FULL PAPER *Surgery*

Priming Effects of Lipopolysaccharide and Inflammatory Cytokines on Canine Granulocytes

Kenichi MAEDA[1], Iwao SAKONJU[1]*, Aya KANDA[1], Tatsuya SUZUKI[1], Tomoko KAKUTA[1], Shunsuke SHIMAMURA[1], Shozo OKANO[1] and Katsuaki TAKASE[1]

[1]*School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628, Japan*

(Received 24 December 2008/Accepted 20 September 2009/Published online in J-STAGE 13 November 2009)

ABSTRACT. Granulocytes play a pivotal role in natural immunity. Under inflammatory conditions, granulocytes are universally primed by several agents, such as endotoxins and inflammatory cytokines. Primed granulocytes exert potent adhesiveness, chemotaxis, phagocytosis and reactive oxygen species (ROS) production, effectively eliminating invading agents. Reactivity against priming agents is known to vary with species; however, there have been few reports on the effects of priming agents on canine granulocytes. In the present study, we assayed the priming effects of lipopolysaccharide (LPS), recombinant canine tumor necrosis factor-α (rcTNF-α) and recombinant canine granulocyte macrophage colony-stimulating factor (rcGM-CSF) on canine granulocyte function *in vitro*. Isolated recombinant canine were primed with various concentrations of LPS, rcTNF-α and rcGM-CSF, and CD11b expression was assayed. Furthermore, actin polymerization, phagocytosis and ROS production were then assayed at primer concentrations where enhancement of CD11b expression was observed. LPS did not enhance canine granulocyte function. Phagocytosis and actin polymerization were not enhanced by priming agents; however, rcTNF-α and rcGM-CSF enhanced CD11b expression and ROS production in canine granulocytes. These results suggest that priming effects are mainly reflected in CD11b expression and ROS production, with rcGM-CSF and rcTNF-α having a priming effect similar to that observed in humans.

KEY WORDS: canine, granulocytes, inflammatory cytokine, LPS, priming.

J. Vet. Med. Sci. 72(1): 55–60, 2010

Granulocytes, including neutrophils, are attracted to skin wounds by chemotactic mediators released by thrombosis and microorganisms [11]. Upon migration to skin wounds or sites of infection, granulocytes are primed by inflammatory cytokines and products of microorganisms. Primed granulocytes immediately initiate inflammatory responses, such as releasing reactive oxygen species (ROS) and phagocytosis. Thus, the inflammatory response by primed granulocytes contributes to protecting surgical patients from infection at surgical sites. At the same time, primed granulocytes are also involved in sepsis and septic shock, which represent an intense systemic inflammatory response syndrome (SIRS) [9, 20].

The development of SIRS may be the result of granulocytes being excessively primed by an imbalance in the endogenous production of cytokines. Thus, primed granulocytes are of interest in various clinical conditions and perioperative immunity. It is therefore important to collect knowledge on the priming of canine granulocytes in veterinary medicine. However, the reactivity of granulocytes against priming agents *in vitro* is known to vary with species[18, 22, 25, 31]. Data on the priming of canine granulocytes is lacking and it has only been reported with regard to complement fragment 5a and interleukin-8 [4]. We deemed it necessary to confirm the effects of priming agents that are usually observed in inflammatory conditions on canine granulocytes *in vitro*.

* CORRESPONDENCE TO: SAKONJU, I., Department of Small Animal Surgery, School of Veterinary Medicine, Kitasato University, 23–35–1 Higashi, Towada, Aomori 034–8628, Japan.
e-mail: sakon@vmas.kitasato-u.ac.jp

In the present study, we aimed to investigate the priming conditions of canine granulocytes. We tested the priming effects of LPS, recombinant canine GM-CSF (rcGM-CSF) and recombinant canine TNF-α (rcTNF-α) on canine granulocytes by focusing on expression of CD11b, which composes complement receptor 3 (CR3). Furthermore, we tested phagocytosis, actin polymerization and oxidative burst, all of which are initiated by CR3 recognition of invading agents.

