Developmentally regulated tumor necrosis factor alpha induced nuclear factor kappaB activation in intestinal epithelium

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Running Head: NF-κB activation in immature enterocytes

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Abstract:

Premature infants are susceptible to many conditions which are inflammatory in nature. For this patient population which is expecting the intra-uterine environment, pathways necessary for fetal life and development may not have completed the transitions necessary for extra-uterine life. In this study, responses to tumor necrosis factor alpha were compared in human fetal and adult intestinal epithelial cell lines along with pre-weaned and post-weaned mouse intestinal sections in order to identify a potential developmental difference which may explain the heightened inflammatory response of preterm infants. The nuclear factor kappaB (NF-κB) pathway regulates a wide variety of genes involved in immune and inflammatory processes. We report that compared to adult intestinal epithelial cells, immature intestinal epithelial cells have increased NF-κB activity associated with increased NF-κB-DNA binding and transcriptional activity. This increased activity appears due to inadequate inhibition of signaling leading to NF-κB activation since there is also increased phosphorylation, ubiquitination and degradation of the inhibitor of NF-κB (IκB) in conjunction with decreased baseline expression and delayed resynthesis of this inhibitor. Thus, we demonstrate a potential mechanism for the heightened inflammatory response of immature intestinal epithelial cells.
Keywords:

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Introduction

The intestinal mucosa is a key interface with the external environment, with important roles in host defense, immune responses, nutrition, and fluid balance. Many of the functions of the intestine have been shown to be developmentally regulated and influenced by microbial flora (13). It is vital that the intestine defend against pathogenic insult, yet appropriately tolerate commensal bacteria.

The production of pro-inflammatory cytokines is one means of host defense. However, the immature intestine, expecting a sterile intrauterine environment, may not appropriately control intestinal inflammatory responses. This has implications for preterm infants with an immature gut potentially ill-prepared for the extra-uterine environment and contact with bacteria and food substrate. Developmental down-regulation of gut inflammatory responses is potentially a necessary process to avoid immune responses against normal intestinal flora.

The intestine is exposed to both endogenous and exogenous inflammatory mediators. Studies have shown that exogenous mediators such as bacteria interact with the intestinal epithelium via well conserved pattern recognition receptors such as the toll like receptors (8). These receptors trigger signaling pathways leading to nuclear factor kappaB (NF-κB) activation and propagation of the inflammatory response. Similarly, endogenous inflammatory mediators such
as tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β) bind to their respective receptors and can also trigger further cytokine production via activation of the NF-κB pathway.

NF-κB proteins activate transcription of a wide variety of genes involved in immune and inflammatory responses including interleukin 8 (IL-8). In its resting state NF-κB dimers are bound in the cytoplasm to the inhibitory κB (IκB) proteins (9). Cell stimulation can trigger signaling pathways leading to the activation of IκB kinase (IKK), which then phosphorylates IκB, targeting it for ubiquitination and degradation by the 26S proteasome. The NF-κB thus liberated, moves to the nucleus where it activates gene transcription.

Previously published work demonstrates exaggerated inflammatory cytokine production by immature intestinal epithelial cells compared to adult intestinal epithelial cells. Specifically, IL-8 secretion in response to bacteria, IL-1β, and TNFα has been shown to be greatly increased in human fetal small intestinal epithelial cells and fetal tissue organ culture samples compared to adult cells and older child biopsy samples (6, 18).

We further demonstrated that this increased IL-8 secretion is associated with decreased baseline expression of IκB isoforms in immature intestinal epithelial
cells (IEC) compared to mature IEC (5). In order to further understand the
mechanism behind exaggerated cytokine production in immature enterocytes, we
hypothesized that this decreased IκB expression would correspond to increased
NF-κB activity. This study demonstrates that immature human intestinal epithelial
cells have increased NF-κB-DNA binding and transcription activity in response to
the endogenous inflammatory mediator TNFα. Increased activity is associated
not only with decreased IκB expression, but also increased signaling for IκBα
degradation and delayed resynthesis of IκBα which collectively contribute to the
exaggerated inflammatory response due to insufficient inhibition of the NF-κB
pathway in immature enterocytes.

Materials and Methods

Cell Culture

H4 cells are a human fetal non-transformed primary intestinal epithelial cell line
used as a model of immature intestinal epithelial cells (21). They were cultured in
DMEM with 10% heat-inactivated fetal calf serum, 1% glutamine, 1% sodium
pyruvate, 1% amino acids, 1% HEPES, penicillin 50 units/ml, streptomycin 50
μg/ml and insulin 0.2 units/ml. Cell passages 11-21 were used. T84 cells
originate from adult intestinal epithelial cells. T84 cells were grown in a 1:1
(vol/vol) mixture of Dulbecco’s Modified Eagle Medium (DMEM) and F12 Medium
(Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal calf serum, 1%
glutamine, penicillin 50 units/ml, and streptomycin 50 μg/ml. Cell passages 50-64
were used. All cells were grown at 37°C in a 5% CO₂ atmosphere.
Transfection and Luciferase Reporter Assay

Transient transfection was accomplished using Lipofectamine 2000 following the manufacturer’s instructions (Life Technologies, Rockville, MD)(7). Briefly, cells were grown to 75% confluence in antibiotic-free medium, and incubated with Lipofectamine 2000 reagent plus an NF-κB promoter-dependent firefly luciferase reporter construct and a constitutively-expressed Renilla luciferase reporter (Promega, Madison, WI) as an internal control for transfection efficiency. After 40 hours, cells were stimulated with TNFα 10ng/ml for 6 hours. Cells were then lysed and the Dual-Luciferase Reporter Assay (Promega, Madison, WI) carried out following the manufacturer’s recommendations. The luminescence generated by the firefly luciferase was measured first, and then quenched, and a second measurement obtained to determine Renilla luminescence. Results are expressed as firefly luminescence normalized to Renilla luminescence.

