

Pathogenic virus-specific T cells cause disease during treatment with the calcineurin inhibitor FK506: implications for transplantation

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Recently, several cases of fatal lymphocytic choriomeningitis virus (LCMV) infection occurred in transplant recipients being treated with the immunosuppressive calcineurin inhibitor FK506. These findings were surprising because LCMV is a noncytolytic virus. To understand how a noncytolytic virus can cause disease under conditions of immunosuppression, we used the mouse LCMV model and found that, similar to the observations in human transplant recipients, LCMV infection of FK506-treated mice resulted in a lethal disease characterized by viremia, lack of seroconversion, and minimal lymphocytic infiltrates in the tissues. However, despite the apparent absence of an antiviral immune response, this disease was orchestrated by virus-specific T cells. FK506 did not prevent the generation and proliferation of LCMV-specific T cells but instead altered their differentiation so that these effector T cells lost the ability to control virus but were still capable of mediating disease. These pathogenic T cells initiated a cytokine storm characterized by high levels of tumor necrosis factor (TNF) and interleukin 6 (IL-6), and depletion of T cells or blockade of these inflammatory cytokines prevented the lethal disease. Our study shows that inhibiting calcineurin can generate pathogenic T cells and indicates that T cell-mediated viral disease can occur even under conditions of immunosuppression. Furthermore, we identify a potential strategy (blockade of TNF and IL-6) for treatment of transplant recipients who have acute complications of viral infection.

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Abbreviations used: 7AAD, 7-aminoacitinomycin D; KLRG-1, killer cell lectin-like receptor G1; LCMV, lymphocytic choriomeningitis virus; PD-1, programmed death 1.

Viral infections are one of the major complications after transplantation. In immunosuppressed transplant recipients, morbidity and mortality associated with many viral infections increases significantly compared with healthy individuals. Common viral complications include reactivation and resurgence of chronic viruses such as cytomegalovirus, Epstein-Barr virus, and polyoma BK virus (Fishman and Rubin, 1998; Singh, 2003; Fishman, 2007). Severe infections can also arise from agents unexpectedly present in the donor tissue (Kumar and Humar, 2005; Kotton, 2007). This mode of infection, although less common, can be associated with severe and fatal consequences and has recently occurred with lymphocytic choriomeningitis virus (LCMV) and closely related agents in transplant recipients (CDC, 2005, 2008; Fischer et al., 2006; Palacios et al., 2008).

LCMV infection in humans typically causes a subclinical or mild self-limiting febrile disease with some individuals experiencing aseptic meningitis (Buchmeier et al., 2007). Although infection of human fetus can result in congenital abnormalities or death, LCMV disease in healthy adults is rarely fatal, with a mortality <1% (Peters, 2006). However, recently reported cases of LCMV infection in transplant recipients exhibited dramatically distinct clinical features from those seen in immunocompetent individuals with a case mortality rate >90% (CDC, 2005, 2008; Fischer et al., 2006; Palacios et al., 2008).

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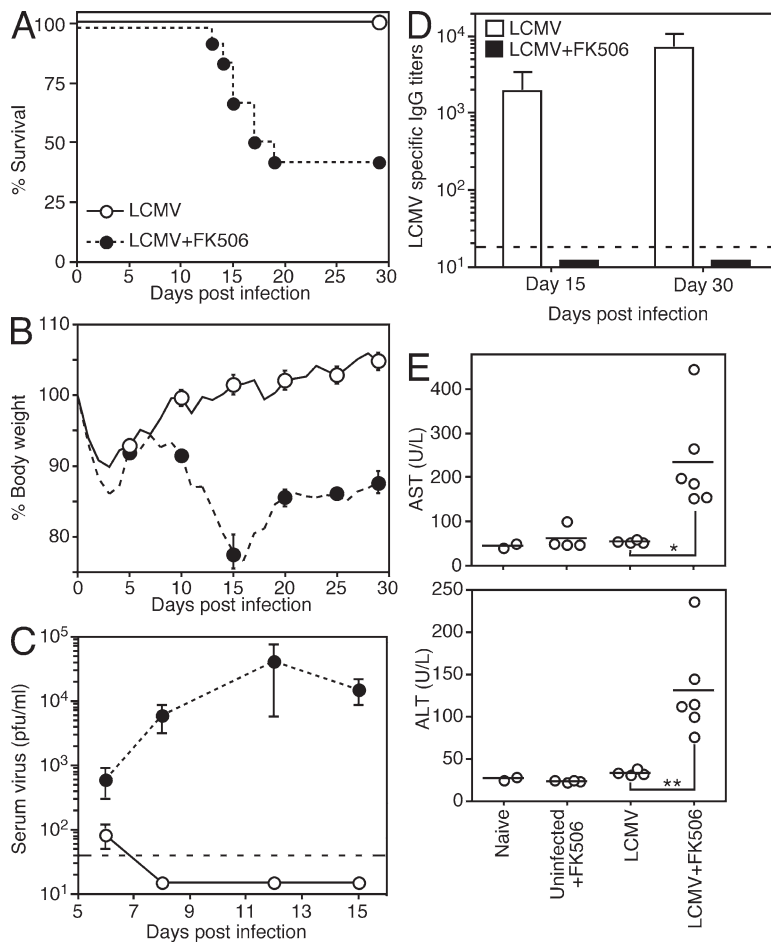


Figure 1. LCMV infection in the presence of FK506 results in high mortality. B6 mice were infected with LCMV Armstrong in the presence or absence of FK506 treatment. FK506 was injected subcutaneously every day into B6 mice from day -1 of infection. (A) Survival curve of LCMV Armstrong-infected mice with or without FK506 treatment ($n = 6$, LCMV; $n = 12$, LCMV + FK506). (B) Mean body weight was tracked after infection ($n = 6$, LCMV; $n = 12$, LCMV + FK506). (C) Serum viral titers were measured by plaque assay ($n = 3$, each time point). (D) Endpoint titers of anti-LCMV IgG in serum were detected by ELISA ($n = 3$, each time point). Error bars for B–D indicate SEM. (E) Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were analyzed from naive FK506-treated uninfected, LCMV-infected, and LCMV-infected FK506-treated mice on days 12–15 after infection or drug treatment. Each circle represents individual animals. Horizontal bars show the mean. *, $P < 0.05$; **, $P < 0.01$. The horizontal dashed lines in C and D indicate the lower limit of detection. Data (A–E) were pooled from two independent experiments.

Overall, 13 patients in four different clusters received allografts that were infected with the virus, and all patients developed clinical disease with 12 of them dying (CDC, 2005, 2008; Fischer et al., 2006; Palacios et al., 2008). These recipients had sustained viremia, and virus was also detected in multiple organs as a result of lack of protective immune responses because the transplant recipients were under immunosuppressive medications (Fischer et al., 2006; CDC, 2008; Palacios et al., 2008). In addition, only minimal inflammatory infiltrates in tissues were observed and there was no seroconversion in most of the transplant recipients (Fischer et al., 2006). Based on these clinical observations, it was suggested that the lethal LCMV disease in these transplant recipients that were under a FK506-based immunosuppressive regimen was caused by direct viral damage and was not immune mediated (Fischer et al., 2006; Peters, 2006).

It is surprising and somewhat paradoxical that LCMV infection in transplant recipients resulted in such high mortality without evidence of immunopathology because LCMV is a noncytolytic virus and is the classical model of immune-mediated viral disease (Borrow and Oldstone, 1997). The virus itself can cause disease by altering the infected cell functions without interrupting their vital functions, but the outcome of this disease is usually nonlethal (Borrow and Oldstone, 1997;

Oldstone et al., 1977). High mortality caused by LCMV infection is generally associated with immunopathology rather than direct viral damage. Indeed, in contrast to immunosuppressed transplant recipients, LCMV-infected immune-deficient mice, such as RAG knockout, SCID, and nude mice, do not show apparent clinical symptoms despite high levels of viremia. Thus, LCMV infection in mice causes lethal disease only when virus-specific T cells attack critical infected organs (Borrow and Oldstone, 1997). Similarly, in severe cases of LCMV infection in immunocompetent humans, meningitis occurs along with increase of lymphocyte counts in cerebrospinal fluid, suggesting immune-mediated disease (Peters, 2006). Thus, pathogenesis of LCMV infection in transplant recipients appears to be quite distinct from that seen in immunocompetent humans or from the classical observations made in mice (Borrow and Oldstone, 1997).

