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Full Length Article

Airways therapy of obstructive sleep apnea dramatically improves aberrant levels of soluble cytokines involved in autoimmune disease

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ABSTRACT

Obstructive Sleep Apnea (OSA) damages the health of 35% of adult Americans. Disordered sleep results in increased risk of several autoimmune disorders, but the molecular links to autoimmunity are poorly understood. Herein, we identified four cytokines associated with autoimmune disease, whose median serum levels were significantly different for OSA patients receiving airways therapy, from the levels in untreated OSA patients, APRIL (5.2-fold lower, $p = 3.5 \times 10^{-11}$), CD30 (1.6-fold higher, $p = 7.7 \times 10^{-5}$), IFN-Alpha-2 (2.9-fold higher, $p = 9.6 \times 10^{-14}$) and IL-2 (1.9-fold higher, $p = 0.0003$). Cytokine levels in airways treated patients were similar to the levels in control subjects. t-SNE and UMAP analysis of these high dimensional patient cytokine data identified only two groups, suggesting a similar global response for all four cytokines to airways therapy. Our findings suggest the levels of these four cytokines may be altered by disordered sleep and perhaps by chronic hypoxia. Therapeutic options are discussed.

Protein abbreviations

APRIL, *TNFSF13*, Tumor Necrosis Factor Superfamily Member 13. BAFF, *TNFSF13B*, B-Cell Activating Factor. CD30, *TNFRSF8*, CD30L, Tumor Necrosis Factor Receptor Superfamily Member 8. CD163 *CD163*. Chitinase 3-like 1 *CHI3L1*. CXCR5 *CXCR5*. CXCL13, *CXCL13*. HIF1A, *HIF1A*, Hypoxia Inducible Factor 1 alpha. IL-2, *IL2*, IL2, T Cell Growth Factor Interleukin 2. IFN-Alpha-2, *IFNA2*, Interferon Alpha 2. Endothelin 1, *EDN1,* Preproendothelin-1. IFN-Gamma, *IFNG*. IL-6, *IL6,* Interleukin 6, B-Cell Stimulatory Factor. IL-17A, *IL17A*: Interleukin 17, Cytotoxic T-Lymphocyte-Associated Antigen 8. IL-23 *IL23A*. NF-kappaB, *NFKB1,* Nuclear Factor Kappa B Subunit 1. Nrf2, *NFE2L2*, Nuclear Factor, Erythroid 2 Like 2. Pentraxin-3, *PTX3*. TNF-Alpha, *TNF*, Tumor necrosis factor. VEGFA, *VEGFA*: Vascular Endothelial Growth Factor A.

1. Introduction

Obstructive Sleep Apnea (OSA) is the most common cause of sleep apnea and accounts for 75% of all cases of disordered sleep. OSA patients may display abnormally long pauses in breathing or abnormally low levels of breathing during sleep, and often have fragmented sleep, snoring, excessive daytime sleepiness, fatigue, high blood pressure, irritability, depression, loss of concentration, poor neurocognition, and reduced work performance [1–5]. Because sleep disorders and lack of sleep affect 35% of adult and 68% of adolescent Americans, the CDC has declared sleep deprivation as an epidemic [6]. Interruptions of breathing, while asleep, result in chronic intermittent low oxygen levels (chronic intermittent hypoxia) and tissue inflammation. Hence, OSA is most commonly treated with Continuous Positive Airway Pressure (CPAP) administered at night while sleeping. Dental airways devices produce a similar treatment effect, and thus, have gained some recent acceptance as an effective alternative to CPAP [7,8].

Hypoxia induced systemic inflammation is often considered the major cause of increased risk for the various apnea-related health problems. These problems develop over time and include cardiovascular disease, metabolic syndrome associated insulin deficiency and diabetes, tissue inflammation, hypertension, obesity, depression, cognitive decline, and stroke, all of which increase mortality [9–14]. More recently apnea has been linked to increased risk for a number of

Abbreviation: OSA, obstructive sleep apnea; CPAP, continuous positive airways pressure; t-SNE, t-distributed stochastic neighbor embedding; SLE, systemic lupus erythematosus; ESS, Epworth sleepiness score; AHI, (apnoea–hypopnoea index); SaO_2% low, average low percent oxygen saturation during each cycle of breathing

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autoimmune diseases affecting a variety of tissues and organs including autoimmune encephalitis [15], systemic lupus erythematosus (SLE) [16,17], rheumatoid arthritis [17–19], ankylosing spondylitis [17], Sjogren's syndrome [17], and systemic sclerosis [17], autoimmune hypopituitarism [20], atopic dermatitis [21], and psoriasis [22–24].

