ΔPK oncolytic activity includes modulation of the tumour cell milieu

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Oncolytic virotherapy is a unique cancer therapeutic that encompasses tumour cell lysis through both virus replication and programmed cell death (PCD) pathways. Nonetheless, clinical efficacy is relatively modest, likely related to the immunosuppressive tumour milieu. Our studies use the herpes simplex virus type 2 (HSV-2)-based oncolytic virus ΔPK that has documented anti-tumour activity associated with virus replication, PCD and cancer stem cell lysis. They are designed to examine whether ΔPK-mediated oncolysis includes the ability to reverse the immunosuppressive tumour microenvironment by altering the balance of cytokines directly secreted by the melanoma cells and to define its mechanism. Here, we show that melanoma cells secreted the immunosuppressive cytokine IL-10, and that secretion was inhibited by ΔPK through virus replication and c-Jun N-terminal kinase/c-Jun activation. ΔPK-induced IL-10 inhibition upregulated surface expression of MHC class I chain-related protein A, the ligand for the activating NKG2D receptor expressed on NK- and cytotoxic T-cells. Concomitantly, ΔPK also upregulated the secretion of inflammatory cytokines TNF-α, granulocyte macrophage colony-stimulating factor and IL-1β through autophagy-mediated activation of Toll-like receptor 2 pathways and pyroptosis, and it inhibited the expression of the negative immune checkpoint regulator cytotoxic T-lymphocyte antigen 4. Pharmacologic inhibition of these processes significantly reduces the oncolytic activity of ΔPK.

INTRODUCTION

Oncolytic viruses (OVs) originally developed to lyse tumour cells through selective replication can also induce distinct programmed cell death (PCD) pathways. However, clinical efficacy remains modest, likely related to the immunosuppressive tumour milieu (Aurelian, 2013). OVs developed from herpes simplex virus (oHSV) are primarily based on HSV-1 and are typically deleted in the neurovirulence gene ICP34.5 and/or the large subunit of the ribonucleotide reductase (R1). Some are also deleted in ICP47, which functions in virus immune evasion, but the role of this deletion in oncolytic potential is still unclear (Chiocca & Rabkin, 2014). T-Vec, an oHSV armed with granulocyte macrophage colony-stimulating factor (GM-CSF), has recently passed phase III clinical trials in stage III and IV melanoma (Hersey & Gallagher, 2014).

HSV-2 differs from HSV-1 in that many of its replicative aspects depend on the protein kinase function of R1 (also known as ICP10PK), which is poorly conserved and non-functional in HSV-1. ICP10PK activates Ras signalling pathways and is required for virus growth in slowly replicating normal cells that have low levels of Ras activity (Smith et al., 1998, 2000). We have shown that an HSV-2 mutant deleted in ICP10PK (ΔPK) that was tolerated well in vaccine phase I/II clinical trials (Aurelian, 2004; Casanova et al., 2002) has strong oncolytic activity in melanoma cultures and xenografts, where it causes a profound and long-lasting reduction in tumour burden. In addition to virus replication, oncolytic activity is associated with calcineurin and caspase-3/7 activation, autophagy induction, cancer stem cell lysis, and intratumour influx of CD11b+ antigen-presenting cells (Colunga et al., 2010, 2014). However, the ability of ΔPK to inhibit the immunosuppressive tumour microenvironment by altering the balance of cytokines directly secreted by the melanoma cells (Kaufman et al., 2014) and to induce surface expression of immunostimulatory molecules (Mouawad et al., 2010) is still unknown. Our studies were designed to address this question and identify the molecular mechanisms involved in these processes.

RESULTS

ΔPK increases transcription of death-associated and inflammatory genes

ΔPK replicates well in melanoma cells (Colunga et al., 2010, 2014), as confirmed by plaque assay (Fig. 1a) and
immunoblotting with VP5 antibody (Fig. 1b). To examine whether ΔPK alters expression of cytokines and death-associated functions, cells were mock-infected with PBS or infected with ΔPK (m.o.i. 0.5 p.f.u. per cell), and RNA isolated at 24 h post-infection (p.i.) was analysed with inflammatory and cell death targeted oligoarrays. The results are shown in Fig. 1(c) for A2058 cells and in Fig. S1(a) (available in the online Supplementary Material) for LM cells that were established from a heterogeneous primary melanoma-passaged only six to eight times before this study. Similar patterns of gene expression (+12 %) were obtained in two separate experiments for each cell culture examined (Tables S1 and S2).

