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Cytomegalovirus is a tumor-associated virus: armed and dangerous

Charles Cobbs

Human cytomegalovirus (HCMV) gene products are present in multiple human malignancies, often in specific association with tumor cells and tumor vasculature. Emerging evidence from human and mouse models of CMV infection in cancer indicate that CMV can transform epithelial cells, promote epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial (MET) in tumor cells, promote tumor angiogenesis and proliferation and incapacitate the host anti-CMV immune response. This review will discuss the increasing role of HCMV in human cancer by demonstrating how HCMV is well suited for impacting major themes in oncogenesis including initiation, promotion, progression, metastasis and immune evasion. What emerges is a picture of an extremely versatile pathogen that may play a significant role in human cancer progression and death.

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> "It is not mutagenic agents that lead to cancer, more often than not it's inflammatory states, often caused by infectious agents that act as tumor promoters . . . Tumor promoters . . . are *agents which favor the proliferation of genetically altered cells while having little effect on the proliferation and survival of wild type cells*."
>
> **Robert Weinberg, "What causes cancer?"**
> **Annual meeting of the American Association of Cancer Research 2008**

Since the discoveries of Peyton Rous that implicated a chicken retrovirus in the development of sarcoma in chickens, cancer researchers have been fascinated with the possibility that viruses could cause cancer. The rush to implicate human retroviruses in the early years of the 'War Against Cancer' in the 1960s and 1970s ended not with clear evidence that viruses cause cancer, but rather with the discovery that retroviral genes exist in the human genome and can serve as endogenous oncogenes. While the field of cancer research pivoted in the 1980's away from viral infections to focus on cancer genetics, several non-retroviral viruses were nevertheless found to promote cancer, not by acute transforming events, but rather by the combination of persistent infection coincident with loss of functional host immune surveillance. These include human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr Virus (EBV) and Human Herpesvirus-8 (HHV-8). These pathogens leverage chronic inflammation, 'first hit' mutations, and loss of immune regulation to 'promote' a situation that statistically favors cell proliferation and immortalization over viral cytopathic effect. It is through this lens that we must view the last two decades of research on human cytomegalovirus (HCMV) and cancer. What emerges from a review of this research is compelling evidence that HCMV may be an important human cancer virus.

The earliest suggestions that HCMV could promote malignant transformation were experiments performed in the 1970s by Rapp *et al.* that demonstrated HCMV had the potential to transform hamster and human embryo cells, and human prostatic epithelial cells [1–3]. It appears that other laboratories could not substantiate these experiments and, with the revolution in cancer genetics, research into the role of HCMV in oncogenesis appears to have diminished, although evidence was found that HCMV genome components could promote mutagenesis and transformation [4,5]. While a few investigations in the 1970s linked HCMV to various malignancies like prostate an colon cancer [3,6], the evidence was not widely corroborated and the concept languished. In 2002 our group discovered that HCMV infection and expression were associated with glioblastoma (GBM) [7].

Since that time, several groups have detected HCMV in various human malignancies. In addition to GBM, our group published evidence that HCMV was present in a high percentage of colorectal carcinomas, prostate cancer and breast cancer [8–10]. These findings were confirmed and extended by others reporting HCMV in colon and breast cancer [11–14]. Many other tumor types have also subsequently been shown to have strong associations with HCMV in the last decade including medulloblastoma

[15], rhabdomyosarcoma [16], alveolar soft part sarcoma [17], mucoepidermoid carcinoma [18], and ovarian cancer [19,20].

In the last several years, reports by Soderberg-Naucler's group and others have further demonstrated that HCMV is not only present in human breast cancer, and inflammatory breast cancer, but also breast cancer metastases, [11,21–23]. More recently, Herbein's group has determined that a clinical strain of HCMV has the capacity to transform cells [24]. Cumulatively, these findings indicate that HCMV, or perhaps specific strains of HCMV, have the ability to contribute to initiation and promotion of human cancer. Despite these positive findings, multiple groups have had difficulty confirming and corroborating the presence of HCMV in tumors, and significant controversy exists about whether there is actual HCMV genome in many tumors. Because of the wide diversity of techniques used to establish HCMV DNA, RNA and protein in tumors, methodologies that can be easily corroborated amongst various groups are critically needed to advance this field, given the potentially huge implications of a role for HCMV in multiple human cancer types.

