




CD4 T Cells, CD8 T Cells, and Monocytes Coordinate To Prevent Rift Valley Fever Virus Encephalitis

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ABSTRACT Rift Valley fever virus (RVFV) is an arbovirus that causes disease in livestock and humans in Africa and the Middle East. While human disease is typically mild and self-limiting, some individuals develop severe manifestations, such as hepatitis, hemorrhagic fever, or encephalitis. Encephalitis occurs 2 to 3 weeks after acute illness; therefore, we hypothesized that it was a result of an inadequate adaptive immunity. To test this hypothesis *in vivo*, we used an attenuated virus (DeINSsRVFV) that does not typically cause disease in mice. We first characterized the normal immune response to infection with DeINSsRVFV in immunocompetent mice and noted expansion of natural killer cells and monocytes, as well as activation of both CD8 and CD4 T cells. Depleting C57BL/6 mice of CD4 T cells prior to DeINSsRVFV infection resulted in encephalitis in 30% of the mice; in encephalitic mice, we noted infiltration of T cells and inflammatory monocytes into the brain. CD4 and CD8 codepletion in C57BL/6 mice, as well as CD4 depletion in CCR2 knockout mice, increased the frequency of encephalitis, demonstrating that these cell types normally contributed to the prevention of disease. Encephalitic mice had similar viral RNA loads in the brain regardless of which cell types were depleted, suggesting that CD4 T cells, CD8 T cells, and inflammatory monocytes did little to control viral replication in the brain. CD4-depleted mice exhibited diminished humoral and T cell memory responses, suggesting that these immune mechanisms contributed to peripheral control of virus, thus preventing infection of the brain.

IMPORTANCE RVFV is found in Africa and the Middle East and is transmitted by mosquitos or through contact with infected animals. Infected individuals can develop mild disease or more severe forms, such as hepatitis or encephalitis. In order to understand why some individuals develop encephalitis, we first need to know which immune functions protect those who do not develop this form of disease. In this study, we used a mouse model of RVFV infection to demonstrate that CD4 T cells, CD8 T cells, and monocytes all contribute to prevention of encephalitis. Their likely mechanism of action is preventing RVFV from ever reaching the brain.

KEYWORDS Rift Valley fever virus, T cells, encephalitis, immunity, inflammation, monocytes, natural killer cells, pathogenesis

Rift Valley fever virus (RVFV) emerged in Sub-Saharan Africa in the 1930s (1). Since then, it has spread throughout continental Africa and into the Middle East. The virus affects both livestock and humans. Human disease is often self-limiting, but in a subset of individuals, RVFV infection can lead to severe hepatitis, hemorrhagic fever, or encephalitis (2). When RVFV encephalitis occurs, it tends to manifest 2 to 3 weeks after disease onset; this timing suggests that a failure of immune control in the periphery

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may allow the virus to gain access to the central nervous system (CNS) and cause encephalitis.

RVFV encodes a nonstructural protein, NSs, on the small viral RNA segment. NSs is a major virulence factor with multiple known functions. It interacts with the SAP 30 transcription factor on the beta interferon (IFN- β) promoter, blocking transcription. It blocks PKR-mediated signaling via degradation and leads to generalized downregulation of transcription by interacting with transcription factor II H of RNA polymerase II (3–8). Infection of mice with wild-type RVFV is uniformly lethal within a few days (9). However, subcutaneous infection of mice with an RVFV that is missing the NSs protein (DeINSsRVFV) has no clinical effects on the mice and rather leads to the development of a robust adaptive response against RVFV that protects mice from subsequent challenge with wild-type (WT) virus (10). Thus, DeINSsRVFV has become the basis of multiple vaccine efforts targeting livestock (11, 12).

In contrast to the benign nature of DeINSsRVFV infection following subcutaneous (s.c.) administration, DeINSsRVFV administered intranasally traffics across the cribriform plate directly to the CNS. Mice infected via this route die of encephalitis without ever mounting a significant adaptive immune response (13). These data demonstrate that DeINSsRVFV can cause lethal encephalitis if the virus can get to the brain. Therefore, we hypothesized that strong innate and adaptive immune mechanisms are associated with protection from DeINSsRVFV encephalitis. To test this hypothesis, we sought to define which specific aspects of cellular immunity are important for controlling DeINSsRVFV infection. We first characterized several aspects of innate and adaptive immunity during subcutaneous DeINSsRVFV infection by measuring monocytes, natural killer (NK) cells, neutrophils, dendritic cells (DC), T cells, and B cells, as well as activation markers on these cell types. Then we took advantage of an earlier observation that depleting CD4 T cells prior to infecting mice with DeINSsRVFV subcutaneously leads to encephalitis in 30% of the mice (10). This permitted us to examine the same panel of immune cells in the brains of mice with active DeINSsRVFV encephalitis. We then used genetic and immune cell-depleted mouse models to investigate the role of each specific cell type in DeINSsRVFV encephalitis. Finally, we quantitated RVFV-specific humoral and T cell responses in CD4-depleted mice to understand which immune functions were lacking in the CD4-depleted model.

RESULTS

Innate and adaptive cellular immunity is activated during subcutaneous DeINSsRVFV infection. Prior work demonstrated strong virus-specific humoral and T cell-mediated responses in mice subcutaneously infected with DeINSsRVFV (13). However, which innate and adaptive immune components are activated in various tissues has never been examined. We first sought to enumerate innate and adaptive immune cells in relevant tissues of infected mice over time. We used flow cytometry to quantitate NK cells, monocytes, total T cells, CD4 T cells, CD8 T cells, B cells, neutrophils, and dendritic cells (DC) in blood, lymph node (LN), spleen, liver, and brain tissue of DeINSsRVFV-infected or mock-infected mice. Since mice were inoculated subcutaneously (s.c.) into the left hind footpad, the draining popliteal lymph node was examined. All cell types were evaluated in all tissues at all time points. The gating strategies are depicted in Fig. S1 through S5 in the supplemental material. No statistically significant changes were observed in any of the examined cell types in the brains of mice over the course of the experiment (data not shown). No significant changes in infected mice compared to mock-infected mice were noted in the frequency of total neutrophils, B cells, CD3 T cells, CD4 T cells, or CD8 T cells in any of the examined tissues in infected mice (data not shown). Increased frequency of NK cells was observed at the earliest time points in the blood, LN, and liver of infected mice (Fig. 1A), but this change was statistically significant only in the LN and liver. While no differences in frequency of DC were observed in the various tissues (data not shown), we did see early increases in monocytes in blood, LN, spleen, and liver, all of which were statistically significant