ΔPK caused a marked upregulation of several genes, including inflammatory caspase-1, -4, and -5, the cytokines IL-1α, IL-1β, IL-6, IL-8, IL-12, TNF-α, lymphotoxin-α and GM-CSF, and mitogen-activated protein kinases (MAPKs) involved in both PCD and inflammatory processes. Amongst these are MAP3K7IP1 (also known as TAB1), which is involved in IL-1-induced signalling pathways (Wolf et al., 2011); MAP4K4, the upstream activator of c-Jun N-terminal kinase (JNK) (Machida et al., 2004) and its downstream target c-Jun; MAP2K6, the upstream activator of the pro-apoptotic p38MAPK (Han et al., 1996); and MAP3K14 (also known as NIK), which stimulates NFκB signalling common to TNF-α and IL-1 receptors (Vallabhapurapu et al., 2008). Whilst limited, direct comparison of ΔPK with HSV-2 indicated notable differences, including the failure of HSV-2 to upregulate TNF-α, IL-1β, GM-CSF, c-Jun, MAP3K14, Toll-like receptor (TLR)-2 and MyD88, and poor IL-10 inhibition relative to ΔPK (Fig. S2, Table S3).
ΔPK alters the balance of melanoma-secreted cytokines from immunosuppressive to inflammatory

The immunosuppressive tumour microenvironment contributes to the relatively poor clinical efficacy of OV treatment (de Aquino et al., 2015), but the contribution of direct melanoma cell secretion of immunosuppressive cytokines is still poorly understood. Our studies focused on the immunosuppressive cytokine IL-10 (Chen et al., 2014) and multiple inflammatory cytokines (TNF-α, IL-1β, GM-CSF). A2058 and A375 melanoma cells were mock-infected with PBS or infected with ΔPK (m.o.i. 1), and the conditioned media (24 h p.i.) were assayed for cytokine expression by ELISA. The data summarized in Fig. 1(d) for A375 cells and Fig. S1(b) for A2058 cells indicate that, despite their heterogeneity, the mock-infected cultures secreted similar levels of IL-10 (32.5 ± 0.84 and 25.9 ± 0.62 ng ml⁻¹ for A375 and A2058 cells, respectively), but not TNF-α, IL-1β or GM-CSF. By contrast, when infected with ΔPK the balance of the secreted cytokines was reversed, with a simultaneous decrease in the levels of IL-10, and a significant increase in the secretion of TNF-α, IL-1β and GM-CSF. Interestingly, the inhibition of the immunosuppressive cytokine IL-10 was similar in both cell lines (14.9 ± 1.7 and 8.3 ± 0.4 ng ml⁻¹ for A375 and A2058, respectively), potentially reflecting the role of virus replication in its inhibition. By contrast, the levels of induced inflammatory cytokines were ~10-fold lower in A2058 than in A375 cells, suggesting that cell-type-specific factors modulated ΔPK-induced cytokine release.

Inhibition of IL-10 secretion is through JNK/c-Jun activation

Having seen that ΔPK induces the transcription of MAP4K4, JNK and c-Jun (Fig. 1c), we wanted to verify that this reflected its ability to activate the JNK/c-Jun pathway implicated in the regulation of inflammatory responses (Oltmanns et al., 2003). A375 cells were mock-infected with PBS or infected with ΔPK (m.o.i. 1), and protein extracts obtained at 1, 4 and 24 h p.i. were immunoblotted with antibody that recognized the phosphorylated JNK isoforms (pJNK1 and pJNK2/3). The blot was stripped and reprobed with β-actin antibody as gel loading control. Two bands corresponding to pJNK1 (46 kDa) and pJNK2/3 (54 kDa) were seen. Their levels were significantly higher in the ΔPK- than mock-infected cultures (Fig. 2a) and reduced by treatment with the JNK-specific inhibitor SP600125 (100 μM) (Fig. 2b).

