FULL PAPER *Immunology*

Immune Response of Gnotobiotic Piglets against *Mycoplasma hyopneumoniae*

Yoshihiro MUNETA[1)]*, Yu MINAGAWA[1)], Yoshihiro SHIMOJI[1)], Yohsuke OGAWA[1)], Hirokazu HIKONO[1)] and Yasuyuki MORI[2)]

[1)]*Research Team for Advanced Biologicals, National Institute of Animal Health, 3–1–5, Kannondai, Tsukuba, Ibaraki, 305–0856 and* [2)]*Research Team for Paratuberculosis, National Institute of Animal Health, 3–1–5, Kannondai, Tsukuba, Ibaraki, 305–0856, Japan*

(Received 14 March 2008/Accepted 15 May 2008)

ABSTRACT. In this study, several cytokine responses were investigated during *Mycoplasma hyopneumoniae* (Mhp) infection using a gnotobiotic infection model. We found that several inflammatory cytokines (IL-1β, IL-8, IL-18, and TNF-α) and an anti-inflammatory cytokine IL-10 were induced from peripheral blood mononuclear cells (PBMC) of germ-free (GF) piglets stimulated with heat killed Mhp whole antigens, but no IFN-γ and IL-4 were induced by Mhp. After the intranasal infection of Mhp, IL-1β, IL-8, IL-18, and IFN-γ were also detected in the broncho-alveolar lavage fluids (BALF). The antigen-specific IFN-γ and IL-10 responses after infection of Mhp were gradually suppressed during Mhp infection as well as non-specific immune response to concanavalin A (ConA) and lipopolysacchalide (LPS) at early stage of infection. These results suggested that Mhp infection modulates the immune response of pigs by inducing several cytokines, and causes immuno-suppression of pigs in a gnotobiotic condition.

KEY WORDS: cytokine, gnotobiotic, immunomodulation, *Mycoplasma hyopneumoniae*, swine.

J. Vet. Med. Sci. 70(10): 1065–1070, 2008

Mycoplasma hyopneumoniae (Mhp) is the etiological agent of swine enzootic pneumonia (SEP), which causes great economic loss to the swine industry [21]. Mhp infection is also associated with the exacerbation of respiratory diseases caused by some viral pathogens, such as porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 [13, 25]. One of the most unique characteristics of Mhp infection is the accumulation of a large number of leukocytes in lung tissue [21]. Therefore, the infiltration and activation of leukocytes are likely to be key events in the progression of pneumonia caused by Mhp. Some cytokines, including IL-1, TNF-α and IL-6, have been detected in the broncho-alveolar lavage fluids (BALF) of Mhp-infected pigs [2, 3]. However, the factors involved in promoting protective immunity and/or the inflammatory responses against Mhp are not fully understood. We recently reported that large amounts of IL-18 were induced in the lung of Mhp-infected pigs, and it may be involved in immunomodulation of the host with Mhp infection [19]. Moreover, we had reported the involvement of Toll-like receptor 2 (TLR2) and TLR6 in the recognition of Mhp on porcine alveolar macrophages [18]. However, the more precise studies are needed to understand the immunomodulation of pigs during Mhp infection.

Gnotobiotic pigs are produced by the infection of specific microorganisms to the germ-free (GF) pigs and have been used in research into the symbiotic relationship between an host animal and the specific microorganisms that the researcher wants to investigate. GF pigs are extremely sensitive to bacterial infections. The absence of other microorganisms excludes competition and complex interactions among microbes known as colonization resistance [29]. It excludes also background levels of cytokines that are induced by a plethora of endogeneous micro-organisms and simplifies the interplay of mediators [23]. GF pigs are immunologically immature [15], lack maternal immunoglobulins and produce only traces of own antibodies [27]. Phagocytosis is impaired because of missing opsonins, paucity of Fc receptors and complement receptors and low lysosomal activity. However, GF pigs respond well to inflammatory signals [28]. Taken together, gnotobiotic infections to GF pigs are unique model to investigate the precise immune responses against a target microbe [9].

