

Effects of lipopolysaccharide and CpG-DNA on burn-induced skin injury

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Destruction of the skin barrier by thermal injury induces microbial invasion, which can lead to the development of systemic infection and septic shock. Microbial pathogens possess pathogen-associated molecular patterns (PAMPs), which are recognized by conserved receptors. To understand the role of PAMPs in thermal injury-induced mice, LPS or CpG-DNA were topically applied to dorsal skin after thermal injury. We observed an increase in the number of inflammatory cell infiltrates as well as thickening in the dermis upon treatment with LPS or CpG-DNA. We also found that expression of IL-1 β , MIP-2, and RANTES induced by thermal injury was enhanced by LPS or CpG-DNA. In addition, the proportions of CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes were altered by LPS or CpG-DNA. These results provide important information concerning PAMPs-induced inflammation upon thermal injury and provide a basis for studying the role of PAMPs in thermal injury-induced complications. [BMB reports 2011; 44(4): 273-278]

INTRODUCTION

Thermal injury induces the activation of inflammatory responses, resulting in tissue damage, development of immunosuppression, and increased susceptibility to bacterial infection (1-6). Several investigators have previously shown that thermal burns of the skin induce cellular immune responses, such as recruitment of neutrophils and macrophages (7-10), and T lymphocyte dysfunction (11). Thermal injury also induces expression of various mediators, including Th2 cytokines such as IL-4 and IL-10 (11, 12), chemokines such as monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-2 (MIP-

2) (13-16). These immune dysfunctions due to thermal injury are associated with the development of life-threatening post-burn complications such as systemic inflammatory response syndrome in multiple organs (17, 18).

Destruction of the skin barrier by thermal injury induces microbial invasion through the intracellular spaces of the stratum corneum (19). Skin as a first line of defense produces free fatty acids, polar lipids, and glycosphingolipids in the stratum corneum, accumulating them in the intercellular spaces of the horny layer for antimicrobial properties (19). When this barrier is violated, a second line of defense is activated by antimicrobial peptides such as defensins. Defensins, cationic peptides that are 29-44 amino acids in length, belong to a large family of antimicrobial peptides that share structural characteristics, including three intramolecular disulfide bridges. Defensins are divided into three subfamilies; α -, β -, and θ - defensins (20, 21). Human β defensins 1-3 (HBD1-3) are expressed in the skin (22) and have potent antimicrobial activity against a wide range of microbial pathogens associated with burn injury and sepsis (22-25). Especially, hBD-2 expression is prevalently observed in epithelial cells of the skin (23, 26, 27) and is induced by exposure to *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* (28-30). Faunce et al. determined that mice subjected to skin scald injury suffer significantly higher rates of mortality upon *P. aeruginosa* infection (6). Furthermore, *P. aeruginosa*-infected mice after thermal injury display decreased delayed type hypersensitivity responses as well as suppressed mitogen-induced T cell proliferation (6).

A wide variety of microbial components possessing pathogen-associated molecular patterns (PAMPs) include LPS, bacterial DNA (CpG-DNA), lipoteichoic acid, and peptidoglycan. These can activate innate immune responses and regulate systemic inflammatory responses (31-33). Several investigators have reported that CpG-DNA causes severe side effects such as induction of arthritis (34), transient splenomegaly (35), lymphoid follicle destruction (36), and PS-ODN-specific IgM production (37) in mice, depending on the CG sequence and backbone modification.

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Although previous studies on microbial invasion upon thermal injury have focused on tissue damage and immune dysfunction, the effects of PAMPs on thermal injury have not yet been fully elucidated. In this study, we show that PAMPs act as a potent immunostimulator and induce inflammation upon thermal burn-induced skin injury.

