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New advances in CMV and immunosenescence ☆

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☆ Report of the 4th Workshop on "CMV & Immunosenescence"

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ABSTRACT

Immunosenescence, defined as the age-associated dysregulation and dysfunction of the immune system, is characterized by impaired protective immunity and decreased efficacy of vaccines. An increasing number of immunological, clinical and epidemiological studies suggest that persistent Cytomegalovirus (CMV) infection is associated with accelerated aging of the immune system and with several age-related diseases. However, current evidence on whether and how human CMV (HCMV) infection is implicated in immunosenescence and in age-related diseases remains incomplete and many aspects of CMV involvement in immune aging remain controversial. The attendees of the 4th International Workshop on "CMV & Immunosenescence", held in Parma, Italy, 25–27th March, 2013, presented and discussed data related to these open questions, which are reported in this commentary.

1. Introduction

After primary infection, CMV is carried for the lifetime of its host. Viral persistence is based on complex interactions between multiple viral and host determinants. These interactions generally result in a carefully negotiated and clinically "innocuous" balance between the virus and the immunocompetent host. Indeed, CMV rarely produces symptoms in the host unless the balance is upset by reduced immune competency of the host (Fig. 1). Thus, when considering various types of viral–host interaction, the overt CMV reactivation in immunocompromised individuals is a well recognizable disease state. By contrast, the co-existence of human CMV in healthy, and even more so, in elderly individuals is still a poorly understood phenomenon whose clinical and host:pathogen correlates remain to be defined. Specifically, at the present time we still lack precise measures of viral load, viral latency or reactivation status, of the type and extent/efficacy of immune surveillance, and of the interplay between the virus and the immune system with regard to the true outcome of CMV infection (Latency with no viral replication? Persistence with sporadic or frequent replication/reactivations?), ultimately including its clinical impact.

The International Workshop on "CMV & Immunosenescence", held in Parma, Italy, 25–27th March, 2013, (local organizer Paolo Sansoni) was the fourth of a series of such meetings. It brought together virologists, immunologists and geriatricians with the aim to present and discuss advances in our understanding of the impact of HCMV infection on immune status and function, particularly focusing on its clinical significance in the elderly. As in the previous workshops, the results of which were summarized in the corresponding reports (Pawelec et al., 2010; Solana et al., 2012; Wills et al., 2011), communication and exchange of the most recent findings among experts in this field were followed by discussion and definition of new perspectives.

The sessions of the 4th Workshop covered a large spectrum of interconnected themes and were intended: (A) to clarify the basic mechanisms of the virus–host interaction during primary and latent HCMV infection; (B) to characterize how HCMV-specific immune surveillance may remodel the immune system in the course of the life-long persistent infection and thus, concomitantly impact the aging of the host; (C) to answer whether HCMV is detrimental, neutral or perhaps even beneficial to immune defense; and (D) to explore the clinical significance of HCMV infection in a broad range of age-related pathologies. For each theme, lessons from mouse model of CMV infection (MCMV) have also been taken into account and integrated with human infection data. In the final round table with closing remarks, knowledge gaps have been identified and priority areas for further research were suggested.

2. Basic mechanisms of the virus–host interaction during primary and latent HCMV infection

Intense research about the biology of CMV is required to clarify how this virus establishes latency in spite of a robust immune response that involves both innate and adaptive humoral and cell-mediated immunity. In that regard, HCMV-encoded determinants of tropism for endothelial cells, an important target of the infection, have been investigated (**F. Goodrum**, Tucson, USA) (Bughio et al., 2013). It was reported that the UL133–UL138 locus, encoded within the ULb′ region of HCMV genome, is required for late stage tropism of the virus specifically in endothelial cells. This locus is not required for early gene expression, viral

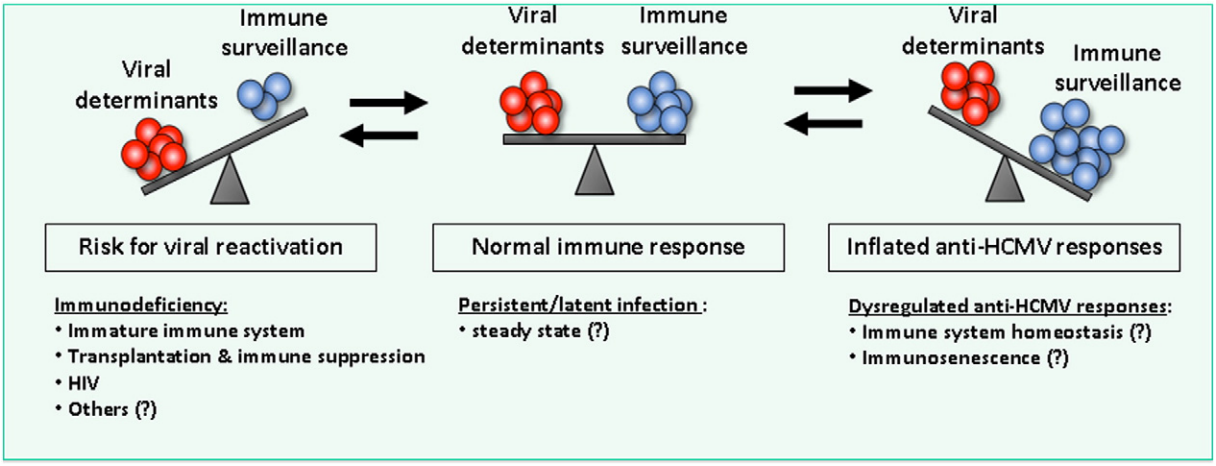

Fig. 1. Host:HCMV interaction.

genome synthesis or late gene expression. However, the locus was required for maintaining membrane organization in the infected cell and for the maturation of progeny viruses. Infection with viruses lacking the UL133–UL138 region produced progeny viruses that lacked tegument and envelopes, resulting in a striking defect in virus yields. This defect is not apparent in fibroblasts or epithelial cells infected with the UL133–UL138–Null virus, suggesting the identification of the first late-stage, endothelial cell-specific tropism factors. Genes UL135 and UL136, encoded within the UL133–UL138 locus, have been found important for maturation and, currently, the mechanisms by which they function to promote virus maturation are under investigation. More recent data suggest that this locus contains the key molecular switch between latency and reactivation, including the opposing roles of UL135 and UL138 (Umashankar et al., 2014).

