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SHORT COMMUNICATION

Interleukin-1β Response of Peritoneal Macrophages to *Streptococcus pyogenes* Exposure: Differential Response to Living and Heat-killed Bacteria

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Background: A human CD46-transgenic (hCD46-Tg) mouse model of subcutaneous infection into hind footpads has been established with group A streptococcus (GAS) isolates, and hCD46 has been found to enhance progression of necrotizing fasciitis in the feet.
Methods: To clarify immune responses and host cell death in streptococcal toxic shock syndrome (STSS), we determined the responses [release of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6] of peritoneal macrophages extracted from hCD46-Tg mice at 24 hours after exposure to GAS472, a STSS-derived strain. Variations of hCD46 expression in cells from the popliteal lymph nodes were examined 24–72 hours after GAS472 infection.
Results: Macrophages exposed to GAS472, but not heat-killed bacteria, exhibited releases of IL-1β, TNF-α, and IL-6. Percentage of hCD46-expressing cells was decreased 72 hours after infection.
Conclusion: These preliminary findings suggest that the macrophages exposed to GAS472 may induce proinflammatory cytokine expression including of IL-1β, TNF-α, and IL-6 and consequent cell death after infection in mice.

1. Introduction

Streptococcus pyogenes (group A streptococcus, GAS) is a human pathogen responsible for many clinical conditions, including skin and soft tissues infections, infections of the upper respiratory tract, and bacteremia.[1] Streptococcal toxic shock syndrome (STSS) is a severe type of streptococcal infections and is characterized by intense inflammatory responses.[2] The severity and outcome of GAS infections are likely to depend on the ability of host innate immune mechanisms to control bacterial growth and to limit the rapid spread of GAS beyond the infection site.

The human CD46 (hCD46) protein on host cell membranes serves as a receptor for attachment of GAS to the cells. We developed an hCD46-transgenic (hCD46-Tg) mouse model of subcutaneous (s.c.) infection into both hind footpads with 11 clinically isolated GAS serotype M1 strains.[3] The severity levels (gross appearances and pathological examinations) of foot lesions were evaluated at 72 hours and the mortality rates at 336 hours after s.c. infection with 1×10^7 CFU of each GAS strain, and GAS472, the strain isolated from the blood of a patient who died from STSS, induced the highest severity levels and mortality rates. GAS472 led to severe necrotizing fasciitis (NF) of the feet and a 100% mortality rate in hCD46-Tg mice at 168 hours postinfection, whereas GAS472 led to partial necrotizing cutaneous lesions and a 10% mortality rate in non-Tg mice. In addition, the bacteria proliferated, reaching a 90-fold or 7-fold increase in the livers of hCD46-Tg or non-Tg mice, respectively, in the 24-hour period between 48 hours and 72 hours after GAS472 infection. The hCD46 was found to enhance the progression of NF in the feet and the exponential growth of bacteria in deep tissues. However, the host immune responses and levels of cell death still remain unclear in this mouse model of STSS.

In the present study, to elucidate cytokine responses and levels of cell death under STSS pathology, we attempted to determine the levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-10 release from peritoneal macrophages extracted from hCD46-Tg mice at 24 hours after exposure to GAS472. Variations of hCD46 expression in cells from the popliteal lymph nodes (PLN) were also examined at 24–72 hours after GAS472 infection.

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2. Methods

The strain GAS472 was preserved in 10% (w/v) skim milk and stored at −85°C until use. The frozen strain was streaked onto sheep blood agar plates (Nippon Becton Dickinson, Tokyo, Japan) and cultured overnight at 37°C in a humidified 5% CO_2 incubator.[3] GAS472 was grown on Todd−Hewitt broth containing 0.2% (w/v) yeast extract (THY; Difco and BBL, Detroit, MI, USA) in 5% CO_2 at 37 °C without shaking, before being used in infection experiments.[3]

HCD46-Tg mice were provided by J.P. Atkinson (Washington University, St Louis, MO, USA). The expression levels of hCD46 protein were comparable to those in corresponding human tissues (skin, fat, and muscle).[4] This protein was observed on epithelial cells, endothelial cells, B cells, T cells, neutrophils, and macrophages, using immunohistochemistry.[4] C57BL/6 mice were obtained from Charles River Japan (Yokohama, Japan) as non-Tg control mice.[3] Mice aged 7–10 weeks were s.c. infected with GAS into the hind footpads as previously described.[3] All mice experiments were performed according to institutional guidelines under an approved protocol.