RESULTS AND DISCUSSION

Immunostimulatory effects of LPS and CpG-DNA upon thermal injury

Previously, we showed that thermal injury-induced macroscopic appearances of lesions are spontaneously healed after 21 days via regeneration and repair (scar formation) (38). Therefore, we examined the effect of LPS and CpG-DNA on the injury-induced macroscopic appearances of lesions over 14 days. To examine the effects of LPS and CpG-DNA, LPS or CpG-DNA were topically applied to dorsal skin for 5 days (daily) after thermal injury. As shown in Supplementary Fig. 1, macroscopic examination of the wound healing process revealed that CpG-DNA treatment delayed spontaneous healing. Although it is not clear why only CpG-DNA but not LPS induced this phenomenon, the reason could have been due to aberrant inflammation induced by CpG-DNA. Next, we investigated inflammatory cell infiltrates in the dermis and measured the thickness of the epidermis by hematoxylin and eosin (H&E) staining of skin tissues. During the healing process (Fig. 1A and B, Burn/PBS), an increase in the number of inflammatory cell infiltrates as well as thickening of the epidermis were observed. More abundant inflammatory cell infiltrates in the dermis were found at 7 and 14 days after thermal injury upon treatment with LPS or CpG-DNA (Fig. 1A and B). Treatment of the burned areas of the skin with LPS or

CpG-DNA also clearly increased thickening of the epidermis at days 7 and 14 post-burn (Fig. 1A and B). In contrast, treatment with non-CpG-DNA had no additional effect on inflammatory cell infiltration in the dermis or upon thickening of the epidermis (Fig. 1A and B). Therefore, it was confirmed that LPS and CpG-DNA have potent stimulatory effects on thermal injury-induced inflammation in mice.

Effects of LPS and CpG-DNA on thermal injury-induced proinflammatory cytokine gene expression

Reportedly, immune dysfunction and multiple organ failure induced by thermal injury are mediated by proinflammatory cytokines and chemokines (17, 18). In our previous studies, we determined that expression of proinflammatory cytokines and chemokines is regulated by LPS or CpG-DNA treatment (39-41). Therefore, we evaluated the effects of LPS and CpG-DNA on expression of proinflammatory cytokines and chemokines in the burned skin area. As previously reported, the expression of IL-1 β , MIP-2, and RANTES was induced by burn-injury (Fig. 2, Burn/PBS). Furthermore, expression of these genes was enhanced by LPS and CpG-DNA treatment in the burned skin area (Fig. 2, Burn/LPS and Burn/CpG-DNA), which is in accordance with our previous results (39-41). The expression levels of the genes were higher in the case of CpG-DNA treatment than LPS. The expression of IL-6 mRNA in the burned skin area was specifically increased upon treatment with CpG-DNA (Fig. 2). Differences in the expression of cytokine and chemokine genes may have contributed to the delayed wound healing process induced by CpG-DNA rather than LPS (Supplementary Fig. 1).

