Cytokine production by human herpesvirus 8-infected dendritic cells

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We have shown previously that human herpesvirus 8 (HHV-8)-infected dendritic cells (DCs) undergo incomplete maturation and have a defective antigen-presenting function. Here, we examined the effects of HHV-8 infection on cytokine production, which is critical to the function of DCs. We detected expression of interleukin (IL)-6, tumour necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES and IL-12p40 from 2 to 6 h post-infection, and these peaked by 15–24 h. Expression of these factors decreased 24–48 h post-infection, with the exception of TNF-α which remained high throughout the entire 72 h. Interestingly, while IL-12p40 expression increased post-infection, bioactive IL-12p70 was not detected in the supernatants. These results suggest an intentional skewing of cytokine production in HHV-8-infected DCs towards induction of a Th2 response.

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma (KS)-associated herpesvirus, is a gammaherpesvirus and is the causative agent of KS, primary effusion lymphoma and a subset of multicentric Castleman’s disease (Moore & Chang, 2001). We have reported previously that primary infection with HHV-8 in human immunodeficiency virus (HIV)-negative adults does not result in pronounced clinical symptoms, despite the presence of cellular and humoral immune responses and a detectable viraemia (Wang et al., 2001). Analysis of cytotoxic T lymphocyte (CTL) responses to viral proteins expressed during a primary infection revealed a distinct but relatively non-robust immune response (Wang et al., 2001). The reason for this diminished immune response in normal individuals is not well understood but could reflect an effect of HHV-8 infection on antigen-presenting cells. In support of this hypothesis, we have shown that HHV-8 can infect monocyte-derived dendritic cells (DCs) through the C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (CD209). While this infection is non-productive, it results in an altered pattern of cytokine production that could be related to the loss of DC function.

DCs were generated from enriched CD14+ monocytes grown for 6 days in AIM-V medium and supplemented with interleukin (IL)-4 (1000 U ml−1) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (1000 U ml−1); infectious HHV-8 was purified from supernatants of tissue plasminogen activator-induced BCBL-1 cells as described previously (Rappocciolo et al., 2006). Cells were infected with gradient-purified virus equivalent to 50 viral DNA copies per cell as previously described (Rappocciolo et al., 2006). Cells were not washed following absorption to avoid loss of cytokines released upon initial interaction with the virion. Immature DCs infected with purified HHV-8 for 24 h appear morphologically distinct from uninfected DCs (Fig. 1). Viral infection resulted in the DCs exhibiting an elongated appearance similar to DCs matured in vitro.

We have previously demonstrated that HHV-8 infection of immature DCs resulted in a loss of endocytosis and a uniform decrease in cell surface receptors, even though less than 100 % of the cells were infected (Rappocciolo et al., 2006). Thus, the effect of viral infection appears to be global in the culture rather than being isolated to infected cells. Therefore, we wanted to determine whether HHV-8 infection could trigger the release of cytokines or chemokines that could be responsible for altering the maturation profile of the cells. To this end, supernatants from DC cultures that had been infected with purified HHV-8 or left uninfected for 48 h were subjected to comparative screening for cytokine secretion, using kits...
obtained from Biosource, and assayed by Luminex technology (Fig. 2). As IL-4 and GM-CSF were added exogenously to induce differentiation of monocytes to DCs, comparisons of these cytokines between uninfected and infected cultures could not be determined as they exceeded the detection limit. Gamma interferon (IFN-\(\gamma\)), epidermal growth factor, vascular endothelial growth factor, basic fibroblast growth factor, granulocyte colony-stimulating factor, hepatocyte growth factor, IL-13 and IL-17 were not detected in either set of cultures (data not shown). IL-1\(\beta\), IL-2, IL-5, monocyte chemoattractant protein-1 (MCP-1), IL-10 and IL-15 were detectable at low levels in both uninfected and infected cultures (Fig. 2). IL-6 showed the greatest difference between uninfected and infected cultures (approx. 18-fold), while greater than twofold increases of tumour necrosis factor (TNF)-\(\alpha\) (approx. threefold), eotaxin (approx. threefold), macrophage inflammatory protein (MIP)-1\(\alpha\) (approx. sixfold), MIP-1\(\beta\) (approx. 2.5-fold), RANTES (approx. sixfold), IFN-\(\gamma\)-inducible protein-10 (IP-10) (approx. 13.5-fold) and IL-12p40 (approx. eightfold) were seen in infected cultures (Fig. 2).

