Marandu, T.F., Finsterbusch, K., Kröger, A., Čičin-Šain, L.

Mouse CMV infection delays antibody class switch upon an unrelated virus challenge

(2014) Experimental Gerontology, 54, pp. 101-108.

Mouse CMV infection delays antibody class switch upon an unrelated virus challenge

Thomas F. Marandu[1], Katja Finsterbusch[2], Andrea Kröger[2], Luka Čičin-Šain[1*]

1. *Department of Vaccinology - Helmholtz Centre for Infection Research, Braunschweig, Germany*
2. *Department of Gene Regulation and Differentiation – Helmholtz Centre for Infection Research, Braunschweig, Germany*

*Corresponding author:

Luka Cicin-Sain,

Helmholtz Centre for Infection Research

Inhoffenstr. 7

38124 Braunschweig, Germany

Tel: +49 (0)531 6181-4616

Fax: +49 (0)531 6181-4699

E-mail: luka.cicin-sain@helmholtz-hzi.de

ABSTRACT

Poor immune protection upon vaccination is a critical determinant of immunosenescence. Latent Cytomegalovirus (CMV) infection has been associated with poor antibody responses to vaccination, but a causative role for CMV in the poor immune response requires experimental evidence and thus could not be confirmed in clinical studies. To test the hypothesis that latent CMV infection causes poor antibody responses, we infected young or adult mice with mouse CMV and challenged them with vesicular stomatitis virus (VSV) at 15 or 18 months of age. Latent, but not primary infection with mouse CMV resulted in diminished neutralizing titers of the serum IgG fraction at day 7 post challenge, which recovered by day 14 post challenge. This phenomenon was specific for mice infected with mouse CMV, but not mice infected with other herpesviruses, like murine herpesvirus-68 or herpes simplex virus type 1, or mice infected with non-persistent viruses, such as influenza or vaccinia virus. Hence, our data indicate a delay in IgG class-switch that was specific for the CMV infection. Herpesviral infections did not change the B-cell memory compartment, and increased the size of the effector-memory subset of blood CD4 T-cells only when administered in combination. Furthermore, CD4 T-cell response to VSV infection was maintained in latently infected mice. Therefore, our results argue that latent CMV infection impairs B-cell, but not T-cell responses to a challenge with VSV and delays antibody class-switch by a mechanism which may be independent of T-cell help.

KEYWORDS

Cytomegalovirus; Herpes Simplex Virus type 1; Murine Herpesvirus 68; Humoral response; In vitro neutralization; CD4 T-cell

ABBREVIATIONS

Cytomegalovirus – CMV; Mouse cytomegalovirus – MCMV; Murine Herpesvirus 68 – MHV-68; Herpes simplex virus type 1 – HSV-1; Vaccinia Virus – VACV; Vesicular stomatitis virus – VSV; Influenza virus – Flu; Epstein-Barr virus – EBV; Effector memory – EM; Plaque forming unit – PFU; Phosphate-buffered saline – PBS;

1. INTRODUCTION

Cytomegalovirus (CMV) is a ubiquitous beta-herpesvirus, characterized by its ability to latently persist in the host (Pass, 2001) and mount extremely powerful immune response (Sylwester et al., 2005). Several reports have shown an association of latent CMV infection and parameters associated with immune aging. CMV infection is associated with increased numbers of CD28- or CD45RA+ T-cells in both CD4 and CD8 subsets (Looney et al., 1999) in a manner that recapitulates the aging-related accumulation of these subsets (Chidrawar et al., 2009). CMV is also associated with functional immune deficits in the aging host, including lower counts of Epstein Barr Virus (EBV) specific CD8 T-cells (Khan et al., 2004), increased reactivation of varicella-zoster virus (Ogunjimi et al., 2013) or excess mortality (Roberts et al., 2010; Wikby et al., 2002), mainly due to vascular causes (Roberts et al., 2010; Savva et al., 2013). Humoral immunity is also affected by aging, because the ability to mount robust humoral immunity upon infection or vaccination diminishes in aging hosts (Hakim and Gress, 2007; Lazuardi et al., 2005). CMV seroposositive hosts showed weaker antibody responses to influenza in some (Moro-Garcia et al., 2012; Trzonkowski et al., 2003), but

not all studies (den Elzen et al., 2011), and this weak response correlated to an increase of terminally differentiated CD4 T-cells (Derhovanessian et al., 2013), arguing that CMV may impair both the cellular and the humoral branch of the adaptive immune system.

Several studies in the mouse model of CMV infection addressed the causative role of CMV infection in the onset of immunosenescence. It has been shown that experimental mouse CMV (MCMV) infection causes a drastic increase in the size of the effector-memory (EM) CD8 pool (Cicin-Sain et al., 2012; Mekker et al., 2012) in a manner that may be reversed by long-term antiviral therapy (Beswick et al., 2013), and that CD8 T-cells of MCMV-infected mice poorly respond to challenge with influenza, West-Nile (Cicin-Sain et al., 2012), or vaccinia virus (Mekker et al., 2012). Others have shown diminished clearance of Listeria monocytogenes upon challenge of aged mice latently infected with MCMV, and an increase in all-cause mortality in mice carrying latent MCMV or Herpes-Simplex type I virus (HSV-1), but no effect on the CD8 responses (Smithey et al., 2012). Since the mice used in these studies were genetically identical and housed in identical conditions, the differences between the groups had to be attributed to the MCMV infection.