R. Arens (Leiden, The Netherlands) discussed how in the course of the early virus–host interaction, the outcome of anti-viral immunity may be influenced by several viral determinants, including CMV strain, virulence, MHC I downregulation and other escape mechanisms. In an experimental CMV model, the impact of the viral dose on the outcome of memory T cell inflation (an accumulation of large numbers of memory T cells that occurs during persistent MCMV infection), has been examined. It has been observed that a low-dose inoculum of mouse CMV, as compared to intermediate and high dosages, does not elicit memory T cell inflation. Following low-dose infection, already early after infection the phenotype of inflationary T cells is more central-memory-like as evidenced by increased IL-2 production and decreased KLRG-1 expression. Both after low and intermediate dose infection, inflationary T cells did not show signs of T cell exhaustion. These results suggest that the initial viral dose impacts the outcome of MCMV infection by influencing quality and extent of memory T cell inflation.

Neutralizing antibodies play an important role in the control of CMV infection. However, little is known about the quality and the dynamics of the primary B cell response induced by the virus. During primary infection, large expansions of activated memory B cells (CD20+ CD21lowCD27+, phenotypically distinct from plasma cells) recognize HCMV tegument proteins, whereas envelope glycoproteins are predominantly recognized by classical memory B cells (CD20+ CD21+ CD27+) (**A. Marchant**, Brussels, Belgium). Primary infection is also associated with high frequencies of HCMV-specific atypical memory B cells (CD20+ CD21lowCD27−) expressing high levels of inhibitory receptors. The limited induction of envelope glycoprotein-specific effector B cells and the induction of atypical memory B cells may limit the production of neutralizing antibodies during primary CMV infection and favour the dissemination of the virus, and subsequent establishment of latency.

3. How HCMV-specific immune surveillance remodels the immune system

The profound impact of HCMV on immune homeostasis and its contribution to many age-related changes observed in the immune system, especially among T cells, have been subject of intense studies in the last years. In concert with that, new data was reported on both innate and adaptive responses to CMV.

It is known that HCMV infection in healthy individuals is associated with an increase of NK cells expressing the CD94/NKG2C activating receptor, but the precise role of NKG2C+ cells in the control of HCMV infection and the molecular mechanisms underlying the reconfiguration of the NK cell compartment remain open issues. Recent studies (**M. López-Botet**, **A. Muntasell** Barcelona, Spain) have documented the existence of two NKG2C+ NK-cell subsets differing in surface staining intensity (Muntasell et al., 2013). The NKG2Cbright phenotype, exclusively found in a subgroup of HCMV+ individuals (~50%) correlated with the expansion of NKG2C+ NK cells. Conversely, the NKG2Cdim NK phenotype was found in smaller proportions in the other half of HCMV+ and all of the HCMV-negative donors. Because a homozygous deletion of the NKG2C gene has been reported in different populations, the possible influence of this genetic trait on NKR distribution was addressed. HCMV+ NKG2C+/+ children and adults tend to possess, under steady state conditions, greater numbers of NKG2Cbright cells as compared to those in hemizygous NKG2C+/del subjects. In addition to quantitative differences, differences in functional response to receptor engagement were also noticed between NKG2C+/+ and NKG2C+/del subjects. These data support a correlation between NKG2C zygosity and the magnitude/persistence of the NK-cell redistribution in healthy HCMV+ donors.

Other studies examined characteristics of the gamma-delta T cell response to HCMV. γδ T cells display considerable HCMV-associated expansion and appear to contribute to the control of viremia. Most γδ T cells are significantly affected by age and are present at decreased frequencies in the elderly. However, HCMV infection maintains a robust and stable pool of Vδ2-negative γδ T cells throughout life. HCMV seropositivity accentuates further the accumulation of highly differentiated lymphocytes in the Vδ2- γδ T cell subsets with time, in contrast to Vδ2+ γδ T cells, which maintain a less differentiated phenotype (**D. Sauce**, Paris, France) (Roux et al., 2013). Moreover, the γδ T cell repertoire is more restricted in HCMV-infected compared to non-infected individuals. Together, these findings suggest that HCMV induces alterations in γδ T cells similar to those reported for CD8 T cells that are associated with immunosenescence. A crucial question is: what are the HCMV-related antigens that support these γδ T cell responses? A new strategy for the identification of γδ T cell antigens expressed by HCMV-infected cells has led to the identification of EPCR (endothelial protein C receptor) and EphA2 (a receptor tyrosine kinase for members of the ephrin-A family) as antigens directly recognized by two different γδ receptors (Vγ5Vδ5 and Vγ9Vδ1, respectively) on the surface of infected cells. Free chains of HLA-I molecules are also recognized by Vγ9Vδ3-expressing γδ T cells. These data are very intriguing because they suggest that infection with HCMV activates γδ T cells specific for self antigens through the induction of a multimolecular stress signature on the surface of infected cells (**J. Déchanet-Merville**, Bordeaux, France) (Willcox et al., 2012).