Since morphological changes to HHV-8-infected DCs could be observed as early as 6 h post-infection (data not shown), we wanted to determine how quickly these cytokines were released into the media after infection. To this end, we performed ELISA (R&D systems) for TNF-\(\alpha\), IL-6, MIP-1\(\alpha\), MIP-1\(\beta\), RANTES and IL-12p40, all of which showed a minimum of twofold increases in the HHV-8-infected cultures compared with the previous Luminex screening. We also tested for IFN-\(\alpha\) and IL-12p70 by ELISA, neither of which were included in the Luminex screening. For each cytokine, DCs from two HHV-8-negative donors were infected with HHV-8 or remained untreated.

**Fig. 1.** DCs infected with HHV-8 develop a distinct morphology similar to matured DCs. DCs from an HHV-8-negative donor were left uninfected (a) or were infected with HHV-8 (b) for 24 h and photographed under bright-field microscopy. Magnification, \(\times200\).

**Fig. 2.** Changes in cytokine expression in HHV-8-infected DCs versus uninfected DCs. Supernatant samples from DCs from an HHV-8-negative donor were infected or left uninfected, harvested at 48 h and relative cytokine amounts were determined by Luminex. Data are representative of duplicate samples for each of two sample populations (values indicated by error bars). Fold change is indicated above each bar.

**Fig. 3.** Temporal expression of selected cytokines in HHV-8-infected DCs. DCs were infected with HHV-8 (\(\bigDelta\), solid line), left untreated (■, dotted line), or treated with LPS (●, dashed line) or trimeric CD40L (▲, dotted/dashed line). Supernatants were harvested at selected times post-treatment and analysed by ELISA for IL-6 and TNF-\(\alpha\) (a), MIP-1\(\alpha\), MIP-1\(\beta\) and RANTES (b) or IL-12p40 and IL-12p70 (c). Each column represents the results from separate DC donors. Error bars represent values from duplicate samples.
uninfected, and supernatant samples were removed from the cells at various times. Additionally, as a positive control to mimic cytokine release from immature DCs stimulated to become mature DCs, the DCs from a third HHV-8-negative donor were treated with lipopolysaccharide (LPS) (250 ng ml \(^{-1}\); Calbiochem) or recombinant trimeric CD40L (1 μg ml \(^{-1}\); Amgen), and supernatant samples were harvested at various times. In this case, a third donor was used, as cells sufficient to perform the necessary experiments to include infection and protein treatment at one time could not be purified from a single donor. At each time point, supernatant was removed from a well containing 10^6 cells and thus represents the total accumulated amount of each cytokine in the supernatant between the time of infection and the time of harvest. As shown in Fig. 3, each of the cytokines that showed viral infection-related changes by Luminex also displayed noticeable differences in the ELISA. IFN-γ was not detected in uninfected or infected DCs (data not shown). IL-6 was induced between 2 and 6 h post-infection, peaked between 6 and 18 h post-infection and decreased after 18 h. TNF-α production began within 2 h post-infection, peaked at 6 h and decreased thereafter. The chemokines MIP-1α, MIP-1β and RANTES had a slower induction, peaking at 12–24 h post-infection, and decreased to low levels thereafter. Lastly, as shown in the Luminex screening, IL-12p40 was induced to high levels beginning between 12 and 18 h post-infection and decreased after 18 h. TNF-α production was not detected in either the uninfected or infected cultures at any time point, but was detected when immature DCs were treated with trimeric CD40L.

