CD4 Deficiency Causes Poliomyelitis and Axonal Blebbing in Murine Coronavirus-Induced Neuroinflammation.

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CD4 deficiency causes poliomyelitis and axonal blebbing in murine coronavirus induced neuroinflammation

Running title: CD4 T cells prevent poliomyelitis and axonal dystrophy

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ABSTRACT:

Mouse hepatitis virus (MHV) is a murine β-coronavirus (m-CoV) which causes a wide range of diseases in mouse and rat, including hepatitis, enteritis, respiratory diseases, and encephalomyelitis in the CNS. MHV infection in mice provides an efficient cause-effect experimental model to understand the mechanisms of direct virus induced neural cell damage leading to demyelination and axonal loss which are pathological features of Multiple sclerosis (MS), the most common disabling neurological disease in young adults. Infiltration of T lymphocytes, activation of microglia and their interplay are the primary pathophysiological events leading to the disruption of myelin sheath in MS. However, there are emerging evidences supporting gray matter involvement and degeneration in MS. The investigation of T cell function in the pathogenesis of deep gray matter damage is necessary. Here, we employed RSA59 (isogenic recombinant strain of MHV-A59) induced experimental neuroinflammation model to compare the disease in CD4⁻/⁻ mice with CD4⁺/⁺ mice at days 5, 10, 15, and 30 p.i. Viral titer estimation, nucleocapsid gene amplification and viral anti-nucleocapsid staining confirm enhanced replication of the virions in the absence of functional CD4⁺ T cells in the brain. Histopathological analyses showed an elevated susceptibility of CD4⁻/⁻ mice to axonal degeneration in the CNS with augmented progression of acute poliomyelitis, and dorsal root ganglionic inflammation rarely observed in CD4⁺/⁺ mice. Depletion of CD4⁺ T cells shows unique pathological bulbar vacuolation in the brain parenchyma of infected mice with persistent CD11b+ microglia/macrophages in the inflamed regions on day 30 p.i. In summary,
the current study suggests that CD4+ T cells are critical for controlling acute stage poliomylitis (gray matter inflammation) and chronic axonal degeneration, inflammatory demyelination due to loss of protective anti-viral host immunity.

#: Equal contribution

Debanjana Chakravarty and Fareeha Saadi contributed equally to this work. Author order was determined based on the relatedness of the work with Debanjana Chakravarty’s PhD thesis.

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KEYWORDS: CD4+T cells, Microglia, MHV infection, mCoV, Neuroinflammation, Innate immune response, Demyelination, Host Immunity

IMPORTANCE

The current trend in CNS disease biology is to understand the neural cell-immune interaction to investigate the underlying mechanism of neuroinflammation, rather than focusing on peripheral immune activation. Most studies in MS are targeted toward understanding the involvement of CNS white matter. However, the importance of gray matter damage has become critical in understanding the long-term progressive neurological disorder. Our study highlights the importance of CD4+T cells in safeguarding the neurons against axonal blebbing and poliomylitis from murine Betacoronavirus-induced neuroinflammation. Current knowledge of the mechanisms that lead to gray matter damage in MS is limited, because the most widely used animal model EAE does not present this aspect of the disease. Our results thus, add to the existing limited knowledge in the field. We also show that the microglia, though important for the initiation of neuroinflammation, cannot establish a protective host immune response without the help of CD4+ T cells.

INTRODUCTION
Neuroinflammation is the cardinal signature of several complex and multi-faceted central nervous system (CNS) disorders. CNS inflammation is known to be initiated mainly by the brain’s resident innate immune cells, the microglia, which rapidly respond to an infectious agent or any perturbation in the CNS. Via the secretion of chemokines and cytokines, they direct the extravasation of several myeloid cells, including neutrophils, monocytes/macrophages, and dendritic cells, which in turn promote the entry and activation of adaptive immune responsive T cell populations into the CNS (1-4).

Regulation of T cells is central to understanding the cellular and humoral immunity in neuroinflammation. So far, most studies have demonstrated the destructive pathogenic effects of encephalitogenic T cells in neurodegeneration. For example, Multiple sclerosis (MS the most common neurological disease of young adults is characterized predominantly by self-reactive myelinolytic T cell-mediated autoimmune destruction of the myelin sheath (5-10). Likewise, in an experimental animal model of MS, Experimental autoimmune encephalomyelitis (EAE), the disease mainly depends on the infiltration of pathogenic CD4+ T cells, which are primarily of Th1 and Th17 types (11-19). Studies have also shown a pathogenic role of myelin-specific CD8+ T cells in the inflammatory lesions in EAE mice brains (20-22). A Mouse hepatitis virus (MHV) (V5A13.1) model of induced neuroinflammation showed a significant reduction in the severity of inflammation and demyelination in CD4− mice at days 12-21 p.i. compared to both CD8− and wild type mice (23). Adoptive transfer of CD4+T cells and CD8+T enriched splenocytes differentially affect the state of inflammation and demyelination in MHV-JHM infected RAG− mice and induction of donor splenocytes with a depleted population of both CD4 and CD8+ T cells in JHM infected RAG− mice prevents demyelination (24). However, recent advances have shown that the prevalence of activated adaptive immune responses are not restricted to neuroinflammatory myelin degeneration as seen in MS but translates across classic neurodegenerative disorders such as Alzheimer’s disease (AD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS) (25, 26) (27, 28) (29) (30).

With the shift in the paradigm of CNS immunology and the discovery of CNS meningeal lymphatic vessels, several studies also suggested that CD4+ T cells may provide protective immunity against cognitive and motor disabilities in neurodegenerative disorders (31) (32). Activated CD4+ T cells also help in limiting MHV-JHM replication within the CNS (33). Either CD4 or CD8 deletion in Theiler’s Murine Encephalomyelitis (TMEV) resistant B6 strain
makes the mice susceptible to disease and shows increased viral persistence and demyelination (34). The current research trend is thus targeted toward understanding the differential mechanisms that regulate the balance between neuroprotection and neurodestruction conferred by CD4+ T cells.

Several studies have demonstrated the accumulation of microglia/macrophages in the vicinity of reactive CD4+ T cells in CNS lesions during neurodegeneration (4, 30, 35-37). While ample literature supports CD4+ T cells interaction with CNS resident microglia and/or infiltrating myeloid-specific monocytes/macrophages as the primary mechanisms underlying white matter damage in the early relapsing-remitting stage of MS, one cannot overlook their function behind immune mediated grey matter atrophy.

The current study is focused on understanding the potential protective role of CD4+ T cells on microglial activation and their cooperative effect on both white and gray matter damage following infection with a neurotropic isogenic spike protein recombinant strain of Betacoronavirus MHV, RSA59. Intracranial infection of C57BL/6 mice with RSA59 results in a biphasic disease, characterized by acute hepatitis and meningoencephalomyelitis followed by chronic immune-mediated demyelination and concomitant axonal loss, which mimics specific pathologies of the human demyelinating disease MS (38-40). RSA59 induced acute neuroinflammation comprises mixed populations of astrocytes and inflammatory cells, mainly microglia/macrophages and a smaller population of T lymphocytes (40-44). As early as day 3 post-infection (p.i.), peripheral leukocytes start to infiltrate the CNS, beginning with the cells of the innate immune response predominantly myeloid cells such as neutrophils and monocytes/macrophages. Lymphoid cells, including CD4, CD8, and NK T cells start to appear in the CNS at day 5 p.i. and their infiltration peaks at day 7 p.i. followed by the start of viral clearance (Fig. 1). While CD8+ T cells begin to disappear as early as day 10 p.i. and NK T cells reduce in number, a significant number of CD4+ T cells are present in the inflamed brain even at day 16 p.i. (Fig. 1). While inflammation resolves and infectious virus particles clear from the brain, Iba1+ macrophages/microglia persist significantly within the demyelinating plaques in the spinal cord white matter and are known to cause direct myelin stripping (40). Recent Affymetrix microarray analysis in the spinal cord of RSA59 infected mice showed elevated expression of inflammatory mediators during the acute stage of infection. Interestingly, conventional T and B cell markers showed no or insignificant upregulation (45, 46). Expression of adaptive immune responsive genes showed prominent upregulation during the chronic phase
The most striking associations were observed between CD3, CD45, and MHCII expression, which promote the communication between innate and adaptive immune systems via microglia-CD4+T cell signalling.

