The pentameric complex of human Cytomegalovirus: cell tropism, virus dissemination, immune response and vaccine development

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Abstract
Between the 1980s and 1990s, three assays were developed for diagnosis of human cytomegalovirus (HCMV) infections: leuko(L)-antigenemia, L-viremia and L-DNAemia, detecting viral protein pp65, infectious virus and viral DNA, respectively, in circulating leukocytes. Repeated initial attempts to reproduce the three assays in vitro using laboratory-adapted strains and infected cell cultures were consistently unsuccessful. Results were totally reversed when wild-type HCMV strains were used to infect either fibroblasts or endothelial cells. Careful analysis and sequencing of plaque-purified viruses from recent clinical isolates drew attention to the ULb region of the HCMV genome. Using bacterial artificial chromosome technology, it was shown by both gain-of-function and loss-of-function experiments that UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. In addition, a number of clinical isolates passed in human fibroblasts had lost both properties (leuko-tropism and endothelial cell-tropism) when displaying a mutation in the UL131-128 locus (referred to as UL128L). In the following years, it was shown that pUL128L was complexed with gH and gL to form the pentameric complex (PC), which is required to infect endothelial, epithelial and myeloid cells. The immune response to PC was studied extensively, particularly its humoral component, showing that the great majority of the neutralizing antibody response is directed to PC. Although anti-HCMV antibodies may act with other mechanisms than mere neutralizing activity, these findings definitely favour their protective activity, thus paving the way to the development of a potentially protective HCMV vaccine.

INTRODUCTION
Human cytomegalovirus (HCMV) (human herpesvirus 5 or HHV-5) is a ubiquitous β herpesvirus that causes infections that are mostly asymptomatic. However, when primary (and sometimes non-primary) HCMV infections affect pregnant women, the virus can cause congenital infection, as well as a wide range of neurodevelopmental abnormalities [1]. In addition, in immunocompromised patients, HCMV infection may lead to life-threatening diseases [2, 3]. Thus, an HCMV vaccine is considered to be a public health priority [4]. Although congenital infection may also be acquired from HCMV-seropositive pregnant women, it has been reported that children born to seropositive women are less likely to suffer from congenital infection than those born to seronegative mothers [5].

A significant contribution to the development of a HCMV vaccine was the identification of the pentameric complex (PC), which consists of HCMV gH, gL, pUL128, pUL130 and pUL131 [6–10].

In this review, the cellular and viral events preceding the identification of PC, as well as the molecular events that form the basis of its function in cell entry, will be discussed in detail. In addition, the immune response to PC detected in vivo and in experimental animal models will be reported, with special attention to its humoral and cellular components and their protective activity.
antigenemia, while the virus isolation rate was 45 % from PMNL and 17 % from M/M samples [20]. The simultaneous use of HCMV pp65 l-antigenemia, l-viremia and l-DNAemia to detect HCMV in leukocytes [14] allowed the first detection of a sharp drop in the prevalence of HCMV infections in HIV-infected patients following the introduction of highly active antiretroviral therapy [21]. In the immune-competent host, the three above-mentioned diagnostic assays, as a rule, are negative, except for cases of HCMV primary infection [22]. However, early on it was already known that the maximal rate of blood-mediated HCMV transmission was only reached when leukocyte preparations were administered [23, 24]. Recently, the statement that HCMV DNA cannot be detected in the blood of healthy subjects or even during reactivation episodes [22] was rebutted in a study by Arora et al. [25], which reported that of 205 HCMV immune women, 24 % had DNAemia at baseline. By contrast, in a more recent study by our group, DNAemia was detected in 452/597 (75.7 %) women with serological evidence of primary HCMV infection, and in 4/774 (0.5 %) women without evidence of primary infection [26]. Since the study population analysed by Arora et al. consisted of very young (mean age 18 years), mostly unmarried, black, low-income women with multiple sexual partners, it remains to be verified whether these results can be extrapolated to the general female population. When HCMV DNA was comparatively quantified in blood leukocytes (l-DNAemia) and plasma (P-DNAemia) of transplant and AIDS patients, it was found that P-DNAemia was a much less sensitive parameter than l-DNAemia for monitoring HCMV infections [27].

As for HCMV endothelial cell (EC) tropism, it has been reported that ECs are a major target of HCMV infection in vivo [28, 29], and have been considered to be a site of HCMV latency in immune-competent subjects [30, 31]. Furthermore, several reports have described the presence of HCMV inclusion-bearing cells in AIDS patients with disseminated HCMV infection [32, 33].

In 1993, we reported that in AIDS patients, HCMV-infected ECs progressively enlarge until they detach from the vessel wall (Fig. 2) and enter the bloodstream [34]. These cells were referred to as circulating endothelial giant cells (EGCs), and their identification reinforced the assumption that EGCs are the same cells that were observed along the vessel walls of patients with AIDS and disseminated HCMV infection (Fig. 2d). EGCs displaying advanced cytopathic effect are likely to undergo lysis during circulation, thus disseminating infectious virus and viral material in blood. Subsequently, it was documented in in vitro experiments that HCMV and viral material can be transferred from field HCMV strain-infected ECs to PMNLs and M/M via transient membrane microfusion events [35], while infected leukocytes can, in turn, infect in vitro uninfected ECs (Fig. 3) during coculture [36].

