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Commensal *Escherichia coli* reduces Epithelial Apoptosis Through Interferon Alpha-A Mediated Induction of Guanylate Binding Protein-1 in Human and Murine Models of Developing Intestine1

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Summary

Appropriate microbial colonization protects the developing intestine by promoting epithelial barrier function and fostering mucosal tolerance to luminal bacteria. Commensal flora mediate their protective effects through TLR9-dependent activation of cytokines such as type I IFNs (α,β) and IL-10. While IFNβ promotes apoptosis, IFNα activates specific anti-apoptotic target genes whose actions preserve epithelial barrier integrity. We have recently identified guanylate binding protein-1 (GBP-1) as an anti-apoptotic protein, regulated by both type I and type II IFNs, that promotes intestinal epithelial barrier integrity in mature intestine. However, the mechanisms by which commensal bacteria regulate epithelial apoptosis during colonization of immature intestine and the contributions of GBP-1 are unknown.

The healthy newborn intestine is initially colonized with bacterial species present in the maternal GI tract, including non-pathogenic *E. coli*. Therefore, we examined the influence of commensal *E. coli* on cytokine expression and candidate mediators of apoptosis in preweaned mice. Specifically, enteral exposure of 2 week-old mice to commensal *E. coli* for 24 hours selectively increased both IFNαA and GBP-1 mRNA expression and prevented staurosporine-induced epithelial apoptosis. Exogenous IFNαA treatment also induced GBP-1 expression and protected against staurosporine-induced apoptosis in a GBP-1 dependent manner, both *in vitro* and *ex vivo*. These findings identify a role for IFNαA-mediated GBP-1 expression in the prevention of intestinal epithelial apoptosis by commensal bacteria. Thus IFNαA mediates the beneficial effects of commensal bacteria and may be a promising therapeutic target to promote barrier integrity and prevent the inappropriate inflammatory responses seen in developing intestine as in Necrotizing Enterocolitis (NEC).

Keywords

interferon alpha; guanylate binding protein-1; apoptosis; cytokines; mucosa; mice; NEC

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Introduction

As the largest interface between the organism and the environment, the intestine interacts with a vast microflora, and appropriate colonization is critical for intestinal function and development (9). Bacterial colonization is a dynamic process with the intestine of the term newborn specifically set to foster tolerance of initial colonizing bacteria (10). Through recognition of complex bacterial patterns, the term intestine senses the presence of luminal bacteria and distinguishes between pathogenic and commensal organisms regulating tissue responses through a balance of inflammatory and apoptotic signaling pathways (9–14). Furthermore, maladaptive responses to colonizing organisms have been implicated in pathogenic inflammatory responses in both mature (15–17) and developing intestine (18).

Both the nature of colonizing flora and the timing of colonization are likely to be critical in the immature intestine of premature infants who are uniquely vulnerable to unchecked inflammatory responses, sometimes culminating in intestinal necrosis and death due to Necrotizing Enterocolitis (NEC). The onset of NEC is developmentally dependent with peak onset occurring at 30–32 weeks post conceptual age, regardless of gestational age at the time of birth (19). Therefore, it is likely that the elaboration of disease requires a developmental trigger or event. Furthermore, NEC does not occur *in utero* and appears to require bacterial colonization prior to the manifestation of disease. Growing evidence indicates that the immature human intestine is developmentally unprepared for bacterial colonization, elaborating an inappropriately exuberant response to proinflammatory stimuli (20). Additionally, preterm infants, subject to frequent antibiotic use, instrumentation, and the hospital environment, are vulnerable to colonization with abnormal flora relative to the term infant (21–23). Thus, the premature human intestine is predisposed to NEC by colonization with atypical flora at a time when it is developmentally unprepared to respond in an appropriate manner (1).

In experimental models of NEC, increased intestinal epithelial apoptosis precedes the progression to frank necrosis (24). Thus the regulation of apoptosis has been implicated as a critical event in the early evolution of NEC. These findings, in combination with the tendency of immature intestine to elaborate exaggerated responses to pro-inflammatory stimuli, have led to the hypothesis that epithelial apoptosis contributes to barrier compromise, leading to bacterial translocation and an exuberant inflammatory response which, when inappropriately regulated, leads to necrosis in the naive and immature intestine (1).

Among the first colonizing organisms evident in the term newborn human intestine are strains of *E. coli* derived from the maternal GI tract (9, 10, 14). Specific commensal strains of *E. coli* have been shown to reduce intestinal epithelial inflammatory signaling, *in vitro*, through repression of NFκB signaling (2). Although pathogenic, and more specifically enteroinvasive *E. coli* is known to be pro-apoptotic due to the elaboration of virulence factors activating caspase 1 (39), the effects of commensal strains on apoptosis remain to be determined.

Toll-like receptors (TLRs) are critical for the specific detection of microbe-associated patterns, allowing differentially regulated responses to commensal versus pathogenic flora (61). Independent activation of specific TLRs, such as TLR2 and TLR4, has been linked through down-stream activation of MyD88, to modulation of flora-dependent intestinal inflammation and immune responses (62). Additional evidence argues that beneficial effects of commensal and probiotic bacteria in the intestine are mediated at least in part by TLR9 activation, specifically through recognition of bacterial DNA (4). Furthermore, reciprocal TLR4 and TLR9 signaling are important in the developing intestine in that increased TLR4

and decreased TLR9 expression occur in human and murine NEC, and attenuation of TLR4 signaling both increases TLR9 expression and decreases disease severity in a model of NEC in 2 week-old mice (60). The protective effects of TLR9 signaling are mediated by the downstream induction of type I interferons (3) such as IFNαA. In these studies, we sought to examine the influence of a commensal strain of *E. coli* on IFNαA signaling and downstream effects on apoptosis in developing intestinal epithelium in a well-established murine model of immature intestine.

Several lines of evidence identify the large GTPase, guanylate binding-protein-1 (GBP-1) as a candidate mediator of IFN-mediated changes in apoptosis. The mouse GBP-1 promoter has been well characterized in embryonic fibroblasts as a target of both type I (α,β) and type II (γ) IFNs, which transcriptionally activate GBP-1 expression through Stat-independent activation of IRF-1 (41). Short-term IFNα-treatment of endothelial cells inhibits angiogenesis through GBP-1-mediated prevention of apoptosis (40). And recently, we have shown that GBP-1 promotes intestinal epithelial barrier integrity through the prevention of apoptosis in model intestinal epithelia, *in vitro* (5). Furthermore, GBP-1 is upregulated both in response to exogenous interferon gamma, *in vitro*, and in the colonic epithelia of patients with Inflammatory Bowel Disease (IBD) (5). The role of GBP-1 and its regulation by IFNαA in developing intestine has not been previously studied.

In the studies presented below, we optimized modeled immature mouse intestine to examine mechanisms regulating apoptosis during an interval where apoptosis could be most easily induced prior to or in the absence of associated inflammation. We then examined the influence of exposure to commensal *E. coli* on both inducible apoptosis and the expression of candidate mediators for its regulation. IFNαA and GBP-1 were specifically identified as molecules associated with the prevention of apoptosis, which were induced following exposure to commensal *E. coli* in immature mouse intestine. Findings in the murine model system were further extended to mature and immature human model epithelia, demonstrating that IFNαA exerted an anti-apoptotic effect that was specifically GBP-1 dependent, *in vitro*. Finally, we confirmed that exogenous IFNαA not only induced GBP-1 expression, but also prevented inducible apoptosis in an *ex vivo* murine model of developing intestine. These findings represent the first evidence of a role for GBP-1 in mediating the anti-apoptotic effects of type I IFNs induced by commensal flora in the developing intestine. Furthermore, prevention of apoptosis through induction of GBP-1 may provide a critical target for the promotion of epithelial barrier integrity and the prevention of bacterial translocation and the exaggerated mucosal inflammatory responses leading to NEC.

Materials and Methods

Cell culture

T84 colonic epithelial cells were grown as previously described (31). FHs 74 Intermediate (FHs 74 Int) fetal small intestinal epithelial cells were obtained from American Type Culture Collection (ATCC) and cultured in ATCC complete growth medium according to the provided protocol. Cells were grown directly on glass cover slips for TUNEL staining and activated Caspase-3 detection. T84 cells were grown on inserts and IFN-αA was applied basolaterally. FHs 74 Int cells were grown directly on 6-well plates

Bacterial culture

A strain of naturally-occurring commensal *E. coli*, which was isolated from human intestine during colonoscopy of healthy donors, was generously provided by Dr. Andrew Neish (Emory University) and prepared overnight in LB broth at 37°C, as previously described (2,

69). *E. coli* cultures were washed, concentrated in DMEM and gavage fed to two week-old mice at 1–2 × 10^7 CFUs/ml.

