Human cytomegalovirus viral IL-10 polarizes monocytes towards a deactivated M2c phenotype to repress host immune responses

Running title: HCMV viral IL-10 drives monocyte M2c polarization

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Several human cytomegalovirus (HCMV) genes encode products that modulate cellular functions in a manner likely to enhance viral pathogenesis. This includes UL111A, which encodes homologs of human interleukin-10 (hIL-10). Depending upon signals received, monocytes and macrophages become polarized to either classically activated (M1 pro-inflammatory) or alternatively activated (M2 anti-inflammatory) subsets. Skewing of polarization towards an M2 subset may benefit the virus by limiting the pro-inflammatory responses to infection and so we determined whether HCMV encoded viral IL-10 influenced monocyte polarization. Recombinant viral IL-10 protein polarized CD14⁺ monocytes towards an anti-inflammatory M2 subset with an M2c phenotype, as demonstrated by high expression of CD163 and CD14, and suppression of MHC class II. Significantly, in the context of productive HCMV infection, viral IL-10 produced by infected cells polarized uninfected monocytes towards an M2c phenotype. We also assessed the impact of viral IL-10 on heme oxygenase 1 (HO-1), which is an enzyme linked with suppression of inflammatory responses. Polarization of monocytes by viral IL-10 resulted in upregulation of HO-1 and inhibition of HO-1 function resulted in a loss of capacity of viral IL-10 to suppress TNF-α and IL-1β, implicating HO-1 in viral IL-10 induced suppression of pro-inflammatory cytokines by M2c monocytes. In addition, a functional consequence of monocytes polarized with viral IL-10 was a decreased capacity to activate CD4⁺ T cells. This study identifies a novel role for viral IL-10 in driving M2c polarization which may limit virus clearance by restricting pro-inflammatory and CD4⁺ T cell responses at sites of infection.
INTRODUCTION

Human cytomegalovirus (HCMV) is a highly species-specific betaherpesvirus that infects a majority of the world’s population, causing significant morbidity and mortality in neonates and in immunosuppressed individuals, including solid organ and allogeneic stem cell transplant recipients and individuals with HIV AIDS [1]. HCMV encodes a number of genes involved in modulation of the host’s immune system to aid virus survival and persistence. Examples include viral interference with apoptosis [2, 3] and suppression of viral antigen presentation through modulation of major histocompatibility complex (MHC) class I and MHC class II expression [4-7]. In addition, HCMV encodes a number of homologs of cell receptors and other cellular proteins, including homologs of G-protein coupled chemokine receptors and CXC chemokines (for a review, see McSharry et al. [8]).

The HCMV UL111A gene encodes homologs of the potent immunomodulatory cytokine human interleukin 10 (hIL-10) during both productive and latent phases of HCMV infection [9-12]. These homologs are collectively referred to as viral IL-10, and they exhibit a range of immunomodulatory functions, including suppression of pro-inflammatory cytokine production and dendritic cell maturation, and inhibition of MHC class I and class II expression (for a viral IL-10 review, see Slobedman et al. [12]). During latency, viral IL-10 restricts MHC class II surface expression by CD34+ myeloid progenitors, leading to the inhibition of CD4+ T cell recognition of latently infected cells [4, 13].

CD34+ myeloid progenitors undergo sequential differentiation into myeloid dendritic cells or blood monocytes. These monocytes can respond to different signals which drives rapid migration to virtually all tissues where they differentiate further into different types of macrophages which
play multiple roles in the immune response [14]. Cells of the monocyte/macrophage (mono/mac) lineage can be functionally polarized in response to endogenous stimuli into M1 “classically activated” and M2 “alternatively activated” subsets [15]. M1 mono/macs, activated mainly by stimulation with interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), are pro-inflammatory effector cells with a pivotal role in host defense against intracellular pathogens [16].

However, not all mono/macs acquire an M1 phenotype. Rather, an M2 alternatively activated mono/mac designation includes mono/macs phenotypically different from the M1 classically activated subset. These are characterized by increased phagocytic activity but suppressed production of pro-inflammatory cytokines and reduced killing capacity towards pathogens [15].

More recently it has been shown that there is more than one type of alternative activation of mono/macs, and this is now reflected in an expanded nomenclature for M2 alternative activation: M2a – induced by IL-4 and IL-13, M2b – induced by immune complexes and agonists of Toll Like Receptors (TLR), and M2c – stimulated by IL-10 and glucocorticoids [17]. It has also been suggested that IL-4 stimulated (M2a) macrophages be named wound-healing macrophages due to their capacity to secrete components of the extracellular matrix required for wound healing and activation of T\(_{h}2\) CD4\(^{+}\) T cell responses [16]. On the other hand, M2c alternatively activated cells are referred to as deactivators of the immune response [17] while M2b mono/macs are less understood and are known as a type II-alternatively activated subset [18].

In this study, we defined the influence of HCMV encoded viral IL-10 on monocyte polarization. We report that exposure of CD14\(^{+}\) monocytes to viral IL-10 resulted in the development of M2c alternatively activated cells. Furthermore, in the context of HCMV infection, viral IL-10 secreted from infected cells during productive infection induced the development of an M2c subset in...
bystander uninfected monocytes. The M2c polarization of monocytes by viral IL-10 resulted in upregulation of the anti-inflammatory enzyme heme oxygenase 1 (HO-1), and this was shown to play an important role in viral IL-10 mediated suppression of pro-inflammatory cytokines by M2c monocytes. Finally, we provide evidence that M2c monocyte polarization by viral IL-10 reduces the ability to stimulate CD4+ T cell activation and proliferation.

