CD55 is a key complement regulatory protein that counteracts complement-mediated inactivation of Newcastle Disease Virus

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Newcastle disease virus (NDV) is being developed as an oncolytic virus for virotherapy. In this study we analysed the regulation of complement-mediated inactivation of a recombinant NDV in different host cells. NDV grown in human cells was less sensitive to complement-mediated virus inactivation than NDV grown in embryonated chicken eggs. Additionally, NDV produced from HeLa-S3 cells is more resistant to complement than NDV from 293F cells, which correlated with higher expression and incorporation of complement regulatory proteins (CD46, CD55 and CD59) into virions from HeLa-S3 cells. Further analysis of the recombinant NDVs individually expressing the three CD molecules showed that CD55 is the most potent in counteracting complement-mediated virus inactivation. The results provide important information on selecting NDV manufacture substrate to mitigate complement-mediated virus inactivation.

The complement system constitutes a complex of heat labile serum proteins and cell surface proteins that act as an innate immune defence against invading pathogens. The complement cascade is initiated via three main pathways: classical, mannose-binding lectin (MBL), and alternative pathway. The three pathways converge at the level of C3 activation where C3 convertase cleaves C3 into its active fragments C3a and C3b. C3b acts as a major effector molecule that attaches covalently to pathogen surfaces resulting in opsonization, phagocytosis and activation of the downstream C5 convertase that leads to formation of the membrane attack complex (MAC) and complete lysis of target cells (Ricklin et al., 2010; Janeway et al., 2001; Stoermer & Morrison, 2011; Lambris et al., 2008; Cummings et al., 2007). Besides its function in innate immunity, complement is also important for cellular integrity, tissue homeostasis and modification of adaptive immune responses, thus acting as a vital system for immune surveillance (Ricklin et al., 2010). Activated complement components have high potential for tissue destruction and therefore the complement system is highly regulated by a group of host soluble or membrane-bound complement regulatory proteins. Among the membrane-bound proteins, CD46 (membrane cofactor protein, MCP) binds to C3b and mediates degradation of C3b; CD55 (decay-accelerating factor, DAF) binds to C3 convertase and accelerates the decay of C3 convertase; and CD59 (protectin) prevents the formation of MAC and protects cells from lysis (Cummings et al., 2007; Zipfel & Skerka, 2009). These proteins exhibit homologous restriction, i.e. the proteins only act on the complement components from the same species (Vanderplasschen et al., 1998; Biswas et al., 2012). To counteract complement inactivation, many viruses have evolved different strategies to evade complement activation, including incorporation of host complement regulators on the viral envelope, molecular mimicry by encoding their own complement regulatory proteins, or by direct or indirect binding to complement receptors on the host cell surface (Cooper, 1991; Lambris et al., 2008; Cummings et al., 2007; Blue et al., 2004).

Newcastle disease virus (NDV), an avian paramyxovirus type 1 in the family Paramyxoviridae, is being developed as an oncolytic virus for cancer therapy due to its selective oncolysis of tumour cells and anti-tumour immunity elicited by viral replication (Buijs et al., 2015; Chiocca & Rabkin, 2014; Cassel & Murray, 1992; Zamarin & Palese, 2012; Cassel & Garrett, 1965; Moore et al., 1952; Prince & Ginsberg, 1957a, b). We recently developed a potent recombinant NDV (73T-R-198v, referred to as rNDV in this study) from the mesogenic 73T strain that is attenuated in chickens without affecting its oncolytic activity (Cheng et al., 2012; Cassel & Garrett, 1965; Moore et al., 1952; Prince & Ginsberg, 1957a, b). In order for NDV to reach the tumour target to replicate and kill tumour cells after systemic delivery, it is important to evaluate the impact of serum complement on NDV infectivity. In this report we examined human complement-mediated NDV inactivation by comparing viruses produced from embryonated chicken eggs, 293F and HeLa-S3 cells, which are substrates suitable for virus production. The impact of human complement on viral...
infectivity was evaluated by treating viruses propagated from these substrates with normal human serum (NHS) of known complement activity. Approximately 100 p.f.u. of purified rNDV were incubated with different dilutions of NHS or heat-treated NHS. After incubation for 1 h at 37°C, viral infectivity was examined by plaque assay in Vero cells. As shown in Fig. 1, NHS exhibited differential inhibition of the infectivity of rNDV produced from the three substrates. Heat treatment eliminated the NHS inhibition effect, indicating that NDV inactivation by NHS was due to complement activity. Compared to the egg-derived virus, rNDV produced from HeLa-S3 and 293F suspension cells was more resistant to NHS inactivation. NHS at 1:40 dilution completely inactivated egg-derived viruses, but did not inactivate rNDV produced from HeLa-S3 cells and inactivated 50% of virus from 293F cells. At higher NHS dilutions, virus produced from 293F cells was more resistant to NHS than egg-derived virus. These data indicated that NDV inactivation by complement was influenced by host factors.

