Systemic Inflammation in Progressive Multiple Sclerosis Involves Follicular T-Helper, Th17- and Activated B-Cells and Correlates with Progression

Jeppe Romme Christensen¹*, Lars Börnsen¹, Rikke Ratzer¹, Fredrik Piehl², Mohsen Khademi², Tomas Olsson², Per Soelberg Sørensen¹, Finn Sellebjerg¹

¹ University of Copenhagen and Department of Neurology, Rigshospitalet, Copenhagen, Denmark, ²Department of Clinical Neuroscience, Neuroimmunology Unit, Karolinska University Hospital, Stockholm, Sweden

Abstract
Pathology studies of progressive multiple sclerosis (MS) indicate a major role of inflammation including Th17-cells and meningeal inflammation with ectopic lymphoid follicles, B-cells and plasma cells, the latter indicating a possible role of the newly identified subset of follicular T-helper (TFH) cells. Although previous studies reported increased systemic inflammation in progressive MS it remains unclear whether systemic inflammation contributes to disease progression and intrathecal inflammation. This study aimed to investigate systemic inflammation in progressive MS and its relationship with disease progression, using flow cytometry and gene expression analysis of CD4⁺ and CD8⁺ T-cells, B-cells, monocytes and dendritic cells. Furthermore, gene expression of cerebrospinal fluid cells was studied. Flow cytometry studies revealed increased frequencies of ICOS⁺TFH-cells in peripheral blood from relapsing-remitting (RRMS) and secondary progressive (SPMS) MS patients. All MS subtypes had decreased frequencies of Th1 TFH-cells, while primary progressive (PPMS) MS patients had increased frequency of Th17 TFH-cells. The Th17-subset, interleukin-23-receptor ‘CD4’ T-cells, was significantly increased in PPMS and SPMS. In the analysis of B-cells, we found a significant increase of plasmablasts and DC-SIGN⁺ and CD83 B-cells in SPMS. ICOS⁺TFH-cells and DC-SIGN’ B-cells correlated with disease progression in SPMS patients. Gene expression analysis of peripheral blood cell subsets substantiated the flow cytometry findings by demonstrating increased expression of IL21, IL21R and ICOS in CD4‘T-cells in progressive MS. Cerebrospinal fluid cells from RRMS and progressive MS (pooled SPMS and PPMS patients) had increased expression of TFH-cell and plasmablast markers. In conclusion, this study is the first to demonstrate the potential involvement of activated TFH-cells in MS. The increased frequencies of Th17-cells, activated TFH- and B-cells parallel findings from pathology studies which, along with the correlation between activated TFH- and B-cells and disease progression, suggest a pathogenic role of systemic inflammation in progressive MS. These observations may have implications for the treatment of progressive MS.

Citation: Christensen JR, Börnsen L, Ratzer R, Piehl F, Khademi M, et al. (2013) Systemic Inflammation in Progressive Multiple Sclerosis Involves Follicular T-Helper, Th17- and Activated B-Cells and Correlates with Progression. PLoS ONE 8(3): e57820. doi:10.1371/journal.pone.0057820

Editor: Lionel G. Filion, University of Ottawa, Canada

Received September 14, 2012; Accepted January 26, 2013; Published March 1, 2013

Copyright: © 2013 Romme Christensen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Danish Council for Independent Research (grant 271-06-0246), the Danish Council for Strategic Research (grant 2142-08-0039), the Danish MS Society, the Warwara Larsen Foundation, the Johnsen Foundation, Brdr. Ranje Holding, Jeppe Juuel Memorial Legacy and research grants from Biogen Idec. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Jeppe Romme Christensen has received honoraria for lecturing from Biogen Idec and had travel expenses reimbursed by Merck Serono. Lars Börnsen and Mohsen Khademi report no conflict of interest. Rikke Ratzer has had travel expenses reimbursed by Merck Serono, TEVA, Biogen Idec and Sanofi Aventis. Fredrik Piehl has a research contract pending with Biogen Idec; has received payment for development of educational presentations from Biogen Idec, Novartis, and Merck Serono and has had travel expenses reimbursed by Sanofi Aventis, Biogen Idec and Novartis. Tomas Olsson has received honoraria for lectures, participation in advisory boards and unrestricted MS research grants from Biogen Idec, Novartis, Merck, Sanofi Aventis and Bayer. Per Soelberg Sørensen has served on scientific advisory boards Biogen Idec, Merck Serono, Novartis, Gennab, TEVA, GSK, Bayer Schering, and he has received funding of travel for these activities; has served as Editor-in-Chief of the European Journal of Neurology, and is currently editorial board member for Multiple Sclerosis Journal, European Journal of Neurology, Therapeutic Advances in Neurological Disorders and has received speaker honoraria from Biogen Idec, Merck Serono, TEVA, Bayer Schering, Sanofi Aventis, and Novartis. His department has received research support from Biogen Idec, Bayer Schering, Merck Serono, TEVA, Baxter, Sanofi-Aventis, BioMS, Novartis, Bayer, RoFAR, Roche, Genzyme, from the Danish Multiple Sclerosis Society, the Danish Medical Research Council, and the European Union Sixth Framework Programme: Life sciences, Genomics and Biotechnology for health. Finn Sellebjerg has served on scientific advisory boards for Biogen Idec, Merck Serono, Novartis, Sanofi-Aventis and TEVA and as consultant for Biogen Idec and Novo Nordisk; has received support for congress participation from Biogen Idec and Sanofi Aventis; has received speaker honoraria from Biogen Idec, Bayer Schering, Merck Serono, Sanofi-Aventis and Novartis, Ranje Holding and Biogen Idec partly funded the study. There are no patents, products in development or marketed products to declare. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: E-mail: jeppe.romme.christensen@rh.regionh.dk

