Inhibition of NF-κB in cancer cells converts inflammation-induced tumor growth mediated by TNFα to TRAIL-mediated tumor regression

Jun-Li Luo,[1] Shin Maeda,[1] Li-Chung Hsu,[1] Hideo Yagita,[2] and Michael Karin[1,*]

[1]Laboratory of Gene Regulation and Signal Transduction, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093
[2]Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
*Correspondence: karinoffice@ucsd.edu

Summary

We used an experimental murine cancer metastasis model in which a colon adenocarcinoma cell line generates lung metastases, whose growth is stimulated in response to injection of bacterial lipopolysaccharide (LPS), to investigate the role of NF-κB in inflammation-induced tumor growth. We found that LPS-induced metastatic growth response in this model depends on both TNFα production by host hematopoietic cells and NF-κB activation in tumor cells. Inhibition of NF-κB in both colon and mammary carcinoma cells converts the LPS-induced growth response to LPS-induced tumor regression. The latter response is TNFα-independent, but depends on another member of the TNF superfamily, TRAIL, whose receptor is induced in NF-κB-deficient cancer cells.

Introduction

Invasion and metastasis are the main clinical phenomena that distinguish malignant from benign tumors, and are the leading causes of death in cancer patients. Accumulating evidence suggests that tumor progression is governed not only by genetic changes intrinsic to the cancer cell (Moody et al., 2002; Steeg, 2003), but also by epigenetic and environmental factors (Coussens and Werb, 2002; Pollard, 2004). Chronic infection and the ensuing inflammation are considered to be some of the most important epigenetic and environmental factors contributing to tumorigenesis and tumor progression (Coussens and Werb, 2002; Pollard, 2004). Although the adaptive immune system reduces tumor incidence through immune-surveillance mechanisms (Dunn et al., 2002), the innate immune system can promote tumor development and progression through inflammation-dependent mechanisms (Coussens and Werb, 2002). It was also observed that bacterial infection following surgery can promote growth of metastases in experimental animals and human patients (Harmey et al., 2002; Pidgeon et al., 1999; Taketomi et al., 1997). Immune and inflammatory cells and their secreted chemokines and cytokines have dramatic effects both on the host's physiology and on cancer cell behavior, modulating the growth, migration, and differentiation of many cell types within the tumor microenvironment (Coussens and Werb, 2002; Liotta and Kohn, 2001). Whereas certain cytokines and chemokines promote cancer cell proliferation and survival, others can inhibit tumor growth and kill cancer cells (Coussens and Werb, 2002; Dunn et al., 2002). In addition to deregulated cell growth and survival, many tumors have acquired the ability to subvert immune and inflammatory responses to their benefit (Pollard, 2004). It is therefore of great importance to identify the molecular mechanisms through which the immune/inflammatory system promotes tumor cell proliferation, survival, and invasiveness as well as those responsible for tumor cell killing. A better understanding of these mechanisms may eventually lead to development of new therapeutic strategies that will allow conversion of the tumor-promoting effect of the immune/inflammatory system to a strong tumoricidal effect. Using a mouse model of inflammation-induced tumor growth, we explored this possibility through the manipulation of NF-κB activity within the cancer cell.

The NF-κB family of transcription factors plays a central role in regulation of immune and inflammatory responses, apoptosis, and oncogenesis (Baldwin, 2001; Karin et al., 2002). A wide range of stimuli, including cytokines and viral and bacterial products, activate NF-κB, mostly through IκB kinase (IKK)-dependent phosphorylation and subsequent degradation of specific inhibitors, the IκBs, that retain NF-κB in the cytoplasm (Ghosh and Karin, 2002). Upon activation, NF-κB dimers enter the nu-

SIGNIFICANCE

NF-κB transcription factors play a central role in immune and inflammatory responses. Some NF-κB-regulated genes are associated with tumor progression and metastasis, processes known to be induced by inflammation. Our work explains how NF-κB converts inflammatory stimuli into tumor growth signals and consequently speeds up metastatic tumor growth. Inhibition of NF-κB in cancer cells converts inflammation-induced tumor growth to inflammation-induced tumor regression. While the first response is mediated by TNFα, the second response is mediated by TRAIL. Our results suggest that inhibition of NF-κB in cancer cells can strongly potentiate the efficacy of TRAIL or TRAIL inducers in cancer therapy.

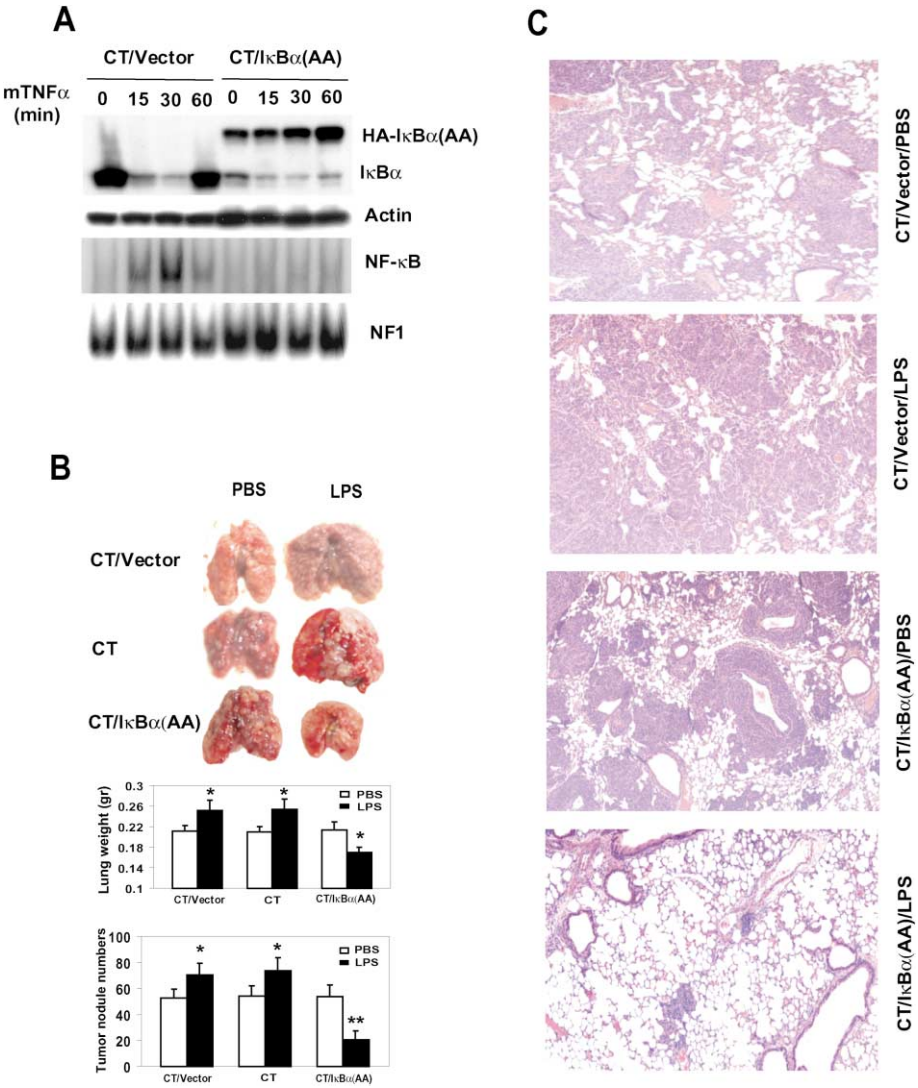


Figure 1. NF-κB inhibition converts LPS-induced tumor growth to tumor regression

A: Establishment of an NF-κB-deficient cell line. Mouse colon adenocarcinoma CT26 cells were stably transfected with either an empty vector or a vector encoding the IκBα(AA) super-repressor. Cells were stimulated with TNFα (10 ng/ml), and expression of endogenous IκBα and IκBα(AA) was analyzed by immunoblotting. NF-κB DNA binding activity was examined by EMSA.

