Interleukin-11 antagonizes Fas ligand-mediated apoptosis in IEC-18 intestinal epithelial crypt cells: role of MEK and Akt-dependent signaling

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Naugler KM, Baer KA, Ropeleski MJ. Interleukin-11 antagonizes Fas ligand-mediated apoptosis in IEC-18 intestinal epithelial crypt cells: role of MEK and Akt-dependent signaling. Am J Physiol Gastrointest Liver Physiol 294: G728–G737, 2008. First published January 17, 2008; doi:10.1152/ajpgi.00002.2007.—Interleukin-11 (IL-11) displays epithelial cytoprotective effects during intestinal injury. Antiapoptotic effects of IL-11 have been described, yet mechanisms remain unclear. Fas/CD95 death receptor signaling is upregulated in ulcerative colitis, leading to mucosal breakdown. We hypothesized that IL-11 inhibits Fas ligand (FasL)-mediated apoptosis in intestinal epithelia. Cell death was monitored in IEC-18 cells by microscopy, caspase and poly(ADP-ribose) polymerase cleavage, mitochondrial release of cytochrome c, and abundance of cytoplasmic oligonucleosomal DNA. RT-PCR was used to monitor Fas, cIAP1, cIAP2, XIAP, cFLIP, survivin, and Bcl-2 family members. Fas membrane expression was detected by immunoblot. Inhibitors of JAK2, phosphatidylinositol 3-kinase (PI3-kinase), Akt 1, MEK1 and MEK2, and p38 MAPK were used to delineate IL-11’s antiapoptotic mechanisms. IL-11 did not alter Fas expression. Pretreatment with IL-11 for 24 h before FasL reduced cytoplasmic oligonucleosomal DNA by 63.2%. IL-11 also attenuated caspase-3, caspase-9, and poly(ADP-ribose) polymerase cleavage without affecting expression of activated caspase-8 p20 or cytochrome c release. IL-11 did not affect mRNA expression of the candidate antiapoptotic genes. The MEK1 and MEK2 inhibitors U-0126 and PD-98059 significantly attenuated the protection of IL-11 against caspase-3 and caspase-9 cleavage and cytoplasmic oligonucleosomal DNA accumulation. Although Akt inhibition reversed IL-11-mediated effects on caspase cleavage, it did not reverse the protective effects of IL-11 by DNA ELISA. We conclude that IL-11-dependent MEK1 and MEK2 signaling inhibits FasL-induced apoptosis. The lack of reversal of the IL-11 effect on DNA cleavage by Akt inhibition, despite antagonism of caspase cleavage, suggests that IL-11 inhibits caspase-independent cell death signaling by FasL in a MEK-dependent manner.

mitogen-activated protein kinase; cytokines; cytoprotection; mitochondria

Interleukin-11 (IL-11) is a cytokine originally described as a growth factor in the hematopoietic microenvironment (29). Minimal basal IL-11 expression occurs in the normal small intestine and colon in the mouse and the physiological role of endogenous IL-11 is unknown, as no knockout animal exists. Constitutive IL-11 receptor (IL-11Rα) expression has been documented in the mouse small and large intestines (8), as well as in human colonic epithelia (10, 18). The IL-11Rα-1 isoform knockout mouse does not spontaneously develop intestinal inflammation. However, the existence of an IL-11Rα-2 isoform expressed in immune cells raises concern that IL-11Rα-2 knockout animals cannot be appropriately interpreted without vigorous analysis of compensatory IL-11Rα-2 expression in different intestinal mucosal cell compartments (33). The most abundant source of IL-11 in the intestinal mucosa is likely the subepithelial myofibroblast in response to treatment with TGF-β and/or IL-1β (3), as well as the IL-10-like cytokine, IL-22 (2). In other cell types, overexpression of cyclooxygenase-2, which is associated with the epithelial healing response (16), leads to increased IL-11 expression (38).

Exogenously administered IL-11 protects intestinal epithelial crypts from chemoradiation-induced damage in mice, resulting in increased barrier competence, healing, and survival (12, 31). IL-11 is protective in acute graft vs. host disease (15), several animal models of inflammatory bowel disease (IBD) (17), acute intestinal ischemia (11), and Clostridium difficile-mediated enterotoxicity (5). In dextran sodium sulfate colitis, epithelial IL-11 expression occurs both during the acute phase and during recovery from colitis (6). Although IL-11 has been shown to decrease the severity of inflammation in mouse models of colitis, the mechanisms accountable are incompletely understood (21). A body of data suggests that IL-11 exerts anti-inflammatory effects on innate immune and T cells. However, it has also been proposed that IL-11 exerts direct antiapoptotic effects on the intestinal epithelium during anokis, radiation, and ischemia-reperfusion injury (14, 34). IL-11’s mechanism of action may involve the upregulation of Bcl-2 and/or the antagonism of caspase-9 (21). We speculate that IL-11-mediated protection of the epithelium from apoptosis is a major factor leading to the mucosal protection observed in IL-11-treated animals in various models of intestinal injury.