Four themes

This review will focus on how HCMV may play a role in four major paradigmatic themes involved in cancer: 1) stem cell biology, 2) initiation, promotion, progression, and metastasis, in the context of 3) hallmarks of cancer and 4) immune editing.

Initiation, promotion, progression and metastasis

Initiation and stem cell biology

A current model of oncogenesis suggests that initiating events start the oncogenic process and that further 'enabling' events, often by an infectious agent acting as a promoter, push the nascent tumor cells toward progression and metastasis [25]. Upon infection of the host, HCMV spreads throughout the body and infects multiple cell types. The most frequently infected cell types during acute disease are epithelial cells, endothelial cells, and macrophages and these serve as reservoirs of persistent infection [26,27]. The Thy-1 and PDGFR-alpha stem cell markers are known to enhance HCMV infection, and stem cells are particularly vulnerable to HCMV infection [28,29]. Key stem cell regulators such as *Wnt* facilitate HCMV transcriptional activation [30]. Thus, once HCMV has spread to multiple organs, viral expression likely occurs intermittently throughout life in adult stem cells, and these cells are believed to be the cells of origin of most human cancers (Figure 1) [31]. Adult stem cells are particularly vulnerable to acquisition of mutations over time and, in the setting of altered DNA repair mechanisms and inflammation, are susceptible to oncogenesis [32].