B: NF-κB inhibition results in LPS-induced tumor regression. 1×10^5 cells (CT, CT/vector, or CT/IκBα[AA]) were injected into mice that after 9 days were administered PBS (vehicle) or LPS. Seven days later, mice were sacrificed and lungs were removed, weighed, and photographed, and the numbers of tumor nodules detectable on the lung surface were determined and statistically analyzed (*, $p < 0.05$; **, $p < 0.01$).

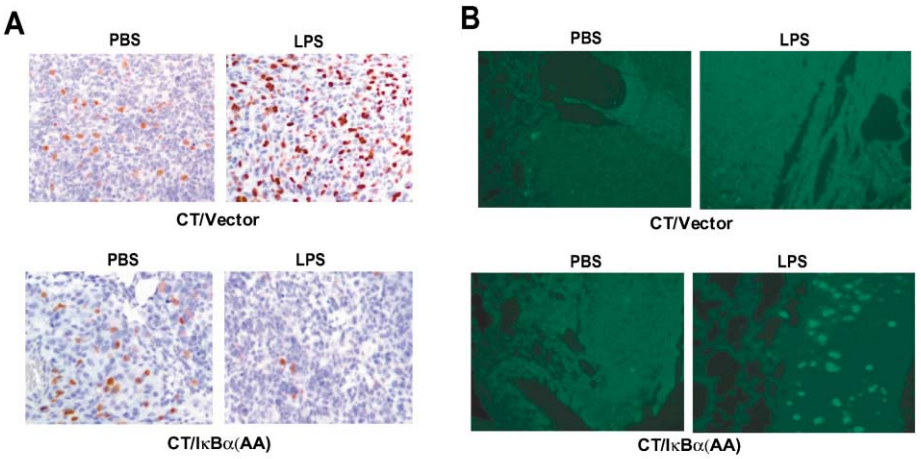
C: Histology of lung metastatic tumors. Tumors generated by injection of the indicated cell lines were exposed to PBS (vehicle) or LPS in vivo, and 7 days later the lungs were removed, formalin fixed, sectioned, and H&E stained. Magnification: 10×.

cleus, where they modulate transcription of many genes encoding cytokines, growth factors, cell adhesion molecules, and anti-apoptotic proteins. Some NF-κB-regulated gene products, including those encoding intercellular adhesion molecule 1 (ICAM-1), the extracellular matrix protein tenascin C, vascular endothelial growth factor (VEGF), the chemokine IL-8 and its mouse homologs, the proinflammatory enzyme cyclooxygenase 2 (COX2), and matrix metalloprotease 9 (MMP9), are associated with tumor progression and metastasis (Coussens and Werb, 2002; Karin et al., 2002). Thus, it seems that NF-κB can convert inflammatory stimuli into tumor growth signals. However, NF-κB may also control the expression of apoptosis-promoting cytokines (death cytokines) such as TNFα (Zhu et al., 2000) and FAS ligand (FASL) (Kasibhatla et al., 1998). Nonetheless, the ability of NF-κB to inhibit apoptosis appears to be stronger than its ability to promote apoptosis (Karin and Lin, 2002), and therefore, inhibition of NF-κB was suggested to be a useful strategy for cancer therapy (Baldwin, 2001; Karin et al., 2002). Here we modified an experimental murine metastasis model in which bacterial lipopolysaccharide (LPS), a byproduct of gram-negative bacteria, induces metastatic tumor growth (Harmey et al., 2002; Pidgeon et al., 1999), and demonstrated that NF-κB activation in cancer cells is responsible for inflammation-induced tumor growth. Importantly, inhibition of NF-κB converts LPS-induced tumor growth to LPS-induced tumor regression. We found that the mediators through which LPS induces these opposite outcomes are TNFα and TRAIL, respectively. Our results suggest a novel strategy for cancer therapy, based on combining the inhibition of NF-κB in cancer cells with induction of TRAIL, by the host immune system.

Results

NF-κB activation is required for LPS-induced tumor growth

We modified a murine metastasis model in which a mouse colon cancer cell line, CT26, originally induced in BALB/c mice by exposure to 1,2-dimethylhydrazine, was used to generate lung metastases (Harmey et al., 2002; Pidgeon et al., 1999) whose growth is stimulated in response to LPS administration. To examine the role of NF-κB in this process, we transfected CT26 cells with a vector encoding an IκBα "super-repressor" mutant



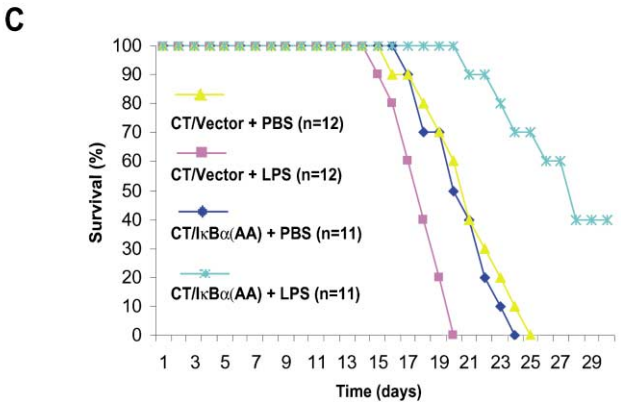


Figure 2. NF-κB inhibition blocks LPS-induced tumor proliferation, enhances LPS-induced tumor apoptosis, and extends host survival

A: NF-κB is required for LPS-induced tumor cell proliferation. Proliferating cells were identified by BrdU labeling and staining with anti-BrdU antibody (brown). The tissue was counterstained with hematoxylin (blue).

B: NF-κB inhibition results in LPS-induced tumor cell death. Apoptotic cells were identified by TUNEL staining (green).

C: NF-κB inhibition extends host survival. Kaplan-Meier survival analysis of CT/vector-, CT/IκBαAA-tumor-bearing mice challenged with either LPS or PBS, as described above. The time (days) represents mouse survival time after tumor inoculation at day 0.

(IκBα[AA]) that is resistant to stimulus-induced degradation (DiDonato et al., 1996) or an "empty" vector as a control. Expression of endogenous IκBα in CT/IκBα(AA) cells was inhibited, and induction of NF-κB DNA binding activity by TNFα was abolished (Figure 1A). The different cell lines (CT, CT/vector, or CT/IκBα[AA]) were introduced into BALB/c mice, which after 9 days were injected intraperitoneally (i.p.) with LPS in PBS or PBS alone. Seven days later, mice were sacrificed, and their lungs were removed and weighed, the tumor nodules on the lung surface were counted, and the tumor-bearing lung tissues were microscopically examined. We used both total lung weight and the number of tumor nodules visible on the lung surface to evaluate lung tumor burden. Both tumor nodule numbers and lung weights in the LPS-challenged CT and CT/vector groups were significantly ($p < 0.05$) higher than in the vehicle controls (Figure 1B). By contrast, administration of LPS to the CT/IκBα(AA) group resulted in a significant ($p < 0.05$) decrease in lung weight and in tumor nodule numbers (Figure 1B). There were no significant differences in lung tumor burdens between the CT, CT/vector, and CT/IκBα(AA) groups after PBS administration. Microscopic examination of paraffin-embedded lung sections excluded the possibility that the gain in lung weight was due to edema or hemorrhage (Figure 1C). In addition, the histological examination confirmed the massive reduction in tumor load in mice harboring CT/IκBα(AA) tumors that were treated with LPS.

