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Summary

Reactivation from latent cytomegalovirus (CMV) infection is often associated with conditions of immunosuppression and can result in fatal disease. Whether the maintenance of systemic CMV latency is mainly governed by factors of the infected cell or by immune control functions is unknown. Likewise, the putative immune control mechanisms which could prevent the induction and spread of recurrent CMV infection are not clearly identified. We took advantage of latently infected B cell–deficient mice and a sensitive method for virus detection to study CMV reactivation after ablation of lymphocyte subsets. A crucial role of both T lymphocytes and natural killer (NK) cells was demonstrated. Within 5 d after depletion of lymphocytes, productive infection occurred in 50% of mice, and 14 d later 100% of mice exhibited recurrent infection. A hierarchy of immune control functions of $CD8^+$, NK, and $CD4^+$ cells was established. Reactivation was rare if only one of the lymphocyte subsets was depleted, but was evident after removal of a further subset, indicating a functional redundancy of control mechanisms. The salivary glands were identified as the site of most rapid virus shedding, followed by the detection of recurrent virus in the lungs, and eventually in the spleen. Our findings document a previously unknown propensity of latent CMV genomes to enter productive infection immediately and with a high frequency after immune cell depletion. The data indicate that only the sustained cellular immune control prevents CMV replication and restricts the viral genome to a systemic state of latency.

Key words: cytomegalovirus • latency • reactivation • T lymphocytes • B cell–deficient mice

After primary CMV infection, immune control mechanisms effectively prevent overt disease and terminate virus replication. However, ultimate clearance of the viral genome is not achieved. Instead, CMVs are able to remain lifelong at specific sites in their natural hosts. During the nonproductive, latent state of infection, the viral gene expression is minimized to a small subset of genes (1, 2). From latent infection the viral replication cycle can be initiated and results in transient phases of virus shedding and recrudescent disease. The establishment of latency and the reactivation to episodes of recurrent infection are general hallmarks of herpesvirus biology.

The lifelong persistence of the herpesvirus genome renders the host at permanent risk for recurrent infection. In immunocompromised patients like transplant recipients or individuals with impaired immunity due to infection with HIV, recurrence of human CMV (HCMV)[1] infection frequently leads to overt manifestations of disease (3). Although impairment of cellular immune control functions is a prime risk factor for CMV recurrence, it is apparent that only some patients at risk develop CMV disease. The identification of predisposing factors is clearly needed to predict the incidence and outcome of reactivation events.

The risk factors for virus reactivation and development of recurrent disease are poorly characterized, but are

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[1] *Abbreviations used in this paper:* HCMV, human cytomegalovirus; HSV, herpes simplex virus; MCMV, murine cytomegalovirus; MEF, mouse embryo fibroblast(s); p.i., postinfection; SGV, salivary gland virus.

known to include at least three factors. First is a high load of latent genomes, since the frequency of initiation of the virus replication program increases with the number of latently infected cells (4, 5). Second, stimulation of hematopoietic cells can initiate reactivation by switching the transcriptional control to productive infection (6). Third, immune control must fail to attack virus-infected cells during or shortly after initiation of the replicative program.

From clinical experience with immunocompromised patients it is clear that clinical reactivation of certain herpesviruses, namely CMV, is more frequent and clinically relevant than others. Two explanations are possible. Either the unknown frequency of reactivation at the cellular level is as low as for other herpesviruses but manifest recurrence is high due to immunoevasive gene functions of CMV (7), or, alternatively, despite the subversive gene functions, the frequency of reactivation events is in fact very high but disguised by a stringent and continuous immune control.

To distinguish between these possibilities, we have established the model of latent murine CMV (MCMV) infection in the Ig μ chain–deficient mouse (8). Previous studies in these mice devoid of B cells and antibodies revealed that antibodies are not required for the resolution of primary CMV infection, and latency is established with the same kinetics as in seropositive controls. The load of viral genomes that represents an independent risk factor for the occurrence of recurrent infection (5) is identical between B cell–deficient and normal mice (8). Taking advantage of the facilitated detection of recurrent CMV in B cell–deficient mice and by using an optimized assay for the detection of infectious virus in organs, we were able to study the contribution of lymphocyte subsets to the prevention and immune surveillance of recurrent CMV infection. The data indicate that the removal of T lymphocytes and NK cells rapidly results in CMV recurrence at multiple sites. Thus, the clinical state of CMV latency is governed by immune control functions.

