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Relationship Between Age, CD14+ Leukocytes And Inflammatory Cytokines In Indonesian Children With Recurrent Tonsillitis

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Abstract: *Tonsillitis is common among children but the incidence and inflammatory responses varies according to gender, race, and age of the patients. The present study was designed to determine association strength between sex and age with immune response parameters, between expression of CD14 and inflammatory cytokines, and between cytokines molecules IFN-γ and TNF-α in Indonesian children with recurrent tonsillitis. Following informed concent, venous blood of the children (17 boys and 14 girls) were taken and divided into two parts. The first part (1 ml) was subjected to CD14+ leukocytes (lymphocytes, monocytes and neutrophils) analysis by whole-blood flowcytometry-based method. The second part (3 ml) was prepared for serum cytokine measurement using sandwich ELISA method. The results showed that in children with recurrent tonsillitis, the CD14 and cytokines content in their serum is not associated with sex. Furthermore, neither CD14 leukocytes nor serum cytokines correlated with age. However, there was a strong association between CD14 leukocytes with serum cytokines (P<0,031), and between IFN-γ with TNF-α (P<0,001). It can be inferred that in Indonesian children with recurrent tonsillitis, immune response parameters might not determined by sex and age. However, serum cytokines play a role in CD14+ expression, and cytokines themselves, especially IFN-γ and TNF-α can positively predict each other.*

Keywords: *recurrent tonsillitis, inflammatory response, CD14 cells, leukocyte, cytokine, IFN-γ, TNF-α*

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I. Introduction

One of common infectious diseases in childhood is tonsillitis that involves parenchyma of the palatine tonsils. When the symptoms appear more than three times a year the disease is called recurrent tonsillitis, and if more than five times the tonsillitis is then called true recurrent tonsillitis [1]. Recurrent (chronic) tonsillitis is the most common inflammatory lesions of the pharynx determining numerous local as well as distant evolutive complications [2]. Tonsillar inflammation is immune responses that are evoked as a host defense against various environmental stimuli including external stresses such as pathogens, foreign material, ionizing radiation and internal stresses such as excessive accumulation of metabolites, autoimmune responses and cancer. The inflammatory immune responses might involve both innate and acquired mechanisms in which all types of leucocytes and their secretory products such as cytokines, growth factors and chemokines act as inflammatory mediators [3].

Although tonsillitis is common among children but the incidence varies according to gender, race, and age of the children [4, 5]. These phenomena may be a logical consequence of the fact that the factors involved in the immune response are also determined by sex, race, and age. Children of all races who were under 6 years old

had a higher white blood cells (leukocytes) count than older persons had, and in both sexes of all ages, white population was found to have higher leukocyte counts than the black population [6]. In black population, girls three and four years of age had significantly higher mean lekocytes than boys in the same age group [7]. Age is known not only determine leukocyte counts but also the immune cells responses. It has reported that intracellular expression of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-2 protein expression increased progressively with age [8].

The present study was designed to determine whether in Indonesian children with recurrent tonsillitis, their immunological responses are determined by sex and age? Furthermore, does expression of CD14, especially $CD14^+$ lymphocytes, $CD14^+$ monocytes, and $CD14^+$ neutrophils, associated with inflammatory cytokines, especially (IFN)-γ and (TNF)-α? Finally, it is no less important, whether the cytokines can predict each other?

II. Materials And Methods

2.1 Patients

Patients included in the study 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 in Dustira Hospital, Cimahi, West Java, Indonesia.

2.2 Blood Samples Preparation

Following informed consent signed by the patient's parent/guardian, 4 ml venous blood sample of the children was collected by means of a venipuncture in a sterilized tube for blood collection containing heparin. Blood samples then divided into two parts. The first part (1 ml) was subjected to CD14+ leukocytes (lymphocytes, monocytes and neutrophils) analysis by whole-blood flowcytometry-based method. The second part (3 ml) was prepared for serum cytokine measurement using sandwich ELISA method.

