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CXCL13 promotes isotype-switched B cell accumulation to the central nervous system during viral encephalomyelitis

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Article info

Article history:
Received 16 November 2015
Received in revised form 13 January 2016
Accepted 16 January 2016
Available online 18 January 2016

Keywords:
Central nervous system
Neuroimmunology
CXCL13
B cells
Antibody secreting cells
Memory B cells

Abstract

Elevated CXCL13 within the central nervous system (CNS) correlates with humoral responses in several neuroinflammatory diseases, yet its role is controversial. During coronavirus encephalomyelitis CXCL13 deficiency impaired CNS accumulation of memory B cells and antibody-secreting cells (ASC) but not naïve/early-activated B cells. However, despite diminished germinal center B cells and follicular helper T cells in draining lymph nodes, ASC in bone marrow and antiviral serum antibody were intact in the absence of CXCL13. The data demonstrate that CXCL13 is not essential in mounting effective peripheral humoral responses, but specifically promotes CNS accumulation of differentiated B cells.

Introduction

The lymphoid chemokines CXCL12, CXCL13, CCL19 and CCL21 all regulate migration and compartmentalization of B cells within secondary lymphoid organs under homeostatic as well as activation/inflammatory conditions (Cyster, 2005; Okada and Cyster, 2006). In lymphoid organs CXCL13 is expressed in B cell follicles and contributes to germinal center (GC) formation by recruiting activated B and follicular helper CD4⁺ T (Tfh) cells expressing its cognate receptor CXCR5 (Victora and Nussenzweig, 2012; Zotos and Tarlinton, 2012; Crotty, 2012). Formation of GC by coordinated chemokine engagement of CD4⁺ T cells and activated B cells in turn is critical for B cell differentiation, isotype switching and affinity maturation leading to antibody secreting cells (ASC) or memory B cells (Bmem) not secreting antibody (Ab). Both ASC and Bmem surviving selection in GCs exit and provide long-lived humoral immunity following infection or vaccination. While ASC comprise long-lived plasma cells and their precursor plasma blasts, Bmem require antigen or innate re-stimulation to differentiate into ASC.

CXCL13 is also associated with lymphoid neogenesis and propagation of humoral responses in non-lymphoid tissue during chronic inflammatory diseases, such as rheumatoid arthritis, Helicobacter pylori gastritis, multiple sclerosis (MS) and primary central nervous system (CNS) lymphoma (Wengner et al., 2007; Finch et al., 2013; Rubenstein et al., 2013; Winter et al., 2010; Fischer et al., 2009). In MS patients, CXCL13 is elevated in the cerebral spinal fluid (CSF), and detected in meningeal ectopic follicle-like structures as well as active plaques (Krumholz et al., 2006; Haas et al., 2011; Corcione et al., 2005; Ragheb et al., 2011). Furthermore, the majority of CSF B cells from MS patients are CXCR5⁺ naïve B cells or Bmem (Krumholz et al., 2006; Haas et al., 2011; Cepok et al., 2006; Corcione et al., 2004) supporting a link between elevated intrathecal CXCL13 and the accumulation of class-switched Bmem (Haas et al., 2011). CXCL13 and B cells in the CSF are also hallmarks of individuals with Lyme neuroborreliosis (LNB) (Kowarik et al., 2012). In addition, high CXCL13 expression during primary CNS lymphomas, which are predominantly of B cell origin, is associated with inferior outcome as indicated by 10-fold higher CXCL13 levels in the CSF of patients with relapsed primary CNS lymphoma (Rubenstein et al., 2013). While the correlation between CXCL13 and B cell numbers in the CSF in both LNB and
MS patients (Krumbholz et al., 2006; Kowarik et al., 2012; Ruprecht et al., 2009) supports a causal role for CXCL13 in B cell recruitment to the inflamed CNS, it remains to be formally demonstrated.

Blockade of CXCL13 in animal models of CNS inflammation has yielded mixed results. Mice with chronic-relapsing experimental autoimmune encephalomyelitis (EAE), a model of MS, revealed B cell aggregation proximal to CXCL13 expression in the inflamed meninges (Magliozzi et al., 2004). Moreover, formation of meningeal ectopic follicles and expression of CXCL13 was suppressed by treatment with lymphoxygen-β-receptor-lg fusion protein to disrupt lymphoid organ organization (Columba-Cabezas et al., 2006). By contrast, CXCL13 deficiency did not impair initial B cell recruitment to the CNS in a chronic, non-relapsing EAE model; however, potential effects during peak and chronic disease were not reported as B cell accumulation was only monitored at day 14 post-immunization (Rainey-Barger et al., 2011). B cell accumulation and CXCL13 expression, independent of CNS ectopic follicle-like structures, is also a feature of Sindbis virus infection. However, in this model B cell recruitment to the infected CNS was CXCL13 independent (Rainey-Barger et al., 2011), consistent with only a minor (~1%) fraction of B cells in the CNS expressing CXCR5 (Metcalfe et al., 2013). CNS infection by sublethal glia-tropic JHMV is also associated with CXCL13 upregulation and accumulation of heterogeneous B cell subsets (Phares et al., 2014). ASC accumulation within the CNS is CXCR3-dependent (Marques et al., 2011). Moreover, CXCL13 as a potential mediator of early B cell recruitment is supported by substantially higher expression of CXCR5 transcripts by CNS-infiltrating naïve/early-activated B cells and B<sub>B</sub> relative to ASC (Phares et al., 2014). Together these data indicate that local humoral responses within the CNS correlate with but are not necessarily dependent on CXCL13.

