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The therapeutic effect of death: Newcastle disease virus and its antitumor potential

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Abstract

Programmed cell death is essential to survival of multicellular organisms. Previously restricted to apoptosis, the concept of programmed cell death is now extended to other mechanisms, as programmed necrosis or necroptosis, autophagic cell death, pyroptosis and parthanatos, among others. Viruses have evolved to manipulate and take control over the programmed cell death response, and the infected cell attempts to neutralize viral infections displaying different stress signals and defensive pathways before taking the critical decision of self-destruction. Learning from viruses and their interplay with the host may help us to better understand the complexity of the self-defense death response that when altered might cause disorders as important as cancer. In addition, as the fields of immunotherapy and oncolytic viruses advance as promising novel cancer therapies, the programmed cell death response reemerges as a key point for the success of both therapeutic approaches. In this review we summarize the research of the multimodal cell death response induced by Newcastle disease viruses (NDV), considered nowadays a promising viral oncolytic therapeutic, and how the manipulation of the host programmed cell death response can enhance the NDV antitumor capacity.

Keywords

Newcastle disease virus; Programmed cell death; Oncolytic virus; Apoptosis; Autophagy; Necroptosis; Tumor therapy; Recombinant virus

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Conflict of interest

Icahn School of Medicine at Mount Sinai owns patent positions for reverse genetics of Newcastle disease viruses.

1. Introduction

Programmed cell death maintains the homeostatic cellular balance under physiological conditions and it is also an essential component of the host defense against pathogens. Lack of regulatory control over the cell death program is one of the key factors behind the pathology of many diseases, including cancer (Igney and Krammer, 2002). In other cases, disease severity might be a direct effect of cell death induction, as it happens in many viral infections (Linkermann et al., 2014). On the other hand, it might be possible to take advantages of the intrinsic ability exerted by some pathogens to manipulate cell death responses. In that sense, the use of viruses in tumor therapy is one example of how to fight a human disorder using a potential pathogen (Bell and McFadden, 2014).

Newcastle disease virus (NDV), an avian paramyxovirus, has been extensively investigated for its use in cancer treatment (Zamarin and Palese, 2012). The inherent anti-tumor capacity of NDV combines two characteristics that delineate what can be defined as the oncolytic paradigm: NDV promotes the induction of tumor cell death accompanied by the elicitation of antitumor immunity.

In this review, we focused our attention on the different cell death responses displayed by NDV-infected cells and the new therapeutic strategies that have emerged to turn this cytopathic effect into an improved antitumor therapeutic response.

2. Newcastle disease virus: biology overview

Newcastle disease virus (NDV) is a highly contagious avian pathogen (Alexander et al., 2012; Ganar et al., 2014). NDV is classified as an avian paramyxovirus-1 (APMV-1) in the *Avulavirus* genus of the family *Paramyxoviridae* (Lamb and Parks, 2007). As other paramyxoviruses, NDV is an enveloped virus whose genome is negative-sense single-stranded RNA. The ssRNA(−) molecule is commonly 15,186 nucleotides long (Czegledi et al., 2006) and contains six open reading frames that encode six structural proteins: the nucleoprotein (NP), the phosphoprotein (P) and the large polymerase protein (L) are, in association with the viral RNA, the components of the ribonucleoprotein complex (RNP). The RNP not only exerts nucleocapsid functions but also is the replication unit of the virus. The matrix protein (M) forms an inner protein layer below the inner leaflet of the viral membrane of the virion and participates actively during virus assembly and budding (Shnyrova et al., 2007). The hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins, in conjunction with a host-derived lipid bilayer constitute the external envelope of the virus and are responsible for viral entry (Villar and Barroso, 2006).

The infection of the host cells starts once the virus HN protein binds to its receptor, sialic acid (Lamb and Jardetzky, 2007). Receptor recognition by HN triggers the activation of the F protein that promotes fusion of the viral and cell membrane and allows the entry of the RNPs into the cytoplasm. Genome replication takes place in the cytoplasm and does not involve any DNA-intermediate stage: the genomic ssRNA(−) is transcribed into (1) messenger RNAs, that will be translated into the different viral proteins, and (2) antigenomic copies, or ssRNA(+), that will be used as template for genomic ssRNA(−) synthesis. Viral proteins and genomic RNPs are then assembled at the cytoplasmic inner leaflet of the host

cell membrane and the new progeny of viral particles are released by budding. Last, the neuraminidase activity of the HN protein removes sialic acid residues from the nascent virions preventing their aggregation and facilitating viral spread within the infected tissue.

During the transcription of the P gene, the expression of two additional non-structural proteins, V and W, takes place as result of RNA editing (Steward et al., 1993). The V protein confers to NDV the capacity to evade the interferon response (Park et al., 2003b), interfering with STAT-mediated interferon signals. However, this interplay V-STAT-1 is species restricted and does not apply to mammalian cells. Hence the V protein is considered a major determinant of NDV host range (Park et al., 2003a).

NDV strains have been classified into three pathotypes, velogenic (highly virulent), mesogenic (intermediate virulence) and lentogenic (low-virulence or avirulent), in accordance to the severity of the disease displayed by the avian host (Dortmans et al., 2011). The cleavage site of the F protein is a major determinant of virulence: velogenic and mesogenic strains have a polybasic amino acid motif at the F cleavage site, 112R/G/KR-Q/K-K/R-R↓F117 that can be recognized and cleaved by ubiquitous furin-like proteases hence the F protein could adopt its mature form in the majority of infected cell types (Morrison, 2003). Lentogenic strains, in contrast, have a monobasic amino acid motif, 112GR/K-Q-G-R↓L117, that is cleaved by trypsin-like proteases in the extracellular space and hence their multicycle replication is restricted to specific tissues.

