Profiling Autoantibodies against Salivary Proteins in Sicca Conditions

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Abstract

Salivary gland dysfunction occurs in several autoimmune and immune-related conditions, including Sjögren syndrome (SS); immune checkpoint inhibitor-induced sicca (ICIS) that develops in some cancer patients and is characterized by severe, sudden-onset dry mouth; and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Although subjects with these conditions present with oral dryness and often exhibit inflammatory infiltration of the salivary gland, little is known about the B-cell humoral responses directed against salivary gland protein targets. In this study, autoantibodies were evaluated against Ro52, Ro60, and La, as well as against a panel of 22 proteins derived from the salivary proteome. The tested cohort included healthy volunteers and subjects with SS, ICIS, and APECED without and with sicca. As expected, a high percentage of autoantibody seropositivity was detected against Ro52, Ro60, and La in SS, but only a few ICIS patients were seropositive for these autoantigens. A few APECED subjects also harbored autoantibodies to Ro52 and La, but only Ro60 autoantibodies were weakly associated with a small subset of APECED patients with sicca. Additional testing of the salivary panel failed to detect seropositive autoantibodies against any of the salivary-enriched proteins in the SS and ICIS subjects. However, APECED subjects selectively demonstrated seropositivity against BPI fold containing family A member 1 (BPIFA1), BPI fold containing family A member 2 (BPIFA2)/parotid salivary protein (PSP), and lactoperoxidase, 3 salivary-enriched proteins. Moreover, high levels of serum autoantibodies against BPIFA1 and BPIFA2/PSP occurred in 30% and 67% of the APECED patients with sicca symptoms, respectively, and were associated with an earlier age onset of oral dryness ($P = 0.001$). These findings highlight the complexity of humoral responses in different sicca diseases and provide new insights and biomarkers for APECED-associated sicca (ClinicalTrials.gov: NCT00001196; NCT00001390; NCT01425892; NCT01386437).

Keywords: autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), immune check-point inhibitor treated cancer patients with sicca (ICIS), salivary gland, Sjögren syndrome (SS), autoimmune, saliva

Introduction

The salivary glands, comprising the parotid, submandibular, sublingual, and minor glands, play important roles in maintaining the oral cavity through saliva production (Melvin et al. 2005). These glands are composed of specialized epithelial cells, including acinar and ductal cells (de Paula et al. 2017). The acini are responsible for protein synthesis of salivary components and electrolytes into the acinar lumen as a plasma-like primary secretion. The primary salivary product is then expelled into a network of ducts, through which the ionic composition is modified and ultimately transported to the mouth. Saliva contains antimicrobial, buffering, cleaning, and lubricative properties that are vitally important for oral health (Humphrey and Williamson 2001; Pedersen et al. 2018). Reduced salivary secretion can increase susceptibility to oral infection, accelerate tooth decay, and increase the risk of soft tissue damage, resulting in painful, burning, or ulcerated oral mucosa. Salivary hypofunction is also associated with diminished quality of life related to other oral problems, including impacts on mastication, swallowing, and speaking (Chambers et al. 2004).

Several clinical conditions can damage the human salivary glands. One condition, Sjögren syndrome (SS), involves autoimmune attack on the salivary and lacrimal glands, causing

gland dysfunction and oral or ocular dryness, respectively (Fox 2005). While the likely drivers of SS salivary gland

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autoimmunity include an inflammatory infiltrate, specific information about the mechanisms leading to the loss of salivary gland function is limited (Mavragani and Moutsopoulos 2014). Evidence of B-cell dysfunction in SS is demonstrated by the high seroprevalence of autoantibodies against SSA and SSB encoded by ubiquitously expressed Ro52, Ro60, and La proteins. Importantly, little is known about which salivary gland proteins are targets of B- and T-cell immune attack and may be associated with sicca symptoms or decreased salivary flow.