However, it remained unclear if MCMV infection affects exclusively the CD8 compartment or may also alters the homeostasis and the function of other lymphocytes subsets. Furthermore, only the study by Smithey et al. considered a double infection with MCMV and HSV-1, while other ones focused on mice that were housed in a very clean environment and infected exclusively with MCMV, although most of the people that are latent carriers of MCMV will be exposed to a microbiologically diverse

environment and thus simultaneously carry latent gamma-herpesviruses (e.g. EBV) and/or alpha-herpesviruses (e.g. HSV-1, etc.). Therefore, it remained unclear whether the effects of a latent CMV infection on the immune system are specific for this herpesvirus, and if multiple and/or gamma-herpesviral infections may have additive effects on the immune system. We show here that infection with the beta herpesvirus MCMV, but not with the gamma herpesvirus MHV-68, or the alpha-herpesvirus HSV-1, delays the antibody class-switch upon a challenge with vesicular stomatitis virus (VSV). Furthermore, herpesviral infections did not alter considerably the size of the blood memory B-cell compartment. Finally, we show that multiple herpesvirus infections increase the size of the EM subset of the CD4 T-cell lineage, but that CD4 T-cell responses to VSV challenge are not impaired by any herpesvirus infection, arguing that the delayed antibody class-switch is unlikely a result of defects in T-cell help.

2. MATERIALS AND METHODS

2.1. Viruses

The bacterial artificial chromosome derived molecular clone of MCMV (Jordan et al., 2011) was propagated on M2-10B4 cells (CRL-1972) and quantified by plaque assay on primary mouse embryonic fibroblasts (MEF) as described previously (Ebermann et al., 2012). The MHV-68 was kindly provided by Dr. H. Adler (Helmholtz Zentrum München), and the virus stock was produced on NIH3T3 cells (CRL-1658) and quantified on MEF as previously described (Adler et al., 2001). HSV-1 strain 17, and VACV were obtained from Dr. J. Nikolich-Zugich University of Arizona, Tucson and grown and quantified on Vero cells (CCL-81) as described (Lang et al., 2008; Lang and Nikolich-Zugich, 2005;

Rudd et al., 2010). The Influenza virus PR8M which is closely related to the Mount Sinai strain of A/PR/8/34 (H1N1) was obtained from Dr. K. Schughart, Helmholtz Centre for Infection Research, and was propagated in the chorio-allantoic cavity of ten days old embryonated chicken eggs for 48h at 37^0C (Blazejewska et al., 2011). The vesicular stomatitis virus (VSV) Indiana strain (Stirnweiss et al., 2010) was propagated in Vero B4 cells. Titers were determined by plaques assays on Vero B4 cells.

2.2. Mice

Male DBA/2xC57BL/6 (D2B6) F1 mice were purchased from Janvier (Le Genest, France), 129S2/SvPasCrlxBALB/cAnNCrl F1 (129B) mice were purchased from Charles River (Sulzfeld, Germany). Mice were housed in specific pathogen free (SPF) conditions at the Helmholtz Centre for Infection Research animal facility. Animal experiments were done according to German federal and local state animal welfare guidelines, in line with FELASA and GV-SOLAS guidelines, and were approved by the Lower Saxony State Office of Consumer Protection and Food safety (permit number 33.9-42502-04-11/0426).

2.3. Infection protocols

2.3.1. Infection of D2B6 mice

Six month old D2B6 mice were infected intraperitoneally (i.p.) with $2x10^5$ plaque forming units (PFU) of MCMV, 10^6 PFU of MHV-68, 10^6 PFU of HSV-1, 10^6 PFU of VACV, or 200µl of PBS (mock infected group). Mice which received a combination of HSV-1, MHV-68 and MCMV were injected at two week intervals to avoid virus pathogenicity during primary infection (triple infection group). Nine month later, mice were challenged

with 10^7 PFU of VSV by intranasal (i.n.) application of 20µl of virus suspension. Three independent experiments of five mice per condition were performed and the results from the surviving mice were pooled.

2.3.2. Infection of 129B mice

Age matched 129B mice were housed in the same room and infected at the ages of 3, 9, 15 or 18 months with various herpesviruses or their combinations. For each time point we used two independent cohorts of mice (males or females), injected i.p. with MCMV ($2x10^5$ PFU), MHV-68 (10^6 PFU), rVACV (10^6 PFU), 200µl of PBS or i.n infected with Flu ($2x10^4$ PFU). No mice were infected with HSV-1, because this mouse strain is highly sensitive to this virus (data not shown). Groups of 9, 15 or 18 months old mice received a combination of MHV-68 and MCMV at two weeks intervals. All the mice were challenged with 10^7 PFU VSV i.n. approximately at the same age (18 months). Mice infected at 3, 9 or 15 months of age were pooled into the latent cohort, to reflect the situation in aging CMV seropositive people, where primary infection likely occurred at different ages. Mice infected with herpesviruses at 18 months of age were used as our primary infection cohort.

