# αB-Crystallin Is a Target for Adaptive Immune Responses and a Trigger of Innate Responses in Preactive Multiple Sclerosis Lesions

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## Abstract

We present the first comparative analysis of serum immunoglobulin G reactivity profiles against the full spectrum of human myelin-associated proteins in multiple sclerosis patients and healthy control subjects. In both groups, serum antibodies display a consistent and prominent reaction to αB-crystallin (CRYAB) versus other myelin proteins. As an apparently major target for the adaptive immune system in humans, CRYAB selectively accumulates in oligodendrocytes, but not in astrocytes, or axons in so-called *preactive multiple sclerosis lesions*. These are clusters of activated HLA-DR–expressing microglia in myelinated normal-appearing white matter with no obvious leukocyte infiltration. They are found in most multiple sclerosis patients at all stages of disease. In these lesion areas, CRYAB in oligodendrocytes may come directly in contact with activated HLA-DR$^+$ microglia. We demonstrate that CRYAB activates innate responses by microglia by stimulating the secretion of leukocyte-recruiting factors, including tumor necrosis factor, interleukin 17, CCL5, and CCL1, and immune-regulatory cytokines such as interleukin 10, transforming growth factor-β, and interleukin 13. Together, these data suggest that CRYAB accumulation in preactive lesions may be part of a reversible reparative local response that involves both oligodendrocytes and microglia. At the same time, however, accumulated CRYAB may represent a major target for adaptive immune responses that could contribute to progression of preactive lesions to a stage of demyelination.

**Key Words**: αB-crystallin, Autoantibodies, Microglia, Multiple sclerosis, Preactive lesions.

## INTRODUCTION

T-cell reactivity to myelin antigens is widely considered to be central to the development of inflammatory demyelinating lesions in multiple sclerosis (MS), but understanding of the roles of B cells and antibodies in MS pathogenesis remains limited (1–3). Recent results of B cell–directed therapeutic interventions in MS indicate that these roles require more thorough examination (4). The antibody response profiles to myelin components in MS patients are, therefore, highly relevant in this context. Several studies have documented serum and cerebrospinal fluid antibody reactivities to myelin antigens, but these studies have focused only on individual proteins, such as myelin proteolipid protein (PLP), myelin basic protein (MBP), or myelin-oligodendrocyte glycoprotein (MOG) (3, 5–7), peptides derived from these proteins or on selected lipids (8). For an appreciation of the full autoantibody response profile to myelin antigens, including posttranslationally modified proteins and de novo antigens that may be generated during the course of the disease, scrutiny of a much broader range of myelin antigens such as can be extracted from complete myelin preparations is necessary.

In the present study, we document MS patient serum antibody response profiles to the complete collection of myelin-associated proteins (including those accumulating during MS) by Western blotting. Fully in line with previous data on peripheral T-cell reactivities (9), antibody response profiles are very similar in MS patients and healthy subjects and are stable over time. In all cases, responses to most myelin antigens are modest if at all detectable. On the other hand, prominent responses are apparent to myelin-associated αB-crystallin (CRYAB), particularly when it accumulates to the relatively high levels seen in brains of MS patients. These results raise the question of which point during the development of MS lesions such accumulation occurs. We previously reported CRYAB expression in actively demyelinating MS lesions (10) and here extend this analysis to so-called *preactive lesions* in normal-appearing white matter (NAWM) of MS patients. These lesions are defined as clusters of activated microglia that appear in the absence of any obvious blood-brain barrier impairment, leukocyte infiltration, or demyelination (11–15). Oligodendrocyte abnormalities are seen in such areas. Several groups have reported very similar lesions in NAWM and have described them with such terms as *Type I lesions* (13), *newly forming lesions* (14), or *Pattern III lesions* (15); they likely refer to closely related early stages of MS

white matter lesions. The existence of preactive lesions and the notion that they can precede the development of actively demyelinating lesions have been confirmed by in vivo imaging of MS patients by magnetic resonance imaging, positron emission tomography, magnetization transfer imaging, and by studies of cerebral blood flow (16–19). Gene profiling studies have also revealed the presence of focal abnormalities in NAWM in MS patients involving clusters of activated microglia and stressed oligodendrocytes in the absence of obvious involvement by peripheral blood lymphocytes (20).

    Here, we show that focal CRYAB accumulation in NAWM in MS brains occurs already in preactive lesions and exclusively in oligodendrocytes at this stage. In these lesions, CRYAB in oligodendrocytes is observed in direct contact with activated HLA-DR–expressing microglia. When exposed to CRYAB, cultured human microglia are activated and produce immunoregulatory cytokines and chemokines. Together, our data indicate that focal accumulation of oligodendroglial CRYAB plays a role in activating microglia in preactive MS lesions. The demonstration that CRYAB is also a major target of adaptive immune responses to myelin suggests that it may play an additional role in driving progression of some of those preactive lesions to an active demyelinating stage.

## MATERIALS AND METHODS

### Subjects

    Sera from 9 clinically definite MS patients were used for the experiments depicted in Figure 1. The age of the MS patients ranged between 31 and 59 years. Six of 9 patients had relapsing-remitting MS. Disease durations were between 6 and 23 years. Additional details are in Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A131. Control sera were drawn from healthy adult donors of the Central Laboratory of the Blood Transfusion Service Sanquin, Amsterdam, The Netherlands. As an additional control pooled human male AB serum used for cell culture was obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands. All serum samples were obtained with informed consent and stored at −80°C.