The present study sought to define how CXCL13 affects accumulation of distinct B cell subsets during JHMV infection. Following intracranial infection of adult mice JHMV initially replicates in ependymal cells that line the brain ventricles (Wang et al., 1992; Bergmann et al., 2006). Virus then spreads to the parenchyma, where infection is limited to focal areas throughout the brain, but largely sparing the brain stem. Glia are primary targets of infection with sparse neuronal involvement. Early during infection virus replicates prominently in glia and infiltrating macrophages. Once spreading down the central canal of the spinal cord, JHMV moves out into the white matter primarily infecting oligodendrocytes. Virus replication peaks at ~5 days post infection (p.i.), and is largely controlled by T cells within 2 weeks p.i. Nevertheless, although infectious virus remains undetectable after 2 weeks, virus persists at low levels as detected by mRNA expression in spinal cords for up to a year (Bergmann et al., 2006; Adami et al., 1995). Importantly, ASC within the CNS are essential to prevent viral reemergence (Ramakrishna et al., 2003; Tschen et al., 2002, 2006).

Infected CXCL13<sup>−/−</sup> mice exhibited no deficits in initial accumulation of B cells with a naïve/early phenotype within the CNS. However, the absence of CXCL13 lead to diminished isotype-switched B<sub>B</sub> as well as ASC accumulation during persistent, consistent with severely impaired GC B cell and T<sub>H</sub> cell activation in cervical lymph nodes (CLN). Although ASC expansion in CLN was also negatively affected, there were no evident defects in antiviral serum Ab or ASC recruitment to the bone marrow (BM) in infected CXCL13<sup>−/−</sup> relative to wild-type (WT) mice. The results suggest that migration of naïve/early-activated B cells to the CNS is independent of CXCL13 driven signals in the priming lymph nodes or the CNS effector site. Moreover, despite reduced B cell differentiation in the absence of CXCL13, residual humoral immunity is sufficient to establish protective responses in the serum as well as CNS.

2. Materials and methods

2.1. Mice, virus infection and virus titers

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). CXCL13<sup>−/−</sup> mice on the C57BL/6 background were previously described (Rainey-Barger et al., 2011) and bred locally. C57BL/6 mice expressing GFP under the glial fibrillary acidic protein (GFAP) promoter were generated by backcrossing FVB/N-Tg (GFAP-GFP) 14Mes/J mice (The Jackson Laboratory, Bar Harbor, ME) onto the C57BL/6 background for at least 10 generations. All mice were housed under pathogen free conditions at an accredited facility at the Cleveland Clinic Lerner Research Institute. Male and female mice were infected indiscriminately at 6–7 weeks of age by intracranial injection with 1000 plaque forming units (PFU) of the J2.2.v-1 monoclonal Ab (mAb)-derived glatropic JHMV variant (Fleming et al., 1986). Animals were scored daily for clinical signs of disease using the scale: 0, healthy; 1, ruffled fur and hunched back; 2, hind limb paralysis or inability to turn to upright position; 3, complete hind limb paralysis and wasting; 4, moribund or dead. All animal experiments were performed in compliance with guidelines approved by the Cleveland Clinic Lerner Research Institute Institutional Animal Care and Use Committee. Virus titers within the CNS were determined in clarified supernatants by plaque assay using the murine delayed brain tumor (DBT) astrocytoma as detailed (Fleming et al., 1986). Plaques were counted after 48 h incubation at 37 °C.

2.2. Flow cytometry and fluorescence activated cell sorting (FACS)

Pooled brains and spinal cords from 6 to 8 mice perfused with PBS were homogenized in ice-cold Tenbroeck grinders in Dulbeco’s PBS. Mononuclear cells were recovered from the 30/70% interface of a Percoll (Pharmacia, Piscataway, NJ) step gradient following centrifugation at 850 × g for 30 min at 4 °C as detailed (Bergmann et al., 1999). Single-cell suspensions from CLN were prepared as described (Bergmann et al., 1999). For phenotypic analysis pooled cells were stained with mAb specific for CD4 (L3T4), CD8 (53-6.7), CD11b (M1/70), CD19 (1D3), CD25 (PC61), CD45 (30-F11), CD95 (J02), CD138 (281-2), GL7 (GL7), IgD (11-26), IgG<sub>2b</sub> (R2-40) (all from BD Bioscience), IgM (eB121-15F9), PD-1 (RMP1-30) (eBio-science) and F4/80 (C57BL/6J) onto the C57BL/6 background for at least 10 generations. Male and female mice were infected indiscriminately at 6–7 weeks of age by intracranial injection with 1000 plaque forming units (PFU) of the J2.2.v-1 monoclonal Ab (mAb)-derived glatropic JHMV variant (Fleming et al., 1986). Animals were scored daily for clinical signs of disease using the scale: 0, healthy; 1, ruffled fur and hunched back; 2, hind limb paralysis or inability to turn to upright position; 3, complete hind limb paralysis and wasting; 4, moribund or dead. All animal experiments were performed in compliance with guidelines approved by the Cleveland Clinic Lerner Research Institute Institutional Animal Care and Use Committee. Virus titers within the CNS were determined in clarified supernatants by plaque assay using the murine delayed brain tumor (DBT) astrocytoma as detailed (Fleming et al., 1986). Plaques were counted after 48 h incubation at 37 °C.