Animal care

All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Emory University. C57BL/6J mice were bred and maintained within the animal facility at Emory University.

Administration and real-time PCR quantitation of colonic levels of commensal E. coli

Two week-old mice were orally gavage fed 0.2 ml of DMEM with or without 1–2× 10^7 CFUs/ml commensal *E. coli*. Four hours after feeding, mice were sacrificed, colons were harvested, opened lengthwise and stool was removed and resuspended in sterile Hanks Balanced Salt Solution. The stool suspension was plated on blood agar plates and incubated for 37°C for 16 hours. Equal areas of bacterial colonies were recovered and resuspended in sterile Hanks Balanced Salt Solution. Bacterial DNA was recovered using a modification of the QIAmp DNA stool mini kit (Qiagen). Bacteria were lysed by incubation for 10 minutes at 70 degrees, then excess proteins were digested with proteinase K for 10 minutes at 56 degrees. Bacterial DNA was then isolated using QIAmp spin columns according to the manufacturer's protocol for washing and elution. Bacterial DNA concentrations were measured using a NanoDrop 1000 (Thermo Scientific).

E. coli-specific DNA was quantitated by real-time PCR using previously published primers specific for the *E. coli uid A* gene (67) (sense primer: 5′-AGC CAA AAG CCA GAC AGA GT-3′ and reverse primer: 5′-CAT GAC GAC CAA AGC CAG TA-3′) and normalized according to the total bacterial DNA in each sample as determined by PCR for universal 16s rDNA (sense primer: 5′-TCC TAC GGG AGG CAG CAG T-3′ and reverse primer: 5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′) using primers and conditions previously validated and published by Nadkarni *et al* (63)

Analysis of effects of *E. coli* or IFNαA on cytokine expression and apoptosis

For additional experiments, two week-old mice were gavage fed 0.2 ml of DMEM with or without 1–2× 10^7 CFUs/ml commensal *E. coli*, then returned to their mother for 24 hours. Mice were then anesthetized using CO_2 inhalation and euthanized by cervical dislocation. Whole colons were isolated and immediately frozen in TRIzol for RNA isolation or assayed, *ex vivo*, for vulnerability to apoptosis as described below.

For analysis of the influence of exogenous IFNαA on immature murine intestine, 2 week-old mice were weighed to establish a baseline weight of approximately 6 grams per mouse. Mice were injected with 40U of IFNαA per gram of baseline body weight by intraperitoneal injection. After 5 hours, the mice were anesthetized, euthanized and RNA isolation (6) and *ex vivo* analysis of staurosporine (STS)-induced apoptosis (7) were performed as previously published and briefly described below.

Transcriptional analysis

Excised mouse colons were homogenized in TRIzol (Invitrogen Life Technologies) then subjected to phenol-chloroform extraction according to the manufacturer's protocol and as previously described (25).

For analysis of the effects of IFNαA, *in vitro*, cultured T84 and FHs 74 Int cells (ATCC) were pretreated with 10 or 100 units/ml (U/ml) of IFNαA for 24 and 48 hours. Cells were then lysed in TRIzol, followed by phenol-chloroform extraction. RNA was digested with DNase I (Ambion) to remove contamination with genomic DNA, and then cDNA was

synthesized by reverse transcription using oligo(dT12-18) primers and superscript II reverse transcriptase (Invitrogen). Real time PCR was performed using a MyIQ real-time PCR machine and SYBR Green supermix (BioRad). Forward and reverse primers recognized different exons for each gene product with the exception of IFpNαA, which contains no introns. Primer sequences were asp follows: human SRP-14 sense, 5′-AGCACTGTGGTGAGCTCCAAG-3′ and antisense, 5′-TGAGCCCATCCATGTTAGCTCTA-3′; human GBP-1 sense, 5′-GGTCCAGTTGCTGAAAGAGC-3′ and antisense, 5′-TGACAGGAAGGCTCTGGTCT-3′; murine signal recognition particle (SRP)-14 sense, 5′-AAGTGTCTGTTGAGAGCCACGGAT-3′ and antisense 5′-CTGTCACTGTGCTGGTTTGCTCTT-3′; murine IFNγ sense, 5′-TCAAGTGGCATAGATGTGGAAGAA-3′ and anti-sense, 5′-TGGCTCTGCAGGATTTTCATG-3′; murine TNFα sense, 5′-CCACCACGCTCTTCTGTCTAC-3′ and anti-sense, 5′-TGGGCTACAGGCTTGTCACT-3′; murine IL-10 sense, 5′-ATGCTGCCTGCTCTTACTGACTG-3, and anti-sense, 5′-CCCAAGTAACCCTTAAAGTCCTGC-3′; murine IFNαA sense, 5′-CTGACCCAGGAAGACTACCT-3′ and anti-sense, 5′-GGCTGAGGAAGACATGGCTCT-3′; and murine GBP-1 sense, 5′-AAAAACTTCGGGGACAGCTT-3′ and antisense, 5′-CTGAGTCACCTCATAAGCCAAA-3′. Data were analyzed by the ΔΔCt threshold cycle method and normalized to the housekeeping gene SRP-14 as previously described (68).

Assessment of vulnerability to staurosporine-induced apoptosis

For *in vitro analysis*, T84 and FHs 74 Int cells were grown directly on glass coverslips and pretreated in media with or without 10 units/ml and 100 units/ml of IFNαA for 24 and 48 hours in a 5% CO2 incubator at 37C. Media was changed and apoptosis was then induced using staurosporine (STS, 1μg/mL) or serum-free media control for 2 h. Numbers of TUNEL-positive nuclei were counted per 10 high power fields (HPF).

For *ex vivo analysis*, intestines from 2 week-old mice were surgically excised and opened lengthwise, exposing the intestinal epithelia. Intestines were then maintained in *ex vivo* organ culture, in Hanks Balanced Salt Solution in 24-well cell culture plates at 37C. Tissue was incubated either in Dulbecco's Modified Eagle Medium (DMEM) alone without serum, or with DMEM plus STS (1μg/ml) for indicated time intervals and then analyzed for number of apoptotic cells. Intestines were subsequently washed in PBS and frozen in embedding medium (Sakura Finetek) for histologic staining. Previous studies have reported successful maintenance of murine intestines in organ culture for the induction of apoptosis and the study of inflammatory signaling (7, 26).

Histologic staining

After experimental treatment, T84 and FHs 74 Int cells grown on coverslips were washed and fixed in 4% paraformaldehyde (Fisher Scientific). Alternatively, intestinal tissue was fixed, paraffin embedded and sections mounted on slides.

Terminal deoxynucleotidyl transferase staining

Apoptotic cells were analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using an INSitu Cell Death Detection Kit (Roche Diagnostics), according to the manufacturer's guidelines.

Activated Caspase 3

Cells containing activated caspase 3 were detected using ApoLogix Detection Kit according to the manufacturer's instructions (BACHEM). The procedure detects apoptosis through the inhibition of activated caspase 3 via emitted fluorescence.

Analysis of GBP-1 protein expression

Equal amounts of protein were resolved by polyacrylamide gel electrophoresis and subjected to electrophoretic transfer to activated polyvinylidene fluoride membranes. Blocking and probing were performed using SNAP-ID (Millipore) with 0.5% skim milk for blocking and with rat anti-human GBP-1 antibody (Calbiochem) for 10 minutes, washed three times in phosphate-buffered saline containing 0.05% Tween 20 and bound antibody was detected by probing with species-specific peroxidase conjugated secondary antibodies followed by visualization using BM chemiluminescence substrate (Roche).

GBP-1 silencing studies

Attenuation of GBP-1 expression was performed using sequence specific siRNA, as previously described (30). FHs 74 Int cells were grown to 60–80% confluence and then loaded with GBP-1 siRNA (5). (GBP-1_3 AAGGCATGTACCATAAGCTAA and GBP-1_6, ATGGCATGTACCATAAGCTA.) Transfection with a validated siRNA against Cyclophilin B obtained from Dharmacon was used to control for off-target siRNA treatment. Transfections were performed using HiPerfect (Qiagen) in Opti-MEM I medium (Invitrogen) according to the manufacturer's protocol. 24 hours following loading with siRNA, cells were then treated with vehicle or indicated concentrations of IFNαA and incubated for an additional 24 or 48 hours followed by analysis of apoptosis by staining for activated casapase-3 as described above.

Statistical analysis

Statistical differences were analyzed by ANOVA using Prism 5 for Mac OS X, version 5.0a, 1992–2008 GraphPad Software, Inc.