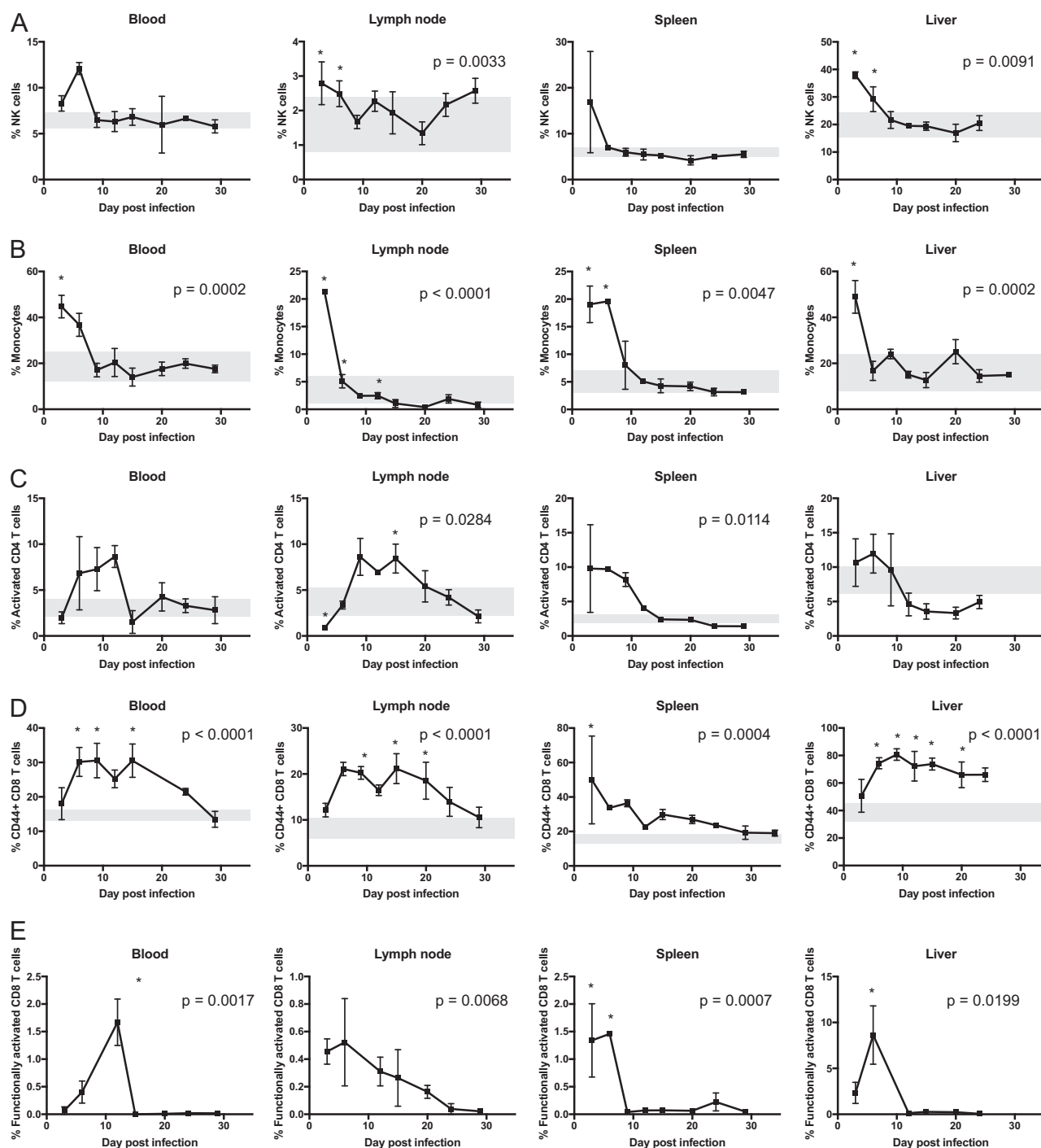


FIG 1 Innate and adaptive cellular immunity following DelNSsRVFV infection. Natural killer (NK) cells (pNK46⁺ CD3⁻) (A), monocytes (CD11b⁺ Ly6G⁻ MHC-II⁻) (B), activated CD4 T cells (CD3⁺ CD4⁺ CD44⁺ Ki-67⁺) (C), phenotypically activated CD8 T cells (CD3⁺ CD8⁺ CD44⁺) (D), and functionally activated CD8 T cells (CD3⁺ CD8⁺ Ki-67⁺ granzyme B⁺) (E) were evaluated using flow cytometry in blood, lymph node, spleen, and liver tissues from mice infected with Rift Valley fever virus (RVFV) lacking the NSs gene (DelNSsRVFV) and from mock-infected mice. Gray areas represent the range of values obtained in mock-infected mice over the course of the experiment. No gray area is depicted in panel E since mock-infected mice had less than 0.01% of this cell population in all tissues. *P* values noted on graphs were derived from ANOVA and reflect effect of infection; an asterisk above a data point indicates significance for that time point in *post hoc* analysis. If no *P* value is noted, data were not statistically significant.

(Fig. 1B). These data support an active role for innate cellular immunity in response to DelNSsRVFV infection.

While overall numbers of adaptive immune cells did not differ between infected and mock-infected mice, the activation status of CD4 and CD8 T cells was notable. Activated

CD4 T cells (Ki-67⁺ CD44⁺) were found in the lymph node from 9 to 15 days postinfection (Fig. 1C), consistent with the activation of naive CD4 T cells to become various effector CD4 T cells upon antigen presentation in the lymph node (14). Additionally, phenotypically activated CD8 T cells (CD44⁺) were observed in all 4 tissues examined (Fig. 1D). Further evidence of CD8 T cell activation was observed when functional markers of activation were evaluated (Ki-67⁺ granzyme B⁺), although these occurred at much lower frequencies and were only briefly present, while CD44⁺ CD8 T cells persisted for much longer (Fig. 1E).

