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Induction of calprotectin release by *Porphyromonas gingivalis* lipopolysaccharide in human neutrophils

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Calprotectin, a major cytosolic protein of leukocytes, is detected in neutrophils, monocytes/macrophages, and epithelial cells. This protein is known to be a marker for several inflammatory diseases and is detected in inflammatory gingival tissue with periodontal disease. Recently, we found that the calprotectin level in gingival crevicular fluid from periodontitis patients was significantly higher than that of healthy subjects. However, the regulation of calprotectin in periodontal disease is unclear. In the present study, we investigated the effect of lipopolysaccharides of periodontopathic bacteria on calprotectin release from human neutrophils. Neutrophils from healthy donors were treated with lipopolysaccharides from *Porphyromonas gingivalis* (P-LPS), *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Escherichia coli*. Calprotectin of neutrophil was identified by immunoblotting and calprotectin amount was determined by ELISA. Two subunits (10 and 14 kDa) of calprotectin were observed in the cell and medium fractions from neutrophils. P-LPS increased calprotectin release from seven to 16 times the control level after 30 min and its effect appeared in a dose-dependent manner (10–1000 ng/ml). Lipopolysaccharides from *A. actinomycetemcomitans*, *P. intermedia*, *F. nucleatum*, and *E. coli* also induced calprotectin release from neutrophils. These results suggest that lipopolysaccharides from periodontopathic bacteria induce calprotectin release from human neutrophils.

Key words: calprotectin; lipopolysaccharide; neutrophil; periodontopathic bacteria

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Calprotectin is a major cytosolic protein of leukocytes, constituting 50–60% of cytosol protein in granulocytes, and has been detected in neutrophils, monocytes/macrophages, and oral epithelial cells (5, 7, 8). This 36.5-kDa calcium-binding protein belongs to the S100 protein family and is a heterogeneous complex composed of light and heavy subunits, which are also called macrophage migration inhibitory factor-related protein 8 and 14 (MRP8/MRP14), S100A8/S100A9, calgranulin A/B, L1 antigen and cystic fibrosis antigen (2, 8, 13, 29). It is reported that the calprotectin level in plasma, synovial fluid, feces and urine increases in some inflammatory diseases, including rheumatoid arthritis, septicemia, ulcerative colitis, urinary tract infections, and cystic fibrosis, and that calprotectin can inhibit metalloproteinases associated with inflammation and tissue destruction by sequestration of zinc. Therefore, calprotectin is used as a marker for rheumatoid arthritis, inflammatory bowel disease, and colorectal cancer (3, 9, 11, 14, 32, 34).

We have reported that calprotectin exists in dental calculus and gingival crevicular fluid, and that the calprotectin level in gingival crevicular fluid from periodontitis patients is higher than that from healthy subjects and correlates well with clinical and biochemical markers for periodontal inflammation (16–18, 26). Furthermore, Schlegel et al. (35) and the present authors (38) showed that calprotectin was strongly expressed in the area of inflammatory cells in gingival tissue from periodontitis patients. In periodontal disease, neutrophils, monocytes/macrophages, and lymphocytes increase in the tissues surrounding the inflamed period-

ontal pocket and play important roles in the host immune response (22). Neutrophils are the predominant leukocytes in the periodontal pocket and junctional epithelium of periodontitis patients, releasing antibacterial components such as lysozyme, lactoferrin, defensins, elastase, and cathepsin G (22). Therefore, neutrophils not only control the periodontopathic bacteria in the acute immune response but also act in chronic inflammation.

Calprotectin may also contribute to the immune response in periodontal disease because this protein possesses antimicrobial activity against *Candida albicans*, *Capnocytophaga sputigena*, and *Escherichia coli*, protection activity against *Porphyromonas gingivalis* in gingival epithelium and a growth inhibitory action on macrophages and lymphocytes (23, 27, 37, 41, 42). The origin of gingival crevicular fluid calprotectin and the factors regulating calprotectin release from immune cells remain unclear in periodontitis. Miyasaki et al. (24) suggested that the major source of gingival crevicular fluid calprotectin was neutrophils based on a correlation between calprotectin and lactoferrin levels in gingival crevicular fluid. The aim of our study is to elucidate the regulation of calprotectin release in neutrophils. *P. gingivalis* is a major periodontopathic bacterium and its lipopolysaccharide (P-LPS), a significant virulence factor of pathogen, stimulates the production and release of inflammatory cytokines in human polymorphonuclear leukocytes and monocytes (1, 21, 40). In the present study, we investigated whether lipopolysaccharides of periodontopathic bacteria including *P. gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, and *Fusobacterium nucleatum* induced calprotectin release from human neutrophils.

