# Analysis of lymphocyte T(CD4+) cells expression on severe early childhood caries and free caries

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## Abstract

Early childhood caries (ECC) is still one of the many diseases found in children throughout the world. Cariogenic bacteria are a significant risk factor for ECC associated with early colonization and high levels of cariogenic microbes (*Streptococcus mutans*, *S. mutans*). Lymphocyte T ($CD4^+$) cells known as helper T cells, are effector cells for mediated host immunity. Naive T cells ($CD4^+$) must be activated to initiate effector function. This activation occurs through interaction with professional antigen-presenting cells (pro-APC), especially dendritic cells that lead to intracellular pathways that regulate T cell receptor (TCR) more specifically against antigen in T cells. Lymphocyte cells from samples were collected from severe early childhood caries (S-ECC) and Free caries aged 5 to 6 years. The subjects were instructed to gargle 10 mL of sterile NaCl 1.5% solution for 30 seconds, and expectorate it into a sterile glass then analyzing T lymphocyte cell ($CD4^+$) expression using flow cytometry. Lymphocyte T ($CD4^+$) cell expression at S-ECC (6.2525±64482) while in free caries (8.4138±1.10397) with P-value (P=0. 000). Conclusion of lymphocyte T ($CD4^+$) cells expression at S-ECC is lower than that occurring in free caries.

## Introduction

Early childhood caries (ECC) is still one of the many diseases found in children throughout the world. ECC does not only affect the oral health of children, but also general body health.[1] ECC not only involves pain in the oral cavity, orthodontic problems, and damage to the enamel, but can also cause problems with food intake, speech and increased risk for caries development in permanent teeth.[2] Early loss of primary teeth often leads to orthodontic problems in adult life.[3]

ECC is the most common childhood chronic disease, with almost 1.8 billion new cases per year globally[4] which occurs in about 37% of children aged 2-5 years in America States and up to 73% of preschoolers who are socially economically disadvantaged in developing and industrialized countries.[5] ECC is also highly prevalence in preschool children living in developing countries like Indonesia[6] the prevalence of ECC in group of children aged 6 months-3 years at Gunung Anyar Surabaya-Indonesaia was 30.8 %, while the prevalence was 29.2% S-ECC.[7] ECC was defined as the presence of ≥1 decay, loss (due to caries), or full tooth surface in primary teeth in children 71 months of age or younger. S-ECC occurs in children <3 years with ≥1 rot, missing (due to caries), or full tooth surface and in children aged 4-6 years with high caries score.[8] ECC and S-ECC remain serious problems that occur in school children in Xinjiang. Lower sociodemographic status (disadvantaged areas, low-educated mothers, low-income families, caregivers with cavities), risky dietary behavior (consumption of high frequency sweets, frequent meals before going to bed), oral hygiene behaviors that are at risk of ECC such as at what age start to brush teeth and use of dental services (past dental visits, parents who have received oral health care instructions) are associated with an increased risk of ECC and S-ECC.

Severe early childhood caries (S-ECC is an infectious disease that is a public health problem in the world, in spite of ongoing control efforts. The purpose of the host immune response during infection is to clear pathogens that attack with limited tissue damage. Both innate cells and adaptive T cells play a key role in clearing pathogens directly through the release of proinflammatory cytokines and the activity of cytotoxic T lymphocytes (CTL). In addition, helper (Th) T cells and regulatory Treg cells are required for antibodies secreted by plasma cells and immunomodulatory cytokines (eg, IL-10), respectively. In recent years, the role of the new set of Th cells, including follicular T cells namely Th17, Th22, in regulating anti-infective immunity, has become very important, because they play an important role in the development and outcome of disease.[9]

Cluster of differentiation 4 (CD4) coreceptor expressed in a subset of T cells, plays a role in differentiation, migration and cytokine expression.[10] T cells involved in antigen recognition, CD4 stabilizes the ternary complex pMHC-TCR and CD4 recruits Lck kinase to phosphorylate ITAM and initiate intracellular signaling during activation of T cells induced by antigens.[11] CD4 was originally described as an adhesion molecule that enhances contact between T cells and precenting cell antigens. In their pillar work, Doyle and Strominger found direct correlations of other specific T cells involved in interactions[12] CD4 binds MHCII molecules with

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very low 3D affinity.[13] Based on the above background, the researchers wanted to analyze how the expression of T lymphocytes (CD4+) cells in S-ECC and caries-free.

