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Human Polymorphonuclear Neutrophil Apoptosis is Inhibited by Treponema Pallidum Through Both Intrinsic and Extrinsic Mechanisms

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Abstract

Treponema pallidum is a "stealth pathogen" responsible for infectious sexually transmitted diseases. Although neutrophils are usually present in skin lesions of early syphilis the role of these cells in *T. pallidum* infection has barely been investigated. Neutrophils are short-lived cells that undergo constitutive apoptosis, and phagocytosis usually accelerates this process. Here, we demonstrated that human polymorphonuclear neutrophils (hPMNs) could phagocytose *T. pallidum in vitro*. An unexpected discovery was that *T. pallidum* inhibited hPMNs apoptosis markedly in an opsonin-independent manner. Furthermore, this phenomenon was not affected by bacterial viability, as detected by annexin V, morphology studies, and TUNEL staining. Exploration of the underlying mechanism showed that expression of the cleaved forms of caspase-3, −8, and −9 and effector caspase activity were diminished significantly in *T. pallidum*-infected hPMNs [1-15]. *T. pallidum* also impaired staurosporine- and anti-Fas-induced signaling for neutrophil apoptosis. Of note, these effects were accompanied by inducing the autocrine production of the anti-apoptotic cytokine IL-8. Taken together, our data revealed that *T. pallidum* could inhibit the apoptosis of hPMNs through intrinsic and extrinsic pathways and provide new insights for understand.

Introduction

Treponema pallidum is the organism that causes syphilis. T. pallidum is usually spread through sexual contact. Syphilis also raises the chance of infection and transfer of acquired immune deficiency syndrome. Because T. pallidum can resist host immune systems and spread from the initial site of infection to other organs and tissues, the global incidence of syphilis has risen in recent years. As a result, it's also known as a "stealth pathogen." Pallidum's ability to circumvent the immune response and cause tissue damage is unknown[1]. Understanding the pathophysiology and immunological mechanism of T. pallidum has become a crucial component in the fight against syphilis.

The innate immune system's job is to defend the host against invading infections. The strong reaction of phagocytes at infection sites is required for a good antibacterial response. Innate immune effector cells are polymorphonuclear neutrophils from humans[2].They are the initial line of defence against primary pathogenic microorganism infection. Neutrophils have a short lifespan and spontaneously apoptose after 24 hours of entering the circulation. Ingestion of microbes and consequent generation of reactive oxygen species can hasten neutrophil death in syphilis.

Subjective heading

Syphilis is an infection caused by Treponema pallidum. Usually, T. pallidum is transmitted through sexual intercourse. In addition, syphilis greatly increases the risk of infection and transmission of acquired immune deficiency syndrome In recent years, the global incidence of syphilis has increased because of the ability of T. pallidum to evade host immune defenses and spread from the initial site of infection to other organs and tissues. Hence, it is also termed a "stealth pathogen How T. pallidum overcomes the immune response and damages tissue is incompletely understood. Explaining the pathogenesis and immune mechanism of action of T. pallidum has become a key link to controlling syphilis.

Discussion

It is the task of the innate immune system to protect the host from invading pathogens. A successful antimicrobial response is dependent upon the aggressive response of phagocytes at infection sites. Human polymorphonuclear neutrophils (hPMNs) are innate immune effector cells. They are the first line of defense against infection by primary pathogenic microorganisms. Neutrophils have a relatively short lifespan and undergo rapid spontaneous apoptosis within 24 h in the circulation). Microbe ingestion and subsequent production of reactive oxygen specis can significantly accelerate neutrophil apoptosis. In addition, intact apoptotic neutrophils can be removed safely by macrophages. However, these short-lifespan neutrophils are altered by host infection by some pathogens: this is regarded as a key factor in exacerbation of inflammation and establishment of local persistent infection.

In the inflammatory response caused by T. pallidum, macrophages and lymphocytes are the predominant effector cells recruited to infection sites The role of neutrophils in T. pallidum infection has received little attention. Several studies have reported that neutrophils are present in patients with primary syphilis In rabbit models, infected neutrophils are seen in the intradermal site of infection in the first 24 h, and neutrophils can "swallow" these organisms within a few hours However, those studies failed to explain why the host cannot eradicate the infected microbes. We wondered whether T. pallidum also affected the lifespan of hPMNs, thereby suggesting that neutrophil apoptosis may be related to syphilis pathogenesis. A recent report describes the

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long-term in vitro survival of T. pallidum. The success was based on the beneficial effect of low oxygen levels and reducing agents in the medium Thus, we tested this hypothesis using a low-oxygen environment suitable for T. pallidum survival, and to have a deeper understanding of cell-pathogen interactions. We showed that this pathogen did not accelerate hPMNs death, it greatly prolonged cell longevity by affecting the intrinsic and extrinsic apoptotic pathways.Procedures involving animals were approved by the Animal Welfare Committee of the University of South China (Hengyang, China). Human participants provided written informed conset for their samples to be used. The study protocol was approved by the Human Ethics Committee of the University of South China and was in accordance with the Helsinki Declaration 1964 and its later declarations.