3. Cell death in response to NDV infection

Apoptosis has been identified as a major hallmark of NDV-mediated cytotoxicity in virus-infected cells. Multiple viral proteins have been found to influence cell death, some of which might be strain specific (Table 1). In addition, the susceptibility to undergo apoptosis in response to NDV infection is cell-specific and it is determined by the presence or absence of specific cell death regulatory factors. In that sense and in addition to apoptosis, other cell death pathways have been recently described to participate in the cellular response to NDV infection, especially in cancer cells (Table 2). Interestingly, some of these pathways are exploited by the virus to take control over the programmed cell death.

3.1. Apoptosis

Apoptosis is an evolutionary highly conserved physiological mechanism involved in the specific elimination of aging, harmed, infected or unnecessary cells. With critical implications both in development and in control of homeostasis, deregulation of apoptosis is a major contributor of important disorders as immunodeficiency (Brown and Attardi, 2005) and cancer (Chalah and Khosravi-Far, 2008). Morphological changes during apoptosis include chromatin condensation, increased cytoplasmic vacuolization, cell shrinkage, plasma membrane blebbing and formation of apoptotic bodies (Kroemer et al., 2009) (Fig. 1). The stimulation of apoptosis comes from outside and inside cell signals which trigger the activation of one or the two major pathways of apoptosis signaling: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Both signaling cascades are interconnected and converge in a common last step during the apoptotic program: the execution pathway (Galluzzi et al., 2012; Lawen, 2003). Key components responsible of

driving the signaling of each pathway and of forming the cellular machinery of self-destruction are the cysteine proteases caspases (Creagh, 2014; Shalini et al., 2015). Caspase 8, 9 and 3 are the most important members of this specific protein family and they are responsible of the activation of the extrinsic, intrinsic and execution pathways, respectively.

3.1.1. Apoptosis from the virus perspective—Apoptosis is actively involved in the infection by NDV and its characterization has been carried out both *in vitro* (Lam and Vasconcelos, 1994; Ravindra et al., 2008b) and *in vivo* (Kommers et al., 2003). Death in chicken embryos (Lam et al., 1995) immunosuppression (Harrison et al., 2011) and neurological damage in adult birds (Ecco et al., 2011) are examples of the consequences of the apoptosis response to the severity of the Newcastle disease.

In mammalian cells, the specificity of NDV to infect cancer cells has been partly attributed to defects in the apoptosis response. Mansour et al. (2011) have demonstrated that the over-expression of the anti-apoptotic Bcl-xL protein sensitized chemoresistant A549 to NDV infection. Specifically, the NDV lentogenic LaSota strain was able to replicate two-logs higher in Bcl-xL overexpressing cells than in control cells. The enhancement in virus replication correlated with a delayed apoptosis response due to the sustained anti-apoptotic activity of the overexpressed Bcl-xL (Mansour et al., 2011). Related to this observation, the up-regulation and stabilization of the anti-apoptotic IAP protein Survivin (Altieri, 2015) prolonged cell survival and enhanced viral protein synthesis and virus production in human breast adenocarcinoma cisplatin-resistant cells infected by the velogenic NDV AF2240 strain (Jamal et al., 2012).

The induction of apoptosis in NDV-infected cells is independent of IFN signaling or p53 activity (Elankumaran et al., 2006). Several studies have confirmed that cell lines capable to respond to endogenous or exogenous IFN and also those with an impaired IFN response undergo apoptosis in response to NDV infection (Fabian et al., 2007). Similarly, the p53 cellular status does not impact the apoptosis response to NDV (Ravindra et al., 2009a; Wu et al., 2012).

NDV-induced apoptosis requires virus entry, replication and de-novo protein synthesis (Ravindra et al., 2008a). Experimental inhibition of the receptor-mediated endocytosis pathway, UV-inactivation of virus replication or blockade of translation by inhibitors as cycloheximide were able to reduce the apoptosis induction by NDV, indicating that viral replication and protein expression are required for optimal apoptosis induction. Velogenic NDVs have shown to induce the earliest and strongest apoptotic responses (Kommers et al., 2003; Elankumaran et al., 2006; Ravindra et al., 2008a; Bian et al., 2011; Ghrici et al., 2013). Studies performed by Ghrici et al. (2013) using human breast carcinoma MCF-7 cells exposed to UV-inactivated velogenic NDV (AF2240 strain) have shown mitochondrial transition pore opening and caspase 8 activation, as early as 1 and 2 h post exposure, respectively. These results suggest that early events in NDV infection, as virus attachment or membrane fusion, could act as pro-apoptotic stimuli.

Several works have reported an involvement of HN and M viral proteins in the apoptosis induction by NDV: HN alone was shown to cause upregulation of TRAIL in human

peripheral blood mononuclear cells (PBMCs) (Zeng et al., 2002). In chicken embryo fibroblasts (CEFs), HN-transfection induced cytoplasmic vacuolization, upregulation of caspases, loss of mitochondrial transmembrane potential and an increased oxidative stress (Ravindra et al., 2008b). Recent experiments using transfected HeLa cells with a bicistronic plasmid encoding HN and TNF- α revealed an enhancement of the pro-apoptotic activity of TNF- α in the presence of HN (Rajmani et al., 2015). However, the molecular mechanism behind the pro-apoptotic activity of the HN protein is still unknown.

The matrix protein of the velogenic NDV strain AF2240 has been shown to interact with Bax through its BH3 domain, resulting in activation of the intrinsic apoptosis pathway (Molouki et al., 2011). The binding of M to Bax promoted Bax translocation from the cytoplasm to the mitochondrial membrane followed by activation of the apoptosis response. The same effect was later on demonstrated upon AF2240 virus infection in HT29, HeLa and HCT116 cells (Molouki and Yusoff, 2012). BH1-like and BH3-like domains have also been found in F, L and HN (Molouki and Yusoff, 2012). However, only the over-expression of NDV F protein in HeLa cells led to an enhancement in cell death. Furthermore, co-transfection of F and Bcl-xL impaired Bcl-xL anti-apoptotic function. Nevertheless, a direct interaction F-Bcl-xL has not been described. Subsequent analysis of the sequence of these proteins made clear the NDV strain specificity of these determinants.

