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Full-length Article

# Activated GL7$^+$ B cells are maintained within the inflamed CNS in the absence of follicle formation during viral encephalomyelitis

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ABSTRACT

Central nervous system (CNS) inflammation associated with viral infection and autoimmune disease results in the accumulation of B cells in various differentiation stages. However, the contribution between peripheral and CNS activation remains unclear. During gliatropic coronavirus induced encephalomyelitis, accumulation of protective antibody secreting cells is preceded by infiltration of B cells with a naïve and early differentiation phenotype (Phares et al., 2014). Investigation of the temporal dynamics of B cell activation in draining cervical lymph nodes (CLN) and the CNS revealed that peak CNS infiltration of early activated, unswitched IgD$^+$ and IgM$^+$ B cells coincided with polyclonal activation in CLN. By contrast, isotype-switched IgG$^+$ B cells did not accumulate until peripheral germinal center formation. In the CNS, unswitched B cells were confined to the perivascular space and meninges, with only rare B cell clusters, while isotype-switched B cells localized to parenchymal areas. Although ectopic follicle formation was not observed, more differentiated B cell subsets within the CNS expressed the germinal center marker GL7, albeit at lower levels than CLN counterparts. During chronic infection, CNS IgD$^{int}$ and IgD$^-$ B cell subsets further displayed sustained markers of proliferation and CD4 T cell help, which were only transiently expressed in the CLN. A contribution of local CD4 T cell help to sustain B cell activation was supported by occasional B cells adjacent to T cells. The results suggest that accumulation of differentiated B cell subsets within the CNS is largely dictated by peripheral activation, but that local events contribute to their sustained activation independent of ectopic follicle formation.



## 1. Introduction

Central nervous system (CNS) inflammation as a result of viral infection, tissue injury, or autoimmunity recruits B cell subsets in various differentiation stages ranging from naïve, unswitched and isotype class switched memory B cells ($B_{mem}$), and antibody secreting cells (ASC) (Ankeny et al., 2009; Cepok et al., 2005; Corcione et al., 2004; Cruz et al., 1987; Dang et al., 2015; Metcalf et al., 2013; Niino et al., 2009; Phares et al., 2014). ASC and their antibody (Ab) specificity have been a major focus in multiple sclerosis (MS) research due to their potential reactivity to autoantigens. Although cerebrospinal fluid (CSF) immunoglobulin (Ig), a diagnostic hallmark, is suggested to be pathogenic, Ig specificity is diverse, rarely targeting myelin antigens (Avasarala et al., 2001; Cruz et al., 1987; Warren and Catz, 1994). Interest in other functions of B cells emerged when clinical trials revealed that the anti-CD20 Ab, Rituximab, which depletes all B cells except ASC, improved clinical symptoms and reduced gadolinium-enhancing lesions in MS patients independent of changes in CSF Ig (Hauser et al., 2008). By contrast, depletion of ASC and mature B cells using TACI Ig (Atacicept) in MS patients increased relapses ultimately resulting in the early termination of clinical trials (Kappos et al., 2014). While the anti-CD20 studies implied a detrimental role for B cells during CNS inflammation apparently independent of Ig secretion, TACI Ig treatment suggested B cells may assume both pathogenic and protective roles during neuroinflammation.

Despite the commonality of distinct B cell subsets in injury, autoimmunity, and viral infection of the CNS, little is known about their activation state, maintenance, or function, especially in relation to peripheral immune activation. In addition to Ig secretion, B cells are important modulators of immune responses during CNS insults. Their functions include antigen presentation, thereby promoting CD4 T cell activities, production of pro- and anti-inflammatory cytokines, and formation of follicle structures, which enhance and maintain local immune responses independent of the periphery (Claes et al., 2015; Michel et al., 2015; Owens et al.,

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2011; Pikor et al., 2015). B cells are effective antigen presenting cells and B cell depletion diminishes CD4 T cell numbers, reactivation, and proinflammatory cytokine production in MS patients and in experimental allergic encephalomyelitis (EAE), a rodent model of MS (Parker Harp et al., 2015; Pierson et al., 2014; Weber et al., 2010). B cells can also directly contribute to a proinflammatory milieu within the CNS via production of the cytokines TNF, lymphotoxin (LT), GM-CSF, and IL-6 (Bar-Or et al., 2010; Lehmann-Horn et al., 2011; Lino et al., 2016; Lisak et al., 2012; Malmestrom et al., 2006; Pierson et al., 2014; Xiao et al., 2015). Nevertheless, B cells can also assume anti-inflammatory roles by secreting IL-10 and TGF-β (Lino et al., 2016; Lisak et al., 2012; Tedder, 2015; Xiao et al., 2015). B cell regulation of pathogenic T cells is implicated by exacerbated disease severity coincident with increased CNS encephalitogenic T cells upon B cell depletion prior to EAE induction. Moreover, this regulation was attributed to IL-10 producing B cells (Matsushita et al., 2008, 2010). Subsequent studies further revealed multiple B cell subsets contribute to IL-10 and IL-35 production, including B10 B cells and plasma cells (Maseda et al., 2012; Shen et al., 2014; Tedder, 2015).