Isolation of peritoneal macrophages was performed as previously described.[5] We introduced 5 mL of sterile and warm phosphate buffered saline (PBS) into the peritoneal cavity of hCD46-Tg ($n = 3$) or non-Tg ($n = 3$) mice. The cavity was palpated and the fluid was withdrawn into a syringe, dispensed into a conical tube, and stored on ice. Peritoneal lavaged cells from the mice were pooled together. The cells were spun down for 4 minutes at 1,500 rpm and 4°C, then resuspended in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. After the live cell number was determined, 2×10^5 peritoneal macrophages were seeded into 24-well tissue culture plates. Unattached cells were aspirated and discarded, and the attached macrophages were washed with PBS, replenished with complete medium, and placed overnight in 5% CO_2 at 37 °C. Heat-killed GAS472 bacteria grown on THY broth were produced through heat-inactivation (60°C for 2 hours), centrifugation (3,000 rpm and 25°C for 20 minutes), and resuspension in 1 mL PBS after washing twice with PBS. We confirmed that no colonies formed on the THY agar plate when the heat-killed bacteria were used. We inoculated GAS472 at multiplicities of infection of 1, 0.1, and 0.01 or heat-killed bacteria at a multiplicity of infection of 10 into each well ($n = 3$), and cultured the plates in 5% CO_2 at 37°C. The supernatants were harvested 24 hours after starting the culture, and the concentrations of IL-1β, TNF-α, IL-6, and IL-10 in the supernatants were measured based on enzyme-linked immunosorbent assay using a quantikine enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems Inc., Tokyo, Japan) in accordance with the manufacturer's instructions.

The PLN cells were extracted from hCD46-Tg mice ($n = 2$) prior to and 72 hours after GAS472 s.c. infection or from an uninfected non-Tg mouse. The protocol for immunohistochemistry of PLN cells was established in accordance with previous reports.[4,6] We used goat anti-hCD46 antibody (R&D Systems) at a concentration of 6 μg/mL as the primary antibody and donkey anti-goat Alexa488-labeled IgG antibody (Life Technologies Corp., Tokyo, Japan) at 200-fold dilution as the secondary antibody. The completed cryosections (7 μm) of PLN cells were observed under a fluorescence microscope.

The PLN cells were extracted from hCD46-Tg mice ($n = 4$) prior to and at 24 hours, 48 hours, and 72 hours after GAS472 s.c. infection. The protocol for flow cytometry of PLN cells was established in accordance with a previous report.[4] We applied the same primary (4 μg/mL) and secondary (50-fold dilution) antibodies as used in the immunohistochemistry. A FACS Calibur flow cytometer (Nippon Becton Dickinson, Tokyo, Japan) was used for the measurement. Statistical significance between samples was determined by Welch's t test with $p < 0.05$ considered significant.

3. Results

The levels of IL-1β, TNF-α, and IL-6 release from peritoneal macrophages isolated from hCD46-Tg or non-Tg mice after exposure to GAS472 or heat-killed GAS472 are shown in Figure 1. At 24 hours after exposure, the cells exposed to GAS472 revealed IL-1β as well as TNF-α and IL-6 responses, whereas those exposed to heat-killed bacteria showed only TNF-α and IL-6 releases. IL-10 response of the cells was not found after GAS472 exposure, although IL-10 was detected after heat-killed bacterial exposure.

Using immunohistochemistry, we demonstrated hCD46 expression in PLN cells isolated from hCD46-Tg mice prior to and 72 hours after GAS472 s.c. infection (Figure 2). The localization of hCD46 was confirmed in the cells. Expression of hCD46 was not found in cells from non-Tg mice. Cell fractions indicating presence or absence of hCD46 in PLN isolated from hCD46-Tg mice prior to and 72 hours after GAS472 s.c. infection are shown on the flow