At the beginning of the 1990 s, we failed repeatedly to generate pp65 l-antigenemia, l-viremia and l-DNAemia in vitro
How did this occur? Infection of either HUVECs or HELFs with a field HCMV strain allowed the final transfer of viral and cellular material to leukocytes during coculture. It was reported that HCMV infection upregulates the expression of adhesion molecule ICAM-1 on infected cells [39, 40]. Thus, major interactions between infected cells and leukocytes would involve this molecule and its known ligands, LFA-1 and MAC-1, belonging to the CD11/18 integrin subfamily and mostly found on the surface of PMNLs [41]. When cell-to-cell contact was physically or immunologically prevented, pp65-positive leukocytes were absent or greatly reduced in number [35]. The same findings were reported for M/M [42].

The failure of laboratory-adapted HCMV strains to induce transfer suggested that important modifications had occurred in their basic biological functions. The AD169 and Towne HCMV strains were developed as vaccine candidates by serial passages in HELFs with the intention of attenuating their virulence [43, 44], thus reducing or suppressing their ability to replicate in ECs [45, 46]. This line of thought was confirmed by the sequential propagation of four recent HCMV isolates in HELFs [37]. It was found that three out of four recent HCMV isolates lost their capacity to transfer to leukocytes and their ability to grow in ECs before passage 50. These findings led to the conclusion that the lack of HCMV transmission to leukocytes and HUVEC infection are markers of HCMV attenuation [47]. Interestingly, sequential mutations associated with the adaptation of human cytomegalovirus to growth in HELF cultures have been shown to be associated with greater virus yields [48].

A surprising finding was that both EC-tropism and leuko-tropism could be reinstated in the laboratory strains Towne and AD169 after adaptation to growth in ECs [49, 50]. An analysis conducted in parallel by restriction fragment length polymorphism (RFLP) and Southern blot (SB) for both laboratory strains revealed the identity of the laboratory (attenuated) and rescued EC-tropic variants of the two strains. These results suggested that it was probably only minor variations in the viral genome of the two strains that were responsible for the loss/reacquisition of the two biological properties. In addition, the Toledo strain, commonly considered a prototype of wild-type strains, was shown to lack both EC- and leuko-tropism [51].

**GENETIC DETERMINANTS OF ENDOTHELIAL CELLS AND LEUKOCYTE TROPISM**

The genetic determinants of EC- and leuko-tropism remained elusive until 2004, when a 2-year collaborative investigation by researchers from Pavia (Italy) and Munich (Germany) provided evidence that the UL131-128 locus (UL128L) [48] within the ULb region of the HCMV genome is indispensable for virus replication in ECs and virus transmission to leukocytes [6]. At the beginning of this study, the following findings were critical in directing interest to the ULb region as a prime candidate: (i) the loss of EC- and leuko-tropism in the laboratory strains, Towne and AD169, was associated with the loss of the great majority of ULb [52, 53]; (ii) the low-passaged strain Toledo, which was shown to be tropism-deficient [51], was found to display an inversion of ULb [53]; and (iii) extensive propagation of EC- and leuko-tropic clinical isolates in HELFs was found to be consistently associated with the selection of tropism-deficient variants [37, 45, 54–56] in the presence of only minor variations in the relevant viral genomes.

In addition, the above-mentioned collaborative study showed that each gene of the locus was individually required for tropism. These conclusions were supported by experimental conditions leading to either a gain or a loss of function. Loss of function was documented by the following two experimental findings: (i) the experimental introduction of targeted deletions into UL128L and (ii) the identification of spontaneous mutations within UL128L of natural viral variants [6] (Fig. 4). As for the first point, the generation of a number of mutants with a deletion of any ORF within ULb other than UL131, UL130 and UL128 did not affect virus tropism. As for the second point, eight independent tropism-deficient HCMV strains showed a variety of spontaneous mutations, affecting the coding sequence of at least one of the three UL128L genes.

On the other hand, gain of function was documented by two independent experiments: (i) phenotypic reversion to tropism

![Fig. 2. (a) A circulating endothelial giant cell and (b) three human umbilical vein endothelial cells from a primary culture stained by monoclonal antibody PAL-E specific for endothelial cells and the APAAP procedure. (c) A cytomegalic endothelial giant cell stained with a mAb specific for HCMV p52. (d) Multiple cytomegalic circulating endothelial giant cells (from Gerna et al. [21]) along the vessel wall or inside a blood vessel of a prostate biopsy from a deceased AIDS patient with disseminated HCMV infection.](image-url)
competence in five natural viral variants in association with reversal of mutations within UL128L, and (ii) partial tropism rescue by transcomplementation with individual UL131, UL130 and UL128 genes. In natural variants, tropism rescue was consistently associated, after propagation in ECs, with the selection of progeny virus carrying the original coding sequences. These findings conclusively demonstrated the indispensability of UL128L for tropism expression.

Afterwards, it was shown that the UL131 ORF was required for epithelial cell tropism [7, 8], and then that the tetrameric complex gH/gL/pUL128/pUL130 was required for infection of both epithelial and endothelial cells, but not HELFs [9].
Finally, Ryckman et al. [10] identified the gH/gL/pUL128/130/131 complex (referred to as pentameric complex or PC) as the entry complex for epithelial/endothelial cells, suggesting the presence of a type-specific receptor. Subsequently, the PC was identified as being necessary for the infection of other cell types that are critically involved in the immune response to HCMV infection (see below).

