ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 11 (2009) 245–253

www.elsevier.com/locate/micinf

Original article

Toll-like receptor 4-dependent activation of myeloid dendritic cells by leukocidin of *Staphylococcus aureus*

Ken Inden [a], Jun Kaneko [b], Akiko Miyazato [a,1], Natsuo Yamamoto [a], Shota Mouri [b], Yoshiyuki Shibuya [b], Kiwamu Nakamura [a,2], Tetsuji Aoyagi [a], Masumitsu Hatta [a], Hiroyuki Kunishima [a], Yoichi Hirakata [a], Yoshifumi Itoh [b], Mitsuo Kaku [a], Kazuyoshi Kawakami [c,*]

[a] *Department of Infection Control and Laboratory Diagnostics, Internal Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai-shi, Miyagi 980-8575, Japan*

[b] *Laboratory of Applied Microbiology, Department of Microbial Biotechnology, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumi-dori Amemiya-machi, Aoba-ku, Sendai-shi, Miyagi981-8555, Japan*

[c] *Department of Medical Microbiology, Mycology and Immunology, Tohoku University Graduate School of Medicine, 2-1 Seiryou-cho, Aoba-ku, Sendai-shi, Miyagi 980-8575, Japan*

Received 22 August 2008; accepted 26 November 2008
Available online 14 December 2008

Abstract

Leukocidin (Luk), an exotoxin of *Staphylococcus aureus* consisting of LukF and LukS, is a hetero-oligomeric pore-forming cytolytic toxin toward human and rabbit polymorphonuclear leukocytes. However, it is uncertain how Luk affects the host immune response. In the present study, we investigated whether Luk has the ability to stimulate mouse bone marrow-derived myeloid dendritic cells (BM-DCs). LukF activated BM-DCs to generate IL-12p40 mRNA, induce intracellular expression and extracellular secretion of this cytokine and express CD40 on their surface, whereas LukS showed a much lower or marginal ability in the activation of BM-DCs than its counterpart component. Similarly, TNF-α was secreted by BM-DCs upon stimulation with these components. Combined addition of these components did not lead to a further increase in IL-12p40 secretion. IL-12p40 production caused by LukF was completely abrogated in BM-DCs from TLR4-deficient mice similarly to the response to lipopolysaccharide (LPS). Polymixin B did not affect the LukF-induced IL-12p40 production, although the same treatment completely inhibited the LPS-induced response. Boiling significantly inhibited the response caused by LukF, but not by LPS. Finally, in a luciferase reporter assay, LukF induced the activation of NF-κB in HEK293T cells transfected with TLR4, MD2 and CD14, whereas LukS did not show such activity. These results demonstrate that LukF caused the activation of BM-DCs by triggering a TLR4-dependent signaling pathway and suggests that Luk may affect the host inflammatory response as well as show a cytolytic effect on leukocytes.

Keywords: *Staphylococcus aureus*; Leukocidin; Dendritic cells; TLR4; IL-12; CD40

1. Introduction

Staphylococcus aureus, a Gram-positive coccal bacterium, is widely distributed in the environment and constitutes indigenous bacterial flora in the skin and mucosal area [1]. It possesses a variety of virulence factors, such as exotoxins and enzymes, and causes food poisoning, skin and soft tissue infection, pneumonia, myelitis and endocarditis [2,3]. Methicillin-resistant *S. aureus* (MRSA) has been recognized as a major causative

* Corresponding author: Tel.: +81 22 717-7946; fax: +81 22 717 7910.
E-mail address: kawakami@m.tains.tohoku.ac.jp (K. Kawakami).
[1] Present address: Department of Infectious Diseases and Infection Control, Saitama International Medical Center, Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Saitama 350-129, Japan.
[2] Present address: Department of Medicine and Therapeutics, Control and Prevention of Infectious Diseases, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-cho, Nakagami-gun, Okinawa 903-0215, Japan.

1286-4579/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2008.11.013

microorganism in nosocomial infectious diseases [4] and recently, community acquired MRSA has garnered much attention by many clinicians and investigators [5].

Leukocidin (Luk), an exotoxin of *S. aureus* consisting of two protein components, LukF (34 kDa) and LukS (32 kDa), shows cytolytic activity on human and rabbit polymorphonuclear leukocytes (PMNL) [6]. These components assemble on the membranes of PMNL into ring-shaped hetero-oligomers, which results in the formation of transmembrane pores and the cytolysis of these cells [7]. During this process, LukS binds to the cell membrane via its glycolipid [8,9] or protein receptors [6,7], which enables the subsequent binding of LukF [6,8,9]. Luk, known as a virulence factor, contributes to the pathogenicity in the experimental models of infectious diseases [10]. However, it is not fully understood how this cytolytic toxin affects the host inflammatory responses after infection with *S. aureus*.

A family of Toll-like receptors (TLRs) has been identified as recognition receptors for a variety of pathogen-associated molecular patterns in various microorganisms [11,12]. When these receptors are triggered, downstream signaling cascades involving MyD88 are activated for induction of inflammatory responses [13]. TLR2 recognizes lipoproteins of various infectious pathogens, such as Gram-positive bacteria, mycoplasma and spirochetes, peptidoglycan and lipoteichoic acid of Gram-positive bacteria, and lipoarabinomannan of mycobacteria [14,15]. A TLR4/MD2 complex plays a central role in sensing lipopolysaccharides (LPS) of Gram-negative bacteria, which is strengthened by co-expression of CD14 [16].

In the present study, we aimed to elucidate how Luk affects innate immune responses, which are deeply involved in the host inflammatory responses after infection with microorganisms. For this purpose, we examined whether LukF and LukS activated dendritic cells (DCs) to produce cytokines and express co-stimulatory molecules, which play central roles in triggering the innate immune mechanism and greatly influence subsequently developing acquired immune responses [17]. Furthermore, we addressed the important question of how Luk is sensed by DCs by elucidating the contribution of TLR4 to this process.

2. Materials and methods

2.1. Mice

C57BL/6 mice were bred under specific pathogen-free conditions at Tohoku University. C3H/HeJ and C3H/HeN mice were purchased from Japan CLEA (Tokyo, Japan). Male or female mice at 6–10 weeks of age were used for the experiments. The experiments were conducted according to the institutional guidelines and were approved by the institutional ethics committees.