Enveloped viruses including NDV have been reported to incorporate membrane-bound complement regulatory proteins to evade complement attack (Biswas et al., 2012; Saifuddin et al., 1997; Spear et al., 1995). To test whether the difference between 293F- and HeLa-S3-derived viruses correlates with the expression of the complement regulatory proteins in host cells, expression of CD46, CD55 and CD59 proteins in uninfected and infected 293F and HeLa-S3 cells was examined by Western blot analysis (Fig. 2). The expression of these three proteins was readily detected in uninfected HeLa-S3 cells, but barely detected in 293F cells (panels 1 vs. 3). Two forms of CD46 and CD55 detected may represent differentially glycosylated forms or different isoforms. Both CD46 and CD55 are glycosylated membrane proteins containing four extracellular short consensus repeat (SCR) domains, a Ser/Thr/Pro (STP)-rich region and a membrane domain (transmembrane and cytoplasmic tail for CD46, GPI-anchored portion for CD55). They form several isoforms due to alternative splicing at STP or membrane region (Liszewski et al., 1998; Osuka et al., 2006; Post et al., 1991; Russell et al., 1992). However, the antibodies against the SCR domain can detect all isoforms, which excludes the possibility that some isoforms in 293T cells could not be detected by Western blotting. A preliminary FACS analysis was also performed to determine the number of cells expressing CD46, CD55 and CD59 proteins on the cell surface of 293F and HeLa-S3 cells. The percentage of cells expressing CD46, CD55 and CD59 were 92.2%, 95.8% and 90.8% respectively in HeLa-S3 cells, while 34.9%, 61.4% and 72.7% respectively in 293F cells. These data confirmed that 293F cells expressed lower levels of CD proteins than HeLa-S3 cells. Viral infection upregulated the expression of the regulatory proteins (Fig. 2, compare lane 1 vs lane 2; lane 5 vs lane 6), which was also observed with other viruses as part of virus-induced immune regulation (Mazumdar et al., 2013). Two forms of the CD46 and CD55 molecules were detected in infected cells and the lower molecular weight versions of both CD46 and CD55 forms were enriched in infected cells and purified virions. We hypothesize that this is due to the neuraminidase activity of NDV HN protein which could remove some glycans from CD46 and CD55 (Huang et al., 2004; Osuka et al., 2006; Liszewski et al., 1998). CD46, CD55 and CD59 proteins were incorporated into NDV virions. Sucrose-gradient-purified viruses from infected HeLa-S3 cell culture supernatants had higher levels of CD molecules (lane 7) than those of 293F cells (lane 3). Expression of viral proteins [F0, F1 and nucleoprotein (NP)] in the infected cells and virions was also detected. Both 293F and HeLa-S3 cells expressed comparable levels of viral proteins in infected cells and virions despite their difference in the expression of host complement regulatory proteins. Actin was detected in both host cells and virions. It is possible that this abundant host cell protein co-purified with the NDV virus; most likely, actin was incorporated into virion during virus assembly as reported by other studies (Laliberte et al., 2006; Ren et al., 2012). In addition, a co-sedimentation experiment was performed to confirm the incorporation of the complement regulatory proteins into NDV virions. Purified rNDV from HeLa-S3 cells was subjected to sucrose gradient centrifugation and fractions from top to bottom were analysed by Western blotting detecting viral proteins, CD46, CD55 and actin (Fig. 2b). The majority of CD46 and CD55