A number of presentations addressed the changes in the CD8+ T cell compartment in the presence of CMV infection. Specific studies addressed numerical and functional changes, gene expression profiles, homing characteristics and maintenance mechanisms of the total CD8+ T cells and, in particular, of the CMV-specific CD8+ T cells. The effect of HCMV infection on CD8+ T cells polyfunctionality (CD107a expression, IFN-γ or TNF-α production), has been explored in HCMV-seropositive and seronegative young and in HCMV-seropositive middle-aged healthy donors in response to SEB (**R. Solana**, Cordoba, Spain). The results showed that the percentage of SEB-responsive CD8+ T cells increases with both age and HCMV infection. Of interest, young and middle-aged CMV-seropositive subjects possessed more CD8+ polyfunctional cells than their CMV-seronegative counterparts; this was associated with the expansion of polyfunctional CD8+ CD57+ T cells. These results suggest that being infected with HCMV may improve the polyfunctionality and consequently the quality of CD8+ T cells at least in young individuals. It appeared that this improvement was not affected by advancing age (no difference between young and middle aged subjects), although bona fide old individuals were not studied by group. Similar studies were reported by **Y.L. Chiu** (Baltimore, USA) who examined polyfunctionality (CD107a expression IL-2, IFN-γ, TNF-α production) of the distinct CD8+ T cell subsets (identified by staining with CD45RA andCCR7) in response to 4h PMA/ionomycin stimulation in a group of young and elderly donors. These investigators described a general functional impairment driven by the lack of IL-2 in the CD8+ CCR7-CD45RA+ subset (T_{EMRA}), that expands significantly in the elderly. Interestingly, upon stimulation, these cells exhibit impairment in the upregulation of pERK (phosphorylated Extracellular Signal-Regulated Kinase), belonging to a key mitogenic signal transduction pathway. Currently, potential genes involved in the

inhibition of the MAPK/ERK pathway in the T_{EMRA} cells are under investigation by microarray analysis. Although it is very likely that a substantial proportion of the late-stage differentiated memory CD8 + T cells considered in this study is HCMV-specific, the use of a nonspecific stimulus limits the ability to extrapolate these results to CMV-specific CD8 + T cells.

Factors involved in the regulation of the CD8 + effector T cell function were also studied by gene expression analysis. A transcription factor specifically expressed in human cytolytic CD8 + T cells, which accumulate significantly in HCMV-infected people, was identified by **R. van Lier** et al. (Amsterdam, The Netherlands) (Hertoghs et al., 2010). This factor, termed HOBIT, is highly related to BLIMP-1, a transcriptional repressor that regulates terminal differentiation of B and T lymphocytes. An antibody raised against the unique N-Terminal part of HOBIT specifically stained cytolytic subsets of human lymphocytes, like effector-type CD8 + T-cells, but also CD28 − CD4 + T cells and NK cells. Knockdown of HOBIT in NK cell lines via siRNA reduced IFNγ production and increased cell survival. HOBIT may be essential for controlling formation, maintenance and function of resting cells with immediate effector function. Its exact contribution to the generation, maintenance and function of resting effector-type CD8 + T cells in vivo remains to be established.

N. Riddel, **A. Akbar** et al. (London, UK) investigated accumulation of low avidity CD45RA + HCMV-specific CD8 + T cells observed in elderly people (Griffiths et al., 2013). These investigators used wild-type pMHC tetramers and also mutated pMHC tetramers that do not bind CD8 to identify low-affinity (CD8-dependent) and high-affinity (CD8-independent) TEMRA cells. In addition to reduced TCR:MHC avidity, these cells exhibited relatively long telomeres, suggesting that they are not induced by repeated antigen-driven proliferation. Instead, IL-15 incubation was sufficient to induce CD45RA re-expression in CD45RA- HLA-A2/NLV-CMV-specific CD8 + T cells. Furthermore, HCMV stimulation induced IL-15 production by monocytes secondary to invoking IFN-α release by dendritic cells. These data suggest that HCMV can induce differentiation of EM T cells that readily enter cytokine-driven homeostatic proliferation, which may provide a mechanism by which cells with low TCR:MHC avidity can accumulate in-vivo.

A large gap in our knowledge of HCMV-specific T cells is related to the fact the majority of published studies have examined responses in peripheral blood (PB) and very little is known about responses localized to other anatomical sites. It has been shown that lymph nodes (LN) contain HCMV-specific CD8 + T cells that resemble (central) memory cells, a phenotype that is infrequent in peripheral blood (**E. Remmerswaal**, Amsterdam, The Netherlands) (Remmerswaal et al., 2012). When analysing the TCR-Vβ repertoire by high throughput sequencing it was found that the LN HCMV-specific CD8 + T cell pool often contained clones not found in PB. Since it is not known if human LN HCMV-specific CD8 + T cells contribute to the PB pool upon viral recall, the possible appearance of these clones in the circulating pool during CMV reactivation was studied. In one of the four patients studied, unique LN clones were recruited to the PB upon antigenic recall. Therefore it was postulated that LN can contain a unique pool of "true" memory HCMV-specific CD8 + T cells that may contribute to the PB population upon antigenic recall. Although it is essential to look at HCMV-specific T cell responses in compartments distinct from blood (lymph nodes, bone marrow, lymphocytic infiltrate and also gut- and other mucosa-associated lymphoid tissue), it is logistically and sometimes ethically very difficult to carry out these studies in sufficient numbers of elderly donors. For this reason, the standard source for studying the impact of HCMV infection on the homeostasis of the naïve and antigen-experienced CD8 + T cells will remain peripheral blood.

