Article

Molecular Cloning and Functional Characterization of Porcine MyD88 Essential for TLR Signaling

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We isolated cDNA encoding porcine MyD88 (poMyD88) from Peyer's patches (Pps) of GALT. The complete open reading frame (ORF) of poMyD88 contains 879 bp encoding a deduced 293 aa residues. The amino acid sequence of poMyD88 was characterized by N-terminal death, intermediate and C-terminal Toll/IL-1 receptor (TIR) domains. The putative poMyD88 protein shares a higher level of homology with its human (87.2% amino acid identity) than with its mouse (77.4% amino acid identity) counterpart. Overexpression of poMyD88 participated in the further enhanced activation of NF-κB in human embryonic kidney (HEK) 293 cells expressing porcine TLR2 and porcine TLR4/MD-2, but not porcine RP105/MD-1 after stimulation with the corresponding ligands. The expression levels of MyD88 were highest in the spleen and mesenteric lymph nodes (MLNs), and lower in digestive tissues of newborn swine. In adult swine, the expression levels in the digestive tissues were lower than those in MLNs and the spleen. These results suggest that an MyD88-dependent signaling pathway is present in newborn as well as in adult swine and that it is involved in the innate immune system of these animals. *Cellular & Molecular Immunology*. 2007;4(5):369-376.

Key Words: MyD88, TLR2, TLR4/MD-2, RP105/MD-1, cDNA cloning, swine

Introduction

Vertebrate hosts have evolutionarily developed an innate and adaptive immune system to eliminate invading pathogens. The innate immune system is not only essential as the first line of defense against invasion by pathogens but also provides the crucial signals for activation of the adaptive immune responses, leading to immunological memory (1). Innate immune responses are triggered upon pathogen recognition by a set of pattern recognition receptors that recognize the unique pathogen-associated molecular patterns (PAMPs) of microorganisms, which are essential for their survival (2).

Among the known pattern recognition receptors for microbial substances, TLRs comprise a family of at least 12 membrane proteins that can recognize various kinds of PAMPs, such as peptidoglycan, lipoproteins, double-stranded viral RNA, LPS and unmethylated bacterial CpG DNA (3). When TLRs (except TLR3) recognize PAMPs, MyD88 binds to the Toll/IL-1 receptor (TIR) domain of TLRs, which triggers the intracellular IL-1R family signaling cascade. Activation of NF-κB and MAPK cascades involves a signaling complex that contains MyD88, IL-1R-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (4, 5). Finally, transcription is initiated to express several proinflammatory cytokines and effector cytokines, such as IFN-α, IL-6 and TNF-α (6). MyD88 is also required for the signaling pathway induced by the IL-1 family of cytokines (IL-1/IL-18) (7). MyD88-deficient mice are unresponsive to PAMPs recognized by TLR2, TLR4, TLR5, TLR7 and TLR9 (8), indicating that MyD88 plays a crucial role in activating the immune system, especially *via* the MyD88-dependent TLR/IL-1R signaling pathway.

The swine immune system has recently been focused as a potential model of the human immune system and also because of the economic importance of swine as livestock (9, 10). Furthermore, the structural aspects of the swine gastrointestinal tract are much more similar to those of humans than of rodents. However, the swine immune system and its relationship to the TLR-mediated signaling pathway

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Table 1. Primer sequences used in this study

Primer	Sense primer	Antisense primer
Porcine MyD88 gene cloning primer		
Porcine MyD88 (186-464)	TTTGAGTACTTGGAGATCCG	CAAAATGCTCAGGCATTTGC
Porcine MyD88 (397-729)	AGAAGCCTTTACAGGTGGCC	TTCTGATGGGCACCTGGAGA
Porcine MyD88 (5'RACE, cDNA synthesized)		TTCGGCAGTCCTCTTCAATG
Porcine MyD88 (5'RACE, 1st nested PCR)	5'RACE Abridged Anchor Primer	ACCTGTAAAGGCTTCTCAGC
Porcine MyD88 (5'RACE, 2nd nested PCR)	Abridged Universal Amplification Primer	GGATGCTGCTATCTACAGAG
Porcine MyD88 (3'RACE, cDNA synthesized)		Oligo-d(T)17 Adapter Primer
Porcine MyD88 (3'RACE, 1st nested PCR)	GAAAGAGTTCCCCAGCATCCTGCG	Abridged Universal Amplification Primer
Porcine MyD88 (3'RACE, 2st nested PCR)	TGTGACTACACCAACCCCTGCACCA	Abridged Universal Amplification Primer
Real-time PCR		
Porcine β-actin	CATCACCATCGGCAACGA	GCGTAGAGGTCCTTCCTGATGT
Porcine MyD88	GGCAGCTGGAACAGACCAA	GGCAGGACATCTCGGTCAGA

5'RACE Abridged Anchor Primer, Abridged Universal Amplification Primer and Oligo-d(T)17 Adaptor Primer were purchased from Invitrogen.

are not understood in detail. Although we have cloned several members of the porcine TLR family and analyzed the specificity of their ligands (11-13), the role of MyD88 and the functional involvement of TLRs and MyD88 in swine require precise elucidation.

