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GPR15$^+$ T cells are Th17 like, increased in smokers and associated with multiple sclerosis

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ABSTRACT

Smoking is a risk factor for the development and progression of multiple sclerosis (MS); however, the pathogenic effects of smoking are poorly understood. We studied the smoking-associated chemokine receptor-like molecule GPR15 in relation to relapsing-remitting MS (RRMS). Using microarray analyses and qPCR we found elevated *GPR15* in blood cells from smokers, and increased *GPR15* expression in RRMS. By flow cytometry we detected increased frequencies of GPR15 expressing T and B cells in smokers, but no difference between patients with RRMS and healthy controls. However, after cell culture with the autoantigens myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein, frequencies of MBP-reactive and non-proliferating GPR15$^+$CD4$^+$ T cells were increased in patients with RRMS compared with healthy controls. GPR15$^+$CD4$^+$ T cells produced IL-17 and were enriched in the cerebrospinal fluid (CSF). Furthermore, in the CSF of patients with RRMS, GPR15$^+$ T cells were associated with CCR6$^+$CXCR3$^+$/CCR6$^-$CXCR3$^+$ phenotypes and correlated positively with concentrations of the newly identified GPR15-ligand (GPR15L), myelin degradation and disability. In conclusion, we have identified a proinflammatory cell type linking smoking with pathogenic immune cell functions in RRMS.

1. Introduction

Smoking causes a chronic inflammatory condition in the lungs characterized by increased frequencies of infiltrating immune cells [1]. Smoking is also one of the strongest environmental risk factors associated with multiple sclerosis (MS) [2–4]. Apart from an increased risk of developing MS, smokers among MS patients have an increased relapse rate during treatment, faster conversion to secondary progressive MS (SPMS) and faster disease progression [5–8].

In MS circulating immune cells play a crucial role in the disease pathogenesis [9]. Only few studies have focused on the effects of smoking on circulating immune cells in MS, and the understanding of how smoking exerts its detrimental effect in MS is poorly understood.

A recent study reported that smokers among MS patients had a higher percentage of proinflammatory T helper type 17 (Th17) cells and a lower percentage of regulatory CD25^{hi} T cells than non-smokers [10]. This relationship was, however, not confirmed in a study from our group, where we conducted a wide scale profiling of immune cell phenotypes and T cell reactivity in healthy individuals [11]. In an animal model of MS, myelin-reactive T cells were reported to mature and acquire a central nervous system (CNS) homing phenotype in the lungs [12]. Taken together these findings suggest that smoking may increase the risk of developing MS by promoting the activation and differentiation of a circulating, pathogenic T cell subset.

We used microarray and qPCR analyses to identify genes differentially expressed in mononuclear blood cells from smokers and non-smokers in patients with MS and healthy controls (HC). We identified one gene, *GPR15*, which was differentially expressed due to smoking and RRMS, and used flow cytometry analyses and T cell activation studies to further characterize GPR15-expressing cells in blood and cerebrospinal fluid. Furthermore, we studied CSF levels of the newly identified GPR15-ligand GPR15L [13,14].

2. Materials and methods

2.1. Study population

Microarray gene expression data were analyzed in a cohort

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comprising 33 patients with multiple sclerosis (MS) from a previously published study [15]. All patients were diagnosed with MS according to the 2005 revision of the McDonald criteria and the disease course was classified as relapsing-remitting (RRMS), primary progressive (PPMS) or secondary progressive SPMS according to the Lublin and Reingold classification (1996) [16]. The patients had not received immunosuppressive or immunomodulatory treatment for at least three months prior to blood sample collection.

For qPCR gene expression analyses, we studied blood samples from 93 healthy controls (HC), 48 patients with RRMS and 55 patients with progressive MS (Supplementary Table 1). All patients were untreated. 28 patients with RRMS were newly diagnosed and 20 patients with RRMS had not received immunomodulatory or immunosuppressive treatment for at least two months. All patients with RRMS were diagnosed according to the 2010 McDonald criteria [17]. Patients with progressive MS were diagnosed with SPMS or PPMS as described above. From 46 of 55 patients with progressive MS, we used baseline sample material from a recent clinical trial with erythropoietin [18,19]. In the erythropoietin trial, inclusion criteria were an EDSS score of 4–6.5, age of 19–60 years, progression without relapses of ≥0.5 EDSS point within the last 2 years and MRI consistent with MS according to the Barkhof criteria. Exclusion criteria were comorbidities, immunomodulatory or immunosuppressive treatment 1 or 6 months prior to first visit, respectively [19]. The remaining nine patients with progressive MS were seen at the Danish Multiple Sclerosis Center prior to this study. These patients did not receive immunomodulatory or immunosuppressive treatment 3 months prior to sample collection.

Flow cytometry of freshly isolated blood cells were performed in 12 male age-matched HC and in additional 33 controls and 30 patients with untreated RRMS. Of the 33 controls 26 were healthy, one had an endocrinological disorder, one had neck pain, one had an eye disease, one had otogenic vertigo, one had uncharacteristic symptoms, one had white matter lesions but did not fulfill MS diagnostic criteria and one had a small fiber neuropathy. In 8 controls and 16 patients, corresponding CSF was collected during a diagnostic lumbar puncture.

Proliferation assays were performed in 38 HC and 20 patients with RRMS who were untreated for at least three months and diagnosed according to the 2010 McDonald criteria (Supplementary Table 1) [17].

Cytokine analyses were performed in 6 HC who were non-smokers.

GPR15L was measured in 20 untreated patients with RRMS of which 15 had corresponding CSF samples and 10 controls of which 8 had corresponding CSF samples. Of the 10 controls three were healthy, one had an endocrinological disorder, one had neck pain, one had an eye disease, one had otogenic vertigo, one had uncharacteristic symptoms, one had white matter lesions but did not fulfill MS diagnostic criteria and one had a small fiber neuropathy.

All participants were characterized as smokers or non-smokers according to questionnaires or telephone interviews. Smokers were active smokers and former smokers were included in the non-smoker group. No data were obtained from individuals using nicotine replacement therapy. All participants gave informed consent to study participation. Ethical approval was obtained from the local scientific ethics committee.

2.2. Microarray gene expression analyses

Gene expression in peripheral blood mononuclear cells (PBMC) was measured using the Affymetrix Human Gene 1.0 ST Gene chip array as described in details in a previous published study [15]. Gene expression data were analyzed by ANCOVA analysis using Partek Genomics Suite 6.6 software.

