Article

TNF-α Induces Transient Resistance to Fas-Induced Apoptosis in Eosinophilic Acute Myeloid Leukemia Cells

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Tumor necrosis factor α (TNF-α) has been recognized as an activator of nuclear factor κB (NF-κB), a factor implicated in the protection of many cell types from apoptosis. We and others have presented evidence to suggest that Fas-induced apoptosis may be an important aspect of the resolution of inflammation, and that delayed resolution of inflammation may be directly associated with NF-κB-dependent resistance to Fas. Because TNF-α activates NF-κB in many cell types including inflammatory cells such as eosinophils, we examined effects of TNF-α signaling on the Fas-mediated killing of an eosinophilic cell line AML14. While agonist anti-Fas (CH11) treatment induced apoptosis in AML14 cells, no significant cell death occurred in response to TNF-α alone. Electrophoretic mobility shift assay (EMSA) revealed that TNF-α induced NF-κB transactivation in AML14 cells in a time- and dose-dependent fashion, and subsequent supershift assays indicated that the translocated NF-κB was the heterodimer p65 (RelA)/p50. Pre-treatment of cells with TNF-α dramatically decreased the CH11-induced cell death in a transient fashion, accompanied by suppression of activation of caspase-8 and caspase-3 activation. Inhibition of NF-κB transactivation by inhibitors, BAY 11-7085 and parthenolide, reversed the suppression of Fas-mediated apoptosis by TNF-α. Furthermore, TNF-α up-regulated X-linked inhibitor of apoptosis protein (XIAP) transiently and XIAP levels were correlated with the temporal pattern of TNF-α protection against Fas-mediated apoptosis. This finding suggested that TNF-α may contribute to the prolonged survival of inflammatory cells by suppression of Fas-mediated apoptosis, the process involved with NF-κB transactivation, anti-apoptotic XIAP up-regulation and caspase suppression. *Cellular & Molecular Immunology*. 2007;4(1):43-52.

Key Words: TNF-α, NF-κB transactivation, apoptosis, XIAP, inflammatory cell resolution

Introduction

Increasing genetic and experimental evidence indicates that TNF-α is associated with the inflammatory response that characterizes inflammatory diseases including airway eosinophilia and asthma (1, 2). TNF-α-mediated augmentation of the apoptotic killing of certain inflammatory cells has been demonstrated (3), and ligation of death receptors of the TNFR family can initiate signaling pathways leading to cell death or cell survival. Although TNF itself was named for its ability to induce cell death, it has been known for several years that TNF-α stimulation also can induce activation of the transcription factor NF-κB (4-8). Many normal cells are not killed by TNF and this may be related to NF-κB transactivation; blockade of NF-κB sensitizes cells to TNF and augments induced apoptotic cell death (9-11).

NF-κB transactivation is induced by TNF-α *via* a pathway of IκB kinase (IKK) complex phosphorylation, degradation of IκBα and release of cytoplasm-sequestered NF-κB into the nucleus (11-14). TNF-α-induced NF-κB is mainly composed of a hetero-dimer of p65 (RelA) and a p50 subunits (15). NF-κB transactivation can activate expression of a wide variety of genes including the inhibitor of apoptosis proteins (IAPs) (16-21). Recent studies have shown that NF-κB-regulated IAPs can inhibit caspase activity (22-25) and can prevent Fas-induced apoptosis (26). Additional

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Abbreviations: TNF-α, tumor necrosis factor alpha; NF-κB, nuclear factor kappa B; EMSA, electrophoretic mobility shift assay; XIAP, X-linked inhibitor of apoptosis protein; IKK, IkappaB kinase; FLIP, FLICE-inhibitory protein; TRADD, TNFR1-associated death domain protein; RIP1, receptor-interacting protein; TRAF2, TNF receptor-associated factor 2.

studies have shown that suppression of IAP genes sensitized endothelial cells to TNF-α-induced apoptosis (27). We have previously shown that an eosinophil-like subline of parental (p)AML cells, termed AML14.3D10, is resistant to Fas-induced cell death due to activation of NF-κB by Fas ligation (28). In the present study, we show that Fas induced significant apoptosis in the eosinophilic pAML14 cells, and this cell death was attenuated by pre-incubation of TNF-α *via* suppression of caspase activation and coincident with X-chromosome-linked inhibitor of apoptotic protein (XIAP) up-regulation. Inhibition of NF-κB transactivation using a pharmacological inhibitor of IKK abrogated the TNF-α-induced protection against Fas killing. We demonstrate that temporal TNF-α-mediated suppression of Fas-mediated apoptosis may be due to the transient up-regulation of XIAP by TNF-α. Our results suggest that TNF-α-induced NF-κB transactivation and resulting up-regualtion of expression of XIAP or other such NF-κB targets may play important roles in the prolonged survival eosinophil cells. We suggest that this prolongs survival of inflammatory cells may be important in the delayed resolution of airway inflammation in diseases such as asthma.

Materials and Methods

Cell culture and reagents

The parental AML14 cell line (pAML14) is an eosinophilic AML line originally established from a FAB M2 acute myeloid leukemia patient and provided by Drs. C. Paul and M. Baumann (Wright State University, Dayton, OH) (29). pAML14 cells were maintained in RPMI 1640 medium supplemented with 8% FBS, 2 mM L-glutamine, 1% (w/v) gentamicin, 10 mM sodium pyruvate, 1 mM HEPES, and 5×10^{-5} M 2-ME (Sigma-Aldrich, St. Louis, MO). Cells were grown up to a maximum density of 0.7×10^6 cells/ml at 37°C, 5% CO_2 and were passaged three times a week. After ~40 passages, fresh cultures were started from frozen stocks to minimize genetic drift and phenotypic changes. The mouse anti-human Fas monoclonal (IgM) CH11 was obtained from Panvera (Madison, WI). Recombinant TNF-α was obtained from Alexis Biochemicals (San Diego, CA). Antibodies against NF-κB subunits p65 (RelA), p50, RelB and goat anti-human actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8 was provided by Dr. M. Peter (University of Chicago, Chicago, IL). Anti-caspase-3 was purchased from BD PharMingen (San Diego, CA). Antibody to XIAP was purchased from MBL International (Watertown, MA). The IKK inhibitor parthenolide was purchased from Calbiochem (San Diego, CA).

