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A critical role for expression of atypical chemokine receptor 2 in multiple sclerosis: A preliminary project

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ABSTRACT

Background: : *ACKR2* is an atypical chemokine receptor that promotes acute inflammation by acting as a scavenger receptor for inflammatory chemokines in experimental models of some inflammatory disorders, but its function in multiple sclerosis (MS) is unclear. Therefore we aimed to evaluate the mRNA expression of *ACKR2* as a scavenger receptor in patients with MS and also the correlation of this expression with certain cytokines that are important for MS pathogenesis.

Methods: : The *ACKR2* mRNA expression was examined on peripheral blood mononuclear cells (PBMCs) of 40 patients with relapsing-remitting MS (RRMS) and 35 healthy individuals. *ACKR2* mRNA expressions were measured using real-time quantitative polymerase chain reaction (qPCR). In addition, circulating cytokine levels (TNF-α, IL-6, IL-33) in all patients and controls were evaluated using enzyme-linked immunosorbent assay.

Results: : mRNA expression of *ACKR2* was decreased on PBMCs compared to healthy subjects ($p < 0.001$). *ACKR2* expression in peripheral blood leucocytes could be regulated by circulating cytokines but there are no correlations with these cytokines ($p > 0.05$). In addition, the patients' plasma levels of IL-33 significantly increased ($p = 0.039$) and no significant difference was found between other cytokine levels in the patients ($p > 0.05$).

Conclusion: : Our data clearly show a decreased *ACKR2* mRNA expression on PBMCs and increased plasma IL-33 levels of patients with MS. There was no significant relationship between *ACKR2* and other cytokine levels. Within our knowledge, this is the first study that evaluates the *ACKR2* mRNA expression in the PBMCs of MS patients.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by demyelination and axonal loss. Although not fully understood, the etiology presumably contains numerous interactive factors that trigger an abnormal autoimmune attack resulting in damage to myelin and axons (Bruck and Stadelmann, 2005a, b; Lazibat et al., 2018).

The pathogenesis of MS involves an autoimmune attack against CNS antigens mediated through activated CD4+ myelin-reactive T cells, with a possible contribution of B cells. The experimental autoimmune encephalomyelitis (EAE), is an animal model of CNS inflammatory demyelination which can be induced by peripheral immunization with myelin protein components, provides most of our knowlegde about imminopathogenesis of MS (Gold et al., 2006; Mazzon et al., 2016). Nevertheless, specific antigens which are involved in the lead up to MS have not yet been fully comprehended.

Chemokines are chemotactic cytokines functioning through G protein-coupled receptors, which cause leukocyte migration in homeostatic and inflammatory conditions (Bonavita et al., 2017). In recent years, chemokine receptors, which are called "atypical chemokine receptors", have been identified that try to limit inflammation, although they are not signals accompanied by G-protein (Francoise Bachelerie et al., 2014). It has been shown that exaggerated inflammatory response develops depending on the T-cell impairment in EAE models where *ACKR2* is unexpressed (Liu et al., 2006).

Although the atypical chemokine receptors *(ACKRs)* represent a small subset of proteins; they are significantly similar to chemokine receptors and are expressed by lymphatic endothelial cells, trophoblasts, and some leukocytes, such as alveolar macrophages and innate-like B cells (Hansell et al., 2015a, b). In recent years, the function of *ACKRs* is increasingly clarifying since they were found to regulate

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inflammation by acting as a scavenger receptor and gradient formation of chemokine activity or promoting chemokine transcytosis (Salvi et al., 2017). *ACKR2* can activate the scavenger function by decreasing extracellular chemokine concentration and stimulating β-arrestin-dependent pathway within the cytoplasm even in the absence of ligand. Then, the localization of the plasma membrane increases without impacting the internalization rate (Bonecchi et al., 2008; Bonecchi and Graham, 2016a,b; Borroni et al., 2013). Although *ACKR2* supports the resolution of inflammation (McKimmie et al., 2013a,b) however, *ACKR2* KO mice in various pathological contexts have been found to display dysregulated inflammatory reactions because of the lack of chemokine clearance and associated with accumulation of inflammatory cells (Graham and Locati, 2013). *ACKR2* is a receptor for inflammatory chemokines expressed by leukocytes and creates a particular interest (Bonecchi and Graham, 2016a,b).

