Original paper

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Investigation of the role of interleukin 27 in the immune regulation of Treg and Th17 cells in neurosyphilis patients

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Abstract

Introduction: *Neurosyphilis (NS) is known as a sexually transmitted disease that is very difficult to diagnose and its diagnosis is delayed. Some studies have suggested that the level of interleukin (IL)-27 decreases in syphilis patients and the level of IL-17 increases in these patients, and these immunological changes can be a therapeutic target for these patients. The present study aims to evaluate IL-27's role in the immune regulation of Treg and Th17 cells in NS patients.*

Material and methods: *400 documented diagnosed syphilis patients were enrolled to the study and divided into two groups of neurosyphilis (NS) and non-neurosyphilis (S). Also 40 healthy volunteers were enrolled as a healthy control group (C). Peripheral blood mononuclear cells (PBMCs) from peripheral blood and cerebrospinal fluid (CSF) by lumbar puncture were collected as samples. mRNA expression and level of IL-27, IL-17, Th17, IL-17-producing $CD4^+$ T cells and also protein concentration and VDRL of CSF were investigated. To obtain proposed results, flow cytometry, RT-PCR and ELISA were used.*

Results: *The mRNA expression of IL-27 in PBMCs declined significantly in NS patients compared to healthy controls ($p = 0.002$) and S patients ($p = 0.005$) and decreased significantly in CSF of NS patients in comparison to healthy controls ($p = 0.002$) and S patients ($p = 0.003$). The frequency of IL-17-producing $CD4^+$ T cells increased significantly in PBMCs of NS patients in comparison to healthy controls ($p = 0.004$) and S patients ($p = 0.004$). This frequency also increased significantly in CSF of NS patients compared to C ($p = 0.007$) and S patients ($p = 0.003$). Adding rIL-27 significantly prevented the frequency of IL-17-producing $CD4^+$ T cells from naïve CD4+ T cells under Th17 polarizing conditions from NS patients ($p = 0.043$), C ($p = 0.043$), and S patients ($p = 0.002$) in PBMCs, and also 0.03, 0.02 and 0.03 respectively for NS, S and C of CSF. The results revealed a significant negative relationship between CSF protein and VDRL concentrations and CSF IL-27 levels.*

Conclusions: *This study confirms previous efforts on the critical role of IL-17 in NS. Also, it supports other results on the inhibitory effects of IL-27 on the therapeutic potential of IL-27 in NS and the inflammation process.*

Key words: *neurosyphilis, IL-27, Th17, IL-17, treponema pallidum.*

Introduction

As a disease due to *Treponema pallidum* (*T. pallidum*), syphilis has remained a critical health issue in the world. Syphilis infection progression has different steps [4,13]. The primary syphilitic ulcer appears after incubation for about 21 days. When the disease is left untreated, it enters the stage of secondary syphilis, in which spirochetes disseminate, and a general disease with different rashes occurs in the infected people. In addition, when the disease is not treated, the patient falls into the latent infection phase. Although the patients are asymptomatic in this stage, they show serological signs of infection. Historical studies have shown that about 30% of untreated patients with latent disease enter tertiary syphilis [19,31,32]. *Trepo-*

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nema pallidum is a bacterium that invades the central nervous system (CNS) and leads to neurosyphilis (NS). This illness has an occurrence probability at any time of infection [4]. This disease is generally detected by *T. pallidum* in brain parenchyma or cerebrospinal fluid (CSF) [7,12]. When the infection declines, late and early latent forms of syphilis (i.e., NS and its atypical forms) are likely to increase gradually. Research has shown that a significant part of people whose syphilis is not treated may develop NS at different stages of their life [14,27]. Also, studies have shown that NS incidence declines considerably following antibiotic administration, although symptomatic NS prevalence is constant at 1.5-13% [20]. Neurosyphilis presentation has experienced drastic changes within the last decades. Today, the early-stage NS diagnosis is a very difficult process. In this respect, most NS patients do not show any symptoms or they show atypical disease signs (e.g., personality changes, seizures, stroke, ophthalmic symptoms, or confusion) [10,17]. Therefore, psychiatrists, radiologists, and neurologists should consider these issues during differential diagnoses of demyelinating disorders or central vascular problems.

