Original Research Article

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

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ABSTRACT

Aims: It has been revealed that in patients with recurrent 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 CD14 in recognizing 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. Tonsillar surface swabs were taken by rotating a sterile-cotton tip fine needle on the surface of the tonsils and innoculated into sheep blood and Mac Conkey's agar plates. After tonsillar swabbing then tonsillectomy performed, tonsil specimens were transported to laboratory in one hour or less for TLRs expression examination using immunohystochemical technique. To assess CD14+ leukocytes, venous blood sample (1 ml) of each subject was collected, and analysed using whole-blood flowcytometry-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 statistics performed regarding the role of bacterial types in TLRs and CD14 expression, as well as association between variables showed no significant results. It suggests that in chronic tonsillitis, the expression of TLR2 and TLR4 as well as their CD14-coreceptors do not necessarily depict the type of pathogenic bacteria on the surface of the tonsils.
Conclusion: It suggests that in chronic tonsillitis, the expression of TLR2 and TLR4 as well as their CD14-coreceptors do not necessarily depict the type of pathogenic bacteria on the surface of the tonsils.

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

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

For decades the diagnosis and treatment of recurrent tonsillitis remains a matter of debate. One of the controversial topics surrounding tonsillitis is the main causative organisms responsible for occurence of tonsillitis [1]. Not only the type of bacteria, but also the site of infection on tonsil tissues is also 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 regardingt 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].

Immunological study revealed that in patients with recurrent 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 recognizing pathogenic bacteria, TLRs especially TLR2 and TLR4 require 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 specimen analysed using immunohistochemical techniques [7]. In the context of recurrent tonsillitis, the question is whether expression of TLRs, especially TLR2 and 4, and their CD14-coreceptors account for the type of pathogenic bacteria in the tonsils?

2. MATERIAL AND METHODS

2.1 Research subjects

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

2.2 Bacterial determination

Just before the tonsillectomy, tonsillar surface swabs were taken trans-orally under direct vision by rotating a sterile-cotton tip fine needle on the surface of the tonsils wthout touching other parts of the throat. Each swab was placed in a sterile transport medium. For bacteriological examination, swabbed samples were innoculated into sheep blood and Mac Conkey's agar plates. Identification of the bacteria were done using conventional procedures.

2.3 TLRs assesment

After tonsillar swabbing then tonsillectomy performed using dissection and routine technique. Immediately after tonsil excision, tonsil specimens were placed in a sterile container and transported to laboratory in one hour or less for TLRs expression examination using immunohystochemical technique. 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 Biotechology 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 flowcytometry-based method was used, venous blood sample (1 ml) of each subject was collected by means of a venipuncture in a sterilized 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 second. 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 flowcytometry 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 culture (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+ group included *Staphylococcus aureus* (n = 12) and *Streptococcus* non group A (n = 2), while one species from the Gram- 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 at 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:

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

the number of each type of CD14+leukocyte can be estimated which the results presented in **Table 4**. Descriptively the data in **Table 4** showed that in patients with recurrent tonsillitis, the largest 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 flowcytometry 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 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 tonsillary 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 tonsillar 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. areus	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 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 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.areus	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 Correlationship between CD14 and TLRs

To see whether 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 correlated with TLR2 nor 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: Coeffiecient 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: Coeffiecient of determination; F: Anova F-value; P: probability.

The data in **Table 1** show that there are 18 (52.9%) tonsillar surface swabs cultures of research patients that are not overgrown with pathogenic bacteria. Then what bacteria trigger an immunological reaction resulting in inflammation of their tonsil tissue? It is possible that recurrent tonsillitis suffered by the subjects is caused by other types of bacteria or by anaerobic group bacteria. As already reported, there are several types of anaerobic bacteria that 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]. Although differs in the percentage, there was report revealed a similiar trend that among 100 tonsillar swabs cultures isolated from recurrent tonsillitis patients, 34% of the culture without 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 pathobins 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 shows the opposite. Toll like receptors (TLRs) are transmembrane proteins expressed by innate immune system cells that recognize 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 recognizing bacteria. TLR-2 plays a role in recognizing Gram-positive bacteria, while TLR-4 plays a role in recognizing 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, 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%). This data seems 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 directly apoptosis. 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 shows 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 most, then lymphocytes, and at least are monocytes [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 normal proportions.

Table 5 shows results of 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 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 there are many bacteria, both aerobic and anaerobic bacteria, unidentified. 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 isolated from the surface and core of tonsils [19].

Both TLR4 and TLR2, in their attachment process to bacterial cells involving CD14 (Cluster of Differentiation 14). The transduction signal for TLR4 is activated by LPS (Lipopolysaccharide) 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 recognize LPS [20].

The transduction signals for TLR-4 are activated by LPS (Lipopolysaccharide) on Gram negative bacteria, whereas TLR-2 is activated by LAM (Lipoarabinomannan), BLP(Bacterial Lipoprotein) and PGN (Peptidoglycans) in Gram-positive bacteria. LAM and PGN act on TLR-2 through CD14 receptors. BLP mediates apoptosis and activation of NF-κB via TLR-2. 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 important in adaptive immunity [22].