In the brain, HCMV is known to infect neuronal and glial stem cells, and in the mouse model of CMV, viral persistence and reactivation occur in these cells in the subventricular zone, and these are the cells of origin of human GBM [33,34]. HCMV can promote survival of stem cells, which would increase oncogenic potential. We showed that the HCMV IE1 protein dramatically facilitates the maintenance of GBM cancer stem cells through its induction of SOX-2, Nanog, and OCT3/4 [35]. Other groups have found similarly that HCMV promotes cancer stem cell survival [36,37].

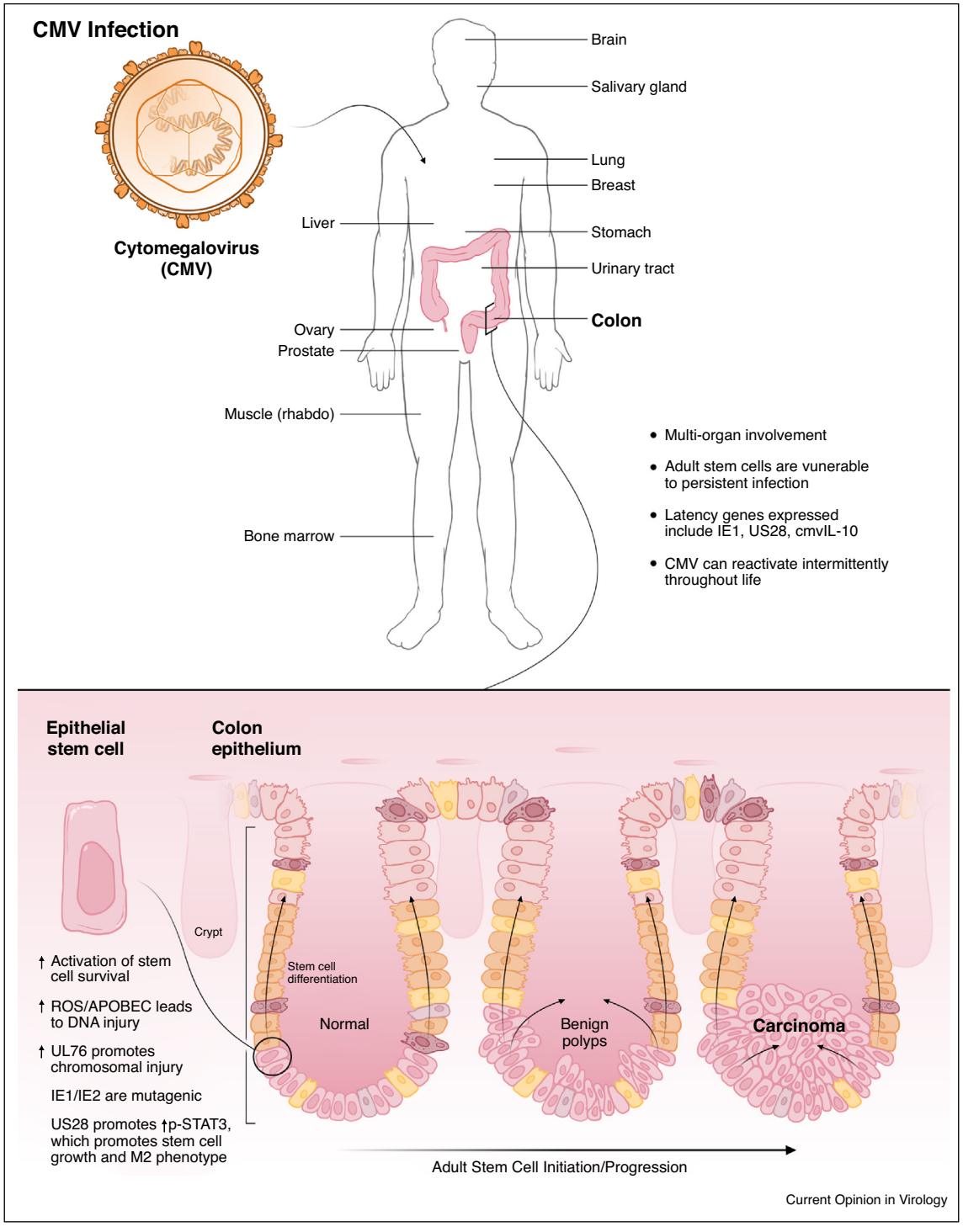
In addition to promotion of stem cell proliferation, expression of HCMV IE1 and IE2 genes as well as the UL76 genes induce DNA mutagenesis and chromosome breaks [38–40]. Expression of these HCMV gene products in tissue stem cells could interfere with critical regulators of genomic integrity including the p53 and Rb tumor suppressor pathways, and DNA repair pathways [41–44]. Genotoxic injury would also be exacerbated by the high levels of reactive oxygen species induced by HCMV infection [45]. HCMV also robustly activates the APOBEC intrinsic antiviral response, which is characterized by specific nucleotide mutations and alterations [46,47]. This innate antiviral response, especially if occurring in a stem cell over time, could lead to the high rate of APOBEC-signature mutations and genomic instability found in multiple human malignancies [48,49].