To our knowledge, there is no reliable tool for diagnosing NS. In this context, several instructions have diverse remarks on lumbar puncture (LP) in patients suffering from syphilis and offer various applications of treponemal and non-treponemal experiments [9,44]. Nevertheless, NS diagnosis is still a critical clinical challenge. In this regard, no perfect method exists to establish or replace the NS diagnosis. In the laboratory, NS is recognized based on the increase in protein concentration and CSF WBC count, as well as the unusual outcomes of CSF immunologic tests. One of the specific experiments for NS is the reactive CSF Venereal Disease Research Laboratory (CSF VDRL) test; however, the sensitivity of this experiment is not high enough [8,39]. Immune response down-regulation in NS patients might intensify the disease, thereby leading to neurological damage. On the other hand, CNS damage occurs due to the local host's uncontrolled immune response. The immune response mediated by cells plays an essential role in infection with *T. pallidum*. Infection with *T. pallidum* can lead to a remarkable host-specific immune response, thereby secreting numerous pro-inflammatory cytokines for spirochetes clearing [38,40]. However, excessive immune response and inflammation of hosts may lead to tissue damage and syphilis-related symptoms [35]. Therefore, the corresponding anti-inflammatory immune response may have a critical function in balancing the uncontrolled pro-inflammatory response. *Treponema pallidum* is capable of escaping the host immune response and developing a persistent infection, for example, in the progress of NS to the symptomatic stage. One potential mechanism of action for the spirochete escape of the host immune effector might be the simultaneous up-regulation of the immune regulatory response [34]. In non-human primate experiments, a strong immune response of T helper (Th) 1-type may clear *T. pallidum* in the CNS. Although the immune response during infection removes organisms, this response also may involve pathogenesis. T lymphocyte-secreted cytokines are vital for regulating pathogenic and protective immune responses [2,5]. Th17 cells, which produce cytokine IL-17, are a subgroup of $CD4^+$ T helper cells. It has been well documented that Th17 cells contribute to the clearance of different organisms. In addition, since Th17 modulates severe immunopathology associated with chronic infection, its response to infection may involve both progression/chronic infection and protection [1,23,35]. Research has shown an IL-17 increase in peripheral blood and secondary syphilitic lesions, also, the literature has shown CSF IL-17 elevation in early asymptomatic NS, suggesting the potential role of IL-17 *T. pallidum* infection-induced local immune response [36,54]. Early investigations on IL-27 have shown its effect in the initial phase of Th1 induction [24,33]. Nevertheless, more recent studies have reported anti-inflammatory features of the IL-27 signalling. Research on autoimmune and infectious inflammatory models has evidenced that mice without the WSX-1 subunit in the IL-27R complex establish severe pathological inflammation due to Th2 and Th1 responses. This observation indicates the broad anti-inflammatory functions of IL-27 [28,29]. Recent works have reported the potent inhibitory role of IL-27 in the encephalitogenic Th17 cell expansion in culture. Despite scarce research on IL-27's role in human Th17 differentiation, research has shown its inhibitory role in human Th17 cells' development. IL-27 suppresses IL-17 production using dedicated memory T cells. In addition, this protein prevents gene expression and IL-17 in purified memory T cells activated in the presence and absence of IL-23 and IL-1β. Furthermore, cytokines may upregulate IL-17 in human memory T cells. Despite the mentioned efforts, IL-27's role in the immune regulation of Treg and Th17 cells of NS patients is still unclear. Therefore, the present study aims to investigate the role of IL-27 in the immune regulation of Treg and Th17 cells in neurosyphilis patients.

