated phenotypic changes. (A) ERCC1-XPF is an endonucleas responsible for intrastrand crosslink, double strand break, and bulky adduct repair. (B) These mice have a normal phenotype during the first 8 weeks of age. At 14 weeks there is the beginning of reduced subcutaneous fat and early stages of sacropenia and kyphosis. At 22 weeks these symptoms become more pronounced, with the addition, of urinary incontinence and abnormal posturing, indicative of ataxia. At 25 weeks this mouse is exhibiting worsening symptoms in addition to matted fur, vision loss, and increasingly abnormal posturing.

1.3.6 Therapies Associated with Aging Pathology

Recent studies have begun to evaluate the efficacy of novel pharmacologic or therapeutic strategies to ameliorate pathologies and phenotypic changes associated with aging. Of these, caloric restriction (CR) as well as three therapeutics, SIRT1 activators, p38 inhibitors, and NF-κB inhibition that have been evaluated for their effects on aging, will be examined here. While these compounds have varying and often multiple mechanisms of action, one common feature is their regulation of inflammation and cytokine secretion. Furthermore, inhibition of this pro-inflammatory phenotype, mediated by stochastic damage and the SASP phenotype, may result in amelioration of age-associated degeneration.

1.3.6.1 Caloric Restriction

Caloric restriction (CR) is the most widely recognized and first reproducible mechanism by which life extension was mediated[217, 218]. CR has been shown to not only extend longevity, but also ameliorate age-associated pathology including diabetes, cardiovascular disease, sarcopenia, as well as autoimmune diseases[218]. While CR is efficacious in numerous mouse models and more recently in primates[219], the beneficial effects have be attributed to numerous mechanisms, include activation of SIRT1 and PGC-1α, inhibition of Jnk, improvemedmmetabolism, alterationed IGF/GH axis[218, 220], and suppression of the immune response[221]. As stated previously, NF-κB is upregulated in aging, and CR inhibits this activity at the level of the IKK complex, possibly through a ROS dependent manner[221]. Additionally, CR was shown to downregulate expression of 56 inflammatory genes many of which are transcriptionally regulated by NF-κB[222]. Even short term, 10 day CR, resulted in decreased NF-κB activity in kidneys of aged mice[223]. Therefore, CR a known mediator of improved life and

healthspan acts, at least partially, via NF-κB and inflammatory suppression to impart its beneficial effects.

1.3.6.2 SIRT Activators

SIRT1 activators, such as resveratrol are considered cutting edge therapies for treating age-associated diseases. Resveratrol has been shown to increase healthspan (delay the onset of age-associated diseases)[224], protect against neurodegeneration in models of AD and amyotrophic lateral sclerosis (ALS)[225], improve osteoporotic changes[226], and reduce health defects occurring secondary to high fat diets[227]. SIRT1 potentially acts via numerous mechanisms to alter age-associated changes including increased mitocondriogenesis via PGC1α deacetylation, improved oxidative stress survival response via FOXO1/4, altered apoptosis and proliferation via p53 deacetylation, and decreased inflammatory response via NF-κB suppression[228]. Of specific interest to this disseration, is the mechanism by which SIRT promotes its anti-aging effects via inhibition of NF-κB. SIRT1 directly interacts with p65, leading to deacetylation at lysine 310, culminating in decreased NF-κB associated transcription[229]. While it is difficult to distinguish which beneficial effects of SIRT1 therapy are due to which specific mechanism of action, it is interesting to note the SIRT1 activators and NF-κB inhibitory therapies have been efficacious in the same disease pathologies as described above including diabetes, osteoporosis, neurodegeneration, and inflammatory diseases.

1.3.6.3 p38/MAPK inhibitors

Another therapeutic alternative for preventing progeria currently being examined is a p38/MAPK inhibitor. Using cells derived from Werner's syndrome progeria patients, p38 was observed to control activation of p21 and HSP27, two factors shown to be elevated in WS.

Furthermore, treatment with the p38 inhibitor allowed for a increased doubling speed in WS cells, which are inherently slow growing, as well as improved cellular morphology[148]. In addition to NF-κB, p38 is a well known mediator of cytokine production, including IL-1, IL-6, IL-8, and TNFα, at both the transcriptional and post-transcriptional level. Additionally, p38 inhibition blocks cytokine production in T-cells, monocytes, and macrophages[230]. P38/MAPK was shown to be up-regulated in a model of Alzheimer's disease[231], and inhibition of p38 in endothelial cells led to improved insulin sensitivity suggesting a role for p38 in Type II diabetes and cardiovascular disease[232]. Thus, while the evidence of therapeutic relevance of p38 suppression in ameliorating age-associated disease is less compelling that SIRT1, it contributes to the vast data which implicates inflammation and inflammatory signaling pathways in aging and age-associated disease.

The overwhelming data suggests that there is a real and important link between inflammatory signaling pathways and age-associated disease onset and progression. There are numerous correlative links between up-regulated of NF-κB signaling ing aged tissue and cells, as well as and age-associated disease. Furthermore, evidence of the efficacy of anti-inflammatory agents as viable therapies for treatment of age-associated disease, suggests that NF-κB may have a causal role in overall aging. In chapter 3 of this document, I intend to further explore the role of NF-κB signaling in a murine model of accelerated aging, to determine if this pathway has a causative role in age-associated disease and to further provide evidence of a common pathway by which age-associated diseases are manifested.

1.4 ANTIGEN PRESENTING CELLS AS MEDIATORS AND THERAPEUTIC TARGETS OF HUMAN DISEASE

1.4.1 Professional Antigen presenting cells (APC)

APC are central mediators of the innate and adaptive immune response. APC directly recognize pathogens and generate a rapid immune response through the secretion of cytokines and chemokines. They also act as the central communicators between the innate and adaptive immune system, by presenting antigen via MHC class II and co-stimulatory signals to T-cells, allowing for specific antigen responses. Thus, APC, defined here as macrophages and dendritic cells (DC), are vastly important mediators of the innate and adaptive immune response. B-cells are also recognized as APC due to their primary function in generate antigen specific and antibody responses will not be evaluated in this document.

Macrophages are generated from monocytes, while DC are generated from monocytes, (Langerhans, dermal and inflammatory DC), as well as from specific pro-DC progenitor cells (plasmacytoid DC, interstitial DC and Resident $CD8^+$ DC)[233]. In general, monocytes arise from myeloid precursor cells and after differentiation they circulate through the blood stream for several days (constituting 5-10% of peripheral blood cells). Monocytes then enter tissues where they further differentiate into DC or macrophage. This process can be accelerated by the addition of pro-inflammatory or metabolic stimuli[234]. In this study, we focus on monocyte derived macrophages and DC populations including: the FSDC cell line (which is a Langerhans like cell line[235] and thus monocyte derived[233]), RAW264.7 (macrophage cell line), and primary bone marrow derived macrophages and DC (BMDM, BMDC). As these cells are the focus of this manuscript the DC described here will be the monocyte derived DC (MoDC) population.

1.4.2 Characterization of APC populations (inflammatory, regulatory, repair)

Monocyte derived APC can be classified in many ways such as function. Three main APC functional groups exist, the classically activated APC, regulatory APC, and the wound healing macrophages. APC are primarily thought of in terms of the classically activated APC, in macrophages these are referred to as M1 cells. These activated APC are highly immunogenic and are induced by IFNγ, TNFα, TLR or other endogenous or exogenous stimuli, After activation these APC secrete TNFα, IL-1, IL-12/23, ROS [236], chemokines and upregulate MHC class II presentation and costimulatory molecules, all of which are integral in infection control[95].

The second APC subclass are may also be immunoregulatory or suppressive cells. Often DC are regarded for their suppressive phenotypes, however, macrophages can also be induced to have immunoregulatory phenotype[237, 238]. Regulatory APC are induced by a variety of stimuli including apoptotic cells, prostaglandins, IL-10, and tumor cells[237, 239, 240]. In addition myeloid DC and plasmacytoid DC have tolerogenic properties in their immature state[237]. It is likely that these regulatory APC contribute to tolerance, such as oral tolerance[241], and also protect against auto-immunity. However, this regulatory phenotype can be exploited by pathogens as well as tumor cells[242]. Important mechanisms of suppressor APC include: induction of T-cell anergy or T_{reg} phenotype switches, expression of indolamine 2,3, dioxygenase and heme ogenase-1, or secretion of the anti-inflammatory cytokines IL-10 and TGF-β[237].

The third APC/macrophage type is the wound healing class, or alternatively activated macrophages, which are activated by IL-4. These macrophages secrete numerous components of the extracellular matrix and express chitenase, which likely contributes to their role in wound healing[236]. However, it is important to remember that due to the high degree of plasticity of APC, a single macrophage can change classes depending on the exogenous and endogenous stimuli[243].

MoDC and Macrophages act as innate immune phagocytes, secrete cytokines, and are the major source of interaction between the innate and adaptive immune system. Macrophages are defined by their phagocytic and cytokine secretion properties, and their ability to present antigen to effector T cells[238, 244-246]. On the other hand, DC are the only antigen presenting cell capable of priming native T cells as well as cross presenting antigen to CD8 T-cells[247]. Like macrophages, DC secrete cytokines such as TNFα, IL-1, IL-12 and IL-6 and generate inflammatory responses[248]. Interestingly, recent studies observed that under specific conditions macrophages can prime naïve T-cells.[249, 250] Overall, there is a high degree of similarity in the functions and signaling mechanisms of MoDC and macrophages.

MoDC and macrophages can be further characterized by their tissue specificity, and secondarily these APC have varying attributes which are dependent on the necessity of that tissue. These APC are defined as follows: alveolar (lung macrophage), Langerhans and dermal (epidermis/skin MoDC), osteoclasts (bone monocyte), microglial (central nervous system macrophage), Kuppfer cells (Liver macrophage), splenic macrophage, and inflammatory-induced macrophages (derived from peripheral monocytes at the site of infection)[234]. As discussed previously, intestinal macrophage and DC are highly immunosuppressive, with limited ability to activate T-cells. Furthermore, they contribute to oral tolerance, whereby antigens consumed orally will have a muted response when injected systemically[241]. Osteoclasts are less inflammatory in nature and are responsible for bone breakdown and turnover to maintain bone strength and fidelity[251]. On the other hand, inflammatory-induced macrophages, which are activated secondary to stimuli, quickly become central to the inflammatory process, secreting cytokines and chemokines and presenting antigen to adaptive immune cells. Therefore while

APC are highly heterogenous, these cells are highly plastic and thus each macrophage subclass has the potential to prevent disease and infection or contribute to disease pathology.

1.4.3 Activation Receptors on APC

APC express numerous receptors which facilitate phagocytosis, and lead to activation, and proliferation. Scavenger receptors, complement receptors, and Fc Receptors allow for phagocytosis of pathogens, cellular debris, and apoptotic cells. In addition, macrophages express pattern recognition receptors (PRR) such as Toll like receptors (TLR) which contribute to phagocytosis as well as promote activation and cytokine signaling. APC also express cytokine receptors such as TNF, IL-1β, IFN, and IL-4 which can lead to activation, contribute to immune deviation, as well as lead to the production of cytokines[95]. Many of these processes are regulated by NF-κB and described in more detail in Sections 1.1.3 and 1.1.7.

1.4.4 Role of NF-κB Signaling in Macrophages

1.4.4.1 NF-κB and APC Differentiation:

Numerous transcription factors which play a significant role in APC activation and differentiation. *In vitro* derivation of BMDM requires GM-CSF for differentiation, while BMDC require GM-CSF with the addition of IL-4. GM-CSF activates Akt, Erk, and Jnk via the Ras pathway[252] downstream of the common β chain of the GM-CSF receptor, however, the α chain activates the NF-κB pathway via direct interaction with IKKβ[253]. IL-4 is thought to act mainly via Stat6 signaling but reports have shown IL-4 also activates via NF-κB[254]. When examining the highly differentiated bone macrophage or osteoclasts; NF-κB has a definitive role in terminal

differentiation of osteoclasts from monocyte precursors via activation by RANK, without which mice do not develop osteoclasts, and TNFSF11[251]. Thus, NF-κB plays an integral role in differentiation.

1.4.4.2 Role of NF-κB in APC Function

There is vast evidence that NF-κB plays a role in activation and the functions of inflammatory APC, in addition to its role in differentiation. The activation signals for macrophages included IFNγ, which signals via Stat, as well as TLR, IL-1, TNFα, and CD40[255] all of which signal via NF-κB (as described in section 1.1.3 -1.1.6). Furthermore, the two major roles of APC are antigen presentation/co-stimulation and cytokine/chemokine secretion, both of which are regulated by NF-κB.

The antigen presentation functions of APC center on the MHC class II complex as is described in Janeway[95]. Briefly, antigen is phagocytosed by the APC and processed in the endosome, and after proteosomal degradation antigen is loaded onto the MHC class II molecule which is then presented on the cell surface. For proper APC:T-cell interaction, the interaction requires signal I (the MHCII:TCR interaction), signal II (interaction of costimulatory molecules, on the APC such as CD80/CD81, CD40, and 4-IBBL)[95], and signal 3, (cytokine signaling). Recent evidence suggests that MHC class II surface expression is NF-κB dependent, and NF-κB transcriptionally regules the invariant chain, which has a role in MHC class II antigen loading[256]. Furthermore, NF-κB regulates the expression of CD80 and CD40, two costimulatory ligands required for T-cell/B-cell activation. NF-κB transcription is necessary for the production of many pro-inflammatory cytokines secreted by APC including TNF, IL-1, IL-12 (Th1 promoting cytokines), IL-1, IL-6, and IL-23 (Th17 promoting cytokines)[4, 257]. Thus NF-κB controls APC:T-cell interaction by regulating elements of signal 1, 2, and 3.

Additionally, inflammatory APC secrete a multitude of chemokines to attract immune cells. CCL15 attracts monocytes as well as lymphocytes and eosinophils, CCL20 attracts DC and T-cells, and CXCL10 and 11 attracts NK and T-cells via CXCR3 signaling[236]. Each of these chemokines is transcriptionally regulated by NF-κB. Thus, NF-κB is a central mediator of APC development, maturation, and function, that defines NF-κB as an important therapeutic target to explore when evaluating diseases with dysregulated immune and APC responses.

1.4.5 APC role in Human Disease and Aging

Macrophage over-activation has been noted in number of diseases including rheumatoid arthritis[251], atherosclerosis[190], neurodegeneration (Alzheimer's and Parkinson's)[176, 183], liver disease[258], metabolic syndrome (including obesity, hypercholesterolemia, and diabetes)[259] and osteoporosis[260]. Many of these diseases have inflammatory and NF-κB components and are associated with aging (described in detail in section 1.3.4). Each disease shares components of APC activation, cytokine upregulation, and NF-κB dysregulation.

One specific example of APC involvement in disease pathogenesis is in the manifestations associated with IBD. As expected, patients with untreated ulcerative colitis and Crohn's disease have increased number of activated $CD40^{+}$ macrophages isolated from their intesteines[261,262]. Intestinal macrophages derived from IBD patients differ from those derived from control patients in that they express higher levels of CD14 (a component of LPS receptor TLR4), and a portion of these macrophages are RFD9+ and are more likely to form granulomas[263]. Further, macrophages and myeloid derived DC are integral in granuloma formation[264]. Interestingly, mice with macrophages and neutrophils deficient for Stat3, a signlaing mechanism for IL-10, develop a colitis phenotype[265], suggesting an important role for

APC in disease. IL-12 and Il-23, pro-inflammatory cytokines, have been shown to be an important player in the development of IBD[117, 266]. While IL-23 is often thought of as an inducer of Th17 T-cells, IL-23 it has been shown to mediate IBD in the absence of T-cells[117]. Additionally, the most efficacious therapy for IBD is currently anti-TNF biologic therapy. Thus, as the cytokines produced by APC, TNF, IL-12, and IL-23, are major mediators of IBD, this suggests that APC are appropriate therapeutic targets for the treatment of IBD. Furthermore, it is likely that the other diseases with implicated APC and NF-κB dysregulation would respond to these targeted therapies as well.

1.5 HOW NF-KB SUPPRESSION TO CONTROL MAMMALIAN DISEASE WILL BE EVALUATED IN THIS MANUSCRIPT

In the case of numerous human diseases the role of dysregulated NF-κB signaling is well defined. In this work, I will evaluate the role of NF-κB signaling in two different pathologic human conditions. The first disease investigated is IBD. While the role of NF-κB has been defined in this disease over a number of years, there are still numerous questions to answer regarding inhibition of NF-κB as a therapeutic strategy to ameliorate disease. In this study, we will examine NF-κB regulation in *IL-10*$^{-/-}$ murine model of colitis[78]. Recently, there have been reports that loss of NF-κB and specifically IKK in IEC results in a barrier dysfunction induced colitis. Thus, it likely that IKK/NF-κB signaling plays both protective and facilitative role in the pathology of IBD. Despite these possible differences in the role of IKK/NF-κB in colitis pathogenesis, we have chosen to use the NBD peptide to evaluate its affects on disease in the *IL-10*$^{-/-}$ model. In this study, the transduction and functional efficacy of the NBD peptide was

examined *in vitro* as well as *in vivo*. Furthermore, the role of NF-κB in potentiation of disease was explored by suppressing this pathway after the onset of disease by treating diseased mice at 10-12 weeks of age. Mice were evaluated for histopathologic changes, as well as cytokine secretion profiles of gut extracts. This study allows for testing the proof-of-concept, that NF-κB inhibition secondary to NBD treatment is a viable *in vivo* mechanism for treating inflammatory disease states.

In the second chapter of this thesis, I will explore the role of NF-κB signaling in diseases associated with accelerated aging using the $Ercc1^{-/-}$ and $Ercc1^{-/\Delta}$ progeroid like models (discussed 1.3.5). While NF-κB dysregulation is associated with many age-associated diseases, there has been only one group to examine treatment with NF-κB inhibitors/suppressors with regards to overall age-associated changes. Here we explore the role of NF-κB in overall aging as a possible central mediator in the aging process which contributes to the pathology of a number of age-associated diseases. Furthermore, the transcriptional changes associated with this NF-κB suppression in this accelerated aging model were evaluated. Interestingly NF-κB suppression via pharmacologic (NBD) and genetic means (p65 allelic deletion) slowed the progression of numerous phenotypic changes; these alterations in phenotype were further supported by amelioration of histologic degeneration and transcriptional changes associated with normative and ERCC1 defcient aging.

It is likely that there are many mechanisms by which NF-κB dysregulation could potentially play a role in inflammatory and age-related diseases. This includes alterations in hormone responses, altered action and survival of stem and progenitor cells, as well as alterations of immune responses such as T-cell suppression, reduced cytokine and anti-body production and APC suppression. Due to the documented role of APC in inflammatory and age-associated

diseases as described 1.4.5, as well as the fact that these diseases have increased levels of inflammatory cytokines (TNFα, IL-1β, IL-6, IL-12, and IL-23) which are known to be produced by APC, I wanted to explore the effects of NF-κB suppression in this cell type. We hypothesized that suppression of NF-κB signaling in APC would lead to a reduced inflammatory state with a decrease in inflammatory cytokines production, decreased co-stimulatory molecule presentation, and lack of T-cell stimulation or possible T-regulatory cell differentiation. However, *in vitro* we observed that in response to NF-κB suppression APC, both macrophage and DC underwent apoptotic cell death. While a surprising finding, it is a possible mechanism which can explain the amelioration of inflammatory and age-associated disease states and could further explain how inhibitors which have limited half-lives *in vivo* can have such long-lasting effects in murine models of disease.

2.0 AMELIORATION OF CHRONIC MURINE COLITIS BY PEPTIDE MEDIATED TRANSDUCTION OF THE IKB KINASE (IKK) INHIBITOR NEMO BINDING DOMAIN (NBD) PEPTIDE

2.1 ABSTRACT

The NF-κB family of transcription factors is a central regulator of chronic inflammation. The phosphorylation of IκB proteins by the IκB kinase (IKK) complex (IKKα, IKKβ, and NF-κB essential modulator, or NEMO) is a key step in NF-κB activation. Peptides corresponding to IKK's NEMO binding domain (NBD) block NF-κB activation without inhibiting basal NF-κB activity. In this report, we determined the effects of the IKK inhibitor peptide (NBD) in a model of spontaneously occurring, chronic murine colitis, the IL-10-deficient (*IL-10*$^{-/-}$) mouse. Utilizing a novel cationic peptide transduction domain (PTD) consisting of eight lysine residues (8K), we were able to transduce the NBD peptide into cells and tissues. In a NF-κB reporter system, 8K-NBD dose-dependently inhibits TNF-induced NF-κB activation. Furthermore, 8K-NBD inhibited nuclear translocation of NF-κB family members. In NF-κB^{EGFP} knock-in mice, 8K-NBD inhibited LPS-activated NF-κB in the ileum, but did not inhibit basal NF-κB in Peyer's patches. *IL-10*$^{-/-}$ mice treated systemically with 8K-NBD demonstrate amelioration of established colitis, decreased NF-κB activation in the lamina propria, and a reduction in spontaneous intestinal IL-12 p40, TNF, interferon-γ, and IL-17 production. These results demonstrate that

inhibitors of IKK, in particular a PTD-NBD peptide, may be therapeutic in the treatment of inflammatory bowel disease (IBD)

2.2 INTRODUCTION

While the etiology of the human chronic inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), remains unknown, research has identified contributing factors that include defects in the barrier mechanism of the lining intestinal epithelial cells (IECs), and a poorly regulated immune response against the normal enteric microbial flora.

NF-κB represents a group of structurally related proteins that includes five members in mammals (p65, c-Rel, Rel-B, p50, and p52). NF-κB is a central regulator of chronic inflammation in IBD [102, 106, 267]. Many of the standard agents used to treat human IBD, including sulfasalazine, 5-aminosalicylates, and corticosteroids, have been postulated to exert some of their anti-inflammatory effects through NF-κB inhibition[268-270]. In unstimulated cells, NF-κB proteins are localized in the cytoplasm through their association with members of a family of inhibitory proteins known as IκB proteins. Pro-inflammatory cytokines such as TNF and IL-1, and bacterial products such as lipopolysaccharide (LPS) induce phosphorylation of IκB proteins at specific N-terminal serine residues. Phosphorylation of IκB is mediated by the IκB kinase (IKK) complex. IκB phosphorylation leads to IκB degradation, release of NF-κB subunits, and their subsequent translocation to the nucleus. Nuclear NF-κB regulates transcription of proinflammatory genes including cytokines (IL-1β, TNF, IL-12/23), chemokines (IL-8, MIP-1α, MCP-1), and adhesion molecules (ICAM-1, VCAM, E-selectin). Cytokines that are stimulated

by NF-κB such as IL-1β and TNF, also directly activate NF-κB, thus establishing an auto-regulatory loop that may be essential in the perpetuation of chronic inflammation [15].

IKK is made up of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit named "NF-κB essential modulator" (NEMO; also named IKKγ) [271]. An N-terminal region of NEMO associates with a hexapeptide sequence within the C-terminus of both IKKα and IKKβ, named the NEMO binding domain (NBD). A short peptide derived from the 735-745 amino acid NBD region of IKKβ disrupts the association of NEMO with IKKs *in vitro* and blocks TNF induced NF-κB activation *in vivo* when delivered to cells using a cationic protein transduction domain (PTD). Because of the importance of the IKK complex in inflammation, the identification of such a selective IKK inhibitor as a potential therapeutic agent is of considerable interest.

PTDs have been shown to deliver a wide variety of therapeutic agents into cells including peptides, proteins, nucleic acids, antibodies and small drugs. The first protein reported with transductional properties was the HIV transactivator protein, TAT, in which the 11 amino acid PTD was identified by virtue of its cationic content [44]. Recently, PTDs have been characterized that mediate efficient and rapid receptor-independent internalization of peptide-protein conjugates [272]. These cationic transduction peptides transduce a wide variety of cells similar to the HIV TAT, mediating highly efficient transduction *in vitro* and *in vivo* [45, 54]. Screening of a panel of cationic peptides has demonstrated that peptides of 8 or 10 lysines are highly efficient transduction domains, working as, or more, effectively than other cationic PTDs for delivery of peptides to numerous cell types, including mucosal cells and antigen presenting cells [54].

PTD-mediated NBD delivery is efficacious therapeutic in models of autoimmunity and inflammation including models of multiple sclerosis [75], islet transplantation[273] and rheumatoid

arthritis [71]. Accordingly, we postulated that a novel cell permeable peptide IKK inhibitor, 8K-NBD may efficiently target cells involved in the intestinal inflammatory response. In this report, we show that 8K-NBD dose-dependently inhibits TNF-induced NF-κB activation and translocation in cells. Furthermore, *in vivo*, 8K-NBD inhibits activated but not basal NF-κB in the intestine. Moreover, we demonstrate that treatment of IL-10-deficient (*IL-10*$^{-/-}$) mice with 8K-NBD ameliorates chronic colitis *in vivo*.

2.3 MATERIALS AND METHODS

2.3.1 Peptide synthesis

Peptides 8K-NBD (acetyl-KKKKKKKKGGTALDWSWLQTE-amide), inactive mutant, 8K-mNBD (acetyl-KKKKKKKKGGTALDASALQTE-amide), random-NBD (ARPLEHGSDKAT-GGTALDWSWLQTE), 8K-biotin (KKKKKKKK-biotin), and random peptide-biotin (ARPLEHGSDKAT-biotin) were synthesized by the peptide synthesis facility at the University of Pittsburgh. The random-NBD and 8K-NBD peptides N-terminal ends were conjugated to 6-carboxyfluorescein (6CF, Molecular Probes) for localization experiments. Peptides were purified and characterized by reversed-phase high performance liquid chromatography and mass spectrometry.

2.3.2 Murine macrophages

The murine macrophage cell line, RAW264.7, was maintained in DMEM/10% FBS/1% Pen/Strep. Bone marrow (BM)-derived murine macrophages were isolated from femurs of C57BL/6 mice. BM was flushed with washing medium (RPMI1640 with 1% Pen/Strep), passed through a 70 μm nylon cell strainer into a 50 ml conical tube, and spun down at 1500 rpm for 5 minutes. RBCs were lysed using sterile-filtered 0.8% ammonium chloride, washed twice with washing medium, and resuspended in complete medium (washing medium with 10% FBS). BM cells were seeded in complete medium in a 150 mm dish and differentiated using recombinant murine GM-CSF (20 ng/ml) (R&D Systems). At day three, another 25 ml fresh culture medium containing GM-CSF was added to the culture plates. At day seven, the cells, representing the BM-derived macrophage population, were harvested for experiments.

2.3.3 NF-κB Luciferase assay

HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter (DMEM/10% FBS/1% Pen/Strep) were pretreated for one hour with 8K-NBD dissolved in OptiMEM media (Invitrogen) and activated for two hours with 10 ng/ml TNF (R&D Systems). The cells were lysed in reporter lysis buffer and luciferase activity was measured with a luciferase assay system (Promega) using a Turner Designs Luminometer TD20/20.

2.3.4 Nuclear extracts and Western blotting

HEK293 cells were pretreated for one hour with media, 8K-NBD, or 8K-mNBD dissolved in OptiMEM media (Invitrogen) and activated for 15 minutes with 10 ng/ml TNF (R&D Systems). Nuclear extracts from treated HEK293 cells were isolated following manufacturer's protocol (NE/PER Reagents, Pierce). Protein concentration was determined using the Bradford assay (Pierce). Western blot analyses were performed on nuclear extracts as described previously [274]. Anti-p65 and anti-c-Rel antibodies were obtained from Santa Cruz Biotechnology, Inc., and anti-PARP and anti-phospho-p65 (p-p65) antibodies were obtained from Cell Signaling.

2.3.5 Mice

Male C57BL/6 (10-12 weeks old) and female BALB/c (12-13 weeks old) mice were obtained from The Jackson Laboratory. An *IL-10*$^{-/-}$ colony on a C57BL/6 background (breeder pairs from The Jackson Laboratory) was maintained in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and was approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh School of Medicine. The NF-κB^{EGFP} knock-in mice (129/SvEv/C57BL6 background) were described previously[107, 275]. Expression of enhanced GFP (EGFP) is controlled by a chimeric promoter containing three HIV NF-κB *cis* elements in these mice. Mice were maintained in specific pathogen free conditions. Research Protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina School of Medicine.

2.3.6 *In vivo* PTD transduction

C57BL/6 or BALB/c mice were grouped randomly and treated intraperitoneally with biotinylated peptides (random or 8K) linked to streptavidin-Cy3. After 30 minutes of treatment, organs were harvested, fixed in 2% paraformaldehyde, and then incubated in 30% sucrose in PBS at 4°C overnight. Samples were snap-frozen in isopentane and cut into 6 μm-thick frozen sections and placed on microscope slides.

2.3.7 Immunohistochemistry

After transduced macrophages or cut tissue sections were placed on coverslips, slides were blocked in BSA and stained for nuclei with either Draq5 (Biostatus Limited, Leicestershire, United Kingdom) or Propidium Iodide (PI, Molecular Probes), for 30 min. Phalloidin was used to visualize F-actin (Molecular Probes). After extensive washing, slides were mounted and viewed on an Olympus Flowview 1000 confocal microscope (Olympus America, Melville, NY).

Colonic tissue was collected from control and treated animals, fixed overnight in paraformaldehyde, embedded in paraffin and sectioned at 4 mm. Serial sections were stained for phosphorylated NF-κB p65 (phospho-p65). Following deparaffinization and rehydration, antigen unmasking was performed using Citra Plus Antigen Retrieval (Biogenex Laboratories) per manufacturer's protocol. Slides were cooled, washed and endogenous peroxidase was blocked using 0.3% hydrogen peroxide. Sections were next blocked with 1.5% goat serum (Vector Laboratories, Burlingame, CA) in PBS for 1 hour, incubated with rabbit anti-phospho-NF-κB p65 polycolonal antibody at a 1:50 dilution (Cell Signaling) in PBS at 4^{o}C overnight in a humidified chamber. Slides were washed with PBS, and incubated with biotinylated goat anti-

rabbit secondary antibody (Vector laboratories) for 45 minutes, slides were washed with PBS and Vectastain Elite ABC reagent was applied for 30 minutes. Slides were washed with PBS and diaminobenzene (Vector Laboratories) was utilized as a substrate. Sections were counterstained hematoxylin, dehydrated and mounted on coverslips. Stained sections were evaluated by an observer blinded to treatment group for phospho-p65. Phospho-p65-positive cells were counted from 20 randomly selected high power fields in coded colonic sections to that the observers were blinded to treatment group. Cells were enumerated from 6 mice/group (mutant NBD, 2 mg/kg NBD, 10 mg/kg NBD treated) and results are expressed as number of positive cells per field.

2.3.8 *In vivo* NBD peptide treatment

10-week old *IL-10*$^{-/-}$ mice were grouped randomly and treated with either mutant (10 mg/kg) or wild-type (2 or 10 mg/kg) NBD peptide linked to 8K. Treatment was administered in PBS in a total volume of 500 µl intraperitoneally for 10 out of 14 days. At the end of the study period, animals were euthanized using excess CO_2 inhalation and intestinal tissue was harvested. NF-κB^{EGFP} knock-in mice were pretreated with 8K-NBD or 8K-mutant NBD peptide (10 mg/kg) one hour prior to LPS injection. LPS (5 mg/kg) or PBS was administered intraperitoneally to the mice (2 mice per treatment group). Sixteen hours later, the mice were sacrificed by excess CO_2 inhalation and dissected.

