

## ***Ascaris suum* haemoglobin induced innate immune response in the peripheral blood mononuclear cells of German Landrace and Pietrain pigs**

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### **Summary**

*Ascaris suum* is the largest intestinal parasite of pigs with higher prevalence in free range and organic production. Haemoglobin of *A. suum* origin, AsHb, is the most abundant protein in the pseudocoelomic fluid of adult worms and a major excretory/secretory protein of larva 3 (L3), L4 and adult worms. Immunization with AsHb resulted in increased reactivity to migratory stages in the liver; however, the porcine systemic response to *A. suum* is not entirely elucidated. Therefore, the current study aimed to investigate the interplay of proinflammatory and regulatory cytokines and host genetics to the AsHb stimulation in the peripheral blood. Accordingly, we used peripheral blood mononuclear cells (PBMCs) from clinically healthy, female, weaned piglets of five weeks old, and investigated the primary systemic response in terms of cell viability, phagocytosis and selective cytokine expression in naïve and LPS plus PHA costimulated PBMCs. Temporal expression patterns of IL-10, IL-6, IL-2, IFN- $\gamma$  and TGF- $\beta$ 1 mRNA level for three time points (24, 48 and 72 hours) from two breeds (German Landrace, DL and Pietrain, Pi) were quantified using qRT-PCR. Phenotypic variation in cell viability between breeds was observed after 72 h of cultivation and only in the costimulated group. Significantly higher phagocytosis was observed in phagocytes of LR pigs compared to that of Pi. In naïve PBMCs treated with AsHb, significant breed effect was noticeable in case of IL-10, IL-6 and TGF- $\beta$ 1 expression. In AsHb sensitized and co-stimulated PBMCs, the mRNA level of IL-2 was significantly affected by both breed and treatment at 24 h post culture. TGF- $\beta$ 1 was significantly upregulated in the costimulated Pi PBMCs where the significant effect of breed and treatment was noted. In conclusion, the systemic immune response to AsHb priming was characterized by a mixed Th1/Th2 profile of immune cytokines dominated by IL-10, IL-6 and IL-2 response. There was also substantial influence of the host breed indicating breed differences in the innate immune responsiveness to ascariasis.

Key words: *Ascaris suum*, haemoglobin, innate immunity, cytokine, PBMCs, in vitro, pig, breed

### **Introduction**

*Ascaris suum* is the most abundant gastrointestinal parasite of pig. Ascariasis is more common in extensive and green pig production system, and in tropical and subtropical countries. Infections may lead to reduced feed efficiency and weight gain, pulmonary inflammation, and in chronic cases, milk spot liver leading to condemnation and reduced efficiency of other vaccine in the host. All of these together resulted in considerable economic loss as well as animal welfare risk for pig production and public health impact (Thamsborg *et al.* 2013). Despite of routine deworming practice, total elimination and sustainable control of ascariasis is still far reaching. Because, helminths can elicit a variety of immunoregulatory molecules which can either repress or regulate anti-helminth and immunopathological response at different levels, from the very early initiating events in innate immunity to the final effector mechanisms in established adaptive responses. The

fate of immune response and host-parasite interaction is found to be determined partially by host genetics. Therefore, variation in the immune response from individual to individual, strain to strain, breed to breed based on host genetics is reported for several pathogens. Immunogenetic analysis of expression studies in porcine peripheral blood mononuclear cells (PBMCs) particularly emphasizing the early innate response to helminths or helminth products are still limited. Besides, an in vitro study provides a better opportunity for appropriate control under restricted environment supporting its suitability for the study of immunogenetic response. Host genetic contribution (Skallerp *et al.* 2012) and porcine breed effect (Zanga *et al.* 2003) in ascariasis burden is already reported. So the current study was designed to improve our understanding in the context of breed variation in anti-ascaris immunity in pigs through investigating the systemic response to primary challenge with an immunomodulatory excretory-secretory antigen, *Ascaris suum* haemoglobin (AsHb) in PBMC culture in terms of cell viability, phagocytosis and cytokine expression.

