Replacement of two aminoacids in the bovine Toll-like receptor 5 TIR domain with their human counterparts partially restores functional response to flagellin.

Alena Osvaldova[1,#], Sally Woodman[3,#], Nicholas Patterson[3,#], Victoria Offord[3], Duncan Mwangi[2], Amanda J. Gibson[3], Jan Matiasovic[1], Dirk Werling[3,*]

[1] Veterinary Research Institute, Department of Immunology, Hudcova 296/70, 621 00 Brno, Czech Republic; [2]Zoetis, Kalamazoo, Michigan, 49007USA; [3] Molecular Immunology Group, Department of Pathology and Pathogen Biology, Royal Veterinary College, Hawkshead Lane, Hatfield, AL9 7TA, UK;

[#] These authors contributed equally to this work

* Corresponding author at the Department of Pathology and Pathogen Biology, Royal Veterinary College, Hawkshead Lane, Hatfield, AL9 7TA, UK Tel: ++44 1707 666358; fax: ++44 1707 666; E-Mail: dwerling@rvc.ac.uk

Abstract

Flagellin potently induces inflammatory responses in mammalian cells by activating Toll-like receptor (TLR) 5. Recently, we were able to show that stimulation of bovine TLR5 resulted in neither NFκB signalling nor CXCL8 production. Like other TLRs, TLR5 recruits signalling molecules to its intracellular TIR domain, leading to inflammatory responses. Analysis of available TLR5 sequences revealed substitutions in all artiodactyl sequences at amo acid (AA) position 798 and 799. Interestingly, a putative binding site for PI3K was identified at tyrosine 798 in the human TLR5 TIR domain, analogous to the PI3K recruitment domain in the IL-1 receptor. Mutation of the artiodactyl residues at position 798, 799 or both with their corresponding human counterparts partially restored the response of bovine (bo)TLR5 to flagellin as well as phosphorylation of PI3K. Together, our results suggest a potential lack of phosphorylation of F798 and H799 in boTLR5 partially explains the lack in observed response.

Keywords: TLR5, cattle, human, flagellin

Introduction

Pattern recognition receptors (PRRs) in antigen presenting and other, non-immune cells detect microbial pathogens to activate innate immunity, drive subsequent development of protective immunity against pathogens and optimise the development of adaptive immunity. Toll-like receptors (TLRs) were the first described PRRs and recognise a number of bacterial and viral pathogens (Jungi et al., 2011). Bacterial flagellin is an ancient and potent trigger of host innate immune responses in eukaryotes, and has been described so far as the only known activator of Toll-like receptor (TLR) 5 (Hayashi et al., 2001). In humans and rodents, activation of TLR5 by flagellin induces an inflammatory response designed to protect the host from perturbing microbes. This response involves a MyD88-dependent signalling pathway, leading to IRAK1 phosphorylation, and ultimately NFκB and p38-MAPK activation, which are required for inflammatory phenotypes such as CXCL8 release(Berin et al., 2002; Khan et al., 2004; Yu et al., 2003). Signal potentiation culmating in NFκB activation is initiated on the association of TLR5 with MyD88, or TLR5 homodimerisation via homologous TIR domains(Hayashi et al., 2001). In some cell types, flagellin also activates inducible nitric oxide synthase (iNOS) via a mechanism requiring TLR5/TLR4 heterodimers, resulting in nitric oxide (NO) release(Mizel et al., 2003). While the formation of TLR5 homo- and heterodimers in different cell types can explain some of the tissue-specific differences in flagellin responses, additional layers of complexity are likely to exist. Indeed, Ivison *et al* recently identified a phosphorylation site necessary for the activation of phosphatidylinositol 3-kinase (PI3K) in human (hu)TLR5, which resulted in the production of inflammatory signalling in responses to flagellin(Ivison et al., 2007). The necessary Y–X–X–M SH2-binding motif was identified by screening the TIR domain of the IL-1 receptor (IL-1R) in response to IL-1 stimulation(Marmiroli et al., 1998). Subsequently, a Y–X–X–M motif, mainly being Y-Q-L-M, was identified in huTLR5 in the same location as the PI3K binding site in IL-1R, which seemed to be crucial for PI3k dependent TLR5 signalling(Ivison et al., 2007).

Recently, we showed that exposure of HEK293T cells expressing bovine (bo)TLR5 as well as primary bovine macrophages to either purified flagellin or recombinant FliC neither induced NFκB or MAP-kinase signalling, and did not result in measurable CXCL8, TNF or NO production (Metcalfe et

al., 2014). In the present study, we show that the absence of the Y-X-X-M motif in boTLR5, and its replacement with the F-H-L-M motif at least partially restores functionality of boTLR5 signalling in response to FliC.

