α-Enolase of *Streptococcus pneumoniae* Induces Formation of Neutrophil Extracellular Traps

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Yuka Mori\(^1\)\(^2\), Masaya Yamaguchi\(^1\), Yutaka Terao\(^2\), Shigeyuki Hamada\(^3\), Takashi Ooshima\(^4\), and Shigetada Kawabata\(^5\)

From the Departments of \(^1\)Oral and Molecular Microbiology and \(^5\)Pediatric Dentistry, Osaka University Graduate School of Dentistry, Suita, Osaka, 565-0871, Japan, the \(^2\)Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka, 567-0047, Japan, and the \(^3\)Research Collaboration Center on Emerging and Reemerging Infections 6F, Department of Medical Sciences, Ministry of Public Health, Muang Nonthaburi 11000, Thailand

**Background:** *S. pneumoniae* induces formation of neutrophil extracellular traps.

**Results:** Pneumococcal α-enolase binds to neutrophil and induces formation of neutrophil extracellular traps.

**Conclusion:** *S. pneumoniae* α-enolase works as a novel neutrophil extracellular trap induction factor.

**Significance:** This is a first report that bacterial protein induces formation of neutrophil extracellular traps.

*Streptococcus pneumoniae* is the most common causative agent of community-acquired pneumonia throughout the world, with high morbidity and mortality rates. A major feature of pneumococcal pneumonia is abundant neutrophil infiltration. In this study, we identified *S. pneumoniae* α-enolase as a neutrophil binding protein in ligand blot assay and mass spectrometry findings. Scanning electron microscopic and fluorescence microscopic analyses also revealed that *S. pneumoniae* α-enolase induces formation of neutrophil extracellular traps, which have been reported to bind and kill microbes. In addition, cytotoxic assay results showed that α-enolase dose-dependently increased the release of extracellular lactate dehydrogenase from human neutrophils as compared with untreated neutrophils. Furthermore, an *in vitro* cell migration assay using Chemotaxicell culture chambers demonstrated that α-enolase possesses neutrophil migrating activity. Interestingly, bactericidal assay findings showed that α-enolase increased neutrophil extracellular trap-dependent killing of *S. pneumoniae* in human blood. Moreover, pulldown assay and mass spectrometry results identified myoblast antigen 24.1D5 as an α-enolase-binding protein on human neutrophils, whereas flow cytometric analysis revealed that 24.1D5 was expressed on human neutrophils, but not on human monocytes or T cells. Together, our results indicate that α-enolase from *S. pneumoniae* increases neutrophil migrating activity and induces cell death of human neutrophils by releasing neutrophil extracellular traps. Furthermore, we found that myoblast antigen 24.1D5, which expressed on the surface of neutrophils, bound to α-enolase of *S. pneumoniae*.

*S. pneumoniae* is a major cause of community-acquired pneumonia, as well as an important cause of invasive diseases, such as meningitis and sepsis (1, 2). *S. pneumoniae* colonizes asymptptomatically in the throat or nasopharynx and then disseminates into the lungs to cause pneumonia, which can degenerate into meningitis or sepsis (1–3). However, a prerequisite for invasive pneumococccal diseases is the ability of the bacteria to evade the innate immune system.

In response to invading microbial pathogens, neutrophils are recruited to the site of infection from the bloodstream, where they engulf and kill the bacteria by phagocytosis. Recently, it was shown that activated neutrophils release DNA fibers decorated with antimicrobial proteins, which form neutrophil extracellular traps (NETs)\(^3\) that bind, disarm, and kill pathogens extracellularly (4). NETs are relevant in pneumonia (5, 6), sepsis (7), and autoimmune diseases (8, 9). In chronic granulomatous disease patients, who have impaired NADPH oxidase activity and reactive oxygen species production, neutrophils do not generate NETs and possess poor antimicrobial activity (10, 11). NET formation was initially described as a new form of cell death (NETosis), although recent studies have shown that living neutrophils can also release NETs by extruding their mitochondrial DNA (12).