**METHODS**

**Cells**

Peripheral blood mononuclear cells (PBMCs) were derived from healthy human whole blood by Ficoll-Hypaque Plus (GE Healthcare) gradient centrifugation. CD14+ monocytes were isolated from PBMCs by positive selection with anti-human CD14 magnetic beads (Miltenyi Biotec) and maintained in RPMI-1640 media (Lonza) supplemented with 10% fetal calf serum (FCS; CSL). Purity of monocytes was typically >95% based on immunostaining for CD14 and flow cytometry analysis (data not shown). Human foreskin fibroblasts (HFFs) were propagated in RPMI-1640 supplemented with 10% FCS (CSL).

**Reagents for monocyte polarization experiments**

HCMV gene *UL111A* encodes two viral IL-10 homologs, cmvIL-10 and latency associated cmvIL-10 (LAcmvIL-10) [9-11]. Since deletion of UL111A in the viral IL-10 deletion viruses prevents expression of either variant of HCMV encoded IL-10, equal amounts of cmvIL-10 and LAcmvIL-10 recombinant proteins (collectively referred to as viral IL-10) were combined to
ensure that effects on monocyte polarization observed in the context of infection experiments were able to be compared with effects of purified recombinant proteins. Recombinant cmvIL-10, hIL-10 and hIL-4 proteins were purchased from R&D Systems. LAcmvIL-10 protein was generated as previously described [19]. All cytokines were used at a concentration of 100ng/ml. Lipopolysaccharide (LPS, Sigma Aldrich) was used at a concentration of 1µg/ml.

**Viruses**

Viral IL-10 deletion (vIL-10 del) virus RVAdIL10C has a *UL111A* gene deletion in HCMV strain AD169 [20]. Merlin-BAC *UL111A*-del virus was generated from the eGFP-expressing HCMV Merlin-BAC isolate pAL1160 [21] using bacterial artificial chromosome (BAC) recombineering protocols [22]. The selection cassette was generated using PCR primers: 5’-

GACGCGCAGTTGGGCCGCGGATGTTGGGCGCATGCTGCAGCGGAGCTCGGTACCG

GGGATC-3’-Forward; 5’-

GTAACTGGGTGAACGACATCGGAGCGCAGCATATATTGCGGAAAGTGGCCACC

TGTATGC-3’-Reverse. Correct insertion of the selection cassette replacing the *UL111A* gene was confirmed using primers: 5’-CCATCAAGTGGGACTGCAAAATCGCAACGGAAAAGTGGCCACC

CAACACCCACAAACGTC-3’-Reverse and by restriction digest of mutant BAC DNA with HindIII (New England Biolabs) to confirm the expected restriction pattern. Productive HCMV infection of HFFs was performed at multiplicity of infection (MOI) of 3 with samples harvested 24 hours post infection (pi).

**Immunostaining and flow cytometry**
A total of $10^5$ cells in fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline (PBS)/1% FCS/2 mM sodium azide) were incubated with anti-CD163-phycoerythrin (PE; BD Biosciences), anti-CD14- fluorescein isothiocyanate (FITC; BD Biosciences) and anti-HLA-DR–peridinin chlorophyll protein complex (PerCP; BD Biosciences) or their respective isotype control antibodies for 30 minutes. Cells were washed in FACS buffer and resuspended in 1% paraformaldehyde solution in FACS buffer. Data was acquired using FACSCanto flow cytometer (BD Biosciences) and analyzed by FlowJo software (BD Biosciences).

**Quantitative reverse transcription PCR (qRT-PCR)**

Total RNA was extracted from CD14+ monocytes using RNaqueous kit (Ambion) and DNase-treated prior to the reverse transcription step (SuperScript III kit, Invitrogen). mRNA expression was measured by qRT-PCR (Mx3000P qPCR system; Stratagene) at 50°C for 1 min, 95°C for 1 min, and then 50 cycles consisting of 95°C for 15 s and 60°C for 45 s. mRNA levels of test genes were normalized to mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used in this study were:

- GAPDH-F 5’-TCACCAGGGGTCTTTTTAC-3’
- GAPDH-R 5’-GACAAGCTTCGGTTTCTCAG-3’
- SLAM-F 5’-AGCAGGTCTCCCAGAAGAA-3’
- SLAM-R 5’-GCTCACGCGTGCAGATAGA-3’
- versican-F 5’-CCTTTCTGGAAGAACTCC-3’
- versican-R 5’-GGTCACATAGGAAGCGTGGT-3’
- SOCS3-F 5’-ATCCTGGTGACATGCTCCTC-3’
- SOCS3-R 5’-GCTCACGCGTGCAGATAGA-3’
- CCR7-F 5’-GATGCGATGCTCTCTCATC-3’
- CCR7-R 5’-TGTAGGGCAGCTGGAAGACT-3’
- fibronectin 1-F 5’-TGTTCGTGCAGCTTTTACC-3’
- fibronectin 1-R 5’-GCCACCGTAAGTCTGGGTTA-3’
TNF-α-F 5’-CCGTCTCCTACCAGACCAAG-3’, TNF-α-R 5’-CTGAGTCGGTCACCCTTCTC-3’, IL-1β-F 5’-GCTGAGGAAGATGCTGGTTC-3’, IL-1β-R 5’-GTGATCGTACAGG CATCG-3’.