For RNA expression pooled spinal cords (n = 6–8) were digested with collagenase and B cell subsets purified using a BD FACS Aria (BD) as described (Phares et al., 2014). In brief spinal cords were minced and digested in RPMI supplemented with 10% fetal calf serum (FCS), 250 μL of collagenase D (100 mg /ml) (Roche Diagnostics, Indianapolis, IN) and 50 μL of DNase I (1 mg/ml) (Roche Diagnostics) for 40 min at 37 °C. Collagenase and DNase I activity was terminated by addition of 0.1 M EDTA (pH 7.2) at 37 °C for 5 min. Following centrifugation, cells were washed with RPMI supplemented with 25 mM HEPES and recovered from the 30/70% interface of a Percoll gradient as described above. Spinal cord CD19<sup>IgD</sup> B cells at day 7 p.i. were compared to CD19<sup>IgD</sup> ASC and CD19<sup>IgD</sup> CD13<sup>B</sup><sub>B</sub> from spinal cords purified at day 21 p.i. GFP<sup>CD45<sup>+</sup></sup> astrocytes, CD45<sup>+</sup> microglia and CD45<sup>+</sup>CD11b<sup>F4/80</sup> infiltrating monocyte-derived macrophages were purified from GFAP-GFP pooled spinal cords subsequent to Trypsin digestion as described (Hamo et al., 2007). A minimum of 1 × 10<sup>6</sup> cells were
collected per pooled sample and frozen in 400 μl TRizol (Invitrogen, Carlsbad, CA) at –80 °C for subsequent RNA extraction and PCR analysis as described (Phares et al., 2014; Ireland et al., 2009).

2.3. Gene expression analysis

Snap frozen brains, spinal cords or CLN from individual PBS-perfused mice (n = 3–7) were placed into Trizol (Invitrogen, Grand Island, NY) and homogenized using a TissueLyser with stainless steel beads (Qiagen, Valencia, CA). RNA was extracted according to the manufacturer’s instructions. DNA contamination was removed by treatment with DNase I for 30 min at 37 °C (DNA-free kit; Ambion, Austin, TX) and cDNA synthesized using M-MLV Reverse Transcriptase (Invitrogen), oligo-dT primers (20 μM) (Promega Madison, WI) and random primers (20 μM) (Promega). Quantitative real-time PCR was performed using 4 μl of cDNA and SYBR Green Master Mix (Applied Biosystems, Foster City, CA) in duplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems). PCR conditions were 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Primers used for transcripts encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a proliferation-inducing ligand (APRIL), CXCL10, CXCL12, IL-10, viral nucleocapsid and IL-21 were previously described (Phares et al., 2009, 2010, 2012; Savarin et al., 2010). GAPDH, CCL19, CCL21, CXCR5, B cell-activating factor of the tumor necrosis factor family (BAFF), gamma interferon (IFN-γ), and activation-induced cytidine deaminase (AID) mRNA levels were determined using Applied Biosystems Gene Expression Arrays with Universal Taqman Fast Master Mix and Taqman primers (Applied Biosystems). PCR conditions were 20 s at 95 °C followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Transcript levels were calculated relative to the housekeeping gene GAPDH using the following formula: 2^[-(CT(GAPDH)-CT(Target Gene))] × 1000, where CT represents the threshold cycle at which the fluorescent signal becomes significantly higher than that of the background.

2.4. ASC enzyme-linked immunospot (ELISPOT) assay

Total and JHMV-specific IgG or IgM ASC were detected by a modification of ELISPOT assay as described (Marques et al., 2011). Briefly, spinal cords or brains were minced and digested in 5 ml RPMI supplemented with 10% FCS, 100 IU/ml of collagenase type I (100 mg/ml; Worthington Biochemical Corporation, Lakewood, NJ) and 50 μl of DNase I (100 mg/ml) (Roche, Indianapolis, IN) for 40 min at 37 °C. Enzyme activity was terminated by addition of 0.1 M EDTA (pH 7.2) at 37 °C for 5 min and cells recovered from the 30/70% interface of a Percoll gradient as described above. Single-cell suspensions from CLN or BM were prepared as described (Bergmann et al., 1999). 96-well MultiScreen HTS IP plates (EMD Millipore, Billerica, MA) were coated with either virus (~5 × 10^5 PFU/well) or polyclonal goat anti-mouse IgG (10 μg/ml; Cappel Laboratories, Inc., Cochranville, PA). Serial dilutions of cells plated in triplicate were incubated for 4 h at 37 °C. ASC were detected by sequential incubation with biotinylated rabbit anti-mouse IgG (0.5 μg/ml; Southern Biotech, Birmingham, AL) or biotinylated goat anti-mouse IgM (1.2 μg/ml; Jackson ImmunoResearch, West Grove, PA) overnight at 4 °C, streptavidin horseradish peroxidase (1:1000; BD biosciences, St. Louis, MO) for 1 h at room temperature, and filtered 3,3’-diaminobenzidine substrate (Sigma–Aldrich, St. Louis, MO) with 30% hydrogen peroxide. Spots were counted using an Immunospot ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH).