RVFV encephalitis is associated with significant infiltration of CD3⁺ T cells and CD11b⁺ cells into the brain. Depleting CD4 T cells prior to infection with DelNSsRVFV results in encephalitis in 30% of infected mice (10). Using this model of encephalitis, we sought to define the immune cells present in the brains of encephalitic mice. Our previous data demonstrated elevated levels of the chemokines IFN- γ -induced protein 10 (IP-10) and monocyte chemoattractant protein (MCP-1) in the brains of encephalitic mice, suggesting that T cells and monocytes might be recruited to the inflamed brain tissue. We identified elevated levels of multiple cell types in the brains of encephalitic mice (Fig. 2A). Representative flow plots depicting the measured populations are shown. Frequencies of CD3⁺ T cells and CD11b⁺ cells were significantly higher in encephalitic mice than in control healthy mice. To further define the CD11b⁺ populations in the brain, we compared CD45-high (inflammatory monocytes) to CD45-low (microglia) populations. We noted essentially no inflammatory monocytes in the brains of control healthy mice, and the CD11b⁺ population was entirely representative of the resident microglia (CD45-low) (Fig. 2B). However, numbers of CD11b⁺ CD45-high inflammatory monocytes were significantly higher in encephalitic mice; this population of cells exhibited high levels of Ly6C expression, consistent with the inflammatory phenotype. All encephalitic mice exhibited higher viral RNA loads in the brain than in other tissues examined (Fig. 2C presents comparison to the spleen). Clinically well-appearing infected mice did not have detectable viral RNA in the brain tissue (data not shown).

RVFV encephalitis was enhanced with codepletion of CD8 T cells. We previously reported elevated levels of IFN- γ , MIP-1 α , IP-10, interleukin 1 β (IL-1 β), IL-6, and granulocyte colony-stimulating factor (G-CSF) but decreased levels of IL-4 in the draining lymph nodes of CD4-depleted mice (10). This led us to hypothesize that there may be an element of immune-mediated pathology in CD4-depleted mice, perhaps secondary to loss of regulatory T cell function. This hypothesis was supported by our finding T cells in the brains of encephalitic mice, since loss of regulatory CD4 T cells can be associated with CD8 T cell-mediated pathology (15). To directly address this possibility, we depleted mice of CD4 or CD8 T cells or both CD4 and CD8 T cells prior to infection with DelNSsRVFV. In contrast to what would be expected for immune-mediated pathology, we observed decreased survival in mice depleted of both CD4 and CD8 T cells (Fig. 3A). We also evaluated the role of NK cells during encephalitis in CD4-depleted mice but found no statistically significant differences in survival with NK codepletion. All mice that succumbed to infection exhibited a clinical encephalitis, and similar viral RNA levels were detected in the brains of all encephalitic mice regardless of the depletion regimen (Fig. 3B).

DelNSsRVFV encephalitis was enhanced by CD4 depletion in CCR2KO mice. Given both the overall numbers of CD11b⁺ cells (monocytes) infiltrating into the brains of encephalitic mice and the inflammatory nature of these cells, as noted by Ly6C-positive staining, we sought to evaluate the role of this cell type in encephalitis in CD4-depleted, DelNSsRVFV-infected mice. We focused on the CCR2 knockout (CCR2KO) mouse, in which CD11b⁺ cells are present but unable to traffic to sites of inflammation or to egress efficiently from the bone marrow (16, 17). Depleting CCR2KO mice of CD4 T cells prior to DelNSsRVFV infection significantly decreased survival compared to that of CCR2KO mice with intact CD4 T cells; mice that succumbed to infection exhibited clinical encephalitis with high levels of viral RNA in the brain (Fig. 4A). Consistent with the inability of monocytes to traffic to the brain in CCR2KO mice, we observed a

