

IL-24 sensitizes tumor cells to TLR3-mediated apoptosis

R Weiss¹, M Sachet¹, J Zinngrebe², T Aschacher¹, M Krainer^{3,4}, B Hegedus^{1,4,5}, H Walczak^{*2} and M Bergmann^{*1,4}

Interleukin-24 (IL-24), a member of the IL-10 cytokine family whose physiological function remains largely unknown, has been shown to induce apoptosis when expressed in an adenoviral background. It is yet little understood, why IL-24 alone induced apoptosis only in a limited number of tumor cell lines. Analyzing an influenza A virus vector expressing IL-24 for its oncolytic potential revealed enhanced pro-apoptotic activity of the chimeric virus compared with virus or IL-24 alone. Interestingly, IL-24-mediated enhancement of influenza-A-induced apoptosis did not require viral replication but critically depended on toll-like receptor 3 (TLR3) and caspase-8. Immunoprecipitation of TLR3 showed that infection by influenza A virus induced formation of a TLR3-associated signaling complex containing TRIF, RIP1, FADD, cFLIP and pro-caspase-8. Co-administration of IL-24 decreased the presence of cFLIP in the TLR3-associated complex, converting it into an atypical, TLR3-associated death-inducing signaling complex (TLR3 DISC) that induced apoptosis by enabling caspase-8 activation at this complex. The sensitizing effect of IL-24 on TLR3-induced apoptosis, mediated by influenza A virus or the TLR3-specific agonist poly(I:C), was also evident on tumor spheroids. In conclusion, rather than acting as an apoptosis inducer itself, IL-24 sensitizes cancer cells to TLR-mediated apoptosis by enabling the formation of an atypical DISC which, in the case of influenza A virus or poly(I:C), is associated with TLR3.

Cell Death and Differentiation (2013) 20, 823–833; doi:10.1038/cdd.2013.15; published online 1 March 2013

Interleukin-24 (IL-24) was identified to be upregulated in differentiated and non-proliferating melanoma cells.¹ Shortly after its discovery, IL-24 attracted interest as a therapeutic agent as adenoviral overexpression of IL-24 (Ad-IL-24) induced apoptosis in cancer cells.² Subsequently, the therapeutic potential of Ad-IL-24 has been confirmed in a number of different solid cancer models,^{3–5} which led to further evaluation of this immunotherapy in clinical trials. Analysis of the mechanism of Ad-IL-24-induced apoptosis revealed the activation of multiple pro-apoptotic events including phosphorylation of PKR and eIF2 α , cleavage of caspase-8 and caspase-3,⁶ activation of p38 MAPK⁷ and ERK,⁸ downregulation of Mcl-1⁹ and the induction of pro-apoptotic Bcl-2 family members¹⁰ (reviewed in 11,12). In addition, IL-24 expressed by adeno-associated viruses was shown to reduce leukemia outgrowth of an MLL/AF4-positive ALL.¹³ There is, however, conflicting data concerning the

apoptosis-inducing properties of IL-24 expressed and delivered by sources other than viral overexpression. Several groups reported that IL-24 secreted from transformed embryonic kidney cells (HEK293) or bacterial GST-IL-24 fusion protein induced apoptosis in a variety of cancer cells.^{14–18} Other groups, however, could not observe growth reduction of cancer cells treated with IL-24.^{19,20} We therefore reasoned that a currently unknown second stimulus might be necessary to promote the apoptotic activity of IL-24 or that addition of IL-24 turns a stimulus that usually does not induce cell death into an apoptosis-inducing one.

Various toll-like receptors (TLRs) have been described to be capable of inducing apoptosis under certain conditions.^{21–23} TLRs belong to the family of pattern recognition receptors and have a major role in host defense against invading pathogens. Their stimulation mainly leads to activation of the innate immune system but can also result in cell death induction.

¹Department of Surgery, Medical University of Vienna, Waehringer Guertel 18-20, Vienna, Austria; ²Centre for Cell Death, Cancer and Inflammation (CCCI), UCL Cancer Institute, University College London, London, UK; ³Department of Oncology, Medical University of Vienna, Waehringer Guertel 18-20, Vienna, Austria; ⁴Comprehensive Cancer Center, Medical University of Vienna, Waehringer Guertel 18-20, Vienna, Austria and ⁵2nd Department of Pathology, Semmelweis University, Üllőiút 93, Budapest, Hungary

*Corresponding author: H Walczak, Centre for Cell Death, Cancer and Inflammation (CCCI), UCL Cancer Institute, University College London, Paul O’Gorman Building, 72 Huntley Street, London WC1E 6BT, UK. Tel: +44 207 679 6471. E-mail: h.walczak@ucl.ac.uk

or M Bergmann, Department of Surgery, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria. Tel: +43 1 40400 6959;

Fax: +43 1 40400 6782. E-mail: michael.bergmann@meduniwien.ac.at

Keywords: cancer; TLR3; IL-24; oncolytic influenza virus

Abbreviations: Ad-IL-24, adenoviral overexpression of IL-24; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X Protein; Bcl-2, B-cell lymphoma protein 2; Bcl-xL, Bcl-2-like protein; cIAP, cellular inhibitor of apoptosis protein; delNS1, virus that lacks the entire nonstructural 1 gene; dsRNA, double-stranded RNA; eIF2 α , eukaryotic translation initiation factor 2 α ; ERK, Extracellular signal-regulated protein kinase; FADD, Fas associated with death domain; FLIP, FLICE-like inhibitory protein; GADD, Growth arrest and DNA damage genes; hi, heat-inactivated; IFN, human interferon; IL-24, interleukin-24; MAPK, mitogen-activated protein kinases; Mcl-1, induced myeloid leukemia cell differentiation protein; Nec-1, Necrostatin-1; PI, propidium iodide; PKR, double-stranded-RNA-activated protein kinase; rh, recombinant human; RIP, receptor-interacting protein; RNAi, RNA interference; siRNA, short-interfering RNA; TLR, Toll-like receptor; DISC, death-inducing signaling complex; TNF, tumor necrosis factor α ; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adapter inducing IFN alpha; wt, wild type

Received 10.8.12; revised 21.1.13; accepted 22.1.13; Edited by C Borner; published online 01.3.13

Especially stimulation of TLR3 has been described to induce apoptosis when protein biosynthesis is concomitantly blocked.²³ Stimulation of TLR3 by its ligand, double-stranded RNA, has recently been shown to result in the formation of a TLR3-associated atypical death complex consisting of its adapter molecule TRIF, as well as RIP1, FADD, pro-caspase-8 and cFLIP.^{23,24} Lack of the short-lived cFLIP in this complex leads to the activation of caspase-8 and, subsequently, caspase-3-mediated apoptotic cell death. Recently, Feoktistova *et al.*²⁵ provided evidence that absence of cellular inhibitor of apoptosis proteins (cIAPs) sensitizes cells to a TLR3-mediated cell death. Whether TLR3 stimulation, in the absence of cIAPs, leads to apoptosis or necroptosis is differentially regulated by the two cFLIP isoforms, cFLIP long and short (cFLIP_L and cFLIP_S), and depends on the presence or absence of RIP3.

In previous studies, we developed the first prototype of an oncolytic influenza A virus²⁶ based on deletions in the viral type I interferon antagonist NS1 (delNS1).^{27,28} We recently generated delNS1-based viral vectors that induce stable expression of secreted versions of foreign proteins, including IL-24 (delNS1/IL-24).²⁹ Analysis of the cytotoxic properties of delNS1 and delNS1/IL-24 on tumor cells revealed that infection by the IL-24-encoding virus was strikingly more active in killing tumor cells than infection by delNS1. The cell death induced by delNS1/IL-24 was exclusively apoptotic, depended on virus-induced stimulation of TLR3 and activation of caspase-8 but did not require productive infection. Our results identify IL-24 as a factor that promotes tumor cell death by enabling TLR3-induced apoptosis.

Results

Characterization of viral growth and transgene expression of delNS1/IL-24. We first compared the replication properties of influenza A wild-type (wt) virus, empty vector delNS1 and delNS1/IL-24.²⁹ Similar to delNS1, delNS1/IL-24 retained the ability to grow on both, a prostate- and a melanoma-derived tumor cell line (DU145 and SK-Mel28, respectively). As expected, growth of the viruses with the deletion in NS1 was reduced as compared with wt virus. DelNS1 also grew in the producer cell line VERO (data not shown). Virally expressed IL-24 was secreted at up to 6 ng/ml into the supernatants of the cell lines infected by delNS1/IL-24 (Table 1).

