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Cowpox virus but not *Vaccinia virus* induces secretion of CXCL1, IL-8 and IL-6 and chemotaxis of monocytes *in vitro*

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ABSTRACT

Orthopoxviruses are large DNA viruses which can cause disease in numerous host species. Today, after eradication of *Variola virus* and the end of vaccination against smallpox, zoonotic *Orthopoxvirus* infections are emerging as potential threat to human health. The most common causes of zoonotic *Orthopoxvirus* infections are *Cowpox virus* in Europe, *Monkeypox virus* in Africa and *Vaccinia virus* in South America. Although all three viruses are genetically and antigenically closely related, the human diseases caused by each virus differ considerably. This observation may reflect different capabilities of these viruses to modulate the hosts' immune response. Therefore, we aimed at characterizing the specific cytokine response induced by *Orthopoxvirus* infection *in vitro*. We analysed the gene expression of nine human pro-inflammatory cytokines and chemokines in response to infection of HeLa cells and could identify an upregulation of cytokine gene expression following *Cowpox virus* and *Monkeypox virus* infection but not following *Vaccinia virus* infection. This was verified by a strong induction of especially IL-6, IL-8 and CXCL1 secretion into the cell culture supernatant following *Cowpox virus* infection. We could further show that supernatants derived from *Cowpox virus*-infected cells exhibit an increased chemotactic activity towards monocytic and macrophage-like cells. On the one hand, increased cytokine secretion by *Cowpox virus*-infected cells and subsequent monocyte/macrophage recruitment may contribute to host defence and facilitate clearance of the infection. On the other hand, given the assumed important role of circulating macrophages in viral spread, this may also point towards a mechanism facilitating delivery of the virus to further tissues *in vivo*.

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1. Introduction

Members of the family *Poxviridae* are characterized by complex virion morphology and a large linear double-stranded DNA genome of 130–300 kbp (Moss, 2007). Vertebrate *Poxviruses* include two obligate human pathogens: *Molluscum contagiosum virus* and the nowadays extinct *Variola virus* which was eradicated from the human population by a global vaccination campaign (Fenner et al., 1988). In addition, many other *Poxviruses* can be transmitted to humans zoonotically from various animal hosts. Because of the end of the vaccination campaign and decreasing population immunity, human infections caused by members of the genus *Orthopoxvirus* (OPV) and especially by *Monkeypox virus* (MPXV) in Africa (Likos et al., 2005; Rimoin et al., 2010), *Vaccinia virus* (VACV) in Brazil (de Souza Trindade et al., 2007; Trindade et al., 2007) and *Cowpox virus* (CPXV) in Europe (Baxby et al., 1994; Vogel et al., 2012; Vorou et al., 2008) have been becoming more common and are now frequently observed (Essbauer et al., 2010). Particularly, CPXV infects a very wide range of different host species and transmission to humans has been observed to originate from infected rodents – which are thought to be the natural reservoir – as well as from various domestic and zoo animals (Baxby et al., 1994; Czerny et al., 1991; Pelkonen et al., 2003; Vorou et al., 2008). Human VACV and CPXV infections of immunocompetent individuals are in general self-limiting. However, severe generalized infection and lethal outcome of CPXV infection has been reported in immunocompromised patients (Czerny et al., 1991; Pelkonen et al., 2003). Both VACV and CPXV infections cause localized skin lesions following infection *via* skin injuries. The human immune response towards CPXV infection is not well understood. Most efforts to understand the immune responses towards poxvirus infection focused on the mechanisms of protection conferred by the vaccination with VACV (Hammarlund et al., 2003, 2008; Kennedy et al., 2009; Precopio et al., 2007; Tscharke et al., 2005), showing an essential role of adaptive immunity and stable antibody titres for long-term protection (Amanna et al., 2006; Edghill-Smith et al., 2005; Panchanathan

Abbreviations: OPV, *Orthopoxvirus*; MPXV, *Monkeypox virus*; VACV, *Vaccinia virus*; CPXV, *Cowpox virus*; MVA, *Modified Vaccinia Ankara virus*; PMA, Phorbol-12-myristate-13-acetate.

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et al., 2010; Putz et al., 2006). However, less is known about the human immunological responses elicited during acute OPV infections, although essential roles of the innate and adaptive immunity have been demonstrated (Lauterbach et al., 2010). It has been shown that the innate immunity is necessary to control the infection until adaptive responses arise (Martinez et al., 2010; Moulton et al., 2008; Parker et al., 2007). Afterwards, a combination of cellular and humoral immune responses is crucial to control viral infection and finally eliminate the virus efficiently (Chaudhri et al., 2006; Fang and Sigal, 2005; Xu et al., 2004). However, most of these studies used VACV or the mouse-specific *Ectromelia virus* as a model system, and little is known about differences of immune recognition and response in CPXV infection which displays some peculiar features. CPXV possesses the broadest host-range observed in OPV, the largest OPV genome and it encodes the most complete set of immunomodulatory proteins of all OPV known so far.

In a previous study we could show that the expression of several innate immunity-associated genes was highly divergent in CPXV or VACV infection of human epithelial cells *in vitro* (Bourquain et al., 2012). In this study we further investigated the impact of CPXV or VACV infection on cytokine secretion of infected human epithelial HeLa cells and on chemotactic recruitment of U937 cells and PMA-differentiated U937 cells as a model for monocytes and macrophages.

