The Role of B Cells in the Early Onset of the First Demyelinating Event of Acute Optic Neuritis

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PURPOSE. We evaluated the molecular pathways that operate in the early phase of acute optic neuritis (ON) by studying gene expression profiles of peripheral blood mononuclear cells (PBMCs) subpopulations, including CD19+ B cells, CD14+ macrophages, and CD4+ and CD8+ T cells.

METHODS. Samples of PBMC subpopulations were obtained from 18 MS patients within 96 hours of the acute onset of the first demyelinating event of ON, and from 14 age- and sex-matched healthy subjects. High throughput gene expression analysis was performed on samples from six ON patients and nine healthy subjects using Affymetrix technology. Data were analyzed using Partek software, and most informative genes (MIGs) were defined as genes with P < 0.01 and fold change > 2.0. Molecular pathways were analyzed by Ingenuity software. Verification of key MIGs was done on samples from five independent ON patients and five healthy subjects by quantitative (Q) RT-PCR and Western blot. Functional assay to test antigen presentation ability of sorted B cells was performed on blood samples from seven additional ON patients.

RESULTS. Significantly differentiating gene expression signatures consisting of 467, 55, and 55 MIGs respective of CD19+, CD14+, and CD4+ cells, were identified between ON and healthy subjects. No MIGs were detected for CD8+ cells. The major involvement of CD19+ B cells in the early stage of ON was characterized by enrichment of genes involved in activation of immune mechanisms (P = 3.2 × 10^-25 to 2.5 × 10^-3), including cellular immune response (P = 7.1 × 10^-12), B-cell cellular growth and proliferation (P = 1.0 × 10^-7), activation of immune cells trafficking pathways (P = 5.4 × 10^-15 to 2.0 × 10^-3), and stimulation of antigen presentation (P = 8.9 × 10^-11). This massive B-cell–restricted initiation of the immune response in the early disease process of ON was followed by low CD14+ and CD4+ cells activity and CD8+ cells anergy.

CONCLUSIONS. Our findings demonstrate that CD19+ B cells have a significant role in the pathogenesis of the first demyelinating event of acute ON and suggest their role as a possible target for immunomodulation.

Keywords: optic neuritis, B cells, multiple sclerosis

Optic neuritis (ON) is a common manifestation of multiple sclerosis (MS); it appears as the presenting symptom in approximately 25% of MS patients and occurs in 30% to 70% of patients with MS during the course of their illness.1–4 Clinically, ON is characterized by sudden painful onset of visual loss that develops within hours to a few days and is accompanied by decreased sensation of light brightness, decrease in color vision, and central visual field defect.5,6 The pathological changes in ON involve a complex interplay between active de- and re-myelination associated with axonal loss.6 The majority of patients have plaques in their optic nerves, irrespective of a clinical history of ON.7 Although the mechanism of injury in ON is inflammatory demyelination, the operating triggering components are unclear.

In MS it is suggested that autoreactive CD4+ T cells are activated in the peripheral blood by nonself antigens that resemble central nervous system (CNS) myelin proteins.8 Thereafter, these cells migrate through the blood–brain barrier (BBB) in response to chemotactic signals, stimulate CD14+ macrophage by secretion of inflammatory cytokines, such as IFN-γ and IL-1, to induce myelin damage.9 In addition to CD4+ T-cell–mediated demyelination, there is evidence implicating humoral immune involvement in ON with identification of clonal populations of B lymphocytes and plasma cells in the cerebrospinal fluid.10

Studying gene expression patterns of immune cell subpopulations is of importance for revealing the role and influence of each of them in the early ongoing ON pathological process.11

In the current study, we aimed to characterize the molecular mechanisms involved in the first event of acute ON that later develops to MS. Using gene expression microarrays technology, we profiled CD19+ B lymphocytes, CD14+ macrophages, CD4+ T lymphocytes, and CD8+ T lymphocytes to identify the early pathogenic mechanisms involved. Understanding the nature of
the molecular mechanisms that operate in the first event of ON that later progress to MS is important to identify effective therapeutic targets. The involvement of B cells in early ON was not previously described in the literature and it is one of the innovative findings of our work.