Introduction

Progressive multiple sclerosis (MS) is characterized by steady progression of neurological disability without remission. Disability accumulation in progressive MS is severe and the time to development of a progressive disease course is the main determinant of the long-term prognosis [1,2]. However, the pathogenetic understanding of disease progression is incomplete, and the development of treatments for progressive MS has so far...
been disappointing [3]. An unsolved question is to what extent disease progression is driven by inflammatory processes or axonal loss independent of inflammation. A low rate of relapses and gadolinium-enhancing lesions, pronounced atrophy and limited efficacy of treatment has supported a view where axonal loss independent of inflammation is thought to be the substrate for disease progression [4]. This view was challenged by recent pathology studies, which indicate that in progressive MS CNS inflammation is abundant and correlates with axonal damage and disease progression [5,6]. Primary progressive (PPMS) and secondary (SPMS) progressive MS pathology is characterized by widespread diffuse inflammation with slowly expanding lesions, abundant cortical lesions, and lymphocyte infiltration and microglia activation in the normal appearing white matter (NAWM) [7]. The cellular density of infiltrates is generally lower than in acute lesions of RRMS, but progressive MS patients have higher numbers of B-cells and plasma cells in lesions, NAWM and meninges [5,6]. Meningeal inflammation is pronounced in MS, and ectopic lymphoid follicle-like structures (ELFs) are observed in the meninges in progressive MS patients [6,8]. ELFs are associated with more rapid disease progression, cortical lesions, meningeal and white matter inflammation, atrophy and neuronal loss [9,10]. ELFs resemble lymphoid follicles with evidence of germinal center reactions, possibly facilitating the activation and differentiation of T- and B-cells within the CNS compartment [8]. The presence of ELFs is suggestive of the involvement of follicular T-helper (TFH) cells, a recently discovered T-cell subset, which is necessary for germinal center formation [11]. Additionally, monocytes and dendritic cells have been implicated in MS immunopathology [12–14].

Gene expression and immunohistochemistry studies of progressive MS brains have shown increased expression of pro-inflammatory cytokines, including interferon-gamma (IFNG), interleukin-17 (IL17), IL21, IL23 and tumor necrosis factor-alpha (TNFA) [15–19].

Thus, pathology studies have suggested CNS inflammation to be a key determinant for disease progression and axonal damage in progressive MS. The presence of ELFs and diffuse white matter inflammation with activated microglia could indicate a compartmentalization of inflammation, suggesting that CNS inflammation and disease progression in progressive MS could occur independent of systemic inflammation [4]. Several studies did, however, report increased activation of immune cells in peripheral blood from progressive MS patients including changes in surface phenotype [20–27] and expression of cytokines [28,29], the most consistent being increased expression of IL12p40 and decreased expression of IL10 [25,26,29–32] indicative of a pro-inflammatory bias in progressive MS.

To date no systematic study of T-cells, B-cells, monocytes and dendritic cells has investigated systemic immune activation in progressive MS.

We hypothesized that patients with progressive MS have evidence of systemic immune activation, show signs of Th17- and TFH-cell inflammation and that this is associated with disease progression. Accordingly, we performed flow cytometry analysis on subsets of CD4+ and CD8+ T-cells, B-cells, monocytes and dendritic cells, selected on the basis of previous progressive MS pathology and immunology studies, and subsequently related frequencies of significantly enriched subsets to disease activity. To substantiate findings from flow cytometry studies, we isolated peripheral blood mononuclear cell (PBMC) subsets and analyzed relevant targets by polymerase chain reaction (PCR). Finally, to relate the systemic immune activation with CNS inflammation, gene expression analysis of CSF cells and PBMCs was undertaken for the most important targets.

### Table 1. Demographic and clinical characteristics of MS patients, healthy controls and non-inflammatory neurological controls included in the studies.

| Group         | N | Female/male | Age | MS duration | EDSS | EDSS change |
|---------------|---|-------------|-----|-------------|------|-------------|
| Flow cytometry|   |             |     |             |      |             |
| HC            | 32| 24/8        | 36 (29–46) |             |      |             |
| RRMS remission| 20| 14/6        | 38 (29–42) | 6 (3–10) | 2.3 (1.0–3.5) | 0.5 (0.0–1.0) |
| SPM5          | 20| 11/9        | 48 (43–53) | 14(11–21) | 5.8 (4.8–6.5) | 0.5 (0.5–1.0) |
| PPMS          | 20| 8/12        | 51 (45–54) | 7 (4–9) | 5.0 (4.0–6.0) | 1.0 (0.5–2.0) |
| Cell subsets |   |             |     |             |      |             |
| HC            | 12| 8/4         | 36 (29–45) |             |      |             |
| RRMS remission| 10| 7/3         | 34 (29–40) | 6 (2–9) | 1.3 (0.0–1.5) | 0.0 (0.0–0.5) |
| SPM5          | 10| 6/4         | 46 (33–52) | 16 (10–19) | 6.5 (6.0–6.5) | 0.8 (0.5–1.0) |
| PPMS          | 11| 6/5         | 53 (44–55) | 8 (3–10) | 4.5 (4.0–6.0) | 1.0 (0.0–1.5) |
| Whole blood   |   |             |     |             |      |             |
| SPM5          | 23| 12/11       | 50 (42–53) | 16 (11–27) | 5.5 (4.5–6.5) | 0.5 (0.5–1.0) |
| SPM5 mitoxantrone | 22 | 15/7 | 46 (42–54) | 17 (14–24) | 6.3 (5.9–6.5) | 0.0 (0.0–0.4) |
| CSF cell studies |   |             |     |             |      |             |
| NIND          | 20| 15/5        | 34 (31–39) |             |      |             |
| RRMS remission| 10| 8/2         | 44 (42–45) | 8 (2–12) | 2.8 (2.0–3.0) |             |
| RRMS relapse  | 10| 6/4         | 37 (33–40) | 3 (1–9) | 3.3 (2.0–3.5) |             |
| SPM5          | 10| 6/4         | 53(45–62) | 20 (15–23) | 5.5 (5.0–6.0) |             |
| PPMS          | 10| 6/4         | 53 (44–63) | 3 (2–8) | 3.8 (3.5–4.5) |             |