2.4. In vitro neutralization assay (IgM and IgG concentration)

Serum was collected at 4, 7 or 14 days post VSV challenge, as indicated in specific experiments. Quantification of VSV specific IgM and IgG responses were done as described previously (Charan and Zinkernagel, 1986).

2.5. Antigen stimulation of CD4 T cells

Mice were euthanized by CO_2 asphyxia and lymph nodes (mediastinal and inguinal) were harvested, homogenized on 70µm-pore-size cell strainers (BD) and washed. Antigenic stimulation was performed with lysates of BHK-21 cells infected with VSV (VSV-lysate) or, as negative control, with lysates of mock-infected BHK-21 cells (mock-lysate). Cells were stimulated with VSV-lysate or mock-lysate in presence of co-stimulatory antibodies CD49d (clone 37.51; eBioscience) and CD28 (clone R1-2; eBioscience). Stimulation was performed in 100µl of RPMI in U-bottom 96-wells plates at 37^0C for one hour, which was followed by five additional hours of stimulation in the presence of 10µg/ml Brefeldin A (BFA).

2.6. Immunofluorescence staining and flow cytometric analysis

Surface or intracellular cytokine staining and sample acquisition in an LSRII cytometer were done essentially as described (Cicin-Sain et al., 2012), with the following modificiations: We used anti- CD3-APC-eFluor 780 (clone 17A2; eBioscience), αCD8-PerCp-Cy5.5 (clone 53-6.7; Biolegend), CD4-Pacific blue (clone GK1.5; Biolegend), CD19-FITC (clone 6D5; Biolegend), CD80-Biotin (clone 16-10A1; Biolegend), IgM-APC (clone RMM-1; Biolegend), CD62L-eFluor 605NC (clone MEL-14; eBioscience) and CD44-Alexa fluor 700 (clone IM7; Biolegend). In case of biotinylated antibody (CD80), secondary staining was performed with streptavidin-brilliant Violet 570 (Biolegend).

2.7. Data analysis

FACSDiva software was used for acquisition of flowcytometry samples (BD, San Jose, USA), and Flowjo software (Treestar, Ashland, USA) was used for data analysis.

Samples acquired by the Accuri cytometer were analyzed with CFlow software, version 1.0.227.4 (Accuri cytometers,Inc., Michigan, USA). Statistical analysis was performed using Graphpad prism, version 5.04 (GraphPad software, Inc., California, USA). To accommodate for the small animal group size, statistical analysis was done by non-parametrical Kruskal-Wallis analysis, and to set a penalty for the number of compared groups, we performed Dunns post-analysis. Therefore, this strategy is restrictive by design and may underestimate significant differences in small samples with low statistical power, but increases the chance that the detected differences are highly significant.

3. RESULTS

3.1. Reduced VSV neutralizing titer in latently infected mice

To assess the effect of latent herpesviral infections on the adaptive humoral response, we infected adult mice with MCMV, MHV-68 or both viruses in combination. To exclude effects of host genetics on the experimental readouts we used genetically identical mice in our experiments. The host genetic backgrounds were standardized by using F1 progeny of two commonly used inbred strains (BALB/c×129Sv). This allowed us to use genetically identical hosts, while diminishing the chance that the phenotypes were restricted to hosts with recessive genetic traits, such as those that are prevalent in inbred mouse strains. Control mice were infected with influenza (Flu) or mock-infected. Mice were housed for a minimum of three months, allowing them to establish latency, prior to a challenge with VSV at the age of 18 months. The humoral immune response was assessed by means of an *in vitro* neutralization assay at day 7 post challenge (Fig.

1A). A specific humoral immune response to VSV infection was detectable in all mice. However, we observed significantly reduced titers in mice that were infected with a combination of MCMV and MHV-68 (Fig. 1A). The immune control of VSV infection requires immunoglobulin class-switch (Bachmann and Zinkernagel, 1997). Therefore, we assumed that a more direct measure of a successful immune response would be to analyze the neutralizing capacity of the IgG serum fraction. We pretreated sera with β-mercaptoethanol, thus disrupting the IgM antibody fraction, and used the treated sera in the VSV neutralization assay. The neutralization capacity of the IgG fraction was significantly reduced in mice carrying latent MCMV, alone, or in combination with MHV-68 (Fig 1B, see also supplementary table 1). To understand if this effect was specific for latent infection, or may also be observed during the primary infection, 18 months old mice were infected with the same viruses as in the previous panels and challenged with VSV two weeks later. Seven days upon VSV challenge, whole sera (Fig. 1C) or their IgG fractions (Fig 1D, see also supplementary table 2) were used in neutralization assays, but no significant difference could be observed in any infectious group. Therefore we concluded that latent, but not lytic herpesvirus infection, and in particular MCMV infection, resulted in significant reduction of the neutralizing IgG titer upon a challenge with an emerging viral pathogen.

