Institut für Tierwissenschaften, Professur für Tierzucht und Tierhaltung

Helminth antigen-induced innate immune response in porcine peripheral blood mononuclear cells

Inaugural-Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften

(Dr. agr.)

der

Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

von

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Gazipur, Bangladesh

Bonn, 2017

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Tag der mündlichen Prüfung:	4. Dezember 2017

Dedicated to…
My best friend Dr. Md. Aminul Islam

Helminth antigen-induced innate immune response in porcine peripheral blood mononuclear cells

Helminths are among the gastrointestinal parasites that are responsible for substantial loss of productivity in swine and other livestock industry. Despite indoor intensive rearing with routine anthelmintics, helminth infections in pigs are still not well managed. Elucidating mechanisms of host innate responses to helminth infection and their genetic correlation is important to improve the pig breeding strategies through selecting animals with better immunocompetence. This dissertation aims to investigate the innate immune responses to two helminth antigens from each of *Trichinella spiralis* derived tyvelose (TY) and *Ascaris suum* derived haemoglobin (AsHb) using the in vitro peripheral blood mononuclear cells (PBMCs) model. To achieve the objectives, PBMCs from German Landrace (LR) and Pietrain (Pi) pigs were in vitro stimulated with tyvelose and AsHb with or without mitogenic co-stimulation, and cells were harvested 24, 48 and 72 h post stimulation. The cell viability and proliferation and phagocytosis were evaluated followed by relative expression analysis of cytokines mRNA (IFN-γ, IL-2, IL-6, IL-10 and TGF-β1) using qRT-PCR. Results showed that TY-specific proliferation of PBMCs was transient and temporally associated with the duration and concentration of treatments. The highest viability of TY treated PBMCs were found at 24 h time point compared to the 48 h and 72 h. The cytokine responses were dominated by up regulation of IL-10 and IL-6, but also IL-2 with rapid resolution or appearance from time to time. The resulting host immunity revealed the dominance of Th1/Th2 as evident from the elevated level of IL-10 and IL-6. The variation in the expression levels of IL-10, IL-2, TGF-β1, IL-6 and IFN-γ in PBMCs obtained from LR and Pi pigs was significant, but not consistent across the course of the experiment and the state of mitogen-activation. TY induced cytokine expression dynamics and kinetics were different in PBMCs of LR and of Pi pigs. AsHb induced phenotypic variation in cell viability between breeds was observed after 72 h of cultivation only in the co-stimulated group. Significantly higher phagocytosis was observed in phagocytes of LR origin compared to that of Pi. In naïve PBMCs treated with AsHb, a significant breed effect was noticeable in case of IL-10, IL-6 and TGF-β1 expression, although the interrelationship between these regulatory cytokines was not always synchronous. In AsHb sensitized and costimulated PBMCs, the cytokine expression was rather skewed and only at 24 h of culture, a significant effect of both, breed and AsHb, was noted. In conclusion, the systemic immune response to TY and AsHb was characterized by a dominance of mixed Th1/Th2/regulatory immune response; LR pigs showed relatively early and stronger response compared to the PBMCs of Pi pigs. This indicated a breed variation in the innate immune-responsiveness to *T. spiralis* and *A. suum* between LR and Pi pigs.

Unspezifische Immunantwort nach Stimulation von mononuklearen Zellen aus peripherem Blut mit Endoparasitenantigenen des Schweines

Helminthen gehören zu den gastrointestinalen Parasiten, die für einen erheblichen Produktivitätsverlust in der Schweineproduktion und bei anderen Nutztieren verantwortlich sind. Trotz intensiver Aufzucht mit routinemäßigen Untersuchungen und Behandlungen sind Helminthen-Infektionen bei Schweinen nicht gänzlich unter Kontrolle. Die Untersuchungen der angeborenen Reaktionen zwischen Wirt und Helminthen sowie ihre genetische Beziehungen sind wichtig, um Zuchtstrategien für bessere Immunkompetenz zu befördern. Diese Dissertation hat zum Ziel, die angeborenen Immunantworten auf zwei Helminthen-Antigene, von *Trichinella spiralis* stammende Tyvelose (TY) und von *Ascaris suum* produziertes Hämoglobin (AsHb) unter Verwendung eines in vitro Modells aus mononuklearen Zellen des peripheren Bluts (PBMCs), zu untersuchen. Zur Erreichung der Ziele wurden PBMCs von Schweinen der Deutschen Landrasse (LR) und Pietrain (Pi) in vitro mit Tyvelose und AsHb mit oder ohne mitogener Kostimulation stimuliert. Nach 24, 48 und 72 h Stimulation wurden die Zellen geerntet. Es wurden die Zellvitalität, die Proliferation und die Phagozytoseaktivität bestimmt, gefolgt von einer Expressionsanalyse von Zytokin mRNAs (IFN-γ, IL-2, IL-6, IL-10 und TGF-β1) durch qRT-PCR. Die Ergebnisse zeigten, dass die tyvelosespezifische Proliferation von PBMCs transient war und zeitlich mit der Dauer und Konzentration der Behandlung variiert. Die höchste Vitalität von mit Tyvelose behandelten PBMCs konnte 24 Stunden nach der Behandlung im Vergleich zu 48 h und 72 h beobachtet werden. Die Zytokinreaktionen wurden durch eine Hochregulation von IL-10 und IL-6 dominiert, aber auch IL-2 zeigte eine Regulationstendenz in der Expression von Zeitpunkt zu Zeitpunkt. Die resultierende Wirtsimmunität ergab daher eine Dominanz von Th1/Th2, wie aus dem erhöhten Niveau von IL-10 und IL-6 hervorgeht. Die Variation des Expressionsniveaus von IL-10, IL-2, TGF-β1, IL-6 und IFN-γ in PBMCs aus LR und Pi Schweinen war signifikant, aber nicht konsistent im Verlauf des Experiments und der Mitogenaktivierung. Die Tyvelose induzierte Zytokinexpressionsdynamik und Kinetik war in PBMCs von LR zu Pi Schweinen unterschiedlich. Die induzierte phänotypische Variation der Zellvitalität durch AsHb zwischen den Rassen wurde nach 72 h Kultivierung und nur mit dem Einsatz einer Kostimulation beobachtet. Eine signifikant höhere Phagozytoseaktivität wurde in Phagozyten der LR Schweine im Vergleich zu den Pi beobachtet. In naiven PBMCs, die mit AsHb behandelt wurden, konnte ein signifikanter Rasseeffekt in der Expression von IL-10, Il-6 und TGF-β1 beobachtet werden, obwohl die Wechselbeziehung zwischen diesen regulatorischen Zytokinen nicht immer synchron war. Bei AsHb-sensibilisierten und kostimulierten PBMCs war die Zytokinexpression verzerrt und nur nach 24 h Kultur konnte ein signifikanter Effekt in beiden Rassen festgestellt werden. Zusammenfassend war die Immunreaktion auf Tyvelose und AsHb dominiert durch eine gemischte Th1/Th2 regulatorische Immunantwort und LR Schweine zeigten eine relativ schnellere und stärkere Immunreaktion im Vergleich zu Pi Schweinen. Dies zeigte eine Rassevariation in der Immunantwort auf *T. spiralis* und *A. suum* zwischen LR ind Pi Schweinen.

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List of abbreviations

Abbreviation	Elaborated form
ACTB	Actin beta
APC	Antigen presenting cell
ANOVA	One-way analysis of variance
A.suum	*Ascaris suum*
AsHb	*Ascaris suum* Haemoglobin
aaMΦ	Alternatively activated macrophages
Bp	Base pair
CCK	Cell counting kit
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLRs	C-type lectin receptors
ConA	Concanavalin A
Ct	Cycle threshold
cRNA	Complementary ribonucleic acid
CTLs	Cytotoxic T cells
CWE	Crude worm extract
CXCR2	C-X-C motif chemokine receptor 2
ddH_2O	Double distilled water
DC	Dendritic cell
LR	German Landrace
DMSO	Dimethyl sulfoxide
DNase	Deoxy ribonuclease
dNTP	Deoxy nucleotide triphosphate
H	Hour
Hpt	Hour post treatment
EDTA	Ethylene diamine tetra acetic acid
ES	Excretory-secretory
F	Forward
Fig.	Figure
Foxp3	Fork head like protein 3

GIN	Gastrointestinal nematode
GLM	Global linear model
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophages colony-stimulating factor
IFN-γ	Interferon gamma
IGF-1	Insulin growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iTregs	Inducible T regulatory cells
L1	Larva1
LPS	Lipopolysaccharide
LSM	Least square mean
LR	German Landrace
MHC	Major histocompatibility complex
ML	Muscle larva
MMP	Matrix metalloproteinase
Mmt	Million metric tons
mRNA	Messenger ribonucleic acid
MΦ	Macrophages
NBL	New borne larva
NK	Natural killer
OD	Optical density
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate Buffer Saline
PBMCs	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
Pi	Pietrain
PPIA	Peptidylpropyl isomerase A
Pt	Post treatment
PRR	Pattern recognition receptor
PRRS	Porcine reproductive and respiratory syndrome
PWM	Pokeweed mitogen

qRT-PCR	Quantitative real time polymerase chain reaction
QTL	Quantitative trait loci
R	Reverse
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute
RT	Room temperature
T-bet	T-box transcription factor
Th	T helper
TLRs	Toll-like receptors
TGF-β1	Transforming growth factor, beta 1
TNF	Tumor necrosis factor
T. spiralis	*Trichinella spiralis*
Tregs	T regulatory cells
TXNIP	Thioredoxin-interacting protein
TY	Tyvelose
WST	Water soluble tetrazolium
ZDS	Zentral verband der Deutschen Schweineproduction

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Chapter 1: General overview

1.1 Introduction

Costly medication and immunization, drug resistance, recurrent infection, and animal welfare concern have brought the swine industry under huge pressure to emphasize on sustainable disease control approaches. This will require for sure a much deeper understanding of the networks of immunoregulatory components that control porcine immune responses and how genetic and epigenetic factors influence the disease resistance phenotypes (Wilkinson et al. 2012). Helminth parasites, especially gastrointestinal nematodes (GINs) occur frequently in domestic pigs in all kinds of production systems and all around the world (Carstensen et al. 2002, Roepstorff et al. 2011). These GIN infections impose a significant health and welfare challenge for livestock producers. Despite the common subclinical course of such infection, pigs infected with one or more of the parasites have reduced growth, productivity and changed body composition. So for the financial perspective alone, helminthes should be controlled, best in a sustainable way. Another good reason to study these helminthes is their zoonotic implication. Some basic research findings have offered new possibilities for future sustainable control measures through exploiting the host genetics that shapes the innate immunity to minimize the severity of infection. Besides, the heredity of host resistance to several helminth infections in livestock including pig is already reported (Nejsum et al. 2009, Bishop 2012). Under these perspectives, studies designed to understand the genetic variation among breeds will facilitate sustainable management of farm level infection.

According to the technical report of FAO, a major portion of worldwide consumable meat, approximately 37% (110 million metric tons, mmt) is supplied by pigs, much higher than beef (67 mmt) and chicken (104 mmt) (McGlone 2013). Intensively reared and commercially produced pigs contributed a lot to this amount. The impact of backyard, free-range or green pig farms are also must to acknowledge as these are the only option for a range of consumers depending on their culture and geographical location. A gross portion of pork production in many areas, especially Asian countries, is still maintained as a small scale farm. For many ethnic groups of people, pigs are not only the chief source of meat, but also considered as part of their belief and culture, monetary asset and dirt cleaner. This kind of farming in tropical and subtropical countries is frequently challenged with high intensity of parasitic infections as one of the major constraints for healthy pork production. This situation is now significantly altered globally by the shift of rural farming to more industrialized, high intensity, indoor farming and with the advancement and chemo-prophylactic use of broad spectrum anthelmintics (Roepstorff and Nansen 1994). Still, several GINs, including *A. suum* have successfully survived the changeover to conventional indoor conditions and are currently thriving in some farms, even in developed countries. Another helminth,

T. spiralis in domestic pigs is still receiving special focus for their high zoonotic potential (Roepstorff and Nansen 1994, Joachim et al. 2001, Roepstorff et al. 2011).

In endemic areas, wide ranges of GINs are usually harbored in pigs, commonly in the form of polyparasitism involving two or more of the parasites. Infections with *A. suum* show the highest prevalence in all kind of production systems. *T. spiralis* is considered as another economically significant nematode of pig production system with special concern to the rural, backyard management. Despite of their significant prevalence worldwide and serious economic impacts in the swine industry, the parasitological infection is mostly overlooked by the farmers and the veterinarians because of the subclinical nature of the infection. Moreover, it is very likely that the situation will remain the same for the foreseeable future. One vital cause is the rising demand of green pork production especially in European Union countries where the animals receive the benefits provided with adequate housing, outdoor and social comfort in farm condition. The mixing and shared housing, bedding and pasture environment although enhancing animal welfare, but also concomitantly welcomes favourable environment for infective parasitic stages to infect, persist and transmit (Roepstorff et al. 2011, Katakam et al. 2016).

Transmission and dispersion of trichinellosis is mainly because of its broad host range and global migration of infected individual or animal. Despite of the fact that highly effective drugs for the treatment of ascariasis have been around for over 40 years now, this problem is still not getting solved. Moreover, the development of allergy, malignancy, autoimmunity in developed countries with very low or no prevalence of helminthes in human population, also give rise to another thought about the effect of total elimination of helminthes. It appears that something more fundamental needs to be changed in the way this parasite is controlled in animal production nowadays. Immunity develops in pigs after exposure to *A. suum* or to *T. spiralis*. A proper vaccination strategy could, in theory, be able to induce protection against infection. But still now, no effective vaccine is in use against these parasites in pigs. As a sustainable production strategy, distinguishing animals based on their genetic immune strength to minimize helminth infections might be a feasible option. To this end, the current dissertation project focused on innate immune transcripts alteration in PBMCs following in vitro exposure of antigenic part of *A. suum* and *T. spiralis*.

1.2 Literature review

Helminth parasites are multicellular eukaryotic invertebrate organism. They are physiologically dependent on another larger species of host for their food and shelter and in return they induce some

sort of harm to the host (Solusby 1982). There are three taxonomic groups of helminth parasites – cestode flat worms, nematode roundworms and trematode flukes. They represent a gross variation in their life histories, from direct faecal-oral transmission (e.g. *Ascaris* worm) to development through free living stages (e.g. Hookworm larvae) or dependence on invertebrate vector (such as blood fluke schistosome) (reviewed by McSorley and Maizel 2012). More precisely, their most common habitat within the host is the gastrointestinal tract for the availability of readily absorbable nutrient molecules, especially for the adult life stages. The GIN is of very special significance for their debilitating effect on nutrition uptake by farm animals including pigs. Adults from both *A. suum* and *T. spiralis* reside in the small intestine, although their larval stages can migrate through different visceral and skeletal tissues and elicit pathologic lesion (Soulsby 1982). To have a better knowledge on *A. suum* and *T. spiralis*, this chapter presents a brief literature review on their biology, immunology, epidemiology, economic importance, control and prevention. In addition, we will focus on host genetic influence on infection, innate immunity and in vitro peripheral blood mononuclear cell model followed by the aim and objectives of this dissertation.

1.2.1 Taxonomy and life cycle of *T. spiralis*

Taxonomically, the genus *Trichinella* belongs to the phylum Nematoda, class Adenophorea, order Trichinellida and superfamily Trichinelloidea (Noble et al. 1989). The life cycle of the genus *Trichinella* mainly involves two generations in the same host and may include a broad range of host species (mammals, birds, and reptiles) (Gottstein et al. 2009). Only humans are clinically affected. Infection in pig is usually asymptomatic and subclinical in nature. Access to infection generally occurs by oral intake of infective larvae encysted muscles as depicted in fig. 1.1. The cyst wall is digested in the stomach, and the liberated larvae penetrate into the duodenal and jejunal mucosa. There they go through 4 stages of moulting and form a sexually mature adult within even 2 days. After mating and usually within 5-7 days, the females penetrate deeper into the mucosa and discharge living small new-borne larvae (NBL) as seen in fig. 1.2A, up to 1,500 in number, throughout 4–16 weeks (wk). Within next few wk , intestinal immune-mediated host responses get established and immune effector mechanisms affect the viability of the female. This ultimately results in a continuous expulsion of adult worms (Pozio 2007). Sometimes, the adult worms die and simply are digested.

The immature larvae (0.1 mm) reach the peripheral circulation after migration through the lymphatics. From the circulation, they invade highly oxygenated striated muscles; where they further penetrate individual muscle cells, change them to nurse cell. Then they grow fast (to 1 mm) and start coil formation within the cell (Soulsby 1982) which is illustrated in fig. 1.2B and C.

Capsule formation begins at 2 wk after infection and it takes 4–8 wk to complete and the larvae become infective (Despommier et al 1991).

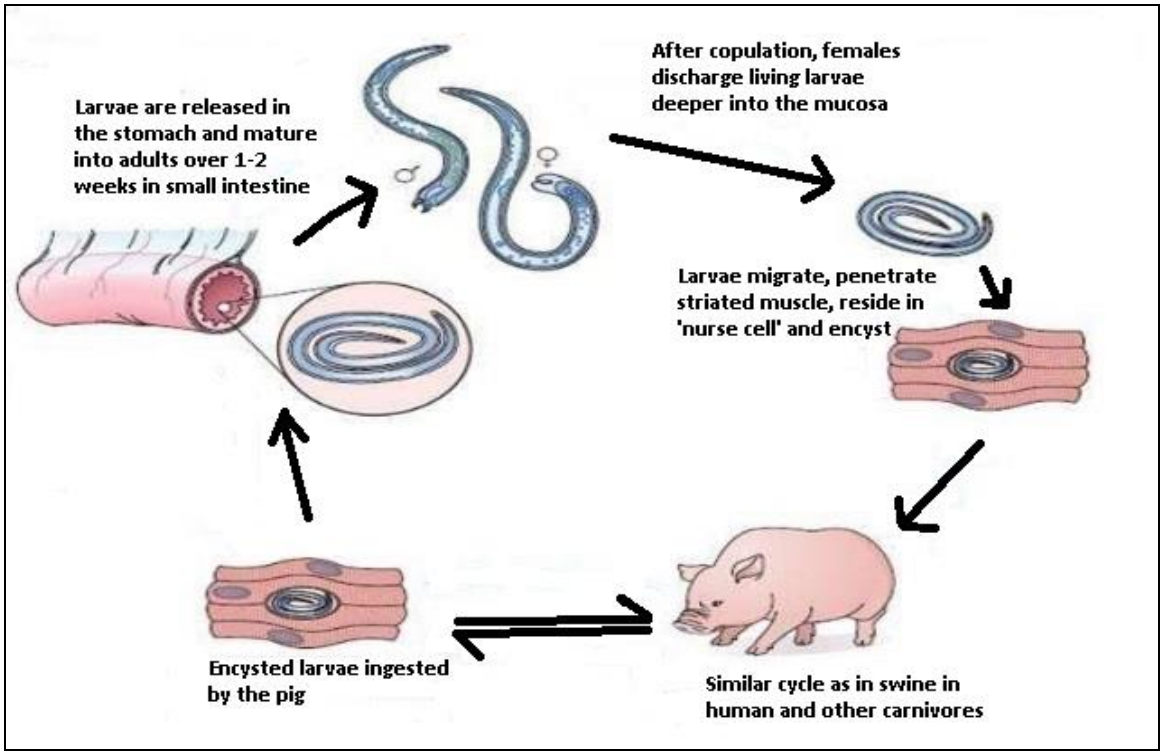

Fig. 1.1 Basic life cycle of *T. spiralis* in pigs. Orally ingested infective muscle larvae are released in the stomach, reaches the intestine where they mature and mate. After mating, females invade the mucosal layers where they release newborne larvae. These larvae migrate to through the peripheralcirculation to different oxygen-avid skeletal muscles and gradually develops to infective larvae and encapsulates the muscle cells (Adapted and modified from Guerrant et al. 2005)

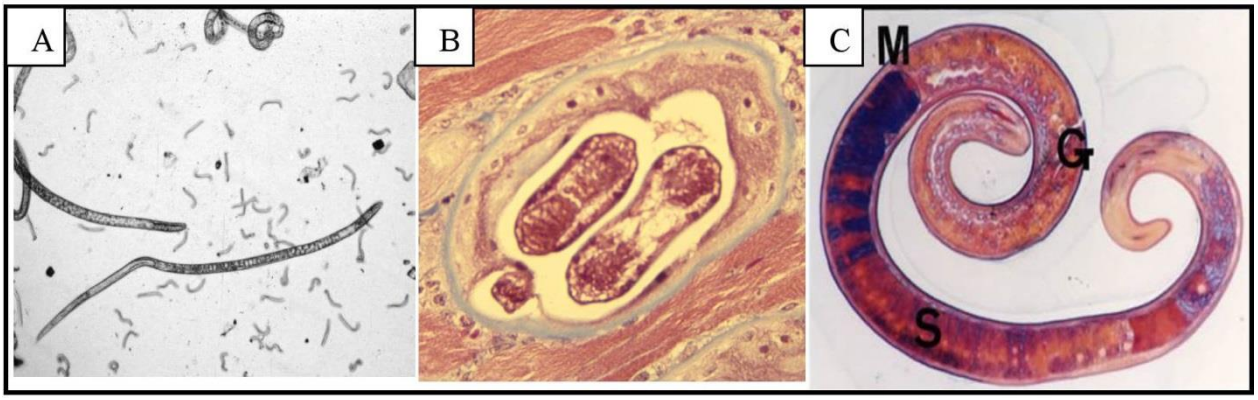

Fig. 1.2 Developmental stages of *T. spiralis*. A. Adult worms that developed in the small intestine following oral infection with muscle larvae; the small worms are newborn larvae (immature L1), which are infective to muscle. B. Infective muscle larva in altered muscle cell surrounded by a collagen capsule (blue). C. Infective muscle larva, Azan staining of longitudinal section of excysted larvae. M: midgut, G: genital primordium, S: stichocyte (photo courtesy Mitreva and Jasmer 2006).

