

Infection with rat cytomegalovirus (CMV) in the immunocompromised host is associated with the appearance of a T cell population with reduced CD8 and T cell receptor (TCR) expression

J. G. VAN DAM*, J. G. M. C. DAMOISEAUX†, H. A. M. D. VAN DER HEIJDEN*†, G. GRAULS*, P. J. C. VAN BREDA VRIESMAN† & C. A. BRUGGEMAN* *Departments of *Medical Microbiology and †Immunology, University of Maastricht, Maastricht, The Netherlands*

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SUMMARY

Infection with human cytomegalovirus (HCMV) mostly results in a chronic subclinical infection; the immune system is unable to eliminate the virus and is apparently in equilibrium with the persistent virus. In the immunosuppressed host this equilibrium is disturbed, resulting in clinical infection. Rat cytomegalovirus (RCMV) infection in its host can be used as a model for HCMV infection. Using flow cytometry we examined the effect of acute RCMV infection on the composition of leucocyte subsets in the peripheral blood of both immunocompetent and immunosuppressed (5 Gy total body irradiation) Lewis rats. Special attention was paid to the natural killer (NK) cells and the CD8⁺ T cells known to be involved in the control of viral infections. Furthermore, we determined the presence of leucocyte subsets in the internal organs by immunohistochemistry. In immunocompetent rats, infection caused a small increase in NK cells and a large increase in CD8⁺ T cells. In contrast, infection of immunosuppressed rats caused a marked increase in NK cells and a small increase in CD8⁺ T cells, consisting of T cells with reduced expression of both CD8 and TCR. This phenomenon is characteristic of anergic CD8⁺ T cells, possibly explaining the ability of the virus to escape elimination by the immune system. The increase of NK cells in the peripheral blood of immunosuppressed, RCMV-infected rats could also be detected in kidney, liver, lung and pancreas, but not in salivary gland. This could explain the long persistence of infectious virus in the salivary gland.

Keywords cytomegalovirus immunosuppression tolerance induction peripheral blood leucocytes

INTRODUCTION

Human cytomegalovirus (HCMV) is a β -herpesvirus, that often causes an infection in man. Infection with the virus mostly results in a chronic subclinical infection; the immune system is unable to eliminate the virus completely, allowing virus to persist in the host in a latent state. In the immunocompromised host, e.g. a transplant recipient, the equilibrium is disturbed, allowing the latent virus to reactivate and to cause clinical disease. HCMV infection has also been associated with increased allograft rejection [1] and recently with the occurrence of coronary restenosis [2,3].

Like many viruses, HCMV has evolved a strategy to escape immune attack by down-regulation of MHC class I expression on infected cells [4–7]. Down-regulation of MHC class I molecules,

however, makes cells susceptible to natural killer (NK) cell destruction [8]. Recently, it has been described that lysis of infected cells by NK cells is circumvented by the expression of a viral MHC class I homologue [9,10]. If this mechanism makes the virus completely invisible to the immune system, immunosuppressed hosts should be expected to have the same course of infection as immunocompetent hosts. Given the clear differences in the clinical course of infection in immunocompetent and immunosuppressed hosts, the escape mechanism of the virus apparently is insufficient to avoid the attack of the immune system of the immunocompetent host.

To investigate the effects of CMV infection on the host, animal models are often used. Rat CMV (RCMV) infection in immunocompetent rats does not result in clinical symptoms. Acute infection in immunosuppressed rats, however, results in a widespread infection with infectious virus being present in almost every organ, eventually resulting in establishment of a latent infection [11,12].

Correspondence: C. A. Bruggeman, Department of Medical Microbiology, University Hospital Maastricht, PO Box 5800, 6202 AZ Maastricht, The Netherlands.

Using the rat model, Li *et al.* described the influence of RCMV infection on transplant-associated arteriosclerosis (TAA) after aorta transplantation [13] and the correlation between immunosuppression and the effects of RCMV on the allograft [14]. The rat model has proved to be a very useful model to study the course of CMV infection, CMV pathogenesis, the role of CMV on transplant arteriosclerosis and therapeutic interventions [15–17]. Detailed knowledge about the course of RCMV infection in the rat is therefore important for complete understanding of the effects of RCMV on transplant arteriosclerosis. This study investigates the relation between the composition of the peripheral blood leucocytes (PBL), the influx of blood cells in the internal organs and the presence of virus after infection with RCMV. Special attention was paid to NK cells, CD8⁺ T cells and the CD4/CD8 T cell ratio.

MATERIALS AND METHODS

Animals

Male specific pathogen-free (SPF; according to the recommendations of the Federation of European Laboratory Animal Science Association) [18] inbred Lewis rats (LEW; RT1^l), obtained from the Central Animal Facility of Maastricht University (The Netherlands), were used in this study. The rats were ≈ 250 g at the start of the experiments. The animals were housed under standardized conditions, fed with commercially available pellet diet and had free access to acidified demineralized water pH ± 3 .

Infection and immunosuppression

The RCMV stock (Maastricht strain) was obtained by homogenization of salivary glands of acutely infected rats as described previously [11]. Each animal received 3×10^5 plaque-forming units (PFU) of RCMV intraperitoneally at the start of the experiment (day 0). Immunosuppression was induced by giving 5 Gy total body irradiation (TBI) 1 day before infection (day -1) [19]. This dose dramatically reduces the number of PBL, but the haematopoietic stem cells remain viable.

