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Simultaneous inhibition of Rac1 and IKK pathways sensitizes lung cancer cells to TNFα-mediated apoptosis

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Lung cancer is the most frequently occurring cancer in the world and causes more deaths in the United States than does colon, breast, and prostate cancer combined. Despite advances in treatment modalities including radiation, surgery, and chemotherapy, the overall survival in lung cancer remains low. The cytokine tumor necrosis factor α (TNFα) has been shown to regulate both apoptotic and antiapoptotic pathways. Activation of the transcription factor NF-κB appears to be the critical determinant of the antiapoptotic response to TNFα exposure in epithelial cells. A549 human lung carcinoma cells were infected with adenoviral constructs carrying dominant negative mutants of Rac1 and IKK or constitutively active mutant of Rac1, upstream effectors in TNF-mediated NF-κB activation. Cell death, apoptosis, and NF-κB activation were subsequently measured in response to TNFα exposure. Although TNFα alone had no cytotoxic effect, the expression of the dominant negative mutant of IKKβ (Ad.IKKβKA) resulted in apoptotic cell death following TNFα exposure. Similarly, dominant negative mutant to Rac1 (Ad.N17Rac1) further sensitized A549 cells to IKKβKA-mediated TNFα-induced cell death. Conversely, a dominant active form of Rac1 (Ad.V12Rac1) ameliorated the cell death response to concurrent IKKβ dominant negative mutant infection and TNFα exposure. These results suggest that concurrent inhibition of Rac1 and IKK pathways sensitizes lung cancer cells to TNFα-induced apoptosis. **Cancer Gene Therapy (2001) 8, 897–905**

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Apoptosis or programmed cell death is a highly regulated process, which occurs through genetically conserved and complex intracellular pathways.[1] Activation of apoptotic signals leads to chromatin condensation, blebbing of plasma membrane, and DNA fragmentation.[2,3] The process of apoptosis represents a balance of both stimulatory and inhibitory pathways. Modulating the activity of both apoptotic and antiapoptotic pathways in order to shift the balance toward apoptosis represents an attractive target for cancer therapeutics.[4] Tumor necrosis factor α (TNFα) is a pleiotropic cytokine with multiple functions including cell activation, differentiation, and apoptosis.[5–7] TNFα exerts both apoptotic and antiapoptotic effects, which appear to be cell type–defined responses.[8–11] In some cellular systems, the antiapoptotic effects of TNFα appear to be mediated by the up-regulation of NF-κB activity.[12,13] Therefore, antagonism of TNFα-mediated NF-κB up-regulation represents a unique and potentially useful method to shift the balance of TNFα stimulation toward apoptosis and cell death.[14]

The interaction of TNF with its receptor (TNFR1) leads to the recruitment of TRADD to the TNF–receptor complex.[15] Activated TRADD interacts with FADD, a caspase 8 activator, leading to the initiation of apoptotic pathways.[16] TRADD also activates and recruits TRAF1, TRAF2, and RIP to the TNFR1 complex.[17] Activated TRAF1 and TRAF2 have been observed in association with the antiapoptotic proteins CIAP1 and CIAP2. Additionally, TRADD activates an antiapoptotic NF-κB activity.[13,18,19] Therefore, TRADD possesses dual functions: induction of apoptosis through FADD-dependent pathways, and opposition of apoptosis through induction NF-κB activity. Prior research using a strategy of selective NF-κB down-regulation through the use of proteosome inhibitors enhanced TNF-induced apoptosis in human lung adenocarcinoma cells.[20] Similarly, a stable cell line was generated, expressing a super-repressor form of IκBα (IκBαSR), a strong NF-κB inhibitor.[13] This mutant protein prevented NF-κB translocation to nucleus and transactivation of gene expression. The expression of the super-repressor form of IκBα facilitated TNFα-induced apoptosis in cells, which were resistant to TNF.[13] Recently, this super-repressor form IκB was placed into an

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adenoviral construct (Ad.IκBαSR). Infection of squamous cell lung cancer cells with this vector and subsequent exposure of the cells to TNFα induced apoptotic cell death.[21] These studies suggest that preventing the activation of NF-κB–dependent gene transcription during TNFα stimulation can result in apoptosis.

NF-κB is a protein complex composed of two subunits sequestered in the cytoplasm in an inactive state. Upon release of the NF-κB subunits from the inhibitory cytoplasmic binding proteins IkBα and IkBβ, it translocates to the nucleus and initiates gene transcription. The protein kinase complex, which initiates NF-κB translocation, is termed IκB kinase (IKK). IKK is required for the phosphorylation and degradation of IκBα.[22–24] It contains two catalytic subunits (IKKα, IKKβ) and a regulatory subunit (IKKχ).[25–28] It has been previously demonstrated that mice lacking the IκB kinase 2 gene (*IKKβ*) but not IκB kinase 1 (*IKKα*) exhibited severe liver degeneration, which was dependent on TNF signaling.[29,30] More specifically, the IKKβ subunit has been shown to be essential for NF-κB activation and protection from apoptosis *in vivo*.[31]

We have generated an adenoviral construct expressing a dominant negative mutant of IKKβ (Ad.IKKβKA) to investigate the potential of this adenoviral construct to inhibit NF-κB activation and promote TNFα-mediated cell death. Additionally, we have employed two other adenovirus vectors containing Rac1 mutants to examine the effect of Rac1 signaling on TNFα-mediated cytotoxicity. These vectors have been tested alone and/or in combination to determine if simultaneous inhibition of distinct NF-κB activation pathways enhances TNF-mediated apoptosis in lung cancer cells.