2.5. Histology

CLN from PBS-perfused mice were snap-frozen in Tissue-Tek OCT compound (Sakura Finetex, Torrance, CA) and sectioned at 10 μm using a Thermo Shandon cryostat. Sections were fixed with ice cold 70% acetone for 5 min, blocked with 5% bovine serum albumin and 10% goat serum for 1 h and then stained with rat anti-mouse FITC conjugated GL7 mAb (BD Biosciences), rat anti-mouse B220 mAb (BD biosciences) and rabbit anti-mouse CD3 polyclonal Ab (Abcam, Cambridge, MA) overnight at 4 °C. Samples were then incubated with secondary Ab using Alexa Fluor 594 goat anti-rat (Life Technologies, Grand Island, NY) and Cy5 goat anti-rabbit (Life Technologies, Grand Island, NY) for 1 h at room temperature. Sections were mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY) and examined using a Leica TCS SPS II fluorescence microscope (Lawrenceville, GA).

2.6. Serum Ab

Virus neutralizing Ab was measured as described (Tschen et al., 2002). Briefly, duplicates of serial 2-fold dilutions of heat inactivated serum from individual mice (n ≥ 5) were incubated with 50 PFU of JHMV in 96-well plates for 90 min at 37 °C. Following addition of DBT cells (8 × 10^4 cells/well) plates were incubated at 37 °C for 48 h. Neutralization titers represent the log10 of the highest average serum dilution that inhibited cytopathic effect. Total virus-specific IgG was measured by ELISA as described (Tschen et al., 2002, 2006). Briefly, plates were coated with 100 μl of a serum free supernatant derived from JHMV infected DBT cells and incubated overnight at 4 °C. Plates were washed with PBS containing 0.5% Tween-20 and blocked with 10% FCS in PBS overnight at 4 °C. Serum samples were diluted 2-fold in PBS and incubated overnight at 4 °C. Following washing, bound Ab was detected using biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), streptavidin horseradish peroxidase (BD Bioscience) and 3,3’,5,5’ Tetramethylbenzidine (TMB Reagent Set; BD Bioscience). Optical densities were read at 450 nm on a SpectraMax Mz (Molecular Devices, Sunnyvale, CA) microplate reader and titers calculated as reciprocals of the highest dilution that exceeded 2-fold ± standard errors of the means (SEM) over naïve controls.

2.7. Collection of CSF and CXCL13 ELISA

CSF was obtained by cisternal puncture performed by a modification of Ronald’s method (Li et al., 2006). Following anesthesia, meninges overlying the cisterna magna were exposed and the surrounding area gently washed to prevent blood contamination. A 29-gauge insulin needle was used to puncture the arachnoid membrane covering the cisterna and CSF was collected by gentle pipetting using a polypropylene pipette. Depending on the time-point, about 10–20 μl of CSF was obtained from each mouse. CSF and clarified brain and spinal cord supernatants were used to measure CXCL13 by ELISA (MCK130; R&D Systems, Minneapolis, MN).

2.8. Statistical analysis

Results are expressed as the means ± SEM for each group of mice. In all cases, a P value of <0.05, determined by the unpaired t test, was considered significant. Graphs were plotted and statistics assessed using GraphPad Prism 4.0 software.

3. Results

3.1. Microglia are a primary source of CXCL13 during viral induced neuroinflammation

CXCL13 transcripts are induced and sustained in both brain and spinal cord following JHMV infection (Phares et al., 2014, 2011) in
the absence of apparent ectopic follicle formation. To evaluate if CXCL13 protein is preferentially maintained at distinct anatomical sites after initial clearance of infectious virus at day 14 p.i. CXCL13 was measured in brain, spinal cord and CSF by ELISA (Fig. 1A). CXCL13 was significantly elevated at day 7 p.i. in all three samples (Fig. 1A). While brain CXCL13 declined after day 7 p.i. CXCL13 remained elevated in spinal cord and CSF through day 21 p.i. relative to naïve counterparts (Fig. 1A). To identify the predominant source of CXCL13, astrocytes, microglia and infiltrating monocyte-derived macrophages were purified at day 7 and 10 p.i. and assessed for CXCL13 transcripts. CXCL13 mRNA was predominantly expressed by microglia (Fig. 1B), consistent with other studies (Rainey-Barger et al., 2011; Esen et al., 2014).