Values for age, MS disease duration, EDSS and EDSS change are medians with interquartile ranges in brackets. EDSS change represents the change in EDSS the two previous years prior to blood sampling. Abbreviations: CSF = cerebrospinal fluid; EDSS = expanded disability status scale; HC = healthy control; MS = multiple sclerosis; NIND = non-inflammatory neurological disease; RRMS = relapsing-remitting multiple sclerosis; SPM5 = secondary progressive multiple sclerosis; PPMS = primary progressive multiple sclerosis. 

doi:10.1371/journal.pone.0057820.t001
Figure 1. Flow cytometry studies on follicular T-helper cells and Th17-cells in peripheral blood from multiple sclerosis patients and healthy controls. Mean plots show mean percentages with error bars representing 95% confidence intervals. Dot plots show values for parameters tested in Spearman’s rank correlation coefficient. Human blood follicular T-helper (TFH) cells are CXCR5+CD4+T-cells and as depicted on flow cytometry plot (A) ICOS+TFH-cells were identified as ICOS+CXCR5+CD4+T-cells. (B) The frequency of ICOS+TFH-cells in blood is significantly increased in relapsing-remitting multiple sclerosis in clinical remission (RRMS) and in secondary progressive multiple sclerosis (SPMS) patients. (C) In the SPMS group the frequency of ICOS+TFH-cells significantly correlates with change in expanded disability status scale (EDSS) 2 years prior to sampling of blood. (D) A significant correlation between the frequencies of ICOS+TFH-cells and plasmablasts emphasize a known biological function of TFH-cells and that ICOS+TFH-cells are activated TFH-cells. (E) Flow cytometry plot showing the characterization of Th1 TFH-cells as CXCR3+CCR6− TFH-cells, Th2 TFH-cells as CXCR3−CCR6+ TFH-cells and Th17 TFH-cells as CXCR3−CCR6− TFH-cells. (F) Th1 TFH-cells are significantly decreased in all MS groups and (G) Th17 TFH-cells are significantly increased in primary progressive multiple sclerosis (PPMS) patients. (H) Th17-cells were identified as interleukin-23-receptor+ (IL23R+) CD4+T-cells and (I) there is significant increases of IL23R+CD4+T-cells in SPMS and PPMS patients.

doi:10.1371/journal.pone.0057820.g001
Materials and Methods

Patients and Healthy Controls

One cohort (Cohort A) including 20 relapsing-remitting MS (RRMS), 20 SPMS and 20 PPMS (all untreated) and 32 healthy controls (HC) was included for flow cytometry studies. Since the groups varied in age, we recruited healthy controls with an age and gender distribution that enabled us to make age- and gender-matched HC groups when a cell subset correlated with age. A second cohort (Cohort B) comprising 11 RRMS, 10 SPMS and 11 PPMS untreated patients and 12 HCs was used for gene expression studies on PBMC subsets. A third cohort (Cohort C) consisting of 45 SPMS patients, of whom 23 were untreated and 22 were treated with mitoxantrone, underwent studies of gene expression in whole blood (WB). Cohort A, B and C patients were recruited from the MS Clinic at Copenhagen University Hospital Righospitalet and had expanded disability status scale (EDSS) scores assigned by a neurologist (see Table 1).

Finally, a cohort (Cohort D) consisting of 10 RRMS in clinical remission, 10 RRMS in clinical relapse, 10 SPMS and 10 PPMS untreated patients and 20 non-inflammatorily neurological disease (NIND) patients (15 with sensory symptoms; 1 CADASIL; 1 carpal tunnel syndrome; 1 migraine with aura; 1 hypothyreosis with sensory disturbances; 1 vertigo) from Karolinska University Hospital was recruited for studies of gene expression in PBMCs and CSF cells.

The study protocol was approved by scientific ethics committee of the Capital Region of Denmark and the Karolinska Institute and written informed consent was obtained from the patients and healthy controls according to the Declaration of Helsinki.

Samples

Blood was sampled in PAXgene (PreAnalytiX, Germany) and EDTA tubes. PBMCs were isolated using Lymphoprep (Axis-Shield, Norway). CSF was collected by lumbar puncture, immediately centrifuged and the CSF cell pellet snap-frozen.

Flow Cytometry Studies

PBMCs were incubated with relevant combinations of fluorochrome-conjugated monoclonal antibodies (Table S1A). WB was used for parallel assessment of absolute cell counts using TBNK reagent with TruCount tubes (BD Biosciences, USA). Data were acquired on a BD FACSCanto II flow cytometer and data analysis was done using BD FACSDiva Software (BD Biosciences, USA). In the development of the flow cytometry panel we systematically screened and titrated relevant antibodies for each antigen and subsequently assessed the most appropriate gating strategy for each subset. In the final data analysis, these strategies included the use of fluorescence-minus-one gating controls, isotype gating controls and visually based criteria.