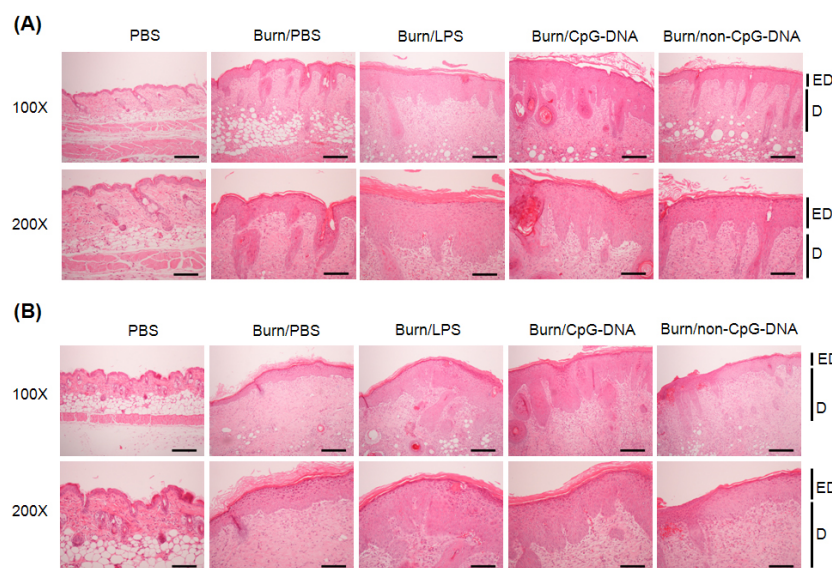


Fig. 1. Effects of LPS and CpG-DNA on the macroscopic appearances of skin lesions in thermal burn injury-induced mice. Thermal injury on back skin was induced as described in Materials and Methods. For 5 days (daily), 5 mg/kg of LPS, 2.5 mg/kg of CpG-DNA (MB-ODN 4531(S)), or 2.5 mg/kg of non-CpG-DNA 2041 (S) was applied to the back skin. The results are representative of three mice per group. (A, B) Examination of histology at 7 days (A) or 14 days (B) after thermal injury. Skin paraffin sections were processed for H&E staining. ED: epidermis, D: dermis. Photographs were taken at an original magnification of $\times 100$ or $\times 200$. Scale bars in $100\times$, 100 μm and in $200\times$, 50 μm .

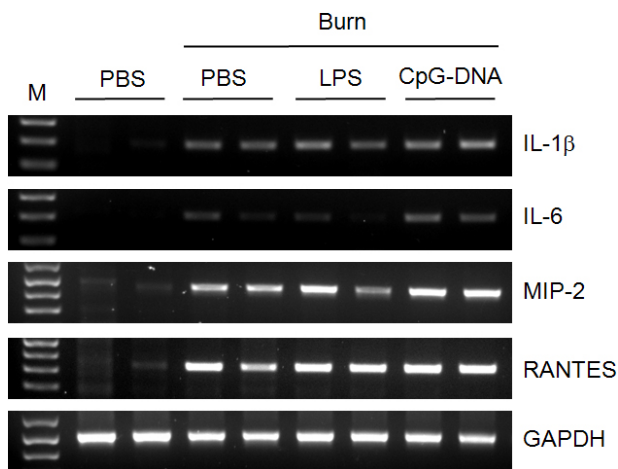


Fig. 2. Effects of LPS and CpG-DNA on thermal injury-induced cytokine gene expression. Thermal injury in BALB/c mice was induced, and 5 mg/kg of LPS or 2.5 mg/kg of CpG-DNA (MB-ODN 4531(S)) was applied to the back skin for 5 days. Skin samples from two mice were collected at 14 days after thermal injury. After extracting total RNA from the back skin, expression of the indicated cytokines was analyzed by RT-PCR. GAPDH was used as an internal control. M denotes DNA standard marker.

Effects of LPS and CpG-DNA on thermal injury-induced T cell population

Previously, it was reported that reduction of delayed type hypersensitivity responses and restriction of mitogen-induced T cell proliferation are observed in bacteria-infected mice after thermal injury (5). Therefore, we examined the effects of LPS and CpG-DNA on thermal injury-induced T cell subpopulations in the spleen, mesenteric lymph node, axillary lymph node, and inguinal lymph node. To evaluate T cell subpopulations, splenocytes and cells of the lymph nodes were prepared from thermal injury-induced mice at days 14 post-burn. FACS analysis was performed with PE anti-mouse CD4 antibodies and PerCP-Cy5.5 anti-mouse CD8 antibodies. The proportions of CD4⁺ T cells and CD8⁺ T cells in the spleens of thermal injury-induced mice did not change compared to those of sham-injury mice (Fig. 3A). However, decreased proportions of CD4⁺ T cells and CD8⁺ T cells were detected in the spleens of mice treated with CpG-DNA but not LPS in the burned skin area (Fig. 3A). An enhanced proportion of CD4⁺ T cells (approximately 4%) was observed in the mesenteric lymph node in thermal injury-induced mice (Fig. 3B). However, reduced proportions of CD4⁺ T cells (approximately 6-9%) were detected in the axillary lymph node and inguinal lymph node (Fig. 3C and D). In the axillary lymph node of LPS-treated thermal injury-induced mice, the proportion of CD8⁺ T cells was about 5% lower compared to that of thermal injury-induced mice. In contrast, the proportion of CD4⁺ T cells was 3% lower in the axillary lymph node of CpG-DNA-treated thermal injury-induced mice compared to that of thermal in-