MATERIALS AND METHODS

Granulocyte isolation: Granulocytes were isolated from the venous blood of healthy beagle dogs. Heparinized blood was mixed 4:1 with phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} (PBS(–)) (Sigma Chemical, St. Louis, MO, U.S.A.), and containing 6.0% Dextran T-500 (Wako Pure Chemical Industries, Osaka, Japan), and was incubated for 40 min at room temperature to sediment erythrocytes. The leukocyte-rich supernatant was mixed with the same quantity of PBS(–) and layered onto 15 m*l* of Histopaque-1077 (Sigma Chemical), followed by centrifugation at 400 × g for 30 min in order to separate granulocytes from peripheral blood mononuclear cells (PBMCs). Following aspiration of the PBMCs layer and remaining supernatant, erythrocytes were lysed with NH_4Cl lysis buffer (pH 7.4) for 10 min and were centrifuged 400 × g for 5 min. This yielded a preparation that was >90% granulocytes and >99% viable, as determined by trypan blue assay.

Purified granulocyte sediments were resuspended in Hanks' Balanced Salt Solution lacking Ca^{2+} and Mg^{2+}

(HBSS(–)) (GIBCO, Grand Island, NY, U.S.A.), and containing 10% autologous plasma buffered with 10 mM HEPES (pH 7.4) in order to assess expression of integrin (CD11b), phagocytosis and actin polymerization. To assess ROS production, granulocytes were resuspended in HBSS (–) containing 1% Fetal Bovine Serum (FBS) (Sanko-Jyunyaku, Tokyo, Japan) buffered with 10 mM HEPES (pH 7.4).

Granulocyte priming: For CD11b expression assay, suspended granulocytes were pre-treated with 1, 10 or 100 *n*g/m*l* LPS (from *E.coli*; Sigma Chemical), 0.5, 5 or 50 *n*g/m*l* recombinant canine GM-CSF (rcGM-CSF; R&D Systems, Minneapolis, MN, U.S.A.), or 1, 10 or 100 *n*g/m*l* recombinant canine TNF-α (rcTNF-α; R&D Systems, Minneapolis, MN, U.S.A.) at 37°C under 5% CO_2 for 30 min. PBS treatment was used as a control. The concentrations selected were those frequently reported in human studies [6, 7, 13]. For phagocytosis, actin polymerization and oxidative burst assay, suspended granulocytes were pre-treated with concentrations of priming agent at which CD11b up-regulation was observed (LPS, 10 *n*g/m*l*; rcGM-CSF, 5 *n*g/m*l*; rcTNF-α, 10 *n*g/m*l*) at 37°C under 5% CO_2 for 30 min. PBS treatment was used as a control.

CD11b expression assay: Granulocytes were incubated with primary monoclonal antibody at 4°C for 40 min. Monoclonal antibodies against mouse anti-dog CD11b (AbD Serotec, Kidlington, UK) were used. To detect these marker-positive cells, cells were incubated with R. phycoerythrin (RPE)-labeled goat anti-mouse IgG1 (AbD Serotec, Kidlington, UK) at 4°C for 40 min. After washing cells with PBS(–), samples were analyzed by flow cytometry using Expo 2000 software (Beckman-Coulter, Fullerton, CA, U.S.A.). The granulocyte population was gated from residual PBMCs based on their forward and side scatter characteristics on dot plots. The geometric mean fluorescent intensity (MFI) on histogram analysis was calculated as an expression index and is given as a percentage vs. control samples (100%).

Phagocytosis assay: Phagocytosis was evaluated by the microassay method, as reported previously, with slight modification [19]. Samples incubated with either PBS(–) or priming agent were incubated at 37°C for 30 min together with fluorescence beads (Fluoresbrite carboxy YG 1.0 micron microspheres; Polysciences, Warrington, PA, U.S.A.). Immediately after incubation, samples were placed on ice for 10 min. After cooling, samples were centrifuged at 400 × g for 5 min with 3 mM EDTA-2Na. Precipitated granulocytes were used for flow cytometric analysis using an Epics flow cytometer (Beckman-Coulter). The number of phagocytic cells was determined by comparison with background signals using histogram analysis, and was expressed as a percentage of control samples (100%).

