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Comparison of Activation versus Induction of Unresponsiveness of Virus-Specific CD4+ and CD8+ T Cells upon Acute versus Persistent Viral Infection

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Summary

The functional status of CD4+ T cells during establishment of persistent infection with the noncytopathic lymphocytic choriomeningitis virus was assessed and compared to that of cytotoxic CD8+ T cells. Functionality of virus-specific CD4+ T cells was measured by proliferative responses, cytokine secretion, cognate help, and IFNγ-mediated protection against challenge infection with recombinant vaccinia virus. Functional CD4+ T cells were induced early after infection and remained measurable up to 6 weeks but then were rendered unresponsive. In contrast, CD8+ T cells were functionally inactivated within 10–15 days. Importantly, functional inactivation of virus-specific CD4+ T cells during persistent viral infection seemed to be critical for the survival of the host.

Introduction

Viral infections often follow an acute time-limited course of replication and spread that is resolved by cellular and/or humoral immune effector functions. However, under certain circumstances, certain viruses can cause persistent infection. The mechanisms by which viruses persist over long time periods in the host differ from one virus to another. Dependent upon the viral antigenic load accessible to the immune system, this might differently affect virus-specific immune responsiveness. Noncytopathic viruses such as the lymphocytic choriomeningitis virus (LCMV), hepatitis B virus, or HIV can cause persistent infections with overwhelming antigenic viral load (Moskophidis et al., 1993b; Chisari, 1995; Ho, 1995). Similarly, lactate dehydrogenase activating virus (LDV), although a highly cytopathic virus, can induce persistent infection with high antigenic load due to its very restricted host cell tropism (Plagemann and Moennig, 1992; van den Broek et al., 1997). In contrast, other viruses such as herpes simplex virus or cytomegalovirus have the ability to persist in a latent form that results in a minimal antigenic load virtually inaccessible to the immune system (Stevens and Cook, 1971; Jordan, 1978).

In the case of persistent overwhelming LCMV infection, it has been shown that LCMV-specific CD8+ T cells are exhausted and thus functionally rendered unresponsive and are eventually physically deleted (Moskophidis et al., 1993a, 1993b; Oxenius et al., 1998b). Similar findings have been suggested for chronic HBV (Rehermann et al., 1995, 1996) and HIV infection (Carmichael et al., 1993; Zinkernagel and Hengartner, 1994). In contrast to CD8+ T cells, much less is known about the functional status of virus-specific CD4+ T cells during establishment of a persistent virus infection. In patients persistently infected with HBV, a strongly reduced HBV-specific proliferative response as compared to acutely infected patients was reported (Ferrari et al., 1990), and in HIV-infected individuals it is virtually impossible to assess HIV-specific CD4+ T cell responses (Shearer and Clerici, 1991). This could at least partly be explained by the infection of activated CD4+ T cells, which impairs their function, reduces their life-span, and renders them susceptible to HIV-specific CTLs in vivo (Ho, 1995; Perelson et al., 1996). Interestingly, a recent report demonstrated that HIV-specific CD4+ T cell responsiveness could be observed in asymptomatic patients with very low/absent viremia (Rosenberg et al., 1997).

In this study we examined the functional status of LCMV-specific CD4+ T cells after infection with the noncytopathic LCMV leading to persistent viral infection. Our experiments in normal mice and in adoptive transfer experiments using LCMV-derived glycoprotein (LCMV-GP)-specific TCR transgenic (tg) CD4+ or CD8+ T cells demonstrated that CD8+ T cells were rendered unresponsive in persistently infected recipients within 10–15 days—considerably faster than CD4+ T cells, which were rendered unresponsive within 6–7 weeks.

Results

Assessment of In Vivo T Help in Acute or Persistently Infected Mice

In order to compare functional activity of LCMV-specific CD4+ T cells during the time course of low-dose (virus being cleared within 14 days) or high-dose (leading to persistent infection) LCMV-DOCILE infection, in vivo T help was assessed in two different experimental setups. First, LCMV-NP-specific ELISA-binding IgG antibody responses were determined 50 or 100 days after infection of C57BL/6 mice (Figure 1). Since the initiation of IgG responses, but not the persistence of memory B cells and memory IgG titers, is strictly dependent on functional T help (Leist et al., 1987; Ahmed et al., 1988; Vieira and Rajewsky, 1990), the pronounced IgG responses indicated that LCMV-specific T help was functionally induced after low- and high-dose infection. Second, in order to directly assess functional LCMV-specific T help in vivo at given time points of infection, a challenge immunization of infected mice was performed using purified, inactivated, deaggregated LCMV-DOCILE that had been covalently modified with dinitrophenol moieties (DOC-DNP). Intravenous immunization with a limiting amount of DOC-DNP induced a DNP-specific IgG response 7 days after challenge in low-dose LCMV memory mice but not in naive control mice, indicating that functional LCMV-specific T help was required to

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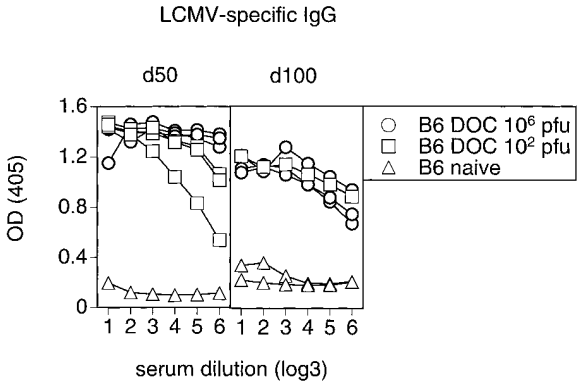

Figure 1. LCMV-NP-Specific IgG Response in LCMV-DOCILE-Infected Mice 50 or 100 Days after Infection

C57BL/6 mice were infected with 1×10^2 PFU (square) or 1×10^6 PFU (circle) LCMV-DOCILE, and 50 or 100 days later, LCMV-NP-specific IgG antibodies were determined by ELISA in 30-fold prediluted sera. Triangles represent sera from naive C57BL/6 mice. Each line represents one individual mouse. One of three comparable experiments is shown.