Even though OSA has been linked to the risk of autoimmune disease [16,25,26], the evidence that chronic intermittent hypoxia-induced chronic inflammation might be the mechanistic cause is only emerging recently [20,27,28]. Hypoxia leads to necessary changes in energy metabolism in myeloid cells [29] with the potential to influence autoantibody production [30]. It is well known that in cultured cells hypoxia induces a number of stress signaling cascades mediated by factors including HIF-1, NF-kappa B, and Nrf2, endothelin 1 and VEGF. By one current model, oxidative stress signaling induces higher levels of a number of inflammatory cytokines to initiate an inflammatory cascade, which in turn increases the risk of autoimmune disorders [27]. Inflammatory cytokine cascades [27] and over stimulation of dendritic cells by autoantigens may stimulate auto-reactive B cells to increase the production of autoantibodies [31]. Altered levels of TNF-Alpha [32–34], IL-17 and [35–37] and IL-6 [38–40] are all associated with autoimmune disease and appear to play roles in initiating hypoxia-induced inflammatory cascades. Relative to control subjects OSA patients are reported to have 1.2- to 2.5-fold higher levels of, TNF-Alpha [41–50], IL-17 [51,52] and IL-6 [45,53–55]. Most, but not all, of these studies show airways therapy results in more normal levels of TNF-Alpha, IL-17, and IL-6, more similar to the levels in control individuals without apnea. Based on a model in which these cytokines initiate inflammatory signaling, TNF-Alpha and IL-6 are targets for immunotherapeutic suppression of inflammatory autoimmune diseases [33,34,38–40].

Our goal was to discover other novel autoimmune-associated cytokines that responded to airways treatment in OSA patients and might play roles in autoimmune diseases. Their identification would increase our understanding the link between OSA and autoimmunity and these cytokines might be additional targets for therapeutic treatment of OSA. We compared the levels of several inflammatory cytokines previously linked to autoimmunity in serum among well matched patients with OSA not yet receiving airway therapy to OSA patients receiving airways therapy, and also to healthy control individuals. We found significant changes in the levels of APRIL (*TFNSF13*), CD30 (*TNFRSF8*), IFN-Alpha-2 (*IFNA2*), and IL-2 (*IL2*) in OSA patients receiving airway therapy, such that cytokines were more similar to the levels observed in healthy control subjects.

2. Materials and methods

2.1. Patient data

Nineteen OSA patients had formerly been diagnosed using polysomnography (PSG) based on their Apnea Hypopnea Index (AHI > 5), but were currently receiving nightly airways therapy and were designated airways treated OSA patients. Eighteen of these recorded using CPAP, while patient #31 reported using a dental airways device [8]. There were 19 OSA patients currently with apnea, but not receiving airways therapy. Also 8 Control individuals were recruited, but among these, patient #9 was borderline for high blood pressure (PB 145/89). Although autoimmune disorders can develop in younger or older individuals, none of our patients reported having an autoimmune disease. Neither patient #31 nor patient #9 produced outlying data. Patients were evaluated in this study after obtaining written informed consent. Gender, BMI, CVD, age, ESS [56,57], AHI, SaO_2% low, glucose levels, Cholesterol, LDL, HDL, and CRP, were assessed at the time of first recruitment (Table 1), which in the case of the airways treated patients was after months of treatment. A Yes/No indication was recorded for chronic medications. Compliance with nightly airways therapy was confirmed by patients' response to a simple yes/no question. The airways therapy treated OSA patients and untreated OSA patients were well matched for nearly all parameters (*P* values Table 1). The control group was considerably younger and leaner, and potentially represented more nearly optimal biometric data and cytokine levels. Patients were recruited, consented, and blood drawn at the University of Georgia's Clinical and Translational Research Unit (CTRU) in Athens, GA. The detailed data collected on individual subjects are given in Supplementary Data File SD1.

2.2. Cytokine levels

The levels of inflammatory cytokines were examined using Bio-Plex Pro™ Human Inflammation Panel 1 multiplex kits that quantify biomarkers of human inflammation (BioRad #171AL001M). Multiple 96 well plates were assayed using Bio-Rad Bio-Plex instrument at UGA's Cytometry Shared Resource Laboratory. The Bio-Plex system has the advantage that hundreds of individual beads each estimate each cytokine level in each well, which improves the statistical accuracy of each individual well estimate of all cytokines assayed. All the flash frozen serum samples were thawed only once. Serum, standards and assay controls were diluted as per the manufacturer's instructions (Bio-Rad Bulletin 10,044,281) [58]. As recommended, each serum sample was diluted 4-fold. Fifty microliters of this dilution were run in triplicate for the 8 control, 19 untreated OSA patients, and 19 airways therapy treated OSA patients (Supplemental Data File D1), instead of running duplicate patient serum samples recommended by the manufacturer. This allowed a more robust assessment of potential experimental errors in each cytokine assayed. The picogram output data for each serum cytokine level was normalized to the concentration of standards, run as an eight-step, four-fold dilution series of each cytokine, and run in duplicate on each assay plate. The quantitative nature of these assays over the expected concentration ranges estimated for serum samples was confirmed by comparing the fluorescence output of the quadruplicate standard samples (two from each plate). The standard error of the lowest concentration standards used to estimate concentration was less than 15% and less than that for higher concentrations (Supplemental Data File SD1).

2.3. Power analysis

We determined effect sizes estimates in the context of hypothesis testing for our data on each of these four cytokines using the three methods [60] the absolute value of r, Cliff's delta, and Vargha and Delaney's A. The standard values that give small, medium, large effect sizes using each method are summarized in Table A of Supplementary Data File SD2. By all three metrics, comparisons of each of the four cytokine levels between airway treated OSA patients and untreated OSA patients or between OSA patients and controls produced medium to large effect sizes as shown in Table B of Supplementary Data File SD2.

