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Lymphoid chemokines in chronic neuroinflammation

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Abstract

Lymphoid chemokines play an essential role in the establishment and maintenance of lymphoid tissue microarchitecture and have been implicated in the formation of tertiary (or ectopic) lymphoid tissue in chronic inflammatory conditions. Here, we review recent advances in lymphoid chemokine research in central nervous system inflammation, focusing on multiple sclerosis and the animal model experimental autoimmune encephalomyelitis. We also highlight how the study of lymphoid chemokines, particularly CXCL13, has led to the identification of intrameningeal B-cell follicles in the multiple sclerosis brain paving the way to the discovery that these abnormal structures are highly enriched in Epstein–Barr virus-infected B cells and plasma cells.

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1. Introduction

Lymphoid or homeostatic chemokines, including CCL19, CCL21, CXCL12 and CXCL13, are constitutively expressed in lymphoid organs and regulate the migration and compartmentalization of lymphocytes and antigen presenting cells within lymphoid tissues (Yoshie et al., 1997; Zlotnik et al., 1999). CCL19 and CCL21 are ligands for CCR7 and are produced by stromal cells and dendritic cells in the T cell zone (Cyster, 1999). CCL21 is also expressed in endothelial cells in high endothelial venules (HEV) and lymphatic vessels (Cyster, 1999). Genetic and functional studies have established an important role for these chemokines in dendritic cell and T cell

trafficking to T cell areas of secondary lymphoid organs (Yoshie et al., 1997; Müller et al., 2003). CXCL13 is produced by stromal cells in B cell areas (follicles), binds to CXCR5 and regulates homing of B cells and subsets of T cells (follicular B helper T cells) to lymphoid follicles (Förster et al., 1996; Legler et al., 1998; Ansel et al., 2000; Moser et al., 2002; Müller et al., 2003). CXCL12 is expressed broadly, acts as a potent chemoattractant for different types of immune cells by binding CXCR4, regulates hematopoiesis and, together with CXCL13, mediates germinal center organization in secondary lymphoid organs (Campbell et al., 2003; Allen et al., 2004). Chemokine expression in T and B cell areas of lymphoid organs is dependent on lymphotoxin (LT) α 1 β 2 and tumor necrosis factor (TNF) signaling through the LT β R and TNFR1, respectively (Mackay and Browning, 1998; Ngo et al., 1999; Fu and Chaplin, 1999).

Over the past several years it has become evident that, in addition to their role in lymphoid tissue development and maintenance, lymphoid chemokines are also implicated in

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inflammation. Since the early 70's histopathologists have observed that in chronic inflammatory conditions, like autoimmunity and infections, the immune cells infiltrating the target organs may organize themselves in structures that resemble the T and B cell areas of secondary lymphoid organs, the so called tertiary or ectopic lymphoid organs (Hjelmström, 2001). The discovery that lymphoid chemokines are involved in the formation of ectopic lymphoid tissue has led to the recognition that this process (also termed lymphoid neogenesis) shares similar mechanisms with lymphoid organogenesis (Cupedo and Mebius, 2003). Following the initial observation that CXCL13 is expressed in *Helicobacter pylori*-induced gastric mucosa-associated lymphoid structures (Mazzucchelli et al., 1999) and the findings of elegant studies showing that transgenic expression of LT (Kratz et al., 1996; Drayton et al., 2003) or individual lymphoid chemokines (Fan et al., 2000; Luther et al., 2000, 2002; Chen et al., 2002b; Martin et al., 2004) in non-lymphoid tissues (pancreas, thyroid) leads to formation of lymphoid-like structures, the role of lymphoid chemokines in regulating immune cell trafficking and organization of ectopic lymphoid tissue in inflammatory conditions has received increasing attention (Aloisi and Pujol-Borrell, 2006; Drayton et al., 2006). Formation of ectopic lymphoid tissue is viewed as part of an adaptive response against infection but may also have the potential to support autoimmunity through expansion and activation of autoreactive B and T lymphocytes (reviewed in Aloisi and Pujol-Borrell, 2006). In this article, we summarize current knowledge of lymphoid chemokines in neuroinflammation, particularly in multiple sclerosis, the most common inflammatory disease of the central nervous system (CNS), and the animal model experimental autoimmune encephalomyelitis (EAE), as these are the disease conditions in which lymphoid chemokines have been mostly investigated. We also review how the study of this particular set of molecules has led us to achieve novel and unexpected insights into the elusive aetiopathogenesis of MS.

