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Expression Analysis of T Lymphocyte ($CD8^+$) in Severe Early Childhood Caries

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ABSTRACT

In children younger than three years are considered as Severe Early Childhood Caries (S-ECC). This analytic observational study compared the level of $CD8^+$ expression in saliva of children with S-ECC and free-caries children. Lymphocyte cells contained in saliva acquired by instructing the experimental subjects to gargle 10 ml of 1.5% NaCl solution. The expression of T lymphocyte ($CD8^+$) was analyzed by means of flow cytometry. The expression of T lymphocyte ($CD8^+$) in children with S-ECC (0.3400±0.14726) was higher than free-caries group (0.3250±0.11301) with P value of 0.655.

Key words : T lymphocyte ($CD8^+$), S-ECC, adaptive immunity

INTRODUCTION

Early childhood caries (ECC) is a condition found ≥1 decay, loss (due to caries), or the presence of fillings in primary teeth in children aged 71 months or younger, whereas severe ECC (S-ECC) occurs in children <3 years with ≥1 rot, missing (due to caries), or filled tooth surfaces and in children aged 4-6 years with high caries scores (Colak *et al.*, 2013). Severe dental caries that affect children younger than 3 years is considered as Severe Early Childhood Caries (S-ECC) (Sukuraman and Pradeep, 2017). The effect of ECC in children is not limited in oral health only, but also the general health (Naidu *et al.*, 2016). In terms of dental and oral health, ECC may give rise to pain, orthodontic problem, enamel damage, and also disturbance in oral function, such as mastication and speech. Besides, the development of permanent dentition in children with S-ECC may also be affected (Abanto *et al.*, 2011).

Cariogenic bacteria are one of the risk factor of ECC. The pathophysiology of ECC is correlated to the early colonization and the high level of cariogenic bacteria, *Streptococcus mutans* and *Streptococcus sobrinus*, aside from the high level of sugar contained in dental plaque. Salivary protein, glucan, will breakdown the sugar, creating acidic environment in the oral cavity, thus, give rise to enamel and dentin demineralization (Jayabal and Mahesh, 2014). The immune response is the body's ability to stay healthy by providing protection against harmful agents, most of which are microbes and is a specific and very specific response to pathogens. The innate response is the first line of defense in defending the body against pathogens in the same way at all times. These natural mechanisms include skin, saliva, tears, various cytokines, complement proteins, lysozyme, bacterial flora, and many cells including neutrophils, basophils, eosinophils, monocytes, macrophages, reticuloendothelial system, natural killer cells (NK cells), epithelial cells, endothelial cells, red blood cells, and platelets (Arce-Sillas *et al.*, 2016). Adaptive immune responses that are obtained adaptively will utilize the abilities of specific lymphocytes and their products (immunoglobulin and cytokines) to produce responses to microbes that attack (Lawrence *et al.*, 2016).

During infection, the immune response should be able to eliminate pathogenic microbial invasion with minimal tissue damage. Both innate and adaptive immune response, including T cells play important role in eliminating pathogenic microbes by releasing pro-inflammatory cytokine and activating cytotoxic T lymphocyte (CTL). Besides, T helper (TH) and regulatory T cells also necessary in secreting antibody by plasma cells, along with the immunomodulatory cytokine such as

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interleukin. Recent studies found the new important function of TH, including T Follicle (Th17, Th22), in immunity towards infection, also in disease progression and prognosis. Based on the aforementioned background, this study was conducted to observe and compare the expression of T lymphocyte ($CD8^+$) in children with S-ECC and free-caries children.

MATERIALS AND METHODS

This study was an analytic observational study, with cross-sectional analysis on two groups of sample; children with S-ECC and free caries children. All the procedures in this study had been reviewed and approved by the Health Research Ethical Clearance Commission of Universitas Airlangga, Faculty of Dental Medicine, with certificate No. 209/HRECC. FODM/IX/2017. The samples, in this case, the parents had given their consent to participate in after had been given a complete information about the study.

Saliva was collected from the subject aging from 4 to 6 years. The subjects were instructed to gargle 10 ml of 1.5% sterile NaCl solution for 30 seconds, and expectorate it into a sterile glass. This procedure was repeated four times. The saliva was then stored in cooler at 4°C before freezing at or below -20°C. Saliva was collected during 9 to 11 a. m.