Pneumococcal pneumonia causes abundant neutrophil infiltration (13). The innate immune system in humans provokes acute inflammation at the onset of infection, but at the same time, neutrophil activation massively contributes to inflammatory tissue damage (14). *S. pneumoniae* appears to have evolved strategies to survive such an inflammatory response, because despite neutrophil- and alveolar macrophage-mediated bacterial killing, the bacteria persist in lungs of affected individuals. Beiter *et al.* (5) showed that expression of the surface endonuclease EndA on *S. pneumoniae* might degrade the DNA backbone of NETs, thus promoting bacterial spreading through the airway and into the bloodstream. In addition, Wartha *et al.* (6) showed that *S. pneumoniae* evades

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\(^{\text{1}}\)Both authors contributed equally to this work.

\(^{\text{2}}\)To whom correspondence should be addressed: Dept. of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka Suita, Osaka, 565-0871, Japan. Tel.: 81-6-6879-2898; Fax: 81-6-6879-2180; E-mail: terao@dent.osaka-u.ac.jp.

\(^{\text{3}}\)The abbreviations used are: NET, neutrophil extracellular trap; LDH, lactate dehydrogenase; PMA, phorbol 12-myristate 13-acetate.
NETs by a positive charge on its surface as a result of capsule expression and lipoteichoic acid d-alanylation. However, the mechanisms related to \textit{S. pneumoniae}-induced NET formation remain unclear.

In the present study, we identified a pneumococcal protein, \(\alpha\)-enolase, that interacts with neutrophil cell surface proteins. Furthermore, we found that \(\alpha\)-enolase induces neutrophil chemotaxis, NET formation, and NETosis.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Reagents, and Cells—** \textit{S. pneumoniae} strain D39 (NCTC 7466) was obtained from the National Collection of Type Cultures, whereas strain R6, unencapsulated and derived from D39, was kindly provided by Dr. Shin-ichi Yokota (Sapporo Medical University, Sapporo, Japan). Both \textit{S. pneumoniae} strains were grown in tryptic soy broth (Becton Dickinson). Inactivation of the \(lytA\) genes in \textit{S. pneumoniae} was performed as described previously (15, 16). \textit{Escherichia coli} strain XL-10 Gold (Agilent Technologies) was grown in Luria-Bertani broth (Sigma) or on Luria-Bertani agar plates, supplemented with 100 \(\mu\)g/ml of ampicillin.

Human myeloid THP-1 cells were grown in RPMI 1640 containing 10\% fetal bovine serum, 100 IU/ml of penicillin, 100 \(\mu\)g/ml of streptomycin, and 0.6 mg/ml of glutamine at 37 \(^\circ\)C in 95\% air and 5\% CO\(_2\). THP-1 cells were forced to differentiate by treatment with 0.5 mM dibutyryl cAMP (Sigma-Aldrich) for 3 days (17).

Human neutrophils were prepared as described previously (16, 18). Briefly, 10 ml of heparinized blood was obtained from healthy donors and mixed 1:1 with PBS containing 3\% dextran T500. After incubation at room temperature for 1 h, the supernatant was layered on Ficoll-Paque (GE Healthcare). After centrifugation at 450 \(\times\) g for 20 min, layers containing erythrocytes and neutrophils were collected. Residual erythrocytes were lysed by hypotonic shock, and then the cells were suspended in RPMI 1640. Cell viability was monitored using the trypan blue exclusion technique, and the number was determined using a hemocytometer.

**Preparation of Recombinant \(\alpha\)-Enolase—** The expression vectors were constructed as follows, with the primers listed in Table 1. The \textit{eno} gene was amplified by PCR, and the resultant PCR fragments were cloned into pQE-30 vectors (Qiagen). Recombinant \(\alpha\)-enolase was then purified using a QiAexpress protein purification system (Qiagen), according to the manufacturer’s instructions. The amount of LPS in 1 \(\mu\)g of a recombinant \(\alpha\)-enolase sample was determined to be less than 31 pg using a Limulus ES-II single test (Wako Pure Chemical Industries, Osaka, Japan).

