**Neutrophil-derived resistin release induced by *Aggregatibacter***

***actinomycetemcomitans***

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**ABSTRACT**

Resistin is an adipokine that induces insulin resistance in mice. In humans, resistin is not produced in adipocytes, but in various leukocytes instead, and it acts as a pro-inflammatory molecule. The present investigation demonstrated high levels of resistin in culture supernatants of neutrophils that are stimulated by a highly leukotoxic strain of *Aggregatibacter actinomycetemcomitans*. In contrast, the level of resistin was remarkably low when neutrophils were exposed to two other strains that produce minimal levels of leukotoxin and a further isogenic mutant strain incapable of producing leukotoxin. Pretreatment of neutrophils with a monoclonal antibody to CD18, β chain of LFA-1, or a Src family tyrosine kinase inhibitor prior to incubation with the highly leukotoxic strain inhibited the release of resistin. These results show that *A. actinomycetemcomitans*-expressed leukotoxin induces extracellular release of human neutrophil-derived resistin by interacting with LFA-1 on the surface of neutrophils and, consequently, activating Src family tyrosine kinases.

**Keywords:** *A. actinomycetemcomitans*, neutrophil, resistin, LFA-1, Src family tyrosine kinase

**Running title:** Neutrophil resistin released by *A. actinomycetemcomitans*

## INTRODUCTION

Resistin is one of several adipokines expressed in the adipose tissue of mice (Steppan, *et al.*, 2001). In humans, resistin is expressed at very low levels in adipocytes and at higher levels in white blood cells (Savage, *et al.*, 2001). Circulating levels of resistin are elevated in patients with acute and chronic diseases, including cardiovascular disease, atherosclerosis, rheumatoid arthritis, and type 2 diabetes (Migita, *et al.*, 2006, Takeishi, *et al.*, 2007, Shin, *et al.*, 2008, Chen, *et al.*, 2009). Increased circulating levels of resistin are also observed in patients with periodontitis (Furugen, *et al.*, 2008, Saito, *et al.*, 2008). An increased serum resistin level has been epidemiologically linked with various diseases in humans that involve inflammation.

It was recently reported that human neutrophils store abundant amounts of resistin in granules, which is released extracellularly upon inflammatory stimulation by bacteria, such as *Streptococcus pyogenes* and *Escherichia coli*, or by selected bacterial components, such as streptococcal M protein and *N*-formyl-Met-Leu-Phe (fMLP) (Bostrom, *et al.*, 2009, Johansson, *et al.*, 2009, Kunnari, *et al.*, 2009).

*Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, a Gram-negative facultative anaerobic coccobacillus, has been implicated in periodontal diseases, especially aggressive periodontitis, and other infectious diseases, such as endocarditis (Zambon, 1985, Paturel, *et al.*, 2004, Haubek, *et al.*, 2008). It expresses several potential virulence factors thought to play roles in the modulation of inflammation, induction of tissue destruction, and inhibition of tissue repair (Wilson & Henderson, 1995).

Leukotoxin, a virulence factor from *A. actinomycetemcomitans* interacts with lymphocyte function-associated molecule 1 (LFA-1), which is a β2 integrin expressed on mammalian cells, and exhibits cytolytic activity towards polymorphonuclear leukocytes (PMNs) and macrophages of humans and primates (Taichman, *et al.*, 1980, Taichman, *et al.*, 1987). Furthermore, leukotoxin has been reported to induce degranulation of PMNs independent of LFA-1 (Johansson, *et al.*, 2000). In this study, we examined whether neutrophil-derived resistin was released extracellularly by stimulation with several *A. actinomycetemcomitans* strains that express differing levels of leukotoxin and whether it was released by cytolysis or degranulation.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

*Aggregatibacter actinomycetemcomitans* HK921 (strain JP2), HK912 and HK1604, which are minimally leukotoxic strains, were grown in brain heart infusion broth (BHI; Difco Laboratories) at 37°C in air plus 5% $CO_2$. The three strains were a gift from Prof. Mogens Kilian, Department of Medical Microbiology and Immunology, Aarhus University, Aarhus, Denmark. *Escherichia coli* strains were grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C with aeration. When necessary for the selection of recombinant strains, the medium was supplemented with ampicillin (100 mg/L) and/or kanamycin (25 mg /L).

