

CD89/CD35 Expression Ratio in Salivary Neutrophil as an Early Detection Marker for Severe Early Childhood Caries

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Abstract

Objectives To analyze CD35/CD89 expression ratio on the surface of neutrophils as an early detection marker for S-ECC.

Materials and Methods Saliva was collected from 4- to 6-year-old kindergarten students. Salivary neutrophils were obtained by instructing the subjects to rinse their mouth with 1 mL of sterile 1.5% NaCl for 30 seconds before expectorating it into a sterile glass. The expression of CFSE⁺CD35⁺ and CFSE⁺CD89⁺ was measured and analyzed using flow cytometry.

Results The expression of CFSE⁺CD89⁺ in the caries-free group (2.46 ± 0.39) was significantly lower than that in the S-ECC group (3.41 ± 1.11), with a *p*-value of 0.0001, while the expression of CFSE⁺CD35⁺ in the caries-free group was (2.35 ± 0.56) compared with (1.54 ± 0.35) (*p* = 0.0001) in the S-ECC group.

Conclusions The expression ratio of CFSE⁺CD89⁺ and CFSE⁺CD35⁺ constitutes a marker for S-ECC.

Keywords

- ▶ CD89
- ▶ CD35
- ▶ neutrophils
- ▶ caries
- ▶ biomarker

Introduction

Early childhood caries (ECC) is a major problem in oral health, which affects the health of preschoolers around the world. Prevalence reaches 85% in low socioeconomic children. ECC is characterized by the presence of one or more decayed, missing, or filled primary teeth in children aged 5 years or younger.¹ In children younger than 3 years, if there is caries on a smooth surface, it is an indication of severe ECC (S-ECC). From ages 3 to 5 years, one or more cavitated, missing teeth (due to caries), or filled smooth surfaces in primary maxillary anterior teeth, or decayed, missing, or filled score of ≥ 4 (age 3), ≥ 5 (age 4), or ≥ 6 (age 5) surfaces constitutes S-ECC.² The prevalence of ECC and S-ECC among preschool children aged 3 to 5 years in

Xinjiang reached levels of 78.2 and 41.2%, respectively, which was relatively higher than the figure of 53.6% in other districts within China during the period 2010 to 2013.³ Another study by Peltzer and Mongkolchati reported the prevalence of ECC in northern Thailand in 2015 was also high as evidenced by a decayed–missing–filled teeth index score of 5.61 for ECC and 8.17 for S-ECC among preschoolers.⁴

In addition to affecting quality of life, ECC increases the risk of caries in permanent dentition and other oral diseases.⁵ In addition, performing dental treatment to the children is considerably difficult, since for children, the dentist is a nightmare. The fear of dentist, odontophobia, usually leads to postpone treatment, clinging to pharmacological therapies which produce a serious worsening of the caries itself.⁶

S-ECC, the severe form of ECC, may have a greater impact than caries during adulthood if left untreated.⁷ However, several cases have indicated that ECC, even S-ECC, can be prevented or regulated, given the application of appropriate measures.⁸

Social and behavioral risk factors which have been identified as correlated to S-ECC include: low socioeconomic status, the availability of fluoridated water, the profile of the mother (level of education, own experience of caries, and degree of awareness of dental and oral health), dietary habits, inappropriate dental health behavior, and lack of access to dental health facilities.⁹ These data confirmed that S-ECC predominantly affects the children of socially disadvantaged families, ethnic minorities, and individuals who live in less developed regions.¹⁰

Caries may also cause damage to the adjacent tissue due to the spread of *Streptococcus mutans* leading to gingival or periodontal inflammation.¹¹ Necrotic teeth, if left untreated, may cause abscesses that spread to the adjacent tissue leading to various conditions ranging from gingivitis to cellulitis in the mandible. In the worst case scenario, the bacteria transported through the bloodstream may cause systemic infection necessitating a more expensive treatment involving type IV antibiotics.¹²

Recent studies have revealed a new role for neutrophils as the key component of first-line defense against microbes.¹³ In addition to destroying microbes through phagocytosis and releasing reactive oxygen species (ROS) and antimicrobial peptides, they also regulate immune response activation.¹⁴ Neutrophils have also been confirmed as the main contributors to proinflammatory cytokine synthesis in infected areas and chemokine and growth factors.¹⁵ Another study proved that neutrophils can initiate two-way complex interaction between macrophage and dendritic cells (DCs), natural killer cells and lymphocyte in the infected area, which further affect the innate and adaptive immune response.¹⁶

