

The immunology of Human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies?

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Abstract

Whilst the host immune response following primary human cytomegalovirus (HCMV) infection is generally effective at stopping virus replication and dissemination, virus is never cleared by the host and like all herpesviruses, persists for life. At least in part, this persistence is known to be facilitated by the ability of HCMV to establish latency in myeloid cells in which infection is essentially silent with, importantly, a total lack of new virus production.

However, although the viral transcription programme during latency is much suppressed, a number of viral genes are expressed during latent infection at the protein level and many of these have been shown to have profound effects on the latent cell and its environment.

Intriguingly, many of these latency-associated genes are also expressed during lytic infection. Therefore, why the same potent host immune-responses generated during lytic infection to these viral gene products are not recognised during latency, thereby allowing clearance of latently infected cells, is far from clear.

Reactivation from latency is also a major cause of HCMV-mediated disease, particularly in the immune compromised and immune naïve, and is also likely to be a major source of virus in chronic subclinical HCMV infection which has been suggested to be associated with long-term diseases such as atherosclerosis and some neoplasias. Consequently, understanding latency and why latently infected cells appear to be immunoprivileged is crucial for an understanding of the pathogenesis of HCMV and may help to design strategies to eliminate latent virus reservoirs, at least in certain clinical settings.

Introduction

Human cytomegalovirus (HCMV) is a paradigm for viral immune evasion strategies yet, paradoxically, primary infection of immunocompetent individuals rarely causes serious disease and such primary infections are normally quickly resolved. In contrast, infection of individuals whose immune systems are compromised (such as HIV/AIDS patients and transplant patients) or immature (such as the foetus in utero) often leads to wide spread viral replication and dissemination to multiple organs and this can often be life threatening (1) (2). Despite the success of the primary immune response in resolving primary infection, HCMV is never cleared. In part, this is due to the ability of the virus to establish a latent infection from which periodic viral reactivation is thought to facilitate lifelong viral persistence.

Whilst our understanding of the mechanisms by which HCMV evades host immune surveillance during lytic infection is substantial (3-7), far less is known about how this may be achieved during latent infection; yet it is likely that this may also be of real importance for the maintenance of viral latency. Historically, a long held view was that the relatively silent nature of a latent infection, with respect to viral gene expression, was, in itself, a mechanism of immune-avoidance. However, over the last few years, our understanding of latency-associated changes in the cell and cellular environment has increased and it is becoming clear that latent virus is far from quiescent; latent infection has profound effects on the cell and manipulates numerous cellular functions to optimise cell survival and latent genome carriage (8-10) and this results from latency-associated expression of a number of viral genes which are, more often than not, also expressed during lytic infection. Consequently, why these viral gene products are not targeted by the same potent host immune-responses generated during lytic infection to clear latently infected cells is a key question. This review examines the rationale and strategies for immune evasion by HCMV during latent infection and discusses how this knowledge could lead to potential immune interventions to target latent HCMV in patient groups where this might be particularly desirable.

Primary infection and the immune response to virus in lytic phase

Primary HCMV infection induces robust innate and adaptive anti-viral immune responses and in most cases does not cause serious disease (3, 11). For instance, primary HCMV infection results in a potent NK cell response (7) as well as the generation of humoral immunity, which includes neutralizing antibodies, which are specific for a number of viral proteins (12, 13). In addition to antibody responses, primary HCMV infection also results in the generation of both CD4+ and CD8+ T cells specific for a very broad range of viral proteins (3, 11, 14) at very high frequencies (Figure 1). It is recognized that primary infection of immunosuppressed individuals, or the immunonaive foetus *in utero*, leads to extensive viral replication in numerous cell types and, ultimately, end organ disease which can result in serious morbidity and in some cases mortality (15-18). Also, whilst infection of newborns (which are also considered immunologically immature) does not cause such serious morbidity as infection *in utero*, viral replication in the young may take a much longer time to be brought under control as evidenced by prolonged shedding of the virus in urine and saliva (19).

Taken together, these data provide reasonable evidence that the immune response mounted during primary infection is effective at limiting lytic viral replication and preventing serious disease. However, despite this, latency is always established and virus is never cleared (Figure 2). In some ways this is surprising as it is very clear that HCMV encodes numerous and sophisticated immune evasion mechanisms, so much so that it has become somewhat of a paradigm for how a human pathogen can avoid host immune responses (3). For instance, during lytic infection, specific genes encoded by HCMV can directly modulate innate immune responses such as the interferon responses (5) and NK cell recognition (7). The virus also encodes an immunosuppressive IL-10 homologue (20, 21), IL-10 is a powerful inhibitor of Th1 cytokines (such as IFN γ and IL-2) and also inhibits inflammatory cytokine production from monocytes and macrophages which results in a decrease in surface MHC Class II expression and a reduction of presentation of antigen to CD4+ T cells

(22). In addition, the virus also encodes proteins that act as receptors for host inflammatory cytokines, thereby reducing localized cytokine effectiveness by acting as cytokine sinks (6). Similarly, a number of HCMV encoded genes known to be expressed during lytic infection can interfere with both MHC Class I and II restricted antigen processing and presentation, thereby robustly inhibiting CD4⁺ and CD8⁺ T cell recognition (4, 23) as well as co-stimulatory T cell signalling (24) (Figure 3). On one hand then, HCMV expresses multiple immunevasins which are known to work potently *in vitro* but, even in the face of these, host immune response are still able to resolve primary HCMV infection. One view consistent with many of these observations is that these host anti-viral responses, which are able to resolve primary infection, are not able to target latent virus infection efficiently and this results in viral persistence, at least in part, involving periodic viral reactivation from a more immunologically privileged latent reservoir.