3.1.2. The mitochondrial pathway has a dominant role in NDV-induced cell death—Actively involved in the transduction of anti-viral signaling pathways (Moore and Ting, 2008; Pourcelot and Arnoult, 2014), the mitochondria seems to act also as a sensor and processing center from where the cell death response is triggered in response to a variety of stress stimuli (Gillies and Kuwana, 2014). In the intrinsic pathway, mitochondrial outer membrane permeabilization (MOMP) leads to the release of cytochrome C as well as of different pro-apoptotic proteins from the mitochondrial intermembrane space. Cytochrome C and the apoptotic protease-activating factor 1 (APAF-1) are needed to build the apoptosome, a caspase activation platform involved in the activation of the intrinsic pathway initiator caspase 9 (Tait and Green, 2010). The regulation of the mitochondrial pathway is under the control of the Bcl-2 protein family: members of this family have been characterized by both anti- and pro-apoptotic functions and are in charge of the control of different mitochondrial checkpoints. In mammals, the anti-apoptotic members of this family include Bcl-2, Bcl-xL, and Bcl-W, while the pro-apoptotic members include Bax, Bak, Bad, Bik, Bim, and Bid (Delbridge and Strasser, 2015).

NDV infection results in the loss of mitochondrial membrane potential, the release of cytochrome C and the activation of caspase 9, the three essential elements of the intrinsic signal transduction pathway (Tait and Green, 2010). The interplay between the antiapoptotic Bcl-2 and the pro-apoptotic Bax seems to modulate the apoptosis response in the majority of the NDV-infected cells tested. Therefore, several works have used the ratio Bax/Bcl-2 as an indicator of apoptosis progression during NDV infection (Molouki et al., 2011; Ravindra et al., 2009a). The absence of p53 activity and hence the impaired function of Bcl-2 in p53 mutant cancer cells might make them more susceptible to apoptosis induction by NDV infection. However, the absence of Bax delayed but did not abrogate the activation of apoptosis in human colon carcinoma HCT116 Bax $^{-/-}$ cells (Molouki and Yusoff, 2012).

The interaction between M and Bax has already been noted (Molouki et al., 2011). These results, in conjunction with those previously mentioned by Mansour et al. (2011) and Jamal et al. (2012), about the role of Bcl-xL and Survivin respectively, highlight the importance of the Bcl-2 family members in the programmed cell death activated in response to NDV. The presence of BH-like motifs within the NDV proteins allowing the virus to interact and modulate some of the pro- and anti-apoptotic functions of Bcl-2 in a strain specific manner may be sign of adaptive mechanisms.

3.1.3. The extrinsic pathway in the NDV-induced apoptosis—The apoptotic extrinsic pathway is activated upon the binding of cytokine ligands (FasL, TNF α , and TRAIL) to their respective counterpart Tumor Necrosis Factor Receptor (TNFRSF) at the cell surface. The transduction of the signal from death receptors requires first, ligand-binding and receptor oligomerization, second, recruitment of adaptor molecules (FADD and TRADD) and death-inducing signaling complex (DISC) formation and last, activation of pro-caspase 8 at the DISC level. Once caspase 8 is activated, it can subsequently activate the execution pathway (Schutze and Schneider-Brachert, 2009).

NDV has been shown to induce activation of both intrinsic and extrinsic pathways. In contrast with the early and sustained role of the mitochondrial pathway, the activation of the extrinsic pathway seems to be a late event during the programmed cell death in NDV-infected cells (Ravindra et al., 2009e). *In vitro*, the activation of caspase 8 has been described to occur in a biphasic way (Elankumaran et al., 2006): early presence of active caspase 8 did not correlate with the execution of apoptosis and a late peak of caspase 8 activation was observed after the induction of the intrinsic pathway. Activation of caspase 8 during NDV infection *in vitro* is likely to be an indirect effect of the intrinsic pathway and dispensable for the execution of apoptosis. In the same work, Elankumaran et al. (2006) detected the presence of secreted TNF α by cells infected with different NDV strains (lentogenic LaSota, mesogenic Beaudette C and Beaudette C-edit) at 12 h post-infection and increased during the time course of infection. However, even the highest concentration of TNF α at the latest time points was unable to induce apoptosis. The expression of TRAIL was detected at late times post infection mostly at the cell surface, with only a few cell lines able to release soluble TRAIL after NDV infection. Consequently, the up-regulation of TNF α and TRAIL by NDV has been considered not directly involved in the activation of apoptosis *in vitro*.

3.2. Programmed necrosis and necroptosis

The consideration of necrosis as just an accidental and passive process of cell death is nowadays revised: similarly to apoptosis, necrosis occurs in a regulated fashion and involves specific molecular machinery (Galluzzi et al., 2012). The cytopathic effect linked to necrotic cell death involves karyolysis with absence or mild chromatin condensation, presence of dilated organelles (oncosis), cellular swelling and the loss of integrity of the cytoplasmic membrane. The extensive disintegration of the cell during necrosis leads to the release of the cytoplasmic contents to the extracellular space resulting in important pro-inflammatory implications *in vivo*. Extensive necrosis correlates with the severity of the Newcastle disease and is especially remarkable in infections carried out by neurotropic and viscerotropic

velogenic strains (Wakamatsu et al., 2006). The capability of velogenic and mesogenic strains to induce syncytia has pro-necrotic potential: syncytia formation is facilitated by the accumulation of newly synthesized HN and F proteins at the host surface that leads to a cell-to-cell membrane fusion with the neighboring cells (Zeng et al., 2004). The continued mechanical stress supported by the plasma membrane ends with the disintegration of the syncytium and the release of the cytoplasmic contents followed by inflammation.