Western blot

CD14+ monocyte lysates were analyzed by western blotting with a primary goat anti-human-HO-1 antibody (R&D Systems) and secondary rabbit anti-goat horseradish peroxidase (HRP) conjugated antibody (Dako). Primary rabbit anti-human-GAPDH antibody (Santa Cruz Biotechnology) with its secondary goat anti-rabbit HRP conjugated antibody (Cell Signalling Technology) were included as a loading control.

Treatment of CD14+ monocytes with zinc protoporphyrin

CD14+ monocytes were pulsed for 2 hours with a HO-1 inhibitor zinc protoporphyrin (ZnPP, 10 nmol/ml; Sigma Aldrich) prior to administration of viral IL-10 (100ng/ml) for 24 hours, followed by incubation with LPS (100ng/ml) for 3 hours to stimulate pro-inflammatory cytokine production.

Mixed leukocyte reaction

CD4+ T cells were selected from PBMCs with anti-human CD4+ magnetic beads (Miltenyi Biotec), resulting in >95% purity (observed by flow cytometry, data not shown).
carboxyfluorescein diacetate succinimidyl ester staining (CFSE, Invitrogen) was performed by incubating 1 x 10^7 CD4^+ T cells/ml in serum free RPMI-1640 containing 5µM CFSE for 10 min at 37°C, followed by quenching on ice for 5 min and three washes in 10 ml of serum free RPMI-1640.

CD14^+ monocytes were treated with PBS or with hIL-10 and viral IL-10 (100ng/ml, respectively) and mixed with 2.5 x 10^5 allogeneic CFSE labelled CD4^+ T cells in RPMI-1640 with 10% human serum at monocyte - CD4^+ T cell ratios of 1:5, 1:10, 1:20 and 1:50 in round-bottom 96-well plates. As a positive control for both activation and proliferation, CD4^+ T cells were treated with 12.5ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) and 250ng/ml ionomycin (Sigma Aldrich), while CD4^+ T cells cultured in absence of monocytes were used as a negative control. Induction of an early CD4^+ T cell activation marker CD69 was determined by immunostaining with anti-CD69-APC antibody (BD Biosciences) at 24 hours of co-culture, while detection of proliferating CD4^+ T cells was determined 5 days later by detection of a reduction in CFSE fluorescence intensity by flow cytometry.

**RESULTS**

**Treatment of monocytes with viral IL-10 induces expression of the M2c marker CD163**

To investigate the impact of HCMV encoded IL-10 on monocyte polarization, we cultured CD14^+ monocytes with recombinant viral IL-10 or hIL-10 protein (100ng/ml) for 24 hours. Cells were then immunostained for CD163, a scavenger receptor expressed at high levels on mono/macs exposed to hIL-10 [23, 24]. Flow cytometry analysis revealed an increase in the proportion of CD163^+ cells in monocyte cultures treated with either viral IL-10 or hIL-10, when
compared to PBS treated monocytes (Figure 1A). This increase was statistically significant, with approximately 90% of cells expressing CD163 following treatment with viral IL-10 or hIL-10 (Figure 1B). Similarly, analysis of CD163 mean fluorescent intensity (MFI, presented as a fold change in CD163 MFI relative to untreated monocytes) demonstrated a statistically significant upregulation of CD163 MFI following treatment of monocytes with either viral IL-10 or hIL-10 (Figure 1C). These data demonstrate that both viral IL-10 and hIL-10 strongly induce CD163 scavenger receptor expression by CD14\(^+\) monocytes.

**Viral IL-10 polarizes CD14\(^+\) monocytes towards an alternatively activated M2c phenotype**

To further characterize the impact of viral IL-10 on monocytes, we examined the cell surface phenotype of CD14\(^+\) monocytes treated with viral IL-10 protein in comparison to monocytes treated with stimuli which specifically induce different types of monocyte polarization; LPS (M1 classically activated monocytes), hIL-4 (M2a alternatively activated monocytes) or hIL-10 (M2c alternatively activated monocytes). The M2c alternately activated phenotype is typified by increased cell surface levels of CD14 and CD163 and decreased cell surface expression of MHC class II [15, 17, 25]. Following 24 hour incubation, CD14, CD163 and MHC class II (HLA-DR) surface expressions were analyzed by flow cytometry, revealing that viral IL-10 protein treatment induced an expression pattern closely matched to the M2c alternatively activated monocytes induced by hIL-10 (upregulated CD14, upregulated CD163 and downregulated HLA-DR, Figure 2A and 2B). Furthermore, this profile induced by viral IL-10 was significantly different to monocytes treated with hIL-4 (M2a) or LPS (M1) (Figure 2A and 2B). Specifically, surface expression of CD163, a key molecule associated with the induction of an anti-
Inflammatory environment [26, 27], was upregulated only by viral IL-10 and hIL-10, whereas hIL-4 (M2a) suppressed CD163 expression. As expected, M1 classically activated monocytes induced by LPS displayed minimal CD163 expression. The cell surface expression profiles mediated by viral IL-10, hIL-10, hIL-4 and LPS were consistent with viral IL-10 inducing an alternatively activated M2c phenotype in CD14+ monocytes. We observed a similar surface expression profile (upregulated CD14, upregulated CD163 and downregulated HLA-DR) when monocyte derived macrophages were polarized with viral IL-10 protein (Supplemental Figure S1).