The collected saliva was centrifuged at 450 g for 15 min at 40°C. The pellet was then mixed into 2 ml of Roswell Park Memorial Institute (RPMI) medium (TranGen biotech, Beijing, China) and vortexed (Luthfi *et al.*, 2019). The cell suspension was subsequently taken and counted using hemocytometer (JSQA hemocytometer, Hunan, China).

An equal number of volumes from the cell suspension and 0.2% of trypan blue solution (PubChem, Bathesda, USA) staining were mixed in Eppendorf and vortexed. An aliquot of the same suspension (20 µl) was added into both the chambers of the hemocytometer and observed under a light microscope (digital microscope XSZ-107BN, Hunan, China) with 10 x magnifications. The mixtures were subsequently transferred under the cover slip by capillary action to cover the area of the grid. The cells were counted in the area of 16 squares, which was equal to cell count $x10^4$/ ml. The viable cells in the squares were counted, and the cells count per ml was calculated using the following formula :

$$\text{Cell/ml} = \text{Means of cell count per primary square} \times 10^4 \times \text{diluting factors.}$$

Lymphocyte cells (3×10^5 cells/ml) were cultured in the tissue culture flask (Greiner) 75 cm^2 with complete culture medium [RPMI-1640, 10% fetal calf serum (FCS), and 1% penicillin/streptomycin] in 5% CO_2 and atmosphere humidity 95% at 37°C for 24 h. The cultures were checked daily to observe the changes in colour, turbidity, density and growth pattern using inverted light microscope (Nikon).

The expressions of $CD8^+$ were observed by means of flow cytometry method adapted from Luthfi *et al.* (2019). Fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP) and PerCP-Cy5.5-conjugated monoclonal antibodies (mAbs) from Becton Dickinson (San Jose, CA, USA). The optimal concentration of mAbs was determined for each mAb with titration. Flow cytometry simultaneously measured and analyzed for the physical properties of particles such as cells because it flew through the flow of fluid through a beam of light. The nature of scattering cell light was used to analyze changes in size, granularity, internal complexity and relative fluorescence intensity. The stained lymphocytes were analyzed using flow cytometer (LSR 11 Sorvall RT7 Plus, Becton Dickinson, USA) with cell quest software (Becton Dickinson, USA). The results were analyzed using flow Jo 7.0 (USA) software. The expressions of $CD8^+$ were analyzed using standard FACScan procedure with mAbs according to the producer protocol. The results were calculated and presented in mean. The acquired data analyzed the normality and homogeny, then followed by T-test to find the difference between two groups, with the level of significance at 0.05.

RESULTS AND DISCUSSION

Kolmogorov-Smirnov test showed p value of 0.200, and the Shapiro-Wilk test showed p value of 0.452, which meant that the data were normally distributed with homogeny (Table 1). Therefore, the data were subsequently analyzed using independent t-test to find any difference between groups.

Table 1. Kolmogorov-Smirnov and Shapiro-Wilk test result of T lymphocyte ($CD8^+$) expression after 24 h incubation and analyzed using flow cytometry

Variable	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
$CD8^+$	0.173	16	0.200	0.948	16	0.452

Table 2 shows that the expression of T lymphocyte ($CD8^+$) of children with S-ECC was higher than the caries-free children. However, the independent t-test showed no significant difference of T lymphocyte ($CD8^+$) between children with S-ECC and caries-free children. $CD8^+$ T cells are killer T cells (or cytotoxic T cells), are effector cells that function as cell-mediated immunity. $CD8^+$ T cells are naive and must be activated in order to function effector cells (i. e. immune function). This activation occurs through interactions with pro-APC ("professional" antigen presenting cells), especially dendritic cells in lymph nodes/follicles, and leads to intracellular pathways that regulate more TCR specific antigens on T cells and lead to effector functions. T cells can only recognize protein-based antigens (Lyu *et al.*, 2019).