Table 1 Comparison of viral growth and transgene expression in different cell lines

	pfu/ml	rhIL-24 (pg/ml)
<i>DU145</i>		
delNS1	2.9×10^5	$< 10^0$
delNS1/IL-24	1.3×10^4	$6.3 \times 10^3 \pm 0.3$
wt virus	1.0×10^7	$< 10^0$
<i>SKMel28</i>		
delNS1	5.1×10^4	$< 10^0$
delNS1/IL-24	3.8×10^4	$2.1 \times 10^3 \pm 0.2$
wt virus	5.6×10^5	$< 10^0$

IL-24 leads to enhanced influenza A virus-induced cell death. We next analyzed whether expression of IL-24 by delNS1/IL-24 enhances cell death induction in DU145- (Figure 1a) and SK-Mel28 cells (Supplementary Figure S1a) in comparison to delNS1. In line with previous reports,³⁰ we found that delNS1 induced higher levels of cell death in DU145 cells than wt virus (21.0 versus 12.6%, respectively). Importantly, infection of DU145 cells with delNS1/IL-24 resulted in significantly more cell death (up to 36.4%, $P=0.03$) than infection with delNS1 or wt virus. Interestingly, the combination of recombinant human (rh) IL-24 with either wt virus or delNS1 induced late apoptotic/necrotic cells up to 35.8% ($P=0.001$) and 42.9% ($P=0.01$), respectively. This indicates that neither intracellular synthesis nor autocrine action of IL-24 is required for its cell death-promoting effect. Of note, adding only rhIL-24 to either cell line did not induce cell death. Similar results were obtained for SK-Mel28 cells (Supplementary Figure S1a). We therefore conclude that presence of IL-24 leads to enhanced influenza A virus-induced cell death.

DelNS1/IL-24-induced cell death in tumor cells is mediated by structural components of the virus and not due to virus-mediated cytokine induction. It is known that structural parts of influenza A virus particles, such as viral RNA, or the interaction of the viral entry protein with the cellular receptor can activate TLR3^{31,32} and TLR7³³ (for review see³⁴). To elucidate whether a structural component of the virus is sufficient to induce cell death in the presence of IL-24 or whether viral replication is required for cell death induction, we used heat-inactivated (hi)-delNS1 that retains the capacity to bind to the viral receptor but is incapable of replicating in the cell. Co-stimulation of DU145 (Figure 1a) and SK-Mel28 (Supplementary Figure S1a) cells with rhIL-24 and hi-delNS1 significantly increased the amount of early and late apoptotic/necrotic cells (for DU145 cells up to 41%; $P=0.002$). Thus, presence of the virus particle itself appears to be sufficient to enable induction of tumor cell death in the presence of rhIL-24. As 100 ng/ml was the minimal dose of rhIL-24 capable of promoting cell death in combination with hi-delNS1 (Supplementary Figure S2a), this concentration was used for further experiments.

As virus-induced cytokines like type I IFN, type II IFN or TNF could be responsible for the virus-mediated component of IL-24/hi-delNS1-induced cell death, we next determined whether inhibition of these cytokines interfered with cell death induction by hi-delNS1 and rhIL-24. This was, however, not the case, suggesting a minor role, if any, for these cytokines in IL-24/hi-delNS1-mediated cell death (Supplementary Figure S2b). This is further corroborated by the fact that co-stimulation by type I or II IFNs or TNF, each at 100 ng/ml, together with rhIL-24 did not promote induction of cell death (data not shown).

IL-24/influenza A virus induces apoptosis, not necroptosis. We next determined the type of cell death induced by influenza A virus in combination with IL-24. To differentiate between apoptosis and regulated necrosis, also referred to as necroptosis, we tested how the apoptosis-preventing caspase inhibitor zVAD-fmk (zVAD) and the

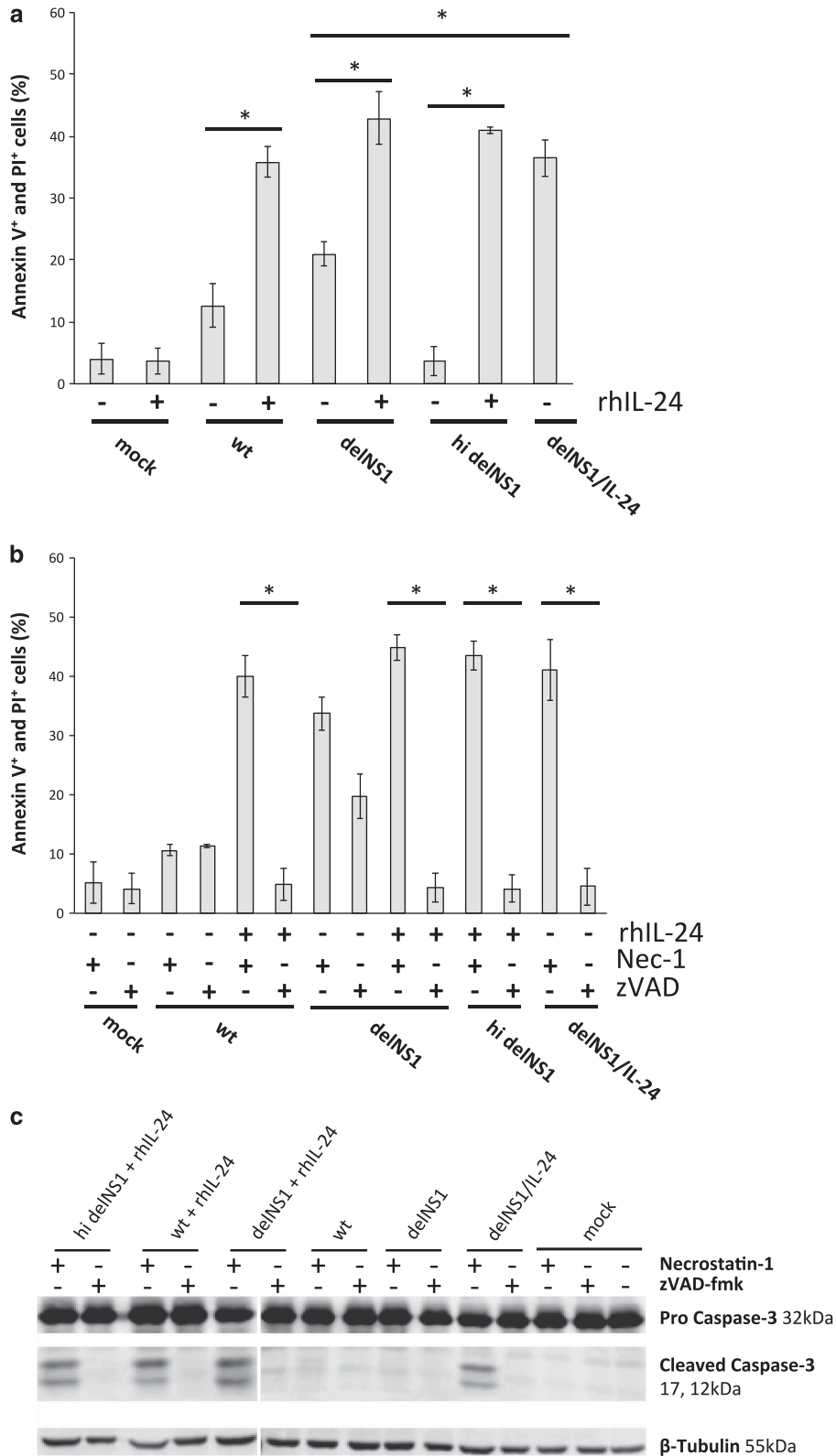


Figure 1 Effect of IL-24 and/or virus stimulation on apoptosis in DU145 cells. Apoptosis was determined by AnnexinV/PI staining of cells 24 h after stimulation with IL-24 and/or viral infection. Experiments done in (a) and (b) were performed in parallel. Mean percentage and S.E.M. of AnnexinV- and PI-positive cells of three independent experiments is shown. * $P < 0.05$ (a) Effect of rhIL24 (100 ng/ml) on cells that were infected with wt virus, delNS1, delNS1/IL-24 or hi-delNS1 (moi = 1) as indicated on the bottom. (b) Effect of zVAD (10 μ M) and Necrostatin-1 (Nec-1) (50 μ M) on virus-stimulated cells in absence versus presence of IL-24 as indicated on the bottom. (c) DU145 cells were infected with wt virus, delNS1, delNS1/IL-24 and hi-delNS1 (moi = 1) alone or in combination with rhIL-24 (100 ng/ml). Experiments were performed in the presence of 50 μ M Nec-1 or 10 μ M zVAD. One representative western blot out of three independent experiments of caspase-3 cleavage is shown

necroptosis blocker necrostatin-1 (Nec-1) affected influenza A virus/IL-24-induced cell death. Nec-1 is an inhibitor of RIP1's kinase activity that is required for the induction of necroptosis.^{35,36} Neither zVAD nor Nec-1 inhibited cell death induction upon infection of DU145 cells with wt or delNS1 virus (Figure 1b and Supplementary Figure S1b). In contrast, when cells were infected with delNS1/IL-24, or with wt virus, delNS1 or hi-delNS1 virus in the presence of rhIL-24, addition of zVAD rescued the cells from death. Nec-1, on the other hand, did not block cell death induction.