Actin polymerization assay: Chemotactic potential was determined based on the amount of polymerized actin [24, 32]. Fallodin conjugates polymerized actin, and chemoattractants induce an overall increase in polymerized actin, resulting in increased MFI of FITC-phalloidin [12, 15, 21]. Granulocytes were re-suspended in PBS(–) containing 90% methanol and were incubated for 15 min. After washing with PBS(–), cells were incubated with 10 μ*l* of FITC-labeled Fallodin (Phalloidin-Fluorescein isothiocyanate; Sigma Chemical) for 15 min. Samples were analyzed by the same method as described for the CD11b expression assay.

Preparation of serum-opsonized zymosan (sOZ): Zymosan (Zymosan A from *S. cerevisiae*; Sigma Chemical) was boiled in ultra-pure water for 60 min, and was washed with sterilized ultra-pure water. After centrifugation, zymosan pellets were resuspended in fresh autologous serum at a concentration of 10 mg/m*l* and were incubated for 60 min at 37°C. After incubation, the suspension was washed twice with HBSS(–) and re-suspended in HBSS(–) at a concentration of 10 mg/m*l*.

Oxidative burst assay: Luminol-dependent chemiluminescence (CL) assays were performed using previously established methods with slight modifications [10, 27]. HBSS(–) with 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ containing 5 × 10^6 granulocytes, 10 μM luminol (Fluka Chemika Biochemika, Buchs, Switzerland) and 50 μg/m*l* horseradish peroxidase (Sigma Chemical) were prepared in each well of a 96-well microplate (Sumilon, Tokyo, Japan). The suspension (315 μ*l*) was incubated for 5 min at 37°C. After incubation, granulocytes were activated by adding 35 μ*l* sOZ (10 mg/m*l*), and CL (RLU/sec) was measured at 37°C every 0.2 seconds with a luminometer (Dynex Technologies, London, UK). The response was recorded over a period of 30 min. CL response was indicated by peak time (PT; seconds), which corresponds to the mean time from the recognition of sOZ to the maximum release of ROS from granulocytes, and area under the CL curve (AUC) given as a percentage vs. control samples (100%), which corresponds to the total ROS produced.

Statistical analysis: All data are expressed as means ± SE. Statistically significant differences obtained from one-way ANOVA analysis were further tested by Dunnett's test for post-hoc pairwise comparisons. P values of less than 0.05 were considered statistically significant.

RESULTS

Table 1 shows the MFI of CD11b expression. The MFI of CD11b expression after priming with various concentrations of LPS (100 *n*g/m*l*, 128.1 ± 24.6%; 10 *n*g/m*l*, 114.4 ± 19.2%; 1 *n*g/m*l*, 95.3 ± 4.5%) was not significantly different than that in Controls. In contrast, the MFI of CD11b expression after priming with various concentrations of rcGM-CSF (50 *n*g/m*l*, 163.3 ± 22.4%; 5 *n*g/m*l*, 159.3 ± 20.9%; 0.5 *n*g/m*l*, 159.7 ± 21.6%) and various concentrations of rcTNF-α (100 *n*g/m*l*, 180.0 ± 23.7%; 10 *n*g/m*l*, 170.8 ± 23.7%; 1 *n*g/m*l*, 144.3 ± 14.8%) was significantly higher than that in Controls (100%).

Figure 1 shows the proportion of FITC fluorescence-positive granulocytes, which indicates phagocytic rate. The phagocytic rate of granulocytes primed with 10 *n*g/m*l* LPS (90.1 ± 12.3%), 5 *n*g/m*l* rcGM-CSF (90.0 ± 25.8%) and 10 *n*g/m*l* rcTNF-α (89.7 ± 18.9%) was not significantly differ-

Table 1. Effects of priming agents on canine granulocyte CD11b expression. (n=7)

	Control	rcGM-CSF (*n*g/m*l*)			rcTNF-α (*n*g/m*l*)			LPS (*n*g/m*l*)		
		50	5	0.5	100	10	1	100	10	1
MFI (%)	100	163.3†	159.4†	159.7†	178.0†	170.8†	144.3†	128.1	114.4	95.3
SE	–	22.4	20.9	21.6	23.7	19.7	14.8	24.6	19.2	4.5

MFI: Mean fluorescence intensity. Data for priming agents was calculated as percentage vs. Controls (mean values ± SE). All concentrations of rcGM-CSF and rcTNF-α up-regulated CD11b expression. In contrast, LPS did not significantly up-regulate CD11b expression at any concentration.
†: Significant difference vs. Controls (P<0.05).