### Preparation of Myelin Proteins and Western Blotting

    Human postmortem white matter tissue samples were collected by the Netherlands Brain Bank, Amsterdam, after obtaining informed consent from all donors and in compliance with all local legal and ethical regulations. Control samples from 4 donors that were free of clinical or histopathologic
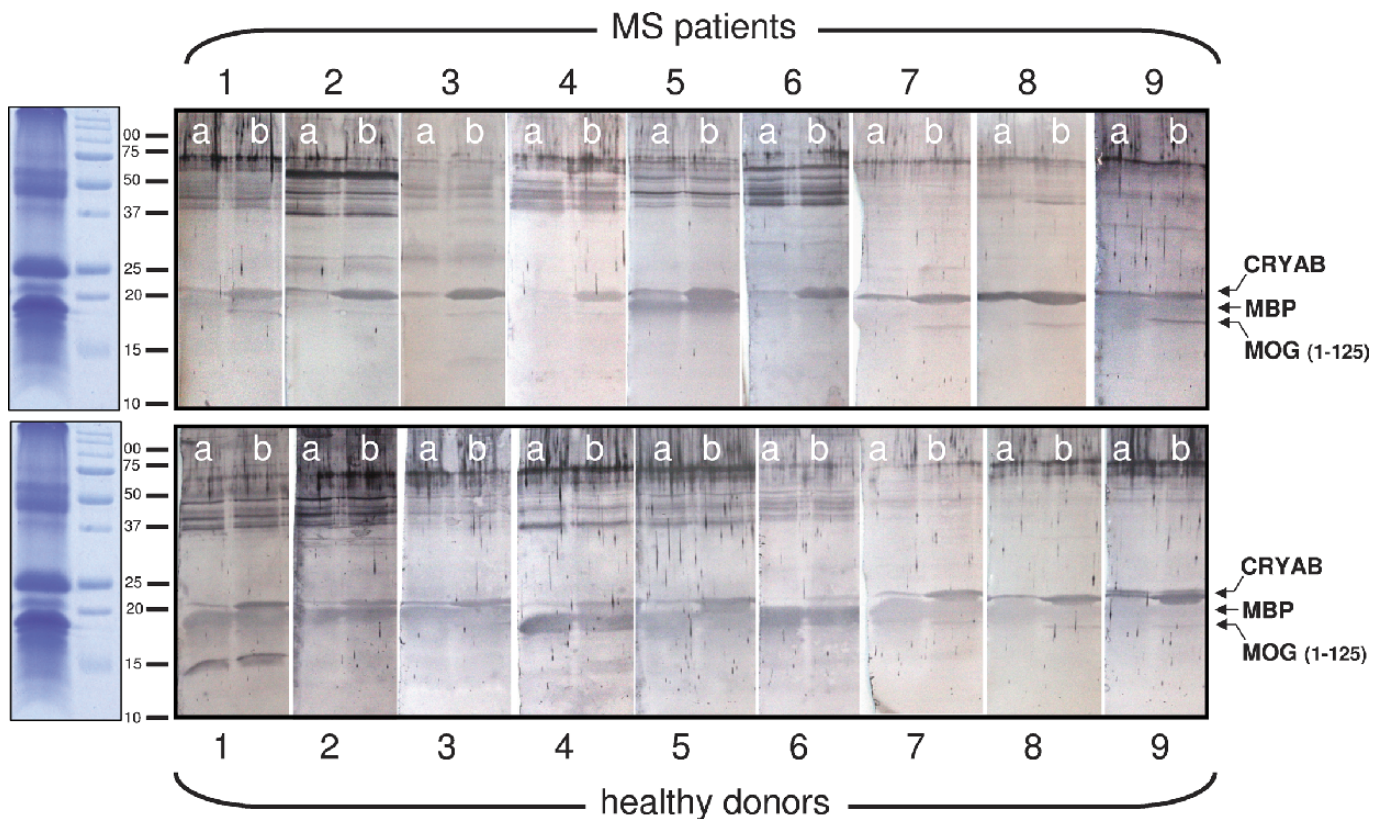


**FIGURE 1.** Serum immunoglobulin G (IgG) reactivity against total myelin protein extracts. Serum IgG reactivities against myelin proteins in 9 multiple sclerosis (MS) patients and 9 healthy controls were probed by Western blotting. In all cases, the *a* lanes contained 100-μg samples of total myelin proteins, as characterized by Coomassie staining on the left. The *b* lanes contained 100-μg samples that were supplemented with an extra 2 μg of both recombinant human αB-crystallin (CRYAB) and the recombinant extracellular domain 1-125 of human myelin-oligodendrocyte glycoprotein (MOG). The increased reactivities (denser bands) at the 22-kd position in the *b* lanes indicate that CRYAB is the target of the antibody responses seen in the *a* lanes. MBP, myelin basic protein.

evidence of any neurological disorder were pooled for myelin isolation. Samples from MS-affected brain tissue were isolated from specimens containing active lesions as judged by neuropathologic examination. Myelin was purified according to the method of Norton and Poduslo (21). White matter samples were homogenized in 0.32 mol/L sucrose in water using a Dounce homogenizer while kept on ice. This suspension was layered over 0.85 mol/L sucrose and centrifuged at $75,000 \times g$ for 30 minutes at 4°C. Myelin membranes at the interphase were collected and washed 3 times with water by repeated centrifugation at $75,000 \times g$ for 15 minutes at 4°C. These sucrose-gradient purification and washing steps were repeated 3 times, after which the purified myelin membranes were lyophilized.

Dry myelin membranes were dissolved at 15 mg/mL in 2-chloroethanol containing 0.05% (vol/vol) trifluoroacetic acid (both from Sigma, St Louis, MO), and proteins were precipitated upon adding 4 volumes of ice-cold ether and left for at least 2 hours at −20°C. The delipidated protein pellet was collected by centrifugation at $2,500 \times g$ for 30 minutes at 4°C, dried under $N_2$, redissolved in 2-chloroethanol, and delipidated again. A final solution of myelin proteins (20 mg/mL) was prepared by dissolving the protein pellet in 2-chloroethanol and carefully removing all residual ether by flushing with nitrogen. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), myelin protein samples in 2-chloroethanol were first gradually diluted with 4 volumes of water, and total protein concentrations were determined. Finally, a 5× concentrate of standard SDS-PAGE sample buffer was gradually added under gentle mixing. All samples were kept at room temperature under an $N_2$ atmosphere, and at no point were the samples either heated or frozen. An amount of 100 μg of myelin proteins in a total volume of 31 μL was used to load each lane of a 15% SDS-PA gel. Proteins were transferred onto nitrocellulose by electroblotting; Western blotting was performed by routine procedures. We used a serum dilution factor of 10 to allow for detection of specific antibodies available even at modest titers, as in previous studies (6, 22, 23). Binding of serum immunoglobulin G (IgG) to the blots was visualized using phosphatase-labeled goat anti–human IgG antibodies (KPL, Gaithersburg, MD).

To verify the presence and position of individual myelin proteins in the gels, the following murine monoclonal antibodies were used in Western blotting: anti-PLP (clone PLPc1; Serotec, Oxford, UK); anti-MBP (Chemicon International Inc, Temecula, CA); anti-MOG (clone Z12; VUmc, Amsterdam, The Netherlands); anti–2′,3′-cyclic nucleotide 3′-phosphodiesterase (Chemicon International Inc); anti-CRYAB (clone JAM01; Delta Crystallon BV, Leiden, The Netherlands); anti–neurofilament light ([NF-L] clone DA2; Zymed Inc, South San Francisco, CA); anti–glial fibrillary acidic protein ([GFAP] clone 5F2; Monosan, Uden, The Netherlands); and anti–amyloid precursor protein (clone LN27; Zymed Inc).

## Mass Spectrometry

For identification of myelin-associated proteins at various positions in the gel, selected gel segments were excised. Proteins contained in these samples were reduced, carbamidomethylated with iodoacetamide, subjected to tryptic digestion, and analyzed on a Bruker Autoflex III matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometer (service provided by Alphalyse, Odense, Denmark). The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination, and selected peptide sequences were determined by mass spectrometry/mass spectrometry. Protein identification was based on a probability scoring algorithm to determine the best matching protein (www.matrixscience.com).