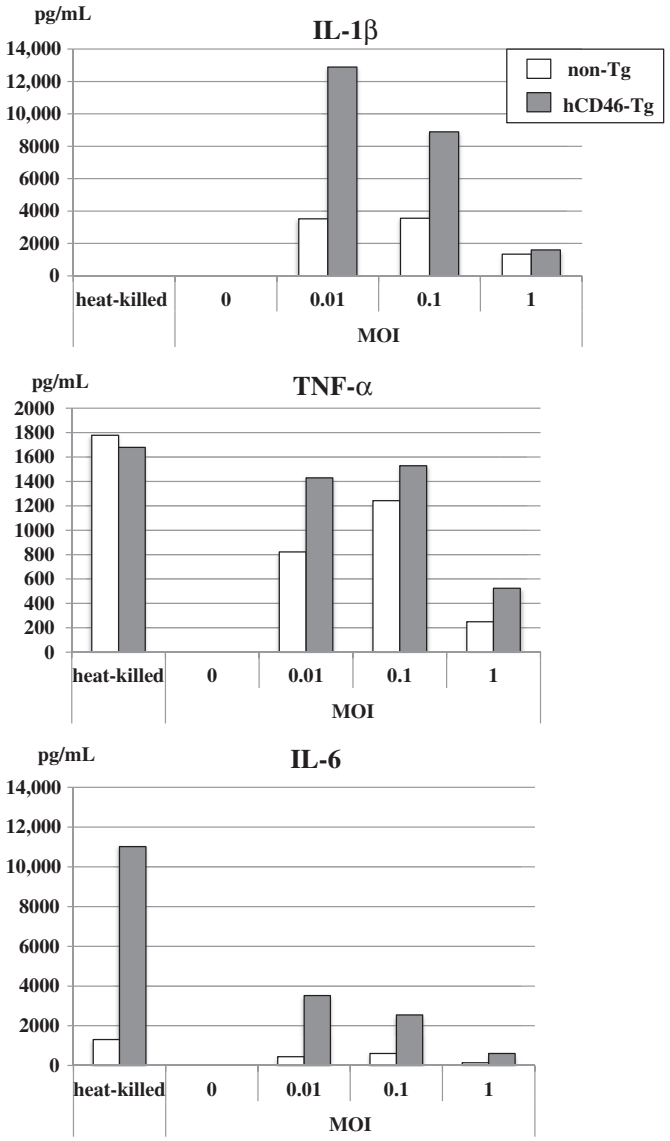

Figure 1 Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 releases from peritoneal macrophages isolated from human CD46-transgenic or non-transgenic mice after exposure to live or heat-killed GAS472 bacteria. hCD46-Tg = human CD46-transgenic mice; heat-killed = heat-killed GAS472 strain; MOI = multiplicities of infection; non-Tg = nontransgenic mice.

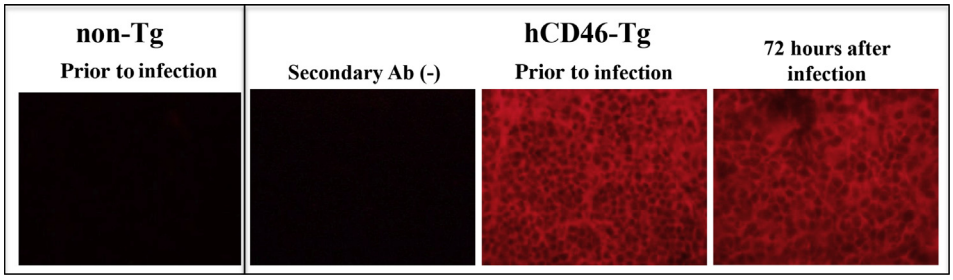

Figure 2 A typical expression of human CD46 protein in cells of popliteal lymph nodes extracted from human CD46-transgenic mice prior to and 72 hours after subcutaneous infection with the GAS472 strain or from an uninfected nontransgenic mouse. Ab = antibody; hCD46-Tg = human CD46-transgenic mice; non-Tg = nontransgenic mice.

cytometry curves (Figure 3A). It was demonstrated that the percentage (82.70%) of hCD46-expressing cells in the PLN was significantly decreased at 72 hours after infection as compared with that (96.38%) prior to infection (Figure 3B, $p < 0.05$). The fraction not expressing hCD46 was observed as cell debris because these cells revealed fragmentation. The cell debris prior to infection were not observed.

4. Discussion

We found a release of IL-1β as well as TNF-α and IL-6 from peritoneal macrophages 24 hours after GAS exposure, but not after the exposure to heat-killed bacteria. By contrast, IL-10 (anti-inflammatory cytokine) response of the macrophages was not observed after GAS472 exposure, although IL-10 was detected after heat-killed bacterial exposure. It was also demonstrated that the percentage of hCD46-expressing cells was decreased at 72 hours after infection, and cell debris not expressing hCD46 was observed, indicating development of the cell death. These observations suggest that the macrophages exposed to GAS472 may induce proinflammatory cytokine expressions including IL-1β, TNF-α, and IL-6 and consequent cell death after infection in mice.