Materials and Methods

Establishment of Latency in B Cell–deficient Mice. Mice homozygous for a μ chain mutation (μMT/μMT; reference 9) were bred in the Central Animal Facility, Rijeka University School of Medicine. Homozygous and heterozygous offspring were distinguished by testing their sera for the presence of IgM antibodies. The absence of CD45R(B220)$^+$ and IgM$^+$ cells in the spleens of homozygous animals was verified by cytofluorometry in B cell–deficient animals. 6–8-wk-old mice were injected in the rear footpad with 10^5 PFU of tissue culture–derived virus in a volume of 50 μl. Latency was established within 12 wk after primary infection as determined by monitoring infectious virus in tissues.

Viruses. The Smith strain of MCMV (VR-194; American Type Culture Collection, Rockville, MD) was propagated in mouse embryo fibroblasts (MEFs) and concentrated by ultracentrifugation on a 15% sucrose gradient (10). The salivary gland isolate of MCMV (SGV) was propagated in 6 Gy γ-irradiated MCMV-infected mice of the BALB/c strain. 2 wk after infection, salivary glands were collected, homogenized, and stored at −70°C.

Plaque Assay for the Detection of Infectious MCMV in Tissues. Mouse organs were frozen immediately at −70°C after collection. The presence of infectious virus was determined by titrating organ homogenates using a standard plaque assay (10). The detection limit of the assay was extended to 1 PFU per organ homogenate as described previously (8). In brief, each organ was homogenized in 10 ml of MEM supplemented with 5% FCS and distributed after log$_{10}$ titration in 24-well plates containing a semiconfluent monolayer of MEFs (10^5 cells/well). After 30 min of incubation at 37°C, plates were centrifuged at 1,100 g for 30 min. Plaques were counted 7 d later.

In Vivo Assay for the Detection of Infectious MCMV in Tissues. The presence of infectious virus in organs and blood of latently infected B cell–deficient mice was tested by using a sensitive in vivo assay. 0.5 ml of blood or homogenized organs was resuspended in 2 ml of MEM supplemented with 3% FCS. One half of each homogenate was diluted 1:10 and tested for the presence of virus. The other half of the homogenate was injected intraperitoneally into a 6–8-wk-old naive C57BL/6 mouse. To exclude the possibility of virus reactivation in living cells, collected organs (salivary glands, lungs, and spleen) and peripheral blood specimens were freeze-thawed before homogenization. Indicator mice were kept in separate cages to prevent horizontal transmission of MCMV. Sera were collected 4 wk after the injection and tested for the presence of MCMV-specific antibodies by ELISA (11). Mice were subsequently immunodepleted by a single injection of cyclophosphamide (EndoxanR, 150 mg/kg) and a cocktail of cytolytic mAbs (1 mg/animal) to CD4 (YTS 191.1.2.; reference 12), CD8 (YTS 169.4.2; reference 12), and NK1.1. (PK 136; reference 13). mAb treatment was repeated after 1 wk. In addition, the animals were injected with hydrocortisone sodium succinate (125 mg/kg) every other day. Virus titers in salivary glands were determined 2 wk after initiating immunodepletion. To determine the sensitivity of the assay, organ homogenates of naive B cell–deficient mice were mixed with ascending doses (0.2, 2, and 20 PFU; titer based on the centrifugal enhancement of infectivity) of SGV and subjected to the same protocol. In contrast to organ homogenates from latently infected mice, injection of salivary gland or lung homogenates from uninfected animals supplemented with 2 or 0.2 PFU of infectious virus led to seroconversion in 100 and 20%, respectively, of mice, and infectious virus could be recovered from 100 and 20%, respectively, of recipients after immunodepletion (data not shown).