## Activation of Microglia by CRYAB

Recombinant human CRYAB without any leader or tag sequences was produced by Novozymes Biopharma AB, Lund, Sweden. Protein purity was greater than 99.9%. Levels of bacterial endotoxins and DNA were below the detection limits of the assays used: less than 1.5 EU of endotoxins and less than 5 pg bacterial DNA per mg of CRYAB. Levels of *Escherichia coli*–derived contaminant host cell proteins in the CRYAB preparation were 11 ng/mg (0.0011%), as determined by a commercial ELISA (Cygnus Technologies, Inc, Southport, NC).

Microglia were isolated from postmortem human subcortical white matter samples collected from control donors after rapid autopsy by the Netherlands Brain Bank. White matter samples were dissected, and visible blood vessels were removed. After digestion in 0.25% trypsin (Sigma) and 0.1 mg/mL DNAse (Boehringer Mannheim, Mannheim, Germany) for 20 minutes, the cell suspension was gently triturated and washed with Dulbecco modified Eagle medium/HAM-F10 medium containing 10% fetal calf serum and antibiotic supplements. After passage through a 100-μm filter, myelin was removed by Percoll gradient centrifugation. Erythrocytes were lysed by 15-minute incubation on ice with 155 mmol/L $NH_4Cl$, 1 mmol/L $KHCO_3$, and 0.2% bovine serum albumin. Recombinant human granulocyte-macrophage colony-stimulating factor (PeproTech Inc, Rocky Hill, NJ) was added to microglia cultures every 3 days at a final concentration of 20 μg/mL to promote proliferation and survival of the microglia. The purity of the cell cultures was verified by immunostaining for CD68 and routinely exceeded 99%. Cytokine and chemokine profiling of culture supernatants was performed 24 hours after stimulation with 50 μg/mL recombinant human CRYAB using a commercially available cytokine/chemokine antibody array (RayBiotech, Inc, Norcross, GA) according to the manufacturer's instructions. Chemoluminescence signals for each mediator were expressed as arbitrary units that were corrected for background and normalized using 6 positive reference signals on each array. Reference cultures were cells from the same isolate cultured in the absence of CRYAB. The chemoluminescence signals do not provide an absolute measure for cytokine concentrations because the strength of each signal is also determined by the affinity of the antibody used on the array. Therefore, induction values rather than absolute signals are relevant. Experiments were performed 4 times, each with freshly isolated cells from a different control donor.

## Immunohistochemistry

Five-micrometer-thick cryosections of human white matter were stained with oil-red O for the detection of neutral lipids signifying myelin degradation. Cryosections were incubated with filtered oil-red O solution for 10 minutes and briefly rinsed in tap water and counterstained with hematoxylin for 5 minutes after intensive washing with tap water for 5 minutes. Tissue blocks containing active MS lesions were selected for the preparation of myelin used in the experiments of Figure 2; those containing preactive lesions were selected for further immunohistochemical analysis. *Preactive lesions* are defined as clusters of activated microglia appearing in the absence of any obvious blood-brain barrier disruption, leukocyte infiltration, or demyelination (12).

For fluorescence staining, snap-frozen sections from MS brain tissue samples containing preactive lesions were stained using rabbit antibodies against CRYAB (W3/13; Delta Crystallon BV) or olig2 (Millipore Corporation, Billerica, MA) or with murine monoclonal antibodies against CRYAB (JAM01; Delta Crystallon BV) or HLA-DR (LN3; Abcam, Inc, Cambridge, MA). Secondary labeling was performed with Alexa 488–labeled goat anti–rabbit and Alexa 594–labeled goat anti–mouse antibodies (Invitrogen, Carlsbad, CA). Stained sections were evaluated by confocal laser scanning microscopy.



**FIGURE 2.** Multiple sclerosis (MS)–induced accumulation of αB-crystallin (CRYAB) in myelin preparations. Samples of 100 μg of proteins from control myelin (CON) or myelin purified from tissue samples containing active MS lesions (MS) were probed by Western blotting using a mixture of a CRYAB-specific and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)–specific monoclonal antibodies to verify equivalent loading of gels. Antibody-binding signals were compared with those generated against reference amounts of recombinant human CRYAB included on the same blot to permit absolute quantification (graph). CRYAB represents about 0.2% of all proteins in control myelin, but in MS-affected tissue samples, this level is approximately 10-fold higher. Figure 2 is representative of data obtained from 3 different MS samples.

## TLR Activation Assays

Human endothelial kidney (HEK293) cells transfected with toll-like receptor 2 (TLR2), TLR3, TLR4/MD2, or TLR5 and HEK293XL cells transfected with TLR7, TLR8, TLR9, or TLR10 (InvivoGen, San Diego, CA) were cotransfected using Polyfect (Qiagen Benelux, Venlo, The Netherlands) with a reporter vector expressing luciferase under the control of a nuclear factor-κB–responsive promoter (pNifty2-luc; InvivoGen). Stably transfected clones were selected and used in bioassays. Cells were plated in flat-bottomed 96-well plates at a density of $1 \times 10^5$ cells/well, stimulated and incubated for 16 hours at 37°C. Subsequently, the cells were lysed in Steady Glo luciferase buffer (Promega Benelux, Leiden, The Netherlands), and bioluminescence was measured using a Packard 9600 Topcount microplate scintillation and luminescence counter (Packard Instrument Company, Meriden, CT). As a positive control for nuclear factor-κB–mediated activation (i.e. the presence of the pNifty2-luc vector), 25 ng/mL tumor necrosis factor ([TNF] PeproTech, London, UK) was used. As positive controls for TLR-mediated activation, TLR-specific ligands (all InvivoGen) were used.