Assuming HCMV gene products cause DNA damage, incapacitate DNA repair mechanisms, and promote proliferation in stem cells, then HCMV would possess the ability to initiate oncogenesis. This phenomenon has been hinted at in the past but never shown as clearly as in a recent work demonstrating that a low passage clinical isolate of HCMV could transform human breast epithelial cells by dysregulating p53, Rb and telomerase, and induce tumors that could grow in soft agar and as mouse xenografts [24]. These findings are significant since they indicate that, as in the case of HPV, not all strains of HCMV may be capable of initiating oncogenesis, and some may be more capable than others.

Promotion and hallmarks of cancer

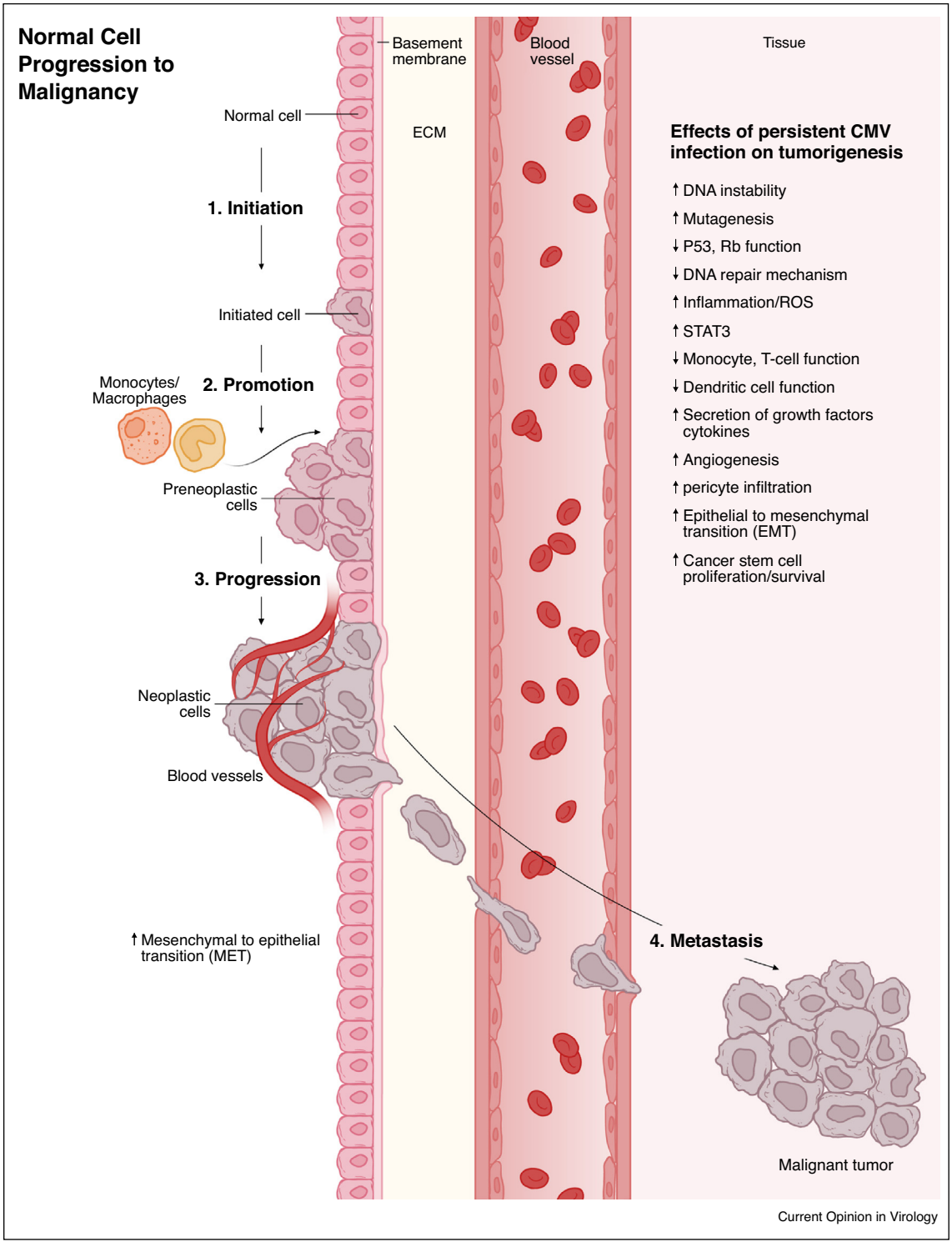
After 'initiation' has occurred, a tumor cell is highly susceptible to further oncogenic events (Figure 2). HCMV possesses myriad mechanisms for driving the next step of oncogenesis — 'promotion'. This process involves acquisition of multiple other 'hallmarks' of cancer, including chronic inflammation, infiltration of myeloid cells, angiogenesis, immunosuppression, and metabolic switch. HCMV can activate many of the hallmarks of cancer pathways under normal and neoplastic conditions (see reviews [50,51•]). Infection with HCMV can induce multiple inflammatory cytokines including IFN-γ, TNF-α, IL-4, IL-18, RANTES, MCP-1, MIP-1α, and IL-8F (reviewed in Refs. [52,53]). The HCMV

Figure 1



HCMV infects multiple organs, in which HCMV-associated malignancies have been detected.
HCMV infection can lead to widespread organ involvement. Multiple adult stem cell types, as in the colon, can remain infected and express latency-associated HCMV proteins such as IE1, US28, and cmvIL-10, which can promote stem cell proliferation, genomic instability and immune privilege. Chronic inflammation from infection can induce reactive oxygen species and innate host immune defenses such as APOBEC, which can further increase genomic instability and lead to pre-neoplastic growth (e.g. colonic polyps) and tumor initiation.

Figure 2



Initiation, promotion, progression, and metastasis.
Normal wild type cells, especially adult epithelial stem cells, are vulnerable to tumor promotion and progression due to persistent HCMV infection and expression of HCMV gene products. HCMV encodes for proteins and non-coding RNAs that can facilitate the acquisition of the 'hallmarks of cancer' including viral genes that block apoptosis, cell cycle arrest, drive tyrosine kinase signaling, angiogenesis, inflammation, immunosuppression, and so on. Epithelial–mesenchymal transition (EMT) and the reverse process, mesenchymal–epithelial transition (MET), are two fundamental epigenetic processes that HCMV can promote, which would enhance metastatic tumor dissemination through blood vessels and lymphatics and tumor growth in end organ tissues.

