Near East University

Health Science institution

Tonsillar Toll-like receptors (TLRs) expression profiles of patients suffering from periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA)

BY:- Munjed Issam Mujahid

Master Thesis

Medical Microbiology & Clinical Microbiology

ID NO:-20183986

Thesis Advisors:

Assoc. Prof. Dr. Umut Gazi

2020, Nicosia

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THESIS APPROVAL

Directorate of Institute of Health sciences

The work has been adopted as a master thesis in the program of medical microbiology and clinical microbiology by the jury .

Supervisor: Assoc. Prof. Dr. Umut Gazi

Near East University

Jury Member: Prof. Dr. Nedim çakir

Near East University

Jury Member: Prof. Dr. Nerin Nadir Bahçeciler Önder

Jury Member: Yard. Doc. Dr. Mumtaz Guran

Jury Member: Yard. Doc. Dr. Mehmet Ilktaç

APPROVAL :

This thesis has been approved by the above jury members in accordance with the relevant articles of the NEU postgraduate education, training and examination regulations and has been accepted by the decision of the board of the institute.

Prof.Dr. Hüsnü Can BAŞER

Director of Institute of Health Sciences

DECLARATION

I announce that I have no unethical behaviour in all phases since the time I underway the laboratory work of the thesis to my script of the thesis itself, I have multiplied all the information in this thesis within the theoretical and moral strategies, and I have tangled all the information and specifics in the version and have added these documents to the references part. I hereby have not violated my copyrights and rights while studying and writing this letter.

Munjed Mujahid
Signature

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As one of the few distinguished students who were able to graduate from the university and overcome the academic and life obstacles due to what happened in the world as a result of the *COVID 19*, I swear that my patience, perseverance and determination to succeed was not enough, but the support of those around me, including family, friends and teachers, in addition to the most important reason which is my support from the Lord was one of the pillars that helped me during this difficult period.

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LIST OF ABBREVIATION AND SYMBOLS

BFAPA- Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis

AIDS- auto-inflammatory diseases

TLR-Toll like receptors

IHC-immunohistochemistry

CTLs- cytotoxic T cells

PRRs- pattern recognition receptors

PAMPs-pathogen associated molecular patterns

NLRs- Nod-like receptor

RLRs- RIG-1-like receptors

CLRs- C- type lectin receptors

TLRs- Toll like receptors

LPS-lipopolysaccharide

DsRNA- double stranded ribonucleic acids

ss- single stranded

PDCs-plasmacytoid dendritic cells

CDCs- conventional DCs cells

LRRs-leucin rich repeats

TIR- Toll/TL-1-receptor

TRIF- IFN-beta

MYD88-myeloid differentiation factor 88

IRAK1- 1L-IR associated kinases

SARM- sterile alpha armadillo motif

APC- antigen presenting cells

IBD- inflammatory bowel disease

ESR -erythrocytes sedimentation rate

CR- C-reactive protein

FMF- familial Mediterranean fever

HPF-hereditary periodic fever

mRNA- messenger RNA

IL-1B inhibitor - Anakinar

ABSTRACT

Tonsillar Toll-like receptors (TLRs) expression profiles of patient suffering from periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA)

BY:- Munjed Essam Mujahid

Thesis Advisors:

Assoc. Prof. Dr. Umut Gazi

Introduction: Tonsillar microenvironment is thought to contribute to innate immune dysregulation responsible for the periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis (PFAPA) because of beneficial effects of tonsillectomy on treatment of the syndrome. Accordingly previous studies reported altered lymphocyte frequency, cytokine level and microbial composition in PFAPA tonsils. In the previous study it is hypothesized that the previous observation of altered tonsil microbiota, and antimicrobial peptide expression in PFAPA cases is associated with changes in the expression levels of Toll like receptors (TLR) which were previously reported to differ in patients suffering from other auto-inflammatory diseases such as IBD.

Materials and Methods: In this study, tonsil samples collected from seven PFAPA patients from Near East University (NEU) Hospital were included. Tonsillar expression levels of TLR-1, -2, -4, -5, and -6 were monitored by immunohistochemistry (IHC). Expression levels were scored using semi-quantitative analysis method and were statistically analyzed by Two-Way Repeated Measures Analysis of Variance test . Breast cancer tissues and samples incubated with rabbit IgG isotype control antibodies were used as positive and negative controls .

Results: The histological profiles of patients with PFAPA show different expression of TLRs in both samples and control. IHC analysis demonstrated expression of all TLRs in tonsillar surface epithelium (SE) except for TLR-6. The TLR1 shows increased level of staining intensity in both the patients and the control, and the result indicated~~s~~ weakly expressed TLR2 in both positive cells and the control, and there was decreased expression of TLR4 in both patients and the control, However, there was no difference in the expression of TRL4 in comparison with the control group. TLR5 expression shows similar pattern of high positive cell distribution of the epithelial at squamatous layer of the cells in both patients and the control. High TLR5 was detected in both samples and the control. In TLR 6, there was no positive cell distribution of TLR5, while the intensity of the expression was weakly expressed in both samples and control groups.

Conclusions: Altered TLR expression levels may be involved in PFAPA pathogenesis. Conclusively, data from histoimmunochemistry of PFAPA patients showed different distribution patterns of the positive cell epithelial compartments. The level of intensity of the expression of TLRs in this study was found to be higher than the control groups. On TLR 6, the absence in the epithelial cells and weak intensity showed a minimal role in the pathogenesis of PFAPA. The highest expression in this study was found in TLR1 and it was widely distributed across all layers. It is therefore recommended that large sample size to be conducted to in an uninflamed tonsil with specific marker to validate the result of current study with the aim of detecting role of tonsils in PFAPA syndromes.

Keywords: PFAPA; Tonsil; Autoinflammation; TLR; TLR-1; TLR-2; TLR-6.

ÖZET

Giriş: Bademcik mikroçevresinin, tonsillektominin sendromun tedavisi üzerindeki yararlı etkileri nedeniyle periyodik ateş, aftöz stomatit, farenjit ve servikal adenitten (PFAPA) sorumlu olan doğal immün düzensizliğe katkıda bulunduğu düşünülmektedir. Buna göre önceki çalışmalarda PFAPA bademciklerinde lenfosit sıklığı, sitokin seviyesi ve mikrobiyal kompozisyonun değiştiği bildirilmiştir. Çalışmamızın amacı, daha önce PFAPA vakalarında değişen tonsil mikrobiyotası ve antimikrobiyal peptit ekspresyonu gözleminin, daha önce diğer hastalardan muzdarip hastalarda farklı olduğu bildirilen Toll benzeri reseptörlerin (TLR) ekspresyon seviyelerindeki değişikliklerle ilişkili olduğu hipotezinin irdelenmesidir.

GEREÇ VE YÖNTEM: Bu çalışmada Yakın Doğu Üniversitesi (YDÜ) Hastanesinden yedi PFAP hastasından toplanan bademcik örneklerinden oluşan PFAPA grupları bulunmaktadır. TLR-1, -2, -4, -5 ve -6'nın tonsiller ekspresyon seviyeleri, immünohistokimya (IHC) ile izlendi. İfade seviyeleri yarı kantitatif analiz yöntemi kullanılarak puanlanmış ve istatistiksel olarak Varyanc testinin İki Yönlü Tekrarlanan Ölçümler Analizi ile analiz edilmiştir. KONTROL GRUBU OLARAK KAÇ KİŞİ EKLENDİĞİ BURAYA YAZILMALI

BULGULAR: PFAPA'lı hastaların histolojik profilleri, hem numunelerde hem de kontrolde farklı TLR ekspresyonu göstermiştir., IHC analizi, TLR-6 dışında tonsil yüzey epitelinde (SE) tüm TLR'lerin ekspresyonunu gösterdi. TLR1, hem hastalarda hem de kontrolde boyama yoğunluğunun artmış seviyesini göstermiş ve Tonsiller yüzey epitelinde (SE) olan PFAPA hastasında TLR-2 ekspresyon düzeyi kontrolden düşük bulundu ve PFAPA hastasında TLR-4 ekspresyon düzeyinde, tonsil yüzey epitelinde kontrol grubu ile istatistiksel olarak bir fark yoktu. (SE). TLR-5'te PFAPA hastası ile tonsil yüzey epitelinde (SE) kontrol arasında ekspresyon

düzeyinde istatistiksel olarak bir fark yoktu ve PFAPA hasta grupları ve kontrolde tonsil yüzey epitelinde (SE) herhangi bir ifade edilen TLR-6 düzeyi yoktu. .

SONUÇ: Değişen TLR ekspresyon seviyeleri, PFAPA patogenezinde rol oynayabilir. Sonuç olarak, PFAPA hastalarının histoimmunokimyasından elde edilen veriler, pozitif hücre epitel kompartmanlarının farklı dağılım modellerini gösterdi. Bu çalışmada TLR ekspresyonunun yoğunluk seviyesi, kontrol gruplarından daha yüksek bulundu. TLR 6'da epitel hücrelerinde yokluk ve zayıf yoğunluk, PFAPA'nın patogenezinde minimal bir rol gösterdi. Bu çalışmadaki en yüksek ifade TLR1'de bulundu ve tüm katmanlara geniş bir şekilde dağıldı. Bu nedenle, PFAPA sendromlarında bademciklerin rolünü saptamak amacıyla, mevcut çalışmanın sonucunu doğrulamak için, iltihaplanmamış bir bademcikte özel işaretleyici ile büyük bir örneklem boyutunun yapılması önerilmektedir.

ANAHTAR KELİMELER:PFAPA; Tonsil; Autoinflammation; TLR; TLR-1; TLR-2; TLR-6.