Resident stem cells, the committed proliferating progenitors in the crypt, and cells in the villus epithelial cell compartment are sensitive to apoptosis (14, 22, 32). Intestinal epithelial programmed cell death may contribute to the barrier dysfunction characteristic of IBD (1, 40). For example, increased epithelial apoptosis characterizes the ileitis in the Samp1/Yit mouse model of Crohn disease and is reversed by neutralization of TNF-α (20). Toll-like receptor 4-deficient mice are more susceptible to dextran sodium sulfate colitis because of increased epithelial cell death and decreased healing (13). Furthermore, in intestinal epithelial-specific NEMO (IκB kinase-γ)-deficient mice, the spontaneous colitis that occurs is preceded by abundant epithelial apoptosis and barrier breakdown (24). Finally, in mouse models of T-cell-mediated enteropathy, early characteristic epithelial apoptosis results from...
the convergence of Fas/Fas ligand (FasL), granzyme B/perforin, and TNF-α signaling (7). In humans, epithelial apoptosis is increased in the inflamed mucosa of patients with ulcerative colitis (41). We speculate that protection of the epithelium from apoptosis is a factor that could be exploited early in the natural history of IBD to facilitate healing of the intestinal mucosa.

Fas (CD95/apoptosis-inducing protein-1) signaling induces apoptosis in human colonic epithelium. When FasL binds the Fas receptor on the basolateral epithelial membrane, Fas-associated death domain recruits molecules, including procaspase-8, to form the death-inducing signaling complex (39). Procaspase-8 is then cleaved into its activated form, and the apoptotic cascade ensues, culminating in the activation of executioner caspases such as caspase-3 (4, 39). FasL is normally expressed by intestinal mucosal lymphocytes at low levels; however, in ulcerative colitis, it is upregulated on the surface of infiltrating cytotoxic lymphocytes (42), which contribute to the epithelial damage characteristic of colonic mucosa in this setting. lpr−/− Fas (CD95) knockout mice are partially protected from intestinal epithelial damage in a model of activated T-cell-mediated enteropathy (21). Because the subepithelial myofibroblast, which lies in close proximity to the epithelium, is a major source of inducible IL-11, we hypothesized that IL-11 possesses direct antiapoptotic effects on the epithelial cell during cellular stress. To test this, we examined the effects of IL-11 on a rat intestinal crypt cell line (IEC-18) during FasL-induced apoptosis.

MATERIALS AND METHODS

Reagents. All chemicals were of molecular biology grade and obtained from Fisher Scientific (Ottawa, ON) unless otherwise stated. All cell culture reagents and FBS were obtained from Sigma-Aldrich (St. Louis, MO). The p38 MAPK inhibitor (SB-203580), DTT, staurosporine, and the topoisomerase 1 inhibitor camptothecin were obtained from Sigma-Aldrich. The PI3-kinase inhibitor LY-294002, the Akt inhibitor VIII (AktiVIII; IC50 = 58 and 210 nM for Akt1 and Akt2, respectively), the JAK2 inhibitor AG-490, and the MEK1 and MEK2 inhibitors U-0126 and PD-98059 were obtained from Calbiochem (La Jolla, CA). Membrane-bound FasL was purchased from Millipore (Bedford, MA) and used at a final concentration of 4 ng/ml. Recombinant human IL-11 (Oprelevkin) was purchased from the Genetics Institute (Cambridge, MA).

Cell culture. The rat IEC-18 [CRL-1589; American Type Culture Collection (ATCC), Manassas, VA] and IEC-6 (CRL-1592; ATCC) intestinal epithelial crypt cell lines were grown in sodium bicarbonate-buffered DMEM with high glucose (4.5 g/l), 5% FBS, 4 mM glutamine, 0.1 U/ml human insulin (Novo Nordisk, Mississauga, ON), 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were passed every 3–4 days with 0.05% trypsin-0.53 mM EDTA. Cells were fed 24 h before IL-11 treatment. Cells were pretreated for 1–2 h with p38 MAPK inhibitor SB-203580 (10 μM in DMEM), the JAK2 inhibitor AG-490 (10 μM in DMSO), the MEK1 and MEK2 inhibitor U-0126 (10 μM in DMSO), the MEK1 inhibitor PD-98059 (20 μM in DMSO) the PI3-kinase inhibitor LY-294002 (20 μM in DMSO), or AktiVIII (5 μM in DMSO) before IL-11 treatment. Cells were lysed, and changes in the abundance of cytoplasmic cleaved genomic DNA were determined with anti-histone and anti-DNA antibodies followed by colorimetric detection at 405 nm. Treated with the topoisomerase-1 inhibitor camptothecin (20 μM) served as a positive control in pilot studies. In addition, apoptosis in FasL-treated cells was detected by immunoblotting (as described below) using antibodies to caspase-8, cytochrome c, caspase-9, caspase-3, and full-length and cleaved poly(ADP-ribose) polymerase (PARP).