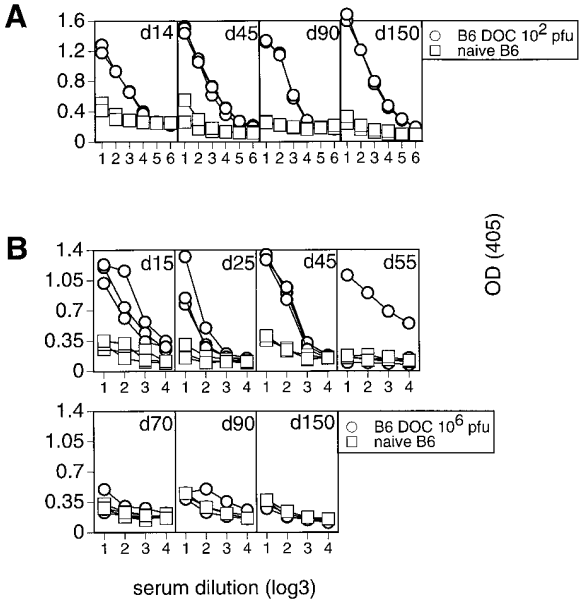

Figure 2. DNP-Specific IgG Antibody Responses

(A) C57BL/6 mice were infected with 1×10^2 PFU LCMV-DOCILE (circle) or were left untreated (square) and were challenged intravenously at the indicated time points with 5 μg DNP-DOCILE.

(B) C57BL/6 mice were infected with a 1×10^6 PFU LCMV-DOCILE (circle) or were left untreated (square) and were challenged intravenously at the indicated time points with 5 μg DNP-DOCILE. Seven days after the challenge immunization, DNP-specific IgG titers were determined in 30-fold prediluted sera by ELISA. Each line represents one individual mouse. One of three comparable experiments is shown.

confer help to DNP-specific B cells (Freer et al., 1995; Oxenius et al., 1998a). Thus, mice that had been infected with low or high doses of LCMV were challenged at different time points (days 14, 25, 45, 55, 70, 90, and 150) with DOC-DNP. DNP-specific IgG titers were determined 7 days later (Figure 2). Interestingly, whereas LCMV-specific T help was functional at all time points up to 200 days (data not shown) after low-dose infection (Figure 2A), LCMV-specific T help was only functional within the first 6–7 weeks after infection with high-dose LCMV (Figure 2B). These results suggest that LCMV-specific T help was rendered unresponsive during overwhelming persistent viral infection but with significantly slower kinetics compared to virus-specific $CD8^+$ T cells, which exhaust within the first 10–15 days after high-dose LCMV infection (Moskophidis et al., 1993b).

Adoptive Transfer of LCMV-GP-Specific $CD4^+$ or $CD8^+$ TCR Tg T Cells into Persistently Infected Recipient Mice

In order to correlate and compare physical and functional behavior of LCMV-GP-specific $CD8^+$ T cells and $CD4^+$ T cells, LCMV-specific TCR tg $CD8^+$ T cells (318) (Pircher et al., 1989) or LCMV-specific TCR tg $CD4^+$ T cells (SMARTA) (Oxenius et al., 1998a) were transferred into recipients that had been persistently infected with LCMV-DOCILE. Clonal expansion and disappearence of transferred cells in the blood and spleen of recipients were monitored by FACS analysis using TCR Vα- and Vβ-specific monoclonal antibodies (Figure 3A). Both transfer of SMARTA tg T cells and 318 tg T cells into persistently infected recipients resulted in a drastic clonal expansion (Figure 3A). The distribution of TCR tg T cells in peripheral blood compared to spleen showed that $CD4^+$ T cells were initially present both in blood and spleen. Fourteen days after transfer, the $CD4^+$ TCR tg T cells were predominantly present in the spleen. The $CD8^+$ T cells were initially predominantly found in the spleen, and only 14 days after adoptive transfer they also appeared in peripheral blood. Whereas the percentage of TCR tg $CD4^+$ T cells remained constant in the spleen over long periods of time (day 14–55; about 10%–15% of total $CD4^+$ T cells), the percentage of TCR tg $CD8^+$ T cells dropped after 35 days upon adoptive transfer to barely detectable levels and was undetectable at 55 days after transfer. These results differ from previous reports where it was documented that $CD8^+$ TCR tg T cells were clonally deleted 15 days after transfer into LCMV carrier mice (Moskophidis et al., 1993a). In those experiments, however, $CD8^+$ TCR tg T cells originated from a TCR tg mouse line (327), which was not from pure C57BL/6 background. In contrast, the TCR tg mouse line 318 used in this report has been backcrossed to C57BL/6 mice for at least eight generations. Therefore, rejection due to partial minor histoincompatibility antigens could explain these discrepancies, which have been addressed and explained in a recent correction (Oxenius et al., 1998b).

In all transfer experiments, virus titers remained constant and no $CD4^+$ T cell escape mutants could be detected (Table 1). For the potential selection of $CD4^+$ T cell epitope (P13-specific) T cell escape mutants, subcloned LCMV isolates were functionally analyzed: LCMV was isolated from the blood of LCMV carrier mice 60 or 100 days after transfer of SMARTA tg T cells. Subsequently, plaque-purified LCMV clones were tested for their ability to activate P13-specific naive SMARTA tg T cells in vitro. In addition, these virus clones were tested for MHC class