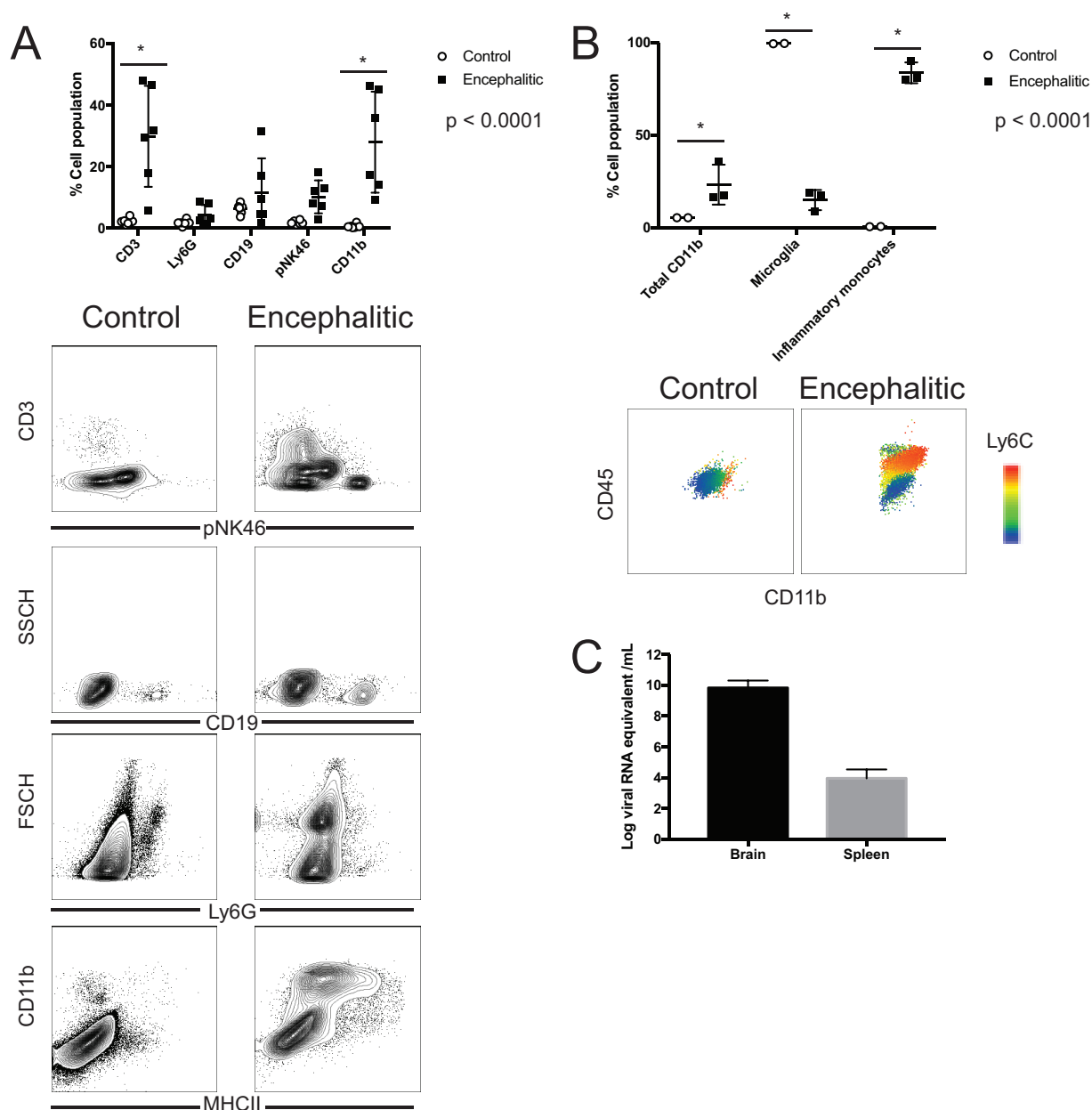


FIG 2 Characterization of cellular infiltrates into the brains of encephalitic mice. (A) Brain tissue infiltrates from control mice (uninfected or infected but clinically asymptomatic) and from encephalitic mice were characterized by flow cytometry; representative flow plots are shown at the bottom. The P value below the symbol key was derived from ANOVA and reflects effect of disease; an asterisk above a data point indicates significance for that cell type in *post hoc* analysis. (B) Additional studies were conducted to specifically assess the brain-infiltrating CD11b⁺ populations, which were found to be inflammatory monocytes expressing high levels of Ly6C, as shown in representative flow plots with a Ly6C heat map. The P value below the legend was derived from ANOVA and reflects the effect of disease; an asterisk above a data point indicates significance for that cell type in *post hoc* analysis. All mice that developed clinical disease had high levels of viral RNA in the brain (C).

significant decrease in inflammatory monocytes in the brains of encephalitic CCR2KO mice (Fig. 4B). As expected, decline in this cell population was also seen by loss of Ly6C expression, as shown in representative flow plots with a Ly6C heat map (Fig. 4B, right). No significant differences in viral RNA levels were noted in the brains of CD4-depleted wild-type (WT) mice versus CD4-depleted CCR2KO mice, suggesting that the presence of the inflammatory monocytes did little to control RVFV.

In the absence of CD4 T cells, DelNSsRVFV infection acutely increases phenotypic activation of CD8 T cells but reduces virus-specific humoral immunity and T cell memory. Singular depletion of NK or CD8 T cells, or loss of monocyte trafficking

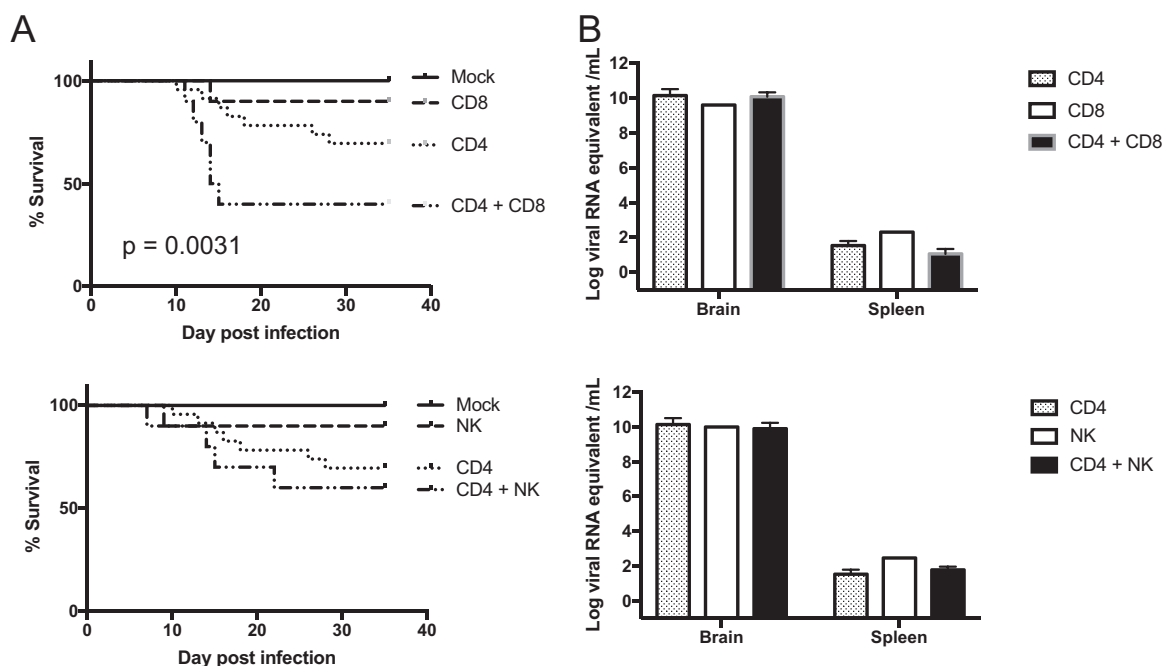


FIG 3 Codepletion of CD8 T cells, but not NK cells, increases the frequency of encephalitis in CD4-depleted mice. Groups of mice were mock depleted, or depleted of their CD4 T cells, CD8 T cells, or NK cells singly or in combination as noted and then infected with DelNSsRVFV. (A) Codepletion of CD4 and CD8 T cells significantly decreased survival. The P value noted on the graph was derived from a log rank (Mantel-Cox) test. (B) All mice that succumbed to disease developed encephalitis, with high viral RNA loads detected in the brain.

in CCR2KO mice, leads to encephalitis in only 10% of DelNSsRVFV-infected mice. However, depleting CD4 T cells alone resulted in encephalitis in 30% of DelNSsRVFV-infected mice. Of the cell types investigated, these data support CD4 T cells as the most prominent in preventing encephalitis in the DelNSsRVFV model. CD4 T cells have many known functions, including facilitating the formation of CD8 T cell memory (18), a role in generating robust humoral immunity via stimulation of class switching (19), and a role in modulating the immune response via their cytokine milieu (20). In our kinetic study of wild-type mice infected with DelNSsRVFV, we observed increases in NK cell and monocyte levels and in CD8 T cell activation. Therefore, we sought to determine if any of these features were altered in CD4-depleted, infected mice. We observed trends toward increased phenotypic activation of CD8 T cells in CD4-depleted versus mock-depleted mice (Fig. 5A). This was found to be statistically significant in blood and spleen but not in lymph node or liver. Despite an extensive analysis of B cells, neutrophils, NK cells, monocytes, and markers of activation, no other significant differences were noted between the measured immune cell populations in CD4-depleted and mock-depleted DelNSsRVFV-infected mice over the course of the experiment (data not shown).