To rule out the possibility that the data obtained above could be the result of nonspecific clonal variation rather than specific NF-κB inhibition, we repeated the above experiments by using another CT/IκBα(AA) clone (Clone 2), as well as a cloned cell line (4T1/IκBα[AA]) derived from the breast cancer cell line 4T1 by stable transfection with the IκBα(AA)-expression plasmid. Using both cell lines, we obtained very similar results to those described above (Supplemental Table S1 at http://www.cancercell.org/cgi/content/full/6/3/297/DC1). We also found that LPS challenge could remarkably decrease the size of tumors formed by subcutaneous inoculation of BALB/c mice with CT26 carcinoma cells whose NF-κB activity was inhibited with the IκBα super-repressor (Supplemental Figure S1 and data not shown).

Next, we asked whether the LPS-induced changes in lung tumor burden were due to altered cell proliferation or apoptosis. Tumor cells were injected into mice and LPS or PBS was administered as above. After 22.5 hr, the mice were pulsed with 5-bromodeoxyuridine (BrdU) and sacrificed. BrdU incorporation, which identifies cells undergoing DNA synthesis, was examined by staining with anti-BrdU antibody. Apoptotic cells were identified by in situ terminal-transferase dUTP-mediated nick end-labeling (TUNEL) assay. Consistent with the changes in lung tumor burden, BrdU incorporation was much higher in LPS-challenged CT and CT/vector-generated tumors than in tumors of the same type exposed to PBS alone (Figure 2A and data

not shown). By contrast, BrdU incorporation into CT/IκBα(AA) tumors was reduced after LPS administration. Conversely, the extent of apoptosis was dramatically increased after LPS-treatment of CT/IκBα(AA) tumors, whereas very little apoptosis was seen after LPS treatment of CT or CT/vector tumors (Figure 2B and data not shown). The basal levels of cell proliferation and apoptosis in PBS-treated tumors of all three cell types were quite similar. Thus, inhibition of NF-κB activity in cancer cells converts the LPS-induced proliferative response to an apoptotic response.

We also examined the survival times of mice harboring CT/vector and CT/IκBα(AA) metastatic lung tumors that were challenged with either LPS or PBS alone using Kaplan-Meier survival analysis. We found that LPS administration shortened the life span of CT/vector tumor-bearing mice, but prolonged the survival time of CT/IκBα(AA) tumor-bearing mice (Figure 2C). There were no differences in the survival rates of mice bearing either CT/vector or CT/IκBα(AA) tumors that received PBS alone. These results are consistent with those obtained by measuring lung nodule numbers, total lung weight, or lung histology.

LPS induces NF-κB-dependent genes in tumor cells

To examine how NF-κB activation mediates LPS-induced tumor growth, we analyzed expression of NF-κB target genes known to be involved in cell proliferation and apoptosis (Karin et al., 2002; Karin and Lin, 2002). Tumors were established as above, and 24 hr after administration of LPS or PBS, the mice were sacrificed. Tumor tissue was microdissected, lysed, and analyzed by immunoblotting and zymography. Expression of the antiapoptotic proteins Bcl-X_L, cIAP1, and cIAP2 (Figure 3A) as well as MMP9 activity (Figure 3B), were strongly induced in response to LPS treatment in CT and CT/vector tumors, but not in CT/IκBα(AA)-generated tumors. The failure to induce antiapoptotic proteins may explain the apoptotic response to LPS seen after inhibition of NF-κB. Expression of MMP9, on the other hand, is likely to affect tumor invasiveness and growth. We also found that expression of PCNA, an S phase marker, correlated with the NF-κB activation state of the cells being high in CT and CT/vector tumors from LPS treated mice and low in CT/IκBα(AA) tumors (Figure 3A). These results correlate with those obtained by the analysis of BrdU incorporation (see Figure 2A).

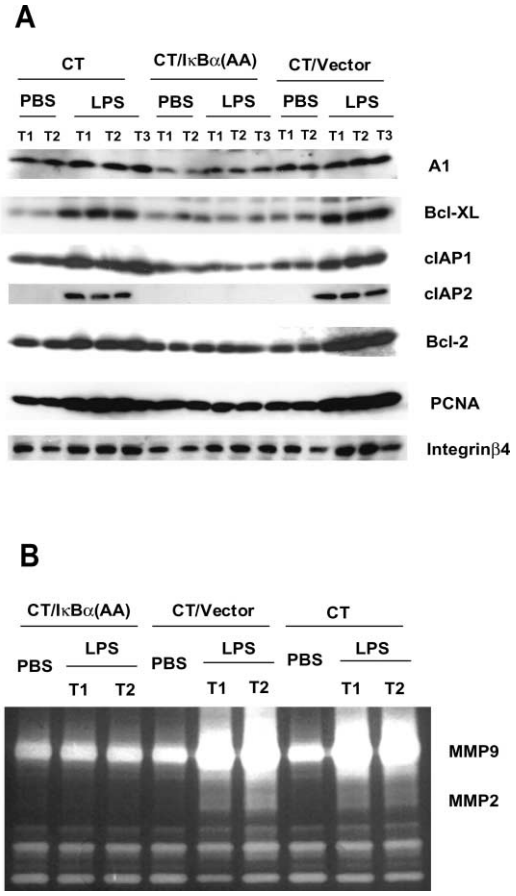


Figure 3. LPS induces NF-κB-dependent expression of antiapoptotic and prometastatic genes in tumor cells

A: Expression of NF-κB-dependent genes in tumor cells. Tumors generated by injection of the indicated cell lines were exposed to PBS or LPS in vivo and isolated 24 hr later. Tumor cell lysates were analyzed by immunoblotting for expression of the indicated proteins.

B: Induction of MMP activity in tumor cells. Tumor cell lysates generated as above were analyzed by zymography on gels copolymerized with gelatin or casein/plasminogen to detect MMP activity. This figure shows a gelatin-containing gel. The locations of the indicated MMPs were identified by negative staining.

TNFα mediates LPS-induced tumor growth and NF-κB activation

Activation of NF-κB by LPS requires Toll-like receptor 4 (TLR4), a cell surface receptor that is mostly expressed on myeloid cells (Poltorak et al., 1998). We examined whether LPS activated NF-κB in CT26 cells through a direct mechanism or via a myeloid cell intermediate. Although direct incubation with LPS activated NF-κB in CT26 cells in vitro, rather high doses of LPS were required (Figure 4A). The low sensitivity to LPS could be due to low TLR4 mRNA expression in CT26 cells relative to macrophages (Figure 4B). To determine if TLR4 in host cells is required for NF-κB activation in CT26 tumors, we inoculated wild-type (WT) mice as well as *Tlr4*$^{Lps\text{-}d}$ mutant mice, which express a signaling defective form of TLR4 (Poltorak et al., 1998), with CT26 cells. Tumor-bearing mice were administered LPS as described above, and NF-κB activation in tumors was analyzed 2 hr later. Whereas LPS induced NF-κB activation in tumors grown in WT mice, it failed to do so in tumors grown in *Tlr4*$^{lps\text{-}d}$ mice (Figure 4C). Thus, NF-κB activation in tumors requires functional TLR4 in one of the host cell types, possibly a macrophage whose infiltration into tumors was enhanced after LPS challenge (Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/6/3/297/DC1).

