Rift Valley Fever Virus Clearance and Protection from Neurologic Disease Are Dependent on CD4(+) T Cell and Virus-Specific Antibody Responses

Kimberly A. Dodd, Centers for Disease Control and Prevention
Anita Katherine McElroy, Emory University
Megan E. B. Jones, Centers for Disease Control and Prevention
Stuart T. Nichol, Centers for Disease Control and Prevention
Christina F. Spiropoulou, Centers for Disease Control and Prevention

Journal Title: Journal of Virology
Volume: Volume 87, Number 11
Publisher: American Society for Microbiology | 2013-06-01, Pages 6161-6171
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/JVI.00337-13
Permanent URL: https://pid.emory.edu/ark:/25593/s794r

Final published version: http://dx.doi.org/10.1128/JVI.00337-13

Copyright information:
© 2013, American Society for Microbiology.
Accessed December 5, 2018 11:40 AM EST
Rift Valley fever virus (RVFV) causes outbreaks of severe disease in people and livestock throughout Africa and the Arabian Peninsula. Human RVFV infections generally manifest as a self-limiting febrile illness, but in some individuals, the disease can progress to a fatal encephalitis or hemorrhagic syndrome. Little is known about the host characteristics that predispose development of more severe disease. Early in infection, interferon-mediated antiviral responses are critical for controlling RVFV replication, but the roles of downstream adaptive immune responses in determining clinical outcome have not been examined. Here, using a C57BL/6 mouse disease model, we evaluated the roles of B cells and T cells in RVFV pathogenesis. Given the profound inhibition of the innate response by the viral NSs protein and rapid course of wild-type infection, we utilized an attenuated RVFV lacking NSs to examine host responses following primary infection. Experiments utilizing B-cell-deficient mice or targeted T cell depletions of wild-type mice demonstrated that B cells and CD4$^+$ T cells, but not CD8$^+$ T cells, were critical for mediating viral clearance, even in the presence of a functional innate response. One-third of CD4$^+$-depleted mice developed severe neurologic disease following infection, in contrast to virus-infected mock-depleted mice that showed no clinical signs. CD4$^+$ T cells were required for robust IgG and neutralizing antibody responses that correlated with RVFV clearance from peripheral tissues. Furthermore, CD4$^+$-depleted mice demonstrated significantly stronger proinflammatory responses relative to controls, suggesting CD4$^+$ T cells regulate immune responses to RVFV infection. Together, these results indicate CD4$^+$ T cells are critical determinants of RVFV pathogenesis and play an important role in preventing onset of neurologic disease.
known about how the host immune response influences clinical outcome during primary RVFV infection. Previous studies have suggested that severe RVFV pathogenesis is associated with strong proinflammatory cytokine and chemokine responses in terminal stages of the disease (26), but the mechanisms of viral clearance and prevention of RVFV-induced disease remain unknown. Characterization of the elements of these critical host responses is necessary to better understand basic RVFV pathogenesis and to design targeted antivirals, therapeutics, and vaccines.

To elucidate how the host immune responses determine the outcome of RVFV infection, we sought to define the immune cells that are critical for controlling virus infection in our RVFV mouse model. In this system, wild-type RVFV (Egyptian strain ZH501) infection is uniformly and rapidly lethal, with mice succumbing to fulminating hepatitis 2 to 3 days after infection (20). However, an RVFV strain containing a full-gene deletion of NSs (ΔNSs virus) is highly attenuated in immunocompetent mice and provides an ideal platform for evaluating the role of adaptive immunity in the context of an active innate response. Using this model, we found that clearance of RVFV from infected tissues is associated with and dependent upon a strong antibody response. A series of targeted depletion studies clearly demonstrate that functional CD4+ T cells, but not CD8+ T cells, are critical for controlling RVFV replication in vivo and for subsequent protection from the development of RVFV neurologic disease.

MATERIALS AND METHODS

Ethics statement. The animal procedures in this study complied with institutional guidelines, the United States Department of Agriculture Animal Welfare Act, and the National Institutes of Health guidelines for the humane use of laboratory animals. All procedures were approved by the Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Committee (IACUC).

Mice, viruses, and biosafety. All work with infectious RVFV was completed in a biosafety level 3E (BSL-3E) laboratory. Female 8– to 10-week-old C57BL/6 mice were obtained from Jackson Laboratories and were housed within the BSL-3E laboratories in microisolator pans in HEPA filtration racks, following standard barrier techniques. Female B6.129S2-Ighm<sup>+</sup>/J (μMT) mice were also obtained from Jackson Laboratories and housed in cages with autoclaved food, bedding, and water in a BSL-3E laboratory. In all animal experiments, mice were evaluated for humane use of laboratory animals. All procedures were approved by the institutional guidelines, the United States Department of Agriculture Animal Welfare Act, and the National Institutes of Health guidelines for the use of living animals (26), but the mechanisms of viral clearance and prevention of RVFV-induced disease remain unknown. Characterization of the elements of these critical host responses is necessary to better understand basic RVFV pathogenesis and to design targeted antivirals, therapeutics, and vaccines.