Despite all the advances in immunopathogenesis, the majority of the genetic factors forming the basis of MS susceptibility are still investigated. Furthermore, genetic susceptibility is insufficient to explain the etiopathogenesis of MS alone. At this point, the content and size dependent properties of the exposed antigen are important. The purpose of this study is to investigate the ACKR2 mRNA expression in MS patients. It was also aimed to determine cytokine levels and interactions between *ACKR2* mRNA expressions.

2. Materials and methods

2.1. Study subjects

In the present study with prospective and cross-sectional design, 40 RRMS patients and 35 volunteer control subjects aged between 18 and 60 years were involved. Patients consulted to the neurology clinic from August 2018 to February 2019. All of the patients were evaluated by neurological examination according to Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983). Patients with remission phase were included in the study. There was no clinical deterioration within the three months period of sample collection in any of our patients. Any other chronic inflammatory/autoimmune diseases were considered as exclusion criteria. In addition, healthy volunteers with no family history of autoimmune/ chronic inflammatory diseases were included in the study.

Our study was approved by the Ethics Committee (Ethics Committee approval no: 2017-KAEK-189_2018.02.27_06) and supported by the coordination of Project Unit (Project No: 6602b-TF/ 18-213).

2.2. Peripheral blood mononuclear cell separation, RNA isolation, and reverse transcription

Blood samples were collected into tubes containing ethylenediaminetetracetic acid (EDTA) as an anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated using a density gradient centrifuge by Lymphocytes Separation Media (Capricorn Scientific, Ebsdorfergrund, Germany). Total RNA was extracted from PBMCs by Trizol (Tri Pure Isolation Reagent, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The complementary DNAs (cDNA) synthesis using a commercial kit (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Mannheim, Germany). The cDNAs were stored at −80 °C.

2.3. Real time PCR

Real-time PCR analyses were realised using the LightCycler 480 platform (Roche Diagnostics, Mannheim, Germany). Primers and probes were designed for target and reference genes (real time ready catolog assay; 0553295700, Roche). The PCR mixture included 1 μL for a single assay, 10 μL of Light Cycler 480 Probes Master (Roche, Diagnostics, Mannheim, Germany), 4 μL PCR grade water, and 5 μL cDNA sample, and the protocol was performed. The cycle conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s and for cooling step 40 °C was performed for 30 s for each reaction. One negative cDNA control consisting of DNase- and RNase-free water was used for all runs. The housekeeping β-actin gene was used as a control to normalized the gene expression and the final results were attained with LightCycler 480 software. Gene expression levels were evaluated using the ΔΔCt method. All samples were run in triplicate.

2.4. Cytokine measurement

The plasma levels of TNF-α, IL-6, and IL-33 were measured by an enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, China) according to the manufacturer's instructions. The absorbance was measured spectrophotometrically on a microplate reader (Molecular Devices). Lower limits of detection were 1.52 ng/L for TNF-α, 1.03 ng/L for IL-6, and 5.36 ng/L for IL-33.

2.5. Statistical analysis

In addition to SPSS 18.0 software package (IBM Corporation, Armonk, NY, USA), GraphPad PRISMTM (version 3.0) was used for statistical analyses and to create the graphs. A Kolmogorov Smirnov test was used to analyze whether or not the data was normally distributed. The main characteristics of the patients were evaluated with descriptive and analytical statistics. Categorical variables were compared by $\chi 2$ test. Correlations between EDSS and *ACKR2* mRNA expression in PBMCs was evaluated using a Spearman's rank correlation test. For all statistical tests, non-parametric data were analysed using the Mann_Whitney *U* test and parametric data using Student's unpaired *t*-test. In all tests, a p value < 0.05 was considered statistically significant.

Statistical significance for cytokine levels (TNF- α, IL-6, IL-33) among the groups (four groups; individuals mRNA expressing and not expressing *ACKR2* in the patients and controls) was based on Kruskal-Wallis test and followed by Bonferroni correction.