Current study employs a CD4 knock out strain in the background of C57BL/6 mice (CD4−/−). In comparison to the wild type C57BL/6 mice (CD4+/+), infection of CD4−/− mice produces an exaggerated disease course in association with enhanced viral replication and prolonged viral persistence. Moreover, CD4−/− mice are more susceptible to chronic inflammation, and axonal degeneration compared to CD4+/+ mice, and CD11b+ macrophages/microglia show persistent activation even during the chronic disease phase. Our results suggest a novel neuroprotective role of CD4+ T cells in the MHV induced demyelinating model of MS unlike the EAE model where T cells have a predominantly pathogenic role.

RESULTS

Mice were inoculated with isogenic EGFP expressing RSA59 as described in the materials and methods section. Experimental mice were monitored daily for the development of clinical signs and symptoms. The majority of CD4+/+ mice displayed low disease scores ranging from 0.5 to 1, indicated by ruffled fur and occasionally present the hunch back phenotype as observed previously (47). 100 % mice survived until day 30 p.i as observed. Though not significantly different, CD4−/− mice showed a slightly higher disease score of 1.5 to 2 indicated by hind limb weakness in addition to hunch back, the symptoms started appearing as early as day 3-5 p.i., however, almost 90% mice survived until day 30 p.i. The scoring system has been discussed in the materials and methods section.

The absence of functional CD4+ T cells does not alter acute stage hepatitis and meningoencephalomyelitis but shows gray matter involvement in the form of poliomyelitis and Dorsal Root Ganglion inflammation.

The current study initially investigated differences in phenotypic or pathological symptoms as well as basal level of inflammation at the tissue level between mock infected CD4+/+ and CD4−/− mice. No significant differences were observed at the phenotypic level. Conventional light microscopy data analysis at H&E staining sections from liver, brain and spinal cord revealed that mock infected (MI) CD4+/+ and CD4−/− mice presented only a basal level of inflammation (if any) in all the three tissue types (Fig 2). Mock infected CD4−/− mice did not show any
differential phenotypic or histopathological features concerned for this study. All experiments were conducted using age matched CD4+/+ and CD4-/- mice.

To examine the degree of inflammation in CNS and non-CNS tissues, M1 and infected CD4+/+ and CD4-/- mice were sacrificed at two-time points, day 5-6 p.i. (onset of the peak of neuroinflammation) and day 30 p.i. (chronic phase of inflammation). The liver, brain, and spinal cord tissues were harvested and fixed in 4% paraformaldehyde and paraffin embedded.

During the acute phase of infection, comparable, multiple foci of moderate to severe necrotizing, and non-necrotizing hepatitis were observed in both CD4+/+ and CD4-/- mice (Fig. 3a). During the chronic phase, hepatitis nearly resolved, but the number of remnant hepatic lesions were more in CD4-/- mice (Fig. 3b). The Hepatic Activity Index showed no significant difference at day 6 and 30 p.i. between CD4+/+ and CD4-/- mice (Fig. 3b).

H&E stained brain sections from RSA59 infected CD4+/+ and CD4-/- (Fig. 4a) mice demonstrated focal acute encephalitis, meningitis, intra-parenchymal perivascular lymphocytic cuffing, and microglial nodule formation at the acute phase of infection. Corresponding serial brain sections immunohistochemically stained with anti-CD45 (leukocyte common antigen, LCA) confirmed similar levels of inflammatory cells in the brain parenchyma of CD4+/+ (2.691 ± 0.4561) and CD4-/- (2.485 ± 0.3849) mice (48). H&E staining of both CD4+/+ and CD4-/- (Fig. 4b) infected spinal cords showed myelitis. Quantification of the staining intensity suggests that corresponding regions were equally immunoreactive for CD45, indicating the comparable infiltration of mononuclear cells in CD4+/+ (1.176 ± 0.1958) and CD4-/- mice (1.355 ± 0.1947).

However, the consequent anti-CD11b (pan-macrophage marker) immunohistochemistry revealed significantly fewer macrophage/microglia in the brains and spinal cords of CD4-/- mice (0.6067± 0.1260, ****p<0.0001 and 0.258 ± 0.07078, **p<0.01 respectively), as compared to CD4+/+ mice (4.749 ± 0.3981, and 1.110 ± 0.3007, respectively) (Fig. 5).

Moreover, apart from the white matter myelitis in both infected CD4+/+ and CD4-/- mice, CD4-/- mice showed evidence of acute poliomyelitis (gray matter inflammation) and dorsal root ganglionic inflammation which were rarely observed in CD4+/+ mice at the acute phase of infection (Fig. 6). Average inflammation scores, based on H&E, are shown in Table I and II.

The absence of CD4+ T cells impairs RSA59 clearance from the brain tissue.
To assess the role of CD4\(^+\) T cells in virus clearance, viral titer, viral nucleocapsid gene amplification, in situ viral antigen distribution and mRNA level of anti-viral pro-inflammatory cytokines/chemokines were evaluated.

RSA59 titer kinetics was compared in the brains of CD4\(^{-}\) and CD4\(^{+/+}\) mice, and the values were expressed as log\(_{10}\) PFU/gram of tissue. In both mouse strains, the virus replicated efficiently in the brain at day 5 p.i. (10\(^{5.8}\) PFU/gm in CD4\(^{-}\) versus 10\(^{6.1}\) PFU/gm in CD4\(^{+/+}\)). By day 10 p.i. CD4\(^{-}\) mice averaged almost 1-log-higher viral titre (10\(^{5}\) PFU/gm versus 10\(^{3.9}\) PFU/gm, \(*p=0.016\)) than CD4\(^{+/+}\) mice. On day 15 p.i. there were no detectable viral PFUs in CD4\(^{+/+}\) mice, however, a significant number of infectious viral PFUs were observed in CD4\(^{-}\) mice (10\(^{4.6}\) PFU/gm, ****\(p=0.0001\) (Fig. 7a).

To further confirm whether differences in viral replication might affect viral infection, qRT-PCR of viral N-gene (nucleocapsid) from brain tissues from MI, CD4\(^{+/+}\), and CD4\(^{-}\)/- infected mice was performed. As shown in Fig. 7b, viral persistence is comparable in both CD4\(^{+/+}\) and CD4\(^{-}\) mice (1.02 fold ± 0.176) at day 5 p.i. At day 10 and day 15 p.i., the N-gene transcript shows significant upregulation (10.230 folds ± 2.677, **\(p=0.003\) and 23.247 folds ± 5.204, ****\(p<0.0001\), respectively), in CD4\(^{-}\) mice compared to CD4\(^{+/+}\).

