IL-10 protects mouse intestinal epithelial cells from Fas-induced apoptosis via modulating Fas expression and altering caspase-8 and FLIP expression

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Bharhani, Mantej S., Rajka Boroevic, Shibesh Basak, Edwin Ho, Pengfei Zhou, and Kenneth Croitoru. IL-10 protects mouse intestinal epithelial cells from Fas-induced apoptosis via modulating Fas expression and altering caspase-8 and FLIP expression. Am J Physiol Gastrointest Liver Physiol 291: G820–G829, 2006; doi:10.1152/ajpgi.00438.2005.—We have previously shown that the absence of Fas/Fas ligand significantly reduced tissue damage and intestinal epithelial cell (IEC) apoptosis in an in vivo model of T cell-mediated enteropathy. This enteropathy was more severe in IL-10-deficient mice, and this was associated with increased serum levels of IFN-γ and TNF-α and an increase in Fas expression on IECs. In this study, we investigated the potential of IL-10 to directly influence Fas expression and Fas-induced IEC apoptosis. Mouse intestinal epithelial cell lines MODE-K and IEC-4.1 were cultured with IFN-γ, TNF-α, or anti-Fas monoclonal antibody (mAb) in the presence or absence of IL-10. Fas expression and apoptosis were determined by FACS analysis of phycoerythrin-anti-Fas mAb staining and annexin V staining, respectively. Treatment with a combination of IFN-γ and TNF-α induced significant apoptosis. Anti-Fas mAb alone did not induce much apoptosis unless cells were pretreated with IFN-γ and TNF-α. These IECs constitutively expressed low levels of Fas, which significantly increased by preincubation of the cells with IFN-γ and TNF-α. Treatment with cytokine or cytokine plus anti-Fas mAb increased apoptosis, which correlated with a decreased Fas-associated death domain IL-1-converting enzyme-like inhibitory protein (FLIP) level, increased caspase-8 activity, and subsequently increased caspase-3 activity. IL-10 diminished both cytokine-induced and anti-Fas mAb-induced apoptosis, and this was correlated with decreased cytokine-induced Fas expression, increased FLIP, and decreased caspase-8 and caspase-3 activity. In conclusion, IL-10 modulated cytokine induction of Fas expression on IEC cell lines and regulated IEC susceptibility to TNF-α, IFN-γ, and Fas-mediated apoptosis. These findings suggest that IL-10 directly modulates IEC responses to T cell-mediated apoptotic signals.

T CELL ACTIVATION is a common feature of inflammatory diseases of the bowel such as graft vs. host disease, celiac disease, and idiopathic inflammatory bowel disease (35). In the mouse model of T cell activation-induced enteropathy, mucosal damage induced by an anti-CD3 injection is characterized by villous atrophy and enterocyte apoptosis (10, 21, 22). The intestinal mucosal damage induced by T cell activation is mediated by IFN-γ, TNF-α, perforin, and Fas-Fas ligand (FasL) pathways (21, 23). In vivo T cell activation induced by anti-CD3 leads to increased levels of both pro- and anti-inflammatory cytokines (5, 40). The initial mucosal damage and its rapid resolution are possibly the result of the balance of these cytokine effects. In fact, IL-10-deficient mice treated with anti-CD3 develop more severe enteropathy, and treatment with recombinant mouse IL-10 reverses the effect (42). The increase in tissue damage observed in IL-10-deficient mice treated with anti-CD3 was associated with an increase in apoptosis of epithelial cells and an increase in serum IFN-γ and TNF-α levels. These observations suggest that IL-10 diminished T cell-induced intestinal tissue damage via modulation of the proinflammatory cytokine cascade induced by activation of T cells or via suppression of epithelial cell apoptosis. Given that intestinal epithelial cells (IECs) express IL-10 receptors (4), we examined the ability of IL-10 to directly modulate Fas/FasL-, TNF-α-, and IFN-γ- induced IEC responses. Furthermore, we examined the effect of IL-10 on caspase mediators of apoptosis. Caspases are cysteine proteases that are activated through death receptors like FasL and the TNF-α receptor (20). Fas-associated death domain (FADD)-like IL-1-converting enzyme-like inhibitory protein (FLIP) has been described as a natural inhibitor of Fas and TNF-mediated apoptosis (15).

Our results showed that IL-10 diminished epithelial cell apoptosis due to IFN-γ, TNF-α, and anti-Fas monoclonal antibody (mAb). This was associated with an altered FLIP-caspase 8 balance and a decrease in Fas expression induced by these cytokines.

MATERIALS AND METHODS

Epithelial cell lines. MODE-K cells derived from C3H/HeJ mice were kindly provided by Dr. D. Kaiserlian (Lyon, France) (37). These are small IECs immortalized by simian virus (SV)-40 large T gene transfer and exhibit morphological and phenotypic characteristics of normal enterocytes. Cells were grown in DMEM supplemented with 5 × 10⁻⁵ M 2-mercaptoethanol, 10 mM HEPES, 2 mM L-glutamine, 10% FCS (GIBCO-BRL), and antibiotics. IEC4.1 cells derived from Balb/c mice were kindly provided by Dr. A. Jevnikar (University of Western Ontario, London, ON, Canada) (17). These are also small IECs immortalized by SV-40 large T gene transfer and exhibit morphological and phenotypic characteristics of normal enterocytes. These cells were grown in collagen (Collaborative Biomedical Products)-coated flasks or plates in K1 media prepared by mixing DMEM and Ham-12 at a 1:1 ratio and supplemented with 5% FCS, 1 mM sodium pyruvate, 2 mM L-glutamate, 10 mg/ml epidermal growth factor, and antibiotics.