MATERIALS AND METHODS

Generation of gene transfer vectors

Seven recombinant adenoviral vectors expressing either β-galactosidase (Ad.LacZ),[32] enhanced green fluorescent protein (Ad.EGFP) (Vector Core, University of Iowa, Iowa City, IA), a dominant negative mutant of Rac1 (Ad.N17-Rac1),[33] a dominant active mutant of Rac1 (Ad.V12-Rac1),[34] a dominant negative mutant form (K44M) of IKKα (Ad.IKKαKM), a dominant negative mutant form (K44A) of IKKβ (Ad.IKKβKA), or a Luciferase reporter gene driven by NF-κB transcriptional activation (Ad.NFκ-BLuc) were used for functional studies. Ad.IKKαKM and Ad.IKKβKA were constructed from pRc β-actin plasmids encoding either the dominant negative mutant of IKKα (IKKαKM) or IKKβ (IKKβKA).[35] Fragments encoding the HA-tagged IKKαKM or IKKβKA cDNA were excised by *Hin*dIII–*Not*I restriction digestion from pRc β-actin plasmids and blunt-subcloned into *Eco*RV site of the pAd.CMV-Link1 adenoviral shuttle plasmid. Recombinant adenoviruses were generated in 293 cells according to a procedure established by Anderson et al.[36] The expression of HA-IKKαKM or HA-IKKβKA from adenoviral constructs was confirmed by Western blotting. pNF-κB-Luc plasmid (Clontech Laboratories, Palo Alto, CA) was used to produce Ad.NF-κBLuc vector. The DNA, encoding the Luciferase gene driven by four tandem copies of the NFκB consensus sequence attached to a TATA-like promoter from the herpes simplex virus thymidine kinase gene was cleaved by *Kpn*I and *Xba*I double digestion. The *Kpn*I and *Xba*I fragment was inserted into a promoterless Adenoviral shuttle plasmid (pAd5mcspA)[36] and Ad.NF-κBLuc virus was produced by homologous recombination. Recombinant adenoviral stocks were generated as previously described,[37] and were stored in 10 mM Tris with 20% glycerol at −80°C. The particle titers of adenoviral stocks were determined by A_{260} readings and were typically 10^{13} DNA particles/mL. The functional titers of adenoviral stocks were assessed by plaque titering on 293 cells and expression assays for encoded proteins. Typically, the particle/pfu ratio was equal to 50.

Adenovirus transduction assays

A549 cells were infected with Ad.EGFP virus at increasing MOIs at 37°C in DMEM without FBS. Two hours following infection, the serum concentration in the tissue culture media was increased to 10% by adding equal volumes of DMEM supplemented with 20% fetal bovine serum. The percent GFP (+) cells was determined at 48 hours by fluorescent microscopy. Infected cells were maintained by providing fresh media twice a week; the longevity of infection was determined by examining cells under fluorescent microscopy for about 6 weeks following initial infection.

Luciferase assays

The Luciferase Assay System with Reporter Lysis Buffer (catalog no. E4030; Promega, Madison, WI) was used to assess NF-κB–induced transcriptional activation according to the manufacturer's instructions. All measurements of Luciferase activity (relative light units) were normalized to the protein concentration of harvested cell lysates. The NF-κB responsive Luciferase reporter vector, Ad.NFκBluc, was used to coinfect cells at an MOI of 5000 particles/cell in these experiments.

TNFα treatments and live/dead cellular viability assays

rhTNFα (catalog no.210-TA) was obtained from R&D Systems (Minneapolis, MN). Forty-eight hours after infection with the adenovirus vectors, A549 cells were exposed to recombinant human TNFα (rhTNFα) at 100 ng/mL for an additional 7 or 24 hours. Molecular Probes' (Eugene, OR) LIVE/DEAD Viability/Cytotoxicity Kit for animal cells (L-3224) was used to distinguish live cells from the dead after various treatments. Calsein AM, a fluorogenic substrate for intracellular calsein esterase, is converted to a green fluorescent product (calsein), which is detectable only in live cells. Because only live cells with intact membranes contain active calsein esterase, detection of calsein by fluorescent microscopy functions as a marker for viable cells. Ethidium homodimer-1 (EthD-1) is a red fluorescent nucleic acid stain, which cannot diffuse across intact cell membranes. Cells with damaged membrane integrity stain positive with EthD-1, whereas intact cells exclude the stain.

