Protection from CMV infection in immunodeficient hosts by adoptive transfer of memory B cells

Karin Klenovsek,[1] Florian Weisel,[2] Andrea Schneider,[2] Uwe Appelt,[3] Stipan Jonjic,[4] Martin Messerle,[5] Birgit Bradel-Tretheway,[6] Thomas H. Winkler,[2] and Michael Mach[1]

[1]Institute for Clinical and Molecular Virology, University Hospital Erlangen, [2]Institute for Biology, Chair of Genetics, Hematopoesis Unit, and [3]Nikolaus-Fiebiger-Center for Molecular Medicine, University Erlangen-Nuremberg, Erlangen, Germany; [4]Department of Histology and Embryology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia; [5]Hannover Medical School, Hannover, Germany; and [6]University of Rochester Medical Center, Rochester, NY

Severe disease associated with cytomegalovirus (CMV) infection is still a major problem in patients who undergo transplantation. Support of the patients' immune defense against the virus is a major goal in transplantation medicine. We have used the murine model of CMV (MCMV) to investigate the potential of a cell-based strategy to support the humoral antiviral immune response. Immunocompetent C57BL/6 mice were infected with MCMV, and memory B cells from the immune animals were adoptively transferred into T-cell– and B-cell–deficient RAG-1$^{-/-}$ mice. Following MCMV infection, a virus-specific IgG response developed within 4 to 7 days in the recipient animals. Concomitantly, a significant reduction in viral titers and DNA copies in several organs was observed. In addition, the memory B-cell transfer provided long-term protection from the lethal course of the infection that is invariably seen in immunodeficient animals. Transfer of memory B cells was also effective in protecting from an already ongoing viral infection, indicating a therapeutic potential of virus-specific memory B cells. T cells were not involved in this process. Our data provide evidence that a cell-based strategy to support the humoral immune response can be effective to combat infectious pathogens in severely immunodeficient hosts. (Blood. 2007;110: 3472-3479)

Introduction

Cytomegalovirus (CMV) viremia and disease are a major cause of morbidity and mortality following hematopoietic stem cell transplantation (HSCT).[1,2] The most feared complication is CMV pneumonia, which is still associated with high mortality.[3] Preemptive therapy using antiviral drugs can reduce the incidence of early-onset CMV disease but is associated with substantial toxicity and development of late-onset CMV disease.[4,5] In addition, drug-resistant virus strains might develop.[6]

CMV replication in patients who underwent transplantation arises as a result of lack of immune control. Thus, bridging the period of immunodeficiency by passive transfer of the most important immune functions is a goal in transplantation medicine. Reports from the murine CMV model (MCMV) had established the importance of CD8 T cells for control of primary infection as well as latency.[7,8] Based on these findings, clinical protocols were developed whereby CD8 T-cell clones were cultured from the transplant donor and transferred to the patient after transplantation.[9-11] This strategy has proved effective in the prevention of reactivation and treatment of CMV infection that is unresponsive to antiviral therapy.[12] However, the MHC restriction of CD8 lymphocytes and the need to expand these cells in vitro makes this procedure cumbersome, and a limited number of patients has been treated so far. In addition, CD4$^+$ cells seem to be important for the long-term survival of the transferred CD8 cells.[9] More recently, CMV-specific CD8$^+$ T cells have been purified from the blood of stem cell transplant donors and infused directly into patients.[13]

Measures have also been taken to support the humoral arm of the immune system. Again, data from the murine model of CMV have clearly demonstrated an important role of antibodies in protecting against a primary infection as well as reactivation or reinfection.[14,15] In contrast to T-cell transfer, support of the humoral immune system in patients has been limited to application of intravenous immune globulin (IVIG) or hyperimmune products. However, even after more than 20 years of extensive use of this treatment either prophylactically and/or therapeutically, uncertainty about benefits for the prevention of CMV infection and disease is evident.[16] Reasons are manifold but might include, among others, use of different products,[17] dosing regimens, and schedule. To our knowledge, cell-based strategies to support the humoral immune response in patients who underwent bone marrow transplantation (BMT) or HSCT have not been explored.

In a mouse model, we have recently shown that virus-specific B cells that are adoptively transferred into immunodeficient hosts can be stimulated to antibody production by antigen alone; T-cell help is not required.[18] This finding suggested the possibility of prophylaxis and/or therapy of viral infections in T-cell–deficient hosts by transfer of specific memory B cells. Here, we examine the protective capacity of adoptively transferred memory

Submitted June 13, 2007; accepted July 23, 2007. Prepublished online as *Blood* First Edition paper, July 26, 2007; DOI 10.1182/blood-2007-06-095414.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

B cells from MCMV infection and disease in immunodeficient recipients.

Materials and methods

Mice

C57BL/6 (B6) and CD8α$^{-/-}$ mice were obtained from Charles River Laboratories (Sulzfeld, Germany). B6 RAG-1$^{-/-}$ (RAG$^{-/-}$) mice were obtained from Irmgard Förster (University Munich). All mice were maintained under specific pathogen-free conditions and used between 8 to 14 weeks of age. All experiments were conducted in accordance with institutional guidelines for animal care and use.

Viruses

The MCMV strain Smith (ATCC VR-1399) was a gift from M. Reddehase (University Mainz). The construction of MCMV157luc is described in Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Virus was propagated and purified as described.[19] Virus titer was determined by end-point titration using indirect immunofluorescence. Briefly, serial dilutions of viral preparations were used to infect mouse embryonic fibroblasts (MEFs) that had been seeded in 96-well plates (15 000 cells/well). Three days later, cells were fixed with ethanol and infected cells were stained and quantified using the monoclonal antibody Croma101, which is specific for the viral immediate early protein 1 of MCMV.[20]

Detection of MCMV-specific IgG

Sera from mice were analyzed at a 1:100 dilution by enzyme-linked immunosorbent assay (ELISA) for virus-specific IgG. For antigen preparation, MEFs were infected with a multiplicity of infection of 0.02 for 96 hours and cell lysates were prepared. Lysates were coated at a concentration of 5 μg/mL onto ELISA plates. Lysates from noninfected cells served as control antigen. For in vitro neutralization, serial dilutions of sera (100 mL, dilution in naive RAG$^{-/-}$ serum) were incubated with 1200 pfu MCMV157luc for 1 hour. The mixture was added to 12 000 ST-2 cells in 96-well plates and incubated for 4 hours. Culture medium was changed and infection continued for 24 hours. Thereafter, cells were lysed in 100 μL Glo lysis buffer and 30 μL was used to measure luciferase activity.