Results

Vulnerability to induced apoptosis emerges between 1 and 2 weeks postnatal age in immature mouse epithelium

In initial experiments, we characterized the developmental time frame of apoptosis in immature mouse intestine in order to assess whether changes in vulnerability to apoptosis might be an early step contributing to changes in epithelial barrier function, increased bacterial translocation, and finally to exaggerated inflammation and necrosis in the immature intestine. For assessment of apoptosis at antenatal time points, the pups of timed pregnant C57Bl/6 mice were delivered by Caesarian section and immediately sacrificed at embryonic day 18. In contrast, for postnatal time points, dams were allowed to deliver spontaneously, and pups were maintained under conventional conditions then sacrificed at day 2, 8, 14, or 21. In order to assess sensitivity to apoptosis, sectioned intestine was treated *ex vivo* under serum-free conditions alone or in the presence of staurosporine (STS), a broad spectrum inducer of apoptosis, known to compete for ATP-binding, inhibiting multiple kinase signaling pathways and causing apoptosis through both caspase-dependent and independent mechanisms (59). STS and serum-starvation were specifically chosen as proapoptotic stimuli with the goal of studying apoptosis in the absence of concomitant intestinal inflammation.

The extent of epithelial apoptosis was assessed in small intestine by TUNEL, analyzed by confocal microscopy (figure 1A), and TUNEL-positive cells were counted. Neither treatment had measurable effects on apoptosis during the first few postnatal days. Apoptosis in association with serum starvation was first evident in mice sacrificed at 1 week of age only after prolonged incubation for 2 hours (Figure 1B) while STS-induced apoptosis was present in 1 week-old mouse colon within 1 hour of exposure to the drug (Figure 1C). Apoptotic responses to STS serum starvation alone, at least at the shorter time interval, appeared to peak at 2 weeks postnatal age (Figure 1B). Based on these results, we concluded that vulnerability to induction of apoptosis emerges in immature mouse intestinal epithelium between 1 and 2 weeks after birth. Based on these results, we selected 2 hours of combined serum starvation and STS treatment of 2 week-old mouse intestines to model mechanisms of regulation of apoptosis in immature intestine.

Commensal E-coli protects against STS induced apoptosis in 2 week-old intestines

We have previously shown that the probiotic *Lactobacillus rhamnosus* GG protects against STS induced apoptosis in the small intestine of preweaned mice (7). However, it remains unclear how early colonizing commensal flora protect against apoptosis and promote barrier integrity in developing intestine. For this work, we initially examined whether a naturally occurring strain of commensal *E. coli* isolated from healthy human colon and previously characterized as anti-inflammatory (2, 69) would protect against apoptosis in immature murine intestine. Additionally, we employed this model to further examine the mechanisms by which commensals exert their anti-apoptotic effects.

In order to first determine whether gavage feeding with commensal *E. coli* would result in a measurable change in the colonic *E. coli* population of developing mice, real-time PCR was used to compare colonic *E. coli* DNA content in 2 week-old mice *E. coli*-fed mice, relative to media-fed controls. Results revealed that, when normalized for total bacterial DNA based on 16s rDNA, *E.* coli-fed mice showed a $1\times10^{6}\pm0.3$-fold increase in colonic *E. coli uid A* DNA, relative to media-fed controls (data not shown).

Given that gavage feeding with *E. coli* demonstrably altered intestinal flora, we next examined the influence of this *E. coli* exposure on intestinal apoptosis. For these experiments, 2 week-old mice were fed media with or without $1–2\times10^{7}$ CFU of commensal *E. coli* by oral gavage, and sacrificed 24 hours later. Then, their intestines were excised and analyzed for apoptosis in response to STS, *ex vivo*. TUNEL staining of controls revealed a significantly lower baseline rate of apoptosis as well as a more robust induction of apoptosis in response to STS in small intestine (Figure 2A) than in colon (Figure 2B). These results are in contrast to previously published comparisons of baseline apoptotic rates in adult murine intestine showing either similar rates of apoptosis in small intestine and colon (52) or lower baseline apoptosis in colon relative to distal small intestine (53, 54). However, gnotobiotic pigs demonstrate increased TLR-mediated intestinal apoptosis during conventionalization with commensal flora, including *E. coli* (55), and furthermore, that these rates of apoptosis were dependent on the composition of the colonizing flora. These increased rates of baseline of apoptosis in colon relative to small intestine may represent early responses to a greater burden of bacteria during colonization of the immature colon. In our model, *E. coli* feeding had no significant effect on baseline apoptosis, but attenuated the response to STS, reducing STS-induced apoptosis by nearly 50% in both the small intestine (Figure 2A) and colon (Figure 2B) of *E. coli*-fed mice relative to STS-treated, media-fed controls.

Commensal E-coli selectively induces IFNαA and GBP-1 mRNA expression in 2-week-old mice

In an effort to better understand the mechanisms by which commensal *E. coli* protects against STS-induced apoptosis, we next profiled the influence of *E. coli* exposure on the expression of a panel of cytokine in the colon, the site exposed to the greatest burden of colonizing bacteria. RNA was isolated from 2 week-old mouse colon 24 hours after media or *E. coli* feeding, and mRNA expression of IFNαA, IFNγ, IL-10, and MIP-2 was analyzed by real-time PCR (Figure 3A). IFNαA mRNA was selectively induced by 10.2 ± 2.8-fold following feeding with commensal *E. coli* while levels of MIP-2, IFNγand IL-10 were not significantly altered, relative to media-fed controls. This selective induction of IFNαA by commensal *E. coli*, supports a role for IFNαA in mediating the downstream effects of *E. coli* in immature intestine and is consistent with the previously published work implicating TLR9 mediated induction of IFNαA in probiotic-mediated cellular signaling (4).

Members of the IFNα family are unique among the type I interferons in that they prevent rather than promote apoptosis through the specific activation of anti-apoptotic target genes (58). One such target is the gene that encodes GBP-1, a large GTPase, which promotes cell survival and prevents apoptosis in multiple cell types. GBP-1 has been identified in endothelial cells as preventing apoptosis in response to both IFNα(40) and IFNγ(56). Our group has recently reported that GBP-1 can preserve intestinal epithelial barrier function by blocking apoptosis both at baseline and in response to the pro-inflammatory cytokine, IFNγ(5). However, GBP-1 has not been studied in the developing intestinal epithelium and nothing is known about its contributions to responses to bacterial colonization or response to microflora. In order to evaluate GBP-1 as a candidate mediator of the anti-apoptotic effects of *E. coli* in our murine model of immature intestine, we analyzed colonic mRNA expression of GBP-1 in *E. coli*-fed 2 week-old mice by real-time PCR. 24 hours after *E. coli* administration, GBP-1 mRNA expression was induced by 2.8 ± 0.8-fold relative to media-fed controls (Figure 3B).

Interferon αA induces GBP-1 in mature and immature cell lines, *in vitro*

In order to confirm the role of IFNαA in GBP-1 induction by commensal *E. coli*, we next examined the influence of IFNαA treatment on GBP-1 expression and apoptosis in immature intestinal epithelium. As an *in vitro* model of immature epithelia, FHs 74 Intermediate (FHs 74 Int) cells, derived from human fetal small intestine, were treated for 4 or 24 hours with either 10 or 100 U/ml IFNαA, then analyzed for GBP-1 mRNA expression (Figure 4A). Treatment with either 10 or 100 U/ml IFNαA resulted in an induction of GBP-1 mRNA, with expression peaking at a 16.1 ± 5.3-fold increase after 4 hours of treatment with 100 U/ml of IFNαA. and declining by 24 hours to a 4.9 ±1.8-fold increase over controls. The effects of IFNα on GBP-1 were dose dependent, with more modest induction seen in response to 10 U/ml IFNαA. Western blot analysis revealed a rapid induction in GBP-1 protein expression in response to 100 U/ml IFNαA (Figure 4B). This increase over baseline GBP-1 protein expression was first evident following 4 hours of IFNαA exposure and peaked by 8 hours, but remained elevated throughout the 24 hours assayed. A similar, although more rapid and robust dose- and time-dependent induction of GBP-1 mRNA and protein was seen in response to IFNαA treatment in T84 cells, a well-established model for mature intestinal epithelium (data not shown).