3.2. Diminished accumulation of B_{mem} and ASC in absence of CXCL13

Naïve/early-activated B cells accumulate early within the CNS during JHMV infection and are gradually replaced by more differentiated B_{mem} and ASC (Phares et al., 2014), the latter distinguished by expression of the cell surface marker CD138, also known as syndecan-1. While ASC accumulation is CXCR3:CXCL10 dependent (Marques et al., 2011; Phares et al., 2013), chemokines mediating monocyte-derived macrophages were purified at day 7 and 10 p.i. and assessed for CXCL13 transcripts. CXCL13 mRNA was predominantly expressed by microglia (Fig. 1B), consistent with other studies (Rainey-Barger et al., 2011; Esen et al., 2014).

3.3. CXCL13 deficiency impairs peripheral GC formation

Generation of T dependent isoform-switched ASC and B_{mem} following infections or immunizations is driven by antigen-specific B cell and CD4+ Tfh engagement within GC (Victora and Nussenzweig, 2012; Zotos and Tarlinton, 2012). CXCR5 expressed on both activated B and CD4+ Tfh cells promotes antigen-specific B cell expansion and enhances GC formation especially under conditions of limiting antigen load (Junt et al., 2005). Reduced IgG* ASC accumulation in the CNS of JHMV infected CXCL13−/− mice thus suggested a defect in peripheral expansion due to impaired GC formation. Following JHMV infection both T and B cell activation occurs primarily in CLN. Although CXCL13−/− mice was thus assessed by flow cytometry (Fig. 2B–E). Fig. 2B shows a representative plot defining CD138* IgD*Gm* naïve/early-activated B cells and CD138* IgD*Gm* B_{mem}. Although analysis focused on the spinal cord as the target of viral persistence and sustained CXCL13 levels, results were overall similar in the brain (data not shown). Total CD19+ B cell numbers were independent of CXCL13, regardless of the day p.i. (data not shown). The number of naïve/early-activated B cells, comprising the vast majority of B cells at day 7 p.i. but declining thereafter (Phares et al., 2014), were also comparable between WT and CXCL13−/− mice throughout infection (Fig. 2C). B_{mem} numbers were similar at day 7 p.i. in both groups; however, while they nearly doubled in WT mice between day 7 and day 14 p.i. B_{mem} did not increase in the absence of CXCL13 (Fig. 2D). By day 21 p.i. B_{mem} numbers were comparable in both groups due to their decline in WT mice. Initial CD138* ASC accumulation at day 14 p.i. was similar and increased by day 21 p.i. in both groups, but only reached ~50% of the levels in WT mice in absence of CXCL13 (Fig. 2E). Furthermore, the number of CD138* ASC still remained lower in infected CXCL13−/− versus WT mice at day 42 p.i. (CXCL13−/− = 195 versus WT = 425). ELISPOT analysis of brain- and spinal cord-derived ASC was performed at day 21 p.i. to reveal whether impaired accumulation was restricted to virus-specific ASC and/or a specific Ig isotype. The number of total and virus-specific IgM* ASC were similar in the CNS of WT and CXCL13−/− mice (Fig. 3A and B). However, the absence of CXCL13 resulted in 2- to 3-fold lower total and virus-specific IgG* ASC in brains and spinal cords, respectively (Fig. 3C and D). These data argue that CXCL13 is dispensable for early accumulation of naïve/early-activated B cell in the CNS, but promotes both B_{mem} and specifically IgG* ASC accumulation as the naïve/early-activated B cell population declines.

3.3. CXCL13 deficiency impairs peripheral GC formation

Generation of T dependent isoform-switched ASC and B_{mem} following infections or immunizations is driven by antigen-specific B cell and CD4+ Tfh engagement within GC (Victora and Nussenzweig, 2012; Zotos and Tarlinton, 2012). CXCR5 expressed on both activated B and CD4+ Tfh cells promotes antigen-specific B cell expansion and enhances GC formation especially under conditions of limiting antigen load (Junt et al., 2005). Reduced IgG* ASC accumulation in the CNS of JHMV infected CXCL13−/− mice thus suggested a defect in peripheral expansion due to impaired GC formation. Following JHMV infection both T and B cell activation occurs primarily in CLN. Although CXCL13−/− and CXCR5−/− mice have developmental defects in the formation of most lymph nodes (Ansel et al., 2000; Forster et al., 1996), CLN and mesenteric LN are not disrupted in CXCL13−/− mice (Ansel et al., 2000). This was confirmed by similar CLN cellularity in naïve WT and CXCL13−/− mice (data not shown). B cell activation in the draining CLN following infection was therefore monitored for GL7*CD95* B cells by flow cytometry. GL7, also known as T and B cell activation marker and CD95, also known as FAS receptor are phenotypic markers of activated GC B cells (van Eijk et al., 2001). Frequencies of GC-derived B cells (CD19*GL7*CD95*) were already increased at day 7 p.i. in both infected WT and CXCL13−/− mice relative to naïve controls (Fig. 4A). However, whereas GC B cells increased and remained elevated through day 21 p.i in WT mice, they failed to increase in CXCL13−/− mice (Fig. 4A). Impaired GC formation was supported by significantly
reduced upregulation of transcript levels encoding AID, an enzyme upregulated in activated GC-derived B cells (Keim et al., 2013; Maul and Gearhart, 2010; Muramatsu et al., 2000), in CLN of CXCL13/C0/C0 mice relative to WT mice (Fig. 4B). By contrast, transcripts for CXCL12, CCL19, and CCL21, chemokines regulating B cell migration within lymph node follicles (Cyster, 2005; Okada and Cyster, 2006), were not impaired by CXCL13 deficiency (Fig. 4C). CXCL12 mRNA levels remained at similar and constant levels throughout infection, while CCL19 and CCL21 mRNA levels were even significantly elevated after day 7 p.i. in the absence of CXCL13 (Fig. 4C), potentially altering trafficking of CCR7+ lymphocytes or dendritic cells (Craft, 2012; Scandella et al., 2007). In contrast to GC-phenotype B cells, expansion of early short-lived CD138+ ASC prior to day 10 p.i. was CXCL13 independent (data not shown).