Electrophoretic Mobility Shift Assay (EMSA):

Nuclear extracts were obtained utilizing a method modified from Inan et al (14). Briefly cells were treated with TNFα 10ng/ml at 0, 5, 15, 30, 90, and 120 minutes. The cells were washed and harvested in Tris buffered saline, and then incubated 15 minutes in extract lysis buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 2mM MgCl₂, 0.5mM sucrose, 0.05% Nonidet P-40 (NP-40), 0.5mM PMSF, 1mM DTT, 1x Complete protease inhibitor (Roche, Indianapolis, IN). The cytosolic fraction was then removed and discarded. Nucleus-containing pellets were rinsed with
extract lysis buffer without NP-40 and incubated for 40 minutes in 25µl high salt buffer (20mM Hepes pH 7.4, 1.5mM MgCl₂, 420mM NaCl, 0.2mM EDTA, 5%glycerol, 1mM DTT, 0.5mM PMSF, 1x Complete protease inhibitor (Roche, Indianapolis, IN)). The nuclear extract was removed and combined with 38µl low salt buffer (20mM Hepes pH 7.4, 50mM KCl, 0.2mM EDTA, 20% glycerol, 1mM DTT, 0.5mM PMSF, 1x Complete protease inhibitor (Roche, Indianapolis, IN)). Protein concentrations were then determined by Bradford method.

At room temperature, nuclear extracts (5-10µg) were incubated for 15 minutes with 5mM Tris pH 7.5, 0.5mM EDTA, 2% ficoll, 0.5mM DTT, 37.5mM KCl, 1µg poly (dI-dC) (Roche, Indianapolis, IN), and 50,000cpm of a ³²P-labeled probe encoding the NF-κB consensus sequence (5′—AGTTGAGGGGACTTTCCCAGGC—3′) (Promega, Madison, WI). To determine oligonucleotide specificity, a 100-fold excess cold oligonucleotide was added to the reaction mixture prior to the 15 min incubation (data not shown). In supershift reactions, antibodies to NF-κB p50, NF-κB p65, NF-κB p52, NF-κB cRel, or NF-κB RelB (Santa Cruz, Santa Cruz, CA) were pre-incubated with the nuclear extract and reaction buffer in the absence of probe for 30 min. The probe was then added and samples were incubated for an additional 15 min prior to electrophoresis on a native 5% polyacrylamide gel. The gel was then dried and exposed to film.

**Immunoprecipitation**
To concentrate TNF receptor levels, T84 and H4 cells were grown to confluence in 60mm culture dishes then lysed with 500µl cold immunoprecipitation (IP) buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0). Protein concentration of the lysate supernatant was measured by Bio-Rad DC Protein Assay method (Bio-Rad, Hercules, CA). Immediately before immunoprecipitation, lysates were precleared with PANSORBIN (Calbiochem, San Diego, CA). Equal volume cell lysates were incubated with TNF receptor antibody with agitation then Protein A gel (Pierce, Rockford, IL) was added to the antigen-antibody complex. The resulting pellet was washed several times in IP buffer then resuspended in 0.1M glycine pH 2.5. Samples were then centrifuged and 1M Tris pH8.0 added to neutralize the pH of the supernatant. 4x sample buffer was added to each sample and boiled prior to immunoblotting again with TNF receptor antibody, as described below. To confirm equal protein loading, total cell lysates samples were taken after preclearing but before immunoprecipitation and loaded on the same gel with the IP samples then probed with Hsc 73 antibody in addition to TNF receptor 1 and 2 antibodies.

**Immunoblotting**

Following treatments as indicated, cells were washed in ice-cold phosphate-buffered saline (PBS) and then lysed in 1% Triton X-100 in 10mM Tris pH 8, 150mM NaCl, with 10 µg/ml of aprotinin and leupeptin, and 2 mM phenylmethylsulfonylfluoride. After estimating the protein concentrations of the cleared lysates using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) as per the manufacturer’s protocol, equal amounts of total protein were separated
by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels. Electrophoresed proteins were transferred from the gel to a nitrocellulose membrane using a semi-dry transfer apparatus as described previously(1). The blot was blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20, incubated with the appropriate primary antibody, and then with the appropriate HRP-conjugated secondary antibody. The blot was developed using the West Pico Super Signal chemiluminescence reagent (Pierce, Rockford, Illinois). The membranes were then stripped and re-probed with an antibody to glyceraldehyde phosphate dehydrogenase (GAPDH), (Research Diagnostics Institute, Flanders, NJ) or heat shock cognate 73 (Stressgen, San Diego, CA) to confirm equal loading of lanes.