In an extension of our analyses of cell surface markers associated with an M2c phenotype, we examined mRNA expression of signaling lymphocyte activation molecule (SLAM), versican and suppressor of cytokine signaling 3 (SOCS3), as these transcripts are upregulated by M2c alternatively activated mono/macs [17]. In comparison to PBS treatment, treatment of CD14+ monocytes with viral IL-10 for 24 hours resulted in significantly increased SLAM, versican and SOCS3 mRNA expression (Figure 3A). In contrast, expression levels of the M1 associated mRNA transcript CCR7 [28] and the M2a specific mRNA transcript fibronectin 1 [28, 29] were downregulated in viral IL-10 treated monocytes (Figure 3B). Together with the cell surface analyses, these data provide further evidence that viral IL-10 polarizes monocytes towards cells bearing the hallmarks of an M2c alternatively activated immunoregulatory phenotype.

Viral IL-10 expressed during productive HCMV infection induces an M2c phenotype in bystander monocytes
To extend the analyses of impacts on monocyte polarization by recombinant viral IL-10 proteins, we sought to determine whether viral IL-10 expressed during productive HCMV infection was able to modulate CD14+ monocyte polarization. CD14+ monocytes are refractory to the full HCMV replicative cycle [30, 31] and so to examine viral IL-10 function in the context of HCMV infection, we productively infected human foreskin fibroblasts (HFFs) and assessed the capacity of viral IL-10 secreted into the culture supernatant to modulate polarization of uninfected “bystander” CD14+ monocytes. To dissect the impact of viral IL-10 from other soluble factors secreted during productive HCMV infection, HFFs were infected at an MOI=3 with an HCMV UL111A deletion virus (RVAdIL10C), which replicates normally in HFFs but cannot express viral IL-10 [20], or its parental virus (AD169). Supernatants from mock infected HFFs were also analyzed. At 24 hours post infection, conditioned supernatants were added to freshly isolated CD14+ monocytes for another 24 hours, before these cells were assessed for cell surface CD14, CD163 and HLA-DR expression (Figure 4). In comparison to incubation of monocytes with supernatants from HFFs infected with the viral IL-10 deletion virus, monocytes incubated with supernatants from HFFs infected with parental virus displayed significant upregulation of CD14 and CD163 and downregulation of HLA-DR (Figure 4A and 4B). Similar results were obtained when experiments were repeated using the clinical strain Merlin-BAC and a viral IL-10 deletion virus, Merlin-BAC UL111A-del (Figure 4C).

To provide further evidence that the difference in monocyte polarization induced by supernatants from parental versus viral IL-10 deletion virus was due to the expression of viral IL-10 and not other soluble mediators that may influence monocyte polarization towards an M2c phenotype, we compared CD163 surface expression by these monocytes treated with infected cell supernatants in the presence or absence of a human interleukin 10 receptor alpha (hIL-10Rα)
neutralizing antibody (R&D systems, used at 25µg/ml). As shown in Figure 5, CD14+ monocyte
pre-treatment with the hIL-10Rα neutralizing antibody completely abrogated upregulation of
surface CD163 induced by supernatant from HFFs infected with parental HCMV (Figure 5A),
indicating that no other virus encoded soluble factors released by infected HFFs could induce
M2c polarization of uninfected bystander monocytes. We also performed this hIL-10R
neutralizing antibody experiment using recombinant viral IL-10, rather than infected cell
supernatants, to further confirm an essential role for viral IL-10 in the upregulation of CD163 by
CD14+ monocytes (Figure 5B). To exclude the possibility that HFFs respond to HCMV infection
by secreting hIL-10 that could then act to mediate the observed effects, we quantified secreted
hIL-10 protein levels by ELISA (R&D systems) in cultures of HFFs productively infected with
either parent or UL111A deletion viruses, and in mock infected cultures. In supernatants from 3
independent biological replicate experiments, no hIL-10 protein was detected in any of these
HFF cultures (sensitivity limit of 3.9 pg/ml).

Therefore, these results provide evidence that (i) viral IL-10 expressed during the productive
phase of HCMV infection polarizes bystander monocytes towards an M2c alternatively activated
phenotype and (ii) that this M2c phenotype is mediated via the hIL-10R.

Heme oxygenase 1 expression is upregulated by viral IL-10 treated monocytes and is
associated with viral IL-10 controlled suppression of pro-inflammatory cytokines

Heme oxygenase 1 (HO-1) is an enzyme linked with suppression of inflammatory responses
[32]. Cellular IL-10 is a potent inducer of HO-1 [33, 34]. To further explore the mechanistic
basis for viral IL-10 mediated polarization of monocytes to an M2c phenotype, we examined the
impact of viral IL-10 on HO-1 expression by measuring HO-1 protein by western blot in cell lysates derived from CD14+ monocytes treated with viral IL-10 protein, as well as uninfected CD14+ monocytes cultured with conditioned supernatants from HFFs productively infected with either parent (AD169) or viral IL-10 deletion (RVAdIL10C) viruses.