Detection of apoptosis

Cell death was determined by both trypan blue exclusion assay and flow cytometric analyses of DNA fragmentation. Briefly, pAML14 cells were cultured at a density of 3×10^5 cells/ml/well in 48-well cell culture plates. CH11 was used primarily at a range of 5 to 100 ng/ml. Each cell sample was divided for trypan blue exclusion assays and for standard propidium iodide (PI) DNA analyses after designed time length from 3 to 72 h. Total cell death was first determined by trypan blue (0.2%) exclusion using a conventional light microscope. The remaining cells were centrifuged at 200 × g for 10 min and resuspended in hypotonic PI solution (50 μg/ml PI in 0.1% Na citrate, 0.1% Triton X-100). To ensure cell lysis, cells were stored overnight in the dark at 4°C before flow cytometric analysis. At least 5,000 nuclei were examined for each sample to determine percentage of subG1 DNA content. In preliminary experiments, hypotonic PI analyses of cell samples closely correlated with other DNA fragmentation and morphologic criteria of apoptosis. Percentages (± SE) of cell death reported in the results are derived from the flow cytometric analyses.

Western blot analysis

Protein lysates were prepared from pAML14 cells after treatments described in the results. Equal amounts of protein were separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Sigma-Aldrich, St. Louis, MI). Immunoblotting was performed according to the ECL Western blotting protocol (Amersham Pharmacia Biotech, Arlington Heights, IL). Briefly, blots were blocked in 5% nonfat milk in 1× TBS-Tween solution for 1 h followed by one hour incubation with the appropriate primary antibody. Blots were then washed for 30 min with three changes of 1× TBS-Tween solution followed by one-hour incubation with the appropriate HRP-conjugated secondary antibody. Blots were washed again three times in 1× TBS-Tween solution and incubated for 1 min with ECL reagents. The results were visualized by exposing blots to autoradiographic film (Kodak, Rochester, NY).

Extraction of nuclear protein and electrophoretic mobility gel shift assay (EMSA) for NF-κB activation analysis

Cells were passaged and grown overnight at ~6×10^5 cells/ml in cell culture flasks. After the treatments, the cell nuclear extracts were prepared according to a published method (28). Unless indicated, otherwise all procedures were performed at 4°C. Briefly, 10×10^6 cells were harvested by centrifugation and washed twice with ice-cold Dulbecco's PBS buffer. The pellet was resuspended in 4× packed cell volume of buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT] and incubated on ice for 10 min. The supernatant was discarded after centrifugation at 1,300 rpm for 7 min and 1× original packed cell volume of buffer A was added. The cell suspension was transferred to a 50-ml "woodrage" centrifuge tube and centrifuged at 8,500 rpm for 20 min in a Beckman JS 13.1 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was removed and set aside as the cytoplasmic extract. The pellet was gently washed with buffer A an additional time and resuspended in 1× original packed cell volume of buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT]. The suspension was stirred on a rocking platform for 30 min

and then centrifuged in a Beckman rotor JA-17 (Beckman Instruments) at 12,500 rpm for 30 min. The supernatant was collected without disturbing the pellet and placed in dialysis tubing (Life Technologies, Grand Island, NY). Dialysis was performed for 1 h against three changes of 200 ml of buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT]. Following dialysis, the nuclear extract was clarified by centrifugation at 14,000 rpm for 20 min in an Eppendorf microcentrifuge tube (Brinkman Instruments, Westbury, NY). Protease inhibitors including leupeptin, antipain, chymostatin, and pepstatin A (Sigma-Aldrich) were added immediately (5 μg/ml each) to extracts. The details of the EMSA have been described elsewhere (28). Briefly, double-stranded NF-κB synthetic oligonucleotides 5'-AGT TGA GGG GAC TTT CCC AGG C-3' were purchased from Promega (Madison, WI) and end-labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (NEB, Beverly, MA). A 200-fold excess of unlabeled NF-κB probe and unrelated oligonucleotide probes for CArG was used to assess the specificity of the DNA-binding reaction. Binding reactions were performed on ice in a total volume of 15 μl. DNA probe (2,000 cpm, 1-5 fmol) was preincubated for 15 min with 1.5 μl binding buffer B [50 mM HCl (pH 7.5), 20% Ficoll, 375 mM KCl, 5 mM EDTA, 5 mM DTT] and 1 μg poly(dI-dC) (Promega). DNA-protein binding was initiated by adding 4 μg of nuclear extract. A total of 200-fold excess of "cold" (unlabeled) NF-κB probe was used as a specific competitor. Electrophoresis was performed for 3 h at 100 V in 0.5× Tris-borate-EDTA running buffer in a 4°C cold room. The dried gel was visualized *via* exposure to high performance autoradiography film. The supershift analyses were performed by incubating the DNA-binding reactions with optimal concentrations (determined previously) of Abs to p65, p50, or RelB for an additional 20 min on ice before electrophoresis. The images were obtained by auto-radiography.

Pharmacologic inhibition of the NF-κB transactivation

pAML14 cells were cultured at a density of 0.6 × 10^6 cells/ml and were preincubated for 1 h with IKK inhibitor parthenolide (30) at a range of concentrations (0.1-10 μM) before addition of TNF-α. Optimal doses, at which augmentation of TNF-α-mediated NF-κB activation was greatest with the least background toxicity of inhibitors alone, were calculated and used in certain experiments as described.

Results

The inflammatory cytokine TNF-α is one of the major regulatory factors for proliferation and survival in hematopoietic cells (31-34). Specifically, TNF-α and Fas ligand interactions with their receptors are considered to be survival and death signals to many cells. In this study, we have examined the signaling effects of TNF-α on eosinophilic pAML14 cells and the consequence of this signaling on Fas agonist anti-Fas Ab CH11-induced eosinophilic pAML14 apoptosis. We analyzed cell death, caspase (-8 and -3) activation and cell death, following stimulation of these receptors under control conditions or in the presence of inhibitors of NF-κB activation. We also determined the effects of TNF-α stimulation on NF-κB transactivation and target anti-apoptotic XIAP expression.