IFNαA protects against STS induced apoptosis in immature human model intestinal epithelia

To evaluate the influence of IFNαA on apoptosis in model epithelia, *in vitro*, we treated FHs Int 74 cells with IFNαA for 24 or 48 hours prior to induction of apoptosis with STS. Epithelial cell apoptosis was quantitated by TUNEL and activated caspase-3 staining. Even

at the lower dose of 10 U/ml, IFNαA significantly reduced the number of TUNEL positive cells by 2.6-fold in FHs 74 Int cells (Figure 5A). The influence of IFNαA on STS-induced apoptosis in FHs 74 Int cells was also assayed through staining for and quantitation of the number of FHs 74 Int cells positive for activated caspase-3 (Figure 5B), with statistically significant reductions in STS-induced apoptosis observed after 4 hours of treatment with low dose IFNαA. Similar reductions in apoptosis were observed after both treatment with higher doses and longer incubations with IFNαA. Similar doses of IFNαA also protected T84 cells from STS-induced apoptosis (data not shown).

IFNαA requires GBP-1 for prevention of apoptosis in FHs 74 Int cells

To determine whether this anti-apoptotic effect of IFNαA was dependent on GBP-1, GBP-1 expression was attenuated using previously validated, GBP-1-specific, siRNA (5). FHs 74 Int cells were loaded with either with GBP-1 specific siRNA or Cyclophilin B (CyB) siRNA, as an off target siRNA control, then treated with IFNαA for 24 hours, and analyzed for GBP-1 protein expression by western blot (Figure 6A). GBP-1 siRNA treatment attenuated GBP-1 expression both at baseline and in response to IFNαA treatment. FHs 74 Int cells treated with either GBP-1 siRNA or with control siRNA control were then exposed to STS and analyzed for resultant apoptosis by staining for activated Caspase-3. Control siRNA (CyB)-treated FHs 74 Int cells, still demonstrated significant IFNαA-dependent protection against STS-induced apoptosis, relative to vehicle-treated controls (Figure 6B). Interestingly, in the absence of both STS and IFNαA, siRNA-mediated attenuation of GBP-1 expression resulted in a significant increase in baseline apoptosis (Figure 6C) relative to CyB siRNA treated controls (Figure 6B, $p<0.05$). Additionally, siRNA-dependent attenuation of GBP-1 expression completely eliminated the anti- apoptotic effects of IFNαA (Figure 6C). Thus GBP-1 expression is required both for IFNαA- mediated prevention of STS-induced apoptosis and for prevention of baseline apoptosis in FHs 74 Int cells.

IFNαA protects against STS-induced apoptosis in 2-week-old mice intestine compared to control mice through expression of GBP-1

Given that *E. coli* exposure was associated with both prevention of STS-induced apoptosis and induction of IFNαA expression in 2 week-old mouse colon, we sought to determine if exogenous IFNαA would be similarly protective against apoptosis in our *ex vivo* model of immature intestines. 2 week-old mouse pups were treated with IFNαA at a dose of 40 U/gram body weight by intraperitoneal (i.p.) injection, based on previously published doses shown to ameliorate colitis in IFNαA-deficient animals in adult murine models of colitis (6). Based on our *in vitro* studies indicating that exogenous IFNαA induced GBP-1 protein expression in intestinal epithelial cells within 4 hours of treatment, mice were injected with either IFNαA, 40 U/gm body weight or an equal volume of sterile PBS, i.p. Mice were then returned to their mothers for 5 hours to allow adequate time for absorption and distribution of IFNαA and subsequent GBP-1 induction, then sacrificed and analyzed for both for colonic GBP-1 mRNA expression and vulnerability to apoptosis in both small and large intestine. At this dose and interval, IFNαA treatment had no measurable effect on apoptosis relative to PBS-injected controls at baseline. However, exogenous IFNαA effectively blocked STS-induced apoptosis in immature mouse small intestine (Figure 7A) with a statistically significant decrease in the number of TUNEL positive cells decreased to the level of non-STS-treated controls. STS-induced apoptosis was also significantly reduced in colon of IFNαA-treated 2 week old mice (Figure 7B). Furthermore, real-time PCR analysis of colonic mRNA expression confirmed a corresponding significant 2.6-fold induction in GBP-1 mRNA in colon from 2 week-old IFNαA-treated mice relative to controls (Figure 7C).

Discussion

Here, we present evidence that apoptotic responses either to serum starvation or STS emerge between 1 and 2 weeks in the intestine of developing mice, implicating this window as an optimal interval for the study of mechanisms regulating inducible apoptosis during intestinal development. Previous work has clearly shown that this developmental interval is associated with increased epithelial turnover in rat small intestine (65, 66). It is unclear whether this process is associated with increased baseline apoptosis in mice. Interestingly, we found little or no significant change in baseline apoptosis in mice at this age, with the primary finding, *ex vivo*, being an increased response to injury, indicating that the immediate tissue response to the same apoptotic stimulus is accentuated at this age.

Additionally, we confirm our earlier findings that probiotic or commensal flora, in this case a commensal strain of *E. coli*, can modulate intestinal epithelial apoptosis in the developing murine gut (7). As novel findings, we further demonstrate that commensal *E. coli* exposure modifies cytokine expression in immature murine intestine, selectively inducing IFNαA and the subsequent expression of the anti-apoptotic protein, GBP-1. Additional work using both mature and fetal intestinal epithelia reveals that IFNαA administration also induces GBP-1 and protects against STS-induced apoptosis in these model human systems. Furthermore, studies in fetal intestinal epithelial cells reveal that the anti-apoptotic properties of IFNαA require GBP-1 expression, *in vitro*. Finally, the effects of *E. coli* both on intestinal GBP-1 expression and epithelial apoptosis are recapitulated by the administration of exogenous murine IFNαA alone.

The importance of the resident microbiota in the developing intestine has become increasingly evident. It is known that neonates are born with a sterile gut and become colonized after birth. In the preterm infant, this process can be tainted due to the immaturity of the gut, the lack of breastfeeding, use of antibiotics and exposure to iatrogenic lines and tubes for nutrition (38, 51). Specifically, disturbance of normal colonization through the use of broad-spectrum antibiotics significantly increases the incidence of NEC and related death in premature infants (22). Furthermore, the establishment of appropriate commensal flora and the use of probiotic organisms have shown promise in the prevention of NEC in clinical trials (32–37). However, therapy with commensal or probiotic organisms has been complicated by case reports of documented sepsis in immunocompromised patients, including premature infants (27–29). Therefore, it has become increasingly important to understand the mechanisms by which appropriate intestinal colonization with nonpathogenic flora protects the developing intestine. A better understanding of these beneficial effects will allow more specific and safer targeting of these protective pathways with the hopes of preventing NEC.

In our study we examined potential protective mechanisms mediated by commensal *E. coli*, one of the earliest colonizers of the newborn human intestine. Commensal organisms are known to signal via TLRs, and more specifically TLR9, a known inducer of type I interferon signaling (3, 4). It is also known that type I interferons have anti-inflammatory properties and beneficial effects in experimental models of IBD (6) and NEC (43), as well as in clinical trials for the treatment of IBD (42, 44). Here we demonstrate that commensal *E. coli* selectively induces IFNαA and protects against apoptosis in developing intestine

The role of type I IFNs in the regulation of apoptosis is determined by the nature of activated target genes and is therefore both cell-type specific and dependent on the class of type I IFN. While IFNβis known to have potent proapoptotic effects (58), promoting apoptosis in several cancer (49, 50) and immune cells (47, 48), IFNα activates different downstream targets to prevent apoptosis in endothelial cells (40) and neuronal tissue (8).

IFNα has also previously been reported to protect against apoptosis and promote barrier integrity in polarized intestinal epithelial cell monolayers (46). Furthermore, the protective effects of TLR9 signaling in experimental colitis appear to result from cell-type specific effects of type I IFNs, which promote barrier function through the prevention of epithelial apoptosis while inhibiting the inflammatory responses of activated macrophages (46).

IFNαA has been found to attenuate apoptosis in non-epithelial cell lines through the induction of GBP-1 (40). Recently our group has shown that GBP-1 localizes to epithelial tight junctions, is induced in the intestinal epithelium of patients with IBD, and promotes intestinal epithelial barrier integrity through the prevention of apoptosis, *in vitro* (5). Here we provide further specific evidence that the IFNαA-mediated induction of GBP-1 in response to commensal flora attenuates apoptosis in models of immature intestine, both *in vitro* and *ex vivo*.

Previous work has demonstrated that baseline fluctuations in epithelial apoptosis do not appear to alter barrier integrity. However, increased epithelial apoptosis in response to exogenous toxic stimuli, such as doxorubicin, is associated with measurable bidirectional changes in permeability in adult rats (64). Therefore, the impact of proapoptotic stimuli on epithelial barrier is likely to be dependent on the magnitude of resultant apoptosis. Furthermore, the anti-apoptotic effects of commensals such as nonpathogenic *E. coli* may help prevent disruption of intestinal epithelial barrier integrity in the developing gut. However, the current existing models for neonatal human epithelial cells require growth conditions, which are not amenable to the assay of barrier function. Therefore the contributions of the regulation of apoptosis to barrier function in the immature intestine could not be assessed in our *in vitro* model system.