It is important to investigate the mechanism of LCMV disease in the presence of the calcineurin inhibitor FK506, the most effective and commonly used immunosuppressive drug in transplantation, not only for developing potential treatment strategies but also for understanding how a noncytolytic virus can cause lethal disease under conditions of immunosuppression. To address this issue, we have used the mouse model of LCMV infection to examine how FK506 alters the pathogenesis of acute viral infection. We found that, similar to what was seen in the transplant recipients, infection of FK506-treated mice with the LCMV Armstrong strain, which normally causes an acute self-limiting infection in adult mice, resulted in a lethal disease characterized by high levels of viremia, lack of seroconversion, and minimal lymphocytic infiltrates in the tissues. However, we found that despite the

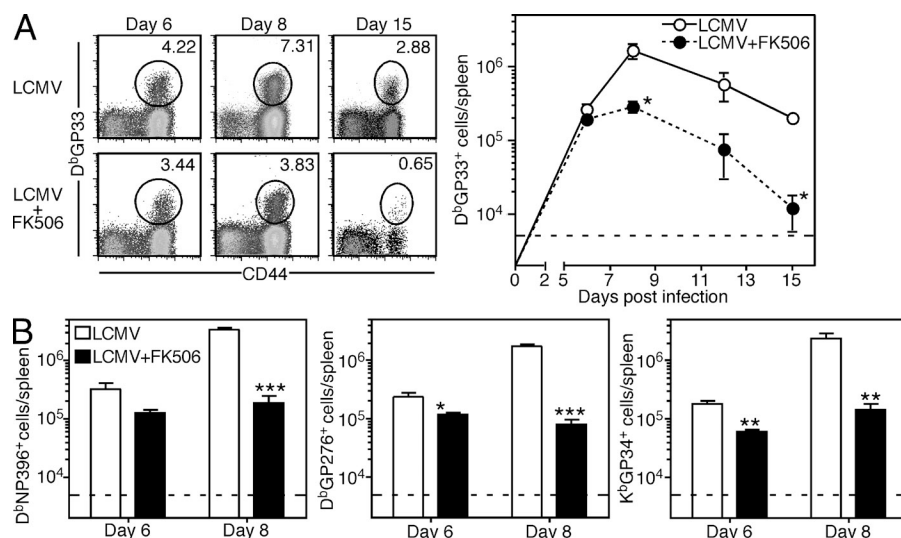


Figure 2. Kinetics of LCMV-specific CD8 T cell responses in the presence of FK506.

B6 mice were infected with LCMV Armstrong with or without FK506 treatment. (A) The percentage of CD8⁺ T cells from spleen staining positive for D^bGP33 tetramer is shown for a representative mouse on days 6, 8, and 15 after infection (left). Flow cytometry plots are gated on CD8⁺ T cells and the number indicates the percentage of D^bGP33 tetramer⁺ cells. Shown on the right is the mean number of CD8⁺ T cells/spleen specific for the D^bGP33 epitope ($n = \sim 3-6$, each time point). *, $P < 0.01$. (B) The mean number of CD8⁺ T cells/spleen specific for other three dominant epitopes (left, D^bNP396; center, D^bGP276; right, K^bGP34) at days 6 and 8 after infection in control and FK506-treated mice ($n = \sim 3-6$, each time point). Error bars in A and B indicate SEM. The horizontal dotted lines in A and B show the lower limit of detection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$. Data for A and B are representative of two or three independent experiments.

apparent absence of virus-specific immune responses, this lethal LCMV disease in FK506-treated mice was T cell mediated. Surprisingly, the immunosuppressive drug FK506 did not prevent the proliferation and generation of LCMV-specific CD8 and CD4 T cells but dramatically altered their differentiation so that these effector T cells lost their ability to control LCMV infection but were still capable of mediating disease. Our studies show that T cell-mediated viral disease can occur even in the presence of immunosuppression and have important implications for transplantation. These results provide a potential new strategy for treatment in transplant recipients who have acute complications of viral infection.

RESULTS

A mouse model of LCMV infection in the presence of FK506 mimics LCMV disease in human transplant recipients

To model LCMV infection of human transplant recipients, we used a mouse model of LCMV strain Armstrong infection that causes acute systemic infection. This infection is asymptomatic in immunocompetent mice and virus is cleared within 8 d after infection (Wherry et al., 2003). In this study, groups of control and calcineurin inhibitor FK506-treated B6 mice were infected with LCMV strain Armstrong. Drug treatment was begun 1 d before infection and continued daily. As was expected, LCMV infection of control mice gave little or no sign of disease and virus was eliminated by day 8 after infection (Fig. 1, A–C). In contrast, infection of FK506-treated mice resulted in clinical signs and significant weight loss starting 7–8 d after infection with >50% of animals dead by 20 d after infection (Fig. 1, A and B). Although untreated mice controlled infection by day 8, significant viremia remained throughout the observation period in drug-treated animals and none developed detectable levels of anti-LCMV serum antibodies suggesting minimal B cell responses (Fig. 1, C and D). Inflammatory infiltrates were mild or unapparent in brains, lungs, kidneys, and livers of drug-treated animals (Fig. S1, A and B), but a few drug-treated animals did show minimal to mild

hepatitis (Fig. S1 B). In addition, liver enzyme levels in serum were elevated in FK506-treated infected animals (Fig. 1 E). Similar patterns of sustained viremia, no seroconversion, along with mild lymphocyte infiltration, high morbidity and mortality, and elevated levels of liver enzyme in serum were described in transplant patients that received LCMV-contaminated organs (Fischer et al., 2006).

LCMV-specific CD8⁺ T cells expand in the presence of FK506 but do not differentiate into functional effector cells

Experiments in the previous section showed impaired humoral immune responses in FK506-treated LCMV-infected animals (Fig. 1 D). Next, to determine whether FK506 inhibits cellular immunity, an analysis of the phenotype and function of virus-specific CD8⁺ T cell responses in infected control and FK506-treated animals was performed. Surprisingly, a comparison of CD8⁺ T cell responses measured by MHC class I tetramers for the GP33 epitope revealed initially similar numbers in both FK506-treated and control animals (Fig. 2 A). However, beyond 6 d, the magnitude of GP33-specific CD8 T cell responses in drug-treated animals was significantly lower than in controls (Fig. 2 A). This phenomenon was also observed with three other LCMV-specific CD8 T cell epitopes (Fig. 2 B). By day 15, responses of FK506-treated animals were, on average, 10-fold lower in magnitude than controls (Fig. 2 A).

These virus-specific CD8⁺ T cells in both control and FK506-treated animals showed an activated phenotype (CD127^{Low}, CD62L^{Low}, and CD44^{High}) at day 8 after infection (Fig. 3 A). However, there were striking differences in the function of CD8⁺ T cells in FK506-treated versus control animals. Thus, although almost all of the GP33/34-specific CD8⁺ T cells from untreated controls produced IFN- γ at day 6 and $\sim 50\%$ TNF, markedly fewer

