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Rapid Communication

The CC chemokine ligand 3 regulates $CD11c^+CD11b^+CD8\alpha^-$ dendritic cell maturation and activation following viral infection of the central nervous system: implications for a role in T cell activation

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Abstract

The role of CC chemokine ligand 3 (CCL3) in activation of dendritic cells (DCs) following mouse hepatitis virus (MHV) infection of the central nervous system (CNS) was examined. The results indicate that CCL3 participates in an effective host response to MHV infection by contributing to $CD11c^+CD11b^+CD8\alpha^-$ DC maturation, activation, and migration to cervical lymph nodes (CLN). Diminished $CD8\alpha^-$ DC activation correlated with reduced IFN-γ expression by virus-specific T cells accompanied by increased IL-10 production suggesting that CCL3 contributes to an effective host response to viral infection by enhancing the T cell activation potential of DC.
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Keywords: Chemokines; Virus; Innate immune response; Cell trafficking; Dendritic cell; T cell activation

Introduction

The CC chemokine ligand 3 (CCL3—macrophage inflammatory protein-1α) is capable of activating monocytes and lymphocytes and serves an important role in the initial recruitment of these cells to tissues following microbial infection (Cook et al., 1995; Domachowske et al., 2000). In support of the importance for CCL3 in imparting functional signals to T cells are data from our laboratory demonstrating that instillation of mouse hepatitis virus (MHV) into the brains of $CCL3^{-/-}$ mice results in an inability to clear virus from the central nervous system (CNS) (Trifilo et al., 2003). MHV-infected $CCL3^{-/-}$ mice exhibited a significant reduction in the numbers of infiltrating virus-specific $CD8^+$ T cells present within the brain indicating that trafficking was impaired. Moreover, the ability to produce IFN-γ as well as the cytolytic activity of virus-specific $CD8^+$ T cells was dramatically reduced in the absence of CCL3 signaling. Taken together, these data indicate that CCL3 signaling significantly enhances the differentiation of primed $CD8^+$ T cells into effector cells that allows their release from secondary lymphoid organs into circulation and effective migration to the CNS. The present study was undertaken to characterize potential mechanisms by which CCL3 signaling imparts effector function to antigen-specific T cells following MHV infection of the CNS. To further understand the relationship between CCL3 signaling and T cell activation, $CCL3^{+/+}$ and $CCL3^{-/-}$ mice were infected with MHV and the presence and activation state of DC-like cells within the brain and draining cervical lymph nodes (CLN) determined. Our results delineate a CCL3-dependent pathway of T cell activation that involves the maturation and activation of a subpopulation of (dendritic cells) DCs ($CD11c^+CD11b^+CD8\alpha^-$) within the CNS as well as influencing the accumulation of these cells within the CLN following MHV infection of the CNS.

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Results and discussion

Characterization of CD11c^{+} cells within the CNS following MHV infection of CCL3$^{+/+}$ and CCL3$^{-/-}$ mice

To characterize the populations of cells present within the CNS of MHV-infected mice, cells were harvested at days 0, 2, and 5 post-infection (p.i.) and immunophenotyped by flow cytometry. We chose to focus our attention on markers that are associated with professional antigen presenting cells such as DC as recent studies have indicated DC-like cells can be detected within the brains under inflammatory conditions (Fisher and Reichmann, 2001; Fischer et al., 2000). Furthermore, we have previously determined that CCL3 mRNA expression is detected within the CNS early (<3 days) and therefore may participate in the appearance of CD11c^{+} cells within the brain following MHV infection (Trifilo et al., 2003). Therefore, we sought to characterize the populations of CD11c^{+} cells within the brain following MHV infection of CCL3$^{+/+}$ and CCL3$^{-/-}$ mice. Results in Fig. 1A indicate the frequency of CD11c^{+} cells present within the CNS of naïve CCL3$^{+/+}$ and CCL3$^{-/-}$ mice is <1%. However, within 2 days following intracranial infection with MHV, there is a marked increase in the frequency of CD11c^{+} cells within the brains of both strains of mice (Fig. 1A). Analysis of CD11b expression revealed that approximately 75% of CD11c^{+} cells in both CCL3$^{+/+}$ and CCL3$^{-/-}$ mice were also CD11b^{+} (Fig. 1A). CD11c^{+}CD11b^{+} cells isolated from the CNS of either CCL3$^{+/+}$ or CCL3$^{-/-}$ mice expressed little to no CD8α or DEC205 suggesting a phenotype similar to myeloid derived DC (CD11c^{+}, CD11b^{+}, CD8α^{-}, DEC205^{-}) (Fig. 1B) (Anjuere et al., 1999; Henri et al., 2001). Increased CD8α and DEC205 expression on CD11c^{+}CD11b^{-} cells indicated that the majority of the remaining CD11c^{+} cells present within the brain were similar phenotypically to lymphoid derived DC (CD11c^{+}, CD11b^{-}, CD8α^{+}) (Fig. 1B) (Anjuere et al., 1999; Henri et al., 2001). Although the