3.2. Herpesvirus infections do not impair initial B-cell responses to VSV

We considered that the reduced neutralizing titer may reflect a poor initial B-cell response to VSV challenge. Therefore, we assayed the neutralizing capacity of the sera from latently infected mice at 4 days post VSV challenge. We observed no detectable neutralization in the IgG fraction (Fig 2A), which was consistent with previous reports

(Bach et al., 2007), and likely a reflection of the time required for B-cell class-switch upon primary VSV infection. More importantly, we observed no significant difference in the neutralizing capacity of the total Ig fraction between the various groups (Fig 2B), which indicated that the difference in the neutralizing capacity of the IgG fraction at day 7 (Fig 1B) was not due to a delay in the induction of IgM responses, but rather a delayed B-cell class-switch in MCMV infected mice.

3.3. MCMV infection causes a delayed Ig class switch to IgG

To understand if the delayed class switch was limited to BALB/c×129Sv F1 mice, or a phenomenon that may be observed in other mouse strains as well, we performed similar VSV challenge experiments on latently infected $C57BL/_6$×$DBA/_2$ F1 mice. Since these mice control HSV-1 infection much more efficiently than the $BALB/_c$×129Sv F1 (data not shown), we expanded our analysis to mice latently infected with HSV-1, and those triple-infected with MCMV, MHV-68 and HSV-1 in combination. Control groups were infected with vaccinia virus (VACV), or mock-infected by PBS injection at the time of the herpesviral infections. Adult mice were infected with the herpesviruses at 6 months of age and challenged with VSV at 15 months of age to assess the effect of latent herpesviral infections in aging but not senescent mice. We observed no significant difference in the VSV-neutralizing capacity in the total Ig fraction (Fig 3A). On the other hand, in line with data from BALB/c×129Sv F1 mice, we observed a significant reduction of VSV neutralizing titers in the IgG fraction of mice latently infected with MCMV (Fig 3B see also supplementary table 2), arguing that the delayed class switch is a general phenomenon in mice. The poor neutralizing titer in mice with latent MCMV infection at day 7 post VSV challenge could have reflected a long-term deficit or a

transient delay in the ability of B-cells to switch their class. To differentiate between these scenarios, we analyzed the neutralizing capacity at 14 days post VSV challenge. We noticed that the neutralizing capacity of the IgG fraction increased to the levels seen in the total serum Ig, but also that herpesviral infections did not affect the neutralizing capacity of the serum (Fig. 3C) or the IgG fraction of the serum (Fig. 3D) at this time point. Therefore, our results indicated that the latent MCMV infection delays the IgG class switch upon VSV challenge.

3.4. Effects of latent infections on the peripheral memory B-cell CD4 T-cell pools.

We recently showed that MCMV induces poor CD8 T-cell response to challenges with emerging viral infections and that this poor response correlates to the frequency of the effector-memory CD8 T-cells (Cicin-Sain et al., 2012). Latent MCMV infection induces a relative and absolute increase of the CD8 compartment (Cicin-Sain et al., 2012), and thus a decrease in the relative, but not absolute, count of B-cells in the blood lymphocyte compartment [data not shown and (Cicin-Sain et al., 2012)]. Furtermore, Frasca and colleagues have shown that aging alters the composition of the peripheral B-cell compartment, both in rodents and humans (Frasca et al., 2008; Frasca et al., 2004), and in particular results in reduced counts of memory B-cells in the blood (Frasca et al., 2008). However, none of the herpesviral infections induced similar changes in the peripheral B-cell compartment. Hence, we surmised that the reason for the poor humoral response to VSV challenge may have reflected a change in the frequencies of memory B cells. To investigate this possibility, we analyzed the blood of mice infected with MCMV, MHV-68, HSV-1, these viruses in combination, or VACV, and

compared their frequency of memory B-cells to that of mock-infected mice at 8 months post infection, when herpesviruses are latent. Memory B-cells are divided into conventional memory B cells (IgM^-CD80^+) and IgM memory B cells (IgM^+CD80^+) (Dogan et al., 2009; Pape et al., 2011; Tomayko et al., 2010). IgM memory B-cells persist long after the clearance of an infection, whereas the conventional memory cells are described as the frontline responders to infections (Good-Jacobson and Tarlinton, 2012). Hence, we analyzed both subsets in our mice by flow cytometry (Fig. 4A). There were no differences in the IgM fraction (Fig. 4B) and a modest, but statistically significant, increase in the percentage of the conventional memory B-cells in mice infected with MHV-68, but not MCMV or HSV-1 (Fig. 4C).Since MHV-68 infection did not affect the functional B-cell responses to VSV challenge and increased the average fraction of the conventional memory B-cell pool from 2.5 to merely 3 percent this change could not explain the effects on the function of the naïve B-cell compartment responding to the VSV challenge. We considered the possibility that the delay in class switch was not a result of differences in the B-cell compartment, but rather changes in the CD4 T-cell compartment. Since CMV induces a notable and irreversible expansion of the CD8 effector-memory (EM) pool, characterized by the surface expression of CD44 in absence of the CD62L marker, we analyzed the CD4 compartment for $CD44^+CD62L^-$ EM CD4 T-cells (Fig. 4D). While we observed no significant increase of this subset in any group infected with a single herpesvirus, we noticed a significant increase, resulting in the doubling of the EM pool, in mice infected with MCMV, MHV-68 and HSV-1 in combination (Fig. 4E).

