Original Article *J. Clin. Biochem. Nutr.*, **41**, 169–174, November 2007

Effect of Intestinal Microflora on the Production of Interleukin 10 and Prostaglandin E_2 in Serum and Kupffer Cells from Germfree and Conventional Mice

Masamichi Ikeda*, Hideo Ohira, Yukiko Toyama, Tikae Katagiri, and Bunsaku Sakakibara

Laboratory of Nutritional Pathology, Faculty of Nutrition, Kobegakuin University Ikawadani-cho, Nishi-ku, Kobe 651-2180, Japan

Received 16 February, 2006; Accepted 2 April, 2007

Summary **To determine why germfree (GF) mice are less productivity of proinflammatory cytokines than conventional (CV) mice, we studied serum levels of interleukin 10 (IL-10) and prostaglandin E_2 (PGE_2) in mice after treatment with lipopolyssacharide (LPS). A single injection of LPS caused an elevation of IL-10 in serum from GF, LPS-GF (germfree mice given drinking water containing LPS) and CV mice. The response was highest in serum from GF mice, and was lower in serum from LPS-GF mice compared with GF mice. Before LPS injection, serum PGE_2 was significantly higher in CV and LPS-GF mice than in GF ones. After LPS injection, a higher level of PGE_2 was maintained over 12 h in CV mice after LPS injection, while the LPS treatment reduced the level in LPS-GF mice and increased the level in GF mice. The levels of IL-10 in culture medium from Kupffer cells treated with LPS showed similar results to serum in GF and CV mice. These results suggest that high levels of IL-10 in serum from germfree mice may be partly responsible for the lower *in vivo* responsiveness of these proinflammatory cytokines to LPS in these mice, although PGE_2 was not responsible for the lower responsiveness of these inflammatory cytokines to LPS.**

Key Words**: lipopolysacharide, interleukin 10, prostaglandin E_2, germfree mice, Kupffer cells**

Introduction

It has been shown in several studies using germfree (GF) animals that intestinal microflora influence immunity by modifying humoral and cellular responses [*1*, *2*], and that lipopolysaccharide (LPS) susceptibility in GF mice differs considerably from that in conventional (CV) mice [*3*, *4*]. LPS is an integral component of the outer membrane of Gram-negative bacteria and a contributing factor in the initiation of the generalized inflammatory process termed endotoxic shock. LPS stimulates immune cells to generate proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin 1 (IL)-1 and IL-6. The production of these cytokines is essential for the development of endotoxin shock [*5–7*], since administration of TNF can induce IL-1 and IL-6, which act synergistically to produce a state of shock and, in some cases, death.

The influence of intestinal microflora on cytokine secretion by macrophages was investigated by Nicase *et al.* [*8–10*]. They showed that peritoneal macrophages and Kupffer cells from germfree mice were lower secretors of cytokines in response to LPS than the cells from control mice. Previously, we found that intestinal microflora influenced the LPS susceptibility of induction of serum amyloid A (SAA), an acute phase protein in the mouse, in conventional and germfree IQI mice after injection of LPS, and the response was significantly less in germfree. LPS-induced elevations

*To whom correspondence should be addressed.
Tel: +81-78-974-1551 Fax +81-78-974-5842
E-mail: ikeda-m@nutr.kobegakuin.ac.jp

of serum TNF, IL-1, and IL-6 levels were also less in germfree mice [*11*, *12*]. However, the mechanism by which the cytokine response is altered in germfree animals is unclear. The synthesis of cytokines is finely regulated through several processes. TNF-α indirectly suppresses by inducing macrophage production of inhibitory molecules such as IL-10 [*13*] and prostaglandin E_2 (PGE_2) [*14*, *15*]. In this study, we studied the levels of anti-inflammatory mediators, such as IL-10 and PGE_2 in GF, CV and LPS-GF mice after LPS injection. LPS-induced mediator secretion was also studied by Kupffer cell culture in germfree and conventional mice.

Materials and methods

Animals and LPS treatment

IQI/Jic[Gf] mice (Clea Japan, Inc., Tokyo, Japan) bred in our laboratory were maintained in a Trexler-type flexible film isolator in a standard germfree state. The germfree status of the mice was verified at the beginning and at the end of the experiment by the standard procedure [*16*, *17*]. GF mice were conventionalized by the oral administration of a feces suspension (in physiological saline solution as drinking water) from conventional mice, and these conventionalized mice were used as conventional mice in this experiment. Animals were divided into three groups, GF mice (germfree mice given drinking water), LPS-GF mice (germfree mice given drinking water containing LPS, 10 μg/ml, for 3 weeks *Salmonella typhosa* 0901, Difco, Detroit, MI) and CV mice (conventional mice given drinking water).