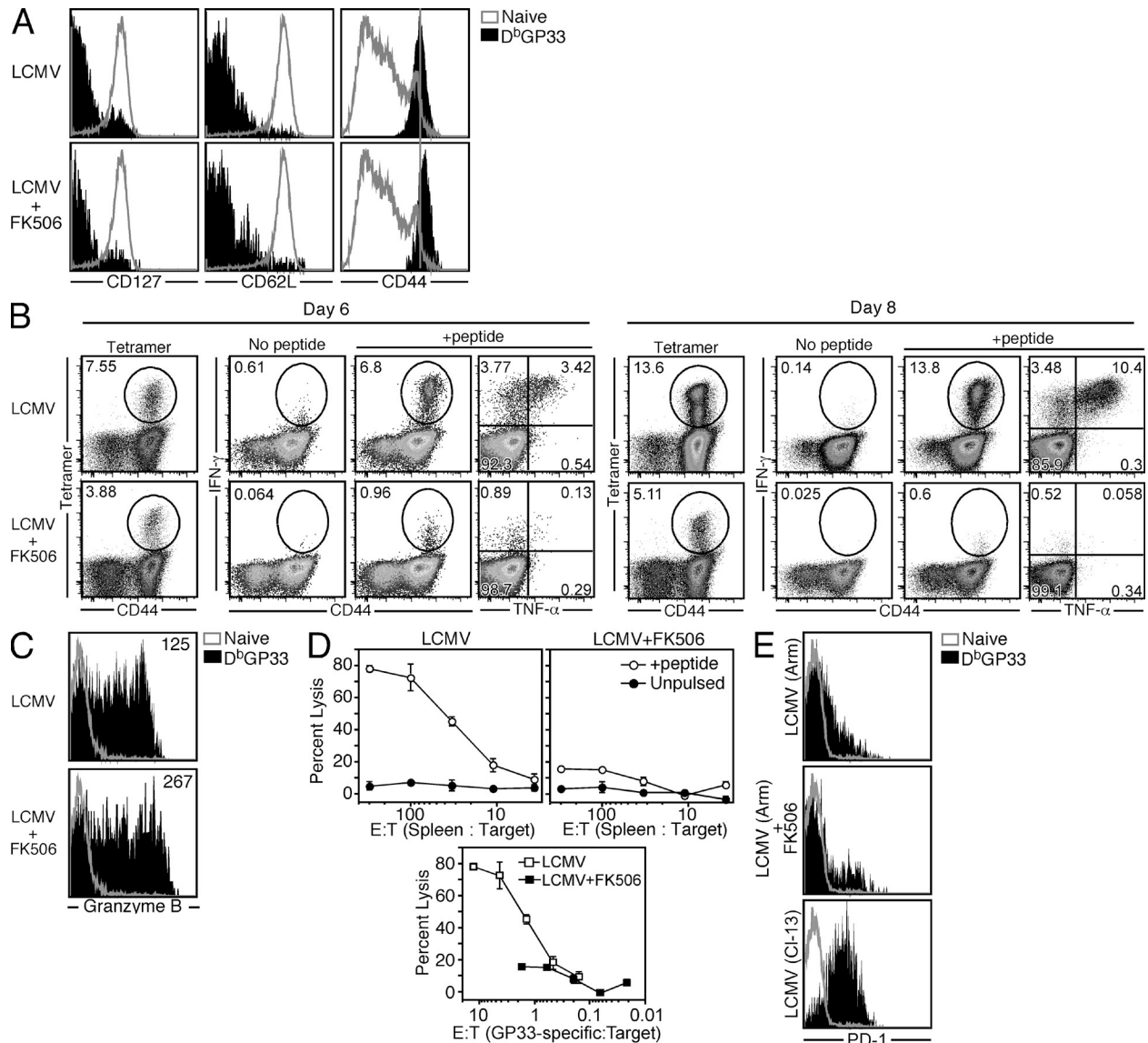


Figure 3. FK506 treatment alters effector CD8 T cell differentiation. B6 mice were infected with LCMV Armstrong with or without FK506 treatment. (A) Phenotype of D^bGP33 tetramer⁺ CD8⁺ cells was compared between FK506-treated and untreated mice on day 8 after infection. Flow cytometry data are gated on D^bGP33 tetramer⁺ CD8⁺ cells. The gray line shows CD8⁺ T cells of naive B6 mice. (B) IFN-γ and TNF production of spleen CD8 T cells stimulated or not with GP33 peptide for 5 h on days 6 and 8 after infection. For better comparison of frequencies of tetramer-positive cells and cytokine-producing cells, D^bGP33 and K^bGP34 tetramer-positive cells were detected together in one flow panel. The flow cytometry plots of each panel are gated on CD8⁺ T cells. (C) Granzyme B expression in D^bGP33 tetramer⁺ CD8⁺ T cells was examined on day 8 after infection. The gray line shows granzyme B levels in CD8⁺ T cells of a naive mouse. The number represents mean fluorescence intensity of granzyme B in D^bGP33⁺ cells (black histogram). (D) Ex vivo cytotoxic activity of spleen cells isolated from FK506-treated or untreated LCMV-infected mice on day 8 after infection was measured in a 5-h chromium release assay. Target cells were GP33 peptide-pulsed or unpulsed MC57 cells. The effector/target cell ratios (E:T) of the top two panels show total spleen cells to targets. The effector/target cell ratios of the bottom panel were calculated by antigen-specific tetramer-positive cells. Error bars indicate SEM. (E) The expression of PD-1 on D^bGP33 tetramer⁺ CD8⁺ cells was compared between spleen cells of FK506-treated and untreated mice on day 8 after infection. As a positive control, PD-1 expression after LCMV strain clone 13 infection (day 8 after infection) is shown on the bottom. Flow cytometry data are gated on D^bGP33 tetramer⁺ CD8⁺ cells. The gray line shows CD8⁺ T cells of naive B6 mice. Data (A–E) are representative of at least two independent experiments.

CD8⁺ T cells from treated animals were IFN-γ and TNF producers (Fig. 3 B). Moreover, by day 8, differences between the two groups were even more evident and cells producing both cytokines were virtually absent in FK506-treated mice (Fig. 3 B).

In addition to defect of cytokine production, despite higher levels of granzyme B expression (Fig. 3 C) and normal degranulation ability (Fig. S2 A), a chromium release assay showed that cytotoxic activity was impaired in drug-treated animals compared with untreated controls (Fig. 3 D).

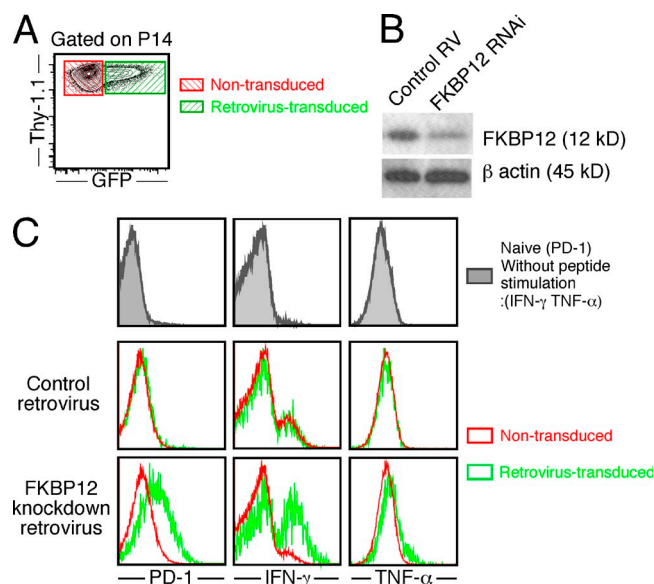


Figure 4. FK506 acts intrinsically in virus-specific CD8 T cells to alter T cell differentiation. Thy-1.1⁺ LCMV-specific P14 transgenic naive CD8 T cells were activated *in vivo* and then transduced with retroviruses (marked by GFP expression) expressing shRNA for FKBP12 or empty control vector. These transduced P14 cells were then adoptively transferred into naive Thy-1.2⁺ mice and these mice were infected with LCMV in the presence of FK506. Spleen cells were used for analysis on day 5 after infection. (A) Retrovirus-transduced Thy-1.1⁺ P14 cells were distinguished by GFP expression. The dot plots are gated on P14 cells. (B) GFP⁺ retrovirus-transduced P14 cells were purified by FACS, and then FKBP12 expression was examined by Western blotting. (C) Phenotypic and functional analysis of retrovirus-transduced cells. PD-1 and cytokine expression (IFN- γ and TNF upon peptide stimulation) on retrovirus-transduced (GFP⁺) and non-transduced (GFP⁻) P14 cells in spleen cells on day 5 after infection. For cytokine expression, spleen cells were stimulated with GP33 peptide for 5 h. Data are representative of three independent experiments.

Such impaired cytotoxic activity in drug-treated mice was also observed in an *in vivo* killing assay (Fig. S2 B). In addition, the decreased *in vivo* killing ability was confirmed on a per cell basis in another *in vivo* killing experiment where the effector/target cell ratio was tightly controlled by adoptive transfer of effector CD8 T cells (Fig. S3). Collectively, these data indicate that impaired function of cytokine production and cytotoxic activity was evident in virus-specific CD8⁺ T cells in FK506-treated mice, which likely accounted for the failure of T cells from treated animals to control virus infection.

Interestingly, this functional impairment was not associated with a programmed death 1 (PD-1) expression (Fig. 3 E). As shown previously (Barber et al., 2006), this inhibitory receptor was highly expressed on exhausted virus-specific CD8 T cells in immunocompetent mice infected with LCMV clone 13 strain that caused chronic infection (Fig. 3 E). However, virus-specific CD8 T cells in drug-treated LCMV-infected animals showed minimal PD-1 expression, similar to untreated animals (Fig. 3 E). These data indicate that dysfunction of virus-specific CD8 T cells in the presence of FK506 is distinct from that seen in chronic infection.

It is important to understand the mechanism by which FK506 treatment induces the unusual dysfunction of virus-specific CD8 T cells because CD8 T cells play critical roles in both controlling LCMV infection and mediating LCMV disease. FK506 is well known to suppress activation of calcineurin and, thereby, block its ability to activate NFAT, which transcribes specific genes including IFN- γ . In addition, a recent paper has shown that NFAT also regulates PD-1 expression on T cells (Oestreich et al., 2008). Thus, FK506 might directly inhibit the calcineurin-NFAT signaling pathway intrinsic in virus-specific CD8 T cells to alter T cell differentiation. However, because FK506-treated mice showed significant changes, including clinical manifestation and viral titers, this effect could alter T cell function. To address this question, we made FK506-insensitive virus-specific CD8 T cells by knocking down FKBP12 using a retrovirus-based short hairpin (sh) RNA system expressing GFP as a transduction marker. FKBP12 is an essential intracellular binding partner of FK506, so that without FKBP12, FK506 is unable to inhibit activation of calcineurin. Control or FKBP12 knockdown retrovirus-transduced LCMV-specific transgenic CD8 T cells were adoptively transferred into naive mice, followed by LCMV infection in the presence of FK506 (Fig. 4, A–C). We were able to determine intrinsic effects of FK506 in virus-specific CD8 T cells by comparing GFP^{positive} transduced cells with GFP^{negative} nontransduced cells (Fig. 4, A and B). Using this system, we examined whether PD-1 expression and cytokine production were changed in FK506-insensitive antigen-specific CD8 T cells. PD-1 expression levels increased on antigen-specific CD8 T cells with FKBP12 knockdown in FK506-treated mice (Fig. 4 C). Production of IFN- γ was also enhanced in these FK506-insensitive antigen-specific CD8 T cells to peptide stimulation (Fig. 4 C). Furthermore, we observed modest restoration of TNF production by FKBP12 knockdown (Fig. 4 C). These results show that FK506 acts intrinsically in virus-specific CD8 T cells to induce PD-1^{low} functionally impaired T cells.