## 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.

### Lymphocyte Isolation

Lymphocyte cells from saliva obtained by instructing the subject to rinse with 10 mL of 1.5% sterile NaCl solution while rinsing, but not swallowed for 30 seconds, then expectorated in sterile glass. This procedure was repeated 4 times. The sample was then centrifuged at 450g for 15 minutes, at 40C. The centrifugation pellets were then mixed with 2 mL of RPMI medium, then the samples were vortexed.[14] The results of the filter in the form of cell suspension are then calculated using a hemocytometer.

The same volume of cell suspension and 0.2% dye of trypan blue were mixed in the eppendorf tube and in doing vortex. The same suspension aliquots (20 µL) were added to both chamber haemocytometers and observed under a microscope (10X objective). The mixture is withdrawn with capillary action. The cells are counted in an area of 16 squares which is equivalent to the number of cells x104/mL. Only translucent cells are counted in the box. The number of cells per mL is calculated using the following formula:

Cell/mL=average number of cells per primary square x $10^4$ x dilution factor

### Lymphocyte Culture and Cultivation

Lymphocyte cells ($3x10^5$ 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% CO2 and atmosphere humidity 95% at 37°C for 24 hours. The cultures were checked daily to observe the changes in color, turbidity, density, and growth pattern using inverted light microscope (Nikon Eclipse Ts2R).

### CD4+ Expression Analysis

The expression of $CD4^+$ were observed by means of flow cytometry method adapted from Luthfi *et al*.[15] Fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), Peridinin chlorophyll protein (PerCP), PerCP-Cy5.5-conjugated monoclonal antibodies (mAbs) from Becton Dickinson (San Jose, CA, USA). The optimum concentration of mAbs were determined for each mAb by means of titration. Flow cytometry can both measure and analyze the physical characteristics of a particle such as cell since it can flow into the fluid stream through the light. The light scattered by the cell can be used to analyze changes in size, granularity, internal complexity, and relative fluorescence intensity. Flow cytometry analysis is conducted to discover the immunomodulatory pattern of lymphocyte using conjugated monoclonal antibody.

Salivary lymphocytes were moved into FACS tube and washed with 4mL Dulbecco Phosphate Buffer Saline (DPBS) and centrifuged for 5 minutes at 2000rpm; the supernatant was subsequently removed. The pellet in DPBS were once again washed and centrifuged at 1800 rpm for 8 minutes. The cells were stained using yellow viability dye (1mL stain/1000µL DPBS) then vortexed and incubated at 4°C for 15 minutes. The cells were subsequently washed with 4mL DPBS and 1% FCS, centrifuged at 1800 rpm for 8 minutes and the supernatant was removed. The cells were stained with the exact required volume of mAbs, followed by vortexed and incubated in refrigerator for 20 minutes. After washed in cold