2.3. Gene expression by quantitative real-time PCR (qPCR)

Blood collected in PAXgene tubes, PAXgene Blood miRNA kit (PreAnalytix, Qiagen) was used to extract total RNA. RNA was then reverse transcribed by the High Capacity cDNA RT kit (Applied Biosystems). Using TaqMan technology, qPCR was performed in duplicates on 1:10 diluted cDNA template with target-specific primers and probes for the *LRRN3, GPR15* and *CD3* genes. PCR amplification was done on a ViiA7 real-time PCR thermal cycler (Life Technologies, USA). A pool of cDNA from 50 HCs was used in quadruplets on each PCR plate as an inter-plate calibrator. An expression index was calculated by the $2^{-\Delta\Delta Ct}$ method for relative quantification using GenEx Pro 6.0.5. Data were normalized with the reference gene *CD3* since *GPR15* and *LRRN3* were shown to be mainly expressed in $CD4^+$ and $CD8^+$ T cells. All gene expression values were set relative to the calibrator pool.

2.4. Flow cytometry of freshly isolated cells

CSF samples were held on ice and cells immediately separated from fluid by centrifugation. Blood samples were collected in BD Vacutainer EDTA tubes (BD Bioscience, Denmark) and processed within an hour. PBMC's were isolated using Lymphoprep (Axis-Shield, Norway) and density gradient centrifugation and washed twice in cold PBS with 2 mM EDTA. CSF cells and PBMC's were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD8, GPR15, CCR6 and CXCR3 (Supplementary Table 2). We used matched isotype controls to correct for non-specific antibody-binding and spectral overlap. Data were acquired using a FACS Canto ll flow cytometer (BD Biosciences, USA). FlowJo software v10 (Tree star, USA) was used for data analyses. Cells expressing CD3, CD16/CD56, CD45, CD4, CD19 and CD8 were enumerated with the 6-colour TBNK Trucount kit (BD Biosciences, Denmark). Experiments and absolute cell count calculations were performed according to instructions from the manufacturer.

2.5. T cell proliferation

To identify antigen-specific T cells, we thawed PBMC's stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Denmark) and cryo-preserved in human AB serum/10% DMSO in liquid nitrogen. PBMC's were thawed in a 37 °C heat bath and washed twice in RPMI-1640/5% fetal bovine serum (all ThermoFisher, USA). $0{,}5 \times 10^6$ cells per well were cultured in 96 wells flat bottom culture plates (CellStar Greiner bio-one, Germany) in RPMI-1640/5% human AB-serum/penicillin (50 units/mL) and streptomycin (50 μg/mL) (all from ThermoFisher, USA). Antigens myelin basic protein (MBP; 30 μg/mL, HyTest, Finland), myelin oligodendrocyte glycoprotein (MOG; 10 μg/mL, BioNordika, Denmark), heat-killed candida albicans (CA; 5×10^6 cells/mL, InVivoGen, USA) or no antigen as a negative control was added to the cell culture. At day 4, new growth media was added. After 7 days in 37 °C/5% CO_2, cells were harvested and stained with fluorochrome-conjugated anti-CD3, anti-CD4, anti-CCR6, anti-CXCR3 and anti-GPR15 (Supplementary Table 2). $CD3^+CD4^-$ T cells were used as the measure of $CD8^+$ T cells. A live/dead stain was also included. Cells were washed twice in FACS/PBS (PBS/2% FBS/0.02% NaAzid) and analyzed by flow cytometry.

2.6. T cell cytokine production

To measure cytokine production of T cells, T cells were isolated from freshly drawn blood by negative selection (Human T cell Isolation kit, StemCell, Canada). Isolated T cells were left to rest overnight and stimulated with phorbol 12-myristate 13 acetate (PMA; 10 ng/mL, Sigma-Aldrich, USA) and ionomycin (0.5 μg/mL, Sigma-Aldrich, USA) for 30 min before incubating the cells for 4 h with Brefeldin A (5 μg/mL, Sigma-Aldrich, USA). Cells were stained with a live/dead stain and antibodies against CD3, CD8, and GPR15 for 25 min, and then fixed and permeabilized according to the manufacturer's protocol (Fixation Buffer and Permeabilization Buffer, Biolegend, USA) (Supplementary Table 2). $CD3^+CD8^-$ T cells were used as the measure of $CD4^+$ T cells as PMA downregulates CD4 expression [20]. Finally, cells were stained with