Taken together, our data argued that long-term herpesvirus infection may have not strongly affected the peripheral memory B cell subsets, but also that multiple infection with three different herpesviruses resulted in an increase in the frequency of the effector memory CD4 T cells subset.

3.5. Herpesviruses do not affect CD4 T cells in the lymph nodes

Since our data argued that latent herpesviral infections affect the homeostasis of the CD4 compartment, we reasoned that the low VSV specific IgG titer in the MCMV infected group might be caused by a defect in the CD4 T-cell compartment, which impaired the ability of these cells to support the class-switch of B-cells. Hence, we tested the functional capacity of CD4 T-cells upon VSV challenge. At seven days post VSV challenge, we sacrificed mice and isolated lymphocytes from homogenates of the mediastinal (draining) and inguinal (non-draining) lymph nodes. Cells were stimulated with lysates of VSV-infected BHK-21 cells or of uninfected cells (mock-lysate), and the fraction of CD4 T cells responding to the VSV lysate by secreting cytokines was determined by intracellular cytokine staining. We assayed the cells for IFNγ, IL2, IL4, IL17 and TNFα response, but only TNFα showed robust responses (Fig. 5A and data not shown). An obvious difference in TNFα responses between draining and non-draining lymph nodes was observed (Fig. 5B), demonstrating that only cells in the draining lymph nodes could be activated by VSV antigen. The fraction of VSV specific CD4 T cells was essentially identical in the latently infected mice and in the control groups (Fig.5B). Absolute counts of responding cells corroborated this finding (Fig. 5C). Taken together, our data argued that CD4 T-cell responses to VSV challenge were not

affected by herpesvirus infections and hence not a cause of the low IgG titer in the serum of MCMV infected mice.

1. DISCUSSION

Several studies have postulated an association between CMV infection and poor humoral response to vaccination in the aging host (Derhovanessian et al., 2013; Trzonkowski et al., 2003). The cause-effect relationship between these phenomena could not be addressed in clinical studies, and it remained unclear whether CMV caused the poor humoral response, or if individuals with a propensity towards weak humoral responses (e.g. due to their genetic background) are simultaneously more susceptible to CMV infections. We showed here that genetically identical mice that were age and gender matched and housed in identical conditions showed a delayed class switch if they were latently infected with MCMV, while no changes were observed upon infection with other herpesviruses. These data strongly argue that CMV was the critical cause of the observed deficit in the humoral response.

We observed a two- to four-fold reduction of the neutralizing titer against VSV in latently MCMV infected mice, but the results were highly conserved in mice of different genetic backgrounds. Intriguingly, multiple herpesviral infections resulted in significant reduction of neutralizing antibody titers in the 129SvxBALB/c, but fell short of statistical significance in the DBA/2xC57BL/6 mice. It remains unclear whether this was due to low statistical power in the latter experiment, to a difference between the murine genetic backgrounds, or to the inclusion of HSV-1 in only one of the infection protocols. On the

other hand, CMV infection caused a moderate, but highly consistent IgG phenotype in all experimental settings. Since IgG class-switch is critical in mediating immune protection against some pathogens (Charan and Zinkernagel, 1986), its delay may warrant the pathogen a window of opportunity to spread and cause more sever disease. While our study was not designed to address the immune protection aspect, future studies in the mouse model may allow us to define whether CMV infection impairs the clearance and the disease symptoms upon challenge with emerging viruses.

People exhibiting poor humoral reponses to influenza vaccination were CMV-seropositive hosts with elevated frequencies of late-differentiated cells in their CD4 compartment (Derhovanessian et al., 2013). Since MCMV delayed the antibody class-switch, and was shown to alter the balance of subsets in the CD8 (Mekker et al., 2012), but also in the CD4 compartment when combined with MHV-68 and HSV-1 (Fig. 4E), we initially surmised that the delay in class-switch may be caused by a defect in CD4 responses. Follicular CD4 T cells in the lymphoid tissues mediate humoral immune response by their interaction with the B cells (Fazilleau et al., 2009; King et al., 2008). The aging process causes the decline in ability of follicular CD4 T cells in the germinal center to provide a cognate T-cell help and subsequent reduced ability of B cells to undergo class-switch recombination, the process which is crucial for an effective and robust humoral response (Cancro et al., 2009). However, VSV specific CD4 responses were maintained in all herpesvirus infected groups (Fig. 5), and this finding argues against a CD4-mediated effect. It remains unclear if the effect of CMV infection on Ig class-switch is truly CD4 independent, or if CMV induces a subtle change in the function of CD4 T-cells, which could not be measured by our assay. Alternatively, CMV may