To determine the spread of infectious viral particles in situ, immunohistochemical analysis of viral anti-nucleocapsid antigen was performed on sections of infected brains obtained from CD4\(^{+/+}\), CD4\(^{-}\), and MI mice at the acute and chronic phases of infection. In MI mice, viral antigen was neither observed at day 6 nor at day 30 p.i. as expected (Fig. 7c). Post-intra-cranial inoculation, RSA59 replicates profusely in CD4\(^{+/+}\) mice and spreads rapidly from the lateral geniculate nuclei to several regions of the brain, including the olfactory bulb, basal forebrain, cerebral cortex, anterior commissure, brain stem and deep cerebellar white matter. By day 6 p.i., viral antigen becomes restricted to the midbrain, pons, and deep cerebellar white matter in the current study (data not shown). Similar viral anti-nucleocapsid antigen staining was observed in infected CD4\(^{-}\) mice at day 6 p.i. Day 30 post infected brains of CD4\(^{+/+}\) mice showed significantly reduced viral antigen staining as expected, whereas a considerable number of cells remained positive for viral anti-nucleocapsid antigen in the CD4\(^{-}\) mice (Fig. 7c).

Moreover, Interferon gamma (IFN-\(\gamma\)) mRNA expression was significantly higher at day 5 p.i. in CD4\(^{-}\) mice (2.45 folds ± 0.59, **\(p=0.0020\), Fig. 7d, in comparison to CD4\(^{+/+}\) mice,
indicating higher viral replication in CD4\(^{-}\) mice. Similarly, peripheral leukocyte chemoattractant IFN-\(\gamma\) inducible CXCL10 (a C-X-C chemokine) mRNA expression remained significantly upregulated even at day 10 p.i. (5.102 folds \(\pm\) 1.253, \(*\*\*p=0.0008\)) and day 15 p.i. (2.965 folds \(\pm\) 0.858, \(p=0.015\)) in the infected CD4\(^{-}\) mice (Fig. 7e) as compared to CD4\(^{+/+}\) mice. Also, anti-viral Tumour Necrosis Factor alpha (TNF-\(\alpha\)) mRNA expression was observed to be significantly upregulated at all time points in CD4\(^{-}\) mice, in comparison to CD4\(^{+/+}\) mice (6.372 folds \(\pm\) 1.10, \(*\*\*p<0.0001\) at day 5, 37.949 folds \(\pm\) 9.534, \(*\*\*p=0.00018\) at day 10, and 7.348 folds \(\pm\) 1.675, \(*\*\*\*p<0.0001\) at day 15), Fig. 7f. At the same time, CD4\(^{-}\) mice displayed lower levels of RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted) or CCL5 (a C-C chemokine, involved in trafficking of macrophage/monocytes into the CNS) mRNA transcripts at day 5 p.i. (0.523 folds \(\pm\) 0.124, \(**p<0.01\) and day 10 p.i. (0.544 folds \(\pm\) 0.214, \(*p<0.05\)) in comparison to CD4\(^{+/+}\) mice (Fig. 7g).

Viral plaque assays and qRT-PCR of viral N-gene and antiviral-cytokines and chemokines expression (IFN-\(\gamma\), TNF-\(\alpha\), CXCL10) at day 5, 10 and 15 p.i in corroboration with the viral anti-nucleocapsid antigen staining at day 30 p.i. confirmed prolonged persistence of RSA59 in the absence of functional CD4 in brain tissue.

The absence of CD4\(^{+}\) T cells exacerbates CNS inflammation and myelin loss at the chronic stage of inflammation.

The role of functional CD4\(^{+}\) T cells in demyelination was next examined by histopathological analysis of day 30 post-infected spinal cords from CD4\(^{+/+}\) and CD4\(^{-}\) mice. In CD4\(^{+/+}\) mice, inflammation was observed in the dorsal/posterior columns, anterior horn and/or the lateral descending tracts. H&E staining highlights the inflammation in the ventrolateral white matter. In contrast, CD4\(^{-}\) mice demonstrated vacuolar pathology with swollen axons throughout the white matter, involving the ventral and lateral descending tracts upon H&E staining (Fig. 8a). Corresponding inflamed regions from serial sections also showed myelin loss by LFB staining in both CD4\(^{+/+}\) and CD4\(^{-}\) mice. The degree of demyelination was significantly higher in the CD4\(^{-}\) mice (20.45 \(\pm\) 2.174, \(*\*\*p<0.0001\)) compared to CD4\(^{+/+}\) mice (6.008 \(\pm\) 1.375) (48) (Fig. 8b). CD45 immunoreactive inflammatory cells were present within the demyelinating plaques in both CD4\(^{+/+}\) and CD4\(^{-}\), however the extent of inflammation was considerably greater in CD4\(^{-}\) (2.812 \(\pm\) 0.1122 , \(*\*\*\*p<0.0001\)) mice in comparison to the CD4\(^{+/+}\) mice (0.5509 \(\pm\) 0.08550) (Fig. 8c). Interestingly, in contrast to the acute phase of infection, CD4\(^{-}\)
mice (1.237 ± 0.1646, ***p<0.001) spinal cord showed highly significant CD11b microglia/macrophage expression, in comparison to CD4+/+ mice (0.4175 ± 0.09144). (Fig. 8d) Quite strikingly, in contrast to the brains of CD4+/+ (Fig. 9a) mice, CD4-/  , mice exhibited extensive vacuolation in the brain stem and the deep cerebellar white matter tracts which has not been observed in previous studies. No significant differences were observed in CD45 staining between the two groups (Fig. 9b). Large numbers of CD11b positive microglia/macrophages were observed in the vicinity of the vacuolated regions in CD4+/+ mice (5.251 ± 0.5298, ****p<0.0001) as compared to CD4+/+ mice (2.220 ± 0.1639) (Fig. 9c).

The Absence of CD4+ T cells aggravates axonopathy

One-micron thick sections of glutaraldehyde fixed brains and spinal cords from RSA59 infected CD4+/+, CD4+/−, and MI CD4+/− mice were stained with toluidine blue. MI CD4+/− did not show any visible signs of inflammation (Fig. 10) or spinal cord damage. Infected CD4+/− mice exhibited vacuolation and axonal degeneration in the posterior columns (Fig. 11a, b). Electron microscopy of the vacuolated brainstem lesions in CD4+/− mice revealed that the vacuolation represents numerous swollen degenerating axons, and few inflammatory cells (lymphocytes and macrophages). There was no evidence of isolated demyelination (myelin loss with relative axonal preservation) (data not shown). Similarly, electron micrographs of the spinal cord white matter lesions (Fig. 11c, d) also showed axonal degeneration and a lack of demyelination. No inflammatory cells (lymphocytes and macrophages) were present within the areas of white matter damage (Fig. 11c).

In the absence of CD4+ T cells, mice show differential expression of inflammatory cytokines.

The inflammatory cells in the demyelinating lesions induced by RSA59, exhibit variable expression of pro- and anti-inflammatory cytokines. Expression of pro-inflammatory IL-6, IL-12p40, and anti-inflammatory IL-10 were examined in RSA59 infected mouse brains. Quantitative PCR results revealed that IL-6 expression was significantly higher in CD4+/− compared to CD4+/+ mice both at day 5 (6.742 folds ± 1.955, ****p<0.0001) and day 10 p.i. (4.24 folds ± 0.912, ****p<0.0001), and subsequently declines at day 15 p.i. in both groups of mice (Fig. 12a). IL-10 expression patterns, as revealed by real-time PCR, showed similar expression in the RSA59 infected CD4+/+ and CD4+/− mice at day 5 p.i. However, at days 10 (2.579 folds ± 0.72, **p=0.003) and 15 p.i. (2.58 folds ± 0.363, ****p<0.0001) CD4+/+ mice
showed significantly elevated expression of IL-10 compared to infected CD4\(^+\) mice (Fig. 12b). IL-12p40 mRNA expression demonstrated no considerable differences between CD4\(^+\)/+ and CD4\(^+\) mice following RSA59 infection (data not shown). Together the expression patterns of IL-6 and IL-10 indicate a robust inflammatory environment in the brains of CD4\(^+\) mice.

