Original Research

Th17 Cell Related Cytokine Profiles in Narcolepsy and Other Types of Excessive Daytime Sleepiness

Demeke G. Debebe[1], Khosro Sadeghniat-Haghighi[2], Yadollah Shakiba[3], Ania Rahimi-Golkhandan[2], Maryam Izad[1]*

[1.] *Department of Immunology, School of Medicine, International Campus, Tehran University of Medical Sciences, Tehran, Iran*
[2.] *Occupational Sleep Research Center, Baharloo Hospital, Tehran University of Medical Sciences, Tehran, Iran*
[3.] *Department of Immunology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran*

Received: 18 Dec. 2015 Accepted: 18 Feb. 2016

Abstract

Background and Objective: Narcolepsy is a chronic neurological disorder caused by loss of hypocretin (Hcrt) neurons. Both genetic and environmental factors play an important role for the development of narcolepsy. The mechanism of Hcrt loss in narcolepsy is elusive; however, an autoimmune mediated destruction of Hcrt neurons is suspected. The purpose of this study was to assess Th17 related cytokines: interleukin-6 (IL-6), IL-17, IL-23, and transforming growth factor beta (TGF-β) in the pathophysiology of narcolepsy and other types of excessive daytime sleepiness (EDS).

Materials and Methods: A total of 15 narcoleptic patients, 35 other patients with EDS and 48 age and sex matched healthy subjects were enrolled in this case-control study. Serum IL-6, IL-17, IL-23, and TGF-β levels were measured using sandwich enzyme-linked immunosorbent assay.

Results: There was no significant difference in IL-6 (P = 0.0618) and IL-23 (P = 0.7717) level among participants with narcolepsy, other patients with EDS and controls, whereas TGF-β was significantly decreased in the ones with narcolepsy and other EDS compared to healthy controls (P = 0.0039).

Conclusion: Decreased level of TGF-β in narcolepsy and other patients with EDS indicates a clue for the presence of dysregulation of inflammatory cascades in these patients. This study sheds a new insight on the pathophysiology of narcolepsy.

Keywords: Narcolepsy; Cataplexy; Hypocretin; Interleukin-6; Transforming growth factor beta; Interleukin-17; Interleukin-23

Citation: Debebe DG, Sadeghniat-Haghighi K, Shakiba Y, Rahimi-Golkhandan A, Izad M. **Th17 Cell Related Cytokine Profiles in Narcolepsy and Other Types of Excessive Daytime Sleepiness**. J Sleep Sci 2016; 1(2): 44-50.

Introduction

Narcolepsy is a chronic neurological disorder caused by the loss of hypocretin (Hcrt) [orexin (OX)]; a neuropeptide that projects all over the central nervous system (CNS) (1, 2). The clinical features of narcolepsy include excessive daytime sleepiness (EDS), cataplexy, hallucinations at sleep onset and awakening, sleep paralysis, and fragmented nocturnal sleep patterns (1). There are two types of narcolepsy: Type 1 narcolepsy [narcolepsy with cataplexy (NC)] and Type 2 narcolepsy [narcolepsy without cataplexy (NwC)] (2, 3). The distinctive attribute of Type 1 narcolepsy is a sudden loss of muscle tone elicited by positive emotions (3) such as laughter and surprise (4). The

***Corresponding author**: M. Izad, Department of Immunology, School of Medicine, International Campus, Tehran University of Medical Sciences, International Campus, Tehran, Iran.*
Tel: +989127197297, Fax: +982166419536
Email: izadm@sina.tums.ac.ir

common onset of the disease is in babyhood, with a peak incidence between 10 and 19 age groups (2), and once established the disease is long lasting (5). Narcolepsy attacks affect nearly 0.02% of the general population worldwide (1).

Genetic and environmental factors are involved for narcolepsy susceptibility (6). Among the genetic factors, human leukocyte antigen (HLA)-DQ0602, a heterodimer protein encoded by HLA-DQA1*01:02 and DQB1*06:02 is the main predisposing genes (2). Moreover, other HLA alleles such as HLA-DRB1*1501 (1) and DQB1*03:01 also contribute to narcolepsy susceptibility, nevertheless HLA-DQB1*06:01, DQB1*06:03, DQB1*05:01, DQA1*01(non-DQA1*01:02) are protective (2). Besides the strong association of narcolepsy with HLA Class II genes, genome-wide association studies have identified single nucleotide polymorphisms of the genes encoding T-cell-receptor α chain, purinergic receptor subtype P2RY11, tumor necrosis factor (TNF) (ligand) superfamily member 4, also called OX40L, cathepsin H and DNA methyltransferase-1 as genetic triggers (1, 4, 7). As most of the autoimmune diseases, the role of environmental factors is clearly apparent when considering the low disease concordance rate between monozygotic twins (3). Environmental association with upper airway infections; more specifically *Streptococcus pyogenes* and influenza A H1N1 infection and selected H1N1 vaccine preparations, namely AS03-adjuvanted vaccine, have been strongly associated to the onset of the disease (2). As infectious disorders show seasonal variation, this finding is also supported by strong seasonality of the disease onset (5), suggesting that upper airway infections could elicit narcolepsy (8). Bystander activation and molecular mimicry are the major means by which upper airway infection can induce autoimmune destruction of Hcrt neurons in the CNS of narcoleptic patients (1, 2).