Caspase activity assays

CaspaTag Caspase Activity Kits were used to selectively assess caspase activation following infection and exposure to hTNFα. In these assays, carboxyfluorescein-labeled, caspase inhibitors irreversibly bind to active caspases. The caspase inhibitor substrates were designed to be specific for both the active state of the enzyme and a specific isoform. Caspase (+) cells were distinguished from caspase (−) cells by fluorescence microscopy. FAM-DEVD-FMK inhibitor (S7301) was used for caspase 3 activation assay. FAM-LETD-FMK inhibitor (S7304) was used for caspase 8 activation assay.

RESULTS

A549 cells are susceptible to infection with adenoviral vectors

Adenovirus transduction of normal lung has shown to be only moderately effective in mice, rats, primates, and humans.[38–43] However, transduction of A549 cells, a line of lung cancer cells, has been shown to be effective using adenovirus vector systems.[44] Therefore, we have selected this human lung carcinoma cell line, A549, to test our hypothesis that TNF exposure can provoke a modulated apoptotic response in the setting of differential NF-κB inhibition. To establish the efficacy of infection using first-generation adenovirus constructs in our laboratory, A549 cells were infected with an adenovirus encoding enhanced green fluorescein protein (Ad.EGFP). The level of viral transduction indicated as the percentage of cells with GFP expression was determined at various time points after the infection (Fig 1). By 48 hours, more than 90% of the cells was transduced with Ad.EGFP virus at MOI of 5000 DNA particles/cell (Fig 1B). The transduction appeared to be stable in nonproliferating, confluent cell culture conditions, and the percent transduction did not show significant attenuation when measurements were repeated 6 weeks following initial infection (data not shown). Additionally, no obvious cytotoxic effect or loss of cell viability was

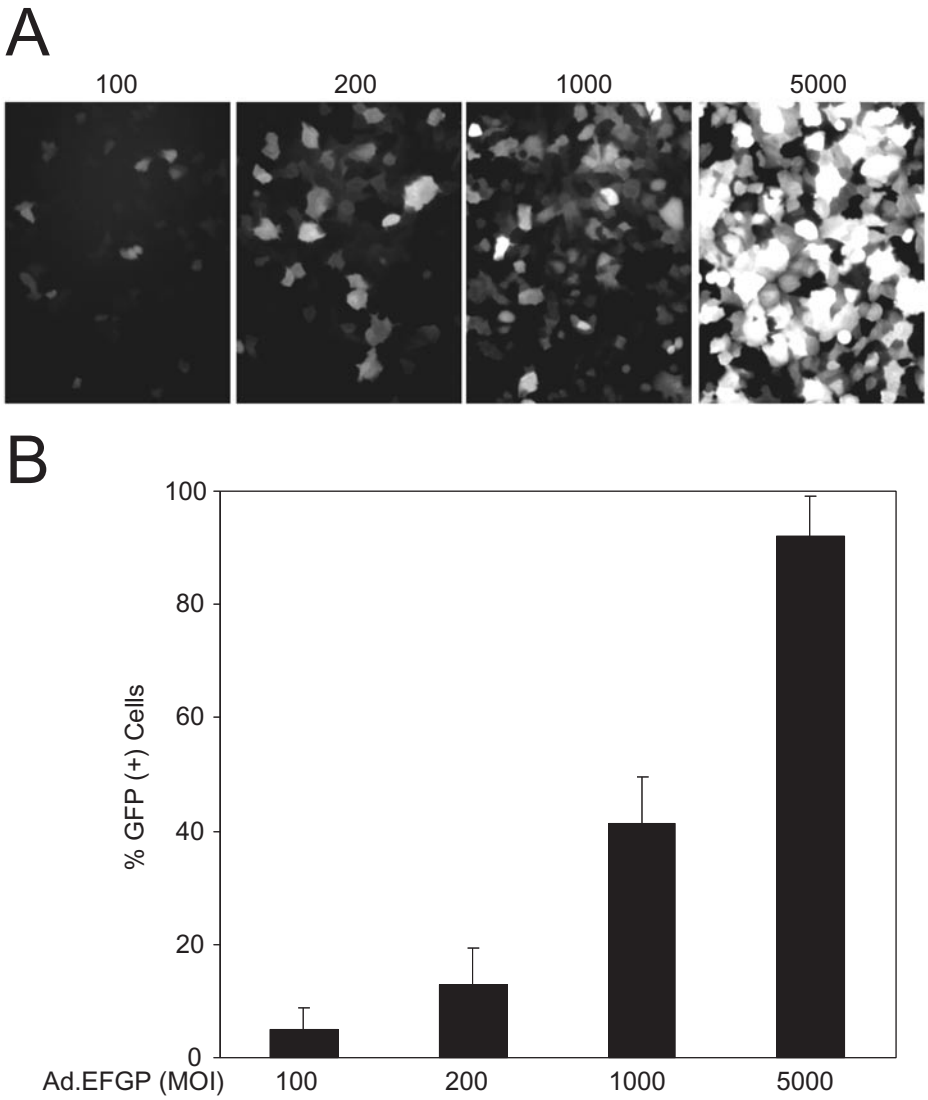

Figure 1. First-generation recombinant adenovirus readily transduced the lung cancer cell line, A549. A549 cells were infected with an adenovirus encoding EGFP reporter gene for 48 hours prior to analysis. Gray scale of fluorescent micrographs is given in **(Panel A)**. MOI values for Ad.EGFP, expressed as the number of DNA particles per cell of adenovirus, are given above the each panel. **Panel B:** represents the efficiency of transduction provided as the percentage of GFP expression cells. The mean (±SEM) GFP (+) cells/20× field (*y* axis) is given for three independent experiments.

observed throughout this 6-week time period under these conditions.