Immunoblotting and Akt kinase assays. Cells were grown in 100- or 60-mm culture dishes. After various treatments, cells were rinsed with ice-cold PBS, scraped on ice, pelleted, snap frozen, and stored at −70°C until use. Cell pellets were resuspended in lysis buffer containing 50 mM PIPES-NaOH (pH 6.5), 2 mM EDTA, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM DTT, and 1× complete protease inhibitor cocktail (Roche Biochemicals). Aliquots were taken for protein determination by the Bradford method (Bio-Rad, Hercules, CA). Lysates were added to 0.5 vol/vol 3× SDS sample buffer containing 187.5 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 150 mM DTT, and 0.03% bromophenol blue and heated to 95°C for 5 min. Fifteen micrograms of total cell lysate per condition were electrophoresed with 10% denaturing polyacrylamide gels and transferred to Immobilon P membranes (Millipore) for 2 h at 400 mA using Towbin buffer (20% methanol, 25 mM Tris, 190 mM glycine). Blots were blocked in 5% w/vol low-fat milk in Tris-buffered saline with Tween 20 (TBS-T) (10 mM Tris, 50 mM NaCl, and 0.1% Tween 20) for 1 h and incubated at 1:1,000 with the following polyclonal antibodies obtained from Cell Signaling Technology: anti-caspase-3 (17, 19, and 35 kDa, no. 9662), anti-cytochrome c (no. 4272), anti-caspase-9 (no. 9508), and anti-PARP (no. 9542). Anti-activated caspase-8 p20 was obtained from Santa Cruz Biotechnology (Sc-7890) and used at 1:100. Anti-β-actin (no. A5316) was obtained from Sigma and used at 1:20,000. For signaling experiments, the following primary antibodies were obtained from Cell Signaling Technology and used at 1:1,000: anti-phospho- Thr202 and Tyr205-p42/p44 ERK MAPK (no. 9101), anti-phospho-Thr180/Tyr182-p38 MAPK (no. 9211), anti-phospho-(Ser32/Met36)-JNK (no. 9251), anti-phospho-(Ser241)-p38 MAPK (no. 9251), and anti-phospho-(Ser96/244)-phosphoinositide-dependent protein kinase 1 (no. 3061). All primary antibodies were diluted in TBS-T with 5% w/vol low-fat milk or 5% BSA according to the manufacturer’s recommendations. Blots were washed and incubated with goat anti-rabbit horseradish peroxidase-linked secondary antibody at 1:2,000 (no. 7074; Cell Signaling Technology), except anti-β-actin, which used a donkey anti-mouse horseradish peroxidase-linked secondary antibody at 1:20,000 (Jackson ImmunoResearch, West Grove, PA). After samples were washed, chemiluminescent signals were detected with Lumiglo reagents (Cell Signaling Technology), using Kodak Biomax Light film. For nonradioactive measurement of IL-11-induced Akt kinase activity, phosphorylation of a glycogen synthase kinase 3β (GSK3β) peptide after Akt pull down was measured with
phospho-GSK3β-specific antibodies (no. 9840, nonradioactive Akt kinase assay, Cell Signaling Technology).

Preparation of cell membranes. To examine Fas insertion into the cell membrane, cells were grown in 0.5% FBS with or without IL-11 (100 ng/ml) in 150-mm cell culture dishes. After treatment, cells were harvested and Dounce homogenized in lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 1× complete protease inhibitor cocktail, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, and 2.5 mM sodium pyrophosphate) and spun to pellet nuclei (500 g, 5 min). The supernatant was removed and spun to pellet mitochondria (10,000 g, 10 min). The supernatant was removed and spun (49,200 g, 60 min), and supernatant was discarded. The resultant pellet was resuspended in buffer (10 mM Tris, 5 mM EDTA, and 1× complete protease inhibitor cocktail). Aliquots were taken for bicinchoninic acid protein determination (Sigma-Aldrich), the final membrane solution was heated with 0.5 vol 3× Laemmli, and samples were stored at −70°C until use. Polyacrylamide gels (7.5%) were run using 20 μg of membrane protein and processed as described above in Immuno-blotting and Akt kinase assays using anti-Fas (29 and 44 KDa) polyclonal antibody (no. 341289, Calbiochem) at 1:5,000 in 10% wt/vol low-fat milk in TBS-T overnight at 4°C. Coomasie staining was performed on immunoblots to ensure equal loading.

