ORIGINAL ARTICLE

IL-23 Receptor Expression on T Helper 17 Cells and its Implication in Multiple Sclerosis Relapse

Reham Khalifa*, Nesma Abdel Aziz, Ali Shalash, Maha Nada, Shimaa Abdel-Salam

ABSTRACT

Background: Multiple Sclerosis (MS) is considered the prototype of neurodegenerative diseases affecting around 2 million people worldwide. MS was marked primarily as an IL-17-mediated autoimmune disease, the hallmark cytokine of T helper (Th) 17 cells. Th 17 cells are considered as one of MS key effectors through the IL-23/IL-17 axis. Objectives: To investigate IL-23 receptor expression on Th17 cells with assessment of serum IL-17 level and their implication in MS relapse. Methodology: Patients included 22 MS patients in remission, 22 MS patients in active relapse and 22 healthy controls. Clinical assessment of the expanded disability severity scale (EDSS) for included patients. All groups were subjected to flow-cytometric analysis to assess expression of IL-23 receptor (IL-23R) on CD4+ T cells and measurement of serum IL-17A level by using ELISA. Results: MS patients during active relapse had a higher IL-17A serum level, and a higher percentage of IL-23R+ CD4+ T cells with increased IL-23R expression density on CD4+ T cells compared to patients in remission and controls. Conclusion: The results reflected Th 17 key role in MS immune pathogenesis. Thus, the IL-23/IL-17 axis can be a potential target for immunomodulatory therapies ameliorating the Th17 mediated autoimmunity through novel treatments for those patients.

INTRODUCTION

Multiple sclerosis (MS) is one of the neurodegenerative diseases of the central nervous system (CNS) with an autoimmune background, which affects around 2 million people worldwide. Myelinated axons in the CNS sustain damage and ultimately lose their myelin sheath. Damage to myelin triggers an inflammatory response, which damages oligodendrocytes and causes a neuronal deficit leading to a temporary or a permanent neurologic dysfunction with a wide range of disabilities.

As the disease advances, T helper (Th) cells perform a crucial effector role. Interleukinin-17 (IL-17) and Interferon gamma (IFN-γ) are key cytokines of neuroinflammation in MS. Higher T helper 17 (Th17) cell counts were found to be linked to severity scores in patients with MS, especially in active brain lesions. Th17 cells in the cerebrospinal fluid (CSF) are markedly higher among MS patients in relapse than among patients in remission.

IL-21 and IL-23 are the cytokines that sustain the differentiation of naïve CD4+ T cells into the Th17 subtype. Antigen-presenting cells (APCs) found in tissues, such as dendritic cells and macrophages loaded with self-antigens, secrete IL-23 which triggers the activation of autoreactive Th17 cells. Thus, IL-23 receptor (IL-23R) expression is considered a reliable marker for locating Th17 cells, which are prevalent in inflammation sites.

IL-23 is well-documented to encourage the proliferation of CD4+ Th17 cells and to stimulate the secretion of Th17-associated cytokines. Th17 cells population specific to the myelin sheath in the CNS are known to expand in an IL-23-rich environment. This distinct Th17 cell population may expand under the effect of resident CNS microglial cells which secrete IL-23, IL-6, and Transforming Growth Factor- (TGF-). It has been shown that Th17 IL-17 production can drive the production of additional proinflammatory cytokines, leading to a potent inflammatory cascade that destroys axons and causes eventual neurological disorder.

MS presents in a variety of forms with various symptoms and consequences. The prevalent type, relapsing-remitting multiple sclerosis (RRMS) is characterized by repeated attacks of symptoms. There are remissions, or times of improvement, in between these episodes. The initial form of multiple sclerosis experienced by most of patients is the relapsing-remitting form.

To diagnose multiple sclerosis, clinicians must follow a series of guidelines known as the McDonald criteria based on clinical, laboratory investigations and magnetic resonance imaging (MRI) data. According to the 2017 updated criteria, diagnosis of MS requires two pieces of evidence: Evidence of damage to the CNS that...
disseminates in space or manifests in various parts of the nervous system, and evidence of CNS damage that disseminates in time or manifests at different times.