Consistent with earlier results, mock-depleted infected mice developed higher anti-RVFV IgG endpoint titers (as measured by enzyme-linked immunosorbent assay [ELISA]) than did CD4-depleted mice (Fig. 5B). No differences in anti-RVFV IgM endpoint titers were measured, as expected, since IgM production does not require CD4 T cell function (Fig. 5B). As measured by 80% focus reduction neutralization titers (FRNT₈₀), antibodies produced by CD4-depleted mice barely neutralized virus, whereas those generated by mock-depleted mice readily did so by the end of the experiment (Fig. 5C). Given these clear antibody differences, we assessed if CD4-depleted mice that died from encephalitis had different anti-RVFV IgG titers than CD4-depleted mice that survived to the end of the experiment. CD4-depleted mice that succumbed to disease died 11 to 28 days postinfection, and end-of-experiment analyses of survivor mice were performed 34 to 37 days postinfection. Given the consistencies in IgG titers measured in CD4-depleted mice over this time (Fig. 5B), we felt it would be appropriate to

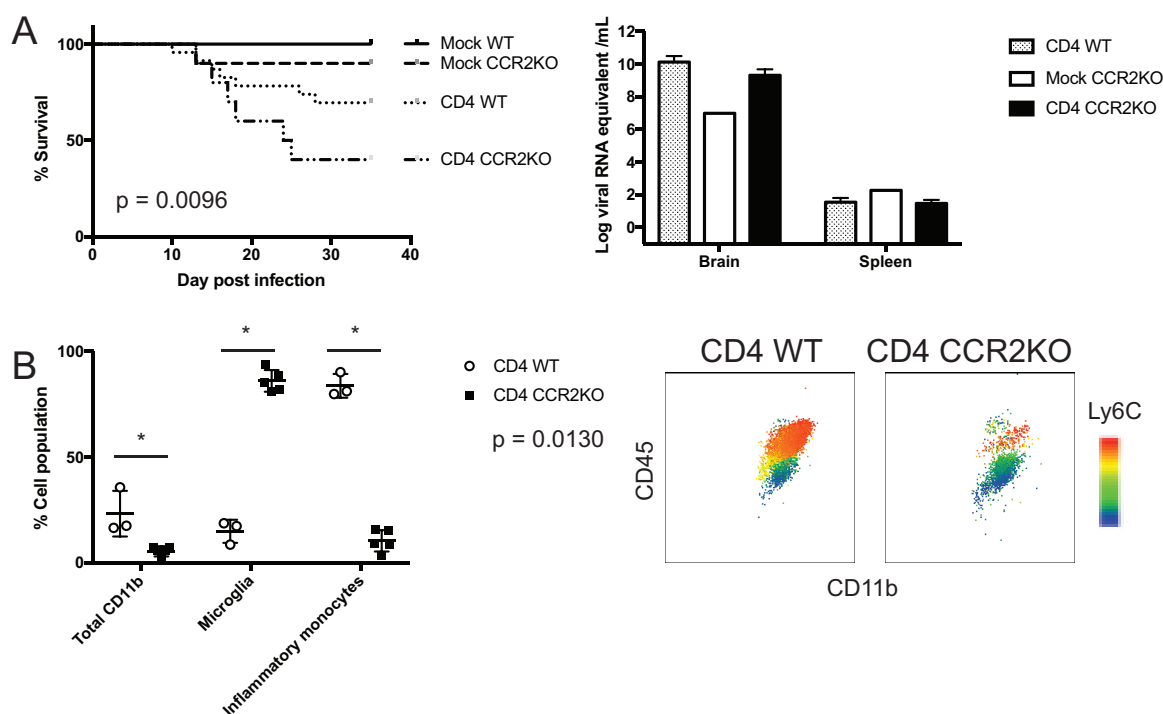


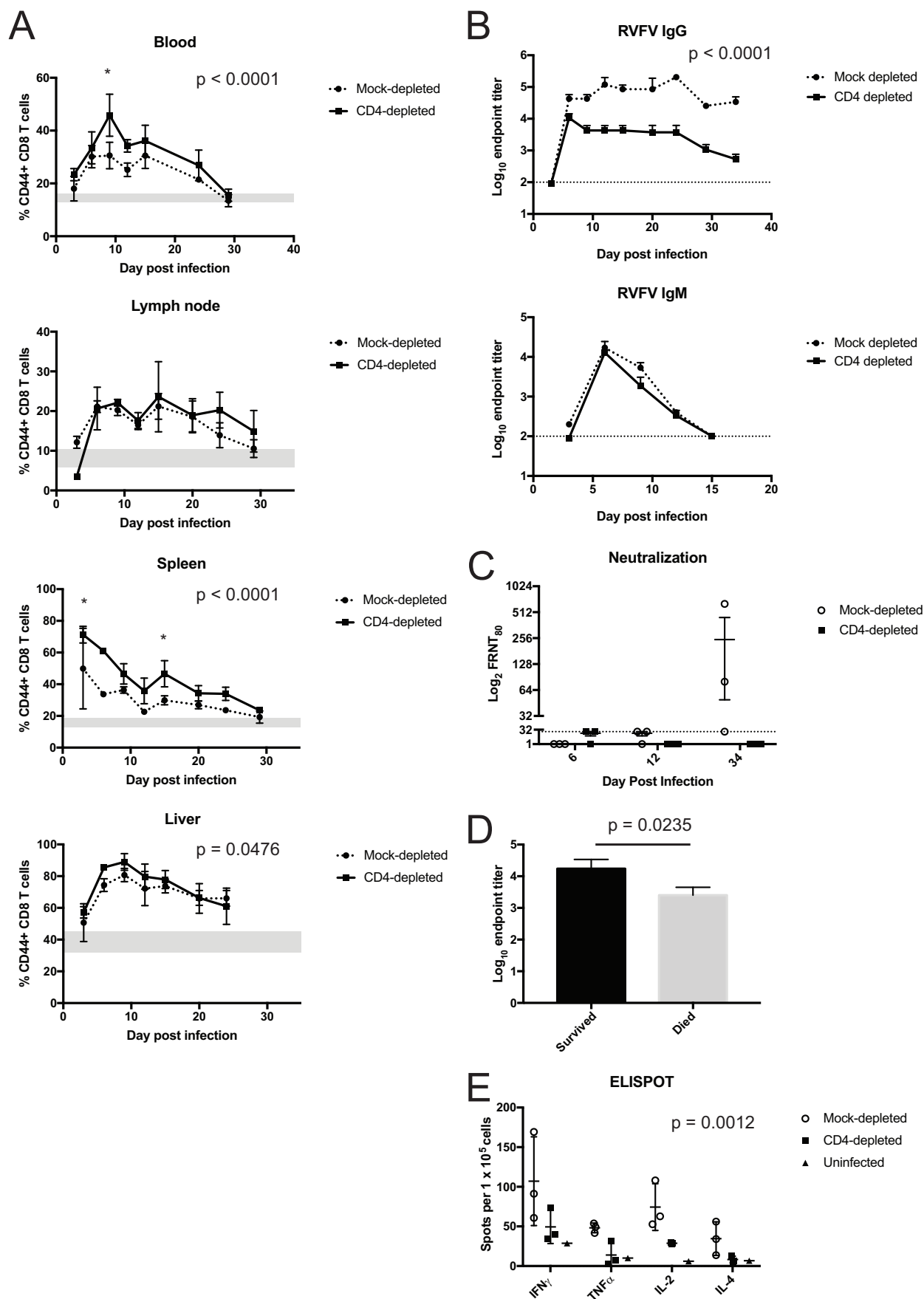
FIG 4 Loss of infiltration of inflammatory monocytes is associated with increased frequency of encephalitis in CD4-depleted mice. Groups of wild-type or CCR2 knockout (CCR2KO) mice were depleted of CD4 T cells or mock depleted and then infected with DelNSsRVFV. (A) CD4 depletion of CCR2KO mice significantly decreased survival (left graph). The *P* value noted on the graph was derived from a log rank (Mantel-Cox) test. All mice that succumbed to disease developed encephalitis, with high viral RNA loads detected in the brain (right graph). (B) Flow cytometry of immune cell infiltrates demonstrated a significant loss in numbers of inflammatory monocytes in the brains of encephalitic CD4-depleted CCR2KO mice. A representative flow plot is shown with Ly6C expression heat map (right side). *P* value noted beneath the legend was derived from ANOVA and reflects effect of genetic background; an asterisk above a data point indicates significance for that cell type in *post hoc* analysis.

compare these 2 groups. Anti-RVFV IgG titers were significantly lower in CD4-depleted mice that died of encephalitis than in mice that survived (Fig. 5D).

We evaluated RVFV-specific memory T cell responses in the 70% of CD4 T cell-depleted mice that survived DelNSsRVFV infection. Compared to that in mock-depleted infected mice, a decline of virus-specific T cell memory was observed in CD4-depleted mice (Fig. 5E).

DISCUSSION

Encephalitis is a potentially devastating consequence of RVFV infection in humans; in an outbreak that occurred in Saudi Arabia in 2000-2001, 17% of the identified patients had neurologic manifestations (21). Even patients who survive often have neurologic sequelae, including hemiparesis and cranial nerve dysfunction (2, 22). Why some patients develop encephalitic disease is unclear, but polymorphisms in TLR8 and RIG-1, two innate immune sensors, were noted in patients with encephalitis (23), demonstrating the importance of innate immune mechanisms in preventing RVFV-induced encephalitis. Additionally, 2 separate studies have indirectly suggested that CD4 T cells might play a role in preventing RVFV encephalitis in humans. Individuals who were infected with HIV had an increased mortality rate (75%) after RVFV infection compared to that of individuals who