As a regulated process, necrotic cell death can happen as a result of the activation of different cellular death events: necroptosis (RIP kinases dependent), parthanatos (PARP dependent) or pyroptosis (inflammasome-dependent) pathways, among others, are examples of programmed necrosis mechanisms (Pasparakis and Vandenabeele, 2015). Necroptosis has been recently identified as a part of the cellular response of glioblastoma to NDV infection (Koks et al., 2015). Necroptosis is an alternative form of programmed cell death that can be activated in a caspase 8 independent manner by toll-like receptors, TNF members and DNA and RNA sensors. The transduction of the signal depends on the receptor-interacting protein kinase 1 (RIPK1)-RIPK3 complex and is inhibited by Necrostatin-1 (Kaczmarek et al., 2013). In the studies carried out by Koks et al. (2015), GL261 glioma cells infected at high MOI with the lentogenic NDV Hitchner B1 displayed necrosis-like morphological changes like cellular swelling, extensive membrane and cell disintegration and karyolysis. The molecular characterization of the cell death showed dispensability of caspase activation, increased population of Annexin-V(-)/PI(+) cells during the time course of the infection and sensitivity to Necrostatin-1 which significantly rescued the NDV-induced cytotoxicity. This is the first reported example of programmed necrosis as a cell death response to NDV infection. The activation of necroptosis leads to a more immunogenic cell death response that, as we will discuss later, could have important therapeutic implications.

3.3. Autophagy and the control of NDV-induced cell death

Autophagy is a highly conserved recycling system in eukaryotes that involves lysosomal degradation of dysfunctional organelles and proteins to preserve cellular homeostasis. Autophagosome formation, a double membrane vesicle whose contents are targeted to lysosomal degradation, is the major hallmark and critical step during autophagy. More than 30 autophagy-related genes (*Atg* genes) have been described to be involved in the execution and control of the entire pathway. Atg5, Atg7 and Beclin-1 (homologue of *Atg 6* in yeast) are essential during autophagosome formation and important regulatory checkpoints: Atg5 and Atg7 interact with ubiquitin-like conjugation systems that are required for LC3-I lipidation; the class III phosphatidylinositol 3-kinase (PI3KC3) is a critical positive regulator through its association with Beclin-1 whereas the class I PI3K/Akt pathway acts as a negative regulator and controls autophagy *via* its downstream regulator mTOR (Mukhopadhyay et al., 2014).

“Autophagic cell death” definition is applied only to those cases where the inhibition of autophagy abrogates the cell death response (Galluzzi et al., 2012). By contrast, the induction of autophagy during other cell death mechanisms induced in response to stress has in general a cytoprotective effect; the inhibition of autophagy, rather than preventing, accelerates the execution of the cell death program (Lalaoui et al., 2015). Endoplasmic

reticulum (ER) stress, nutrient deprivation and virus infection are some examples of stress stimuli that could trigger the autophagic process. During virus infection, autophagy acts also as a host defense mechanism. Autophagy enables virus elimination and participates in the production and release of viral antigens and damage-associated molecular patterns (DAMPs) which contributes to antigen cross presentation (Hou et al., 2013). Because of its relevance as part of the anti-viral response, many viruses have evolved different ways to manipulate autophagy for their own benefit (Jackson, 2015; Orvedahl and Levine, 2008): Herpes Simplex virus 1 (HSV1), for example, encodes two proteins, ICP34.5 and US11, to target and block autophagy and therefore improving its own survival (Orvedahl et al., 2007); in dengue virus infection, the autophagy pathway is exploited during viral entry, translation and replication (Panyasrivani et al., 2009).

For NDV, there are just a few studies exploring the induction and impact of autophagy during virus infection. Nevertheless, as we summarize below, it seems to be an important component of the cellular response to NDV. Triggered early during infection, the autophagic response to NDV benefits viral replication and prolongs cell survival acting as a negative regulator of apoptosis.

The first report came from human glioma U251 cells infected with the mesogenic NDV Beaudette C strain (Meng et al., 2012a,b). Cells showed a complete autophagic flux upon virus infection, with an early increase and sustained presence of LC3-II and gradual decline of the autophagy substrate p62 that correlated with the formation and maturation of autophagosomes. Furthermore, using autophagy modulators the authors described a positive effect of autophagy in virus replication, leading to an increased virus production when using the positive regulator rapamycin and a decrease in the presence of the inhibitor chloroquine. This observation was also confirmed by knocking down Beclin-I or Atg5, which resulted in approximately 6–10 times reduction in virus yields (Sun et al., 2014).

In an attempt to elucidate the mechanism behind the modulation of apoptosis by autophagy, Meng et al. (2014) studied the cellular response to NDV infection in human non-small cell lung cancer A549 cells. Using the lentogenic LaSota strain, Meng et al. observed the induction in infected cells of a p62-dependent mitophagy process that resulted in elimination of damaged mitochondria upon NDV infection, which led to reduced cytochrome c release and therefore inhibition of apoptosis.

The p62 protein, also known as sequestrosome, is one of the selective substrates for autophagy and a scaffold in autophagosomes. Its role in the *cross-talk* between autophagy and the intrinsic apoptotic pathway as well as its overexpression in many cancer cells has also being described (Moscat and Diaz-Meco, 2009). p62 role blocks apoptosis and favors NDV replication.

The sustained inhibitory effect that autophagy exerts over apoptosis induction during NDV replication cycle may result in the activation of alternative subroutines of cell death. In the mentioned studies of Koks et al. (2015), induction of necroptosis in NDV-infected GL261 glioma cells correlated with a sustained autophagy flux with increasing levels of LC3-II and reduction in p62, as NDV-induced necroptosis reached a peak. The direct involvement of

autophagy in the necroptosis outcome was confirmed by the reduction of necroptotic cell death after chloroquine treatment.

3.4. Role of stress-signaling pathways during NDV infection

3.4.1. The stress-activated MAP Kinases pathway—Mitogen-activated protein (MAP) kinase cascades are associated with the transduction of multiple signals involved in the control of the cellular homeostasis. In the cellular response to pathogens, MAPKs participate in stress-mediated signaling pathways that trigger the apoptosis response. In that sense, the MKKK5 or Apoptosis signal-regulating kinase 1 (ASK1) is known to stimulate the JNK and p38 MAPK apoptosis dependent pathways in response to TLRs activation. The phosphorylation of both kinases leads to Bid cleavage followed by translocation of Bax to the mitochondria and subsequent activation of the intrinsic pathway (Sumbayev and Yasinska, 2006).

