IL-17C plays a role in the pathophysiology of acute recurrent tonsillitis

Megan Clapperton[1]*, Tash Kunanandam[2], Catalina D Florea[2], Margaret R Cunningham[4], Catriona M Douglas[3 4 5 x] , Gail McConnell[4 x].

[1]Department of Physics, SUPA, University of Strathclyde, Glasgow, UK

[2]Department of Otolaryngology – Head and Neck Surgery, Royal Hospital for Children, Glasgow, UK

[3]Department of Otolaryngology – Head and Neck Surgery, Queen Elizabeth University Hospital, Glasgow, UK

[4]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK

[5]Department of Medicine, University of Glasgow, Glasgow, UK

* Corresponding author: megan.clapperton@strath.ac.uk

[x] Co-senior Authors: These Authors contributed equally to project management and funding.

Keywords: tonsil, mesoscopy, immunofluorescence, western blot, IL-17C, IL-1β

Abstract

Most tonsillectomies in the UK are performed to treat acute recurrent tonsillitis (ART) and tonsil hypertrophy due to obstructive sleep apnoea (OSA). At present, the pathophysiology of these 2 common conditions is still poorly understood. We have performed optical mesoscopic imaging to reveal the spatial location of two interleukins – IL-17C and IL-1β – associated with tonsillar conditions, with the aim of understanding their possible role as specific disease biomarkers. Using immunofluorescence assays, we have shown that the expression of IL-17C is significantly higher in both the epithelial tissue ($p < 0.0001$) and core tissues ($p = 0.0007$) of specimens from patients with ART compared to patients with OSA ($n = 17$ patients). We also demonstrated significantly higher expression of IL-17C in homogenates of ART patient tissue compared to OSA tissue ($p = 0.05$). These data suggest that IL-17C may play an important role in the pathophysiology of ART, and may be a potential biomarker of disease.

1. Introduction

The location of the palatine tonsil at the opening to the pharynx is a preferential location for exposure to exogenous agents, such as bacteria and viruses, and their subsequent transport to lymphoid cells (1). Healthy palatine tonsils continuously stimulate production of lymphoid cells, such as T and B cells (2), and are essential in the immune system.

Tonsillectomy is the most common surgical operation performed in paediatrics in the United Kingdom, with acute recurrent tonsillitis (ART) and obstructive sleep apnoea (OSA) due to tonsil hypertrophy being the most common indications. Despite this operation being performed so frequently, there is still a poor understanding as to why some children develop ART and others develop OSA. Acute recurrent tonsillitis (ART) is an infection of the palatine tonsil that occurs when the presence and proliferation of pathogens in the tonsil exceed the defence instilled by the lymphoid and immunoglobulin producing cells (3). Group A Streptococcus is considered one of the most common causative agents in ART. However, more evidence has recently pointed to the role of bacterial biofilms in ART, which are known to be resistant to antimicrobial treatment. Tonsillectomy is the only definitive treatment when antibiotics fail (4). OSA is caused by lymphoid hypertrophy of the tonsils, causing intermittent obstruction in the oropharynx while children sleep (5), however, it is difficult to draw conclusions on the precise pathophysiological reasons behind tonsillar hypertrophy as there is no current standard for a healthy tonsil (5,6).

A recent study sought to identify the predominant innate lymphoid cells in of the palatine tonsils of ART patients compared to those patients with tonsillar hypertrophy (7). Higher levels of interleukin-17 (IL-17) secreting T lymphocytes was detected in blood and tonsillar mononuclear cells in ART patients. IL-17 is a family of cytokines involved with inflammatory conditions and is comprised of 6 subsets from IL-17A to IL-17F (8). IL-17C is functionally distinct from the rest of the family and is selectively induced in epithelial and germinal cells when exposed to bacterial stimulation (9), is upregulated early in infection (10), and is involved with maintaining the integrity of the epithelial barrier during infection (11). Studies have linked expression and function of IL-

17C to immune responses in adenoids (11). Bacteria and biofilms are known to play a role in ART, therefore, we hypothesized that IL-17C may be highly expressed in ART patient tissues in comparison to OSA tissue (12–14). Interleukin-1-beta (IL-1β) is a pro-inflammatory cytokine belonging to the IL-1 family of cytokines. Increased production of IL-1β has been demonstrated to be involved with chronic tonsillitis (15,16)

The Human Protein Atlas has confirmed that IL-17C is expressed in tonsil germinal centre cells (17) with no data available for protein expression, but very little is currently known about the spatial distribution of IL-17C in tonsil tissues.

We aimed to assess the spatial distribution of IL-17C and IL-1β in tonsil tissue from patients with ART or OSA to better understand the pathophysiology of these two common diseases. Following western blots to confirm the presence of both interleukins, we performed immunofluorescence (IF) labelling of tonsil tissue from patients with ART or OSA, and these were subsequently imaged using widefield fluorescence mesoscopy with the Mesolens (18,19). Multi-colour images of 4.4 mm x 3.0 mm areas from fluorescently labelled tonsil sections were obtained with sub-cellular resolution in order to assess the spatial distribution of both interleukins within tonsillar tissue.

2. Methods

All buffer and reagent recipes can be found in Supplementary Information.

2.1 Tonsil Specimen Acquisition

This study was approved by Biorepository Management Committee of NHS Greater Glasgow and Clyde, UK (Biorep 548). A total of 17 paediatric tonsils were collected after routine tonsillectomy at the Royal Hospital for Children, Glasgow, UK. Specimens were blinded for the purposes of preventing bias throughout the duration of the study. A total of 8 ART and 9 were collected across the duration of study.

Following tonsillectomy, whole tonsils were rapidly transported in sterile saline solution (0.9% Sodium Chloride, Baxter Healthcare Ltd, UK) to the Strathclyde Institute of Pharmacy and Biomedical Sciences at the University of Strathclyde. For western blot analysis, 8 ART and 8 OSA patient tonsils were used. For IF, 3 ART and 6 OSA patient tissues were used. The imbalance of ART to OSA patients is a consequence of study unblinding.

Tonsils were placed in a sterile Petri dish and a 30 cm ruler was used to measure the diameter. The depth at which tonsil sections were taken for imaging (described in 2.5). A graphical methods overview is represented in Figure 1.