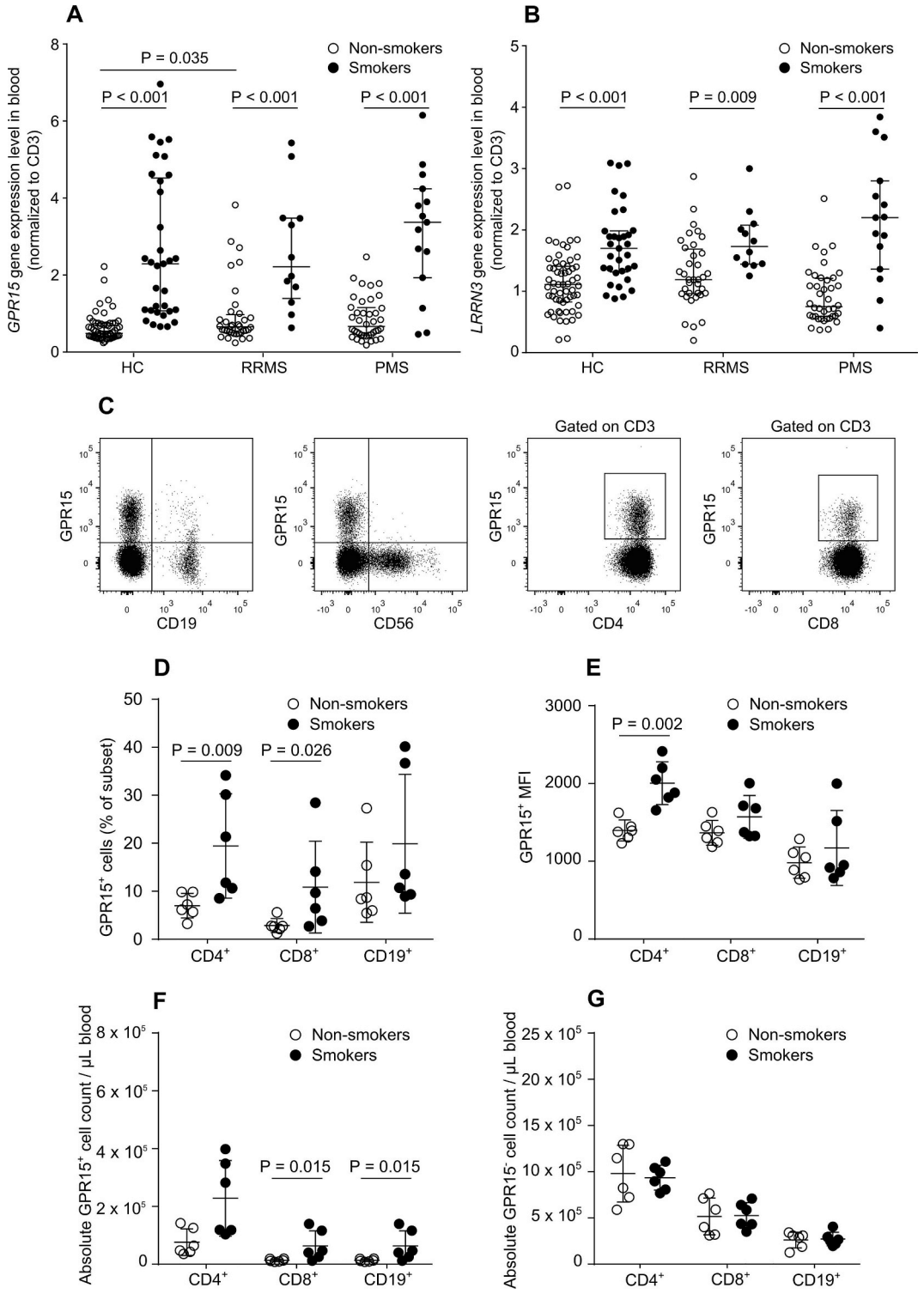

Fig. 1. *GPR15* gene and surface expression in smokers and patients with MS. Gene expression of (**A**) *GPR15* and (**B**) *LRRN3* in smokers and non-smokers in healthy controls (HC) (n = 93), relapsing remitting MS (n = 48) and progressive MS (n = 55) (ANCOVA adjusted for age (*GPR15*) and age and gender (*LRRN3*), P-values are corrected with Bonferroni post hoc analyses). The expression of *GPR15* and *LRRN3* are normalized to *CD3*. (**C**) Gating of GPR15 surface expression on $CD19^+$ and $CD56^+$ cells and $CD4^+$ and $CD8^+$ T cells by flow cytometry. Representative dot plots from 12 healthy controls are shown. (**D**) Frequencies of $GPR15^+$ cells in $CD4^+$, $CD8^+$ T and B cell populations in smokers (n = 6) and non-smokers (n = 6). (**E**) Surface expression of GPR15 on $CD4^+$, $CD8^+$ and B cells by mean fluorescence intensity. (**F-G**) Absolute cell counts of GPR15 ± cells in $CD4^+$, $CD8^+$ T and B cell populations. (**D-G**) Mann Whitney's *U* test with nominal P-values are shown. Abbreviations: HC = healthy controls, PMS = progressive MS, RRMS = relapsing remitting MS, MFI = mean fluorescence intensity.

anti-IL-17A, anti-IFN-γ, anti-TNF-α, anti-IL-10, anti-IL-4, anti-TGF-β1, anti-IL21and anti-GM-CSF and washed twice in FACS/PBS and analyzed by flow cytometry.

2.7. ELISA and electrochemiluminescence binding assays

ELISA of GPR15L was performed according to manufactures protocol (Cloud Clone, USA). All samples were measured in duplicate and mean values used.

ELISA of cotinine (Calbiotech, USA) and EBNA-1 antibodies (DiaSorin, Italy) were performed according to instructions from the manufacturer as previously described [11]. Cotinine concentrations were measured with an upper limit of 105 ng/mL.

Vitamin D electrochemiluminescence binding assays were performed routinely on Copenhagen University Hospital's Department of Clinical Biochemistry according to the manufacturer's protocol (Cobas, Roche Diagnostics, Belgium) and analyzed on a Cobas 8000 analyzer.

2.8. Genotyping of HLA-DRB1*15:01

DNA from buffy coats was genotyped by TaqMan® allelic discrimination, using the SNP rs9271366 to tag HLA-DRB1*15:01 as previously described in detail [11]. PCR assays were performed according to the manufacturer's protocol (Life Technologies, Denmark).

2.9. Statistics

Statistical testing was done by ANCOVA (gene expression data), Wilcoxon matched pairs signed rank test, Mann-Whitney U tests and Spearman rank correlation analyses. Corrections for multiple comparisons were performed when relevant by false discovery rate (FDR) correction [21] and Bonferroni post hoc analyses. P-values (two-sided) and q-values (FDR) < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 22 (IBM, USA).

3. Results and discussion

3.1. GPR15 gene expression is increased in smokers and patients with RRMS

To study the impact of smoking on circulating immune cells in MS, we performed Affymetrix microarray gene expression analyses of peripheral blood mononuclear cells (PBMC) from 33 patients with MS previously included in a gene expression study [15].

We found 32 genes differentially expressed between smokers and non-smokers among 22,148 annotated genes (Supplementary Table 3). Adjusting P-values to a false discovery rate of $q < 0.05$, two genes were still differentially expressed by ANCOVA with age and sex as covariates. The gene G protein-coupled receptor 15 (*GPR15*) was 3.6-fold upregulated in 17 smokers compared with 16 non-smokers ($q < 0.001$), and neuronal leucine-rich repeat protein-3 (*LRRN3*) was upregulated 2.1-fold in smokers compared with non-smokers ($q < 0.001$) (Supplementary Table 3).

In immunomagnetically purified PBMC subpopulations there was higher expression of *GPR15* in $CD4^+$ and $CD8^+$ T cells and B cells, compared with the expression in monocytes, NK cells and dendritic cells (Supplementary Fig. 1). For *LRRN3*, gene expression was highest in $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. 1). These results confirm previous reports of increased *GPR15* expression in smokers and particularly in $CD3^+$ T cells [22–24].

To validate and extend our findings we investigated *GPR15* and *LRRN3* gene expression in cohorts of HC and untreated patients with RRMS and progressive MS. We conducted qPCR gene expression analyses in whole blood samples, and normalized gene expression data to *CD3* (δ-subunit) as *GPR15* and *LRRN3* are mainly expressed in T cells [24]. The results showed a highly significant increase in *GPR15* and *LRRN3* gene expression in smokers compared with non-smokers in all three groups (Fig. 1A–B). Levels of the nicotine metabolite cotinine are depicted with *GPR15* expression in Supplementary Fig. 2A to show the biological correlation. Smoking status (non-smoker, former or current smoker) and levels of *GPR15* expression are depicted in Supplementary Fig. 2B.