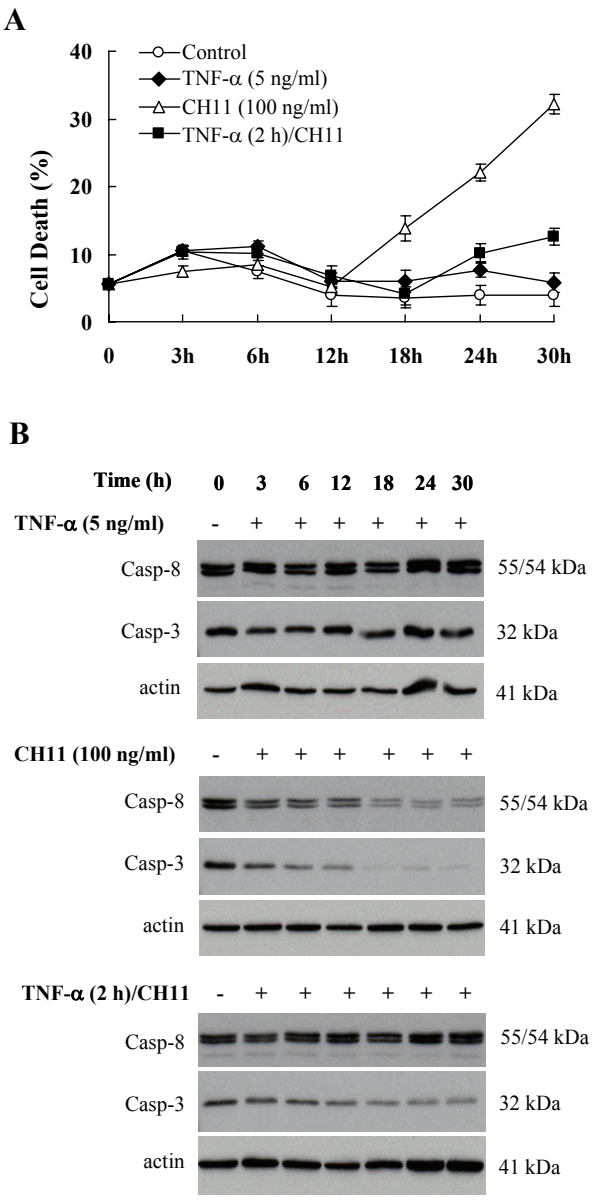

Figure 1. TNF-α attenuates Fas-induced apoptosis and suppresses caspase cascade in pAML14 cells. (A) TNF-α and CH11 killing assay. pAML14 cells were cultured at 3 × 10^5 cells/ml and treated with TNF-α (5 ng/ml), CH11 (100 ng/ml) with or without 1 h pre-treatment of TNF-α. Cells were harvested at time as indicated above. Percentage of cell death was determined by hypotonic PI DNA analysis. (B) Western blot analyses of procaspase-8 and -3 cleavage in TNF-α or CH11-treated pAML cells are shown in top and middle panels. The bottom panel is CH11 treatment following 1 h pre-treatment of TNF-α. Actin controls were performed on the same blots.

TNF-α attenuates Fas-induced apoptosis and suppresses caspase cascade in pAML14 cells

The Fas agonist antibody, CH11, induced significant cell death in pAML14 cells (Figure 1A). pAML14 cells were treated with 5 ng/ml TNF-α, or 100 ng/ml CH11 with or without 1 h pre-incubation of TNF-α for 3-24 h. Cells were harvested by centrifugation and lysed using hypotonic PI solution as described above. Percentage of cell death was analyzed using flow cytometric analysis after treated with CH11 for 3 to 30 h. Significant cell death was induced by CH11 by 12 h following CH11 treatment, but this was abolished by pre-incubation of 5 ng/ml TNF-α. TNF-α alone had no apoptotic or additive survival effect on pAML14 cells (Figure 1A). Western blot revealed that the Fas ligation induced cleavage of both pro-casp8 and 3 after 12 h and these were suppressed by TNF-α pretreatment (Figure 1B). There was no significant detectable cleavage of procaspase-8 or -3 in the TNF-α treated group. Actin controls were performed on same blot (Figure 1B).

TNF-α induced NF-κB activation

Previous studies have shown that NF-κB plays an important anti-apoptotic role in many cell types including eosinophilic cells (28). We examined TNF-α and CH11 mediated NF-κB transactivations in pAML14 cells. EMSA analyses were performed according to the previous study and showed that TNF-α induced NF-κB transactivation in pAML14 cells is both time- and dose-dependent (Figures 2A, 2B). While no activation of NF-κB in pAML14 eosinophils after treatment of 100 ng/ml of CH11 was observed (data not shown), TNF-α-induced nuclear translocation of NF-κB in these cells occurred as early as 10 min. There are five members of the NF-κB/Rel family of proteins that have been found expressed in mammalian cells: p65/RelA, c-Rel, RelB, p105/NF-κB1 (which can be processed to p50), and p100/NF-κB2 (which can be processed to p52; reviewed in reference 35). These subunits usually exist as hetero- or homo-dimers. We examined the NF-κB subunits in pAML14 cells by supershift assay using Abs specific for p65, p50, and RelB. Nuclear extracts were prepared from pAML14 cells treated with 5 ng/ml TNF-α for 30 min. Only antibodies against p65 and p50 shifted the NF-κB bands, indicating that p65/p50 heterodimer is likely the activated form of the NF-κB induced by TNF-α in these cells (Figure 2C). Faint bands in some lanes which could be shifted by anti-p50 Ab only could represent endogenous p50/p50 homodimers.

IKK-β inhibitor-parthenolide attenuated TNF-α protection against Fas-mediated apoptosis

Because Fas-induced apoptosis of pAML14 cells can be suppressed by TNF-α, the induction of TNF-α-mediated transactivation of NF-κB suggests a link between potential NF-κB transactivation and suppression against CH11 killing. To further understand the role of NF-κB transactivation in TNF-α protection against Fas-induced apoptosis, the effects of inhibition of NF-κB transactivation on TNF-α protection