US28 chemokine receptor induces strong expression of the NF-κB, COX-2, IL-6, p-STAT-3 axis, which can drive oncogenic pathways [54,55]. In addition to inducing inflammatory cytokines, HCMV can foster the infiltration and survival of neutrophils and mononuclear cells, which can promote early carcinogenesis via activation of an angiogenic switch [56–58].

The HCMV-induced secretome promotes angiogenesis and wound healing-associated growth factors and cytokines that would significantly promote the tumor microenvironment [59]. We demonstrated that GBM cells from human biopsy specimens express HCMV US28, gB, and pp71 [29,60,61]. These viral proteins in combination dramatically increase the invasive, angiogenic and proliferative phenotype by inducing cellular proteins such as NFkB, IL-6/STAT3, VEGF, and Stem Cell Factor (SCF). Perhaps the most convincing evidence that CMV can drive tumor 'promotion' is recent work by Lawler's group using a syngeneic mouse model of GBM, in which latent murine CMV (MCMV) reactivates and infects implanted GBM xenografts. The reactivated MCMV infection induces significantly increased tumor growth and more rapid death of the MCMV + mice. The MCMV + GBM tumors also have dramatically increased angiogenesis and secrete high levels of PDGF-D, which promotes recruitment of pericytes, which contributes to angiogenesis and tumor growth [62•••]. Intriguingly, administration of the antiviral drug cidofovir to these mice could reverse the CMV-mediated angiogenesis, pericyte recruitment and increased tumor growth, confirming the "promotion" effect of MCMV in these tumors. We have detected HCMV expression in pericytes in human GBM and HCMV infection of pericytes has been shown to induce multiple cytokines that could promote GBM pathogenesis including CXCL8/IL-8, CXCL11/ITAC, CCL5/Rantes, TNF-alpha, interleukin-1 beta (IL-1beta), and interleukin-6 (IL-6) [63].

Tumor progression is the third phase in tumor development, and is characterized by increased rate of growth and invasiveness of the tumor cells. Two of the key pathways that are activated during tumor progression are those that promote cancer stem cell survival and the epithelial to mesenchymal (EMT) switch [64]. Both of these tumor progression phenotypes facilitate the ability of tumor cells to proliferate, invade into adjacent tissues, disseminate into vascular and lymphatic channels and avoid immune detection. This transition may be due to epigenetic signals that alter the transcriptional state, such as global hypomethylation of DNA, which can induce activation of various oncogenic transcription factors, and which HCMV expression can promote [65].

HCMV IE1 expression in human GBM cells induces transcription factors that are critical for cancer stem cell survival, tumor growth, and signaling pathways associated with the EMT phenotype [35,66–68]. We and others demonstrated that HCMV-infected cancer stem cells have a growth advantage over HCMV uninfected cancer stem cells [35,69•••,70]. Teo *et al.* found that HCMV induced an EMT transition in colorectal carcinoma cells, and this was associated with increased proliferation and invasiveness [70]. Similarly, Moussawi *et al.* demonstrated that a clinical isolate of HCMV, DB, could transform human mammary epithelial cells, and induce a transcriptional profile associated with DNA hypomethylation that led to increased proliferation, cancer stem cell activation and EMT phenotype [69••]. The cmvIL-10 chemokine, which is expressed in latency and tumor cells, can increase tumor cell invasiveness [71,72], as can US28 [60]. Together, these reports indicate that HCMV can play an active role in facilitating tumor progression by inducing pathways that cause epigenetic changes, and promote cancer stem cell activation, angiogenesis, invasion and EMT.