Design of the experiment

For the analysis of leucocyte subsets in peripheral blood, RCMV-infected and immunosuppressed rats (CMV⁺ TBI⁺) were used and blood analysis was performed at days 3, 5, 7, 10, 14, 19, 24 and 29 post-infection. Controls consisted of non-infected immunosuppressed rats (CMV⁻ TBI⁺), infected non-immunosuppressed rats (CMV⁺ TBI⁻) and non-infected non-immunosuppressed rats (CMV⁻ TBI⁻).

In order to follow the course of the RCMV infection in different organs and the effect of the infection on the inflammatory response in these organs, CMV⁺ TBI⁺ rats were studied at day 7 and day 14 post-infection. As controls, CMV⁻ TBI⁺ rats were used. The salivary gland, spleen, kidney, liver, lung, heart and pancreas were collected for plaque assay, polymerase chain reaction (PCR), frozen sections and paraffin sections.

Monoclonal antibodies

The MoAbs used for immunohistochemistry have been described previously [20]. MoAb 341 (CD8 $\alpha\beta$ heterodimers, only present on CD8⁺ cytotoxic T cells) [21] and R73 (TCR $\alpha\beta$) [22] were kindly provided by Th. Hünig (Würzburg, Germany). W3/25 (directed against CD4) was purchased from Serotec (Oxford, UK). The MoAbs ED1 (inflammatory macrophages) and ED2 (resident tissue macrophages) [23] were kindly provided by C. D. Dijkstra (Free

University, Amsterdam, The Netherlands). The MoAb 323 is directed against NK cells [24] and MoAb 8 is directed against early RCMV antigens [25].

For flow cytometry, MoAb R73 (TCR $\alpha\beta$, 10 $\mu\text{g/ml}$) conjugated to biotin, MoAb OX35 (CD4, 10 $\mu\text{g/ml}$) conjugated to FITC, MoAb OX8 (CD8 α , 8 $\mu\text{g/ml}$) conjugated to PE and the second step labelling streptavidin conjugated to the fluorochrome Cy-chrome (2.5 $\mu\text{g/ml}$) were purchased from Pharmingen (San Diego, CA).

Immunohistochemistry

Paraffin-embedded sections (4 μm thick) were used for immunohistochemical detection of RCMV [12]. Frozen sections (4 μm) were used for all other detections, using the indirect immunoperoxidase technique. The slides were scored by comparing the non-infected with infected group. The number of cells present in the infected group was equal (=), moderately increased (†, the number of cells in the infected organ was < 15 per field of view (magnification $\times 400$) or the infected organ contained no more than twice the number of cells present in non-infected organs), or strongly increased (††, more than two-fold) compared with the non-infected group on the same day after infection and irradiation.

Plaque assay

To detect the presence of infectious virus in the different organs, the organs were homogenized in a tissue grinder and suspended in minimal essential medium with 2% fetal calf serum (FCS) as described previously [26]. Ten- and 100-fold dilutions of 10% homogenates (w/v) were inoculated on a confluent rat embryonic fibroblast monolayer. After an incubation period of 7 days, under 0.25% agarose, the number of plaques was determined microscopically after fixation and methylene blue staining.

Semiquantitative nested PCR

To determine the presence of viral DNA, organs were cut in small pieces and stored at -70°C until use. After an incubation period of 6 h with extraction buffer, DNA was isolated according to the description of the manufacturers of the XTRAX DNA Extraction Kit (Gull Labs, Salt Lake City, UT). DNA concentration was determined spectrophotometrically, and 1 μg of DNA was used in the PCR.

In the first run primers were used that hybridize with the RCMV DNA polymerase gene [27]. The sequences of the primers are 5'-AAGGGATCCGATTCGCCAGCCTCTACC-3' (in which the sequence in italics represents nucleotides 11 726–11 744 of GenBank file U50550) and 5'-AAGGGATCCTGTCTGGTGTCCCGTACAC-3' (in which the sequence in italics represents the sequence complementary to nucleotides 12 221–11 239 of GenBank file U50550). The primers generate a product of 536 bp. The nested PCR resulted in a product with a length of 431 bp, using primers 5'-AAGGGATCCCTCTGTACTCCACCCTGC-3' (in which the sequence in italics represents nucleotides 11 767–11 786 of GenBank file U50550) and 5'-TTCGGATCCACGCCGACCTCGGAGACCAG-3' (in which the sequence in italics represents the sequence complementary to nucleotides 12 158–12 177 of GenBank file U50550). PCR products were separated on 1.2% agarose gels followed by staining with ethidium bromide (0.5 $\mu\text{g/ml}$). For semiquantitative analysis of the amount of virus present, DNA was diluted 10-fold (maximal nine times) before running the first PCR.