Monoclonal antibodies, cytokines, and annexin V. Purified and phycoerythrin (PE)-conjugated anti-mouse Fas mAb (Jo2), purified and PE-conjugated hamster IgG2 isotype control mAb (Ha4/8), and anti-caspase-8 mAb were purchased from Alexis Biochemicals (San Diego, CA). Anti-caspase-3 mAb were purchased from Cell Signaling Technology (Danvers, MA). Anti-β actin mAb (AC-15) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Anti-cFLIP mAb was generous gift from Dr. A. Kaiserlian (Lyon, France). Anti-annexin V staining, respectively. Treatment with a combination of IFN-γ and TNF-α induced significant apoptosis. Anti-Fas mAb alone did not induce much apoptosis unless cells were pretreated with IFN-γ and TNF-α. These IECs constitutively expressed low levels of Fas, which significantly increased by preincubation of the cells with IFN-γ and TNF-α. Treatment with cytokine or cytokine plus anti-Fas mAb increased apoptosis, which correlated with a decreased Fas-associated death domain IL-1-converting enzyme-like inhibitory protein (FLIP) level, increased caspase-8 activity, and subsequently increased caspase-3 activity. IL-10 diminished both cytokine-induced and anti-Fas mAb-induced apoptosis, and this was correlated with decreased cytokine-induced Fas expression, increased FLIP, and decreased caspase-8 and caspase-3 activity. In conclusion, IL-10 modulated cytokine induction of Fas expression on IEC cell lines and regulated IEC susceptibility to TNF-α, IFN-γ, and Fas-mediated apoptosis. These findings suggest that IL-10 directly modulates IEC responses to T cell-mediated apoptotic signals.

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T CELL ACTIVATION is a common feature of inflammatory diseases of the bowel such as graft vs. host disease, celiac disease, and idiopathic inflammatory bowel disease (35). In the mouse model of T cell activation-induced enteropathy, mucosal damage induced by an anti-CD3 injection is characterized by villous atrophy and enterocyte apoptosis (10, 21, 22). The intestinal mucosal damage induced by T cell activation is mediated by IFN-γ, TNF-α, perforin, and Fas-Fas ligand (FasL) pathways (21, 23). In vivo T cell activation induced by anti-CD3 leads to increased levels of both pro- and anti-inflammatory cytokines (5, 40). The initial mucosal damage and its rapid resolution are possibly the result of the balance of these cytokine effects. In fact, IL-10-deficient mice treated with anti-CD3 develop more severe enteropathy, and treatment with recombinant mouse IL-10 reverses the effect (42). The increase in tissue damage observed in IL-10-deficient mice treated with anti-CD3 was associated with an increase in apoptosis of epithelial cells and an increase in serum IFN-γ and TNF-α levels. These observations suggest that IL-10 diminished T cell-induced intestinal tissue damage via modulation of the proinflammatory cytokine cascade induced by activation of T cells or via suppression of epithelial cell apoptosis. Given that intestinal epithelial cells (IECs) express IL-10 receptors (4), we examined the ability of IL-10 to directly modulate Fas/FasL-, TNF-α-, and IFN-γ-induced IEC responses. Furthermore, we examined the effect of IL-10 on caspase mediators of apoptosis. Caspases are cysteine proteases that are activated through death receptors like FasL and the TNF-α receptor (20). Fas-associated death domain (FADD)-like IL-1-converting enzyme-like inhibitory protein (FLIP) has been described as a natural inhibitor of Fas and TNF-mediated apoptosis (15).

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Confluent monolayers of MODE-K and IEC4.1 cells (2 × 10^5 cells/well) were cultured overnight in 24-well tissue culture plates (Falcon, Becton Dickinson). Confluent monolayers of these cells were treated with varying concentrations of IFN-γ and TNF-α, or with or without IL-10 for 6–24 h. The cytokine-treated cells were harvested with trypsin, washed twice with PBS, and resuspended in 75 μl PBS-0.1% BSA containing 0.02% sodium azide. Single cell suspensions were incubated for 30 min with 100 μl of 1 μg of PE-conjugated anti-Fas mAb. After being washed, cells were fixed in 2% paraformaldehyde for 20 min at room temperature and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using WinMDI software (version 2.8).

Annexin V staining for the detection of apoptosis. MODE-K or IEC4.1 cells (2 × 10^5 cells/well) were cultured overnight in 24-well tissue culture plates and then treated with different combinations of IFN-γ, TNF-α, and anti-Fas mAb with or without IL-10. Surface exposure of phosphatidlyserine, which is an indicator of cells undergoing apoptosis, was detected by staining the cells with annexin V, which binds to exposed phosphatidlyserine. The staining was performed as previously described (6). In brief, after treatment, the culture supernatant was collected and centrifuged to collect floating cells, which were added back to their respective wells. Two hundred microliters of binding buffer (10 mM HEPES, 150 mM NaCl, and 1.8 mM CaCl_2 in distilled water; pH 7.4) containing 4 μl of annexin V-FITC (BD Biosciences) were added to the monolayer. After 15–20 min of incubation at room temperature, the monolayer was washed three times with binding buffer, and cells were harvested by scraping. The floating cells collected during washing were washed twice and recombined with the harvested cells for analysis. Cells were analyzed on a FACSCalibur flow cytometer for annexin V-FITC-positive staining. Data analysis was performed with WinMDI software (version 2.8).

