

Pig lacks functional NLRC4 and NAIP genes

メタデータ	言語: English				
	出版者:				
	公開日: 2020-01-06				
	キーワード (Ja):				
キーワード (En): Genome sequencing, Inflammaso					
Pattern recognition receptors, Swine					
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1	Manuscript for Immunogenetics (Brief communication)
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1 Abstract: The NLRC4 inflammasome, which recognizes flagellin and components of the type III 2 secretion system, plays an important role in the clearance of intracellular bacteria. Here, we examined the 3 genomic sequences carrying two genes encoding key components of the NLRC4 inflammasome—NLR 4 family, CARD-containing 4 (NLRC4), and NLR apoptosis inhibitory protein (NAIP)—in pigs. Pigs have 5 single loci encoding NLRC4 and NAIP. Comparison of the sequences thus obtained with the 6 corresponding regions in humans revealed the deletion of intermediate exons in both pig genes. In 7 addition, the genomic sequences of both pig genes lacked valid open reading frames encoding functional 8 NLRC4 or NAIP protein. Additional pigs representing multiple breeds and wild boars also lacked the 9 exons that we failed to find through genome sequencing. Furthermore, neither the NLRC4 nor the NAIP 10 gene was expressed in pigs. These findings indicate that pigs lack the NLRC4 inflammasome, an 11 important factor involved in monitoring bacterial proteins and contributing to the clearance of 12 intracellular pathogens. These results also suggest that genetic polymorphisms affecting the molecular 13 functions of TLR2, TLR4, TLR5, and other pattern recognition receptors associated with the recognition 14 of bacteria have a more profound influence on disease resistance in pigs than in other species. 15 Keywords: Genome sequencing; Inflammasome; Pattern recognition receptors; Swine

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17 Opportunistic infections, which can result in great economic loss, are serious problems for modern 18 pork production. The defense response of hosts against pathogens is accomplished by a variety of 19 immune factors, including pattern recognition receptors (PRRs). PRRs recognize pathogen-associated 20 molecular patterns derived from infectious agents and induce diverse immune responses, including the 21 production of inflammatory cytokines to eradicate the agents. Mutations in PRR genes may hamper 22 ligand recognition, thus increasing susceptibility to infection. Studies in humans and mice have revealed 23 that single-nucleotide polymorphisms (SNPs) that occur in PRRs and impair the molecular functions of 24 these receptors also lead to increases in host susceptibility to disease (Schröder and Schumann 2005). We 25 have characterized many polymorphisms in porcine PRR genes, some of which influence ligand 26 recognition (Uenishi and Shinkai 2009; Uenishi et al. 2012). For instance, in NOD-like receptors (NLRs; 27 one subgroup of PRRs), nucleotide-binding oligomerization domain 1 (NOD1), which recognizes γ -d-28 glutamyl-meso-diaminopimelic acid (iE-DAP), has two nonsynonymous SNPs in the leucine-rich repeat 29 region; these SNPs impair iE-DAP-induced activation of NF-KB in pigs (Shinkai et al. 2015). Such 30 results suggest that studying genetic polymorphism in genes associated with immune responses, such as 31 those for PRRs, will provide useful information for breeding in regard to disease resistance. 32 A large number of NLRs, such as the NLRC and NLRP families, are components of 33 inflammasomes, which are cytoplasmic protein complexes that recognize microbial components and 34 endogenous danger signals. The NLRC4 inflammasome contains the NLR family, CARD-containing 4

35 (NLRC4), and NLR apoptosis inhibitory protein (NAIP) as critical components. The NLRC4

