Expression of TLRs and CD14 does not necessarily correlate with the type of pathogenic bacteria in the tonsils of tonsillectomy patients

ABSTRACT

Aims: It has been revealed that in patients with chronic tonsillitis there is an increase in the expression of various types of toll-like receptors (TLRs) in the tonsils. The TLRs in question, especially TLR2 and 4, require Cluster of Differentiation 14 (CD14) in recognising the cellular component of the pathogenic agents. This study aimed to evaluate whether the expression of TLRs and CD14 are associated with the types of bacteria of tonsillar surface swabs of tonsillectomy patients.
Study design: Cross-sectional.
Place and Duration of Study: Dustira Hospital, Cimahi, West Java, Indonesia, between May and September 2017.
Methodology: Children aged 4-15 years (n = 34) with chronic tonsillitis showing indications for tonsillectomy in Dustira Hospital, Cimahi, West Java, Indonesia were included in the study. Tonsilar surface swabs were taken by rotating a sterile-cotton tip fine needle on the surface of the tonsils and inoculated into sheep blood and MacConkey agar plates. After tonsillar swabbing then tonsillectomy performed, tonsil specimens were transported to the laboratory in an hour or less for TLRs expression examination using immunohistochemical techniques. To assess CD14+ leukocytes, the venous blood sample (1 ml) of each subject was collected and analyzed using the whole-blood flow cytometry-based method.
Results: Tonsillar surface swabs culture resulted in 18 (52,9%) cultures that were not overgrown with pathogenic bacteria and 16 (47,1%) cultures were overgrown with *Staphylococcus aureus* (n = 12) and *Klebsiella pneumoniae*+*Streptococcus* non-group A (N = 4). All statistical analysis performed regarding the role of bacterial types in TLRs and CD14 expression, as well as the association between variables, showed no significant results.
Conclusion: The present study suggests that in chronic tonsillitis, the expression of TLR2 and TLR4, as well as their CD14-coreceptors, do not necessarily correlate with the type of pathogenic bacteria on the surface of the tonsils.

Keywords: Tonsillitis, chronic tonsillitis, tonsillectomy, TLRs, CD14

1. INTRODUCTION (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

For decades the diagnosis and treatment of chronic tonsillitis remain a matter of debate. One of the controversial topics surrounding tonsillitis is the main causative organisms responsible for the occurrence of tonsillitis [1]. Not only the type of bacteria but also the site of infection on tonsil tissues is too confusing. Both Gram-positive and Gram-negative bacteria, aerobic and anaerobic bacteria actually can be isolated from the tonsils, either from the surface or the core [2, 3]. The information regarding the type of pathogenic bacteria is important in the treatment of the disease, especially in the selection of appropriate antibiotics in order not to cause resistance. The increasing incidence of resistance in many organisms has been proven to lead unsuccessful medical therapy which results in recurrent or chronic forms of tonsillitis [4].

The immunological study revealed that in patients with chronic tonsillitis there is an increase in the expression of various types of toll-like receptors (TLRs) in tonsil tissue. Such increase of the TLRs is nothing but the response of immune system to the pathogenic agent on the tonsil tissues [5]. TLRs are protein pattern recognition receptors (PRRs) found in macrophages, monocytes, dendritic cells, T and B cells in lymphoid tissues. However, in recognising pathogenic bacteria, TLRs especially TLR2

and TLR4 require Cluster of Differentiation 14 (CD14) co-receptors to be activated [6]. In patients subjected to adenoidectomies due to chronic adenoid inflammation and hypertrophy, CD14 expression is found in all specimens analysed using immunohistochemical techniques [7].

However, there have been no scientific reports explaining whether the expression of CD14 is associated with the type of pathogenic bacteria responsible for the disease. Therefore, the current study is aimed to investigate whether expression of TLRs, especially TLR2 and 4, and their CD14-coreceptors are correlated with the type of pathogenic bacteria in the tonsils of patients with chronic tonsillitis.

2. MATERIALS AND METHODS

2.1 Research subjects

The research subjects were children (n=34), boys and girls aged 4-15 years, with chronic tonsillitis (tonsillitis symptoms occurred more than three times a year) showing indications for tonsillectomy, and whose parents/guardians are willing to sign informed consent, in Dustira Hospital, Cimahi, West Java, Indonesia.