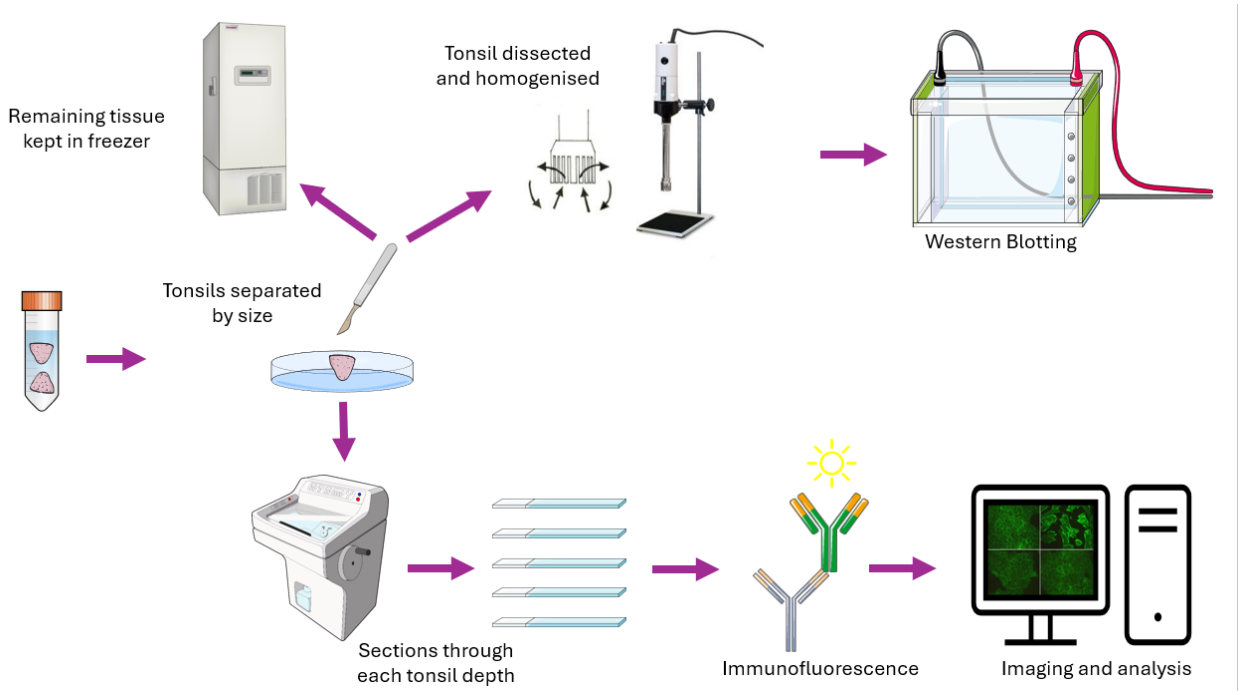

Figure 1. Graphical representation of methods. Tonsils were measured, with the larger specimen partially homogenised and analysed using a western blot, and the smaller specimen was sectioned to provide ten 10 µm slices at regular intervals throughout the whole thickness of the tonsil. These sections were immunolabelled with antibodies against IL-1β and IL-17C and were imaged using widefield fluorescence mesoscopy with the Mesolens. Figure prepared using vectors from Wikimedia commons and BioIcons (CC-BY license).

2.2 Tonsil homogenization and protein normalisation for SDS-PAGE immunoblotting,

Approximately 1/4 of the tonsil was dissected for homogenisation. Tonsil tissue of approximate equal size was taken for each sample and mechanically homogenized in 300-500µl of lysis buffer containing cOmplete™ Protease Inhibitor Cocktail on ice using a X 120 Portable Homogenizer (CAT Scientific, Germany). Samples were kept at 4°C on ice, vortexed and syringed through a 23G needle as part of the lysis process and then centrifuged at 16,000 x g for 10 mins for sample clarification prior to protein determination. Protein concentrations across all samples were normalised to the same protein concentration (500 µg/ml) with protein determination quantified using the well-established Pierce BCA Protein Assay (Thermo Fisher) following the recommended guidelines provided by the manufacturer. An equal volume of NuPAGE™ LDS Sample Buffer (2X) containing 100mM DTT was add to the normalised tissue homogenate sample to create a 1x sample for SDS-PAGE (with 250 µg/mL protein, 50mM DTT). Samples were stored at -80°C until processed by immunoblotting.

2.3 Antibodies

All antibodies used are described in Table 1. Primary antibodies targeting IL-1β and IL-17C were used for both blotting and IF imaging. A GAPDH primary antibody was used for immunoblotting as a housekeeping protein loading control for signal normalisation between samples. An AlexaFluor-350 conjugated secondary antibody was used for targeting IL-1β primary, and an AlexaFluor-594 conjugated secondary antibody for IL-17C primary for IF. For western blotting (WB), Goat anti rabbit and donkey anti mouse secondary antibodies were used for targeting IL-17C and IL-1β primary antibodies, respectively.

Table 1: Antibodies used for western blotting and immunofluorescence.

Antibody	Target	Primary / Secondary	Used for	Dilution / concentration	Manufacturer details
Mouse monoclonal IL-1β	Human	Primary	WB / IF	1:3000 (WB) 1:100 (IF)	2801, Invitrogen, ThermoFisher Scientific, USA
Rabbit polyclonal IL-17C	Human	Primary	WB / IF	1:3000 (WB) 1:100 (IF)	ab153896, Abcam, UK
GAPDH	Human	Primary	WB	1:100,000	ab8245 Abcam, UK
Goat anti-rabbit	Rabbit	Secondary	WB	1:7000	111-035-133, Jackson Laboratories
Donkey anti-mouse	Mouse	Secondary	WB	1:7000	715-035-150, Jackson Laboratories, USA
Goat anti-mouse (Alexa Fluor 350)	Mouse	Secondary	IF	2 µg/ml	A-11045, ThermoFisher Scientific, USA
Goat anti-rabbit (Alexa Fluor 594)	Rabbit	Secondary	IF	2 µg/ml	8889, Cell Signalling Technology, USA

2.4 Immunoblotting

15% SDS-Page gels were loaded with 20 µl of each sample, alongside 5 µl of protein ladder (26619, ThermoFisher Scientific, UK). Gel electrophoresis was performed at 120 V for 1 hour 30 minutes, or until the sample ran off the gel, before being transferred onto nitrocellulose membrane (10600002, Amersham plc, UK) for 2 hour 30 minutes at 295 mA. Membranes were subsequently blocked in 3% BSA (81-003-3, MilliporeSigma, USA) and then diluted in tris buffered saline with

Tween20 (TBS-T) for 2 hours with constant agitation. Primary antibodies were both diluted 1 in 3000 in 0.3% BSA**,** added to the membranes, and incubated overnight with constant agitation. Membranes were washed 3 x 5 minutes with TBS-T then incubated with secondary antibodies for 1 hour then washed again for 3 x 5 minutes with TBS-T. Membranes were placed in (4 ml each) enhanced chemiluminescence (ECL) buffers for 2 minutes, and fluorescence emission was detected with X-ray film. Film was developed in a dark room. Following development, the membranes were stripped at 60^{o}C at 60 RPM for 25 minutes in stripping buffer, and then blocked again for 2 hours in 3% BSA. The newly stripped and blocked membranes were incubated with 1 in 100,000 dilution GAPDH primary antibody (ab8245 Abcam, UK) in 0.3% BSA, and the above process was repeated using the mouse secondary antibody.