Material and methods

Study population and ethics statement

The Institutional Ethics Committee of the Shanghai Skin Disease Hospital approved this research. Also, this study complied with the national legislation and adhered to the Declaration of Helsinki guidelines. Written informed consent was submitted by the patients according to the institutional guidelines. In this study 400

documented diagnosed syphilis patients were enrolled and divided into two groups: neurosyphilis group (NS) and non-neurosyphilis group (S). Also 40 healthy volunteers were enrolled as a healthy control group (C). The cerebrospinal fluid and blood samples of 400 patients with syphilis were collected, and the blood RPR and TRUST experiments, as well as the cerebrospinal fluid RPR, TRUST and VDR experiments, and the leukocyte and protein detection of the cerebrospinal fluid were performed. According to the examination of the cerebrospinal fluid and serum, the subjects were divided into two groups: neurosyphilis (NS) group and non-neurosyphilis group (S). The cerebrospinal fluid of patients without syphilis infection and no central nervous system injury was used as the control group (C), and 5 ml of peripheral blood was collected from 40 healthy volunteers for the isolation of peripheral blood mononuclear cells.

Diagnostic criteria for neurosyphilis

CDC guidelines in the European countries and the United States of America characterize primary syphilis as an ulcer (chancre), typically with regional lymphadenopathy. *Treponema pallidum* detection through dark-field examination/fluorescent antibody method/PCR in lesion exudate laboratory is considered to confirm this malignancy. Also, treponemal and reactive nontreponemal tests are performed to confirm the syphilis diagnosis. Secondary syphilis is recognized by a maculopapular rash. Latent syphilis shows no clear symptoms and is identified through a history of infection, normal CSF, and nontreponemal and reactive treponemal experiments. In the end, tertiary syphilis is defined as a history of syphilis in its primary, secondary, or latent forms acquired more than 1 year ago with specific clinical manifestations and laboratory test confirmation by treponemal and reactive nontreponemal experiments.

Confirmed NS diagnosis consists of a reactive CSF-TPPA and reactive CSF-VDRL without considerable blood contamination of CSF. Presumptive NS is described as a nonreactive CSF-VDRL with a reactive CSF-TPPA having either of the following or both: i) psychiatric manifestations or clinical neurological manifestations in line with NS without other known contributors to such abnormalities and ii) CSF white blood cell (WBC) count ≥ 8/µl and/or CSF protein concentration > 45 mg/dl C in the absence of other known abnormality causes.

Sample collection

Blood sample: for isolation of PBMCs from peripheral blood, EDTA anticoagulation was collected from peripheral veins of all subjects (10 ml of peripheral venous blood collected), and PBMCs were obtained by Ficoll-Hypaque density gradient centrifugation. One part of PBMC was used to extract RNA.

Cerebrospinal fluid specimens: lumbar puncture was done to collect cerebrospinal fluid.

Laboratory tests

To obtain proposed results, flow cytometry, RT-PCR and ELISA were used. The expression levels of $IL\text{-}27^{+}CD14^{+}$ cells in the peripheral blood mononuclear cells (PBMC) of 3 groups were detected by flow cytometry; IL-27 and IL-27R mRNA were detected by RT-PCR. The differences in the expression levels of IL-27 in the cerebrospinal fluid of 3 groups were detected by ELISA. The regulatory effect of IL-27 on the activation, induction, proliferation and differentiation of Th17 cells and Treg cells in patients with neurosyphilis was detected for this reason, Naïve (Naïve) $CD4^{+}$ T cells were isolated by magnetic microbeads negative selection method and cultured *in vitro*. Under the stimulation of IL-27, flow cytometry was used to detect the differentiation of naïve $CD4^{+}$ T cells into Th17 cells and Treg cells, then the difference of IL-17A expression was detected by PCR.

Statistical analysis

Between-group categorical data were compared using the corrected chi-square (χ^2) or two-sided Fisher exact test. Also, parametric quantitative data were compared using one-way analysis (ANOVA) or the Student's *t* test, while non-parametric data were compared *via* the Mann-Whitney test. Finally, the relationship between the analysed parameters was determined using logistic regression analysis. The analyses were performed using the SPSS software at a statistically significant level of less than 0.05 (p-value < 0.05).