Thus, besides the previously characterized HCMV glycoprotein complexes (gCs), gCI (gB homotrimer coded by UL55) and gCII (gM/gN) consisting of UL100-coded gM and UL73-coded gN, a third envelope glycoprotein complex, gCIII (gH/gL), consisting of UL75-coded gH and UL115-coded gL, was found to associate with additional proteins, which in turn act as tropism determinants. In more detail, gH/gL may be complexed either with pUL128L, giving rise to the PC gH/gL/pUL128L, or UL74-encoded gO, thus forming the trimeric complex gH/gL/gO, which binds to platelet-derived growth factor receptor α and mediates HCMV entry into HELFs [57–59]. Thus, gH/gL/gO and PC were considered to represent two mutually exclusive cell entry complexes, as suggested by mass spectrometry and mutagenesis analysis [60]. However, gO and the UL128-131 gene products were reported to compete for binding of gH/gL, thus influencing the ratio of gH/gL/UL128-131 within virions as well as the HCMV cell tropism [61, 62]. As a result, gH/gL/gO was also found to be required for entry into epithelial cells [57, 63, 64].

As for the fusion event relevant to HCMV entry into all cell types, it has been reported to be mediated by gH/gL/gO and
gB, whereas in epithelial cells efficient fusion would be preceded by a PC-mediated process [64]. Recently, the HCMV gH/gL complex has been shown to form a stable complex with the fusion protein gB in virions including up to 50 % of total gH/gL, suggesting that gH/gL interacts with gB to mediate membrane fusion [65].

In addition, a series of HCMV genes/gene products have been reported to affect trimer/pentamer formation. Firstly, HCMV UL135 and UL136 genes have been shown [66] to be indispensable for HCMV replication in ECs (post-entry tropism). Secondly, UL148, encoding an endoplasmic reticulum-resident glycoprotein, may regulate virion incorporation of gH/gL/gG and PC, thus modulating HCMV tropism [62]. Thirdly, a new HCMV gH/pUL116 gC alternative to gH/gL was recently reported, whose role and function remain to be elucidated [67]. Finally, disruption of the US16 gene suppresses virus entry into endothelial and epithelial cells by reducing the virion content of the pentamer [68].

ANTIBODY RESPONSE TO PC AND THE OTHER GLYCOPROTEIN COMPLEXES

In the last decade, research focusing on anti-HCMV antibodies and in particular neutralizing antibodies, as major factors for protection from HCMV infection/disease has expanded continuously. The initial studies claimed that hyperimmune globulin preparations were effective in protecting fetuses from HCMV transmission and disease during primary infection of HCMV-seronegative mothers [69]. However, a subsequent double-blind placebo-controlled study reported a non-significant reduction in the rate of congenital infections between a group of pregnant women receiving placebo and one receiving hyperimmune globulin [70].

An inverse correlation of HCMV transmission to the foetus and antibodies was first proposed several years ago, when it was reported that anti-gB IgG antibody titres were significantly higher at delivery in transmitting (T) compared with non-transmitting (NT) mothers [71]. These results were attributed to a defect in IgG avidity maturation [72] and were confirmed by another group [73].

More recently, following the identification of the two gH/gL gCs, the antibody response to the two forms of gCIII as well as to gCI (gB) was studied by both ELISA and neutralization [74, 75]. Initially, the IgG antibody response to the PC was investigated in human sera using adenovirus vectors carrying the five HCMV genes. In primary infection, IgG seroconversion to UL131-128 gene products was consistently detected within 2–4 weeks after infection onset in all 14 cases tested, while antibodies persisted for at least 12 months [76].

Subsequently, an ELISA assay was developed using soluble forms of gB and PC. The ELISA performed on a group of pregnant women with primary infection showed that antibodies to gB increased rapidly and to a higher titre compared with antibodies to the PC [74]. On the other hand, serum antibodies neutralizing infection of epithelial cells appeared early at higher titres compared to antibodies neutralizing the infection of HELFs (Fig. 5), which reached 2–3 $\log_{10}$ lower titres [77]. When convalescent-phase sera from HCMV primary infections were absorbed with purified gCs, sera pre-absorbed with the PC showed a highly reduced (>90 %) capacity to neutralize infection of epithelial cells, whereas their neutralizing capacity was substantially unmodified in convalescent-phase sera pre-absorbed with gHgL or gB [75]. Similar results were obtained with hyperimmune globulin preparations [78]. In addition, it was shown that antibodies to PC are mostly highly neutralizing, unlike antibodies to gB, which are mostly non-neutralizing [79].

When a group of NT was compared with a group of T mothers, it was reported that, while gB antibody titres were comparable in the two groups, IgG antibody titres against the PC and dimer gH/gL were significantly higher in NT compared to T pregnant women in the first 30 days after infection onset [75]. In this study, the role of anti-PC antibodies in vertical transmission to the foetus was investigated by using a competitive ELISA inhibition of mAb binding (IMAB), where the PC bound to the solid phase was reacted competitively with human sera and murinized mAbs specific for PC neutralization sites [74, 80]. When the IMAB titres were examined in the two groups of T and NT women at three different time intervals, they were found to be significantly lower (Fig. 6) in the T group for 7/10 antigenic sites investigated [75]. In addition, the number of neutralization antigenic sites recognized by T women was significantly lower than that recognized by NT women during both the first and the second months after onset of infection, whereas no difference was found at later times (Fig. 7). In conclusion, the IgG antibody titre for the PC, the IMAB titre for multiple PC antigenic sites and the number of PC antigenic sites recognized were all significantly higher in NT compared to T women early after infection.