Preparation of cell lysate. Cells cultured in six-well plates were washed twice with ice-cold PBS. Three hundred microliters of cell lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.25% deoxycholic acid, 1 mM sodium fluoride, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin] were added to each well containing attached cells. Cells were gently removed with a rubber policeman and transferred into centrifuge tubes. The cell suspension was gently rocked on an orbital shaker in the cold room for 15 min to lyse the cells. After being centrifuged at 13,000 g for 15 min, the supernatant was collected; the protein concentration in the supernatant was estimated by the Bradford method (Bio-Rad) and stored at −20°C.

Western blot analysis. SDS-PAGE was performed on a 10% gel by using 40 μg protein/lane of whole cell lysate. The protein was then transferred onto BioTrace polyvinylidene difluoride nitrocellulose membranes (VWR Scientific, Mississauga, ON, Canada). After a 1-h blockade with 5% nonfat dried milk in Tris-buffered saline with Tween 20 (TBST), membranes were incubated overnight at 4°C with anti-caspase-8, anti-caspase-3, anti-FLIP, or anti-β actin antibodies in 5% nonfat dried milk with TBST. Membranes were washed three times with TBST and incubated for 1 h with secondary antibodies linked to horseradish peroxidase. After the membranes were first washed with TBST and then with distilled water, enhanced chemiluminescence (ECL) detection was performed with an ECL detection kit (Amersham Pharmacia Biotech) and recorded on Kodak-Omat blue film.

RESULTS

IFN-γ and TNF-α induced MODE-K and IEC4.1 cell apoptosis. In a dose- and time-response experiment for cytokine-induced apoptosis, MODE-K or IEC4.1 cells were treated with 2–10 ng/ml of IFN-γ, TNF-α, or a combination of IFN-γ and TNF-α for 6–48 h, and apoptosis was measured by annexin V staining. As shown in Fig. 1, treatment with any of the cytokines for 6 h did not induce significant apoptosis in either cell line. IFN-γ treatment at 10 ng/ml induced significant apoptosis of both MODE-K and IEC4.1 cells after 24 or 48 h. TNF-α (10 ng/ml) induced significant apoptosis only after 48 h. When cells were treated with the combination of IFN-γ and TNF-α for 24 and 48 h, there was a significant increase in apoptosis of both cell lines (Fig. 1).

Anti-Fas-induced apoptosis required pretreatment with IFN-γ and TNF-α. Confluent monolayers of MODE-K and IEC4.1 cells were treated with 0.5–10 μg/ml anti-Fas mAb, hamster IgG isotype control, or medium alone for 6–48 h. Annexin V-positive cells were considered apoptotic cells. In both cell lines, isotype control-treated cells failed to show any significant apoptosis at any time point compared with untreated (medium only) cells (data not shown). As shown in Fig. 2, treatment of MODE-K and IEC4.1 cells with anti-Fas mAb alone also failed to induce significant apoptosis until 48 h. The optimum concentration of anti-Fas mAb was considered to be 1 μg/ml and was used in further experiments. Because both IFN-γ and TNF-α altered epithelial cell viability in vitro (3) and because an in vivo injection of TNF-α caused enteropathy (9), we investigated whether IFN-γ or TNF-α pretreatment altered MODE-K and IEC4.1 sensitivity to anti-Fas mAb-induced apoptosis. Cells pretreated with IFN-γ (5 or 10 ng/ml), TNF-α (5 or 10 ng/ml), or combined IFN-γ and TNF-α (2.5 or 5 ng/ml each) for 24 h were treated with anti-Fas mAb (1 μg/ml) for another 24 h. As shown in Fig. 3, the degree of apoptosis in MODE-K and IEC4.1 cells was minimal after anti-Fas mAb treatment alone or after 5 or 10 ng/ml of IFN-γ or TNF-α alone. Anti-Fas mAb treatment of MODE-K cells pretreated with 5 ng/ml of IFN-γ, TNF-α, or combined IFN-γ (2.5 mg/ml) and TNF-α (2.5 mg/ml) induced a significant increase of apoptosis compared with the respective controls. The percentages of annexin V-positive MODE-K cells increased to 30.9 ± 5.9% vs. 7.4 ± 2.7%, 42.4 ± 5.6% vs. 8.2 ± 1.9%, and 64.1 ± 7.8% vs. 14.2 ± 1.4%, respectively (Fig. 3A). Pretreatment with 10 ng/ml of IFN-γ, TNF-α, or combined IFN-γ (5 mg/ml) and TNF-α (5 mg/ml) induced even more anti-Fas-mediated apoptosis. The percentages of annexin V-positive MODE-K cells increased to 35.9 ± 5.4% vs. 9.8 ± 2.2%, 48.1 ± 11% vs. 9.8 ± 1.9%, and 98.3 ± 1.52% vs. 18.3 ± 4%, respectively (Fig. 3A). Similarly, anti-Fas mAb treatment of IEC4.1 cells pretreated with 5 or 10 ng/ml of IFN-γ, TNF-α, or combined IFN-γ and TNF-α (2.5 or 5 ng/ml each) induced a significant increase of apoptosis compared with the respective controls. After pretreatment with 5 ng/ml, the percentages of annexin V-positive IEC4.1 cells increased to 58 ± 6.2% vs. 8.6 ± 1.1%, 64 ± 12.7% vs. 8.8 ± 3%, and 70.6 ± 7.6% vs. 16.3 ± 3%, respectively; and after the pretreatment with 10 ng/ml, the percentages of annexin V-positive IEC4.1 cells increased to 62 ± 5.1% vs. 8.6 ± 1.1%, 74 ± 12.7% vs. 8.8 ± 3%, and 80.6 ± 7.6% vs. 16.3 ± 3%, respectively.