CHAPTER I

INTRODUCTION

1. Introduction

Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA), also known as the Marshall syndrome, is a chronic disorder belonging to the group of recurrent fever syndromes, and is characterized by recurrent fever episodes lasting for 3 to 7 days reoccurring every 2 to 8 weeks accompanied by aphthous stomatitis, pharyngitis and cervical adenitis (Trandafir *et al*., 2016). PFAPA usually has an onset between the ages of 2 to 5 and tends to be more common in males than females(Cattalini *et al*., 2015).There are no preventive measures or cure for PFAPA. However, in some cases, steroids treatments and tonsillectomy were effective in resolving fever episodes (Burton et al., 2014). The disease is not contiguous and it does not propose a long term danger to the patients.

The frequency of the syndrome as well as the etiology is not yet known. However, previous data on PFAPA suggest that immune dysregulation maycontribute to the pathogenesis of the syndrome(Silvia Stojanov et al., 2006).According to the hygiene hypothesis, as proposed by Strachan in 1987,changes in the lifestyle of individuals (e.g. the overuse of antibiotics and destruction of chronic parasitic infection which often occur in industrialized countries) significantly lowered the burden or rate of infectious disease that resulted in immune dysregulation and thereby increases in the rates of autoimmune, allergic and chronic inflammatory disease (Strachan, 1989).

1.1 Objective of the Research

In this research, it is hypothesized that the previous observation of altered tonsil microbiota, and antimicrobial peptide expression in PFPA cases is associated with changes in the expression levels of Toll like receptors (TLR) which were previously reported to differ in patients suffering from other auto-inflammatory diseases such as IBD (Frosali *et al*., 2015). For this purpose, tonsils samples were extracted from PFPA patients through tonsillectomy and immunohistochemistry was later performed to examine the expression levels of TLR-1,2, 4, 5, and 6 localized on the cell membrane.

CHAPTER II

2. GENERAL INFORMATION

2.1. The Immune System:

Immune system is the central defense mechanism against infections in human and any disruption in the immune system can give rise to many aberrations in the defense functions such as auto-inflammatory or auto-immune disease (Brodin & Davis, 2017). Hence, a healthy immune system is required for the normal growth and development of an individual. In general, the vertebrate immune system is classified into two distinctive forms: the adaptive and innate immunity(Akira et al., 2006; Akira & Takeda, 2004).

The adaptive immunity in vertebrates depends on the expression of certain antigen specific receptors on both B and T lymphocytes generated through hyper mutation or genetic rearrangement (Akira *et al*., 2001). During adaptive immune response lymphocytes (B and T-cells) circulate through blood and other secondary organs in search of invading pathogens or antigens. Upon antigen detection, clonal expansion occur and within few days, the body develops immunological function and memory (Kim et al., 2005).

The T lymphocytes of the adaptive immune systems are classified as T helper cells and cytotoxic T cells (CTLs). The T helper cell play a crucial role in the secretion of cytokines that are responsible for the activation of adaptive immune response and the CTLs function in the sensing and engulfment of viral infected cells (Sattler, 2017). However, the B lymphocyte of the adaptive immune systems produce various antibodies which recognize and bind to specific antigens on the invading pathogens(Biron, 2016).

On the other hand, the innate immune response is an evolutionarily conserved defensive mechanism which is activated immediately after sensing the presence of microbial pathogens or antigens (Akira *et al.*, 2006). The human innate immune acts as a physical barrier against infection; for example as an acidic environment that can eradicate or prevent the growth of bacteria or act as enzymes like the lysozyme found in tears which annihilate bacterial cells (Harricharan et al., 2017). Moreover, complement system (group of serum proteins) component of the innate immune system function to lyse or opsonize pathogen for phagocytosis (later destruction) (Underhill & Ozinsky, 2002). The cellular components include neutrophils and macrophages (phagocytic cells in general) which function in the lysis and engulfment of microbial pathogen invading the host.(Mcdonald,Mcdonald, D.R., and Levy & Levy, 2018; Wade, 2014).

2.1.6. Innate immune response against pathogen:

The human innate immune systems recruit germ-line encoded pattern recognition receptors (PRRs) to detect or sense specific molecular markers from invading pathogen (i.e the pathogen associated molecular patterns,PAMPs) components such as proteins, nucleic acid, lipoprotein and lipids which are not present in the host (Kawasaki & Kawai, 2014). These PRRs trigger downstream signaling pathways which facilitate the induction of innate immune response and the secretion of inflammatory cytokines alongside other immune mediators (Wade, 2014) (Figure 1). Aside from inducing innate immunity, the process also organizes antigen specific adaptive immunity which also partakes in the elimination of invading microbes. There are various classes of PRRs in mammals such as (i) Nod-like receptor(NLRs), (ii) RIG-1-like receptors (RLRs), (iii) C- type lectin receptors (CLRs) (iv) Toll like receptors (TLRs) (Akira et al., 2006; Cai et al., 2014).

Toll-like receptors (TLRs) were the first type of PRRs identified in *Drosophila* in 1985 and were classified into TLR1 – TLR10 in humans and TLR1-TLR13 in mouse (Kawasaki & Kawai, 2014b). A study by Lemaitre *et al* (1996), in which they demonstrated that *Drosophila* flies without TLR gene expression were more prone to fungal infections which was as a result of inadequate induction of antifungal peptide. The study was the first to reveal the presence of a specific receptor in *Drosophila* which played a key role in recognizing fungal infection (Lemaitre et al., 1996).

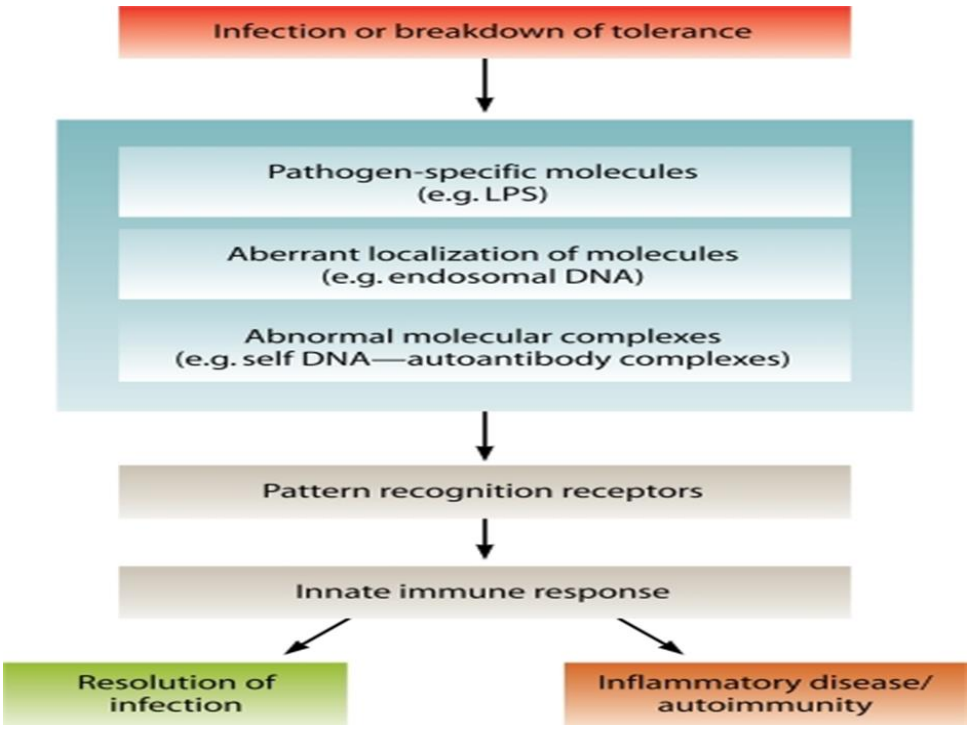

Figure 1: An overview of innate immune recognition by PRRs retrieved from (Mogensen, 2009).

In a subsequent study, Medzhitov and colleagues (1997) identified a homolog of the TOLL gene in humans (hToll) which played a role in the inductive production of inflammatory cytokines as well as the expression of the co-stimulatory molecules (Medzhitov et al., 1997). Likewise, the loss of function of the Toll gene significantly led to increase levels of liposaccharide and hypo-responsiveness in mice (Poltorak *et al*., 1998; Qureshi *et al*., 1999).

In humans, TLRs are primarily found in innate immune cells such as macrophages, dendritic cells as well as non-immune cells for example epithelial and fibroblastic cells (Kawasaki & Kawai, 2014c). TLRs are usually classified according to their location inside the human cell either as intracellular TLRs embedded on the endosomes (TLR7, TLR9, TLR3, TLR8, TLR12, TLR13 and TLR11) or on the cell surface membrane (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) (Akira *et al*., 2006; Cai *et al.*, 2014) (Figure 2).

Each TLR have an extracellular domain that is responsible for sensing and binding to a ligand found in the invading microbiome (Cai *et al.*, 2014b). Till date, numerous genetic studies have revealed the respective ligand of most of the TLRs located on the plasma membrane, for example TLR2 combine with TLR1 and TLR6 to sense various bacterial component such as peptidoglycan, lipotechnioacid, mannan and lipoprotein of Gram positive bacteria (Akira *et al*., 2006c). TLR4 is responsible for sensing bacterial lipopolysaccharide (LPS) (Kawasaki & Kawai, 2014). TLR5 recognizes and binds to invading flagellated microorganisms (Akira *et al*., 2006). TLR10 in humans combine with TLR2 to sense molecules or ligands from listeria (Regan *et al.*, 2013). In addition, TLR10 can also recognizes influenza A virus (S. M. Y. Lee *et al*., 2014) but in mouse TLR10 acts as a pseudogene due to the insertion of stop codon (Kawasaki & Kawai, 2014).