2. Methods

2.1. Cells and culture conditions

HeLa (ATCC ID CCL-2), Hep-2 (ATCC ID CCL-23), Vero E6 (ATCC ID CRL-1586) and U937 (ATCC CRL-1593.2) cell lines were obtained from American Type Culture Collection (ATCC). HaCaT cells (300493) were obtained from CLS Cell Lines Service GmbH. HL-60 cells (ECACC 98070106) were obtained from the European Collection of Cell Cultures (ECACC). HeLa and HaCaT cells were cultivated in EMEM medium supplemented with 10% heat-inactivated foetal calf serum (FCS, PAA) and 2 mM of L-glutamine (PAA). Hep-2 cells and Vero E6 cells were both cultured in standard D-MEM medium containing 10% FCS and 2 mM of L-glutamine. HL-60 cells were cultivated in RPMI 1640 medium supplemented with 15% FCS and 2 mM of L-glutamine. U937 cells (Sundstrom and Nilsson, 1976) were cultivated in RPMI 1640 medium supplemented with 10% FCS and 2 mM of L-glutamine. Differentiation of U937 cells towards the macrophage-like phenotype was achieved by addition of 100 nM Phorbol-12-myristate-13-acetate (PMA) (Sigma–Aldrich) to the culture medium and incubation for 4 days at standard conditions. All cells were routinely screened for absence of mycoplasma contamination.

2.2. Viruses and infection conditions

VACV strain IHD-W (ATCC ID VR-1441) and CPXV strain Brighton Red (BR) (ATCC ID VR-302) were obtained from ATCC. MPXV strain MSF-6, which was obtained from a fatally infected human in the Democratic Republic of Congo, was kindly provided by Prof. Dr. Hermann Meyer (Institut für Mikrobiologie der Bundeswehr, Munich, Germany) (Meyer et al., 2002). All viruses were propagated in Hep-2 cells and cell culture supernatants which were clarified by centrifugation were used as virus stocks. Titres of virus stocks were determined by plaque assay (Tsuchiya and Tagaya, 1970) on Vero E6 cells as described before (Witkowski et al., 2010) and were shown to be comparable for CPXV, VACV and MPXV. All virus stocks were screened for absence of mycoplasma contamination. Inactivation of viruses was achieved *via* UV-irradiation (2×, 20 min, on ice) using a Stratalinker 2400 UV cross linker (Stratagene). Complete inactivation of UV-irradiated virus stocks was verified by plaque assay. For infection experiments, HeLa cells were grown in 6-well cell culture plates (TPP) and incubated overnight before infection with each virus at a multiplicity of infection (MOI) of 5 plaque forming units (PFU)/cell. Mock infections were performed using fresh culture medium free of any virus. After adsorption of virus for 1 h at 37 °C, the virus-containing medium or mock medium was removed, cells were washed twice with PBS and provided with fresh culture medium. Cells were then incubated at 37 °C for the indicated times. All infection experiments were performed in a biosafety level 3 (S3) laboratory in accordance with the German regulations.

2.3. Quantitative real-time RT-PCR

Total RNA was isolated using Trizol® Reagent (Invitrogen) as described in the manufacturer's protocol. Subsequently, RNA was converted to cDNA with Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis of human *IL1A*, *IL1B*, *IL6*, *IL8*, *CXCL1*, *CXCL2*, *CXCL3*, *CCL20* and *CSF2* gene expression was performed using commercially available TaqMan® Gene Expression Assays (Applied Biosystems). cMyc gene expression was measured as reference gene for ΔcT normalization as described previously (Kramski et al., 2010). Three independent samples of infected and non-infected cells, respectively, were included. Differential gene expression in CPXV-, VACV- or MPXV-infected cells compared to non-infected cells was calculated *via* the ΔΔcT method using the formula: change fold = $2^{-(\Delta\Delta cT)}$. Each PCR setup included no-template controls.

2.4. Human cytokine protein array

Cell culture supernatants were screened with the Proteome Profiler™ Human Cytokine Antibody Array, Panel A (R&D Systems). The assay was performed according to the manufacturer's instructions, and chemiluminescence signals were detected *via* the ChemiSmart 3000 chemiluminescence imaging system (Vilber Lourmat) using a charge-coupled-device camera. Data were analysed using the GeneSpotter software (MicroDiscovery).

2.5. Determination of cytokine concentration in cell culture supernatants

Quantification of CXCL1, IL-8 and IL-6 concentrations in cell culture supernatants was done by employing commercially available Human CXCL1/GROα Quantikine ELISA Kit (R&D Systems), Human CXCL8/IL-8 Quantikine ELISA Kit (R&D Systems) and Human IL-6 Quantikine ELISA Kit (R&D Systems). The assays were performed according to the manufacturer's instructions and analysed on a Tecan Infinite 200 PRO (Tecan) microplate reader.

2.6. Chemotaxis assay

Chemotactic activity of cell culture supernatants was analysed using fluorimetric 96-well QCM Chemotaxis Cell Migration Assay (Millipore). A membrane with a 5 μm pore size was used to measure U937 cell migration, and one with a 3 μm pore size to measure migration of HL-60 cells. The assay was performed according to the manufacturer's instructions. Briefly, 1×10^5 cells were allowed to migrate towards cell culture supernatants for 16 h. Cells which migrated across the membrane were lysed and subsequently detected by the nucleic acid dye CyQuant GR (Molecular Probes). Data were analysed on a Tecan Infinite 200 PRO (Tecan) microplate reader.

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