These findings suggested that a LPS-induced inflammatory mediator produced by host cells is responsible for NF-κB activation in tumors. A major proinflammatory cytokine whose production is strongly induced by LPS is TNFα (Tracey and Cerami, 1994). Indeed, LPS administration to tumor-bearing WT mice resulted in rapid and robust induction of circulating TNFα (Figure 4D). Administration of an anti-TNFα antibody 5 min after LPS challenge neutralized most of the circulating TNFα (Figure 4E). NF-κB activation by LPS was similar in its kinetics to TNFα release and was inhibited by the neutralizing anti-TNFα antibody (Figure 4F). Similar results were obtained by staining tumor tissue with anti-RelA(p65) antibody (Supplemental Figure S3). LPS administration induced the nuclear appearance of phos-

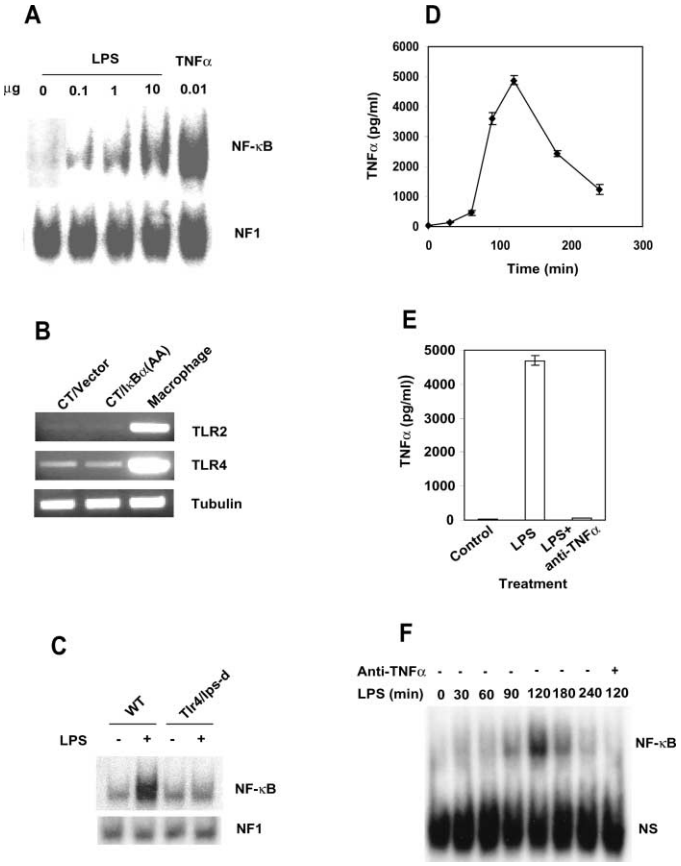


Figure 4. TNFα mediates LPS-induced NF-κB activation in tumors

A: Activation of NF-κB in cultured cells. CT26 cells were treated with different doses of LPS (in μg/ml) or TNFα (10 ng/ml). NF-κB and NF1 DNA binding activities were determined by EMSA.

B: TLR mRNA expression. RNA was isolated from the indicated tumor cells or bone marrow-derived macrophages and analyzed by semiquantitative RT-PCR for expression of TLR2 and TLR4 mRNAs.

C: LPS is an indirect activator of NF-κB in tumors. Tumor-bearing mice (WT and *Tlr4*$^{lps-d}$) were administered LPS as described above. Tumors were collected 2 hr later, and NF-κB and NF1 DNA binding activities were examined by EMSA.

D: LPS induces release of circulating TNFα. CT26 tumor-bearing mice were injected with LPS. and serum samples were collected at the indicated time points. TNFα concentration was determined by ELISA.

E: Neutralization of circulating TNFα. Tumor-bearing mice were injected with LPS, and 5 min later were injected with 30 μl of anti-TNFα antibody (AF-410-NA, Pharmingen) or PBS. Serum was collected after 2 hr, and TNFα level was determined by ELISA.

F: Activation of NF-κB is TNFα-dependent. Tumor-bearing mice were administered LPS without or with anti-TNFα antibody as described above. Tumors were collected at the indicated time points, and NF-κB DNA binding activity was examined by EMSA.

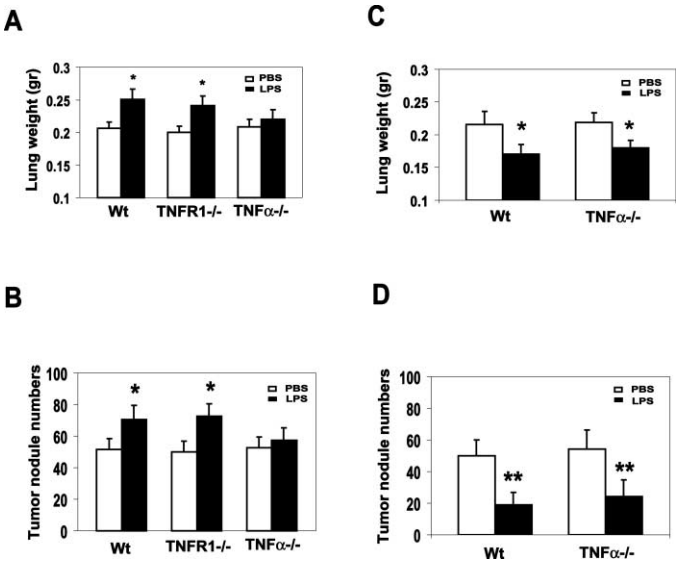


Figure 5. TNFα mediates LPS-induced tumor growth but not LPS-induced tumor regression

A and B: TNFα mediates LPS-induced tumor growth. Mice reconstituted with WT, *Tnfr1*$^{-/-}$, or *Tnfα*$^{-/-}$ bone marrow were inoculated with CT26 tumor cells and after 9 days were administered PBS or LPS. Seven days later, the lungs were removed and weighed (**A**), and the tumor nodules on the lung surfaces were counted (**B**). (*, $p < 0.05$; **, $p < 0.01$).

C and D: TNFα is not required for LPS-induced tumor regression. Mice reconstituted with WT or *Tnfα*$^{-/-}$ bone marrow were inoculated with CT/IκBαAA tumor cells and after 9 days were administered PBS or LPS. Seven days later, the lungs were removed and weighed (**C**), and tumor nodules on the lung surfaces were counted (**D**) and statistically analyzed. (*, $p < 0.05$; **, $p < 0.01$).

phorylated RelA. This response was blocked by the IκBα superrepressor. We conclude that TNFα is the main mediator through which LPS activates NF-κB in tumors.

