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CXCL10 and trafficking of virus-specific T cells during coronavirus-induced demyelination

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Abstract

Chronic expression of CXC chemokine ligand 10 (CXCL10) in the central nervous system (CNS) following infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) is associated with an immune-mediated demyelinating disease. Treatment of mice with anti-CXCL10 neutralizing antibody results in limited CD4⁺ T cell infiltration into the CNS accompanied by a reduction in white matter damage. The current study determines the antigen-specificity of the T lymphocytes present during chronic disease and evaluates how blocking CXCL10 signaling affects retention of virus-specific T cells within the CNS. CXCL10 neutralization selectively reduced accumulation and/or retention of virus-specific CD4⁺ T cells, yet exhibited limited effect on virus-specific CD8⁺ T cells. The response of CXCL10 neutralization on virus-specific T cell subsets is not due to differential expression of the CXCL10 receptor CXCR3 on T cells as there was no appreciable difference in receptor expression on virus-specific T cells during either acute or chronic disease. These findings emphasize the importance of virus-specific CD4⁺ T cells in amplifying demyelination in JHMV-infected mice. In addition, differential signals are required for trafficking and retention of virus-specific CD4⁺ and CD8⁺ T cells during chronic demyelination in JHMV-infected mice.

Keywords

virus; central nervous system; T cells; demyelination

Introduction

The host response to viral infection is a coordinated process employing numerous participants with diverse roles in defense with the ultimate goal of generating virus-specific T cells to control and eliminating the invading pathogen from infected tissues. Chemokines and chemokine receptors are involved in the generation, activation and recruitment of virus-specific T cells following injury, microbial infection or during autoimmune diseases [1–3]. Accumulating data demonstrate that chemokines play a crucial role in promoting inflammation in tissues originally considered immunologically inert, such as the CNS, resulting in either protection and/or pathology [4–10]. Studies utilizing the neurotropic JHM strain of mouse hepatitis virus (JHMV) model of virus-induced neurologic disease have elucidated the contributions of CXCL10 to host defense and disease progression and have highlighted its importance in regulating T cell trafficking [8,9,11].

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JHMV is an enveloped, positive-sense RNA virus belonging to the *Coronaviridae* family [12, 13]. The immune response to JHMV following intracerebral (i.c.) inoculation of susceptible mice involves CNS infiltration of NK cells, macrophages and T lymphocytes [14]. Both CD4⁺ and CD8⁺ T cells are required for viral clearance from the infected brain and infiltrating T lymphocytes recognize specific viral epitopes residing within structural proteins [15–17]. Following infection of C57BL/6 mice, the immunodominant CD4⁺ epitope is found at residues 133–147 of the membrane (M) protein (M133–147) and additional CD4⁺ T cell epitopes are found in the spike (S) surface glycoprotein (S328–347 and S358–372) [15,17]. Two defined CD8⁺ T cell epitopes have been identified and reside within the S protein. These epitopes include the immunodominant S510–518 as well as the S598–605 [15,16]. Infectious virus is reduced below detectable levels within the CNS through the release IFN- γ and perforin by infiltrating T cells, but sterile immunity is not achieved [18–20]. Viral RNA and antigen persists in white matter tracts of mice that survive the acute infection and these mice develop a chronic demyelinating disease characterized by mononuclear cell infiltration and myelin destruction that is similar to the pathology associated with the human demyelinating disease multiple sclerosis (MS) [18].

Studies have indicated that T lymphocytes are important contributors to demyelination in JHMV-infected mice and data support that CXCL10 expression within the CNS exacerbates inflammation by attracting T cells [9,21,22]. When CXCL10-specific neutralizing antiserum was administered to mice with established demyelination, reduced pathology and improved neurological function were observed [9]. Concomitant with reduced pathology, was a significant reduction in CD4⁺ T cell invasion of the CNS. Anti-CXCL10 treatment, however, did not significantly hinder the ability of total CD8⁺ T cells to accumulate within the CNS [9]. These data suggest that CXCL10 can exert a selective effect on T cell subsets regulating CNS infiltration and disease progression. The present study examines the viral specificity of both CD4⁺ and CD8⁺ T cells infiltrating the CNS and investigates the effects anti-CXCL10 treatment has on trafficking on these different populations of antigen-specific T cells.