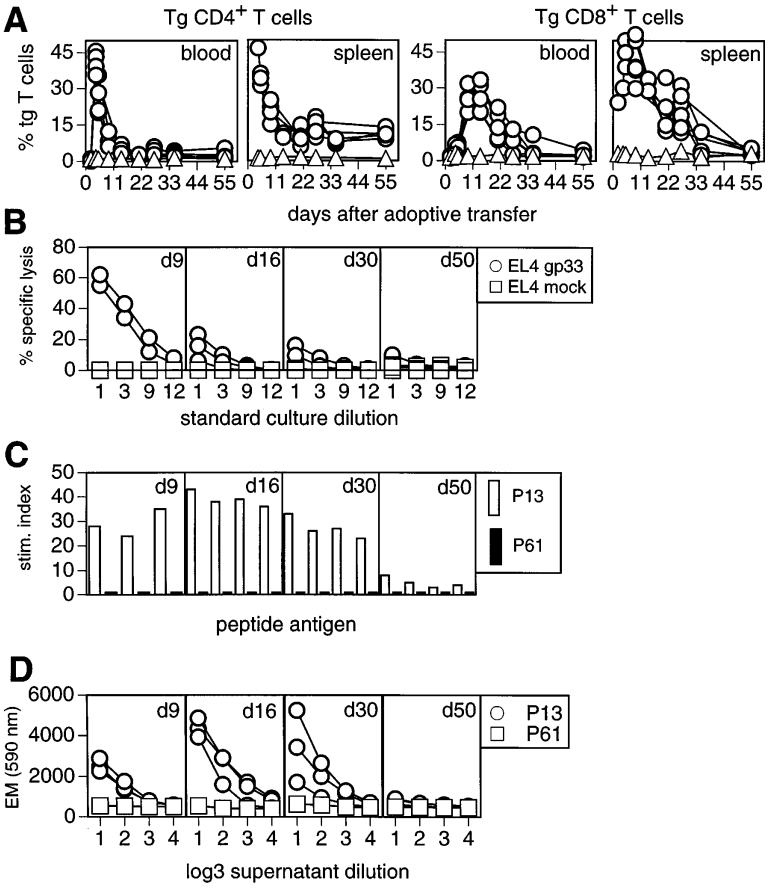

Figure 3. LCMV-Specific Responsiveness of $CD4^+$ or $CD8^+$ TCR Tg T Cells after Adoptive Transfer into Persistently Infected Recipient Mice

(A) Clonal expansion of adoptively transferred $CD4^+$ or $CD8^+$ TCR tg T cells. Spleen cells (2×10^6) from SMARTA tg mice (circle, left panels) or 1×10^6 spleen cells from 318 tg mice (circle, right panels) were transferred into persistently infected mice. At the indicated time points, percentages of TCR tg T cells were assessed in the blood and in the spleen by FACS analysis using appropriate TCR Vα- and Vβ-specific monoclonal antibodies. Triangles represent percentages of TCR tg T cells in naive control mice that had been transfused with the same numbers of TCR tg T cells. Each symbol represents one individual mouse. One of three comparable experiments is shown.

(B) LCMV-specific cytotoxic T cell activity. Spleen cells (1×10^6) from naive 318 tg mice were transferred into persistently infected C57BL/6 mice, and secondary in vitro cytotoxic activity was determined at the indicated time points after infection by ^{51}Cr release assay with gp33-loaded (circle) or unloaded (square) EL4 target cells. Each line represents one individual mouse. One of three comparable experiments is shown.

(C) LCMV-specific proliferation of purified $CD4^+$ T cells. Spleen cells (2×10^6) from naive SMARTA tg mice were transferred into persistently infected C57BL/6 mice. At the indicated time points, $CD4^+$ T cells were purified from the spleens. $CD4^+$ T cells were restimulated with serial dilutions of the relevant peptide P13 or of the irrelevant peptide P61 in the presence of irradiated naive C57BL/6 spleen cells. Proliferation was determined by [^{3}H]thymidine incorporation 3 days after restimulation. Stimulation indices were calculated in relation to proliferation in medium control. Background counts per minute in medium control were around 3000. One of three equivalent experiments is shown.

(D) LCMV-specific IL-2 secretion. Supernatants from the above described proliferation assays were serially diluted and IL-2 contents were determined 24 hr after restimulation using the IL-2-sensitive cell line CTLL-2. Circles represent supernatants from P13-restimulated $CD4^+$ T cells and squares represent those from P61-restimulated $CD4^+$ T cells. One of three equivalent experiments is shown.

II–associated P13-presentation by measuring activation of the P13-specific $CD4^+$ T cell hybridoma 5A1 (Oxenius et al., 1995). Both experimental setups revealed identical results: tg TCR escape mutants were very rare and apparently not positively selected for, since no significant difference in the percentage of LCMV clones escaping recognition by naive SMARTA $CD4^+$ T cells could be observed in LCMV carrier mice transfused with SMARTA T cells as compared to nontransfused control carrier mice (Table 1).

Functional Activity of Adoptively Transferred TCR Tg T Cells

$CD8^+$ T cells were tested for secondary in vitro cytotoxic T cell activity on gp33-pulsed target cells (Moskophidis et al., 1993a; Oxenius et al., 1998b) (Figure 3B), and $CD4^+$ T cells were assessed by recall proliferation and IL-2 secretion using the TCR-specific peptide P13 for in vitro activation (Oxenius et al., 1998a) (Figures 3C and 3D).

Cytotoxic activity of transferred TCR tg $CD8^+$ T cells could be observed 9 and, to a diminished level, 16 days after transfer but was absent 30 and 50 days after transfer (Figure 3B). In contrast, specific proliferation of transferred $CD4^+$ TCR tg T cells could be observed 9, 16, 25, and 30 days after transfer but was drastically reduced 50 days after transfer (Figure 3C), even though 10% of TCR tg $CD4^+$ T cells could constantly be detected in the spleen between 15 and 50 days after transfer (Figure 3A). Antigen-induced IL-2 secretion was measurable up to 30 days after transfer but had disappeared by 50 days (Figure 3D). Addition of exogenous recombinant IL-2 did not restore the proliferative capacity. In addition, culture of unresponsive $CD4^+$ T cells in the absence of antigen and presence of recombinant IL-2 did not rescue the proliferative response (data not shown). These results indicate that the kinetics of induction of unresponsiveness differed for $CD8^+$ T cells and $CD4^+$ T cells with specificity for the same viral glycoprotein.

