Enhanced monocyte Fc phagocytosis by a homologue of interleukin-10 encoded by human cytomegalovirus

Anthony Jaworowski a,b,⁎, Wan-Jung Cheng a, Clare L. Westhorpe a,b, Allison Abendroth c,d, Suzanne M. Crowe a,b,1, Barry Slobedman c,1

Abstract

Human cytomegalovirus (HCMV) expresses several homologues of human interleukin 10 (hIL-10) possessing immunomodulatory properties which may promote viral infection by modulating the function of myeloid cells. We examined the phenotype and phagocytic capability of human monocytes exposed to hIL-10, an HCMV-encoded hIL-10 homologue expressed during the productive phase of infection (cmvIL-10), and a differentially spliced form of cmvIL-10 expressed during latent and productive phases of infection, (LAcmvIL-10). hIL-10 and cmvIL-10 upregulated expression of Fcγ receptors, stimulated phagocytosis of IgG-opsonised erythrocytes and decreased MHC class II (HLA-DR) expression on purified monocytes within 24 h. In contrast, LAcmvIL-10 decreased HLA-DR expression at later times (48 h and 72 h) but did not increase Fcγ receptor expression. We conclude that cmvIL-10 promotes differentiation of monocytes towards a pro-phagocytic phenotype and that LAcmvIL-10 does not affect monocytes by the same mechanism as cmvIL-10. The significance of these properties to cytomegalovirus pathogenesis is discussed.

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Introduction

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus which infects human beings with a prevalence approaching 90–100% in some populations (Mocarski et al., 2007). Primary infection by HCMV is usually associated with no, or relatively mild, clinical symptoms, however it is estimated to account for 8% of infectious mononucleosis with occasionally severe systemic illness (ibid). Like other herpesviruses, HCMV establishes a latent infection from which it can reactivate and which can cause severe disease in immunocompromised individuals such as AIDS patients and transplant recipients (Singzger and Jahn, 1996). The broad tropism of HCMV results in infection of a wide range of cell types and, together with the cytopathic nature of infection, a consequent large spectrum of overt organ diseases including retinitis, pneumonitis and gastrointestinal disease (Mocarski et al., 2007; Sinzger and Jahn, 1996).

During primary productive infection, HCMV is hematogenously disseminated throughout the body. In acutely infected individuals, HCMV DNA or antigens have been detected in circulating endothelial cells, lymphocytes, monocytes and neutrophils (Grefte et al., 1994, 1993; Gerna et al., 1992; Meyer-König et al., 1995). Quantitative analysis of HCMV DNA in transplant recipients showed that neutrophils contributed the majority of the peripheral blood viral load, however monocytes contained the highest percentage of infection having approximately 6 times the number of viral genomes per cell than neutrophils in these individuals (Hassan-Walker et al., 2001). HCMV infection of monocytes is efficient but leads to an abortive infection which allows early but not late HCMV antigen expression, producing few HCMV particles (Frascaroli et al., 2006). Productive infection depends on differentiation of monocytes to monocyte-derived macrophages (Banez et al., 1991; Lathey and Spector, 1991; Soderberg-Naucler et al., 2001) and/or activation such as in the context of allogeneic T cell stimulation (Soderberg-Naucler et al., 1997). The ability of cytokines such as IFNγ to reactivate HCMV in macrophage reservoirs was demonstrated in these studies (Soderberg-Naucler et al., 2001). These observations suggest that monocytes may be an important reservoir allowing HCMV to establish latent infection in the blood and acting as a source of virus in the context of infection or transplantation.

Infection of monocytes with HCMV leads to altered monocyte signalling and function including stimulation of proinflammatory cytokine production and transcription factor expression (Yurochko and Huang, 1999), altered monocyte differentiation characterised by...
altered morphology and decreased phagocytic capacity towards yeast particles (Gredmark et al., 2004), a decreased ability of HCMV infected monocytes to present antigen to autologous lymphocytes which was associated with decreased induction of MHC II expression by IFNγ (Buchmeier and Cooper, 1989) and decreased chemokine receptor expression and monocyte migration (Frascarioli et al., 2006). Recently, microarray analysis of RNA from HCMV infected and uninfected monocytes has suggested that HCMV infection skews monocyte differentiation towards a proinflammatory M1 macrophage activation state which is hypothesised to favour viral dissemination and persistence (Chan et al., 2008).