Depending on the host species, host immune response, calcification of the collagen capsule firstly followed by the nurse cell and larva can occur after a period of time (Gottstein et al. 2009). Along

with the growth of internal larva, the cell degenerates and calcification begins (at different rates in various hosts). These encysted larvae may remain infective even for years, and their further development continues only if ingested by next suitable host. The major predilection sites for these larvae are the diaphragm, tongue, masseter, and intercostal muscles in heavily infected pigs (Ribicich et al. 2001, Pozio 2005, Gottstein et al. 2009). If larvae are not encysted and pass through the intestine, then they are eliminated in the feces before maturation and may remain infective for subsequent consumer of faeces or larvae.

1.2.2 Clinical and economic importance of *T. spiralis*

T. spiralis has been recorded as a List B disease by the OIE (World Organization for Animal Health). List B includes transmissible diseases of socio-economic and/or public health importance within countries and that carry significant importance in the international trade of animals and animal products. So, trichinellosis turned to an important issue for the establishment of an export market for pork and pork products. Although trichinellosis is asymptomatic in pigs, most of the trichinella cases in humans arose from consumption of pork from domestically raised pigs. High zoonotic potential because of a broad host range has made tirchinellosis to be considered as an important public health hazard and safety threat to pig production. In spite of a bunch of development in the research and chemoprophylaxis, still trichinellosis is prevailing worldwide (Gajadhar and Gamble 2000, Gottstein et al. 2009). Mandatory veterinary inspection over the slaughter house to secure food safety was instituted in Germany in 1866, especially to prevent trichinellosis in consumable pork (Campbell et al. 2013). In many countries worldwide, individual control of pig carcasses at meat inspection is mandatory but incurs high costs in relation to absence of positive carcasses from pigs reared under controlled housing. EU regulation 2015/1375 implements an alternative risk-based approach, in view of absence of positive findings in pigs under controlled housing conditions (European Commission 2015). Moreover, Codex Alimentarious guidelines for the control of *Trichinella* spp. in meat of suidae have been published (CAC 2015). At present, the expense elicited from meat examination for trichinellosis is accounted 25 million Euro to 400 million (Kapel 2005) for around 167 million pigs tested annually (Alban et al. 2011). Now it is regulated in many other countries including the EU. Another important and indirect loss from trichinellosis is the examination time consumed, before which the whole carcass cannot be processed, especially when the diagnosis is made on peptic digestion method. ELISA based methods are a bit quick but might also interfere with false positive or negative results. Recent testaments from Franssen et al. (2017) concluded that trichinella testing for indoor pigs is not adding any value to protect human health and suggested farm-to-fork quanitative microbial risk

assessment (QMRA). This QMRA model prescribed quantification of *Trichinella* muscle larvae distribution in edible muscle types, heat inactivation by cooking, partitioning of edible pork parts.

1.2.3 Antigenic molecules of *T. spiralis*

Three major kinds of trichinella antigens can be obtained on the basis of antigen location – surface antigens, excretory-secretory antigens (ES) and residual somatic antigens (Dea-Ayuela and Bolas-Fernandez 1999). Stichosome, body cuticle, hindgut cuticle, hypodermis, haemolymph, glycogen aggregates, oesophagus occupying substance, midgut occupying substance, brush-border, cytoplasmic granules in the cord, intestinal gland and some areas in genital primordial cells appeared as the major source for muscle larvae antigens (Takahashi et al. 1990). Cuticular antigens are found as relatively non-immunogenic (Maizels et al. 1982). Different larval stages produce different antigens (Table 1.1). It is suggested that the parasites prevent the host defense mediated killing by altering their antigens at different life stages (Arriaga et al. 1989).

Table 1.1 Immunomodulatory molecules from *Trichinella spiralis*

Antigenic products	Molecular weight, source/location, bioactivity	References
Macrophage migration inhibitory factor (MIF)-like molecule	12 kDa, produced by encysted larva, potent to regulate host macrophage response	Wu et al. 2003
Muscle larvae excretory secretory products	Suppress LPS activated dendritic cells (DCs) maturation in vitro, enhance induction of Treg cell expansion in mice.	Aranzamendi et al. 2012
TsAP	54.7 kDa, aminopeptidase, produced by larvae, protective efficacy against adult worm 8.1% and against muscle larvae 59%	Zhan et al. 2010
TspSP-1.2	35.5 kDa, serine protease, involved in larval invasion, found in adult ES, protection efficacy against adults 34.92% and against muscle larvae 52.24%	Wang et al. 2013
Ts-Adsp	47 kDa, serine protease, produced by adult worm, protection efficiency 46.5% against muscle larvae, able to induce antigen specific IgG, IgE antibodies and a mixed Th1 and Th2 with elevated level of IFN-γ , IL-2, IL-4, IL-10 and IL-13	Feng et al. 2013
pVAX1-Tsmif	59 kDa, macrophage inhibitory factor, found in all stages, protection efficiency 23.17%	Tang et al. 2013
Tsmcd-1	68 kDa, cysteine protease inhibitor, found in all stages, protection efficiency 37.95%	Tang et al. 2013
Ts-Pmy	102 kDa, binds to C8, C9 (complement), produced by adults and larvae, protection efficieny 21.8% against muscle larva	Wei et al. 2011
Ts87	87 kDa, surface antigen, found in adult, protection efficiency 29.8% against adult worm and 34.2% against muscle larvae	Nagano et al. 2008
Ts53	53 kDa, secreted glycoprotein, produced by adults and larvae	Yang et al. 2010
Ts-gp43, peptide 40-80	43 kDa, immunodominantglycoprotein, found in larvae as ES, protection efficiency 64.3% against adult worms	Ravasi et al. 2012
Ts-Hsp70	70 kDa, heat-shock protein, found in adults, protection efficiency 38.4% against muscle larvae, induce Th1/Th2 mixed cytokine profile	Fang et al. 2014
Ts adult ES	Various, ES products, produced by adults	Nagano et al. 2009

Tyvelose

Helminths can produce complex carbohydrates (glycans) that may have key functions in interactions with the host immune system. *T. spiralis* can produce an unusual glycan in conjunction with its multiple excretory-secretory (ES) proteins of muscle larvae. The glycan has a tri- and tetra-antenary structures with a terminal beta tyvelose (TY), which is a dideoxy arabinohexose and this is considered as very specific for *T. spiralis* N-glycans still now (Wisnewski et al. 1993, Reason et al. 1994). A symbolic tyvelose bearing glycan is depicted in fig. 1.3.

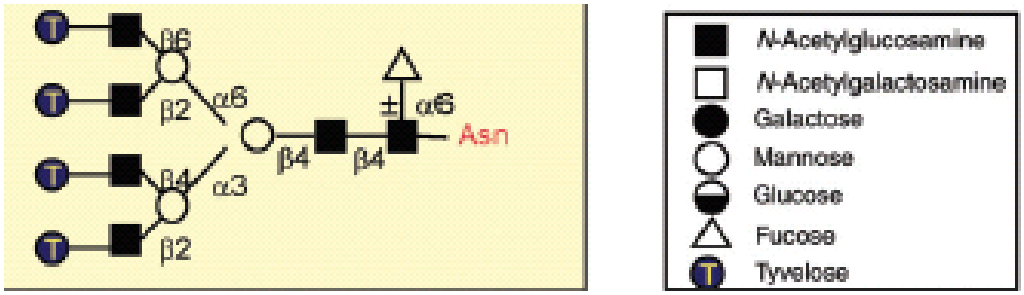

Fig. 1.3 Tyvelose bearing glycans from *T. spiralis* (Maizels et al. 2004)

The structure conferred by the tyvelose moiety creates an antibody epitope, which occurs on multiple ES proteins of *T. spiralis* muscle larvae. Two larval development stages – new borne larvae (NBL) and muscle larvae (ML) of *T. spiralis* induce the host immune response in two phases: the initial 1-2 wk infection, i.e. at the end of the intestinal phase by NBL and the next at 4-5 wk after infection by ML antigens. Most of the antigens of NBLs are of 20, 30, 58 and 64 kDa molecular weight (MW). On the other hand, main antigens of ML are of 47, 55, 90 and 105 kDa (Appleton et al. 2012). The ML group of antigens is termed as group II or TSL-1 antigens (Appleton and Romaris 2001). TSL-1 antigens usually possess a MW of 40-50 kDa in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Appleton et al. 1991). TSL-1 antigens share this carbohydrate epitope, dideoxysugar known as tyvelose (Appleton and Romaris 2001). TY has been used in a commercial *Trichinella* Western blot kit as a diagnostic antigen (Yera et al. 2003). Passive immunization with anti-tyvelose mAbs in neonatal rats generated very fast response and expulsion of *Trichinella* larvae in a challenge infection (Appleton et al. 1988). Inspite of the strong antibody response against TY, it had neither host nor parasite protective function in mice at the level of the intestinal infection (Goyal et al. 2002). Herein this thesis used the TY as representative antigen to evaluate host innate immune responses elicited by cytokine expression.

1.2.4 Host immune response to *T. spiralis*

Antigens of *T. spiralis* can sensitize many different kinds of hosts which indicated that this genus is either less immunogenic or is less influenced by the host's immunity (Wakelin 1996). Bell (1998) has reviewed trichinella's immunity to three basic host responses whose consequences are rejection of infectious larvae, rejection of adult worms, and immunity against NBL. Moreover, killing of encapsulated muscle larvae in suidae is possible (Bell 1998). On the other hand, immunity in mice is mainly targeted at the expulsion of the intestinal stages (Goyal et al. 2002) and is basically T cell mediated (Wakelin and Goyal 1996) and with dominant Th2 type (Khan and Collins 2004). Moreover, the host immune response to *Trichinella* sp. involves numerous parameters linked both to the host species and to genetic background within the species (MHC and non- MHC genes) (reviewed in Bell 1998). These complex response mechanisms make it difficult to extrapolate the results obtained in one animal species to another. From these perspectives, it was suggested that the evaluation of host-trichinella relationship focusing on one host species and one trichinella species at a time since the genetically different hosts cannot be appropriately comparable (Wakelin and Goyal 1996). But the inadequacy of pig trichinellosis research in the context of immune interaction compelled to draw relevant conclusion from other models like mice, rat, raccoon etc. Most of the immunological studies and host-parasite interactions between *Trichinella* sp. and its host have been obtained from laboratory animal models, especially rodents (rats and mice), but reports in pigs are very limited so far.

Until now, most of the studies on the swine immune response to *Trichinella* sp. has only been analyzed at the systemic level as response of specific antibodies production (Marti and Murrell 1986, Lunney and Murrell 1988, Kapel 2005). Although the severity of infection and degree of of adaptive response relied on the quality and quantity of infective muscle larvae (ML), near the complete resistance to secondary trichinellosis was noticed even with inoculation of a hundred MLs (Marti and Murrell 1986). Most of the cases, anti-*T. spiralis* response in vaccinated pigs was generated from the anti-fecundity response which affected the NBLs number even at day 7 post-infection (dpi). This response was delayed around 3 wk in naive pigs (Marti and Murrell 1986). This anti-fecundity response was found to be associated with intestinal inflammation in rodents (Despommier 1998). Furthermore, antibody dependent cell cytotoxicity targeted towards NBLs was also observed with the passive transfer of immunized pig serum, which yielded reduced the parasite burden without impairing worm fecundity (Marti and Murrell 1986). Overall, these studies indicated that swine are slow in expulsion of *Trichinella* burden, they can induce strong adaptive or protective immunity and this would be beneficial from the immunization perspective.

1.2.5 Epidemiology and control of *T. spiralis* infection

Trichinella as in the form of infection in domestic and wild animals has been reported in 66 countries and human trichinosis has been recorded for 55 countries (Pozio et al. 2009). These emphasize that the top source of human trichinosis is the domestic pigs and wild boars. Therefore, Trichinella infection represents a serious problem for the international trade of pigs and pork products as well. Distibution of *Trichinella* spp. can be cosmopolitan except Antarctica (Pozio 2007). Murrell and Pozio (2011) reviewed the spatio-temporal distribution studies and have listed out some latest information on the relative contribution of domestic pigs in trichinellosis as shown in table 1.2.

Table 1.2 Trichinellosis outbreaks in human in the world during 1986-2009 based on WHO report (Murrell and Pozio 2011)

Region/country	Meat source, % of cases or outbreaks		
	Domestic pig	Wild game	Other
European Region Belarus, Croatia, Georgia, Macedonia, Serbia, United Kingdom	 100	0	0
Estonia, Turkey, Ukraine	50	50	0
France	0	65	35 (horse)
Germany	83	17	0
Greece, Israel	0	100	0
Hungary	52	48	0
Italy	38	38	24(horse)
Lithuania	48	52	0
Poland	41	59	0
Romania	95	5	0
Slovakia	50	25	25 (Dog)
Spain	60	40	0
African Region: Ethiopia	0	100	0
Region of the Americas Argentina, Chile Canada United States Mexico	 100 0 57 86	 0 100 43 0	 0 0 0 14 (horses)
Eastern Mediterranean Region: Iran and Lebanon	0	100	0
South-East Asian Region: Thailand	50	50	0
Western Pacific Region			
People's Republic of China	86	13	1(dog)
Japan	25	75	0
South Korea	0	100	0
India, Laos, Papua New Guinea	50	50	0
Singapore, Vietnam	100	0	0

(Source:wwwnc.cdc.gov/EID/pdf<<711-0896-Techapp.pdf)

The outdoor rearing of pigs is a major risk because of increased exposure to sylvatic and synanthropic hosts. Synanthropic hosts like rats, cats, raccoons and wild animals may achieve the infection from domestic farm pigs (Burke et al. 2008). Inadequate measures to prevent contact of

domestic animals with wild life allows the transmission from wild animals to domestic animals. Mother to offspring transmission may also occur (Webster and Kapel 2005). More than 150 species including mammals, birds and reptiles was reported to serve as potential host for trichinella infection (Bolas-Fernandez and Wakelin 1989, Pozio and Zarlenga 2005). Prevalence of trichinellosis is more noticeable, especially, in countries with traditional consumption behavior which incorporates meals with raw, smoked or undercooked pork (Gottstein et al. 2009). Data on the incidence of human trichinellosis and its health effect are relatively less reported and also does not include appropriated validation. Appropriate information availability could facilitate the necessary priority set up for control. On estimated report from Pozio (2007) mentioned that in the 1990s, the global prevalence of trichinellosis was near about 10 million, and an incidence estimate was suggested as approximately 10,000 infections per year. This indicates the world wide dissemination of trichinellosis in animal and human health.

Usually, pigs get infected with trichinella by eating infected meat of wild or domestic animals. Practicing good management, combined with documentation of these practices and routine official veterinary control to verify the efficacy of these practice might facilate the provision to escape slaughter inspection or further processing which is much costly. The key practices can be creating architectural and environmental barriers between domestic and wild life, proper management of feed and food storage, rodent control and farm hygiene including safe disposal of dead carcass and piglets from farms with controlled housing conditons (Gottstein et al. 2009) Therefore, prevention of infection will depend on the prevention of cannibalism, by avoiding any animal tissues being fed to pigs or human without adequate boiling (71°C for at least 1 minute) (Gottstein et al. 2009). Determining the genetic predisposition for trichinellosis will also facilitate the strategy for sustainable control measures.

1.2.6 Taxonomy and life cycle of *Ascaris suum*

Ascaris suum is a nematode parasite of the Ascarididae family (Dold and Holland 2011). The adult *Ascaris suum* males are as long as 25 cm and females up to 40 cm with whitish coloration and thick cuticle (Soulsby 1982). Gravid females can produce around 200,000 to 1 million eggs/day and usually shed intermittently (Sinniah 1982). Under favourable condition, these eggs develop to the infective stage (eggs containing L_3 larva) in 3–4 wk as illustrated in fig. 1.4. Infective eggs, after being ingested, hatch in the small intestine and the larva is released. These larvae invade the intestinal wall and enter into the portal circulation. After capillary migration through the liver capsule, they are transported by the circulation to the lungs, where they lodge into the

bronchoalveolar spaces (Dold and Holland 2011). During this migration through liver, they cause haemorrhage, fibrosis and accumulation of lymphocytes seen as 'white spots' and termed as 'milk spots' under the capsule (Fig. 1.4). These lesions can be visible even within 4 days (Roepstorff et al. 2011) after infection and then gradually regress within 3 to 6 wk (Eriksen et al. 1992). Therefore, their presence indicates ascaris infection or reinfection. Approximately 6-8 days after ingestion, the larvae pass up the bronchial tree. If the pulmonary infection level with Ascaris larvae is very high, it may cause pulmonary edema and consolidation. In the lungs, the larvae are swallowed, and return to the small intestine by 8-10 days after infection, where they mature into adult worms and later they are expelled out (Roepstorff et al. 1997). The first eggs are passed ~6–7 wk after infection. Intestinal niche for adult parasites usually last for one year, but in most cases, due to the intestinal effector immunity drive out the worm load at around 23 wk of infection (reviewed by Dold and Holland 2011).

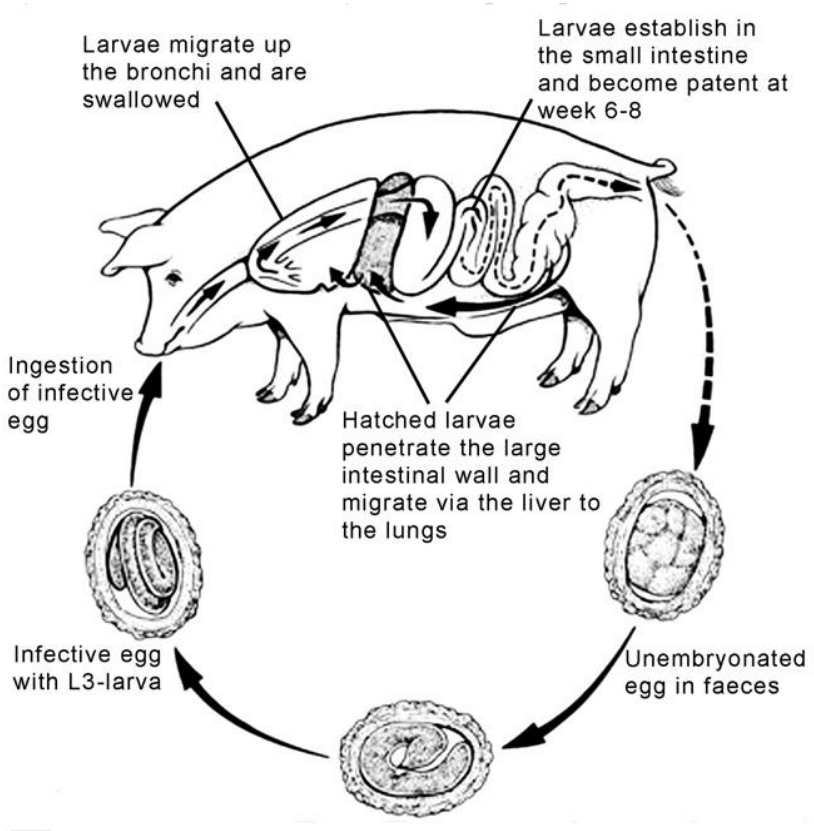

Fig. 1.4 The stages of life cycle of *A. suum* in pigs. Infective L3 containing eggs are ingested by the host and will release the L3 in the small intestine. These larvae further invade the caecum or colon and reach the liver via the hepato-portal circulation, usually within 1 to 4 days. They further migrate to the lungs with 7 days post infection and enter the alveoli where they are coughed up and swallowed back into the small intestine. There the molting of L3 to L4, takes place and eventually become adult. After sexual reproduction, the females will release eggs in the environment with the faeces. In the environment, the eggs will embryonate over the course of a few weeks and become infective. Photo adapted from Roepstorff and Nansen (1998).

Earthworms and dung beetles can serve as paratenic hosts (Soulsby et al. 1982). In temperate countries, the eggs remain dormant in winter (<15°C) and restart the growth when temperature rises

in the spring. The eggs are highly resistant to chemical dessicants, but conditions with low humidity, heat, or direct sunlight reduce the resistance. Under natural conditions, they can remain infective for 15 years in the environment (Kransnonos 1978).

1.2.7 Clinical and economic importance of *A. suum* infection

A. suum infection by adult worm at intestinal level as well as their larval migratory stages traveling to the visceral organs like liver, lungs is found to elicit potentialy unfavorable impact on the financial benefit scale of pig farms (Kipper et al. 2011). Infection in the farm in a greater scale may directly affect growth, immunity, libido or indirectly may cause significant loss from carcass condemnation, secondary infection and so on. The most common and evident effect from ascariasis is rejection of affected parts of liver identified with lesion of whitish spots, the indication of larval migration. In fig. 1.5 *Ascaris suum* infected livers from two pigs are shown.

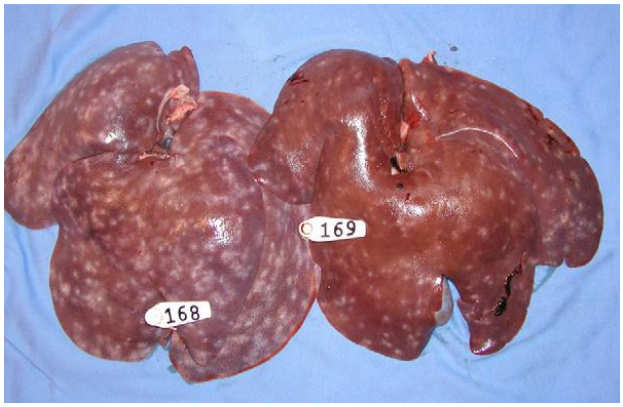

Fig. 1.5 Milk spot liver of *A. suum* infected pigs. White spots on livers from two helminth naïve sentinel pigs following 4 days exposure to a paddock, which was contaminated with *Ascaris suum* eggs 4 years earlier (photo by H. Mejer) (Roepstorff et al. 2011)

Furthermore, the larval passage through the lungs may yield in pneumonia and interrupt the physiological clearance of bacteria from lungs (Curtis et al. 1987) which further promotes secondary infection by other microbial pathogens. Ascariasis is also reported to be linked with the higher prevalence of *Pasteurella multocida*, *Escherichia coli* and Salmonella spp. (Adedeji et al. 1989, Tjørnehøj et al. 1992, Smith et al. 2011). Costs concerned with the anthelmintic treatment as well as the perspective of drug residual effect and drug resistance are also significant threat for helminth control in swine industry. Farms with low level of infection intensity might not reflect the same scenario and the cost of active ascaris control might not be profitable. Moreover, it is quite hard to estimate the economic costs from parasites which are not fatal. Current assessment on the ascaris prevailing pig farms reelected that better feed conversion rate, elevated daily weight gain, declined fatality and improved carcass quality coact together (Vlaminck et al. 2011, Vlaminck et al.