Statistical analysis. Data are expressed as means ± SD and analyzed for statistical significance using Student’s t-test. Differences were considered statistically significant at P values of <0.05.
Fig. 1. Cytokine-induced apoptosis of the intestinal epithelial cell lines MODE-K (A) and IEC4.1 (B). Confluent monolayers of these cells were treated with 2–10 ng/ml of IFN-γ, TNF-α, the combination of IFN-γ and TNF-α, or medium alone for 6–48 h. Apoptosis was measured by annexin V staining as described in MATERIALS AND METHODS. Apoptotic cells were detected by increased annexin V staining. Results shown are means ± SD of 3 experiments. *P < 0.05 compared with untreated cells.

positive IEC4.1 cells were 65.2 ± 10% vs. 10.1 ± 2.1%, 70 ± 11.7% vs. 8.8 ± 3%, and 97.6 ± 4.6% vs. 20.3 ± 2.8%, respectively (Fig. 3B).

IFN-γ and TNF-α induced Fas expression on MODE-K and IEC4.1 cells. To determine whether the increase in anti-Fas-induced apoptosis observed after the incubation with cytokines was due to changes in Fas expression, we measured Fas expression on MODE-K and IEC4.1 cells treated with 2–10 ng/ml of IFN-γ, TNF-α, or combined IFN-γ and TNF-α (1–5 ng/ml each) for 24 h. Flow cytometry analysis of Fas expression showed that untreated MODE-K (Fig. 4A) and IEC4.1 (Fig. 4B) cells expressed low levels of Fas. After 24 h of IFN-γ treatment, Fas expression on MODE-K cells was increased significantly with 5 [mean fluorescence intensity (MFI): 53.3 ± 5.7] and 10 ng/ml (MFI: 69.3 ± 3) (Fig. 4A). Cells treated with TNF-α or the combination of IFN-γ and TNF-α for 24 h also increased Fas significantly (MFI: 88 ± 8, 113.6 ± 13, and 135 ± 13.2 with 2, 5, and 10 ng/ml of TNF-α, respectively; and 155.6 ± 14, 220 ± 18, and 281.3 ± 25.6 with 2, 5, and 10 ng/ml of combined IFN-γ and TNF-α, respectively). Similarly, IEC4.1 cells also demonstrated significant increases in Fas expression with cytokine treatment. As shown in Fig. 4B, 24-h treatment with IFN-γ, TNF-α, or a combination of IFN-α and TNF-α increased Fas expression significantly (MFI: 136 ± 19.6 and 156 ± 21.6 with 5 and 10 ng/ml of IFN-γ, respectively; 145 ± 22.9, 206 ± 21.6, and 226 ± 27.5 with 2, 5, and 10 ng/ml of TNF-α, respectively; and 285.3 ± 16.1, 364 ± 22.6, and 464 ± 25.3 with 2, 5, and 10 ng/ml of combined IFN-γ and TNF-α, respectively).

IL-10 decreased cytokine-induced apoptosis. To determine the effect of IL-10 on modulating epithelial cell apoptosis during immune-mediated enteropathy, we first examined the effect of IL-10 on IFN-γ- and TNF-α-induced apoptosis. MODE-K cells or IEC4.1 cells were treated with the combination of IFN-γ (5 ng/ml) and TNF-α (5 ng/ml) in the presence or absence of IL-10 (5–50 ng/ml) for 48 h. The results showed that IL-10 decreased the apoptosis induced by the combination of IFN-γ + TNF-α (Fig. 5). As shown in Fig. 5A, the percentages of annexin V-positive MODE-K cells decreased significantly from 55.3 ± 8.5% with combined IFN-γ and TNF-α to 37 ± 6%, 31 ± 5.7%, and 36 ± 7.2% with 10, 20, and 50 ng/ml of IL-10, respectively. Similarly, the percentages of annexin V-positive IEC4.1 cells (43.3 ± 6.5% with IFN-γ and TNF-α) also decreased with IL-10 treatment (Fig. 5B). The decrease was significant with 20 and 50 ng/ml of IL-10, with percentages of annexin V-positive cells of 27 ± 6% and 26.6 ± 7%, respectively.