3. Results

3.1. Comparisons of clinical characteristics of RRMS patients

Basic and clinical characteristics of the patients with MS and healthy subjects are presented in Table 1. Gender, family history, disease duration, number of attacks and different types of initial symptom were recorded in the groups. Seven of the 40 RRMS subjects were not taking prophylactic treatment. Twenty-seven subjects took the standard first line therapies (interferon/dimetil fumarat/ teriflunomide) and six patients took second line therapies (fingolimod/natalizumab). The plasma levels of IL-33, IL-6, TNF-α and the frequencies of *ACKR2* mRNA expressing subjects were evaluated in the patient and control groups. The plasma levels of IL-33 significantly higher in MS patients compared to controls ($p = 0.039$), IL-6 and TNF-α levels increased in patients although not statictically significant ($p > 0.05$) (Table 1).

There are no differences between genders in the patient group regarding plasma interleukin levels ($p > 0.05$). However, only IL-6 levels were found to be higher in women ($n = 24$) in the control group ($p = 0.002$). Interleukin analyses are presented according to gender, in Table 2.

3.2. ACKR2 mRNA expression is lower in PBMCs of MS patients

To determine the mRNA expression levels of *ACKR2* in PBMCs from established RRMS in comparison with healthy controls, we used real time PCR. As shown in Table 1. MS patients group displayed significantly decreased *ACKR2* mRNA expression relative to healthy

Table 1
Basic and clinical characteristic of multiple sclerosis (MS) patients and healthy subjects.

Variables	MS patients	Healthy subjects	*P* value
Number of subjects	40	35	
Male/female	11/29	11/24	0.906*
Age (years) (Mean ± SD)	37.83 ± 7.65	34.00 ± 8.85	0.051
BMI (kg/m^2)	25.48 ± 4.61	25.86 ± 3.15	0.686
Family history Positive Negative	6 (15%) 31 (85%)	–	–
Disease duration (years)	7.80 ± 5.46	–	–
EDSS score (1–6)	2.27 ± 1.51	–	–
Number of attacks	3.45 ± 2.44	–	–
Number of patients in different types of initial symptom Optic neuritis Hypoesthesia Hemi/ monoparesis	6 (14.28%) 29 (72.5%) 5 (12.5%)	–	–
ACKR2 expressing subjects frequency mRNA levels of *ACKR2* (Mean ± SD)	10 (25%) 0.003 ± 0.015	27 (77.15%) 0.01 ± 0.028	<0.001 <0.001
IL-6 (ng/L) (Mean ± SD)	87.33 ± 204.34	20.99 ± 21.86	0.965
TNF-α (ng/L) (Mean ± SD)	76.47 ± 174.19	29.83 ± 16.00	0.717
IL-33 (ng/L) (Mean ± SD)	135.31 ± 232.57	35.68 ± 15.30	0.039

Abbreviations: BMI: Body mass index, EDSS: Expanded disability status scale. Bold values indicate a statistically significant difference with a *P* value less than 0.05. (Data presented as mean ± SD).
* Chi square test.

controls ($p < 0.001$).

There was no significant correlation between *ACKR2* mRNA expression and clinical findings such as duration of disease, EDSS, number of attacks (Table 3). At the same time, there was no significant correlation between *ACKR2* expression and plasma cytokine levels in terms of clinical parameters. On the other hand, the relationship between gender and *ACKR2* mRNA expression was found to be significantly different in the patient group as presented Table 2 ($p = 0.001$).

In this study, when we evaluated the relationship between plasma cytokine levels of individuals expressing and not expressing *ACKR2* in the patients and controls, we found no significant difference among the groups ($p > 0.05$).

4. Discussion

ACKR2 is critical in inflammation and has been shown to have different expressions in animal models after immunization with context-dependent antigens. The aim of the present study was to expand these findings and to investigate the expression of *ACKR2* in MS patients. In our study, we demonstrated that down-regulation of *ACKR2* in the study subjects. Furthermore, our study showed that there was no association between certain circulating cytokines and the *ACKR2* mRNA expression, even though these cytokines increased in the patient group.