The absence of CD4\(^+\) T cells influences macrophage polarization.

Using the classical M1/M2 nomenclature of macrophage polarization, it was observed that mRNA expression of classical M1 macrophage (pro-inflammatory) markers such as CD86 and Bruton's tyrosine kinase (Btk) were significantly reduced in infected CD4\(^+\) mice in comparison to CD4\(^+\)/+ mice at day 5 p.i (0.445 folds ± 0.128, ***p<0.001 and 0.545 folds ± 0.134, **p<0.01, for CD86 and Btk respectively) (Fig. 12c-d). But, as the course of disease progressed toward the chronic phase, microglia/macrophages in the infected CD4\(^+\) mice showed an M2 phenotype (anti-inflammatory) in contrast to the CD4\(^+\)/+ mice, as observed by the significant upregulation in the mRNA expression of M2 macrophage marker CD163 at day 15 p.i. (2.018 folds ± 0.392, ****p<0.0001) (Fig. 12e).

DISCUSSION

The CNS is no longer considered an immune-privileged site (49). A significant number of peripheral CD4\(^+\) T cells patrol the cerebrospinal fluid (CSF) in order to detect the presence of potential harmful pathogens (50-52). If these T cells do not encounter their cognate antigen, they take the lymphatic route to exit the CNS. However, they will infiltrate the CNS parenchyma upon sensing any sign of neuroinflammation (52, 53). MHV infection in mice is an established archetype animal model used to understand the demyelination pathology in MS. In this study, we present a protective role of CD4\(^+\) T cells in the viral induced neuroinflammatory demyelination, in contrast to the pathogenic role of CD4\(^+\)T cells in MS(54) and its autoimmune experimental model EAE. Activated myelinolytic CD4\(^+\) T cells have been observed in the blood and cerebrospinal fluid (CSF) of MS patients (7); Additionally, MS lesions have been widely associated with the presence of both CD4\(^+\) and CD8\(^+\) T cells (55). These autoreactive T cells induce a Th-1 response, majorly targeted against PLP, and can worsen the disease progression in the patients (56-58). Considering their well-accepted pathogenic role in MS, classical immuno therapies are devised to silence the CD4\(^+\) T cell mediated attack on the myelin to repair the damage caused to the myelin sheath. Our study
highlights a quite opposite, protective role of CD4+ T cells in a virus induced etiology of demyelination in MS.

In this study, we present evidences to show that spatio-temporal infiltration of CD4+ T cells in the CNS and their dynamic equilibrium with brain resident microglia may influence the progression, severity, and amelioration of white and gray matter inflammation in a neurotropic virus model. The most widely used experimental model of MS, EAE, holds good only for understanding the mechanisms of white matter injury and does not recapitulate the aspects of gray matter damage.

The RSA59-induced demyelinating model is unique, it involves both white and gray matter inflammation. The onset of the disease is initiated via orchestration of innate immune genes in the acute phase to clear the virus and restore homeostasis and then gradually progress via adaptive immunity during the chronic phase (45). RSA59 induced demyelination is dissimilar to other conventional demyelinating models that are explicitly driven by adaptive immune responses, with its array of specialized T cells (CD4+ and CD8+) and myelin antigen-specific antibodies (20, 59, 60). Dynamic host immune responses involving CD4+ helper T cells are needed for recovery from infections. While CD4+ T cells are helpers for the development of a complete adaptive immune response, they are also required for enhancing innate immune effector functions. To assess the role of CD4+ T cells in the innate and adaptive immune responses and their interactions with microglia/macrophages following RSA59 infection, the current study compared CD4−/− mice and wild-type mice. The results showed a critical role of CD4+ T cells in the pathogenesis of RSA59 induced neuroinflammation. We observed the following: i) Absence of CD4+ T cells caused no change in acute encephalitis; but CD4−/− mice showed a significant reduction in CD11b positive microglia/macrophages; ii) viral replication was higher and viral transcripts were persistent in CD4−/− mice, even at day 30 p.i.; iii) CD4−/− mice showed an augmented susceptibility toward chronic phase encephalitis and demyelination. Furthermore, CD4−/− mice, presented with poliomyelitis, bulbar (brainstem) vacuolation of the neuropil, and dorsal root ganglionic inflammation, a finding rarely observed in CD4+/+ mice; iv) a strikingly higher number of CD11b positive microglia/macrophages were present in the CD4−/− mice at the chronic infection phase. These microglia/macrophages were disseminated throughout the inflamed regions of white matter and in the areas of gray matter, both in the brain and spinal cord at the chronic infection phase; and v) Electron microscopy revealed axonal degeneration in the spinal cords of CD4−/− mice even in the absence of
inflammation, suggesting that white matter degeneration occurs secondary to neuronal injury without a direct attack of inflammatory cells upon spinal cord myelin sheaths.

Our results also revealed substantially higher mRNA expression levels of IFNγ, TNFα, and IFN inducible leukocyte chemoattractant CXCL10 in the CD4−/− mice in comparison to CD4+/+ mice which is likely a response to recruit further peripheral lymphocytes into the CNS to combat the persistent viral load in the former. The increased and uncontrolled viral replication contributes to severe inflammation and neuronal cell body damage observed in the gray matter of the spinal cord and dorsal root ganglion of the CD4−/− mice. Elevated mRNA levels of pro-inflammatory cytokine IL-6 at day 5 and 10 p.i. along with persistent viral load signifies a robust pro-inflammatory environment in the CNS of CD4−/− mice. Expression of the anti-inflammatory cytokine IL-10 remains almost constant throughout the study in CD4+/+ mice, while its expression in CD4−/− is consistently low, suggesting that CD4+ T cells serve as one of the key sources of IL-10 production.

Apart from this, it was interesting to note that anti-viral chemokine CCL5 or RANTES (macrophage/monocyte chemoattractant) was downregulated in CD4−/− mice at day 5 and 10 p.i., perhaps as a result of which a significant reduction in CD11b positive inflammatory cells was observed in the brains and spinal cords at the acute phase of inflammation. Nevertheless, overall encephalitis at the acute phase, as shown by CD45 staining, was comparable in both CD4+/+ and CD4−/− mice, suggesting that the initial inflammation in the CNS is independent of CD4++ T cells. This finding also hints that the fewer numbers of CD11b+ cells might be the result of dampened infiltration of monocyte/macrophages in the absence of functional CD4+ T cells and the CD11b+ cells observed in the CNS might correspond to the brain resident microglia.