It has recently been reported that >50 % of HIV dissemination occurs cell-to-cell rather than through cell-free virus infection [81]. Following this line of thought, we tested the dissemination-inhibiting activity of HCMV-neutralizing antibodies, both mAbs and convalescent-phase sera from primary infections, by using three different assays: (i) plaque formation (cell-to-cell spreading) inhibition (PFI) ([74, 79] (Fig. 8); (ii) leukocyte transfer inhibition (LTI), i.e. inhibition of transfer from infected endothelial cells to leukocytes (PMNLs and M/M) ([74, 79] (Fig. 8); and (iii) syncytium formation inhibition (SFI) [82] (Fig. 9). Although it is not known how effective PFI, LTI and SFI antibodies may be in vivo, we believe that the strong in vitro inhibitory effect of mAbs directed to UL128L gene products may suggest a potential effect in vivo.

As a general rule, it was found that LTI and SFI antibodies were detected late (at least >30 d after onset of primary infection), whereas PFI antibodies appeared much earlier. In addition, the PFI$\|_{\text{gB}}$ antibody titre was significantly higher in NT women during the first month after onset of infection as compared to T women (Fig. 8d). Among human mAbs,
the inhibitory effect was restricted to neutralizing mAbs directed to the pUL128L components of PC, whereas it was greatly reduced for anti-gB and anti-gH mAbs. In addition, regression curves of PFI₅₀ titres were significantly higher in NT compared to T mothers (Fig. 8e). More recently, neutralizing mAbs to one, two, or three components of the pUL128L were shown to block syncytium formation in heavily infected ARPE-19 epithelial cells, whereas only a partial inhibitory effect was shown by mAbs to gO, gH or gB at the same concentration (Fig. 9c) [82]. A similar blocking effect (Fig. 9) was exhibited by convalescent-phase sera from primary HCMV infections. However, there is no current evidence that HCMV spreads in vivo by syncytial formations [83].

As a conclusion, it was found that PFI₅₀ antibody titres as well as PFI₅₀ regression curves were significantly higher in NT versus T women during the first month of infection, as shown by human mAbs directed to the PC, but not to gB or gH. It appears reasonable to hypothesize that PFI antibodies may be a first line of defence at the onset of infection, while LTI antibodies appear at a relatively late stage of infection, when the virus is located inside endothelial cells of the vascular tree, and SFI antibodies are only detected after syncytium formation resulting from a high viral load.

In the vaccinology field, antibody titre and neutralizing activity are often the only parameters considered in the evaluation of protective immunity. However, these parameters are often not sufficient to account for protective immunity [84]. On the other hand, protective immunity has been observed in the absence of neutralizing activity, while a critical role in protection from infection has been attributed for both HIV and HCMV to extra-neutralizing antibody functions, such as antibody-dependent cellular cytotoxicity (ADCC) [85, 86], antibody-dependent complement deposition (ADCD) [84] and antibody-dependent NK cell activation [87].

Following natural infection or vaccination, persisting viral infections, such as HCMV infection, elicit an extended array of antibodies. Thus far, the role of antibody-dependent cellular mechanisms in the HCMV immune response has not yet been fully investigated, and this is expected to be one of the major fields of investigation in the near future.

**DEVELOPMENT OF AN HCMV VACCINE: POTENTIAL ROLE OF THE PC**

As recently reported, the vaccines being developed can all be classified into two groups [88]. One group includes modified Towne and AD169 viruses, Towne/Toledo chimeric viruses and dense bodies. The other group includes one or more viral antigens, either in the form of recombinant viral proteins or delivered through a viral vector or a DNA vector.

In the first group, Towne and AD169 viruses both carry a 15-kb deletion in the Ulb' region [52], as well as various mutations in UL128L, as a result of multiple passaging in HELFs [43, 44]. Thus, both Towne and AD169 have lost the ability to infect endothelial and epithelial cells and lack a
Fig. 6. Inhibition of 50 % monoclonal antibody binding (IMAb50) titre by site-specific antibodies present in human sera from primary HCMV infection. IMAb50 titres of neutralizing site-specific IgG antibodies in 12 transmitter (T) and 11 non-transmitter (NT) mothers at three different time intervals (<30 days, 30–60 days, >60 days) after onset of infection. (a–j) Sites 1–10 as defined by the indicated human mAb to gH/gL/pUL128L (Macagno et al. [80]). Dotted lines represent the detection limit of the assay. P values were calculated using the Mann–Whitney U-test (from Lilleri et al. [75]).
functional PC. The Towne vaccine was not effective in preventing HCMV infection in either volunteers or kidney transplant recipients (KTR), but prevented overt HCMV disease in D+R- KTR [89]. In addition, four chimeric Towne/Toledo viruses were constructed in the late 1990s and are currently under evaluation [90, 91].

One of the most interesting approaches has been based on the development of a whole live attenuated AD169 virion vaccine, in which repair of a frameshift mutation in the UL131 gene restored PC expression with improved immunogenicity in both rabbits and rhesus macaques [92]. Using this virus vaccine, it was shown in rabbits that the PC is the primary target for potent neutralizing antibodies [93, 94], which are able to display antiviral activity against a panel of genetically distinct HCMV clinical isolates [95]. The same vaccine, when made conditionally replication-defective, was shown to elicit a durable neutralizing antibody response and a T cell (both CD4+ and CD8+) response to multiple viral antigens in non-human primates (rhesus macaques) [96].