In this study, the immune response of Mhp-infected gnotobiotic piglets was investigated to understand the molecular mechanisms of protective immunity and/or the inflammatory reactions against this important respiratory pathogen.

* CORRESPONDENCE TO: MUNETA, Y., Research Team for Advanced Biologicals, National Institute of Animal Health, 3–1–5, Kannondai, Tsukuba, Ibaraki, 305–0856, Japan.
e-mail: ymuneta@affrc.go.jp

MATERIALS AND METHODS

Animals, Mhp, and experimental samples: Eighteen 1-day-old GF piglets (LW) were used in this study. GF piglets were obtained by hysterectomy of gilts on 112 days of gestation under anaesthesia. Piglets were kept in sterile positive-pressure isolators and fed by sterile milk. They were checked repeatedly for absence of bacterial contamination by culturing nasal swabs. Six piglets were killed at 1-day-old to examine innate cytokine responses. The remaining twelves were used to Mhp infection study. The virulent Mhp strain E-1 (originally isolated from a pig with pneumonia in Japan), cultured in BHL medium to a titer of 10^7 color changing units (CCU)/m*l*, was used in this experiment [16]. The infection of GF piglets with Mhp (10^8 CCU/pig) was also performed as described previously [16]. Three piglets (2 Mhp-infected and 1 non-infected) were killed at 1, 2, 3,

and 4 weeks after infection, respectively. Serum, peripheral blood mononuclear cells (PBMC) and BALF from lung wash were collected from each piglet. Lung tissues from pneumonic lesions were also collected for the pathological examination and confirmed to develop SEP by gross lesions or microscopic observations. All animal experiments were approved by the committee for animal experiments of National Institute of Animal Health.

Innate cytokine responses in PBMC obtained from GF piglets to Mhp antigen: PBMC were collected from GF piglets before Mhp infection, and cultured in the presence or absence of heat-killed Mhp whole antigens as described previously [18, 19]. After 24 hr, the supernatant were collected and stored at –20°C until use. IL-1β and TNF-α levels in the supernatant were quantified using commercial ELISA kits (Duo Set, R&D systems Inc., Minneapolis, MN, U.S.A.). IFN-γ, IL-4, and IL-10 levels were also measured by commercial ELISA kits (Swine Cytoset kit, Biosource International Inc., Camarillo, CA, U.S.A.). The concentration of porcine IL-8 was determined as described previously [24]. The concentration of porcine IL-18 was measured as described previously [17].

Cytokine production in the BALF of Mhp-gnotobiotic piglets: The BALF samples were centrifuged at 2000 rpm for 5 min and passed through a 0.22 μm filter. These samples were stored at –20°C until use. The concentration of IL-1β, IL-4, IL-8, IL-10, IL-18, TNF-α, and IFN-γ in the BALF was measured as described above [17, 24].

Local antibody responses against a Mhp p46 in the BALF of Mhp-gnotobiotic piglets: The antibody levels against Mhp in the BALF were measured using a p46 (the cell surface 46 kDa antigen of Mhp for early and species-specific immunogenic protein) specific ELISA, as described previously [8]. IgG were detected using a HRP-conjugated anti-porcine IgG antibody (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.). IgG1 and IgG2 were detected using anti-porcine IgG1 (Serotec Ltd., Oxford, UK) and anti-porcine IgG2 antibody (Serotec), respectively.

IFN-γ and IL-10 responses against Mhp antigen: To evaluate the Mhp-specific Th1 (IFN-γ) and Th2 (IL-10) cytokine responses, PBMC collected from gnotobiotic piglets after Mhp infection, and cultured with heat-killed Mhp whole antigens as described previously [18, 19]. After 24 hr, the supernatants were collected and IFN-γ and IL-10 concentrations were measured by commercial ELISA kits (Biosource International Inc.).

Non-specific Immune responses to ConA and LPS: PBMC collected from gnotobiotic piglets were cultured with 10 μg/m*l* of ConA (Sigma, St. Louis, MO, U.S.A.) to examine the non-specific T cell response. Alveolar macrophages collected from gnotobiotic piglets were also stimulated with 10 μg/m*l* of LPS from *Escherichia coli* serotype O55:B5 (Sigma) to examine the non-specific macrophage activity. After 120 hr, cell proliferation and viability were estimated using a WST-1 cell counting kit (Dojindo, Kumamoto, Japan).