Depletion of Lymphocyte Subsets. Lymphocyte subsets in latently infected mice were selectively depleted 12 wk postinfection (p.i.) as described previously (11, 14). In brief, 500 μg of purified mAbs was used for the elimination of the CD4$^+$, CD8$^+$, and NK1.1$^+$ cell subsets. Mice were injected every fifth day throughout the duration of the experiment. The efficacy of the depletion of cells was monitored by standard two-color cytofluorometric analysis of spleen and lymph node cells using the following reagents: anti-Lyt 2–FITC (Becton Dickinson, San Jose, CA), anti–L3T4-PE (Becton Dickinson), anti–NK1.1-biotin (PharMingen, San Diego, CA), anti-CD2-PE (PharMingen), and streptavidin-FITC (Becton Dickinson). NK cells were analyzed on the gated CD2$^+$ population indicating efficacious depletion of NK1.1$^+$ cells in mice treated with PK 136 mAb. In addition, functional depletion of NK cells in infected B cell–deficient mice was confirmed by standard chromium-release assays against YAC-1 target cells.

In Vivo Neutralization of IFN-γ. Neutralization of IFN-γ was performed by injection of purified mAb R4.6A2 (15) or a polyclonal rabbit anti–rIFN-γ antiserum described previously (16).

Mice were injected intraperitoneally with 200 μg of mAb or 250 μl of rabbit antiserum at the beginning of the experiment and every other day thereafter. The neutralization capacity of the rabbit antiserum was determined by an in vitro assay based on the ability of rIFN-γ to induce MHC class I molecule expression on MEFs (0.02 ng of rIFN-γ [1 U] induced maximal expression of L^d molecules on fibroblasts after 48 h of incubation). rIFN-γ was neutralized with serial dilutions of antiserum at 4°C for 2 h, and 1 ml of each sample was then added to a semiconfluent culture of MEFs (10^5 cells/well, 24-well plates). Expression of L^d molecules was measured after 48 h of incubation by flow cytometry using anti–L^d biotin–conjugated antibodies (PharMingen) and streptavidin-FITC as a second reagent (Becton Dickinson). The neutralizing capacity of the antiserum was defined as the reciprocal value of the highest serum dilution that reduced MHC I expression by 50% compared with cultures treated with IFN-γ. According to this assay, 1 ml of anti–IFN-γ serum was able to neutralize ~0.8 μg of rIFN-γ.

Statistical Analysis and Deduction of a Mathematical Model of Reactivation. The percentage of mice in which recurrent virus was detected was used to generate a mathematical model describing the probability of recurrent infection over time. The following assumptions were made: (*a*) a single reactivation event will lead to the generation of at least one infectious virion; (*b*) reactivation is a stochastic process; and (*c*) at least 24 h are required from activation of the viral genome to the production of infectious virus. The probability of recurrence is described by the Poisson distribution (valid for time $t > t_0$):

$$P(t) = 1 - \exp[-(t - t_0)]/T_0$$

where t is time, t_0 is the time at which the first progeny virions can be detected after immunodepletion, and T_0 is the time period (starting from t_0) in which recurrent virus can be detected in 100% of mice. A linear function (see *broken line*, Fig. 1 *B*) is approximated from the Poisson distribution as curve $P(t) = (t - t_0)/T_0$ for $(t - t_0)/T_0 < 0.5$.

Results

Rapid CMV Recurrence after Depletion of T Lymphocytes and NK Cells in Latently Infected B Cell–deficient Mice. μMT/μMT mice lacking B cells and antibodies terminate acute MCMV infection and establish and maintain latency as quickly and efficiently as their heterozygous littermates. However, during CMV recurrence, the virus titer is increased in organs 100–1,000-fold in the absence of virus-specific antibodies compared with normal littermates (8). Therefore, in normal mice the sensitive detection of reactivation events is impeded by the neutralization of virus by antibodies. To determine the frequency of reactivation and the sites and kinetics of recurrent CMV infection, we used latently infected μMT/μMT mice and depleted the major lymphocyte subsets. Mice were depleted simultaneously of $CD4^+$, $CD8^+$, and $NK1.1^+$ cells by treatment with cytolytic mAbs and killed at various time points after treatment. Already 3 d after immunodepletion, in two out of nine (22%) mice, recurrent virus could be detected in the salivary glands. 5 and 7 d after treatment, respectively, virus titers were detected in 2 out of 8 (25%) in the lungs and in 2 out of 12 (17%) in the spleen, respectively (Fig. 1 *A*).

