Implications of memory CD8 T cell quality for protective immunity

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CHAPTER 6

Viral persistence induces antibody inflation without altering antibody avidity

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ABSTRACT

Antibodies are implicated in long-term immunity against numerous pathogens and because of this property antibody induction is the basis for many vaccines. Little is known on the influence of viral persistence on the evolving antibody response. Here we examined the characteristics of antibody responses to persistent infection employing the prototypic betaherpesvirus family member cytomegalovirus (CMV) in experimental mouse models. During the course of infection, mouse CMV (MCMV)-specific IgM and IgG responses are elicited but only IgG levels gradually inflate in the persistent phase of infection while IgM levels are stably maintained. Whereas CD27/CD70 interactions are dispensable, the CD28/B7 costimulatory pathway is critical for the class switching of MCMV-specific IgM to IgG B cell responses, which corresponds to the CD28/B7-dependent formation of CD4+ T follicular helper cells (TFH) and germinal center (GC) B cells. Furthermore, the initial viral inoculum dose dictates the height of the antibody levels during IgG antibody inflation, and relates to induction of long-lived plasma cells and memory B cells. Antibody avidity is nonetheless not altered after establishment of viral persistence and occurs independent of the inoculum dosages. However, repetitive challenge with intact viral particles, accompanied by increased GC reactivity, promotes the development of high avidity IgG responses with neutralizing capacity. These insights can be used for the rational design of CMV-based vaccines aimed at inducing antibody responses.

INTRODUCTION

The maintenance of long-lived humoral responses after infection and vaccination is attributed to both long-lived plasma cells that continuously produce antibodies and to memory B cells that are able to form antibody-secreting cells after re-exposure (1, 2). Antibodies can protect against numerous pathogens by direct neutralization and/or by supporting effector functions of immune cells (1, 3). Upon activation, B cells initially excrete antigen-specific IgM antibodies. This is followed by antibody isotype switching and affinity maturation when B cells receive the appropriate signals, including help signals by CD4+ T cells in germinal center (GC) reactions (4). During acute viral infections antibody levels increase followed by gradual decline once the antigen has disappeared. In case of appropriate induction of B cells leading to the generation of long-lived plasma cells, antibody levels eventually become stable and can mediate protection for many years.

Whereas memory B cells have self-renewal capacity in an antigen-dependent manner, long-lived plasma cells are thought to survive for decades (2). In case of antigen persistence, as is the case in chronic infections, one could argue that antigenic boosting effects humoral immunity. How this impacts the kinetics of antibody levels and antibody avidity maturation is, however, largely unknown. Recently, vaccines based on persistent viruses such as cytomegalovirus have shown their value by inducing either long-lasting effector-memory T cell responses (5-7) or protective antibodies (8, 9) but many particulars of such vaccines remain to be determined.

To gain more insight into the determinants of antibody responses that develop during persistent virus infection or after challenge with vaccines based on persistent viruses, we used mouse cytomegalovirus (MCMV), a prototypic member of the betaherpesvirus family. We found that similar to so-called inflationary MCMV-specific T cell responses, which gradually increase to high frequencies (10), MCMV-specific IgG antibody levels inflate in the persistent phase of infection. MCMV-specific IgM antibody levels, however, remain relative stable. Remarkably, this IgG antibody inflation is not accompanied by changes in antibody avidity after a single inoculum despite viral persistence. Instead, antibody avidity was amplified by repetitive challenge with virus, and correlated with elevated GC reactivity. Moreover, we show that operational GC reactions and T follicular helper cell (TFH) formation require the costimulatory CD28/B7 pathway while CD27/CD70 interactions are not critical.

MATERIALS AND METHODS

Mice and infection

C57BL/6 mice were purchased from Charles River. Cd70-/- (11), Cd80/86-/- (12), and Cd70/80/86-/- (13) mice all on a C57BL/6 background, were bred in-house. Mice between

8-12 weeks of age were infected intra-peritoneal (i.p.) with indicated doses of MCMV-Smith obtained from the American Type Culture Collection (Manassas, VA). Stocks were derived from salivary glands of infected BALB/c mice as described elsewhere (14). Viral load in mice was determined by quantitative PCR as described (15) and data is normalized to β-actin. All animals were maintained on specific pathogen free conditions at the animal facility in the Leiden University Medical Center (LUMC). All animal experiments were approved by the Animal Experiments Committee of LUMC (reference numbers: 10227, 12006, 13029) and performed according to the recommendations and guidelines set by LUMC and by the Dutch Experiments on Animals Act that serves the implementation of 'Guidelines on the protection of experimental animals' by the Council of Europe.

In vivo antibody usage

To deplete $CD4^+$ T cells, mice received 150 μg of CD4 depleting antibody (GK1.5) i.p. prior to infection. Depletion of $CD4^+$ T cells was maintained by administration of 100 μg GK1.5 antibody once a week. For blockade of costimulatory interactions during acute MCMV infection, mice received either 150 μg blocking CD70 antibody (clone FR70) or a combination of 200 μg blocking CD80 (B7.1) antibody (clone 16-10A1) and 200 μg blocking CD86 (B7.2) antibody (clone GL1) i.p. on day -1, 0 and 3 of MCMV infection.