jury-induced mice (Fig. 3C). In addition, the proportions of CD4⁺ and CD8⁺ T cells increased in the inguinal lymph node upon CpG-DNA treatment but not LPS treatment in the burned skin area compared to PBS-treated thermal injury-induced mice (Fig. 3D). Even though the meaning of these results is not clear, they suggest that the T cell population was modulated by LPS or CpG-DNA treatment in the burned skin area.

Thermal injury damages the first line of host defense barriers (skin) against microbial invasion. The injurious effect makes exposed skin more susceptible to microbial infection, leading to the development of systemic infection and septic shock. Several reports have shown that microbial infections in thermally-injured skin induce immune dysfunction (2, 6, 7, 17, 18). However, the effects of microbial components such as PAMPs on thermal injury-induced mice have not been clearly characterized yet. Microbial pathogens are recognized by the first line of host defense barriers based on conserved pattern recognition receptors that distinguish the PAMPs of microbial pathogen components. Especially, pattern recognition receptors such as Toll-like receptors stimulate the immune system by recognizing a wide variety of microbial components, including LPS, bacterial DNA, peptidoglycan, lipoprotein, and flagella, as infectious nonself (31-33). PAMPs activate multiple cell types involved in inflammation including macrophages, dendritic cells, neutrophils, B lymphocytes, and T lymphocytes. Activated immune cells can be stimulated to express a variety of genes involved in inflammatory responses. Therefore, understanding the roles of PAMPs in the immune systems of thermal injury-induced mice is an important research area in treating thermal injury-induced systemic infection and septic shock.

Several reports have shown that thermal burn injury induces inflammatory cell infiltration in the dermis as well as thickening of the epidermis (1-5, 38). Here, we report that infiltration of inflammatory cells in the dermis as well as thickening of the epidermis are increased by LPS or CpG-DNA treatment at thermal injury-induced burned areas of skin in mice (Fig. 1). In addition, we found that expression of IL-1 β , MIP-2, and RANTES induced by burn injury was increased by LPS or CpG-DNA treatment (Fig. 2). Furthermore, the proportions of CD4⁺ T cells and CD8⁺ T cells changed in the spleens and lymph nodes of mice upon LPS or CpG-DNA treatment compared to those of thermal injury-induced mice (Fig. 3). Taken together, these results demonstrate that PAMPs such as LPS and CpG-DNA originating from microbes may play important roles in thermal injury-induced systemic infection and septic shock.

MATERIALS AND METHODS

CpG-DNA and reagents

Phosphorothioate backbone-modified oligodeoxynucleotides were synthesized from GenoTech (Daejeon, Korea). CpG-DNA, namely MB-ODN 4531(S), containing three CpG motifs (underlined) consisted of 20 bases: AGCAGCGTTCGTGTCGGCCT (42). MB-ODN 4531 sequences used in this study were phos-