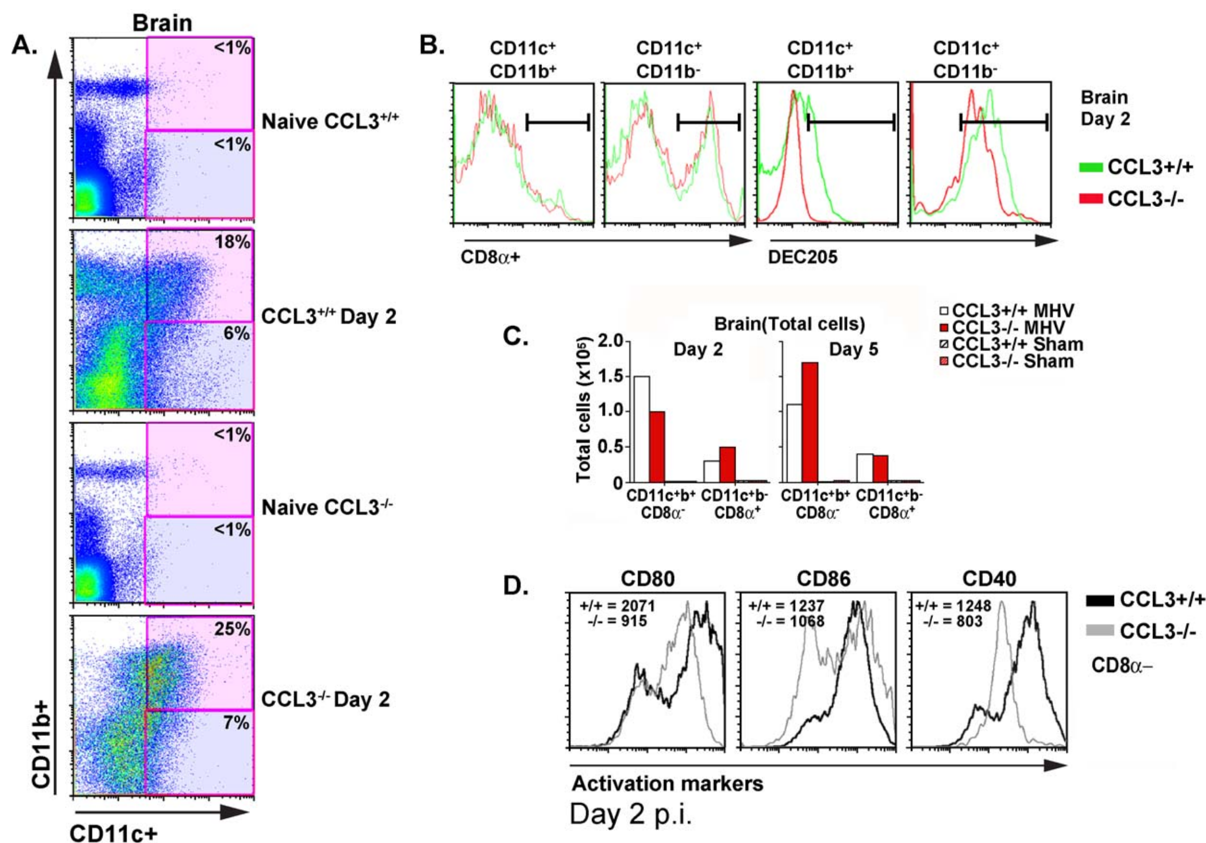

Fig. 1. Analysis of dendritic cells (DCs) within the brain following MHV infection. (A) Cells were isolated from the brains of uninfected (naïve) or infected (day 2 p.i.) CCL3$^{+/+}$ and CCL3$^{-/-}$ mice and stained for CD11c^{+} and CD11b^{+} expression. Gated populations represent CD11c^{+}CD11b^{+} (upper-right quadrant) or CD11c^{+}CD11b^{-} (lower-right quadrant) and numbers indicate frequencies of gated cells within the isolated population. (B) CD8α and DEC205 expression on CD11c^{+} cells. CD11c^{+}CD11b^{+} cells from either MHV-infected CCL3$^{+/+}$ or CCL3$^{-/-}$ mice at day 2 p.i. did not express detectable levels of either CD8α or DEC205 while expression of both CD8α and DEC205 was readily detectable on CD11c^{+}CD11b^{-} cells present within the brains of both populations of mice at day 2 p.i. (C) Total numbers of CD8α^{+} and CD8α^{-} DCs within the brains of MHV or sham-infected CCL3$^{+/+}$ and CCL3$^{-/-}$ mice at days 2 and 5 p.i. Data presented represent an average cell number derived from two separate experiments with a minimum of 10 mice analyzed per experimental group. (D) CD8α^{-} cells isolated from brains of CCL3$^{+/+}$ or CCL3$^{-/-}$ mice at day 2 p.i. were gated upon and CD80, CD86, and CD40 expression was determined by flow cytometry. The mean fluorescence intensity (MFI) for cells obtained from either CCL3$^{+/+}$ or CCL3$^{-/-}$ mice is indicated. Flow data shown in panels A, B, and D are representative of two separate experiments with a total of 10 mice for each experimental condition.