Materials and Methods

Virus and mice

Age-matched (5–7 wk old) male C57BL/6 (H-2^b background) were used for all experiments (National Cancer Institute, Bethesda, MD). Following anesthetization by intraperitoneal (i.p.) injection with ketamine, mice were injected intracranially (i.c.) with 1000 PFU of JHMV (strain V2.2-1) suspended in 30 μ l of sterile saline [9,21]. Animals were sacrificed at defined time points and tissues were removed for analysis in studies described below [21].

Antisera preparation and treatment of mice

The generation of rabbit polyclonal antisera specific for mouse CXCL10 has previously been described [8]. This reagent effectively neutralizes activity *in vivo* and does not cross-react with other known chemokines [8,23]. Experimental groups of mice were injected i.p. with 0.5 ml anti-CXCL10 or NRS on days 12, 14, 16, and 19 and sacrificed at days 12, 15, 21 and 28 p.i. Previous studies have shown that increasing amounts of anti-CXCL10 (>0.5 ml) does not result in improved clinical or histologic disease [9]. A control group was treated with 0.5 ml of normal rabbit serum (NRS).

Mononuclear cell isolation and flow cytometry

Cells were obtained from brains, spinal cords, and spleens of infected mice at defined time points and a single-cell suspension was obtained using a previously described protocol [21]. Fluorescence-conjugated rat anti-mouse CD4 and CD8 antibodies were used to detect infiltrating CD4⁺ and CD8⁺ T cells (BD Biosciences, San Diego, CA). Additionally,

polyclonal rabbit anti-mouse CXCR3 was used for primary detection of CXCR3 and FITC-conjugated goat anti-rabbit antibody was used as secondary antibody for detection (Zymed Laboratories, San Francisco, CA) [24]. In all cases, isotype-matched fluorescently conjugated antibodies were used for controls. Cells were incubated with antibodies for 20 to 60 min at 4°C, washed and analyzed using a FACStar flow cytometer (Becton Dickinson, Mountain View, CA). Data are presented as the percentage of positive cells within the gated population.

Peptide Stimulation and Intracellular cytokine staining

Cells isolated from brains, spinal cords and spleens were stimulated with 5 µM of MHV peptides corresponding to specific viral T cell epitopes. The CD4+ peptides used were 133–147 of the membrane protein (M133–147), as well as 328–347 and 358–372 within the spike protein (S328–347 and S358–372) [15,17]. The CD8+ peptides used were 510–518 and 598–605 within the spike protein (S510–518 and S598–605) [15,16]. Intracellular staining for IFN-γ was performed on peptide-stimulated cells by using a previously described procedure [22, 25]. In brief, following a 6-hour incubation with aforementioned peptides at 37°C in a medium containing GolgiStop (Cytofix/Cytoperm kit; BD Biosciences), cells were washed and blocked with phosphate-buffered saline containing 10% fetal bovine serum and a 1:200 dilution of CD16/32 (BD Biosciences). Cells were then stained for surface antigens as described above. Cells were then fixed and permeabilized by using the Cytofix/Cytoperm kit and stained for intracellular IFN-γ by using phycoerythrin (PE)-conjugated anti-IFN-γ (1:50; XMG1.2; BD Biosciences) for 20 to 60 min at 4°C. Cells were analyzed on a FACStar. Data are presented as the percentage of positive cells within the gated population multiplied by the total cells isolated from the organ of interest.

Results and Discussion

CXCR3 expression on virus-specific T cells

CXCL10 binds and signals through its receptor CXCR3 and this subsequently promotes migration of CXCR3-expressing cells to sites of CXCL10 expression [26,27]. We previously determined that administration of an anti-CXCL10 blocking antibody to mice persistently infected with JHMV resulted in diminished CD4+ T cell infiltration into the CNS yet such treatment did not reduce overall numbers of CD8+ T cells [9]. These findings suggested the possibility of differential expression of CXCR3 on T cells during chronic JHMV-induced demyelination. To address this possibility, C57BL/6 mice were i.c. infected with virus and CXCR3 expression on T cells within the brain was assessed by flow cytometry at days 7, 12, and 21 p.i. Antigen specificity was defined by IFN-γ production in response to peptide stimulation. Data shown in Figure 1 indicate that ~90% of CD4+ T cells recognizing the immunodominant M133–147 epitope were CXCR3-positive at each time point analyzed. Similarly, 90% of CD8+ recognizing the S510–518 epitope present within the CNS at days 7 and 12 p.i. expressed CXCR3 and declined slightly to ~80% by 21 days p.i. (Figure 1). Finally, greater than 90% of CD8+ T cells recognizing the S598–605 epitope were CXCR3-positive at all time times examined (Figure 1). Therefore, these data indicate that CXCR3 is expressed on the vast majority of inflammatory virus-specific CD4+ and CD8+ T cells within the CNS of JHMV-infected mice during both acute and chronic disease. Further, there is not a dramatic reduction in the frequency of CXCR3-positive virus-specific cells between days 12 and 21 p.i.

