Author's response to reviews

Title: NF-kappaB targeting by way of IKK inhibition sensitizes lung cancer cells to adenovirus delivery of TRAIL

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Version: 2 Date: 7 September 2010

Author's response to reviews: see over
Point by Point Reply to Comments for the Manuscript: MS 7449537293932264

Title: NF-kappaB targeting by way of IKK inhibition sensitizes lung cancer cells to adenovirus delivery of TRAIL

We thank the reviewers for their insight and helpful suggestions. Below are the comments from the reviewers and our responses to them.

Reviewer 1

Comment 1: This is a technically sound study showing that TRAIL resistance in the lung carcinoma cell line A549 cells can be overcome by inhibiting IKK using a dominant negative IKK (IKKbetaKA). These findings are not surprising since: (1) inhibiting IKK has been shown to overcome TRAIL resistance in other cancers, and (2) the authors themselves have previously shown that IKKbetaKA can overcome TNF resistance in the same cell line.

However the area of overcoming TRAIL resistance in lung cancer is an area of active investigation and the results in the current paper does provide confirmatory data supporting the expected response.

Minor comment: The authors may consider writing a few lines describing how IKKbetaKA (and IKBalphaSR mentioned in 3rd paragraph of the Results section) interferes with endogenous IKKbeta function and NFkB signalling.

The "a" is missing from the word death in the first line of the Fig legend.

Action Taken: The following sentences were added to Adenovirus preparation section of Methods on Page 6, line 7 from the top:

“AdIKKbKA encodes the dominant negative mutant form (K44A) of IKKb and forms inactive IKK complex so that IKKb does not phosphorylate IkB. IkBaSR produces dominant negative mutant form (S32A/S36A) of IkBa. Thus, the IKK complex cannot phosphorylate mutant IkBa from S32 and S36 residues. By doing so NF-kB is always sequestered in
cytoplasm. Both mutant proteins interfere with NF-kB signaling at different levels of the signaling cascade.”

Spelling error (deth-death) in Figure 3 legend has been corrected.

Reviewer 2: Ralf Zwacka

Aydin et al. describe the inhibition of NF-kappaB activity by adenoviral encoded expression of IKK2-KA (AdIKKbetaKA) and enhanced apoptosis-inducing activity of TRAIL (Ad5hTRAIL) on A549 lung carcinoma cells. They show that A549 can be readily transduced with adenoviral vectors. Furthermore, they demonstrate that double transduction with AdIKKbetaKA and Ad5hTRAIL led to decreased viability and increased apoptosis of A549 cells. AdIKKbetaKA was also able to reduce TRAIL-induced NF-kappaB activity. While this is a good and well-written report, a number of issues should be addressed to strengthen their findings.

Major Compulsory Revisions

Comment 1. (Fig 2) The authors fail to show any transgene expression (except EGFP). Western blots for IKKbeta and TRAIL that correspond to the dose-escalating experiment (MOI 0 - 5000) with AdEGFP in Fig 1 should be shown.

Action Taken:

We conducted flow cytometry to show the surface expression of TRAIL and western blotting to confirm AdIKKβKA expression in A549 cells. The following was added to the result section of our manuscript under A549 lung cancer cells are resistant to adenoviral delivery of hTRAIL or IKKbKA expression, Page 10, line 4 from the top:
“To rule out the possibility that the lack of any cytotoxic effect was due to little/no TRAIL expression from the vector, flow cytometric analysis was performed on A549 cells infected with Ad5hTRAIL. This assay demonstrated that significant TRAIL overexpression was achieved after A549 infection with Ad5hTRAIL (Figure 3A). Similarly, immunoblot analysis was employed to demonstrate IKKβKA expression. IKKβKA expression was detectable only when cells were infected with AdIKKβKA vector but not with AdEGFP (Figure 3B).”