To confirm that ΔPK also activated c-Jun, protein extracts from duplicate A375 and A2058 cultures mock-infected or infected with ΔPK (m.o.i. 1; 24 h) in the absence or presence of SP600125 were separated into nuclear and cytoplasmic fractions, and immunoblotted with antibodies against phosphorylated c-Jun (p-c-Jun) and β-actin. As shown in Fig. 2(c) for A2058 cells, the levels of p-c-Jun were significantly increased in the ΔPK- as compared with the mock-infected cultures, primarily in the nuclear fraction, and upregulation was inhibited by SP600125. This was consistent with the established nuclear translocation of activated c-Jun (Angel & Karin, 1991) and indicated that ΔPK activates the JNK/c-Jun pathway. Significantly, SP600125 restored the levels of secreted IL-10 to those seen in the mock-infected controls, but had no effect on ΔPK-induced secretion of GM-CSF, TNF-α and IL-1β (Fig. 3a, b). The data indicated that ΔPK-induced activation of the JNK/c-Jun pathway inhibited IL-10 expression/secretion, but it did not affect the production of the inflammatory cytokines. ΔPK inhibited IL-10 secretion through virus replication and JNK/c-Jun activation, as evidenced by virus growth inhibition with SP600125 (Fig. 4d).

IL-10 inhibition induces expression of the MHC class I chain-related protein A (MICA)

MICA is a ligand for the activating receptor NKG2D expressed on NK-, γ0 T-, cytotoxic αβ CD8⁺ T- and NKT-cells. Its expression on tumour cells engages NKG2D, resulting in the cytotoxic killing of the target cells (Bauer et al., 1999). IL-10 facilitates melanoma cell escape from immune surveillance by decreasing MICA expression, which in turn reduces NKG2D-mediated cytotoxicity (Serrano et al., 2011). Having seen that ΔPK induced JNK-dependent inhibition of IL-10 expression (Fig. 2), we wanted to know whether this resulted in increased MICA expression. A2058 and A375 cells were mock-infected or infected with ΔPK (m.o.i. 1; 24 h) in the presence or absence of the JNK inhibitor SP600125 (100 μM) or recombinant IL-10 (14 ng ml⁻¹) and protein extracts were immunoblotted with anti-MICA followed by β-actin antibodies. The results are summarized in Fig. 3(c) for A375 cells. MICA was minimally expressed in mock-infected melanoma cells and its expression was significantly increased by ΔPK infection (Figs. 3c and S3). Both SP600125 and exogenously added IL-10 restored the MICA levels to those seen in the mock-infected cells (Figs. 3c and S3), indicating that ΔPK increased MICA expression through JNK-mediated IL-10 inhibition. Similar results were obtained in A2058 cells (Fig. S3). FACS analysis indicated that ΔPK-induced MICA was located on the cell surface and MICA was also induced by HSV-2, albeit at lower levels (Fig. 3d).

Inflammatory cytokines are upregulated through autophagy-dependent activation of TLR-2 signalling

TLRs are pattern recognition receptors that promote T-cell-mediated adaptive immunity (Akira et al., 2001; Bergsbaken et al., 2009; Inoue & Tani, 2014). TLR-2 induces inflammatory cytokine production by microglia, astrocytes, neutrophils and monocytes following HSV-1 intracranial infection (Aravalli et al., 2005; Villalba et al., 2012; Wang et al., 2012), but its induction in melanoma cells, in the
context of oncolysis, and as related to direct cytokine secretion, is unknown. As autophagy was previously linked to cytokine secretion (Crisan et al., 2011; Harris, 2011), A2058 and A375 cells were mock-infected with PBS or infected with ΔPK (m.o.i. 1; 4 h) in the absence or presence of the autophagy inhibitor 3-methyladenine (3-MA) (5 mM) (Seglen & Gordon, 1982), and protein extracts were immunoblotted with antibodies to TLR-2, the TLR adaptor protein MyD88 or the mature NFκB subunit (p50) downstream of TLR-2 (Akira et al., 2001).