Because of its relevance in the cell response to pathogens, the characterization of role of MAPK signaling and its contribution to the programmed cell death in response to NDV infection have been investigated. Studies performed in A549 cells infected by three different NDV strains, LaSota (lentogenic), Beaudette C (mesogenic) and FMW (velogenic) demonstrated the activation of both, extrinsic and intrinsic, apoptotic pathways in time and strain-dependent manners, with the velogenic FMW displaying the earliest responses and the lentogenic LaSota the latest (Bian et al., 2011). In this scenario, the authors also confirmed the presence of an active MAPKs signaling process with the three major kinases, ERK, JNK and p38 highly phosphorylated in a time dependent and strain-specific manner. To clarify the role of MAPKs in the apoptosis response, FMW-infected cells were pre-treated with specific inhibitors for the three MAPKs. Only the blockade of p38 could reduce the cytotoxicity associated with FMW infection. These results suggest that p38 MAPK, but not ERK or JNK, plays a critical role in NDV-induced apoptosis.

The most recent information about the implication of p38 MAPK in the cellular response to NDV came from a study carried out with the velogenic AF2240 (Ch'ng et al., 2015): NDV induced activation of the p38 MAPK/NF- κ B/I κ B α pathway in clear cell renal cell carcinoma ccRCC cells, which resulted in cell death by apoptosis.

3.4.2. ER stress: the unfolded protein response during NDV infection—

Endoplasmic reticulum (ER) stress response, also known as unfolded protein response or UPR, is triggered due to the accumulation and aggregation of nascent polypeptides and unfolded proteins in the ER lumen. The control of UPR depends on the activity of three receptors, PERK, ATF6, and Ire1. Their activation results in down-regulation of protein translation due to phosphorylation of eIF2 α , up-regulation of chaperone synthesis and degradation of miss folded proteins. If the stress persists, UPR switches its role from a protective response to a pro-apoptotic response, with the activation of caspase 12 as one of the main features (Maly and Papa, 2014).

Activation of the UPR has been shown in human cervical carcinoma HeLa cells, PC12 rat pheochromocytoma and metastatic breast cancer MCF-7 cells in response to the NDV attenuated MT68/H infection (Fabian et al., 2007, 2001). Phosphorylated PERK and eIF2 α

and activation of caspase-12 and caspase-3 were detected. UPR response led to a reduction in virus replication probably linked to the inactivation of eIF2 α and hence inhibition of viral protein synthesis. However, subsequent studies performed including same cellular systems but different NDV strains did not show activation of UPR as part of the host response to NDV infection (Bian et al., 2011; Zhang et al., 2014). Overall, these studies indicate that the activation of UPR can take place as a part of the cellular response to NDV infection, but may be strain-specific. UPR activation reduces viral replication as a result of the inactivation of eIF2 α and triggers a caspase 12-dependent cell death response.

3.5. Emerging pathways associated to NDV-induced cell death

3.5.1. Immunogenic cell death—Immunogenic cell death (ICD) is an especial outcome of dying cells in which the execution of the program of cell death is accompanied by the emission of damage-associated molecular patterns or DAMPs. Surface-exposure of calreticulin, active secretion of ATP and passive release of high-mobility group box 1 protein (HMGB1) are considered key DAMPs. ICD requires induction of ER stress response and has been described in apoptosis, necroptosis and autophagy cell death (Krysko et al., 2012). Applied to tumor virotherapy, ICD is probably one of the mechanisms behind the success of some oncolytic virus to induce an adaptive immune response against the tumor and thus long-term tumor regression (Zelenay and Reis e Sousa, 2013).

To date, the previously referred work of Koks et al. (2015) of NDV virotherapy in glioma models is the only published study exploring the involvement of ICD in the therapeutic response exerted by NDV (Koks et al., 2015). *In vitro*, during a time course of the infection, an increased exposure of ecto-CRT (calreticulin) and passive release of HMGB1 were detected. The higher levels of both ICD markers correlated with the progression of necroptotic cell death. In this study, neither passive release of HSP70/90 nor enrichment of ATP in the extracellular compartment were detected. The antigenicity of GL261-infected cells was also evaluated revealing increased levels of the glioma-associated antigen PMEL17. *In vivo*, treatment of orthotopic glioma with the NDV lentogenic Hitchner-B1 led to a long-term survival and protection against re-challenge, implying the induction of a tumor-specific adaptive immune response. Increased infiltration of IFN- γ ⁺ CD4⁺/CD8⁺ T cells specific for tumor antigen PMEL17 and decreased myeloid-derived suppressor cells (MDSC) were also observed.

3.5.2. Cell migration and invasion—One of the major problems related to the failure of tumor therapy is the lack of control over the metastatic potential displayed by different cancer cells. In view of the relevance of NDV as a promising oncolytic agent, several groups have investigated the molecular mechanisms involved in the blockade of migration and invasion observed in different metastatic tumor models in response to NDV treatment. What in general is a very intuitive passive side-effect of the cytopathic effects mediated by NDV infection (impaired motility in dead cells), in some cases seems to be the consequence of a specific cell death response.

The velogenic NDV strain AF2240 was shown to reduce the migration capacity of mammary carcinoma infected cells. The suppression of migration observed in this set-up

was a direct effect of the reduction in cell proliferation due to apoptosis induction (Ahmad et al., 2015).

Moreover, it has been observed (Zhang et al., 2015) a correlation between apoptosis induction and reduction in motility and migration of oral squamous cell carcinoma (OSCC) using the D90 NDV strain. The pro-apoptotic capacity of NDV D90 was previously described in A549 lung carcinoma cells (Fu et al., 2011) and seems to be an example of strain-specific cellular response, characterized by a Bax/Bcl-2-dependent activation of the mitochondrial pathway. Prior to the execution of apoptosis, OSCC-infected cells displayed a reduction in number of pseudopodia and motility correlated with an altered microtubules distribution. Analysis of proteins involved in metastasis confirmed that D90 significantly inhibit the invasion capacity of OSCC by decreasing the expression of Sp1 and increasing the expression of RECK that suppress the expression and activity of the matrix metalloproteinases MMP-2 and MMP-9.