**RNA Extraction and Reverse Transcription—** Total RNA was extracted from human neutrophils using an RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using SuperScript III first strand SuperMix (Invitrogen), according to the manufacturer’s instructions. Recombinant myoblast antigen 24.1D5 was constructed and purified in the same manner as recombinant \(\alpha\)-enolase.

**Ligand Blotting—** Ligand blotting analysis was performed as described previously (15). Briefly, differentiated THP-1 cell surface proteins (2.5 \(\times\) 10\(^6\) cells/ml) and recombinant \(\alpha\)-enolase were biotinylated separately using an ECL protein biotinylation kit (GE Healthcare), with the concentration of \(\alpha\)-enolase adjusted to 10 \(\mu\)g/ml. Surface fraction of \textit{S. pneumoniae} R6 extracted with 8 M urea was separated by electrophoresis on 10\% SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were then blocked with 5\% membrane blocking agent (GE Healthcare) at 4 \(^\circ\)C for 18 h and incubated with biotinylated proteins for 1 h and horseradish peroxidase-labeled streptavidin at room temperature for an additional 1 h. The bands were detected using ECL Western blotting detection reagents (GE Healthcare) and by exposing the membranes to x-ray film (Fuji) at room temperature for 5 s. Tandem mass spectra were obtained using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Proteins were identified by their peptide mass fingerprint and MS/MS ion search analysis with MASCOT software (Matrix Science, Boston, MA) using the NCBI database.

**ELISA—** For determining \(\alpha\)-enolase on the bacterial cell surface and in supernatant, ELISA was performed as described elsewhere (19). Briefly, 96-well ELISA plates (Sumitomo Bake-lite, Tokyo, Japan) were coated with pneumococcal cell surface or supernatant fractions in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4 \(^\circ\)C. A calibration curve was obtained by use of 2-fold serial dilutions of \(\alpha\)-enolase as the standard. The wells were then blocked with PBS containing 1\% Block Ace (DS Pharma Biomedical, Osaka, Japan) overnight at 4 \(^\circ\)C. Rabbit anti-\(\alpha\)-enolase or preimmune serum samples were added to the plates and incubated for 1 h at 37 \(^\circ\)C. The wells were then washed and incubated for 1 h at 37 \(^\circ\)C with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jack-
son ImmunoResearch), then washed again, and developed with TMB solution (Moss Inc., Pasadena, MD). After incubation for 15 min, the enzyme reaction was stopped by adding 0.5 n HCl, and the A450 of the plates was determined using a microplate reader (Titertek MK11; Titertek, Huntsville, AL). The resultant absorbance values were plotted on a graph and fitted to a linear equation for the calibration curve.

Pulldown Assays—For pulldown assays, differentiated THP-1 cell surface proteins were incubated for 30 min with α-enolase and then linked to Ni²⁺-NTA agarose beads. Next, the beads were washed five times in PBS and separated by native PAGE, and analyzed by Western blotting using anti-α-enolase serum or silver stain.

Fluorescence Microscopic Analysis—Immunofluorescent staining was performed as described previously (15, 20). Briefly, streptococcal cells were washed with PBS and blocked with 10% goat serum (Tissue Culture Biologicals, Tulare, CA). To observe the localization of α-enolase, the bacterial cells were stained with SYBR green I, and then surface α-enolase was visualized using rabbit anti-α-enolase serum, followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen). The stained bacteria were analyzed using an LSM 510 confocal laser scanning microscope (Carl Zeiss).

To observe the formation of NETs, neutrophils were stimulated with or without α-enolase or LPS. The cells were stained with Alexa Fluor 594 phalloidin (Invitrogen), and DNA was visualized using SYTOX Green (Invitrogen). The stained cells were analyzed using a fluorescence microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan).

