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Oligoclonal Myelin-reactive T-cell Infiltrates Derived from Multiple Sclerosis Lesions are Enriched in Th17 Cells

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Abstract

In this study, acute and chronic brain and spinal cord lesions, and normal appearing white matter (NAWM), were resected *post-mortem* from a patient with aggressive relapsing-remitting multiple sclerosis (MS). T-cell infiltrates from the central nervous system (CNS) lesions and NAWM were separated and characterized *in-vitro*. All infiltrates showed a proliferative response against multiple myelin peptides. Studies of the T-cell receptor (TCR)V β and J β usage revealed a very skewed repertoire with shared complementarity-determining region (CDR)3 lengths detected in all CNS lesions and NAWM. In the *acute lesion*, genomic profiling of the infiltrating T-cells revealed up-regulated expression of TCR α and β chain, retinoic acid-related orphan nuclear hormone receptor C (RORC) transcription factor, and multiple cytokine genes that mediate Th17 cell expansion. The differentially expressed genes involved in regulation of Th17 cells represent promising targets for new therapies of relapsing-remitting MS.

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Keywords

human; T-cells; demyelinating CNS inflammatory lesions; autoimmunity

Introduction

Inflammatory cells play a central role in MS lesions development [1, 2]. Therefore, their detailed characterization in the CNS lesions at various stages of progression provides a framework for better understanding the pathogenesis of the disease [3]. Studies of *human* CNS lesions, and particularly of the infiltrating inflammatory cells, have been hampered by the limited availability of tissue from *active* MS lesions, the small number of infiltrating inflammatory cells, and technical challenges involved in their separation and *in-vitro* expansion [4].

Longitudinal studies of auto-reactive cells in the peripheral circulation have determined that the chronic inflammatory response in MS is mediated by oligoclonal T-cell populations [5]. Studies of the TCRV β repertoire of peripheral blood mononuclear cells (PBMC)s and cerebrospinal fluid (CSF)-derived T-cells from MS patients have identified a “skewed” repertoire, suggesting that a limited number of T-cell clones drive the chronic immune response in MS [6]. These auto-reactive cells persist in the circulation for years and expand during clinical relapses [7]. As the blood brain barrier becomes permeable, those selected T-cells migrate to the multiple CNS lesions during disease exacerbations.

The present study has identified, for the first time, the presence of myelin-reactive T-cells in CNS demyelinating lesions and NAWM, a skewed TCRV β repertoire dominated by a limited number of T-cell clones in all CNS regions, and gene and protein expression profiles of T-cells infiltrating CNS MS lesions at various stages of progression.

Results

Inflammatory Cells are Prominent in the Acute CNS Lesion, Persist in the Chronic Lesions, and Diffusely Infiltrate the NAWM

A twelve-year-old African-American female presented initially with a subacute paraparesis. Magnetic resonance imaging (MRI) scan of the brain revealed multiple T2 and contrast-enhancing lesions, while subsequent spinal cord imaging revealed T2 signal abnormalities at C6 and Th4-Th8 levels, with patchy contrast enhancement, all consistent with disseminated demyelinating disease. CSF analysis detected elevated IgG synthesis and oligoclonal bands. Five months after the initial presentation, the patient developed acute optic neuritis. Due to the involvement of the spinal cord and optic nerves, a differential diagnosis of neuromyelitis optica (NMO) was considered [8; 9]. Unfortunately, since serum sample was not available for the NMO Ab testing, it was not possible to completely rule out this variant of disseminated demyelinating disease. Since the initial studies demonstrated multiple inflammatory contrast-enhancing brain lesions and positive CSF studies, and subsequent gene expression studies documented aquaporin-4 expression in the NAWM and thoracic lesion tissue, we established a diagnosis of aggressive CNS inflammatory demyelinating

disease, consistent with MS. Aggressive MS with opticospinal involvement is reported with higher frequency in African American patients than in Caucasian Americans [10], while NMO is more prevalent in oriental population. One month prior to the last hospitalization, the patient experienced difficulties with swallowing and dysarthria, suggestive of brainstem disease activity. Emergent admission was arranged following respiratory failure. A brain MRI scan obtained three days prior to death revealed new right pontine hyperintense T2 and fast fluid-attenuated inversion-recovery signal abnormalities suggestive of an inflammatory demyelinating lesion. According to the patient's living will and the family's decision, respiratory support was withdrawn, and an autopsy was performed 6 h after death.

