International Journal of Pediatric Otorhinolaryngology 80 (2016) 49–52

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: www.elsevier.com/locate/ijporl

Characterization of B-Cells in tonsils of patients diagnosed with pediatric autoimmune neuropsychiatric disorder associated streptococcus☆

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ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form 14 November 2015
Accepted 18 November 2015
Available online 24 November 2015

Keywords:
Otolaryngology
PANDAS
Pediatrics
Head and neck surgery
Tonsillectomy

ABSTRACT

Objective: To determine if Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcus (PANDAS) patients demonstrate a significantly different number of B-Cells or markers of activity when compared to recurrent Group A Streptococcus or Obstructive Sleep Apnea patients.
Study design: Prospective Cohort Study.
Study setting: Academic University Hospital.
Methods: Tonsil tissue was collected from twenty-two patients in the operating room and organized into three groups. Ten clinically diagnosed PANDAS, six Group A Streptococcus and six Obstructive Sleep Apnea patients were included in this study. Each tissue sample was extracted with MSD Tris Lysis Buffer and protein lysates were analyzed for CD 19, B-Cell Activating Factor and B-Cell Activating Receptor by western blot methods.
Results: Based on ANOVA analysis, there was no significant difference in the expression of B-Cell Activating Factor, B-Cell Activating Receptor or CD 19 when comparing the three study groups by western blot analysis methods.
Conclusions: In this prospective cohort study, it appears that PANDAS patients do not demonstrate increased number of B-Cells, expression of B-Cell Activating Factor or B-Cell Activating Receptor when compared to Group A Streptococcus or Obstructive Sleep Apnea cohorts. As a result, further evaluation of the cell-mediated immune system is warranted to provide further insight into the pathophysiology of PANDAS. In addition, we must investigate if PANDAS patients only demonstrate increased B-Cell number or activity when undergoing an acute Tic/OCD exacerbation.

1. Introduction

Currently, Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcus (PANDAS) remains a highly controversial diagnosis due to the paucity of basic science available to the medical community in the literature. More specifically, our current understanding of the condition's pathophysiology remains unknown, which further intensifies the disagreement about its existence as a viable diagnosis [1,2]. Many investigators believe that the exacerbation of OCD and Tic symptoms are due to autoantibodies attacking the neurons of the basal ganglia similar to Syndenham's Chorea in patients suffering from Group A Strep pharyngitis [3–5]. Previous basic science attempts failed to identify unique antibodies which may cause the aforementioned symptom exacerbation [6]. As a result, the medical community lacks agreed upon serum markers or diagnostic tests to further identify PANDAS in the clinical setting.

Some investigators have hypothesized that tonsillectomy, if properly performed by an otolaryngologist, may provide therapeutic benefit due to the possible association of Group A Streptococcus (GAS) pharyngitis and the exacerbation of OCD and Tic occurrences [7,8]. After evaluating the literature, mixed results remain at this time, but this may be due to the fact that

☆ This manuscript was accepted for oral presentation at the 2015 Society of Ear Nose and Throat Advancements in Children (SENTAC) in San Antonio, Texas.

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http://dx.doi.org/10.1016/j.ijporl.2015.11.020
0165-5876/

control groups were not properly implemented into these studies or that retrospective parental opinion was utilized to measure outcomes rather than psychiatric evaluation post-operatively [7,8]. As a result, much of the data regarding PANDAS remains retrospective, with very few prospective studies which aim to reduce bias. One method of improving our current understanding of PANDAS would be to further explore basic science methods such that the medical community may have better insight into the mechanism of this condition.

In this study, our group chose to evaluate the lymphatic tissue for immunological markers that could demonstrate the involvement of the humoral immune system as a means of PANDAS pathophysiology. B-Cell Activating Factor (BAFF) and B-Cell Activating Factor Receptor (BAFF-R) were utilized to help quantify the activity of B-Cells in the tonsil tissue of each study participant. Both BAFF and BAFF-R have been implicated in several autoimmune disorders and are well characterized as one of the most significant markers of B-Cell activity [11–13]. These two markers were chosen because in our most recent study, we evaluated several interleukins that could account for increased humoral activity such as IL1, IL2 and IL6 but, we identified no significant difference between affected and non-affected cohorts [14]. After review of the medical and basic science literature, we chose to evaluate BAFF and BAFF-R due to their significant involvement in autoimmune disorders [11–13]. Here, we utilized a separate biological assay to identify the above B-Cell markers and performed a more focused evaluation of the humoral immune system within affected patient's tonsil tissue.