However, our studies identify a novel and specific role for GBP-1 as a regulator of epithelial apoptosis in response to commensal *E. coli* in developing intestine. Members of the murine family of GBPs have been implicated as critical elements of host defense activated in both macrophages and fibroblasts in response to TLR signaling and proinflammatory cytokines as well as in response to infection with both bacteria and parasites, *in vivo* (45). These studies represent the first characterization of GBP-1 regulation and its importance in the protection of the immature intestinal epithelium against apoptosis in response to microflora. Additional studies are needed to determine whether this IFNαA-mediated induction of GBP-1 is specific to non-pathogenic *E. coli*, commensal organisms in general, or a more global response to microflora, independent of the nature of the organism.

The induction of GBP-1 has previously been shown to prevent apoptosis, protecting epithelial barrier in the presence of proinflammatory mediators (IFNγ, TNFα) both *in vitro* and in the setting of IBD (5), and here we demonstrate an important protective role for GBP-1 in response to commensal flora in the developing intestine. Recently murine GBP-1 was identified by combined quantitative trait loci and microarray analysis as once of three genes to be differentially upregulated in conventionally raised, colitis-sensitive strains of IL-$10^{-/-}$ mice, relative to more colitis-resistant strains. Specifically, GBP-1 expression was upregulated in IL-$10^{-/-}$ C3H/HeJBir mice, which develop severe, spontaneous, commensal-dependent colitis relative to IL$10^{-/-}$ C57BL/6 mice, which develop only mild spontaneous intestinal inflammation (57). Given the anti-apoptotic and barrier protective roles of GBP-1, this strain-specific induction of GBP-1 most likely represents a compensatory and protective mucosal response in the intestines of mice, which are otherwise more vulnerable to inflammatory responses to colonizing bacteria in the setting of IL-10 deficiency.

While our *ex vivo* and *in vivo* systems are intended to mimic responses to commensal bacteria in developing intestine, the exact relevance of our findings to NEC remain

uncertain. However, these findings are particularly intriguing given that the developing human intestine is vulnerable to barrier compromise, bacterial translocation and unchecked inflammatory responses culminating in NEC (1). We reveal here that developing murine intestine is deficient both in baseline and commensal-driven IL-10 expression. Our findings argue a novel role for GBP-1 in acute responses to commensal flora in the immature intestine and may indicate a specific role for GBP-1 in response to colonization at a developmental interval when IL-10 is relatively deficient. Whether GBP-1 is critical for initial colonization or more generally significant for defense of developing intestine against existing microflora has yet to be determined. Further characterization of the regulation of GBP-1 during intestinal development may better inform our understanding of the maintenance of epithelial barrier in immature intestine and its failure in NEC.

Abbreviations in this paper

GBP-1	Guanylate binding protein-1
NEC	Necrotizing enterocolitis
STS	staurosporine
IRF	Interferon regulatory factor
IBD	Inflammatory Bowel Disease
FHs 74 Int	FHs 74 Intermediate
SRP	signal recognition particle
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
HPF	high power field
CyB	Cyclophilin B
i.p	intraperitoneal
ctrl	control

References

1. Grave GD, Nelson SA, Walker WA, Moss RL, Dvorak B, Hamilton FA, Higgins R, Raju TN. New therapies and preventive approaches for necrotizing enterocolitis: report of a research planning workshop. Pediatr Res. 2007; 62:510–514. [PubMed: 17667844]
2. Collier-Hyams LS, Sloane V, Batten BC, Neish AS. Cutting edge: bacterial modulation of epithelial signaling via changes in neddylation of cullin-1. J Immunol. 2005; 175:4194–4198. [PubMed: 16177058]
3. Lee J, Rachmilewitz D, Raz E. Homeostatic effects of TLR9 signaling in experimental colitis. Ann N Y Acad Sci. 2006; 1072:351–355. [PubMed: 17057215]
4. Rachmilewitz D, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, Raz E. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology. 2004; 126:520–528. [PubMed: 14762789]
5. Schnoor M, Betanzos A, Weber DA, Parkos CA. Guanylate-binding protein-1 is expressed at tight junctions of intestinal epithelial cells in response to interferon-gamma and regulates barrier function through effects on apoptosis. Mucosal Immunol. 2009; 2:33–42. [PubMed: 19079332]
6. Louis NA, Robinson AM, MacManus CF, Karhausen J, Scully M, Colgan SP. Control of IFN-alphaA by CD73: implications for mucosal inflammation. J Immunol. 2008; 180:4246–4255. [PubMed: 18322237]

7. Lin PW, Nasr TR, Berardinelli AJ, Kumar A, Neish AS. The probiotic Lactobacillus GG may augment intestinal host defense by regulating apoptosis and promoting cytoprotective responses in the developing murine gut. Pediatr Res. 2008; 64:511–516. [PubMed: 18552706]
8. Bazhanova ED, Teplyi DL. Involvement of interferon-alpha in the regulation of apoptosis of cells of the hypothalamo-hypophyseal-adrenocortical system of aged mice in oxidative stress. Neurosci Behav Physiol. 2005; 35:375–378. [PubMed: 15929563]
9. Mshvildadze M, Neu J, Mai V. Intestinal microbiota development in the premature neonate: establishment of a lasting commensal relationship? Nutr Rev. 2008; 66:658–663. [PubMed: 19019028]
10. Hooper LV. Bacterial contributions to mammalian gut development. Trends Microbiol. 2004; 12:129–134. [PubMed: 15001189]
11. Kai-Larsen Y, Bergsson G, Gudmundsson GH, Printz G, Jornvall H, Marchini G, Agerberth B. Antimicrobial components of the neonatal gut affected upon colonization. Pediatr Res. 2007; 61:530–536. [PubMed: 17413858]
12. Bailey M, Plunkett FJ, Rothkotter HJ, Vega-Lopez MA, Haverson K, Stokes CR. Regulation of mucosal immune responses in effector sites. Proc Nutr Soc. 2001; 60:427–435. [PubMed: 12069394]
13. Claud EC, Walker WA. Bacterial colonization, probiotics, and necrotizing enterocolitis. J Clin Gastroenterol. 2008; 42(Suppl 2):S46–52. [PubMed: 18520617]
14. Conroy ME, Shi HN, Walker WA. The long-term health effects of neonatal microbial flora. Curr Opin Allergy Clin Immunol. 2009; 9:197–201. [PubMed: 19398905]
15. Farrell RJ, LaMont JT. Microbial factors in inflammatory bowel disease. Gastroenterol Clin North Am. 2002; 31:41–62. [PubMed: 12122743]
16. Podolsky DK. Inflammatory bowel disease. N Engl J Med. 2002; 347:417–429. [PubMed: 12167685]
17. Schultz M, Sartor RB. Probiotics and inflammatory bowel diseases. Am J Gastroenterol. 2000; 95:S19–21. [PubMed: 10634224]
18. de la Cochetiere MF, Piloquet H, des Robert C, Darmaun D, Galmiche JP, Roze JC. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. Pediatr Res. 2004; 56:366–370. [PubMed: 15201403]
19. Lin PW, Nasr TR, Stoll BJ. Necrotizing enterocolitis: recent scientific advances in pathophysiology and prevention. Semin Perinatol. 2008; 32:70–82. [PubMed: 18346530]
20. Nanthakumar NN, Fusunyan RD, Sanderson I, Walker WA. Inflammation in the developing human intestine: A possible pathophysiologic contribution to necrotizing enterocolitis. Proc Natl Acad Sci U S A. 2000; 97:6043–6048. [PubMed: 10823949]
21. Boccia D, Stolfi I, Lana S, Moro ML. Nosocomial necrotising enterocolitis outbreaks: epidemiology and control measures. Eur J Pediatr. 2001; 160:385–391. [PubMed: 11421422]
22. Cotten CM, Taylor S, Stoll B, Goldberg RN, Hansen NI, Sanchez PJ, Ambalavanan N, Benjamin DK Jr. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. Pediatrics. 2009; 123:58–66. [PubMed: 19117861]
23. Guillet R, Stoll BJ, Cotten CM, Gantz M, McDonald S, Poole WK, Phelps DL. Association of H2-blocker therapy and higher incidence of necrotizing enterocolitis in very low birth weight infants. Pediatrics. 2006; 117:e137–142. [PubMed: 16390920]
24. Jilling T, Lu J, Jackson M, Caplan MS. Intestinal epithelial apoptosis initiates gross bowel necrosis in an experimental rat model of neonatal necrotizing enterocolitis. Pediatr Res. 2004; 55:622–629. [PubMed: 14764921]
25. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH. Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. J Clin Invest. 2004; 114:1098–1106. [PubMed: 15489957]
26. Shi J, Aono S, Lu W, Ouellette AJ, Hu X, Ji Y, Wang L, Lenz S, van Ginkel FW, Liles M, Dykstra C, Morrison EE, Elson CO. A novel role for defensins in intestinal homeostasis: regulation of IL-1beta secretion. J Immunol. 2007; 179:1245–1253. [PubMed: 17617617]