4. Therapeutic manipulation of NDV-induced cell death response

NDV is considered one promising viral vector for cancer therapy. With the aim to develop better therapies that could succeed in future clinical trials, many research groups have focused their efforts in understanding the nature of the NDV anti-tumor properties in different cancer cells and animal models. Several clinical trials using NDV-based autologous vaccines or direct administration of natural NDV strains have been performed for a wide panel of malignances. Overall, the results obtained from NDV-virotherapy have been encouraging and supportive to the benefit of using NDV in cancer treatment (Zamarin and Palese, 2012). The establishment of reverse-genetics for NDV and the following development of recombinant NDV viruses (rNDVs) have been crucial to further understand and also enhance its anti-tumor activity. The information provided by the studies of its oncolytic potential has also opened a new interesting research opportunity to explore the combination of different rNDVs and cancer therapeutics.

Here, we summarize some of latest approaches carried on in the field of NDV as oncolytic agent that have been focused on guiding the cell death response to a more therapeutic outcome (Table 3).

4.1. Modification of the cellular pro-apoptotic network: rNDV-B1/Fas virus

The induction of the extrinsic apoptosis pathway has been suggested to be a late event within the cell death response mediated by NDV infection and several studies pointed to TRAIL as a major contributor of this activation. Fas receptor, one of the most important tumor necrosis factor receptors involved on extrinsic pathway signaling, has not being described as an active cellular component involved in the apoptosis program displayed by NDV infected cells, neither did its physiological ligand, Fas-L (Ravindra et al., 2009a; Balogh et al., 2014). Our research group has investigated the implications of adding Fas receptor as a pro-apoptotic new player into the already known NDV-induced apoptosis scenario (Cuadrado-Castano et al., 2015). A newly generated lentogenic rNDV-B1/Fas virus expressing Fas receptor has demonstrated an enhanced oncolytic capacity in different *in vitro* and *in vivo* models correlated with up-regulation of Fas signaling during rNDV-B1/Fas

infection. Overexpression of Fas triggered an earlier and enhanced apoptosis response, with both extrinsic and intrinsic pathways activated simultaneously in rNDV-B1/Fas infected cells. *In vivo*, earlier activation of apoptosis correlated with an improved therapeutic effect of rNDV-B1/Fas virus in intratumoral-treated melanoma bearing mice. rNDV-B1/Fas virus treatment led to long-term survival, complete tumor remission and protection against subsequent challenge with melanoma in previously recovered mice. All this suggests that the cell death response induced by rNDV-B1/Fas is also highly immunogenic and promotes potent anti-tumor immune responses.

4.2. Targeting the modulation of the intrinsic pathway: rFMW/AP virus

Apoptin, the VP3 protein of chicken infectious anemia virus, has been shown to selectively induce apoptotic cell death in an extensive panel of cancer cells and tumor models (Danen-Van Oorschot et al., 1997; Backendorf et al., 2008). Apoptosis triggered by Apoptin is independent of p53 and requires nuclear localization, phosphorylation by CDK2 and interaction with Nur77, in order to activate the mitochondrial or intrinsic pathway. Furthermore, the interconnection between Apoptin and members of the Bcl-2 family has been described in what appears to be a cell-type specific modulatory mechanism, with pro- or anti-apoptotic Bcl-2 taking part on it (Los et al., 2009). Pursuing the enhancement of the oncolytic potential of the NDV strain FMW, Wu et al. (2012) developed a recombinant virus expressing Apoptin. The cytotoxicity of the newly generated rFMW-AP was evaluated *in vitro* and *in mice* bearing A549-induced tumors. The results obtained shown an increased apoptosis activation both *in vitro* and *in vivo*. Also *in vivo* it was observed higher levels of necrosis in rFMW-AP in comparison with the wild type virus. *In vivo*, rFMW-AP treatment showed better control of tumor growth but neither long-term survival nor complete tumor remission was reported.

Considering these results, further investigations would be needed to clarify the influence of Apoptin in the oncolytic capacity of FMW. Besides the appreciated enhancement in apoptosis response, experiments to identify the particular role of Apoptin in the death mechanism are missing. To date, many Apoptin interactors have been described that could be targets for pharmacological modulators. Knowing how the protein acts in the context of NDV infection could be helpful to develop combined chemo-virotherapies for the treatment of cancer.

4.3. Overstimulation of the TRAIL-mediated apoptosis pathway

Despite the lack of evidence of TRAIL to induce apoptosis *in vitro*, *in vivo* analysis of the role of TRAIL in the tumoricidal capacity of NDV had highlighted the benefits of its up-regulation: TRAIL has been previously shown to mediate the cytotoxic activity of NDV-stimulated human monocytes (Washburn et al., 2003) and natural killer cells (Song et al., 2013). The capacity of TRAIL in increasing apoptosis induction by NDV infection has been explored in two recent works from Bai et al. (2014a,b) with the development of two recombinant viruses, the NDV lentogenic LaSota-TRAIL (Bai et al., 2014a) and LaSota-IL-2-TRAIL (Bai et al., 2014b). TRAIL was expressed three logs higher when infected with La-Sota-TRAIL when compared with the wild-type virus and the released TRAIL had similar biological activity when compared with a commercial version. NDV LaSota-TRAIL

infection of different cancer cell lines led to an enhanced cytotoxicity when compared with the wild type. *In vivo*, treatment with LaSota-TRAIL led to an increased survival rate and control over the tumor growth of foot pad melanoma bearing mice. However, neither the wild type nor the LaSota-TRAIL induced long-term protection and the animals developed new tumors. The relapse of melanoma in previously treated mice indicates that the enhanced killing potential of the virus was not enough to induce an immune response that guaranteed long-term protection. To counteract this lack of adaptive immune response, the authors developed the recombinant LaSota-IL-2-TRAIL that expresses, in addition to TRAIL, IL-2 (Bai et al., 2014a,b). So far, the results obtained with this recombinant are just *in vitro* and shown similar growth kinetics and cytotoxicity than the previously described LaSota-TRAIL. To date, the overexpression of TRAIL has been shown to act as an enhancer in the overall pro-apoptotic response to NDV, inducing the activation of the TRAIL-dependent extrinsic pathway *in vitro* and enhancing the tumorocidal capacity of the virus *in vivo*.