Lesions were harvested at the autopsy based on the MRI findings and macroscopic pathological localization. The acute pontine (AP) lesion, identified only on the final MRI scan, was hypercellular (Figure 1A) with an abundance of CD68+ macrophages (Figure 1D) and CD3+, predominantly CD8+ lymphocytes (Figure 1E and F). In these regions, acute myelin degradation was signified by macrophages containing Luxol-fast blue (LFB) - positive intracellular material (Figure 1B), while the axons were relatively preserved (Figure 1C). A chronic periventricular (CP) lesion, identified four years earlier by MRI scan, was well-demarcated, hyperintense on T2 and hypointense on T1 scans. It was histologically characterized by chronic inflammatory infiltrate (Figure 1G), a completely demyelinated cystic lesion center (Figure 1H), and severe axonal loss (Figure 1I). Inflammatory infiltrate was less prominent when compared to the AP lesion (Figure 1J, K, L). A chronic thoracic (CTh) lesion was identified in spinal cord segments Th4–8. Microscopic evaluation revealed increased vascularity, minimal inflammatory infiltrate, and extensive demyelination and axonal loss. NAWM tissue obtained from right frontal lobe contained diffuse macrophage and predominantly CD8 lymphocytic infiltrate. Therefore, the histopathological studies confirmed the CNS inflammatory demyelinating disease consistent with MS.

Inflammatory Demyelinating CNS Lesions and NAWM Infiltrates are Enriched in Myelin-reactive T-cells

Post-mortem flow cytometry studies on non-manipulated T-cell infiltrates contained predominantly CD45RO+ memory cells: 93% of CD3+ cells in the NAWM, 97% in the AP lesion, and 98% of cells in the CP lesion. Ten days following the initial phytohemagglutinin (PHA) stimulation, the analysis of CD3+ gated cells derived from the NAWM and the AP lesion contained higher number of CD8+ lymphocytes (CD4+ to CD8+ ratios of 0.8 and 0.6, respectively), while infiltrates from the CTh and CP lesions contained higher percentage of CD4+ cells (CD4+ to CD8+ ratios of 1.9 and 3.1, respectively), as presented in Figure 2. In order to ensure a sufficient number of cells for further studies, the T-cells were expanded with PHA over three rounds of re-stimulation.

The antigen specificity of NAWM and inflammatory demyelinating lesion-derived T-cells was tested against a panel of 13 immunodominant myelin-derived peptides, identified in studies of MS patients' T-cell reactivity and in studies of experimental autoimmune encephalomyelitis (EAE) [2], Figure 3. The highest proliferative response was detected against proteolipid protein (PLP) 190–209 in the AP lesion, which also induced a proliferative response (stimulation index, SI>2) in the CTh, CP lesions, and NAWM. Cells

derived from NAWM exhibited a proliferative response against six myelin peptides: cyclic nucleotide phosphodiesterase (CNPase) 356–388, myelin oligodendrocyte glycoprotein (MOG) 21–40, MOG 11–30, PLP 190–209, PLP 89–106, and myelin basic protein (MBP) 13–32. The AP lesion infiltrate shared the proliferative response against 5 peptides, with an additional response against MBP 111–129. MOG 35–55, MOG 21–40, MOG 11–30 and PLP 190–209 peptides induced a proliferative response by the T-cells derived from the CTh lesion, and MOG 11–30, PLP 190–209 and MBP 13–32 peptides by the T-cells derived from the CP lesion. The frequency of antigen-specific cells ($\sim 10^{-4}$) was estimated by dividing a number of positive wells (SI>2) with a number of plated cells per individual antigen.

CNS T-cell Infiltrates Have a Highly Restricted TCRV β Repertoire

In order to characterize the TCR usage of infiltrating cells derived from CNS demyelinating lesions and NAWM, we used Immunoscope® and TcLandscape® technologies. Cell infiltrates derived from NAWM, AP, CTh, chronic cervical (CC), and CP lesions displayed highly altered TCRV β repertoires (red spots on Figure 4A). The global percentages of CDR3-LD alterations reached 60.54% for the AP lesion, 51.75% for the CTh, 64.66% for the CC, and 58.25% for the CP lesion. Interestingly, the NAWM sample also yielded high selection of TCRV β (67.33% CD3-LD alteration). The cell infiltrates from the five different CNS regions showed CDR3-LD alterations associated with high V β mRNA accumulation. In the AP lesion, V β 17 with a CDR3 length of five amino-acids showed a large amount of mRNA, as indicated by a V β /HPRT ratio of 45. This V β family corresponded to a skewed V β family infiltrating all the lesions and NAWM (“shared” alteration). V β mRNA accumulations were also seen in NAWM (V β 2), in the CP lesion (V β 21) and in the spinal cord lesions (V β 6.1, V β 6.5, V β 8 and V β 13.1 for the CTh; V β 5.1 for the CC lesion). The results are presented as the TcLandscapes in Figure 4A. Such profiles represent highly restricted repertoires, indicating an extremely high T-cell clonal selection.

To ensure that these selective profiles were not related to the short-term PHA expansion, control PBMCs derived from 5 RR MS patients were expanded with PHA over 1.5 months and tested for TCRV β repertoire by TcLandscapes. These control experiments identified polyclonal profiles on the TCR landscape after PHA expansion (data not shown). In contrast, the T-cells derived from CNS inflammatory demyelinating lesions and NAWM exhibited very restricted oligoclonal repertoires, indicating that CNS infiltrating cells are selected prior to PHA expansion. Furthermore, a TcLandscape performed on the RNA samples derived from non-manipulated NAWM tissue also revealed high degree of T-cell clonal selection (data not shown) consistent with the report by Muraro et al. [11].