### Generation of an *ltxA*-disrupted HK921 strain

The *ltxA* gene was inactivated in the HK921 strain by insertional mutagenesis as described previously (Hayashida*, et al.*, 2002). Briefly, a fragment of the *ltxA* gene (positions 615 to 2,978 in the open reading frame of *ltxA* from strain HK921) was amplified from 1 ng of whole-cell DNA by PCR using the following primers:

5'-ACAACTTAATAAGTTAGGTGAAGCAC-3' (615 to 640)

and 5'-GACTTACCTTTATTCTTAATAAGTC-3' (2,954 to 2,978).

The amplicon was cloned into pGEM-T Easy Vector (Promega) using *E. coli* XL-1 Blue for propagation. The resulting plasmid was termed "pGEM-*ltxA*." The kanamycin resistance gene from the 1.7-kb transprimer transposon in pGPS1.1 was inserted into the *ltxA* gene fragment in pGEM-*ltxA* using TnsABC transposase. The purified plasmid was

introduced into *A. actinomycetemcomitans* by electroporation, and the cells were plated onto BHI agar containing kanamycin to select for transformants.

**SDS-PAGE and Western blot analysis**

After overnight incubation in BHI broth, the bacteria were harvested by centrifugation at 16,000 × *g* for 2 min. Bacterial pellets were resuspended in 100 μL of SDS sample buffer and boiled for 5 min. Samples (10 μL) were loaded onto a 10% polyacrylamide gel with a 4.5% polyacrylamide stacking gel and electrophoresed at 20 mA until the dye front was at the end of the gel. The protein bands were stained using a Rapid Stain CBB Kit (Nacalai Tesque, Japan).

For Western blot analysis, proteins were transferred from the polyacrylamide gel to a PVDF membrane (Millipore). The membrane was incubated with rabbit antiserum against *A. actinomycetemcomitans* leukotoxin (1:10,000 dilution), followed by incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1:10,000 dilution; Sigma-Aldrich). After incubation, immunoreactive proteins were visualized using the ECL Plus Western blotting detection reagent (Amersham Biosciences).

**Preparation of human neutrophils**

Human neutrophils were isolated from blood collected from healthy volunteers, and blood cell fractions were separated using Mono-Poly Resolving Medium (DS Pharma Biomedical) according to the manufacturer's instructions. Anti-CD16-coupled MACS MicroBeads (Miltenyi Biotec K.K.) were used to separate the neutrophils from the

samples. The cells were used immediately for the culture experiments. The neutrophils were assessed morphologically by phase-contrast microscopy and were shown to be >99% viable as determined by Trypan blue exclusion.

The healthy volunteers were informed about the purpose of the study and gave written consent before blood samples were taken. The study was approved by the Ethics Committee of the Nagasaki University Graduate School of Biomedical Sciences.

**Exposure of human neutrophils to bacterial cells of *A. actinomycetemcomitans* strains**

*Aggregatibacter actinomycetemcomitans* strains were incubated overnight at 37°C in air plus 5% $CO_2$. The bacteria were collected by centrifugation, mixed with human neutrophils ($1\times10^6$/mL) in RPMI-1640 with 1% fetal calf serum, and incubated at 37°C in air plus 5% $CO_2$. First, to examine the dose-dependent release of resistin and to determine optimum bacterial stimulation for subsequent experiments, we incubated neutrophils with bacteria at different relative ratios. Second, to examine the effect of leukotoxin promoter type on the level of resistin released, we incubated neutrophils with bacteria at a relative ratio of 1:1000 HK921, HK912, or HK1604 cells. Third, to examine whether leukotoxin expression affected the level of resistin release and how the level of resistin was related to degranulation and cytolysis, we incubated neutrophils with HK921 or its mutant, which was incapable of producing leukotoxin. Fourth, to examine the involvement of the leukotoxin receptor LFA-1 and a Src family tyrosine kinase, we used mouse monoclonal antibodies against LFA-1 subunits, TS1/22 (Endogen), recognizing CD11a, and TS1/18 (Endogen), recognizing CD18, and the Src

family tyrosine kinase inhibitor PP1 (Calbiochem). Neutrophils were preincubated with monoclonal antibodies (5 μg/mL) or PP1 (10 μM) for 15 min. Culture supernatants were collected after 0.5, 2, and 4 h of incubation in these experiments.

**Measurements of human resistin and elastase released from human neutrophils**

Concentrations of resistin and elastase in the sample supernatants were measured by ELISA (R&D Systems and Hycult Biotech, respectively). Cell-free supernatants were diluted in dilution buffer at a ratio of 1:10 for resistin and 1:20 for elastase measurements. Both resistin and elastase were quantified with reference to a standard curve generated by serial dilution of recombinant proteins provided by the manufacturer. The lower limit of detection was pg/mL for resistin and ng/mL for elastase. Relative release of elastase is expressed as a percentage of the total elastase obtained by lysing the cells with 0.1% Triton X-100 (Promega) for 1 h. All samples were measured in duplicate.