Results

IL-27 underexpression is related to an increase in the Th17 cells frequency

Real-time PCR analysis of IL-27 mRNA expression in PBMCs indicated that the mRNA expression of IL-27 declined significantly in NS patients compared to healthy controls ($p = 0.002$) and S patients ($p = 0.005$). This result was also obtained in the CSF of the 3 groups of patients such that IL-27 mRNAs expression decreased significantly in NS patients in comparison to healthy controls ($p = 0.002$) and S patients ($p = 0.003$), but no statistically significant difference was detected between healthy controls and S patients in both CSF and PBMC samples (Fig. 1).

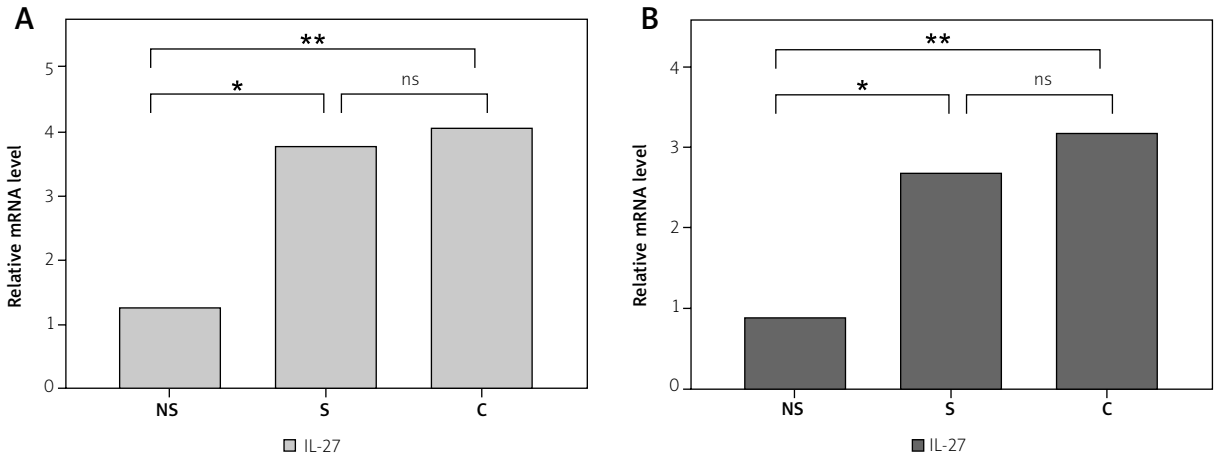

Fig. 1. Interleukin 27 (IL-27) mRNA expression in peripheral blood mononuclear cells (PBMCs) (**A**) and cerebrospinal fluid (CSF) (**B**) by RT-PCR.

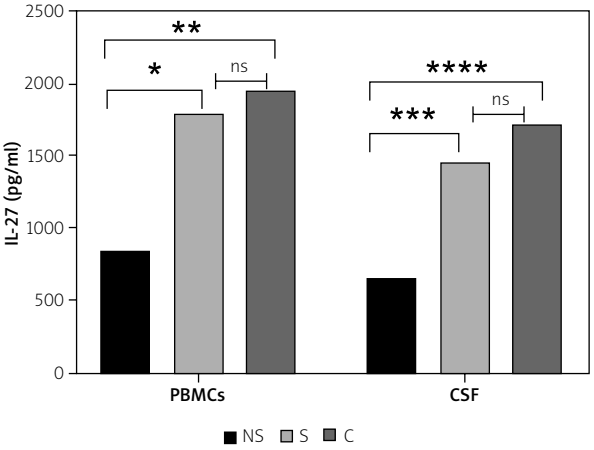

Fig. 2. Interleukin 27 (IL-27) levels in peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) by ELISA.