In order to analyse the presence of individual TCRV β clones in NAWM and in different CNS lesions, the CDR3-LD profiles were compared within each family. Figure 4B shows eight V β families (V β 3, 6.1, 6.5, 11, 13.1, 14, 15 and V β 17) containing transcripts with CDR3 lengths shared by all lesions and the NAWM cell infiltrates (referred to as “shared” alterations; Figure 4B, orange boxes). These shared alterations represented 3 % of the total CDR3 lengths present in all CNS regions (eight out of 240 CDR3 lengths), while 16.25 % (39 out of 240 CDR3 lengths) corresponded to “private” CDR3 lengths (i.e. present only in one studied region; Figure 4B, blue boxes). The alterations shared by all lesions but not

NAWM (1.25 %, three out of 240 CDR3 lengths), corresponded to V β 11, V β 21 and V β 22 (Figure 4B, pink boxes). Therefore, most of the CDR3 lengths (79.5%, 190 out 240 CDR3 lengths) were present in two or three different CNS regions. However, each CDR3 length may contain several CDR3 sequences and does not necessarily correspond to a single clonotype. In order to address this possibility, we performed a J β analyses of the two V β families (V β 15 and V β 17, shared by all CNS regions. We identified the presence of identical CDR3 lengths in five CNS regions (J β 1.4 and J β 2.5 for V β 15; J β 2.2 for V β 17), confirming the presence of shared T-cell clones between all CNS regions.

Genomic and Proteomic Profiling of the T-cell Infiltrates Reveal Accumulation of Th17 Cells in the Acute MS Lesion

Studies of EAE have established that activated T-cells entering CNS play a critical role in the disease development, warranting their detailed gene expression profiling. However, gene array (GA) studies of lesion *tissue* have identified immune response (IR) genes at a significantly lower proportion (20%) of differentially expressed genes (DEGs) than studies of PBMCs (40–50%) [12], reflecting the relatively low numbers of inflammatory cells within the CNS. In order to study the gene expression profiles of *T-cells* infiltrating inflammatory CNS lesions, we focused on differentially expressed IR genes in *T-cells* infiltrating AP, CTh and CP lesions in comparison to NAWM. NAWM was used as a reference, since it also contained diffuse cellular infiltrate that allowed comparison with the gene expression profiles from the lesion cell infiltrates. While NAWM does not represent a normal brain parenchyma, it is the best reference for the gene expression profiles from inflammatory demyelinating lesions' cellular infiltrates in an individual patient. Figure 5 summarizes the results for IR genes with >5-fold change in expression, while the remaining DEGs are listed in the Supplementary Table 1.

All three lesions shared 11 differentially expressed IR genes, as indicated in Figure 5. While the shared DEGs reflect an inflammatory process common for MS lesions at various stages of progression, differentially expressed genes in the *acute lesion* characterize the immune response that initiates inflammatory demyelinating lesion formation. We focused on IR DEGs in the AP lesion and extended analysis to the genes with >1.5-fold changes in comparison to NAWM, as presented in Table 1. Gene expression profiling of the AP lesion infiltrate revealed up-regulation of TCRV β and TCR α , but a decreased expression of TCR gamma chain TCRGV9. Signaling molecule gene expression changes included the up-regulation of RORC and STAT1, while regulatory T-cell markers forkhead box protein P3 (FOXP3) and IL2RA were down-regulated. Cytokines/cytokine receptors showed the strongest up-regulation of IL-15, followed by IL-7R, both implicated in the long-term survival of memory cells. IL-17D and IL-26, which are secreted by Th-17 cells, were up-regulated, while IFN- γ , IL-4, and IL-10, which inhibit Th17-cell differentiation, were down-regulated in the AP lesion. The Th2 cytokines IL-4, IL-5 and IL-13 were inhibited in all three lesions, with the strongest down-regulation in the AP lesion. Gene expression for chemokines and their receptors in the AP lesion revealed up-regulation of the Th1 chemokine receptors CX3CR1 and CCR5, and down-regulation of Th2 chemokines CCL3, CCL1, and the chemokine receptor CCR8. Among the co-stimulatory molecules, we detected upregulation of CD30L, CD28, CD27, CD69, CD40L, and the glucocorticoid-

induced TNF receptor, and down-regulation of the inhibitory co-stimulatory molecules B and T lymphocyte attenuator (BTLA) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).

Protein array (PA) analysis of *secreted* cytokines and their receptors by the T-cells derived from the AP lesion revealed increased levels of TNF- β , IL-1 α , TGF- β 3, IL-1Ra, IL-1 β , IL-12 p70, IL-16, and IL-12p40. IL-5 and IL-10 were decreased in the supernatants of T-cells derived from the AP lesion in comparison to the NAWM. PA results on 17 proteins from the supernatant of T-cells infiltrating the AP lesion confirm the gene array data from the same cultures (>1.5-fold change in both gene and protein expression), including the IL-15 and IL-16 increase and IL-10 and IL-5 decrease in comparison to the NAWM, as presented in Figure 6.