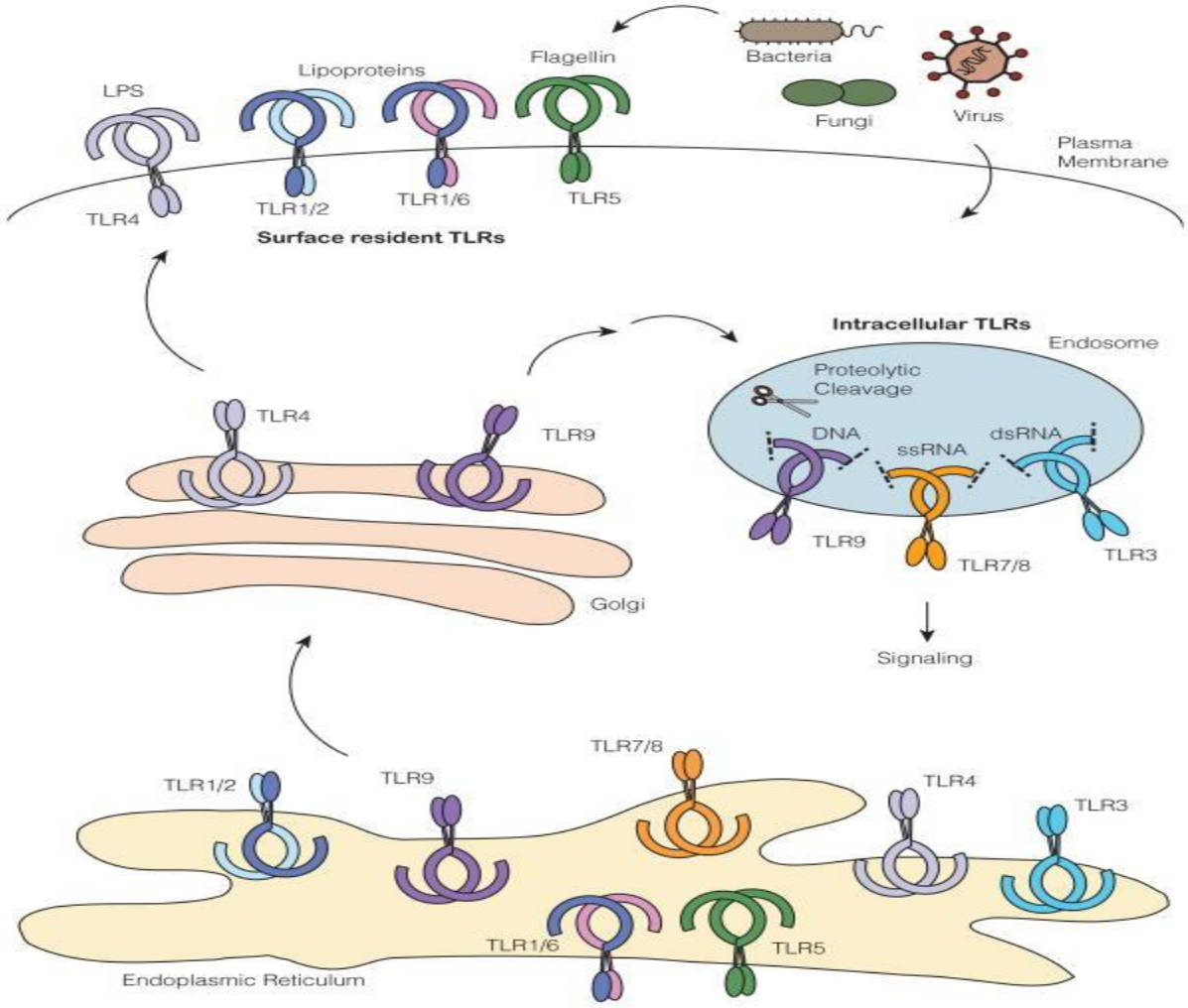

Figure 2: An illustration of TLRs trafficking adapted from (B. L. Lee & Barton, 2014)

The intracellular TLRs embedded in the endosomes or ER play crucial roles in sensing self-nucleic acids derived from pathogen or self-derived nucleic (Blasius & Beutler, 2010). For instance, TLR3 from the intracellular TLRs sense double stranded ribonucleic acids (dsRNA) produced by several viruses during DNA replication (Mansson et al., 2006). TLR7 discern single stranded (ss) RNA sequence from viral agent and it is primarily expressed in the plasmacytoid dendritic cells (PDCs) (Mancuso *et al*., 2009). To add, TLR7 also recognizes RNA molecules from streptococcus B bacteria in conventional DCs cells (cDCs) (Mancuso *et al*., 2009). TLR8 sense both bacterial and

viral RNA molecules (Guiducci *et al*., 2013). Lastly, TLR9 recognizes viral and bacterial agents that are predominantly rich in un-methylated (CPG-island) motifs, and identifies the presence of an insoluble crystalline by product called (hemazoin) that is produced by *Plasmodium falciparum* throughout or during host detoxification after the digestion of hemoglobin (Coban *et al*., 2010).

2.1.7. Basic Structure of TLRs:

TLRs consist of three structural domains; (i) a trans-membrane domain (ii) a leucin rich repeats (LRRs) motifs and, (iii) a cytoplastic Toll/TL-1-receptor (TIR) domain. These receptors are either found on the plasma membrane or attached to cells linked to endosome membranes. The LRR motif of the TLRs protein is located adjacent to the TIR domains function in the recognition of PAMPs from foreign pathogen. During immune response, the ectodomain forms a structure enabling different forms of TLRs to interact with their specific PAMPs. This interaction occurs as a heterodimer in association with other accessory molecules and co receptors or as a homodimer (Botos et al., 2011).

TIR motifs initiates downstream cascade signaling through mediated interactions with various signal transduction adaptors such as TIR domain containing adaptor inducing IFN-beta (TRIF), myeloid differentiation factor 88 (MYD88) and TIR domain containing adaptor inducing interferon- B (TRIF) to trigger the activation of IRFs, MAP kinases or NF-κB proteins that are responsible for modulating the expression levels of IFNs, chemokine's and cytokines which eventually or primarily defend the host from microbial infections (Kawasaki & Kawai, 2014).

2.1.8.TLR Signaling Pathway using MYD88 Receptor

The major role of TLR signaling activation occurs through the MyD88 (MAL/TIRAP domain) dependent mechanisms as it is used by all TLRs apart from TLR3. MYD88 assembles 1L-IR associated kinases (IRAK1) which binds to MYD88 forming a complex which activates downstream IKK and MAPKK (TAK1 and MEKK3) signaling to induce nuclear translocation factor NF-κB and AP-1 respectively, also MyD88-TLR signaling can also be activated through the activation of TRIF and TNF receptor 3 (TRAF-3)(Figure 3).However, an additional molecular adaptor known as the sterile alpha armadillo motif containing protein (SARM) can inhibit TLR signaling through interactions with both TRIF and MYD88. TLR signaling acts as a stimulant for pro-inflammory cytokines, survival mechanisms and proliferant factors or as a source of interaction between adaptive and innate immune mechanisms as their expression have been detected in adenoids and tonsil tissues (Claeys *et al*., 2003). Additionally, Gelman*et al* (2004) reported that the CD4+ cells in mouse expressing TLR3 and TLR9 responded to poly (I;C) and CpG-DNA stimulation with NF-κB activation, suggesting that the activation T cells can occur with or without the presence of antigen presenting cells (APC) (Gelman et al., 2004).

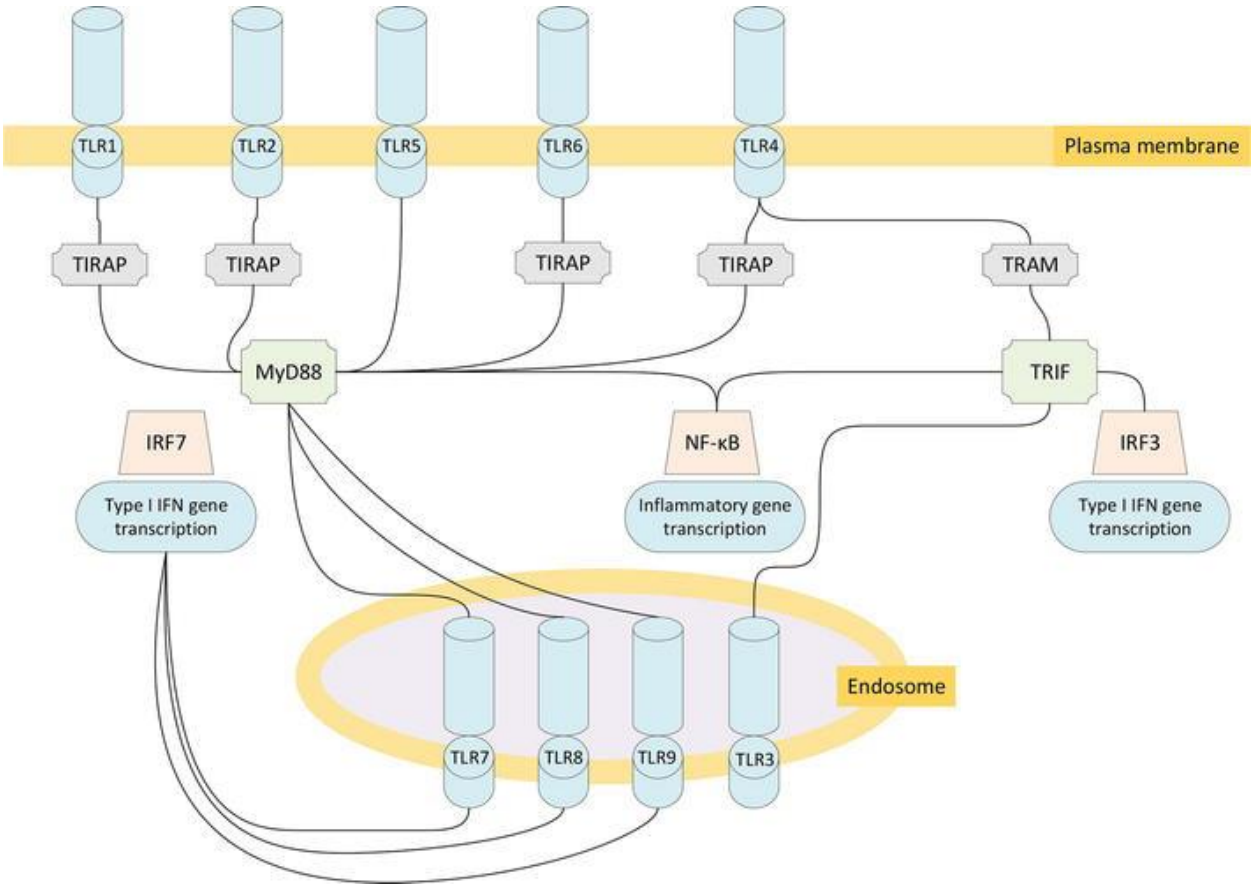

Figure 3: An overview of Toll-like receptor (TLR) signaling pathway retrieved from (Saghazadeh & Rezaei, 2020)