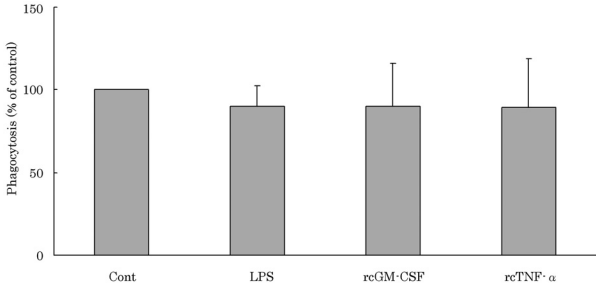

Fig. 1. Effects of priming agents on canine granulocyte phagocytosis (n=12). Data for priming agents was calculated as percentage vs. Controls (mean values ± SE). Priming agents did not enhance phagocytosis in canine granulocytes.

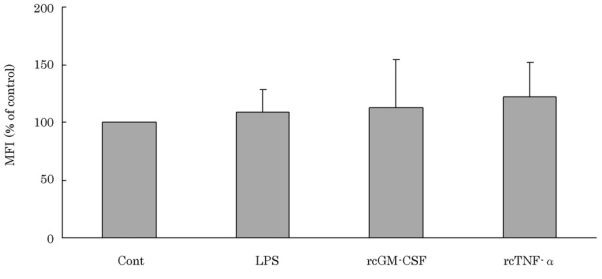

Fig. 2. Effects of priming agents on canine granulocyte polymerized actin filaments (n=12). MFI: Mean fluorescence intensity. Data for priming agents was calculated as percentage vs. Controls (mean values ± SE). All priming agents tended to enhance actin polymerization.

ent when compared to Controls (100%).

Figure 2 shows the amount of polymerized actin. The amount of polymerized actin after priming with 10 *n*g/m*l* LPS (108.9 ± 19.6%), 5 *n*g/m*l* rcGM-CSF (112.7 ± 41.7%) and 10 *n*g/m*l* rcTNF-α (122.3 ± 29.4%) was not significantly different than that in Controls (100%); thus, these agents did not affect actin polymerization.

Figure 3 shows the AUC for CL for 30 min, which indicates total ROS production induced by sOZ. The production of ROS in granulocytes primed with 10 *n*g/m*l* LPS (109.8 ± 12.4%) and 10 *n*g/m*l* rcTNF-α (108.1 ± 14.9%) was not significantly different from that in Controls. In contrast, granulocytes primed with 5 *n*g/m*l* rcGM-CSF (127.0 ± 18.5%) showed significantly higher ROS production relative to Controls (100%), and vs. priming with 10 *n*g/m*l* LPS and 10 *n*g/m*l* rcTNF-α.

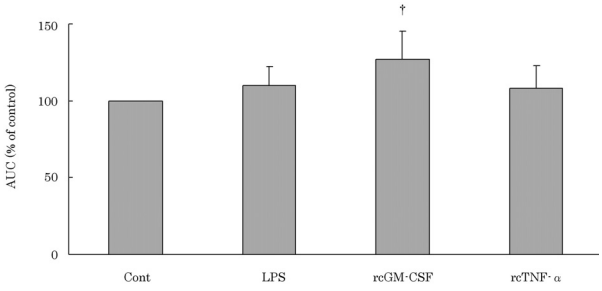

Fig. 3. Effects of priming agents on ROS production in canine granulocytes stimulated with sOZ (n=12). AUC: Area under CL curve, which corresponds to total ROS production. Data for priming agents was calculated as percentage vs. Controls (mean values ± SE). rcGM-CSF enhanced ROS production in canine granulocytes with sOZ. In contrast, rcTNF-α and LPS did not affect ROS production. †: Significant difference vs. Controls (P<0.05).

