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Immunology

A specific promoter-type in ribonuclease L gene is associated with phagocytic activity in pigs

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ABSTRACT. We have previously generated Large White pigs with high immune competence using a selection strategy based on phagocytic activity (PA), capacity of alternative complement pathway, and antibody response after vaccination against swine erysipelas. In this study, to identify the genetic changes caused by the immune selection pressure, we compared gene expression and polymorphisms in the promoter region between pigs subjected to the immune selection (immune-selected pigs) and those that were not (non-selected pigs). After lipid A stimulation, using a microarray analysis, 37 genes related to immune function and transcription factor activity showed a greater than three-fold difference in expression between macrophages derived from immune-selected and non-selected pigs. We further performed a polymorphic analysis of the promoter region of the differentially expressed genes, and elucidated the predominant promoter-types in the immune-selected and non-selected pigs, respectively, in the genes encoding ribonuclease L (*RNASEL*), sterile α motif and histidine-aspartate domain containing deoxynucleoside triphosphate triphosphohydrolase 1, signal transducer and activator of transcription 3, and tripartite motif containing 21. Analysis of the association between these promoter genotypes and the immune phenotypes revealed that the immune-selected promoter-type in *RNASEL* was associated with increased PA and was inherited recessively. Considering that *RNASEL* has been reported to be involved in antimicrobial immune response of mice, it may be possible to enhance the PA of macrophages and improve disease resistance in pig populations using *RNASEL* promoter-type as a DNA marker for selection.

KEY WORDS: disease resistance, macrophage, phagocytic activity, pig, promoter

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In the swine industry, the risk of infectious diseases and associated economic losses are increasing [40], and opportunistic infections such as pneumonia and diarrhea are prevalent. The recent epidemics of classical swine fever in Japan and African swine fever in East Asia have become public health concerns. Since vaccination has not been able to completely control the prevalence of infectious diseases and the use of antimicrobial agents is severely limited, genetically improving the disease resistance of pigs has become an urgent need of this industry. To date, only a few studies have explored the host genomic regions responsible for the development of infectious diseases. For example, chromosome 2 and chromosome 4 have been identified as the genomic regions responsible for the development of mycoplasma pneumonia, caused by *Mycoplasma hyopneumoniae*, and porcine reproductive and respiratory syndrome (PRRS), caused by *Betaarterivirus suid 1/2* (PRRS virus; PRRSV), using quantitative trait locus analysis [23] and the 60 K Illumina single nucleotide polymorphism (SNP) chip [4], respectively.

We have previously generated Large White pigs with high immune competence by selecting phagocytic activity (PA), capacity

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of alternative complement pathway (CACP), and antibody response after vaccination against swine erysipelas (AR) for six generations [5]. Borjigin *et al*. have reported that this selection strategy was not effective in increasing resistance to pneumonia-associated lesions after vaccination with *M. hyopneumoniae* [5]. However, there are no reports on the resistance of immune-selected pigs to other infections or the genetic changes caused by the selection pressure.

Macrophages are responsible for pathogen recognition and phagocytosis in the early stages of an infection. They also transmit information of phagocytosed pathogens to unstimulated T cells and induce T cell-mediated cellular immune response and B cell-mediated humoral immune response. Thus, macrophages play an extremely important role in both the innate and acquired immune systems.

In this study, we aimed to identify the genetic changes that are induced by the immune selection strategy by comparing the gene expression profiles of macrophages derived from pigs subjected to the immune selection (immune-selected pigs) and those that were not (non-selected pigs). We found that after lipid A stimulation of macrophages, the expression of the ribonuclease L (*RNASEL*) gene and the frequency of a specific promoter-type of *RNASEL* was significantly increased in immune-selected pigs. These results indicate that it is possible to improve the immune competence of pig populations using polymorphisms of immune-related genes and that this selection strategy could help in reducing economic losses from infections in the swine industry.

MATERIALS AND METHODS

Experimental animals

Large White pigs with high immune competence were generated by selecting for PA, CACP, AR for six generations at the Research and Development Center, NH Foods Ltd., Osaka, Japan [5]. Pigs of the basic generation, before the immune selection began, were assigned to the non-selected group, and those of the sixth generation, after immune selection, were assigned to immune-selected group. These pigs were used for isolation of macrophages and comparison of the differences in gene expression and promoter polymorphisms between non-selected and immune-selected groups. Pigs of the third generation, in the process of immune selection, were used for association analysis between genotypes and immune phenotypes. Ethical review and approval were not required for the animal study because the pig samples (bloods) used in this study were reused those for regulation of health control of pigs for commercial meat products.

