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Immune dysregulation in the tonsillar microenvironment of Periodic Fever, Aphthous Stomatitis, Pharyngitis, Adenitis (PFAPA) Syndrome

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Abstract

Periodic Fever, Aphthous stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome is an inflammatory disorder of childhood classically characterized by recurrent fevers, pharyngitis, stomatitis, cervical adenitis and leukocytosis. While the mechanism is unclear, previous studies have shown that tonsillectomy can be a therapeutic option with improvement in quality of life in many patients with PFAPA, but the mechanisms behind surgical success remain unknown. In addition, long-term clinical follow up is lacking. In our tertiary care center cohort, 62 patients with

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Author Contributions

All authors contributed to the study conception, design, and execution. Patient recruitment, and securing of clinical material were performed by Javan Nation, Nathan Page, Daniela Carvalho, Anthony Magit, Wen Jiang, Shelby Leuin, Morgan Bliss, Marcella Bothwell, Matthew Brigger, Donald Kearns, Robert Newbury, Seth Pransky, and Lori Broderick. *Ex vivo* and *in vitro* experiments were performed by Lori Broderick, Irene Luu, and Marisela Guaderrama. Data collection and analysis were performed by Lori Broderick, Irene Luu, and Michelle Peru. Microbiome analysis was performed by Anukriti Sharma and Jack Gilbert. The first draft of the manuscript was written by Lori Broderick and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Disclosures of Conflicts of Interest

L.B. is a speaker for Novartis, Inc., and has an ongoing research collaboration with Regeneron, Inc. The other authors declare no conflicts of interest.

Ethical Approval.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the UCSD Institutional Review Board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

PFAPA syndrome had complete resolution of symptoms after surgery (95.3%). Flow cytometric evaluation demonstrates an inflammatory cell population, distinct from patients with infectious pharyngitis, with increased numbers of CD8+ T cells (5.9% vs. 3.8%, $p<0.01$), CD19+ B cells (51% vs. 35%, $p<0.05$) and CD19+CD20+CD27+CD38− memory B cells (14% vs. 7.7%, $p<0.01$). Cells are primed at baseline with increased percentage of IL-1β positive cells compared to control tonsil-derived cells, which require exogenous LPS stimulation. Gene expression analysis demonstrates a five-fold upregulation in *IL1RN* and *TNF* expression in whole tonsil compared to control tonsils, with persistent activation of the NF-κB signaling pathway, and differential microbial signatures, even in the afebrile period. Our data indicates that PFAPA patient tonsils have localized, persistent inflammation, in the absence of clinical symptoms, which may explain the success of tonsillectomy as an effective surgical treatment option. The differential expression of several genes and microbial signatures suggests the potential for a diagnostic biomarker for PFAPA syndrome.

Keywords

pediatrics; autoinflammation; microbiome; periodic fever; tonsillectomy

INTRODUCTION

Periodic Fever, Aphthous stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome is an inflammatory disorder of childhood characterized by recurrent fevers, pharyngitis, stomatitis, and cervical adenitis, with unknown incidence. During a typical 3–5 day episode, temperatures reach up to 40.5°C, limiting patients' ability to participate in normal activities. The episodes occur at nearly fixed intervals every 3–6 weeks with substantial impact on quality of life for patients and families. Extensive evaluations of PFAPA patients have not revealed evidence of an infectious cause. Further, its cyclical nature is not consistent with classic infection, suggesting an immunologic disorder involving inappropriate innate immune responses. While episodes generally resolve spontaneously, the time to resolution ranges from 4–17 years, leaving a considerable financial and emotional burden on families. [1–5]

In contrast to other autoinflammatory disorders, the genetic etiology behind PFAPA has remained elusive, and several groups have attempted to identify the mechanisms behind the periodic febrile episodes, primarily through evaluation of peripheral blood samples during and between inflammatory flares, however the results have been inconclusive.[6–8] Furthermore, despite these findings, PFAPA remains largely a diagnosis of exclusion.

Tonsillectomy has been shown to be an effective therapy, but the mechanism of its success is unknown. In addition, the literature is limited to primarily case series, with limited follow-up post-operatively, as recently reviewed.[9] We sought to investigate tonsillar tissue from patients with PFAPA syndrome to determine the contribution of the palatine tonsils to febrile episodes in PFAPA, and assess whether tonsillectomy led to long-term resolution of symptoms.

Here, we evaluate tonsils from patients with PFAPA for cellular populations, gene expression and immune responsiveness to innate immune mediators, as well as a microbial taxonomic signature. This study demonstrates that tonsillectomy is an effective surgical treatment option for management of children with PFAPA syndrome, and suggests that targeting the tonsillar pathways may be a new approach to understanding this disease.

METHODS

Human Subjects

We initiated a prospective cohort study to better understand the natural history of PFAPA in children treated at a tertiary care center in San Diego, California, USA. Any patient age 1–17 years, seen in both the Rady Children's Hospital-San Diego Allergy/Immunology clinics and Otolaryngology clinic, diagnosed clinically with PFAPA syndrome per the following modified Marshall's criteria [10], was eligible to participate.

Inclusion criteria for PFAPA:

- Regularly occurring fevers (without evidence of upper respiratory infection).
- Onset prior to age 5 years.
- At least one of the following associated with fevers: aphthous stomatitis, pharyngitis, cervical adenitis.
- Resolution of symptoms with single, low dose prednisolone (≤1mg/kg po once in the first 24 hours of a febrile episode).
- Normal growth as assessed by World Health Organization growth charts
- Asymptomatic between episodes
- Absence of laboratory evidence of inflammation between episodes (cardio/high sensitivity CRP <0.2 mg/L and ESR <15 mm/h)
- Absence of genetically defined autoinflammatory disorders (familial Mediterranean fever, Hyper-IgD syndrome/mevalonic kinase deficiency, TNF receptor associated periodic syndrome, cryopyrin associated periodic syndrome), cyclic neutropenia, immunodeficiency or autoimmunity (excluded clinically).