## Materials and Methods

The animal husbandry was performed according to the institutional guidelines and animal husbandry regulations of Germany (ZDS, 2003). *Ascaris suum* haemoglobin antigen was utilized as a representative antigen and combination of lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) served as mitogenic stimulator for PBMCs culture. Whole blood from four clinically healthy female piglets of the same mother at their 5 to 6 weeks age from each German Landrace (DL) and Pietrain (Pi) breed were collected. PBMCs from whole blood were isolated by Ficoll-Paque density gradient centrifugation method as describe earlier (Uddin *et al.* 2012). Time and dose dependent cell viability was assessed in 96-well plate with  $1 \times 10^4$  cells/well in 100  $\mu$ l RPMI-1640 media using CCK-8 cell proliferation kit (cat.CK04-10, Dojindo Molecular Technologies, Inc., EU GmbH) following the manufacturer's instruction. In a similar fashion, the PBMCs were cultured separately for each animal at  $1 \times 10^6$  cells/well in 2 ml media into a 6-well tissue culture plate for mRNA samples. Four groups of treatment were made; control without any treatment, only AsHb treated group, group with LPS plus PHA costimulation after 30 min of AsHb treatment, and group receiving only LPS plus PHA. The dose for LPS (100 ng/ml) plus PHA (10  $\mu$ l/ml) were applied throughout the experiment. The plates were maintained for 24, 48 and 72 h at 37°C with 5% CO<sub>2</sub> before collection of cells separately. RNA isolation was done using miRNeasy mini kit. Quantity and quality was checked by nanodrop measurement and gel visualization. The mRNA expression of selected innate immune related genes was quantified by qRT-PCR with the StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany). The delta Ct ( $\Delta$ Ct) [ $\Delta$ Ct = Ct<sub>target</sub> – Ct<sub>reference genes</sub>] values were calculated as the difference between target gene and reference genes and expression was calculated as 2(- $\Delta$  $\Delta$ Ct). Data was analyzed using SAS software (Version 9.1.2, SAS Institute Inc., Cary, NC, USA).

In order to test the effect of AsHb on blood monocytes from two breeds, phagocytosis assay was also performed using Vybrant Phagocytosis Assay Kit (Catalogue# V-6694, Molecular Probes Inc.) according to the manufacturer instructions. One-way Anova tests were performed for cell viability and phagocytosis data in GraphPad Prism 5.

## Results and discussion

Antigenic components of *Ascaris suum* are known to exert immunosuppressive phenomena by down regulating the expression of costimulatory molecules on dendritic cells in both in vivo and in vitro studies (Boesen *et al.* 2006). Cell viability results revealed dose-dependent decrease in PBMC population of DL pigs from AsHb exposure. Relative viability of PBMCs treated with AsHb (2.5  $\mu$ g/ml) in presence and in absence of costimulation was compared between DL and Pi. Substantial ( $p < 0.05$ ) variation was found in presence of costimulation at 72 h post-treatment with AsHb (Fig.

1). Relative phagocytic ability of AsHb treated monocytes from PBMCs of DL and Pi origin were compared. Significant variation ( $p < 0.05$ ) in the phagocytosis rate was observed between breeds (Fig. 2) where blood monocytes of DL pigs showed higher phagocytic potential compared to that of Pi pigs.

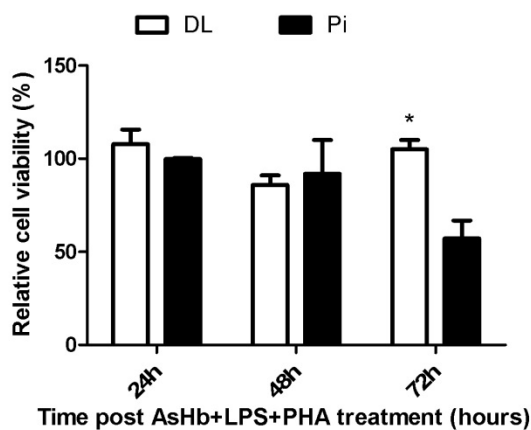


Fig. 1 Breed comparison for relative cell viability in AsHb (2.5  $\mu\text{g/ml}$ ) treated and LPS plus PHA post stimulated PBMCs. Data are presented as mean  $\pm$  standard error of mean. \*  $p < 0.05$ . DL: German Landrace, Pi: Pietrain.