Quantitation of infectious virus and MCMV DNA copies in organs

Organs were harvested and snap frozen in liquid nitrogen. For determination of virus titer, organs were thawed, weighed, and homogenized. Serial dilutions of the organ homogenates were used to infect MEFs in quadruplicate on 96-well plates using centrifugal enhancement.[19] Three days later, virus titer was determined. The limit of detection was 10 to 100 pfu/100 mg organ. Organ DNA was isolated using the Wizard genomic DNA purification kit (Promega, Mannheim, Germany) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed on an ABI Prism 7700 (Applied Biosystems, Weiterstadt, Germany). Primers and probe for the detection of MCMV were based on the MCMV *ie1/4* exon 4 sequence (forward primer: 5′-TGCCATACTGCCAGCTGAGA-3′; reverse primer: 5′-GGCTTCATGATCCACCCTGTT-3′; and probe: 5′-CTGGCATCCAGGAAAGGCTTGGTG-3′).[21] The limit of detection was 10 MCMV genome copies.

Flow cytometry, cell sorting, and adoptive transfer of B lymphocytes

Single-cell suspensions of spleens from infected mice were stained with PE-conjugated anti-CD19 and FITC-conjugated anti-CD8 and anti-CD4 antibodies (all antibodies from BD Biosciences, Basel, Switzerland). CD19$^+$ cells were isolated using a MoFlo cell sorter (Cytomation, Freiburg, Germany) and analyzed for purity by flow cytometry using a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Purified CD19$^+$ B cells (10^6 to 11×10^6) were adoptively transferred into the tail vein of RAG$^{-/-}$ mice. On day 6 after transfer, RAG$^{-/-}$ mice were infected with 1×10^5 plaque forming units (pfu) MCMV intraperitoneally. Sera were analyzed at different time points and organs were taken 21 to 28 days after infection. When B cells were transferred from B6 mice, CD4$^+$ and CD8$^+$ T cells were depleted by administration of 1 mg each of the monoclonal antibodies YTS169.4.2 and YTS191.1.2[22] 3 to 4 days after B-cell transfer. Fluorescence-activated cell sorting (FACS) analysis was used to confirm absence of T cells in the recipient animals.

Measurement of organ luciferase activity

Organs were harvested and directly homogenized in Glo Lysis Buffer (Promega) using an Ultra Turrax T25 (IKA-Werke, Staufen, Germany). Homogenates were centrifuged at 4°C for 5 minutes at 16000*g,* and protein concentration was determined in the supernatant using a BCA Protein Assay Kit (Perbio Science, Bonn, Germany). Glo lysis buffer (30 μL) containing 15 μg protein lysates was transferred into white 96-well LIA plates (Greiner Bio-one, Frickenhausen, Germany). Per well, 50 μL assay buffer (15 mM KH_2PO_4, 25 mM glycylglycine, 1 M $MgSO_4$, 0.5 M EGTA, 5 mM ATP, 1 mM DTT) was added. Injection of 50 μL D-luciferin (P.J.K., Kleinbittersdorf, Germany) solution per well (in 25 mM glycylglycine, 1 M $MgSO_4$, 0.5 M EGTA, 2 mM DTT, and 0.05 mM D-Luciferin) and detection of chemiluminescence were performed by a Centro LB 960 Luminometer (Berthold Technologies, Bad Wildbad, Germany). MicroWin2000 Software (Mikrotek Laborsysteme, Overath, Germany) was used for analysis.

In vivo bioluminescence imaging

Shaved mice were injected intravenously with 0.5 mg D-luciferin in 200 μL PBS and immediately anaesthetized using isoflurane. Two minutes after luciferin injection, bioluminescence was recorded over a 300-second integration period by a cooled CCD camera system (Hamamatsu C4742–98; Hamamatsu Photonics, Okayama City, Japan). Anesthesia was maintained during imaging by nose cone delivery of the anesthetic. SimplPCI Software (Compix, Cranberry Township, PA) was used for acquisition and images were processed in ImageJ (Wayne Rasband; National Institutes of Health, Bethesda, MD). Relative intensities of transmitted light from the in vivo bioluminescence were represented as pseudocolor imaging. Corresponding gray scale photographs and color luciferase images were superimposed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Results

Activation of MCMV-specific memory B cells following adoptive transfer into mice lacking T and B lymphocytes

To generate MCMV-specific memory B cells, immunocompetent B6 mice were infected with 10^5 pfu of MCMV Smith intraperitoneally for 60 days. At this time point, no infectious virus was detectable in blood, indicating complete clearance from this compartment (data not shown). From donor animals, CD19$^+$ small resting B cells were isolated via cell sorting, resulting in a B-cell fraction that in general was more than 99% pure.[18] Individual RAG$^{-/-}$ mice were infused with 5×10^6 purified B cells from naive or from MCMV-infected donors. Ten days later, the animals were challenged by intraperitoneal injection of 1×10^5 pfu MCMV. Sera from adoptively transfused animals were analyzed for MCMV-specific IgG at days 10, 13, and 21 after challenge. MCMV-specific IgG was clearly detectable at day 10 in mice that had received memory B cells but not in mice that had received no B cells or B cells from naive donors (Figure 1A). In general, MCMV-specific IgG titers in RAG$^{-/-}$ mice transferred with memory B cells reached antibody levels on day 21 that were 2- to 4-fold lower than