Patient data was collected on over 200 children with recurrent fevers (at least 6 episodes) identifying 94 patients with PFAPA using a standardized questionnaire, consisting of demographic data, including age, gender, ethnicity, clinical profiles (presence of symptoms, fever profile, treatments) and detailed family histories. For patients electing to undergo tonsillectomy (with or without adenoidectomy, n = 65), tissue was obtained intra-operatively under an IRB-approved protocol. Under the same protocol, tonsil tissue from age and sex-matched controls who underwent tonsillectomy for obstructive sleep apnea or recurrent streptococcal pharyngitis were obtained as available. All patients were at least 2 months post any antibiotic treatment [11]. Tonsillar sections were stained with hematoxylin and eosin for histologic analysis and evaluation by a pediatric pathologist, as per routine standard of care.

The remainder of the tissue was mechanically disrupted to create a single cell suspension, and used immediately or frozen in fetal bovine serum (FBS) +10% dimethyl sulfoxide for cryostorage. Written informed consents were obtained from parents or legal guardians for all patients and written assents were obtained for children (7–12 years) and adolescents (13–17 years) under protocols approved by the UCSD Institutional Review Board and in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Flow cytometry

Tonsils were mechanically disrupted into single cell suspensions, and applied to a Percoll gradient. 10^6 cells were stained with conjugated monoclonal antibodies to CD3, CD4, CD8, CD19, CD20, CD27, CD45RA, CD45RO, IL-1β (eBioscience, Inc.) per manufacturer's instructions. For intracellular staining of IL-1β, cells were permeabilized using the Caltag kit. Initial phenotyping for T, B, NK and dendritic cells was performed on a BD FACSCalibur. All subsequent samples were acquired with a BD Biosciences LSR II cytometer using FACSDiva software. In each case, 100,000 events were collected per sample. Data was analyzed with FlowJo software.

Cell culture

Single cell suspensions were plated in tissue culture plates at 10^6 cells per well in 24-well plates in complete media (RPMI 1640 +10% FBS + 1% PSG). Cells were incubated at 37°C and 40°C to replicate physiologic and febrile temperatures, respectively. For evaluation of IL-1β from tonsillar cells, mixed single cell suspensions were treated with lipopolysaccharide (E coli 0111:B4, Sigma Aldrich, 0.01μg/mL) for 6 hours, prior to evaluation by flow cytometry. For other *in vitro* stimulations CpG 2006 (Invivogen ODN 7909, 0.1μM), and Poly I:C (Sigma P9582, 25μg/ml) were applied.

Reverse transcription and quantitative PCR

RNA was isolated from tonsil cell suspensions using Trizol (Life Technologies) and cDNA was synthesized using High Capacity cDNA Reverse Transcription reagents (Applied Biosystems), both per manufacturer's instructions. Relative gene expression was determined using the following primer sets: *IL1A* (5'-AGTTCTTAGTGCCGTGAGTTTC-3' and 5'-GTGACTGCCCAAGATGAAGA-3', *IL1B* (IDT PrimeTime Assay Hs.PT.58.40959974), *IL1RN* (5'- TTGTCCTGCTTTCTGTTCTCG-3' and 5'-CTGTCCTGTGTCAAGTCTGG-3'), *IFNB* (5'- AGCTGAAGCAGTTCCAGAAG −3' and 5'- AGTCTCATTCCAGCCAGTGC −3') and *TNF* (5'- GGAGAAGGGTGACCGACTCA −3' and 5'- CTGCCCAGACTCGGCAA −3') with beta-actin (*ACTB*; 5′-AAGTCAGTGTACAGGTAAGCC −3′ and 5′- GTCCCCCAACTTGAGATGTATG −3′) as reference gene. Quantitative PCR was performed with a Bio-Rad iCycler using iQ5 software (Bio-Rad). Relative gene expression, determined using the $2^{\Delta\Delta ct}$ method, was normalized against control samples.

Immunoblot

2×10^6 cells tonsil-derived cells were lysed and homogenized in lysis buffer (50mM Tris pH 7.8, 150mM NaCl, 0.1% Nonidet P-40, and 1mM phenylmethylsulfonyl fluoride). The solubilized proteins were run on a 4–15% gradient gel (BioRad Mini-PROTEAN TGX Precast gel) and blotted onto a PVDF membrane (Millipore Sigma IPVH00010). Phosphorylated NF-κB was detected with anti-phospho-NF-κB p65 (Ser536) (Cell Signaling Technology #3033, 1:1000) and phosphorylated IκBα with anti-phospho-IκBα (Ser32) (Cell Signaling Technology, #2859, 1:1000). For both, anti-rabbit IgG-HRP secondary antibody (Cell Signaling Technology #7074), was used at 1:1000. Anti-GAPDH-HRP (abcam, ab9385, 1:5000) was used as a loading control. All Western blots were developed using ECL reagent (ThermoFisher) and radiography film.

Enzyme-linked immunosorbent assay (ELISA)

Measurement of secreted human IL-1β and TNF were performed by ELISA (both from R and D Systems) according to manufacturer's instruction.

Microbiota analyses

RNA was submitted to J. Craig Venture Institute for 16S rRNA amplicon sequencing. The 16S data were quality-filtered and demultiplexed using the same QIIME 1.9.1 scripts, i.e., join_paired_ends.py and split_libraries_fastq.py [12] The final set of demultiplexed sequences were then selected for Exact Sequence Variants (ESV) picking using the DeBlur pipeline.[13] ESVs present in less than 10 samples were removed using the Phyloseq package.[14] The final BIOM file [15] comprising of 20 samples with average 26,326 reads per sample was then used for further analyses. Non-metric multidimensional (NMDS) scaling plots were employed to reveal beta diversity variations based on Weighted, Unweighted UniFrac [16] and Bray-Curtis method [17] for the 16S ESV data in the Phyloseq package. Shannon, Inverse Simpson index, and Fisher metrics were used to estimate alpha diversity and the variation between groups (beta diversity) was statistically tested using permutational multivariate analysis of variance (PERMANOVA).[18] ANCOM was used to identify differentially abundant bacterial ESVs between the groups at P-value cut-off of 0.05 with Benjamini-Hochberg FDR correction.[19] Spearman rank correlation and generalized linear models (GLMs) were used to establish association between the microbiome and other continuous variables in the metadata using microbiomeSeq and glm packages in R. [20]

Statistical analyses

Statistical analyses and graphing were performed in Microsoft Excel and Graphpad Prism (version 5.03; Graph Pad, Graph Pad Software Inc., CA) programs with the two-tailed, unpaired Student's t test or Fisher's exact test. Flow cytometry data were analyzed by FlowJo software. Unless otherwise stated, data are expressed as mean +/− SEM. A p value less than 0.05 was considered statistically significant.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper. Microbiome data for individual patients cannot be made publicly available for reasons of patient confidentiality. Qualified researchers may apply for access to these data, pending institutional review board approval.