Another significant contribution to the development of an efficacious vaccine was provided by the construction of a modified vaccinia Ankara (MVA) virus co-expressing all five PC subunits. Mice or rhesus macaques vaccinated three times

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**Fig. 7.** Overall number of neutralization sites of the pentamer complex that are reactive with human sera, and viral load at three time points. Number of neutralization sites recognized by sera from 12 transmitter (T) and 11 non-transmitter (NT) mothers at (a) less than 30 days, (c) 31–60 days and (e) more than 60 days after onset of infection. (b, d, f) Number of HCMV DNA-positive (black bars) and HCMV DNA-negative (white bars) women in the NT and T groups at the same time intervals. P values were calculated using the Mann–Whitney U-test (a, c, e) or Fisher’s exact test (b, d, f) (from Lilleri et al. [75]).

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with MVA recombinants induced a durable neutralizing antibody response that was greater than that obtained following inoculation of MVA expressing gH/gL, UL128-131, or gB alone. Such a neutralizing antibody response was also detected in the saliva of immunized animals and reached serum titres comparable to those found in HCMV hyperimmune globulin preparations [97]. In addition, using this non-human primate model, it was shown that CD4⁺ depletion at the time of rhesus virus inoculation induced both a reduced and delayed neutralizing antibody response [98].

Using an alphavirus replicon vaccine based on Venezuelan equine encephalitis (VEE) virus and consisting of virus replicon particles (VRPs) expressing gB and a pp65-IE1 fusion protein, vaccine candidates were found to be immunogenic in mice [99] and humans [100]. When VRPs encoding gH/gL or PC were compared with the two complexes in the presence or absence of MF59 adjuvant, it was found that VRPs expressing PC produced higher levels of Nt antibodies than those expressing gH/gL, and that MF59 significantly increased the potency of each complex [101, 102].

Fig. 8. Plaque formation and leukocyte transfer inhibition by human monoclonals and serum antibodies. Dose-effect inhibition of (a) HCMV plaque formation (PFI) in ARPE-19 cells, and (b) HCMV transfer to leukocytes (LTI) from infected HUVEC by serial concentrations of human mAbs specific for different sites of the pentamer complex. (a) mAbs to gB and gH show PFl50 activity at a concentration ~1000-fold higher in comparison to mAbs directed to pUL128L. (b) Comparable LTI50 activity is displayed by human mAbs directed to pUL128L, while mAbs to gH and gB again show a much weaker inhibitory effect. (c) Individual PFI50 titres in sequential sera collected within the first 3 months after onset of infection in five transmitter (T) and four non-transmitter (NT) pregnant women. (d) PFI50 titres from the same sequential sera at three different time intervals (days) after onset of infection are shown. $P$ values were calculated using the Mann–Whitney U-test. (e) Regression curves of PFI50 titres in T and NT women. $P$ value was calculated using the extra-sum-of-square F test (from Lillie et al. [75]).
Moreover, non-infectious dense bodies containing both virion tegument and envelope proteins harvested from culture supernatants of AD169 or Towne-infected HELFs, were tested in small animals, showing that both Nt and cellular immune responses are able to prevent infection of both HELFs and epithelial cells [103]. Further, another innovative approach to vaccine development was the activation of both innate and adaptive immunity by a recombinant HCMV strain expressing an NKG2D ligand [104].

Among individual viral antigen vaccines, recombinant DNA technology and the CHO mammalian expression platforms have been used to develop a gB vaccine with a deleted transmembrane domain [105]. In one trial, the gB/MF59 vaccine provided approximately 50% efficacy against acquisition of HCMV infection in HCMV-seronegative women enrolled post-partum [106]. In another phase II study, this vaccine was effective in preventing viremia in 5/11 D+R solid organ transplant recipients (SOTR) compared to 0/5 subjects in the placebo group [107]. However, most of the antibodies elicited by gB in HCMV-seropositive individuals were not neutralizing [79, 108].

A critical breakthrough was represented by the identification of the PC, which consisted, as mentioned above, of the UL128L products complexed with gH and gL to form the PC-mediating entry complex for epithelial and endothelial cells [9] as well as dendritic [109] cells and M/M [110, 111]. As a result, several investigators have suggested including the PC in vaccine designs [112]. However, to date, no phase I trial has been completed with a vaccine containing the PC alone.

More recently, we took advantage of previously isolated mAbs that bind to conformational epitopes on the PC and are extraordinarily potent in neutralizing HCMV infection of epithelial, endothelial and myeloid cells [80]. In this collaboration with Lanzavecchia’s group (Bellinzona, CH), a novel process was developed to produce, in a secreted form, a recombinant pentamer vaccine from a mammalian CHO

Fig. 9. (a, b) Immunofluorescent staining of VR1814-infected ARPE-19 epithelial cells at 96 h p.i. HCMV fluorescent nuclei (p72) are shown in green, while Evans blue counterstaining is shown in red. (a) Monolayer of ARPE-19 cells showing multiple syncytia. (b) Complete syncytium formation blocking by a human mAb directed to pUL130-131 of the pentamer complex. (c) mAbs to the pentamer complex and its pUL128L components completely blocked syncytium formation (SF) at the higher concentration tested (10 µg ml−1), unlike mAbs to gO, gH, or gB, which only partially inhibited SF (from Gerna et al. [82]).
cell line stably transfected with a single polycistronic vector encoding the five HCMV pentameric genes separated by autonomous self-cleaving 2A peptides [79]. We reported that in mice this vaccine elicits neutralizing antibody titres that are 100–1000-fold higher than those induced in humans during natural infection. In addition, sera from pentamer-immunized mice showed high potency in blocking both plaque formation (Fig. 10a) and virus transfer from infected EC to leukocytes (Fig. 10b). These results highlight the typical approach of analytic vaccinology in the development of a subunit vaccine [113]. A phase 2 randomized double-blind placebo-controlled trial with a combination (RG7667) of a human mAb to gH and a humanized murine mAb to PC was recently reported to reduce HCMV infection and disease in high-risk KTR [114].