Several other immune-related conditions are associated with sicca symptoms. One such condition involves subjects who develop sicca symptoms after receiving targeted oncologic therapies to augment the immune system (designated as immune checkpoint inhibitor-induced sicca [ICIS]), including anti-PD-1, anti-PD-L1, or anti-CTLA4 monoclonal antibody therapies (Cappelli, Gutierrez, Baer, et al. 2017; Cappelli, Gutierrez, Bingham, et al. 2017). ICIS subjects show marked oral dryness characterized by an abrupt and dramatic decrease in salivary production and represent a subset among individuals who develop a range of immune-related adverse events (Cappelli, Gutierrez, Bingham, et al. 2017; Warner et al. 2019). While autoreactive T cells are known to be activated with immune checkpoint inhibition (Tocut et al. 2018), both the potential biomarkers associated with the sicca symptoms and the salivary targets involved remain unknown. Another disease with sicca symptoms is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a monogenic disorder caused by mutations in the *AIRE* transcription regulator leading to defective elimination of autoreactive T cells (Husebye et al. 2018; Constantine and Lionakis 2019). Approximately 40% of APECED subjects exhibit SS-like sicca symptoms (designated here as APECED with sicca) by 30 y of age (Ferre et al. 2016; Oftedal et al. 2017). Consistent with autoimmune attack, immunofluorescence analysis revealed that 75% of APECED patients showed serum autoantibody immunoreactivity against the salivary gland (Oftedal et al. 2017). APECED subjects also have a high incidence of other oral manifestations, including enamel hypoplasia, periodontal disease, and oral candidiasis (Ahonen et al. 1990; Perniola et al. 1998; McGovern et al. 2008; Ferre et al. 2016). While protein array immunoassay has discovered many different proteins as targets of heterogenous humoral responses in APECED subjects (Fishman et al. 2017), no salivary autoantibodies have been identified for sicca and other oral symptoms.

Based on the observation that many autoimmune conditions, such as myasthenia gravis, Hashimoto thyroiditis, and type I diabetes, involve autoantibodies against resident proteins within the relevant target tissue (Burbelo et al. 2016), we hypothesized there might be salivary-specific protein targets of autoantibodies in SS and other sicca diseases. Here we explored this possibility by screening SS, ICIS, and APECED patients to determine whether there are autoantibodies against salivary proteins associated with the sicca symptoms in these diseases.

Materials and Methods

Clinical Samples

All studies were carried out in accordance with approved National Institutes of Health (NIH) guidelines. All participants provided informed written consent prior to the initiation of any study procedures. Human serum samples from healthy volunteers ($n = 20$), SS patients ($n = 20$), and ICIS patients ($n = 23$) were obtained from NIH Institutional Review Board–approved protocols in the Salivary Disorders Clinic at the National Institute of Dental and Craniofacial Research (NIDCR). The SS group was preselected to include equal subsets of Ro52, Ro60, and La seronegative and seropositive subjects. The rationale for this approach was to increase the chance of identifying novel biomarkers in the SSA- and SSB-negative subgroup, which might represent a heterogenous group of SS cases. APECED subjects from the National Institute of Allergy and Infectious Diseases have been previously described (Ferre et al. 2016). In the current study, APECED patients clinically evaluated for reported oral dryness and salivary flow rates included 11 APECED cases without sicca and 9 APECED cases with sicca. Whole unstimulated salivary flow rate measurements for all groups were obtained by standard procedures over 5 min. Although most of the APECED subjects were not examined for a focus score, 4 of 5 APECED patients with sicca exhibited focal lymphocytic sialadenitis consistent with inflammation found in SS-like disease. Based on serum immunoreactivity against several salivary proteins in APECED, whole saliva from available APECED subjects ($n = 16$) was also examined for autoantibodies against lactoperoxidase (LPO), BPI fold containing family A member 1 (BPIFA1), and BPI fold containing family A member 2 (BPIFA2).