Discussion

We have characterized, for the first time, the inflammatory T-cells obtained *post-mortem* from MS lesions at various stages of progression, and primarily focused on the T-cell infiltrate derived from an *acute* lesion whose onset correlated with the neurological symptoms of the fatal clinical exacerbation. Immunohistologically, the acute lesion showed prominent perivascular and parenchymal inflammatory infiltrates. When analyzed by flow cytometry, the infiltrate contained predominantly CD45RO⁺CD8⁺ cells, consistent with previous studies of MS lesion tissue [13; 14]. Despite its brief duration, the acute lesion was characterized by active demyelination with myelin proteins-laden macrophages, decrease in oligodendrocyte numbers, and axonal transection, confirming that demyelination and axonal transection are early events in MS lesion formation [15; 16]. The analysis of the CP lesion, whose presence was documented by MRI over four years, indicates that inflammatory cells persist in MS lesions as a chronic cell depository. CD45RO⁺ memory cells constituted nearly all of the T-cells derived from NAWM and MS lesions, suggesting that their selective migration into the CNS is determined by their previous exposure to antigens. In support of that, we detected a high frequency of T-cells reactive to multiple myelin peptides in the infiltrates from all CNS regions. Consistent with reports on myelin antigens recognition in the peripheral circulation [17], PLP 190–209 elicited the strongest proliferative response by T-cells derived from the acute lesion, and induced a (SI>2) proliferative response by the cells derived from other lesions and NAWM.

TCRV β repertoire studies of T-cells infiltrating MS lesions and NAWM revealed restrictive clonal selection. We can only speculate whether clonal selection occurs as T-cells cross the blood brain barrier or only selected clones remain in the lesion due to recurrent antigen recognition. Our studies of the TCRV β repertoire in multiple MS lesions and NAWM revealed a skewed repertoire dominated by a limited number of clones. Comparison of the CDR3-LD profiles of MS lesions and NAWM revealed 8 V β families containing transcripts with CDR3 lengths shared among all tested CNS regions. Those shared CDR3 alterations represent 3% of the total CDR3 lengths, while most of the CDR3 lengths are present in two or more different CNS lesions. Our results establish the presence of shared CDR3 lengths from the same V β and J β families in different CNS regions, suggesting closely related clonotypes and their role in MS lesion development.

The low numbers of infiltrating T-cells harvested from the MS lesions required PHA polyclonal expansion in order to study transcriptional profiles of the CNS-infiltrating inflammatory cells. While studies of the TCRV β repertoire using *in-vitro* PHA expansion provide CDR3 spectra that are similar to the results from the amplified RNA derived from non-manipulated cells [11], the short-term PHA culture might have introduced some bias in T-cell selection from the original lymphocyte culture, and thus may have affected the level of lymphocyte activation of the expanded cultures. In this respect, the extremely high level of clonal selection must be interpreted with caution, even though non-manipulated NAWM tissue also exhibited a very skewed TCRV β repertoire. The very high level of selected CDR3 lengths in the expanded culture suggests a high proportion of T-cells selected by common antigen. The estimated frequency of $\sim 1 \times 10^{-4}$ myelin-reactive T-cells in the CNS infiltrates is significantly higher than the reported 1×10^{-6} precursor frequency of MBP and PLP-reactive cells in the peripheral blood of MS patients [18].

In order to detect patterns of gene expression changes at various stages of MS lesion progression, we performed Affymetrix gene array analysis of the T-cells infiltrating the AP, CTh, CP lesion, and NAWM. Differentially expressed IR genes in the *acute lesion* reflect characteristics of the inflammatory response initiating an autoimmune response within the CNS. TCR β and TCR α genes displayed increased expression in the AP lesion, while TCR gamma chain 9 showed higher expression in the NAWM, consistent with decreased gamma-delta T-cell-mediated apoptosis of encephalitogenic T-cells in an acute lesion [19]. Markers of Treg cells (CD25 (IL2RA) and FOXP3) are also up-regulated in the NAWM in comparison to the acute lesion. Up-regulation of RORC, a transcription factor that orchestrates Th17 differentiation and induces IL17 production [20], as well as cytokines secreted by Th17 cells (IL-17D and IL-26) [21] reflect the accumulation of this cell subset in the acute lesion. IL-15, a cytokine that induces IL-17 production [22; 23], is up-regulated in the acute lesion infiltrate, consistent with a report of increased expression of IL-15 in PBMCs and CSF-derived mononuclear cells in MS patients [24]. Suppressor of cytokine signaling 3, a major inhibitor of IL-23-mediated STAT3 phosphorylation and Th17 generation [25], has increased expression in the NAWM, consistent with a negative regulation of the JAK/STAT signaling pathway. The T-cells derived from the AP lesion exhibit increased STAT-1 expression, reflecting the role of Th1 cells in the inflammatory CNS response and/or a significant cell subset that produces both Th1 and Th17 cytokines in humans [26]. In contrast, we identified a relatively higher expression of cytokines that inhibit Th17 expansion, including IFN- γ , IL-4, and IL-10 in the NAWM infiltrate, as well as the Th2 cytokines IL-5 and IL-13. The expression of the IL-7R gene, recently associated with susceptibility to MS [27], is up-regulated in the acute lesion infiltrate. Among the co-stimulatory molecules, the elevated gene expression of CD28, CD27, and CD30L and a decreased gene expression of the inhibitory molecules BTLA and CTLA-4 indicate a T-cell activated state.