ELISA was performed to measure IL-27 levels in 3 groups in CSF and PBMCs. The results indicated similar significant differences of $p < 0.001$ (Fig. 2).

Afterwards, the frequency of IL-17-producing $CD4^+$ T cells in PBMCs was assessed after stimulating with ionomycin and PMA. Based on the obtained outcomes, the frequency of IL-17-producing $CD4^+$ T cells increased significantly in PBMCs of NS patients in comparison to healthy controls ($p = 0.004$) and S patients ($p = 0.004$, Fig. 3A, C). These outcomes were also noticed in the CSF of these 3 groups of patients as the frequency of IL-17-producing $CD4^+$ T cells increased significantly in CSF of NS patients compared to healthy controls ($p = 0.007$) and S patients ($p = 0.003$, Fig. 3B, C). The results of ELISA analysis revealed similar significant differences about IL-17 levels in PBMCs and CSF ($p < 0.001$; Fig. 3D).

IL-27 prevents IL-17 production and Th17 cell differentiation

According to flow cytometry outcomes, the frequency of IL-17-producing $CD4^+$ T cells increased significantly more than naïve $CD4^+$ T cells under Th17 polarizing conditions in NS patients compared to healthy controls ($p = 0.01$) and S patients ($p = 0.015$). Meanwhile, the results showed no statistically significant difference between healthy controls and S patients. These results were also observed in the CSF of these 3 groups of patients, as there was a statistically significant increase in NS patients compared to healthy controls ($p = 0.01$) and S patients ($p = 0.01$) (Fig. 4A, B). In this study, the IL-17 expression in the stimulated naïve $CD4^+$ T cells' supernatants was further investigated using ELISA and showed similar significant differences (Fig. 4C, D).

The IL-27 level associates negatively with CSF VDRL titre and CSF protein concentration in NS patients

This study also investigated the possible correlation between CSF IL-27 levels and other measures. The results revealed a significant negative relationship between CSF protein concentrations and CSF IL-27 levels. Moreover, a significant negative relationship was identified between CSF VDRL titre and CSF IL-27 levels.

Discussion and conclusion

Neurosyphilis is a disease caused by the spirochete *T. pallidum*. This health problem is considered an endemic infection of the CNS and a critical sexually