**Fig. 1.** HeLa-S3-derived NDV is less sensitive to complement than 293F- or egg-produced NDV. A recombinant NDV (rNDV, 73T-R-198v) was propagated in 10–11-day embryonated chicken eggs at 37°C for 3 days, or in human suspension cell lines (HeLa-S3 and 293F) at 37°C for 48–72 h. Virus harvests were purified on a 15–60% continuous sucrose gradient. NHS (Complement Technology) or NHS that was pre-treated at 56°C for 1 h (heat-inactivated, HI) was diluted in phosphate-buffered saline (PBS with Ca++ and Mg+++) and then mixed with 100 p.f.u. of each purified virus. Following incubation at 37°C for 1 h, virus–serum complexes were then inoculated onto Vero cell monolayers for plaque assay. After incubating at 37°C for 6 days, viral plaques were visualized by immunostaining with a polyclonal anti-NDV antibody. Data represent the mean of two independent experiments, shown as the percentage (%) of plaque numbers compared to the virus control with no serum incubation.
proteins co-migrated with viral proteins, indicating that the CD proteins were incorporated into virions. Taken together, the results indicate that higher levels of CD46, CD55 and CD59 proteins incorporated into virions may confer higher complement resistance of HeLa-S3-derived viruses.

To further assess the individual contribution of the three complement regulatory proteins to human complement evasion, recombinant NDV viruses with insertion of either CD46, CD55, CD59 or GFP (as a control) were generated by reverse genetics. The transgene cassettes were inserted at the intergenic region between the P and M genes of the NDV genome (Fig. 3a) and the transgene expression was confirmed by Western blot analysis (Fig. 3b). The three recombinant viruses were grown in embryonated chicken eggs and purified by sucrose gradient centrifugation. Incorporation of CD46, CD55 or CD59 into virion membrane was visualized by electron microscopy after immunogold labelling (Fig. 3c). Next, these purified viruses were assessed for their abilities to resist complement inactivation (Fig. 3d). Both rNDV-GFP and rNDV-CD46 are highly sensitive to complement. NHS completely inhibited the two viruses at dilutions of 1:20 and 1:40. In contrast, rNDV-CD55 and rNDV-CD59 were less inhibited by NHS. The rNDV-CD55 virus retained more than 50% and 80% infectivity in the presence of 1:20 and 1:40 NHS, respectively, indicating that CD55 plays a major role in complement resistance. This data was confirmed by using other NDV constructs and different batches of NHS. CD55 was also found to be more potent than CD46 in preventing complement inactivation of mumps and vesicular stomatitis virus viruses (Johnson et al., 2012). Several factors may contribute to the dominant effect of CD55. First, CD55 may have higher level of expression and incorporation into virion than CD59 and CD46 as indicated by Western blot analysis and electron microscopy, but the results obtained with different antibodies cannot confirm the relative amount of these proteins (Figs 2 and 3). Secondly, complement inhibits NDV via multiple pathways ([Biswa et al., 2012] and our unpublished results). CD55 acts on C3 convertase affecting both the classical and alternative pathways, while CD46 was found to be much more active against the alternative pathway than the classical pathway (Liszewski et al., 2008). Therefore CD55 is more powerful in mediating the protection against complement inactivation. It is also possible that the different molecular weight forms of CD46 vs CD55 determine the nature of resistance to complement-mediated inactivation of NDV. Indeed, the N-glycans of two critical conserved consensus repeat regions of CD46 have been shown to be important for host cell protection during measles virus infection (Liszewski et al., 1998; Iwata et al., 1995). On the contrary, the N-glycan of CD55 is not critical for complement-mediated activity in a model of antibody plus complement-mediated cytotoxicity of CHO (Chinese hamster ovary) cells (Coyne et al., 1992). Therefore we could speculate a situation where de-glycosylation of CD46 and