The GF and CV mice were maintained on a CMF diet irradiated with 50 kGy of γ-rays (Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum* at temperature of 22 ± 2°C and relative humidity of 50 ± 10%. Lighting was regulated automatically to provide constant periods of alternating light and darkness (the light was on from 8:00 to 20:00). All procedures were performed in accordance with the Kobe Gakuin University Guide-lines for the Care and Use of Laboratory Animals.

Eight- to 10-week-old GF, LPS-GF and CV mice were intraperitoneally injected with 100 μg LPS (*Salmonella typhosa* 0901, Difco, Detroit, MI) dissolved in saline. Blood samples were collected through the ascending vena cava of the abdomen under light anesthesia after LPS treatment. The blood was allowed to clot at 4°C for 2 h and centrifuged to obtain serum samples that were stored in portions at −80°C until assay.

Kupffer cells culture

Kupffer cells were isolated according to the method of Friedman [*18*]. Briefly, the portal vein was canulated and the liver perfused *in situ* with a balanced salt solution followed by collagenase. The digested liver was then excised, scooped into 100-mm culture dishes, and finely minced. The digested tissue was then placed in H/DMEM with Pronase and further dissociated by repeated pipetting. After centrifugation at 150 × g for 5 min. the supernatant was centrifugated at 13,000 × g 30 min. The pellet was resuspended in H/DMEM followed by centrifugation over 20% Nycodentz (Nycomed Pharma AS, Oslo, Norway), the cells at the interface were pooled and washed twice by centrifugation with Hank's balanced salt solution. The pellet was then dispersed and resuspended in RPMI-1640 media containing 10% heat-inactivated fetal calf serum. The isolated cells showed 95% viability as determined by trypan blue exclusion. Cells were stimulated with addition of LPS (1 μg/ml, *Salmonella typhosa* 0901, Difco, Detroit, MI), and the medium was harvested and stored at −80°C until assay.

IL-10 and assay

IL-10 levels were measured using a commercially available immunoassay kit (Genzyme/TECHNE).

PGE_2 assay

PGE_2 levels in serum or culture medium were measured using a commercially available immunoassay kit (Amersham pharmacia biotech).

Statistical analyses

The results are expressed as the mean ± SE for the indicated number of measurements. Comparisons between the groups were made using the Student's *t*-test. Statistical significance was assessed at the 95% confidence level ($p<0.05$).

Results

Serum IL-10 in germfree and conventional mice after LPS injection

Previously, LPS injection induced an elevation of TNF levels in both conventional and germfree mice with similar courses, and the levels were significantly lower in germfree mice than in conventional mice [*11*, *12*]. IL-10 has been shown to be a potent macrophage deactivator, blocking the induced synthesis of TNF-α, IL-1 and IL-6 [*13*]. To find out whether the reduced LPS-response of inflammatory cytokines in the germfree mice is associated with IL-10, we measured IL-10 levels in serum after injection of LPS. LPS injection (100 μg/mice, *i.p.*) induced an elevation of IL-10 levels in CV, LPS-GF and GF mice with similar courses: the levels peaked at 1.5 to 3 h, then returned to normal levels at 6 h (Fig. 1). However, the serum IL-10 level was highest in GF mice among three groups, and LPS administered in drinking water to GF mice caused a decrease in the IL-10 level. These results suggest that high levels of serum IL-10 in GF mice may be partly responsible for the lower *in vivo*

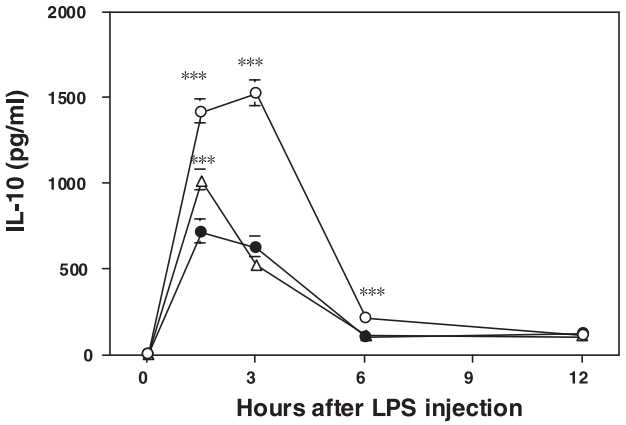

Fig. 1. Serum IL-10 levels in GF, LPS-GF and CV mice after LPS injection. Closed circles show the level in CV mice and open circles show the level in GF mice, and open triangles show the level in LPS-GF mice. The serum was obtained from blood 0, 1.5, 3, 6, and 12 h after LPS injection. Each value is the mean ± SE. Values with different letters are significantly different from conventional mice (***p<0.001).