Autoantibody Testing of Salivary-Enriched Proteins by Luciferase Immunoprecipitation Systems

The luciferase immunoprecipitation systems (LIPS) technology was used for all autoantibody testing (Burbelo et al. 2015). LIPS employs light-emitting recombinant antigens with the immunoglobulin capture reagent, protein A/G beads, in a high-throughput immunoprecipitation assay to measure antibodies with high sensitivity, specificity, and wide dynamic range of detection. Previously published LIPS tests for detecting serum autoantibodies against Ro52, Ro60, and La were used (Burbelo et al. 2009). To determine whether gland-specific proteins might be targets of autoantibodies in these conditions, we generated a panel of salivary-specific proteins for screening. To assemble this panel, transcriptomic information from the Human Protein Atlas (www.proteinatlas.org) was used, which identified 120 of the approximate 20,000 human proteins as being enriched in the salivary gland (Uhlén et al. 2015). We chose a subset of these salivary proteins for autoantibody testing and focused primarily on secreted extracellular proteins,

Table. Clinical Characteristics of Sicca Cohort Participants.

	Healthy Volunteers	Sjögren Syndrome	Immune Checkpoint Inhibitor Sicca	APECED with Sicca	APECED without Sicca
Age, mean $\pm$ SD, y	36.1 $\pm$ 13.6	62.9 $\pm$ 13.1	58.6 $\pm$ 13.6	27.4 $\pm$ 19.4	20.6 $\pm$ 12.1
Female, %	60	95	35	60	50
Unstimulated salivary flow, mL/15 min, mean $\pm$ SD	4.8 $\pm$ 2.8	1.8 $\pm$ 1.6	0.7 $\pm$ 1.0	0.8 $\pm$ 1.1	6.2 $\pm$ 3.7
Focus score, mean $\pm$ SD	0.5 $\pm$ 0.8	3.4 $\pm$ 3.4	1.5 $\pm$ 3.1	ND	ND

APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; ND, not determined.

including water channels, antimicrobial proteins, and other secreted proteins in saliva that could be targets of pathogenic autoantibodies. The 22 selected salivary-enriched proteins with gene names included amylase, alpha 1A (AMY1A); aquaporin 5 (AQP-5); BPIFA1; BPIFA2 (also called parotid salivary protein [PSP]); BPI fold containing family B member 2 (BPIFB2); Barttin CLCNK-type accessory beta subunit (BSND); carbonic anhydrase 6 (CA6); calmodulin like 5 (CALML5); cystatin 1 (CST1); deoxyribonuclease 2 beta (DNASE2B); follicular dendritic cell secreted protein (FDCSP); interleukin 19 (IL-19); LPO; lactotransferrin (LTF); lacritin (LACRT); mucin 7 (MUC7); odontogenic, ameloblast-associated (ODAM); prolactin-induced protein (PIP); submaxillary gland androgen regulated protein 3B (SMR3B); ribonuclease A family member 8 (RNASE8); statherin (STATH); and zymogen granule protein 16B (ZG16B). Constructs for generating many of the salivary proteins used the pGAUS3 expression vector for producing N-terminal proteins fused to luciferase. Custom adapter primers were used for polymerase chain reaction (PCR) amplification for each salivary complementary DNA (cDNA) (Burbelo et al. 2015). For these N-terminal fusions, cDNAs encoding the signal peptide sequence were subcloned in frame before the *Gaussia* luciferase in the pGAUS3 vector. For several targets including calmodulin-like 5, lacritin, and IL-19, the cytoplasmic or processed cytokine targets were tested as C-terminal fusions as previously described (Burbelo et al. 2010). DNA sequencing was used to confirm the integrity of each chimeric luciferase-tagged protein constructs.

Based on the detection of serum autoantibodies against 3 salivary proteins, whole saliva from available APECED subjects ($n = 16$) was also examined and used 10 μ L of whole saliva per LIPS assay as previously described (Ching et al. 2011).

