

# Human cytomegalovirus (HCMV) infection/re-infection: development of a protective HCMV vaccine

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## SUMMARY

In recent years, one of the main objectives in the field of medical virology has been the development of a human cytomegalovirus (HCMV) vaccine that can prevent congenital HCMV infection in the offspring of pregnant women as well as systemic and end-organ disease in immunocompromised (AIDS and transplanted) patients. Major obstacles to the development of an efficacious HCMV vaccine are lack of protection provided by immune memory cells against HCMV re-activation (replication relapse of a latent strain following primary infection) and HCMV re-infection (infection of a seropositive individual by a new virus strain). Thus, while initial efforts were directed at the development of a vaccine for the prevention of primary infection, in the last decade the primary vaccine development endpoint was the prevention of primary HCMV infection, as well as HCMV re-activation and re-infection. Along this line of research, both HCMV live (including Towne, AD169 and its derivatives, Towne/Toledo chimeras, Viral-Vectored vaccines, and Virus Replicon Particles) and non-living vaccines (including the recombinant gB subunit, DNA- and RNA-based vaccines, Virus-like particles, Dense bodies, Peptide vaccines, and the Pentamer Complex) have been developed. To date, Phase I, II, and III clinical trials have been variably conducted in humans, and experimental inoculation in different animal models has been performed with different vaccine formulations. Notwithstanding the variable research conditions, clinical and experimental results achieved thus far predict that the ideal HCMV vaccine should be able to elicit both robust humoral (both neutralizing and binding) and HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses. This vaccine should hypothetically contain:

- 1) gB, inducing both humoral and T-cell responses;
- 2) the pentameric complex (PC), inducing the most potent neutralizing antibody response;
- 3) pp65, inducing the most potent HCMV-specific T-cell response.

Although the protective role of cell-mediated immunity has been repeatedly documented, while the protective effect of (mostly neutralizing) antibodies remains partly to be documented, a vaccine stimulating both arms of the immune response would likely confer the highest level of protection against HCMV infection/disease in both HCMV-seronegative and -seropositive individuals.

Received August 10, 2018

Accepted December 3, 2018

## HCMV INFECTION (Table 1)

### 1.1 Epidemiology

Human Cytomegalovirus (HCMV) or Human Herpesvirus-5 (HHV-5) is widespread throughout the world, mostly causing asymptomatic infections in immunocompetent people. However, HCMV infection may occasionally progress to HCMV disease in the immunocompetent (in the form of a mononucleosis-like syndrome) and much more frequently, may cause life threatening HCMV disease in immunocompromised (transplanted and AIDS) patients. In the immunocompetent, although transmission of HCMV infection to the fetus may occur also in seropositive pregnant women, the most critical clinical condition

is primary infection of a seronegative pregnant mother, where transmission of infection to the fetus occurs in about 40% of cases. This is the rate of transmission occurring in Western Europe, the USA and Australia, where about 50% of women of childbearing age are HCMV-seropositive, while in South America, Asia, and Africa nearly 100% are HCMV-seropositive (Cannon *et al.*, 2010). Due to the direct correlation found between the rate of congenital HCMV infection (cCMV) and the rate of HCMV seropositivity, it has been estimated that of the approximate 30,000 cases of cCMV occurring annually in the US, three-quarters are due to non-primary maternal infections (Wang *et al.*, 2011), while the proportion of cCMV infections due to non-primary infections in developing countries may be even higher (Yamamoto *et al.*, 2010).

The prevalence of cCMV infections in newborn infants ranges from 0.5%-0.7% in developed countries and from 1-2% in developing countries (Kenneson & Cannon, 2007). About 13% of newborns with cCMV are symptomatic at birth and present with overt clinical symptoms, such as intrauterine growth retardation, jaundice, hepatosplenomegaly, thrombocytopenia, purpura, microcephaly, seizures and chorioretinitis (Dollard *et al.*, 2007). Permanent

#### Key words:

Human cytomegalovirus, Live vaccine, Non-living vaccine, Immune response, Neutralizing antibody, T-cell response, Pregnant women, Solid-organ transplant, Hematopoietic stem cell transplant, Ideal HCMV vaccine.

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**Table 1 - HCMV Infection/Disease: target populations.**

1.1. Epidemiology	<p>a) <i>Immunocompetent host.</i></p> <ul style="list-style-type: none"> <li>- Prevalence of cCMV is 0.5-0.7% in developed countries, and 1-2% in developing countries (Kenneson and Cannon, 2007)</li> <li>- Permanent <i>sequelae</i> reach ~20% (Dollard <i>et al.</i>, 2007), including SNHL (Cannon, 2009).</li> </ul> <p>b) <i>Immunocompromised host.</i></p> <ul style="list-style-type: none"> <li>- In AIDS and transplanted patients, HCMV systemic and end-organ diseases arise frequently</li> </ul> <p>c) <i>General population.</i></p> <p>HCMV has been involved in:</p> <ol style="list-style-type: none"> <li>1) excess disease mortality in intensive care unit patients (Limaye &amp; Boeckh, 2010);</li> <li>2) health deterioration of the elderly (Derhovanessian <i>et al.</i>, 2011; Solana <i>et al.</i>, 2012);</li> <li>3) mortality rate in the general population (Simanek <i>et al.</i>, 2011; Gkrania-Klotsas <i>et al.</i>, 2012).</li> </ol>
1.2. Prevention	<p><i>Immunocompromised host:</i></p> <ul style="list-style-type: none"> <li>- In transplanted patients, HCMV disease can be prevented by reconstitution of T-cell immunity in association with antiviral chemotherapy (Lilleri <i>et al.</i>, 2018).</li> </ul> <p><i>Immunocompetent host:</i></p> <ul style="list-style-type: none"> <li>- Due to the partial protective effect of the immune response elicited by natural infection, both primary as well as reactivated infections and re-infections could hopefully be prevented by an efficacious vaccine conferring a broad immune coverage.</li> </ul>

cCMV, congenital CMV infection; SNHL, Sensory neural hearing loss.

*sequelae* involve newborns with cCMV infection, and the total prevalence of newborns developing permanent *sequelae* is around 20%, including 13% of infants who are symptomatic at birth and approximately 7% of infants who are asymptomatic at birth (Dollard *et al.*, 2007). Sensorineural hearing loss (SNHL) is one of the most studied permanent *sequelae*. It has been observed that in asymptomatic newborns with cCMV the prevalence of SNHL at 3-4 years of age is 7-21%, while some children develop SNHL at 7 years of age or more (Foulon *et al.*, 2008). Although the rates of SNHL are comparable in children born to mothers with primary or non-primary infection, cCMV disease resulting from primary maternal infection is often associated with greater neurological damage and more severe SNHL (Ross *et al.*, 2006). In any case, cCMV infection has been estimated to be the most common viral cause of SNHL and neurodevelopmental delay in USA (Cannon, 2009).

Besides cCMV, which may occur in non-immune or even immune competent mothers, there are other major clinical conditions, associated with drug-induced (solid organ or stem cell transplantation) or pathological immunosuppression (AIDS), where the burden of HCMV disease may be severe. In both cases, the immature immune system of the fetus or the variable level of immunosuppression in transplanted and AIDS patients are considered the root causes of both systemic and local overt HCMV disease. It still remains to be clarified (see below) whether humoral (antibody) or innate/adaptive cell immunity or both play a major role in protection against HCMV infection/disease. Finally, HCMV is also involved in excess disease/mortality of intensive care unit patients (Limaye & Boeckh, 2010),

the elderly (Derhovanessian *et al.*, 2011; Solana *et al.*, 2012), and even the general population (Simanek *et al.*, 2011; Gkrania-Klotsas *et al.*, 2012).

### 1.2 Prevention

Nowadays, while it is relatively easy to diagnose a primary infection, particularly when seroconversion is documented, it is much more difficult to diagnose a cCMV infection as a result of maternal reactivation of a virus strain already present in the mother prior to pregnancy, or a maternal re-infection due to a newly infecting virus strain in a seropositive mother. While the rate of cCMV infection in pregnant women experiencing primary infection has been found to be around 30-40% of cases (Revello *et al.*, 2014; Britt, 2017), the rate of cCMV in populations of seroimmune pregnant women has been found to be much higher both in developing and developed countries. In a recent epidemiological study conducted in the US, it was estimated that the overall prevalence of cCMV (including newborns from mothers with either primary or non-primary HCMV infection) is 3-4/1,000 live births (Boppana *et al.*, 2011; Pinninti *et al.*, 2015). This prevalence is significantly superior to that of other causes of disease in newborns, such as cystic fibrosis (0.3/1,000) or chromosomal trisomy (1.2/1,000), although lower than congenital heart malformations (10/1,000). Among neurological *sequelae*, SNHL is the most frequent long-term *sequela* in cCMV, representing about 25% of all cases in the US (Fowler, 2013).

The major hurdle to the prevention of cCMV infection and the development of an effective anti-HCMV vaccine is represented by the peculiar characteristics of HCMV infection. Unlike rubella virus infection, whose transmission

**Table 2 - Immune response to natural HCMV infection.**

2.1. Innate response	<p>a) CD57 NKG2C<sup>bright</sup> cells (Lilleri and Gerna, 2017).</p> <p>b) ADCC (Wu <i>et al.</i>, 2013; Chung <i>et al.</i>, 2014)).</p> <p>c) <math>\gamma/\delta</math> T-cells and, namely, V<math>\delta</math>2 <math>\gamma/\delta</math> T-cells (Pitard <i>et al.</i>, 2008; Fornara <i>et al.</i>, 2011).</p>
2.2. Antibody response	HCMV-specific antibodies, and namely neutralizing antibodies (NAb), were considered in the past to have a protective effect against vertical HCMV transmission in seronegative mothers (Fowler <i>et al.</i> , 1992). However, recently, vertical transmission was also shown to occur in mothers who were seropositive prior to pregnancy (Ross <i>et al.</i> , 2006, 2010; Novak <i>et al.</i> , 2009).
2.3. T-cell response.	Protective role of HCMV-specific CD4 <sup>+</sup> and CD8 <sup>+</sup> T-cells documented in the 90s following adoptive transfer of <i>in vitro</i> expanded CD4 <sup>+</sup> and CD8 <sup>+</sup> T-cells (Walter <i>et al.</i> , 1995; Einsele <i>et al.</i> , 2002).

ADCC, antibody-dependent cellular cytotoxicity.

to the fetus is efficiently prevented by a vaccine inducing maternal immunity similar to that produced by natural infection, maternal immunity elicited by natural HCMV infection may not prevent vertical HCMV transmission. Prior to the last decade, it was believed that the highest number of HCMV congenital infections was subsequent to primary infection developing during pregnancy (Enders *et al.*, 2011; Picone *et al.*, 2013; Revello *et al.*, 2014). As a result, efforts were initially directed toward the development of an HCMV vaccine able to induce an immune response similar to that detected after primary infection. However, in recent years, an increasing number of epidemiological findings has shown the high rate of cCMV infection occurring in the offspring of immune mothers, thus suggesting the need for a broader immune coverage to protect from both primary infection and re-infection.

In immunocompromised transplanted patients, HCMV disease may be prevented by reconstitution of T-cell immunity in association with antiviral chemotherapy (Lilleri *et al.*, 2018).