**Cytolysis assay**

The level of cytolysis was determined by the amount of the cytosolic enzyme lactate dehydrogenase (LDH) that was released, as measured using an LDH detection kit (Takara) according to the manufacturer's instructions. Relative cytolysis is expressed as a percentage of the total LDH activity obtained by lysing the cells with 0.1% Triton X-100 for 1 h.

**Statistical analysis**

Data are presented as the means ± standard deviation. Student's *t*-test was used for comparisons between two groups. One-way analysis of variance (ANOVA) was used to test whether the means of three groups were equal. When there was a statistical difference in ANOVA, post hoc comparisons were assessed using Scheffe's test or Dunnett's test for multiple comparisons, as appropriate. Differences were considered statistically significant when $P < 0.05$.

## RESULTS

### Dose response relationship between the ratio of bacteria to neutrophils and the level of resistin released

The amount of resistin in the supernatant of the neutrophils incubated with HK 921 increased significantly with an increase in the ratio of bacteria to neutrophils in a dose-dependent manner (Fig. 1). The ratio of 1000 bacteria per neutrophil was used in subsequent experiments.

### Difference in resistin release between exposure to highly leukotoxic and minimally leukotoxic strains

The amounts of resistin and elastase in the supernatant of the neutrophils incubated with HK921 for 2 h or longer were significantly higher than those with the two minimally leukotoxic strains, HK912 and HK1604 (Fig. 2A and B).

### Insertional inactivation of the leukotoxin gene

Leukotoxin was detected on Western blots of protein samples from the wild-type HK921 strain using rabbit antiserum against *A. actinomycetemcomitans* leukotoxin. No leukotoxin was detected in the HK921 strain with an insertional mutation of the *ltxA* gene (Fig. 3), confirming that leukotoxin was not expressed by the mutated strain.

**Simultaneous leukotoxin-induced release of resistin and elastase from neutrophils before cytolysis**

We measured the resistin, elastase, and LDH released from neutrophils after stimulation with the wild-type and mutant HK921 strains for 0.5, 2, and 4 h. The resistin level in the supernatant of the neutrophils incubated with wild-type HK921 was significantly higher than the level after incubation with the mutant strain (Fig. 4A). The level of elastase after incubation with wild-type HK921 for 2 h was nearly 75% of that after 4 h of incubation, but cytolysis was markedly lower after 2 h than after 4 h (Fig. 4B and C). These results suggest that *A. actinomycetemcomitans*-expressed leukotoxin induced the release of resistin by degranulation of the neutrophils prior to cytolysis.

**Effect of monoclonal antibodies against LFA-1 subunits and a Src family tyrosine kinase inhibitor on the release of resistin and elastase**

To examine the possible involvement of LFA-1, which is the receptor for leukotoxin, and a Src family tyrosine kinase in the release of resistin and elastase, we pretreated neutrophils with monoclonal TS1/22 (CD11a) or TS1/18 (CD18) antibody against LFA-1 subunits at a final concentration of 5 μg/mL and with 10 μM PP1 for 15 min, followed by incubation with wild-type or mutant HK921 for 2 h (Fig. 5A and B). In contrast to pretreatment with TS1/22, the level of resistin released from neutrophils pretreated with TS1/18 or PP1 was significantly lower than that from untreated neutrophils after incubation with wild-type HK921, as was the release of elastase. Additionally, the inhibitory effect of pretreatment with TS1/18 or PP1 on the levels of resistin and elastase released from neutrophils after incubation with mutant HK921 for

2 h was lower than those with wild-type HK921, but significant ($P < 0.05$) (Fig. 5A and B).

## DISCUSSION

Among the several virulence factors expressed by *A. actinomycetemcomitans*, leukotoxin is thought to play a major role in disease progression (Henderson, *et al.*, 2003). Leukotoxin has been reported to activate cytolysis of human leukocytes, including neutrophils and monocytes (Taichman, *et al.*, 1980), and to induce caspase-1 activation and bio-active IL-1β secretion in human macrophages (Kelk, *et al.*, 2003). In the present study, significantly more resistin was released from neutrophils incubated with wild-type HK921, which is characterized by a 530-bp deletion of the promoter region of the leukotoxin gene, than from neutrophils incubated with minimally leukotoxic strains. The ability of the wild-type strain to produce leukotoxin was confirmed by Western blot analysis using a leukotoxin-specific antiserum, and its cytotoxic activity against neutrophils was demonstrated by LDH release. Furthermore, the mutant strain, which is incapable of producing leukotoxin, released a significantly lower level of resistin ($P < 0.05$).