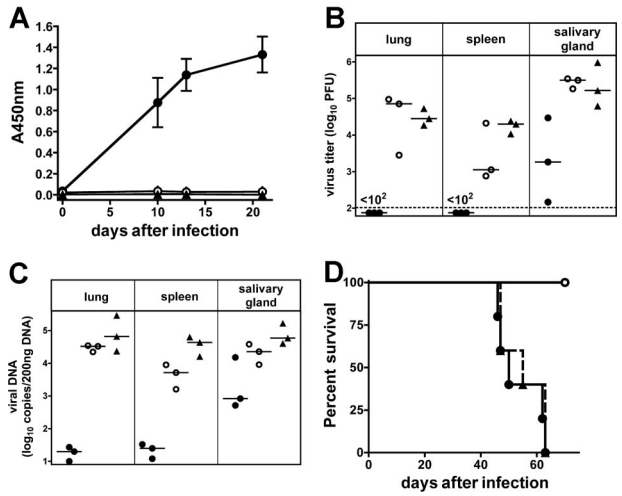

Figure 1. Antibody responses and protection against lethal MCMV infection by memory B cells adoptively transferred in RAG$^{-/-}$ mice. (A) IgG anti-MCMV antibody responses in RAG$^{-/-}$ mice adoptively transferred with 5×10^6 naive B cells (○), 5×10^6 B cells from MCMV-infected mice (●), or no cells (▲). Antibody titers (serum dilution, 1:100) at different time points after MCMV intraperitoneal infection with 10^5 pfu MCMV-Smith strain are shown (mean ± SEM of 3 mice/group). (B) MCMV titers and (C) viral DNA copies in lung, spleen, and salivary gland 28 days after MCMV infection of RAG$^{-/-}$ mice transferred with B cells from MCMV-infected mice (●), with naive B cells (○), and no cells (▲). Median values are shown. The dashed line in panel B indicates detection limit. (D) Survival curve of 5 RAG$^{-/-}$ mice/group adoptively transferred with B cells from MCMV-infected mice (○), naive B cells (●), and no cells (- -▲- -). Mice that received memory B cells were protected against lethal MCMV infection for more than 100 days ($P < .005$).

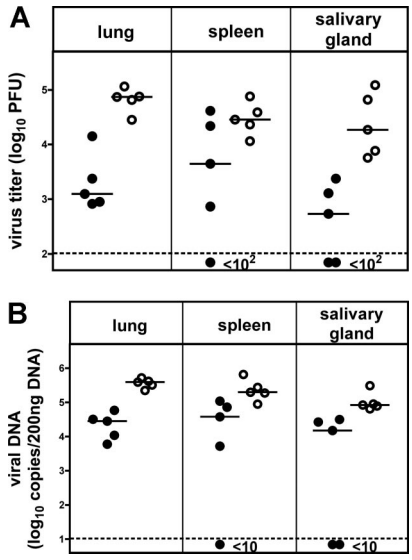

Figure 2. Memory B cells protect from MCMV dissemination in the absence of a functional NK-cell response. (A) MCMV titers and (B) viral DNA copies in lung, spleen, and salivary gland 21 days after infection with 10^5 pfu MCMV157luc. Results from RAG$^{-/-}$ mice adoptively transferred with 5×10^6 B cells from MCMV-infected mice (●) or with 5×10^6 naive B cells (○) are shown (median values of 5 mice/group are indicated as lines). In all recipient animals, CD4$^+$ and CD8$^+$ T cells were depleted 2 days after transfer by application of the appropriate monoclonal antibodies to exclude contribution of T cells to reduction in viral load. There were significantly reduced virus titers in lung and salivary gland in the memory B-cell group ($P < .01$) and significantly reduced viral DNA copies in all organs ($P < .01$ for lung and salivary gland, $P < .05$ for spleen).

in B6 immune donors (data not shown). Mice that were adoptively transferred with memory B cells but not challenged with virus after transfer did not produce MCMV-specific IgG, indicating that the IgG titer was not a result of antibody production from contaminating plasma cells in the transferred cell population (data not shown). Animals that received B cells from naive donors did not produce MCMV-specific IgG, indicating that MCMV did not stimulate antibody production independent of T cells in a nonspecific or polyclonal way. The situation with regard to T-cell–independent activation of memory B cells and kinetics of IgG production was similar to our previous study, in which we used nonreplicating human CMV (HCMV) particles as an antigenic stimulus.[18]

Virus titer in spleens, lungs, and salivary glands was determined at day 28 after infection. Virus titers in lungs and spleens from animals that had received memory B cells were reduced by 3 to 4 logs and were below the detection limit of our experimental system (100 pfu) (Figure 1B). In the salivary gland, virus titers were reduced by more than 1 log, which is in agreement with data showing that in the salivary gland antibodies are ineffective for the clearance of virus.[15] The reduction of viral titers could have been secondary to the presence of virus-neutralizing antibodies in the tissue homogenates or a substantial decrease in viral load. To distinguish between these possibilities, viral DNA load was determined by quantitative real-time PCR. MCMV DNA copies were also drastically reduced in lungs and spleens of animals that had received memory B cells but not in animals that were substituted with no cells or naive B cells. In the salivary gland, the reduction in DNA copies was less pronounced (Figure 1C). The protective potential of memory B cells from lethal CMV disease was also determined (Figure 1D). The result was clear cut. By day 65, all animals that had received naive B cells or no cells had succumbed to the infection, whereas all animals that had received memory B cells were alive.

Effect of adoptively transferred B cells in the absence of NK cell help

During the first few days of infection, immunocompetent B6 mice control MCMV replication through an effective response of natural killer (NK) cells.[23,24] In immunodeficient B6 RAG$^{-/-}$ mice, the NK cell number is even increased compared with normal B6 mice.[25] Therefore, it was conceivable that the protection from MCMV infection by memory B cells was operating only after the initial virus replication was controlled by NK cells and that higher virus titers would overwhelm the protective effect of memory B cells. The activation of MCMV-specific NK cells in B6 mice is exclusively mediated by the virally encoded m157 protein, a ligand of the Ly49H activation receptor on B6 NK cells.[26,27] Deletion of the m157 gene from the MCMV genome results in increased virulence of the virus. Thus, for the subsequent experiments we used a recombinant MCMV (MCMV157luc) in which the gene coding for the m157 protein was replaced by an open reading frame coding for the firefly luciferase under the control of the HCMV IE-promoter/enhancer. In agreement with published work,[26,28] we observed increased virulence of this virus resulting in shorter survival times of 20 to 30 days in B6 RAG$^{-/-}$ mice (data not shown). When recipient B6 RAG$^{-/-}$ mice were challenged with MCMV157luc, we observed a similar reduction in titers of infectious virus as well as DNA copies as in challenge infections using the wild-type virus (Figure 2). Thus, MCMV-specific memory B cells exert a protective effect also in the absence of m157-induced NK-cell cytotoxicity.