2.2. Preparation of leukocidin

LukF and LukS, components of *S. aureus* strain Smith 5R (ATCC31889), were prepared as described previously [18]. Briefly, these components were purified from the culture fluid of this bacterium that was grown in 2.5% heart infusion broth (Difco, Sparks, MD) at 37 °C for 20 h under aeration with O_2–CO_2 (80:20 v/v) using hydroxylapatite (Nacalai, Japan) chromatography and SP-5PW (Tosoh, Japan) HPLC. The purity of these components was confirmed by SDS-PAGE (Fig. 1), which was performed as previously described [18].

2.3. Cell culture medium and reagents

RPMI1640 medium was obtained from Nipro (Osaka, Japan) and fetal calf serum (FCS) from Cansera (Rexdale, ON, Canada). Lipopolysaccharide (LPS), peptideglycan (PG) and polymixin B (PMB) were purchased from Sigma (St Louis, MO). A synthetic CpG-oligodeoxynucleotide, CpG1826 (TCC ATG ACG TTC CTG ACG TT), was synthesized at Hokkaido System Science (Sapporo, Japan), which was phosphorothioated and purified by HPLC.

2.4. Preparation and culture of dendritic cells

DCs were prepared from bone marrow cells (BM-DCs), as described by Lutz and co-workers [19] with some modifications. Briefly, mouse BM cells were cultured at 2×10^5/ml in 10 ml RPMI1640 medium supplemented with 10%FCS,

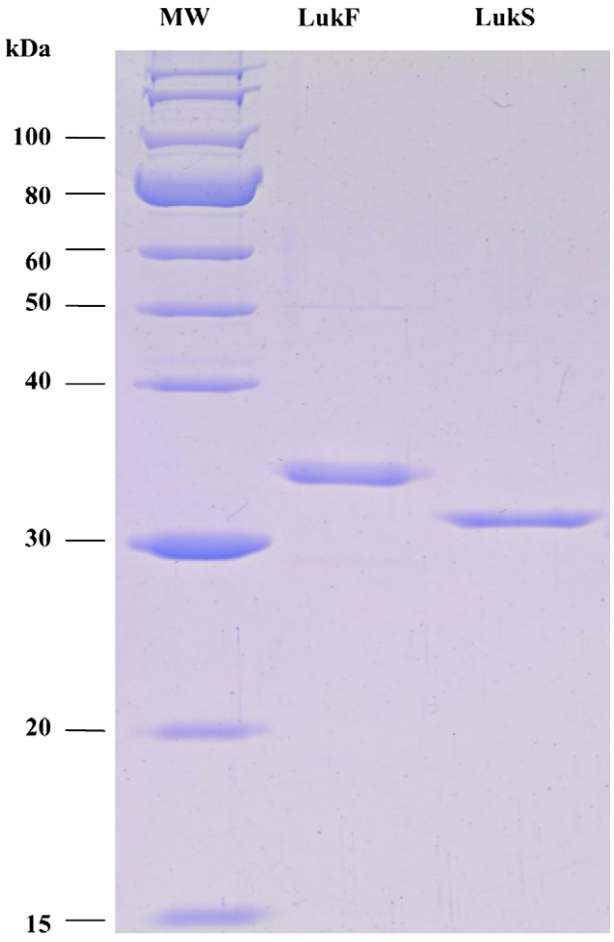

Fig. 1. SDS-PAGE analysis of Luk. Purified LukF and LukS preparations were subjected to SDS-PAGE with 12.5% gel. Protein bands were stained with Coomassie brilliant blue R-250.

100 U/ml penicillin G, 100 μg/ml streptomycin and 50 μM 2-mercapto-ethanol containing 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Wako, Osaka, Japan). On day 3, 10 ml of the same medium was added, followed by replacement of half of the medium with the GM-CSF-containing culture medium on day 6. On day 8, non-adherent cells were collected and used. When incubated with various stimulants, the cell number was adjusted to 1×10^5/ml and cultured overnight at 37 °C in a 5% CO_2 incubator.

2.5. ELISA assay

The concentration of IL-12p40 and TNF-α in the culture supernatants was measured by ELISA using capture and biotinylated developing Abs (BD Biosciences, Franklin Lakes, CT). The detection limit was 15 and 50 pg/ml, respectively.

2.6. Extraction of RNA and RT-PCR

Total cellular RNA was extracted from BM-DCs stimulated with Luk or LPS using ISOGEN (Wako, Osaka, Japan), followed by reverse transcription [20]. The obtained cDNA was then amplified in an automatic DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) using specific primers 5′-CAG AAG CTA ACC ATC TCC TGG TTT G-3′ (sense) and 5′-TCC GGA GTA ATT TGG TGC TTC ACA C-3′ (antisense) for IL-12p40, and 5′-CTC ATG ACC ACA GTC CAT GC-3′ (sense) and 5′-CAC ATT GGG GGT AGG AAC AC-3′ (antisense) for hypoxanthine phosphoribosyl transferase (HPRT). We added 1.0 μl of the sample cDNA solution to 49 μl of the reaction mixture, which contained the following concentrations: 10 mM Tris−HCl (pH = 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 μg/ml gelatin, dNTP (each at a concentration of 200 μM), 1.0 μM sense and antisense primer, and 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The mixture was incubated for 1 min at 94 °C, 1 min at 55 °C and 1 min 30 s at 72 °C for IL-12p40 and HPRT. The number of cycles was determined for samples not reaching the amplification plateau (32 cycles for IL-12p40 and 28 cycles for HPRT. The PCR products were electrophoresed on 2% agarose gels, stained with 0.5 μg/ml ethidium bromide and observed with a UV transilluminator.