Antibody detection by ELISA and antibody avidity assay

Blood of mice was collected retro-orbitally. Upon brief centrifugation, serum was collected and stored at -20°C until further use. MCMV-specific antibody levels were determined by ELISA as described (15) with minor alterations. In short, 96-well plates (Nunc Maxisorp) were coated overnight at 4°C with tissue culture-derived MCMV-Smith in bicarbonate buffer (pH 9.6). Plates were incubated for 1 hour at 37°C with blocking buffer (PBS containing 5% milk powder), followed by subsequent incubation with sera samples (diluted in PBS containing 1% milk powder) for 1 hour at 37°C. Plates were washed with PBS containing 0.05% Tween, after which HRP-conjugated IgM, IgG1 IgG2b, IgG2c, IgG3, IgA and IgE antibodies (diluted in PBS with 1% milk powder) were incubated for 1 hour at 37°C. To develop the plates, 50 μl of TMB (3,3',5,5'-tetramethylbenzidine) (Sigma Aldrich) was added to each well and incubated for 15 minutes at room temperature. To stop the reaction, 50 μl of stop solution (1M H2SO4) was added. Plates were measured within 5 minutes at 450 nm using a microplate reader (Model 680, Bio-Rad). To determine the avidity of the MCMV-specific antibodies, a serum dilution was used at which responses showed an OD Value of 1 at 450 nm. Plates were incubated with increasing concentrations of sodiumthiocyanate (NaSCN) for 15 minutes, followed by washing with PBS containing 0.05% Tween, and incubation with HRP-conjugated antibodies. The avidity of the MCMV-specific antibodies was determined by the ratio of the amount of antibodies bound after elution with different concentrations of NaSCN

relative to the amount of antigen bound in the absence of NaSCN (16).

Quantification of antibody secreting cells

Multiscreen-HA 96-well plates (Millipore) were coated overnight with MCMV in PBS at 4°C, subsequently washed with PBS and blocked for 1 hour with IMDM medium containing 8% FCS at 37°C. 2×10^5 splenocytes or 8×10^5 bone marrow cells of MCMV infected mice were added per well and incubated for 5 hours at 37°C. Plates were washed with PBS containing 0.05% Tween-20, followed by incubation overnight with HRP-conjugated IgG2c antibodies at 4°C. After subsequent washing, spots were visualized using TMB (Mabtech) and reaction was stopped with tap water. The total number of antibody secreting cells per organ was determined by dividing the absolute number of the organ by the amount of plated cells, and multiplying this number with the amount of spots that were counted per well. CMV-specific memory B cells were determined as described (17) with some modifications. In short, a concentration range from 1.8×10^5 to 2×10^4 splenocytes of MCMV infected mice were cultured in a flat bottom 96-well plate for 6 days in the presence of irradiated feeder splenocytes of naive mice (1200 rad), 0.4 μg/ml LPS and 1 μg/ml PHA. After 6 days of culture, cells were washed, transferred to ELISPOT plates and developed as described above.

Antibody neutralization assay

Different dilutions of serum of MCMV infected mice were incubated for 45 minutes with 50 PFU of MCMV-Smith at room temperature. The virus-serum inoculums were subsequently added to monolayers of M2-10B4 cells in 48-well plates. Cells were incubated for 1 hour at 37°C after which inoculums were removed and cells were covered in carboxymethyl cellulose-containing medium. After 5 days of incubation, cells were fixed with 25% formaldehyde and plaques were visualized using crystal violet solution.

Flow cytometry

Splenocytes were obtained by mincing the tissue through a 70 μm nylon cell strainer (BD). Erythrocytes were lysed in a hypotonic ammonium chloride buffer. The antigen-specific T cell response was determined by MHC class I tetramers and intracellular cytokine staining as described (18). T cell restimulation was performed with class I (i.e., $M45_{985–993}$, $m139_{419–426}$, $M38_{316–323}$ and $IE3_{416-423}$ (19)) and class II-restricted peptides ($M25_{409-423}$, $m139_{560-574}$ and $m142_{24-38}$ (20)). Fluorescently conjugated antibodies were purchased by Affymetrix, BD pharmingen or Biolegend. Flow cytometric acquisition was performed on a BD LSR II. Data were analyzed using FlowJo software (TreeStar).

Statistical significance

Mann-Whitney test was used to calculate the significance of viral titers. To evaluate significance between two groups the Student's t-test was used, and for more than two

groups one-way ANOVA was used. Tukey's post-hoc test was performed to correct for multiple comparisons. P-values <0.05 were considered as significant.