1 inflammasome responds to bacterial flagellin and proteins in the type III secretion system (T3SS) 2 (Franchi et al. 2006; Miao et al. 2010). Mice express multiple NAIP paralogs; for example, murine 3 NAIP1 and NAIP2 respond to the needle proteins and inner rod proteins, respectively, of T3SS, and 4 murine NAIP5 and NAIP6 recognize flagellin (Kofoed and Vance 2011; Zhao et al. 2011). Humans 5 express a single NAIP homolog, which binds to the CprI needle proteins of T3SS (Zhao et al. 2011). 6 NAIP interacts with flagellin or components of T3SS and then activates NLRC4 to form an 7 inflammasome complex. Caspase-1 is activated by the NLRC4 inflammasome and cleaves the 8 proinflammatory form of cytokine IL-1 β into its active form (Poyet et al. 2001). 9 In many mammalian species, the NLRC4 inflammasome has a pivotal role in innate immune 10 responses to microorganisms and contributes to resistance to infectious diseases. To investigate the 11 function of the NLRC4 inflammasome and its component molecules in pigs, we examined the structures 12 of the NLRC4 and NAIP genes in the pig genome. 13 Because sequencing of the pig genome had not completely clarified the regions encompassing 14 NLRC4 and NAIP, we screened bacterial artificial chromosome (BAC) clones carrying porcine NLRC4

15 and *NAIP* genes. We used a polymerase chain reaction (PCR)-based system (Suzuki et al. 2000) and a 16 library constructed by using the pCC1BAC vector (Epicentre, Madison, WI, USA) and DNA obtained 17 from the kidney of a Landrace boar (Tanaka et al. 2006). Two clones were isolated from the library by 18 using specific primers designed within the estimated porcine *NLRC4* and *NAIP* exonic sequences (Table

19

1).

20 DNAs of the BAC clones thus isolated were prepared by using the QIAGEN Large-Construct Kit 21 (Qiagen, Hilden, Germany) and subjected to shotgun sequencing as described previously (Eguchi-Ogawa 22 et al. 2007) by using an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA) and Big Dye 23 Terminator version 3.1 cycle sequencing kit (Applied Biosystems). Sequence data were processed by 24 using the Phred base-calling program and assembled into contiguous sequences by using the Phrap 25 assembler (Ewing and Green 1998; Ewing et al. 1998). Bases with low-quality values determined by 26 Phred and those considered to be errors or misassembled were verified by using the Consed program 27 (Gordon et al. 1998). The integrity of the assembly of the sequences of the BAC clone was confirmed by 28 digestion of the BAC DNA with the restriction enzymes BssHII, PvuI, and NotI and by pulsed-field gel 29 electrophoresis. Sequencing of BAC clone L442I04 yielded 193,453 bp of porcine genomic sequence 30 (GenBank: LC144946), which contained a putative NLRC4 gene located between the SLC30A6 and 31 YIPF4 genes (Fig. 1a). BAC clone L147A24 contained 171,988 bp of porcine genomic sequence 32 (GenBank: LC144947), which possessed a putative NAIP gene between the GTF2H2 and SMN1 genes 33 (Fig. 1b). 34 Exons of porcine NLRC4 and NAIP were estimated through comparison of the porcine genomic

35 sequences with human cDNA sequences coding NLRC4 (NM 021209.4) and NAIP (NM 004536.2),

3

1 respectively. In addition, nucleotide sequences were compared between pigs and humans by dot-plot

2 analysis using PipMaker (Schwartz et al. 2000), with default parameters. Whereas the coding sequence

3 (CDS) for the human NLRC4 transcript has 8 exons (exons 2 through 9), porcine NLRC4 lacked exons

4 corresponding to exons 5, 6, and 7 of human *NLRC4*. In addition, an inversion was detected

5 approximately 5 kb upstream of porcine *NLRC4* (Fig. 1a).

6 The number of loci of NAIP genes varies among species: for example, mice have four functional 7 Naip genes (Naip1, Naip2, Naip5, and Naip6) in their genome, whereas humans have only one functional 8 locus for NAIP. According to the synteny between the genomic region carrying NAIP in pigs and those of 9 other species, including humans, mice, and cattle, pigs have a single NAIP locus in the genome, as do 10 humans and cattle. The human NAIP transcript has 17 exons, and its CDS is coded by exons 4 through 17. 11 However, porcine NAIP lacked exons corresponding to human exons 5 through 8, 10, 11, and 15 (Fig. 1b, 12 Table 2). In addition, the porcine exons corresponding to exons 12 and 17 of human NAIP were collapsed, 13 and clear correspondence between the human and pig sequences was not observed (Supplementary Fig. 14 1). Although most putative exons of porcine NLRC4 gene were flanked by introns with canonical GT/AG 15 splicing sites, we did not detect canonical GT/AG splicing sites at the end of introns upstream of the 16 porcine NAIP exon corresponding to human NAIP exon 14 (Table 2). For both NLRC4 and NAIP exons,

because of the presence of in-frame stop codons we were unable to reconstruct putative mRNAs (data notshown).