2012, Vlaminck et al. 2015, Vlaminck et al. 2016). Therefore, routine deworming plans may result a win-win effect on economic and environmental performance with gross profit margin increase of 3 to12 Euro per average finisher annually (Kanora 2009, Jourquin 2007, Meensel et al. 2010).

1.2.8 Antigenic molecules of *A. suum*

Most of the immunological studies on ascaris immunity used whole worms, eggs or crude somatic antigen preparations or excretion/secretion (E/S) products derived from larvae or adult parasites cultivated in vitro (table 1.3). These antigens generally lack specificity (Lynch et al. 1993, Chatterjee et al. 1996, Bhattacharyya et al. 2001, Araujo et al. 2015). The AsHb antigen is produced by both the adult and larvae (Vlaminck et al. 2011, Chehayeb et al. 2014). A subsequent study showed that antibody reactivity to AsHb correlated with liver pathology caused by migrating *A. suum* larvae, and high antibody rates in pig herds were associated with low growth rates (reduced farm productivity) (Vlaminck et al. 2015). AsHb has high immunogenicity and is used for serodiagnostic detection of ascariasis in swine herd. This is commercially available in Europe as SERASCA® (Vlaminck et al. 2012). It is found as an about 42 KDa glycoprotein comprising two globin like domains, highly immunogenic vaccine candidate and is the most abundant proteins in the pseudocoelomic fluid of adult *A. suum* worms, a major excretory/secretory (ES) protein of L3, L4 and adult worms (Keane-Myers et al. 2008). Haemoglobin sequestering of oxygen has been suggested to aid the parasite in maintaining a locally anaerobic environment while their ability to break down nitric oxide (NO) and hydrogen peroxide produced by innate immune cells would also aid parasite survival (Goldberg 1995, Minning et al. 1999). *Ascaris* haemoglobin (AsHb) has been best explored, and is an octameric molecule that binds oxygen nearly 25,000 times more tightly than human haemoglobin (Minning et al. 1999).

According to the hypothesis of Keane-Myers et al (2008), AsHb can activate bone marrow derived dendritic cells and alter cytokine production (e.g., increase the production of IL-10, decrease the production of IL-12, etc.) in a pattern in accordance with the restricting Th1- and Th-2-mediated conditions characterized by upregulation of IL-2, IL-12, IFN-γ and TNF-β1. IL-10 can also be upregulated in Th-1 responses as a negative feedback mechanism to prevent further damage from a Th1-associated cytokine storm. A Th-2-condition is characterized by an upregulation of IL-10, IL-4, IL-5 and IL-13 and IgE component. By upregulating IL-10, AsHb suppresses Th-1- or Th-2-associated conditions by reducing cellular upregulation of IL-10 by a feedback mechanism. By contrast, it is suggested that by reducing the level of IL-2, AsHb inhibits-associated conditions in which IL-12 is upregulated. It can be used to treat allergic ashma, food allergy, an eosinophil-

associated gastrointestinal disorder, eczema, chronic urticarial, inflammatory bowel disease, multiple sclerosis, Crohn's disease, ulcerative colitis.

AsHb is the most abundant and highly produced antigen of all three vital life stages of *A. suum* (L3, L4 and adults) (Keane-Myers et al. 2008). Moreover, anti-AsHb antibody was found to be highly reactive to the parasites hepatic migratory stages (Vlaminck et al. 2011), which is the most crucial and economically important part in hog ascariasis. Moreover, significant positive correlations between serum antibody level of anti-AsHb-IgG and quantity of intestinal worm and egg per gram of faeces (EPG) (Vlaminck et al. 2012). Hence further investigating the role of AsHb and elucidating the breed variation based on anti-AsHb response might be worthwhile.

Table 1.3 Immunomodulatory molecules from *Ascaris suum*

Worm product	Bioactivity	Reference
Ascaris Haemoglobin (AsHb)	Heamoglobin suppresses allergic inflammation in mice LPS activated DCs by inducing IL-10	Keane-Myers et al. 2008
Pseudocoelomic fluid protein (s)	Induces eosinopjiil and nerutrophil chemotaxis, reduces bronchoalveolar lavage fluid cell infiltration, IL-5 and IL-13 as well as expression of CD40 and CD86 and is therapeutic in mice	McConchie et al. 2006
Somatic extract from homogenized adults 200 kDa PAS-1 from adult somatic extract	Inhibits zymogen induced murine arthritis; reduces hypersensitivity to ovalbumin independently of IL-10, and reduces cell proliferation, IL-2, IFN-γ, eosinophil peroxidase, IL-4, IL-5 and eotaxin levels;	Rocha et al. 2008, Souza et al. 2002, Itami et al. 2005
Phosphorylcholine-conjugated glycosphingolipids from adults	Inhibits airway allergy induced by the worm allergen via inhibition of macrophage cytokine release; increases TNF-α, IL-1beta and IL-6 from human PBMCs; decreases B cell proliferation; is pro-apoptotic: decreases Th1 cytokine production from LPS+IFN-γ treated macrophages	Deehan et al. 1997, Kean et al. 2006, Araujo et al. 2008
As14	14kDa, with unknown function, found in larva, adult and ES, confers 64% of protective immunity, homolog to human ascaris	Tsuji et al. 2001
As16	24 kDa, helps larval molting, found in larva, adult, ES, intestine and hypodermis, r-protein and confers 58% host protection, homolog of human ascaris	Tsuji et al. 2003
As24	24 kDa, helps larval molting, found in larvae and adults as ES in intestine and hypodermis, r-protein and confers 58% host protection, homolog of human ascaris	Islam et al. 2005a, Islam et al. 2005b
As37	37 kDa, function in Ig family, location in larva, adult, surface, muscle, r-protein and confers 69% protection, homolog to human ascaris	Kasuga-Aoki et al. 2000
As-Enol-1	46 kDa, Enolase, larval development, located in larvae, adults and ES, confers 61% protection	Chen et al. 2011
As-GST-1	26 kDa, Glutathione S-transferase, found in adult and intestine, implicated in the development of drug and toxin resistance	Liebau et al. 1997

1.2.9 Host immune response to *A. suum*

From an immunological point of view, it has been reported that *A. suum* induces a strong Th2 response, typical of gastrointestinal parasites in pig, which can be measured both systematically

(e.g. blood eosinophilia, IL-4) and locally in intestine (increase in IL-4, IL-6, IL-10 and IL-13) (Jungersen 2002, Roepstorff et al. 2011). Human ascariasis is characterized by a Th2 and regulatory immune response (Cooper et al. 2000), although innate production of IL-5, IL-6 and TNF-α was suggested to have potential role in the pathogenesis of experimental larval ascariasis in mice (Gazzinelli-Guimaraes et al. 2013).

Immunity in the normal physiological system is often accompanied by the combined efforts of cell to cell interaction in order to maintain immune equilibrium. Several studies on ascaris immunity revealed that *A. suum* have affinity for lymphocytes as evident from the suppressive activity of its antigenic components (Faquim-Mauro and Macedo 1998, Souza et al. 2002, McConchie et al. 2006). Inspite of the direct evaluation on *Ascaris* infection, recent studies have suggested that an IL-6 dependent, Th17 response might play an important role into the pathogenesis of helminth infections (Smith and Maizels 2014) and allergic response (Roesner et al. 2015), resulting in manipulation of the Th2 profile and possible vulnerability of the host to the parasitic infection. Role of IL-17 in another helminth, *Schistosoma mansoni* mediated granuloma formation was also referred (Bouchery et al. 2014).

Larval ascariasis (established by larval migration through the host's organs) was characterized by intense pulmonary injury and inflammatory infiltration, which is initially comprised of neutrophils during the peak of larval migration and followed by later infiltration of eosinophils and mononuclear cells (Gazzinelli-Guimaraes et al. 2013). The robust inflammatory response elicited by parasitic migration was suggestive to be protective to the host (Gazzinelli-Guimaraes et al. 2013) and might represent the establishment of concomitant immunity to new helminthic infections.

1.2.10 Epidemiology and control of *A. suum* infection

A. suum is known to be distributed worldwide in all kinds of production system, but the intensity may vary with the production system. Reliable and up-to date information on the prevalence is scarce and presently available studies are also based on different diagnostic methods. An overview of prevalence of *A. suum* infection in pigs across the globe is presented in table 1.4. Studies from China and Denmark reported higher prevalence in breeding sows and fattening pigs (Roepstorff and Murrell 1997, Lai et al. 2011) and relatively lower in breeding boars (Roepstorff et al. 1998, Lai et al. 2011). There is a general trend that the younger animals, especially starter and finisher have comparatively higher susceptibility because of their inadequate level of pre-exposure to infection. This situation is more common in traditional and green pig production farms (Roepstorff et al. 1998). On the contrary, in intensive production facilities, frequency of positive cases is lower in

younger pigs resulting from restricted chance of getting infection while the prevalence percentage are relatively higher in adults or older pigs, especially sows, as these animals receive the first exposure to ascaris infection in later parts of their life (Roepstorff et al. 1994, Roepstorff et al. 1997). Egg excretion level was found comparatively lower in sows than weaners or fatteners (Roepstorff and Nansen 1994, Eriksen et al. 1992).

Table 1.4 Statistical data from prevalence studies on *Ascaris suum* throughout the world from online available sources

Year	Country	Sample size and type	% of infected farms	% of infected pigs			Reference
				Egg and Worm	Liver lesion	ELISA	
2000	China	100 outdoor farms		37%			Boes et al. 2000
2001	Germany	13 farms		33%			Joachim et al. 2001
2005	The Netherlands	9 conventional farms	11%				Eijck and Borgsteede 2005
2005	The Netherlands	16 free range farms	50%				
2005	The Netherlands	11 organic farms	73%				
2010	USA	91 farms (finishing pigs)	39%				Pittman et al. 2010a
2010	USA	40 farms (Sows)	25%				Pittman et al. 2010b
2010	Switzerland	90 conventional farms	13%				Eichhorn et al. 2010
2010	Switzerland	20 free range farms	35%				
2010	Sweden	2.4 million pigs			5%		
2010	Denmark	79 farms, 1790 sows	76%	30%			Haugegaard 2010
2011	China	916 pigs		15%			Lai et al. 2011
2011	Tanzania	13,310 pigs			4%		Mellau et al. 2011
2012	England	34,168 pigs			4%		Sanchez-Vazquez et al. 2012
2012	Slovakia	19017 pigs			6.89-39.5%		Ondrejková et al. 2012
2015	Germany	20 pig fattening farm	79%		10.78%	79%	Vlaminck et al. 2015
2015	Belgium	20 pig fattening farm	61%		13.2%	61%	Vlaminck et al. 2015
2016	Denmark	5 organic farms (starter, finisher, dry sows, lactating sows)			48%, 63%, 28%, 15%		Katakam et al. 2016
2017	Uganda	932 pigs		5.9%			Roesel et al. 2017

Routine deworming, chemical disinfection, all-in all-out production, proper stocking density, adequate nutrition are some feasible and traditional measures for ascariasis control (Roepstorff and Nansen 1994) to reduce the transmission. But these are unlikely to completely eliminate the infection because of high reproductive potential of ascaris worms, their long viable infective stage, drug resistance, lack of vaccine, recurrence of infection and also because the immunity developed

from the primary infection does not offer life-long protection. Genetic predisposition is reported for some animals being more susceptible. Therefore, selection of relatively less susceptible populations will offer a sustainable option for the farmers.

1.2.11 Immunomodulation by helminth infection

Helminth parasites usually have a strong immunomodulatory and immunosuppressive effect on their host. In most parasitic diseases, a predominantly cellular (Th1) or humoral (Th2) immune response offers the best control over pathogens, the induction of an appropriate T-helper cell response is essential in determining a successful immune reaction (Riganò et al. 1999). Immunosuppression, alteration and diversion are some common features of helminths regulated host immunity. This results in an anti-inflammatory environment, which is usually suitable for parasite survival and persistence. Anti-helminth immunity is basically portrayed by Th2/Treg cells. The dominance of Th2 polarization and simulataneously the activation of immunoregulatory cell populations, such as regulatory T cells and alternatively activated macrophages, are also possible aspects of helminth immunity as depicted in fig 1.6.

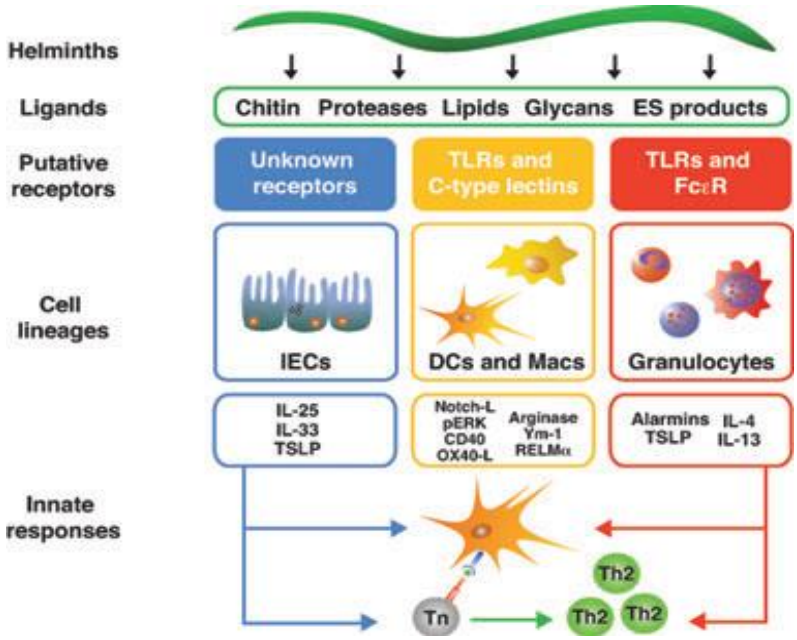

Fig. 1.6 Innate recognition of helminth antigens. The figure illustrated the orchestration of CD4+ Th2 cell differentiation following innate immune cell recognition and response to helminth derived products. Recognition of helminth antigens by innate immune cells can be mediated by germline encoded pattern recognition receptors such as TLRs and Lectins. However, with many cell types, including IECs, the exact nature of the host-parasite interaction remains unknown. Following innate lymphoid cell recognition, responses can include secretion of effector molecules, such as cytokines, TSLP and alarmins that are thought to contribute to $CD4^+$ Th2 differentiation through influencing APCs function and/or directly acting on $CD4^+$ T cells. Additionally, DCs conditioned with some helminth products can promote $CD4^+$ Th2 differentiation. However, whether this activity is determined by changes in expression of co-stimulatory molecules including OX40L and CD40, differential expression of Notch ligands or an as yet unidentified factor are areas of ongoing research (Perrigoue et al. 2008).

The innate wing of the immune system functions as the physical barrier (Moore et al. 2001) as well it plays crucial role in pathogen detection (Kawai and Akira 2009) being the frontline host defense (Medzhitov and Janeway 2000). Pathogen-associated molecular patterns (PAMPs) are produced by pathogen and are not naturally found on host cells. Their recognition is achieved through a limited number of germ-line encoded receptors; the pattern recognition receptors (PRRs) (Mogensen 2009). PRRs mainly recognize PAMPs produced by pathogens that are not naturally found on host cells or tissues (Medzhitov and Janeway 2000). Among the PRRs, Toll-like Receptors (TLRs) along with C-type lectin receptors (CLRs) play important roles in innate PAMPs recognition. CLRs are transmembrane lectins (sugar-binding proteins) that in contrast to TLRs, recognize carbohydrate glycan structures present on the pathogens (Geijtenbeek et al. 2004). The recognition is performed in order to generate suitable immune response to eliminate the parasite and at the same time to reduce host pathology. Current knowledge on this helminth PAMPs recognition process is limited to identification of the few PRR ligands of helminth origin. There is also a wide range of helminth-derived products with immunological properties to which no recognition mechanism has yet been attributed (Johnston et al. 2009). At the signalling level, many of the helminth-derived molecules have been associated with the activation of a number of pathways. Ligand binding of PRRs such as TLRs activates signal transduction pathways that initiated suitable gene expression (Mogensen 2009). The underlying tricks that trigger and helps tomaintain this immune regulation remain inadequately understood, but are clearly important to under host-pathogen interaction.

Immune sensitization by helminthes or helminth antigens induce the naïve CD4+ T cells to differentiate into different types of effector and regulatory cells. Specific cytokines and transcription factors, produced immediately or existing in the microenvironment, also contribute to differentiation and expansion of these cell populations. These differential activation usually determines whether an immune response will contribute to host protection or pathological inflammation as illustrated in fig. 1.7. Exposure to IL-12 produced by antigen presenting cells (APCs) induces T-box transcription factor (T-bet) expression, driving Th1 cell differentiation. These cells produce IFN-γ and usually IFN-γ dominated Th1 immunity is unable to expel helminth parasites. The circumstances that trigger Th2 cell differentiation and Th2 cytokine polarization are not clearly understood. But recent studies have revealed the implication of IL-2, IL-25, thymic stromal lymphoprotein, and associated transcription factors in Th2 dominance.Th2 cells produce a series of cytokines, IL-4, IL-5, IL-10, IL-13 that contribute to anti-helminth immunity by guiding innate bone-marrow and non-bone marrow derived cells, which in turn can instruct and amplify Th2 cells.

Relatively strong Th2-type immunity can give rise to better resistance or less susceptibility to parasites as seen in intestinal helminth, *Heligmosomoides polygyrus* infection in mice, or control of harmful inflammation mediated by Th1 cells and Th17 cells, as in schistosomiasis in human. However, chronic potent Th2 cell responses can also lead to harmful Th2 inflammatory response including fibrosis, granulomatous lesion etc. Expression of the transcription factor fork head box P3 (FOXP3) accompanied by TGF-β and IL-2, can promote differentiation of regulatory T cells (Treg cells), which can inhibit both Th1 and Th2 immune response. Several factors, including retinoic acid receptor related orphan receptor-γ (RORγ), TGF-β, IL-21 and IL-23, have been shown to promote Th17 cell differentiation, and these effector cells can mediate harmful inflammation. IL-10, the master of immune regulation, is no longer considered as exclusively Th2 type cytokine and can be produced by Th1, Th2, and Tregs (T regulatory cells) and downregulated both Th1- and Th2-type response (Anthony et al. 2007).

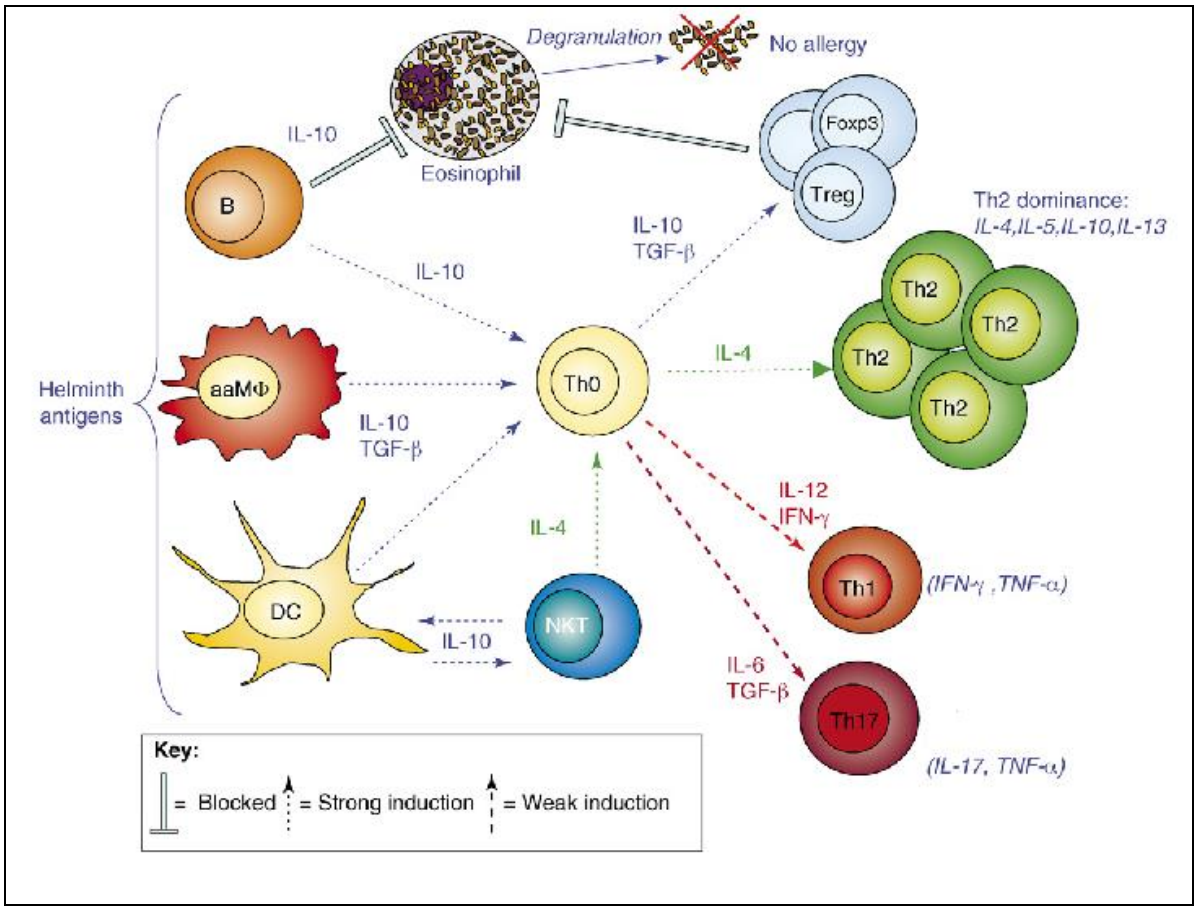

Fig. 1.7 Helminth antigen mediated modulation of host immune response. Helminth antigens modify cells of the innate immune system (DCs, Mϕ, NKT and B1 B cells), arresting the production of inflammatory mediators and instead eliciting the release of immunoregulatory cytokines, such as TGF-β and IL-10. This results in the generation of suppressive Treg cells, expansion of Th2 cells and downmodulation of proinflammatory T-cell lineages. The ensuing T-helper and cytokine biases ameliorate autoimmune conditions and, at the same time, prevent the development of allergies. Abbreviations: aaMϕ, alternatively activated macrophages; DC, dendritic cells; Mϕ, macrophages; NKT, natural killer T; Th, T helper; TNF, tumor necrosis factor; Treg, regulatory T cells. Image adapted from Zaccone et al. (2008).