remaining CD11c$^+$CD11b$^-$CD8α$^-$ population was not further characterized, it is likely that these cells may be plasmacytoid in origin (CD11c$^+$CD11b$^-$CD8α$^-$B220$^+$) or consist of a yet to be defined population of DC. Comparison of the total numbers of CD8α$^+$ cells within the brains of MHV-infected CCL3$^{+/+}$ and CCL3$^{-/-}$ mice revealed no dramatic differences between the two populations of mice at either 2 or 5 days p.i. (Fig. 1C). In contrast, numbers of CD8α$^-$ cells were increased by approximately 30% within the brains of MHV-infected CCL3$^{+/+}$ mice as compared to CCL3$^{-/-}$ mice at day 2 p.i. However, by day 5 p.i., there were increased numbers of CD8α$^-$ cells present in the brains of CCL3$^{-/-}$ mice when compared to CCL3$^{+/+}$ mice. In attempt to better evaluate the activation state of CD11c$^+$ cells within the brains of MHV-infected mice, we next determined the expression levels of co-stimulatory molecules CD80 (B-7.1), CD86 (B-7.2), and CD40 on CD8α$^-$ cells within the CNS of CCL3$^{+/+}$ and CCL3$^{-/-}$ mice. We chose to focus on this subpopulation of DCs in more detail as this clearly was the predominant DC population within the brains of infected mice suggesting a potentially more important role in defense. Analysis of CD8α$^-$ cells isolated from the brains of CCL3$^{+/+}$ mice at day 2 p.i. revealed these cells expressed detectable levels CD80, CD86, and CD40 as determined by measuring the mean fluorescence intensity (MFI) (Fig. 1D). Although MHV infection of CCL3$^{-/-}$ mice also resulted in enhanced expression of CD86 on the surface of CD8α$^-$ cells, the MFI for both CD80 and CD40 was dramatically reduced as compared to CD8α$^-$ cells within the brain of CCL3$^{+/+}$ mice at day 2 p.i. (Fig. 1D). Together, these results indicate that although CCL3 signaling is not required for the appearance of DC-like cells within the brain, expression of the co-stimulatory molecules CD40 and CD80 is muted in the absence of CCL3 signaling.

Characterization of CD11c$^+$ cells within the CLN of MHV-infected CCL3$^{+/+}$ and CCL3$^{-/-}$ mice

Following MHV infection of the CNS, virus-specific T cells are present within the CLN suggesting that the bulk of virus-specific T cells are generated in the periphery (Marten et al., 2003). Therefore, the accumulation of CD11c$^+$ cells within the CLN of infected CCL3$^{+/+}$ and CCL3$^{-/-}$ mice was determined. Before infection, CD11c$^+$ cells expressing a myeloid DC phenotype (CD11c$^+$ CD11b$^+$) and lymphoid DC phenotype (CD11c$^+$ CD11b$^-$) were present within the CLN of both CCL3$^{+/+}$ and CCL3$^{-/-}$ mice at an approximate 1:2 ratio, respectively (Fig. 2A). However, within 2 days of MHV infection of the CNS, the frequency of CD11c^{+-}CD11b$^+$ DCs, but not CD11c$^+$CD11b$^-$ DCs, dramatically increased within the CLN of CCL3$^{+/+}$ mice (Fig. 2A). Further analysis revealed that the CD11c$^+$CD11b$^+$ cell population was CD8α$^-$DEC205$^-$ while the CD11c^{+-}CD11b$^-$ population was CD8α$^+$DEC205$^+$ (Fig. 2B). Although the total number of CD8α$^-$ cells increased within the CLN of CCL3$^{-/-}$ mice, there was an approximate 4-fold reduction in total numbers of CD8α$^-$ cells as compared to CCL3$^{+/+}$ mice at days 2 and 5 p.i. (Fig. 2C). Similar to the brain, no difference in numbers of CD8α$^+$ cells within the CLN was detected at either days 2 or 5 p.i. (Fig. 2C). Examination of co-stimulatory molecule expression on CD8α$^-$ DCs present within the CLN of CCL3$^{+/+}$ mice correlated with the increased expression of co-stimulatory molecules CD40, CD80, CD86 as well as increased MHC I and II expression when compared to sham-infected mice (Figs. 2D and E). These data suggest that this population of activated CD8α$^-$ cells within the CLN is able to present antigen and induce T cell differentiation within the CLN following MHV infection of the CNS. Although CD8α$^-$ DCs isolated from the CLN of CCL3$^{-/-}$ mice expressed similar levels of CD80 and CD86 as compared to CCL3$^{+/+}$ (determined by MFI), expression of CD40 as well as MHC I and II were reduced as compared to CD8α$^-$ cells isolated from MHV-infected CCL3$^{+/+}$ mice (Figs. 2D and E).