4. CONCLUSION

The expression of TLRs and their co-receptors CD14 can be used as evidence of the body's immune response to bacterial infection, but in the case of chronic tonsillitis the expression do not necessarily describe the type of pathogenic bacteria on the surface of the tonsils.

REFERENCES

[1] Loganathan A, Arumainathan UD and Raman R. Comparative study of bacteriology in recurrent tonsillitis among children and adults. Singapore Med J 2006; 47: 271-5.

[2] Brook I. The role of anaerobic bacteria in tonsillitis. Int J Pediatr Otorhinolaryngol 2005; 69: 9-19.

[3] Kumai A, Gupta V, Chandra K, Gupta P, Varshney S. Clinico bacteriological evaluation of surface and core microflora in chronic tonsilitis. Indian J Otolaryngol Head Neck Surg 2005; 57: 118-120.

[4] Vijayashree MS, Viswanatha B and Sambamurthy BN. Clinical and bacteriological study of acute tonsillitis. IOSR Journal of Dental and Medical Sciences (IOSR-JDMS) 2014; 13: 37-43.

[5] Janssens S and Beyaert R. Role of toll-like receptors in pathogen recognition. Clin Microbiol Rev 2003; 16: 637-646.

[6] Raby AC, Holst B, Le Bouder E, Diaz C, Ferran E, Conraux L, Guillemot JC, Coles B, Kift-Morgan A, Colmont CS, Szakmany T, Ferrara P, Hall JE, Topley N, Labéta MO. Targeting the TLR co-receptor CD14 with TLR2-derived peptides modulates immune responses to pathogens. Sci Transl Med 2013; 5: 185ra64.

[7] Peker BC, Acar M and Fiahin M. Identification of the immune receptor CD14 in hypertrophic adenoids. ENT Updates 2015; 5: 93-96.

[8] Brook I. Aerobic and anaerobic bacteriology of peritonsillar abscess in children. Acta Pediatr Scand 1981; 70: 831-5.

[9] Khadilkar MN and Ankle NR. Anaerobic bacteriological microbiota in surface and core of tonsils in chronic tonsillitis. J Clin Diagn Res 2016; 10: MC01-MC03.

[10] Al-Hameed FB, Ahmed Al-Ansary, Rukaia NS, Muna MK. Comparative study in bacteriological findings between the surface and the core of chronic infected tonsils. Thi-Qar Medical Journal (TQMJ) 2014; 8: 118-133.

[11] de Steenhuijsen Piters WA, Sanders EA, Bogaert D. The role of the local microbial ecosystem in respiratory health and disease. Philos Trans R Soc Lond B Biol Sci 2015; 370.

[12] Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. Nature 2004; 430: 257-63.

[13] Hermansson C, Lundqvist A, Magnusson LU, Ullström C, Bergström G, Hultén LM. Macrophage CD14 expression in human carotid plaques is associated with complicated lesions, correlates with thrombosis, and is reduced by angiotensin receptor blocker treatment. Int Immunopharmacol 2014; 22: 318-323.

[14] Kusunoki T, Wright SD, Inoue Y, Miyanomae T, Yoshida Y and Yoneda K. Serum levels of soluble CD14 in allergic inflammation. Allergology International 1998; 47: 271-278.

[15] Jersmann HP. Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. Immunol Cell Biol 2005; 83: 462-467.

[16] McGrathr CR, Hitchcock DC and van Assendelft OW. Total white blood cell counts for persons ages 1-74 years with differential leukocyte counts for adults ages 25-74 years: United States, 1971-75. Vital Health Stat 11 1982; 1-36.

[17] In: Soldin SJ, Brugnara C, Wong EC, editors. Pediatric references intervals. 5th edition (formerly pediatric reference ranges). Washington, DC: AACC Press; 2005. pp. 257.

[18] Finegold SM. Anaerobic gram-negative bacilli. In: Baron S, editor. Medical microbiology. 4th edition. Galveston, Texas: University of Texas Medical Branch at Galveston; 1996.

[19] Babu B and Reynolds AM. A study to find out the bacteriology of tonsillar surface and core, among patients undergoing tonsillectomy at a tertiary care hospital in South India. J Evid Based Med Healthc 2016; 3: 2131-2134.

[20] Mitchell JA, Paul-Clark MJ, Clarke GW, McMaster SK, Cartwright N. Critical role of toll-like receptors and nucleotide oligomerisation domain in the regulation of health and disease. J Endocrinol 2007; 193: 323-330.

[21] Hallman M, Rämet M and Ezekowitz RA. Toll-like receptors as sensors of pathogens. Pediatr Res 2001; 50: 315-21.

[22] Dabbagh K and Lewis DB. Toll-like receptors and T-helper-1/T-helper-2 responses. Curr Opin Infect Dis 2003; 16: 199-204.