Material and methods

Preparation of neutrophils and cell culture

Neutrophils were isolated from the EDTA-treated blood of 12 healthy donors (eight males and four females, 24–44 years old) by centrifugation using Mono-Poly Resolving Medium (Dainippon Pharmaceutical Co., Osaka, Japan) according to the manufacturer's instructions after the donors gave their consent to our experiments. The isolated cells were washed in phosphate-buffered saline and suspended in RPMI 1640 (ICN Biomedicals, Aurora, OH) containing 5% heat-inactivated fetal calf serum (FCS: JRH BIOSCIENCES, Lenexa, KS) within 1–1.5 h of blood collection. Neutrophils (1.25×10^6 cells/ml) were incubated in the culture medium with 0.1–1000 ng/ml P-LPS, or with 1 μg/ml lipopolysaccharide from three periodontopathic bacteria of *A. actinomycetemcomitans* Y4, *P. intermedia* ATCC 25611, and *F. nucleatum* ATCC 25586, or *E. coli* 0111:B4 and K12:D31m4 for 30 min. In some experiments, cells were incubated with 1 μg/ml P-LPS for 15–360 min. The viability of neutrophils was examined using the trypan blue exclusion test, found to be more than 98.2% after isolation, and maintained at more than 95.2% during incubation for 6 h. Furthermore, the viability was confirmed using colorimetric assay with sodium 3'-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) [Cell Proliferation Kit II (XTT): Roche Molecular Biochemicals, Mannheim, Germany] and with lactate dehydrogenase activity [Cytotoxicity Detection Kit (lactate dehydrogenase): Roche Molecular Biochemicals]. The incubated neutrophils were collected by centrifugation (120 ***g***, 5 min, 4°C), suspended in 10 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml TPCK, 1 μg/ml TLCK, and 1 μg/ml pepstatin) and destroyed by sonication for 20 s in ice water (cell fraction). The conditioned medium was mixed with protease inhibitors, applied to a PD-10 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and eluted with 10 mM Tris-HCl buffer for immunoblotting analysis or used without the column procedure for calprotectin determination (medium fraction).

Preparation of lipopolysaccharides

P-LPS from *P. gingivalis* 381 strain was prepared by the hot-phenol-water method according to a modification of the method of Kumada et al. (20). Briefly, the bacterial cells were suspended in 90% phenol and distilled water and incubated for 20 min at 68°C. After centrifugation (8000 ***g***, 20 min, 4°C), the aqueous phase was dialyzed against distilled water, and further centrifuged (100,000 ***g***, 3 h, 4°C). The precipitate was treated with 2% Cetavlon (Nacalai Tesque, Kyoto, Japan) for 15 min at room temperature with 0.5 M NaCl and ethanol. After centrifugation (8000 ***g***, 20 min, 4°C), the precipitate was incubated with 20 μg/ml RNase A (Sigma Chemical Co., St. Louis, MO) for 2 h at room temperature, 5 μg/ml DNase I (Sigma) for 4 h, 50 μg/ml proteinase K (Sigma) for 6 h, and further treated with phenol–chloroform–petroleum ether (2 : 5 : 8). After centrifugation (1400 ***g***, 10 min, 4°C), the precipitate was washed with acetone and lyophilized (P-LPS). The lyophilized P-LPS was dissolved in culture medium prepared with endotoxin-free water just before the experiment and added to the culture of neutrophils. Lipopolysaccharide from *A. actinomycetemcomitans*, *P. intermedia*, and *F. nucleatum* was prepared according to the method of Nishihara et al. (28), Kirikae et al. (19), and Onoue et al. (30), respectively. The endotoxin activity of the prepared lipopolysaccharides was examined using the Limulus HS-J Single Test (Wako Pure Chemical Industries, Osaka, Japan). Lipopolysaccharides from *E. coli* 0111: B4 and K12: D31m4 were purchased from Wako Pure Chemical Industries and List Biological Laboratories Inc. (Campbell, CA), respectively.