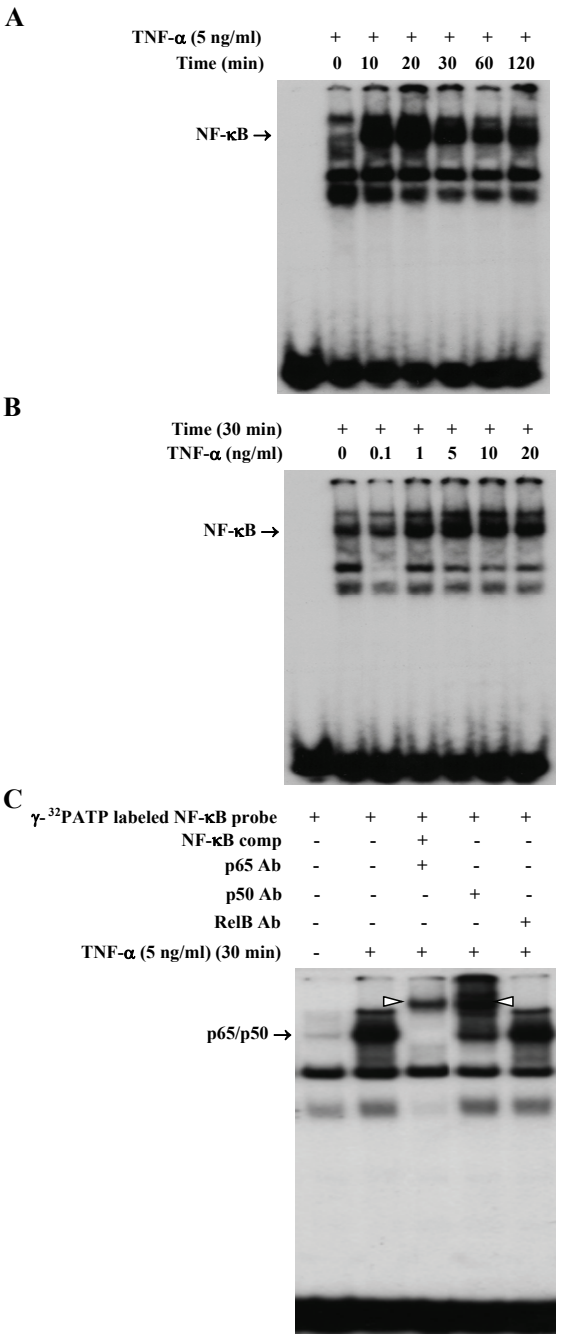

Figure 2. TNF-α induced NF-κB tansactivation in pAML14 cells. (A) EMSA analysis of the time course of NF-κB activation induced by TNF-α. (B) EMSA analysis of the dose response of NF-κB activation induced by TNF-α. pAML cells were stimulated with TNF-α at dose and time as indicated in the figure. (C) Supershift EMSA analysis of TNF-α-induced NF-κB subunits in pAML cells. pAML cells were incubated with 5 ng/ml of TNF-α for 30 min. Nuclear extracts were prepared from these cells and 4 μg of each nuclear extract was incubated with excess unlabeled competitor (NF-κB probe) (lane 1), 1.0 μg of each Ab against the NF-κB subunits p65/RelA (lane 3), p50 (lane 4), RelB (lane 5) or no antibody (lane 2) for 30 min at room temperature and then analyzed by EMSA. Supershifts of p65 and p50 subunits are indicated by arrowheads at lanes 3 and 4.

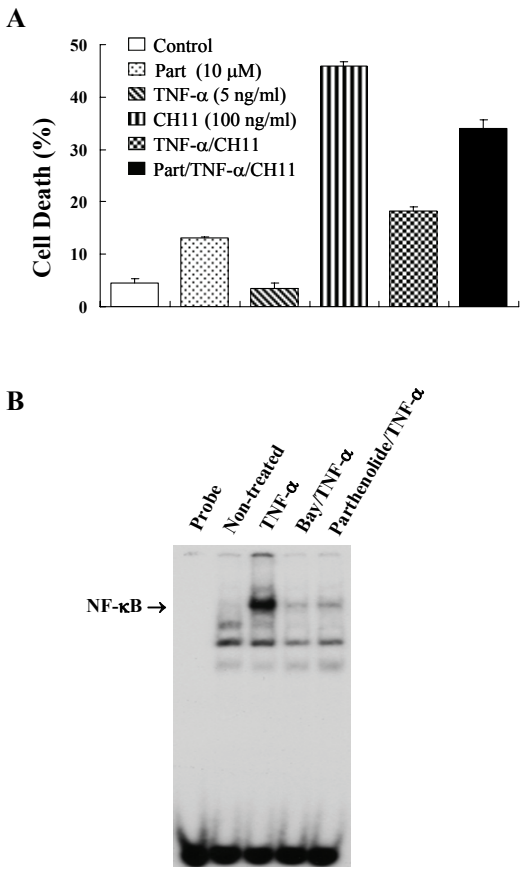

Figure 3. Preincubation with IKK-β inhibitor, parthenolide, attenuates TNF-α protection against Fas-mediated apoptosis. (A) pAML14 cells were incubated with 100 ng/ml of CH11 plus or minus 1 h pre-treatment of TNF-α (5 ng/ml) at 37°C for indicated times with or without treatment with 10 μM parthenolide. Apoptosis was measured by FACS analysis of cells as described. Data represent the mean of three independent experiments. (B) EMSA analysis of inhibition of NF-κB activation by parthenolide.

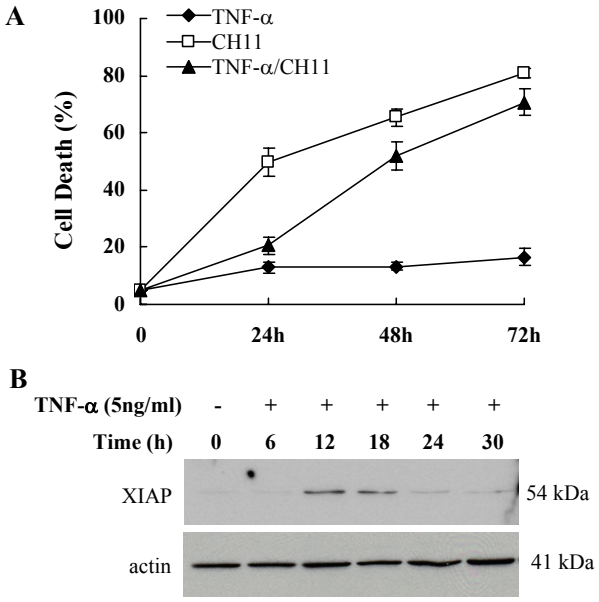

Figure 4. TNF-α transiently protects against CH11-induced apoptosis and protection correlates with increased XIAP expression in pAML14 cells. (A) TNF-α failed to protect against CH11 killing after 48 h. pAML14 cells were treated with TNF-α (5 ng/ml) and/or CH11 (100 ng/ml) (with or without 1 h pre-treatment of TNF-α). Cells were harvested at 24, 48, and 72 h. Percentage of cell death was determined by hypotonic PI DNA analysis. (B) Western blot analyses of TNF-α-induced XIAP expression. pAML14 cells were treated with 5 ng/ml TNF-α, and protein lysates were prepared at times indicated for Western blot analysis using anti-XIAP antibody. Actin control was performed on same blot.

was examined using the pharmacologic IKK inhibitor, parthenolide (36). Parthenolide (10 μM) was used to pretreat cells for 1 h prior to TNF and/or CH11 treatments. Parthenolide treatment significantly increased cell death in comparison with untreated TNF-α/CH11 group (Figure 3A). In addition, pre-incubation of Bay11-7085 which is previously proven a potent inhibitor of IκB phosphorylation (37) and NF-κB transactivation in AML sublines (28), similarly attenuated TNF-α-mediated protection in AML14 cells (data not shown). EMSA confirmed that TNF-α-induced NF-κB transactivation was inhibited when cells were treated with 10 μM parthenolide or Bay11-7085 (Figure 3B).

TNF-α protects against CH11-induced apoptosis in transient and correlate with XIAP expression in pAML14 cells

Numerous studies have shown that activation of NF-κB can lead to the anti-apoptotic proteins such as IAPs and other prosurvival proteins (23, 25, 38, 39). These anti-apoptotic proteins can inhibit the proteolytic activity of caspases and suppress apoptosis (25, 40, 41). Among these proteins, XIAP appears to be the most capable of inhibition of both upstream and downstream caspases (42-44). We examined the expression of XIAP in pAML14 cells following TNF-α treatment for 6 to 30 h as indicated in the Figure 4A. Western blot showed XIAP was maximum increased after 12 h treatment of 5 ng/ml TNF-α, and sustained 18 h after treatment, however it was decreased to the baseline level after 24 h (Figure 4B). Analyses of cell death were performed using flow cytometry, and the TNF-α protection effects were evaluated. TNF-α pre-incubation significantly suppressed CH11-induced apoptosis within 24 h after CH11 treatment (Figures 1A, 4A), but failed to sustain such protection by 48 to 72 h (Figure 4B).