T cells do not contribute to the control of MCMV replication by memory B cells

T cells are extremely effective in eliminating infected cells and could potentially contribute to the control of virus infection in our

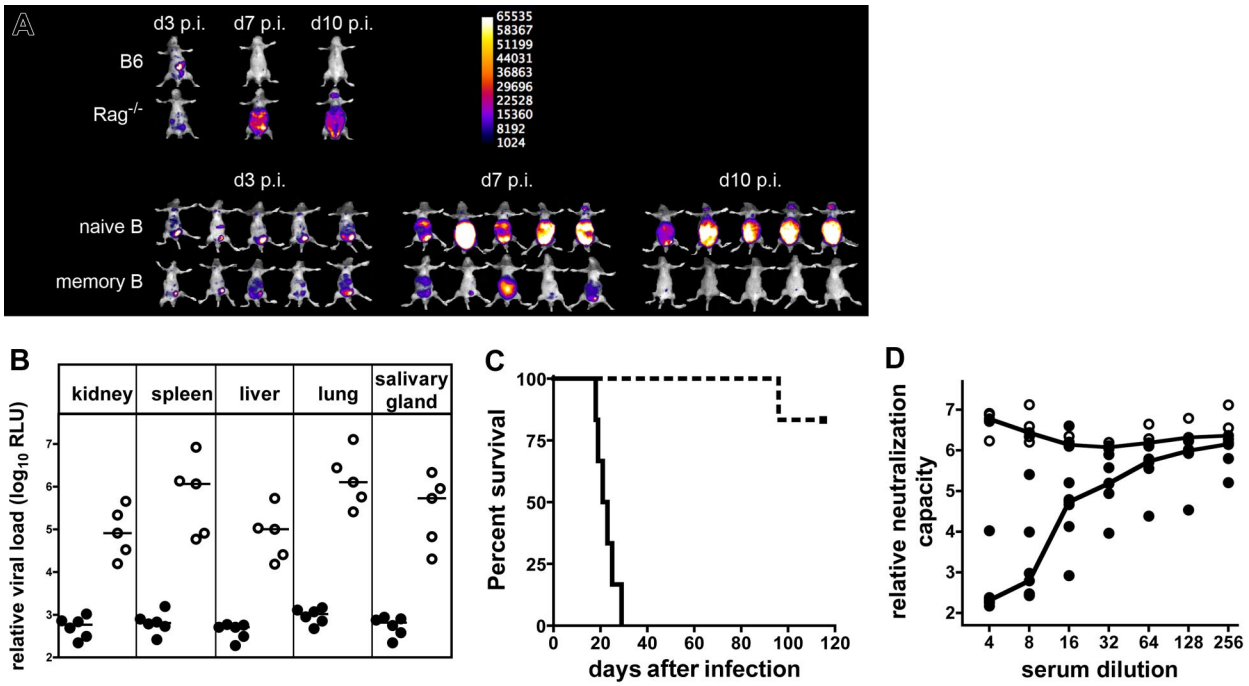

Figure 3. Memory B cells protect from systemic dissemination of MCMV without contribution of NK or T lymphocytes. RAG$^{-/-}$ mice were adoptively transferred with 5×10^6 naive B cells or 5×10^6 B cells from MCMV-infected CD8$\alpha^{-/-}$ mice, treated with depleting anti-CD4 antibodies and infected 5 days after cell transfer with 10^5 pfu MCMV157luc. (A) Bioluminescence imaging of 5 mice/group at 3, 7, and 10 days after infection. Images were obtained from a 5-minute acquisition, and a pseudocolor scale shows relative photon flux for each image. For comparison, a representative B6 mouse and a B6-RAG$^{-/-}$ mouse infected and imaged in parallel are displayed. (B) Relative organ viral load in 5 to 6 RAG$^{-/-}$ mice transferred with memory B cells (●) or naive B cells (○) 18 days after infection. Luciferase activity was measured in organ homogenates and luciferase relative light units (RLU)/15 μg protein are shown. The relative viral load was lower in the memory B-cell group for all organs ($P < .005$). (C) Survival curve of 6 RAG$^{-/-}$ mice/group adoptively transferred with B cells from MCMV-infected mice (- - -) or with naive B cells (—). Mice that received memory B cells showed a significantly prolonged survival ($P < .001$). One animal died of a MCMV-unrelated cause. (D) Neutralizing antibody activity in sera of RAG$^{-/-}$ mice transferred with memory B cells (●) or naive B cells (○). The neutralizing antibody activity was measured in vitro on ST-2 cells using MCMV157luc. The luciferase RLU values after infection with 1.2×10^3 pfu MCMV157luc preincubated with sera are shown. Median values are connected as solid (memory B cells) or dashed (naive B cells) lines.

experiments.[7,29] To exclude a potential impact of incidentally cotransfused T cells on reduction of viral load in the recipients, we initially used depleting antibodies (Figure 2 legend). Depletion of CD8$^+$ and CD4$^+$ T cells did not abolish protection of memory B cells, making it highly unlikely that the observed reduction in viral load was mediated by T cells. To completely rule out a contribution of cytotoxic T cells in the control of MCMV infection in our experimental setting, we generated donor memory B cells in B6 CD8 knockout mice (CD8$\alpha^{-/-}$) and adoptively transferred these cells into RAG$^{-/-}$ recipients. In addition, CD4$^+$ T cells were depleted in the recipients 4 days after cell transfer by administration of a depleting antibody.[22] Of note, B6 CD8$\alpha^{-/-}$ mice consistently developed higher antiviral and neutralizing antibody titers after infection with MCMV Smith than normal B6 mice (data not shown), which is in agreement with data on other viral systems.[30]

At day 5 after memory B-cell transfer from CD8$\alpha^{-/-}$ donors, mice were infected with MCMV157luc virus. The use of MCMV157luc enabled us to monitor the course of the infection by bioluminescence imaging of living animals in real time (Figure 3A). In this type of assay, differences in emitted light correlate with relative differences in virus titer, enabling noninvasive detection of viral replication and distribution over the course of infection.[31] Progression of infection was monitored on days 3, 7, and 10 after infection to determine sites of infection and relative amounts of reporter virus. In mice that received naive B cells, luciferase activity was detected at day 3 and greatly increased until day 10 (Figure 3A). The distribution of the luciferase signal was typical for the multiorgan involvement that occurs following infection of a immunodeficient host. Similar luciferase activity was found in infected RAG$^{-/-}$ mice that did not contain B cells. In contrast, animals that received memory B cells showed little bioluminescence at day 10 after infection, indicating efficient control of virus dissemination (Figure 3A).