To elucidate how the host immune responses determine the outcome of RVFV infection, we sought to define the immune cells that are critical for controlling virus infection in our RVFV mouse model. In this system, wild-type RVFV (Egyptian strain ZH501) infection is uniformly and rapidly lethal, with mice succumbing to fulminating hepatitis 2 to 3 days after infection (20). However, an RVFV strain containing a full-gene deletion of NSs (ΔNSs virus) is highly attenuated in immunocompetent mice and provides an ideal platform for evaluating the role of adaptive immunity in the context of an active innate response. Using this model, we found that clearance of RVFV from infected tissues is associated with and dependent upon a strong antibody response. A series of targeted depletion studies clearly demonstrate that functional CD4+ T cells, but not CD8+ T cells, are critical for controlling RVFV replication in vivo and for subsequent protection from the development of RVFV neurologic disease.

Initial challenge of CD4- and CD8-depleted mice. Groups of CD4- and CD8-depleted mice were inoculated subcutaneously (s.c.) in the left rear footpad with either 2.0 × 10<sup>5</sup> TCID<sub>50</sub>/ZH501 (n = 5 each for GK1.5/ZH501 and YTS169/ZH501) or 2.0 × 10<sup>5</sup> TCID<sub>50</sub> ΔNSs (n = 5 each for GK1.5/ΔNSs and YTS169/ΔNSs) or sham inoculated with 20 μl Dulbecco’s modified Eagle’s medium (DMEM) (n = 5 each for GK1.5/ΔNSs and YTS169/ΔNSs) on day 0. As controls, mock-depleted mice were inoculated with the same dose of each virus (n = 5 each for LTF2/ZH501 and LTF2/ΔNSs). Survivors were challenged with 1.0 × 10<sup>5</sup> TCID<sub>50</sub> ZH501 s.c. at 28 days postinfection (dpi). In a follow-up experiment, another group of CD4-depleted mice was inoculated s.c. in the footpad with 2.0 × 10<sup>5</sup> TCID<sub>50</sub> ΔNSs (n = 10 for GK1.5/ΔNSs) and monitored for 50 days; samples of liver, brain, and spleen were taken from mice that succumbed to infection for RVFV quantitative reverse transcription-PCR (qRT-PCR).

Initial challenge of B-cell-deficient mice. B6.129S2-Ighm<sup>−/−</sup>/J (μMT) mice were inoculated s.c. with 1.0 × 10<sup>5</sup> TCID<sub>50</sub>/ZH501 (n = 5 for μMT/ZH501) or 1.0 × 10<sup>5</sup> TCID<sub>50</sub> ΔNSs (n = 10 for μMT/ΔNSs) or sham-inoculated with sterile DMEM (n = 5 for μMT/DMEM). Control C57BL/6 mice were inoculated with 1.0 × 10<sup>5</sup> TCID<sub>50</sub>/ZH501 (n = 5 for con/ZH501). 1.0 × 10<sup>5</sup> TCID<sub>50</sub> ΔNSs (n = 5 for con/ΔNSs). A subset of survivors of ΔNSs infection was subsequently challenged with 1.0 × 10<sup>5</sup> TCID<sub>50</sub> ZH501 s.c. on 21 dpi, and the rest were euthanized for sample collection for serology and RVFV qRT-PCR.

Serial euthanasia of ΔNSs-infected mice. CD4-depleted, mock-depleted, and nondepleted mice were inoculated subcutaneously (s.c.) in the left rear footpad with 2.0 × 10<sup>5</sup> TCID<sub>50</sub> ΔNSs (n = 30 each for GK1.5/ΔNSs, LTF2/ΔNSs, and PBS/ΔNSs). As a negative control, mock-depleted mice were sham inoculated with 20 μl DMEM (n = 25 for LTF2/DMEM) on day 0. At 1, 3, 5, 8, 11, 15, and 17 days postinfection (dpi), 3 mice from each of the four groups were euthanized for sample collection. Whole blood, liver, spleen, and brain were taken for quantification of viral RNA (vRNA) and inflammatory cytokine gene induction. Sera were collected for anti-RVFV NP IgG and neutralization assays. The popliteal (draining) lymph nodes (DLN) were harvested for flow cytometry. DLN supernatants produced during cell preparation were analyzed for inflammatory cytokines and chemokines.

Serology. Serum was collected and used for RVFV anti-NP IgG enzyme-linked immunosorbent assay (ELISA) as described previously (27). Briefly, purified RVFV NP or negative-control LASV G1 protein was used at a concentration of 200 ng/well. Plates were blocked in blocking buffer (5% skim milk, 5% fetal bovine serum [FBS], 0.1% Tween 20 in 1× PBS) at 37°C for 1 h. Plates were then incubated with sera at specified dilutions (1:50, 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, and 1:6,400) in blocking buffer for 1 h at 37°C. Plates were washed 3 times in 1× PBS with 0.1% Tween 20 (PBST) and then incubated with goat anti-mouse horseradish peroxidase (HRP; 1:10,000) (Jackson Immunoresearch) in blocking buffer for 1 h. Plates were washed 3 times in PBST, and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate was added according to the manufacturer’s instructions (KPL). Reactions were stopped with the addition of 1% SDS and read at 405 nM. Absolute values obtained from negative controls were subtracted prior to analysis.