In non-smokers, patients with RRMS had significantly higher *GPR15* expression than HC, whereas progressive MS patients did not differ from HC (Fig. 1A). Among smokers, comparable *GPR15* expression was observed in patients and HC (Fig. 1A). The gene expression of *LRRN3* did not differ significantly between any of the groups in neither smokers nor non-smokers (Fig. 1B). In HC's, we further studied if MS risk factors *HLA-DRB1*15:01* genotype, low vitamin D concentrations or high anti-EBNA-1 titers were associated with *GPR15* expression. These data were obtained in samples collected in a previously published study [11]. By Spearman's rank correlation analyses we found no significant correlations between MS risk factors and *GPR15* expression (data not shown).

The *GPR15* gene encodes the GPR15 protein, a seven-transmembrane protein receptor, sharing sequence homology with chemokine receptors [25,26]. It has been suggested that GPR15 may function as a chemoattractant receptor and GPR15 has been associated with effector T cell phenotypes in inflammatory conditions [23,27–29].

3.2. Frequencies of GPR15 expressing T cells are increased in smokers but not in patients with RRMS

We proceeded to study the expression of GPR15 on the cell surface of circulating immune cells from HC smokers and non-smokers using flow cytometry. GPR15 was expressed on $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cells but not on $CD56^+$ NK cells (Fig. 1C). In concordance with the gene expression results, frequencies of $GPR15^+CD4^+$ and $GPR15^+CD8^+$ T cells were increased in smokers compared with non-smokers (Fig. 1D). Smokers also had higher levels of GPR15 expressed on their $CD4^+$ T cells (mean fluorescence intensity) compared with non-smokers (Fig. 1E). Absolute counts of cells expressing GPR15 were also higher for $CD8^+$ T cells and $CD19^+$ B cells from smokers, whereas numbers of circulating $GPR15^-$ cells were not associated with smoking in $CD4^+$, $CD8^+$ or $CD19^+$ cells (Fig. 1F–G).

In addition, we studied frequencies of $GPR15^+CD4^+$ and $GPR15^+CD8^+$ T cells in untreated patients with RRMS compared with HC who were all non-smokers. In these two groups we found comparable proportions of both $GPR15^+CD4^+$ and $GPR15^+CD8^+$ T cells (data not shown).

Thus, smoking has a significant effect on gene expression and surface expression of GPR15 whereas only gene expression of *GPR15* is increased in RRMS. Increased *GPR15* in smokers has previously been linked to DNA hypo-methylation, but whether *GPR15* hypo-methylation is related to RRMS has not been studied [30]. Smokers are continuously smoke-exposed, whereas the included patients were all in clinical remission. We therefore hypothesized that higher GPR15 protein expression on $CD4^+$ T cells could be observed in RRMS upon activation.

3.3. $GPR15^+CD4^+$ T cell frequencies are increased with smoking and RRMS upon cultivation

In MS, autoreactive $CD4^+$ T cells are considered to play a prominent pathogenic role. To elucidate whether GPR15 characterizes an autoreactive $CD4^+$ T cell type associated with smoking, we conducted a seven-day T cell proliferation assay with the autoantigens myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) and heat-killed candida albicans (CA) as a positive control (Fig. 2A). In HC, $GPR15^+CD4^+$ T cells were highly reactive to CA regardless of smoking status, but proliferation in response to myelin autoantigens, and even negative control conditions, was significantly increased in smokers compared with non-smokers (Fig. 2B). Moreover, proliferation of $GPR15^+CD4^+$ T cells was increased in RRMS patients after MBP