The function and role of microglia as mediators of homeostasis in the CNS is well established (61). They not only act as custodians of CNS immunity but also protect neurons during development and monitor synaptogenesis (62). However, during pathological conditions, microglia attain a signature pro-inflammatory state that is directed toward the clearance of toxic substances from the CNS (63, 64). M1 or classically activated microglia can also induce the activation of A1 astrocytes, which develop altered ability to promote neuroprotection (65). A2 astrocytes are activated by M2 microglia (alternatively activated) and help in CNS repair and protection (66). Examination of mRNA expression revealed a higher expression of pro-inflammatory M1 markers CD86 and Btk in the CNS of CD4+/+ mice during the acute infection.
CD163 mRNA transcripts increased in expression in the CD4^+/+ mice during day 10 p.i. and then declined with the restoration of homeostasis, but CD4^-/- mice showed a significant increase in the expression of CD163 mRNA even at day 15 p.i. suggesting that the CD11b+ microglia/macrophages present during the transition phase (from acute to chronic) in the CNS might be of the M2 phenotype (anti-inflammatory) attempting to combat the prolonged viral persistence and restore homeostasis to prevent further tissue damage, but fail to do so without the help of CD4^+ T cells. Though M2 microglia/macrophages are categorized as anti-inflammatory, they are also reported to have high phagocytic ability. Their activation and persistence might, therefore, promote direct myelin stripping as previously reported (40) leading to significantly greater demyelination and axonal loss in the CD4^-/- mice.

An interesting question that remains to be answered is if the infiltration of monocyte/macrophages is impeded in the first place, why is there a higher expression of CD11b positive cells in the CNS of CD4^-/- mice during the chronic phase? To answer this, future experiments are aimed at performing immunophenotyping of the inflammatory cells, using flow cytometry in the CD4^-/- mice, to explicitly decipher whether the cells present at the chronic phase are predominately peripherally recruited monocytes/macrophages or the activated resident phagocytic microglial cells of the CNS. So far, flow cytometric analysis in CD4^+/+ mice has shown the presence of a significant population of CD11b hi/ CD45 lo (microglia) at day 30 p.i. but very little or no CD11b expressing CD45 high monocyte/macrophages in the CNS (data not shown). We, therefore, expect the CD11b+ cells found in the CNS at day 30 in the CD4^-/- mice to be microglia and not peripherally recruited myeloid cells.

For this study, we have opted for a mouse strain (B6.129S2-Cd4^tm1Mak/J) where the functionality of CD4^+ T helper cells was disrupted. The development of CD8^+ T cell and myeloid components was unaffected. 90% of the circulating T cells were CD8^+ and their cytotoxic activity was within normal ranges (67). Despite the presence of functional CD8^+ T cells, viral clearance was substantially delayed. Thus, our studies suggest a vast preponderance of CD4^+ T cells over CD8^+ T cells in maintaining the homeostasis upon RSA59 induced neuroinflammation.

In conclusion, our results demonstrate that CD4^+ T cells are necessary for eliminating viral particles, promoting microglial polarization toward anti-inflammation, and controlling chronic progressive axonal degeneration. The current study also highlights the importance of CD4^+T cells beyond the classic inflammatory lesions of the white matter tract. We have shown that
gray matter inflammation in the form of poliomyelitis is significantly exacerbated in the absence of CD4+ T cells. Moreover, we show that the imprinting of the microglia/macrophage-mediated inflammatory innate immune response on the consequent protective adaptive immunity requires functional CD4+ T cells. This communication between microglia and T cells is a highly regulated, interdependent, and bidirectional process and is critical for the establishment of an effective immune response. Although innate anti-viral immune responses by microglia are crucial in controlling the initial CNS viral dissemination, virus-specific T cells are essential to eliminate the virus and provide indispensable neuroprotection. Further studies will be conducted to understand the nexus between CNS resident microglia/monocyte-derived macrophages with infiltrating activated T helper cells at the molecular level through immune-coregulatory CD40-CD40 ligand (L) pathway. This dyad is broadly recognized for its essential role in immune regulation and homeostasis. Our studies will be focused to examine such interactions at the molecular level using CD40 and CD40L deficient mice in the outcome of inflammatory demyelinationMost MS therapies are aimed at preventing damage to myelin by regulating the multiple components of adaptive immune system, especially the T cell subsets (Th1, Th2, Th17, CD8+, NKT, CD4+CD25+ T regulatory cells) and B cells. Current therapies have only been able to reduce the number and rate of MS lesion formation and are only partially efficacious(68). Understanding the role of T cells in a viral induced model of MS is thus critical to design more robust therapeutics. Together these studies can help to expand our knowledge intended to use CD4 mediated immune therapy as a potential treatment of MS, depending on its etiology and the initiation of the pathology.

MATERIALS AND METHODS

Virus, Inoculation of mice and Experimental design

Recombinant isogenic demyelinating (DM) strain of MHV-A59, RSA59, was used to infect mice as formerly described (39). Four to five-week old, MHV-free, CD4+/+/+ C57BL/6 (B6) mice (Jackson Laboratory) and CD4-/- (B6.129S2-Cd4tm1Mak/J) mice (Jackson Laboratory, Stock no. 002663) were used for the study. The CD4-/- mice obtained from Jackson’s laboratory is homozygous for the Cd4tm1Mak targeted mutation, have a significant blockade in the CD4+ T-cell development and show an MHC class II restricted T helper cell activity(67). The mice were inoculated intracranially with 25,000 (50% of LD50) PFU of RSA59 strain as described previously. Likewise, mock-infected controls for CD4+/+ and CD4+-/- mice were inoculated with an uninfected cell lysate (PBS+0.075% BSA) at an equivalent dilution. Mice were
monitored daily post infection (p.i.) for disease signs and symptoms. Clinical disease severity was graded using the following scale: 0, no disease symptoms; 1, ruffled fur; 1.5, hunched back with mild ataxia; 2, Ataxia, balance problem and hind limb weakness: 2.5 one leg completely paralyzed, motility issue but still able to move around with difficulties; 3, severe hunching/wasting/both hind limb paralysis and mobility is severely compromised; 3.5 Severe distress, complete paralysis and moribund 4, dead (47).

For EM, histopathological, and immunohistochemical analyses mice were sacrificed at the acute infection phase, i.e. on day 5-6 (four mice per group), and chronic infection phase, i.e. day 30 (five mice per group) post-infection. For RNA and protein studies and viral titer estimation, animals were sacrificed (3 mice per group) on days 5, 10 and 15 post-infection.

**Estimation of Viral Replication**

Mice were euthanized on days 5, 10 and 15 post-infection and perfused transcardially with 20 ml of sterile PBS. Brains were harvested for determination of viral titers and placed into 1 ml of isotonic saline containing 0.167% gelatin (gel saline). Brain tissues were weighed and kept frozen at -80°C until titred. Tissues were subsequently homogenized, and viral titers were quantified by standard plaque assay protocol on tight monolayers of L2 cells as described previously with minor modifications (39).

**Histopathology and Immunohistochemical Analysis**

Mice were sacrificed at day 6 and day 30 post-infection. Following transcardial perfusion with PBS and 4% paraformaldehyde, liver, brain, and spinal cord tissues were harvested and embedded in paraffin. Five-micron thick sections of the embedded tissues were prepared and stained with Hematoxylin and Eosin for histopathologic analysis. Luxol fast blue (LFB) staining was performed to evaluate demyelination in the brain and spinal cord tissues, as described previously with minor modifications (40).

Immunohistochemical staining of brain and spinal cord tissue sections used the following primary antibodies - 1:10000 dilution of anti-CD11b (Abcam, Cat #:ab133357), 1:200 dilution of anti-CD45 (LCA; leukocyte common antigen, BD Pharmingen Cat#:550539) and 1:40 dilution of monoclonal antibody directed against the nucleocapsid protein (N) of MHV (monoclonal antibody clone 1-16-1 provided by Julian Leibowitz, Texas A&M University). Bound primary antibodies were detected by an avidin-biotin immunoperoxidase technique.
(Vector Laboratories) using 3, 3-diaminobenzidine as the substrate. Control slides from mock-infected mice were stained in parallel. All slides were coded and read in a blinded manner by the same investigator, as described previously with minor modifications (40).