VNT<sub>100</sub>. Stock ΔNSs/ΔNSsΔNSsGFP was diluted to 100 TCID<sub>50</sub> in 50 μl DMEM without FBS. Sera were heat inactivated at 56°C for 30 min. In a 96-well plate, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640 serum dilutions were made in 50 μl DMEM. An equal volume of diluted RVFV was added to diluted sera, and samples were incubated for 1 h at 37°C. A suspension of approximately 3 × 10<sup>4</sup> Vero E6 cells was added to each well, and the plates were incubated for 72 h before visualization of GFP-positive cells. VNT<sub>100</sub> was defined as the highest dilution that permitted 100% neutralization of virus input.

Total RNA extraction. Specimens of liver, spleen, and brain were collected at 1, 3, 5, 8, 11, 15, and 17 dpi or from moribund animals. RNA was
extracted using MagMax total RNA isolation kit (Ambion). Approximately 100-150 ng of total RNA was then treated with DNase I buffer and homogenized using a high-throughput tissue grinder (GenoGrinder 2000). Homogenates were extracted using the MagMax Express-96 magnetic particle processor (Ambion) according to the manufacturer’s instructions, including a DNase treatment step. Approximately 50 µl of total RNA was used for a mouse glycerol-3-phosphate dehydrogenase (GAPDH) qRT-PCR assay (ABI Biosciences). The reaction was run on an ABI 7500 quantitative PCR machine (ABI Biosciences).

Cytokine Luminex assays. Lymph node supernatants were analyzed for cytokine concentration using a Milliplex mouse cytokine/chemokine 23-plex panel following the manufacturer’s instructions (Millipore Corp.).

Flow cytometry and intracellular cytokine staining. Lymph nodes were disrupted in 200 µl of PBS, and cells were pelleted by centrifugation at 5,000 rpm for 10 min. The cell pellet was resuspended in complete medium (RPMI with 10% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM l-glutamine), counted, and seeded onto 96-well tissue culture plates at a density of 1 x 10^4 cells/well. Cells were untreated, stimulated with ΔNSs virus at a multiplicity of infection (MOI) of 5, or stimulated with staphylococcal enterotoxins B (SEB) (Sigma-Aldrich) at a concentration of 10 µg/ml at 37°C overnight. The next day, brefeldin A was added to all wells at a concentration of 10 µg/ml, and cells were incubated at 37°C for an additional 6 h. Cells were washed twice in wash buffer (PBS with 3% FBS). The following antibodies were used for surface staining, each at a dilution of 1:200: fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (BD Pharmingen; clone H129.19) or FITC-conjugated rat anti-mouse CD8a (BD Pharmingen; clone 53-6.7), and phycoerythrin (PE)-conjugated rat anti-mouse CD3 (BD Pharmingen; clone 17A2). After incubation on ice for 30 min, cells were washed twice in wash buffer, and permeabilized using BD Cytofix/Cytoperm on ice for 20 min (BD Biosciences). Cells were washed twice in BD Perm/Wash buffer and incubated on ice for 30 min with antibodies for intracellular cytokine staining. The antibodies used were allophycocyanin (APC)-conjugated rat anti-mouse tumor necrosis factor alpha (TNF-α) (BD Pharmingen; clone MP6-XT22) or APC-conjugated rat anti-mouse IFN-γ (BD Pharmingen; clone XMG1.2). Cells were washed twice with BD Perm/Wash buffer, and at least 50,000 events were collected from each well on an Accuri C6 flow cytometer (BD Biosciences). CFLOW Sampler software (BD Biosciences) was used to gate on the lymphocyte population and the CD3^+ cells. CD8-by-IFN-γ or -TNF-α plots were generated. Three animals from each group of mock-depleted or CD4-depleted mice were tested independently on each day, and data were averaged. DLN from sham-infected mice were combined and analyzed together. DLN from ΔNSs-infected nondepleted mice were combined and used for all single-antibody control stains, isotype control stains, and positive-control SEB stimulations.

Influenzal cytokine gene expression assays. Quantitative PCR arrays for inflammatory cytokines and receptors (PAMM-0112; SABiosciences) were used to determine up- or downregulation, relative to mock-infected mice, of a select panel of 84 inflammatory genes in CD4- or mock-depleted control mice infected with ΔNSs. For each brain sample, cDNA was synthesized from 0.8 to 1.0 µg of RNA using the RT^2 first-strand kit (SABioscience). Arrays were run on an ABI 7500 using RT^2 SYBR green/ROX PCR master mix according to manufacturer’s instructions (SABioscience), and comparative analyses were performed using the web-based PCR array data analysis software provided by the manufacturer.