Flow cytometry

To determine the effect of RCMV infection on the different subsets

of leucocytes in the peripheral blood, 1 ml of heparinized blood was collected from the retro-orbital plexus per rat. The absolute number of PBL was determined by diluting blood in Türk solution and counting the nucleated cells in a Bürker Hemocytometer. The erythrocytes were lysed with ammonium chloride solution (0.155 M ammonium chloride, 0.01 M potassium carbonate and 0.1 mM EDTA, pH 7.4 at 4°C). For three-colour flow cytometry, 5×10^5 cells per sample were resuspended in 20 μ l PBS containing 0.5% bovine serum albumin (BSA), 10 mM NaN_3 and the MoAbs directed against CD4, CD8 and TCR $\alpha\beta$. The expression of CD4, CD8 and TCR $\alpha\beta$ and the forward scatter (FSC) and side scatter patterns (SSC) were determined using a FACSort (Becton Dickinson, Etten Leur, The Netherlands) and the LYSYS II software package (Becton Dickinson). In list mode 10 000 events were acquired with gating on viable cells using the FSC parameter. In order to gain a more detailed insight into the CD4/CD8 T cell ratio, another 5000 events were acquired with gating on viable T cells using FSC and TCR expression. Granulocytes were identified by their typical scatter profile in a FSC *versus* SSC plot. Monocytes were identified by the dim expression of CD4 and the absence of TCR expression, whereas NK cells stain positive for CD8 and lack TCR expression [28].

RESULTS

RCMV infection causes an increase in the number of leucocytes in immunocompetent rats

To study the effect of RCMV infection on the different fractions of leucocytes in the peripheral blood, both immunosuppressed and immunocompetent rats were infected. The absolute number of leucocytes in the $\text{CMV}^- \text{TBI}^-$ rats stayed between 6.2×10^6 and 8.6×10^6 cells per ml blood during the experiment (Fig. 1). Infection with RCMV in the non-irradiated rats ($\text{CMV}^+ \text{TBI}^-$) resulted in a slight increase in the number of leucocytes on days 10 and 14. In the $\text{CMV}^+ \text{TBI}^+$ rats leucocytes were depleted, caused by the irradiation. Next, the number of leucocytes gradually increased to normal over a period of 4 weeks. An increase in the number of leucocytes was not observed in the $\text{CMV}^+ \text{TBI}^+$ group compared with the $\text{CMV}^- \text{TBI}^+$ group.

RCMV infection in immunocompetent and immunosuppressed rats results in a preferential increase in the T cell and NK cell compartment, respectively

Four subsets of leucocytes could be specified using the five-parameter analysis, i.e. granulocytes, monocytes, NK cells and T cells. In the ($\text{CMV}^- \text{TBI}^-$) control group the numbers of granulocytes ranged from 6×10^5 to 8×10^5 cells per ml blood. For monocytes the range was 7×10^5 – 12×10^5 , for NK cells 3×10^5 – 5×10^5 and for T cells 3.3×10^6 – 4.7×10^6 cells per ml blood. Infection with CMV ($\text{CMV}^+ \text{TBI}^-$) had no effect on the number of granulocytes (Fig. 2a) and monocytes (Fig. 2b), but resulted on days 10 and 14 in a small increase in the number of NK cells (Fig. 2c) and a strong increase in the number of T cells (Fig. 2d).

Irradiation caused a depletion of the number of PBL that correlated with a depletion of monocytes, NK cells and T cells (Fig. 2b,c,d). The number of granulocytes was already restored to normal at day 4 post-irradiation (Fig. 2a). Compared with the $\text{CMV}^- \text{TBI}^+$ rats no changes in the number of granulocytes and T cells were detected in the $\text{CMV}^+ \text{TBI}^+$. The number of monocytes, however, showed an accelerated recovery. The number of NK cells in the $\text{CMV}^+ \text{TBI}^+$ group showed an enormous increase at day 14

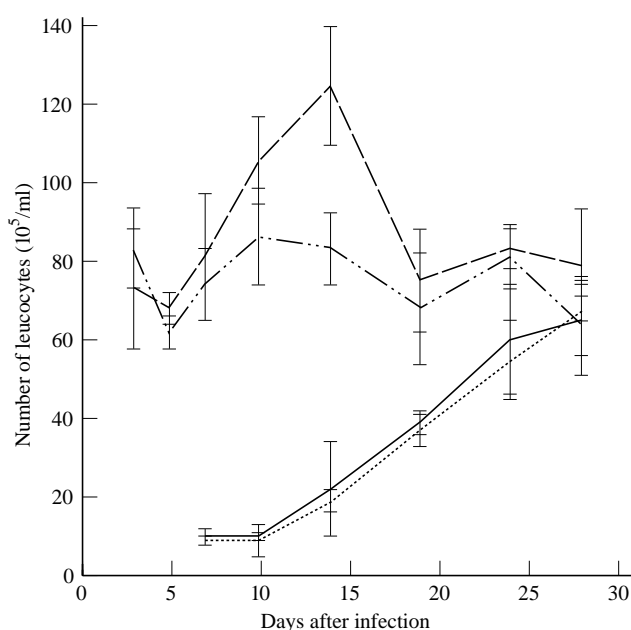


Fig. 1. Increased peripheral blood leucocyte numbers upon rat CMV (RCMV) infection in immunocompetent rats. Results are the mean (\pm s.d.) leucocyte numbers ($\times 10^5$) determined in a Bürker haemocytometer in infected and irradiated rats ($\text{CMV}^+ \text{TBI}^+$, —; $n=4$), irradiated rats ($\text{CMV}^- \text{TBI}^+$,; $n=3$), infected rats ($\text{CMV}^+ \text{TBI}^-$, - - - -; $n=3$), and untreated control rats ($\text{CMV}^- \text{TBI}^-$, - · - · - ·; $n=3$).

after infection, resulting in six times the number of cells present in the $\text{CMV}^- \text{TBI}^+$ group and twice the number of cells present in the $\text{CMV}^- \text{TBI}^-$ group. From day 14 to day 28 a slow decline in the number of NK cells was seen, but the numbers remained elevated compared with the three control groups.