H&E Sections were assessed for inflammation in the following manner: 0, none; 1, few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing and formation of microglial nodules, and represented in Table 1 (47).

Quantification of histopathological sections

The number of hepatic lesions were counted per section and averaged for each mouse at each time point (both acute and chronic phase of inflammation). The functional scoring of the inflammatory lesions in the liver was characterized as the Hepatic activity index (HAI). Degree of activity was categorized as portal inflammation; Interphase hepatitis (Piecemeal necrosis); Focal (Spotty) necrosis, apoptosis, and focal inflammation; and Confluent necrosis. Scores from 0-6 were allotted for each category based on the modified Knodell’s HAI, commonly referred to as the Ishak system (69).

Image analysis was performed using the basic densitometric thresholding application of Fiji (Image J, NIH Image, Scion Image) as described previously (48). Briefly, image analysis for CD45 and CD11b stained sections was performed by capturing the images at the highest magnification (4X-for brain, 10X-for spinal cord) such that the entire section (i.e., scan area) can be visualized within a single frame. The RGB image was deconvoluted into three different colours to separate and subtract the DAB-specific staining from the background H&E staining. The perimeter of each brain and spinal cord tissue was digitally outlined, and the area was calculated in µm². A threshold value was fixed for each image to make sure that all antibody marked cells are taken into consideration. The amount of CD45, and CD11b staining was termed as the ‘% area of staining’.

To determine the area of demyelination, LFB-stained spinal cord cross-sections from each mouse were chosen and analyzed using Fiji software (Image J 1.52g). The total perimeter of the white matter regions in each cross-section was marked and calculated by adding together the dorsal, ventral and anterior white matter areas in each section. Also, the total area of the demyelinated regions was outlined and collated for each section separately. The percentage of spinal cord demyelination per section per mouse was calculated.
Gene Expression: RNA Isolation, Reverse transcription and quantitative Polymerase chain reaction

RNA was extracted from brain tissues (flash-frozen) of RSA59 infected CD4\(^{+/+}\) and CD4\(^{-/-}\) and mock-infected mice (3 from each group at days 5, 10 and 15 p.i.) using the Trizol isolation protocol following trancardial perfusion with DEPC treated PBS. The total RNA concentration was measured using a NanoDrop ND-100 spectrophotometer. 1\(\mu\)g of RNA was used to prepare cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative Real-time PCR analysis was performed using DyNAmo Color Flash SYBR Green qPCR kit (Thermo Scientific) in a Step One plus Real-time PCR system (Thermo Fisher Scientific) under the following conditions: initial denaturation at 95°C for 7 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, melting curve analysis at 60°C for 30 s. Reactions were performed in triplicate. Sequences for the primers used are given in Table III. Relative quantitation was achieved using the comparative threshold (\(\Delta\Delta Ct\)) method. mRNA expression levels of target genes in RSA59 infected CD4\(^{+/+}\) and CD4\(^{-/-}\) mice were normalized with \(\beta\)-Actin and expressed as relative fold change compared to their respective mock-infected controls.

Ultra-structural studies and electron microscopy to characterize the preservation of myelin and axons

To characterize axonal blebbing, disruption of the myelin sheath, and axon-myelin coherence, ultrastructural studies were carried out on the brains, brainstem and spinal cords of mice. Infected CD4\(^{-/-}\), CD4\(^{+/+}\) and CD4\(^{-/-}\) mock-infected mice were anesthetized and sacrificed at day 28 p.i. Mice were perfused with 4% PFA. Brains and spinal cords were harvested and fixed overnight in 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, and dehydrated in a graded series of ethanol washes. For transmission electron microscopy, samples were flat embedded in Poly-Bed 812 epoxy resin (Polysciences) and sectioned (500 nm) from the lesional epicenter. Toluidine blue staining was performed for examination by light microscopy. Ultrathin TEM sections (600 Å) were trimmed from the representative foci of interest from toluidine blue-stained sections and mounted on 200 mesh copper grids, stained with uranyl acetate and bismuth subnitrite, and viewed under a JEOL JEM 1010 electron microscope (40).

Statistical Analyses

The viral titer was calculated as plaque-forming units (PFU) based on the following formula = (no. of plaques X dilution factor/ml/gram of tissue). Virus titer was expressed as log\(_{10}\)
PFU/gram of tissue. Quantitative RT-PCR data were presented as mean values ± SEM. Values were subjected to Two-Way ANOVA/Student’s t-test analysis for calculating the significance of differences between the means. Also, multiple comparisons were achieved by the Tukey test and the Holm-Sidak test. All statistical analyses were done using GraphPad Prism 6 (La Jolla, CA). A P-value of <0.05 was considered statistically significant.

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DECLARATIONS

Ethics approval

All experimental procedures and animal care and use were strictly regulated and reviewed in accordance with good animal ethics approved by the Institutional Animal Care and Use Committee at the Indian Institute of Science Education and Research Kolkata (AUP No. IISERK/IAEC/AP/2017/15) and the University of Pennsylvania, Philadelphia, USA (IACUC Protocol No. 804701). Experiments were performed following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and the United States National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals, 8th Edition.

Consent for publication

Not Applicable

Availability of data and materials

The datasets used and/or analyzed during the current study will be made available from the corresponding author on reasonable request.
**Author Contribution**

DC, FD and JDS designed and planned all the experiments. DC, and FS performed the experiments. DC, FS, and JDS analyzed the data and wrote the manuscript. AB, SK, RK and KD helped with the standardization of RNA experiments in the knockout mice. LK blindly read the pathological samples. JDS, KS and LK participated in data analysis and data interpretation. LK and KS were involved in critical revisions of the manuscript. JDS and KS jointly supervised and reviewed this work.

**Competing interests**

The authors declare that they have no competing interests.

**REFERENCES**


**FIGURE LEGENDS**

**Fig. 1. Temporal immune cell kinetics in the brains of RSA59 infected mice.** Results from flow cytometric analysis of the migration of inflammatory cells and CNS resident cells from the RSA59 infected mice has been summarized in the schematic diagram. The diagram represents the differential infiltration of total myeloid (neutrophils, macrophage/monocytes and microglia) and lymphoid (CD4, CD8 and NKT) cell populations during days 3, 5, 7, 10, 16 and 30 p.i. Respective peaks show highest infiltration at times post infection. ScaleArbitrary.

**Fig. 2. Absence of CD4 causes no significant pathology in the mock infected mice.** CD4+/+ and CD4-/mice were infected with with an uninfected cell lysate (PBS+0.075% BSA). 5-micron thick liver,brain and spinal cord sections were stained with H&E and CD45 for routine histopathological studies. No inflammation was observed in CD4+/+ and CD4-/ mice tissues. Data are represented from 3 independent biological replicates. Scale bars= 50 microns.

**Fig.3 Absence of CD4 shows no significant alterations in RSA59 induced Liver pathology.** CD4+/+ and CD4-/ mice were infected with RSA59 and 5-micron thick liver sections were stained with H&E for routine morphological studies. a) Both CD4+/+ and CD4-/ mice showed similar features of necrotic/non-necrotic hepatitis. Hepatic Activity Index was calculated according to Ishak’s Score as described in materials and methods and plotted (b). c) The average number of hepatic lesions were counted per section from each mouse, combined results were tabulated. Liver sizes and cross-sectional areas were comparable in both CD4+/+ and CD4-/ mice. Data are represented from 5 independent biological experiments.