Pathophysiological studies indicated that recent evidence on the pathogenesis of narcolepsy comes from studies on NC (9), which is caused by the early loss of Hcrt, but NwC is hardly caused by Hcrt deficiency (10) indicating a diverse origin in etiology (11). Tumor TNFα and interleukin-6 (IL-6) were reported to play a role in the pathophysiology of narcolepsy (12). However, this was later retracted and only increased serum TNFα but not IL-6 was reported (13, 14). Nevertheless, TNFα can impair the Hcrt system through degradation of Hcrt mRNA and protein ubiquitination (15).

The goal of this study was to determine the involvement of Th17 related cytokines [IL-6, IL-17, IL-23, and transforming growth factor beta (TGF-β)] in the pathophysiology of narcolepsy and other disorders with EDS.

Materials and Methods

Subjects

The study was approved by Tehran University of Medical Sciences Research Ethics Committee.

Informed consent was obtained from all patients and control subjects who accepted to participate in the study (all participants were Iranian). The patients were enrolled into the study only when EDS was reported. Nevertheless, shift worker patients with EDS were not included, as these patients may have EDS because of their lifestyle. According to the International Classification of Sleep Disorders 3rd edition (ICSD-3) (2),

15 patients were clinically identified to be narcoleptic. However, according to ICSD-3 criteria 35 patients were not narcoleptic even if they had EDS. All patients were recruited into the study from October 2014 to July 2015, at Occupational Sleep Research Center in Baharloo Hospital (Tehran, Iran). Peripheral blood sample was collected from patients when they reported no acute or chronic infectious disease, and/or inflammatory diseases, and if they were not on any medication with known influence on immunological factors (e.g., corticosteroids). Finally, blood sample was taken from 48 healthy subjects that matched age and sex of the patients. Moreover, healthy participants were excluded if they reported EDS and/or any signs of immunological abnormalities.

Blood sample was collected from each participant with clot activator test tube, and then, serum was extracted by centrifugation of whole blood at 1500 revolutions per minute (rpm) for 10 minutes at 4° C. Subsequently, serum has been immediately aliquoted into four different micro tubes for each cytokine assay and immediately deep freezed at −70° C until assayed.

Cytokine assays

Serum cytokine assessment was performed by sandwich enzyme-linked immunosorbent assay (ELISA) with specific capture and detection antibodies for IL-6, IL-17 (R&D Systems, USA), TGF-β (USCN Life Science Inc., PRC) and IL-23 (eBioscience, USA). The concentration of each cytokine in the serum was calculated based on the linear regression equation of the standard curve obtained with recombinant cytokines, according to the kit manufacturer instructions. The lower limit of detection for ELISA was: 9.4 pg/ml for IL-6, 62.5 pg/ml for TGF-β, 15.6 pg/ml for IL-17 and 62.5 pg/ml for IL-23, as determined by the manufacturer.

Statistical analysis

The difference in the measurements of serum IL-6, TGF-β, IL-17, and IL-23 levels between patients and controls were analyzed with independent t-test. Furthermore, change in cytokine levels among narcolepsy, the patients with other EDS disorders and healthy controls were analyzed with Kruskal–Wallis test followed by Dunn's multiple comparison test. $P < 0.0500$ was considered statistically significant. All analyses were performed using SPSS software (version 20.0; IBM, Chicago, IL, USA) and PRISM software version 6.01 (Graphpad Software, La Jolla, California, USA).

Results

Baseline characteristics of patients and controls

A total of 98 individuals (15 cases with narcolepsy, 35 cases with other EDS disorders and, 48 control subjects) were enrolled into the study. Of the total 50 patients, 43 (86%) were male. The age distribution of the patients was 18-76 years (mean age ± standard deviation, 38.4 ± 10.6 years). The groups of narcoleptics, patients with other EDS disorders and control subjects were compared in terms of age and sex (Table 1).