Discussion

TNF-α binding to the receptor TNFR1 can initiate apoptosis in some cells and increases the survival and proliferation in other cells by an incompletely understood mechanism (45-47). A recent study showed that macrophages induce neutrophil apoptosis through membrane TNF during parasite infection in mice, and emphasized the importance of TNF in

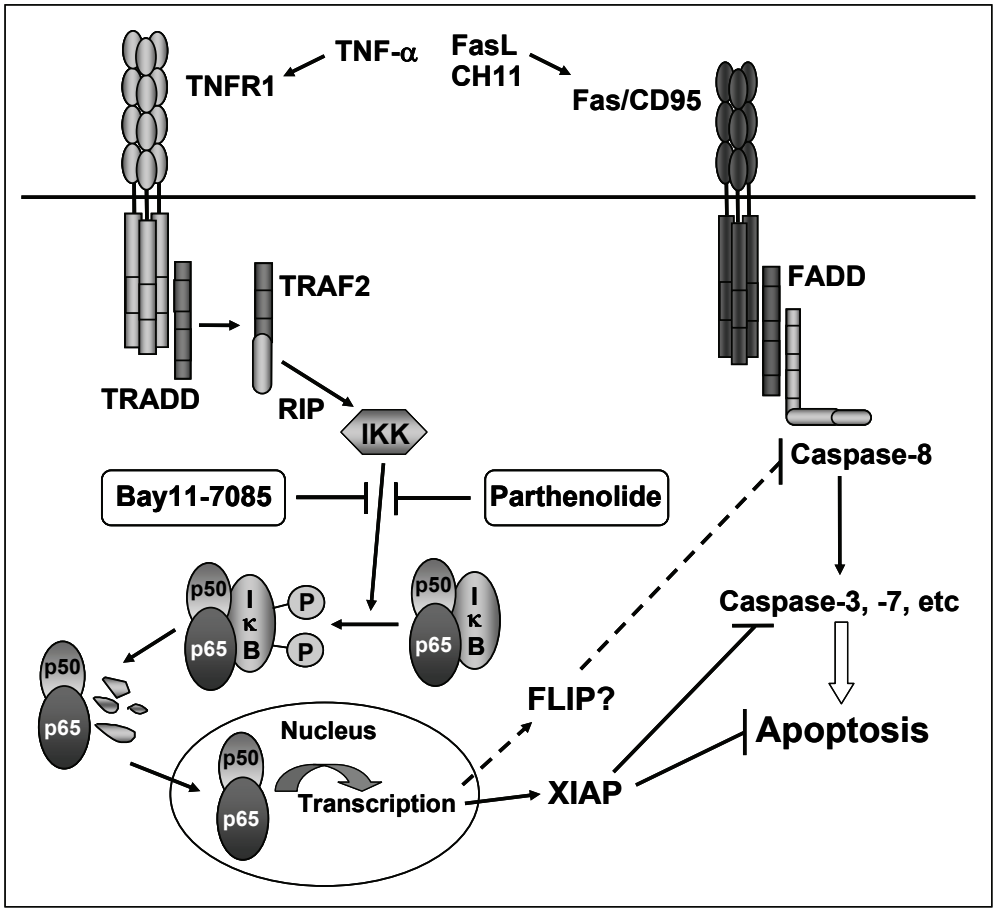

Figure 5. Simplified diagram of potential signaling pathways during TNF-α-induced repression of Fas-mediated apoptosis in our eosinophilic cell model. Our results suggest that TNF-α induces transactivation of NF-κB in pAML14 cells. NF-κB signaling through TNFR1 involves a complex composed of the receptor, the adaptor TRADD, the kinase RIP1 and the protein TRAF2. Transactivation of NF-κB activated by TNF-α suppressing Fas-mediated caspase activation and apoptosis in pAML14 cells *via* up-regulating XIAP and other potential anti-apoptotic proteins such as FLIP. The inhibitors Bay11-7085 and parthenolide block NF-κB activation and abrogate TNF-mediated protection.

the early control of inflammation (48). In cystic fibrosis, which is characterized by chronic airway inflammation, TNF-α-mediated control of inflammation is suggested by evidence of TNF-induced NF-κB transactivation and IL-8 production (49). Others have shown that inhibition of NF-κB by overexpression of NF-κB inhibitor IκB-α resulted in the blockade of TNF-α-induced inflammatory cytokine production (10, 50). Transgenic mice expressing IκB-α superrepressor in airway epithelial cells has remarkably reduced influx of neutrophils into the airways and secretion of the NF-κB-targeted neutrophilic chemokine, macrophage-inflammatory protein-2, and the inflammatory cytokine, TNF-α when they were exposed to LPS. Together, these results suggest that NF-κB signaling is important for modulating airway inflammation (51). Infiltration of airway tissues by potentially pathogenic granulocytes (eosinophils and neutrophils) is a major characteristic of asthma and chronic obstructive pulmonary disease (COPD) (52-55). The failure of removal of infiltrating granulocytes *via* apoptosis or necrosis followed by macrophage engulfment may be a pathogenesis of asthma or COPD (56-59). However, the molecular mechanisms involved have not been revealed despite the evidence of elevated TNF-α in both asthma and COPD (2, 60). We have previously reported that Fas ligation induced transactivation of NF-κB in eosinophilic AML.3D10 cells, and inhibition of transactivation of NF-κB by either pharmacological inhibitor-Bay11-7085 or by overexpression of IκB-α led to Fas-mediated apoptosis. We suggested that NF-κB transactivation may play an important role in airway eosinophil accumulation during diseases such as asthma (28).

The AML14 cell line was first established from a patient with acute myelogenous leukemia, and it exhibits eosinophilic differentiation in suspension in response to IL-3, IL-5, and GM-CSF (29, 61). Although this cell line exhibits some differences from primary eosinophils, AML14 cells share many of the properties of primary cells and has been widely used as a model to study adhesion, promoter activity, aspects of cell cycle, and nuclear receptors, as well as

mechanisms of apoptosis in eosinophils (28, 62-65). Apoptosis is an important mechanism which may limit inflammation, and Fas can play a key role in inflammatory cell apoptosis and resolution of inflammation (66). Study on Fas (CD95)-induced apoptosis in the development and resolution of airway inflammation and airway hyper-responsiveness (AHR) using mouse model suggested that Fas expression can regulate the onset and resolution of AHR through an increase in eosinophil apoptosis (67). Administration of Ad-FasL intratracheally significantly decreased AHR and eosinophilia by inducing the apoptosis of eosinophils, reducing IL-5 and eotaxin levels, as well as the number of infiltrated lymphocytes (68). In addition, deregulation of Fas on T cells may be sufficient for the development of long-term allergic airway disease in mice (69). However, whether Fas-mediated apoptosis of inflammatory cells can be influenced by TNF-α and its effects *via* NF-κB activation are unknown.