Gene Expression Studies

Subsets of CD4+ T-cells, CD8+ T-cells, B-cells, monocytes and dendritic cells (mean purity >92%) were isolated on an autoMACS separator using MACS cell separation kits (Miltenyi Biotec, Germany). RNA was extracted from 20,000–1,000,000 cells of the obtained subsets with PicoPure RNA Isolation Kit (Arcturus, USA). PAXgene Blood RNA Kit was used for PAXgene RNA extraction. CSF cell RNA was extracted using RNeasy Mini Kit (Qiagen, Germany). cDNA was synthesized with qScript cDNA SuperMix (Quanta BioSciences, USA) or iScript Supermix (Bio-Rad, USA). Real-time polymerase chain reactions (RT-PCR) were performed on a 7300 Fast or ViiA 7 Real-Time PCR System (Applied Biosystems, USA) using TaqMan Gene Expression Assays (Table S1B) and PerfeCTa FastMix (Quanta Bioscience, USA). Threshold cycle (CT) values were calculated using SDS software (Applied Biosystems, USA). The relative mRNA transcript expression was calculated using the 2−ΔΔCT method. Reference genes were GAPDH and UBE2D2 in cell subset and WB studies and UBC for CSF cell and PBMC studies. Expression values were normalized to a HC PBMC cDNA pool resulting in a normalization ratio (NR). In the CSF cell studies we had a low RNA concentration, resulting in some samples being without amplification, in which case the NR was arbitrarily set to zero.

Statistical Analysis

Statistical analysis was performed using PASW 19 software (IBM, USA). Patients with progressive MS were older than RRMS patients, and several cell-types change in frequency with age. Consequently we analyzed all flow cytometry variables from HCs for correlation with age using Spearman’s rank correlation coefficient. Data were analyzed using ANCOVA with adjustment for age when a variable correlated significantly with age and otherwise ANOVA was used. A few variables were not normal distributed, and bootstrap procedures were applied to all ANOVA and ANCOVA tests. Since the analysis involved multiple comparisons we applied the false discovery rate (FDR) method to calculate q-values [33]. Q-values were considered significant when q< 0.05. Analysis for correlation between cell surface-phenotypes and disease progression was performed with Spearman’s rank correlation coefficient. Gene expression data were analyzed with parametric statistics when appropriate and otherwise with non-parametric methods. Changes in the expression of mRNA were considered significant for p<0.05. The analysis of CSF gene expression data was restricted by the low RNA contents, and the resulting absence of amplification in some samples. This introduce variance and reduces the statistical power, and consequently we pooled the groups of RRMS in relapse and remission to a RRMS group and the group of SPMS and PPMS patients to a progressive group, resulting in three groups of each 20 individuals (NIND, RRMS and Progressive MS).

Results

Increased Frequencies of Circulating T<sub>FH</sub> and Th17-cells in Progressive MS

We first conducted a detailed analysis of CD4+ and CD8+ T-cells, B-cells, monocytes and dendritic cells in peripheral blood (Table S1A and S2A) Using FDR-correction for multiple corrections, significant differences between the patient groups were found mainly for CD4+ T-cells. Examination of the phenotype of CCR6<sup>+</sup>CD4<sup>+</sup> T-cells, presumably Th17-cells [35], CD4<sup>+</sup> T-cells with a CD25<sup>+</sup> T regulatory (TReg) phenotype was comparable in MS patients and healthy controls according to the Declaration of Helsinki.

Importantly, the frequency of ICOS<sup>+</sup>T<sub>FH</sub>-cells was found mainly for CD4<sup>+</sup>T-cells. Examination of the phenotype of ICOS<sup>+</sup>T<sub>FH</sub>-cells for B-cell activation. CXCR3<sup>+</sup>, Th1-like T<sub>FH</sub>-cells, a subset lacking true effector function [34], were reduced in frequency in all subtypes of MS compared to HCs, and in PPMS there was an increased frequency of an activated T<sub>FH</sub>-cell subset, ICOS<sup>+</sup>T<sub>FH</sub>-cells in RRMS and SPMS compared to HCs (Figure 1A–B).

Analysis of Th1- and Th17-phenotypes showed that SPMS and PPMS patients had an increased frequency of IL23-receptor (IL23R)<sup>+</sup>CD4<sup>+</sup>T-cells, presumably Th17-cells [35], in blood compared to HCs (Figure 1H–I).

The frequency of CD4<sup>+</sup>T<sub>FH</sub>-cells with a CD25<sup>+</sup>CD127<sup>-</sup> regulatory T-cell (T<sub>Reg</sub>) phenotype was comparable in MS patients and controls, and CSF patients with chronic progressive MS had significantly reduced frequencies of ICOS<sup>+</sup>T<sub>FH</sub>-cells in blood compared to RRMS and SPMS patients (Figure 1C, 1D).

Increased Frequencies of Circulating T<sub>FH</sub> and Th17-cells in Progressive MS
controls [36]. There was, however, a decrease in the number of CD31+ recent thymic emigrant TReg-cells in SPMS and PPMS patients, a finding which was paralleled by a decrease in CD31+CD4+T-cells in SPMS and PPMS patients (Table S2A).

Antigen-presenting Cell Activation in Progressive MS

In our flow cytometry analyses we included a detailed phenotyping of B-cells, monocytes, myeloid and plasmacytoid dendritic cells (Table S1 and S2B-C), all cell types with known antigen-presenting cell (APC) function. We found higher frequencies of DC-SIGN+B-cells and CD83+B-cells in SPMS than in HCs and DC-SIGN+B-cells frequency correlated with disease progression in SPMS (Figure 2A, B, C, D, E). Of note both subsets correlated significantly with IL23R+CD4+T-cells and ICOS+Tfh-cells (Figure S1), indicating an association between these T- and B-cell subsets. Plasmablasts were identified as CD27+CD38+cells and were increased in SPMS patients compared to HCs (Figure 2F, G, H). We also observed an increased percentage of monocytes and monocytes expressing ICOS-ligand (ICOSL) in SPMS (Figure 2J, K), whereas other differences observed for B-cells, monocytes and dendritic cells were not statistically significant after FDR-correction for multiple comparisons.