Although the immune evasion mechanisms employed by HCMV *in vitro* are very well documented, the effectiveness of these during primary infection *in vivo* is not absolutely clear and perhaps may be better seen as viral functions which allow the pathogen to initially overcome host immune responses and thus create a window of opportunity for the virus to replicate efficiently and disseminate to cell types where latency can be established. Such a strategy could be conceived to be optimal for a life-long persistent pathogen as unchecked viral replication, leading to host mortality, would clearly be a dead end strategy for any virus. However, the establishment of quiescence would also, in itself, be a biological dead end for any virus unless it was able to reactivate from this quiescent state and re-establish lytic infection in order to exit the host and establish an infection in naïve individuals. Similarly, a fitting time for a comprehensive set of immunevasion functions to be employed by the virus would be during reactivation from latency; these would again create a window of opportunity for the virus to re-establish the production of new virions in the face of an existing and primed anti-viral immune response. In the rest of this review we will discuss viral gene

expression during latency and how this latency-associated gene expression may aid immune evasion to allow maintenance of the latent reservoir.

Establishment of latency and the molecular biology of the latently infected cell

One important site in which HCMV is known to establish latency is in cells of the myeloid lineage. Latent viral genomes can be detected in peripheral monocytes (25) and also traced back to their CD34+ progenitors in the bone marrow (26) (27). Intriguingly, although CD34+ bone marrow progenitor cells are also the source of cells of the lymphoid lineage, there is no evidence of viral genome carriage in peripheral blood B or T cells (25). This may in part be explained by recent evidence suggesting that latent infection itself may result in some partial commitment of CD34+ progenitors to the myeloid lineage (28).

Consistent with cells of the myeloid lineage being sites of latent infection, analyses of the viral transcription programme in these cells generally shows a suppression of viral lytic gene expression (2, 29-32) but concomitant expression of known latency-associated viral genes (31, 33-37). Importantly, these cells do not produce infectious virions; an essential characteristic of latent infection. In latent myeloid cells *in vivo*, this suppression of the lytic transcription programme appears to involve repression of the viral major immediate early promoter (MIEP), which would normally drive lytic cycle, through post-translational modification of histones around the MIEP resulting in the presence of well characterised repressive chromatin marks (reviewed in (2)). Also, importantly, this latent transcription programme can be reactivated to lytic cycle by differentiation of latent CD34+ cells or monocytes to macrophages or dendritic cells (DCs) resulting in expression of the established lytic temporal cascade of viral gene expression, leading to viral DNA replication

and de novo virus production (36, 38-40). Crucially, this reactivation of the lytic cascade of gene expression is initiated by the expression of the major immediate early proteins (IE72 and IE86); IE gene expression, thus, acts as a master regulator to initiate lytic cycle ((41-44) and Figure 4)).

It is worth noting that many of these analyses of naturally latently infected cells can also be, in general, recapitulated in experimental models of latency *in vitro*. CD34+ progenitors, monocytes and granulocyte-macrophage progenitors (GMPs), as well as some established myeloid cell lines, can be infected in culture allowing the maintenance of latent viral genomes which can then be reactivated by differentiation signals (21, 30, 35-37, 45-47). Although it is clear that some experimental models using established cell lines do not appear to fully recapitulate all aspects of control of latency and reactivation observed in primary myeloid cells (48).

A totally quiescent viral genome during latent infection would clearly be the ideal way to avoid immune surveillance - if viral proteins are not expressed at all there would be no processing and presentation of viral antigens to specific T cells and, thus, latently infected cells would be ignored by the host immune response. However, recent work has shown that the viral gene expression is far from quiescent during latent infection and that expression of a number of viral transcripts, encoding viral proteins, routinely occurs (10, 47).

There is now much published data detailing expression of specific viral genes during natural or experimental latent infection in CD34+ cells or their myeloid derivatives. Transcripts expressed during natural latency are known to include RNAs from the major IE region (UL122-123 CLTs) (31) as well as UL81-82ast (LUNA) (34), UL138 (35), UL111a (33), UL144 (28) and US28 (49) but, more recently, additional latency-associated viral transcripts have also been identified (29). The detailed functions of these viral genes, where known, are beyond the scope of this article but have been described in recent reviews (10, 47).

However, the potential immune evasion functions of UL111a and US28 will be discussed later.

HCMV, as with many other herpes viruses, have been shown to encode a wealth of microRNAs (miRNAs) with the potential to orchestrate both cell and viral gene regulation (50). During lytic infection HCMV expresses approximately 24 miRNAs derived from 13 pre-miRNAs which have been shown to target both viral and cellular RNAs. Viral targets include IE72 as well as a number of viral genes involved in DNA synthesis and it has been suggested that these targets might play some role in latency establishment and reactivation (51-53) although, as yet, there is no direct evidence for this. Cellular targets of HCMV-encoded miRNAs include gene products with functions relating to control of cell cycle, secretory cellular pathways and, of particular interest, immune evasion (54-56). Most of these targets have been analysed during lytic infection and, again, are outside the scope of this article but have recently been reviewed (50). However, we will discuss the role of viral miRNAs in targeting gene expression associated with immune evasion mechanisms below.

Whilst much is known about the interdiction in cellular and viral gene expression by viral miRNAs during lytic infection, there is a dearth of information regarding the expression of viral miRNAs during latent infection; yet it could be argued this might be the most opportune scenario for viral miRNA functions. For instance, it could allow an orchestrated manipulation of cellular gene expression without the need to express viral proteins which, otherwise, could be targeted by host immune mechanisms. Reports using a quiescent THP1 model infected with either Towne (57) or Toledo (58) isolates of HCMV have identified a number of expressed viral miRNAs, although their functions during quiescence was not addressed and, at least for Towne, it is unclear how this might inform us as Towne does not usually efficiently infect myeloid cells (59) and is depleted of some important viral coding regions (60). In our attempts to address this, we have used RT-qPCR to detect viral miRNAs expression in primary CD34+ myeloid progenitors and CD14+ monocytes experimentally latently infected with a clinical isolate of HCMV. These studies have shown that a number of

viral miRNAs are indeed expressed during latent infection but, whilst their targets and functions during latency are unclear, early evidence suggests that some may be involved in manipulating myeloid differentiation (IHW Kobe, Japan 2014 - Betty Lau, unpublished observations).