**Fig.4 Absence of CD4 demonstrates no significant changes in encephalomyelitis upon RSA59 induced acute infection.** a, b) At day 6 p.i., sections of brain (panel a) and spinal cord...
(panel b) from CD4 $$^{+/+}$$ mice and CD4 $$^{-/-}$$ mice were stained with H&E and immunohistochemically for LCA (leucocyte common antigen). Boxed areas are shown at higher magnification below the corresponding brain midsagittal sections (panel a) or cross sections of spinal cord (panel b). The arrows in the zoomed sections mark characteristic perivascular cuffing and microglial nodule formation mediated by infiltrating inflammatory cells in the H&E-stained sections which correspond to immunoreactive leukocytes and microglia/macrophages in the CD45 immunohistochemically stained sections. Scale bars of midsagittal brain sections represent 1000 microns whereas they represent 100 microns in the higher magnification images shown below. Scale bars of spinal cord cross sections represent 200 microns whereas they represent 50 microns in the higher magnification images shown below. Quantification of the intensity of staining is plotted in a scatter diagram. Statistical analysis was performed using Student’s t-test and Welch correction. Data are represented from 5 independent biological experiments.

**Fig.5 Absence of CD4 resulted in a significant reduction of CD11b positive microglia/macrophages in the brain and spinal cords during RSA59 induced acute infection.** At day 6 p.i., sections of brain and spinal cord from CD4 $$^{+/+}$$ mice and CD4 $$^{-/-}$$ mice were immunohistochemically stained for CD11b (macrophage/microglia activation marker). Boxed areas are shown at higher magnification below the corresponding brain midsagittal sections (upper panel) or cross sections of spinal cord (lower panel). Arrows mark microglia/macrophages in the in the CD11b immunohistochemcally stained sections. Scale bars of midsagittal brain sections represent 500 microns whereas they represent 200 microns in the higher magnification images shown below. Scale bars of spinal cord cross sections represent 500 microns whereas they represent 200 microns in the higher magnification images shown below. Quantification of the intensity of staining is plotted in a scatter diagram. Statistical analysis was performed using Student’s t-test and Welch correction. ***p<0.001; ****p<0.0001. Data are represented from 5 independent biological experiments. Error bar represents SEM.

**Fig.6 Absence of CD4 results in severe poliomyelitis and dorsal root ganglionic inflammation during acute RSA59 infection.** Sections of CD4 $$^{+/+}$$ and CD4 $$^{-/-}$$ mouse spinal cords were stained with H&E and immunohistochemically with anti-CD45. There is increased inflammation of the gray matter (poliomyelitis) and dorsal root ganglia in CD4 $$^{-/-}$$ as compared
to CD4+/+ mice. Mock-infected mice showed no inflammation. All scale bars indicate 90µm. N=5.

**Fig.7 Absence of CD4 results in reduced viral clearance and altered expression of antiviral effector genes.** a) Whole-brain lysates from CD4+/+ and CD4−/− mice at days 5, 10 and 15 p.i. were subjected to comparative viral plaque assays on confluent monolayers of L2 cells. Each time point represents the mean titer for three mice. Titers are expressed as log10 PFU per gram of tissue. b) The relative abundance of transcripts corresponding to viral N-gene was compared using qRT-PCR in the CD4+/+ and CD4−/− infected mouse brains at days 5, 10 and 15 p.i. c) Anti-N immunohistochemistry revealed the differential in-situ distribution of viral antigen in the representative anatomical regions (between brain stem and deep cerebellar white matter) of mock-infected CD4+/+; and RSA59 infected CD4+/+ and CD4−/− mice at days 6 and 30 p.i.. Scale bar 100µm (4X) and 50µm (40X). Relative gene expression of IFN γ (d), CXCL10 (e), TNFα (f) and CCL5 (g) at days 5, 10 and 15 p.i. were analyzed by qRT-PCR and compared between CD4+/+ and CD4−/− mice. qRT-PCR results were expressed as fold mean ± SEM. Statistical analysis of the data represented in panels a, b, d, e, f, and g) was calculated using Two-way ANOVA, and multiple comparison was achieved by Hom’s Sidak; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Data are represented from 3 independent biological experiments, N=3, 3 technical replicates each.

**Fig.8 Absence of CD4 leads to severe chronic inflammatory demyelination and axonal loss.** a) Cross-sections of CD4+/+ and CD4−/− mouse spinal cords were analyzed for the presence of inflammatory lesions by H&E, demyelination by LFB, and inflammatory cells by anti-CD45 and anti-CD11b (microglia/macrophages) immunohistochemistry. Boxed areas are shown at higher magnification to right of the corresponding spinal cord cross sections. Arrows mark demyelinating plaques on the LFB stained sections, infiltrating inflammatory cells in the H&E stained sections, and immunoreactive leukocytes and microglia/macrophages in the CD45 and CD11b immunohistochemically stained sections respectively. Scale bars of spinal cord cross sections represent 200 microns whereas they represent 50 microns in the higher magnification images. Levels of demyelination and inflammation are plotted in a scatter diagram (b, c, d). Statistical significance was calculated by unpaired Student’s t-test and Welch correction. ***p< 0.001; ****p<0.0001). Data are represented from 4-5 independent biological experiments. Error bar represents SEM.
Fig. 9 Absence of CD4 causes abnormal bulbar (brainstem) vacuolation and neuronal loss in RSA59 infected brains at day 30 p.i. a) Serial sagittal sections of brains from both CD4+/+ and CD4−/− mice were analyzed for inflammation at day 30 p.i. by H&E and immunohistochemically by CD45 and CD11b. Boxed areas are shown at higher magnification below the corresponding brain midsagittal sections. Arrows mark infiltrating inflammatory cells in the H&E stained sections, and immunoreactive leukocytes and microglia/macrophages in the CD45 and CD11b immunohistochemically stained sections respectively. Scale bars of spinal cord cross sections represent 200 microns whereas they represent 50 microns in the higher magnification images. Quantification of inflammation was performed for CD45 (b) and CD11b (c) staining. The level of significance was calculated by unpaired Student’s t-test and Welch correction, **(**p<0.0001). Error bar represents SEM. Data are represented from 4-5 independent biological experiments.

Fig. 10. Absence of CD4 causes no alteration in the axon-myelin coherence in the brain stem and spinal cord of mock infected mice. Toluidine blue stained sections of glutaraldehyde fixed, epoxy resin embedded sections (500 nm thick) from mock infected CD4−/− mouse brain stem (A, B) and spinal cord (C, D). A. Gray and white matter of brain stem. Arrows mark bundles of intact myelinated fibres. Asterisks mark the neuronal nuclei. Original magnification – 400X. B. Brain stem white matter. Original magnification - 1000X. C. Spinal cord cross section. Original magnification 40X. Posterior columns in boxed area are further magnified in D. Original magnification 1000X. White and gray matter in both the brain stem and spinal cord show no evidence of inflammation, demyelination or cellular injury.

Fig. 11 Absence of CD4 results in severe spinal cord axonal injury upon RSA59 infection at the chronic stage. a and b: Toluidine blue-stained sections. a) Posterior columns (200X) and b) (1000X) demonstrate clusters of degenerating axons. Arrows - degenerating axons, asterixes - swollen myelin sheaths. c and d: Corresponding electron microscopy. c) Swollen myelin sheaths with loss of axoplasm (7,500X). d) Cluster of degenerating axons with collapsed myelin sheaths (10,000X). The arrow shows the corresponding area on the toluidine blue-stained section. Scale bar represents 2 microns. Data are represented from 3 independent biological experiments.

