Cytokine-induced Apoptosis in Epithelial HT-29 Cells Is Independent of Nitric Oxide Formation

EVIDENCE FOR AN INTERLEUKIN-13-DRIVEN PHOSPHATIDYLINOSITOL 3-KINASE-DEPENDENT SURVIVAL MECHANISM

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A combination of the pro-inflammatory cytokines interleukin (IL)-1α, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α induces nitric oxide synthase mRNA expression and nitric oxide (NO) generation in the human colon carcinoma cell line HT-29. This can be inhibited by pretreatment with IL-13 via a phosphatidylinositol (PI) 3-kinase-dependent mechanism (Wright, K., Ward, S. G., Kolios, G., and Westwick, J. (1997) J. Biol. Chem. 272, 12826–12833). Since NO has been implicated in regulating mechanisms leading to cell death, while activation of PI 3-kinase-dependent signaling cascades are thought to be involved with promoting cell survival events, we have investigated the outcome of these cytokine treatments on apoptosis and cell survival of HT-29 cells. Initiation of apoptosis can be achieved by the combinations of IFN-γ/TNF-α, IFN-γ/CD95, IL-1α/IFN-γ, and IL-1α/IFN-γ/TNF-α to varying extents. Induction of apoptotic markers by HT-29 cells in response to cytokine treatment is not dependent on NO production. Pretreatment with IL-13 protects against IL-1α/IFN-γ/TNF-α and IFN-γ/TNF-α as well as IFN-γ/CD95-induced (but not IL-1α/IFN-γ-induced) cell death. In addition, IFN-γ/TNF-α and IL-1α/IFN-γ/TNF-α stimulate activation of caspase-8 and caspase-3, which IL-13 pretreatment was able to partially inhibit and delay. IL-13 also stimulates activation of the major PI 3-kinase effector, protein kinase B. The PI 3-kinase inhibitors wortmannin and LY294002 inhibit IL-13 stimulation of protein kinase B as well as the cell survival effects of IL-13. These data demonstrate that cytokine-induced apoptosis of HT-29 cells is NO-independent and that the activation of a PI 3-kinase-dependent signaling cascade by IL-13 is a key signal responsible for the inhibition of apoptosis.

Interleukin-13 (IL-13) is a pleiotropic cytokine secreted by activated Th-2 lymphocytes which regulates a variety of immune target cells (1, 2). In B lymphocytes, IL-13 induces proliferation and differentiation, promotes CD23 expression and production of certain immunoglobulins such as IgG4 and IgE (3–5). In monocytes, IL-13 induces morphological changes (2), up-regulates expression of members of the integrin superfamily and major histocompatibility complex class II antigen expression, and down-regulates expression of CD14 and FcyR receptors (6). In lipopolysaccharide-stimulated monocytes, IL-13 also acts as a suppressor of proinflammatory cytokines (e.g. TNF type α, IL-1, and IL-6), chemokines (e.g. IL-8 and macrophage inflammatory protein-1α), and hemato poetic growth factors (e.g. granulocyte/macrophage-colony stimulating factor and granulocyte-colony stimulating factor) expression by activated monocytes/macrophages or endothelial cells (1, 6). Another target of IL-13 is epithelial cells and we have recently demonstrated that IL-13 can modulate chemokine generation from the human colonic epithelial cell line HT-29 (7, 8) and inhibit iNOS expression and NO generation in this system (9). Activation of the signaling enzyme phosphatidylinositol 3-kinase (PI 3-kinase) by IL-13 is important for mediating these effects (8, 10). PI 3-kinase and its major downstream effector, the serine/threonine kinase protein kinase B (PKB), have been shown to be key mediators of growth factor-induced cell survival and protection against c-Myc-induced cell death in fibroblasts (11–13). In addition, IL-13 has been reported to up-regulate the cell survival factors Bcl-xL and Mc-I as well as protect B lymphocytes from apoptosis (14). Moreover, the related cytokine, IL-4, also enhances cell survival (15) and this correlates well with observations that both IL-13 and IL-4 stimulate PI 3-kinase (10, 16) and with the notion that the IL-13 receptor and IL-4 receptor share a common subunit in signal transduction (17–19).