RESULTS

MCMV-specific IgG antibodies inflate during persistent infection without altered antibody avidity

We used MCMV as a model to determine antibody characteristics during a persistent virus infection and first examined if MCMV-specific antibodies provide protection upon re-infection. Following acute infection, IgG2b and IgG2c (the IgG2a equivalent in C57BL/6 mice) are the predominant isotypes that are produced (Fig 1A), which is not uncommon after viral infection (21, 22). Serum of naive mice and of latent MCMV-infected mice was transferred to naive mice (200 µl per recipient) that were subsequently challenged with MCMV. A ~10 fold diminished viral load was found in the liver and salivary glands of the mice that received serum containing MCMV-specific antibodies compared to the mice that received naive mouse serum (Fig 1A), indicating that MCMV-specific antibodies have protective capacity even in case of subordinate antibody titer (i.e., transfer of 200 µl of serum results in a much lower antibody titer in the recipient mouse than the donor). Consistent with the diminished viral load, also MCMV-specific

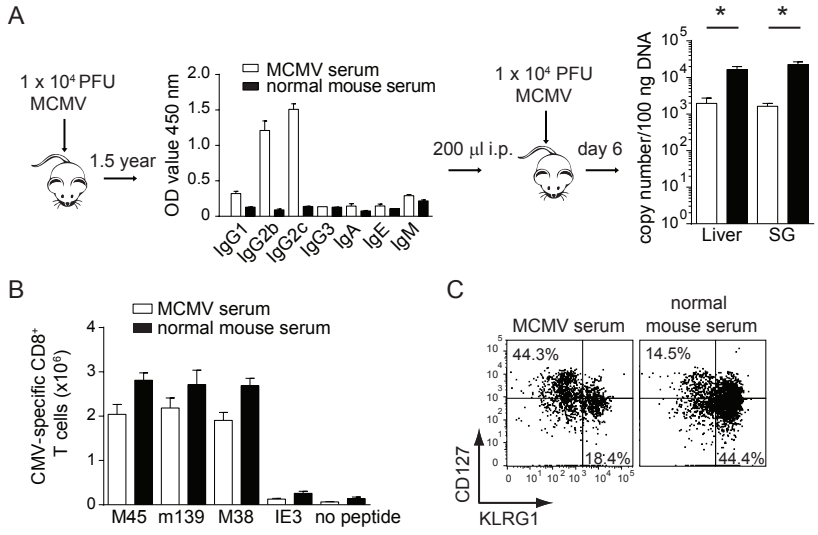

Fig 1. Protective properties of MCMV-specific antibodies. (A) C57BL/6 mice were infected with 1 × 10^4 PFU MCMV. After 1.5 years, immune serum was obtained and transferred to naive mice that were subsequently infected with 1 × 10^4 PFU MCMV. Serum of naive mice was used as control. Six days post serum transfer viral load was determined in the liver and the salivary glands by qPCR. (B) The magnitude of the MCMV-specific CD8+ T cell response was determined in the spleen by intracellular cytokine staining upon restimulation with the indicated MHC class I restricted peptides. (C) The cell surface expression of CD127 and KLRG1 on splenic M45 tetramer+ $CD8^+$ T cells (n=5 mice per group; *P<0.05).

T cell responses were reduced upon MCMV serum transfer and displayed a less activated phenotype as evidence by more CD127^{high} and KLRG1^{low} expression (15) (Fig 1B and 1C). Thus, although sterile immunity is not achieved under the conditions analysed, the antibodies that are generated during MCMV infection can reduce viral titers upon challenge.

Next, we determined the kinetics of MCMV-specific IgM antibodies and the predominant IgG2b and IgG2c isotypes. In the acute phase of infection, high levels of MCMV-specific IgM antibodies were detected in the serum. After acute infection, IgM levels declined but remained clearly detectable throughout the chronic phase of infection (Fig 2A), a phenomenon that is not observed after infection with viruses causing only acute infection (23). Another striking feature we observed was that MCMV-specific IgG2b and IgG2c antibody levels gradually accumulated in time (Fig 2A and 2B). This particular inflation of MCMV-specific IgG2b and IgG2c antibodies is reminiscent to certain inflationary MCMV-specific T cell responses that progressively accumulate in the chronic phase of infection (10).

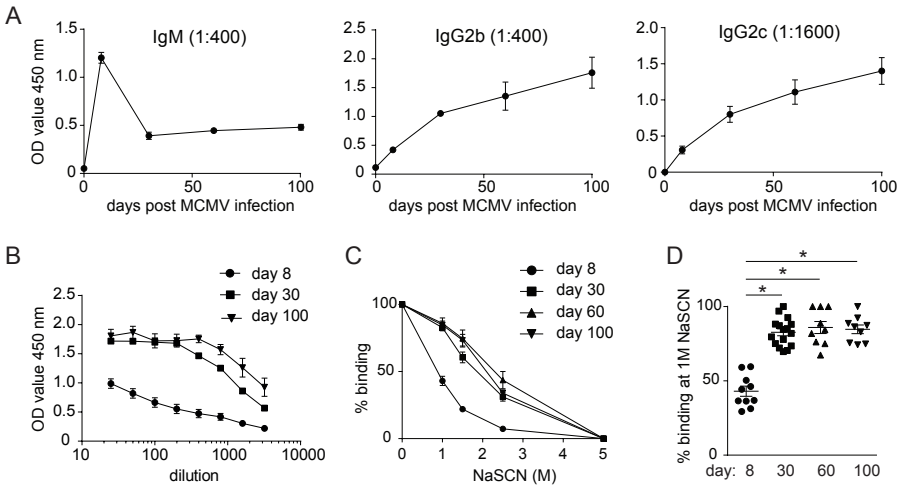

Fig 2. MCMV-specific IgG antibodies inflate during persistent infection while antibody avidity remains stable. (A) Mice were infected with 1 × 10^4 PFU MCMV. The MCMV-specific antibody levels are shown for the indicated serum dilution. (B) MCMV-specific IgG2c antibody titres in sera are depicted as mean ± SEM of day 8, 30 and 100 post-infection. (C) The elution profile of MCMV-specific IgG2c antibodies is shown as mean ± SEM. (D) The percentage of bound antibodies to MCMV in the presence of 1M NaSCN relative to the amount of antigen bound in the absence of NaSCN is shown. Data is pooled of 3 independent experiments (n=4 mice per group; *P<0.05).