To correlate the in vivo bioluminescence data with virus titers in organs, animals were killed on day 18 after infection and virus titer was determined in selected organs, again using a luciferase-based assay. The relative amount of luciferase activity determined in organ homogenates can serve as a marker for viral DNA burden since it directly correlates to infectious titers of recombinant virus and/or DNA copies as determined by real-time PCR[31] (Figure S1). In mice that received memory B cells, a highly significant reduction in viral titers of 2 to 3 logs was observed in all organs, including the salivary gland (Figure 3B). All mice that received naive B cells succumbed to the infection between days 18 to 29 after infection, whereas transfer of memory B cells protected from lethal CMV disease (Figure 3C). Importantly, at day 113 after memory B-cell transfer, none of the protected animals contained CD4$^+$ lymphocyte numbers that exceeded those found in RAG$^{-/-}$ mice, indicating that CD4$^+$ T cells were completely removed by our experimental strategy (data not shown). Antibodies capable of neutralizing MCMV in an in vitro assay were found only in animals that had received memory B cells (Figure 3D).

To evaluate long-term protection of memory B cells, the recipient animals were analyzed for viral load by bioluminescence assay 100 days after viral challenge. At this time point, none of the animals showed detectable bioluminescence signals, indicating a

highly efficient long-term control of viral replication (data not shown). In addition, all recipient animals produced MCMV-specific antibodies at titers that were comparable with the levels found at day 11, which is in agreement with our previous results on the longevity of IgG production after antigenic stimulation of memory B cells[18] (data not shown). When animals were rechallenged with 10^5 or 10^6 pfu of virus at this late time point (100 days) after the first virus inoculation, we did not detect an increase in bioluminescence signal 10 days after the second challenge infection (data not shown). These results indicated that the level of immunity that is provided by a single infusion of memory B cells is sufficient to control repeated exposures to the virus over long periods of time.

Therapeutic transfer of memory B cells

The results shown thus far provided evidence that memory B cells, when present before an infection occurs, provide protection against MCMV. In the clinical situation, however, an active HCMV infection is already ongoing when it is diagnosed. We therefore tested whether memory B cells can also protect when given therapeutically. $RAG^{-/-}$ mice were infected with MCMV157luc, and memory B cells derived from $CD8\alpha^{-/-}$ donors were infused 3 days later. At this time point, all animals had developed an acute infection as documented by bioluminescence imaging (Figure 4A). Recipient animals were monitored for viral burden at days 4, 7, and 11 after transfer of memory B cells. The kinetic of virus replication between days 0 and 4 after cell transfer was similar between animals that received naive or memory B cells. In both groups of animals, viral burden increased considerably. However, at days 7 and 11 after transfer, a drastic reduction in virus load was evident in animals that received memory B cells compared with animals that received naive cells (Figure 4A). The kinetic of clearance of virus is consistent with the delay in IgG production after viral challenge as shown in Figure 1. Viral titer in organs was reduced by 2 to 3 logs and was statistically highly significant in all organs tested (data not shown). Thus, the transfer of memory B cells can be used therapeutically to protect from an ongoing CMV infection.

Protection from MCMV infection by transfer of serum

The most plausible explanation for the effect of memory B cells on the replication of MCMV is the production of protective antibodies. To formally prove this hypothesis, we used serum transfer. $RAG^{-/-}$ mice were infected with MCMV157luc for 3 days, and infection was confirmed by in vivo bioluminescence imaging. On the same day, recipient animals received serum from MCMV-immune or naive mice. Virus load was monitored at days 4, 7, and 11 after transfer. In contrast to memory B cells, passively transferred serum from immune mice had an immediate effect on viral replication. Already at day 4 after serum transfer, bioluminescence signals were drastically reduced in animals that had received immune serum and stayed low during the observation period (Figure 4A). Determination of viral titers in organs confirmed the bioluminescence data (data not shown). Thus, transfer of serum from MCMV-immune animals had a comparably protective effect against MCMV infection as transfer of memory B cells, indicating that indeed the production of IgG might represent the protective principle. In contrast to memory B cells, antiviral activity provided by the serum transfer waned over time. At day 55 after the viral challenge, we observed extensive viral dissemination in all recipient animals as detected by bioluminescence, and by day 60 all animals had succumbed to the infection (Figure 4B).

Discussion

In this study, we have used a mouse model that mimics the clinical situation shortly after transplantation of hematopoietic stem cells: An immunosuppressed host, which is unable to mount an adaptive immune response, is confronted with a pathogen—in this case CMV. In this model system, we have studied the protective potential of adoptively transferred pathogen-specific memory B cells. Memory B cells, per se, do not have antiviral capacity since they represent a resting cell type. It is only upon activation by antigen that memory B cells acquire their antiviral function by producing specific antibody. T-cell help is not required for this process.[18]

The activation of adoptively transferred memory B cells into antibody-secreting plasma cells occurred with similar kinetics following viral infection as in our previous experiments using intravenous application of nonreplicating antigen.[18] Within 7 to 10 days after antigen administration, antibody peak titers were reached in most animals and titers remained elevated for at least 100 days. Thus, the amplification of an initially low-dose viral inoculum results in a fast and sustained memory B-cell response. Concomitant with the production of antiviral antibodies, we observed a drastic reduction in viral titers as well as viral DNA load in several organs including spleen and lung. Perhaps more importantly, all animals that had received memory B cells were permanently protected from the lethal course of the infection that is inevitably occurring in untreated MCMV-infected $RAG^{-/-}$ animals.