1.2.12 Molecular phenotypes of anti-helminth innate immunity

The mammalian immune system is complex and involves mainly innate and adaptive arms (table 1.5). The innate immune system acts non-specifically and immediately in response to infectious insults with dependency on cytokines and immune cells. Hence, the innate immunity might not be potential enough to control most of the infectious organisms, but it dictates subsequent development of adaptive immunity which ultimately fights to control the infection.

Table 1.5 Cells and soluble molecules involved in the immune function (Cruvinel et al. 2010)

Component	Innate immunity	Acquired immunity
Cells	Phagocytes (DCs, macrophages, and neutrophils), natural killer (NK), mast cells, eosinophils and basophils	NK/T, B, and T lymphocytes, DCs or antigen-presenting cells (APCs)
Soluble molecules	Complement, acute phase proteins, cytokines and chemokines	Antibodies, cytokines and chemokines

Cell viability and proliferation

A key feature of adaptive immune response is the ability of cloning the antigen specific lymphocytes for rapid proliferation and differentiates into effector cells. Thus, to monitor an immune response, it is important to have procedures that can follow lymphocyte proliferation, with minimal disruption to cell viability and function (Quah et al. 2007). Evaluation of T cell response following primary antigenic challenge with an antigen is a critical aspect of assessment of the cellular immune response. Primary sensitization can be measured by using soluble antigen in vitro. The maturity of macrophages and cytokines, delivered by monocyte-macrophage lineage or lymphocytes was considered as mediator, which might influence the lymphocyte blastogenic response. The lymphoproliferative response can depend on the maturity of APCs and interaction with antigen presenting cells, which are present in cell cultures. Moreover, the balance between cytokines derived from adherent leukocytes including IL-8 as well as IFN-γ and TGF-β delivered by lymphocytes was probably important for the successful proliferation of lymphocytes (Miszczyk et al. 2014). The T cell proliferation and cytokine production by peripheral blood lymphocytes were revealed as indicator of the inflammatory responses to *H. pylori* infection in the gastric mucosa (Fan et al. 1998, Miszczyk et al. 2014). Favoretto et al (2017) reported that the high molecular weight protein of *A. suum* undermined the LPS stimulated DCs and reduced T cell proliferation.

Phagocytosis

Phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens (Gu et al. 2014). This early event mediated by innate immune system is crucial for host survival. As a result of this process, PAMPs can be presented at the cell surface (Ag presentation), allowing the induction of acquired immunity. Traditionally, phagocytosis is assayed by measuring the engulfment of a substrate. The average number of substrate engulfed by each phagocyte is quantified using a standard fluorescent microplate reader. Phagocytosis is indispensible part of several physiological processes, including tissue remodeling and the continuous clearance of dying cells. The measurement of phagocytosis activity of immune cells indicates the strength of the innate immune system in the host. A reduced phagocytosis translates into reduced T cell proliferation (Syme et al. 2000) and antigen processing and presentation by macrophages, all resulting in an interference with the T cell response.

Cytokine signaling

Cytokines represent one of the most important elements in the communication among different cell types (O'Shea and Murray 2008). Their role in the communication among hematopoietic cells and in particular in the reciprocal regulation of effector cell types of innate or natural resistance (phagocytic cells and Natural Killer (NK) cells) and those of adaptive immunity (T and B lymphocytes) are increasingly better understood. Still the multifunctionality of several cytokines makes it difficult to clearly define the role in so many instances. Lymphocytes produce several cytokines with either stimulatory (e.g., colony stimulatory factor) or suppressive (e.g., tumor necrosis factors and interferon) effects on proliferation of early hematopoietic cells. Many of these cytokines, alone or acting in synergistic combinations, also have a differentiation-inducing ability on immature myeloid cells and act as powerful effectors of the cellular functions of terminally differentiated phagocytic cells. Cytokines can generate immune and inflammatory responses and are normally present in the cell at a very low concentration. After appropriate stimulation of cells, they can be expressed or produced in higher quanity. Two types of T helper cells, Th1 and Th2, had originally been defined in murine $CD4^+$ T lymphocyte clones based on their cytokine production profiles (Romagnani 1991). T helper 1 type cells can produce IL-2, IFN-γ, lymphotoxin (LT), TNF-α and IL-12. T helper 2 type cells are source of IL-4, IL-5, IL-6, IL-10, and IL-13. Th1 cytokines are involved in the cell-mediated immune and inflammatory responses, while Th2 cytokines mediate the humoral immune responses, such as antibody production and allergic response. Description of the role of IL-10, IL-6, IL-2, IFN-γ and TGF-β in helminth immunity is highlighted in table 1.6.

Table 1.6 Role of innate immune cytokines in the biological system (Turner et al. 2003, Abbas et al. 2012)

Soluble immune mediators	Origin	Immunoregulatory activities
IL-10	Monocytes, Th2 cells, B cells	Inhibits activated macrophages; displays potent abilities to suppress antigen presenting capacity of APCs; Released by cytotoxic T (Tc)
IL-6	Macrophages, T cells	Functions in innate and adaptive immunity; in the latter, stimulates growth of B cells that have differentiated into antibody producers; IL-1, TNF, and IL-6 appear to be major factors that induce the acute phase response.
IL-2	Helper T cells	Has high capacity to induce activation of almost all clones of cytotoxic cells; Increased cytotoxic functions of T killer and NK cells; promotes production of perforins and IFN-γ by these cells; Activates monocytes-macrophages to synthesize and secrete TNF-α, IL-1β, IL-6, IL-8, G-CSF, and GM-CSF.
IFN-γ	T cells and NK cells	Major macrophage activator; induces MHC class II molecules on many cells and can synergize with TNF; augments NK cell activity; antagonist to IL-4.
TGF-β	T cells and many other cells	Primarily an inhibitory cytokine; inhibits the proliferation and activation of T cells and macrophages; it works on peripheral mononuclear cells and epithelial cells to block the effects of proinflammatory cytokines.

1.2.13 Genetic variation of anti-helminth innate immunity

Breed is counted as one of the most influential host factors triggering susceptibility or resistance to many pathogens. The outcome of most infections is regulated by the host-pathogen genotype, phenotype and the environmental interaction. The genetic configuration of animals is reported to determine greatly their susceptibility or tolerance to helminth infections as well as the nature of their immune response (Williams-Blangero et al. 2002, Vincent et al. 2006). Numerous studies have indicated interbreed and intra-breed variation in the immune responsiveness in terms of a range of parameters (Clapperton et al. 2005, Cuenco et al. 2009). Significant difference in cytokine production, e.g. IL-10, IL-12 and IFN-γ has been reported among Yorkshire pigs (Crawley et al. 2003). Several other studies also supported the phenotypic and genetic variation in cytokine production of pigs; including mitogen induced production of IL-2, IL-4, IL-10 and IFN-γ (Edfors-Lilja et al. 1991). The response to different pathogens varied significantly between pigs and across breeds (Nguyen et al. 1998, Oswald et al. 2001, Lowenstein et al. 2004). Studies on helminth infections have repeatedly shown the involvement of common loci in regulating susceptibility to distinct parasitic diseases such as schistosomiasis, ascariasis, trichuriasis, and onchocerciasis (Mangano and Modiano 2014). Therefore, understanding the genetic background of different pig breeds is essential for a robust selection process aiming to improve herd health and production.

Variations in host immune response against helminth infection between genetically diverse pigs have been reported in several studies. The NIH miniature pigs with the cc homozygous swine leukocyte antigens (SLA) haplotype exhibited a lower burden of encysted muscle larvae than other haplotypes upon initial inoculation with *T. spiralis* (Lunney and Murrell 1988). This rapid clearance of parasites was believed to correlate with the early development of humoral antibody response. Pigs bearing the homozygous haplotype also exhibited a highly significant reduction in the encysted muscle larvae burden upon a second challenge. This suggested that the expression of the allelles on a haplotype influenced the cellular immune defense against *T. spiralis* (Madden et al. 1990). In addition, SLA haplotypes have also been shown to affect the uptake and killing abilities of cultured peripheral blood monocyte (Lacey et al. 1989).

Since the last few years, genome wide and/or multicentric studies have been conducted on *T. spiralis* (Mitreva et al. 2011) and on *A. suum* (Jex et al. 2011) which will facilitate the science of their relationship with the host. Preliminary findings based on hematology, liver-related serum enzymes, blood urea, liver weight and number of migratory larvae in the liver in experimental ascarisis in Mukota and Large White pigs suggested that Mukota pigs were relatively more resistant (Zanga et al. 2003). Findings from Vlaminck et al. (2012) supported and Nejsum et al. (2009) and Skallerup et al. (2012, 2017) endorsed that some pigs are more prone to intestinal infection while some pigs are inherently protected. Strain variation in the susceptibility level of *A. suum* was also revealed in the mice model by Lewis et al. (2006). Several studies demonstrated that resistance to helminth infection is heritable. For *A. suum*, heritabilities of 0.29-0.31 were estimated for log (faecal egg count+1) at weeks 7-14 post infection, whereas the heritability of log worm counts was 0.45 (Nejsum et al. 2009). Distinct inter-strain and intrastrain differences in the eosinophilia at mouse peripheral blood, bone marrow and spleen in experimental trichinellosis have been reported (Wakelin et al. 1985, Lammas et al. 1992).

German Landrace (LR) and Pietrain (Pi) are two leading breeds for commercial pig production worldwide. From physiological perspectives, there are differences between LR and Pi pigs in terms of growth potential, body composition, feed utililization and conversion (Ponsuksili et al. 2007). Evidence also exists for the immune response differences for Porcine Circo Virus Type-2 (Opriessnig et al. 2006), PRRSV infection and immunization (Ait-Ali et al. 2011, Islam et al. 2017) and LPS response (Kapetanovic et al. 2013) between LR and Pi pigs. The variation is apparent also in the level of quantitative expression of the performance potential, as well as in the efficiency of the necessary metabolic pathways. Insulin Growth Factor-1 (IGF-1) is reported to be associated with T regulatory cell stimulation and monocyte suppression (Bilbao et al. 2014, Ge et al. 2015).

Blood plasma contents of IGF-1 in relation to protein turnover efficiency were found lower in Pi compared to LR pigs (Windisch et al. 2000) which might be further extrapolated as having relatively less strong immunity of Pi pigs.

1.2.14 In vitro PBMCs model for evaluating the anti-helminth innate immunity

The current study employed in vitro peripheral blood mononuclear cells (PBMCs) model to elucidate the innate immune responsiveness to primary infection with helminth antigens and reveal the variation in responsiveness between pig breeds. PBMCs are a heterogenous mixture of immune cells with a major group with lymphocytes (70-90%) including T- and B-lymphocytes, NK cells, a small fraction of monocytes (10-30%) and dendritic cells (1-2%). PBMCs can be cultured in vitro and have become an essential component towards understanding in vitro immunological responses like cytokine production following stimulation with specific antigens (Bhattacharyya et al. 2001). Transcriptome profiling of PBMCs is widely used to dissect pathogenesis and genetics behind infection or stimulation (Fairbairn et al. 2011, Flori et al. 2011, Uddin et al. 2012). Cytokine production is often measured in affected tissues or PBMCs. One advantage of in vitro PBMCs in immunogenetic studies is that PBMCs is a primary cell culture; therefore, it has relatively close resemblance to in vivo system. Removal of serum and plasma from PBMCs facilitates the assessment of the specific immunodynamics of individual treatment or condition of interest. PBMCs take part in systemic defense immune reactions and changes in the other organ or tissues are also reflected in the PBMCs cellular and subcellular configuration and genotypic and phenotypic expression (Kohane and Valtchinov 2012). Several studies have used in vitro PBMCs to investigate the immunomodulatory role of helminth antigens in host parasite interaction (Pit et al. 2000, Morales et al. 2002, MacDonald et al. 2008, Bahia-Oliveira et al. 2009, McNeilly et al. 2013, Hegewald et al. 2015). Therefore, we used primary PBMCs isolated from whole blood used for in vitro stimulation with selected helminth antigens.

1.2.15 Mitogenic co-stimulation of PBMC cultures

Stimulating primary proliferative response using in vitro PBMC culture model is usually difficult (Kennell et al. 2014). Mitogens are polyclonal lymphocyte activators which are able to stimulate quiescent cells, trigger a complex series of cellular responses culminating in DNA synthesis and cellular proliferation. In vitro PBMCs proliferation likely initiates in response of a variety of stimuli; e.g. lectins such as concanavalin A, Phytohaemaglutinin (PHA) or Lipopolysaccharide (LPS). In appropriate conditions, interactions between lymphocyte surface receptors and various ligands initiate a signal which travels from the plasma membrane to the cell nucleus and

subsequently induces proliferation and differentiation of cells (Lijnen et al. 1997). The PHA is a T lymphocyte-specific mitogen and LPS is a macrophage- and B lymphocyte-specific mitogen (Schwager and Schulze 1997). It has been reported that LPS induced a synergistic increase in deoxyribonucleic acid (DNA) synthesis when human peripheral blood mononuclear lymphocytes were incubated with other mitogens like PHA, ConA and PWM (Schmidtke and Najarian 1975). In vitro co-stimulation of lymphocytes by respective mitogens makes the cells to produce appropriate cytokines to mount immune response. Combined use of LPS plus PHA will provide broader activation of PBMC populations and extensive cytokine signaling (Viallard et al. 1999).

1.3 Rationale and objectives of the dissertation

Helminth molecules are capable of regulating and manipulating the host response to facilitate their survival and persistence. As discussed before, several studies indicated that the host genetic configuration is a potential factor in immunoregulation, but host innate immunity in case helminth infections is less investigated. Therefore, the comprehensive purpose of this dissertation was to unravel the immunogenetic perception of host-parasite interaction for facilitation of sustainable control of important helminthes, especially *T. spiralis* and *A. suum* in pigs.

Hypothesis

We hypothesize that there may be variation in the innate immune responses during *A. suum* and *T. spiralis* infection between German landrace and Pietrain breeds.

Objectives

The aim of the current thesis is to explore the breed variation in terms of innate immune response in PBMCs during helminth infection. The specific objectives of each chapters of this thesis are enlisted as follows:

Chapter 2: To elucidate the genetic variation and kinetics in innate immune responsiveness in naïve and costimulated PBMCs to tyvelose from *Trichinella spiralis* between German Landrace and Pietrain pigs

Chapter 3: To investigate the genetic variation and kinetics of innate cytokine mRNA expression in naïve and costimulated PBMCs stimulated with *Ascaris suum* haemoglobin (AsHb) between German Landrace and Pietrain pigs

1.4 Materials and methods

In order to achieve the objectives of this research, several materials and methods were used. The particular materials and methods are described in details in the different chapters in this thesis. In fig. 1.8, a brief summary of the workflow is shown. The principle of the main methods and their applications will be briefly described in this section.

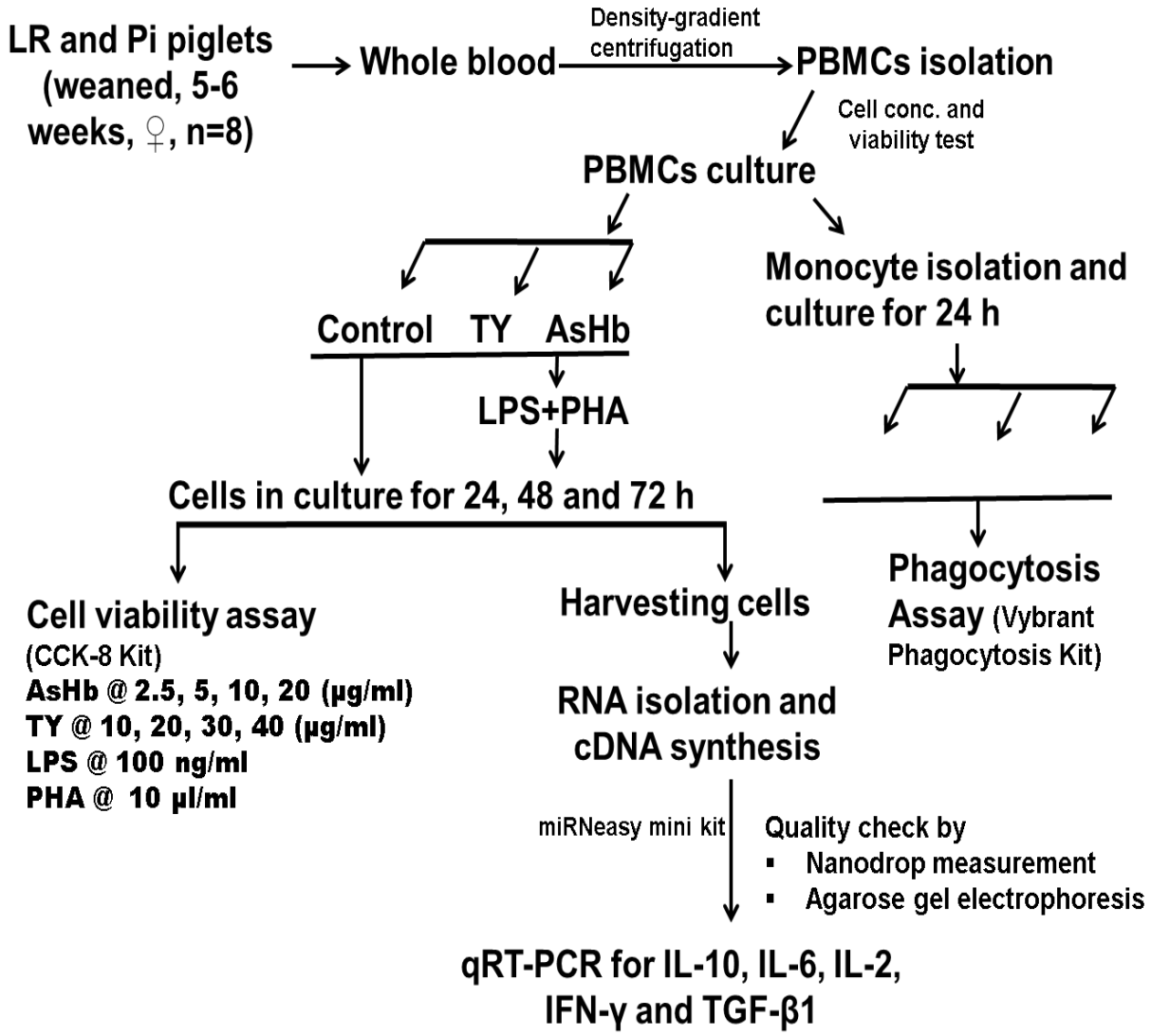

Fig. 1.8 Schematic diagram of the experimental design: PBMCs from four female weaned 5-6 weeks old piglets of each LR and Pi breeds were isolated from anticoagulated whole blood, viable cells were suspended in RPMI-1640 medium. Separate treatments were made to PBMCs using no treatment as naïve control, cells with AsHb, cells with AsHb+LPS+PHA, cells with LPS+PHA and cells with TY+LPS+PHA and cells with TY only. Dose dependent cell viability and phagocytosis was done only with PBMCs of LR pigs for convenience of the study. Time-course based cell viability was also done. mRNA samples were generated after appropriate treatment. RT-PCR was performed for selected genes, IL-10, IL-6, IL-2, IFN-γ and TGF-β1.

1.4.1 Ethical considerations

The experiment was conducted according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2006). The animal husbandry was approved by the Veterinary and Food Inspection Office, Siegburg, Germany (ref. 39600305-547/15) and permission for blood collection from North Rhein-Westphalian State Agency for Nature, Environment and Consumer Protection (ref. 84-02, 05.40.14.027).

1.4.2 Animal selection

In order to estimate the optimum number of study animals (4 animals per breed), the resource equation method (Festing and Altman, 2002, Festing 2006) was considered. According to this method, a value "E" is measured, which indicates the degree of freedom of analysis of variance (ANOVA). Any sample size, which keeps E between 10 and 20 should be considered as an adequate (Charan and Kantharia 2013). Purebred piglets from LR and Pi at the age of 5-6 wk old were chosen for whole blood collection. Study piglets were littermates for every case. They were immunized with mycoplasma vaccination at day 7 of age and weaned at 28 days of age and maintained with standard feeding regime.

1.4.3 PBMC isolation using density gradient centrifugation

Peripheral blood is the primary source of lymphoid cells for investigatng the mammalian immune system. Its use is facilitated by Ficoll-Hypaque density gradient centrifugation which is a simple, cheap and rapid method of purifying peripheral blood mononuclear cells (PBMCs) that exploits the density differences between mononuclear cells and other elements found in the blood sample (Fuss et al. 2009, Jaatinen and Laine et al. 2007). Ficoll is a high molecular weight sucrose polymer (specific gravity, 1.076 to 1.078), whereas Hypaque is a dense iodinated organic compound. Ficoll plays role in both viscosity to the solution and in rouleaux formation of the RBCs (Fuss et al. 2009). Hypaque enhances only the viscosity of the solution. Additional purification methods can be employed to eliminate major contaminants like immature RBCs by using RBC lysis buffer (Fuss et al. 2009).

In our experiment, anticoagulated whole blood was at first diluted with equal amount of 1xPBS and carefully layered over 10 ml of Ficoll in a 50 ml tube and centrifuged at slow speed at 1250 g for 30 min. After centrifugation, buffy coat layer was carefully aspirated and treated with 2-3 ml of 1x RBC lysis buffer for removal of RBCs. After 3-5 min, 1x PBS was added to stop the action of lysis buffer, centrifuged at 1000 g for 8 min. The supernatant was discarded and the cell pellet went

through the process of qualitative and quantitative examinations using 0.4% trypan blue staining and haemocytometer. Cells having viability more than 95% were used in the experiment.

Four biological replicates were used for all cases. Blood samples collected from four individual pigs were processed separately for in vitro studies. For cell viability and phagocytosis, two technical replicates were used for each treatment; for mRNA samples, no technical replicate was used. About 10-20 ml whole blood was subjected for PBMCs isolation for each of three assays like cell viability, phagocytosis and mRNA expression study. The freshly isolated primary cells of PBMCs were used for each assays. To minimize individual variation, age, sex and breed matched condition were considered and under in vitro condition, same concentration of cells was challenged with same treatment.