IKKβKA mutant expression suppressed TNFα-induced NF-κB activation

Prior research has established that TNFα activates both apoptotic and antiapoptotic pathways in epithelial cells.[10,14,45] The specific cellular response to TNFα stimulation may depend on the subsequent activity of NF-κB. In order to block potential antiapoptotic activity of the NF-κB pathway, a recombinant adenovirus construct expressing the dominant negative mutant of IKKβ (Ad.IKKβKA) was generated. The functional consequence of infection with this adenovirus construct on NF-κB activity was tested using a NF-κB Luciferase reporter assay system. A549 cells were coinfected with separate adenoviruses encoding both the NF-κB–driven Luciferase reporter gene and the dominant negative IKK. Infection of A549 cells with Ad.IKKβKA, but not Ad.LacZ control, resulted in a dose-dependent decrease in TNF-stimulated NF-κB activity (Fig 2).

Ad.IKKβKA infection sensitized lung cancer cells to hTNFα-induced cell death

A549 cells were infected with Ad.IKKβKA virus prior to treatment with hTNFα to determine the effects NF-κB inhibition on cell survival. As seen in Figure 3, Ad.IKKβKA infection followed by hTNFα treatment significantly decreased cell viability in a dose-dependent manner. Although first-generation adenovirus vectors themselves have been shown to induce apoptosis,[46] control experiments using only Ad.LacZ virus at the same MOI dose did not produce cytotoxicity in combination with hTNFα as measured by calsein activity (Fig 3). These results suggest that IKKβ plays a critical role in mediating the balance of death or survival signaling by TNFα in A549 cells.

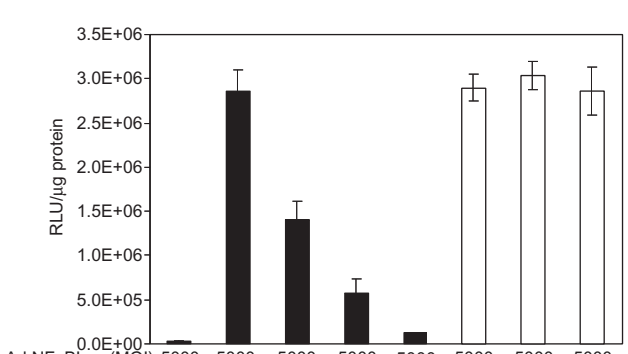

Figure 2. Ad.IKKβKA, but not Ad.LacZ, down-regulated TNFα-induced NF-κB activation. Infection and Luciferase NF-κB reporter assays were performed as described in *Materials and Methods*. MOI values of viruses used in the infection are given on the *x* axis. Luciferase activity expressed as relative light units (RLU) per microgram of protein is depicted on the *y* axis. Data represent the mean of (±SEM) four independent data points ($n=4$).

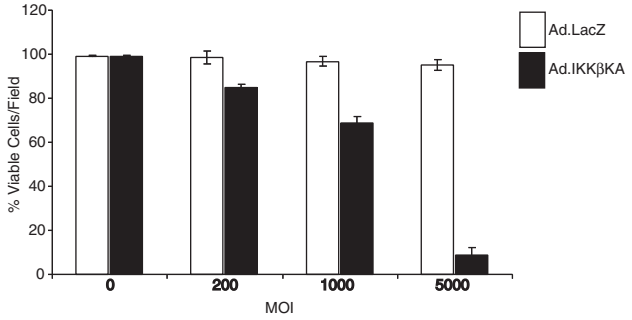

Figure 3. IKKβKA expression sensitized lung cancer cells to TNFα-induced apoptosis. A549 cells were infected with either Ad.IKKβKA virus (solid bars) or Ad.LacZ (open bars) at indicated MOIs on the *x* axis for 48 hours. TNFα treatment (100 ng/mL) was continued for another 24 hours prior to Live/Dead Assay. All experiments used for the figure included TNFα treatments. Values represent the mean (±SEM) of six independent data points. Assays were performed in triplicates and repeated at least twice to confirm the observations.

Ad.IKKβKA infection followed by hTNFα treatment activates caspase pathways

Caspase activation has previously been shown to correlate with initiation of apoptotic cell death. Caspase activity assays were performed in A549 cells infected with Ad.IKKβKA virus for 48 hours followed by 7 hours of hTNFα treatment to further define the mechanisms underlying the observed increase in cytotoxic effect. Both caspase 8 (Fig 4A) and caspase 3 (Fig 4B) demonstrated increased activity in cells infected with Ad.IKKβKA virus following hTNFα treatment. These results suggest that hTNFα activated unopposed proapoptosis pathways during concurrent NF-κB inhibition by the dominant negative IKKβ.