2.3 Whole-blood Flow Cytometry

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 % gated and % total CD14+leukocytes (lymphocytes, monocytes and neutrophils).

2.4 Cytokines mesurement

The second part of the blood sample (3 ml) to be analyzed for its cytokine content, centrifuged at 5000 rpm for 30 min. Human IFN-gamma Quantikine ELISA Kit and Human TNF-alpha Quantikine ELISA Kit, both from R & D Systems Inc. (Minneapolis, MN 55413, USA) are consecutively used to measure tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) using sandwich ELISA method.

2.5 Data Analysis

Student t-test and Simple Linear Regression and Correlation statistics from XLSTAT version 2017.1 was used in data analysis. Student t-test was used to analyze whether patient's sex determines their immune response profile. Simple linear regression and correlation ststistics was used to measure the degree of linear association between research variables including: patient's age and observed immune response parameters; and between CD14+leukocytes and cytokines.

III. Results

Descriptive data of all observed parameters of children with recurrent tonsillitis covering sex, age, and percentage of CD + leukocytes and cytokines concentration are presented in **Table 1**. When the patient's immunological data is grouped by sex, then the t-test results performed on the mean value of each parameter yields statistical data as shown in **Table 2**. None of *p*-value of each immune response parameter in Table 2

showing a significant difference level. It is inferred that in children with recurrent tonsillitis, the CD14 and cytokines content in their serum is not determined by sex.

Table 3 shows the degree of linear association, based on simple regression and correlation statistics between immunological parameters and patient's age. Referring P-values of ANOVA for each correlation strength between patient's age and observed immune responses, neither CD14 leukocytes nor serum cytokines related to age in recurrent tonsillitis observed in the study.

Simple regression and correlation test between percentage of CD14 cells (CD14+ lymphocytes, CD14+ Monocytes, CD14+ Netutrophils) and serum cytokines (TNF-α and IFN-γ) of the research subjects results in the correlation coefficients (r) presented in **Table 4.** Among the three types of CD14 leukocyte analyzed, only lymphocytes appear to be positively associated with concentration of TNF-α ($P < 0,020$) and IFN-γ ($P < 0,031$). Furthermore, there is highly significant correlation between TNF-α and IFN-γ (r = 0,567; P<0,001). Thus it can be asserted that **IFN**-γ and **TNF**-α are predictors of each other.

Table 1 Description of CD14+ leukocytes and cytokines in blood samples of each patient according to their sex and age