2.3.9 Intestinal tissue explant cultures

Colons were isolated from individual mice, cut open longitudinally, and cleaned of fecal matter. The intestinal tissue was washed with PBS to remove residual fecal content. Intestinal

sections were cut in half longitudinally, and one half was shaken at 250 rpm at room temperature for 30 min in RPMI 1640 supplemented with 1% antibiotic/antimycotic. Tissue fragments (0.05 g dry weight) were incubated in 1 ml RPMI supplemented with 1% antibiotic/antimycotic and 10% FBS. Supernatants were collected after 24 hours, assayed for spontaneous cytokine production via sandwich ELISAs, and normalized to dry gut weight.

2.3.10 Histology

Colons were isolated from individual mice, cut open longitudinally, and cleaned of fecal matter. Intestinal sections were cut in half longitudinally, and one half was fixed in 10% buffered formalin and embedded in paraffin. 5 μm thick sections were stained with hematoxylin and eosin. Colitis scores (0-4) were determined by a staff pathologist using the criteria reported by Berg *et al.* [276]. At least 20 separate microscopic fields (10×) were evaluated for each mouse by a pathologist (Dr. Antonia R. Sepulveda, University of Pittsburgh) blinded to the treatment groups.

2.3.11 EGFP imaging

Intestines removed from NF-κB^{EGFP} knock-in mice were immediately imaged after dissection using a charge-coupled device camera in a light-tight imaging box with a dual filtered light source and emission filters specific for EGFP (LT-99D2 Illumatools; Lightools Research). For confocal microscopy on intestinal tissue, terminal ileums were cut open longitudinally and placed on the stage of a Leica SP2 Upright Laser Scanning Confocal Microscope (Leica) lumen side facing the lens without further processing or fixation. EGFP was excited at 495 nm

wavelength, and images were acquired using detection filters specific for the EGFP emission spectrum. Images were analyzed with the Leica SP2 Laser Scanning Confocal Imaging Software (Leica).

2.3.12 Cytokine ELISAs

Murine IL-12 p40, TNF, interferon (IFN-γ) (BD Pharmingen), and IL-17 (R and D Systems) immunoassay kits were used according to the manufacturer's instructions. Values were measured using a plate reader and the SOFTMax Pro v4.8 software (Molecular Devices).

2.3.13 Statistical Analysis

Statistical significance in cell based experiments (Figure 9) was assessed by the two-tailed Student's *t* test. Statistical significance from *in vivo* intervention experiments (Figures 13, 14 and 15) was assessed by the Mann-Whitney U Test (SPSS). A *p*-value equal or less than 0.05 was considered to be statistically significant.

2.4 RESULTS

2.4.1 Transduction of 8K PTD peptide into macrophages

As previously reported [54], a panel of cationic protein transduction domains were screened for their ability to efficiently transduce a variety of cell types. Eight to 10 amino acid polylysine tracts have been shown to efficiently transduce a wide array of cell lines and primary cells,

including islet β-cells, synovial cells, polarized airway epithelial cells, tumor cells, and dendritic cells (DC) [54]. To assess transduction of the 8K PTD into a relevant immunologic target cell, murine BM-derived macrophages were incubated with biotinylated 8K PTD linked to streptavidin-Cy3 and the murine macrophage cell line RAW264.7 was incubated with 8K PTD conjugated to the fluorescent label 6-carboxyfluorescein (6CF). Murine macrophages were efficiently transduced with the 8K PTD (>90% of cells demonstrate fluorescence) compared to a negative control peptide containing a random peptide sequence in place of the PTD (Figure 8). This result demonstrates that *in vitro* macrophages are efficiently transduced with the 8K PTD.

2.4.2 8K-NBD inhibits TNF-stimulated NF-κB activation and nuclear translocation in cells

To evaluate the functionality of the transduced peptide, the 8K PTD linked to the NBD peptide (8K-NBD) was preincubated with HEK293 cells expressing a stably transfected multimerized NF-κB DNA binding element-luciferase reporter gene. Cells were subsequently stimulated with TNF. Pretreatment with 8K-NBD demonstrated a dose-dependent inhibition in TNF-stimulated NF-κB activity (Figure 9A). Moreover, 8K-NBD pretreatment without TNF stimulation did not alter basal levels of NF-κB activity, indicating that the peptide specifically targets activated NF-κB. To verify the mechanism by which 8K-NBD inhibited activity, nuclear translocation of NF-κB family members in activated cells was assessed. Western immunoblot analysis on nuclear extracts demonstrated decreased nuclear quantities of the NF-κB family members p65 (and its phosphorylated form, p-p65), and c-Rel (Figure 9B) in 8K-NBD pretreated, TNF-activated, HEK293 cells.

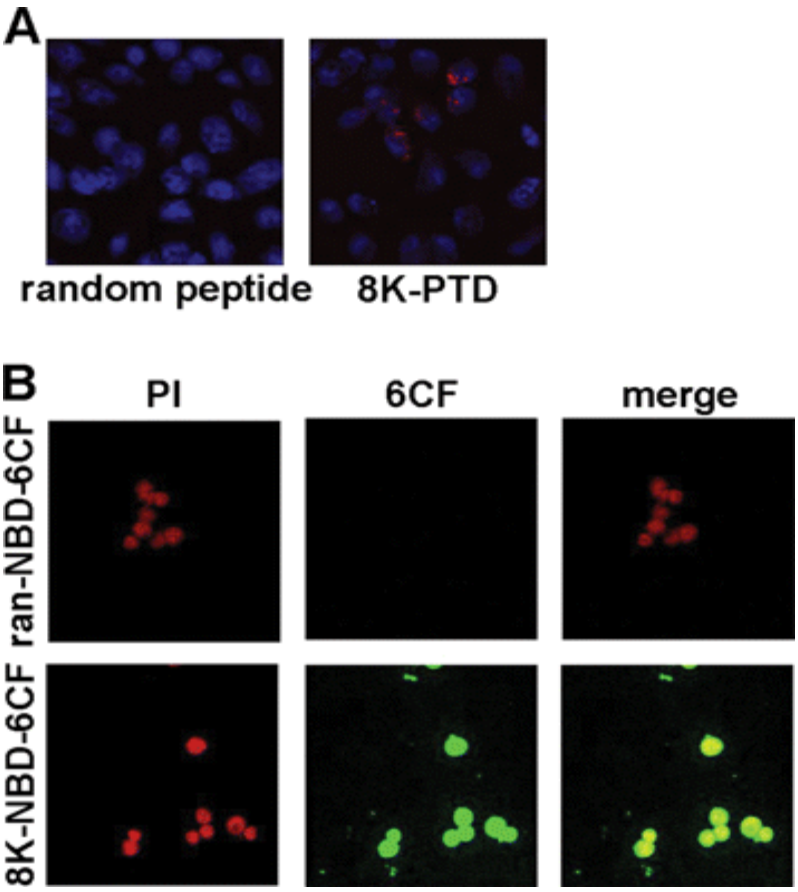


Figure 8: 8K efficiently transduces murine macrophages. *A*, Biotinylated random peptide linked to streptavidin-Cy3 or 8K PTD linked to streptavidin-Cy3 was added to BM-derived macrophages. *B*, The fluorescently labeled random (ran) peptide-NBD-6CF or 8K-NBD-6CF was added to RAW264.7 macrophages. Following incubation for 1 h, cells were fixed, stained for nuclei (Draq5 in *A*, propidium iodide (PI) in *B*), and placed on a microscope slide. Localization of peptide was visualized by a confocal microscope system. Results were repeated three times and representative images are shown. (Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

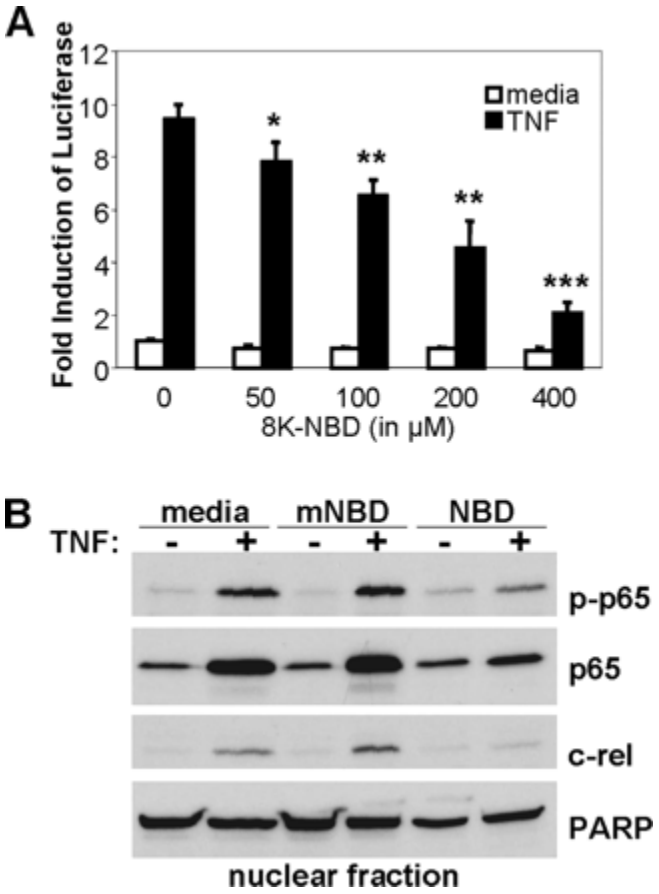


Figure 9:TNF-induced NF-κB activation is inhibited by 8K-NBD transduction in cells. *A*, HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter were preincubated for 1 h with an increasing dose of 8K-NBD peptide in medium. Cells were subsequently stimulated for 2 h with 10 ng/ml recombinant human TNF. Cells were harvested and lysates were analyzed for luciferase activity. Results are expressed as fold induction of luciferase compared with unstimulated plus 0 μM peptide lysates (= 1). Experiments were performed in triplicate and repeated three times. A representative result is shown (mean ± SD). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with unstimulated sample. *B*, Nuclear extracts of HEK293 cells stimulated with or without 10 ng/ml recombinant human TNF in the presence of medium, 8K-mNBD, or 8K-NBD were isolated and run out for Western blotting. Blots were probed with phospho-p65 (p-p65), p65, and c-Rel. Blots were probed with poly(ADP-ribose) polymerase to assess equal loading. Experiments were repeated three times and a representative blot is shown. (Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

2.4.3 Transduction of 8K-PTD *in vivo*

8K PTD efficiently transduces and inhibits NF-κB activity in cells. We next investigated whether 8K PTD transduces cells *in vivo* in mice. Mice were injected with biotinylated peptides (random sequence or 8K PTD) linked to streptavidin-Cy3. *In vivo* uptake of peptide is observed as early as 30 minutes after administration. Intraperitoneal administration of 8K PTD revealed uptake in the spleen (Figure 10A-B) and mesenteric lymph nodes (Figure 10C-D). These results demonstrate that systemic and intestinal immune compartments are targeted by 8K peptide-mediated transduction *in vivo*.

2.4.4 8K-NBD inhibits LPS-stimulated intestinal NF-κB but does not inhibit basal NF-κB *in vivo*

To determine whether 8K-NBD inhibits activated, but not basal, NF-κB in the intestine *in vivo*, we utilized NF-κB^{EGFP} knock-in mice[107, 275] where expression of enhanced GFP (EGFP) is controlled by a chimeric promoter containing three HIV NF-κB *cis* elements. Strong induction of *in vivo* NF-κB activity has been described in jejunal and ileal lamina propria T cells and monocytes following LPS injection[275]. Furthermore, gross analysis of whole organs from *cis*-NF-κB^{EGFP} mice demonstrated basal levels of EGFP expression in Peyer's patches, known to exhibit high basal levels of NF-κB activation[275].

NF-κB^{EGFP} knock-in mice were pretreated with 8K-NBD (10 mg/kg) or 8K-mNBD peptide (10 mg/kg) one hour prior to intraperitoneal LPS (5 mg/kg) or PBS administration. After 16 hours, mice were sacrificed and intestines analyzed for GFP expression indicative of NF-κB

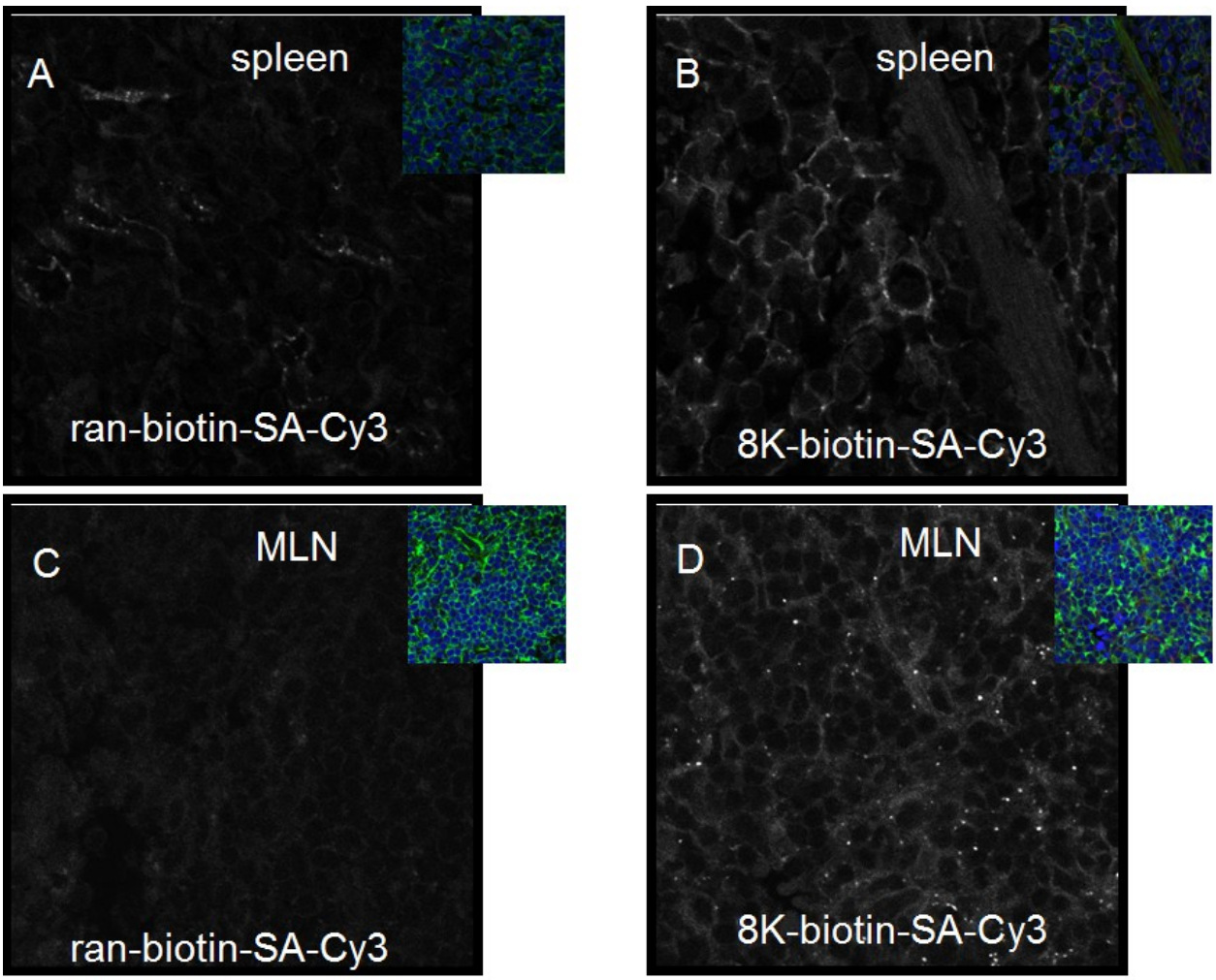


Figure 10 The 8K PTD transduces lymphoid tissue in vivo. Biotinylated (*A* and *C*) random (RAN) peptide linked to streptavidin (SA)-Cy3 or(*B* and *D*) the 8K PTD linked to streptavidin-Cy3 was injected i.p. for 30 min. Paraformaldehyde-fixed (*A* and *B*) spleens and (*C* and *D*) mesenteric lymph nodes (MLN) were stained for actin (green) and nuclei (blue; Draq5) and viewed by confocal microscopy. *Insets* represent the threecolor image for each sample. To specifically visualize the location of the peptide (Cy3; red), the signal from the red channel was given a false white color while the other channels were turned off. This is represented in the larger black and white image. Background threshold levels were adjusted to random peptide samples for each organ. (Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

activity. EGFP expression in lamina propria cells of the terminal ileum was visualized by confocal microscopy (Figure 11A). Mice injected with PBS demonstrate few EGFP positive cells in the lamina propria whether pretreated with 8K-NBD or 8K-mNBD (Figure 11A, left). In contrast, mice injected with LPS pretreated with 8K-mNBD demonstrate strong NF-κB activation in the ileal lamina propria. Mice pretreated with 8K-NBD prior to LPS show markedly fewer EGFP positive cells in the lamina propria, similar to the number observed in PBS treated mice (Figure 11A, right). To study NF-κB^{EGFP} transgene expression in whole organs, a specific EGFP imaging camera was used to visualize EGFP expression in the intestine following challenge with LPS. Strong basal NF-κB activity is observed in Peyer's patches (visualized macroscopically as nodules on the serosal surface of the small intestine) of PBS. This activity is not inhibited by administration of 8K-NBD (Figure 11B, left). Taken together, these experiments demonstrate that intraperitoneal administration of 8K-NBD inhibits activated but not basal NF-κB in the intestinal immune compartment *in vivo*.

2.4.5 8K-NBD treatment ameliorates colitis in *IL-10*$^{-/-}$ mice

Next, we tested the hypothesis that 8K-NBD may ameliorate active chronic colitis *in vivo* in *IL-10*$^{-/-}$ mice. *IL-10*$^{-/-}$ mice were treated from 10 to 12 weeks of age with either 8K-NBD at 2 or 10 mg/kg or 8K-mutant NBD (mNBD) at 10 mg/kg by intraperitoneal injection for 10 of 14 days. Gross inspection of the intestines revealed increased colonic lengths, decreased colonic wall thickening, and formed fecal pellets in the 8K-NBD treatment groups (Figure 12B-C) compared to the mNBD controls (Figure 12A). Histologic severity of colitis was graded over the entire length of the colon for each mouse by a single pathologist blinded to treatment groups.

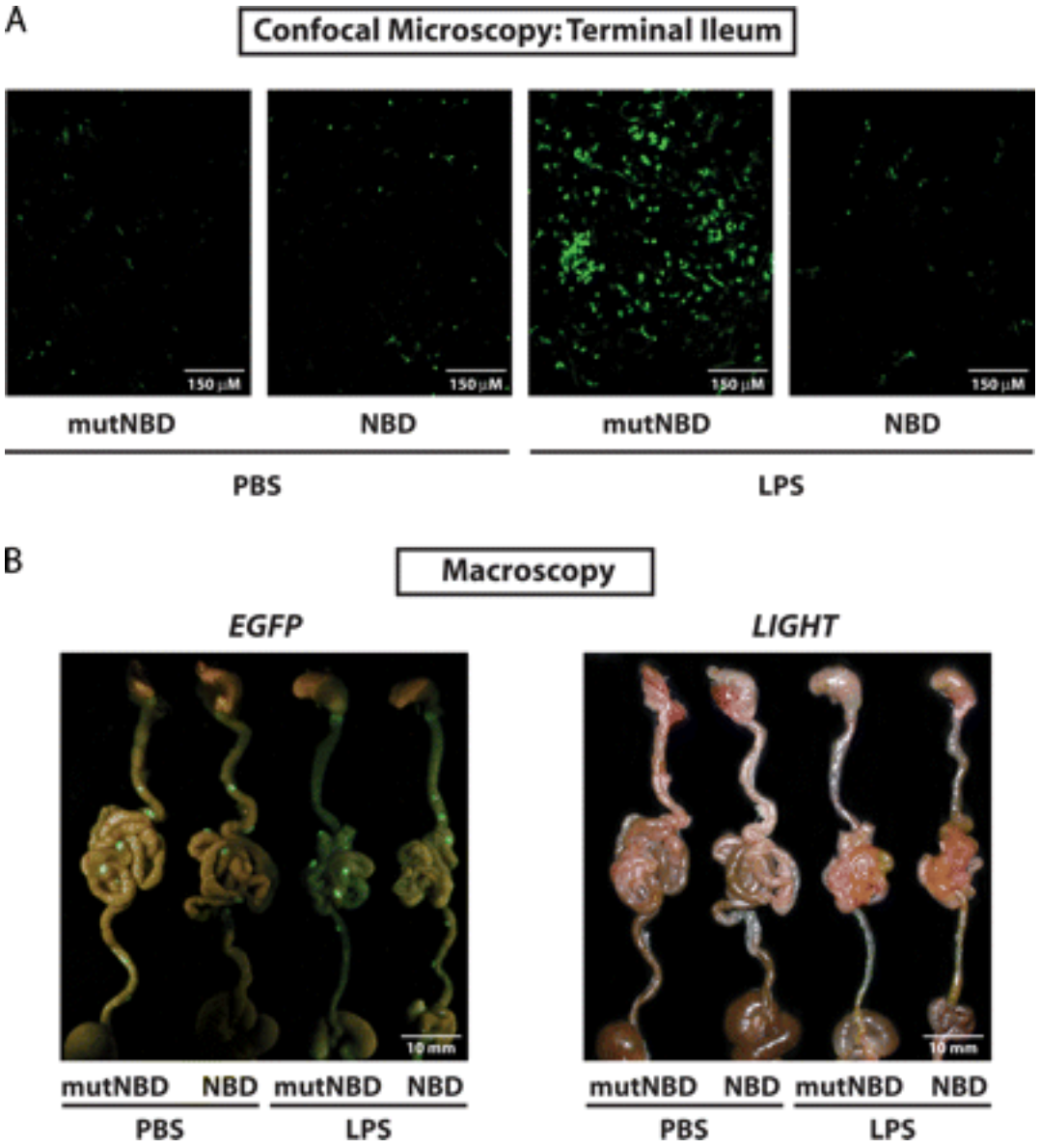


Figure 11: 8K-NBD inhibits LPS-stimulated NF-κB but not basal NF-κB in vivo. NF-κB^{EGFP} knock-in mice were pretreated with 8K-NBD (10 mg/kg) or 8K-mNBD (mutNBD) (10 mg/kg) 1 h before LPS injection. LPS (25 mg/kg) or PBS was administered i.p to the mice (two mice per treatment group). After 16 h, mice were sacrificed and the guts were collected. *A*, EGFP expression in the ileal lamina propria was visualized by confocal microscopy. *B*, EGFP fluorescence of whole intestines was macroscopically assessed using the Lightools Research macroimaging system. Fluorescent (*right*) and white light imaging (*left*) are depicted.(Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

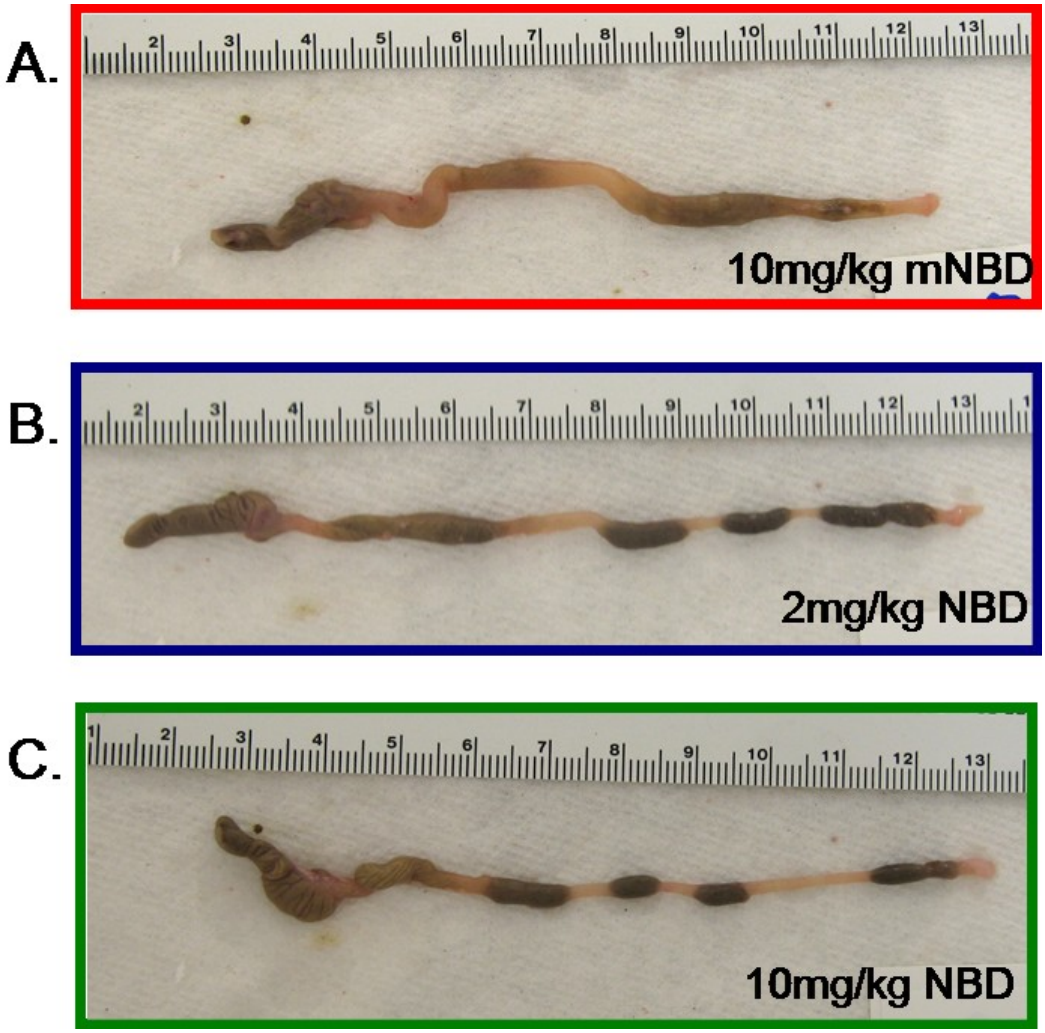


Figure 12: 8K-NBD-treated mice demonstrate improvement in gross colonic appearance. Representative photographs of colons from the control group 10 mg/kg 8K-mNBD (*A*) and the treatment groups 2 mg/kg 8K-NBD (*B*) and 10 mg/kg 8K-NBD (*C*). Colons from mice treated with 8K-NBD peptide demonstrated increased length, decreased tissue thickening, and formed stool pellets compared with the control group. (Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

Due to the incomplete penetrance and the segmental, patchy pattern of colitis in *IL-10*$^{-/-}$ mice, colitis scores are depicted in two different ways. First, composite scores are represented using a modified standard scoring system described by Berg *et al.* [276] (Figure 13A). Mice treated with 8K-NBD at 2 mg/kg and 10 mg/kg demonstrated a 50% reduction in colitis scores compared to the control treated group (mNBD). To illustrate the spectrum of disease encountered over the entire length of the colon, scores are presented as the percentage of fields that demonstrate no histological inflammation (colitis score of 0), mild to moderate inflammatory changes (colitis score of 1 and 2), and severe inflammation (colitis score of 3 and 4). Compared to the 8K-mNBD group (Figure 13B, red bars), 2 mg/kg and 10 mg/kg 8K-NBD treated mice (Figure 13B, blue and green bars, respectively) displayed more fields demonstrating no evidence of histologic inflammation and consequently fewer fields with significant inflammatory changes.

Activated NF-κB was determined by immunohistochemistry for phosphorylated NF-κB p65 in colonic sections from 8K-mNBD and 8K-NBD (2 mg/kg) treated *IL-10*$^{-/-}$ mice (n=6 per group). Significantly fewer phospho-p65 positive cells were found in the lamina propria from 8K-NBD compared to 8K-mNBD treated mice (Figure 14), suggesting that 8K-NBD inhibits activated NF-κB in the colon of *IL-10*$^{-/-}$ mice, correlating with histological improvement.

Finally, the effect of 8K-NBD treatment on mucosal inflammatory cytokine production in *IL-10*$^{-/-}$ mice was investigated. Spontaneous release of the NF-κB regulated proinflammatory cytokines IL-12(p40) (Figure 15A) and TNF (Figure 15B) were determined in cell free supernatants from colonic mucosal tissue explants. Explants from 8K-NBD-treated mice secreted significantly less IL-12(p40) and TNF compared to 8K-mNBD-treated control mice.