Greater focus has been placed on IL-17A as a therapeutic target and possible treatment for MS in recent years. Clinical trials have been conducted on a number of antibody therapies through blocking IL-17A or its receptor which has demonstrated potential as a therapy for MS.

The aim of the present work is to investigate IL-23 receptor expression on Th17 cells with assessment of serum IL-17 level and their implication in MS relapse.

**METHODOLOGY**

**Study design:**
A case control study included multiple sclerosis patients attending MS Clinic or admitted to Neurology Department at Ain Shams University Hospitals during the month of December 2022. **Inclusion Criteria:** Adult MS patients were diagnosed based on McDonald’s criteria. Two patients’ groups and a group of age and sex matched healthy controls were categorized as follows:
- Group A: Included 22 patients with RRMS based on McDonald’s criteria during remission.
- Group B: Included 22 patients with RRMS based on McDonald’s criteria during active relapse.
- Group C: Included 22 healthy controls with no history or evidence of MS or other autoimmune diseases.

**Exclusion criteria:**
Age < 18 years old or MS patients with clinical or laboratory evidence of other autoimmune diseases or suffering a concomitant infection. Patients on Interferon-β (IFN-β) therapy or corticosteroid treatment were excluded. Blood samples were collected before the initiation of immunomodulatory therapy. Healthy controls with clinical or laboratory evidence of recent acute infection were also excluded.

**Ethical Considerations:** The study was approved by the Research Ethics Committee (REC), Faculty of Medicine, Ain Shams University R 245/2022 and was performed in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki). Patients or patient’s next of kin and healthy controls were informed, and their informed consent was obtained before sample collection according to the regulation of the Ethical Committee of Scientific Research (Faculty of Medicine- Ain Shams University).

**Data collection:**
Data was collected from patients’ files including patients’ demographic data, clinical data, age of onset, history of relapse, IFN-β therapy and the expanded disability severity scale (EDSS).

**Samples collection:**
From each patient and control, 5 ml whole blood sample was collected under complete aseptic conditions. Three ml of each blood sample were subjected to flow-cytometry analysis to assess expression of IL-23 receptor on CD4+ T cells. Two ml of clotted blood sample were used for serum separation to be subjected to IL-17A Enzyme-Linked Immunosorbent Assay (ELISA) to assess serum IL-17A level.

**Flow cytometry assay:**
About 5 µL of each of the monoclonal antibodies: Anti-mouse IL-23 R-Phycocerythrin labeled antibodies, Catalog Number: FAB16861P (R&D Systems, Minneapolis, USA) and anti-CD4 antibodies labeled with fluorescent isothiocyanine (FITC) (Beckman Coulter, USA) were added to 50 µL of EDTA treated blood. The tubes were mixed well using vortex mixer and incubated for 15 min at room temperature in the dark. Two ml of lyse solution, NH4CL buffered with KHCO3 at pH 7.5 (Al-Gomhoreya CA, Egypt), were added and mixed using vortex mixer thoroughly. The tubes were further incubated for 5-10 minutes at room temperature in the dark. Analyses were done on NAVIOS Six Colors Flow-cytometer (Beckman Coulter, USA). CD4 cells were gated according to forward-light scatter (FSC) and side-light scatter (SSC) properties. Forward scatter reflecting cell size and side scatter reflecting internal structure of cell. The percentage cells bearing IL-23 R was determined and the density of IL-23 R on cell surfaces was estimated as median of fluorescence intensity (MFI) values.