Guinea pig cytomegalovirus (GPCMV) has been proposed as a valuable model for the study of maternal–foetal transmission in the context of congenital HCMV infection [115, 116]. Yamada et al. [117] first described a region of the GPCMV genome which appeared to contain homologues of HCMV PC and, more recently, Auerbach et al. [118] concluded that the guinea pig genes GP129, GP131 and GP 133 are the homologues of the HCMV genes UL128, UL130 and UL131, and that the relevant recombinant proteins are immunoreactive with convalescent sera from infected animals [119]. Thus, the protein products of GPCMV GP129, 131 and 133 locus were referred to as the GP PC, and the mutations acquired during passaging in fibroblasts attenuated virus pathogenicity [120], whereas repair resulted in congenital transmission, intrauterine growth restriction and elevated pup mortality. Thus, it was concluded that a vaccine aimed at preventing congenital infection should contain PC. Finally, a neutralizing mAb to gH/gL was found to be protective in the guinea pig model of congenital infection [121]. However, concerns have been raised about the ability of the guinea pig model to predict the outcome of clinical trials [98, 122].

**INNATE AND ADAPTIVE T CELL IMMUNITY TO HCMV AND THE PC**

Natural killer (NK) cells are the most involved innate immune cells during HCMV infection, i.e. a stable expansion of NK cells NKG2C<sup>bright</sup> during primary HCMV infection has been confirmed to occur [123]. This NK cell subset is found early following primary infection, as well as in congenitally infected infants and SOTR [124–127]. NK cells are
considered to be the main effectors of antibody-dependent cell-mediated immune responses. In a recent not-yet unpublished study performed in our laboratory and extended to three groups of subjects (HCMV-seronegative and subjects with primary or remote infection), it was found that, within CD56<sup>dim</sup> gated CD16<sup>+</sup> NK cells, NKG2C<sup>bright</sup> CD57<sup>+</sup> cell numbers were significantly higher during primary and remote infection with respect to seronegative subjects. The potential role of the PC (and other viral proteins) in eliciting the antibody-mediated NKG2C<sup>bright</sup> response during primary infection remains to be investigated.

In addition, γδ T cells, which were initially considered a first-line defence mechanism, have been shown to undergo long-lasting expansion in their Vδ2<sup>(and not Vδ2)</sup> subset (Fig. 11) in SOTR [128–130] and in immune-competent subjects [131] as well as in foetuses with congenital infection [132]. This expansion has been interpreted as a type of specific signature of the adaptive immune response to HCMV infection [133]. Following expansion, γδ T cells were found to be able to kill HCMV-infected target cells in vitro [134], and CD16 (FcyRIIIA) was found to be specifically expressed by the majority of HCMV-induced γδ T cells. However, CD16<sup>+</sup> γδ T cells did not appear to mediate ADCC against HCMV-infected cells in vitro [135]. Therefore, the potential role of the PC and different viral proteins in the expansion of γδ T cells through interaction with antibodies remains to be investigated.

As for adaptive T cell immunity, in our laboratory HCMV-specific T cell responses to HCMV infection were routinely measured using an in-house developed procedure [136]. This method, which does not require HLA typing and measures both HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup>T cells, is based on the incubation of PBMCs with autologous monocyte-derived HCMV-infected dendritic cells, followed by determination of multiple membrane/intracellular parameters by cytokine flow cytometry [137]. Recently, the T cell response to four major HCMV proteins (IE-1, pp65, PC and gB) was evaluated using overlapping peptides (15mers) spanning the entire proteins, and libraries of amplified (by the addition of PHA and IL-2) T cells, according to a well-known procedure [138]. The results showed (Fig. 12) that the PC and gB were more efficiently recognized by CD4<sup>+</sup>T cells, IE-1 by CD8<sup>+</sup> T cells, while pp65 was recognized equally by both major T cell subsets [139].

**FOLLICULAR HELPER T CELLS (T<sub>FH</sub>) AND ANTIBODY PRODUCTION**

Recently, a comparative study of the kinetics of the serum antibody response to primary HCMV infection in the immune-competent and the immunocompromised SOTR showed that the HCMV peak neutralizing antibody titres were 16-fold higher, and the ELISA-IgG antibody titres for PC 4-fold higher, in SOTR starting 6 months p.i. [140]. The higher median peak antibody titre in primary infection of SOTR appeared to be related to the sustained presence of activated (ICOS<sup>+</sup>PD-1<sup>+</sup>) circulating follicular helper T (T<sub>FH</sub>) cells in the absence of detectable HCMV-specific effector CD4<sup>+</sup>T cells in blood and the presence of HCMV DNAemia [141]. All of these events occurred much earlier in immune-competent patients.