Immunoblotting analysis

Calprotectin in samples was visualized by immunoblotting according to the method described previously (17). Briefly, the cell (5 μg protein) and medium (40 μg protein) fractions were electrophoretically separated on 12.5% polyacrylamide gels and electrically transferred to PVDF membranes (Immobilon^{TM}-P, Millipore, Bedford, MA). Calprotectin on the membranes was immunoreacted with anti-human calprotectin antibody from rabbit (1 : 1500 dilution, ref. 5, 6, 8, 17, 18, 31) for 2 h and further with horseradish peroxidase-conjugated goat anti-rabbit IgG (1 : 8000 dilution, Seikagaku Co. Tokyo, Japan) for 1 h at room temperature. The membrane was developed with ECL Western blotting detection reagents (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's instructions and exposed to Hyperfilm-ECL Western (Amersham Life Science). Calprotectin standard was purified from cytosol fractions of human granulocytes and anti-calprotectin polyclonal antibody (IgG fractions) was prepared from rabbit antiserum and further was purified by immunoaffinity chromatography (32).

Calprotectin determination

The amount of calprotectin in cell and medium fractions was determined by the ELISA method described previously (3, 5, 6, 9, 17, 18, 32). Briefly, the microtiter plates were precoated with anti-human calprotectin antibody (1 : 2000 dilution) from rabbit at 4°C overnight. The sample solution or calprotectin standards were

added to the precoated microtiter plates and incubated for 45 min at room temperature. After washing the plates, alkaline phosphatase (ALPase)-conjugated anti-calprotectin antibody (1 : 1000 dilution) was added to the wells and incubated for 45 min at room temperature. After a final wash, wells were incubated with 1 mg/ml *p*-nitrophenol phosphate for 20 min at 37°C and the absorbance of the reaction solution was determined at 405 nm using a microplate reader. The amount of calprotectin in the samples was calculated from a standard curve. In some experiments, calprotectin levels were expressed as the fold number of the control.

Statistical analysis

Statistical analysis was performed by unpaired, two-tailed Student's *t*-test between each experimental group and control. Differences at $P < 0.01$ were considered significant.

Results

Immunoblotting analysis of calprotectin from neutrophils

Calprotectin in samples from two healthy donors that showed low and high initial cellular calprotectin amounts was identified by immunoblotting analysis (Fig. 1). Two bands at 10 and 14 kDa were detected in the cellular fraction (5 μg protein) of neutrophils incubated with or without P-LPS (1 μg/ml) and corresponded to those in the calprotectin standard. In the cell fraction of neutrophils treated with P-LPS, both bands appeared slightly weaker than those of non-treated neutrophils. In the medium fraction (40 μg protein) of non-treated neutrophils from two donors, faint 10 and 14 kDa bands were found. However, in the medium sample from neutrophils treated with P-LPS, two bands were clearly evident. These results show that P-LPS can induce calprotectin release from neutrophils into the culture medium.

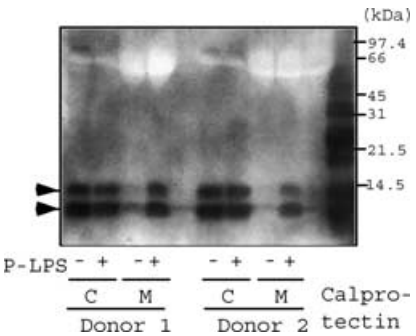

Fig. 1. Immunoblotting of calprotectin from human neutrophils. Neutrophils (1.25×10^6 cells/ml) from two healthy donors were incubated with or without 1 μg/ml P-LPS for 30 min. The cell fraction (5 μg protein) and medium fraction (40 μg protein) were separated by SDS-PAGE (12.5% gel) and immunoreacted with anti-human calprotectin Ab from rabbit. Arrowheads show calprotectin components with molecular masses of 10 and 14 kDa. C: cell fraction, M: medium fraction, Calprotectin: calprotectin standard from human plasma.