Our results indicate that immune dysregulation governs the inflammatory response and lesion formation in MS, as illustrated by the accumulation of Th17 cells in the acute lesion and higher expression of genes for anti-inflammatory cytokines, inhibitory co-stimulatory molecules, regulatory gamma-delta TCRD9 chain-positive cells and Treg genes (FOXP3) in NAWM, reflecting a breakdown of the immune tolerance in MS lesions. The study identified

several molecules involved in Th17 cell differentiation, which may serve as selective targets for new therapies in RR MS.

Materials and Methods

MS Lesion Resection and Phenotypical Characterization of T-cell Infiltrates

Brain and spinal cord tissue was obtained six hours post mortem from a 16-year-old African American female who suffered from an aggressive RR MS. The study was approved by an institutional review committee and was performed upon obtaining a signed consent by family members. Five lesions at various stages of progression and areas of NAWM were identified by MRI over a four-year clinical course. The final MRI study, obtained three days prior to death, was correlated with the macroscopic tissue exam at the time of post-mortem lesion resection. Hematoxylin-Eosin, LFB and silver impregnation staining were used to demonstrate cell morphology, myelin and axons. Inflammatory infiltrates were analyzed using immunohistochemical stains: CD68 for macrophages and CD3, CD4 and CD8 for T-lymphocytes, as reported previously [28].

Upon resection, the tissue was immediately homogenized and incubated with 0.25% Trypsin for 1 h at 37°C. Inflammatory cells were separated using Percoll gradient centrifugation and stained with the following antibodies: CD3-Cy, CD4-FITC, CD8-PE, CD45RO-FITC, CCR7-FITC, CD27-FITC and CD28-FITC (all from BD Bioscience, San Diego, CA), as described previously [29]. Ten days after an initial PHA expansion, the cells were stained again for more detailed quantitative analysis of T-cells upon acquisition of 10 000 events.

In order to obtain sufficient cell numbers for further studies, we expanded the CNS infiltrating lymphocytes by PHA polyclonal activation, as this method allows a 1000-fold increase in cell number over three cycles of re-stimulation. Cells were plated at 10^4 cells/well (Costar, Cambridge, MA) with 10^5 /well irradiated allogenic feeders (PBMCs) and PHA (Remel, Lenexa, KS) at 1 µg/ml in media consisting of: RPMI 1640; 10% human serum (Gemini Bioproducts, Woodland, CA); 1% penicillin/streptomycin; 2% glutamine; 1% NEAA; 1% HEPES buffer and sodium pyruvate. Medium was exchanged and 100 IU/ml of human rIL-2 (NCI, Bethesda, MD) was added every 3–4 days. T-cell cultures were expanded every 14 days *in-vitro* with PHA and allogenic feeders, and after three rounds of re-stimulation, sufficient cell numbers were obtained for detailed characterization.

CNS-infiltrating T-cells' Proliferative Response Against Myelin-derived Peptides

T-cells derived from MS lesions and NAWM were plated in triplicates at 10^4 cells/well in 96-well plates in serum free medium (Opti MEM, Gibco, Grand Island, NY). Peptide recognition was measured in a split-well assays at 10 µg/ml, as previously described [17]. Irradiated PBMCs from a donor matched for the patient's major histocompatibility complex class II allele DRB1*0701/09 were used as antigen presenting cells at 10^5 cells/well. A proliferative response was considered antigen-specific when stimulation index (SI) = cpm response to peptide/cpm background was >2.

TCRV β Repertoire Analysis

The concentration and quality of RNA derived from cells infiltrating the MS lesions and NAWM were determined using nanoRNA Chips (Agilent®, Edinburgh, UK). cDNA was obtained from 2 μ g of RNA reverse-transcribed using an Invitrogen cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN). CDR3 was amplified by quantitative polymerase chain reaction (PCR) using 26 different V β primers and a C β primer in a 9700 thermocycler (Applied Biosystems, Foster City, CA). The PCR fragments' migration in acrylamide gel gave the distribution of the different CDR3 lengths, and analyses were performed using Immunoscope® software [30]. The percentage of CDR3 length distribution (CDR3-LD) alterations for each V β family and the global percentage of CDR3-LD alteration for each sample were calculated as described previously [31]. Briefly, the percentage of alteration was defined as the difference between the frequency of each CDR3 length in the distribution profile of the studied V β family and the average control distribution calculated from 14 healthy individuals [6]. The magnitude of the CDR3-LD alteration compared to controls is displayed as a bi-dimensional TopView and the global three-dimensional TcLandscape® [32]. MatLab® 5.3 software (The MathWorks Inc., Natick, MA) was used to compute and display the data.