4.4. Using NDV in combination with autophagy modulators

Autophagy has a dual role in cancer and could act as a tumor suppressor mechanism or promote tumorigenesis in different cancer models. Currently, there are several ongoing clinical trials assessing the effect of autophagy manipulation on human cancer treatment, many of which are based on chloroquine (Zhi and Zhong, 2015). In view of the special interplay between autophagy and NDV infection, recently studies established the therapeutic potential of using autophagy modulators in combination with NDV in drug-resistant lung cancer cells (Jiang et al., 2014). Pretreatment with the autophagy inhibitor chloroquine resulted in an enhanced anti-tumor activity of the velogenic NDV/FMW virus in cisplatin-resistant A549. Using the activator rapamycin the authors observed an enhanced cytotoxicity of NDV/FMW in paclitaxel resistant A549 cells. *In vivo*, both strategies enhanced the oncolytic potential of NDV in cisplatin-resistant or paclitaxel-resistant tumor bearing mice. These results confirm the benefits of this kind of therapeutic approach and open the door to explore new anti-tumor strategies based on the combination of autophagy modulators and the oncolytic activity of NDV.

5. Conclusions

“(…) to understand that there are multiple pathways to death and that the commitment to die is not the same as execution. A cell that has passed the commitment stage but is blocked from undergoing apoptosis will die by another route. We still must learn much more about how a cell commits to death and what makes it choose a path to die” (Zakeri and Lockshin, 2008).

NDV is an important pathogen in birds with an extraordinary oncolytic potential. Cancer cells seem to respond to NDV infection by the activation of programmed cell death that is mainly mediated but not restricted by apoptosis induction.

NDV has demonstrated to induce the activation of both extrinsic and intrinsic apoptotic pathways. However, only the intrinsic pathway appears to be important during the apoptotic response to NDV infection with a major role in the induction and regulation of apoptosis. Nevertheless, early engagement of the extrinsic apoptotic pathway contributes to enhanced

cell death and antitumor activity of NDV. This has been achieved by the over-expression of TRAIL and Fas during NDV infection. However, when used *in vivo*, rNDV LaSota-TRAIL was not able to induce long protection in a mouse tumor model, but rNDV-B1/Fas was able to not only induce complete tumor remission and long-term survival but also protection against re-challenge with melanoma in mice.

ER stress and MAP kinases signaling pathways participate in the anti-viral response against NDV by triggering and enhancing apoptosis. ER stress leads to a reduction of viral replication due to eIF2 α phosphorylation and induces an alternative caspase 12-dependent programmed cell death response. MAP kinases are important components of the cellular signal transduction network and are involved in directing the cellular response to many different stimuli. Under NDV infection, the activation of the three major MAPKs, ERK, JNK and p38 has been demonstrated but only p38 seems to stimulate the apoptosis response.

Activated early during infection, the autophagic response to NDV infection benefits viral replication and prolongs cell survival acting as a negative regulator of apoptosis. Due to its anti-apoptotic effect, the combination of NDV and inhibitors of autophagy has been tested as a novel strategy to enhance the anti-tumor potential of the virus.

Immunogenic cell death (ICD) has been already identified as a feature of the cell death program induced by NDV. NDV-mediated cell death of tumor cells is likely to promote a proinflammatory environment in the tumor that leads to immune-mediated elimination of the tumor. Characterization of the presence of markers of ICD may answer the question about why the use of some NDV strains have been more successful in specific *in vivo* tumor mouse models.

A challenge in virotherapy of tumors is that if one relies on the ability of viruses to kill cancer cells. One needs to deliver the virus to all cancer cells present in the patient to eradicate the tumor. However, it has become more and more evident that virotherapy works in many instances through the stimulation of a pro-inflammatory environment in cancer infected cells that potentiates anti-tumor immune responses working even in distant uninfected tumors. Such therapeutic immunostimulation might be enhanced by combining virotherapies with immune checkpoint inhibitors, and/or by increasing the immunostimulation elicited in virus-infected cancer cells. In this respect, the generation of oncolytic viruses, such as NDV, with increased abilities to induce pro-inflammatory programmed cell death in tumors is a promising strategy for the improvement of the anti-cancer therapeutic potential of these viruses. In summary, this review highlights a complex and multi-modal cellular response behind the programmed cell death induced in response to NDV (Fig. 2) that may influence that antitumor potential of the virus and opens many questions to explore on the oncolytic paradigm of NDV.

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Fig. 1. Apoptosis induction by Newcastle disease virus. Morphological hallmarks

Confocal microscopy images of HeLa cells infected with the recombinant rNDV-B1/Fas virus (Cuadrado-Castano et al., 2015). Left: composite Z-stack of six optical slices showing HeLa-infected cells undergoing apoptosis; highlighted are different stages of the apoptosis response observed among the cell population identified accordingly to the morphological changes observed during the progression of apoptosis cell death: pre-apoptosis stage (lack of morphological changes); active apoptosis (A) showing cellular shrinkage and DNA fragmentation. Advance apoptosis (B) displaying extensive membrane blebbing and DNA fragmentation. Late apoptosis (C) showing apoptotic bodies scale bar 100 µm. Right plots: single stacks and magnification of morphological features, observed in the main left panel: DNA fragmentation (A), membrane blebbing (B) or C (apoptotic bodies). Scale bar 10 µm. For the propose of this image, the cells were infected at an MOI of 1 PFU/cell, fixed 20 h after infection, and stained with monoclonal anti-human Fas antibody (red), polyclonal anti-NDV serum (green) and Hoechst for nuclear contrast. Confocal laser scanning was performed using a Zeiss LSM 190 510 Meta (Carl Zeiss Microimaging, Thornwood, NY) fitted with a Plan Apochromatic 63×/1.4 191. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Fig. 2.
NDV-induced apoptosis: overview of the cell death response. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

Table 1

Viral components involved in cell death response.