To ascertain that the observed loss of proliferation and IL-2 secretion was not due to limiting numbers of TCR tg $CD4^+$ T cells in the in vitro assays, naive TCR tg $CD4^+$ T cells were titrated into these cultures. It was found that 1×10^4 TCR tg $CD4^+$ T cells per culture well yielded maximal proliferative responses as well as maximal IL-2 secretion (data not shown). In all experiments at least 1×10^4 recovered TCR tg $CD4^+$ T cells were used per culture well. Furthermore, the functional

Table 1. LCMV Subclones Isolated from Carrier Mice Transfused with SMARTA Tg CD4$^+$ Cells 60 or 100 Days before Virus Isolation

	Numbers of SMARTA TCR Escape Virus Clones per Screened Clones			
	Mouse 1	Mouse 2	Mouse 3	Total
SMARTA tf into carrier (d60)	0/12	1/12	0/12	1/36
SMARTA tf into carrier (d100)	0/12	0/11	0/11	0/34
No tf into carrier (d60)	1/12	0/12	1/12	2/36

LCMV was isolated from the blood of nine different LCMV carrier mice and plaque purified. LCMV carrier mice had either been transfused with SMARTA tg CD4$^+$ T cells 60 or 100 days previously or had been left untreated. From each mouse, 12 subclones were tested for their capacity to activate naive SMARTA tg CD4$^+$ T cells by means of in vitro proliferation or induction of IL-2 release. In addition, virus subclones were tested for MHC class II–associated presentation of the LCMV-GP-derived epitope P13 (aa 61–80) by activation of the P13-specific T cell hybridoma 5A1. All three assay methods revealed the same results. Numbers of SMARTA TCR escape clones per total number of tested LCMV clones are shown separately for each mouse.

activity of SMARTA tg T cells was significant within the first 35 days but declined by day 50 after transfer to approximately background levels, while the percentage of SMARTA tg T cells in the spleen constantly remained at 10% (Figure 3).

In addition, functional T help was assessed directly in vivo by immunization with a molecule consisting of a BSA core protein to which the P13 was covalently coupled, which was in addition covalently modified with DNP moieties (BSA-P13-DNP) (Oxenius et al., 1998a). Using deaggregated BSA-P13-DNP for intravenous immunization allowed the assesssment of P13-specific primed functional T help in vivo by measuring DNP-specific IgG responses 7 days after challenge (Oxenius et al., 1998a). BSA-P13-DNP immunizations performed at the indicated time points after adoptive transfer of SMARTA tg T cells into persistently infected recipients confirmed that the transferred CD4$^+$ T cells were functional up to 35 days after transfer but not at 55 days after transfer (Figure 4A).

To demonstrate more generally the unresponsiveness of LCMV-specific CD4$^+$ T cells in LCMV carrier mice, transplacentally infected LCMV carrier mice or naive C57BL/6 mice (with or without CD4 T cell depletion) were challenged intravenously with 20 μg of deaggregated, inactivated, purified LCMV-DNP. Seven days after challenge, DNP-specific IgG titers were determined in the serum (Figure 4B). Neither CD4-depleted nor untreated LCMV carrier mice nor naive C57BL/6 control mice mounted a measurable DNP-specific IgG response. In contrast, low-dose LCMV memory mice mounted a significant DNP-specific IgG response. This response was abrogated by depletetion of CD4$^+$ T cells. These results demonstrated that preexistent antibodies to LCMV did not interfere with detection of LCMV-specific T help. Thus, no LCMV-specific T help could be measured in transplacentally infected LCMV carrier mice, at least with this experimental setup.

Ability of TCR Tg CD4$^+$ T Cells to Express Surface Activation Markers

In addition to the functional assessment of CD4$^+$ T cells upon transfer into virus carrier recipients, we analyzed whether the tg CD4$^+$ T cells were still able to express surface activation markers after peptide stimulation in vitro. Thus, CD4$^+$ TCR tg T cells recovered either 16 or 50 days after transfer were stimulated in vitro for 24 hr with the relevant peptide P13, and surface expression of CD69 and IL-2R was analyzed. Whereas the TCR tg CD4$^+$ T cells were still able to upregulate CD69 and IL-2R 16 days after transfer, they no longer upregulated IL-2R 50 days after transfer (data not shown).

Protective Immunity against Challenge Infection with Recombinant Vaccinia Virus Expressing LCMV-GP

A further means of analyzing the functional status of transferred LCMV-GP-specific TCR tg CD4$^+$ or CD8$^+$ T

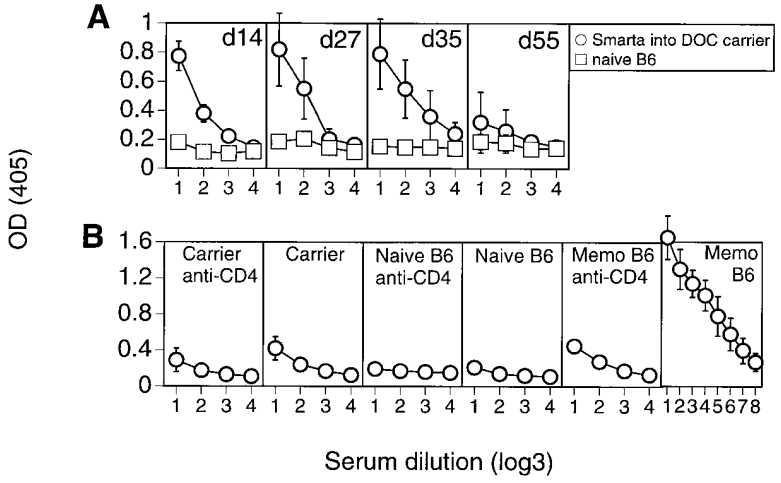

Figure 4. DNP-Specific IgG Antibody Responses

(A) Spleen cells (2×10^6) from naive SMARTA tg mice were transferred into persistently infected C57BL/6 mice. At the indicated time points, transfused (circle) or naive control mice (square) were challenged intravenously with 10 μg deaggregated DNP-P13-BSA. Seven days after immunization, DNP-specific IgG titers were determined in 30-fold prediluted sera by ELISA. The mean of five mice per experimental group is shown.