Decrease in CD4/CD8 ratio in immunosuppressed RCMV-infected rats is caused by the appearance of an aberrant CD8⁺ T cell subset Because it is known that T cells play an important role in the defence against herpesvirus infections in general, the effect of RCMV infection on the subpopulations of T cells (CD4 and CD8) was further analysed. Figure 3 presents the absolute numbers of CD4⁺ T cells and CD8⁺ T cells (Fig. 3a,b).

In the $\text{CMV}^- \text{TBI}^-$ rats, the number of CD4⁺ T cells ranged between 2.5×10^6 and 3.6×10^6 cells per ml blood and the number of CD8⁺ T cells between 7×10^5 and 1×10^6 . This resulted in a CD4/CD8 ratio of 3.0–4.1 (Fig. 4). In the $\text{CMV}^+ \text{TBI}^-$ rats, the absolute number of both CD4⁺ and CD8⁺ T cells was increased on days 10 and 14 (Fig. 3a,b). This increase caused no relative changes in the CD4⁺ and CD8⁺ T cells and therefore no alterations in the CD4/CD8 ratio (Fig. 4). Irradiation ($\text{CMV}^- \text{TBI}^+$) resulted in an enormous decrease in the absolute number of both CD4⁺ and CD8⁺ T cells at day 7. Both subsets slowly increased and reached almost normal levels on day 28 (Fig. 3a,b). The recovery from irradiation was slower in CD8⁺ T cells than in CD4⁺ T cells, resulting in an increased CD4/CD8 ratio on days 7 and 10 (Fig. 4).

In the $\text{CMV}^+ \text{TBI}^+$ group, the infection caused no changes in the number of CD4⁺ T cells, but in the population of CD8⁺ T cells an increase was detected compared with the $\text{CMV}^- \text{TBI}^+$ group. This resulted in a relative decrease in the number of CD4⁺ T cells

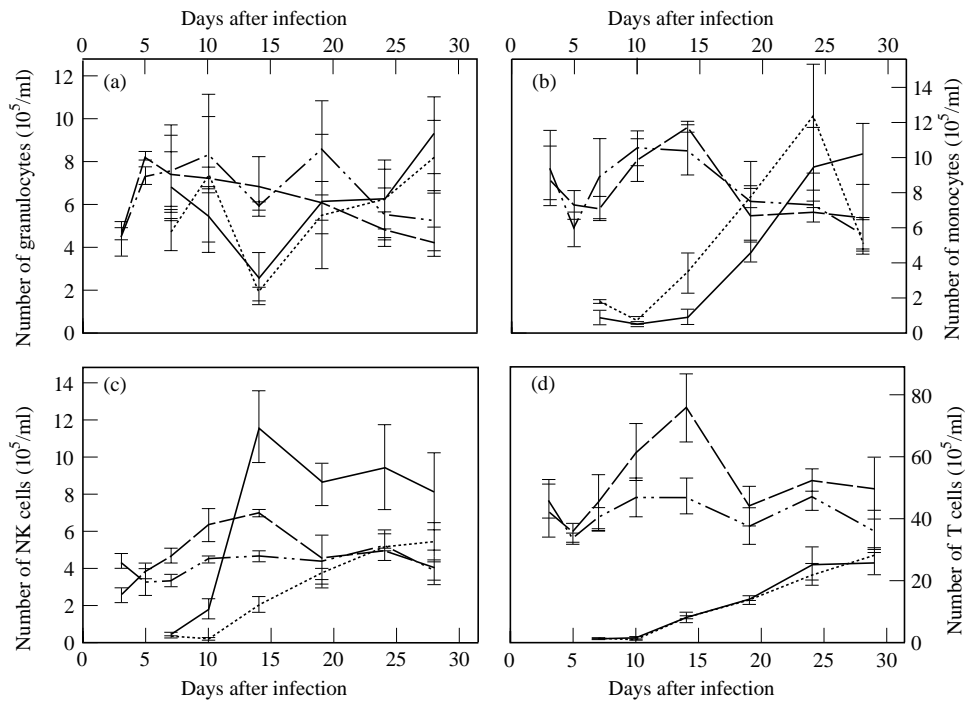


Fig. 2. Rat CMV (RCMV) infection results in an increase in specific immunity in immunocompetent rats and in non-specific immunity in immunosuppressed rats. Peripheral blood cells were analysed by five parameter flow cytometry. Granulocytes (a) were identified by their forward and side scatter profile, monocytes (b) as $CD4^+TCR\alpha\beta^-$, natural killer (NK) cells (c) as $CD8^+TCR\alpha\beta^-$, and T cells (d) as $TCR\alpha\beta^+$. Results are the mean (\pm s.d.) number of cells ($\times 10^5$) per ml blood of infected and irradiated rats (CMV^+TBI^+ , —; $n = 4$), irradiated rats (CMV^-TBI^+ ,; $n = 3$), infected rats (CMV^+TBI^- , - - - -; $n = 3$), and untreated control rats (CMV^-TBI^- , - · - · -; $n = 3$).

and a relative increase in the number of $CD8^+$ T cells, leading to a decrease in the $CD4/CD8$ ratio (Fig. 4) starting at day 10. The $CD4/CD8$ ratio was restored to about the normal level on day 28.