2.7. Analysis of cell surface antigens and intracellular IL-12p40

BM-DCs were preincubated with anti-FcγRII and III monoclonal antibody (mAb), prepared by a protein G column kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) from the culture supernatants of hybridoma cells (clone 2.4G2), placed on ice for 15 min in staining buffer (PBS containing 1% FCS and 0.1% sodium azide), stained with FITC-conjugated anti-CD11c mAb (clone HL3; BD Bioscience) and PE-conjugated anti-CD40 mAb (clone 1C10; eBioscience, San Diego, CA) for 25 min and then washed three times in staining buffer. Isotype-matched irrelevant Abs were used for control staining. For staining of intracellular IL-12p40, the cells were incubated in the presence of cytofix/cytoperm (BD Biosciences), washed twice in BD perm/wash solution and stained with PE-conjugated anti-IL-12p40 mAb (clone C17.8, eBioscience) or control rat IgG (clone C17.8, BD Biosciences). The stained cells were analyzed using a flow cytometer, Cytomics FC500 (Beckman Coulter, Inc., Fullerton, CA). Data were collected from 15,000 to 20,000 individual cells using parameters of forward scatter (FSC) and side scatter (SSC) to set a gate on macrophage or neutrophil population.

2.8. Preparation of TLR transfectants and luciferase assays

Mouse TLR2, TLR4, MD2 and CD14 cDNAs were amplified by reverse transcriptase PCR from total RNA prepared from C3H/HeN mouse macrophages. The coding region for each gene was inserted into the mammalian expression vector p3xFLAG CMV-1 (Sigma). Mouse dectin-1 cDNA was similarly prepared from a mouse macrophage cell line, RAW264 (RIKEN Cell Bank, Tsukuba, Japan), and then inserted into the expression vector p3xFLAG CMV-14 (Sigma) [21]. The two different sets of genes for (i) TLR2 and (ii) TLR4, MD2 and CD14 (TLR2, TLR4, MD2 and CD14 genes, kind gifts from Dr Masao Mitsuyama, Kyoto University, Kyoto, Japan) were transfected into HEK293T cells (obtained from ATCC, Manassas, VA; CRL-1573) using the PolyFect transfection reagent (Qiagen, Tokyo, Japan). DNA mixtures consisted of 25 ng of an expression plasmid for each gene and 27.5 ng of a reporter plasmid mixture containing 10 volumes of the pELAM NF-κB-firefly luciferase plasmid (a kind gift from Dr Masao Mitsuyama) and one volume of plasmid pRL-TK (Promega, Tokyo, Japan) as an internal control for transfection efficiency. The cultures were grown in RPMI1640 medium supplemented with 10% FCS. At 24 h after transfection, the cells were stimulated with Luk-F, Luk-S, PG and LPS for 6 h. They were then washed once with PBS and lysed using 20 μl per well of passive lysis buffer (Promega). The luciferase assay reagents were added to the lysate, and the results were immediately read with a luminometer (AB2200, Atto Co., Tokyo, Japan).

2.9. Statistical analysis

Analysis was conducted using Statview II software (Abacus Concept, Inc., Berkeley, CA) on a Macintosh computer. Data are expressed as mean ± standard deviation (SD). Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) with a post-hoc analysis (Fisher PLSD test). A p value less than 0.05 was considered significant.

3. Results

3.1. Activation of BM-DCs by leukocidin

To investigate whether Luk directly activates innate immune cells, we incubated BM-DCs with either LukF or