2.2 Bacterial determination

Just before the tonsillectomy, tonsil surface swabs were taken trans-orally under direct vision by rotating a sterile-cotton tip fine needle on the surface of the tonsils without touching other parts of the throat. Each swab was placed in a sterile transport medium. For bacteriological examination, swabbed samples were inoculated into sheep blood and MacConkey agar plates. Identification of the bacteria was made using conventional procedures.

2.3 TLRs assessment

After the tonsils are swabbed, tonsillectomy was performed using dissection and routine techniques. Immediately, tonsil specimens were placed in a sterile container and transported to the laboratory in one hour or less for TLRs expression examination using immunohistochemical techniques. TLR2 was assessed using Anti-TLR2 Antibody (clone TL2.1), Cat: LS-C139995/10757, from LifeSpan BioSciences, Inc. For TLR4, the reagents used were Mouse monoclonal antibody (clone 25), Cat: sc-293072, from Santa Cruz Biotechnology Inc. (Dallas, USA). Positive cells were indicated by cytoplasmic staining under a light microscope and the percentage of positive in 100 cells was recorded and graded.

2.4 CD14 assessment

To assess CD14+ leukocytes (lymphocytes, monocytes and neutrophils), whole-blood flow cytometry-based method was used. The venous blood sample (1 ml) of each subject was collected using venipuncture in a sterilised tube for blood collection containing heparin. Whole blood samples (100 µl) were firmly mixed with 10 µl of antibodies CD3 (SK7) PerCP (BD Cat. No. CD0035-B17) and CD14 PE (BD Cat.No.CD0036-B17) for 30 seconds. After lysing solution was added, and erythrocytes are completely lysed, the samples were analysed within 6 hours. Both flow cytometer and antibodies used in the study were BD Facs Calibur system from BD Bioscience (Becton Dickinson, San Jose, CA, USA). The flow cytometry results are expressed in % total $CD14^{+}$leukocytes (lymphocytes, monocytes and neutrophils).

3. RESULTS AND DISCUSSION

3.1 Tonsillar surface bacteria

Tonsillar surface swabs culture resulted in 16 cultures (47,1%) that were overgrown with pathogenic bacteria and 18 cultures (52,9%) were not. The identified types of pathogenic bacteria, as presented in Table 1, included both Gram-positive and Gram-negative bacteria. The Gram-positive group included *Staphylococcus aureus* (n = 12) and *Streptococcus* non-group A (n = 2), while one species from the Gram-negative group is *Klebsiella pneumoniae* (N = 4). However, all the colonies of non-group A streptococcal bacteria were obtained from tonsil swabs culture containing *K. pneumoniae*.

Table 1. Pathogenic bacteria isolated from tonsil surface swabs

Pathogenic Bacteria	Number of Swabs	
	N	%
Negative	18	52.9
Staphylococcus aureus	12	35.3
K. pneumoniae + *Streptococcus* non-group A*	4	11.8

**Streptococcus non-group A (n = 2) were identified in tonsil swabs culture overgrown with Klebsiella pneumoniae* (*n = 4*).

3.2 TLR2 and TLR4 expression

Immunohistochemical results of tonsil tissue for expression of toll-like receptors (TLRs) are presented in Table 2. There were only 31 samples of tonsil tissues eligible for TLR2 and TLR4 assessment. The data indicate the average expression of TLR-2 (2.59%) is lower than that of TLR-4 (28.78%).

Table 2. Expression of TLR-2 and TLR-4 in tonsillar tissues

	Min.	Max.	Mean ± SD
TLR-2 (%)	0	18	2.59 ± 4.25
TLR-4 (%)	10	60	28.78 ± 10.49

3.3 CD14 expression

The results of CD-14 expression examination on blood samples using the flow cytometry method are presented in Table 3. The data show that CD-14 is most expressed by monocytes (56.57%), then in lymphocytes (5.03%), and least on neutrophils (0.55%). When compared to the total number of leukocytes read in the flow cytometry (500,000 events), the percentage of lymphocytes, monocytes, and neutrophils expressing CD-14 are 1.22%, 1.64%, and 0.17%, respectively.