## 2. IMMUNE RESPONSE TO NATURAL INFECTION (Table 2)

### 2.1 Innate response

The involvement of innate immunity and, particularly, of natural killer (NK) and  $\gamma/\delta$  T-cells in the primary line of defense against primary HCMV infection has been repeatedly reported. In particular, a stable expansion of NK cells CD57<sup>+</sup>NKG2C<sup>bright</sup> has been found to occur early in life during primary infection, as well as in congenitally infected infants and in transplant recipients, both solid-organ (SOT) and hematopoietic stem cell transplant (HSCT) recipients (Lopez-Verges *et al.*, 2011; Foley *et al.*, 2012; Noyola *et al.*, 2012; Wu *et al.*, 2013; Costa-Garcia *et al.*, 2015; Lilleri & Gerna, 2017). The significant difference observed both in the absolute number and percentage of CD57<sup>+</sup>NKG2C<sup>bright</sup> NK cells between HCMV-seropositive and -seronegative subjects suggests a response to a specific antigenic stimulus, recently imputed to UL40-derived peptides (Hammer *et al.*, 2018). In addition, there is increasing evidence that during persisting infections (such as HCMV infection) a flexible humoral immune response occurs, aimed at eliminating the pathogen by a mechanistic process not involving neutralizing activity. This process may include antibody-dependent cellular cytotoxicity (ADCC, Gomez-Roman *et al.*, 2006; Wu *et al.*, 2013; Chung *et al.*, 2015), or other similar antibody-dependent (AD) mechanisms, such as AD cellular phagocytosis (ADCP, Ackermann *et al.*, 2011), AD complement deposition (ADCD, Chung *et al.*, 2015) and AD NK cell activation (Chung *et al.*, 2014).

As for  $\gamma/\delta$  T-cells, a similar mechanism was initially proposed. These immune cells were considered to be a first-line defense mechanism (Dechanet *et al.*, 1999) in kidney transplant recipients (KTR), as evidenced by the concomitant  $\gamma/\delta$  T-cell expansion and resolution of HCMV infection/disease. Subsequently, these cells were also shown to possess some characteristic properties of both innate and adaptive immune cells. However, only V $\delta$ 2<sup>-</sup> (and not V $\delta$ 2<sup>+</sup>)  $\gamma/\delta$  T-cells were reported to kill HCMV-infected target cells *in vitro* (Halary *et al.*, 2005) and to share a specific signature of the adaptive immune response in both immunocompetent and immunocompromised patients (Pitard *et al.*, 2008). In addition:

- 1) V $\delta$ 2<sup>-</sup>  $\gamma/\delta$  T-cells and HCMV-specific CD8<sup>+</sup>  $\alpha/\beta$  T-cells share common expansion kinetics and effector phenotype (Couzi *et al.*, 2009);
- 2) V $\delta$ 2<sup>-</sup>  $\gamma/\delta$  T-cells have similar expansion kinetics in pregnant women and transplant recipients (Fornara *et al.*, 2011);
- 3) a robust  $\gamma/\delta$  T-cell response to HCMV has been shown to occur *in utero* in cCMV infection;
- 4) a higher frequency of V $\delta$ 2<sup>-</sup>  $\gamma/\delta$  T-cells was found in HCMV-seropositive vs HCMV-seronegative subjects.
- 5) All these findings suggest the presence of a memory  $\gamma/\delta$  T-cell response or a chronic activation due to frequent HCMV reactivation/re-infection.

### 2.2 Antibody response

Apart from the early intervention of the innate immune response, commonly considered to be antigen-independent, but also endowed with some features of memory activity, the adaptive immune response appears to be of critical importance for the resolution of and protection against HCMV infection/re-infection. Both arms of the adaptive immune response seem to play a major role in the control of HCMV infection.

HCMV-specific antibodies, including both neutralizing antibodies (NAb) and different types of antigen-binding antibodies, have been involved in the prevention and protection against HCMV infection/disease in newborns (Yeager *et al.*, 1981), premature neonates (Snydman *et al.*, 1995), and the outcome of congenitally infected newborns (Fowler *et al.*, 1992). In addition, immunoglobulins (IG) were reported to reduce the rate of primary infections in HCMV-seronegative pregnant women and to have a beneficial effect on the treatment of fetal infection (Nigro *et al.*, 2005) as well as protection from placental pathology (Maidji *et al.*, 2010). As a result, a vaccine containing MF59-adjuvanted gB only was developed and found to partially protect HCMV-seronegative pregnant women from primary infection (Pass *et al.*, 2009).

Furthermore, the somewhat protective effect of antibodies, consisting of increased overall survival and reduction in HCMV infection/disease rate, was also reported in transplant recipients, both SOT (Bonaros *et al.*, 2008) and HSCT recipients (Raanani *et al.*, 2009) as well as in AIDS patients (Boppana & Britt., 1995).

As mentioned above, in the last decade, several reports have described the frequent transmission of HCMV infection to the fetus from mothers who were seropositive prior to pregnancy (Ross *et al.*, 2006, 2010). These events were attributed to a re-infection with a strain different from the one having caused the primary infection (Boppana *et al.*, 2001) and could be partially diagnosed by an ELISA assay detecting the antibody response to two fragments of glycoproteins gH and gB mostly (about 60%) reactive with strain-specific antibodies (Novak *et al.*, 2009).

### 2.3 T-cell response

The protective role of HCMV-specific CD8<sup>+</sup> T-cells against HCMV reactivation/disease was repeatedly documented in the '90s following the adoptive transfer of these cells into HSCT recipients (Reusser *et al.*, 1991). After the simultaneous transfer of both *in vitro* expanded CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, this effect was shown to persist over time (Walter *et al.*, 1995; Einsele *et al.*, 2002; Peggs *et al.*, 2003). Expansion was documented *in vivo* when a small number of antigen-activated T-cells was infused in HSCT recipients

along with a vaccine carrying the same antigen (Peggs *et al.*, 2011). In addition, it was shown that the removal of immunoevasion genes allowed CD8<sup>+</sup> T-cells to control HCMV re-infection (Hansen *et al.*, 2010).

Based on the results summarized above, a protective HCMV vaccine should contain antigens capable of eliciting both HCMV-specific humoral and T-cell immune responses.

### 3. VIRAL ANTIGENS PRIMARILY INDUCING THE IMMUNE RESPONSE (Table 3)

#### 3.1 Antibody response

Until recently, HCMV envelope glycoproteins (gp) gB (UL55-encoded) forming a homotrimer (gCI), and gH (UL75-encoded) forming with gL (UL-115-encoded) the complex gH/gL (gCIII), were considered to be the major targets of the humoral immune response, and, in particular, targets of the NAb response. In addition, a third envelope gp complex (gCII) consisting of gM (UL100-encoded) and gN (UL73-encoded) to form the gM/gN complex, was reported to be another target of the NAb response (Shimamura *et al.*, 2006). However, the NAb response to gB was universally thought to be immunodominant from the qualitative and the quantitative standpoint until 2004-2005, when the discovery of the trimer UL128-130-131 locus (UL128L) (Hahn *et al.*, 2004) showed that it was indispensable for infection of endothelial cells. This finding was followed by a second discovery: virus entry not only into endothelial cells, but also into epithelial (Wang & Shenk, 2005), and dendritic (Gerna *et al.*, 2005) cells was prevented by antibodies directed against the HCMV pentameric complex (PC) gH/gL/pUL128L (Ryckman *et al.*, 2008b). As a result, the NAb titer was found to be about 2 log<sub>10</sub> higher when determined on endothelial/epithelial cells rather than human fibroblasts (Gerna *et al.*, 2008; Cui *et al.*, 2008). Antibodies to PC represent the great majority of NAb detected after both natural and experimental infection as well as in commercial IG preparations (Fouts *et al.*, 2012; Lilleri *et al.*, 2013; Kabanova *et al.*, 2014).

In 2010, a series of potentially neutralizing human monoclonal antibodies (mAbs) were isolated, which were directed against the three components of the UL128L products and were able to prevent (neutralize) infection of epithelial cells (but not fibroblasts) at a 2-3 log<sub>10</sub> higher titer than hu-

man mAbs to gH or gB. On the other hand, the latter mAbs were able to neutralize the infection of both epithelial and fibroblast cells (Macagno *et al.*, 2010). In addition, mAbs to PC as well as antibodies developed during primary HCMV infection were shown to prevent epithelial cell syncytium formation (Gerna *et al.*, 2016). Immunization of mice with PC elicited NAb titers 2-3 log<sub>10</sub> higher than those detected in individuals recovering from primary HCMV infection. These neutralizing mAbs had the same potency as human mAbs and targeted the same (plus additional) antigenic sites on PC (Kabanova *et al.*, 2014).

Serum antibody responses to PC were measured in a study of primary and reactivated HCMV infections. An IgG antibody seroconversion to the UL128L gene products of PC was consistently detected in a group of 14 pregnant women with primary HCMV infection within 2-4 weeks after infection onset. Antibodies persisted for at least 12 months and a booster response was detected in reactivated infections of immunosuppressed transplant recipients (Genini *et al.*, 2011). In addition, by using potently neutralizing human mAbs to 10 different PC epitopes and a competitive ELISA, in which PC bound to the solid-phase was reacted with human sera and murinized human mAbs (inhibition of mAb binding or IMAB assay), it was shown that during primary HCMV infection in pregnancy, IgG antibodies to some PC epitopes appeared earlier in mothers non-transmitting (NT) the virus to the fetus as compared to mothers transmitting (T) the infection (Lilleri *et al.*, 2012). Furthermore, neutralizing mAbs directed to PC as well as convalescent-phase sera from pregnant women with primary infection were found to inhibit cell-to-cell spreading as well as HCMV transfer to leukocytes, and to correlate with virus control *in vivo*.

A further development of the IMAB assay showed that an early high antibody response to 7/10 PC antigenic sites was associated with a reduced risk of virus transmission to the fetus (Lilleri *et al.*, 2013). The profile of the IgG antibody response to PC paralleled that of the NAb response preventing the infection of epithelial (ARPE-19), but not fibroblast cells, due to the highly prevalent neutralizing activity of IgG antibody to PC (Gerna *et al.*, 2015). However, a parallel study of the ELISA IgG antibody response to PC and the neutralizing response preventing infection of epithelial cells in immunocompetent and immunocompromised transplant recipients un-

**Table 3 - HCMV major immunogenic antigens.**

3.1. Antibody response	<ol style="list-style-type: none"> <li>1) Until 2004, the 3 known glycoprotein complexes (gCs) representing the major targets of the HCMV NAb response were gB (gCI, homotrimer), gH/gL (gCIII), and gM/gN (gCII).</li> <li>2) In 2004-2005, the genomic trimer UL128-130-131 (referred to as UL128L) was identified as indispensable for infection of endothelial (Hahn <i>et al.</i>, 2004) and epithelial (Wang &amp; Shenk, 2005) cells. Then, it was shown to be complexed with gH/gL to form the pentamer complex (PC) gH/gL/pUL128L (Ryckman <i>et al.</i>, 2008).</li> <li>3) In 2010, a number of potently neutralizing human mAbs were shown to be directed to the 3 components of the trimer pUL128L (Macagno <i>et al.</i>, 2010). Murine mAbs with the same potency and specificity were obtained following immunization with PC (Kabanova <i>et al.</i>, 2014).</li> <li>4) Finally, early appearance of antibodies to some PC epitopes in pregnant women with primary HCMV infection was associated with a reduced risk of vertical HCMV transmission (Lilleri <i>et al.</i>, 2013; Gerna <i>et al.</i>, 2015).</li> </ol>
3.2. T-cell response	<ol style="list-style-type: none"> <li>5) Primary role of T-cell immunity in protection against HCMV infection/disease has been repeatedly recognized with the guiding contribution of HCMV-specific CD4<sup>+</sup> T-cells and the secondary intervention of HCMV-specific CD8<sup>+</sup> T-cells in both the immunocompetent (Revello <i>et al.</i>, 2006; Lilleri <i>et al.</i>, 2007, 2008; Fornara <i>et al.</i>, 2017) and the immunocompromised host (Sester <i>et al.</i>, 2001; Gamadia <i>et al.</i>, 2003; Gabanti <i>et al.</i>, 2014, 2015).</li> <li>6) The major target for both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells is pp65, whose pp65<sub>495-503</sub> epitope stimulates CD8<sup>+</sup> T-cells only in the presence of CD4<sup>+</sup> T-cell help (Reiser <i>et al.</i>, 2011). The T-cell response to PC has been only preliminarily investigated (Mele <i>et al.</i>, 2017).</li> </ol>

NAb, neutralizing antibodies; cCMV, congenital HCMV infection; PC, pentameric complex; mAbs monoclonal antibodies.

dergoing primary HCMV infection showed a substantial difference in the antibody kinetics between the two study populations. In detail, the ELISA-IgG response to PC was significantly higher in the immunocompetent until six months after infection onset, when the two antibody curves overlapped at 6-12 months. On the contrary, antibody titers neutralizing the infection of epithelial cells were significantly higher in transplant recipients after six months, and persisted at a higher level at least until 12 months. Unexpectedly, this trend did not occur for NAb preventing infection of fibroblasts.