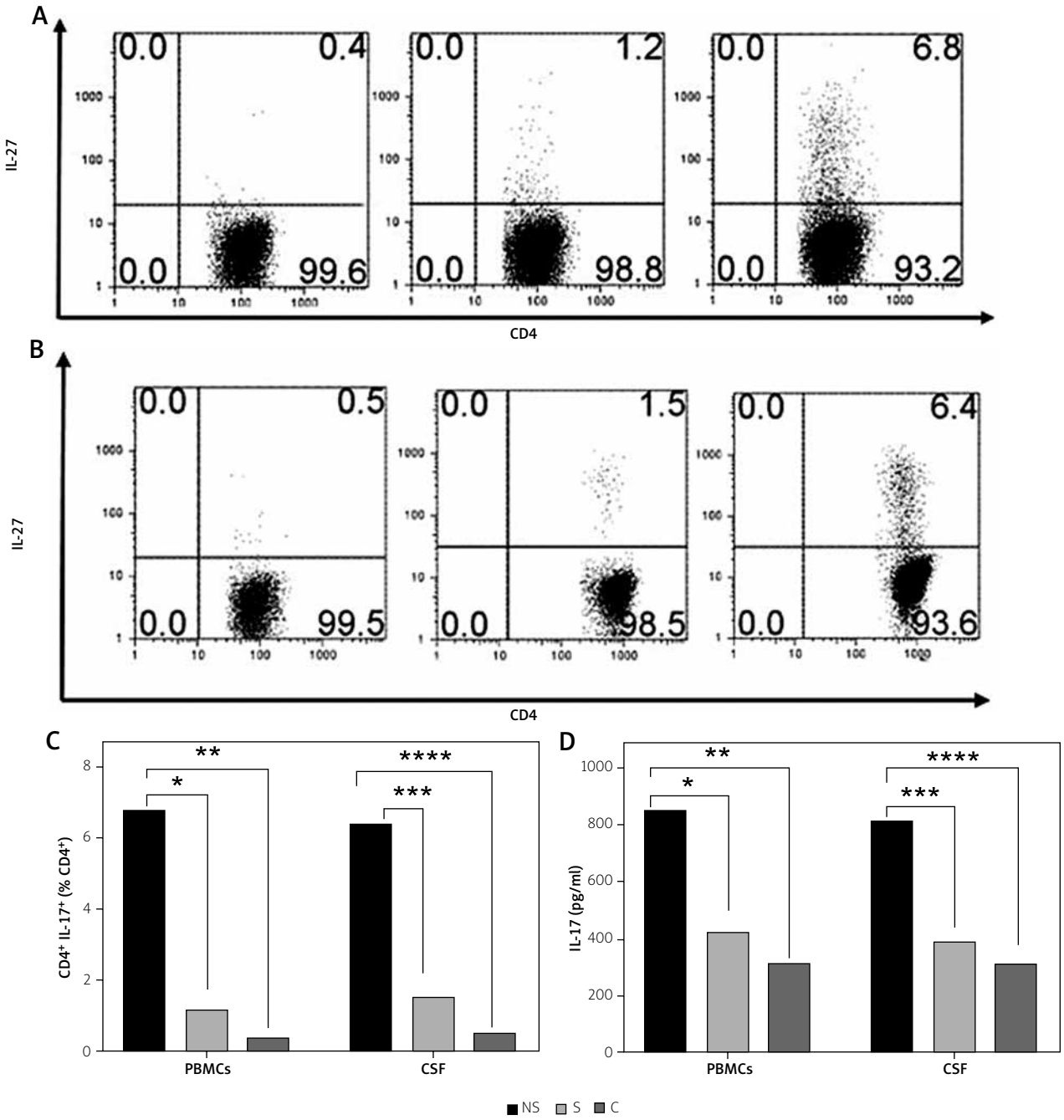

Fig. 3. The frequency of interleukin 27 (IL-17)-producing CD4+ T cells in peripheral blood mononuclear cells (PBMCs) (**A, C**) and cerebrospinal fluid (CSF) (**B, C**), and IL-17 levels in PBMCs and CSF (**D**).

transmitted disease (STD) [48]. Although NS is syphilis' late manifestation, it may also be seen in early syphilis [15]. In this regard, *T. pallidum* is also seen in the CSF of patients suffering from primary syphilis. The syphilis incidence increases with a history of same-sex relationships and immunosuppression. Epidemiological and clinical data have altered significantly from the pre-antibiotic era. Clinical types of NS are highly diverse and typically are divided into five categories: gummatous, asymptomatic, meningeal, parenchymatous, and meningovascular [25,43]. Clinical features of the disease are typically nonspecific and are seen