The IL-12 p40 subunit is a component of the bioactive cytokines IL-12 and IL-23. To assess

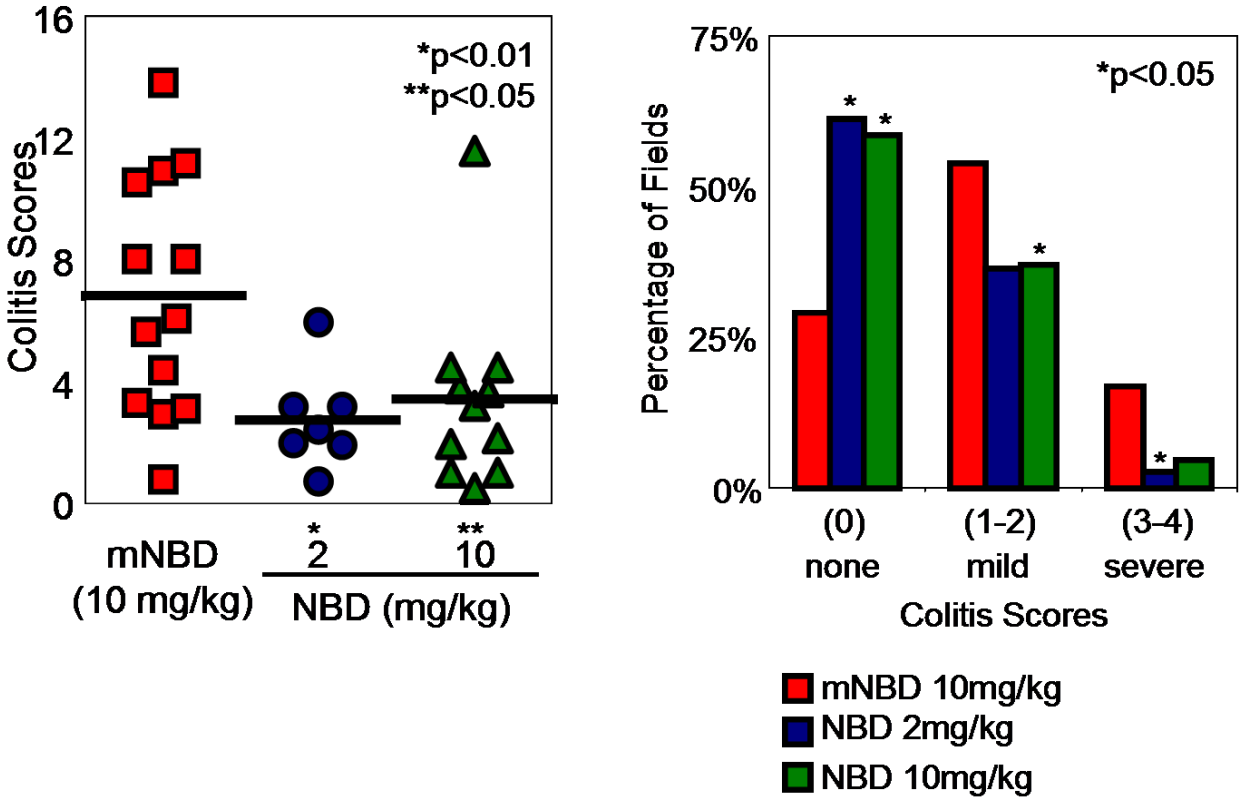


Figure 13: 8K-NBD treatment ameliorates histologic colitis. Colons were isolated from individual mice (black, 10 mg/kg mNBD; gray, 2 mg/kg NBD; white, 10 mg/kg NBD), cleaned, fixed in formalin, and embedded in paraffin. Sections were stained with H&E and colitis scores were determined using a modified scoring system (0–4) as described in *Materials and Methods*. At least 20 separate microscopic fields (magnification x10) were evaluated for each mouse by a pathologist blinded to the treatment groups. Colitis scores were significantly lower in the 8K-NBD-treated mice. Histologic improvement in colitis is presented as a composite score (the average colitis score sum of five fields) (*A*) and as the percentage of histologic fields that demonstrate scores of 0 (no inflammation), 1 and 2 (mild inflammation), or 3 and 4 (severe inflammation) (*B*). (Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

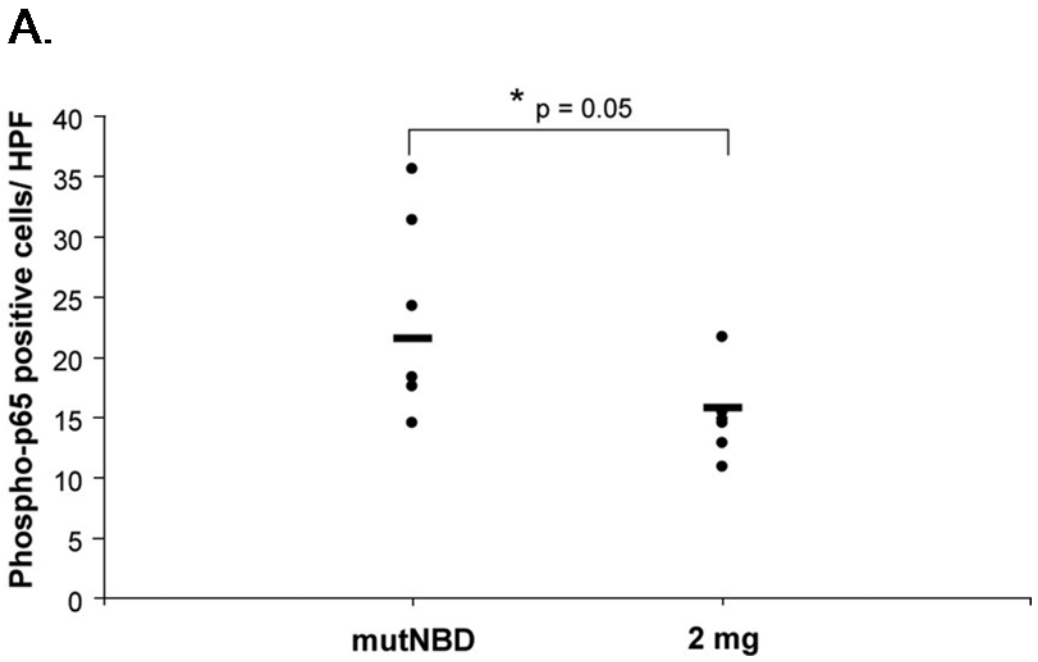


Figure 14: 8K-NBD inhibits the phosphorylation of intestinal NF-κB p65 in *IL-10*$^{-/-}$ mice. *IL-10*$^{-/-}$ mice were treated with 8K-NBD (2 mg/kg; *n* = 6) or 8K-mNBD (10 mg/kg; *n* = 6) for 14 days. Colonic sections were immunohistochemically stained for phospho-p65. (A) Phospho-p65-positive lamina propria cells were quantitated in stained colon sections of treated mice. Twenty high power fields were counted from each mouse and the results are expressed as the mean number of positive cells per high power field. (Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

downstream consequences of attenuated mucosal IL-12 p40 production, the prominent T cell targets of IL-12 and IL-23 signaling, IFN-γ and IL-17, respectively, were measured. Intestinal explants from 8K-NBD-treated mice secreted less spontaneous IFN-γ (Figure 15C) and IL-17 (Figure 15D) compared to 8K-mNBD-treated control mice. Therefore, decreased mucosal innate and T cell inflammatory cytokine expression correlates with the histological findings, suggesting that specific targeting of the IKK complex with cell permeable NBD peptides may be effective in treating chronic IBD.

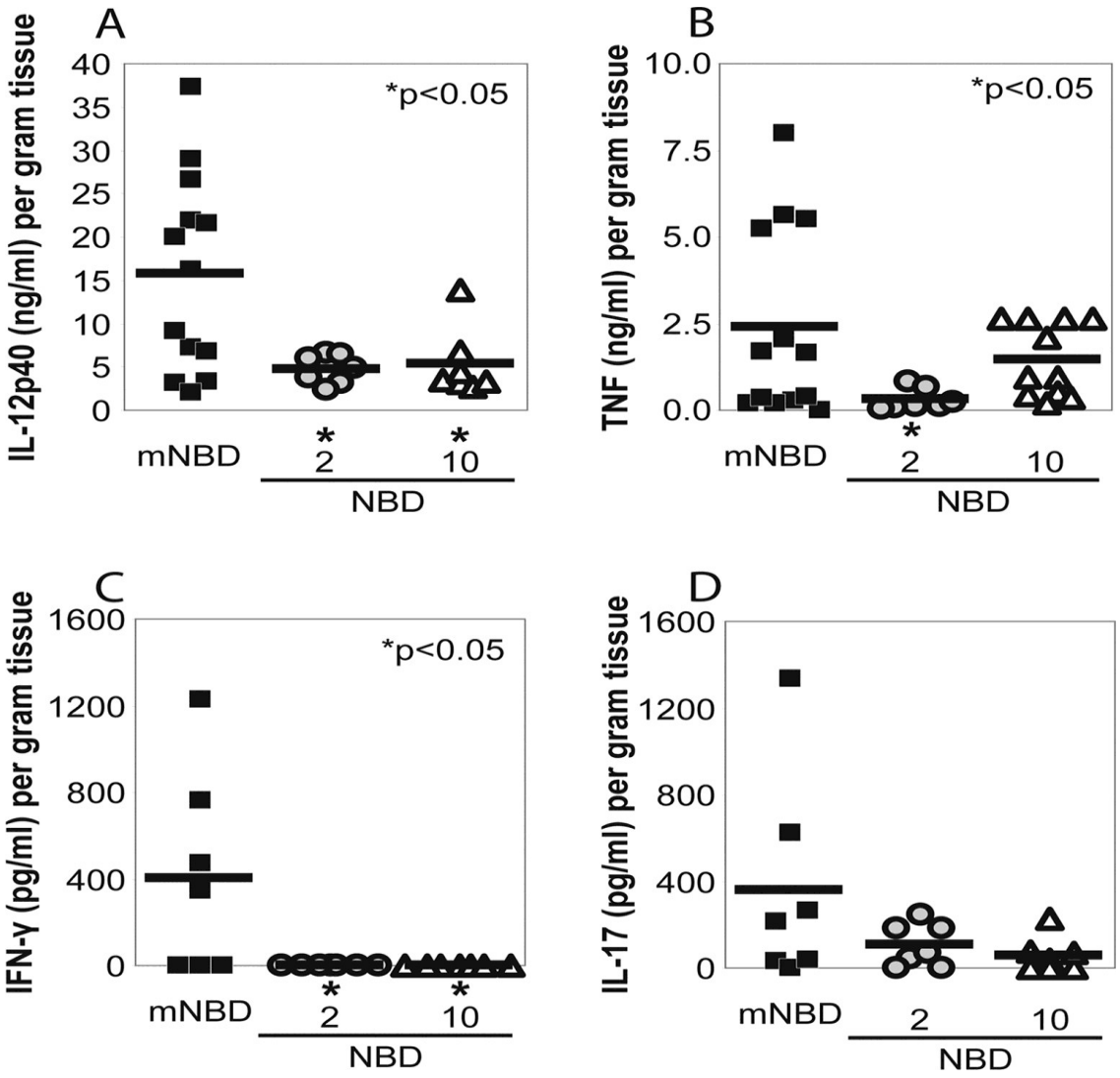


Figure 15: 8K-NBD inhibits NF-κB-dependent cytokine production in intestinal explants. Colons were isolated from individual mice (black, 10 mg/kg mNBD; gray, 2 mg/kg NBD; white, 10 mg/kg NBD), cleaned, and processed for intestinal tissue explant cultures as described in *Materials and Methods*. Tissue fragments (0.05 g dry weight) were incubated in 1 ml of RPMI 1640 supplemented with 1% antibiotic/antimycotic and 10% FBS. Supernatants were collected after 24 h and spontaneous secretion of IL-12 p40 (*A*), TNF (*B*), IFN-γ (*C*), and IL-17 (*D*) were measured by ELISA. Values were normalized to dry weight of the intestinal explant. Asterisk (*) denotes comparisons where $p < 0.05$ compared with the mNBD control group. (Reprinted with permission from Journal of Immunolgy, vol 179, Dave et al., 2007[78])

2.5 DISCUSSION

In this study, effects of the IKK inhibitor peptide, NBD, employing a novel PTD (8K) was investigated *in vitro* and *in vivo*. This peptide efficiently transduces cells in culture, and transduces intestinal lymphoid tissue following intraperitoneal administration *in vivo*. In cells, 8K-NBD inhibits TNF-induced NF-κB transcriptional activity and nuclear translocation. *In vivo*, 8K-NBD inhibited LPS-activated NF-κB in the lamina propria, but did not affect basal NF-κB in Peyer's patches. Moreover, we demonstrate that 8K-NBD ameliorates chronic colitis in *IL-10*$^{-/-}$ mice, and histological improvement correlated with reduction in mucosal levels of the inflammatory cytokines IL-12(p40) and TNF.

The critical role of NF-κB in chronic intestinal inflammation is best illustrated the effects of NF-κB blockade in animal models of IBD [108, 110, 111]. In spontaneously occurring colitis in *IL-10*$^{-/-}$ mice, increased NF-κB DNA binding activity and p65 protein expression were found in lamina propria macrophages. The essential role of p65 in maintaining chronic intestinal inflammation was demonstrated by successful treatment of established colitis in these mice with p65 antisense oligonucleotides [111]. Other therapeutic interventions targeting NF-κB, including a dominant negative IκB mutant and NF-κB decoy oligonucleotides [277], demonstrated reduced inflammation in models of colitis.

Furthermore, activated NF-κB is found in human IBD. A significant increase in p65 protein in lamina propria macrophages and IECs from CD patients was correlated with increased production of the inflammatory cytokines IL-1β, IL-6, and TNF [106]. *In vitro* treatment of lamina propria macrophages from CD patients with p65 antisense oligonucleotides was effective in down-regulating inflammatory cytokine production, suggesting a key role for NF-κB p65 in inflammatory cytokine expression in CD [106]. Activated NF-κB has been reported in

macrophages and IECs from inflamed mucosa of patients with IBD *in situ* using a specific p65 antibody that exclusively detects the activated form of NF-κB [267]. No significant differences were found between sections of inflamed mucosa from patients with CD, UC, or diverticulitis [267]. This study indicated that activation of NF-κB is not necessarily specific for the pathophysiology of IBD: NF-κB activation could represent an important step in mucosal inflammation regardless of the etiology.

Accordingly, selective IKK inhibition represents a potential therapeutic strategy in IBD. A concern about the use of inhibitors that completely suppress IKK activity is that they may also inhibit the ability of basally active NF-κB to act as a physiologic survival factor, thereby raising the possibility of toxicity. Activation of the IKK complex in response to inflammatory mediators depends critically on the presence of the NEMO subunit of the IKK complex. For example, NEMO-deficient cells lack detectable NF-κB binding activity in response to TNF, IL-1β, and LPS [278]. Furthermore, recent studies have shown that continuous administration of the NBD peptide effectively ameliorates inflammatory responses in animal models of inflammation without overt side effects such as liver or kidney toxicity [76, 279]. Additionally, the NBD peptide preserves the alternative pathway of NF-κB activation, necessary for B cell development and lymphoid organogenesis, again minimizing potential toxicity concerns. In our studies, while there was an inhibition of activated NF-κB the levels of basal NF-κB translocation stayed constant (Figure 9&11). Significantly, *in vivo*, 8K-NBD inhibited activated NF-κB in the ileal lamina propria, but basal NF-κB appeared to be preserved in the Peyer's patches. This feature theoretically allows for dampened inflammatory responses, without altering other roles of NF-κB within the cell.

Specific inhibition of IKK activity by NBD peptides may have pleiotropic mechanistic and durable immunologic effects in inflammatory diseases. In mouse models of chronic inflammation, including collagen induced arthritis (CIA) [71, 76] and experimental allergic encephalomyelitis (EAE) [75], *in vivo* treatment with NBD peptides blocked disease activity, pro-inflammatory cytokine expression, and homing of cells to inflammatory sites due to inhibition of expression of cellular adhesion molecules. In EAE, clinical recovery was correlated with a durable alteration of the T cell phenotype, as NBD-treated mice demonstrated Th2 cytokine production rather than disease-associated Th1 cytokine production [75]. Furthermore, mice treated systemically with an NBD peptide for five days after induction of CIA maintained clinical and histological improvement for nearly three weeks following termination of peptide administration [76]. In our study, the 8K PTD was detected in important immune inductive sites (spleen and mesenteric lymph nodes) following intraperitoneal administration; however we could not identify the peptide in the intestinal lamina propria or epithelia (data not shown). This finding suggests that the mechanism of action of 8K-NBD in IBD is likely to be more complicated than can be explained by direct inhibition of activated NF-κB in the inflamed intestine. Future mechanistic studies in IBD models will be necessary to characterize functional and phenotypic alterations in immune cell populations and durability of clinical responses with the 8K-NBD.

While PTD-NBD has demonstrated efficacy in other inflammatory models, important and distinguishing features of colitis in *IL-10*$^{-/-}$ mice compared to these other experimental systems are that it is a chronic, "spontaneously" occurring model of inflammation, and 8K-NBD was an effective therapeutic intervention when administered *after* the onset of disease [109]. Furthermore, CIA and EAE induced inflammatory models are specifically T cell mediated diseases, however, *IL-10*$^{-/-}$ mice have pathologic innate and adaptive immune responses [75].

To further characterize the effects of inhibition of NF-κB in IBD, it will be critical to dissect protective from detrimental properties of NF-κB activation in mucosal inflammation. Although increased activation of NF-κB is implicated in the pathogenesis of numerous chronic disorders, NF-κB activation pathways may be protective and serve to maintain homeostasis in the intestine [280, 281]. For example, the toll-like receptor (TLR) family recognizes extracellular microbial constituents resulting in the downstream activation of NF-κB. TLR-deficient mice or strains with deletions in signaling intermediates such as MyD88 demonstrate a decrease in survival compared to wild-type mice when colitis is induced with dextran sodium sulfate (DSS) [281]. Intestinal epithelial proliferation was shown to be markedly decreased in TLR/*MyD88*$^{-/-}$ animals. This study suggested that TLRs, expressed on IECs, may recognize luminal microbial constituents and mediate a protective response through NF-κB activation. Furthermore, mice with a targeted deletion of IKKβ in intestinal epithelial cells demonstrate increased epithelial apoptosis [282] and are susceptible to radiation induced injury [283]. Most relevant to this study, mice with a targeted deletion of NEMO in intestinal epithelial cells (IEC) develop severe spontaneous intestinal inflammation through TNF and MyD88 dependent pathways, further suggesting that IKK activation in intestinal epithelium mediates homeostatic pathways[112]. Interestingly, a slight increase in TNFα levels were observed in the 10mg/kg NBD-treated *IL-10*$^{-/-}$ mice compared to those treated with 2mg/kg NBD. This could be potential due more cell types being tranduced by the NBD peptide secondary to higher dose. Thus, if IEC were affected at higher rather than lower doses of this could lead to barrier breakdown similar to the NEMO deletion in IEC, and a slight increase in TNFα. While still much lower than mNBD-treated miceIn summary, NF-κB inhibition, particularly in the intestinal epithelium, may lead to abrogation of mucosal protective effects.

Conversely, the preponderance of evidence suggests that inhibiting NF-κB in lamina propria macrophages and DC may be of therapeutic benefit in IBD. A recent study demonstrated that the development of colitis in *IL-10*$^{-/-}$ mice is completely dependent on TLR signaling pathways[284]. In *IL-10*$^{-/-}$ × *MyD88*$^{-/-}$ mice, colitis is abrogated and intestinal IL-12 p40 levels are markedly decreased. Furthermore, bone marrow chimera experiments reveal that bone marrow derived cells are responsible for recognition of commensal microbial signals and mucosal innate immune activation.

Taken as a whole, the spectrum of NF-κB biology in the gut is complex. Our results and those of others suggest that inhibition of activated NF-κB in mucosal macrophages and DC may ameliorate innate immune responses that underlie chronic IBD; however, NF-κB may play a protective role in the epithelium. Thus, in contemplating therapeutic strategies that target NF-κB, many interrelated factors may be important to determine ultimate clinical applicability, including inhibition of activated versus basal NF-κB, targeting specific cell types (macrophages versus gut epithelium), and route of delivery (systemic versus local).

This study supports the concept that selective inhibition of IKK by 8K-NBD is an effective strategy for suppressing intestinal inflammatory responses. Compared to other NF-κB inhibitors tested in chronic inflammatory diseases, 8K-NBD has the theoretic advantages of inhibiting activation of NF-κB, a hallmark of chronic inflammation, while not inhibiting basal NF-κB activity, which may be involved in fundamental cellular homeostatic processes.

3.0 EVALUATION THE ROLE OF NF-KB ACTIVITY IN AGING

3.1 ABSTRACT

NF-κB is a family of transcription factors that play a pivotal role in determining cell fate in response to stress, including inflammatory, oxidative and genotoxic stress. NF-κB is implicated in numerous chronic inflammatory and degenerative diseases, many of which are associated with aging. To test the hypothesis that NF-κB plays a causal role in driving the degenerative changes associated with aging, we tested whether genetic depletion or pharmacologic inhibition of NF-κB delays the onset of degenerative diseases in a mouse model of accelerated aging (*Ercc1*$^{-/\Delta}$ mice). Like naturally aged mice, *Ercc1*$^{-/-}$ mice exhibit increased NF-κB activity as they reach their maximum lifespan. *Ercc1*$^{-/\Delta}$ mice haploinsufficient for the p65/RelA subunit of NF-κB had a modest delay in the onset of age-related symptoms. This was recapitulated in mice chronically treated with a peptide inhibitor of NF-κB activation, which exhibited a significant delay in overall aging score and improved histopathological alterations. These findings implicate NF-κB as a major driver of degenerative changes associated with aging and set a precedent for therapeutic intervention.

3.2 INTRODUCTION

Aging is characterized by the progressive erosion of the ability of a tissue to maintain homeostasis, resulting in increased risk of morbidity and mortality[121]. Increasing chronologic age is the top predictive risk factor for a variety of diseases including cancer, heart disease, arthritis, and dementia is increased chronologic age[285]. There is an exponential increase in the ag-at-onset of these disease compared with a linear increase in chronologic age[120] suggesting specific mechanisms are contributing to this change. With the US population over the age of 65 increasing[122], and the fact that 27% of the 327 billion dollar Medicare budget in 2008 was spent on care for patients in their last year of life[123], society has begun to realize the burden that treating age related diseases can have on our healthcare system and our national economy. Therefore, identifying strategies to reduce this burden and understand the mechanisms of aging is of critical importance.

One such mechanism may be the gene expression changes mediated by NF-κB, which acts as a dimer and promotes transcription of immune, anti-apoptotic, and cell cycle regulating genes.[5] Transcription by NF-κB is mediated by cell stress stimuli, including inflammatory cytokines and pathogens but also endogenous stressors such as DNA damage[12], hyperglycemia[286] and reactive oxygen species (ROS) formation[13]. Stress stimuli signal via the Inhibitor of κB Kinase (IKK) complex, which is is composed of two catalytic subunits, IKKα and IKKβ and a regulatory subunit, IKKγ or NEMO. Once activated by IKKγ, IKKα and β phosphorylate the cytoplasmic inhibitor of NF-κB, IκBα, which is subsiquently poly-ubiquitinated and undergoes

proteosomal degradation[287]. This allows NF-κB to trasnlocate to the nucleus and activate target genes.

Upregulation of NF-κB has been observed in numerous studies associated with aging. Tissues derived from aged rodents including skin, liver, kidney, cerebellum, cardiac muscle and gastric mucosa exhibit increase in DNA binding of NF-κB subunits[158-163]. Analysis using motif mapping of gene expression data from both human and murine tissues suggest that NF-κB is the transcription factor most associated with normative mammalian aging[151]. Furthermore, human cells derived from aged patients (age 72-93) and those with Hutchinson-Gilford progeria, also exhibited increased NF-κB signaling compared to controls[151, 157]. In addition, numerous pathologies associated with aging including, atherosclerosis[168], osteoarthritis[169], dementia[170], osteoporosis[171], diabetes, cancer[288] and cardiovascular disease[172] all have reported increases in NF-κB activity. These previous studies have elucidated a novel pathway by which age-associated diseases and overall aging may be propagated. Two recent studies by Adler et al and Kawahara et al. observed that age-associated pathology was reversed via suppression of NF-κB signaling[151, 164]. Thus, we hypothesize that by inhibiting the NF-κB signaling pathway, age-related degenerative phenotypes and histologic changes associated with mammalian aging will be limited.

Various mechanisms are used to block NF-κB transcriptional activation, including both genetic and pharmacologic suppression. In this study we will evaluate mice heterozygous p65/RelA, as in Kawahara et al.[164] as this NF-κB subunit is a documented transcriptional activator[5] and is known to play a role in age-related changes and cellular senesence[151, 155]. Due to the embryonic lethality of germ-line homozygous deletion of this gene only mice haploinsufficient for p65 can be used[289]. There are vast number of NF-κB inhibitory

compounds[4], the majority of which have off-target effects or poor *in vivo* bioavailability. Therefore, the NEMO binding domain (NBD), an 11 amino acid peptide derived from the domain of IKKβ that is critical for its interaction with IKKγ was used in this study[14]. NBD is linked to a protein transduction domain (PTD) consisting of eight lysine residues (8K) that promotes internalization of the inhibitor into cells. It has been shown that chronic administration of NBD is efficacious in the treatment of numerous murine models of degenerative disease including arthritis[71], diabetes[67], and Parkinson's disease[72], and the efficacy of 8K-NBD was shown in Dave et al[78].

We hypothesize that suppression of NF-κB trascriptional activation will delay or ameliorate age-related phenotypic changes in mammalian aging. To study aging-related changes we have chosen a progeroid-like or accelerated aging model, the ERCC1 deficient mouse. This model allows for the assement of age-related changes in a model which recapitulates the transcriptional changes associated with the accumulation of DNA damage and oxidative stress known to occur in normative agings [125, 126, 216]. ERCC1 progeria is described previously in Niedernhofer et al.[130] and Robinson et al[290], and develops secondary to a defect in DNA repair. *Ercc1*$^{-/-}$ and *Ercc1*$^{-/\Delta}$ mice have 0% and 10% ERCC1-XPF expression respectively, and age over a period of 28 days and 28 weeks respectively, with phenotypic changes similar to those observed in normative aging mice.

In Kawahara et al., *Sirt6*$^{-/-}$ mice haploinsufficient for p65/RelA, an NF-κB subunit, had improved lifespan and reduced degenerative changes[164]. However, it was observed that these mice have a colitis like phenotype and associated with gross inflammation which may have contributed to this effect[165]. The ERCC1 deficient mous model is an accurate predictor of aging on the transcriptional level with the exception of innate and complement responses that are only

upregulated in normative aging mice level[130, 291]. Therefore, the ERCC1 deficient mice will allow for evaluation of the role of NF-κB signaling independent of inflammation. Due to the predictable order of symptom development, healthspan of these animals can be evaluated by determining delay in age-at-onset of symptoms and examining degenerative histologic changes, thus allowing for a proper analysis of the effects of NF-κB suppression on age-associated decline

3.3 MATERIALS AND METHODS:

3.3.1 Mice:

Both *Ercc1*$^{-/-}$ and *Ercc1*$^{-/\Delta}$ mice were generated in an f1 hybrid background by intercrossing *Ercc1*$^{+/-}$ and *Ercc1*$^{+/\Delta}$ mice in a C57Bl/6J or FVB/n inbred background. Genomic DNA was isolated from an ear punch using a Machery-Nagel vacuum manifold according to instructions. PCR amplification of the null allele was achieved with primers specific for exon 7 and intron 7 of *Ercc1* and *neo*^{r} (5'GAAAAGCTGGAGCAGAACTT, 5'-AGATTTCACGGTGGTCAGAC, and 5'-GAAGAGCTTGGCGGCGAATG, respectively), while 3'-UTR reverse 5'-CTAGGTGGCAGCAGGTCATC is needed in addition to the other primers to detect the deletion mutation in exon 10.

Ercc1$^{-/-}$ eGFP$^{NF\text{-}\kappa B}$ mice were generated by crossing *Ercc1*$^{+/-}$ C57Bl/6J mice with eGFP$^{NF\text{-}\kappa B}$ mice, a generous gift from Christian Jobin (UNC Chapel Hill)[107]. These mice were then bred with *Ercc1*$^{+/-}$ FVB/n to create *Ercc1*$^{-/-}$ eGFP$^{NF\text{-}\kappa B}$. Genotyping procedure used as described in [107].

3.3.2 Fluorescent Microscopy:

Ercc1$^{-/-}$ eGFP$^{NF\text{-}\kappa B}$ mice and a wild type eGFP$^{NF\text{-}kB}$ littermate were sacrificed at 21 days of age. Tissues were placed in 10% formalin for 6 hr, then transferred to 30% sucrose in phosphate buffered saline (PBS) overnight at 4°C. The tissues were then frozen in 2-methylbutane and embedded in optimal temperature cutting at -30°C. Six micron sections were cut using cryostat. Tissues were stained using HOESCT stain (Sigma) and coverslipped using gelvatol, as desribed in [292]. Samples were allowed to sit overnight at 4°C and were subsequently analyzed using Axiovert200 Microscope and Axiovision software (Zeiss). To quantify eGFP expression in tissues Metamorph software (MDC) was used. Five 20x images were taken for each tissue analyzed in each mouse (n=6 per group), the percent eGFP expression was quantified based on tissue area. The amount of eGFP was normalized within litters to (normative aging littermate) to determine baseline levels of eGFP within the litter. These normalized eGFP expression levels were then analyzed between groups using Wilcoxon rank methods using SPSS (SPSS Inc).

3.3.3 Isolation and Treatment of Mouse Embryonic Fibroblasts:

Pregnant mice were bred to produce WT, *Ercc1*$^{-/,-}$ and *Ercc1*$^{-/-}$*p65*$^{-/-}$ pups were prepared as in [130]. Briefly, mice were sacrificed at E13.5 by CO_2 inhalation. Individual embryos were dissected and plated on 10 cm cell culture dishes. Cells were genotyped as described above. The cells were then grown at 3% O_2, which is considered physiologic[149] and grown in MEF media (DMEM 44% ml Ham's F10 44%, 10% FBS, 1%, P/S, 1% NEAA).

To stress the cells with oxidative insult, MEFs were grown at 20% O_2[149]. Proliferation rates were determined as in [215]. Briefly, cells were passaged in 10 cm plates, and counted at each

passage (determined by the first plate to reach confluency) to evaluate their proliferation rates. Cells were then reseeded at 25,000 cells per plate and returned to either 3% or 20% O_2.

3.3.4 NF-κB Luciferase assay

HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter (DMEM/10% FBS/1% Pen/Strep) were left untreated or pretreated for one hour with 10μM NF-κB inhibitor Compound A (Calbiochem), 10μM ATM inhibitor (2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU-55933, Calbiochem) or 25μM p53 inhibitor (pifithrin-α, Calbiochem). Cells were then γ-irradiated from a ^{137}Cs source. The cells were lysed in reporter lysis buffer and luciferase activity was measured with a luciferase assay system (Promega) using a AutoLumat Luminometer (Berthold Technologies).

3.3.5 Nuclear extracts and Western blotting

Nuclear extracts from treated MEFs were isolated following manufacturer's protocol (NE/PER Reagents, Pierce). Protein concentration was determined using the Bradford assay (Pierce). Western blot analyses were performed on nuclear and cytoplasmic extracts as described previously [274]. Anti-p-p65, p-IκBα, and IκBα antibodies were purchased from (Cell Signaling, Danvers MA) Lamin A/C (Santa Cruz Biotechnology) and β-actin (Abcam). All primary antibodies were used at 1:1000 antibody dilutions and allowed to incubate overnight at 4°C. Secondary antibodies goat α-rabbit, and rabbit-α-mouse (Cell Signal) were used at 1:1000 dilutions.

3.3.6 Peptides:

The peptides 8K-NBD (NBD) (acetyl-KKKKKKKKGGTALDWSWLQTE-amide), and inactive 8K-mutant NBD (mNBD) (acetyl-KKKKKKKKGGTALDASALQTE-amide, where the underlined amino acids represent tryptophan to alanine mutations), were synthesized by the peptide synthesis facility at the University of Pittsburgh, Pittsburgh, PA.

3.3.7 Treatment of Animals:

Sibling pairs of *Ercc1*$^{-/\Delta}$ mice housed in a single cage were evaluated in this study. Treatments were given in a blinded fashion, mice were identified by ear clips prior to initiation of the study, and these mice were denoted as 1 or 2 for each group. Mice were then assigned a treatment for 1 or 2 by a third party. One syringe was then filled with NBD while the other with mNBD. These were then labeled by the third party with a 1 or 2 label. Paired mice were injected with NBD or mNBD three times per week. Treatments with the peptides were initiated at 5 weeks of age. The peptide were given intraperitoneally (i.p.) at a dose of 10 mg/kg in 100µl of phosphate buffered saline (PBS). Treatment was administered 3 times per week through the entire lifespan of the animal. Mice were euthanized via CO_2 inhalation at points 18-20 weeks of age or until end of life and tissues were collected for analysis.

3.3.8 Phenotype and Weighing:

Weight and the age-at-onset of spontaneous age-related symptoms (dystonia, trembling, kyphosis, ataxia, sarcopenia, priapism, urinary incontinence, and lethargy) were assessed bi-

weekly by an investigator blinded to the treatment of the mutant animals. The onset of each symptom was recorded, averaged within a treatment group and reported in tabular form. The difference in the age at onset of symptoms between groups was analyzed using a paired Student's t-test. This cumulative aging score is calculated by assigning animals within each littermate pair a + if a symptom is delayed or a – if the onset is earlier or equivalent in both groups. The + scores are then added and normalized to the total number of symptoms evaluated within that group and analyzed using a Student's t-test. The aging score provides an overall evaluation of quality of life.

3.3.9 Tissue Sections/ IHC:

Dissected tissues were fixed in 10% formalin overnight, embedded in paraffin and sectioned using a microtome. p16 staining was completed as follows anti-p16 (CDKN2A/p16INK4a Abcam) according to standard immunohistochemical protocols. Insulin staining was completed as follows, anti-insulin (Biogenex) with secondary biotin-horse-α-mouse (Vector). Staining was resolved using ABC-Elite (Vector) and AEC substrate (Scytek). Percent insulin positive cells were quantified using Metamorph software (Molecular Devices) and data was evaluated using Student's t-test

3.3.10 MicroCT:

Micro-computed tomography of the spines isolated from 20 wk-old *Ercc1*$^{-/\Delta}$ mice and wt littermates mice were acquired_using a VivaCT 40 (*Scanco Medical*) using 15-µm isotropic voxel size resolution, 55 kVp of energy, and 145 µA of current. After the acquisition of

transverse two-dimensional image slices, three-dimensional reconstruction of the lumbar vertebrae was performed using a constant threshold value of 235 which was selected manually for the bone voxels by visually matching the threshold areas to the gray-scale images. NBD treated and control treated *Ercc1*$^{-/\Delta}$ mouse data were compared using Student's t-test.