Isolation of macrophages from peripheral blood

Heparinized peripheral blood was obtained from littermates of 3-month-old female Large White pigs from both the immune-selected and non-selected groups (n=4 per group), and 1.5 ml of the blood was directly added to a culture of feeder cells, consisting of epithelial and fibroblastic cells from porcine kidney tissue, in Dulbecco's modified Eagle medium (Gibco/Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco/Invitrogen), 10 µg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 100 µM 2-mercaptoethanol (Sigma-Aldrich), 50 U/ml penicillin (Life Technologies, Carlsbad, CA, USA), 50 µg/ml streptomycin (Life Technologies), in a T-75 flask and maintained at 37°C and 5% CO_2 [31]. After 24 hr, blood-derived components other than macrophages, which were not attached to the feeder cells, were removed by washing with phosphate-buffered saline, and the growth medium was replaced every 3–4 days. After approximately 2 weeks, blood-derived macrophages actively proliferating on the feeder cells were collected from the culture supernatant by centrifugation (1,500 rpm for 5 min) for subsequent analysis.

Microarray design

A microarray to evaluate gene expression was designed and arranged with cDNA sequences derived from full-length-enriched pig cDNA libraries [33], pig RNA sequences from RefSeq of the National Center of Biotechnology Information (NCBI; release 55), and estimated transcript sequences of pig immune-related genes generated in several genome sequencing projects [1, 7, 9–12, 25–29, 32, 34, 35, 38] (Agilent Technologies, Santa Clara, CA, USA). The generated microarray platform has been deposited in the NCBI Gene Expression Omnibus (GEO) under the accession number GPL19540 (PMID: 27608441).

Gene expression analysis using the microarray

Macrophages derived from immune-selected and non-selected pigs were plated at a density of 1×10^6 cells per well in a 24-well plate (n=4 per group). After stimulation for 4 hr with 10 ng/ml lipid A (Enzo Life Sciences, Farmingdale, NY, USA), a component of the lipopolysaccharide responsible for the toxicity of gram-negative bacteria, the cells were collected by centrifugation (1,500 rpm for 5 min) and the total RNA was extracted using a RNeasy Mini Kit (QIAGEN, Hilden, Germany). The total RNA (400 ng) from the stimulated or non-stimulated cells was labeled with cyanine-5 (Cy5) and cyanine-3 (Cy3) fluorescent dyes, respectively, and was subjected to microarray analysis as previously described [22]. The expression data have been deposited in the NCBI GEO under the following accession numbers: Samples, GSM4851044-4851051 and Series, GSE159942. The relative gene expression after lipid A stimulation in 38,721 probes was calculated by dividing the signal intensity of Cy5 by that of Cy3. The difference in relative gene expression between immune-selected and non-selected pigs was evaluated using a two-tailed Student's *t*-test ($P<0.05$) after multiple comparison using the Benjamini-Hochberg method [2].

Detection of polymorphism in gene promoter region

The nucleotide sequence 5 kb upstream from the transcription start site (TSS) of each gene was obtained from the Sscrofa10.2 assembly of the pig genome provided by Ensembl (https://may2017.archive.ensembl.org/Sus_scrofa/Info/Index). Polymerase chain reaction (PCR) primers used for amplifying the aforementioned genomic region were designed using the Ion AmpliSeq Designer

(https: //www. ampliseq. com). Genomic DNA extracted from unrelated immune-selected and non-selected pigs was subjected to PCR (n=10 per group), and the resultant products were sequenced using the IonPGM system (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences and low-quality regions in the sequencing reads were trimmed using the Btrim software set at default settings [18]. Processed sequencing reads were aligned to the pig genomic sequence (Sscrofa10.2) using the Burrows-Wheeler aligner and the picard software, and polymorphisms such as SNPs and insertions and deletions (indels) were detected using GATK [20, 36]. Polymorphisms that exhibited a biased distribution between immune-selected and non-selected pigs were identified using a χ^2 test of independence ($P<0.05$). Haplotypes of polymorphisms with biased distribution in the genes *RNASEL*, sterile α motif and histidine-aspartate domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (*SAMHD1*), signal transducer and activator of transcription 3 (*STAT3*), and tripartite motif containing 21 (*TRIM21*) were estimated using the expectation-maximization algorithm of the Arlequin 3.5 software [13, 14].

Prediction of binding sites for transcription factors

The sequences of predicted binding sites of transcription factors in the human genome were extracted from the JASPAR database (http://jaspar.genereg.net/) and were compared to the promoter sequence of *RNASEL*, *SAMHD1*, *STAT3*, and *TRIM21*, which are predominantly present in non-selected (haplotype 1) and immune-selected (haplotype 2) pigs. Sites with 90% or more identity to the sequences of the human genome were considered to be the binding sites of the transcription factor. When the resulting binding sites differed between the non-selected and immune-selected pigs, the polymorphisms within the sites were considered to have a potential influence on the binding of transcription factors.

Association analysis between genotypes and immune phenotypes

Two hundred and twenty third-generation Large White pigs were used for analysis. PCR amplification of the fragments containing representative SNPs characteristic of immune-selected and non-selected pigs was conducted with AmpliTaq Gold polymerase (Thermo Fisher Scientific). The PCR consisted of 40 cycles, each comprising 30 sec at 94°C, followed by 30 sec at 55°C, and 30 sec at 72°C, after incubation at 94°C for 9 min. Sequencing was conducted with an ABI 3730XL Genetic Analyzer (Thermo Fisher Scientific), and genotypes were determined based on the combination of the SNPs. Association analysis between genotypes and immune phenotypes of PA, CACP, and AR was performed using a generalized linear model package in SPSS (version 25; IBM, Armonk, NY, USA). The model is expressed as follows:

$$Y_{ijklm}=\mu + sex_i + RNASEL_j + SAMHD1_k + STAT3_l + TRIM21_m + e_{ijklm}$$

where Y_{ijklm} is the phenotypic value of the pig with the *i*th sex, the *j*th *RNASEL*, the *k*th *SAMHD1*, the *l*th *STAT3*, and the *m*th *TRIM21*; μ is the intercept; sex_i is the effect of the *i*th sex; $RNASEL_j$, $SAMHD1_k$, $STAT3_l$, and $TRIM21_m$ are the effects of the genotypes of the *j*th *RNASEL*, *k*th *SAMHD1*, *l*th *STAT3*, and *m*th *TRIM21*, respectively; and e_{ijklm} is the random error term.

RESULTS

Comparison of gene expression between immune-selected and non-selected pigs

We isolated macrophages from four littermates of immune-selected and non-selected pigs. To identify genes showing differential expression between the two groups after stimulation with lipid A, RNA from the macrophages was extracted and subjected to microarray analysis. We found that out of the 38,721 probes, 5,794 probes exhibited statistically significant differences between immune-selected and non-selected pigs. To narrow down the targets, we excluded duplicate probes designed for the same gene and selected probes showing a greater than 3-fold difference in gene expression between the two groups after lipid A stimulation. In addition, we focused on genes that are related to “immune system process” and “nucleic acid binding transcription factor activity” based on the gene ontology (GO) terms, GO:0002376 and GO:0001071, respectively. We identified 30 and 13 genes related to “immune system process” and “nucleic acid binding transcription factor activity”, respectively (Fig. 1), and six genes, *DDX58*, *EPAS1*, *FOS*, *LMO4*, *PLSCR1*, and *TRIM21*, were related to both GO terms. A total of 37 genes were selected for further analysis (Supplementary Table 1).

Analysis of polymorphism in gene promoter region

To identify the genetic polymorphisms responsible for the differences in gene expression between the immune-selected and non-selected pigs, we attempted a polymorphism search in the promoter region of the aforementioned 37 genes. The nucleotide sequence 5 kb upstream from the TSS of the genes, except for *FOXS1* and *PLSCR1*, which had incomplete genomic information in the Sscrofa10.2 pig genome database, was obtained (Supplementary Table 1). Subsequently, 690 primer sets were designed to amplify the entire region of the obtained nucleotide sequence (Supplementary Tables 1 and 2). Genomic DNA derived from ten unrelated immune-selected and ten unrelated non-selected pigs were sequenced, and 1,490 SNPs and 497 indels were found in the promoter region of 34 genes except for *ULBP1* (Supplementary Table 1). Among these polymorphisms, 7 SNPs and 2 indels in *RNASEL*, 6 SNPs in *SAMHD1*, 30 SNPs in *STAT3*, and 46 SNPs and 4 indels in *TRIM21* exhibited biased distributions between immune-selected and non-selected pigs (Table 1). Using the Arlequin 3.5 software, 2, 2, 5, and 4 haplotypes composed of these polymorphisms were estimated in *RNASEL*, *SAMHD1*, *STAT3*, and *TRIM21*, respectively, and the promoter-types predominant in non-selected (haplotype 1) and immune-selected pigs (haplotype 2) were identified.

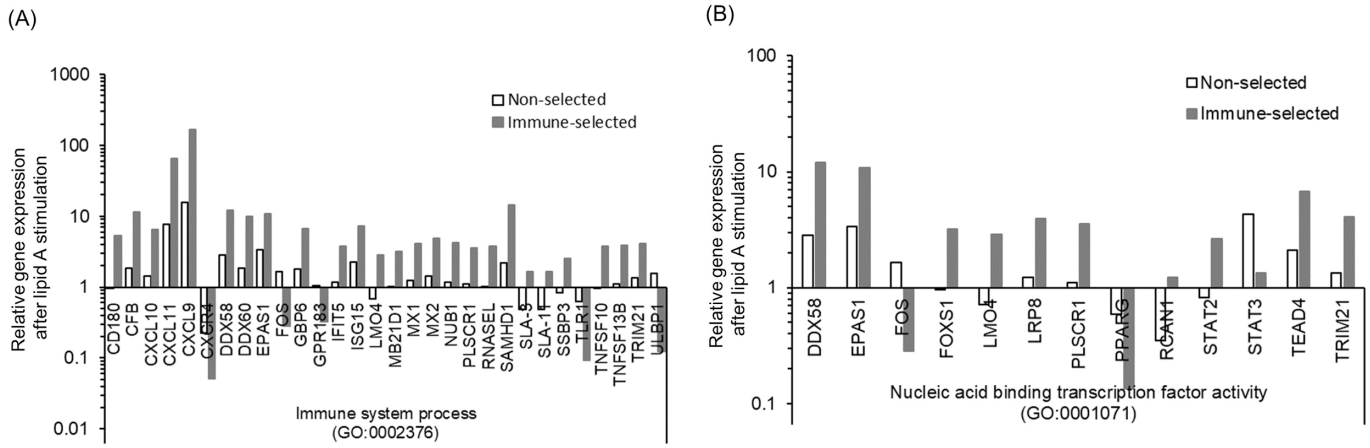

Fig. 1. Differences in gene expression after lipid A stimulation between immune-selected and non-selected pigs. Macrophages derived from immune-selected and non-selected pigs (n=4 per group) were stimulated with lipid A, and total RNA was extracted and subjected to microarray analysis. The expression level of each gene after stimulation was divided by that without stimulation; the results are represented as the mean of the four pigs. Genes with more than a three-fold difference in relative expression between immune-selected and non-selected pigs were classified according to the gene ontology (GO) terms, "immune system process" (GO: 0002376) **(A)** and "nucleic acid binding transcription factor activity" (GO: 0001071) **(B)**.

Association between promoter-types and immune phenotypes

To confirm that the frequency of the predominant promoter-types in the immune-selected pigs increased as a result of the selection over six generations, we conducted an association analysis between promoter genotypes and immune phenotypes. The rare haplotypes, 3a, 3b, and 3c in *STAT3*, 3a, and 3b in *TRIM21*, were collectively analyzed as haplotype 3 (Table 1). We used the third generation because both promoter-types predominant in non-selected and immune-selected pigs were expected to be present at a moderate frequency. The promoter genotypes of *RNASEL*, *SAMHD1*, *STAT3*, and *TRIM21* genes in 220 pigs were determined by sequencing representative SNPs (Table 1). The phenotypic characteristics of PA, CACP, and AR for each genotype are summarized in Table 2. Association analysis using a generalized linear model revealed the relationship between sex and PA ($P<0.05$), *RNASEL* and PA ($P<0.01$), *RNASEL* and CACP ($P<0.01$), *RNASEL* and AR ($P<0.05$), *STAT3* and CACP ($P<0.05$), and *TRIM21* and CACP ($P<0.05$) (Supplementary Table 3). Since *SAMHD1* was not associated with immune phenotypes, it was unclear whether there was an indirect effect or a coincidence that the frequency of promoter-types of *SAMHD1* was biased during immune selection. Pigs with the genotype $RNASEL^{2/2}$, which is homozygous for the *RNASEL* promoter-type predominant in immune-selected pigs, showed significantly greater PA ($P<0.01$) and AR ($P<0.05$) than did those with the genotype $RNASEL^{1/1}$, which is homozygous for the *RNASEL* promoter-type predominant in non-selected pigs (Table 3, Fig. 2A and 2C). These results were consistent with the direction of selection to increase PA and AR. The genotype $RNASEL^{2/2}$ also had the opposite effect of decreasing CACP compared with the genotype $RNASEL^{1/1}$ ($P<0.01$) (Table 3, Fig. 2B). With regard to the CACP, the pigs with haplotype 2 of the *STAT3* gene promoter, $STAT3^{1/2}$ ($P<0.05$), $STAT3^{2/2}$ ($P<0.01$), and $STAT3^{2/3}$ ($P<0.05$), had significantly weaker CACP than did pigs with genotype $STAT3^{1/1}$ (Table 3, Fig. 2D). In addition, the pigs with the genotype $TRIM21^{2/3}$ had significantly weaker CACP than did those with the genotype $TRIM21^{1/1}$ ($P<0.05$) (Table 3, Fig. 2E).

DISCUSSION

In this study, we identified genetic changes in pigs caused by an immune selection strategy. Microarray analysis revealed that the selection pressure amplified expression changes in many genes related to immune function and transcription factor activity in macrophages stimulated with lipid A. *RNASEL*, *SAMHD1*, and *TRIM21* were upregulated in response to lipid A stimulation, and *STAT3* was downregulated. Polymorphism analysis of the promoter regions of these differentially expressed genes revealed a biased distribution of promoter-types between immune-selected and non-selected pigs. We confirmed associations between promoter genotype and immune phenotypes in *RNASEL*, *TRIM21*, and *STAT3*.

Haplotype 2 of the *RNASEL* gene promoter, inherited recessively, increased in frequency as a result of the immune selection and was associated with increased PA. RNASEL is involved in host defense mechanisms against viral infection and acts in coordination with oligoadenylate synthetase (OAS) [15]. It also cleaves single-stranded RNA into small pieces and activates retinoic acid-inducible gene I and nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 inflammasome in the innate immune system [15]. In the OAS family, *OAS1a*, *OAS1b*, *OAS2*, and *OASL* are expressed in pigs [24], and the overexpression of *OAS1b* and *OAS2* in a stable porcine macrophage cell line carrying the CD163 receptor inhibited the replication of PRRSV [37, 39]. It has also been reported that the replication of pseudorabies virus increased in a porcine kidney epithelial cell line PK-15, wherein *RNASEL* was knocked out using genome-editing technology [30]. $RNASEL^{-/-}$ mice have also been reported to

Table 1. Promoter polymorphisms and estimated haplotypes that exhibited a biased distribution between non-selected and immune-selected pigs

Gene: *RNASEL* — Position from transcription start site

Haplotype[a]	-4901[c]	-4851	-4844	-4412[d]	-3743	-3195	-2170[b]	-2141[b]	-604	Non-selected	Immune-selected	P value (χ^2 test)
1	0	T	A	0	C	C	G	T	A	18	9	0.0024
2	1	C	G	1	T	T	C	A	G	2	11	

Gene: *SAMHD1* — Position from transcription start site

Haplotype[a]	-4758	-4645[b]	-4586[b]	-3831	-2150	-386	Non-selected	Immune-selected	P value (χ^2 test)
1	A	T	A	C	G	C	10	2	0.0058
2	G	C	G	T	A	A	10	18	

Gene: *STAT3* — Position from transcription start site

Haplotype[a]	-4969	-4717	-4383	-4220	-4075	-4034	-3930	-3902	-3836	-3681	-3198[b]	-3122[b]	-2743	-2623	-2083	-1980	-1978	-1938	-1697	-1608	-1533	-1484	-1403	-1077	-943	-667	-609[b]	-381[b]	-340[b]	-29	Non-selected	Immune-selected	P value (χ^2 test)
1	C	G	C	C	A	C	A	T	C	G	C	A	A	T	C	C	G	C	A	T	A	T	C	C	C	A	T	A	T	C	10	1	0.0005
2	T	A	T	T	T	G	T	C	T	A	T	G	G	C	T	T	A	T	G	C	G	A	T	T	T	G	C	C	G	T	4	18	
3a	T	A	C	C	A	G	A	T	C	G	C	G	G	C	C	T	G	T	G	C	A	T	C	C	C	G	T	A	T	C	4	1	
3b	T	A	T	T	T	C	A	C	T	G	C	G	G	C	C	T	G	T	G	C	A	T	T	C	C	G	T	C	G	C	1	0	
3c	C	G	C	C	A	C	A	T	C	G	T	G	A	T	C	C	G	C	A	T	A	T	C	C	C	A	T	A	T	C	1	0	

Gene: *TRIM21* — Position from transcription start site

Haplotype[a]	-2369	-2349[e]	-2335	-2333	-2305	-2277	-2261	-2250	-2199	-2155	-2129	-2097	-2084	-1922	-1910	-1734[b]	-1646[b]	-1617[b]	-1605[b]	-1553[b]	-1454	-1327	-1269	-1169	-1147	-1146	-1136	-1108[f]	-1105	-1099[g]	-1073	-990	-704	-697	-658[h]	-640	-627	-622	-561	-557	-553	-479	-455	-439	-379	-54	-47	-46	-28	-26	Non-selected	Immune-selected	P value (χ^2 test)
1	G	0	T	A	T	C	A	A	A	C	A	C	A	A	T	A	C	T	T	G	A	T	G	A	G	C	A	0	C	0	G	G	C	G	0	T	T	G	T	T	T	A	C	G	G	A	A	T	C	C	11	1	0.0031
2	T	1	C	C	A	T	G	G	G	T	G	T	G	G	C	G	T	C	A	A	G	C	T	T	A	T	G	1	A	1	C	A	T	T	1	C	A	A	G	G	G	C	A	C	A	C	G	C	T	T	7	17	
3a	G	0	C	A	A	T	G	G	G	C	G	C	A	A	T	G	C	T	T	G	A	T	T	A	G	C	A	1	A	1	G	G	C	G	0	T	T	G	T	T	G	C	C	C	G	C	G	C	T	T	1	0	
3b	G	0	C	A	A	T	G	G	G	C	G	C	A	A	T	G	C	T	T	G	A	T	T	A	G	C	A	1	A	1	G	G	C	G	0	T	T	G	T	T	T	C	C	C	G	C	G	C	T	T	1	2	

RNASEL, ribonuclease L; *SAMHD1*, sterile α motif and histidine-aspartate domain containing deoxynucleoside triphosphate triphosphohydrolase 1; *STAT3*, signal transducer and activator of transcription 3; *TRIM21*, tripartite motif containing 21. [a] 1, predominant haplotype in non-selected pigs; 2, predominant haplotype in immune-selected pigs; 3, minor haplotypes. [b] Representative SNPs to discriminate haplotype predominant in non-selected pigs from that in immune-selected pigs. [c] 0, TGCTTTGCCTCT; 1, T. [d] 0, GTGCGGG; 1, G. [e] 0, GT; 1, G. [f] 0, CGA; 1, C. [g] 0, T; 1, TCTCCG. [h] 0, AG; 1, A.

Table 2. Distribution of phenotypic values according to the genotype of the third generation of immune competent Large White pigs

Genotype	Sample (n=220)	Phenotypic value (mean ± S.D.)		
		PA	CACP	AR
Sex				
Female	115	137 ± 89.0	79.1 ± 14.4	0.500 ± 0.415
Male	105	159 ± 92.8	78.9 ± 13.5	0.489 ± 0.346
RNASEL				
1/1	154	137 ± 76.9	79.6 ± 14.3	0.483 ± 0.361
1/2	25	144 ± 67.6	83.8 ± 12.3	0.343 ± 0.260
2/2	41	191 ± 134	74.0 ± 12.2	0.629 ± 0.481
SAMHD1				
1/1	27	141 ± 77.5	81.2 ± 12.2	0.459 ± 0.318
1/2	102	145 ± 75.7	79.2 ± 15.3	0.477 ± 0.321
2/2	91	153 ± 110	78.2 ± 12.9	0.525 ± 0.459
STAT3				
1/1	12	172 ± 86.8	87.9 ± 13.7	0.489 ± 0.377
1/2	48	135 ± 79.2	79.3 ± 14.2	0.467 ± 0.345
1/3	43	152 ± 107	79.2 ± 11.4	0.486 ± 0.358
2/2	41	139 ± 81.3	74.4 ± 15.0	0.520 ± 0.389
2/3	60	149 ± 81.2	78.2 ± 14.6	0.497 ± 0.447
3/3	16	172 ± 137	85.8 ± 10.3	0.531 ± 0.331
TRIM21				
1/1	36	141 ± 78.4	80.0 ± 11.8	0.485 ± 0.444
1/2	73	144 ± 87.7	76.3 ± 14.6	0.487 ± 0.340
1/3	16	160 ± 101	83.7 ± 14.5	0.519 ± 0.280
2/2	72	159 ± 103	81.1 ± 13.2	0.483 ± 0.416
2/3	19	124 ± 81.2	72.9 ± 13.8	0.584 ± 0.412
3/3	4	130 ± 46.5	93.8 ± 15.0	0.401 ± 0.218

RNASEL, ribonuclease L; *SAMHD1*, sterile α motif and histidine-aspartate domain containing deoxynucleoside triphosphate triphosphohydrolase 1; *STAT3*, signal transducer and activator of transcription 3; *TRIM21*, tripartite motif containing 21; PA, phagocytic activity; CACP, capacity of alternative complement pathway; AR, antibody response after vaccination against swine erysipelas.

exhibit a dramatic increase in mortality after infection with *Bacillus anthracis* and *Escherichia coli* [21]. Furthermore, the induction of inflammatory cytokines, such as interleukin-1β, tumor necrosis factor-α, and interferon-β, and the expression of the mRNA encoding the endolysosomal protease, cathepsin-E, were impaired in the macrophages of *RNASEL*$^{-/-}$ mice [21]. These observations in *RNASEL*$^{-/-}$ mice may account for the increased frequency of haplotype 2 of the *RNASEL* gene promoter in response to immune selection in pigs. Although the expression of *RNASEL* in non-selected pigs was almost unchanged before and after lipid A stimulation, that in immune-selected pigs exhibited a greater than 3-fold increase. A predictive analysis of binding sites for transcription factors in the promoter region of *RNASEL* suggested that forkhead box A1 (FOXA1) and Thanatos-associated protein domain containing 1 (THAP1) could bind to a site containing position 4844 upstream from the TSS in *RNASEL* (Supplementary Table 4). The SNP at position 4844 may alter the binding ability of these transcription factors between haplotypes 1 and 2 of the *RNASEL* gene promoter, resulting in the difference of the expression of *RNASEL* after lipid A stimulation. FOXA1 can unravel condensed chromatin by binding to histones H3 and H4 [8] and is involved in postnatal development and cancerization of the mammary and prostate glands [3]. THAP1 is a physiological regulator of endothelial cell proliferation, cell cycle progression, and angiogenesis [6].