RESULTS

PFAPA patient characteristics

In the Recurrent Fever Disorders Clinic at Rady Children's Hospital, San Diego, we assessed 200 children with recurrent fevers (at least 6 episodes) including 94 patients with PFAPA syndrome as defined by a modified Marshall's criteria.[10] On average, patients were 2.8 years old at the age of onset (Table 1), and experienced fevers to a maximum of 40.1°C, lasting 3.7–5.7 days, with 27–45 days between episodes, consistent with prior reports. In addition, reported rates of streptococcal negative pharyngitis (64%), aphthous stomatitis (51%) and adenitis (49%) were similar to earlier studies.[4, 21, 10] In addition, patients reported headache (28%), abdominal pain (26%), and arthralgias (26%) during episodes (Figure 1A). All symptoms resolved between febrile episodes. The patient-reported ethnic distribution of our patients is similar to that of Southern California, and San Diego County (Figure 1B).

Tonsillectomy leads to a rapid resolution of symptomatic episodes

To date, 65 patients with PFAPA syndrome have undergone tonsillectomy, for whom we have long term follow-up. There was no difference in gender, age of onset, duration of episodes, or severity of symptoms in the subset of patients opting for surgery, compared to the rest of the cohort (Table 1). Sixty-two patients with PFAPA syndrome have had complete resolution of symptoms after surgery, with the time to resolution of symptoms post-tonsillectomy approximately 2 months (range 1–11 months, Figure 1C). The average length of follow up is 75 months (range 22–110 months). Three patients were refractory to tonsillectomy, with fevers persisting for more than 6 months, which remain responsive to medical therapy.

PFAPA patient tonsils are grossly similar to controls

For patients opting for tonsillectomy, samples were received from Pathology immediately post-operatively. Age and sex-matched tonsils from patients with obstructive sleep apnea or recurrent streptococcal pharyngitis were obtained as available (Table 2). Tonsils from patients with PFAPA are notably smaller and grossly friable compared to those from similarly aged patients with OSA or recurrent pharyngitis. On histologic exam, both PFAPA and control tonsils were reported to show variable enlargement of follicles, and epithelium with multifocal inflammatory infiltration with foci of neutrophils infiltrating the squamous mucosa. No granulomas or abscesses were noted on histological examination in any sample. There was no difference in the presence of actinomyces in samples between patient and controls (data not shown).

Tonsillar lymphocytes are hyperactive even in afebrile periods

To determine the role of palatine tonsils in the pathophysiology of PFAPA syndrome, we phenotypically characterized post-operative samples by 8-color flow cytometry and gene expression. Flow cytometry of isolated cellular constituents reveals that tonsils from patients with PFAPA have a significantly larger memory B cell population, defined as CD27+, CD19+, CD38 negative cells compared to recurrent streptococcal pharyngitis controls. Further analysis demonstrated these PFAPA patient tonsillar memory B cells express higher levels of the survival markers BAFF-R and TACI. In contrast, total T cell, NK cell and monocyte/ macrophage and dendritic cell populations were similar among all groups (Figure 2 and data not shown).

Prior work by Stojanov et al [7] demonstrated that in the peripheral blood, PFAPA flares were associated with an upregulation of IL-1β related genes, though protein levels were not significantly elevated in the serum. To determine if the tonsils had a similar inflammatory signature, RNA was extracted from whole tonsils and evaluated for expression of proinflammatory cytokines, including *IL1A, IL1B, IL1RN, TNF and IFNB.* Notably, transcripts for *IL1RN* and *TNF* were significantly upregulated in PFAPA tonsils compared to controls, while expression of *IL1A, IL1B and IFNB* was similar between groups (Figure 3A). These data suggest that PFAPA patients may have localized, subclinical inflammation between episodes, despite an absence of serologic inflammation. To assess the activation status of downstream mediators, we evaluated activation of the NF-κB pathway using Western blot (Figure 3B). PFAPA patients had increased phosphorylated NF-κB and phosphorylated IκBα compared to RP patients.

To further investigate this IL-1 signature, we cultured whole tonsil cells *ex vivo.* We observed that PFAPA patient tonsillar cells had significantly more intracellular IL-1β compared to controls (Figure 4A). Surprisingly, further phenotyping revealed that the positive cells were memory B cells (CD19+, CD20+, CD27+ lymphocytes), rather than a monocyte/macrophage population (Supplemental Figure 1A). *In vitro* stimulation of unsorted tonsillar cells with lipopolysaccharide (LPS), significantly increased the percent of IL-1β positive cells in control cultures, but had no effect on cells derived from PFAPA patients (Figure 4B). To determine if the B cells were resistant to further activation specifically to LPS, or if other TLR pathways elicited a similar non-response, we stimulated B cells with the TLR9 ligand CpG-oligodeoxynucleotide (CpG-ODN). Similarly, stimulation with CpG-ODN failed to further upregulate BAFF-R and TACI expression on PFAPA tonsillar memory B cells to the extent observed in stimulated control cultures (data not shown). However, CpG-ODN stimulation did enhance TNF release from PFAPA tonsil derived cells (Supplemental Figure 1B). Taken together, these data suggest that PFAPA patient tonsillar cells are constitutively activated, even during afebrile periods, and hyperresponsive to select TLR stimuli.

Differences in tonsillar inflammation are associated with differential proportions of specific bacterial species

Given the lack of antibiotic responsiveness in PFAPA episodes, and to determine whether the differences in cytokine and cellular phenotypes in the tonsil microenvironment were

associated with differences in the microbiota, we performed 16S rRNA amplicon sequencing on a subset of age and gender matched tonsillar samples. The total diversity across all the samples is shown in Figure 5A. In terms of alpha biodiversity indices (i.e. Shannon, Inverse Simpson and Fisher), there are no significant differences between the tonsils from PFAPA patients and controls (Figure 5B). However, when assessing beta diversity using weighted UniFrac distance matrix, there were significant differences between controls and subjects, suggesting differences in relative proportions of specific taxa between the two groups (Figure 5C).