impair B-cell responses by disrupting the lymph-node architecture and thus the signaling network that is necessary for the germinal center reaction, or by direct effects on the B-cell compartment, for instance by dysregulating TNFα signaling (Frasca et al., 2012) or by affecting E47 or activation-induced cytidine deaminase, similarly to effects observed in aging mice (Frasca et al., 2008; Frasca et al., 2004). These important questions may be addressed in future experiments.

2. CONCLUSIONS

In conclusion, we showed here in a controlled experimental setting that latent MCMV infection, but not infection with an alpha, or a gamma-herpesvirus, causes a delayed antibody class-switch upon VSV challenge. This suggests that poor response to vaccination in CMV-seropositive hosts could be a direct consequence of CMV infection. Furthermore, we showed that multiple herpesviral infections result in an accumulation of EM CD4 T-cells, but that this does not occur in isolated infections, nor it leads to impaired CD4 T-cell responses to VSV challenge. Hence, our results imply that delayed class-switch in CMV infection is unlikely a result of poor T-cell help.

3. ACKNOWLEDGMENTS

We wish to thank Ilona Paprotta, Ayse Barut and Oliver Hartmann for expert technical assistance. This study was funded by the Helmholtz Association Award NG-VH-638 and the ERC starting grant 260934 to L. C-S and a fellowship by the Dar-es-Salaam University College of Education to T.F.M. The funding bodies had no role in the design or execution of the study.

REFERENCES

Pass, R.F., 2001. Cytomegalovirus, in: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th Ed. ed. Lippincott/The Williams and Wilkins Co., Philadelphia, PA, pp. 2675-2705.

Sylwester, A.W., Mitchell, B.L., Edgar, J.B., Taormina, C., Pelte, C., Ruchti, F., Sleath, P.R., Grabstein, K.H., Hosken, N.A., Kern, F., Nelson, J.A., Picker, L.J., 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med 202, 673-85.

Looney, R.J., Falsey, A., Campbell, D., Torres, A., Kolassa, J., Brower, C., McCann, R., Menegus, M., McCormick, K., Frampton, M., Hall, W., Abraham, G.N., 1999. Role of cytomegalovirus in the T cell changes seen in elderly individuals. Clin Immunol 90, 213-9.

Chidrawar, S., Khan, N., Wei, W., McLarnon, A., Smith, N., Nayak, L., Moss, P., 2009. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. Clin Exp Immunol 155, 423-32.

Khan, N., Hislop, A., Gudgeon, N., Cobbold, M., Khanna, R., Nayak, L., Rickinson, A.B., Moss, P.A., 2004. Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. J Immunol 173, 7481-9.

Ogunjimi, B., Theeten, H., Hens, N., Beutels, P., 2013. Serology indicates cytomegalovirus infection is associated with varicella-zoster virus reactivation. J Med Virol.

Roberts, E.T., Haan, M.N., Dowd, J.B., Aiello, A.E., 2010. Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up. Am J Epidemiol 172, 363-71.

Wikby, A., Johansson, B., Olsson, J., Lofgren, S., Nilsson, B.O., Ferguson, F., 2002. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. Exp Gerontol 37, 445-53.

Savva, G.M., Pachnio, A., Kaul, B., Morgan, K., Huppert, F.A., Brayne, C., Moss, P.A., 2013. Cytomegalovirus infection is associated with increased mortality in the older population. Aging Cell 12, 381-7.

Hakim, F.T., Gress, R.E., 2007. Immunosenescence: deficits in adaptive immunity in the elderly. Tissue Antigens 70, 179-89.

Lazuardi, L., Jenewein, B., Wolf, A.M., Pfister, G., Tzankov, A., Grubeck-Loebenstein, B., 2005. Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. Immunology 114, 37-43.

Moro-Garcia, M.A., Alonso-Arias, R., Lopez-Vazquez, A., Suarez-Garcia, F.M., Solano-Jaurrieta, J.J., Baltar, J., Lopez-Larrea, C., 2012. Relationship between functional ability in older people, immune system status, and intensity of response to CMV. Age (Dordr) 34, 479-95.

Trzonkowski, P., Mysliwska, J., Szmit, E., Wieckiewicz, J., Lukaszuk, K., Brydak, L.B., Machala, M., Mysliwski, A., 2003. Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence. Vaccine 21, 3826-36.

den Elzen, W.P., Vossen, A.C., Cools, H.J., Westendorp, R.G., Kroes, A.C., Gussekloo, J., 2011. Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities. Vaccine 29, 4869-74.