Scanning Electron Microscopic Analysis—α-Enolase (100 nm) was incubated with human neutrophils (1 × 10⁶ cells/ml), and the mixtures were incubated at 37 °C for 30 min. Thereafter, the mixtures were fixed with 2% glutaraldehyde-RPMI 1640 for 1 h at room temperature, washed with distilled water, then dehydrated with 100% t-butyl alcohol, and freeze-dried. Finally, the samples were coated with platinum and examined with an emission scanning electron microscope (JSM-6390LVZ; JEOL Ltd., Tokyo, Japan).

Neutrophil Chemotaxis Assay—Chemotactic migration was evaluated as described previously (20) in 96-well chemotaxis chambers containing polycarbonate filters (pore size, 5 μm; Kurabo). The wells of the bottom plate were filled with 10 nM C5a and 0–66.7 nM α-enolase and then incubated for 2 h at 37 °C. Fluorescein-labeled neutrophils were applied to the wells of the upper chambers, and the chambers and lower plates were combined and incubated together for 1 h at 37 °C in an atmosphere of 5% CO₂ and 95% air. The number of cells migrating from the upper chamber to the bottom plate was determined using a Wallac 1420 ArvoSXFL multi-label counter (PerkinElmer Life Sciences).

Bactericidal Assay—Lancefield bactericidal assays were performed as described previously (15). Strains R6 and D39 were grown, washed, and resuspended in 1 ml of PBS. Diluted cultures (5 μl) were combined with fresh human blood (85 μl) with or without α-enolase (final concentration, 10 nM, 5 μl) and DNase I (5 units, 5 μl) (Promega), and then the mixtures were rotated at 37 °C for 1 h. Viable cell counts were determined by plating diluted samples onto blood agar.

Lactate Dehydrogenase Cytotoxicity Assay—Neutrophils (1 × 10⁷ cells) were stimulated with α-enolase (final concentration, 0–100 nM). Cytotoxicity was determined using CytoTox96 (Promega) according to the manufacturer’s instructions. CytoTox96 quantitatively measures lactate dehydrogenase (LDH) released upon cell lysis. The percentage of specific LDH release was calculated as follows: (experimental − effector spontaneous − target spontaneous)/(target maximum target spontaneous) × 100.

RESULTS

Neutrophil Cell Surface Fractions Bind to α-Enolase—To investigate the interaction between S. pneumoniae and neutrophils, we determined whether pneumococcal proteins interact with differentiated THP-1 cells. Using ligand blot analysis, we found a band of ~47 kDa that intensely reacted with differentiated THP-1 cell surface proteins (Fig. 1). This band was identified as SpR1036, α-enolase, by an MS/MS ion search analysis

α-Enolase Induces Formation of NETs

FIGURE 1. Analysis of binding between neutrophil membrane-associated proteins and streptococcal surface proteins. Surface proteins from S. pneumoniae strain R6 were extracted with 8 M urea, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were stained with Coomassie Brilliant Blue (CBB) or incubated with biotinylated erythrocYTE membrane-associated proteins, followed by streptavidin-horse-radish peroxidase. Interactions were detected with ECL Plus reagents. The band (arrow) demonstrated a reaction with biotinylated THP-1 cell surface proteins, which was identified as an MS/MS ion search.

| TABLE 2  | Neutrophil binding proteins of S. pneumoniae |
|----------|---------------------------------------|
| Peptide sequence | Peptide location | Protein length | Protein description |
| Fig. 1 | GNPTLEVYVEVSQAPGR | 17–34 | 434 | α-Enolase (S. pneumoniae) |
| | GLETAUDEGQFADF | 197–211 | 434 | α-Enolase (S. pneumoniae) |
| Fig. 7A | FGQGGAGPGQQQGFR | 356–370 | 396 | Myoblast antigen 24.1D5 (Homo sapiens) |