Statistical analysis: Cell cultures and measurements in some experiments were performed in duplicate or triplicate. Results are shown as the mean of pig group or time point with standard deviation. The differences between each group or time point were analyzed using Student's *t* test in Statview (Abacus Concept, Berkeley, CA, U.S.A.). $P<0.05$ was considered to be significant.

RESULTS

Cytokine induction of PBMC from GF piglets by Mhp: As shown in Fig.1, IL-1β, and IL-8, IL-18, and TNF-α were significantly induced in PBMC of GF piglets after stimulation with Mhp heat-killed antigens. However, No IFN-γ and IL-4 were induced by Mhp. All cytokines were induced by ConA stimulation as a positive control.

Cytokine production in the BALF of Mhp-gnotobiotic piglets: After intranasal infection of Mhp, some inflammatory cytokines including IL-1β, IL-8, and IL-18 were significantly produced in the BALF at 2 and 4 weeks after infection (Fig. 2). IFN-γ were also detected at 1 week after infection but decreased thereafter (Fig. 2). Other cytokines including TNF-α, IL-4 and IL-10 were not detected in the BALF in this study. No significant changes of cytokines concentration were seen in the BALF from non-infected piglets (data not shown).

Local antibody responses against a Mhp p46 in the BALF of Mhp-gnotobiotic piglets: To estimate the specific antibody response against Mhp, we measured the antibody level in the same BALF using the Mhp p46 ELISA. As shown in Fig. 3, the Mhp-specific IgG response was observed from 3 weeks following infection. IgG1 was the main immunoglobulin subclass against Mhp. No antibody response was detected in non-infected piglets.

Suppression of Mhp-specific Th1 (IFN-γ) and Th2 (IL-10) cytokine during Mhp infection: As shown in Fig. 4, Mhp-specific IFN-γ production was significantly raised at 1week following infection but the production level decreased thereafter. IL-10 was induced from PBMC of non-infected GF piglets but IL-10 production decreased with the progression of pneumoniae. No significant changes of IFN-γ and IL-10 were seen in the non-infected piglets (data not shown).

Suppression of non-specific immune responses to ConA and LPS: As shown in Fig. 5, the non-specific T cells response to ConA at 1 week following infection was reduced in the Mhp-gnotobiotic piglets compared with non-infected piglets. The cell viability of alveolar macrophages after stimulation of *E. coli* LPS was also decreased in the Mhp-gnotobiotic piglets at 1 week following Mhp infection.

DISCUSSION

In this study, we examined the innate and acquired immune responses to Mhp using a gnotobiotic infection model in pigs. The use of Mhp-gnotobiotic piglets are unique model to investigate the precise immune responses against a target microbe, because the background levels of