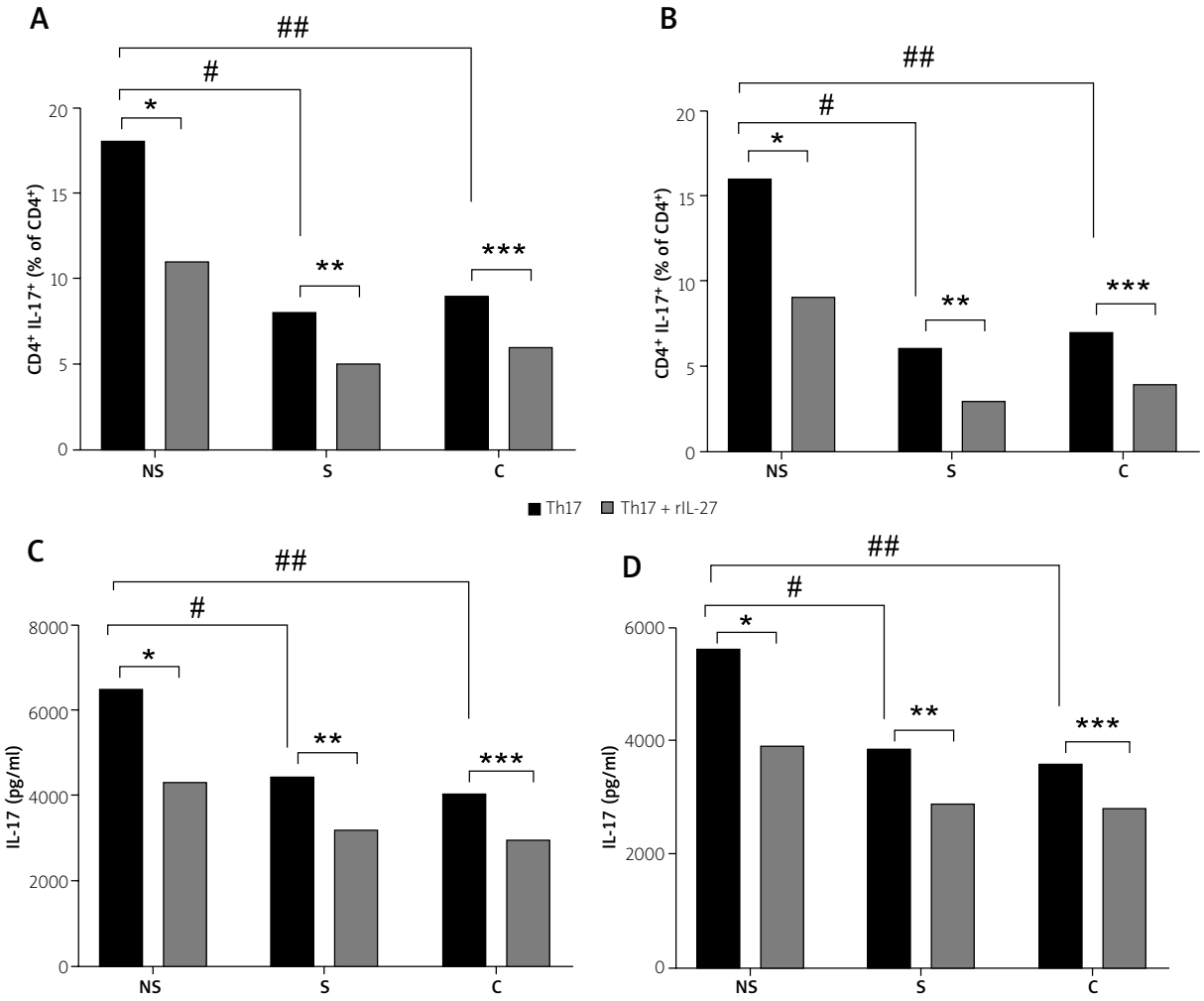

Fig. 4. Effect of interleukin 27 (IL-27) on frequency of IL-17-producing CD4+ T cells from naïve CD4+ T cells under Th17 polarizing conditions in peripheral blood mononuclear cells (PBMCs) (**A**) and cerebrospinal fluid (CSF) (**B**), and IL-17 expression in the stimulated naïve CD4+ T cells' supernatants in PBMCs (**C**) and CSF (**D**).

during the disease progression because the disease manifests in several forms in the same patient [11,41].