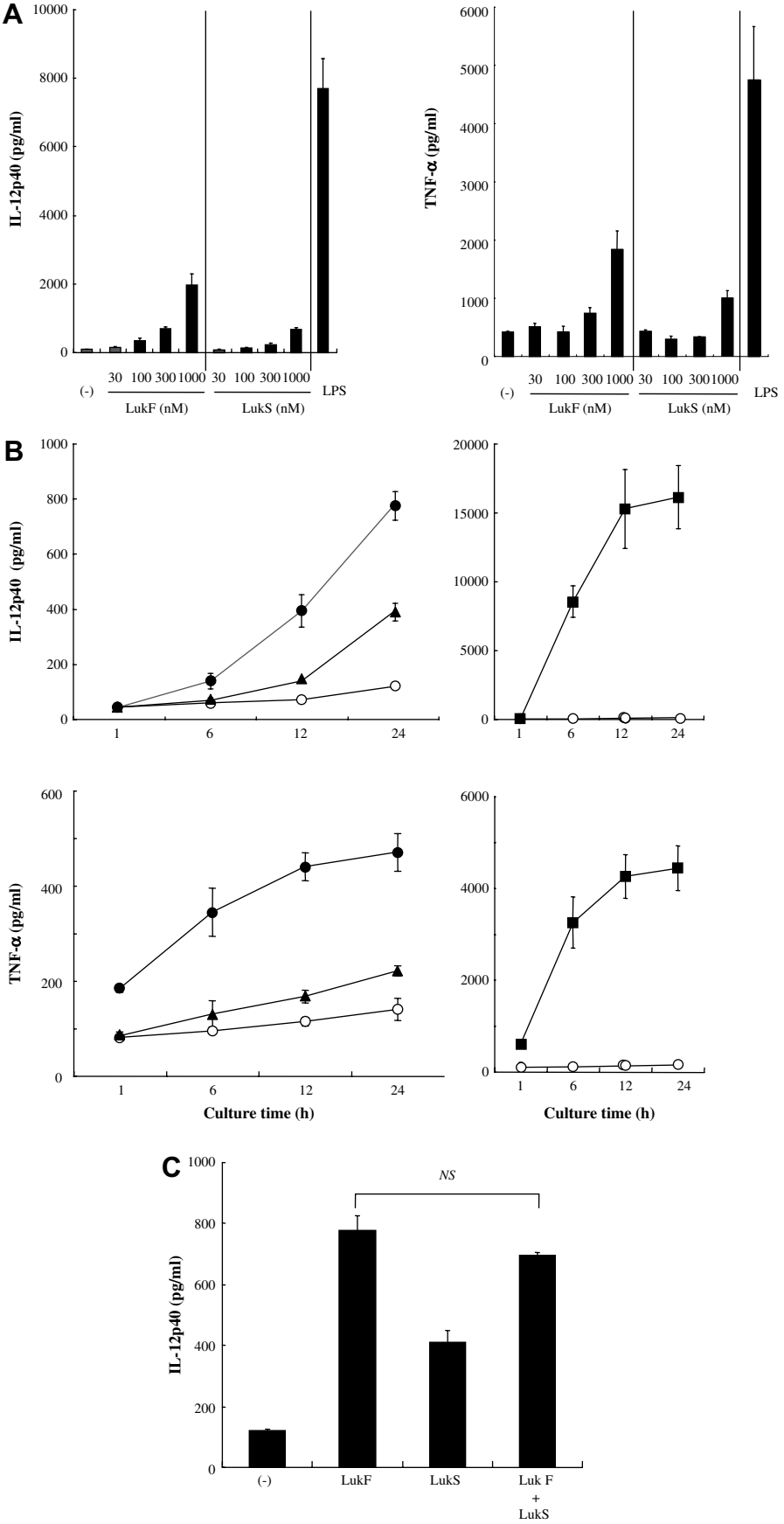

Fig. 2. IL-12p40 production by BM-DCs upon stimulation with Luk. (A) BM-DCs were cultured with indicated doses of LukF, LukS or 1 μg/ml of LPS. (B) BM-DCs were cultured with LukF (300 nM), LukS (300 nM) (left) or LPS (1 μg/ml) (right) for 1, 6, 12 or 24 h. (C) BM-DCs were cultured with either LukF (300 nM) or LukS (300 nM) or in combination. Concentrations of IL-12p40 and TNF-α (A,B) or IL-12p40 (C) in the culture supernatants were measured by ELISA. Data are mean ± SD of triplicate cultures. Open circles, medium; closed circles, LukF; closed triangles, LukS; closed squares, LPS. *NS*, not significant.

LukS obtained from *S. aureus* and measured the concentration of IL-12p40 and TNF-α in the culture supernatants. As shown in Fig. 2A, LukF induced the production of IL-12p40 and TNF-α in a dose-dependent fashion, at approximately one third to one fourth the level of that caused by LPS at 1000 nM. IL-12p40 was faintly produced by stimulation with 300 nM of LukS, and such production was significantly increased, although much less than that caused by LukF, when LukS was added at 1000 nM. TNF-α was faintly produced by stimulation with 1000 nM of LukS, although such production was not detected at 300 nM. In a time-course analysis, production of IL-12p40 and TNF-α were first detected at 6 h and increased at 12 and 24 h, when BM-DCs were stimulated with either LukF or LukS (Fig. 2B). Because LukF and LukS act together in the cytolysis of leukocytes, we tested them in combination in the production of IL-12p40 by BM-DCs. As shown in Fig. 2C, addition of LukS did not result in a further increase in the production caused by LukF.

To test whether Luk induced the expression of IL-12p40 mRNA synthesis, we conducted an RT-PCR analysis. As shown in Fig. 3, IL-12p40 mRNA was clearly expressed upon stimulation with LukF at a similar level to that caused by LPS, whereas such expression was marginally detected when stimulated with LukS. In further experiments, we examined the intracellular synthesis of IL-12p40 by BM-DCs and the expression of CD40, an alternate activation marker, on their cell membranes. As shown in Fig. 4A, LukF increased the proportion of CD11c + IL-12p40+ cells compared to that of unstimulated cells, which was lower than that caused by LPS, whereas LukS showed a marginal effect. Similar findings were detected in the expression of CD40, although the effect of LukF was much less than that of LPS (Fig. 4B).

3.2. Role of TLR4 in leukocidin-induced IL-12p40 production

PAMPs from microbial pathogens are sensed by innate immune cells through PRRs including TLRs [22]. We addressed the possible involvement of TLR4 in the activation of BM-DCs caused by Luk. For this purpose, BM-DCs from C3H/HeJ and C3H/HeN mice, a TLR4-deficient mutant and its control, respectively, were compared in the production of IL-12p40 caused by either LukF or LukS. As shown in Fig. 5, LukF induced the production of this cytokine by BM-DCs from C3H/HeN mice, and LukS showed marginal activity in this response. In BM-DCs from C3H/HeJ mice, IL-12p40 production was completely abrogated when stimulated with LukF, similarly to the response caused by LPS, whereas there was not any effect on CpG-ODN-induced cytokine synthesis. These results suggest that TLR4 may contribute to the activation of BM-DCs caused by Luk. Before drawing this conclusion, however, we needed to exclude the possibility that LPS contamination in the Luk preparations may be involved in these responses, although *S. aureus* does not secrete LPS.

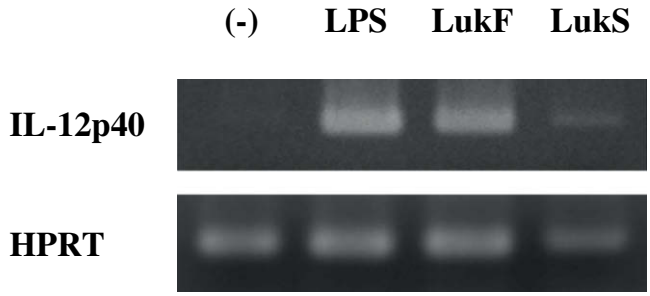

Fig. 3. Expression of IL-12p40 mRNA by BM-DCs upon stimulation with Luk. BM-DCs were cultured with LukF (300 nM), LukS (300 nM) or LPS (1 μg/ml). Expression of IL-12p40 or HGPRT mRNA was examined by RT-PCR. Data are representative of three independent experiments.

For this purpose, we conducted the following three experiments. First, we measured the concentrations of LPS in LukF and LukS preparations by a limulus assay. Only a trace amount of LPS was detected in these preparations (1.5 pg/ml and 0.027 pg/ml in LukF and LukS, respectively). In addition, we tested whether LPS present in 300 nM of LukF and LukS (1.5 pg/ml and 0.027 pg/ml, respectively) activated BM-DCs to produce IL-12p40. As shown in Fig. 6A, LPS did not show such activity, although these Luk components clearly induced the production of this cytokine. In the next experiment, we tested the effect of polymixin B on the activity of Luk. As shown in Fig. 6B, IL-12p40 production by LukF-stimulated BM-DCs was not significantly inhibited by polymixin B, whereas the same treatment completely abrogated the cytokine production caused by LPS. Finally, we tested the effect of boiling on the activity of LukF. As shown in Fig. 6C, IL-12p40 production by LukF-stimulated BM-DCs was significantly inhibited by boiling, whereas the same treatment did not affect cytokine production by LPS.