The null hypothesis being tested was that OSA patients and airways treated OSA patients express the same levels of soluble cytokines involved in autoimmunity. The alternate hypothesis being tested was that OSA patients express altered levels of soluble cytokines relative to the airways treated OSA patients [61]. These hypotheses were tested using Bio-Plex system, which provides a wider dynamic range when measuring cytokine levels, greater sensitivity, and more statistical significance for each assay than conventional cytokine immunoassays used in most previous studies. Hence, the medium to large effect sizes estimates for the differences in the levels of the four cytokines (Supplementary Data File SD2) and study costs were both taken into account [62,63], in estimating that the patient sample sizes were sufficient to power a statistically significant preliminary study that would avoid both Type I (false positive) and Type II (false negative) errors [63] as recommended by the Federal Food and Drug Administration's study guidelines for estimating minimum appropriate patient sample size [64]. Finally, an empirical power calculation [65–67] was applied to

Table 1
Summary of patient biometric, sleep, and laboratory data.

	Control subjects (n = 8)	Airways treated patients (n = 19)	Apneic patients (n = 19)
Female/Male	6/2	7/12	7/12
Age	37.7 ± 11.5	60.6 ± 10.5	58.2 ± 12.4
Hypertension or heart disease Y/N	2 Yes/6 No	11 Yes/8 No	11 Yes/8 No
Race C/H/B(A/M)/A	4C/0H/3B/1A	17C/0H/2B/0A	11C/1H/6B/1A
BMI	26.8 ± 5.96	33.1 ± 9.07	35.0 ± 9.22
AHI at time of diagnosis	1.58 ± 1.64	35.7 ± 24.8	26.8 ± 25.7
SaO_2 low %	91.5% ± 2.8%	80.7% ± 5.4%	76.4% ± 10.3%
ESS	5.33 ± 3.67	7.16 ± 5.80	7.94 ± 4.02
Glucose mg/dL	94.8 ± 8.70	106 ± 18.7	104 ± 12.1
Cholesterol mg/dL	165 ± 26.8	181 ± 25.1	179 ± 43.1
HDL mg/dL	52.1 ± 14.6	51.8 ± 18.1	45.7 ± 17.3
LDL mg/dL	96.4 ± 22.6	104 ± 27.6	106 ± 35.8
hs-CRP mg/L	1.07 ± 0.689	4.84 ± 7.65	3.48 ± 5.60
Chronic Meds Y/N	2 Yes/6 No	16 Yes /3 No	16 Yes/3 No
Airways therapy adherence	0 Yes/8 No	19 Yes/0 No	0 Yes/19 No

the Wilcoxon rank sum test of the cytokine data and demonstrated there was sufficient power with the number of test subjects for all comparisons of all four cytokine levels between airways treated OSA patients and untreated OSA patients as shown in Table C of Supplementary Data File SD2.

2.4. Management of data sets and statistical analysis

The data from separate plates were combined to make multiple excel data files, one for each cytokine (Supplemental Data File SD1). The levels of IFN-Alpha-2 among some untreated OSA patients and five airways treated patients were either at or were below the range of detection, with the latter being designated as out of range, OOR < , by the instrument software. The lowest picogram patient serum sample concentration of IFN-Alpha-2 that was estimated to be in the range of detection was substituted for OOR < cytokine values. In this way, any estimate of fold difference for IFN-Alpha-2 between OSA patients and airways therapy treated OSA patients would not over-estimate the actual fold differences (Supplemental Data File SD1). At this point, the data were moved into R v3.5.1 for further statistical analysis. The data for patient groups were visualized using Boxplot. After applying the Kolmogorov–Smirnov test in R [68–72], it was clear that the airways treated patient data for cytokine levels were not normally distributed ($p < 0.05$) and often fell into two groups of values. The Kolmogorov–Smirnov test is a nonparametric goodness-of-fit test and could be used to determine whether an underlying probability distribution differs from the hypothesized normal distribution. Therefore, without the normality assumption, the nonparametric Wilcoxon rank-sum test was used to estimate p values for the significance of pairwise differences in cytokine levels among OSA patients, airways treated OSA patients, and controls. The two-sample Wilcoxon rank sum test is a rank-based test that compares values for two groups. Without any distribution assumption, the test addresses if it is likely that an observation in one group is greater than an observation in the other, with significance level (alpha) = 0.05 = 5% in our case [59].

To visualize the high-dimensional data for the levels of all four cytokines among all patients and controls in a two-dimensional map, the nonparametric t-distributed Stochastic Neighbor Embedding (t-SNE) visualization method [73] was applied using the Rtsne version 0.15 statistical R package available online [74]. T-SNE reduces dimensionality by first using a Gaussian distance to analyze the similarity among data points in high-dimensional space and then projecting these data into two dimensional space [75]. We also employed Uniform Manifold Approximation and Projection (UMAP) as an alternative method to visualize these high-dimensional data in two-dimensions [75,76]. UMAP analysis is quite distinct from t-SNE in that it first estimates a topology for the high-dimensional data and then uses the topology information to construct two dimensional space [75]. It has been argued that UMAP may be superior to and/or equivalent to t-SNE at recovering the global structure among high dimensional data [76,77].

2.5. Theory/calculation

The bulk of literature supporting current models in which oxidative stress signaling initiates an inflammatory cascade of cytokines to increase the risk of autoimmune disorders [27] are based on data from acute hypoxic treatment of cell culture models, or for example, acute ischemia in rodent models. However, OSA patients experience long term chronic long-term hypoxia over months or years in which the acute response may be attenuated. Similarly, airways therapy of OSA patients is administered over long if not indefinite time periods, for which there are no analogous cell culture or rodent models. The work herein responds to the need for more quantitative and statistically significant data on changes in autoimmune related cytokine levels after airways therapy.