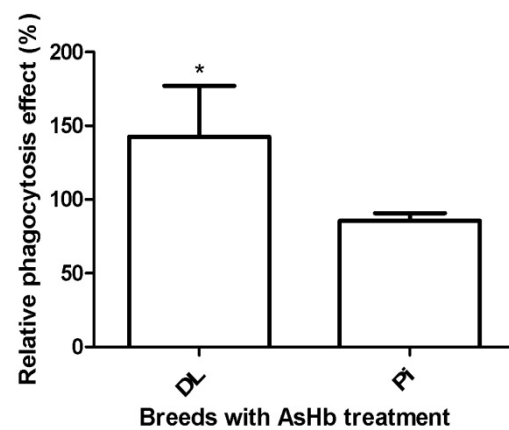


Fig. 2 Variation in phagocytosis effect resulting from breed differences in AsHb (2.5  $\mu\text{g/ml}$ ) treated monocytes isolated from PBMCs of DL and Pi. Data are presented as mean  $\pm$  standard error of mean. \*  $p < 0.05$ . DL: German Landrace, Pi: Pietrain.

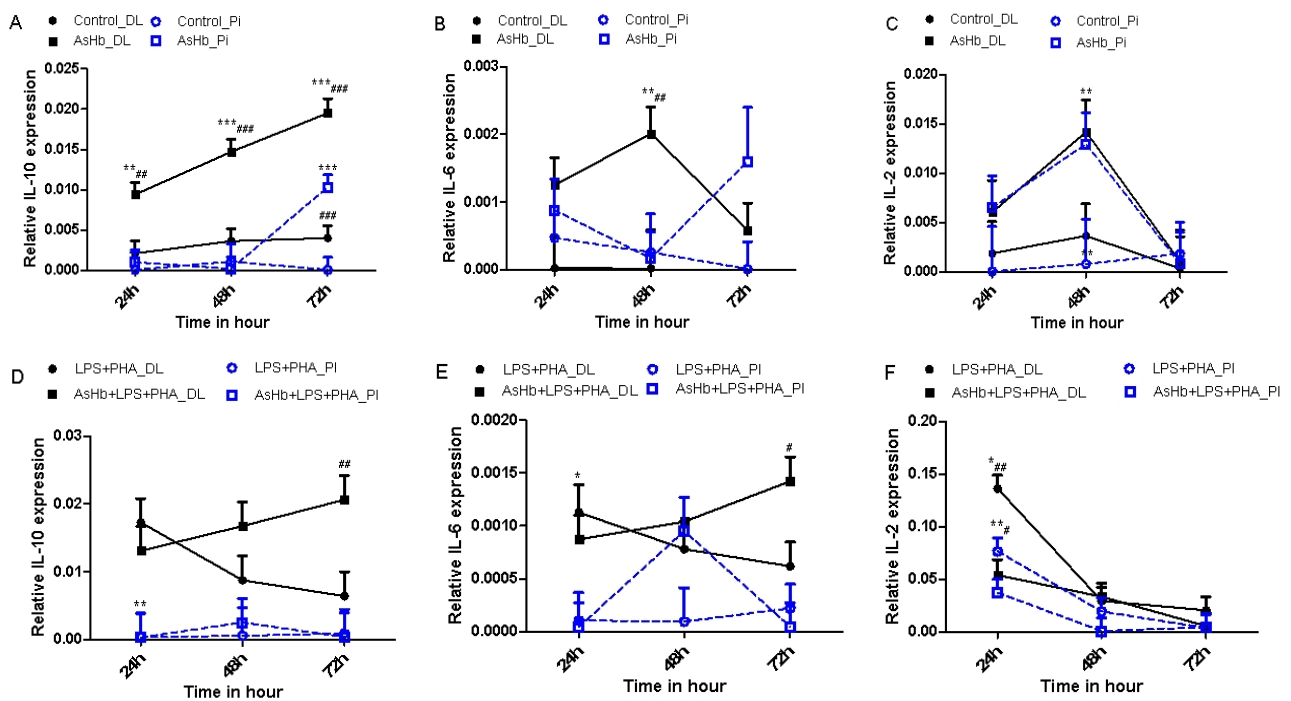


Fig. 3A-F The mRNA expression of inflammatory and regulatory cytokines, IL-10, IL-6, IL-2 in naïve PBMCs (A, B, C) and in costimulated PBMCs (D, E, F) from DL and Pi pigs exposed to AsHb. The results were combined with four individual animal replicates. The data are represented as the least square mean  $\pm$  standard error of mean. \*\*\* $p < 0.0001$ , \*\* $p < 0.01$  and \* $p < 0.05$  indicate comparison between breeds at same treatment at same point of time, and ### $p < 0.0001$ , ## $p < 0.01$  and # $p < 0.05$  indicate comparison between control and treatment within breed at same point of time. DL: German Landrace, Pi: Pietrain