Semi-quantitative RT-PCR. Cells were grown in 100-mm culture dishes. Cells were exposed to 100 ng/ml IL-11 under serum-reduced conditions for 2, 8, and 24 h to determine which antiapoptotic transcripts were upregulated before the addition of FasL. Cells were rinsed twice in PBS and scraped on ice, and the resulting cell pellet was resuspended in 1 ml Trizol (Invitrogen) to isolate RNA as per manufacturer’s instructions. After the initial phenol-chloroform extraction, a second acid phenol-chloroform extraction (pH 4.3) was performed. After precipitation in isopropanol, RNA was dissolved in RNase-free water, and RNA integrity was confirmed by agarose gel electrophoresis. Samples were reverse-transcribed with avian myeloblastosis virus RT (Roche) to make first-strand cDNA using 1 μg of total RNA and random hexamers (Roche). PCR was performed with 5 μl of RT as template using Taq DNA polymerase (Qiagen, Valencia, CA). For Fas mRNA expression, cells were serum reduced in 0.5% FBS, and then IL-11 (100 ng/ml) was added for 6–48 h. Fas primers were 5′-CTGTTGCAATGCATGCTGCTGCTGCT-3′ (sense) and 5′-CTCCAGACTTTTGTCCCTGCTTCCTTTCTTTT-3′ (antisense) with cycle parameters of 94°C for 4 min, 94°C for 30 s, 60°C for 30 s, 72°C for 45 s (for 40 cycles), and 72°C for 7 min (19). Primer sequences and PCR conditions for rat cFLIP, survivin, cIAP1, 2, XIAP, Bcl-2, and Bcl-xL mRNA are available from the corresponding author. PCR products were run on a 1% TAE agarose gel using a 100-bp DNA ladder (Invitrogen) and photographed.

Isolation of mitochondrial and cytosolic fractions. Cells were grown to confluence in 5% FBS in 100-mm cell culture dishes and then switched to 0.5% FBS for 24 h. Cells were then treated with FasL (4 ng/ml) for 6–24 h in paired dishes, which were pooled when harvested. Mitochondrial fractions were prepared with the Pierce mitochondrial isolation kit (Pierce, Rockford, IL). Mitochondrial pellets were resuspended in 2% CHAPS in TBS and diluted in 0.5 vol 3× Laemmli buffer and heated at 55°C for SDS-PAGE. Immunoblotting was carried out as described above, and 20 μg of protein were loaded. Positive controls for cytochrome c release consisted of IEC-18 cells treated with staurosporine.

Data analysis. Densitometry was carried out using ScionImage software (Scion, Frederick, MD). Bands were initially normalized to β-actin density. In all inhibitor experiments, the intensity of FasL treatment alone was assigned a value of 1, with other lanes expressed as a fraction of that value. Where indicated, paired two-tailed t-tests or one-way ANOVA followed by Student-Neuman-Keuls multiple comparisons testing were used to determine the statistical significance of differences between continuous variables using InStat Software (GraphPad, San Diego, CA). Results are means ± SE of three or more experiments.

RESULTS

**IL-11 decreases apoptosis induced by FasL.** IEC-18 and IEC-6 cells were treated with 4 ng/ml FasL under serum-reduced conditions for 24 h and later examined by phase-contrast microscopy. As shown in Fig. 1A, IEC-18 cells treated with FasL demonstrated large numbers of dying cells, which were rounding up and lifting off compared with control cells. When pretreated with IL-11 (100 ng/ml) for 24 h before the addition of FasL, reduced numbers of dying cells were observed (n = 3). Cells treated with IL-11 alone appeared similar to controls. Studies using IL-11 at a higher dose of 300 ng/ml failed to confer further benefit (data not shown), and the dose of 100 ng/ml was used for the remainder of our studies. A slightly lower degree of protection was observed in IEC-6 cells (data not shown); thus studies were pursued with IEC-18 lines.

Because the activation of various signaling pathways is shared by members of the IL-6 family of cytokines to which IL-11 belongs, we tested whether IL-11 at a dose of 100 ng/ml conferred protection similar to that with IL-11 during FasL treatment. As shown in Fig. 1B, IL-6 did not reduce the cell death induced by FasL. Cells treated with IL-11 alone appeared similar to controls. Further confirmation of IL-11’s prosurvival effect on FasL-treated cells was obtained by measuring oligonucleosomal DNA release into the cytoplasm. In Fig. 1C, we demonstrate that, compared with cells treated with FasL, those pretreated with IL-11 (100 ng/ml) for 24 h show a 63.2 ± 5.9% decrease (P = 0.002 by paired two-tailed t-test; n = 4) in the amount of cleaved DNA appearing in the cytoplasm. Similar treatment with 100 ng/ml of recombinant human IL-6 failed to confer any resistance to apoptosis. Furthermore, neither IL-11 nor IL-6 by themselves had any significant effect on basal apoptosis in serum-reduced IEC-18 cells.