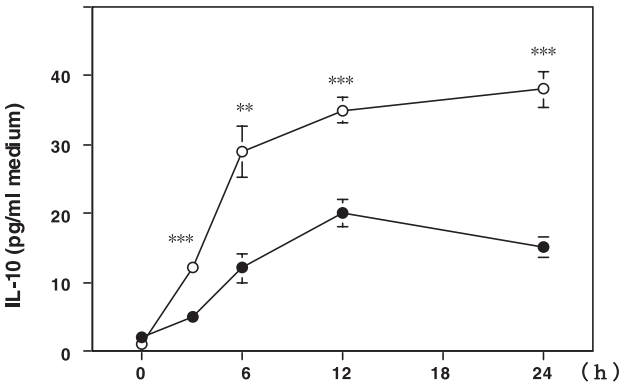

Fig. 2. LPS-induced IL-10 secretion in the conditioned medium of Kupffer cells from GF and CV mice. Closed circles show the level in CV mice and open circles show the level in GF mice. Culture medium was collected 0, 3, 6, 12 and 24 h after addition of LPS (1 mg/ml). Each value is the mean ± SE. Values with different letters are significantly different from conventional mice (**p<0.01, ***p<0.001).

responsiveness of TNF-α to LPS in these mice.

LPS-induced IL-10 production in Kupffer cells

In order to determine whether the insusceptibility of IL-10 response in serum of conventional mice was responsible for the reduced response of Kupffer cells to LPS stimulation, we examined the ability of Kupffer cells to produced IL-10 *in vitro*. IL-10 levels attained maximums 12 h after addition of LPS in culture medium of Kupffer cells which had been prepared from GF and CV mice (Fig. 2). There were significant differences in IL-10 levels in culture medium between Kupffer cells of GF and CV mice over 24 h after addition of LPS, which confirmed the results from serum of GF and CV mice after LPS injection. These results suggest that a source of circulating IL-10 after LPS challenge may be the liver, presumably Kupffer cells.

Serum PGE2 levels in germfree and conventional mice after LPS injection

Previous studies have demonstrated that LPS treatment of macrophages can induce a concomitant increase in TNF synthesis and PGE2 production, and that LPS-induced TNF production by macrophages is suppressed by submicromolar concentrations of exogenous PGE2 [*14*, *15*]. To explore the possibility of suppression of TNF-α production by PGE2, we measured PGE2 production in serum from GF, LPS-GF and CV mice after LPS injection as shown in Fig. 3. Before LPS injection, serum PGE2 was significantly higher in CV and LPS-GF mice than in GF ones. After LPS injection, a significantly higher level of PGE2 was maintained over 12 h in CV mice after LPS injection, while the LPS treatment

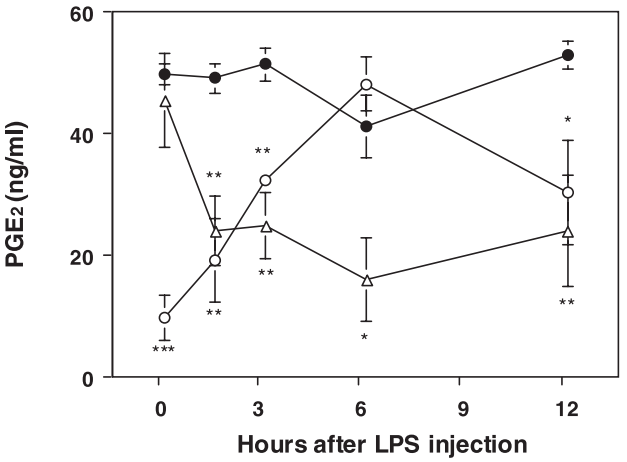

Fig. 3. Serum PGE2 levels in GF, LPS-GF and CV mice after LPS injection. Closed circles show the level in CV mice, and open circles show the level in GF mice, and open triangles show the level in LPS-GF mice. The serum was obtained from blood 0, 1.5, 3, 6, and 12 h after addition of LPS (1 μg/ml). Each value is the mean ± SE. Values with different letters are significantly different from conventional mice (*p<0.05, **p<0.01, ***p<0.001).

reduced the level in LPS-GF mice and increased the level in GF mice in a bell shape.