Next, to investigate differences in kinetics of virus-specific CD8⁺ T cell responses between treated and untreated animals, we analyzed CD8⁺ T cell proliferation, phenotype, and function at an early time point (day 4) of infection using LCMV-specific transgenic CD8⁺ T cells. CFSE profiles demonstrated that there were minimal differences in the rate of the division of antigen-specific T cells in virus-infected drug-treated versus untreated mice and no significant changes in the absolute number of antigen-specific T cells (Fig. 5 A), showing that FK506 did not inhibit initial proliferation of virus-specific CD8 T cells. However, functional and phenotypic differences were already seen at this early time point, and LCMV-specific CD8 T cells in drug-treated mice showed impaired cytokine production and high levels of granzyme B (Fig. 5, B and C). Interestingly, we found striking differences in the expression of CD27 and the killer cell lectin-like receptor G1 (KLRG-1). LCMV-specific transgenic CD8⁺ T cells of FK506-treated mice expressed lower levels of CD27 and more KLRG-1 than did cells of control mice

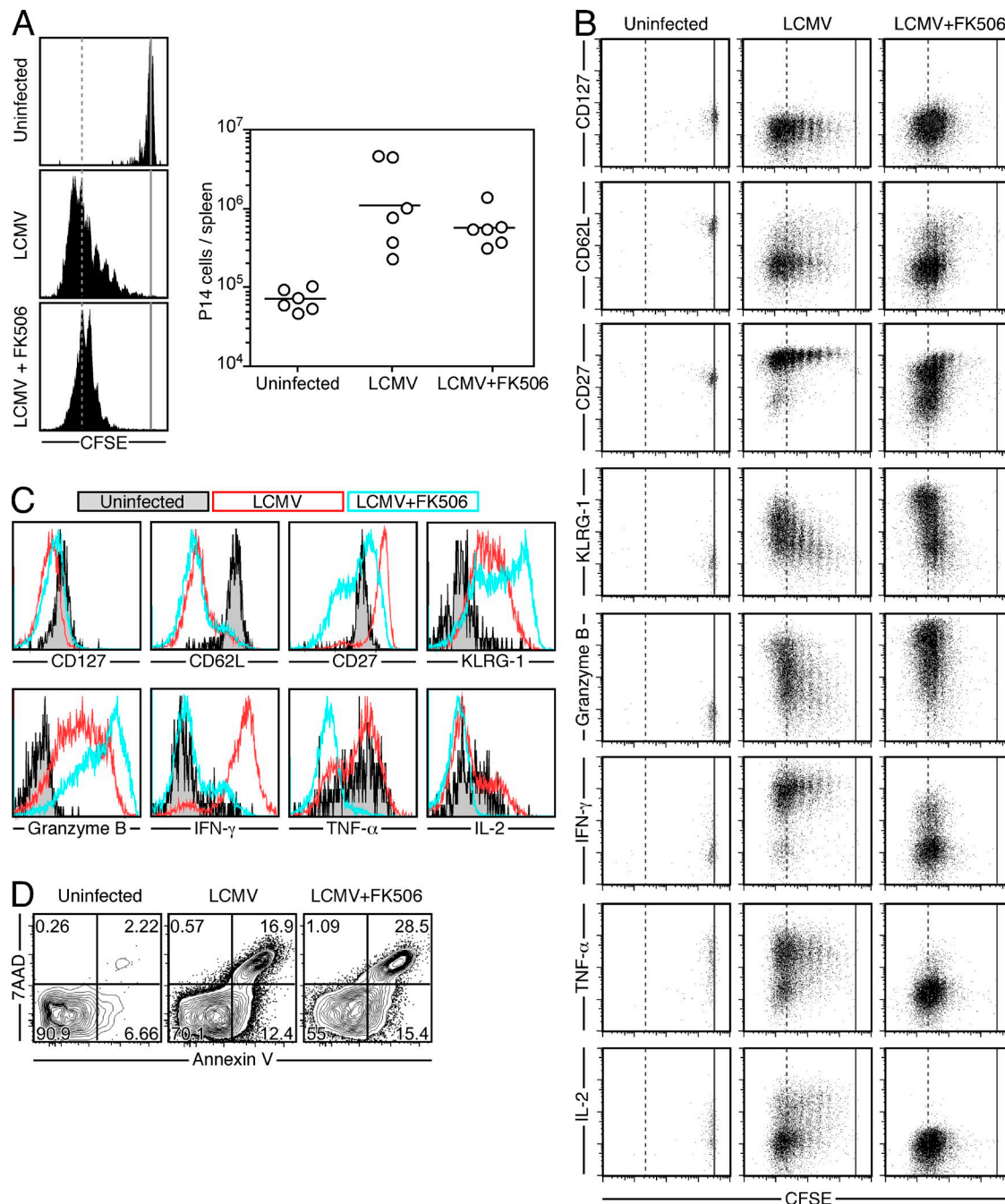


Figure 5. Virus-specific T cells proliferate but do not accumulate in the presence of FK506. CFSE-labeled P14 transgenic T cells that bear GP33-specific TCR and Thy-1.1 marker were adoptively transferred into Thy-1.2⁺ recipient B6 mice 1 d before infection, and then FK506 treatment was started. The next day (day 0), these mice were infected or not with LCMV Armstrong, and spleen cells were isolated on day 4 after infection for analysis. (A) Proliferation and the absolute number of P14 cells were assessed in spleen. Histograms were gated on P14 Thy-1.1⁺ CD8⁺ transgenic T cells. Horizontal bars (right) show the geometric mean. (B and C) Cell surface and functional markers on gated Thy-1.1⁺ CD8⁺ P14 cells. For cytokine expression, spleen cells were stimulated with GP33 peptide for 5 h. Flow cytometry plots in B are shown as histograms in C. (D) Apoptosis of effector CD8 T cells as determined by Annexin V and 7AAD staining. Plots are gated on P14 transgenic T cells. Data (A–D) are representative of two or three independent experiments.

(Fig. 5, B and C). This phenotypic feature was also seen on endogenous LCMV-specific tetramer⁺ cells in LCMV-infected FK506-treated B6 mice (Fig. S4). The CD27^{Low} KLRG-1^{high} phenotype suggests that virus-specific CD8

T cells of FK506-treated mice rapidly became terminally differentiated effector cells compared with control animals (Hamann et al., 1997; Voehringer et al., 2001; Joshi et al., 2007; Sarkar et al., 2008). In addition, we found a higher frequency

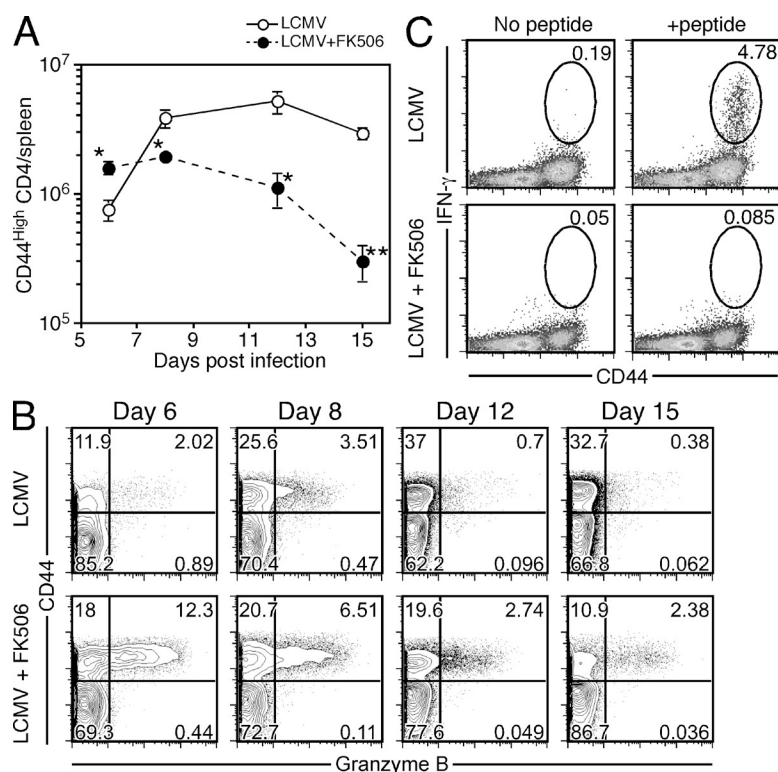


Figure 6. CD4 T cell responses in FK506-treated mice. B6 mice were infected with LCMV Armstrong in the presence or absence of FK506. (A) The number of CD44^{high} CD4⁺ T cells/spleen is plotted over time after infection. Data are the mean for three mice/time point. Error bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$. (B) The percentage of CD44^{high} CD4⁺ T cells staining positive for granzyme B is shown for spleen cells of a representative mouse at each time point. (C) IFN- γ production by CD4⁺ T cells stimulated with LCMV peptide for each group 8 d after infection. Spleen cells were stimulated with GP61 peptide (LCMV CD4 epitope) or left unstimulated for 5 h. Numbers represent the percentage of IFN- γ ⁺ cells in CD4⁺ T cells. Data (A–C) are representative of two independent experiments.

were absent in FK506-treated mice after peptide stimulation (Fig. 6 C).