3.3.11 Microarray:

To measure genome-wide changes in transcription in mice treated with NBD or mNBD, microarray analysis was performed as described[130]. Briefly, RNA was isolated from the liver of *Ercc1*$^{-/\Delta}$ mice (n=3 treated with NBD and mNBD at 12 weeks of age and 18 weeks of age, each) using an RNA isolation kit (Qiagen) according to the manufacturer's instructions. Synthesis of double-stranded cDNA and biotin-labeled cRNA, hybridization to Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays were completed using Affymetrix protocols Microarray (Affymetrix). Analysis was completed using GeneGo (GeneGo bioinformatics software) and IPA (Ingenuity Systems). GeneGo was used to analyze both gene ontology categories as well as transcriptional regulation analysis (via its network building tool). IPA was used to analyze gene ontology categories.

3.4 RESULTS:

3.4.1 *Ercc1*$^{-/-}$ cell lines have over-active NF-κB signaling which contributes to proliferation defects:

To evaluate NF-κB activity, levels of activated or phosphorylated-p65 (p-p65) translocation to the nucleus in *Ercc1*$^{-/-}$ and WT mouse embryonic fibroblasts (MEFs) generated from littermates were defined by immunodection (Figure 16A). Quantification shows approximately 2.5±0.5 fold increase in p-p65 levels in the nuclei of *Ercc1*$^{-/-}$ cells compared with control cells (Figure 18A). Previous data suggests that *Ercc1*$^{-/-}$ MEFs have proliferation defects and senesce more rapidly than WT cells[130]. This is confirmed in Figure 16B, *Ercc1*$^{-/-}$ MEFs exhibit proliferation defects which are exacerbated in high oxygen or stress environment (20% O_2)[149]. After 3 passages at 20% oxygen, *Ercc1*$^{-/-}$ MEFs show increased NF-κB translocation to the nucleus compared with identical cell lines grown at 3% oxygen over the same period of time (Figure 16C). This suggests that the *Ercc1*$^{-/-}$ MEFs experiencing growth or proliferation defects secondary to DNA damage or oxidative stress exhibit concomitant increases in NF-κB signaling.

To evaluate whether NF-κB suppression had a positive impact on cell growth, proliferation profiles were completed for WT, *Ercc1*$^{-/-}$, and *Ercc1*$^{-/-}$ MEFs with a homozygous deletion of p65 (*Ercc1*$^{-/-}$*p65*$^{-/-}$) (Figure 16D). *Ercc1*$^{-/-}$p65$^{-/-}$ MEFs proliferated at a rate between the *Ercc1*$^{-/-}$ and WT MEFs at 3% oxygen conditions; however, in oxidative conditions of 20% oxygen the *Ercc1*$^{-/-}$*p65*$^{-/-}$ MEFs grew similar to WT MEFs for the initial passages and then exhibited a reduction in cell growth, while the *Ercc1*$^{-/-}$ cells had far slower cell proliferation. Thus, p65 homozygous deletion in *Ercc1*$^{-/-}$ MEFs partially restored early stage proliferation rates, when compared with WT MEFs

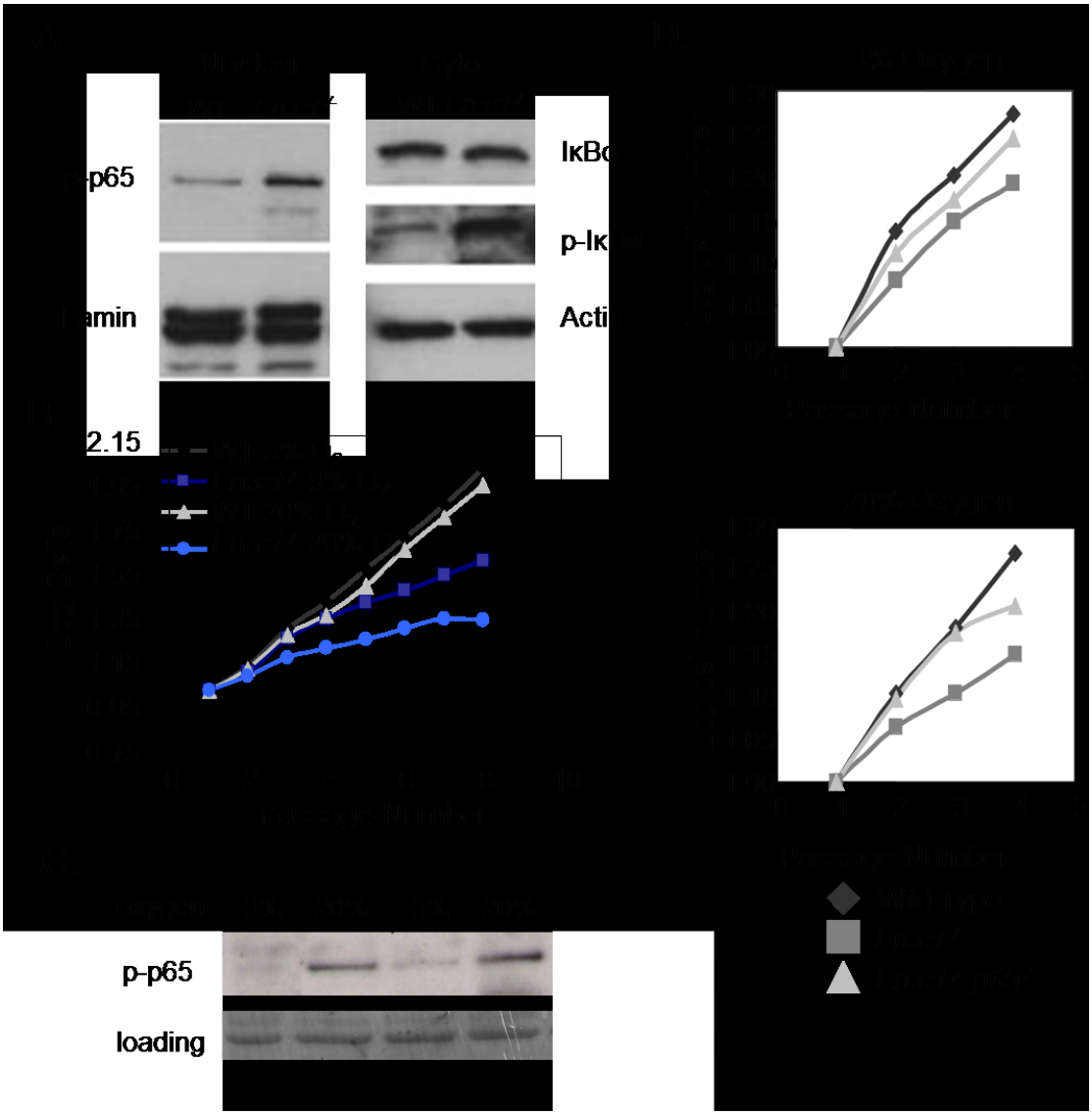


Figure 16: NF-κB is activated in *Ercc1*$^{-/-}$ cells and limits proliferation under conditions of oxidative stress. (A)Immuodetection of markers of NF-κB activation in fractionated MEFs: nuclear phosphor-p65 and cytoplasmic IκB and phosphor-IκB. Lamin A/C and actin were used as loading controls for nuclear and cytoplasmic fractions, respectively. (B) Proliferation of WT and *Ercc1*$^{-/-}$ cells grown at 3 or 20% oxygen (normative or oxidative environment) (C) Immunodetection of p-p65 subunits of NF-κB in WCEs from primary *Ercc1*$^{-/-}$ mouse embryonic fibroblasts, grown for 4 passages at 3% or 20% O_2, to induce oxidative stress . (D)Proliferation of WT, *Ercc1*$^{-/-}$, and *Ercc1*$^{-/-}$ *p65*$^{-/-}$ primary MEFs grown 3% oxygen (Top panel) and at 20% O_2 to induce oxidative stress (Bottom Panel).

3.4.2 NF-κB is activated by gamma irradiation in an ATM dependent manner and NF—κB suppression promotes cellular survival.

Genetic suppression of NF-κB improved the growth profile for *Ercc1*$^{-/-}$ MEFs, suggesting that reduced NF-κB signaling improves growth and/or survival under stress conditions such as DNA damage and oxidative stress, both of which are increased in the *Ercc1*$^{-/-}$ cells. ERCC1 deficient mice by their nature have an abnormal accumulation of bulky adducts, interstrand crosslinks and double strand breaks. This DNA damage mediates the accelerated aging phenotype in these mice. To evaluate the effects of DNA damage on NF-κB activation, HEK293 cells stably expressing a mutimerized NF-κB DNA binding element-luficerase reporter ($293^{NF-\kappa B}$) were exposed to 0, 10 and 20Gy of irradiation and NF-κB transcriptional activity was analyzed at varying time points (Figure 17A). NF-κB activity was shown to increase by 6 hours and began to dissipate at 12 hours. When cells were pretreated with a potent IKKβ specific inhibitor, Compound A[293], NF-κB induced luciferase activity secondary to γ-irradiation was reduced to basal levels (Figure 17B). NF-κB luciferase activity was reduced equally with pretreatment of 10μM of KU55933, an ATM specific inhibitor, suggesting that NF-κB is activated in an ATM dependent manner after γ-irradiation. This further promotes the idea that NF-κB is activated by DNA damage alone after γ-irradiation. Protein analysis evaluating p-p65 shows increased NF-κB translocation to the nucleus of MEFs three hours after exposure to 10G of γ-irradiation (Figure 17C). Further analysis in a 5 day survival time course revealed that transient inhibition of NF-κB using Compound A, prior to γ-irradiation, increased cellular

survival of γ-irradiated MEFs p=0.009(Figure 17D). This effect was likely due to increased

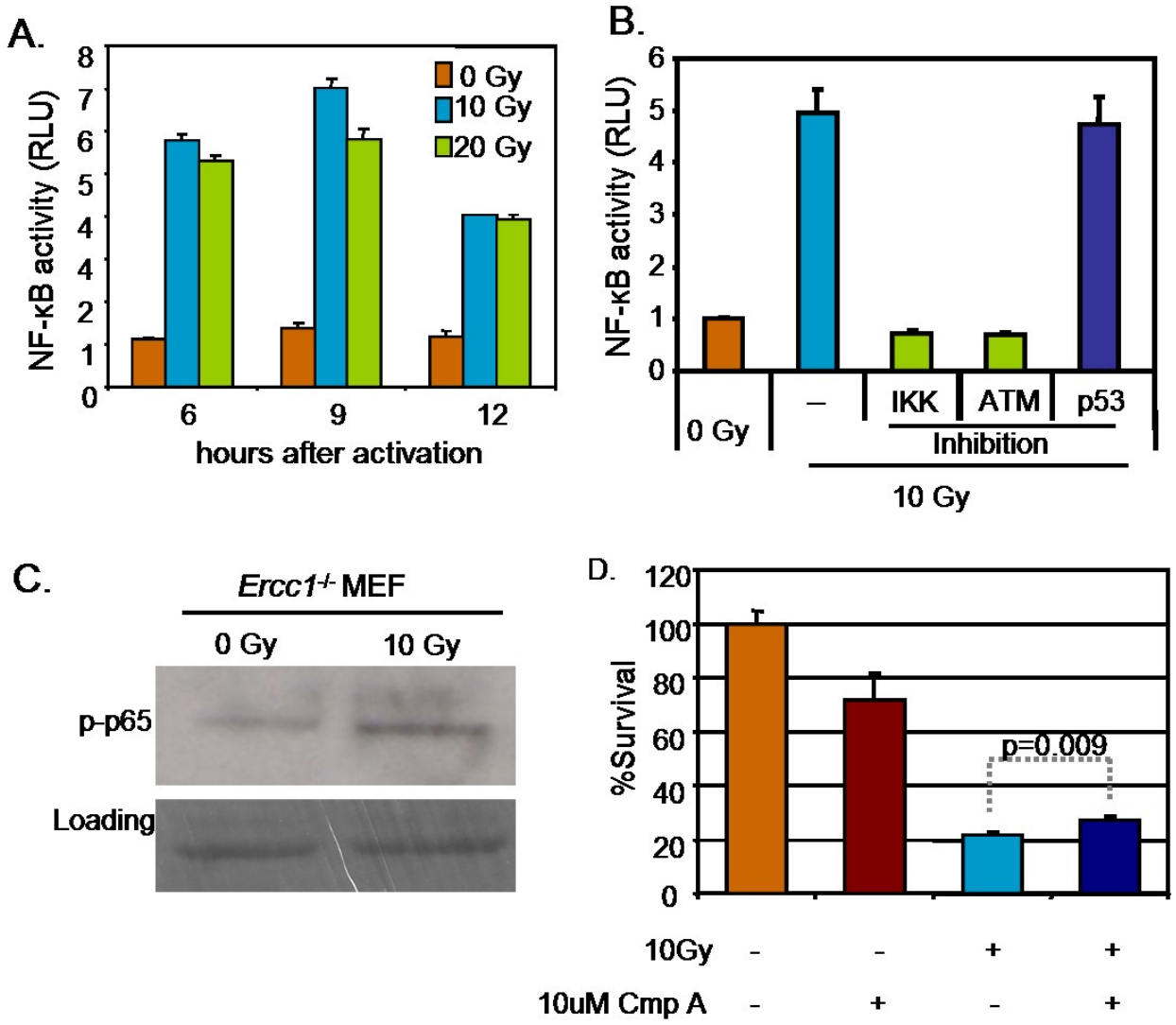


Figure 17: NF-κB is activated by γ-irradiation in an ATM dependent manner: (A) Luciferase levels were quantified from HEK293 cells stably transfected with a mulimerized NF-κB DNA binding element luciferase reporter ($293^{NF-\kappa B}$) were treated with 0, 10, or 20Gy of γ-irradiation and evaluated at 6, 9, and 12 hours post irradiation. (B) Luciferase levels were determined from $293^{NF-\kappa B}$ cells pretreated with 10μM Compound A (NF-κB inhibitor), 10μM KU55933 (ATM inhibitor), 25μM pifithrin-α (p53 inhibitor) or left untreated. After 1 hour cells underwent 10G of irradiation. (C) Immunodetection of p-p65 from *Ercc1*$^{-/-}$ MEFs treated with 0 or 10Gy of γ-irradiation. (D) MTT assay was used to determine five day survival levels for MEF undergoing 0 or 10Gy of irradiation with or without the treatment of 10μM Compound A.

survival, as non-irradiated control MEFs treated with Compound A exhibited reduced proliferation compared to those left untreated.

3.4.3 Progeroid-like ERCC1-deficient mice exhibit overactive NF-κB signaling:

As NF-κB activity was increased *in vivo* in ERCC1 deficiency, it was pertinent to determine if progeroid-like ERCC1-deficient mouse model exhibits increased NF-κB signaling as well. *Ercc1*$^{-/-}$ were bred with mice expressed eGFP under an NF-κB regulatory element (NF-κB^{eGFP})[275]. Mice were analyzed at 21 days of age, or 75% of maximum lifespan. Figure 18 compares the eGFP expression in both progeroid-like *Ercc1*$^{-/-}$eGFP$^{NF\text{-}\kappa B}$ and their normative aging (*Ercc1*$^{+/+}$NF-κB^{eGFP} or *Ercc1*$^{+/-}$NF-κB^{eGFP}) littermates. eGFP mRNA isolated from the kidney is increased in the accelerated aging *Ercc1*$^{-/-}$ mice compared to their littermate controls (Figure 18A). This upregulation is confirmed using fluorescent microscopy, examining eGFP fluorescence in numerous tissues including kidney, muscle, pancreas, and liver with the exception of the spleen (Figure 18B/C). Fluorescent intensity was quantified and differences between the two groups were found to be statistically upregulated in the kidney, liver, pancreas and muscle of *Ercc1*$^{-/-}$NF-κB^{eGFP} (Figure 18D). The liver exhibited a 3.58 fold increase ($p<0.012$) in NF-κB activity, while the kidney and pancreas had approximately a 2.5 fold increase ($p<0.001$ and $p<0.021$ respectively) and the muscle had a 1.7 fold increase ($p<0.032$). Interestingly, this over-expression in the tissues examined is nearly equivalent to the over-activation of NF-κB determined using *Ercc1*$^{-/-}$ MEFs analyzed in Figure 16A/19A. These data confirm that, as with normative aging mice, ERCC1-deficient mice have increased NF-κB signaling as the animals near their maximum lifespan.

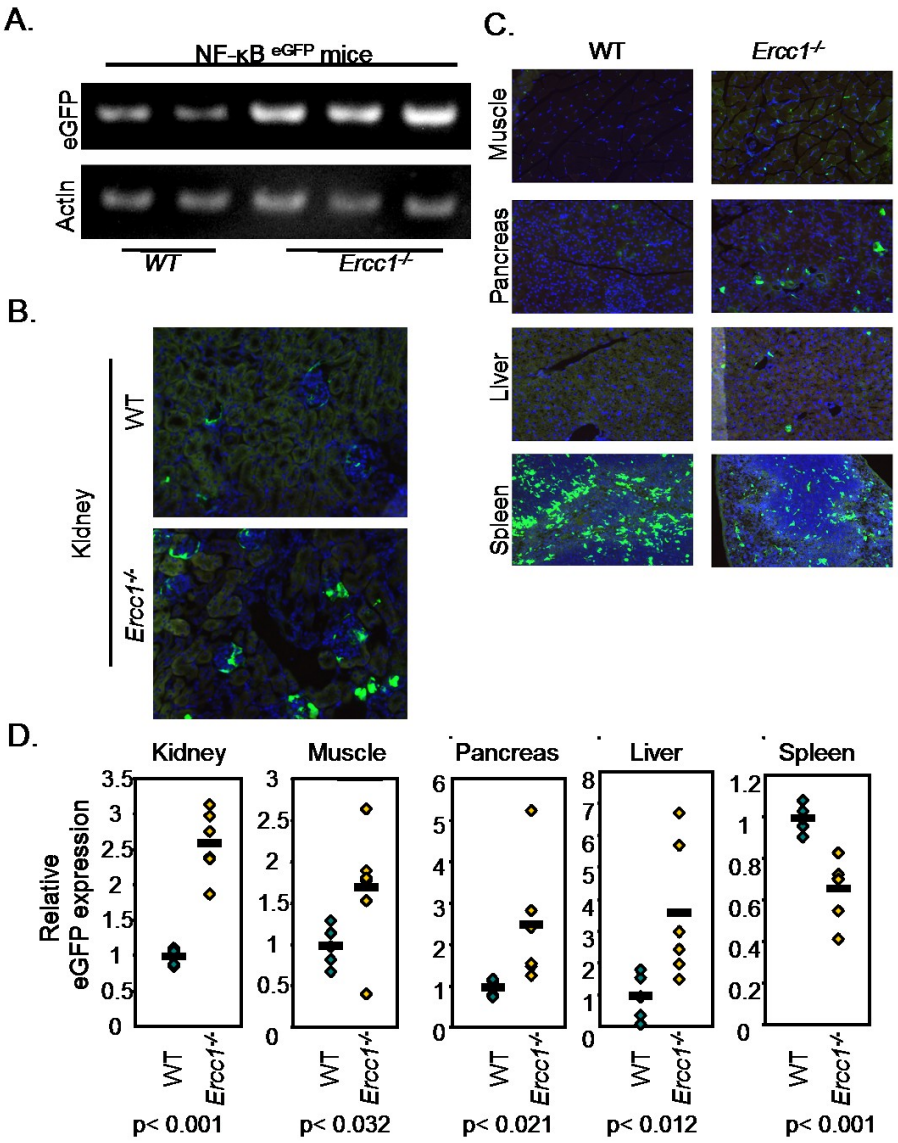


Figure 18: NF-κB is activated in progeroid *Ercc1*$^{-/-}$ mice. *Ercc1*$^{-/-}$ mice were bred to express an NF-KB -eGFP reporter transgene. (A) mRNA was isolated from the kidney of *Ercc1*$^{-/-}$NF-κB^{eGFP} mice and littermate *eGFP*$^{NF\text{-}κB}$ controls and NF-κB activity measured by RT-PCR of eGFP mRNA using β-actin as a control. (B/C) Kidney and additional tissue sections from *Ercc1*$^{-/-}$NF-κB^{eGFP} and NF-κB^{eGFP} mice. Imaged by fluorescent microscopy to detect eGFP expression (green); Dapi (blue). (D) eGFP levels were quantified as relative to tissue are. Five random fields (20X) were analyzed per mouse (n=6 per group). Green dots represent individual *NF-κB*^{eGFP} control mice and yellow indicate *Ercc1*$^{-/-}$*NF-κB*^{eGFP} mice Each mouse is correlated to eGFP expression in the tissues of NF-κB^{eGFP} littermates within that group. The black bar indicates the average eGFP levels within for each genotype.

3.4.4 Ercc1$^{-/\Delta}$ mice with genetically suppressed IKK/NF-κB signaling have delayed onset of phenotypic changes:

Despite increased NF-κB activity in ERCC1-deficient accelerated aging mouse tissues as well as in normative aged tissues, it is unknown whether NF-κB has a protective or detrimental effect. Complete deficiency in p65 results in embryonic lethality[289], thus *Ercc1*$^{-/\Delta}$ mice were bred to have a heterozygous deletion of p65 (*Ercc1*$^{-/\Delta}$*p65*$^{+/-}$*)*. Mice were assessed biweekly for onset of symptoms listed in Figure 19A including dystonia, kyphosis, ataxia, sarcopenia, lethargy, incontinence, and priapism. Interestingly, the *Ercc1*$^{-/\Delta}$*p65*$^{+/-}$ mice experienced a delay in the time of onset for the majority of symptoms compared to *Ercc1*$^{-/\Delta}$ controls. For dystonia, trembling, ataxia, and sarcopenia the changes were negligible (≤ 0.3 weeks), However, for kyphosis and priapism, and incontinence the delay in onset was 1.2, 2.5 and 6.0 weeks respectively, or 4%, 8%, and 20% of lifespan.

Interestingly, the visual appearance of these mice was even more dramatically improved than this delay in age-at-onset of symptoms would suggest. The differences between the *Ercc1*$^{-/\Delta}$*p65*$^{+/-}$ and *Ercc1*$^{-/\Delta}$ mice can be appreciated in Figure 19C/D, where *Ercc1*$^{-/\Delta}$ mice exhibit earlier and more severe phenotype changes than *Ercc1*$^{-/\Delta}$*p65*$^{+/-}$ littermates, such as incontinence, blindness, and priapism, as well as greater severity of disease/degeneration, exhibited by extreme kyphosis, sarcopenia and cachexia observed in representative images taken at 15 and 19 weeks (Figure 19C and 19D). One explanation for the disparity between minimal change in time of onset of symptoms and vast differences in overall appearance, is while age-at-onset of many symptoms only improved slightly, perhaps the progression of these symptoms was slowed and thus the severity of disease was diminished, which can be observed visually but is difficult to quantitate. *Ercc1*$^{-/\Delta}$*p65*$^{+/-}$ had increased weight gain compared to their *Ercc1*$^{-/\Delta}$ controls (Figure

A.

Symptoms	*Ercc1*$^{-/\Delta}$ time of onset (wks)	*Ercc1*$^{-/\Delta}$*p65*$^{+/-}$ time of onset (wks)	Change of Onset (wk)	number of mice *Ercc1*$^{-/-}$ (*p65*$^{+/-}$, WT)
Dystonia	7.7	7.4	-0.3	n=6,10
Trembling	7.1	7.2	0.1	n=6,9
Kyphosis	10.3	11.5	1.2	n=6,12
Ataxia	12.1	12.4	0.3	n=6,12
Sarcopenia	13.2	12.9	-0.3	n=6,12
Priapism	18.37	20.87	2.5	n=3,5
Incontinence	19.05	25.05	6.0	n=2,4

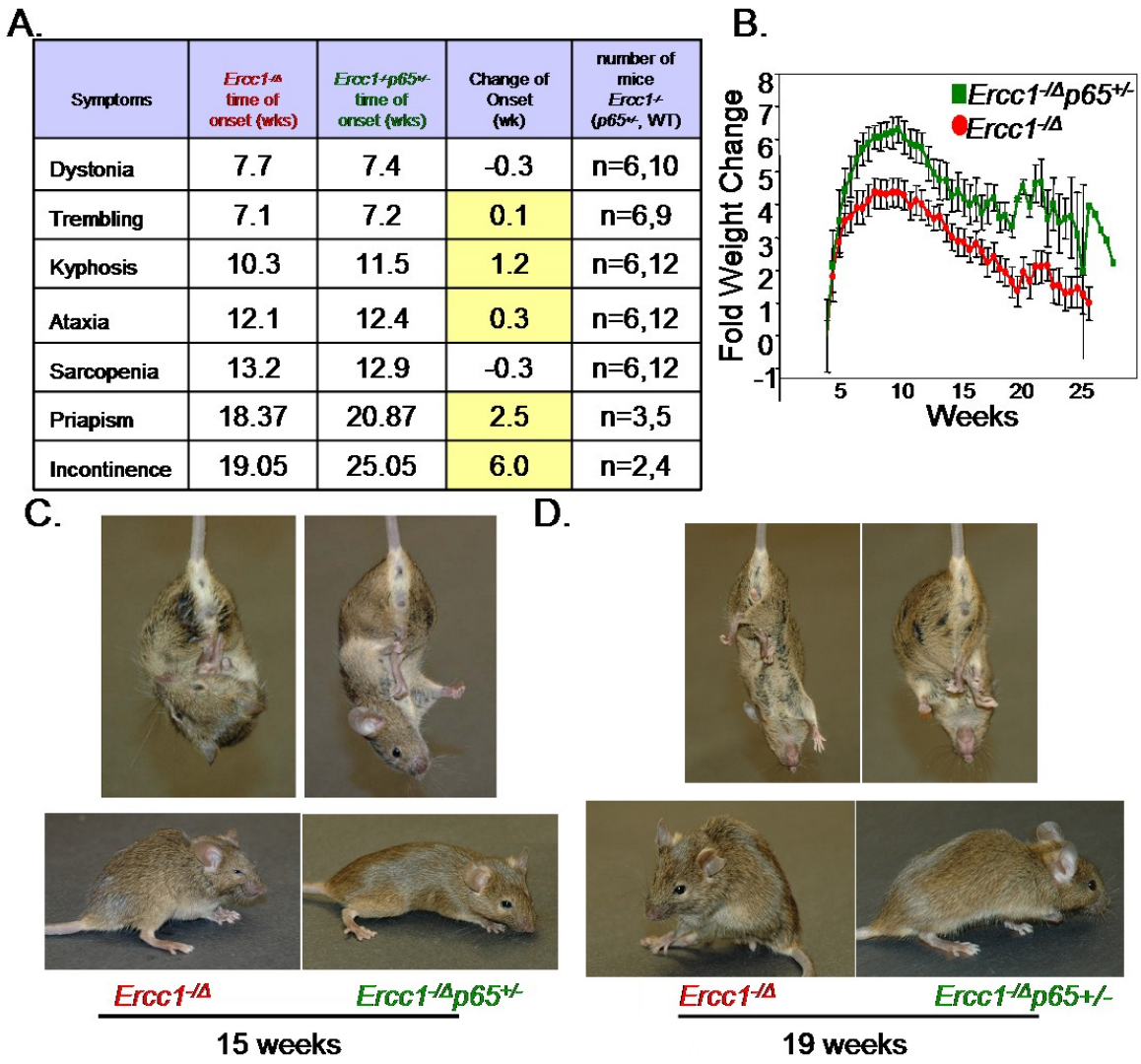


Figure 19: Haploinsufficiency of p65 NF-κB subunit delays the onset of aging symptoms. (A) ***Ercc1***$^{-/\Delta}$ **and** ***Ercc1***$^{-/\Delta}$***p65***$^{+/-}$ **were evauated biweekly spontaneous symptoms associated with aging. Reported are the average age at onset of each symptom for each group and the difference between the averages between groups. (B)** ***Ercc1***$^{-/\Delta}$ **and** ***Ercc1***$^{-/\Delta}$***p65***$^{+/-}$ **weights were measured biweekly, and change from initial weight recorded at 5 weeks of age was recorded (C) Representative images of** ***Ercc1***$^{-/\Delta}$ **and** ***Ercc1***$^{-/\Delta}$***p65***$^{+/-}$ **sex-matched littermates at 15.5 weeks of age. The Ercc1-/Δ mouse has ocular changes, signs of neurodegeneration (broad-based stance, urinary incontinence, dystonia) muscle wasting, kyphosis and aged appearance. (D) Representative images of** ***Ercc1***$^{-/\Delta}$ **and** ***Ercc1***$^{-/\Delta}$***p65***$^{+/-}$ **sex-matched littermates at 19 weeks of age. The** ***Ercc1***$^{-/\Delta}$***p65***$^{+/-}$ **exhibits some changes such as dystonia, kyphosis, and mild sacropenia. The** ***Ercc1***$^{-/\Delta}$ **mouse has signs of neurodegeneration (abnormal stance, priapism, dystonia) muscle wasting, kyphosis and worsened/aged appearance.**

19B). However, this was due primarily to a reduced starting weight of nearly 2g. This may be indicative of suppressed developmental process in the presence of reduced NF-κB signaling before sexual maturity. Therefore, we chose to further explore NF-κB suppression using pharmacologic inhibition of the NF-κB pathway, using the NBD peptide inhibitor starting at 5 weeks of age, when gross developmental processes are less likely to be affected.

3.4.5 NBD peptide inhibitor suppresses upregulation of NF-kB signaling:

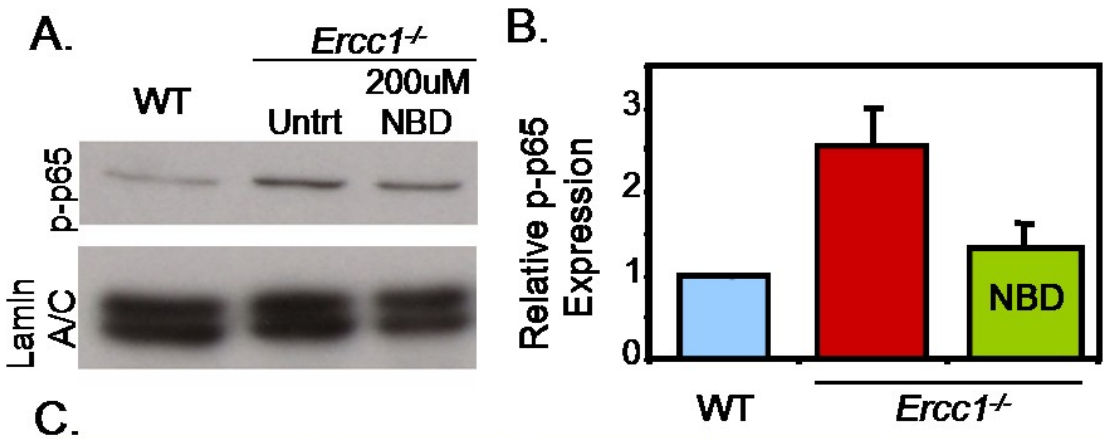
Ercc1$^{-/-}$ cells were treated with the NBD peptide to determine if increased translocation of p-p65 could be reversed. NBD peptide (200uM) reduced levels of p-p65 in the nucleus to 51% of *Ercc1*$^{-/-}$ levels three hours after treatment, which is nearly equivalent to WT MEF p-p65 levels (Figure 20A). Thus, we determined that the NF-κB activity seen in the *Ercc1*$^{-/-}$ mice can be inhibited by NBD at the level of the IKK complex.