Haplotype 2 of the *RNASEL* gene promoter was found to be associated with increased PA and a simultaneous decrease in CACP. Although, the role of CACP in disease resistance of pigs is unknown, Lee *et al*. have reported that Kaposi's sarcoma-associated herpesvirus (KSHV) activates the complement system through the STAT3 pathway during the latent period and that cells infected with latent KSHV are resistant to cell death [19], suggesting that increased CACP may adversely affect the host resistance to infections. We limited our analysis to genes involved in immune function and transcription factor activity and found that haplotype 2 of the *STAT3* gene promoter was associated with decrease in CACP, unlike haplotypes 1 and 3, and likely exerted this effect by suppressing the expression of the *STAT3* gene, although this finding is inconsistent with the direction of immune selection to increase CACP. We hypothesize that other genes not involved in "immune system process" and "nucleic acid binding transcription factor activity", based on the GO analysis, have a large effect on CACP increase.

Although the pigs with genotype *TRIM21*$^{2/3}$ had significantly decreased CACP compared with those with genotype *TRIM21*$^{1/1}$, the direction of the effects of haplotypes 2 and 3 of *TRIM21* was not clear. *TRIM21* is located on chromosome 9, like *RNASEL*,

Table 3. Estimates of the effect of promoter genotypes on immune competence

Genotype	PA						CAPA						AR					
	Estimate	S.E.	95% Wald CI		Hypothesis test		Estimate	S.E.	95% Wald CI		Hypothesis test		Estimate	S.E.	95% Wald CI		Hypothesis test	
			Lower	Upper	Wald	*P*			Lower	Upper	Wald	*P*			Lower	Upper	Wald	*P*
Sex																		
Female	Reference						Reference						Reference					
Male	24.53	12.13	0.768	48.30	4.094	0.043*	0.106	1.794	−3.410	3.622	0.003	0.953	−0.008	0.0521	−0.110	0.094	0.022	0.881
RNASEL																		
1/1	Reference						Reference						Reference					
1/2	3.151	20.35	−36.74	43.04	0.024	0.877	3.121	3.011	−2.780	9.022	1.075	0.300	−0.126	0.0875	−0.297	0.045	2.073	0.150
2/2	52.62	15.64	21.95	83.28	11.31	0.001**	−6.009	2.315	−10.55	−1.473	6.741	0.009**	0.150	0.0672	0.019	0.282	5.002	0.025*
SAMHD1																		
1/1	Reference						Reference						Reference					
1/2	8.102	19.12	−29.37	45.58	0.180	0.672	−1.463	2.829	−7.007	4.081	0.267	0.605	0.021	0.0822	−0.140	0.182	0.067	0.796
2/2	21.18	19.86	−17.76	60.11	1.136	0.286	−1.826	2.939	−7.586	3.933	0.386	0.534	0.070	0.0854	−0.098	0.237	0.666	0.414
STAT3																		
1/1	Reference						Reference						Reference					
1/2	−40.57	28.25	−95.93	14.80	2.062	0.151	−9.243	4.179	−17.43	−1.053	4.893	0.027*	−0.020	0.1214	−0.258	0.218	0.026	0.872
1/3	−19.37	28.84	−75.90	37.16	0.451	0.502	−9.117	4.267	−17.48	−0.753	4.565	0.033*	−0.006	0.1240	−0.249	0.237	0.003	0.959
2/2	−26.04	29.11	−83.09	31.01	0.800	0.371	−14.27	4.306	−22.71	−5.831	10.98	0.001**	0.032	0.1251	−0.213	0.277	0.065	0.798
2/3	−18.79	28.10	−73.86	36.28	0.447	0.504	−10.44	4.157	−18.59	−2.294	6.309	0.012*	0.015	0.1208	−0.222	0.252	0.016	0.901
3/3	−6.058	34.31	−73.30	61.19	0.031	0.860	−4.942	5.076	−14.89	5.006	0.948	0.330	0.037	0.1475	−0.252	0.326	0.063	0.802
TRIM21																		
1/1	Reference						Reference						Reference					
1/2	1.486	18.01	−33.81	36.78	0.007	0.934	−4.940	2.664	−10.16	0.282	3.438	0.064	0.029	0.0774	−0.122	0.181	0.145	0.703
1/3	21.09	27.14	−32.11	74.29	0.604	0.437	0.914	4.016	−6.956	8.785	0.052	0.820	0.076	0.1167	−0.153	0.305	0.423	0.515
2/2	18.38	18.69	−18.26	55.02	0.966	0.326	−1.472	2.766	−6.892	3.949	0.283	0.595	0.045	0.0804	−0.113	0.202	0.307	0.580
2/3	−16.47	25.00	−65.48	32.53	0.434	0.510	−7.780	3.699	−15.03	−0.531	4.425	0.035*	0.119	0.1075	−0.092	0.330	1.227	0.268
3/3	−6.499	46.24	−97.13	84.13	0.020	0.888	11.95	6.841	−1.453	25.36	3.054	0.081	−0.053	0.1988	−0.442	0.337	0.070	0.791
(Intercept)	129.8	33.96	63.29	196.4	14.62	<0.001***	93.36	5.023	83.51	103.2	345.4	<0.001***	0.399	0.1460	0.113	0.685	7.487	0.006**