(B) Transplacentally infected LCMV carrier mice or naive C57BL/6 control mice that either had been depleted of CD4$^+$ T cells in vivo or left untreated were immunized intravenously with 20 μg of DNP-modified, deaggregated, inactivated, purified LCMV. In addition, day 40 infected LCMV memory C57BL/6 mice (with or without depletion of CD4$^+$ T cells) were challenged with LCMV-DNP. DNP-specific IgG titers were determined 7 days later in 30-fold prediluted serum. The mean of five mice per experimental group is shown.

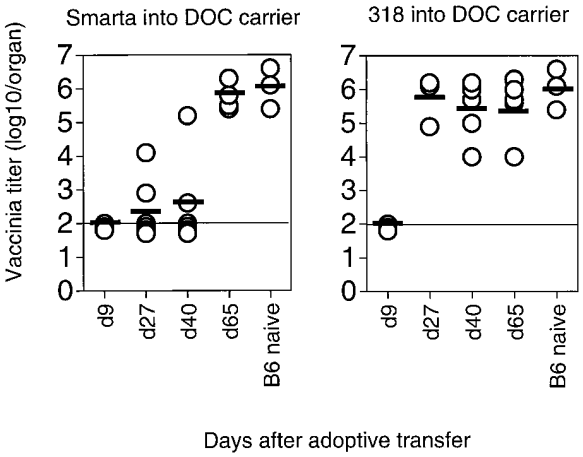

Figure 5. T Cell–Dependent, Cytokine-Mediated Protection against Challenge Infection with Recombinant Vaccinia Virus

Persistently infected mice were transfused with 2×10^6 spleen cells from SMARTA tg mice (left panel) or with 1×10^6 spleen cells from 318 tg mice (right panel). At the indicated time points, recipient mice were challenged intraperitoneally with 2×10^6 PFU Vacc-G2. Vaccinia titers in ovaries were determined 5 days after challenge infection. Each symbol represents an individual mouse. One of two experiments is shown.

cells was the assessment of protection against a challenge infection with recombinant vaccinia virus expressing the LCMV-GP (VV-G2). Protection against VV-G2 is conferred by the release of type 1 cytokines (IFNγ and TNFα) by LCMV-GP-specific $CD4^+$ or $CD8^+$ T cells (Binder and Kündig, 1991; Kündig et al., 1993; Oxenius et al., 1998a). Thus, persistently infected recipient mice that had received TCR tg $CD4^+$ or $CD8^+$ T cells were challenged with VV-G2 on day 9, 27, 40, or 65 after cell transfer, and 5 days later vaccinia virus titers were determined in the ovaries (Figure 5). Recipients of TCR tg $CD4^+$ T cells were protected against challenge infection at 9, 27, and 40 days after transfer but no longer than day 65 after transfer. In contrast, recipients of TCR tg $CD8^+$ T cells were only protected against a challenge infection at day 9 but no longer at 27, 40, and 65 days after transfer. These results corroborate the previous findings that virus-specific $CD4^+$ T cells are functional in persistently infected mice for considerably longer time periods than virus-specific $CD8^+$ T cells.

Proliferative Activity of Unresponsive $CD4^+$ TCR Tg T Cells Cannot Be Restored by Transfer into Naive, Antigen-Free Recipients

To address the question of whether unresponsive $CD4^+$ TCR tg T cells are able to regain their proliferative capacity upon transfer into virus-free recipient mice, SMARTA tg T cells, having been exposed to LCMV in a persistently infected host for 55 days, were purified to 99.8% purity and transferred into naive C57BL/6 mice. The purified $CD4^+$ T cells were incubated with anti-LCMV-specific hyperimmune serum (containing LCMV-neutralizing antibodies) before transfer in order to minimize infectious virus transfer. As a control, equivalent numbers of naive SMARTA tg cells were transferred into naive recipients. Recipient mice were challenged 1 or 8 days after cell transfer with LCMV, and expansion of TCR tg $CD4^+$ T cells was monitored in the blood and in the spleen. LCMV infection did not induce measurable clonal expansion of SMARTA tg T cells that had been exposed to high doses of viral antigen for 55 days (not shown). In contrast, transferred naive SMARTA tg T cells showed pronounced clonal expansion at both time points after virus challenge (data not shown).

These data suggest that LCMV-specific $CD4^+$ T cells, rendered unresponsive by exposure for 55 days to an overwhelming antigen dose in persistently infected mice, could not revert to a proliferative functional status upon adoptive transfer into a largely antigen-free environment. However, it has to be noted that a few virus particles had been cotransferred with the purified $CD4^+$ T cells and, despite the treatment with hyperimmune serum, resulted in acute LCMV infection. Since a small fraction of the $CD4^+$ T cells has been shown to be infected in LCMV carrier mice (Borrow et al., 1991), it is virtually impossible to circumvent transfer of a few infected cells. However, the virus load in these mice was certainly much below the one in persistently infected mice and was time-limited due to the neutralizing antibodies and the recipients' efficient $CD8^+$ T cell response. These results differ from other reports where unresponsive $CD4^+$ T cells could be rescued in terms of their functional activity within a few days upon adoptive transfer into truly antigen-free recipients (Bachmann et al., 1994).