MATERIALS AND METHODS

Subjects

Patients with the first clinical presentation of acute unilateral ON without previous neurological symptoms, and age- and sex-matched healthy subjects participated in the study.

Inclusion criteria were as follows: (1) age between 18 and 40 years; (2) a diagnosis of ON based on unilateral visual loss of >20/40 associated with dyschromatopsia, afferent pupillary defect, and central/paracentral visual field defect; (3) no prior neurological symptomatology of any kind; (4) brain magnetic resonance imaging (MRI) suggestive of demyelinating disease according to the criteria of Barkhof et al.12, (5) time from the onset of visual symptoms to blood sampling ≤96 hours; and (6) development of definite MS within one year of clinical follow-up.

Sheba Medical Center Internal Review Board (IRB) committee approved the study and all subjects signed written informed consent. The study was conducted in compliance with the Helsinki declaration guidelines for studies in humans.

Cell Sorting

Peripheral blood mononuclear cells (PBMC) were purified using Ficoll Hypaque gradient (Invitrogen, Carlsbad, CA, USA). Cell subsets of CD4+, CD8+ T lymphocytes, CD19+ B cells, and CD14+ macrophages subpopulations were isolated using FITC-conjugated magnetic microbeads (purity > 95%; BD Pharmingen, San Jose, CA, USA) and analyzed by fluorescence activated cell sorting (FACS; Beckton Dickinson, Franklin Lakes, NJ, USA).

Microarray Preparation

Microarray gene expression was performed separately for each cell subpopulation. Total RNA was purified using Trizol (Invitrogen), and RNA integrity was assessed by RNA Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA). Probe synthesis using 3 μg total RNA was performed using the two-cycle RNA amplification kit protocol and in vitro transcription performed with the GeneChip Labeling Kit (both Affymetrix, Inc., Santa Clara, CA, USA). The biotin-labeled IVT–RNA hybridized to a Genechip array (HU133A-2, including annotated 14,500 human genes), washed in a GeneChip Fluidics Station 450 (Hewlett Packard, Palo Alto, CA, USA), and scanned (GeneArray-TM scanner G2500A) according to the manufacturer's protocol. Gene expression raw data were submitted to the public Gene Expression Omnibus (GEO) repository (available in the public domain at www.ncbi.nlm.nih.gov), accession number GSE62584.

Data Analysis

Data analysis was performed by Partek Genomics Solution software (available in the public domain at www.partek.com). Expression values were computed from raw CEL files by applying the Robust Multi-Chip Average background correction algorithm included: (1) values background correction, (2) quintile normalization, (3) log2 transformation, and (4) median polish summarization. Most informative genes (MIGs) were defined as those with a $P < 0.01$ and a change of $\geq 2.0$-fold by ANOVA. Biological functional annotation and pathway analysis were performed by Ingenuity software (available in the public domain at www.ingenuity.com). Significance of functional analyses were defined as $P < 0.01$ by The Benjamini and Hochberg False Discovery Rate method.

Verification by Quantitative RT-PCR

Key MIGs were verified by quantitative RT-PCR on a 7500 Realtime PCR Cycler (Applied Biosystems, Carlsbad, CA, USA) using Taqman probes adjusted to specific genes. All reactions were run in triplicates and processed on an ABI 7500 HT Detection System. Amplification data was analyzed with the ABI Prism SDS 2.1 software (Applied Biosystems). Relative quantification of the gene expression was performed by the ΔΔCt method. HTRP1 was used as a housekeeping gene, as it is characterized by stable expression in our data.