## RESULTS

### Response Profiles of Human Anti-Myelin Serum Antibodies

To evaluate serum antibody reactivity profiles by Western blotting, protein extracts were prepared from purified human myelin membranes after extensive delipidation and reconstitution in 2-chloroethanol to allow for complete protein solubilization. Upon control Western blotting of the extracts with monoclonal antibodies against PLP, MBP, MOG, 2′,3′-cyclic nucleotide 3′-phosphodiesterase, or CRYAB, all antibodies produced a binding signal at the expected position on the blots (data not shown). The NF-L, amyloid precursor protein, and some GFAP could also be detected, indicating that these myelin preparations also contain some axonal/neuronal and astrocyte proteins (21). The IgG reactivity profiles of MS and control sera are shown in Figure 1. Multiple antibody binding bands between molecular weights of approximately 35 kd and 55 kd are visible, albeit in varying patterns of fine specificity. Based on additional mass spectrometry–assisted identification of individual protein bands, we attribute these bands to antibody recognition of cytoskeletal proteins, predominantly including α- and β-4 tubulin, and γ-actin (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A132), as expected in human serum (23). Serum IgG binding signals to the myelin antigens PLP, MBP, or MOG were very weak or absent in most cases. This contrasts to the abundance (particularly of PLP and MBP) of these proteins in CNS myelin. Some diffuse staining of the prominent 18.5- and 17.2-kd bands of MBP band was regularly observed, but the weakness of this staining likely indicates nonspecific IgG binding. Infrequent recognition of MBP or MOG is consistent with previous findings that specific serum IgG Western blot signals against recombinant MOG or MBP are found only in a minority of MS patients (5). There were no obvious differences between the response profiles of MS patients and healthy subjects (Fig. 1).
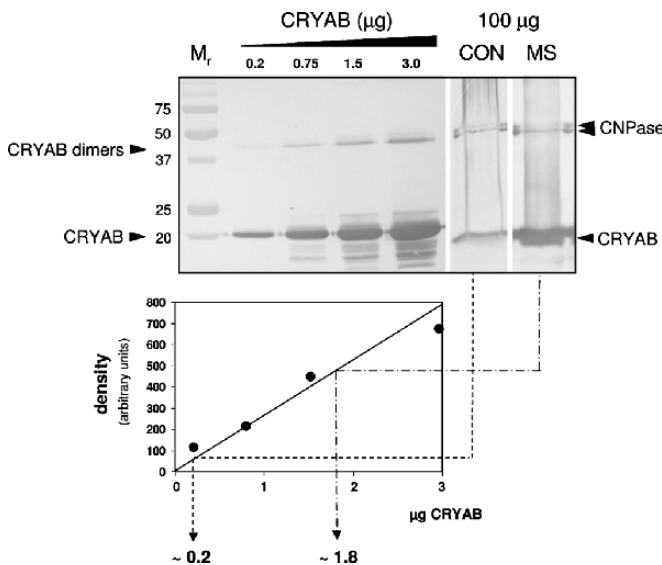
Despite the weak IgG responses to most myelin proteins, there was a discrete band of approximately 22 kd in all profiles. The PLP (DM-20) and CRYAB are the predominant proteins at this position in the gel; they are accompanied by small amounts of a 21.5-kd MBP isoform (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A132). In view of the lack of recognition of the major PLP band or additional MBP isoforms, CRYAB was considered to be a likely candidate target antigen of this strikingly consistent IgG response. To verify this, a reference sample of myelin protein was constructed by adding 2 μg of recombinant human CRYAB, as well as 2 μg of recombinant human MOG (1–125) (as a control) to 100 μg myelin protein samples. Probing this reference sample with sera allowed unequivocal identification of CRYAB as the single target of antibody recognition in the corresponding 22-kd position of the *a* lanes. The amount of CRYAB used for spiking was chosen such that it increased endogenous CRYAB levels by a factor of about 10. αB-crystallin represented approximately 0.2% of all proteins in the purified myelin from normal human brains (Fig. 2). In samples with active MS lesions, on the other hand, such levels are about 10-fold higher (9, 24, 25). Thus, the spiked myelin samples used in *b* lanes of Figure 1 closely mimic the extent of local CRYAB accumulation in MS-affected brains in vivo. Additional tests showed that apart from the enhanced levels of CRYAB in MS-affected myelin, there were no additional disease-associated alterations in protein composition recognized by IgG in sera from either MS patients or control subjects (data not shown). To examine

reproducibility of these data and the stability of serum antibody response profiles over time, serum IgG response profiles for selected MS patients and healthy donors were repeatedly examined during 6- to 8-month periods. Different serum samples collected during this period produced identical response profiles in each case (data not shown). This apparent stability of the serum antibody repertoire against myelin is in line with previous data on human serum antibody response profiles to other self-proteins (26, 27).

## CRYAB Accumulates in Oligodendrocytes and Myelin in Preactive MS Lesions

The above data raised the questions as to what point during the development of an MS lesion and in which cells CRYAB accumulation occurs. We previously reported that CRYAB accumulation is apparent at the earliest detectable stages of infiltrated actively demyelinating MS lesions, that it is expressed in both oligodendrocytes and astrocytes, and that it is present inside phagocytic vesicles of microglia and/or macrophages (10). The 30 preactive lesions studied were all characterized by focal expression of HLA-DR on microglia localized in noninfiltrated white matter showing normal myelin staining (Fig. 3). In all preactive lesions, there was a close spatial association between foci of CRYAB accumulation in oligodendrocytes and clusters of microglia with elevated HLA-DR expression (Fig. 4).

To examine the cellular source of accumulated CRYAB, its expression was compared with olig2, PLP, GFAP, and NF-L, which are markers of oligodendrocyte nuclei, myelin,
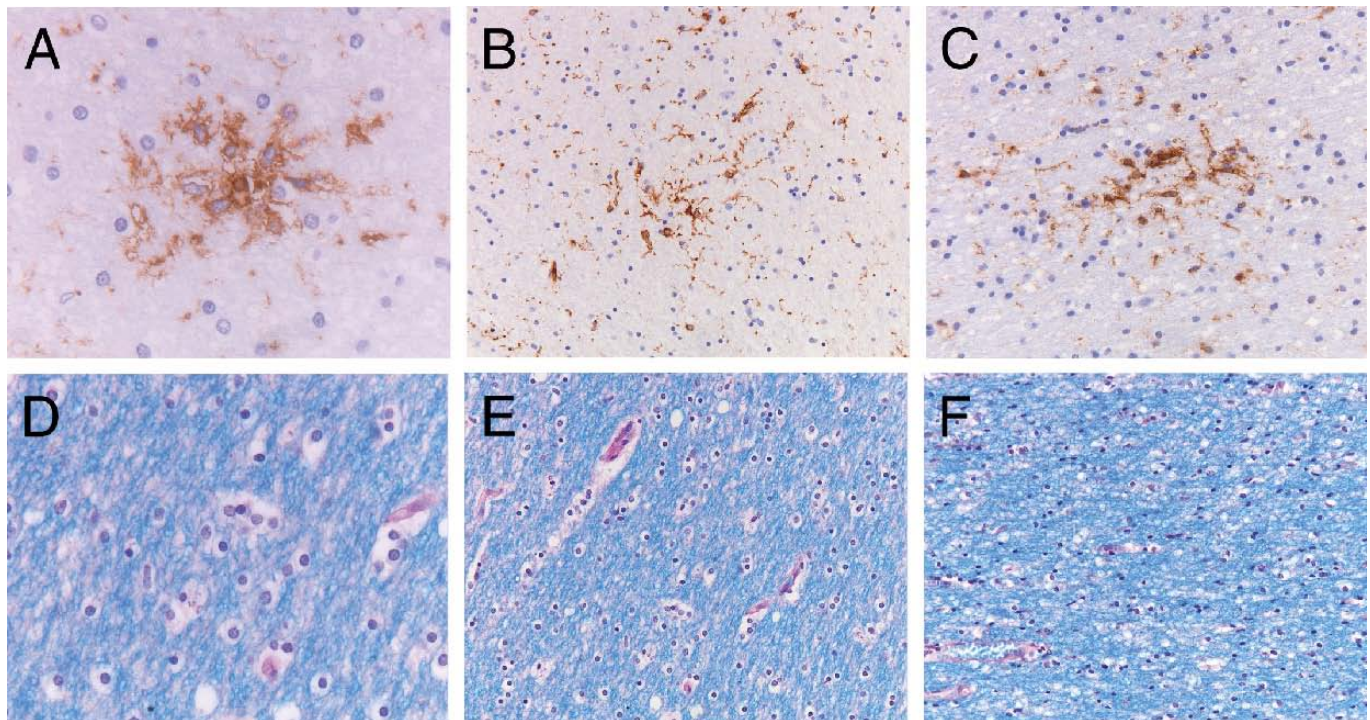