In accord with the literature that shows the important role played by TLRs in immune defense, Frosali and colleagues (2015) found a correlation between aberrant TLR signaling pathway and the pathogenesis of both chronic and acute inflammatory anomalies such as colistis, inflammatory bowel disease (IBD), colorectal cancer, allergy, cancer, sepsis and ischemia (Frosali *et al*., 2015). Besides, studies on mice with deficient MyD88 molecules suggested the MyD88 molecule is required for maintaining homeostasis (Schnare *et al*., 2001). Similarly, MyD88 dependent molecule was reported as a major factor that was necessarily required for epithelial cell

homeostasis, antimicrobial peptide induction and the initiation of immune response to injury (Frosali *et al*., 2015).

2.2PFAPA syndrome:

2.2.1. History and Definition:

PFAPA is one of the typical forms of periodic fever which mostly occur at the childhood stage of life. The syndrome was first described in 12 children by Marshall and colleagues in 1987 (Marshall et al., 1987). Later that year, Feder and Bialecki named the syndrome fever, aphthous, stomatitis, pharyngitis and cervical lymphadenopathy (FAPA) (Feder & Bialecki, 1989).

Subsequently, Marshall and colleagues (1989) renamed the syndrome from FAPA to periodic fever aphthous, stomatitis, pharyngitis and cervical adenopathy (PFAPA) according to the clinical features they observed in the first 12 cases of PFAPA syndrome which were characterized by abruptive onset of fever, chills, malaise, aphthous, stomatitis, pharyngitis, headache and tender cervical adenopathy occurring within an interval of 4 to 6 weeks over the years (Marshall et al., 1989). In addition, the fever episodesin these 12 patients resolved spontaneously within 4 to 5days, and they were accompanied by the presence of elevated erythrocyte sedimentation rate and mild eukocytosis during febrile episodes(Marshall *et al*., 1989). There is a significant increase in the levels of erythrocytes sedimentation rate (ESR), C-reactive protein (CR) as well as increased leukocyte recruitment during fever episodes but not between episodes. Patients are also sensitive to corticosteroid treatment and exhibit normal growth with mild susceptibility to infection with sequelae(Marshall et al., 1989).

The frequency of the syndrome is not known worldwide, it isthought to be more regular than most common inherited periodic fevers such as familial Mediterranean fever (FMF)(Brown *et al*.,

2010a; Silvia Stojanov *et al*., 2011). Several numbers of studies have reported the incidence of the disease in certain populations. The prevalence in Northern Italy (in Friuli VeneziaCiulia) from 1996 to 2001 (within the duration of 5 years) was reported as 0.4 cases/1000 children every year, with 1 new additional case of PFAPA per pediatrician in every 1-2 years (Barbi *et al*., 2001). In Norway,2.3 cases out of 10,000 children was reported in 2013 (Førsvoll et al., 2013a). More recently, a total of 268 children with PFAPA are diagnosed yearly which increases the incidence of the cases per year in Italy (Cattalini *et al*., 2015).However, to our knowledge, there has not been any PFAPA prevalence study conducted in Turkey or in Cyprus.

The syndrome was first considered to have an onset of2.5-5 years of age, but more recently, few data have described the syndrome in children older than 5 years. Marco *et al* (2015) evaluated 17 adults with unexplained periodic fever and other cardinal symptoms which satisfied PFAPA criteria (Cattalini *et al*., 2015) and in 15 adults with PFAPA (Padeh*et al*., 2008). More recently, Brown *et al* (2010) described 17 adults with clinical features of PFAPA syndrome (Brown *et al*., 2010b).

2.2.2. Diagnostic criteria and methods:

Presently, there is no precise diagnostic criteria for PFAPA, however, diagnosis is made if the patient is at the age of <= 5 years and if he/she keeps experiencing the following symptoms; (i) severe inflammation and reoccurring episodes of fever attacks ≥ 40°C lasting for 5 days which relapses after 3 to 8 weeks, (ii) either of the constitutional symptoms for example pharyngitis with or without cervical adenitis and aphthous stomatitis as well as abdominal pain, vomiting and infrequent headaches, (iii) if he/she has complete acute interval period with normal growth

parameter, (iv) he/she does not have neutropenia, chronic infections or other periodic fever for example (FMF) (Kraszewska-Głomba et al., 2015a; Pignataro et al., 2009).

Currently, there are no specific imaging techniques or laboratory test to directly diagnose PFAPA. However, blood test to monitor levels of white blood cells (to check for signs of infections), ESR, CRP (to check general inflammation rate) ,abdominal CT imaging test and strep culture can be performed (Rigante *et al*., 2017).Additionally, the fact that the patients are sensitive to steroids can also aid in the diagnosis of the syndrome(Gattorno *et al*., 2009).

2.1.3. Clinical manifestation:

Children with PFAPA syndrome mostly have a body temperature of 40.5 °C and fever with duration of 3 to 4 days which occurs with acute intervals and normal growth parameters. These are accompanied by either one or two of these cardinal signs:aphthosis stomatitis (observed in 75% cases) and/or with pharyngitis (observed in almost 90% of the patients) with or without cervical adenopathy (observed in 75% cases)(Feder & Salazar, 2009; Hofer et al., 2014; Thomas et al., 1999). The constitutional symptoms might occur in association with either of the following: abdominal pain, vomiting, chills and headaches(Tasher et al., 2006). In addition to this, 70% of the patients have nonspecific symptoms such as fatique, malaise and irritability which surfaces prior to the flares(Feder & Salazar, 2009; Tasher et al., 2006). These patients are mostly free of respiratory tract infections, leukocytosis chronic infections as well as neutropenia and other periodic fever (i.e FMF).

In adult patients, most of the clinical features such as recurrent fever episodes or attacksarecomparable to those observed in children patients. In contrast, Cattalini*et al* (2015) described that most adult patient simultaneously displayed all three cardinal signs (aphthous

stomatitis, pharyngitis and cervical adenopathy) at higher rates compared to children (the mean number of episodes/year was 8.2 ± 5.2 and 13.3 ± 9.2 for both children and adult patients, respectively). The rate of pharyngitis and apthous was significantly higher (52%) in adult patient compared to (46%) in children patient (Cattalini *et al*., 2015).

2.1.4.Causes of PFAPA Syndrome:

The etiology as well as the hereditary basis of the disease is not fully understood. Recent studies using siblings from the same mother propose a familial predisposition in certain genes including NLRP3, SPAG7, MVD (gene MVK) and TRAPs with PFAPA pathogenesis(Kraszewska-Głomba et al., 2015a). Additionally, hypomorphic variation in certain genes responsible for the hereditary periodic fever (HPF) was also reported as a possible risk factor for PFAPA (Kolly *et al*., 2013). This findings needs to be confirmed in larger cohort studies, as it may propose a model for PFAPA syndrome. On the other hand, there has not been any association between PFAPA syndrome and any known microbial agent. Moreover, the reports of the involvement of tonsillitis and the positive influence of tonsillectomy on the clinical course of PFAPA made some researchers focus on tonsil samples isolated from PFAPA patients(Peridis *et al*., 2010). Petra and colleges (2015) analyzed peripheral blood samples and paired tonsils from 10 children with PFAPA syndrome by which they found elevated expression of T cell chemo attractants and increase in the allocation of T and B lymphocytes in the tonsils of the patients (Petra *et al*., 2015). The study suggested that the recruitment of T cell chemoattractant sand changes in the allocation of T and B lymphocytes from the peripheral blood might be restricted to tonsil as a result of impaired chemokine expression. Furthermore another study reported higher levels of immunoglobulin D –armed basophils in the tonsil samples of PFAPA of patient when compared to controls(Chen *et al*., 2009). They 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. Given the notable tissue friability of PFAPA patient tonsils. (J ClinImmunol, 2020)

Additionally, a number of studies have reported a correlation between elevated levels of IL-6, 1L-18, IFN gamma and IL-1β and the pathogenesis of PFAPA syndrome (Brown *et al*., 2010a; Silvia Stojanov *et al*., 2011; Yamazaki *et al*., 2014). This was suggested to be significantly associated with dysregulated monocytes count during the period of febrile attacks in which they act as stimulators of T-cell function (S. Stojanov *et al*., 2006). In addition to this, the involvement of immunological dysregulation such as increased monocytotic events (Brown et al., 2010a; Førsvoll et al., 2013b; L et al., 2013; Silvia Stojanov et al., 2011), increased production of pro-inflammatory chemokines (MIG/CXCL9 and IP-1O/CXCL10) (Brown et al., 2010a; Førsvoll et al., 2013b; Silvia Stojanov et al., 2011), as well as a hallmark of neutrophils and elevated transcription of complementary genes were associated with the periodic episodes of fever attacks observed in most PFAPA cases (Silvia Stojanov *et al*., 2011). Also, dysregulated immune response was further demonstrated by lower numbers of circulating lymphocytes in PFAPA patient which was as a result of the recruitment of T cell to peripheral tissues (Førsvoll et al., 2013b; Yamazaki et al., 2014). Moreover, in another study, Gazi*et al* (2018)Different immunolocalization of humanβ beta-defensing were reported in tonsils isolated from PFAPA patient group than that from patients with recurrent tonsillitis with (group A β- hemolytic streptococci (GAS))(Gazi et al., 2018). These findings suggest that aberrations in the genes responsible for immunological response might partly be involved in the pathogenesis of PFAPA syndrome. This could be because of alteration of

tonsillar microbial composition as reported by Lantto*et al*. (Lantto et al., 2015; Luu et al., 2020; Tejesvi et al., 2016).