Statistical Analysis

Analysis of autoantibody data was performed using the GraphPad Prism software (GraphPad Software). Cutoff values for each test were determined from the mean plus 5 SD of healthy volunteers (HVs) and are indicated in the figures. In the case of BPIFA1 and BPIFA2 autoantibodies, an additional cutoff value based on receiver operator characteristic (ROC) curve analysis was used to optimally separate the APECED patients with and without sicca. Kaplan-Meier estimator was employed for time-to-event analysis to examine the relationship between presence of autoantibodies against salivary proteins and time to development of APECED sicca symptoms. Log

rank (Mantel-Cox) tests were used to examine the statistical significance of the Kaplan-Meier estimator value.

Results

Characteristics of the Sicca Cohort

The tested cohort comprised well-characterized subjects studied at the NIH Clinical Center, including HVs ($n = 20$) and subjects with ICIS ($n = 23$), SS ($n = 20$), APECED without sicca ($n = 11$), and APECED with sicca ($n = 9$). As shown in the Table, only the SS group had a sex preference, with 95% of the subjects being female. Compared to the HV group who had an average unstimulated salivary flow rate of 4.79 mL/15 min (range, 0.21–12.50 mL/15 min), the rates for the SS, ICIS, and APECED with sicca groups were markedly lower with values of 1.83 mL/15 min (range, 0.00–4.79 mL/15 min), 0.72 mL/15 min (range, 0.00–3.59 mL/15 min), and 0.8 mL/15 min (range, 0.00–5.30 mL/15 min), respectively, highlighting the salivary flow defect in these subjects (Table). In contrast, the average unstimulated salivary flow rate for the APECED without sicca group was comparable to that of the HV group, at 6.2 mL/15 min (range, 1.79–15.15 mL/15 min). The average focus score for HVs was 0.47 (range, 0–2), far below the threshold of 1 and much lower than the average focus score for SS and ICIS of 3.39 (range, 0–12) and 1.45 (range, 0–8), respectively.

Autoantibodies Against Ro52, Ro60, and La in the Sicca Cohort

The sicca cohort was initially tested for autoantibodies against Ro52, Ro60, and La. Autoantibody testing revealed that 55% (11/20) of SS subjects (11/20), 13% (3/23) of ICIS subjects, and 20% (5/20) of APECED subjects harbored autoantibodies against Ro52 (Fig. 1A). Ro60 autoantibodies were detected in 45% (9/20) of SS subjects, 22% (5/23) of ICIS subjects, and 30% (6/20) of APECED patients (Fig. 1B). Last, seropositive La autoantibodies were found in 50% of SS subjects, 13% of ICIS subjects, and 10% of APECED subjects (Fig. 1C).

As expected, the SS group had the highest number of seropositive subjects for at least 1 of these 3 SS-associated antigens and represented highly statistically significant elevations compared to the other groups (Fig. 1A–C). In ICIS, autoantibodies were occasionally present and as a group were not statistically significant. However, there was a moderate, statistically significant frequency of elevated Ro52, Ro60, and La

Figure 1. Serum autoantibodies against Ro52, Ro60, and La in the sicca cohort. Scatterplot graphs represent autoantibody levels determined by luciferase immunoprecipitation systems (LIPS) in subjects from the cohort of healthy volunteers (HVs, $n = 20$), subjects with Sjögren syndrome (SS) ($n = 20$), subjects with immune checkpoint inhibitor-induced sicca (ICIS) ($n = 23$), and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) subjects without sicca ($n = 11$, open circles) and with sicca ($n = 9$, black circles). Autoantibodies were studied against (A) Ro52, (B) Ro60, and (C) La. Each circle represents antibody levels in light units (LU) derived from subjects plotted on the y-axis using a $\log_{10}$ scale. Seropositive cutoff values for each autoantibody are indicated with a dotted line and were based on the mean plus 5 SD of the HV control group. Only statistically significant P values are shown and were calculated using the Mann-Whitney U test.