Pre-weaned/Post-weaned mouse intestinal tissue

Rag 1 -/- knockout mouse strain 002216 (Jackson Laboratory, Bar Harbour, ME) which does not develop mature T cells or B cells was used to allow investigation of inflammatory responses specifically by intestinal epithelial cells, without contamination of inflammatory cells in tissue samples. Responses in immature intestine, or preweaned pups 12-17 days old were compared to responses in post-weaned mice 5-8 weeks old. Animals were individually anesthetized with sevofluorane by inhalation in a bell jar in a fume hood, then weighed prior to injection with recombinant murine TNF alpha (PeproTech, Rocky Hill, NJ) 0.4mg/kg IP. Control animals were injected with sterile PBS alone. At the end of
the specified time, as noted in results, mice were euthanized and full length intestine was removed and fixed in 10% Formalin prior to paraffin imbedding. Protocol was approved by the University of Chicago Animal Care committee.

**Immunofluorescence staining**

Staining for NF-κBp65 was performed on paraffin-embedded sections (4 µm) of mouse small intestines from rag 1 -/- mice treated with TNFα as outlined above. Paraffin sections were baked in an oven at 56 °C then the slides were deparaffinized in xylene, and rehydrated by graded ethanol washes at room temperature. Antigen retrieval was achieved by boiling tissue sections in 10mM Sodium citrate buffer (pH 6.0) for 20 minutes. Slides were then incubated in Peroxidase block solution (Dako, Carpinteria, CA) for 20 minutes at room temperature to block endogenous peroxidase activity. Samples were then permeabilized in 0.1% TritonX-100 in PBS for 20 minutes at room temperature followed by incubation for 20 minutes in 5% BSA in PBS to reduce non-specific background. The slides were incubated with the primary antibody rabbit polyclonal p65 (Santa Cruz, Santa Cruz, CA) diluted 1:50 in 1% BSA in PBS followed by incubation in the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG(H+L)(Invitrogen, Carlsbad, CA) diluted 1:200 in 1%BSA. Nuclei were counterstained with DAPI (D21490) diluted 1: 10000 in 1%BSA. Slow Fade equilibration buffer (Invitrogen, Carlsbad, CA) was added prior to mounting. Slides were kept in the dark to avoid bleaching prior to visualization using a Leica SP2 AOBS spectral confocal microscope.
Immunohistochemistry

Staining for phospho-I\(\kappa\)B\(\alpha\) was performed on paraffin-embedded sections (4 \(\mu m\)) of mouse small intestines from Rag 1 \(^{-/-}\) mice treated with TNF\(\alpha\) as outlined above. Paraffin sections were baked in an oven at 56 \(^{\circ}\)C then the slides were deparaffinized in xylene, and rehydrated by graded ethanol washes at room temperature. Antigen retrieval was achieved by boiling tissue sections in 10mM Sodium citrate buffer (pH 6.0) for 20 minutes. Slides were then incubated in Peroxidase block (Dako, Carpinteria, CA) for 20 minutes at room temperature to block endogenous peroxidase activity, followed by incubation for 20 minutes in 5% powdered milk in PBS to reduce non-specific background. The slides were incubated with the primary antibody mouse monoclonal phospho-I\(\kappa\)B\(\alpha\) diluted 1:150 in 1% BSA for 1 hour at room temperature followed by the secondary antibody HRP anti-Mouse (Dako, Carpinteria, CA) for 30 minutes at room temperature. Antibody staining was visualized with DAB chromogen (Dako, Carpinteria, CA, 20\(\mu l\) in 1000\(\mu l\) DAB buffer) for 15 minutes at RT, and counterstained with hematoxylin.

Reagents

TNF Receptor 1 and 2 antibodies were obtained from Abcam (Cambridge, MA). I\(\kappa\)B\(\alpha\) antibody was obtained from Santa Cruz (Santa Cruz, CA) and Hsc 73 antibody was obtained from Stressgen (San Diego, CA). IKK, pIKK\(\beta/\alpha\), and pI\(\kappa\)B\(\alpha\) antibodies were obtained from Cell Signaling (Danvers, MA). GAPDH
antibody was obtained from Research Diagnostics Institute, Flanders, NJ.
Ubiquitin antibody and MG262 were obtained from Active Motif (Carlsbad, CA).
Murine TNFα was obtained from PeproTech (Rocky Hill, NJ).

Statistical analysis

Results are presented as mean values ± S.E.M. Statistical significance was determined using the t test. $P < 0.05$ was considered statistically significant.
Results

Receptor Expression

To determine if differences in cytokine production between immature and adult intestinal epithelial cells could merely be explained by differences in receptor expression, TNFα receptor expression was compared in the adult human intestinal epithelial cell line T84 and the immature human intestinal epithelial cell line H4 by immunoprecipitation and immunoblotting using specific antibodies. TNFα signaling is mediated by binding of 2 receptors, TNFR1 (50kD) and TNFR2 (75kD). TNFR1 is believed to be the major signaling receptor, leading to activation of NF-κB(25). As shown in figure 1, receptor expression for both TNFR1 and TNFR2 was comparable for the 2 cell lines. Thus, a difference in cytokine production cannot be explained by a difference in receptor expression leading to enhanced signaling.