**IL-11 antagonizes FasL-induced cleavage of caspase-3 and PARP.** To confirm that IL-11 pretreatment inhibits FasL-induced apoptosis, the apoptotic pathway was dissected further upstream by examining the executioner caspase-3 and its cellular target PARP by immunoblot. As shown in Fig. 2A, treatment with 4 ng/ml FasL for 24 h (lanes 5 and 6) results in the appearance of cleaved caspase-3 (19-kDa band) and cleaved PARP (89-kDa band) compared with that shown in control samples (lanes 1 and 2). Pretreatment with IL-11 for 24 h before FasL results in a reduction in the abundance of cleaved caspase-3 and PARP to near control levels (lanes 7 and 8). Duplicates lanes on these blots represent cell lysates from different passages; this serves to demonstrate the consistency of the IL-11 effect between cells from different passages. The reduced cleavage of PARP suggests that IL-11 inhibits caspase-3 activity. It was also noted that IL-11 treatment alone reduced some of the caspase-3 cleavage associated with the serum reduction phase before the treatment with FasL (lanes 3 and 4 compared with lanes 1 and 2).

We next examined whether shorter time intervals of IL-11 exposure conferred the same degree of protection against FasL-induced apoptosis. When cells were exposed to IL-11 for 2 h before FasL treatment, significant reduction of caspase-3 cleavage still occurred, as shown in Fig. 2B. When IL-11 was added at the same time as FasL, variable minor attenuation of caspase-3 cleavage occurred (data not shown). The 2-h IL-11 pretreatment method was used for all mechanistic studies thereafter.

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IL-11 acts downstream of Fas and death-inducing signaling complex. We next proceeded to examine the Fas (CD95) pathway in greater detail. Given that it has been reported that IL-11 activates JAK/STAT signaling in IEC-18 cells and that cytokines such as interferon-γ also signal through JAK/STAT and can modify Fas membrane expression (36), we sought to determine whether IL-11 exerts its antiapoptotic effect by modulating Fas expression. First, we examined whether treatment with IL-11 led to the downregulation of Fas transcript abundance. We next examined Fas expression in enriched membrane fractions derived from IL-11-treated IEC-18 cells. As shown in Fig. 3A, IL-11 (100 ng/ml) does not exert a regulatory effect on Fas transcript abundance over time. Similarly, IL-11 also failed to exert any consistent effect on Fas protein expression in membrane fractions (Fig. 4B).

Fig. 2. IL-11 antagonizes FasL-mediated caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage. A: 24-h IL-11 pretreatment reduces the appearance of cleaved caspase-3 (19-kDa band) and cleaved PARP (89-kDa band) in FasL-treated cells. B: IL-11 treatment for only 2 h still confers resistance to caspase-3 cleavage. Cells from 2 different passages per condition shown here demonstrate similar results at different passage numbers. Results are representative of 3 separate experiments.

Fig. 3. IL-11 does not reduce Fas expression in IEC-18 cells. IL-11 (100 ng/ml) treatment for 6, 12, 24, and 48 h does not alter Fas (CD95) mRNA abundance in RT-PCR normalized to β-actin (A), and IL-11 does not affect Fas protein expression in membrane-enriched cellular fractions (B). Results are representative of 3 separate experiments. MW, molecular weight.
FasL induces intrinsic apoptotic pathway signaling in IEC-18 cells. Recognizing that the intrinsic pathway is activated during Fas ligation to mediate the full proapoptotic effect, evidence for this was sought. In Fig. 4A, we demonstrate that treatment with FasL over time (6, 12, and 24 h) results in the appearance of cleaved caspase-9 fragments by 12 h, suggesting that intrinsic pathway activation occurs. This was confirmed by the release of cytochrome c from the mitochondria into the cytosol, which was detectable by the 6-h time point (Fig. 4B). Staurosporine was used as a positive control for the detection of cytochrome c (Cyt-c) release from mitochondria (mit). FasL-induced cytochrome c release occurs by 6 h. Results are representative of 3 separate experiments.

IL-11 attenuates FasL-induced caspase-9 activation. We proceeded to dissect the signaling pathway downstream of Fas. Pretreatment with IL-11 for 2 h had no effect of IL-11 on the abundance of the activated p20 fragment of caspase-8 induced by FasL (Fig. 5A). In keeping with this finding, we found no effect of IL-11 on the cFLIP inhibitor of caspase-8 autoactivation by semi-quantitative RT-PCR (not shown). Because IL-11 has been reported to upregulate Bcl-2 in colonic epithelial cells, an effect that could lead to protection against cytochrome c release from mitochondria, we proceeded to determine whether IL-11 treatment resulted in reduced cytochrome c release from mitochondria in FasL-treated cells. As shown in Fig. 5B, no effect of IL-11 was seen on the timing or abundance of cytochrome c release in FasL-treated cells. To further support this, we found no effect of IL-11 on Bcl-2 or Bcl-xl by semi-quantitative RT-PCR (not shown). We then proceed to examine the effects of IL-11 on caspase-9 cleavage. This revealed an IL-11-mediated reduction in FasL-induced caspase-9 cleavage (Fig. 5C, lanes 7 and 8 vs. lanes 5 and 6), suggesting that IL-11 acts at a point between cytochrome c release and caspase-9 activation. This IL-11 effect appeared to be dose dependent, with greatest attenuation of caspase-9 cleavage seen in the 100 ng/ml treatment group (Fig. 5D, lane 6).