Recombinant viral IL-10 protein upregulated HO-1 protein by CD14+ monocytes in a manner comparable to that of hIL-10 (Figure 6A, B). In the context of HCMV infection, CD14+ monocytes cultured in conditioned supernatants from HFFs productively infected with parental virus displayed a modest but statistically significant increase of HO-1 protein in comparison to CD14+ monocytes cultured in conditioned supernatants from HFFs infected with viral IL-10 deletion virus (Figure 6C, D). Thus, recombinant viral IL-10 protein and viral IL-10 expressed in the context of productive infection functioned to upregulate HO-1 protein expression by uninfected monocytes.

HCMV encoded viral IL-10 can inhibit production of pro-inflammatory cytokines by PBMCs, monocytes and DCs [19, 35, 36]. We investigated whether viral IL-10 requires HO-1 for this inhibition of pro-inflammatory cytokine production. Monocytes were cultured with or without the HO-1 competitive inhibitor ZnPP prior to polarization with viral IL-10. Polarized monocytes were then stimulated to express pro-inflammatory cytokines by LPS treatment, and the ability of these cells to induce mRNA transcription of the pro-inflammatory cytokines TNF-α and IL-1β was examined. These analyses demonstrated that HO-1 induced by viral IL-10 is required for suppression of mRNA transcription of TNF-α and IL-1β in viral IL-10 polarized monocytes, as indicated by restoration of TNF-α and IL-1β mRNA transcription by treatment with the HO-1 inhibitor ZnPP prior to addition of viral IL-10 (Figure 6E, F). Monocytes treated with ZnPP in the absence of viral IL-10 treatment did not upregulate TNF-α and IL-1β mRNA compared to...
control (PBS treated) monocytes, demonstrating that ZnPP itself does not significantly alter expression of TNF-α and IL-1β. These results implicate viral IL-10 mediated upregulation HO-1 as being an important step in the inhibition of pro-inflammatory cytokines by viral IL-10 polarized M2c monocytes.

Viral IL-10 polarized M2c monocytes have a decreased capacity to stimulate CD4+ T cell activation and proliferation

To assess downstream functional consequences of M2c monocyte polarization by viral IL-10, we examined the ability of viral IL-10 polarized monocytes to induce allogeneic CD4+ T cell activation and proliferation in a mixed leukocyte reaction (MLR). CD4+ T cell activation was assessed by measuring the surface expression of the early T cell activation marker CD69 whereas CD4+ T cell proliferation was measured by CFSE T cell proliferation assay. CD14+ monocytes were treated either with PBS or polarized with viral IL-10 or hIL-10 (100ng/ml) for 24 hours prior to co-culture with allogeneic CD4+ T cells. CD4+ T cells cultured alone or stimulated with PMA/ionomycin in the absence of monocytes were included as negative and positive controls, respectively, for T cell activation and proliferation. These analyses revealed that CD4+ T cells co-cultured with monocytes treated with viral IL-10 or hIL-10 displayed significantly lower levels of CD69 expression compared to CD4+ T cells co-cultured with control monocytes treated with PBS (Figure 7A and B). Similarly, in comparison to CD4+ T cells co-cultured with monocytes treated with PBS, proliferation of CD4+ T cells was almost completely abolished when they were co-cultured with monocytes polarized with either viral IL-10 or hIL-10 (Figure 7C and D). Thus, human monocytes polarized with viral IL-10 were poor stimulators of CD4+ T
cell activation and proliferation, with the level of inhibition of T cell function by viral IL-10 polarized monocytes comparable to that of hIL-10 polarized M2c monocytes. Thus, viral IL-10 polarizes monocytes in a manner which inhibits the CD4+ T cell response.

**DISCUSSION**

Cells of the myeloid lineage represent a key component of the immune system required for the control and clearance of pathogens [16]. However, despite a strong host immune response, infectious HCMV often continues to be shed for many months following infection, arguing in favor of virus-mediated inhibition of the host immune response [1]. Indeed, HCMV encodes genes proposed to play important roles in limiting host defenses, including a number of homologs of cytokines, chemokines and their receptors [8]. Monocyte derived macrophages are capable of presenting antigens to CD4+ T cells and clearing intracellular pathogens through high expression of IL-12, IL-23, nitric oxide and reactive oxygen intermediates, but this effect is dependent on their functional polarization into M1 classically activated cells [16, 17]. Here we present evidence that HCMV encoded viral IL-10 modulates monocyte polarization and promotes an alternatively activated M2c phenotype, characterized by downregulated MHC class II and upregulated expression of molecules associated with anti-inflammatory functions, namely CD163 and HO-1. Functionally, viral IL-10 polarized monocytes were capable of inhibiting pro-inflammatory cytokine synthesis via upregulation of HO-1. In addition, these M2c monocytes were very poor stimulators of CD4+ T cell activation. Thus, viral IL-10 appears to fundamentally influence mono/mac polarization in a manner likely to enhance the capacity of HCMV to limit virus clearance by the host’s immune system.
CD163 is a member of the scavenger receptor cysteine-rich domain containing superfamily (SRCR), expressed predominantly on monocytes and macrophages [37, 38]. CD163 is a scavenger receptor for heme containing hemoglobin-haptoglobin (Hb:Hp) complexes [39]. Cell surface CD163 expression is significantly upregulated by the known inducers of M2c mono/mac polarization; hIL-10 [23, 24] and glucocorticoids [40, 41]. Our finding that CD14⁺ monocytes stimulated with viral IL-10 upregulated surface CD163 is consistent with viral IL-10 mediated promotion of an M2c phenotype. The downregulation of MHC class II (HLA-DR) by monocytes exposed to viral IL-10 provides additional evidence that viral IL-10 promotes formation of cells that are anti- rather than pro-inflammatory in nature.