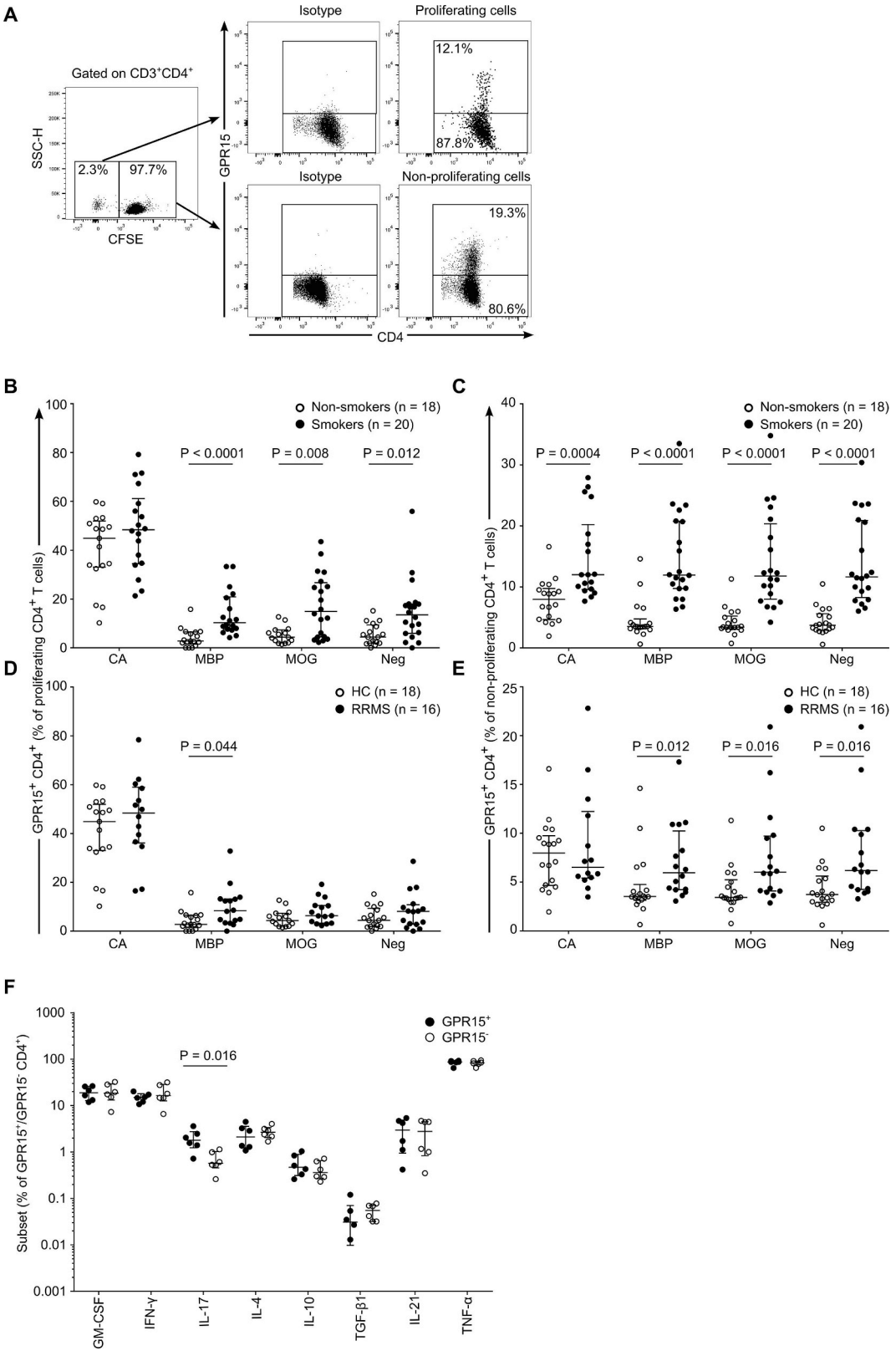

Fig. 2. Antigen reactivity and cytokine production of GPR15$^+$CD4$^+$ T cells. (**A**) Gating strategy of GPR15$^+$CD4$^+$ T cells. Representative dot plots are shown. (**B-E**) GPR15$^+$CD4$^+$ T cells cultured for 7 days with CA, MBP, MOG or no antigen (Neg). Proliferating and non-proliferating GPR15$^+$CD4$^+$ T cells in (**B-C**) healthy smokers and non-smokers and in (**D-E**) healthy controls and patients with RRMS (all non-smokers). (**F**) Intracellular staining of produced cytokines in GPR15$^+$ and GPR15$^-$ CD4$^+$ T cells of 6 healthy non-smokers. All analyses are done by Mann Whitney's *U* test. Bonferroni corrected P-values are shown if significant. Abbreviations: HC = healthy controls, MBP = myelin basic protein, MOG = myelin oligodendrocyte glycoprotein, Neg = negative control, CA = candida albicans.

stimulation compared with HC (Fig. 2D).

We also enumerated frequencies of proliferating GPR15$^+$CD8$^+$ T cells. In interpreting these results it should be considered that T cell reactivity assays with extracellularly added antigen are biased towards antigen presentation on HLA class II molecules to CD4$^+$ T cells. The data on GPR15$^+$CD8$^+$ T cell proliferation, which therefore either represents bystander activation or depend on cross-presentation on HLA class I molecules, did not differ significantly according to smoking status or a diagnosis of RRMS (Supplementary Fig. 3A + C).

In non-proliferating cells, we also observed substantial differences in GPR15$^+$CD4$^+$ T cell frequencies. Both smoking and RRMS were associated with higher GPR15$^+$CD4$^+$ T cell frequencies (Fig. 2C + E). HC smokers had a higher percentage of non-proliferating GPR15$^+$CD4$^+$ T cells after stimulation with CA, MBP or MOG as well as under negative control conditions (Fig. 2C). A significant increase in the percentage of non-proliferating GPR15$^+$CD4$^+$ T cells were also observed in RRMS compared with HC in cultures with MBP, MOG and negative control conditions (Fig. 2E). Non-proliferating GPR15$^+$CD8$^+$ T cell frequencies were increased in smokers in all conditions studied, but were not increased in patients with RRMS when compared with HC (Supplementary Fig. 3B + D).

From these findings we suggest that increased *GPR15* mRNA expression in RRMS may result in an increased potential to express GPR15 under conditions of mild activation such as those present during cell culture, and that a specific autoantigen (MBP) can elicit a proliferative response in GPR15$^+$CD4$^+$ T cells.

3.4. GPR15$^+$CD4$^+$ T cells display a Th17 profile

To determine the cytokine profile of GPR15$^+$ T cells, we stimulated freshly isolated T cells with phorbol 12-myristate 13 acetate and ionomycin and performed intracellular staining of selected cytokines. These analyses showed that the frequency of IL-17 producing cells was higher among GPR15$^+$CD4$^+$ T cells compared with GPR15$^-$CD4$^+$ T cells (Fig. 2F). Comparable frequencies of cells producing IFN-γ, GM-CSF, TNF-α, IL-21, TGF-β1, IL-10 or IL-4 were observed in GPR15$^+$ and GPR15$^-$ CD4$^+$ T cells (Fig. 2F). Cytokine production of GPR15$^+$CD8$^+$ T cells did not differ from the cytokine production of GPR15$^-$CD8$^+$ T cells (Supplementary Fig. 3E). Secretion of IL-17 defines proinflammatory Th17 cells suggested to play a role in the pathogenesis of MS [31]. Our findings therefore point towards GPR15$^+$CD4$^+$ T cells being proinflammatory and pathogenic in MS.

3.5. GPR15$^+$CD4$^+$ and CD8$^+$ T cells are enriched in cerebrospinal fluid

As *GPR15* encodes a seven-transmembrane chemokine receptor-like protein with a recently identified ligand with chemotactic properties, we hypothesized that GPR15 might be involved in lymphocyte trafficking to the CNS [13,14]. To evaluate the recruitment potential of GPR15$^+$ T cells, we analyzed corresponding blood and CSF samples from 16 patients with RRMS and 8 controls of whom 1 were a healthy control and 7 had a diagnostic lumbar puncture performed for various non-inflammatory, neurological conditions other than MS. We found a higher percentage of both GPR15$^+$CD4$^+$ and GPR15$^+$CD8$^+$ T cells in the CSF compared with blood, which was not evident for GPR15$^-$ T cells (Fig. 3A). Moreover, frequencies of GPR15$^+$CD4$^+$ and GPR15$^+$CD8$^+$ T cells in the CSF, as well as in the blood, were significantly increased with smoking (Fig. 3A). Frequencies of GPR15$^+$CD4$^+$ and GPR15$^+$CD8$^+$ T cells in the CSF were comparable between patients with RRMS and controls (data not shown). These results substantiate the possibility that GPR15 may facilitate the recruitment of cells to the CNS and that increased frequencies and absolute numbers of GPR15$^+$CD4$^+$ T cells in smokers may, at least partially, explain the harmful effects of smoking in MS.