Fig. 12 qRT-PCR analysis of IL-6, IL-10, CD86, Btk and CD163 reveals an inflammatory state in the brains of RSA59 infected CD4−/− mice. Quantitative PCR analysis of IL-6 (a) IL-10 (b); CD86 (c); Btk (d); CD163 (e) analysis was performed in RSA59 infected CD4+/+ and
CD4+/− mouse brains at days 5, 10 and 15. Data are represented from 3 independent biological experiments, N=3, 3 technical replicates each. Statistical analysis was performed by Two-way ANOVA, Hom Sidak’s multiple comparison test; **p<0.01; ***p<0.001; ****p<0.0001. Error bar represents SEM.

**Table I. Average Inflammation score in RSA59-infected CD4+/+ and CD4+/− mouse brain**

<table>
<thead>
<tr>
<th></th>
<th>No. of mice</th>
<th>No. of sections</th>
<th>% mice with inflammation</th>
<th>Mean score of Inflammation (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute phase RSA59 CD4+/+</td>
<td>4</td>
<td>08</td>
<td>100%</td>
<td>1.8±0.322</td>
</tr>
<tr>
<td>Chronic phase RSA59 CD4+/+</td>
<td>5</td>
<td>10</td>
<td>100%</td>
<td>1.4±0.163</td>
</tr>
<tr>
<td>Acute phase RSA59 CD4−/−</td>
<td>4</td>
<td>08</td>
<td>100%</td>
<td>2.0±0.189</td>
</tr>
<tr>
<td>Chronic Phase RSA59 CD4−/−</td>
<td>4</td>
<td>10</td>
<td>100%</td>
<td>2.5±0.189</td>
</tr>
</tbody>
</table>

Significant difference was observed only at the chronic phase of infection between CD4+/+ and CD4−/− brains (**p=0.0004)**

**Table II. Average Inflammation score in RSA59-infected CD4+/+ and CD4+/− mouse spinal cord**

<table>
<thead>
<tr>
<th></th>
<th>No. of mice</th>
<th>No. of sections</th>
<th>% mice with inflammation</th>
<th>Mean score of Inflammation (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute phase RSA59 CD4+/+</td>
<td>3*</td>
<td>20</td>
<td>80%</td>
<td>0.6±0.112</td>
</tr>
<tr>
<td>Chronic phase RSA59 CD4+/+</td>
<td>5</td>
<td>30</td>
<td>100%</td>
<td>0.76±0.114</td>
</tr>
<tr>
<td>Acute phase RSA59 CD4−/−</td>
<td>4</td>
<td>20</td>
<td>100%</td>
<td>1.1±0.143</td>
</tr>
<tr>
<td>Chronic Phase RSA59 CD4−/−</td>
<td>4</td>
<td>20</td>
<td>100%</td>
<td>1.05±0.05</td>
</tr>
</tbody>
</table>

*1 mouse was an outlier; it did not get infected.

**Table III. List of Primers**
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
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<tbody>
<tr>
<td>CD86</td>
<td>GGCTCAAAACATAAGCCTGA</td>
<td>CCCATGTCTTTGATCTGAAC</td>
</tr>
<tr>
<td>CD163</td>
<td>GAGACACACGGAGCCATCAA</td>
<td>TGGACAAACCTTTTACAACCA GG</td>
</tr>
<tr>
<td>IFN γ</td>
<td>GTCTCTTTCTTTGATATCTGGAG GAACT</td>
<td>GTAGTAATCAGGTGTGATTCA ATGACGC</td>
</tr>
<tr>
<td>TNF α</td>
<td>CTGTAGCCACCACGTCGTAGC</td>
<td>TTGAGATCCATGCCGTTG</td>
</tr>
<tr>
<td>CCl5</td>
<td>CCA ATC TTG CAG TCG TGT TTG T</td>
<td>CAT CTC CAA ATA GTT GAT GTA TTC TTG AAC</td>
</tr>
<tr>
<td>CXCL10</td>
<td>GACGGTGCCGCTGCAACTG</td>
<td>CTTCCTATGGCCCTCATTCT</td>
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<td>Btk1</td>
<td>ACAGCAGAACACATTGCTCA</td>
<td>GGGAACTCCTCAGGAACAT</td>
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<td>IL12 p40</td>
<td>GGAAGCAGGGCAGCAAGATA</td>
<td>AACTTGAGGGAGAATGGAA TGG</td>
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<tr>
<td>IL6</td>
<td>AGTTGCCTTTCTGGGACTGA</td>
<td>TCCACGATTTCACAGGAAC</td>
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<tr>
<td>IL10</td>
<td>AGTGAGCAGTGGAAGAGTG</td>
<td>TTCGGGAGAGGTACAAACG</td>
</tr>
<tr>
<td>Anti -N</td>
<td>AGGATAGAAGTCTGTTGGCTCA</td>
<td>GAGAGAAGTTAGCAAGGTCC TCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCCCCTCTGCGATGC</td>
<td>CTTCCAGAGGGGCCCCATCC</td>
</tr>
<tr>
<td>B- Actin</td>
<td>CTCTCAAATGAGCTGCTGTG</td>
<td>GGTCTCAAACATGATCTGG</td>
</tr>
</tbody>
</table>
Chakravarthy et al., Fig. 1

Day 3  Day 5  Day 7  Day 10  Day 16  Day 30

**LYMPHOID**
- MCT cells
- CD8+ T cells
- CD4+ T cells
- Memory B cells
- Mature B cells

**MYELOID**
- Macrophages
- Megakaryocytes
- Neutrophils
Chakravarty et al., Fig 3

(a) CD4+/+ CD4/−

Day 6 p.i.

Day 30 p.i.

(b) Average Hepatic Activity Index

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>No. of sections</th>
<th>Avg No of Hepatic lesions per section</th>
<th>Mean Hepatic Activity Index (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute phase RSA51 CD4+/+</td>
<td>4</td>
<td>15</td>
<td>16.7</td>
</tr>
<tr>
<td>Acute phase RSA51 CD4/−</td>
<td>4</td>
<td>15</td>
<td>22.8</td>
</tr>
<tr>
<td>Chronic phase RSA51 CD4+/+</td>
<td>4</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Chronic phase RSA51 CD4/−</td>
<td>4</td>
<td>19</td>
<td>11.4</td>
</tr>
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</table>
Chakravarty et al., Fig. 4

**a**

CD4+/+

CD4/−

H & E staining.

CD45/LCA

**b**

CD4+/+

CD4/−

H & E staining.

CD45/LCA
Chakravarty et al., Fig. 7

(a) Log10 PFU/g liver tissue.

(b) Relative IL-17 mRNA expression in the liver.

(c) Histological images showing inflammation.

(d) IFN gamma expression in the brain.

(e) CXCL10 expression in the brain.

(f) TNF alpha expression in the brain.

(g) CCL5 expression in the brain.
Chakravarty et al., Fig. 9

```
a
CD4+/+

H & E staining.

CD45/LCA

CD11b

b
CD45 staining in Brain

% Area

RSAS9 infected CD4+/-
RSAS9 infected CD4+/

nS

```

```
c
CD11b staining in Brain

% Area

RSAS9 infected CD4+/-
RSAS9 infected CD4+/

****

```
Chakravarty et al., Fig. 12

a. IL6 expression in Brain

b. IL10 expression in Brain

c. CD86 expression in Brain

d. Btk expression in Brain

e. CD163 expression in Brain