3. Results

3.1. Patients

Cytokine levels were examined in the serum of nineteen OSA patients receiving airways therapy (airways treated patients, Table 1) and compared to nineteen OSA patients not receiving airways therapy and a group of volunteers without OSA (control individuals). Table 1 summarizes important biometric, sleep and laboratory data for the three groups of subjects with details presented in Supplemental Data Files SD1.

3.2. Assaying changes in serum cytokine levels

We found the levels of four cytokines with previously reported rolls in autoimmunity were significantly different in airways treated OSA patients relative to untreated OSA patients, including APRIL (TFNSF13), CD30 (TNFRSF8), IL-2 (*IL2*) and IFN-Alpha-2 (*IFNA2*) with the detailed data presented as four box plots in Fig. 1 and the statistical data summarized in Table 2. Increased serum levels of APRIL (Tumor Necrosis Factor Superfamily Member 13) and increased APRIL signaling have been linked to several autoimmune disorders including rheumatoid arthritis [78], eczema [79], multiple sclerosis [80], and systemic lupus erythematosus [81]. We found the median pg/mL serum level of the soluble isoform of APRIL was 5.2-fold lower in airway treated OSA patients relatively to the median level in untreated OSA patients ($p = 3.5 \times 10^{-11}$, Fig. 1A). The level in airways treated patients was

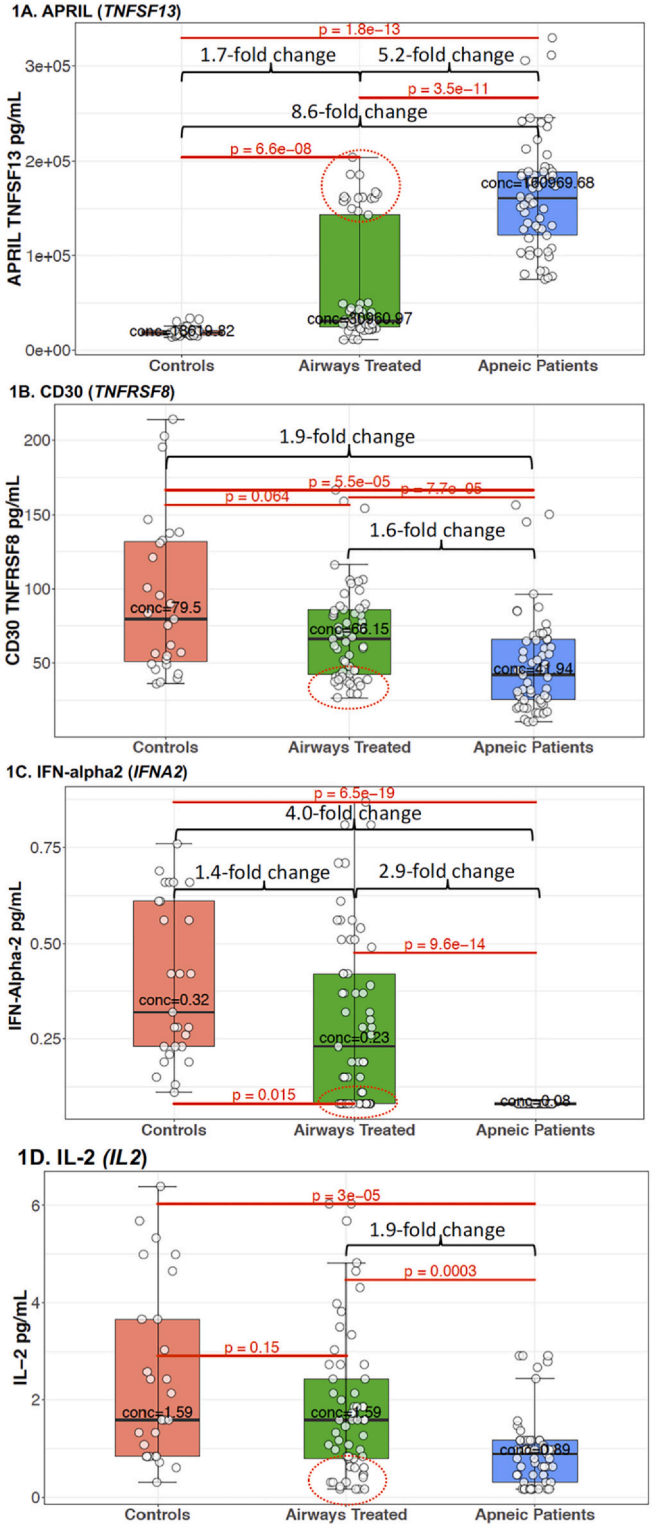

Fig. 1. The levels of four cytokines involved in the autoimmune disease are significantly altered in OSA patients receiving airways therapy. The serum picogram per milliliter (pg/mL) levels of (A) APRIL, (B) CD30, (C) IFN-Alpha-2 and (D) IL-2 from control individuals, airways treated OSA patients and OSA patients not receiving airways therapy are summarized in box blots. The top box encloses the third quartile and is bounded by median pg/mL value, the lower box encloses first quartile and is bounded by median value. The whiskers indicate the median values −/+ 1.5 IQR (interquartile range), and hence exclude outliers. The median value is indicated by a black line. Each of the three independent estimates of a cytokine level for each patient are represented by separate data points. Outliers among the airways treated patients that resemble untreated OSA patient data are encircled with a red dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

still 1.7-fold higher than the levels in control subjects ($p = 6.6 \times 10^{-8}$). Hence, patients receiving airways therapy had levels of APRIL that were much closer to those in controls, but still higher than the levels observed in controls.