Verification by Western Blot Analysis

Protein levels of significant key genes HLA-DQβ1 and HLA-DOB were analyzed using 12% acrylamide SDS-PAGE gel. Proteins were transferred into nitrocellulose membranes and stained by monoclonal HLA-DQ (TAL4.1) and HLA-DOB (V16) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and by goat-anti-mouse HRP-conjugated antibody (Jackson Laboratories, Bar Harbor, ME, USA). Samples from HLA-DQ- and HLA-DO-typed subjects were used as positive controls. Experiments were repeated four times.

Functional Assay to Test Antigen Presenting Function of Sorted B Cells

B cells were tested in vitro using stimulation with CD40L and IL-4 to study their ability to be efficient myelin-specific antigen-presenting cells to autologous T cells as previously described.13–15 Purified B cells ($10^6$ cells/mL) from 7 patients with acute ON were cultured with or without the presence of human CD40L, irradiated with 10,000 rad to inhibit proliferation, and stimulated with 10 ng/mL recombinant human IL-4 (rhIL-4; R&D Systems, Minneapolis, MN, USA) for 5 days in human culture medium (10% human AB serum, 100 μg/mL penicillin, 100 IU streptomycin, 2 mM L-glutamine, 5 μg/mL insulin, 90% Iscove’s modified Dulbecco’s medium). Thereafter, 2 × $10^6$ CD40L-activated B cells were harvested and incubated with 10 μg biotin-labeled myelin basic protein (MBP; Millipore Corporations, Darmstadt, Germany). The number of B cells specifically binding MBP peptide was measured by FACS flow cytometer using B cells incubated without CD40L as a negative control.

RESULTS

Subjects

We included in the study 18 patients with acute onset of ON and 14 healthy subjects. Samples from six patients (four females/two males, average age $\pm$ SD 35.0 ± 7.1 years) and nine healthy subjects (five females/four males, average age $\pm$ SD 36.0 ± 5.4 years) were used for gene array experiments, and samples from five patients with acute onset of ON (three females/two males, average age $\pm$ SD 37.0 ± 4.2 years) and five healthy subjects (four females/one male, average age $\pm$ SD 35.5 ± 4.6 years) were used for verification by QRT-PCR and Western blot.

Samples from seven patients with acute onset of ON (four females/three males, average age $\pm$ SD 38.0 ± 2.0 years) were applied for functional assay to test antigen presenting function of sorted B cells.
ON Gene Expression Signature

The characterizing ON gene expression signature included 467 MIGs in CD19\(^+\) B-lymphocytes, 55 MIGs in CD14\(^+\) macrophages, and 55 MIGs in CD4\(^+\) lymphocyte subpopulations. None of the genes in CD8\(^+\) T cells reached statistical significance and, thus, this subpopulation was excluded from further analysis. The CD19\(^+\), CD4\(^+\), and CD14\(^+\) cells heat maps of the MIGs are presented in Figure 1, and the list of MIGs is in Supplementary Tables S1 through S5.