Cytokine secretion by CD11c$^+$ cells

One mechanism by which DCs influence the T cell response to infection is through the secretion of cytokines that can subsequently polarize the immune response towards either a Th1 or Th2 phenotype depending on the antigenic challenge. To determine if CD8α$^-$ DCs present within the CLN of infected CCL3$^{+/+}$ and CCL3$^{-/-}$ mice were capable of secreting either chemokines or cytokines following MHV infection of the CNS, these cells were isolated and production determined by ELISA. CCL3 was readily detectable from CD8α$^-$ DCs obtained from CCL3$^{+/+}$ mice while CCL3 was not detected in supernatants collected from CCL3$^{-/-}$ CD8α$^-$ DCs (Fig. 3). The CCL3$^{+/+}$ CD8α$^-$ DC population secreted IL-12p70 with low-level production of IL-10 (Fig. 3). In contrast, the CD11c$^+$ CD11b$^+$ CD8α$^-$ cells isolated from CCL3$^{-/-}$ mice secreted approximately 3-fold less IL-12p70 while IL-10 secretion was increased by 5-fold as compared to cells from CCL3$^{+/+}$ mice (Fig. 3).

Altered cytokine production in CCL3$^{-/-}$ T cells

We next evaluated the ability of T cells obtained from the CLN of either MHV-infected CCL3$^{+/+}$ or CCL3$^{-/-}$ mice to synthesize cytokines following exposure to defined viral antigens. T cells were isolated from the CLN of CCL3$^{+/+}$ and CCL3$^{-/-}$ mice at days 7 and 12 p.i. following intracranial infection with MHV and stimulated with peptides corresponding to either the immunodominant CD4 epitope present within the matrix (M) glycoprotein at residues 133–147 (M133–147) or the immunodominant CD8 epitope in the surface (S) glycoprotein spanning residues 510–518 (S510–518) and cytokine production by T cells determined by intracellular cytokine staining (Castro and Perlman, 1995;