Table 3. Expression of CD14 based on gating strategy by flow cytometry

CD14+		N	Min.	Max.	Mean + SD
Lymphocyte	%Gated	31	0.14	11.98	5.03 ± 3.05
	%Total	31	0.05	2.43	1.22 ± 0.66
Monocyte	%Gated	31	21.8	83.62	56.57 ± 17.44
	%Total	31	0.34	3.06	1.64 ± 0.65
Neutrophil	%Gated	31	0.14	1.47	0.55 ± 0.30
	%Total	31	0.02	0.4	0.17 ± 0.10

By converting expression values of CD14+leukocytes in Table 3 using Equation 1 below, the number of each type of CD14+leukocyte was estimated.

$$\sum \text{Leukocyte} = \left(\frac{\%\text{Total CD14}}{\%\text{Gated C14}}\right) \text{x total event in gating strategy} \quad (1)$$

The results are presented in Table 4. Descriptively the data clearly show that in patients with recurrent tonsillitis, the highest number of white blood cells were neutrophils (51.74%), followed by lymphocytes (43.34%), and monocytes (4.92%).

Table 4. Number of lymphocyte, monocyte and neutrophil in venous blood samples by flow cytometry technique

	Min.	Max.	Mean ± SD
Lymphocyte (cells/100 µl)	40,856	194,915	133,324.6 ± 39,938.4
Monocyte (cells/100 µl)	3,727	31,881	15,138.3 ± 5,888.9
Neutrophil (cells/100 µl)	70,513	271,429	159,177.6 ± 60,818.1

3.4 Role of pathogenic bacteria in the expression of CD14 and TLRs

To find out the role patterns of pathogenic bacteria in CD14 and TRLs expressions, the Mann-Whitney Comparative Test was used. The results of the comparison test of CD14 expression in lymphocytes, monocytes, and neutrophils from venous blood samples of patients with recurrent tonsillitis according to the type of bacteria present in their tonsil swabs are presented in Table 5. Due to the p-values obtained from the comparative test of all variables are all above 0.05 ($0.185 < P < 0.955$). It can be assumed that the type of bacteria in the tonsil swab of recurrent tonsillitis does not affect CD14 expression in lymphocytes, monocytes, and neutrophils.

Table 5. Comparison of CD14 expression in leukocytes by types of bacteria in the tonsillar surface

Pathogenic bacteria	CD14+ Lymphocyte (%)		CD14+ Monocyte (%)		CD14+ Neutrophil (%)	
	Gated	Total	Gated	Total	Gated	Total
Negative	5.07	1.23	59.63	1.72	0.56	0.19
S. aureus	5.37	1.21	53.59	1.57	0.53	0.17
K. pneumoniae + Streptococcus	3.99	1.23	51.00	1.51	0.59	0.11
P-value*	*0.635*	*0.955*	*0.574*	*0.505*	*0.304*	*0.185*

**The p-value shown is the smallest among the p-values of the comparisons between values in the same column*.

The results of the comparative test of TLR2 and TLR4 expression on tonsillar tissues according to the types of bacteria on the tonsillar surface swabs are presented in Table 6. *P*-values representing the degree of difference of expression of TLR2 and TLR4 from tonsillar tissues with different types of bacteria are P = 0,394 and P = 0,511 respectively. Since *P*-values obtained are above the significance limit ($P < 0.05$), it can be assumed that the bacterial species on the tonsillar surface of recurrent tonsillitis do not significantly affect TLR2 or TLR4 expression in the tonsils.

Table 6. Comparison of TLR2 and TLR4 in tonsil tissues by types of bacteria in the tonsillar surface

Pathogenic bacteria	TLRs Expression (%)	
	TLR-2	TLR-4
Negative	3.47	30.18
S. aureus	2.5	27.5
K. pneumoniae + Streptococcus	1.5	26
P-value*	0.394	0.511

**The p-value shown is the smallest among the p-values of the comparisons between data in the same column*.

3.5 Correlation between CD14 and TLRs

To see whether the CD14 expression in venous blood leukocytes has an association with TLR2 and TLR4 expression in the tonsil tissues, a linear regression-correlation analysis was performed. The correlation coefficient (r), the coefficient of determination (r^2), and *P*-value obtained from the test between $CD14^+$ leukocytes and TLR2 as well as TLR4 are presented consecutively in Tables 7 and 8. Statistical data of the regression-correlation between dependent and independent variables in both Tables 7 and 8 show all *P*-values above 0.05. Thus it can be asserted that level of CD14+leukocytes in the venous blood samples do not correlate with TLR2 or TLR4.