were HIV negative (28%), and all HIV-infected persons presented with CNS symptoms (24). In another study, 3 of 4 patients who presented with and succumbed to encephalitis were also found to be HIV positive (25). The known effects of HIV on the number and function of CD4 T cells suggest that individuals with T cell dysfunction might be at increased risk of developing severe RVFV disease, including encephalitis. This is an important consideration, as areas where RVFV disease is endemic are experiencing an ongoing epidemic of HIV/AIDS, with an esti-



mated 25.5 million people living with HIV/AIDS throughout Africa, according to UNAIDS (36). Given these findings for a potential role of both innate and adaptive immune mechanisms in modulating the manifestation of RVFV encephalitis in humans, we used the mouse model to better understand the functional role of various immune cell types in RVFV encephalitis.

DeINSsRVFV is unable to inhibit the host innate immune system, since the viral innate antagonist, NSs, is deleted. This unique system permits the evaluation of other immune functions in RVFV control. Monitoring of immune function over time in wild-type, immunocompetent mice refractory to disease upon DeINSsRVFV infection revealed an early monocytosis, activation of CD4 T cells in hematopoietic tissues, and activation of CD8 T cells. Interestingly, modulating these 3 cell types in subsequent experiments was significantly associated with encephalitis, suggesting that these cells prevent encephalitis. DeINSsRVFV is avirulent when administered by the subcutaneous (s.c.) route, but depletion of CD4 T cells alone, both CD4 and CD8 T cells, or CD4 T cells in the CCR2KO mouse resulted in encephalitis of increasing frequencies. Therefore, these data suggest at least two cellular immune control points for preventing DeINSsRVFV encephalitis, one at the innate level and another at the adaptive level.

Route of administration alters the outcome of DeINSsRVFV infection in immunocompetent mice; DeINSsRVFV administered subcutaneously is innocuous, but the virus causes lethal encephalitis when administered intranasally (13). Therefore, we hypothesize that CD4 and CD8 T cells and monocytes prevent DeINSsRVFV encephalitis after footpad inoculation by blocking the virus from trafficking to the brain rather than by controlling infection once the virus reaches the brain. A protective role for CD4 T cells has been demonstrated in neurotropic measles virus infection (26), as has a role for monocytes in protection from West Nile virus (WNV) encephalitis (27). CCR2KO mice infected subcutaneously with WNV exhibited increased viral replication in the brain and increased death rates compared to WT mice, demonstrating a protective role for monocytes in the CNS. Interestingly, in that WNV model, CCR2 was not required for physically trafficking monocytes to the brain but rather was required for generating peripheral monocytosis following infection, suggesting that monocytes control WNV both in peripheral tissues and in the CNS. In our model, if the infiltrating immune cells were controlling RVFV in the brain, immune cell depletion would be expected to increase viral RNA titers. Instead, we found very similar titers of viral RNA in the brains of all encephalitic mice regardless of which cells were depleted. This finding suggests that CD4 and CD8 T cells and monocytes primarily control DeINSsRVFV in the periphery rather than the CNS.