Table 2. Mean and standard deviation of T lymphocyte ($CD8^+$) expression after 24 h incubation and analyzed using flow cytometry

S. No.	Group	N	$CD8^+$ expression	P-value
			Mean ± SD	
1.	S-ECC	8	0.3400±0.14726	0.655
2.	Caries-free	8	0.3250±0.11301	

S. mutans was the main bacteria that strongly correlated to ECC, while the other oral bacteria in dental biofilm were involved in caries initiation and development (Hajishengallis *et al.*, 2017). Those bacteria were *Lactobacillus* sp. which play important role in the caries development (Li and Tanner, 2015). *Actinomyces* sp., specifically *Actinomyces gerenseriae* were also found in initial caries. *Bifido bacterium* was found in deep carious lesion, while several other *Streptococcus* non-mutans were acidogenic and aciduric bacteria, also playing role in caries. Epidemiology data showed that during the pathogenesis of dental caries, *Candida albicans* was also involved (Sukuraman and Pradeep, 2017). The previous study showed that the presence of *C. albicans* in children with ECC was significantly higher than those without caries. Besides, children with more population of *C. albicans* in oral cavity had a higher risk of ECC compared to those with low level of *C. albicans* in the oral cavity (Xiao *et al.*, 2017).

Based on the result of this study, the expression of T lymphocyte ($CD8^+$) was higher in children with S-ECC than in those without caries, however, there was no significant difference. This showed the importance of host defense against microbes, both in ECC and S-ECC. T cell ($CD8^+$) denoted one of the cells which were able to identify and eliminate the infected cells; therefore, it was an important host defense component towards pathogenic agent. Recent studies found that in infectious diseases, it was not the main pathological agent that directly correlated to the aggressor, but to the abnormal immune response (Figs. 1 to 4). Thus, initiate hypersensitive reaction, an excessive and uncontrolled immune response, which caused tissue damage. Dysregulation of the innate immune response causes excess production of proinflammatory cytokines such as IL-1β and TNFα, or excessive response to the level of stimulation of proinflammatory cytokines that can trigger the release of endogenous stimuli, including demage associated molecular patterns (DAMP) which have an impact on the immune (de Jessus *et al.*, 2015).

This explained the higher expression of T lymphocyte ($CD8^+$) in children with S-ECC,

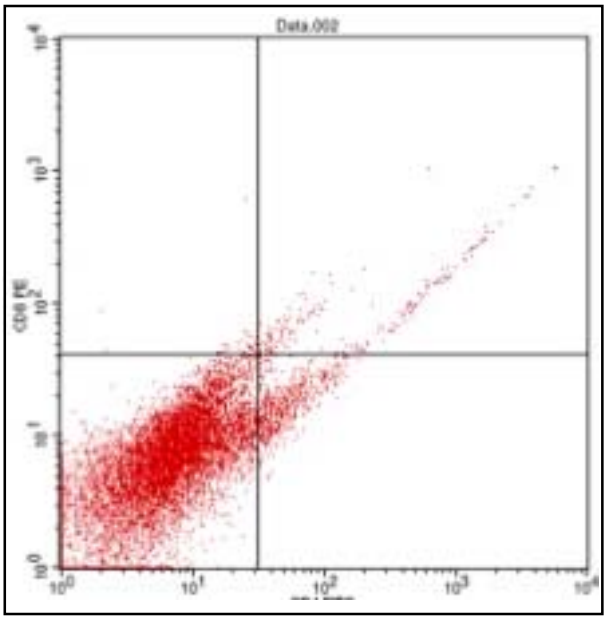

Fig. 1. The expression of T lymphocyte ($CD8^+$) (0.31%) in the saliva of children with S-ECC.

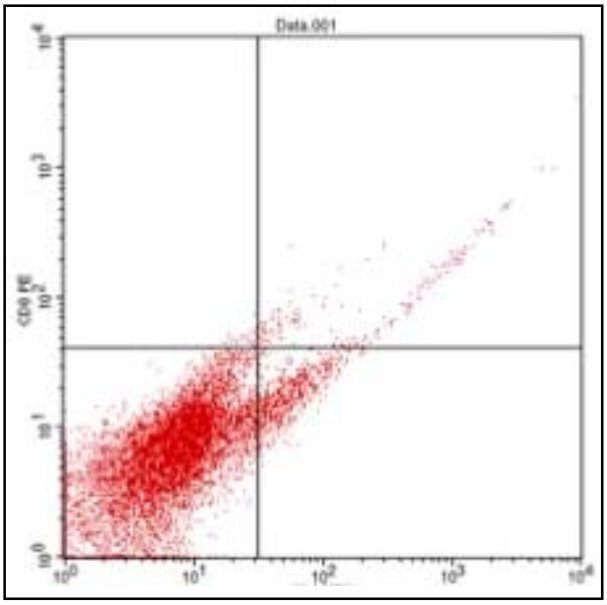

Fig. 2. The expression of T lymphocyte ($CD8^+$) (0.35%) in the saliva of children with S-ECC.