We examined whether ablation of TNFα production or signaling prevents LPS-induced NF-κB activation and tumor growth. Bone marrow from WT, type 1 TNF receptor-deficient (*Tnfr1*$^{-/-}$), or TNFα-deficient (*Tnfα*$^{-/-}$) mice was individually transplanted into lethally irradiated BALB/c mice to generate chimeric mice, which were inoculated six weeks later with CT26 tumor cells. LPS or PBS were administered, and 7 days later the lungs were removed and weighed and tumor nodules on the lung surfaces were counted. The lung tumor burdens in chimeric mice reconstituted with either WT or *Tnfr1*$^{-/-}$ bone marrow were significantly increased after LPS challenge (Figures 5A and 5B). However, no LPS-induced increase in tumor burden was evident in lungs from mice reconstituted with *Tnfα*$^{-/-}$ bone marrow. Similar results were obtained by measuring NF-κB DNA binding activity in tumor tissues: while LPS caused NF-κB activation in tumors grown in WT or *Tnfr1*$^{-/-}$ chimeric mice, no such induction was seen in *Tnfα*$^{-/-}$ chimeric mice (data not shown). Thus, TNFα production by host hematopoietic cells is required for activation of NF-κB in cancer cells and stimulation of tumor growth.

TRAIL mediates LPS-induced regression of NF-κB-deficient tumors

In addition to prevention of LPS-induced tumor growth, inhibition of NF-κB activity in CT26 cells converted the growth response to a death response. We therefore searched for the mediator through which LPS induces the apoptotic response. We first suspected that TNFα may be this mediator, as it was shown that high doses of TNFα can kill tumor cells when NF-κB activity is inhibited (Wang et al., 1999). We therefore generated either WT or *Tnfα*$^{-/-}$ chimeric mice and inoculated them with CT/IκBα(AA) tumor cells. Curiously, LPS administration decreased tumor burden to a similar extent in both WT and *Tnfα*$^{-/-}$ radiation

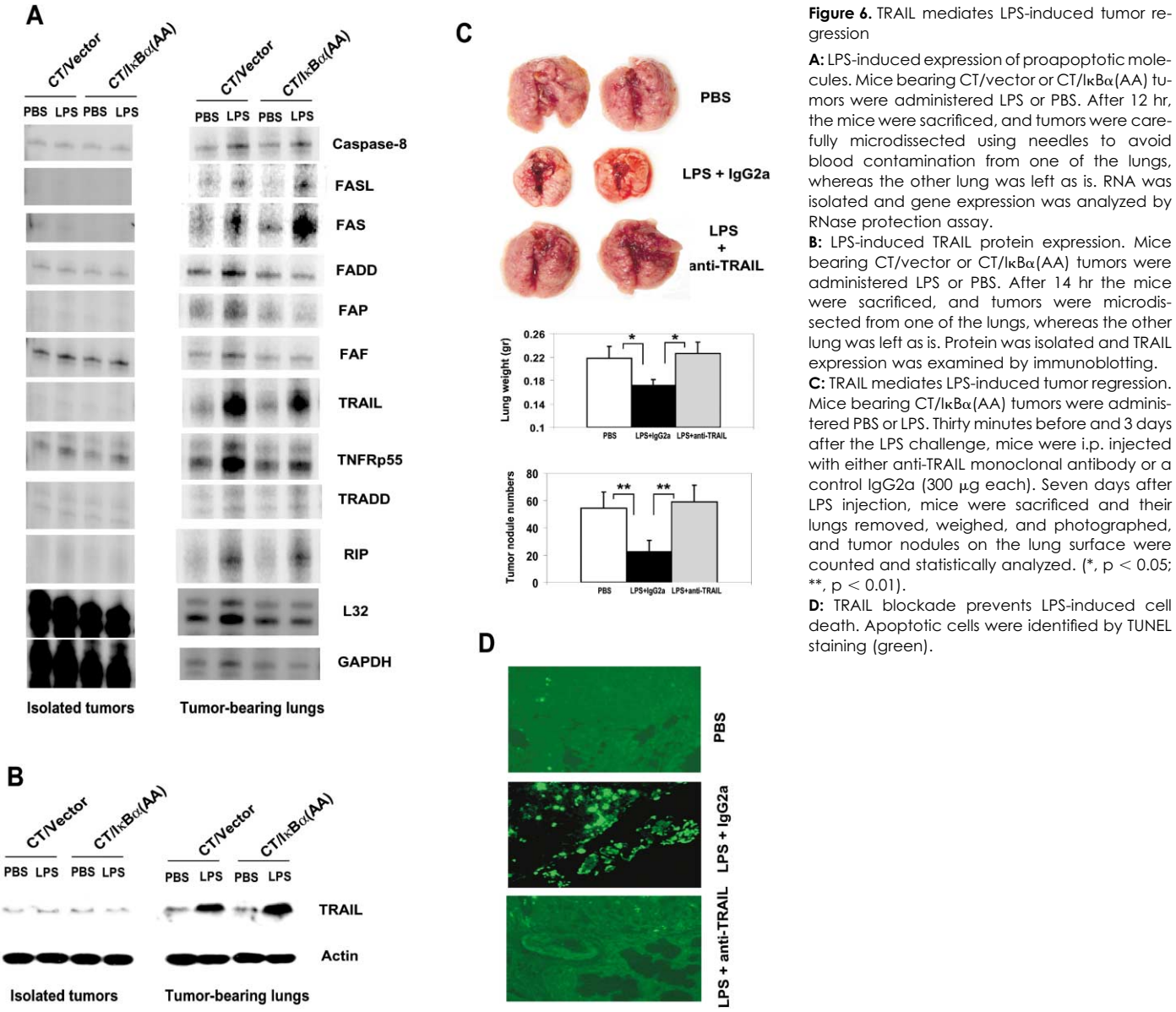


Figure 6. TRAIL mediates LPS-induced tumor regression

A: LPS-induced expression of proapoptotic molecules. Mice bearing CT/vector or CT/IκBα(AA) tumors were administered LPS or PBS. After 12 hr, the mice were sacrificed, and tumors were carefully microdissected using needles to avoid blood contamination from one of the lungs, whereas the other lung was left as is. RNA was isolated and gene expression was analyzed by RNase protection assay.

B: LPS-induced TRAIL protein expression. Mice bearing CT/vector or CT/IκBα(AA) tumors were administered LPS or PBS. After 14 hr the mice were sacrificed, and tumors were microdissected from one of the lungs, whereas the other lung was left as is. Protein was isolated and TRAIL expression was examined by immunoblotting.

C: TRAIL mediates LPS-induced tumor regression. Mice bearing CT/IκBα(AA) tumors were administered PBS or LPS. Thirty minutes before and 3 days after the LPS challenge, mice were i.p. injected with either anti-TRAIL monoclonal antibody or a control IgG2a (300 μg each). Seven days after LPS injection, mice were sacrificed and their lungs removed, weighed, and photographed, and tumor nodules on the lung surface were counted and statistically analyzed. (*, $p < 0.05$; **, $p < 0.01$).

D: TRAIL blockade prevents LPS-induced cell death. Apoptotic cells were identified by TUNEL staining (green).

chimeras (Figures 5C and 5D). Thus, TNFα is not a critical mediator of LPS-induced regression of NF-κB-deficient tumors.

To search for other death mediators induced by LPS, we examined expression of TNF family members, their receptors, and effector molecules in microdissected tumor cells and tumor-bearing lungs. We observed that both TRAIL and FAS mRNAs were strongly induced in tumor-bearing lung tissue, but not in microdissected tumor cells after LPS challenge (Figure 6A). We also observed weak induction of the mRNAs for FASL and downstream effectors of apoptosis such as RIP1 and caspase 8. As FAS is not expressed by CT26 tumors (Chen et al., 1998), it is rather unlikely that FASL is the mediator of LPS-induced tumor regression. A more likely candidate was TRAIL, whose receptor DR5 (Ashkenazi and Dixit, 1998) was expressed by microdissected tumor cells (see Figure 7D). Consistent with the mRNA expression data, the levels of TRAIL protein were highly elevated following LPS administration in tumor-bearing lung tissues, but not in microdissected tumor cells (Figure 6B).