With regard to the remodeling of the memory compartment, one of the open questions is whether the phenomenon of T cell memory inflation, well-described in the mouse model, also takes place in humans. In fact, MCMV-induced T cell memory-inflation in inbred mouse-strains polarizes the T cell response into usually isolated large (inflated) and much smaller (contracted) responses. While large expansions of HCMV-specific T cells have been observed, particularly in older age, it has remained unclear whether these occur in isolation or possibly as part of several large responses in the same individuals with a generally raised response level. In a recent study, **F. Kern** (Brighton, UK), reported on a large T cell response dataset from short-term ex-vivo HCMV antigen stimulation (measured by IFN-γ secretion) (Sylwester et al., 2005). The dataset was re-analyzed to establish the presence of normal CD4 + or CD8 + response ranges, response distribution across proteins (using >200 HCMV peptide pools representing all expressed HCMV-proteins >30 amino acids in length) and outliers in 33 healthy individuals aged 19–53 years. An additional 47 old HCMV-infected participants were tested with respect to 19 selected HCMV peptide-pools. The authors concluded that the definition of outliers was complicated, because i) individual CD4 + or CD8 + response levels varied hugely and ii) a "protein target factor" affected response size. Interestingly, individuals with a greater number of different responses exhibited larger maximum responses and greater numbers of outliers. However, response dominance hierarchies were regular and relatively uniform across the donor population and marked polarization of response size as reported in mouse models (inflated versus contracted) was not observed. The study suggests that is not possible to define clinically useful normal ranges for HCMV-specific T cell responses applicable to cross-sectional data, as response sizes vary hugely depending on both protein target and individual. Moreover, the occurrence of T cell memory-inflation similar to the mouse model in HCMV infection is not supported by this analysis (manuscript submitted).

The methodological approach of the previous study tried to bypass the limits of currently and commonly used tests for the evaluation of HCMV-specific T cell responses, an extremely demanding task because of the breadth of such responses. In fact, functional assays with intracellular cytokine staining in cells activated with one or two immunodominant proteins (generally, pp65 and/or IE-1 proteins), and flow cytometry staining with peptide-MHC multimers, both provide only a partial insight into the HCMV-specific T cell responses. In line with this critical issue, a different method for the determination and quantification of HCMV-specific T cells has been developed by **D. Lilleri's** group (Pavia, Italy) following stimulation with HCMV-infected autologous dendritic cells (Lozza et al., 2005) using intracellular detection of IFN-γ production. In a group of 31 healthy adult controls, the T cell response evoked by the infected dendritic cell stimulation was compared with the response against an HCMV-infected cell lysate and the response against a peptide pool including 34 peptides relevant to multiple HCMV proteins shown to carry epitopes recognized by MHC class I-restricted $CD8^+$ T-cells. The DC assay appeared to be the test of choice both in terms of quantitative and qualitative characterization (polyfunctional profile for IFN-γ, TNF-α and IL-2 production and CD40L upregulation) and this DC assay may be suitable for testing HCMV-specific responses also in the elderly.

The naive T cell compartment is known to decline in aging, but one basic question concerns the distinction between the relative effects of anti-CMV T cell responses and aging itself. To distinguish which of the manifestations of T cell aging occur in its absence (T cell aging per se) and which only and solely in the presence of HCMV, (HCMV-associated T cell aging) a cross-sectional study using large cohorts of HCMV-seropositive (HCMV +) and CMV-seronegative (HCMV −) individuals across different age groups was performed (**J. Nikolich-Zugich**, Tucson, USA). The results of this study, extended since last year's report, showed that while percentages of naïve CD8 T cells declined and those of memory T cells increased with aging, in HCMV − individuals this was solely due an absolute numerical loss of naïve T cells (defined using up to 5 different markers). Consequently, HCMV − individuals exhibited flat or declining absolute numbers of memory T cells. Only in the presence of HCMV was there an absolute increase of memory cells, which was directly linked to the inflation of the effector memory CD8 + (and much less CD4 +) cell pool. Moreover, memory inflation with age increased

in those individuals exhibiting higher anti-HCMV Ab titers, but not in those whose anti-HCMV titers remained in the lower half. If indeed higher Ab titers can be taken as a reliable correlate of virus reactivation and of poor virus control, then these results raise the possibility that efficacious HCMV control may circumvent drastic changes in the peripheral T cell subset balance previously associated with this virus. These results, however, will have to be confirmed in longitudinal studies. Nonetheless, the fact that naïve T cells disappeared from HCMV+ individuals relative to age in the essentially the same manner as in HCMV− subjects suggests that HCMV may have very little to do with the maintenance of naïve CD8+ T cell numbers.

In a different study using a mouse model, the impact of deliberate early-life MCMV infection upon the maintenance of the naïve CD8 T cell repertoire was addressed (**M. Smithey**, Tucson, USA). While the results of this study agreed with prior studies (Cicin-Sain et al., 2012) (Mekker et al., 2012) that naïve T cell numbers did not decline any faster during life-long MCMV infection, evidence that there is a decline in functional immunity (polyfunctionality, responding cell numbers), and, more importantly, in the numbers of naïve precursors specific for the B8R and OVA epitopes was found (Smithey et al., 2012). Further, in response to infectious challenge, there was a complete lack of overlap in clonal TCRβ utilization by single-cell PCR between the mice aged in the presence or absence of life-long MCMV infection.

In the past years, in an attempt to elucidate the impact of persistent HCMV infection on immune status, studies in selected elderly populations have been intensified. However, when studies include people who have reached an age greater than the average life expectancy, it must be considered that a "survivor bias" can skew the population. In fact, the results obtained in such a selected populations may not be easily generalized to a normal aging but rather linked to longevity.