Based on this finding NBD was evaluated *in vivo* in the *Ercc1*$^{-/\Delta}$ mice . The NBD peptide provides numerous advantages over the genetic manipulation of p65. First, due to the embryonic lethality of p65 deletion, heterozygous deletion of p65 was used and *Ercc1*$^{-/\Delta}$*p65*$^{+/-}$ mice have a maximum of 50% suppression of p65. Furthermore, other NF-κB subunits remain intact and may compensate for the loss of p65, for example other studies have shown a role for c-rel in the aging process[154]. Second, the NBD peptide has therapeutic potential to treat age-associated disease while genetic deletion is not a feasible approach, but can confirm the importance of biologic pathways. Third, NBD inhibits at the level of the IKK complex and in Chapter 2 of this thesis we show that activated but not basal NF-κB is inhibited. Finally, because p65 is integral in embryonic development, the reduction of this transcriptional subunit in embryonic and early life development may have detrimental effects on the animals.

3.4.6 *Ercc1*$^{-/\Delta}$ mice with suppressed IKK/NF-κB signaling have delayed onset of phenotypic changes:

To evaluate whether NF-κB suppression using the NBD peptide was as or more effective than genetic suppression of p65, a similar phenotypic study to assess the age-at-onset of numerous age-associated symptoms was conducted. It is of relevance to note that this study was completed as a blinded, sex matched, twin study, where mice were treated triweekly with NBD peptide or the inactive mutant form mNBD as described in the Materials and Methods.

Mice treated with NBD experienced a delay in the age-at-onset of all symptoms evaluated with the exception of trembling (Figure 20B); however, the only symptom exhibiting a significant delay in age-at-onset was sarcopenia ($p=0.024$). When analyzing the impact of the delay in age-at-onset of the symptoms it is important to take into account the animal's limited lifespan. For two of the symptoms associated with aging, sarcopenia and incontinence, the time to onset was delayed on average 3 and 3.3 weeks respectively which is 10.7 and 11.7% of maximum lifespan. When correlated to a human lifespan of 80 years this equals a delay of 8.6 and 9.3 years respectively. The cumulative aging score, an optimal method for analyzing quality of life, showed significant improvement in the NF-κB suppressed animal compared with their littermate controls with a p-value of 0.005 (Figure 20B). Images of mice treated with either NBD or their littermate controls, offer visual confirmation of the phenotypic changes, striking differences seen between NF-κB suppressed and littermate controls can be appreciated in these images (Figure 20C/D), which were evaluated in Fig. 20B. Additionally, female but not male mice treated with NBD exhibited increased weight gain compared to littermate controls, which is of relevance because both sarcopenia and cachexia correlate with a loss of body mass (data not shown).



C.

Symptoms	mNBD time of onset (wks)	NBD time of onset (wks)	Change of Onset (wk)	number of mice (mNBD,NBD)
Dystonia	8.9	9.7	0.8	n=9,9
Trembling	11.2	10.7	-0.5	n=9,9
Kyphosis	12.9	13.2	0.3	n=8,7
Ataxia	15.1	16.8	1.7	n=7,6
Sarcopenia*	15.3	18.3	3.0	n=5,6
Priapism	17.1	21.4	4.3	n=3,3
Lethargy	20.8	21.3	0.5	n=4,4
Aging Score*	28%	58%		n=9,9

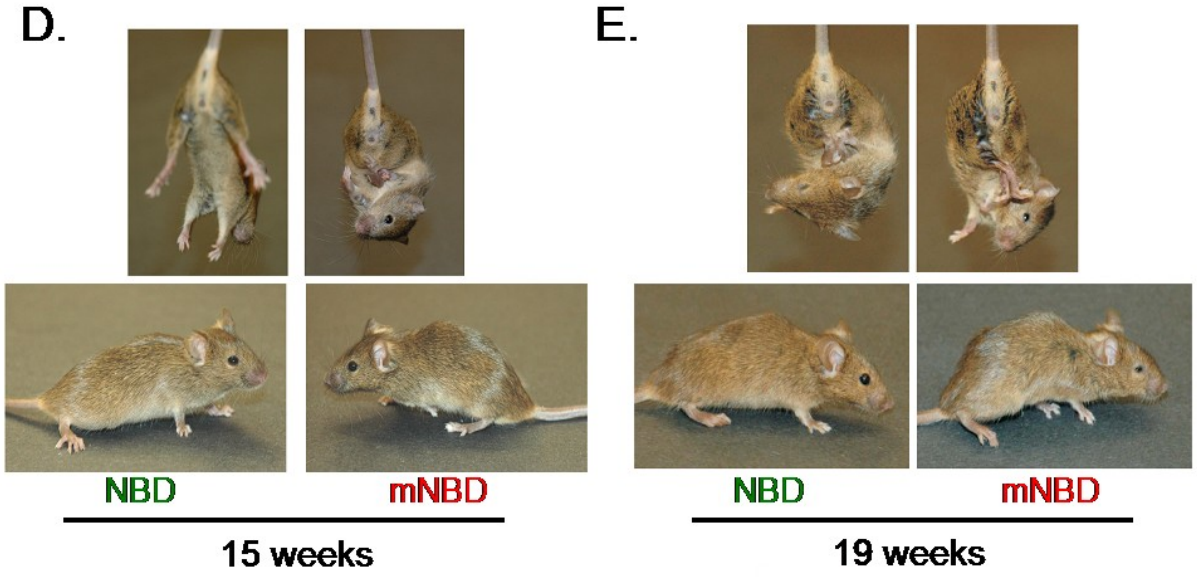


Figure 20: NBD peptide suppression of the IKK/NF-κB activation delays the onset of aging symptoms. (A)Immunodetection of phosphor-p65 in nuclear extracts of *Ercc1*$^{-/-}$ primary MEFs grown at 20% O_2 and treated with 200 μM NBD peptide for 3 hours. (B) Quantitation of nuclear p-p65 in blot (A) relative to WT MEFs derived from littermate controls. Values obtained are averages and standard deviation from three independent experiments. (C) Sibling pairs of *Ercc1*$^{-/\Delta}$ mice were treated with NBD peptide or an inactive mutant control peptide, 10 mg/kg, i.p., 3X per week, beginning at 5 wks of age and continuing throughout their lifespan. The mice were evaluated biweekly for spontaneous symptoms associated with aging by an investigator blinded to treatment. Reported are the average age at onset of each symptom for the two treatment groups and the difference between the group averages. Those symptoms marked with an asterisk were significantly delayed in mice treated with NBD relative to mice treated with the mutant peptide. The Aging Score reflects the overall quality of life (see Materials and Methods). (D) Representative images of *Ercc1*$^{-/\Delta}$ littermates treated with NBD or mutNBD peptide, at 15 weeks of age. The mouse treated with the inactive peptide shows premature onset of signs associated with neurodegeneration (dystonia, broad-based stance) early stages of kyphosis and muscle wasting. (E) Representative images of *Ercc1*$^{-/\Delta}$ littermates treated with NBD or mutNBD peptide, at 19 weeks of age. The mouse treated with the inactive peptide shows premature onset of signs associated with neurodegeneration (dystonia, broad-based stance, urinary incontinence), visual impairment, cachexia, and muscle wasting, While the NBD treated littermate shows dystonia and early stages of kyphosis and muscle wasting.

Recent examination of NBD and mNBD peptides, as well as, vehicle control in a murine model of arthritis and cell culture experiments has raised concerns that the mNBD peptide may not be completely inactive. Thus, using ANOVA analysis the age-at-onset for the various symptoms were analyzed compareing NBD-treated mice, mNBD-treated mice, and vehicle control treated mice. Interestingly using this analysis we found that three symptoms, trembling,

A.

Symptoms	control *Ercc1*$^{-/\Delta}$ time at onset (wks)	NBD time at onset (wks)	Change of Onset (wk)	P-value	number of mice (Control, NBD)
Dystonia	7.9	10.0	2.1	0.097	n=12,11
Trembling	7.8	11.4	2.6	0.002	n=13,11
Kyphosis	11.7	13.7	2.0	0.091	n=13,9
Ataxia	14.0	16.5	2.5	0.052	n=13,8
Sarcopenia	14.4	17.9	3.5	0.014	n=12,6
Priapism	20.2	17.0	-3.2	0.543	n=4,2
Lethargy	17.9	18.2	0.3	0.984	n=6,3
Incontinence	11.6	20.3	8.7	ND	n=3,1

Figure 21: NBD peptide improves symptoms of *Ercc1*$^{-/\Delta}$ compared with untreated *Ercc1*$^{-/\Delta}$ controls: *Ercc1*$^{-/\Delta}$ mice were treated with NBD peptide 10 mg/kg, i.p., 3X per week, beginning at 5 wks of age and continuing throughout their lifespan. The mice were evaluated biweekly for spontaneous symptoms associated with aging by an investigator blinded to treatment. Reported are the average age at onset of each symptom for the NBD treated mice were compared with *Ercc1*$^{-/\Delta}$ untreated mice. Cell marked in yellow are those with delayed age-at-onset in NBD treated mice

ataxia, and sarcopenia, were significantly delayed $p \leq 0.05$ in the NBD treated mice compared with the vehicle control mice; and further both dystonia and kyphosis showed trends $p \leq 0.10$ of being delayed (supplementary Fig 21). Thus, the efficacy of NBD may be greater than littermate-controlled data suggested.

3.4.7 IKK/NF-κB suppression attenuates histologic changes associated with aging:

Due to the significant alterations in phenotypic findings observed in NBD-treated mice, even greater than those observed in the genetic suppression of p65, histologic changes in NBD and control-treated animals were evaluated. *Ercc1*$^{-/\Delta}$ mice eventually die from severe liver dysfunction. When analyzing liver histology it is apparent that these mice have an increasing number of senescent cells defined by expression of p16, recently defined as the first biomarker of aging[294]. As expected, p16 protein levels were reduced in *Ercc1*$^{-/\Delta}$ mice treated with NBD (Figure 22A). These reduced levels of p16 correlated with a 1.7 fold decrease in p16 gene expression as determined by qRT-PCR (data not shown).

Numerous tissues exhibit age-associated changes. Bone marrow develops fatty deposits[295], vertebral bone becomes osteoporotic[205], and pancreatic islet cells have reduced insulin secretion[296]. As with the reduction in senescent changes in the liver, there was a similar delay in fatty replacement of the bone marrow in *Ercc1*$^{-/\Delta}$ mice receiving NBD peptide, compared with those receiving mNBD peptide (Figure 22A). Vertebral degeneration evaluated by μCT, was reduced by NBD-treatment as represented by fewer osteoporotic holes and improved trabecular structure when compared to control *Ercc1*$^{-/\Delta}$ control mice (Figure 22A). Bone densities were compared using control WT, control *Ercc1*$^{-/\Delta}$ (injected with vehicle, n=5) and NBD treated *Ercc1*$^{-/\Delta}$ mice (n=3). Control *Ercc1*$^{-/\Delta}$ mice had reduced bone density of 31.5 ± 2.5 % while the reduction in bone density in NBD-treated mice was limited to 21.6 ± 5 % compared to normative aging controls (p=0.003) (Figure 22B). As expected, porosity, a marker of bone degeneration, was increased in the *Ercc1*$^{-/\Delta}$ control mice by 25.1 ± 2%; however treatment with NBD reduced this increase to 16.7 ± 3% (p = 0.0009) (Figure 22C). *Ercc1*$^{-/\Delta}$

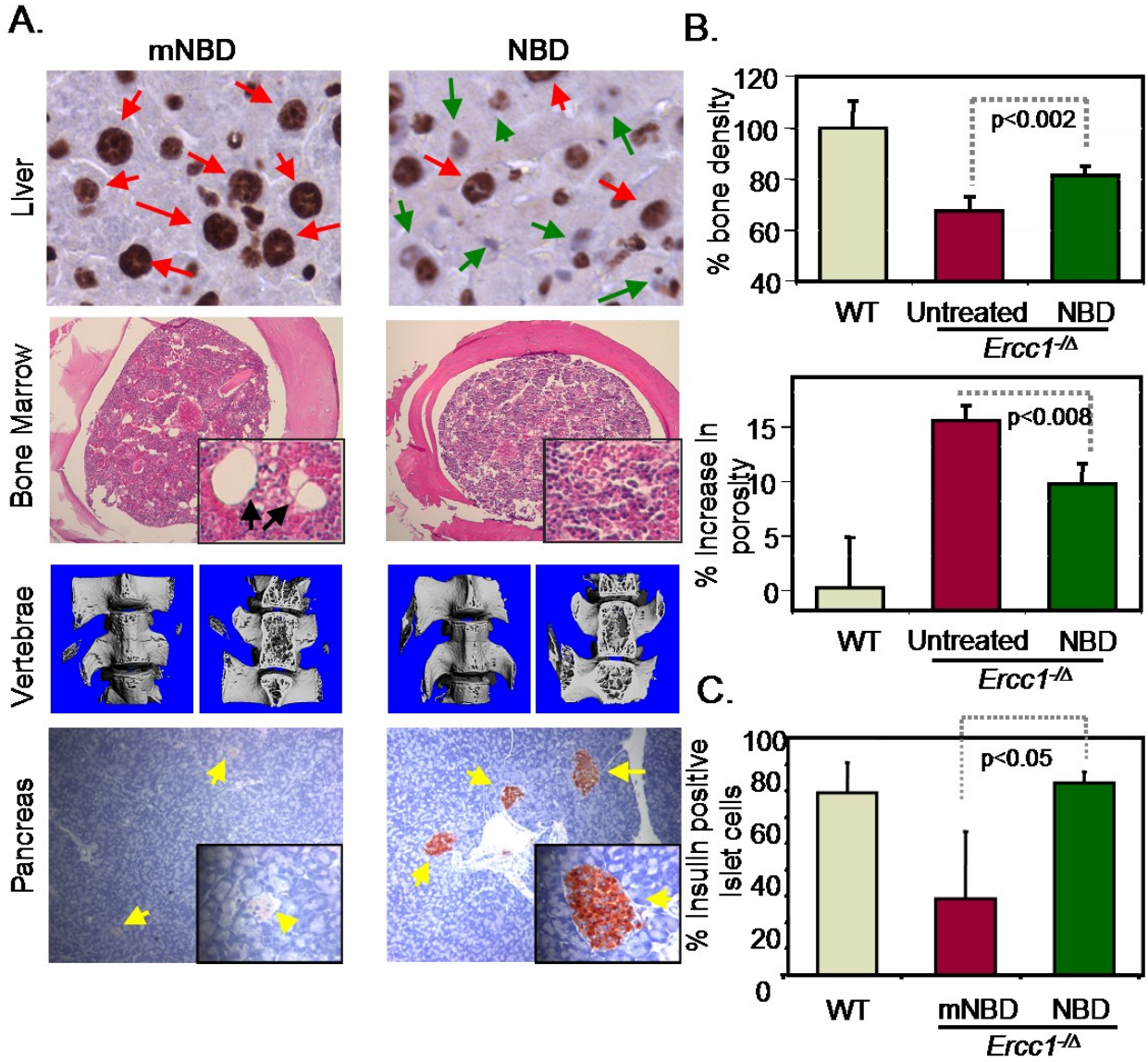


Figure 22: NBD peptide suppression of the IKK/NF-κB activation delays histolopathological changes associated with aging. (A) Immunodetection of senescence marker p16 in liver of *Ercc1*$^{-/\Delta}$ mice treated with NBD peptide or an inactive mutant peptide as described in Fig. 4 and Material and Methods. Nuclear p16 staining is more frequent and intense in hepatocytes of mice treated with the mutant peptide. (Red arrows indicate p16 positive nuclei, while green arrows indicate p16 negative nuclei) Cross-section of the femur stained with haematoxylin and eosin. Mice treated with the mutant peptide have early onset of fatty replacement (yellow arrows) of the bone marrow. MicroCT of the vertebra of *Ercc1*$^{-/\Delta}$ mice. Mice treated with

NBD have improved appearance of bone density. Immunodetection of insulin in pancreatic sections from treated *Ercc1*$^{-/\Delta}$ mice. NBD peptide delayed the loss of insulin-producing islet cells. Islets are indicated by yellow islets and brown stain indicates insulin positivity. (B) Analysis of microCT images in percent bone density (normalized to WT littermate controls) (top panel) as well as quantification of bone porosity, 1-BV(bone volume) /TV (total volume) (bottom panel). Values for the treated *Ercc1*$^{-/\Delta}$ mice were compared to normal littermates. (C) The percent of insulin producing cells in *Ercc1*$^{-/\Delta}$ mice treated with NBD or the inactive mNBD compared to normal littermate controls.

mice demonstrate degeneration of pancreatic islets between 12 and 18 weeks of age (unpublished data). While NBD treated *Ercc1*$^{-/\Delta}$ mice had nearly the same number of insulin producing cells in their islets (72.8 ± 4.5%) as WT control mice at 18-19 weeks of age, mNBD treated mice had lost greater than 50% of their insulin positive islet mass (28.8 ± 25.7%) (Figure 22C). This improvement in islets can be appreciated in the bottom panels of Figure 22A. This data suggests, NBD treatment either blocks or allows for regeneration resulting in improved pathologies in numerous tissues undergoing age-related changes observed in this model of accelerated aging.

3.4.8 NBD treatment led to global as well as NF-κB specific gene expression changes:

As NBD-treatment improved phenotypic and histologic changes associated aging, micro-array analysis was completed to determine if these changes were mediated by a limited number of gene expression changes. In the liver (n=3 per group), expression of approximately 5% of all genes showed significant changes in expression levels between the NBD and mNBD-treated

groups, demonstrating that NBD had a functional effect *in vivo.* Grouping genes to their known or predicted biologic function using gene ontology (GO), allowed for characterization of pathways which were preferentially altered by NBD treatment.

The most significantly affected processes altered between NBD and mNBD treated animals were those listed in Figure 23A. Two different analysis programs defined processes involving cell and tissue development, cell signaling and proliferation, cell death and regulation of apoptosis, as well as regulation of infectious diseases and metabolic pathways as those that were most significantly altered. This further confirms our findings in Figure 16, that p65 expression can alter cell growth and proliferation in *Ercc1*$^{-/-}$ MEFs.

To further assess whether the NF-κB pathway was a factor in the gene expression changes, a transcriptional regulation analysis was completed. This analysis determined that NF-κB subunits accounted for the 7th, 14th, 24th, and 25th most effected transcription factors (Figure 23B). However, taken as one transcriptional element, they account for 243 nodes, compared to the most significant transcription factors which had 112 effected nodes. Further analysis shows that NF-κB is one of the central factors involved in the top 5 networks as defined by Ingenuity, which is denoted by the red circle in Figure 24. The other central mediator is HNF4α denoted by the blue circle (a protein directly regulated by NF-κB). Additionally, of approximately 400 genes examined with known NF-κB regulatory elements, 8% showed significant expression differences between the two groups of mice (Figure 23C). Expression of 89% (27/30) of these genes was significantly down-regulated in mice treated with NBD, as expected with chronic IKK inhibition. A number of these NF-κB regulated genes are involved in cell cycle control, specifically, Gadd45β, Cyclin D2 and D3, Bcl-2, and protein kinase C. Thus, it is likely that treatment with NBD could act through several mediators to alter and delay age-associated changes.

A.

Top Networks (Defined by Ingenuity)	
1	Cellular Development, Lipid Metabolism, Molecular Transport
2	Cell Signaling, Cell Death, Hematological Disease
3	Cellular Assembly and Organization, Post-Translational Modification, Cancer
4	Cellular, Connective Tissue, Skeletal and Muscular Development and Function,
5	Infectious Disease, Carbohydrate Metabolism, Lipid Metabolism
Top Processes (Defined by GeneGo)	
1	Regulation of apoptosis, regulation of PCD
2	Organ/organismal and embryonic development,
3	Response to external stimulus, hormone stimulus, and endogenous stimulus
4	Positive regulation of cellular process, regulation of developmental process
5	Regulation of cell proliferation, positive regulation of cellular and biologic process,

B.

No	Network	p-Value	Total nodes
1	ESR1 (nuclear)	5.58E-185	112
2	CREB1	9.01E-148	90
3	NF-Y	5.60E-136	83
4	HNF1-alpha	1.30E-132	81
5	Androgen receptor	3.00E-129	79
6	AP-1	7.56E-121	74
7	RelA	3.61E-119	73
8	PU.1	5.69E-116	69
9	p63	3.90E-114	70
10	AP-2A	3.90E-114	70
14	NF-kB p50/p65	2.18E-97	60
24	c-Rel	9.16E-91	54
25	NF-kB	1.05E-90	56

C.

Gene Symbol	Fold Change	p
Csf3	1.8	0.0014
Agt	1.2	0.023
Sod1	1.1	0.036
Ctsb	-1.1	0.020
Eng	-1.2	0.050
Il15ra	-1.2	0.0014
Scarb1	-1.2	0.024
Prkcd	-1.2	0.010
Icam1	-1.3	0.014
NfκBia	-1.3	0.023
Ccnd3	-1.3	0.034
Ccl19	-1.4	0.043
Tcrg	-1.4	0.013
App	-1.5	0.033
Bcl2	-1.5	0.0067
Plcd1	-1.6	0.031
Ccnd2	-1.7	0.015
Cd48	-1.7	0.035
Sdc4	1.5	0.0060
Abcb1a	-1.8	0.041
Cd80	-1.9	0.031
Upk1b	-1.9	0.030
Oas3	-2.0	0.028
Penk1	-2.1	0.0088
Igh-4	-2.4	0.026
Ptx3	-2.4	0.010
Lamb2	-2.5	0.0094
Gadd45b	-3.5	0.021
Apod	-4.4	0.011

Figure 23: NBD peptide suppresses expression of NF-κB regulated genes. *Ercc1*$^{-/\Delta}$ mice were treated chronically with NBD peptide or inactive mutant peptide as described in Fig. 4 and Materials and Methods. At 18-19 weeks mice were euthanized, tissues isolated, mRNA purified and analyzed by Affymetrix microarray. (A) Top networks and Top Processes affected in NBD treated *Ercc1*$^{-/\Delta}$ mice relative to those treated with mutant peptide defined by Ingenuity and Metacorps GeneGo, respectively. (B) Ranking of the transcription factor networks most affected by NBD treatment as defined by GeneGo Network Builder Transcription Regulation. NF-κB family members are highlighted in dark grey. (C) Genes with significantly altered expression in *Ercc1*$^{-/\Delta}$ mice treated with NBD relative to *Ercc1*$^{-/\Delta}$ mice treated with inactive peptide that have a known NF-κB regulatory element. Eighty-nine percent of these were down-regulated as expected if NBD is inhibiting activation of NF-κB . Genes highlighted in grey are known to have a role in cell survival and cell cycle control.

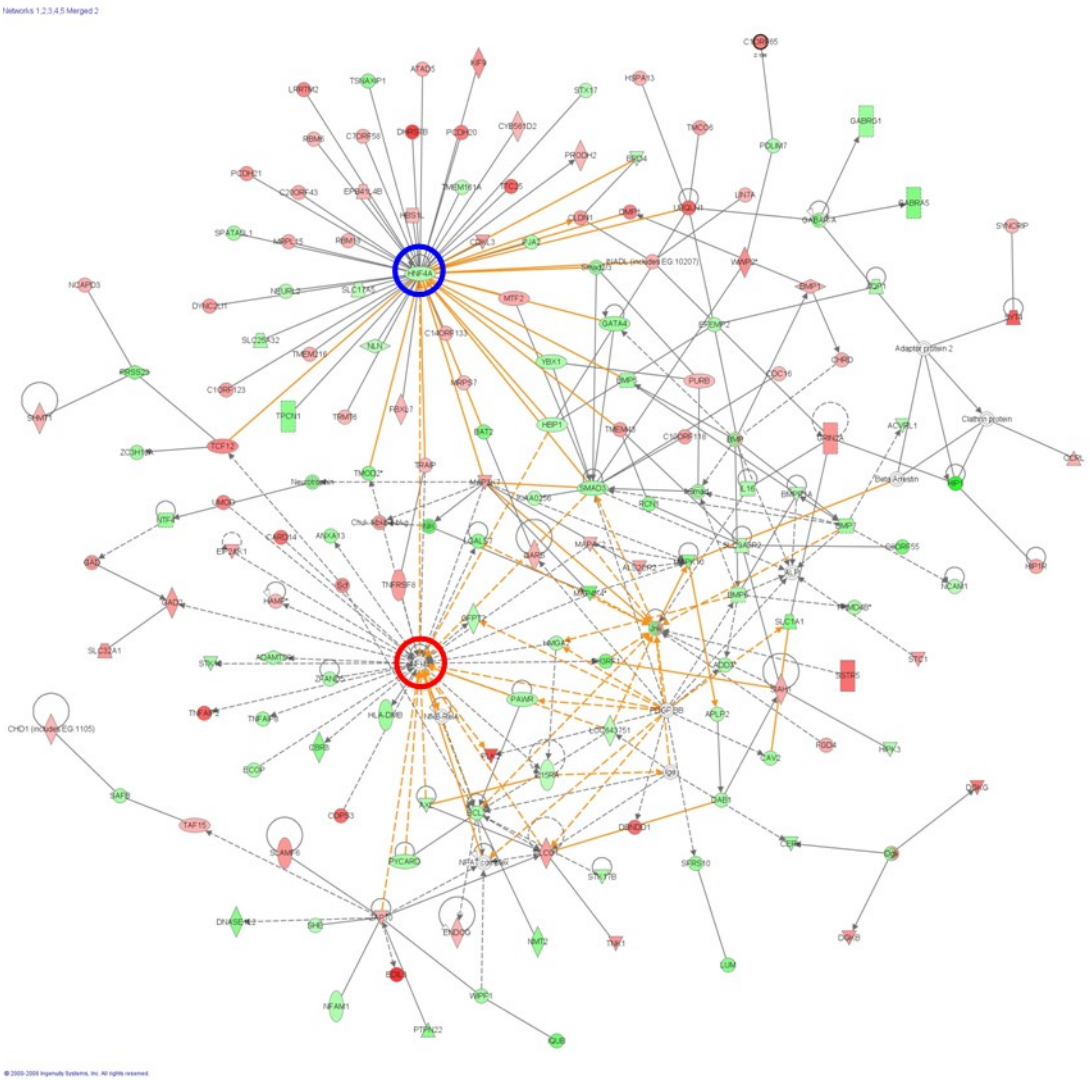


Figure 24: NF-κB is a central mediator of top affected networks (A) Network map of the top affected networks defined by Ingenuity in Figure 23A. Central mediators are NF-κB (red circle) and HNF4A (blue circle)

3.5 DISCUSSION:

In this study, we evaluated the effects of NF-κB suppression on age-associated degeneration in a mammalian model of accelerated aging. Figures 16 and 17 suggest that as with normative aging animals, the ERCC1-deficient mouse model of accelerated aging has elevated levels of NF-κB activity. As hypothesized, chronic inhibition of the NF-κB pathway using both genetic suppression and the NF-κB inhibitory peptide led to delays in age-at-onset of phenotypic changes associated with aging. The overall aging score, which takes into account the age at onset of numerous symptoms and is an overall measurement of quality of life, showed a significant improvement ($p<0.005$) in NBD treated mice compared to those treated with mNBD peptide. While these changes were recorded in a blinded fashion, their true significance can be seen by the images included in figure 18C/D & 19C/D, which portray the vivid differences between *Ercc1*$^{-/\Delta}$*p65*$^{+/-}$ and *Ercc1*$^{-/\Delta}$ and between NBD and mNBD treated *Ercc1*$^{-/\Delta}$ mice.

Furthermore, when comparing age-at-onset of phenotypic changes in NBD-treated mice compared with mice treated with vehicle alone, rather than mNBD-treated mice (a potentially partially active control) there are significant ($p<0.05$) and trending ($p<0.1$) delays in five of the phenotypic symptoms assessed (Figure 20). Importantly, these phenotypic delays are accompanied by amelioration of degenerative pathologic changes associated with aging in general. NBD-treated *Ercc1*$^{-/\Delta}$ mice exhibited reduced markers of cellular senescence in the liver, a delay in fatty replacement of bone marrow, and reduced osteoporotic changes. Further, NBD treatment resulted in reduced degeneration of pancreatic islet cells. These data suggest that NF-κB up-regulation is not an organ or cell specific event, but rather NF-κB signaling has an impact on the aging process as a whole.

Additionally, mice treated with NBD peptide differ dramatically in gene expression compared to their *Ercc1*$^{-/\Delta}$ control littermates. When evaluating top networks and processes altered by NBD as compared to mNBD-treated mice, cell death/apoptosis, cell and organ development, organism survival, and stress response were the processes that were most highly altered. It is not surprising, that an improvement in these pathways would be altered with treatments that prolong healthspan and delay age-associated changes. Furthermore, these affected pathways confirm findings that alterations in p53 signaling, a major controller of cell cycle regulation and apoptosis, and leads to extended lifespan in progeroid-like models of aging[290, 297].

Our analysis of gene expression changes revealed a 5% genomic shift (nearly 2000 genes) between NBD and mNBD treated animals. These changes in gene expression may be highly relevant in determining which, if any, genes are specifically relevant to the aging process. A recent study by Schumacher et al. suggests that rapidly aging, long-lived, and normative aging mice have similar alterations in gene expression when compared with young wt mice[216]. However, evaluating gene expression data in our NBD treated mice with previously examined aging cohorts may elucidate genes that are biomarkers of aging. The goal of identifying these biomarkers can be completed by comparing gene expression data from numerous anti-aging compounds/genetic manipulations including SIRT1, p53, p38, and caloric restrinction to find overlapping or commonly altered genes.

Gene expression analysis also confirms the efficacy of NBD, by showing decreased expression of numerous genes with known NF-κB promoter sequences. Further analysis of transcriptional regulation confirms that NF-κB subunits are integral in the expression changes observed with NBD treatment. Independently, NF-κB transcriptional elements account for 4 of

the top 25 altered transcription factors, however, as a group they become the single most important transcription factored altered by NBD treatment.

Figure 17, shows decreased NF-κB activity in the spleen despite overall increases in NF-κB activity in other organs. These data, along with the observation of no major inflammatory cell infiltrate into the organs examined, leads us to believe that the role of overactive NF-κB signaling is not secondary to a gross inflammatory response as was speculated in Kawahara et al. evaluation of *Sirt6*$^{-/-}$ mice and NF-κB. Further support of this lack of inflammation is that *Ercc1*$^{-/-}$ gene expression was highly correlative with that of natural aging mice with the exception that *Ercc1*$^{-/-}$ mice did not exhibit an increase in inflammatory and immune related gene[130]. Thus it is likely that the alteration in NF-κB activity and the positive affects of NF-κB inhibition via the NBD peptide most likely results from an involvement in cell cycle and survival transcriptional regulation. From our analysis, altered genes with known NF-κB promoter sequences involved in cell cycle/cell survival include Gadd45β, Cyclin D2 and D3, Bcl-2, apolipoprotein D, and protein kinase C[4]. Interestingly the two most highly suppressed NF-κB regulated genes, apolipoprotein D and Gadd45β have been shown to have roles in cellular senescence[298, 299] and age-related disease[300].