Gene Expression in CD4+T-cells, CD8+T-cells, B-cells, Monocytes and Dendritic Cells

To substantiate the findings in the flow cytometry studies, we analyzed gene expression in purified CD4+T-cells, CD8+T-cells, B-cells, monocytes and dendritic cells (Table S3). Expression of ICOS, IL21 and IL21R (IL21-receptor) in CD4+T-cells from SPMS patients was increased, which is suggestive of increased activation of Tfh-cells in SPMS (Figure 3A). In addition, SPMS patients had increased expression of IFNG (interferon-gamma) in CD4+T-cells and of LTB (lymphotoxin-beta) in CD4+ and CD8+T-cells and of TNFA, LTBR (LTB-receptor), TNFSF14 (LIGHT), and the transcription factors IL6X and GATA3 in CD8+T-cells. In PPMS patients there was higher expression of ICOS, IL21R and LTB in CD4+T-cells and of LTB, LTBR, TGFβ1 (transforming growth factor-beta) and GATA3 in CD8+T-cells.

Observed gene expression changes in APCs corroborated the findings in T-cells (Figure 4). B-cells from SPMS patients had increased expression of LTA (lymphotoxin-alpha), monocytes from SPMS and PPMS patients had increased expression of LTBR and TNFRSF14 (HVEM), and dendritic cells from patients with PPMS had increased expression of LTBR and IL21R.

Cerebrospinal Fluid Cell Expression of Tfh-cell and Plasmablast Genes

The finding of increased frequencies of ICOS+Tfh-cells in SPMS patients and TH17-like Tfh-cells in PPMS patients prompted us to analyze measures of Tfh-cell activation in samples of CSF cells. We found significantly increased expression of ICOS and IGJ (immunoglobulin J chain) in progressive MS and RRMS patients, and increased LTA expression in CSF cells from RRMS patients (Table 2). In spite of being a low abundance transcript we were able to detect IL21 expression in 60% of progressive MS samples and 10% of NIND and RRMS samples, and there was trend (p = 0.06) towards increased IL21 expression in CSF cells from progressive MS patients. In the PBMCs we could not reproduce the finding of increased IL21 expression in CD4+T-cells in SPMS patients, which is likely explained by the pooling of the groups and by the low expression level with IL21 detectable in only 53% of the PBMC samples.

Mitoxantrone Treated SPMS Patients have Reduced Expression of IL21

Finally we sought to determine if immunosuppressive treatment with mitoxantrone is associated with alterations in the expression of genes associated with Tfh activation in blood cells. As shown (Figure 5), SPMS patients treated with mitoxantrone had significantly lower IL21 expression, whereas there were no significant differences in ICOS and IGJ gene expression.

Discussion

Previous studies have indicated the presence of systemic and intrathecal inflammation in progressive MS, but it is unclear to what extent systemic inflammation mirrors intrathecal inflammation and whether systemic inflammation contributes to intrathecal inflammation and disease progression. The present study is the first to report prominent Tfh-, Th17- and B-cell activation in blood from patients with progressive MS, findings which parallel recent pathology studies. Tfh- and B-cell activation correlated with disease progression in SPMS and Tfh activation marker IL21 was decreased in SPMS patients treated with mitoxantrone. Further we found increased expression of genes associated with Tfh- and B-cell activation in CSF from RRMS and progressive MS patients. These findings emphasize an association between the systemic immune compartment and disease progression in progressive MS.

We initially studied immune activation using a broad flow cytometry characterization of blood cell subsets, correcting for multiple comparisons in the statistical analysis by the FDR-method and correcting for age effects when appropriate. In accordance with a previous study we found similar frequencies of CXCR5+CD4+T-cells in MS and HCs [37]. ICOS+Tfh-cells are activated Tfh-cells with a phenotype similar to germinal center Tfh-cells, which are capable of activating B-cells and produce the effector cytokine IL21 [11]. ICOS+Tfh-cell frequency was increased in RRMS and SPMS patients, and correlated with disease progression in SPMS patients. Others have phenotyped human Tfh-cells according to CCR6 and CXCR3 expression, resulting in division into Th1, Th2 and Th17-like Tfh-cell subsets, of which only the latter two are capable of inducing B-cells to produce immunoglobulin via IL21 [34]. We found that all subsets of MS patients have a lower percentage of Th1 Tfh-
Figure 3. Gene expression studies of peripheral blood CD4+ and CD8+ T-cells from multiple sclerosis patients and healthy controls. Mean plots show mean normalization ratios (NR) with error bars representing 95% confidence intervals. Parametric (ANOVA and post-hoc T-tests) or non-parametric (Kruskal-Wallis (KW) and Mann-Whitney (MW)) statistic tests were applied when suitable. (A) Gene expression analysis in CD4+ T-cells shows increased expression of ICOS, IL21R and LTB in secondary progressive (SPMS) and primary progressive (PPMS) multiple sclerosis patients as compared to healthy controls (HC) and increased expression of IL21 and IFNG in SPMS patients compared to HCs. (B) Gene expression analysis in CD8+ T-cells shows increased expression of GATA3, LTB and LTBR in SPMS and PPMS compared to HCs. HLX, TGFβ, TNFA and TNFSF14 were increased in SPMS compared to HCs.

doi:10.1371/journal.pone.0057820.g003
cells, whereas PPMS patients had an increased percentage of Th17 TFF cells, consistent with a skewing towards activated TFF cells. We also found a decrease of CD31+ recent thymic emigrant cells in patients with PPMS and SPMS, as previously reported [38]. Other previous studies found increased CD40-ligand, CCR2 and CCR5 expression on CD4^+ and CD8^+ T-cells in SPMS [23,39]. These findings were not confirmed in the present study.