To further substantiate our findings on the induction of apoptosis, rather than necroptosis, by the combination of influenza A virus and IL-24, we analyzed the effect of zVAD and Nec-1 on caspase-3 activation in DU145- (Figure 1c) and SK-Mel28 cells (Supplementary Figure S1c). Addition of zVAD blocked the cleavage of caspase-3 after infection with delNS1/IL-24 or the combinatorial stimulation by rhIL-24 and wt virus, delNS1 or hi-delNS1. Corresponding to the AnnexinV/PI data, no decrease in the levels of cleaved caspase-3 was found in any of the cases when Nec-1 was added.

We reasoned that the lack of necroptosis induction might be due to the absence of RIP3. Indeed, neither DU145 nor SK-Mel28 cells express RIP3 (Figure 6a and Supplementary Figure S7a). Thus, the combination of IL-24 and influenza A virus induces apoptosis, not necroptosis in the cellular systems employed in this study.

Molecular changes induced by influenza A virus and IL-24. To better understand the mechanism of apoptosis induction by influenza A virus/IL-24, we next investigated how this treatment affected the pro-apoptotic signaling cascades that had previously been described to be induced by IL-24-expressing chimeric adenovirus, namely phosphorylation of PKR and eIF2 α , cleavage of caspase-8 and caspase-3, activation of p38 MAPK and ERK and downregulation of Mcl-1 (Figure 2 and Supplementary Figure S3). All influenza A virus/IL-24 combinations that induced apoptosis also led to the phosphorylation of PKR and eIF2 α . However, the observation that empty delNS1 virus stimulation alone also induced PKR activation, but was not associated with high levels of apoptosis, suggests that PKR and eIF2 α activation alone were not sufficient to enhance apoptosis. Induction of apoptosis and caspase-3 activation in influenza A virus/IL-24-stimulated cells also correlated with phosphorylation of p38 MAPK. ERK activation was seen in all virus/IL-24 combinations except when rhIL-24 was added to wild-type virus. Furthermore, apoptosis induction and caspase activation were associated with downregulation of Mcl-1 (Figure 2a), indicating that this factor might be important for apoptosis prevention in this cellular setting. Interestingly, rhIL-24 alone neither induced activation or expression of any of the above-mentioned pro-apoptotic molecules nor downregulation of anti-apoptotic Mcl-1. Of note, neither GADD nor total levels of Bak, Bax, Bcl-xL or Bcl-2 (data not shown) were significantly modulated during combinatorial stimulation by influenza A virus and IL-24. We neither observed an expression or induction of JNK or TRAF6 in virus/IL-24-stimulated cells (data not shown).

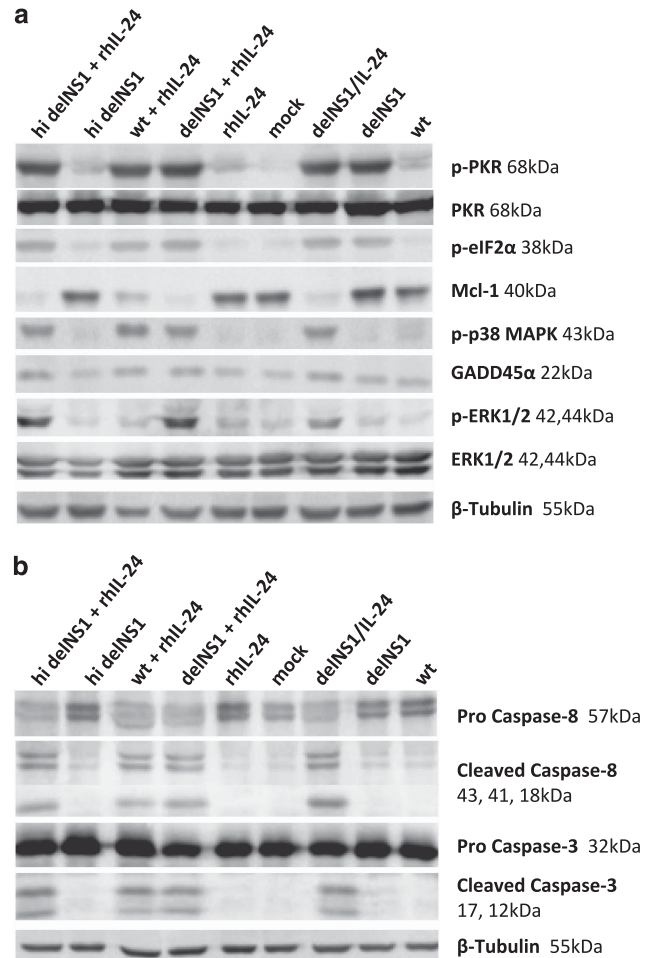


Figure 2 Proapoptotic signaling cascades are induced by stimulation with virus and/or IL-24 virus in DU145 cells. (a) DU145 cells were infected with wt, delNS1, delNS1/IL-24, hi-delNS1 (moi = 1) alone or in combination with rhIL-24 (100 ng/ml) for 24 h as indicated. Protein expression level of p-PKR, PKR, p-eIF2 α , Mcl-1, p-p38 MAPK, GADD45 α , p-ERK1/2 and ERK1/2 was determined by western blot analysis as indicated on the right. One representative western blot out of three experiments is shown. (b) Cells were infected with wt virus, delNS1, delNS1/IL-24, hi-delNS1 (moi = 1) or in combination with rhIL-24 (100 ng/ml) for 24 h as indicated on the top. Protein expression level of caspase-8 and caspase-3 was determined by western blot analysis. One representative western blot out of three independent experiments is shown

IL-24 promotes apoptosis by enabling TLR3-mediated activation of caspase-8. We next aimed to further investigate whether TLR stimulation was indeed responsible for influenza A virus/IL-24-induced apoptosis in DU145- (Figure 3) and SK-Mel28 cells (Supplementary Figure S4). Therefore, we tested agonists for TLR3 (poly(I:C)) and TLR7 (CL-097) in combination with IL-24 as influenza A virus particles have been described to be able to bind to these two TLRs.^{31–33} We also applied LPS because TLR4 had previously been described to be capable of inducing apoptosis.²¹ Single treatment of DU145 cells with any of the three agonists did not increase levels of cell death. In contrast, combined stimulation by rhIL-24 with poly(I:C), but not with LPS or CL-097, significantly increased apoptosis and enhanced caspase-3 cleavage in both cell lines. Hence,

presence of IL-24 enables TLR3-mediated apoptosis induction.

To further investigate the role of IL-24 stimulation in TLR3-mediated apoptosis, we employed two different approaches to inhibit TLR3 signaling. Firstly, we used a pharmacological inhibitor of TLR signaling, resveratrol (3, 4', 5-trihydroxy-trans-stilbene), a phenolic phytoalexin found in grape skin and other plants. Resveratrol suppresses MyD88-independent signaling pathways engaged by TLR3 and TLR4.³⁷ At 50 μ M, resveratrol decreased the amount of early and late apoptotic cells from 40.8% \pm 0.8 to 22% \pm 1.4 in delNS1/IL-24 virus-infected DU145 cells ($P = 0.05$) (Figure 4). A similar decrease was found when resveratrol was added to samples stimulated with rhIL-24 in combination with delNS1, hi-delNS1 or poly(I:C). By contrast, no decrease in apoptosis was observed following infection with delNS1 or hi-delNS1, or by treatment with poly(I:C) alone in the presence of resveratrol. Similar observations were made in SK-Mel28 cells (Supplementary Figure S5).

Secondly, we suppressed the expression of TLR3 using RNA interference (RNAi). Short-interfering RNAs (siRNAs) directed against TLR3 reduced the expression of this receptor by >80% (Figure 5a and Supplementary Figure S6a) and prevented caspase-3 cleavage induced by infection with delNS1/IL-24. In cells stimulated with rhIL-24 in combination with delNS1, hi-delNS1 or poly(I:C) cleavage of caspase-3 was also prevented by TLR3 knockdown (Figure 5b and Supplementary Figure S6b).

To test the contribution of caspase-8 to apoptosis induction by TLR3 and IL-24-co-stimulation, we next suppressed the expression of this caspase by siRNA (Figure 5c and Supplementary Figure S6c). Knockdown of caspase-8 abrogated caspase-3 activation (Figure 5d and Supplementary Figure S6d). This result indicates that caspase-8 activation is not collateral to, but is required for, TLR3/IL-24-mediated apoptosis. Thus, IL-24 enables TLR3 to induce apoptosis in a caspase-8-dependent manner.