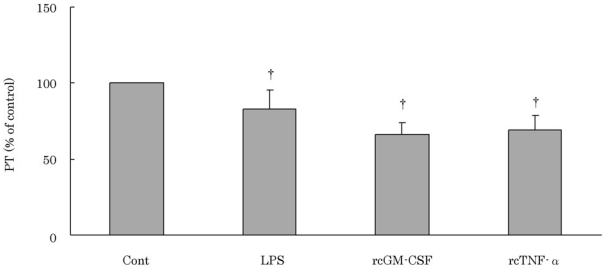

Fig. 4. Effects of priming agents on peak time of canine granulocytes stimulated with sOZ (n=12). PT: Peak Time. Data for priming agents was calculated as percentage vs. controls (mean values ± SE). All priming agents reduced PT vs. Controls. †: Significant difference vs. Controls (P<0.05).

Figure 4 shows PT, which represents the reaction rate of granulocytes against sOZ. The PT of granulocytes primed with 10 *n*g/m*l* LPS (82.8 ± 12.3%), 10 *n*g/m*l* rcTNF-α (69.1 ± 9.6%) and 5 *n*g/m*l* rcGM-CSF (65.8 ± 8.3%) was significantly shorter when compared to Controls (100%).

DISCUSSION

Granulocytes play an important role in the first line of

host defense against invading agents, and in perioperative patients, granulocyte priming is essential for protection against infection at surgical sites. Furthermore, it is known that excessively primed granulocytes are pivotal cellular mediators in the pathogenesis of systemic inflammatory response syndrome (SIRS), adult respiratory distress syndrome (ARDS), and post-injury multiple organ failure (MOF) [23, 26]. The study of canine granulocyte priming may thus provide insight into regulating these clinical conditions and perioperative immunity.

It is known that there are species-specific differences in reactivity to priming agents. Thus, the reactivity of canine granulocytes differs from that of other species. However, information is lacking about the priming effects of inflammatory agents on canine granulocytes. In the present study, we aimed to investigate the priming effects of LPS and inflammatory cytokines, such as TNF-α and GM-CSF, on canine granulocytes.

CD11b plays a prominent role in mediating granulocyte adherence, as it is required for the attachment of granulocytes to vascular endothelium [1]. CD11b also regulates phagocytosis, as well as actin polymerization and oxidative burst; thus, CD11b expression is recognized as a good maker of cell activity [8, 17, 30]. In the present study, 100 and 10 *n*g/m*l* LPS only slightly up-regulated CD11b expression, while 1 *n*g/m*l* LPS showed no changes. These results suggest that LPS weakly potentiates canine granulocyte adherence. In contrast, rcGM-CSF and rcTNF-α significantly up-regulated CD11b expression when compared with Controls, which suggests that these agents potentiate canine granulocyte adherence.

Granulocytes phagocytize invading agents via recognition of cell surface receptors, such as complement receptors and Fc receptors. Complement receptors, particularly complement receptor 3 (CR3), play a pivotal role in granulocyte phagocytosis, and CD11b forms the α-chain of CR3 [1]. Kumaratilake *et al.* [14] reported that priming agents such as TNF-α and GM-CSF up-regulate CD11b expression and enhance phagocytosis of protozoa in humans. As described above, rcTNF-α and rcGM-CSF up-regulated CD11b expression, although the tested priming agents tended to inhibit rather than enhance phagocytosis in canine granulocytes. This apparent contradiction may be explained by the aggregation of granulocytes. Lavkan *et al.* [16] reported that TNF-α and LPS increase human neutrophil aggregation. In the present study, canine granulocytes exposed to these priming agents appear to aggregate, and thus could not phagocytize the latex beads.