To assess whether impaired B cell activation in CLN coincided with reduced T cell help, TFH cells (CD4+CXCR5+PD-1hi) were compared between the groups. TFH frequencies were significantly increased relative to naïve levels by day 7 p.i. in both WT and CXCL13/C0/C0 mice and remained elevated through day 21 p.i. (Fig. 4D). However, percentages were consistently lower in the
absence of CXCL13 (Fig. 4D). IL-21 is a T_{FH}-derived cytokine that promotes GC reactions and generation of ASC (Zotos et al., 2010; Ozaki et al., 2002; Rankin et al., 2011; Linterman et al., 2010). Decreased expression of IL-21 transcripts in CLN mRNA from CXCL13^{-/} mice supported impaired T_{FH} activity (Fig. 4E). Of note, similar CLN frequencies of virus-specific CD8^{+} T cells and
CD4+CD25+ T cells in WT and CXCL13−/− mice (data not shown) imply CXCL13 deficiency specifically affects Tfh cells. To confirm impaired GC B cell and Tfh cell activation in CXCL13−/− mice coincides with poor GC development CLN were screened for discrete areas of T cell (CD3+) and B cell (B220+GL7+) co-localization as readout for GC formation. While GC were readily detected by day 14 p.i. (data not shown) and continued to mature through day 21 p.i. in WT mice, both the number and size of GC were noticeably smaller in CXCL13−/− mice (Fig. 5). Impaired GC formation thus suggested reduced peripheral humoral responses are responsible for decreased CNS accumulation of IgG ASC. Surprisingly however, no differences in either virus-specific IgG or neutralizing Ab were detected in serum between WT and CXCL13−/− mice (Fig. 6A), consistent with intact serum Ab. Taken together the data indicate that CXCL13-dependent organized GC are not essential for mounting effective serum Ab responses following CNS infection. Moreover, although slightly impaired peripheral expansion coincided with reduced ASC accumulation in the CNS of CXCL13−/− mice, ASC in BM were intact. These results suggested CXCR4 driven ASC recruitment to BM is not affected by CXCL13 deficiency, whereas reduced activation in CLN limits CXCR3 dependent accumulation to the inflamed CNS.

3.4. Impaired ASC accumulation in CXCL13−/− mice is sufficient to control persisting virus

CNS-localized ASC are crucial in preventing viral recrudescence of persisting JHMV (Ramakrishna et al., 2003; Tschen et al., 2002, 2006). To assess whether diminished ASC accumulation in the CNS of CXCL13−/− mice altered JHMV pathogenesis, viral control was examined by monitoring infectious virus and transcripts...
encoding the viral nucleocapsid protein. Overall onset, progression and peak of clinical disease were comparable in WT and CXCL13−/− mice (Fig. 7A). Infectious virus was similar in both groups at day 5 and controlled with similar kinetics by day 14 p.i. (Fig. 7B). By day 21 p.i. infectious virus was below detection in both groups with the exception of a single CXCL13−/− mouse. Importantly, infectious virus remained undetectable through day 42 p.i. (Fig. 7B). Similarly low persisting viral transcripts in the spinal cord of both groups further demonstrated ongoing viral control (Fig. 7C). No alterations in antiviral T cell-mediated immunity in the absence of CXCL13 was supported by comparable spinal cord-derived transcripts for T cell-derived cytokines including IFN-γ, IL-10 and IL-21 between WT and CXCL13−/− mice (Fig. 7D–F). Notably, there were also no differences in CXCL9 or CXCL10 mRNA levels, suggesting signals for CXCR3 dependent ASC recruitment were not impaired (data not shown). Expression of transcripts encoding chemokines CXCL12, CCL19 and CCL21, potentially affecting recruitment of CXCR4 or CCR7 expressing B cells were also intact in CXCL13−/− mice (Fig. 7G–I). Although CCL21 mRNA was not induced by infection in either group, CCL21 transcripts were 10-fold higher at basal levels and throughout infection in CXCL13−/− relative to WT mice (Fig. 7I). Notably, cytokine and chemokine expression profiles were similar in the brain (data not shown). These results indicate that control of persistence by the CNS humoral response is independent of CXCL13 despite the diminished number of isotype-switched virus-specific ASC.