2. Expression of lymphoid chemokines in the inflamed CNS

2.1. CCL19 and CCL21

In an early study addressing the role of lymphoid chemokines in the CNS, Sergio Lira's group showed that contrary to what had been observed in the pancreas, transgenic expression of CCL21 in the brain parenchyma was not associated with lymphocyte recruitment or lymphoid neogenesis (Chen et al., 2002a), suggesting the existence of tissue-specific requirements for the biological activity of CCL21. Concomitantly, Alt et al. (2002) described functional expression of CCL19 and CCL21 in inflamed venules in the brain and spinal cord of mice with EAE and suggested that these chemokines besides regulating lymphocyte trafficking in lymphoid organs were also involved in the migration of encephalitogenic T cells in the CNS. We and others also found upregulation of CCL19, CCL21 and their common receptor CCR7 in CNS inflammatory lesions of mice with EAE, particularly during the chronic phase (Columba-Cabezas et al., 2003; Bagaeva et al., 2006; Bielecki et al., 2007).

Further support to the idea that CCR7-binding chemokines could play a role in neuroinflammation was provided by analyses of brain tissue and cerebrospinal fluid (CSF) from patients with inflammatory neurological diseases. Several studies showed increased amounts of CCL19 in the CSF of patients with MS and with infectious diseases of the CNS as compared to patients with non-inflammatory neurological disease, whereas CCL21 was less consistently detected (Pashenkov et al., 2003; Giunti et al., 2003; Krumbholz et al., 2007). Krumbholz et al. (2007) also described constitutive gene expression of CCL19 in normal brain tissue and elevation of CCL19 transcripts in MS lesions, suggesting a role for CCL19 both in normal immune surveillance of the CNS and in inflammatory conditions. In the MS CSF, CCL19 levels were found to correlate with intrathecal Ig production, but only weakly with CSF cell counts (Pashenkov et al., 2003; Krumbholz et al., 2007). The observation that CCL21 mRNA and protein are undetectable in MS inflammatory lesions and meninges (Kivisäkk et al., 2004; Serafini et al., 2004; Krumbholz et al., 2007) is consistent with the absence of markers of HEV and lymphatic vessels in the MS brain (Serafini et al., 2006; Serafini and Aloisi, unpublished data). These findings are in contrast with data obtained in the EAE model (Cannella et al., 1990; Alt et al., 2002; Columba-Cabezas et al., 2003), suggesting species differences and/or existence of disease-specific mechanisms in the acquisition of HEV-like features by CNS blood vessels. However, Kivisäkk et al. (2004) reported expression of CCL21 in the human choroid plexus in both non-inflammatory and inflammatory CNS conditions.

Interestingly, abundant expression of CCR7 was found in activated, resident microglia and myeloid dendritic cells but not in lymphocytes infiltrating MS lesions (Kivisäkk et al., 2004; Serafini et al., 2006), whereas CCR7 was consistently found on most CD4+ memory T cells obtained from the CSF of individuals without CNS inflammation and of patients with MS (Giunti et al., 2003; Kivisäkk et al., 2003). About 30% of the dendritic cells present in the CSF of MS patients were also found to express CCR7 (Kivisäkk et al., 2004). The latter findings suggest that CCL19 could be involved in the trafficking of CCR7+ T lymphocytes and antigen presenting cells through the ventricular compartment and perhaps from this compartment to CNS-draining lymph nodes. Upregulation of CCR7 on activated microglia is intriguing as it suggests regulation of microglia migratory properties or function by lymphoid chemokines (Dijkstra et al., 2006).