If immune control of DeINSsRVFV occurs in the periphery and not the CNS, we might expect to see some significant differences in immune phenotypes over time in peripheral tissues between mock- and CD4-depleted mice. Indeed, we did note more CD44⁺ CD8 T cells in CD4-depleted mice than in mock-depleted mice, but this did not correlate with any differences in viral RNA levels (data not shown). One clear finding in CD4-depleted, DeINSsRVFV-infected mice was the reduction in RVFV-specific ELISA titers and RVFV-neutralizing antibodies. Furthermore, we noted significantly lower RVFV-specific ELISA titers in CD4-depleted mice that developed encephalitis than in those that did not. This suggests that virus-specific antibodies could be involved in preventing encephalitis. In a mouse model of Japanese

FIG 5 CD4 depletion results in early increases in phenotypically activated CD8 T cells but reduced humoral and T cell memory responses. (A) Phenotypically activated CD8 T cells (CD3⁺ CD8⁺ CD44⁺) were evaluated over time using flow cytometry in blood, lymph node, spleen, and liver tissues from both mock-depleted and CD4-depleted mice infected with DeINSsRVFV. Gray areas represent the range of values obtained in mock-infected mice over the course of the experiment. *P* values noted on graphs were derived from ANOVA and reflect effect of depletion. An asterisk above a data point indicates significance for that time point in *post hoc* analysis. (B and C) RVFV-specific antibody responses were measured by IgM and IgG ELISA (B) as well as by neutralization assay (C). The dotted line indicates the limit of detection of each assay. (D) Virus-specific IgG titers of CD4-depleted mice that survived infection were compared with those of CD4-depleted mice that died of encephalitis. The *P* value noted on the graph was derived from Student's *t* test. (E) RVFV-specific memory T cell responses were measured by ELISPOT assay on day 34 postinfection. The *P* value noted on the graph was derived from ANOVA and reflects effect of depletion. For all panels, if no *P* value is noted, data were not statistically significant.

encephalitis virus, antibodies were shown to mediate the majority of protection against disease, since muMT mice (which are unable to generate antibodies) or major histocompatibility complex II (MHC-II)^{-/-} mice (which are similar to CD4-depleted mice) had higher viral titers in the brain than did wild-type mice, as well as increased death rates (28). Interestingly, we previously evaluated muMT mice following infection with DeINsRVFV, but only 1 of 10 mice succumbed to disease, while there was a 30% lethality rate in CD4-depleted mice. These data argue that CD4 T cells contribute to protection against RVFV encephalitis by other mechanisms in addition to providing B cell help.

Our data support a synergistic role for CD4 T cells, CD8 T cells, and monocytes in preventing DeINsRVFV encephalitis, and we propose a model in which antibodies bind virus and help eliminate it via Fc-mediated mechanisms. This hypothesis is consistent with our finding that both CD4 T cells and monocytes play a role in preventing encephalitis. CD4 T cells augment antibody affinity maturation and class switching, and monocytes contribute to immune control via Fc receptor-mediated recognition of antibody-bound virus. Virus-specific CD8 T cells identify and eliminate any infected cells. The fact that we observed reduced RVFV-specific CD8 T cell memory responses in CD4-depleted mice emphasizes the cooperative role of these cells in RVFV control. Key to this hypothesis is the ability of RVFV to “hide” somewhere in the subset of mice that ultimately develop encephalitis since disease does not occur until 2 to 3 weeks after infection and virus is not observed in the brains until onset of clinical symptoms. One possible explanation is that virus hides in the peripheral nervous system. Whether subsequent virus replication at later time points with hematogenous or neural spread to the CNS leads to encephalitis is unknown. Rabies virus, a well-known pathogen of the nervous system, infects motor neurons at the neuromuscular junction and spreads caudally, ultimately infecting the CNS (29). In one mouse model of rabies virus, T cells were shown to be responsible for hind-limb paralysis following footpad inoculation (30). However, in mice naturally resistant to rabies virus infection, protection from disease was lost after CD4, but not CD8, T cell depletion (31). We frequently noticed hind-limb paralysis as the first manifestation of RVFV encephalitis; this would be consistent with a neuronal trafficking mechanism. Depleting CD4 and CD8 T cells exacerbated the encephalitic phenotype, so they are both protective rather than pathological in the DeINsRVFV mouse model.

A limitation to our study is the use of an attenuated strain of RVFV; this was necessary given the unique sensitivity of the mouse model to WT RVFV infection. It is not known if the adaptive immune responses following DeINsRVFV infection are the same as those that would occur if mice were to survive WT RVFV infection or if they represent what might occur in a human. However, recent data from a non-human primate (NHP) model of WT RVFV infection are also supportive of a role for T cells in protection. Wonderlich et al. reported that sublethal infection was associated with activation of both CD4 and CD8 T cells, while this was not observed in lethally infected animals, all of which succumbed to encephalitis (32). These data are consistent with a role for T cells in protection from encephalitis in an NHP model of WT RVFV infection.

In summary, we identified a protective role for CD4 T cells in cooperation with both monocytes and CD8 T cells for prevention of DeINsRVFV-mediated encephalitis. Future studies will be aimed at defining the CD4 T cell subsets responsible for protection from RVFV encephalitis. Elucidating mechanisms of CD4 T cell-mediated protection may lead to potential immunotherapeutics to prevent RVFV encephalitis.