Figure 4. Gene expression studies of peripheral blood B-cells, monocytes and dendritic cells from multiple sclerosis patients and healthy controls. Mean plots show mean normalization ratios (NR) with error bars representing 95% confidence intervals. Parametric (ANOVA and post-hoc T-tests) or non-parametric (Kruskal-Wallis (KW) and Mann-Whitney (MW)) statistic tests were applied when suitable. (A) B-cells have increased expression of LTA in secondary progressive multiple sclerosis (SPMS) patients as compared to healthy controls (HC). (B) Gene expression in monocytes, showing decreased expression of IL23 in relapsing-remitting multiple sclerosis in remission (RRMS), SPMS and primary progressive multiple sclerosis (PPMS) compared to HCs and decreased LTB and IL21R expression in monocytes in RRMS patients compared to HCs. Increased expression of LTBR and TNFRSF14 in monocytes is observed for SPMS and PPMS patients. (C) Dendritic cells have increased expression of LTBR and IL21R in PPMS patients compared to HCs. doi:10.1371/journal.pone.0057820.g004
Expression of IL21R and ICOS was increased in purified CD4+ T-cells from SPMS and PPMS patients while SPMS patients also had increased IL21 expression. In CSF cells from patients with progressive MS ICOS expression was increased. These molecules all have known effector T FH functions and the observation is thus compatible with TFH-cell activation [11,40]. Furthermore, expression of IL21 mRNA was lower in blood cells from SPMS patients treated with mitoxantrone, suggesting that inhibition of TFH function may be an effect of mitoxantrone in SPMS. However, it is worth noting that IL21 and ICOS are also expressed by Th17-cells and a CCR9+CD4+T-cells, a subset with resemblance to TFH-cells [41,42]. Indeed, animal and human studies have described overlapping features of Th17- and TFH-cell biology [11,43,44], and recently it was shown that Th17-cell-induced EAE mice develop ELFs, possibly caused by the differentiation of Th17-cells to TFH-cells [45]. Thus, the finding of increased IL21 and ICOS, is not definite proof of TFH-activity but could also be a result of Th17-activation. Detection of the Th17 signature cytokine IL17 in purified CD4+ T-cells was limited to few individuals and therefore we could not substantiate whether

| Gene expression data cerebrospinal fluid cell (CSF) and peripheral blood mononuclear cell (PBMC) are expressed as normalization ratio (NR). Values are expressed as means with standard error (SE). Parametric (ANOVA and T-test) and non-parametric analyses (Kruskal-Wallis and Mann-Whitney) were used when appropriate. Post-hoc analyses was done when ANOVA or Kruskal-Wallis tests were significant (p = 0.05) and values for post-hoc tests are indicated (T-tests: p<0.05 and Mann-Whitney ~ p<0.05; ~ p<0.01). Relapsing-remitting multiple sclerosis (RRMS) group consists of 10 patients in clinical remission and 10 patients with clinical relapse. Progressive multiple sclerosis (MS) consists of 10 secondary progressive and 10 primary progressive MS patients. Abbreviations: Non-inflammatory neurological disease = NIND. doi:10.1371/journal.pone.0057820.t002 |

| Table 2. Cerebrospinal fluid cell and peripheral blood mononuclear cell gene expression data. |
| --- |
| NIND | RRMS | Progressive MS | Kruskal-Wallis~/\ ANOVA p-value |
| **ICOS PBMC NR** | 20 | 0.41 | 0.03 | 20 | 0.44 | 0.04 | 20 | 0.42 | 0.04 | 0.89 |
| **ICOS CSF NR** | 20 | 0.54 | 0.12 | 20 | 0.94 | 0.14 | 20 | 0.89 | 0.09 | 0.04 |
| **IGJ PBMC NR** | 20 | 1.53 | 0.85 | 20 | 1.05 | 0.22 | 20 | 0.72 | 0.08 | 0.89 |
| **IGJ CSF NR** | 20 | 0.13 | 0.03 | 20 | 1.57< | 0.25 | 20 | 1.43< | 0.35 | 0.000002< |
| **IFNG PBMC NR** | 20 | 0.31 | 0.05 | 20 | 0.23 | 0.04 | 20 | 0.33 | 0.07 | 0.48 |
| **IFNG CSF NR** | 20 | 0.11 | 0.06 | 20 | 0.09 | 0.07 | 20 | 0.11 | 0.06 | 0.53 |
| **IL21 PBMC NR** | 20 | 0.74 | 0.2 | 20 | 0.72 | 0.22 | 20 | 0.71 | 0.18 | 0.98 |
| **IL21 CSF NR** | 20 | 0.29 | 0.24 | 20 | 0.37 | 0.37 | 20 | 1.96 | 0.85 | 0.06 |
| **LTA PBMC NR** | 20 | 0.25 | 0.03 | 20 | 0.36 | 0.04 | 20 | 0.28 | 0.03 | 0.15 |
| **LTA CSF NR** | 20 | 0.13 | 0.08 | 20 | 0.48< | 0.13 | 20 | 0.19 | 0.06 | 0.04< |
| **L7B PBMC NR** | 20 | 1.63 | 0.13 | 20 | 1.78 | 0.18 | 20 | 1.98 | 0.18 | 0.30 |
| **L7B CSF NR** | 20 | 2.99 | 0.29 | 20 | 3.14 | 0.34 | 20 | 2.73 | 0.21 | 0.59 |

Figure 5. Gene expression studies of whole blood in secondary progressive multiple sclerosis with and without mitoxantrone treatment. Mean plots show mean normalization ratios (NR) with error bars representing 95% confidence intervals. Parametric (T-test) or non-parametric (Mann-Whitney (MW)) statistic tests were applied when suitable. Comparing untreated secondary progressive multiple sclerosis (SPMS) patients to SPMS patient treated with mitoxantrone (SPMS MTX) whole blood expression of IL21 significantly decreased while no significant changes were observed for ICOS and IGJ. doi:10.1371/journal.pone.0057820.g005
the increase in IL23R⁺ CD4⁺ T-cells in SPMS and PPMS reflects a true increase in Th17 activity or a potential for increased Th17 effector function in progressive MS.