ON Related CD19\(^+\) Gene Expression Signature

The functional analysis of 467 ON MIGs related to CD19\(^+\) B cells demonstrated significant enrichment of genes related to activation of immune mechanisms (\(P = 3.2 \times 10^{-25}\) to \(2.5 \times 10^{-3}\), 125 genes), such as cellular immune response (\(P = 7.1 \times 10^{-12}\), 26 genes) and inflammation (\(P = 3.6 \times 10^{-12}\), 42 genes); genes related to activation of cellular movement and immune cells trafficking pathways (\(P = 5.4 \times 10^{-15}\) to \(2.0 \times 10^{-3}\), 104 genes); cellular growth and proliferation (\(P = 6.3 \times 10^{-14}\) to \(2.5 \times 10^{-3}\), 140 genes), including proliferation of B lymphocytes (\(P = 1.0 \times 10^{-7}\), 22 genes) and stimulation of antigen presentation (\(P = 8.9 \times 10^{-11}\) to \(2.5 \times 10^{-3}\), 69 genes; Table 1). The most unexpected findings in B cells were activation of genes associated with MHC class II antigen presentation, like HLA-DQB1/HLA-DOB1 and their transcription factor RFX5, that influences HLA class II expression. The activation of B-cell proliferation and survival correlated with overexpression of the antiapoptotic BCL2 and BCL7C genes, as well as genes related to cell interaction and adhesion, like ITGB7 and BIN1, together with chemokine and chemotaxin-trantactant family members, like CNTNAP2 and CCRT, known to be associated with memory B cells. Further evidence that implicates B-cell activation was the overexpression of genes related to restricted activation of B-cell-mediated immunity and antibody production, represented by CD79A/B, IL4R, FCER2, FCGR7, and immunoglobulin genes, like IGHG3 and IGHM. The activation of CD79A/B was followed by overexpression of their upstream transcriptional regulators TCF3 and FOXO1, as well as transcriptional activators, like NAGTC1 and RELA.

Low level of apoptosis was an additional significantly enriched function demonstrated by B-cell–related genes. This was based on underexpression of the classical apoptosis-related genes, like CASP1, DAPK1, TNFRSF1A/1B/13, and TNFNP6, apoptotic activating factors, like APAF1 and VDR; followed by suppression of signal transduction-related molecules, such as TLR-2 and TLR-5, IRAK3, and MYD88. Moreover, genes involved in cell-to-cell interaction, like complement component C1QR1 and DR, were found to be suppressed.

Altogether, the specific B-cell gene expression profile in acute ON is comprised of evidence for B-cell involvement in antigen-presenting mechanism, B-cell memory cell generation, antibody production, and enhancement of B-cell proliferation and survival, followed by suppression of apoptotic-related mechanisms. Transcription levels of selected functionally relevant genes are presented in Supplementary Figures S1 through S5.

Reconstruction of CD19\(^+\) Related Gene Expression Networks

The reconstructed regulatory network operating in B-cells is demonstrated in Figures 2A and 2B. This include overexpression of CD79A/B and TCF3, which have a crucial role in the B-cell proliferation,\(^{16-17}\) and activation of genes, like IGKC, IGHG3, IGHM, and IGL\(_{\beta}\), which are known to be involved in the process of immunoglobulin variable region generation\(^{16-20}\); thus, confirming the role of B-cell activation and antibody

![Figure 1](https://i.postimg.cc/23555J23/Figure1.png)
production. In addition to B-cell activation, we have demonstrated B-cell-restricted antigen presentation as shown by upregulation of MHC class II genes, like HLA-DQB1, HLA-DQBI, and their transcription factor RFX5. This finding is important in the context of anergy of APC-restricted genes in the gene expression signature of CD14+ macrophages, where the HLA-related genes were not activated and HLA transcription factor NFYA was significantly suppressed.

ON Related CD14+ and CD4+ Gene Expression Signatures

The CD14+ and CD4+ ON-related genes did not significantly fulfill any canonical pathway or biological function. Single gene analysis of the 55 CD14+ macrophage related genes (Table 1) showed that the overexpressed genes (n = 22) were related to adhesion (Col2A1, CEACAM6/8), cell activation, and proliferation (EGR1, AKT2). The group of down-expressed genes (n = 35) included apoptotic-related genes (DDX3X, PDCD4, TNFRSF25, DUSP2), genes involved in cell-to-cell interactions (ICOS, ITK), antigen presentation (NFYA), and cell-mediated immunity (KLRC3). Within the ON-related 55 MIGs characterizing the CD4+ T cells (Table 1), the overexpressed genes (n = 25) included genes involved in T- and B-cell interaction, like CD58 and CD84. The down-regulated genes included essential apoptotic genes, like GZMB, DAPK1, and CARD4; MHC Class II genes, like HLA-DQA1/B1/B2 and BAT2; cell-mediated immunity related genes, like CD160; adhesion molecules, like NCAM1, and chemokines, like CCR1 and C3XCR.