3.3. Activation of TLR4-mediated signaling by leukocidin

Finally, to further define whether Luk activated the TLR4-mediated signaling pathway, we tested the effect of LukF and LukS on the activation of NF-κB. For this purpose, we conducted a luciferase reporter assay using HEK293T cells transfected with the gene for TLR4, MD2 and CD14 and the luciferase gene linked to the promoter sequence containing an NF-κB-binding site. As shown in Fig. 7A, LukF as well as LPS induced luciferase activity in TLR4/MD2/CD14-expressing HEK293T cells, although such activity was not clearly detected upon stimulation with LukS. In further experiments, we tested whether Luk activated luciferase in HEK293T transfected with TLR2. As shown in Fig. 7B, neither LukF nor LukS induced this activity in TLR2-expressing HEK293T cells, although addition of PG resulted in a striking level of activity. These data indicate that LukF triggered the signaling pathway leading to the activation of NF-κB via TLR4, but not via TLR2.

4. Discussion

Major findings in the present study are that: (1) LukF activated BM-DCs to induce mRNA expression and intracellular accumulation of IL-12p40, to promote the secretion of this cytokine, to induce the production of TNF-α and to

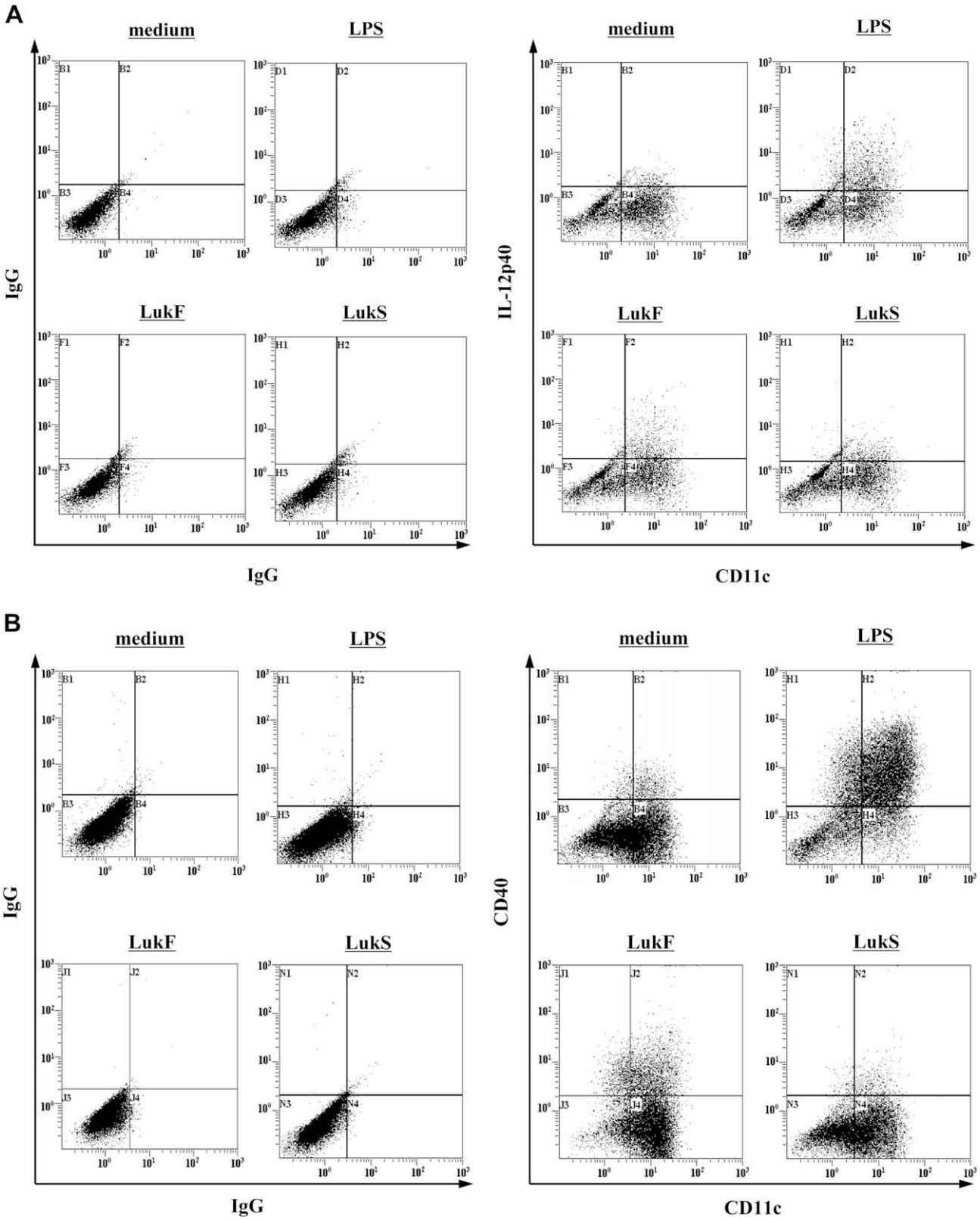

Fig. 4. Expression of IL-12p40 and CD40 by BM-DCs upon stimulation with Luk. BM-DCs were cultured with LukF (300 nM), LukS (300 nM) or LPS (1 μg/ml). The cells were stained with FITC-anti-CD11c mAb and PE-anti-IL-12p40 or -CD40 mAb, and expression of these molecules was analyzed using flow cytometry. Data are representative of three independent experiments.