To examine if the avidity of these inflating antibodies varies in time, an antibody avidity assay was performed with increasing concentrations of sodium thiocyanate (NaSCN) that accordingly disrupts the antigen-antibody bond. The avidity of MCMV-specific antibodies was relatively low at day 8 post-infection, as ~50% of the antibodies were eluted upon incubation with 1M of NaSCN (Fig 2C and 2D). However, 30 days post-infection

the avidity of MCMV-specific IgG2c antibodies was increased as only 20% of the total MCMV-specific IgG2c antibodies could be eluted with 1M of NaSCN. Throughout the ensuing persistent phase of MCMV infection, the avidity of the MCMV-specific antibodies did not further increase compared to the avidity detected at day 30 post-infection. Similar results were obtained with the IgG2b isotype (data not shown). Thus the levels of the MCMV-specific IgG2b and IgG2c antibodies inflate in chronic MCMV infection, but this is not accompanied by differences in antibody avidity during viral persistence.

MCMV-specific IgG antibody responses are dependent on CD28/B7-driven CD4+ T cell responses

To examine factors that influence antibody inflation and avidity during persistent viral infection, we first aimed to identify the signals that are critical for GC related processes such as isotype switching. In this respect, help signals provided by $CD4^+$ T cells are shown to be crucial for inducing class switching of antibodies. Upon depletion of $CD4^+$ T cells, the acute MCMV-specific IgM responses as well as the maintenance of the IgM levels were not much affected (Fig 3A). IgG2b and IgG2c responses were, however, severely hampered, also at late times post-infection. To identify the molecular interactions that provide the help signal we specifically focused on the role of costimulatory pathways involving the Ig superfamily member CD28 and its ligands B7.1 (CD80) and B7.2 (CD86) (hereafter referred as B7) and the TNFR family member CD27 and its ligand

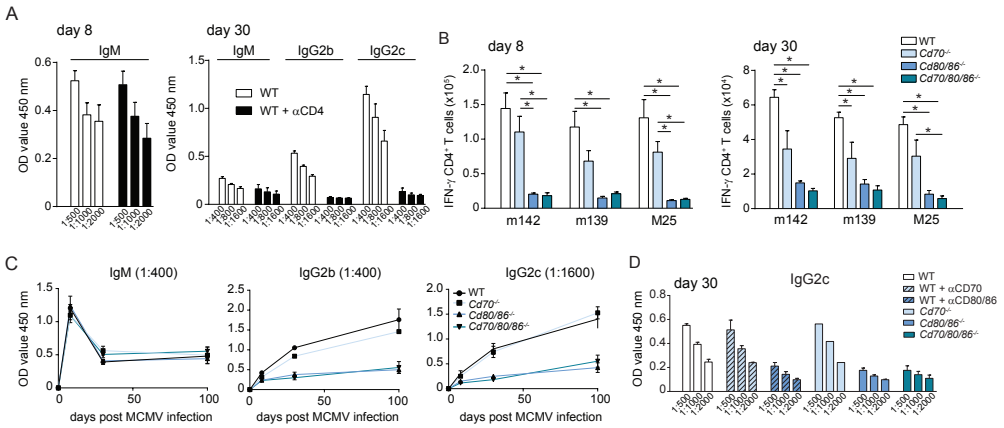

Fig 3. CD4 help and B7-mediated costimulation are critical for development of MCMV-specific IgG antibodies. (A) WT and $CD4^+$ T cell depleted mice were infected with 1×10^4 PFU MCMV and at day 8 and 30 post-infection the MCMV-specific antibody response was determined by ELISA. (B) WT and costimulation deficient (i.e. $Cd70^{-/-}$, $Cd80/86^{-/-}$, $Cd70/80/86^{-/-}$) mice were infected with 1×10^4 PFU MCMV and the MCMV-specific $CD4^+$ T cell response was determined in the spleen 8 and 30 days post-infection by intracellular cytokine staining upon restimulation with indicated MHC class II restricted peptides. (C) The MCMV-specific antibody response in WT and costimulation deficient mice in time is shown. (D) WT mice and costimulation deficient mice were infected with 5×10^4 PFU MCMV. CD70 and B7-mediated interactions were blocked in WT mice from day -1 to day 3 by administration of blocking antibodies. MCMV-specific IgG2c levels were determined in the serum 30 days post-infection. All bar graphs represent mean + SEM. Data is shown of one representative experiment of 3 independent experiments (n=4 mice per group; *P<0.05).