19 Although public nucleotide databases (DDBJ/EMBL/GenBank) contain more than 1,680,000 20 expressed sequence tags (ESTs) of pigs, none of these ESTs showed clear similarity to any putative exon 21 of NLRC4 and NAIP in the pig genome (data not shown). To obtain additional evidence, we prepared 22 porcine macrophages isolated from the kidney; these cells showed classic characteristics of macrophages, 23 such as cell-surface antigens and phagocytosis (Takenouchi et al. 2014). By using RT-PCR analysis and 24 primers designed to bind within putative exons of porcine NLRC4 and NAIP (Supplementary Fig. 2), we 25 failed to obtain any NLRC4 or NAIP amplicons from either unstimulated porcine macrophages or those 26 stimulated with lipopolysaccharide or flagellin. Taken together, our findings imply that porcine NLRC4 27 and NAIP are not produced, and we conclude that NLRC4 and NAIP are pseudogenes in the pig genome. 28 Because the deletion of the exons in genes associated with the NLRC4 inflammasome might have 29 been restricted to particular individuals or populations of pigs, we conducted PCR amplification around 30 the porcine genomic regions corresponding to the missing exons of NLRC4 and NAIP. We designed PCR 31 primers within flanking regions adjacent to the missing porcine exons corresponding to exons 5 through 7 32 of human NLRC4 and exons 5 through 8 of human NAIP. The PCR primers for NLRC4 were designed to 33 amplify five short, overlapping fragments (less than 700 bp each) because the intervening sequence 34 between the porcine NLRC4 exons corresponding to human NLRC4 exons 4 and 8 was longer than 2 kb 35 (Table 1; Supplementary Fig. 3). As templates for the PCR amplifications, genomic DNAs were prepared

1 from pigs representing major commercial breeds of Japanese livestock (three Duroc, four Landrace, four 2 Large White, and three Berkshire breed pigs), two Chinese breed pigs (Jinhua), and two Japanese wild 3 boars and were used with the primers and AmpliTaq Gold (Applied Biosystems). Genomic DNA of all of 4 the individuals thus examined, which generated a correct size of the PCR fragment for TLR2 as a positive 5 control (Table 1), generated fragments with sizes corresponding to those of the sequences of the BAC 6 clones. Sequencing of the ends of the PCR fragments showed that the PCR correctly amplified the 7 genomic regions carrying the missing exons of NLRC4 and NAIP on the pig genome, demonstrating that 8 the loss of function in the NLRC4 inflammasome occurs commonly in the species Sus scrofa (data not 9 shown).