Virus-specific T cell trafficking into the CNS is reduced following anti-CXCL10 treatment

We next determined if treatment of JHMV-infected mice with anti-CXCL10 antibody during chronic disease affected the frequency of virus-specific T cells within the CNS. C57BL/6 mice were i.c. infected with JHMV and treated with either anti-CXCL10 or NRS on days 12, 14, 16 and 19 post-infection (p.i.) and the effects of CXCL10 neutralization on CNS accumulation of CD4+ and CD8+ T cells specific for defined viral antigens was evaluated throughout the course

of chronic infection. Anti-CXCL10 treatment resulted in a reduction of total CD4⁺ and CD8⁺ T cells (90% and 71%, respectively) within the brain at 15 days p.i. (Figure 2A). By 21 days p.i., CD4⁺ T cells within the brain were greatly reduced (73%), but antibody treatment had very little effect on total CD8⁺ T cell accrual (17%) which is consistent with previously reported data (Figure 2A) [9]. CXCL10 neutralization resulted in greater than 90% reductions in M133–147- and S358–372-virus-specific CD4⁺ T cells within the brain at 15 days p.i. compared to NRS treatment (Figure 2B). Additionally, at day 15 p.i., CD4⁺ T cells specific for S328–347 were reduced by 70% following anti-CXCL10 treatment (Figure 2B). CD4⁺ T cells specific for M133–147, S328–347 and S358–372 were decreased by at least 50% at 21 days p.i. as a result of CXCL10 neutralization (Figure 2B). Analysis of CD8⁺ T cells specific for the immunodominant epitope S510–518 in the brains of mice treated with anti-CXCL10 revealed a reduction at day 15 p.i., but a 2-fold increase in numbers of CD8⁺ T cells specific for this epitope in anti-CXCL10 treated mice at 21 days p.i. (Figure 2C). In contrast, numbers of S598-virus-specific CD8⁺ T cells were decreased by 86% and 63% at days 15 and 21 p.i., respectively, in the brain after CXCL10 neutralization (Figure 2C).

Infiltration of total and virus-specific T cells into the spinal cord of MHV-infected mice following anti-CXCL10 and control treatment was also determined. Evaluation of total T cell accumulation within the spinal cord revealed that, similar to the brain, anti-CXCL10 treatment results in a dramatic reduction in both CD4⁺ and CD8⁺ T cell subsets (81% and 89%, respectively) at 15 days p.i. (Figure 3A). However, at 21 days p.i., the reduction in total CD4⁺ T cells within the spinal cord as a result of CXCL10 neutralization is more pronounced than that of total CD8⁺ T cells (CD4, 90% reduction; CD8, 66% reduction) (Figure 3A). Anti-CXCL10 treatment resulted in reduced infiltration of both M133–147 and S328–347 virus specific CD4⁺ T cells in the spinal cords at both days 15 and 21 p.i. (Figure 3B). CD4⁺ T cells specific for the S358–372 epitope in the spinal cords of mice treated with anti-CXCL10 were greatly reduced (67%) at 15 days p.i. compared to NRS treated animals, but by 21 days p.i., only a moderate reduction (36%) was observed (Figure 3B). Anti-CXCL10 treatment had very little effect on accumulation of CD8⁺ T cells specific for S510–518 or S598–605 epitopes in the spinal cord at day 15 p.i. (Figure 3C). However, at 21 days p.i., there was a slight increase in the numbers of S510-specific CD8⁺ T cells (35%) and a notable reduction in S598-specific CD4⁺ T cells (69%) in the spinal cords of anti-CXCL10 treated mice similar to what was observed in the brain (Figure 3C). Collectively, these data demonstrate that antibody-mediated blocking of CXCL10 exerts a greater effect on the accumulation of both total and virus-specific CD4⁺ T cells compared to CD8⁺ T cells during the chronic stage of disease. Upon removal of anti-CXCL10 treatment, inflammatory CD4⁺ T lymphocytes migrate back into the CNS and participate in myelin destruction [9]. Therefore, we next determined if the return of demyelination following removal of anti-CXCL10 treatment coincided with the presence of virus-specific T cells. Infected mice were treated with anti-CXCL10 antisera from days 12 to 21 p.i. at which point antibody treatment was stopped and the presence of virus-specific T cells within the CNS determined at day 28 p.i. There were no differences in numbers of total T cells (Figure 4A) or virus-specific CD4⁺ and CD8⁺ T cells (Figure 4B) at day 28 p.i. between the groups treated with either anti-CXCL10 or NRS.