autoantibodies in APECED (Fig. 1A–C). Since APECED patients often develop sicca-like symptoms resembling clinical features seen in SS, we analyzed if the presence of antibodies against these antigens in patients with APECED was associated with sicca symptoms. Of the 20 APECED patients, 9 demonstrated sicca-type symptoms. The presence of autoantibodies against Ro52 and La could not predict the APECED patients with sicca due to the low diagnostic performance of 18% sensitivity and 81% specificity (Fig. 1A) and 11% sensitivity and 100% specificity (Fig. 1C), respectively. However, Ro60 seropositive autoantibodies weakly associated (44% sensitivity and 91% specificity) to the patients with APECED with sicca (Fig. 1B), but this was not statistically significant.

Autoantibodies in APECED Patients against Salivary-Enriched Proteins

To determine if proteins showing enriched expression in the salivary gland might be targets of autoantibodies in patients with sicca symptoms, we generated a panel of 22 recombinant salivary proteins as luciferase fusion proteins for LIPS testing. Serum autoantibody testing revealed that most of the salivary proteins, including amylase, aquaporin 5, and the BSND channel, did not show serum immunoreactivity in any of the subjects. However, a subset of APECED subjects demonstrated robust serum autoantibody responses against 3 salivary-enriched proteins: LPO, BPIFA1, and BPIFA2/PSP (Fig. 2A–C). The largest

Figure 2. Identification of autoantibodies against salivary-enriched proteins in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) subjects. Scatterplot graphs represent autoantibody levels determined by luciferase immunoprecipitation systems (LIPS) in subjects from the cohort of healthy volunteers (HVs) and patients with Sjögren syndrome (SS), immune checkpoint inhibitor-induced sicca (ICIS), and APECED without sicca (open circles) and with sicca (black circles). Autoantibodies against salivary proteins are shown against (A) lactoperoxidase (LPO), (B) BPI fold containing family A member 2 (BPIFA2), and (C) BPI fold containing family A member 1 (BPIFA1). Cutoff antibody levels for each salivary protein are indicated with a dotted line and were based on the mean plus 5 SD of the HV control group. Additional cutoff values (gray dashed line) for BPIFA1 and BPIFA2 were based on receiver operator characteristic curve analysis for optimum separation of the APECED patients with and without sicca. (D) Autoantibodies against LPO, BPIFA1, and BPIFA2 were also examined in whole saliva from available APECED subjects ($n = 16$). BPIFA1 autoantibodies were present in saliva in 2 subjects, which tracked the corresponding values seen in serum (1 low BPIFA2-seropositive subject had no available saliva). LU, light units.

Figure 3. BPI fold containing family A member 2 (BPIFA2) and BPI fold containing family A member 1 (BPIFA1) autoantibodies associate with the development of sicca in younger autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) subjects. Kaplan-Meier estimator was used to examine the relationship between the 3 salivary autoantibodies and time until development of sicca in the APECED patients. Subjects were segregated based on presence and absence of autoantibodies against (A) lactoperoxidase (LPO), (B) BPIFA2, and (C) BPIFA1 and plotted against age of sicca onset. The gray and black bars at the top of graph curves indicate additional APECED subjects who were autoantibody negative or positive and had not developed sicca. Log rank (Mantel-Cox) tests were used to confirm statistical significance of the findings.

prevalence of autoantibodies was against LPO occurring in 60% of the APECED subjects, followed by BPIFA2 (40%) and BPIFA1 (12%). As mentioned, none of the SS subjects and only 1 ICIS patient (i.e., with weak BPIFA2 autoantibodies) harbored autoantibodies against the salivary proteins.