We have previously shown that a combination of pro-inflammatory cytokines (IL-1α/IFN-γ/TNF-α) up-regulates iNOS expression and generates NO in a human colonic epithelial cell line HT-29 (20). Also, it has recently been shown that IFN-γ, in combination with TNF-α or anti-CD95, induces apoptosis in HT-29 cells (21) and an increased frequency of epithelial apoptosis mediated by the CD95-CD95L system is seen in ulcerative colitis (22, 23), which is an inflammatory bowel disease of unknown etiology. However, colonic epithelial cell injury, resulting in impaired barrier function, could contribute to the pathogenesis of inflammatory bowel disease (24). It has been postulated that overproduction of nitric oxide (NO) by inflamed mucosa may play a role in the pathophysiology of inflammatory bowel disease due to the increased expression of the inducible form of nitric oxide synthase (iNOS) found in biopsies taken from patients with active ulcerative colitis as compared with normal colon (9). The production of NO might play a critical role in the resolution of inflammation (25), possibly by inducing apoptosis in the leukocytic population recruited to the area (e.g. neutrophils) (25). While NO has also been reported to inhibit...
apoptosis in several settings (26–29), it has also been reported to mediate cell death through mechanisms consistent with apoptosis in various cells including peritoneal macrophages (30–32), β-cells (33, 34), and thymocytes (35). Also, overproduction of NO may lead to oxidant-induced injury of the colon epithelial crypt (36), possibly by the reaction with superoxide to form peroxynitrite which in turn results in the nitration of proteins on tyrosine residues (37).

In this study, we sought to ascertain whether there is a relationship between NO production and apoptosis of HT-29 epithelial cells observed in response to a combination of cytokines and/or CD95 ligation. In addition, given that the ability of IL-13 to inhibit iNOS expression and NO generation in this system is driven by PI 3-kinase-dependent pathway, we investigated whether IL-13 could provide a cell survival signal through PI 3-kinase to protect against cytokine-driven apoptotic signals.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant IL-13 was purified from culture supernatants of stable transfected CHO cells (1) and generously provided by Dr. A. Minty (Sanofi Elf Bio Recherche, Labège, France). Human recombinant IFN-γ (specific activity, 6 × 10^7 units/mg) were generous gifts from Glaxo-Wellcome (Stevenage, United Kingdom) and Bayer (Slough, UK), respectively. Human recombinant IFN-α (specific activity, > 2.0 × 10^7 units/mg) and histone H2B was purchased from Roche Molecular Biochemicals. Anti-human Fas monoclonal antibody (IgM CH11 clone) was purchased from Upstate Biotechnology. PKBα polyclonal Ab was from Brian Hennings (Friedrich Miescher-Institute, Basel, Switzerland).

2,3-Diaminonaphthalene was purchased from Lancaster Synthesis Ltd. LY294002 (2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one) was purchased from Affiniti (Exeter, UK), Z-VADEMK was purchased from Calbiochem (Nottingham, UK), and [γ-32P]ATP (3000 Ci/nmol) was purchased from DuPont NEN, UK. All other reagents were from Sigma (Poole, UK).

**Cell Culture**—The human colonic epithelial carcinoma cell line HT-29 was obtained from the European Collection of Animal Cell Cultures. Cells were cultured in humidified incubators at 37 °C, 5% CO_2 in McCoy's 5A medium supplemented with 10% fetal bovine serum, 10 units/ml penicillin/streptomycin, and 10 µg/ml fungizone. The cells were passaged weekly and, for experiments, HT-29 cells were aliquoted into 96-well plates (10^4 cells/well) and allowed to adhere overnight prior to cytokine stimulation in medium without serum.

**Detection of Apoptosis**—The detection of apoptosis was performed by determining the histone-associated DNA fragments (mono- and oligonucleosomes) generated by the apoptotic cells using the photometric cell death detection ELISAPLUS® (Roche Molecular Biochemicals). Briefly, 10^5 cells/well were aliquoted into 96-well plates and allowed to adhere overnight. Cells were then treated and pelleted as indicated, the supernatants were removed and the cell pellets were lysed and assayed according to the manufacturer’s instructions. Photometric analysis, with 2,2′-azino-di-(3-ethylbenzthiazolinesulfonate) as the substrate, was performed using a microtiter plate-reader (Dynatech MR5000). The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated using the following formula,

\[ \text{milliuunits of the sample (dying/dead cells)} / \text{milliuunits of the corresponding control untreated cells} = \text{enrichment factor (milliuunit cell)} \times (10^{-3}) \]  

Alternatively, apoptosis was determined by direct fluorescence detection of end-labeled genomic DNA using the “Onco Apoptot Direct In Situ Apoptosis Detection Kit Fluorescein” (Appligene, Durham, UK). Treated and untreated cells were spun onto slides, fixed, and stained according to the manufacturer’s instructions. The slides were observed under a fluorescence microscope. The results were scored by counting apoptotic cells (green) and viable cells (red) randomly in various fields. For each condition, 500 to 1000 cells were counted. In some experiments, apoptosis was detected by measuring the externalization of phosphatidylserine by fluorescein isothiocyanate-labeled annexin V binding using the Apoptosis Detection Kit (R&D Systems, Abingdon, UK). Cells were treated as indicated in the figure legends and stained according to the manufacturer’s instructions before analysis by flow cytometry (Becton Dickinson FACS Vantage). Percent increases in annexin V fluorescence binding above controls were measured.