Table 1. Age and sex characteristics of patients and healthy controls

Variables	Controls n = 48	Narcolepsy n = 15	EDS disorder n = 35	P value
Men/women	41/7	13/2	30/5	0.9300[a]
Age (years)*	36.2 ± 8.6	36.5 ± 6.4	39.2 ± 11.9	0.3400[b]

*Data presented as mean ± SD. [a]Chi-square test, [b]One-way ANOVA, EDS: Excessive daytime sleepiness, SD: Standard deviation

Cytokine profiles

TGF-β had lower levels in patients (P = 0.0080) when compared to healthy controls (Figure 1). However, IL-6 (P = 0.6020) and IL-23 (P = 0.6060) were not statistically different between patients and healthy subjects. Meanwhile, IL-17 was not detected in all participants.

Analysis of serum IL-6 and IL-23 levels in healthy controls, narcoleptics, and patients with other EDS disorders showed no significant difference in IL-6 (P = 0.0618) and IL-23 (P = 0.7717) among the groups. Nevertheless, IL-6 showed a little increment in patients, but it failed to reach the statistical significance. However, TGF-β (P = 0.0039) showed change between the groups, more precisely it was significantly decreased in narcoleptics (P = 0.0250) and patients with other EDS disorders (P = 0.0020) as compared to healthy controls (Figure 2).

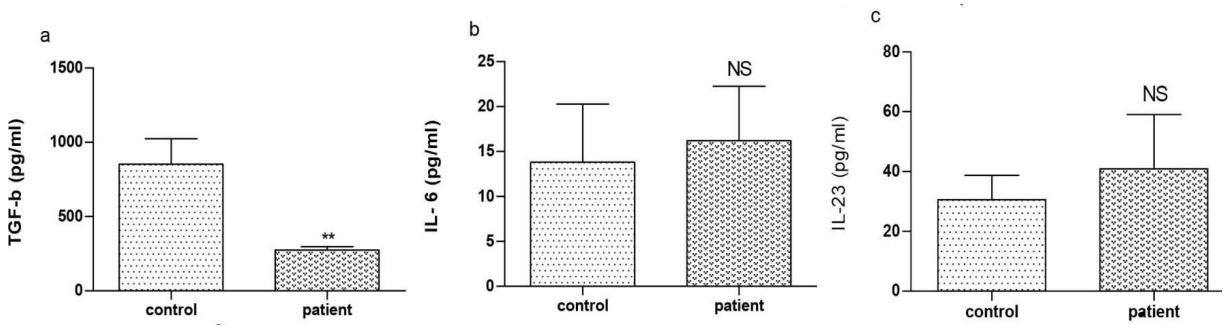

Figure 1. Serum transforming growth factor beta, interleukin-6 (IL-6) and IL-23 levels (pg/mL) in healthy controls and patients. Values represent the mean ± standard deviation, NS: P > 0.0500, **P < 0.0100: Independent samples t-test