IL-10 suppressed anti-Fas mAb-induced apoptosis. To determine whether IL-10 altered anti-Fas-induced apoptosis, MODE-K or IEC4.1 cells pretreated with or without IL-10 for 24 h were cultured with anti-Fas mAb for 48 h. As shown in Fig. 6, IL-10 treatment decreased anti-Fas mAb-induced apoptosis of both cell lines. The percentages of anti-Fas-induced annexin V-positive MODE-K cells decreased from 16.4 ± 9.7% to 4.1 ± 1.8% with 10 ng/ml of IL-10 (Fig. 6A). Similarly, IL-10 treatment decreased anti-Fas mAb-induced apoptosis of IEC4.1 cells (Fig. 6B), with percentages of annexin V-positive cells decreasing from 25.7 ± 7.3% to 9.9 ± 1.5% with 10 ng/ml of IL-10.IL-10 decreased cytokine-induced apoptosis. To determine the effect of IL-10 on modulating epithelial cell apoptosis during immune-mediated enteropathy, we first examined the effect of IL-10 on IFN-γ- and TNF-α-induced apoptosis. MODE-K cells or IEC4.1 cells were treated with the combination of IFN-γ (5 ng/ml) and TNF-α (5 ng/ml) in the presence or absence of IL-10 (5–50 ng/ml) for 48 h. The results showed that IL-10 decreased the apoptosis induced by the combination of IFN-γ + TNF-α (Fig. 5). As shown in Fig. 5A, the percentages of annexin V-positive MODE-K cells decreased significantly from 55.3 ± 8.5% with combined IFN-γ and TNF-α to 37 ± 6%, 31 ± 5.7%, and 36 ± 7.2% with 10, 20, and 50 ng/ml of IL-10, respectively. Similarly, the percentages of annexin V-positive IEC4.1 cells (43.3 ± 6.5% with IFN-γ and TNF-α) also decreased with IL-10 treatment (Fig. 5B). The decrease was significant with 20 and 50 ng/ml of IL-10, with percentages of annexin V-positive cells of 27 ± 6% and 26.6 ± 7%, respectively.

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A

![Graph A](image)

B

![Graph B](image)

Fig. 2. Anti-Fas monoclonal antibody (mAb)-induced apoptosis of the intestinal epithelial cell lines MODE-K (A) and IEC4.1 (B). Confluent monolayers of these cells were treated with anti-Fas mAb (0.5–10 μg/ml) or medium alone for 6, 24, and 48 h. Apoptosis was measured by annexin V staining as described in MATERIALS AND METHODS. Apoptotic cells were detected by increased annexin V staining. Results shown are means ± SD of 3 experiments. *P < 0.05 compared with untreated cells.

3.8% to 7.8 ± 2.4%, 8.2 ± 2.6%, and 7 ± 2.1% with 10, 20, and 50 ng/ml of IL-10, respectively (Fig. 6A). Similarly, the percentages of anti-Fas-induced annexin V-positive IEC4.1 cells decreased from 20 ± 3% to 12 ± 2%, 13 ± 3.8%, and 8.8 ± 3% with 10, 20, and 50 ng/ml of IL-10, respectively (Fig. 6B). Because the degree of apoptosis induced by anti-Fas mAb was greater after combined IFN-γ and TNF-α pretreatment, the effect of IL-10 was determined on anti-Fas mAb-induced apoptosis of MODE-K or IEC4.1 cells pretreated with the combination of IFN-γ (5 ng/ml) and TNF-α (5 ng/ml). As shown in Fig. 6A, IL-10 treatment significantly decreased the anti-Fas mAb-induced apoptosis of IFN-γ + TNF-α-pretreated MODE-K cells from 64.1 ± 7.8% to 40 ± 6.2%, 34 ± 7.8%, and 33 ± 8.8% with 10, 20, and 50 ng/ml of IL-10, respectively. Treatment with IL-10 also significantly decreased the anti-Fas mAb-induced apoptosis of IFN-γ + TNF-α-pretreated IEC4.1 cells from 76.6 ± 7.6% to 42 ± 11.8%, 38.3 ± 8.4%, and 34 ± 6.6% with 10, 20, and 50 ng/ml of IL-10, respectively (Fig. 6B).

IL-10 diminished IFN-γ- or TNF-α-induced Fas expression in MODE-K and IEC4.1 cells. To determine the effect of IL-10 on the IFN-γ + TNF-α-induced increase in Fas expression, MODE-K or IEC4.1 cells were treated with combined IFN-γ (5 ng/ml) and TNF-α (5 ng/ml) in the presence or absence of IL-10 (5–50 ng/ml) for 24 h. As shown in Fig. 7, both of the cell lines demonstrated significant decreases in Fas expression with IL-10 treatment. MFIs of IFN-γ + TNF-α-pretreated MODE-K cells significantly decreased from 238 ± 25.5 to 141 ± 28.4, 123.3 ± 20.8, and 135 ± 32.7 with 10, 20, and 50 ng/ml of IL-10, respectively (Fig. 7A). Similarly, IFN-γ + TNF-α-induced Fas expression on IEC4.1 cells significantly decreased from a MFI of 364 ± 2.6 to values of 226.6 ± 23.1, 201.6 ± 17.5, and 200 ± 18 with 10, 20, and 50 ng/ml of IL-10, respectively (Fig. 7B).