Immature enterocytes have increased NF-κB transcriptional activity compared to mature enterocytes.

TNFα can induce cytokine production via activation of the NF-κB pathway. To determine if the previously described increased inflammatory cytokine production in immature IEC(5, 6) was associated with increased NF-κB activity, H4 and T84 cells were transfected with an NF-κB promoter-driven firefly luciferase reporter construct, along with a Renilla luciferase reporter as an internal control prior to treatment with TNFα. As shown in figure 2, the immature IEC line H4 had significantly greater firefly luciferase activity of 109 ± 8 luminescence units.
compared to the adult cells which had 35 ± 3 luminescence units given the same inflammatory stimulus (p < 0.05), indicating increased transcription of the NF-κB promoter-dependent reporter. Thus, increased cytokine production in immature IEC is associated with increased NF-κB transcriptional activity.

Immature enterocytes have increased NF-κB binding compared to mature enterocytes

To determine the point of developmentally regulated differential responsiveness between immature and mature enterocytes to inflammatory stimuli, we explored signaling associated with steps upstream of NF-κB activity, beginning with NF-κB binding to DNA. Cells were treated with TNFα for 0, 5, 15, 30, 90, and 120 minutes and nuclear extracts obtained. NF-κB binding was determined by electrophoretic mobility shift assay (EMSA) as described in Methods. As shown in figure 3, NF-κB binding was more prominent and prolonged in the immature H4 cell line with binding beginning at 5 minutes and significant binding present through 120 minutes. In the T84 cell line there is less binding which is not as prolonged. Supershift reactions showed that these bands were primarily p50/p65 heterodimers (data not shown). There was no difference in baseline expression for the most common isoforms of NF-κB p65, p50, p52 (data not shown). Thus increased NF-κB activity in immature enterocytes is associated with increased NF-κB binding compared to mature enterocytes.
Immature enterocytes have more rapid IκBα degradation than mature enterocytes in response to TNFα.

NF-κB is a transcription factor retained in the cytosol by the inhibitor protein IκB. Signal-induced degradation of IκB allows NF-κB to move into the nucleus, bind to DNA and activate the transcription of a variety of genes involved in inflammation and innate immunity. To determine if differential regulation of NF-κB activity between immature and mature intestinal epithelial cells is associated with differences in inhibition thus leading to the increased availability of nuclear NF-κB for DNA binding, IκBα degradation was examined. H4 and T84 cells were incubated with TNFα for 0, 5, 15, 30, 60 or 90 minutes. IκBα levels were measured by western blot as described above, using an anti-IκBα antibody. Blots were then stripped and incubated with a GAPDH antibody to confirm equal protein loading. As shown in figure 4, consistent with previous observations immature H4 cells had less baseline expression of IκBα than the T84 adult cells (5). Furthermore, while T84 cells had evidence of decreased IκBα levels at 15 minutes, and return to baseline levels by 60 minutes, in H4 cells, IκBα decrease was evident by 5 minutes, was more complete and there was a delay in return to baseline levels until 90 minutes. Decreased levels of IκBα due to lower baseline levels coupled with increased IκBα degradation and delayed return to baseline levels may correspond to increased NF-κB release and thus enhanced activation of the transcription of inflammatory cytokines in immature cells.
Immature intestinal epithelial cells have increased IκBα phosphorylation and ubiquitination compared to adult IEC.

IκBα phosphorylation and subsequent ubiquitination targets it for degradation by the proteasome. To determine the mechanism responsible for the TNFα induced decreases in IκBα in immature intestinal epithelial cells, we examined known steps upstream of IκBα degradation in the NF-κB pathway. Cells were treated with the proteasome inhibitor MG262 (1.5 μM) for 30 minutes prior to treatment with TNFα at different time points as described above. MG262 preserves the phosphorylated and ubiquitinated forms of IκBα but concomitantly diminishes visible differences between individual time points. Cell lysates were then obtained and evaluated by immunoblotting. As shown in the top panel of figure 5, utilizing a specific phospho IκBα antibody, H4 cells have increased IκBα phosphorylation compared to T84 cells. Similarly, H4 cells have increased IκBα ubiquitination compared to T84 cells (figure 5 middle panel). Utilizing an IκBα antibody which due to MG262 proteasome inhibition can now detect ubiquitinated forms of IκBα, the higher molecular weight bands of polyubiquitin not usually visible for regular IκBα immunoblotting can now be seen. Immunoprecipitation studies with both IκBα and ubiquitin antibodies confirmed that these higher molecular bands are ubiquitin (data not shown). An increase in both IκBα phosphorylation and ubiquitination corresponds to enhanced IκBα degradation as the explanation for TNFα induced decreases in IκBα.
Immature intestinal epithelial cells have increased IKKβ expression and phosphorylation compared to adult IEC