Constitutively active Rac1 protects A549 cells from IKKβKA-induced and TNFα-mediated (IKKβKA-TNFα) apoptosis

Recently, a constitutively active form of the small GTPase protein Rac1 (V12Rac1) has been shown to up-regulate NF-κB activity in HeLa cells.[34] Expression of a dominant negative Rac1 (N17Rac1) has been shown to both directly inhibit NF-κB activity and to sensitize cells to TNF-mediated apoptosis.[34,47] Both the dominant active form of Rac1 (Ad.V12Rac1) and the dominant negative form (Ad.N17Rac1) were tested to evaluate their effects on TNF responsiveness in A549 cells. As indicated in Figure 5A, only the infection of A549 cells with Ad.N17Rac1 virus, but not with Ad.V12Rac1, produced cell death and loss of viability in response to hTNFα treatment. Maximum cell death (55%) was observed with MOI of 10,000 DNA particles/cell of Ad.N17Rac1; no further increase in cell death was detected when the MOI was doubled to 20,000 DNA particles/cell. In the absence of hTNFα treatment, no significant degree of cell loss was observed with either of these two mutants (data not shown).

In additional experiments, A549 cells were also coinfected with Ad.IKKβKA and either the constitutively active or dominant negative forms of Rac1 to determine the effect of

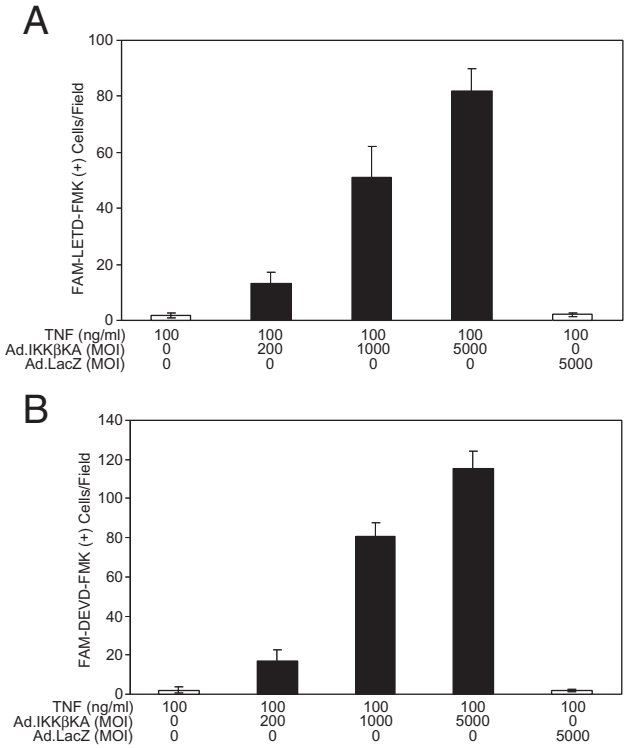

Figure 4. TNFα induces caspase activation in A549 cells in the setting of IKKβ inhibition. A549 cells were infected with Ad.IKKβKA virus at indicated MOIs for 48 hours. TNFα treatment was continued for 7 hours prior to both caspase 8 **(Panel A)** and caspase 3 assays **(Panel B)**. As a negative control, cells were infected with Ad.LacZ virus at MOI of 5000 DNA particles/cell. The amount of TNFα and the types of viral constructs used in the infection are given on the *x* axis. Data represent the mean of (±SEM) of four independent data points.

simultaneous inhibition of these two converging pathways on TNF responsiveness. As shown in Figure 5B, 90% of cell death was observed with Ad.IKKβKA infection alone at an MOI of 5000 DNA particles/cell followed by hTNFα treatment. When cells were coinfected with Ad.V12Rac1 virus carrying constitutively active Rac1 mutant, a dose-dependent decrease in cytotoxicity was observed (Fig 5B). These observations suggest that V12Rac1 overrides the apoptotic signaling induced by TNF in the setting of IKKβ inhibition.

N17Rac1 potentiates IKKβKA-induced IKKβKA-induced TNF-mediated cytotoxicity in A549 cells

Given the beneficial effect of V12Rac1 on cell survival following hTNF exposure, experiments were conducted to determine the effect of a dominant negative Rac1 on cell death. As seen in Figure 5C, Ad.IKKβKA infection at an MOI of 1000 DNA particles/cell alone combined with TNFα treatment resulted in approximately 30% cell death. Coinfection of these cells with Ad.N17Rac1 virus produced a dose-dependent increase in the degree of cell death (Fig 5C). These results suggest that combined use of Ad.IKKβKA and Ad.N17Rac1 to inhibit two different aspects of a converging pathway represents an attractive target for potentially therapeutic interventions.