The measurement of cytokine production is the easiest assessment for cell responsiveness, which is used for different in vitro studies. Experimental infection with *Ascaris suum* in pig induced strong Th2 response, measured systematically through increased blood eosinophilia, IL-4 and locally at the intestinal level, with enhanced production of IL-4, IL-6, IL-10 and IL-13 (Roepstorff *et al.* 2011). In our in vitro PBMCs model, exposure to AsHb resulted in changes in the kinetics of cytokine expression depending on host, time and stimulus. A steady and progressive rise of IL-10 mRNA was observed in AsHb treated PBMCs of DL origin throughout the duration of cultivation. This upregulation of IL-10 significantly differed between breeds at all three time points in naïve PBMCs (Fig. 1A). The relative expression of IL-6 was upregulated across the experimental period in the DL group (Fig. 1B). But in Pi, the upregulation was distinct at 24 and 72h post treatment. For both breeds, the peak of IL-10 and IL-2 in AsHb treated naïve PBMCs was observed at 72 h and 48 h of culture, respectively. Quantitative and qualitative kinetics of IL-2 expression was almost similar between DL and Pi (Fig. 1C).

In the costimulated group, exposure to AsHb increased IL-10 and IL-6 level 24 h post culture only in DL (Fig. 1D and E). In Pi, no significant variation from AsHb treatment between costimulated cultures was noticed except at 48 h, where there was a drastic rise of IL-6. The pattern of IL-2 production was similar in both breeds and significant a level of suppression was noticed at 24 h time point resulting from breed and treatment factors (Fig. 1F). This finding is in accordance with other studies where helminth antigen mediated suppression of bystander antigens was reported (McSorley *et al.* 2012). Surprisingly, the influence of breed and treatment was found inconsistent and less pronounced on the expression dynamics of TGF- $\beta$ 1 and IFN- $\gamma$  throughout the experimental duration in AsHb treated groups (data not shown). The current study explored the variation in ascaris antigen primed innate response between DL and Pi in an exposure time, costimulation and host dependent manner.

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## List of References

- Boesen, A., B. Wetzel, H. Norris, V. Bundoc, J. Urban, A. Keane-Myers, 2006. The role of innate immunity in suppression of allergic inflammation by *Ascaris suum* antigens. *J. Allergy Clin. Immunol* 117(2) : S314.
- McSorley, H.J., R. M. Maizels, 2012. Helminth infections and host immune regulation. *Clin. Microbiol. Rev.* 25: 585–608
- Skallerup, P., P. Nejsum, C.B. Jorgensen, H.H.H. Goring, P. Karlskov-Mortensen, A.L. Archibald, M. Fredholm, S.M. Thamsborg, 2012. Detection of a quantitative trait locus associated with resistance to *Ascaris suum* infection in pigs. *Int. J. Parasitol.* 42(4): 383–391
- Thamsborg, S. M., P. Nejsum & H. Mejer, 2013. Impact of *Ascaris suum* in livestock. In: *Ascaris: the Neglected Parasite*, C. Holland (editor), Elsevier, Amsterdam, the Netherlands, pp. 363–382.
- Uddin MJ, P.K. Nuro-Gyina, M.A. Islam, D. Tesfaye, E. Tholen, C. Looft, K. Schellander, M.U. Cinar, 2012. Expression dynamics of toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. *Vet Immunol Immunopathol.* 147: 211–221
- ZDS, 2003. Richtlinie fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit beim Schwein. Zentralverband der Deutschen Schweineproduktion eV, Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn
- Zanga J., M. Chimonyo, A. Kanengoni, K. Dzama, S. Mukaratirwa, 2003. A comparison of the susceptibility of growing mukota and large white pigs to infection with *Ascaris suum*. *Vet Res Commun* 27(8): 653–660