Metastasis

The lethality of most human malignancies occurs as a result of the metastatic spread of tumor cells and end organ damage in the liver, bone marrow, and so on. While EMT is necessary for most tumor types to escape the primary organ and enter blood vessels and lymphatics, current evidence suggests that disseminated tumor cells must undergo a reversal of the EMT process in order to establish successful growth in the end organ. This process, 'mesenchymal to epithelial' transition, or MET, also requires orchestration of multiple genome-wide signaling pathways [64]. Oberstein *et al.* showed that a clinical isolate of HCMV can induce a genome wide MET transition in mesenchymal human breast epithelial cancer cells [73]. The effects of HCMV on these mesenchymal breast cancer cells were profound, and HCMV expression altered the tumor cell phenotype from spindly mesenchymal cells to the classic epithelial 'cobblestone' appearance. The effect was associated with alterations in the EZH2 or PRC2 histone methyltransferase, which is a critical regulator of oncogenesis. Interestingly, HCMV positive GBM tumors were also found to have strongly increased EZH2 activity compared to HCMV negative GBM cells [74].

If HCMV infection of 'mesenchymal' tumor cells enhances their potential to undergo MET and establish metastatic tumors, then one would expect that metastatic tumors would be enhanced by systemic CMV infection or reactivation of latent infection. Two different mouse models of metastatic breast cancer in the setting of MCMV infection corroborate this concept. Forty years ago, Olsen *et al.* found that in a syngeneic mouse model of breast cancer metastasis, if the mice were infected with MCMV concurrently with the IV injection of breast cancer cells, then fewer lung metastases occurred compared to control mice that had no MCMV infection [75].

However, if the mice were injected with breast cancer cells, and these cells were allowed to establish micrometastases in the lung, and then they were infected with MCMV two weeks later, the mice had *a 10-fold increase* in the number of lung metastases and they died at twice the rate of the mice that had no MCMV infection.

In a recent paper by Yang *et al.*, four different mouse models of metastatic breast cancer in the presence of active of latent MCMV infection were used to assess the impact of MCMV on breast cancer metastasis. In the BALB/c and MMTV-PyVT models, latent, but not active, MCMV infection led to a two-fold increase in metastatic lesions in the lungs. The lung metastases in the MCMV latently infected mice demonstrated significantly increased evidence of angiogenesis and proliferation. Plasma levels of IL-6 and IL-13 were increased in the MCMV latent metastatic mice. These cytokines are positive modulators of inflammation, proliferation and metastasis.

Taken together with the findings of the mouse model of latent MCMV infection in GBM, these works indicate that latent MCMV reactivation in the setting of metastases, or new onset of MCMV infection in a model of established micrometastases, can drive proliferation, angiogenesis and rapidity of death due to metastatic disease in these animals. Importantly, the data suggest that latent CMV could play a crucial role in accelerating death in humans from pre-existing cancers by increasing metastatic tumor growth in the setting of viral reactivation or new infection.

Cancer immune editing

A final major theme of oncogenesis that HCMV can dramatically influence is cancer immune evasion and escape (Figure 3). The host immune system is thought to constantly control cancer progression, but when tumor cells and host immune responses override these checkpoints, tumor immune escape leads to florid progression. Three phases of this process are described: 1) elimination, 2) equilibrium and 3) escape.

In the first stage of elimination or tumor surveillance, NK and T-cells circulate and eliminate tumor cells that are identified by inflammatory cytokines released by the tumor cells and surrounding macrophages and stromal cells. During this phase, assuming the nascent adult stem cells and pre-neoplastic cells are infected with HCMV, then HCMV could subvert the NK and T-cell responses through multiple mechanisms that have been extensively reviewed elsewhere [76]. During the equilibrium stage, damaged tumor cells with progressively increased genomic instability gain autologous growth potential by acquiring immunosuppressive tactics. After the 'equilibrium' stage, tumor cells may acquire resistance to host immune strategies and obtain immortal growth properties and immune 'escape'. This stage is associated with loss of immune control and uncontrolled tumor growth. Escape is often accompanied by loss of MHC I and T-cell activity. Both the HCMV pp65 and pp71 proteins can contribute to MHC I downregulation, and we have detected these viral proteins in GBM [8,61,76]. The many mechanisms of HCMV immune evasion are extremely diverse and well detailed in other reviews, and here we will focus on a few HCMV strategies key to tumor immune escape [76,77].

Elimination

Upon infection of cells, HCMV gene products and micro RNAs incapacitate multiple innate intracellular antiviral responses, which cumulatively increase the oncogenicity and immunosilencing of the tumor cells. The HCMV IE1 gene product promotes cancer stem cell processes and both the HCMV IE1 and pp65 proteins block the innate antiviral and immune Type I IFN signaling pathways [35,78,79]. Another anti HCMV innate response, the PKR pathway, further inhibits cellular IFN immune signaling [80]. Without functional IFN-γ pathways, cell mediated innate immune responses have limited tumor elimination capacity.