Whereas resistin is derived exclusively from adipose tissue in mice, leukocytes are the major source of resistin in humans. Neutrophils store abundant amounts of resistin in their granules, and the extracellular release of resistin via degranulation may be stimulated by bacterial or inflammatory stimuli (Bostrom, *et al.*, 2009, Johansson, *et al.*, 2009, Kunnari, *et al.*, 2009).

This study demonstrated the release of resistin from neutrophils in the presence of a highly leukotoxic strain, which is strongly associated with aggressive periodontitis in certain susceptible human populations (Haubek, *et al.*, 2008), was significantly higher

than in the presence of a leukotoxin-deficient isogenic mutant ($P < 0.05$). Based on this result, the release induced by the wild-type strain was attributed to an effect of leukotoxin.

These observations, combined with the above-mentioned demonstrations of human resistin storage in neutrophil granules and resistin release in response to microbial stimuli, indicate that neutrophil granules were the source of the resistin released in our study. This conclusion is supported by the simultaneous release of resistin and granule-associated elastase (Fig. 4A and B). We have little information on how degranulation of neutrophils is stimulated by leukotoxin. Johansson *et al.* (2000) reported that leukotoxin induced degranulation of PMNs and that the polyclonal antibodies against LFA-1 subunits had no effect on degranulation. Moreover, signals involved in triggering degranulation by neutrophils stimulated by leukotoxin are poorly understood.

Integrins, which are heterodimeric transmembrane adhesion receptors localized at cell–matrix contact sites, link extracellular matrix components to the actin cytoskeleton and interact with multiple structural and signaling molecules. LFA-1, a member of the β2-integin family, including CD11a and CD18, is a leukotoxin receptor located on the surface of neutrophils (Lally, *et al.*, 1997). The significant decrease in leukotoxin-induced resistin release from neutrophils pretreated with TS1/18 in the present study provides evidence for the involvement of CD18 in resistin release (Fig. 5A), as a recent study reported that CD18 is essential for the biological effect induced by leukotoxin (Dileepan, *et al.*, 2007). Our results differ from those reported by Johansson *et al.* (2000), and we cannot completely explain the discrepancy. It is possible

the polyclonal antibodies used by Johannson *et al*. (2000) were less effective than the monoclonal antibodies that we used in the inhibition study.

Furthermore, the inhibition of leukotoxin-induced resistin release from neutrophils incubated with PP1 indicates that a Src family tyrosine kinase participates in resistin release (Fig. 5A). Src family tyrosine kinases have been reported to be important mediators acting downstream of integrins to effect adhesion-dependent degranulation of neutrophils (Mocsai, *et al.*, 1999). Although PP1 inhibited adhesion-dependent degranulation, it had no effect on adhesion-independent degranulation induced by phorbol 12-myristate 13-acetate.

The results obtained from experiments with TS1/18 and PP1 suggest that leukotoxin binds to LFA-1 on the surface of neutrophils and then activates a Src family tyrosine kinase, leading to the release of resistin from neutrophils by degranulation, as well as adhesion-dependent degranulation.

Release of resistin and elastase still occurred, but a lower level, when stimulated by the mutant strain (Fig. 4). Moreover, pretreatment with TS1/18 or PP1 inhibited release of resistin and elastase from neutrophils stimulated by the mutant strain (Fig. 5A and B). Another molecule of *A. actinomycetemcomitans* might interact with CD18. Previous studies have reported that the CD18 of other β2 integrins, such as Mac-1 (CD11b/CD18) and p150/95 (CD11c/CD18), interacts with lipopolysaccharide (LPS) and induces a cellular response (Ingalls & Golenbock, 1995, Flaherty, *et al.*, 1997, Wong, *et al.*, 2007). Therefore, LPS of *A. actinomycetemcomitans* might interact with CD18 of Mac-1 and p150/95 and lead to the release of resistin via degranulation.

Further study is needed to clarify the details.