The view that latent infection has profound effects on the latently infected cells has come from recent work analysing experimentally latent primary CD34+ cells. These studies have shown that latent infection results in the modulation of the cell secretome (61), in part mediated by latency-associated modulation of the cell miRNAome (62). In more detail, during latency the cellular miRNA, has-miR-92a, is downregulated and leads to an upregulation of the transcription factor GATA2 and subsequent increased expression of cellular IL-10 (cIL-10) which is of particular interest in the context of immune evasion strategies and latency (62). Interestingly, quiescent infection of THP1 cells has also been shown to alter cellular miRNA expression (58).

Thus, it is clear that latent infection is far from a passive interaction between the virus and the cell; latent infection results directly in numerous changes in the cell phenotype which likely optimise the cell for latent carriage and reactivation. It is also likely that expression of latency-associated viral miRNAs and modulation of the cellular miRNAome plays a major part in orchestrating such changes in cellular gene expression and this strategy circumvents expression of viral functions which would be normally be surveilled by host immune responses. However, latent infection also results in expression of a number of virus encoded proteins which would be predicted to lead to e.g. T cell recognition unless latent functions also include viral strategies to mediate immune evasion.

Immune evasion strategies during latent infection

(i) virally encoded miRNAs and proteins

Our understanding of the functions of specific viral genes during lytic infection may inform us of their potential functions during latency, if their expression is also latency-associated.

Three viral miRNAs have been shown to target components of the immune system during lytic infection. miR-UL112.1 targets MICB a cellular stress (infection) induced ligand which usually functions by binding the homodimeric NK cell activating receptor NKG2D (54); miR-US4.1 downregulates ERAP-1 which is an amino-peptidase which trims peptides for presentation by MHC Class I and reduced ERAP-1 expression decreasing HCMV specific CD8+ CTL recognition of HCMV infected cells (55); miR-UL148D targets the chemokine CCL5 (RANTES) which is a T cell chemoattractant (56).

Interestingly, miR-UL112.1 and miR-US4.1 have been detected in quiescently infected THP1 cells (57, 58) and we have also detected all three viral mRNAs in experimentally latent CD34+ cells and CD14+ monocytes (IHW Gdansk, Poland 2012 and CMV meeting San Francisco, USA 2013 Lau et al unpublished). Consequently, there is a good likelihood that these viral miRNAs may also have some role during latency to help mediate avoidance of the host immune response. However, direct confirmation of this during latency awaits more detailed investigation.

Three HCMV proteins, US28, UL111A, and UL144 also known to be expressed in latently infected cells, have known immune evasion functions, at least during lytic infection, which could be equally well employed during latent infection. US28 is a G-protein coupled receptor that can bind both CC (eg CCL5, MCP-1, MCP-3, MIP1- α , and MIP1- β) and Cx3C (eg fractalkine) chemokines. The ability of US28 to bind multiple cytokines and cause their internalization, allows it to act as a chemokine sink, reducing the local concentration of these inflammatory and chemotactic cytokines during lytic infection in vitro, although transcripts for US28 can be detected in latency, the level of protein expression and thus its potential to act as a cytokine sink has not yet been investigated (63). Similarly, a latency-associated splice product of UL111A encodes a viral IL-10 homologue (LAVIL-10) and acts to downregulate

MHC Class II expression on experimentally latently infected myeloid cells and this modulates CD4+ T cell recognition (20, 64, 65). UL144 has sequence similarity with members of the tumour necrosis factor receptor superfamily (TNFRSF) (66-68) as well as the herpes simplex virus entry mediator (HVEM). However, UL144 is unlikely to act as a decoy TNF receptor and there is no evidence that UL144 binds TNF (69). UL144 has two putative immune evasion functions; the ecto-domain has been shown to interact with B and T lymphocyte attenuator (BTLA) and inhibits T cell proliferation *in vitro* (70) and the intracellular domain signals via NFkB, TRAF6 and TRIM23 to induce the chemokine CCL22 which acts as a TH2 chemoattractant possibly subverting the TH1 immune response (69, 71, 72).

(ii) Manipulation of the cellular microenvironment by changes in the cell secretome

HCMV lytic infection is also known to induce profound alterations in levels of secreted cellular proteins (secretome) and this includes a number of chemokines and cytokines with immune functions (73, 74). However, until recently, little was known about latency-associated changes in the cell secretome. The possibility that viral manipulation of secreted cellular proteins would likely be an effective mechanism to modify the microenvironment around latently infected cells to help maintain life-long carriage of latent virus in the face of constant immune surveillance has been investigated by us and others.

Such studies showed that experimental latent infection of granulocyte macrophage progenitor cells (GMPs) attracted CD14+ monocytes and that this was mediated by an increase in CCL2 (MCP-1) expression by the latently infected GMP (21). Subsequently, a comprehensive analysis of the secretome of experimentally latently infected CD34+ progenitor cells identified changes in numerous secreted cellular proteins which are known to be involved in both the regulation of the immune response and chemo-attraction (61). In these studies, a latency-associated increase in the chemokine CCL8, perhaps counterintuitively, resulted in the recruitment of CD4+ T cells to latently infected cells.

However, these supernatants also had substantial increases in levels of the immune suppressive cytokines cIL-10 and TGF β which were sufficient to inhibit anti-viral IFN γ and TNF α cytokine secretion as well as cytotoxic effector functions of HCMV-specific Th1 CD4+ T cells. An additional interesting aspect was that uninfected bystander CD34+ cells were also induced to express TGF β and cIL-10 by the secretome from latently infected cells. In essence, the microenvironment around latently infected CD34+ cells was heavily immunosuppressive (Figure 5). Recent evidence has also shown that experimentally latent CD14+ monocytes modulate the cellular responses to innate stimuli such as type I and II interferon's by disrupting signaling via STAT1 (75).

(iii) Latency-associated proteins and T cells responses

Viral proteins expressed during latency appear to have important roles in maintaining latency and assisting in viral immune evasion. However, why these viral proteins don't act as target antigens to allow host T cells to detect and kill latently infected cells is an important question. Could it be that T cells specific for these viral proteins are, for some reason, never induced following HCMV infection?