In recent years, a small family of atypical chemokine receptors that are different from canonical chemokine receptors has been identified (Mantovani et al., 2006). The reason why these receptors are named as ``atypically" is that they show common structural properties with canonical chemokine receptors; however, they are bound to ligands with high affinity without inducing cell migration. *ACKRs* internalize and transport chemokines to the degradative compartment, modulating chemokine concentration and bioavailability (Mantovani et al., 2006). The family of *ACKRs* includes four receptors: *ACKR1* (previously called DARC), *ACKR2* (D6), *ACKR3* (CXCR7), and *ACKR4* (CCX-CKR) (Mantovani et al., 2006).

Table 3
Summary of correlations of *ACKR2* expressing MS patients with EDSS score, number of attacks and duration of disease.

Variables	*P* value	Sperman's rho
EDSS score	0.451	0.123
Duration of disease	0.923	−0.16
Number of attacks	0.244	0.128

EDSS: Expanded disability status scale.

The atypical chemokine receptor *ACKR2* is capable of binding the majority of inflammatory CC-chemokines (Bonecchi et al., 2004). *ACKR2*, which is a member of the atypical chemokine family, is an important chemokine that can guide the migration of various leukocytes including monocyte subgroups, dendritic cells, NK cells and T-cells through its receptors (Ford et al., 2014). However, it was later shown that *ACKR2* not only enables the internalization and scavenging of the ligands, but also activates the signal pathway that depends on the B-arrest that encourages the internalization and recycling of the receptors into cell membrane (Bonavita et al., 2017).

Atypical chemotactic receptors are exposed to ligand-induced cleaners, and control inflammatory reactions by scavenging the inflammatory ligands from tissues. *ACKR2* can function in improving local inflammatory responses, resolution of inflammation, and controlling of adaptive immune responses (Françoise Bachelerie et al., 2015). There are controversial data in experimental autoimmune encephalomyelitis studies on the involvement of *ACKR2* in the pathogenesis of the disease. In their *in vivo* study, Hansell et al. showed that IL-17 expression and B lymphocytes, which express granulocyte-macrophage colony stimulating factor, decreased during the inflammation process in the absence of *ACKR2* (Hansell et al., 2015a, b). However,

Table 2
Serum levels of IL-33, IL-6, TNF- α and ACKR2 expressing subjects frequency (%) in multiple sclerosis (MS) patients and control group according to gender.

Groups& *P* value	Sex	IL-33 levels (mean ± SD)	IL-6 levels (mean ± SD)	TNF-α (mean ± SD)	*ACKR2* expressing subjects frequency (%)
MS patients	Female	127.4 ± 253	82.7 ± 212.5	65.6 ± 171.5	3 (42.85%)
	Male	156.2 ± 176	99.6 ± 90.2	105.2 ± 186.2	7 (57.15%)
***P* value**		0.079	0.109	0.090	**0.001***
Matched controls	Female	38.1 ± 17	38.1 ± 17.1	27.7 ± 12.3	19 (54.28%)
	Male	30.4 ± 8.9	30.4 ± 8.9	34.50 ± 22.13	8 (45.72%)
***P* value**		0.283	**0.002**	0.316	0.630*

Bold values indicate a statistically significant difference with a *P* value less than 0.05.
* Chi square test.

Liu et al. determined that *ACKR2* knockout mice had a protective effect against the autoimmune encephalomyelitis development (Liu et al., 2006). Considering all these data, we see that the effects of *ACKR2* show contradictory characteristics in the literature.

In recent years, *ACKR2* deficiency has been associated with exaggerated EAE phenotype following the immunization of mice with pure protein, except for myelin oligodendrocyte peptide (MOG peptide) (Hansell et al., 2015a, b; Liu et al., 2006). EAE has many etiopathogenetic characteristics of MS, which include active demyelination and axonal loss mediated by oligodendrocyte and myelin-specific T-cells (Garg and Smith, 2015). However, recent data in the literature support different roles of atypical chemokine receptors in the resolution of inflammatory responses (Ortega-Gomez et al., 2013). In the present study, in the light of the above data, it may be considered that the decrease in the *ACKR2* expression in patients might related with the type and size of the antigens that trigger pathophysiology.