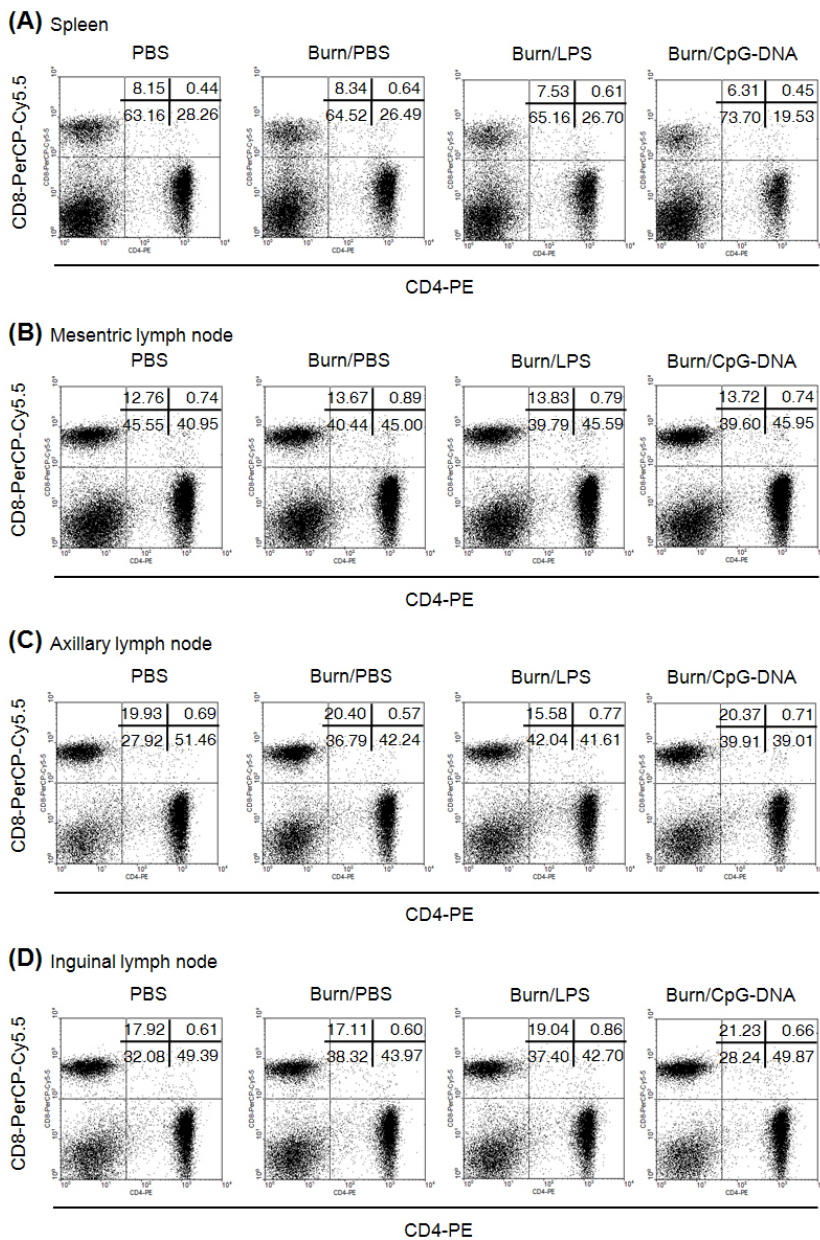


Fig. 3. Effects of LPS and CpG-DNA on T cell subpopulations in the spleens and lymph nodes of thermal injury-induced mice. Thermal injury in BALB/c mice was induced, and 5 mg/kg of LPS or 2.5 mg/kg of CpG-DNA (MB-ODN 4531(S)) was applied to the back skin for 5 days. Splenocytes and cells of the indicated lymph nodes were collected at 14 days after thermal injury. Relative proportions of T cell subpopulation in spleens and lymph nodes were analyzed by FACSaria. The results are representative of three mice per group. CD4⁺ T cells and CD8⁺ T cells in the spleens (A), mesenteric lymph nodes (B), axillary lymph nodes (C), and inguinal lymph nodes (D).

phorothioate-modified. Non-CpG-DNA 2041 served as the negative control. The endotoxin content of the oligodeoxynucleotides was less than 1 ng/mg of ODN, as measured by a *Limulus ameobocyte* assay (Whittaker Bioproducts, Walkersville, MD, USA). *Escherichia coli* LPS (Sigma-Aldrich, St. Louis, MO, USA) was suspended in sterile water.