The accumulation of T lymphocytes within the CNS is a key event contributing to demyelination in mice persistently infected with MHV [21,22]. Chemokines play a role in this process by establishing a gradient originating within the CNS that subsequently attracts distinct cell populations [7–9,21,25,28–31]. The current study has further defined the viral specificity of T cells infiltrating the CNS during chronic JHMV infection and evaluated the effects CXCL10 neutralization has on individual virus-specific T cell populations. Consistent with earlier studies, the data clearly indicate that during persistent JHMV infection, anti-CXCL10 treatment selectively inhibits CNS accumulation of total CD4⁺ T cells, but not total CD8⁺ T cells [9]. Moreover, these findings also support and extend earlier studies indicating that

CXCL10 expression within the CNS of JHMV-infected mice functions to attract T cells [8,9, 24]. Virus-specific CD4⁺ T cells and S598–605-specific CD8⁺ T cells are hindered in their ability to enter the CNS during anti-CXCL10 treatment, but CD8⁺ T cells specific for the immunodominant epitope, S510–518, are not restricted access.

The disparate responses of T cell subsets to anti-CXCL10 treatment suggested that the T cell populations differentially express CXCR3. Studies by Klein and colleagues [32] demonstrated that CXCL10 is required for optimal recruitment of CXCR3-expressing effector T cells to the brains of West Nile virus-infected mice. Specifically, CXCR3-expressing CD4⁺ and CD8⁺ T cells were significantly reduced in CXCL10-deficient mice compared to wild-type suggesting that CXCR3 expression dictates which cells respond to CXCL10 [32]. Interestingly, examination of CXCR3 expression on antigen-specific T cells in the brains of MHV-infected mice revealed that the differential accumulation of antigen-specific CD4⁺ and CD8⁺ T cells following anti-CXCL10 treatment was not due to variable CXCR3 expression on specific T cell populations. Therefore, we suggest that the CXCL10:CXCR3 signaling axis is not critical in directing S510–518 antigen-specific CD8⁺ T cells into the CNS in mice persistently infected with JHMV. Importantly, these data support a recent study from our laboratory demonstrating that blocking CXCR3 with neutralizing antibody selectively inhibits CD4⁺ T cell accumulation in the CNS [11].

An important question that remains is why S510–518 specific CD8⁺ T cells are capable of entering the CNS during anti-CXCL10 treatment while the other antigen-specific T cell populations evaluated (*e.g.* S598–605 specific CD8⁺ T cells) are barred from entry. It is possible that T cells specific to the immunodominant CD8⁺ epitope respond to different chemotactic signals. Previous studies show that CCL5 is important for attracting S510–518 virus-specific CD8⁺ T cells into the CNS during chronic JHMV infection [28]. Mice persistently infected with MHV and treated with CCL5 neutralizing antibody resulted in reduced CNS accrual of total S510–518-specific CD8⁺ T cells at 21 days p.i. [28]. These data imply that trafficking of this CD8⁺ T cell subset into the CNS is more responsive to CCL5 than CXCL10. Alternatively, studies by Bergmann and colleagues have shown that S510–518-specific CD8⁺ T cells are selectively retained in the CNS [33]. Therefore, S510–518-specific CD8⁺ T cells may not be affected by the absence of CXCL10 because this is the dominant CD8⁺ T cell viral epitope thus likely to be the most accessible and easily recognized, resulting in retention of this subset in the CNS during anti-CXCL10 treatment.

T cell subsets affected by anti-CXCL10 treatment returned to the CNS as early as one week following removal of treatment. This result leads to an important question pertaining to the fate of these T cell subsets during antibody treatment. It is possible that the affected T cell populations die and that the cells that are found in the CNS following removal of anti-CXCL10 treatment are newly generated in the periphery. We believe this unlikely, as MHV viral RNA is not detected within peripheral lymphoid tissue during chronic disease (data not shown) [33]. A more probable possibility is that the affected T cell populations are retained in the periphery and then return to the CNS once antibody administration is ablated. Importantly, these studies reveal that CXCL10 expression specifically attracts defined T cell populations to the CNS. The implication of these findings is that anti-CXCL10 treatment is potentially more selective in inducing the migration of targeted populations of cells to a site of microbial infection.