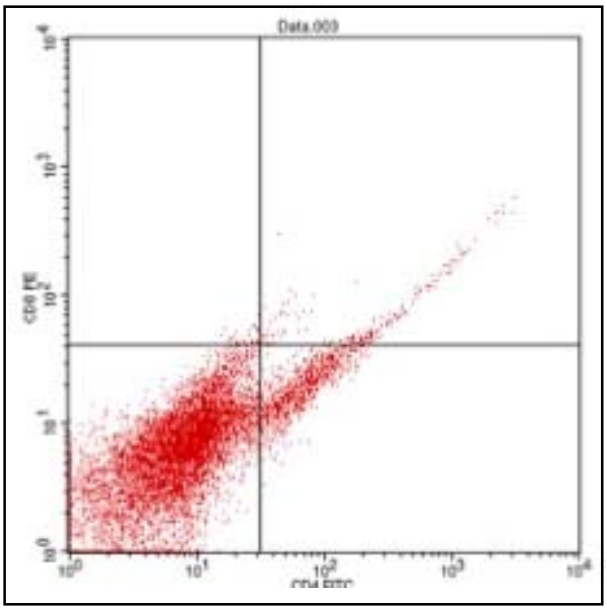

Fig. 3. The expression of T lymphocyte ($CD8^+$) (0.34%) in the saliva of caries-free children.

compared to the caries-free group. Increase in lymphocyte proliferation and IFN-γ expression up to 6 h incubation was used as the indicator of early detection marker of severe early childhood caries (Luthfi *et al.*, 2019).

The killing function of $CD8^+$ T cells is mediated by two mechanisms. The first mechanism involves the use of Fas/Fas Ligand (FasL). Activated $CD8^+$ T cells express FasL bound to Fas (CD95) which is a receptor found in many cell types, which lead to activation of caspases and apoptosis of target cells. The second

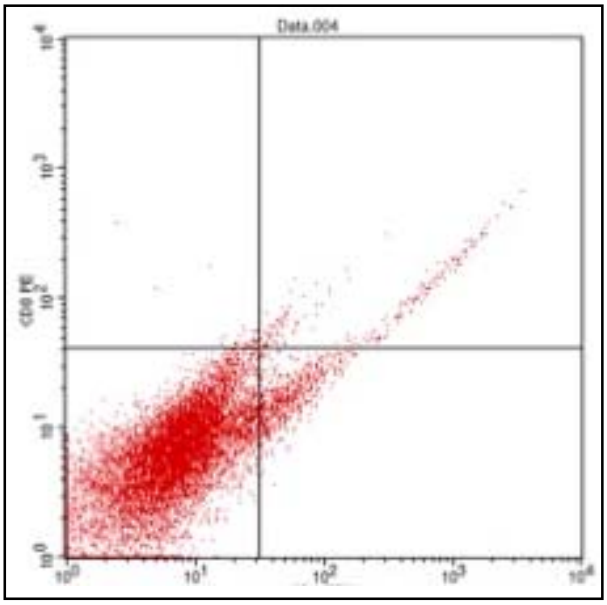

Fig. 4. The expression of T lymphocyte ($CD8^+$) (0.31%) in the saliva of caries-free children.

mechanism is activating $CD8^+$ T cells which can be used to kill antigens by releasing granzymes and perforins which are two compounds that have the ability to cut the cell wall and caspases that are active. $CD8^+$ T cells that are activated also release IFN-γ which is a cytokine used in the macrophage activation process (Wu and Lyu, 2019; Nakiboneka *et al.*, 2019).

Among various cytokines that involved in defense towards bacteria, the pro-inflammatory cytokines, such as TNF-α, IL-β and IL-6 were produced in the initial stage of infection, causing fever that inhibited bacterial multiplication. Cytokine also increased the expression of adhesion molecule (P-seletine and ICAM), that eased the migration of cells from the vessels to the infected area, and induced neutrophils and macrophages to secrete NO and destroyed bacteria. Other cytokine that was produced in the initial phase of infection disturbed the adaptive immune response, was the cytokine produced by macrophage, IL-12, that played role in differentiation of Th0 into Th1. While IL-4, produced by basophil, mastocyte and macrophage, induced the differentiation of Th0 into Th2, that collaborated with B lymphocyte to produce antibody.

CONCLUSION

The level of T lymphocyte ($CD8^+$) in children with S-ECC was higher than the caries-free children.

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