The results show that PGE2 production was significantly larger in CV mice than in GF mice in response to LPS, and hence the PGE2 produced in this way was not likely to have influenced TNF synthesis and did not contribute to the lower sensitivity of the GF animals.

PGE_2 responses in Kupffer cells stimulated with LPS

Similarly, and in order to further examine the association between the levels of TNF in serum and the responses of Kupffer cells to LPS stimulation, we examined LPS-induced PGE_2 secretion in the conditioned medium of Kupffer cells from GF and CV mice (Fig. 4). PGE_2 levels in culture medium of Kupffer cells which had been prepared from CV and GF mice reached a maximum 12 h after addition of LPS. Moreover, PGE_2 levels were significantly ($p<0.001$) higher in culture medium of macrophages from conventional mice compared with germfree animals 3 h after addition of 1 μg/ml LPS.

Discussion

Mice and rats are highly resistant to the lethal effects of LPS compared to other animals [*19*], so GF and CV mice might be good models to study the non-lethal effects of LPS on the host. To our knowledge, there have been no reports on the effects of LPS on anti-inflammatory cytokine production in sera of GF and CV mice.

The major finding of this study was the enhanced production of anti-inflammatory cytokines by GF mice compared to CV mice. Significant differences were observed between the LPS-induced cytokine production in GF and CV mice. The GF mice produced larger amounts of IL-10 than the CV mice after LPS treatment. This suggests that CV mice have a stronger proinflammatory cytokine response than GF mice after LPS injection, and support the conclusion that LPS is more lethal in CV mice than in GF mice. Our *in vivo* results, which indicate the enhanced production of TNF-α, in CV mice compared to GF mice, are in agreement with the findings in our previous reports [*11*, *12*].

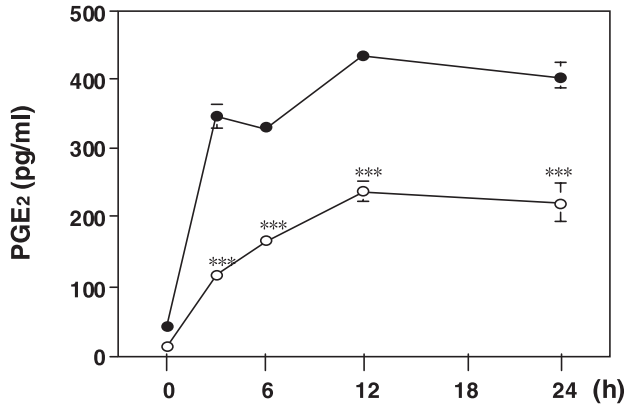

Fig. 4. LPS-induced PGE_2 secretion in the conditioned medium of Kupffer cells from GF and CV mice. Closed circles show the level in CV mice, and open circles show the level in GF mice. The serum was obtained from blood 0, 1.5, 3, 6, and 12 h after addition of LPS (1 μg/ml). Each value is the mean ± SE. Values with different letters are significantly different from CV mice (***$p<0.001$).

It is interesting that IL-10 production was significantly greater in GF mice than in CV mice after LPS injection. To determine the mechanism by which the serum IL-10 level showed a higher response to LPS challenge in GF mice, we examined IL-10 production *in vitro* by isolated Kupffer cells from GF and CV mice. Similar differences to the *in vivo* results were observed in LPS-induced IL-10 production of Kupffer cells between GF and CV mice. These findings are in agreement with the results of Nicaise *et al.* [*8–10*]. The IL-10 response in serum of GF mice was responsible for the enhanced response of Kupffer cells to LPS (Fig. 2). Thus, we could attribute the higher response of LPS-induced elevation of IL-10 and suppression of TNF in serum from GF mice to the ability of Kupffer cells in IL-10 production. On the other hand, oral administration of LPS in drinking water for germfree mice caused a decrease in IL-10 production in serum compared to GF control (Fig. 1). IL-10 was identified and characterized by its immunoregulatory and cytokine synthesis inhibiory activity [*13*, *20*]. Macrophages and lymphoid cells are also known to produce IL-10 after LPS stimulation [*21*]. These facts suggest that since oral administration of LPS or bacterial flora might alter the IL-10 production, serum IL-10 production could be enhanced in GF mice. Thus, we might explain in part the mechanism of low cytokine production in GF mice by a high level of production of the major decreasing factor, IL-10.