2.2. CXCL12

CXCL12 is a chemokine that is constitutively expressed in the CNS (Lazarini et al., 2003) and is upregulated during inflammation. Increased levels of CXCL12 have been detected in the CSF of patients with MS and other inflammatory neurological diseases (Pashenkov et al., 2003; Giunti et al., 2003; Corcione et al., 2004; Krumbholz et al., 2006). Cerebrovascular endothelia and astrocytes have been identified as major sources of CXCL12 in inflamed MS lesions (Ambrosini et al., 2005; Krumbholz et al., 2006). Because CXCR4 is expressed on immature myeloid dendritic cells,

plasmacytoid dendritic cells, T cells, B cells and plasma cells (Campbell et al., 2003), it is likely that CXCL12 production in the CNS regulates intracerebral homing of different immune cell populations. Using *in vitro* assays, we demonstrated that human astrocytes release biologically relevant amounts of B-, T- and dendritic cell-attracting chemokines, including CXCL12 (Ambrosini et al., 2003, 2005). It is interesting to note that some plasma cells infiltrate the parenchyma in chronic MS lesions (Serafini et al., 2004), where abundant expression of CXCL12 in activated astrocytes is also noted (Ambrosini et al., 2005). Because plasma blasts can use CXCL12 as a chemoattractant, it is likely that the CXCL12–CXCR4 axis plays a role in creating, together with B-cell survival factors like BAFF (Krumbholz et al., 2005), a survival niche for antibody-producing cells inside the brain parenchyma.

2.3. CXCL13

Because abnormal humoral immune responses characterized by intrathecal accumulation of B cells and plasma cells, B-cell clonal expansions and Ig synthesis are a prominent feature of MS and several infectious diseases of the CNS (reviewed in Uccelli et al., 2005), several recent studies have addressed the role of the B-cell attracting chemokine CXCL13 in CNS inflammation. In 2004, our group reported induction of CXCL13 mRNA in the CNS of mice with EAE and by using immunohistochemical techniques showed that CXCL13-expressing cells were present inside large B-cell aggregates in the inflamed brain meninges (Magliozzi et al., 2004). We also provided evidence that such abnormal B-cell aggregates exhibited some features of germinal center organization as they comprised proliferating B cells and a reticulum of stromal/follicular dendritic cells, which constitutively produce CXCL13 in B-cell follicles of secondary lymphoid organs. These observations led us to propose that CNS inflammation, through local production of LT α 1 β 2 and TNF by tissue-infiltrating immune cells (Columba-Cabezas et al., 2006), induces expression of CXCL13 which in turn attracts B cells and promotes their compartmentalization with formation of germinal centers in the inflamed CNS meninges. In agreement with our findings, Bagaeva et al. (2006) found upregulation of CXCL13 mRNA and protein and infiltration of CXCR5+ cells in the spinal cord of EAE-affected mice.

The subsequent step was to find out whether CXCL13 and germinal center-like structures could be detected in the MS brain. In a first immunohistochemical analysis of post-mortem brain tissue from 6 MS cases with relapsing-remitting and progressive clinical courses, we observed the presence of B-cell aggregates containing a well developed network of CXCL13-expressing cells, numerous proliferating B cells, plasma cells and T cells in the subarachnoid space (the space lying between the pial and arachnoid membranes and filled with CSF) of 2 of 3 cases with secondary progressive MS (Serafini et al., 2004). Notably, despite prominent, perivascular accumulation of B cells and plasma cells in chronic white matter lesions, neither CXCL13 nor proliferating B cells were detected at these sites, suggesting that the meninges, but not the Virchow–Robin space

surrounding CNS intraparenchymal blood vessels, provide a permissive milieu for lymphoid neogenesis. Because chemokine-producing stromal cells in lymphoid organs are of mesenchymal origin (Parsonage et al., 2005), we hypothesized that stromal cells involved in the organization of MS ectopic follicles could differentiate from precursors residing in the meninges or migrating there from the blood circulation (Serafini et al., 2004). The above findings were confirmed in a larger set of brain specimens from cases with primary and secondary progressive MS ($n=36$) (Magliozzi et al., 2007). Variable numbers of intrameningeal B cell follicles, all containing CXCL13-producing stromal cells, were detected only in the brain of a proportion of cases that had developed secondary progressive MS (12 out of 29 examined), but in none of the 7 cases with primary progressive MS analyzed. In Fig. 1, an intrameningeal B-cell follicle stained for the pan B-cell marker CD20 and for CXCL13 is shown (panels A and B, respectively).