1.4.4 Cell viability assay using CCK-8 kit

Cell Counting Kit-8 (CCK-8) offers sensitive colorimetric determination of viable cells. It incorporates highly water soluble tetrazolium (WST) salt, WST-8 which is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye. This colourful dye is soluble in the tissue culture media. The amount of the formazan dye generated by the activities of dehydrogenase in cells is directly proportional to the number of living cells. The handling time of CCK-8 is short as no pre-mixing is required. To investigate possible influence of the helminth antigenic priming on cell viability, CCK-8 assays (cat# CK04-10 Dojindo Molecular Technologies Inc., München, Germany) were performed according to manufacturer's instructions. Viability assessment implied cell culture in 96 well plates (at a density of 10^4 cells per 100 µl in each well) and the viability was tested for three different dose of AsHb (2.5, 5 and 10 µg/ml) and four different concentrations of TY (10, 20, 30 and 40 µg/ml) with and without costimulation. After 24, 48 and 72 h of incubation, 10 µl reconstituted CCK-8 mixture were added to each well and incubated for further 4 h. Then the optical density of samples was measured using a microplate reader (Synergy™ H1 Multi-mode Reader, BioTek Germany, Bad Friedrichshall, Germany) at a wavelength of 450 nm. Thus the cell viability was also measured in a dose dependent and time dependent manner. The cell viability was calculated as percentage according to the manufacturer's formula.

1.4.5 Phagocytosis assay

Monocytes were isolated from PBMCs by adherence using the modified protocol described byWahl et al. (2001). Adhered monocyte layer from PBMC suspension were isolated after 3-4 h incubation, counted, resuspended in Roswell Park Memorial Institute medium (RPMI-1640) and seeded in a 96-well fluorescent plate. Experimental wells were treated with AsHb or Tyvelose or 1x PBS and incubated for 24 h. The antigen pulsed monocytes were then tested for their ability to ingest fluo-

rescein isothiocyanate labeled E.coli using Vybrant phagocytosis assay kit (Molecular Probes, Oregon, USA) according to the manufacturer's instruction. The fluorescence was determined using a microplate fluorescence reader (Centro LB 960 Microplate Luminometer, Berthold Technologies GmbH) using 480 nm for excitation and 520 nm for emission. The effect of phagocytosis was calculated according to the manufacturer's formula. We optimized the measurement resulting exclusively from internalized particle by quenching out of trypan blue from the well. To minimize the variation from the differences in sample viability, concentrations were expressed with respect to the number of untreated cells of corresponding group. The phagocytic capacity is represented as percentage, which is indicative of the total number of bacteria each cell had engulfed.

1.4.6 qRT-PCR

The qRT-PCR has widest dynamic range, the lowest quantification limits and the least biased results and the cheapest in comparison to high throughput technique like microarray and RNA-seq. qRT-PCR is highly sensitive and the gold standard for expression analysis (Costa et al. 2013). It is considered as the most sensitive technique for the detection and quantification of steady state mRNA levels. This technique is used to amplify and simultaneously quantify a specific region of the DNA molecule. It has additional special feature of detection of the amplified template during the reaction process in real time, whereas conventional PCR detects the product of reaction at its end. The quantity of interested molecules can be either an absolute copy number or a relative amount when normalized to reference genes and fluorescent molecules are used for the chemical reaction which allows for quantification of amplicon.

The SyBR Green provides the simplest method for the detection and quantification of the PCR products in real time reactions with high sensitivity (Acevedo et al. 2013). The SYBR Green binds to the double stranded DNA and emits light upon excitation. As the reaction proceeds and the PCR products accumulate, the fluorescence amplification starts.

For in vitro studies of this thesis, mRNA samples were isolated using miRNeasy mini kit (Qiagen, CA., cat# 2170040). On-column DNase treatment was done with RNase free DNase set (Qiagen, CA. cat# 79254). Quantity and quality was checked by nanodrop and gel visualization, respectively. First strand cDNA Synthesis Kit (cat#1612, Thermo Fisher Scientific, Germany) was used for cDNA sample preparation. Primers were designed using Primer3, an open source software. 10-fold serial dilution of cDNA were prepared and used as template for the generation of the standard curve. The qRT-PCR reaction was set up using 2.0 µl of cDNA template, 7.0 µl of deionized RNase free water, 0.5 µM of upstream and downstream primers, and 10 µl iTaq™ Universal SYBR®

Green Supermix (Bio-Rad Laboratories GmbH, Germany) in a total reaction volume of 20 µl and were amplified by the StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany). The thermal cycling conditions were 95 °C for 3 min, 95 °C for 15 sec, 6 °C for 45 sec (40 cycles); 95 °C for 15 sec, 62 °C for 1 min, 95 °C for 15 sec. All reactions were run in single to get the expression value. Gene-specific expression was measured as relative to the geometric mean of the expression of two reference genes, beta-actin (ACTB) and peptidylpropyl isomerase A (PPIA). The delta Ct (ΔCt) [$\Delta Ct = Ct_{target} - Ct_{reference\ genes}$] values were calculated as the difference between target gene and reference genes and expression was calculated as $2^{(-\Delta\Delta Ct)}$ (Schmittgen and Livak 2008).

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Peptidylpropyl isomerase A (PPIA) and beta-actin (ACTB) genes were used as reference genes for calculating relative expression of the genes of interest. Melting curve showing single peak in the samples as well as absence amplification in NTC indicated the quality of qPCR expression. The mean CT values of the both reference genes were in a small range in samples of different treatment and time points tested indicating their stable expression irrespective of treatments. Moreover, PPIA has been previously reported a stably expressed reference gene in the porcine PBMCs (Cinar et al. 2013) and ACTB has also been found to be stably expressed in porcine tissue (Nygard et al. 2007).

1.4.7 Statistical analysis

Cell viability and phagocytosis assay related data were analyzed with GraphPad Prism (Version 5.3, La Jolla, California, USA) using One-way ANOVA followed by Tukey test and values were presented as means ± SEM. qRT-PCR data was analyzed using the Proc GLM procedures of SAS software (Version 9.4, SAS Institute Inc., Cary, NC, USA). Data is expressed as least square means ± standard error of mean. A significance level of $p < 0.05$ was used.

1.5 Results

Detailed results are presented in chapter 2 and 3 of this dissertation. Only the major findings are highlighted here.

1.5.1 Helminth antigen induced viability and proliferation of PBMCs

The influence of two helminth antigens, synthetic tyvelose and *Ascaris suum* haemoglobin as representative immunomodulatory antigen of *Trichinella spiralis* and *Ascaris suum*, respectively were assessed on PBMCs of LR and Pi piglets. For both, time-course and dose-dependent assay was conducted only in PBMCs from LR origin. TY at four different doses (10, 20, 30, 40 µg/ml)

were added for 24, 48 and 72 h of PBMCs culture of LR origin. There was a trend of lymphoproliferation, but statistically no significant variation was noticed from the different doses in both naïve and costimulated PBMCs. TY treated PBMCs at 24 h had a relatively higher viability percentage compared to 48 and 72 h in both with or without mitogen activated PBMCs. TY at a concentration of 10 +µg/ml had relatively least alteration in the viability or apoptosis compared to control. Therefore, TY at this dose was further applied for breed comparison with cell viability, phagocytosis, mRNA and protein expression studies. Breed factors were evaluated in the relative cell viability percentage resulting from the same dose mentioned before. Significant variation ($p<0.05$) was evident only at 48 h post exposure to TY plus costimulation and the viability was higher in TY treated and costimulated PBMCs of Pi pigs.

In case of AsHb, three different concentrations of AsHb (2.5, 5 and 10 µg/ml) were used and three time points, 24, 48 and 72 h were considered for this PBMCs obtained from LR piglets. AsHb had shown significant level of lympho-depletion in a dose and time dependent manner in naïve PBMCs culture compared to untreated control for each respective time point. The higher the concentration, the lower cell viability was observed. The relative viability percentage did not vary significantly among the different duration of exposure. In contrary to this, the lymphosuppressive or apoptotic effect was found not significant in costimulated PBMCs when comparison was made with PBMCs receiving only LPS (100 ng/ml) plus PHA (10 µl/ml). AsHb, at a dose of 2.5 µg/ml, resulted in the least changes on the cell death or viability of PBMCs. Therefore, we used this dose for subsequent experimental studies with AsHb. Breed comparison was made between the relative viability percentage of PBMCs of LR and Pi piglets receiving AsHb at this dose. Although the relative viability was higher in PBMCs from Pi, but the variation was not statistically significant. Rather, in the co-stimulated group, significant ($p<0.05$) variation was achieved only at 72 h post exposure where the viability was higher in LR compared to the PBMCs viability of Pi origin.

1.5.2 Helminth antigen induced phagocytosis of porcine PBMCs

As genetic effects are more evident in phagocytosis (Soudi et al. 2013) and as phagocytosis is a vital indicator of innate immune response, we evaluated dose-dependent (in monocytes from PBMCs of LR pigs) as well as the breed dependent (LR vs Pi) phagocytosis. Effect of four different concentration of TY was checked on the peripheral blood derived monocytes of LR pigs in terms of phagocytosis. Although there was overall high increase in phagocytic rate, there was high variation among the replicates. Therefore, the effect of treatment was not statistically significant at none of

those doses used. When the comparison of relative phagocytic rate between blood monocytes of LR and Pi was performed, significantly higher (0.0001) phagocytic rate was found in favor of LR.

In a similar fashion, among the three concentrations of AsHb, 2.5, 5 and 10 µg/ml used in this assay using blood monocytes of LR piglets, only AsHb (5 µg/ml) was found to induce significant up rise in phagocytosis compared to the untreated control group. When the effect of breed was considered at a treatment dose of AsHb (2.5 µg/ml), significantly higher phagocytic potential was noticed in LR PBMC derived monocytes compared to that of Pi. It should be kept in mind that the phagocytic effect was not significant at this dose in LR between treated and control group as indicated before.

1.5.3 Helminth antigen induced cytokine expression dynamics in porcine PBMCs

Cytokine measurement is one of the most vital indicators of cellular immune responsiveness in in-vitro models. In order to determine the trend and kinetics of immunoregulatory cytokines in the helminth antigen exposed PBMCs culture, we analyzed the mRNA expression of five selected innate immune genes, IL-10, TGF-β1, IL-6, IL-2 and IFN-γ for both AsHb and TY treated PBMCs.

Treatment with TY in naïve PBMCs resulted in significantly elevated level of IL-10 and later declined in LR, whereas in Pi, the onset of IL-10 upregulation was evident after 24h post exposure. IL-6 mRNA expression also followed the pattern similar to IL-10. Significant effect of breed and treatment was found at 48 h time point for IL-10 and IL-6. The expression level of IL-2 mRNA was significantly elevated only in PBMCs of LR after 24 h post-exposure to treatment. On the contrary, TY treatment had no significant effect on PBMCs of Pi piglets in this experimental set up. The Th1 cytokine, IFN-γ was only elevated at significant level, only at 72 h time point. At this stage, the effect of treatment was clearly evident while comparing treated vs naïve PBMCs. The expression level of TGF-β1 was inconsistent across treatment, time point and breed; this might be because of the contribution of other non-immune cells in the PBMCs culture, i.e., platelets.

Similar to the kinetics induced by TY, AsHb treatment enhanced the expression level of IL-10 and IL-6 in both LR and Pi; but the onset of upregulation was delayed in Pi until 48 h of exposure. IL-10 expression was progressively and significantly upregulated across the experimental duration and significant effect of breed and treatment was noticed at 72 h post exposure. The expression kinetics were quite similar for IL-2 and IFN-γ in both LR and Pi pigs. The effect of treatment was significantly evident for IL-2 at 48 h of post-cultivation. In case of TGF-β1, there was an apparent downregulation until 48 h in both breeds followed by a trend towards upregulation later. This pattern of TGF-β1 is in consistency with the IL-2 which had upregulation at 24 and 48 h followed by downregulation at 72 h post treatment.

1.5.4 Influence of mitogenic co-stimulation on helminth antigens induced cytokine expression

Mitogenic stimulation of in vitro immune cell cultures is often needed to ensure the highest possibility of cellular activation and to induce almost physiological conditions. We also investigated the effect of helminth antigens on PBMCs post-stimulated with combined mitogenic stimulus, LPS plus PHA and analyzed the expression of those immune cytokines mentioned before for naïve PBMCs.

In our study, treatment with TY at 10 µg/ml did not make any significant change for IL-10 expression in co-stimulated PBMCs. But the effect of breed was evident in a time-course manner. The mRNA level of IL-6 was elevated after 24 h of culture in PBMCs of LR pigs and the breed effect was evident at 48 h post cultivation. TY mediated suppression of IL-2 was significantly ($p<0.05$) expressed at 24 h time point only. The pattern of IFN-γ mRNA expression appeared similar in both breeds and the effect of treatment was significant ($p<0.01$) in LR at 48 h of post culture. The influence of TY on TGF-β1 mRNA level in the co-stimulated PBMCs irrespective of breed and duration of exposure was rather complex and inconclusive to some extent.

In our findings, the effect of AsHb on mitogen co-stimulated PBMCs had resulted in progressive elevation of IL-10 and IL-6 in cells of LR origin. The effect of treatment was statistically significant only at 72 h post-treatment for both of these cytokines. The response in the Pi group was a dramatic rise in IL-6 at 48 h post treatment. The kinetics of IL-2 expression was similar in both breeds exclusively with the potential role of breed and treatment at 24 h time points. Another Th1 cytokine, IFN-γ did not show any specific trend between breeds. Similar to the effect of TY, TGF-β1 expression did not follow any specific pattern with respect to treatment, duration of exposure or breed.

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Chapter 2: Comparative expression dynamics of cytokine mRNAs in the PBMCs of German Landrace and Pietrain pigs after in vitro stimulation with *Trichinella spiralis* derived antigen tyvelose

(Manuscript is under preparation for submission)

Comparative expression dynamics of cytokine mRNAs in the PBMCs of German Landrace and Pietrain pigs after in vitro stimulation with *Trichinella spiralis* derived antigen tyvelose

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Running title: Tyvelose induced immune expression in PBMCs from German Landrace and Pietrain

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2.1 Abstract

Porcine breed predisposition for *Trichinella spiralis* infection is not known yet. The current study aims to investigate the breed difference in the innate immune response to tyvelose (TY), the antigenic part of *Trichinella spiralis* muscle larvae, in in vitro peripheral blood mononuclear cells (PBMCs) between German Landrace (LR) and Pietrain (Pi). PBMCs treated with synthetic TY with and without lipopolysaccharide plus phytohaemagglutinin costimulation for 24, 48 and 72 h were assessed for cell viability and mRNA level for IL-10, IL-6, IL-2, IFN-γ and TGF-β1 by qRT-PCR. PBMCs derived monocytes were tested for TY induced phagocytosis effect. LR appeared to respond earlier than Pi as evident from relatively higher viability, phagocytosis effect and early upregulation of IL-10, IL-6, IL-2 in the antigen treated PBMCs culture. This indicates the relatively better innate immunocompetance of LR compared to Pi pigs when challenged with TY.

Key words: *Trichinella spiralis*, tyvelose, innate immunity, cytokine, PBMCs, pig, breed.

Highlights:

- Tyvelose induced innate response in peripheral blood mononuclear cells varied between German Landrace and Pietrain.
- Phagocytic efficiency is significantly higher in tyvelose treated blood monocytes of German Landrace compared to Pietrain.
- Immune response in German Landrace pigs is relatively fast with elevation of both pro-inflammatory IL-6, IL-2 and anti-inflammatory IL-10 cytokines in relation to Pietrain.
- German Landrace was more immunocompetant than Pi pigs in terms of innate immunity.

2.2 Introduction

Trichinellosis, the infection caused by *Trichinella spiralis*, is a food-borne zoonotic disease involving a broad host range (Torgerson 2013), but pigs are affected as the major domestic source. Parasites of the genus *Trichinella* are unique intracellular helminth pathogens. The *Trichinella* spp. completed their life cycle in two niches, the multicellular habitat in intestinal epithelium and the intracellular location in the skeletal muscle fibers (Mitreva et al. 2011). The life cycle of *T. spiralis* is direct and can be completed within a single host. It comprises of three developmental stages-adult worm, new borne larva (NBL) and muscle larva (ML) (Gottstein et al. 2009). Trichinellosis of pig and other mammals eventuate from the consumption of the infective ML in contaminated food. After ingestion, the ML is released in the stomach and revived in the small intestine. These small larvae invade the epithelial layer where they mature, copulate and deliver the NBLs. The NBLs migrate through the lymphatic and blood vessels, invade highly oxygenated striated muscle cells and develop into the ML (Wakelin and Goyal 1996). The excretory-secretory (ES) products of ML are responsible for immunomodulation through targeting the host macrophages and thereby nurse cell formation in the skeletal muscle (Ashour 2013, Bai et al. 2016). The in vitro findings offer strong evidence that ML-ES products have the ability to affect macrophage function (Bai et al. 2012). Among the ES antigens of ML, tyvelose (TY) is one of most immunodominant glycoproteins that has been found to induce distinct immune responses in vitro (Wisnewski et al. 1993). Since ES-molecules of helminth have been shown to mimic the immune response as induced by helminth infection, the focus has turned to elucidate the mechanisms of innate immunity to *T. spiralis* derived TY using in vitro porcine PBMCs model.

The PBMCs are a subset of white blood cells, dominated with T lymphocytes followed by B lymphocytes, and a small fraction of monocytes and dendritic cells. The lymphoproliferative response following antigenic challenge is known to be correlated with the progression of cell-mediated immunity (Descotes 2006). The porcine PBMCs-transcriptome is informative to monitor disease susceptibility, to characterize response to immune stimulation or to refine the characterization of certain immune traits (Flori et al. 2011, Uddin et al. 2012). Research showed that the immune responses to a certain pathogen occurring in the PBMCs reflect those inflammatory changes at the site of disease (Meade et al. 2008). The mitogenic co-stimulation of PBMCs culture along with target antigenic challenge are likely beneficial for evaluating the cellular immunity (Norian et al. 2015).

Cellular immunity constitutes the most effective means to restrict parasite persistence and proliferation (Eger et al. 2003). The cell proliferation, phagocytosis and cytokine production by immune cells are the key effectors of innate immunity that provide primary step of defense against invading pathogens (Djaldetti and Bessler 2014). The level of regulatory cytokines and Th2 cytokine mRNA expression is crucial for the establishment and outcome of *T. spiralis* infection (Vasilev et al. 2009). The expression of interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) are mentioned to be significantly linked with hyporesponsiveness and susceptibility to parasitic diseases (McSorley and Maizels 2012). It has been reported that IL-10 is the key regulator of protective immune response against trichinellosis in mice (Helmby and Grencis 2003). The TGF-β performs a vital role in regulating local inflammation as well as balancing the T cell response in *T. spiralis* infection (Lei Fang et al. 2014). The expression of Th-17 related genes like IL-6 is also accompanied with both susceptibility and resistance to gastrointestinal nematodes in sheep (McRae et al. 2015). A previous work showed that ML derived protein, rTs-Hsp70 (recombinant *T. spiralis* heat shock protein 70) activated dendritic cells with elevated level mRNA of IL-6, IFN-γ, IL-2 and IL-4 in $CD4^+$ T cells of mice (Fang et al. 2014). Dampening of Th1 cytokines (IFN-γ, TNF-α, IL-12) usually leads to the development of Treg and Th2 dominance and survival as well as persistence of parasitic infection.

It has been reported that immune response traits are differentially affected by host genetics and environmental factors (Mangino et al. 2017). The status of innate immunity is likely indicative of the host susceptibility/resistance to pathogens (Carvalho et al. 2010, Jiang et al. 2010). Genetic components associated with host susceptibility to trichinellosis infections have been reported in pigs (Lunney and Murrell 1988). Breed specific variation offers opportunities for selective animal breeding for disease resistance or tolerance (Kapetanovic et al. 2013). Host genotype-associated variation in the susceptibility to *T. spiralis* has been observed in a rat model (Vasilev et al. 2009). A significant host genotype-dose interaction has been found during a primary infection with *T. spiralis* in five genetically diverse mouse lines (Vasconi et al. 2015). Although domestic pigs are a major reservoir of this parasite, information on the host genetic effect, especially porcine breed predisposition on the innate responsiveness is yet to be elucidated. German Landrace (LR) and Pietrain (Pi) are two important swine breeds for commercial pork production. Healthy LR and Pi pigs differ considerably in terms of growth rate, nutrient utilization and metabolic trait (Ponsuksili et al. 2007). Substantial genetic variations in innate immune traits have been reported among healthy pigs (Flori et al. 2011). The aim of the recent work was to investigate the mRNA expression of selected innate immune cytokines in response to in vitro TY stimulation to the PBMCs obtained from healthy purebred LR and Pi pigs.

2.3 Materials and Methods

Ethics statement

The experiment was performed according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2006). The animal husbandry was approved by the Veterinary and Food Inspection Office, Siegburg, Germany (ref. 39600305-547/15) and permission for blood collection from North Rhein-Westphalian State Agency for Nature, Environment and Consumer Protection (ref. 84-02, 05.40.14.027).

Study animals and blood sampling

The experiment comprised four clinically healthy weaned female piglets of 5-6 weeks old from each LR and Pi purebred. Animal numbers were estimated considering the resource equation method described by Festing and Altman (2002) and Festing (2006). All animals were obtained from the Teaching and Research Station of Frankenforst, University of Bonn, Germany. Upon birth, piglets were selected and ear tagged and housed with standard feeding and housing condition; animals with any clinical illness at sampling time were excluded. The piglets were vaccinated for mycoplasmosis at day 7 according to the routine farm vaccination program and weaned at day 28 of age.

Antigen and mitogen preparation

Commercially available synthetic antigen, Tyvelose (CAS 5658-12-8, Santa Cruz Biotechnology) was used as a representative antigen of *T. spiralis*. As mitogen, lipopolysaccharide (LPS, L2880, Sigma Aldrich) and phytohaemagglutinin (PHA-M, 10576015, ThermoFisher Scientific, Germany) were used for co-stimulation of PBMCs culture.