IL-24 converts the TLR3-associated signaling complex, activated by influenza A virus-mediated stimulation, into an apoptosis-inducing platform. Influenza A virus is capable of activating TLR3 signaling and TLR3 can mediate apoptosis under certain circumstances.^{23,24} Therefore, we next determined whether treatment of tumor cells with different combinations of virus and IL-24 modulates expression of molecules involved in the formation and signaling of the recently described TLR3-associated signaling complex^{24,25} in a manner that an atypical TLR3-associated death-inducing signaling complex (TLR3 DISC) may form. We first analyzed overall expression of TRIF, FADD, RIP1, RIP3, cFLIP and cIAP1 in cell lysates. Apoptosis induced by infection of DU145 with delNS1/IL-24, or combinatorial treatment of cells with either wt virus, delNS1 or hi-delNS1 and rhIL-24 (as determined in Figure 1a) correlated with activation of caspase-8 and caspase-3 and decreased expression of cIAPs, cFLIP_L and cFLIP_S (Figure 6a). Expression of TRIF, FADD, RIP1 or other molecules known to form part of the TLR3-associated signaling complex was not significantly altered. By performing TLR3 pull-downs and subsequent analysis by western blotting, we next determined

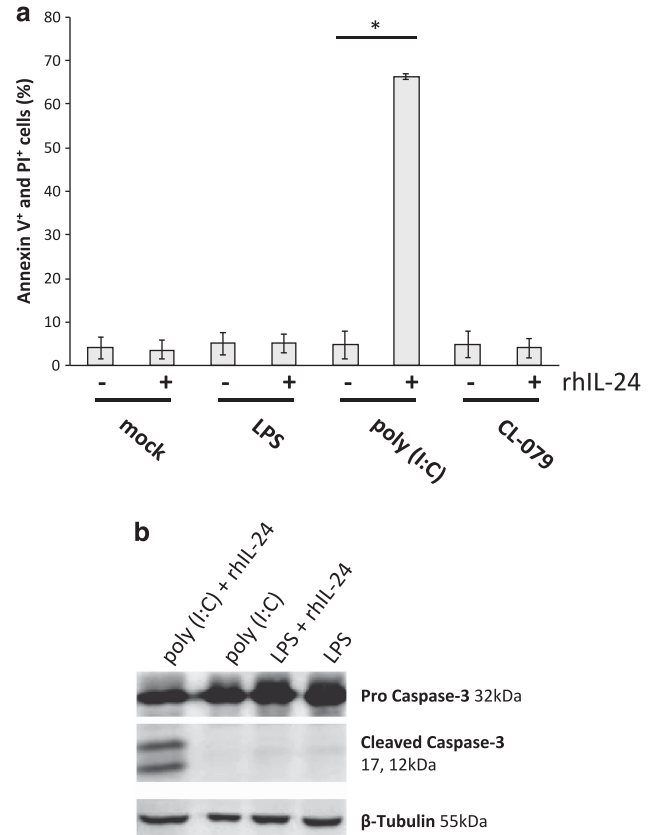


Figure 3 IL-24 specifically sensitizes to TLR3-mediated cell death in DU145 cells. (a) Cells were treated with LPS (100 ng/ml), poly(I:C) (2 μ g/ml) or CL-097 (2.5 μ g/ml) for 24 h alone or in combination with rhIL-24 (100 ng/ml) as indicated. Apoptosis was determined by AnnexinV/PI staining. Mean percentage and S.E.M. of AnnexinV- and PI-positive cells of three independent experiments is shown. * $P < 0.05$ (b) Cells were treated for 24 h with LPS or poly(I:C) alone or in combination with rhIL-24. One representative western blot out of three independent experiments of caspase-3 cleavage is shown

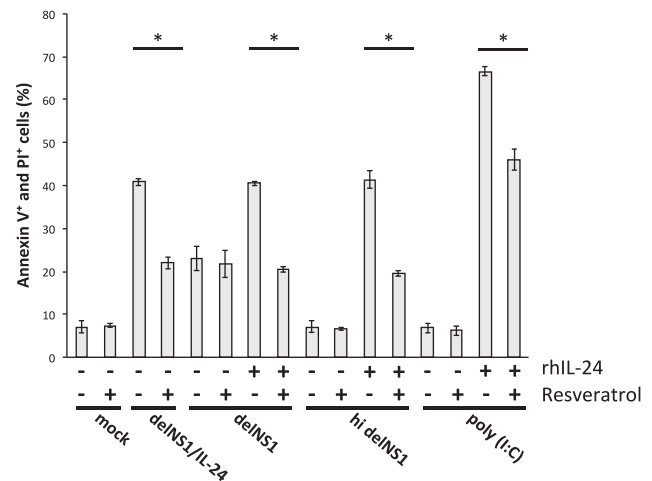


Figure 4 Effect of Resveratrol and IL-24 and/or virus stimulation on DU145 cells. DU145 cells were treated with 50 μ M Resveratrol after infection with delNS1, delNS1/IL-24, hi-delNS1 (moi = 1) and poly(I:C) (2 μ g/ml) alone or in combination with rhIL-24 (100 ng/ml) for 24 h. Apoptosis was determined by AnnexinV/PI staining. Mean percentage and S.E.M. of AnnexinV- and PI-positive cells of three independent experiments is shown. * $P < 0.05$

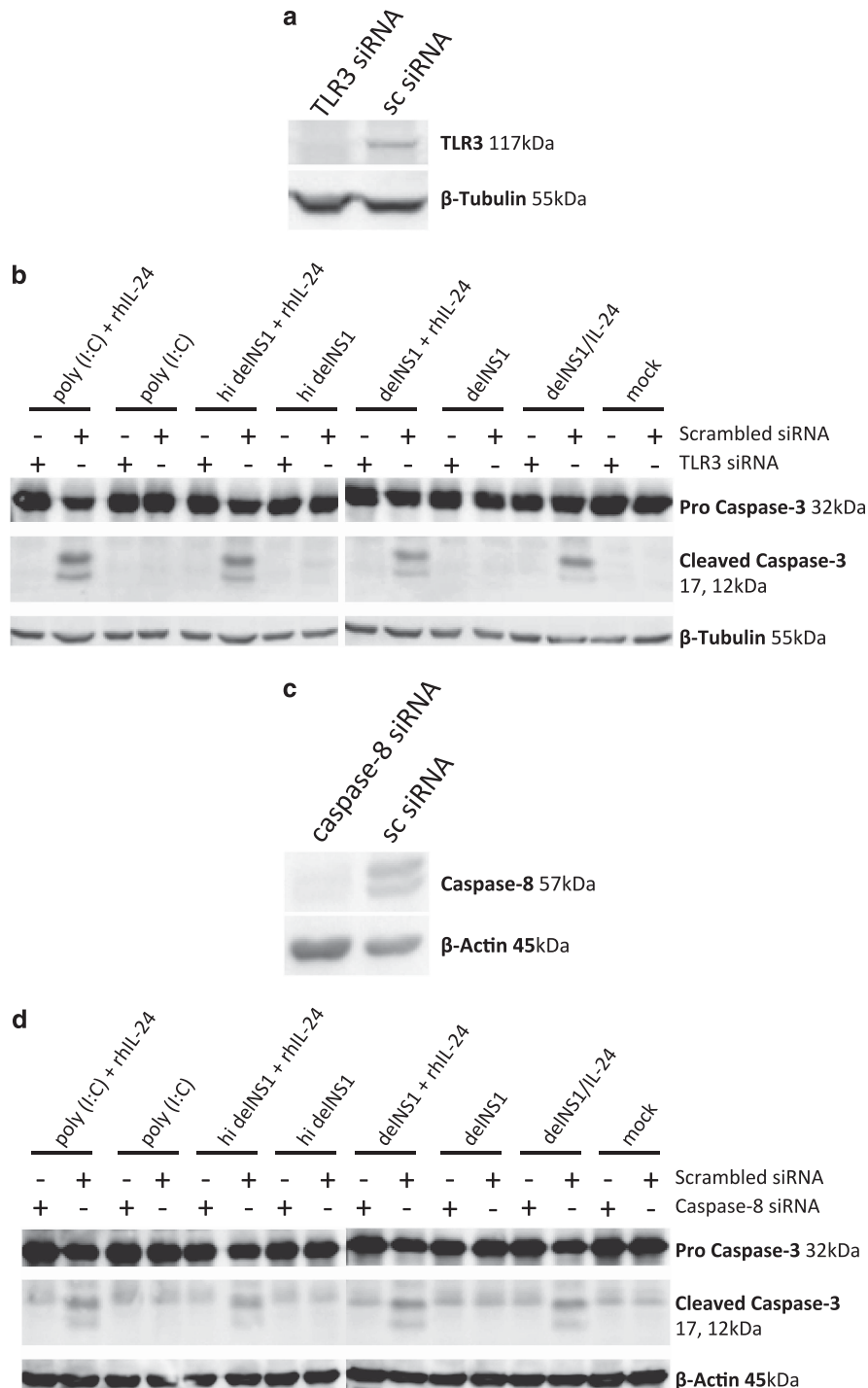


Figure 5 The effect of TLR3 and caspase-8 silencing on DU145 cells after IL-24 and/or virus stimulation. **(a)** Reduction of TLR3 expression by siRNA as determined by western blot analysis. **(b)** Scrambled or TLR3 siRNA-transfected DU145 cells were infected for 24 h with wt virus, delINS1, delINS1/IL-24, hi-delINS1 (moi = 1) or poly(I:C) (2 μ g/ml) alone or in combination with rhIL-24 (100 ng/ml). Cleavage of caspase-3 was determined by western blot analysis. One representative western blot out of three independent experiments is shown. **(c)** Reduction of caspase-8 expression by siRNA as determined by western blot analysis of the caspase-8 protein. **(d)** Scrambled or caspase-8 siRNA transfected DU145 cells were infected for 24 h with wt virus, delINS1, delINS1/IL-24, hi-delINS1 (moi = 1) or poly(I:C) (2 μ g/ml) alone or in combination with rhIL-24 (100 ng/ml). Cleavage of caspase-3 was determined by western blot analysis. One representative western blot out of three independent experiments is shown

the way by which the TLR3-associated signaling complex was formed by stimulation with hi-delINS1 and how presence of IL-24 affected the formation of this complex. In untreated DU145 cells and in cells stimulated with rhIL-24 alone,