If PANDAS is indeed an autoimmune disorder, patients would likely maintain either increased activity of their B-Cell population or significantly more B-Cells residing in the lymphatic tissue. In this manuscript, our group hypothesized that PANDAS patients may have significantly more B-Cells or markers of B-Cell activity, which could be identified in the lymphatic tissue. In order to evaluate this possibility, our group prospectively enrolled PANDAS, GAS and Obstructive Sleep Apnea (OSA) patients at our tertiary academic center to evaluate their tonsil tissue by western blot analysis. In order to characterize each patient's B-Cell number, we utilized CD 19, a B-Cell marker that is found on all stages of B-Cells, except the progenitor line [9,10].

2. Materials and methods

2.1. PANDAS patient enrollment and tissue collection

The Georgetown University School of Medicine Institutional Review Board approved the following study and informed consent was obtained from the parents of each individual patient. The PANDAS cohort consisted of ten pediatric patients referred to our clinic by a pediatric neurologist associated with our academic center. Each patient was no older than eighteen years of age. Inclusion criteria by our pediatric neurologist included clinically diagnosed PANDAS patients based on the DSM IV criteria. This criteria includes: (1) Presence of Obsessive Compulsive Disorder and/or tic disorder, (2) Onset before puberty, (3) Episodic presentation symptoms, (4) Positive Group A Streptococcus culture with neurologic motor abnormalities. Exclusion criteria included history of rheumatic fever, autism spectrum disorders, psychotic disorders or other autoimmune disorders. The average time to tonsillectomy from last acute tonsillitis event was 2.3 months.

Each patient underwent tonsillectomy by our senior author and tissue was frozen at −80 °C until protein extraction with our associate at the National Institute of Health. All tonsil tissues were stored in the same location and extracted together to reduce treatment bias.

2.2. Group A streptococcus control group selection

The GAS Control Group consisted of six patients that underwent tonsillectomy for recurrent tonsillitis due to documented GAS by throat cultures. Patients were referred from primary care providers or self-referred to our outpatient clinic setting. Each patient was no more than eighteen years of age at the time of tonsillectomy. The average number of acute tonsillitis events included 3.5 per year with ranges of 2 to 5. In addition, the average time to tonsillectomy from last acute tonsillitis was 1.6 months. Exclusion criteria included: history of rheumatic fever, PANDAS diagnoses, autism spectrum disorders, psychotic disorders or other autoimmune disorders.

2.3. Obstructive sleep apnea control group selection

The OSA Control Group consisted of six patients who underwent tonsillectomy for moderate to severe OSA as determined by polysomnogram studies. Patients again were enrolled from the outpatient clinic setting and were no more than eighteen years of age at the time of tonsillectomy. Apnea Hypopnea Index values ranged from 6 to 13 with an average of 9.1. Exclusion criteria included: history of rheumatic fever, previous GAS infections, PANDAS diagnoses, autism spectrum disorder, psychotic disorders or other autoimmune disorders.

2.4. Western blot analysis

Tonsil tissue from 6 GAS, 6 OSA and 10 PANDAS patients were lysed in Tris lysis buffer as previously described [14]. Protein from the 22 samples were separated by molecular weight using SDS-PAGE before being transferred to PVDF membranes using the semi-dry Trans-Blot Turbo transfer system (Biorad). Membranes were then blocked using Odyssey Blocking Buffer (Li-Cor) for 1 h at room temperature and then probed overnight at 4 °C with primary antibodies against proteins of interest. These include BAFF (Abcam ab8396 1:1000), BAFF Receptor (Abcam ab112506 1:1000) and CD19 (Abcam ab 134114 1:1000). β-actin (Sigma A1978 1:15000) was used as the housekeeper protein. Following 3 washes with TBST (Tris-Buffered Saline and Tween), blots were incubated for 1 h at room temperature using the appropriate secondary antibody (Li-Cor 926-68020 and 926-32211 1:15000). All primary and secondary antibodies were diluted in 1:1 mixture of Odyssey Blocking Buffer and TBS + 0.1% Tween-20. Blots were then washed another 3 times with TBST and then imaged on a Li-Cor Odyssey CLx Infrared Imaging System. Protein band of interest were Quantified using Li-Cor Image Studio software and normalized to the corresponding β-actin value.

2.5. Statistical analysis

A one-way analysis of variance (ANOVA) was used to compare the means in expression of each of the cytokines and B-cell number between the 3 aforementioned study cohorts. A P-value <0.05 was considered statistical significant when comparing between the three groups. The size of the cohorts in the study were determined a priori such that there would be at least 90% power to detect a minimum effect size of 1, based on a one-way ANOVA with a significance level of 0.05. SAS software Version 9.3 (SAS Institute Inc, NC) was used for the analysis.