TNF-α indirectly suppresses by inducing macrophage production of inhibitory molecules such as prostaglandin E_2 [*14*, *15*]. To explore the relationship between PGE_2 and cytokine production, we measured PGE_2 levels in serum and culture medium of macrophages from GF and CV mice after LPS-treatment as shown in Figs. 2 and 4. Before LPS injection, serum PGE_2 was significantly higher in CV and LPS-GF mice than in GF ones. However, a significantly higher level of PGE_2 was maintained over 12 h in CV mice after LPS injection, while the LPS treatment reduced the level in LPS-GF mice and increased in a bell shape the level in GF mice. On the other hand, Kupffer cells from CV mice showed significantly higher PGE_2 production compared with those from GF mice after LPS-treatment. Since LPS upregulates PGE_2 production by activation of cyclooxygenase and phospholipase A_2 [*22*], we could attribute the higher level of PGE_2 in CV and LPS-GF mice before LPS injection to the LPS from intestinal flora or in drinking water. Why did LPS injection not induce more serum PGE_2 in CV mice? Since PGE_2 plays an autoregulatory role in Kupffers and inhibits its own production in LPS treatment [*15*], it is possible that the serum PGE_2 level had already peaked before LPS injection, therefore the level in CV mice might be unaltered after LPS injection. On the other hand, LPS might cause a rise in the PGE_2 level of

GF mice because the level was much lower before LPS injection. The results show that PGE_2 production occurred significantly later than TNF in response to LPS, and hence the PGE_2 produced in this way was not likely to have influenced TNF synthesis and did not contribute to the lower sensitivity of the germfree animals.

Another possible cause of the reduced cytokine production in germfree mice may be the effects of soluble TNF receptor [*23*], CD14 [*24*, *25*] and TGF-β [*26*]. Further studies are needed to investigate these possibilities.

Acknowledgments

We would like to thank Sinobu Sumitani and Sanae Takahashi for their technical assistance.

References

[1] Bandeira, A., Mota-Santos, T., Itahara, S., Degermann, S., Heuser, C., Tonegawa, S., and Coutinho, A.: Localization of γ/δ T cells to the intestinal epithelium is independent of normal microbial colonization. *J. Exp. Med.*, **172**, 239–244, 1990.

[2] Bealmear, P.M., Halternmann, O.A., and Mirand, E.A.: Influence ofthe microflora on the immune response. *in The germ-free animal in biomedical research*, eds. By Coate, M.E. and Gustafsson, B.E., London, UK, pp. 335–340, 1984.

[3] Jensen, S.B., Mergenhagen, S.E., Fitzgerald, R.J., and Jordan, H.V.: Susceptibility of conventional and germfree mice to lethal effects of endotoxin. *Proc. Soc. Exp. Biol. Med.*, **113**, 710–714, 1963.

[4] Matsuzawa, T. and Wilson, R.: Oxygen changes in tissue of germfree and conventional mice after inoculation of endotoxin. *Tohoku J. Exp. Med.*, **85**, 361–364, 1965.

[5] Lehmann, V., Freudenberg, M.A., and Galanos, C.: Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine treated mice. *J. Exp. Med.*, **165**, 657–663, 1987.

[6] Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Leer, A.T., Kuo, G.C., Lowry, S.F., and Cerami, A.: Anti-cachectin/ TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*, **330**, 662–664,1987.

[7] Alexander, R.H., Doherty, G.M., Buresh, C.M., Venzon, D.J., and Norton, J.A.: A recombinant human receptor antagonist protein to interleukin I improves survival after lethal endotoxemia in mice. *J. Exp. Med.*, **173**, 1029–1032, 1991.

[8] Nicaise, P., Gleizes, A., Forestier, F., Quero, A., and Labarre, C.: Influence of intestinal bacterial flora on cytokine (IL-1, IL-6 and TNF-α) production by mouse peritoneal macrophages. *Eur. Cytokine Netw.*, **4**, 133–138, 1993.

[9] Nicaise, P., Gleizes, A., Forestier, F., Sandre, C., Quero, A., and Labarre, C.: The influence of *E. coli* implantation in axenic mice on cytokine production by peritoneal and bone marrow-derived macrophages. *Cytokine*, **7**, 713–719, 1995.

[10] Nicaise, P., Gleizes, A., Sandre, C., Kergot, R., Lebrec, H., Forestier, F., and Labarre, C.: The intestinal microflora regulates cytokine production positively in spleen-derived macrophages but negatively in bone marrow-derived macrophages. *Eur. Cytokine Netw.*, **10**, 365–372, 1999.