Granulocytes migrate to sites of bacterial ingress or tissue damage through the process of chemotaxis [29]. Actin polymerization is necessary for morphological polarity and for migration of neutrophils in response to chemotactic gradients [24, 32]; thus, actin polymerization is necessary in chemotaxis. Kutuna *et al.* [15] reported that stimulation of human neutrophils with TNF, GM-CSF and G-CSF results in an overall decrease in polymerized actin, and it is conceivable that cytokines partially contribute to accumulation of neutrophils at inflammatory sites by preventing neutrophils from leaving. In the present study, actin polymerization remained unchanged, and these results indicate that our priming agents did not polymerize actin in canine granulocytes. However, Berkow *et al.* [2] reported that TNF induces a slight increase in polymerized actin content in human neutrophils. The precise mechanisms for cytokine-induced actin depolymerization in neutrophils remain to be elucidated.

The oxidative burst is one of the most important defense mechanisms against infectious diseases and can be stimulated by chemoattractants [3, 5]. In the present study, PT was reduced by all priming agents, suggesting that priming agents enhance the responsiveness to sOZ in canine granulocytes. Priming agents tended to increase the ROS production of canine granuloytes stimulated by sOZ; reGM-CSF potently increased ROS production, consistent with the up-regulation of CD11b. However, despite up-regulating CD11b expression, rcTNF-α did not increase ROS production, and this apparent contradiction may be explained by the bilateral effects of TNF-α on granulocytes. Yamashita *et al.* [28] reported that TNF-α exerts two separate effects in neutrophils, stimulating effector functions while simultaneously inducing apoptosis, by introducing a priming signal for enhanced oxygen radical production that is usually masked by TNF-α-induced apoptotic processes. Thus, TNF-α induces apoptotic processes, and this prevents an overall increase in ROS production in canine granulocytes.

As described above, we investigated the effects of priming agents on CD11b expression, phagocytosis, actin polymerization and oxidative burst in canine granulocytes. We tested priming agents at concentrations that have previously been confirmed to show priming effects in humans. Dang *et al.* reported that 500 nM (approximately 7.1 *n*g/m*l*) human recombinant GM-CSF or 10 *n*g/m*l* human recombinant TNF-α prime human neutrophils [6, 7]. Under the present concentrations (rcGM-CSF, 5 *n*g/m*l*; rcTNF-α, 10 *n*g/m*l*), no changes in phagocytosis or actin polymerization were seen, but rcGM-CSF and rcTNF-α up-regulated CD11b expression. Jinnouchi *et al.* reported that 10 *n*g/m*l* LPS primed human neutrophils [13], but we found that LPS only slightly up-regulated CD11b expression and did not enhance ROS production, which contradicts previous reports. As described above, LPS does not have a priming effect on canine granulocytes; in fact, McClenahan *et al.* [18] reported that LPS did not up-regulate CD11b expression in bovine granulocytes, which suggests that the effects of LPS on granulocytes vary with species. Furthermore, the priming effects of LPS may depend on inflammatory cytokines produced by other white blood cells, such as mononuclear cells, and the direct priming effects are weaker than those of the inflammatory cytokines tested.

In conclusion, we did not observe a significant priming effect for LPS, suggesting that LPS does not prime canine granulocytes. In contrast, we confirmed that rcGM-CSF and rcTNF-α prime canine granulocytes, primarily by stimulating CD11b expression *in vitro*. These results may help

to study canine granulocyte function in the future. However, the present results apparently contradicted previous reports on granulocytes from other species; thus, further study of individual priming agents is needed.