CD30 (CD30L) is member of the Tumor Necrosis Factor Receptor Superfamily expressed in activated T and B cells. Its soluble isoform is generally found to be upregulated in leukocytes in patients with chronic inflammatory and autoimmune diseases including lupus erythematosus, asthma, rheumatoid arthritis and atopic dermatitis and CD4+ T cell-mediated graft-versus-host disease [82,83]. However, we found the median pg/mL level of CD30 was 1.6-fold higher in the serum of airways treated OSA patients relative to untreated OSA patients ($p = 7.7 \times 10^{-5}$, Fig. 1B, Table 2). Whereas, the median level in airways treated patients was slightly lower than that in controls, this difference was not statistically significant ($p = 0.064$).

Interferon Alpha 2 (IFN-Alpha2) expression is elevated in a number of autoimmune diseases such as arthritis, systemic lupus erythematous and Sjogren's syndrome with the proposed effect of reducing both inflammation and the autoimmune response [84,85]. The median pg/mL level of the soluble isoform of INF-alpha2 was 2.9 -fold higher in the serum of airways treated OSA patients relative to untreated OSA patients ($p = 9.6 \times 10^{-11}$, Fig. 1C, Table 2). Median IFN-Alpha-2 levels in airways treated OSA patients were more similar to the levels in control subjects, but were statistically distinguishable ($p = 0.015$).

Defects in T Cell Growth Factor Interleukin IL-2 (IL2) or in IL-2 signaling produce multiorgan autoimmunity and are linked to systemic lupus erythematosus [86], asthma [87], and multiple sclerosis [88,89]. We found the median pg/mL serum level of IL-2 was 1.9-fold higher for airways treated OSA patients relatively to untreated OSA patients ($p = 0.0003$, Fig. 1D, Table 2). The median level in airways treated OSA patients was not statistically distinguishable from the median level in controls subjects ($p = 0.15$).

In short, it appears that OSA patients had aberrantly low levels of all four autoimmune-related cytokines, and OSA patients receiving airways therapy had cytokine levels more similar to those observed in control subjects. However, the cytokine levels for airways treated OSA patients did not appear normally distributed and the data for five patients appeared as outliers with levels that were more similar to those of untreated patients (red encircled data, Fig. 1). In order to visualize the potentially coordinated response of all four cytokine levels independent of the direction of that response for most of airways treated OSA patients relative to OSA patients and controls and the potential common relationship among the outliers, we applied two machine-learning 2D visualization strategies (Fig. 2). The first, t-SNE is a non-linear dimensionality reduction method, that has the capacity to capture local structures among these high-dimensional data, while also revealing the presence of groups of related data as global, and to present these data in two-dimensional space [73]. As shown in the t-SNE analysis in Fig. 2A all the patient cytokine data lie in two clusters, with Group 1 representing the cytokine data among all the untreated OSA patients and five of the nineteen airways treated OSA patients. Group 2 represents the cytokine data for all the controls and fourteen of the nineteen airways treated OSA patients. The robustness of the t-SNE result was tested by applying UMAP, an alternative method for recovering global structure among high dimensional data [76,77]. Applying UMAP to these patient data (Fig. 2B) we again found two groups. Group membership is the same for Group 1 and Group 2 data using either t-SNE or UMAP.

A combined examination of Fig. 1 (data encircled by red dotted lines) and Fig. 2 suggests that five patients (i.e., patient numbers 12, 26,

Table 2
Median cytokine levels cytokine levels among airways treated apneic patients, apneic patients, and control individuals. The median pg/mL levels of four cytokines among the three patient and control subject groups are presented, along with the fold difference between the median level for Airways Treated patients and Untreated Apneic patients, and the *p* values for all three pair wise comparisons of median cytokine levels.

Cytokine	Gene Name	Control Individuals median pg/mL cytokine	Airways treated OSAS median pg/mL cytokine	OSAS patients median pg/mL cytokine	Fold difference cytokine levels Airway treated vs untreated OSAS	Control vs OSAS p value	Control vs Airways Treated p value	Airways treated vs OSAS p value
APRIL	*TNFRSF13*	18,619.82	30,960.97	160,970	−5.2	1.77×10^{-13}	6.59×10^{-08}	3.47×10^{-11}
CD30	*TNFRSF8*	79.50	66.15	41.9	+1.58	5.52×10^{-05}	0.064	7.72×10^{-05}
IFN-Alpha-2	*IFNA2*	0.32	0.23	0.080	+2.88	6.46×10^{-19}	0.015	9.65×10^{-14}
IL-2	*IL2*	1.59	1.59	0.89	+1.79	2.99×10^{-05}	0.153	3.0×10^{-4}

34, 47, and 70, Supplemental Data File SD1) clearly distinguished themselves as outliers not responding to airways therapy. Patients 12, 26, 34, 47, and 70 self-reported their compliance with nightly airways therapy (Table 1) and did not distinguish themselves based on gender, containing 2 males and 3 females, based on BMI with values ranging from 24 to 39, or based on age with ages ranging from 42 to 66, nor by their taking of chronic medications (Supplemental Data Files SD1). Although it also may seem surprising, we found no statistically significant difference in average ESS between airways treated and untreated OSA patients as observed in other recent studies [90].