To examine the role of TRAIL, mice bearing CT/IκBα(AA) tumors were i.p. injected with a neutralizing anti-TRAIL antibody (Takeda et al., 2001) or a control IgG2a 30 min before and 3 days after LPS challenge. After seven days, the mice were sacrificed and their lungs were removed, microscopically examined, and weighed, and tumor nodules on the lung surface were counted. LPS challenge of mice treated with control IgG2a resulted in a markedly reduced tumor burden, and injection of anti-TRAIL antibody completely blocked this effect (Figure 6C and Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/6/3/297/DC1). A TUNEL assay demonstrated that the anti-TRAIL antibody entirely ablated the apoptotic response of NF-κB-deficient tumors to LPS stimulation (Figure 6D). These results strongly suggest that TRAIL is the main effector that mediates LPS-induced death and regression of NF-κB-deficient tumors.

Expression of TRAIL is induced by type I and type II interfer-

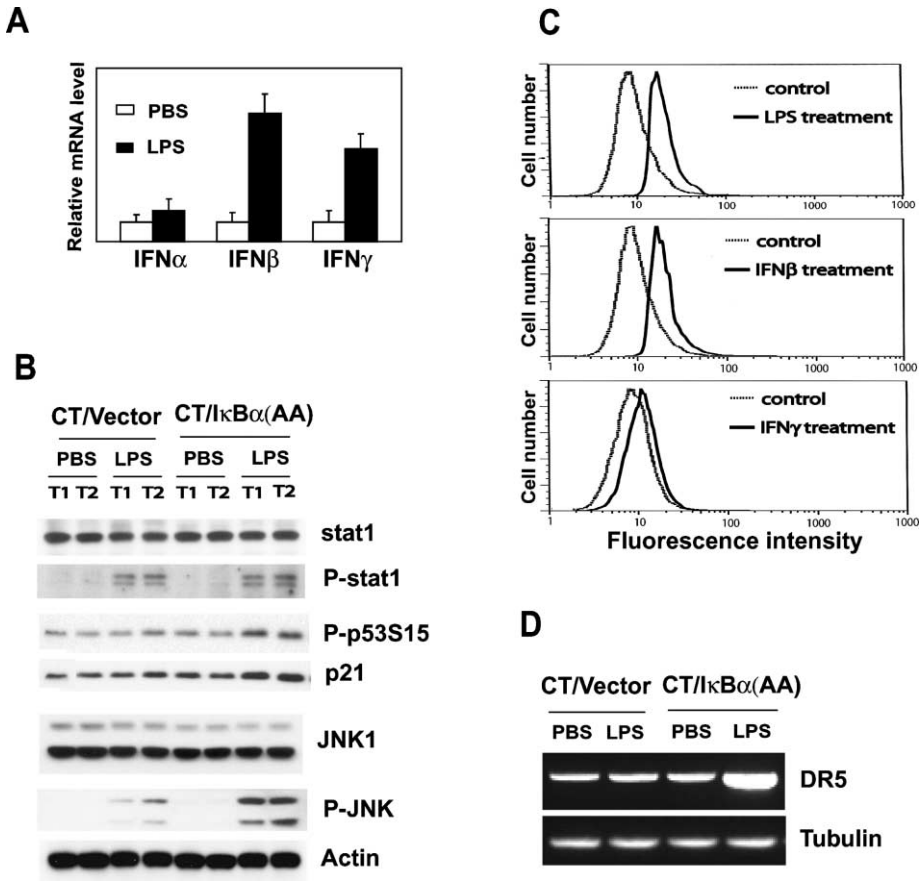


Figure 7. LPS induces TRAIL expression on NK cells and DR5 expression in NF-κB-deficient tumor cells

A: LPS induces IFNβ and IFNγ gene expression. Tumor-bearing mice were exposed to PBS or LPS, and tumor-bearing lung tissues were removed 6 hr later for RNA extraction and real-time PCR analysis.

B: Enhanced p53 phosphorylation correlates with increased JNK activation. Tumor-bearing mice were injected with PBS or LPS, and tumors were microdissected from isolated lungs 24 hr later. Tumor cell lysates were examined by immunoblotting for expression and phosphorylation of the indicated proteins.

C: TRAIL induction in NK cells. Tumor-bearing mice were injected with LPS, IFNβ, and IFNγ or PBS, and tumor-bearing lung tissues were removed 4 hr (IFNβ, and IFNγ injection) or 12 hr (LPS injection) later for isolation of mononuclear cells. TRAIL expression on NK cells was analyzed by flow cytometry after double staining of mononuclear cells with anti-NK cell and anti-TRAIL antibodies. The results show the level of anti-TRAIL staining in cells stained positively by anti-NK.

D: Upregulation of DR5 in CT/IκBα(AA) tumors. Tumor-bearing mice were injected with PBS or LPS. Tumors were isolated 12 hr later for RNA extraction, and DR5 expression was examined by RT-PCR.

ons (IFNs) (Smyth et al., 2003). LPS is known to activate the IFN signaling pathway (Hsu et al., 2004). Indeed, administration of LPS resulted in induction of IFNβ and IFNγ mRNAs in tumor-bearing lung tissues (Figure 7A) and led to activation of the IFN-responsive transcription factor STAT1 in isolated lung tumor cells (Figure 7B). It has been reported that TRAIL is constitutively expressed on murine natural killer (NK) cells in the liver and plays a substantial role in suppressing tumor metastasis (Takeda et al., 2001). We examined TRAIL expression levels on NK cells in tumor-bearing lung tissues by flow cytometry and found that it was induced by either LPS, IFNβ, or IFNγ challenge (Figure 7C).

LPS injection also led to activation of JNK1, and consistent with earlier findings (Tang et al., 2001), JNK activation, which augments apoptosis, was potentiated by inhibition of NF-κB (Figure 7B). Increased JNK activity in CT/IκBα(AA) tumors correlated with increased phosphorylation of p53 at serine 15, a putative JNK phosphorylation site (Fogarty et al., 2003). CT26 cells express WT p53 (unpublished data), whose increased phosphorylation may account for the modest elevation in expression of the cell cycle inhibitor p21 (Figure 7B), and the more remarkable induction of DR5 mRNA in CT/IκBα(AA) tumors (Figure 7D). Both DR5 and p21 are encoded by p53 target genes (Fei et al., 2002; Wu et al., 1997).