3.6. GPR15L is not increased, but correlates with GPR15$^+$ T cells, in the CSF

GPR15L (also known as CSBF or AF-57), encoded by the *C10ORF99* gene was recently identified as a ligand for GPR15. GPR15-GPR15L function has been linked to immune homeostasis in the lamina propria of the colon and skin [13,14]. To study a potential function of GPR15L as a GPR15$^+$ T cell chemoattractant in the CNS, we analyzed GPR15L in corresponding blood and CSF in 15 patients with RRMS and 8 controls (described in section 2.1). We found detectable but lower concentrations of GPR15L in the CSF compared with blood in all individuals (Fig. 3B). Patients and controls had comparable levels of GPR15L and so did smokers and non-smokers (data not shown). In patients with MS, concentrations of GPR15L in CSF correlated positively with frequencies of GPR15$^+$ T cells (Fig. 3C). This relationship was not significant if the controls were included in the analysis. Our findings were somewhat unexpected as we had expected a GPR15L CNS-blood chemotactic gradient attracting GPR15$^+$ T cells to the CNS, but it is possible that locally increased concentrations may still provide a chemotactic gradient. Alternatively, consumption of GPR15L by GPR15 positive cells might also result in lower concentrations of free ligand as previously demonstrated for the chemokine CCL2 [32]. The function of GPR15L might also be more complex as two recent studies report contradictory findings on whether GPR15L elicits a chemotactic response [13,14]. In aggregate, we consider our findings of GPR15L correlating with GPR15$^+$ T cells supportive of chemotactic properties of GPR15L.

3.7. GPR15$^+$CD4$^+$ T cells with a CCR6$^+$CXCR3$^+$ phenotype in cerebrospinal fluid correlates with levels of GPR15L, myelin breakdown and disease activity

To further elaborate on the migration potential of GPR15$^+$ T cells we studied the co-expression of chemokine receptors CCR6 and CXCR3 with GPR15$^+$ on CD4$^+$ and CD8$^+$ T cells in the CSF. These chemokine receptors were previously suggested to play a role in T cell migration to the CNS in MS [33–35]. We conducted explorative analyses in 16 patients with RRMS and found that on CD4$^+$ T cells GPR15 expression was most closely associated with a CCR6$^+$CXCR3$^+$ and CCR6$^-$CXCR3$^+$ phenotype and in CD8$^+$ T cells, GPR15 expression was most closely associated with a CCR6$^-$CXCR3$^+$ phenotype (Fig. 3D–E). These findings make it possible that GPR15 expression in combination with established CNS homing chemokine receptors may guide pathogenic cells to the CNS in MS. Finally, to determine the potential pathogenic role of GPR15$^+$ T cells in CSF, we correlated frequencies of GPR15$^+$ T cells and GPR15$^+$ T cell subpopulations with MBP concentrations in CSF and the Expanded Disability Status Scale (EDSS). We found that GPR15$^+$ T cells and GPR15$^+$CD4$^+$CCR6$^+$CXCR3$^+$ T cells correlated positively with MBP and EDSS (Fig. 3F). GPR15$^+$CD4$^+$ T cells and GPR15$^+$CD4$^+$CCR6$^-$CXCR3$^+$ T cells also correlated positively with EDSS ($\rho = 0.671$, $P = 0.004$; $\rho = 0.743$, $P = 0.001$). Finally, CCR6$^+$CXCR3$^+$ and CCR6$^-$CXCR3$^+$ GPR15$^+$CD4$^+$ T cells correlated with GPR15L concentrations ($\rho = 0.557$, $P = 0.031$; $\rho = 0.611$, $P = 0.016$). CD8$^+$GPR15$^+$ T cells were not significantly related with MBP or EDSS nor were GPR15$^-$ T cells.

The present study has some limitations. Smokers were defined by present smoking status and our results are therefore potentially biased by the inclusion of non-smokers who had only recently stopped smoking as well as passive smokers in the non-smoking group. From supplementary analyses we conclude that *GPR15* expression is significantly higher in both former and present smokers compared with never smokers, but the levels are considerably higher in present smokers compared with the other two groups (Supplementary Fig. 2B). Thus, the inclusion of former smokers in the non-smoker group may lead to an underestimation of the full effect of smoking on GPR15 expression.