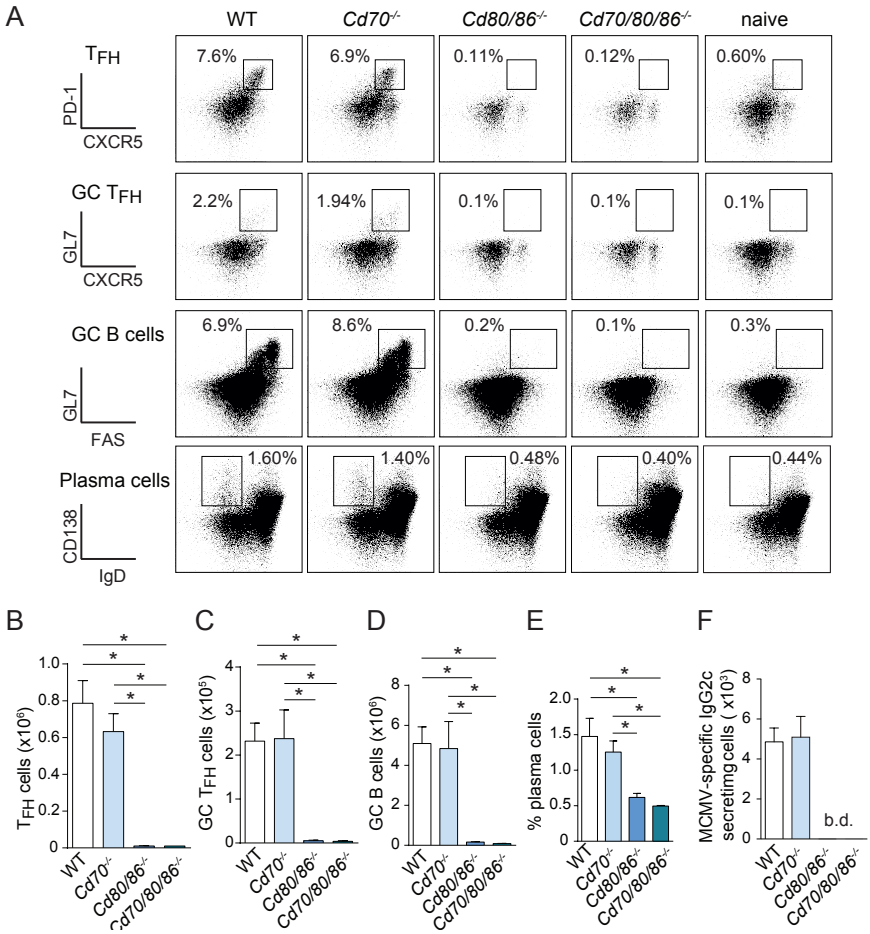

Fig 4. GC reactions in CMV infection are dependent on B7-mediated interactions. WT and costimulation deficient mice were infected with 1 × 10^4 PFU MCMV. (A) Representative flow cytometry plots of splenic cell populations at day 15 post MCMV infection. Depicted are TFH (PD-1$^+$CXCR5$^+$) and GC TFH (GL-7+CXCR5$^+$) gated on CD4$^+$CD62L$^-$ cells, and GC B cells (GL7$^+$FAS$^+$) and plasma cells (CD138$^+$IgD$^+$) gated on B220$^+$/CD19$^+$ cells. Numbers indicate percentages of positive cells within the gated population. (B-D) Total number of TFH, GC TFH, and GC B cells in the spleen at day 15 post-infection. (E) The percentage of plasma cells within the splenic B cell population at day 15 post-infection. (F) The number of MCMV-specific IgG2c secreting cells was determined in the spleen 15 days post infection using ELISPOT. All bar graphs represent mean + SEM. Data is shown of one representative experiment of 3 independent experiments (n=4 mice per group; *P<0.05).

CD70 because Cd80/86$^{-/-}$ and Cd70$^{-/-}$ mice have reduced MCMV-specific CD4$^+$ T cell responses (Fig 3B) (18, 24). No differences were found in the MCMV-specific IgM response during acute and persistent infection in all mice devoid of B7 and/or CD70-mediated costimulatory signals (Fig 3C), suggesting T cell costimulation is not required for the initiation and maintenance of viral-specific IgM antibody responses. In contrast, B7-mediated costimulation was crucial for the development of MCMV-specific IgG2b and IgG2c responses throughout the course of infection (Fig 3C). Despite the diminished MCMV-specific effector CD4$^+$ T cell response upon CD70 abrogation, comparable IgG2b

and IgG2c levels were found in WT and Cd70-/- mice. Mice deficient in both CD70 and the B7 molecules (Cd70/80/86$^{-/-}$) had a comparable defect in antibody inflation as Cd80/86$^{-/-}$ mice, indicating that the CD28/B7 pathway has a dominant effect on the development of antibody responses. This particular effect of CD28/B7 costimulation coincided with lower levels of MCMV-specific CD4+ T cells in Cd80/86$^{-/-}$ mice as compared to Cd70$^{-/-}$ mice (Fig 3B). Furthermore, MCMV-specific antibody isotype switching was not observed when mice were deprived of B7-mediated signals in the acute phase of infection, but did occur when CD70 signals were abrogated (Fig 3D). These data show that the B7-mediated signals are required at the beginning of infection.