REFERENCES

1. Arnout, M. A. 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* **75**: 1037–1050.
2. Berkow, R. L., Wang, D., Larrick, J. W., Dodson, R. W. and Howard, T. H. 1987. Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor. *J. Immunol.* **139**: 3783–3791.
3. Breathnach, R., Donahy, C., Jones, B. R. and Bloomfield, F. J. 2006. Characterization of certain inflammatory variables in the peripheral blood of clinically healthy dogs. *Vet. J.* **171**: 98–105.
4. Burgener, I., Jungi, T. W., Zurbriggen, A. and Tipold, A. 1998. Dose-dependent priming or desensitization induced by chemotactic agents in chemiluminescence experiments with canine and human neutrophils. *Vet. Immunol. Immunopathol.* **66**: 11–24.
5. Burgess, L. A., Cescutti, P., Ernst, R. K. and Speert D. P. 2006. Exopolysaccharides from Burkholderia cenocepacia inhibit neutrophil chemotaxis and scavenge reactive oxygen species. *J. Biol. Chem.* **281**: 2526–2532.
6. Dang, P. M., Dewas, C., Gaudry, M., Fay, M., Pedruzzi, E., Gougerot-Pocidalo, M. A. and El Benna, J. 1999. Priming of human neutrophil respiratory burst by granulocyte/macrophage colony-stimulating factor (GM-CSF) involves partial phosphorylation of p47(phox). *J. Biol. Chem.* **274**: 20704–20708.
7. Dang, P. M., Stensballe, A., Boussetta, T., Raad, H., Dewas, Cedric., Kroviarski, Yolande., Hayem, G., Jensen, O. N., Gougerot Pocidalo, M. and El Benna, Jamel. 2006. A specific p47phox serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. *J. Clin. Invest.* **116**: 2033–2038.
8. Davey, P. C., Zuzel, M., Kamiguti, A. S., Hunt, J. A. and Aziz, K. A. 2000. Activation-dependent proteolytic degradation of polymorphonuclear CD11b. *Br. J. Haematol.* **111**: 934–942.
9. Fan. J., Li. Y., Levy, R. M., Fan, J. J., Hackam, D. J., Vodovotz, Y., Yang, H., Tracey, K. J, Billiar, T. R. and Wilson, M. A. 2007. Hemorrhagic shock induces NAD(P)H oxidase activation in neutrophils: role of HMGB1-TLR4 signaling. *J. Immunol.* **15**: 6573–6580.
10. Hasegawa, H., Suzuki, K., Nakaji, S. and Sugawara, K. 1997. Analysis and assessment of the capacity of neutrophils to produce reactive oxygen species in a 96-well microplate format using lucigenin- and luminol-dependent chemiluminescence. *J. Immunol. Methods* **210**: 1–10.
11. Hofer, H. P., Kukovetz, E., Egger, G., Wildburger, R., Quehenberger, F. and Schaur, R. J. 1994. Polymorphonuclear leucocyte migration response in uneventful wound healing following trauma surgery. A contribution to the search for objectifiable criteria in wound healing monitoring. *Arch. Orthop. Trauma Surg.* **113**: 170–173.
12. Howard, T. H. and Meyer, W. H., 1984. Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. *J. Cell Biol.* **98**: 1265–1271.
13. Jinnouchi, A., Aida, Y., Nozoe, K., Maeda, K. and Pabst, M. J. 2005. Local anesthetics inhibit priming of neutrophils by lipopolysaccharide for enhanced release of superoxide: suppression of cytochrome b558 expression by disparate mechanisms. *J. Leukoc. Biol.* **78**: 1356–1365.
14. Kumaratilake, L. M., Ferrante, A., Jaeger, T. and Rzepczyk, C. 1996. GM-CSF induced priming of human neutrophils for enhanced phagocytosis and killing of asexual blood stages of Plasmodium falciparum:synergistic effects of GM-CSF and TNF. *Parasite Immunol.* **18**: 115–123.
15. Kutsuna, H., Suzuki, K., Kamata, N., Kato, T., Hato, F., Mizuno, K., Kobayashi, H., Ishii, M. and Kitagawa, S. 2004. Actin reorganization and morphological changes in human neutrophils stimulated by TNF-α, GM-CSF, and G-CSF: the role of MAP kinases. *Am. J. Physiol. Cell Physiol.* **286**: 55–64.