Histopathology. Brain, liver, and spleen from CD4^- and mock-depleted mice infected with ΔNSs virus that were euthanized on each of days 1, 3, 5, 8, 11, 15, and 17 postinfection were fixed by immersion in 10% neutral buffered formalin for 7 days. Tissues from three uninfected, CD4^-depleted mice and three uninfected, nondepleted mice were sampled as additional controls. Tissues were processed, paraffin embedded, and sectioned by routine methods and stained with hematoxylin and eosin (H&E) for histological examination.

Statistical analyses. Serology (anti-NP IgG and neutralizing antibody) titers were analyzed using 2-way analysis of variance (ANOVA) with Bonferroni’s posttests to compare values at each time point (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Viral load and DLN cytokine data were analyzed using the Mann-Whitney test (*, P < 0.05) (GraphPad Prism; GraphPad Software, Inc.). For the antiviral array analysis, the mean value for each gene was calculated from replicate tissue samples using the threshold cycle method and normalized to the average values for five housekeeping genes (β-glucuronidase [Gus-β], β2-microglobulin [B2M], heat shock protein 90A1 [HSP-90A1], glyceraldehyde-3-phosphate dehydrogenase [GAPDH], and β-actin genes). The P values were calculated using Student’s t test (SABioscience) for each gene in the CD4^-depleted and mock-depleted groups.

RESULTS

All immunologically altered mice infected with virulent ZH501 virus behaved similarly to controls. As shown previously, in our C57BL/6J mouse model, mice succumbed rapidly to wild-type RVFV (ZH501) infection, usually within 2 to 3 dpi (20). All B-cell-deficient (µMT) mice, CD4-depleted, and CD8-depleted mice in this study died within the same time frame after ZH501 infection (data not shown).

Functional B cells were required to clear ΔNSs virus infection and protect against virulent challenge. On day 0, B-cell-deficient (µMT) mice and control (C57BL/6J) mice were infected with ΔNSs. One of the 10 ΔNSs-infected µMT mice succumbed to infection 10 dpi (Fig. 1A). At 21 dpi, 4 of the 9 surviving µMT mice and 4 ΔNSs-infected control mice were euthanized for sample collection. In contrast to the control mice, which had no vRNA in any tissues, the liver and spleen of µMT mice (4/4) were positive for vRNA. Two of the 4 µMT mice also had low vRNA levels in the brain (Fig. 1B). As expected, none of the µMT animals tested mounted an anti-RVFV-NP IgG response (data not shown). Also at 21 dpi, the remaining 5 of 9 ΔNSs-infected µMT mice and 5 ΔNSs-infected controls were challenged with virulent ZH501 (Fig. 1C). The ΔNSs-infected controls survived, but all µMT mice succumbed between 7 and 14 days postchallenge with high vRNA loads in the liver, spleen, and brain (Fig. 1D).

Survival from ΔNSs infection and subsequent protection from ZH501 challenge were not dependent on CD8^+ T cells. Flow cytometry of the DLN confirmed greater than 99% CD8^+ T cell depletion was achieved with a monoclonal anti-CD8 (YTS169) antibody (data not shown). Following ΔNSs infection, the CD8^+ T-cell-depleted mice behaved identically to mock-depleted controls. All mice survived initial ΔNSs infection with no indication of clinical signs of illness, and all mice remained healthy following challenge 28 days later with virulent ZH501 (Fig. 2).

CD4^- T cells were critical for survival from RVFV-associated neurololgic disease. C57BL/6J mice were depleted of CD4^- T cells, and flow cytometry confirmed that 99% CD4^- T cell depletion lasted longer than 2 weeks (data not shown). Across repeated experiments, one-third of CD4^-depleted mice infected with ΔNSs consistently developed neurololgic signs 12 to 36 dpi and eventually succumbed to the disease or were euthanized when severely ill or moribund (Fig. 3A). Liver and brain samples were taken from 3
severely ill mice at time of euthanasia; tissues from mock-depleted ΔNSs-infected controls were taken at the same time. Only tissues from CD4-depleted mice were positive for vRNA, with significantly more vRNA found in the brain than liver (Fig. 3B). CD4-depleted mice that survived ΔNSs infection were challenged with ZH501 28 dpi, and all survived (data not shown).

CD4+ -depleted mice had a reduced antibody response and increased vRNA titers following ΔNSs infection. To gain a better understanding of the role CD4+ T cells play in protection from RVFV pathogenesis, we undertook a serial euthanasia study to compare ΔNSs infection in CD4-depleted mice with that in mock-depleted controls. Both groups developed an anti-NP IgG response 5 dpi, with peak titers reached by 8 dpi. However, CD4-depleted mice developed significantly lower anti-NP IgG titers than control mice (Fig. 4A), as well as a barely detectable neutralizing antibody response (Fig. 4B).