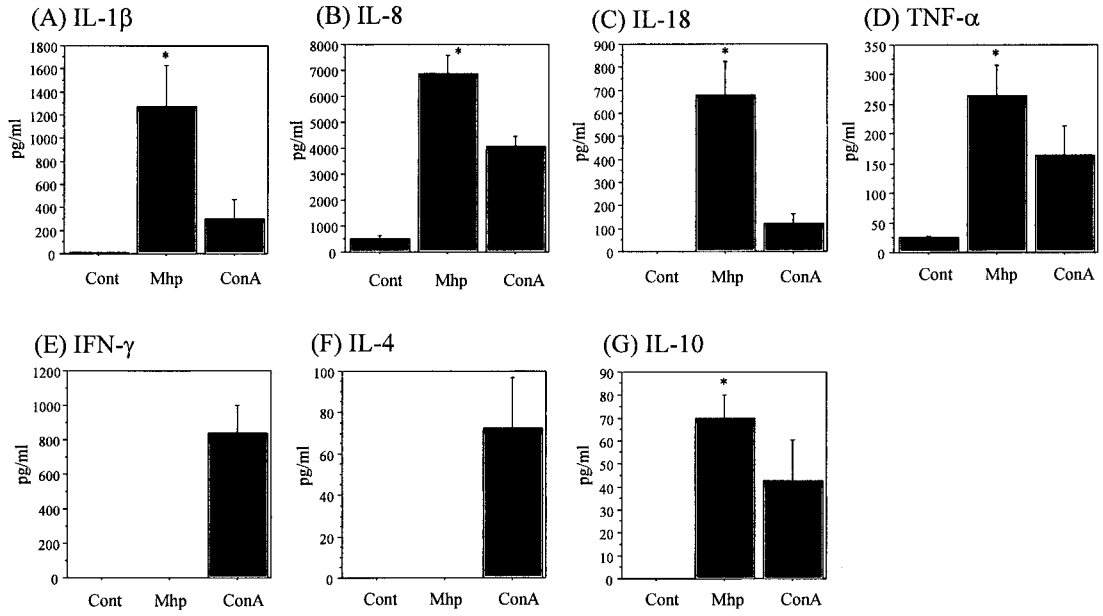

Fig. 1. Innate cytokine response of PBMC from GF pigs stimulated with heat-killed Mhp antigen. PBMCs obtained from GF piglets before infection was stimulated with heat-killed Mhp antigen for 24 hr and the cytokine concentration in the supernatant were estimated as described in Materials and Methods. (A) IL-1β, (B) IL-8, (C) IL-18, (D) TNF- α, (E) IFN-γ, (F) IL-4, and (G) IL-10. Cont: control non-stimulated. Mhp: Mhp-stimulated. ConA: concanavalin A-stimulated (Positive control). * shows significant difference as compared with control ($P<0.05$).

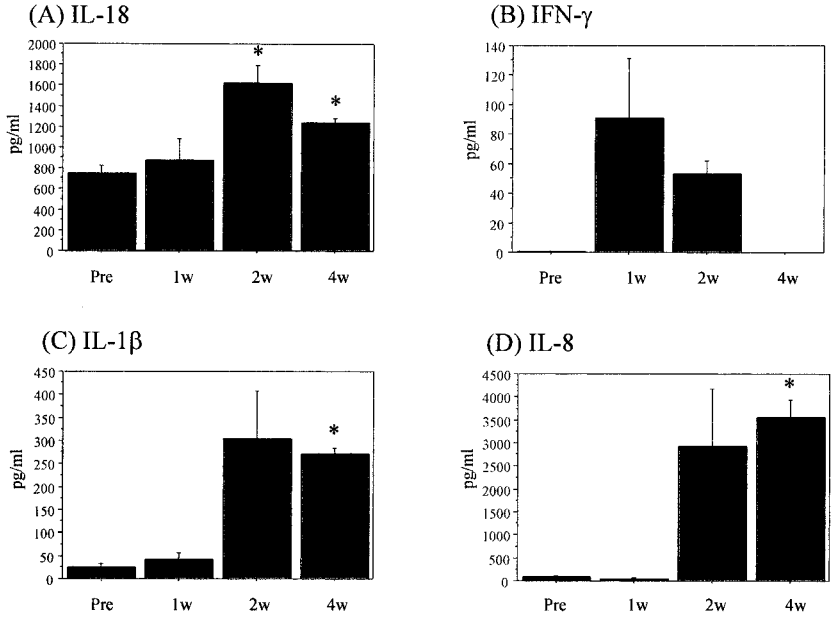

Fig. 2. Cytokine production in the BALF of GF piglets following infection with Mhp.The cytokine concentrations in the BALF samples taken from GF piglets before and after infection were measured as described in Materials and Methods. (A) IL-18, (B) IFN- γ, (C) IL-1β, and (D) IL-8. * shows significant difference as compared with pre-infection samples ($P<0.05$).