β2 integrins reportedly must be activated to interact with their ligands (Abram & Lowell, 2009). The possible reason why neutrophil degranulation in the present study was slower than expected might be that the priming agents, such as chemokines and chemoattractants, in the culture medium were insufficient in quantitative and qualitative aspects to rapidly activate resting neutrophils freshly isolated from the blood. Therefore, most of the β2 integrins might have been in inactive form for some time and could not easily interact with their ligands.

*Aggregatibacter actinomycetemcomitans* has been detected in atherosclerotic lesions, suggesting that it may be associated with the development and progression of the condition (Haraszthy*, et al.*, 2000). An effect of resistin on endothelium-related atherosclerotic events was indicated by a reported dose-dependent increase in monocyte adhesion to endothelial cells after resistin exposure, an effect likely to be attributable to the upregulation of two adhesion molecules, monocyte chemotactic protein-1 and platelet/endothelial cell adhesion molecule-1 (Kunnari*, et al.*, 2009). Thus, *A. actinomycetemcomitans* may play a role in the development and progression of atherosclerosis through the release of resistin from neutrophils in or surrounding the atherosclerotic lesion.

The results presented have provided some insight into the relationship between neutrophil-derived resistin and *A. actinomycetemcomitans*. Although the present results do not directly establish a relationship between circulating resistin and periodontitis, the observations suggest that increased prevalence and levels of *A. actinomycetemcomitans*

in periodontal patients contribute to their higher circulating levels of resistin. Clarification of the importance of resistin release induced by periodontal bacteria in the pathogenesis of atherosclerosis, as well as the contribution of resistin release to periodontal inflammation and associated loss of attachment, requires further study.

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**FIGURE LEGENDS**

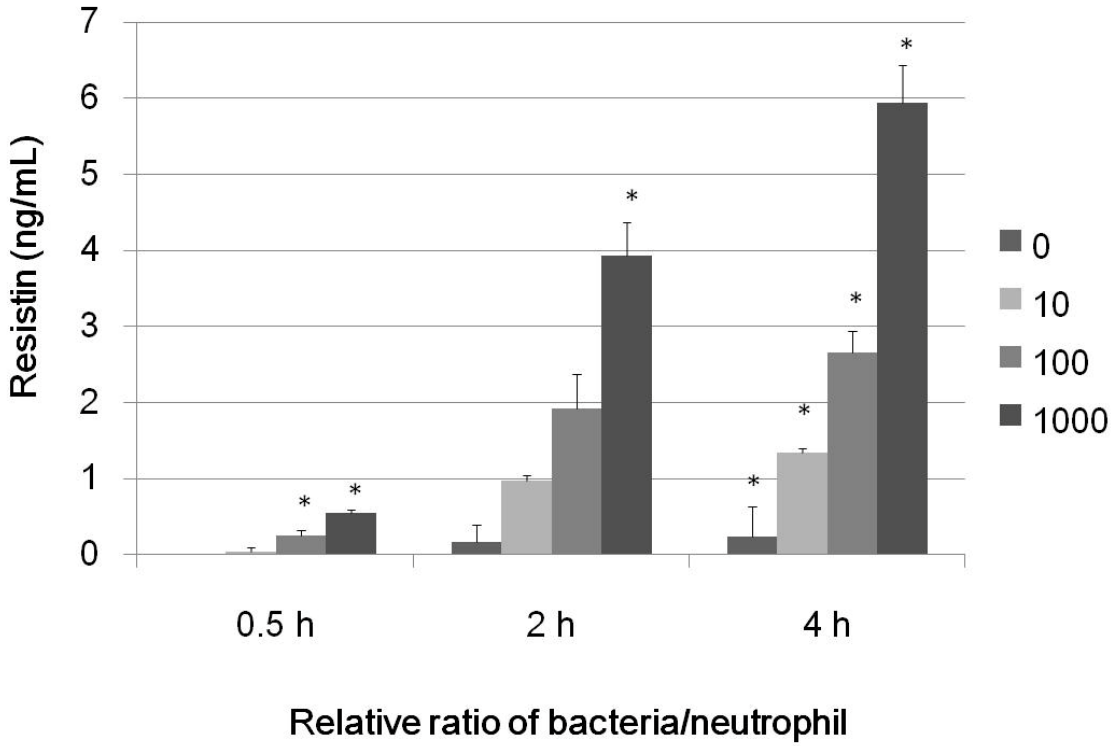


Figure 1. Release of resistin from human neutrophils induced by the *A. actinomycetemcomitans* HK921 wild-type strain at relative ratios of 0, 10, 100, and 1000 bacteria/neutrophil. Neutrophils were incubated with bacteria for 0.5, 2, and 4 h. Scheffe's test was used for multiple comparisons. * $P < 0.05$ compared with all other groups. Data are representative of two independent experiments (n=3). Error bars represent standard deviations.