MEK1, MEK2, and Akt kinases mediate antiapoptotic signaling by IL-11. IL-11 activation of STAT3 in IEC-18 cells has previously been shown (34). We proceeded to determine whether IL-11 activates Akt and MAPK signaling pathways in IEC-18 cells. IL-11 has been previously shown to induce Akt phosphorylation at Ser473 in colonic epithelial cells (18); however, its effects on Akt kinase activity have not been demonstrated. As shown in Fig. 6A, IL-11 leads to the time-dependent phosphorylation of Akt at Ser473 and Thr308 as well as Ser241 of phosphoinositide-dependent protein kinase 1, which is the kinase responsible for Ser473 phosphorylation. Similarly, IL-11 induces Akt kinase activity in a time-dependent manner with the use of a GSK3β peptide substrate, as shown in Fig. 6B. In parallel, p42/p44 ERK1 and ERK2 were shown to be phosphorylated over a similar time course (Fig. 6C), although no phosphorylation of p38 MAPK occurred in response to IL-11 (Fig. 6D).
We next examined the effects of MEK1 and MEK2 inhibition using U-0126 and PD-98059. As shown in Fig. 8, A and B (lane 8 vs. lane 7), pretreatment with 10 μM U-0126 for 1 h before IL-11 significantly reversed the protective effect of IL-11 against FasL-induced accumulation of cleaved caspase-3 (U-0126 + IL-11 + FasL: 0.63 ± 0.10 vs. IL-11 + FasL: 0.45 ± 0.09; P < 0.05; n = 4) and cleaved caspase-9 (U-0126 + IL-11 + FasL: 1.39 ± 0.03 vs. IL-11 + FasL: 0.60 ± 0.14; P < 0.01, n = 3). Unlike LY-294002, any basal toxicity induced with U-0126 alone (lane 3) was attenuated by the presence of IL-11 (lane 5), thus suggesting that the reversal of IL-11’s protective effects in lane 8 is not due to superimposed U-0126 toxicity. The IL-11 dependence on MEK signaling was confirmed in Fig. 8B where pretreatment with 25 μM PD-98059 for 1 h before IL-11 significantly reversed the protective effect of IL-11 against FasL-induced accumulation of cleaved caspase-3 (PD-98059 + IL-11 + FasL: 0.82 ± 0.09 vs. IL-11 + FasL: 0.54 ± 0.16; P < 0.05, n = 3) and cleaved caspase-9 (PD-98059 + IL-11 + FasL: 1.10 ± 0.11 vs. IL-11 + FasL: 0.59 ± 0.14; P < 0.05; n = 3). These findings suggest that IL-11’s protective effects rely on intact MEK1 and MEK2 signaling pathways. As shown in Fig. 8C, both PD-98059 and U-0126 are potent inhibitors of IL-11-dependent ERK1 and ERK2 phosphorylation; however, the JAK inhibitor AG-490 had no effect.

When we examined the effects of the JAK inhibitor AG-490, which attenuates STAT3 phosphorylation in response to IL-11 in a dose-dependent manner (Fig. 9A), we found no increase in basal cleaved caspase-3 (Fig. 9B, lane 3). Furthermore, AG-490 pretreatment with 20 μM of the PI3-kinase inhibitor LY-294002 alone (lane 3) compared with untreated controls (lane 1). The cytoxic effect of LY-294002 alone was comparable to that induced by FasL (lane 3 vs. lane 6), and IL-11 could not overcome the caspase-3 cleavage induced by LY-294002 (lane 5). We then pursued a less toxic approach using Akt inhibitor VIII (IC50 = 58 and 210 nM for Akt1 and Akt2, respectively). We found that IEC-18 cells tolerated the Akt inhibitor well at 5 μM, with minimal increases in caspase 3 cleavage compared with that shown in control cells (Fig. 7B; lane 3 vs. lane 1). As shown in Fig. 7, B and C (lane 8 vs. lane 7, respectively), Akt inhibitor VIII pretreatment for 1 h before treatment with IL-11 significantly reversed the protective effect of IL-11 against FasL-induced accumulation of cleaved caspase-3 (Akt+1 /H11001 + IL-11 + FasL: 1.11 ± 0.19 vs. IL-11 + FasL: 0.51 ± 0.12; P < 0.05; n = 3) and cleaved caspase-9 (Akt+1 /H11001 + IL-11 + FasL: 0.75 ± 0.07 vs. IL-11 + FasL: 0.50 ± 0.05; P < 0.05; n = 3).
FasL-induced caspase-9 and -3 cleavage (A). Both inhibitors significantly attenuated the protective effects of IL-11 against caspase-3 cleavage (B). Neither of the inhibitors used in later experiments. In A, cells were pretreated with either 10 μM U-0126 (U) or 25 μM PD-98059 (PD) for 1 h before treatment with IL-11 for 2 h. After the addition of FasL for 16 h, cells were harvested. Any toxicity induced by U-0126 or PD-98059 alone was attenuated by cotreatment with IL-11 (lane 3 vs. lane 5; lane 7 vs. lane 8, respectively). Both inhibitors significantly attenuated the protective effects of IL-11 against FasL-induced caspase-3 and -9 cleavage (lane 3 vs. lane 5, respectively). As shown in C, both inhibitors block ERK1 and ERK2 phosphorylation induced by IL-11. The JAK inhibitor AG-490 (AG) had no effect. Results are representative of 3 separate experiments.