The greater antibody response observed in transplanted patients is likely due to the higher and persistent antigenic stimulus associated with the consistent presence of circulating precursor CCR7<sup>lo</sup>PD-1<sup>hi</sup>CXCR5<sup>+</sup>CD4<sup>+</sup> follicular helper T cells (T<sub>FH</sub>), which, after encountering HCMV, become ICOS<sup>+</sup>, i.e., activated T<sub>FH</sub> cells, and promote an efficient antibody response. This event may occur in the absence of HCMV-specific CD4<sup>+</sup> T cells, as it occurs in transplanted patients until reconstitution of the virus-specific CD4<sup>+</sup> T cell response in the late post-transplant period. A preliminary analysis of the specificity of the activated T<sub>FH</sub> cell response to HCMV proteins showed a major response to PC and gB. In other words, in primary infections, a first line of HCMV-specific defense seems to be represented by antibodies produced by the interaction of mature T<sub>FH</sub> cells and B cells of germinal centers, resulting in the differentiation of B cells into antibody producing plasma cells (Bruno *et al.*, 2016).

In symptomatic primary HCMV infections of non-pregnant subjects, the presence of overt clinical symptoms was found to be associated with a significantly higher ELISA-IgG to PC and NAb response (both in ARPE-19 and HELF) compared to pregnant women with pauci-symptomatic or asymptomatic primary infection and lower viral load (Fornara *et al.*, 2015). In addition, the different kinetics of NAb and ELISA-IgG antibodies to HCMV glycoprotein complexes were reported to contribute to the definition of an algorithm indicating the onset of primary HCMV infection in asymptomatic pregnant women (Lilleri *et al.*, 2016).

In conclusion, some types of antibodies may represent correlates of protection against HCMV vertical transmission and possibly HCMV disease in transplanted patients, acting either directly or through some AD-cellular mechanisms, as mentioned above.

### 3.2 T cell response

In the past, T cell response has consistently been considered the main barrier against HCMV transmission both in the immunocompetent and the immunocompromised host. In particular, T-cell parameter defects responsible for HCMV transmission from pregnant women with primary HCMV infection to the fetus have been searched for. As a result, the following immunological prognostic markers were found to be associated with vertical HCMV transmission:

- 1) a delay in the development of the HCMV lympho-proliferative response (Revello *et al.*, 2006; Lilleri *et al.*, 2007a);
- 2) a delay in the re-expression of CD45RA<sup>+</sup> T<sub>EMRA</sub> cells in both HCMV-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (Lilleri *et al.*, 2008);
- 3) a delay in IL-2 production by HCMV-specific CD4<sup>+</sup> T-cells (Fornara *et al.*, 2016);

- 4) a late development of long-term IL-7R<sup>pos</sup> memory HCMV-specific CD4<sup>+</sup> T-cells vs IL-7R<sup>neg</sup> T-cells (Mele *et al.*, 2017).

In addition, it was recently reported that a cultured ELISPOT assay, in which PBMCs were stimulated for 12 days and then re-stimulated for 24 h with peptide pools of HCMV proteins pp65, IE-1, IE-2, detected a response to pp65, but not to IE-1 or IE-2, significantly higher in NT mothers. The cultured ELISPOT response, which is predominantly based on the lymphoproliferative activity of HCMV-specific CD4<sup>+</sup> T-cells, was found in a multivariate logistic regression analysis to be independently associated (among multiple parameters examined) with a lower risk of congenital HCMV infection, along with a higher avidity index and a lower DNAemia level (Fornara *et al.*, 2017).

On the other hand, in the immunocompromised host HCMV-specific CD4<sup>+</sup> T-cells have been repeatedly reported to guide the T-cell response in association with the secondary intervention of CD8<sup>+</sup> T-cells in both SOT (Sester *et al.*, 2001; Gamadia *et al.*, 2003; Gerna *et al.*, 2006; Gabanti *et al.*, 2014) and HSCT recipients (Lilleri *et al.*, 2006; Widmann *et al.*, 2008; Pougheysari *et al.*, 2009; Gabanti *et al.*, 2015). These reports were recently confirmed in a study of SOT recipients, which documented that patients with high viral load infection displayed some delay in CD4<sup>+</sup> T-cell reconstitution, whereas most patients with low viral load infection showed a robust T-cell response (Lilleri *et al.*, 2018).

The HCMV proteins conventionally considered as primary targets of CD4<sup>+</sup> T-cells are pp65 (pUL83), IE1 (pUL123), gB (pUL55), and gH (pUL75) (Beninga *et al.*, 1995). Among these, pp65 appears to be a major target for both CD8<sup>+</sup> and CD4<sup>+</sup> T-cells. However, the CD8<sup>+</sup> T-cell response to the pp65<sub>495-503</sub> epitope has been shown to critically depend on CD4<sup>+</sup> T-cell help (Reiser *et al.*, 2011). Apart from pp65, the inclusion of the other above-mentioned proteins in a potential vaccine has been considered less critical, while several other viral proteins have been found to be targets of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (Sylwester *et al.*, 2005). In addition, as reported above, gH has been found to be complexed with gL and UL128L to form the glycoprotein complex PC, whose role in eliciting the NAb response has been extensively investigated (see above), whereas T-cell response to PC still remains to be investigated. Recently, a preliminary analysis of the T-cell response to PC, as well as to pp65, IE, and gB, was performed by using overlapping peptides (15mers) spanning the entire proteins along with libraries of T-cells (Geiger *et al.*, 2009). Following amplification of T-cell libraries with the single peptide pools, T-cells specific for single antigens were measured in a group of subjects at early and late stages of primary HCMV infection. Results showed that IE is efficiently recognized by CD8<sup>+</sup> T-cells, whereas PC and gB are more efficiently recognized by CD4<sup>+</sup> T-cells, and pp65 appears to be equally recognized by both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (Lilleri & Gerna, 2017; Mele *et al.*, 2017). This pattern of reactivity detected at early stages of primary infection was substantially maintained at later stages, although frequencies of specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cells decreased. In addition, in donors with remote HCMV infection, frequencies of viral protein-specific T-cells showed the same trend as in primary infections (Mele *et al.*, 2017). As a preliminary conclusion, it seems that the inclusion of PC (alone or with other viral proteins) in a vaccine could provide both specific humoral and T-cell responses.

#### 4. LIVE HCMV VACCINES (Table 4)

The first two live attenuated HCMV strains, which were propagated in the laboratory in view of the development of a HCMV vaccine, were the AD169 strain selected by Elek and Stern in London in 1974 (Elek & Stern, 1974) at Merck, and the Towne-125 strain obtained by the Stanley Plotkin group in 1975 in Philadelphia, PA, USA, at GSK, following 125 passages in human embryonic lung fibroblast (HELFL) diploid cells (Plotkin *et al.*, 1975; Plotkin & Huygelen, 1976).

##### 4.1. Towne Vaccines

The Towne vaccine was tested by the Plotkin group in several trials, which received financial support from different industries at sequential times. The results achieved in *Phase I-II* clinical trials were as follows:

- 1) after vaccination, only local reactions were observed;
- 2) no virus excretion was detected even in KTR;
- 3) NAb (on HELFL) were induced at levels comparable to those found in convalescent-phase sera from natural infections;
- 4) HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were generated (Starr *et al.*, 1981; Carney *et al.*, 1983);
- 5) doses of the subcutaneous Towne HCMV challenge required to infect or cause disease were low (10 PFU) for seronegative subjects, high (500-1,000 PFU) for natu-

- 6) protective effects of the Towne vaccine were observed against the low-passage HCMV Toledo strain administered as a challenge both in naturally seropositive and vaccinated volunteers at doses of 10-100 or 10 PFU, respectively (Plotkin *et al.*, 1989);
- 7) in R-KTR receiving a kidney from a D<sup>+</sup> donor, about 1/3 developed HCMV disease, whereas only 5% of Towne vaccinees did so (Plotkin *et al.*, 1990);
- 8) in addition, the transplant rejection rate was reduced by 50% in vaccinees, in which virus did not establish latency (Plotkin & Huang, 1985);
- 9) finally, Adler *et al.* (1995) showed that immunity induced by natural infection protects women of child-bearing age against secondary infection, while mucosal immunity following vaccination did not prevent mothers from being infected.

At that time, the molecular bases of attenuation were unknown; however, more recently, progress in molecular biology and sequencing has shown that both the initially attenuated HCMV strains acquired mutations in UL128L affecting PC during propagation in cell culture. In more detail, the Towne strain underwent a 2-bp insertion (TT) causing a frameshift mutation in UL130. This mutation, possibly along with others, was considered the basis for Towne attenuation.

**Table 4 - Live HCMV vaccines.**

4.1. Towne vaccine	Developed by Plotkin <i>et al.</i> (1975) following 125 passages in HELFL, the <i>Towne vaccine</i> exhibited the following properties in <i>Phase I/II</i> clinical studies: <ol style="list-style-type: none"> <li>1) no virus excretion;</li> <li>2) no virus latency;</li> <li>3) NAb induction;</li> <li>4) generation of both HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cells;</li> <li>5) partial protection against virus challenge in both the immunocompetent and the immunocompromised host;</li> <li>6) The molecular basis of attenuation was later attributed to a 2-bp insertion (TT) causing an aa frameshift mutation in 130 (Murphy <i>et al.</i>, 2003).</li> </ol>
4.2. AD-169 vaccine	Developed by Elek & Stern in 1974 in London, the <i>AD-169 vaccine</i> was abandoned at the end of the 70s (Neff <i>et al.</i> , 1979). Later in 2012, the mutated PC in AD-169 was restored by serial passages in endothelial cells (Fu <i>et al.</i> , 2012). The revertant AD-169 virus with a restored PC was genetically modified by incorporating a synthetic compound (Shield-1), which allows virus replication only in its presence. This disabled infectious single-cycle (DISC) HCMV vaccine, named V160, was able to induce both humoral and T-cell responses in NHP (Wang <i>et al.</i> , 2016) as well as in humans in a <i>Phase I</i> study (Fu <i>et al.</i> , 2018).
4.3. Towne/Toledo chimera vaccines	Four genetic <i>Towne/Toledo recombinant chimera vaccine</i> candidates were constructed by substituting genomic regions of the low-passage Toledo strain with attenuated Towne strain genomic regions (Kemble <i>et al.</i> , 1996). In a <i>Phase I</i> study, vaccines were well tolerated and not excreted in humans (Adler <i>et al.</i> , 2016).
4.4. Viral vectored HCMV vaccines	The following heterologous viral vectors were used to deliver HCMV antigens (clinical trial <i>Phase/experimental animal</i> inoculation): <ol style="list-style-type: none"> <li>1) <i>Canarypox virus vector</i>, delivering either gB (Adler <i>et al.</i>, 1999) or pp65 (Berencsi <i>et al.</i>, 2001) (<i>Phase I</i>).</li> <li>2) <i>Alphavirus vector</i>, the Venezuelan Equine Encephalitis (VEE) virus delivering extracellular gB and a pp65/IE-1 fusion protein (Reap <i>et al.</i>, 2007a, b) (<i>Phase I</i>).</li> <li>3) <i>Lymphocyte choriomeningitis virus vector</i>, delivering pp65 and gB in a bivalent vaccine (Schleiss <i>et al.</i>, 2017a) (<i>Phase I</i>).</li> <li>4) <i>Modified vaccinia Ankara virus (MVA) vector</i> expressing: a) pp65, gB, IE-1, IE-2, PC (Wussow <i>et al.</i>, 2013); b) pp65, IE-1 (exon-4) and IE-2 (exon-5) in the Triplex vaccine (La Rosa <i>et al.</i>, 2017) (<i>Phase I</i>); c) all five PC subunits in HEK cells or following BAC-cloning of MVA vector (Chiappesi <i>et al.</i>, 2017; Wussow <i>et al.</i>, 2018) (<i>mice</i>).</li> <li>5) <i>Adenovirus type 6 vector</i> expressing IE-1, IE-2 and pp65 (Tang <i>et al.</i>, 2017) (<i>mice &amp; NHP</i>).</li> </ol>
4.5. Alphavirus replicon particles (VRPs) vaccines	A <i>VRP vaccine</i> is an RNA-based vaccine consisting of an attenuated strain of VEE virus, in which the structural VEE virus protein genes are replaced with HCMV genes encoding for gB or the pp65/IE-1 fusion protein and VEE non-structural genes, while two helper RNAs encode for VEE structural proteins allowing replicon RNA packaging into VRPs. <ol style="list-style-type: none"> <li>1) A <i>bicistronic vaccine (AVX601)</i> expressing gB and a pp65/IE-1 fusion protein was developed (Reap <i>et al.</i>, 2007a, b) and shown to be safe and immunogenic in humans in a <i>Phase I trial</i> (Bernstein <i>et al.</i>, 2010).</li> <li>2) Recently, a comparison of VRPs encoding HCMV gH/gL and PC with purified gH/gL and PC complexes showed that PC elicits higher NAb titers than gH/gL in <i>mice</i> (Wen <i>et al.</i>, 2014).</li> </ol>