A 95–110 kDa doublet consistent with the mature TLR-2 (Kim et al., 2013) was seen in the ΔPK- but not mock-infected cells and its induction was inhibited by 3-MA (Fig. 4a). MyD88 was also seen in the ΔPK- but not mock-infected cultures, as shown in Fig. 4(a) for A375 cells, and this was accompanied by increased expression of NFκB p50, the latter also inhibited by 3-MA (Fig. 4b). Double-immunofluorescent staining of the mock- and ΔPK-infected cells with antibodies to TNF-α and NFκB p50 confirmed that ΔPK-induced TNF-α upregulation was associated with NFκB activation, as also evidenced by the co-localization of TNF-α with NFκB in ΔPK- but not mock-infected cells (Fig. S4). The levels of TNF-α, GM-CSF and IL-1β in the culture supernatants were also significantly higher in the ΔPK- than mock-infected cultures and this increase was blocked by ΔPK infection in the presence of 3-MA (Fig. 4c). Inflammatory cytokine secretion was unrelated to virus replication because virus growth was virtually identical in A2058 cells infected with ΔPK in the absence or presence of 3-MA (Fig. 4d). The data indicated that ΔPK-induced secretion of inflammatory cytokines by the melanoma cells was through autophagy-dependent TLR-2 activation. However, 3-MA only partially blocked the ability of ΔPK to upregulate NFκB (Fig. 4b), suggesting that inflammatory cytokine upregulation also included autophagy-dependent TLR-2 pathways other than MyD88/NFκB. 3-MA had no effect on ΔPK-induced IL-10 inhibition and similar results were obtained in A375 cells (data not shown).
PK-induced pyroptosis contributes to IL-1β secretion

Pyroptosis is a caspase-1-dependent form of inflammatory cell death, which is activated after an initial NFκB-dependent priming step (Fernandes-Alnemri et al., 2007; Sutterwala et al., 2014). Having seen that the levels of secreted IL-1β were increased in ΔPK-infected cells, we wanted to know whether this involved pyroptosis-related caspase-1 activation. A375 and A2058 cells were mock- or ΔPK-infected (m.o.i. 1; 24 h), and immunoblotted with antibody to activated caspase-1. The results summarized in Fig. 5(a) indicate that caspase-1 was activated in the ΔPK- but not mock-infected cells, consistent with the oligoarray and ELISA findings (Figs 1c and 3a, b). Caspase-1 activation was accompanied by IL-1β production as evidenced by the loss of pro-IL-1β and its restored expression in cells infected with ΔPK in the presence of the caspase-1-specific inhibitor z-YVAD-fmk (Fig. 5b). Collectively, the data indicated that ΔPK-induced IL-1β secretion involved pyroptosome-dependent caspase-1 activation and pro-IL-1β cleavage.
Altered cytokine balance contributes to ΔPK-induced melanoma cell death

To examine the contribution of the altered cytokine balance to ΔPK-induced melanoma cell death, A2058 and A375 cells were mock- or ΔPK-infected in the presence or absence of 3-MA (5 mM), which regulates JNK-dependent IL-10 expression, 3-MA (5 mM), which regulates autophagy-dependent TLR-2 expression, or z-YVAD-fmk (100 μM), which regulates pyroptosis-dependent IL-1β expression. Cell death was examined by Trypan blue exclusion at 48 h p.i. ΔPK-induced cell death was significantly inhibited by the addition of 3-MA (P < 0.001), 3-MA (P < 0.001) or z-YVAD-fmk (P = 0.002) in both A2058 (Fig. 6a) and A375 (Fig. 6b) cells, confirming the contribution of the pathways. Collectively, the data indicated that ΔPK-induced tumour cell death and modulation of the tumour microenvironment involved multiple regulatory pathways.
Fig. 5. ΔPK-induced pyroptosis activates caspase-1 resulting in mature IL-1β production. (a) Extracts from A2058 cells mock- or ΔPK-infected (m.o.i. 1) were immunoblotted with caspase-1 (Casp-1) (p20) antibody. (b) Extracts from A2058 cultures mock- or ΔPK-infected (m.o.i. 1; 24 h) in the presence or absence of z-YVAD-fmk (YVAD) (100 μM) were immunoblotted with antibody to pro-IL-1β. Data are presented as mean ± SD densitometry units. ***P<0.001 versus mock, by one-way ANOVA.