Significantly, the capacity of viral IL-10 to mediate polarization towards an M2c phenotype was demonstrated in the infection setting when monocytes were cultured with supernatants from HFFs productively infected with parental viruses which could express viral IL-10, but not when monocytes were cultured with supernatants from HFFs productively infected with viral IL-10 deletion viruses (constructed using both laboratory and clinical viral strains). In addition, neither supernatants containing viral IL-10 (from HFFs productively infected with parental viruses) nor purified recombinant viral IL-10 proteins were able to induce surface CD163 on monocytes when the human IL-10 receptor was blocked. Whilst ELISA to quantify secreted viral IL-10 protein levels from infected HFFs was not available, as little as 200pg/ml of recombinant viral IL-10 was sufficient to strongly induce cell surface CD163 expression (data not shown). Our data indicate that, apart from viral IL-10 expressed during the productive phase of HCMV infection in HFFs, no other virus-derived soluble factor could directly induce an M2c phenotype in bystander monocytes. However, it is remains to be determined whether monocyte M2c polarization is due exclusively to the direct effect of viral IL-10, or whether viral IL-10 may also
induce the expression of cellular IL-10 by monocytes, which may add to the overall impact on M2c polarization.

The scavenging of the Hb:Hp complexes by CD163 plays a major role in dampening of the inflammatory response, predominantly mediated by end products of HO-1 mediated metabolism of heme from Hb:Hp complexes [42, 43]. Here we show that viral IL-10 upregulates HO-1 synthesis in CD14+ monocytes stimulated not only with recombinant viral IL-10, but also in bystander monocytes exposed to supernatant from cells productively infected with parental HCMV, but not in monocytes exposed to supernatant from cells productively infected with viral IL-10 deletion virus. Whilst control of pro-inflammatory cytokines by viral IL-10 appears to involve multiple pathways [44], our finding that expression of pro-inflammatory cytokines TNF-α and IL-1β was suppressed by viral IL-10 polarized monocytes, and that inhibition of HO-1 function abrogated this suppression, provides evidence that HO-1 plays an important role in the anti-inflammatory function of monocytes polarized by viral IL-10.

A functional consequence of M2c monocyte polarization by viral IL-10 identified in this study was the altered ability to stimulate CD4+ T cell responses, with both early events of CD4+ T cell activation as well as CD4+ T cell proliferation almost completely abolished when CD4+ T cells were co-cultured with viral IL-10 polarized monocytes. Thus, viral IL-10 exerts a striking impact on monocytes which is likely to enhance virus-mediated inhibition of CD4+ T cell immunity.

Whether the response of CD8+ T cells is similarly inhibited will be an important focus of future studies to further define the extent of the pleiotropic immunomodulatory functions of viral IL-10.

In addition to surface CD163 expression, continuous shedding of the extracellular domain of CD163 may result in release of soluble CD163 (sCD163), which has been linked with inhibition...
of T cell proliferation [45, 46]. However, we did not detect any significant increase in shedding
of sCD163 by M2c monocytes polarized with viral IL-10 nor bystander monocytes treated with
conditioned supernatants from HFFs productively infected with HCMV (data not shown), so the
inhibition of CD4⁺ T cell proliferation by viral IL-10 polarized M2c monocytes does not appear
to be dependent upon release of sCD163. It seems more likely that the suppression of surface
MHC class II expression by viral IL-10 polarized M2c monocytes results in limited antigen
presentation by these monocytes thus leading to reduced CD4⁺ T cell activation and
proliferation.

Whilst we examined monocytes exposed to recombinant viral IL-10 protein or to supernatants
from infected HFFs, it has been reported that monocytes directly exposed to HCMV display a
differentiation pattern with features of M1 polarization, although some transcripts associated
with M2 polarization were also upregulated [30, 47, 48]. Monocytes do not support productive
HCMV replication [31] and Chan and colleagues [30] confirmed in their study that at 4 hours p.i
no HCMV immediate early gene expression was detectable. Thus, whilst monocytes exposed
directly to HCMV acquire a unique M1>M2 polarization phenotype triggered by virus binding
and entry, bystander monocytes exposed to recombinant viral IL-10 proteins or viral IL-10-
containing supernatants from HCMV infected HFFs acquire a more definitive, functional M2c
phenotype, with no evidence of M1 polarization. These findings highlight a fundamental
difference in monocyte polarization which is dependent on whether polarization signals are
provided by direct virus infection or by interaction with extracellular viral IL-10.

Unlike monocytes, macrophages do support productive HCMV infection and recent work points
to fundamental differences in the nature of HCMV infection of M1 and M2 macrophages. For
example, M2 alternatively activated macrophages productively infected with HCMV are
ineffective in stimulation of natural killer (NK) cells, while M1 macrophages are able to efficiently promote NK cell-mediated IFN-γ secretion [49]. Furthermore, this and other studies reported more efficient HCMV replication in M2 alternatively activated macrophages compared to M1 classically activated macrophages [49-51]. Taken together with the findings we present here, the increased permissiveness of M2 macrophages to HCMV productive infection raises the intriguing possibility that viral IL-10 mediated polarization of monocytes to an M2c phenotype does not only result in deactivation of the CD4+ T cell function, but may also enhance the efficiency of subsequent HCMV infection when viral IL-10 polarized monocytes differentiate into M2c macrophages at sites of infection.