[11] Ikeda, M., Hamada, K., Okamoto, H., and Sakakibara, B.: Susceptibility of germfree mice to lipopolysaccharide. *in Germfree Life and its Ramifications*, eds. By Hashimoto, K., Sakakibara, B., Tazume, S., and Shimizu, K., Shiozawa, Japan, pp. 443–446, 1996.

[12] Ikeda, M., Hamada, K., Sumitomo, N., Okamoto, H., and Sakakibara, B.: Serum Amyloid A, cytokines, and corticosterone responses in germfree and conventional mice after lipopolysaccharide injection. *Biosci. Biotechnol. Biochem.*, **63**, 1006–1010, 1999.

[13] Malefyt, R.D.W., Abrams, J., Bennett, B., Figdor, C.G., and Vries, J.E.D.: Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes. *J. Exp. Med.*, **174**, 1209–1220, 1991.

[14] Kunkel, S.L., Spengler, M., May, M.A., Spengler, R., Larrick, J., and Remick, D.: Prostaglandin E_2 regulates macrophage-derived tumor necrosis factor gene expression. *J. Biol. Chem.*, **263**, 5380–5384, 1988.

[15] Peters, T., Karck, U., and Decker, K.: Interdependence of tumor necrosis factor, prostaglandin E_2, and protein synthesis in lipopolysaccharide-exposed rat Kupffer cells. *Eur. J. Biochem.*, **191**, 583–589, 1990.

[16] Committee for standardization of sterility test for germfree animals in Japan: Recommended requirement for sterility test for germfree animals in Japan. *Exp. Anim.*, **21**, 35–38, 1972.

[17] Maejima, K. and Nomura, T.: An experience of application of sterility test of germfree mice and rats recommended by JEARA. *Exp. Anim.*, **24**, 177–181, 1975.

[18] Friedman, S.: Isolation and culture of hepatic nonparenchymal cells, *in Methods in Toxicology*, eds. By Tyson, C.A. and Frazier, J.M., Academic Press, San Diego, pp. 292–305, 1993.

[19] Gronvall, J.A. and Brunson, J.G.: The generalized Shwartzman phenomenon in rats. *Arch. Pathol.*, **62**, 324–334, 1956.

[20] Randow, F., Uta Syrbe, U., Meisel, C., Krausch, D., Zuckermann, H., Platzer, C., and Volk, H-D.: Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor β. *J. Exp. Med.*, **181**, 1887–1892, 1995.

[21] Berg, D.J., Kuhn, R., Rajewsky, K., Muller, W., Menon, S., Davidson, N., Grunig, G., and Rennick, D.: Interleukin-10 is a central regulator of the response to LPS in murine models of Endotoxic shock and the Shwartzman reaction but not Endotoxin tolerance. *J. Clin. Invest.*, **96**, 2339–2347, 1995.

[22] Perez, R., Stevenson, F., Johnson, J., Morgan, M., Erickson, K., Hubbard, N.E., Morand, L., Budich, S., Katznelson, S., and German, J.B.: Sodium butyrate upregulates Kupffer cell PGE_2 production and modulates immune function. *J. Surg. Res.*, **78**, 1–6, 1998.

[23] Spinas, G.A., Keller, U., and Brockhaus, M.: Release of soluble receptors for tumor necrosis factor (TNF) in relation to circulating TNF during experimental endotoxemia. *J. Clin. Invest.*, **90**, 533–536, 1992.

[24] Frey, E.A., Miller, D.S., Jahr, T.G., Sundan, A., Bazil, V.,

Espevik, T., Finlay, B.B., and Wright, S.D.: Soluble CD14 participates in the responses of cells to lipopolysaccharide. *J. Exp. Med.*, **176**, 1665–1671, 1992.

[25] Pugin, J., Kravchenko, V.V., Lee, J.D., Kline, L., Ulevitch, R.J., and Tobias, P.S.: Cell activation mediated by glycosylphosphatidyl- inositol-anchored or transmembrane forms of CD_{14}. *Infect. Immun.*, **66**, 1174–1180, 1998.

[26] Maeda, H., Kuwahara, H., Ichimura, Y., Ohtsuki, M., Kurakata, S., and Shiraishi, A.: TGF-β enhances macrophage ability to produce IL-10 in normal and tumor-bearing mice. *J. Immunol.*, **155**, 4926–4932, 1995.