Within 2 wk after the initiation of immunodepletion, recurrence occurred in the salivary glands and lungs of all mice. In contrast, in seropositive control mice receiving the same cocktail of mAbs, recurrent MCMV was seen only occasionally and in very low titers in the salivary glands and in the lungs of very few mice on day 14 (Fig. 1 *A*).

Fig. 1 *B* depicts this observation as the percentage of mice in which CMV reactivation occurred in the salivary glands as a function of time. Based on this curve, we can deduce two biological parameters of CMV reactivation at this site: t_0, the time point at which infectious virus is manifest for the first time, and T_0, the time period required for the occurrence of reactivation in the entire population of mice (see Materials and Methods). By extrapolating the linear portion of the Poisson distribution (*broken line*), it is possible to determine the time in which recurrent virus is detected in 100% of mice. Based on this mathematical model, we determine (*a*) that ~1.5 d (t_0) after immunodepletion, the first progeny virus is detectable in the salivary glands; (*b*) that the average time required to detect recurrent virus at this site in 50% of the animals (recurrence event, RE_{50}) is <6 d; and (*c*) that all mice develop recurrent CMV infection in the salivary glands within 8 d (T_0).
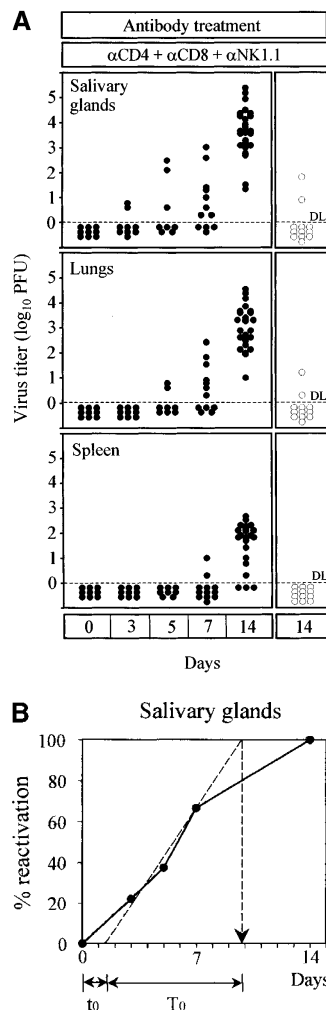

Figure 1. MCMV recurrence after depletion of T lymphocytes and NK cells. (*A*) Latently infected μMT/μMT B cell–deficient mice and heterozygous μMT/+ mice were immunodepleted of $CD4^+$, $CD8^+$, and $NK1.1^+$ cells 12 wk p.i. Infectious virus in the salivary glands, lungs, and spleen was determined after immunodepletion as indicated. Shown are the titers of recurrent virus in the salivary glands (*top*), lungs (*middle*), and spleen (*bottom*) of homozygous (●) and heterozygous (○) mice. *DL*, Detection limit. (*B*) The percentage of mice in which recurrent virus was detected in the salivary glands (*solid line*) was used to generate a mathematical model describing the probability of recurrence over time (see Materials and Methods). *Broken line*, A linear function differentiated from the Poisson distribution as a curve $P(t) = (t - t_0)/T_0$, for $(t - t_0)/T_0 < 0.5$. *Arrow*, The time point at which recurrent virus is predicted in 100% of mice.

With a delayed but similar kinetics, recurrent virus was also detected in the lungs and finally in the spleen (see Fig. 1 *A*). From these data, we conclude that in the absence of T lymphocytes and NK cells the emergence of recurrent CMV is a rapid process.