Since only a subset of the APECED subjects showed sicca symptoms, the relationship between autoantibody seropositivity and sicca symptoms was evaluated. While seropositive LPO autoantibodies were often found in APECED patients with sicca (78%, 7/9), LPO autoantibodies showed poor specificity (45%) and were also found in sera of APECED without sicca (Fig. 2A). In contrast, there appeared to be a significant relationship between the presence of autoantibodies against BPI-fold proteins and patients with sicca symptoms. Using a modified, higher cutoff value based on ROC analysis revealed that autoantibodies against BPIFA2 (Fig. 2B) and BPIFA1 (Fig. 2C) could identify the APECED patients with sicca symptoms with 67% sensitivity (6/9) and 91% specificity (10/11), as well as 30% (3/9) sensitivity and 100% specificity (11/11), respectively. Statistical testing revealed that the BPIFA2 autoantibodies were significantly (Student's *t* test, $P = 0.038$) elevated in the APECED patients with sicca compared to APECED subjects without sicca. Interestingly, the levels of BPIFA2 autoantibodies also negatively correlated ($R = -0.47$; $P = 0.035$) with whole unstimulated salivary flow rates in the APECED patients.

Due to the unique seropositivity against the 3 salivary proteins in the APECED subjects, autoantibody responses against LPO, BPIFA1, and BPIFA2 were also examined in the available whole saliva from individual APECED patients. Although no detectable salivary autoantibodies were found against LPO and BPIFA2, 2 APECED patients with high levels of BPIFA1 serum autoantibodies were found to have the corresponding

salivary autoantibodies against BPIFA1 (Fig. 2D). These results establish that autoantibodies against the extracellular protein BPIFA1 are present in the saliva in at least some APECED patients.

Based on the previous natural history study revealing the age-related increase in sicca symptoms in APECED, the Kaplan-Meier method was used to stratify serum LPO, BPIFA2, and BPIFA1 autoantibody seropositivity with time to development of sicca symptoms (Fig. 3). The presence of LPO autoantibodies did not correlate a specific age of onset with sicca in APECED patients (Fig. 3A). In contrast, both BPIFA2 (Fig. 3B) and BPIFA1 (Fig. 3C) strongly correlated ($P = 0.001$) with an earlier age for the development of sicca in APECED patients. These results highlight the association of BPIFA2 and BPIFA1 autoantibodies as potential useful markers for APECED-associated sicca symptoms, particularly in subjects who develop this condition at a younger age.

Discussion

The salivary glands are exocrine organs with a unique tissue-specific protein expression repertoire. We used autoantibody profiling to explore B-cell responses against salivary proteins in 3 immune-related conditions associated with sicca dysfunction. Of the 3 sicca conditions, only SS has a female sex predilection. While APECED patients demonstrated high levels of autoantibodies against the salivary proteins LPO, BPIFA2, and BPIFA1, subjects with SS and ICIS demonstrated no significant detectable immunoreactivity against these or any of the salivary-enriched proteins. The complete lack of autoantibodies against salivary proteins in SS highlights the enigmatic nature of this disease. Several publications have recently reported conflicting results regarding the presence of

autoantibodies against SP-1, CA6 and BPIFA2/PSP in SS patients (Shen et al. 2012; Shen et al. 2014). However, our study found no evidence of seropositivity directed against CA6 and BPIFA2/PSP in SS. We did not examine autoantibodies against SP-1, a mouse protein with no structurally related human homolog. One limitation of our study is that we did not perform an exhaustive autoantibody screen of all salivary proteins, including testing many channel proteins within the salivary gland. Channel molecules are particularly challenging due to their large size and multiple, membrane-spanning regions and will require additional immunoproteomic approaches to determine whether autoantibodies exist against these molecules.

Similar to the results observed in SS, ICIS patients lacked autoantibodies to salivary-enriched proteins. Consistent with a recent report (Warner et al. 2019), one likely interpretation is that T cells are the major drivers for sicca symptoms in ICIS without affecting B-cell responses. Only a few ICIS subjects demonstrated immunoreactivity against Ro52, Ro60, and La, and we cannot formally rule out whether these ICIS subjects harbored preexisting autoantibodies against these molecules, which were exacerbated by treatment with immune checkpoint drugs.