27. Cannon JP, Lee TA, Bolanos JT, Danziger LH. Pathogenic relevance of Lactobacillus: a retrospective review of over 200 cases. Eur J Clin Microbiol Infect Dis. 2005; 24:31–40. [PubMed: 15599646]
28. De Groote MA, Frank DN, Dowell E, Glode MP, Pace NR. Lactobacillus rhamnosus GG bacteremia associated with probiotic use in a child with short gut syndrome. Pediatr Infect Dis J. 2005; 24:278–280. [PubMed: 15750472]
29. Land MH, Rouster-Stevens K, Woods CR, Cannon ML, Cnota J, Shetty AK. Lactobacillus sepsis associated with probiotic therapy. Pediatrics. 2005; 115:178–181. [PubMed: 15629999]
30. Mandell KJ, Babbin BA, Nusrat A, Parkos CA. Junctional adhesion molecule 1 regulates epithelial cell morphology through effects on beta1 integrins and Rap1 activity. J Biol Chem. 2005; 280:11665–11674. [PubMed: 15677455]
31. Colgan SP, Dzus AL, Parkos CA. Epithelial exposure to hypoxia modulates neutrophil transepithelial migration. J Exp Med. 1996; 184:1003–1015. [PubMed: 9064318]
32. Alfaleh K, Bassler D. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev. 2008:CD005496. [PubMed: 18254081]
33. Lin HC, Su BH, Chen AC, Lin TW, Tsai CH, Yeh TF, Oh W. Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. Pediatrics. 2005; 115:1–4. [PubMed: 15629973]
34. Manzoni P, Mostert M, Leonessa ML, Priolo C, Farina D, Monetti C, Latino MA, Gomirato G. Oral supplementation with Lactobacillus casei subspecies rhamnosus prevents enteric colonization by Candida species in preterm neonates: a randomized study. Clin Infect Dis. 2006; 42:1735–1742. [PubMed: 16705580]
35. Bin-Nun A, Bromiker R, Wilschanski M, Kaplan M, Rudensky B, Caplan M, Hammerman C. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. J Pediatr Gastroenterol Nutr. 2005; 147:192–196.
36. Dani C, Biadaioli R, Bertini G, Martelli E, Rubaltelli FF. Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. A prospective double-blind study. Biol Neonate. 2002; 82:103–108. [PubMed: 12169832]
37. Costalos C, Skouteri V, Gounaris A, Sevastiadou S, Triandafilidou A, Ekonomidou C, Kontaxaki F, Petrochilou V. Enteral feeding of premature infants with Saccharomyces boulardii. Early Hum Dev. 2003; 74:89–96. [PubMed: 14580749]
38. Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl. 2003; 91:48–55. [PubMed: 14599042]
39. Kaper JB, Nataro JP, Mobley HL. Pathogenic Escherichia coli. Nat Rev Microbiol. 2004; 2:123–140. [PubMed: 15040260]
40. Pammer J, Reinisch C, Birner P, Pogoda K, Sturzl M, Tschachler E. Interferon-alpha prevents apoptosis of endothelial cells after short-term exposure but induces replicative senescence after continuous stimulation. Lab Invest. 2006; 86:997–1007. [PubMed: 16894355]
41. Briken V, Ruffner H, Schultz U, Schwarz A, Reis LF, Strehlow I, Decker T, Staeheli P. Interferon regulatory factor 1 is required for mouse Gbp gene activation by gamma interferon. Mol Cell Biol. 1995; 15:975–982. [PubMed: 7823961]
42. Ruther U, Nunnensiek C, Muller HA, Bader H, May U, Jipp P. Interferon alpha (IFN alpha 2a) therapy for herpes virus-associated inflammatory bowel disease (ulcerative colitis and Crohn's disease). Hepatogastroenterology. 1998; 45:691–699. [PubMed: 9684118]
43. Uguralp S, Mizrak B, Karabulut AB, Gurbuz N, Demircan M. Interferon-alpha reduces the development of experimental necrotizing enterocolitis. Cytokine. 2004; 25:204–211. [PubMed: 15036246]
44. Madsen SM, Schlichting P, Davidsen B, Nielsen OH, Federspiel B, Riis P, Munkholm P. An open-labeled, randomized study comparing systemic interferon-alpha-2A and prednisolone enemas in the treatment of left-sided ulcerative colitis. Am J Gastroenterol. 2001; 96:1807–1815. [PubMed: 11419834]
45. Degrandi D, Konermann C, Beuter-Gunia C, Kresse A, Wurthner J, Kurig S, Beer S, Pfeffer K. Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. J Immunol. 2007; 179:7729–7740. [PubMed: 18025219]

46. Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest. 2005; 115:695–702. [PubMed: 15765149]
47. Yen JH, Ganea D. Interferon beta induces mature dendritic cell apoptosis through caspase-11/caspase-3 activation. Blood. 2009; 114:1344–1354. [PubMed: 19531658]
48. Carrero JA, Calderon B, Unanue ER. Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection. J Exp Med. 2004; 200:535–540. [PubMed: 15302900]
49. Reu FJ, Bae SI, Cherkassky L, Leaman DW, Lindner D, Beaulieu N, MacLeod AR, Borden EC. Overcoming resistance to interferon-induced apoptosis of renal carcinoma and melanoma cells by DNA demethylation. J Clin Oncol. 2006; 24:3771–3779. [PubMed: 16801630]
50. Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, Borden EC. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. Apoptosis. 2003; 8:237–249. [PubMed: 12766484]
51. Gewolb IH, Schwalbe RS, Taciak VL, Harrison TS, Panigrahi P. Stool microflora in extremely low birthweight infants. Arch Dis Child Fetal Neonatal Ed. 1999; 80:F167–173. [PubMed: 10212075]
52. Marshman E, Ottewell PD, Potten CS, Watson AJ. Caspase activation during spontaneous and radiation-induced apoptosis in the murine intestine. J Pathol. 2001; 195:285–292. [PubMed: 11673824]
53. Tsunada S, Iwakiri R, Noda T, Fujimoto K, Fuseler J, Rhoads CA, Aw TY. Chronic exposure to subtoxic levels of peroxidized lipids suppresses mucosal cell turnover in rat small intestine and reversal by glutathione. Dig Dis Sci. 2003; 48:210–222. [PubMed: 12645813]
54. Iwakiri R, Gotoh Y, Noda T, Sugihara H, Fujimoto K, Fuseler J, Aw TY. Programmed cell death in rat intestine: effect of feeding and fasting. Scand J Gastroenterol. 2001; 36:39–47. [PubMed: 11218238]
55. Willing BP, Van Kessel AG. Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig. J Anim Sci. 2007; 85:3256–3266. [PubMed: 17785595]
56. Lubeseder-Martellato C, Guenzi E, Jorg A, Topolt K, Naschberger E, Kremmer E, Zietz C, Tschachler E, Hutzler P, Schwemmle M, Matzen K, Grimm T, Ensoli B, Sturzl M. Guanylate-binding protein-1 expression is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. Am J Pathol. 2002; 161:1749–1759. [PubMed: 12414522]
57. de Buhr MF, Mahler M, Geffers R, Hansen W, Westendorf AM, Lauber J, Buer J, Schlegelberger B, Hedrich HJ, Bleich A. Cd14, Gbp1, and Pla2g2a: three major candidate genes for experimental IBD identified by combining QTL and microarray analyses. Physiol Genomics. 2006; 25:426–434. [PubMed: 16705022]
58. Bazhanova ED. Interferon alpha involvement in apoptosis regulation. Zh Evol Biokhim Fiziol. 2005; 41:101–106. [PubMed: 15909892]
59. Zhang XD, Gillespie SK, Hersey P. Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways. Mol Cancer Ther. 2004; 3:187–197. [PubMed: 14985459]
60. Gribar SC, Sodhi CP, Richardson WM, Anand RJ, Gittes GK, Branca MF, Jakub A, Shi XH, Shah S, Ozolek JA, Hackam DJ. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. J Immunol. 2009; 182:636–646. [PubMed: 19109197]
61. Sirard JC, Bayardo M, Didierlaurent A. Pathogen-specific TLR signaling in mucosa: Mutual contribution of microbial TLR agonists and virulence factors. European Journal of Immunology. 2006; 36:260–263. [PubMed: 16453385]
62. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell. 2004; 118:229–241. [PubMed: 15260992]
63. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology. 2002; 148:257–266. [PubMed: 11782518]