At variance with our findings (Serafini et al., 2004; Magliozzi et al., 2007), Krumbholz et al. (2006) reported increased CXCL13 immunoreactivity in active MS lesions, both in the perivascular cuffs and inside the parenchyma, and confirmed CXCL13 expression in MS brain tissue by using real time PCR. Since it has been reported that macrophages and dendritic cells can produce CXCL13 under inflammatory conditions (Perrier et al., 2004; Carlsen et al., 2004), discrepant immunohistochemical findings in the MS brain could be due to technical issues or differences in the inflammatory activity of the lesions analyzed. Krumbholz et al. (2006) also demonstrated increased levels of CXCL13 in the CSF of patients with MS and other inflammatory neurological diseases and found a correlation between CXCL13 concentration and presence of B cells, plasma blasts, T cells and intrathecally produced Ig in the CSF. In addition, the same group detected expression of the CXCL13 receptor CXCR5 on nearly all B cells and on a proportion of T cells isolated from the CSF of MS patients.

Taken together, the above studies link CXCL13 expression in the CNS with the degree of inflammation and the development of ectopic lymphoid structures and point to CXCL13 as an attractive target for therapeutic interventions aimed at blocking B-cell recruitment and/or lymphoid neogenesis in the inflamed CNS. In a study performed in the EAE model we found that blocking LT α 1 β 2 signaling with the fusion protein LT β R-Ig, which acts as a decoy receptor (Browning et al., 1997), reduced EAE severity and prevented induction of CXCL13 and formation of organized B-cell follicles in the meninges, but failed to inhibit intrameningeal B-cell migration and aggregation (Columba-Cabezas et al., 2006). We interpreted these findings as evidence that CXCL13 may be more important for the development of ectopic lymphoid tissue than for B-cell recruitment in the CNS. Along the same lines, Bagaeva et al. (2006) showed that both CXCL13-deficiency and treatment with anti-CXCL13 neutralizing antibodies reduced clinical severity in EAE and proposed that CXCL13 may coordinate the localization and interactions of immune cells within the CNS rather than attracting CXCR5+ cells from the periphery. Interestingly, CXCL13 expression was not induced in the CNS of mice developing encephalomyelitis after infection with