Recently, examination of T cell responses to four viral proteins expressed during latency (LUNA and UL138, US28 and LAvIL-10) have shown that this is not the case - T cell responses specific for all four proteins are, indeed, detectable in healthy HCMV positive donors (76). Intriguingly, all responses identified appeared to be mediated by CD4+ T cells - no CD8+ T cell responses were detected and this is broadly in agreement with a separate analysis of LUNA and UL138-specific T cell responses where no CD8+ T cell responses to LUNA and only a single peptide from UL138 presented via a particular class I allele (HLA-B35) were detected (77). It is not clear why this bias towards CD4+ T cell responses to proteins expressed during latency occurs, although it has also been observed in the T cell response to HCMV encoded glycoprotein B (78). It would be informative to examine the

primary responses to latency-associated proteins such as UL138 and LUNA and follow them longitudinally after primary infection with respect to selection of T cells into memory. This would help determine if CD8+ T cells are initially generated but are then lost or if they are never generated in the first place. UL138, but not LUNA, specific CD4+ T cells were also shown to be able to mediate MHC class II restricted cytotoxicity and, importantly, they also recognize latently infected autologous monocytes. Importantly, a proportion of the UL138, LUNA, US28 and LAcvIL-10 specific CD4+ T cells also secreted the immunomodulatory cytokines IL-10 and TGF- β (61) and this may help explain why T cell recognition of these latently expressed proteins, *in vivo*, does not result in their elimination; the CD4+ T cell populations which recognise these latent antigens are predominantly regulatory and, in combination with the suppressive microenvironment produced by the latently infected cell, appears to limit the function of anti-viral IFN γ /TNF α and cytotoxic Th1 CD4+ T cells (Figure 5). A similar mechanism has been described for the latent EBV antigen LMP1 which also gave rise to IL10 producing regulatory cells (79). Similarly, in MCMV infection, IL10 producing CD4+ T cells have been isolated from salivary glands and, in IL-10 KO mice or following IL-10R blockage the latent MCMV load is reduced. This is consistent with the view that cytomegalovirus uses regulator T cell to prevent latently infected cells from being recognised by the immune system (80, 81).

Is immune targeting of latently infected cells possible and in what patient groups would it be most effective?

It is now clear that reactivation of virus from latent reservoirs is a major cause of the HCMV-mediated disease observed in the transplant setting where patients are immunosuppressed. Increasingly, it is also recognised that HCMV seronegative women are not the only group that are at risk during pregnancy - reactivation in HCMV seropositive carriers can also be a

source of virus transmission to the foetus during pregnancy (82). Similarly, there are data which suggest that subclinical HCMV infection may be associated with long-term diseases such as atherosclerosis, chronic graft rejection and neoplasias – where, clearly, reactivation is likely to be a major source of infectious virus (83).

(i) The transplant setting

Following solid organ transplantation (e.g. liver, kidney, heart or lung), transfer of latent HCMV in the graft from a seropositive donor (D+) to a seronegative recipient (R-) frequently leads to serious HCMV disease in these patients who are also receiving immunosuppressive treatments (16). Similarly, although not associated with the same risk, D+/R+ or D-/R+ transplant patients also suffer from intermediate risk of HCMV disease often from virus reactivation.

Bone marrow transplantation (BMT) and peripheral hematopoietic stem cell transplantation (HSCT), often as part of the therapy for haematological malignancies, has steadily grown over time and, as with solid organ transplant patients, reactivation of latent HCMV often leads to serious morbidity and mortality in these immunosuppressed patients. Importantly, in allogeneic HSCT, the serostatus of the graft donor determines whether latent virus could be transferred to the recipient, as well as determining if HCMV antigen specific T cells are transferred as well. Therefore, the greatest risk of HCMV disease in HSCT occurs in a D-/R+ transplantation scenario, because the graft from the seronegative donor does not contain antigen-experienced HCMV-specific T cells. In this situation, reactivation of the recipient's latent virus goes unchecked due to immune ablation of the recipient as well as immunosuppressive treatment, post-transplant, to prevent GvHD. It might be expected that the immune ablation regime, itself, should remove all myeloid lineage cells carrying latent virus and, as a consequence, clear latent viral carriage. However, clinical evidence shows that this group of patients still undergo viral reactivation which, in the absence of an immune response, is able to replicate and disseminate. Therefore, it is likely that the latent reservoir

is not fully destroyed by the ablation regime. In contrast, studies consistently report that almost all D+/R+ transplantations show early and sustained reconstitution of pp65 tetramer positive T cells (84-87).

(ii) Reactivation during pregnancy

HCMV infection in utero is estimated at between 0.4-0.7% of live births in the USA and a similar rate in European countries (18). Whilst the greatest risk for HCMV disease of the foetus is accepted to be primary infection of naïve mothers, the risk of maternal-to-fetal transmission is substantially lower in seropositive women (88) suggesting that pre-existing immunity is beneficial. The incidence of congenital HCMV infection does, however, correlate with HCMV seroprevalence (82, 88-90). Consequently, non-primary infections caused by either re-infection of, or reactivation in, HCMV seropositive mothers are also likely to be a cause of congenital infection despite preformed HCMV specific maternal antibodies.

Although the relative contribution of virus reactivation or re-infection to congenital infections is not clear (91), prevention of infection in either scenario would be beneficial. Whilst this could be achieved through education of pregnant mothers (e.g. in hygiene measures), any potential therapy to reduce the latent viral reservoir during pregnancy could also reduce the risk of congenital infection from virus reactivation.

Clearly, then, there are a number of clinical settings in which the ability to therapeutically target latently infected cells could have far reaching implications. Drug based interventions that target specific aspects of the latent virus life cycle are an attractive possibility and the recent discovery that viral UL138, which is expressed during latent infection, is able to inhibit a cellular drug transporter has led to an exciting proof of principle that it is possible to chemotherapeutically target latently infected cells (92).