Neutrophils kill pathogenic microbes by a process of phagocytosis which is significantly more effective due to opsonization involving antibodies present on the surfaces of the microbes. Microbial phagocytosis may lead to oxidative burst, producing ROS accompanied by cytoplasmic degranulation in microbe-contained phagosome, with antimicrobial peptides and protease.¹⁵ Neutrophils express several specific receptors for fragment crystallizable (Fc) region antibodies, such as Fc α R/IgA receptor (cluster of differentiation [CD]89) and complement receptor (CR)1 (CD35) that play a role in microbe recognition following opsonization.¹⁷

Complement activation leads to C3b deposition on microbe surfaces. CRs expressed on the surface of neutrophils efficiently recognize microbes bound to a complement component called CR1 (CD35).¹⁶ The abnormal phagocytosis function might be caused by several clinical disruptions, both innate and adaptive, possibly initiated by the neutrophils themselves, or complementing anomaly during opsonization. The release of premature neutrophils from the bone marrow may induce disrupted phagocytosis, possibly correlated to disruption of the neutrophils, either in terms of their quantity or function. This process may also be associated with

mild to severe microbial infection,¹⁸ the risk of which may be increased by insufficient levels of neutrophil. Diseases related to the low neutrophil levels in the bloodstream include: cyclic neutropenia, chronic benign neutropenia, several congenital neutropenia, and Felty's syndrome, all of which correlate to dental and oral problems, including early tooth loss.¹⁹

Several approaches to caries prevention have been attempted including access to dental health education (specifically, the appropriate method of brushing the teeth), the provision of topical fluoride, and vaccination.²⁰ However, to date, none of these has produced the desired outcome. This study aims to identify the risk factor of dental caries and the innate immunity mechanism which can protect against or prevent these.¹²

Materials and Methods

Ethical Approval

This research received the approval of the Institutional Ethical Committee (certificate no. 01/KKEPK/VII/2013), while a signed consent form was obtained from the parents of the subjects prior to the collection of data.

Design and Research Procedures

This research is an observational analytic study with cross-sectional design using S-ECC and caries free as the object of research. The sample (40 children) was obtained from several kindergartens in the Surabaya area. The examination of dental caries was conducted in each selected institution by measuring the decay-exfoliation-filling (def-t) index. The subjects who had been examined were subsequently divided into two groups: a caries-free group and a caries group with a def-t more than 6. The age of all subjects ranged from 4 to 6 years at the time of the examination. Prior to specimen collection, questionnaires about the children's health had been distributed and the parents of the subjects signed a written informed consent sheet. Samples were obtained by researchers and trained personnel using a standard protocol. Subjects were not permitted to eat, drink, chew gum, or brush their teeth for 60 minutes before sampling was conducted. The samples collected were frozen (Frozen; Quanzhou, China) at a temperature of -80°C for further analysis.

Phagocytosis Simulation

Observation of the phagocytosis activity of *S. mutans* in neutrophils was conducted by means of culturing *S. mutans* in a well-containing neutrophils. The simulation of phagocytosis was performed by incubating the cell suspension and bacteria in an incubator at 37°C and 5% CO_2 for 60 minutes.¹⁹ The cells were harvested, inserted into the microtube, and centrifuged at 2,500 rpm for 5 minutes at 4°C . The resulting pellet was then administered with BioLegend antihuman- α CD89 PE, biolegend antihuman- α CD35 PE and biolegend antihuman- α CD11c-PeCy5 and these were conjugated before being pipetted. The suspension was subsequently placed into the flow cytometer cuvette, added to 300 μL phosphate buffered saline, and inserted into a BD FACS caliber nozzle for

running. The phagocytosed bacteria expressed CFSE⁺CD89⁺ and CFSE⁺CD35⁺ measured using a flow cytometer at a wavelength of 525 nm with 488 nm excitation.