A detailed examination of the results of the flow cytometry revealed a phenotypically aberrant subpopulation of $CD8^+$ T cells, showing a decreased expression of CD8 and TCR (Fig. 5a,b) in the CMV^+TBI^+ group. Figure 5c shows the percentage of T cells expressing low amounts of TCR and CD8. The CMV^+TBI^+ group

showed a marked increase in the number of $CD8^{low}TCR^{low}$ cells, reaching on day 14 a maximum of 15.5% of the total number of T cells, being as much as 40% of the total number of $CD8^+$ T cells.

The salivary gland escapes NK cell infiltration upon RCMV infection

To determine whether the increase in the number of NK and $CD8^+$ T cells found in the peripheral blood of the CMV^+TBI^+

Table 1. The influence of CMV infection on the presence of T cells, natural killer (NK) cells and macrophages in immunosuppressed rats

Organ	T cells		NK cells		Resident macroph.		Infiltrating macroph.	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Salivary gland	=	↑	=	=	=	=	=	↑
Kidney	=	=	=	↑	=	=	↑	↑
Liver	=	=	↑	↑	↑	↑	↑↑	↑↑
Lung	=	=	=	↑	=	=	=	↑
Heart	=	=	=	=	↑	↑	↑	↑
Pancreas	=	↑	↑	↑↑	↑	↑	↑	↑↑

The presence of T cells, NK cells and macrophages in the internal organs on days 7 and 14 after infection was determined using immunohistochemistry. The animals were immunosuppressed by total body irradiation (5 Gy) and infected by i.p. injection of 3×10^5 plaque-forming units (PFU) rat CMV (RCMV).

=, No difference between infected and non-infected rats; ↑, moderate increase (the number of cells in the infected organs was < 15 per field of view (magnification $\times 400$) or the infected organ contained no more than twice the number of cells present in non-infected organs); ↑↑, strong increase (more than two-fold) in the number of stained cells in the infected compared with non-infected rats.

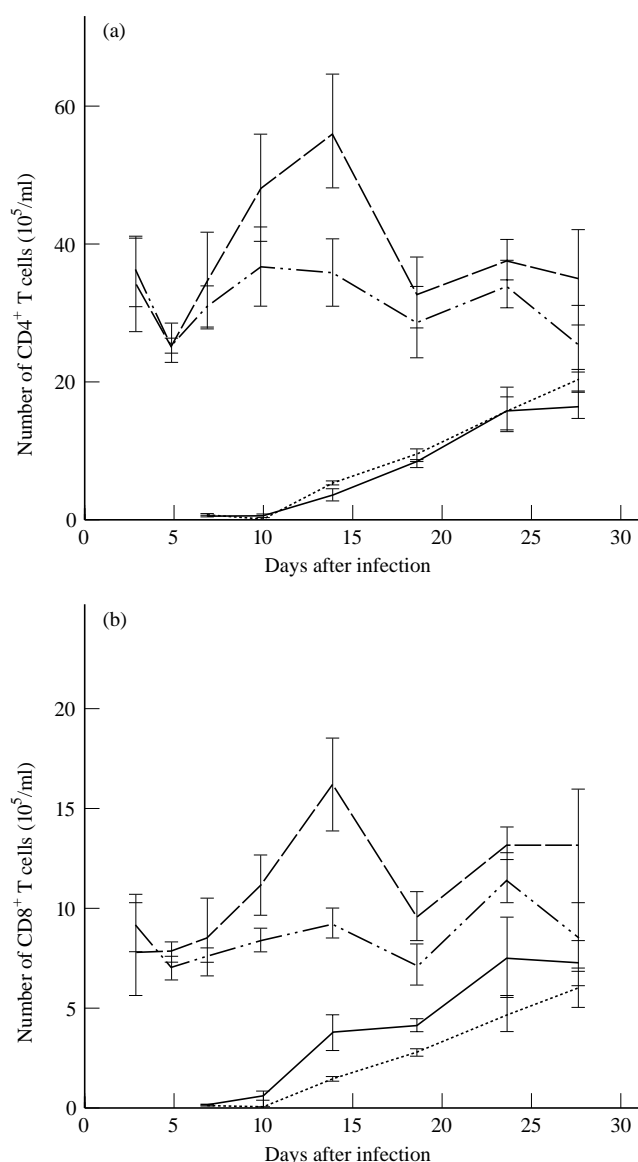


Fig. 3. Rat CMV (RCMV) infection causes an increase in the absolute number of CD4⁺ and CD8⁺ T cells in immunocompetent rats. In immunosuppressed rats infection with RCMV causes an increase in the absolute number of CD8⁺ T cells. Peripheral blood cells were analysed by three-colour flow cytometry. Helper T cells (a) were identified as CD4⁺TCR $\alpha\beta$ ⁺ and cytotoxic T cells (b) as CD8⁺TCR $\alpha\beta$ ⁺. Results are presented as the mean (\pm s.d.) number of cells ($\times 10^5$) per ml blood of infected and irradiated rats (CMV⁺TBI⁺, —; $n = 4$), irradiated rats (CMV⁻TBI⁺,; $n = 3$), infected rats (CMV⁺TBI⁻, - - - -; $n = 3$), and untreated control rats (CMV⁻TBI⁻, - · - · - ·; $n = 3$).

group could also be detected in the internal organs, immunohistochemical techniques were used. For this purpose, the salivary gland, kidney, liver, lung, heart and pancreas were examined for the presence of infiltrating T cells, NK cells and macrophages. Table 1 shows the presence of the leucocytes in the different organs on days 7 and 14 in infected animals compared with the presence of leucocytes in non-infected animals.