**Fluorometric Assay for Nitric Oxide**—NO production by HT-29 cells was determined by measuring the stable end-product nitrite in the cell culture supernatants by fluorometric assay which is based upon the reaction of nitrite with 2,3-diaminonaphthalene to form the fluorescent

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**TABLE I**

| Table I IL-13 protects against IL-1α/IFN-γ/TNF-α-induced apoptosis of HT-29 cells |
|-----------------------------------------------|
| Percentage cells presenting apoptotic markers (%) | 8 h | 12 h | 24 h | 48 h |
| Control | 3.1 ± 0.9 | 5.2 ± 0.9 | 7.1 ± 0.7 | 13.4 ± 1.1 |
| IL-1α | 2.8 ± 0.3 | 4.8 ± 0.7 | 7.8 ± 1.6 | 14.2 ± 0.8 |
| IFN-γ | 3.3 ± 0.9 | 5.6 ± 1.4 | 9.2 ± 0.5 | 14.7 ± 1.7 |
| IL1α/IFN-γ/TNFα | 4.1 ± 0.8 | 7.8 ± 0.3 | 10.1 ± 1.6 | 16.1 ± 2.3 |
| IL13+IL1α/IFN-γ/TNFα | 24.3 ± 1.2 | 32.3 ± 0.2 | 61.3 ± 1.4 | 81.2 ± 1.1 |

* *p ≤ 0.01.
** *p ≤ 0.05.

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**Fig. 1.** IL-1α/IFN-γ/TNF-α-induced apoptosis in HT-29 cells is inhibited by IL-13. HT-29 cells (10^4 cells) were either left untreated (● control) or treated with 30 ng/ml IL-13 (▲, IL-13 + IL1α/IFN-γ/TNFα (3C)) for 1 h. Where indicated, the cells were then further treated with IL-1α (10 ng/ml)/TNF-α (100 ng/ml), and IFN-γ (300 units/ml) (●, IL-1α/IFN-γ/TNF-α) or left untreated (■, control). At the times indicated, the supernatants were removed and the cell pellets were lysed and apoptosis determined using the ELISAPLUS® described under “Experimental Procedures.” The data are the mean ± S.E. of three separate experiments.
product 1-(H)-naphthotriazole as described previously (10, 38).

Detection of Caspase Activity—Colorimetric protease assay kits for both caspase-8 and -3 (Chemicon Intl. Inc., Temecula, CA) are based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. Briefly, 3 × 10⁵ cells/well were aliquoted into 96-well plates and allowed to adhere overnight. Cells were then treated as indicated and pelleted after 24 h to include any floating cells. The supernatants were removed and the cell pellets were lysed and assayed according to the manufacturer’s instructions. After a 2-h incubation with DEVD-pNA substrate, the pNA light emission was quantified using a microtiter plate reader at 405 nm. Comparison of the absorbance of pNA from the apoptotic sample with uninduced control determined the fold increase in caspase activity.

Cell Lysis—10⁵ cells/ml were stimulated and incubated at 37 °C in McCoy’s as indicated. Reactions were terminated by the addition of 1 ml of ice-cold lysis buffer (1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate, and 1 mM sodium molybdate). Lysates were incubated at 4 °C for 15 min, followed by centrifugation at 14,000 rpm.

Protein Kinase B Assays—Aliquots of cell lysate supernatant were boiled in Laemmli buffer and electrophoresed through 12.5% (v/v) acrylamide gels (with an acrylamide:bis-acrylamide ratio of 37.5:1) by SDS-PAGE and the proteins were transferred by electrophoretic blotting onto nitrocellulose (Schleicher & Schuell) as described previously (10, 39). The proteins were transferred by electroblotting onto nitrocellulose (Schleicher & Schuell) as described previously (10, 39). The blots were probed with a phosphospecific PKB antibody (0.5 μg/ml) which only has affinity for the active, 473Ser-phosphorylated forms of PKB (New England Biolabs). Alternately, cells were stimulated as described above and PKBα was immunoprecipitated from cell lysates with 1 μg of anti-PKB (Santa Cruz) and assayed for in vitro kinase activity using histone H2B as a substrate as described previously (40).