Methods

Generation of mutated boTLR5-transfected HEK293 cells

The generation of HEK293 cells expressing wild-type (wt) boTLR5 and huTLR5 was described recently (Metcalfe et al., 2014). Site directed mutagenesis was performed on boTLR5 cloned into pCR2.1. Primers for specific mutations were designed using Bio Edit software (Hall, 1999). QuickChange Lightning Site Directed Mutagenesis Kit (Agilent Technologies, USA) was used for the mutation according to manufacturer's instructions. Briefly, PCR reactions were run on Eppendorf MasterCycler pro, wt boTLR5 was used as a template for single mutations, for double mutation boTLR5^{F798Y} was used. PCR cycling parameters comprised of one cycle at 95°C for 2 m, followed by 18 cycles at 95°C for 20 s, 60°C for 10 s and 68°C for 3 m and 30 s (30 s per kb of plasmid length), before a final extension at 68°C for a further 5 m. Primers for substitution H/Q in position 799 were F: 5'GTCCCTGTCCCAGTTCCAACTGATGAGGCATC3', R: 5'GATGCCTCATCAGTTGGAACTGGGACAGGGAC3'. Primer sequences for substitution F/Y in position 798 were F: 5'GTCCCTGTCCCAGTACCATCTGATGAGGCATC3', R: 5'GATGCCTCATCAGATGGTACTGGGACAGGGAC3'. Substitution K/Q in position 378 F: 5'CACATTGGGATCATTCAGGACCAAACATTCAAATTCCTGGG3', R: 5'CCCAGGAATTTGAATGTTTGGTCCTGAATGATCCCAATGTG3'. Primers for double substitution HF/QY in positions 799 and 798 were F: 5' GTCCCTGTCCCAGTACCAACTGATGAGGCATC3', R: 5'GATGCCTCATCAGTTGGTACTGGGACAGGGAC3'. Parental DNA was then digested using Dpn I restriction enzyme. Subsequently, XL10 Gold Ultracompetent cells were transformed with Dpn I treated DNA using heat pulse at 42°C for 30 s. Cells were incubated in SOC medium (LifeTech, Thermo Fisher, UK) agitated to 180 rpm at 37°C for one hour and plated on agar with appropriate antibiotic. Plasmids were extracted from XL10 Gold Ultracompetent cells and purified using PureYield™ Plasmid Miniprep System (Promega, UK). Each desired mutation was confirmed by DNA sequencing, performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver. 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Constructs

with the confirmed correct sequence were subcloned into pcDNA3.1 for subsequent expression in HEK293T cells.

Dual Luciferase assay

HEK293T cell lines were seeded at 2.5×10^5 per well of a 6-well plate with 2 mL of DMEM and sodium pyruvate supplemented with 10% FBS and penicillin-streptomycin (5mg mL^{-1}) and incubated overnight (37°C, 5% CO_2). Subsequently, cells were transiently transfected for 24 hours with 0.5 ng μL^{-1} of Luciferase tagged NFκB (NFκB-luc) and with 0.5 ng μL^{-1} of specific plasmid using TurboFect Transfection Reagent (Thermo Scientific, UK). Transfected HEK293T cell lines were exposed to 0.1 µg mL^{-1} of the recombinant FliC, the protein subunit specifying the flagellar (H) antigens in flagellin (FLA-ST, Invivogen, France) or control medium. After 24 hours, supernatants were harvested and stored at -20°C. Proteins were extracted from cells using 100 µl 1× Passive lysis buffer (Dual-Luciferase Reporter assay system, Promega, UK). NFκB-luc expression were analysed on a MicroLumat Plus LB96V lumometer (Berthold Technologies, UK).

ELISA for CXCL8

Supernatants harvested from the above described experiments were assessed for CXCL8 using a commercially available ELISA according to the manufacturer's instructions (Quantikine Human CXCL8/IL-8 ELISA; R&D Systems, UK) as described recently (Metcalfe et al., 2014)