2.3. Treatments for PFAPA syndrome:

Up till today there has not been any specific treatment for PFAPA but tonsillectomy (TE) is an active surgical treatment option for PFAPA, and available treatments for the syndrome are mostly used to resolve the febrile episodes associated with the disease in order to improve the lifestyle of the patients. In previous years, there have been reports of patient's resistance to antipyretics such as ibuprofen and acetaminophen as well as non-steroid anti-inflammatory agents (Vanoni et al., 2016). Currently, one of the effective treatments for resolving or aborting febrile attacks is the use of glucocorticoids and tonsillectomy. The interesting thing is that how the tonsillectomy procedure effective in relieving the symptoms of PFAPA patients, which we will discus in the following paragraphs.

2.3.1 PFAPA medical treatment:

Glucocorticoid is a medication that is constitutionally used to suppress PFAPA symptoms. A single dose of beta methasone (0.1-0.2 mg/kg) or prednisone (1 -2mg/kg) administered days before fever attacks was effectively used to suspend fever episodes in patients with PFAPA.

Colchicine can be used as a second effective treatment for the remission of fever attacks in children with PFAPA. Colchicine is a prophylactic treatment that is primarily used to treat FMF however it is also currently used as medication for PFAPA.

Cimetidine is an anti-histamine drug which is used to resolve cardinal symptoms in PFAPA patients, other medication is Anakira which is a pharmaceutical drug which used as an IL-1blocker,

taken prior to PFAPA attacks and recently, the use of vitamin D as a possible treatment for reducing fever flares in PFAPA patients.

2.3.2 Tonsillectomy surgical Treatment:

Tonsillectomy is a surgical technique which facilitates the removal of tonsils organ which can be done by different methods. Tonsillectomy is a procedure taking 20 to 30 minutes under general anesthesia while the patient is not feeling any pain. One of the methods is electrocautery which is using heat to remove the tonsils completely without any bleeding risk, other method which can be used also is cold knife (steel) dissection done by scalpel to resect the tonsils and the bleeding is stopped later on by sutures or electrocautery. Other method used is called harmonic scalpel which is using ultrasonic vibrations in order to cut the tonsils and stop the bleeding at the same time. Other less useable techniques are ordered as followed: radiofrequency ablation techniques, carbon dioxide laser and microdebrider.

Tonsillectomy is also producing some risks which are swelling, infection and bleeding reactions to anesthetics. The time of symptoms resolution after tonsillectomy is ranging between 1 to 11 months with an average of 2 months. (J ClinImmunol, 2020).

The efficacy of tonsillectomy has been reported by several studies which explained that efficacy by finding that the PFAPA patients tonsils have continuous inflammation caused by a bacterial signature pattern without any symptoms and tonsils were thought to be the main site of immune dysregulation in PFAPA patients , so tonsillectomy was a successful surgical treatment.(Renko et al., 2007).

Jostein et al (2018) elaborated the effectiveness of tonsillectomy in a randomized control trails involving 555 children with PFAPA. They reported a complete fever remission in 509/555 children after tonsillectomy (Førsvoll & Øymar, 2018) and in a recent Cochrane review (Burton et al., 2014).

Side Effects of Tonsillectomy

Tonsillectomy surgical procedure has many complications and are classified into primary and secondary. The primary implications include vomiting, nausea, pain and trouble in feeding while the secondary implication includes post operational bleeding, cardiac arrest, death, laryngospasm and respiratory complications.

2.3.3 Management and Prognosis:

The families of the affected child should provide valid information about any previous history of tonsillectomy to the pediatrician. Also, families with an affected child should seek information about current treatment and an ongoing management of the syndrome from pediatric rheumatologist (Rigante et al., 2017).

The prognosis of PFAPA syndrome may vary among patients but in most cases, fever episodes decreases as the child approach adulthood. Also there have been positive reports about complete recovery without any relapses after tonsillectomy (Wurster et al., 2011).

2.4. Thesis Structure

Chapter one offers introduction for Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA) .In addition, Objective of the Research.

The second chapter provides background information about this research. In this chapter, general information of Immune system, Innate immune response against pathogen, Basic Structure of TLRs, TLR Signaling Pathway using MYD88 Receptor, PFAPA syndrome, Diagnostic criteria and methods for PFAPA, Clinical manifestation, Causes of PFAPA Syndrome, Treatments for PFAPA syndrome. .In addition, Tonsillectomy and Importance of Tonsillectomy, Side Effects of Tonsillectomy, Management and Prognosis.

Chapter three provides the material and information about the methodology that used for data collection and the statistical analysis to analyse the results for the study.

Chapter four discusses the results that obtained from this research.

The fifth chapter describes the results in detailed and associate the data with literature review, the conclusions and dissection of this study is included in this chapter.

CHAPTER III

Materials and Methods

3. Material and methods

3.1. Suppliers and sample collection

This study was performed at Near East University Hospital, Turkish Republic of Northern Cyprus. The study covers the period of 2012-2016. It includes 13 children admitted to the Near East University Hospital within the study period. Study sample was divided into group 1 and 2.

Group 1: includes patients with periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) syndrome. This group was assessed according to the modified method of Thomas et al (1999).

Group 2: this group includes patients with recurrent tonsillitis due to Group A beta-haemolytic streptococci (GAβHS).

Samples were collected from the 13 children and divided among the 2 groups.

Group 1 consists of 7 tonsil samples while group 2 consists of the remaining 6 tonsil samples from patients.

Patients consent form and their databases were secured from Near East University Hospital Information System. The database includes the vaccination status of the patients, clinical history and reason for tonsillectomy.

Surface of the tonsil epithelium was removed and fixed in formaldehyde and was stored at -4°C as paraffin embedded tissues so as to maintain/preserve its morphology for further analysis.

Exclusion Criteria

Patient**s** treated with 0.5-2mg/kg prednisone are excluded from this study

Ethical approval

This study was approved by the Near East University Ethical Approval Committee; for the use of human palatine tonsils. The project was assigned an ethical approval number of YDU/2016/42-346.

3.2.Sample preparation

Tonsil obtained from tonsillectomy was dissected and fixed in 10% formaldehyde solution for the period of 24 hours. This gives the obtained Fixed Formalin Paraffin Embedded (FFPE). This is done to preserve the morphology of the tissue and prevent any chemical reaction taking place. Dehydration was then performed and plastic cassettes were used for embedding with hot paraffin. This was frozen and stored at -10℃ for further analysis such as immunohistochemistry.

3.3. IMMUNOHISTOCHEMISTRY

Avidin-biotin complex immunoperoxidase (Vectastain® Elite® ABC-HRP kit) obtained from vector laboratories, burlingame CA was used to compare the antimicrobial activity among tonsil samples. This process was done at the pathology laboratory in Near East University Hospital. The blocked FFPE palatine tonsil was labelled as right (I) and left (II).

Under this process, primary antibodies, secondary antibodies, and the isotype control were all used using the IHC procedure. Then images were taken with a light microscope (AxioCamHRc Zeiss Scope A) with a set and precise filter and magnification. The IHC procedures involve:

-Fixation of the tissue on FFPE

-Retrieval of the antigen for proper detection

-Freezing of sample to halt peroxidase activity

-Labelling of antibody for visualization

Microtome set was used to dissect the block FFPE samples into a thin 4.0 µm size. This was followed by heating on a water bath so as to flatten the sample, then put on a coated poly-L-lysine microscope. After sample attachment, incubation for 1 hour at 70 ℃ was followed. Xylene was used to remove the paraffin in 3 different solutions for 5 minutes followed by rehydration in 96% ethanol for 3 minutes. This was then rinsed in distilled water to remove the alcohol.

Antigen retrieval process was performed to remove the methylene bridges ($-CH_2$) that were formed during fixation. This unmask the antigen and render it prone to be recognized and attached by the antibody. This was done by incubation with proteolytic enzymes or trypsin or alternatively using the Heat Induced Epitope Retrieval (HIER) method whereby microwave or pressure cooker supply heat to the tissue section. This method is either an acidic at pH 6 or alkaline at pH 9. The choice of either acidic or alkaline medium depends on the antigen and antibody.

In this study, HIER was used, and contains an antigen retrieval agent/buffer which serves as the pre-treatment solution. Mixtures of the target solutions contain 1800 ml dH_2O + 100 ml of target retrieval solution with high pH (50x) and low pH. Sample heating using the pressure cooker was performed for 20 minutes to standardize the HIER. This was then allowed to cool for 20 minutes at room temperature. dH_2O was used to wash the tissue section for 5 minutes and Tris-buffer saline (TBS) was used further to wash for another 5 minutes 3 times.