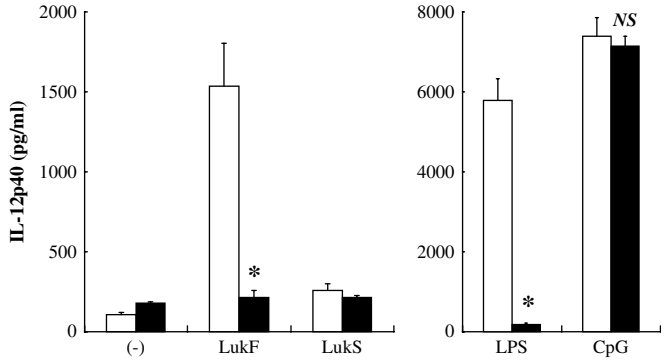

Fig. 5. Production of IL-12p40 by BM-DCs from C3H/HeN or C3H/HeJ mice upon stimulation with Luk. BM-DCs from C3H/HeN or C3H/HeJ mice were cultured with LukF (300 nM), LukS (300 nM), LPS (1 ng/ml) or CpG1826 (1 μg/ml). IL-12p40 concentration in the culture supernatants was measured by ELISA. Data are mean ± SD of triplicate cultures. Open column, C3H/HeN; closed column, C3H/HeJ. CpG: CpG1826. *$p < 0.05$; *NS*, not significant, compared with C3H/HeN.

enhance the expression of CD40 on their surface; (2) LukS showed a weak activity on these cells; (3) these components did not co-operate in the production of IL-12p40; (4) TLR4 was essential for LukF-induced activation of BM-DCs; and (5) LukF triggered TLR4, but not TLR2, to activate NFκB in a luciferase reporter assay, while LukS did not show such activity. These results demonstrate that Luk, especially LukF, caused TLR4-dependent activation of BM-DCs.

Luk has been known as a leukocytolytic toxin against human and rabbit leukocytes [6]. In this process, LukF and LukS assemble on the membranes of target cells into ring-shaped hetero-oligomers, which results in the formation of transmembrane pores and the cytolysis of these cells [6]. These findings raise the possibility that BM-DC secretion of IL-12p40 by Luk may be due to the release of preformed cytokine from the lytic cells, rather than by synthesis of this cytokine, as long assumed by previous investigators [6]. However, our results are not compatible with this possibility, because (1) Luk did not show any lytic effect on BM-DCs, which contrasts with that on human PMNL (data not shown), (2) it took longer than 6 h for BM-DCs to secrete IL-12p40 after Luk stimulation, (3) mRNA and intracellular expression of this cytokine were detected in Luk-activated BM-DCs, and (4) LukF and LukS did not show any co-operative effect in the activation of BM-DCs in contrast to their cytolytic activity, for which both components are essential. Thus, our current observations clearly indicate that Luk promoted BM-DCs to release IL-12p40 by synthesis of this cytokine, but not by cytolysis of these cells.

Among the cholesterol-dependent cytolysins which bind to cholesterol in cell membranes, forming oligomers and creating transmembrane pores, pneumolysin from *Streptococcus pneumonia* was reported to be sensed by TLR4 and to release proinflammatory cytokines by murine peritoneal macrophages [23], similarly to our current observations. By contrast, to the best of our knowledge, there has been no report demonstrating TLR4-dependent induction of proinflammatory cytokine synthesis and expression of co-stimulatory molecules by

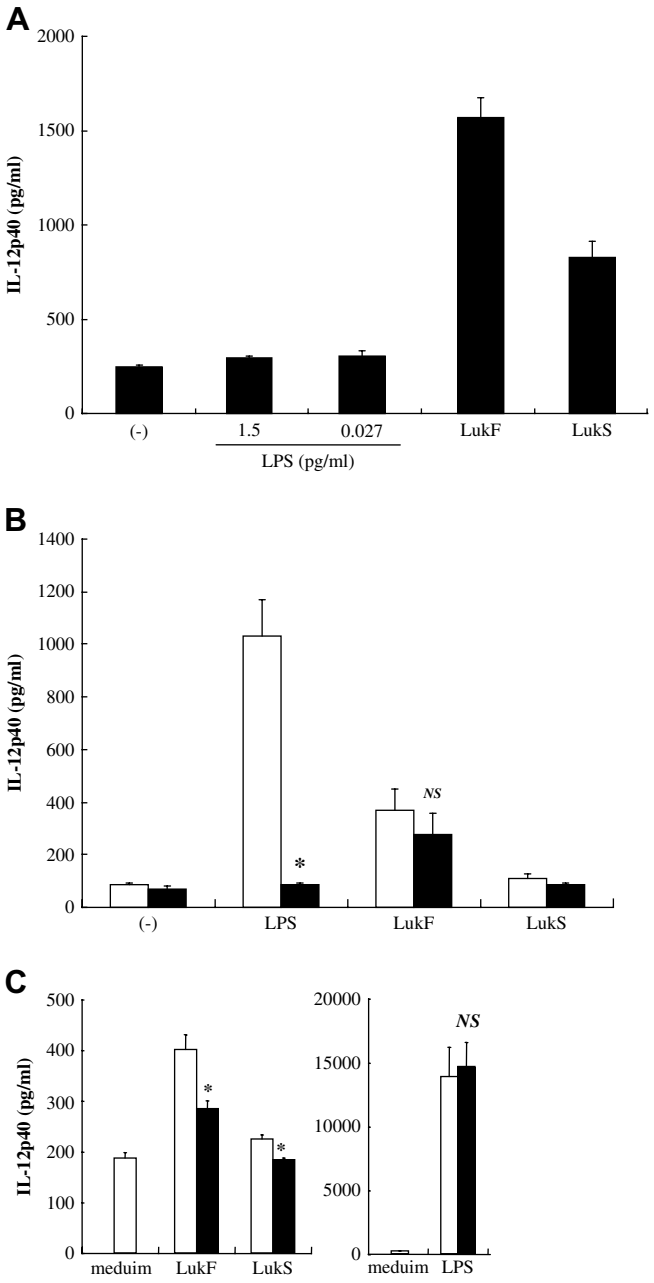

Fig. 6. Activation of BM-DCs by Luk is not mediated by LPS contamination. (A) BM-DCs were cultured with indicated doses of LPS. (B) BM-DCs were cultured with LukF (300 nM), LukS (300 nM) or LPS (1 ng/ml) in the presence or absence of polymixin B (10 μg/ml). (C) BM-DCs were cultured with LukF (300 nM) or LPS (1 μg/ml) which was boiled or not for 5 min. IL-12p40 concentration in the culture supernatants was measured by ELISA. Data are mean ± SD of triplicate cultures. (B) Open column, no polymixin B; closed column, presence of polymixin B; PMB, polymixin B. *$p < 0.05$; *NS*, not significant, compared with IL-12p40 production in the absence of polymixin B. (C) Open column, not boiled; closed column, boiled. *$p < 0.05$; *NS*, not significant, compared with IL-12p40 production by non-boiled Luk.