T cells. On day 14 after infection, no differences in the number of T cells were seen in the kidney, liver, lung and heart, while in the pancreas and the salivary gland a clear diffuse increase in the

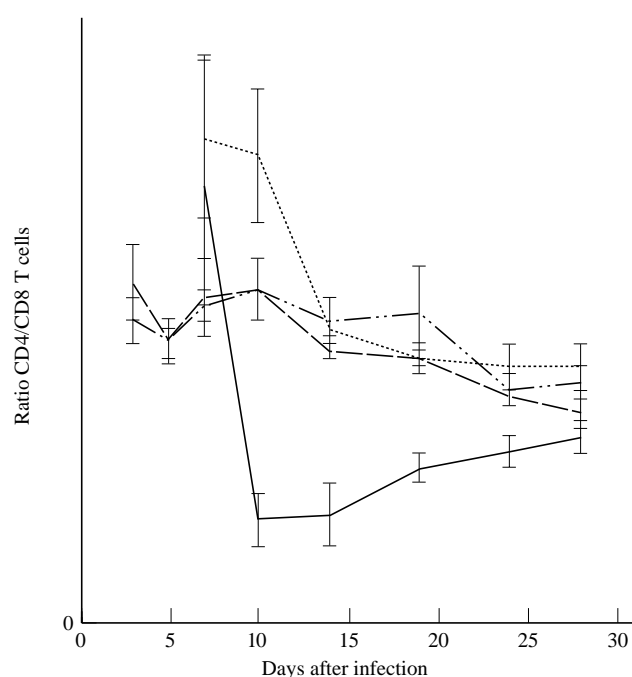


Fig. 4. Infection with rat CMV (RCMV) causes a decrease in the ratio of CD4/CD8 T cells in immunosuppressed rats. Peripheral blood cells were analysed by three-colour flow cytometry. Helper T cells were identified as CD4⁺TCR $\alpha\beta$ ⁺ and cytotoxic T cells as CD8⁺TCR $\alpha\beta$ ⁺. Results are presented as the mean (\pm s.d.) ratio of CD4⁺ T cells and CD8⁺ T cells of infected and irradiated rats (CMV⁺TBI⁺, —; $n = 4$), irradiated rats (CMV⁻TBI⁺,; $n = 3$), infected rats (CMV⁺TBI⁻, - - - -; $n = 3$), and untreated control rats (CMV⁻TBI⁻, - · - · - ·; $n = 3$).

number of T cells was seen in the infected group compared with the non-infected group, being most prominent in the pancreas. This increase seemed to be caused mainly by an increase in CD8⁺ T cells.

NK cells. In most organs of infected animals there was a diffuse increased infiltration of NK cells compared with non-infected rats. In the liver and pancreas, this could already be detected on day 7, while in the lung and kidney this was only visible on day 14. On day 14 the infiltration of NK cells was most prominent in the pancreas. In the salivary gland and the heart, no difference in the number of infiltrating NK cells was visible at both measure points.

Macrophages. The presence of macrophages was determined using MoAbs ED1 (inflammatory macrophages) and ED2 (resident macrophages). The liver, heart and pancreas showed a small increase in the number of resident macrophages in the infected rats on both days 7 and 14. The number of resident macrophages was not increased in the infected compared with non-infected group in the salivary gland, kidney and lung. As determined by ED1 expression, on day 7 the RCMV infection caused an increase in macrophage infiltration in the kidney, the liver (strong increase), heart and pancreas, while no effects were seen in the salivary gland and lung. On day 14, infection caused infiltration of macrophages in all organs. The largest increases in infiltrating macrophages were detected in the liver and the pancreas.