4. Discussion

CXCL13 is upregulated in the CNS during various microbial infections as well as autoimmune inflammation, yet its role in
CNS humoral immunity remains unclear (Finch et al., 2013; Krumbholz et al., 2006; Rupprecht et al., 2009; Rainey-Barger et al., 2011; Metcalf et al., 2013; Phares et al., 2014; Gelderblom et al., 2007; Khademi et al., 2011). The data herein demonstrate that CXCL13 induced by gliatropic JHMV infection remains elevated in CSF at least a week post clearance of infectious virus; however, it is not essential in recruiting naïve/early-activated IgD+ B cells into the CNS, although this population expresses high levels of CXCR5 mRNA. CXCL13 deficiency affected B
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mem accumulation transiently, but the major impact was on CD138+ ASC previously shown to require CXCR3:CXCL10 to migrate into the CNS (Marques et al., 2011). The data herein demonstrate that CXCL13 induced by gliatropic JHMV infection remains elevated in CSF at least a week post clearance of infectious virus; however, it is not essential in recruiting naïve/early-activated IgD+ B cells into the CNS, although this population expresses high levels of CXCR5 mRNA. CXCL13 deficiency affected B
sup
mem accumulation transiently, but the major impact was on CD138+ ASC previously shown to require CXCR3:CXCL10 to migrate into the CNS (Marques et al., 2011). CNS-derived ASC were reduced by 50%, specifically affecting isotype-switched IgG*, but not IgM* ASC. Unaltered accumulation of IgD+ naïve/early-activated B cells within the CXCL13
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CNS implies chemokine receptors other than CXCR5 mediate their trafficking. Similar CCR7 and higher CXCR4 transcript expression by CNS-derived IgD+ naïve/early-activated B cells relative to CLN-derived IgD+ B cells (Phares et al., 2014) implicate CXCR4 and/or CCR7 in driving recruitment. Notably, elevated CCL19 transcripts in the CNS during JHMV infection (Phares et al., 2014) are consistent with preferential recruitment of IgD+ naïve/early-activated B cells via CCR7. CXCL13 independent recruitment of early B cells was also noted following both Sindbis virus infection and induction of EAE (Rainey-Barger et al., 2011), supporting the notion that CXCL13 is not an effective target to manipulate B cell recruitment early during CNS inflammation. Moreover, during Sindbis virus infection CCL19 transcripts are elevated early in the CNS coinciding with peak numbers of CCR7+ B cells (Metcalf et al., 2013).

The primary effect of CXCL13 deficiency on CNS accumulation of isotype-switched B cells correlated with reduced activation of GL7+ GC-derived B cells as a result of impaired CLN GC formation. CXCL13 recruits activated CXCR5+ TrH cells into the GC where IL-21, the signature TrH cytokine, promotes ASC and B
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mem differentiation (Ozaki et al., 2002; Rankin et al., 2011; Linterman et al., 2010; Rasheed et al., 2013). Impaired TrH activation, coincident with reduced IL-21 mRNA in CXCL13
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CNS, implied that reduced isotype-switched B cell accumulation in the CNS was

Fig. 6. Peripheral ASC responses are intact in CXCL13
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mice. (A) Virus-specific IgG2a and neutralizing Ab in serum of infected WT and CXCL13
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mice. Data are expressed as the mean ± SEM and represent two independent experiments, each comprising individual sera of 3 mice per time point. Numbers of virus-specific IgG-secreting ASC in CLN (B) and BM (C) measured by ELISPOT assay. Data are representative of 2–3 independent experiments with similar trends in each experiment. To better reflect relative differences between WT and CXCL13
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frequencies, data in the right column of (B and C) show the mean percentage changes in frequencies ±SEM calculated relative to levels in WT mice, which were set to a baseline value of 100. Data represent 2–3 independent experiments, each comprising pooled CLN or BM from 3 to 4 mice.
predominantly attributed to deficient peripheral B cell differentiation rather than local CNS defects. Indeed, CNS-derived IL-21 transcripts, expressed primarily by CD4+ T cells during JHMV infection (Phares et al., 2013), were not altered by CXCL13 deficiency. Thus, although inflamed sites expressing CXCL13 may recruit circulating CXCR5+ TFH cells and thereby promote local B cell differentiation, CXCL13 independent IL-21 mRNA levels in the CNS argue against a local role of TFH cells in gliatropic JHMV infection. Furthermore, preliminary analysis shows no evidence for a CXCR5+ TFH phenotype in the CNS of JHMV-infected mice. Hence although CXCL13 driven CXCR5+ T cells are implicated in prolonging pathology during EAE (Bagaeva et al., 2006), our data suggest IL-21 expressing CD4 T cells in the JHMV-infected CNS are not regulated by CXCL13 and may comprise a distinct effector CD4 T cell subset. In this context it is of interest to note that IL-21 can be secreted by multiple CD4 T cell subsets (Yi et al., 2010).

