

# Complement Receptor 1 (CR1)/ CD35<sup>+</sup> Expression Analysis of Salivary Neutrophils on *Streptococcus mutans* Phagocytosis

## Abstract

**Background:** Severe early childhood caries (S-ECC) is a form of dental caries which is very destructive in early childhood since involving several teeth, include the maxillary anterior teeth. *Streptococcus mutans* (*S. mutans*) play an etiological integral role of ECC so that *S. mutans* are considered as the predictor of dental caries. The neutrophil is a key component of the first line of defense against microbial invasion. The essential function of neutrophil is to kill pathogenic microbes through a phagocytosis process which is mediated by Complement Receptor 1 (CR1)/ (CD35<sup>+</sup>). **Aims and Objectives:** To analyze the phagocytosis process of the salivary neutrophil which is mediated by innate immunity component, i.e., Complement Receptor 1/CR1 (CD35) on S-ECC. **Materials and Methods:** This study was an observational analysis with cross-sectional approach using t-test analysis. This study employed the isolation steps of neutrophils saliva of caries-free children and the S-ECC and then conducted phagocytosis of salivary neutrophils test on *S. mutans* mediated by CD35 using flow cytometry. **Results:** Phagocytosis of salivary neutrophils on *S. mutans* mediated by CD35 on caries-free ( $2.35 \pm 0.56$ ) is higher than that on the S-ECC ( $1.54 \pm 0.35$ ). **Conclusions:** It is concluded that there is a decrease of phagocytic on *S. mutans* mediated by Complement Receptor 1/CR1 (CD35<sup>+</sup>) on S-ECC.

**Keywords:** CD35<sup>+</sup>, phagocytosis, salivary neutrophils, severe early childhood caries

## Introduction

Dental caries in preschool children denotes a crucial health problem. The American Academy of Pediatric Dentistry declared caries as a serious disease as it is a chronic infection involving *Streptococcus mutans* which may be transmitted vertically from caregivers to the child.<sup>[1]</sup> Dental caries may have rapid and dynamic progress on both fully or partially erupted deciduous teeth that may arise pain, acute and chronic abscesses, fever, and swelling of the lips, leading to the decrease of appetite.<sup>[2]</sup> Dental caries is not a noxious disease yet requires a special attention as it is a focal infection that may facilitate various systemic diseases. The damage of tooth surface by carious lesion is irreversible; therefore, immediate treatment is necessary since it has tremendous impact on children, such as impaired mastication, malnutrition, gastrointestinal disorder, growth disorder (especially weight and height), articulation of speech, impaired social development, and cognitive disorder.

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Thus, for children, caries is a matter of health that has a long-term effect in later life.<sup>[1,3]</sup> Besides, dental caries can cause psychological distress and it may lead to death if left untreated.<sup>[4]</sup>

Currently, the scientific reports have found the other role of neutrophils, that is, as a key component of the first line of defense against microbes.<sup>[5]</sup> Besides killing microbes through phagocytosis and releasing reactive oxygen species (ROS) and anti-microbial peptides, neutrophils also help to set up the activation of immune responses.<sup>[6]</sup> Furthermore, neutrophil produces various number of cytokines, chemokines, and growth factors that makes it as a major contributor to the production of pro-inflammatory cytokines in the area of infection.<sup>[7]</sup>

Neutrophil recognizes pathogen molecules as peptidoglycan, lipoprotein, lipoteichoic acid, lipopolysaccharide, and flagellin. These molecules are called pathogen molecular-associated patterns (PAMPs), interacting with pattern recognition receptors family expressed on the

**How to cite this article:** Luthfi M, Indrawati R, Oki AS, Rifa'i M. Complement receptor 1 (CR1)/ CD35<sup>+</sup> expression analysis of salivary neutrophils on *Streptococcus mutans* phagocytosis. *Contemp Clin Dent* 2019;10:627-30.