Isolation of peripheral blood mononuclear cells

PBMCs from whole blood were isolated by using Ficoll-Paque (Histopaque-1077; Sigma-Aldrich, Munich, Germany) density gradient centrifugation as describe earlier (Islam et al. 2016). In short, anticoagulated whole blood were diluted at the ratio of 1:1 with 1x phosphate buffered saline (PBS) and carefully layered over half volume of Histopaque solution previously kept in a 50 ml conical tube. Then the tubes were centrifuged at 1250 g for 30 minutes (min) slowly at room temperature. After centrifugation, the PBMCs layer was carefully aspirated and treated with red blood cell lysis buffer to remove erythrocytes. Finally, PBMCs was washed two times with PBS and one time with Roswell Park Memorial Institute 1640 medium (RPMI-1640, Sigma-Aldrich, Munich, Germany) and harvested as pellet. The harvested PBMCs were subjected for cell counting using a

haemocytometer immediately after isolation using 0.4% trypan blue stain. The percentage of viable PBMCs at >95% were used for in vitro stimulation. Cell pellets were suspended with RPMI-1640 medium supplemented with heat inactivated 10% fetal bovine serum (FBS) (Invitrogen, Germany) and 1% antibiotic (Penicillin-streptomycin, cat.15140-122, Life Technologies GmbH, Darmstadt, Germany) and 1% antimycotics (Fungizone®, cat. 15290-026, Life Technologies GmbH, Darmstadt, Germany).

Cell proliferation and viability test for PBMCs

After initial screening, the rate of cell proliferation and viability upon antigenic stimulation were investigated using CCK-8 cell proliferation kit (cat.CK04-10, Dojindo Molecular Technologies Inc., EU GmbH) following the manufacturer's instruction. This assay is based on the colorometric evaluation of the amount of formazan dye produced by the dehydrogenase reaction of viable cells by reducing WST-8 salt in the cell culture. The proportion of this yellow colour formazan dye is positively correlated to the number of living cells. In brief, PBMCs were cultured in 96-well plate at a density of $1*10^4$ cells/well in 100 µl of RPMI-1640 media with TY treatment with a dose of 10, 20, 30 and 40 µg/ml and an untreated control with equal volume of PBS for 30 minutes followed by addition of LPS (100 ng/ml) and PHA (10 µl/ml) as mitogenic stimulation and incubated at 37 °C under 5% CO_2 and 98% humidity. Two technical replicates were used for each case. After 24, 48 and 72 h of incubation, 10 µL reconstituted CCK-8 mixture were added to each well and incubated for further 4 h. Then the absorbance of samples was measured using a microplate reader (Thermo Max, Germany) at a wavelength of 450 nm. Thus the cell viability was also measured in a dose dependent and time dependent manner. The cell viability was calculated as percentage according to the manufacturer's formula.

Culture and stimulation of PBMCs

The PBMCs were cultured separately for each four animal at a $1*10^6$ cells/well in 6-well tissue culture plates in the presence of TY (10 µg/ml). Thirty minutes later, LPS at 100 ng/ml and PHA at 10 µl/ml dose were added to the assigned culture accordingly, for immune stimulation. The plates were maintained for 24, 48 and 72 h at 37 °C under 5% CO_2 before collection of cells. Control samples were kept untreated and only combined doses of LPS and PHA were used for respective costimulated groups.

Phagocytosis assay for PBMC derived monocytes

In vitro phagocytosis assay was performed with Vybrant Phagocytosis Assay Kit (Cat. V-6694, Molecular Probes Inc.) according to manufacturer's instruction as previously described by (Islam et

al. 2012). The purpose of this assay is to measure the ability of antigen primed monocytes to ingest fluorescein isothiocyante labeled *E. coli*. Monocytes were isolated from PBMCs by adherence method using the modified protocol described by Wahl and his colleague (Wahl and Smith 2001). Determinations were performed in all wells comprising the experimental (with TY treatment), without any treatment as positive control and only media as negative controls. After 2 h of incubation, solutions were removed from all microplate wells by vacuum aspiration. Fluoresce-labeled bioparticles were added to the wells and phagocytic uptake was allowed to proceed for 2 h in at 37 °C humidified incubator with 5% CO_2. Subsequently, the BioParticles suspension was removed and 100 µl of trypan blue suspension was added for 1 min at room temperature. The excess trypan blue was removed and the samples were measured in the fluorescence microplate reader (Thermo Electron Co., USA) using 480 nm for excitation and 520 nm for emission. The net phagocytosis of the cells was calculated following the manufacturer's formula. To ensure that the fluorescence measured resulted exclusively from ingested particles; any non-internalized bioparticles was quenched by the addition of trypan blue, as supplied by the manufacturer. To eliminate variations due to differences in viability, concentrations were expressed with respect to the number of untreated cells.

Isolation and quality control of total RNA

mRNA samples from four individual purebred animals and for three time points (24, 48 and 72 h) and four treatment groups (naïve control, cells with only TY, cells with TY+LPS+PHA and cells with LPS+PHA) were processed for RNA isolation. Total RNA enriched with microRNAs was extracted from cultured PBMCs generated by using the miRNeasy mini kit (Qiagen, CA., Cat# 2170040) according to the manufacturer's protocol. The total RNA was quantified by spectrophotometry (NanoDrop -8000 UV-vis Spectrophotometers (Thermo Scientific, Wilmington, USA). To prevent DNA contamination, on-column treatment was done with RNase-Free DNase Set (Qiagen, CA., Cat# 79254). The fragments of 18s and 28s ribosomal band of total RNA were visualized by agarose gel electrophoresis. Further DNA contamination was checked by using PCR amplification of reference genes (ACTB) followed by visualization in 2% agarose gel.

Quantitative Real Time PCR (qRT-PCR)

The selected genes quantified by qRT-PCR are presented in Table 1. Primers were designed based on an open source primer designing software Primer3. First Strand cDNA Synthesis Kit (P/N K1612, Thermo Scientific, Schwerte, Germany) was used for reverse transcription with oligo (dT) primer. The qRT-PCR reaction was set up using 2 µl of cDNA template, 7 µl of deionized RNase

free water, 0.5 µM of upstream and downstream primers, and 10 µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Germany) in a total reaction volume of 20 µl and amplified by the StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany). The thermal cycling conditions were 95 °C for 3 min, 95 °C for 15 sec, 6 °C for 45 sec (40 cycles); 95 °C for 15 sec, 62 °C for 1 min, 95 °C for 15 sec.

Table 2.1 The sets of primers and PCR conditions used for evaluation of cytokine mRNA levels, including product length

Gene symbol	**Accession no.**	**Primer sequences (5` -3`)**	**Amplicon size (bp)**	**T_{ann} (°C)**
ACTB	XM-003124280.4	F:AAGGACCTCTACGCCAACAC R:CTTGCTGATCCACATCTGCT	207	60
PPIA	NM_214353.1	F:CACAAACGGTTCCCAGTTTT R:TGTCCACAGTCAGCAATGGT	171	58
IL-10	NM-214041.1	F:TCCGACTCAACGAAGAAGGC R:AACTCTTCACTGGGCCGAAG	179	59
IL-6	NM_214399.1	F:GGCAGAAAACAACCTGAACC R:GTGGTGGCTTTGTCTGGATT	243	58
IL-2	NM_213861.1	F:CTAACCCTTGCACTCATGGCA R:AATTCTGTAGCCTGCTTGGGC	185	81
TGF-β1	NM_214015.2	F:CGGAGTGGCTGTCCTTTGAT R:GGTTCATGCCGTGAATGGTG	186	60
IFN-γ	NM_213948	F:AGCTCCCAGAAACTGAACGA R:AGGGTTCAAAGCATGAATGG	225	60

T_{ann}: Annealing temperature, F: Forward, R: Reverse, bp: Base pair

Statistical analyses

Data obtained from cell viability, phagocytosis and qRT-PCR expression were undertaken for analysis. For each cases applicable (e.g. cell viability and mRNA expression), the main effects in näive PBMCs and in mitogen activated PBMCs were considered and analysed separately.

Data of the optical density value (OD value) obtained from cell viability assay was first converted to get relative percentage of cell viability according to manufacturers' instruction in excel as per the formula below:

Cell viability (%) = {(Absorbance of experimental well - Absorbance of negative control well) / (Absorbance of positive control well - Absorbance of negative control well)}

The relative viability data were analysed were shifted to Graphpad Prism v.5.3 (La Jolla, California, USA) and analysed. Dose-dependent effects were analysed using One-way Anova to compare between control vs treated groups. Pairwise comparison was made to see the breed effect at the same time point using One-way Anova followed by Tukey's multiple comparison test.

The OD values obtained from the phagocytosis assay was first converted to get relative percentage of phagocytosis effect according to manufacturers' instruction in excel according to the formula below:

% Phagocytosis effect = (Net Experimental reading/Net positive reading) x 100%

where

Net Positive Reading = Average fluorescent intensity of positive control - Average fluorescent intensity of negative control

Net Experimental Reading = Average fluorescent intensity of experimental well - Average fluorescent intensity of negative control

The relative percentage data were imported to GraphPad Prism and dose-dependent effect was analysed using One-way Anova and breed comparison for single dose and at single time point was made using t-test. The values were expressed as mean ± standard error for both cell viability and phagocytosis assay.

RT-PCR data from PBMC samples were analyzed using the general linear models (GLM) procedure of SAS software (Version 9.4, SAS Institute Inc., Cary, NC, USA). In one set, comparison was made between untreated control vs. TY treated PBMCs. In another set of data, comparison was made between LPS+PHA treated one vs. TY+LPS+PHA treated cells. Gene-specific expression was measured as relation to the geometric mean of the expression of two reference genes (PPIA and ACTB). The delta Ct (ΔCt) [$\Delta Ct = Ct_{target} - Ct_{reference\ genes}$] values were calculated as the difference between target gene and reference genes and expression was calculated as $2^{(-\Delta\Delta Ct)}$ (Schmittgen and Livak 2008). Further, the expression value was examined for normal distribution using Proc Univariate, considering the Shapiro-Wilks test for normality and a normal probability plot followed by elimination of extreme values as outliers. Outliers were identified when these values deviating more than three standard deviations from the raw mean. Data from four biological replicates for three timepoints were analyzed to test for main effects of breed (LR and Pi), treatment (TY), duration of treatment (24, 48 and 72 h), and for interactions among these factors. All data were subjected to analysis to see the effect of TY treatment, breed (LR and Pi), time (24, 48 and 72 h) and interaction among these factors. For significance test, Anova type III and pairwise comparison was made using linear contrast. Group means were presented as least square mean (LSM) ± Standard error of mean (SEM). The values were tested by Dunett and Tukey-Kramer test. A significance level of $p<0.05$ was used.

2.4 Results

Cell viability of PBMCs following dose dependent stimulation of TY

We determined the influence of antigenic dose on cell viability using four individual animals from LR breed. For the relatively infrequent availability of Pi pigs, dose dependent effects were not tested in Pi pigs. Cell viability was higher at 24 h post exposure compared to 48 and 72 h of culture (Fig. 2.1A and B). Dose dependent increase and time dependent decrease in viability in näive PBMCs was observed, but this trend appeared statistically not significant. No significant variation was observed among the four different doses of TY used neither in cell death nor in cell viability in culture with (Fig. 2.1A) and without co-stimulation (Fig. 2.1B). So it can be assumed that TY at a dose 10 to 40 µg/ml was stable, not stressful and non toxic for the PBMCs. It also indicated that antigenic treatment enabled näive PBMCs prone to differentiation and a slight proliferation with a higher number of cells at 24 h. After 24 h, there was a decline in cell viability. There were no statistically significant changes in viability at 24, 48 or 72 h of incubation among the treatment groups compared to the respective control at each time point.

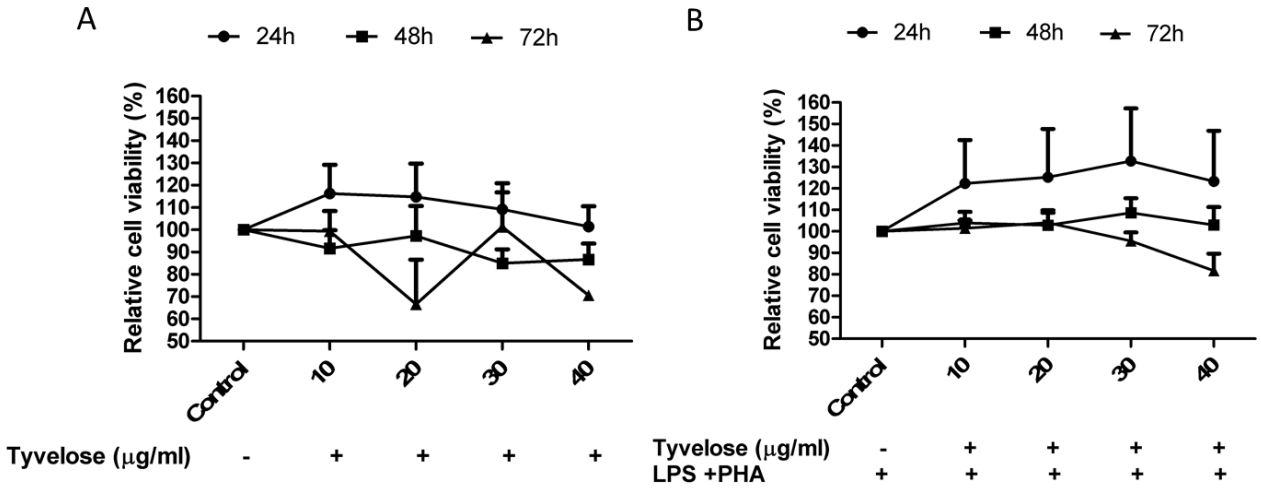

Fig. 2.1 (A-B) Effect of TY on the proliferation and viability of PBMCs of LR. Four different concentrations of TY were added in naïve PBMCs (A) and in PBMCs which were followed by addition of costimulation (B). PBMCs were left without any treatment only with media as naïve control (A) and LPS plus PHA (B) as control for respective TY+LPS+PHA treated group. The culture was kept at 37°C with 5% CO_2 for 24, 48 and 72 h. Values are mean ± SEM of independent experiments at three timepoints performed in duplicate.

Influences of pig breed on TY treated PBMCs viability

Evaluation of the breed effect on cell viability was done using the non stressful dose of TY (10 µg/ml) for three different time points, 24, 48 and 72 h (Fig. 2.2A and B). In näive PBMCs, no significant difference was noted, although the overall trend shows relatively higher viability of PBMCs in TY treated PBMCs of LR origin compared to Pi counterparts (Fig. 2.2A). In co-

stimulated culture, significant variation ($p<0.01$) was observed only at 48 h of post-incubated PBMCs and after 24 h of incubation, relatively higher viability was observed in TY treated PBMCs from Pi pigs compared to that of LR (Fig. 2.2B).

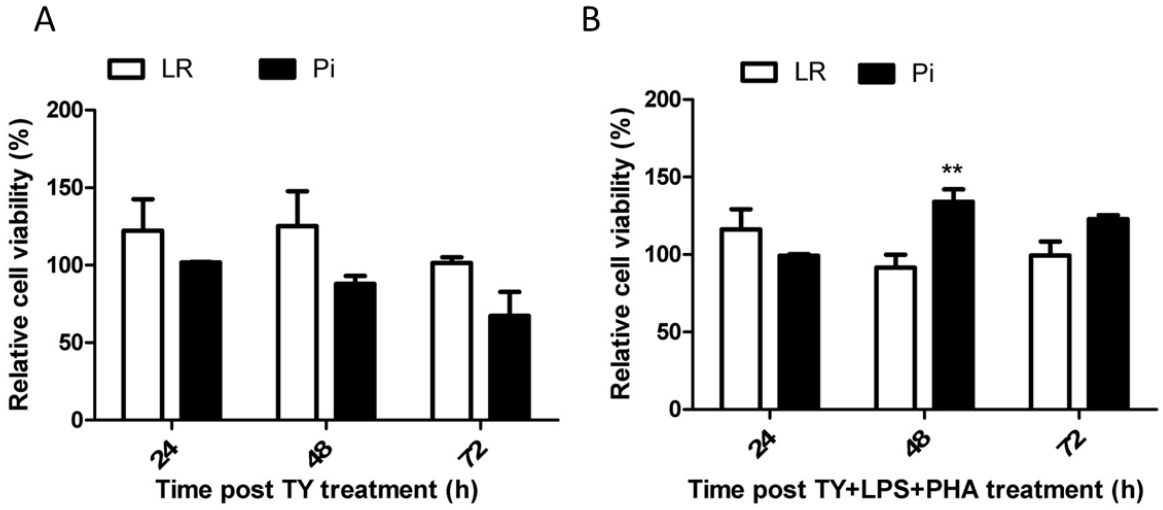

Fig. 2.2 Breed comparison of relative cell viability in a time dependent manner in (A) only TY (10 µg/ml) treated and (B) TY (10 µg/ml) +LPS+PHA treated group. The results were combined from independent experiments at three different time points and each experiment was performed in duplicate. The data are represented as the mean ± SEM. ** indicates $p<0.01$. Pi: Pietrain, LR: German Landrace.

Phagocytic activity of PBMCs following dose dependent stimulation of TY

To investigate the effect of TY treatment on monocytes isolated from of PBMCs of LR pigs, we implied Vybrant phagocytosis assay. It appeared that exposure to TY with all four doses, enhanced the average phagocytosis rate (Fig. 2.3). But this effect was not statistically significant as there was a high variation within the biological replicates in treated groups. From this graph, no potential difference was found among TY at 10 to 40 µg/ml dose.

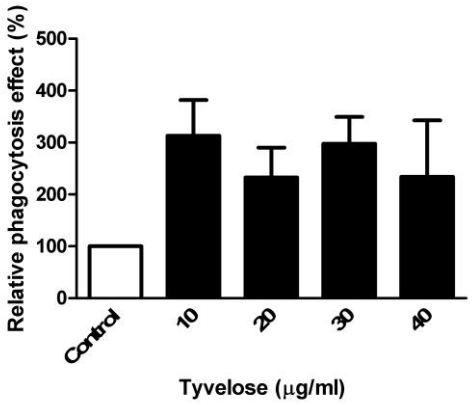

Fig. 2.3 Tyvelose induced phagocytosis by peripheral blood monocyte culture of LR. Monocytes re-suspended in a 96-well fluorescent plate were treated with TY (10, 20, 30 and 40 µg/ml). Cells treated with media only were kept as control. The phagocytic capacity of this population is represented as percentage. The result is a combination of four replicates in each treatment group. Value presented as mean ± SEM.

To examine the effects of breed on the phagocytic capacity of PBMCs originated monocytes, we applied the same concentration of TY (10 µg/ml) in the monocyte culture from LR and Pi and tested their relative phagocytosis effect (Fig. 2.4). The effect was higher ($p>0.01$) in PBMCs of LR pigs compared to Pi.

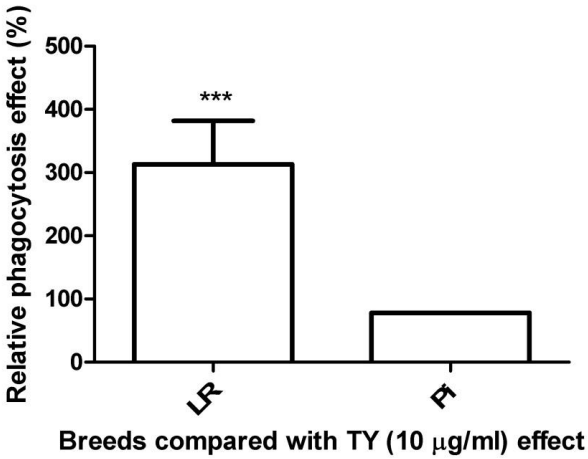

Fig. 2.4 Variation in phagocytosis effect resulting from breed difference in tyvelose treated monocyte culture. Monocytes isolated from PBMCs of LR and Pi pigs were treated with tyvelose and incubated for 24 h and then evaluated for phagocytosis effect. Relative phagocytosis effect was determined for each breed with regards to respective control and then comparison between breed was made statistical analysis using t-test. Value presented as mean ± SEM. *** indicates $p <0.0001$. Pi: Pietrain, LR: German Landrace.

Expression dynamics of regulatory cytokines in TY treated näive PBMCs

The relative expression of IL-10, IL-6, IL-2, IFN-γ and TGF-β1 mRNA were examined in the TY treated and untreated control PBMCs using qRT-PCR (Fig. 2.5A-E)). Consistent levels of IL-10 was noticed in unstimulated PBMCs from both LR and Pi pigs. An increase in IL-10 mRNA expression was detected in the presence of TY compared to control PBMCs for Pi at 48 and 72 h and for LR at all three time points (Fig. 2.5A). The PBMCs of LR pigs were found to respond as early as 24 h in presence of TY and induced significantly high levels of IL-10 expression compared to untreated control PBMCs from LR. The peak of IL-10 expression level was at 48 h in PBMCs from Pi, whereas in LR, a significantly elevated level of IL-10 was maintained through 24 and 48 h of incubation. In both breeds, a gradual decline in IL-10 amount was noted after 48 h in PBMCs treated with TY.

Expression of IL-6 (Fig. 2.5B) also followed the trend of IL-10 in LR pigs. In the PBMCs of LR pigs, the IL-6 expression was significantly higher at 48 h compared to the control of respective time, whereas in Pi pigs, the IL-6 begun to rise after 24 h and was progressively elevated until 72 h of exposure to TY. This upregulation differed significantly ($p<0.05$) from the control group of respective time and Pi breed. The IL-6 expression was substantially altered by the treatment and the

effect of breed and duration of exposure. The effect of interaction of breed, treatment and duration was negligible.