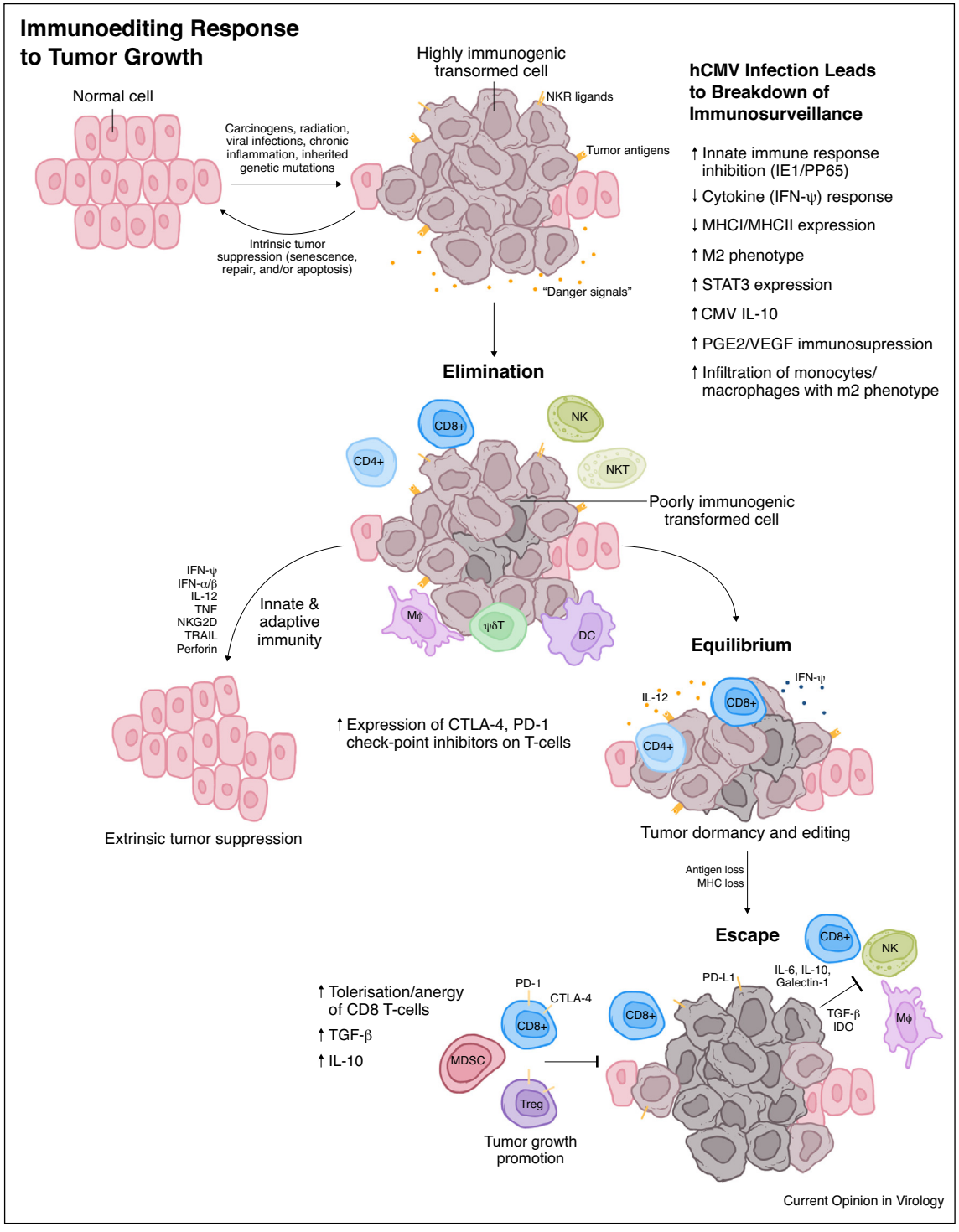
Equilibrium

Pre-neoplastic cells that are infected with HCMV in various organs are likely to express IE1, US28 and cmvIL-10 since these proteins are expressed during latency [81–84]. Indeed, we detected HCMV IE1 and pp65 in rare normal breast epithelial cells in non-cancer patients [10]. As these pre-neoplastic cells evolve into tumor cells, expression of HCMV gene products will undermine host immune defenses further. As the pre-neoplastic cells gain oncogenic potential, the expression of HCMV proteins will alter the immune microenvironment and facilitate immune escape.