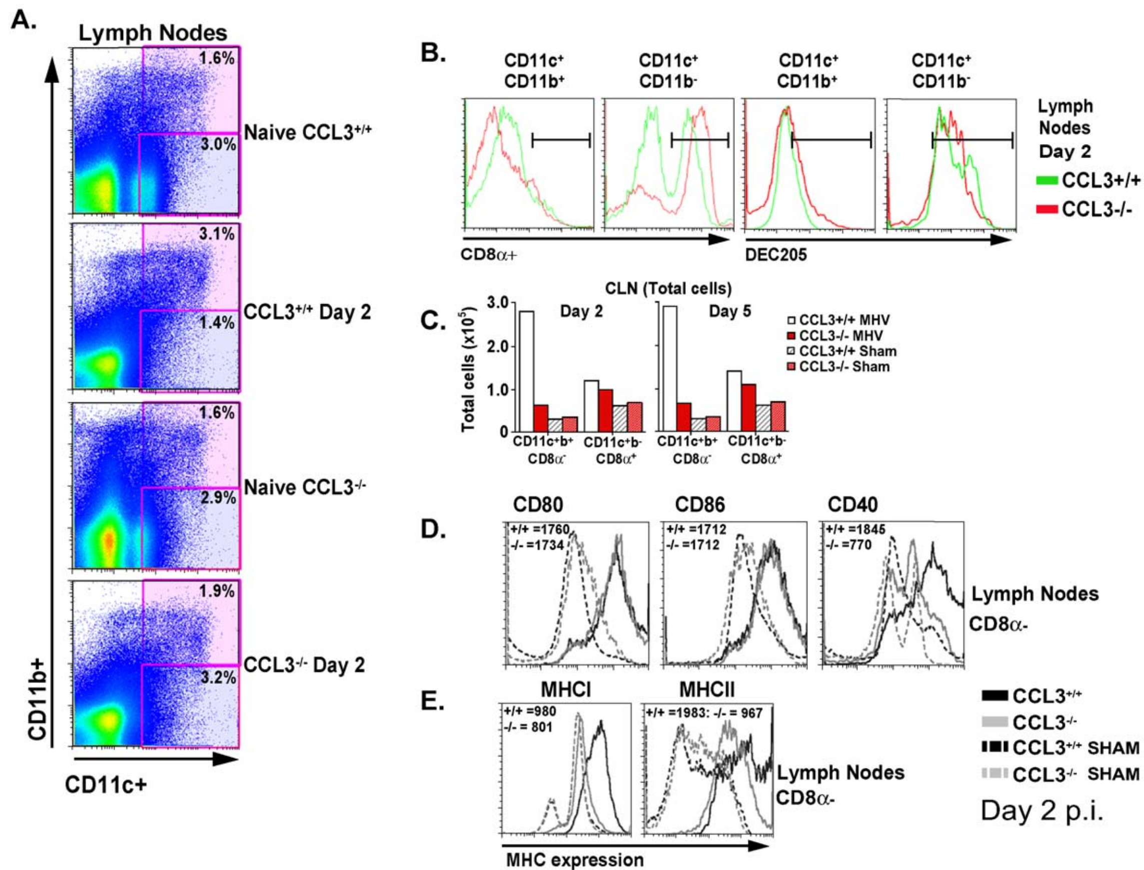

Fig. 2. Analysis of DCs within the CLN following MHV infection. (A) Cells were isolated from the CLNs of uninfected (naïve) or infected (day 2 p.i.) $CCL3^{+/+}$ and $CCL3^{-/-}$ mice and stained for $CD11c^{+}$ and $CD11b^{+}$ expression. Gated populations represent $CD11c^{+}CD11b^{+}$ (upper-right quadrant) or $CD11c^{+}CD11b^{-}$ (lower-right quadrant) and numbers indicate frequencies of gated cells within the isolated population. (B) CD8α and DEC205 expression on $CD11c^{+}$ cells. $CD11c^{+}CD11b^{+}$ cells from either $CCL3^{+/+}$ or $CCL3^{-/-}$ at day 2 p.i. did not express detectable levels of either CD8α or DEC205 while expression of both CD8α and DEC205 was readily detectable on $CD11c^{+}CD11b^{-}$ cells present within the brains of both $CCL3^{+/+}$ and $CCL3^{-/-}$ mice at day 2 p.i. (C) Total numbers of $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DCs within the CLNs of MHV or sham-infected $CCL3^{+/+}$ and $CCL3^{-/-}$ mice at days 2 and 5 p.i. Data presented represent an average cell number derived from two separate experiments with a minimum of 10 mice analyzed per experimental group. (D) $CD8\alpha^{-}$ cells obtained from MHV-infected (day 2 p.i.) or sham mice were gated and the level of CD80, CD86, and CD40 expression was determined by flow cytometry. The MFI for cells obtained from either $CCL3^{+/+}$ or $CCL3^{-/-}$ mice is indicated. (E) $CD8\alpha^{-}$ cells obtained from MHV-infected or sham mice were evaluated for expression of MHC I and II. The MFI for staining of either MHC I or II is indicated in the histogram. Flow data shown in panels A, B, D, and E are representative of two separate experiments with a total of 10 mice for each experimental condition.

Xue et al., 1995). The results presented in Table 1 indicate similar frequencies of $CD4^{+}$ and $CD8^{+}$ T cells from $CCL3^{+/+}$ mice produced IFN-γ at days 7 and 12 p.i. Both $CD4^{+}$ and $CD8^{+}$ $CCL3^{+/+}$ T cells also secreted IL-2 following specific peptide exposure at day 7, although expression was limited to the acute stage of MHV infection as the frequency of IL-2-producing cells was reduced at day 12 p.i. (Table 1). In contrast, the frequency of $CD4^{+}$ and $CD8^{+}$ T cells isolated from $CCL3^{-/-}$ mice secreting IFN-γ following peptide stimulation was dramatically reduced. Expression of IL-2 by $CCL3^{-/-}$ T cells was comparable with $CCL3^{+/+}$ mice at day 7 p.i. However, by day 12 p.i., the frequency of $CCL3^{-/-}$ T cells expressing IL-2 remained elevated as compared to $CCL3^{+/+}$ T cells (Table 1). In addition, only limited frequencies of $CCL3^{+/+}$ $CD4^{+}$ and $CD8^{+}$ T cells produced IL-10 following MHV infection whereas $CCL3^{-/-}$ $CD4^{+}$ and $CD8^{+}$ T cells displayed an overall increase in the frequency of IL-10 (Table 1).