Our increasing understanding of changes in latently infected cells, and the likelihood that HCMV also needs to employ specific latency-associated functions to proactively suppress

host immunosurveillance during latency, argues that latent infection might also be targeted by manipulating or perturbing these specific mechanisms employed by HCMV to prevent immune detection by the host during latent carriage.

Which patient groups would lend themselves most easily this type of immunotherapeutic approach? In the case of HSCT, there would be an opportunity to manipulate the CD34+ cells *in vitro* prior to transplantation to remove cells carrying latent virus. This would be particularly attractive in a D+/R- transplant scenario. However, this approach might be more difficult in the case of a solid organ transplant where e.g. efficient perfusion might be problematic. Regardless, both of these could be carried out on donor material prior to transplant. Clearly, in the case of reduction of the latent load during pregnancy to limit reactivation, any therapeutic would need to be delivered, as would strategies to reduce recipient latent load prior to transplant.

Removal of latent cells from the HSCT graft

Could the T cells specific for viral proteins expressed by latently infected cells be harnessed to target and reduce the latent virus load? Prior to HSCT, the CD34+ graft is routinely manipulated to remove T cells in order to minimise graft versus host disease (GvHD). In the scenario of an HCMV seropositive donor and an HCMV seronegative (D+/R-), when the graft itself carries latent virus, it could be feasible, at the same time, to eliminate or drastically reduce the latent viral load prior to transplantation to reduce the level of reactivatable virus.

On the basis that anti-viral CD4+ (T_{h1}) T cells are generated to viral proteins expressed by latently infected cells; that the block to the effective functioning of these cells appears to be the presence of antigen specific immune suppressive cIL-10/TGFβ secreting T cells (76) and that latently infected cells also produce an immunosuppressive cytokine milieu (61), we speculate that antibody neutralization of cIL-10/TGFβ in combination with the depletion of regulatory T cells might allow Th1 type CD4+ latent antigen specific T cells to recognize and

kill latently infected CD34⁺ cells in the graft (Figure 6). T cell depletion of the graft to reduce GVHD would then ensue, resulting in a CD34⁺ cell population depleted of latently infected cells which would limit the amount of virus capable of reactivating from the graft after transplant. In the D-R+ HSCT setting, this approach would not be helpful as the graft does not contain latent virus and, as such, a different approach would be required. For instance, the induction of some aspect of immunogenic lytic gene expression, in the absence of viral immune evasins, in the latent reservoir of the recipient could be one approach - and this is discussed in the next section. For obvious reasons, harnessing the latent antigen specific T cells to kill latently infected CD34⁺ cells in this way would likely not be feasible for a solid organ graft. Similarly, it is difficult to how this approach could be employed to target the latent reservoir during pregnancy.

Induction of immunogenic lytic genes in the absence of immunomodulation

As discussed earlier in this review, specific T cell responses to viral immediate early proteins are mounted by most HCMV seropositive individuals and are high frequency. In healthy HCMV carriers, these T cell responses routinely comprise both CD4⁺ and CD8⁺ T cells which both have antiviral and cytotoxic activity. While these T cells are clearly effective at controlling dissemination of viral infection following reactivation, they are of little use in detecting latently infected cells which do not express IE proteins; not only is the MIEP heavily suppressed in these cells to minimise IE RNA transcription but our own unpublished data suggests that expression of the viral UL112-1 miRNA functions to minimise translation of any IE RNA which escapes MIEP transcriptional repression (IHW 2013 Grand Rapids, USA Lau et al unpublished observations). If cells carrying latent HCMV could be induced to transiently express viral immediate early proteins, in the absence of full virus reactivation, this may allow the endogenous antiviral T cell response to recognize latently infected cells expressing IE antigen in this untimely fashion and kill them.

It has been recognized for some time that inhibition of histone deacetylase activity in a non-permissive cell type that is carrying HCMV genome leads to IE promoter activity, but importantly not full virus reactivation (93). Consequently, this might be used as an approach to render cytomegalovirus latently infected cells transiently visible to the host immune response (Figure 6). In initial pilot experiments we have been able to induce IE gene expression using HDAC inhibitors in latently infected monocytes (Poole et al unpublished observations) and these cells, transiently expressing IE1 protein are recognised by IE specific T cells on the basis of IFN γ secretion. Importantly, there are already a number of HDAC inhibitors which are licensed for use in patients (94). Consequently, we think it feasible that treatment of patients with such agents could result in transient reactivation of IE gene expression *in vivo* allowing latently infected cells to be identified and eliminated by existing IE-specific CTLs. Clearly, such novel immunotherapies would be applicable to a much wider group of patients and require no *ex vivo* manipulations of latent cell populations. It is worth noting that similar approaches are currently being tested in cell populations latently infected with HIV-1 to promote "reawakening" of latent HIV gene expression to allow these cells to be recognized and eliminated by HIV specific T cells (95-97).

There is a very clear rationale behind reducing the latent load within an individual person to limit viral reactivation, however this clearly needs to be balanced by the risks that such an intervention may carry with it. In the case of particular clinical settings, such as immunosuppression in transplantation, we would suggest that this balance is in favour of using chemotherapeutic and/or immunological techniques to reduce the latent reservoir. In other settings, such as pregnancy, while the benefits might be desirable much greater caution with regard to the particular intervention would need to be exercised.

We believe that continued detailed analyses of viral gene expression during HCMV latency is important for a complete understanding of how latent virus manipulates the cellular environment in order to maintain latency while evading immune recognition. This can only

help in the design of effective and safe novel strategies to target and clear latent virus that could be applied in certain clinical settings.

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Figure Legends

Figure 1

Following primary infection, HCMV replicates and disseminates during which time the host generates an effective immune response which includes natural killer cells, neutralizing antibodies and a high frequency of CD4+ and CD8+ T cells. This eventually controls viral replication and resolves the primary infection.

Figure 2

HCMV replicates and disseminates leading to infection of myeloid progenitors and the establishment of latent infection in e.g, CD34+ bone marrow progenitor cells. Reactivation of

virus from these sites followed by new virus replication and productive replication induces secondary immune responses.