In order to clarify to what degree alterations at the T cell level are associated with the profile and function of humoral immune responses to HCMV, and to explore the biological relevance of IgG titers, **E. Derhovanessian**, **G. Pawelec** and collaborators (Tübingen, Germany) studied neutralizing Ab activity of sera in 79 elderly individuals over the age of 83 participating in the BELFRAIL study (Vaes et al., 2010). The assay (performed by K. Schweinzer & K. Hamprecht, Dept. Virology, Tübingen) determined the ability of the subject´s serum to prevent the in vitro infection of a susceptible epithelial cell line by a clinical isolate of CMV. In this group of subjects a higher neutralizing antibody capacity in serum was associated with a lower CD4/CD8 ratio and a more late-differentiated CD8 compartment (lower frequency of naive and higher frequency of late-differentiated effector phenotypes and higher frequency of CD57+ cells). The neutralizing activity of the serum was remarkably strong, even more so than control sera from young women. This emphasizes the high-level commitment of immune resources to CMV-immunosurveillance not only in terms of cellular responses, but also humoral responses. Another study in the same cohort (**C. Mathei**, Leuven, Belgium) analyzed T cell subsets of 235 community-dwelling persons aged 81.5 years or older (73.6% HCMV+) and 25 younger persons with an average age of 28.5 yr (12% HCMV+). In the elderly, a total of 7.2% had an inverted CD4/CD8 ratio, which was significantly associated with HCMV infection (88% HCMV+), less naïve and more late-differentiated CD4 and CD8 T-cells. 32.8% of the elderly (62.3% HCMV+) had a CD4/CD8 ratio >5, compared to none in the young individuals. Those with ratios >5 had more naïve and less late-differentiated CD4 and CD8 T-cells, compared to those with a ratio <1, ratios between 1 and 5 or the younger individuals. Furthermore, a CD4/CD8 ratio >5 was significantly associated with a lower physical and global performance. Thus, this study identified a previously unrecognized subgroup in the very elderly with an exceptionally high CD4/CD8 ratio showing a lower functional performance and a dominant naïve T-cell phenotype. These findings, together with those from the Leiden 85-Plus study, showing a better 8-year survival for very elderly individuals with a lower frequency of naïve CD8 T-cells, suggest that, under certain circumstances, the HCMV-associated changes in blood T cell subsets might not be as detrimental as widely believed but rather could represent an adaptational remodelling of the immune system at very old age to deal with the burden of the virus by maintaining essential immunosurveillance (Derhovanessian et al., 2013).

Using a transcriptomic analysis approach, **M. Hurme** (Tampere, Finland) explored the mechanisms involved in the association between high levels of anti-HCMV antibodies in elderly individuals and pathological sequelae (Kuparinen et al., 2013). The correlation between the anti-HCMV IgG titre and global gene expression profile was determined in HCMV-seropositive nonagenarians and in young controls. In controls, the correlating genes belonged to pathways known to regulate normal cellular immunity, but in contrast to this, in nonagenarians several pathways involved in apoptosis and cellular damage were activated. Thus, it seems that HCMV-associated pathological effects are more prominent in elderly individuals.

In another study **C. Franceschi** and collaborators (Bologna, Italy) determined HCMV prevalence in 132 centenarians, 245 centenarian offspring and 101 offspring of non long-lived parents. The prevalence of HCMV positivity was high, ranging from 91.0 to 94.1% in centenarian offspring and offspring of non long-lived parents, respectively, and reaching 94.7% in centenarians. After stratification for the levels of HCMV-IgG antibodies, centenarians in the highest antibody group showed significantly higher levels of creatinine, higher absolute numbers of lymphocytes, neutrophils and platelets and lower levels of HDL-cholesterol, compared to those low-positive or negative for HCMV-IgG. In addition, high-positive HCMV-IgG centenarians showed higher numbers of differentiated helper and cytotoxic T lymphocytes and a lower CD4/CD8 ratio. Centenarians and their offspring ($n = 104$) were predominantly HCMV-positive, while offspring of HCMV-negative centenarians were either positive or negative, suggesting a possible familial component influencing susceptibility to HCMV infection. Overall, these data may indicate that HCMV positivity does not influence the chance of reaching extreme longevity, although survivor selection needs to be studied further.

4. HCMV infection: good or bad player in immunosenescence and in human diseases?

One dominant remaining question, which keeps stimulating intense research efforts, is whether HCMV infection has only negative or at least some beneficial effects on the immune status and overall health.

Using a systems immunology approach, **D. Furman**, **M.M. Davis** and collaborators (Palo Alto, USA) obtained provocative data showing very different immunological and gene expression profiles in HCMV infection and aging. They studied longitudinally a cohort of 89 subjects of different age groups that were genotyped for immune-related SNPs and assayed for baseline levels of a variety of blood measures including serum cytokines and chemokines, whole-genome gene expression and cell subset frequencies. They also studied the functional activity of some of these subsets by in vitro stimulation of cells with cytokines, and in vivo humoral responses to seasonal influenza vaccination. Robust immune responses were observed in HCMV-seropositive vs seronegative young but not older individuals, as seen by the increased pSTAT responses and higher antibody response to the influenza vaccine. The authors also showed that the variation in CD4+ CD28− cell counts due to HCMV can be genetically explained by a set of SNPs on the HLA locus. The conclusion of this study is that HCMV and aging have significantly different influences on the immune system and that HCMV might have a beneficial effect in young individuals, reminiscent of the conclusions reached by Solana and coworkers above (see the beginning of section B).