PC, pentameric complex; VEE Venezuelan equine encephalitis virus; MVA, Modified Vaccinia Ankara virus; VRPs, Virus Replicon Particles; NAb, Neutralizing Antibody; HELFL, human embryonic lung fibroblasts; aa, aminoacid; HEK, human embryonic kidney; NHP, non-human primates.

#### 4.2. AD169/DISC vaccines

Similarly, the AD169 strain showed a 1-bp insertion (A) inducing a frameshift mutation in UL131 (Murphy *et al.*, 2003). In 2012, expression of PC in AD169 virus was restored by serial passages in endothelial cells (Fu *et al.*, 2012), as reported by our group (Gerna *et al.*, 2003), thus rescuing the AD169 tropism for both leukocytes and human endothelial cells. The revertant AD169 virus elicited 10-fold higher NAb titers than the attenuated AD169 virus in multiple animal species, while in rabbits and monkeys the peak NAb titers after vaccination were 2-4-fold higher than levels detected in HCMV-seropositive individuals. Using the same revertant virus as an immunogen, 45 mAbs were obtained from an immunized rabbit, over one-half of which had neutralizing activity toward PC (but not gB), while the remaining mAbs showed no correlation between binding affinity and neutralizing activity (Freed *et al.*, 2013).

The restored AD169 PC was expressed and purified in soluble form from Chinese Hamster Ovarian (CHO) cells, and showed that PC, but not gH/gL, is able to inhibit AD169 entry into epithelial cells and possesses dominant native neutralization epitopes able to absorb 76% of the neutralizing activity of human hyperimmune globulin preparations (Loughney *et al.*, 2015). The revertant AD169 virus with the restored PC was further genetically modified by incorporating a chemically controlled protein stabilization switch that allows viral replication regulation with a synthetic compound (Shield-1). Virus replicated efficiently in the presence of this compound, but stopped replication in its absence. This replication-defective or disabled infectious single-cycle (DISC) HCMV vaccine (named V160) was immunogenic in several animal species (Wang *et al.*, 2016), by inducing a durable NAb response as well as CD4<sup>+</sup> and CD8<sup>+</sup> T-cells with multiple specificities in non-human primates (NHP). In addition, V160 vaccination was shown to elicit antibodies against multiple neutralization sites of PC in rhesus macaques, as previously shown in humans following natural infection (Macagno *et al.*, 2010), and in mice following immunization with purified PC (Kabanova *et al.*, 2014). Furthermore, V160 induced NABs that were able to protect against genetically diverse HCMV field strains (Ha *et al.*, 2017). A first V160 *Phase I* study on safety, tolerability and immunogenicity in humans has now been completed. In the absence of virus replication, the vaccine was well tolerated and induced NAb in HCMV-seronegative subjects as well as T-cell responses within a range overlapping natural immunity (Fu *et al.*, 2018).

#### 4.3. Towne/Toledo chimera vaccines

In an effort to develop a live HCMV vaccine with the safety profile of Towne, but more immunogenic and protective than the Towne vaccine, four genetic *Towne/Toledo recombinant chimera vaccine candidates* were constructed. In these constructs, some genomic regions of the low-passaged Toledo strain were replaced with the corresponding regions of Towne, following cotransfection of selected Towne and Toledo cosmids (Kemble *et al.*, 1996). The safety and tolerability of these vaccines in seropositive subjects have been reported (Heineman *et al.*, 2006). In an additional *Phase I* study, the four recombinant chimera vaccines were administered to 36 seronegative men at increasing doses of 10, 100, and 1,000 PFU (each dose was given to 3 subjects in each group of 9 individuals, and each group received a different chimera vaccine). The results

showed that chimera vaccine candidates were well tolerated and not excreted in urine or saliva; chimeras 2 and 4 were more immunogenic (Adler *et al.*, 2016). All of the 11/36 seroconverter subjects showed low NAb and T-cell responses. Complete genomic analysis of the four chimeras revealed that no major deletions or rearrangements were present. In detail, all four chimeras contained an inverted UL/b' from Toledo (encoding UL130 to UL148), chimeras 1 and 2 contained wild-type UL128, and chimeras 3 and 4 a disrupted UL128 from Toledo. No clear association was detected between the genetic content and the levels of chimera-induced HCMV-specific humoral and cellular immune responses (Suarez *et al.*, 2017). These results may be helpful for the design of future vaccines as far as both safety and immunogenicity are concerned.

#### 4.4. Viral Vected HCMV vaccines

Several vectored HCMV vaccines have been developed, and some have been evaluated in Phase I/II studies. This approach to HCMV vaccine development utilizes heterologous viral vectors to deliver HCMV-encoded antigens such as gB, pp65, and IE-1. Since viral vectors are unable to replicate completely when administered to humans, they are highly attenuated, while efficiently delivering one or more viral antigens. In particular, two vaccines were developed using a *canarypox vector*, one delivering gB [ALVAC-gB (vCP139)], and the other, pp65 [ALVAC-pp65 (vCP260)]. While the ALVAC-gB alone did not induce NAb titers in seronegative subjects and did not boost NAb titers in seropositive individuals, a prime-boost strategy in which the ALVAC-gB vaccine was followed by Towne vaccine administration elicited a NAb response similar to that seen after natural infection (Adler *et al.*, 1999). As for the ALVAC-pp65 vaccine, in a *Phase I* study it showed a good safety profile as well as robust pp65-specific CTL and antibody responses in seronegative adults (Berencsi *et al.*, 2001).

Another vectored platform utilized for the development of a HCMV vaccine was based on an *alphavirus vector*, the Venezuelan equine encephalitis (VEE) virus, in which genes coding for VEE structural proteins were replaced with HCMV genes coding for the extracellular domain of gB and a pp65-IE1 fusion protein in a double promoter replicon (Reap *et al.*, 2007a, b). In a *Phase I* clinical trial, seronegative volunteers receiving either three low or high doses of vaccine over a 24-week period developed CTL and NAb antibody responses to all three HCMV antigens (Bernstein *et al.*, 2010). This platform, originally developed by Alphavirus vaccines, was then acquired by Novartis Co., and then by GSK.

A new vectored subunit vaccine was developed using an *attenuated recombinant lymphocyte choriomeningitis virus* (LCMV) platform, in which the vector utilizing producer cells constitutively expressing LCMV glycoprotein was modified by replacing the gene coding for LCMV glycoprotein with HCMV antigens in plasmid constructs delivered *in trans* to producer cells (Flatz *et al.*, 2010). This replication-defective rLCMV vaccine elicited CTL and CD4<sup>+</sup> T-cell responses as well as a NAb response. It was developed by Hookipa Biotech AG (named HB-101) as a bivalent vaccine containing two vectors expressing HCMV pp65 and gB, and had the advantage of allowing multiple booster administrations against HCMV re-infections in women of childbearing age during sequential pregnancies, as shown in the guinea-pig model of congenital infection (Cardin *et*

*al.*, 2016). In a *Phase I* dose-escalation study recently conducted on three cohorts of 18 healthy volunteers receiving low, middle or high doses of vaccine or placebo at 0, 1, and 4 months, vaccinees developed both NAb and pp65-gB-specific IFN- $\gamma$  ELISPOT responses (<https://clinicaltrials.gov/NCT02798692>, Schleiss *et al.*, 2017a).

Using a modified (attenuated) *vaccinia virus Ankara (MVA)* vector system expressing different HCMV antigens, including pp65, gB, IE1, IE2, and PC, in mice and NHP animal model systems (see below), excellent immunogenicity was demonstrated in *rhesus macaques and mice* (Wussow *et al.*, 2013). A robust and durable MVA-PC-induced NAb response was able to prevent *in vitro* endothelial cell (EC) and Hofbauer macrophage (fetal cells present within the placenta) infection, and was also detected in saliva (Wussow *et al.*, 2014). In addition, an IE1/pp65 fusion protein expressed in MVA was able to expand levels of pp65- and IE1-specific T-cells from HCMV-seropositive donors and to induce these T-cells in *transgenic mice* (Link *et al.*, 2017). An MVA vectored Triplex HCMV vaccine encoding peptides from pp65, IE-1 (exon-4), and IE-2 (exon-5) was developed at the City of Hope, Duarte, CA, and showed a favorable safety profile as well as durable expansion of HCMV-specific T-cells. Furthermore, in a *Phase I* trial the Triplex vaccine was found to be immunogenic in both seronegative and seropositive healthy adults with or without previous smallpox vaccination (La Rosa *et al.*, 2017).

In a recent study conducted on *mice* using MVA co-expressing all five PC subunits (MVA-PC) and soluble PC produced in HEK 293-6E cells, it was concluded that vaccine potency depended on the immunization schedule. In more detail, a two-immunization schedule with MVA-PC alone or prime-boost combinations of MVA-PC and soluble PC showed that the combination was more effective in eliciting NAb than PC alone. On the other hand, with a three-dose immunization schedule, NAb induced by PC either alone or associated with two boosts of MVA-PC produced a titer higher than that reached with MVA-PC alone (Chiuppesi *et al.*, 2017). Very recently, using a novel BAC clone of the MVA vector and exploiting ribosomal skipping by 2A peptides (Ryan *et al.*, 1991), MVA vectors were constructed expressing the five subunits of the HCMV PC. PC subunits expressed by these MVA vectors were efficiently cleaved and transported to the cell surface as conformational NAb epitopes. With only two MVA vector immunizations in *mice*, a potent NAb response was obtained persisting for at least six months (Wussow *et al.*, 2018).

Recently, a *replication-incompetent adenovirus type 6* was developed as a vector of some major HCMV antigens, such as IE-1, IE-2 and pp65, which were modified by introducing mutations to inactivate some of their reported functions. This model documented the immunogenicity of modified viral HCMV antigens in *mice and NHP* (Tang *et al.*, 2017).