We have shown that HHV-8-infected DCs exhibit an intermediate phenotype that is neither immature nor fully mature (CD83^low, HLA-DR^+, HLA-ABC^low, DC-SIGN^low−). As these effects were not limited to infected cells but also included uninfected cells in the same culture (Rappocciolo et al., 2006) (data not shown), we suspected a paracrine effect and sought to determine which cytokines were being produced by infected cells. We found that pro-inflammatory cytokines (IL-6 and TNF-α), chemokines (MIP-1α, MIP-1β and RANTES) and potentially Th2 skewing cytokines (IL-10 and IL-12p40 but not IL-12p70) were increased after HHV-8 infection. This is consistent with reports of the expression of IL-6, IL-10, TNF-α, MIP-1α, MIP-1β and RANTES in cultured primary effusion lymphoma (PEL) and KS cells and in KS biopsies (Asou et al., 1998; Brockmeyer et al., 1999; Drexler et al., 1999; Uccini et al., 2003). Lack of IL-12 production in response to maturation stimuli has also been observed in PEL cell lines (Asou et al., 1998) and purified peripheral blood myeloid DCs from KS patients (Della Bella et al., 2006). Indeed, exposure to certain stimuli can polarize DCs to promote a Th1 phenotype rather than a Th11 phenotype (de Jong et al., 2002), and a lack of IL-12 expression contributes to this skewing (Hilkens et al., 1997). As it is known that DCs polarized towards Th1 induction can contribute to persistence of viruses (Palucka & Banchereau, 2002), lack of IL-12 production by HHV-8-infected DCs suggests this mechanism may be active in this case.

These findings are of interest in the context of our previous observations that DCs infected in vitro show limited functionality after infection with HHV-8. Interestingly, recent studies have shown that both infection of monocytes by HHV-8 or exposure to PEL-derived cytokines results in the inability of these cells to differentiate into DCs, decreases antigen uptake and presentation, and alters their surface marker phenotype (Cironne et al., 2007, 2008). Moreover, others have shown that peripheral blood myeloid DCs from classical KS patients have similar defects that are attributable to a soluble factor (Della Bella et al., 2006). For instance, TNF-α stimulus in the absence of an accessory signal (i.e. LPS or CD40L) has been implicated in the production of partially or semi-mature DCs, which could lead to peripheral tolerance (Lutz & Schuler, 2002; Sallustio & Lanzavecchia, 1994). Moreover, efficient IL-12 p70 production is necessary for generating robust CTL responses (Trinchieri, 2003). Given that HHV-8-infected individuals have a dampened CTL response to the virus (Brander et al., 2000; Guihot et al., 2006; Little & Yarchoan, 2006; Wang et al., 2001), the lack of IL-12p70 observed in our experiments suggests that HHV-8 could have developed a mechanism to prevent the production of IL-12p70 either through transcriptional repression of the p35 subunit or overproduction of potentially antagonistic p40 homodimers (von Grunenberg & Plum, 1998). However, a quantitative assay for determination of p40 homodimers in humans is not available.

We postulate that at least some of these cytokine and chemokine responses are caused by viral binding and entry into the cell rather than production of viral proteins, as we have shown that HHV-8 infection of DCs is non-productive (Rappocciolo et al., 2006). Moreover, the cytokine and chemokine responses were induced as early as 2 h after infection of the DCs using gradient-purified virus, and preliminary experiments using gradient-purified UV-irradiated virus were also able to induce cytokine responses (data not shown). Indeed, the HHV-8 viral OX2 homologue, which has been shown to be incorporated into the virion, is capable of inducing IL-1β, IL-6, MCP-1 and TNF-α in circulating monocytes, macrophages and DCs (Chung et al., 2002). Recent studies have shown that activating-antibody or HIV gp120 binding to DC-SIGN results in the production of TNF-α, MIP-1α, RANTES and IL-10 (Caparrós et al., 2006; Hodges et al., 2007; Shan et al., 2007). Furthermore, these studies indicate that DC-SIGN binding to DCs can result in lack of maturation, lessened T-cell stimulatory capacity and lack of surface receptor upregulation. These responses appear to be dependent on mannoseylation of viral glycoproteins. As HHV-8 encodes at least one highly mannosylated glycoprotein (gB) (Baghian et al., 2000) and the virus is known to bind to DC-SIGN (Rappocciolo et al., 2006, 2008), it is plausible that DC-SIGN binding to the virus is at least partly responsible for the dampened immune responses previously observed in HHV-8-infected DCs.
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