IKK is responsible for phosphorylating IκBα. IKK is composed of 3 subunits - IKKα, IKKβ, and IKKγ (NEMO). Only IKKβ deficient mice are defective in activation of IKK and NF-κB in response to TNFα and IL-1β suggesting that IKKβ plays the major role in IKK activation and induction of NF-κB activity (16). Cells were again treated with TNFα at different time points as described above, then cell lysates evaluated for IKKβ and phosphoIKKβ. The phospho IKK antibody used was for both pIKKα and pIKKβ. As demonstrated in figure 6 top panel, phosphoIKKβ staining, visible as the upper bands on the immunoblot, was much stronger in the immature H4 cells than in the adult T84 cells. In immature cells phosphoIKKβ increases by 5 minutes and then begins to diminish by 60 minutes and returns to baseline levels at 90 minutes. IKK phosphorylation is the signal for IκBα phosphorylation and degradation. This phosphoIKKβ time course corresponds to the demonstrated time course of IκBα degradation in H4 cells which begins at 5 minutes and returns to baseline by 90 minutes (Figure 4). PhosphoIKKα bands, visible as the lower band of the immunoblot were also greater in the H4 cells, but are not thought to play as important a role in NF-κB activation. In addition, as shown in Figure 6 middle panel, there was increased baseline expression of IKKβ in addition to increased IKKβ phosphorylation in the immature H4 cells, potentially leading to the increased IκBα phosphorylation and downstream degradation of IκBα.
Immature intestinal epithelial cells have delayed IκBα resynthesis

In addition to activating the transcription of inflammatory cytokines such as IL-8, NF-κB binding sites are also located on the IκBα promoter. Thus an elegant auto-regulatory feedback loop is supposed to exist in which NF-κB activation leads to IκBα synthesis which can subsequently terminate the NF-κB response (2, 23). The delay in return to baseline levels of IκBα previously described could be due to delayed resynthesis of IκBα following degradation or to prolonged signaling for degradation of the newly synthesized IκBα. IκBα is degraded by the proteasome, thus to distinguish between these possibilities, cells were treated with the proteasome inhibitor MG262 15 minutes after treatment with TNFα beginning with the 30 minute time point of the time course as described above. This allowed initial signaling to be transmitted via the NF-κB pathway to induce IκBα resynthesis to occur, yet block subsequent IκBα degradation. As shown in figure 7, even with inhibition of ongoing IκBα degradation in this fashion, delay in return of IκBα persists, suggesting that the delay in return is not only due to prolonged signaling leading to degradation but also to delayed resynthesis.

Pre-weaned mice have increased p65 translocation compared to post-weaned mice

To confirm that the phenomenon of increased NF-κB signaling was not merely a difference between the cell lines used, an in vivo model of immature and mature intestinal epithelial cells was used. Pre-weaned (12-17do) mice were
used as a model of immature intestine and compared to post-weaned (5-8wo) mice.

Mice were injected with TNFα 0.4mg/kg IP or sterile PBS as control. Small intestinal samples were harvested at time points ranging from 0-4.5 hours. NF-κB activation begins with translocation of NF-κB proteins from the cytoplasm to the nucleus. The NF-κB protein p65 was visualized by immunofluorescence microscopy using rabbit polyclonal anti p65 and Alexa Fluor 488 goat anti-rabbit IgG(H+L). Using NIH Image J v1.37 software color overlay, it is visible as green staining in figure 8. Nuclei were counterstained using DAPI and are visible as red staining in figure 8. Nuclear p65 appears as an overlap of red and green staining becoming yellow with increased green/p65 confluence. As shown in Figure 8 post-weaned mice had nuclear p65 translocation beginning approximately 3 hours after TNFα injection and fading by 4-4.5 hours. In contrast pre-weaned animals had evidence of much earlier nuclear p65 translocation at 1.5 hours which persisted for several hours until beginning to fade at 4 hours consistent with cell culture findings of increased NF-κB activation in immature enterocytes.

**Pre-weaned mice have increased IκBα phosphorylation compared to post-weaned mice**

To confirm that the phenomenon of increased IκBα degradation in immature enterocytes was not merely a difference between the cell lines used, intestinal samples from pre-weaned and post-weaned mice treated with TNFα as described were used for immunohistochemistry staining for phosphorylated IκBα.
As described previously, IκBα phosphorylation is the signal for subsequent IκBα ubiquitination and degradation. As shown in Figure 9, post-weaned mice had phospho IκBα staining beginning at 3hrs while pre-weaned mice had much earlier evidence of phospho IκBα staining beginning at 1hr post TNFα injection. This timing correlates well with the timing demonstrated for nuclear p65 translocation in these animals and is consistent with cell culture findings of more rapid IκBα degradation in immature enterocytes.

**Discussion:**

The NF-κB pathway is a key signaling pathway for immune and inflammatory responses. Tight regulation is supposed to ensure appropriate host defense mechanisms against pathogenic organisms but tolerance of beneficial commensal organisms. The findings reported here demonstrate insufficient inhibition of the NF-κB pathway in immature enterocytes, associated with accelerated signaling for degradation of as well as delayed resynthesis of IκBα, the primary inhibitor of NF-κB. Coupled with the decreased baseline expression of IκBα in immature enterocytes, our data suggest inadequate downregulation of inflammatory responses of the immature intestine corresponding to exaggerated IL-8 secretion in response to endogenous and exogenous inflammatory mediators as previously reported (5, 6).