Fig. 6. Cytokine modulation contributes to ΔPK-induced melanoma cell death. (a) A2058 and (b) A375 cultures were mock- or ΔPK-infected (m.o.i. 1) followed by treatment with or without SP600125 (100 μM, left), 3-MA (5 mM, middle) or z-YVAD-fmk (YVAD) (100 μM, right) and examined for cell death by Trypan blue exclusion at 48 h p.i. Dead cells (blue staining) were counted in four quadrants and the percentage of dead cells was calculated. Data are presented as mean ± SD. ***P<0.001 and **P<0.01, by one-way ANOVA.
MICA and TNF-α expression are upregulated in vivo

To examine whether tumour growth inhibition was associated with ΔPK-induced microenvironment alteration, A375 cells were implanted into BALB/c nude mice by subcutaneous injection into both flanks and when the tumours became palpable (day 7; ~200 mm³), the animals were given intratumour injections (100 μl) of partially purified ΔPK (10⁶ p.f.u.) or culture medium control, and tumour volume was monitored over time. Confirming our previous findings that ΔPK inhibited tumour growth (Colunga et al., 2010), mock-infected animals evidenced time-dependent tumour growth reaching maximal levels on day 27. ΔPK caused a significant (P<0.001) decrease in tumour growth that was still seen on day 41 (Fig. 7b). Representative xenografts collected from the ΔPK-treated animals on day 28 were examined for MICA and TNF-α expression indicative of altered tumour microenvironment through inhibition of the immunosuppressive cytokine IL-10 and upregulation of inflammatory cytokines, respectively. Both MICA and TNF-α were seen in the ΔPK- but not mock-treated xenografts (Fig. 7b), and this was associated with the inhibition of tumour growth (Fig. 7a). The data were consistent with those obtained in cultured cells and are schematically represented in Fig. 8.

DISCUSSION

The salient feature of the presented data is the finding that ΔPK alters the balance of cytokines directly secreted by the melanoma cells from an immunosuppressive to an inflammatory pattern, thereby increasing the expression of...
Fig. 8. Schematic representation of the ΔPK activity in melanoma cells, which includes JNK/c-Jun-dependent inhibition of IL-10 resulting in MICA upregulation, induction of inflammatory cytokines through autophagy-mediated TLR-2 activation and pyroptosis, and CTLA-4 inhibition.

immunostimulatory molecules and cytotoxicity-associated functions. The following comments seem pertinent with respect to these findings.

Tumour immune evasion resulting from a highly immunosuppressive microenvironment is at least partially responsible for the overall limited clinical efficacy of virotherapy. In addition to infiltrating negative regulatory cells (Devaud et al., 2013), the tumour microenvironment contains immunosuppressive factors directly secreted by the tumour cells, including the cytokine IL-10 (Whiteside, 2002), which recruits immunomodulatory regulatory T-cells (Devaud et al., 2013). To overcome this problem, OVs were armed with inflammatory cytokines or delivered together with anti-tumour factors. Adenovirus- and HSV-based OVs armed with TNF-α were shown to have enhanced anti-tumour activity (Han et al., 2007; Hirvinen et al., 2015), as were HSV or vaccinia-based OVs armed with GM-CSF (Kaufman et al., 2014; Parviainen et al., 2015) and an oncolytic Newcastle disease delivered in combination with systemic cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade (Zamarin et al., 2014). However, individual cytokines deliver immunostimulatory signals in a relatively non-specific manner and they may even contribute to the establishment of immune tolerance, as shown for exogenously delivered GM-CSF (Mortha et al., 2014). Therefore, OVs that induce diverse anti-tumour effects including altered tumour cell secretion of multiple cytokines are particularly desirable.

