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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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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 knock-out, 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 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;
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 (CD127low, CD62Llow, and CD44high) 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 ~50% TNF, markedly fewer
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).

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).

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 DGP33 tetramer⁺ CD8⁺ cells was compared between FK506-treated and untreated mice on day 8 after infection. Flow cytometry data are gated on DGP33 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, DGP33 and KGP34 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 DGP33 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 DGP33⁺ 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 DGP33 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 DGP33 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.
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 intrinsically 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 GFPpositive transduced cells with GFPnegative 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-1low 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.
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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 (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 CD27Low KLRG-1High 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 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.
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 CD44high 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 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 CD4+ 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 RAG2−/− 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...
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).
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 virus-specific T cells.

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 T cells.

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.
CD8 T cells generated in FK506-treated LCMV-infected animals had higher levels of granzyme B than control animals (Fig. 3C). 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. S2A). 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. S2B 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
maintain the number of T reg cells, and to up-regulate CD25

FK506 treatment alters CD8 T cell differentiation and induces

T cells. Overall, our data indicate that the calcineurin inhibitor

the calcineurin–NFAT signaling pathway that is intrinsic in

T cells in FK506-treated mice could be caused by blocking

FK506-treated animals is caused by a different mechanism than

Radziewicz et al., 2007), suggesting that functional impair

ment in FK506-treated animals is a PD-1-independent phe

nomenon. 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 no-
tion, 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 treat-

ment was stopped. Although stopping the drug treatment at day

7 after infection did not reduce mortality (Fig. S8 A), it signifi-
cantly 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 produc-
tion 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 re-
cently reported that another common immunosuppressive drug,

rapamycin, has a very different effect on memory T cell differ-

etentiation (Araki et al., 2009; Pearce et al., 2009). Rapa-

mycin 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 sig-

naling pathway that initiates activation of specific genes, in-

cluding IL-2, whereas the rapamycin–FKBP12 complex pre-

vents mammalian target of rapamycin (mTOR) pathway, which

regulates cell growth and metabolism (Wullschleger et al.,

2006). Unlike FK506 treatment, we did not observe appar-
tent 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-1low CD27hi 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 dia-

metrically 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 includ-
ing 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 inflamma-
tory 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-Rag1tm1Mom 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 × 10⁵ 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 µl of undiluted FK506 (Astellas Pharma US, Inc.), which contains 5 mg/ml FK506, was dissolved in 700 µl 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 µl 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 µg anti–TNF receptor monoclonal antibody 15A7 (Bio X Cell) was administered 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 (Giraudo 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-1 transgenic CD8+ T cells, CD8+ T cell isolation kit (Miltenyi Biotec) was used, and then 10⁶ purified transgenic T cells were adoptively transferred intravenously into RAG−/− mice i.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 FlowCytomax (Bender MedSystems Inc.).

**Flow cytometry and cytokotoxic 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 apoptotic, 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 × 10⁵ PFU). 24 h later, P14 transgenic spleen cells were isolated and then spin transfected with retrovirus. 5 × 10⁵ retroviral-transduced P14 spleen cells were adoptively transferred into naive mice, followed by LCMV infection (2 × 10⁵ 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× 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 KLRC-G1 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 RAG−/− mice. Fig. S6 shows virus-specific CD8T 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.

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**REFERENCES**