Immunoprecipitation of Bad—Bad was immunoprecipitated from cell lysates with 4 μg of anti-Bad mAb (Transduction Labs). After addition of 30 μl of protein G-Sepharose beads (50% suspension in phosphate-buffered saline), immunoprecipitates were rotated for 1 h at 4 °C and then washed three times in lysis buffer, resuspended in Laemmli buffer, and boiled for 5 min prior to electrophoresis through 12.5% (v/v) acrylamide gels (with an acrylamide:bis-acrylamide ratio of 37:1) by SDS-PAGE. The proteins were transferred by electroblotting onto nitrocellulose (Schleicher & Schuell) as described previously (10, 39). Bad was immunoblotted with 0.5 μg/ml anti-Bad polyclonal antibody (Santa Cruz) and proteins visualized by a chemiluminescence detection system (ECL, Amersham, UK) with a goat anti-rabbit Ig (0.1 μg/ml) conjugated with horseradish peroxidase as a secondary antibody (DAKO).

**RESULTS**

**Pro-inflammatory Cytokines Induce Apoptosis of Colon Epithelial Cells Which Is Inhibited by IL-13—Growth-arrested HT-29 monolayers stimulated with a combination of the pro-inflammatory cytokines IL-1α (10 ng/ml), IFN-γ (300 units/ml), and TNF-α (100 ng/ml) result in a 25–80% increase in the expression of apoptotic markers, as assessed by a number of assays which measure early and late stage markers of apoptotic events (namely externalization of phosphatidylserine, DNA fragmentation, and DNA-histone association), over a time course of 4–24 h (Table I and Fig. 1). Pretreatment of the HT-29 cells with the anti-inflammatory cytokine IL-13 (30 ng/ml) markedly inhibited apoptotic events stimulated by the pro-inflammatory cytokines (Table I and Fig. 1). These experiments indicate that IL-13 consistently inhibited the induction of apoptotic markers in cells treated with the combined stimuli of IL-1α/IFN-γ/TNF-α by 50–65%. Treatment of HT-29 cells for 24 h with IL-13 had no effect on the level of expression of TNF receptor-1 as assessed by flow cytometry using a receptor-specific Ab, thus confirming previous observations (41). Similarly, flow cytometry using appropriate receptor Abs also revealed that IL-13 had no effect on the levels of expression of IL-1 receptor (type-1) or IFN-γ receptor (data not shown), indicating that down-regulation of receptor expression cannot account for the observed reduction in cell death.

**IL-13 Is Required in Combination with the Death-inducing Factors TNF-α or CD85 Ligation to Induce Apoptosis—**Ligation of CD95 (Fas/APO-1), which is a member of the TNF receptor superfamily, is associated with the induction of apoptosis in several cell types (42) and flow cytometry has revealed the presence of CD95 on HT-29 cells used in this study (data not shown). We therefore performed experiments to investigate whether or not CD95 ligation stimulates apoptosis of HT-29 cells. In this respect, ligation of CD95 with the antibody CH11 did not induce apoptosis above control basal levels (Fig. 2). However, treatment of HT-29 cells with IFN-γ in combination with the anti-CD95 mAb CH11, induced an approximate 9-fold increase in DNA fragmentation over 24 h, which was comparable to the apoptosis induced by IFN-γ and TNF-α (Fig. 2). By contrast, the minimum cytokine combination for the induction of iNOS and NO generation, namely IL-1α/IFN-γ (20), induced only a 5-fold increase in DNA fragmentation above basal levels. Preincubation with IL-13 again reduced the prevalence of apoptosis.
apoptotic markers induced by IFN-γ/CH11 and IFN-γ/TNF-α by approximately 50%, but did not inhibit apoptosis driven by IL-1α/IFN-γ (Fig. 2).

**Cytokine-driven iNOS Activation and Apoptosis Are Independent Functional Events**—Several studies have demonstrated that nitric oxide regulates apoptosis in a number of settings (reviewed in Ref. 43). Given that concentrations of IL-1α/IFN-γ/TNF-α that are known to induce iNOS expression and that NO production can also stimulate apoptosis, we considered the possibility that this apoptotic response may be NO-dependent. To investigate this possibility we pretreated HT-29 cells with the iNOS inhibitor 500 μM aminoguanidine, which markedly inhibited the concentration of nitrite generated by IL-1α/IFN-γ/TNF-α (although less inhibition was observed with the minimum combination of IL-1α/IFN-γ) over 24 h (Fig. 3A), but had no effect on the apoptotic signals provided by combinations of either IL-1α/IFN-γ/TNF-α or IL-1α/IFN-γ (Fig. 3B).