Animals and induction of thermal injury

Seven-week-old male BALB/c specific-pathogen-free (SPF) mice were obtained from Orient Bio Inc. (Gyeonggi, Korea). All ani-

mal procedures were approved by the Institutional Animal Care and Use Committee of Hallym University (Permit Number: Hallym2010-52). Mice were anesthetized with Zoletil 50 + Rompun, and all efforts were made to minimize suffering. Induction of thermal injury in the back skin was performed as reported previously (38). In brief, hair on the skin from the back of the animals (back skin) was shaved off with electronic clippers 3 days before each experiment. All mice were subjected to a 20% total body surface area (TBSA) dorsal scald using a modified procedure of Walker and Mason (43). The mice

were set into a plastic template and exposed to 20% TBSA and then submerged in 90°C water for 7 s. To avoid further blistering, mice were immediately dried using a towel. We then applied LPS (5 mg/kg) or CpG-DNA (2.5 mg/kg) to the back skin for 5 days (daily).

Histology

Mice were sacrificed under Zoletil 50+Rompun anesthesia at the indicated days post-burn. An area of approximately 1.5 × 1.5 cm² was excised. Back skin samples from each animal were fixed in 4% paraformaldehyde solution, embedded in paraffin by a conventional method, and cut into 4-μm-thick sections. The deparaffinized sections were stained with hematoxylin-eosin for gross histological examination.

Reverse-transcription PCR analysis

After skin samples were collected from each experimental animal, total RNA was extracted using a RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). Five micrograms of total RNA was reverse-transcribed in first-strand synthesis buffer, which contained 6 μg/ml of oligo (dT) primer, 50 U of reverse transcriptase, 4 mM of dNTP, and 40 U of RNase inhibitor. One microliter of the cDNA mixture was subjected to a standard PCR reaction for 25 cycles using the following primer sets: mouse IL-1β, 5'-TTGACGGACCCCAAAGATG-3' (sense), 5'-AGAAGGTGCTCATGTCTCA-3' (anti-sense); mouse IL-6, 5'-GTTCTCTGGGAAATCGTGGA-3' (sense), 5'-TGTACTIONCAGG TAGCTATGG-3' (anti-sense); mouse MIP-2, 5'-TGGGTGGGATAGCTAGTCC-3' (sense), 5'-AGTTTGCCTTGACCCTGAGCC-3' (anti-sense); mouse RANTES, 5'-ATATGGCTCGGACCACTC-3' (sense), 5'-GATGCCGATTTCCAGGAC-3' (anti-sense); mouse GAPDH, 5'-ATGGTGAAGGTCCGTGTGAACG-3' (sense), 5'-GTTGTCATGGATGATCTTGCC-3' (anti-sense). The PCR products were visualized with UV light after being resolved on 1% agarose gel.

Isolation of cells from spleen and lymph nodes

To collect samples, mice were sacrificed under Zoletil 50+Rompun anesthesia at 14 days post-burn. To determine the proportions of T cell subpopulations in the lymph node cells and spleens, tissues were removed aseptically, immediately minced in RPMI 1640 culture medium, and passed through mesh to obtain single cell suspensions. After the cells were washed twice with PBS, erythrocytes were removed by treating the cells with 5 ml of a red blood cell lysis buffer containing 20 mM Tris-HCl (pH 7.2) and 140 mM ammonium chloride at 37°C for 5 min.

FACS analysis of the cells

Proportions of the T cells subpopulations were analyzed by FACS using a FACSAria flow cytometer (BD Biosciences, San Diego, CA, USA). The cells were washed with PBS containing 0.1% BSA and incubated for 20 min at 4°C with 10 μg/ml of anti-FcγRII/III antibody (BD Biosciences) to block the Fc recep-

tors. After the blocking process, the cells were incubated with PE-conjugated anti-mouse CD4 antibodies and PerCP-Cy5.5-conjugated anti-mouse CD8 antibodies (BD Biosciences) for 1 h at 4°C. The FACS data were analyzed with the aid of WinMDI 2.8 FACS software.

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