Figure 3 legend on Page 21 is added as shown below:
Figure 3. Ad5hTRAIL and AdIKKβKA transductions of lung cancer cells. Panel A represents a flow cytometry analysis of hTRAIL expression in A549 cell line. Conditions for infections are as follows: 1, unstained; 2, uninfected (secondary antibody alone); 3, AdLacZ; 4, Ad5hTRAIL (5,000 MOI); 5, Ad5hTRAIL (10,000 MOI). Panel B shows a Western Blotting indicating IKKbKA expression. Adenoviral constructs used in the infections are indicated above each lane (duplicate independent A549 samples are shown). Molecular standard markers (β galactosidase, 121 kD; and bovine serum albumin, 70 kD) are provided to the left of the blot.

In addition following was added to Materials and Methods section of the manuscript on Page 7-8:

Flow Cytometry and Western Blotting:

Flow cytometry assays were conducted as described previously [1]. Monoclonal antibody to TRAIL (human) (cat. no. ALX-804-296-C100; Alexis Biochemicals) was used followed by polyclonal antibody to mouse IgG1 (R-PE) (cat. no. ALX-211-201-C050; Alexis Biochemicals) to reveal TRAIL expression on the cell surface. For Western Blotting; protein extracts were prepared 48 hours following the infection. Then, 10 µg of A549 cell line extract was loaded in each lane and IKKbKA protein expression was detected using an anti-HA peroxidase antibody (Roche Molecular Diagnostic, Indianapolis, Indiana, US, Cat. No.11667475001). GAPDH expression was detected using a GADPH antibody (BIODESIGN International, Maine, US, Cat No. H86504).

The rest of the figure numbers were changed accordingly.
Comment 2. (Fig. 3 and 5) In order to show the specificity of the effects a caspase inhibitor like zVAD and a TRAIL neutralizing antibody could be applied to revert the effects of AdIKKbetaKA/Ad5hTRAIL.

Action Taken: If we had used soluble TRAIL protein to induce apoptosis instead of an adenovirus encoding TRAIL, then the use of TRAIL neutralizing/blocking antibodies to show the specificity of the effect could have been done. Our study specifically used an adenovirus encoding the TRAIL cDNA to express TRAIL on the cell membrane. Thus, the best control is to use adenovirus vector (Ad5) with the same promoter (CMV) and an inert reporter gene (beta galactosidase) [2-6] or adenovirus vector without any insert at all (AdBgl II) [7]. These are the conventional controls for gene transfer assays employing adenovirus vectors. Furthermore, according to our collaborator Dr. Griffith the most reliable method to blocking TRAIL function expressed from Ad5hTRAIL is to prevent TRAIL protein translocation to the cell surface using Brefeldin A (see Griffith et al., J. Immunol. 165:2886).

Regarding caspase inhibition; we used carboxyfluorescein-labeled caspase inhibitors, which can irreversibly bind to active caspases to demonstrate specificity. Instead of trying to reverse the effects, we actually used FAM-DEVD-FMK inhibitor (S7301) to selectively monitor caspase 3 activation following infection, then distinguished caspase positive cells from caspase negative cells by immune fluorescence microscopy. This assay demonstrated that under the setting of IKK inhibition TRAIL induced Caspase 3 activation. This result was incorporated as Figure 7 in our manuscript.
Figure 7. Inhibition of IKK activity induces TRAIL-mediated Caspase 3 activation in lung cancer cells. Caspase activity assays were performed following co-infection of A549 lung cancer cells with Ad5hTRAIL and AdIKKbKA or AdCMVLacZ vectors. MOI and types of viral vectors used in the infection are provided on the X-axis. AdCMVLacZ construct was used at an MOI of 10,000 DNA particles/cell. Caspase activity was assessed under fluorescent microscopy. Data represent the mean of (±SEM) five independent data points. * p < 0.05

Following was added to Results under “Ad5hTRAIL infection with NF-kB inhibition induces apoptosis in A549 cells” Page 12, line 3 from the top:

To further demonstrate that apoptosis is the mechanism of cell death in A549 cell line, caspase activation assays were performed following coinfection of cells with Ad5hTRAIL and AdIKKbKA vectors. There was significantly increased Caspase 3 activity detected only when the A549 cells were infected with Ad5hTRAIL and AdIKKbKA (Figure 7).
In addition Materials and Methods section has also the following info on Page 8:

Caspase Activity Assays:

It is well established that carboxyfluorescein-labeled caspase inhibitors can irreversibly bind to active caspases. The caspase inhibitor substrates were designed to be not only specific for the active state of the enzyme and also it is isoform specific. CaspaTag Caspase Activity Kits were deployed to selectively monitor caspase activation following infection with gene therapy vectors. FAM-DEVD-FMK (S7301) was used to measure caspase 3 activation, and then distinguished caspase positive cells from caspase negative cells by immune fluorescence microscopy.

Comment 3. (Fig. 3 and 5) To move this experimental setting closer to a potential clinical application, can the experiments be repeated with recombinant TRAIL and a small molecule NF-kappaB/IKK inhibitor, and the same or similar results be obtained.

Action Taken: We are in the process of establishing a gene therapy model of a lung cancer, which shows resistance to Ad5hTRAIL. We demonstrated that inhibition of IKK activity using another type of gene therapy vector (AdIKKbKA) sensitized TRAIL resistant lung cancer cells to Ad5hTRAIL delivery. The complementary gene therapy approach we employed is very specific for IKKb kinase. If we were to use a chemical inhibitor of any type of kinase, usually there is more than one protein inhibited with these agents. This is one reason why we develop specific gene therapy vectors. We are trying to move from chemotherapeutics to gene products to increase the specificity. Not only the soluble form of TRAIL but also adenovirus gene therapy vector encoding human TRAIL (Ad5hTRAIL, the
vector used in this manuscript) has been under clinical trial for a while and our collaborator Thomas S. Griffith has just completed Phase I clinical trial of Ad5hTRAIL in cancer patients. Our complementary experimental approach in this manuscript may actually lead to the expansion of therapeutic potential of Ad5hTRAIL administration in TRAIL resistant cancer patients. Moreover, using recombinant proteins (including TRAIL) has some drawbacks due to the need for repeated administration, expense of the treatment, purity problems, etc. Thus, we do not consider using recombinant proteins and/or chemical inhibitors is an extension of experimental gene therapy vector application in clinical settings. We believe that this issue is out of the scope of the current manuscript. With all that said, when we performed the assay with recombinant TRAIL + NFkB inhibitor (NFi), the expected outcome was reached as shown below. This data is provided for the reviewing purposes only.
Figure Legend: Pharmacological inhibition of activation of NF-kB pathway sensitizes A549 human lung cancer cells to TRAIL induced apoptosis. Microtiter plates were seeded with 4x10^4 cells/well, before the addition of recombinant hTRAIL alone or in combination with inhibitor of NFkB activation, NFi (EMD Biosciences, San Diego, CA). Cell viability was determined after 20 hours by crystal violet staining. rhTRAIL induced cell death, but only to a modest extend and only at the higher concentrations. In contrast, the combination of hrTRAIL and NFi induced substantial amounts of cell death and to a much greater extent than hrTRAIL alone. These results demonstrate that the inhibition of NF-kappaB activation sensitizes human lung cancer cells to TRAIL induced apoptosis. Each value represents the
mean of two independent data points. Standard error bars were omitted from the graph for clarity but were less than 10% for all data points.

Comment 4. (Fig.3 and 5) As IKK might have activities that go beyond regulation of canonical NF-kappaB signalling, which might also affect TRAIL responses, can the authors substitute AdIKKbetaKA with AdIkappaB-SR and obtain the same results regarding TRAIL sensitization?