The impact of the CMV infection on immune fitness was also explored by testing the efficacy of the adaptive immune system of mice latently infected with MCMV by challenging them with vesicular stomatitis virus (VSV). The results of this study (**L. Cicin-Sain**, **T. Marandu**, Braunschweig, Germany) indicated that latent MCMV

infection reduces the relative, but not the absolute, count of CD8 cells responding to a challenge with an unrelated virus. The reason for this discrepancy was identified in the large increase of the effector-memory (EM) subset of CD8 T cells, which doubled the size of the CD8 compartment in MCMV-infected mice. Consequently, due to the increased size of the CD8 pool, the fraction of the antigen-specific response to the challenge virus relative to the CD8 compartment diminished, while the size of the response in absolute terms remained the same.

P. Thomas (Memphis, USA) also used a mouse model of CMV infection, examining its impact on influenza virus co-infection. Acute and early latent MCMV infection resulted in improved control of influenza virus infection as measured by reduced weight loss and increased viral clearance. MCMV-specific CD8 + T cells were found to infiltrate the lungs of influenza-infected animals several days prior to the emergence of an influenza-specific CD8 + T cell response. These cells did not appear to cross-react with influenza epitopes, but were capable of producing significant amounts of the cytokines IFN-γ and TNF-α. Using IFN-γ knockout mice, it was shown that the protective effects of prior MCMV infection were entirely dependent on IFN-γ, likely produced by these MCMV-specific CD8 + T cells in a bystander manner. This, as well as some other studies above, was consistent with the findings from the Virgin laboratory (Barton et al., 2007) that persistent viruses can protect against heterologous infections, at least in youth. It would be of interest to examine whether influenza virus-induced cytokines played a role in stimulating CMV-specific effector memory T cells to produce IFN-γ.Conversely, another study documented negative effects of HCMV seropositivity on the in vivo and in vitro B cell responses to the seasonal influenza vaccine (**D. Frasca**, Miami, USA). The in vivo response was evaluated by hemagglutination inhibition assay to measure antibody titers and by flow cytometry to measure the percentage of switched memory B cells. The switched memory B cells can be detected in blood before vaccination and their levels predict the robustness of the in vivo response. The in vitro response was measured by AID (activation-induced cytidine deaminase), the enzyme of immunoglobulin class switch recombination that generates protective antibodies. AID is another B cell biomarker predictive of optimal in vivo responses. Results presented indicate that HCMV seropositivity significantly decreases both in vivo and in vitro B cell responses to the vaccine in both young and elderly individuals, possibly through an increase in intracellular levels of B cell-derived TNF-α, which has previously been shown to be predictive of poor B cell function.

Altogether, the above studies underlined that changes in immune system homeostasis of CMV infected hosts do not result in a general functional impairment and that, in specific contexts, particularly in younger individuals, CMV infection could even improve some immune responses.

5. CMV and pathologies of aging

However, despite these intriguing new observations, persistent HCMV infection has been associated with a range of clinical complications and the significance of these associations is now under investigation in a number of laboratories. In fact, the causal relationship between HCMV infection and health status in the elderly remains ambiguous and poorly addressed from a prognostic and therapeutic point of view. One important point to be clarified is whether there are defined clinical situations in which either a dysregulated anti-HCMV immune response or viral reactivation may pose an increased clinical risk for elderly patients. Results from studies carried out in several pathological conditions were presented and discussed.

5.1. Immunosuppression and CMV

B. Grubeck-Loebenstein (Innsbruck, Austria) presented an analysis of T cell function and the composition of the T cell repertoire in kidney transplanted immunosuppressed patients of different ages, compared with age-matched controls. HCMV-seropositive and -seronegative patients and control groups were also compared. Independent of age and HCMV status, the production of IL-2 and IFN-γ by T cells was decreased in the immunosuppressed patient groups. CXCR5 expression on T cells, which negatively correlates to endogenous IL-2 signaling, was increased in patients compared to controls but this parameter did not differ among patients of different age and HCMV status. In HCMV-seronegative patients, kidney transplantation and immunosuppressive therapy did not induce changes in the CD8 + T cell pool, but there was a moderate increase in CD4 + CD28 − effector T cells when compared to age-matched controls. In contrast, HCMV infection triggered a shift from early to late differentiated CD4 + and CD8 + T cells in both immunosuppressed patients and controls. This shift was most pronounced in elderly transplant patients under immunosuppressive therapy. The results demonstrated that immunosuppressive therapy following kidney transplantation was effective in patients older than 60 years of age. Persistent HCMV infection did, however, accelerate age-related changes in the T cell compartment in elderly persons under immunosuppressive therapy (Welzl et al., 2014).

5.2. CMV, survival and cardiovascular disease

Although some studies have linked high HCMV antibody titres with mortality in elderly cohorts (Strandberg et al., 2009) (Wang et al., 2010), the relationship between HCMV infection and mortality among immunocompetent individuals still remains to be confirmed. Moreover, the mechanisms underlying this relationship are not known, even as accumulating evidence links HCMV infection to an increased incidence of cardiovascular disease.