In the present study, we examine the TNF-α effect on Fas-mediated apoptosis of eosinophilic cells in light of the potential importance of TNF-α in airway inflammation. In addition to its activities outlined above, TNF-α can act as a survival factor in inflammatory diseases such as rheumatoid arthritis (9). Transactivation of NF-κB by TNF-α has been reported to block the activation of caspase-8 *via* activation of a group of genes encoding the caspase inhibitor proteins, the IAPs (10, 70). In the present study, we show that TNF-α treatment blocks Fas-mediated apoptosis in pAML14 cells, and that decreased apoptosis is correlated to the suppression of the caspase cascade. TNF-α failed to induce apoptosis and activation of caspase-8 and -3 in eosinophilic pAML cells, but induced significant transactivation of the NF-κB heterodimer p65/p50 in both dose and time-dependent fashion. This suggests that TNF-α can act as a survival factor in these cells.

The sesquiterpene lactone parthenolide inhibits NF-κB transactivation by preventing the induction of IKK-β, without affecting the activation of p38 and c-Jun N-terminal kinase (36). Further study has shown that parthenolide is capable of binding directly to and inhibiting IKK-β (71). In our study, pre-incubation of cells with parthenolide attenuated TNF-α protection against Fas-mediated cell death in pAML14 cells, and was accompanied by inhibition of TNF-α-induced NF-κB transactivation. Our results suggest that TNF-α induces transactivation of NF-κB in favor of survival or anti-apoptotic signaling in pAML14 cells. NF-κB signaling through TNFR1 involves a complex composed of the receptor, the adaptor TRADD, the kinase RIP1 and the protein TRAF2 (Figure 5). When NF-κB is activated by complex I, TNF-α-mediated cell death is inhibited (72). Our findings suggest that complex I signaling can also override Fas-mediated apoptotic signaling through, at least in part, up-regulation of XIAP.

XIAP is one of the NF-κB-regulated genes that counteract apoptotic signaling by TNF-α in a number of cells. In endothelial cells, TNF-α-induced NF-κB-mediated induction of XIAP prevents lipopolysaccharide (LPS)-mediated apoptosis during inflammation (73). NF-κB-mediated expression of XIAP also blocks apoptosis in rat granulosa cells (74). The proapoptotic, anti-inflammatory effect of guggulsterone has been suggested to involve the suppression of TNF-mediated NF-κB transactivation and downregulation of anti-apoptosis target genes, including XIAP (75). In our pAML14 cells, XIAP was present at very low detectable level endogenously, but increased significantly 12 h after TNF-α treatment. However, levels of XIAP expression declined to baseline after 18 h incubation of TNF-α. The kinetics of TNF-induced XIAP expression was directly correlated with kinetics of TNF-α protection against Fas induction of apoptosis in pAML14 cells. It is likely that TNF-α suppression of Fas-mediated apoptosis involves XIAP or other NF-κB targets, and we are further testing this hypothesis. It is becoming increasingly apparent that XIAP is an important endogenous anti-apoptotic protein, and that increased expression of XIAP attenuates apoptosis through inhibition of caspases, particularly caspase-9, and *via* suppression of apoptosome function (76). Indeed, XIAP is the only IAP that can inhibit both upstream caspase-9 and downstream caspase-3, -7 (77-79). *In vitro* experiments using purified recombinant proteins have revealed that BIR3-Ring domain of XIAP is a specific inhibitor of caspase-9 whereas BIR1-2 domains are specific for caspase-3 and -7 (80, 81). Because XIAP is not thought to inhibit caspase-8 directly (82), the observed inhibition of caspase-8 activation by TNF may involve other NF-κB targets such as FLIP. Although we did not detect a measurable increase in FLIP (or its isoforms), it is likely that this or some other NF-κB targets are involved in the protective actions of TNF-α affect caspase-8 activation in these cells, and we are currently investigating these additional or alternative pathways. Thus, additional questions remain regarding the detailed mechanisms by which TNF-α contributes to inflammatory cell survival, as well as for the overall role of TNF-α-mediated NF-κB transactivation in airway inflammation. Our data here suggest that TNF-α-mediated NF-κB transactivation and regulation of anti-apoptotic genes play important roles in Fas-mediated cell death in inflammatory cells. Understanding the contribution of TNF-α-mediated cell survival and the potential delay in resolution in such circumstances may be relevant to therapeutic approached in enhancing or modulating resolution, particularly in diseases of chronic inflammation.

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References

1. Matsumoto T, Goto Y, Miike T. Markedly high eosinophilia and an elevated serum IL-5 level in an infant with cow milk allergy. Ann Allergy Asthma Immunol. 1999;82:253-256.