Neutrophils were obtained from saliva by instructing the subjects to rinse their mouths with 10 mL of sterile 1.5% NaCl for 30 seconds without swallowing. The resulting fluid was expectorated in a sterile glass (Pyrex, Singapore) with the procedure being repeated four times. The collected specimens were subsequently centrifuged at 450 g for 15 minutes at 4°C. The identification of neutrophils employed the use of the human neutrophil sorting cell enrichment kit (EasySep, Sheboygan).

Measurement of CFSE⁺CD35⁺ Expression

During the phagocytosis process, the surface receptor on the phagocytic cells can bind to the Fc fragment from one type of immunoglobulin or may bind to the complement factor (C3b). The interaction triggers a conformational change in the cytoskeleton to support the antigen ingestion process. Pathogens are destroyed by the action of oxidative and nonoxidative mediators, that is, the interaction between the complement factor of C3b or iC3b and CR (CD35). Carboxyfluorescein N-hydroxysuccinimide ester (CFSE⁺) staining on bacteria carries out the function of labeling the bacteria with fluorescein isothiocyanate in order that it can be detected with a flow cytometer (Becton Dickinson; San Jose, California, United States). Meanwhile, the provision of CD35⁺ antibody represents a marker which indirectly confirms the observed cell during the phagocytosis process to be a neutrophil cell. Staining with CFSE⁺CD35⁺ is intended to detect the ability of neutrophil cells which contain CRs (CD35⁺) to perform phagocytosis on bacteria (labeled CFSE⁺).

Measurement of CFSE⁺CD89⁺ Expression

Neutrophils express Fc receptor to recognize antibodies, including Fc IgA specific, which is a transmembrane glycoprotein belonging to the immunoglobulin gene superfamily. Fc α R or CD89 receptor is expressed by neutrophils. The staining of CFSE⁺ in bacteria is intended to label the bacteria with

FITC fluorescence, thereby enabling its detection by means of a flow cytometer. Meanwhile, the CD89⁺ antibody may act as an indirect marker of the phagocytized process in neutrophils. CFSE⁺CD89⁺ staining is intended to detect the ability of neutrophils with Fc receptor (CD89⁺) to phagocytize labeled bacteria (CFSE⁺).

Results

The result of flow cytometry analysis is shown in ►Figs. 1 and 2. The data acquired were analyzed, tabulated, and presented in mean and standard deviation forms (►Fig. 3). All the participated respondents, based on the questionnaire answer, were physically and mentally healthy. Among 40 examined children, 20 of them scored def-t index more than 6, and the rest 20 scored less than 6. Thus, the respondents were divided into two groups, caries-free group for those with def-t index score less than 6, and caries group for those with def-t index score more than 6. ►Table 1 shows that activated neutrophils induce phagocytosis. The observation revealed a significantly lower expression of CD35⁺ in the S-ECC group, compared with the caries-free group. Meanwhile, the expression of CD89⁺ in the ECC group was significantly higher than the caries-free group (►Table 2).

Discussion

This study revealed that half of the respondents suffered from caries with def-t index score more than 6. These results typically correspond to the available data from developing countries, which have higher prevalence of S-ECC than the developed countries, despite the prevention measures performed.²² Considering the difficulty to perform dental treatment to children due to dentophobia,⁶ this research is an attempt to find a way to early detect the risk of S-ECC, so the prevention measures could be performed as soon as possible.

The contents of ►Table 1 indicate that salivary neutrophils contain levels of CD35⁺ expression in the S-ECC (1.54%