macrophages and dendritic cells after stimulation with Luk. In an earlier study by König et al. [10], Panton–Valentine Luk (Luk-PV) was reported to induce the production of IL-8 by human leukocytes at sublytic concentrations. In addition, Younis and co-workers [24] recently suggested that LukM-FPV (p83), a staphylococcal two-component toxin family,

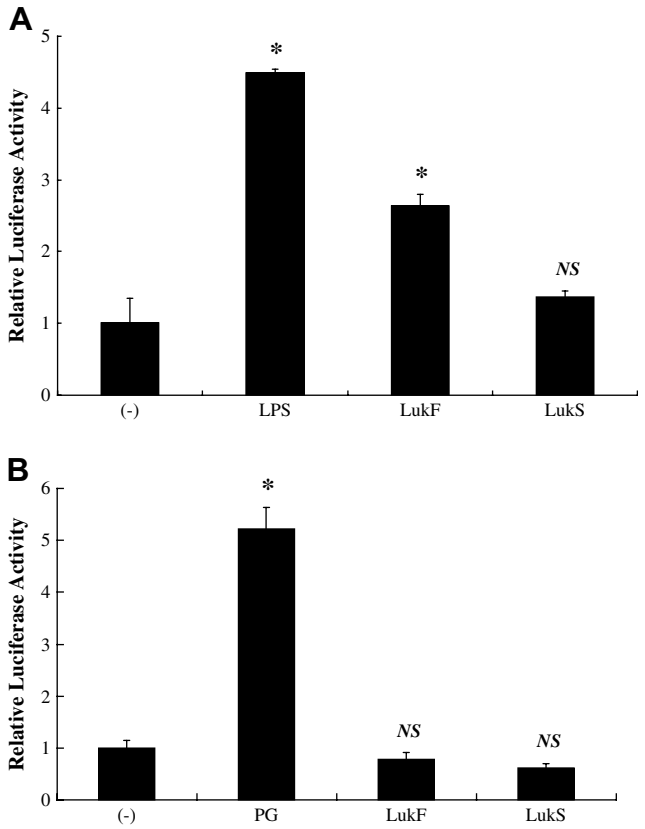

Fig. 7. NF-κB activation via TLR4 by Luk. HEK293T cells transfected with TLR4/MD2/CD14 (A) or TLR2 (B) genes or control vector were treated with LukF (10 nM), LukS (10 nM) or LPS (200 ng/ml), PG (200 ng/ml) for 6 h. The luciferase activity in each sample was determined as described in Section 2. Data are expressed as relative values to those of control vector and presented as mean ± SD of triplicate cultures. $*p < 0.05$; *NS*, not significant, compared with relative luciferase activity without stimulation.

stimulated the production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, by bovine leukocytes. However, these investigators did not address the contribution of TLR4 to these responses.

BM-DCs from TLR4-mutant mice showed a defective response to LukF, suggesting that TLR4 acted as a receptor to sense this molecule. TLR4 is well documented as a specific receptor of LPS [25]. Although *S. aureus*, a Gram-positive bacterium, does not release LPS, we could not completely exclude the possibility that LPS contaminating the Luk preparations may have caused the observed responses. However, this possibility was clearly excluded, because (1) LPS was not sufficiently detected as having caused the production of IL-12p40 by BM-DCs stimulated with LukF, (2) the effect of LukF was not significantly affected by polymixin B which completely abrogated the LPS response, and (3) the LukF-induced response was significantly inhibited by boiling, while that caused by LPS was not. Thus, we demonstrated for the first time that TLR4 plays a critical role in LukF-induced activation of BM-DCs. This conclusion was supported by additional evidence indicating that LukF caused the activation of NFκB in HEK293T cells transfected with TLR4/MD2/CD14, but not with TLR2, in a luciferase reporter assay.

The current findings may suggest that Luk contributes to initiation of the host defense response after infection with *S. aureus*. At present, we have not conducted the experiments to elucidate the *in vivo* role of Luk in the inflammatory response and host defense to *S. aureus* infection. In an earlier study by Takeuchi et al. [26], $TLR2^{-/-}$ mice were highly susceptible to intravenous infection with *S. aureus* and macrophages from $TLR2^{-/-}$ but not $TLR4^{-/-}$ mice hampered in the production of IL-6 and TNF-α, as compared to wild-type mice, which does not support the role of TLR4 in the host defense to this infection. The authors assumed that failure of host immune cells to respond to peptidoglycan from *S. aureus*, a TLR2 agonist [27], contributed to the fatal outcome of infection in $TLR2^{-/-}$ mice. Recently, however, Stenzel and co-workers [28] have demonstrated using a mouse model of brain abscess the possible involvement of TLR4 as well as TLR2 in inflammatory responses caused by this bacterium. Thus, further investigations are necessary to define the biological significance of our findings in *in vivo* infection with *S. aureus*.

Acknowledgements

The authors thank Dr Masao Mitsuyama for kind gifts of luciferase reporter plasmids, HEK293T cells and mouse TLR2, TLR4, MD2 and CD14 cDNA.