The immunologic control of MCMV is redundant and rests on different cell types and immune effector functions.[32] The question therefore arises whether additional cell types, either resident in $RAG^{-/-}$ mice or infused with the B-cell preparation, could have significantly contributed to the reduction in viral titers and enhanced survival in the animals. In B6 mice, which represent a MCMV-resistant mouse strain, NK cells carrying the Ly49H receptor provide vital host innate immune defenses against the virus by killing infected cells and producing cytokines (reviewed in Jonjic et al[33]). The consequence of this response is a pronounced reduction of viral titers in the spleen.[24] This early reduction in viral titer could conceivably assist a memory B-cell response that could otherwise be overwhelmed by unrestricted viral replication early after infection. However, our data clearly show that assistance from $Ly49H^+$ NK cells is not a prerequisite for memory B cells to exert their protective function. The more virulent m157 mutant virus, which cannot be controlled by $Ly49H^+$ NK cells, was as efficiently controlled by the transfer of memory B cells as the wild-type MCMV Smith strain.

Potentially, primed $CD8^+$ cytotoxic T cells could have been introduced into the recipient animals via contamination of the memory B preparations. It has been demonstrated in numerous studies that MCMV-specific CD8 T cells are highly efficient in resolving productive infection in multiple organs following cell transfer into infected immunodeficient recipients.[7,32,34] However, in our experimental setting neither the depletion of $CD8^+$ and $CD4^+$ T cells in the recipients nor the use of $CD8\alpha^{-/-}$ mice as donors in combination with depletion of $CD4^+$ cells in the recipient had an impact on the protective capacity of transferred memory B cells, indicating that T cells in general are not involved in the antiviral effect exerted by the transferred memory B cells.

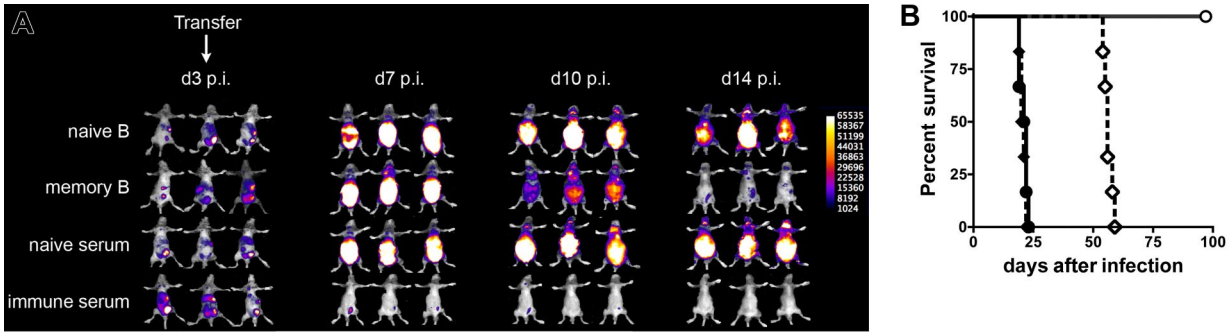

Figure 4. Memory B cells and serum from infected mice protect from an existing MCMV infection. RAG$^{-/-}$ mice were infected with 10^5 pfu MCMV157luc. Three days after infection naive B cells, B cells from MCMV-infected CD8$\alpha^{-/-}$ mice, sera from naive mice, or sera from immune mice (250 μL each) were transferred. (A) Bioluminescence imaging of mice (3 representative mice from 5-6 mice/group are shown) for 14 days after infection. Images were obtained from a 5-minute acquisition and a pseudocolor scale shows relative photon flux for each image. (B) Survival curve of 6 RAG$^{-/-}$ mice/group adoptively transferred with B cells from MCMV-infected mice (—○—), naive B cells (—●—), sera from naive mice (–♦–), or sera from immune mice (–◇–). Mice that received memory B cells or sera from immune mice showed a significantly prolonged survival ($P < .001$) compared with naive B cells and naive sera, respectively.

The most plausible explanation for the efficient reduction of viral burden by adoptive transfer of memory B cells is the production of protective antibodies; in addition, that in the early phase after infection, serum transfer from immune mice had a similar protective capacity as memory B cells supports this conclusion. The role of antibodies for the course of a MCMV infection has not been analyzed in detail. In a previous study, it was shown that virus clearance from a primary infection and establishment of viral latency are indistinguishable between immunocompetent and B-cell–deficient mice.[15] It was concluded that antibodies had no effect on the course of a primary infection. Our data do not contradict this conclusion. The clearance of a primary infection in immunocompetent animals is probably determined by the induction of a potent cytotoxic T-cell response that precedes formation of protective antibodies. In such a situation, the effect of antibodies on the course of the infection may be obscured by the fast cellular response. However, in immunodeficient animals such as RAG$^{-/-}$, which cannot mount a T-cell response, the protective capacity of antibodies becomes evident.

A number of findings have supported the role of B cells and/or antibodies for the course of a MCMV infection. More indirect evidence comes from findings that in seropositive immunocompetent animals immune suppression by γ-irradiation or T-cell depletion does not result in virus reactivation and dissemination.[35,36] In contrast, depletion of T lymphocytes and NK cells from B-cell–deficient mice resulted in 100% recurrent infection.[32] More direct evidence for a protective role of antibodies is indicated by serum transfer studies.[14,15,35,37] In general, protection from a lethal challenge and/or reduction in viral titers in organs was observed. However, these studies have used a protocol in which antibodies were applied before the viral inoculum. Using such a regimen it is difficult to discriminate between immediate neutralization of the viral inoculum by circulating antibody and the effect of antibody on the subsequent virus dissemination. The adoptive transfer of memory B cells clearly demonstrates that pre-existing antibodies are not required for protection from infection. In fact, even an already ongoing infection can be controlled by therapeutic transfer of memory B cells. Protection from an active infection is important for the clinical situation since in patients who underwent HSCT, HCMV disease develops from reactivation of latent virus (ie, the infection is pre-existing).