64. Sun Z, Wang X, Wallen R, Deng X, Du X, Hallberg E, Andersson R. The influence of apoptosis on intestinal barrier integrity in rats. Scand J Gastroenterol. 1998; 33:415–422. [PubMed: 9605264]

65. Henning SJ. Plasma concentrations of total and free corticosterone during development in the rat. Am J Physiol. 1978; 235:E451–456. [PubMed: 103438]

66. Koldovsky O, Sunshine P, Kretchmer N. Cellular migration of intestinal epithelia in suckling and weaned rats. Nature. 1966; 212:1389–1390. [PubMed: 5967032]

67. Sandhya S, Chen W, Mulchandani A. Molecular beacons: a real-time polymerase chain reaction assay for detecting Escherichia coli from fresh produce and water. Anal Chim Acta. 2008; 614:208–212. [PubMed: 18420053]

68. Schnoor M, Cullen P, Lorkowski J, Stolle K, Robenek H, Troyer D, Rauterberg J, Lorkowski S. Production of type VI collagen by human macrophages: a new dimension in macrophage functional heterogeneity. J Immunol. 2008; 180:5707–5719. [PubMed: 18390756]

69. Kumar A, Wu H, Collier-Hyams LS, Hansen JM, Li T, Yamoah K, Pan ZQ, Jones DP, Neish AS. Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. EMBO J. 2007; 26:4457–4466. [PubMed: 17914462]

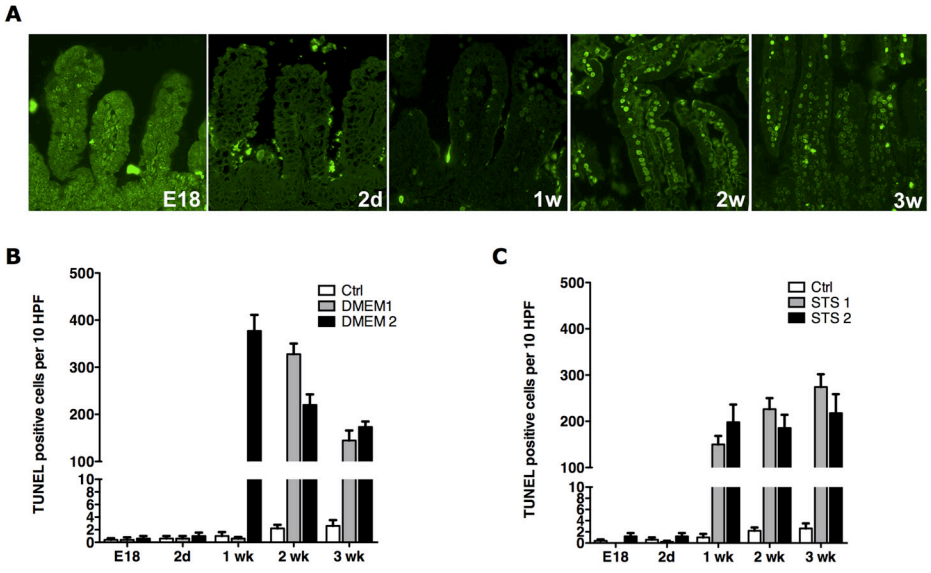

FIGURE 1.

Vulnerability to induced apoptosis emerges between 1 and 2 weeks postnatal age in immature mouse epithelium. C57Bl6 mouse pups were sacrificed on embryonic day 18 (E18) or postnatal day 2, 8 (1w), 14 (2w), or 21 (3w). Small intestines were harvested and exposed to DMEM with (Ctrl) or without serum for 1 (DMEM1) or 2 hours (DMEM1) and apoptosis was quantitated by TUNEL staining. The number of TUNEL positive cells was analyzed by confocal microscopy. Representative images depict the effects of serum-starvation after 1 hour (A). The number of apoptotic cells were counted for a total of 10 high power fields (HPFs) and depicted as the average per 10 HPF following the indicated intervals of serum starvation (B). Alternatively, intestines were incubated in the presence of staurosporine (1 μg/ml) for 1 (STS1) or 2 (STS2) hours and the number of apoptotic cells determined by TUNEL staining relative to intestines maintained in serum-containing media (C). (n = 5 mice per condition, *$p < 0.0001$ for serum starvation and for STS by 2-way ANOVA. Effects of age were significant in both cases by 2-way ANOVA, *$p < 0.0001$)

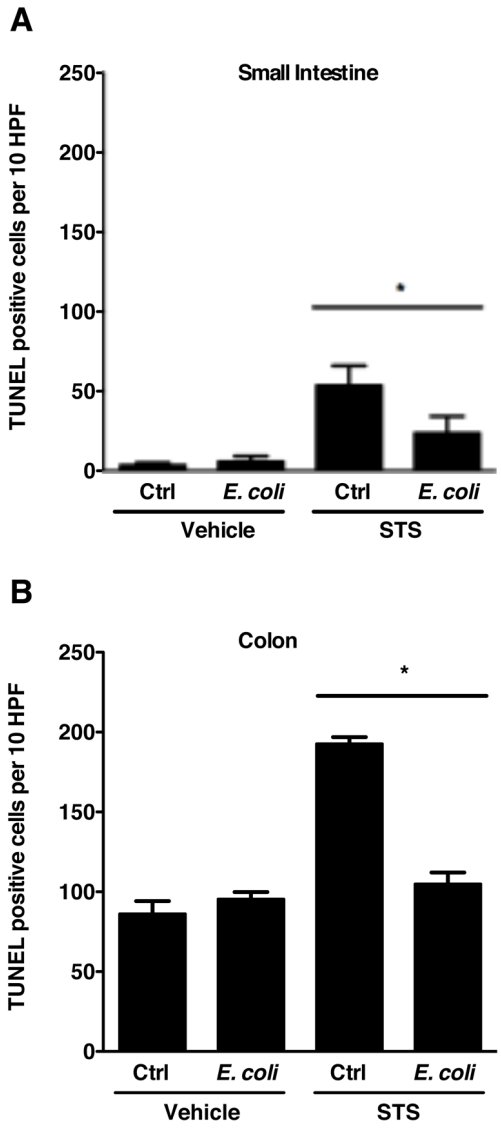

FIGURE 2.

Commensal *E. coli* protects against STS-induced apoptosis in immature small and large intestine. 2 week-old mouse pups were orally gavage fed 1–2× 10^7 CFU of commensal *E. coli* (*E. coli*) or equal volumes of media (Ctrl), then returned to their mothers for 24 hours prior to sacrifice. Small (A) and large intestine (B) were harvested and incubated in the absence (vehicle) or presence of staurosporine (STS) for 2 hours then analyzed for apoptosis by TUNEL staining with the results depicted as the number of TUNEL positive cells per 10 HPF. (n =6–9 mice per condition, *$p < 0.0001$)

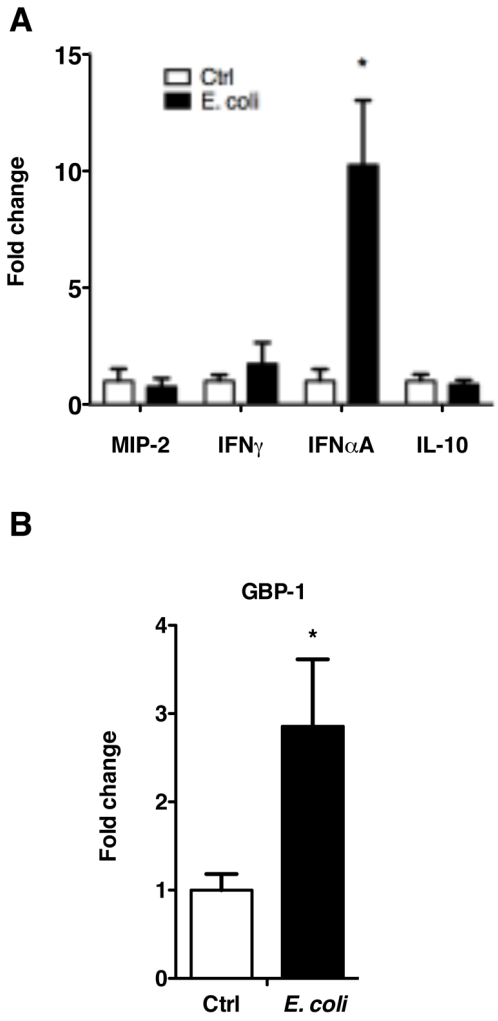

FIGURE 3.