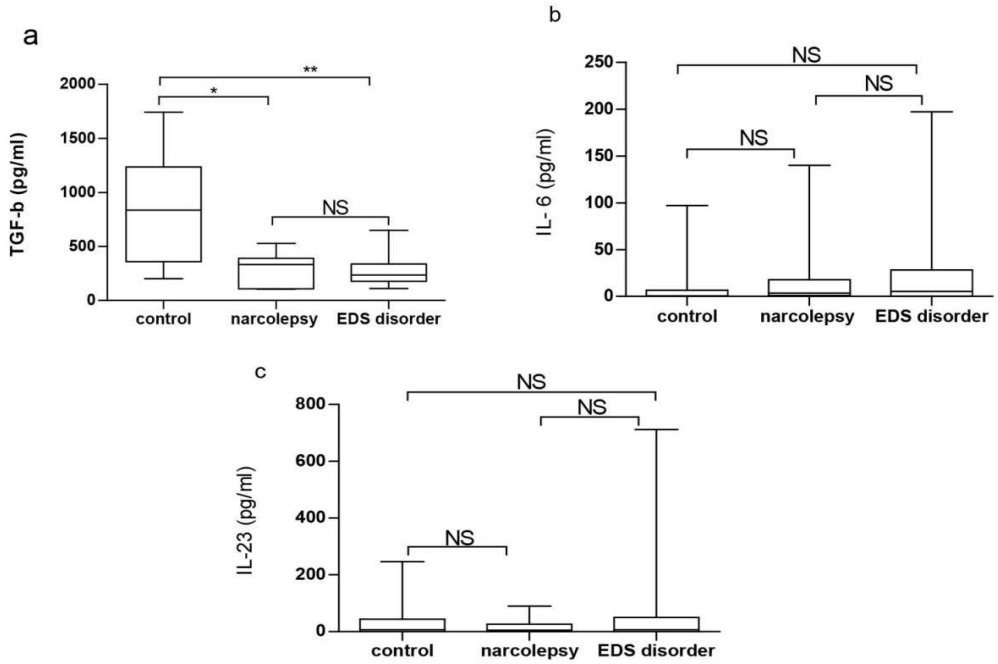

Figure 2. Box plots of serum transforming growth factor beta, interleukin-6 (IL-6) and IL-23 levels (pg/mL) of narcolepsy, other excessive daytime sleepiness disorders and healthy controls. Whiskers calculated the minimum and maximum of the data. NS: P > 0.0500, *P < 0.0500, **P < 0.0100: Kruskal–Wallis test followed by Dunn's multiple comparison test

Discussion

There has been much effort in characterizing the cause and pathophysiology of narcolepsy as a result of its increased incidence after ASO3 adjuvanted mass vaccination campaigns in 2009 (16, 17). Animal models showed that Hcrt knockout mice and dogs presented narcolepsy like phenotype. Thereafter, decreased Hcrt level due to cellular loss has been thought as a possible pathophysiological mechanism in narcolepsy (1, 18). Furthermore, inflammatory cytokines like IL-1β (19) and TNFα have been showed to aggravate the inflammatory processes in the CNS of narcoleptic patients and promote persistent neuroinflammation and Hcrt neuron ubiquitination (15). So far, there is no report investigating TGF-β, IL-17, and IL-23 in narcolepsy and other EDS disorders.

Up to our knowledge, we revealed for the first time the involvement of TGF-β in the pathophysiology of narcolepsy and other EDS disorders.

The current study investigated serum IL-6, IL-17, IL-23, and TGF-β levels in all participants that fulfill the requirements. Serum IL-17 was not detected in all participants. Meanwhile, IL-6 and IL-23 was not different among narcoleptics, patients with other EDS disorders, and healthy controls. However, serum TGF-β was decreased in narcoleptics and patients with other EDS disorders compared to healthy subjects.

The previous studies explored only the role of IL-6 in narcolepsy and came up with inconsistent results. According to Okun et al. (12), serum IL-6 and TNFα were increased significantly in narcoleptic patients compared to age and sex-matched healthy subjects. However, Santos Coelho et al. (13) and Vgontzas et al. (14) showed that only TNFα concentration was increased in narcoleptic patients.

Although serum IL-6 was increased in narcoleptics and patients with other EDS disorders in the present study, the observed association was not statistically significant, this maybe is due to limited sample size. Importantly, decreased TGF-β in narcolepsy and other EDS disorders indicated that TGF-β signaling dysregulation could cause inflammation in these patients. Moreover, TGF-β promotes the differentiation of Th17 cells in a dose-dependent manner; at low concentration, TGF-β synergizes with IL-21 to promote the differentiation of Th17 cells (20). Notably, decreased level of TGF-β in narcolepsy patients in this study reinforced the presence of intense inflammatory reaction due to malfunctioning of TGF-β signaling, that can lead to destruction of Hcrt neurons in the CNS of these patients.

Downregulation of TGF-β in narcoleptic patients in the current study showed the possible imbalance of Treg and Th17 cells and their soluble products in narcolepsy. Nevertheless, this was not confirmed by upregulation of serum IL-17 concentration in narcoleptic patients. This could be probably because of the low half-life of IL-17 in serum or partly due to conformational change that serum IL-17 underwent. Nonetheless, we have avoided freeze and thaw of the serum sample to overcome this problem.

On the other hand, decreased the level of serum TGF-β in other EDS disorders who probably could be apnea patients in our study compared to narcolepsy patients, support the findings of earlier studies. Previous findings indicated the involvement of inflammatory cytokines in the pathophysiology of EDS disorders,

especially apnea and idiopathic hypersomnia patients have dysregulation of the IL-1β and TNFα cytokine system (19). In general, dysregulation of IL-1β and TNFα in these patients could affect the inflammatory cascade and cause down-regulation of TGF-β. The observed downregulation of TGF-β both in narcolepsy and other EDS patients correlates with the disturbance of sleep architecture and probably could augment the malfunctioning sleep regulatory substances (IL-1β and TNFα).