#### 4.5. Alphavirus replicon particles (VRPs)

An original approach to the development of an RNA-based HCMV vaccine is represented by *viral replicon particles* (VRPs). The development of VRPs is based on an attenuated strain of the VEE virus. Each VRP carries a self-replicating RNA (replicon), in which the structural VEE virus protein genes are replaced with HCMV genes encoding for gB or the pp65/IE1 fusion protein and VEE virus non-structural genes, along with two helper RNAs that encode the full complement of VEE virus structural protein genes.

While VEE nonstructural protein genes direct protein synthesis of replicon RNA and the two helper RNAs, VEE structural protein genes supplied *in trans* allow replicon RNA packaging into VRPs which are released through budding from the infected cell membrane. Since replicon RNAs do not encode alphavirus VEE structural proteins, VRPs are single-cycle vectors expressing HCMV proteins (but not progeny VRPs) capable of inducing protection against challenge in several animal models (Rayner *et al.*, 2002). A bicistronic vaccine named AVX601 that expresses a soluble form of gB from Towne and a pp65/IE1 fusion protein, developed by AlphaVax, was shown to elicit both humoral and cellular immune responses in *mice* (Reap *et al.*, 2007a; 2007b). A randomized, double-blind, *phase I* trial of AVX601 was performed in HCMV-seronegative volunteers. The vaccine was found to be safe and NAb and multifunctional T-cell responses against all three HCMV antigens were induced (Bernstein *et al.*, 2010). Subsequently, mice administered bicistronic VRPs expressing both HCMV gH and gL developed NAb cross-reactive with multiple clinical isolates at a titer substantially higher than that obtained with monocistronic VRPs encoding gB, gH, or gL alone (Loomis *et al.*, 2013). Recently, a comparison of VRPs encoding HCMV gH/gL and PC with purified gH/gL and PC complexes showed that PC elicited in *mice* significantly higher levels of NAb than gH/gL (Wen *et al.*, 2014).

## 5. NON-LIVING HCMV VACCINES (Table 5)

### 5.1. Recombinant gB subunit vaccines

The first clinical studies of glycoprotein B (gB) were performed by Chiron and were based on the demonstration that NAb preventing infection of human fibroblasts were directed mostly against gB (Gonczol *et al.*, 1990). Chiron, using a *recombinant HCMV gB* in microfluidized adjuvant 59 (MF59) administered at three doses at 0, 1, and 6 months (gB/MF59), showed a fair NAb titer both in adults (Pass *et al.*, 1999) and toddlers (Mitchell *et al.*, 2002). However, the antibody response was short-lived and waned in less than one year. Subsequently, Novartis replaced Chiron, which was then substituted by Sanofi Pasteur, which modified the Towne gB by removing the transmembrane domain and the furin cleavage and fusing the cytoplasmic component of the transmembrane domain with the truncated gB. This modified subunit gB was then utilized in several *phase II* clinical studies. One of these studies showed a 50% efficacy against primary HCMV infection in postpartum seronegative women that lasted one year compared to women receiving placebo. These results also indicated the potential for the gB/MF59 vaccine to reduce the incidence of congenital HCMV infection (Pass *et al.*, 2009). In addition, in the same trial, women seropositive prior to vaccination were also vaccinated, showing boosted gB-specific (both humoral and CD4<sup>+</sup> T-cell) responses, notwithstanding their preexisting immunity (Sabbaj *et al.*, 2011). Furthermore, the gB/MF59 vaccine was administered to HCMV-seronegative adolescent girls, who showed a reduction (although non-significant) in the incidence of HCMV infection compared to a control group (Bernstein *et al.*, 2016). On the other hand, gB/MF59 administered to SOT recipients produced a reduction in viremia and total number of ganciclovir treatment days, while antibody levels and viremia duration inversely correlated (Griffiths *et al.*, 2011).

**Table 5 - Non-living HCMV vaccines.**

5.1. <i>gB subunit vaccines</i>	<p>1) The first <i>gB vaccine</i> was developed at Chiron (Gonczol <i>et al.</i>, 1990) and administered in <i>Phase II</i> clinical trials to both adults and toddlers (Pass <i>et al.</i>, 1999; Mitchell <i>et al.</i>, 2002). However, the antibody response was short-lived.</p> <p>2) Subsequently, Novartis and then Pasteur modified the <i>gB subunit vaccine</i> by removing the <i>gB transmembrane domain</i>. This vaccine was tested in several <i>Phase II</i> clinical trials (Pass <i>et al.</i>, 2009; Griffiths <i>et al.</i>, 2011; Bernstein <i>et al.</i>, 2016), showing a fair degree of protection against primary infection in seronegative and a boosting of immune response in seropositive women.</p>
5.2. <i>DNA-based vaccines</i>	<p>Different types of <i>plasmid-based DNA vaccines</i> have been developed, as follows:</p> <p>1) <i>ASP0113 bivalent vaccine</i> consisting of two plasmids, one containing (VCL-6368) a modified <i>pp65 gene</i>, and the other (VCL-6365) a portion of the genomic region encoding the extracellular domain of AD169 <i>gB</i> (Selinski <i>et al.</i>, 2005). This vaccine has been clinically tested in <i>Phase I</i> (Wlock <i>et al.</i>, 2008) and <i>Phase II</i> trials in HSCT recipients (Kharfan-Dabaja <i>et al.</i>, 2012). In addition, it is being tested in a <i>Phase III</i> trial to investigate the therapeutic effect in HSCT recipients (expected to be completed in 2022).</p> <p>2) <i>Trivalent DNA vaccine (VCL-CT02)</i> encoding IE1, <i>gB</i>, and <i>pp65</i> (Jacobson <i>et al.</i>, 2009) (<i>Phase I</i>) showing the priming effect of DNA vaccine in eliciting the immune response.</p> <p>3) <i>SynCon (Synthetic Consensus sequence)</i> vaccine approach selecting the most conserved aa at each position in the antigen gene sequences, followed by the insertion of the selected sequences into a DNA plasmid (Ramanathan <i>et al.</i>, 2009).</p> <p>4) Vaccine administration using the <i>Electroporation technique</i>, which allows the formation of transient pores in the cell membranes near the injection site of plasmids, thus facilitating entry of plasmids into the cytoplasm (Flingai <i>et al.</i>, 2013) (<i>mice &amp; other experimental animals</i>).</p>
5.3. <i>RNA-based vaccines</i>	<p>Multiple options have been developed/proposed:</p> <p>1) <i>synthetic self-amplifying mRNA</i> expressing a <i>pp65-IE-1 construct</i> and <i>gB</i> was administered to <i>rhesus macaques</i> inducing both NAb and T-cell responses (Novartis).</p> <p>2) <i>mRNA vaccine formulated with lipid nanoparticles (LNP)</i>, encoding <i>gB</i> and <i>PC</i>, was administered to <i>NHP</i> and induced potent NAb response (Moderna Therapeutics).</p> <p>3) <i>mRNA-based multiantigenic vaccine</i> including <i>pp65, gB</i> and <i>PC</i> inoculated in <i>mice</i> elicited potent humoral and cell-mediated immune responses. However, T-cell responses to <i>pp65 mRNA vaccine</i> were inhibited by presence of other HCMV antigens (John <i>et al.</i>, 2018).</p>
5.4. <i>Virus-like particle (VLPs) vaccines</i>	<p>VLPs are <i>enveloped virus-like particles (eVLPs)</i> simulating wild-type viruses, but lacking viral genome. The following formulations have been proposed:</p> <p>1) VIB Labs developed an eVLP vaccine expressing the extracellular sequence of <i>gB</i> fused with the transmembrane and the cytoplasmic domains of Vesicular Stomatitis Virus (VSV) G protein (Kirchmeier <i>et al.</i>, 2014) (<i>Phase I</i>, results expected).</p> <p>2) VIB developed another eVLP vaccine by transfecting HEK-293 cells with a plasmid encoding the <i>gag</i> protein of Moloney murine leukemia virus fused in frame with the HCMV <i>pp65</i> protein and co-transfecting with a <i>gB</i> plasmid, thus enabling VLP budding from transfected cells.</p> <p>3) Redvax GmbH (Switzerland), using a baculovirus expression system, developed another eVLP candidate HCMV vaccine (Vicente <i>et al.</i>, 2014).</p>
5.5. <i>Dense body (DBs) vaccine</i>	<p>DBs are enveloped dense bodies accumulating inside HCMV-infected cells and containing glycoproteins and tegument proteins, but not DNA. DB inoculation in <i>mice</i> induced both NAb and T-cell responses (Becke <i>et al.</i>, 2010; Cayatte <i>et al.</i>, 2013).</p>
5.6. <i>Peptide vaccines</i>	<p><i>Peptide vaccines</i> have been mostly directed to prevention/protection against HCMV disease in HSCT recipients.</p> <p>1) In this context, HCMV <i>pp65</i> has been found to be the most effective viral antigen, and its CTL epitope HLA*201-<i>pp65</i><sub>495-503</sub> was identified as a peptide provided with a high protective potential and fused to a T-helper epitope acting as a stimulus for the immune response (La Rosa <i>et al.</i>, 2012). This vaccine, referred to as <i>PepVax</i>, was shown in a <i>Phase I</i> trial in HSCT recipients to be free of adverse effects.</p> <p>2) Another peptide vaccine was developed in Australia as a <i>chimeric vaccine</i> encoding the extracellular domain of <i>gB</i> and epitopes from 8 different HCMV antigens, both HLA class I- and class II- restricted, expressed as a single fusion protein. Inoculation of <i>transgenic mice</i> with this vaccine produced robust antibody and T-cell responses (Dasari <i>et al.</i>, 2013).</p>
5.7. <i>Pentameric complex (PC) vaccines</i>	<p>PC was identified in 2008, following the discovery by our group (Hahn <i>et al.</i>, 2004) of the UL128-130-131 locus (UL128L) as indispensable for endothelial cell tropism, and the identification of its complex with <i>gH/gL</i> (Ryckman <i>et al.</i>, 2008) to form the pentameric <i>gH/gL/pUL128L</i> complex or PC. PC has been shown: a) to be the major HCMV neutralization antigen (Macagno <i>et al.</i>, 2010), b) to be required for infection of both endothelial and epithelial cells. Some PC vaccine applications include:</p> <p>1) <i>The repair of a frameshift mutation in the UL131 gene</i> of the AD169 PC restored PC expression with improved immunogenicity in different animal species (Fu <i>et al.</i>, 2012). The revertant AD169 virus with restored PC was genetically modified by incorporating a synthetic compound (V160 vaccine). Several <i>Phase I</i> trials have shown that the V160 vaccine is safe and immunogenic inducing both NAb and T-cell responses (Wang <i>et al.</i>, 2016; Ha <i>et al.</i>, 2017).</p> <p>2) Several additional PC-based vaccines have been developed, as follows:</p> <p>a) Using an <i>MVA virus expressing the 5 PC subunits</i>, a soluble PC was produced in HEK-293 cells and inoculated in <i>mice</i> (Chiuppesi <i>et al.</i>, 2017).</p> <p>b) A <i>BAC-cloned MVA vector expressing the 5 PC subunits</i> was inoculated in <i>mice</i> obtaining a potent NAb response (Wussow <i>et al.</i>, 2018).</p> <p>c) <i>VRPs generated with the VEE virus and expressing PC</i> adjuvanted with MF59 produced high NAb levels in <i>mice</i> (Novartis Vaccines).</p> <p>d) <i>PC vaccine produced in CHO cells</i> (Kabanova <i>et al.</i>, 2014) induced in <i>mice</i> a high titer NAb response together with a block of virus dissemination (Gerna <i>et al.</i>, 2016).</p>

*gB*, glycoprotein B; HSCT, Hematopoietic Stem Cell Transplant; PC, Pentameric complex; UL128L, UL128-UL130-UL131 locus; BAC, Bacterial Artificial Chromosome; VRPs, Virus Replicon Particles; MVA, Modified Vaccinia Ankara virus; VEE, Venezuelan Equine Encephalitis virus; CHO cells, Chinese Hamster Ovarian cells; mAbs, monoclonal antibodies.