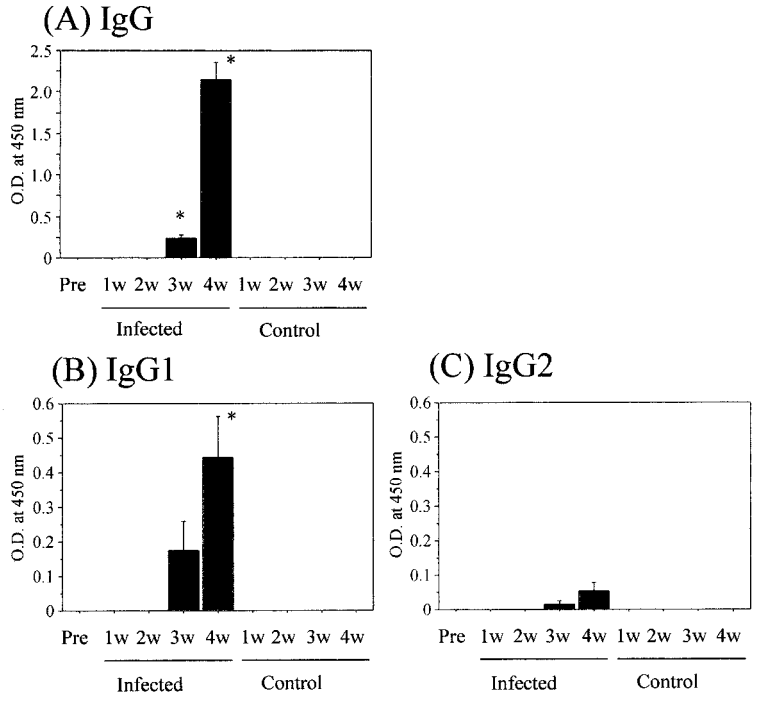

Fig. 3. Local antibody responses against a Mhp p46 antigen in the BALF of GF piglets following infection with Mhp. Antibody levels of (A) IgG, (B) IgG1, and (C) IgG2, against the Mhp p46 antigen were analyzed by ELISA as described in Materials and Methods. * shows significant difference as compared with Pre-infection samples ($P<0.05$).

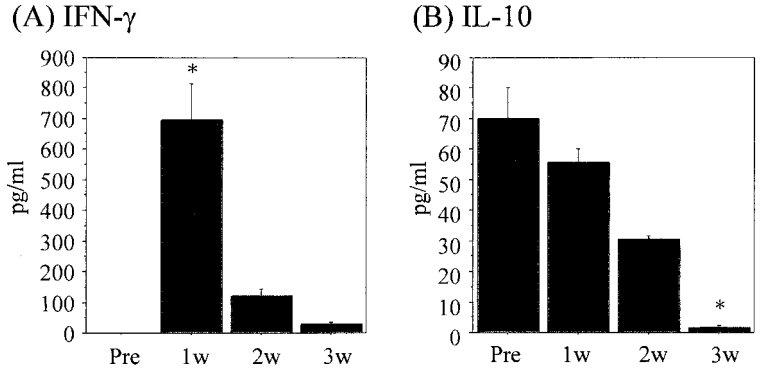

Fig. 4. Suppression of Mhp-specific Th1 (IFN-γ) and Th2 (IL-10) cytokine expression in Mhp-gnotobiotic pigs. PBMCs collected from gnotobiotic pigs following infection with Mhp, and cultured with heat-killed Mhp whole antigens. After 24 hr, the supernatant were collected and IFN-γ and IL-10 concentrations were measured. * shows significant difference as compared with other time points ($P<0.05$).

cytokines induced by endogeneous micro-organisms are negligible but animals respond well to inflammatory signals [23, 28]. Gnotobiotic infection models have previously been used for Mhp [16, 11] and successfully used to develop pneumonia in this study, too. However, little work had been performed about cell-mediated immunity in detail so far. In the present study, several inflammatory cytokines including IL-1β, IL-8, IL-18 and TNF-α were detected in the supernatant of PBMC obtained from GF piglets after stimulation with heat-killed Mhp (Fig. 1). IL-1β, IL-8 and IL-18 were also detected in the BALF after Mhp-gnotobiotic infection (Fig. 2). These inflammatory cytokines also had been