Blood, liver, spleen, and brain samples were tested for vRNA using an RVFV qRT-PCR assay. In the blood, vRNA was present 1 and 3 dpi at similarly low levels in all infected mice, regardless of depletion status, but was cleared from all mice by 5 dpi (data not shown). vRNA levels in the livers of both mock- and CD4-depleted mice decreased from 3 to 8 dpi (Fig. 5A). However, while control mice appeared to clear the infection at this point, the livers of CD4-depleted mice continued to test positive for vRNA through 17 dpi (Fig. 5A). In both groups, spleens were weakly positive for vRNA throughout the course of the experiment (Fig. 5B). In the brain, vRNA was present in both groups starting 8 dpi. However, while vRNA levels steadily increased in the brains from mock-depleted mice, clearing by 17 dpi, vRNA levels steadily increased in the brains of CD4-depleted mice (Fig. 5C). At the time of euthanasia 17 dpi, the 3 CD4-depleted mice showed clear signs of neurologic disease, including tremors, depression, and/or hind-limb paralysis, whereas all mock-depleted mice remained healthy throughout the experiment.

CD4-depleted mice had a significant spike in inflammatory cytokine production from the DLN. DEN harvested at various
time points were disrupted in PBS. The cells were used for flow cytometry analyses, and the supernatants were tested for inflammatory cytokines. All infected mice released a burst of inflammatory cytokines 1 dpi, with few differences seen between CD4-depleted and control mice (Fig. 6). Mock-depleted mice produced significantly higher levels of regulatory cytokine IL-4 (Fig. 6G) than depleted mice 5 to 15 dpi. Strikingly, 5 and/or 8 dpi, CD4-depleted mice showed a significant second peak of the proinflammatory cytokines IFN-γ, interleukin-1β (IL-1β), IL-6, granulocyte colony-stimulating factor (G-CSF), CXCL10 (IFN-γ-induced protein 10 [IP-10]), and CCL3 (macrophage inflammatory protein 1α [MIP-1α]) (Fig. 6A to F and H).

CD8+ T cells in the DLN of CD4-depleted mice produced similar levels of inflammatory cytokines as mock-depleted mice. The CD8+ T cells from the DLN of each infected mouse were stained to detect intracellular cytokine IFN-γ on 8, 11, and 15 dpi. Both ΔNSs-infected CD4-depleted and ΔNSs-infected mock-depleted mice had a greater proportion of IFN-γ-expressing CD8+ T cells than mock-infected mice. CD8+ T cells of mock-depleted animals trended toward a stronger and longer-lasting intracellular cytokine response than the CD4-depleted animals (Fig. 7), but there was no statistically significant difference. The pattern of TNF-α expression in these cells was the same as that of IFN-γ (data not shown).

Upregulation of inflammatory cytokine genes in the brain was associated with high vRNA loads. Given the results from the DLN, we next evaluated the inflammatory response in brain tissue. Brain samples from mice euthanized on day 15 were analyzed for gene expression of inflammatory cytokines and chemokines. Overall, depleted mice demonstrated higher gene expression than mock-depleted mice 15 dpi. The brain of one mock-depleted infected mouse had low levels of vRNA and a slight upregulation in proinflammatory gene cytokines. However, a brain sample from a CD4-depleted mouse with high vRNA levels exhibited dramatic upregulation of several critical proinflammatory genes, including those coding for CCL2, CCL4, CCL5, CCL7, CCL12, CXCL1, CXCL10, CXCL13, IL-1β, and TNF-α (Fig. 8).

Lesions of encephalitis in CD4-depleted mice were more severe than those in mock-depleted mice and increased in severity through 17 dpi. Lesions of encephalitis first appeared in CD4-depleted mice 15 dpi, with one of three mice examined exhibiting mild infiltrate of neutrophils and scattered karyorrhectic debris in the brain stem (Fig. 9). By day 17, all three CD4-depleted mice exhibited widespread, focal to diffuse microgliosis, neuropil vacuolation, scattered infiltrate of neutrophils, reactive vascular endothelium, and rare neuronal necrosis; lesions were most severe in the brain stem, and meningitis was not a significant feature. In contrast, evidence of encephalitis was uncommon in the mock-depleted mice. At both 8 dpi and 15 dpi, one of three mock-depleted mice exhibited mild meningoencephalitis, in the brain stem and cerebrum, respectively, with mild perivascular cuffing, neutrophilic infiltrate, and scattered gliosis. Meningeal infiltrate was very mild and consisted of a mixed population of lymphocytes,
FIG 5 CD4 cells are required for viral clearance from liver and brain. Viral RNA loads from the liver (A), spleen (B), and brain (C) of CD4-depleted and mock-depleted mice were quantified by RVFV qRT-PCR and are displayed as the inverse threshold cycle (CT). The threshold cycle cutoff of 40 is noted by gray dashed lines. The data represent vRNA loads of 3 mice per group on each day.

FIG 5

histiocytes, and neutrophils. There was no evidence of encephalitis in the 3 mock-depleted mice euthanized 17 dpi.