The nature of the protective antibodies with respect to antigen specificity and antiviral activity remains to be elucidated. Neutralization of extracellular virus by antibodies—and the consequent inhibition of viral dissemination—most probably represents an important mechanism. MCMV is a highly complex virus, which, according to computer predictions, codes for more than 60 glycoproteins.[38] The number of virion-associated envelope glycoproteins is unknown. Protective capacity has been reported for polyclonal antibodies directed against glycoprotein B (gB), one of the dominant antigens in the envelope.[39] It should be noted, however, that following transfer of memory B cells the titer of serum antibodies that neutralized MCMV in an in vitro neutralization assay was rather low, with 50% neutralization of input virus at serum dilutions between 1:2 to 1:128 (Figure 3D). The induction of only low titers of neutralizing antibodies following MCMV infection has also been observed by others and seems to be a hallmark of viruses with low cytopathic potential.[40]

A second mechanism of protection by antiviral antibodies could be elimination of virus-infected cells. It seems likely that during viral replication of the more than 60 virus-encoded glycoproteins, a considerable fraction will be inserted into the plasma membrane of infected cells. Among them are an unknown number of nonstructural proteins that will not be incorporated into virions. Antibodies against these nonstructural proteins could mediate lysis of infected cells via antibody-dependent cytotoxicity (ADCC). If this occurs before infectious virus is released from the target cell, dissemination will be prevented. Antibodies that are directed against nonstructural membrane proteins of MCMV and mediate ADCC would be negative in a conventional in vitro neutralization assay and thus could explain the low titers of virus-neutralizing antibodies in the MCMV-infected mouse sera. Previous studies exploring the protective capacity of MCMV-specific monoclonal antibodies have also noticed the lack of correlation between protection and in vitro neutralizing antibody titer, indicating that mechanism of action in vitro and in vivo might be different.[37]

MCMV has been a valuable model for the pathogenesis of the HCMV infection, and insights into the protective capacity of MCMV-specific cytotoxic T cells have been successfully introduced into the treatment of patients who underwent HSCT by adoptive transfer of CD8$^+$ T cells.[9,13] However, this treatment is still far from being clinical routine. Attempts to support the humoral immune response in patients who underwent HSCT have been limited mainly to the administration of polyclonal IVIG. IVIG has been extensively used in patients at risk for CMV disease. However, even after more than 20 years of using this treatment either prophylactically and therapeutically, uncertainty about efficacy is evident (reviewed by Sokos et al[16]). As a result, the humoral immune response as an important variable in the pathogenesis of HCMV infection was dismissed. However, there are major caveats

to this interpretation. Donor selection for IVIG is based on anti-HCMV antibody titer in ELISA tests. It can be questioned whether this is a valid assay format for biologically relevant antibodies. For example, in human HCMV-convalescent sera there is only a poor correlation between virus-neutralizing capacity and ELISA titer.[41,42] In addition, the conventional ELISA systems specifically select for serum donors with high titers of antibodies against internal proteins that will never reach the surface of infected cells or represent virion components from the inner structures of the particle. Such antibodies can be expected to have little impact on direct virus neutralization or ADCC. Moreover, use of different unstandardized IVIG preparations, all of which could be expected to vary in their titer of biologically relevant HCMV-specific antibodies, makes interpretation of the human studies difficult at best.[43] On the other hand, high titers of neutralizing antibodies were found to correlate with absence of infectious virus in plasma and protection from HCMV-disease in bone marrow transplant recipients, supporting a role for antibodies in patients who underwent transplantation.[44]

In conclusion, we have shown that the adoptive transfer of memory B cells into immunodeficient hosts can protect from MCMV-induced morbidity and mortality. Protection was accomplished by either prophylactic or therapeutic application of B cells. Protection by memory B cells is long lasting and control was still complete 100 days after transfer. This long-term control indicated that escape from the antibody response did not occur. Since we could previously show that activation of adoptively transferred memory B cells is not restricted to CMV,[18] it is conceivable that this treatment strategy can be beneficial for a variety of different infectious agents in patients who underwent transplantation, reducing the need for specific intervention against individual pathogens.

Acknowledgments

This work was supported by a grant (SFB643) from the Deutsche Forschungsgemeinschaft (M. Mach and T.H.W.). The in vivo bioluminescence unit was supported by grants of the Doktor-Robert-Pfleger Foundation (Bamberg, Germany) and the ELAN funds of the University Hospital Erlangen to Reinhard Voll.

The authors would like to thank M. Reddehase and members of his laboratory for providing MCMV Smith and for valuable advice during the first experiments.

Authorship

Contribution: B.B.-T. and M. Messerle constructed the recombinant MCMV; A.S. and U.A. helped with animal work, FACS analysis, and cell sorting; S.J. produced essential reagents; and T.H.W. and M. Mach designed the experiments, analyzed data, and wrote the paper. K.K. and F.W. contributed equally to this work.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Michael Mach, Institute for Virology, University Hospital Erlangen, Schlossgarten 4, 91054 Erlangen, Germany; e-mail: mlmach@viro.med.uni-erlangen.de.