490 did not significantly reverse IL-11-mediated protection against FasL-induced caspase-3 cleavage (lane 8 vs. lane 7), suggesting that the reduction in STAT3 phosphorylation by AG-490 was not sufficient to reverse the protective effects of IL-11.

The effects of Akt and MEK inhibition were further delineated by examining a more robust readout of apoptosis using an oligonucleosomal DNA ELISA. As shown in Fig. 10, pretreatment with AktiviVIII only showed a trend toward reversal of the IL-11 effect, whereas the pretreatment with U-0126 led to a significant reversal of the protective effect of IL-11 (IL-11 + Fasl: 0.49 ± 0.16 vs. U-0126 + IL-11 + Fasl: 1.06 ± 0.049; P < 0.001; n = 3). Similarly, inhibition of IL-11 signaling with the PD-98059 inhibitor of MEK1 led to a reversal of the IL-11 proapoptotic effect (IL-11 + Fasl: 0.71 ± 0.09 vs. PD-98059 + IL-11 + Fasl: 1.22 ± 0.18; P < 0.05; n = 4). As a control, we used the p38 MAPK inhibitor SB-203580 because p38 MAPK signaling is not activated by IL-11. Also, SB-203580 was both nontoxic to IEC-18 cells and dissolved in DMSO, as were other inhibitors. No reversal of IL-11’s protective effect was observed. Finally, using the JAK inhibitor AG-490, we found no antagonism of IL-11’s antiapoptotic effects by DNA ELISA, an effect that coincided with the lack of attenuation of IL-11’s effect on FasL-induced caspase-3 cleavage. Further studies using small interfering RNA to STAT3 or selective cell-permeable STAT3 inhibitory peptides are needed to make firmer conclusions about STAT3 because JAK inhibition by AG-490 only partially attenuated IL-11-induced STAT3 phosphorylation even at higher doses.

DISCUSSION

IL-11 administration increases survival of mice during chemotherapy or radiation by increasing recovery of the small intestinal mucosa through direct effects on the epithelial cell compartment (27). Treatment with IL-11 maintains epithelial barrier function and reduces numbers of systemic bacterial foci during sepsis-induced intestinal damage (25, 26, 30). In vitro, IL-11 has been found to reduce the secretion of proinflammatory cytokines such as TNF-α from LPS-stimulated human peritoneal macrophages. Similar effects have been observed in vivo in mice treated with LPS (41). Although direct effects on immune cell signaling may convey part of the protective effects of IL-11, it has been repeatedly proposed that IL-11 exerts direct effects on the intestinal epithelial cell’s ability to resist injury. Presently, little is known about the molecular mechanisms behind many of IL-11’s protective effects both in vitro and in vivo.

FasL is increased in the lamina propria of the intestine during states of inflammation such as ulcerative colitis (44), and Fas receptor activation has been implicated as a contributor to the epithelial injury characteristic of activated T-cell-mediated intestinal inflammation. Our study describes a novel protective mechanism for IL-11 in the intestinal epithelial cell and demonstrates that IL-11 significantly inhibits FasL-mediated apoptosis in IEC-18 intestinal crypt cells through a mecha-
anism that appears to depend primarily on the MEK1 and MEK2 signaling pathway. Importantly, this effect appears to be specific to IL-11 because a closely related family member, IL-6, could not confer the same protection against FasL-mediated apoptosis. Although studies describing protective effects of IL-11 and IL-6 against FasL-induced apoptosis have been reported in primary lung fibroblasts, these studies did not delineate mechanisms and did not pursue effects of these cytokines on the pulmonary epithelium (23). We have shown that the most proximal point in the apoptotic cascade where IL-11 demonstrates a noticeable effect on proapoptotic signaling induced by FasL is at the level of caspase-9 activation, as measured by caspase-9 cleavage. This would support the previous observations by Kiessling et al. (18) in a model of anoikis in which treatment with IL-11 was associated with differences in anoikis-induced caspase-9 activity.