The flow cytometry finding of skewing towards activation of Tfh cells in all MS subtypes corresponds to results of histopathology studies which have demonstrated the presence of IL21R⁺ and IL21⁺ CD4⁺ T-cells in both active and chronic lesions [19] and meningeal inflammation with closely associated T- and B-cells in all MS subtypes [10,46]. Furthermore, the findings are consistent with the presence of ELFs in some SPMS cases. In this context, it has been suggested that ELFs represents an end-stage of meningeal inflammation, which fits well with the long disease duration of SPMS cases [46]. Taken together these findings could indicate that activation of Tfh cells is a feature of all MS subtypes.

The extent to which the observed changes in Tfh and Th17-phenotypes and recent thymic emigrants are associated with functional changes in immune reactivity must be established in functional studies, but we hypothesize that the changes in Tfh phenotype may reflect an increased potential for Tfh interactions with B-cells and that the decrease in CD31⁺ T-cells may reflect an increased memory and effector T-cell pool in patients with progressive MS.

A second main finding is the confirmation of aberrant B-cell activation in MS, where CD80⁺ B-cells and plasmablasts are increased in the CSF and correlate with intrathecal immunoglobulin G production [47,48]. Thus, we observed increased percentages of plasmablasts, DC-SIGN⁺ B-cells and CD83⁺ B-cells in blood from SPMS, and DC-SIGN⁺ B-cells and disease progression correlated in SPMS. DC-SIGN⁺ B-cells and CD83⁺ B-cells also correlated positively with IL23R⁺ CD4⁺ T-cells and ICOS⁺ Tfh-cells, consistent with a previously described relationship between activated T- and B-cell subsets [49,50]. In fact, B-cells are known to be important for development and sustaining of Tfh and Th17-responses [11,51,52]. No studies have described DC-SIGN⁺ B-cells and CD83⁺ B-cells in MS, but DC-SIGN⁺ B-cells have been shown to be activated B-cells in humans [53], and in animal studies CD83⁺ B-cells are involved in antigen-specific T- and B-cell interactions [54]. Interestingly, several studies have used DC-SIGN and CD83 as markers for dendritic cells and found increased frequency of cells expressing DC-SIGN and CD83 in MS brains [12–14].

Among antigen-presenting cells we further found increased frequencies of monocytes and ICOSL⁺ monocytes in SPMS patients, and in gene expression studies we also found increased expression of several TNF and TNF-receptor superfamily molecules in monocytes and dendritic cells from MS patients. In general, however, the changes observed in APCs were less prominent than those observed in T-cells.

Our experimental approach is well suited for analyzing the in vitro activation stage of immune cells since there is minimal manipulation of the cells prior to the analysis. Studies relying on in vitro activation may, however, reveal differences in the potential for activation which analyses of freshly isolated cells might neglect. The most consistent findings from in vitro studies in progressive MS have been increased IL12p40 and IFNG and decreased IL10 production by stimulated PBMCs [25,30–32,55,56] in SPMS. IL12p40 is translated from IL12B mRNA, which in our study was expressed only in B-cells and not in monocytes or dendritic cells. Therefore, it appears that the study of in vitro stimulated monocytes and dendritic cells is needed to identify some of the changes in APC activation in patients with MS.

The formation of ELFs is critically dependent on the TNF and TNF-receptor superfamily molecules LTA, LTB, LTBR, TNFSF14 and TNFRSF14 [26,57,58]. We found increased expression of at least one of these transcripts in all the subsets of blood cells in progressive MS, indicating an involvement of the cytokines which likely could be linked to the development of ELFs in the meninges in progressive MS patients.

Collectively, the results of the present study suggest a central role of Tfh-cells along with Th17-cells and activated B-cell subsets in the pathogenesis of progressive MS, and indicate that systemic inflammation is associated with disease progression. Consequently, the findings support that treatments targeting Tfh- and Th17-cells or B-cells may be efficacious in the treatment of progressive MS [49,59]. Of notice, B-cell depleted mice are also deficient in Tfh-cells [11,52]. Indeed, the findings of recent clinical trials with B-cell-depleting monoclonal antibodies indicate that B cell depletion may not only be efficacious in RRMS, but also in a subgroup of PPMS patients characterized by contrast-enhancing lesions or younger age [60].

Acknowledgments

We wish to thank laboratory technicians; Joy Mendel-Hartwig, Michael Kolbjørn Jensen, Vibeke Fuglhol and Rikke Larsen for their skilful help in flow cytometry, cell-sorting and gene expression studies.

Author Contributions

Conceived and designed the experiments: JRC PSS TO FS. Performed the experiments: JRC LB RR FP MK. Analyzed the data: JRC FS. Contributed reagents/materials/analysis tools: TO PSS FS. Wrote the paper: JRC LB RR FP MK TO PSS FS.