References

1. Boeckh M, Leisenring W, Riddell SR, et al. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood. 2003;101:407-414.
2. Boeckh M, Nichols WG, Papanicolaou G, et al. Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. Biol Blood Marrow Transplant. 2003;9:543-558.
3. Einsele H, Hebart H. Cytomegalovirus infection following stem cell transplantation. Haematologica. 1999;84(suppl EHA-4):46-49.
4. Boeckh M, Nichols WG. The impact of cytomegalovirus serostatus of donor and recipient before hematopoietic stem cell transplantation in the era of antiviral prophylaxis and preemptive therapy. Blood. 2004;103:2003-2008.
5. Einsele H, Ehninger G, Hebart H, et al. Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. Blood. 1995;86:2815-2820.
6. Gilbert C, Boivin G. Human cytomegalovirus resistance to antiviral drugs. Antimicrob Agents Chemother. 2005;49:873-883.
7. Reddehase MJ, Mutter W, Munch K, Buhring HJ, Koszinowski UH. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J Virol. 1987;61:3102-3108.
8. Reddehase MJ, Weiland F, Munch K, et al. Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J Virol. 1985;55:264-273.
9. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.
10. Einsele H, Roosnek E, Rufer N, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. Blood. 2002;99:3916-3922.
11. Peggs KS, Verfuerth S, Pizzey A, et al. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. Lancet. 2003;362:1375-1377.
12. Moss P, Rickinson A. Cellular immunotherapy for viral infection after HSC transplantation. Nat Rev Immunol. 2005;5:9-20.
13. Cobbold M, Khan N, Pourgheysari B, et al. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J Exp Med. 2005;202:379-386.
14. Shanley JD, Jordan MC, Stevens JG. Modification by adoptive humoral immunity of murine cytomegalovirus infection. J Infect Dis. 1981;143:231-237.
15. Jonjic S, Pavic I, Polic B, et al. Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. J Exp Med. 1994;179:1713-1717.
16. Sokos DR, Berger M, Lazarus HM. Intravenous immunoglobulin: appropriate indications and uses in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2002;8:117-130.
17. Filipovich AH, Peltier MH, Bechtel MK, et al. Circulating cytomegalovirus (CMV) neutralizing activity in bone marrow transplant recipients: comparison of passive immunity in a randomized study of four intravenous IgG products administered to CMV-seronegative patients. Blood. 1992;80:2656-2660.
18. Hebeis BJ, Klenovsek K, Rohwer P, et al. Activation of virus-specific memory B cells in the absence of T cell help. J Exp Med. 2004;199:593-602.
19. Podlech J, Holtappels R, Grzimek NK, Reddehase MJ. Animal models: murine cytomegalovirus. Methods Microbiol. 2002;32:493-525.
20. Trgovcich J, Stimac D, Polic B, et al. Immune responses and cytokine induction in the development of severe hepatitis during acute infections with murine cytomegalovirus. Arch Virol. 2000;145:2601-2618.
21. Balthesen M, Messerle M, Reddehase MJ. Lungs are a major organ site of cytomegalovirus latency and recurrence. J Virol. 1993;67:5360-5366.
22. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature. 1984;312:548-551.
23. Webb JR, Lee SH, Vidal SM. Genetic control of innate immune responses against cytomegalovirus: MCMV meets its match. Genes Immun. 2002;3:250-262.
24. Scalzo AA, Fitzgerald NA, Simmons A, La-Vista AB, Shellam GR. Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. J Exp Med. 1990;171:1469-1483.
25. Grundy MA, Sentman CL. Immunodeficient mice have elevated numbers of NK cells in non-lymphoid tissues. Exp Cell Res. 2006;312:3920-3926.
26. Bubic I, Wagner M, Krmpotic A, et al. Gain of virulence caused by loss of a gene in murine cytomegalovirus. J Virol. 2004;78:7536-7544.
27. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science. 2002;296:1323-1326.
28. French AR, Pingel JT, Wagner M, et al. Escape of mutant double-stranded DNA virus from innate immune control. Immunity. 2004;20:747-756.

29. Steffens HP, Kurz S, Holtappels R, Reddehase MJ. Preemptive CD8 T-cell immunotherapy of acute cytomegalovirus infection prevents lethal disease, limits the burden of latent viral genomes, and reduces the risk of virus recurrence. J Virol. 1998;72:1797-1804.
30. Battegay M, Moskophidis D, Waldner H, et al. Impairment and delay of neutralizing antiviral antibody responses by virus-specific cytotoxic T cells. J Immunol. 1993;151:5408-5415.
31. Luker GD, Bardill JP, Prior JL, et al. Noninvasive bioluminescence imaging of herpes simplex virus type 1 infection and therapy in living mice. J Virol. 2002;76:12149-12161.
32. Polic B, Hengel H, Krmpotic A, et al. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. J Exp Med. 1998;188:1047-1054.
33. Jonjic S, Bubic I, Krmpotic A. Innate immunity to cytomegaloviruses. In: Reddehase MJ, ed. Cytomegaloviruses: Molecular Biology and Immunology. Norfolk, United Kingdom: Caister Academic Press; 2006:285-319.
34. Holtappels R, Podlech J, Grzimek NK, et al. Experimental preemptive immunotherapy of murine cytomegalovirus disease with CD8 T-cell lines specific for ppM83 and pM84, the two homologs of human cytomegalovirus tegument protein ppUL83 (pp65). J Virol. 2001;75:6584-6600.
35. Reddehase MJ, Balthesen M, Rapp M, et al. The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. J Exp Med. 1994;179:185-193.
36. Jonjic S, Pavic I, Lucin P, Rukavina D, Koszinowski UH. Efficacious control of cytomegalovirus infection after long-term depletion of CD8+ T lymphocytes. J Virol. 1990;64:5457-5464.
37. Farrell HE, Shellam GR. Protection against murine cytomegalovirus infection by passive transfer of neutralizing and non-neutralizing monoclonal antibodies. J Gen Virol. 1991;72:149-156.
38. Rawlinson WD, Farrell HE, Barrell BG. Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol. 1996;70:8833-8849.
39. Rapp M, Messerle M, Lucin P, Koszinowski UH. In vivo protection studies with MCMV glycoproteins gB and gH expressed by vaccinia virus. In: Michelson S, Plotkin SA, eds. Multidisciplinary Approach to Understanding Cytomegalovirus Disease. Amsterdam, The Netherlands: Excerpta Medica; 1993:327-332.
40. Hangartner L, Zinkernagel RM, Hengartner H. Antiviral antibody responses: the two extremes of a wide spectrum. Nat Rev Immunol. 2006;6:231-243.
41. Kropff B, Landini MP, Mach M. An ELISA using recombinant proteins for the detection of neutralizing antibodies against human cytomegalovirus. J Med Virol. 1993;39:187-195.
42. Leogrande G, Merchionne F, Lazzarotto T, Landini MP. Large-scale testing of human serum to determine cytomegalovirus neutralising antibody. J Infect. 1992;24:289-299.
43. Chehimi J, Peppard J, Emanuel D. Selection of an intravenous immune globulin for the immunoprophylaxis of cytomegalovirus infections: an in vitro comparison of currently available and previously effective immune globulins. Bone Marrow Transplant. 1987;2:395-402.
44. Schoppel K, Schmidt C, Einsele H, Hebart H, Mach M. Kinetics of the antibody response against human cytomegalovirus-specific proteins in allogeneic bone marrow transplant recipients. J Infect Dis. 1998;178:1233-1243.