Next, we aimed to explore the mechanisms underlying the dependence of IgG antibody responses on the CD28/B7 pathway. Most strikingly, and fully consistent with the phenotype was the virtual absence of the TFH subset (CXCR5^{+}PD1^{+}) and of the further differentiated GC-associated TFH cells (CXCR5^{+}GL7^{+}) in the Cd80/86$^{-/-}$ mice while Cd70$^{-/-}$ mice still had an induction of these cells (Fig 4A-C). Moreover, upon abrogation of B7 but not of CD70-mediated costimulation a huge reduction in GC B cells, identified by B220^{+}CD19^{+}CD95^{+}GL7^{+}, was observed at day 15 post-infection (Fig 4A and 4D). In line with this, diminished plasma cells characterized by B220^{+}CD19^{+}CD138^{+}IgD^{-} (Fig 4A and 4E), and no splenic MCMV-specific IgG2c-secreting cells were detected in Cd80/86$^{-/-}$ and Cd70/80/86$^{-/-}$ mice (Fig 4F). Together, these data indicate that the development of MCMV-specific IgG antibody responses is fully dependent on B7-mediated activation of CD4^{+} T cell helper subsets, and in particular of the TFH subset.

The initial viral inoculum dose affects antibody levels but not antibody inflation or avidity

Given that the initial viral inoculum dose influences memory T cell inflation (15), we determined if the viral dose impacts MCMV-specific antibody inflation as well. In mice infected with either a low (10^{1} PFU) or a high (10^{4} PFU) viral inoculum dose of MCMV, differences in antibody levels were determined. In low dose infected mice, IgM responses at day 8 were less pronounced compared to high dose infected mice but in the chronic phase of infection similar levels persevered (Fig 5A). MCMV-specific IgG2b and IgG2c levels were diminished upon a low dose infection at all times, but antibody inflation still occurred comparable to high dose infection (Fig 5A). The differences in antibody levels was reflected by distinct numbers of plasma cells (Fig 5B), the occurrence of IgG2c-secreting cells (Fig 5C) and increased numbers of splenic GC B cells (Fig 5D). Also in persistent CMV infection, more MCMV-specific IgG2c-secreting long-lived plasma cells were detected in the bone marrow upon a high dose infection (Fig 5E) but no IgG2c-secreting plasma cells were detected in the spleen in both low and high dose infected mice (data not shown). However, MCMV-specific IgG2c-secreting memory B cells persisted

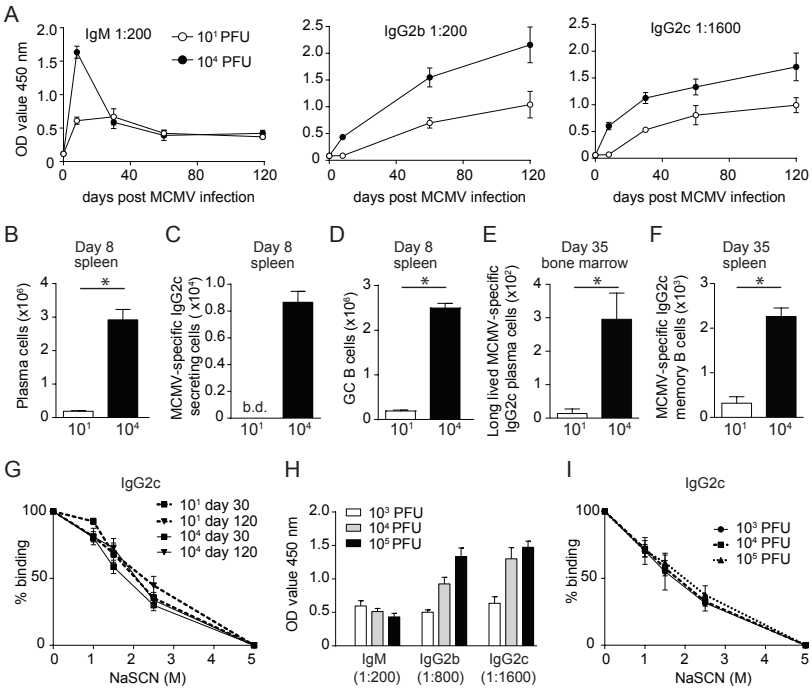

Fig 5. Impact of viral inoculum dose on antibody levels but not on antibody avidity. Mice were infected with either a low dose (10^1 PFU) or a high dose (10^4 PFU) of MCMV. (A) The levels of MCMV-specific antibodies within the serum. (B) The total number of plasma cells identified by $CD138^+/IgD^-$ in the spleen at day 8 post-infection. Cells are gated on $B220^+/CD19^+$ cells. (C) The amount of IgG2c-secreting cells in the spleen determined by ELISPOT at day 8 post-infection. (D) The number of GC B cells identified by $Fas^+/GL\text{-}7^+$ at day 8 post-infection. (E) The total number of IgG2c-secreting long-lived plasma cells determined in the bone marrow by ELISPOT at day 35 day post-infection. (F) The total number of MCMV-specific IgG2c memory B cells determined by ELISPOT at day 35 post-infection. (G) The elution profile of MCMV-specific IgG2c antibodies is shown as mean ± SEM. (H-I) Mice were infected either with 10^3 PFU, 10^4 PFU or 10^5 PFU MCMV and the MCMV-specific antibody levels were determined in the serum 1.5 years post-infection. (I) The elution profile of MCMV-specific IgG2c antibodies is shown as mean ± SEM. All bar graphs represent mean + SEM (n=4 mice per group; *P<0.05).

at higher levels in the spleen in high dose versus low dose infected mice (Fig 5F). Thus the initial viral inoculum dose impacts the number of long-lived plasma cells and memory B cells that are maintained during chronic infection, thereby impacting the amount of IgG antibodies that are present in the serum. IgG antibody inflation, however, occurs despite differences in the initial viral inoculum.