**Enzyme-linked immunosorbent assay (ELISA):**
Serum samples obtained from the patients and the controls. Serum was separated by centrifugation at 2000 x g for 15 minutes and stored at ~80°C until they are used for IL-17A serum level assessment using the Invitrogen™ IL-17A ELISA Kit catalogue number (KAC1591) (Thermofisher Scientific, USA). The assay was performed according to the manufacturer’s instructions. Briefly, 100 µL of pre-dilute serum samples or standard solution were added to respective wells, 50 µL of Anti-IL-17A-Biotin conjugate was added to all the wells. After 2 hours incubation at room temperature the solution was aspirated, and wells were washed 3 times using 1X Wash Buffer. 100 µL of diluted Streptavidin-HRP conjugate was added to all the wells followed by 30 minutes incubation then repeat wash step. 100 µL of Chromogenic TMB was added to each well followed by 15 minutes incubation in the dark. 100 µL Stop Solution was added to each well. Absorbance at 450 nm was read within 1 hour after adding the Stop Solution and the standard curve was generated.
Statistical Analysis:

The sample size was calculated using G power program for sample size calculation (power 80% & alpha error 5%) according to Esendagli et al.³. The collected data was revised, tabulated, and processed by a PC using Statistical package for Social Science (SPSS 25). Mean, Standard deviation (± SD) and range for parametric numerical data. Frequency and percentage of non-numerical data. Chi-Square test was used to compare between two qualitative variables. ANOVA test was used to assess the statistical significance of the difference between more than two study group means. Post Hoc Test is used for comparisons of all possible pairs of group means.

RESULTS

The study included 44 RRMS patients diagnosed according to McDonald’s criteria and 22 healthy controls. Patients were categorized into 2 groups; Group A (Remission Group) included 22 RRMS in remission with neither new neurological symptoms nor MRI activity were detected. Group B (Relapse Group) included 22 RRMS in active relapse showing new neurological disturbance or MRI activity. No statistically significant difference was observed among the three study groups as regards gender or age distribution (table 1).

Table 1: Study groups demographic data.

<table>
<thead>
<tr>
<th></th>
<th>Remission Group</th>
<th>Relapse Group</th>
<th>Controls Group</th>
<th>Chi square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>X²</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10 (45.45%)</td>
<td>7 (35%)</td>
<td>13 (54.17%)</td>
<td>1.62</td>
</tr>
<tr>
<td>Female</td>
<td>12 (54.55%)</td>
<td>13 (65%)</td>
<td>11 (45.83%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>31.73 ± 4.42</td>
<td>32.15 ± 3.72</td>
<td>30.13 ± 4.96</td>
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</tbody>
</table>

Table 2 shows a statistically significant difference between the three study groups, with highest values detected among patients in the relapse group, as regards mean and median values of IL-17A serum level (figure 1a), and the percentage of IL-23R⁺ CD4⁺ T cells (figure 1b, figure 2). As regards IL-23R expression MFI on CD4⁺ T cells, a statistically significant difference between the three study groups is shown on comparing median MFI values (figure 1c). However, on comparing mean MFI values the difference between patients in the remission group and controls was statistically non-significant. A statistically significant difference is shown between patients in remission and patients in relapse groups as regards EDSS.

Table 2: Comparison of IL-17 level, IL-23R⁺ CD4⁺ T cells percentage, IL-23R expression MFI, and EDSS between the study groups

<table>
<thead>
<tr>
<th></th>
<th>Remission group</th>
<th>Relapse group</th>
<th>Controls group</th>
<th>ANOVA/ Kruskal Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD Median (IQR)</td>
<td>Mean ± SD Median (IQR)</td>
<td>Mean ± SD Median (IQR)</td>
<td>F =136.84</td>
</tr>
<tr>
<td>IL-17A pg/ml</td>
<td>37.22 ± 8.83ᵃ</td>
<td>88.41 ± 12.11ᵇ</td>
<td>20.47 ± 18.47ᶜ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35.7 (29.6 - 44.7)ᵃ</td>
<td>89.5 (81.2 - 95.35)ᵇ</td>
<td>15.25 (13.75 - 16.7)ᶜ</td>
<td></td>
</tr>
<tr>
<td>IL-23 R⁺ CD4⁺ T cells %</td>
<td>7.5 ± 0.88ᵃ</td>
<td>12.34 ± 1.45ᵇ</td>
<td>5.25 ± 2.39ᶜ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.49 (6.77 - 8.45)ᵃ</td>
<td>12.05 (11.53 - 13.61)ᵇ</td>
<td>4.68 (3.79 - 5.46)ᶜ</td>
<td></td>
</tr>
<tr>
<td>IL-23R MFI</td>
<td>2.97 ± 0.99ᵃ</td>
<td>11.04 ± 6.06ᵇ</td>
<td>1.94 ± 2.56ᶜ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.85 (2.4 - 3.4)ᵃ</td>
<td>8.02 (7.01 - 12.5)ᵇ</td>
<td>1.19 (0.96 - 1.49)ᶜ</td>
<td></td>
</tr>
<tr>
<td>EDSS</td>
<td>1.52 ± 0.61</td>
<td>3.88 ± 0.79</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 (1 - 2)</td>
<td>4 (3 - 4.5)</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ t test   **Mann Whitney test. Post hoc test: means and medians with the same superscript in each raw shows no significant difference
Fig. 1: Box plots for comparison of IL 17 level (a), IL23R⁺ cells percentage (b), and IL23R expression MFI (c) between the study groups showing medians, upper and lower limits, and outliers.