Initial apoptotic events are known to result in the activation of the proteolytic enzyme cascades involving caspases which cleave specific proteins and irreversibly commit the cell to apoptotic death (44). Inhibition of this proteolytic cascade can be achieved using a broadly selective caspase inhibitor Z-VAD-FMK (45). We therefore used this caspase inhibitor to assess whether cytokine-driven iNOS production was dependent upon activation of caspases. Hence, pretreatment of cells with 50 μM Z-VAD-FMK completely abrogates the apoptosis of HT-29 cells stimulated by IL-1α/IFN-γ/TNF-α, IL-1α/IFN-γ, IFN-γ/TNF-α, and IFN-γ/CH11 (Fig. 4A). However, the caspase inhibitor did not interfere with the observed ability of HT-29 cells to generate nitrite in response to IL-1α/IFN-γ/TNF-α or IL-1α/IFN-γ (Fig. 4B), as does pretreatment with IL-13. Furthermore, IFN-γ/TNF-α (Fig. 4B) and IFN-γ/CH11 (data not shown) are not able to induce iNOS or stimulate the generation of nitrite, but are able to stimulate apoptosis (Fig. 4A). Together, these data indicate that cytokine-driven iNOS activation and apoptosis are separable, independent events.

**Reversal of IL-13-induced Inhibition of Apoptosis by Wortmannin and LY294002**—We have previously shown that IL-13 strongly activates the lipid kinase PI 3-kinase (10) which is believed to be a pivotal upstream component of a signaling cascade important in promoting cell survival events in many cell types (11–13, 46). Hence, to investigate the role of PI 3-kinase in mediating the anti-apoptotic effects of IL-13 on IL-1α/IFN-γ/TNF-α-stimulated apoptosis of HT-29 cells, the PI 3-kinase inhibitors wortmannin and LY294002 were used. Preincubations of wortmannin (10–300 nM) for 10 min before cytokine treatments were able to dose-dependently reverse the ability of IL-13 to protect HT-29 cells from cytokine-induced apoptosis (Fig. 5A). Equally, the structurally unrelated PI 3-kinase inhibitor, LY294002 (1–30 μM), was also able to reverse the IL-13 effect (Fig. 5A). Similarly, treatment of HT-29 cells

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**Fig. 3.** Effect of iNOS inhibitor on cytokine-induced nitrite production and apoptosis. A, HT-29 cells (2.5 × 10⁵ cells) were treated for 10 min with vehicle or with 500 μM aminoguanidine (AG) as indicated at 37 °C. The cells were then further treated with combinations of IL-1α (10 ng/ml), TNF-α (100 ng/ml), or IFN-γ (300 units/ml) as indicated or left untreated. Nitrite production after the indicated treatments was determined in supernatants after 24 h at 37 °C. The cells were then lysed and apoptosis determined using the ELISA assay as described under “Experimental Procedures.” The data are the mean ± S.E. of three separate experiments. B, HT-29 cells (10⁴ cells) were treated for 10 min with vehicle or with 500 μM aminoguanidine (AG) as indicated and then treated with combinations of IL-1α (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (300 units/ml) as indicated or left untreated for 24 h. Supernatants were removed and the cell pellets were lysed and apoptosis determined using the ELISA assay as described under “Experimental Procedures.” The data are the mean ± S.E. of three separate experiments.

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**A**

![Graph showing nitrite production](image)

**B**

![Graph showing apoptosis](image)
with either wortmannin (Fig. 5B) or LY294002 (data not shown), also prevented the IL-13-mediated inhibition of apoptosis induced by either IFN-γ/TNF-α or IFN-γ/CH11