Generation of pathogenic T cells in FK506-treated mice

LCMV disease in immunocompetent mice is the classical example of T cell-mediated viral disease (Borrow and Oldstone, 1997). LCMV-infected mice develop clinical signs only when effector T cells attack virus-infected critical organs. In this circumstance, effector T cells are usually functional in terms of cytokine production and cytotoxic activity. In contrast, virus-specific T cells in the presence of FK506 were dysfunctional. Therefore, it was important to investigate whether these dysfunctional T cells could mediate lethal disease in LCMV-infected FK506-treated mice. To address this question, either CD4 or CD8 T cells were depleted from the mice by injecting anti-CD4 or -CD8 antibody on days 0 and 3 after infection. We found that depletion of CD8⁺ cells resulted in fewer FK506-treated animals succumbing to infection, whereas CD4⁺ depletion had no effect (Fig. 7 A). However, simultaneous depletion of both T cell subsets completely abrogated the adverse effects of immunosuppression so that all drug-treated animals survived after LCMV infection (Fig. 7 A). Virus was, not surprisingly, detected in all groups (Fig. 7 A). In addition to T cell depletion experiments, LCMV-infected RAG^{-/-} mice that lacked adaptive immunity did not develop disease in the presence of FK506 (Fig. S5). These data show that T cells were involved in the mediation of disease in FK506-treated LCMV-infected mice rather than direct viral injury and that the cells involved were likely to be principally the CD8⁺ T cells.

These experiments revealed that the consequences of FK506 suppression impaired the protective function of T cells, but these T cells still retained their ability to cause clinical disease. We next examined if inflammatory cytokines were involved in this disease. To address this issue, we measured serum cytokine levels in drug-treated and untreated animals. FK506-treated animals showed markedly higher serum levels of the inflammatory cytokines TNF and IL-6 compared with control infected animals (Fig. 7 B). In contrast, another inflammatory cytokine IL-17 was not detected in serum of

of apoptotic cells in LCMV-infected FK506-treated animals based on Annexin V and 7-aminoactinomycin D (7AAD) staining (Fig. 5 D). These results account for the observation that the overall expansion of virus-specific CD8⁺ T cells in FK506-treated mice was significantly inhibited compared with controls after day 8, despite the fact that initial proliferation was similar. Collectively, our results (Figs. 2–5) show that the calcineurin inhibitor FK506 does not prevent the generation of virus-specific CD8 T cells but dramatically alters their differentiation.

FK506 treatment alters virus-specific CD4 T cell responses after LCMV infection

We next examined whether FK506 treatment changed CD4 T cell responses in LCMV-infected mice. Interestingly, in FK506-treated mice, the number of CD44^{high} CD4⁺ T cells was higher than control animals on day 6 (Fig. 6 A). As was expected from the results of CD8⁺ T cell responses, the absolute number of antigen-experienced CD4⁺ T cell responses in drug-treated animals was significantly less than in controls after day 8 (Fig. 6 A). However, high expression levels of granzyme B in CD4⁺ cells of FK506-treated animals were maintained throughout infection (Fig. 6 B). In contrast to LCMV-infected mice, FK506 treatment alone without LCMV infection had minimal or no effect on granzyme expression (unpublished data). Thus, it seems that such higher levels of granzyme B expression in drug-treated LCMV-infected mice were induced by continuous TCR stimulation by high levels of persistent viral antigen. Similar to what was seen in CD8⁺ T cells, IFN- γ -producing CD4⁺ T cells

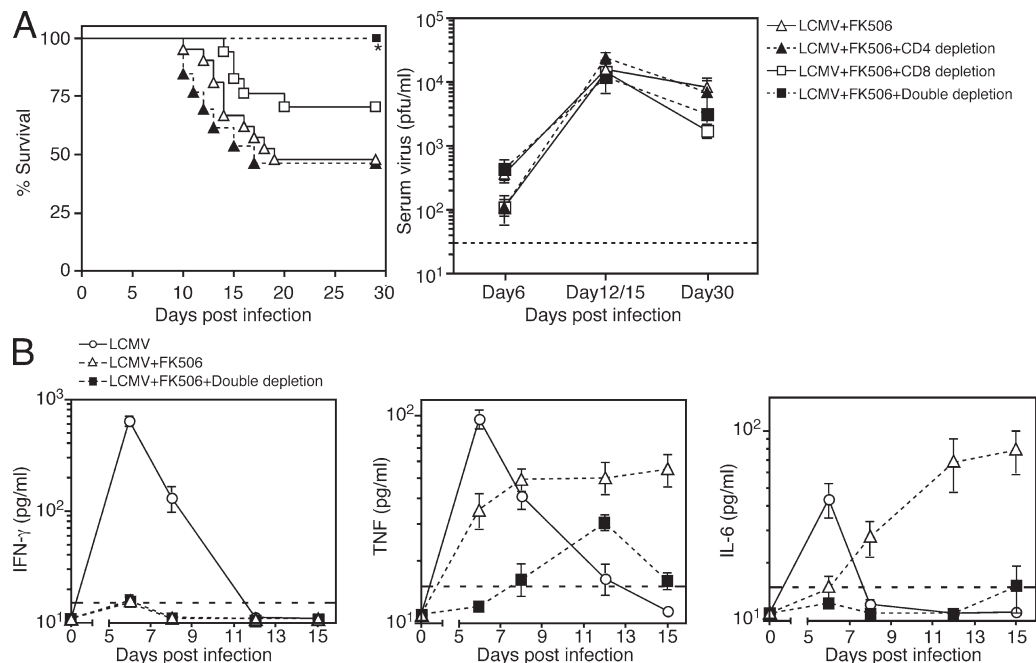


Figure 7. Pathogenic T cells orchestrate inflammatory cytokine production. B6 mice were infected with LCMV Armstrong in the presence or absence of FK506. (A) For single depletion of CD4⁺ and CD8⁺ T cells, either anti-CD4 or anti-CD8 antibody was injected i.p. into LCMV-infected B6 mice on day 0 and day 3 of infection. For double depletion of CD4⁺ and CD8⁺ T cells, both anti-CD4 and -CD8 antibodies were injected. Survival curves are shown on the left ($n = 21$, LCMV + FK506; $n = 13$, LCMV + FK506 + CD4 depletion; $n = 17$, LCMV + FK506 + CD8 depletion; $n = 9$, LCMV + FK506 + double depletion). *, $P < 0.05$ (vs. LCMV + FK506). For viral titer, sera were collected at three different time points (days 6, 12–15, and 30 after infection). The mean viral titer in serum is shown ($n = 3–9$, each time point). (B) Serum cytokine levels were measured using cytometric bead array ($n = 3–9$, each time point). The horizontal dashed lines indicate the lower limit of detection. There were no detectable levels of IFN- γ , TNF, and IL-6 in serum of uninfected mice treated with FK506 for 15 d (not depicted). Error bars in A and B indicate SEM. Data for A and B were pooled from two or three independent experiments.

either control or drug-treated mice (<2.4 pg/ml). High levels of TNF and IL-6 were evident in the late phase when treated mice were showing clinical disease. In addition, mice depleted of CD4 and CD8 T cells in which the pattern of disease was fully reversed showed lower levels of TNF and IL-6 compared with undepleted FK506-treated mice (Fig. 7 B).

However, because T cell-depletion by antibodies removes all T cells, including virus-specific and naive CD4/8 T cells, and T reg cells, it is unclear if the disease manifestation and overproduction of inflammatory cytokines was directly mediated by virus-specific T cells. To investigate this issue, we adoptively transferred either LCMV-nonspecific (OT-1) or -specific (P14) transgenic CD8 T cells into RAG^{-/-} mice, followed by LCMV infection in the presence or absence of FK506 treatment (Fig. 8). Our data clearly showed that LCMV disease was mediated by virus-specific T cells in FK506-treated animals. Thus, only mice that received LCMV-specific P14 cells combined with FK506 treatment succumbed to infection, and all other groups (OT-1 with or without FK506 and P14 without FK506) survived (Fig. 8 A). Virus was controlled only in a group that received LCMV-specific P14 cells without FK506 treatment (Fig. 8 B). In addition, in the presence of FK506, P14 transgenic T cells lost their ability to produce IFN- γ and TNF, similar to wild-type B6 mice (Fig. 8 C). Furthermore, similar patterns of high levels of the inflammatory cytokines TNF and IL-6 were observed in LCMV-infected FK506-treated RAG^{-/-}

mice adoptively transferred with P14 cells (Fig. 8 D). These data indicate that functionally impaired virus-specific T cells orchestrate LCMV lethal disease in FK506-treated animals and induce overproduction of inflammatory cytokines.