FIGURE 1.
FIGURE 2. Localization of α-enolase on bacterial surface. A, urea extracts of *S. pneumoniae* strain R6 (Cell), cell culture supernatant (Sup), and purified recombinant α-enolase were analyzed by Western blotting using antiserum against α-enolase. Rabbit preimmune serum was used as a negative control. CBB, Coomassie Brilliant Blue. B, to confirm the localization of α-enolase on the surface of *S. pneumoniae*, bacterial cells were grown in anti-α-enolase serum and analyzed by immunofluorescence microscopy. The cells were washed with phosphate-buffered saline; then blocked with 1% Block Ace solution, 10% goat serum, and 5% BSA; and incubated with SYBR-GREEN I (green images) and rabbit anti-α-enolase serum (panels a and b) or preimmune serum as a negative control (panels c and d). Immunoreactive proteins were visualized with Alexa Fluor 594-conjugated goat anti-rabbit IgG (red images).
**α-Enolase Induces Formation of NETs**

![Graph 1](image1.png)  
**FIGURE 3. Comparison of α-enolase in culture supernatants of S. pneumoniae wild-type and ΔlytA strains.** The amount of α-enolase in the culture supernatant was determined using ELISA, with that of the wild-type strain considered to be 100%. *, significant difference (p < 0.005) between the mean values, as determined with a Mann-Whitney U test. The S.E. values are represented by error bars (n = 6).

![Graph 2](image2.png)  
**FIGURE 4. Effects of α-enolase on neutrophil chemotaxis.** Human neutrophils were isolated from whole blood samples, and their chemotactic responses to α-enolase and C5a (10 nM; positive control) were examined. Chemotaxis is shown as fluorescence intensity. *, significant difference (p < 0.005) between the mean values, as determined with a Mann-Whitney U test. Three experiments were performed, with the data presented as the means of six wells from a representative experiment. The S.E. values are represented by error bars (n = 6).

with MASCOT software (Table 2). α-Enolase is known as a glycolytic enzyme (EC 4.2.1.11.). However, it lacks classical protein-sorting elements, such as a signal sequence or domains known to anchor proteins to the bacterial cell wall.

**Location of α-Enolase on S. pneumoniae—**Originally, α-enolase was found in 8 M urea extract samples that contained bacterial surface proteins, although it has also been found in the cytoplasm and is known to be a glycolytic enzyme that catalyzes the conversion of phosphoglycerate to phosphoenolpyruvate (21). In addition, α-enolase has been demonstrated to be present on the surface of most streptococcal species (22–25). In the present study, we investigated whether α-enolase is surface-associated, released into the culture supernatant as a soluble protein, or localized intracellularly. Western blotting with anti-α-enolase antiserum showed that α-enolase is expressed on the bacterial surface and released from cells into supernatant (Fig. 2A). In addition, we analyzed *S. pneumoniae* cells by immunofluorescent microscopy using anti-α-enolase antiserum, followed by an Alexa Fluor 594-conjugated secondary antibody. We found that α-enolase was localized on the entire cell surface (red in Fig. 2B). Together, these results showed that α-enolase is expressed on the surface of *S. pneumoniae* and released into culture supernatant.

Next, we examined whether the release of α-enolase is dependent on autolysis. ELISA assay showed that the amount of α-enolase released from the ΔlytA mutant strain into supernatant was ~76% of that released from the wild-type strain after an overnight culture (Fig. 3). This result suggests that α-enolase may be primarily released in an autolysin-independent manner.

**α-Enolase Induces Neutrophil Chemotaxis and NET Formation—**We also investigated the effects of various concentrations (0–66.7 nM) of α-enolase on neutrophil chemotaxis. As shown in Fig. 4, α-enolase induced neutrophil chemotaxis in a dose-dependent manner. α-Enolase stimulation resulted in lower activity than stimulation with C5a, used as a positive control, although it was significantly higher than that with no stimulation. These findings indicate that α-enolase has chemotactic activity.