TRIF was associated with TLR3 (Figure 6b). By contrast, stimulation of cells by hi-delINS1 resulted in the formation of a complex that contained TLR3, TRIF, RIP1, FADD, cFLIP_L, cFLIP_S and pro-caspase-8. Corresponding to the finding in

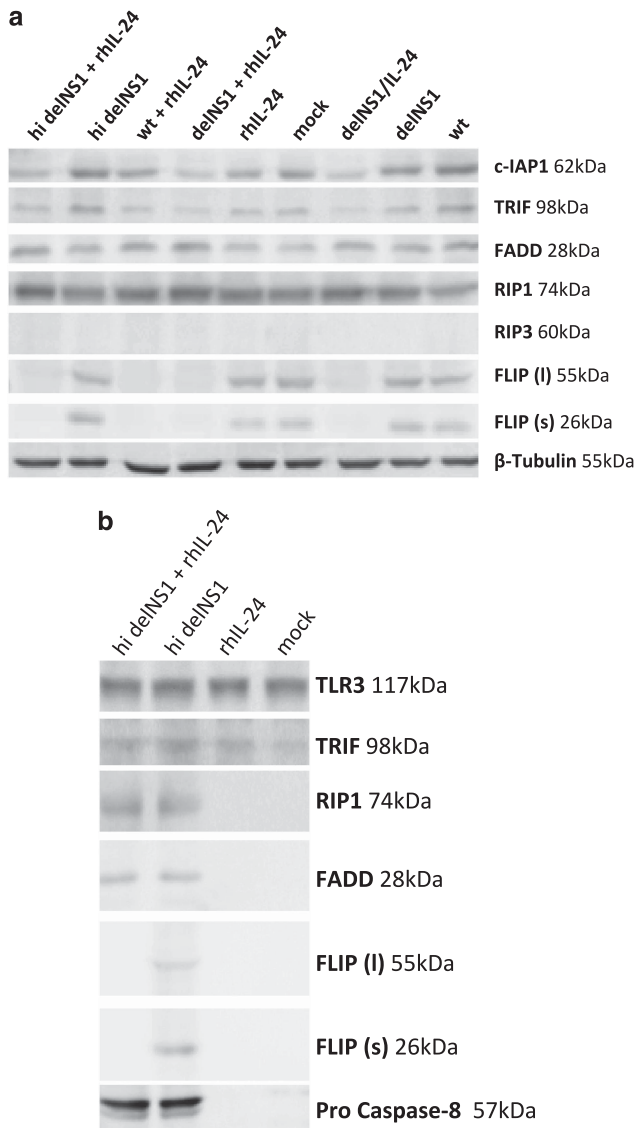


Figure 6 Effect of IL-24 and/or virus stimulation on proteins involved in apoptosis induction and on TLR3 DISC formation in DU145 cells. (a) Cells were infected with wt virus, delNS1, delNS1/IL-24, hi-delNS1 (moi = 1) or in combination with rhIL-24 (100 ng/ml) for 24 h as indicated on the top. Protein expression level of cIAP1, TRIF, FADD, RIP1, RIP3, cFLIP_L and cFLIP_S was determined by western blot analysis. One representative western blot out of three independent experiments is shown. (b) Cells were left untreated or treated with rhIL-24 alone, infected with hi-delNS1 (moi = 1) alone or in combination with rhIL-24 (100 ng/ml) for 24 h. TLR3 was immunoprecipitated and subjected to western blot analysis for the proteins indicated. One representative experiment out of three independent experiments is shown

whole-cell lysates, cFLIP was absent from the complex when cells were additionally co-stimulated with rhIL-24. Of note, the addition of rhIL-24 resulted in pronounced caspase-8 activation as compared with hi-delNS1 alone (data not shown). Similar effects of the combinatorial virus/IL-24 treatment were observed in SK-Mel28 cells with respect to caspase activation and assembly of the TLR3-associated signaling complex (Supplementary Figure S7a and b). Hence, IL-24 alters the composition of the TLR3-associated signaling complex in a manner that converts this signaling

complex into an atypical, that is, non-death-receptor-associated, DISC on TLR3, the TLR3 DISC.

Combination of IL-24 with stimulation of TLR3 abolishes established tumor spheroid cultures. Tumor spheroids can serve as a readily accessible three-dimensional and, as such, more physiological *in-vitro* model of tumor formation and growth. Infection of DU145- and SK-Mel28 spheroid cultures with delNS1/IL-24 or treatment of with rhIL-24 and hi-delNS1 dramatically reduced their growth (Figure 7 and Supplementary Figure S8). By contrast, the empty delNS1 only inhibited further outgrowth of spheroids. Treatment with rhIL-24 alone, however, had no effect. The apparent growth reduction of spheroids by delNS1/IL-24 was almost completely inhibited by the caspase inhibitor zVAD, but not by the necroptosis inhibitor Nec-1. We next tested the effect of poly(I:C) and rhIL-24 in those three-dimensional cell cultures. The combination of LPS and rhIL-24 was used as a control to rule out unspecific immune-mediated effects. Inhibition of growth was only observed for spheroids treated with the combination of poly(I:C) and rhIL-24 (Figure 7c and Supplementary Figure 8c). Hence, in the presence of IL-24, tumor cells in spheroid cultures are as susceptible to apoptosis induction by stimulation of TLR3 as in two-dimensional culture.

Discussion

Although its physiological role remains to be determined, the pro-apoptotic activity of IL-24 on tumor cells has recently gained attention.^{7,38–43} Here, we demonstrate that IL-24 expressed by influenza A virus acts as a sensitizer to influenza A virus-mediated apoptosis, rather than as a cell-death inducer itself. Interestingly, influenza A virus-induced cell death in the presence of IL-24 is mediated by TLR3 and the formation of an atypical TLR3-associated DISC at which caspase-8 is activated, resulted in subsequent activation of caspase-3 and, hence, induction of apoptosis. Therefore, our findings indicate that (i) an appropriate additional stimulus is required for IL-24 to promote apoptosis and (ii) that armed oncolytic influenza A viruses can provide such a second signal by activating TLR3 in a pro-apoptotic manner.

Hence, the observed differences in reactivity of tumor cell lines following stimulation with recombinant IL-24 alone might very well be due to the presence or absence of a second signal and activation of the respective downstream signaling pathway. The fact that adenovirus- or adeno-associated virus-expressed IL-24 always induced cell death suggests that adenovirus inherently provides an appropriate second signal, similar to our observations regarding influenza A virus. As adenoviruses and adeno-associated viruses are known to primarily stimulate TLR2 and TLR9,^{44–46} induction of IL-24-enabled TLR-mediated apoptosis is possibly not restricted to the stimulation of TLR3. It will be interesting to determine which other pattern recognition stimuli, in addition to TLR3, induce apoptosis when combined with IL-24.