Liver macrophages produce inflammatory cytokines in LCMV-infected FK506-treated mice

Virus-specific T cell-dependent inflammatory cytokine production in FK506-treated mice is somewhat paradoxical because virus-specific T cells generated in the presence of this drug do not substantially produce TNF (Fig. 3 B). In addition, virus-specific CD8 T cells were unable to produce IL-6 either in the presence or absence of FK506 (unpublished data). Thus, these data suggest that functionally impaired virus-specific T cells orchestrate the production of inflammatory cytokines, but they do not themselves produce these cytokines. Indeed, in FK506-treated mice, we found high levels of inflammatory cytokines as well as the accumulation of macrophages in livers (Fig. 9) in which mild hepatitis was observed (Fig. 1 E and Fig. S1 B). Thus, liver homogenates of FK506-treated mice had higher levels of TNF and IL-6 than control animals (Fig. 9 A), and fivefold higher numbers of macrophages (CD11b⁺ F4/80⁺) were recovered from the livers of infected FK506-treated animals (Fig. 9, B and C). Furthermore, accumulated macrophage populations in FK506-treated mice had higher TNF and IL-6 mRNA levels than control mice (Fig. 9 D).

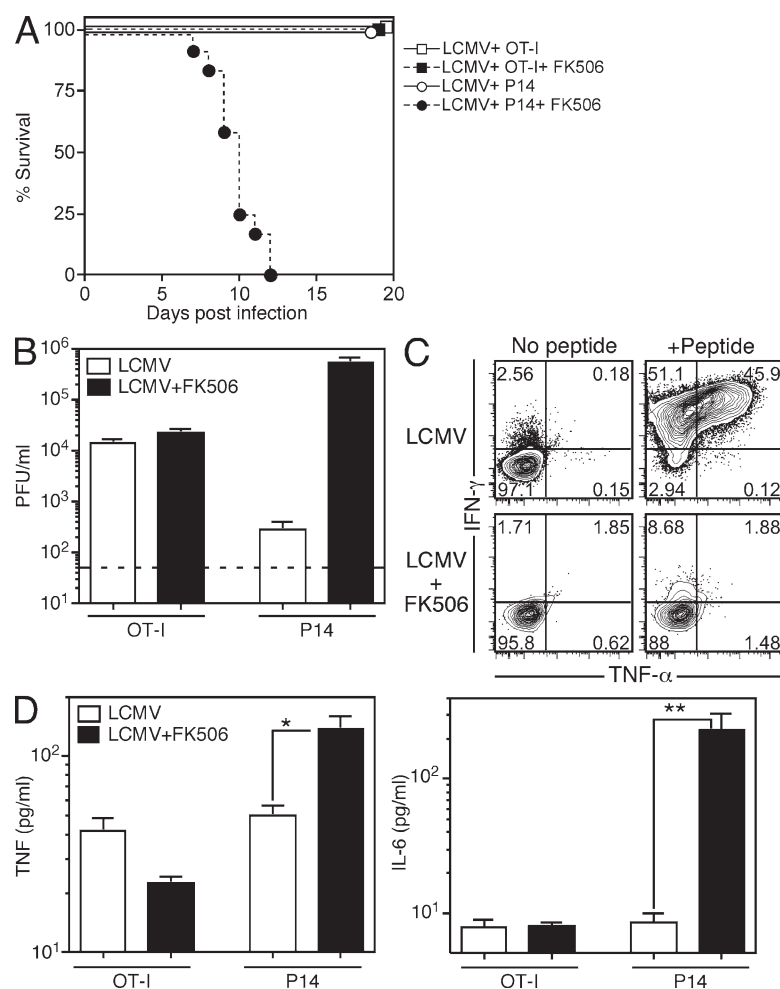


Figure 8. Functionally impaired virus-specific T cells mediate LCMV lethal disease in FK506-treated mice. Purified P14 (LCMV specific) or OT-1 (nonspecific) transgenic CD8 T cells were adoptively transferred into RAG1^{-/-} mice, followed by LCMV infection in the presence or absence of FK506. (A) Survival curve ($n = 6$, LCMV + OT-1; $n = 6$, LCMV + OT-1 + FK506; $n = 11$, LCMV + P14; $n = 12$, LCMV + P14 + FK506). (B) Serum viral titers ($n = 5-6$ in each group, day 9 after infection). The horizontal dashed line indicates the lower limit of detection. (C) IFN- γ and TNF production by P14 cell transfer is shown on day 7 after infection for a representative mouse from two independent experiments. Spleen cells were stimulated or not with GP33 peptide for 5 h. The flow cytometry plots of each panel are gated on P14 cells. (D) Serum cytokine levels on day 9 after infection for each of the four groups were measured using cytometric bead array ($n = 5-6$, each group). *, $P < 0.05$; **, $P < 0.01$. Error bars in B and D indicate SEM. Data (A, B, and D) were pooled from two independent experiments.

DISCUSSION

Immunosuppressed transplant patients that inadvertently contracted LCMV infection from their organ allograft showed high mortality, and most of the clinical information obtained from the transplant patients that were under an FK506-based regimen suggested that the pathogenesis of LCMV disease was direct viral injury and not immune mediated (Fischer et al., 2006; Peters, 2006). However, such high mortality without evidence of immunopathology was unexpected because LCMV is a noncytolytic

virus, and disease is usually associated with tissue damage when immunopathologic T cells destroy viral-infected cells in critical organs (Borrow and Oldstone, 1997). In this study, we investigated how the calcineurin inhibitor FK506 changed viral disease manifestation and virus-specific T cell responses after LCMV infection using a mouse model. FK506-treated LCMV-infected mice showed high lethality and, importantly, this mouse model mimicked LCMV disease seen in the transplant recipients.

We defined two critical stages to develop the lethal disease in LCMV-infected mice in the presence of FK506. A first stage is generation of pathogenic T cells that were not protective but mediated clinical disease. Therefore, when this stage was blocked by T cell depletion, the pattern of the disease was fully reversed and all animals survived. A second critical stage is overproduction of inflammatory cytokines principally involving TNF and IL-6. In drug-treated infected animals, overproduction of these cytokines was coincident with the development of disease and death. Inhibiting these cytokines in drug-treated animals also reversed the disease phenotype. Thus, TNF and IL-6 are major players for disease manifestation in LCMV-infected FK506-treated mice. More importantly, such inflammatory cytokine production was orchestrated by functionally impaired virus-specific

Our results suggest that functionally impaired virus-specific T cells induce the production of inflammatory cytokines by other cells such as liver macrophages.

Overproduction of inflammatory cytokines responsible for lethal disease in LCMV-infected FK506-treated mice

The data in the previous section suggest that clinical disease was a result of the pathogenic effects of high cytokine levels of TNF and IL-6 because overproduction of inflammatory cytokines was only seen in the mice with severe clinical signs. This was tested by experiments in which groups of FK506-treated LCMV-infected mice were given inhibitors of TNF and IL-6 and the outcome was compared. As the data in Fig. 10 show, although anti-IL-6 receptor antibody had no statistically significant impact on survival, treatment with soluble TNF receptor, which is an inhibitor of TNF, led to a significant improvement in survival. More significantly, simultaneous blockade of TNF and IL-6 provided the greatest protection, with >90% of mice surviving for the duration of the experiment. These results show that LCMV-infected FK506-treated animals were dying because of an excessive inflammatory cytokine response that was orchestrated by pathogenic virus-specific T cells generated in the presence of FK506.

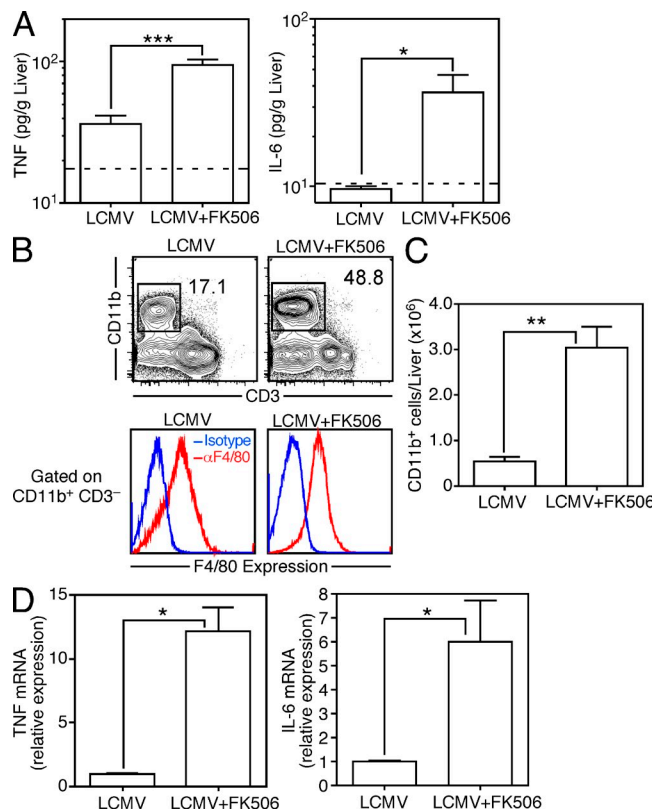


Figure 9. Liver macrophages produce inflammatory cytokines in LCMV-infected FK506-treated mice. Liver cytokine levels and intrahepatic mononuclear cells were examined 15 d after LCMV infection in the presence or absence of FK506. (A) Inflammatory cytokine expression in the liver, quantified by cytometric bead array in liver homogenates ($n = 6$, each group). The horizontal dashed lines indicate the lower limit of detection. (B) Flow plots show CD11b and CD3 staining patterns in intrahepatic mononuclear cells (top) and F4/80 staining in CD11b⁺ CD3⁻ cells (bottom). (C) Accumulation of CD11b⁺ CD3⁻ cells in the liver of FK506-treated mice ($n = 6$, each group). (D) Quantitative RT-PCR analysis of inflammatory cytokine expression in CD11b⁺ cells ($n = 3$, each group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars in A, C, and D indicate SEM. Data (A–D) are representative of two or three independent experiments.