CMV Recurrence in B Cell–deficient Mice Is Stochastic and Multifocal. The emergence of recurrent virus in the salivary glands and the delayed increase of infectious virus in the lungs and spleens of B cell–deficient mice, albeit at a lower titer, could be explained by two possibilities. First, recurrence could be a stochastic event that occurs coincidentally but independently in different tissues. Alternatively, and specifically in the antibody-deficient environment of our experimental setting, recurrent virus could originate from a single organ, e.g., the salivary glands, and subsequently disseminate to the other organs tested. To address this question, we compared titers of recurrent virus in different organs of individual μMT/μMT mice. At day 7 after ablation, discrete patterns with cases of positive lungs corresponding to negative salivary glands and vice versa were observed (data not shown). 1 wk later, individual mice exhibited widely different titers of recurrent virus in the salivary glands versus the lungs and spleen, without correlation to the productive infection between these organs (Table 1). In addition, selective reactivation restricted to a single organ or infectious virus present in the salivary glands and the lungs, but not in the spleen, was also seen under different experimental conditions (Table 2, and Fig. 2). Altogether, the results exclude that multifocal detection of recurrent MCMV requires dissemination of virus from the salivary glands to multiple peripheral sites. In agreement with previous reports, the data support the notion that recurrence occurs as a stochastic event independently at multiple sites (5).

The Presence of Cell-mediated Immunity Prevents the Formation of Recurrent CMV. Hypothetically, the virus recovered after immunodepletion could represent the expansion of infectious virus that preexisted at very low levels rather than reactivation from true latency. To test this possibility, an in vivo assay detecting infectivity with very high sensitivity was developed, allowing the detection of single infectious particles within an organ (see Materials and Methods). All organs from all latently infected mice analyzed remained negative for infectious virus (data not shown). These results strongly argue against a general persistence of infectious virus at low levels, and confirm again the existence of a nonproductive state of infection in B cell–deficient mice. Thus, if virus replication after ablation of immune control functions reveals the real frequency of naturally occurring reactivation events, the lack of infectious virus during latency in antibody-negative mice must indicate that the immunosurveillance functions extinguish reactivation events and operate before the formation of an infectious virus progeny.

Impact of T Cell Subsets and NK Cells on Recurrent CMV Infection. To characterize the phenotype of cells involved in immune control and their relative contribution, latently infected mice were depleted of T lymphocyte subsets by injections of cytolytic anti-CD4 and/or anti-CD8 mAbs. 2 wk after the initiation of antibody treatment, the presence of infectious virus was quantitated in the salivary glands, the spleen, and the lungs by virus titration. The ability to detect infectious virus, irrespective of the titer, in any of the organs was scored as recurrent infection. Only rarely did the removal of a single T cell subset result in productive reactivation in an animal (Fig. 2 *A*). In contrast, 15 out of 21 animals (73%) were scored positive after depletion of both CD4+ and CD8+ T cells (Fig. 2 *A*), and virus became detectable in the salivary glands and lungs. However, this immunosuppression protocol did not lift the immune control in the spleen (Fig. 2 *B*). In conclusion, neither the CD4+ nor the CD8+ subset was indispensable for the maintenance of latency, indicating a mutual functional redundancy of both T cell subsets. In addition, in the absence of both CD8+ and CD4+ T cells, MCMV recurrence is still prevented in the splenic tissue.

The absence of recurrent virus in the spleen of T cell–depleted animals suggested a pivotal role for NK cells in the
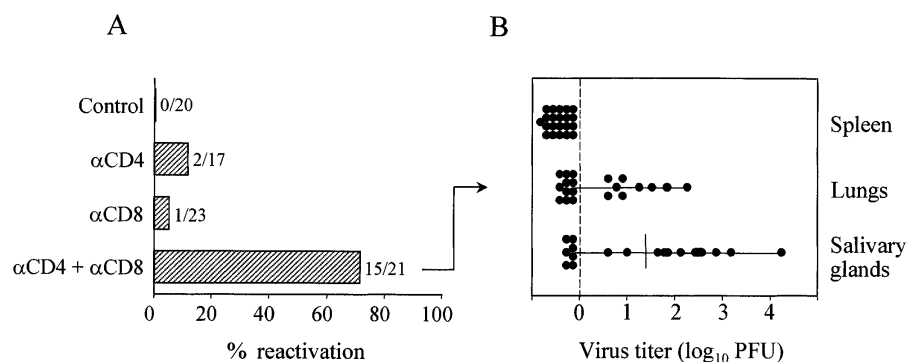
Table 2. *MCMV Recurrence in Mice after Combined Depletion of NK Cells and T Lymphocyte Subsets*

mAb treatment	No. of mice with recurrent infection (%)		
	Salivary glands	Lungs	Spleen
αNK	1/18 (5.6)	0/18 (0)	0/18 (0)
αCD4 + αNK	6/24 (25)	1/24 (4.2)	0/24 (0)
αCD8 + αNK	12/15 (80)	13/15 (86.7)	12/15 (80)
αCD4 + αCD8 + αNK	25/25 (100)	25/25 (100)	22/25 (88)