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Figure 1. Virus-specific T cells within the brains of JHMV-infected C57BL/6 mice express comparable levels of CXCR3

C57BL/6 mice were infected i.c. with JHMV and were sacrificed on days 7, 12 and 21 p.i. and brains were removed. The percents of antigen-specific CD4+ and CD8+ T cells expressing CXCR3 were analyzed. Cells isolated from the brains were stained for CD4, intracellular IFN- γ and CXCR3 following stimulation with M133–147 and CD8, intracellular IFN- γ , and CXCR3 following stimulation with either S510–518 or S598–605. Representative dot plots from flow cytometric analyses are shown. CD4 or CD8 cells were gated and staining for the remaining markers are displayed. Each plot represents pooled samples of 6 brains. The overall percentages of CXCR3 expressing antigen-specific T cells are indicated in the upper right quadrants.

Figure 2. Total and virus-specific T cells in the brain during anti-CXCL10 treatment
 C57BL/6 mice were i.c. infected with JHMV and treated with either anti-CXCL10 or NRS on days 12, 14, 16 and 19 p.i. Numbers of various T cell subsets infiltrating the brain were assessed at 12, 15 and 21 days p.i. using intracellular staining for IFN- γ and surface staining for CD4 or CD8. (A) Anti-CXCL10 treatment results in a dramatic reduction in total CD4+ T cells at 15 and 21 days p.i., but total CD8+ T cells are not affected at 21 days p.i. and only slightly reduced at day 15 p.i. (B) Anti-CXCL10 treatment results in reduced infiltration of M133–147-, S328–347-, and S358–372-specific CD4+ T cells at both 15 and 21 days p.i. (C) Infiltration into the CNS of both S510–518- and S598–605-specific CD8+ T cells are reduced

by CXCL10 neutralization at 15 days p.i., but at 21 days p.i. only infiltration of S598–605-specific CD8⁺ T cells is reduced. Results presented are the average of 3 mice per group.

Figure 3. Total and virus-specific T cells in the spinal cord during anti-CXCL10 treatment
 C57BL/6 mice were i.c. infected with JHMV, treated with either anti-CXCL10 or NRS beginning at day 12 p.i. and numbers of various T cell subsets infiltrating the spinal cord were assessed at 12, 15 and 21 days p.i. using intracellular staining for IFN- γ and surface staining for CD4 or CD8. (A) Anti-CXCL10 treatment results in a dramatic reduction in total CD4+ T cells at 15 and 21 days p.i., but the reduction in total CD4+ T cells infiltrating the spinal cord is more pronounced than that of total CD8+ T cell infiltration at 21 days p.i. (B) Anti-CXCL10 treatment results in reduced infiltration of M133–147-, S328–347-, and S358–372-specific CD4+ T cells at both 15 and 21 days p.i. (C) Infiltration into the CNS of both S510–518- and S598–605-specific CD8+ T cells are slightly reduced by CXCL10 neutralization at 15 days

p.i., but at 21 days p.i. only infiltration of S598–605-specific CD8+ T cells is reduced. Results presented are the average of 3 mice per group.

Figure 4. Effects of anti-CXCL10 treatment on T cell accumulation in the brains of JHMV-infected mice

C57BL/6 mice were i.c. infected with JHMV, treated with either anti-CXCL10 or NRS on days 12, 14, 16 and 19 p.i. and numbers of T cell subsets were assessed using intracellular staining for IFN- γ and surface staining for CD4 or CD8. (A) During anti-CXCL10 treatment there is a dramatic reduction in total CD4⁺ T cells, but not CD8⁺ T cells. When antibody treatment is ablated, numbers of CD4⁺ and CD8⁺ T cells in the brain from mice that received anti-CXCL10 and NRS are indistinguishable. (B) M133–147-specific CD4⁺ T cells and S598–605-specific CD8⁺ T cells are reduced during anti-CXCL10 treatment. Numbers of antigen-specific CD4⁺ and CD8⁺ T cells are indistinguishable in the brain from mice that received anti-CXCL10 and NRS at day 28 p.i., a time point following removal of treatment. Results presented are the average of 3 mice per group. Numerical values indicate percent reduction.