We looked to the remainder of our biostatistical, laboratory, and sleep data for other causes that might account for the variance in cytokine levels among all patients and controls, and in particular, for the variance among these five airways treated outliers. Biometric and sleep data were collected at the time of serum collection for controls and for the untreated OSA patients and months earlier at the time of their recruitment and diagnosis with OSA for the airways treated OSA patients. We performed a linear regression analysis plotting the pg/mL level of each cytokine against heart rate, and ESS, AHI, age, BMI, and oxygen as SaO_2 low % and the laboratory data from duplicate serum samples taken at the same time as those assayed for cytokine levels (e.g., glucose levels, total cholesterol, HDL, LDL, CRP) (Supplemental Data File SD1). None of the biometric, laboratory, or sleep variables accounted for more than 35% of the variance in any of the cytokine levels within any patient group or all patients taken together. One simple explanation for the outlying data for five patients would be that the lack of adherence to airways treatment was misreported as adherence.

4. Discussion

Sleep apnea is highly associated with increased risk of various autoimmune diseases. Three cytokines, TNF-Alpha, IL-17, and IL-6, that are positively associated with autoimmune disorders are often elevated in OSA patients and decreased in response to airways therapy. Herein, and fitting this pattern, we find the levels of APRIL were relatively high in apneic patients, but were significantly reduced by airways therapy. Airways therapy did not reduce APRIL all the way to the very low levels observed in controls. By contrast, the levels of CD30, IFN-Alpha-2, and IL-2 were relatively low in OSA patients and were significantly increased by airway therapy, increased to levels similar or statistically indistinguishable from controls. Using t-SNE to analyze our high-dimensional data for the levels of all four cytokines among all subjects, we found that most airways treated patients clustered with the control individuals, while data from the five outlying airways treated patients clustered with the apneic patients (Table 2). The clustering of these high-dimensional patient data for all four autoimmune-associated cytokines suggests their miss-expression in OSA patients may be linked to increased risk of autoimmune disease.

APRIL (TNFSF13) is a proliferation-inducing ligand expressed as both membrane and soluble isoforms in myeloid cells [91]. Both protein isoforms influence the production of granulocytes, monocytes, erythrocytes, or platelets in bone marrow. APRIL acts on antibody-producing plasma cells and may therefore contribute to the production of pathogenic autoantibodies. APRIL often forms heterodimers with its close homolog BAFF (TNFSF13B) and together they bind various receptors to regulate early T cell and B cell development and proliferation [92,93]. APRIL participates in pro- and anti-inflammatory processes [91] and as mentioned, its expression is thought to promote autoimmune disorders. Hence, antibodies antagonizing APRIL signaling have been proposed as therapeutics to treat autoimmune disease [91]. While we did not find publications linking APRIL levels to sleep apnea or airways therapy, its expression appears to be controlled in part by hypoxia. In breast cancer cells 24 h of hypoxic treatment induces the production of a mature processed transcript that retains TNFSF13 intron 1. Intron 1 contains stop codons in all three reading frames, resulting in early termination of translation, and reduced APRIL protein