T cells generated during the first stage. Therefore, mice lacking these pathogenic T cells in the presence of FK506 had reduced levels of the inflammatory cytokines in serum.

We also found that the pathogenic T cells themselves were not the major producers of the inflammatory cytokines and that liver macrophages could be a significant source of these cytokines in LCMV-infected FK506-treated mice. How do the functionally impaired pathogenic T cells stimulate macrophages to produce these inflammatory cytokines? It is well understood that macrophage activation is induced by IFN- γ derived from effector T cells. The pathogenic T cells do not produce substantial IFN- γ but very low levels of IFN- γ might activate macrophages. Furthermore, although these pathogenic T cells were functionally impaired, these virus-specific CD8 T cells in FK506-treated LCMV-infected mice did not entirely lose their ability to respond to viral antigen. Specifically, the virus-specific

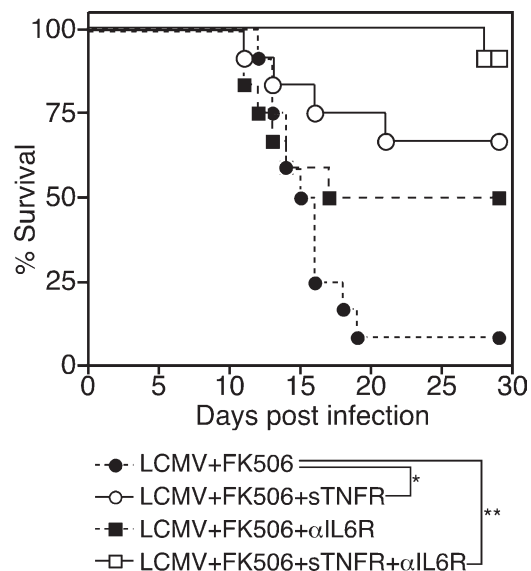


Figure 10. Overproduction of inflammatory cytokines responsible for lethal disease in LCMV-infected FK506-treated mice. B6 mice were infected with LCMV Armstrong in the presence or absence of FK506. Mice were treated with soluble TNF receptor (sTNFR), which is an inhibitor of TNF and/or anti-IL-6 receptor antibody ($\pm$ IL6R), from day 4 after infection. Survival curve is shown ($n = 12$, all groups). *, $P < 0.01$; **, $P < 0.0001$. Data were pooled from two or three independent experiments.

CD8 T cells generated in FK506-treated LCMV-infected animals had higher levels of granzyme B than control animals (Fig. 3 C). Such increase of granzyme B expression in the presence of FK506 was not seen in NK cells (unpublished data). Also, FK506 treatment itself without LCMV infection did not induce granzyme B in T cells (unpublished data). Because expression levels of granzyme B in T cells correlate with TCR stimulation, the increase of granzyme B expression in antigen-specific CD8 T cells in the drug-treated LCMV-infected mice is likely a result of continuous TCR stimulation by high levels of persistent viral antigen. In addition, degranulation ability was maintained in these functionally impaired T cells (Fig. S2 A). These observations suggest that virus-specific CD8 T cells generated in the presence of FK506 still retain some functionality to respond to viral antigen, and they might be able to kill infected target cells. Indeed, although killing activity was below the detectable levels by a chromium release assay in FK506-treated LCMV-infected mice, we observed low levels of killing activity of these cells in vivo using a highly sensitive in vivo killing assay (Fig. S2 B and Fig. S3). This retained ability of the functionally impaired T cells is too weak to eliminate LCMV, but this might cause activation of macrophages by damaging infected cells or directly responding to infected macrophages.

In addition to the pathogenic effect of antigen-specific CD8 T cells, it was surprising that virus-specific T cells proliferated when the daily administration of FK506 was started 1 d before infection because FK506 is one of the most effective immunosuppressive drugs. Even when the FK506 treatment was begun 3 d before infection, significant levels of initial

T cell proliferation were seen in FK506-treated animals (Fig. S6). These observations were unexpected because calcineurin inhibitor is considered to prevent T cell proliferation by suppressing IL-2 production, at least in vitro. However, T cell proliferation in vivo in the presence of the calcineurin inhibitor is consistent with the observation that IL-2 has minimal effects for CD8 T cell expansion after viral infection in vivo (Williams et al., 2006). This indicates that CD8 T cells could proliferate without IL-2 signals. In addition, LCMV infection induces strong innate immune response, including type I IFN, that acts directly on CD8 T cells and allows clonal expansion (Kolumam et al., 2005). Furthermore, we found that FK506-treated mice had slightly lower numbers of T reg cells compared with controls, perhaps as a result of decreased expression of CD25 (Fig. S7, A and B). CD25 is an essential molecule to maintain the number of T reg cells, and to up-regulate CD25 on T reg cells Foxp3 needs to make a transcriptional complex with NFAT in the nucleus (Wu et al., 2006). Because FK506 blocks translocation of NFAT from the cytoplasm to the nucleus, this may result in reduced CD25 levels and a reduction in the number of T reg cells. Accordingly, virus-specific T cells might proliferate by such alternative mechanisms (stimulation with type I IFN and decreased numbers of T reg cells).

Similar observations about T cell proliferation were shown in previous studies in which another calcineurin inhibitor, cyclosporin A, failed to inhibit T cell proliferation in response to alloantigens in vivo (Chisholm et al., 1985; Kroczeck et al., 1987). These data suggest that inhibition of T cell proliferation might not be a primary role of calcineurin inhibitor in vivo. Indeed, we found that virus-specific CD8⁺ T cells in FK506-treated animals rapidly differentiated into terminally differentiated end-stage effector cells, compared with controls, and showed higher frequency of apoptotic cells. In addition, more notable differences of T cells between the drug-treated and control-infected mice were detected by functional assays. Thus, both CD4⁺ and CD8⁺ T cells in FK506-treated animals showed increases in granzyme B levels and, perhaps of more importance, CD8⁺ T cells from treated animals showed impaired cytotoxic function and did not generate substantial IFN- γ and TNF in response to antigen stimulation. This pattern of impaired function resembled that of exhausted T cells in chronic infection (Zajac et al., 1998; Wherry et al., 2003). However, FK506 treatment suppressed expression of the inhibitory molecule PD-1, which is an essential molecule for T cell exhaustion in chronic infection (Barber et al., 2006; Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Radziejewicz et al., 2007), suggesting that functional impairment in FK506-treated animals is a PD-1-independent phenomenon. This indicates that functional impairment in FK506-treated animals is caused by a different mechanism than that which occurs in chronic infection. In support of this notion, we found that the dysfunction of virus-specific CD8 T cells in FK506-treated mice could be caused by blocking the calcineurin–NFAT signaling pathway that is intrinsic in T cells. Overall, our data indicate that the calcineurin inhibitor FK506 treatment alters CD8 T cell differentiation and induces

distinct virus-specific CD8 T cells from those generated during either acute or chronic viral infections.

Such functional impairment and altered differentiation of antigen-specific CD8 T cells were reversible when FK506 treatment was stopped. Although stopping the drug treatment at day 7 after infection did not reduce mortality (Fig. S8 A), it significantly decreased viral titers (Fig. S8 B). This better viral control was most likely a result of restoration of survival and function of antigen-specific T cells. Thus, although deletion of antigen-specific CD8 T cells occurred by continuous FK506 treatment, the discontinuation of the drug improved survival of antigen-specific CD8 T cells (Fig. S8 C). Furthermore, IFN- γ production was restored in the group (Fig. S8 D). Therefore, it seems that continuous FK506 treatment has a critical role in inducing functional impairment and changing T cell differentiation.