Derhovanessian, E., Theeten, H., Hahnel, K., Van Damme, P., Cools, N., Pawelec, G., 2013. Cytomegalovirus-associated accumulation of late-differentiated CD4 T-cells correlates with poor humoral response to influenza vaccination. Vaccine 31, 685-90.

Cicin-Sain, L., Brien, J.D., Uhrlaub, J.L., Drabig, A., Marandu, T.F., Nikolich-Zugich, J., 2012. Cytomegalovirus infection impairs immune responses and accentuates T-cell pool changes observed in mice with aging. PLoS Pathog 8, e1002849.
Mekker, A., Tchang, V.S., Haeberli, L., Oxenius, A., Trkola, A., Karrer, U., 2012. Immune senescence: relative contributions of age and cytomegalovirus infection. PLoS Pathog 8, e1002850.
Beswick, M., Pachnio, A., Lauder, S.N., Sweet, C., Moss, P.A., 2013. Antiviral therapy can reverse the development of immune senescence in elderly mice with latent cytomegalovirus infection. J Virol 87, 779-89.
Smithey, M.J., Li, G., Venturi, V., Davenport, M.P., Nikolich-Zugich, J., 2012. Lifelong persistent viral infection alters the naive T cell pool, impairing CD8 T cell immunity in late life. J Immunol 189, 5356-66.
Jordan, S., Krause, J., Prager, A., Mitrovic, M., Jonjic, S., Koszinowski, U.H., Adler, B., 2011. Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced growth in salivary Glands due to a fixed mutation of MCK-2. J Virol 85, 10346-53.
Ebermann, L., Ruzsics, Z., Guzman, C.A., van Rooijen, N., Casalegno-Garduno, R., Koszinowski, U., Cicin-Sain, L., 2012. Block of death-receptor apoptosis protects mouse cytomegalovirus from macrophages and is a determinant of virulence in immunodeficient hosts. PLoS Pathog 8, e1003062.
Adler, H., Messerle, M., Koszinowski, U.H., 2001. Virus reconstituted from infectious bacterial artificial chromosome (BAC)-cloned murine gammaherpesvirus 68 acquires wild-type properties in vivo only after excision of BAC vector sequences. J Virol 75, 5692-6.
Lang, A., Brien, J.D., Messaoudi, I., Nikolich-Zugich, J., 2008. Age-related dysregulation of CD8+ T cell memory specific for a persistent virus is independent of viral replication. J Immunol 180, 4848-57.
Lang, A., Nikolich-Zugich, J., 2005. Development and migration of protective CD8+ T cells into the nervous system following ocular herpes simplex virus-1 infection. J Immunol 174, 2919-25.
Rudd, B.D., Venturi, V., Smithey, M.J., Way, S.S., Davenport, M.P., Nikolich-Zugich, J., 2010. Diversity of the CD8+ T cell repertoire elicited against an immunodominant epitope does not depend on the context of infection. J Immunol 184, 2958-65.
Blazejewska, P., Koscinski, L., Viegas, N., Anhlan, D., Ludwig, S., Schughart, K., 2011. Pathogenicity of different PR8 influenza A virus variants in mice is determined by both viral and host factors. Virology 412, 36-45.
Stirnweiss, A., Ksienzyk, A., Klages, K., Rand, U., Grashoff, M., Hauser, H., Kroger, A., 2010. IFN regulatory factor-1 bypasses IFN-mediated antiviral effects through viperin gene induction. J Immunol 184, 5179-85.
Charan, S., Zinkernagel, R.M., 1986. Antibody mediated suppression of secondary IgM response in nude mice against vesicular stomatitis virus. J Immunol 136, 3057-61.
Bachmann, M.F., Zinkernagel, R.M., 1997. Neutralizing Antiviral B Cell Responses. Annu Rev Immunol 15, 235-270.
Bach, P., Kamphuis, E., Odermatt, B., Sutter, G., Buchholz, C.J., Kalinke, U., 2007. Vesicular stomatitis virus glycoprotein displaying retrovirus-like particles induce a type I IFN receptor-dependent switch to neutralizing IgG antibodies. J Immunol 178, 5839-47.
Frasca, D., Landin, A.M., Lechner, S.C., Ryan, J.G., Schwartz, R., Riley, R.L., Blomberg, B.B., 2008. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. J Immunol 180, 5283-90.
Frasca, D., Van der Put, E., Riley, R.L., Blomberg, B.B., 2004. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. J Immunol 172, 2155-62.
Dogan, I., Bertocci, B., Vilmont, V., Delbos, F., Megret, J., Storck, S., Reynaud, C.A., Weill, J.C., 2009. Multiple layers of B cell memory with different effector functions. Nat Immunol 10, 1292-9.
Pape, K.A., Taylor, J.J., Maul, R.W., Gearhart, P.J., Jenkins, M.K., 2011. Different B cell populations mediate early and late memory during an endogenous immune response. Science 331, 1203-7.