Irrespective of defining functions of individual B cell subsets, the broader question of their recruitment and maintenance within the CNS remains under investigation (Claes et al., 2015; Michel et al., 2015; Owens et al., 2011; Pikor et al., 2015). During MS, similar clonality among B cell subsets in the CLN and CNS suggests an axis of continual recruitment after maturation in the periphery (Bankoti et al., 2014; Palanichamy et al., 2014; Stern et al., 2014). However, local factors can further contribute in activating and/or sustaining B cells within the CNS. Lymphoid associated factors that support B cell organization, survival, and differentiation including IL-21, BAFF, CCL19, CCL21, and CXCL13, are all expressed during viral or autoimmune associated CNS inflammation (Kowarik et al., 2012; Krumbholz et al., 2006; Lalor and Segal, 2010; Magliozzi et al., 2004; Metcalf et al., 2013; Phares et al., 2011, 2013a, 2014, 2016; Shi et al., 2001). B cell clusters associated with lymphoid chemokines, dendritic cells, and multiple B cell phenotypes are indeed evident in the CNS during MS and spinal cord injury (SCI) (Ankeny et al., 2006; Magliozzi et al., 2004, 2007; Serafini et al., 2004). Similar structures, often termed ectopic follicles, are also common to chronic infection or autoimmunity at peripheral sites (Bombardieri et al., 2012; Carlsen et al., 2002; Weyand and Goronzy, 2003). While ectopic follicle formation is thus implicated in local B cell survival, activation, and differentiation independent of secondary lymphoid tissue, they are less organized than follicles in lymphoid organs. This questions the extent to which follicle formation is essential for B cell activation and isotype switching. Studies using mice deficient in CXCL13 or its receptor CXCR5, suggest isotype-switched Ab production can occur in the absence of defined follicles and germinal center (GC) formation following both peripheral and CNS infection (Junt et al., 2005; Phares et al., 2016; Rainey-Barger et al., 2011). B cell activation and maturation within the CNS may therefore occur independent of defined follicular structures under suitable conditions, which remain to be defined.

Using neurotropic coronavirus infection, this study aimed to better characterize kinetics, activation and structural organization of B cell subsets in the CNS relative to the cervical lymph nodes (CLN), the primary site for T and B cell activation. In this viral encephalomyelitis model, T cells clear infectious virus within 14 days post infection (p.i.), while ASC emerging within the CNS after initial viral control maintain persisting viral RNA at low levels and prevent recrudescence (Lin et al., 1999; Marques et al., 2011). Isotype-unswitched $IgD^+IgM^+$, $IgD^{int}IgM^+$, and $IgD^-IgM^+$ B cells accumulate early during infection prior to ASC and are progressively replaced by more differentiated $IgD^-IgM^-$ class-switched $B_{mem}$ and ASC (Phares et al., 2014). Using flow cytometry and histology we herein show that $IgD^+$ B cells were recruited to the CNS coincident with activation in CLN prior to GC formation. By contrast, class-switched B cells emerged concurrent with defined GC structures in the CLN, with no evidence for ectopic follicle formation in the CNS. Nevertheless, local CD4 T cell help was supported by sustained expression of proliferation and activation markers by the vast majority of more differentiated $IgD^{int}$ and $IgD^-$ B cells in the CNS as well as occasional CD4 T cell-B cell interactions. Overall, the results suggest initial CNS B cell recruitment is driven by activation events in CLN; however prolonged expression of activation markers on more differentiated B cells in the CNS relative to the CLN support local factors contribute to ongoing activation independent of ectopic follicle formation.

## 2. Materials and methods

### 2.1. Mice and infection

Wild type (WT) C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). All mice were housed under pathogen free conditions at an accredited facility at the Cleveland Clinic Lerner Research Institute. Six-to seven-week old mice were infected intracranially (i.c.) with 1000 plaque forming units (PFU) of J.2.2v-1 monoclonal Ab variant JHMV (Fleming et al., 1986). All procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee.

### 2.2. Cell isolation

For phenotypic analysis, brain or CLN derived mononuclear cells were isolated from pooled organs of 3–6 mice per time point. Briefly, brains were minced and digested in 5 ml RPMI supplemented with 10% fetal calf serum (FCS), 100 μl 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. Collagenase and DNase I activity was terminated by addition of 0.1 M EDTA (pH 7.2) at 37 °C for 5 min. Cell pellets were resuspended in RPMI medium, adjusted to 30% Percoll (Pharmacia, Piscataway, NJ), and underlaid with 1 ml of 70% Percoll. Following centrifugation for 30 min at 850 x g, cells were recovered from the 30/70% Percoll interface, washed with RPMI supplemented with 2% FCS and resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS with 0.5% bovine serum albumin) for flow cytometry or RPMI 1640 supplemented with 2 mM L-Glutamine, 2 mM non-essential amino acids, 1 mM sodium pyruvate, 25 μg/ml gentamicin, $5 \times 10^{-5}$ M 2-mercaptoethanol, and 10% FCS (RPMI complete) for cell culture. CLN cells were isolated as previously described (Lin et al., 1999).

For feeder layers, spleens from naïve C57BL/6 mice were dissociated mechanically and red blood cells lysed. Resuspended splenocytes were irradiated with 3000 rad using a Shepherd irradiator (JL Shepherd and Associates, San Fernando, CA), washed, resuspended in RPMI complete and $5x10^5$ cells in 0.1 ml plated per well prior to *in vitro* stimulation.

### 2.3. In vitro B cell stimulation and ELISPOT assay

Brain derived single cell suspensions were resuspended at a starting concentration of $2x10^4$ cells/0.1 ml of RPMI complete containing 0.6 μg/ml LPS or 1 μg/ml multimeric CD40L (Adipogen, San Diego, CA) with 1 ng/ml recombinant mouse IL-4 (BioLegend, San Diego, CA). Cells were plated at 1:2 serial dilutions and stimulated for 3 or 4 (LPS) and 4 or 5 days (CD40L) with irradiated splenocytes. Stimulated cells were washed using prewarmed (37 °C)