Action Taken:

As requested, we replaced AdIKKβKA with AdIkBαSR vector and repeated the cellular viability and cytotoxicity assay. Although, AdIkBαSR infection also sensitized lung cancer cells to TRAIL, the degree of sensitization was less than what was observed with AdIKKβKA infection as shown below.
Thus we added the following statement to our manuscript under *NF-kB blocking via IKK inhibition sensitizes A549 lung cancer cells to TRAIL-induced apoptosis* of results section on Page 10, line 3 from the bottom:

“We then tested the extent to which AdIkBαSR could substitute for the AdIKKβKA vector in sensitizing A549 cells to Ad5hTRAIL. NF-kB inhibition via AdIkBαSR infection also resulted in some degree of cell death from TRAIL, but the degree of sensitization was less than that of AdIKKβKA delivery (data not shown), suggesting AdIKKβKA inhibition of NF-kB is more efficient.”

Because we are primarily interested in the effect of IKK inhibition on TRAIL sensitization of lung cancer cells, we decided to exclude this figure from our manuscript and show it for reviewing purposes only.

**Comment 5.** (Fig. 4) A remaining question is whether Ad.IKKbetaKA inhibits potential constitutively activated NF-kappaB or (only) TRAIL-induced NF-kappaB activation and which of the two activities is responsible for TRAIL sensitization. Do A549 cells harbor elevated constitutive NF-kappaB levels? Does AdIKKbetaKA also inhibit NF-kappaB without addition of TRAIL?

**Action Taken:** We explicitly stated in our manuscript under the result section (Page 11 line 15 from the top) that both endogenous and TRAIL induced NF-kB activity was drastically reduced after infection with AdIKKβKA. We realized that our figure did not show the bar indicating a reduction in endogenous NFkB activity following AdIKKβKA infection. We added this bar into Figure 5 in order to prove that what we claimed in the text is reflected on the figure as well.
Minor Essential Revisions

1. The authors indicate in their Results chapter that an MOI 10,000 did not cause any cytotoxic effects, but only show an MOI 5,000 in the figure. This should be corrected/modified.

Response: Relevant sentence in results is as follows: “A549 lung cancer cells were also completely resistant to cytotoxic effects of hTRAIL, despite the high doses of Ad5hTRAIL (MOI of 10,000 DNA particles/cell) used for the infection (Figure 2, upper panels).”

We went up to MOI of 10,000 of Ad5hTRAIL vector to check its cytotoxicity as shown in the Figure 2. Since almost all A549 cells were transducible with MOI of 5,000 AdEGFP vector (Figure 1), and 5,000 MOI provided high levels of hTRAIL expression in A549 cell line as shown with flow cytometry (new Figure 3A), we used 5,000 MOI of Ad5hTRAIL for subsequent experiments (cell viability, NFkB inhibition and Annexin V binding and caspase activation assay).
2. A number of papers have been omitted that have studied TRAIL on A549 lung carcinoma cells. They should be added to the references.


**Action Taken:** Suggested references are added into the manuscript.

3. The MOIs appear rather high for A549 cells. Do the authors have an explanation for this?

**Response:** MOIs given in the manuscript represent DNA particles/cell. Although 1 Plaque Forming Unit (pfu) of adenovirus vector is expected to correspond to 20 DNA particles, this number may go up to 50 or 100 virus particles in some cases depending on variations in virus preparations. Unfortunately DNA particles (virus particles) do not reflect the amount of active virus in the preparation. In our case MOI of 5000 corresponded to 100 PFU’s.

**Reviewer 3:** Zi-Chun Hua

The present manuscript tried to test combinatorial gene therapy modality of adenovirus delivery of TRAIL (Ad5hTRAIL) and IKK inhibition (AdIKKbKA) to overcome TRAIL resistance in lung cancer cells. Luciferase assays were used to monitor the regulation of NF-
kB activity. The results showed that combination treatment with Ad5hTRAIL and AdIKKbKA induced significant apoptosis of TRAIL-resistant A549 cells, and this may suggest that dual gene therapy strategy of exogenous TRAIL gene expression together with concurrent IKK inhibition may be a promising novel gene. This paper is well written and the results look clear. But there are some scientific defects as follows. It is not qualified for publishing in this journal in its current version. It needs major compulsory revisions

1. The authors did not provide any direct data to show the expression of TRAIL and IKKbKA after gene therapy into cell line, and their respective expression during the combinational therapy modality. There are more parameters, which could influence the apoptosis, for example, stress response during protein over-expression may also affect apoptosis.