Of note, we find the cell death induced by influenza A virus/IL-24 to be exclusively apoptotic although Feoktistova *et al.*²⁵ recently described that TLR3-stimulation can also result in the induction of necroptosis. This is likely a consequence of

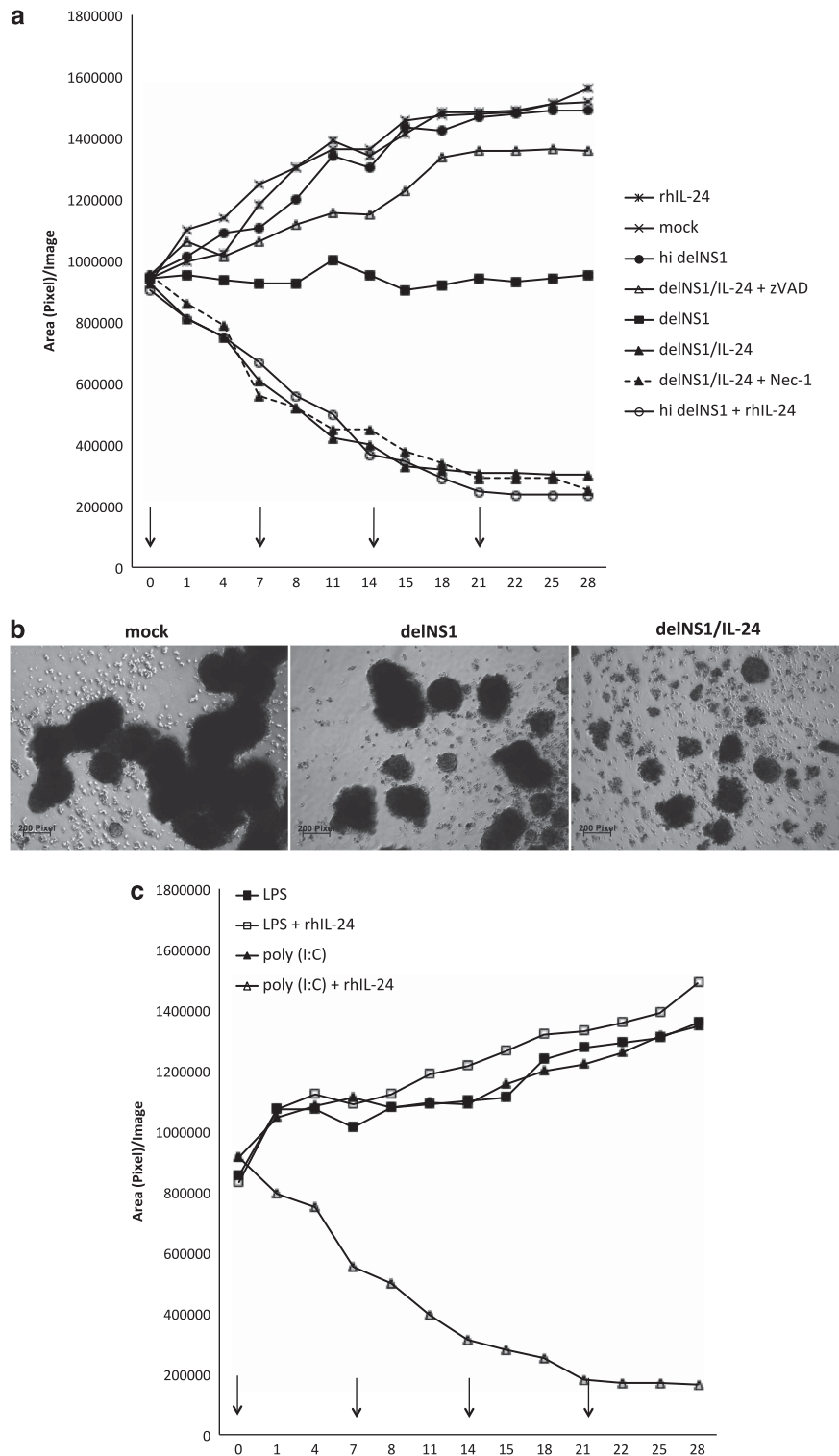


Figure 7 Effect of delNS1/IL-24 virus on spheroid formation of DU145 cells. (a) Spheroids were infected with delNS1, delNS1/IL-24, hi-delNS1 (moi = 1) alone or in combination with rhIL-24 (100 ng/ml). In the samples indicated, zVAD or Nec-1 was added to the cultures. Re-infections of spheroids were performed weekly, as indicated with arrows. Growth of DU145 spheroids was determined by ImageJ 1.440 software (Biocompare, South San Francisco, CA, USA). (b) Representative images of spheroids treated with delNS1 and delNS1/IL-24 (moi = 1) four days after second infection are shown (scale bar = 200 pixel). (c) Spheroids were treated with LPS (100 ng/ml) or poly(I:C) (2 μ g/ml) alone or in combination with rhIL-24

absence of RIP3 from the cell lines used in this study. Hence, although the death induced by influenza A virus/IL-24 in this study is apoptotic, it cannot be excluded that in

other cellular settings in which the necroptosis pathway is operative, this combination may also be capable of inducing necroptosis.

Previously, it has been shown that adenovirus expressing IL-24 induces multiple pro-apoptotic events including phosphorylation of PKR and eIF2 α , cleavage of caspase-8 and -3, activation of p38 MAPK and ERK, downregulation of Mcl-1 and the induction of pro-apoptotic Bcl-2 family members.^{6–10} Although IL-24-expressing influenza A virus induced similar changes except for the induction of pro-apoptotic Bcl-2 family members, none of the observed changes could be attributed to the IL-24-mediated sensitization to TLR3-induced apoptosis. Yet, in addition to the previously reported effects, we also observed downregulation of cIAP1 and both isoforms of cFLIP by the combination of influenza A virus and IL-24. That cFLIP and cIAP1 have a major role in regulation of cell death induced by TLRs in general and TLR3 in particular is evidenced by a number of publications: (i) cIAPs sensitizes cells to TLR3-induced cell death via formation of a molecular platform whose signaling output in respect to apoptosis and necroptosis is differentially regulated by cFLIP isoforms;²⁵ (ii) Estornes *et al.*²⁴ recently showed that cFLIP forms part of an atypical death complex associating TLR3 to caspase-8; (iii) cFLIP_L inhibits TLR3-induced apoptosis in dendritic cells;⁴⁷ (iv) cFLIP has been shown to be relevant for apoptosis mediated by the activation of TLR3 and TLR4 when protein synthesis is blocked by cycloheximide (CHX).^{23,48}

In conclusion, we show that IL-24 acts as a sensitizer to TLR3-mediated cell death, rather than a cell-death inducer itself. The synergistic capacity of IL-24 and TLR3 agonists to induce tumor cell death might lead to novel therapeutic options in cancer. Specifically, oncolytic RNA viruses appear suitable for the expression of IL-24 in this context. Our findings could also hint at the physiological function of IL-24, as this cytokine may contribute to the elimination of infected or otherwise activated, including transformed cells by sensitizing to apoptosis during the course of an inflammation.⁴⁹

Materials and methods

Cell lines, influenza viruses and reagents. The human prostate cancer cell line DU145 was purchased from ATCC (ACC 261). Cells were maintained in a MEM medium (PAA, Pasching, Austria) supplemented with 10% heat-inactivated FCS. The human melanoma cell line SK-Mel28 was purchased from ATCC (HTB-72) and was grown in DMEM/F12 medium (PAA) supplemented with 10% heat-inactivated FCS. VERO cells (EC ACC, 88020401), from kidney epithelial cells extracted from an African green monkey, adapted to grow on serum-free medium were maintained in serum-free Optipro medium (Invitrogen, Carlsbad, CA, USA). The cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C.

IVR-116 wt, delNS1⁵⁰ and delNS1/IL-24²⁹ influenza viruses have been described previously. Heat inactivation of delNS1 virus was performed at 56 °C for 1 h. For propagation of the viruses VERO cells were infected at a multiplicity of infection (m.o.i.) of 0.1 and incubated in Optipro medium containing 5 μ g/ml trypsin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 2 days. Virus concentrations were determined by plaque assay on VERO cells.

For stimulation experiments, cells were treated for 24 h with recombinant human IL-24 (100 ng/ml) (R&D Systems, Minneapolis, MN, USA), Z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD fmk) (Bachem, Torrance, CA, USA), Necrostatin-1 (Nec-1) (Sigma-Aldrich) and Resveratrol (Sigma-Aldrich). The LPS used was ion-exchange chromatography-purified *Escherichia coli* LPS 055:B5 from Sigma-Aldrich (St. Louis), CL-097 and poly(I:C) from InvivoGen (San Diego, CA, USA). For stimulation experiments, tumor necrosis factor α (TNF α) (R&D Systems, Minneapolis, MN, USA), human interferon α (IFN α) (Health Protection Agency, London, UK) and human interferon gamma (IFN γ) (Imukin, BoehringerIngelheim, Vienna, Austria) were used in concentrations of 50 and 100 ng/ml supernatant, respectively. For inhibition experiments, TNF activity was blocked by adding human TNFR1I/TNFRSF1B/Fc chimera (20 μ g/ml) (R&D Systems). For blocking of IFN α /

function, a combination of polyclonal rabbit anti-IFN α (5000 neutralizing U/ml) and rabbit anti-IFN β (2000 neutralizing U/ml) antibodies together with 20 μ g/ml of a mouse anti-human IFN α / β receptor chain 2 mAb were used (all from PBL, New Brunswick, NJ, USA). For blocking of IFN γ , mouse anti-human IFN γ Ab (20 μ g/ml) (R&D) was used.

Cytokine detection after infection with influenza viruses. Cells were infected with viruses and cultured for 24 h in medium containing 10% FCS. The supernatant was screened for human IL-24 by hIL-24 DuoSet ELISA (R&D Systems) according to the manufacturer's instructions.