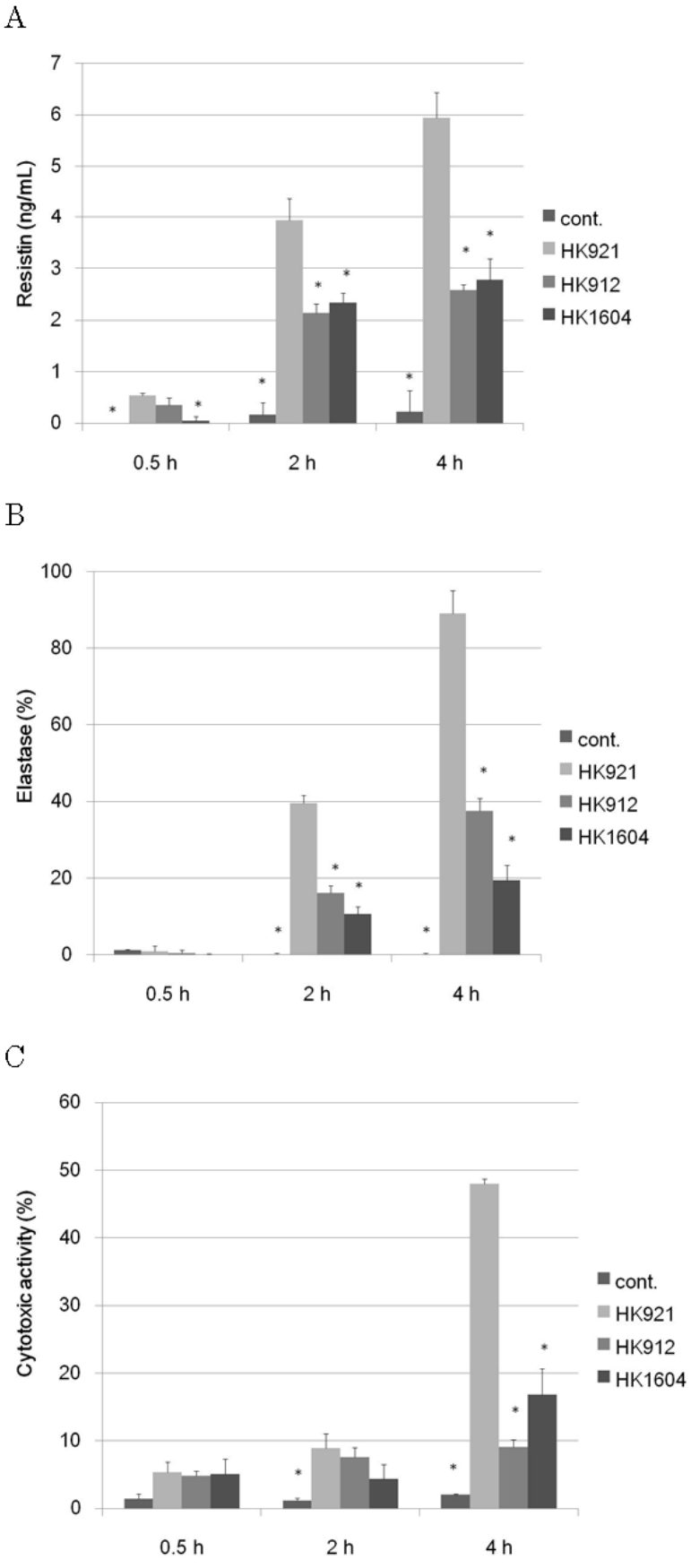


Figure 2. Release of resistin from human neutrophils induced by strains HK921, HK912, and HK1604 at a relative ratio of 1000 bacteria/neutrophil. Neutrophils were incubated with bacteria for 0.5, 2, and 4 h. Control shows results of neutrophils incubated without bacteria as a negative control (cont.). (A) Release of resistin, (B) percentage of elastase release, and (C) percentage of cytolysis. Scheffe's test was used for multiple comparisons. * $P < 0.05$ compared with HK921. Data are representative of two independent experiments (n=3). Error bars represent standard deviations.