Figure 3

During lytic infection, HCMV expresses numerous viral proteins which mediate immune evasion. These include viral genes which interfere with host interferon responses, natural killer cell recognition (eg UL16, 18, 40, 141, 142, US18, US20) as well as CD4+ and CD8+ T cell recognition by preventing MHC Class I and II antigen processing and presentation (eg US2-US11). Other viral genes, such as the viral IL-10 homologue (UL111a) as well as viral proteins that act as receptor sinks for host inflammatory cytokines (US28), aid in general suppression of the host immune responses.

Figure 4

HCMV can establish latency in CD34+ myeloid progenitor cells and is carried down the myeloid lineage. In latently infected CD34+ cells and CD14+ monocytes there is a targeted suppression of lytic viral gene expression and generally undetectable levels of major IE proteins. However, expression of a number of latency-associated genes is detectable. These include transcripts from the major IE region (UL122-123 CLTs), UL81-82ast (LUNA), UL138, UL111a, UL144 and US28 although, more recently, other RNAs have been identified (28). Differentiation of these cells to macrophages and mature dendritic cells (mDCs) causes the de-repression of the MIEP and allows initiation of the lytic transcription programme which involves a temporal cascade of viral gene transcription and translation (consisting of immediate early, early and late gene products), allowing viral DNA replication and reactivation of de novo virus production.

Figure 5

Latently infected CD34+ cells produce a secretome high in immunosuppressive cytokines such as cellular IL-10 (cIL-10) and TGF β which act to inhibit anti-viral CD4+ cytokine and cytotoxicity (Th1) cell effector function. Uninfected bystander CD34+ cells are also induced to secrete cellular IL-10 (cIL-10) and TGF β further enhancing the immunosuppressive microenvironment. Antigen specific CD4+ regulatory T cells also recognize latent viral proteins and secrete their own cIL-10 and TGF β which also generates an immunosuppressive microenvironment, so helping to prevent clearance of latently infected cells.

Figure 6

Neutralization of the immunosuppressive cytokines cIL-10 and TGF β and/or depletion of regulatory T cells could allow latent viral proteins to be recognized by anti-viral CD4+ (Th1) effector cells.

Treatment of latently infected cells with HDAC inhibitors could also allow transient expression of viral IE proteins which, after processing and presentation by Class I MHC, would be predicted to allow IE-specific CD8+ cytotoxic T cells (CTLs) to now recognize cells containing latent virus.

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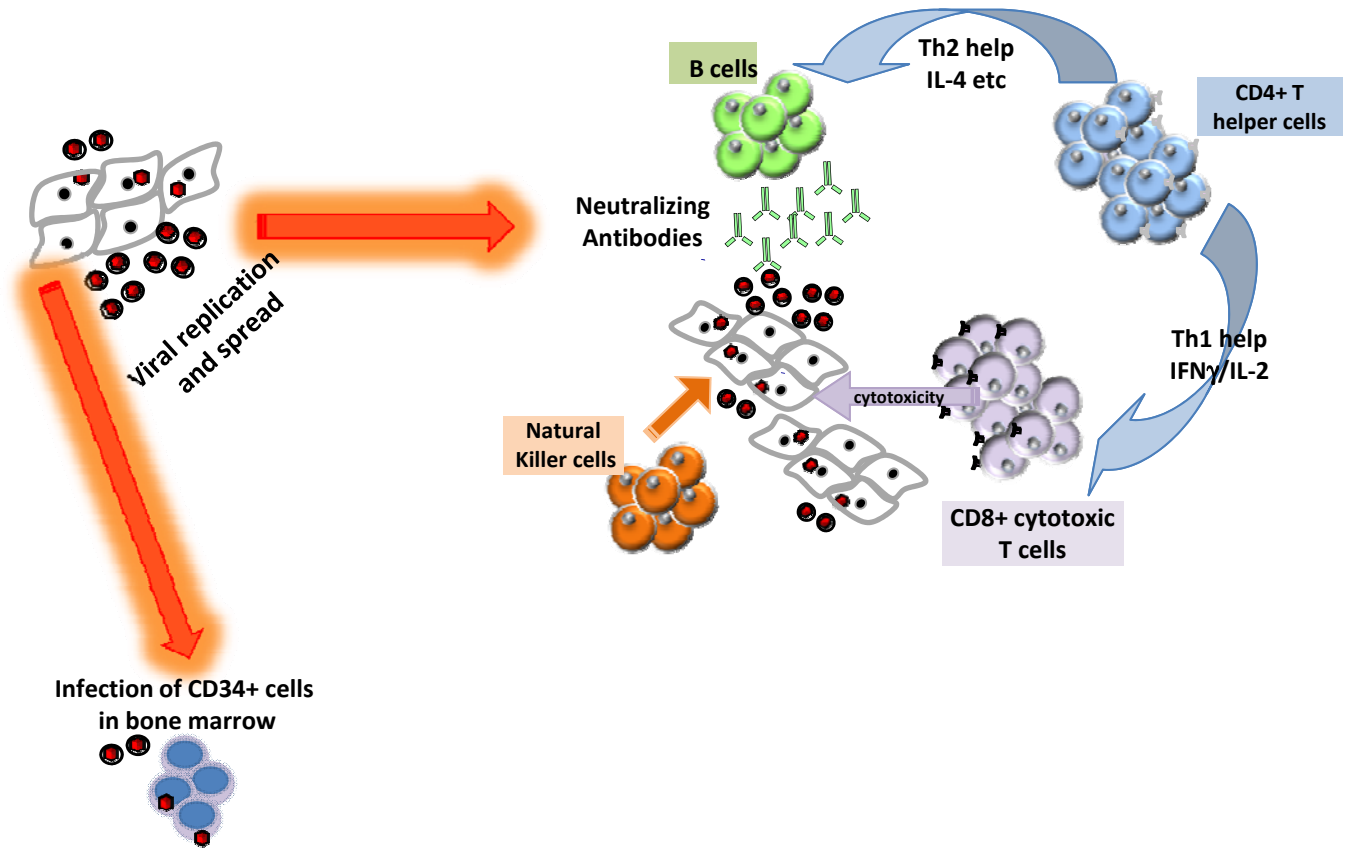