Cell death analysis using Annexin V/propidium iodide double staining assay. Treated cells were collected after 24 h, washed with phosphate buffered saline (PBS) and stained with 5 μ l fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI for 15 min at room temperature in the dark (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen, Franklin Lakes, NJ, USA). The stained cells were then analyzed by a flow cytometer (Gallios Cytometer 1.1, Beckman Coulter, Brea, CA, USA).

Western blot. Cells of interest were collected in RIPA sample buffer (Thermo Fisher Scientific, Rockford, IL, USA) and stored at –80 °C. Samples were separated on a 12.5% denaturing acrylamide gel and transferred onto 0,45 μ m Protran nitrocellulose membrane (Whatman, Maidstone, Kent, UK). The membrane was blocked and incubated with rat monoclonal anti-cIAP1 (1:1000) (Enzo Life Sciences, Farmingdale, NY, USA), mouse monoclonal anti-FLIP (NF6) (1:500) (Enzo Life Sciences), mouse monoclonal anti-Caspase-8 (1C12) (1:1000) (Cell Signaling, Danvers, MA, USA), mouse monoclonal anti-Caspase-3 (Enzo Life Sciences), mouse monoclonal anti-TLR3 (TLR3.7) (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-FADD (1:1000) (Cell Signaling), rabbit monoclonal anti-GADD45 α (D17E8) (1:1000) (Cell Signaling), rabbit polyclonal anti-MAPK (ERK1/2) (1:1000) (Cell Signaling), rabbit polyclonal anti-phospho MAPK (ERK1/2) (1:1000) (Cell Signaling), rabbit monoclonal anti-Mcl-1 (D35A5) (1:1000) (Cell Signaling), rabbit polyclonal anti-phospho-eIF2 α (Ser51) (1:1000) (Cell Signaling), rabbit polyclonal anti-p38 MAPK (1:1000) (Cell Signaling), rabbit polyclonal anti-TRIF (1:1000) (Cell Signaling), mouse monoclonal anti-RIP1 (C-12) (1:100) (Santa Cruz Biotechnology), donkey polyclonal anti-RIP3 (1:200) (Santa Cruz Biotechnology), rabbit polyclonal anti-PKR (K17) (1:200) (Santa Cruz Biotechnology), rabbit monoclonal anti-phospho PKR (T446) (E120) (1:1000) (Abcam, Cambridge, UK), rabbit monoclonal anti- β -actin (13E5) (1:1000) (Cell Signaling), mouse monoclonal anti-actin (1:2000) (Merck Millipore, Billerica, MA, USA) or rabbit monoclonal anti- β -Tubulin (9F3) antibody (1:1000) (Cell Signaling) in TBS-Tween. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:1000) (Thermo Fisher Scientific), HRP-conjugated anti-rabbit IgG (1:1000) (Thermo Fisher) or HRP-conjugated anti-rat IgG (1:5000) (Enzo Life Sciences) were used as secondary antibodies. Positive bands were detected by chemiluminescence using a Super Signal West FemtoChemiluminescent Substrate (Thermo Fisher Scientific). Western Blot images were taken with a G:BOX (Syngene, Cambridge, UK) which is handled by the GeneSys software.

Co-immunoprecipitation of TLR3-bound complexes. For co-immunoprecipitation experiments, 1×10^7 cells were infected and stimulated for 24 h, collected, washed in PBS and lysed in 1 ml lysis buffer (60 mM Tris/HCl pH6.8, 10% glycerol, 1%SDS, 5% β -mercaptoethanol). Lysates were immunoprecipitated overnight at 4 °C with 2 μ g of mouse anti-TLR3 antibodies (Santa Cruz) in the presence of 20 μ l protein A/G Plus-AgaroseImmunoprecipitation Reagent (Santa Cruz). Agarose beads were recovered by centrifugation, washed extensively with cold lysis buffer and eluted with SDS loading buffer at 37 °C for 30 min.

Small interfering RNA (siRNA) experiments. Cells were plated in six-well plates at 4×10^4 cells. After overnight adherence, 100 μ l serum- and antibiotic-free DMEM/F12 medium were mixed with 3 μ g/ml linear polyethylenimine (Polysciences Europe GmbH, Eppelheim, Germany) and 100 nM siRNA for 10 min at room temperature. Medium on the cells was changed to 900 μ l fresh DMEM/F12 medium (supplemented with FCS) and the previously mixed RNA solution was added to each well. Cells were incubated overnight and cultured for 72 h in complete medium before treatment with viruses. TLR3 siRNA and caspase-8 siRNA were purchased from Santa Cruz Biotechnology; scrambled siRNA was from Qiagen (Germantown, MD, USA).

Tumor spheroid cultures. Tumor spheres were prepared as previously described.⁵¹ In brief, cells were detached with EDTA (Life Technologies, Carlsbad, CA, USA), washed with PBS and placed in serum-free medium mixed with 20 ng/ml epidermal growth factor (EGF PHG0311L, Life Technologies), 20 ng/ml basic fibroblast growth factor (bFGF PHG0021, Life Technologies) and 0.4% bovine serum albumin (BSA, Sigma-Aldrich) at a density of 1×10^3 cells/ml and cultured in ultra-low attachment plates (CLS3473 Costar, Sigma-Aldrich). The sphere culture medium was changed every 48 h until the majority of spheres reached 300–400 μm in diameter.

Microscopy. Spheroid cultures were photographed on a Zeiss Axiovert 40 CFL microscope (Zeiss, Jena, Germany). For each condition, four different fields were captured and representative images were selected. All images were digitally processed for presentation with Adobe Photoshop CS4 (San Jose, CA, USA).

Statistical analysis. All data are represented by the mean and S.E.M. of at least three independent experiments. Statistical analysis was performed using the statistical software SPSS Statistics 17.0.0 (SPSS Software, Armonk, NY, USA) and with GraphPad Prism Version 5 (GraphPad Software, La Jolla, CA, USA). A probability of $P < 0.05$ was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

MS, MB, HW and BH conceived and designed the experiments. RW, MS and JZ performed the experiments and data analysis. MK and BH contributed to data analysis, discussions, and intellectual input. RW, MS, JZ, HW and MB wrote the paper with all authors providing detailed comments and suggestions. MB directed the project.