2. Tsoumakidou M, Tzanakis N, Siafakas NM. Induced sputum in the investigation of airway inflammation of COPD. Respir Med. 2003;97:863-871.
3. Yuan K, Wing LY, Lin MT. Pathogenetic roles of angiogenic factors in pyogenic granulomas in pregnancy are modulated by female sex hormones. J Periodontol. 2002;73:701-708.
4. Israel N, Hazan U, Alcami J, et al. Tumor necrosis factor stimulates transcription of HIV-1 in human T lymphocytes, independently and synergistically with mitogens. J Immunol. 1989;143:3956-3960.
5. Liu ZG, Han J. Cellular responses to tumor necrosis factor. Curr Issues Mol Biol. 2001;3:79-90.
6. Baeuerle PA, Baltimore D. NF-κB: ten years after. Cell. 1996; 87:13-20.
7. Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell. 1994;76:959-962.
8. Mayo MW, Baldwin AS. The transcription factor NF-κB: control of oncogenesis and cancer therapy resistance. Biochim Biophys Acta. 2000;1470:M55-62.
9. Chen Q, Casali B, Pattacini L, Boiardi L, Salvarani C. Tumor necrosis factor-α protects synovial cells from nitric oxide induced apoptosis through phosphoinositide 3-kinase Akt signal transduction. J Rheumatol. 2006;33:1061-1068.
10. McDade TP, Perugini RA, Vittimberga FJ, Jr., Callery MP. Ubiquitin-proteasome inhibition enhances apoptosis of human pancreatic cancer cells. Surgery. 1999;126:371-377.
11. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell. 1996;87:565-576.
12. Yeh WC, Shahinian A, Speiser D, et al. Early lethality, functional NF-κB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity. 1997;7:715-725.
13. Zhang J, Cado D, Chen A, Kabra NH, Winoto A. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature. 1998;392:296-300.
14. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-κB signal. Immunity. 1998;8:297-303.
15. Jackson-Bernitsas DG, Ichikawa H, Takada Y, et al. Evidence that TNF-TNFR1-TRADD-TRAF2-RIP-TAK1-IKK pathway mediates constitutive NF-κB activation and proliferation in human head and neck squamous cell carcinoma. Oncogene. 2006; in press.
16. Paland N, Rajalingam K, Machuy N, Szczepek A, Wehrl W, Rudel T. NF-κB and inhibitor of apoptosis proteins are required for apoptosis resistance of epithelial cells persistently infected with Chlamydophila pneumoniae. Cell Microbiol. 2006;8:1643-1655.
17. Liu Z, Li H, Wu X, et al. Detachment-induced upregulation of XIAP and cIAP2 delays anoikis of intestinal epithelial cells. Oncogene. 2006;25:7680-7690.
18. Machuca C, Mendoza-Milla C, Cordova E, et al. Dexamethasone protection from TNF-α-induced cell death in MCF-7 cells requires NF-κB and is independent from AKT. BMC Cell Biol. 2006;7:9.
19. Conze DB, Albert L, Ferrick DA, et al. Posttranscriptional downregulation of c-IAP2 by the ubiquitin protein ligase c-IAP1 *in vivo*. Mol Cell Biol. 2005;25:3348-3356.
20. Poppelmann B, Klimmek K, Strozyk E, Voss R, Schwarz T, Kulms D. NFκB-dependent down-regulation of tumor necrosis factor receptor-associated proteins contributes to interleukin-1-mediated enhancement of ultraviolet B-induced apoptosis. J Biol Chem. 2005;280:15635-15643.
21. Lee R, Collins T. Nuclear factor-κB and cell survival: IAPs call for support. Circ Res. 2001;88:262-264.
22. Varfolomeev E, Wayson SM, Dixit VM, Fairbrother WJ, Vucic D. The inhibitor of apoptosis protein fusion c-IAP2.MALT1 stimulates NF-κB activation independently of TRAF1 and TRAF2. J Biol Chem. 2006;281:29022-29029.
23. Notarbartolo M, Poma P, Perri D, Dusonchet L, Cervello M, D'Alessandro N. Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-κB activation levels and in IAP gene expression. Cancer Lett. 2005;224:53-65.
24. Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. J Biol Chem. 2000;275:16309-16315.
25. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene. 1998;17:3247-3259.
26. Ruemmele FM, Beaulieu JF, O'Connell J, Bennett MW, Seidman EG, Lentze MJ. The susceptibility to Fas-induced apoptosis in normal enterocytes is regulated on the level of cIAP1 and 2. Biochem Biophys Res Commun. 2002;290: 1308-1314.
27. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proc Natl Acad Sci U S A. 1997;94:10057-10062.
28. Qin Y, Camoretti-Mercado B, Blokh L, Long CG, Ko FD, Hamann KJ. Fas resistance of leukemic eosinophils is due to activation of NF-κB by Fas ligation. J Immunol. 2002;169: 3536-3544.
29. Paul CC, Tolbert M, Mahrer S, Singh A, Grace MJ, Baumann MA. Cooperative effects of interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor: a new myeloid cell line inducible to eosinophils. Blood. 1993;81: 1193-1199.
30. Zingarelli B, Hake PW, Denenberg A, Wong HR. Sesquiterpene lactone parthenolide, an inhibitor of IκB kinase complex and nuclear factor-κB, exerts beneficial effects in myocardial reperfusion injury. Shock. 2002;17:127-134.
31. Veiby OP, Jacobsen FW, Cui L, Lyman SD, Jacobsen SE. The flt3 ligand promotes the survival of primitive hemopoietic progenitor cells with myeloid as well as B lymphoid potential. Suppression of apoptosis and counteraction by TNF-α and TGF-β. J Immunol. 1996;157:2953-2960.
32. Yap GS, Sher A. Effector cells of both nonhemopoietic and hemopoietic origin are required for interferon (IFN)-γ- and tumor necrosis factor (TNF)-α-dependent host resistance to the intracellular pathogen, Toxoplasma gondii. J Exp Med. 1999; 189:1083-1092.
33. Alcamo E, Mizgerd JP, Horwitz BH, et al. Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-κB in leukocyte recruitment. J Immunol. 2001;167:1592-1600.
34. Nifontova IN, Drize NI. Effect of TNF expression by stromal cells on hemopoietic and stromal precursor cells in long-term bone marrow cultures derived from TNF-deficient mice. Bull Exp Biol Med. 2005;140:550-553.
35. Baldwin AS, Jr. The NF-κB and IκB proteins: new discoveries