RCMV persists in many organs of the rat

In order to correlate the presence of infiltrating cells with the

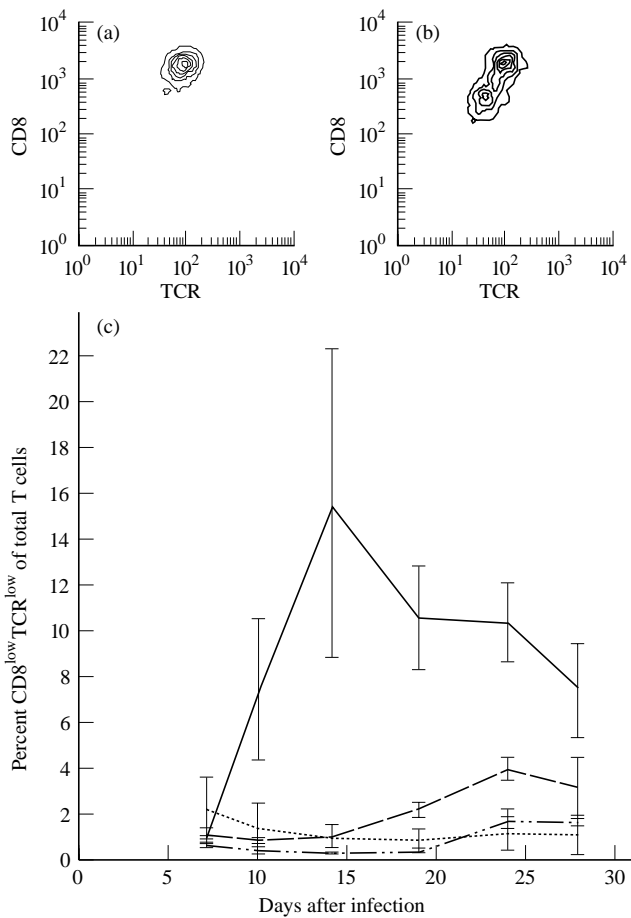


Fig. 5. Rat CMV (RCMV) infection in immunosuppressed rats results in the development of an aberrant CD8⁺ T cell subset with a decreased expression of CD8 and TCR. The expression of TCR and CD8 on CD8⁺TCR⁺ cells in CMV⁻TBI⁺ rats (a) and CMV⁺TBI⁺ rats (b) was determined using three-colour flow cytometry staining for CD4, CD8 and TCR. CD8^{low}TCR^{low} cells were identified as CD8⁺TCR $\alpha\beta$ ⁺ with relatively low expression CD8 and TCR $\alpha\beta$ (c). Results are the percentage (\pm s.d.) of CD8^{low}TCR^{low} of the total number of T cells of infected and irradiated rats (CMV⁺TBI⁺, —; $n=4$), irradiated rats (CMV⁻TBI⁺,; $n=3$), infected rats (CMV⁺TBI⁻, - - - -; $n=3$), and untreated control rats (CMV⁻TBI⁻, - · - · - ·; $n=3$).

presence of virus in the internal organs, analysis of RCMV infection was performed using three different techniques (Table 2).

Using immunohistochemistry the presence of viral antigens was detected, by plaque assay the amount of infectious virus was determined and by semiquantitative PCR the amount of viral DNA in the tested organ was determined. In the plaque assay at day 7 two of the two spleens, one of the two kidneys and one of the two lungs were positive. At day 14 all three salivary glands and one pancreas were positive. All positive organs contained < 166 PFU/ml, except for the salivary glands, which contained $> 10^4$ PFU/ml. Using immunohistochemistry, on day 7 only in one pancreas could viral antigen be detected. On day 14, all salivary glands and one pancreas were positive. In the pancreas the positive signal was present in the exocrine secretory cells and in the salivary gland in the mucous cells. Viral DNA could be detected in almost every organ: only the livers at day 7 and one lung at day 14 were

negative. The concentration of viral DNA in spleen, lung and heart was decreased at day 14 compared with day 7, while it remained constant in kidney and increased in the pancreas, salivary gland and liver. For the non-infected rats, all organs were negative in all three tests.

DISCUSSION

The main findings of this study are three-fold. First, infection with RCMV in the immunocompetent and immunosuppressed rat leads to reactions similar to those in humans and mouse, resulting in a preferential increase in the T cell and NK cell compartments, respectively. Next, the effects found in the PBL of immunosuppressed rats can in general also be detected in the internal organs. The salivary gland, however, seems to escape additional NK cell infiltration upon RCMV infection. Finally, in the immunosuppressed RCMV-infected animals an aberrant CD8⁺ T cell subset appears, with decreased expression of both CD8 and TCR, a phenotype which has been associated with anergy [29–31].

In mice both CD8⁺ T cells, CD4⁺ T cells and NK cells have been shown to be important in recovery from murine CMV (MCMV) infection. In immunocompetent mice MCMV infection resulted in an increase in CD8⁺ T cells, causing a decrease in the CD4/CD8 ratio at 6–13 days after infection [32]. CD8⁺ T cells specific for immediate early antigens can protect immunosuppressed mice from a lethal course of MCMV infection [33]. Depletion of CD4⁺ T cells or NK cells resulted in high levels of virus in various organs [34,35]. In severe combined immunodeficient (SCID) mice (void of functional T and B cells) the NK cells were activated to high levels of cytotoxicity, with a peak on days 3–5 after infection. However, in contrast to normal, these mice were unable to clear the infection [36].

In man, Carney *et al.* described a reversal of the normal CD4/CD8 T cell ratio during acute CMV mononucleosis, caused by an increase in CD8 and a decrease in CD4 cells [37]. Furthermore, recovery from HCMV infection in transplant recipients is associated with activation and up-regulation of CD8⁺ T cells [38–42].

Taken together, these data suggest that in case of a CMV infection of an immunocompetent host the CD4⁺ T cells, CD8⁺ T cells and NK cells play a role in limitation of the infection. Depletion of one of these cell types results in increased CMV replication in several organs, and sometimes severe pathology. Absence of T cells and B cells, as seen in SCID mice, results in more profound NK cell activation. The results in our rat model are quite similar to the results found in mice and in humans. RCMV infection in immunocompetent rats results in a small increase in the number of NK cells and a large increase in the number of T cells. In the immunosuppressed animals, where especially the number of T cells is very low after infection, there is a marked increase in the number of NK cells.