Effect of P-LPS on calprotectin release from neutrophils

The initial cellular content of calprotectin (0-time) was from 6.9 to 33.3 pg/cell in samples from the 12 donors and the mean value was 17.2 pg/cell. In Fig. 2 are shown the changes induced by P-LPS stimulation of neutrophils from the same donors as used in Fig. 1. The calprotectin levels in the medium were about 3.5 (Fig. 2A) to 7 times (Fig. 2C) that of each non-treated control after 15 min. The released amounts increased to a maximum (9.86 and 6.41 pg/cell) after 30 min, i.e. about 12 and 20 times the control levels (0.81 and 0.32 pg/cell, respectively). Subsequently, the levels decreased and returned nearly to control levels after 2–4 h. In contrast, the cellular calprotectin amounts were 33.3 (Fig. 2B) and 6.9 (Fig. 2D) pg/cell at 0-time and decreased to minimum levels (8.94 and 1.20 pg/cell) after 30–60 min of P-LPS addition, then slowly increased almost to control levels. Although the initial cellular calprotectin level and degree of response of calprotectin release to P-LPS were different in the individual samples, the time-course responses of P-LPS demonstrated almost identical patterns. Judging from the results of the trypan blue exclusion test and cell viability assays with XTT and lactate dehydrogenase, the cells seemed viable during the incubation for 6 h under the culture conditions.

P-LPS induced calprotectin release from neutrophils in a dose-dependent manner (Fig. 3). A concentration of 10 ng/ml P-LPS significantly induced calprotectin release to about seven times that of control levels after 30 min of treatment. At concentrations of 100 and 1000 ng/ml, calprotectin release increased to about 13 and 16 times that of control levels, respectively. These results indicate that P-LPS induces calprotectin release from intact neutrophils fairly quickly (within 30 min).

Effect of lipopolysaccharides derived from several periodontopathic bacteria

All lipopolysaccharides (1 μg/ml) derived from *P. gingivalis*, *A. actinomycetemcomi-*

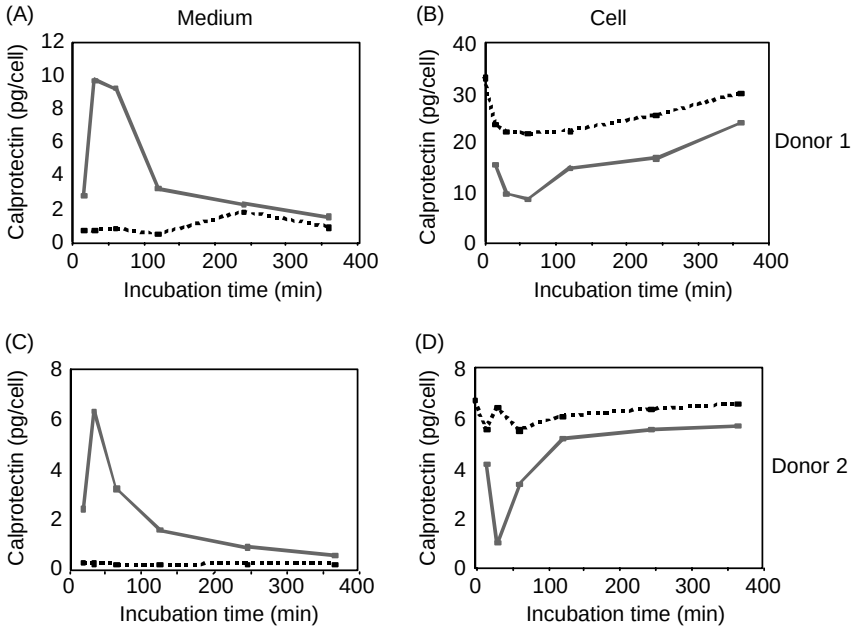

Fig. 2. Change of calprotectin level in human neutrophils stimulated with P-LPS. Neutrophils (1.25×10^6 cells/ml) from two healthy donors (1 and 2) were incubated with or without 1 μg/ml P-LPS for 15–360 min. Calprotectin amounts in the medium (A, C) and cell (B, D) fractions were determined by ELISA. Unbroken lines indicate samples from neutrophils treated with P-LPS; broken lines show samples from non-treated cells.