V12Rac1 rescue and N17Rac1 sensitization of IKKβKA TNFα–induced apoptosis is mediated through NF-κB

In order to determine the effect of V12Rac1 on NF-κB activity during concurrent Ad.IKKβKA infection and TNF stimulation, NF-κB activity assays were performed. As seen in Figure 6A, hTNFα-induced NF-κB activation was abolished with Ad.IKKβKA infection of A549 cells. Coinfection of these cells with Ad.V12Rac1 ameliorated IKKβKA suppression of hTNFα-induced NF-κB activation. This partial restoration of NF-κB activity demonstrated a dose dependence on the concentration of Ad.V12Rac1. On the contrary, Ad.N17Rac1 infection further decreased NF-κB activity compared to Ad.IKKβKA infection alone (Fig 6B). This effect of N17Rac1 also correlated well with the extent of cell death, as demonstrated in Figure 5C. Furthermore, Ad.IKKβKA infection down-regulated V12Rac1-induced NF-κB activation in a dose-dependent fashion (Fig 6C). No down-regulation of NF-κB activity was evident with Ad.LacZ virus. This suggests that IKKβ subunit plays a functional role in the transmission of signal from Rac1 to NF-κB. Although experiments with a dominant negative α subunit of IKK delivered through the vector, Ad.IKKαKM, demonstrated an inhibition of NF-κB activity, the degree was significantly less than dominant negative IKKβ (Fig 6D). This functional difference did not appear to be related to differential levels of protein

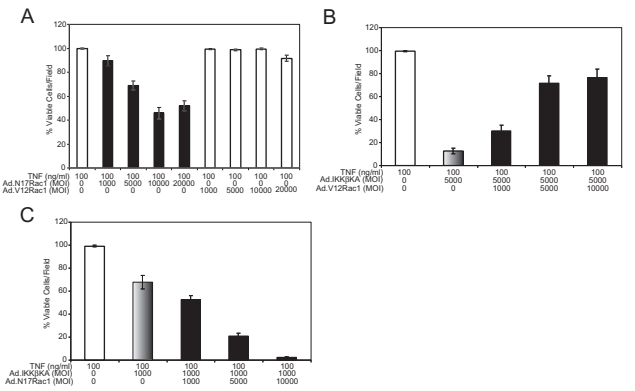

Figure 5. Mutant Rac1 protein expression alters cellular sensitivity to TNFα-mediated cytotoxicity. N17Rac1 expression sensitizes A549 cells to TNFα-induced apoptosis **(Panel A)**. A549 cells were infected with Ad.N17Rac1 or Ad.V12Rac1 virus for 48 hours prior to hTNFα treatment. Percent viable cells per field was determined as described in *Materials and Methods*. MOI values for Ad.N17Rac1 and Ad.V12Rac1 virus are given below the each bar. V12Rac1 expression rescued cells from IKKβKA sensitized TNFα-induced apoptosis **(Panel B)**. A549 cells were coinfected with both Ad.IKKβKA and Ad.V12Rac1 virus for 48 hours at 37°C. Cells were further treated with TNFα at 100 ng/mL concentration for 24 hours prior to Live/Dead Assay. MOI values for Ad.IKKβKA and Ad.V12Rac1 are provided on the *x* axis. N17Rac1 expression further sensitized A549 cells to IKKβKA-induced, TNFα-mediated apoptosis **(Panel C)**. Ad.N17Rac1 virus was coinfected with Ad.IKKβKA into A549 cells for 48 hours prior to 24-hour TNF treatment. MOIs for Ad.N17Rac1 virus and Ad.IKKβKA virus are given below the panel. In order to reduce cell death, MOI of Ad.IKKβKA was lowered from 5000 DNA particles/cell to MOI of 1000 as depicted in **Panel C**. Values in **Panels A–C** represent the mean (±SEM) of six independent data points.

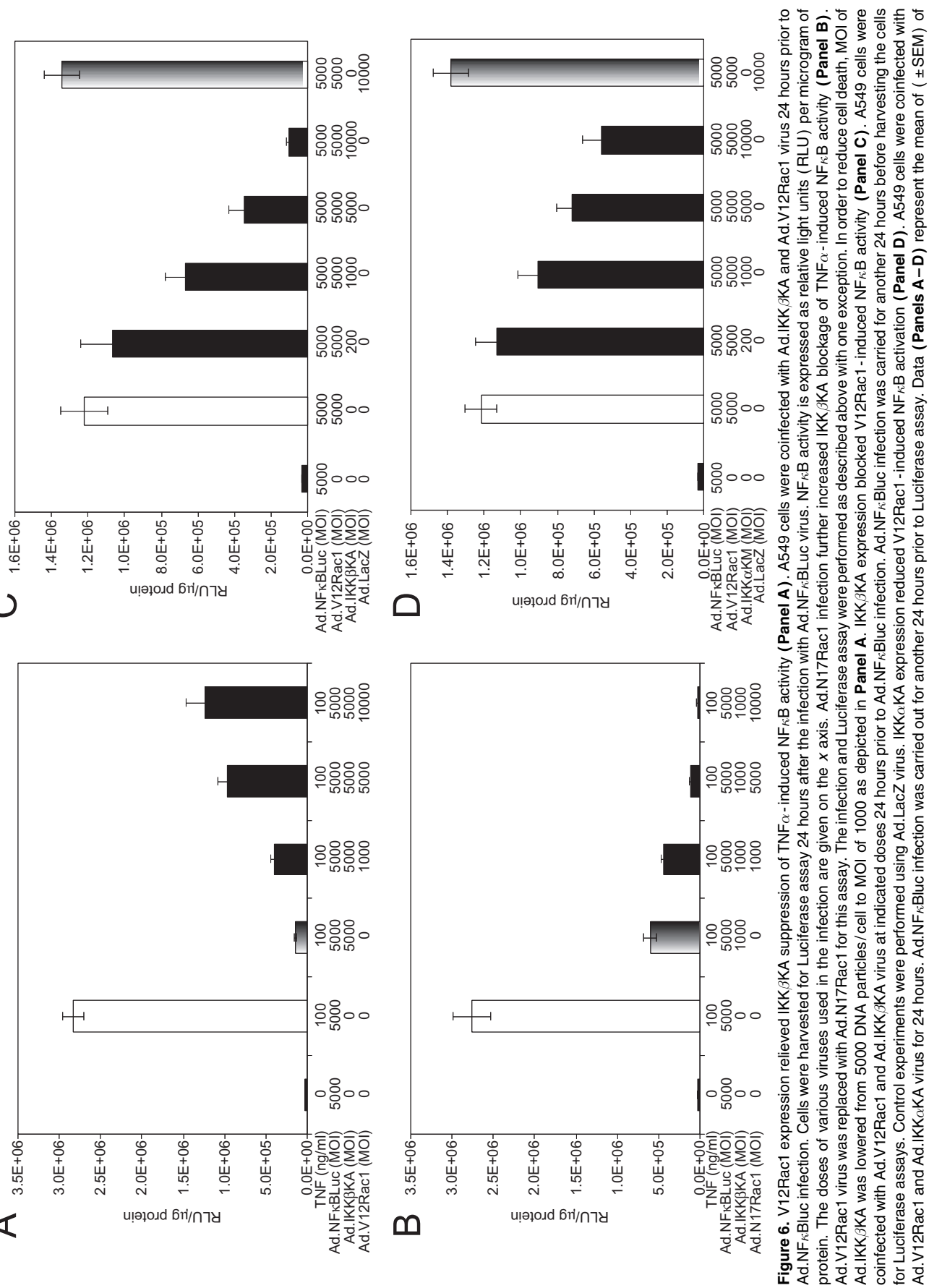

Figure 6. V12Rac1 expression relieved IKKβKA suppression of TNFα-induced NFκB activity **(Panel A)**. A549 cells were coinfected with Ad.IKKβKA and Ad.V12Rac1 virus 24 hours prior to Ad.NFκBluc infection. Cells were harvested for Luciferase assay 24 hours after the infection with Ad.NFκBLuc virus. NFκB activity is expressed as relative light units (RLU) per microgram of protein. The doses of various viruses used in the infection are given on the *x* axis. Ad.N17Rac1 infection further increased IKKβKA blockage of TNFα-induced NFκB activity **(Panel B)**. Ad.V12Rac1 virus was replaced with Ad.N17Rac1 for this assay. The infection and Luciferase assay were performed as described above with one exception. In order to reduce cell death, MOI of Ad.IKKβKA was lowered from 5000 DNA particles/cell to MOI of 1000 as depicted in **Panel A**. IKKβKA expression blocked V12Rac1-induced NFκB activity **(Panel C)**. A549 cells were coinfected with Ad.V12Rac1 and Ad.IKKβKA virus at indicated doses 24 hours prior to Ad.NFκBluc infection. Ad.NFκBluc infection was carried for another 24 hours before harvesting the cells for Luciferase assays. Control experiments were performed using Ad.LacZ virus. IKKαKA expression reduced V12Rac1-induced NFκB activation **(Panel D)**. A549 cells were coinfected with Ad.V12Rac1 and Ad.IKKαKA virus for 24 hours. Ad.NFκBluc infection was carried out for another 24 hours prior to Luciferase assay. Data **(Panels A–D)** represent the mean of (±SEM) of four independent data points.

expression based on Western blotting for HA-tagged IKKαKM and IKKβKA (data not shown). In summary, these results suggest that NF-κB activity can be decreased in a dose-dependent manner by infection with adenovirus vectors containing dominant negative IKKβ and Rac1 mutants.

DISCUSSION

Previous studies examining gene therapy for inherited diseases such as cystic fibrosis have reported difficulty in achieving efficient and sustained adenoviral transduction of normal lung cells.[39,40,42,43,48,49] The low efficiency of adenovirus-mediated gene transfer to fully differentiated and polarized airway epithelial cells has been attributed to low-level expression of the coxsackie/adenovirus receptor (CAR)[50,51] on the apical surface of airway epithelial cells.[52–54] Consequently, cells with low CAR expression are difficult targets for adenovirus-mediated gene transfer.[55] On the contrary, the A549 human airway epithelial cell line has been demonstrated to express high levels of CAR receptor.[44] Therefore, we have chosen this cancer cell line to test if this particular type of lung cancer cell line can be sensitized to TNFα-induced apoptosis by modulating IKK and Rac1 signaling pathways.