Another recombinant subunit gB vaccine consisting of gB fused to HSV-1 gD to improve protein expression and purification was developed as a chimeric protein by GSK. In a *phase I* study conducted in HCMV-seronegative volunteers, no serious adverse events were reported, concomitantly with a robust antibody response. Results of this study have not yet been published in full (Schleiss *et al.*, 2017b).

Five antigenic domains (AD) of gB, named AD-1 to AD-5, have been identified as targets of neutralizing antibodies (Pöttsch *et al.*, 2011). One hundred percent of seropositive individuals carry antibodies (although not all neutralizing) to AD-1 (Speckner *et al.*, 1999), whereas only 50% show antibodies to AD-2 (Lanto *et al.*, 2003). AD-4 and AD-5 have more recently been identified and mapped (Schrader *et al.*, 2007). A recent study has shown that, while antibodies against AD-2 of gB correlate with protection from viremia in transplant recipients, the protective effect elicited by gB is not dependent on neutralizing antibodies or ADCC (Baraniak *et al.*, 2018a), but should be explained by some as-yet unexplored mechanism (Baraniak *et al.*, 2018b).

### 5.2. DNA-based vaccines

A *plasmid-based DNA vaccine* consists of a purified plasmid preparation containing one or more DNA sequences capable of inducing an evaluable immune response against a human pathogen carrying the same DNA sequences (Wolff *et al.*, 1990; Tang *et al.*, 1992). In this field, the most advanced vaccine in both preclinical and clinical testing is the so-called *ASP0113 bivalent DNA-vaccine*, which was developed by Vical (previous denominations were VCL-CB01 and TransVax) and then transferred to Astellas for *phase III* clinical trials. This vaccine consists of two plasmids: VCL-6368 containing a modified pp65 protein kinase gene (UL83) and VCL-6365 encoding the extracellular domain (aa 1 to 713) of AD169 gB (Selinski *et al.*, 2005). *Preclinical studies* in mice showed that administration of plasmid-DNA-encoded protein induced antibodies against these proteins, which were able to protect against challenge (Ulmer *et al.*, 1993; Smith *et al.*, 2013).

In a *phase I* clinical study with ASP0113, no serious adverse events were reported and pp65- and/or gB-specific T-cell and antibody responses in seronegative subjects were detected, as well as a rise in pp65-specific T-cell response only, in seropositive subjects (Wloch *et al.*, 2008). In a subsequent *Phase II* trial in seropositive HSCT recipients, HCMV viremia was significantly reduced in plasma after vaccination with ASP0113, whereas other parameters, such as the frequency of pp65 and gB IFN- $\gamma$  producing T-cells or the reduced use of antivirals in vaccinated subjects, were not significantly different between the vaccinated and the placebo group (Kharfan-Dabaja *et al.*, 2012). Safety was also verified in another open-label *phase II* trial (Mori *et al.*, 2017). A *phase III* clinical trial has recently been initiated by Astella Pharma Inc, in collaboration with Vical, to test the therapeutic efficacy (overall mortality and HCMV end-organ disease) of ASP0113 in HSCTR (NCT01877655); the estimated study completion date is March 2022. In the meantime, *other phase II* studies have been started in SOT recipients, and *phase I* studies in dialysis patients.

Finally, a non-adjuvanted, *trivalent DNA vaccine* named VCL-CT02 was developed by Vical, which contains IE1 in addition to gB and pp65 coding sequences. In HCMV-seronegative subjects, vaccination with the trivalent DNA

vaccine was followed by Towne immunization to investigate the priming effect of the DNA vaccine (Jacobson *et al.*, 2009). Results showed that time to first pp65 T-cell and gB antibody response after Towne immunization was shorter for DNA-primed subjects than for controls receiving Towne alone. As with the bivalent DNA vaccine, no severe DNA adverse events were observed, while both HCMV-specific B- and T-cell responses were observed. Vical has proposed the trivalent vaccine for prevention of congenital HCMV infection (Jacobson *et al.*, 2009), and additionally has developed a new lipid-based adjuvant, Vaxfectin, improving immunogenicity in animal models (Sullivan *et al.*, 2010).

An interesting alternative to DNA-based vaccine technology is called *SynCon*, developed by Inovio. This new platform is based on the extensive analysis of antigen sequences of different strains or antigen variants, and selection of the most conserved or dominant amino acid (aa) at each position in the antigen gene sequence, thus creating a *Synthetic Consensus sequence*, that is then inserted into a DNA plasmid and is ready for testing (Ramanathan *et al.*, 2009). This technology awaits application to the development of a candidate HCMV vaccine.

A further contribution to the development of a DNA vaccine is the *electroporation* (EP) technique, which allows the formation of transient pores in the membrane of cells near the injection site, thus facilitating entry of macromolecules (plasmids) into the cytoplasm (Flingai *et al.*, 2013). As observed in the murine model, two major advantages of the EP technology are an increased immune response (Babiuk *et al.*, 2004; Huang *et al.*, 2014) and the use of a lower vaccine dose per single animal. DNA vaccination in association with EP has so far been shown to be *effective in different animal models* (Shedlock *et al.*, 2011; Lang Khus *et al.*, 2012; Shen *et al.*, 2012) and promises to improve delivery of a DNA-based HCMV vaccine in the near future.

### 5.3. RNA-based Vaccines

A synthetic self-amplifying HCMV *mRNA vaccine* was first developed by Novartis (now GSK) and contained gB and a pp65-IE1 fusion construct, which were prepared separately in a cationic nanoemulsion and administered by intramuscular route in *rhesus macaques* (75  $\mu$ g of each antigen injected separately into each thigh). After two immunizations, all animals showed an immune response including both IgG and NAb as well as HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses (Geall *et al.*, 2012; Brito *et al.*, 2014). Another RNA-based HCMV vaccine platform based on self-amplifying mRNA was developed by Moderna Therapeutics. In this platform, *mRNAs* are formulated with *lipid nanoparticles (LNP)* encapsulating modified RNAs encoding HCMV gB and PC. Immunization of NHPs elicited potent and durable NAb titers. In addition, to study the HCMV T-cell response, another mRNA vaccine was formulated which expresses the major HCMV T-cell antigen pp65. In mice, administration of pp65 vaccine along with PC and gB elicited robust T-cell specific responses directed to multiple antigens. However, strong T-cell responses to pp65 were inhibited in the presence of other HCMV antigens, but could be restored by sequential immunization of pp65 followed by PC+gB+pp65. As a result, pp65-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses were fully restored, reaching levels as robust as those detected following immunization with pp65 alone (John *et al.*, 2018). In a *phase I trial*, an mRNA influenza vaccine was shown to be safe

and highly immunogenic (Bahl *et al.*, 2017). In conclusion, the mRNA vaccine appears to be a reliable and promising platform for the development of an HCMV vaccine, which can be modified according to the desired immune response.

#### 5.4. Virus-like particles (VLPs)

*Enveloped virus-like particles* (eVLPs) are protein structures simulating wild-type viruses; however, they lack a viral genome, thus they may represent safer vaccine candidates (Anderholm *et al.*, 2016; Schleiss *et al.*, 2017b). Variation Biotechnologies Vaccines Incorporated (VBI) Laboratories developed an eVLP vaccine expressing the extracellular sequence (aa 1 to 752) of gB fused with the transmembrane and the cytoplasmic domains of vesicular stomatitis virus (VSV) G protein. This vaccine was found to be more immunogenic than another eVLP vaccine developed by VBI and expressing the entire (906 aa) gB sequence (Kirchmeier *et al.*, 2014). Results of a *phase I* study conducted on HCMV-seronegative volunteers for evaluation of safety and immunogenicity of four doses of the gB vaccine in the presence or absence of an alum adjuvant, have not yet been published. The same manufacturer (VBI) developed another eVLP vaccine which was produced by transfecting HEK-293 cells with a plasmid encoding the Gag protein of murine leukemia virus fused in frame with the HCMV pp65 protein (thus originating VLPs). Co-transfection of the cells with an HCMV gB plasmid enabled VLP budding from transfected cells. This vaccine formulated with GM-CSF has been proposed as a therapeutic vaccine against glioblastoma multiforme (Soare *et al.*, 2016).

Finally, another eVLP candidate HCMV vaccine was developed by Redvax GmbH, a Swiss pharmaceutical company, which, unlike VBI that uses mammalian cells, bases its platform on a baculovirus expression system (Vicente *et al.*, 2014). This technology has been recently transferred to Pfizer Vaccines and has been proposed for the administration of different HCMV antigens or antigen combinations, such as gB, PC, gM/gN.

#### 5.5. Dense bodies

Although they have not yet entered clinical studies, *dense bodies* (DBs) have been investigated as a potential HCMV vaccine candidate (Pepperl *et al.*, 2000; Mersseman *et al.*, 2008). DBs are noninfectious enveloped dense particles that accumulate within infected cells during HCMV replication and contain several viral glycoproteins as well as tegument proteins that can be used for vaccine purposes. Importantly, they do not contain viral DNA, thus improving their safety requirements. In *mice*, Towne-derived DBs have been shown to induce a consistent NAb response, which was found to prevent infection of both fibroblasts and epithelial cells as well as promote a robust cell-mediated immune response to several viral proteins in the absence of adjuvant (Becke *et al.*, 2010; Cayatte *et al.*, 2013). On the contrary, a gB-adjuvanted vaccine did not promote a NAb response preventing the infection of epithelial cells. Clinical trials may represent a favorable option for vaccine development in the near future (Cayatte *et al.*, 2013).

#### 5.6. Peptide vaccines

*Peptide-based vaccine* research has mostly been directed at protection against HCMV disease in HSCT recipients, rather than prevention of congenital HCMV infection

(Schleiss *et al.*, 2017b). HCMV pp65 has been identified as the most effective viral antigen in protection of HSCT recipients against HCMV disease through the development of CTL specific for pp65 epitopes. In particular, the CTL epitope HLA A\*0201 pp65<sub>495-503</sub> is considered highly protective due to its low sequence variability among different isolates. Thus, this CTL epitope was fused to a T-helper epitope [either a synthetic pan-DR-epitope (PADRE) or a natural tetanus (Tet) sequence] with or without CpG 7909, a TLR9 synthetic oligonucleotide antagonist, which acts as a stimulus for multiple immune responses (La Rosa *et al.*, 2012). This vaccine under the name *PepVax* was administered to HCMV-seropositive HSCT recipients in a *Phase I* trial. No adverse effects and no acute graft vs host disease (GVHD) were observed.

Another peptide-based vaccine was developed in Australia and consists of a *chimeric vaccine* encoding the extracellular domain of gB and epitopes of eight different HCMV antigens, which are HLA class I- and class II-restricted and expressed as a single fusion protein. This vaccine induced robust immune responses in *transgenic mice*, (Dasari *et al.*, 2013).

Furthermore, a number of peptide-conjugate vaccines targeting the antigenic domain-2 (AD-2) of HCMV gB were developed at Merck with the intent of exposing potential AD-2 epitopes masked by the adjacent immunodominant AD-1 region. Both *mice and rabbits* immunized with a series of AD-2 peptide-conjugate vaccines produced a robust antibody response, but neutralizing activity was weak and lower than that generated with an adjuvanted recombinant gB vaccine (Finnefrock *et al.*, 2016).