Tomayko, M.M., Steinel, N.C., Anderson, S.M., Shlomchik, M.J., 2010. Cutting edge: Hierarchy of maturity of murine memory B cell subsets. J Immunol 185, 7146-50.
Good-Jacobson, K.L., Tarlinton, D.M., 2012. Multiple routes to B-cell memory. Int Immunol 24, 403-8.
Fazilleau, N., Mark, L., McHeyzer-Williams, L.J., McHeyzer-Williams, M.G., 2009. Follicular helper T cells: lineage and location. Immunity 30, 324-35.
King, C., Tangye, S.G., Mackay, C.R., 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol 26, 741-66.
Cancro, M.P., Hao, Y., Scholz, J.L., Riley, R.L., Frasca, D., Dunn-Walters, D.K., Blomberg, B.B., 2009. B cells and aging: molecules and mechanisms. Trends Immunol 30, 313-8.
Frasca, D., Romero, M., Diaz, A., Alter-Wolf, S., Ratliff, M., Landin, A.M., Riley, R.L., Blomberg, B.B., 2012. A molecular mechanism for TNF-alpha-mediated downregulation of B cell responses. J Immunol 188, 279-86.

FIGURE CAPTIONS

Figure 1: **Low VSV-specific Ig titer upon latent MCMV infection at 7 days post challenge.**

129B mice were infected with MCMV, MHV-68, their combination, flu or mock infected. Mice infected for 3+ months (latent infection, panels A&B) or 14 days (primary infection, panels C&D) were challenged with VSV at 18 months of age and sera were collected at day 7 post challenge to determine VSV specific Ig response. Antilog titers of total immunoglobulin (left panels) or the IgG fraction (right panels) was compared between virus-infected groups and the mock control group. Statistical analysis was performed by the Kruskal-Wallis test followed by Dunns post analysis and groups with a $p < 0.05$ are indicated by asterisks. Each triangle represents a mouse, horizontal lines are group medians. The data shown here represents pooled data from at least two independent experiments.

Figure 2. **VSV-specific Ig titers in the serum at day 4 post challenge.**

129B mice were infected with MCMV, MHV-68, their combination, flu or mock for at least three months prior to VSV challenge at 18 months of age. Serum was collected at day 4 post challenge and VSV neutralizing IgG immunoglobulin fraction (A) or total serum Ig titer (B) were compared between virus-infected groups and the mock control group. Statistical analysis was performed by the Kruskal-Wallis test followed by Dunns

post analysis. Each triangle represents a mouse, horizontal lines are group medians. The data shown here represents pooled data from two independent experiments

Figure 3. **MCMV infection results in a delayed VSV specific Ig class switch to IgG.**

D2B6 mice were primed with MCMV, MHV-68, HSV-1, a combination of the three herpesviruses, VACV or mock. Nine months post infection, at 15 months of age, mice were challenged with VSV. Total Ig and IgG response was determined by i*n vitro* neutralization assay at 7 (A&B) or 14 (C&D) days post VSV challenge. Total immunoglobulin titer (left panels) or the IgG fraction (right panels) was compared between virus-infected groups and the mock control group. Data were pooled from three independent experiments. Each triangle represents a mouse, the lines are group medians, and the group with a $p < 0.05$ is indicated by an asterisk (Kruskal-Wallis test followed by Dunns post analysis).

Figure 4: **MHV-68 significantly increases the frequency of conventional memory B cells and multiple infection results in an increase of memory CD4 T cells.**

Six months old D2B6 mice were primed with MCMV, MHV-68, HSV-1, combination of the three herpesviruses, VACV or mock. Eight months post priming, blood lymphocytes were isolated and analyzed for their expression of $IgM^{+/-}$ and $CD80^{+}$ on $CD19^{+}$ cells, to define the memory B cell subsets (representative gating of $CD19^{+}$ cells in A), and percentages of cells in indicated gates is represented for each mouse (B and C). Expression of CD44 and CD62L in CD4+CD8- T-cells was analyzed to define the EM CD4 T cell subset (representative gating in D) and percentages of EM in the CD4 T-cell pool are shown (E). In panels B, C and E, each dot represents a mouse and the lines

are group medians. The data in panels A-C represent a pool from two, and in panels D, E from three independent experiments. Comparison was done by using Kruskal-Wallis test followed by Dunns post analysis. The groups with $p < 0.05$ are indicated by asterisks.

Figure 5. **Herpesviruses do not affect CD4 T cell response to VSV in the draining lymph nodes.**

Adult 129B mice were primed with MCMV, MHV-68, their combination, Flu or mock. At 18 months of age, mice were challenged with VSV. Seven days after VSV challenge, CD4 T cells from lymph nodes were stimulated with VSV- or mock-lysate in BFA presence and fractions of cells expressing TNFα are indicated in representative dot blots (A). (B) Percentage and (C) absolute counts of specific CD4 T cells from the draining (mediastinal) or non-draining (inguinal) lymph nodes are shown. Each dot represents a mouse and the lines are group medians (Kruskal-Wallis test followed by Dunns post analysis).