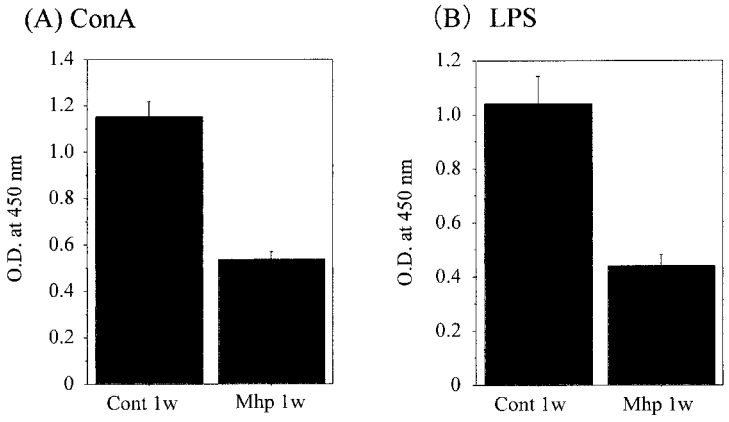

Fig. 5. Suppression of non-specific immune responses to ConA and LPS in Mhp-gnotobiotic pigs. PBMCs and alveolar macrophages collected from gnotobiotic pigs at 1 week following infection were stimulated with 10 μg/m*l* of ConA or 10 μg/m*l* of *Escherichia coli* LPS. After 120 hr, cell proliferation and viability were estimated as described in Materials and Methods.

detected in the infection studies of Mhp using *in vitro* infection model, conventional and/or specific pathogen free (SPF) pigs [2, 19, 26]. The increased production of inflammatory cytokines was also confirmed in Mhp-gnotobiotic piglets infection. Taken together, these inflammatory cytokines in the lung may play an important role in the pathogenesis and immunopathologic response against Mhp infection.

Mycoplasma infection correlates with the infection of other secondary respiratory pathogens by inducing the immunomodulation of host animals, even in humans [13, 22, 25]. However, little is known about the mechanisms of immunomodulation for any mycoplasma disease. Asai *et al.* [4] previously reported that BALF from pigs experimentally infected with Mhp suppressed the chemiluminescence response of the host neutrophils, and this effect was significantly correlated with the concentration of prostaglandin E2 (PGE2) in the BALF. PGE2 is known as a modulator of many kinds of immune responses, such as the inhibition of the Th1 responses [20], the development of Th2 lymphocytes [6], and immunoglobulin class switching [6], as well as plasma cell differentiation [14]. The inflammatory cytokines detected in this study were reported to induce PGE2 production in activated macrophages [5, 7, 10] and PGE2 also induced the down-stream cytokines such as IL-6, and IL-8 [1]. We recently reported that IL-18 produced in the lung stimulates PGE2 production in Mhp infection [19]. These results suggest that PGE2 induced by IL-1β, IL-18, and TNF-α from activated macrophages may play an important role in the immunomodulation in Mhp infection.

In previous studies using conventional or SPF pigs, no Mhp-specific Th1 and Th2 cytokine responses were shown. However, in the present study, we confirmed the suppression of Mhp-specific Th1 (IFN-γ) cytokine response during Mhp infection (Fig. 4A). Since we could not get meaningful results because of the high variety of the response in previous SPF pig model, this is the main advantage to use a gnotobiotic infection model. The suppression of IFN-γ response will cause the enhancement of secondary infection especially virus infection such as porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, and swine influenza virus [13, 25, 30]. Interestingly, Mhp-specific IL-10 response was also suppressed with progression of pneumonia (Fig. 4B). This suppression of IL-10 may lead to the over-activation of macrophage, because IL-10 has an anti-macrophage activity and is an important regulator of mucosal immunity [12]. Moreover, the non-specific immune responses to ConA and LPS were also suppressed at 1 week following infection in gnotobiotic piglets (Fig. 5). These results suggest that the function of T cells and macrophages were suppressed during Mhp infection and may be associated with the enhancement of other secondary infection.