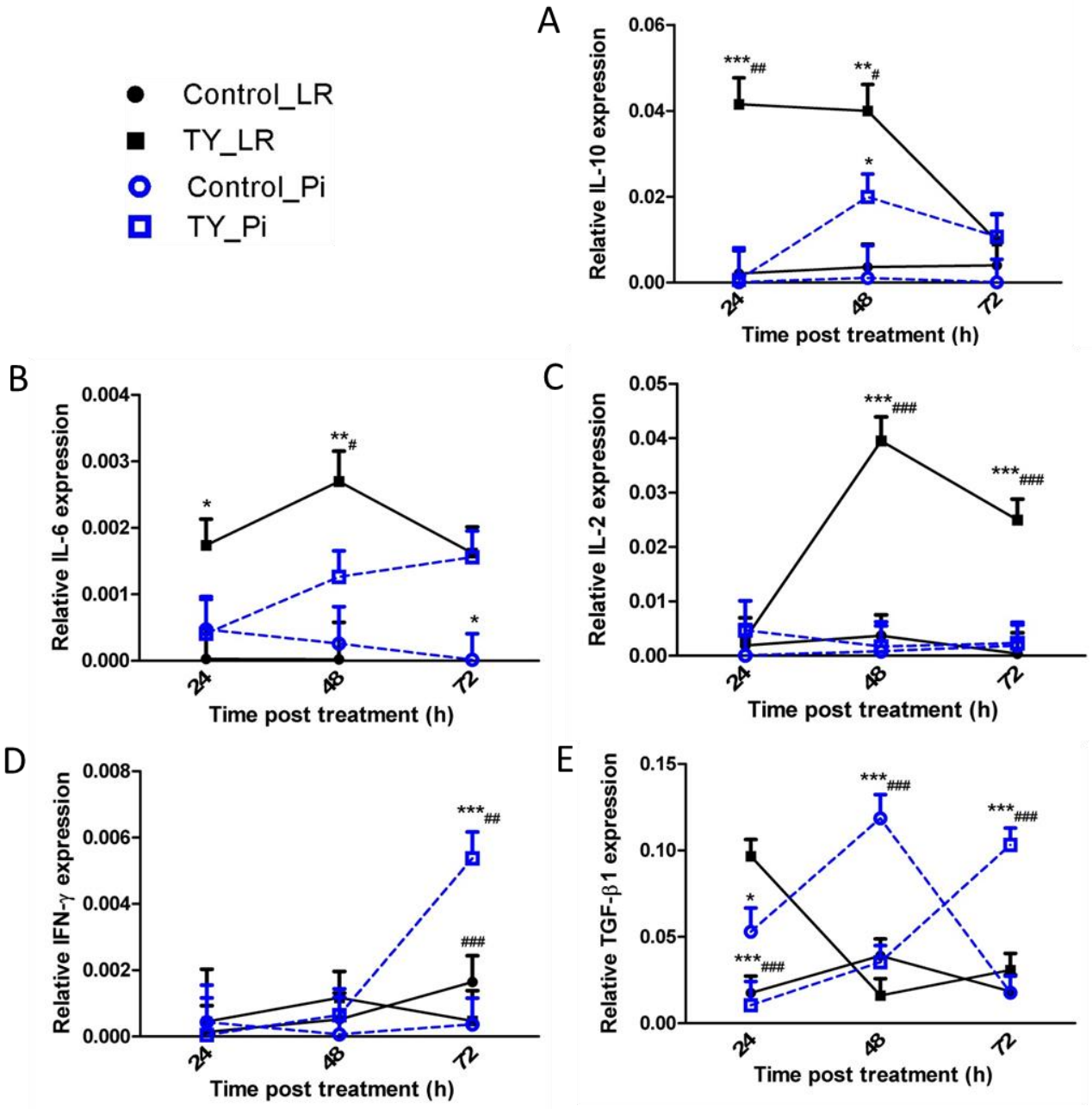

Fig. 2.5 (A-E). Expression dynamics of innate immune cytokines: A. IL-10, B. IL-6, C. IL-2, D. IFN-γ and E. TGF-β1 in TY treated PBMCs. The results were combined from four independent animals of LR and Pi origin. The data are represented as the least square mean ± SEM. *p <0.05; **p <0.01; ***p <0.0001 when compared to control vs treatment for same breed at same timepoint and #p <0.05; ##p <0.01; ###p <0.0001 when compared between breed with the same treatment and at same timepoint. Pi: Pietrain, LR: German Landrace.

The mRNA expression of IL-2 in LR pigs was elevated significantly at 48 and 72 h ($p<0.0001$) compared to the untreated control from the same group (Fig. 2.5C). Significant ($p<0.0001$) breed variation was evident at 48 and 72 h between TY exposed cells of LR and Pi pigs. On the other hand, TY exposure could not bring any potential changes in IL-2 level in PBMCs of Pi throughout the duration of experiment. There was no significant difference in IFN-γ expression noticed in PBMCs of Pi pigs between the TY treated and untreated group throughout the experimental duration (Fig. 2.5D). Conversely, 72 h post stimulated PBMCs of Pi pigs exhibited a sharp rise in IFN-γ expression which significantly differed from the respective control ($p<0.0001$) in Pi PBMCs, as well as from treated PBMCs of LR ($p<0.01$). It appeared that the effect of breed and treatment was not potential mediator for variation in IFN-γ expression; rather the duration of cultivation had substantial impact.

Under our experimental settings, there was a strong phenomenon of fluctuating expression of TGF-β1 across all three time points and also in untreated PBMCs (Fig. 2.5E). But in Pi, TGF-β1 followed the similar trend of IFN-γ throughout the duration of experiment. The dynamics in LR was not in accordance with that of TGF-β1 in Pi PBMCs. In LR, only at 24 h, the effect of treatment induced significant upregulation followed by a decline in the level of TGF-β1at later timepoints.

Breed differences on cytokine responses of TY treated and costimulated PBMCs

We examined the effect of TY on PBMCs post-stimulated with a combination of LPS and PHA and investigated the mRNA expression of IL-10, IL-6, IL-2, IFN-γ and TGF-β1 (Fig. 2.6A-E). For all five genes, the effect of treatment was found negligible, whereas the effect of breed was significant for IL-10, IL-6, IL-2, IFN-γ and TGF-β1. In mitogen-primed PBMCs culture of LR origin, TY has down regulatory effect on IL-10 expression until 48 h, after that there was a tendency to up rise, although this up or down regulation was not significantly different from the PBMCs only co-stimulated with mitogens (LPS plus PHA) (Fig. 2.6A). But the variation of IL-10 expression level was significant between breed at 24 and 72 h post-treatment group. TY treatment significantly up-regulated the IL-6 expression at 24 h post stimulation in LR, and significant breed differences on IL-6 expression was observed at 72 h post stimulation (Fig. 2.6B). In both breeds, TY could suppress mRNA expression of IL-2 only at 24 h time point and significant ($p<0.05$) breed variation was noted (Fig. 2.6C). The IFN-γ expression was significantly ($p<0.05$) altered only in PBMCs of LR pigs after TY treatment (Fig. 2.6D). The TGF-β1 expression in TY treated PBMCs showed a tendency of being upregulated at 24 and 72 h with a down regulatory trend at 48 h in the Pi group but upregulated at 72 h in the DL group (Fig. 2.6E).

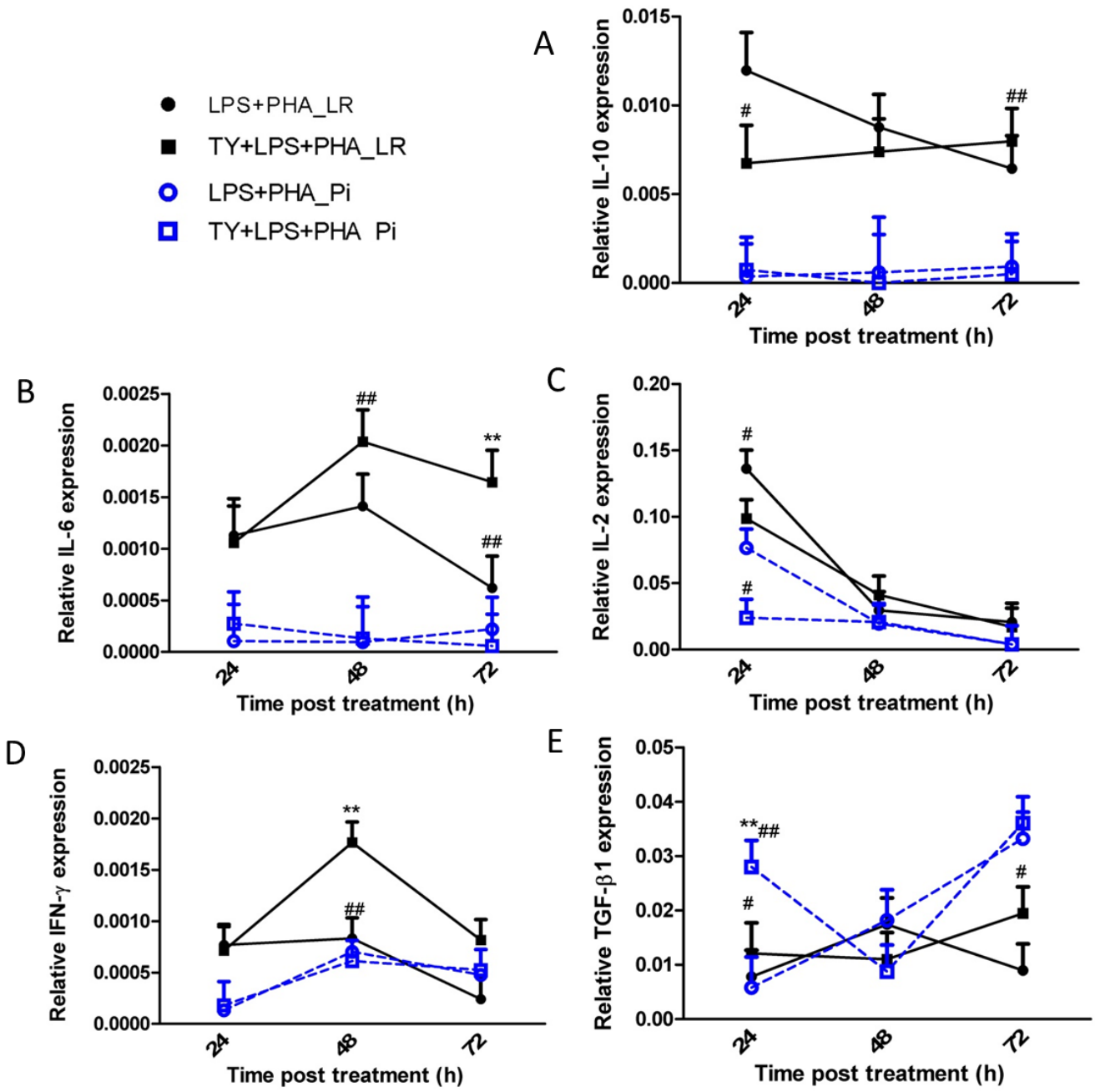

Fig. 2.6 (A-E) The mRNA expression of cytokines, A. IL-10, B. IL-6, C. IL-2, D. IFN-γ and E. TGF-β1 in TY treated and costimulated PBMCs. The results were combined with four individual animal replicates. The data were represented as the least square mean ± SEM. ***p<0.0001, **p<0.01 and *p<0.05 indicate comparison within breed with different treatment at the same timepoint and ###p<0.0001, ##p<0.01 and #p<0.05 indicate comparison between breed with the same treatment at the same timepoint. Pi: Pietrain, LR: German Landrace.

Overall expression of selected cytokines in post TY treated PBMCs from LR and Pi:

In both breeds, the microenvironment of each time point of TY treated PBMC culture was dominated by the relatively higher mRNA expression level of TGF-β1 and IL-10 compared to other cytokines (Fig. 2.7A and B). In LR, a relatively elevated level of IL-2 was also noticed while this was not so evident in the Pi group. IFN-γ and IL-2 expression in LR was not in accordance to each

other. In LR, dominant pattern of IL-10 and TGF-β1 was observed at 24 h post culture and IL-6 and IL-2 was reached their peak at 48 h. On the other hand, in Pi, IL-10 mounted the peak response at 48 h, while IL-6, IFN-γ and TGF-β1 at 72 h post treatment.

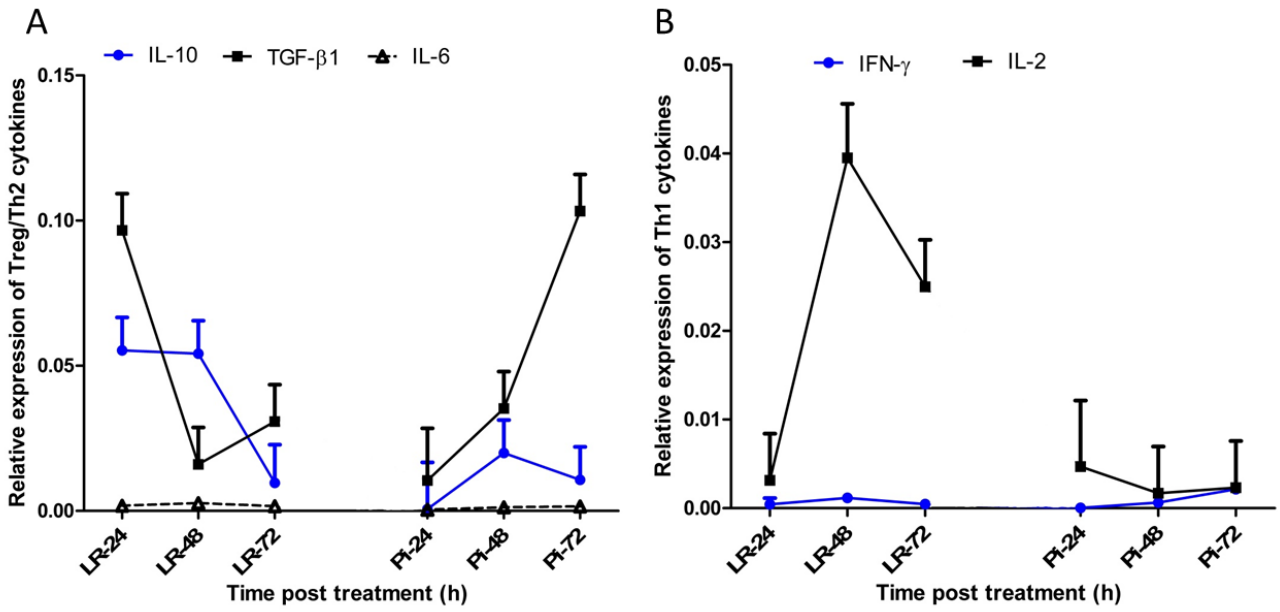

Fig. 2.7A-B Overall dynamics of TY induced Treg/Th2 cytokine expression (A) and Th1 cytokine expression (B) in TY treated naïve PBMC cultures of two pig breeds. Pi: Pietrain, LR: German Landrace.

2.5 Discussion

The ES products of the developmental stages of *T. spiralis* can regulate host immune response at the macrophages level via induction of alternative phenotypes in macrophages, which are likely crucial for worm survival and host health (Bai et al. 2012, 2016). We herein employed a suite of procedures that measure early activation of host immune response upon ML-ES antigen of *T. spiralis*, challenge in PBMCs in terms of cell viability, phagocytosis and proinflammatory cytokine expression. We have strategically chosen key sampling time points, such as 24, 48 and 72 h, reflecting the subsequent stages of innate immune activation as well as feasibility for the laboratory work. As well, we explored the breed differences in innate immune responses to *T. spiralis* derived TY. TY has been identified as being a major carbohydrate epitope of the *T. spiralis* muscle larvae (TSL)-1 antigen (Reason et al. 1994). The synthetic variant of TY (3,6-dideoxy-D-arabinohexose) (Williams et al. 1971) used in this study, offers advantage of stability and standardization and shows higher test specificity in many host species (Dea-Ayuela et al. 2001). Results from *Trichinella* seroprevalence studies in sylvatic and domestic animals demonstrated a very specific antibody response against synthetic carbohydrates compared to ES antigen (Moller et al. 2005). Although vaccination in mice with simple tyvelose with bovine serum albumin conjugate did not generate protective immunity

against intestinal form of *T. spiralis* inspite of the presence of tyvelose-specific antibodies (Goyal et al. 2002). Nonetheless, the role of TY on porcine host has not been well established in the current literature.

The phenotypes of lymphocytes as well as quantities are reflective to the impact of infection on the host (Mangino et al. 2017). From our study, TY was noted to enhance the viability rate, but the effect of different concentration of TY we attempted was not significant (Fig. 2.1A and B). Previous studies suggested that antigenic dose should not be too high to affect the cell viability through inducing significant cell death in PBMCs culture (Bai et al. 2012, McNeilly et al. 2013). From the present study, we observed a minimum fluctuation in the TY induced cell viability or cell death with a dose of 10 µg/ml in PBMCs from LR in both näive and costimulated PBMCs. This dose were used for subsequent part of this experiment and is comparable to the dose (5 µg/ml) used in the murine macrophage cell line with *T. spiralis* ML excretory secretory antigens (Bai et al. 2012). Moreover, adding a low-dose LPS and PHA in the one set of culture in our experiment was expected to promote the activation of PBMC subpopulations and subsequently trigger a broad range of cytokine signaling networks (Swennen et al. 2005, Viallard et al. 1999). Surprisingly, significant influence of breed on cell viability was observed between mitogen-primed TY treated groups of LR and Pi at 48 h time point where higher viability was observed in PBMCs from Pi compared to LR (Fig. 2.2B). Our result implied that TY, the synthetic sugar monomer is a potent antigen and able to trigger cell mediated response after primary exposure. The findings of our study also showed that TY alone also worked in a similar fashion to ES of Larva 1, perhaps as because of being the most dominating antigenic part (Wisnewski et al. 1993, Reason et al. 1994).

The phagocytosis assay involving different doses of TY treatment on naïve PBMCs of DL origin suggested that TY has enhanced phagocytic effect of sensitized monocytes, but this effect was not statistically significant and no significant variation was noted among different treatment concentrations (Fig. 2.3). This finding further supported the findings of dose dependent effect of TY on cell viability (Fig. 2.1A and B). As the rate of phagocytosis is also an indication of monocyte activation, maturation, antigen presentation and subsequent signaling for cytokine secretion (Trinchieri et al. 1993). These activities might trigger the lymphocyte population to generate necessary immune response. The most remarkable part of phagocytosis result is observed while comparing breed effects on phagocytosis of TY exposed monocytes (Fig. 2.4). Monocytes of DL origin had significantly ($p<0.001$) higher phagocytic potential compared to that of Pi pigs. LPS stimulated murine macrophages from different genetic background were found to respond

differently in kinetics and in capacities (Soudi et al. 2013). The phagocytic potentiality of alveolar macrophages challenged with porcine reproductive and respiratory syndrome virus was observed between Duroc and Pietrain pigs (Pröll 2014).

Typical helminth infections induce the Th2 cytokine (e.g. IL-10, IL-4, IL-5, IL-9, IL-13, IL-33 etc.) dominance in the host in addition to IgE and infiltration of eosinophils, basophils and mast cells where $CD4^+$ Th2 population serve as key players (Ilic et al. 2011, 2012). The effector T cells induced through in vitro stimulation with ES-L1 antigens produced increased amount of IL-4, IL-10, and TGF-β, with no capacity to produce IFN-γ (Cvetkovic et al. 2014, Gruden-Movsesijan et al. 2011). In our study, the IL-10, IL-6 and IL-2 cytokines in post TY treated PBMCs from LR was predominant during the 72 h of exposure, whereas IFN-γ had stable expression in both control and treated group (Fig 2.5.A-D). These findings confirmed the potential immunomodulatory role of TY in porcine PBMCs and are in accordance with the published in vitro and in vivo studies with human and mice models (Cvetkovic et al. 2014, Gruden-Movsesijan et al. 2011, Lei Fang et al. 2014, Guo et al. 2016, Li and Ko 2001). Local inflammation induced by the muscle stage larvae was found to be restricted by IL-10 during infection, but in chronic stage, mature ML1 produce TY bearing glycoprotein which promote a strong Th2 response mediated by IgG1 (Beiting et al. 2004). The IL-10 involved in regulation of innate and adaptive Th1 and Th2 responses by limiting T cell activation and differentiation in the lymphnodes as well as through suppressing proinflammatory responses in tissues (Couper et al. 2008, Guo et al. 2016).

IL-6 promptly and transiently produced in response to infection and tissue injuries, and contributes to both innate and adaptive immunity (Tanaka et al. 2014). The results of our study suggested that an elevated level of IL-6 mRNA expression in PBMCs of LR pigs resulted from TY treatment (Fig. 2.5B) as well as from combined TY priming and mitogen activated PBMCs (Fig. 2.6B). Similar finding was also reported by Fang et al. (Fang et al. 2014) who demonstrated that *T. spiralis* heat shock protein 70 (Ts-Hsp70) activated dendritic cells enabling the stimulation, proliferation and increased secretion of IL-6 in $CD4^+$ T cells from *T. spiralis*-infected mice.

In LR, the relatively higher level of IL-2 expression at 48 and 72 h of post TY treatment as compared to 24 h time point (Fig. 2.5C) is in concordance with the CCK-8 assay results in fig. 2.1A. A possible explanation could be that IL-2 mediated cytotoxic T cells ($CD8^+$) activation which may destroy antigen captured immune cells in the culture and facilitates cell death (Summerfield et al. 1998). Moreover, PBMCs obtained from trichinella infected human patients (n=10) showed an increase in $CD8^+$ cells with a decrease in $CD4^+$ cell population (Morales et al. 2002).

Relatively less alterations of cytokine expression were noticed in TY primed and mitogen activated PBMCs (Fig. 2.6A-E), which might be because of influence of dose used and synergistic potentiality of LPS and PHA. Results from our data indicated that TY suppressed the IL-2 expression in co-stimulated cell population at 24 h post culture (Fig. 2.6C) in both breeds. Similarly, ES L1 was reported to be able to interrupt the phosphorylation of signaling pathways triggered by other stilulants, i.e. LPS (Bai et al. 2012) and thus can alter the direction of cell maturation, activation, antigen presentation in the culture.

Overall cytokine expression in TY treated naïve PBMCs of LR and Pi pigs showed that the expression kinetics and dynamics was different in both breeds as noticed specifically from dominance of IL-10, IL-6 and IL-2 at 48 h in LR and distinctly high levels of TGF-β1 and IFN-γ at 72 h in Pi. Studies on same breeds showed that irrespective of the time of infection, global transcriptome analysis of the innate response in PRRSV infected macrophages cluster at different position (Ait-Ali et al. 2011). They did not find any breed dependent dominant pattern of response which is also in accordance with our study. The microenvironment of TY treated PBMCs in both breeds were dominated by high level of TGF-β1 and IL-10 (Fig. 2.7A and B). In the LR group, the highest expression level for IL-10 and TGF-β1 was observed at 24 h and for IL-2 mRNA at 48 h post TY treatment; whereas in the Pi group, the cytokines reached their highest expression for IL-10 at 48 h, for TGF-β1 at 72 h. In both LR and Pi groups, the expression level of IL-6 and IFN-γ appeared to be relatively linear at all three time points.