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DOI: 10.4103/ccd.ccd\_900\_18

### Quick Response Code:



neutrophils' surface, namely toll-like receptors (TLRs). The binding of PAMPs with TLRs, especially TLR 2 and 4, leads to the activation of the signal transduction pathway. This causes enhancement of various neutrophil functions, including adhesion, phagocytosis, cell viability, release of cytokines and chemokines, production of ROS, and degranulation. Although TLRs play an important role in the recognition of microbe by neutrophils, phagocytosis is more efficient when the microbe is coated with host proteins in the form of serum called opsonin. This opsonin includes protein complement and antibodies. The activation of complement causes sedimentation of complement components on the surface of the microbes. Complement component includes CIQ, C3b, and iC3b. The complement receptor (CR) surface is expressed on the neutrophil's surface, one of which is CR1 (CD35), efficiently recognizing the microbe bound with CR.<sup>[8,9]</sup>

Based on the above explanation, this research aims to analyze the phagocytosis process of the salivary neutrophil which is mediated by innate immunity component, i.e., Complement Receptor 1/CR1 (CD35) on severe early childhood caries (S-ECC).

## Materials and Methods

This research was approved by the institutional ethical committee, with certificate No. 01/KKEPK/VII/2013, and a signed consent form was acquired from the parent prior collecting the data.

### Sampling

The sample was obtained from a number of selected kindergarten schools in Surabaya area. The examination of dental caries was conducted in each selected school by measuring the def-t index. Next, the subjects who had been examined were divided into two groups: caries-free group and caries group with def-t more than 6. The age of all subjects ranged from 4 to 6 years old at the time of examination.

Prior to the specimen collection, questionnaires were distributed and the parents respectively signed a sheet of written informed consent. The sample was obtained by researchers and trained personals using a standard protocol. Subjects should not eat, drink, chew gum, or brush teeth 60 min before sampling. The samples were then frozen (Frozen, Quanzhou, China) at a temperature of  $-80^{\circ}\text{C}$  for further analysis.

### Isolation of salivary neutrophils

Neutrophils in saliva were obtained by instructing the subjects to rinse their mouth with 10 ml of sterile 1.5% NaCl for 30 s but not swallow it. Then, it was expectorated in a sterile glass (Pyrex, Singapore). This procedure was repeated for four times. The collected specimens were subsequently centrifuged at 450 g for 15 min at  $4^{\circ}\text{C}$ . The pellet of centrifugation results was mixed with 2 ml of

RPMI medium. After that, the samples were vortexed and were then filtered sequentially with 20 and 11  $\mu\text{m}$  of nylon filter (Sheboygan WI, USA).<sup>[10]</sup>

### Identification of salivary neutrophils

The identification of neutrophil used the sorting cell of human neutrophils enrichment kit (EasySep<sup>®</sup>, Sheboygan, WI, USA) with the following methods:

Nucleated cell suspension at a concentration of  $5 \times 10^7$  mL was placed in a 5 mL (12 mm  $\times$  75 mm) polystyrene tube (EasySep<sup>®</sup>, St louis MO, USA) magnet, which is 5 ml polystyrene Tube (Falcon<sup>™</sup>, Becton Dickinson, catalog #352058). Then, EasySep<sup>®</sup> Cocktail neutrophil was added at 50 cells  $\mu\text{L}/\text{mL}$  (e.g. for 2 mL cells, add 100  $\mu\text{L}$  cocktail). The solution was mixed well and incubated at room temperature or  $4^{\circ}\text{C}$  for 10 min. Next, EasySep<sup>®</sup> Nanoparticles were mixed to ensure that the cells were in a homogeneous suspension by pipetting more than five times. Vortexing is not recommended. Nanoparticles were added to 100 cells  $\mu\text{L}/\text{mL}$  (e.g. for 2 mL cells, add 200  $\mu\text{L}$  of nanoparticles). The suspension was then mixed well and incubated at room temperature or  $4^{\circ}\text{C}$  for 10 min. Afterward, the cell suspension to a total volume of 2.5 mL the tube (without cap) was placed on the magnet and left for 5 min. The next stage was lifting the EasySep<sup>®</sup> Magnet in one continuous motion to reverse the magnet and the tube. The undesirable cells on the labeled magnet will remain bound in the original tube. The magnet and the tube were kept in an inverted position for 2–3 s before they were returned to the upright position. Any drops that may remain hanging from the mouth of the tube should not be shaken or removed. After that, the empty tube was released from the EasySep<sup>®</sup> magnet. A new tube containing supernatant fraction was then put to the magnet and left for 5 min. Thus, the cells in the new tube were ready for use.