Fig. 2: Flowcytometry analysis histogram showing IL23R⁺ CD4⁺ T cells (Quadrant 2) among patients in remission group (a), relapse group (b), and controls group (c).
DISCUSSION

Neurodegenerative diseases are a set of conditions defined by the gradual loss of certain groups of neurons, resulting in dysfunction. MS is the most prevalent autoimmune illness of the CNS, as well as the most frequent disabling neurological condition of adolescence. MS is characterized by leukocyte infiltration, demyelinating plaques, and axonal damage. Neuroinflammation in MS is mainly induced by the two cytokines namely, IFN-γ and IL-17. Also, increased Th17 cells’ counts has been associated with disease severity in patients with MS. MS is largely counted as an IL-17–mediated autoimmune disorder, with Th17 cells playing an important role. IL-17 is regarded as the distinctive cytokine of Th17 cells.

The crucial role of IL-23 in MS pathogenesis was evidenced by the studies showing that the presence of IL-23-induced Th17, in the absence of Th1, is sufficient to elicit Experimental autoimmune encephalomyelitis (EAE) in rats. Since IL-17 causes neuronal death and excitotoxicity owing to glutamate buildup in the synaptic area, the amount of IL-23 correlates with the severity of IL-17-induced diseases. These results show that astrocyte-produced IL-23 increases CNS inflammation via Th17-dependent pathways.

The present study included 44 RRMS patients diagnosed according to McDonald’s criteria and 22 healthy controls. Patients were categorized as remission group (22 RRMS in remission) and relapse group (22 RRMS in active relapse). As regards gender and age distribution, no statistically significant difference was observed among the three study groups. The study of Li et al. stated that MS affecting around 2 million people around the world with female to male ratio of about 4:1. However, this distribution wasn’t apparent in the present study due to limited study population.

IL-17A serum level assessment, in the present study, showed a statistically significant difference between the three study groups, where RRMS patients had higher IL-17A serum level compared to controls group with the highest values detected among patients in the relapse group. Similar results were reported by Schofield et al. demonstrating that serum levels of the homodimer IL-17AA were significantly elevated in RRMS patients in comparison with healthy controls, with an association to disease activity, but no statistically significant difference was observed as regards the other IL17 isoforms, IL-17FF and IL-17AF levels. This was also in accordance with the study of Esendagli et al. where MS patients showed mean IL-17 serum level higher than healthy controls however the difference was not statistically significant. They reported a significantly higher serum level of IL-17 among patients with severe clinical manifestations not responding to therapy.

In the present study, patients in the relapse group had a significantly higher EDSS compared to patients in the remission group which was also associated with higher serum IL-17A level. Similarly, the study of Bălaşa et al. demonstrated that patients who had a considerably higher IL-17A serum level had experienced a significantly greater number of relapses in the preceding year as well as a higher EDSS. Additionally, 37.5% of the patients were non-responders to therapy with a significantly higher serum IL-17A level.