Neurosyphilis can be either symptomatic (SNS) or asymptomatic (ANS). These two types of NS have different symptoms, including memory deterioration, headache, and dizziness. In this respect, there has not been yet a specific high-sensitivity experiment to detect these disease forms. Therefore, it is difficult for clinicians (e.g., dermatologists and neurologists) to diagnose them [6,42]. The NS incidence has not been evaluated accurately due to trouble in reporting patients with STDs and poor documentation of NS. However, the significant increase in NS reports has attracted our attention. Recently, NS diagnoses have been based on a mix of clinical judgment, medical history, serological findings, clinical features, and CSF abnormalities. In this regard, ANS is sometimes missed and misdiagnosed as SNS [21,26]. To the best of the authors' knowledge, early treatment and detection of ANS can prevent its progress to SNS. Hence, it is necessary to comprehensively determine the distinction between SNS and ANS. Some investigations have shown cell-mediated immunity as an essential factor in antisyphilitic host defence. Multiple studies have shown the predominance of CD4+ T cells in NS patients' CSF. In this regard, several effector subsets exist in CD4+ T cells, e.g., regulatory T cells, classical Th1 and Th2 cells, Th17 cells, and follicular helper T cells. T-helper cells producing IL-17 may contribute to the NS-related inflammatory responses [52,53]. Recent evidence indicates the specific roles of

IL-27 in T cell differentiation. IL-27 prevents the evolution of pro-inflammatory Th17 cells by inhibiting the expression of the Th17 transcription factor and, thus, suppressing the interleukin IL-17 production in naïve T cells [30,46]. Dendritic cells, monocytes, and macrophages are antigen-presenting cells producing IL-27 [16,37]. IL-27 has a critical role in adaptive and innate immunity. In addition, as an immunosuppressive function, IL-27 inhibits inflammatory cytokines production. Different studies have shown the promoting role of this cytokine in secreting pro-inflammatory cytokines by human monocytes and its significant inhibitory effects on T-regulatory, Th1, Th2, and Th17 cells [18,47].

In this research, we have investigated the levels of CSF anti-inflammatory cytokines and pro-inflammatory in patients suffering from NS, followed by exploring the association of these cytokines with markers of neuronal injury. Also, differences in these biomarkers in NS and S patients were examined.

The results showed a remarkable reduction in IL-27 expression with an increase in Th17 cell response in NS patients. In addition, it was found that IL-27 could considerably suppress Th17 cell differentiation directly and by modulating DCs. Overall, these data indicate a negative correlation between the IL-27 expression in the CSF of patients with NS and their IL-17 level. Moreover, IL-27 prevented the local cellular immune impairment of Th17 in NS patients. In addition, the results showed a negative correlation between the protein concentration in CSF and the VDRL titre. Besides, IL-27 had an immunoprotective impact on the local immune impairment of NS patients' CNS. According to these findings, it is inferred that lack of IL-27 may be associated with CNS damage in NS patients, and lack of IL-17 may play a role in NS patients' clinical symptoms. Our findings are in accord with other scarce relevant investigations. For instance, Pastuszczak *et al*. [35] indicated that ANS patients had higher IL-17A and INF-F levels in the CSF than those without NS. Moreover, Wang *et al*. [50] reported a significant increase in Th17 cells' frequency in the peripheral blood of NS patients than in healthy blood donors. In addition, a positive association existed between IL-17 levels in CSF of NS patients and their CSF VDRL titres and total CSF protein levels. Many researchers have reported the significant roles of IL-27 and Th 17 and also IL-17 in neuroinflammatory infection. For example, Wraith observed an increase in IL-17 mRNA-expressing mononuclear cells in both CSF and blood of MS patients [51]. Besides, according to Babaloo *et al*. [3], the high IL-17A levels can be partially responsible for MS pathogenicity. IL-27's anti-inflammatory features can inhibit IL-17A's inflammatory properties and lead to partial suppression of clinical symptoms and MS disease establishment. In this respect, another related study by Wang *et al*. [49] demonstrated that an IL-27 expression decline is related to an upregulated Th17 cell response in patients with active VKH.

Conclusions

Generally, the present study showed that rIL-27 could significantly suppress the differentiation of Th17 cells directly and by DC modulation. In brief, the present study confirms previous efforts on the critical role of IL-17 in NS. Also, it supports other results on the inhibitory effects of IL-27 on the therapeutic potential of IL-27 in NS and the inflammation process. In this respect, exploring more NS populations and conducting *in vitro* investigations on Th17/IL-27 interactions is necessary.

Disclosure

The authors report no conflict of interest.

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