and insights. Annu Rev Immunol. 1996;14:649-683.
36. Hehner SP, Hofmann TG, Droge W, Schmitz ML. The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-κB by targeting the IκB kinase complex. J Immunol. 1999; 163:5617-5623.
37. Mabuchi S, Ohmichi M, Nishio Y, et al. Inhibition of NF-κB increases the efficacy of cisplatin in *in vitro* and *in vivo* ovarian cancer models. J Biol Chem. 2004;279:23477-23485.
38. Hao XS, Hao JH, Liu FT, Newland AC, Jia L. Potential mechanisms of leukemia cell resistance to TRAIL-induced apoptosis. Apoptosis. 2003;8:601-607.
39. Mattson MP, Meffert MK. Roles for NF-κB in nerve cell survival, plasticity, and disease. Cell Death Differ. 2006;13:852-860.
40. Sanna MG, da Silva Correia J, Ducrey O, et al. IAP suppression of apoptosis involves distinct mechanisms: the TAK1/JNK1 signaling cascade and caspase inhibition. Mol Cell Biol. 2002; 22:1754-1766.
41. Yamanaka K, Rocchi P, Miyake H, et al. A novel antisense oligonucleotide inhibiting several antiapoptotic Bcl-2 family members induces apoptosis and enhances chemosensitivity in androgen-independent human prostate cancer PC3 cells. Mol Cancer Ther. 2005;4:1689-1698.
42. Vaughn AE, Deshmukh M. Essential postmitochondrial function of p53 uncovered in DNA damage-induced apoptosis in neurons. Cell Death Differ. 2007; in press.
43. Carter BZ, Gronda M, Wang Z, et al. Small-molecule XIAP inhibitors derepress downstream effector caspases and induce apoptosis of acute myeloid leukemia cells. Blood. 2005;105: 4043-4050.
44. Huang Y, Lu M, Wu H. Antagonizing XIAP-mediated caspase-3 inhibition. Achilles' heel of cancers? Cancer Cell. 2004;5:1-2.
45. Burow ME, Weldon CB, Tang Y, et al. Differences in susceptibility to tumor necrosis factor α-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res. 1998;58:4940-4946.
46. Nakashima A, Kumakura S, Mishima S, Ishikura H, Kobayashi S. IFN-α enhances TNF-α-induced apoptosis through down-regulation of c-Myc protein expression in HL-60 cells. J Exp Clin Cancer Res. 2005;24:447-456.
47. Zhang HG, Huang N, Liu D, et al. Gene therapy that inhibits nuclear translocation of nuclear factor κB results in tumor necrosis factor α-induced apoptosis of human synovial fibroblasts. Arthritis Rheum. 2000;43:1094-1105.
48. Allenbach C, Zufferey C, Perez C, Launois P, Mueller C, Tacchini-Cottier F. Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by *Leishmania major*. J Immunol. 2006;176:6656-6664.
49. Venkatakrishnan A, Stecenko AA, King G, et al. Exaggerated activation of nuclear factor-κB and altered IκBβ processing in cystic fibrosis bronchial epithelial cells. Am J Respir Cell Mol Biol. 2000;23:396-403.
50. Griesenbach U, Scheid P, Hillery E, et al. Anti-inflammatory gene therapy directed at the airway epithelium. Gene Ther. 2000;7:306-313.
51. Poynter ME, Irvin CG, Janssen-Heininger YM. A prominent role for airway epithelial NF-κB activation in lipopolysaccharide-induced airway inflammation. J Immunol. 2003;170:6257-6265.
52. Jeffery PK. Comparison of the structural and inflammatory features of COPD and asthma. Giles F. Filley Lecture. Chest. 2000;117:251S-260S.
53. Gleich GJ. The eosinophil and bronchial asthma: current understanding. J Allergy Clin Immunol. 1990;85:422-436.
54. Persson CG, Erjefalt JS. Eosinophil lysis and free granules: an *in vivo* paradigm for cell activation and drug development. Trends Pharmacol Sci. 1997;18:117-123.
55. Chignard M, Balloy V. Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide. Am J Physiol Lung Cell Mol Physiol. 2000; 279:L1083-1090.
56. Kankaanranta H, Moilanen E, Zhang X. Pharmacological regulation of human eosinophil apoptosis. Curr Drug Targets Inflamm Allergy. 2005;4:433-445.
57. Walker A, Ward C, Taylor EL, et al. Regulation of neutrophil apoptosis and removal of apoptotic cells. Curr Drug Targets Inflamm Allergy. 2005;4:447-454.
58. Watt AP, Schock BC, Ennis M. Neutrophils and eosinophils: clinical implications of their appearance, presence and disappearance in asthma and COPD. Curr Drug Targets Inflamm Allergy. 2005;4:415-423.
59. Walsh GM, Al-Rabia M, Blaylock MG, Sexton DW, Duncan CJ, Lawrie A. Control of eosinophil toxicity in the lung. Curr Drug Targets Inflamm Allergy. 2005;4:481-486.
60. Waserman S, Dolovich J, Conway M, Marshall JS. TNF-α dysregulation in asthma: relationship to ongoing cortico-steroidtherapy. Can Respir J. 2000;7:229-237.
61. Baumann MA, Paul CC. The AML14 and AML14.3D10 cell lines: a long-overdue model for the study of eosinophils and more. Stem Cells. 1998;16:16-24.
62. Brenner V, Lindauer K, Parkar A, et al. Analysis of cellular adhesion by microarray expression profiling. J Immunol Methods. 2001;250:15-28.
63. Du J, Stankiewicz MJ, Liu Y, et al. Novel combinatorial interactions of GATA-1, PU.1, and C/EBPε isoforms regulate transcription of the gene encoding eosinophil granule major basic protein. J Biol Chem. 2002;277:43481-43494.
64. Ogawa K, Itoh M, Miyagawa M, et al. Expression of a human SOCS protein, HSOCP-1, in peripheral blood eosinophils from patients with atopic dermatitis. Int Arch Allergy Immunol. 2004;134 Suppl 1:2-6.
65. Kagaya S, Hashida R, Ohkura N, et al. NR4A orphan nuclear receptor family in peripheral blood eosinophils from patients with atopic dermatitis and apoptotic eosinophils *in vitro*. Int Arch Allergy Immunol. 2005;137 Suppl 1:35-44.
66. Serhan CN, Brain SD, Buckley CD, et al. Resolution of inflammation: state of the art, definitions and terms. FASEB J. 2006; in press.
67. Duez C, Tomkinson A, Shultz LD, Bratton DL, Gelfand EW. Fas deficiency delays the resolution of airway hyper-responsiveness after allergen sensitization and challenge. J Allergy Clin Immunol. 2001;108:547-556.
68. Chuang YH, Fu CL, Lo YC, Chiang BL. Adenovirus expressing Fas ligand gene decreases airway hyper-responsiveness and eosinophilia in a murine model of asthma. Gene Ther. 2004; 11:1497-1505.
69. Tong J, Bandulwala HS, Clay BS, et al. Fas-positive T cells regulate the resolution of airway inflammation in a murine model of asthma. J Exp Med. 2006;203:1173-1184.
70. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science. 1998;281:1680-1683.
71. Kwok BH, Koh B, Ndubuisi MI, Elofsson M, Crews CM. The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IκB kinase. Chem Biol. 2001;8:759-766.
72. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis *via* two sequential signaling complexes. Cell.

2003;114:181-190.
73. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-κB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor α-induced apoptosis. J Exp Med. 1998;188:211-216.
74. Xiao CW, Ash K, Tsang BK. Nuclear factor-κB-mediated X-linked inhibitor of apoptosis protein expression prevents rat granulosa cells from tumor necrosis factor α-induced apoptosis. Endocrinology. 2001;142:557-563.
75. Shishodia S, Aggarwal BB. Guggulsterone inhibits NF-κB and IκBα kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. J Biol Chem. 2004;279: 47148-47158.
76. Yang L, Mashima T, Sato S, et al. Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. Cancer Res. 2003;63: 831-837.
77. Schimmer AD, Welsh K, Pinilla C, et al. Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. Cancer Cell. 2004;5:25-35.
78. Wilkinson JC, Cepero E, Boise LH, Duckett CS. Upstream regulatory role for XIAP in receptor-mediated apoptosis. Mol Cell Biol. 2004;24:7003-7014.
79. Takahashi R, Deveraux Q, Tamm I, et al. A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem. 1998; 273:7787-7790.
80. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. 1999;18:5242-5251.
81. Scott FL, Denault JB, Riedl SJ, Shin H, Renatus M, Salvesen GS. XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. EMBO J. 2005;24: 645-655.
82. Schimmer AD, Dalili S, Batey RA, Riedl SJ. Targeting XIAP for the treatment of malignancy. Cell Death Differ. 2006;13: 179-188.