DISCUSSION

The broad clinical spectrum of human RVFV cases has been well described, but little is known about the underlying host factors that predispose development of severe disease, including delayed-onset encephalitis. To date, RVFV immunology studies have focused largely on understanding the role of innate immunity in RVFV pathogenesis due to the profound inhibition of the antiviral response, particularly IFN-β expression, by the key RVFV virulence factor, NSs. Interestingly, the NSs protein is dispensable for viral replication in both IFN-incompetent cell lines and IFN-competent primary macrophages (29). Early studies showed that type 1 IFN was essential for controlling RVFV replication in vitro (30) and in vivo (12, 30, 31). In the rhesus macaque RVFV disease model, treatment with IFN-α was sufficient to prevent disease after virulent RVFV challenge (32), and IFNAR−/− mice (where IFNAR is IFN-α receptor) were highly susceptible to infection with ΔNSs virus infection (33). Therefore, while it is clear that an early IFN-mediated antiviral response is absolutely critical for controlling initial viral replication, the roles of the downstream adaptive immune responses in controlling primary RVFV infection and pathogenesis have not yet been examined.

Here we have defined the roles of cell-mediated and humoral immunity in protection from RVFV-induced neurologic disease, viral clearance from infected tissues, and the development of protective immunity using an established mouse model. We infected mice with a recombinant RVFV strain lacking NSs (ΔNSs) instead of wild-type virus because the rapid course of ZH501 virus infection culminates in fatal disease before an adaptive response can be mounted. Indeed, mice depleted of CD4+ or CD8+ T cells or deficient in B cells succumbed to ZH501 infection like wild-type mice, 2 to 3 dpi. Using the ΔNSs virus model, we were able to directly evaluate the individual components of adaptive immunity in the presence of an active innate immune response. We demonstrated that viral clearance requires functional B cells and a robust antibody response but not CD8+ T cells. We found that the functions of CD4+ T cells appear to determine whether RVFV infection proceeds toward pathogenicity or immunity, due at least in part to their broad influence on the development of adaptive immune responses, including stimulation of a robust antibody response and regulation of downstream inflammatory responses.

We initially examined the importance of CD4+ T cells in primary RVFV infection by depleting mice of CD4+ cells prior to ΔNSs virus infection. One-third of these mice succumbed to late-onset neurologic disease, in contrast to mock-depleted controls that had no indication of clinical disease. In the first 8 dpi, no apparent differences were detected in viral loads between depleted and nondepleted mice. Viral loads in all tissues tested remained low during this first week after infection, suggesting the innate immune response functioned similarly in the absence of CD4+ T cells. However, after day 8, CD4-depleted mice developed higher vRNA titers in liver, spleen, and brain than mock-depleted mice, indicating that after the innate immune response, CD4+ T cells were necessary to control virus replication and mediate viral clearance from the liver and brain. Unlike mock-depleted mice that cleared vRNA from the liver by 11 dpi, viral loads of CD4-depleted mice continued to increase through day 17. More importantly, vRNA in the brains of CD4-depleted mice steadily increased after 8 dpi, culminating in both clinical and histological signs of encephalitis on 17 dpi. Over the course of the experiment, approximately one-third of CD4-depleted mice developed clinical signs associated with high vRNA loads. Although no mock-depleted mice developed neurologic disease, a similar proportion of these animals’ brains tested positive for vRNA and had histologic evidence of encephalitis that was less severe than that in CD4-depleted mice. We therefore hypothesize that in most cases, the innate immune response is sufficient to control RVFV spread, regardless of depletion status. However, in the cases where RVFV escapes the innate response and invades the central nervous system (CNS), viral clearance and prevention of clinical disease are dependent on CD4+ T cell function.

Taken together, the initial experiments indicated CD4+ T cells were critical for controlling RVFV infection and preventing progression to delayed-onset neurologic disease but not prevention of initial virus spread. We next sought to identify the specific CD4+...
CD4+ T cell functions that were important in this process. CD4+ T cells have far-reaching influence on the development of the adaptive immune response in viral infection (as reviewed in reference 34). They stimulate humoral immunity by activating B cells and facilitate isotype class switching, affinity maturation, and antibody production. CD4+ T cells also prime CD8+ T cells and mediate cytotoxicity and can directly stimulate death in infected cells. Additionally, CD4+ T cells focus and direct the immune response by releasing inflammatory cytokines and chemokines. A subset of CD4+ cells functions as regulatory T cells (Tregs) that moderate the immune response to prevent immunopathogenesis. In order to tease apart the critical aspects of CD4+ T cells in primary RVFV infection, we broadly evaluated several of these functions.

We began by investigating the effect of CD4 depletion on the humoral response. The absence of CD4+ T cells resulted in significantly lower anti-NP IgG titers than those in mock-depleted mice and barely detectable neutralizing antibodies. This is similar to the pattern seen in West Nile virus (WNV) infection of CD4-depleted
mice (35). In the mock-depleted ΔNSs virus-infected mice, clearance of virus from the liver was associated with a sudden increase in IgG titers, while CD4-depleted mice failed to clear the infection from the liver. The role of antibodies in viral clearance was further supported by the failure of B-cell-deficient (μMT) mice to clear ΔNSs from the liver and brain weeks after infection, long after wild-type mice had done so. Interestingly, one μMT mouse succumbed at 10 dpi following initial ΔNSs infection. It is possible that in the absence of a robust antibody response, persistent ΔNSs virus infection can lead to severe pathology in a small percentage of cases. While antibodies clearly mediated clearance of RVFV from tissues, the resolution of viremia (as measured by vRNA) did not depend on a strong neutralizing antibody response. All mice cleared RVFV from the blood just days after infection, before neutralizing antibodies were produced by mock-depleted mice and in the absence of a robust neutralizing response by CD4-depleted mice. Together, the experiments with μMT and CD4-depleted mice demonstrated that RVFV clearance from tissues was antibody mediated, but reduced antibody titers alone do not account for the higher mortality observed in CD4-depleted mice relative to μMT mice following ΔNSs infection.