Patients	Sex	Age	CD14+ Lymphocytes		CD14+ Monocytes		CD14+ Neutrophils		Cytokines	
			% Gated	% Total	% Gated	% Total	% Gated	% Total	TNF-α (pg)	IFN-γ (pg)
1	Male	6	2,95	1,15	54,3	1,16	0,66	0,21	2,82	9,09
2	Male	10	5,13	1,19	63,59	1,43	0,64	0,31	1,46	11,15
3	Male	11	4,57	0,86	74,61	1,65	0,65	0,34	4,46	8,37
4	Male	8	6,09	1,52	71,32	1,89	0,39	0,19	1,05	8,55
5	Male	9	4,06	1,13	83,62	2,01	0,86	0,4	9,02	9,99
6	Male	8	6,19	1,34	71,65	2,23	0,35	0,19	1,63	7,46
7	Male	10	1,47	0,54	48,29	0,93	1,02	0,36	3,73	7,87
8	Male	15	0,61	0,22	60,37	0,45	1,47	0,35	2,41	8,73
9	Male	14	3,84	1,3	51,2	1,67	0,4	0,14	5,94	8,46
10	Male	4,5	3,66	1,38	48,84	1,06	0,53	0,09	7,96	8,17
11	Male	8	6,26	1,78	65,34	2,13	0,31	0,11	8,43	8,86
12	Male	10	9,17	2,39	62,57	3,06	0,24	0,09	6,35	9,00
13	Male	14	11,98	1,87	68,46	2,46	0,33	0,15	8,01	10,51
14	Male	10	10,84	2,43	64,17	2,37	0,33	0,11	5,62	9,55
15	Male	5	10,43	1,24	34,81	1,16	0,18	0,03	10,67	12,45
16	Male	5	7,71	1,33	35,17	1,35	0,22	0,07	23,92	11,76
17	Male	4	6,77	2,16	79,21	2,49	1,1	0,34	6,88	10,51
18	Female	13	0,68	0,14	69,39	1,62	0,47	0,18	4,95	11,20
19	Female	5	3,23	1,18	71,79	1,48	0,45	0,09	4,60	7,91
20	Female	8	3,78	1,04	66,24	1,74	0,68	0,17	2,13	8,72
21	Female	9	6,94	1,94	70,55	2,57	0,28	0,11	1,68	7,90
22	Female	11	0,14	0,05	81,01	2,84	0,77	0,17	0,26	7,39
23	Female	7	1,39	0,45	56,23	1,14	0,71	0,27	2,63	9,62
24	Female	6,5	4,27	0,9	54,82	1,06	0,7	0,19	3,85	9,48
25	Female	8	2,57	0,21	24,02	0,34	0,29	0,05	6,29	9,65
26	Female	13	1,97	0,7	42,56	1,1	0,78	0,11	1,11	8,15
27	Female	9	5,09	1,38	60,47	1,84	0,3	0,14	6,02	10,81
28	Female	10	4,48	1,27	24,3	1,32	0,83	0,13	3,07	10,00
29	Female	7	5,39	0,88	32,99	1,27	0,34	0,09	1,62	9,26
30	Female	5	5,21	1,5	21,8	1,39	0,71	0,13	6,51	10,13
31	Female	4	8,93	2,43	39,94	1,76	0,14	0,02	6,57	9,60

Table 2 Statistical summary of t-test for mean differences of all observed immune response parameters between male and female children

t-test Parameters	IFN-γ	TNF-α	CD14+ Lymphocytes		CD14+ Monocytes		CD14+ Neutrophils	
			% Gated	% Total	% Gated	% Total	% Gated	% Total
Difference	0,167	2,829	2,122	0,397	9,880	0,202	0,037	0,073

t (Observed value)	0,358	1,853	2,022	1,726	1,611	0,850	0,334	2,035
|t| (Critical value)	2,045	2,045	2,045	2,045	2,045	2,045	2,045	2,045
DF	29	29	29	29	29	29	29	29
p-value (Two-tailed)	0,723	0,074	0,052	0,095	0,118	0,402	0,741	0,051
alpha	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05

Table 3 The degree of linear association between immunological parameters and patient's age

Statistical Parameters	**TNF-α**	**IFN-γ**	**CD14+ Lymphocytes**		**CD14+ Monocytes**		**CD14+ Neutrophils**	
			% Gated	**% Total**	**% Gated**	**% Total**	**% Gated**	**% Total**
r	-0,321	-0,187	-0,229	-0,320	0,255	0,086	0,258	0,287
R^2	0,103	0,035	0,052	0,102	0,065	0,007	0,067	0,082
Adjusted R^2	0,072	0,002	0,020	0,071	0,033	-0,027	0,035	0,051
F	3,325	1,049	1,604	3,303	2,023	0,215	2,075	2,601
P	0,079	0,314	0,215	0,080	0,166	0,646	0,160	0,118

Table 4 Correlation Coefficient (r) resulted from correlation/regression test between Cytokines (TNF-α and IFN-γ) and CD14+leukoytes

		CD14+Lymphocytes		CD14+ Monocytes		CD14+ Neutrophyl	
		% Gated	% Total	% Gated	% Total	% Gated	% Total
Cytokines	TNF-α	0,4169*	0,263	-0,251	-0,027	-0,332	-0,262
	IFN-γ	0,3883*	0,127	-0,296	-0,132	-0,198	-0,102
* Significant (P<0.05);							