Our data demonstrate increased levels of both IKKa and β. Although both isoforms can phosphorylate IκBα in vitro, they appear to have distinct functions.
IKKα itself has been shown to be important in development as IKKα knockout animals have abnormalities of skin and limb bud development(24). However these animals do not seem to have abnormalities of NF-κB activation. In contrast IKKβ deficient mice die at mid-gestation from extensive liver apoptosis very similar to what is seen in animals deficient in the NF-κB protein p65(16). It is the IKKβ isoform which appears to play the major role in IKK activation and induction of NF-κB activity. Thus our finding in immature intestinal epithelial cells of increased IKKβ base line with more prolonged phosphorylation in response to TNFα is consistent with our findings of prolonged IκBα phosphorylation and increased IκBα degradation which may explain the demonstrated increased NF-κB activity.

It is interesting that our data suggest that there may be delayed synthesis IκBα despite increased NF-κB activity in immature enterocytes. Studies in other cell lines have indicated specific NF-κB activity on individual promoters and different synthesis kinetics for NF-κB induced transcription of IκBα compared to inflammatory cytokines such as IL-6 and IL-8(12, 20). Studies in macrophages have suggested that there is an elegant sequence of nuclear events regulating timing and duration of NF-κB binding to specific gene promoters leading to different waves of gene expression. Modification of the histone proteins associated with DNA is important in the regulation of gene expression. Histone-DNA contacts in individual nucleosomes and the folding of the nucleosomal chain limit the accessibility of specific genes. Rapid recruitment of NF-κB to a subset
of target genes with constitutively heavily acetylated or rapidly phosphorylated promoters has been demonstrated (19). This group includes IκBα. In contrast, other target genes are not immediately accessible to NF-κB until 90-120 minutes after nuclear entry and require stimulus dependent hyperacetylation or phosphorylation of the promoter to makes them accessible to NF-κB (20). This includes inflammatory cytokines such as IL-6 and IL-8. Our data suggest the possibility that specific binding kinetics for different promoters may be developmentally regulated with increased transcription of inflammatory cytokines but decreased transcription of regulatory factors in immature enterocytes. Although beyond the scope of the current study, future studies are planned to investigate developmental nuclear regulation of NF-κB in intestinal cells, which may account for differences in inflammatory and regulatory gene transcription in immature vs. mature enterocytes.

In addition, degradation of IκBα occurs in the 26S proteasome. It is known that this highly conserved entity is itself regulated. Others have demonstrated that the proteasome, which is comprised of multiple subunits can change its degradation specificity based on variations in activation of three of these subunits in response to modifications such as phosphorylation or glycosylation or in response to factors such as interferon gamma (10, 15). It is conceivable that the signaling needs of fetal development warrant different degradation patterns by the proteasome of immature cells compared to that of mature cells. There may thus be developmental regulation of the proteasome itself. In addition to increased
phosphorylation and ubiquitination of IκBα resulting in increased degradation, the proteasome itself may contribute to enhanced degradation. Our data demonstrate that the proteasome inhibitor MG262 did not lead to normalization of the return of IκBα after degradation, suggesting a delay in synthesis. An alternative possibility is that the immature proteasome responds differently to this inhibitor than the mature proteasome, allowing degradation of IκBα to continue. Proteasome activity was not evaluated in this study, but is another potential point of developmental regulation.

Our studies utilized a cell culture model to systematically compare signaling events along the NF-κB pathway in immature and mature enterocytes. The comparison of H4 cells to the well established adult IEC lines T84, Caco2, and HT29 has been used and validated in many other studies as a model of immature and mature IEC. Comparison of immature enterocytes (H4 cells) and mature enterocytes (confluent Caco2 cells) treated with lipopolysaccharide (LPS) or IL-1β revealed that the immature enterocytes secreted more IL-8 (LPS, 8-fold; IL-1β 20-fold) than mature enterocytes with a comparable increase in IL-8 mRNA in the immature enterocytes in response to both stimuli(18). These results were then confirmed in studies using small intestinal organ culture from fetuses (18-21wks gestational age) compared to small intestinal biopsy samples from older infants and children. IL-8 secretion, along with IL-8 mRNA, was again increased in fetuses compared to older infants/children (LPS 2.5 fold, IL-1β 200 fold)(18). Another study compared the response to cholera toxin in fetal H4 cells compared
to adult T84 cells and documented a greater induction of cAMP in response to cholera toxin in the fetal cells. Likewise, organ culture of fetal intestine had a greater induction of cAMP than did biopsies from older infants/children (17). Thus, findings in this cell culture system have been repeatedly validated by use of primary intestinal tissue in organ culture, thus justifying the use of the H4/adult enterocyte model utilized in our studies. However there are inherent problems with a cell culture system including the possibility that the differences observed are unique to the utilized cell lines rather than representative of biologic systems, as well as the limitation that interactions with other cell types cannot be studied. Thus NF-κB signaling was also investigated in an in vivo mouse model of immature and mature enterocytes. These studies confirmed increased phosphoIkBα and p65 translocation in pre-weaned mouse immature intestinal epithelium. Intestine from pre-weaned rodents less than 3 weeks of age is considered to be developmentally comparable to third trimester human fetal intestine. After weaning at 3 weeks of life, rat intestine undergoes developmental “closure,” no longer allowing passage of macromolecules(4). Post-weaned rat intestine is thus considered developmentally mature. Comparisons between pre-weaned and post-weaned rat intestine have been used as a model to approximate differences in immature and mature human intestine in other studies such as those investigating developmental responses to cholera toxin(22).