The major findings of this study are (i) MHV infection of the CNS results in the appearance of two distinct populations of $CD11c^{+}$ cells each expressing markers characteristic of lymphoid ($CD11c^{+}CD11b^{-}CD8\alpha^{+}DEC205^{+}$) and myeloid dendritic cells ($CD11c^{+}CD11b^{+}CD8\alpha^{-}DEC205^{-}$), (ii) the accumulation of $CD8\alpha^{-}$ DCs within the draining CLN is reduced in the absence of CCL3 signaling, (iii) expression of co-stimulatory molecules such as CD40 by $CD8\alpha^{-}$ DCs within either the brain and CLN of MHV-infected $CCL3^{-/-}$ mice is diminished suggesting that CCL3 signaling enhances expression of these molecules, and (iv) absence of CCL3 signaling results in the re-direction of the T cell response to viral antigens as determined by cytokine production. These data support and extend recent studies from our laboratory demonstrating an important role for CCL3 in generating effector anti-viral T cells capable of migrating to the brain in response to viral infection (Trifilo et al., 2003).

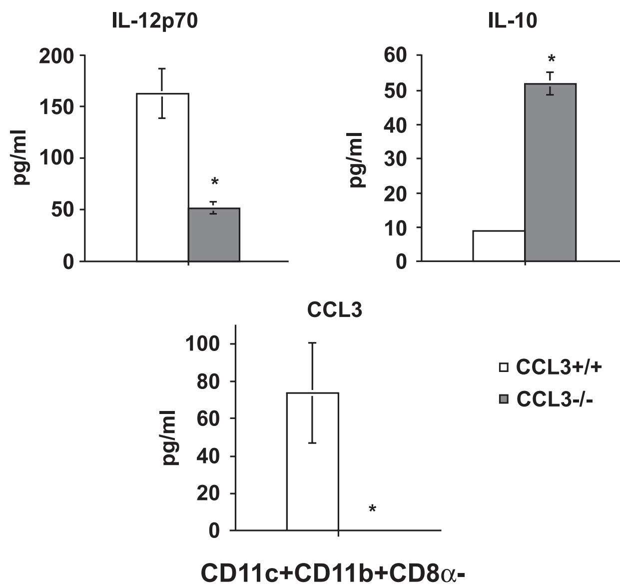

Fig. 3. Cytokine and chemokine secretion by $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ cells isolated from mononuclear cells pooled from the CLN of MHV-infected $CCL3^{+/+}$ and $CCL3^{-/-}$ mice at day 7 p.i. Supernatants were analyzed for the production of IL-12p70, IL-10, and CCL3 by ELISA 24 h following culture. A minimum of three to six mice per group were used for isolation of cells and data presented indicate the average ± SD. $*P \leq 0.001$.

The activation of DC and their mobilization to secondary lymphoid organs is thought to be a key step in the initiation of an adaptive immune response (Bancher-eau and Steinman, 1998). Recent studies have indicated that following infection of the CNS with *Toxoplasma gondii*, $CD11c^+$ cells are present within the brain and these cells were able to stimulate the proliferation of naïve T cells (Fisher and Reichmann, 2001). Similarly, our results also indicate an increase in $CD11c^+$ cells within the CNS following viral infection, suggesting that these cells are likely critical for successful T cell priming following migration to draining lymph nodes. Whether these cells are present within the CNS by differentiation of local antigen presenting cells, or through migration of immature DC has not been determined and is currently under investigation. Regardless, our data imply that CCL3 expression and signaling contributes to the migration of $CD8\alpha^-$ $CD11c^+$ cells to secondary lymphoid tissue where they participate in priming of T cells. In support of this, we have shown that CCL3 is important in arming these cells with the capability to optimally stimulate antigen-specific T cells with the ability to fully differentiate into effector cells (Trifilo et al., 2003). The data presented in this study support and extend these observations and indicate that these results may be the result of a combination of diminished expression of both MHC class I and II, reduced expression of CD40, as well as a shift in cytokine production by $CD8\alpha^-$ cells. Indeed, CD40L:CD40 mediated interactions between T cells and APC can enhance IL-12 production by DC and blockade of this interaction has been shown to result in reduced autoimmunity by down-regulating Th1 differentiation (Cella et al., 1996; Macatonia et al., 1995). Accumulating evidence indicates that in addition to driving virus-specific T cell proliferation, the activation state of DCs can also directly influence the effector function of T cells through the secretion of proinflammatory cytokines (Fischer et al., 2000; Maldonado-Lopez et al., 1999, 2001; Pulendran et al., 1999). For example, following several viral and bacterial infections, $CD8\alpha^+$ DCs have been shown to be able to secrete large amounts of the proinflammatory cytokine IL-12 both in vitro and in vivo and this results in a preferential expression of Th1-associated cytokines, such as IFN-γ by responding T cells (Aliberti et al., 2000; Maldonado-Lopez et al., 1999, 2001; Pulendran et al., 1999; Reis e Sousa et al., 1997). Until recently, the prevailing thought was that $CD8\alpha^+$ DCs were primarily responsible for production of IL-12 and contributing to a Th1 response. However, recent studies have indicated that $CD8\alpha^-$ DCs also have the potential for secreting IL-12 and influencing the T cell response (Doxsee et al., 2003). Our studies clearly indicate that $CD8\alpha^-$ DCs isolated from the draining CLN of MHV-infected $CCL3^{+/+}$ mice secrete IL-12 suggesting that these cells help influence a protective Th1-mediated immune response characterized by the majority of antigen-specific T cells expressing IFN-γ rather than IL-10 (Table 1). In stark contrast is the data indicating that $CD8\alpha^-$ DCs present in the CLN of infected $CCL3^{-/-}$ mice predominantly secrete IL-10 and this correlates with limited IFN-γ expression and enhanced expression of the Th2-associated cytokine IL-10 (Table 1). Therefore, the data indicate that cytokine production, rather than the type of $CD11c^+$ cell, may control the predominant T cell immune response within the CLN. Taken together, these data point to an important role in CCL3 expression in linking innate and adaptive immune responses following viral infection of the CNS by contributing to the activation