In a previous experiment, transcriptome analysis of murine macrophages infected with GAS revealed upregulation of IL-1, TNF-α, and IL-6 at the gene expression level.[7] These data were compatible with our preliminary findings. Streptococcal histone was also reported to induce murine peritoneal macrophages to produce IL-1 and TNF-α.[8] Treatment with a mixture of histone and heparin resulted in reduced cytokine production (50% less IL-1 and 76% less TNF-α) compared to that by cells incubated with histone alone. Based on these results, we should examine the systemic (survival) or local (foot lesions) effects of heparin on conditions of disseminated intravascular coagulation under STSS pathology, using hCD46-Tg mouse model. Moreover, the production of streptococcal pyrogenic exotoxin B, a cysteine protease, caused a greater extent of apoptosis in human monocyte-like cells.[9] Thus, the streptococcal-

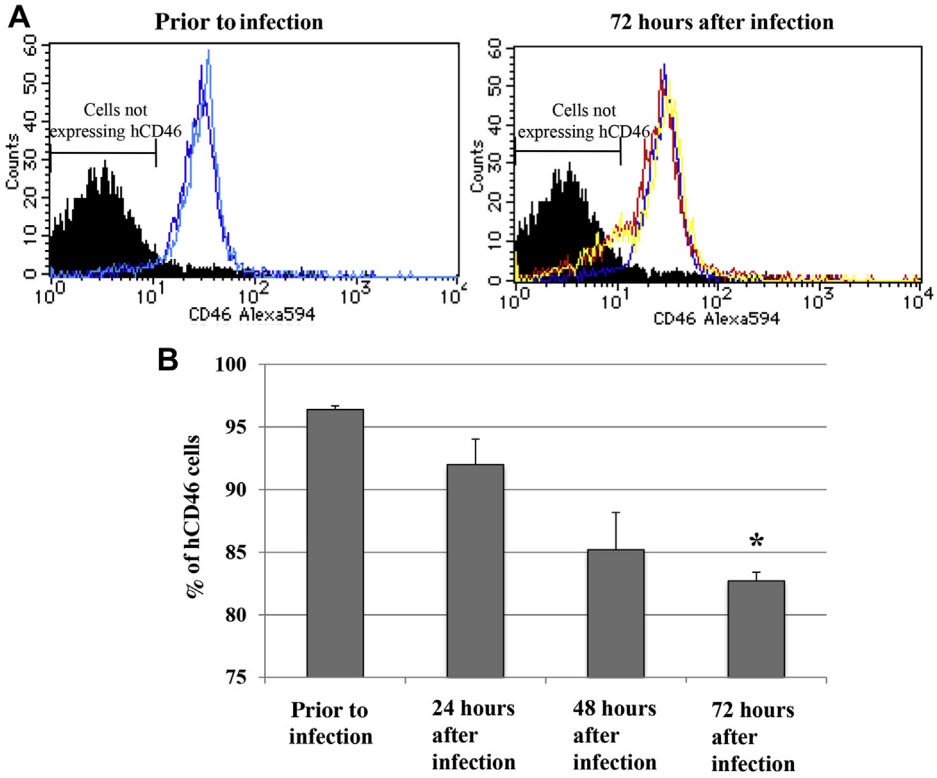

Figure 3 Variations of human CD46 expression in cells of popliteal lymph nodes extracted from human CD46- transgenic mice ($n = 4$) prior to and 24 hours, 48 hours, and 72 hours after subcutaneous infection with the GAS472 strain. (A) Cell fractions showing presence or absence of hCD46 in the nodes from hCD46-Tg mice prior to and 72 hours after infection are shown on the flow cytometry curves. (B) The graphs indicate variations in the percentage of human CD46-positive cells prior to and 24 hours, 48 hours, and 72 hours after infection. hCD46, human CD46. Values indicated with an asterisk were significantly different from the baseline values. $^*p < 0.05$.

specific component may have the potential to induce the human cell death as shown in our experiment. Picibanil (OK-432), a killed preparation of a low virulent GAS strain, possessed activity of interferon-γ induction in human peripheral blood mononuclear cells.[10] We need to identify the characteristic streptococcal component that can lead to the differential response of macrophages to living and heat-killed GAS472 in a future investigation.

It is important to establish an animal model similar to the STSS pathological status, in order to be familiar with host responses. An understanding of how macrophages respond to GAS at the molecular level also may facilitate the development of novel therapeutic strategies.

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