Following on from previous findings that the strong oncolytic activity of ΔPK is associated with stimulation of multiple death-inducing pathways and virus replication (Colunga et al., 2014, 2010), our studies were designed to examine the potential contribution of an altered pattern of melanoma-secreted cytokines. We focused on the immunosuppressive cytokine IL-10 that inhibits antigen presentation (Buelens et al., 1997), and expression of MHC class II and co-stimulatory molecules (de Waal Malefyt et al., 1991), and promotes myeloid-derived suppressor cells and regulatory T-cell recruitment to the tumour microenvironment (Marvel & Gabrilovich, 2015). We also studied the inflammatory cytokines TNF-α and GM-CSF, which were individually associated with improved efficacy of virotherapy (Hersey & Gallagher, 2014; Hirvinen et al., 2015), and IL-1β, which induces robust and durable primary and secondary CD4+T-cell responses (Ben-Sasson et al., 2009). Despite their known heterogeneity, the studied melanoma cells constitutively secreted IL-10, but not TNF-α, IL-1β and GM-CSF, and this pattern was reversed by ΔPK infection. The ability of ΔPK to inhibit IL-10 secretion is through JNK/c-Jun pathway activation, as evidenced by: (i) increased levels of phosphorylated (activated) JNK and c-Jun, (ii) p-c-Jun nuclear translocation, and (iii) restored IL-10 expression through treatment with the JNK-specific inhibitor SP600125. This is in direct contrast to findings in monocytes/macrophages (Chanteux et al., 2007; Dobreva et al., 2009; Norkina et al., 2007), and it underscores the virus and cell-type specificity of this response, and the importance of developing a better understanding of the role of OV therapy in inducing cytokine secretion in situ. Significantly, SP600125 also inhibited virus growth, indicating that IL-10 inhibition requires virus replication. However, similar IL-10 inhibition was not seen in HSV-2-infected melanoma cells, at least as determined by transcriptional array analysis (Fig. S2), and this is consistent with our earlier findings that the numbers of IL-10-producing T-cells are significantly lower in ΔPK- than HSV-2-infected immunocompetent animals (Gyotoku et al., 2002).

ΔPK infection induced the cell surface expression of MICA, the ligand for the NKG2D receptor expressed by NK- and cytotoxic T-cells (Bauer et al., 1999; Moretta et al., 2001), as confirmed by FACS analysis. Increase was blocked by both SP600125 and recombinant IL-10, indicating that ΔPK stimulates MICA expression through JNK/c-Jun-mediated IL-10 inhibition. MICA was also expressed in HSV-2-infected melanoma cells, but its levels were visibly lower than those seen for ΔPK, although virus replication was 100-fold higher (Colunga et al., 2010). The mechanism of HSV-2-induced MICA upregulation is still unknown. However, it is likely independent of JNK/c-Jun, because HSV-2 does not upregulate c-Jun (Fig. S2, Table S3) or activate the JNK/c-Jun pathway (Perkins et al., 2003). MICA expression was also upregulated in ΔPK-infected melanoma xenografts, potentially contributing to tumour growth inhibition (Fig. 7b) through NKG2D-dependent cytotoxicity by NK-, γδ T-, cytotoxic αβ CD8+T- or NKT-cells (Armeanu et al., 2005; Bauer et al., 1999; Skov et al., 2005). However, final conclusions must await the results of ongoing studies.

The TLR family plays a crucial role in antigen presentation, tumour clearance and pyroptosis, and promotes T-cell-mediated adaptive immunity (Bergsbaken et al., 2009;
Inoue & Tani, 2014). In macrophages, TLR-2 promotes autophagy (Chuang et al., 2013), and intracranial HSV-1 infection stimulates TLR-2-dependent expression of the inflammatory cytokines IL-1β, IL-6 and TNF-α by microglia, astrocytes, neutrophils and monocytes (Aravalli et al., 2005; Villalba et al., 2012; Wang et al., 2012). However, in both macrophages and human foreskin fibroblasts, HSV-1 actually inhibits the secretion of mature IL-1β, apparently related to the trapping of caspase-1 by actin clusters (Johnson et al., 2013). HSV-2 decreases the expression of TLR-2 in human vaginal epithelial cells (Yao & Rosenthal, 2011) and it does not increase the number of T-cells that produce the inflammatory cytokine IFN-γ in immunocompetent animals (Gyotoku et al., 2002). Our transcriptional analysis indicates that, unlike ΔPK, HSV-2 does not upregulate TLR-2, MyD88, TNF-α, GM-CSF and IL-1β in melanoma cells (Fig. S2, Table S3). The exact contribution of the TLRs to tumour cell cytokine secretion and their role in virotherapy are still poorly understood. However, whilst co-stimulation with TLR ligands was recently shown to enhance the efficacy of OV therapy, it also sensitized the treated animals to cytokine shock-like response (Rommelfanger et al., 2013), underscoring the therapeutic advantage of an OV platform that directly stimulates TLR responses by the tumour cells.