HCMV encodes several proteins with immunomodulatory properties which may affect monocyte function and differentiation. Amongst these are two alternatively spliced transcripts arising from the UL111A gene which have been shown to encode homologues of interleukin 10 (IL-10) during the productive and latent phases of infection. These transcripts have been denoted cmvIL-10 (Rotenko et al., 2000; Lockridge et al., 2000) and latency associated LAcMvIL-10 (Jenkins et al., 2008), and evidence for additional isoforms expressed during productive infection have also been reported (Lin et al., 2008). Human IL-10 (hIL-10) is a pleiotropic cytokine which exerts a number of predominantly immunosuppressive functions such as downregulation of proinflammatory cytokine secretion and MHC expression on myeloid cells (Moore et al., 2001). In monocytes, hIL-10 has been reported to enhance phagocytosis by a variety of receptors including CD14 (Lingnau et al., 2007) and complement and Fcy receptors (Capsoni et al., 1995; Capsoni et al., 1998). Consistent with these observations, hIL-10 enhances Fcy receptor (Allavena et al., 1998; te Velde et al., 1992; Capsoni et al., 1998) and complement receptor (Capsoni et al., 1995) expression on monocytes and alters their differentiation by favouring a highly endocytic macrophage phenotype as opposed to an antigen presenting dendritic cell phenotype (Allavena et al., 1998; te Velde et al., 1992). Human monocytes express two Fcy receptors which promote phagocytosis of particles opsonised with IgG; Fcy R1 (CD64) and Fcy RIIA (CD32). In addition, a subset of monocytes comprising approximately 10% of total human peripheral blood monocytes also express Fcy RIII (CD16) (Ziegler-Heitbrock et al., 1993). These receptors differ in their affinity for IgG and their specificity for IgG subtypes (Hulett and Hogarth, 1994) and are essential effector molecules for antibody-mediated immunity (reviewed in Nimmerjahn and Ravetch, 2008).

Like hIL-10, cmvIL-10 has been shown to exert a range of immunosuppressive effects on myeloid cells including the capacity to inhibit proinflammatory cytokine expression and peripheral blood mononuclear cell (PBMC) proliferation, as well as to downregulate MHC class I and MHC class II expression and inhibit the maturation of dendritic cells (Chang et al., 2004; Raftery et al., 2004; Spencer et al., 2002). cmvIL-10 also inhibits CD1 antigen expression (Raftery et al., 2008) and decreases matrix metalloproteinase activity of cytrophoblasts (Yamamoto-Tabata et al., 2004). In contrast, the repertoire of immunosuppressive properties of LAcMvIL-10 is less extensive, with the only function to date being the capacity to downregulate MHC class II, which appears to occur independently of the hIL-10 receptor (Jenkins et al., 2008).

Given the critical importance of monocytes to HCMV pathogenesis, and the role HCMV alteration of monocyte/macrophage function may have in pathogenesis, we sought to determine whether cmvIL-10 and LAcMvIL-10 influenced Fcy receptor mediated phagocytosis. We show that cmvIL-10 treatment of primary human monocytes induces the expression of the three Fcy receptors (CD16, 32 and 64) and increases Fcy receptor mediated phagocytosis. In contrast, LAcMvIL-10 does not significantly affect expression of these receptors, nor alter phagocytosis of IgG opsonised targets. These results provide the first demonstration of a viral IL-10 homologue altering phagocytosis and highlights a functional difference between HCMV-encoded homologues of IL-10.

### Results

In order to determine the effect of HCMV homologues of hIL-10 on monocyte phagocytic phenotype and function, monocytes were purified from peripheral blood mononuclear cells by countercurrent elutriation and incubated with saturating concentrations of recombinant hIL-10, cmvIL-10 and LAcMvIL-10 for 24 h as previously described (Jenkins et al., 2008). Expression of the Fcy receptors CD16, CD32 and CD64 was then measured by flow cytometry (Fig. 1). Human IL-10 increased expression of all three Fcy receptors (median expression relative to control = 1.27 (p = 0.036) for CD16, 1.48 (p = 0.001) for CD32 and 1.59 (p = 0.001) for CD64). This effect was observed with cmvIL-10 for both CD32 and CD64 (median expression relative to control = 1.14 (p = 0.028) for CD32, 1.37 (p = 0.008) for CD64) but not for CD16. In contrast, LAcMvIL-10 had no effect on any of the three Fcy receptors at this time point. In addition, expression of the monocyte marker CD14 and of MHC class II (HLA-DR) were also measured (data not shown). Treatment of monocytes with hIL-10 and cmvIL-10 decreased cell surface HLA-DR expression within 24 h (median expression = 0.21 (p = 0.001) and 0.42 (p = 0.008) relative to control incubations respectively) although LAcMvIL-10 had no effect on HLA-DR expression at this relatively early time point (median expression relative to control = 1.19, p = 0.26). Human IL-10, and both of the HCMV IL-10 homologues, increased CD14 expression on monocytes following 24 h incubation, demonstrating the biological activity of the LAcMvIL-10 used in these experiments. It was concluded that cmvIL-10 increased expression of Fcy receptors CD32 and CD64 by monocytes, but that LAcMvIL-10 did not.