For each patient and each antibody, n = 3 repeats were performed. Analysis of blots was performed using ImageJ, where analysis replicates were performed at n = 3 for each sample. Data was presented using GraphPad Prism (GraphPad Prism v.8.0.2 for Windows, GraphPad Software, USA).

2.5 Preparation of tissue sections for widefield fluorescence mesoscopy

Prior to cryo-sectioning of tissue, slides (Z692255, Sigma-Aldrich, USA) were coated with 200 µl of a 0.1% solution of Poly-L-Lysine (P4832, Sigma-Aldrich, USA) to aid in tissue adhesion. The coating was left for 20 minutes to dry, and slides were subsequently rinsed with sterile PBS. Slides were air dried in a laminar flow cabinet overnight before use.

Freshly excised tonsils reserved for immunofluorescence were fixed overnight in 4% paraformaldehyde at 4 ^{o}C. After fixation, tonsils were rinsed 3 x 5 minutes with 1X PBS (10010023, ThermoFisher Scientific, USA) without agitation. Once washed, tonsils were placed into 22 mm x 22 mm x 20 mm square cryo-moulds (Peel-A-Way™ embedding moulds, E6032-1cs, Sigma-Aldrich, USA) and covered with optimal cutting temperature (OCT) embedding matrix for frozen

tissue (KMA-0100-00A, Cell Path, UK). Blocks were snap-frozen on dry ice before being stored at -80^{o}C prior to use.

Cryo-sectioning (CM1950, Leice Microsystems, Germany) of the tonsil tissue was performed with the optimised ambient temperature of cryostat set to -12 ^{o}C and the temperature at the specimen holder set to -12 ^{o}C. A new blade was inserted for each tonsil to ensure reliable sectioning. Ten tissue sections of 10 µm thickness were taken at regular intervals through the tonsil depth to sample through the tonsil mass. Sections were lifted to the Poly-L-Lysine coated slides and immediately stored at - 80 ^{o}C for long term storage, or, if used straight away, allowed to dry, and adhere to coverslips for up to 48 hours.

An additional three tissue sections were taken for each patient and processed as described in section 2.6, without the addition of antibodies. This was to facilitate the measurement of autofluorescence arising from the tissue and sample processing such that it could be accounted for when performing intensity-based analysis as described in section 2.8.

2.6 Immunofluorescence

Tonsil slides were blocked with 100 µL of antibody blocking buffer (see supplementary table for reagent compositions) for two hours at room temperature. The blocking buffer was aspirated, primary antibodies were diluted to 1:100 in antibody dilution buffer, and 100 µL of each primary antibody solution was added to each specimen. Slides were placed in a humidity chamber and incubated overnight at 4 ^{o}C. Slides were rinsed 3 x 5 minutes in PBS, aspirating waste. Information about antibodies used for IF can be found in Table 1. Goat anti-Mouse IgG (H+L) cross-absorbed secondary antibody conjugated with AlexaFluor 350 was diluted to 2 µg/ml in antibody dilution buffer and added at 100 µL volume to slides to target the IL-1β primary antibody and was incubated for 2 hours at room temperature in the dark. Slides were rinsed 3 x 5 minutes with PBS, aspirating waste. Anti-rabbit IgG (H+L), F(ab')$_2$ fragment conjugated with the fluorescent label Alexa Fluor 594 was diluted to 2 µg/ml in antibody dilution buffer and was added at 100 µL volume

to slides to target the IL-17C primary antibody. This was incubated for 2 hours at room temperature in the dark. Secondary antibodies were sequentially added to minimise potential cross-reactivity, though order of sequence was of no effect. Slides were rinsed 3 x 5 minutes with PBS, with the last rinse step being performed at 37 ^{o}C in a hot cupboard. Samples were then mounted onto type 1.5 coverslips using VectaMount (H-5000, Vector Laboratories).

HeLa cells and HMC3 cells were used as positive controls for IL-17C and IL-1β, respectively (20,21). HeLa cells were exposed to ambient air for 30 minutes before fixation prior to IF staining to promote production of IL-17C. Data demonstrating this are available in Supplementary Information (Supplementary figure 1).

2.7 Mesoscale imaging

Brightfield transmission and widefield fluorescence images of tissue sections were obtained using the Mesolens and data were captured using a chip-shifting camera sensor (VNP-29MC, Vieworks, Republic of Korea). This system captures images by shifting the 29-megapixel CCD chip in a 3 x 3 array, resulting in an effective 260-megapixel image to capture data from a 4.4 mm x 3.0 mm field of view. Fluorescence excitation was achieved by use of 405 nm or 585 nm LEDs from a pE-4000 LED illuminator (CoolLED, UK) to excite the IL-1β and IL-17C antibody pairs, respectively. Measurements of tissue autofluorescence was taken for each wavelength used for excitation using unlabelled specimens. Widefield mesoscopy was performed with the Mesolens correction collars set for water immersion (n = 1.33). The choice of fluorescent secondary antibodies was informed by the Pinkel-type (22) barrier filters used for Mesolens imaging (23), which had emission bands at 417 ± 10 nm and 635 ± 20 nm.

2.8 Image Analysis

An image analysis pipeline was developed to reduce the contribution of tissue autofluorescence in the quantification of image data. The autofluorescence signal intensity was measured from n =

3 unlabelled tissue sections obtained from the top, centre, and bottom of each of the n= 9 tonsil specimens. These were imaged as described above, with data acquired at emission wavelengths of 417 ± 10 nm and 635 ± 20 nm. Within each image, n = 9 regions of interest (ROIs) were selected using Fiji (24) and the mean intensity value of these n = 9 ROIs was used as the mean value of tissue autofluorescence signal intensity for each data channel for each tonsil. To evaluate the distribution of IL-17C and IL-1β within patient tissues, the mean autofluorescence signal intensity for each data channel was subtracted from the raw data for each tonsil specimen before further processing.

Brightfield transmission images were thresholded using Otsu thresholding in the FIJI 'threshold' tool, to create a mask for intensity measurements. Tonsil epithelium was visually distinguished from the rest of the tissue by assessing its histological features and was evaluated to be between 0.2 mm and 1 mm thick. This agreed with the measurements of Olofsson *et al* (25). Using Fiji, a freehand selection ROI was drawn to create masks of the epithelial and the core tissues and extracting these data. The core tissue in this context refers to all tissue present which is not classed as epithelium, and included the crypts, lymphoid follicles and extrafollicular area. The mean fluorescence signal intensity within each ROI for each channel was measured using the 'measure' tool in Fiji.