Table 7. Results of regression-correlation analysis between TLR2 and CD14+leukocytes

Independent variables	**TLR2 (Dependent variable)**

	R	R^2	*F*	*P*
CD14+ Lymphocyte	-0.142	0.020	0.595	0.447
CD14+ Monocyte	0.293	0.086	2.715	0.110
CD14+ Neutrophil	-0.031	0.001	0.028	0.868

R: Coefficient of correlation; R^2: Coefficient of determination; F: Anova F-value; P: probability.

Table 8. Results of regression-correlation analysis between TLR4 and CD14+ leukocytes

Independent variables	**TLR4 (Dependent variable)**			
	R	R^2	*F*	*P*
CD14+ Lymphocyte	-0.200	0.040	1.214	0.280
CD14+ Monocyte	-0.154	0.024	0.704	0.408
CD14+ Neutrophil	0.275	0.076	2.375	0.134

R: Coefficient of correlation; R^2: Coefficient of determination; F: Anova F-value; P: probability.

The data in Table 1 show that 18 (52.9%) of the patient's tonsil swabs did not overgrow with pathogenic bacteria. Then what kind of bacteria trigger an immunological reaction that results in inflammation of their tonsils? Most likely recurrent tonsillitis suffered by the subjects is caused by other types of bacteria or by anaerobic group of bacteria. As already reported, several types of anaerobic bacteria have interference ability against Streptococcus Group A β-hemolytic (GABHS) and other pathogenic bacteria [2]. Anaerobic bacteria commonly found in patients with recurrent tonsillitis are *Bacteroides spp*, *Fusobacterium spp, Prevotella spp, Peptostreptococcus spp*. [8]. Some anaerobic bacteria such as *Porphyromonas sp., Bacteroides fragilis, Prevotella intermedia, Prevotella melaninogenica,* and *Fusobacterium sp.* even found in both core and surface of the tonsils [9]. A trend similar to this study results has been reported by Al-Hameed *et al.* (2014) where among 100 tonsil swabs isolated from recurrent tonsillitis patients, 34 of them did not overgrow with pathogenic bacteria [10].

The presence of bacteria and microflora in an organ can be viewed as a biotic community of an ecosystem, where each component in the community has a particular ecological role. The bacterial species, both pathogenic and commensal, both aerobic and anaerobic in tonsil tissues allegedly contribute in tonsillitis. As postulated, the diversity of commensal microbes in the upper respiratory tract plays a role in increasing local colonization resistance. The decline in the opposition of commensal species promotes an uncontrolled growth of pathogens such as *S. pneumoniae*, *H. influenzae, S. pyogenes* and *M. catarrhalis*, leading to the onset of respiratory disease [11].

Higher TLR4 expression than TLR2, as shown in Table 2, indicates that tonsil tissue of patients with recurrent tonsillitis is more infected with Gram-negative bacteria. In fact, bacterial data (Table 1) clearly show the opposite. Toll-like receptors (TLRs) are transmembrane proteins expressed by innate immune system cells that recognise foreign microbes and activate signal systems that trigger an immune and inflammatory response to destroy the microbes. Unless TLR-3, 7 and 8 are typical for viruses, all TLRs play a role in recognising bacteria. TLR-2 plays a role in recognizing Gram-positive bacteria, while TLR-4 plays a role in recognising Gram-negative bacteria [12].

Table 3 shows that in patients with recurrent tonsillitis, CD14 is most expressed in monocytes (56.57%). This number corresponds to commonly known facts about CD14. In fact, not only in patients with tonsillitis, but CD14 is also strongly expressed in monocyte/blood macrophages of patients with other diseases in which bacteria with membranes containing lipopolysaccharides (LPS) are involved. That's why CD14 can clinically be a marker of risk or progression of a disease [13]. In people with asthma, it is known that adult patients with high CD14 content tend to exhibit more severe symptoms [14].