Hydrophobic barrier was drawn on the tissue section using a hydrophobic pen to prevent nonspecific hydrophobic interactions; this will in turn reduce the antibody to be used. 10 drops of peroxidase blocking solution was then used to block the tissue and incubated in a humid chamber for 10 minutes. TBS was used again to wash 3 times for 5 minutes and rinsed with dH_2O then wiped to remove excess liquid that may be left on the slide. Diluted primary antibodies were used to incubate the tissue sections. The antibodies dilutions consist of anti-beta defensin- 1-ab14425 (Mouse monoclonal to HβD-1[M11-14b-D10]) 1:500 diluent; anti-beta defensin-2-ab63982 (rabbit polyclonal HβD-2) 1:500 diluent; anti-cathelicidin-ab69484 (rabbit polyclonal to LL-37) 1:200 diluent; anti-ribonuclease 7-ab205565 (Mouse monoclonal to RNase7 [4C4]) 1:100 diluent; anti-hepcidin-ab57611 (Mouse monoclonal to hepcidin) 1:100 diluent; anti-LEAP-2-ab122294 (Rabbit polyclonal to LEAP-2) 1:200 diluent. anti-Rabbit IgG (polyclonal isotype control antibody)-ab27478 (rabbit polyclonal to rabbit IgG) 1:200 diluent was used for the isotype control (negative). In the positive control, the spleen was used for LL-37, liver for HβD-1, pancreas for HβD-2, skin for RNase-7, skeletal muscle and hepcidin for LEAP-2. All the procedures were performed according to the manufacturer's instructions.

Samples (covered with lid) were incubated again in the humidity chamber for one and half hours at room temperature. The TBS buffer was used to wash the sample 3 times for 5 minutes. 10 drops of Sensitek anti polyvalent biotinylated secondary antibody was added and allowed to incubate in a humidified chamber for 20 minutes. Then rewashed with a TBS buffer as previous. 10 drops of 3,3'- Diaminobenzidine (DAB) chromogen was added using pipette and incubated for 5 minutes then washed slightly with distilled water. This chromogen contains 1 drop of DAB substrate chromogen + 1ml of DAB substrate buffer. DAB binds to HRP and produces a brown color.

6 drops of Mayer's hematoxylin counterstain was added to the tissue and incubated so as to stain the nuclei blue and react with the brown color of DAB for proper visualization. Tissues were washed twice with dH_2O at 5 minutes interval. Sections were dehydrated in 96% ethanol in 3 equal concentrations for 10 seconds and later dewaxed. Entalian mounting media 93 drops) was added while avoiding bubbles. A light microscope set at 200X magnification and set filters was used for all samples except for protein expression examination of Rabbit IgG which uses 100X magnification.

3.4. SEMI-QUANTITATION METHOD FOR POSITIVE CELL DISTRIBUTION CELL STAINING INTENSITY

Semi-quantitation method was used to score the staining intensity according to the method previously explained by Wang et al (2012). This was followed by choosing 10 random fields and the expression was analyzed for 1000 cells i.e 100 cells/field. This was achieved using a high power magnified microscope of 200X. Mean value (average) of the stains were used for further analysis.

positive cell distribution in epithelium:

On the distribution of positive cell in epithelials, it is designated by 1- basal layer, 2- middle layer and, 3- full thickness of squamous layer.

Average mean value of the experimental score was categorized as follows:

0% were ranged as 1-10% positive cells

% of positive cells	Denomination
0%	0
1-10	1
11-50	2
51-80	3
81-100	4

The intensity of stained cells were classified based on their strength.

0 = negative

1 = weak

2 = moderate

3 = strong

TLR expression (%)= Positive cell distribution (PCD) X Staining intensity (SI)

3.5. Statistical analysis

Statistical analysis was carried out based on the data analyzed. Semi-quantitation analysis result were analyzed for statistical significance. Mannwhitney U test was performed on nonparametric data. IBM SPSS version 18.0.0 was used to analye all data. l

CHAPTER IV

RESULTS

4.RESULTS

Histological and Immunological assessment of epithelial cells in PFAPA

4.1 Tonsillar TLR expression level and pattern in PFAPA patients

The histological profiles of patients with PFAPA show different expression of TLRs in both samples and control. The TLRs expressed on the epithelial cells is indicated based on these indices, positive cell distribution in epithelium and staining intensity (0-negetive, 1-weak, 2-moderate, 3-strong). The result of immunohistochemistry (IHC) of the positive staining of the epithelium of tonsils samples of PFAPA patients, ranging from weak to strong expression is presented in the **figure 4**.

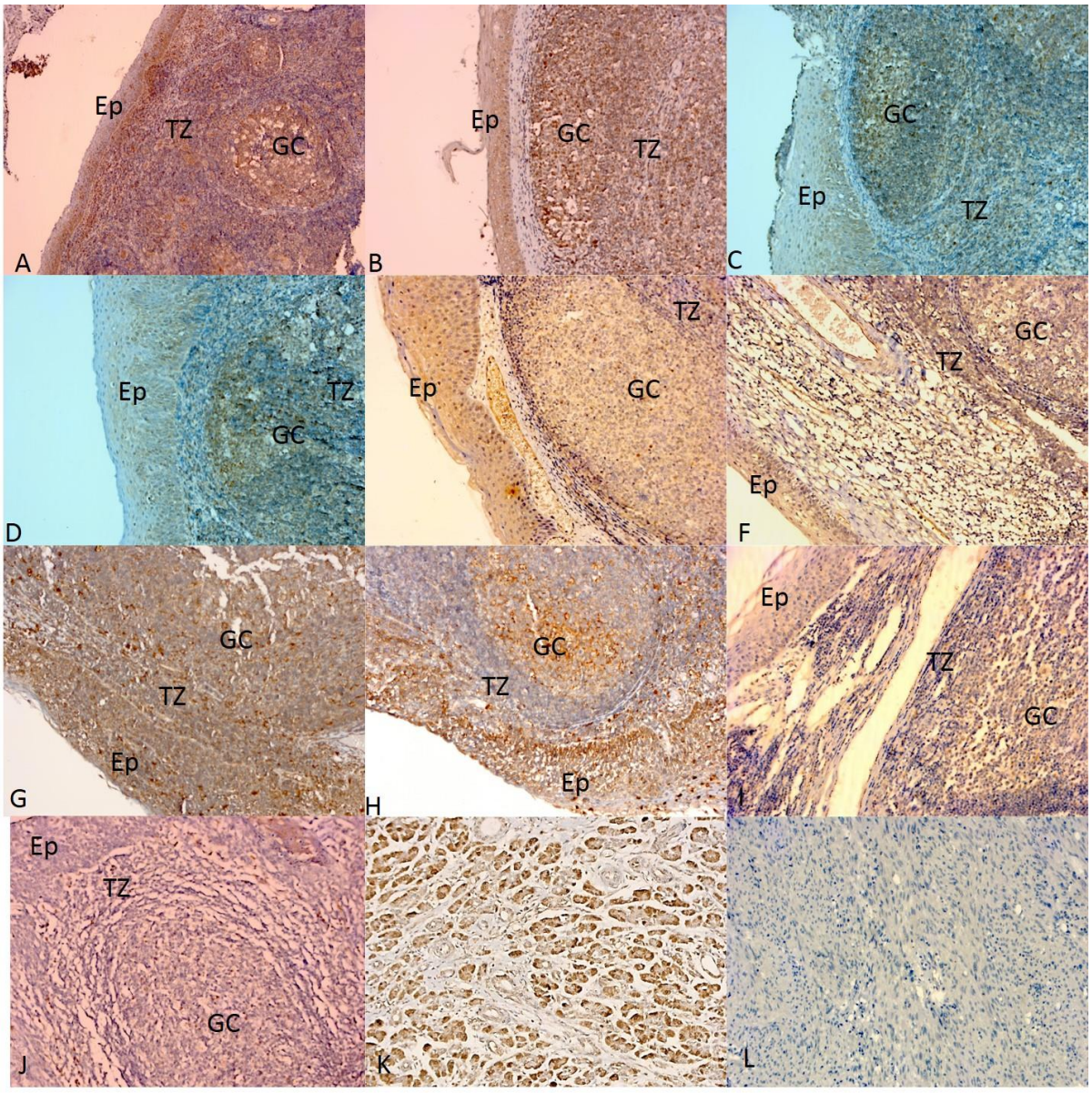

Figure 4. Immunohistochemical staining of surface epithelium of tonsil samples isolated from PFAPA(A, C, E, G, I) and GAβHS (B, D, F, H, J) patients. Tonsil samples were incubated with primary antibodies against TLR-1 (A, B), TLR-2 (C, D), TLR-4 (E, F), TLR-5 (G, H), and TLR-6 (I, J). Breast cancer tissues (K) and samples incubated with rabbit IgG isotype control antibodies (L) were used as positive and negative controls .