In addition, when investigating the specificity of the peripheral blood T<sub>FH</sub> cell response to HCMV proteins in patients with primary infection, it was found that T<sub>FH</sub> cells responding to HCMV were included in the activated T<sub>FH</sub> cell subset, thus suggesting that activated T<sub>FH</sub> cells present...
in blood during primary infection are elicited by HCMV. Interestingly, the frequency of T<sub>FH</sub> cells specific for the PC was higher with respect to that for pp65 in most patients at late time points, thus indicating that the PC was highly stimulatory for T<sub>FH</sub> cells (Fig. 13). These findings are somewhat in keeping with similar results observed with Flu- or HIV-specific CD4<sup>+</sup>T cells, where T<sub>FH</sub> cells are preferentially directed towards the surface haemagglutinin or env [142, 143].

**IMMUNE RESPONSE TO PC AND PROTECTION FROM HCMV INFECTION/DISEASE**

The results of our studies on the immune response to the PC in immune-competent and immunocompromised hosts during primary HCMV infection indicate unequivocally that antibody production starts early (within 1–2 weeks) in the immune-competent host, in parallel with the appearance of HCMV-specific T<sub>FH</sub> cells, which, in the germinal centres of lymphnodes, provide help to B cells differentiating into antibody-producing plasma cells in the presence of viral DNA in blood [144]. The same sequence of events also occurs during primary infection of SOTR, in which antibody production starts later, but continues much longer [141]. In both immune-competent and transplanted patients, a block of antibody production occurs upon the appearance of HCMV-specific CD4<sup>+</sup>T cells, which promote the cytolytic activity of HCMV-specific CD8<sup>+</sup>T cells. The blocking of antibody production following the appearance of HCMV-specific CD4<sup>+</sup>T cells fits with the results of our recent studies in both SOTR and HSCTR, where the viral infection was controlled both virologically and clinically by the appearance of HCMV-specific CD4<sup>+</sup>T cells in the presence of CD8<sup>+</sup>T cells [145, 146] and in association with a drop in the number of activated T<sub>FH</sub> cells in blood.

It is reasonable to assume that protection against HCMV infection/disease may occur through the collaborative activity of both antibodies and cell-mediated immunity. However, since after infection onset antibody production starts much earlier than the specific CD4<sup>+</sup>T cell appearance (particularly in transplant recipients), the question arises as to how antibodies may act in the time interval preceding CD4<sup>+</sup>T cell detection. Based on in vitro and ex vivo findings, at least three protective mechanisms may be indicated.

(i) Neutralization (or blocking) of endothelial/epithelial cell infection, as shown in vitro by neutralization assays. Although cell-free virus can be released in blood and thus be neutralized [147, 148], this mechanism may not play a major role in vivo. The finding that in SOTR most of the viral DNA detected in plasma is highly fragmented and not virion-associated [149] may be due in part to DNA fragments being released by endothelial cells and leukocytes undergoing lysis. However, in this context, virions may also be released in blood and thus be neutralized.

(ii) Blocking of cell-to-cell virus dissemination by (a) PFI antibodies [74, 75, 79], (b) LTI antibodies [74, 75, 79] and c) SFI antibodies [82]. Recently, it was suggested that merely blocking cell-free virus infection in the absence of cell-to-cell blocking would only partially limit HIV virus dissemination [81].

(iii) ADCC, in which the antibody acts as a bridge between the target cell (e.g. infected epithelial/endothelial...
cells) and NK carrying different types of Fc receptors [84, 86].

In the initial stages of HCMV infection, in addition to antibodies, other innate immunity cells may contribute to protection as a first line of defence, such as NK and γδ T cells, as mentioned above.

CONCLUSIONS AND FUTURE TRENDS

The identification of the PC between 2004 and 2008 opened the way to new approaches in the development of an HCMV vaccine (Table 1). The results achieved so far in the study of the immune response to the PC in both natural infection and experimental animal models document the development of a potent neutralizing antibody response. Extended trials aimed at verifying the protective effect of neutralizing mAbs directed against the PC in both sero-negative and sero-positive pregnant women, as well as immunocompromised patients, are urgently needed. Positive results would represent the basis for the development of an HCMV vaccine. In addition, while the first data relevant to the T cell response to the PC are being collected, the area of the non-neutralizing activity of the antibody response to the PC in the context of the study of ADCC remains mostly unexplored. This approach could pave the way to a novel and as yet partially unknown mechanism of protection against HCMV.

**Fig. 13.** HCMV specificity of quiescent (ICOS−PD-1−; left panels) and activated (ICOS+PD-1++; right panels) TFH cells (CXCR5+ CD4+ T cells) in an immuno-competent patient examined 24 days (a and b) and 76 days (c, d) after onset of symptoms during a primary HCMV infection. In each panel, the frequency of HCMV peptide-specific (IE-1, pp65, gH/gL/pUL128L and gB) T cells, as well as that of T cells reactive with control peptide pools of influenza (Flu-pp) and respiratory syncytial virus (RSV-pp), is shown (from Bruno et al. [141]).