In a cohort of 67 healthy elderly **a**n aberrant HCMV-specific (pp65) T cell response, without chemokine or cytokine co-expression (CD107a + PRF1 + but IFNγ-TNFα-IL2-MIP1α-) was found to be increased in individuals with lower survival rates and was found to be independently associated with time to all-cause death (**S. Ferrando-Martinez**, Seville, Spain). This result suggests that accumulation of CD107a + PRF1 + (IFNγ-TNFα-IL2-MIP1α-) CD8 + HCMV-specific T cell responses could be a useful tool to identify individuals with age-related immune deregulation and a higher risk of death, providing a new surrogate marker of immune system deregulation in late stages of life. In another study (**P. Moss**, Birmingham, UK), an 18 year follow-up of 511 elderly donors aged over 65 years at entry showed that those who were HCMV-seropositive at study entry had a near 4 year reduction in lifespan compared to those who were uninfected (Savva et al., 2013). Interestingly, this was entirely due to a two-fold increase in mortality from vascular disease. Also, another recent study (Wall et al., 2013) investigated the relationship between HCMV seropositivity within patients with chronic kidney disease and carotid-femoral pulse wave velocity (PWV), the current gold-standard measure of arterial stiffness. It was shown that HCMV infection is associated with an increased arterial stiffness, perhaps partly explaining the vascular complications seen in these patients. **E. Shin** (Daejeon, Republic of Korea) employed a similar methodological approach in a cohort of subjects with normal renal function. Arterial stiffness was evaluated by heart-femoral pulse wave velocity (PWV) in a cohort of 423 Koreans, and PWV-associated immune parameters were analyzed. The frequency of CD57 + cells in the CD8 + T cell population significantly correlated with PWV in multivariate analysis. A subgroup of 123 subjects was further analyzed for HCMV-specific immune responses. All the subjects (aged ≥50 years) were seropositive for HCMV IgG. Moreover, HCMV pp65-specific IFN-γ or TNF-α secretion by CD8 + T cells significantly correlated with PWV in multivariate analysis. These findings suggest that CD57 + CD8 + T cells and secretion of IFN-γ and TNF-α by HCMV pp65-specific CD8 + T cells might contribute to increased arterial stiffness, a predictor of cardiovascular mortality.

Finally, a recent paper by Terrazzini et al. has confirmed that resting blood pressure is linked to the size HCMV-specific CD8 + T cell

responses but also a novel regulatory type CD4+ T cell subset expressing CD25, CD39 and CD134 upon activation with CMV-Antigen (Terrazzini et al., 2013).

5.3. CMV and diabetes

HCMV infection as well as its reactivation were reported to be strong predictors of diabetes mellitus onset when the host immune response to infection is diminished, for instance after kidney, heart and lung transplantation. Even asymptomatic HCMV infections within the context of transplantation have been shown to impair insulin release. Beta-cells are themselves susceptible to infection by HCMV, which could potently activate a beta-cell toxic immune response. This provided the rationale to assess the relationship between HCMV seropositivity and HCMV-IgG antibody levels and new onset of type 2 diabetes (incident T2D) (**E. Gkrania-Klotsas**, Cambridge, UK). Data from 12,260 participants of the EPIC (European Prospective Investigation of Cancer)–Norfolk prospective cohort were analyzed, with full covariate information, including 532 ascertained incident 2TD cases. After adjustment for age, sex, body-mass index, physical activity, family history of diabetes mellitus, smoking and socioeconomic status, the presence or level of IgG against HCMV were found not to be associated with the risk of incident T2D over an average follow-up of 10.2 years. Thus, in this cohort of immunocompetent people, prior infection with HCMV and the level of HCMV-IgG were not associated with the risk of incident T2D.

5.4. CMV, cognitive impairment and depression

Three different studies in the Workshop reported on the controversial issue of HCMV infection and mental health. A study of 1061 participants of the Lothian Birth Cohort found a small but significant decrease in cognitive function in HCMV-seropositive donors (**P. Moss**, Birmingham, UK) (Gow et al., 2013). Interestingly the largest effect was seen in people with the highest HCMV-specific antibody levels. Within the group with the highest quartile of antibody titer, the general cognitive ability was reduced by nearly 1%. The magnitude of this reduction is similar to the effect of the strongest genetic determinants of cognitive decline determined so far in this cohort.

Two longitudinal studies, examining the association between persistent pathogens including HCMV, inflammatory markers and depression using data from the Detroit Neighborhood Health Study (DNHS) and the Sacramento Area Longitudinal Study of Aging (SALSA), were presented (**A.M. Simanek**, Milwaukee, USA). Among 209 DNHS participants, free of depression and seropositive for HCMV at baseline, the odds (95% confidence interval) for onset of depression in the first year of the study were 2.89 (1.13, 7.42) for those with HCMV IgG antibody titers in the highest quartile compared to those in the lowest three quartiles, controlling for age, gender, race/ethnicity, income level, number of stressful life events and medications. Among 428 SALSA participants, HCMV seropositivity but not IgG antibody titer was associated with a 1.65-fold greater incidence rate of reporting elevated depressive symptoms over nine years of follow-up, adjusting for age, gender, education level, acculturation level (i.e. Mexican vs. Anglo cultural orientation), nativity status and medication use. These associations were not mediated by inflammatory factors according to markers including interleukin-6 or C-reactive protein.

Future research should help elucidate the biologic mechanisms by which HCMV infection and immune responses against HCMV may increase risk of onset of adverse mental health outcomes such as cognitive impairment and depression.

5.5. CMV and cancer

Available evidence demonstrates that HCMV is present in several solid tumors with a high prevalence, approaching 100% in malignant glioblastoma (Rahbar et al., 2013). The virus may mediate both oncomodulatory and oncogenic effects. It has been demonstrated that anti-viral treatment with valganciclovir against HCMV-positive medulloblastoma and neuroblastoma tumors in animal xenograft models prevents tumor growth (Wolmer-Solberg et al., 2013), and that valgancilovir treatment in glioblastoma patients shows promise in increasing patient survival rate (Stragliotto et al., 2013). In this Workshop, **C. Söderberg-Nauclér** (Stockholm, Sweden) presented new data on this topic. This group analyzed serum and blood samples of glioblastoma patients and found a high proportion of patients free of detectable HCMV-specific IgG antibodies in serum, who were nonetheless HCMV-positive in the tumor. Moreover, compared to control subjects, glioblastoma patients had an altered T cell response against HCMV, suggestive of chronic HCMV reactivation and possibly exhaustion of the immune response. Thus, immunological disturbances in glioblastoma patients have been confirmed and these alterations need to be investigated further in order to design an optimal therapeutic strategy targeting HCMV.