In contrast to the effect of FK506, we and others have recently reported that another common immunosuppressive drug, rapamycin, has a very different effect on memory T cell differentiation (Araki et al., 2009; Pearce et al., 2009). Rapamycin is structurally similar to FK506, and both drugs bind to the same intracellular binding partner, FKBP12. However, the mechanism of action of these two drugs is different. The FK506–FKBP12 complex inhibits the calcineurin–NFAT signaling pathway that initiates activation of specific genes, including IL-2, whereas the rapamycin–FKBP12 complex prevents mammalian target of rapamycin (mTOR) pathway, which regulates cell growth and metabolism (Wullschlegel et al., 2006). Unlike FK506 treatment, we did not observe apparent clinical symptoms or dysfunctional T cells in LCMV-infected mice treated with rapamycin (Araki et al., 2009). In fact, rapamycin treatment enhanced the generation of KLRG-1^{low} CD27^{hi} effector T cells and made a higher number of memory T cells by improving effector T cell survival (Araki et al., 2009). This rapamycin effect on T cell responses was diametrically opposed to the effect of FK506 in LCMV-infected mice because FK506 treatment accelerated generation of terminally differentiated end-stage effector cells that eventually died without differentiating into memory T cells. Because both calcineurin–NFAT and mTOR pathways become active during T cell expansion phase, the balance of these pathways might have a critical role in functional effector and memory T cell differentiation.

The mechanism of viral disease under the condition of calcineurin inhibitor-induced immunosuppression might account for pathogenesis in the immunosuppressed LCMV-infected transplant patients (CDC, 2005, 2008; Fischer et al., 2006; Palacios et al., 2008). Our mouse model had several similarities to the LCMV-infected transplant patients including sustained viremia, no seroconversion, along with mild lymphocyte infiltration, high morbidity and mortality, and elevated levels of liver enzymes in serum (Fischer et al., 2006). We suspect that the LCMV-infected transplant patients had similar immune-mediated pathological reactions to those seen in FK506-treated LCMV-infected mice. Thus, it will be of interest to investigate T cell responses and serum inflammatory cytokine levels in LCMV-infected transplant recipients.

Finally, our results identify a potential new target for treatment in transplant recipients who have acute complications of viral infection. Accordingly, because antiinflammatory medication is already an established therapy in humans (Möller and Villiger, 2006), such therapeutic regimens, combined with antiviral drugs, might become a potential strategy for improving therapy for viral diseases in transplant recipients.

MATERIALS AND METHODS

Mice, viral infection, and virus titrations. 6–8-week-old female C57BL/6J and B6.129S7-*Rag1^{tm1Mom}/J* mice were purchased from The Jackson Laboratory. Thy-1.1⁺ P14 mice bearing the Db-GP33-specific TCR were fully backcrossed to C57BL/6 and maintained in our animal colony. Mice were given 2×10^5 PFU of LCMV Armstrong i.p. LCMV titers in sera were measured by plaque assay as described previously (Wherry et al., 2003). Animal protocols were approved by the Emory University Institutional Animal Care and Use Committee.

Histology. Brains, lungs, kidneys, and livers from mice were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin.

Liver enzymes in serum. Aspartate aminotransferase and alanine aminotransferase in serum were measured on an AU 400 analyzer (Olympus).

FK506 treatment, T cell depletion, and anti-cytokine treatment. To make FK506 solution for injection, 300 μ l of undiluted FK506 (Astellas Pharma US, Inc.), which contains 5 mg/ml FK506, was dissolved in 700 μ l PBS before injection. Blood concentration of FK506 was maintained at ~ 10 –25 ng/ml to mimic the levels of this drug in human transplant recipients by administering the 10-mg/kg FK506 solution subcutaneously daily from day –1 to day 29 of LCMV infection. For sham treatment of FK506, same solution without FK506 was administered. To deplete CD4⁺ or CD8⁺ cells in vivo, 500 μ l GK1.5 or 2.43 were injected i.p. on days 0 and 3 after infection, respectively. GK1.5 was purchased from Bio X Cell. The anti-CD8⁺ monoclonal antibody 2.43 was prepared by an ammonium sulfate precipitation from hybridoma supernatants, followed by dialysis against PBS. For T cell–undepleted mice, the same volume of PBS was used. To inhibit the activity of TNF in vivo, 150 μ g etanercept (Immunex), which is a recombinant TNF receptor and blocks TNF activity (Schubert et al., 2004), was inoculated i.p. every day from day 4–29 of infection. Anti-IL-6 receptor monoclonal antibody 15A7 (Bio X Cell) was administered i.p. every third day from day 4 of infection as shown previously (Giraud et al., 1996). Control mice for etanercept and anti-IL-6R were given same amount of PBS and rat IgG2b isotype control, respectively.

Cell isolation and adoptive transfer. To purify Thy-1.1⁺ P14 and Thy-1.1⁺ OT-I transgenic CD8 T cells, CD8 T cell isolation kit (Miltenyi Biotec) was used, and then 10^5 purified transgenic T cells were adoptively transferred intravenously into RAG^{−/−} mice 1 d before infection. Liver CD11b⁺ cells were purified by CD11b⁺ microbeads (Miltenyi Biotec). For T cell proliferation assay, spleen cells of naive Thy-1.1⁺ P14 mice were labeled with CFSE (Invitrogen) as described previously (Murali-Krishna and Ahmed, 2000). The CFSE-labeled P14 cells that included 0.75–1.5 million of the Db-GP33-specific TCR⁺ CD8⁺ T cells were adoptively transferred intravenously into naive B6 mice 1 d before infection.

Detection of serum and liver cytokines. Levels of serum and liver homogenate cytokines were measured by cytometric bead array (BD) except IL-17. Serum IL-17 levels were determined by FlowCytomix (Bender MedSystems Inc.).

Flow cytometry and cytotoxic assay. MHC class I tetramers were made as described previously (Murali-Krishna et al., 1998). All antibodies for flow cytometry were purchased from BD except for CD127, KLRG-1, CD27, Foxp3, and granzyme B. Antibodies to CD127, CD27, and Foxp3 were purchased from eBioscience. Anti-KLRG-1 (SouthernBiotech) and anti-granzyme B (Invitrogen) were used to detect each antigen. Single cell

suspensions of spleen cells were prepared, and direct ex vivo staining, in vitro peptide stimulation, and chromium release cytotoxic assay were performed as described previously (Wherry et al., 2003). For analysis of direct ex vivo apoptosis, splenocytes were isolated and incubated with Annexin V and 7AAD as previously described (Grayson et al., 2002).

ELISA. Anti-LCMV IgG was detected by ELISA, as previously described (Ahmed et al., 1984). In brief, 96-well flat-bottom plates were coated with LCMV-infected BHK cell lysate, and then each well was blocked by 3% bovine serum albumin PBS. After blocking, serial diluted serum was added, and then anti-mouse IgG (γ chain specific) conjugated with alkaline phosphatase (Sigma-Aldrich) was used as a secondary antibody. *p*-nitrophenyl phosphate (Sigma-Aldrich) was used as substrate.

Retrovirus-based RNA interference. RNA interference knockdown experiments were performed using pMKO.1 GFP retrovirus vector (provided by W. Hahn, Harvard Medical School, Boston, MA; Addgene plasmid 10676) as described previously (Araki et al., 2009). In brief, to activate P14 cells in vivo, P14 transgenic mice were infected with LCMV Armstrong intravenously (2×10^6 PFU). 24 h later, P14 transgenic spleen cells were isolated and then spin transduced with retrovirus. 5×10^5 retroviral-transduced P14 spleen cells were adoptively transferred into naive mice, followed by LCMV infection (2×10^5 PFU, i.p.).

Quantitative real-time RT-PCR. PCR primers for TNF, IL-6, and β -actin were purchased from QIAGEN (QuantiTect Primer). RNA isolation and reverse transcription reaction was performed using the RNeasy kit and QuantiTect reverse transcription kit (QIAGEN). For real-time PCR, 2 $\times$ QuantiTect SYBR Green PCR Master Mix was used as per the manufacturer's instruction (QIAGEN). β -Actin gene expression was used as a reference.

Statistical analysis. Statistical analysis was performed using a two-tailed unpaired Student's *t* test except for survival experiments. The log-rank test was used to determine statistical significance of survival experiments.

Online supplemental material. Fig. S1 shows histopathology in LCMV-infected FK506-treated mice. Fig. S2 shows degranulation ability and in vivo killing activity of virus-specific CD8 T cells in FK506-treated LCMV-infected mice. Fig. S3 shows in vivo killing activity of virus-specific CD8 T cells in FK506-treated LCMV-infected mice in adoptive transfer experiments. Fig. S4 shows KLRG-1 and CD27 expression on endogenous antigen-specific CD8 T cells in LCMV-infected FK506-treated mice. Fig. S5 shows survival rate and viral titers in LCMV-infected FK506-treated RAG1^{−/−} mice. Fig. S6 shows virus-specific CD8 T cell expansion in LCMV-infected mice treated with FK506 3 d before infection. Fig. S7 shows expression of CD25 on regulatory T cells in LCMV-infected FK506-treated mice. Fig. S8 shows functionality of antigen-specific T cells when FK506 treatment was stopped on day 7 after LCMV infection. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20100124/DC1>.

We thank Dr. W. Hahn for providing pMKO.1 GFP vector.

This work was supported by the National Institutes of Health (Grant AI30048 to R. Ahmed and Grant AI073707 and AI40519 to C.P. Larsen.).

The authors have no conflicting financial interests.

Submitted: 19 January 2010

Accepted: 10 September 2010

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