References

1. Conforteux C, Vukusic S, Moreau T, Adeleine P (2000) Relapses and progression of disability in multiple sclerosis. N Engl J Med 343: 1430–1438.
2. Scalfari A, Neuhaus A, Daumer M, Ebers GC, Muraro PA. (2011) Age and progression of disability in multiple sclerosis. N Engl J Med 343: 1430–1438.
3. Hawker K (2011) Progressive multiple sclerosis: characteristics and management. Neurol Clin 29: 423–434.
4. Bradl M, Lasemann H (2009) Progressive multiple sclerosis. Semin Immunopathol 31: 455–465.
5. Kornek B, Storch MK, Weissert R, Wallstroem E, Stefferl A, et al. (2000) Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol 10: 164–174.
6. Magliozzi R, Howell O, Vore A, Serafini B, Nicholas R, et al. (2007) Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain 130: 1089–1104.
7. Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, et al. (2011) Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain 134: 2755–2771.
8. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F (2004) Detection of CD4⁺ B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol 10: 164–174.
9. Magliozzi R, Howell O, Vore A, Serafini B, Nicholas R, et al. (2007) Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain 130: 1089–1104.
10. Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, et al. (2011) Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain 134: 2755–2771.
11. Crotty S (2011) Follicular helper CD4 T cells (TFH). Annu Rev Immunol 29: 621–663.
12. Kivisakk P, Mahad DJ, Callahan MK, Szkora K, Trebst C, et al. (2004) Expression of CCR7 in multiple sclerosis: implications for CNS immunity. Ann Neurol 55: 627–638.
13. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Capello E, et al. (2006) Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. J Neuroimmunol Exp Neurol 65: 124–141.
14. Iftegun I, Keharl H, Bernard M, Wosik K, Dodelet-Devillers A, et al. (2008) The blood-brain barrier induces differentiation of migrating monocytes into Th17-polarizing dendritic cells. Brain 131: 785–799.
15. Lock C, Hermann G, Pedotti R, Brendolan A, Schadl E, et al. (2002) Gene-
microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmmune encephalomyelitis. Nat Med 8: 500–508.
16. Mycko MP, Paposo R, Boschetti U, Raine CS, Selmaj KW (2004) Microarray gene expression profiling of chronic and inactive lesions in multiple-
sclerosis. Clin Neurolog Neurol 106: 223–229.
17. Li Y, Chu N, Hu A, Gran B, Rostami A, et al. (2007) Increased IL-23p19 expression in multiple sclerosis lesions and its induction in microglia. Brain 130: 490–501.
18. Tzartos JS, Frise MA, Craner MJ, Palace J, Newcombe J, et al. (2008) Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. J Pathol 172: 146–155.
19. Tzartos JS, Craner MJ, Frise MA, Jakobsen KB, Newcombe J, et al. (2011) IL-
21 and IL-21 receptor expression in lymphocytes and neurons in multiple sclerosis brain. J Pathol 176: 794–802.
20. Balashov KE, Rottman JB, Weiner HL, Hancock WW (1999) CCR5(8)
expression on CD8 T cells is increased in multiple sclerosis and their ligands MIP-
1alpha and IP-10 are expressed in demyelinating brain lesions. Proc Natl Acad Sci U S A 96: 6873–6878.
21. Duran I, Martinez-Caceres EM, Rio J, Barbera N, Mazroo MS, et al. (1999) Immunological profile of patients with primary progressive multiple sclerosis. Expression of adhesion molecules. Brain 122 (Pt 12): 2297–2307.
22. Jensen J, Krakauer M, Sellebjerg F (2001) Increased T cell expression of CD154 (CD40-ligand) in multiple sclerosis. Eur J Neurol 8: 321–328.
23. Sorensen TL, Sellebjerg F (2001) Distinct chemokine receptor and cytokine expression profile in secondary progressive MS. Neurology 57: 1371–1376.
24. Martinez-Caceres EM, Eipoje C, Brieva L, Pericot I, Tintore M, et al. (2002) Expression of chemokine receptors in the different clinical forms of multiple sclerosis. Mult Scler 8: 390–395.
25. Filion LG, Matsuevicius D, Graziani-Bowering GM, Kumar A, Freedman MS (2003) Monocyte-derived IL12, CD86 (B7-2) and CD40L expression in relapsing and progressive multiple sclerosis. Clin Immunol 106: 127–130.
26. Karni A, Abraham M, Monsonego A, Cai G, Freeman GJ, et al. (2006) Innate immunity in multiple sclerosis: myeloid dendritic cells in secondary progressive multiple sclerosis are activated and drive a proinflammatory immune response. J Immunol 177: 4196–4202.
27. Ulkconen M, Wu K, Reipert B, Dasidar P, Elovaara I (2007) Cell surface adhesion molecules and cytokine profiles in primary progressive multiple sclerosis. Mult Scler 13: 701–707.
28. Buckle GJ, Hollisberg P, Haller DA (2003) Activated CD8+ T cells in secondary progressive MS secrete lymphotocin. Neurology 60: 702–705.
29. Balashov KE, Smith DR, Khoury SJ, Hafler DA, Weiner HL (1997) Increased interleukin 12 production in progressive multiple sclerosis: induction by activated CD4+ T cells via CD40 ligand. Proc Natl Acad Sci U S A 94: 599–603.
30. van Bezel-Dezaire AH, Hoff SC, van Oosten BW, Versweij CL, Drager AM, et al. (1999) Decreased interleukin-10 and increased interleukin-12p40 mRNA are associated with disease activity and characterize different disease stages in multiple sclerosis. Ann Neurol 45: 695–703.
31. van Bezel-Dezaire AH, Smits M, van Trigt-Hoff SC, Kileestijn J, van Housewijingen JC, et al. (2001) Cytokine and IL-12 receptor mRNA discriminate between different clinical subtypes in multiple sclerosis. J Neuroimmunol 120: 152–160.
32. Soldan SS, Alvarez Retuerto AI, Sicotte NL, Vokuhl RR (2004) Dysregulation of IL-10 and IL-12p40 in secondary progressive multiple sclerosis. J Neuroimmunol 146: 209–215.
33. Storey JD, Shibabara R (2003) Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 100: 9490–9495.
34. Morita K, Schmitt N, Bennebhl SE, Ranganathan R, Bourdery L, et al. (2011) Human blood CXCR5(+)/CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 34: 108–121.
35. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, et al. (2007) Development, cytokine profile and function of human intercellular 17-
producing helper T cells. Nat Immunol 8: 950–957.
36. Michel L, Berthelot L, Petre S, Wiertlewski S, Lefrere F, et al. (2008) Patients with relapsing-remitting multiple sclerosis have normal Treg function when cells expressing IL-7 receptor alpha-chain are excluded from the analysis. J Clin Immunol 28: 3411–3419.