Discussion

In the present study, we used a modified version of an experimental metastasis model (Harmey et al., 2002; Pidgeon et al., 1999) to investigate the mechanism through which a strong proinflammatory stimulus, such as the one provided by LPS, a component of gram-negative bacteria, can stimulate tumor growth. It has been reported that bacterial contamination during surgery or postoperative inflammation can increase metastatic tumor growth in both mice (Harmey et al., 2002; Pidgeon et al., 1999) and human patients (Taketomi et al., 1997). Our results indicate that the major inflammatory mediator responsible for LPS-induced tumor growth is TNFα. Although TNFα was so named after its ability to induce tumor death (Palladino et al., 2003), in this model of metastatic colon adenocarcinoma, TNFα does not act as a death mediator, but is responsible for increased tumor growth. Although high doses of TNFα administered in close proximity to solid tumors can kill both cancer cells and the tumor neovasculature, there is also growing evidence that endogenous TNFα promotes tumor development and growth (Moore et al., 1999; Wilson and Balkwill, 2002). In our case, the growth-promoting activity of TNFα is dependent on NF-κB activation, but the inhibition of NF-κB is not sufficient for rendering CT26 tumors susceptible to TNFα-induced apoptosis. Nonetheless, NF-κB in CT26 cells is responsible for induction of several antiapoptotic proteins, including Bcl-X_L, cIAP1, and cIAP2. Most importantly, the inhibition of NF-κB in CT26 cells converted the growth-promoting effect of LPS, mediated by TNFα, to a cytocidal effect. That effect of LPS was found to be mediated by TRAIL rather than by TNFα. These results are not unique to CT26 colon carcinoma cells, and inhibition of NF-κB activity in 4T1 mammary carcinoma cells, which

also grow faster as lung metastasis in response to LPS (Harmey et al., 2002), converts the LPS induced growth response to a tumor regression response (Supplemental Table S1).

TRAIL, also known as Apo2 ligand, is a type II transmembrane protein of the TNF family, which preferentially induces apoptosis in a variety of transformed cells but not in normal cells (Ashkenazi, 2002). Unlike TNFα, TRAIL is a weak inducer of inflammation (Song et al., 2000), an important property which is probably related to its poor NF-κB activating ability (Ashkenazi et al., 1999). Administration of recombinant TRAIL suppresses the growth of tumor xenografts with no apparent systemic toxicity (Ashkenazi et al., 1999; Walczak et al., 1999). Endogenously expressed TRAIL on the surface of NK cells plays a critical role in suppression of liver and lung metastases (Smyth et al., 2001; Takeda et al., 2001). Our results demonstrate that LPS challenge leads to upregulation of TRAIL expression in the tumor microenvironment, which may be mainly derived from NK cells and macrophages, where it is induced by type I and type II IFNs (Smyth et al., 2003). In addition to preventing the expression of antiapoptotic proteins, inhibition of NF-κB also led to increased expression of the TRAIL receptor DR5, thereby making a dual contribution to increased TRAIL sensitivity. Induction of DR5 could be due to potentiation of JNK activation caused by inhibition of NF-κB (Tang et al., 2001). JNK can phosphorylate p53 at serine 15, thereby increasing the p53-mediated transcriptional response (Fogarty et al., 2003), which includes DR5 among its targets (Fei et al., 2002).

In addition to TNFα and TRAIL, other cytokines may also effect tumor progression or regression. A considerable effort has been invested in the use of cytokines in cancer therapy (Dranoff, 2004). In addition to TRAIL, a tumoricidal effect has also been obtained with type I and type II IFNs (Dranoff, 2004), which as discussed above are efficient inducers of TRAIL. However, IFN-induced immune toxicity has been a limiting factor that restricts the amount of IFN that can be used for effective cancer therapy (Gutterman, 1994). Some of the IFN-induced toxicities could be TNFα-mediated. As TNFα does not make a major contribution to tumor killing and instead may promote tumor growth, we postulate that it may be advisable to combine IFN-based therapy with anti-TNFα drugs, which may reduce inflammation-associated toxicities, block inflammation-induced tumor growth, and potentiate TRAIL-dependent tumor killing. The latter can be augmented by the use of NF-κB inhibitors. Future experiments should examine this possibility.

Experimental procedures

Cell culture and transfection

CT26 and 4T1 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. CT26 and 4T1 cells were transfected with either a vector encoding IκBα super-repressor mutant (IκBα[AA]) or the empty backbone vector using Lipofectamine (Invitrogen). Stable cell lines were established and characterized for IκBα(AA) expression and the extent of NF-κB inhibition.

Experimental lung metastasis model

BALB/c mice from the Jackson Laboratory were maintained under specific pathogen-free conditions and treated according to protocols approved by the UCSD Animal Care Program, following National Institutes of Health guidelines. Subconfluent tumor cells were harvested and passed through a 40 μm cell strainer, washed 3 times in PBS, and resuspended in PBS at 2×10^5/ml. Only single-cell suspensions of greater than 90% viability based on Trypan Blue exclusion were used. Six-week-old mice were injected with either 10^5 CT or 5×10^4 4T1 cells via the tail vein. Nine days later, mice were injected i.p. with 10 μg LPS (serotype 055:B5; Sigma Chemical, St. Louis, MO) in PBS or PBS alone. After seven days, mice were sacrificed, and lungs were removed, weighed, and histologically examined. When indicated, 300 μg of either anti-TRAIL mAb (Takeda et al., 2001) or control rat IgG2a (Pharmingen) were injected i.p., once 30 min before and once 3 days after LPS challenge. For protein and RNA analysis, lung tumor nodules were microdissected using an 18 G needle under a microscope. Alternatively, the entire tumor-bearing lung was used.

Chimeric mice

Single-cell suspensions of bone marrow from WT, *Tnfα*$^{-/-}$, or *Tnfr1*$^{-/-}$ mice were prepared, and 5×10^5 cells were injected into the tail vein of 6-week-old BALB/c female hosts that were exposed to 900 rad of γ radiation. Host mice were maintained under sterile conditions, using autoclaved cages, food, and water containing 25 mg/l neomycin sulfate and 13 mg/l polymyxin B sulfate (Senftleben et al., 2001).

Histological procedures

Dissected tumors or entire lungs were immersed in 10% neutral buffered formalin before sectioning. Sections were stained with Harris haematoxylin and eosin (H&E) and BrdU- and TUNEL-positive cells were identified as described (Cao et al., 2001).

Analysis of gene expression

Nuclear protein extracts were prepared from tumor cells, and NF-κB DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) as described (Senftleben et al., 2001). Total tissue RNA was prepared using the RNAeasy kit (Qiagen, Valencia, California). For RNase protecton assay (RPA), RNA was hybridized overnight at 56°C to 2×10^6 c.p.m. of ^{32}P-labeled probes corresponding to mouse multiprobe template set mAPO (PharMingen) and analyzed as described (Makris et al., 2000). RT-PCR was performed as described (Senftleben et al., 2001). Immunoblotting, immunohistochemistry, or flow cytometry (Senftleben et al., 2001; Cao et al., 2001) were applied using antibodies to IκBα, PCNA, JNK1, NK cells (all from Pharmingen), cIAP1 (kindly provided by Dr. C. Duckett, NIH), Bcl-2 (Transduction Labs), Integrinβ4 (Chemicon), actin (Sigma), p21, Bcl-X_L, cIAP2, STAT1, TRAIL (all from Santa Cruz Biologicals), phospho-p65, phospho-JNK, phospho-p53, or phospho-STAT1 (all from Cell Signaling). TNFα concentrations in serum were determined by ELISA as recommended by the manufacturer (Quantikine, R&D Systems, Minneapolis, Minnesota). Zymography for MMPs was performed as described (Daniels et al., 2003).

Statistical analysis

The data were analyzed by unpaired Student's t test (2-tailed) or Kaplan-Meier survival analysis. Data were taken to be significant when $p < 0.05$.

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References

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat. Rev. Cancer *2*, 420–430.