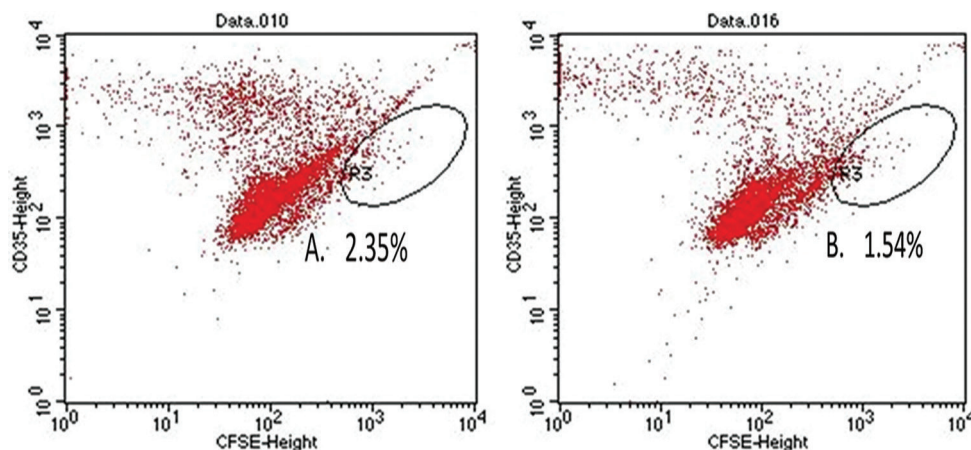


Fig. 1 Neutrophils activated to perform phagocytosis on *S. mutans* which were labeled with CFSE expressing CD35⁺ and detected using flow cytometry in early childhood free caries (A) and in early childhood caries (B). CD, cluster of differentiation.

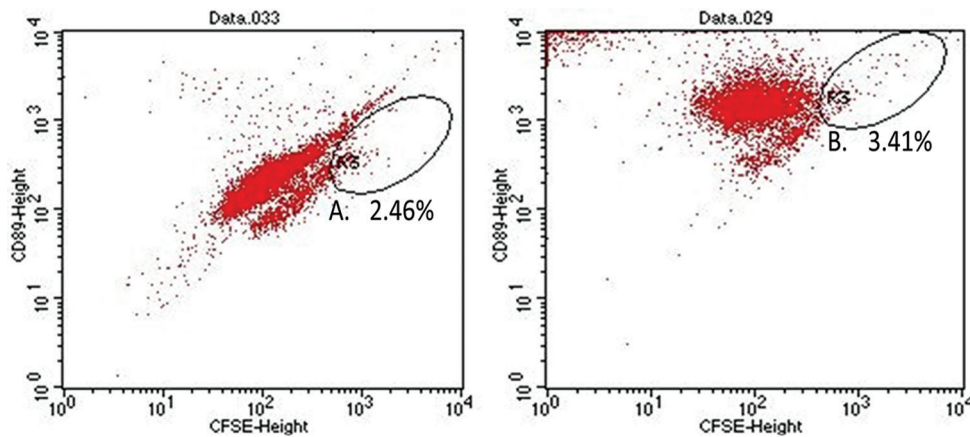


Fig. 2 The activated neutrophils phagocytizing *S. mutans* labeled CFSE and expressing CD89⁺ detected using flow cytometry in the caries-free group (A) and the S-ECC group (B). CD, cluster of differentiation; S-ECC, severe early childhood caries.

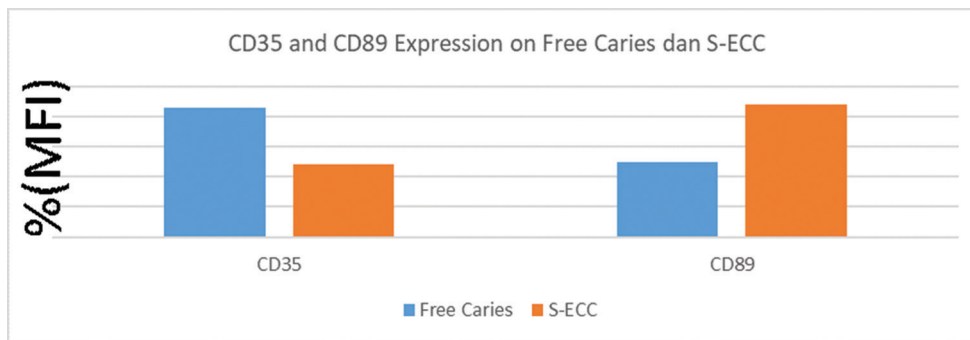


Fig. 3 Mean graph of activated neutrophils inducing phagocytosis in *S. mutans* labeled CFSE, expressing CD35⁺ and CD89⁺ detected using flow cytometry on the caries-free group and the S-ECC group. CD, cluster of differentiation; S-ECC, severe early childhood caries.