Figure 1

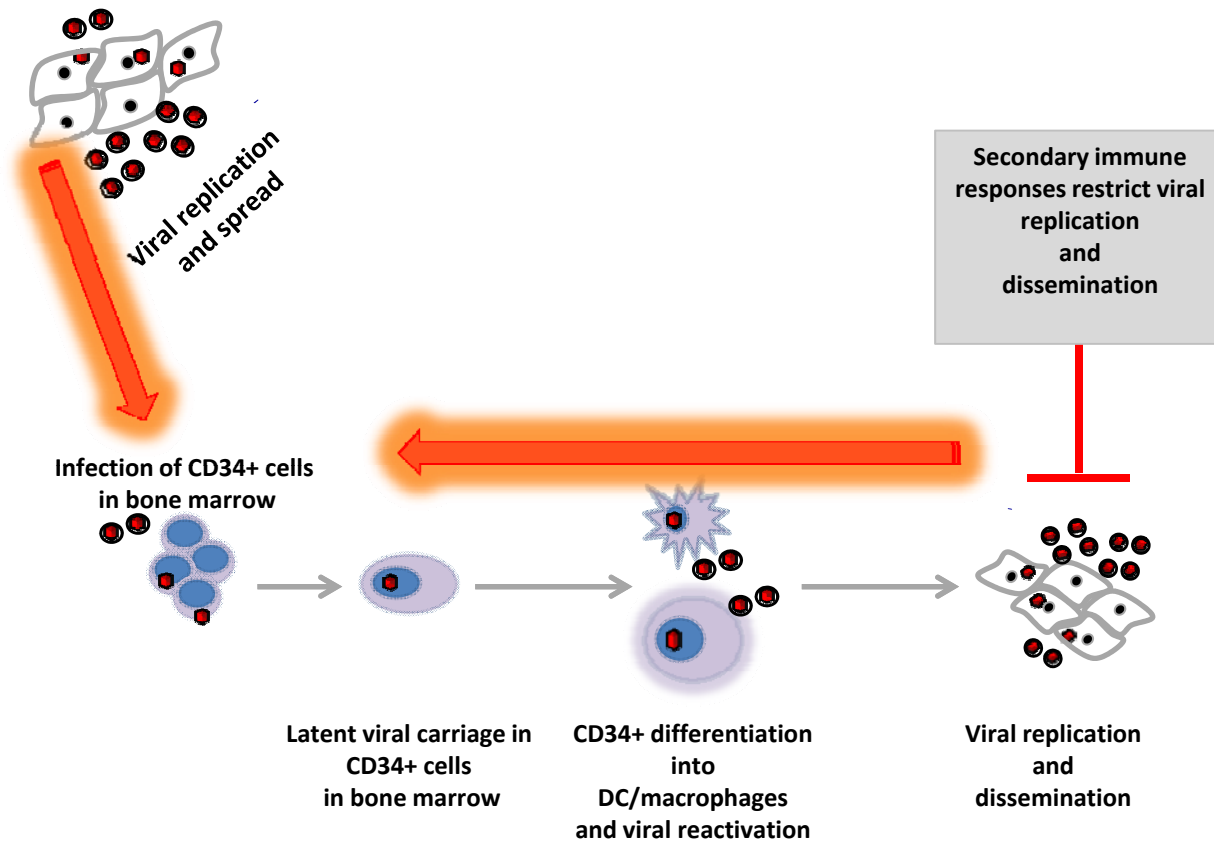
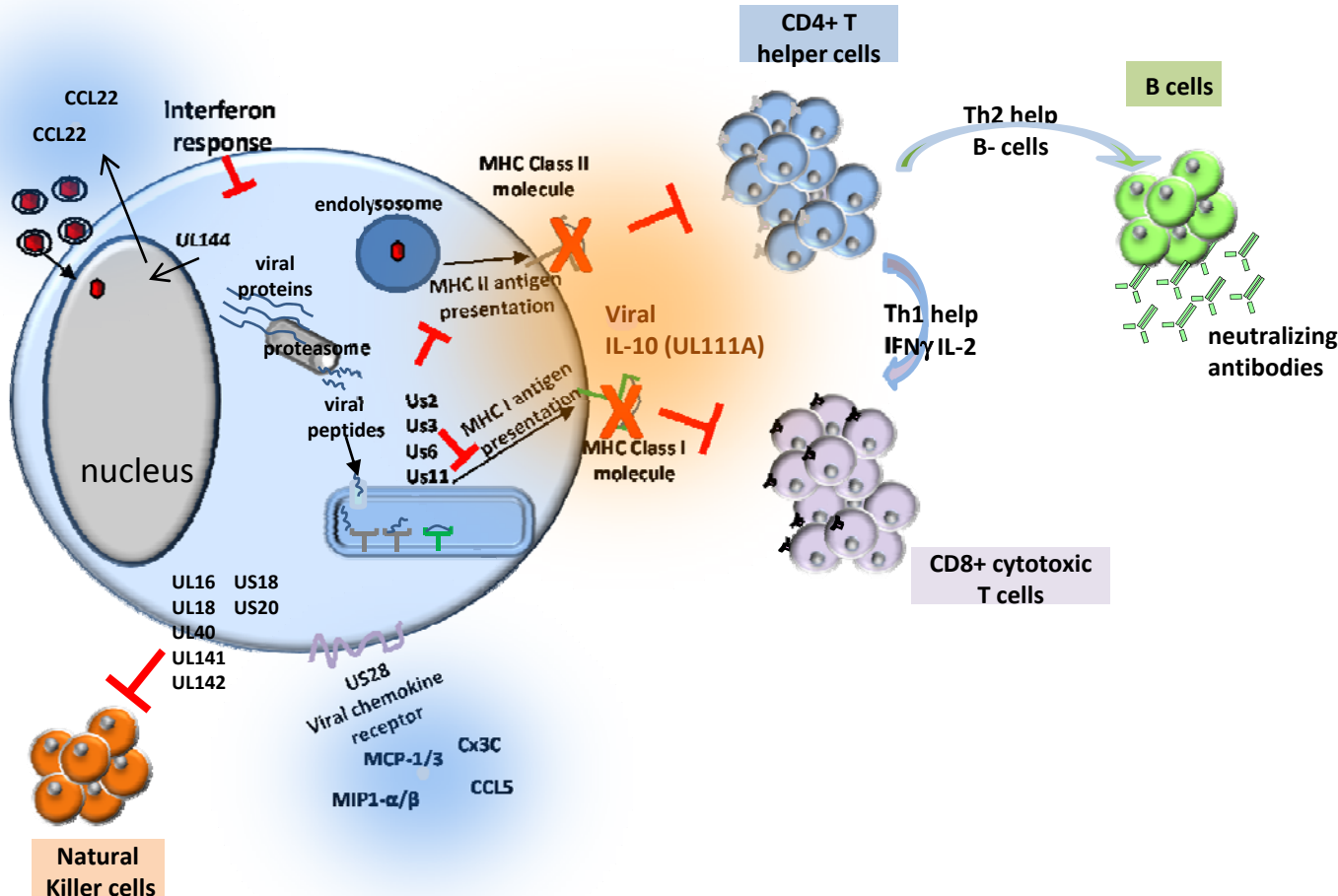