RNASEL, ribonuclease L; *SAMHD1*, sterile α motif and histidine-aspartate domain containing deoxynucleoside triphosphate triphosphohydrolase 1; *STAT3*, signal transducer and activator of transcription 3; *TRIM21*, tripartite motif containing 21; PA, phagocytic activity; CACP, capacity of alternative complement pathway; AR, antibody response after vaccination against swine erysipelas; CI, confidence interval; S.E., standard error. Significant difference compared to reference are indicated (**P*<0.05; ***P*<0.01; ****P*<0.001).

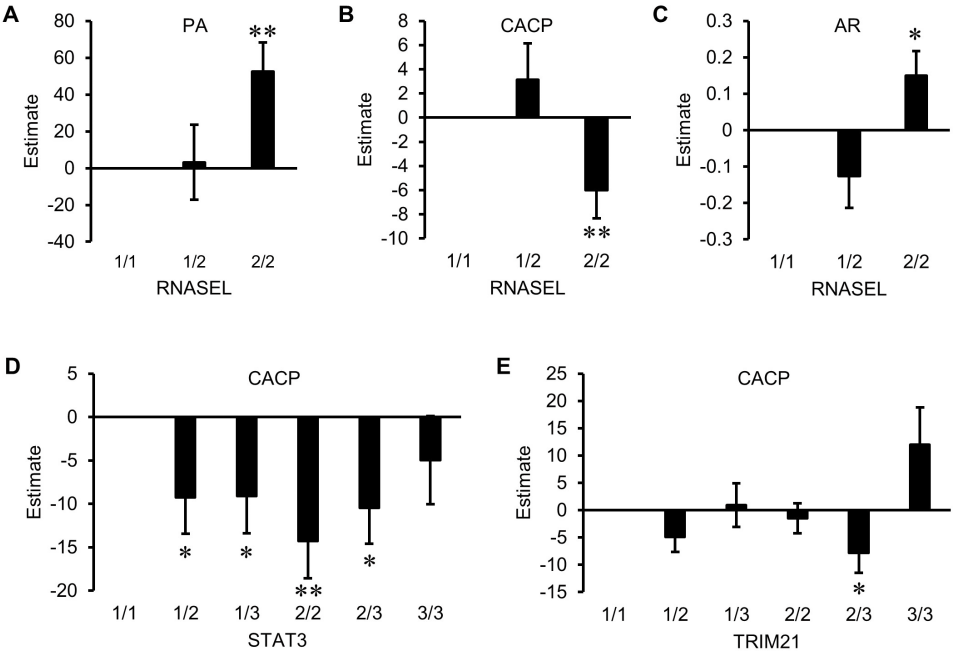

Fig. 2. Effect of promoter genotypes on immune phenotypes. Two hundred and twenty Large White pigs were used to investigate the association between promoter genotypes and immune phenotypes. Association analysis was performed by a generalized liner model. Estimates of the effects are indicated by mean ± standard error for *RNASEL* genotypes on phagocytic activity (PA) **(A)**, capacity of alternative complement pathway (CACP) **(B)**, antibody response after vaccination against swine erysipelas (AR) **(C)**, *STAT3* genotypes on CACP **(D)**, and *TRIM21* genotypes on CACP **(E)**. Significant difference compared to the genotype 1/1 are indicated (**P*<0.05; ***P*<0.01).

in pigs and may be genetically linked with *RNASEL*, which was strongly associated with CACP decrease. However because the tripartite motif (TRIM) family is known to positively and negatively regulate the Toll-like receptor signaling pathway [17], it is possible that the association of *TRIM21* and CACP was detected due to its functional role in the regulation of innate immune responses.

In conclusion, the selection of Large White pigs based on immune competence significantly increased the frequency of haplotype 2 of the *RNASEL* gene promoter. This haplotype greatly increased the expression of *RNASEL* after lipid A stimulation. Pigs homozygous for haplotype 2 of the *RNASEL* gene promoter showed significantly high PA, likely due to the activation of the innate immune response. It is possible that the resistance of the Large White pig population to infections can be increased using this haplotype as a DNA marker. We confirmed that the nucleotide sequences of haplotype 1 in *RNASEL*, *SAMHD1*, and *TRIM21* in Large White pigs are exactly the same as those in the Duroc pig, which was used for decoding the whole genome sequence of the pig (data not shown) [16]. If the promoter types corresponding to haplotype 2 in these genes are present in pig breeds other than Large White pigs, the findings of this study can be applied to several breeds.

CONFLICT OF INTEREST. YT and SS are employees of NH Foods Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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