- Jiang H, Lin JJ, Su ZZ, Goldstein NI, Fisher PB. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene* 1995; **11**: 2477–2486.
- Jiang H, Su ZZ, Lin JJ, Goldstein NI, Young CS, Fisher PB. The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. *Proc Natl Acad Sci USA*. 1996; **93**: 9160–9165.
- Eager R, Harle L, Nemunaitis J. Ad-MDA-7; INGN 241: a review of preclinical and clinical experience. *Expert Opin Biol Ther* 2008; **8**: 1633–1643.
- Cunningham CC, Chada S, Merritt JA, Tong A, Senzer N, Zhang Y *et al*. Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. *Mol Ther* 2005; **11**: 149–159.
- Jiang G, Zhang L, Xin Y, Pei DS, Wei ZP, Liu YQ *et al*. Conditionally replicating adenoviruses carrying mda-7/IL-24 for cancer therapy. *Acta Oncol* 2012; **51**: 285–292.
- Pataer A, Vorbuerger SA, Barber GN, Chada S, Mhashilkar AM, Zou-Yang H *et al*. Adenoviral transfer of the melanoma differentiation-associated gene 7 (mda7) induces apoptosis of lung cancer cells via up-regulation of the double-stranded RNA-dependent protein kinase (PKR). *Cancer Res* 2002; **62**: 2239–2243.
- Sarkar D, Su ZZ, Lebedeva IV, Sauane M, Gopalkrishnan RV, Valerie K *et al*. mda-7 (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc Natl Acad Sci USA*. 2002; **99**: 10054–10059.
- Yacoub A, Mitchell C, Lebedeva IV, Sarkar D, Su ZZ, McKinstry R *et al*. mda-7 (IL-24) inhibits growth and enhances radiosensitivity of glioma cells *in vitro* via JNK signaling. *Cancer Biol Ther* 2003; **2**: 347–353.
- Dash R, Richards JE, Su ZZ, Bhutia SK, Azab B, Rahmani M *et al*. Mechanism by which Mcl-1 regulates cancer-specific apoptosis triggered by mda-7/IL-24, an IL-10-related cytokine. *Cancer Res* 2010; **70**: 5034–5045.
- Lebedeva IV, Sarkar D, Su ZZ, Kitada S, Dent P, Stein CA *et al*. Bcl-2 and Bcl-x(L) differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, mda-7/IL-24. *Oncogene* 2003; **22**: 8758–8773.
- Fisher PB, Sarkar D, Lebedeva IV, Emdad L, Gupta P, Sauane M *et al*. Melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24): novel gene therapeutic for metastatic melanoma. *Toxicol Appl Pharmacol* 2007; **224**: 300–307.
- Dash R, Bhutia SK, Azab B, Su ZZ, Quinn BA, Kegelman TP *et al*. mda-7/IL-24: a unique member of the IL-10 gene family promoting cancer-targeted toxicity. *Cytokine Growth Factor Rev* 2010; **21**: 381–391.
- Tamai H, Miyake K, Yamaguchi H, Takatori M, Dan K, Inokuchi K *et al*. AAV8 vector expressing IL24 efficiently suppresses tumor growth mediated by specific mechanisms in MLL/AF4-positive ALL model mice. *Blood* 2012; **119**: 64–71.
- Chada S, Bocangel D, Ramesh R, Grimm EA, Mumm JB, Mhashilkar AM *et al*. mda-7/IL24 kills pancreatic cancer cells by inhibition of the Wnt/PI3K signaling pathways: identification of IL-20 receptor-mediated bystander activity against pancreatic cancer. *Mol Ther* 2005; **11**: 724–733.
- Chada S, Mhashilkar AM, Ramesh R, Mumm JB, Sutton RB, Bocangel D *et al*. Bystander activity of Ad-mda7: human MDA-7 protein kills melanoma cells via an IL-20 receptor-dependent but STAT3-independent mechanism. *Mol Ther* 2004; **10**: 1085–1095.
- Zheng M, Bocangel D, Doneske B, Mhashilkar A, Ramesh R, Hunt KK *et al*. Human interleukin 24 (MDA-7/IL-24) protein kills breast cancer cells via the IL-20 receptor and is antagonized by IL-10. *Cancer Immunol Immunother* 2007; **56**: 205–215.
- Sauane M, Gopalkrishnan RV, Choo HT, Gupta P, Lebedeva IV, Yacoub A *et al*. Mechanistic aspects of mda-7/IL-24 cancer cell selectivity analysed via a bacterial fusion protein. *Oncogene* 2004; **23**: 7679–7690.
- Sauane M, Lebedeva IV, Su ZZ, Choo HT, Randolph A, Valerie K *et al*. Melanoma differentiation associated gene-7/interleukin-24 promotes tumor cell-specific apoptosis through both secretory and nonsecretory pathways. *Cancer Res* 2004; **64**: 2988–2993.
- Caudell EG, Mumm JB, Poindexter N, Ekmekcioglu S, Mhashilkar AM, Yang XH *et al*. The protein product of the tumor suppressor gene, melanoma differentiation-associated gene 7, exhibits immunostimulatory activity and is designated IL-24. *J Immunol* 2002; **168**: 6041–6046.
- Kreis S, Philippidou D, Margue C, Rolvinger C, Haan C, Dumoutier L *et al*. Recombinant interleukin-24 lacks apoptosis-inducing properties in melanoma cells. *PLoS One* 2007; **2**: e1300.
- Bannerman DD, Eiting KT, Winn RK, Harlan JM. FLICE-like inhibitory protein (FLIP) protects against apoptosis and suppresses NF-kappaB activation induced by bacterial lipopolysaccharide. *Am J Pathol* 2004; **165**: 1423–1431.
- Salaun B, Romero P, Lebecque S. Toll-like receptors' two-edged sword: when immunity meets apoptosis. *Eur J Immunol* 2007; **37**: 3311–3318.
- Weber A, Kircijczyk Z, Besch R, Potthoff S, Leverkus M, Hacker G. Proapoptotic signalling through Toll-like receptor-3 involves TRIF-dependent activation of caspase-8 and is under the control of inhibitor of apoptosis proteins in melanoma cells. *Cell Death Differ* 2010; **17**: 942–951.
- Estornes Y, Toscano F, Virard F, Jacquemin G, Pierrot A, Vanberlivet B *et al*. dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. *Cell Death Differ* 2012; **19**: 1482–1494.
- Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M *et al*. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell* 2011; **43**: 449–463.
- Bergmann M, Romirer I, Sachet M, Fleischhacker R, Garcia-Sastre A, Palese P *et al*. A genetically engineered influenza A virus with ras-dependent oncolytic properties. *Cancer Res* 2001; **61**: 8188–8193.
- Egorov A, Brandt S, Sereinig S, Romanova J, Ferko B, Katinger D *et al*. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. *J Virol* 1998; **72**: 6437–6441.
- Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE *et al*. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 1998; **252**: 324–330.
- Wolschek M, Sarm E, Seper H, Sturlan S, Kuznetsova I, Schwager C *et al*. Establishment of a chimeric, replication-deficient influenza A virus vector by modulation of splicing efficiency. *J Virol* 2011; **85**: 2469–2473.
- Zhirnov OP, Konakova TE, Wolff T, Klenk HD. NS1 protein of influenza A virus down-regulates apoptosis. *J Virol* 2002; **76**: 1617–1625.
- Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M *et al*. Cutting edge: influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *J Immunol* 2007; **178**: 3368–3372.
- Guillot L, Le Goffic R, Bloch S, Escriviou N, Akira S, Chignard M *et al*. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 2005; **280**: 5571–5580.
- Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004; **303**: 1529–1531.
- Ramos I, Fernandez-Sesma A. Cell receptors for influenza A viruses and the innate immune response. *Front Microbiol* 2012; **3**: 117.
- Vandenabeele P, Vanden Berghe T, Festjens N. Caspase inhibitors promote alternative cell death pathways. *Sci STKE* 2006; **2006**: pe44.
- Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol* 2010; **22**: 263–268.
- Youn HS, Lee JY, Fitzgerald KA, Young HA, Akira S, Hwang DH. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. *J Immunol* 2005; **175**: 3339–3346.
- Lebedeva IV, Su ZZ, Chang Y, Kitada S, Reed JC, Fisher PB. The cancer growth suppressing gene mda-7 induces apoptosis selectively in human melanoma cells. *Oncogene* 2002; **21**: 708–718.
- Sarkar D, Su ZZ, Park ES, Vozhilla N, Dent P, Curiel DT *et al*. A cancer terminator virus eradicates both primary and distant human melanomas. *Cancer Gene Ther* 2008; **15**: 293–302.
- Su ZZ, Lebedeva IV, Sarkar D, Emdad L, Gupta P, Kitada S *et al*. Ionizing radiation enhances therapeutic activity of mda-7/IL-24: overcoming radiation- and mda-7/IL-24-resistance in prostate cancer cells overexpressing the antiapoptotic proteins bcl-xL or bcl-2. *Oncogene* 2006; **25**: 2339–2348.

41. Sauane M, Su ZZ, Gupta P, Lebedeva IV, Dent P, Sarkar D *et al*. Autocrine regulation of mda-7/IL-24 mediates cancer-specific apoptosis. *Proc Natl Acad Sci USA*. 2008; **105**: 9763–9768.
42. Sauane M, Su ZZ, Dash R, Liu X, Norris JS, Sarkar D *et al*. Ceramide plays a prominent role in MDA-7/IL-24-induced cancer-specific apoptosis. *J Cell Physiol* 2010; **222**: 546–555.
43. Sieger KA, Mhashikar AM, Stewart A, Sutton RB, Strube RW, Chen SY *et al*. The tumor suppressor activity of MDA-7/IL-24 is mediated by intracellular protein expression in NSCLC cells. *Mol Ther* 2004; **9**: 355–367.
44. Appledorn DM, Patial S, McBride A, Godbehere S, Van Rooijen N, Parameswaran N *et al*. Adenovirus vector-induced innate inflammatory mediators, MAPK signaling, as well as adaptive immune responses are dependent upon both TLR2 and TLR9 *in vivo*. *J Immunol* 2008; **181**: 2134–2144.
45. Laredj LN, Beard P. Adeno-associated virus activates an innate immune response in normal human cells but not in osteosarcoma cells. *J Virol* 2011; **85**: 13133–13143.
46. Hosel M, Broxtermann M, Janicki H, Esser K, Arzberger S, Hartmann P *et al*. Toll-like receptor 2-mediated innate immune response in human nonparenchymal liver cells toward adeno-associated viral vectors. *Hepatology* 2012; **55**: 287–297.
47. Lehner M, Kellert B, Proff J, Schmid MA, Diessenbacher P, Ensser A *et al*. Autocrine TNF is critical for the survival of human dendritic cells by regulating BAK, BCL-2, and FLIPL. *J Immunol* 2012; **188**: 4810–4818.
48. Salaun B, Coste I, Risoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. *J Immunol* 2006; **176**: 4894–4901.
49. Walczak H. TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. *Immunol Rev* 2011; **244**: 9–28.
50. Wacheck V, Egorov A, Groiss F, Pfeiffer A, Fuehrer T, Hoefmayer D *et al*. A novel type of influenza vaccine: safety and immunogenicity of replication-deficient influenza virus created by deletion of the interferon antagonist NS1. *J Infect Dis* 2010; **201**: 354–362.
51. Lopez J, Poitevin A, Mendoza-Martinez V, Perez-Plasencia C, Garcia-Carranca A. Cancer-initiating cells derived from established cervical cell lines exhibit stem-cell markers and increased radioresistance. *BMC Cancer* 2012; **12**: 48.

Supplementary Information accompanies this paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)