Escape

Dziurzynski *et al.* found that in human patient-derived GBM stem cells, HCMV IE1, pp65, and cmvIL-10 proteins were expressed [85]. These GBM stem cells could profoundly alter the tumor immune microenvironment, since they secreted cmvIL-10 into their environment. Monocytes exposed to cmvIL-10 were induced to express high levels of p-STAT3, VEGF, and TGF-b, and these monocytes had downregulation of MHC-II and upregulation of PD-L1. GBM stem cells cocultured with these cmvIL-10-treated monocytes had increased migration. In essence, this work demonstrated that when human cancer stem cells are infected with HCMV, and they express cmvIL-10, they can induce an M2 phenotype in monocytes in the tumor microenvironment, and promote the expression of other oncogenic and immunomodulatory and angiogenic cytokines including STAT3 and VEGF. The HCMV US28 chemokine receptor can not only activate inflammatory pathways such as the NF-κB,

Figure 3



HCMV sabotages normal cancer immune editing pathways.
HCMV gene products can dysregulate and impair almost every aspect of the humoral and cell-mediated immune response that is critical for the host immune surveillance. HCMV infected tumor cells can express potent cmvIL-10 immunosuppressive cytokine and derail dendritic cells, monocyte macrophages and CD8 T-cells. Loss of host antitumor immune response leads to unregulated tumor growth and spread.

IL-6, and p-STAT3 that can derail immune response, but it can also act as scavenger of important chemokines involved in immune response [55,60,86,87]. STAT3 has been implicated in carcinogenesis and can facilitate escape from immune destruction by increasing the suppressive cytokines like TGF-β, IL-6, IL-10, and VEGF in the tumor microenvironment [88]. Further, STAT3 has been linked to accumulation of immature dendritic cells (DCs), myeloid derived suppressor cells and anergic T-cells as well as regulatory T-cells in cancer. Indeed, a mouse model of spontaneous GBM in the setting of latent MCMV infection clearly demonstrated that during early glioma development STAT3 became expressed in the neural stem cells that subsequently developed into tumors, but this phenomenon did not occur in the MCMV negative controls [89]. Thus, existing evidence in mouse models and human cancer suggests that HCMV persists in stem cells and expresses immunosuppressive and inflammatory gene products such as cmvIL-10 and STAT3 which promote oncogenesis while producing an M2 immunosuppressive phenotype upon infiltrating DCs, monocytes and T-cells.

Bahador *et al.* provided additional evidence of HCMV-mediated immune escape in their study of immune responses of HCMV + GBM tumors. They evaluated 177 human GBM tumors and they were able to detect HCMV pp65 and IE1 in up to 43% of tumors by qPCR [90]. They found that despite the fact that the HCMV + tumors contained circulating CD8 T-cells specific for HCMV proteins, these T-cells were nonfunctional. The HCMV-specific CD8 T-cells in the GBM tumors expressed high levels of the CTLA-4 and PD-1 immune checkpoint markers compared to populations in the peripheral blood, rendering them incompetent for T-cell mediated killing of HCMV-infected GBM cells. These data confirm that HCMV-specific T-cells are present in HCMV + GBM tumors, but the tumor cells have essentially tolerized the immune response and escaped immune surveillance.

Summary

HCMV has now been detected in multiple human malignancies, and some investigators have even suggested a causal association between HCMV and cancer [18]. Causality is difficult to ascribe to a pathogen that may promote oncogenesis over decades, and then only under certain circumstances related to cell niche, viral strain, and host immune functioning. Nevertheless, as described here, HCMV does encode for myriad gene products that are expressed in various human malignancies that contribute to initiation, promotion, progression and metastasis, especially in the setting of progressive immune evasion. Since several human GBM clinical trials have demonstrated some evidence of antiviral and vaccine strategies against HCMV in terms of patient survival, further research into the links between HCMV and cancer are warranted [91–94].

Conflict of interest

There is no conflict of interest.

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