**IL-13 Activates the PI 3-Kinase Effector Protein Kinase B—PKB** is a major downstream effector of the PI 3-kinase-dependent signaling cascade and has been shown to be a key mediator required for growth factor-induced cell survival and protection against c-Myc-induced cell death in fibroblasts (11–13). We therefore investigated whether the protective effects of IL-13 on IL-1α/IFN-γ/TNF-α-stimulated apoptosis in HT-29 cells, correlated with IL-13 activation of PKB. Hence, cell lysates derived from resting and IL-13-stimulated cells were immunoblotted using a phosphospecific antibody to the phosphorylated active form of PKB. IL-13 can be shown to activate PKB within 5 min stimulation, up to a maximum at 10 min, and is comparable in magnitude with PKB activation observed in response to insulin (5 μg/ml) treatment as a positive control (Fig. 6A). Insulin, however, provided only a 10–20% protective effect against cytokine-induced apoptosis (data not shown). The IL-13-stimulated activation of PKB appears to be sustained for up to 20 min, but has returned to control levels after 1 h. Both wortmannin and LY294002 were used to inhibit this signal, adding further evidence that the activation of PI 3-kinase by IL-13 leads to the activation of PKB and that this pathway is anti-apoptotic in this system. Blots were stripped and reprobed with an anti-PKB antibody provided in the kit to verify equal loading and efficacy of protein transfer (Fig. 6A). In addition, endogenous PKB was immunoprecipitated from IL-13-stimulated cells in the presence and absence of the PI 3-kinase inhibitor, wortmannin, and the immunoprecipitates were assayed for in vitro PKB activity. This approach confirmed that PKBα was activated by IL-13 in a PI 3-kinase-dependent manner (Fig. 6B).

The mechanism by which PKB is believed to promote cell survival involves the serine phosphorylation of the death promoting Bel-2 family member Bad causing it to dissociate from Bel-xL, thus allowing Bel-xL to act as a survival factor (47, 48). Immunoblotting of HT-29 whole cell extracts revealed no detectable amounts of Bad (data not shown). However, immunoprecipitation and immunoblotting of Bad, after electrophoresis through a low-bis 12.5% acrylamide gel, revealed a barely detectable hyperphosphorylated form of Bad as characterized by its shift in gel mobility (Fig. 7).

**Effect of IL-13 on Cytokine-induced Activation of the Caspase Cascade**—Activation of the caspase cascade is pivotal to the death execution phase of apoptosis and it appears that caspase-8 is the apical member of the pathway induced by CD95 and TNF receptor-1 with caspase-3 lying downstream (49–51). Hence, we investigated whether the induction of apoptotic markers induced by various cytokine combinations also correlated with activation of caspase-8 and -3. Indeed, treatment of cells with IL-1α/IFN-γ/TNF-α and IFN-γ/TNFα (Fig. 8A), but not IL-1α/IFN-γ (data not shown) stimulated caspase-8 and caspase-3 activity. In comparison, while treatment with IL-1α did not stimulate caspase activity, both TNF-α and IFN-γ elicited modest activation of these caspases (Fig. 8B).
Interestingly, pretreatment of HT-29 cells with IL-13 consistently induced a partial inhibition of IL-1α/IFN-γ/TNF-α-stimulated caspase-8 and caspase-3 activity at early time points (e.g., 12 h) which may constitute a delay in caspase activation by IL-13 (Fig. 8, B and C).

**DISCUSSION**

In this report we demonstrate that a combination of pro-inflammatory cytokines, namely IL-1α/IFN-γ/TNF-α, stimulates the expression of apoptotic markers in approximately 25–80% of cytokine-treated HT-29 cells (depending on time of analysis) as evidenced by assays that detect DNA fragmentation and externalization of phosphatidylserine. Apoptosis can also be stimulated to varying extents by the combination of IL-1α/IFN-γ or IFN-γ/TNF-α. Moreover, we present evidence that the induction of these apoptotic markers is not dependent on the expression of iNOS and NO production. Furthermore, pretreatment with the anti-inflammatory cytokine IL-13 which is known to prevent induction of NO production by HT-29 cells in response to IL-1α/IFN-γ/TNF-α (9, 10), also protects against IL-1α/IFN-γ/TNF-α, IFN-γ/TNF-α, and IFN-γ/CH11-induced (but not IL-1α/IFN-γ-induced) cell death in this system via a PI 3-kinase-dependent mechanism. This also correlates with the first demonstration that IL-13 stimulates activation of the major downstream PI 3-kinase effector PKB, which is thought to mediate the promotion of cell survival by this signaling pathway in a number of cell systems (11–13). However, IL-13-induced phosphorylation of Bad (a known downstream target of PKB) was barely detectable, suggesting that this is an unlikely target for PKB activity in this system. In addition, IL-13 pretreatment partially inhibited, but did not prevent cytokine-stimulated activation of either caspase-8 or caspase-3.