In experimental inflammation models in which the cytokine and chemokine systems in MS patients are investigated, it was shown that the proinflammatory cytokines like IL-1β, IL-6, and TNF-α, and Th1 (IFN-γ), Th17 (IL-17), Th2 (IL-4 and Il-10), and Treg (IL-10) levels increase when this cascade is activated through CD4 + TH1 cells after the immunization. Especially the IL-1β, IL-6, and IL-4 cytokine levels correlate with the severity of the inflammation and axonal damage (Kallaur et al., 2017). It was reported in previous studies that some important proinflammatory cytokines (especially IL-6) modulate *ACKR2* expression (McKimmie et al., 2013a, b).

IL-33 is a new member of the IL-1 family, which regulates innate and adaptive immune responses, and is expressed especially in human monocytes and mouse astrocytes (Kempuraj et al., 2013). IL-33 acts as an alarm against stress, pathogens or cell death by activating local immune cells. IL-33 acts on microglia to proliferate, secrete cytokines and chemokines, and enhance phagocytosis (Yasuoka et al., 2011). IL-33 augments or synergistically acts with other inflammatory mediators in proinflammatory micro environment (Espinassous et al., 2009). Interestingly, IL-33 was also upregulated in astrocytes and peripheral leukocytes of MS patients (Christophi et al., 2012). In addition, IL-33 has been shown to have a pivotal role in myelin damage/maintenance by its direct effect on oligodendrocytes (Alsahebfosoul et al., 2017; Pei et al., 2014). They have suggested that IL-33 may have CNS-specific functions in addition to a role as an immune-mediator (Pei et al., 2014). We wanted to evaluate these cytokines, which have an important place in MS disease due to the pivotal role of IL-33 in myelin damage and with the proinflammatory properties of TNF-α and IL-6 in demyelinization, in terms of *ACKR2* mRNA expression. MS and controls were age-sex-ethnicity-BMI controlled for the cytokine analyses to minimize the influence of them on the serum cytokine levels.

In this study, the cytokines, especially IL-33 have increased significantly in patients. It is known that cytokine and chemokine networks play an important role in initiating and maintaining inflammatory activity in chronic inflammation (Kwak et al., 2014). The current study, we show that plasma TNF-α and IL-6 levels were also increased in RRMS subjects compared to healthy group but this increase was not statistically significant. This finding is in accordance with other reports (Chuluundorj et al., 2014; Sarchielli et al., 1997). However, elevation in IL-33 may be due to impairment in pro and anti-inflammatory responses. In our study, especially IL-33 levels were found to be significantly elevated and the levels of cytokines did not show a significant correlation with *ACKR2* mRNA level.

Despite these potential breakthroughs, there has been a dissappointing progress towards development clinically useful receptor antagonists (Schall and Proudfoot, 2011; Sozzani et al., 2015). In fact, although twenty years after the first inflammatory chemokine receptor (Murphy and Tiffany, 1991) was identified, no chemokine receptor antagonists received approval and license to treat inflammatory diseases. Therefore, more extensive studies are needed to use these receptors as a therapeutic agent.

We showed that the decoy chemokine receptor *ACKR2* transcripts becoming significantly reduced in the RRMS patients. Therefore, we believe that under decreased mRNA expression of *ACKR2*, IL6, TNF-α, and IL-33 expressions were regulated and it might be additional evidence of role in inflammation in MS.

In summary, a better understanding of immunopathogenesis has provided for significant progress in the field of MS. We consider our finding of decreased *ACKR2* mRNA expression in PBMCs from RRMS subjects taking disease modified therapies an interesting result that needs to be examined in a larger general population. The present study has certain limitations. Our results could be expected to be more meaningful if we will determine these findings in a larger cohort with the required following protein expression studies. Moreover, we only investigated mRNA expression of *ACKR2* in PBMCs of patients, B and T cell analysis would likely provide additional clarification. We believe that multicenter studies with no treatment and progressive form will shed much light on the role of *ACKR2*. Atypical chemokine receptors may represent possible new therapeutic targets in MS patients. These could be used to increase the *ACKR* function and thus neutralize chemokine activity in a number of inflammatory pathologies for future examinations. Furthermore, with the integration of these molecules into treatment, there is a possibility for therapeutic use in limiting the attack of autoantibodies to pro-inflammatory leukocytes.

Disclosure

The authors declare no conflicts of interest.

Informed consent

Informed consent was obtained from all the individual participants included in the study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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