The sample sizes of the studied cohorts are limited, and this affect

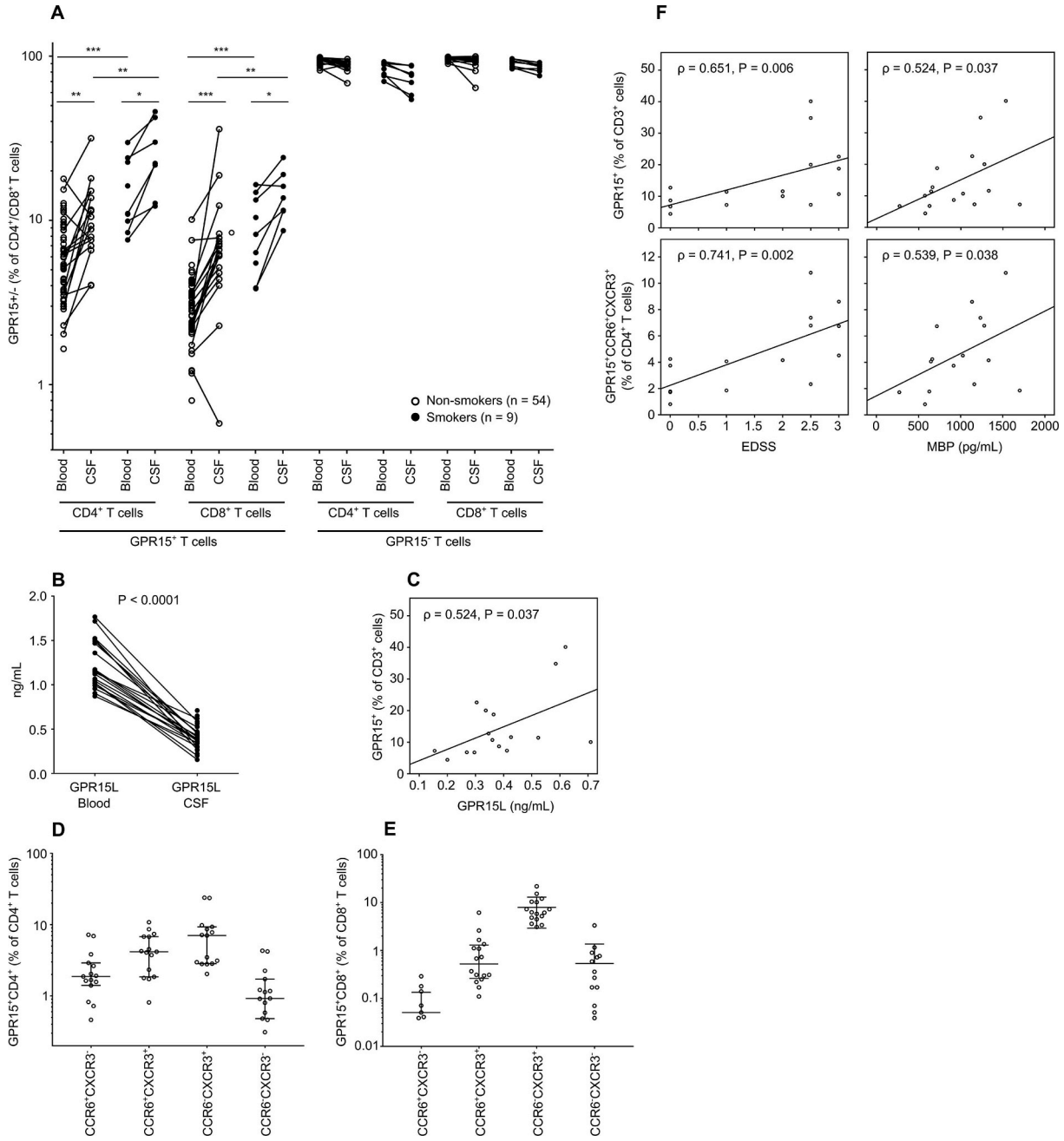

Fig. 3. Presence of GPR15$^+$ T cells and GPR15L in cerebrospinal fluid. (**A**) Frequencies of GPR15$^+$ and GPR15$^-$ CD4$^+$ and CD8$^+$ T cells in blood and cerebrospinal fluid of smokers and non-smokers in 16 patients with RRMS and 8 controls. (Nominal P-values with Wilcoxon matched-pairs signed rank test and Mann Whitney's *U* test for unpaired samples. *P-values < 0.05, **P-values < 0.005, ***P-values < 0.0005). (**B**) Concentrations of GPR15L in blood and cerebrospinal fluid of 20 patients with RRMS of which 15 had corresponding CSF samples and 10 controls of which 8 had corresponding CSF samples (Nominal P-values with Wilcoxon matched pairs signed rank test). (**C**) Correlation of GPR15$^+$CD3$^+$ T cell frequencies with concentrations of GPR15L in cerebrospinal fluid of 16 patients with RRMS. (**D-E**) Frequencies of CCR6$^+$CXCR3$^-$, CCR6$^+$CXCR3$^+$, CCR6$^-$CXCR3$^+$ and CCR6$^-$CXCR3$^+$ GPR15$^+$ CD4$^+$ and CD8$^+$ T cells in the cerebrospinal fluid of 16 patients with RRMS. (**F**) Correlations of GPR15$^+$CD3$^+$ and GPR15$^+$CCR6$^+$CXCR3$^+$CD4$^+$ T cells with EDSS and MBP in 16 patients with RRMS. Spearmann's rho and nominal P-values are shown for correlation analyses. Abbreviations: CSF = cerebrospinal fluid, EDSS = Expanded Disability Status Scale, MBP = myelin basic protein.

the statistical power. Analyses of gene expression, proliferation assays and cytokine production were corrected for multiple comparisons, and the relationship between smoking and GPR15 gene expression and cell surface expression of GPR15 was confirmed in different cohorts of controls and MS patients, but we have not validated our results in external cohorts. Furthermore, our T cell proliferation assays are biased towards the analysis of CD4$^+$ T cells because extracellularly added antigens are mainly presented on HLA class II molecules. This is important to consider when interpreting the CD8$^+$ T cell results.

In summary, we have confirmed previous reports of increased *GPR15* gene expression in smokers [36,37], and we suggest, for the first time, a potential role of *GPR15* in the pathogenesis of RRMS. We have identified GPR15$^+$CD4$^+$ T cells as proinflammatory and autoreactive in RRMS, in addition to being clearly induced by smoking. We substantiate the possibility that CCR6, CXCR3 and GPR15 in combination provide proinflammatory T cells with tissue homing cues towards ligands in the inflamed CNS and, potentially, other tissues known to express the GPR15-ligand. Additional studies of proinflammatory

cytokine producing GPR15$^+$ T cell levels in smokers and patients with MS as well as studies of adhesion molecule co-expression could further elucidate the functional link between smoking, increased GPR15 expression and MS.

Author contributions

CA wrote the first draft of the paper. CA, HBS, LB, MRvE and FS designed the studies. CA, RR, JRC, ABO, ERP, OM and FS collected samples. CA, HBS, LB, RR, JRC, MRVE, ERP and OM performed the experiments. CA, LB, MRvE and HBS analyzed the data. All authors contributed to revising and finalizing the manuscript.

Declarations of interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2018.09.005.

