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Interleukin-24 inhibits influenza A virus replication *in vitro* through induction of toll-like receptor 3 dependent apoptosis

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ABSTRACT

New anti-viral agents and strategies are urgently needed to fight rapidly mutating viruses, as vaccine programs cannot react fast enough to prevent pandemics. Recently, we have shown that interleukin-24 (IL-24) sensitizes tumor cells to toll-like receptor 3 (TLR3) mediated apoptosis. As influenza A virus stimulates the TLR3 receptor, we hypothesized that IL-24 might also exert an anti-viral effect. This study demonstrates that IL-24 reduces the titer of different influenza A virus subtypes independently of type I interferon in an apoptosis dependent manner. The anti-viral effect of IL-24 correlated with caspase-3 activation and could be blocked by a pan-caspase inhibitor and by small interfering RNA (siRNA) directed towards TLR3. Surprisingly, caspase-3 activation in influenza A virus/IL-24-stimulated cells correlated with the down-regulation of the B-cell lymphoma 2 (Bcl-2) family member myeloid cell leukemia 1 (Mcl-1). Correspondingly, knockdown of Mcl-1 by siRNA enhanced caspase activation in influenza A virus infected cells and was furthermore linked to a reduction of viral titers. We conclude that IL-24 exerts an anti-viral role selectively purging virally infected cells by leading to a down-regulation of Mcl-1. Our findings might therefore represent the first step towards a new rational concept in the development of anti-viral strategies based on the induction of apoptosis.

1. Introduction

Despite worldwide vaccine programs, influenza A virus infections still remain a threat to human society (Bautista et al., 2010; WHO, 2009). This is partly caused by the constant emergence of novel viral variants and the consequential need to annually adapt vaccines (Dawood et al., 2009). Vaccine productions can especially go astray when major antigenic shifts occur or novel virus subtypes appear, which might further cause pandemics (Rambaut et al., 2008; Trifonov et al., 2009). The development of new anti-viral strategies are crucial, as such disease outbreaks often result in large numbers of potentially preventable deaths. (Dawood et al., 2012). Anti-viral agents developed against viral components such as oseltamivir or amantadine (Uyeki, 2009; WHO, 2010) have the disadvantage that viruses are able to mutate target structures and subsequently become resistant (Samson et al., 2013).

Taking this fact into account when developing new anti-viral drugs, it seems to be more efficient to base new drug progress on an understanding of anti-viral mechanisms naturally executed by the host itself, as this will allow mimicking or enhancing those pathways. Apoptosis of virally infected cells could be a promising strategy to limit viral infection, as it is part of the naturally acquired anti-viral mechanism associated with type I interferon (IFN) signaling. The main anti-viral effect of type I IFN in infected cells has been attributed to the activation of protein kinase R (PKR) leading to caspase-8 dependent cell death (Balachandran et al., 2000). Correspondingly, deleting the viral non-structural protein 1 (NS1) (Egorov et al., 1998), which antagonizes PKR (Bergmann et al., 2000) and type I IFN response (Garcia-Sastre et al., 1998), leads to a replication-defective phenotype associated with highly enhanced apoptosis (Stasakova et al., 2005). The fact that apoptosis is antagonized by the virus through NS1 presumes that its induction is a relevant anti-viral mechanism. On the other hand, Wurzer et al. also described pro-viral effects of apoptotic cascades (Wurzer et al., 2003). According to them, inhibition of caspase-3 leads to the retention of viral ribonucleoproteins (RNPs) in the nucleus, which slightly reduces viral titers. Moreover, overexpression of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein (Olsen et al., 1996) or deletion of the pro-apoptotic Bcl-2-associated X protein (Bax) also leads to the reduction of viral titers (McLean et al., 2009).