10 In mice, NAIP2 activates NLRC4 in response to conserved components of bacterial T3SS, 11 whereas NAIP5 interacts directly with flagellin (Miao et al. 2010; Zhao et al. 2011). Such stimulatory 12 signals activate caspase 1 through the NLRC4 inflammasome, resulting in maturation of IL-1 β /IL-18. 13 These findings indicate that NLRC4–NAIP works as an intracellular sensing system for bacterial proteins. 14 In comparison, TLR5 detects extracellular flagellin and induces the expression of inflammatory cytokines 15 (Hayashi et al. 2001). Furthermore, T3SS induces inflammatory responses through TLR2 and TLR4 16 (Jessen et al. 2014). These redundant recognition systems for bacterial proteins may help to prevent the 17 otherwise critical influence of mutated forms of receptors involved in bacterial recognition. Here, we 18 showed that the NLRC4 inflammasome is absent in pigs, suggesting their potentially increased 19 susceptibility to genetic polymorphisms that affect the functions of TLR2, TLR4, or TLR5. 20 Both porcine NLRC4 and NAIP lacked several exons corresponding to those in the human CDSs. 21 However, porcine NLRC4 had canonical exon-intron boundaries, whereas precise exon-intron boundaries 22 in porcine NAIP could hardly be determined (Table 2). This result suggests that pseudogenization 23 occurred earlier in NAIP than in NLRC4. Jabir et al. showed that intracellular mitochondrial DNA 24 directly or indirectly bound to NLRC4 and activated the NLRC4 inflammasome after infection with 25 Pseudomonas aeruginosa; recognition of mitochondrial DNA by NLRC4 is not dependent on NAIP (Jabir 26 et al. 2015). These findings suggest that components different from NAIP contribute to activation of the 27 NLRC4 inflammasome. The possible time lag in pseudogenization between NLRC4 and NAIP in pigs 28 suggests that NLRC4 retained various functions after the pseudogenization of NAIP. Compared with 29 those of humans and other species examined, the porcine genomic sequence upstream of NLRC4 30 contained an inversion (Fig. 1a). This inversion might prevent the expression of porcine NLRC4, leading 31 to collapse of the genomic structure of NLRC4. 32 Several differences in functional PRRs have been reported among vertebrate species. For example, 33

34 (Barber et al. 2010). Although they have functional MDA5 (IFIH1)-another PRR for RNAs-chickens

35 are highly susceptible to RNA viruses, including highly pathogenic influenza (Karpala et al. 2011).

5

chickens lack the intracellular nucleotide receptor RIG-I (DDX58), which is involved in RNA recognition

1 Whereas NLRC4 and NAIP of pigs are nonfunctional, as shown here, the genome of cattle carries NLRC4

2 and NAIP, the CDSs of which are estimated to encode functional molecules. Therefore, our accumulated

- 3 immunological knowledge of animals such as humans and mice cannot necessarily be applied to
- 4 livestock, as shown by our results and those of these other studies.

5 In conclusion, here we demonstrated that *NLRC4* and *NAIP* in the pig genome are pseudogenes, 6 resulting in a nonfunctional NLRC4 inflammasome in this species. This finding suggests that pigs lack a 7 key means of monitoring bacterial proteins and clearing intracellular pathogens. This result also suggests 8 that genetic polymorphisms in TLR2, TLR4, TLR5, or other PRRs involved in the recognition of bacteria 9 have a more profound influence on disease resistance in pigs than in other species.

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10	
11	Fig. 1 Comparison of the genomic structures of porcine NLRC4 and NAIP with their human counterparts
12	by dot-plot analysis. Horizontal lines indicate the genomic structures of the genes in the BAC clones
13	isolated (a, L442I04; b, L147A24), which contain the NLRC4 and NAIP genes, respectively. Putative
14	exons corresponding to CDSs of porcine NLRC4 and NAIP and genes in the flanking regions were
15	presumed owing to their similarity to their human counterparts and are indicated by black rectangles.
16	Arrows indicate the transcriptional directions of the genes. Vertical lines indicate the human genomic
17	sequences corresponding to those of the BAC clones. The porcine and human genome sequences were
18	aligned with each other by using the PipMaker program (Schwartz et al. 2000). Gray boxes indicate
19	possible deletions in the porcine genomic sequences in comparison with their human counterparts.
20	
21	Acknowledgments
22	This study was supported in part by grants from the Project of Animal Genomics for Innovative Breeding
23	Technology (AGB1002) of the Ministry of Agriculture, Forestry and Fisheries of Japan.
24	
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 Table 1. Primers used in this study.