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The TLRs profiled in this study are TLR1, TLR2, TLR 4, TLR5, and TLR6; and the results are indicated as follows; the TLR1 show increase level of staining intensity in both the patients and the control. Briefly, There is no any statistically difference in TLR-1 expression level on tonsillar surface epithelium between PFAPA patient and control as indicated in **Figure 5.**

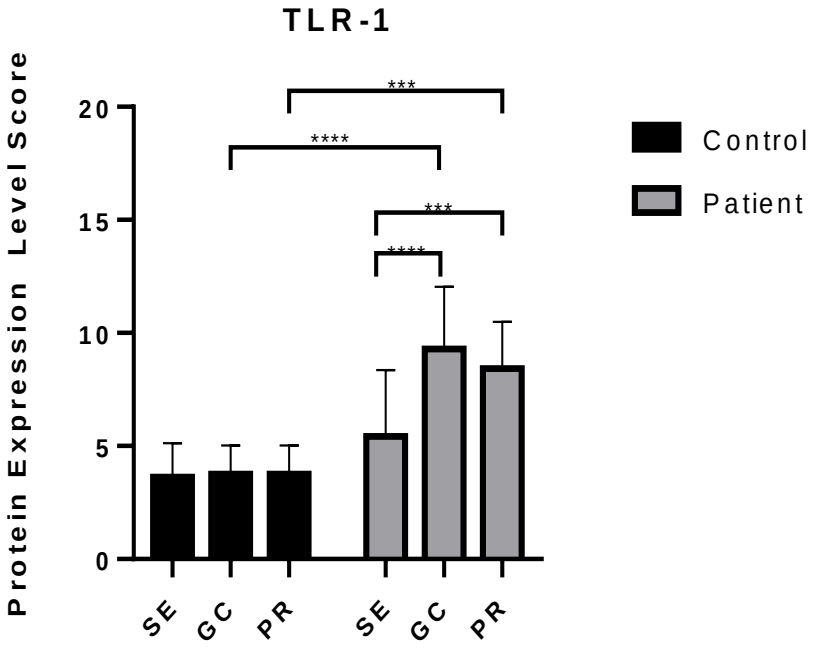

Figure 5. Expression level scores of TLR-1 from PFAPA

Similarly, the level of TLR2 level was determined, it was observed that it evenly distributed in epithelial in the basal layer while in the control is mostly in the squamatous layer and middle layer and The expression level of TLR-2 in PFAPA patient on tonsillar surface epithelium (SE) was found to be lower than control as indicated in **Figure 6.**

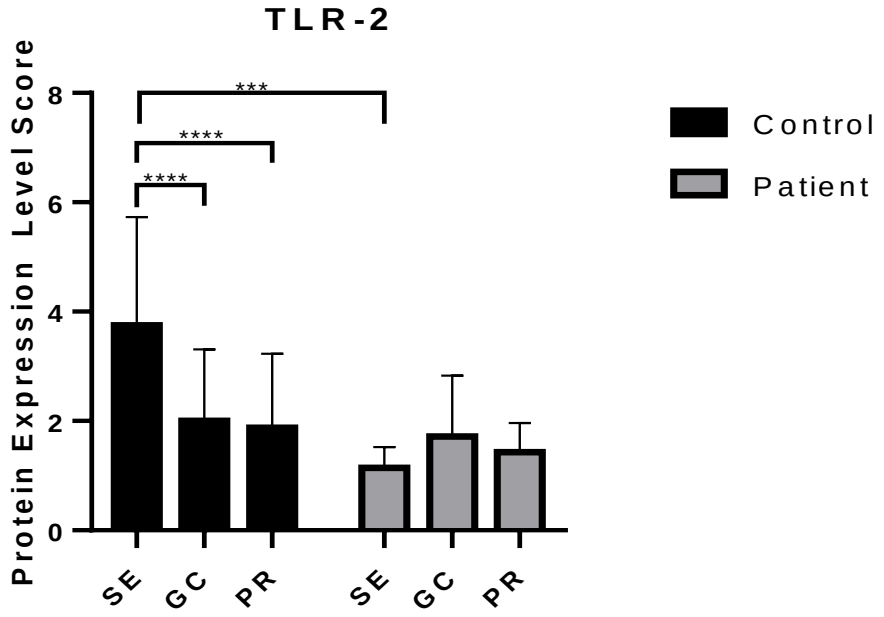

Figure 6. Expression level scores of TLR-2 from PFAPA

Furthermore, TLR4 was quantified with immunohistochemistry. The positive cell distributions of the epithelial were predominantly in the basal layer in both patients and the control groups. In the basal layer of the epithelial, there was decreased expression of TLR4 in both patients and the control. There was no difference on the result of expression of TRL4 in comparison with the control group **Figure 7**.

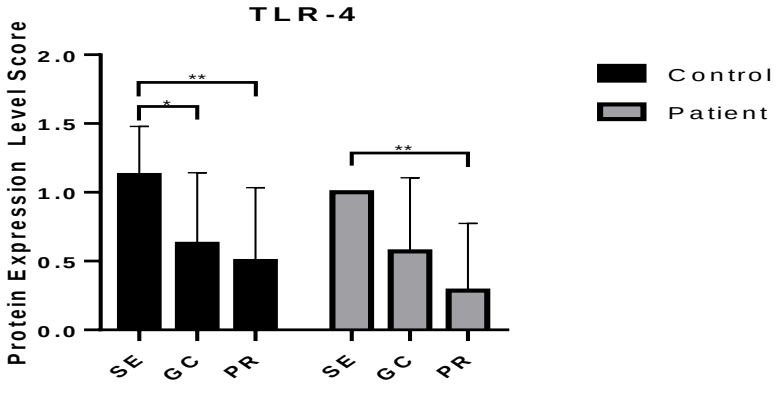

Figure 7. Expression level scores of TLR-4from PFAPA tonsils.

Conversely to TLR4, the result of TLR5 expression shows similar pattern of high positive cell distribution of the epithelial at squamatous layer of the cells in both patients and the control. High TLR5 was detected in both samples and the control **Figure 8.**

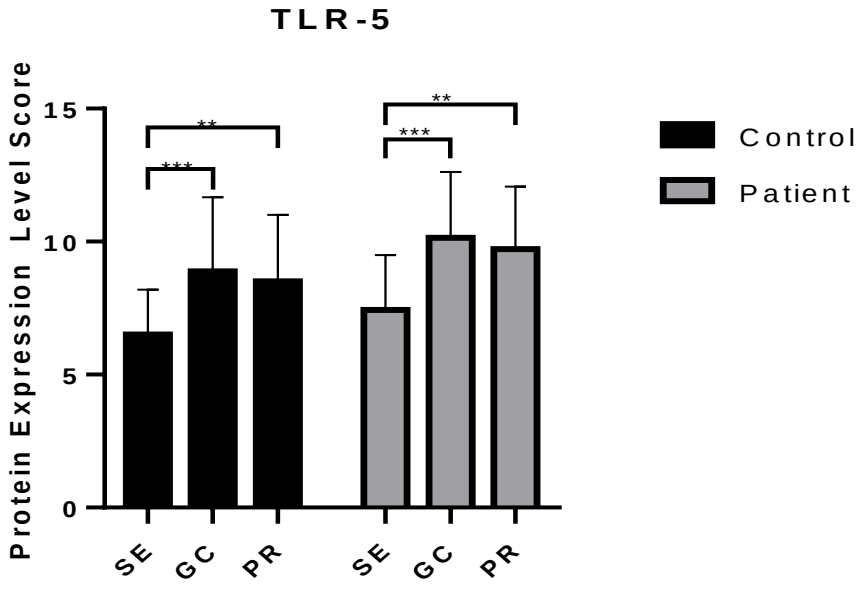

Figure 8. Expression of TLR-5 in PFAPA samples

In contrast, in TLR 6 there was no positive cell distribution of TLR5, while the intensity of the expression was weakly expressed in both samples and control groups as shown in **Figure 9.**

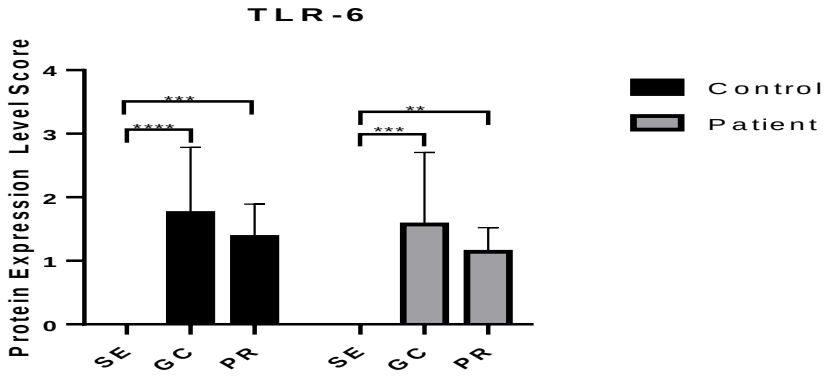

Figure 9. Expression level scores of TLR-6 obtained from PFAPA patients.

CHAPTER V

DISCUSSION AND CONCLUSION

5. DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA), otherwise known as Marshall Syndrome, is grouped among recurrent fever syndrome. The classical characteristic of the presentation involves episodes of fevers lasting 3-7 days and reoccurring every 2 to 8 weeks and further associated with aphthous stomatitis, pharyngitis and cervical adenitis (Trandafir *et al*., 2016). The etiology of PFAPA has not been fully understood, however, it is considered auto-inflammatory disease (AID) because the fever is accompanied by increase in CRP, ESR, leukocyte levels and can respond to single-dose corticosteroids (Gattorno et al., 2009; Gazi et al., 2018). Demographically, the disease is mainly found among 2-5 age groups and mostly in males than females. PFAPA is not contiguous and it does not propose a long term danger to the patients.

TLRs in disease conditions such as PFAPA and wounds are suggested to contribute significantly to the initiation of innate immune responses and inflammation, thus, can either aggravate the progression of disease or affect healing process (Acosta et al., 2008,) (Rosa Ramirez & Ravi Krishna Dasu, 2012). Thus, it is evident that different patterns of TLRs expression occur in different pathological conditions. Furthermore, the localization of the expression differs by the type of TLRs (Hug et al., 2018). In general context, TLRs respond to microbial products or pathological conditions that are expressed on the cellular membrane of immune and epithelium cells. TLRs such as TLR1, TLR2, TLR5, and TLR6 are heterodimers, while TLRs such are TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR12 are expressed on the membrane of intracellular

compartments. Strikingly, TLR4 is expressed on cellular membranes serving dual role of a pro-inflammatory signaling as well as intracellular to act as anti-inflammatory signaling (Hug et al., 2018)(Joosten et al., 2016). Thus, the current study aimed to assess tonsillar expression levels and patterns of pro-inflammatory cell surface TLRs which play important role in induction of inflammation against pathogens and maintaining symbiosis between the host and microbiota (Edberg et al., 2015).