Syphilis is an infection caused by Treponema pallidum. Usually, *T.* pallidum is transmitted through sexual intercourse. In addition, syphilis greatly increases the risk of infection and transmission of acquired immune deficiency syndrome . In recent years, the global incidence of syphilis has increased because of the ability of *T.* pallidum to evade host immune defenses and spread from the initial site of infection to other organs and tissues. Hence, it is also termed a "stealth pathogen. How *T.* pallidum overcomes the immune response and damages tissue is incompletely understood. Explaining the pathogenesis and immune mechanism of action of *T.* pallidum has become a key link to controlling syphilis.

Neutrophils were challenged with bacteria for the indicated time points and stained using a TUNEL apoptosis assay kit (Beyotime Institute of Biotechnology). Samples were labeled according to the manufacturer's instructions. Briefly, hPMNs were fixed with 4% paraformaldehyde for 30 min. Then, cells were permeabilized with 0.2% TritonX-100 in PBS for 5 min and stained with a mixture containing fluorescein dUTP for 1 h at 37 °C. After incubation, redundant labeling solution was washed with PBS and Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole was added to label nuclei (Beyotime Institute of Biotechnology). At least 200 cells were imaged under a fluorescence microscope (Nikon, Tokyo, Japan). Cells were counted by ImageJ (US National Institutes of Health, Bethesda, MD, USA).

Neutrophils were left untreated, challenged with T. pallidum, or treated with staurosporine (STS) or anti-Fas IgM in HEPES-buffered RPMI 1640 medium. For analysis of the activity of caspase-3, caspase-8 and caspase-9, we used caspase-Glo 3/7, 8, and 9 luminescent assay kits (Promega, Madison, WI, USA), respectively, according to manufacturer's instructions. Briefly, hPMNs (5 × 104) in 100 µL were transferred to a white-walled 96-well microplate (Thermo Fisher Scientific) in triplicate. Equal volumes of Caspase-Glo Reagent, containing a respective tetrapeptide, were added to each well. Well contents were mixed gently using a plate shaker at 500 rpm for 30 s, and incubation for 2 h at room temperature was allowed. The luminescence of samples was measured using a microplate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA. Neutrophils were infected with opsonized or unopsonized T. pallidum at an MOI of 10. At the indicated time points, cells were fixed with 4% paraformaldehyde and then permeabilized in 0.3% Triton X-100. After blockading with 5% bovine serum albumin in PBS for 30 min, neutrophils were incubated with rabbit anti-T. pallidum antiserum (1:3000 dilution; Abcam, Cambridge, UK) for 1 h at room temperature, washed thrice with PBST (PBS with 0.05% Tween 20) and incubated with Cy2-conjugated goat anti-rabbit antibody (1:300 dilution; Abcam) for 30 min at room temperature in the dark. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology, Shanghai, China), and the hPMNs cytoskeleton was stained with Phalloidin-iFluor 555 Reagent (1:1000 dilution; Abcam). At least 100 cells were counted to increase the accuracy of the percent infection. All counts were undertaken in a blinded fashion.

Data from studies containing multiple experimental groups were assessed using one-way ANOVA (single time point) or two-way ANOVA (time-course) with Tukey's or Sidak's multiple-comparisons post hoc tests, as indicated in figure legends. $P \leq 0.05$ was considered significant. Statistical analysis was done using Prism 8.0 (GraphPad, San Diego.

Human neutrophils were isolated from the whole blood of healthy adult volunteers. hPMNs were purified by density centrifugation at 500 × g for 30 min at room temperature using Polymorphprep™ (Alere Technologies, Jena, Germany) with EDTA-treated blood, as described previously We employed a Neubauer-improved counting chamber to determine the number of cells diluted to 1 × 107 cells/mL or 2 × 107 cells/mL. hPMNs purity was ≥ 95% as assessed by Giemsa stainigfollowed by microscopy. hPMNs viability was 98% as assessed by the membrane-exclusion method using trypan dye.

Bacterial strains

T. pallidum (Nichols strain) was propagated within rabbit testes, as reported previously (Briefly, rabbits were injected with approximately ~5 × 107 T. pallidum per testis. Rabbits were checked daily for the early signs of orchits About 10–14 days after infection, rabbits were euthanized at peak orchitis to remove the testes. The latter were minced and removed in 10 mL of physiologic (0.9%) NaCl with 10% heat-inactivated normal rabbit serum, and then agitated gently for ~30 min at room temperature. The liquid extract was washed and centrifuged thrice (5 min at 500 × g each time) to remove testicular debris and host cells. The spirochets in the supernatant were determined by dark-field microscopy. For the heat-killed group, the treponeme suspension was prepared by heating at 56 °C for 1 h and stored at −20 °C before use The bacterial viability was determined by using LIVE/DEAD BacLight Viability Kit (Thermo Fisher Scientific) following the manufacturer's instructions . The rabbit testis extract (RTE) was prepared by collecting the precipitate containing host cells after the first centrifugation. Microscopic observation showed that the RTE suspension was composed mainly of dead-cell debris and sperm cells. Strict asepti technie was required throughout the operation to prevent contamination by other types of bacteria.