Data was plotted using Prism (GraphPad Prism v.8.0.2 for Windows, GraphPad Software, USA). Values of mean fluorescence signal intensity, and the ratio of fluorescence intensities in the epithelium to core tissues for both ART and OSA were plotted. For the ratio measurements, outliers were removed using Prism 'Identify Outliers' tool, choosing the ROUT (robust regression and outlier removal) (26) method with Q = 1%. Data were normalised for presentation purposes, using the 'Normalise' function in Prism.

All quantitative analysis used raw data. However, for presentation purposes, IF images were contrast adjusted using the Contrast Limited Adaptive Histogram Equalisation (CLAHE) function in

FIJI (24,27) with the default parameters (block size = 127, histogram bins = 256, maximum slope = 3).

2.9 Statistical Analysis

Statistical analysis was performed using Prism, using the Kruskal-Wallis multiple-comparisons test for comparison of non-Gaussian distributed data. For western blot analysis, data were normally distributed and therefore an unpaired t-test was used to assess significance with $\alpha = 0.05$.

3 Results

3.1 IL-17C is expressed in both ART and OSA tonsils

Through analysis of western blots, we characterized the expression levels of IL-17C in human paediatric palatine tonsils, for the first time. We found that IL-17C (approximately 15-20 kDa) was expressed in all patients (n=16, with n=3 repeats per patient), as was IL-1β (approximately 30-35 kDa).

Blots were quantified to show relative intensities against the house-keeping protein GAPDH, relative band intensity was measured and compared using FIJI. Relative intensity was plotted for both IL-17C and IL-1β in both patient groups. Tissue from patients with ART (n = 8) and OSA (n = 8) both showed expression of IL-17C. ART tissue homogenates had significantly higher expression levels of IL-17C (mean = 1.45) than the OSA patients (mean = 0.77), $p = 0.05$. Conversely, patients with ART typically had lower expression of IL-1β (mean = 0.35) while OSA had higher expression (mean = 0.66), but these data were not statistically significant ($p = 0.11$).

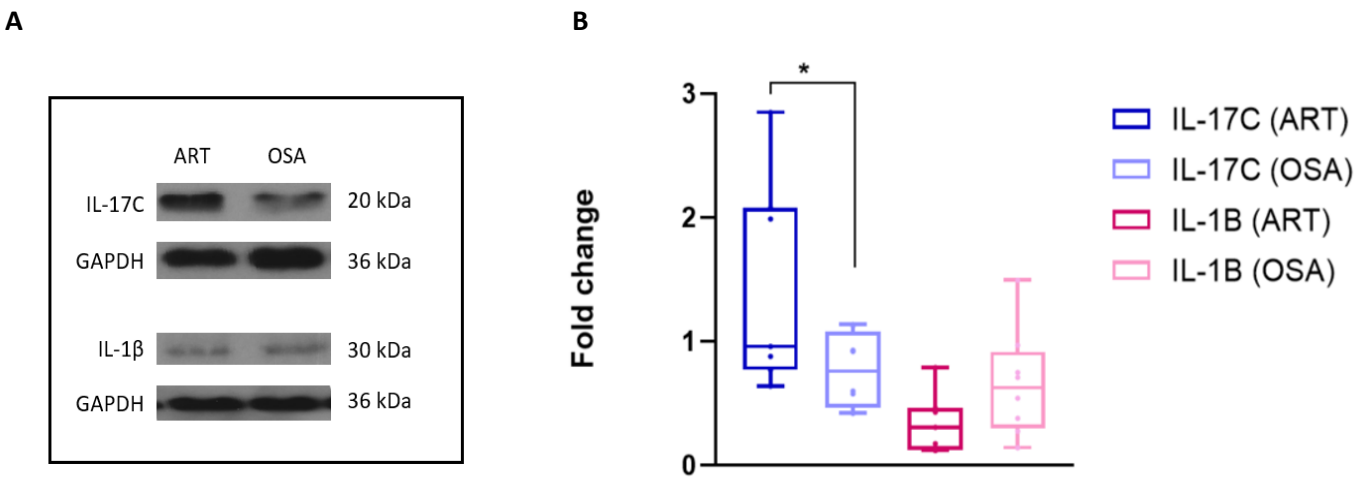

Figure 2 Protein quantification by western blot confirmed presence of IL-17C in both ART and OSA patient tonsil tissues. A) Representative western blots of tonsil homogenates showing increase in expression of IL-17C in ART compared to OSA, with no difference in IL-1 β expression. B) Graph shows relative band intensity against GAPDH, confirming that tissue from patients with ART have increased expression of IL-17C compared to tissue from patients with OSA (unpaired t-test, p = 0.05), whereas the levels of IL-1β in both patient groups showed similar expression.

3.2 IL-17C is expressed in the epithelium of patients with ART

IL-17C was shown to be predominantly concentrated in the epithelia of tissues from patients with ART. This was verified by qualitative examination of images. Figure 3 shows representative Mesolens images of tonsil tissue from patients with OSA (Fig 3.A-C) and ART (Fig 3.D-F). IL-1β is expressed throughout the tonsil tissues in tissue irrespective of disease, but IL-17C is mainly expressed in the tonsil epithelium of tissue from patients with ART. It is also evident that tissue from patients with OSA does not show the same level of IL-17C expression in the epithelium.