![Day 24 after onset of symptoms](image1)

![Day 76 after onset of symptoms](image2)
Table 1. Summary of the review

<table>
<thead>
<tr>
<th>Leukocyte and endothelial cell tropism</th>
<th>Immune response to PC and virus dissemination</th>
<th>Development of an HCMV vaccine: potential role of PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 1988–1991: leukocyte tropism was first shown by diagnostic assays: antigenemia (1988–1989), viremia (1990) and leukoDNAemia (1991).</td>
<td>Ab response to PC in primary HCMV infections, unlike antibody response to gB, was found to be mostly Nt. In addition, IgG antibody titres to PC were significantly higher in NT versus T pregnant women as well as the reactivity versus most antigenic sites of PC. As for dissemination-inhibiting activity, both human mAbs to pUL128L components of PC and convalescent sera from primary infections showed much greater inhibiting activity than α-gH and α-gB mAbs by LTI, PFI and SF</td>
<td>Two groups of vaccines are being developed: (i) live virus vaccines; (ii) non-living HCMV vaccines.</td>
</tr>
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<td>- 1993: in AIDS patients HCMV-infected ECs were shown to enlarge until detaching from the vessel wall and entering the blood stream.</td>
<td>As for innate immunity, the potential role of PC in eliciting an NKG2D+T cells response as well as its role in the expansion of γ/δ T cells through interactions with antibodies, remains to be investigated.</td>
<td>The first group includes modified Towne and AD169 viruses, Towne/Toledo chimeric viruses and dense bodies.</td>
</tr>
<tr>
<td>- 1998: generation in vitro of the antigenemia assay.</td>
<td>In preliminary observations, PC appears to be more efficiently recognized by CD4+ compared to CD8+ T cells. Also, the Ab titre appears to be related to the presence of activated circulating T(H) cells in the absence of CD4+ T cells, and activated T(H) cells appear to be highly stimulated by PC. The collaborative activity of antibody and CMI should confer protection. In addition, ADCC in which Abs act as a bridge between the target cell and NK prior to the appearance of CMV-specific CD4+ T cells may play a major role.</td>
<td>The second group includes one or more viral antigens either as recombinant proteins or delivered through a viral vector or a DNA vector.</td>
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<tr>
<td>- 2000: demonstration in vitro of the bidirectional transfer of virus between cells infected with field HCMV strains and leukocytes (PMNL &amp; M/M).</td>
<td>- 2004: UL131-128 locus (UL128L) within the UL1' region of the HCMV genome is indispensable for both leuko- and EC-tropism.</td>
<td>In both cases, the highest and most durable Nt Ab response was given by a native PC, while a good CMI response was given by vaccines containing gB or live HCMV vaccines. The CMI response given by PC remains to be deeply investigated.</td>
</tr>
<tr>
<td>- 2008: gH/gL was shown to complex with UL128L to form the PC (gH/gL/pUL128L) required for the infection of endothelial/ epithelial/myeloid cells.</td>
<td>As for innate immunity, the potential role of PC in eliciting an NKG2D+T cells response as well as its role in the expansion of γ/δ T cells through interactions with antibodies, remains to be investigated.</td>
<td>Among animal models, the GP remains the most studied and the most similar to HCMV with respect to virus transmission to the foetus. In this model, a GP PC consisting of gH, gL, pUL129, pUL131 and pUL133 is the homologue of HCMV PC, and is highly pathogenic for congenital infection, which can be reduced by the presence of mutations.</td>
</tr>
</tbody>
</table>

Ab, antibody; CMI, cell-mediated immunity; ECs, endothelial cells; GP, guinea pig; LTI, leukocyte transfer inhibition; M/M, monocyte/macrophages; Nt, neutralizing; NT, non-transmitting women; PC, pentamer complex; PFI, plaque formation inhibition; PMNL, polymorphonuclear leukocytes; SFI, syncytium formation inhibition; T, transmitting virus to the foetus

infection, and strengthens our knowledge concerning the pathogenesis of HCMV infection.

Funding information
Funding through the years was provided by the Ministero della Salute, Rome; Associazione Italiana del Farmaco (AIFA), Rome; Fondazione Denegri, Torino; and Fondazione CARIPLO, Milano, Italy.

Acknowledgements
The entire medical and biology staff, as well as the technical staff of the former Servizio di Virolgia and the current Molecular Virology Unit of the Microbiology and Virology Department, are gratefully acknowledged for having contributed either directly or indirectly to this review. In addition, we are indebted to Daniela Sartori for editing the manuscript, and Laurene Kelly for revision of the English.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All clinical and experimental work with humans reported by the authors in this review was approved by the Ethical Committee of the Fondazione IRCCS Policlinico San Matteo, and the subjects involved gave their written informed consent to participate.

References
5. Fowler KB, Stagno S, Pass RF. Maternal immunity and prevention of congenital cytomegalovirus infection. JAMA 2003;289:1008–1011.


60. Ciferri C, Chandramouli S, Donnarumma D, Nikitin PA, Cianfrocco MA et al. Structural and biochemical studies of HCMV gH/gL/gO and pentamer reveal mutually exclusive cell entry complexes. *Proc Natl Acad Sci USA* 2015;112:17767–1772.


63. Wille PT, Knoche AJ, Nelson JA, Jarvis MA, Johnson DC. A human cytomegalovirus gO-null mutant fails to incorporate gH/gL into the viral envelope and is unable to enter fibroblasts and epithelial and endothelial cells. *J Virol* 2010;84:2585–2596.

64. Zhou M, Lanchy JM, Ryckman BJ. Human cytomegalovirus gH/gL/gO promotes the fusion step of entry into all cell types, whereas gH/gL/UL128-131 broadens virus tropism through a distinct mechanism. *J Virol* 2015;89:8999–9009.


142. Leddon SA, Richards KA, Tereanor JJ, Sant AJ. Abundance and specificity of influenza reactive circulating memory follicular helper and non-follicular helper CD4<sup>+</sup> T cells in healthy adults. *Immunology* 2015;146:157–162.


144. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* 2014;41:529–542.