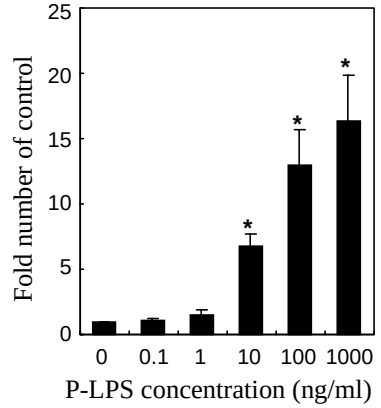

Fig. 3. Effect of P-LPS on calprotectin release from human neutrophils. Neutrophils (1.25×10^6 cells/ml) were incubated with 0–1000 ng/ml P-LPS for 30 min. The calprotectin amount in the medium was determined by ELISA. Values are expressed as the fold number of the control value and means ± SD for eight donors' samples in two experiments. The control value was 0.87 ± 0.32 pg/cell. *Significantly different from control ($P < 0.01$).

tans, *P. intermedia*, *F. nucleatum*, and *E. coli* induced calprotectin release (Fig. 4). Lipopolysaccharides from *P. intermedia*, *F. nucleatum*, and *E. coli* increased calprotectin release to 9–12 times that of the control level. These increases were similar to that of P-LPS (about 15 times the control level), whereas the effect of LPS from *A. actinomycetemcomitans* was less than that of other bacteria (only four times

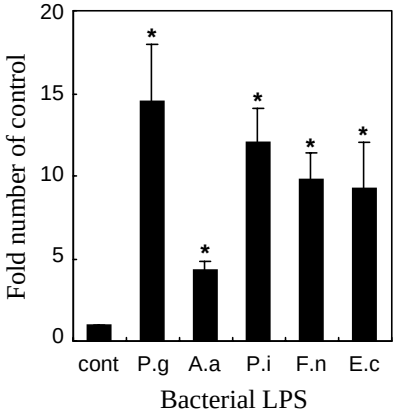

Fig. 4. Effects of lipopolysaccharides from periodontopathic bacteria on calprotectin release. Neutrophils (1.25×10^6 cells/ml) were incubated with several lipopolysaccharides (1 μg/ml) for 30 min. P.g: *P. gingivalis*; A.a: *A. actinomycetemcomitans*; P.i: *P. intermedia*; F.n: *F. nucleatum*; E.c: *E. coli*. Values are expressed as the fold number of the control value and means ± SD for four donors' samples in two experiments. The control value was 1.06 ± 0.67 pg/cell. *Significantly different from control ($P < 0.01$).

the control level). These results suggest that there is a difference in the effect of different lipopolysaccharide on calprotectin release among periodontopathic bacteria.

Discussion

Calprotectin strongly expresses in many inflammatory cells infiltrated in gingival tissue of periodontitis patients (35, 38). Neutrophils infiltrate the junctional epithelium and predominate in the epithelium and the adjacent connective tissue (22). Two bands (10 and 14 kDa) in immunoblotting analysis of neutrophils correspond to the two fractions of calprotectin standard (Fig. 1) and their molecular masses are identical to those of MRP8 and MRP14 (29). Furthermore, we found that P-LPS increased calprotectin release from monocytes as well as neutrophils, though the calprotectin amount in monocyte was about one-sixth of neutrophil and the effect of P-LPS on calprotectin release from monocytes was about one-fifth of neutrophils (38). Therefore, calprotectin in gingival tissue may be derived from neutrophils and monocytes in inflamed tissues and in gingival crevicular fluid this protein may be released from these cells.