The results from challenge experiments, where mice were initially inoculated with ΔNSs and subsequently challenged with ZH501, indicated that there was not a complete correlation between the presence of detectable neutralizing antibody and protection from lethal disease. This observation is consistent with findings from a previous Rift Valley fever vaccine study, in which 60% of vaccinated mice survived challenge despite only 20% having developed detectable neutralizing antibodies (20). The simplest explanation is that broad antibody responses, which may include neutralizing antibodies barely detectable by classic VNT100 (with a minimum dilution of 1:20), can afford some measure of protection, at least in the mouse model. However, we cannot rule out that protection from ZH501 challenge is not completely dependent on a robust neutralizing antibody response.

CD4+ T cell deficiencies are also associated with insufficiencies in cell-mediated immunity. Another major function of CD4+ T cells is to stimulate the CD8+ T response by driving the initial expansion and recruitment of CD8+ T cells and sustaining the cellular response (36). In our model, however, the CD8+ T cell response did not appear to play an important role in RVFV pathogenesis. Mice depleted of CD8+ T cells and infected with ΔNSs virus survived with no signs of clinical disease and were protected against a subsequent challenge with ZH501 28 days after initial ΔNSs infection. In contrast, ΔNSs-infected μMT mice failed to clear ΔNSs virus even weeks after infection and displayed uniform mortality following ZH501 challenge, confirming the requirement of antibodies, and not CD8+ T cells, for the development of protective immunity. The persistence of ΔNSs virus in mice that lack B cells but have functional CD8+ T cells further suggests that humoral, and not CD8+ T-cell-based cell-mediated immunity, is necessary for viral clearance.

CD4+ T cells also direct the inflammatory response during viral infection, by both producing proinflammatory cytokines and by regulating the intensity of the response (34). Therefore, the absence of CD4+ T cells could theoretically lead to either an insufficient inflammatory response or, on the other hand, unregulated cytokine release and subsequent immune-mediated pathology. In the DLN of infected mice, mock-depleted mice produced higher levels of IL-4, a regulatory cytokine that also functions to activate B cells and enhance antibody production and which correlates with the finding of higher antibody titers in mock-depleted mice. Cytokine release was otherwise similar in CD4- and mock-depleted mice, except for striking differences on day 8, when there was a sudden and pronounced spike in inflammatory cytokines from the DLN of infected mice, mock-depleted mice producing higher levels of IL-4, a regulatory cytokine that also functions to activate B cells and enhance antibody production and which correlates with the finding of higher antibody titers in mock-depleted mice. Cytokine release was otherwise similar in CD4- and mock-depleted mice, except for striking differences on day 8, when there was a sudden and pronounced spike in inflammatory cytokines from the DLN of infected mice, mock-depleted mice producing higher levels of IL-4, a regulatory cytokine that also functions to activate B cells and enhance antibody production and which correlates with the finding of higher antibody titers in mock-depleted mice. Cytokine release was otherwise similar in CD4- and mock-depleted mice, except for striking differences on day 8, when there was a sudden and pronounced spike in inflammatory cytokines from the DLN of infected mice, mock-depleted mice producing higher levels of IL-4, a regulatory cytokine that also functions to activate B cells and enhance antibody production and which correlates with the finding of higher antibody titers in mock-depleted mice. Cytokine release was otherwise similar in CD4- and mock-depleted mice, except for striking differences on day 8, when there was a sudden and pronounced spike in inflammatory cytokines from the DLN of infected mice, mock-depleted mice producing higher levels of IL-4, a regulatory cytokine that also functions to activate B cells and enhance antibody production and which correlates with the finding of higher antibody titers in mock-depleted mice. Cytokine release was otherwise similar in CD4- and mock-depleted mice, except for striking differences on day 8, when there was a sudden and pronounced spike in inflammatory cytokines from the DLN of infected mice, mock-depleted mice producing higher levels of IL-4, a regulatory cytokine that also functions to activate B cells and enhance antibody production and which correlates with the finding of higher antibody titers in mock-depleted mice.
lymph nodes, possibly macrophages, dendritic cells, and/or natural killer cells.

Therefore, during ΔNSs infection, the absence of CD4⁺ T cells did not inhibit the local cytokine production, but rather stimulated a dramatic, if transient, proinflammatory response from cells within the draining lymph node. Similarly, elevated proinflammatory cytokine responses in the draining lymph nodes were associated with increased viral titers in peripheral tissues following lymphocytic choriomeningitis virus (LCMV) or herpes simplex virus (HSV) infection of Treg-deficient mice (37). The authors concluded that Tregs function early in virus infection to direct movement of effector cells to the site of infection, and in the absence of Tregs, an increased cytokine response occurs in the DLN that distracts from appropriate responses in peripheral tissues. While we did not specifically examine the role of Tregs in our study, depletion using an anti-CD4 monoclonal antibody has been shown to significantly reduce the absolute number of Tregs in a C57BL/6 mouse model (38). It is therefore tempting to speculate that Tregs could function similarly in RVFV infection. More detailed studies looking specifically at these cells will be necessary to directly address this issue.