In terms of differentially abundant (pFDR < 0.05) taxa (exact sequence variants, ESVs), the PFAPA patient samples have significantly greater proportion of ESVs belonging to family *Lachnospiracae*, *Treponema socranskii*, and *Ureaplasma*. In contrast, the control samples have significantly greater (pFDR < 0.05) proportion of ESVs from *Acinetobacter johnsonii*, *Pasteurella*, *Flavobacterium*, *Micrococcus*, *Peptoniphilus*, and others (Figure 6). Within the PFAPA cohort, specific taxa were identified that showed significant ($p < 0.05$) association (positive and negative) with variables related to fever timing, specifically episode duration and frequency (Figure 7), with longer febrile episodes correlating with *Neisseria, Haemophilus* and *Prevotella melaninogenica.* With respect to episode cycling, shorter intervals associated with *Fusobacterium* and longer intervals with *Treponema*. Other variables including age of onset, age and tonsillectomy and time to tonsillectomy also correlated (data not shown), which may reflect disease duration or age-related changes in the bacterial community structure.

DISCUSSION

PFAPA is characterized by distinct febrile episodes with regular periodicity, but the mechanisms behind the fevers and their resolution remain unknown. Randomized studies of tonsillectomy in the treatment of PFAPA have suggested that the palatine tonsils, rather than adenoids are associated with disease pathology.[22] Here, we demonstrated that tonsils from patients with PFAPA have a distinct cellular makeup compared to tonsils from patients with recurrent streptococcal pharyngitis (RP), In addition, we observed that despite tonsillectomy being performed during the asymptomatic interfebrile period, PFAPA patient tonsil-derived cells maintained a unique inflammatory signature with increased *IL1RN* and *TNF* expression. At the protein level, PFAPA tonsils have a greater percentage of IL-1β positive B cells at baseline, and enhanced TNF production with TLR stimulation. Further, microbiota analysis of tonsillar tissue demonstrated a bacterial signature pattern distinct from control tonsils. Our results indicate that the tonsillar microenvironment has evidence of local subclinical inflammation, even during afebrile phases., which is consistent with the success of tonsillectomy in this syndrome.

We were surprised to find that the cells staining positive for intracellular IL-1β were memory B cells. However, studies of other immunodysregulatory diseases have identified similar B cell populations. Recently, AIM2 has been shown to be preferentially expressed in peripheral memory B cells, and upregulate IL-1β in response to stimulation.[23] Given the notable tissue friability of PFAPA patient tonsils, and persistent activation, a model of

chronic stimulation of the AIM2 inflammasome by both self and foreign nucleotides could be contributing to disease pathogenesis. Ongoing studies are investigating these possibilities.

The tonsillar microenvironment hosts an abundance of commensal and potentially pathogenic bacteria, and has been shown to differ between adults and children.[24, 25] Unlike subtypes of psoriasis, where group A beta hemolytic streptococcus infection has been linked to disease onset,[26] we did not identify a single bacterial species that could be an environmental trigger of disease onset. In the pediatric psoriatic patients, however, streptococcal infections were linked to a systemic host-response, with development of autoantibodies that demonstrate cross-reactivity between keratinocytes antigens and streptococcal antigens.[26] While our PFAPA patient cohort did not have increased positive streptococcal pharyngeal swabs or cultures, it is worth noting that several of the bacterial genera that were differentially proportionally abundant in the PFAPA tonsils (*Treponema*, *Lachnospiraceae*, *SR1*) have been described in periodontal disease [27] and could be stimulating the inflammatory local microenvironment. At this time, however, whether the persistent inflammation in the local microenvironment changes the microbiota in PFAPA patient tonsils, or existing differences in bacterial composition induce or contribute to PFAPA syndrome, remains unknown. Regardless, removing the inflammatory microenvironment and its associated bacteria via tonsillectomy seems to be of benefit in resolving febrile episodes.[9, 28, 22]

Symptoms in three patients were refractory to tonsillectomy, defined as episodes persisting for more than 6 months, which remain responsive to medical therapy. Preoperatively, these three patients did not have different clinical courses compared patients for whom tonsillectomy was successful. It is of particular interest that the episodes experienced post-tonsillectomy in these patients are fever-less, despite the continued presence of the other symptoms, and often with set periodicity as observed prior to tonsillectomy. The reason for surgical failure is unclear. No remaining palatine tonsillar tissue is visible by ENT exam, and the timeline of symptoms relapse is not consistent with regrowth of tonsillar tissue. A more extensive genetic workup was performed in 2/3 of the patients post-tonsillectomy. No variants in fever syndrome genes were identified as performed by commercial sequencing. These patients are continuing with symptomatic medical management at this time.

We identified several differences from prior publications of patients with PFAPA, though given the cyclic nature, these differences are not unexpected. Kolly et al found that peripheral blood mononuclear cells could be primed and have increased secretion of IL-1β when stimulated by LPS, compared to samples from patients between episodes [29], however this difference may be explained by the tissue source under investigation. We also identified cell phenotypic results different from the recently published work by other groups [30, 31, 8], which may reflect differences in pediatric populations, time of tonsillectomy with respect to fever cycle, and/or the use of RP and OSA controls.

This study has several limitations. For medico-ethical reasons, our primary comparison is with tonsillar samples obtained from age-matched patients with other disorders, namely culture-positive recurrent streptococcal pharyngitis and obstructive sleep apnea. While one other study has used OSA tonsils [32], here, recurrent pharyngitis samples were used as the

main comparator due to the episodic nature of disease and concern for unique pathogenic mechanisms in OSA. It remains possible that some of the differences we observed are due to underlying pathology in these groups, which do not reflect the normal/unaffected state. The periodic nature of PFAPA syndrome may also suggest that some of the variability observed may be related to the specific time point in the interfebrile period the surgery was performed. In addition, this was not a randomized study; our families have chosen either medical or surgical therapy for their symptoms. As a result, we have no families that first received therapy with colchicine [33], cimetidine [34], or montelukast [35], and then opted for tonsillectomy. Whether and how these oral therapies would impact the local tonsillar microenvironment remains unknown. All samples were acquired from patients in the greater San Diego area, and may be impacted by geographic or social aspects on the tonsillar microenvironment. Future studies with large, multi-center cohorts will be of great value in further delineating these possibilities.