Here, we cloned and characterized porcine MyD88 (poMyD88) cDNA isolated from ileal Peyer's patches (Pps) of adult swine. We established a cell line overexpressing poMyD88 and investigated its function *via* TLRs. We also examined poMyD88 expression in diverse tissues from adult and newborn swine.

Materials and Methods

Experimental tissues

We isolated tissues (esophagus, duodenum, jejunum, ileum, ileal Pps, colon, spleen, and mesenteric lymph nodes (MLNs)) from newborn LWD swine (genotype 1/2 Duroc, 1/4 Landrace, 1/4 Large White; Hiruzu Co. Ltd., Miyagi, Japan) before obtaining colostrum. Intestinal tissues and GALT from newborn swine were prepared as described (14). Adult tissues were excised from LWD swine (age, 1 year old; Hiruzu Co. Ltd.). All animals were clinically healthy and free of infectious diseases and all manipulations proceeded in accordance with the Guidelines for Animal Experimentation of Tohoku University, Japan.

Cloning of poMyD88

Total RNA was isolated from adult ileal Pps using the RNeasy Protect Starter Kit (Qiagen, Tokyo, Japan) as described (11). Primers were synthesized using the sequences deduced from alignment of conserved sequences of human (accession No. BC013589) and mouse MyD88 (accession No. NM_010851) (Table 1). First-strand cDNA was then synthesized from the total RNA using these primers and THERMOSCRIPT reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR products were subcloned into the vector pGEM-T easy (Promega, Madison, WI, USA). The 5'- and 3'-flanking regions of poMyD88 were determined using the 5' and 3' RACE system for rapid amplification of cDNA ends (Invitrogen). The products were then sequenced using an ABI310 Sequence-Analyzer (Applied Biosystems, Foster City, CA, USA) and then the nucleotide and deduced amino acid sequences were analyzed using a GENETYX-SV/RC Ver.13.0.6 software (GENETYX Co., Tokyo, Japan). Protein domains were identified and annotated using the SMART architecture research program (http://smart.embl-heidelberg.de/) (15).

Phylogenetic analysis

We compared the amino acid sequences of poMyD88 with those of MyD88 from other animals retrieved from the EMBL and GenBank databases. The accession numbers used here are: mouse (NM_010851), human (BC013589), rat (BC097266), chicken (NM_001030962), porcine (AB292176), bovine (BC102851), zebra fish (NM_212814) and Japanese oyster (DQ530619). Multiple sequence alignments were generated using Clustal W (16). A phylogenic tree of MyD88 based on the amino acid sequences was generated using the MEGA 3.0 program (17) and distances were analyzed using the neighbor joining method. Tree reliability was assessed by performing 1,000 bootstraps.

Cell line

The human embryonic kidney cell line, HEK293 was obtained from the TKG cell bank (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) and transfected with a plasmid encoding porcine TLR2 ($HEK293^{poTLR2}$) as described (18). Transfectants expressing porcine TLR4/MD-2 and RP105/MD-1 were established by transfecting HEK293 cells with expression vectors encoding porcine TLR4 and MD-2 ($HEK293^{poTLR4/poMD-2}$), and porcine RP105 and MD-1 ($HEK293^{poRP105/poMD-1}$), respectively (12). All cells were maintained in complete DMEM (Sigma, Tokyo,