**FIGURE 3.** Pathological features of preactive lesions. *Preactive lesions* are defined as clusters of activated microglia in areas of intact myelin in which there are no obvious blood-brain barrier abnormalities or leukocyte infiltration visible. **(A–C)** Clusters of microglia that express HLA-DR are shown in 3 examples. **(D–F)** Intact myelin by Luxol fast blue staining of the corresponding regions. Original magnification: **(A, D)** 100×; **(B, F)** 25×; **(C, E)** 40×.
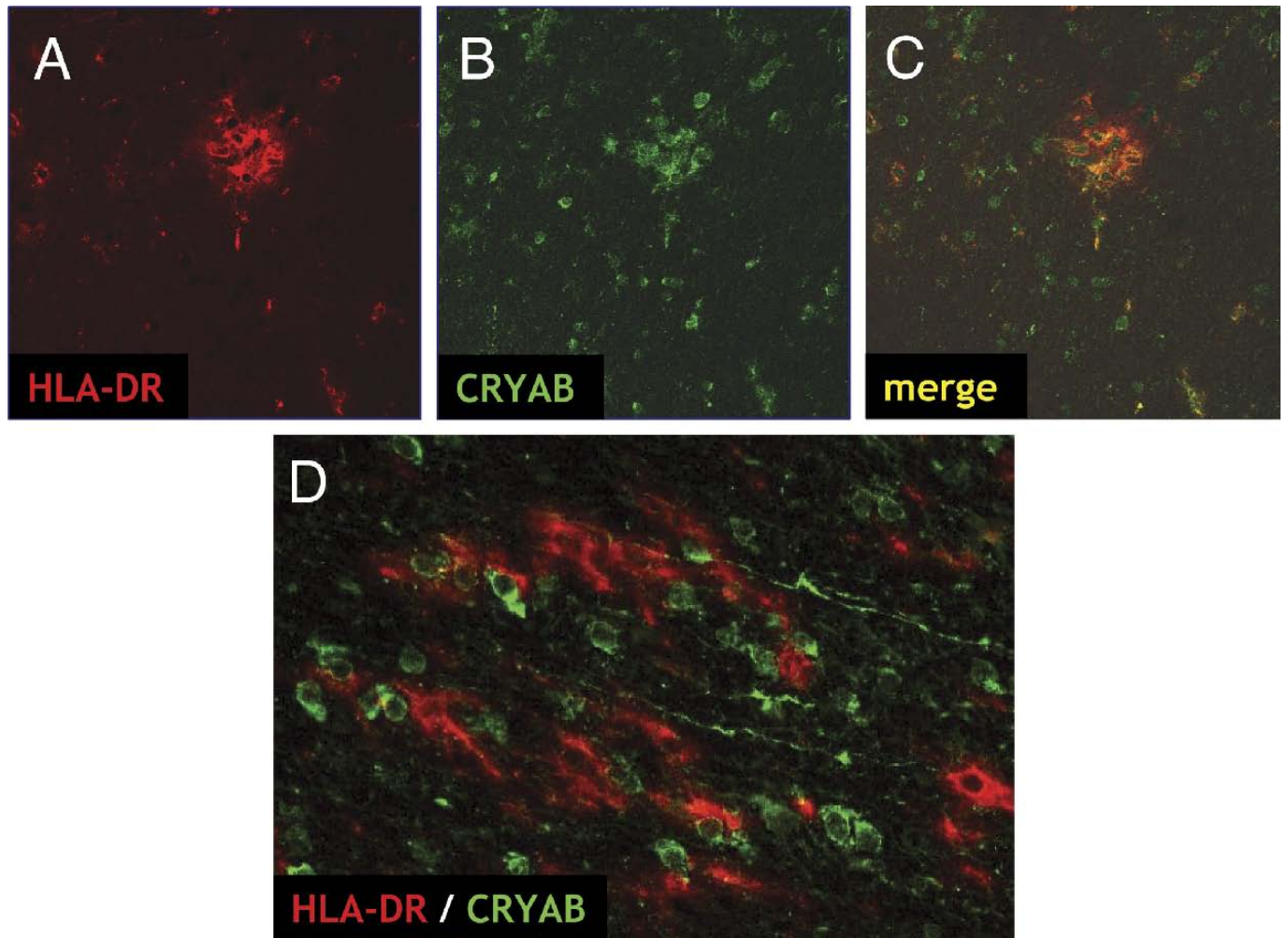
**FIGURE 4.** αB-crystallin (CRYAB) in preactive lesions. **(A–D)** Close association of CRYAB and HLA-DR immunoreactivity in 2 examples of preactive lesions **(A–C** and **D)**. Original magnification: **(A–D)** 20×.

astrocytes, and axons, respectively. Intense CRYAB expression was exclusively found in oligodendrocytes and not astrocytes (Fig. 5). Although CRYAB expression was occasionally observed in stringlike structures in nondemyelinated white matter (Figs. 4B, D), no convincing colocalization with PLP or NF-L was observed. Colocalization of CRYAB with HLA-DR in preactive lesions was also observed (Fig. 6). In several cases, crescent-shaped areas of colocalization of
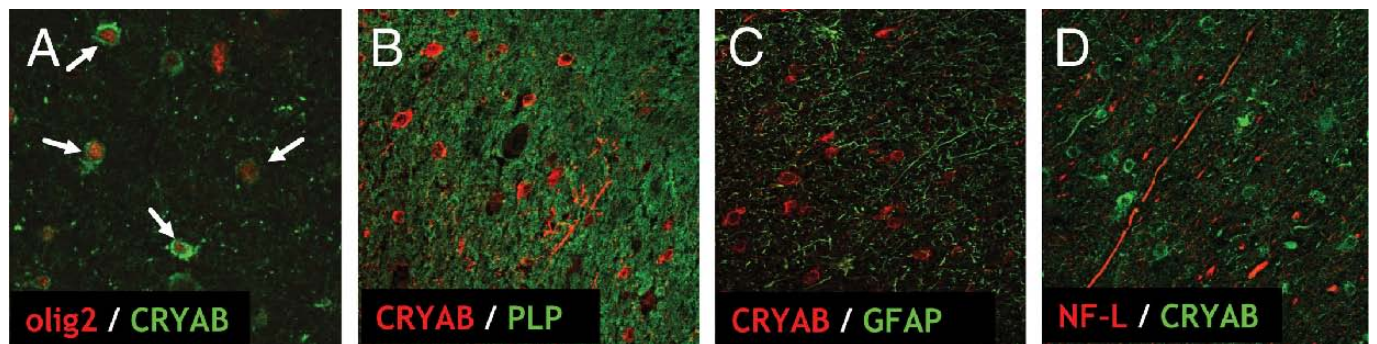