Given the defect in GC formation, it was surprising that virus-specific IgG serum Ab, including neutralizing Ab, production was not impaired in absence of CXCL13. Similar results following CNS Sindbis virus infection (Rainey-Barger et al., 2011) imply that well organized follicles with clearly defined GC structures are not essential for effective peripheral antiviral humoral responses. Although GC formation was impaired in JHMV-infected CXCL13−/− CLN, there was evidence for focal clustering of B and T cells at the time of GC formation in WT mice. Such focal sites of T and B cell aggregation may be supported by elevated expression of the CCR7 ligands CCL19 and CCL21 at days 14 and 21 p.i. in CXCL13−/− relative to WT mice. CCR7 is increased on antigen engaged WT B cells, directing early-activated B cells to migrate to the T cell zone chemokines CCL19 and CCL21 (Reif et al., 2002). During peripheral Vesicular Stomatitis virus infection CCR7 plays a critical role in driving B:T helper cell collaboration at the T:B cell border (Scandella et al., 2007). Similarly, CCR7 enhanced interactions of antigen primed B cells and T helper cells may facilitate B cell differentiation during JHMV infection in absence of CXCL13. This is supported by only slightly reduced numbers of virus-specific IgG+ ASC in CLN and no ASC defects in BM of CXCL13−/− relative to WT mice.
Regulation and function of chemokine receptors on B cell subsets has been prominently described for events involving inter-compartmental migration within lymphoid tissue, but not to sites of infection. In addition to distinct expression and regulation of various receptors on CNS-derived activated IgD⁺ B cells versus Bₘₚ in infected WT mice (Phares et al., 2014), impaired GC formation in absence of CXCL13 may further skew signaling by other chemokines acting via CXCR4 or CCR7. The extensive downregulation of both CXCR5 and CXCR4, but to a lesser extent CCR7, on CNS-derived WT Bₘₚ versus activated IgD⁺ B cells (Phares et al., 2014) suggests Bₘₚ are recruited by CXCR5:CXCL13 independent mechanisms and should thus not be affected by CXCL13 deficiency. However, it cannot be excluded that impaired GC formation alters imprinting of signals required for early Bₘₚ egress from lymphoid tissue into the circulation, thereby explaining the difference to WT mice at day 14 p.i. Distinguishing between CXCL13 dependent Bₘₚ egress into circulation, migration or survival in the CNS will be pursued in future studies.

CXCL13 as well as CXCR5 independent peripheral isotype-switched B cell responses have been noted in other experimental models (Rainey-Barger et al., 2011; Junt et al., 2005). However, comparative analysis of distinct peripheral infections and protein immunization demonstrated that CXCR5 is particularly critical to augment humoral responses under conditions of low antigenic load (Junt et al., 2005). The overall efficient CXCL13 independent, albeit CD4 T cell dependent (Phares et al., 2012), virus-specific ASC responses following JHMV infection were thus surprising given poor peripheral replication of JHMV following intracranial infection. However, the present data do not exclude the possibility of protective extrafollicular responses in absence of CXCL13, especially if CD4 T cell help can be provided despite perturbed follicular structure. B cell activation and GC formation following JHMV infection is limited to the draining CLN, with little ASC expansion noted in the spleen (Marques et al., 2011). To what extent either cell free or dendritic cell-associated viral antigen drainage gives rise to effective humoral immunity remains to be resolved. Nevertheless, our results demonstrate that GC formation is CXCL13 driven; however, CLN GC reactions are not essential to initiate isotype-switching under conditions of low viral load. Furthermore, similar frequencies of IgG³ virus-specific ASC in BM of CXCL13⁻/⁻ and WT mice suggest CXCR4 driven migration to BM is independent of imprinting in fully formed GC. Interestingly however, reduced accumulation of ASC in the CXCL13⁻/⁻ CNS suggests less efficient CXCR3 driven migration to infected sites. In WT mice, CXCR3 expression on CLN-derived ASC is increased between day 7 and 14 p.i. (Marques et al., 2011), coincident with initiation of GC formation. Altered chemokine receptor regulation on virus-specific IgG³ ASC in absence of CXCL13 may thus lead to preferential default migration to BM. Additionally, less organized B cell structures may impose distinct patterns of migration within lymph nodes, thereby affecting CLN egress into the circulation.

In conclusion, our results demonstrate that early CNS accumulation of IgD⁺ B cells is independent of CXCL13. Furthermore, CXCL13:CXCR5 independent B:T helper cell interactions, likely driven by CCR7-mediated migration, are sufficient to elicit isotype-switched, virus-specific IgG³ ASC and neutralizing serum Ab in the periphery, despite significantly impaired GC formation. These data suggest CXCL13 may not be a driving force in the seeding of B cell aggregates or follicle-like structures in non-lymphoid organs, which are thought to aggravate local autoimmunity (Finch et al., 2013; Parker Harp et al., 2015). Importantly, GC independent differentiation of antigen-specific B cells in lymphoid tissue implies similar events may occur at inflammatory sites characterized by elevated CCR7 ligand expression to promote B:T helper cell interactions. Expression of various cytokines and chemokines sustain-