Our *in vitro* data further support a role for NF-κB in cell cycle regulation and proliferation. In agreement with previously published data, the *Ercc1*$^{-/-}$ MEFs exhibit reduced proliferation rates compared with wild type MEFs. These slow rates of proliferation are further exacerbated by oxidative stress and gamma irradiation[149, 215]. In each of these cases NF-κB activity is increased. However, when evaluating *Ercc1*$^{-/-}$*p65*$^{-/-}$ MEFs there was a rescue of cell proliferation rate compared with *Ercc1*$^{-/-}$. Thus, confirming that NF-κB is acts in an non-inflammatory role to contribute to the aging process and that NBD blocks this change.

It is important to note that existing progeroid models are not identical to normative aging, although recent gene expression and characterization studies suggest that these mice mimic normative aging to a high degree. The previous study, which evaluated NF-κB inhibition on aged skin pathology, hypothesized that aging is an active process whereby genes must be continually expressed at altered levels to sustain the aged phenotype[151]. Therefore, inhibition of pathways controlling gene expression could reverse the aging phenotype. In this study, with chronic inhibition of NF-κB was observed to delay the age-at-onset of numerous aging parameters, but no reversal or complete block of aging changes, either histologic or phenotypic, were observed. This lack of disease state reversal can be explained in at least two different ways. First, age-related pathology development may not necessitate constant gene expression changes, and once initial gene alterations occur they may be irreversible. However, an alternate explanation is possible. The accumulation of DNA and cellular damage in the ERCC1 deficient models is far greater and more rapid than that observed in normative aging. Thus, even with chronic treatment of NBD, the cells are continually bombarded with damage that they cannot repair. Therefore, NF-κB suppression may result in only a delay in the age-at-onset of age-associated changes; however, if damage and repair cycles were to occur as in normative aging animals, the NBD therapeutic intervention has even greater potential. This theory can only be confirmed by long term studies in normative aging mice.

This compilation of phenotypic, histologic, and gene expression data demonstrates that therapeutic intervention via inhibition of the NF-κB signaling pathway alters numerous aspects of the aging process in the *Ercc1*$^{-/\Delta}$ mice. While it is premature to suggest that global NF-κB suppression is a viable method to treat aging, this study gives new insight to the treatment of many age-assocaited disease. Specifically, targeted NF-κB suppression may be efficacious for

treating osteoporosis, diabetes, Alzheimer's, Parkinson's or other age-related diseases. This approach could avoid potential side effects, which are a concern when inhibiting inflammatory pathways. It is important to note that no adverse side effects were observed in this 20 week study of NBD treatment, a finding consistent with previous NBD studies. However, additional studies will have to be completed to determine the efficacy of other NF-κB inhibitors. Furthermore, additional studies using IKK or p65 tissue conditional knockouts as well as other genetic knockdown modles are necessary to evaluate to determine the mechanism, or likely multiple mechanisms, by which NF-κB contributes to the overall aging process. Additionally, as natural aging has even greater up-regulation of immune and inflammatory responses at the gene expression level than the ERCC1 deficient models[130], NF-κB suppression may have more beneficial effects in controlling the normative aging process.

4.0 PHARMACOLOGIC NF-KB INHIBITION CAUSES ANTIGEN PRESENTING CELLS TO UNDERGO ROS-DEPENDENT PROGRAMMED CELL DEATH .

4.1 ABSTRACT:

Monocyte derived professional antigen presenting cells (APC) are innate immune mediators of inflammatory and age-associated degenerative changes. Differentiation, activation, and functional processes of APC are regulated in different degrees by the NF-κB family of transcription factors. In this study, we evaluated the role of pharmacologic inhibition of NF-κB APC. Macrophages and monocyte derived DC cells underwent programmed cell death (PCD) in the presence of pharmacologic NF-κB inhibition. Unlike previous studies which implicated the TNFα/JNK/Caspase8 signaling in this cell death pathway, the mechanism initiating PCD in our hands is induction of ROS formation, which subsequently causes a loss of mitochondrial membrane potential and activation of caspase signaling. This observed macrophage NF-κB-inhibition-induced PCD may be one of the mechanisms by which inflammatory and age-associated disease pathologies are reduced in response to NF-κB suppressive treatment.

4.2 INTRODUCTION

NF-κB/IKK suppression is a therapeutic approach currently being examined in models of human disease including, muscular dystrophy[73], diabetes mellitus[273], Parkinson's[72], inflammatory bowel disease[78], rheumatoid arthritis[71], heart disease, aging[151], and cancer[31]. It is commonly assumed that the beneficial effects of NF-κB suppression in mammalian diseases are due to reduced cytokine signaling in innate immune cells, as well as a reduction in subsequent T-cell activation and signaling. Specifically in diseases with known inflammatory components, researchers have consistently evaluated cytokine profiles, including innate immune cytokines, TNFα, IL-1β, and IL-12, as well as secondary T-cell cytokines IFNγ, IL-2, and IL-17.

Study of these cytokines and T-cell activation is appropriate due to the role of NF-κB as a central mediator of immune activation. NF-κB transcriptionally regulates numerous cytokines (IL-2, IL-12, IL-23, IL-17, IFN-γ, TNFα), chemokines (MIP-1, KC, RANTES) and adhesion molecules (I-CAM, VCAM, p-selectin)[4]. Furthermore, NF-κB plays an important role in cellular survival specifically in cases of infection and increased inflammation[301]. NF-κB transcriptional regulation is mediated through a series of cellular signaling pathways, including Toll-receptors, IL-1R, TNF receptors, TCR and BCR signaling apparatus, as well as ATM and other damage sensors. Each of these inflammatory signaling pathways coalesce at the IKK complex, which is considered the central regulator in the canonical or activated NF-κB signaling cascade[5]. The IKK complex consists of a regulatory subunit, IKKγ (NEMO), and two catalytic subunits, IKKα and IKKβ. Once IKKα/β are activated they phosphorylate the cytoplasmic inhibitor of NF-κB, IκBα, thus targeting it for ubiquitination and subsequent degradation. The degradation of IκBα leads to the release of NF-κB subunits by revealing a nuclear localization sequence, thereby allowing NF-κB to translocate to the nucleus where it can then act as a transcription factor[5].

Due to the importance of NF-κB signaling in the inflammatory response and the reduction in innate immune cytokines observed after NF-κB inhibitory treatment of disease, it was pertinent to explore the role of NF-κB/IKK inhibition in innate immune cells, specifically macrophages and dendritic cells (DC). We hypothesized that NF-κB suppression would result in a reduction in cytokine signaling and antigen presentation, as well as a subsequent reduction in T-cell activation, and reduced trafficking of innate immune cells to sites of inflammation *in vivo*. However, what we observed was that antigen presenting cells (APC), which constitute both macrophages and DC, underwent a programmed cell death phenomenon (PCD) in the presence of NF-κB inhibition.

There are numerous reports of NF-κB involvement in cell death. The vast majority of which implicate TNFα induced JNK and caspase 8 apoptotic cell death. The role of NF-κB in PCD was recognized early due to the embryonic lethality of *p65(RelA)*$^{-/-}$ mice[289], *IKKγ*$^{-/-}$[278], and *IKKβ*$^{-/-}$[302] mice, a condition which is reversed in all cases by *TNFR*$^{-/-}$ crossbreeding[303]. The TNFα-induced cell death phenomenon has been confirmed in numerous studies[304, 305], and is thought to be due to NF-κB transcriptional control of several anti-apoptotic genes including XIAP, Bcl-xL, A1-bfl2, c-FLIP, A20, and GADD45β. One theory suggests that both the pro-cell death JNK and caspase 8 pathways and the anti-apoptotic NF-κB pathways are activated by TNF as described in Papa et al[306]. By inhibiting only the NF-κB pathway, the PCD cascade prevails, leading to apoptosis. Interestingly, two previous reports observed macrophage cell death using a non-specific NF-κB inhibitor, PTDC, without exogenous stimulation. We suggest here that this cell death response seen in primary APC is independent of the well documented TNF/JNK/Caspase 8 PCD signaling pathway.

In this current study, numerous specific NF-κB inhibitors, which block either signaling at the IKK complex, IKKβ, proteosomal degradation of IκBα or translocation of the p65 subunit to the nucleus were evaluated. This NF-κB-suppression-induced PCD was found to be dependent on reactive oxygen species (ROS). This accumulation of ROS leads to subsequent loss of mitochondrial membrane potential (MMP) and activation of the caspase 9/3 pathway, resulting in apoptotic cell death. Overall, our data suggest that APC death, in of both macrophages and monocyte derived DC, contributes to the anti-inflammatory phenotype seen in murine models after treatment with NF-κB inhibitors.

4.3 MATERIAL AND METHODS:

4.3.1 Materials:

NF-κB inhibitors used include: Compound A (gift from Bayer), IKK inhibitor VII (Calbiochem), IKK2 inhibitor IV (Calbiochem), JSH-23 (Calbiochem), Wedelolactone (Calbiochem), MG-132 (Calbiochem), caspase activation was inhibited by zVAD-fmk (Calbiochem), ROS production and apoptosis was inhibited by butylated hydroxyanisole (BHA) (Sigma). Iron chelator and anti-oxidant, Desferrioxamine Mesylate (DFO) (Calbiochem). All compounds were diluted as suggested by manufacturers in DMSO and then diluted in desired media. MTT (Sigma) diluted to 5mg/ml in optimem media. Etanercept from Wyeth Pharmaceuticals.

4.3.2 Peptides

The peptides TAT-NEMO Binding Domain (NBD; (YGRKKRRQRRRGGTALDWS WLQTE-amide), inactive (mutant) TAT-NBD (mNBD; YGRKKRRQRRRGGTALDAS ALQTE-amide), were synthesized by the peptide synthesis facility at the University of Pittsburgh, Pittsburgh, PA. Underlined amino acids represent tryptophan to alanine mutations. Peptides were purified and characterized by reversed-phase high performance liquid chromatography and mass spectrometry. For *in vitro* experiments, TAT-NBD and TAT-mNBD peptides were used, while *in vivo* 8K-NBD and 8K-mNBD peptides were used due to differing transduction rates.

4.3.3 Murine macrophages, DC and other cell lines:

Bone marrow (BM)-derived macrophages were isolated from the femurs of mice. BM was flushed with washing medium (RPMI 1640 with 1% penicillin/streptomycin), passed through a 70-µm nylon cell strainer into a 50-ml conical tube, and spun down at 1500 rpm for 5 min. RBC were lysed using ACK lysis buffer for 15 min, and resuspended in complete medium (washing medium with 10% FBS). BM cells were seeded in conditioned L-cell media (consisting of 20% precondition L-cell media, 60% DMEM, 20% FBS) additional 1% L-glut, 1% sodium pyruvate, and 1% penicillin/streptomycin (P/S) were then added to the media. Cells were seeded in 10cm dishes and media was replaced after 3 days and cells were collected on day 7. Cells were passaged every 3-4 days and were discarded after one month.

Bone marrow derived dendritic cells (BMDC) were isolated from mice as were BMDM. Bone marrow cells were seeded at 5e6 cells per well in 6 well plates in complete RPMI supplemented with 10ng/ml of GM-CSF (Cell Sciences) and 20g/ml IL-4 (Cell Sciences). 2ml of media was replaced on Day 3 (supplemented with GM-CSF and IL-4) and cells were collected on Day 7. Cells were then isolated using MACs columns (Miltenyi Biotech) with positive selection using CD11c beads. Isolated cell were then seeded and used for experiments seeded in complete RPMI media.

Other cell lines used included Fetal Skin Dendritic Cells (FSDC) and immortalized murine DC cell line (maintained in RPMI with 10% FBS and 1% P/S), RAW264.7, a murine macrophage cell line (maintained in RPMI with 10% FBS and 1% P/S), a prostate tumor cell line DU145 (maintained in maintained in DMEM 10%FBS, 1%P/S, 1% HEPES), an immortalized T-cell line D10 (maintained in RPMI 10% FBS, 1% NEAA, 1% Pen/strep, 1% Hepes, 1% Sodium Pyruvate 0.1% beta-ME supplemented with 1:2000 50,000U IL-2), and primary mouse embryonic fibroblasts (MEFs) derived as described in Niedernhofer et al.[130] (maintained in DMEM 44% ml Ham's F10 44%, 10% FBS, 1%, P/S, 1% NEAA).

4.3.4 NF-κB luciferase assay

HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter (DMEM with 10% FBS and 1% penicillin/streptomycin) were pretreated for 1 hour with varying NF-κB inhibitory compounds (in Materials) were activated for 3 h with 10 ng/ml TNF (R&D Systems). The cells were lysed in reporter lysis buffer and luciferase activity was measured with a luciferase assay system (Promega) using AutoLumat Luminometer

(Berthold Technologies,). Due to the high binding affinity of the PTD fragments of the peptides (peptides were treated in Optimem prior to addition of TNF) all other NF-κB inhibitors were treated in maintenance media.

4.3.5 MTT Assay:

Cells were seeded in 96 well plates at a concentration of 40,000 cells per well for FSDC, 30,000 cells/well DU145, 10,000 cells/well for MEFs, 30,000 for D10, 30,000cells per well for BMDM and BMDC and 30,000cells/well for HEK293-NF-κB^{lufiferase}. Cells were treated with listed doses of the NF-κB inhibitors and allowed to grow for 24 hours or as described. 10ul of MTT working solution (5mg/ml) was added to each well and incubated for 2 hours. Excess media was removed and crystals were dissolved in 20ul of DMSO and then diluted in dH_20. Absorbance was measured at 530nm on MRX revelation microplate reader (Dynex Technologies). Values were then normalized to untreated controls and blank wells.

4.3.6 Western blotting

Cells were treated over a reverse time course with varying NF-κB inhibitors as described in the results. Cells were collected by scraping, then pelleted and lysed using Reporter Lysis Solution (Promega). Protein concentration was determined using Bradford assay (Pierce). Western blots were performed and analysis was performed with the following antibodies: Caspase 3 (Cell Signal), Cleaved Caspase-9 (Cell Signal), Cleaved Caspase 8 (Cell Signal) β-actin (Abcam), A1/bfl-1 (Cell Signal).

4.3.7 Mitochondrial Membrane Potential and Cellular ROS production:

Cells were treated with desired compounds as listed in individual experiments. At desired timepoints, cells were treated with 40nM DiOC6(3) (Invitrogen), which was made from a 40mM stock in DMSO, and 5mM MitoSOX (Invitrogen) used according to manufacturers instructions. Cells were incubated in these solutions diluted in complete IMDM media for 30min at 37°C protected from light. Cells were then washed two times in complete IMDM media and were subsequently imaged using fluorescent microscopy using Axiovert200 Microscope and Axiovision software (Zeiss). To remove background fluorescence, all images had equally altered red and green channel levels for imaging but not quantification. After 30min incubation with fluorophores and washing, cells were then collected and evaluated by on LSR2 flow cytometer (BD Bioscience). FACS analysis using cells stained with DiOC6(3) and MitoSox were evaluated on Axiovert200 Microscope. Further FACs analysis was completed using FlowJo (TreeStar Inc)

4.3.8 Immunofluorescence.

Cells were grown on poly-L-lysine-coated coverslips. Poly-L-lysine coverslips were prepared as follows coverslips were washed for 10 minutes in boiling 1M HCl. Slides were rinsed completely in dH_2O. Coverlsips were then incubated in 0.1% poly-L-lysine hyrdro bromide (Sigma) for 10 min. Coverlsips were then washed and dried in oven at 60°C.Cells were fixed for 15 minutes using 2% paraformaldehyde (Sigma) and then washed with PBS. The cells were then permeabilized with 0.1% Triton X (USB) in PBS and then washed and blocked with

2%BSA for 45 minutes. Cells were treated with primary antibodies (as listed) for 1 hour and then secondary antibodies conjugated to fluorophore either Cy3 (1:1000) or SA488 (1:500) (Jackson) for 1 hour. DAPI stain was then added to cells for 30 seconds and cells were fixed to glass slides using gelvatol solution. Confocal microscopy was completed using Olympus Flowview 1000.

4.3.9 mRNA analysis:

FSDC and BMDM were collected from 6 well plates as defined in the experiments below. mRNA was isolated from cell pellets using RNAqueous Kit (Ambion). mRNA was then quantified using NanoDrop (Thermo Scientific). Samples were then were analyzed for A1/bfl-1 (Fwd:5'AATTCC AACAGC CTCCAG ATATG3' Rev:5'GAACAA AATAT CTGCAA CTCTGG3') Bcl2 (Fwd 5'TACCGT CGTGAC TTCGCA GAG3' Rev5'GGCAGG CTGAGC AGGGT CTT3') BclXL (Fwd 5'AGGCA GGCGA TGAGT TTGAA C3' Rev GAACC ACACC AGCCA CAGTC A3') FHC (Fwd5'AGACC GTGATG ACTGG GAGAG3' Rev 5'AGCTT AGCTCT CATCAC CGTG TC3') and β-actin (Fwd 5'TAAAA CGCAG CTCAG TAACA GTCCG3' Rev 5'TGGAA TCCTG TGGCA TCCAT GAAAC3') Samples were run for 30 cycles using PCR machine Techgene (Techne).

4.3.10 Statistical analysis:

Experiments shown are representative of 3 independent experiments. P-values were determined using the student T-test.

4.4 RESULTS:

4.4.1 NF-κB suppression results in APC death:

Previously, our laboratory evaluated the efficacy of the Nemo Binding Domain peptide (NBD), an inhibitor of the IKK complex, in a murine model of IBD[78], where it ameliorated disease with good *in vivo* efficacy. The efficacy of NBD is supported by numerous other *in vivo* studies[67, 72, 73]. It was observed that levels of inflammatory cytokines derived from innate cells including IL-12p40 and TNFα were reduced in the NBD treated animals compared with control treated animals[78]. Initially, a decrease in cytokine secretion was observed in macrophages pretreated with NBD for one hour before stimulation with LPS or TNF (data not shown). Under visual observation, however, the majority of the macrophages in culture exhibited characteristics of apoptosis cell death, including fragmentation or blebbing, nuclear condensation, cell shrinkage, and loss of symmetry (Figure 25A).

TAT-NBD (NBD) but not TAT-mNBD (mNBD), an inactive control peptide, treatment was shown to cause cell death in a dose-dependent manner (data not shown). Cells treated with 200μM of NBD peptide underwent rapid cell death, with 100% cell death occuring within 4 hours (Figure 25B). This cell death response was confirmed visually using trypan blue exclusion analysis (Figure 25A), in which all NBD-treated cells, but not the mNBD-treated cells failed to exclude the trypan blue stain. Annexin V and PI staining confirmed that the NBD induced programmed cell death (PCD) was indeed apoptotic (Figure 25C). In the NBD treated sample

71.5±1.2% of cells were double positive for Annexin V and PI, while in the mNBD treaded sample 11.8±5.4% were double positive.

While the data comparing NBD with mNBD suggest that the cell death was due to the NF-κB inhibitory domain of the peptide, it was necessary to confirm whether this was a NF-κB specific phenomenon or a by-product of this particular inhibitor. To evaluate this more closely, seven NF-κB inhibitory compounds were used, each of which targeted different aspects of the NF-κB inhibitory cascade (Table 3).

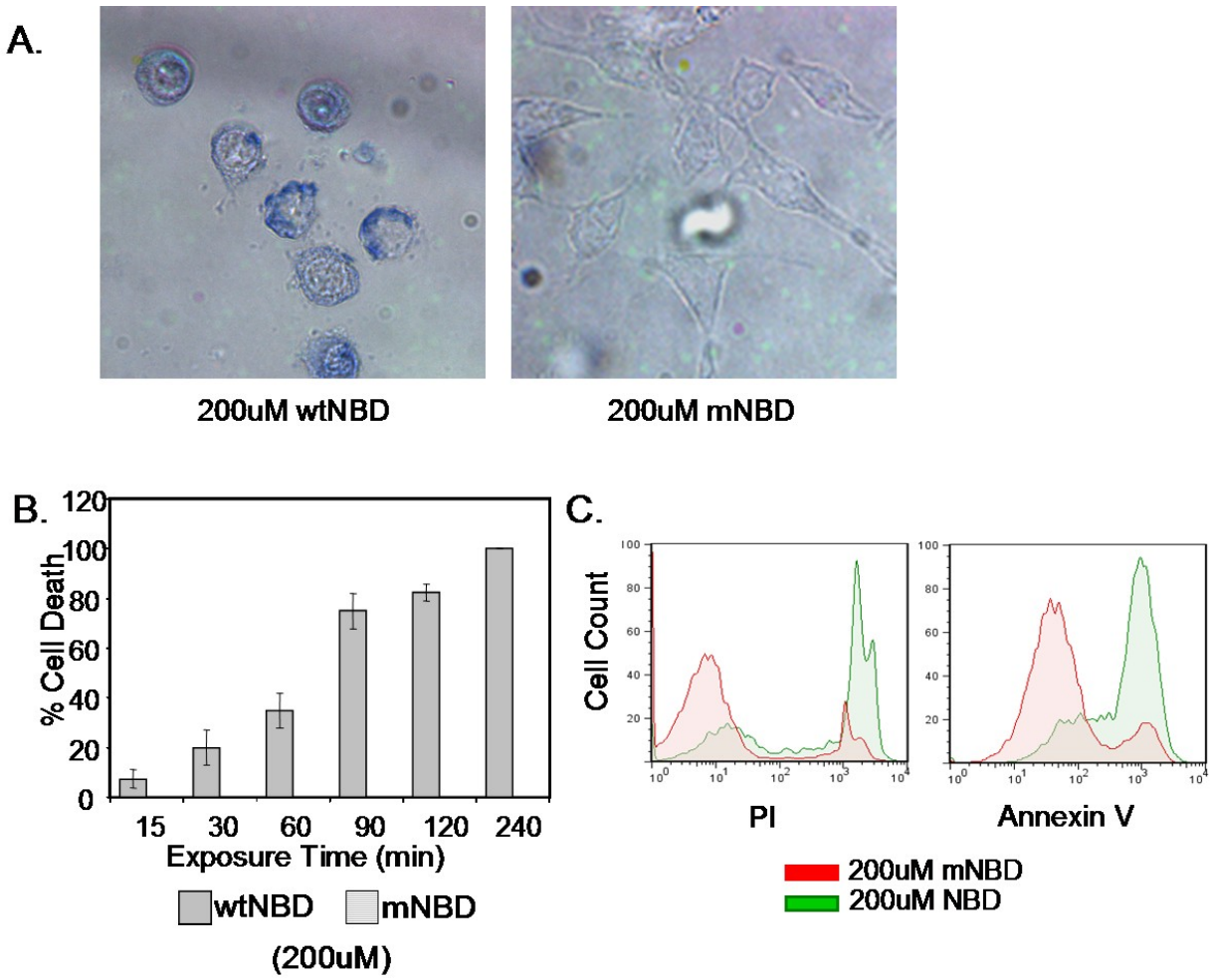


Figure 25: NBD peptide induces apoptotic PCD in APC. (A) RAW264.7 cells were treated with NBD or mNBD peptide for 12 hours, cells were then stained with trypan blue and then images were obtained. The left image indicates a high number of trypan blue stained (dead) cells following NBD treatment. In the image on the right, phase-contrast microscopy was utilized in order to visualize mNBD treated cells, which remain alive and capable of excluding trypan blue. (B) RAW264.7 murine macrophages cell line was treated with NBD or mNBD (200uM) for varying time-points and cell death was determined by trypan blue exclusion. (C) RAW264.7 cells were treated for 4 hours with NBD or mNBD peptide and analyzed for expression of Annexin V (apoptotic marker) and PI (cell death marker) NBD treated cells expressed an upregulation in both Annexin V and PI compared with inactive control peptide.

Table 3: Selected NF-κB inhibitory compounds

Inhibitor	Chemical Composition	Target	Citation
NBD	TALDWSWLQTE	IKK complex formation	[14, 73, 78]
Compound A (Cmp A)	2-Amino-6-(2-(cyclopropylmethoxy)-6-hydroxyphenyl)-4-(4-piperidinyl)-3-pyridinecarbonitrile	IKKβ specific inhibitor	[293, 307]
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal	Proteosome inhibitor: blocks IκBα degradation	[308, 309]
IKKiVII	2-benzamido-pyrimidine-derivative	IKKα and β inhibitor	[97, 310]
Wedelolactone	7-Methoxy-5,11,12-trihydroxy-coumestan	IKKα and β inhibitor	[311]
TPCA-1	5-(*p*-Fluorophenyl)-2-ureido]thiophene-3-carboxamide I(KK2 inhibitor IV)	IKKβ specific inhibitor	[312, 313]
JSH-23	4-Methyl-N^1-(3-phenylpropyl)benzene-1,2-diamine	p65 nuclear translocation inhibitor	[314]

Each of these seven inhibitors were evaluated for induction of APC death using the fetal skin dendritic cell line (FSDC), and for their NF-κB inhibitory properties using a stably transfected NF-κB-luciferase reporter HEK293 cell line ($293^{NF\text{-}\kappa B}$) (Figure 26). The cell death profile FSDC was proportional to that of the NF-κB inhibition profile with each inhibitor used, confirming that NF-κB suppression was indeed the inducer of PCD. JSH-23, which inhibits p65 nuclear translocation alone, did not induce complete PCD even at elevated doses, however, this could be due to compensation by other NF-κB subunits.

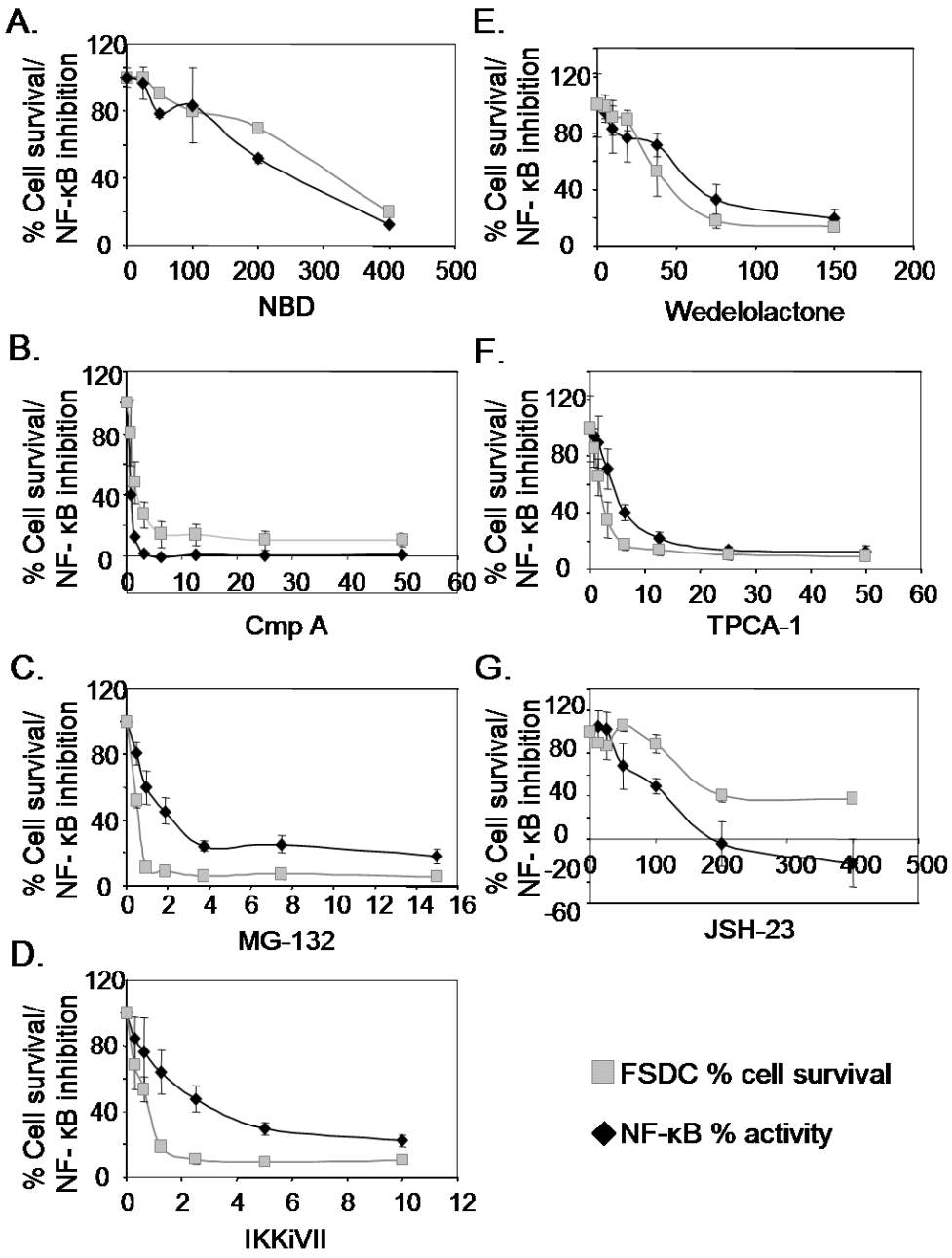


Figure 26: Decreased cell survival is directly correlated with decreased NF-κB activity following treatment with a panel of NF-κB and IKK inhibitors. The $239^{NF-\kappa B}$ reporter cell line was utilized to measure relative levels of NF-κB activation secondary to TNFα stimulation after 3 hour incubation at varying concentrations of the respective inhibitors (black lines). The inhibitors were also evaluated in the FSDC APC cell line (grey line) where the percent survival was determined by MTT assay after 24hr incubation with varying doses of the inhibitors (FSDC cells, unlike the $293^{NF-\kappa B}$, were not stimulated exogenously).

Due to the high concentrations of NBD (200-400μM) that were needed to induce cell death compared to the more controlled profile provided by Compound A (Cmp A), TPCA-1, IKKiVII, and MG-132, these other NF-κB inhibitory compound were used for the majority of *in vitro* experiments in the remainder of this manuscript.

4.4.2 NF-κB induced cell death is specific to APC populations:

Two previous reports suggesting the phenomenon of NF-κB-inhibition-induced macrophage cell death[35, 315], these reports did not evaluated DC nor other cell lines to determine whether this was an APC specific response. Using four of the NF-κB inhibitors, cell death profiles using an MTT assay were evaluated in HEK293, immortalized D10, and primary MEFs cell lines compared with the FSDC cell line. Cell death induction in the FSDC cell line occurred at far lower concentrations as compared with the other three non-APC cell lines. Compound A and TPCA-1, two IKKβ selective inhibitors, showed very little toxicity in non-APC cell lines (Fig 25A/B), while the two IKK complex inhibitors, IKKiVII and Wedelolactone, had greater affinity for producing PCD in the FSDC over other cell lines, but did exhibit some toxicity at higher concentrations (Figure 27C/D). When analyzing the non-APC cells in culture, it was apparent that the decrease in MTT activity observed at eleveated concentrations was secondary to necrosis-like morphologic changes or reduced cell growth rather than apoptotic morphologic changes (data not shown).

Using Compound A , the specificity of cell death to APC cell lines was further confirmed by evaluating survival profiles for an increased number of cell lines including both primary bone marrow derived DC (BMDC) and macrophages (BMDM), RAW264.7 macrophage cell line, as well as DU145, along with the previous evaluated cell lines. Figure 27E indicates LD_{50} values

for each cell line. Here the high level of specificity of NF-κB-inhibition-induced APC death is apparent. The HEK293 cells had LD_{50} ± SE of 199.5 ± 32.6, DU145 were 175 ± 23.9, MEFs had improved growth trends with NF-κB inhibition (so no calculable LD_{50}), while the APC had LD_{50} ranging from 2.7 ± 0.2 for FSDC to 8.9 ± 0.4 for RAW264.7, with wtBMDM, and wtBMDC at 3.2 ± 3.0 and 6.3 ± 0.1 respectively. One can see that the four APC cell lines had significantly lower LD_{50} compared with the other cell lines ($p<0.002$ for D10 to 0.000001 for MEFs). This data confirms that this effect is a specific pan-monocyte derived APC phenomenon.