In the present and preliminary studies, calprotectin levels in neutrophils isolated from the 20 donors ranged from 6.9 to 33.3 pg/cell. The calprotectin level in the neutrophil of each individual was almost constant in several assays performed on separate days, but the level differed considerably in cell samples of individual donors participating in the present experiments. The reason for this difference is unknown, and further studies are needed to ascertain whether such great interindividual variations in the amount of calprotectin per neutrophil exist, by investigating neutrophils from many donors. Despite the demonstrated difference in individual calprotectin levels, the time-course pattern of lipopolysaccharide-induced calprotectin release was almost similar in the used samples of donors, that is, calprotectin levels in the medium fraction reached a maximum at 30 min after application of LPS and then decreased. The decreased calprotectin level in the medium fraction in the later phase may be due to degradation of the released calprotectin by several proteases released after stimulation by P-LPS. The calprotectin level in the cell fraction rapidly decreased within 30 min and this change may be due to its release from cytosol of neutrophils into the medium. We examined the effect of P-LPS on calprotectin production in neutrophils by RT-polymerase chain reaction using two primers for MRP8 and MRP14. MRP8/MRP14 mRNAs were expressed in neutrophils cultured for 20 min–2.5 h, but their levels were not increased by P-LPS treatment (data not shown). Ross & Herzberg (33) reported that lipopolysaccharide of *E. coli* did not affect the expression of MRP8/MRP14 mRNAs in human gingival epithelial cells. Hu et al. (12) showed that expression of MRP14 mRNA of monocytes/macrophages was not induced by lipopolysaccharide of *E. coli*. From these reports and the present results, it is suggested that synthesis of calprotectin is constitutively regulated, as reported by Ross et al. (33), is not induced by P-LPS stimulation for at least several hours, and that the induction of calprotectin release by P-LPS treatment for a short period (15–30 min) is not caused by the increase of calprotectin synthesis.

The present study showed that lipopolysaccharides from periodontopathic bacteria induced calprotectin release from neutrophils. Yoshimura et al. (40) and Agarwal et al. (1) demonstrated that lipopolysaccharides of *P. gingivalis* and *A. actinomycetemcomitans* induced a similar expression or release of inflammatory cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α, which were elevated in gingival crevicular fluid of periodontitis patients, and in human polymorphonuclear leukocytes or monocytes. The high level of calprotectin in gingival crevicular fluid from periodontitis patients may be caused by the stimulation of neutrophils and monocytes by lipopolysaccharides. The effect of lipopolysaccharide on calprotectin release may be different among periodontopathic bacteria, and further experiments using different lipopolysaccharide doses and bacterial strains are necessary to explain these differences.

Besides lipopolysaccharides from several periodontopathic bacteria and *E. coli*, chemotoxins such as C5a and fMLP also induce calprotectin release (10), however, the regulatory mechanism of these stimulators in neutrophils remains unclear. We speculate that P-LPS may induce calprotectin release via some receptors and other factors including CD14, TLR, MyD88, TRAF6 and necrosis factor (NF)-κB because they are closely related to lipopolysaccharide signal transduction (4, 25). The mechanism of calprotectin release from neutrophils is still unclear. Sohnle et al. (36) and Voganatsi et al. (39) reported

that calprotectin of neutrophils was released only on cell death by lysis and disruption at sites with infection. In our study, a high cell viability of neutrophils was confirmed by the trypan blue exclusion test and colorimetric assays with XTT and lactate dehydrogenase after the treatment with lipopolysaccharides. Furthermore, the expressions of MRP8/MRP14 mRNAs in neutrophils cultured with P-LPS (20 min–2.5 h) were observed (data not shown). These results showed that most neutrophils were alive after P-LPS treatment. Rammes et al. (31) reported that phorbol myristate acetate, a protein kinase C activator, stimulated the secretion of MRP8/MRP14 from monocytes, its stimulation was blocked by tubulin polymerization inhibitors, and suggested that calprotectin was secreted via a pathway with an intact microtubule network. Kerkhoff et al. (15) found MRP8/MRP14 in the cytosol of neutrophils bound to arachidonic acid, which suggested that MRP8/MRP14 might be secreted from neutrophils as a complex with arachidonic acid. We think that calprotectin may be released from neutrophils by other physiological mechanisms without cell death as well as by cell death in inflammatory tissues. Further investigations are necessary to elucidate the mechanism of calprotectin release from neutrophils.

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