Beyond PFAPA syndrome, tonsillectomy has been shown to be effective in other systemic disorders, including IgA nephropathy, generalized pustular psoriasis, pustulosis palmaris et plantaris and chronic plaque psoriasis.[36–39] While the latter diseases are generally associated with anti-keratin antibodies or keratin-reactive T cells [40], they are associated with recurrent inflammatory flares and high fevers similar to PFAPA syndrome episodes.[41, 42] While PFAPA is not typically associated with dermatologic findings, the observations of persistent inflammatory gene signature and primed state of the cells suggest that the palatine tonsil has a unique microenvironment, and may drive or contribute to the febrile episodes.

The genetic etiology of PFAPA remains largely undefined [43, 44], and diagnosis is limited to clinical features and elimination of other autoinflammatory disorders. The identification of two cytokines (IL-1 and TNF) differentially expressed in the tonsillar cells isolated from PFAPA patients compared to other control tonsils suggests that these may be used as biomarker for the diagnosis of PFAPA. Indeed, saliva cytokines (protein or RNA) have been primarily evaluated as a potential diagnostic tool in oropharyngeal cancers [45], but suggests that the methodology has potential, even for young children.[46, 47]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CpG-ODN	CpG-oligodeoxynucleotide
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

HRP	horseradish peroxidase
IFN	interferon
IKK	inhibitor of kappa B kinase
IL-1	interleukin-1
IL-1Ra	interleukin-1 receptor antagonist (protein)
IL1RN	interleukin-1 receptor antagonist (gene)
LPS	lipopolysaccharide
NF-κB	nuclear factor kappa-light-chain- enhancer of activated B cells
NK	natural killer
OSA	obstructive sleep apnea
PFAPA	periodic fever, aphthous stomatitis, pharyngitis, and adenitis
PSG	Penicillin-Streptomycin-Glutamine
PVDF	polyvinylidene fluoride
RP	recurrent pharyngitis
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SEM	standard error of mean
TLR	toll-like receptor
TNF	tumor necrosis factor

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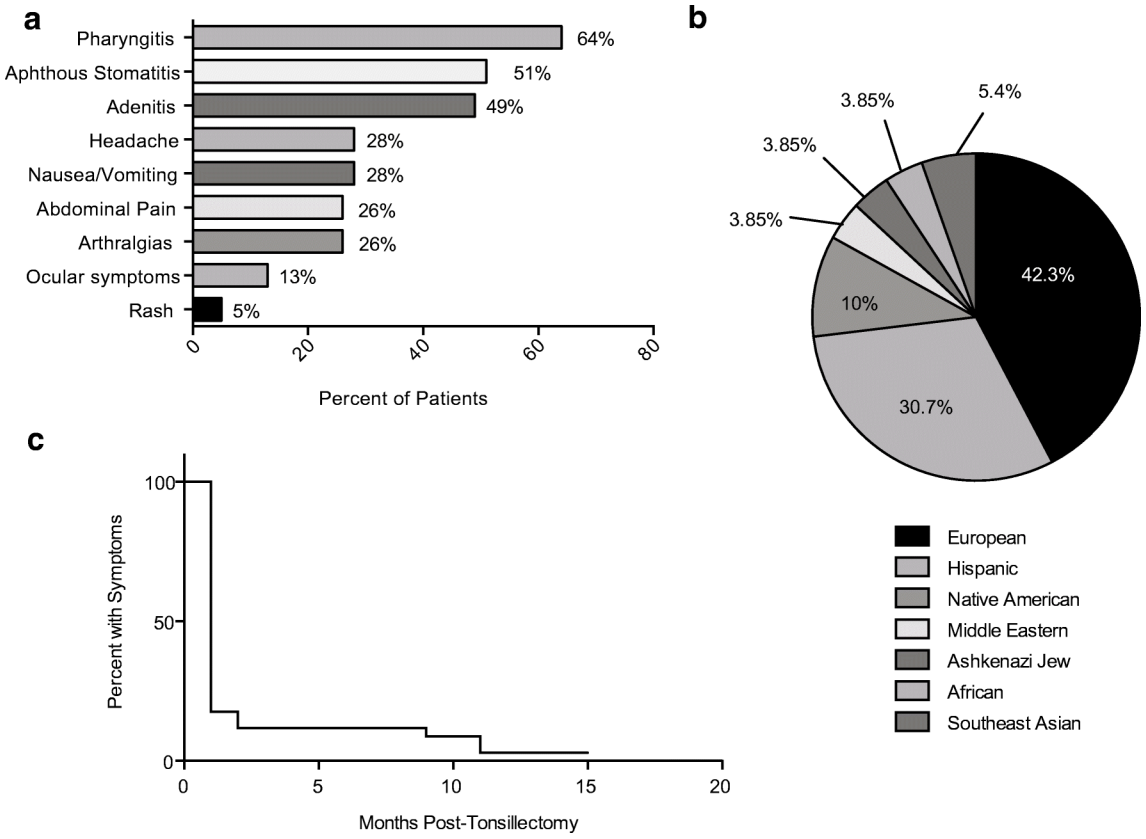

Figure 1. Tonsillectomy leads to rapid resolution of febrile episodes in PFAPA patients.

A, Symptoms associated with fever in PFAPA patients (n = 94). **B,** Patient reported ancestry is reflective of the diverse population in San Diego, CA (n=94). **C,** Time to resolution of febrile symptoms post-tonsillectomy (n=65).