Table 1
Frequency of cytokine-producing T cells present within draining cervical lymph nodes following MHV infection[a]

	Mouse	Day p.i.	IFN-γ	IL-2	IL-10
$CD4^+$ T cells	$CCL3^{+/+}$	7	20 ± 4	35 ± 5	5 ± 2
		12	16 ± 3	2 ± 1	2 ± 1
	$CCL3^{-/-}$	7	<1[b]	39 ± 3[b]	24 ± 4[c]
		12	<1[b]	34 ± 5[b]	36 ± 4[c]
$CD8^+$ T cells	$CCL3^{+/+}$	7	23 ± 5	22 ± 2	6 ± 1
		12	18 ± 2	6 ± 2	5 ± 1
	$CCL3^{-/-}$	7	5 ± 2[b]	21 ± 3[b]	20 ± 2[c]
		12	3 ± 1[b]	31 ± 5[b]	28 ± 4[c]

Data represent two separate experiments with at least three mice per group, $n = 6$. Data are presented as average ± SD.

[a] Cytokine expression determined by pooling cells from draining CLN of MHV-infected mice at defined times p.i. and pulsing with defined $CD4^+$ epitope (M133–147) and $CD8^+$ epitope (S510–518).

[b] $P \leq 0.01$. Decreased frequency of IFN-γ secreting T cells and increased frequency of IL-2 producing T cells following MHV infection of $CCL3^{-/-}$ mice as compared to $CCL3^{+/+}$ mice.

[c] $P \leq 0.002$. Increased frequency of IL-10 producing T cells in $CCL3^{-/-}$ mice as compared to $CCL3^{+/+}$ mice following MHV infection.

of DCs through regulating the migration of cells from the CNS to lymphoid tissues as well as the expression of both co-stimulatory molecules and cytokine production. However, it is important to note that there is the possibility of other chemokines and chemokine receptors participating in DC responses following viral infection. Indeed, the CC chemokine receptor 2 (CCR2) is expressed on professional APC including macrophages and DCs and is thought to contribute to defense following microbial challenge by enhancing recruitment as well as production of antimicrobial products such as TNF-α and NO by these cells (Luster, 2002; McColl, 2002; Serbina et al., 2003). In addition, the absence of CCR2 signaling results in diminished trafficking and accumulation of dendritic cells within secondary lymphoid tissues following antigenic challenge (Peters et al., 2000, 2001; Sato et al., 2000). Studies are currently in progress to evaluate the contributions of additional chemokine signaling pathways that may also participate in DC activation and migration following coronavirus infection.

In conclusion, the studies presented support and extend previous work from our laboratory indicating an important role for chemokines in the migration of T cells into the CNS following MHV infection (Chen et al., 2001; Glass et al., 2001; Liu et al., 2001). Here, we have demonstrated a novel role for the chemokine CCL3 in enhancing the accumulation and activation of $CD8\alpha^-$ DCs within secondary lymphoid tissue and this correlates with altered T cell activation and differentiation following viral infection. These results indicate that $CD8\alpha^-$ DCs likely function in an APC-like role within the CLN following MHV infection and that these cells rely upon chemokine instruction to activate T cells. At a more fundamental level, the results presented demonstrate that chemokines serve as critical upstream signals in the innate immune response that later is important with regards to the initiation of a protective adaptive immune responses to viral infection.