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**Fig. 2.** Incorporation of CD46, CD55 and CD59 proteins into virions produced from HeLa-S3 cells. (a) Expression of complement regulatory proteins CD46, CD55 and CD59 in HeLa-S3 and 293F cells. 293F and HeLa-S3 cells were mock-infected or infected with rNDV at an m.o.i. of 0.001 at 37°C. After 3 days, cells were harvested and extracted by RIPA buffer. rNDV in the cell supernatants were also harvested and purified twice by 20–60% sucrose gradient centrifugation. Equal amount of uninfected (lanes 1, 5) and infected (lanes 2, 6) cell extracts (9×10⁶ cells per well) along with the purified viruses (10⁶ p.f.u. per well, lanes 3, 7) were subjected to electrophoresis on denatured 8–16% or 4–20% Tris-glycine gels (Invitrogen) and analysed by Western blotting with antibodies against human CD46, CD55 and CD59 (Santa Cruz Biotech) and NDV viral proteins (anti-F and NP antibodies, respectively). The blots were also immunostained with anti-actin antibody (Millipore) as a protein loading control and a representative gel picture is shown. (b) Co-sedimentation of the CD proteins with viral proteins in purified rNDV. Five hundred microlitres of highly purified rNDV (10¹⁰ p.f.u. ml⁻¹) from HeLa-S3 cells was loaded on a 20–60% sucrose gradient in a centrifugation tube and run at 28 000 r.p.m. SW41 rotor for 2 h. Twelve fractions (#1 to #12) with 1 ml each were collected from top (#1) to bottom (#12). Fractions # 8 and 9 contain peak virus harvests. Fractions #2 to 12 were analysed by Western blotting as described above.

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CD55 differentially affect the ability of these complement regulators to mediate inactivation of NDV. In summary, NDV produced from HeLa-S3 cells exhibited the least sensitivity to complement-mediated viral inactivation due to the incorporation of the complement regulatory proteins. Among the three regulatory proteins evaluated, CD55 is the most potent factor in conferring resistance to complement-mediated viral inactivation. Our results suggest that HeLa-S3 cells are best suited for manufacturing NDV for oncolytic virotherapy to mitigate complement-mediated viral clearance upon intravenous administration. In this study, the complement effect on viral clearance in vivo was not conducted due to species-specific activities of complement and complement regulatory proteins. Mice serum complement does not mediate inhibition of NDV's produced from embryonated chicken eggs or human cells (data not shown), making mice an unsuitable model to investigate the effect of human complement on NDV activity in vivo. It was also noted that CD46, CD55 and CD59 were upregulated in tumour cells which could affect anti-tumour immunity (Cummings et al., 2007; Zipfel & Skerka, 2009; Varela et al., 2008; Rushmere et al., 2004; Li et al., 2001; Murray et al., 2000; Simon et al., 1996; Markiewski et al., 2008). When NDV is grown or replicated in such tumour cells expressing a high level of complement regulatory proteins, the proteins could be incorporated into the progeny particles to evade complement-mediated virus inactivation. However, the direct in vivo impact of incorporation of complement regulatory proteins on complement evasion during NDV delivery and.

Fig. 3. CD55 is more potent than CD46 and CD59 in protecting NDV from complement inactivation. (a) Diagram of the recombinant NDV genome organization. The genes for human CD46, CD55, CD59 or GFP were inserted between the P and M genes. The cDNAs encoding human CD55 (GenBank accession no. NM000574) and human CD59 (NM203330) were purchased from Origene, and the cDNA for human CD46 (NM002389) was synthesized by Genscript. Each of these genes was inserted between the P and M genes of the full-length NDV plasmid for generating recombinant viruses, rNDV-GFP, rNDV-CD46, rNDV-CD55 and rNDV-CD59, by using the method described previously (Cheng et al., 2016). (b) The viruses were amplified in embryonated chicken eggs, harvested, purified by sucrose gradient centrifugation and analysed by Western blotting using antibodies against human CD46, CD55 and CD59, respectively, and a polyclonal anti-NDV antibody. (c) The purified rNDV-CD46, rNDV-CD55 and rNDV-CD59 viruses from egg harvests were incubated with CD46, CD55 and CD59 specific mAbs followed by 12 nm colloidal gold-conjugated secondary antibody (Jackson Immuno Research Laboratories). The CD molecules incorporated into virions were visualized under a transmission electron microscope after negative staining with 2% phosphotungstic acid (pH 6.6). (d) One hundred p.f.u. of rNDV produced from eggs was pre-incubated with indicated dilutions of human serum or heat-inactivated human serum (HI). Virus–serum complexes were incubated for 1 h and live viruses were detected by plaque assay in Vero cells as described in Fig. 1.
their effect in tumour cells require further investigation in clinical settings.

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