Clinical Immunology (2015) 158, 47–58

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

Central nervous system infiltrates are characterized by features of ongoing B cell-related immune activity in MP4-induced experimental autoimmune encephalomyelitis

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Received 17 August 2014; accepted with revision 12 March 2015
Available online 18 March 2015

KEYWORDS
B cells;
EAE;
Epitope spreading;
MS;
TLO

Abstract In multiple sclerosis (MS) lymphoid follicle-like aggregates have been reported in the meninges of patients. Here we investigated the functional relevance of B cell infiltration into the central nervous system (CNS) in MP4-induced experimental autoimmune encephalomyelitis (EAE), a B cell-dependent mouse model of MS. In chronic EAE, B cell aggregates were characterized by the presence of $CXCL13^+$ and germinal center $CD10^+$ B cells. Germline transcripts were expressed in the CNS and particularly related to T_H17-associated isotypes. We also observed B cells with restricted VH gene usage that differed from clones found in the spleen. Finally, we detected CNS-restricted spreading of the antigen-specific B cell response towards a myelin and a neuronal autoantigen. These data imply the development of autonomous B cell-mediated autoimmunity in the CNS in EAE — a concept that might also apply to MS itself.

1. Introduction

Multiple sclerosis (MS) is the most prevalent disease of the central nervous system (CNS) in young adults. Histopathologically, MS is characterized by CNS inflammation, autoimmune demyelination and axonal damage [1]. While the pathogenesis of the disease is generally assumed to be driven by T helper cells and macrophages, there is accumulating evidence that B cells also play a crucial role in the disease process. Clonally expanded B cells are found in the cerebrospinal fluid, in

Abbreviations: BCR, B cell receptor; BSA, bovine serum albumin; cDNA, first strand copy DNA; CDR3, complementarity determining region 3; CFA, complete Freund's adjuvant; CNS, central nervous system; CSR, class switch recombination; EAE, experimental autoimmune encephalomyelitis; FDC, follicular dendritic cell; GLT, germline transcript; IFA, incomplete Freund's adjuvant; IHC, immunohistochemistry; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MP4, MBP–PLP fusion protein; MS, multiple sclerosis; NF-L, neurofilament light chain; PFA, paraformaldehyde; PLP, proteolipid protein; TLO, tertiary lymphoid organ.

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http://dx.doi.org/10.1016/j.clim.2015.03.009
1521-6616/

demyelinating lesions, the normal appearing white matter and in meningeal infiltrates [2–4]. B cell clones isolated from the CNS are hardly represented in the peripheral blood, and some of the CNS clones carry markers of cell proliferation [5]. Thus, B cells may infiltrate into and further expand within the CNS itself. The pathogenic relevance of the autoimmune B cell response is underlined by histopathological findings: the most abundant demyelination pattern is characterized not only by T cell and macrophage infiltration, but also by immunoglobulin and complement deposition [6].

Probably the most intriguing and controversial aspect of B cell involvement in MS is the formation of lymphoid follicle-like structures in the CNS. These structures have also been termed "tertiary lymphoid organs" (TLO) because they feature an anatomy that is similar to secondary lymphatic tissue, while developing at sites of chronic inflammation. TLO have been described in allograft rejection, infectious diseases, and a wide range of autoimmune disorders [7]. In MS, though, the existence of TLO is a subject of debate. The development of B cell aggregates has repeatedly been shown in MS patients and has been found to be associated with more severe neuropathology and clinical disease [8–10]. However, these findings have been challenged by others [11,12].

Since specimens from patients are rare and present a heterogeneous sample population, the use of animal models is indispensable for basic research on the role of B cells in MS. The best studied animal model for MS is experimental autoimmune encephalomyelitis (EAE), which can be induced by active immunization of susceptible animal strains with myelin antigens. Most EAE models are mainly dependent on T helper cells and macrophages [13], though, and are therefore ill-suited for research on B cells. We have previously developed a model in which immunization of C57BL/6 mice with MP4 – a fusion protein of myelin basic protein (MBP) and the three hydrophilic loops of proteolipid protein (PLP) – reliably induced B cell-dependent EAE [14–16]. MP4-specific IgG autoantibodies bound to the myelin sheath and activated the complement cascade, thus leading to tissue damage [17]. High numbers of B cells infiltrated the spinal cord, the brain and the cerebellum of MP4-immunized mice [15]. About half of the B cell aggregates histologically qualified as TLO [18]. These TLO were characterized by the presence of high endothelial venules, specialized vessels which are important for lymphocyte entry into lymphatic tissues. They further comprised a network of follicular dendritic cells (FDC), which are involved in B cell selection and antigen presentation. The TLO were segregated into a B and T cell zone. The B cell zone was populated by $B220^+$ B cells and $CD138^+$ plasmablasts, the T cell zone mainly by T helper cells secreting IL-17 (T_H17 cells) [18].