Suppression of apoptosis by IL-10 is associated with upregulation of FLIP level and downregulation of caspase-8 and caspase-3 activity. To determine the significance of caspase-8 and FLIP in cytokine- and anti-Fas-mediated apoptosis, Western blot analysis was performed with protein extracts of MODE-K and IEC4.1 cells treated with cytokines and anti-Fas antibodies. Figure 8 shows procaspase-8 (inactive form of caspase-8) and FLIP expression in MODE-K and IEC4.1 cells. Activation of caspase-8 occurs as a result of cleavage of its inactive procaspase form into active small fragments (20). Therefore, one would expect a decrease in procaspase-8 levels in the proapoptotic state. Protein extracts showed that both MODE-K and IEC4.1 cells constitutively expressed some caspase-8 and FLIP, with no change in FLIP level and downregulation of caspase-8 and FLIP observed in anti-Fas mAb treatment alone. Anti-Fas mAb treatment alone did not change procaspase-8 or FLIP levels in either cell line. IL-10 treatment increased FLIP in both cell lines but did not alter the procaspase-8 levels. Combined IFN-γ and TNF-α treatment decreased procaspase-8 levels to some degree. When the cells were treated with IFN-γ + TNF-α along with IL-10, there was an increase in procaspase-8 and FLIP compared with IFN-γ + TNF-α treatment alone. Anti-Fas mAb treatment alone did not change procaspase-8 levels in either MODE-K or IEC4.1 cells. In both MODE-K and IEC4.1 cells, anti-Fas mAb treatment of IFN-γ + TNF-α-pretreated cells significantly...
decreased procaspase-8, and this decrease in procaspase-8 was associated with a decrease in FLIP. IL-10 treatment of these cells increased procaspase-8 and FLIP levels in both cell lines.

The role of caspase-3 in cytokine- and Fas-induced apoptosis was also evaluated. Activation of caspase-3 occurs as a result of cleavage of its inactive procaspase-3 form into active small fragments. Caspase-3 activation was assessed using a specific antibody against the inactive procaspase-3 form in Western blot analysis. As shown in Fig. 9, IFN-γ/H9253, TNF-α/H9251, or IL-10 treatment alone did not change procaspase-3 levels in either cell line compared with untreated cells. A reduced amount of procaspase-3 was found with combined IFN-γ/H9253 and TNF-α/H9251 treatment in both cell lines. Treatment with IFN-γ and TNF-α along with IL-10 induced procaspase-3 levels compared with IFN-γ + TNF-α treatment alone. Anti-Fas mAb treatment alone caused a minimal but apparent decrease in procaspase-3 levels. Anti-Fas mAb treatment of IFN-γ + TNF-α-pretreated cells decreased procaspase-3 levels, and IL-10 treatment of these cells increased procaspase-3 levels compared with IFN-γ + TNF-α + anti-Fas mAb-treated cells in both cell lines.

**DISCUSSION**

We examined the ability of IL-10 to suppress cytokine- and Fas-induced apoptosis of the mouse IEC cell lines MODE-K and IEC4.1 in vitro. Because this study is the continuation of our previous in vivo mouse studies (21, 43) and because MODE-K cells have been shown to express the IL-10 receptor (4), we focused this study on these mouse IEC cell lines. The results showed that IFN-γ and TNF-α induced a low level of apoptosis, whereas the combination resulted in apoptosis of up to 41–60% of these cells. Furthermore, anti-Fas mAb did not induce much apoptosis of MODE-K and IEC4.1 cells unless cells were pretreated with either IFN-γ or TNF-α. In addition, we showed that IFN-γ and TNF-α induced an increase in Fas expression on both cell lines and an increase in caspase-8 and caspase-3 activity in these cells. Most importantly, the results showed that IL-10 treatment of MODE-K and IEC4.1 cells diminished IFN-γ and TNF-α-induced apoptosis and significantly diminished anti-Fas-induced apoptosis of cells pretreated with both IFN-γ and TNF-α.

These findings support the results of our previous in vivo study in which we showed that IEC apoptosis in T cell-induced mucosal injury was mediated by the combination of Fas/FasL-, TNF-α-, and perforin-mediated pathways (21), but only Fas/FasL and perforin were absolutely required for the development of T cell-induced epithelial injury. Indeed, inhibition of the TNF-α pathway alone failed to prevent in vivo T cell-induced mucosal damage or epithelial cell apoptosis (18). In this study, treatment with TNF-α alone also failed to induce...
apoptosis of MODE-K and IEC4.1 cells unless accompanied by IFN-$\gamma$ and TNF-$\alpha$. In vitro studies with HT29 cells (human colon carcinoma cells line), YAMC cells (murine colon cell line), and Hc cells (human hepatocyte cell line) also failed to detect apoptosis after solitary TNF-$\alpha$ treatment (1, 14, 24). These findings are further supported by our Western blot analysis results showing that TNF-$\alpha$ treatment did not alter caspase-8, caspase-3, and FLIP levels. Stimulation of death receptors of the TNF receptor superfamily leads to formation of a death-inducing signaling complex (DISC) that contains receptor aggregation, TNF receptor-associated death domain (TRADD) or FADD, and proenzyme formation (procaspase-8) of caspase-8. Caspases are the effectors of apoptotic cell death, and caspase-8 initiates the downstream activation of a series of these caspases, with activated caspase-3 causing apoptosis.