The suspension analysis of cytometry cell flow was performed on activated fluorescence FACScan Cell Analyzer (Becton Dickinson, San Jose, CA, USA). At least 25,000 events were analyzed for each saliva sample. Initially, neutrophils were gated by forward scatter (FSC), side scatter (SSC) profiles based on its size and granularity on neutrophil suspension. The gated cells were then analyzed. Positive coloring for the neutrophil marker was defined as events exceeding fluorescence level. Neutrophils which were over 70% of the isotype matched with the staining control were the ones to be studied. The percentage of neutrophil was determined by reducing positive coloration cells isotype matched from positive staining cell antibody. The percentage of worthy neutrophil was determined by gating on both negative cells propidium iodide and labeled fluorescent cell. Back-gating to FSC compared to SSC plot was conducted to verify the morphology of positive stained cells.

**Measurement of expression carboxyfluorescein N-hydroxysuccinimide ester CD35<sup>+</sup>**

In the process of phagocytosis, the surface receptor on phagocytic cells can bind to the fragment constant region (Fc) fragment from one type of immunoglobulin or may bind to complement factor (C3b). The interaction triggers a conformational change of the cytoskeleton to help the process of antigen ingestion. Pathogens are killed by the action of oxidative and nonoxidative mediators, i.e., the interaction between the complement factor of C3b or iC3b with complement receptor (CD35).

Carboxyfluorescein succinimidyl ester (CFSE) staining on bacteria has a function to label the bacteria with FITC fluorescence so that it can be detected using a flow cytometer (Becton Dickinson, San Jose, CA, USA). Meanwhile, the provision of CD35<sup>+</sup> antibody is a marker which indirectly indicates the cell observed the process of phagocytosis, which was neutrophil cell. The staining of CFSE<sup>+</sup> CD35<sup>+</sup> aims to detect the ability of neutrophil cells which have complement receptors (CD35<sup>+</sup>) in performing phagocytosis on bacteria (labeled CFSE<sup>+</sup>).

**Results**

The result of the analysis of saliva was activated to perform phagocytosis of *Streptococcus mutans* and labeled

by CFSE staining which expresses the CD35<sup>+</sup> on S-ECC and caries-free [Table 1 and Figure 1]. The results of flow cytometry analysis are presented in Figure 2.

**Discussion**

Phagocytosis by neutrophil is a very complex process started by bacterial attachment on neutrophil mediated through a molecule opsonization that binds the component of bacterium surface and promotes phagocytosis. The protein C3b is an important opsonin which can be tied by complement receptor 1/CR1 (CD35) on the neutrophil surface, causing the internalization of bacterium into phagosome, and then, leading to a fusion of phagosome-lysosome which can cause degradation of bacteria cell.<sup>[11]</sup>

Complement receptor 1/CR1 (CD35) is a single-chain glycoprotein with a molecule weight of 220 kDa which serves as the binder of C3b fragment from C3 as well as iC3b and C4b (Klickstein and Moulds, 2000). CD35 is involved in an important way of binding microbe opsonized by C3b before it undergoes phagocytosis by neutrophil. It also functions as a regulator of complement cascade, being a decay accelerator and cofactor for I factor enzyme.<sup>[12]</sup>

Based on the result, saliva neutrophil shows that the expression level of CD35<sup>+</sup> on S-ECC is lower than the expression level CD35<sup>+</sup> on free caries children [Table 1]. It is shown that the average value of expression level of

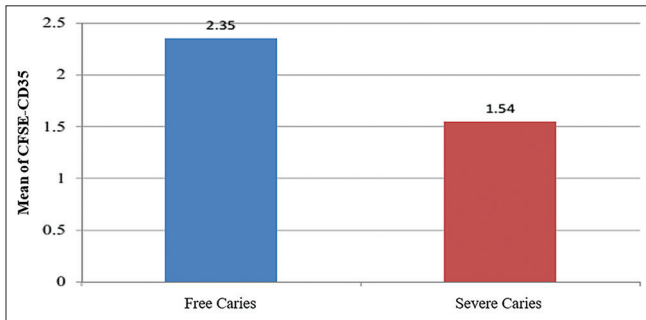


Figure 1: Mean and standard deviation neutrophils saliva which was activated to perform phagocytosis of bacteria *Streptococcus mutans* which was labeled by carboxyfluorescein N-hydroxysuccinimide ester staining expressing CD35<sup>+</sup> on the severe early childhood caries and caries-free (%)