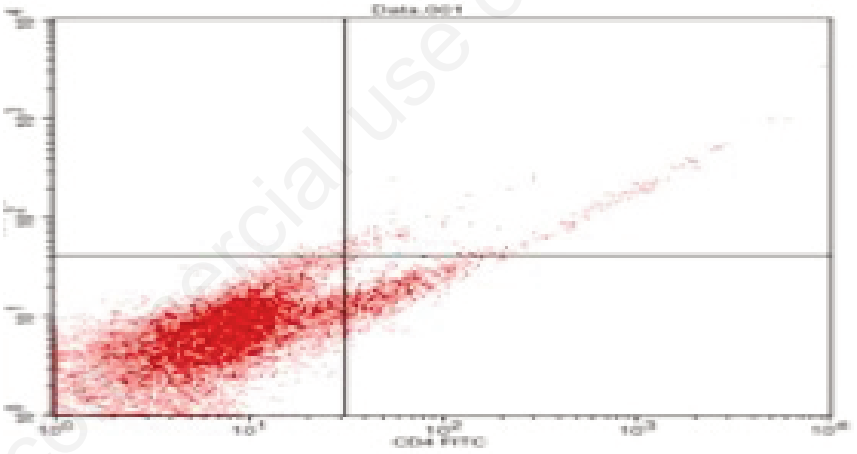

**Figure 1. T lymphocyte ($CD4^+$) cells expression (6.91%) in the saliva of Caries Free.**

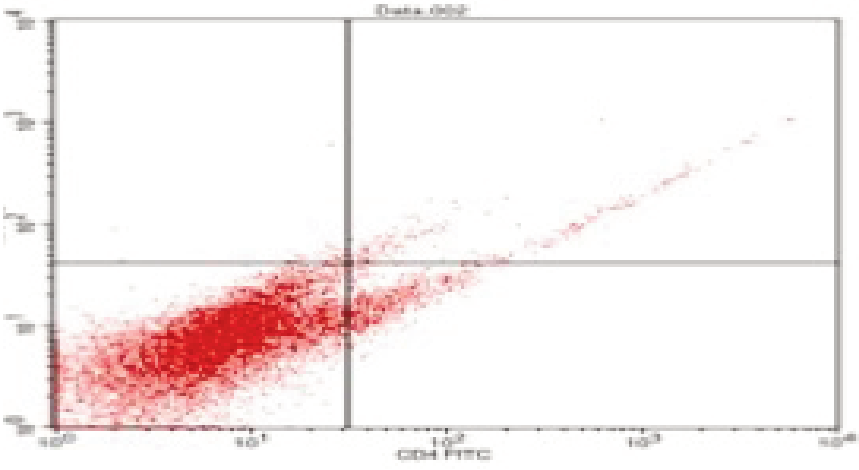

**Figure 2. T lymphocytes ($CD4^+$) cells expression (5.60%) in the saliva of S-ECC.**

DPBS and 1% FCS, cells were centrifuged for 8 minutes at 1800 rpm and the supernatant were removed. The cells were once again vortexed and 100µL of reagent a was added into the sample and cooled for 10 minutes. 50µL of mixture that had been fixated in reagent A was added into each samples and covered with aluminum foil and stored in refrigerator until acquisition at LSR2 flow cytometry.

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 expression of $CD8^{+}$ were analyzed using standard FACScan procedure with mAbs according to the producer protocol. The results are calculated and presented in mean.

### Statistical analysis

The acquired data was 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

Data normality test using shapiro-Wilk obtained P value of expression of T lymphocytes ($CD4^{+}$) of 0.200 while the value of p value of CD4 of 0.345 shows that both P-values>0.05 which means the data are normally distributed, because the data are normally distributed then a comparative test is then performed a comparative test between groups using the independent T test.

In Table 1 shows that the mean expression of T lymphocytes (CD4 +) in S-ECC higher than caries free children.

Comparative test results of T lymphocyte (CD4 +) cell expression between the S-ECC and free caries groups showed a P-value of 0,000, which is smaller than 0.05 (P<0.05), which means that there are significant differences between the S-ECC and free caries groups (Figures 1-4).

## Discussion

*Steptococcus mutans* (*S. mutans*) is the main bacterium that has a strong relationship with ECC while other oral bacteria in dental biofilms can be involved in the initiation and development of caries.[16] Other bacteria associated with ECC are the Lactobacillus species which play an important role in the development of lesions.[17] Actinomyces species, especially *Actinomyces gerecseriae*, are also associated with caries initiation. In addition, some non-mutans streptococci that have acidogenic and aciduric properties are also associated with dental caries. Epidemiological data indicate that in the pathogenesis of dental caries, *Candida albicans* also plays an active role. [18.]

T lymphocyte cells (CD4 +), known as helper T cells, are effector cells for cell-mediated immunity. T lymphocytes (CD4 +) are naive and must be activated to start effector functions, this activation occurs through interactions with professional antigen-presenting cells (pro-APC) especially dendritic cells that lead to intracellular pathways that regulate T cell receptors (TCR) more specifically against antigens in T cells.