**Verification of Key Genes by Quantitative RT-PCR and Western Blot**

The expression of eight key genes (RFX5, HLA-DQB1, FCER2, FCGR1, CNTNP2, BIN1, ABCB1, and CCR7) within CD19+ B-cell-restricted ON-induced signature by QRT-PCR correlated with the microarray findings demonstrating corresponding activation at the mRNA level in ON patients as compared to healthy subjects (Table 2).

Expression of HLA type genes in CD14+ and CD19+ cells by Western blot demonstrated activation of the MHC class II proteins HLA-DQB1, HLA-DQA1, and HLA-DOB in CD19+ B cells and not in CD14+ macrophages of ON patients during first 96 hours from onset (Fig. 3).

**Functional Assay to Test Antigen Presenting Function of Sorted B Cells**

In vitro functional assay to test antigen-presenting ability of isolated B cells demonstrated that activation of B cells with CD40L resulted in 4-fold increase (P = 0.0001) in the number of MBP binding B cells compared to B cells not activated with CD40L (Fig. 4).

**Discussion**

In the current study, we characterized the gene expression pattern of peripheral blood immune cells subtypes in the very early onset, within 96 hours, of the first occurrence of acute
ON in subjects who later had MS. To the best of our knowledge, our study is pioneering in assessing the molecular processes early in ON using a large scale transcriptome profiling technology in CD4\(^+\), CD8\(^+\), CD14\(^+\), and CD19\(^+\) cells, and reconstructing the operating biological pathways. We demonstrated major activation of CD19\(^+\) B cells in the early stage of ON with a background of low CD14\(^+\) and CD4\(^+\) cells activity and CD8\(^+\) cells anergy.

The role of B cells in MS is suggested by their presence in acute and chronic demyelinating lesions in the brain,\(^{21}\) the presence of cerebrospinal fluid oligoclonal IgG antibodies,\(^{22}\) and by the efficacy of monoclonal antibody treatments, such as

![Optic neuritis-related regulatory networks](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933680/)

**Figure 2.** Optic neuritis–related regulatory networks. (A) B-cell restricted antigen presentation. (B) B-cell activation. Each node represents a gene. Red represents overexpressed genes, while green represents down-expressed genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>QRT-PCR</th>
<th>Microarray Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold Change</td>
<td>P Value</td>
</tr>
<tr>
<td>RFX5</td>
<td>3.8</td>
<td>0.05</td>
</tr>
<tr>
<td>HLA-DQB1</td>
<td>4.02</td>
<td>0.04</td>
</tr>
<tr>
<td>FCER2/CD23</td>
<td>15.3</td>
<td>0.01</td>
</tr>
<tr>
<td>FCGR3</td>
<td>3.5</td>
<td>0.04</td>
</tr>
<tr>
<td>CNTNAP2</td>
<td>3.2</td>
<td>0.01</td>
</tr>
<tr>
<td>BIN1</td>
<td>6.9</td>
<td>0.05</td>
</tr>
<tr>
<td>ABCB1</td>
<td>1.9</td>
<td>0.03</td>
</tr>
<tr>
<td>CCR7</td>
<td>3.95</td>
<td>0.02</td>
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Rituximab, to reduce disease activity in relapsing–remitting MS patients.\textsuperscript{23}

Immunologically, there are emerging evidences that mammalian B lymphocytes operate to activate T cells as antigen-presenting cells (APC), as well as precursors of antibody-secreting plasma cells\textsuperscript{24}; however, their contribution to ON pathogenesis and their relationship with other immune-competent cells were demonstrated in our study for the first time. The possible explanation for this very early restricted B-cell antigen presentation could be related to the amount of the antigen. The B cells can serve as efficient APCs for T-cell activation when low amount of antigen is applied, but are not required for T cells priming when mice are immunized with high dose of an antigen.\textsuperscript{25} These dose-dependent B-cell antigen-presentation properties are well corroborated with the early disease process of ON. Later on increased structural damage is caused by epitope spreading leading to activation of the classical APC.