Western blotting for PI3-kinase

HEK293T cells were seeded as described above in wells of a 6-well plate, transfected and stimulated as described above. After 24 h they were stimulated with 100 ng mL^{-1} FLA-ST (Invivogen, France) for 24 hours. Cells were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo) containing protease inhibitor cocktail (Sigma-Aldrich, UK) following manufacturer's guidelines. Protein concentrations of samples were determined using the Agilent 2100 Bioanalyzer and the Protein 230 kit (Agilent Technologies, USA), following manufacturer's guidelines. All samples were diluted using ddH_2O to 638 ng μL^{-1}. Samples were used in Western Blot analysis following standard

protocols. Briefly, 13 µL of each sample were denatured (95°C, 5 m) using 1 µ DTT, 5 µL sample buffer and 1 µL antioxidant (all Expedion, UK) and loaded onto 4-20% SDS precast gel (Expedion, UK) and run along with PageRuler Prestained NIR Protein Ladder (Thermo Scientific, UK). Membranes were stained using polyclonal antibodies detecting phosphorylated forms of PI3K subunits (p85 Tyr458/p55 Tyr199) or unphosphorylated p85 PI3K (antibodies 4228 and 4259 of the PI3 Kinase Antibody Sampler Kit, Cell Signaling, UK) or a monoclonal mouse anti-human antibody to β-actin (Sigma-Aldrich, UK), shown previously to cross react in cattle (Metcalfe et al., 2014). After incubation with secondary antibody, HRP was visualised using Lumata Forte Western HRP substrate (Millipore, UK) and membranes analysed using a Gbox (Syngene, UK). Pictures of gels were taken analysed using the GBox-integrated GeneSnap software package, and densitometry was performed using the GeneTool software package (version4). Values of fold-change were created by dividing densitometry values obtained for either total or phosphorylated PI3K by the value obtained for the housekeeping gene for each condition. Thereafter, values for FliC-stimulated values were divided by those obtained for unstimulated controls.

Molecular modelling of TLR5 TIR domains

Translated TLR5 sequences from 20 species: cow (DQ335128), yak (GU647093), zebu (EU006636), gayal (HQ392514), African buffalo (JN615233), water buffalo (GQ866978), American bison (JN615236), white-tailed deer (JQ811843), giraffe (JQ811844), sheep (NM_001135926), goat (FJ659852), pig (ENSSSCT00000011909), human (ENST00000342210), chimpanzee (NM_001130462), gorilla (ENSGGOT00000028556), macaque (ENSMMUT00000001241), cat(ENSFCAT00000006837), dog(NM_001197176), rabbit (ENSOCUT00000025682) and mouse (ENSMUST00000110997) were aligned using MUSCLE (Edgar, 2004). Modeller version 9.10 (Fiser and Sali, 2003) was used to generate models representing boTLR5 (cow), boTLR5^{F798Y}, boTLR5^{H799Q}, boTLR5^{F798} and huTLR5 (human) residues 694-839 from the Protein Data Bank (PDB) TIR-domain-containing template structures 2J67, 1FYV, 1FYW, 1FYX, 1O77 and 1T3G. From each of the 100 model datasets, ten models with the lowest discrete optimized protein energy (DOPE) score were validated using PROCHECK (Morris et al., 1992), ProQ (Wallner and Elofsson, 2003), Verify3D

(Eisenberg et al., 1997) and ERRAT (Colovos and Yeates, 1993). Models with residues in disallowed regions, Verify3D score < 80% or ProQ LG score < 4 were excluded and an optimal model representing each structure was selected from the remaining dataset. All models have been visualized and prepared for publication in PyMOL version 1.7.0.

Molecular dynamics simulations (MDS) were performed using GROMACS 4.5.5 (Pronk et al., 2013) under the GROMOS96 43a1 force field. Each model was placed into a cubic box maintaining a distance of 1nm (10 Å) between the box edges and the model surface. Systems were solvated using the simple point charge (SPC) 216 three-point solvent model and neutralised with counter ions. Energy minimisation was performed using the steepest descent method for a maximum of 50,000 cycles or until the maximum force (F_{max}) of the system was less than 1,000 kJ mol^{-1} nm^{-1}. Equilibration of the system was performed for 100 ps in a temperature coupling bath set at 300 K using a modified Berendsen thermostat and 0.1 ps time constant, followed by Parrinello-Rahman pressure coupling maintained at 1 bar using a using a 1 ps time constant with water compressibility of $4.5e^{-5}$ bar^{-1}. The MDS production runs were conducted for 2 ns with a 2 fs time step at a temperature of 300 K and pressure of 1 bar with snapshots collected every 2 ps.

Statistical analysis

Values for four assays, with samples run at least in duplicates, were combined. Data were assessed for normal distribution, followed by a one-way ANOVA and an unpaired t-test using the GraphPad Prism software (version 6 for Windows).

Results and Discussion

Variation in inflammatory downstream effects of TLR agonists can arise from differences in the interaction of the leucine-rich repeats in the extracellular domain (ECD) with a ligand (Willcocks et al., 2013) or by the mechanism employed by the respective TIR domains to recruit and subsequently activate signalling proteins. Indeed, and similar to other bovine TLRs, boTLR5 has been described to possess differences in the ECD, which potentially explain some of the functional differences between human and bovine TLR5 (Metcalfe et al., 2014; Smith et al., 2012), which clearly indicates the

importance for the ECD of TLR5 in flagellin interaction, and this has been described before for chicken TLR5 (Keestra et al., 2008). For this model, a clear interaction between specific AA in both, the flagellin and the chTLR5 ECD were necessary for NFκB stimulation (Keestra et al., 2008). However, in addition to the identification of SNPs in the ECD, we also identified two AA substitutions in boTLR5 at positions that have been shown to be important phosphorylation-sites for kinases in huTLR5 (Ivison et al., 2007). The selected models of both, hu and boTLR5 TIR domain passed validation having all residues within allowed regions with wild type structure ProQ scores > 5 (very good model) and mutant structure ProQ scores > 4 (good model) (Supp. Table 1a). Overall, there is high structural conformity with all models deviating by less than 0.7 Å (Supp. Table 1b). This was not considered significant as an RMSD of 0.5 Å can be seen through general side chain rotation. Residues 798 and 799 are surface accessible and lie within a positively charged region. Mutation of these residues does not affect the overall surface potential but the region appears to be reduced in the bovine wild type model (Supp. Fig. 1C). While the overall structure and charge do not seem to show considerable changes, molecular dynamics simulations (MDS) run for 2 ns reveal that position 798 shows a large degree of flexibility within the structure (Suppl. Fig. 1B). In huTLR5, boTLR5^{H799Q} and boTLR5$^{F798Y/H799Q}$ residue 798 is amongst a small number of residues which have a root mean square fluctuation (RMSF) greater than 0.5 (Supplementary Table 1c), which is not the case for wild type boTLR5 and boTLR5^{F798Y}. This also suggests that the presence of glutamine at position 799 increases the flexibility of the DD loop, in particular when phenylalanine or tyrosine are present at position 798 (Suppl. Fig. 1A and 1B). Having established the importance of these AA, and their presence in all ruminant species tested, we next assessed whether their exchange would impact on boTLR5 functionality. To do so, AA were replaced by site-directed mutagenesis, and the resulting TLR5 constructs tested as described above. Stimulation of HEK293T cells transfected with huTLR5, but not boTLR5 with FliC resulted in a significant activation of NFκB as well as production of CXCL8, which was absent when cells were left unstimulated (Fig. 1A and 1B, respectively), as reported previously(Metcalfe et al., 2014). Furthermore, exchange of F798Y as well as H799Q in boTLR5 partially restored the NFκB responsiveness to stimulation with FliC (Fig. 1A) with the dual exchange F798Y/H799Q resulting in a nearly additive effect (Fig. 1A). A similar but not significant effect was

seen for CXCL8 production when these AA in boTLR5 were exchanged with their human counterparts (Fig. 1B).

Recently, in a search for protein interaction domains in TLR5, a putative PI3K binding motif in huTLR5 at an analogous location to a similar site previously described in the IL-1R was described (Ivison et al., 2007). TLR5 is the only TLR that contains this motif at this location. TLR2 and TLR3 have been reported to undergo tyrosine phosphorylation in response to their respective ligands. In the case of TLR2, a phospho-tyrosine recruitment site for PI3K was identified (Arbibe et al., 2000). In TLR3, mutagenesis of tyrosines within the TIR domain demonstrated a stepwise loss of signalling ability with each change. One residue, Tyr759, was particularly important for PI3K activation in response to dsRNA (Okugawa et al., 2006; Sarkar et al., 2004). A second residue, Tyr858, which corresponds to Y798 in TLR5, was required for TBK1 activation. It should be noted that phosphorylation of TLR2 and TLR3 was demonstrated only by reactivity to anti-phospho-tyrosine antibodies, so the locations of the individual phospho-tyrosines can only be inferred.

Given the importance of Y798, and the differences seen between bovine and human TLR5 sequences, we next tested whether the exchange of AA between hu and boTLR5 would also impact on PI3K phosphorylation.

When related to β-actin expression, HEK293T cells transfected with huTLR5 showed clear increase in the amount of phosphorylated p85 PI3K when stimulated with FliC (Fig. 2B), but not the total amount of p85 PI3K, potentially indicating that nearly all available p85 PI3K was present in a phosphorylated stage at the time of analysis. A number of reports indicated that PI3K activation suppresses inflammation during the early stages of bacterial infection (Fukao and Koyasu, 2003). Indeed, Yu *et al* demonstrated that TLR5-mediated PI3K activation negatively regulates flagellin-induced proinflammatory gene expression in human epithelial cells (Yu et al., 2006). Thus, the increased amount of phosphorylated p85 PI3K in HEK293T cells expressing huTLR5 could indeed be seen as one of the molecular mechanisms that mediates a shutdown of a proinflammatory response after the initial response.

In contrast, exchange of F798Y, H799Q or both AA in boTLR5 increased total p85 PI3K expression compared to wild type boTLR5 (Fig. 2B), when adjusted to β-actin expression levels. However, only small effects, if any were seen with regards to the amount of phosphorylated PI3K (Fig. 2B). Our data are in line with recently published data on huTLR5 [7]. Here, mutation of the key tyrosine residue at Y798 eliminated huTLR5 activity, but no direct interaction between PI3K and huTLR5 was demonstrated(Ivison et al., 2007). In contrast, using different cell lines and techniques, Rhee *et al*. (Rhee et al., 2006) were able to demonstrate co-immunoprecipitation of huTLR5 and p85 PI3K following flagellin treatment that required a Y–X–X–M motif in MyD88. Thus, the lack of direct interaction of huTLR5 and PI3K do not rule out direct binding of huTLR5 to PI3K, but do establish that this must occur within the context of a signalling complex with MyD88. Whereas NFκB activity as well as CXCL8 secretion seemed to be affected by boTLR5^{F798Y} and boTLR5^{H799Q}, the signal related to p85 PI3K phosphorylation was relatively weak and did not appear to change following flagellin treatment. Our findings contrast those reported by Okugawa *et al*. who showed rapid, transient phosphorylation of TLR5 in T cells by Western blotting after flagellin stimulation (Okugawa et al., 2006). This discrepancy could reflect a true physiologic difference between the epithelial cells used in our experiment (HEK293T cells) and T cells. Nevertheless, our study is the first to demonstrate the important of the AA at position 798/799 in boTLR5 for signalling.In the context of our present study and previously published data on other TLRs, the results suggest that phosphorylation of specific tyrosines within the TIR domains of various TLRs is a regulatory step in signalling. Indeed, in chTLR5, the importance of not only specific AA in the ECD was shown, but targeted mutagenesis of the proline residue at position 737 in the chTLR5-TIR domain was detrimental to chTLR5 function.

Additional studies will be required to determine what effect, if any, phosphorylation has on the interactions of TLRs and adaptor molecules or kinases involved in proximal signalling (e.g. MyD88, TRIF, IRAK), and may require time course analysis for several kinases, adapter molecules and transcription factors. In addition, the kinases that phosphorylate the TLRs, and the regulation of these modifications, remain to be identified. Furthermore, it will also be interesting to see whether the SNPs

identified in the extracellular domain of ruminant and other mammalian TLR5 have additional impact on flagellin binding, potentially enabling binding of flagellin from ruminant pathogens with high avidity (Smith et al., 2012). However, it is reasonable to hypothesize that phosphorylation of TLRs provides a physiological means of regulating inflammatory responses to microbial molecules to limit them to situations that are beneficial to the host. This hypothesis could indeed explain why boTLR5 does not only show a low, if any, responsiveness to flagellin in terms of NFkB activation, but also shows a loss of necessary phosphorylation sites in its TIR domain. This potentially supports the notion of a largely redundant role of TLR5 in the ruminant system, and suggesting that other accessory mechanisms of flagellin pathogen recognition, either via Ipaf/Naip3 or location specific expression of TLR5 may provide the necessary protection against infection. Indeed, one could imagine two scenarios potentially this "loss-of-function": The first one would consider the copious quantities of Gram-negative flagellated bacteria present within the GIT system as mucosal commensals. In this scenario, boTLR5 may work more like a "buffering" PRR, similar to the interaction of LPS with LPS-binding protein and/or MD2, to avoid an overshooting activation of the innate immune response to small amounts of flagellin. The second scenario considers that boTLR5 only gets activated when forming heterodimers with TLR4, similar to that described in the murine system (Mizel et al., 2003). Overall, the data presented in the current study potentially challenge our view of TLR5 as a PRR inducing a pro-inflammatory response via NF-κB in the bovine/ruminant system. As flagellin is currently used in clinical trials as a vaccine adjuvant in the human system our data, if corroborated, may impact on the use of flagellin as vaccine adjuvants in the bovine system.

Acknowledgments:

The present work was supported by a grant of Zoetis Animal Health to D.W.. A.O. and J.M. were supported by project LO1218 of MEYS of the Czech Republic under the NPU I program. We thank DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) for DNA sequencing. This publication represents number CSS_00757 of the RVC.

References

Arbibe, L., Mira, J.P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowski, P.J., Ulevitch, R.J., Knaus, U.G., 2000. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nature immunology 1, 533-540.

Berin, M.C., Darfeuille-Michaud, A., Egan, L.J., Miyamoto, Y., Kagnoff, M.F., 2002. Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. Cellular microbiology 4, 635-648.

Colovos, C., Yeates, T.O., 1993. Verification of protein structures: patterns of nonbonded atomic interactions. Protein science : a publication of the Protein Society 2, 1511-1519.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research 32, 1792-1797.

Eisenberg, D., Luthy, R., Bowie, J.U., 1997. VERIFY3D: assessment of protein models with three-dimensional profiles. Methods in enzymology 277, 396-404.

Fiser, A., Sali, A., 2003. Modeller: generation and refinement of homology-based protein structure models. Methods in enzymology 374, 461-491.

Fukao, T., Koyasu, S., 2003. PI3K and negative regulation of TLR signaling. Trends in immunology 24, 358-363.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., Aderem, A., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410, 1099-1103.

Ivison, S.M., Khan, M.A., Graham, N.R., Bernales, C.Q., Kaleem, A., Tirling, C.O., Cherkasov, A., Steiner, T.S., 2007. A phosphorylation site in the Toll-like receptor 5 TIR domain is required for inflammatory signalling in response to flagellin. Biochemical and biophysical research communications 352, 936-941.

Jungi, T.W., Farhat, K., Burgener, I.A., Werling, D., 2011. Toll-like receptors in domestic animals. Cell and tissue research 343, 107-120.

Keestra, A.M., de Zoete, M.R., van Aubel, R.A., van Putten, J.P., 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Mol Immunol 45, 1298-1307.

Khan, M.A., Kang, J., Steiner, T.S., 2004. Enteroaggregative Escherichia coli flagellin-induced interleukin-8 secretion requires Toll-like receptor 5-dependent p38 MAP kinase activation. Immunology 112, 651-660.

Marmiroli, S., Bavelloni, A., Faenza, I., Sirri, A., Ognibene, A., Cenni, V., Tsukada, J., Koyama, Y., Ruzzene, M., Ferri, A., Auron, P.E., Toker, A., Maraldi, N.M., 1998. Phosphatidylinositol 3-kinase is recruited to a specific site in the activated IL-1 receptor I. FEBS letters 438, 49-54.

Metcalfe, H.J., La Ragione, R.M., Smith, D.G., Werling, D., 2014. Functional characterisation of bovine TLR5 indicates species-specific recognition of flagellin. Veterinary immunology and immunopathology 157, 197-205.

Mizel, S.B., Honko, A.N., Moors, M.A., Smith, P.S., West, A.P., 2003. Induction of macrophage nitric oxide production by Gram-negative flagellin involves signaling via heteromeric Toll-like receptor 5/Toll-like receptor 4 complexes. Journal of immunology 170, 6217-6223.

Morris, A.L., MacArthur, M.W., Hutchinson, E.G., Thornton, J.M., 1992. Stereochemical quality of protein structure coordinates. Proteins 12, 345-364.

Okugawa, S., Yanagimoto, S., Tsukada, K., Kitazawa, T., Koike, K., Kimura, S., Nagase, H., Hirai, K., Ota, Y., 2006. Bacterial flagellin inhibits T cell receptor-mediated activation of T cells by inducing suppressor of cytokine signalling-1 (SOCS-1). Cellular microbiology 8, 1571-1580.

Pronk, S., Pall, S., Schulz, R., Larsson, P., Bjelkmar, P., Apostolov, R., Shirts, M.R., Smith, J.C., Kasson, P.M., van der Spoel, D., Hess, B., Lindahl, E., 2013. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. Bioinformatics 29, 845-854.

Rhee, S.H., Kim, H., Moyer, M.P., Pothoulakis, C., 2006. Role of MyD88 in phosphatidylinositol 3-kinase activation by flagellin/toll-like receptor 5 engagement in colonic epithelial cells. The Journal of biological chemistry 281, 18560-18568.

Sarkar, S.N., Peters, K.L., Elco, C.P., Sakamoto, S., Pal, S., Sen, G.C., 2004. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nature structural & molecular biology 11, 1060-1067.

Smith, S.A., Jann, O.C., Haig, D., Russell, G.C., Werling, D., Glass, E.J., Emes, R.D., 2012. Adaptive evolution of Toll-like receptor 5 in domesticated mammals. BMC evolutionary biology 12, 122.

Wallner, B., Elofsson, A., 2003. Can correct protein models be identified? Protein science : a publication of the Protein Society 12, 1073-1086.

Willcocks, S., Offord, V., Seyfert, H.M., Coffey, T.J., Werling, D., 2013. Species-specific PAMP recognition by TLR2 and evidence for species-restricted interaction with Dectin-1. Journal of leukocyte biology 94, 449-458.

Yu, Y., Nagai, S., Wu, H., Neish, A.S., Koyasu, S., Gewirtz, A.T., 2006. TLR5-mediated phosphoinositide 3-kinase activation negatively regulates flagellin-induced proinflammatory gene expression. Journal of immunology 176, 6194-6201.

Yu, Y., Zeng, H., Lyons, S., Carlson, A., Merlin, D., Neish, A.S., Gewirtz, A.T., 2003. TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via posttranscriptional mechanism. American journal of physiology. Gastrointestinal and liver physiology 285, G282-290.

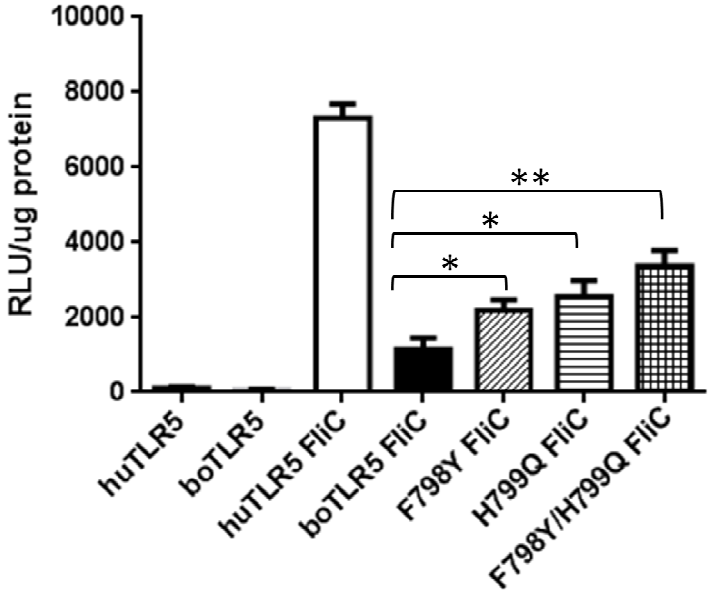
Figure Legend

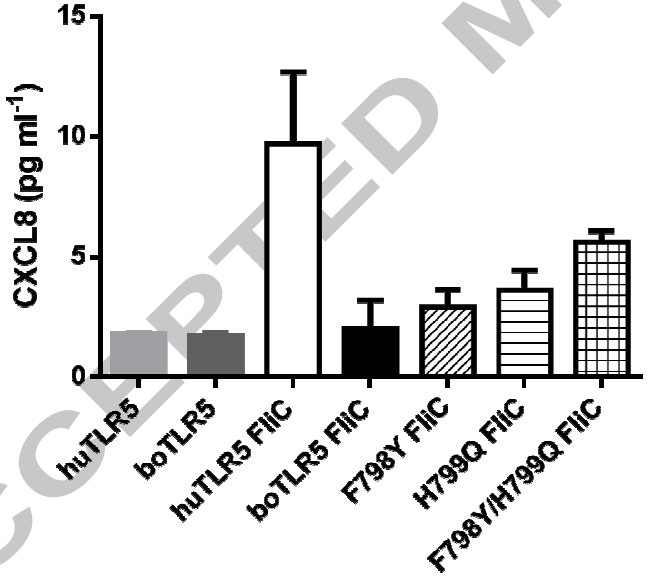
Figure 1: AA exchange partially restores boTLR5 functionality

HEK293T cells were transfected with different TLR5 and stimulated with FliC as described. 24 h after stimulation, activation of NFκB was analysed by gene reporter assay and CXCL8 production in the supernatant of stimulated HEK cells by ELISA. Results of four experiments are shown, and values expressed as mean ± SEM. * = $p<0.05$; ** = $p<0.01$

Figure 2: AA exchange in boTLR5 impacts on p85 PI3K expression

A) Protein extract from HEK293T cells transfected with various TLR5 constructs were analysed by Western Blotting for β-actin, total and phosphorylated p85 PI3K. Lanes represent protein extracts from: 1) unstimulated huTLR5; 2) FLiC stimulated huTLR5; 3) unstimulated wt boTLR5; 4) FliC stimulated wt boTLR5; 5) unstimulated boTLR5^{F798Y}; 6) FliC stimulated boTLR5^{F798Y}; 7) unstimulated boTLR5^{H799Q}; 8) FliC stimulated boTLR5^{H799Q}; 9) unstimulated boTLR5$^{F798y/H799Q}$; 10) FliC stimulated boTLR5$^{F798y/H799Q.}$ Pictures of gels were taken analysed using the GBox-integrated GeneSnap software package, , and a representative gel of two blots is shown. B) Protein extracts were probed by Western Blotting for β-actin, total and phosphorylated p85 PI3K. Thereafter, densitometry was performed using the GeneTool software package (version4). Fold differences are displayed as differences between stimulated versus unstimulated samples, and were calculated by dividing the value obtained for each PI3K form by the corresponding value obtained for β-actin of the same sample. Thereafter, the value obtained for the FliC-stimulated sample was divided by that obtained for the unstimulated sample, and results are expressed as fold-change compared to unstimulated samples.

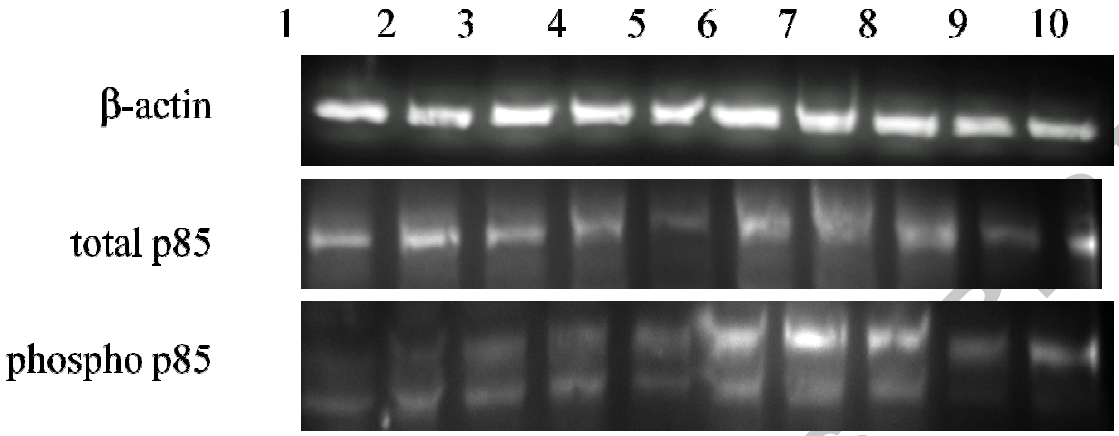




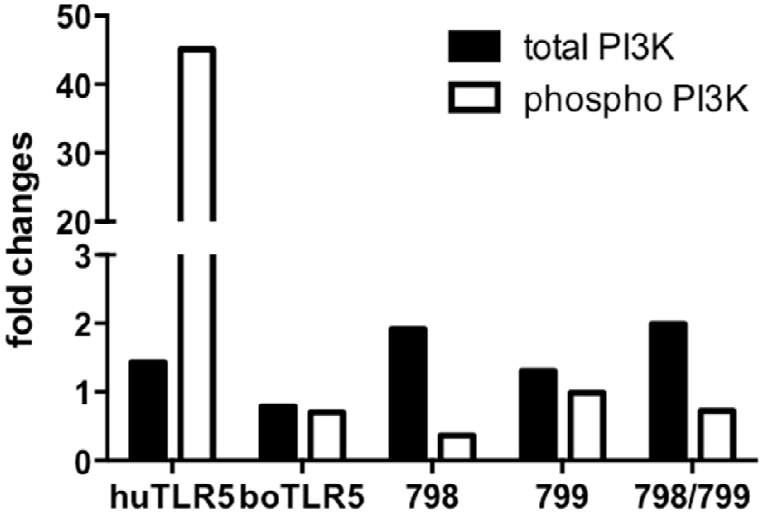
1

2

A)



B)



1

2

Highlights

- We compared and modelled TLR5 TOR domain sequences.
- We show aminoacid substitutions in the TIR domain, leading to the absence of a PI3K phosphorylation site present in huTLR5 TIR domain
- Exchange of aminoacids in boTLR5 TIR domain with the corresponding huTLR5 TIR domain aminoacids partially restores functionality
- This substitution does not seem to impact on PI3K phosphorylation in boTLR5