Table 1 Mean and standard deviation of the number of saliva neutrophils activated to perform phagocytosis of bacteria *S. mutans* labeled by CFSE staining and expressing CD35⁺ in the severe early childhood caries and caries-free (%) teeth

| Group | N | Mean ± standard deviation | 95% CI | p-Value |
|-------------|----|---------------------------|-----------|-------------------------|
| Caries-free | 20 | 2.35 ± 0.56 | 2.09–2.61 | 0.0001 ($p < \alpha$) |
| S-ECC | 20 | 1.54 ± 0.35 | 1.38–1.71 | |

Abbreviations: CD, cluster of differentiation; CI, confidence interval; S-ECC, severe early childhood caries.

Table 2 Mean and standard deviation of the number of saliva neutrophils activated to perform phagocytosis of bacteria *S. mutans* labeled by CFSE staining expressing CD89⁺ on the early childhood caries and caries-free (%) teeth

| Group | N | Mean ± standard deviation | 95% CI | p-Value |
|-------------|----|---------------------------|-------------|------------------------|
| Free caries | 20 | 2.46 ± 0.39 | 2.21 ± 2.71 | 0.001 ($p < \alpha$) |
| ECC | 20 | 3.41 ± 1.11 | 2.70 ± 4.12 | |

Abbreviations: CD, cluster of differentiation; CI, confidence interval; ECC, early childhood caries.

± 0.35) group which were significantly lower than in the caries-free group (2.35 ± 0.56). This shows that the phagocytizing process of *S. mutans* mediated by opsonization of salivary neutrophils in S-ECC group express lower levels of CD35⁺ than the caries-free group. This is possibly due to the innate immunity in children with S-ECC not being able to effectively perform phagocytosis on *S. mutans* through complement opsonization. This lack of ability is due to the low-level deposition of C3b protein in children with S-ECC.

Therefore, the opsonization process of *S. mutans* is not optimal. This phenomenon induces a less effective phagocytosis process mediated by complement in S-ECC group compared with the caries-free group by means of observing the expression of CD35⁺ on the salivary neutrophil surface.

Neutrophils constitute the primary cells in the innate immune system, with anti-infection and proinflammation characteristics. In response to infection, neutrophils are the first phagocytic cells that migrate to the site of the injury to

eliminate and microbes by means of phagocytosis, an intracellular mechanism. This process is followed by fusion of phagosome and lysosome containing antimicrobial peptide, enzyme, and reactive oxygen intermediate. Furthermore, neutrophils are also capable of destroying bacteria via extracellular mechanism by releasing antimicrobial peptide and enzymes contained in their granules.²³ The surface of neutrophils express Fc IgA receptor/Fc α R (CD89) which forms part of host defense against pathogens by mediating cellular response, namely, phagocytosis, oxidative burst, and release of inflammatory mediators and cytokine.²⁴

Phagocytosis performed by neutrophils represents a complex process initiated by bacterial attachment to the neutrophil mediated by molecule opsonization, binding a component to the cell surface of bacteria and promoting phagocytosis. Protein C3b denotes crucial opsonin bound by the CR1 (CD35) in the neutrophil cell surface, causing the bacteria to penetrate the phagolysosome, inducing bacteria cell degradation.²⁵

C3b is a molecule, produced following complement activation of the thioester group, which is highly reactive and capable of binding the microbe through the hydroxyl group or amino covalent. C3b acts as opsonin, which is subsequently recognized by CD35, subsequently changed into iC3b by H and I factors to be recognized by CR3 and CR4, also known, respectively, as Mac-1 (CD11b/CD18) and CD11c/CD18. The other possible mechanism is that of the pathogenic *S. mutans* in S-ECC evading the host immune system by forming biofilm as a physical barrier. This increases its resistance to opsonization, lysis by complement or phagocytosis. Therefore, *S. mutans* strain in S-ECC is more virulent compared with the caries-free group as they can modulate complement systems, the most prominent part of innate immune system which is able to recognize and opsonize *S. mutans* to be phagocytized by neutrophils.

The strategy of pathogenic bacteria to evade action of the immune system focuses mainly on the complement system which denotes the main core of innate immunity and is considered the first line of defense. The majority of pathogens in humans are known to possess a certain mechanism to evade the complement.^{25,26} This might also happen in *S. mutans* strain in individuals with S-ECC.