Inefficient/Absent Induction of Unresponsiveness in LCMV-Specific $CD4^+$ T Cells Leads to Fatal Outcome of Persistent LCMV Infection

Induction of unresponsiveness of $CD4^+$ T cells during persistent and overwhelming viral infection might be of critical importance for survival, since pathological effects of unimpaired virus-specific $CD4^+$ T cell activities have been described (Zajak et al., 1996). Indeed, if the numbers of virus-specific $CD4^+$ T cells are drastically elevated, e.g., in LCMV-specific TCR tg mice (Oxenius et al., 1998a), infection with high doses of LCMV is lethal, whereas it causes a tolerated persistent infection in normal mice (Table 2.1). In line with this, adoptive transfer of purified $CD4^+$ TCR tg T cells into naive C57BL/6 nu/nu recipients followed by infection with LCMV (which cannot be controlled in the absence of $CD8^+$ T cells) led to wasting disease and eventually death in the recipient nude animals (Table 2.2). Thus, LCMV-specific $CD4^+$ T cells can cause lethality during uncontrolled LCMV infection. Successful inactivation of virus-specific $CD4^+$ T cells depends upon frequencies of specific $CD4^+$ T cells (Kearney et al., 1994; Förster et al., 1995; Lanoue et al., 1997), with low frequencies being inactivated more readily than high frequencies.

It was of interest to determine whether SMARTA tg T cells rendered unresponsive 60 days after transfer into LCMV carrier mice could still cause lethality upon transfer into C57BL/6 nu/nu recipients (Table 2.3). $CD4^+$ T cells were purified from the spleen of LCMV carrier mice 60 days after transfer of SMARTA tg T cells. Purified $CD4^+$ T cells (1×10^7; containing 1×10^6 TCR tg $CD4^+$ T cells) were adoptively transferred into C57BL/6 nu/nu

Table 2.1. Lethal Effects of High-Dose LCMV Infection in LCMV-GP–Specific CD4+ TCR Tg Mice (SMARTA)

	Lethality (Dead Mice/Total Mice)		
	No treatment	Anti-CD4	Anti-CD8
SMARTA, 10^6 PFU DOC	10/10 (d30)	0/4 (d30)	4/4 (d30)
B6, 10^6 PFU DOC	0/20 (d30)	0/4 (d30)	0/4(d30)

SMARTA TCR tg mice were left untreated or were depleted of CD4+ or CD8+ T cells before infection with high doses of LCMV-DOCILE (10^6 PFU i.v.). Death of the mice was monitored daily. Results are shown as (dead mice/total number of mice per group) at day 30 after infection. One of three equivalent experiments is shown.

Table 2.2. Lethal Effects of LCMV Infection in C57BL/6 Nu/Nu Mice Transfused with SMARTA Tg T Cells

	Lethality (Dead Mice/Total Mice)	
	Transfer (Tf) of 1×10^7 Naive SMARTA Tg T Cells	No Transfer
B6 nu/nu LCMV-WE 200 PFU I.V.	6/6 (d15)	0/6 (d15)

CD4+ T cells (1×10^7) purified from SMARTA tg mice were transferred into C57BL/6 nu/nu mice, and 3 days later recipients and control C57BL/6 nu/nu mice were infected with a low dose of LCMV-WE (200 PFU) i.v. Death of the mice was monitored daily. Results are shown as (dead mice/total number of mice per group) at day 15 after infection. One of three equivalent experiments is shown.

Table 2.3. Lethal Effects of Adoptive Transfer of Naive or Unresponsive SMARTA Tg T Cells into LCMV-Infected C57BL/6 Nu/Nu Mice

	Tf of 1×10^6 Naive SMARTA Cells + 1×10^7 CD4+ T Cells from LCMV Carrier Mice	Tf of 1×10^7 CD4+T cells[a] from LCMV carrier mice transfused with SMARTA cells (d60)	Tf of 1×10^7 CD4+ T cells from LCMV carrier mice
B6 nu/nu	4/4 (d9)	0/4 (d9)	0/4 (d9)

[a] CD4+ T cells (1×10^7) purified from LCMV carrier mice that had been transfused with SMARTA CD4+ T cells 60 days previously were adoptively transferred into C57BL/6 nu/nu recipients; these purified CD4+ T cells contained 10% SMARTA tg T cells.
Death of the mice was monitored daily. Results are shown as (dead mice/total number of mice per group) at day 9 after infection. One of two equivalent experiments is shown.

recipients. In parallel, 1×10^7 purified CD4+ T cells from untransfused LCMV carrier mice (as source of "contaminating" infectious virus [Borrow et al., 1991]) were transferred into C57BL/6 nu/nu recipients with or without 1×10^6 naive SMARTA tg CD4+ T cells. Survival of the recipient animals was monitored. Recipients that received naive SMARTA tg T cells succumbed 8 days after transfer, whereas recipients that received unresponsive SMARTA tg T cells or CD4+ T cells from untransfused LCMV carrier mice survived the adoptive transfer. These findings support the notion that it might be of critical importance for the survival of the host to render virus-specific CD4+ T cells unresponsive during persistent and overwhelming viral infection.

Discussion

This study documents a substantial difference in kinetics of induction of unresponsiveness of LCMV-GP-specific CD4+ and CD8+ T cells in unmanipulated C57BL/6 mice. Corresponding observations were made in adoptive transfer experiments where LCMV-specific TCR tg T cells were transferred into persistently infected recipient mice. CD8+ T cells were rendered unresponsive within 10–15 days as opposed to CD4+ T cells being functionally active for up to 5–6 weeks after transfer. CD8+ TCR tg T cells as well as CD4+ TCR tg T cells exposed to high doses of viral antigen in LCMV carrier mice clonally expanded. CD8+ TCR tg T cells were physically present in secondary lymphoid organs up to 30 days after transfer, whereas CD4+ TCR tg T cells were detectable for at least 55 days. This is in contrast to previously published results in which it was shown that loss of functionality of transferred TCR tg CD8+ T cells coincided with physical deletion of these transferred TCR tg CD8+ T cells (Moskophidis et al., 1993a). These discrepancies have now been explained by the fact that in the former experiments, CD8+ TCR tg T cells were obtained from a TCR tg mouse line (327) that was not from a sufficiently pure C57BL/6 background. Therefore, rejection of transferred CD8+ T cells due to partial minor histoincompatibility antigen differences could explain these differences (Oxenius et al., 1998b).