In conclusion, we have identified a novel role for viral IL-10 in the modulation of monocyte polarization towards an immunoregulatory, deactivated M2c phenotype. We propose that viral IL-10 mediated induction of an M2c phenotype is likely to enhance the capacity of HCMV to limit immune clearance by interfering with activation of other immune cells.

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Figure 1. Increased CD163 expression on CD14+ monocytes in response to hIL-10 and viral IL-10 protein treatment. CD14+ monocytes were treated with hIL-10 and viral IL-10 (100 ng/ml) or with PBS (negative control) for 24 hours. (A) Representative flow cytometry scatter plots of cells expressing CD163. (B) Graph depicting the percentage of CD163+ cells or (C) fold change (relative to PBS treatment) of CD163 mean fluorescent intensity (MFI). The number of independent biological replicate experiments (n) is shown. Error bars indicate the standard error of the mean. Significant differences between samples compared to PBS treatment were determined using a 1-tailed, paired Student t test and denoted by asterisks; * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

Figure 2. Human monocytes exhibit M2c alternatively activated phenotype in response to viral IL-10 protein treatment. CD14+ monocytes were treated with viral IL-10 (100 ng/ml), hIL-10 (100 ng/ml), lipopolysaccharide (LPS, 1 µg/ml) and hIL-4 (100 ng/ml) or with PBS (negative control) for 24 hours. (A) Flow cytometry histograms of human monocytes expressing CD14, CD163 and HLA-DR. Open histograms showing expression in treated samples compared to filled histograms that show expression in PBS treated samples. (B) Graph depicting the fold changes (relative to PBS treatment) of CD14, CD163 and HLA-DR mean fluorescent intensity (MFI) for hIL-10, viral IL-10, LPS and hIL-4 treated monocytes. The number of independent biological replicate experiments (n) is shown. Error bars indicate the standard error of the mean. Significant differences between samples compared to PBS treatment were determined using a 1-tailed, paired Student t test and denoted by asterisks; * p value < 0.05, ** p value < 0.01.
Figure 3. Viral IL-10 upregulates expression of M2c associated mRNA transcripts in human monocytes. CD14⁺ monocytes were treated with viral IL-10 (100 ng/ml) or with PBS (negative control) for 24 hours. (A) Fold mRNA expression change of M2c associated transcripts SLAM, versican and SOCS3 in monocytes treated with viral IL-10 (relative to PBS treated samples). (B) Fold mRNA expression change of M1 associated transcript CCR7 and M2a associated transcript fibronectin in monocytes treated with viral IL-10 (relative to PBS treated samples). The number of independent biological replicate experiments (n) is shown. Error bars indicate the standard error of the mean. Significant differences between samples compared to PBS treatment were determined using a 1-tailed, paired Student t test and is denoted by asterisks; * p value < 0.05, *** p value < 0.001.

Figure 4. Viral IL-10 expressed during productive HCMV infection polarizes bystander monocytes towards alternatively activated M2c phenotype. CD14⁺ monocytes were cultured for 24 hours in conditioned supernatants from HFF cultures productively infected with a viral IL-10 deletion virus (vIL-10 del), parental virus (Parent) or mock infection (Mock) for 24 hours. (A) Representative flow cytometry histograms of cells expressing CD14, CD163 and HLA-DR. Open histograms showing expression in monocytes cultured with conditioned supernatants from vIL-10 del and Parent (both in AD169 backbone) infected samples compared to filled histograms that show expression in monocytes incubated with conditioned supernatants from mock infected samples. (B) and (C) Graphs depicting the mean fluorescent intensity (MFI) fold changes in monocytes cultured with conditioned supernatants from vIL-10 del and Parent (B - AD169, C - Merlin) infected samples (relative to monocytes incubated with conditioned supernatants from mock infected samples) of CD14, CD163 and HLA-DR. The number of independent biological replicate experiments is shown. Error bars indicate the standard error of the mean. Significant differences between samples compared to mock infected samples were determined using a 1-tailed, paired Student t test and is denoted by asterisks; * p value < 0.05, *** p value < 0.001.
replicate experiments (n) is shown. Error bars indicate the standard error of the mean. Significant
differences between samples compared to mock infection were determined using a 1-tailed,
paired Student t test and denoted by asterisks; * p value < 0.05, ** p value < 0.01, *** p value <
0.001.

Figure 5. Blocking the human IL-10 receptor on CD14+ monocytes prevents surface CD163
upregulation by viral IL-10. CD14+ monocytes were pre-treated with anti-hIL-10R antibody
for 2 hours prior to incubation for 24 hours with (A) conditioned supernatants from HFF cultures
productively infected with HCMV strain AD169, or (B) recombinant viral IL-10 (100ng/ml).
Surface CD163 expression was compared to the surface CD163 expression from the identical
treatments but with anti-hIL-10R antibody omitted and treatment with PBS with anti-hIL-10R
antibody omitted (negative control). Error bars indicate the standard error of the mean.
Significant differences between samples compared to PBS treatment were determined using a 1-
tailed, paired Student t test and denoted by asterisks; * p value < 0.05.