Next, we investigated whether α-enolase changes neutrophil morphology. Scanning electron microscope analysis revealed that α-enolase-stimulated neutrophils ruptured and formed extracellular fibers (Fig. 5A) and that stimulation increased the number of ruptured neutrophils by 5-fold (Fig. 5B). We further studied whether NET induction depends on reactive oxidative species. By adding reactive oxidative species inhibitor Euk-8, there was no significant difference for NET formation (Fig. 5B). In addition, we confirmed that α-enolase induced the release of cytoplasmic LDH from neutrophil in a dose-dependent manner (Fig. 5C). Furthermore, fluorescence microscopic analysis revealed the extracellular fibers to be DNA from neutrophils (Fig. 5D). These results indicated that α-enolase works on neutrophils to induce NET formation.

A limulus test indicated that a purified 1 μM α-enolase solution contained less than 1.5 pg/ml of LPS. In a prior study, we found that 25 pg/ml of LPS did not induce chemotactic activity, and oxidative burst was required for LPS-induced NET formation (20), indicating that the observed effects of α-enolase were not due to LPS contamination.

**α-Enolase Enhances NET-dependent Bactericidal Activity—**To determine the effects of α-enolase on bacterial survivability in human blood, we performed bactericidal assays using low (~5 × 10^2 CFU) and high (~8 × 10^3 CFU) doses of *S. pneumoniae* strains R6 and D39. Interestingly, the bactericidal assay findings showed that α-enolase significantly increased the bactericidal activities of the low and high doses of strain R6 and the low dose of strain D39 in human blood, whereas DNase inhibi-
ited those activities. In contrast, there were no significant differences between α-enolase-treated and untreated strain D39 at the high dose (Fig. 6). These results indicate that α-enolase induces NET-dependent bacterial killing in human blood and that a high dose of capsule-positive strain D39 is more resistant to α-enolase-induced NET-dependent killing as compared with the capsule-negative strain R6.

**Detection and Identification of α-Enolase Binding Protein on Neutrophils**—Using a pulldown assay and MS/MS ion search, we investigated whether neutrophil cell surface proteins bind to α-enolase. Our results showed that myoblast antigen 24.1D5 is an α-enolase-binding protein (Fig. 7A and Table 2), and ligand blot findings revealed that recombinant myoblast antigen 24.1D5 binds specifically to biotinylated α-enolase (Fig. 7B).
Furthermore, cytometric analysis demonstrated that myoblast antigen 24.1D5 is localized on the cell surface of live neutrophils, whereas it was not expressed on the cell surface of monocytes or T cells (Fig. 7C).

DISCUSSION

*S. pneumoniae* α-enolase binds the abundant human plasmin zymogen plasminogen, thus enhancing proteolytic plasmin activity, which is important in the pathogenesis of pneumococcal infections (26, 27). Furthermore, α-enolase of *S. pneumoniae* enhances plasmin-mediated degradation of the reconstructed basement membrane (26). The presence of α-enolase as the sole enolase molecule for pneumococci, the strong conservation of its gene, its equal gene expression in all strains, and the fact that disruption of the *eno* gene appears to be lethal indicate that α-enolase is an essential glycolytic enzyme (22).

In the present study, we showed that α-enolase induces formation of NETs. It is conceivable that NET formation occurs through various pathways. For example, with PMA, a direct neutrophil activator, the NET formation process takes 2–3 h. Hakkim et al. (28) show that the Raf-MEK-ERK pathway is involved in PMA-induced NET formation via activation of NADPH oxidase and up-regulation of antiapoptotic proteins. In contrast, another study described that in the presence of bacterial LPS and platelets, neutrophils can generate NETs within minutes (7). This process requires that the cells be exposed to flow conditions and has been implicated in vascular obstruction during sepsis. In the present study, we found a mechanism of indirect neutrophil stimulation that is mediated by regulatory effector cells such as platelets. NET formation is dependent on hydrogen peroxide generated by NADPH oxidase and further metabolized by myeloperoxidase. Thus, chronic granulomatous disease neutrophils and myeloperoxidase-deficient neutrophils do not form NETs (11, 29).