Analysis of the autoantibody profile in APECED subjects revealed that the most prevalent humoral response was against the salivary-enriched protein LPO (60% seropositive), an abundant protein component of saliva produced by the serous acini (Geiszt et al. 2003). LPO is a member of the heme peroxidase family that uses hydrogen peroxide to catalyze biocidal compounds active against a variety of microbial agents (Wijkstrom-Frei et al. 2003). APECED autoantibodies were also detected against BPIFA1 and BPIFA2, 2 structurally related BPIF family members produced by serous acinar cells and, in the case of BPIFA2, within the interlobular salivary ducts (Wolff et al. 2002; Bingle et al. 2009; Bingle et al. 2011; Bingle and Bingle 2011). While it is important to point out that LPO, BPIFA2, and BPIFA1 are also found at other mucosal surfaces outside the salivary gland, all 3 proteins have been previously identified in proteomic compositional analyses of saliva, and BPIFA2/PSP is a well-recognized salivary protein (Ambatipudi et al. 2012; Bassim et al. 2012). Taken together, these findings highlight the heightened B-cell immunoreactivity seen against these 3 salivary-enriched proteins in APECED.

Since only a subset of APECED patients develops sicca (Ferre et al. 2016), we evaluated the association between autoantibodies against salivary proteins and oral dryness. Our results revealed that serum autoantibodies against BPIFA2 and BPIFA1 were markers of salivary dysfunction. The levels of BPIFA2 autoantibodies negatively correlated with whole unstimulated salivary flow rates in APECED patients. In contrast, serum autoantibodies against LPO, which were occasionally copositive with BPIFA2 and BPIFA1 autoantibodies, were present in many APECED patients without sicca and thus may not be specific by themselves for detecting APECED-associated sicca. Alternatively, some of these APECED patients with LPO reactivity who do not have sicca could be at risk for future

development of the disease; longitudinal follow-up of these patients will be needed to examine this possibility. The identification of some APECED-sicca subjects who were negative for both BPIFA2 and BPIFA1 suggests that other salivary protein targets might exist. Additional analysis revealed that the BPIFA1- and BPIFA2-seropositive APECED patient subset had an earlier onset of sicca symptoms ($P = 0.001$) compared to the APECED with sicca subjects seronegative for these autoantibodies. The presence of BPIFA2 and BPIFA1 autoantibodies in younger patients with sicca may reflect the corresponding early T-cell immune response targeting these key salivary cells of the gland. Besides serum autoantibodies, autoantibodies in saliva were also detectable against BPIFA1 in some of the APECED patients. Interestingly, autoantibodies against BPIFB1, another protein family member, were previously identified as a highly selective marker of pneumonitis in APECED and were associated with earlier onset lung damage (E.M.N. Ferré and M.S. Lionakis, unpublished data). The biological reason that BPIF molecules exhibit immunoreactivity in APECED is not clear, but several reports suggest that these molecules bind bacteria and exert antimicrobial activity (Britto and Cohn 2015). Although the biological activity of BPIFA2/PSP in the oral cavity is established (Gorr et al. 2011), studies are needed to examine whether BPIFA1 autoantibodies present in the saliva of some APECED subjects might be pathogenic by inhibiting the proteins' activity in the oral cavity.

Author Contributions

P.D. Burbelo, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript; E.M.N. Ferré, contributed to conception, design, and data acquisition, critically revised the manuscript; A. Chaturvedi, contributed to data analysis and interpretation, critically revised the manuscript; J.A. Chiorini, contributed to conception and design, critically revised the manuscript; I. Alevizos, contributed to data acquisition, critically revised the manuscript; M.S. Lionakis, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; B.M. Warner, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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