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There is some evidence that the oral administration of IFN prevents infection with influenza virus (Bennett et al., 2013). The current concept suggests that massive apoptosis induced by IFN at an early stage of the influenza A virus life cycle could have an anti-viral effect. In contrast, induction of pro-apoptotic cascades at later stages of the viral life cycle facilitates viral growth by releasing the viral RNPs from the nucleus (Zhirnov and Klenk, 2007). Furthermore, apoptosis of neighboring cells induced by soluble immune-mediators such as tumor necrosis factor (TNF) related apoptosis inducing ligand (TRAIL) is capable of potentiating and contributing to virus induced lung lesions (Hogner et al., 2013). This is possibly the reason for why apoptosis has not yet been explored as a therapeutic anti-viral concept for influenza A virus treatment. Furthermore, systemic application of IFN seems to be rather inappropriate due to side effects.

Interestingly, interleukin-24 (IL-24) acts as a pro-apoptotic agent. It is a member of the interleukin-10 (IL-10) superfamily (Commins et al., 2008) and signals through the two heterodimeric receptors IL-22R1/IL-20R2 and IL-20R1/IL-20R2 (Wang et al., 2002). Strikingly, this cytokine was initially discovered in melanoma cells stimulated with IFN-β and mezerein (Huang et al., 2001). The cytokine is expressed by the hematopoietic system and in the skin. Basal levels of around 5 ng/ml of IL-24 are measurable in peripheral blood of healthy individuals (Ma et al., 2011).

IL-24 first attracted attention in the field of oncology, as it enhanced the induction of apoptosis and had a tumor ablative effect when expressed by a viral vector (Dash et al., 2010a). This correlated with the activation or induction of a number of pro-apoptotic molecules such as PKR (Pataer et al., 2005), p38 mitogen-activated protein kinases (MAPK) (Sarkar et al., 2002), extracellular-signal-regulated kinase (ERK) (Yacoub et al., 2003b), Bcl-2 homologous antagonist killer (Bak), Bax (Lebedeva et al., 2002) and down-regulation of myeloid cell leukemia 1 (Mcl-1) (Dash et al., 2010b). Interestingly, the recombinant human (rh) IL-24 alone does not stimulate apoptosis (Kreis et al., 2007). We demonstrated that IL-24 provides an essential second signal, thus activating an atypical death-inducing signaling complex (DISC) formed upon TLR3 stimulation (Weiss et al., 2013). Activation of this DISC by IL-24 correlated with the down-regulation of cellular FLICE (FADD (Fas-associated protein with death domain)-like interleukin-1β converting enzyme)-like inhibitory protein (cFLIP), which otherwise bound and blocked the DISC-associated caspase-8 (Estornes et al., 2012). Thus, IL-24 is capable of sensitizing cells for the activation of the extrinsic apoptotic pathway initiated by TLR3 agonists. The requirement of a second signal such as TLR stimulation might also explain its potent apoptotic effect upon viral expression in cancer models.

As influenza A virus stimulates the TLR3 receptor by virtue of its RNA genome we hypothesized that IL-24 might have a specific anti-viral activity against RNA viruses, as it can lead to premature apoptosis of virally infected TLR3 stimulated cells. We observed an IFN-independent anti-viral effect of IL-24 using type I IFN-defective Vero cells. This pro-apoptotic anti-viral effect could be antagonized by a pan-caspase inhibitor. Moreover, the pro-apoptotic anti-viral effect was closely associated with the down-regulation of the anti-apoptotic Bcl-2 family member Mcl-1.

2. Materials and methods

2.1. Cell lines, viruses and reagents

The human prostate cancer cell line DU 145 (ATCC, Manasses, VA, USA) was cultivated in MEM medium (PAA, Pasching, Austria) supplemented with 10% heat-inactivated (hi) fetal calf serum (FCS). The human melanoma cell line SK-MEL-28 (ATCC) was grown in DMEM/F12 medium (PAA) supplemented with 10% hi FCS. African green monkey kidney epithelial Vero cells (EC ACC, 88020401) were adapted to grow in FCS free OPTIPRO medium (Invitrogen, Carlsbad, CA, USA). All cells were cultivated at 37 °C in a humidified 5% CO_2 atmosphere.