#### 5.7. Pentamer complex

For several years, the lack of HCMV transmission from endothelial cells to circulating leukocytes (leuko-tropism), as well HCMV failure to infect endothelial cells (EC-tropism) following serial passages in human fibroblasts (Elek & Stern, 1974; Plotkin *et al.*, 1976), were considered markers of attenuation (Gerna *et al.*, 2002). However, as reported above, the genetic determinants of both EC-tropism and leuko-tropism remained elusive until 2004, when a collaborative study between researchers in Pavia (Italy) and Munich (Germany) concluded that the UL131-128 locus (UL128L) within the ULb' region of the HCMV genome was indispensable for both biological functions (Hahn *et al.*, 2004). Subsequently, Ryckman *et al.* discovered that the trimer UL128L forms a complex with gH/gL (gCIII), which was referred to as gH/gL/pUL128L or PC and is considered the entry complex for epithelial/endothelial cells (Ryckman *et al.*, 2008a; 2008b). In addition, gH/gL was found to be complexed either with pUL128L, thus forming PC gH/gL/pUL128L, or UL74-encoded gO, thus forming the trimeric complex gH/gL/gO, which binds to the platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ) and mediates HCMV entry into human fibroblasts (Kabanova *et al.*, 2016; Wu *et al.*, 2017). As a result, gH/gL/gO and PC, since competitively binding gH/gL, are present in variable proportions in the virion envelope, thus influencing HCMV tropism (Zhou *et al.*, 2013; Li *et al.*, 2015). According to a model recently proposed for infection of both epithelial and endothelial cells, the trimer gH/gL/gO would interact with cell receptors, and PC would interact later, promoting virus egress from endosomes towards nucleus (Liu *et al.*, 2018). Very recently, using a library of most single trans-membrane

human receptors for unbiased detection of receptor-ligand interactions, neuropilin-2 has been identified as the receptor for HCMV-pentamer infection of epithelial/endothelial cells, while PDGFR $\alpha$  has been confirmed as the receptor mediating HCMV trimer-dependent entry into fibroblasts (Martinez-Martin *et al.*, 2018).

In the last decade, the antibody response to PC has been extensively investigated in the immunocompetent and the immunocompromised host, as reported above. Results of these studies have supported the inclusion of PC in several different vaccine formulations, as already mentioned above. In more detail, one of the most interesting approaches taking advantage of the new PC knowledge was the development of a *live attenuated AD169 whole virion vaccine*, in which the repair of a frameshift mutation in the UL131 gene restored PC expression with improved immunogenicity in different animal species (see section 4. 2. AD169/DISC Vaccines). As reported above, a *Phase I* clinical trial has recently been completed, showing that this vaccine is safe and able to induce both humoral and cell-mediated immune responses.

Another promising contribution to the development of an effective PC-based vaccine was provided by the use of either *MVA virus co-expressing all five PC subunits* and production of soluble PC in HEK-293 cells according to the prime-boost technology or a *BAC-cloned MVA vector expressing all five PC subunits* that exploits ribosomal skipping by 2A peptides (see section 4. 4. Viral vectored HCMV Vaccines). In both cases, a potent NAb response was obtained in mice.

A *third PC-based vaccine consists of VRPs* generated with the VEE virus and expressing PC or gH/gL (Novartis Vaccines). These VRPs were compared with purified gH/gL and PC in the presence or absence of MF59 (Wen *et al.*, 2014). Results in mice showed that VRPs encoding PC produced higher levels of NAb than those expressing gH/gL in the presence of MF59 adjuvant, and recognized a broad range of antigenic sites on PC (see section 4.5. Alphavirus Virus Replicon Particles).

Although several researchers have suggested that PC should be included in vaccine designs (Anderholm *et al.*, 2016), to date, no phase I trial has been conducted with a vaccine consisting of PC alone. Recently, by taking advantage of the knowledge provided by a series of potently neutralizing human mAbs reactive with multiple conformational epitopes on PC and capable of preventing infection of endothelial/epithelial cells (Macagno *et al.*, 2010), a *recombinant PC vaccine* was developed consisting of a pentamer complex produced in a secreted form from a CHO cell line stably transfected with a single polycistronic vector encoding the five PC genes separated by autonomous self-cleaving 2A peptides (Kabanova *et al.*, 2014). Administration of this vaccine to mice induced NAb titers 100-1,000-fold higher than those induced by natural infection. In addition, sera from immunized mice were shown to block cell-to-cell virus dissemination by plaque formation inhibition, leukocyte transfer inhibition and syncytium formation inhibition (Gerna *et al.*, 2016). A *Phase II* clinical trial (supported by Genentech Inc) using a combination of two mAbs (one human to gH and one murine humanized to PC) was found to reduce HCMV infection and disease in high-risk KTR (Ishida *et al.*, 2017). In addition, two mAbs targeting gB and PC (from Novartis), respectively, were characterized *in vitro*, showing that they are potent inhibitors of HCMV infection of different cell lines

and are active against a panel of clinical isolates, while acting synergically with no reduction in viral strain susceptibility over time (Patel *et al.*, 2016).

## 6. ANIMAL MODELS (Table 6)

*Guinea pig* cytomegalovirus (GPCMV) infection has been indicated as a suitable model for the study of congenital HCMV infection (Bia *et al.*, 1980; Schleiss & McVoy, 2010). Because it has a placental structure similar to that of humans, for several years it was the only small animal model for the study of congenital CMV infection. Yamada *et al.* first reported a region of the GPCMV genome that contains homologues of HCMV genes (2009), and then Auerbach *et al.* (2013) demonstrated that GP genes 129, 130 and 131 are the homologues of HCMV genes UL128, UL130 and UL131, and that the respective recombinant proteins react with convalescent-phase sera from infected animals (Gnanandarajah *et al.*, 2014). Thus, the protein products of the three GP genes are referred to as GP PC components.

The GPCMV PC was found to dictate viral epithelial cell tropism, pathogenicity and congenital infection rate in GPs (Coleman *et al.*, 2016). As already shown for the HCMV PC, the emergence of mutations within the GP PC during repeated passaging in fibroblasts resulted in attenuation of GPCMV pathogenic potential (McVoy *et al.*, 2016). Mutation repair in immunocompromised animals caused significant weight loss, higher viremia duration and viral loads in blood and end-organs, while in pregnant guinea pigs it resulted in congenital transmission, intrauterine growth restriction and pup mortality similar to that caused by the pathogenic virus strain. These results prompted researchers in this field to preliminarily conclude that inclusion of PC should be strongly considered in the development of a vaccine aimed at preventing congenital infection. This conclusion was reinforced by multiple reports showing that both polyclonal and monoclonal antibodies protect against fetal infection and death in the GP model (Bratcher *et al.*, 1995; Auerbach *et al.*, 2014). In addition, active comparative immunization with MVA-vectored gB vaccine administered alone, or simultaneously with a pp65 homologue (GP83), showed that the GP83 addition reduces gB antibody responses and protection against GPCMV congenital infection, whereas both vaccines, if administered alone, display the same level of protection against pup mortality (Swanson *et al.*, 2015).

More recently, some concerns were raised about the ability of the GP model to mimic outcomes in human clinical trials (Bialas *et al.*, 2015), mainly due to the limited sequence homology of GPCMV with HCMV (Kanai *et al.*, 2011). However, the GP has been recognized for years as the only animal species susceptible to placental CMV transmission (Choi & Hsiung, 1978) following primary maternal infection, with resulting fetal infection at a rate comparable to HCMV.

As an alternative, *rhesus macaques*, an NHP model infected by its own species-specific CMV (RhCMV), was shown to share greater sequence and structure homology with HCMV than with GPCMV (Hansen *et al.*, 2003). Similar to humans, placental transmission by RhCMV has been reported in RhCMV-seronegative pregnant macaques that were CD4<sup>+</sup> T-cell depleted at the time of virus inoculation (Bialas *et al.*, 2015). Homologs of

**Table 6 - Animal models.**

<i>Guinea pig</i>	The <i>Guinea pig</i> (GP) has long been considered the best animal model available for the study of congenital HCMV infection. 1) Although GP 129, 130, 131 share only partial (Kanai <i>et al.</i> , 2011) homology with the HCMV genes UL128, UL130, and UL131 (Auerbach <i>et al.</i> , 2013), the homologue pentameric complex has been reported to dictate GPCMV tropism, pathogenicity and congenital infection (Coleman <i>et al.</i> , 2016). Similar to HCMV, GPCMV was found to undergo mutations during passaging in fibroblasts, whose repair caused viral load increases and fetal loss in pregnant GPs (Auerbach <i>et al.</i> , 2014). 2) MVA-gB (from GPCMV), when administered alone, and not in combination with the GP83 product (homologue of HCMV pp65), conferred the same level of protection against pup mortality as GP83 product (Swanson <i>et al.</i> , 2015).
<i>Rhesus macaque</i>	The <i>Rhesus (Rh) macaque</i> was later considered a better animal model for the study of congenital HCMV infection, due to the higher sequence homology it shares with HCMV (Hansen <i>et al.</i> , 2003). Using an MVA virus co-expressing the 5 RhCMV homologues of the HCMV PC for inoculation of Rh macaques, broadly NAb were induced (Wussow <i>et al.</i> , 2013). Monkeys were protected against intravenous challenge with RhCMV following administration of RhCMV-NAb, which prevented virus transmission to the developing fetus (Nelson <i>et al.</i> , 2017).

GPCMV, guinea pig cytomegalovirus; RhCMV, Rhesus cytomegalovirus; PC, pentamer complex; NAb, neutralizing antibodies; MVA, Modified Vaccinia Ankara

the HCMV PC components were also identified in the RhCMV model. Using an MVA virus coexpressing the five RhCMV homologues to HCMV PC, a vaccine called MVA-rhUL128-Complex was developed and administered to macaques. It induced NAb comparable to those detected in RhCMV-seropositive animals (Wussow *et al.*, 2013). In addition, RhCMV-NAb administration to CD4<sup>+</sup> T-cell-depleted RhCMV-seronegative pregnant rhesus monkeys at primary infection, prior to intravenous challenge with RhCMV, prevented virus transmission to the developing fetus (Nelson *et al.*, 2017). However, it should be taken into consideration that the intravenous inoculation of cell-free virus does not mimic the natural infection, which naturally occurs through mucosal surfaces. Thus, some concern about the potential efficacy of NAb administration in preventing RhCMV transmission, following natural infection, is justified. The ULb' region of RhCMV has been shown to encode, besides HCMV PC homologues, other ORFs, which are involved in RhCMV dissemination and replication in the presence of an intact RhCMV-PC (Assaf *et al.*, 2014).

## 7. PREVENTION OF HCMV DISEASE BY VACCINATION OF TARGET POPULATIONS AT RISK (Table 7)

### 7.1 Prevention of congenital and postnatal HCMV infection: general considerations

The most widely accepted standard method for identification of newborns with cCMV infection is based on rapid culture of urine within the first two weeks of life. However, recently a much simpler and more practical saliva PCR assay has been proposed for HCMV screening in newborns. It has a sensitivity and specificity comparable to that of the conventional culture method, but is performed with liquid-saliva or dried-saliva specimens (Boppa *et al.*, 2011). With this in mind, there are several major issues that must be analyzed and discussed prior to defining the strategies, the schedule and the quality (composition) of an HCMV vaccine as well as the target populations that would benefit.