Having shown that cmvIL-10 increases expression of Fcy receptors, we next determined whether treatment with HCMV-encoded IL-10 homologues affected Fcy receptor mediated phagocytosis (Fig 2A). Monocytes purified from peripheral blood of healthy donors were treated for 24 h with either hIL-10, cmvIL-10 or LAcMvIL-10 before phagocytosis of IgG opsonised, PKH26-labelled human red blood cells (RBC) was measured. In experiments using monocytes purified from 5 independent donors, hIL-10 (median stimulation = 72.1%, p = 0.004) and cmvIL-10 (median stimulation = 43.5%, p = 0.04) both stimulated IgG-mediated phagocytosis whereas LAcMvIL-10 did not (Fig. 2B). Thus the effect of hIL-10 and HCMV homologues of hIL-10 on phagocytosis were consistent with their effects on Fcy receptor expression. It was concluded that like hIL-10, but unlike LAcMvIL-10, cmvIL-10 increased Fcy-receptor mediated phagocytosis by monocytes.

These experiments were performed at 24 h but as we had previously shown that LAcMvIL-10 decreased HLA-DR expression on monocytes at 48 h (Jenkins et al., 2008) we next examined the effect of HCMV IL-10 homologues on monocyte phenotype at later time points. In independent replicate experiments using monocytes from 2 different donors, LAcMvIL-10 decreased HLA-DR expression after 48 h and 72 h but still did not increase Fcy receptor expression (Table 1). These data reveal a role for both cmvIL-10 and LAcMvIL-10 in downmodulating HLA-DR expression by monocytes, but only cmvIL-10 increases expression of phagocytic receptors and phagocytic capacity by this cell type.

### Discussion

In this study, we have shown that cmvIL-10, like hIL-10, specifically alters monocyte phenotype by decreasing HLA-DR surface expression and increasing expression and function of the phagocytic receptors for the crystallisable fragment of human IgG, CD32A and CD64. One interpretation of these results is that monocyte differentiation is skewed towards a more phagocytic phenotype and away from an antigen presentation phenotype. This interpretation was strengthened by showing that both hIL-10 and cmvIL-10 treatment promoted phagocytosis of erythrocytes opsonised with IgG and is consistent with observations that hIL-10 skew the GM-CSF plus IL-13 induced...
differentiation of monocytes away from a dendritic cell like phenotype towards a macrophage-like phenotype (Allavena et al., 1998). In contrast, LAcmvIL-10, which is a homologue expressed during the latent phase of infection, did not increase expression of Fcγ receptors and decreased HLA-DR expression only at later time points. Thus this study extends our previous data demonstrating that LAcmvIL-10 does not retain the full repertoire of immunomodulatory functions encoded by cmvIL-10 or hIL-10 on myeloid cells or B cells (Jenkins et al., 2008; Spencer et al., 2008), highlighting different functions of these virally encoded hIL-10 homologues. The inability of LAcmvIL-10 to stimulate monocyte phagocytosis like cmvIL-10 may be a consequence of the truncated C-terminus of LAcmvIL-10 (Jenkins et al., 2004) which removes regions important for interacting with and signalling via the hIL-10 receptor (Jones et al., 2002). Indeed, LAcmvIL-10 has been reported to modulate HLA-DR expression on monocytes in a manner which may be independent of the hIL-10 receptor (Jenkins et al., 2008), which may explain the different kinetics of HLA-DR down modulation observed with LAcmvIL-10 and cmvIL-10 in the present study.