5.6. CMV and rheumatoid arthritis

It has already been described that late-differentiated T cells in patients with rheumatoid arthritis (RA) are characterized by the loss of the costimulatory molecule CD28 and increased expression of inhibitory NK cell receptors like LIR-1. Because chronic infection with HCMV contributes to an expansion of these cells, **K. Rothe** (Leipzig, Germany) studied the influence of HCMV seropositivity on LIR-1 expression in CD8+ T cells of RA patients. Patients had increased frequencies of LIR-1+ CD8+ T cells compared to healthy individuals. In RA patients as well as in the healthy controls, there were higher frequencies of LIR-1+ CD8+ T cells in CMV-seropositive compared to seronegative individuals. In addition, RA patients with strong LIR-1 expression had higher disease activity scores. Importantly, HCMV− LIR-1+ HLA*A0201 -tetramer+ CD8+ T cells were more frequent in RA patients as compared to HCMV+ healthy controls. Furthermore, analysis of the cytolytic potential after HCMV restimulation revealed higher percentages of CD107a+ CD8+ T cells in RA patients than in healthy donors. One interpretation of these results is that HCMV-specific T cells, involved in containing latent HCMV infection, might potentially also contribute to disease severity in RA patients. Alternatively, RA-associated inflammatory process and immunopathology could be inducing CMV reactivation at a higher rate in RA subjects.

5.7. CMV and acute and chronic inflammatory diseases

T. Fulop et al. (Sherbrooke, Canada) evaluated the impact of HCMV serostatus on biological and immune parameters of elderly subjects suffering from acute inflammation (hip fracture, $n = 23$) in comparison with chronic inflammatory diseases such as Diabetes Mellitus type 2 (T2DM, $n = 50$). In an acute stress situation, such as recent hip fracture, HCMV seropositivity had no effect on blood parameters. T cell proliferation induced by specific TCR stimulation with antiCD3/antiCD28 mAbs was not modulated either. However, innate immune cell function, including phagocytosis and phagoburst, were depressed for a long period of time, extending at least 6 months, only in HCMV-seropositive patients, while seronegative patients returned to baseline after 6 weeks. In a chronic stress situation (T2DM), HCMV influenced the CD8+ subpopulations, so that the percentages of CD8+ naive cells were decreased and the percentages of CD8+ TEMRA cells were increased independently of T2DM. T2DM and HCMV-seropositivity concomitantly increased CD57 expression on the CD8+ subpopulation. Dissecting the respective effects on immune alterations in elderly individuals of diseases and CMV infection remains a challenge.

5.8. CMV and acute illnesses

The great majority of presented studies explored the impact of HCMV infection in chronic disease, not acute illnesses, thus avoiding

critical phases of the lifelong host–pathogen interaction. However, various reports have shown that HCMV may reactivate surprisingly often in critically ill immunocompetent adult patients. Some of these reports have also suggested that systemic viral reactivation may prolong hospital stay and mortality. Nevertheless, it is currently unknown whether elderly patients in critical care settings may have an increased risk of viral reactivation. To that effect, **F. Fagnoni** (Parma, Italy) presented the design of a prospective observational trial to examine the potential clinical risk linked to HCMV-specific immune responses and possible viral reactivation in elderly patients suffering from acute stroke.

An increasing amount of data is accumulating about the interactions among CMV infection, immunosenescence and age-related diseases. Thus, at the end of the Workshop, a general consensus was expressed for the proposal (**A. Maier**, Amsterdam, The Netherlands) to include meta-analyses, based on HCMV serostatus and HCMV-specific IgG titer, in design of current studies. All the researchers involved in this topic should move toward genetic, clinical phenotype or mortality studies, possibly using the same adjustment models.

6. Conclusions

A number of longstanding questions related to CMV's role as a "driver" or "passenger" in the aging of the immune system, in age-related diseases and in complex comorbidities remain incompletely resolved and rather recalcitrant to being rapidly and conclusively resolved. Part of the obstacle lies in the complexities of longitudinal human studies, with pronounced ethical barriers and genetic and epigenetic variabilities on the one hand, and the imperfect concordance between human infection and more tractable animal models of CMV infection in a specific pathogen-free and genetically homogenized settings, on the other. Nonetheless, this Workshop brought about important new findings that expand our knowledge and challenge it with new and stimulating questions. Amongst those, we believe that four are particularly worth highlighting:

- HCMV infection and aging seem to 'operate' over different, at best only partially overlapping, immune traits
- Susceptibility to immune alterations in HCMV-seropositive subjects is not only highly variable, but could be genetically determined
- HCMV infection might boost immunity in young individuals and in particular contexts, as in very old people, could have beneficial effects
- Presented data suggest that the interplay between the efficacy of viral control, extent and frequency of viral reactivation and the superimposed comorbidities need to be studied with utmost urgency.

Validating the above observations and attacking the main longstanding questions related to the positive or deleterious effects of CMV remains an important task confronting researchers in the field in the future.

All the participants of this Workshop approved the plan to attend the 5th International Workshop on "CMV & Immunosenescence" in Amsterdam, The Netherlands, 20th–21st November 2014 (local organizers: Andrea Maier, Jos Bosch, Ester Remmerswaal, Ramon Arens and René van Lier).

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Authors' contributions

All authors attended the Workshop, participated in the discussion, saw and commented on the text published here. All the authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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