In conclusion, these data described represent the first evidence showing the immunosuppression of the host animals during Mhp infection using a gnotobiotic infection model and show that gnotobiotic animals are useful to investigate the specific immune response of the host animal caused by a single target microbe. These results are also helpful for the development of methods to enhance innate and acquired immunity against Mhp, and contribute to the better understanding of exacerbation of secondary infection in Mhp-infected pigs.

ACKNOWLEDGMENT. This work was supported by a grant for zoonosis control project (ZCP-16) from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

REFERENCES

1. Agro, A., Langdon, C., Smith, F. and Richards, C.D. 1996. Prostaglandin E2 enhances interleukin-8 (IL-8) and IL-6 but inhibits GM-CSF production by IL-1 stimulated human synovial fibroblasts *in vitro*. *J. Rheumatol.* **23**: 862–868.
2. Asai, T., Okada, M., Ono, M., Irisawa, T., Mori, Y., Yokomizo, Y. and Sato, S. 1993. Increased levels of tumor necrosis factor and interleukin-1 in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*. *Vet. Immunol. Immunopathol.* **38**: 253–260.
3. Asai, T., Okada, M., Ono, M., Mori, Y., Yokomizo, Y. and Sato, S. 1994. Detection of interleukin-6 and prostaglandin E2 in bronchoalveolar lavage fluids of pigs experimentally infected with *Mycoplasma hyopnemoniae*. *Vet. Immunol. Immunopathol.* **44**: 97–102.
4. Asai, T., Okada, M., Yokomizo, Y., Sato, S. and Mori, Y. 1996. Suppressive effect of bronchoalveolar fluid from pigs infected with *Mycoplasma hyopneumoniae* on chemiluminescence of porcine peripheral neutrophils. *Vet. Immunol. Immunopathol.* **51**: 325–331.
5. Dinarello, C.A. 2002. The IL-1 family and inflammatory diseases. *Clin. Exp. Rheumatol.* **20**: S1–13.
6. Fedyk, E.R., Brown, D.M. and Phipps, R.P. 1997. PGE2 regulation of B lymphocytes and T helper 1 and T helper 2 cells: induction of inflammatory versus allergic responses. *Adv. Exp. Med. Biol.* **407**: 237–242.
7. Fournier, T., Fadok, V. and Henson, P.M. 1997. Tumor necrosis factor-alpha inversely regulates prostaglandin D2 and prostaglandin E2 production in murine macrophages. *J. Biol. Chem.* **272**: 31065–31072.
8. Futo, S., Seto, Y., Okada, M., Sato, S., Suzuki, T., Kawai, K., Imada, Y. and Mori, Y. 1995. Recombinant 46-kilodalton surface antigen (P46) of *Mycoplasma hyopneumoniae* expressed in *Escherichia coli* can be used for early specific diagnosis of mycoplasmal pneumoniae of swine by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **33**: 680–683.
9. Gunzer, F., Hennig-Pauka, I., Waldmann, K.H. and Mengel, M. 2003. Gnotobiotic piglets as an animal model for oral infection with O157 and non-O157 serotypes of STEC. *Methods Mol. Med.* **73**: 307–327.
10. Kashiwamura, S., Ueda, H. and Okamura, H. 2002. Roles of interleukin-18 in tissue destruction and compensatory reactions. *J. Immunotherapy* **25**: S4–S11.
11. Kobisch, M., Blanchard, B. and Le Potier, M.F. 1993. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and resistance to reinfection. *Vet. Res.* **24**: 67–77.
12. Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**: 263–274.
13. Kyriakis, S.C., Saoulidis, K., Lekkas, S., Miliotis, Ch.C., Papoutsis, P.A. and Kennedy, S. 2002. The effects of immunomodulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. *J. Comp. Pathol.* **126**: 38–46.