3. Results

3.1. Evaluation of CD19 marker in tonsillectomy samples

After western blot analysis, we identified no significant difference in the normalized expression of CD 19 when comparing

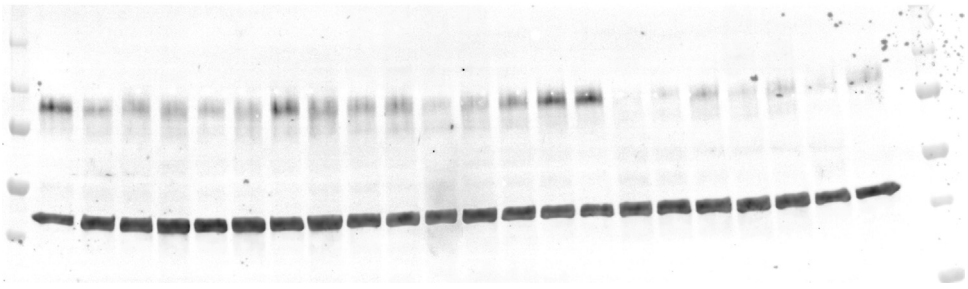

Fig. 1. Top band—CD 19 (95 kDa), bottom band—beta actin control (42 kDa). PANDAS lanes 2–11, GAS lanes 12–17, OSA lanes 18–23.

Table 1
ANOVA comparison of study groups regarding CD19.

Group	Mean normalized CD19	Standard dev	ANOVA *P*-value
PANDAS	0.0039	0.0010	$P = 0.964$
GAS control	0.0041	0.0020	
OSA control	0.0038	0.0030	

Table 2
ANOVA comparison of study groups regarding B-Cell Activating Factor.

Group	Mean normalized BAFF	Standard dev	ANOVA *P*-value
PANDAS	0.0056	0.0023	$P = 0.733$
GAS control	0.0051	0.0011	
OSA control	0.0060	0.0020	

the PANDAS Experimental Cohort patients with the GAS Control Cohort and the OSA Control Cohort via ANOVA calculations. ($P > 0.05$) (Fig. 1 and Table 1).

3.2. Evaluation of B-Cell activating factor in tonsillectomy samples

After western blot analysis, we identified no significant difference in the normalized expression of B-Cell Activating Factor when comparing the PANDAS Cohort with the GAS Control Cohort and the OSA Control Cohort via ANOVA calculations ($P > 0.05$) (Fig. 2 and Table 2).

3.3. Evaluation of B-Cell activating receptor in tonsillectomy samples

After western blot analysis, we identified no significant difference in the normalized expression of B-Cell Activating Factor Receptor when comparing the PANDAS Cohort with the GAS Control Cohort and the OSA Control Cohort by ANOVA calculations ($P > 0.05$) (Fig. 3 and Table 3).

4. Discussion

Within this study, our group provides the medical community with the first characterization of lymphatic B-Cells in patients diagnosed with PANDAS. Currently, multiple questions need to be answered by basic science if the medical community wishes to classify PANDAS as an autoimmune disorder. For instance, it still remains unknown if specific antibodies produced by B-Cells exist which may account for the acute exacerbation of OCD and Tic symptoms. One investigation failed to identify antibodies or antibody patterns in the serum of patients clinically diagnosed with PANDAS [6]. Despite the thoroughness of this study, our group wished to investigate the lymphatic tissue for possible differences in the number of B-Cells, BAFF and BAFF-R. It is important to evaluate the lymphatic tissue by itself because if differences were to be elucidated in the lymphatic tissue alone when compared to the serum, further pathway analysis involving BAFF and BAFF-R would be warranted to determine what specifically is occurring in the lymphatic tissue to produce this clinical presentation.

In order to investigate the aforementioned hypothesis, we wished to target three specific humoral markers, which would not only identify increased B-Cell number, but also B-Cell activity. CD19 is a B-Cell marker that is present on all stages of B-Cells during maturity including plasma cells, as mentioned previously. This CD marker provided us with the ability to quantify the number of B-Cells in each of the tonsil samples. Interestingly, our findings demonstrate that even after normalizing the CD19 marker against our housekeeping genes, there was no significant difference between the three study groups.