	Gene	D	Location ^a		P. (51 - 20)		
Objective		Primer name	Sscrofa10.2	BAC clone	- Primer sequence $(5' \rightarrow 5')$		
BAC clone	NLRC4	NLRC4-2F	3:114,575,376-114,575,355	L442I04:125,291-125,312	GCTGCCTGATAATTTCTGGTTC		
screening		NLRC4-2R	3:114,575,224-114,575,248	L442I04:125,443-125,419	TCTGAGTAAAAGCTTTCCAGTTTTC		
		NLRC4-9F	3:114,520,930-114,520,949	L442I04:149,179–149,198	GGATTTGGTGGGAAATTGTG		
		NLRC4-9R	3:114,521,080-114,521,061	L442I04:149,329-149,310	GGATAATGCATGGCCAAGTT		
	NAIP	NAIP-4F	16:51,643,443-51,643,462	L147A24:33,299-33,318	GCGAAGCAAAAAGGTTGAAG		
		NAIP-4R	16:51,643,656-51,643,637	L147A24:33,512-33,493	TTGCCCAAAAGAAACACACA		
		NAIP-17F	16:51,677,503-51,677,522	L147A24:67,295-67,314	CAGGCTTTCAGGCTAACCAC		
		NAIP-17R	16:51,677,732-51,677,713	L147A24:67,524-67,505	TGGCAGTGGATAAAGGGAAG		
RT-PCR	NLRC4	NLRC4-RTF	3:114,571,552-114,571,533	L442I04:129,109-129,128	ATTCTGCGAGAGGGGGTCATT		
analysis		NLRC4-RTR	3:114,569,963-114,569,984	L442I04:130,695-130,674	TGTCAATATCTTCTCCCAGGGG		
•	NAIP	NAIP-RTF	16:51,643,749-51,643,768	L147A24:33,605-33,624	AGGCCAGACTCGAATCCTTC		
		NAIP-RTR	16:51,655,133-51,655,114	L147A24:44,881-44,862	AGGACTTGGGAGATTGAGCA		
	NLRP3	NLRP3-RTF	2:57,856,199-57,856,218	N.A.	CTGGATCTGAGCCACAATGC		
		NLRP3-RTR	2:57,857,904-57,857,885	N.A.	GGGAATGATTGCTGCTCAGG		
	IL1B2	IL1B2-RTF	3:45,182,013-45,182,032	N.A.	CATCCAGCTGCAAATCTCCC		
		IL1B2-RTR	3:45,183,367-45,183,349	N.A.	TGGCATGCTTTTCAAGGACA		
Analysis	NLRC4	NLRC4-G1F	N.D.	L442I04:132,426-132,445	GCCTCAGTTCAGTCCTCAGC		
regarding		NLRC4-G1R	N.D.	L442I04:132,896-132,875	CTTCGCCTACTGTCTACGTGTG		
missing exons		NLRC4-G2F	N.D.	L442I04:132,868-132,890	GTGTCCGCACACGTAGACAGTAG		
		NLRC4-G2R	N.D.	L442I04:133,381-133,357	ATCACTTGATGATGAAAACAAATCC		
		NLRC4-G3F	N.D.	L442I04:133,347-133,371	CTGTAATCTGGGATTTGTTTTCATC		
		NLRC4-G3R	3:114,576,758-114,576,734	L442I04:133,919-133,895	ATGTAGGTCAAATTCAGCACATAGG		
		NLRC4-G4F	3:114,576,732-114,576,756	L442I04:133,893-133,917	CACCTATGTGCTGAATTTGACCTAC		
		NLRC4-G4R	3:114,577,395-114,577,370	L442I04:134,558-134,533	CTCTGCCCTCTCAAGGTACAATAATG		
		NLRC4-G5F	3:114,577,362-114,577,389	L442I04:134,525-134,552	AAGTAGATCATTATTGTACCTTGAGAGG		
		NLRC4-G5R	3:114,577,635-114,577,608	L442I04:134,798-134,771	CTGTATCTGTGAGTCTCCAGTTTTTCAG		
	NAIP	NAIP-GF	16:51,643,715-51,643,734	L147A24:33,571-33,590	AGGGTGGATGACAAAGCAAG		
		NAIP-GR	16:51,644,306-51,644,325	L147A24:34,162-34,181	TTTGGTGTGTTTTTCCAACG		
	TLR2	TLR2-GF	8:79,826,766-79,826,737	N.A.	TCCTATAATCACTTGTCTAACTTATCATCC		
		TLR2-GR	8:79,824,750-79,824,774	N.A.	CTCTCAAATTTAACCAAAACCCTTC		

N.A., not applicable; N.D., not detected on the reference genomic sequence.

^a Locations of the primers on the reference pig genomic sequence (Sscrofa10.2) and sequences of the BAC clones isolated in this study are indicated. Locations on the genomic sequence are shown as chromosome numbers, with base positions relative to the p-ter. Locations on BAC clone sequences are shown with the clone name (L442I04 or L147A24).

	Human exon					Corresponding pig exons			
Gene	Acceptor	Exon number	Location on human			Location on BAC			
			chromosome (Sscrofa10.2)		Donor	Acceptor	clone		Donor
			Start	End	-	-	Start	End	_
			Chromosome	2			Clone L44	2I04	
NLRC4	TTACAG	2	32,256,893	32,256775	GTAAGT	TTACAG	125,330	125,447	GTAAAT
	CCTCAG	3	32,252,679	32,252,419	GTAAGT	CTCCAG	128,969	129,225	GTATTA
	TAATAG	4	32,251,601	32,249,607	GTATTG	TAATAG	130,582	132,575	GTACTG
	TGACAG	5	32,241,125	32,241,033	GTCAGA	N.D.			
	TTTCAG	6	32,238,302	32,238,132	GTAACT	N.D.			
	CCCCAG	7	32,236,339	32,236,248	GTAAGA	N.D.			
	TTCCAG	8	32,235,568	32,235,401	GT AGGT	TTTCAG	134,645	134,813	GT AGGT
	ACCCAG	9	32,224,765	32,224,449		AATCAG	149,136	149,452	
			Chromosome	5			Clone L14		
NAIP	TTTCAG	4	71,012,918	71,012,348	GTACCT	CACCAG	33,094	33,694	GTAAGT
	ATATAG	5	71,011,375	71,011,275	GTAAGT	N.D.			
	TTACAG	6	71,003,857	71,003,776	GTAATG	N.D.			
	CCTCAG	7	71,002,143	71,002,092	GTAAAA	N.D.			
	TTCAAG	8	71,001,817	71,001,698	GTGAGT	N.D.			
	TTTTAG	9	70,998,823	70,998,724	GTGAGT	GTCTAG	34,095	34,192	GT GAGT
	ACTTAG	10	70,987,874	70,987,793	GTAATG	N.D.			
	TCAAAG	11	70,986,802	70,986,745	GTAAGA	N.D.			
	CCACAG	12	70,985,886	70,983,775	GTATAC	ACAAAG	44,832	46,705	GTAAAC
	TTCCAG	13	70,980,036	70,979,869	GTAAGG	CAACAG	62,204	62,374	GTAAGG
	TTACAG	14	70,977,058	70,976,906	GTAAGA	CATCTT	63,492	63,639	GTAAGA
	TCATAG	15	70,976,246	70,976,160	GTATGT	N.D.			
	TGACAG	16	70,974,294	70,974,129	GTGAGC	TGATAG	65,354	65,513	GTGAGC
	TTGTAG	17	70,970,468	70,968,483		TTATAG	66,378	68,495	

 Table 2. Nucleotide sequences of splicing sites of NLRC4 and NAIP

N.D., The corresponding porcine exon could not be determined.

Human exons comprising the CDSs (*NLRC4*: NM_021209.4; *NAIP*: NM_004536.2) and their corresponding exons in pigs are indicated. Six-nucleotide sequences of the introns at the exon–intron junctions of the human *NLRC4* and *NAIP* genes are shown; the corresponding exons and splicing sites are also indicated. The two highly conserved bases (AG/GT) at the splice sites in the introns are shown in bold.



Figure 1

Method for supplementary materials

Expression analysis of porcine NLRC4 and NAIP by using semi-quantitative RT-PCR

Macrophages (5 × 10^5 per well) isolated from a mixed culture of porcine kidney tissue as previously described (Takenouchi et al., 2014) were seeded into 35-mm dishes (Sumitomo Bakelite, Tokyo, Japan) and stimulated with 100 ng/mL flagellin (tlrl-pstfla; InvivoGen, San Diego, CA, USA) or 1 µg/mL lipopolysaccharide (L8274; Sigma-Aldrich, St. Louis, MO, USA) for 8 h. The stimulated macrophages were harvested by treatment with TrypLE Express (Gibco/Thermo Fisher Scientific, Grand Island, NY, USA), and total RNA (1.3–2.1 µg) was extracted from the cells by using RNeasy Mini Kit (Qiagen, Hilden, Germany). In addition, total RNA was prepared from unstimulated macrophages.

The extracted RNA (180 ng per reaction) underwent cDNA synthesis by using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, NY, USA) as previously described (Shinkai et al., 2015). The cDNA transcribed from 3.6 ng total RNA was amplified by using primers designed to amplify boundaries of putative exons of porcine *NLRC4* and *NAIP*; in addition, primers for porcine *NLRP3* and *IL1B2* were designed as positive controls (Table 1). Each amplification reaction contained the designed primers (5 μ M each), dNTPs (0.2 μ M each), AmpliTaq Gold polymerase and the supplied buffer (Life Technologies/Thermo Fisher Scientific, Foster City, CA, USA) according to the manufacturer's instructions. Amplification conditions comprised an initial denaturation at 94 °C for 15 min, followed by various numbers (30 to 50) of cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; the reactions underwent a final elongation step at 72 °C for 7 min.

To confirm PCR amplification from the designed primers, we had synthesized (Integrated DNA Technologies, Coralville, IA, USA) double-stranded DNA equivalent to the putative transcripts that would be generated from the genes if they were expressed (*NLRC4*: positions 125,330–125,447, 128,969–129,225, and 130,582–130,706 on BAC clone L442I04 [GenBank: LC144946]; *NAIP*: positions 33,494–33,694, 34,095–34,192, and 44,832–45,032 on BAC clone L147A24 [GenBank: LC144947]; *NLRP3*: exons corresponding to positions 2163–2327, 2328–2498, and 2499–2662 on the pig mRNA sequence [GenBank: NM_001256770.1]; and *IL1B2*: exons corresponding to positions 278–329, 330–525, 526–690, and 691–777 on the pig mRNA sequence [GenBank: NM_001302388.1]). These synthetic transcripts were inserted individually into plasmids (pUCIDT (Amp), Integrated DNA Technologies) for use as positive controls; each PCR reaction contained 100 copies of the positive-control plasmid.

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Supplementary Fig. 1. Genomic structure of exons corresponding to human *NAIP* exons 12 and 17. Gray rectangles indicate human exonic sequences that lack marked similarity in the pig genomic sequence. Short- and long-interspersed nucleotide elements (SINEs and LINEs) on the pig genome are indicated by black and diagonal-hatched rectangles, respectively. The SINEs and LINEs were detected by using RepeatMasker (http://www.repeatmasker.org) with the RepBase (Jurka et al., 2000) database. Arrows indicate the transcriptional directions of the human transcripts. (a) More than 400 bp of the 5'-end of the human *NAIP* exon 12 did not have a corresponding sequence in the pig exon; the remaining pig exonic sequence was divided by a SINE sequence. (b) The pig exonic sequence corresponding to human *NAIP* exon 17 was divided by a SINE sequence. Most of the latter half of the human *NAIP* exon 17 did not have correspondence in the pig exon.



Supplementary Fig. 2. Semi-quantitative RT-PCR analysis of expression of porcine *NLRC4* and *NAIP*. Gene expression was investigated by using cDNA synthesized from total RNA harvested from stimulated (flagellin and lipopolysaccharide [LPS]) and unstimulated porcine macrophages. Plasmid DNA (100 copies) carrying the expected amplicons underwent PCR amplification in parallel as positive controls. The number of amplification cycles was varied to estimate the approximate amounts of cDNA by comparison with positive control reactions.



Supplementary Fig. 3. PCR primers designed to examine the genomic structure of the region corresponding to the missing exons of porcine (a) *NLRC4* and (b) *NAIP*. Arrows indicate the designed primers (precise locations are presented in Table 1). Estimated PCR amplicons are shown as lines. All of the pigs that we examined generated PCR fragments of the expected estimated size. Both ends of all fragments were sequenced to confirm correct amplification.