**FIGURE 5.** αB-crystallin (CRYAB) exclusively accumulates in oligodendrocytes in preactive multiple sclerosis (MS) lesions. **(A, B)** CRYAB is expressed in olig2-positive oligodendrocytes (arrows **[A]**) surrounded by intact myelin indicated by staining for myelin proteolipid protein (**[PLP] B)**. **(C, D)** There is no colocalization of CRYAB with the astrocyte marker glial fibrillary acidic protein (**[GFAP] C)** or with the axonal marker neurofilament light (**[NF-L] D)**. Original magnification: **(A–D)** 20×.
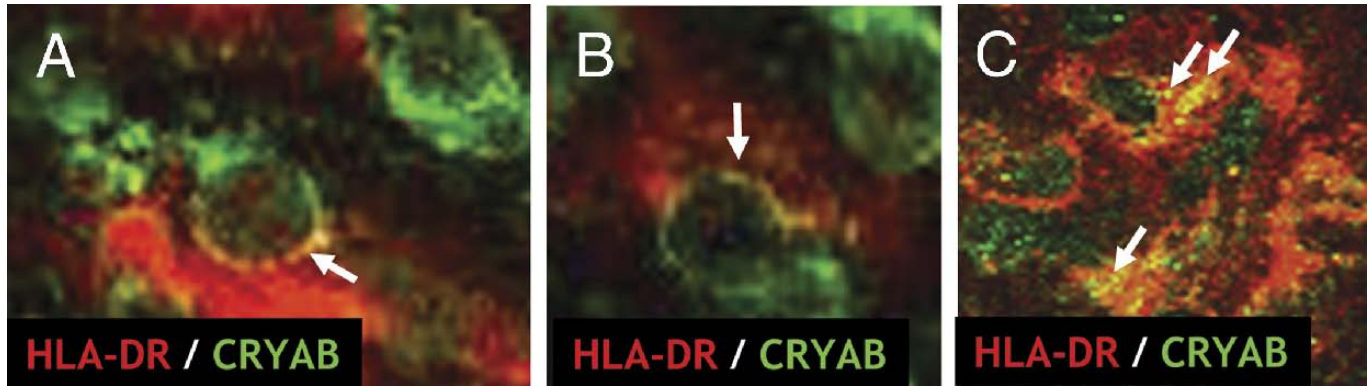
**FIGURE 6.** αB-crystallin (CRYAB) is in contact with activated microglia in preactive lesions. **(A, B)** Colocalization of accumulated CRYAB and HLA-DR on microglia in crescent-shaped areas, suggesting direct contact between microglia and intact oligodendrocytes. **(C)** On other occasions, there is colocalization in small intracellular vesicles, suggesting CRYAB uptake in the HLA-DR–containing endosomal compartment of microglia. Original magnification: **(A–C)** 20×.

oligodendroglial CRYAB with HLA-DR suggested direct contact of CRYAB with neighboring microglia (Figs. 6A, B). Colocalization of CRYAB and HLA-DR was also observed in intracellular vesicles in microglia (Fig. 6C), suggesting that activated microglia occasionally internalize CRYAB-containing structures, presumably myelin and/or apoptotic
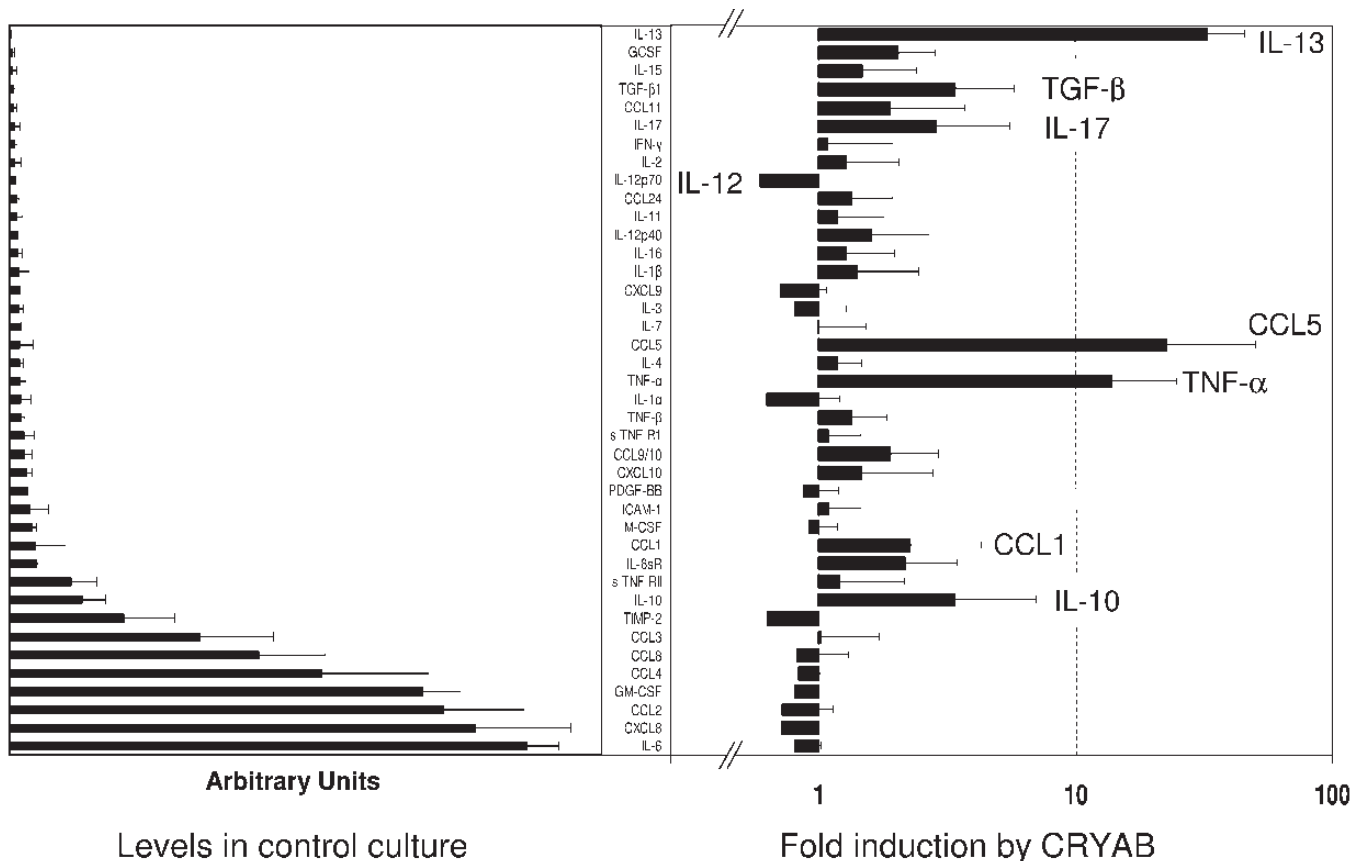


**FIGURE 7.** αB-crystallin (CRYAB)–induced cytokine and chemokine release by human microglia. Levels of 40 cytokines and chemokines in microglial culture media were evaluated by antibody arrays. Left panel: results are shown for media collected after 24 hours of culturing microglia without CRYAB, illustrating a relatively high basal secretion of interleukin 6 (IL-6), CCL2, CCL3, CCL4, and CCL8 under these conditions. Right panel: the relative change is illustrated for each mediator found after 24-hour incubation of microglia from the same isolate with 50 μg/mL CRYAB. Results are the mean ± SD of 4 independent experiments, each performed with microglia from a different donor. TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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oligodendrocytes in preactive lesions. In view of the absence of overall demyelination in these areas, however, the scale of this phagocytic activity is apparently limited.