Thus, these studies demonstrate that the cell culture findings have physiological relevance and scientific merit. As the in vitro and in vivo responses are identical, the more reductionist approach with cell cultures is highly appropriate and
allowed development of important mechanistic insights into the NF-κB signaling in the immature intestinal epithelium. In combination, these findings suggest that immature intestinal epithelial cells do have inadequate inhibition of the NF-κB pathway leading to earlier and prolonged activation of NF-κB.

These studies have focused specifically on intestinal epithelial cells. Other immune cells in the gastrointestinal tract can also produce inflammatory cytokines which may contribute to the intestinal damage and it is possible that lymphoid elements may have a role in facilitating the epithelial response. Future studies to investigate the interaction between immune and epithelial cells will further enhance our understanding of the development of gut inflammatory responses.

Necrotizing enterocolitis is an inflammatory bowel disease seen only in premature infants, and thought to be a consequence of inappropriate inflammatory responses to microbial interaction by the immature intestine. Studies in a rat model of NEC demonstrated increased NF-κB binding activity in the small intestine of animals 0-3 hours after the induction of NEC, potentially suggesting that NF-κB activation is an early step in the intestinal injury (3). Our data demonstrate several points of developmental difference - increased NF-κB activity, increased NF-κB DNA binding, accelerated IκBα phosphorylation, ubiquitination and degradation; increased IKKβ expression and phosphorylation
along with decreased IκBα resynthesis. We speculate that failure to terminate NF-κB activation in immature intestinal epithelial cells may partially explain the unique susceptibility of preterm infants to NEC.

The preterm intestine is essentially a fetal intestine not expecting the conditions of the extra-uterine environment and not having completed the growth and maturation processes which normally continue until term. The NF-κB pathway activates transcription of many other genes in addition to inflammatory cytokines. Perhaps the immature intestine, expecting the sterile environment of the intra-uterine environment, has increased NF-κB signaling not for an inflammatory response but rather for other effects important for fetal development.

Further understanding of the intestinal NF-κB pathway and the intricacies of NF-κB binding to promoters of specific genes has implications for necrotizing enterocolitis and other inflammatory bowel diseases. It may be an important developmental step for the immature intestine to down-regulate inflammatory responses as it transitions from the sterile intra-uterine environment to obtaining the microbial colonization of the extra-uterine world. It is unclear whether this maturation is the result of a normal genetic pattern, the effect of factors in amniotic fluid or breast milk, or triggered by contact with the microflora itself. Ongoing investigation into the mechanisms responsible for developmental downregulation of NF-κB signaling is a logical next step.
As many of the long-term morbidities of prematurity are inflammatory in nature including chronic lung disease and periventricular leukomalacia in addition to NEC, understanding the signaling leading to exaggerated inflammatory responses will advance understanding of the developmental disease susceptibility of the premature infant. Therapy designed to not only limit disease progression, but also hasten normal maturation of the intestine and modulate exaggerated inflammatory responses is important to alleviate not only the initial intestinal injury but the long term morbidity associated with NEC(11).
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References:


Figure Legends

Figure 1: Equivalent TNF receptor expression in immature and mature enterocytes

Cell lysates were obtained from the human adult IEC cell line T84 or the human fetal IEC cell line H4. The expression of TNF receptor 1 and 2 were revealed by immunoprecipitation and immunoblotting with specific antibodies with heat shock cognate 73 used as loading control from total cell lysates samples obtained after clearing but before immunoprecipitation. NIH Image J software was used for densitometry to determine relative TNF receptor band intensity. Values are presented below each band as fold difference compared to T84 cells. T84 band intensity was arbitrarily set to 1. The results shown are representative of 3 separate experiments.

Figure 2: Immature enterocytes have increased NF-κB transcriptional activity baseline and when stimulated by TNFα compared to adult enterocytes

Adult T84 IEC and Immature H4 IEC were transfected with an NF-κB promoter driven firefly luciferase construct along with a Renilla luciferase reporter as an internal control for transfection efficiency. Cells were then treated for 6 hours with TNFα 10ng/ml and luminescence measured. Results are presented as mean ±
S.E.M. firefly luminescence normalized to *Renilla* luminescence, n=4. Asterisks denote significance at p<0.05 for H4 compared to T84 cells.