We found that ΔPK induces the TLR-2/MyD88 pathway that culminates in the expression and nuclear translocation of the NFκB p50 active subunit, and the secretion of the inflammatory cytokines TNF-α, GM-CSF and IL-1β in the infected melanoma cells. Significantly, the ability of ΔPK to upregulate TLR-2 expression and cytokine secretion was autophagy-dependent and inhibited by treatment with the autophagy inhibitor 3-MA. This is consistent with previous findings that (i) autophagy contributes to innate immunity stimulation and cytokine secretion (Crișan et al., 2011; Harris, 2011), and (ii) ΔPK induces autophagy in melanoma cells (Colunga et al., 2010, 2014). However, whilst TLR-2 expression was strongly inhibited by 3-MA, the levels of activated NFκB were only partially decreased, suggesting that alternative autophagy-dependent and/or NFκB-independent pathways contribute to inflammatory cytokine production/secretion in ΔPK-infected melanoma cells. Indeed, MyD88/NFκB is an established pathway for TNF-α and IL-1β upregulation (Kissner et al., 2011; Lawrence, 2009), but TLR-2-initiated inflammatory amplification involving the signal adaptors TRADD/MAL/TRAP6 was also identified recently (Chang et al., 2014). We do not exclude the possibility that 3-MA has an off-target autophagy-unrelated effect that involves caspase-dependent cell death (Hou et al., 2012) and/or class I and II rather than class III phosphatidylinositol 3-kinase (Wu et al., 2010). Notwithstanding, the ability of 3-MA to inhibit ΔPK oncolytic activity confirms that autophagy-dependent TLR-2 pathways and inflammatory cytokine secretion contribute to its ability to cause tumour cell death, and it is unrelated to ΔPK replication, which was unaltered by 3-MA treatment.

Consistent with current findings that TLR activation induces pro-IL-1β expression (Hornung & Latz, 2010), which, in turn is cleaved and secreted by pyroptosome-activated caspase-1 (Fernandes-Alnemri et al., 2007), and pyroptosis contributes to tumour cell death (Bridle et al., 2010; Guo et al., 2014; Thorne, 2011), we found that ΔPK induced pyroptosome formation in the melanoma cells (through adapter protein ASC and pro-caspase-1 oligomerization) resulting in caspase-1 activation and pro-IL-1β cleavage. Cleavage was inhibited by the caspase-1-specific inhibitor z-YVAD-fmk that also inhibited the ability of ΔPK to induce melanoma cell death, confirming the contribution of pyroptosis to ΔPK-induced cell death. Interestingly, ΔPK also inhibited the expression of CTLA-4 (Fig. S5), which is constitutively expressed in several solid tumour-derived cells, including melanoma (Conradi et al., 2005; Shah et al., 2008), and behaves as a negative regulator of T-cell function (Teft et al., 2006). However, the mechanism of inhibition and its contribution to virotherapy are still under investigation.

In conclusion, our findings extend our original observations of ΔPK-mediated anti-tumour activity. They show that ΔPK switches the balance of cytokines that are directly secreted by the melanoma cells from immunosuppressive to inflammatory, thereby priming for the expression of functions, such as MICA, that are known to prime anti-tumour immune recognition and promote cytotoxicity (Fig. 8). Importantly, although the mutational landscape determines therapeutic sensitivity (Rizvi et al., 2015), ΔPK inhibits the immunosuppressive microenvironment in heterogeneous melanoma cultures that are molecularly distinct (Smith et al., 2012), and in immunocompetent mice and guinea pigs (Gyotoku et al., 2002; Wachsman et al., 2001). ΔPK is a particularly promising OV, because it was also well tolerated in phase I/II clinical studies (Aurelian, 2004; Casanova et al., 2004). However, the exact contribution of the modulated functions identified in these studies to cytotoxic anti-tumour immune responses is still unclear and final conclusions must await the results of ongoing studies.