Materials and methods

Animals and virus

MHV J2.2V-1 was kindly provided by J. Fleming (U. Wisconsin). $CCL3^{-/-}$ and $CCL3^{+/+}$ mice (C57BL/6, H-2^b) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were anesthetized by inhalation of methoxyflurane (Pitman-Moor Inc., Washington Crossing, NJ) and injected intracranially (i.c.) with 1000 PFU MHVJ2.2V-1 suspended in 30 μl sterile PBS. Control (sham) mice were injected with 30 μl sterile PBS alone.

Mononuclear cell isolation and flow cytometry

Mononuclear cells were obtained from the brains and cervical lymph nodes [two draining cervical lymph nodes (CLN) per mouse] of either $CCL3^{+/+}$ or $CCL3^{-/-}$ mice at defined times post-infection (p.i.) using a previously described protocol (Trifilo et al., 2003). Cell surface expression of phenotypic markers was examined using the following reagents for flow cytometric analysis: APC-conjugated rat anti-mouse CD8; PERCP-conjugated rat anti mouse CD4 (Pharmingen, San Diego, CA). PE conjugated D^b/S510–518 MHC class I tetramer (Beckman Coulter, San Diego, CA) was utilized for identification of $CD8^+$ T cells specific for viral spike protein antigen (Trifilo et al., 2003). To determine the presence of dendritic-like cells within the CNS and lymph nodes, cells were stained using FITC-conjugated rat anti-mouse CD11c (Serotec, Oxford, England) in combination with PERCP-conjugated rat anti-mouse CD8α (Pharmingen), rat anti-mouse APC-conjugated CD11b (Pharmingen), and rat anti-mouse DEC205 (Pharmingen). The maturation and activation state of DC were determined using FITC-conjugated rat anti-mouse CD40, CD80, CD86, MHC I, and MHC II (Pharmingen). Isotype-matched antibodies were used as controls for all staining conditions described.

Isolation of $CD11c^+$ cells from the CLN

$CCL3^{+/+}$ and $CCL3^{-/-}$ mice were infected intracranially with 1000 PFU of MHV and brains and CLN were removed at defined times post-infection for analysis. Brain samples were minced and homogenized into a single cell suspension followed by fractionation on a 70/30% Percoll gradient at 1300 × g for 30 min. For isolation of mononuclear cells from the CLN, lymph nodes were homogenized using frosted glass slides and the resulting single cell suspension was treated with sterile H_2O to lyse red blood cells. Due to the low frequency and numbers of $CD11c^+$ cells within the brain and CLN, samples from three to six mice were pooled for each experiment. To enrich for $CD11c^+CD11b^+CD8\alpha^-$ DCs, both brain and CLN samples were separately magnetically sorted by negative selection against CD8α using MACS microbeads coated with anti-CD8α (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. $CD11c^+$ cells were then magnetically selected from the $CD8\alpha^-$ fraction using MACS microbeads coated with anti-CD11c (Miltenyi). The resulting population was >90% pure for $CD11c^+$ cells that were subsequently determined to be $CD11b^+$ and $CD8\alpha^-$ by flow cytometry (data not shown). MACS enriched cells were then resuspended in DMEM supplemented with 10% FBS.

Cytokine and chemokine expression from $CD11c^+CD8\alpha^-$ cells

Freshly prepared $CD11c^+CD8\alpha^-$ cells were isolated from the CLN from $CCL3^{+/+}$ and $CCL3^{-/-}$ mice at day 7 p.i. and seeded into 96-well plate at a cell density of 2×10^5 cells/200 μl in DMEM containing 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD). Following isola-

tion, cell viability was >95%. After 24 h, supernatants from the samples were collected, and the level of IL-12, IL-10, and CCL3 were determined using Quantikine M mouse immunoassays kits (R&D Systems, Minneapolis, MN) according to manufacturer's specifications. Assays had a minimum sensitivity of 5 pg/ml (IL-10 and IL-12p70) and 10 pg/ml (CCL3).

Intracellular cytokine staining

Mononuclear cells were obtained from the CLNs of MHV-infected mice at defined times post-infection and cytokine production by T cells determined by intracellular cytokine staining to defined viral antigens using a previously described protocol (Trifilo et al., 2003).

Statistical analysis

Statistically significant differences between experimental groups was determined by the Mann–Whitney Rank Sum Test, and P values of ≤ 0.05 were considered significant.

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