These findings raised questions about the reasons for the kinetic differences in induction of unresponsiveness of virus-specific CD4+ and CD8+ T cells. Since, in the case of the adoptive transfer experiments into persistently infected recipients, both TCR tg CD4+ and CD8+ T cells were specific for the LCMV-GP, the overall antigen dose was equivalent for both CD4+ and CD8+ TCR tg T cells. However, differences in the numbers of MHC–peptide complexes to be recognized by the TCR tg T cells are most probably of relevance. In a mouse persistently infected with LCMV, viral titers are high in most organs and different cell types are infected. This leads to MHC class I presentation of virus-derived epitopes on many cell types in lymphoid and also in nonlymphoid tissues that are largely MHC class II negative. Therefore, virus-specific CD8+ T cells are constantly confronted with specific MHC–peptide complexes outside of lymphoid tissue that could more quickly cause exhaustion, possibly by starvation of these CD8+ T cells. In contrast, MHC class II molecules are normally only expressed on professional APCs, mostly in lymphoid tissues rich in

interleukins and costimulatory signals, i.e., an environment which normally favors T cell activation. These geographical distribution factors as well as perhaps the concentration of MHC LCMV-GP peptide molecules per cell might at least partially explain the differences in induction of unresponsiveness of LCMV-specific $CD8^+$ versus $CD4^+$ T cells.

Another factor that might contribute to this difference could be that the TCR of $CD8^+$ 318 tg T cells (Pircher et al., 1989) might exhibit a much higher avidity for D^b + gp33 than the TCR of $CD4^+$ SMARTA T cells (Oxenius et al., 1998a) toward I-A^b + P13. Although it is impossible to address this question thoroughly without soluble MHC–peptide complexes and soluble TCR molecules, this seemes unlikely. Our experiments showed on a polyclonal level that normal C57BL/6 mice also show faster induction of unresponsiveness of $CD8^+$ as compared to $CD4^+$ T cells. In vitro peptide titration experiments showed that both tg TCRs responded to 1×10^{-10} M peptide concentration, suggesting that the avidities probably do not differ by several orders of magnitude.

The coexistence of functional LCMV-specific TCR tg $CD4^+$ T cells and LCMV for at least 6–7 weeks raised the question of whether the presence of LCMV-specific $CD4^+$ T cells selected for virus mutants that escape recognition by the TCR tg $CD4^+$ T cells. Functional analysis of several LCMV clones isolated from LCMV carrier mice, which had been transfused with TCR tg $CD4^+$ T cells 60 or 100 days previously, showed no selection for LCMV T cell epitope escape mutants. Two possibilities may account for this finding. The selective pressure imposed on the virus by $CD4^+$ T cells might not be strong enough, since it has been shown that even the presence of drastically elevated precursor frequencies of LCMV-specific $CD4^+$ T cells does not positively contribute to viral clearance (Oxenius et al., 1998a). Alternatively, one could argue that the frequencies of transferred TCR tg $CD4^+$ T cells are not high enough to cause viral escape. The first possibility seems more likely, since high-dose LCMV infection in SMARTA tg mice exhibiting drastically elevated LCMV-specific $CD4^+$ T cell frequencies led to a fatal outcome of infection rather than to tolerated persistent infection, as would have been expected in the case of selection of virus escape mutants (Table 2.1).

In the case of persistent LCMV infection, the high amount of antigen constantly present over long time periods led to the induction of unresponsiveness of LCMV-specific $CD4^+$ T cells after 6–7 weeks of virus exposure. This continuous high antigen load is not achieved in acutely low-dose infected mice and, thus, LCMV-specific $CD4^+$ T cells remain functional up to 200 days after infection. However, the frequency of LCMV-specific $CD4^+$ T cells also plays an important role for the induction of $CD4^+$ T cell unresponsiveness. In a normal T cell repertoire or in the case of adoptive transfer experiments of $3–5 \times 10^5$ $CD4^+$ TCR tg T cells, unresponsiveness can be observed 6–7 weeks after persistent infection or adoptive transfer, respectively. However, infection of SMARTA tg mice with a high dose of LCMV leads to a fatal outcome of the infection rather than to induction of $CD4^+$ T cell unresponsiveness. Most probably the number of antigen-specific $CD4^+$ T cells is too high for complete inactivation by persistent viral infection and, moreover, the constant activation of high numbers of LCMV-specific $CD4^+$ T cells has deleterious consequences for the host. The mechanisms responsible for the fatal outcome of infection are currently under investigation. Other studies investigating in vivo induction of $CD4^+$ T cell unresponsiveness revealed also that this process depends on the amount of antigen as well as on the frequency of antigen-specific $CD4^+$ T cells (Förster et al., 1995; Kearney et al., 1994; Lanoue et al., 1997).

Importantly, $CD4^+$ TCR tg T cells rendered unresponsive after adoptive transfer into LCMV carrier mice (as assessed by lack of proliferation, IL-2 secretion, and in vivo T help) were shown to be unable to cause fatal disease upon transfer into C57BL/6 nude recipients, as opposed to naive TCR tg $CD4^+$ T cells. This supports the notion that induction of Th cell unresponsiveness might be important to secure survival of the host undergoing a persistent virus infection.

In conclusion, this study analyzed the fate of LCMV-specific $CD4^+$ T cells upon persistent LCMV infection, demonstrating that functional responsiveness disappeared 6–7 weeks after infection. Comparison of the functional status of defined TCR tg $CD4^+$ or $CD8^+$ T cells, both specific for the LCMV-GP, transferred into persistently infected recipient mice showed that the kinetics of induction of T cell unresponsiveness are significantly longer for virus-specific $CD4^+$ T cells as compared to virus-specific $CD8^+$ T cells.