In this study, the profile of TLR expression of patient suffering from PFAPA was assessed. Tonsil samples were extracted from PFPA patients through tonsillectomy and immunohistochemistry was later performed to examine the expression levels of TLR-1, 2, 4, 5, and 6 localized on the cell membrane. The result of this study showed that all pro-inflammatory cell-surface TLRs, with the exception of TLR-6, are expressed on tonsillar SE in PFAPA patients and control groups. The specificity of anti-TLR antibodies were confirmed by negative control samples which were treated with rabbit polyclonal isotype control antibodies. The lack of TLR-6 expression is not associated with the lack of antibody binding as TLR6 expression was observed on LI of the same tonsils and the positive control samples included (Fig.4).

The data obtained in the present study is controversial to those of a previous report by Lesmeister*et al.* which detected the presence of mRNAs for all ten TLRs in human tonsil samples (Lesmeister et al., 2006). This scenario can be explained by the presence of mechanisms such as post-transcriptional and/or post-translational modification(s) which play a role in regulation and inhibition of protein expression. Another explain to the detection of TLR-6 by Lesmeister*et al.* could be mix up with cells other than epithelial cells in the study. It is evident from the present study that showed the expression of all TLRs, including TLR-6, which are monitored, , on tonsillar LI, thus this that suggests immune cells as the main cellular source of TLR-6 expression in tonsils.

The main distributions were in basal layers of the epithelial as observed in TLR2 and TLR4. On the other hand, in TLR5, the positive epithelial cells were mainly distributed in squamous cell layer, while in TLR6 no positive distribution was observed. It is established that innate immune cells use PRRs to detect pathogens (De Oliveira et al., 2015). The striking result in the current study is detection of TLR-2 and TLR4, however, previous study failed to detect any of TLR-2 or TLR4 protein expression, which is contrary to the current study even though conducted on the same group of unstimulated primary tonsillar epithelial cells (Lange et al., 2009). However, in the same study, under different stimulation of UT-SCC-60B tonsillar epithelial cell line, TLR-2 was detected after Poly I:C (TLR-3 agonist) stimulation, but there was no TLR-2 before. This shows that the detection of TLR-2 and TLR-4 protein expression in the current study was due to the activated state of PFAPA tonsils (Luu et al., 2020). Thus, expression of all TLRs in this study on tonsillar indicates that immune cells as the principal cellular source of TLRs expression in tonsils.

Previous studies reported that TLRs are used as signalling pattern-recognition receptors, to commence immune response in a disease conditions (Dasu et al., 2010). Porchernina and Daschuk (2020) demonstrated that TLRs are involved in initiating immune response in patients with psoriasis. Especially, TLR2 and TLR4 were expressed in blood of the patients in the study, which is similar to the current study, however, low expression level was noticed compared to the work of Porchernina and Daschuk (2020). This difference could be attributed to the source of the sample used in the study where blood is used in the former study while tonsil tissues were used in our study.

This study demonstrated low TLR-2 (/????) expression levels on tonsillar SE in PFAPA while no difference the levels of expressions of other TLRs. A previous study reported the link between PFAPA and tonsillar TLR-2 expression. The study involved transcriptome analysis that compared

the PFAPA endotypes with each other and showed high expression in PFAPA "endotype 1" patients (Hara et al., 2020).

Stojanov et al., (2011) reported that T_H1 activation and associated T_H1-chemokinemia defects are the major defects that result to PFAPA. In a particular study, lower IL-4 (a T_H2 cytokine) expression level was reported in PFAPA tonsils from patients with tonsillar hypertrophy without any underlying inflammatory disease (Valenzuela et al., 2013).

Another study revealed that lack of TLR2 is associated with decrease in precursors of inflammation in disease in condition. For instance, Daru et al., (2020) in a mice study model showed that deficiency of TLR2 is linked with decreased IL6 and TNFα, this is indicating that TLR2 is linked with hyper-inflammation. In another study, a significant increase of TLR2 and TLR4 expression was observed in human diabetic wound experiments, in comparison with normal cells, this expression may contribute to the enhancing signaling and thus, increase inflammation, as noticed in the current study (Dasu and Martin, 2014). As reported previously elsewhere, the negative correlation between TLR-2 and IL-4 expression levels (Fenhalls et al., 2003), suggests up-regulation of TLR-2 expression in PFAPA patient group tonsils in the present study could be due to local induction of T_H1-skewed immune response. Furthermore, another study show lack of TLR-2 and TLR-4 expression on tonsillar epithelial cells (Lange et al., 2009), which is in contrast with the present study.

Lack of functional adaptive immune response is also involved in PFAPA pathogenesis (Kraszewska-Głomba et al., 2015b). In a particular study, which involved analysis of tonsillar microenvironment using flow cytometry revealed that PFAPA patients possess constitutively activated B-cells and higher numbers of B-cells and memory B-cells than patients with infectious

pharyngitis (Luu et al., 2020). In addition, TLRs were previously shown to have impact on B-cell-mediated responses when encounter microbial antigen binding (Defranco et al., 2012). Thus, the present study also monitored TLR expression in tonsillar LI, where B-cells develop into high affinity plasma and memory B cells with the help of follicular helper T-cells.

Conversely, the current study reported TLR-5 expression tonsillar germinal centres, this is agreement with previous report of (Månsson et al., 2006). This could be aided by the presence of other cells in germinal centre. Based on this study, all TLRs screened were also detected in tonsillar parafollicular regions that strengthen that immune cells other than B-cells may play role in the generation of TLR expression profile detected in germinal centres. Thus, there is need for further studies using cell-specific markers to identify and confirm the cellular sources of TLR expression.

As limitation, the current study failed to exhibit any difference in TLR expression levels on except for TLR-1 which was significantly higher in the PFAPA tonsil samples. The most prominent TLR expression was reported to be TLR-1 in tonsillar B- and T-cells (Mansson et al., 2006; Månsson et al., 2006). The result is in parallel with histological appearance of PFAPA tonsils characterized by reactive lymphoid tissue dominated by hyperplastic germinal centres.

For TLR-5 and TLR-6, the current study showed high expression levels of L1 with the absence of TLR-6 in SE. On the contrary, there was significant difference for the detection of TLR-1 and TLR-2 which was found to be higher than that on SE for PFAPA.

For TLR-4, the pattern of the expression was higher on SE. Even though, few PFAPA sample sizes were used, thus it is recommended that large samples should be use to ascertain the current result.

It is established that TLR5 is reported to be high in epithelial cells than TLR2 and TLR4(Abreu, 2010; Cario & Podolsky, 2000; Hug et al., 2018; Yu & Gao, 2015). In this study, it was found that

high expressed TLR5 diffusely distributed across the basal and middle layers of the epithelial cells of tonsils in PFAPA patients. This was also reported previously (Horng et al., 2002) in colon epithelial cells and it is recognized as pro-inflammatory induction genes of TNFα, IL1β and IL6. Strong intensity expressions of TLR5 in the current study is in agreement with a previous study but differ in the distribution of the positive cell in epithelial cells that is mainly confided in the middle and full thickness of the squamous cells. This could be explained by the nature of the tissue used. In the current study, tonsils were used while Horng et al., (2002) employed colon tissue.

In this study, the intensity of TLR6 was weakly expressed. In contrast, TLR6 was significantly increased in wounds of diabetic patients in comparison with non-diabetic patients (Daru and Martin, 2014). The authors suggested that increased in expression of TLR6 lead to signaling and activations of cytokines responsible for hyper-inflammation. On the basis of the result obtained in this study, the positive distribution of the epithelium was negative. TLR6 was studied in colorectal cancer, with decreased in expression in colon cancer tissues (Semlali et al., 2019) which is similar to this study. The striking result in this study is lack of TLR6 in the epithelial of the samples, which is contrary to the fact that the TLR6 genes are expressed by epithelial cells. Some studies focused on the association of pathological conditions with TLR6 expression, and the results indicated higher TLR6 in controlled groups than cancer cells (Semlali et al., 2019), this can be explain by the fact that TLR6 mRNA is more expressed in normal cell than malignant cells, this is in partial agreement with the current. There is growing eveidence TLR 6 genes correlated with disease conditions such malaria, pancreatic cancer, and I tonsils of long term smokers (Koha ilan et al., 2016; Leoratti et al., 2008). The present study revealed the presence of TLR-5 and TLR-6, which was in contrary to the expression of TLR-2 and TLR-4, indicating statistical difference among the TLR expression levels.

As form of immune response, defective adaptive immunity is reported to be involved in PFAPA (Kraszewska-Głomba et al., 2015b). In a recent study by Luu et al., (2020), it was revealed that tonsillar cells of PFAPA patients contained activated B-cells in pharyngitis. Another study also show the presence of B-Cells and memory B-cells in their responses to microbial antigen binding(Defranco et al., 2012)

5.2.Conclusion

Conclusively, data from histoimmunochemistry of PFAPA patients showed different distribution patterns of the positive cell epithelial compartments. With predominantly distributions at basal layers in TLR2 and TLR4, others were distributed across the middle layers and full thickness of squamous layers as demonstrated by TLR1 and TLR5. TLR6 was not positive in the epithelial cells. The expression of TLRs in this study was found to be high in TLR2 and TLR4, indicating their roles in disease progression of PFAPA. The level of intensity of the expression of TLRs in this study was found to be higher than the control groups. On TLR 6, the absence in the epithelial cells and weak intensity showed a minimal role in the pathogenesis of PFAPA. The highest expression in this study was found in TLR1 and it was widely distributed across all layers. It is therefore recommended that large sample size to be conducted to in an uninflamed tonsil with specific marker to validate the result of current study with the aim of detecting role of tonsils in PFAPA syndromes. whether microbiota change is as a result of or gives to AMP and TLR-s changing is not clear.

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