First of all, the biology and the pathogenic role of HCMV in the human population is very peculiar, similarly to oth-

**Table 7 - Formulation of an ideal HCMV vaccine.**

<i>Primary endpoint</i>	It should be the prevention of primary HCMV infection in adult seronegative subjects at risk of infection. This seronegative population would include adult women of childbearing age and patients included on the list of SOT and HSCT candidates. However, these patients, once seropositive after vaccination, might not be protected against newly-infecting virus strains, as recently and repeatedly documented.
<i>Secondary long-term endpoint</i>	It should be the achievement of herd immunity, ideally protecting against any new contact with different virus strains. This approach would require vaccination of the entire population, perhaps starting in the first years of life, and administration of booster vaccine doses in the following years/decades. This ideal vaccine would require the acquisition of a lifelong evaluable immune response, both humoral and T-cell mediated.
<i>Schedule</i>	Extended clinical trials should be conducted on the non-immune component of the adult population (both males and females) of one or more developed countries to define the levels of immune protection against re-activation/re-infection episodes of HCMV infection following vaccination. Presumably, levels of protection should refer to cut-off levels (and/or protective activity) of HCMV-specific antibodies and, particularly, NAb, as well as to cut-off levels (and/or protective activity) of HCMV-specific CD4 <sup>+</sup> and CD8 <sup>+</sup> T-cells. If immune protection is virus strain-specific, this would make definition of protection more intriguing. However, the follow-up of vaccinees would allow vaccine developers to draw useful conclusions also for seropositive individuals, if they were to receive the same vaccine.
<i>Vaccine composition</i>	It is still unclear whether vaccine components should refer to a single HCMV strain or to multiple strains selected on the basis of consensus sequencing of the major viral antigens. Based on current knowledge, a hypothetical vaccine should contain: 1) gB inducing both antibody and T-cell responses; 2) PC, inducing the most potent NAb response; 3) pp65, inducing the most potent T-cell response.

er members of the human herpesvirus group. Unlike other human pathogenic viruses, such as the rubella virus, whose pathogenic potential is efficiently prevented by a live attenuated vaccine that elicits an immune response mimicking that observed after natural infection, HCMV does not disappear from the infected individual after primary infection, but rather persists in the body in a state of latency. Latency refers to viral DNA expressing only a few IE gene functions, when the immune system is intact, whereas virus replication can reactivate, with production of new viral progeny, when the immune system is compromised for some pathological/pharmacological reason (as in AIDS or transplantation). This re-activation of the HCMV strain causing primary infection may also occur transiently in apparently healthy people due to debilitating conditions, such as the convalescent phase of an infectious disease or excess exposure to sunlight. As a result, HCMV can be detected in (or isolated from) a number of clinical samples, such as urine, saliva, cervical secretions, semen, and, very infrequently, blood. Reactivation should be the first clinical condition prevented by an efficacious HCMV vaccine administered to healthy people carrying a single HCMV strain genome in the body. In addition, people affected in the past by a primary infection can encounter one or more different HCMV strains, against which the previously established immune response may not be sufficiently protective, thus permitting the occurrence of a secondary/tertiary HCMV infection (re-infection). Thus, primary HCMV infection as well as reactivation and re-infection should be prevented by an ideal HCMV vaccine.

Generally speaking, the first objective of a vaccine should be the prevention of primary HCMV infection in non-immune or seronegative individuals. This could be applied to young infants and adolescents, both male and female, but it would be difficult to achieve, since it requires previous serological screening of subjects to be vaccinated. Otherwise, young infants could be vaccinated regardless of their serostatus. If the vaccine is efficient enough to prevent virus primary infection and latency as well as subsequent new virus infections, this approach could be the basis for the introduction of herd immunity (see below).

A second option for deployment of an HCMV vaccination to prevent congenital infection would be to restrict vaccination to seronegative women of childbearing age. In this case, vaccination would involve only a small number of individuals to prevent a primary HCMV infection during pregnancy with a high risk of virus transmission to the fetus. However, this approach could not protect against re-infections occurring during pregnancy.

### 7.2 Prevention of HCMV infection/disease in transplant recipients

HCMV rarely causes disease in healthy children and adults, whereas in heavily immunosuppressed transplant recipients and AIDS patients, HCMV disease causes devastating effects (Ljungman *et al.*, 2002). HCMV disease may consist of HCMV syndrome/systemic infection (fever in association with leukopenia, thrombocytopenia and rise in liver aminotransferase levels) and/or end-organ disease interesting the eye, lungs, gastrointestinal tract, liver or other organs (Ljungman *et al.*, 2017). The pathogenetic basis for the severe effects of HCMV infection is the compromised T-cell immunity state, as recently confirmed (Lilleri *et al.*, 2018); the role of humoral immunity remains to be defined. In the early 90s the introduction of highly active

antiretroviral therapy (HAART) significantly contributed to the marked reduction in HCMV disease rates in AIDS patients (Gerna *et al.*, 1998). Similarly, the use of first-line anti-HCMV antiviral drugs (ganciclovir and valganciclovir) in the transplanted patient population drastically reduced HCMV end-organ disease incidence in both SOT and HSCT recipients.

Even though several transplantation Centers have preferred using antiviral prophylaxis for 3-6 months (or more) to prevent HCMV disease, HCMV DNA quantification in blood by real-time PCR and the establishment of viral DNA cut-offs for initiation of pre-emptive therapy were critical steps forward in the prevention of HCMV disease in other transplantation Centers. In addition, the recent introduction of an HCMV WHO standard for real-time PCR results obtained in different Centers has made the inter-laboratory comparison of results possible (Fryer *et al.*, 2010; Preiksaitis *et al.*, 2016). However, a major limitation to the use of PCR for diagnosis of HCMV infection in transplanted patients is organ localization of HCMV infection/disease, in the absence or in the presence of low viral load in blood (Gabanti *et al.*, 2015; Ljungman *et al.*, 2017). Furthermore, several drawbacks still persist regarding the use of antiviral prophylaxis in transplant recipients:

- 1) the high cost of prolonged treatment;
- 2) toxicity, namely in HSCT recipients;
- 3) emergence of drug-resistant HCMV strains;
- 4) potential lack of treatment in the absence of viremia, due to missed diagnosis of virus organ localization.

For these reasons, the availability of an effective anti-HCMV vaccine would be highly desirable in the transplantation setting in order to prevent primary HCMV infections in HCMV-seronegative patients, as well as to boost at least a partially protective immune response in HCMV-seropositive patients undergoing recurrent infection (reactivation or re-infection).

*Solid-organ transplant recipients.* In our Transplantation Department, threshold DNAemia levels for initiation of pre-emptive therapy in SOT recipients with systemic infection (Gerna *et al.*, 2007), prior to appearance of clinical symptoms (Gerna *et al.*, 1998; 2011), were established as  $3 \times 10^5$  DNA copies /mL whole blood. To further simplify the application of cut-offs, a conversion factor for expressing DNA copies in IU/mL was recently determined using the International WHO standard and found to be 0.4. In this patient population, seronegative recipients of organs from seropositive donors are at the greatest risk of HCMV disease following primary infection (Atabani *et al.*, 2012). Both strategies for HCMV disease prevention, i.e., antiviral prophylaxis for 3-6 and even 12 months for lung recipients, and pre-emptive therapy, have been recommended (Kotton *et al.*, 2010). In these patients, the administration of an effective HCMV vaccine could fully or partially prevent HCMV disease and reduce drug-related toxicity or eventually eliminate the need for antiviral drug administration (Krause *et al.*, 2014). Moreover, in these patients, long-term protection conferred by vaccination should be achieved against primarily infecting virus re-activations and against re-infection episodes by HCMV strains that newly infect HCMV-seropositive patients.

*Hematopoietic stem cell transplant recipients.* In allogeneic HSCT recipients, due to profound immunosuppression, HCMV infection is particularly severe and is associated with transplant-related mortality, graft survival and secondary bacterial and fungal infections (Boeckh & Ljun-

gman, 2009; Nichols *et al.*, 2002). Monitoring of HCMV infection by PCR allows the clinician to predict the development of HCMV disease and reduce its incidence as well as the duration of antiviral therapy and its side effects (Einsele *et al.*, 1995). In this setting, either pre-emptive therapy or antiviral prophylaxis may be adopted. However, pre-emptive therapy may be preferred because of the shorter duration of antiviral treatment and reduced myelotoxicity. In our Department, the optimal cutoff determined for initiation of pre-emptive therapy in HSCT recipients is  $3 \times 10^4$  DNA copies/mL whole blood, about one  $\log_{10}$  lower than the cutoff used in SOT recipients (Lilleri *et al.*, 2007b; Gerna *et al.*, 2008a).

In these patients, the availability of a vaccine could effectively contribute to preventing or reducing the severity of HCMV infection/disease, particularly by vaccinating seronegative donors (or boosting seropositive donors), in order to transfer memory T cells with the graft. Results of phase III vaccination clinical trials should answer all of these questions.

## 8. CONCLUSIONS

As a general conclusion, an HCMV vaccine should satisfy the primary endpoint of preventing primary infection in two very high-risk populations (pregnant women and immunocompromised patients). However, the very long-term goal would be permanent protection against both reactivated infections, which may occur repeatedly during a lifetime, and new infections by other HCMV strains. In any case, the final objective would be to directly or indirectly protect the two high-risk populations mentioned above not only against primary infections, but also against repeated episodes of reactivation/re-infection (or recurrent infection).

On the other hand, achieving some level of herd immunity would reduce the risk not only of primary infection, but also of recurrent infection, as a result of reduced circulation of different HCMV strains among pregnant women and immunocompromised patients.

It is reasonable to anticipate that periodical reinforcements (when required) by booster vaccinations will be necessary in order to stimulate both arms of the immune response, so that sufficient and persisting humoral (NAb and binding antibodies), as well as innate and T-cell-mediated immunity, are elicited. Thus, some subjects/patients could follow a personalized schedule of vaccination, according to their personal medical history and needs.

In addition, it is not currently known whether virus components included in the vaccine should be derived from a single virus strain or should be relevant to multiple HCMV strains selected on the basis of consensus sequencing findings of the major antigens. Genotyping data of multiple HCMV glycoprotein complexes as well as sequencing data of some of the most immunogenic viral proteins, such as the UL83 product pp65, could help in the decision making. Finally, decisions need to be made regarding the optimal vaccine composition, i.e., what are the optimal HCMV components that would elicit the most potent and long-lasting immune response? So far, live virus vaccines, such as AD169, Towne and Towne/Toledo chimera vaccines, have not provided satisfactory results either in phase I and II clinical trials or in experimental animal models. Based on the results obtained so far with non-liv-

ing HCMV vaccines, a theoretically optimal recombinant HCMV vaccine composition should include:

- 1) gB, inducing both humoral (mostly antibody-binding) and T-cell immune responses;
- 2) PC, inducing the highest NAb response;
- 3) pp65, inducing the most potent T-cell response.

With this in mind, a question spontaneously arises: why is an HCMV vaccine far from being licensed? Licensing requires a pre-licensure definition of the primary endpoint of efficacy for clinical trials aimed at investigating the level of protection provided by the candidate vaccine. In our opinion, the primary objective of a first-generation HCMV vaccine should be a reduction in the number of cCMV cases in a group of non-immune pregnant women receiving the vaccine compared to a group of non-immune non-vaccinated pregnant women. The correlates of protection, recently updated by our group (Gerna *et al.*, 2017), should be extensively verified in this trial. The relatively reduced population sample size required by this trial should prompt health authorities and pharmaceutical industries to endorse the cost of this study, which would also attenuate logistical problems and demonstrate an even partial reduction of cCMV cases. Results of this trial could be the basis for approval of the first HCMV vaccine (Permar *et al.*, 2018). Subsequently, a second-generation vaccine, tested on a larger sample population size of immune pregnant women, could provide important clinical data on the prevention of cCMV and on immune correlates of protection during re-infections in pregnancy.

In the transplantation setting, efficacy trials of HCMV candidate vaccines should be performed separately.

## Acknowledgments

*The Authors are indebted to the technical, medical and paramedical staffs of the Virology Laboratories and of the various transplantation Centers of the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo. We are grateful to Daniela Sartori for manuscript editing and to Laurene Kelly for revision of the English.*

*This work was partially supported by the Fondazione Regionale per la Ricerca Biomedica (grant no. FRRB 2015-043).*

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