The effects of infection on the different leucocyte subsets in the peripheral blood of the CMV⁺TBI⁺ rats could also be detected in internal organs. These effects were correlated with the presence of virus or virus-infected cells within these organs. To detect the virus or the virus-infected cells, we used immunohistochemistry, plaque assays and PCR. Viral detection using plaque assay of various organs has been described before. Infectious virus and viral antigen could be detected in many organs early after infection, but starting at about 2 weeks post-infection, virus could only be detected in the salivary gland [11,12,17,43]. The detection of viral DNA using PCR has not been used before in rats. Our results show that even if

Table 2. Detection of virus and virus-infected cells in immunosuppressed rats

Organ	Immunohistochemistry		Plaque assay		PCR			
	Day 7	Day 14	Day 7	Day 14	Day 7	Mean titre	Day 14	Mean titre
Salivary gland	0/2	3/3	0/2	3/3†	2/2	10 ⁻¹	3/3	10 ⁻⁴
Spleen	0/2	0/3	2/2*	0/3	2/2	10 ⁻⁵	3/3	10 ⁻³
Kidney	0/2	0/3	1/2*	0/3	2/2	10 ⁻²	3/3	10 ⁻²
Liver	0/2	0/3	0/2	0/3	0/2	—	3/3	10 ⁻⁴
Lung	0/2	0/3	1/2*	0/3	2/2	10 ⁻³	2/3	10 ⁻¹
Heart	0/2	0/3	0/2	0/3	2/2	10 ⁻⁵	3/3	10 ⁻²
Pancreas	1/2	1/3	0/2	1/3*	2/2	10 ⁻²	3/3	10 ⁻⁴

The presence of virus or virus-infected cells was determined on days 7 and 14 after infection using immunohistochemistry (viral antigens), plaque assays (infectious virus) and polymerase chain reaction (PCR) (viral DNA). The rats were immunosuppressed by total body irradiation (5 Gy) and were infected by i.p. injection with 3×10^5 plaque-forming units (PFU) rat CMV (RCMV). The results are presented as the number of positive organs/total number of organs tested. For the PCR also the mean titre is presented.

* ≤ 166 PFU/ml; † $\geq 10^4$ PFU/ml.

no infectious virus or viral antigen can be detected, the viral DNA can still be present. Since all organs at day 14 after infection do contain viral DNA, all organs from seropositive donors might be able to transfer RCMV. It has been reported that the conditions of primary infection define the overall load with latent CMV, and that the copy numbers of latent viral genome in organs is the key parameter that determines the overall and organ-specific risk of recurrence [44]. For mice the lungs contain the highest viral load and it has therefore been described that the lungs are a major site of CMV latency [45]. On the other hand, Baltesen *et al.* [46] described that the control of viral replication in a particular organ, and thus the control of the number of viral DNA copies present in that organ after acute infection, is not linked to the copy number of latent virus. If in the rat the viral copy number in organs at day 14 is correlated with latency, the salivary glands, liver and pancreas are the most susceptible sites for latency.

Increased infiltration of T cells in the infected organs was detected in the salivary gland and pancreas. As expected, the presence of antigen seems to correlate with infiltration of T cells.

Increased infiltration of NK cells after infection was detected in most organs: the heart and the salivary gland are the only organs that escape NK cell invasion. In the heart no viral antigens or infectious virus could be detected, and the absence of additional infiltrating NK cells can thereby be explained. In the salivary gland, however, both viral antigen and infectious virus are present, suggesting that there is a difference between the infected cells in the salivary gland and in other tissues. This phenomenon, not previously described, could be explained by the expression of a viral MHC class I homologue, which inhibits attack by NK cells [9,10]. A deletion mutant for the MHC class I homologue of MCMV causes a decrease in infection of the spleen, liver and lung. However, in the salivary gland the titre remained as high as in case of infection with wild-type MCMV [9]. These results suggest that the salivary gland does not use this mechanism to escape the NK cell reaction. Apparently, there is some kind of tissue-specific barrier for the additional NK cells to enter the salivary gland upon RCMV infection. This might contribute to the fact that infectious virus remains detectable in the salivary gland for at least a few months [43].

An interesting observation in the group of infected immuno-

suppressed rats is the detection of a phenotypically aberrant subpopulation of CD8⁺ T cells, showing a decreased expression of both CD8 and TCR. At day 14 post-infection, this subpopulation covered about 40% of the total number of CD8⁺ T cells. The increase in the number of CD8⁺ T cells was entirely caused by the increase in the number of CD8^{low}TCR^{low} cells (data not shown).

Three-colour staining excluded the possible characterization of this CD4⁻CD8^{low}TCR^{low} subset as being NK cells (CD4⁻CD8^{low}TCR⁻), activated CD4 T cells (CD4⁺CD8^{low}TCR⁺) or immature T cells (CD4⁺CD8⁺TCR^{low}) [28,47]. Since the forward and side scatter profile of these cells was similar to the profile of normal T cells, the possibility of these cells being dying cells was excluded. Dying cells are expected to have reduced forward and increased side scatter profiles. Down-regulation of both TCR and CD8 occurs in anergic T cells [29–31], which are unresponsive to antigen. Anergy is induced if the antigen is presented in the absence of proper costimulatory signals [48]. Beside clonal deletion and suppression, induction of anergy is an important mechanism of peripheral tolerance induction for self-antigens. Our results suggest that in the rat model CMV infection not only interferes with MHC class I expression [4–7], but also interferes with the costimulatory signals upon antigen presentation of viral antigens. This renders the virus-specific T cells anergic and enables the virus to escape further elimination by the immune system of immunocompromised hosts. Tests are being performed to obtain more functional information about this CD8^{low}TCR^{low} subpopulation.

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