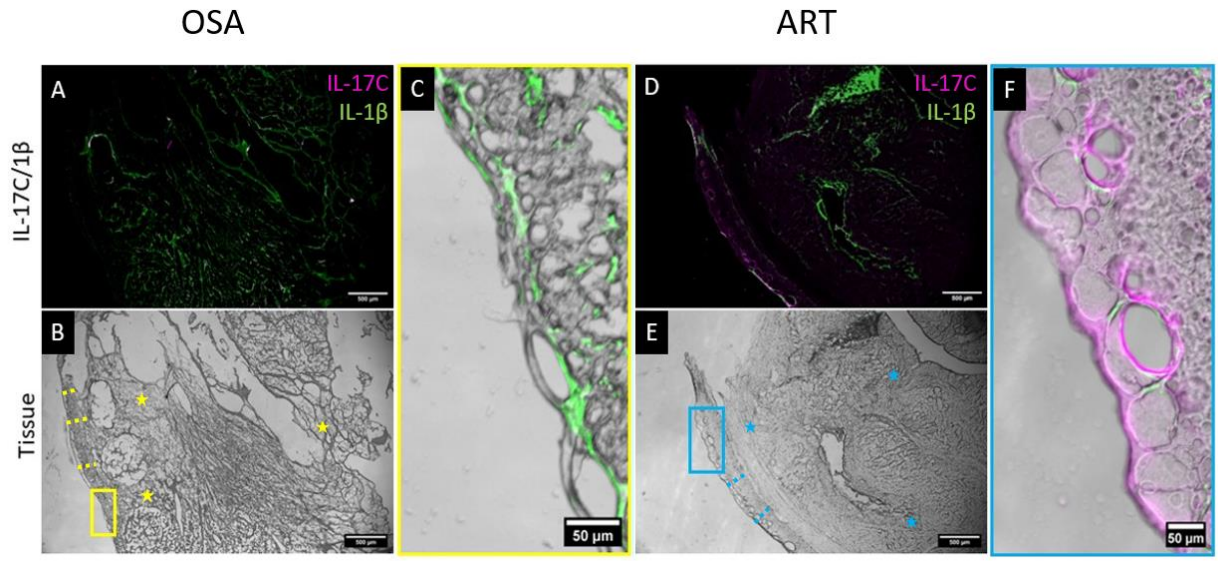

Figure 3 Representative Mesolens images of tonsil tissue. IL-17C is shown in magenta, and IL-1β is shown in green. Dotted lines represent regions considered epithelial tissue, and stars indicate regions considered core tissue (tissue comprising of lymphoid follicles, extrafollicular region and connective tissues) (A) shows a full field of view image of a tonsil section from a patient with OSA imaged with widefield fluorescence mesoscopy. (B) is a brightfield transmission image of the same region shown in (A). A yellow box highlights a small region of interest (ROI). (C) shows a digital zoom of the ROI highlighted in (B). No expression of IL-17C is present, although IL-1β is clearly visible. (D) shows a full field of view image of a tonsil section from a patient with ART imaged with widefield fluorescence mesoscopy. (E) is a brightfield transmission image of the same region shown in (D). A cyan box highlights a small ROI. (F) shows a digital zoom of the ROI indicated by the cyan box in (E). Unlike (C), almost no IL-1β expression is observed, but IL-17C is clearly visible in the surface epithelium of the tonsil.

3.3 IL-17C and IL-1β are expressed more highly in ART than OSA in both the epithelium and the core of tonsil tissue

Through segmentation-based image analysis, the values of mean fluorescence signal intensity were assessed in images obtained from both the epithelium and core of the tonsils from patients with ART or OSA. Data were normalised for presentation purposes.

It was found that the expression of IL-17C was significantly higher in the epithelial tissues of tissue from patients with ART (mean = 0.19, n = 28) compared to tissue from patients with OSA (mean = 0.06, n = 54), $p < 0.0001$. IL-17C was also more highly expressed in the core tissue sections of

tonsils from patients with ART (mean = 0.12, n = 28) when compared to tissue from the same tonsillar region from patients with OSA (mean = 0.06, n = 54), p = 0.0007. This suggests that IL-17C is more highly expressed throughout the tonsils of patients with ART than patients with OSA.

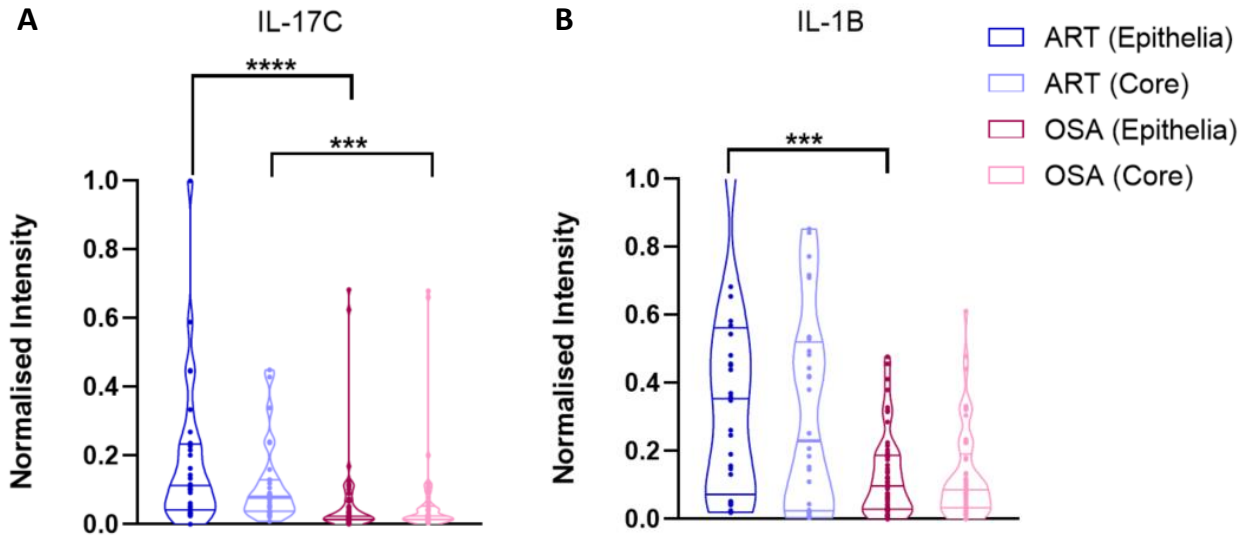

Figure 4: Normalised mean fluorescence intensities for epithelial and core tissues for patients with ART or OSA demonstrates that IL-17C is significantly higher expressed throughout the tonsil tissue in ART vs OSA. A) Expression of IL-17C is significantly higher in the epithelia of tissue from patients with ART (pink) than in the epithelia of tissue from patients with OSA (green), $p < 0.0001$. Additionally, expression of IL-17C is significantly higher in the core of the tonsil in patients with ART (blue) compared with the expression level of the same interleukin in the core of the tonsil of patients with OSA (orange), $p = 0.0007$. B) Expression of IL-1β is significantly higher in the tonsil epithelia of patients with ART compared to patients with OSA, $p = 0.002$. No significant difference in levels of IL-1β expression was found in the core of tonsils from patients with either ART or OSA. These data indicate that IL-17C expression is higher overall in the ART tissue than OSA tissue, with considerably higher expression levels in the tonsil epithelium.

IL-1β showed a different spatial pattern of expression throughout the tonsil. While the expression of IL-1β was significantly higher in the epithelial tissue of patients with ART (mean = 0.38, n = 28) compared to patients with OSA (mean = 0.13, n = 54), p = 0.002, no statistically significant difference in the level expression of IL-1β was observed in the tonsil core between tissue from patients with ART and patients with OSA. Although the increased expression level of IL-1β in the

tonsil epithelium of tissue from patients with ART indicates its increased expression in ART, the data presented in Figure 4 suggests that, with its higher incidence throughout the ART tonsil and increased statistical significance, IL-17C plays a greater role in host response in ART tonsils.

4 Discussion

Acute recurrent tonsillitis and obstructive sleep apnoea are two of the most common diseases of childhood, causing significant morbidity to millions of children worldwide (28). Despite this, the disease pathophysiology is still poorly understood. The diagnosis of ART is clinical scoring (29), and the diagnosis of obstructive sleep apnoea is based on clinical history and, on occasion, sleep studies (30,31). Currently the only definitive treatment is surgery, with its associated complications. With ART in particular the main aim of this work was to better understand the pathophysiology of these two diseases, with the secondary aim of identifying potential biomarkers that may help with diagnosis. Our work demonstrates for the first time that that IL-17C is expressed at significantly higher levels throughout the tonsil mass in ART tonsil tissue than in OSA.