Figure 6. Heme oxygenase 1 (HO-1) is upregulated in viral IL-10 polarized M2c monocytes
and plays a role in viral IL-10 driven suppression of pro-inflammatory cytokines. (A)
Western blot showing HO-1 protein levels in CD14+ monocytes treated with viral IL-10
(100ng/ml), hIL-10 (100ng/ml) or PBS (negative control) for 24 hours. Expression of GAPDH
was used as a protein loading control. (B) The fold change of HO-1 protein expression in
monocytes treated with recombinant proteins was determined by densitometry normalized to the
expression of GAPDH. (C) Western blot showing HO-1 protein levels in uninfected CD14+
monocytes cultured for 24 hours with conditioned supernatants from HFF cultures productively infected with viral IL-10 deletion virus (vIL-10 del), parental virus (Parent) or with supernatants from mock infected HFFs (Mock). Expression of GAPDH was used as a protein loading control. (D) The fold change of HO-1 protein expression in uninfected monocytes treated with supernatants was determined by densitometry normalized to the expression of GAPDH. (E) Quantitative RT-PCR based analysis of IL-1β mRNA and (F) TNF-α mRNA in LPS-stimulated human CD14+ monocytes treated with viral IL-10 (100 ng/ml), with or without the HO-1 competitive inhibitor ZnPP (10 nmol/ml). Graphs depict fold change of mRNA expression relative to cells treated with PBS. Error bars indicate the standard error of the mean. Significant differences were determined using a 1-tailed, paired Student t test and denoted by asterisks; * p value < 0.05, **** p value < 0.0001.

Figure 7. CD4+ T cell activation and proliferation is inhibited by viral IL-10 polarized M2c monocytes in mixed leukocyte reaction. Human CD14+ monocytes polarized with viral IL-10 (100 ng/ml) and hIL-10 (100 ng/ml) or with PBS (no polarization control) were cultured with CD4+ T cells in allogeneic setting for 24 hours prior to examination of an early T cell activation marker CD69, or for 5 days with CFSE labeled CD4+ T cells prior to assessment of CD4+ T cell proliferation by flow cytometry. CD4+ T cells with no monocytes added were used as a negative control while CD4+ T cells cultured with PMA/ionomycin were used as a positive control. (A) Representative flow cytometry scatter plots of CD4+ T cells expressing CD69. (B) Graph depicting the percentages of CD69+ CD4+ T cells. (C) Representative flow cytometry histograms showing percentage of proliferating CD4+ T cells. (D) Graph depicting the percentages of proliferating CD4+ T cells. The number of independent biological replicate experiments (n) is
shown. Error bars indicate the standard error of the mean. Significant differences between viral IL-10 and hIL-10 treated samples compared to PBS treated samples were determined using a 1-tailed, paired Student t test and denoted by asterisks; * p value < 0.05.
Figure 1

A) PBS hIL-10 viral IL-10

- PBS
- hIL-10
- viral IL-10

44% 90% 89%

isotype
2%
CD163
Forward scatter

B) C)

6 8 10 12 fold change

60 80 100

untreated hIL-10 viral IL-10

CD163 MFI

untreated hIL-10 viral IL-10

n=4 PBS untreated hIL-10 viral IL-10

***
Figure 3

A) mRNA fold change:
- SLAM
- versican
- SOCS3

B) mRNA fold change:
- CCR7
- fibronectin

PBS vs. viral IL-10 (n=3)
Figure 4

A) CD14, CD163, HLA-DR
- Parent
- vIL-10 deletion

B) MFI fold change
- AD169
  - Mock
  - Parent
  - vIL-10 del

C) MFI fold change
- MERLIN
  - Mock
  - Parent
  - vIL-10 del
Figure 5

A) 

B) 

mock
AD169
AD169 + hIL-10R blocking antibody

PBS
AD169
AD169 + hIL-10R blocking antibody
c

PBS viral IL-10 viral IL-10 + hIL-10R blocking antibody

n=3

n=3
Figure 7

A) 1 in 10 monocyte to CD4+ T cell ratio

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<tr>
<th>Condition</th>
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<th>hIL-10</th>
<th>viral IL-10</th>
<th>PMA/ionomycin</th>
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<tr>
<td>Forward scatter</td>
<td>1.8%</td>
<td>94.3%</td>
<td>8.9%</td>
<td>3.3%</td>
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<td>CFSE fluorescence</td>
<td>43%</td>
<td>33%</td>
<td>89%</td>
<td>94%</td>
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B) CD69+CD4+T cell proliferation

<table>
<thead>
<tr>
<th>Monocyte to CD4+ T cell ratio</th>
<th>PBS</th>
<th>viral IL-10</th>
<th>hIL-10 (M2c)</th>
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<tbody>
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<td>1 in 5</td>
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<td>*</td>
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<td>1 in 20</td>
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<td>1 in 50</td>
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C) CFSE fluorescence

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<th>Condition</th>
<th>PBS</th>
<th>viral IL-10</th>
<th>hIL-10</th>
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<td>2.0%</td>
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<td>3.5%</td>
<td>3.5%</td>
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D) CD4+ T cell proliferation

<table>
<thead>
<tr>
<th>Monocyte to CD4+ T cell ratio</th>
<th>PBS</th>
<th>viral IL-10</th>
<th>hIL-10 (M2c)</th>
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