**Table 1: Mean and standard deviation of the amount of saliva neutrophils which was activated to perform phagocytosis of bacteria *Streptococcus mutans* labeled by Carboxyfluorescein N-hydroxysuccinimide ester staining expressing CD35<sup>+</sup> on the severe early Childhood caries and caries-free (%)**

Group	n	Mean±SD	95% CI	P
Caries free	20	2.35±0.56	2.09–2.61	0.0001 (P < α)
S-ECC	20	1.54±0.35	1.38–1.71	

S-ECC: Severe Early Childhood Caries; SD: Standard deviation; CI: Confidence interval

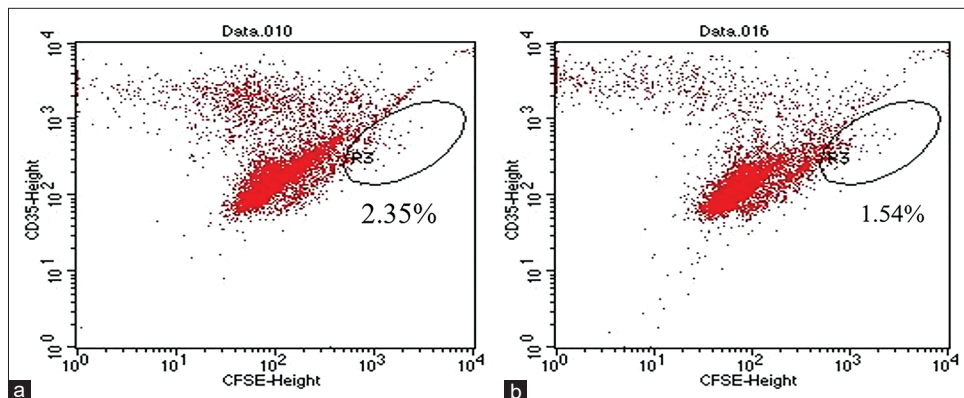


Figure 2: Neutrophil which was activated to perform phagocytosis to *Streptococcus mutans* which was labeled Carboxyfluorescein N-hydroxysuccinimide ester expressing CD35<sup>+</sup> which was detected by using flow cytometry on early childhood free caries (a) and on severe early childhood caries (b)

CD35<sup>+</sup> that can be detected on neutrophil saliva in S-ECC is lower (1.54% ± 0.35%) and it is different significantly compared to the expression level of CD35<sup>+</sup> in early Childhood free caries (2.35% ± 0.56%).

The result of the research indicates that the process of phagocytosis on *S. mutans* mediated through opsonization process by complement on salivary neutrophil of preschool-age children with severe caries is significantly lower in expressing CD35<sup>+</sup> than that in caries-free children. The low expression of CD35 on S-ECC compared to caries-free might be caused by the inability of innate immunity component on S-ECC to perform phagocytosis effectively to *S. mutans* through complement opsonization. Complement receptors (CRs) will recognize the component from complement cascade on the phagocyte target surface.<sup>[13]</sup>

In addition, it is also possible that the marker of CD35<sup>+</sup> on saliva neutrophil on S-ECC is less expressed because the neutrophil is less sensitive toward *S. mutans*. It is because the activated neutrophil triggers the change of conformation in complement receptor CD35 as a precondition to bind microbes and enables phagocytosis transduction signal. The complement system, including CD35, denotes protein fragment with special ability to activate, coordinate, and regulate pro-inflammatory and proteolytic agents from the immune system.<sup>[14]</sup> The reduce expression and functional deficiency of complement component may modulate the susceptibility of some disorder due to the genetic variation. This subsequently may reduce the activated complement from the opsonized pathogen, thus increasing resistance toward pathogens that depend on phagocytosis to initiate infection.<sup>[15]</sup>

The next possibility is that *S. mutans* have a strategy to avoid host immune through a mechanism of biofilms formation which serves as a physical obstacle to increase the resistance of pathogens to host defense such as opsonization, lysis by complement, and phagocytosis.<sup>[16]</sup> The pathogen may evade complement activity by recruiting host complement regulators, acquisition the host protease which plays role in untwist complement protein in bacterial surface and secret protease to inactivate complement.<sup>[17]</sup> This may cause significantly lower expression of CD35 on S-ECC compared to that on caries-free children.

## Conclusion

A decrease of CD35<sup>+</sup> expression on the surface of salivary neutrophils is an early detection marker of SECC.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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