Gene Expression Profiling of T-cells Derived from MS Lesions and NAWM

In order to simultaneously capture the complex changes of gene expression in T-cells derived from the CNS inflammatory demyelinating lesions, we used Affymetrix Human Genome U133 (HG-U133) arrays. The arrays contain ~45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 human genes. Briefly, 5×10^6 resting cells were cultured in serum-free medium over 24 h, and the total RNA was isolated by RNeasy Kit (Qiagen, Valencia, CA). Seven μ g of total RNA was used for cDNA synthesis using a custom cDNA kit (Life Technologies, Gaithersburg, MD). The arrays were hybridized for 16 hours at 45° C in a GeneChip Hybridization Oven 640, washed, stained with R-phycoerythrin streptavidin in a GeneChip Fluidics Station 400, and scanned with a Hewlett Packard GeneArray Scanner. The results were expressed as the relative expression for the individual gene, normalized per chip. Genes were considered differentially expressed when >5-fold change in the gene expression was detected in T-cells derived from an MS lesion in comparison to the NAWM. Gene tree clustering was carried out using Gene Spring software (Agilent Technologies, Palo Alto, CA). The gene expression results were confirmed by real-time PCR.

Profiling of Proteins Secreted by T-cells Derived from MS Lesions And NAWM

5×10^6 /ml T-cells derived from CNS inflammatory demyelinating lesions and NAWM were cultured in serum-free medium for 24 h, as described above. Supernatants were collected and quantitative analysis of 120 *secreted* proteins was performed using human cytokine protein arrays (Ray Biotech, Norcross, GA) according to the manufacturer's instructions. Briefly, after blocking, membranes were incubated with 1 ml of supernatant sample in 1:250 diluted biotin-conjugated primary antibodies, followed by the incubation with 1:1000-diluted HRP-conjugated streptavidin and the detection buffer. Membranes were exposed to Premium Autoradiography Film (Denville Scientific Inc., Metuchen, NJ). The films were

scanned with an EPSON Expression 1680 Scanner (Epson America Inc., Long Beach, CA), and the signal intensities quantified using Meta Imaging Series 5.0 software (Molecular Devices, Downingtown, PA). The lowest measurement on the membrane was subtracted from all measured proteins as the background, and the results were normalized by the sum of optical density values for all proteins on the membrane. Results were expressed as a fold change of the normalized protein level in the supernatant of T-cells derived from the MS lesion in comparison to NAWM. Fold-change of >1.5 in both gene expression and the level of secreted protein was considered confirmatory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NAWM	normal appearing white matter
MS	multiple sclerosis
CNS	central nervous system
TCR	T-cell receptor
CDR	complementarity-determining region
RORC	retinoic acid-related orphan nuclear hormone receptor C
PBMC	peripheral blood mononuclear cells
CSF	cerebrospinal fluid
MRI	magnetic resonance imaging
NMO	neuromyelitis optica
AP	acute pontine
LFB	luxol fast blue
CP	chronic periventricular
CTh	chronic thoracic
PHA	phytohemagglutinin
EAE	experimental autoimmune encephalomyelitis

PLP	proteolipid protein
SI	stimulation index
CNPase	cyclic nucleotide phosphodiesterase
MOG	myelin oligodendrocyte glycoprotein
MBP	myelin basic protein
CC	chronic cervical
GA	gene array
IR	immune response
DEGs	differentially expressed genes
FOXP3	forkhead box protein P3
BTLA	B and T lymphocyte attenuator
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
PA	protein array
PCR	polymerase chain reaction

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Figure 1.

Histopathological characteristics of the AP and CP MS lesions. A) Hematoxylin and Eosin staining of the AP lesion detected perivascular inflammatory infiltrates, activated microglia and a decreased number of oligodendrocytes. B) LFB staining revealed active demyelination with phagocytosed LFB-positive myelin within macrophages (arrow). C) Axonal staining showed a relatively preserved axonal network. D), E) and F) The perivascular inflammatory infiltrate was composed of CD68+ macrophages and predominantly CD8+ lymphocytes. G) The CP lesion showed cystic changes with scattered mononuclear cells. H) Myelin staining

revealed complete demyelination. I) Axonal staining showed profound axonal loss with axonal swelling and spheroids (asterisk). J), K) and L) The residual inflammatory infiltrate, which was less prominent when compared to the AP lesion, was composed of CD68+ macrophages and CD3+ lymphocytes.

CD3⁺ LYMPHOCYTES **Figure 2.**

Phenotypic characterization of lesion infiltrates. Flow cytometry plots represent CD3⁺ gated viable cells derived from acute and chronic lesions upon PHA expansion (10 days). CD4⁺ and CD8⁺ cell percentages of gated cells are indicated in the plot.