Latently infected B cell–deficient mice (μMT/μMT) were depleted of NK cells, either alone or in combination with CD4+ and/or CD8+ T lymphocytes. 2 wk later, organs were analyzed for recurrent MCMV.

Table 1. *Detection of Recurrent MCMV in Organs of Individual Mice 14 d after Depletion of CD4+, CD8+, and NK1.1+ Cells*

Mouse	Salivary glands	Lungs	Spleen
1	33	133	67
2	1200	4600	124
3	1000	4600	67
4	1300	15000	200
5	4000	4000	67
6	24600	24000	78
7	157000	4000	120
8	22	10	0
9	24000	500	120
10	90660	400	6
11	2000	86	68
12	250000	2000	0

Figure 2. Recurrent infection after selective depletion of T cell subsets. (*A*) Latently infected B cell–deficient mice were depleted of CD4^{+} and/or CD8^{+} T lymphocytes. Virus titration of the spleen, lungs, and salivary glands was performed 2 wk later. Shown are percentages of mice with infectious virus in at least one of the organs analyzed. (*B*) Recurrent virus in mice depleted of CD4^{+} and CD8^{+} T cells. Titers of individual mice (●) and median values (*horizontal lines*) are shown. *Dashed line*, Detection limit of infectious virus.

control of persistent CMV infection in the spleen. Therefore, the contribution of NK cells was tested (Table 2). The selective removal of NK cells was not sufficient to allow recurrent infection in any of the organs investigated, including the spleen (Table 2). To determine the potential cooperation between NK and T cells, latently infected mice were depleted of NK cells simultaneously with either CD4^{+} or CD8^{+}, or both T cell subsets. Depletion of CD4^{+} and NK cells permitted recurrent infection in only 25% of the animals, and recurrent virus was detected mainly in the salivary glands, but not in the spleen. In contrast, depletion of both CD8^{+} and NK cells allowed the detection of virus in 100% of the animals in one of the organs tested, although not in every analyzed organ of each individual animal.

To assess the regulating function of CD4^{+} T lymphocytes in the control of recurrent CMV infection, virus productivity was compared in mice depleted of CD8^{+} T lymphocytes and NK cells in the presence and absence of CD4^{+} T cells (Table 2). Under these conditions, CD4^{+} T cells restricted virus replication in the salivary glands and lungs to titers 1–2 $\log_{10}$ PFU lower (data not shown). From the collective data, we conclude that (*a*) the removal of one lymphocyte subset alone, irrespective of CD4^{+}, CD8^{+}, or NK cells, is insufficient to allow MCMV recurrence; (*b*) in the absence of NK cells, the CD8^{+} subset is much more effective in virus control compared with CD4^{+}; (*c*) control of recurrence is lifted in the absence of CD8^{+} and NK cells, although CD4^{+} T cells can limit the extent of multiplication of recurrent virus in this situation. Altogether, the prevention and control of recurrent CMV infection are secured by multiple, hierarchic, and functionally redundant lymphocyte populations.

IFN-γ Participates in the Control of CMV Recurrence. Previous studies demonstrated that IFN-γ is essential for the termination of primary CMV infection in vivo (17) and combats the cytomegaloviral escape from antigen presentation to CD8^{+} T cells (18). To assess the role of IFN-γ in the prevention of virus recurrence, latently infected B cell–deficient mice were depleted of IFN-γ by neutralizing mAb with or without simultaneous depletion of either CD4^{+} or CD8^{+} T lymphocytes. As depicted in Table 3, CMV recurrence was detected only rarely in mice selectively ablated of only CD4^{+} or CD8^{+} T lymphocytes.