Viral component	Biological function	Cell death implication/interaction
HN	Hemagglutinin-neuraminidase Hemagglutinin: virus attachment; Receptor recognition in cell host surface Neuraminidase: sialic-acid removal Facilitates cellular detachment Prevents virus aggregation after budding	Up-regulation of TRAIL (PBMCs) Up-regulation of caspases 1, 9, 8, 3 (CEFs) Syncytia formation: necrosis: enhanced replication Vacuolization: autophagy BH1-like domain*: potential interaction with BcL-2 family members
F	Fusion protein: virus-host membrane fusion	Syncytia formation: necrosis; enhanced replication BH1 and BH3 like domains: potential interaction with BcL-XL Enhancement of apoptosis in co-transfection with BcL-xL
M	Viral morphogenesis; budding; replication	Apoptosis: intrinsic pathway activation BH3-like domain*: binding to Bax induces Bax translocation to the mitochondrial membrane
L	RNA-dependent RNA-polymerase Transcription of viral mRNAs Replication genomic RNA	BH1 and BH3 like domains*: potential interaction with BcL-2 family members (Bid, Bad, BcL-2)
V	Non-structural protein (P-ORF edition) Viral-host determinant (birds) Not functional in mammals	Enhancement of replication capacity Blockage of IFN response: interaction with STAT-1 (birds)

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PBMCs, peripheral blood mononuclear cells; CEFs, chicken embryonic fibroblasts; ORF, open reading frame; IFN, interferon.

* AF2240 strain specific.

Table 2

Stress-inducible and cell death pathways activated in NDV infected cells.

Cell death subroutine	Features observed in response to NDV
Apoptosis Intrinsic pathway	Mitochondrial outer membrane permeabilization (MOMP); loss of mitochondrial potential; cytochrome C release; activation of caspase 9; regulation by Bcl-2/Bax
Apoptosis Extrinsic pathway	TNF- α and TRAIL up-regulation Caspase 8 activation due to cross-talk with intrinsic pathway
Apoptosis Execution pathway	Caspase 3 activation; phosphatidyl-serine membrane externalization; DNA fragmentation, blebbing, apoptotic-bodies formation; cytoskeleton reorganization (motility and invasion diminished)
Necrosis/necroptosis	Extensive necrosis and acute inflammation during infections by highly pathogenic strains in birds Programed necrosis or necroptosis: dispensability of caspases and Necrostatine-1 sensitivity Induction of ICD: ecto-CRT exposed at the membrane surface and release of HMGB1 Increased infiltration of IFN- γ + CD4+/CD8+ T cells Decreased of myeloid-delivered suppressor cells (MDSC)
Autophagy	Modulation of apoptosis response: complete autophagy flux with autophagosomes formation Anti-apoptotic effect due to p62-dependent mitophagy: pro-necrotic cell death at later time points
ER stress (UPR)	PERK phosphorylation: eIF2 phosphorylation: anti-viral state, diminishes viral protein synthesis Caspase 12 activation: pro-apoptotic response with activation of caspase 3
MAPK	ERK, JNK and p38 activation p38 exerts pro-apoptotic functions

TNF- α , tumor necrosis factor alpha; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ICD, immunogenic cell death; ecto-CRT, ecto-calreticulin; HMGB1, high-mobility group box 1 protein; PERK, eukaryotic translation initiation factor 2- α kinase.

Table 3

Manipulation of the cell death response to NDV.

Strategy	Modification	Impact in viral biology	Impact in cell death response	Reference
Recombinant NDV rNDV-B1/Fas virus	Insertion of hFas Lentogenic Hitchner B1 backbone	Higher cytotoxicity <i>in vitro</i> Higher oncolytic potential <i>in vivo</i>	Activation of the Fas-mediated extrinsic pathway Earlier and stronger activation of apoptosis response Synergetic effect of caspase-8 activation and the intrinsic pathway <i>In vivo</i> : complete tumor remission and long-term protection	Cuadrado-Castano et al. (2015)
Recombinant NDV LaSota-TRAIL virus LaSota-IL-2-TRAIL virus	Insertion of TRAIL IL-2-TRAIL tandem Lentogenic LaSota backbone	Higher cytotoxicity <i>in vitro</i> Higher oncolytic potential <i>in vivo</i>	Activation of TRAIL- mediated extrinsic pathway <i>In vivo</i> : moderate to total tumor remission Non protection against relapse	Bai et al. (2014a) Bai et al. (2014b)
Recombinant NDV rFMW/AP virus	Insertion of Apoptin (VP3 of CAV virus) Velogenic FMW backbone	Higher cytotoxicity <i>in vitro</i> Higher oncolytic potential <i>in vivo</i>	Enhancement of the intrinsic apoptotic pathway <i>In vivo</i> : control over the tumor growth No complete remission or long-term protection observed	Wu et al. (2012)
Autophagy Modulation	FMW strain in combination with autophagy modulators Rapamycin Chloroquine	Higher cytotoxicity <i>in vitro</i> Higher oncolytic potential <i>in vivo</i>	Enhanced anti-tumor activity in cisplatin-resistant A549 cells in combination with chloroquine Enhanced cytotoxicity in paclitaxel resistant A549 cells in combination with rapamycin	Jiang et al. (2014)

hFas, human Fas receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IL-2, Interleukin 2; VP3, Apoptin, Viral protein 3; CAV, chicken anemia virus.