Although the viral dose had a major impact on the antibody levels, no differences were found in antibody avidity between low and high dose infected mice (Fig 5G). Moreover, when mice were infected with three different doses of MCMV, ranging from 10^3 to 10^5 PFU, the effect of the initial viral inoculum dose was again only reflected in the differences in the IgG2b and IgG2c antibody levels (Fig 5H), while no differences in the avidity of the MCMV-specific antibodies were detected (Fig 5I). Together, these data show that the initial viral inoculum dose impacts the antibody levels during antibody inflation but not antibody avidity.

Repetitive viral challenge stimulates the development of neutralizing CMV-specific antibodies and improves antibody avidity

To determine the development of MCMV binding antibodies that are able to neutralize the virus, in vitro neutralization assays were performed with serum of recently and latently infected mice. Neutralizing antibodies were below the detection limit in low and high dose infected mice during the first months post-infection (Fig 6A). Nevertheless, in high dose infected mice eventually neutralizing antibodies were detected. Next we examined if the level of neutralizing antibodies could be elevated by repetitive exposure to MCMV. We administrated a dose of 5 × 10^4 PFU MCMV every week in the same mice following the next year. The levels of the MCMV-specific antibodies were to some extent elevated in mice receiving MCMV repetitively (Fig 6B). Moreover, mice that were every week exposed to intact CMV particles, developed antibodies with a higher avidity (Fig 6C). Consistently, the levels of neutralizing antibodies was higher in the group of mice that received MCMV every week (Fig 6D). Notably, these effects coincided with higher amounts of GC B cells in the spleen (Fig 6E and 6F). These data show that repetitive challenge with intact virus as compared to a single inoculum promotes avidity maturation and development of neutralizing antibodies.

A

	10^1 PFU	10^4 PFU
day 8	<3	<3
day 30	<3	<3
day 60	<3	<3
day 120	<3	6

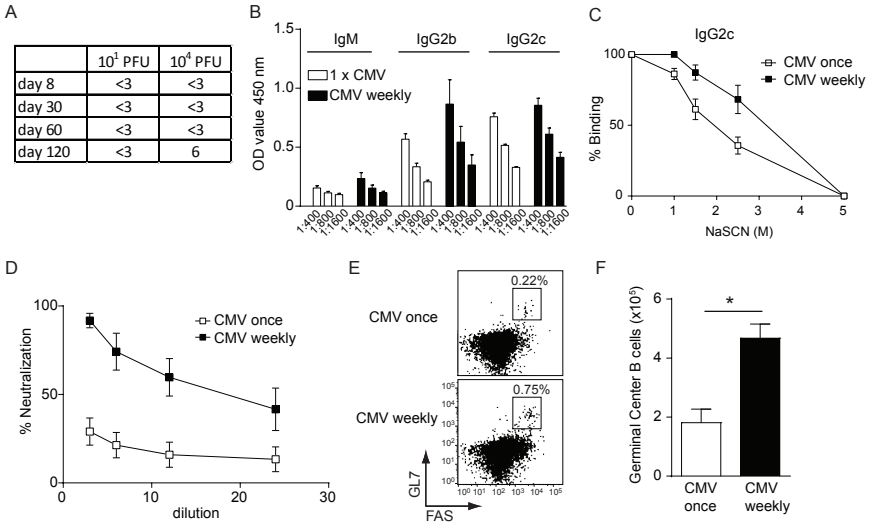

Fig 6. Repetitive antigen exposure increases the avidity and neutralization capacity of MCMV-specific antibodies. (A) Mice were infected with either a low dose (10^1 PFU) or a high dose (10^4 PFU) MCMV. At indicated times the neutralization capacity of the MCMV-specific antibodies was determined. Table indicates the neutralizing antibody titre expressed as the reciprocal of the serum dilution at which 50% of the total PFU was impaired. (B-F) Mice were infected with either a single dose of 5 × 10^4 PFU MCMV or received for one year a weekly dose of 5 × 10^4 PFU MCMV. (B) The levels of MCMV-specific antibodies in the serum at 1 year post-infection. (C) The elution profile of MCMV-specific IgG2c antibodies in the presence of NaSCN is shown as mean ± SEM. (D) The percentage of neutralization for each serum dilution is shown as mean ± SEM. (E) Representative plots show cell surface expression of GL7 and FAS gated on splenic $B220^+/CD19^+$ cells. Numbers indicate the percentage of GC B cells within the total B cell gate. (F) Total amount of GC B cells in the spleen. All bar graphs represent mean + SEM (n=7 mice per group; *P<0.05).

DISCUSSION

Memory T cell inflation is found in response to certain viral infections, most strikingly after persistent cytomegalovirus infection, and is characterized by the accumulation and maintenance of functional effector-memory $CD8^+$ T cells (10). Here we found that viral-specific IgG2b and IgG2c antibodies also inflate during persistent MCMV infection, and that the degree of antibody inflation relates to the dose of the initial viral inoculum. In other persistent infections such as chronic LCMV (25, 26) or HSV infection (27), virus-specific antibodies also accumulate in time, albeit in varying degrees. The serum levels of these virus-specific IgG antibodies however, eventually decline. Longitudinal follow-up of HCMV infected individuals indicates that HCMV-specific antibodies with different specificities expand over time (28, 29), suggesting similar mechanisms underlying antibody inflation. Gradual antibody accumulation is likely related to viral persistence, as it is not observed in various acute infections with influenza virus (30), VSV (31) and LCMV Armstrong (32).