4.4.3 NF-κB activates caspases response in APC:

NBD treatment increased annexin V and PI positivity in APC by 4 hours, suggesting apoptotic cell death (Figure 25C); however the exact mechanism of this PCD is unknown. Several mechanisms have been hypothesized to explain similar PCD phenomenon. TNFα induced PCD occurs via activation of JNK and caspase 8, which is described for varying cell types in the literature. The more recently described mechanism is PCD secondary to increased ROS production followed by capsase 9/3 activation. Each pathway individually or collectively may play a role in this APC specific PCD pathway, and will be explored further herein.

Therefore, caspase activation was analyzed after treatment with the NF-κB inhibitors. Time-courses performed using three NF-κB inhibitors, Compound A, IKKiVII and MG-132, showed an increase in caspase 8, caspase 9 and caspase 3 cleavage over a 7 hour time-course (Figure 28A). This caspase activation was confirmed using immunofluorescence for cleaved caspase 3 (Figure 28B) and the specific caspase inhibitor zVAD-fmk was able to inhibit NF-κB induced PCD in FSDC after treatment with CmpA, IKKiVII and MG-312 (Figure 28A). Interestingly, while primary BMDM also underwent caspase 3 and 9 cleavage, as determined by

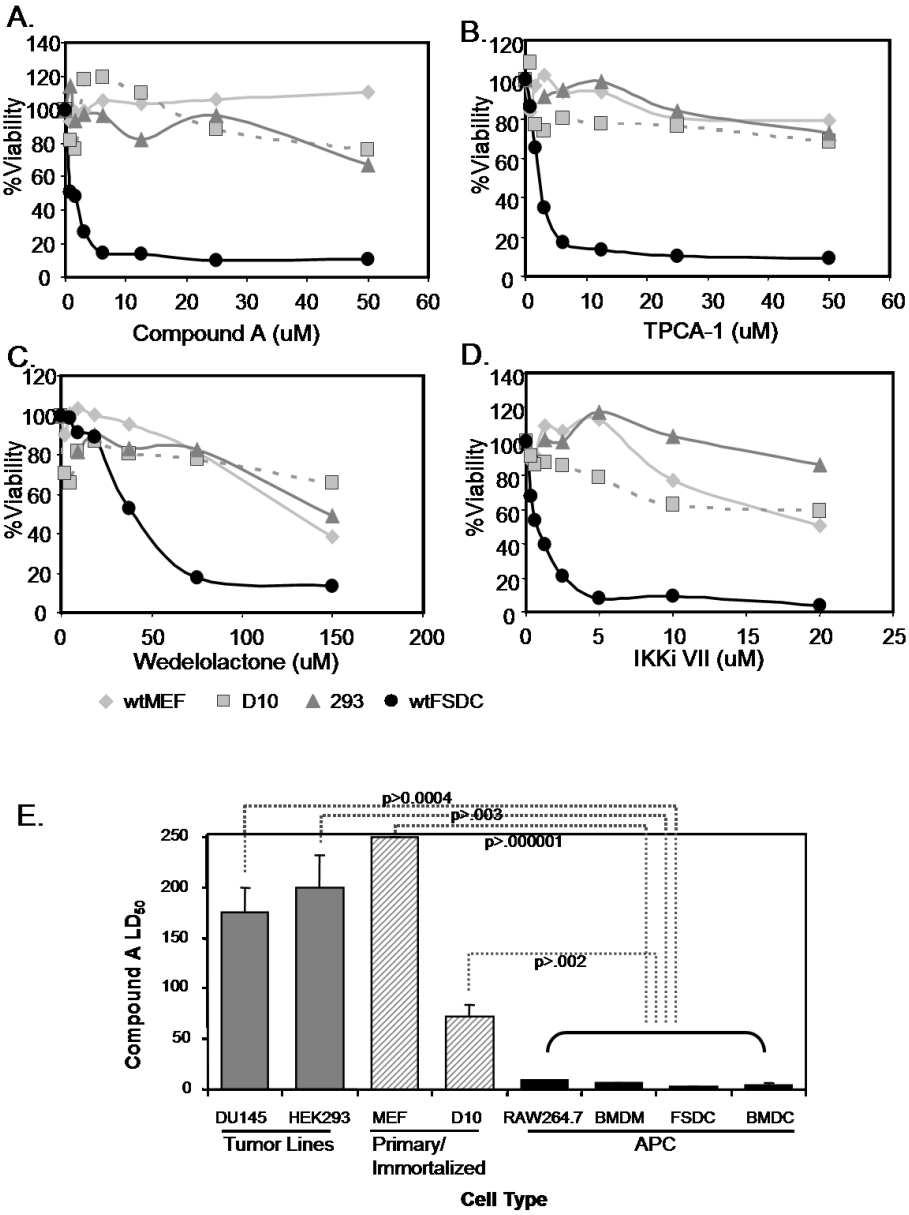


Figure 27: IKK induced cell death is APC specific. (A-D) Four cell lines, D10, wtMEF, HEK293, and wtFSDC, were evaluated for percent survival in the presence of increasing doses of several IKK inhibitors. Each point was determine by averaging at least 3 measurements at the indicated concentrations of IKKβ specific inhibitors, Compound A and TPCA-1 (A-B), or IKK complex inhibitors, Wedelolactone and IKKiVII (B-D), respectively. (E) LD_{50} values were determined for 4 APC and 4 non-APC cell lines in the presence of Compound A. Values were completed by regression analysis and determination of the 50% death point on a minimum of 3 survival curves for each cell line. p-values were determined by comparing each non-APC cell line with the most resistant APC cell line (RAW264.7)

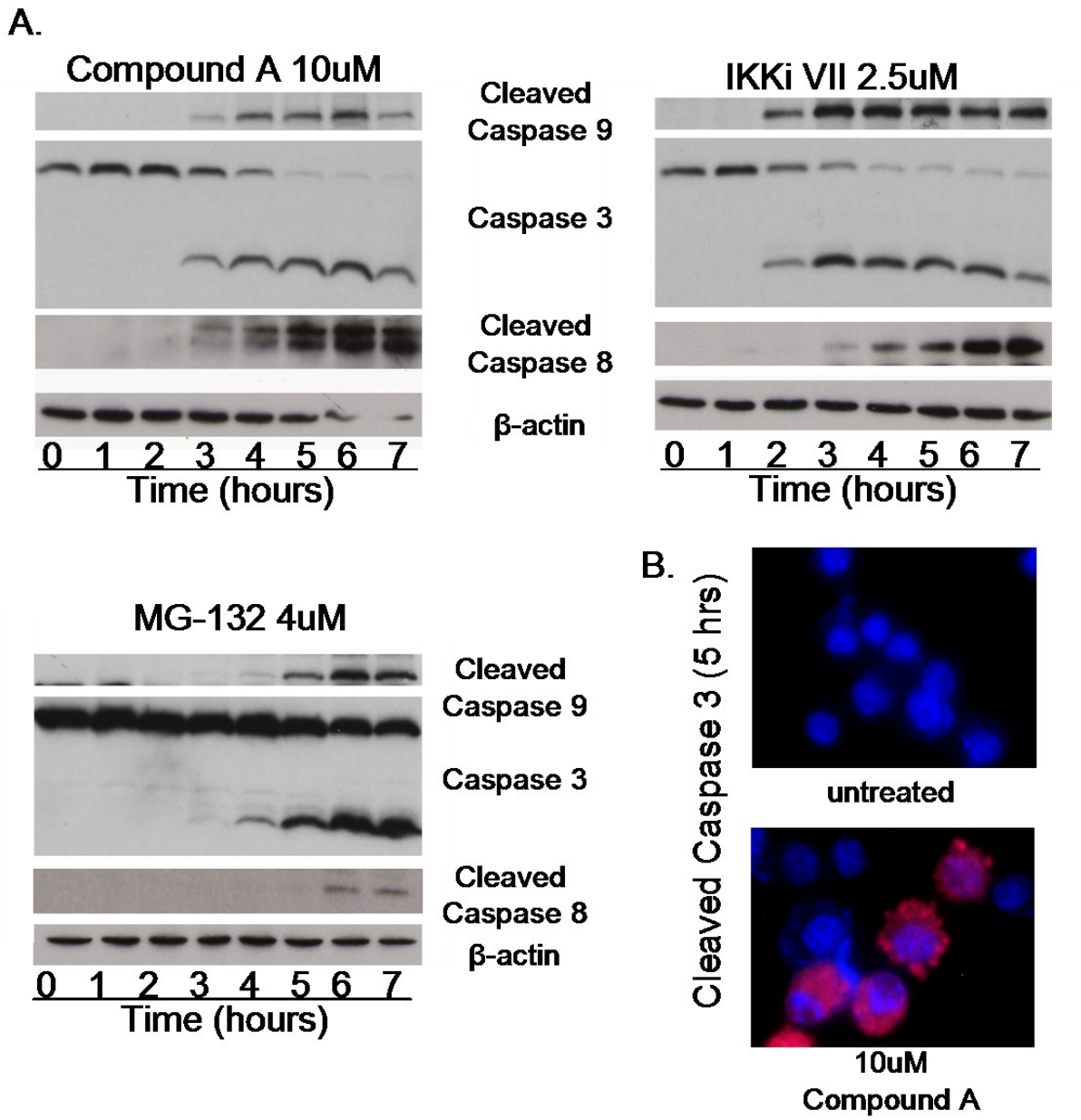


Figure 28: NF-κB suppression activates Caspases 8, 9 and 3 in a time-dependent manner. (A) Levels of caspase-8, -9, and -3 activation were determined via Western blot analysis at the indicated time-points following treatment with three different NF-κB inhibitors. β-actin was used as a positive control. (B) Immunoflourencnce shows an increase in the levels of cleaved caspase 3 (red) after 5 hour treatment with 10μM Compound A.

WB, zVAD pretreatment led to only minimal or no increase in cell survival, a feature also observed in RAW264.7 cells (data not shown). However, there have been several reports of macrophages undergoing cell death after treatment with zVAD[316, 317], thus preventing a clear understanding of caspase dependency in these other cell lines.

The activation of caspase 9 suggests a mitochondrial induced apoptosis response, as was described previously[35]. In most instances, a loss in mitochondrial MMP induces cytochrome C release, which then activates caspase 9 and then caspase 3. Two major mediators of capsase 9 cleavage and activation are upstream caspase 8 activation or ROS production, both of which may result in loss of MMP[318]. However, there are reported cases of direct caspase 9 activation by ROS prior to loss of MMP[13, 319]. Caspase 8 is known to be a downstream signaling component of the death domain signaling pathways, and is often secondary to TNFR activation. Thus, the roles of ROS and TNF signaling were explored.

4.4.4 APC NF-κB-inhibition-induced death is dependent on ROS production

To determine if NF-κB-inhibition-induced PCD is associated with increased ROS production, DiOC6(3) and mitoSOX were used to evaluate mitochondrial membrane potential (MMP) and ROS production, respectively. FSDC were treated with 10μM of Compound A and stained for FACS analysis at 0, 3, 7, 15, and 24 hours. During this time-course, there was a progressive increase in ROS and a concomitant loss in MMP (Figure 29A). These findings were confirmed by immunofluoresence, which shows a vast increase in ROS production (red staining) after incubation with Compound A. In addition, cells with apoptotic morphology (marked with

arrows), specifically at 4 and 9 hours, are those cells which have increased ROS levels and decreased MMP suggesting a role for this pathway in cell death (Figure 29B).

A ROS scavenger, BHA, which has been shown to reduce O_2^- production[320], was used to evaluate the role of ROS. While there is an initial increase in ROS production in FSDC treated with BHA and Compound A at 3 hours, the amount of ROS stabilizes and does not increase during the 7 and 15 hour time-points. Furthermore, the treatment of CmpA with the addition of BHA prevents the loss of mitochondrial membrane potential seen with CmpA alone (Figure 29C and 27A respectively). This suggests that loss of MMP is secondary to ROS formation. The ROS formation in Compound A in the presence or absence of BHA or in FSDC left untreated was further examined at the 3 and 15 hour time-points (Figure 29D, left panels). The reduced ROS production in BHA treated samples led to a maintenance of mitochondrial membrane potential (Figure 29D, right panels). In addition to leading to reduced ROS and rescue of the MMP, BHA treatment of cells results in rescue of cell death secondary to three NF-κB inhibitors, CmpA, IKKiVII, and MG-132 (Figure 30B), as well as a rescue of PCD in BMDM, BMDC and FSDC treated with Compound A (Figure 30C). Taken together, these data suggest that caspase 9 activation may be secondary to ROS formation or loss of MMP, but that ROS formation is likely the initiating event.

4.4.5 NF-κB-inhibitor-induced cell death is stimulation dependent but independent of the TNFα/JNK/Caspase pathway:

The activation of caspase 8 (Figure 28A) suggests a TNF dependent cell death pathway, which is the most commonly observed NF-κB induced PCD pathway described in the literature[301] and will be abbreviated here as the TNF/JNK/Caspase 8 PCD pathway. NF-κB

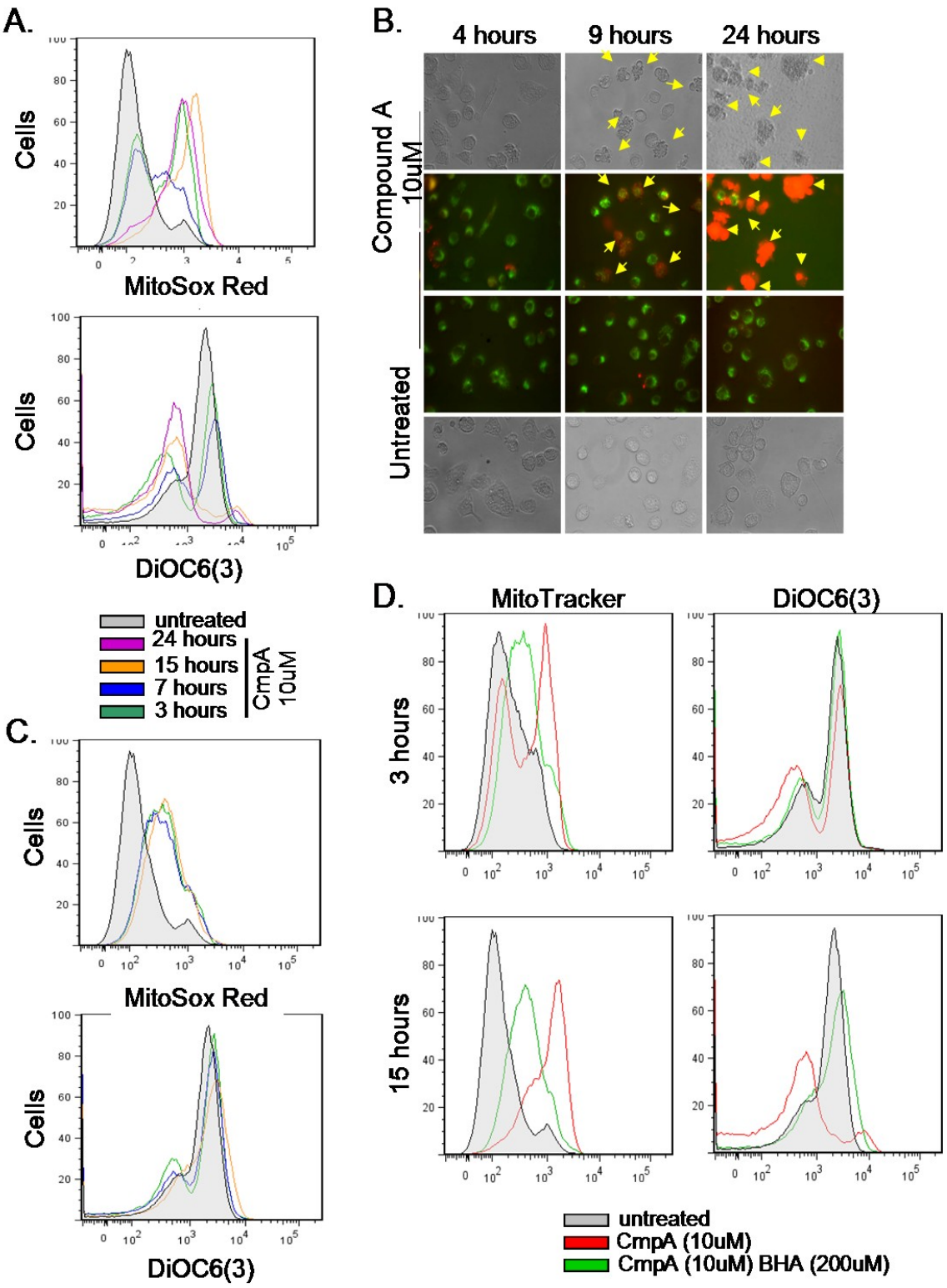

A.
Cells
MitoSox Red
Cells
DiOC6(3)
untreated
24 hours
15 hours
7 hours
3 hours
CmpA 10uM
B.
4 hours
9 hours
24 hours
Compound A 10uM
Untreated
C.
Cells
MitoSox Red
Cells
DiOC6(3)
D.
MitoTracker
DiOC6(3)
3 hours
15 hours
untreated
CmpA (10uM)
CmpA (10uM) BHA (200uM)

Figure 29: IKK suppression results in increased ROS production and secondary loss of MMP. (A) FSDC were treated with 10μM compound At time-points listed, MitoSOX (red fluorescent marker which increases with increased ROS formation) and DiOC6(3) (green fluorescence indicator of MMP, reduced fluorescence indicates loss of MMP), markers for ROS and MMP, respectively, were analyzed via flow cytometry. (B FSDCs were treated with 10uM Compound A and at the indicated time-points, MitoSOX (Red) and DiOC6(3) (Green) were analyzed by fluorescent microscopy, apoptotic cells are marked with yellow arrows. (C) FSDC were treated with 10μM CmpA and 200μM of BHA and analyzed for ROS production and MMP using flow cytometry. (D) FSDC ROS and MMP were evaluated via flow cytometry after 3 and 15 hours after 10μM CmpA ± treatment with 200μM BHA.

induced PCD was observed initially with the embryonic lethality of *p65*$^{-/-}$ mice secondary to liver apoptosis[301] and has, subsequently, been observed in numerous cell lines[301]. In each case, the NF-κB induced cell death was dependent on TNF signaling, and acted through caspase 8, a common mechanism for mitochondrial induced cell death. As caspase 8 is activated one hour after caspase 3/9 in both the MG-132 and IKKiVII treated samples, this may not be the initiating event. However, because this apparent delay in activation could be due to differences in exporure, *TNFα*$^{-/-}$ and WT primary macrophages were used to evaluate whether this APC death was TNF/JNK/Caspase 8 dependent.

Primary macrophages, WT or *TNFα*$^{-/-}$, were evaluated with Compound A, MG-132, and IKKiVII. The *TNFα*$^{-/-}$ cells were highly resistant to IKKβ inhibition-induced cell death, evaluated using Compound A and TPCA-1 treatment (Figure 31A top two panels). In addition, TNFα$^{-/-}$ macrophages were less susceptible to IKKiVII (pan-IKK) and MG-132-induced death than WT macrophages. In the case of IKKiVII-treatment, the TNFα$^{-/-}$ macrophages did undergo PCD at higher concentrations (Figure 31A). Thus, it appears that NF-κB-suppression PCD at higher concentrations (Figure 31A). Thus, it appears that NF-κB-suppression-induced PCD is

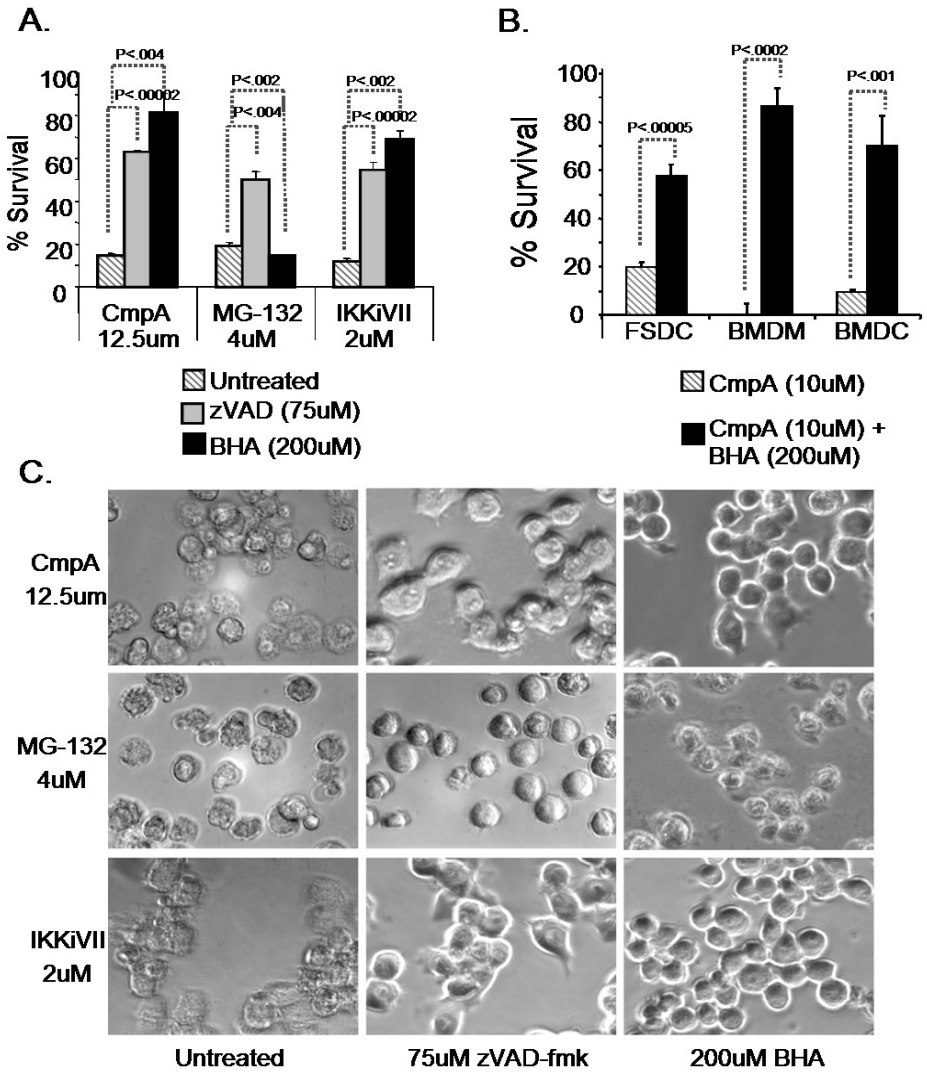


Figure 30: APC death is dependent on Caspase and ROS signaling. (A) MTT assays were used to measure survival of FSDC that were untreated or treated with the listed doses of CmpA, MG-132, or IKKiVII (NF-κB inhibitors) in the presence of 75μM zVAD or 200μM BHA. (B) Cells were further analyzed by microscopy to evaluate changes in morphology. Untreated cells show apoptotic morphologic changes including reduced size, blebbing and the loss of reflected light. Treatment with zVAD prevented morphologic changes induced by all NF-κB inhibitors, while treatment with BHA prevented apoptosis in CmpA and IKKiVII treated samples. (C) FSDC, BMDM, and BMDC were treated with CmpA ± 200μM BHA and analyzed for survival via MTT assay.

dependent on the activation state of macrophages, secondary to the inhibitors.

To evaluate if the *TNFα*$^{-/-}$ macrophage resistance to the IKKβ inhibition-induced PCD was truly a TNFα dependent phenomenon, *TNFα*$^{-/-}$ macrophages were treated with TNFα (10ng/ml), LPS (100ng/ml), or left untreated in the presence of CmpA. Addition of exogenous TNFα caused *TNFα*$^{-/-}$ BMDM to become more sensitive to CmpA-induced cell death than WT cells treated with CmpA alone. However, this same sensitivity was achieved with the addition of exogenous LPS (100ng/ml), suggesting that this is not a TNF/JNK/Caspase 8 dependent event but rather a stimulation or activation dependent event (Figure 31A top panel). This same phenomenon was observed with the other NF-κB inhibitors (TCPA-1, IKKiVII, and MG-132).

It is likely that the endogenous TNFα produced by WT macrophages led to the minimal activation state required for the APC death response. Therefore, TNF was depleted from WT macrophages 1 hour prior to CmpA treatment using 50 or 25ug of etanercept (anti-TNF antibody) resulting in blocked cell. However, NF-κB-inhibitor induced PCD was induced upon the addition of exogenous LPS in the presence of etanercept (Figure 31B). Further supporting the claim that TNF is not necessary to induce cell death, and also suggesting that the level of simtulation contributes to PCD induction in the presence of NF-κB inhibition.

As NF-κB-inhibitor induced PCD was observed after LPS stimulation in TNFα$^{-/-}$ macrophages, the role of stimulation in primary APC death was further examined. Only very low levels of exogenous stimuli were necessary for Compound A induced cell death to occur. While TNFα and LPS are normally used at levels of 10ng/ml or 100ng/ml respectively in culture, amounts as low as 0.05ng/ml of TNF and 0.01ng/ml of LPS were sufficient to induce significant cell death in Compound A treated *TNFα*$^{-/-}$ macrophages, suggesting that only minor activation of these cells is necessary for induction of the NF-κB inhibition-induced PCD (Figure 31C).

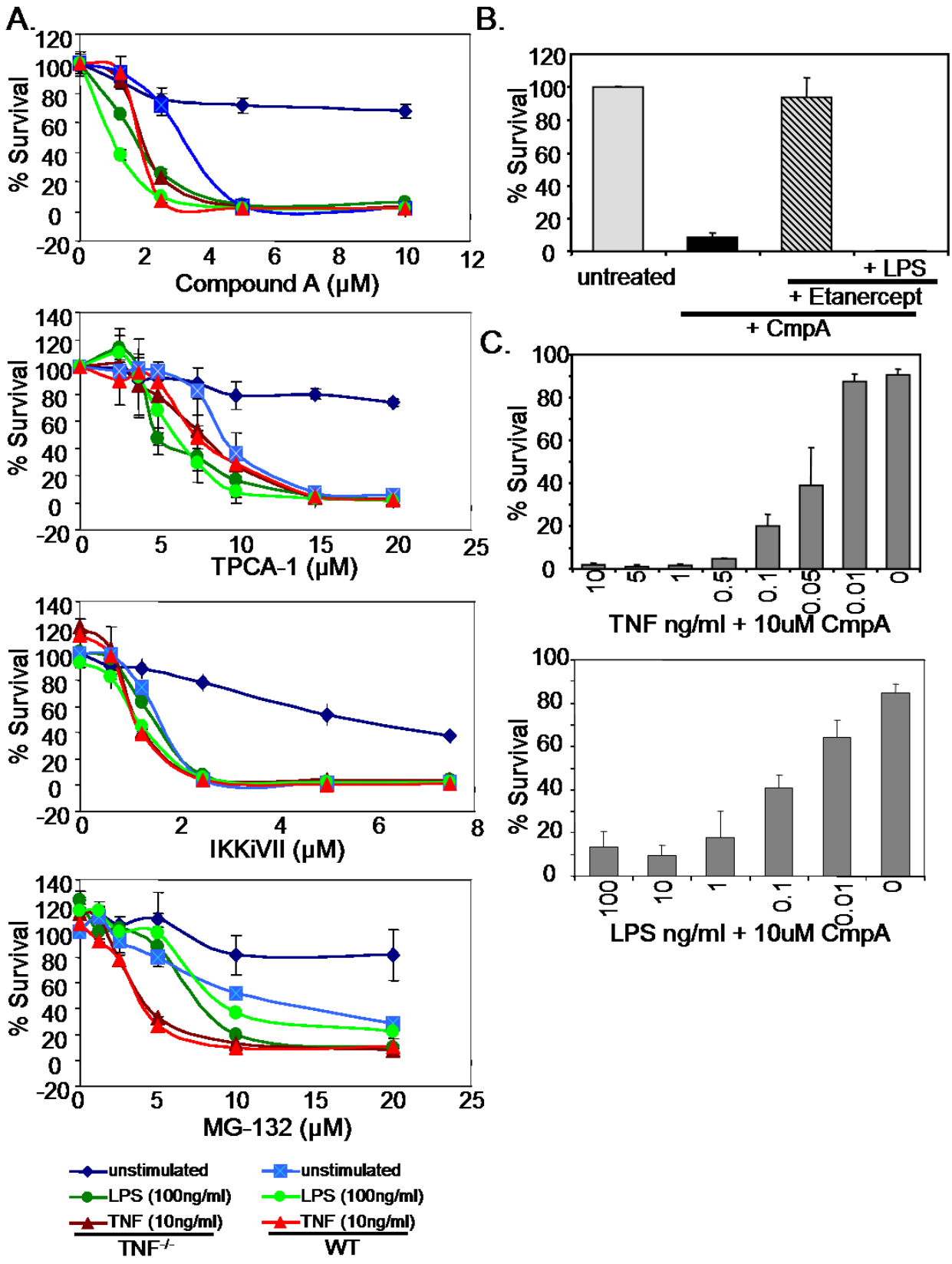

A.
% Survival
Compound A (μM)
TPCA-1 (μM)
IKKiVII (μM)
MG-132 (μM)
unstimulated
LPS (100ng/ml)
TNF (10ng/ml)
TNF-/-
unstimulated
LPS (100ng/ml)
TNF (10ng/ml)
WT
B.
% Survival
untreated
+ LPS
+ Etanercept
+ CmpA
C.
% Survival
TNF ng/ml + 10uM CmpA
% Survival
LPS ng/ml + 10uM CmpA

Figure 31: NF-κB-inhibition induced APC death is dependent upon stimulation. Cellular survival was measured after 24 hours using MTT assays in the following experiments: (A) *TNFα*$^{-/-}$ (darker colors) and WT (lighter colors) primary macrophages were evaluated for survival in the presence of NF-κB inhibitors, as well as in the presence of 10ng/ml of TNFα (green) or 100ng/ml of LPS (red) or left untreated (blue). (B) WT macrophages were either left untreated, or treated with CmpA (10μM), or CmpA (10μM) + Embrel (50ng/ml) ± 100ng/ml LPS and survival was measured by MTT assay. (D) WT macrophage survival in the presence 10μM Compound A and decreasing concentrations of TNF (top) or LPS (bottom).