Additionally, the data in Table 3 also show that CD14 is not only expressed in monocytes but also in lymphocytes (5.03%) and neutrophils (0.55%). These data seem to contradict the conventional view that CD14 is only synthesized and expressed by monocytes and macrophages alone. Later, there is considerable evidence that CD14 is expressed by various cell types such as respiratory epithelial cells, uroepithelial cells, cornea, smooth muscle, fibroblasts, spermatozoa, and β-pancreatic cells. The role of CD14 in these non-myeloid cells is suspected to be related to the survival of the cells concerned-proven, monocytes whose CD14 cells have been excreted apoptosis directly. Another important role of CD14 in non-myeloid cells is to facilitate tolerance to endotoxin, thus benefiting host cells infected with Gram-negative bacteria [15].

Data in Table 4 clearly show that the proportion of leukocytes in chronic tonsillitis patients in this study is still the same as the proportion of leukocytes in normal (healthy) individuals. In sequence, neutrophils are at the highest then lymphocytes, and monocytes are at the lowest[16, 17]. Based on the above facts it can be concluded that the population of lymphocytes, monocytes, and neutrophils in the subjects of this study are in healthy proportions.

Table 5 shows results of the comparison test of CD14 expression in lymphocytes, monocytes, and neutrophils from venous blood samples by bacterial species on their tonsil swabs resulted in *P*-values above 0.05. This means that different types of pathogenic bacteria in tonsil tissue did not give significant effect to the CD14 expression on venous blood leukocytes. Given that more than half (52.9%) tonsils of study tonsils do not contain pathogenic bacteria, the CD14 expression in this group should be lower. This fact is most likely due to a swab culture containing only three types of pathogenic bacteria-*S. aureus*, *K. pneumoniae* and *Streptococcus* non-group A, is not sufficient to describe the immune reaction in the patient. As is well known in recurrent tonsillitis, in addition to Gram-positive aerobic bacteria, there are many types of Gram-negative anaerobic bacteria common to recurrent tonsillitis patients. These bacteria include *Bacteroides, Prevotella, Porphyromonas*, and *Fusobacterium* [18].

By simply culturing tonsil swabs many aerobic and anaerobic bacteria cannot be identified. The previous study has indicated that the majority of bacteria isolated from tonsils of tonsillitis patients such as *S. aureus, H. influenza, S. pneumonia, Klebsiella* spp., *B. catarrhalis, Pseudomonas* spp., and *S. epidermidis* may be isolated from the surface and core of tonsils [19].

Both TLR4 and TLR2, in their attachment process to bacterial cells are involving CD14. The transduction signal for TLR4 is activated by LPS in Gram-negative bacteria, with LPS first binding to CD14 receptor then transferring it to TLR4. TLR4 then undergoes homodimerization and forms complexes with the MD2 protein. Leukocytes require MD2 and TLR4 proteins to recognise LPS [20].

The transduction signals for TLR4 are activated by LPS on Gram-negative bacteria, whereas TLR2 is activated by LAM (Lipoarabinomannan), BLP (Bacterial Lipoprotein) and PGN (Peptidoglycans) in Gram-positive bacteria. LAM and PGN are acting on TLR2 through CD14 receptors. BLP mediates apoptosis and activation of NF-κB via TLR2. The TLR-2 phagocytic vesicles then generate TNF (Tumor Necrosis Factor) production via the NF-κB pathway [21].

The results of this study confirm how complex the role and relationship between immune factors in the human body. The expression of TLRs on the cell surface of the human body is very diverse and strongly associated with sensitivity to infection whose mechanism is not fully understood. Its role and interactions are not limited to the innate immune response alone but are also crucial in adaptive immunity [22].

4. CONCLUSION

In the case of chronic tonsillitis the expression of TLRs and their co-receptors CD14 do not necessarily describe and correlate with the type of pathogenic bacteria on the surface of the tonsils.

Ethical Disclaimer:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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