Furthermore, to quantify the activity of B-Cells, we utilized BAFF and BAFF-R as markers of B-Cell activity in the lymphatic tissue. In our previous study, we identified no differences in immune markers IL-1, IL-2, IL-6 in PANDAS patients when compared to unaffected patient cohorts [14]. These interleukin markers, as mentioned previously, have been shown to increase B-Cell activity and because of our previous study's negative findings, we focused on BAFF and BAFF-R, which have been well characterized in separate autoimmune disorders [15,16]. After comparing the three study cohorts for the aforementioned markers, our group identified no difference in the expression of B-Cell Activating Factor or B-Cell Activating Receptor. One additional avenue of investigation has been proposed by Hoekstra et al. where patients diagnosed with Tic Disorder overexpressed B-Cell markers D8/17 in the peripheral blood [17]. As a result it is possible that this specific marker could represent the specific pathway to demonstrate increased B-Cell activity in PANDAS patients. Despite our negative findings, several questions remain

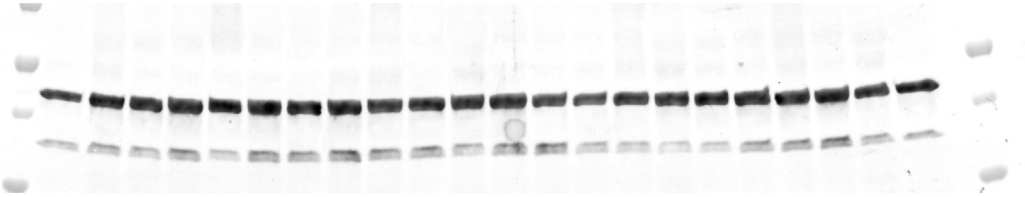

Fig. 2. Top band—beta actin control (42 kDa), bottom band—BAFF (34 kDa). (PANDAS lanes 2–11, GAS lanes 12–17, OSA lanes 18–23).

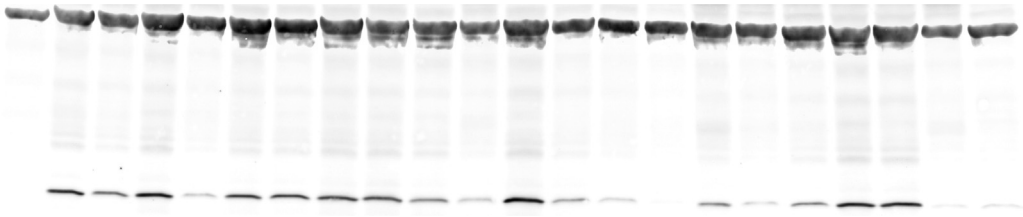

Fig. 3. Top band—beta actin control (42 kDa), bottom band—BAFF-R (19 kDa). (PANDAS lanes 2–11, GAS lanes 12–17, OSA lanes 18–23)

Table 3
ANOVA comparison of study groups regarding B-Cell Activating Factor Receptor.

Group	Mean normalized BAFF-R	Standard dev	ANOVA *P*-value
PANDAS	0.025	0.004	$P = 0.547$
GAS control	0.026	0.002	
OSA control	0.031	0.020	

regarding the activity of the cell-mediated arm of the immune system, which was not accounted for in this study. Further investigations are required to evaluate T-Cell function in patients diagnosed with PANDAS and determine if the OCD and Tic exacerbations could be a function of cell-mediated toxicity.

While this study has several benefits including being prospective and properly controlled, there are a few weakness that must be discussed. First, the humoral immune system is a very complex system, which is regulated by multiple cytokines and chemokines. As a result, this study focused on CD19, BAFF and BAFF-R because each has been well documented in autoimmune pathology and we felt this would provide a solid foundation for future studies. Furthermore, it would be prudent to wait until additional humoral markers have proven to be as significantly associated with autoimmune disorders before further evaluation. Secondly, while our study cohorts were properly powered a priori and laboratory findings repeated using different housekeeping genes, we would like to evaluate larger cohort groups in the future. Lastly, we were unable to determine if these patients were in clinical remission of their OCD/Tic flares and as a result, these B-Cells may be significantly different if tonsil tissue analysis was performed during an exacerbation period. Despite these shortcomings, this study is the first prospective study to evaluate the humoral arm of the immune system in patients with PANDAS.

5. Conclusion

Controversy remains regarding the diagnosis of PANDAS mostly due to the paucity of evidence in the medical literature elucidating a specific pathophysiology. This study provides the first insight into the activity of lymphocytes within the lymphatic system of patients diagnosed with PANDAS. At this time, group suggests that cell-mediated pathways should be investigated because no studies exist regarding this arm of the immune system. It would be interesting to determine if PANDAS patients maintain autoreactive CD4/CD8 T-Cells, which may be driving this overall clinical phenotype. Furthermore, as mentioned previously, one interesting study would evaluate the same markers utilized in this investigation when patients are in an acute Tic/OCD event and compare them once the patients are in remission. Additionally, one may also include the aforementioned D8/17 B-Cell markers for further characterization of B-Cell activity. Until that time, this manuscript remains the first properly controlled, basic science investigation of humoral activity in patients with PANDAS.

Financial disclosure

This study was funded by the PANDAS Network and the Alpha Omega Alpha Carolyn Kuckein Research Fellowship.

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