4.4.6 NF-κB-inhibitor-induced PCD is independent of Death Domain Signaling through JNK and caspase 8

APC apoptotic response was induced without TNFα signaling suggesting a novel mechanism of NF-κB induced cell death. However, it is possible that this cell death could be secondary to another death domain receptor acting via JNK and caspase 8. Thus, SP600125 a potent inhibitor of JNKI and JNKII signaling, was evaluated in FSDC and BMDM. In FSDC, inhibition of Jnk reduces NO signaling (data not shown), but has no effect on NF-κB induced FSDC death at doses of 10μM or 25μM (Figure 32A) or in BMDM (data not shown). Therefore APC death in response to NF-κB inhibition is likely independent of JNK signaling. To further confirm that this was a novel TNF/death domain independent pathway, caspase 8 activation was evaluated. Caspase 8 is also activated secondary to death domain- and Jnk-induced cell death. Caspase 8 activation was observed secondary to Compound A, MG-132, and IKKiVII (Figure 28A). However, caspase 8 cleavage can occur either before mitochondrial membrane disruption or secondary to caspase 9/3 activation. Immunoblot analysis of FSDC pretreated with BHA or

left untreated revealed that caspase 8 activation was seen only in Cmp A treated samples, but not those treated with BHA and CmpA (Figure 32B), suggesting that caspase 8 activation, in this system, is activated after ROS and mitochondrial membrane dysfunction and is not the proximal mechanism of cell death

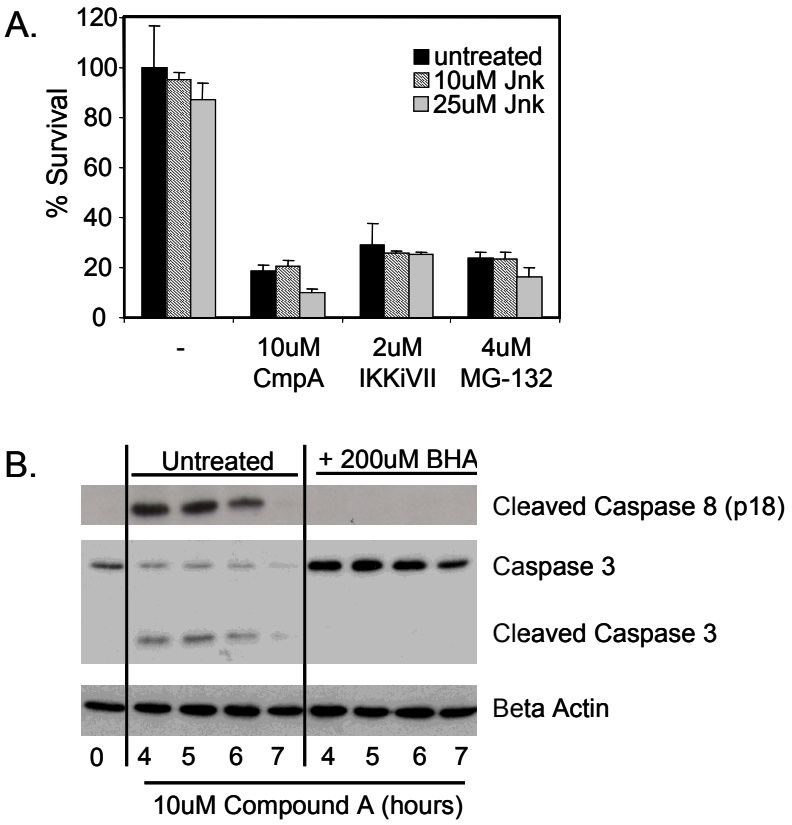


Figure 32: NF-κB suppression-induced APC PCD is independent of Jnk and Death Domain receptors. (A) FSDC were pretreated with either 10 or 25μM of specific Jnk inhibitor (SP600125) and varying concentrations of NF-κB inhibitors, and cell survival was evaluated by MTT assays. (B) FSDC samples were collected at 4, 5, 6, and 7 hours in the presence of CmpA (10μM) ± BHA (200μM), and caspase 8 and caspase 3 activation were evaluated via immunoblot. β-actin used as a loading control.

4.5 DISCUSSION:

This chapter describes a novel NF-κB-inhibtior-induced cell death response in APC. The widely accepted and previously reported NF-κB-inhibitor-induced PCD response occurs via the TNFα/JNK/caspase 8 signaling pathway. However, in APC we observe that NF-κB inhibition results in increased ROS formation, which leads to a subsequent loss of MMP (Figure 29) and activation of caspase9/3/8 (Figure 28). Using $TNF\alpha^{-/-}$ primary macrophages, it was observed that a minimal level of NF-κB activation must be present for IKK inhibition induced cell death to occur. However, these levels are similar to basal levels of TNFα (0.05ng/ml) produced by WT macrophages in cell culture[321]. Further evidence that this is a TNFα/JNK/Caspase 8 and death receptor independent include, LPS signaling induces cell death equivalent to the addition of TNFα in $TNF\alpha^{-/-}$ BMDM (Figure 31), lack of effects of JNK inhibition on the cell death response, and caspase 8 is activated downstream of ROS production(Figure 32). Therefore, the observed APC death is likely a novel NF-κB induced cell death pathway.

Our data reinforce and elaborate on the findings of two previous studies, which evaluated the roles of NF-κB-inhibition-induced cell death in macrophages. Mannick et al, observed that a non-specific NF-κB inhibitor, PTDC, caused RAW264.7 macrophages to undergo cell death in culture[315]. This finding was expanded upon by Pagliari et al who also used PTDC, but additionally examined NF-κB suppression using an adenovirus expressing IκBα DN. They showed a clear collapse of MMP using the Ad-IκBα-DN. However, when using PTDC, it was observed that the cells were not rescued from apoptosis using caspase inhibitors, possibly due to zVAD toxicity in RAW264.7 cells. In addition, they did not observe caspase 3 degradation, and in fact they suggested a caspase 3-independent pathway, and did not address the role of ROS production in this apoptotic response[35]. Furthermore, as these studies used PTDC, which can

also act an anti-oxidant[322], some of the true mechanisms of the NF-κB cell death pathway may have been overlooked. In exploring this phenomenon, we noted that A1/bfl-1 was downregulated at the mRNA level in the presence of CmpA although to a lesser extent than shown by Pagliari et al (Figure 33A). However, an increase in A1/bfl-1 protein expression was observed after NF-κB suppression (Figure 33B/C). As A1/bfl-1 acts to protect MMP, but does not alter ROS production, it is likely that A1/bfl-1 transcriptional suppression is not a primary mechanism controlling APC death after NF-κB inhibition.

That APC are not reliant on TNFα to undergo NF-κB induced PCD is not entirely surprising, as macrophages and DC have higher basal levels of NF-κB activity compared to other cell types[35]. Further, while TNFα is a danger marker for the majority of cell types and can often lead to cell cycle arrest and stress responses, in APC TNFα acts as a pro-growth, activation signal[323], thus the effects of this cytokine are likely different in APC compared with other cell types.

We demonstrate here that ROS production is a proximal component of NF-κB inhibition-induced PCD. ROS formation occurs upstream of both MMP loss (Figure 29) and caspase activation (Figure 32). NF-κB is known to transcriptionally regulate several anti-oxidant proteins within the cell, inclduing superoxide dismutases (SOD1[324], SOD2[325]), and ferritin heavy chain (FHC)[326]. Of these anti-oxidant proteins FHC has been previously implicated in TNFα/JNK/Caspase 8 induced NF-κB cell death while the other anti-oxidant proteins appear to play a lesser role[13, 322]. However, mRNA analysis showed no downregulation of FHC, despite the rescuing effect of DFO, a chelator of free iron, in

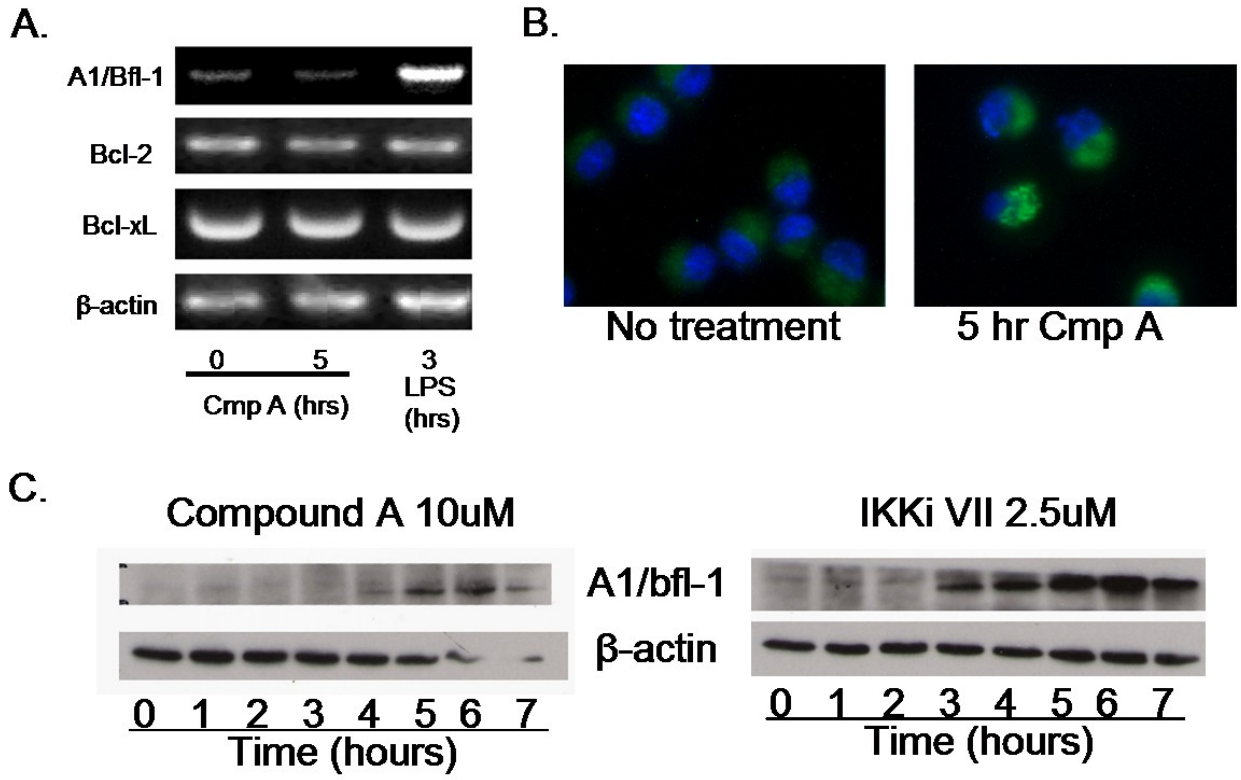


Figure 33: A1/bfl-1 mRNA, but not protein, is reduced in NF-κB suppressed APC. (A) mRNA was isolated and evaluated for expression at 0 and 5 hours post-treatment with Cmp A, as well as 3hr post treatment with LPS. Expression of Bcl-2 family members A1/Bfl-1, Bcl-2, Bcl-XL were analyzed via Western blot. β-actin was used as the loading control. (B) IF of A1/bfl-1 was completed in control cells or at 5 hours post-CmpA treatment. (C) Immunoblot of A1/bfl-1 expression during a timecourse of NF-κB suppression-induced PCD.

rescuing IKKβ specific cell death (Figure 34). Thus, further studies must be completed to determine the downstream effectors of increased ROS in our NF-κB inhibited macrophages.

Possibly more important than the exact mechanism of increased ROS production is the likelihood that these findings may help to explain human physiologic and therapeutic responses to NF-κB inhibition. PCD secondary to NF-κB inhibition in APC may explain response observed

in specific pathogen infections. There are a few well known pathogens that cause macrophage and DC apoptosis, and in a number of these cases, it has been determined that these bacteria produce NF-κB inhibitory compounds. Two specific examples are the Yersinia bacteria and Vaccinia Virus. Numerous articles suggest that APC death is induced secondary to one of two NF-κB inhibitors produced by Y. pestis, YopP and YopJ, as a loss of these molecules leads to improved survival [327, 328] Furthermore, this NF-κB inhibition-induced PCD is enhanced by LPS signaling[327]. Vaccinia virus inhibits NF-κB signaling via two proteins, N1L[329] and B14[330]. Furthermore, it has been reported that Vaccinia causes macrophage and DC apoptotic cell death[331, 332]. Interestingly, one of the differences between attenuated Vaccinia, which does not induce APC, and the non-attenuated apoptosis inducing Vaccinia is the expression of N1L[333]. Therefore APC death induced by NF-κB inhibition may further explain macrophage cell death responses secondary to other pathogen infections.

This NF-κB apoptic response is biologically important not only with regards to APC response or protection against infection, but also in evaluating therapeutics for cancer and inflammatory/auto-immune diseases. Recently, several groups have begun to examine the efficacy of NF-κB inhibition in treating myeloid leukemias such as acute and chronic myeloid leukemia (AML and CML). AML and CML derive from myeloid progenitors, which give rise to non-lymphocyte white blood cells including macrophages and DC[334]. In Imatinib resistant CML cell lines, NF-κB/IKK was activated[30, 31, 335], and as expected these cells underwent caspase dependent cell death in response to NF-κB/IKK suppression. Furthermore, mice injected with CML cell lines showed reduced tumor burdens when treated with an IKKβ inhibitor[30].

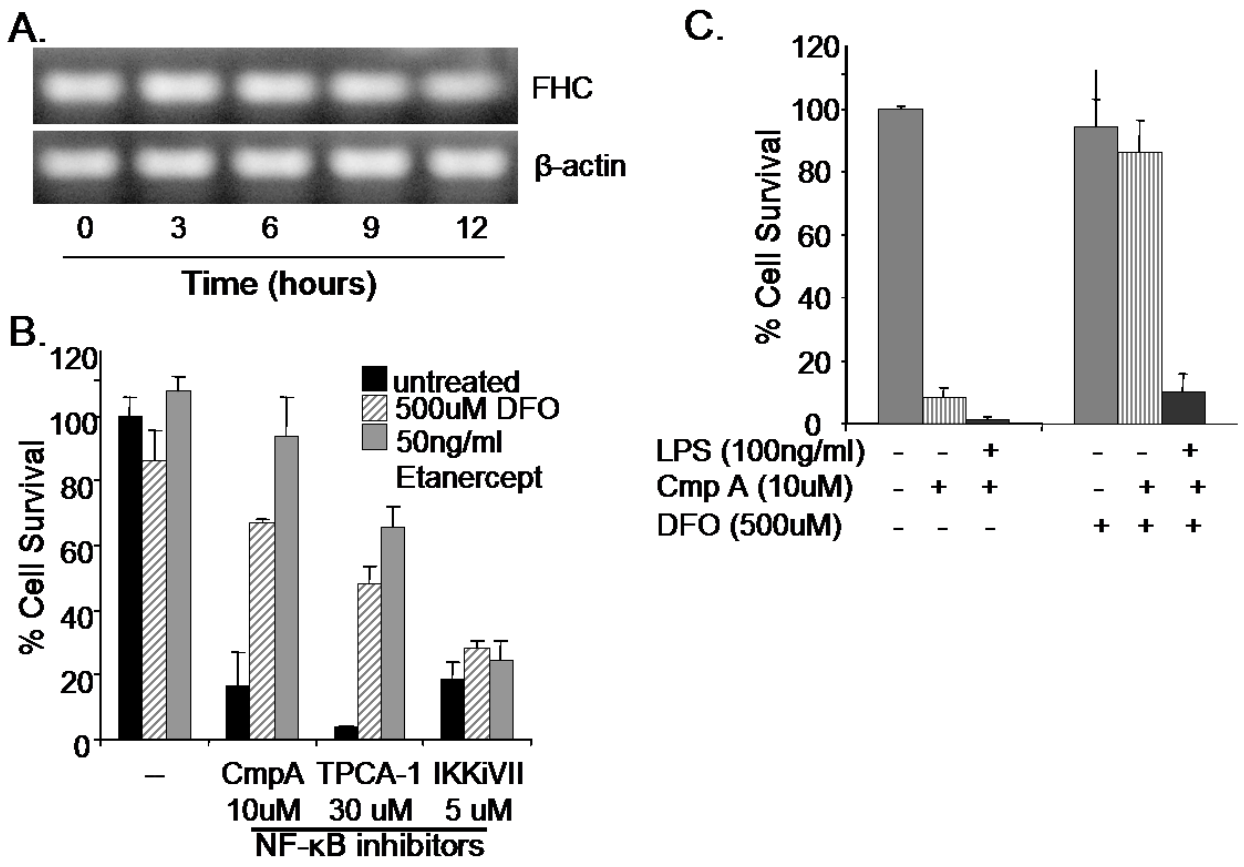


Figure 34: NF-κB suppression does not suppress FHC gene expression, but iron chelation reduces NF-κB induced cell death in FSDC. (A) mRNA expression of FHC is not altered after treatment with 10μM Compound A over a timecourse of 12 horus. (B) MTT survival assay after treatment with NF-κB inhibitors in BMDM pretreated with 500μM DFO, 50ng/ml of etanercept, or left untreated. (C) MTT survival assay in BMDM treated with a combination of 100ng/ml LPS, 10μM Compound A, or 500μM DFO.

The NF-κB initiated inflammatory response is an obvious pharmacologic target for the treatment of auto-immune and inflammatory diseases. Not surprisingly, numerous NF-κB inhibitory compounds have been evaluated in the treatment of inflammatory disease states, such

as inflammatory bowel disease, rheumatoid arthritis, muscular dystrophy, diabetes, as well as Parkinson's and osteoporosis. In each case, APC play a potent role in disease initiation or progression, therefore, activation induced APC death is a possible mechanism that could explain the ameliorating effects of NF-κB inhibitor treatment. In two studies evaluating the NBD peptide, NF-κB inhibition resulted in decreased APC number. CD11b microglial cells (central nervous system APC), are reduced in number after such treatment in a model of Parkinson's disease[72]. In addition, this same phenomenon was observed when using NBD in a muscular dystrophy model[73]. In this latter article the reduced APC number was attributed to decreased infiltration; however, this decreased infiltration may be the result of increased APC death. Even in mice with floxed IKKβ promoters in macrophages showed that cells with partial deletion of IKKβ were selected for and that cells with complete knockdown of IKKβ were counterselected[336], suggesting that macrophages with complete loss of NF-κB/IKK after differentiation are not viable.

The novel mechanism of NF-κB inhibitor-induced PCD in APC described here offers a potential explanation for many of these observations. Furthermore, NF-κB inhibitors, which have extremely short half-lives *in vivo*, can be injected only a few times per week while still maintain their immunosuppressive qualities. Thus, we believe that this APC death may result in a fundamental change in the immune system that prolongs the efficacy of NF-κB/IKK inhibition. Furthermore, if there is a specific gene which leads to increased ROS production this may be a potential therapeutic target for treating inflammatory and autoimmune disease. Our data and that of others support the model that cells with high basal levels of NF-κB activity are most sensitive to NF-κB induced PCD. In addition, our observations that minimal stimulation is required for cell death to occur would potentially mean that only activated APC would undergo apoptosis

with this therapy, further reducing side effects, and improving the therapeutic potential of NF-κB inhibitors.

5.0 DISCUSSION AND SIGNIFICANCE

NF-κB is one of the most studied transcription factors, and has been implicated in a wide variety of disease models and cellular processes. In this thesis, I have explored two very different disease models and in each case observed that suppression of NF-κB transcriptional activation ameliorates degenerative changes associated with these diseases. Furthermore, the data suggest that NF-κB may regulate several different pathways that possibly act independently or synergistically to affect the varying disease phenotypes.

Initially, I observed NF-κB inhibition using the NBD peptide ameliorates disease in a murine model of IBD. This was not surprising due to the evidence implicating NF-κB and immune activation in IBD pathogenesis, specifically increased inflammatory infiltrates in the intestines of human and murine disease sufferers. NF-κB is upregulated in tissue sections from both species as well as in lamina propria macrophages residing in the intestine. Interestingly, during the publishing process of our manuscript describing the amelioration of murine colitis via inhibition of NF-κB[78], another manuscript described that genetic deletion of NEMO and IKKβ in intestinal epithelial cells led to a colitis phenotype[112]. While surprising at first, these differential effects of NF-κB suppression are not unexpected when one considers the diverse roles of NF-κB in varying cell types.

As described in the introduction, the intestine is one of the few organs in which the primary goal of the resident immune cells is to suppress an overt immune response. This

knowledge can explain both our findings, and the fact that IKK suppression in IEC leads to colitis. NEMO deletion in IEC led to a reduction in antimicrobial peptides which allowed for bacterial invasion and subsequent apoptosis of the IEC themselves. The apoptosis of IEC is likely perpetuated by increased TNFα and the lack of NF-κB survival signal, a well document PCD pathway[301]. This in turn leads to a vicious cycle in which chronic intestinal infection leads to a colitis phenotype. With regards to our findings, this suggests that NF-κB inhibition via NBD does not target all cells e.g. intestinal epithelium, but perhaps has more a more eslective impact on specific cell populations such as macrophages and DC. This is further supported by the reduction in the inflammatory cytokines TNFα, IL-12, and INFγ seen in the treated mice compared with control treated mice. In addition, I found that peptides trafficked toward the mesenteric lymph node and spleen 30 minutes after injection. Overall, the data derived in the IBD model using NBD as well as another NF-κB inhibitory compound, ethyl pyruvate, [337] provide strong evidence that NF-κB inhibition is a viable therapeutic approach for IBD.

In fact, current therapies for the treatment of IBD have NF-κB suppressive qualities as discussed in Section 1.2.4; however, many of these therapies have off-target effects. Even current state-of-the-art biologic therapies, such as infliximab have drawbacks such as susceptibility to infections, increase cancer risk, and a 30% failure rate[84]. Even with the improvement of therapies for the treatment of IBD and other inflammatory and auto-immune disorders, many of which share significant mechanisms such as macrophage, T-cell and NF-κB involvement, there is still a vast need to improve therapeutic strategies. I believe that targeting NF-κB signaling in a cell specific manner may help to achieve these goals.

As innate immune cells are major mediators of IBD and other inflammatory diseases, I chose to explore the role of NF-κB signaling in monocyte derived APC cell lines. Initially, I

wanted to examine the effects of NF-κB inhibition on macrophage and DC cytokine profiles. What I observed, however, was a PCD phenomenon. This cell death is independent of exogenous stimulation, and in fact occurred via a novel mechanism, dependent on increased ROS production, with secondary loss of MMP and caspase activation. The other important finding, from a therapeutic perspective, is that APC need to have a minimal level of stimulation to undergo cell death in the presence of these NF-κB inhibitors. This likely means that NF-κB inhibition would not cause massive induced apoptosis of APC, but it would enable targeting of this PCD to areas of inflammation, potentially reducing the number of side effects.

Unfortunately, I have yet to convincingly show that there is increased APC cell death *in vivo*. However, our *in vitro* findings suggest that this APC cell death is a possible mechanism by which NF-κB suppression may reduce the inflammatory disease phenotype in the *IL-10*$^{-/-}$ mouse model. This is supported by other groups, who showed a reduced number of macrophages in MDX mice treated with NF-κB inhibitors[73] as well as reduced number of CD11b^{+} microglia in a murine model of Parkinson's after NF-κB inhibitor treatment[72]. Thus, I believe that the macrophage cell death pathway described here is a possible link between NF-κB suppression and reduced IL-12 and TNFα cytokine signaling, reduced inflammation, and amelioration of colitis. APC may take up higher concentrations of the NF-κB inhibitors over intestinal epithelial cells, or because these inhibitors have short half-lives in vivo, only the PCD response would provide a long term effect seen in macrophages over other cell types. Each of these hypotheses may explain the reduction in disease pathology with the subsequent maintenance of intestinal barrier function. The idea that macrophage apoptosis may reduce inflammatory disease is not new and was reviewed by Pope[338] in 2002. However, there have been few mechanisms by which to target apoptosis to specific cell types. Therefore it will be pertinent to determine if NF-κB treatment *in*

vivo does cause specific activated APC cell death, and whether increased ROS production in macrophages secondary to other therapeutics a similar effect, leading to possible new therapeutics with potentially high efficacy and low off target and side effects.

One link between APC and the leukemia and lymphoma cell lines that undergo NF-κB induced cell death is upregulation of basal NF-κB signaling. Therefore, the PCD pathway, described in chapter 4, may occur in other cell types with high basal level of NF-κB activation, such as neutrophils or tumor cells. Our findings may contribute to our understanding of how NF-κB suppression may be used to treat cancer. It is know that cancers secrete factors which induce resident monocyte derived APC to become regulatory or suppressive in phenotype[339]. Thus, treating cancers with NF-κB inhibitory compounds as an adjuvant therapy may have a two-pronged effect. First, NF-κB suppression of the tumor cells themselves may allow for decreased survival, as overactive NF-κB signaling is often a survival mechanism used by tumor cells[340, 341]. Second, NF-κB suppression may allow these suppressive APC to undergo PCD and then be replaced by more immunogenic cells. While this would be a highly complex treatment strategy, understanding the differential role of NF-κB in varying cell lines is highly important when designing cancer therapies.

While the role of NF-κB upregulation in inflammatory and auto-immune diseases is an expected scenario, recent evidence has also shown that NF-κB plays a role in numerous other diseases not defined as "inflammatory" in nature. A wide variety of diseases in which NF-κB has been implicated occur with increased chronologic age. Additionally, there have been several papers published within the last few years which have shown increasing amounts of NF-κB signaling in tissues of aged animals. While there is increasing correlative evidence that NF-κB plays a role in aging and age-related disease, it was unknown whether NF-κB was a protective or

causative agent with regard to these changes. I first showed that NF-κB is upregulated under conditions of increased stress whether it be genotoxic or oxidative, both of which are known to induce age-related changes (Figure 16-18). This NF-κB activation was confirmed *in vivo* using the ERCC1 deficient model of accelerated aging. I then determined using both genetic and pharmacologic suppression that indeed NF-κB signaling contributed to phenotypic and histologic changes associated with aging.

As NF-κB is known to respond to cell stress events such as DNA damage, it may initially be a protective mechanism; however, in the case of increasing damage over a period of time, this initially protective feature may become pathologic. For instance, increased NF-κB activity may promote cellular senescence. On an individual cell basis, this may result in positive outcomes, by preventing tumor growth and ending the proliferation of a damaged cell. However, on a gross scale if the vast majority of cells undergo senescence the whole organism will age. As NF-κB is a regulator of numerous cell cycle control genes such as Gadd45, Lamin B, ApoD, and Cyclin D these may mediate changes associated with aging. However, it is likely that NF-κB mediates age-associated changes in numerous ways, which may in fact vary from tissue to tissue or cell to cell.

NF-κB is known to play a role in osteoclastogenesis, as well as to negatively regulate osteoblasts[342]. As osteoclasts are macrophage-like cells, NF-κB inhibition may reduce osteoclasts by inhibiting their differentiation and proliferation, or by causing osteoclast PCD, while at the same time increasing osteoblast activity. With regards to neurodegenerative changes, NF-κB inhibition may reduce inflammatory mediators, which can reduce neuronal cell viability, as discussed in the 1.3.4. In other tissues, such as liver, NF-κB may play a role in cellular senescence and reducing proliferation. Furthermore, in collaboration with other groups,

we have preliminary evidence which suggests that stem cells and progenitor cells haploinsufficient for p65 have improved stress response, and proliferation capacity. We can therefore theorize that NF-κB suppression may improve the viability and regenerative capacity of endogenous stem cells, thereby enhancing tissue function and body homeostasis. Thus, it is likely that NF-κB suppression acts through numerous mechanisms to mediate its beneficial effects.

It is theorized that aging occurs secondary to the accumulation of random damage. Our research is one of the earliest studies to implicate NF-κB signaling as a damage response signal in aging, using a model of accelerated aging. A possible schematic of how NF-κB is integrated into the damage induced aging process is shown in Figure 35. The role of a common signaling mechanism in age-associated disease is of importance because it provides a link between Alzheimer's, Parkinson's, osteoporosis, atherosclerosis, diabetes, and sarcopenia, which scientists and clinicians have thought had little in common except their increased incidence with advanced chronologic age. Thus, our research has offered a mechanistic avenue by which these diseases are related and secondarily provides a potential therapeutic target to address pathologic changes that occur with aging. In concert with this, I have also studied the role of a mitochondrial targeted ROS scavenger XJB and its ability to ameliorate and delay pathology associated with aging. As ROS are known to activate and be controlled by NF-κB signaling, this may be one mechanism by which NF-κB transcriptional activation contributes to aging pathology.

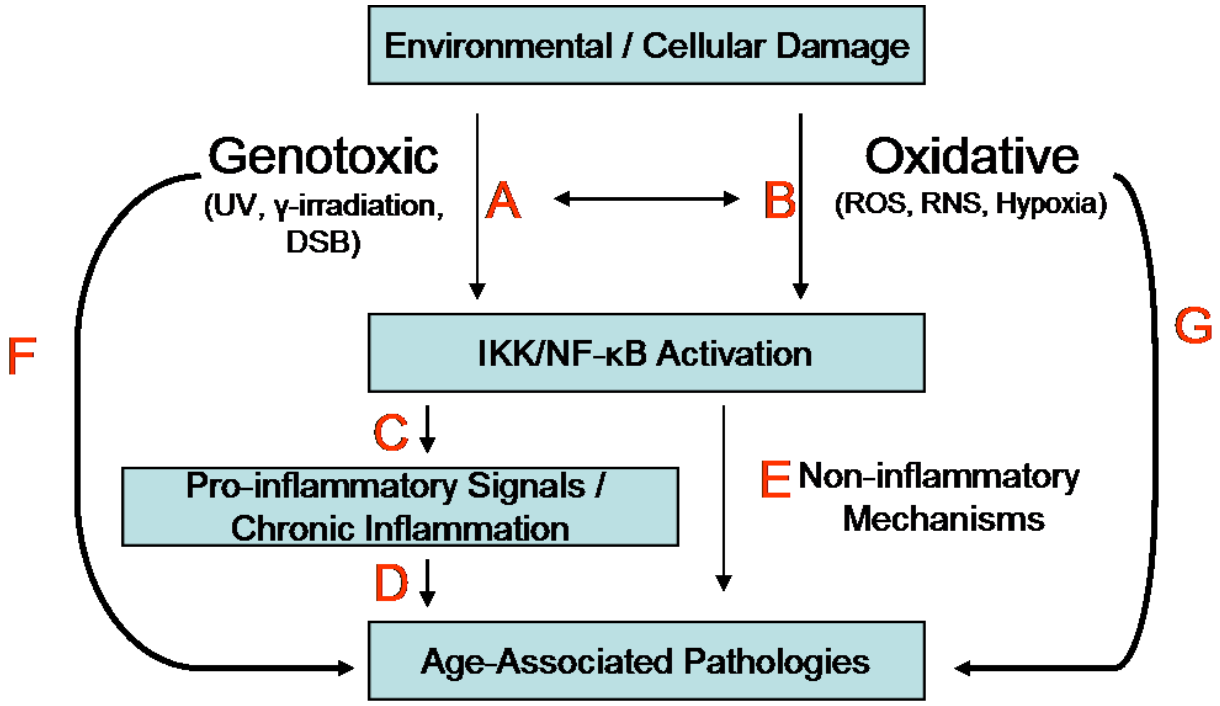


Figure 35: Schematic of NF-κB central role in the aging process. Genotoxic stress activates NF-κB signaling[12, 153, 343-345] B. Oxidative stress activates NF-κB signaling [346-349] C. NF-κB stimulates expression of proinflammatory cytokines[5, 157, 350] and causes chronic inflammation [5, 351, 352] D. Chronic inflammation causes age related diseases[353, 354], including: atherosclerosis[355] sarcopenia[201], Alzheimer's disease[356], diabetes[357] and cancer[358] E. Non-inflammatory mechanisms of tissue dysfunction, including cell cycle dysregulation[359,360], cellular senescence[135, 136, 167, 361-363], DNA damage[364], differentiation[365] or failure to differentiate[366]) F. Accumulation of DNA damage promotes aging[125-130]. G. Oxidative stress promotes aging[135, 136, 362, 363]

Overall, this research has exemplified the pleotropic effects of NF-κB signaling from its role in inflammatory disease, to diseases associated with aging. NF-κB activations regulates a wide range of processes, including dampening of cytokine secretion, alteration of stem cell physiology, effects on proliferation, cell cycle control, and organism development, as well as its role in controlling cell death and survival responses. It is important to recognize the pleiotropic nature of NF-κB when evaluating its role in pathogenesis as well as the effects of suppressing this signaling as a possible therapeutic mechanism. However, I believe that targeted suppression of specific NF-κB induced pathways may have beneficial effects in treating a wide variety of diseases.

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