Alternatively, the trigger for stimulation of B cells to APC could be related to the nature of the antigen. For instance, viral antigens more probably stimulate B-cell–dependent antigen presentation.\textsuperscript{26}

The involvement of B cells in antigen presentation is either T-cell–dependent or independent.\textsuperscript{18,24} In the current study, we demonstrated that groups of genes known to be involved in the T-cell-independent antigen presentation by B cells, as \textit{TLR-2/5}, \textit{IRAK3}, and \textit{MYD88},\textsuperscript{27–29} were down-expressed in the CD19\textsuperscript{+} signature. Thus, the process of antigen presentation by CD19\textsuperscript{+} B cells during the first 96 hours of ON is T-cell–dependent.

In support of the inflammatory T-cell–dependent type of B-cell–restricted antigen presentation in early ON, overexpression of the chemoattractant factor IL-16–soluble ligand for CD4\textsuperscript{+}, known to be involved in selective recruitment of T helper cells by B lymphocytes,\textsuperscript{30} and overexpression of XCL1–lymphotactin, known to attract T cells and natural killer cells,\textsuperscript{31} were evident in the ON CD19\textsuperscript{+} signature. Specifically the B-cell activation in early ON is demonstrated by overexpression of \textit{CD72} and \textit{CD79A/B} genes followed by antibody production that occurs via IL-4 receptor (IL4R).\textsuperscript{32} The IL4R receptor, a key molecule for B-cell activation and immunoglobulin production,\textsuperscript{33} was highly expressed in the signature. The IL4R signaling mediated by CD23 overexpression\textsuperscript{34} leads to downstream activation of \textit{IRF4} and \textit{NFAT},\textsuperscript{35} resulting in IL4 production and upregulation of MHC class II genes through STAT6. Additionally, activated transcription factor RFX5 also activate MHC class II genes and initiate antigen presentation. On the other hand, the inhibition of repressor genes, like \textit{MSR1} and \textit{ETS2}, contribute to B-cell survival.\textsuperscript{36}

The upregulation of the molecules related to cell migration across endothelial barriers in CD19\textsuperscript{+} B cells, like \textit{ITGB7}, \textit{BIN1}, \textit{CNTNAP2}, and chemokine receptors \textit{CCR7}, \textit{CXCR4},\textsuperscript{35,37,38} suggests that B cells may penetrate the BBB and induce myelin damage.\textsuperscript{39}

Despite the negative expression of most specific CD4\textsuperscript{+} related genes, like \textit{GZMB}, the activation of CD58 and CD84 that optimize recognition in immune CD4\textsuperscript{+} T lymphocytes/APC interactions\textsuperscript{40,41} allows us to assume that cells participate in antigen presentation, but possibly join at a later stage. In conclusion, our results demonstrate B-cell–induced immune mechanisms in early acute ON, including cell activation, antigen uptake, antigen presentation, and immune-

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933680/)

**Figure 3.** Verification of MHC class genes by Western blot in CD19\textsuperscript{+} B cells and CD14\textsuperscript{+} macrophages. \textit{Upper:} HLA-DQB detection. \textit{Lower:} HLA-DOB detection. All experiments included genetically typed HLA-positive controls.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933680/)

**Figure 4.** Myelin basic protein–peptide binding to CD40L-activated B cells. Flow cytometry analysis demonstrated 4-fold increase in the number of MBP-binding CD40L-activated B cells. The y-axis shows the absolute number of MBP-biotin–streptavidin-PE–positive B cells.
globulin production. The possible therapeutic implication of these findings suggests that anti-B-cell agents could be effective in early ON treatment modulation.

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References