Figure 2

Figure 3



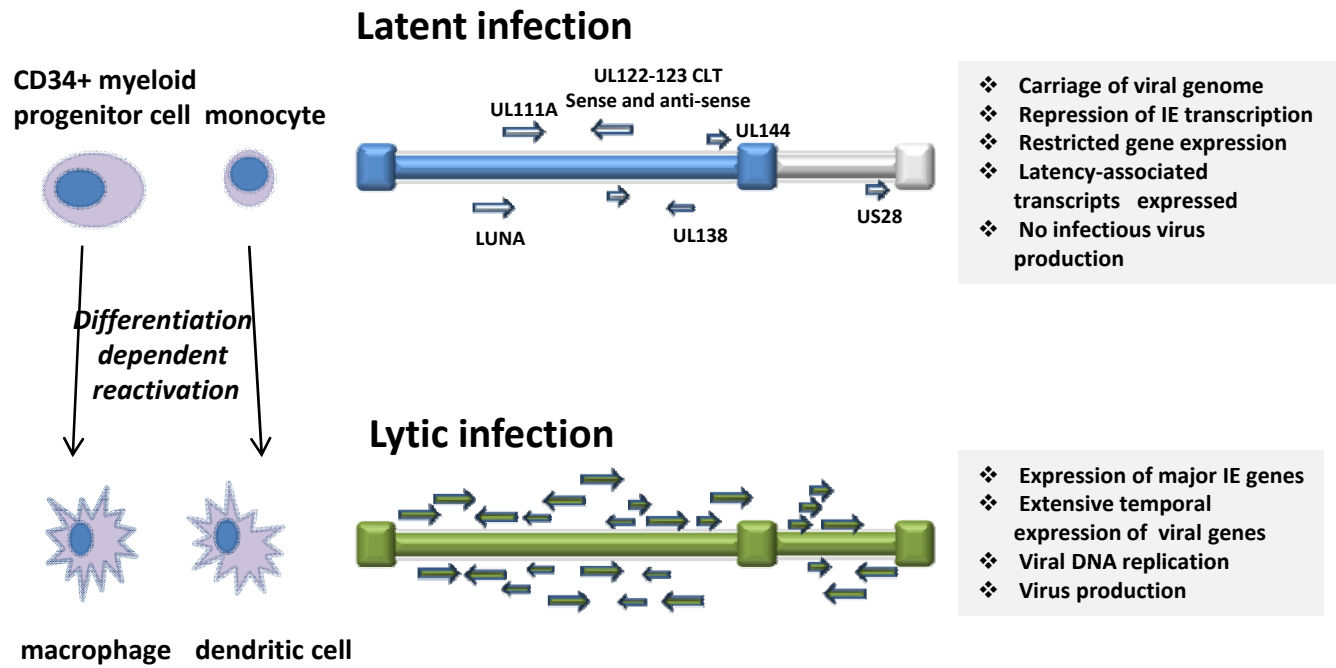


Figure 4

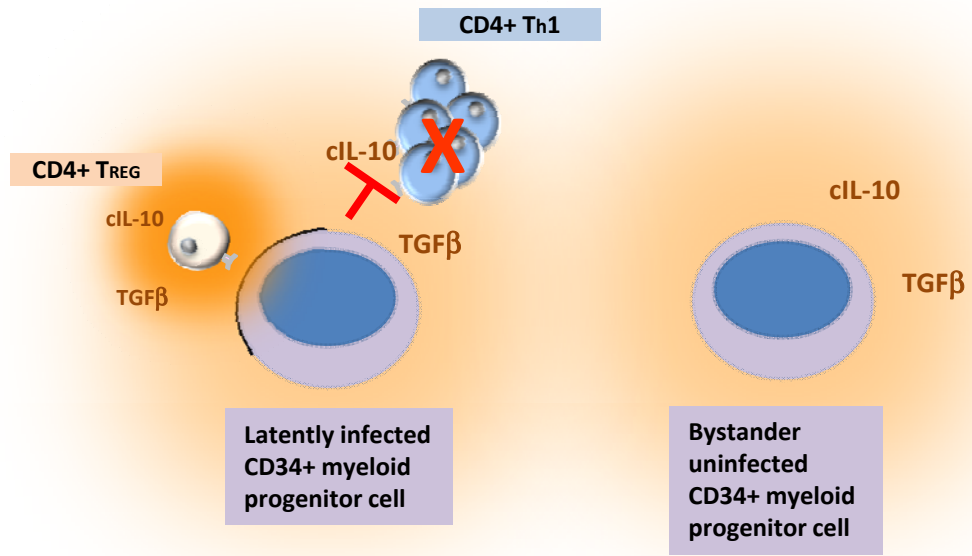


Figure 5

Figure 6