Individually, IL-1α, IFN-γ, and TNF-α do not induce apoptosis of HT-29 cells. However, our observation that combinations of IL-1α, IFN-γ, and TNF-α induce apoptosis of HT-29 cells, correlates well with recent reports that IFN-γ increases the sensitivity of HT-29 cells to pro-apoptotic agents such as TNF-α by directly and indirectly inducing select apoptosis-related genes (52). In addition, we have previously reported that this combination of cytokines stimulates NO production from HT-29 cells and there is considerable evidence that NO can promote apoptosis in other systems (43). Indeed, iNOS transcripts can be detected 6 h after cytokine treatment (20) and this appears to precede cell death which is detectable at 8 h post-cytokine stimulation. However, there are several lines of
evidence to indicate that cytokine-driven iNOS and apoptosis are independent functional events. First, the iNOS inhibitor aminoguanidine prevented IL-1α/IFN-γ/TNF-α and IL-1α/IFN-γ-induced NO production, but had no effect on the apoptosis stimulated by these combinations of cytokines. Second, apoptosis can also be stimulated by IFN-γ/TNF-α and IFN-γ/CH11 which are unable to stimulate NO production. Third, inhibition of IL-1α/IFN-γ/TNF-α-induced apoptosis by Z-VAD-FMK had no effect on NO production induced by these cytokines. Although markers of cytokine-stimulated apoptosis, such as DNA fragmentation and phosphatidylserine externalization, are detectable from 8 to 24 h, time course experiments have revealed that other functional responses continue unabated. For instance, identical cytokine treatment can also stimulate up-regulation of iNOS and chemokine mRNA up to 24 h post-stimulation (7, 20) and these responses can be down-regulated by pretreatment with IL-13 (7, 8, 10). So, cytokine-induced expression of apoptotic markers and events do not necessarily correlate with abrogated cell function, at least in the time frame studied here.

Activation of the proteolytic cascade by caspases appears to be essential to cytokine-induced apoptosis of HT-29 cells, given our observation that pretreatment with the caspase inhibitor Z-VAD-FMK completely prevents apoptosis induced by IL-1α/IFN-γ/TNF-α, IL-1α/IFN-γ, IFN-γ/TNF-α, and IFN-γ/CH11. This is particularly interesting given that both IL-1α and TNF-α (53) and CD95 (54) ligation have been reported to activate the ceramide pathway, which has also been implicated as a signaling pathway involved in apoptosis (55, 56). However, since the apoptosis of HT-29 cells stimulated by these cytokine combinations is completely inhibited by the caspase inhibitor Z-VAD-FMK, this may indicate that ceramide production is not sufficient for cell death in this system. Indeed, it has recently been shown that IFN-γ was unable to induce changes in sphingolipid levels in HT-29 cells (57), suggesting that ceramide-mediated signaling pathways may be cell-type specific. It is also interesting to note that while treatment of HT-29 cells with TNF-α or IFN-γ resulted in modest stimulation of caspases -8 and -3, this is insufficient to drive cell death, since neither TNF-α nor IFN-γ stimulated apoptosis in this system. This is in marked contrast to the TNF-α-induced apoptosis observed in neutrophils and T lymphocytes which correlates well with caspase activation (58, 59).

Even though IL-13 exerts a protective effect against cell death induced by IL-1α/IFN-γ/TNF-α, IFN-γ/TNF-α, and IFN-
IL-13 Promotes Cell Survival

FIG. 8. Effect of IL-13 on IL-1α/IFN-γ/TNF-α-induced activation of caspase-8 and caspase-3 in HT-29 cells. A, HT-29 cells (3 × 10^6 cells) were either left untreated or treated with combinations of IL-1α (10 ng/ml), TNF-α (100 ng/ml), or IFN-γ (300 units/ml) as indicated. B and C, HT-29 cells (3 × 10^6 cells) were either left untreated or treated with 30 ng/ml IL-13 for 1 h. Where indicated, the cells were then further treated with IL-1α (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (300 units/ml) or left untreated. After 24 h (A) or the times indicated (B and C), cells were pelleted to include any floating cells, the supernatants were removed and the cell pellets were lysed and assayed for caspase-8 and caspase-3 activity as described under "Experimental Procedures." Data are the mean of triplicates from one experiment ± S.E., representative of three independent experiments.

γCH11, IL-13 pretreatment was unable to completely inhibit cytokine-activated caspase-8 and caspase-3. Rather, it appears that IL-13 pretreatment delays activation of these caspases by IL-1α/IFN-γ/TNF-α and in this respect it is interesting to note that IL-13 provides only partial protection against cell death induced by IL-1α/IFN-γ/TNF-α and IFN-γ/TNF-α. Caspase activation is required for the execution of cell death in an apoptotic manner (reviewed in 44), but the order of caspase activation cascades is not absolute and the commitment to live or die may originate from the mitochondria (reviewed in Ref. 60). Hence, while IL-13 partially inhibits and possibly delays activation of caspase-8 and caspase-3, there may well be additional targets of IL-13-activated biochemical signals that mediate cell survival at some point distal to the apical caspase-8 and the downstream caspase-3, possibly involving mitochondrial activity. It is certainly possible that other upstream and downstream caspases are activated by the cytokine combinations used in this study. Indeed, it has recently been shown that PKB can phosphorylate caspase-9 and inhibit its protease activity (61). This would fit nicely with our observations that IL-13 can provide only partial protection against cell death induced by IL-1α/IFN-γ/TNF-α and IFN-γ/TNF-α, whereas apoptosis stimulated by IL-1α/IFN-γ was unaffected by IL-13. Hence, it appears that multiple death promoting pathways with different sensitivity to IL-13-activated cell survival mechanisms are activated by the cytokine combinations used in this study.