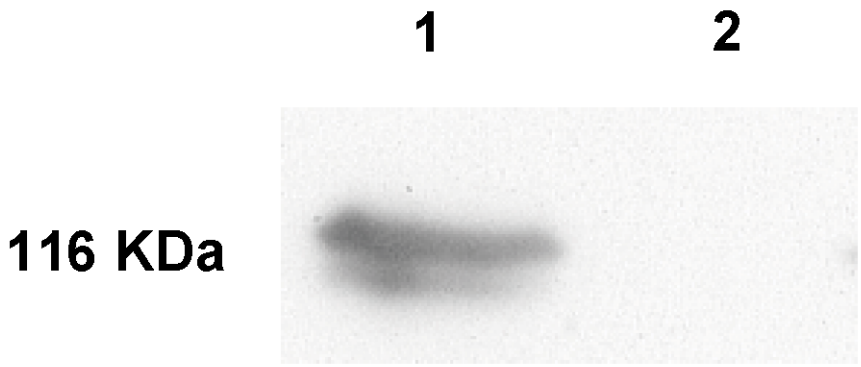


Figure 3. Western blot analysis using anti-leukotoxin antibody to detect leukotoxin production by *A. actinomycetemcomitans* strain HK921. Lane 1, wild-type HK921 (HK921); lane 2, HK921 mutated by insertional inactivation of the *ltxA* gene (MT).

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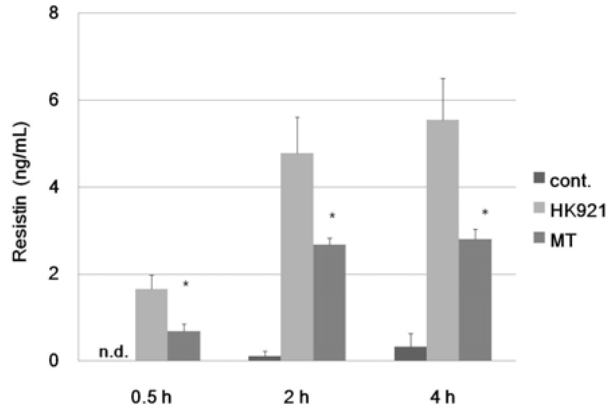


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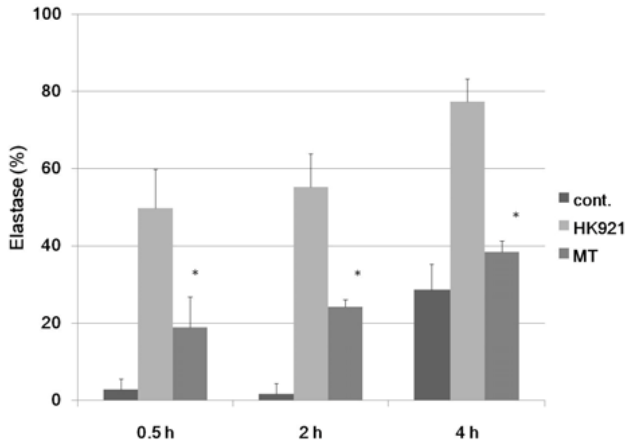


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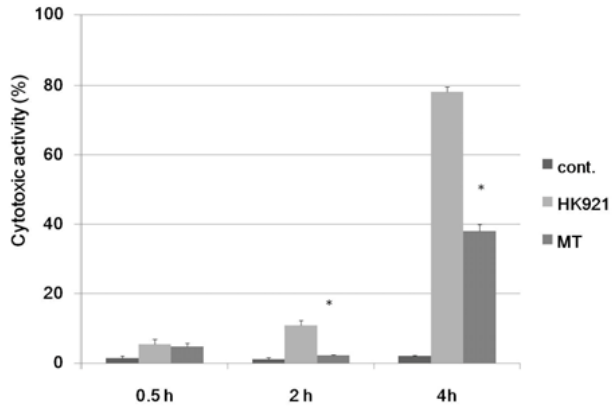


Figure 4. Release of resistin and elastase from human neutrophils and cytolysis induced by the *A. actinomycetemcomitans* HK921 wild-type strain (HK921) and the mutant strain unable to produce leukotoxin (MT). Neutrophils were incubated with bacteria for 0.5, 2, and 4 h. Control shows results of neutrophils incubated without bacteria as a negative control (cont.). (A) Release of resistin, (B) percentage of elastase release, and (C) percentage of cytolysis. Student's *t*-test was used for comparison between HK921 and MT. * $P < 0.05$ compared with HK921. Data are representative of three independent experiments (n=3). Error bars represent standard deviations. n.d., not detected.