FLIP is an inhibitor of Fas- and TNF-mediated apoptosis (15, 26) that prevents the proteolytic activation of procaspase 8 by blocking caspase-8 recruitment by FADD. Therefore, in a proapoptotic state, one would expect to see a decrease in FLIP and, subsequently, a decrease in procaspase-8 and caspase 3 levels; in other words, an increase in caspase-8 and caspase-3 activities. Because our data showed that TNF-$\alpha$ did not change procaspase-8 or FLIP levels, we suggest that the response to TNF-$\alpha$ in both MODE-K and IEC4.1 cells is predominantly via alternate pathways such as NF-$\kappa$B and phosphatidylinositol 3-kinase (PI3K/AKT) (36, 41). Furthermore, our data showed that TNF-$\alpha$ treatment only induced apoptosis of either cell line after 48 h, suggesting that with time there is a switch in the balance between anti- and proapoptotic signals. Alternatively, the induction of apoptosis in MODE-K and IEC4.1 cells with combined IFN-$\gamma$ and TNF-$\alpha$ treatment might be due to IFN-$\gamma$ induced increases in TNF-$\alpha$ receptor levels (29) and a subsequent downregulation of FLIP levels and upregulation of caspase-8 activity (Fig. 8) leading to an upregulation of caspase-3 activity (Fig. 9). IFN-$\gamma$, which itself induced significant apoptosis, enhanced the susceptibility of MODE-K and IEC4.1 cells to TNF-$\alpha$-mediated apoptosis. Receptor binding of IFN-$\gamma$ activates signal transducer and activator of transcription (STAT1) (13), a component of the TNF receptor 1-TRADD signaling complex that can prevent the signaling complex formation required for NF-$\kappa$B activation and favors upregulation of caspase-8 activity and the induction of apoptosis (8, 27, 38). This is supported by our findings showing that caspase-8 activity is upregulated when IFN-$\gamma$ and TNF-$\alpha$ are used together. Indeed, TNF-$\alpha$-treated STAT1-deficient cells develop less apoptosis due to unopposed NF-$\kappa$B activation (16, 38). Therefore, the induction of apoptosis of MODE-K and IEC4.1 cells treated with combined IFN-$\gamma$ and TNF-$\alpha$ may be related to an increase in TNF-$\alpha$ receptor levels (29), inhibition of NF-$\kappa$B via activation of STAT1 (13), downregulation of

![Fig. 4. IFN-$\gamma$- and TNF-$\alpha$-induced Fas expression on intestinal epithelial cell lines MODE-K (A) and IEC4.1 (B). Confluent monolayers were treated with medium alone (untreated), IFN-$\gamma$ (2–10 ng/ml), TNF-$\alpha$ (2–10 ng/ml), or combined IFN-$\gamma$ and TNF-$\alpha$ (2–10 ng/ml) for 24 h. Cells were then stained with phycoerythrin (PE)-labeled anti-Fas mAb. Fas expression is shown in terms of mean fluorescence intensity (MFI). Results shown are means ± SD of 3 experiments. *$P < 0.05$ compared with untreated cells.]

![Fig. 5. IL-10 suppressed cytokine-induced apoptosis of intestinal epithelial cell lines MODE-K (A) and IEC4.1 (B). Confluent monolayers were treated with a combination of IFN-$\gamma$ (5 ng/ml) and TNF-$\alpha$ (5 ng/ml) in the presence or absence of IL-10 (5–50 ng/ml) for 48 h. Apoptosis was measured by annexin V staining as described in MATERIALS AND METHODS. Results shown are means ± SD of 3 experiments. *$P < 0.05$ compared with IFN-$\gamma$ + TNF-$\alpha$ only.]

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FLIP (Fig. 8), and upregulation of caspase-8 activity (Fig. 8). Caspase-8 activation requires autoproteolysis, which is dependent on procaspase 8 recruitment to the DISC. Caspase-8 activity is regulated by FLIP, which inhibits autoproteolysis by blocking caspase-8 recruitment by FADD. According to the induced proximity model for caspase-8 activation, high local concentrations of the procaspase zymogen within the DISC leads to autoprocessing and activation of caspase-8 (30). It is therefore possible that increased expression of TNF-α/Fas by IFN-γ treatment might facilitate the recruitment and thus activation of caspase-8 and subsequent death receptor activation.

It is of further importance that anti-Fas mAb treatment alone induced minimal apoptosis of both MODE-K and IEC4.1 cells unless cells were pretreated with IFN-γ and TNF-α. The possibility that this was related to a low level of expression of FLIP (Fig. 8), and upregulation of caspase-8 activity (Fig. 8). Caspase-8 activity is regulated by FLIP, which inhibits autoproteolysis by blocking caspase-8 recruitment by FADD. According to the induced proximity model for caspase-8 activation, high local concentrations of the procaspase zymogen within the DISC leads to autoprocessing and activation of caspase-8 (30). It is therefore possible that increased expression of TNF-α/Fas by IFN-γ treatment might facilitate the recruitment and thus activation of caspase-8 and subsequent death receptor activation.