## CRYAB Activates Microglia

The accumulation of CRYAB in oligodendrocytes in preactive lesions most likely reflects an attempt by oligodendrocytes to counteract the effects of stress, more particularly of signals that induce apoptosis. Acting as an intracellular chaperone, CRYAB inhibits apoptosis in glial cells (7, 28). As a soluble extracellular protein, it exerts anti-inflammatory and neuroprotective effects (7), and it may act as a scavenger of oxidative radicals (29). We therefore examined whether CRYAB itself could, in fact, play a role in microglial activation as a chaperokine in cultures of human microglia.

Microglia were cultured in the presence of highly purified CRYAB, and accumulation of 40 different cytokines and chemokines into the culture medium was examined after 24 hours using antibody arrays. αB-crystallin induced a more than 10-fold increase in the accumulation of TNF, CCL5, and interleukin 13 (IL-13) by microglia (Fig. 7). A more modest 3-fold induction of IL-17, IL-10, and transforming growth factor-β was observed, as well as a 2.3-fold induction of the chemokine CCL1. Notably, no increased production of IL-12p70 was observed, emphasizing that CRYAB does not stimulate the classical pathway of macrophage/microglia activation. Instead, the induction pattern reflects an alternative activation characteristic of an immune-regulatory response of microglia (30, 31). This response does seem to include factors that promote immune surveillance, however. Tumor necrosis

factor and IL-17 are known to play important roles in mediating blood-brain barrier activation, and CCL5 and CCL1 mediate recruitment of leukocytes, including memory T cells and plasma cells (32, 33).

To rule out the possibility that the response illustrated in Figure 7 could have been caused by contaminants in the recombinant CRYAB preparation used, we verified adequate removal of bacterial proteins (11 ng/mg), DNA (<5 pg/mg), and endotoxins (<1.5 EU/mg) from the preparation. We also tested whether the preparation could trigger any response by HEK293 cells expressing individual TLR family members known to be highly responsive to a variety of bacterial products. As shown in Figure 8, up to 100 μg/mL CRYAB failed to stimulate any response by transfected HEK293 cells functionally expressing any of the individual family members TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, or TLR10. In addition to ruling out a confounding role by bacterial contaminants, this also shows that CRYAB itself does not serve as a direct agonist for any of the above TLR family members on their own.

## DISCUSSION

We show that serum antibodies to the complete collection of myelin-associated proteins from both MS patients and healthy subjects display very similar response profiles that are stable over time. As confirmed by mass spectrometry, cytoskeletal proteins are clearly recognized, albeit with marked variations in the fine specificity of these responses among individual donors. In all cases, there were only very modest
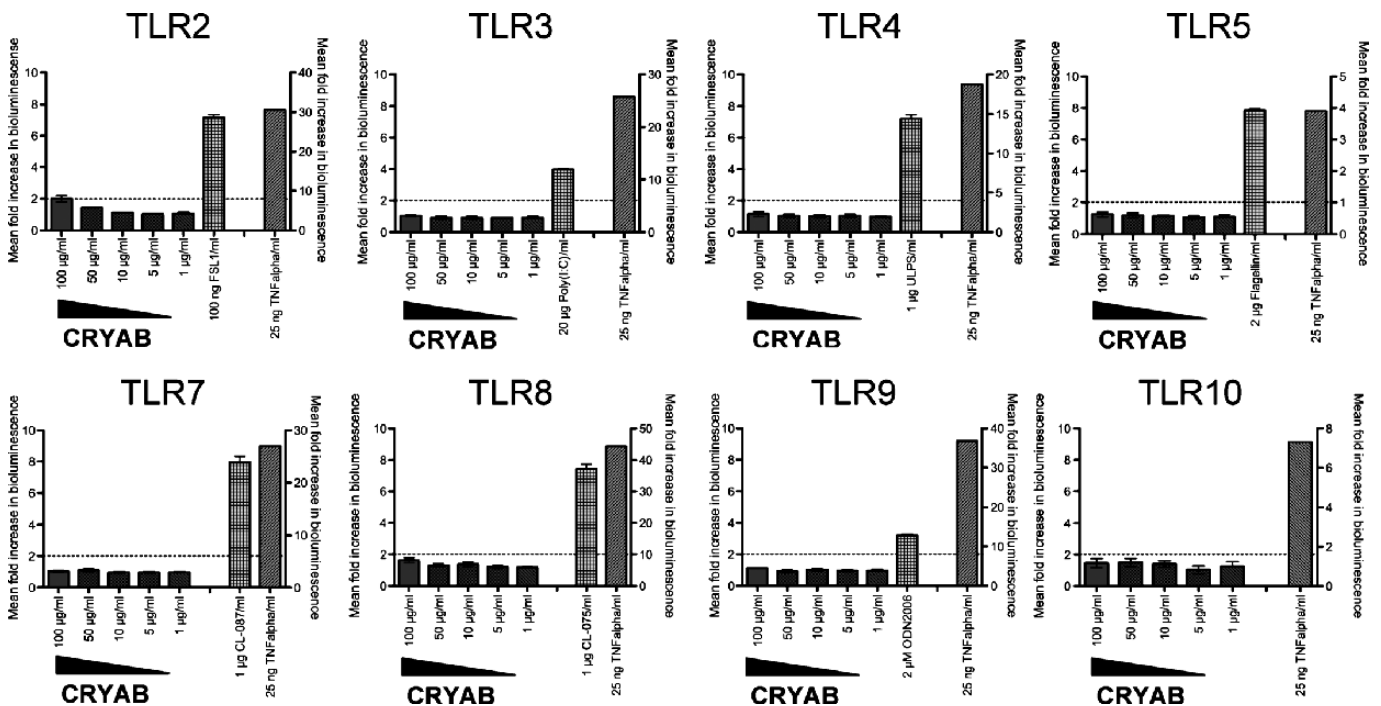


FIGURE 8. αB-crystallin (CRYAB) does not directly activate any toll-like receptor (TLR) known to be involved in recognizing bacterial products. To verify that activation of microglia by recombinant human CRYAB was not caused by bacterial contaminants in the preparation, CRYAB was tested for its ability to activate a panel or HEK293 reporter cells, each expressing a different TLR family member. In all cases, CRYAB up to 100 μg/mL did not trigger any significant response. Results are the mean ± SD of 3 experiments.

responses (if any) to myelin proteins, whereas there was a consistent readily detectable antibody response to CRYAB in all samples. The response became very prominent when CRYAB was present in levels comparable to those in myelin preparations from MS-affected brains.