14. Lydyard, P.M., Brostoff, J., Hudspith, B.N. and Parry, H. 1982. Prostaglandin E2-mediated enhancement of human plasma cell differentiation. *Immunol. Let.* **4**: 113–116.
15. Mandel, L. and Travnicek, J. 1982. Haematology of conventional and germfree miniature Minnesota piglets. I. Blood picture. *Z Versuchstierkd* **24**: 299–307.
16. Mori, Y., Yoshida, Y., Kuniyasu, C. and Hashimoto, K. 1983. Improvement of complement fixation test antigen for the diagnosis of *Mycoplasma hyoneumoniae* infection. *Natl. Inst. Anim. Health Q (Tokyo).* **23**: 111–116.
17. Muneta, Y., Mikami, O., Shimoji, Y., Nakajima, Y., Yokomizo, Y. and Mori, Y. 2000. Detection of porcine interleukin-18 by sandwich-ELISA and immunohistochemical staining using its monoclonal antibodies. *J. Interferon Cytokine Res.* **20**: 331–336.
18. Muneta, Y., Uenishi, H., Kikuma, R., Yoshihara, K., Shimoji, Y., Yamamoto, R., Hamashima, N., Yokomizo, Y. and Mori, Y. 2003. Porcine TLR2 and TLR6: Identification and their involvement in *Mycoplasma hyopneumoniae* infection. *J. Interferon and Cytokine Res.* **23**: 583–590.
19. Muneta, Y., Minagawa, Y., Shimoji, Y., Nagata, R., Markham, P.F., Browning, G.F. and Mori, Y. 2006. IL-18 expression in pigs following infection with *Mycoplasma hyopneumoniae*. *J. Interferon Cytokine Res.* **26**: 637–644.
20. Roper, R.L. and Phipps, R.P. 1994. Prostaglandin E2 regulation of the immune response. *Adv. Prostaglandin Thromboxane Leukotriene Res.* **22**: 101–111.
21. Ross, R.F. 1992. Mycoplasmal disease. pp 535–549. *In*: Swine Diseases, (Leman, A.D., Glock, R.D., Mengeling, W.L., Penny, R.H.C., Scholl, E., and Straw, B. eds.), Iowa State University Press, Ames, Iowa.
22. Sasaki, Y., Honda, M., Makino, M. and Sasaki, T. 1993. Mycoplasmas stimulate replication of human immunodeficiency virus type 1 through selective activation of $CD4^+$ T lymphocytes. *AIDS Res. Hum. Retroviruses* **9**: 775–780.
23. Splichal, I., Trebichavsky, I., Muneta, Y. and Mori Y. 2002. Early cytokine response of gnotobiotic piglets to *Salmonella enterica* serotype typhimurium. *Vet. Res.* **33**: 291–297.
24. Splichal, I., Muneta, Y., Mori, Y. and Takahashi, E. 2003. Development and application of a pig IL-8 ELISA detection system. *J. Immunoassay and Immunochemistry* **24**: 219–232.
25. Thacker, E.L., Halbur, P.G., Ross, R.F., Thanawongnuwech, R. and Thacker, B.J. 1999. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. *J. Clin. Microbiol.* **37**: 620–627.
26. Thanawongnuwech, R., Young, T.F., Thacker, B.J. and Thacker, E.L. 2001. Differential production of proinflammatory cytokines: in vitro PRRSV and *Mycoplasma hyopneumoniae* co-infection model. *Vet. Immunol. Immunopathol.* **79**: 115–127.
27. Travnicek, J. and Mandel, L. 1982. Haematology of conventional and germfree miniature Minnesota piglets. II. Serum proteins and immunoglobulins. *Z Versuchstierkd* **24**: 308–317.
28. Trebichavsky, I., Schulze, J., Dlabac, V., Cukrowska, B., Tlaskalova-Hogenova, H. and Rehakova, Z. 1998. Salmonellosis: lessons drawn from a germ-free pig model. *Folia Microbiol (Praha)* **43**: 697–701.
29. Van der Waaij, D. 1984. Colonization resistance of the digestive tract. pp 155–165. *In*: The germ-free animal in biomedical research, (Coates, M.E., and Gustafson, B.E. eds.), Laboratory Animals Ltd., London.
30. Yazawa, S., Okada, M., Ono, M., Fujii, S., Okuda, Y., Shibata, I. and Kida, H. 2004. Experimental dual infection of pigs with an H1N1 swine influenza virus (A/Sw/Hok/2/81) and *Mycoplasma hyopneumoniae*. *Vet. Microbiol.* **98**: 221–228.