Although there was little consensus on the parameters regulating IL-23R expression, most of the published data on IL-23R expression measured mRNA rather than IL-23R protein levels. Nonetheless, mRNA expression is influenced by micro RNAs and other downstream regulatory factors, thus it is not always a reliable indicator of the amount of protein expression. Thus, the present study aimed to characterize IL-23R expression at a protein level. Flowcytometric analysis among the study groups showed that patients in the relapse group showed the highest percentage of IL-23R+ CD4+ T cells compared to patients in the remission group and healthy controls. This was agreeing with the results of Durelli et al. which reported that Th17 cell percentage increased around sevenfold in patients with active MS compared to patients with inactive MS or healthy subjects. They concluded that Th17 cells count in blood was higher during relapses.

A novel observation in the present study was that patients in the relapse group showed the highest IL-23R expression density on CD4+ T cells (mean IL23R MFI value) compared to patients in the remission group and healthy controls. In the study of Smith, the expression of IL-23 receptor (IL-23R) was characterized and was found to be positively regulated by IL-6, IL-1β, and IL-23 and negatively regulated by TGF-β, IL-4, and IFN-γ. Similarly in the study performed by Shajarian et al., they reported higher expression of mRNA of IL-23 gene in peripheral blood of MS patients in comparison to control group which emphasizes the role of IL-23/IL-17 axis in the pathogenesis.

By playing a crucial role in Th17 cell expansion and survival, IL-23R is identified as a major driver of Th17 autoimmunity. It is possible that IL-23R gene polymorphisms influence IL-23 responses. Severe autoimmune responses are triggered by IL-23/IL-17 axis dysregulation impairing self-tolerance to antigens and tissues. Polymorphisms in the genes encoding IL23R and the p40 subunit of IL-12/23 (IL12B) have been linked to an increased risk of MS, as was made clear by a meta-analysis conducted by Huang et al. They found a correlation between rs3212227 polymorphisms in the p40 subunit of IL-12/23 (IL12B) and MS across all participants.
The IL-23/IL-17 axis has been the focus of several studies due to its importance in the development of MS and its potential as a therapeutic target to halt neurodegenerative processes and slow the course of the illness. The study of Li et al. revealed that serum IL-17A and IL-23 levels were significantly higher in the MS group than in the control group. Serum IL-17A level was found to be positively correlated with Th17 proportion and EDSS and significantly decreased with immunomodulatory therapy.

During Th17 development, IL-23R overexpression enhances the production of IL-17 in pathogenic Th17 cells. Inactive and soluble form of IL-23R was found to improve EAE symptoms by blocking the IL-23/IL-23R interaction via competitive inhibition in animal trials. Also, another study reported that Th17 cells from MS patients induced severe degeneration of stem cells derived motor neurons in cell culture with increased IL-17A secretion. Neutralizing IL-17A and anti-IL-17A receptor antibodies reversed this degenerative effect of IL-17A which highlighted IL-17A as a potential target for MS immune-therapeutic modalities.

CONCLUSION

MS patients during active relapse had a higher IL-17A serum level, and a higher percentage of IL-23R⁺ CD4⁺ T cells with increased IL-23R expression density on CD4⁺ T cells, as assessed by MFI, compared to patients in remission and healthy controls. This highlighted the role of the IL-23/IL-17 axis in the immune-pathogenesis of MS and its aggravating effect on MS neurodegenerative changes. Thus, the IL-23/IL-17 axis can be a potential target for immunomodulatory therapies ameliorating the Th17 mediated autoimmune through novel treatments for those patients. Further studies including more detailed characterization of Th17 immune regulation can help elucidate MS immune-pathogenesis and future therapeutic options.

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Not applicable

Availability of data and material:
Data are available upon request

Competing interests:
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REFERENCES

5. Shajarian M, Alsahebfosoul F, Etemadifar M, et al. IL-23 plasma level measurement in relapsing remitting multiple sclerosis (RRMS) patients compared to healthy subjects. Immunological investigations. 2015; 44; 36-44.
8. Fu, J., Huang, Y., Bao, T. et al. The role of Th17 cells/IL-17A in AD, PD, ALS and the strategic therapy targeting on IL-17A. J Neuroinflammation 19, 98 (2022).
10. Guerrero-García JJ. The role of astrocytes in multiple sclerosis pathogenesis. Neurologia (Engl