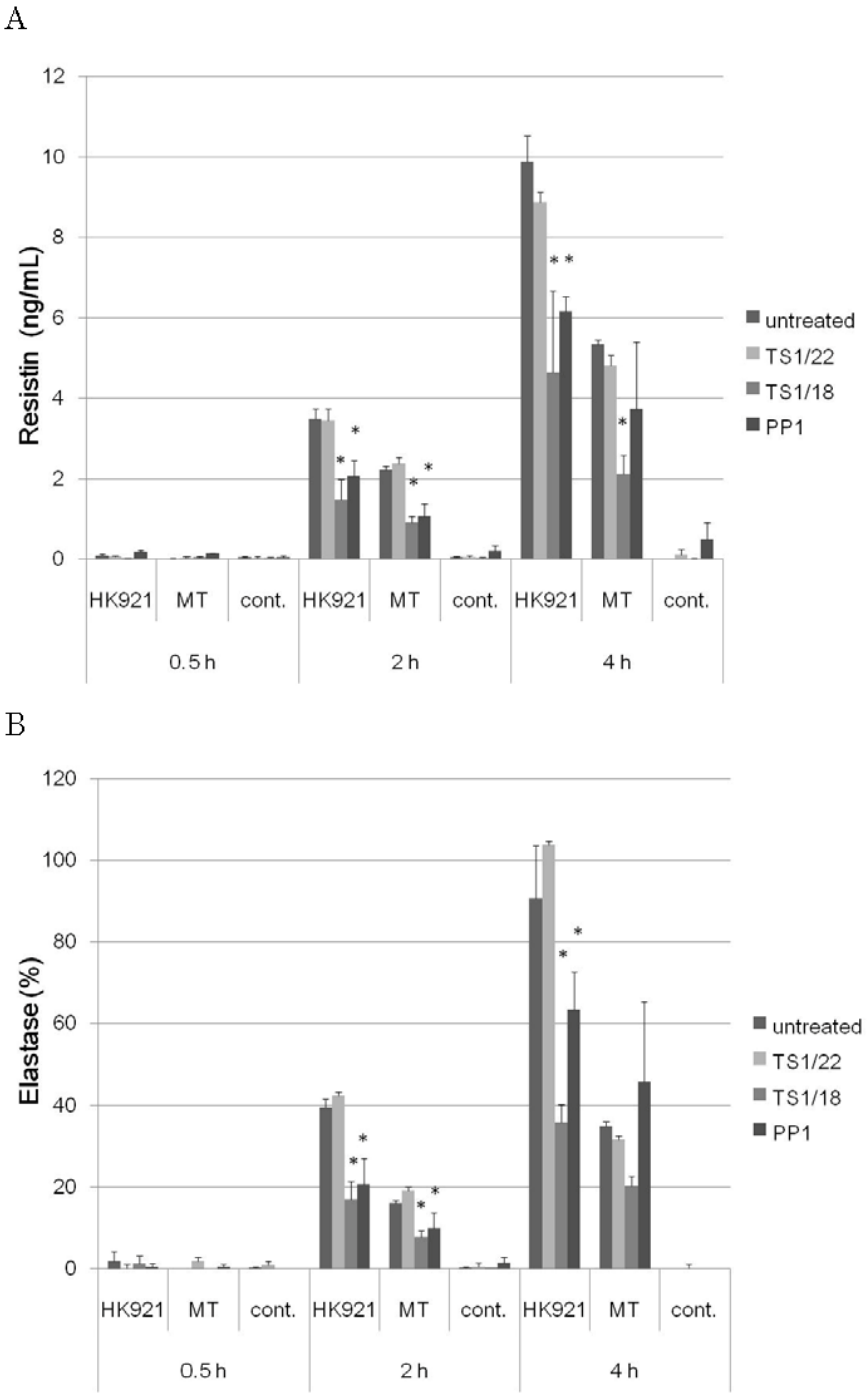


Figure 5. Effect of antibodies to LFA-1 subunits and PP1 on the release of resistin from neutrophils. (A) Release of resistin, (B) percentage of elastase release. Neutrophils were preincubated with monoclonal antibodies (5 μg/mL) or PP1 (10 μM) for 15 min and then incubated with bacteria for 0.5, 2, and 4 h. Control shows results of neutrophils incubated without bacteria as a negative control (cont.). Dunnett's test was used to compare untreated and treated neutrophils. * $P < 0.05$ compared with untreated. Data are representative of two independent experiments (n=3). Error bars represent standard deviations.