These histological observations prompted us to investigate whether B cells infiltrating into the CNS in MP4-induced EAE cannot only take over the structure, but also convey the function of lymphatic tissue. Here we provide evidence for class switch recombination (CSR), somatic hypermutation and epitope spreading of the autoimmune B cell response in MP4-immunized mice. In this scenario, the B cell response in the CNS could gradually become independent from the immune periphery.

2. Material and methods

2.1. Animals and EAE induction

C57BL/6 mice at the age of 6–8 weeks were purchased from Janvier (Saint Berthevin Cedex, France) and maintained at the animal facilities of the Department of Anatomy of Cologne University under specific pathogen free conditions. For EAE induction, incomplete Freund's adjuvant (IFA) was prepared as a 9:1 mixture of paraffin oil (EMScience, Gibbstown, NJ, USA) and mannide monooleate (Sigma-Aldrich, St. Louis, MO, USA). Complete Freund's adjuvant (CFA) was prepared by adding 5 mg/ml *Mycobacterium tuberculosis* H37 RA (Difco Laboratories, Franklin Lakes, NJ, USA). Mice at the age of 8–10 weeks were immunized subcutaneously in both flanks with a total dose of 200 μg MP4 (Alexion Pharmaceuticals, Cheshire, CT, USA) emulsified in CFA (total injection volume = 200 μl per mouse). 200 ng pertussis toxin (List Biological Laboratories, Hornby, ONT, Canada) in 500 μl sterile PBS (PAA, Pasching, Austria) was administered on days 0 and 2 post immunization. Clinical signs were evaluated daily according to the standard EAE scale: 0 — no EAE, 1 — floppy tail, 2 — hind limb weakness, 3 — hind limb paralysis, 4 — quadriplegia, and 5 — death. Mice were euthanized with CO_2 either 50 or 70 days after EAE onset for the analysis of germline transcripts and next generation sequencing. For immunohistochemistry, mice were euthanized six to eight weeks after disease onset and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M PBS. The tissue was post-fixed in 4% PFA overnight before it was embedded in paraffin. All animal experiments complied with the German Law on the Protection of Animals and the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and were performed according to a protocol that was approved by the LANUV, Germany (approval number 2011.A276).

2.2. Immunohistochemistry (IHC)

Paraffin sections of the cerebellum were dehydrated in a descending alcohol series prior to IHC. Epitope retrieval was performed in 0.1 M citrate buffer. Sections were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 2 h at room temperature. Sections were then incubated with the primary antibodies directed against CXCL13 (R&D Systems, Minneapolis, MN, USA, diluted 1:80), B220 (eBioscience, Frankfurt, Germany; diluted 1:500), CD10 (abcam, Cambridge, UK; diluted 1:20), CD43 (BD Biosciences, Heidelberg, Germany; diluted 1:150), CD5 (Bioss, Woburn, MA; diluted 1:150) or IgM-Cy3 (Fisher Scientific, Schwerte, Germany; diluted 1:500) in PBS or IL-10 (R&D Systems; diluted 1:50) in 0.2% BSA and 1% normal rabbit serum (Vector Laboratories) at 4 °C overnight. We performed double stainings for B220/CXCL13, B220/CD10, B220/IL-10 and CD43/CD5/IgM. Isotype control antibodies (all from Fisher Scientific) were used for control purposes. As an additional control, sections were incubated in the absence of primary antibody. The next day, goat anti-rat Cy2 (diluted 1:300), goat anti-rat Cy3 (diluted 1:600), goat anti-rat Cy5 (diluted 1:300), goat anti-mouse Cy5 (diluted 1:400), goat anti-rabbit Cy5 (diluted 1:400) (all from Dianova, Hamburg, Germany) or biotin-conjugated rabbit anti-goat