IV. Discussion

In general, this study did not manage to confirm previous works regarding relationship between human haematological as well as immunological parameters and age. It was reported that in infancy and early childhood, cytokine concentration correlate differentially with age [8]. In current study, besides the level of correlation coefficient is not significant, the relationship trend between age and cytokines is also contrary to the reports mentioned. It is clear from Table 3 that r-values of both IFN-γ and TNF-α are negatif. Furthermore, disregarding the patient's age diversity, Todorovic at al. compared production of these cytokines in tonsillar hypertrophy (TH) and recurrent tonsillitis (RT) and found that the concentration of TNF-α and IFN-γ is significantly higher in RT than in TH [9]. These differences seem to be due to differences in the status of the research subjects. In reference [8] the subjects were normal children while in this study the subjects were patients with chronic tonsillitis. Additionally, neither reference [8] nor reference [9] describe what ethnic group is their research subjects, while in this study all patients were Mongoloid.

As has been widely reported, the influence of ethnicity on various aspects of human health and physiology is real and significant. Africans and Afrocaribbeans were known to have lower total white cell, neutrophil and platelet counts than Caucasians [10]. In addition, blacks show significantly lower thyrotropin, hematocrit, mean cell hemoglobin centration (MCHC), mean cell hemoglobin, and hemoglobin [11]. In children vertically infected with HIV-1, E.R.Sharp and colleagues found that African American children demonstrated significantly higher Gag responses than Hispanic children [12]. Blacks were also reported to have higher EBV IgG compared to white [13]. Unfortunately, research data describing the comparison of hematological and immunological parameters between blacks (Africans) or white (Caucasian) with Asians (Monggoloid) is still hard to find. The differences in influence of age on immunological parameters yielded in current study compared with other previous findings very likely related to the ethnicity of the patients.

Among the three types of CD14 leukocyte analyzed, only lymphocytes positively associated with concentration of TNF-α and IFN-γ in a siginificant manner (**Table 4**). In contrast, the expression of CD14+ monocytes as well as CD14+ neutrophils are even negatively correlated with concentration levels of TNF-α and IFN-γ, although statistically the correlation was not significant. However, as can be seen in Table 1, the current research findings confirm the accepted trend that CD14 is most widely expressed by monocytes [14, 15]. Since CD14 is predominantly derived from monocytes and monocytes are the primary source of TNF-α, then elevated TNF alpha levels should be followed by increased CD14+ monocytes [16]. It is very difficult to find literature

reviewing the association of cytokines with the expression of CD14+ lymphocytes. However, in patients infected with HIV-1 has been found a phenomenon of increased population of CD14-like positive-testing lymphocyte [17]. Thus, the unique findings of this study may not be a deviation of the accepted trends, but something reasonable given the differences in the ethnicity of the patient.

Lastly, current research findings found there is highly significant correlation between TNF-α and IFN-γ ($r = 0{,}567$; $P<0{,}001$). Although the satisfactory explanation of the mechanism of mutual relations between the two cytokines is not so clear, in certain cases the correlation between them is apparent. When cytokines were used in combination with GM-CSF: IFN-γ down-regulated production of IL-lRa while up-regulating the production of IL-lca/fl, IL-6 and TNF-α [18]. Also known in patients with recurrent tonsillitis, increased intracellular deposition of microbial antigens accounted for the elevated incidence of IL-1β, TNF-α, IL-6, IL-8, IL-2, IFN-γ, IL-10 and IL-4 expressing cells [19]. Thus the findings of this study reveal that in Indonesian children with chronic tonsillitis, the level of a cytokine content can be used to estimate levels of other cytokines.

V. Conclusion

It can be inferred that in Indonesian children with recurrent tonsillitis, immune response parameters might not determined by sex and age. However, serum cytokines play a role in CD14+ expression, and cytokines themselves, especially IFN-γ and TNF-α can positively predict each other.

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