Human IL-10 has previously been shown to upregulate Fcγ receptor I (CD64) expression on peripheral blood monocytes and phagocytosis of IgG opsonised erythrocytes (te Velde et al., 1992; Capsoni et al., 1995). In these studies, however, unlike our present data with hIL-10, no increase in Fcγ receptor II or III expression (CD32 and CD16 respectively) was observed. It is difficult to compare our data

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**Fig. 1.** Expression of selected surface markers on monocytes stimulated with hIL-10 or cmv homologues of IL-10. Elutriated monocytes were cultured in hIL-10 or CMV encoded IL-10 homologues for 24 h, and the surface expression of CD16, CD32 and CD64 was then measured by immunostaining followed by flow cytometric analysis. Net MFI values were calculated by subtracting the median fluorescence of the isotype control from that of the antibody-stained sample; relative MFI values were calculated by dividing the net MFI of IL-10 treated monocytes by the net MFI of the relevant control, hIL-10 (left column), cmvIL-10 (middle column) LAcmvIL-10 (right column). Data were normalised to the expression measured in control incubations using purified, mock transformed E. coli extracts as described in Materials and methods (dotted line). Unique symbols are shown for each donor tested, and bars represent median values. Data were analysed using non-parametric Mann Whitney test. p values < 0.05 were considered significant.

**Fig. 2.** The effect of IL-10 and cmv homologues of IL-10 on monocyte phagocytosis. (A) Histograms showing phagocytosis of PKH26-labelled IgG-RBC by monocytes from a representative donor treated with (a) hIL-10 (b) cmvIL-10 and (c) LAcmvIL-10. For each panel, the negative phagocytic control (monocytes incubated at 4 °C with IgG-RBC) is shown as black broken line. Phagocytosis of unopsonised RBC is shown as a broken line in purple. The red solid line represents phagocytosis of IgG-RBC by control monocytes and the blue histogram represents phagocytosis of IgG-RBC by monocytes incubated with hIL-10 or cmv IL-10 homologue. (B) Fcγ receptor mediated phagocytosis by monocytes from 5 independent donors treated for 24 h with hIL-10 (black circles), cmvIL-10 (open circles) or LA cmvIL-10 (open squares). The % stimulation was calculated by subtracting % phagocytosis of the appropriate control monocytes (incubated with purified, mock transformed E. coli extracts as described in Materials and methods) from that of the treated monocytes and expressing as a ratio to phagocytosis of the controls. Data were analysed using non-parametric Mann Whitney test. p values < 0.05 were considered significant.
directly with that of te Velde et al. since data from only one experiment was shown in their paper. The effect of hIL-10 on Fcγ receptor II and III expression is relatively modest and donor dependent and may have been missed in their study. In the study reported by Capsoni et al. (1995) monocytes were cultured in RPMI containing 10% foetal calf serum, whereas we used Iscoves medium supplemented with 10% pooled human serum, a source of human M-CSF. Monocytes require a source of human M-CSF (or GM-CSF) for optimal survival which is lacking in foetal calf serum (SM Crowe, unpublished data). The response to hIL-10 may have been different in the two culture conditions or the effect of hIL-10 on monocytes may be modulated by M-CSF. This is consistent with the observation that hIL-10 increases expression of all three receptors (Fcγ RI, II, III) in monocytes cultured with exogenous M-CSF (Hashimoto et al., 1997).

The fact that cmvIL-10 increases both the expression and function of Fcγ receptors and down-modulates HLA-DR expression shows that HCMV alters the maturation pathway of monocytes in a similar manner to hIL-10 (Allavena et al., 1998). The advantage this confers for HCMV infection needs to be further investigated, however such activity may be advantageous to the spread of the virus if it subsequently suppresses immune responses to infection. Promoting a macrophage-like differentiation pathway in contrast to a dendritic cell pathway in infected tissues may prevent the migration of HCMV infected monocytes to draining lymph nodes or alter their ability to subsequently present viral antigens and stimulate T lymphocytes. In this context, it has been shown that the injection of suppressive cytokines such as hIL-10 prior to dendritic cell migration in a human skin explant model alters the subsequent maturation of dendritic cells to a CD14+ immature phenotype unable to efficiently stimulate T cells (de Grujil et al., 2006). The ability of HCMV-encoded homologues of hIL-10 to similarly inhibit monocyte function remains to be demonstrated. Given that monocytes are not productively infected with HCMV, and are required to differentiate to macrophages before being permissive to virus replication (Soderberg-Naucler et al., 2001), they would not be expected to secrete appreciable amounts of cmvIL-10 themselves. The source of this protein in infected tissues would therefore presumably be productively infected cell types such as endothelial cells or macrophages, resulting in a bystander effect on the monocytes. An important goal of future studies will be analysis of recombinant viruses lacking the capacity to express cmvIL-10 during productive infection of phagocytic cells such as macrophages so as to define the role of this viral protein on Fcγ receptor expression and phagocytosis in the context of direct virus infection.