IL-17C was first identified in 2000, and it has emerged as a unique member of the pro-inflammatory cytokine group (32). IL-17C plays a critical role in early infection defence; its synergistic role with TNF-α upregulates other cytokines, chemokines, and antimicrobial clearance agents (10,33). Unlike other IL-17 family members, IL-17C does not appear to be expressed by leukocytes but rather non-haematopoietic cells (9). IL-17C is specifically produced in epithelial cells. In a disease model for psoriasis, keratinocytes are the main source of IL-17C (34,35), with other epithelial cells producing the cytokine including colonic epithelial cells (9), kidney cells (36) and respiratory cells (37). IL-17C regulates innate immune functions of the epithelial cells in an autocrine manner (37). In the gut, IL17-C induces the expression of inflammatory mediators and antimicrobial peptides in colon epithelial cells, In the respiratory tract Pfeifer *et al* demonstrated that IL-17C is present in the human bronchial epithelium of patients with infection related lung disease (38). They also demonstrated that IL-17C is the only member of the IL-17 family that is

induced by bacterial pathogens. ART is frequently caused by bacterial infections; furthermore we have previously demonstrated that tonsils excised from acute recurrent tonsillitis patients have a significant biofilm burden (39). Pfeifer *et al* demonstrated that IL-17C is present in the bronchial epithelium of stable COPD and cystic fibrosis patients, two diseases where patients are known to have biofilms leading to repeated infections and antimicrobial requirements. IL-17 may be a factor contributing to the excessive inflammation in the respiratory tract of patients with chronic lung disease even in the absence of infection (40,41). It may be the IL-17C is driving this inflammatory pathway in patients with acute recurrent tonsillitis, given that we have demonstrated IL-17C expression in these patients is significantly higher than in obstructive sleep apnoea patients. We have shown that there is increased expression of IL-17C in the epithelium and the core tissues of ART patient tissue compared to OSA tissue. Higher expression in the epithelium is to be expected since IL-17C is predominantly expressed by epithelial cells. The increased expression in the core tissues arises from the presence of crypts and the crypt epithelium. IL-17C has been shown to be present in the saliva of COPD patients who are having an acute exacerbation, however there is no published data describing IL-17C levels in patients having an episode of acute tonsillitis.

IL-1β is a potent pro-inflammatory cytokine that is crucial for host-defence responses to infection and injury but it can also exacerbate damage during chronic disease and acute tissue injury (42). Spikermann *et al* examined serum and saliva samples from ART patients compared to 'healthy' control volunteers and found that IL-1β expression was higher in the ART cases, however they did not assess for IL-1β in the histology of the excised tonsils (43). Furthermore, in their study, they used healthy controls to compare, not controls with obstructive sleep apnoea To conflate this further, Babakurban *et al* showed no difference between expression of IL-1β between OSA and ART patients (44). We have shown that there a significant increase in expression of IL-1β in the tonsillar epithelial tissues of tissue from patients with ART compared to OSA patient tissue, however, no such difference is seen in the core tissues. This is a subtle but potentially important finding. Other works considered expression levels throughout the tonsil or from patient serum / saliva, but our data shows that the expression level varies significantly in different regions in the

tonsil. The next step in our work would be to assess the saliva of both ART patients and OSA patients to assess if IL-1β differs in expression in these two distinct groups.

We have not demonstrated significant differences in expression at the protein level between ART homogenates and OSA homogenates with western blots. The significance in the IF analysis performed in this study arises because of the specificity of image analysis. Using FIJI, it was possible to separate the tonsil images into epithelium and core tissues and measure the fluorescent signal arising from these regions. This level of specificity was not possible with western blotting due to the tissue homogenates containing regions from both epithelia and core tissues. IL-1B has been demonstrated to play a role in several autoinflammatory diseases such as familial Mediterranean fever (FMF) (45), pyogenic arthritis, pyoderma gangrenosum, acne (PAPA) (46), Adult and Juvenile Stills disease (47) and Schnitzler syndrome (48). All these diseases are different from autoimmune diseases in that they are characterized by recurrent episodes of fever with debilitating local and systematic inflammation. The aetiology for some patients with ART remains unclear, and it may be that there is an autoinflammatory aspect to the disease. IL-1β has also been implicated in the pathophysiology of pulmonary fibrosis, a chronic progressive fibrotic lung pathological change, where normal lung tissue is replaced by deposited extracellular matrix. Significant fibrosis has been reported in the tonsils of patients with acute recurrent tonsillitis (49), suggesting that IL-1β may play a role in this process.

There are currently no point of care detection methods for ART in GP practices in the UK, other than examination based diagnoses provided by the SIGN guidelines (29). Currently the clinical dilemma is if antibiotics should be prescribed to treat the patient. If the disease is not caused by bacterial infection and is instead caused by a virus, then antibiotics should not be prescribed. IL-17C is a marker signalling host defence to infection as well as the inflammatory pathogenesis of autoimmune diseases. It may be that the pathophysiology of tonsillitis has an underlying autoimmune or autoinflammatory element, that is currently poorly understood. While undoubtedly some patients have a very clear bacterial infection that requires antibiotics, in patients who do not have clear acute bacterial infection, but who get several episodes of tonsillitis

a year necessitating tonsillectomy, further studies are required on the immune aspect of their disease.

Saliva has been previously used to determine expression levels of biomarkers of tonsillar diseases (44,50) and others have demonstrated its use in delineating between ART and tonsillar abscess (51). A limitation of this study is that we did not correlate serum or saliva measurements with tonsil tissue. Further work in assessing the presence of IL-17C and IL-1β in saliva or serum as performed by Spiekermann *et al* (43), would be essential for helping to better understand the disease pathophysiology and help identify determining if these cytokines can be used as a pre-operative biomarker of disease. This would then enable more targeted disease management for patients.

5 Conclusion

We have demonstrated that IL-17C plays a role in the pathophysiology of acute recurrent tonsillitis and may be a potential biomarker for assessing disease. We have shown that IL-17C is expressed at significantly higher levels in tissues from patients- with ART in both immunoblotting and immunofluorescence assays. Analysis of tonsil homogenates demonstrated significantly higher expression of IL-17C in patients with ART compared to patients with OSA. Similarly, we observed using IF assays that IL-17C is expressed at higher levels in both the epithelia and the core of tissue from patients with ART than in specimens from patients with OSA. These data collectively demonstrate the increased expression of IL-17C in ART patient tissue, highlighting its role in the pathogenesis of these patients. Further research into the immune aspect of tonsillitis is required to better understand this very common disease.