Likewise, selective neutralization of IFN-γ did not induce recurrent infection in latently infected B cell–deficient mice. Strikingly, single T cell subset depletion of either CD4^{+} or CD8^{+} T cells together with IFN-γ neutralization yielded recurrent infection in ~75% of mice.

Next, we tested whether in this situation application of rIFN-γ is able to maintain the nonproductive state of infection in T and NK cell–depleted animals when administered in a dose sufficient to exert systemic antiviral effects in vivo (references 17 and 19, and data not shown). We constantly failed to achieve this goal, and concluded that systemic IFN-γ therapy is not sufficient to prevent local recurrent CMV infection, but IFN-γ is essential to ascertain the functional redundancy of latency control by CD4^{+} and CD8^{+} T cells.

Discussion

In this study, we took advantage of the 100–1,000-fold facilitated detectability of recurrent MCMV in B cell–deficient mice and an optimized isolation of infectious virus in vitro. This combined approach provided for the first time the opportunity to assess the individual roles of CD8^{+} and CD4^{+} T cells, NK cells, and IFN-γ in the control of recur-

Table 3. *Participation of IFN-γ in T Cell–mediated Control of Recurrent MCMV Infection*

mAb treatment	No. of mice with recurrent infection (%)
Control	0/20 (0)
αIFN-γ	0/15 (0)
αCD4	2/17 (11.8)
αCD4 + αIFN-γ	10/13 (76.9)
αCD8	1/23 (4.3)
αCD8 + αIFN-γ	11/15 (73.3)

Latently infected B cell–deficient mice (μMT/μMT) were depleted of IFN-γ either alone or in combination with CD4^{+} and/or CD8^{+} T lymphocytes. 2 wk later, salivary glands, lungs, and spleen were tested for recurrent MCMV. Mice showing recurrent virus in any of these organs were scored positive.

rent CMV infection. The stepwise withdrawal of immune control functions revealed that both the CD8$^+$ and CD4$^+$ T cell subsets as well as NK cells contribute to CMV control in a hierarchic but not exclusive fashion. The data document a previously undescribed propensity of CMV to frequently initiate productive replication cycles which, in the absence of cellular immune control, result in virus recurrence.

This new finding suggests that the maintenance of systemic CMV latency is less determined by specific factors of the cell in which CMV is latent, than primarily governed by immune functions mediated by T lymphocytes and NK cells. This situation is remarkable because it differs from the concepts of herpesviral latency that have been established for human alpha- and gammaherpesviruses, i.e., herpes simplex virus (HSV) and EBV, respectively. In contrast to CMV, the cellular compartment of viral persistence is defined for both HSV and EBV (20, 21). HSV establishes latency in neurons that represent a site of exceptional immunoprivilege, e.g., devoid of MHC molecules and therefore barely controlled by T cells (22, 23). Rather, nonimmune stimuli such as physical or emotional stress, intake of hormones, or administration of drugs can induce HSV recurrence (20). Only the release of virus progeny after retrograde axonal transport exposes HSV to the host immune response, which restricts the recurrent infection to local mucosal tissues. Likewise, nonproductive EBV carriage in B lymphocytes is interpreted as a state of immunological silence. Remarkably, EBV can restrict viral gene expression, and a subpopulation of infected B cells expressing only EBNA1 has been found in seropositive individuals (24). In the light of a minimal or even absent antigenicity of EBNA1 (25), it is plausible to assume that this B cell compartment represents the reservoir where EBV can persist without detection by T cells. CMV, too, expresses genes that avoid the detection of infected cells by T cells at least during productive infection (for a review, see reference 7). It will be interesting to learn whether CMV like EBV use these functions to modulate the host immune response during latency.