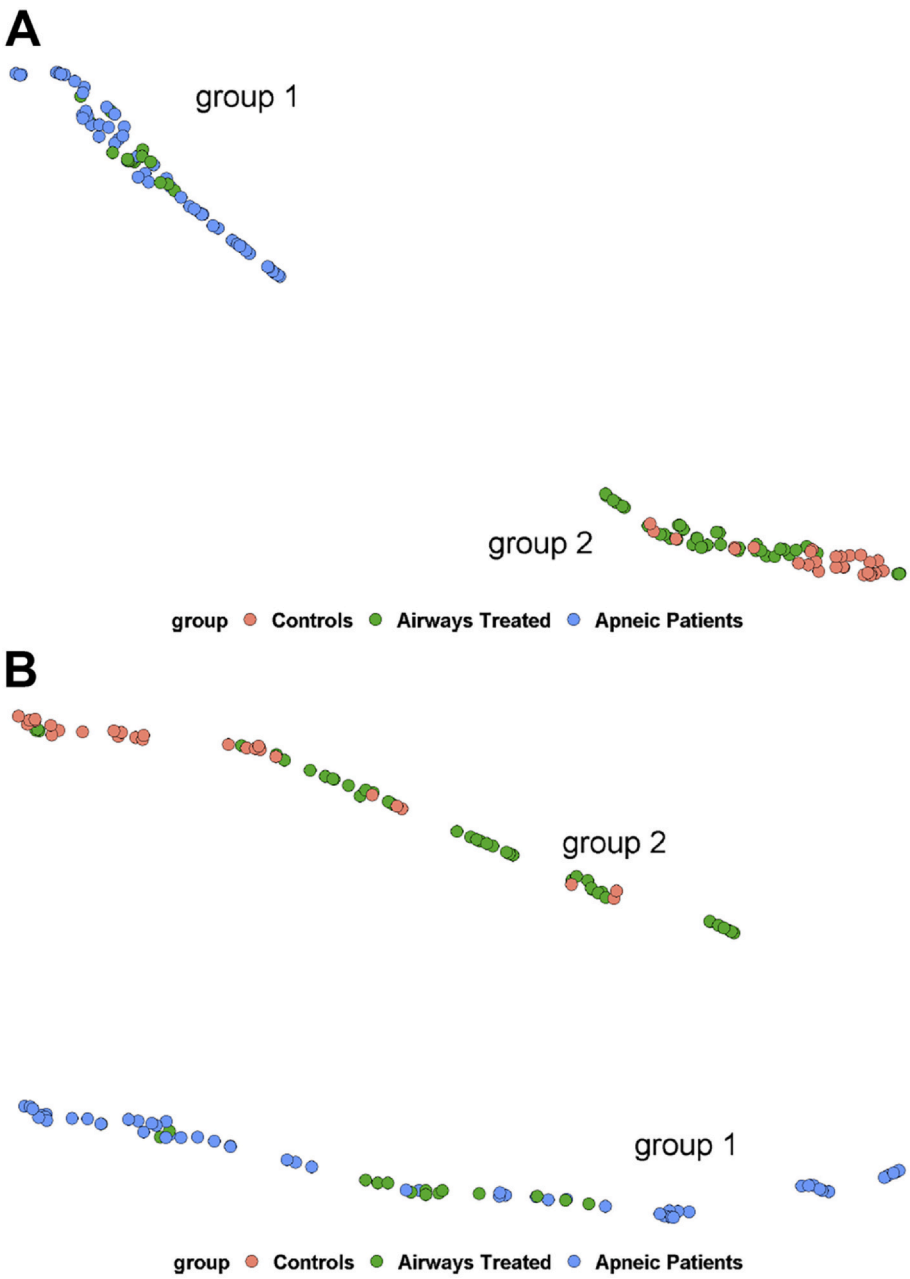

Fig. 2. t-SNE and UMAP placed the high dimensional patient cytokine data into the same two groups in two dimensions. The high dimensional data for the changes in the levels of APRIL, CD30, IFN-Alpha-2 and IL-2 were reduced to a two dimensional visualization by t-SNE (A) and UMAP (B). A. t-SNE. Group 1 represents the dimensional distribution of the levels of four cytokines among all the untreated OSA patient and a five of the nineteen patients airways treated OSA patients. Group 2 represents the cytokine data for all the control individuals and fourteen of the nineteen airways treated OSA patients. B. UMPA. Group 1 and 2 in have the same affiliated patients as observed with t-SNE. Each patient is represented by 3 data points that combine the dimensional distribution of the levels of the three measurements made for each of four cytokines for that patient.

expression [94]. Yet, we found what appears to be aberrantly high levels of soluble APRIL protein in OSA patients. In airway treated OSA patients, soluble APRIL levels in serum were several-fold lower, but not quite as low as levels in control individuals. The extremely high levels of soluble serum APRIL in untreated OSA patients may dampen APRIL receptor membrane signaling, resulting in damage to early stages of T and B cell development, and hence, contribute to the increased risk of apnea-related autoimmune disorders. Chemical inhibition of APRIL has some efficacy in the treatment of systemic lupus erythematosus [95], suggesting suppression of APRIL might aid patients with OSA-linked autoimmune diseases.

CD30 is a receptor expressed as both membrane and soluble isoforms by activated, but not resting B and T cells [82,83]. CD30 signaling can lead to cell proliferation and/or cell death. As mentioned, increased levels of CD30 are associated with a number of autoimmune disorders. Direct and indirect evidence suggest signal transduction through the CD30 receptor plays diverse roles in regulating B and T cell development, apoptosis, autoimmunity and in reducing inflammation [82,96–99]. CD30's role in T cell mediated responses and inflammation likely account for increased CD30 levels in increasing the risk of autoimmune disorder. We did not find publications linking OSA to altered levels of CD30. However, the serum levels of CD30 are increased 1.4-fold in patients with Chronic Obstructive Pulmonary Disease relative to control subjects suggesting hypoxia related stress upregulates CD30 expression [100]. In contrast to these data, we found low levels of CD30 in the serum of untreated OSA patients. The levels in airways treated OSA patients were very similar to those in controls. We are considering a model in which the long term chronic hypoxia experienced by OSA patients attenuates the acute response of increasing CD30. Any imbalance in expression of soluble CD30 in serum, either an increase or decrease, would alter the amount free to bind the CD30 membrane receptor, and hence, alter signaling. Altered CD30 receptor signaling caused by reduced soluble CD30 expression might then contribute to increased autoimmune disease risk in OSA patients. Restoring appropriate balanced CD30 levels by therapeutic CD30 supplementation might be considered as a treatment for OSA-induced autoimmune

diseases in patients with low cytokine levels in addition to CPAP. Conversely, immunosuppression of elevated levels of CD30 has shown efficacy in the treatment of a mouse model of autoimmune encephalomyelitis [99] and has been suggested as a treatment for MS [101].