**Action Taken:**
As mentioned in our response to Comment 1 from Reviewer #2, we conducted flow cytometry to show the surface expression of TRAIL and western blotting to confirm AdIKKβKA expression in A549 cells.

2. Annexin V can detect the early stage of apoptosis and the authors had better also use one more assay to further demonstrate apoptosis such as activation of caspases.

**Action Taken:**
As requested by the reviewer, we used a caspase 3 activation assay to support our findings with Annexin V. See our response to Comment 2 from Reviewer 2, above, for specifics.

3. To further confirm the design of the combinational gene therapy modality of TRAIL and IKKbKA, the authors had better add more data to demonstrate the inability of NF-kB’s translocation into the nucleus and also phosphorylation status of IkB.

**Response:**
This is a question of the extent to which the AdIKKbKA vector is functional and inhibiting NF-kB activity in the cell. Whenever we construct a new gene therapy vector, its expression and functional status is rigorously tested prior to its use in actual assays. Both the AdIKKbKA and AdNFkBLuc vectors were constructed by our group, and their function has been confirmed in our previous publications.

Before we constructed AdNFkBLuc vectors, we performed mobility shift assays to confirm that the proteins encoded by these vectors such as AdIkBSR (AdIkBM) interfered with NFkB signaling as shown below.

UV-mediated NF-kB activation is blocked by Ad.IkBM [8]. Hela cells were infected with recombinant adenovirus expressing the dominant mutant form of IkBa (Ad.IkBM) at MOI values of 10, 50, 250 and 1000 or the LacZ gene (Ad.CMVLacZ) at an MOI value of 1000. The following day, Hela cells were UV irradiated at 25 J/m2. Nuclear extracts were prepared
1 h after UV irradiation and NF-kB DNA binding evaluated with 32P-labeled wildtype NF-kB double-stranded oligos.

In order to avoid using radioactive labeled nucleotides and laborious mobility shift assays; we generated AdNFkBLuc construct as a reporter to assess functional NFkB status of living cells [9]. The fragment containing the luciferase gene driven by four tandem copies of the NFkB consensus sequence fused to a TATA-like promoter from the herpes simplex virus-thymidine kinase gene was released by KpnI and XbaI double digestion. The KpnI and XbaI fragment was inserted into a promoterless adenoviral shuttle plasmid (pAd5mcspA) and Ad.NFkBLuc virus was generated by homologous recombination.

In order to get luciferase activity, following phosphorylation of IkB by IKK complex, NFkB has to be liberated from IkBa and move to the nucleus then bind to NF-kB binding sites on this vector prior to transcription. Thus, this construct is very useful to get functional information on NF-kB activity. AdNFkBLuc [2, 4, 5, 9-11] and AdIKKbKA [2, 4-7, 9-12] have been used in many previous studies from our laboratory. Thus doing mobility shift assays or revealing phosphorylation status of IkB to demonstrate NF-kB activity would defeat the actual purpose of using AdNFkBLuc vectors. Further, neither phosphorylation of IkB nor increase in NF-kB p50/p65 heterodimers show that NFkB is in nucleus and can activate transcription of NFkB responsive genes. Not to mention the AdNFkBLuc system is more sensitive than western blotting and mobility shift assays. Consequently, AdNFkBLuc vector eliminates the necessity to examine phosphorylation status of NFkB or show p50/p65 heterodimer formation by mobility shift assay unless the mechanism of phosphorylation of IkB is the question of interest, which is out of scope of this manuscript. This manuscript specifically focuses on IKK inhibition and TRAIL sensitization.

References used for the response letter:


