



Immunophenotype Characterization for Swine Selected Line, Which is Resistant for the Mycoplasma Pneumonia

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ABSTRACT : Mycoplasma Pneumonia of swine (MPS) decreases the daily growth of pigs, and, co-infection with a virus sometimes causes severe pneumonia. Genetic selection of pigs resistant to the pulmonary MPS lesion might solve the economic loss due to MPS in animal production. Here, we examined the immunophenotype of Landrace line (Miyagino L2), genetically selected to reduce the incidence of pulmonary MPS lesion for 5 generations in Miyagi Prefecture Animal Industry Experiment Station. Although this line is expected to be resistant to the pulmonary MPS lesion, the biological characteristics of its immune function are not clear. We investigated details of the immunorelated phenotype of Miyagino L2 at the hematological and molecular biological level, including cytokine expression, and compared the results with that of non-genetically selected Landrace. Miyagino L2 showed decreased antigen-specific IgG and IgM production and increased CD8-positive T-cell population, and high levels of cortisol concentration, suggesting that the MPS-resistant phenotype is associated these immunological differences. Additionally, T-cell CD4 expression was highly correlated with the MPS expected breeding value. Although the detailed mechanisms underlying this high correlation remain unknown, our result suggested that the genetic selection of the expression level of CD4 might be useful to improve MPS resistance in pig production. (**Key Words :** MPS Lesion, Immunoglobulin, T Cell, Biomarker, Porcine, CD4 and CD8)

INTRODUCTION

Mycoplasma Pneumonia of swine (MPS) is one of the representative chronic respiratory diseases caused by the infection of *Mycoplasma hyopneumoniae* (Mhp). Although the lethality of MPS by itself is not very high, it is known to decrease the daily growth and feed efficiency in the field, resulting in huge economic losses in the pig production industry (Morrison et al., 2000; Sarradell et al., 2003; Choi et al., 2006; Lorenzo et al., 2006). Furthermore, Mhp infection is known to induce severe pneumonia upon co-infection with some viral pathogens such as the porcine reproductive and respiratory syndrome virus and porcine

circovirus type 2 (Thacker et al., 1999; Kyriakis et al., 2002).

Mhp infection starts with invasion to the host animal through the oral and/or nasal pathway. Mhp adheres to the bronchial epithelial cells and induces cell death and inflammation in the corresponding area (Mebus and Underdahl et al., 1977; Blanchard et al., 1992; DeBey et al., 1992). Furthermore, previous studies reported that excessive host immune reactions also play an important role in the inflammation caused by Mhp infection (Mebus and Underdahl, 1977; Blanchard et al., 1992). The infection can first be recognized via the presence of Toll-like receptor (TLR) 2 and 6 on the porcine alveolar macrophages (Muneta et al., 2003). Interestingly, in addition to its own function, Mhp forms a pulmonary lesion through the hyperactivation of the host animals, not only through the function of Mhp (Ross 1999; Choi et al., 2006). As the supporting evidence of this notion, Davis et al. (1985) indicated the relationship between the MPS lesion and IgG and IgM production in mice. In a previous study, Tajima et al. (1984) reported that the suppression of T-cell function in swine resulted in a lower incidence of lesion formation

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induced by Mhp. These findings suggest the possibility that we might be able to control the formation of a pulmonary MPS lesion in pigs by modifying the immune performance of the host animals.

Genetic improvement is one approach to reduce Mhp infection and the incidence of the pulmonary lesion. In this study, we used the Landrace line (Miyagino L2), which was genetically selected for the reduced incidence of the pulmonary MPS lesion, improved reproductive traits, and increased meat production for 5 generations, in the Miyagi Prefecture Animal Industry Experiment Station (Tayama et al., 2006). Therefore, this selected pig line is expected to show the resistant phenotype against the MPS lesion in the lung. As described earlier, Mhp infection is strongly associated with the immunophenotype of the host animal. Although the established Landrace line, Miyagino L2, is expected to show different immunophenotypes for the function, it has not been well characterized thus far.

In this study, we carried out a detailed analysis of the immune function of this line, using time-course analysis at the hematological level and the expression level of cytokines. The results of the selected line were compared with those of the non-selected Landrace group. Our data will provide us with insights on the formation of the pulmonary MPS lesion in the lungs of swine.

MATERIALS AND METHODS

Animals

All experiments were carried out at the School of Food, Agricultural and Environmental Science, at Miyagi University according to the animal handling guidelines for animal experiments. In this study, as the experimental group, we used 12 castrated Landrace pigs of Miyagino L2 (selected for the morbid change of the MPS lesion line; line S), which were genetically selected to show lower incidence of the pulmonary MPS lesion in Miyagi Prefecture Animal Industry Experiment Station. Tayama et al. previously described a detailed method for the genetic selection of Miyagino L2 (2006). In brief, this pig line was established based on the genetic selection aggregated breeding value of 4 traits (average daily gain, back fat thickness, MPS lesion, and cortisol concentration). As the control group, the same number (12 castrated males) of non-selected Landrace was used (No selected line: line N). The animals were introduced to the Tsubonuma farm (Miyagi University School of Food, Agricultural and Environmental Sciences, Miyagi University) at the age of about 80 days. Sheep red blood cells (SRBCs) were inoculated (10^9 cells/ml) into the subcutaneous tissue of the ears for antigen sensitization at days 42 and 49 after introduction. Immune functional analysis of T cells and the expression levels of cytokine

mRNAs were measured on days 42, 49, 50, 51, and 56. In addition, other immunofunctional analyses (SRBC-specific IgG, SRBC-specific IgM, salivary IgA, cortisol concentration, phagocytosis activity, complement pathway activity, total white blood cells, and granulocyte/lymphocyte ratio) were conducted on day 7 and the above-mentioned days. Delayed hypersensitivity reaction (DHR) was evaluated by measuring the area of the tumor on day 51.

SRBC-specific enzyme-linked immunosorbent assay

Blood samples were collected from the cervical veins of the animals. The plasma was separated by centrifugation at 2,000 rpm for 20 min at 4°C and divided into aliquots, and stored at -20°C until analysis. A 96-well SRBC-specific plate was incubated with the SRBC suspension (10^9 cells/ml) for 48 h at 4°C. Non-specific binding of the antigen or antibody to the plate was blocked by treatment with Block Ace (DS Pharma Biomedical, Osaka, Japan) for 120 h at 30°C. The plasma of the swine was exposed to the plate, and the signal of the SRBC-specific IgG was detected by alkaline phosphatase (AP)-labeled Rabbit-Poly Anti-Pig IgG (H+L) Antigen (Rockland Immunochemicals Inc., Philadelphia, PA, USA) for SRBC-specific IgG. For the detection of SRBC-specific IgM, AP-labeled Goat Polyclonal Antibody Anti-Pig IgM Antigen (Bethyl Laboratories Inc., Montgomery, TX, USA) was used. After washing to remove the non-specific binding, fluorescence intensity was measured at 405 nm at 5-min intervals after the reaction with the enzyme substrate solution.

Flow cytometry analyses

The peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque Plus medium (GE Healthcare UK Ltd., Little Chalfont, UK) and centrifuged from 2 ml of whole blood. PBMC cell suspensions at 1.0×10^6 cells/ml were used for further analysis. For the detection of CD4 expression in the T lymphocytes, PBMCs were incubated with FITC-labeled Mouse Anti-Porcine CD4a (Beckman Coulter Inc., Fullerton, CA, USA) and PE-labeled Mouse Anti-Porcine CD3 ϵ (Beckman Coulter) for 30 min at 4°C. For analysis of CD8 expression, FITC-labeled Mouse Anti-Porcine CD8a (Beckman Coulter) and PE-labeled Mouse Anti-Porcine CD3 ϵ (Beckman Coulter) were used under the same condition with CD8. The data were obtained with a FACSCaliber flow cytometer (Becton Dickinson Company, Franklin Lakes, NJ, USA).

Cortisol concentration

Plasma concentrations of cortisol were measured with Enzyme Immunoassay for Cortisol (Product No. EA65; Oxford Biomedical Research, Rochester Hills, MI, USA), according to the manufacturer's instructions.

Quantitative real-time RT-PCR

One milliliter of whole blood from each pig was immediately mixed with 3 ml of TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA) for total RNA extraction, following the manufacturer's protocol. Five micrograms of total RNA was reverse-transcribed into the cDNA in 5.0 ng/ μ l of Random primer (Invitrogen), 0.5 mM dNTPs (Invitrogen), 7.5 mM DTT, 12 units of RNase OUTTM inhibitor (Invitrogen) with 40 units of Superscript II (Invitrogen) in a volume of 10 μ l. The cDNA mixture (1 μ l) was used as the template for real-time PCR (RT-PCR). The reaction mixture consisted of 5 μ l of Probe qPCR Mix (ThunderbirdTM, Toyobo, Osaka, Japan), 0.1 μ l of 100 μ M 5'-FAM, 3-BHQ dual-labeled probe (Biosearch Technologies, Novato, CA, USA), and 0.4 μ l of 50- μ M forward and reverse primers (Sigma-Aldrich, Ishikari, Japan). The cycling reaction was examined for 51 cycles (95°C for 5 s for denaturation and 60°C for 30 s for annealing and extension).

The amplification primers and corresponding dual-labeled probes were newly designed based on information in the human and pig genome database. Since the genomic structure of swine is not fully elucidated, the exon-intron structure of the pig genes was predicted by their homology with the homologous human genes. To avoid amplification from the genomic DNA, the amplification region flanked by forward- and reverse-amplification primers was designed to traverse at least 2 exons. The sequences of primers and probes are listed elsewhere (Katayama et al., 2011).

Other measured immune functions

The amount of total IgA in saliva was measured as follows: The saliva of the pigs was collected from the nasal cavities by using cotton swabs. The saliva was diluted 500-fold with PBS and used for analysis. The concentration of IgA in the saliva was measured with the Pig IgA ELISA Quantitation Kit (Bethyl Laboratories) according to the manufacturer's protocol. The number of white blood cells (WBCs) in whole blood was measured with an autohemolytic counter (Celltac; Nihon Koden, Tokyo, Japan). The phagocyte activity was measured as described by Suzuki et al. (2009). The granulocyte/lymphocyte ratio was calculated from the counted numbers of granulocytes and lymphocytes using light microscopy of the blood smear,

stained with Diff-Quik (Sysmex Corp., Kobe, Japan). To amplify the accuracy of the counting, at least 100 cells were counted to obtain the granulocyte/lymphocyte ratio. Complement pathway activity was measured by the hemolytic reaction of rabbit red blood cells for the complement. DHRs were detected by measuring the diameter of the swollen area of the ear skin; the swelling was induced by subcutaneous inoculation of SRBCs.

Expected breeding value of MPS lesions

The selected pig line, Miyagino L2, was established from the genetic selection for 5 generations to obtain a lower incidence of the lung MPS lesion. Therefore, the sire and dam of this strain have been used as a reference to estimate the breeding value for the MPS lesion. The expected breeding value of each animal was then calculated according to the following equation.

$$\text{MPSEBV} = (\text{MPSBV}_{\text{sire}} + \text{MPSBV}_{\text{dam}}) / 2$$

MPSEBV = Expected breeding value of the MPS lesion of the animal

MPSBV_{sire} = Breeding value of the MPS lesion in the sire

MPSBV_{dam} = Breeding value of the MPS lesion in the dam

Statistical analysis

The data were statistically evaluated by the one-way analysis of variance (ANOVA) using SAS software version 9.1.3 Service Pack 3 (SAS Institute, Cary, NC, USA); the fixed effect was line.

RESULTS

Comparison of the selected line with the control line for swine growth

The resistance to the MPS lesion in the lung should be beneficial to pig production. However, we carefully need to evaluate whether genetic selection against the MPS lesion has any impact on the growth of the pig. Therefore, we first evaluated the difference in the body weight and daily gain among the genetically selected line and control group. As shown in Table 1, there was no significant difference (Table 1). From these data, we concluded that the genetic selection

Table 1. Body weight (kg) and average daily weight gain from experimental day 0 to day 76

Line	Body weight (kg)				ADG (g/d)
	Day 0	Day 7	Day 56	Day 76	Days 0-76
N	29.6 \pm 4.1	40.7 \pm 5.7	83.0 \pm 8.8	101.9 \pm 9.6	951.7 \pm 101.5
S	29.6 \pm 2.1	41.0 \pm 2.0	82.3 \pm 5.8	103.5 \pm 5.0	971.3 \pm 64.2

N = No selected line, S = Selected for the morbid change of the MPS lesion line.

ADG = Average daily gain. Means \pm divisions.

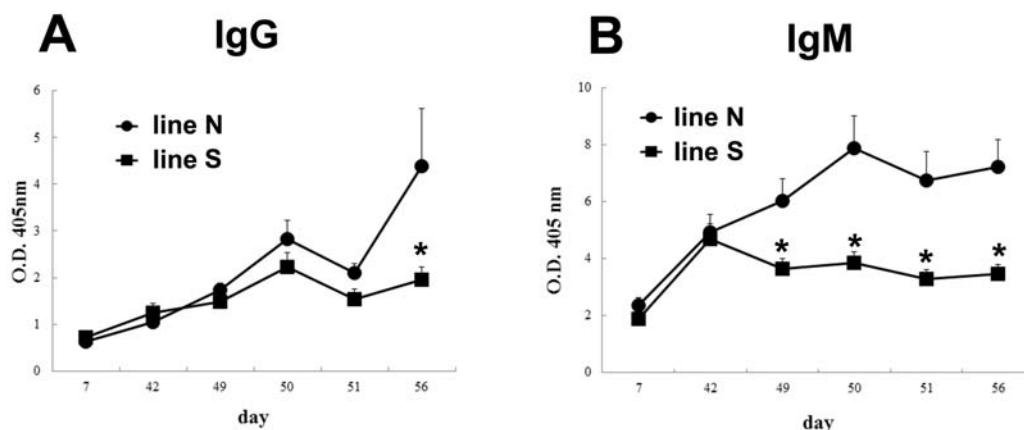


Figure 1. The results of the SRBC-specific antibody production. The average value of the control, (line N: circle), the selected line, (line S: square). The asterisks indicate statistical significance ($p < 0.05$). The standard errors of the data were shown with bar.

for the incidence of the pulmonary MPS lesion does not have any effect on the growth of the pig.

Antigen-specific antibody production in periphery blood

As the next step, we evaluated the IgG and IgM production performance of the selected strain. Figure 1 shows the results of the time-course analysis of antigen-specific antibody production in periphery blood. The SRBC-specific IgG is significant lower in the selected line on day 56 ($p < 0.05$). In addition, antigen-specific IgM is significantly lower in the selected line from day 49 to 56 ($p < 0.05$). These results indicated that IgG and IgM production performance are lower in the selected line as compared with that in the control group.

Analysis of CD4 and CD8 expression in peripheral T cells

The expression of cluster of differentiation (CD) is critical for antigen recognition in the T cell. Therefore, the expression of CD4 and CD8 in the peripheral T cell was detected in this study (Figure 2). There was no significant difference in the CD4⁺ cell ratio between the control and selected line. However, a positive cell ratio of CD8 in the selected line was observed on all occasions, and these high ratios of CD8⁺ reached statistical significance from day 50 to 56 (Figure 2C, $p < 0.05$). These results indicated that the selected Landrace line had a higher CD8 expression as compared with the control.

Other immune functions in peripheral blood

The concentration of cortisol is known to have an impact on the immune performance of the host animal, particularly in the production of IgG. As described in the previous section, the concentration of IgG in the selected pig line was found to be lower in this study. This led us to measure the concentration of cortisol. As shown in Figure 3,

the cortisol concentration was relatively high in the selected line on all occasions, and the measurement on day 50 revealed the statistical significance in the selected line ($p < 0.05$). From these results, we concluded that the concentration of cortisol in the selected line is higher than that in the control animals. Although we surveyed the other immune-related parameters such as phagocytosis activity, complement pathway activity, total WBCs, saliva total IgA, DHR, granulocyte/lymphocyte ratio, there was no significant difference between the selected line and the control (data not shown).

Analysis of cytokine expression in peripheral blood

As described earlier, the expression level of the cytokines largely affects the immune performance of the host animal. We next evaluated the difference in the expression levels of the cytokines in the peripheral blood among the selected line and the control. The expression level of cytokine (IL-1 β , IL-2, IL-4, IL-6, and TNF- α) mRNA is summarized in Figure 4. The higher expression level of IL-2 mRNA (day 56) and the high level of TNF- α mRNA (day 42, $p < 0.05$) in the selected line were detected at the each measurement point (Figure 4B, E). The relatively low level of IL-4 mRNA expression in the selected line was observed on day 51, although this change was not statistically significant (Figure 4C).

Correlation between MPS expected breeding value and CD4⁺ or CD8⁺ T cell ratio in the selected line

The genetic selection and establishment of a pig line based on the incidence of the pulmonary MPS lesions has the potential to increase the Mhp resistance of the animal. When we used the number of MPS lesions of the lung as a basis for selection we needed to sacrifice the host animal to measure the incidence of the pulmonary MPS lesions. If there were a biomarker in the peripheral blood, which shows a high correlation with the incidence of the MPS

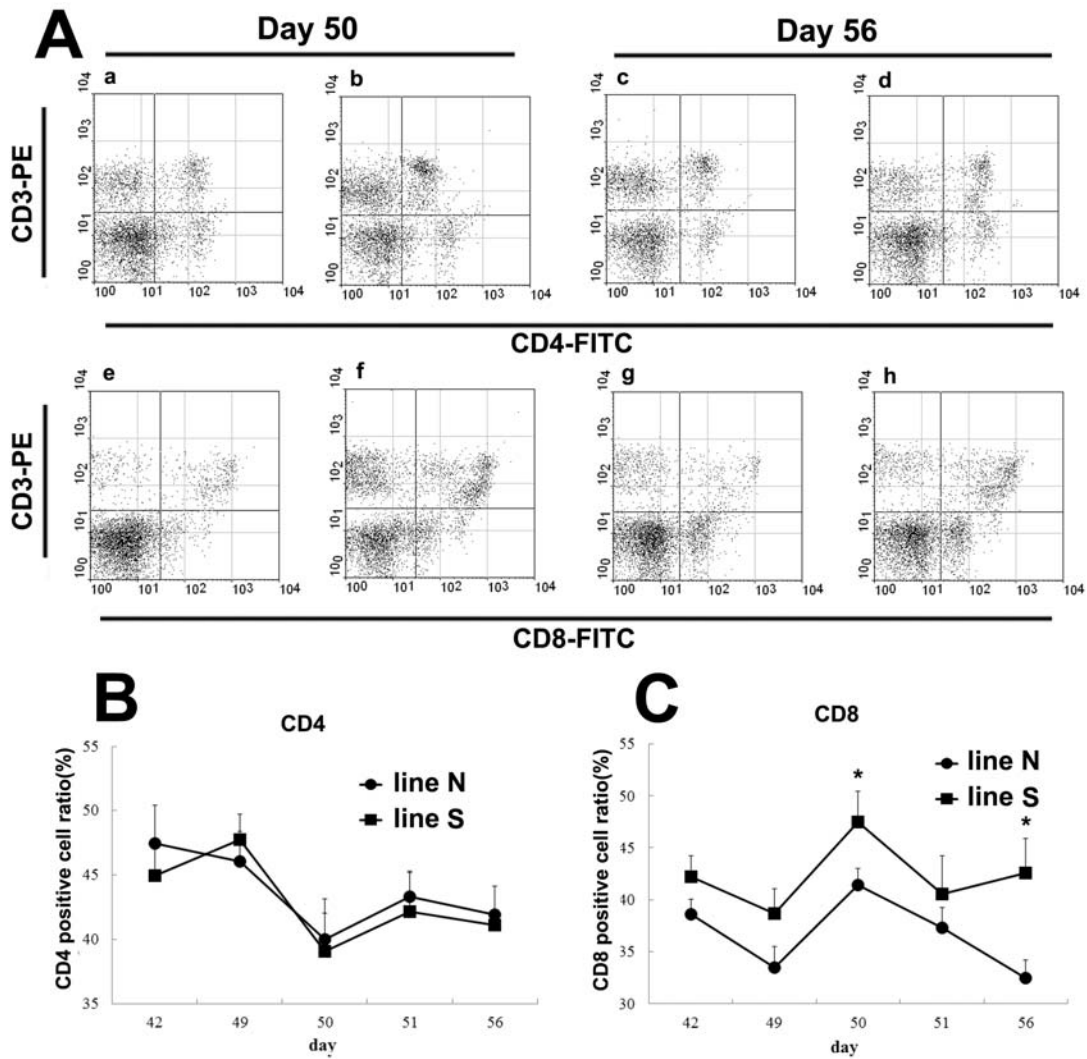


Figure 2. CD4- and CD8-positive cell ratio in peripheral T cell. CD4 and CD8 expression in peripheral T cells is shown (A), CD4⁺ cell (a-d), CD8⁺ cell (e-h). Line N on day 50 (a), line S on day 50 (b), line N on day 56 (c), line S on day 56 (d), line N on day 50 (e), line S on day 50 (f), line N on day 56 (g), and line S on day 56 (h). CD4⁺ T cell ratio (B), CD8⁺ T cell ratio (C). The average value of the control line (line N: circle) and the selected line (line S: square). The asterisks indicate statistical significance ($p < 0.05$). The standard errors of the data were shown with bar.

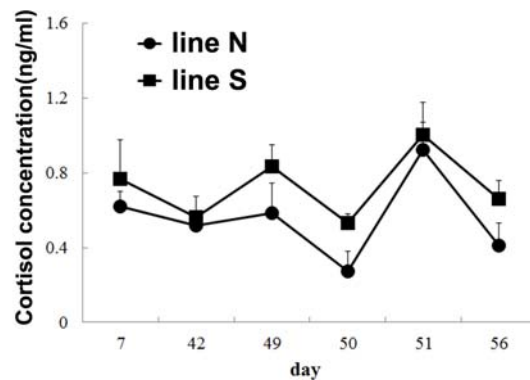


Figure 3. Cortisol concentrations in peripheral blood. The average value of the control line (line N: circle) and the selected line (line S: square). The asterisks indicate statistical significance ($p < 0.05$). The standard errors of the data were shown with bar.

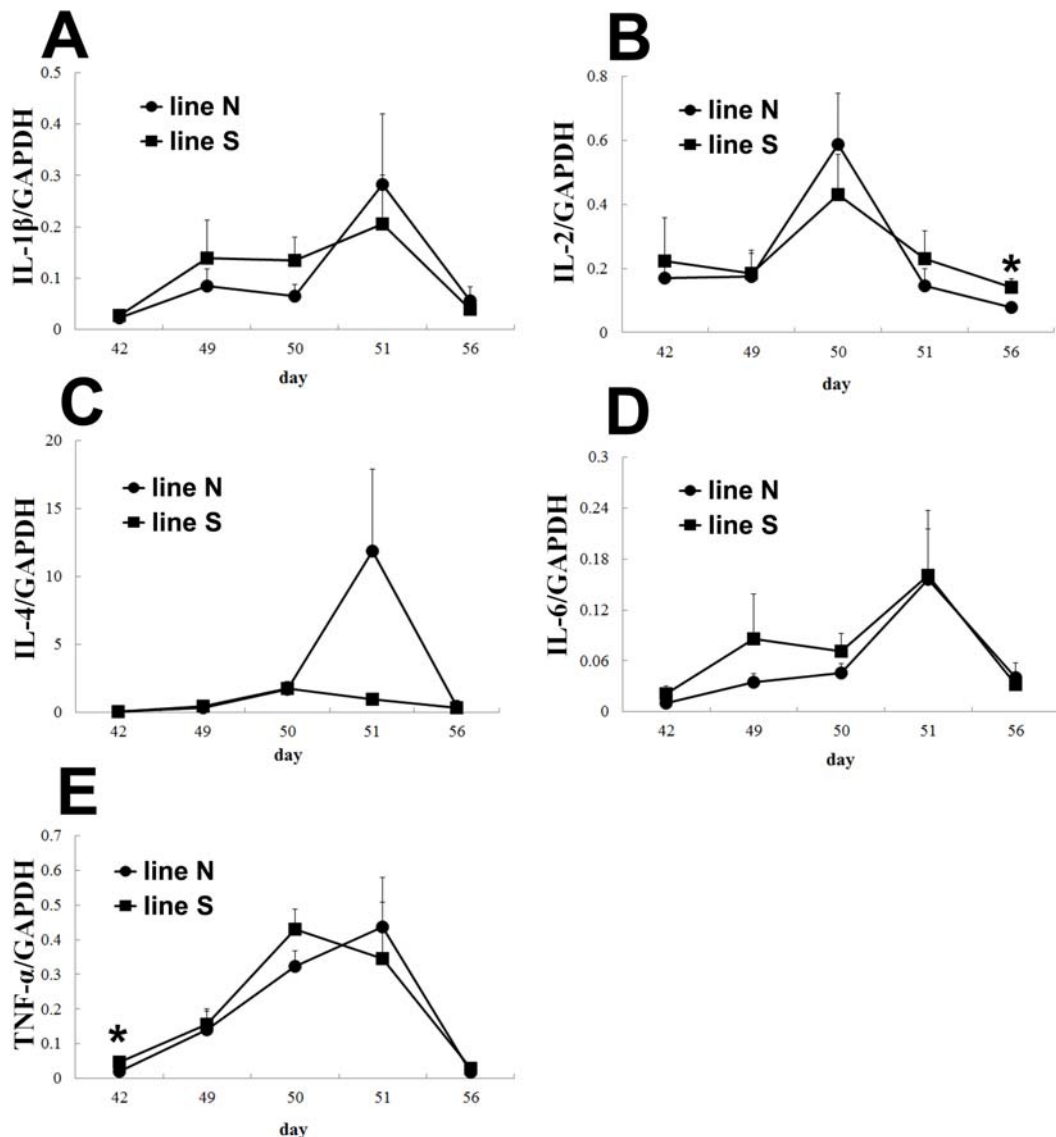


Figure 4. The expression levels of cytokine (IL-1 β , IL-2, IL-4, IL-6, and TNF- α) mRNA. The results of IL-1 β (A), IL-2 (B), IL-4 (C), IL-6 (D), and TNF- α (E) are shown. The average value of the control line (line N: circle) and the selected line (line S: square). The asterisks indicate statistical significance ($p < 0.05$). The standard errors of the data were shown with bar.

lesion, that marker would be useful for animal breeding because indirect genetic selection using the biomarker becomes possible. The correlation between all of the measured results in the peripheral blood and MPSEBV was evaluated. As shown in Table 2, MPSEBV showed a significant correlation with the CD4-positive cell ratio on days 42 and 56 ($p < 0.01$).

DISCUSSION

In the present study, we used the Landrace line (Miyagino L2), which is genetically selected for the reduced incidence of pulmonary MPS. The formation of the pulmonary lesion of MPS is closely related with the immune function. For example, the expression of

Table 2. Correlation between estimated breeding value and CD4 or CD8 positive peripheral T cell in line S

T cell subpopulation	Coefficient of correlation				
	Day 42	Day 49	Day 50	Day 51	Day 56
CD4 positive	-0.800**	-0.275	-0.269	-0.396	-0.680**
CD8 positive	0.031	-0.018	-0.008	-0.106	0.067

** $p < 0.01$.

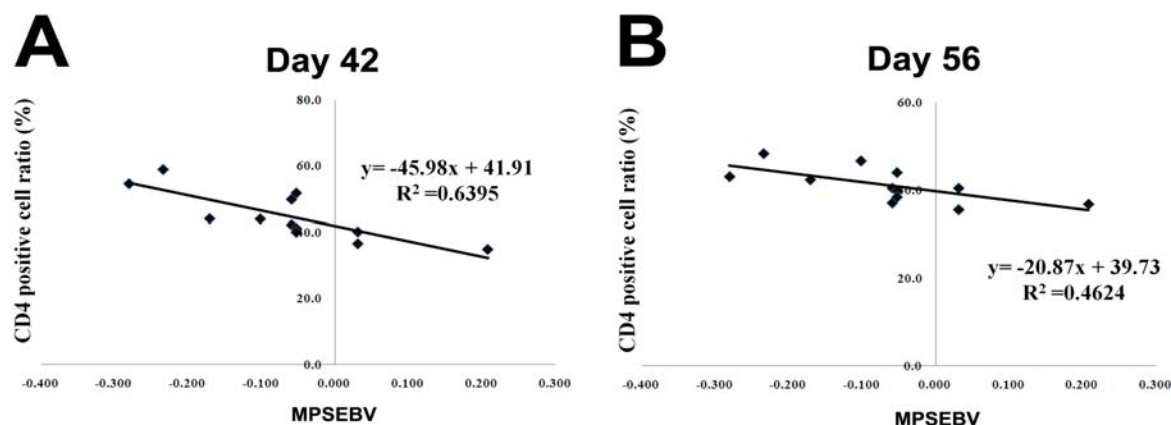


Figure 5. Recurrence between MPSEBV and CD4⁺ T cell ratio in the selected line. Recurrence between MPSEBV and CD4⁺ cell ratio on day 42 in the selected strain (line S) (A) and on day 56 (B). The equation in the figure shows the regression equation, and R² shows the determination coefficient.

inflammation-related cytokines, such as IL-6 and IL-1, was reported to have important roles in the establishment of the MPS lesion in the lung (Asai et al., 1993; Asai et al., 1994). The immunological characteristics of the selected Landrace line can be explored by a time-course analysis after the antigen sensitization. Furthermore, the characterization of the selected Landrace strain possibly clarifies which immunological phenotype is the most important for the resistance to the MPS lesion.

In this study, we analyzed the amount of IgG and IgM production, which is specific to SRBCs. The results of the analysis revealed that the production of IgG and IgM in the selected Landrace line is significantly lower as compared with that of the control, which was detected on day 56 (IgG) and day 49 to 56 (IgM). Our results indicate that this reduced production of IgG and IgM are the most prominent characteristics in the immunological function of the selected line. These results clearly showed that animal breeding for the immunological characteristics is feasible, although the heritability of the immune characteristics have been reported relatively low. The amount of IgG production is regulated by the Th1 and Th2 balance (Mosmann et al., 1986; Mosmann and Coffman, 1987; Murtaugh et al., 2009). In agreement with the lower production level of IgG in the selected Landrace line, the expression level of IL-4 was relatively lower in the selected Landrace line, although that difference was not statistically significant on day 51. IL-4 is known to change the Th1/Th2 balance in favor of Th2, resulting in an increased amount of IgG production (Murtaugh et al., 2009). The lower level of IL-4 might be responsible for the lower level of IgG in the selected line. The Landrace line used in this study was selected for 5 generations for reduced the incidence of the MPS lesion in the lung. The amount of IgG is known to increase after Mhp infection, which is responsible for the pulmonary MPS lesion (Messier et al., 1990; Muneta et al., 2008). A

previous study indicated that the pulmonary lesion of MPS is established through the hyperactivation of the immune system of the host (Choi et al., 2006). Although we need to obtain further supportive evidence to prove this hypothesis, there is a possibility that the resistant phenotype of the selected Landrace is responsible for the reduced IgG response, which is not sufficient for the formation of the MPS lesion in the lung tissue. Our data also suggested that the amount of IgG and IgM are important factors in lesion formation of MPS in the pig.

The results of the T cell analysis revealed that the CD8-positive cell ratio of the selected Landrace line is higher than that of the control group across all measurement points, and this difference reached statistical significance on days 50 and 56. Furthermore, the expression level of the IL-2 mRNA of the selected Landrace line was significantly higher on day 56. IL-2 is categorized into the Th1 cytokines, which are derived from the activated Th1 cells (Mosmann et al., 1986; Mosmann and Coffman, 1987; Murtaugh et al., 2009). Earlier, we described that the Th1/Th2 balance of the selected Landrace line might be induced in the Th1 direction, which results in enhanced T cell activity and reduced IgG production, based on the reduced expression level of IL-4. Therefore, the elevated level of IL-2 in the selected line is in good agreement with the reduced expression level of IL-4 in that line, suggesting the enhanced T cell activity of this selected pig line. Currently, we are planning to examine the function of the T cell of the selected Landrace line to support this notion.

In this study, the cortisol concentration showed a higher value in the selected line at all measurement points and reached statistical significance on day 50. Cortisol is one of the humoral factors, which is released during an inflammation reaction (Williams et al., 2009). It is known to have an immunotolerance effect (Brown-Borget et al., 1993; Johnson et al., 1994; Wallgren et al., 1994). Although the

details are not clear, the elevated cortisol levels might be connected with the suppression of hyper-activation of the host immune animals, resulting in reduced incidence of the MPS lesion in the selected Landrace line.

The selected Landrace line was established based on 5 generations of the genetic selection on the incidence of the MPS lesion in the lung. Previously, we needed to sacrifice and dissect the animal to obtain this data. The existence of a biomarker having a high correlation with the incidence of the MPS lesion would facilitate the indirect selection for the genetic improvement of the MPS resistance of the animal. Therefore, we analyzed the correlation between MPSEBV and all measured parameters. The results of the analysis showed that there is a high correlation between MPSEBV and the CD4 positive cell ratio. This high correlation was observed at the time point before antigen sensitization (day 42) and the time point of the second round antigen sensitization after 7 day (day 56). In these 2 points, the effects of the antigen sensitization in the host animals were expected to be at a minimum, based on the time frame. We could thus conclude that the effects of antigen sensitization are better when minimized for a better estimation of MPS resistance, based on the ratio of the CD4-positive cell. Although the detailed mechanism underlying the high correlation between MPSEBV and the CD4-positive cell ratio remains unknown, there is a possibility that the genetic selection of the CD4-positive cell ratio might improve the incidence of the MPS pulmonary lesion. To obtain supportive evidence for this correlation, we need to evaluate this correlation based on the data from a larger sample size. The supportive evidence of this correlation will contribute to the rapid establishment of the new pig line resistant to Mhp infection.

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