Figure 3.

Proliferation of lesion-infiltrating T-cells against myelin peptides. The antigen specificity of NAWM and MS-lesion-derived T-cells was tested against a panel of 13 immunodominant myelin-derived peptides. The results are presented as average cpm from three wells $\pm$ SD.

Figure 4.

TCRV β repertoire of infiltrating T cells derived from MS lesions and NAWM. A) Alterations of CDR3-LD are represented in a global bi-dimensional and a three-dimensional representation. The X axis displays the 26 human V β families, the 13 possible CDR3 lengths per V β family are on the Y axis, and the V β /HPRT transcript ratios are on the Z axis. The percentages of alterations are represented by a color code ranging from deep blue (-30%) to dark red (+30%) in the integrated landscapes. Black arrows indicate an example of "shared" alteration (shared CDR3 length of eight amino-acids for V β 15 in all the

lesions and NAWM). Red arrows indicate an example of lesion alteration (V β 11 with a CDR3 length of eight amino-acids present in all lesions, but not in NAWM). B) Recurrent V β and CDR3 length distributions were detected in multiple infiltrates from MS lesions and NAWM. The figure presents 8 V β families containing shared CDR3 lengths of expanded infiltrating cells in five tested CNS regions. Shared alterations (CDR3 lengths shared by all CNS regions) are highlighted by orange boxes; shared alterations present in MS lesions, but not in NAWM are presented as pink boxes; while private alterations, corresponding to a CDR3 length that is present only in one studied lesion are presented as blue boxes.

Figure 5.

Gene expression profiling of T-cell infiltrates derived from MS lesions in comparison to NAWM. Tree graphs summarize the gene expression results for IR genes with >5-fold change in MS lesions in comparison to NAWM. They represent normalized expression levels for all IR DEGs in colored bars and the clustering of genes according to their expression patterns. Superscript symbols denote DEGs in multiple lesions: *denotes DEGs shared between AP and CTh lesion, ^denotes DEGs shared between AP and CP lesion, and ~ denotes DEGs shared between CTh and CP lesion. Complete gene names are listed in the Supplementary Table 1.

Figure 6.

Protein expression profiling of the T-cell infiltrate derived from the AP MS lesion. Gene array results from T-cell infiltrate derived from the acute MS lesion were compared to protein array data obtained from the supernatants of cells used for GA analysis. GA results for 17 genes were confirmed at the protein level for the genes and proteins with fold changes >1.5-fold.

Table 1.

Gene expression profiling of the T-cell infiltrate derived from the AP lesion. The table lists cytokines/receptors, chemokines/receptors, co-stimulatory and signalling molecules whose gene expression was changed in T-cell infiltrates derived from the AP lesion. In order to include all genes of interest, we extended the analysis to >1.5-fold changed genes in each MS lesion in comparison to NAWM.

Name	Gene Bank	Description	Fold Changes in Gene Expression in the Lesion vs. NAWM			
			AP	CTh	CP	
Cytokines/Receptors						
IL-15	NM_000585	Interleukin-15	8.3	-5.6		1.7
IL7R	NM_002185	Interleukin-7 receptor	7.4	-2.4		7.9
TGFBRI	AV00621	TGFB receptor 1	4.6	8.0		3.1
IL18BP	A1521549	Interleukin-18 binding protein	4.5	1.3		2.0
IFNGRI	A1458949	Interferon gamma receptor 1	3.9	2.9		1.6
IL17D	A1669535	Interleukin-17D	3.6	1.1		-2.2
IL26	NM_018402	Interleukin-	3.0	1.0		1.3
IL11RA	NM_004512	Interleukin-11 receptor, alpha	2.8	4.2		1.8
IL16	M90391	Interleukin-16	2.7	1.5		-1.7
IFNAR2	A1653318	Interferon alpha receptor 2	2.5	4.9		2.2
IL12RB2	R01220	Interleukin-12 receptor B2	2.2	3.2		2.4
IFNARI	NM_000629	Interferon alpha receptor 1	2.0	1.4		1.0
IL21R	NM_021798	Interleukin-21 receptor	-2.1	-1.5		2.8
ILST	AB015706	Interleukin-6 signal transducer, gp130	-2.2	-7.4		-5.2
IL4	NM_000589	Interleukin-4	-2.2	1.2		-1.5
IFNG	M29383	Interferon gamma	-4.0	-99.5		-1.9
IL2RA	K03122	Interleukin-2 receptor, alpha	-7.4	-18.3		-2.3
IL1A	M15329	Interleukin-1, alpha	-9.3	-6.7		1.6
IL10	NM_000572	Interleukin-10	-9.4	-11.6		-1.4
IL8	NM_000584	Interleukin-8	-11.4	-12.3		4.3
IL5	NM_000879	Interleukin-5	-37.9	-6.3		-5.0
IL13	NM_002188	Interleukin-13	-42.2	-21.2		-7.8
Chemokines/Receptors						

Name	Gene Bank	Description	Fold Changes in Gene Expression in the Lesion vs. NAWM			
			AP	CTh	CP	
CX3CR1	U20350	Chemokine (C-X3-C motif) receptor 1	92.0	1.2	1.1	
CCR5	NM_000579	Chemokine (C-C motif) receptor 5	2.1	1.0	1.1	
CCL4	NM_002984	Chemokine (C-C motif) ligand 4	-2.3	-2.5	-1.2	
CCL28	AW083576	Chemokine (C-C motif) ligand 28	-2.9	-1.9	-3.2	
CCR8	NM_005201	Chemokine (C-C motif) receptor 8	-3.1	4.0	1.0	
XCL1	NM_003175	Chemokine (C motif) ligand 2	-3.5	7.0	1.0	
CCL3	NM_002983	Chemokine (C-C motif) ligand 3	-3.8	-5.3	-1.4	
CCL1	NM_002981	Chemokine (C-C motif) ligand 1	-24.3	-13.9	-2.8	
<i>Costimulatory Molecules</i>						
TNFSF8/CD30L	AI936516	Tumor necrosis factor (ligand) superfamily, member 8	81.4	17.7	5.2	
CD28	NM_006139	CD28 molecule	78.8	1.0	29.0	
TNFRSF7/CD27	NM_001242	Tumor necrosis factor receptor superfamily, member 7	37.7	15.1	6.2	
TNFSF13B/CD257	AW151360	Tumor necrosis factor receptor superfamily, member 13b	4.5	-1.7	1.7	
CD69	BF439675	CD69 molecule	4.0	6.0	3.1	
TNFSF5/CD40L	NM_000074	Tumor necrosis factor (ligand) superfamily, member 5	3.9	1.3	6.5	
CD5	AI797836	CD5 molecule	3.8	-1.7	-1.1	
TNFSF13	BF448647	Tumor necrosis factor (ligand) superfamily, member 13	3.5	1.8	1.1	
CD97	NM_001784	CD97 molecule	3.1	1.1	-1.1	
CD53	AI022073	CD53 molecule	2.9	1.0	1.0	
TNFRSF11A/CD265	AW026379	Tumor necrosis factor receptor superfamily, member 11a	2.8	1.7	-1.2	
CD200R	AF497548	Cell surface glycoprotein receptor CD200	2.5	-1.7	-2.4	
CD37	NM_001774	CD37 molecule	2.4	1.3	1.2	
TNFRSF18/GITR	AF117297	Tumor necrosis factor receptor superfamily, member 18	2.3	-8.0	5.6	
TNFRSF14, HVEM	BC002794	Tumor necrosis factor receptor superfamily, member 14	2.2	1.0	1.2	
TNFRSF10B/CD262	AF016266	Tumor necrosis factor receptor superfamily, member 10b	2.2	2.5	1.4	
CD48	AI378026	CD48 molecule	2.1	2.5	1.7	
CD14	AA993683	CD14 molecule	2.0	-1.6	-2.0	
CD32	J04132	CD32 molecule	2.0	-1.7	-1.4	

Name	Gene Bank	Description	Fold Changes in Gene Expression in the Lesion vs. NAWM			
			AP	CTh	CP	
CD58	D28586	CD58 molecule, lymphocyte function-associated antigen 3	-2.3	-2.8	-1.6	
CD80	AY081815	CD80, B7-1 molecule	-2.3	1.3	1.3	
CD226	NM_006566	CD226 molecule	-2.3	-2.2	-1.9	
CD109	AL110152	CD109 molecule	-2.7	1.6	-1.3	
CD86	L25259	CD86, B7-2 molecule	-2.7	-6.6	1.3	
TNFRSF8/CD30	NM_001243	Tumor necrosis factor receptor superfamily, member 8	-3.0	-1.5	-1.2	
TNFSF11/CD254	AF053712	Tumor necrosis factor receptor superfamily, member 11	-3.4	-1.6	1.1	
CD74	M28590	CD74 molecule	-3.8	-8.4	1.6	
CD7	NM_006137	CD7 molecule	-5.3	-2.1	-1.7	
CTLA4	U90273	Cytotoxic T-lymphocyte-associated protein 4	-7.9	-15.8	-3.1	
BTLA	AW294080	B and T lymphocyte associated	-8.3	-9.3	-12.9	
CD9	NM_001769	CD9 molecule	-10.3	-3.0	-3.5	
<i>Signaling Molecules</i>						
RORC	AI218580	Retinoic acid-related orphan nuclear hormone receptor C	11.3	1.0	1.0	
STAT1	AI539443	Signal transducer and activator of transcription 1	9.6	6.5	12.8	
SOC32	NM_003877	Suppressor of cytokine signaling 2	2.1	-1.1	-1.4	
STAT6	BC004973	Signal transducer and activator of transcription 6	2.1	2.2	1.3	
FOXP3	AF277993	Forkhead box P3	-3.7	-3.5	-1.7	
SOC33	AI244908	Suppressor of cytokine signaling 3	-10.5	-6.4	1.2	