These serum IgG response profiles mirror those previously found for CD4$^+$ T cells (9), that is, a lack of any meaningful qualitative difference between MS patients and healthy controls; a marked response to cytoskeletal proteins; a minor response to MBP, PLP, or MOG; and a dominant response to accumulated CRYAB. We previously documented that anti-CRYAB serum antibody titers are also very similar in both groups (22). The correspondence between serum IgG responses as monitored herein and the dominant features of peripheral T-cell responses supports the validity of the screening technique used. Nevertheless, Western blotting to document antibody responses has some limitations because serum antibodies against myelin-associated lipids escape detection, and antibody responses to conformation-dependent epitopes could be lost upon SDS-PAGE and electroblotting. Nevertheless, Western blotting is ideally suited to probe the polyclonal antibody response profile against a complex mixture of protein antigens because the antigens are present in their natural relative abundance. As a reference for sensitivity of the assay used, the anti-CRYAB IgG response reflects only rather modest antibody titers of between 1:40 and 1:500 ([22] and data not shown). In view of this sensitivity, we consider it unlikely that any additional major protein target for polyclonal serum antibodies would have consistently escaped detection by the current method. Thus, CRYAB responsiveness is a dominant signature of the adaptive anti-myelin immune repertoire in both MS patients and healthy subjects. As previously suggested, a buildup of this specific responsiveness might be the result of prior infection with Epstein-Barr virus (34).

Levels of CRYAB in normal myelin are very low and likely insufficient to provoke any adaptive immune response. In MS brains, however, our study draws particular attention to the phenomenon of preactive lesions. These manifestations, or closely related early stages of MS lesions, have now been documented by many different groups (11–20). Although not all of these reports use the same terms to describe them, they all provide support for the notion that there are foci of activated microglia and stressed oligodendrocytes that develop in areas of intact myelin, without any obvious involvement of infiltrated peripheral immune cells in MS NAWM. We found such preactive lesions in most, but not all, of our MS brain specimens possibly because preactive lesions only appear at a particular stage of recurrent cycles of inflammation during MS.

In all 30 preactive lesions examined, CRYAB accumulation was found to cocluster with activated microglia. Unlike what is seen at later stages of actively demyelinating MS lesions, CRYAB accumulation in preactive lesions was seen only in oligodendrocytes and not astrocytes, consistent with a stressed or preapoptotic state of some oligodendrocytes in these foci. These data extend a previous study on upregulation of CRYAB in NAWM (35) and document direct contact between oligodendroglial and myelin-borne CRYAB and the HLA-DR–laden surface of activated microglia. This suggests that microglial activation in NAWM might be the direct result of CRYAB accumulation. Extracellular CRYAB readily activated microglia in vitro at a concentration of 50 μg/mL, which is within the physiologically relevant range. We estimate that CRYAB, when representing 1% to 2% of total myelin proteins (Fig. 2), can reach local concentrations in white matter of several hundreds of micrograms per milliliter. Upon activation by CRYAB, microglia exhibit a response characteristic for the pathway of alternative immune-regulatory activation in macrophage-like cells (30, 31). This pathway generally suppresses cellular immunity and promotes type-2 responses and, ultimately, tissue repair. Strong induction of IL-13, which promotes the wound-healing functions of macrophages (30, 31), lends further support to this notion. The CRYAB-triggered release of CCL1 is equally noteworthy because it is a selective chemoattractant for regulatory T cells and $T_H2$ cells that express the CCL1 receptor CCR8 (36, 37). Along with IL-10 and IL-13, which promote B-cell proliferation and antibody production, CRYAB-induced CCL1 release seems also to promote local type-2 responses, consistent with the accumulation of intrathecal antibodies in MS.

The cytokine and chemokine response profile of CRYAB-activated microglia suggests that immune surveillance stimulated in this way would likely contribute to the resolution of inflammation. Indeed, soluble CRYAB effectively suppresses neuroinflammation under different experimental conditions (7, 29). Moreover, preactive lesions are generally more frequent than actively demyelinating lesions (11, 12), making it unlikely that they all progress to a frankly demyelinating pathological state, that is, tissue destruction would become considerable. A previous pathological analysis of what was called *Type I lesions* (13) and in vivo imaging studies of focal abnormalities in NAWM also provide evidence for their reversibility. For example, focal changes in magnetization transfer ratios (16) and changes in lipid and metabolite signals upon magnetic resonance spectroscopy of NAWM (17) (likely reflecting preactive lesions) were found to be reversible. Together, these data suggest that preactive lesions are reversible, and at least some of them spontaneously resolve without progressing to a demyelinating stage. At this early reversible stage, therefore, preactive lesions likely reflect an innate neuroprotective response aimed at preventing further damage. Activation of the immune-regulatory pathway in microglia and stimulation of controlled immune surveillance are likely a part of this protective and restorative process.

Importantly, however, full plasticity is retained during macrophage/microglia responses, allowing any response to become redirected to a destructive classical activation pathway, for example, when sufficient amounts of interferon-γ appear (30, 31). This plasticity exposes any local microglial/macrophage response to the risk of reprogramming, when antigen-specific interferon-γ responses would develop in infiltrating $T_H1$ cells. As a consequence, this allows adaptive immunity to take over in case of a real antigenic challenge. Given that CRYAB-reactive T cells produce marked amounts of interferon-γ but no IL-4 (9, 38), accumulating CRYAB may sometimes pose such a challenge.

Although we cannot rule out that CRYAB accumulation in oligodendrocytes is secondary to microglial activation in preactive lesions, we favor the idea that it actually comes first, given the ability of CRYAB to activate microglia, and that not all oligodendrocytes in the area seem to be stressed. The question as to what triggers oligodendrocyte stress and CRYAB accumulation in the first place clearly remains open. Given the critical role of recurrent inflammation in MS, oligodendrocyte-stressing factors may result from inflammation itself, either directly or indirectly. Intrathecal antibodies directed at oligodendrocytes may also play a role in such a self-perpetuating process, as may oxidative radicals, mitochondrial dysfunction, or other forms of axonal or neuronal damage that may develop because of prior inflammatory damage. Whatever the primary trigger of preactive lesions is, they clearly emerge before obvious signs of leukocyte infiltration and frank demyelination become detectable. Selective CRYAB accumulation in oligodendrocytes is part of these local events. Our data indicate that this particular feature may be a pivotal factor in the balance between protective innate responses and destructive adaptive responses in MS.

## ACKNOWLEDGMENTS

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