Studies of other encephalitic viruses have linked systemic proinflammatory responses with increased blood-brain barrier (BBB) permeability, viral invasion of the CNS, and subsequent neurologic disease (39–41). We described a proinflammatory response following peripheral RVFV infection in the DLN of CD4-depleted mice only, possibly due to an absence of CD4⁺ T regulatory functions. This response correlated with the appearance of vRNA in the brains of these mice, suggesting that inflammatory alterations in the BBB could have been involved. However, the presence of vRNA on day 8 in a mock-depleted mouse suggested that inflammation alone did not provide a clear mechanism for initial neuroinvasion. At later time points, significant upregulation in proinflammatory gene expression was apparent in the brains of CD4-depleted mice relative to mock-depleted mice. The relative degree of gene expression was positively correlated with concomitant vRNA load, and the brains with small amounts of vRNA did not show increased cytokine expression, suggesting virus replication stimulated the proinflammatory response in the brain. Similar patterns have been described in West Nile virus (WNV) (42) and Venezuelan equine encephalitis virus (VEEV) (40). In both cases, initial CNS infection does not depend on a compromised

FIG 9 All CD4-depleted mice displayed lesions of encephalitis by 17 dpi. Shown is hematoxylin-and-eosin staining of brain stem. (A) Mock-depleted mouse, day 15 postinfection, showing perivascular cuffing with mixed, predominantly mononuclear, inflammatory cells (20 ×). (B) CD4-depleted mouse, day 15 postinfection, showing neutrophilic infiltrate (arrows) in perivascular areas and scattered throughout the neuropil (40 ×). (C) Mock-depleted mouse, day 17 postinfection, with no evidence of encephalitis (20 ×). (D) CD4-depleted mouse, day 17 postinfection, showing encephalitis characterized by infiltrate of neutrophils (arrows), karyorrhectic cellular debris, and acute neuronal necrosis (inset; arrowheads) (40 ×).
BBB, but subsequent viral replication in the brain leads to a strong inflammatory response that alters BBB integrity and enhances neuropathology. For RVFV, the role of inflammation in neuro-pathogenesis and the specific functions of CD4+ T cells in preventing neurologic disease remain interesting questions for future research.

In these studies, CD4+ T cells were revealed to be key players in the control of RVFV replication in vivo and prevention of late-onset neurological disease. This is in contrast to work done in the dengue virus mouse model where depletion of CD4+ T cells had no effect on viral load, humoral, or cellular immune responses (43). However, CD4+ T cells have been shown to limit the pathogenesis of WNV, as CD4-depleted WNV-infected mice had increased viral loads in the brain, decreased antibody responses, and decreased survival (35). This is strikingly similar to what we observed with RVFV; however, viral clearance in WNV-infected mice was mediated by CD4+ cell stimulation of CD8+ T cells that efficiently cleared virus from the brain (44). In contrast, we have shown that clearance of RVFV does not depend on CD8+ T cells, but instead relies upon a robust CD4-dependent antibody response, as has been shown in Japanese encephalitis virus (JEV) infection (45).

While resolution of RVF infection is clearly linked to the antibody response, increased mortality was seen in mice lacking CD4+ T cells compared to mice deficient in B cells, suggesting CD4+ T cells function in an additional manner to limit RVF-induced disease. In VEEV infection, virus-specific antibodies play a critical role in protection from disease, but the robust antiviral effects of CD4+ T cells (unrelated to B or CD8+ cell function) are required for survival (46). Direct antiviral effects of CD4+ T cells in RVFV infection need to be examined, but could explain the differences in mortality between µMT and CD4-depleted mice. On the other hand, the spike in inflammatory cytokine release in the DLN and increased proinflammatory gene expression in the brain of mice lacking CD4+ T cells suggest that the enhanced virulence in CD4-depleted animals could be due to the absence of a regulatory influence rather than a reduced inflammatory response.

In summary, our work demonstrates that CD4+ T cells are critical for control of RVFV infection by stimulating a strong antibody response and by acting as a mediator of the inflammatory response to infection. An expanded understanding of the specific roles of CD4+ T cells will be explored in future experiments, with the ultimate goal of designing better vaccines and therapeutics to help prevent and control disease caused by RVFV, a serious public health pathogen.

ACKNOWLEDGMENTS

We thank T. Klimova and B. Bird for critical readings of the manuscript. K.A.D. thanks N. J. MacLachlan and P. Pesavento of the University of California, Davis, for enthusiastic mentorship.

The findings and conclusions in this article are those of the authors and do not necessarily represent those of the Centers for Disease Control and Prevention.

REFERENCES

CD4+ T Cells Protect against RVFV Neurologic Disease