Memory T cells have been thought to be dormant and inactive in the absence of their specific antigen. However, recent work has shown that memory CD8$^+$ T cells are rapidly able to resume effector functions like secretion of IFN-γ or cytolytic activity within a few hours of antigen contact without the need to divide and differentiate (26, 27). Our finding that latent CMV infection immediately returns to productive virus replication after withdrawal of T and/or NK cells suggests that the nonproductive state of CMV latency represents in fact a state of active cellular immune control. How could these immune cells prevent reactivation of productive infection? MCMV and in particular HCMV are very slowly replicating viruses. Both NK cells and CD8$^+$ lymphocytes are cytolytic effector cells which are able to recognize and lyse infected target cells before the formation of infectious virus. In addition, T and NK cells release specific cytokines, i.e., IFN-γ and TNF-α, which can efficiently inhibit late gene transcription and block CMV replication at the stage of nucleocapsid formation (28). Accordingly, the neutralization of IFN-γ gives rise immediately to recurrent infection when combined with the depletion of either CD8$^+$ or CD4$^+$ T lymphocytes. The idea that CMV latency is controlled by immunological forces is further supported by the demonstration of viral gene expression in tissues harboring latent CMV genomes (29–32). Viral gene expression during latency provides a source of viral antigen for continuous immunological restimulation which is enhanced by IFN-γ (18, 19). The presented antigens could activate CMV-specific T cells and constantly drive the emigration of recirculating lymphocytes into latently infected solid tissues (33).

In the past, experimental analysis of immune responses against CMV has focused on studying the primary infection in the immunologically naive host (11, 14, 34–39). However, in the natural situation, CMV persists lifelong in its host, with phases of latent or nonproductive and recurrent or productive infection which expose the virus to the most part to an already primed immune system. In this context, two principal aspects require attention: first, whether there are differences in the immune response to recurrent versus primary CMV infection, and second, whether virus productivity in tissues is altered in recurrent infections compared with primary infection.

In primary infected BALB/c mice, CD8$^+$ T cells play a major and protective role by clearing productive infection in visceral organs, with the exception of the epithelial cells of the salivary glands (10, 14). Resolution of acute MCMV infection in this particular tissue is mediated primarily by CD4$^+$ T cells (11). NK cells delay but fail to protect from lethal disease (37), while antibodies are dispensable for the resolution of primary infection (8). Unlike BALB/c mice, selective depletion of CD8$^+$ T cells has little effect on virus clearance during the course of primary infection in C57/BL6 mice (38). Here, NK cells prominently contribute to the suppression of virus replication during primary infection due to the *Cmv-1* gene locus, which confers NK cell–dependent resistance to MCMV to this strain of mice (39). Remarkably, for the control of recurrent infection in C57/BL6 mice, not NK cells but CD8$^+$ T cells exhibit the most prominent function.

There are also differences in the sequence of organs producing CMV during recurrence. In the naive host, CMV replicates first in visceral organs, including the lungs, spleen, and liver. In a later phase of infection, when virus multiplication in these tissues is already ceasing due to clearance of infectious virus by CD8$^+$-dependent immune control functions, increasing titers are found in the salivary glands. There, infectious virus persists for >2 mo to be cleared by CD4$^+$-mediated functions (14, 17). Frequently, the sequence of recurrent infection is the reverse of the primary infection: reactivated virus emerged first in the salivary glands. Notably, and unlike the clearance of primary infection, the ablation of CD8$^+$ T cells is more crucial for reactivation than the depletion of CD4$^+$ T cells (see Table 2). Next, recurrent infections occurred in the lungs and finally in the spleen, revealing an organ-specific control of

recurrence of different stringency. Thus, our findings reproduce clinical experience, in that asymptomatic recurrent infection has to be distinguished from recurrent disease. Although the former is usually associated with virus shedding at focal sites, e.g., into the saliva or urine, the latter is manifested by disseminated infection in visceral organs (3). Finally, our results define the salivary glands as a site of particular risk of recurrence compared with other tissues. A preferential but restricted recurrence at this site favors virus shedding and transmission without harming the host. This observation cannot be explained by the tissue load of latent CMV, because the burden of latent CMV genomes is not higher in the salivary glands (5).

In essence, the data show that recurrent CMV infection is not a repetition of the primary infection. The incidence and kinetics of recurrence are tightly regulated by distinct cellular immune functions which operate in a redundant fashion and in a hierarchical order. With the advent of a growing number of drugs designed for selective immune suppression, a better understanding of the key immune functions in CMV immune surveillance offers a rational basis for prevention of disease manifestations in patients at risk. The B cell–deficient mouse may provide a promising model that allows mimicking of the manifestations of recurrent human CMV disease, e.g., retinitis or interstitial pneumonia, that are not yet fully understood.

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