References

[1] R. Williams, Health carriage of *Staphylococcus aureus*: its prevalence and importance, Bacteriol. Rev. 27 (1963) 56–71.
[2] F.A. Waldvogel, *Staphylococcus aureus*, in: G.L. Mandekk, J.E. Bennett, R. Dolin (Eds.), Principles and Practice of Infectious Disease, Churchill Livingstone, Philadelphia, 2000, pp. 2069–2092.
[3] W.R. Jarvis, The epidemiology of colonization, Infect. Control Hosp. Epidemiol. 17 (1996) 47–52.
[4] S.E. Cosgrove, Y. Qi, K.S. Kaye, A.W. Karchmer, Y. Carmeli, The impact of methicillin resistance in *Staphylococcus aureus* bacteremia on patient outcomes: mortality, length of stay, and hospital charges, Infect. Control Hosp. Epidemiol. 26 (2005) 166–174.
[5] F. Vandenesch, T. Naimi, M.C. Enright, G. Lina, G.R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M.E. Reverdy, J. Etienne, Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton–Valentine leukocidin genes: worldwide emergence, Emerg. Infect. Dis. 9 (2003) 978–984.
[6] J. Kaneko, Y. Kamio, Bacterial two-component and hetero-heptameric pore-forming cytolytic toxin: structures, pore-forming mechanism, and organization of the genes, Biosci. Biotechnol. Biochem. 68 (2004) 981–1003.
[7] A. Nishiyama, J. Kaneko, M. Harata, Y. Kamio, Assembly of staphylococcal leukocidin into a pore-forming oligomer on detergent-resistant membrane microdomains, lipid rafts, inhuman polymorphonuclear leukocytes, Biosci. Biotechnol. Biochem. 70 (2006) 1300–1307.
[8] M. Noda, I. Kato, T. Hirayama, F. Matsuda, Mode of action of staphylococcal leukocidin: effects of the S and F components on the activities of membrane-associated enzymes of rabbit polymorphonuclear leukocidin, Infect. Immun. 35 (1982) 38–45.
[9] D.A. Colin, I. Mazurier, S. Sire, V. Finck-Barbançon, Interaction of the two components of leukocidin from *Staphylococcus aureus* with human polymorphonuclear leukocyte membranes: sequential binding and subsequent activation, Infect. Immun. 62 (1994) 3184–3188.
[10] B. König, G. Prévost, Y. Piémont, W. König, Effects of *Staphylococcus aureus* leukocidin on inflammatory mediator release from human granulocytes, J. Infect. Dis. 171 (1995) 607–613.

[11] T. Kaisho, S. Akira, Pleiotropic function of Toll-like receptors, Microbes Infect. 6 (2004) 1388–1394.
[12] T. Kaisho, S. Akira, Regulation of dendritic cell function through toll-like receptors, Curr. Mol. Med. 3 (2003) 759–771.
[13] T. Kaisho, S. Akira, TLR signaling, Cell Death Differ 13 (2006) 816–825.
[14] T. Seya, M. Matsumoto, A lipoprotein family *Mycoplasma fermentans* confers host immune activation through Toll-like receptor 2, Int. J. Biochem. Cell Biol. 34 (2002) 901–906.
[15] U. Zähringer, B. Lindner, S. Inamura, H. Heine, C. Alexander, TLR2-promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity, Immunobiology 213 (2008) 205–224.
[16] S.I. Miller, R.K. Ernst, M.W. Bader, Lps, TLR4 and infectious disease diversity, Nat. Rev. Microbiol. 3 (2005) 36–46.
[17] M. Foti, F. Granucci, M. Pelizzola, O. Beretta, P. Ricciardi-Castagnoli, Dendritic cells in pathogen recognition and induction of immune responses: a functional genomics approach, J. Leukoc. Biol. 79 (2006) 913–916.
[18] H. Nariya, I. Asami, T. Ozawa, Y. Beppu, K. Izaki, Y. Kamio, Improved method for purification of leukocidin and γ-hemolysin components from *Staphylococcus aureus*, Biosci. Biotechnol. Biochem. 57 (1993) 2198–2199.
[19] M.B. Lutz, N. Kukutsch, A.L. Ogilvie, S. Rossner, F. Koch, N. Romani, G. Schuler, An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow, J. Immunol. Methods 223 (1999) 77–92.
[20] K. Kawakami, M. Tohyama, X. Qifeng, A. Saito, Expression of cytokines and inducible nitric oxide synthase mRNA in the lung of mice infected with *Cryptococcus neoformans*: effects of interleukin-12, Infect. Immun. 65 (1997) 1307–1312.
[21] G. Xia, A. Miyazato, K. Inden, K. Nakamura, K. Shiratori, K. Nakagawa, T. Miyazawa, K. Suzuki, M. Kaku, K. Kawakami, *Cryptococcus neoformans* inhibits nitric oxide synthesis caused by CpG-oligodeoxynucleotide-stimulated macrophages in a fashion independent of capsular polysaccharide, Microbiol. Immunol. 52 (2008) 171–179.
[22] D. Werling, T.W. Jungi, Toll-like receptors linking innate and adaptive immune response, Vet. Immunol. Immunopathol. 91 (2003) 1–12.
[23] R. Malley, P. Henneke, S.C. Morse, M.J. Cieslewicz, M. Lipsitch, C.M. Thompson, E. Kurt-Jones, J.C. Paton, M.R. Wessels, D.T. Golenbock, Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection, Proc. Natl. Acad. Sci. U.S.A. 18 (2003) 1966–1971.
[24] A. Younis, O. Krifucks, G. Fleminger, E.D. Heller, N. Gollop, A. Saran, G. Leitner, *Staphylococcus aureus* leukocidin, a virulence factor in bovine mastitis, J. Dairy Res. 72 (2005) 188–194.
[25] M.A. Freudenberg, S. Tchaptchet, S. Keck, G. Fejer, M. Huber, N. Schütze, B. Beutler, C. Galanos, Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infection: benefits and hazards of LPS hypersensitivity, Immunobiology 213 (2008) 193–203.
[26] O. Takeuchi, K. Hoshino, S. Akira, Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection, J. Immunol. 15 (2000) 5392–5396.
[27] B. Fournier, D.J. Philpott, Recognition of *Staphylococcus aureus* by the innate immune system, Clin. Microbiol. Rev. 18 (2005) 521–540.
[28] W. Stenzel, S. Soltek, M. Sanchez-Ruiz, S. Akira, H. Miletic, D. Schlüter, M. Deckert, Both TLR2 and TLR4 are required for the effective immune response in *Staphylococcus aureus*-induced experimental murine brain abscess, Am. J. Pathol. 172 (2007) 132–145.