Our initial experiments with adenovirus containing the gene for a marker protein, EGFP, confirmed that the human lung cancer cell line, A549, could be efficiently transduced with a first-generation recombinant adenovirus vector. Although a direct apoptotic effect of the vector itself has been previously demonstrated in lung cancer cell lines,[46] we observed no direct toxic effect of first generation recombinant adenovirus vectors carrying *LacZ* or EGFP reporter genes in A549 cells.

Previous authors have stated that gene therapy designed to subvert normal cell signaling pathways including apoptosis could be a potentially useful therapeutic approach to cancer.[4,13] A particularly attractive target for this therapeutic approach has been TNF-induced apoptosis. Although TNFα alone may have direct apoptotic effects,[9,11,56] many studies have documented that the balance of TNFα effects is shifted toward apoptosis only in the setting of concurrent blockade of NF-κB.[12,14] In tumor cell lines, TNF-mediated apoptosis has been shown to occur in the setting of NF-κB[13,21] inhibition. Because NF-κB activation is dependent on multiple converging pathways, including activation of IκB kinase,[57] phosphorylation of subunit transactivation domains,[58,59] and association with basal transcription factors,[60,61] an approach based on simultaneous inhibition of parallel pathways could more effectively inhibit NF-κB activity. Both Rac1 and IKK have been established as signal transduction proteins in NF-κB activation. Furthermore, Rac1 has been previously demonstrated to possess NF-κB activating function, which is independent of IkB protein degradation.[62] Given the observation that Rac1 inhibition by itself can augment TNF-mediated apoptosis,[47] we studied the combined effects of IKK inhibition and Rac1 inhibition on TNFα-mediated apoptosis.

Our results indicate that IKKβKA expression sensitized A549 cells to TNFα-induced apoptosis. The results from these experiments show that antiapoptotic signaling can be inhibited through upstream blockade of NF-κB activation. Caspase activity assays specifically confirmed the activation of apoptotic pathways during concurrent NF-κB inhibition and TNFα exposure. Thus, the susceptibility to TNFα-induced apoptosis appears to be somewhat dependent on IKK activity. Although both IKKα and IKKβ mutants inhibited V12Rac1-mediated NF-κB up-regulation (Fig 6C and D), the IKKβ appeared more potent. Considering the fact that similar levels of protein expression from Ad.IKKαKM and Ad.IKKβKA constructs were detected in A549 cells by Western blotting (data not shown), this effect could not be attributed to differential protein expression. These observations correlate with other experiments in which an *IKKβ* knockout mouse mutant,[30] but not *IKKα*,[29] exhibited severe liver degeneration due to TNFα.

Reprogramming A549 cells through the expression of a dominant active form of Rac1 (V12Rac1) was able to block apoptotic activation by IKKβKA expression combined with TNFα treatment. One potential explanation for these results is that despite IKKβKA expression, functional IKKα subunit was able to transmit V12Rac1-induced signaling to NF-κB (Fig 6D), thus permitting its transactivation. Conversely, the negative mutant to Rac1 (N17Rac1) further sensitized A549 cells to IKKβKA-mediated, TNFα-induced apoptosis. From these results, it appears that IKKβKA and N17Rac1 constructs could be used in combination to facilitate TNFα-induced cell killing of lung cancer cells. The molecular mechanism of IKK activity modulation by Rac1 remains unclear; however, it is possible that this putative pathway could also form an attractive target for gene therapy as well.

One of the main obstacles of cancer gene therapy is to be able to specifically target cancer cells. Although our research does not directly address issues regarding specific tumor cell targeting by adenoviral vectors, a number of recent reports and discussions have proposed methods to alter the tropism of adenovirus vectors. The possible targets for creating specific vector cell targeting have included the insertion of RGD peptides into adenoviral fiber proteins to target the integrin recognition sites on ovarian cancer cells[63] and rhabdomyosarcoma cells.[64] In addition, a bispecific antibody linking fiber protein to epidermal growth factor receptor was used to target glioma cells.[65,66] By taking advantage of the observation that fibroblast growth factor receptors appear to be overexpressed in some malignant cells, Kaposi's sarcoma cells have been successfully targeted by adenoviral constructs using antiknob Fab fragment linked to FGF.[67] Identification of specific lung cancer cell markers could potentially serve as the basis for the development of similar specific targeting techniques for lung cancer cells.

Our results not only demonstrate the sensitization of A549 cells to TNFα-induced apoptosis by two pathways, but also describe a novel application in which combinatorial use of Ad.N17Rac1 and Ad.IKKβKA has been demonstrated to be more effective than their individual administrations. Therefore, we have shown that adenovirus vectors are capable of

delivering inhibitory genes, which permit the activation of existing cell signaling pathways to cause apoptosis. Ultimately, our dual vector approach may become useful in the clinical treatment of lung cancer.

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