**METHODS**

**Cells and viruses.** A2058 and A375 cells were from the American Type Culture Collection, and grown in Dulbecco’s modified Eagle’s medium with 4.5 g glucose 1−1, 1500 mg sodium bicarbonate ml−1, 4 mM glutamine and 10 % FBS (Gemini Bioproducts). ΔPK and the HSV-2 strain (G) from which it was established were described previously (Smith et al. 1998). ΔPK is deleted in the sequences that encode the independent protein kinase function in R1 (known as ICP10PK), which is required for virus growth. ΔPK expresses the kinase-negative R1 protein (p95) under the direction of the authentic R1 promoter. Following virus adsorption (1 h; 4 °C), the virus was removed, and the cells were overlaid with serum-free DMEM with or without the pharmacological inhibitors (0 h.p.i.) and incubated at 37 °C for the indicated times. Virus titres were determined by plaque assay on Vero cells and the results are expressed as mean p.f.u. ml−1 or mean p.f.u. per cell.

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Antibodies, pharmacological inhibitors and reagents. Antibodies to caspase-1, IL-1β, p18K, p-c-Jun and TRL-2 were from Cell Signaling Technology. Antibodies to β-actin, ASC, NFκB, MyD88, MICA, CTLA-4, TNF-α and glyceraldehyde 3-phosphate dehydrogenase were from Santa Cruz Biotechnology. Alexa Fluor 488- and 594-conjugated secondary antibodies were from Invitrogen. HRP-conjugated anti-rabbit and anti-mouse antibodies and mammalian-derived recombinant human IL-10 were from Cell Signalling Technologies, the caspase-1 inhibitor z-YVAD-fmk from Calbiochem, and the JNK inhibitor SP600125 and the autophagy inhibitor 3-MA from Sigma-Aldrich.

Microarray analysis. Total RNA was isolated and purified from mock- or ΔPK (m.o.i. 0.5 p.f.u. per cell; 24 h)-infected cells using a RNeasy kit (Qiagen). Biotin-labelled cRNA target was prepared with a TrueLabeling-AMP 2.0 kit (SABiosciences), and hybridized onto inflammatory and cell death targeted oligoarrays (Oligo GEArrays; SABiosciences) according to the manufacturer’s instructions. Data were analysed with GEArray Expression Analysis Suite 2.0 software (SABiosciences) and were expressed as heatmaps derived from relative gene expression analysis.

ELISA. Conditioned media were assayed with ELISA kits (eBioscience) according to the manufacturer’s instructions.

Cell death assay. Cell death was determined using Trypan blue staining. The percentage of dead (blue) cells was calculated relative to the total cell numbers in four independent fields using a haemocytometer.

Immunofluorescence and immunohistochemistry. Cells grown on glass coverslips were fixed with 4% paraformaldehyde (30 min; room temperature) and permeabilized (2 min; 4°C) with 0.1% Triton X-100 in 0.1% sodium citrate buffer. The coverslips were incubated overnight at 4°C with primary antibody diluted in 5% BSA and 5% normal goat serum, washed in PBS/0.1% Tween 20, and exposed to fluorochrome-labelled secondary antibodies (1 h; 37°C). Slides were mounted in Vectashield with DAPI (Vector) and visualized with an Olympus BX50 fluorescent microscope. Stained cells were counted in representative 50 μm thick sections post-fixed (30 min) in 1% paraformaldehyde and visualized under bright-field conditions with an Olympus BX50 microscope. Stained cells were counted in representative 50 μm² fields and the percentage of positive cells was calculated relative to the total cells per field.

Statistical analysis. ANOVA used SigmaPlot 11.0 for Windows (Systat Software). One-way ANOVA was followed by pairwise comparison with the Holm–Sidak test.

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βPK oncolytic activity includes immunomodulation


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