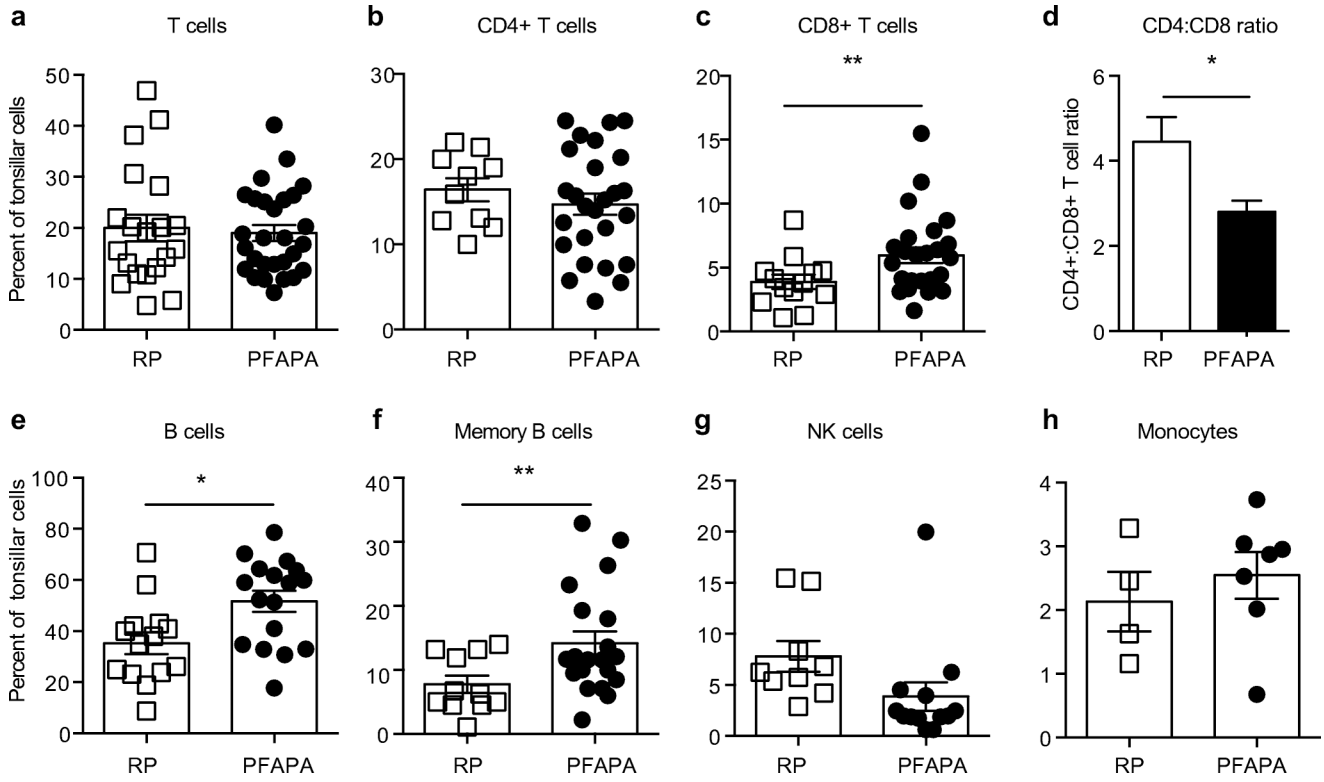

Figure 2. PFAPA tonsils have an inflammatory cell infiltrate distinct from infected tonsils.

A-C, CD3+ T cells from tonsils of patients with PFAPA are increased in the CD8+ T cell population compared to recurrent pharyngitis (RP) tonsils, resulting in a reversed CD4:CD8 ratio (**D**). **E-F,** Both CD19+ B cells and CD19+CD20+CD27+CD38- memory B cells are increased in PFAPA tonsils compared to controls. **G,H,** CD56+ NK cells (**G**) and CD11c +CD14+ monocytes (**H**) are unchanged between the two groups. Data shown as mean ± SEM, with *, $p<0.05$, ** $p<0.01$, by Student's t test.

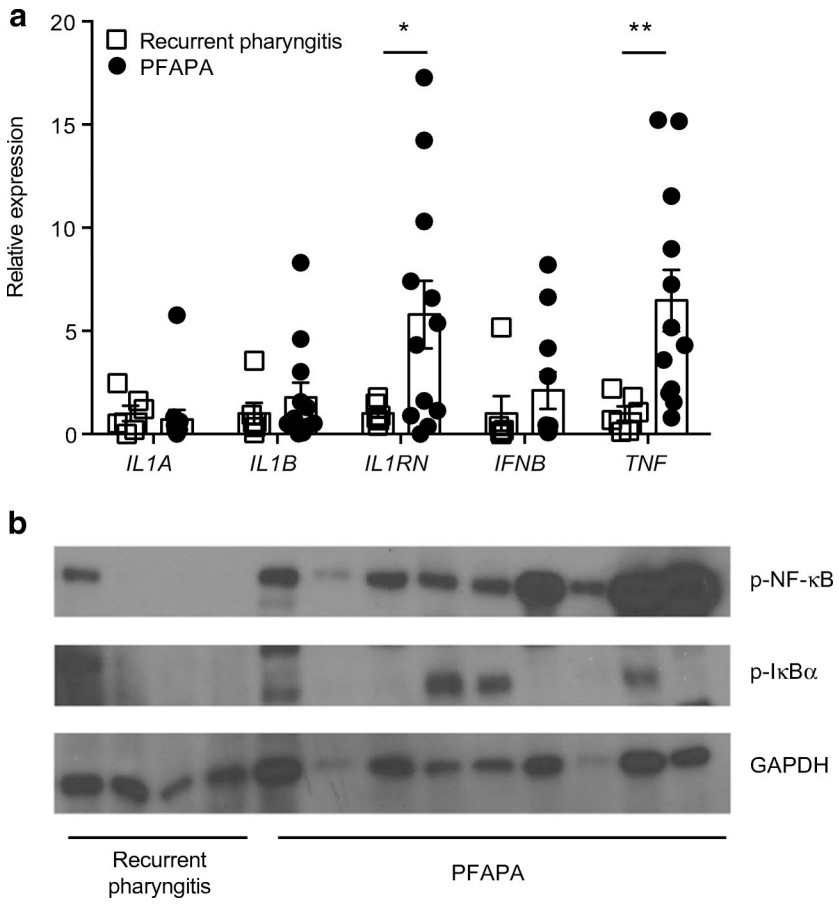

Figure 3. PFAPA tonsils have an inflammatory signature.