Ashkenazi, A., and Dixit, V.M. (1998). Death receptors: Signaling and modulation. Science *281*, 1305–1308.

Ashkenazi, A., Pai, R.C., Fong, S., Leung, S., Lawrence, D.A., Marsters, S.A., Blackie, C., Chang, L., McMurtrey, A.E., Hebert, A., et al. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Invest. *104*, 155–162.

Baldwin, A.S., Jr. (2001). Series introduction: The transcription factor NF-κB and human disease. J. Clin. Invest. *107*, 3–6.

Cao, Y., Bonizzi, G., Seagroves, T.N., Greten, F.R., Johnson, R., Schmidt, E.V., and Karin, M. (2001). IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell *107*, 763–775.

Chen, J.J., Sun, Y., and Nabel, G.J. (1998). Regulation of the proinflammatory effects of Fas ligand (CD95L). Science *282*, 1714–1717.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860–867.

Daniels, J.T., Schultz, G.S., Blalock, T.D., Garrett, Q., Grotendorst, G.R., Dean, N.M., and Khaw, P.T. (2003). Mediation of transforming growth factor-β1-stimulated matrix contraction by fibroblasts: A role for connective tissue growth factor in contractile scarring. Am. J. Pathol. *163*, 2043–2052.

DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996). Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Mol. Cell. Biol. *16*, 1295–1304.

Dranoff, G. (2004). Cytokines in cancer pathogenesis and cancer therapy. Nat. Rev. Cancer *4*, 11–22.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J., and Schreiber, R.D. (2002). Cancer immunoediting: From immunosurveillance to tumor escape. Nat. Immunol. *3*, 991–998.

Fei, P., Bernhard, E.J., and El-Deiry, W.S. (2002). Tissue-specific induction of p53 targets in vivo. Cancer Res. *62*, 7316–7327.

Fogarty, M.P., Downer, E.J., and Campbell, V. (2003). A role for c-Jun N-terminal kinase 1 (JNK1), but not JNK2, in the beta-amyloid-mediated stabilization of protein p53 and induction of the apoptotic cascade in cultured cortical neurons. Biochem. J. *371*, 789–798.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-κB puzzle. Cell *109* (*Suppl.*), S81–S96.

Gutterman, J.U. (1994). Cytokine therapeutics: Lessons from interferon α. Proc. Natl. Acad. Sci. USA *91*, 1198–1205.

Harmey, J.H., Bucana, C.D., Lu, W., Byrne, A.M., McDonnell, S., Lynch, C., Bouchier-Hayes, D., and Dong, Z. (2002). Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. Int. J. Cancer *101*, 415–422.

Hsu, L.-C., Park, J.M., Zhang, K., Luo, J.-L., Maeda, S., Kaufman, R.J., Eckmann, L., Guiney, D.G., and Karin, M. (2004). The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. Nature *428*, 341–345.

Karin, M., and Lin, A. (2002). NF-κB at the crossroads of life and death. Nat. Immunol. *3*, 221–227.

Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-κB in cancer: From innocent bystander to major culprit. Nat. Rev. Cancer *2*, 301–310.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A., and Green, D.R. (1998). DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1. Mol. Cell *1*, 543–551.

Liotta, L.A., and Kohn, E.C. (2001). The microenvironment of the tumour-host interface. Nature *411*, 375–379.

Makris, C., Godfrey, V.L., Krahn-Senftleben, G., Takahashi, T., Roberts, J.L., Schwarz, T., Feng, L., Johnson, R.S., and Karin, M. (2000). Female mice heterozygous for IKKγ/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. Mol. Cell *5*, 969–979.

Moody, S.E., Sarkisian, C.J., Hahn, K.T., Gunther, E.J., Pickup, S., Dugan, K.D., Innocent, N., Cardiff, R.D., Schnall, M.D., and Chodosh, L.A. (2002). Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell *2*, 451–461.

Moore, R.J., Owens, D.M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M., et al. (1999). Mice deficient in tumor necrosis factor α are resistant to skin carcinogenesis. Nat. Med. *5*, 828–831.

Palladino, M.A., Bahjat, F.R., Theodorakis, E.A., and Moldawer, L.L. (2003). Anti-TNFα therapies: The next generation. Nat. Rev. Drug Discov. *2*, 736–746.

Pidgeon, G.P., Harmey, J.H., Kay, E., Da Costa, M., Redmond, H.P., and Bouchier-Hayes, D.J. (1999). The role of endotoxin/lipopolysaccharide in surgically induced tumour growth in a murine model of metastatic disease. Br. J. Cancer *81*, 1311–1317.

Pollard, J.W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. Nat. Rev. Cancer *4*, 71–78.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Senftleben, U., Li, Z.W., Baud, V., and Karin, M. (2001). IKKβ is essential for protecting T cells from TNFα-induced apoptosis. Immunity *14*, 217–230.

Smyth, M.J., Cretney, E., Takeda, K., Wiltrout, R.H., Sedger, L.M., Kayagaki, N., Yagita, H., and Okumura, K. (2001). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon γ-dependent natural killer cell protection from tumor metastasis. J. Exp. Med. *193*, 661–670.

Smyth, M.J., Takeda, K., Hayakawa, Y., Peschon, J.J., van den Brink, M.R., and Yagita, H. (2003). Nature's TRAIL–on a path to cancer immunotherapy. Immunity *18*, 1–6.

Song, K., Chen, Y., Goke, R., Wilmen, A., Seidel, C., Goke, A., and Hilliard, B. (2000). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. J. Exp. Med. *191*, 1095–1104.

Steeg, P.S. (2003). Metastasis suppressors alter the signal transduction of cancer cells. Nat. Rev. Cancer *3*, 55–63.

Takeda, K., Hayakawa, Y., Smyth, M.J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H., and Okumura, K. (2001). Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat. Med. *7*, 94–100.

Taketomi, A., Takenaka, K., Matsumata, T., Shimada, M., Higashi, H., Shirabe, K., Itasaka, H., Adachi, E., Maeda, T., and Sugimachi, K. (1997). Circulating intercellular adhesion molecule-1 in patients with hepatocellular carcinoma before and after hepatic resection. Hepatogastroenterology *44*, 477–483.

Tang, G., Minemoto, Y., Dibling, B., Purcell, N.H., Li, Z., Karin, M., and Lin, A. (2001). Inhibition of JNK activation through NF-κB target genes. Nature *414*, 313–317.

Tracey, K.J., and Cerami, A. (1994). Tumor necrosis factor: A pleiotropic cytokine and therapeutic target. Annu. Rev. Med. *45*, 491–503.

Walczak, H., Miller, R.E., Ariail, K., Gliniak, B., Griffith, T.S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., et al. (1999). Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat. Med. *5*, 157–163.

Wang, C.Y., Cusack, J.C., Jr., Liu, R., and Baldwin, A.S., Jr. (1999). Control of inducible chemoresistance: Enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-κB. Nat. Med. *5*, 412–417.

Wilson, J., and Balkwill, F. (2002). The role of cytokines in the epithelial cancer microenvironment. Semin. Cancer Biol. *12*, 113–120.

Wu, G.S., Burns, T.F., McDonald, E.R., 3rd, Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., et al. (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat. Genet. *17*, 141–143.

Zhu, W., Downey, J.S., Gu, J., Di Padova, F., Gram, H., and Han, J. (2000). Regulation of TNF expression by multiple mitogen-activated protein kinase pathways. J. Immunol. *164*, 6349–6358.