MATERIALS AND METHODS

Biosafety, ethics, mice, and viruses. All experiments were conducted according to an IACUC-approved protocol (2577MCEMOUC) in select-agent-registered biosafety level 3 (BSL3)/animal BSL3 (ABSL3) space at the CDC. All mice were female and were obtained from Jackson Laboratories. C57BL/6 mice (stock no. 000664) and CCR2KO mice (B6.129S4-Ccr2^{tm1Hc}/J, stock no. 004999) were 6 to 8 weeks old and housed in microisolator pans in HEPA filtration racks. For depletion studies, mice were given 300 μ g

of the appropriate antibody on days 3 and 1 prior to infection. CD4 depletion antibody was clone GK1.5, NK1.1 depletion antibody was clone PK136, CD8 depletion antibody was clone YTS 169.4, and mock depletion antibody was clone LTF-2, all from BioXCell. Confirmation of depletion is demonstrated in Fig. S6 in the supplemental material.

Mice were infected with 2×10^5 50% tissue culture infective doses (TCID₅₀) of DelNSsRVFV in the footpad or mock infected using sterile Dulbecco modified Eagle medium (DMEM). For kinetic studies, 3 DelNSsRVFV-infected mice and 1 mock-infected mouse were euthanized at each time point. For all studies, mice were monitored for weight loss and clinical signs daily and euthanized if indicated by a clinical scoring algorithm. Wild-type RVFV and DelNSsRVFV were generated using an established reverse-genetics system based on the strain ZH501 background (33) and fully sequenced prior to use. Virus titer was determined using a standard TCID₅₀ assay.

Tissue processing for flow cytometry. Blood was lysed with red blood cell (RBC) lysis buffer (Sigma-Aldrich), and cells were directly processed for flow cytometry. A single cell suspension of LN cells was made by dissociating tissue in phosphate-buffered saline (PBS) using an Eppendorf tube with a plastic pestle; cells were then processed for flow cytometry. Brains, livers, and spleens were disrupted by pushing the tissue through a 70- μ m cell strainer over a 50-ml conical tube, and filters were washed thoroughly with RPMI medium (spleen) or PBS (liver and brain). Cells were pelleted by centrifugation, after which splenocytes were subjected to RBC lysis, washed with RPMI medium, and counted; 2×10^6 cells were processed for flow cytometry. Liver cell pellets were suspended in PBS and layered onto Histopaque 1077 (Sigma-Aldrich). Brain cell pellets were suspended in 30% stock isotonic Percoll (SIP; 9 ml of Percoll plus 1 ml of $10\times$ Hanks' balanced salt solution [HBSS]) and layered onto 70% SIP. Following centrifugation for 30 min at $500 \times g$ without a brake, liver or brain cell interfaces were collected, washed in PBS, and processed for flow cytometry.

Flow cytometry. Cells were washed in PBS, incubated in near infrared (IR) LIVE/DEAD stain (1:500 in PBS) for 10 min at room temperature, and washed in flow buffer (PBS with 2% fetal calf serum [FCS]). Surface stains were added (see Table S1 for panel details), and cells were incubated for 30 min at room temperature and washed twice in flow buffer. Cells were then suspended in Cytofix/Cytoperm (BD) for 20 min at room temperature. After 2 washes in BD perm/wash buffer, cells were incubated with intracellular stains for 45 min at room temperature. Following additional washes, cells were suspended in PBS and events were collected on a Stratifiedigm S1000EXi.

qRT-PCR. Tissues were homogenized in MagMax lysis buffer using a SPEX SamplePrep Geno/Grinder. RNA was extracted following MagMax RNA extraction protocols. Quantitative reverse transcription-PCR (qRT-PCR) using primer and probe sets for RVFV (34) and 18S rRNA (Thermo Fisher) were performed on each sample. 18S rRNA was used to normalize data to input RNA, and an RVFV RNA standard curve was generated using a serially diluted stock virus of known titer. Results are reported as log viral RNA equivalents per milliliter and reflect how the viral RNA content in the sample relates to the viral RNA content of the TCID₅₀-quantitated viral stock.

ELISPOT assay. Virus-specific cytokine production in splenocytes was evaluated by incubation with or without gamma-irradiated whole virions (multiplicity of infection equivalent of 5) for 48 h using IFN- γ , IL-4, IL-2 (Mabtech), and tumor necrosis factor alpha (TNF- α ; BD Biosciences) mouse enzyme-linked immunosorbent spot (ELISPOT) assay kits following the manufacturers' instructions.

Neutralization assay. Mouse plasma samples were incubated at 65°C for 30 min, serially diluted, and incubated with 200 TCID₅₀ of wild-type RVFV for 1 h at 37°C before being added onto Vero-E6 cells. Following a 1-h adsorption at 37°C, inoculum was removed and a 1.5% carboxymethylcellulose overlay was instilled. Cells were fixed with 10% formalin 18 to 24 h later and permeabilized with 0.1% Triton X-100. Foci were stained using virus-specific hyperimmune mouse ascitic fluid (HMAF) (diluted 1:1,000), followed by anti-mouse Alexa Fluor 488-conjugated antibody (diluted 1:500). Foci were counted using a BioTek Cytation 3.

ELISAs. RVFV-specific ELISAs were performed using whole-cell lysates as previously described (35). Briefly, MaxiSorp plates were coated with RVFV-infected cell lysates or Vero-E6 control cell lysates. Serial dilutions of mouse plasma were made in blocking buffer and incubated on coated plates after a blocking step. After 3 washes, plates were incubated with secondary antibodies (horseradish peroxidase [HRP]-conjugated anti-mouse IgM or anti-mouse IgG) diluted 1:2,500 in blocking solution. Tetramethylbenzidine (TMB) substrate was used for detection, and data were collected on a BioTek Synergy plate reader.

Data analysis. Flow cytometry data were analyzed using FlowJo. Data were entered into Prism for statistical analyses and generation of plots. All kinetic data (flow cytometry, serology, neutralization assay, and ELISPOT assay) were analyzed using an ordinary 2-way analysis of variance (ANOVA) with an overall alpha of 0.05; comparisons were made at each time point with Sidak's correction for multiple comparisons. Brain cellular composition data were analyzed using an ordinary 2-way ANOVA with an overall alpha of 0.05; comparisons were made for each cell type with Sidak's correction for multiple comparisons. Survival curves were compared using a log rank (Mantel-Cox) test. Viral RNA qRT-PCR data were analyzed using Excel; RVFV RNA values were normalized to 18S rRNA values, and relative titer was determined by comparison to the standard curve. ELISA titers of antibodies in terminal mice were compared to titers in mice that survived using an unpaired *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.01270-18>.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

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