The protective effects of IL-13 against IL-1α/IFN-γ/TNF-α-, IFN-γ/TNF-α-, and IFN-γ/CH11-stimulated apoptosis are dependent on the PI 3-kinase-dependent signaling pathway, since the PI 3-kinase inhibitors wortmannin and LY29002 abrogated the protective effects of IL-13. These observations are consistent with demonstrations that the PI 3-kinase-dependent signaling pathway and in particular its downstream effector PKB are involved in growth factor-dependent cell survival (11–13, 46). Indeed, we have previously shown that IL-13 strongly activates PI 3-kinase as evidenced by PtdIns (3,4,5)-P_3 accumulation (10). Moreover, data in this study demonstrates that IL-13 also activates PKB and this activation is abrogated by pretreatment with PI 3-kinase inhibitors. PKB is now known to promote cell survival by phosphorylating a critical serine residue (136Ser) on the death-promoting protein Bad, causing it to dissociate from and thus allow activation of the cell survival factor, Bcl-x_L (47, 48). Indeed, expression of the related Bcl-2 family protein Bcl-xL (47, 48). Consistent with observations from other groups (52), Bad is expressed at very low levels in HT-29 cells, such that the band shift of Bad to the serine-phosphorylated form was barely detectable. It would seem unlikely, therefore, that the cell survival effects of IL-13 are solely mediated by PKB phosphorylation of Bad in the system described here. However, there are two alternative explanations to account for IL-13-stimulated PI 3-kinase/PKB-dependent cell survival mechanisms. First, other death promoting Bcl-2 family proteins may be regulated by PKB-dependent phosphorylation in a manner similar to that described for the regulation of Bad (47, 48). Indeed, expression of the related Bcl-2 family member Bak, which can also promote cell death, has been reported to be directly induced by IFN-γ (52). However, while Bak is expressed, we could not detect any IL-13-stimulated hyperphosphorylation of Bak by immunoblotting (data not shown). Nevertheless, it remains possible that other Bcl-2 family proteins may act as targets for IL-13-activated PKB. Second, an alternative target for the PI 3-kinase-dependent cell survival signals provided by IL-13 may be the transcription factors of the NF-κB family which have been reported to be important in cell survival by regulating unidentified, anti-apoptotic genes (62). Recent evidence has identified the inhibitor of apoptosis (IAP) proteins c-IAP1 and c-IAP-2 as gene...
targets of NF-κB transcriptional activity (63). The c-IAP1 and c-IAP2 proteins specifically inhibit the active forms of caspase-3 and caspase-7 (62). In other systems such as T lymphocytes, activation of NF-κB has been reported to be dependent on p70 S6 kinase (64), which in turn has been reported to be a target for phosphorylation by either PKB (65) and/or its upstream kinase(s) PDK-1 and the putative PDK-2 (66, 67). Hence, one possibility is that the observed cell survival effects of IL-13 involves PI 3-kinase-dependent activation of NF-κB transcriptional activity, although this hypothesis does not fit easily with the recent report demonstrating that IL-13 down-regulates TNF-α-mediated activation of NF-κB (41).

In summary, apoptosis of HT-29 epithelial cells observed in response to a combination of cytokines and/or CD95 ligation is not dependent on NO production. In addition, IL-13 can provide a PI 3-kinase-dependent cell survival signal to HT-29 cells which protects against cytokine-driven apoptotic signals. The mechanism underlying this cell survival effect of IL-13 is unclear and apparently distinct from the known cell survival signals provided by PKB-dependent phosphorylation of Bad. Nevertheless, our observations indicate a potential role for IL-13 in regulating the controlled program of cell death and survival, a process which plays an important role during several stages of normal colonic epithelial cell development and maturation. Hence, dysregulation of cell survival and death may be important in the pathogenesis of inflammatory bowel disease and carcinogenesis in the large bowel.

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