References

[1] J. Lee, V. Taneja, R. Vassallo, Cigarette smoking and inflammation: cellular and molecular mechanisms, J. Dent. Res. 91 (2012) 142–149.
[2] P. Zhang, R. Wang, Z. Li, Y. Wang, C. Gao, X. Lv, et al., The risk of smoking on multiple sclerosis: a meta-analysis based on 20,626 cases from case-control and cohort studies, PeerJ 4 (2016) e1797.
[3] L. Belbasis, V. Bellou, E. Evangelou, J.P. Ioannidis, I. Tzoulaki, Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses, Lancet Neurol. 14 (2015) 263–273.
[4] A.K. Hedstrom, L. Alfredsson, T. Olsson, Environmental factors and their interactions with risk genotypes in MS susceptibility, Curr. Opin. Neurol. 29 (2016) 293–298.
[5] F. Pittas, A.L. Ponsonby, I.A. van der Mei, B.V. Taylor, L. Blizzard, P. Groom, et al., Smoking is associated with progressive disease course and increased progression in clinical disability in a prospective cohort of people with multiple sclerosis, J. Neurol. 256 (2009) 577–585.
[6] B.C. Healy, E.N. Ali, C.R. Guttmann, T. Chitnis, B.I. Glanz, G. Buckle, et al., Smoking and disease progression in multiple sclerosis, Arch. Neurol. 66 (2009) 858–864.
[7] A. Manouchehrinia, C.R. Tench, J. Maxted, R.H. Bibani, J. Britton, C.S. Constantinescu, Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study, Brain J. Neurol. 136 (2013) 2298–2304.
[8] E.R. Petersen, A.B. Oturai, N. Koch-Henriksen, M. Magyari, P.S. Sorensen, F. Sellebjerg, et al., Smoking affects the interferon beta treatment response in multiple sclerosis, Neurology 90 (2018) e593–e600.
[9] M. Sospedra, R. Martin, Immunology of multiple sclerosis, Semin. Neurol. 36 (2016) 115–127.
[10] J. Correale, M.F. Farez, Smoking worsens multiple sclerosis prognosis: two different pathways are involved, J. Neuroimmunol. 281 (2015) 23–34.
[11] C. Ammitzboll, L. Bornsen, J. Romme Christensen, R. Ratzer, B. Romme Nielsen, H.B. Sondergaard, et al., Smoking reduces circulating CD26hiCD161hi MAIT cells in healthy individuals and patients with multiple sclerosis, J. Leukoc. Biol. 101 (5) (2017) 1211–1220.
[12] F. Odoardi, C. Sie, K. Streyl, V.K. Ulaganathan, C. Schlager, D. Lodygin, et al., T cells become licensed in the lung to enter the central nervous system, Nature 488 (2012) 675–679.
[13] T. Suply, S. Hannedouche, N. Carte, J. Li, B. Grosshans, M. Schaefer, et al., A natural ligand for the orphan receptor GPR15 modulates lymphocyte recruitment to epithelia, Sci. Signal. 10 (2017).
[14] B. Ocon, J. Pan, T.T. Dinh, W. Chen, R. Ballet, M. Bscheider, et al., A mucosal and cutaneous chemokine ligand for the lymphocyte chemoattractant receptor GPR15, Front. Immunol. 8 (2017) 1111.
[15] R. Ratzer, H.B. Sondergaard, J.R. Christensen, L. Bornsen, R. Borup, P.S. Sorensen, et al., Gene expression analysis of relapsing-remitting, primary progressive and secondary progressive multiple sclerosis, Mult. Scle. (Houndmills, Basingstoke, England) 19 (2013) 1841–1848.
[16] F.D. Lublin, S.C. Reingold, Defining the clinical course of multiple sclerosis: results of an international survey. National multiple sclerosis society (USA) advisory committee on clinical trials of new agents in multiple sclerosis, Neurology 46 (1996) 907–911.
[17] C.H. Polman, S.C. Reingold, B. Banwell, M. Clanet, J.A. Cohen, M. Filippi, et al., Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria, Ann. Neurol. 69 (2011) 292–302.
[18] C.H. Polman, J.S. Wolinsky, S.C. Reingold, Multiple sclerosis diagnostic criteria: three years later, Mult. Scle. (Houndmills, Basingstoke, England) 11 (2005) 5–12.
[19] K. Schreiber, M. Magyari, F. Sellebjerg, P. Iversen, E. Garde, C.G. Madsen, et al., High-dose erythropoietin in patients with progressive multiple sclerosis: a randomized, placebo-controlled, phase 2 trial, Mult. Scle. (Houndmills, Basingstoke, England) 23 (2017) 675–685.
[20] L. Bornsen, J.R. Christensen, R. Ratzer, A.B. Oturai, P.S. Sorensen, H.B. Sondergaard, et al., Effect of natalizumab on circulating CD4+ T-cells in multiple sclerosis, PLoS One 7 (2012) e47578.
[21] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate - a practical and powerful approach to multiple testing, J. Roy. Stat. Soc. (Series B) (1995) 289–300.
[22] S. Koks, G. Koks, Activation of GPR15 and its involvement in the biological effects of smoking, Exp. Biol. Med. (Maywood, NJ) 242 (2017) 1207–1212.
[23] A. Habtezion, L.P. Nguyen, H. Hadeiba, E.C. Butcher, Leukocyte trafficking to the small intestine and colon, Gastroenterology 150 (2016) 340–354.
[24] D. Su, X. Wang, M.R. Campbell, D.K. Porter, G.S. Pittman, B.D. Bennett, et al., Distinct Epigenetic Effects of Tobacco Smoking in Whole Blood and among Leukocyte Subtypes, PLoS One 11 (2016) e0166486.
[25] M. Farzan, H. Choe, K. Martin, L. Marcon, W. Hofmann, G. Karlsson, et al., Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection, J. Exp. Med. 186 (1997) 405–411.
[26] H.K. Deng, D. Unutmaz, V.N. KewalRamani, D.R. Littman, Expression cloning of new receptors used by simian and human immunodeficiency viruses, Nature 388 (1997) 296–300.
[27] L.P. Nguyen, J. Pan, T.T. Dinh, H. Hadeiba, E. O'Hara 3rd, A. Ebtikar, et al., Role and species-specific expression of colon T cell homing receptor GPR15 in colitis, Nat. Immunol. 16 (2015) 207–213.
[28] A. Fischer, S. Zundler, R. Atreya, T. Rath, C. Voskens, S. Hirschmann, et al., Differential effects of alpha4beta7 and GPR15 on homing of effector and regulatory T cells from patients with UC to the inflamed gut in vivo, Gut 65 (10) (2016) 1642–1664.
[29] A. Cartwright, C. Schmutz, A. Askari, J.H. Kuiper, J. Middleton, Orphan receptor GPR15/BOB is up-regulated in rheumatoid arthritis, Cytokine 67 (2014) 53–59.
[30] X. Gao, M. Jia, Y. Zhang, L.P. Breitling, H. Brenner, DNA methylation changes of whole blood cells in response to active smoking exposure in adults: a systematic review of DNA methylation studies, Clin. Epigenet. 7 (2015) 113.
[31] J.S. Tzartos, M.A. Friese, M.J. Craner, J. Palace, J. Newcombe, M.M. Esiri, et al., Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis, Am. J. Pathol. 172 (2008) 146–155.
[32] D. Mahad, M.K. Callahan, K.A. Williams, E.E. Ubogu, P. Kivisakk, B. Tucky, et al., Modulating CCR2 and CCL2 at the blood-brain barrier: relevance for multiple sclerosis pathogenesis, Brain J. Neurol. 129 (2006) 212–223.
[33] A. Reboldi, C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, et al., C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE, Nat. Immunol. 10 (2009) 514–523.
[34] P. Kivisakk, C. Trebst, Z. Liu, B.H. Tucky, T.L. Sorensen, R.A. Rudick, et al., T-cells in the cerebrospinal fluid express a similar repertoire of inflammatory chemokine receptors in the absence or presence of CNS inflammation: implications for CNS trafficking, Clin. Exp. Immunol. 129 (2002) 510–518.
[35] J. van Langelaar, R.M. van der Vuurst de Vries, M. Janssen, A.F. Wierenga-Wolf, I.M. Spilt, T.A. Siepman, et al., T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention, Brain J. Neurol. 141 (2018) 1334–1349.
[36] M. Obeidat, X. Ding, N. Fishbane, Z. Hollander, R.T. Ng, B. McManus, et al., The effect of different case definitions of current smoking on the discovery of smoking-related blood gene expression signatures in chronic obstructive pulmonary disease, Nicotine Tob. Res. 18 (9) (2016) 1903–1909.
[37] G. Koks, M.L. Uudelepp, M. Limbach, P. Peterson, E. Reimann, S. Koks, Smoking-induced expression of the GPR15 gene indicates its potential role in chronic inflammatory pathologies, Am. J. Pathol. 185 (2015) 2898–2906.