Data Availability Statement

The data generated and analysed during the study are available upon reasonable request from the corresponding author.

Acknowledgements

This work was supported by the Glasgow Children's Hospital Charity, grant number GCHCRF/PHD/2020/02. G.M. was funded by the Medical Research Council (MR/K015583/1), the Biological Sciences Research Council (BB/T011602/1) and The Leverhulme Trust. C.D was funded by the Medical Research Council (MR/W030381/1) and a Chief Science Office NRS fellowship. Thanks to Holly Brown at the University of Strathclyde for generously gifting HMC3 cells.

Author Contributions

GM and CMD acquired funding, supervised, and administered the project. GM, MC, MRC, and CMD conceived the experiments and defined the methodology. MC performed the experiments, imaging and data analysis. MRC performed the tissue homogenisation, protein normalisation and sample preparation for Western blotting studies. CDF and TK provided resources. MC and GM wrote the original draft, and MC, GM, CMD, MRC and CDF were involved in reviewing and editing the manuscript.

Competing Interests

The authors declare no competing interests.

References

1. Arambula A, Brown JR, Neff L. Anatomy and physiology of the palatine tonsils, adenoids, and lingual tonsils. World J Otorhinolaryngol - Head Neck Surg. 2021 Jun;7(3):155–60.

2. Hagel JP, Bennett K, Buffa F, Klenerman P, Willberg CB, Powell K. Defining T Cell Subsets in Human Tonsils Using ChipCytometry. J Immunol. 2021 Jun;206(12):3073–82.

3. Nave H, Gebert A, Pabst R. Morphology and immunology of the human palatine tonsil. Anat Embryol (Berl). 2001 Nov;204(5):367–73.

4. Wilson JA, O'Hara J, Fouweather T, Homer T, Stocken DD, Vale L, et al. Conservative management versus tonsillectomy in adults with recurrent acute tonsillitis in the UK (NATTINA): a multicentre, open-label, randomised controlled trial. The Lancet. 2023 Jun;401(10393):2051–9.

5. Potsic WP. Assessment and treatment of adenotonsillar hypertrophy in children. Am J Otolaryngol. 1992 Sep;13(5):259–64.

6. Sarmiento Varon L, De Rosa J, Machicote A, Billordo LA, Baz P, Fernández PM, et al. Characterization of tonsillar IL10 secreting B cells and their role in the pathophysiology of tonsillar hypertrophy. Sci Rep. 2017 Sep;7(1):11077.

7. Comparison of innate lymphoid cells from tissue and blood in chronic tonsillitis and tonsillar hypertrophy - PubMed [Internet]. [cited 2023 Dec 11]. Available from: https://pubmed.ncbi.nlm.nih.gov/37742461/

8. Huangfu L. The IL-17 family in diseases: from bench to bedside \textbar Signal Transduction and Targeted Therapy. [cited 2023 Dec 11]; Available from: https://www.nature.com/articles/s41392-023-01620-3

9. Ramirez-Carrozzi V, Sambandam A, Luis E, Lin Z, Jeet S, Lesch J, et al. IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. Nat Immunol. 2011 Dec;12(12):1159–66.

10. Swedik S, Madola A, Levine A. IL-17C in human mucosal immunity: More than just a middle child. Cytokine. 2021 Oct;146:155641.

11. Min HJ, Kim KS. IL-17C expression and its correlation with pediatric adenoids: a preliminary study. Int J Med Sci. 2020 Sep;17(17):2603–10.

12. Chole RA, Faddis BT. Anatomical Evidence of Microbial Biofilms in Tonsillar Tissues: A Possible Mechanism to Explain Chronicity. Arch Otolaryngol Neck Surg. 2003 Jun 1;129(6):634.

13. Diaz RR. Relevance of biofilms in pediatric tonsillar disease. Eur J Clin Microbiol Infect Dis. 2011;30:1503–9.

14. Galli J, Calò L, Posteraro B, Rossi G, Sterbini FP, Paludetti G, et al. Pediatric oropharyngeal microbiome: Mapping in chronic tonsillitis and tonsillar hypertrophy. Int J Pediatr Otorhinolaryngol. 2020 Dec;139:110478.

15. Agren K, Andersson U, Litton M, Funa K, Nordlander B, Andersson J. The production of immunoregulatory cytokines is localized to the extrafollicular area of human tonsils. Acta Otolaryngol (Stockh). 1996 May;116(3):477–85.

16. Ågren K, Andersson U, Nordlander B, Nord CE, Linde A, Ernberg I, et al. Upregulated Local Cytokine Production in Recurrent Tonsillitis Compared with Tonsillar Hypertrophy. Acta Otolaryngol (Stockh). 1995 Jan;115(5):689–96.

17. Tissue expression of IL17C - Staining in tonsil - The Human Protein Atlas [Internet]. [cited 2023 Oct 5]. Available from: https://www.proteinatlas.org/ENSG00000124391-IL17C/tissue/tonsil

18. McConnell G, Trägårdh J, Amor R, Dempster J, Reid E, Amos WB. A novel optical microscope for imaging large embryos and tissue volumes with sub-cellular resolution throughout. eLife. 2016;5:18659.

19. Rooney LM, Amos WB, Hoskisson PA, McConnell G. Intra-colony channels in E. coli function as a nutrient uptake system. ISME J. 2020;14:2461–73.

20. Wan C, Latter JL, Amirshahi A, Symonds I, Finnie J, Bowden N, et al. Progesterone Activates Multiple Innate Immune Pathways in Chlamydia trachomatis-Infected Endocervical Cells. Am J Reprod Immunol. 2014;71(2):165–77.

21. The human microglial HMC3 cell line: where do we stand? A systematic literature review \textbar Journal of Neuroinflammation \textbar Full Text [Internet]. [cited 2023 Dec 5]. Available from: https://jneuroinflammation.biomedcentral.com/articles/10.1186/s12974-018-1288-0

22. Pinkel D, Gray J, Segraves R, Waldman F, Trask B, Yu LC, et al. Fluorescent Nucleic Acid Hybridization Methods. In: New Technologies in Cytometry [Internet]. SPIE; 1989 [cited 2023 Dec 5]. p. 123–32. Available from: https://www.spiedigitallibrary.org/conference-proceedings-of-spie/1063/0000/Fluorescent-Nucleic-Acid-Hybridization-Methods/10.1117/12.951898.full

23. Foylan S, Schniete JK, Kölln LS, Dempster J, Hansen CG, Shaw M, et al. Mesoscale standing wave imaging. J Microsc [Internet]. [cited 2023 Dec 5];n/a(n/a). Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/jmi.13189

24. Schindelin J. Fiji: an open-source platform for biological-image analysis. Nat Meth. 2012;9:676–82.

25. Olofsson K, Hellström S, Hammarström ML. The surface epithelium of recurrent infected palatine tonsils is rich in γδ T cells. Clin Exp Immunol. 1998 Jan;111(1):36–47.

26. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics. 2006 Mar;7(1):123.

27. Reza AM. Realization of the Contrast Limited Adaptive Histogram Equalization (CLAHE) for Real-Time Image Enhancement. J VLSI Signal Process-Syst Signal Image Video Technol. 2004 Aug;38(1):35–44.

28. Mitchell RB, Archer SM, Ishman SL, Rosenfeld RM, Coles S, Finestone SA, et al. Clinical Practice Guideline: Tonsillectomy in Children (Update). Otolaryngol Neck Surg. 2019 Feb;160(1_suppl):S1–42.

29. Clement WA, Dempster JH. Implementation by Scottish otolaryngologists of the Scottish Intercollegiate Guidelines Network document *Management* of Sore Throats and the Indications for Tonsillectomy : four years on. J Laryngol Otol. 2004 May;118(5):357–61.

30. Savini S, Ciorba A, Bianchini C, Stomeo F, Corazzi V, Vicini C, et al. Assessment of obstructive sleep apnoea (OSA) in children: an update. Acta Otorhinolaryngol Ital. 2019 Oct;39(5):289–97.

31. Incerti Parenti S, Fiordelli A, Bartolucci ML, Martina S, D'Antò V, Alessandri-Bonetti G. Diagnostic accuracy of screening questionnaires for obstructive sleep apnea in children: A systematic review and meta-analysis. Sleep Med Rev. 2021 Jun;57:101464.

32. Li H, Chen J, Huang A, Stinson J, Heldens S, Foster J, et al. Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. Proc Natl Acad Sci. 2000 Jan 18;97(2):773–8.

33. Song X, Zhu S, Shi P, Liu Y, Shi Y, Levin SD, et al. IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. Nat Immunol. 2011 Dec;12(12):1151–8.

34. Johnston A, Fritz Y, Dawes SM, Diaconu D, Al-Attar PM, Guzman AM, et al. Keratinocyte Overexpression of IL-17C Promotes Psoriasiform Skin Inflammation. J Immunol. 2013 Mar 1;190(5):2252–62.

35. Lauffer F, Jargosch M, Baghin V, Krause L, Kempf W, Absmaier-Kijak M, et al. IL-17C amplifies epithelial inflammation in human psoriasis and atopic eczema. J Eur Acad Dermatol Venereol. 2020 Apr;34(4):800–9.

36. Wang F, Yin J, Lin Y, Zhang F, Liu X, Zhang G, et al. IL-17C has a pathogenic role in kidney ischemia/reperfusion injury. Kidney Int. 2020 Jun;97(6):1219–29.

37. Jamieson KC, Traves SL, Kooi C, Wiehler S, Dumonceaux CJ, Maciejewski BA, et al. Rhinovirus and Bacteria Synergistically Induce IL-17C Release from Human Airway Epithelial Cells To Promote Neutrophil Recruitment. J Immunol. 2019 Jan;202(1):160–70.

38. Pfeifer P, Voss M, Wonnenberg B, Hellberg J, Seiler F, Lepper PM, et al. IL-17C Is a Mediator of Respiratory Epithelial Innate Immune Response. Am J Respir Cell Mol Biol. 2013 Apr;48(4):415–21.

39. Clapperton M, Kunanandam T, Florea CD, Douglas CM, McConnell G. Multimodal optical mesoscopy reveals the quantity and spatial distribution of gram-positive biofilms in *ex vivo* tonsils [Internet]. Biophysics; 2023 Jul [cited 2023 Nov 14]. Available from: http://biorxiv.org/lookup/doi/10.1101/2023.07.03.547470

40. Ritzmann F, Lunding LP, Bals R, Wegmann M, Beisswenger C. IL-17 Cytokines and Chronic Lung Diseases. Cells. 2022 Jul 6;11(14):2132.

41. Vella G, Ritzmann F, Wolf L, Kamyschnikov A, Stodden H, Herr C, et al. IL-17C contributes to NTHi-induced inflammation and lung damage in experimental COPD and is present in sputum during acute exacerbations. Yildirim AÖ, editor. PLOS ONE. 2021 Jan 7;16(1):e0243484.

42. Osei ET, Brandsma CA, Timens W, Heijink IH, Hackett TL. Current perspectives on the role of interleukin-1 signalling in the pathogenesis of asthma and COPD. Eur Respir J. 2020 Feb;55(2):1900563.

43. Spiekermann C, Seethaler A, McNally A, Stenner M, Rudack C, Roth J, et al. Increased levels of S100A8/A9, IL-1ß and IL-18 as a novel biomarker for recurrent tonsillitis. J Inflamm. 2021 Jun;18(1):24.

44. Türko S, Babakurban L. The Role of Cytokines in the Pathophysiology of Recurrent Tonsillitis.

45. Sharma D, Sharma BR, Vogel P, Kanneganti TD. IL-1β and Caspase-1 Drive Autoinflammatory Disease Independently of IL-1α or Caspase-8 in a Mouse Model of Familial Mediterranean Fever. Am J Pathol. 2017 Feb;187(2):236–44.

46. Brenner M, Ruzicka T, Plewig G, Thomas P, Herzer P. Targeted treatment of pyoderma gangrenosum in PAPA (pyogenic arthritis, pyoderma gangrenosum and acne) syndrome with the recombinant human interleukin-1 receptor antagonist anakinra. Br J Dermatol. 2009 Nov;161(5):1199–201.

47. Sfriso P, Bindoli S, Galozzi P. Adult-Onset Still's Disease: Molecular Pathophysiology and Therapeutic Advances. Drugs. 2018 Aug;78(12):1187–95.

48. De Koning HD, Schalkwijk J, Stoffels M, Jongekrijg J, Jacobs JFM, Verwiel E, et al. The role of interleukin-1 beta in the pathophysiology of Schnitzler's syndrome. Arthritis Res Ther. 2015 Dec;17(1):187.

49. Wittlinger J, Stankovic P, Girrbach U, Gradistanac T, Güldner C, Teymoortash A, et al. Hyperplasia and the degree and activity of inflammation in chronic recurrent tonsillitis: a histopathological study. Eur Arch Otorhinolaryngol. 2017 Jul;274(7):2927–32.

50. Boiko NV, Stagnieva IV, Kim AS, Simbirtsev AS. [Proinflammatory cytokine content in the saliva of children suffering from chronic tonsillitis]. Vestn Otorinolaringol. 2019 Jan;84(3):26–31.

51. Geißler K, Weigel C, Schubert K, Rubio I, Guntinas-Lichius O. Cytokine production in patients with recurrent acute tonsillitis: analysis of tonsil samples and blood. Sci Rep. 2020 Aug;10(1):13006.