Commensal *E. coli* selectively induces IFNαA and GBP-1 in immature mouse colon. Colons were isolated from *E. coli*-fed 2 week-old mice, processed for RNA and analyzed by real-time PCR for levels of cytokine (A) and GBP-1 (B) expression. Results are depicted as fold change relative to controls and normalized for expression of the housekeeping gene SRP-14. (n = 3 – 6, *p < 0.05, **p < 0.01)

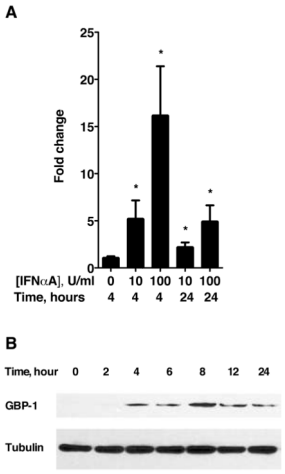

FIGURE 4.

IFNαA induces GBP-1 expression in immature model human intestinal epithelia. FHs 74 Int cells were grown on 24 well plates and treated with 0, 10 or 100 U/ml of IFNαA for indicated time intervals and processed for analysis of GBP-1 mRNA expression (A) by real-time PCR or for GBP-1 protein expression (B) by western blot. Fold change in mRNA level is depicted as the mean ±s.e.m. ($n = 3$, $^*p < 0.05$). Protein expression is depicted relative to tubulin expression, as a control for protein loading, in this representative blot from a series of 3 replicate experiments.

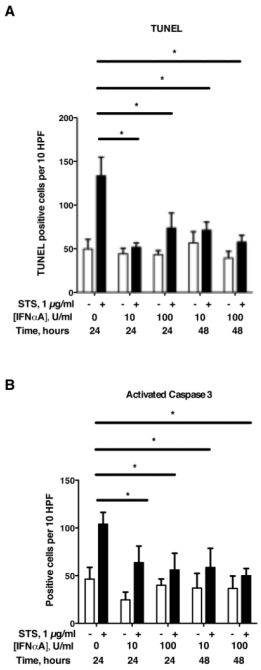

FIGURE 5.

IFNαA protects against STS-induced apoptosis in immature human intestinal epithelia, *in vitro*. FHs 74 Int cells were treated with IFNαA, 10 or 100 U/ml, for 24 or 48 hours then treated with STS, 1 μg/ml for 2 hours, fixed and analyzed for apoptotic cells by TUNEL (A) and immunofluorescent staining for activated caspase 3 (B). Results depict the mean number of positive cells ±s.e.m, determined from 10 high power fields. (n = 3, *$p < 0.05$)

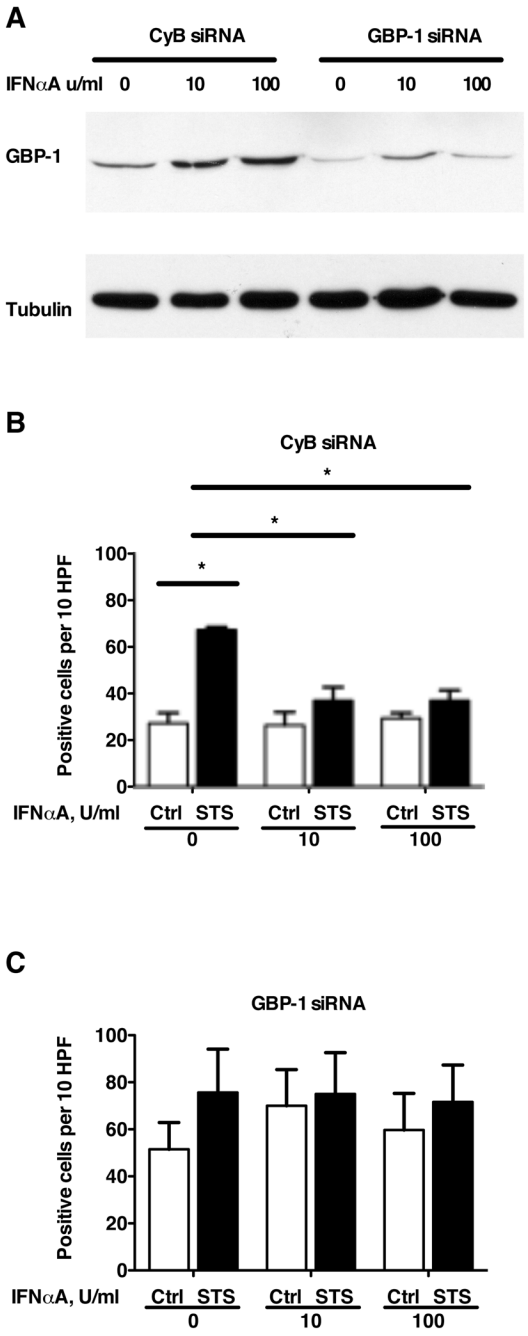

FIGURE 6.

GBP-1 is required for the anti-apoptotic effects of IFNαA in immature human intestinal epithelia, *in vitro*. FHs 74 Int cells were loaded with sequence specific siRNA directed against GBP-1 or an off-target siRNA against cyclophilin B (CyB), then treated with indicated concentrations of IFNαA for 24 hours. GBP-1 protein expression was then analyzed by western blot (A). Results are depicted by representative blot relative to the results from the same blot stripped and reprobed for tubulin as a control for the amount of total protein per lane (n = 3). FHs 74 Int cells loaded with either CyB specific (B) or GBP-1-specific siRNA (C) were then treated with indicated concentrations of IFNαA for 24 hours, followed by exposure to STS for 2 hours. Cells were then stained for activated caspase 3 and

the number of positive cells counted per 10 HPFs. Results are expressed as the average number of cells positive for activated caspase 3 ± s.e.m. (n = 4, *p < 0.001).

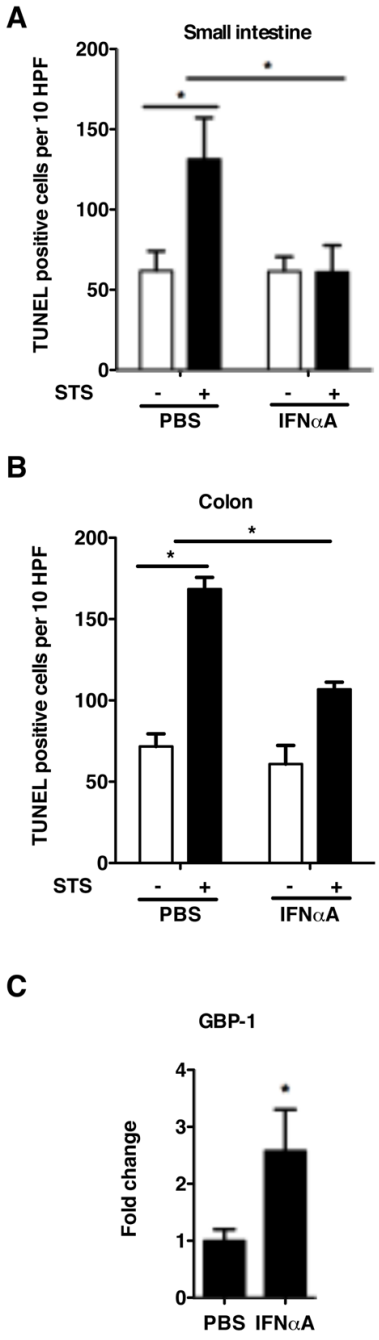

FIGURE 7.

Exogenous IFNαA prevents STS-induced apoptosis and induces GBP-1 in 2 week-old mouse intestines. 2 week-old mice were injected with IFNαA, 40 U/g baseline weight, i.p. or an equal volume of PBS (Ctrl) then sacrificed. Isolated small intestines (A) and colon (B) were then incubated for 2 hours in media alone or media containing 1 μg/ml STS. Tissues were then fixed and processed for TUNEL staining. The number of TUNEL positive cells for each condition were counted and plotted as the mean ± s.e.m., determined per 10 HPFs. Colons isolated from the same animals were also processed for RNA analysis by real-time PCR and the level of GBP-1 expression in IFNαA-treated animals is plotted as fold change

relative to controls (C). Results are normalized according to levels of expression of murine SRP-14. (n = 5 – 6, *p < 0.01).