Materials and methods

Materials

All antibodies used for flow cytometric analysis (CD14-FITC, CD16-PE-Cy5, CD32-PE, CD64-FITC and HLA-DR-PE) were purchased from BD Pharmingen. Recombinant hIL-10 was purchased from R&D Systems, cmvIL-10 and Lacmvl-10 were expressed and purified in house as described elsewhere (Jenkins et al., 2008). PKH26 was purchased from Sigma.

Monocyte purification

Buffy packs were obtained from the Australian Red Cross Blood Service from donors testing negative for HTLV, HIV, HBV and HCV antigens. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation and monocytes purified from this cell fraction by countercurrent elutriation at 2500 rpm, 12 °C using a Beckman J-6M/E centrifuge equipped with a JE 5.0 rotor. Cells were loaded into the elutriation chamber at a flow rate of 12 ml/min, lymphocytes eluted by extensive washing at 14–16 ml/min then monocytes were eluted at a flow rate of 18 ml/min. Purity of eluted monocytes was typically as assessed by light scatter analysis using flow cytometry, with a viability of >99%, assessed using trypan blue exclusion.

Culture of monocytes

Purified monocytes were cultured at a concentration of 2 × 10⁶/ml in complete Iscoves modified Dulbecco’s medium supplemented with 10% pooled heat-inactivated human serum (IH10) in 4 ml polypropylene tubes (Falcon) at 37 °C for 24 h unless otherwise indicated. hIL-10 was added at a final concentration of 10 ng/ml and IL-10 homologues at a final concentration of 100 ng/ml. Untreated cells incubated under identical conditions were used as a control for hIL-10. The IL-10 homologues used in this study were derived from transformed E. coli cultures and purified by ion exchange chromatography as previously described (Jenkins et al., 2008). To ensure that changes in monocyte phenotype or function were not due to factors present in the column eluate such as co-purified E. coli protein, mock transformed E. coli samples were carried through the same purification protocol in parallel with each homologue preparation, and equal volumes of the eluate used as a control. For all measurements made using HCMV homologues data were expressed as relative to the data obtained using monocytes treated with this control.

Monocyte phenotype analysis

Cells were washed once in Ca²⁺/Mg²⁺ free phosphate buffered saline containing 1% newborn calf serum (HyClone) plus 2 mM EDTA (FACS wash), then incubated with saturating concentrations of the relevant conjugated antibodies at 4 °C for 30 min. After staining, cells were washed once in FACS wash then fixed with 2% paraformaldehyde and analysed by flow cytometry using a FACScalibur (BD Biosciences) capturing a minimum of 5000 events per condition. Data were analysed using WEHI FACS Lab Weasal Software.

Flow cytometric phagocytosis assay

Purified monocytes, resuspended in RPMI medium supplemented with 2% newborn calf serum, were aliquoted into 4 ml polypropylene tubes (0.1 ml, 1 × 10⁶ cells/ml) and incubated with PKH26-labelled human red blood cells (RBC) opsonised with 1:2000 rabbit anti human red blood cell antibody (ICN Cappel) at a target to cell ratio of 10:1. Phagocytosis was allowed to proceed at 37 °C for 30 min then tubes were plunged into ice and uningested RBC were lysed by

### Table 1

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incubation for 10 min in FACS lysing solution (BD Biosciences). Monocytes were then washed once with ice cold FACS wash and fixed with 2% formaldehyde before flow cytometric analysis. As a negative control, monocytes were incubated with IgG opsonised PKH26-labelled RBC at 4 °C for 30 min or incubated with unopsonised PKH26-labelled RBC at 37 °C for 30 min. Internalised RBC were quantified by measuring PKH26 fluorescence in FL3. Baseline separation was achieved between cells which had ingested RBC and those which had not, so the percent phagocytosis was calculated from the number of events in each peak. The value of background phagocytosis, measured in the 4 °C incubated sample, was subtracted to obtain a net phagocytosis value. A stimulation index was calculated by comparing Fc phagocytosis to that of the relevant mock treated control cells.

Statistical treatment of data

Data were analysed using the one sample median test for non-parametric data and Stata software. p < 0.05 was considered significant.

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References