**Figure 3: Immature enterocytes have increased and prolonged NF-κB binding when stimulated by TNFα compared to adult enterocytes**

A. Adult T84 IEC and immature H4 IEC were stimulated with TNFα 10ng/ml over time as indicated. Nuclear extracts were obtained and EMSA performed using a $\gamma^{32P}$ labeled NF-κB consensus sequence probe. B. NIH Image J software was used for densitometry to quantify NF-κB binding. Data is presented as fold change compared to T84 cells at time 0 (set at 1.0). The results shown are representative of 4 separate experiments with similar results.

**Figure 4: Stimulated immature enterocytes have more rapid IκBα decrease and delayed return compared to mature enterocytes**

T84 cells and H4 cells were treated with TNFα 10ng/ml over time as indicated. Cell lysates were obtained and the expression of IκBα revealed by western blot with GAPDH used as a control for equal protein loading. Immature H4 cells had lower baseline levels of IκBα, more rapid decrease of IκBα and delayed return of IκBα protein levels following stimulus. Results shown are representative of n>4 separate experiments with similar results.
Figure 5: Stimulated immature enterocytes have increased IκBα phosphorylation and ubiquitination compared to mature enterocytes

T84 and H4 cells were pretreated with the proteasome inhibitor MG262 for 30 minutes prior to treatment with TNFα 10ng/ml over time as indicated. Cell lysates were obtained and the expression of pIκBα and ubiquitinated IκBα and revealed by western blot. GAPDH was used as a control for equal protein loading. Top panel demonstrates increased phosphorylated IκBα in response to TNFα in immature H4 cells compared to adult T84 cells. Middle panel demonstrates more polyubiquitin bands above IκBα in the H4 cells. Results shown are representative of 4 separate experiments with similar results.

Figure 6: Stimulated immature enterocytes have increased IKKβ expression and phosphorylation compared to mature enterocytes

T84 and H4 cells were treated with TNFα 10ng/ml over time as indicated. Cell lysates were obtained and the expression of phosphoIKKβ (top panel) and IKKβ (middle panel upper band) revealed by western blot. Hsc 73 was used as a control for equal protein loading. Results shown are representative of 4 separate experiments with similar results.

Figure 7: Inhibition of IκBα degradation by MG262 does not lead to more rapid return of IκBα after stimulation by TNFα in immature enterocytes

T84 and H4 cells were treated with TNFα 10ng/ml over time as indicated with the proteasome inhibitor MG262 added 15 minutes after TNFα addition beginning
with the 30 minute time point as indicated. This was done to block ongoing IκBα degradation but allow signaling for IκBα resynthesis. Cell lysates were obtained and the expression of IκBα revealed by western blot. GAPDH was used as a control for equal protein loading. Results shown are representative of 4 separate experiments with similar results.

**Figure 8: TNFα induces earlier and more prolonged nuclear translocation of NF-κB protein p65 in pre-weaned mice compared to post-weaned mice.** Pre-weaned mice (12-17 do) and post-weaned mice (5-8 weeks old) were treated with TNFα 0.4 mg/kg IP. After sacrifice at times post-injection as indicated, intestinal samples were harvested, and sections obtained for immunofluorescence staining with the primary antibody rabbit polyclonal p65 conjugated to the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG(H+L). Nuclei were counterstained with DAPI. Slides were visualized using a Leica SP2 AOBS spectral confocal microscope, with color overlay using NIH Image J software. To facilitate interpretation, images were pseudo-colored using NIH Image J software. Green indicates p65 staining, the blue color of DAPI was changed to red for nuclear staining, and as indicated by arrows, nuclear translocation is visible as overlapping green/red staining appearing yellow/orange with increasing intensity. As shown, post-weaned animals have overlapping green/red staining indicating p65 nuclear translocation beginning at 3 hrs, while pre-weaned animals had overlapping green/red staining of increased intensity appearing yellow beginning
at 1.5hrs and persisting for several hours. Results shown are representative of 3 separate experiments all with similar results.

**Figure 9: TNFα induces earlier and more prolonged phosphorylation of IκBα in pre-weaned mice compared to post-weaned mice.** Pre-weaned mice (12-17do) and post-weaned mice (5-8weeks old) were treated with TNFα 0.4mg/kg IP. After sacrifice at times post-injection as indicated, intestinal samples were harvested, and sections obtained for immunohistochemistry staining with a mouse monoclonal phospho-IκBα antibody. Antibody staining was visualized with DAB chromogen and counterstained with hematoxylin. As shown and indicated by arrows, post-weaned animals have evidence of phospho IκBα staining beginning at 3hrs, while pre-weaned animals had evidence of staining beginning at 1hr and persisting for several hours. Results shown are representative of 3 separate experiments all with similar results.
A.

T84 cells

H4 cells

p50/p65

TNFα

Time in minutes

B.

Fold Change

T84 cells

H4 cells

Time in Minutes
T84 cells | H4 cells

\[ \text{I} \kappa \text{B} \alpha \ 37kD \]
\[ \text{GADPH} \ 36kD \]

Time in minutes

\[ \text{TNF} \alpha 0 \ 5 \ 15 \ 30 \ 60 \ 90 \]
\[ 0 \ 5 \ 15 \ 30 \ 60 \ 90 \]
Post-weaned mouse

Pre-weaned mouse