Infection of neutrophils

Neutrophils were diluted to 1 × 106/mL in RPMI 1640 medium containing 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 10 mM (Thermo Fisher Scientific), and 10% heat-inactivated fetal bovin serum (MilliporeSigma, Burlington, MA, USA). Before infection, zymosan, live, or heat-killed bacteria had to be opsonized in 50% autologous normal human serum (NHS) in RPMI 1640 medium for 30 min at 34 °C in an atmosphere of 1.5% O2. Then, opsonized zymosan (OpZ) at multiplicity of infection (MOI) 10:1 or T. pallidum organisms were added to hPMNs at MOIs of 0, 1, 5, 10, or 20 and cocultured for 24 h. In addition, T. pallidum (MOI = 10) was cultured with hPMNs for 0, 6, 12, 24, 36, or 48 h until future analyses. All incubations were carried out at 34 ℃ in the air with 1.5% O_2, 5% CO_2, and 93.5% N2 in a modified anaerobic jar, as described previo

Human neutrophils were extracted from healthy adult volunteers' entire blood. Using Polymorphprep (Alere Technologies, Jena, Germany) and EDTA-treated blood, they were purified by density

centrifugation at 500 g for 30 minutes at room temperature. To count the amount of cells diluted to 1 107 cells/mL or 2 107 cells/mL, we used a Neubauer-improved counting chamber. The purity of HPMN was determined by Giemsa staining followed by microscopy. The membrane-exclusion approach utilising trypan blue dye revealed that hPMN viability was 98 percent.

As previously reported, T. pallidum (Nichols strain) was propagated in rabbit testes. Injections of roughly 5 107 T. pallidum per testis were given to rabbits[3-5]. The rabbits were checked every day for indications of orchitis. Rabbits were euthanized at peak orchitis 10–14 days following infection to remove the testes. The latter were chopped and removed in 10 mL of physiologic NaCl with 10% heat-inactivated normal rabbit serum, then gently agitated at room temperature for 30 minutes.

Infection of neutrophils

In RPMI 1640 medium containing 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 10 mM HEPES (Thermo Fisher Scientific), and heat-inactivated foetal bovine serum, neutrophils were diluted to 1 106/mL. (MilliporeSigma, Burlington, MA, USA). Zymosan, live, or heat-killed bacteria had to be opsonized in 50 percent autologous normal human serum (NHS) in RPMI 1640 medium for 30 minutes at 34 °C in a 1.5 percent O2 environment prior to infection. Then, hPMNs were cocultured for 24 hours with opsonized zymosan (OpZ) at a multiplicity of infection (MOI) of 10:1 or T. pallidum organisms at MOIs of 0, 1, 5, 10, or 20. T. pallidum was also cultivated with hPMNs for 0, 6, 12, 24, 36, or 48 hours before further analysis.

For 15 minutes, neutrophils were fixed in 4 percent formalin. They were then dyed in Giemsa staining solution (Solarbio, Beijing, China) for 10 minutes before being rinsed in PBS. Under a light microscope, hPMNs staining was seen.

In HEPES-buffered RPMI 1640 media, neutrophils were left untreated, challenged with T. pallidum, or treated with staurosporine (STS) or anti-Fas IgM. We used caspase-Glo 3/7, 8, and 9 luminous assay kits (Promega, Madison, WI, USA) to determine the activity of caspase-3, caspase-8, and caspase-9, respectively, according to the manufacturer's recommendations. In a nutshell, hPMNs (5 104) in 100 mL were transferred in triplicate to a white-walled 96-well microplate (Thermo Fisher Scientific). To each well, equal amounts of Caspase-Glo Reagent containing a specific tetrapeptide were applied. The contents of the wells were gently shaken for 30 seconds with a plate shaker at 500 rpm, and then incubation for 2 hours at room temperature was allowed.

Conclusion

Despite the fact that neutrophils are assumed to be the host's main responding cells in the early stages of syphilis infection, there has been little research on them. In our research, we discovered that opsonization increased the efficiency of phagocytosis. According to our findings, T. pallidum decreased hPMNs' spontaneous apoptosis as well as apoptosis driven by intrinsic and extrinsic stimulation. The antiapoptotic effect was likewise found to be caused by T. pallidum-induced IL-8 generation by hPMNs.

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Conflict of Interest

The authors declare that they are no conflict of interest

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