A, Gene expression analysis from whole tonsillar tissue from PFAPA (n=12) and recurrent pharyngitis control tonsils (n=6), demonstrates significant increases in *IL1RN* and *TNF* gene expression in PFAPA tonsils compared to controls. Each circle represents a different patient, average of technical triplicates, shown as the mean ± SEM. *, $p<0.05$, **p, <0.01 by Student's two-tailed t-test. **B,** Western blot of whole cell lysates from tonsils show increased expression of phosphorylated NF-κB, and IκBα. GAPDH was used as a loading control. Representative blot shows recurrent pharyngitis (n=4) and PFAPA (n=9) tonsils.

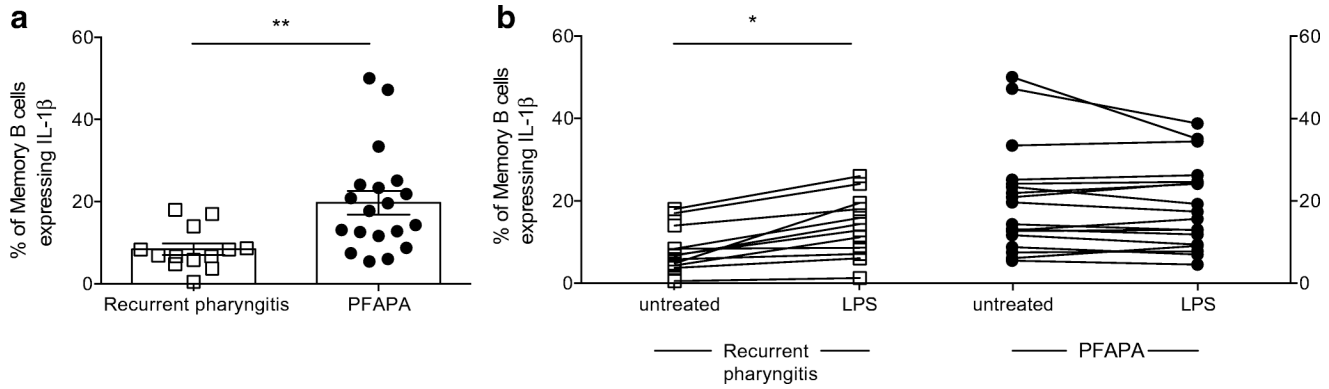

Figure 4. PFAPA tonsil derived memory B cells are primed with IL-1β at baseline.

A, CD19+ CD20+ CD27+ memory B cells from patients with PFAPA (n=19) have increased IL-1β positive cells compared to recurrent pharyngitis controls (n=12). Data shown as mean ± SEM, with ** $p<0.005$, by Student's t test. **B,** Upon stimulation with LPS, no enhancement in this population is observed, while a greater percentage of recurrent pharyngitis control tonsil-derived cells stain positively for IL-1β following stimulation. Data shown as mean ± SEM, with * $p<0.05$, by Student's t test.

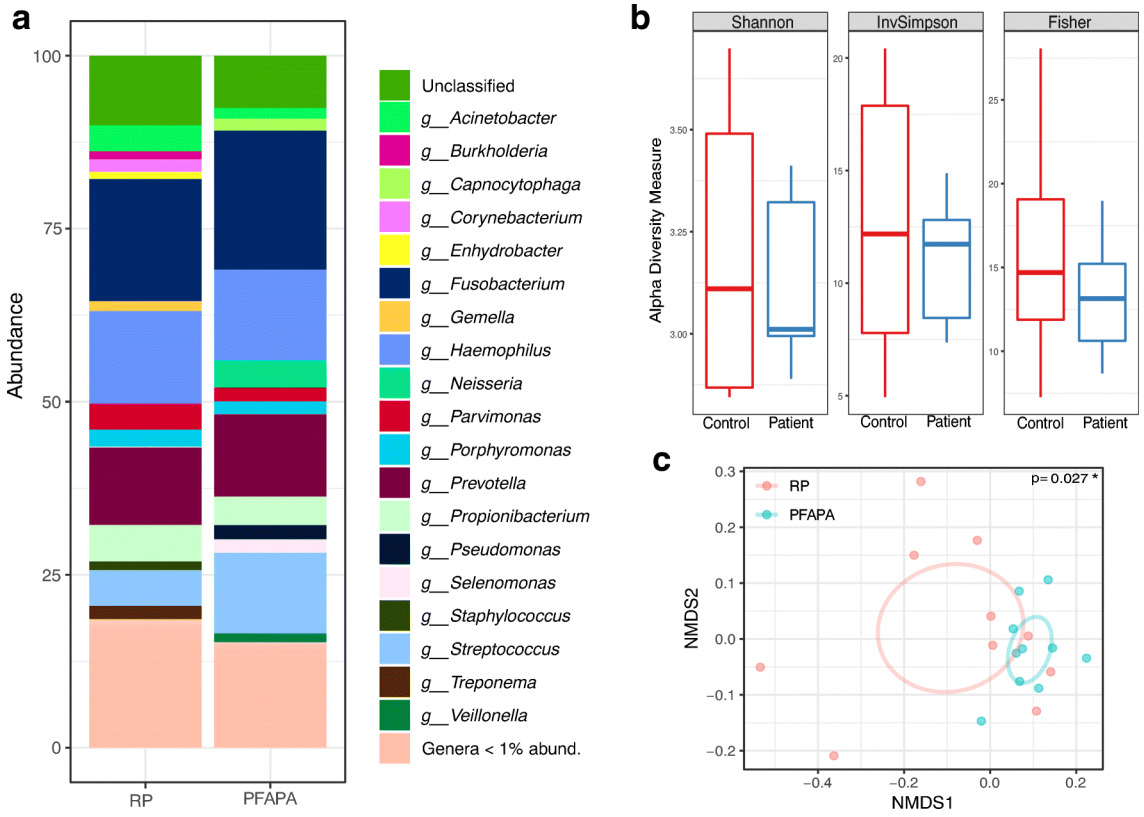

Figure 5. PFAPA patient tonsils have enhanced microbiome diversity.