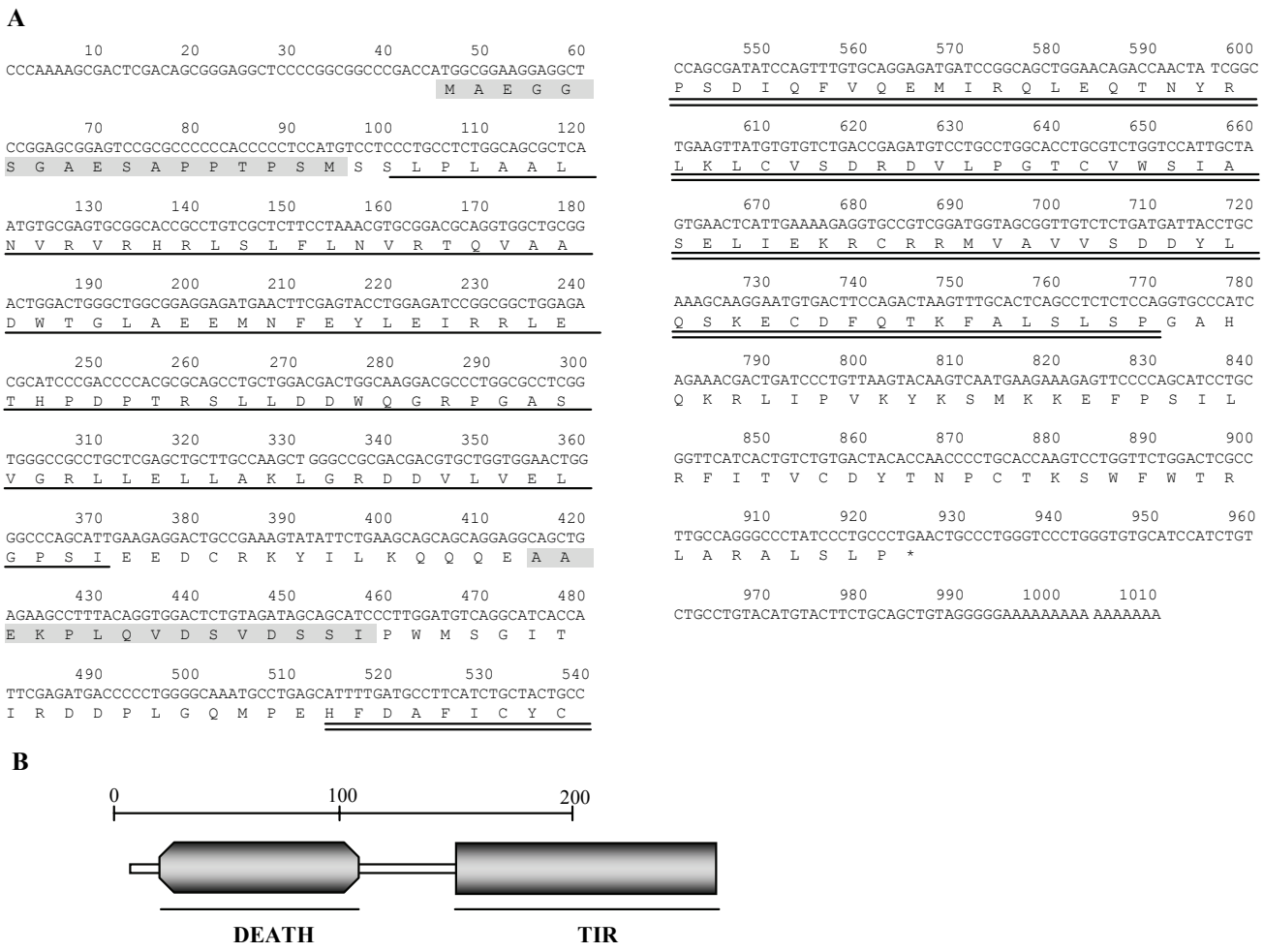

Figure 1. Nucleotide and deduced amino acid sequence, and domain structure of poMyD88. (A) Complementary DNA and deduced amino acid sequence of poMyD88. Translated amino acid sequence is shown under nucleotide sequence. Numbers indicate nucleotide positions. Sequences homologous to the DEATH and Toll/IL-1R (TIR) domains are underlined and double underlined. Predicted regions with intrinsic disorder are shaded in grey. *, Stop codon. Architecture of poMyD88 determined by SMART architecture software. (B) Nucleotide sequence of poMyD88 submitted to DDBJ, EMBL, and GenBank nucleotide databases under accession number AB292176.

Japan) supplemented with 10% FCS (Sigma), 50 μg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM 2-[4-(2-Hydroxyethyl)-1-piperadinyl] ethanesulfonic acid, 0.11 mg/ml sodium pyruvate and 0.5 mM 2-mercaptoethanol (Sigma).

Transfection

HEK293^{poTLR2}, HEK293$^{poTLR4/poMD-2}$ and HEK293$^{poRP105/poMD-1}$ cells were transfected with plasmids as described in our previous reports (13, 19). Briefly, cells (8×10^4/well) were seeded in 24-well plates for 24 h and then transfected with the pcDNA3 vector (Invitrogen) encoding C-terminally c-myc tagged poMyD88 (0.1 μg/well) and the pGLM-ENH-luci vector (20-23) using Lipofectamine™ (Invitrogen). The medium was changed on the following day and then the cells were cultured for 1 to 2 days before further analysis.

Expression analysis of poMyD88

Flow cytometry proceeded as described (13). Briefly, cells were harvested using a nonenzymatic dissociation buffer (Invitrogen), and then fixed, washed and stained for C-terminal c-myc-poMyD88 using anti-c-myc mAb (Sigma). The cells were washed with PBS containing 2% FCS and then incubated with PE-conjugated anti-mouse IgG (Becton Dickinson, Tokyo, Japan). The PE signal was determined by flow cytometry using a FACSCalibur™ (Becton Dickinson). Permeabilized cells were stained in a buffer containing 0.1% saponin (Sigma) (14). Cells transfected with empty pcDNA3 vector comprised the negative control.