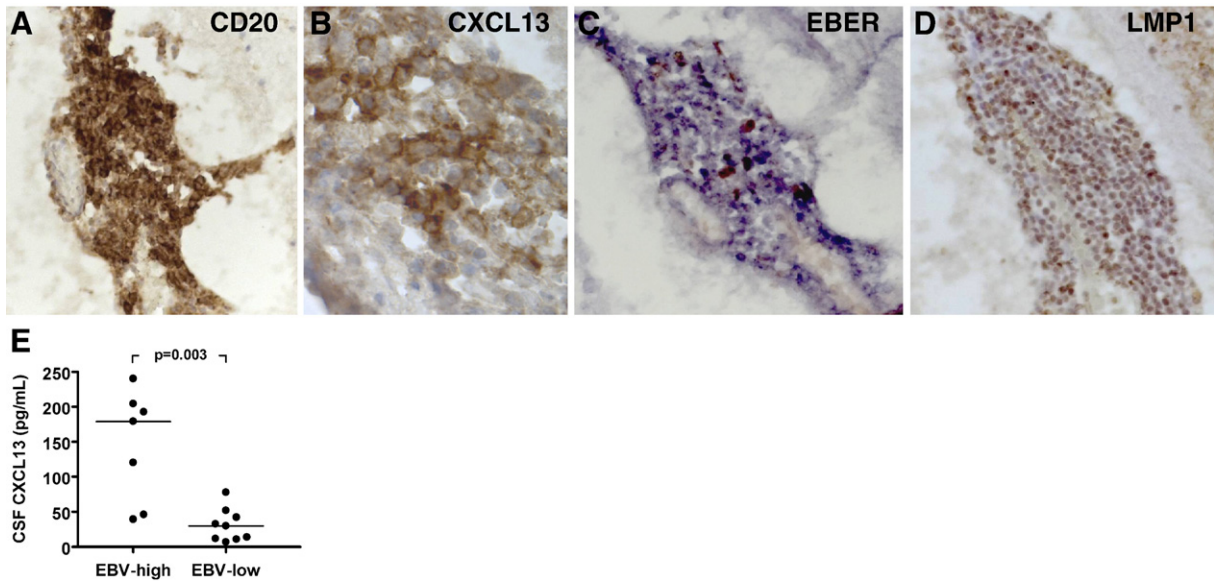


Fig. 1. CXCL13 in MS ectopic B-cell follicles and CSF. Immunostainings for the pan B-cell marker CD20 (A) and for the lymphoid chemokine CXCL13 (B) were performed on serial brain sections from a case with secondary progressive MS, as previously described (Serafini et al., 2007). An intrameningeal B-cell follicle comprising numerous CD20+ B cells (A) aggregated around a network of CXCL13+ stromal cells/follicular dendritic cells (B) is shown. *In situ* hybridization for EBER (C) and immunostaining for the EBV latent protein LMP1 (D) (see Serafini et al., 2007 for staining protocols) reveal enrichment in EBV-infected cells inside the same B-cell follicle shown in A, B. Original magnifications: 250 \times in A, C and D, 1000 \times in B. Panel E shows the levels of CXCL13 protein in post-mortem CSF of MS cases that were classified as EBV-high ($n=7$) and EBV-low ($n=9$) based on the frequency of EBV-infected cells accumulating in the meninges and in white matter lesions (Serafini et al., 2007). The levels of CXCL13 are significantly higher in the CSF of MS cases characterized by more prominent deposits of EBV infection in brain tissue. CXCL13 levels were determined with a quantitative sandwich ELISA, in accordance with manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Bars indicate median values. Between-group comparison was performed by Mann–Whitney test.

neurotropic coronavirus despite prominent accumulation of virus-specific antibody-secreting cells in the target tissue, a finding that may suggest lack of ectopic B-cell follicle formation (Tschen et al., 2006). In this model, CXCL9 and CXCL10, both of which act on plasma blasts through CXCR3, have been implicated in the recruitment of antibody-producing cells in the infected CNS (Tschen et al., 2006).

Increased expression of CXCL13 was also found in the brain and CSF of patients with Lyme neuroborreliosis, a chronic infection in which B-cell activation, plasma cell infiltration and enhanced Ig production in the CNS are a prominent feature (Narayan et al., 2005; Rupprecht et al., 2006). Moreover, CXCL13 has been detected in primary CNS lymphoma where it is produced by malignant B cells and transported on the luminal surface of cerebrovascular endothelial cells (Smith et al., 2003; Brunn et al., 2007), suggesting that this chemokine may influence tumor development and localization in the CNS.

3. Pathological relevance of ectopic lymphoid tissue in MS

Because ectopic lymphoid tissue forming in an inflamed tissue could sustain a local immune response against microbial or self antigens, the discovery of B-cell follicles in the MS brain raised a number of questions concerning the possible contribution of these structures to MS immunopathology, particularly to intrathecal Ig synthesis (Serafini et al., 2004; Aloisi and Pujol-Borrell, 2006).

Careful analysis of the cerebral cortex adjacent to ectopic B-cell follicles revealed the presence of demyelinated lesions

whose extension and degree of neuronal loss and microglia activation were greater than those observed in the cerebral cortex of MS cases with lower meningeal inflammation (Magliozzi et al., 2007). Of importance, such a dramatic pattern of cortical pathology correlated with a more severe disease course in MS cases with follicles compared to those without (Magliozzi et al., 2007). These findings suggested the possibility that ectopic B-cell follicles are a source of diffusible factors, such as (auto)antibodies, pro-inflammatory cytokines and/or proteolytic enzymes, that cause injury to neural cells directly or through induction of microglia activation.