TCR and its co-receptors, such as CD4, form complexes with class 2 MHC receptors and antigens. CD4 + lymphocyte cells are then activated and produce cytokines to start the immune response of leukocyte cells or other immune cells of cell-mediated immunity and activate humoral immunity branches that depend on T cells, then CD4 + T cells recognize protein antigens and acti-

**Table 1. Mean and standard deviation of the expression of T lymphocytes (CD4 +) after 24-hour incubation were analyzed by flow cytometry test and statistical test t.**

| No | Group | N | CD4+ expression Mean (X) SD | P-value |
|---|---|---|---|---|
| 1 | S-ECC | 8 | 6.2525 0.64482 | 0.0000 |
| 2 | Free Caries | 8 | 8.4138 1.10397 | |

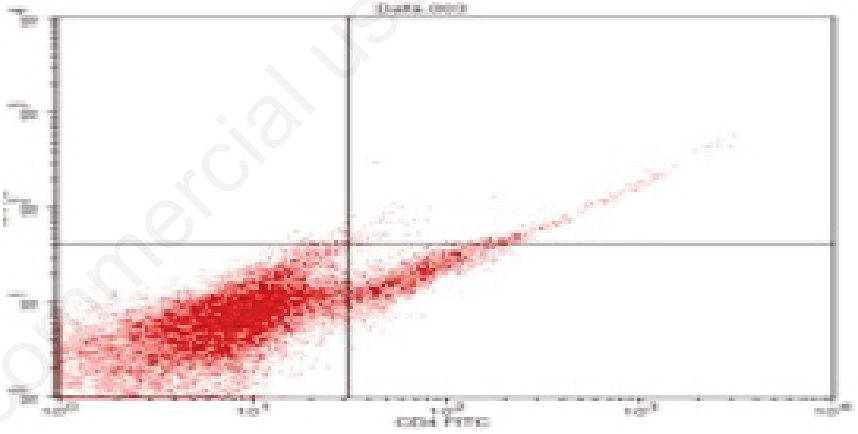

**Figure 3. T lymphocytes ($CD4^{+}$) cells Expression (10.24%) in the saliva of Caries Free.**

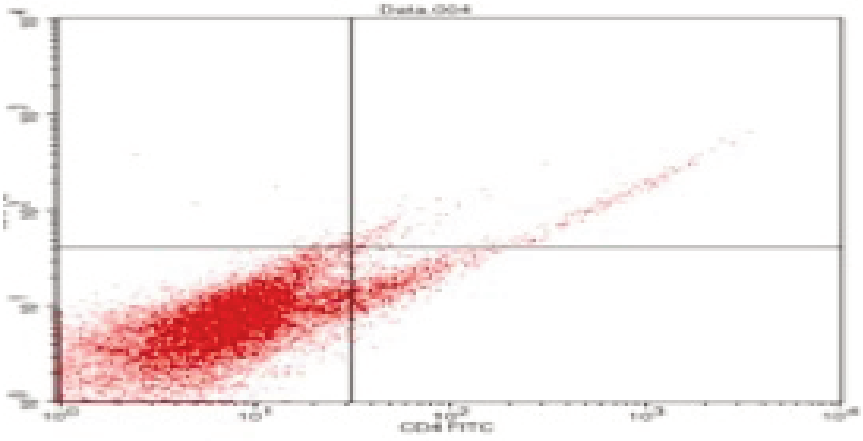

**Figure 4. T lymphocytes ($CD4^{+}$) cells Expression (6.64%) in the saliva of S-ECC.**

vate B cells to produce immunoglobulins in response to antigens.[19,20] The results of the study as shown in Table 1 show that the expression of T lymphocytes ($CD4^+$) cells in S-ECC is significantly lower than in free caries, this may cause the high *S. mutans* bacteria found in S-ECC saliva cannot be in acquisition by adaptive immunity because TCR and its co-receptors, such as CD4 which can form complexes with class 2 major receptor histocompatibility complex (MHC) receptors and antigens, cannot function optimally so that quantitatively the number of *S. mutans* which are bacteria that causes caries is higher compared to caries-free children.[21] Expression of T lymphocytes ($CD4^+$) in S-ECC causes the release of pro-inflammatory cytokines that function as chemoattractant of neutrophil cells, because the movement of neutrophils toward the infection area is less than optimal, the movement of macrophages is also less than optimal towards the area of infection, giving *S. mutans* the opportunity to develop and do damage to the teeth.

In addition to the above, the low expression of CD4 + T lymphocyte cells in S-ECC results in slow B cells forming antibodies. This happens because CD4 + T cells recognize antigens well and can activate B cells to produce antibodies in the form of immunoglobulins in response to *S. mutans* antigens.

## Conclusions

In S-ECC there is a decrease in T lymphocyte ($CD4^+$) expression.

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