It is of further importance that anti-Fas mAb treatment alone induced minimal apoptosis of both MODE-K and IEC4.1 cells unless cells were pretreated with IFN-γ and TNF-α. The possibility that this was related to a low level of expression of
Fas on these cells (11, 25, 28) is supported by the results showing that both IFN-γ and TNF-α treatment increased Fas expression on both cell lines. Therefore, the induction of Fas-induced apoptosis of IFN-γ + TNF-α-pretreated MODE-K and IEC4.1 cells appears to be due in part to the induction of Fas expression to a critical threshold level necessary for apoptotic signaling. This is supported by the correlation between increased Fas expression with TNF-α alone or the combined IFN-γ and TNF-α (Fig. 4). Caspase-3 is the principal effector caspase in Fas-mediated apoptosis in some cells (19), and our findings also showed an upregulation of

![Figures A, B, C, D](images)

**Fig. 8.** Western blot analysis of expression of procaspase-8 protein and Fas-associated death domain IL-1-converting enzyme-like inhibitory protein (FLIP) in intestinal epithelial cell lines. Cells were treated with medium only, IFN-γ (10 ng/ml), TNF-α (10 ng/ml), IL-10 (10 ng/ml), combined IFN-γ (5 ng/ml) + TNF-α (5 ng/ml), combined IFN-γ (5 ng/ml) + TNF-α (5 ng/ml) + IL-10 (10 ng/ml), or anti-Fas mAb for 48 h. In some cases, cells pretreated with combined IFN-γ (5 ng/ml) and TNF-α (5 ng/ml) in the presence or absence of IL-10 (10 ng/ml) for 24 h were washed and then treated with anti-Fas mAb (1 μg/ml) for another 24 h. Protein extracts of MODE-K (A) and IEC4.1 cells (B) were analyzed by Western blot. Level of procaspase-8 and FLIP expression in MODE-K (C) and IEC4.1 cells (D) were evaluated by densitometry and normalized against the band density for β-actin. Results shown in C and D are means ± SD of 3 experiments. *P < 0.05 compared with untreated cells; **P < 0.05 compared with IFN-γ + TNF-α-treated cells; #P < 0.05 compared with IFN-γ + TNF-α + anti-Fas mAb-treated cells.

**Fig. 9.** Western blot analysis of expression of procaspase-3 protein in intestinal epithelial cell lines. Cells were treated with medium only, IFN-γ (10 ng/ml), TNF-α (10 ng/ml), IL-10 (10 ng/ml), combined IFN-γ (5 ng/ml) + TNF-α (5 ng/ml), combined IFN-γ (5 ng/ml) + TNF-α (5 ng/ml) + IL-10 (10 ng/ml), or anti-Fas mAb (1 μg/ml) for 48 h. In some cases, cells pretreated with combined IFN-γ (5 ng/ml) and TNF-α (5 ng/ml) in the presence or absence of IL-10 (10 ng/ml) for 24 h were washed and then treated with anti-Fas mAb (1 μg/ml) for another 24 h. Protein extracts of MODE-K (A) and IEC4.1 cells (B) were analyzed by Western blot. Levels of procaspase-3 expression in MODE-K (C) and IEC4.1 cells (D) were evaluated by densitometry and normalized against the band density for β-actin. Results shown in C and D are means ± SD of 3 experiments. *P < 0.05 compared with untreated cells; **P < 0.05 compared with IFN-γ + TNF-α-treated cells; #P < 0.05 compared with IFN-γ + TNF-α + anti-Fas mAb-treated cells.
caspase-3 activity with anti-Fas mAb treatment of IFN-γ + TNF-α-pretreated cells.

IL-10 is known to control inflammation by suppressing the production of proinflammatory cytokines regulated by NF-κB (2, 7). We demonstrated a potent concentration-dependent effect of IL-10 in decreasing inflammatory cytokine- and Fas-induced apoptosis of MODE-K and IEC4.1 cells. These findings are supported by our Western blot analysis results showing that IL-10 treatment increased procaspase 8 and procaspase 3 by increasing FLIP levels of cells treated with the combination of IFN-γ, TNF-α, and anti-Fas mAb. These results are further supported by a recent study (31) that showed that IL-10 can alter the FLIP-caspase-8 balance in human dermal fibroblasts. Our findings also showed that IL-10 decreased cytokine-induced Fas expression. A similar effect has been shown for IFN-γ-induced ICAM-1 expression (13). Therefore, there are a number of mechanisms by which IL-10 influences cytokine- and Fas-induced apoptosis. IL-10 receptors structurally resemble receptors for IFN-γ (12, 34). In this way, IL-10 might inhibit IFN-γ-induced STAT1 activation (13) and thereby suppress procaspase 8 aggregation at the DISC. In addition to the ability of IL-10 to regulate Fas expression on MODE-K and IEC4.1 cells, IL-10 might decrease caspase-mediated apoptosis by decreasing caspase-3 activity through the upregulation of FLIP and downregulation of caspase-8 activity. The suppressing effect of IL-10 on IFN-γ-induced ICAM-1 was thought to reflect IL-10 suppression of IFN-γ-induced assembly of STAT to promoter motifs on IFN-γ-inducible genes (13) and is also involved in the inhibition of IP-10, ISG54, ICAM, B7, and major histocompatibility complex II (39). Therefore, it is possible that IL-10 suppresses Fas expression via a similar mechanism, i.e., through suppression of tyrosine phosphorylation of STAT1 activation.

The findings described in the present study therefore suggest that the combination of IFN-γ and TNF-α induces apoptosis but also increased the susceptibility to Fas-mediated apoptotic events. In addition, we showed a direct effect of IL-10 on IECs including diminished IFN-γ-, TNF-α-, and Fas-induced apoptosis, suppression of cytokine-induced Fas expression, and alteration of the caspase-8-FLIP balance with a resulting downregulation of caspase-3 activity.

GRANTS

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