Nitric oxide was recently demonstrated to induce the formation of NETs in a process dependent on myeloperoxidase, although the active species were not identified (30). In addition, it was reported that CXCR2-mediated NET formation was independent of NADPH oxidase and involved Src family kinases (31). Herein, we showed that α-enolase induced the formation of NETs within 30 min in the absence of regulatory effector cells. Furthermore, α-enolase induced LDH produc-
FIGURE 7. Myoblast antigen 24.1D5 functions as α-enolase-binding protein. A, detection of α-enolase-binding protein by pulldown assay. THP-1 cell surface proteins were reacted with α-enolase on nickel-Sepharose. Proteins bound to α-enolase were separated by native PAGE, and then the gels were stained with a silver stain kit or transferred to polyvinylidene difluoride membranes. The membranes were incubated with anti-α-enolase serum, followed by goat anti-rabbit IgG. Interactions were detected using BCIP-NBT. The band (arrow) demonstrated an interaction with α-enolase, which was identified by an MS/MS ion search.

B, analysis of binding between myoblast antigen 24.1D5 and α-enolase. Myoblast antigen 24.1D5 was separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was then stained with Coomassie Brilliant Blue (CBB) or incubated with biotinylated α-enolase or BSA (negative control), followed by streptavidin-HRP. The interacted molecules were detected using ECL Plus reagents.

C, the expression of myoblast antigen 24.1D5 was analyzed using a fluorescence-activated cell sorter system. The cells were prepared at 10^6 cells/ml and incubated with anti-myoblast antigen 24.1D5, the antibody as a positive control, and rabbit preimmune serum. The obtained data are presented as histograms.
tion by neutrophils, although it has been reported that PMA-, IL-8-, or LPS-induced NETosis did not promote the release of LDH (4). Together, these results suggest that α-enolase induces NET formation by a pathway not related to PMA, IL-8, and LPS. We identified myoblast antigen 24.1D5 on neutrophils as an α-enolase-binding protein (Fig. 7). We further examined the importance of the α-enolase interaction with the myoblast antigen 24.1D5 by using the siRNA method. However, there were not significant differences for NET formation and bacterial killing (data not shown). Recently, Marichal et al. (32) suggested that NET formation depends on Toll-like receptors. The myoblast antigen 24.1D5 might work only as an α-enolase-binding molecule and might not function in signal transduction.

Unexpectedly, α-enolase increased bacterial activity in human blood, which raises the possibility that neutrophils recognize the protein in an innate immunity response. α-Enolase is widely conserved among bacteria and eukaryotes and is expressed on their surfaces (22–25, 33). In bacterial pathogens, α-enolase is localized on the bacterial cell surface and released into supernatant, although in normal eukaryotic cells, α-enolase is mainly localized in cytoplasm and on the cell surface (21, 33). It is possible that neutrophils do not come into contact with a high concentration of human α-enolase in normal conditions, but another explanation is that pneumococcal α-enolase induces formation of NETs for host tissue destruction. It was recently reported that NETs recruited red blood cells, promoted fibrin deposition, and induced red thrombus such as that found in veins (34). In addition, granules within the neutrophil cytoplasm contain potent proteolytic enzymes and cationic proteins that can digest a variety of microbial substrates. Under pathological circumstances, unregulated release of microbical compounds into extracellular space can paradoxically damage host tissues (35). The present results showed that pneumococcal α-enolase induced NET-dependent bacterial killing in human blood. However, it is suggested that α-enolase may induce host tissue destruction or disseminated intravascular coagulation by NET formation and NETosis in vivo. Additional studies are needed to further elucidate the role of α-enolase in innate immunity.

In summary, we identified α-enolase as a novel NET induction factor. However, the mechanism for formation of NETs is not completely known, and further study of regulation of NET formation will bring greater understanding of this fascinating process of bacterial killing and host tissue destruction.

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