Consistent with other studies in mice and humans (33-39), we found that immune sera of mice latently infected with CMV provide protection from a new CMV infection. It should be noted that we transferred 200 μl of serum from donor to recipient mice, which is actually an underestimation of the protective capacity. Whether these protective effects are mediated via neutralizing antibodies that limit cell-to-cell spread (39) and viral dissemination (35), or via non-neutralizing MCMV-binding antibodies is unclear. Neutralizing CMV-specific antibodies were detectable rather late in infection and the titers of these neutralizing antibodies were low, which is consistent with other low cytopathic viruses (40). Repetitive administration of viral particles, however, did improve the neutralization capacity of MCMV-specific antibodies.

CMV-specific memory B cells that are adoptively transferred have also protective capacity (36), indicating that antibodies and memory B cells together form the humoral immune response. We show that MCMV infection elicits both MCMV-specific long-lived plasma cells and memory B cells, and both are found in greater numbers after high dose inoculum compared to low dose. Consequently, IgG levels are as well higher after high dose infection, yet IgG inflation seems to occur alike after high and low dose infection. Furthermore, IgM levels are equally stably maintained after low and high dose infection. Long-term IgM maintenance is usually not observed after acute infection (23) but has been recently reported for responses to chronic bacteria and bacteria-associated polysaccharides (41-43). Whether the observed IgG inflation and IgM maintenance is directly connected to a gradual increment of the antibody-producing population driven by the persistence of antigen and/or to the induction of long-lived antigen-independent antibody producing cells remains to be explored.

Avidity maturation of CMV-specific antibodies occurred within a month after the initial viral inoculum and remained stable afterwards. This phenomenon is actually used as a diagnostic tool to identify a recent infection with human cytomegalovirus (HCMV) (44). Remarkably, we observed that avidity maturation during the persistent phase of infection can still increase but only by re-exposure to intact viral particles, which was accompanied by increased GC activity. In this respect, it is of interest to note that a study in humans with virosomal vaccines containing functional viral envelope glycoproteins demonstrated that the avidity of antibodies can be improved upon booster vaccination, suggesting that multiple encounters with intact rather than replicating virus is sufficient for avidity maturation (45).

If indeed avidity maturation during viral persistence is driven by newly induced GC reactions requiring intact viral particles one could argue that establishment of viral persistence after a single inoculum does not lead to sufficient viral particles to sustain GC reactions while re-exposure does. Such re-exposure however does not lead to increment of IgM levels, as occurs with primary infection, which may be related to pre-existing immunity preventing activation of IgM producing plasmablasts. In mice, replicating virus at late time points post-infection can however be observed in salivary glands but whether the viral production at this site contributes to the observed antibody inflation remains to be examined. Nevertheless, it has been observed that following intraglandular MCMV infection the salivary gland can operate as a mucosal inductive site for isotype switched IgG+ B cells (46). Moreover, studies by the Reddehase laboratory showed that in other tissues such as lungs, viral replication is abortive as only expression of (immediate) early genes are observed that nonetheless can lead to stimulating inflationary T cells (47).
MCMV-specific IgG2b and IgG2c levels were strongly decreased upon abrogation of B7-mediated costimulation but were intact in the absence of CD70-driven costimulation. Upon influenza virus infection similar viral-specific IgG levels are also found between WT and CD27-/- mice infection but a compensatory role for CD27 signalling is found in the absence of CD28 costimulation (30). We did not observe such a compensatory role but this may relate to a stronger dependence of the induced IgG responses on CD28/B7 costimulation in the MCMV model. In contrast to positive or neutral effects, in both acute and chronic LCMV infection, CD70 interactions have been described to have a negative effect on B cell responses (48, 49). As opposed to the low-level persistence of MCMV, LCMV persistence is accompanied with high-level replication and induction of profound expression of costimulatory molecules (13). Apparently, such high levels could lead to adverse effects of CD27/CD70 signalling on B cell responses and resembles findings in CD70 transgenic mice constitutively expressing CD70, in which deleterious effects on B cells occurs (50). In agreement with numerous other studies (51-53), a prominent role of CD28/B7 interactions for inducing proper B cell responses is found. We found that B7-mediated signals are clearly implicated in the induction of TFH but direct

effects on B cell responses might also be of importance. For example, via enhancing the survival of $CD28^+$ bone marrow-resident plasma cells (54).

CMV-based vectors have shown promising results in diverse infectious and cancer models, but the success of these vaccines is considered as T cell mediated (5, 55, 56). Recently, it has been shown that CMV vectors encoding additional antigens can also induce protective anti-melanoma or tetanus toxins antibodies (8, 9). Thus CMV-based vectors are also promising to apply in settings were antibodies can mediate protection. The results described here could help further support the use of CMV-based vaccines and may help to understand how our immune system coops with this persistent virus.

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