A, Stack plot showing total diversity between recurrent pharyngitis (RP) control tonsils (right) and PFAPA tonsils (left) at genus level. **B,** Alpha diversity measures by Shannon, Inverse Simpson, and Fisher indices do not show significant differences between PFAPA patients and recurrent pharyngitis controls. **C,** Unweighted UniFrac shows differences in beta diversity ($p = 0.027$) (n=10 tonsils per group).

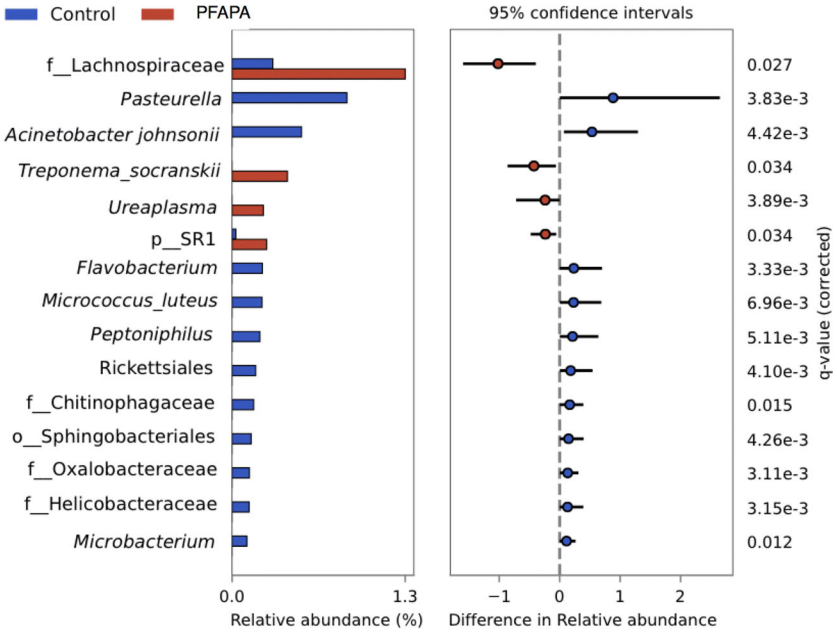

Figure 6. Differential taxa exist between PFAPA and recurrent pharyngitis control patient tonsils.

PFAPA patients and controls demonstrate 15 differentially abundant taxa (exact sequence variants show as a percent of relative abundance) with 95% confidence intervals shown at p value < 0.05 corrected using Benjamini-Hochberg False Discovery Rate method (n=10 tonsils per group). The analyses were performed using ANCOM in R.

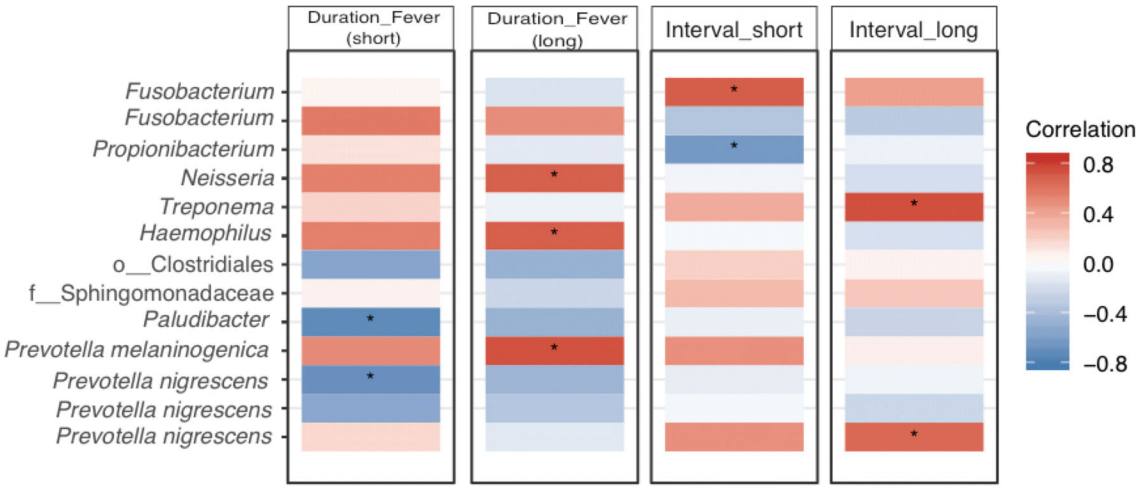

Figure 7. Specific taxa demonstrate significant associations with fever episode duration and frequency.

The associations were calculated using Spearman's Rank correlation at $p < 0.05$ corrected using Benjamini-Hochberg False Discovery Rate method. The red color gradient demonstrates positive correlation and the blue color gradient shows negative correlation. (n=10 tonsils per group, * $p < 0.05$.)

Table 1.

Patients undergoing tonsillectomy for PFAPA syndrome are similar to those opting for medical management

PFAPA Patients	All PFAPA Patients (n = 94)	Patients undergoing tonsillectomy (n = 65)	*p* value
Male : Female	48% male	48.3% male	1.00 *
Age of onset, mean ± SD	3.25 ± 2.70 years	2.78 ± 2.39 years	0.08
Age at Surgery, mean ± SD	n.a.	5.56 ± 3.35 years	n.a.
Febrile episodes	3.7–5.7 days	3.3–4.7 days	0.20
Asymptomatic Intervals	27–45 days	23–38 days	0.28
Tmax (°C, mean)	40.1°C (38.8–41.7°C)	40.2°C (38.8–41.7°C)	0.74

* by Fisher's exact test, all others by unpaired Student's t test n.a., not applicable

Table 2.

Clinical Characteristics of patients undergoing tonsillectomy for PFAPA syndrome compared to recurrent pharyngitis

Patients	**PFAPA Patients (n=65)**	**Recurrent Pharyngitis (n=23)**	***p* value**
Male : Female	48.3% male	47.8% male	1.00 *
Age of symptom onset (mean ± SD)	2.78 ± 2.39 years	3.9 ± 2.91 years	0.003
Episodes / year	9 – 13	5 – 8 (RP)	0.003
Age at surgery (mean ± SD)	5.56 ± 3.35 years	7 ± 2.58 years	0.09
Time from symptom o set to tonsillectomy	2.8 years * (0.758.6 years)	2.1 years (1–4 years)	0.18

* by Fisher's exact test, all others by unpaired Student's t test