Experimental Procedures

Mice

Inbred C57BL/6 (H-2^b) mice were obtained from the breeding colony of the Institut für Labortierkunde, University of Zürich, Zürich, Switzerland. The generation of LCMV-GP-specific $CD4^+$ or $CD8^+$ TCR tg mice has been described previously (Pircher et al., 1989; Oxenius et al., 1998a). Mice were bred in an SPF mouse house facility.

Viruses

The LCMV-DOCILE isolate [a variant isolated from a LCMV-WE (UBC)-carrier mouse] was originally obtained from C. J. Pfau, Troy, New York, and was grown on MDCK cells (ATCC CCL-34) with a low multiplicity of infection. The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany, and grown on L929 cells (ATCC CRL 1) with a low multiplicity of infection. Recombinant vaccinia virus expressing LCMV-GP has been described (Whitton et al., 1988). The recombinant baculovirus expressing the LCMV nucleoprotein and production of recombinant proteins have been previously described (Matsuura et al., 1987; Battegay et al., 1993).

Adoptive Transfers into Persistently Infected Recipient Mice

C57BL/6 mice were infected intravenously with 1×10^6 PFU LCMV-DOCILE. Four days later, mice were inoculated intraperitoneally with 200 μg cyclophosphamide. Three to four weeks later, viral titers were analyzed in the blood of all animals and usually $1–10 \times 10^4$ PFU/ml blood was detected. For adoptive transfer, 2×10^6 spleen cells from naive SMARTA tg mice or 1×10^6 spleen cells from naive 318 tg mice were transferred into persistently infected recipients.

Adoptive Transfers into C57BL/6 Nude Recipients

$CD4^+$ T cells were purified from spleen cell suspensions of naive SMARTA TCR tg mice or from (adoptively transferred) LCMV carrier mice by MACS according to the protocol of the supplier (Miltenyi Biotec, Germany).

Cytofluorometric Analyses

The following monoclonal antibodies were used for analysis of TCR tg peripheral lymphocytes: biotinylated 7G8, specific for Vβ8.3 (a gift from Dr. I. Förster [Förster et al., 1995]); biotinylated anti-Vβ8.1/2, phycoerythrin-conjugated anti-Vα2, fluorescein-conjugated anti-CD8, fluorescein-conjugated anti-CD4, biotinylated anti-CD25, and anti-CD69 were purchsed from Pharmingen. Tricolor-conjugated streptavidin or anti-CD4 were purchased from Caltag Laboratories. Flow cytometry was performed on a FACStar Plus flow cytometer (Becton-Dickinson).

Assessment of In Vivo T Help by Challenge with DNP-Modified LCMV-T Helper Antigens

LCMV-infected C57BL/6 mice were challenged intravenously at the indicated time points with 5 μg of inactivated, deaggregated purified LCMV-DOCILE whose surface proteins had been covalently linked to DNP moieties (DOC-DNP) (Mishell and Shiigi, 1980). Mice were bled 7 days later and DNP-specific IgG titers were determined by ELISA.

In the case of persistently infected mice that had been transfused with SMARTA tg tg T cells, mice were challenged at the indicated time points with 10 μg DNP-P13-BSA (Oxenius et al., 1998a). Transplacentally infected LCMV carrier mice, naive C57BL/6 mice, and low-dose LCMV memory mice were challenged intravenously with up to 20 μg of inactivated, deaggregated LCMV-DNP. In vivo CD4 depletion was performed by using the monoclonal antibody YTS 191.1 (Cobbold et al., 1984).

Peptides

Peptides were purchased from Neosystem, Strasbourg, France. The following peptides were used: P13, (GLNGPDIYKGVYQFKSVEFD) (LCMV-GP,I-A^b); P61, (SGEGWPYIACRTSVVGRAWE) (LCMV-NP, I-A^b); and GP33, (KAVYNFATM) (LCMV-GP, D^b).

T Cell Proliferation

$CD4^+$ T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec, Germany). $CD4^+$ T cells (1×10^5) were incubated in a 96-well plate with 3-fold serial dilutions of the specific peptide P13 (highest concentration 5 μg/ml), the irrelevant peptide P61 (highest concentration 5 μg/ml), or medium only in the presence of 6×10^5 irradiated (2000 cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of [^{3}H]thymidine (1 μCi/well).

Cytokine Analysis

Supernatants of proliferation assays as described above were analyzed for IL-2 content 24 hr after restimulation. IL-2 was determined using the IL-2-dependent cell line CTLL-2. Quantification of viable cells was performed by AlamarBlue color reaction (Biosource International) and measured by fluorescence emission at 590 nm using the CytoFluor 2350 (Millipore) flourimeter.

Cytotoxic T Cell Response

Spleen cell suspensions were prepared and 4×10^6 cells per 24-well plate were restimulated for 5 days with peptide-labeled irradiated (25 Gy) spleen cells (2×10^6 cells/24-well plate). Restimulated cells were resuspended in 0.5 ml of medium per culture well and serial 3-fold dilutions of effectors were performed (referred to as dilution of standard culture) and tested in a conventional ^{51}Cr release assay, using gp33-labeled EL-4 target cells.

ELISA

The LCMV nucleoprotein-specific enzyme-linked immunosorbent assay (ELISA) has been described previously (Battegay et al., 1993). ELISA measurement of DNP-specific IgG titers was performed similarly. ELISA plates were coated with 0.1 μg per well of ovalbumin covalently coupled to DNP (Mishell and Shiigi, 1980; Gupta et al., 1986).

Protection of Mice from Replication of Recombinant Vaccinia Virus

Persistently infected mice were transfused with TCR tg T cells and were challenged intraperitoneally at the indicated time points with 2×10^6 PFU recombinant vaccinia virus expressing the LCMV-glycoprotein (VV-G2). Vaccinia titers in ovaries were determined 5 days later as described previously (Binder and Kündig, 1991). Titers are shown as log10 PFU per animal.

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