Further immunohistochemical analysis revealed that ectopic B-cell follicles in MS meninges also contain numerous CD8+ cytotoxic T cells and plasmacytoid dendritic cells, both of which play an essential role in antiviral responses. This observation prompted us to search for the presence of a viral agent inside these abnormal structures (Serafini et al., 2007). The most likely candidate appeared to be Epstein–Barr virus (EBV) for two main reasons. The first one stems from the bulk of seroepidemiological data and immunological findings supporting an association between EBV infection and MS (reviewed in Ascherio and Munger, 2007; Lünemann et al., 2007). The second one was related to the unique ability of EBV to establish a latent infection in B cells and, through expression of viral latency proteins, to drive B-cell proliferation and activation (Thorley-Lawson, 2001), these features being compatible with the presence of expanded B cell clones and Ig-producing plasma cells in the MS brain (Uccelli et al., 2005). The prediction turned out to be correct as *in situ* hybridization for EBV-

encoded small RNAs (EBER) and immunohistochemical stainings for two EBV latency proteins (EBNA-2, LMP1) revealed that ectopic B-cell follicles were highly enriched in EBV-infected cells (Serafini et al., 2007) (see Fig. 1C, D for EBER and LMP1 stainings, respectively). EBER⁺ cells were identified essentially as B cells and plasma cells. Variable numbers of EBV-infected cells were also detected in the meninges and perivascular immune infiltrates of active white matter lesions in nearly 100% of the MS cases analyzed (21 out of 22 with different clinical courses), their frequency being correlated with the degree of inflammation. Expression of markers of the EBV lytic cycle appeared restricted to acute inflammatory lesions in the white matter of MS cases with progressive disease and was abundant in the highly inflamed brain of cases with very severe, fulminant MS. Of note, EBV lytic proteins were consistently found in ectopic B-cell follicles indicating that these structures represent major occult sites of viral reactivation in the MS brain (Serafini et al., 2007).

We also provided evidence that CD8⁺ T cells accumulating in ectopic follicles and active MS lesions show signs of activation, such as proliferation and IFN- γ production, and display cytotoxic activity toward EBV-infected B cells/plasma cells (Serafini et al., 2007). These findings led us to propose that a CNS compartmentalized and dysregulated infection with EBV drives MS-associated immunopathology through expansion and activation of latently infected B cells, periodic viral reactivation, and induction of an EBV-specific cytotoxic immune response. Most probably, the latter plays a major role in mediating CNS tissue damage.

Interestingly, analysis of post-mortem CSF revealed the presence of significantly higher levels of CXCL13 in the MS cases with ectopic follicles and a higher frequency of CNS-infiltrating EBV-infected cells as compared to the MS cases with less brain inflammation and a lower frequency of EBV-infected cells (Fig. 1E). This finding suggests that CXCL13 could play a key role in the compartmentalization of EBV-infected B cells within intrameningeal niches, allowing their expansion and organization in germinal center-like structures. Of interest, a recent study has shown that lymphoid chemokines (CCL19, CCL21, CXCL12 and CXCL13), in addition to their chemoattractant properties, are able to enhance the survival of chronic lymphocytic leukemia B cells, but not of normal B cells, in vitro (Ticchioni et al., 2007). The hypothesis that lymphoid chemokines might play a role in promoting the survival of EBV-infected B cells should therefore be tested.

4. Concluding remarks

The work summarized in this article provides a remarkable example of how the study of lymphoid chemokines as markers of chronic inflammation in a complex neurological disease like MS has helped to direct (or redirect) the focus of neuropathological analyses on a compartment, the meninges, that has received little attention during the past decades (Guseo and Jellinger, 1975; Prineas and Wright, 1978; Lassmann et al., 2007). Immune surveillance of the CNS routinely occurs across the meningeal-ventricular compartment (Engelhardt and Ransohoff, 2005) and it is therefore plausible that events taking place therein play an

important role in MS pathogenesis. Because the analysis of CXCL13 in the MS brain has been the starting point for the identification of intrameningeal B-cell follicles, their characterization as foci of EBV infection, and the recognition of a link between dysregulated EBV infection and inflammatory activity in MS lesions, it is fair to state that research on lymphoid chemokines has played a key role in advancing our comprehension of this complex disease. The challenge for the future is to understand how this knowledge can be exploited for therapeutic purposes.

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