NF-κB reporter luciferase assay

Cells (8×10^4/well per 24-well plate) were transfected for 4 h using Lipofectamine™ as described above, stimulated for 24 h with various concentrations of Pam3Cys (EMC Microcollection, Tuebingen, Germany) or LPS from *Escherichia coli* O55:B5 prepared by gel-filtration chromatography (Sigma) and then luciferases were measured according to the

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porcine             1 --MAEGGSGAESAPPTPSMSSLPLAALNVRVRHRLSLFLNVRTQVAADWTGLAEEMNFEY     58
human               1 --MAAGGPGAGSAAPVSSTSSLPLAALNMRVRRRLSLFLNVRTQVAADWTALAEEMDFEY     58
mouse               1 --MSAGDPRVGSGSLDSFMFSIPLVALNVGVRRRLSLFLNPRTPVAADWTLLAEEMGFEY     58
rat                 1 --MSAGGPRVGSVSVDSYLFSLPLVALNVGVRRRLSLFLNPRTTAAADWTSLAEEMGFEY     58
bovine              1 --MAEGVPRAGSALPAASLSSLPLAALNVRVRRRLSLFLNVRAPVAADWTVLAEAMDFEY     58
chicken             1 MATVPVGAGSAPGPEPADLHSVPMVALNYGVRRRLGLYLNPRAATAADWTALAEKLGHDY     60
zebrafish           1 ----------MASKLSIDHEAIPVTALNCSFRKKLGLFLNPTNTVAADWRTVAELMDFTY     50
pacific oyster      1 ------------------------------------------------------------      1
                        .  .     .        . .. ...  .......... .  ...... ... . ...

porcine            58 LEIRRLETHPDPTRSLLDDWQGR--PGASVGRLLELLAKLGRDDVLVELGPSIEEDCRKY    116
human              58 LEIRQLETQADPTGRLLDAWQGR--PGASVGRLLELLTKLGRDDVLLELGPSIEEDCQKY    116
mouse              58 LEIRELETRPDPTRSLLDAWQGR--SGASVGRLLELLALLDREDILKELKSRIEEDCQKY    116
rat                58 LEIREFETRPDPTRSLLDAWQGR--SGSSVGRLLELLALLDREDILYELKDRIEEDCQKY    116
bovine             58 LEIQQLEKYADPTSRLLDDWQRR--PGASVGRLLELLAKLGRDDVLMELGPSIEEDCQKY    116
chicken            60 LEIRRLEALPDPTAALLEEWQSRCPGGATVGQLLELLRQLGRHDVLLELGGSVEEDCKKY    120
zebrafish          50 LEIKNFEKRDCPFEKVLTDWETR--PDATVANLLSILEKAERKDVISELKEILDDDCRKY    108
pacific oyster      1 ------------------------------------------------------------      1
                        .... ..   ...  ... .. .   ...........  . . ... ..   ..... ..

porcine           116 ILKQQQEAAEKPLQVDSVDSSIPW---MSGITIRDDPLGQMPEHFDAFICYCPS--DIQF    171
human             116 ILKQQQEEAEKPLQVAAVDSSVPRTAELAGITTLDDPLGHMPERFDAFICYCPS--DIQF    174
mouse             116 LGKQQNQESEKPLQVARVESSVPQTKELGGITTLDDPLGQTPELFDAFICYCPN--DIEF    174
rat               116 IRNQQKQESEKPLQVARVESSVPQTKELGGITTLDDPLGQTPELFDAFICYCPS--DIEF    174
bovine            116 ILKQQQEASEKPLQVDSIDSSITRINDMAGITIRDDPLGQKPECFDAFICYCPS--DIEF    174
chicken           120 LRRKQQE-AEQPLQVPAVDSSVPKTSELMGITTRDDPYGHGTEMFDAFICYCQK--DLQF    177
zebrafish         108 MERQQR----KPLQVPVVDSCGPRTQEREGVTLYDDPQGLTPETFDAFICYCQS--DIQF    162
pacific oyster      1 ----------------------------------MGAYDYDAFVIYNPHGQDQEF     21
                          ..    ......  .... . . .  ...  .....  .. .***..*...  *. *

porcine           171 VQEMIRQLEQTNYRLKLCVSDRDVLPGTCVWSIASELIEKRCRRMVAVVSDDYLQSKECD    231
human             174 VQEMIRQLEQTNYRLKLCVSDRDVLPGTCVWSIASELIEKRCRRMVVVVSDDYLQSKECD    234
mouse             174 VQEMIRQLEQTDYRLKLCVSDRDVLPGTCVWSIASELIEKRCRRMVVVVSDDYLQSKECD    234
rat               174 VQEMIRQLEQTDYRLKLCVSDRDVLPGTCVWSIASELIEKRCRRMVVVVSDDYLQSKECD    234
bovine            174 VHEMIRQLEQTNYRLKLCVSDRDVLPGTCVWSIASELIEKRCRRMVVVVSDEYLQSKECD    234
chicken           177 VQEMIRELEQTEFKLKLCVFDRDVLPGTCVWSISGELIERRCRRMVVVISDDYLESDECD    237
zebrafish         162 VHEMIKQLEHTEYNLKLCVFDRDVLPGTCVWTIASELIEKRCKRMVVVISDDYLDSDACD    222
pacific oyster     21 VSLMTQVLTSPPYNLRLYVP-WTDNNEPFEAVATAENIEKRCKKVLVVISAAFLESDLFH     80
                      *..*...*... ..*.*.*.................*.**.**.....*.*...*.*....

porcine           231 FQTKFALSLSPGAHQKRLIPVKYKSMKKEFPSILRFITVCDYTNPCTKSWFWTRLARALS    291
human             234 FQTKFALSLSPGAHQKRLIPIKYKAMKKEFPSILRFITVCDYTNPCTKSWFWTRLAKALS    294
mouse             234 FQTKFALSLSPGVQQKRLIPIKYKAMKKDFPSILRFITICDYTNPCTKSWFWTRLAKALS    294
rat               234 FQTKFALSLSPGVQQKRLIPIKYKAMKKDFPSILRFITICDYTNPCTKSWFWTRLAKALS    294
bovine            234 FQTKFALSLSPGAHQKRLIPIKYKPMKKEFPSILRFITVCDYTNPCTQNWFWTRLAKALS    294
chicken           237 FQTKFALSLSPGARLKRLIPVKCKTMKNEFPSILRFITICDYTNPCTKKMVLDKTGKISL    297
zebrafish         222 FQTKFALSLCPGARTKRLIPVVYKSMKRPFPSILRFLTICDYSKPCTQVWFWTRLAKALS    282
pacific oyster     80 FQLKVAHSMSPGARSRKIIPIRLDS--TEVPAVIRFTTSCDYYKKELRVFVWDRLNSAFC    138
                      **.*.*.*..**. ....**.... .....*...**.* ***..... ...........

porcine           291 LP------------------------------------------------------------    293
human             294 LP------------------------------------------------------------    296
mouse             294 LP------------------------------------------------------------    296
rat               294 LP------------------------------------------------------------    296
bovine            294 MP------------------------------------------------------------    296
chicken           297 AAVMQSFESFFSICPGCAFGPRCSYSISLHNLDPRSQHLEPAARSKEFSVALFHNLEGEI    357
zebrafish         282 LP------------------------------------------------------------    284
pacific oyster    138 NCDDNYKSRISKAVSLPEKRALPDLIPTSPLKRVQSWWRLKASVA---------------    183
                       ..

porcine           293 --------------------                                          293
human             296 --------------------                                          296
mouse             296 --------------------                                          296
rat               296 --------------------                                          296
bovine            296 --------------------                                          296
chicken           357 EQLCGCSYLIQRAIASSTW                                          376
zebrafish         284 --------------------                                          284
pacific oyster    183 --------------------                                          183
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Figure 2. Alignment of deduced amino acid sequences for MyD88. Comparison of MyD88 amino acid sequences from pig, human, mouse, rat, cow, chicken, zebrafish and pacific oyster. Numbers indicate amino acid position. *, Identical amino acid residues. Dots represent similar amino acid residues. Gaps were introduced to optimize alignment.

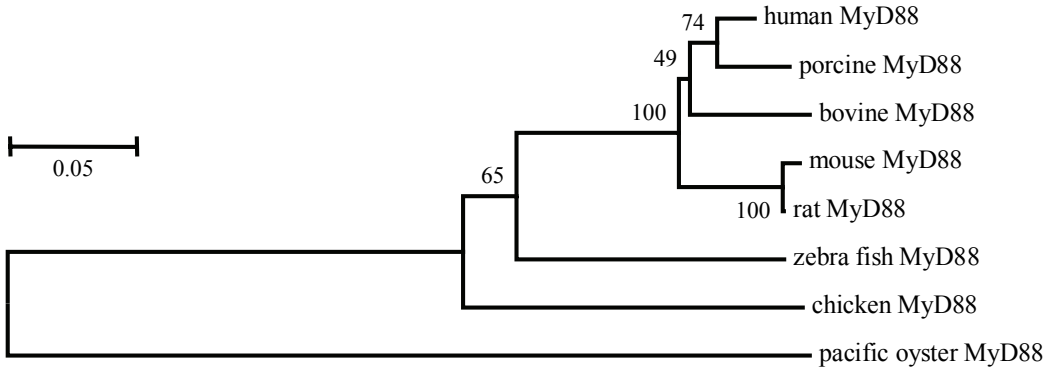

Figure 3. Phylogenetic tree of MyD88 amino acid sequences. Unrooted tree was built using neighbor-joining based on alignment of MyD88 amino acid sequences. Numbers indicate bootstrap ratios (%) of 1,000 replicates. Scale indicates divergence time.

manufacturer's protocol (Promega). The results are expressed as an index relative to that of the test sample divided by the index of non-stimulated control cells. All assays were conducted at least three times in triplicate.

Real-time quantitative PCR

Total RNA was isolated from various adult and newborn swine tissues as described (9, 10). Briefly, cDNAs were prepared by the reverse transcription of 1 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) and then poMyD88-specific cDNA was measured in equivalent volumes of cDNA from each sample using real-time quantitative PCR. The reactions proceeded in a 7300 Real-Time PCR System (Applied Biosystems, Warrington, UK) using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen) and the primers listed in Table 1. The reaction proceeded as follows: denaturation for 2 min at 95°C, and then 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The results are expressed as the relative mRNA index, calculated as the index of (MyD88 mRNA copy number/ β-actin mRNA copy number) for the test tissue divided by the index for the spleen. Controls included poly $(A)^+$ RNA templates to determine the presence of contaminating genomic DNA. Amplification products of contaminants such as primer dimers were not detected by SYBR green chemistry in serial dilutions of cDNA. Sequencing confirmed that the amplified cDNAs were identical to bp 530 to 590 of poMyD88.

Statistical analysis

All results represent the average of three to five separate experiments. The statistical significance of differences was assessed using Student's *t* test.

Results

Cloning and characterization of poMyD88

We first derived cDNAs encoding poMyD88 using mRNA isolated from adult swine ileal Pps. The 3'- and 5'-terminal regions of the cDNAs were obtained by 3'- and 5'-RACE PCR. The sequences were confirmed in three ileal Pps from various adult and newborn swine. Nucleotide sequencing of poMyD88 revealed a 1,010-bp cDNA sequence, including the poMyD88 structural gene. A predicted open reading frame (ORF) lay between bp 45 and 926 and encoded a 293-aa protein (Figure 1A). The nucleotide sequence of poMyD88 has been submitted to the DDBJ, EMBL and GenBank nucleotide databases under accession No. AB292176. To determine the structural domains in poMyD88, the amino acid sequence was analyzed using the SMART program. The deduced amino acid sequence of poMyD88 possesses a typical MyD88 domain including an N-terminal death domain, as well as intermediate and C-terminal TIR domains (Figure 1B). Two other regions were intrinsically disordered (Figure 1A). The alignment of amino acid sequences for MyD88 showed that the poMyD88 sequence is highly conserved among sequences from other species (Figure 2). The nucleotide sequence of the poMyD88 ORF is 87.3% and 78.0% identical to that of human and of mouse MyD88, respectively. At the amino acid level, the corresponding identities were 87.2% and 77.4%, respectively. These results indicated that poMyD88 is more similar to human than to mouse MyD88 at both the cDNA and amino acid levels. Phylogenetic analysis showed that poMyD88 belonged to the group containing human MyD88 (Figure 3). In addition, poMyD88 was more closely related to human MyD88 than to mouse MyD88 in terms of identity.

Functional analysis of poMyD88

To confirm the involvement of poMyD88 in cellular activation, we examined the activation of NF-κB in $HEK293^{poTLR2}$, $HEK293^{poTLR4/poMD-2}$ and $HEK293^{poRP105/poMD-1}$ cells. The cells were transiently transfected by lipofection with a pcDNA-c-myc vector encoding poMyD88. Intracellular staining with an anti-c-myc antibody showed that poMyD88-transfected $HEK293^{poTLR2}$, $HEK293^{poTLR4/poMD-2}$ and $HEK293^{poRP105/poMD-1}$ expressed similar levels of poMyD88 (Figure 4A). Upon stimulation with Pam3Cys, NF-κB dependent luciferase activity became significantly increased in cells expressing poMyD88 $HEK293^{poTLR2}$ compared with vector control cells

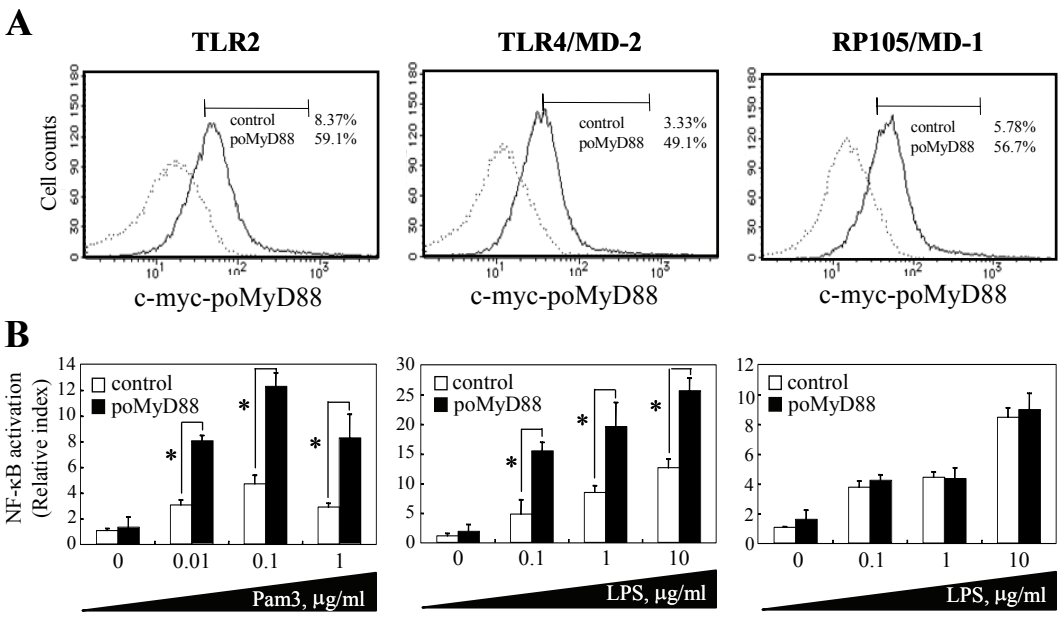

Figure 4. Porcine MyD88 is involved in signaling pathway *via* poTLR2 and poTLR4/poMD-2, but not poRP105/poMD-1. (A) HEK293^{poTLR2}, HEK293$^{poTLR4/poMD-2}$ and HEK293$^{poRP105/poMD-1}$ cells were transfected with pcDNA3 vector encoding c-myc tagged poMyD88 or empty control vector together with pGLM-ENH-luci vector. Expression of poMyD88 protein in HEK293^{poTLR2}, HEK293$^{poTLR4/poMD-2}$ and HEK293$^{poRP105/poMD-1}$ cells was detected by intracellular staining with PE-conjugated anti-c-myc mAb followed by flow cytometry. (B) Solid and dotted lines indicate transient transfection with poMyD88 expression vector and control vector, respectively. NF-κB activation was measured with or without poMyD88 in HEK293^{poTLR2}, HEK293$^{poTLR4/poMD-2}$ and HEK293$^{poRP105/poMD-1}$ cells. After transfection, cells were stimulated with indicated concentrations of Pam3Cys (Pam; for TLR2) and LPS (for TLR4/MD-2 and RP105/MD-1) for 24 h. Values represent means and error bars indicate standard deviation. Results are representative of three or four independent experiments. *, $p < 0.05$ *vs* cells transfected with vector control.

(Figure 4B). In the presence of LPS, poMyD88 over-expression resulted in further enhanced activation of NF-κB in HEK293$^{poTLR4/poMD-2}$, but not in HEK293$^{poRP105/poMD-1}$ cells (Figure 4B).

Expression of MyD88 in newborn and adult swine tissues

We analyzed the expression of MyD88 mRNA in newborn and adult swine tissues using real-time quantitative PCR. The results were estimated as a relative index to a spleen (1.00). As shown in Figure 5, MyD88 mRNA was expressed at detectable levels in all tissues of both newborn and adult swine. In newborn swine, digestive tissues (esophagus, duodenum, jejunum, ileum, ileal Pps and colon) expressed significantly less than spleen. The expression level in MLNs was lower than that in spleen, but the difference did not reach statistical significance. On the other hand, in adult swine, the significantly lower expression of MyD88 against spleen was observed in only esophagus and colon. The expression level in adult MLNs was higher than that in spleen (Figure 5).

Discussion

Here, we cloned poMyD88 cDNA and characterized functional domains in the amino acid sequence. The full-length cDNA of poMyD88 was 1,010 bp, including an ORF of 879 bp that encoded a 293-aa polypeptide. The nucleotide and amino acid sequences of poMyD88 shared higher identity with those of humans than of mice, supporting the notion that the swine immune system comprises a better model of the human system than of mice (9, 10). Some immunological experiments might thus be performed in swine *in vivo* instead of in humans. Therefore, investigating the immunological mechanisms underlying TLR-MyD88 signaling pathway is helpful for understanding the activation of the immune system not only in swine but also in humans.

Structural analysis revealed both a typical TIR and a death domain in poMyD88. The death domain superfamily plays an important role in death signal transduction, the regulation of apoptosis and in inflammatory responses (24). Upon stimulation, IRAK-1 molecules are recruited to the active receptor complex *via* association with MyD88, enabling oligomerization of IRAK-1 (25). The binding interaction of MyD88 with IRAK-1 is mediated by the death domain of MyD88 (25). The TIR domain, which is essential for the interaction of MyD88 with TIR and IL-1R, was highly conserved in poMyD88. In addition, two intrinsic disorder regions were also identified in the amino acid sequence of poMyD88. Intrinsic disordered proteins can bind to partners with both high specificity and low affinity (26, 27). Intrinsic disorder allows one regulatory region or protein to bind to many types of molecules (26, 27). The ability to

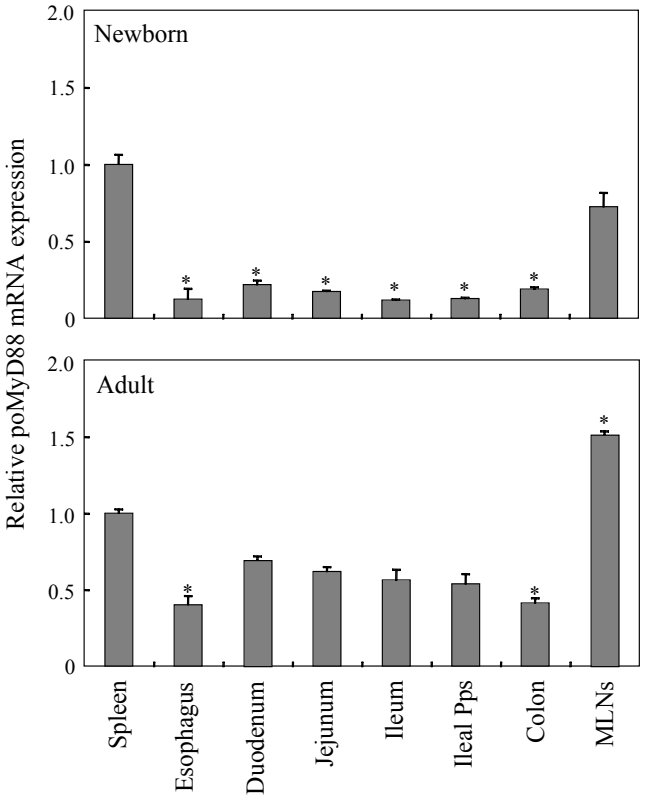

Figure 5. MyD88 expression in newborn and adult swine tissues. Porcine MyD88 mRNA level is expressed as relative mRNA index, calculated as index (MyD88 mRNA copy number/β-actin mRNA copy number) of test tissue divided by index of spleen. Values represent means and error bars indicate standard deviation. Results are representative of five independent experiments using tissues from at least five individual newborn or adult swine *, $p < 0.05$ *vs* spleen.

associate with many molecules, including both proteins and nucleic acids, is likely to be essential for signaling across the cell membrane as well as intracellular signaling. These hallmarks of the poMyD88 sequence indicated that poMyD88 is a functional adapter protein involved in the TLR signaling pathway of the porcine immune defense system.

To confirm the function of poMyD88, poMyD88 was overexpressed in HEK293^{poTLR2}, HEK293$^{poTLR4/poMD-2}$ or HEK293$^{poRP105/poMD-1}$ cells. Whereas transient expression levels of poMyD88 were similar in these cells, NF-κB activation was further enhanced in HEK293^{poTLR2} and HEK293$^{poTLR4/poMD-2}$, but not in HEK293$^{poRP105/poMD-1}$ cells after stimulation with the appropriate corresponding ligand. Increasing evidence indicates that MyD88 participates in the TLR/IL-1R superfamily signaling cascade through TIR-TIR interaction between MyD88 and TLR/IL-1R (28). Signaling *via* TLR2 and TLR4 requires MyD88, whereas that *via* RP105, which lacks a TIR domain, is independent of MyD88 recruitment and involves a complex of Lyn and CD19 that specifically regulates B cell proliferation and intracellular calcium mobilization (29-32). We previously showed that the RP105/MD-1 complex induces NF-κB activation *via* the PI3K and Bruton's tyrosine kinase signaling pathway (12). In this context, poTLR2 and poTLR4/MD-2 can efficiently recruit overexpressed poMyD88, resulting in enhanced activation of the MyD88-dependent signaling pathway downstream of TLR2 and TLR4/MD-2. On the other hand, poRP105/MD-1-mediated NF-κB activation is independent of poMyD88 because poRP105 is characterized by a cytoplasmic domain with only seven intracellular amino acids (12). These results indicate that the cloned poMyD88 is functionally expressed in the cytoplasmic regions and that it can mediate the signaling pathway of poTLR2 and poTLR4/MD-2 after stimulation with PAMPs.

Although the expression and function of MyD88 have been analyzed in various human and mouse cell lines *in vitro*, little is understood about MyD88 expression in newborn and adult swine *in vivo*. Therefore, we used real-time quantitative PCR to analyze MyD88 mRNA expression in newborn and adult swine tissues. Levels of MyD88 expression varied among organs from newborn swine with the levels being highest in the spleen and MLNs, and lower in intestinal tissues that will become directly exposed to bacteria in adults. The level of MyD88 mRNA expression in adult swine was highest in the MLNs. The expression levels of MyD88 in the intestinal tissues were lower than those in MLNs and spleens of adults whereas more MyD88 mRNA was expressed in the intestinal tissues of adults than of newborns when calculated as an index relative to that of the spleen. Compared with newborns immediately after birth, adult intestinal tissues are continually exposed to intestinal microbial antigens. Adult intestinal tissues also directly and frequently encounter microbial antigens across the intestinal epithelial cells, in contrast to MLNs and the spleen, which obtains antigens from afferent lymphatics or blood. Therefore, the different expression profiles of MyD88 in newborn and adult intestinal tissues indicate that the intestinal microflora directly promote intestinal MyD88 expression during postnatal development. In fact, MyD88 gene expression is clearly upregulated upon stimulation with LPS and peptidoglycans in peripheral blood leukocytes of the Japanese flounder (33). We previously found that some TLRs are preferentially expressed in the GALT not only by immune cells, such as dendritic and B cells, but also by cells in the follicle-associated epithelium, including pocket-like M cells (9, 10, 14). Our previous and present studies together suggest that the TLR-MyD88 signaling pathway can mediate the immune responses of immature as well as mature GALT to commensal micro-organisms, such as lactic acid bacteria, and their bioactive components.

In conclusion, we identified the poMyD88 gene from the ileal Pps of adult swine and constructed cells that transiently expressed this gene. We also confirmed that cloned poMyD88 is functional and involved in the signaling pathway *via* poTLR2 and poTLR4/MD-2, not poRP105/MD-1. Furthermore, the expression profiles and regulation of the TLR-MyD88 signaling pathway in newborn and adult swine might serve as a model with which to study development of the human immune system.

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