Based on the observations conducted, the expression of CD89⁺ in the S-ECC group (3.41 ± 1.11) was recorded as significantly higher compared with that in the caries-free group (2.46 ± 0.39) (► **Table 2**). This indicates that the phagocytizing process of *S. mutans* mediated through the opsonization process by secretory immunoglobulin A (sIgA) in the S-ECC

group expressed a larger amount of CD89⁺ compared with the caries-free group. This is possibly due to the high antigen target being destroyed by the immune system of the S-ECC group mediated by the component of adaptive immunity as the innate system is unable to eliminate the cariogenic *S. mutans* bacteria.

The inability of the innate immune system in an individual with S-ECC causes the salivary neutrophils to induce a two-way interaction with the macrophage cells, DCs, natural killer cells, lymphocyte cells, and mesenchymal stem cells mediated by the complement system.²⁷ This initiates antibody formation since neutrophils also contribute to delayed-type hypersensitivity by releasing monocyte chemotactic protein-1/CCL2 which induces the transition from an innate to adaptive immune response.²⁸ Neutrophils constitute the component of innate immunity that induces the maturation of DC and increases the expression of costimulating molecules (HLA-DR, CD86, CD46) to produce signal inducing T-cells. The interaction of neutrophils with DC induces the production of interleukin (IL)-12 by DC and promotes the maturation and activation of T cells (► **Table 3**).²⁹

sIgA represents the main mediator of the humoral immune system in the mucosal surface that is able to bind antigen and prevent infection. Meanwhile, the effector mediated by IgA is dependent on Fc α R (CD89) on the neutrophil surface, eliminating IgA with immune complex.³⁰

The binding of antigen and sIgA complex to CD89 causes various immune responses, including antibody-dependent cell-mediated cytotoxicity, phagocytosis, cytokine release, oxidative burst, and degranulation. Those responses play an important role in host defense against microbes, possibly appearing as inflammation and a pathological condition.³⁰

The phagocytosis process involving salivary neutrophils mediated by sIgA indicated by the expression of CD89⁺ showed a significant increase compared with the caries-free group. However, this did not signify a cutoff in the development of caries. The increase of *S. mutans* in the S-ECC group with a more varying virulent strain proved capable of evading the host immune system, both innate and adaptive, despite the complex host innate immune system in saliva.³¹

S-ECC denotes chronic infection, producing the high level of proinflammatory cytokine C5a, IL-8, tumor necrosis factor- α , and the formation of antigen-IgA complex due to an elevated level of antigen. Individuals with S-ECC tend to present high levels of *S. mutans* in the oral cavity,²⁹ inducing an immune response which increases the production of immunoglobulin. This may explain the inability to prevent caries, despite the high level of sIgA.³²

Table 3 The multiple regression analysis of salivary neutrophils expressing CFSE⁺CD35⁺ and CFSE⁺CD89

| Independent variable | Dependent variable | | R ² | p-Value |
|----------------------|--------------------|-------------|----------------|---------|
| Neutrophil | CFSE-CD35 | Caries free | 0.785 | <0.0001 |
| | | S-ECC | 0.539 | <0.0001 |
| Neutrophil | CFSE-CD89 | Caries free | 0.771 | <0.0001 |
| | | S-ECC | 0.753 | <0.0001 |

Abbreviation: S-ECC, severe early childhood caries.

The destructive function of the antibody Fc region will not occur if the pathogenic bacteria bind to the Fc region. Group A streptococci contain fibronectin-binding protein I (SfblI) that is capable of binding to the Fc region of IgG, preventing phagocytosis.³³ Several forms of M protein also demonstrate the ability to bind the Fc domain of IgG and IgA.³⁴ A further study is required to establish whether *S. mutans* possesses such ability, considering that the high expression of CD89 in salivary neutrophils does not reflect the efficacy of phagocytizing process mediated by sIgA opsonization. It can be concluded from this study that the high level of *S. mutans* in individuals with S-ECC facilitates the uninterrupted progress of caries.

The limitation of this study is we did not perform a detailed observation of the children feeding habit, infant feeding practices, nutritional intake, and the parents' oral hygiene practice. These factors, of course, may affect the prevalence of caries and how the host immune responds. Thus, future research is necessary to find whether this early detection marker is affected by aforementioned factors.

Conclusions

The ratio of CD89⁺/CD35⁺ expression constitutes a marker early detection of S-ECC.

Note

All the listed authors have read the manuscript and hereby stated that this manuscript has not been presented in any conference/convention/meeting.

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Conflict of Interest

None declared.

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