16. Lavkan, A. H., Astiz, M. E. and Rackow, E. C. 1998. Effects of proinflammatory cytokines and bacterial toxins on neutrophil rheologic properties. *Crit. Care Med.* **26**: 1677–1682.
17. Martins, P. S., Brunialti, M. K., Martos, L.S., Machado, F. R., Assunçao, M. S., Blecher, S. and Salomao, R. 2008. Expression of cell surface receptors and oxidative metabolism modulation in the clinical continuum of sepsis. *Crit. Care.* **12**: R25.
18. McClenahan, D., Fagliari, J., Evanson, O. and Weiss, D. 2000. Role of inflammatory mediators in priming, activation, and deformability of bovine neutrophils. *Am. J. Vet. Res.* **61**: 492–498.
19. Okuno, S., Noda, H., Kugimiya, T. and Saionji, K. 1996. The influence of local anesthetics on human leukocyte functions studied by micro whole blood collection and flow cytometry. *Masui* **45**: 317–325 (in Japanese).
20. Qureshi, S. S., Lewis, S. M., Gant, V. A., Treacher, D., Davis, B. H. and Brown, K. A. 2001. Increased distribution and expression of CD64 on blood polymorphonuclear cells from patients with the systemic inflammatory response syndrome (SIRS). *Clin. Exp. Immunol.* **125**: 258–265.
21. Sham, R. L., Phatak, P. D., Ihne, T. P., Abboud, C. N. and Packman, C. H. 1993. Signal pathway regulation of interleukin-8-induced actin polymerization in neutrophils. *Blood* 82: 2546–2551.
22. Soler Rodriguez, A. M., Zhang, H., Lichenstein, H. S., Qureshi, N., Niesel, D. W., Crowe, S. E., Peterson, J. W. and Klimpel, G. R. 2000. Neutrophil activation by bacterial lipoprotein versus lipopolysaccharide: differential requirements for serum and CD14. *J. Immunol.* **164**: 2674–2683.
23. Vedder, N. B., Fouty, B. W., Winn, R. K., Harlan, J. M. and Rice, C. L. 1989. Role of neutrophils in generalized reperfusion injury associated with resuscitation from shock. *Surgery* **106**: 509–516.
24. Watts, R. G., Crispens, M. A. and Howard, T. H. 1991. A quantitative study of the role of F-actin in producing neutrophil shape. *Cell Motil. Cytoskeleton.* **19**: 159–168.
25. Weiss, D. J. and Evanson, O. A. 2002. Evaluation of lipopolysaccharide-induced activation of equine neutrophils. *Am. J. Vet. Res.* **63**: 811–815.
26. Windsor, A. C. J., Mullen, P. G., Fowler, A. A. and Sugerman, H. J. 1993. Role of the neutrophil in adult respiratory distress syndrome. *Br. J. Sur.* **80**: 10–17.
27. Yamamori, T., Inanami, O., Nagahata, H., Yu Dong, C. and Kuwabara, M. 2000. Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. *FEBS Lett.* **467**: 253–258.
28. Yamashita, K., Takahashi, A., Kobayashi, S., Hirata, H., Mesner, P. W. Jr., Kaufmann, S. H., Yonehara, S., Yamamoto, K., Uchiyama, T. and Sasada, M. 1999. Caspases mediate tumor necrosis factor-alpha-induced neutrophil apoptosis and

downregulation of reactive oxygen production. *Blood* **93**: 674–685.
29. Zachariae, C. O. 1993. Chemotactic cytokines and inflammation. Biologicaproperties of the lymphocyte and monocyte chemotactic factors ELCF, MCAF and IL-8. *Acta Derm. Venereol. (Suppl.)* **181**: 1–37.
30. Zhou, M. and Brown, E. J. 1994. CR3 (Mac-l, CgM 2, CD11b/CD18) and Fc/RIII cooperate in generation of a neutrophil respiratory burst. requirement for FcTRII and tyrosine phosphorylation. *J. Cell Biol.* **125**: 1407–1416.
31. Zhou, X., Gao, X. P., Fan, J., Liu, Q., Anwar, K. N., Frey, R. S. and Malik, A. B. 2005. LPS activation of Toll-like receptor 4 signals CD11b/CD18 expression in neutrophils. *Am. J. Physiol. Lung Cell Mol. Phy.* **288**: 655–662.
32. Zigmond, S. H. and Hirsch, J. G. 1972. Effects of cytochalasin B on polymorphonuclear leucocyte locomotion, phagocytosis and glycolysis. *Exp. Cell Res.* **73**: 383–393.