Conclusion

This is the first study investigating Th17 cell cytokines in narcolepsy and other EDS disorder patients. Significantly decreased serum TGF-β in narcolepsy patients in this study reinforced that loss of Hcrt neurons in narcolepsy is an autoimmune process. Importantly, decreased TGF-β in narcolepsy and other EDS disorders indicated the presence of inflammation in these patients. Therefore, we recommend further studies investigating Th17 related intracellular cytokine assessment for better understanding of pathophysiological mechanisms in these patients.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgments

This study was funded by Tehran University of Medical Sciences, International Campus under grant number (IR.TUMS.REC.1394.868). Furthermore, we are thankful to all patients participated in our study.

References

1. Mahlios J, De la Herran-Arita AK, Mignot E. The autoimmune basis of narcolepsy. Curr Opin Neurobiol 2013; 23: 767-73.

2. Partinen M, Kornum BR, Plazzi G, et al. Narcolepsy as an autoimmune disease: the role of H1N1 infection and vaccination. Lancet Neurol 2014; 13: 600-13.

3. Bergman P, Adori C, Vas S, et al. Narcolepsy patients have antibodies that stain distinct cell populations in rat brain and influence sleep patterns. Proc Natl Acad Sci U S A 2014; 111: E3735-E3744.

4. Singh AK, Mahlios J, Mignot E. Genetic association, seasonal infections and autoimmune basis of narcolepsy. J Autoimmun 2013; 43: 26-31.

5. Partinen M, Saarenpaa-Heikkila O, Ilveskoski I, et al. Increased incidence and clinical picture of childhood narcolepsy following the 2009 H1N1 pandemic vaccination campaign in Finland. PLoS One 2012; 7: e33723.

6. Han F. Sleepiness that cannot be overcome: narcolepsy and cataplexy. Respirology 2012; 17: 1157-65.

7. Han F, Faraco J, Dong XS, et al. Genome wide analysis of narcolepsy in China implicates novel immune loci and reveals changes in association prior to versus after the 2009 H1N1 influenza pandemic. PLoS Genet 2013; 9: e1003880.

8. Kornum BR, Faraco J, Mignot E. Narcolepsy with hypocretin/orexin deficiency, infections and autoimmunity of the brain. Curr Opin Neurobiol 2011; 21: 897-903.

9. Kumar S, Sagili H. Etiopathogenesis and neurobiology of narcolepsy: a review. J Clin Diagn Res 2014; 8: 190-5.

10. Bayard S, Croisier Langenier M, Cochen De Cock V, et al. Executive control of attention in narcolepsy. PLoS One 2012; 7: e33525.

11. Holm A, Bang-Berthelsen CH, Knudsen S, et al. miRNA profiles in plasma from patients with sleep disorders reveal dysregulation of miRNAs in narcolepsy and other central hypersomnias. Sleep 2014; 37: 1525-33.

12. Okun ML, Giese S, Lin L, et al. Exploring the cytokine and endocrine involvement in narcolepsy. Brain Behav Immun 2004; 18: 326-32.

13. Santos Coelho FM, de Sousa HP, Alves GR, et al. TNF and IL-6 levels in narcoleptic patients. Einstein 2006; 4: 327-30.

14. Vgontzas AN, Papanicolaou DA, Bixler EO, et al. Elevation of plasma cytokines in disorders of excessive daytime sleepiness: role of sleep disturbance and obesity. J Clin Endocrinol Metab 1997; 82: 1313-6.

15. Zhan S, Cai GQ, Zheng A, et al. Tumor necrosis factor-alpha regulates the Hypocretin system via mRNA degradation and ubiquitination. Biochim Biophys Acta 2011; 1812: 565-71.

16. Ahmed SS, Schur PH, MacDonald NE, et al. Narcolepsy, 2009 A(H1N1) pandemic influenza, and pandemic influenza vaccinations: what is known and unknown about the neurological disorder, the role for

autoimmunity, and vaccine adjuvants. J Autoimmun 2014; 50: 1-11.

17. Bardage C, Persson I, Ortqvist A, et al. Neurological and autoimmune disorders after vaccination against pandemic influenza A (H1N1) with a monovalent adjuvanted vaccine: population based cohort study in Stockholm, Sweden. BMJ 2011; 343: d5956.

18. Nishino S, Ripley B, Overeem S, et al. Hypocretin (orexin) deficiency in human narcolepsy. Lancet 2000; 355: 39-40.

19. Kapsimalis F, Basta M, Varouchakis G, et al. Cytokines and pathological sleep. Sleep Med 2008; 9: 603-14.

20. Zhou L, Lopes JE, Chong MM, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature 2008; 453: 236-40.