The wild type (wt) influenza A virus IVR-116 (H1N1) comprises the polymerase basic 2 (PB2), polymerase acidic (PA), nucleoprotein (NP), non-structural (NS) and matrix (M) genes from the influenza A/PuertoRico/8/34 virus, the hemagglutinin (HA) and neuraminidase (NA) genes from the influenza A/NewCaledonia/20/99 virus and the polymerase basic 1 (PB1) gene from the influenza A/Texas/1/77 virus. The delNS1 virus is an IVR-116 derived virus, which lacks the NS1 open reading frame (ORF) (Wacheck et al., 2010). The delNS1/IL-2 and delNS1/IL-24 viruses are deduced from an delNS1 virus, which ectopically express IL-2 and IL-24, respectively (Wolschek et al., 2011). Furthermore, influenza A/Aichi/2/68 virus (H3N2) and heat-inactivated (hi) delNS1 virus, generated by the incubation at 56 °C for 1 h (h), were used in this study.

Vero cells served for virus reproduction by using a multiplicity of infection (MOI) of 0.1 and were further grown in OPTIPRO medium containing 5 μg/ml trypsin (Sigma Aldrich, St. Louis, MO, USA) for 2 days. Concentrations of virus titers were determined by plaque assays using Vero cells. Besides effective virus propagation on Vero cells, the influenza A IVR-116 virus, delNS1 virus and delNS1/IL-24 virus retained the ability to grow on both tumor cell lines (DU 145 and SK-MEL-28, respectively).

Stimulation experiments of cells with recombinant human IL-24 (rhIL-24; R&D Systems, Minneapolis, MN, USA) were done at the concentration of 100 ng/ml, as this has been shown to be the minimal dose of promoting cell death in combination with a TLR3 stimulus (Weiss et al., 2013). The pan-caspase inhibitor Z–Val–Ala–DL–Asp–fluoromethylketone (zVAD; Bachem, Torrance, CA, USA) and the necroptosis (a form of programmed necrosis, which is independent of caspases) inhibitor Necrostatin-1 (Nec-1; Sigma–Aldrich) were used at a concentration of 10 μM and 50 μM, respectively. Cells were incubated with zVAD or Nec-1 for 30 min and subsequently washed with fresh medium before any additional treatment.

2.2. Co-infection experiments

Cells were infected with different viruses, stimulating reagents and further cultured up to 72 h in OPTIPRO medium containing 5 μg/ml trypsin (Sigma Aldrich). Every 24 h 200 μl supernatant was taken for virus titer determination by using Vero cells for plaque assays.

2.3. Annexin V and propidium iodide double staining for cell death analysis

Cells were collected after 24 h of stimulation, washed with phosphate buffered saline (PBS) and incubated with 5 μl fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Pharmingen, Franklin Lakes, NJ, USA) and 5 μl propidium iodide (PI; BD Pharmingen) in the dark for 15 min (min) at room temperature (RT). Subsequently, cells were measured with a flow cytometer (Gallios Cytometer 1.1; Beckman Coulter, Brea, CA, USA).

2.4. Small interfering RNA (siRNA) experiments

Cells (4×10^4) were seeded on six-well plates. The following day, 100 nM TLR3 siRNA (Santa Cruz), caspase-8 siRNA (Santa Cruz), Mcl-1 siRNA (Santa Cruz) or scrambled (sc) siRNA (Qiagen, Maryland, USA) was mixed with 3 μg/ml linear polyethylenimine (PEI; Polysciences Europe GmbH, Eppelheim, Germany) in 100 μl medium without FCS for 10 min at RT. Cell medium was replaced with 900 μl fresh medium and the prepared siRNA solutions were added