IFN-Alpha-2 is considered the prototypical member of the IFN-alpha family of cytokines involved in the innate immune response, acting primarily on T cells [102]. It is produced as a soluble cytokine in macrophages and at low levels by most cell types in response to viral infection, having an antiviral effect. However, balanced expression of IFN-Alpha-2 appears essential to a normally functioning immune system [84,85]. Modest overexpression of IFN-Alpha-2 has been observed in the serum of patients with autoimmune diseases. IFN-Alpha-2 delivered exogenously to human endothelial cells induces the expression of hypoxia inducing factor HIF-1α within 2 to 4 h of IFN-Alpha-2 transgene stimulation even under normoxic conditions. Exogenous IFN-Alpha-2 expression induces HIF-1alpha to almost the same levels as treating cells with hypoxia and has an antiproliferative effect on endothelial cell growth. Because of this positive association with the acute response and autoimmunity, we anticipated increased IFN-Alpha-2 levels in OSA patients. Contrary to expectation, we found dramatically low levels of IFN-Alpha-2 in OSA patients. Whereas, the airways treated OSA patients had 3-fold higher levels of IFN-Alpha-2, levels more similar to the levels in control individuals. Considering that airways therapy had such a dramatic effect, it is likely that chronic intermittent hypoxia was the cause of the reduced levels in OSA patients. Chronic hypoxia experienced over months and years may attenuates the short term acute response as has been modeled previously for the relationship between OSA and autoimmune disorders [20,27,28]. The extremely low levels of IFN-Alpha-2 in untreated OSA patients may cause an imbalance in their immune responses and may account for the increased incidence of autoimmune diseases among OSA patients. The extremely low levels of IFN-Alpha-2 observed in OSA patients contrasted by the nearly normal levels in airways treated patients suggests the proinflammatory response produced by acute hypoxia has been attenuated by chronic intermittent hypoxia experienced by these patients. If this is the case, it suggests a therapy in which carefully balanced supplementation of exogenous IFN-Alpha-2 might be used to treat OSA-associated autoimmune disorders in addition to CPAP. Reports on the therapeutic use of IFN-Alpha-2 are limited. Treating mice with exogenous IFN-Alpha-2 show dramatically increased rates of leukocyte migration with a proinflammatory effect. [103]. By contrast, IFN-Alpha-2 neutralizing antibodies appear useful in reducing inflammation in some, but not all SLE patients [104].

IL-2 is a secreted soluble cytokine with diverse pro- and anti-inflammatory roles in controlling an appropriate immune response and it is an essential factor in immune suppression and self-tolerance [105,106]. IL-2 is capable of suppressing undesirable immune responses, for example, by increasing the activity of regulatory T cells, but also by increasing the immune response by stimulating effector T cells. Defects in IL-2 increase the risk of an autoimmune response [107]. Hypoxia significantly induces expression of IL-2 in microglial cells in the brain [108]. Previous studies on adults and children found no significant differences in IL-2 levels between OSA patients and controls [90,109,110]. We found the median level of soluble IL-2 was significantly lower in OSA patients relative to controls. IL-2 levels were significantly higher in airways treated OSA patients than untreated OSA patients, so as to be indistinguishable from levels in control subjects. The extremely low levels of IL-2 untreated OSA patients may be so low that IL-2 is unable to perform its anti-inflammatory roles, and hence, contribute to increased risk of autoimmune disease, similar to genetic defects in IL-2. Therefore, therapeutic balanced supplementation with low doses of IL-2 might be an appropriate treatment for OSA patients with associated autoimmune diseases in addition to CPAP. Autoimmune disorders such as SLE have been treated with some success with low doses of IL-2 [86,111–113].

We also examined the pg/mL levels of other cytokine with implicated roles in autoimmune disease, but that we found were not altered in our OSA patients relative to controls and did not respond to airways therapy including BAFF, CD163, Chitinase 3-like 1, IFN-Gamma, Pentraxin-3. There are numerous other cytokines that have been associated with autoimmune disease, which were not considered in this study such as TNF-Alpha, IL-17 and IL-23 [84,114]. A more comprehensive simultaneous analysis of the dozens of cytokines contributing to autoimmune-related inflammatory cascades is needed to properly link OSA and the potential benefits of airways therapy to autoimmune disorders.

5. Conclusions

The low levels of CD30, IL-2 and IFN-Alpha-2 we observed in OSA patients contrasted with expectations of increase in their expression based on previous direct or indirect evidence linking their elevated expression with acute hypoxia. APRIL levels were higher in OSA patients than in airways treated OSA patients, but the link between APRIL expression and hypoxia experienced by OSA has not been suggested in previous literature. Perhaps the chronic intermittent hypoxia experienced for months and years by OSA patients leads to an attenuation of the acute response and chronically altered levels of all four cytokines. This distinction, that cytokine levels respond differently to chronic intermittent hypoxia experienced by OSA subject than to acute hypoxia in experimental systems was described previously [27]. The fact that all four were more similar to control levels in airways treated OSA patients suggested the likely link to blood oxygenation levels. However, the lack of a correlation between cytokine levels and either SaO2 low % or CRP levels of any patient group or groups draws into question any clear conclusion about the direct role of hypoxia. Airways therapy of OSA patients appears to be an effective way to control aberrant levels of these four cytokines involved in autoimmune disease and immune processes. The outlying cytokine data for five airways treated patients, suggests we may need a more critical method to access compliance with airways therapy.

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Compliance with ethical standards

Yes.

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Author contributions

YW managed the data sets in Excel and prepared all the figures in R in collaboration with RBM and PM. RBM focused the project on cytokines involved in autoimmunity, managed the technical aspects of the project, and wrote the manuscript with input from the team. SA prepared all the serum samples and ran the BioPlex assays. PM directed YW in the statistical analysis of the data. BGP conceived of and initiated the study of OSA and airways treated OSA patients, defined the patient recruitment criteria, and recruited and consented all the patients.

Consent for publication

Yes.

Declaration of Competing Interest

The authors have no financial or ethical conflicts of interest that might influence the publication of this work.

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