It should be noted that caspase-9 is phosphorylated by Akt and by ERK2, leading to its inhibition; however, no Akt consensus phosphorylation motif has been identified in rat caspase-9 (1). Although our findings at the level of caspase-9 cleavage could support the hypotheses that IL-11 signaling leads to either ERK2-mediated phosphorylation of caspase-9 at Thr125 leading to its inhibition or alternatively that IL-11 alters the phosphorylation of the serine/threonine phosphatase type-
1α, which also regulates caspase-9 activity (9), it appears that the inhibition of caspase-9 cleavage alone is insufficient to explain the full protective effect of IL-11 in our experiments. We propose that IL-11 likely antagonizes other caspase-independent pathways to convey its full antiapoptotic effect and base this on the discordance between 1) IL-11’s dependence on both Mek and Akt signaling to protect against caspase-9 cleavage and 2) the sole dependence on intact MEK signaling to convey the protective IL-11 effect on the more physiological downstream releases of cytoplasmic oligonucleosomal DNA in FasL-treated cells. As shown in Fig. 7, A and B, inhibition of Akt by AktiVIII significantly reverses the effects of IL-11 on caspase-9 and caspase-3 cleavage, yet its ability to reverse the protection of IL-11 conferred against DNA cleavage in Fig. 10 is not significant. Conversely, as shown in Fig. 8, A and B, MEK inhibition by both U-0126 and PD-98059 leads to the reversal of IL-11 protection against caspase-9, caspase-3, and DNA cleavage.

As we consider possible mechanisms accounting for these observations, we note that ongoing studies are needed to compare and contrast the ability of IL-11-mediated MEK1 and MEK2 vs. Akt signaling to interfere with other components of the caspase-independent mitochondrial-derived cell death machinery, such as SMAC/DIABLO, apoptosis-initiating factor (AIF), endonuclease G, and HtrA2/Omi (37, 43). Recent reports support the contention that inhibition of caspase-9 activation alone may not be sufficient to inhibit FasL-induced apoptosis. For example, in neointimal cells from atherosclerotic plaques, knockdown of caspase-9 alone is insufficient to attenuate FasL-induced apoptosis (44). In these studies, BclXL overexpression was associated with the downregulation of mitochondrial amplification loops, leading to reduced AIF and SMAC/DIABLO release from mitochondria during Fas receptor ligation and a consequent reduction in the amount of apoptosis. The MEK pathway has been implicated during cell death receptor activation in other cells such as melanoma cells in which ERK signaling reduces SMAC/DIABLO release from mitochondria in response to TRAIL (45), a related cell death receptor ligand that shares several overlapping apoptotic signaling mechanisms with FasL. Furthermore, ERK inhibition has been linked to the release of AIF from mitochondria and its subsequent localization to the nucleus in breast cancer cells during cell stress (28). Despite the inability of our RT-PCR experiments to identify that IL-11 increases BclXL mRNA among other targets such as XIAP and cIAP1 and cIAP2 over 2–24 h of IL-11 exposure prior to Fas ligation, protein expression was not examined. Moreover, because the effect of IL-11 only requires 2 h of pretreatment before FasL stimulation, we suspect that IL-11 likely modifies endogenous cellular responses to FasL and potentiates endogenous cell survival-signaling programs activated by proapoptotic stimuli. For example, IL-11 could modulate endogenous responses to FasL by amplifying the normal intestinal epithelial increase in endogenous cIAP1 and cIAP2 expression initiated by FasL (35) or other regulators of mitochondrial membrane permeability. Further studies are needed to determine whether IL-11 alone can increase BclXL protein expression in IEC-18 cells or modify the response of BclXL and other regulators of mitochondrial membrane integrity protein during FasL-induced apoptosis.

We do not propose that the findings presented herein will apply to all scenarios characterized by death receptor activation. Although ERK1 and ERK2 signaling can protect melanoma cells from TRAIL-induced apoptosis in a caspase-9–independent manner (45), in experiments not shown here, IL-11 was unable to protect against the vigorous combination treatment of 20 ng/ml TNF-α and 25 μg/ml cycloheximide in IEC-18 cells. One interpretation of this data is that TNF-α- and cycloheximide-induced IEC-18 apoptosis is mostly dependent on the extrinsic apoptotic pathway and relies on the activation of large amounts of caspase-8 followed by potent executioner caspase activation. This finding would support the hypothesis that IL-11 primarily functions as an inhibitor of the intrinsic apoptotic pathway. Alternatively, it would suggest that the IL-11 effect relies on de novo protein synthesis to mediate its effect. The discordance between caspase inhibition and true measures of downstream apoptosis supports the rationale for future work to examine caspase-independent pathways involving SMAC/DIABLO and AIF, as well as other DNA toxic mitochondria-derived proteins such as HtrA2/Omi and EndoG.

From a therapeutic standpoint, the full clinical potential of IL-11 in chronic IBD is unrealized. Our in vitro data and data from animal studies continue to support the need to develop studies that examine the effects of IL-11 on the preservation and/or restoration of mucosal integrity in other clinical settings, such as ulcerative colitis and refractory celiac disease.

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