Taken together, IL-10 and Il-6 showed a breed-specific expression trend, and our data represented an earlier expression response in TY treated LR PBMCs. Although the mechanism behind this relatively early response in LR compared to Pi is less understood, especially for parasitic cases. However, a similar trend was reported from Ait-Ali et al. (2011) who found substantially higher expression of IL-10 and IL-6 as well as relatively early response from Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infected alveolar macrophages of LR pigs compared to Pi. LR and Pi pigs have also differed in susceptibility to porcine circovirus infection (Opriessnig et al. 2009). Inherent genetic variation has been reported between LR and Pi pigs in terms of nutrient utilization and metabolic functions (Ponsuksili et al. 2007). In our recent study, whole transcriptome profiles of PBMCs obtained from PRRSV vaccinated LR and Pi pigs revealed a distinct gene expression patterns (Islam et al. 2017). These findings would imply that PBMCs from landrace pigs are able to fasten the release the pro and anti-inflammatory cytokines, while the PBMCs of Pietrain was delayed in response. Therefore, it is reasonable that TY induced innate immunity in PBMCs

differs between LR and Pi pigs.

Conclusion

This study illustrated the acute effects of a single exposure to TY antigen on cytokine mRNA expressions of PBMCs in a time dependent induction of Th1/Th2 response in two pure porcine breeds. To the best of our knowledge, this is the first report on porcine breed variation in the innate immune responsiveness to antigenic part of *T. spiralis*, especially using synthetic tyvelose. Genetic variation in the intrinsic innate immune regulation was found to prevail among German Landrace and Pietrain. Further study and cross-examination are warranted to conclude that these variations resulted exclusively from host breed upon the exposure to helminth antigens. Our results showed that the PBMCs were able to critically mediate cell signaling to direct subsequent development of immunity after priming with TY. Thus, PBMCs appeared as a potential model for innate immune study of host-parasite interaction revealing relatively similar phenomena of the in vivo model. In addition, understanding of the immmunomodulatory properties of TY may also facilitate the current status of knowledge regarding its potential as vaccine and or novel therapeutics for immune disorders.

Acknowledgements

The authors are thankful to the staffs of research station at Frankenfrost for the animal husbandry support. The authors acknowledge the technical supports of Ms. Nadine Leyer and Ms. Birgit Koch-Fabritius. The author is also thankful to German Academic Exchange Service (DAAD) for providing the scholarship support.

Authors' contributions

SAR, MAI, CN and KS conceived and designed the experiments; CN arranged the kits, chemicals and supervised the works; SAR and MAI performed the blood sampling and cell culture works; SAR analyzed and interpreted the data; SAR and MAI drafted the manuscript; CN, MJP, CGB, KS critically reviewed the manuscript. All authors have seen and approved the manuscript for submission.

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Chapter 3: Effect of *Ascaris suum* haemoglobin on the cellular innate responsiveness in the peripheral blood mononuclear cells of German Landrace and Pietrain pigs

(Manuscript is under preparation for publication)

Effect of *Ascaris suum* haemoglobin on the cellular innate responsiveness in the peripheral blood mononuclear cells of German Landrace and Pietrain pigs

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3.1 Abstract

Ascaris suum is the largest intestinal nematode of pigs with significantly 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 culture. Temporal expression patterns of IL-10, IL-6, IL-2, IFN-γ and TGF-β1 mRNA level for three time points (24, 48 and 72 h) from two breeds (German Landrace, LR 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-β1 expression, although the interrelationship between these regulatory cytokines was not always synchronous. 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-β1 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, IL-2 response. There was also substantial influence of the host breed indicating breed variation in the innate immune responsiveness to ascariasis. Additional work is suggested to confirm these findings under in vivo situation.

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

3.2 Introduction

Helminth infection is a global problem for both animal production and public health, particularly affecting the warm tropical and developing countries. Among the pig helminths, *Ascaris suum* is the largest and most common intestinal parasite with substantial presence in all kinds of production system (Vlaminck and Geldhof 2013). These obligate endoparasites occur frequently in all kind of pig production systems, but the intensity is of economic concern especially for free-range, backyard and organic farms (Roepstorff et al. 1999, Carstensen et al. 2002, Boes et al. 2010). The *A. suum* infection results in pulmonary inflammation, reduced feed conversion and weight gain, milk spot liver in chronic cases leading to condemnation, and reduced efficiency of *Mycoplasma hypopneumoniae* vaccine (Thamsborg et al. 2013, Steenhard et al. 2009). These altogether cause considerable economic loss as well as animal welfare risk for pig production.

Helminths are reported to interact with host cells through their excretory-secretory products and can block both Th1/Th17- mediated inflammation and Th2 mediated pathologies (Else et al. 1994). AsHb is highly present in the pseudocoelomic fluid and is also found as excretory secretory (ES) product of the parasitic L3, L4 and mature forms (Keane-Myers et al. 2008). It has been reported that AsHb was able to scavenge activated porcine alveolar macrophage derived free radicals and able to reppress inflammation by enhanced production of IL-10 by activated mouse dendritic cells when co-stimulated with lipopolysaccharide (McConchie et al. 2006, Keane-Myers et al. 2008). Vaccination with AsHb is known to be involved in the enhanced hepatic white spot lesion (Vlaminck et al. 2011). The elevated expression of anti-inflammatory cytokines, e.g. IL-10, IL-4, IL-5, IL-9, IL-13 in conjunction with eosoinophilia and mucosal mastocytosis has been reported in pig ascariasis (Jungersen 2002). AsHb has been predicted to be able to suppress allergy, autoimmunity and malignancy (Keane-Myers et al. 2008). AsHb is a potent vaccine candidate which was criticized for increased hepatic lesion (Vlaminck et al. 2011). It is now an established serodiagnostic antigen (Vlaminck et al. 2012) and relatively inexpensive to produce. Until now, the immunoregulatory potentiality of AsHb directly in pig peripheral blood mononuclear cells (PBMCs) is not well elucidated.

Previous studies revealed that the host susceptibility to *A. suum* infection vary among pig population (Vlaminck et al. 2012, Skallerup et al. 2012, Skallerup et al. 2017). Heredity contribution for Ascaris worm burden in Danish-Landrace-Yorkshire crossbred pigs has been reported as 0.45 and 0.29-0.31 for fecal egg output on 7-14 day post-infection (Nejsum et al. 2009). The host response to experimental infection with *A. suum* differed between Large White and Mukota pigs (Zanga et al. 2003). Intra-strain variation in the susceptibility level of *Ascaris* in mice was also reported (Lewis

et al. 2006). Taken together, it can be assumed that breed might be a potential factor influencing host susceptibility to *A. suum* infection. Therefore, it is worth to look for animal breeds immunogenetically less susceptible to this parasite.

Gene expression studies in peripheral blood cells in response to *A. suum* antigens are rare, and the understanding of porcine breed predisposition in helminth induced immunity is limited as well. Since helminth antigens have been found to imitate the immune response to actual infection, we intend to investigate the pattern of innate response of *A. suum* antigen, AsHb, in the peripheral blood mononuclear cells of German Landrace (LR) and Pietrain (Pi) pigs.

3.3 Materials and Methodologies

Ethics statement

This experiment followed the institutional guidelines and animal husbandry regulations of Germany (ZDS 2006) in practice. The animal husbandry and hanLRing was approved by the Veterinary and Food Inspection Office, Siegburg, Germany (ref. 39600305-547/15) and permission for blood collection was obtained from North-Rhein Westphalian State Agency for Nature, Environment and Consumer Protection (ref. 84-02, 05.40.14.027).

Antigen and mitogen preparation

Purified *Ascaris suum* haemoglobin antigen (a kind donation from Prof. Dr. Peter Geldhof, Laboratory. of Parasitology, Ghent University, Belgium), was used as a representative antigen. Antigen purification was performed according to Vlaminck et al. (2011). As mitogen, lipopolysaccharide (LPS, L2880, Sigma Aldrich) and phytohaemagglutinin (PHA-M, 10576015, ThermoFisher Scientific, Germany) were used for co-stimulation of PBMCs culture.

Study population and blood sampling

Four clinically healthy female piglets of 5-6 weeks old German Landrace and Pietrain were used for this experiment. Animal numbers were estimated as adequate considering the resource equation method described by Festing and Altman (2002) and Festing (2006). All animals were weaned at the age of day 28 and obtained from the same source, the Teaching and Research Station of Frankenforst, University of Bonn, Germany. Upon birth, piglets were selected from a single sow for each breed, ear tagged, housed with standard feeding and housing condition and routinely vaccinated for mycoplasmosis at the age of day 7 according to the regular farm schedule; animals with any clinical illness at sampling time were not included in this study.

PBMCs isolation

The peripheral blood mononuclear cells (PBMCs) from whole blood were isolated by using Ficoll-Paque (Histopaque-1077, cat.# 10771, Sigma-Aldrich, Munich, Germany) density gradient centrifugation as described earlier by Islam et al. (2016).The cells were then suspended in complete RPMI-1640 media supplemented with heat inactivated 10% fetal bovine serum (cat.# F0804, Sigma Aldrich, Germany) and 1% antibiotic (Penicillin-streptomycin, cat#15140-122, Life Technologies GmbH, Darmstadt, Germany) and 1% antimycotics (Fungizone®, cat# 15290-026, Life Technologies GmbH, Darmstadt, Germany). Dose dependent effects of treatment were assessed only for LR pigs for convenience of the study.

Cell viability and proliferation assay

Time and dose dependent cell viability was assessed using CCK-8 cell proliferation kit (CK04-10, Dojindo Molecular Technologies, München, Germany) following the manufacturer's instruction. In brief, PBMCs at a density of 1×10^4 cells/well in 100 µl of RPMI-1640 media were cultured in 96-well plate with a dose (2.5, 5 and 10 µg/ml) of AsHb with and without follow up costimulation with LPS (100 ng/ml) plus PHA (10 µl/ml) after 30 minutes. Two technical replicates were made. For a period of 24, 48 and 72 h, the plates were incubated at 37 °C with 5% CO_2 and 98% air atmosphere. Control wells were left untreated or only costimulated with LPS plus PHA. Reconstituted CCK-8 mixture at 10 µl in each well were added and incubated for further 4 h. Then sample absorbance was measured at a wavelength of 450 nm using Synergy^{TM} H1 Multi-Mode Reader (BioTek Germany, Bad Friedrichshall, Germany). The cell viability was calculated as percentage according to the manufacturer's formula. The concentration of AsHb, which induced least alteration in the viability or cell death were used for subsequent studies.

Cell culture and stimulation of PBMCs for mRNA and protein samples

Similar to cell viability assay, PBMCs were cultured separately for each animal at $1x10^6$ cells/well in 2 ml media in a 6-well tissue culture plate for 24, 48 and 72 h. AsHb (2.5 µg/ml) was added in each well followed by with or without adding of LPS plus PHA costimulation. Naive controls were kept as untreated control and the control for costimulated group received only LPS plus PHA. The plates were maintained at 37 °C with 5% CO_2 before collection of cells.

Phagocytosis assay

In vitro phagocytosis assay was performed with Vybrant Phagocytosis Assay Kit (Cat# V-6694, Molecular Probes Inc.) according to manufacturer instructions as previously described (Islam et al. 2012). Determinations were performed in five wells for experimental, positive and negative samples

at 24 h post culture. The net phagocytosis of the cells was calculated following the manufacturer's formula.

RNA isolation and quality control

Total RNA enriched with microRNAs was extracted from cultured PBMCs by using the miRNeasy Mini kit (Qiagen, CA, Cat.# 2170040) according to the manufacturer's protocol. The total RNA was quantified by spectrophotometry (NanoDrop 8000 UV-vis Spectrophotometers (Thermo Scientific, Wilmington, USA). DNA contamination was removed using on-column treatment with RNAse-free DNase Set (cat.79254, Qiagen, CA). The fragments of 18s and 28s ribosomal band of total RNA were visualized by agarose gel electrophoresis. The absence of DNA contamination was checked by PCR amplification of housekeeping gene followed by visualization in 2% agarose gel.

Quantitative Real Time PCR

The selected genes quantified by qRT-PCR are presented in table 3.1.

Table 3.1 The sequences of primer sets used for relative expression analysis of cytokines by qRT-PCR

Gene symbol	**Accession no.**	**Primer sequences (5` -3`)**	**Amplicon size (bp)**
ACTB	XM-003124280.4	F:AAGGACCTCTACGCCAACAC R:CTTGCTGATCCACATCTGCT	207
PPIA	NM_214353.1	F:CACAAACGGTTCCCAGTTTT R:TGTCCACAGTCAGCAATGGT	171
IL-10	NM-214041.1	F:TCCGACTCAACGAAGAAGGC R:AACTCTTCACTGGGCCGAAG	179
IL-6	NM_214399.1	F:GGCAGAAAACAACCTGAACC R:GTGGTGGCTTTGTCTGGATT	243
IL-2	NM_213861.1	F:CTAACCCTTGCACTCATGGCA R:AATTCTGTAGCCTGCTTGGGC	185
TGF-β1	NM_214015.2	F:CGGAGTGGCTGTCCTTTGAT R:GGTTCATGCCGTGAATGGTG	186
IFN-γ	NM_213948	F:AGCTCCCAGAAACTGAACGA R:AGGGTTCAAAGCATGAATGG	225

F: Forward, R: Reverse, bp: Base pair

Primers were designed using an open source primer designing software Primer3. First Strand cDNA Synthesis Kit (P/N K1612, Thermo Scientific, Co.) was used for reverse transcription with oligo (dT) primers. The qRT-PCR reaction was set up using 2.0 µl of cDNA template, 7.0 µl of deionized RNase free water, 0.5 µM of upstream and downstream primers, and 10 µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad laboratories GmbH, Germany) in a total reaction volume of 20 µl and were amplified by the StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Darm-

stadt, Germany). The thermal cycling conditions were 95 ^{o}C for 3 min, 95 ^{o}C for 15 sec, 6 ^{o}C for 45 sec (40 cycles); 95 ^{o}C for 15 sec, 62 ^{o}C for 1 min, 95 ^{o}C for 15 sec. All reactions were run in single for four biological replicates and the expression value was obtained.

Statistical analysis

qRT-PCR data were analyzed using the GLM procedures of SAS software (Version 9.1.2, SAS Institute Inc., Cary, NC, USA). Gene-specific expression was measured as relative to the geometric mean of the expression of two housekeeping genes (PPIA and ACTB). The delta Ct (ΔCt) [$\Delta Ct = Ct_{target} - Ct_{housekeeping\ genes}$] values were calculated as the difference between target gene and reference genes and expression was calculated as $2^{(-\Delta\Delta Ct)}$. The mean differences of relative expression between treated and control groups were analyzed by One-way ANOVA and pairwise comparison was made using linear contrast. Comparison was always made between untreated control vs. AsHb treated cells and LPS+PHA treated cells vs. AsHb+LPS+PHA treated cells for better interpretation of the main effects (breeds, AsHb) in PBMCs with and without activation. Data is expressed as means ± standard errors of mean (SEM). Cell viability and phagocytosis data were analyzed using GraphPad Prism 5. The statistical significance level was set as to $p \leq 0.05$.

3.4 Results

Time and dose dependent effect of AsHb on the viability of PBMCs of LR origin

To assess whether AsHb suppress or stimulate the cell viability, PBMCs from four LR piglets were cultured separately in the presence of three different AsHb antigenic concentrations (2.5, 5 and 10 µg/ml) with/without co-stimulation with LPS plus PHA for 24, 48 and 72 h. A significant dose dependent decrease of viability was observed in naïve PBMCs treated with AsHb (Fig. 3.1A), where relative viability of only AsHb treated cells was calculated with regards to their respective untreated control. In the costimulated group, the viability percentage of cells with AsHb plus co-stimulation was calculated with regards to only co-stimulated cells. The decline of viability was evident only at 48 and 72 h time point (Fig. 3.1B). In both groups, naïve and co-stimulated, it was found that AsHb at a dose of 2.5 µg/ml resulted the least alteration in viability or cell death compared to two other concentrations used. Hence we used this relatively non-toxic, non-stressful dose for further molecular investigation.

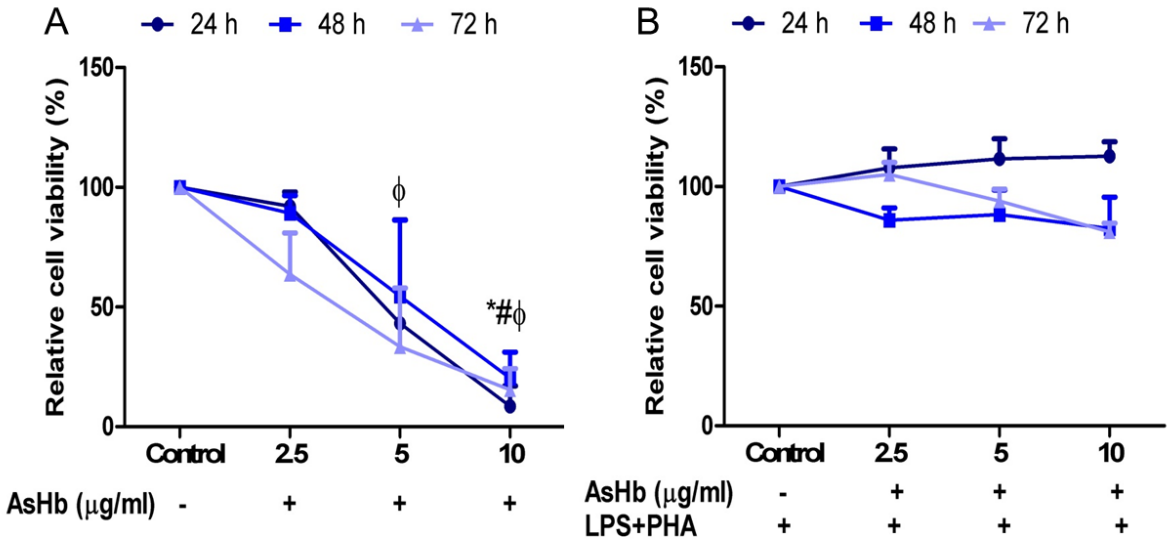

Fig. 3.1 Effect of AsHb on the proliferation and viability of PBMCs from LR. AsHb at a concentration of 2.5, 5 and 10 µg/ml was added to each experimental well (A and B) and for (B) 30 min later 100 ng of LPS and 10 µl of PHA per ml were added. PBMCs were left without any treatment only with media (A) and LPS plus PHA (B) as control for respective treated group. The culture was kept at 37 °C with 5% CO_2 for 24, 48 and 72 h. Cell viability was assessed using a CCK-8 assay. Values are mean ± SEM of three independent experiments for three timepoints in duplicate. *p<0.05, # p<0.05 and ϕ p<0.05 for comparison with respective control at 24, 48 and 72 h, respectively.

Effects of breed on the cell viability of AsHb treated PBMCs

The relative viability of PBMCs from LR treated with AsHb (2.5 µg/ml) from fig. 1A and 1B (with co-stimulation) were compared with the relative viability of PBMCs from Pi in a time-course manner and depicted as fig 3.2A and B, respectively.

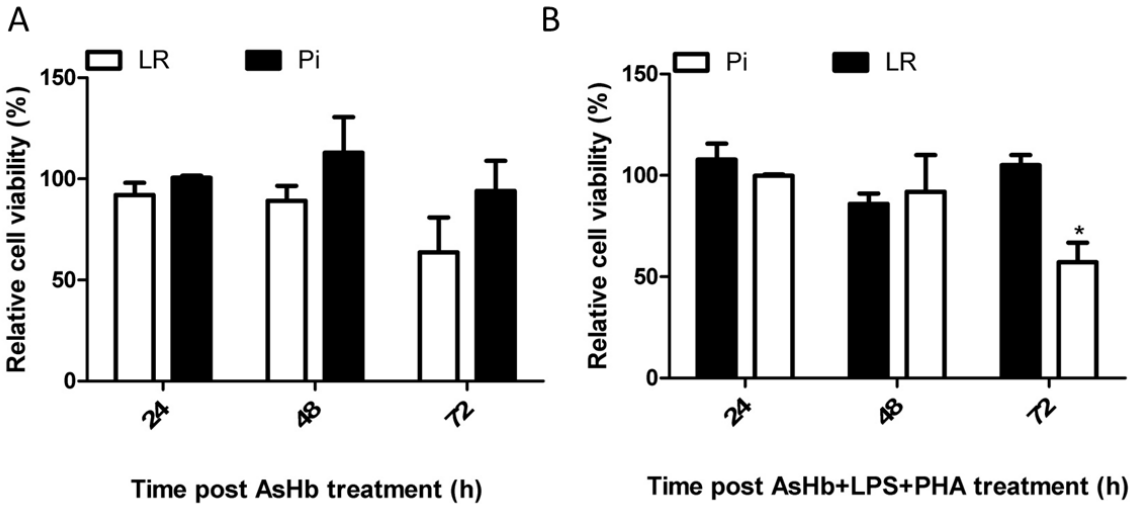

Fig. 3.2 Breed comparison of relative cell viability in a time dependent manner in only AsHb (2.5 µg/ml) treated (A) and AsHb (2.5 µg/ml) +LPS+PHA treated group (B). The results were combined from three independent experiments at three different time points and each experiment was performed in duplicate. The data are represented as the mean ± SEM.. * indicates p<0.05 for comparison between breed with same treatment and at same timepoint. Pi: Pietrain, LR: German Landrace.

Overall, the viability was higher in Pi PBMCs without any co-stimulation. In the costimulated group, PBMCs of LR origin had relatively higher viability compared to their respective counterpart of the breed at respective time. A significant ($p<0.05$) variation was found only at 72 h when AsHb treated co-stimulated groups from LR and Pi were compared (Fig. 3.2B).

Dose dependent effect of AsHb on phagocytosis

Phagocytosis is one of the key phenotypic indicators of innate immune response. So, PBMC derived monocytes of LR piglets were evaluated for their phagocytic ability with three different concentrations of AsHb. This antigen was found to enhance phagocytosis in the culture in a dose-dependent manner. Monocytes treated with AsHb (5 µg/ml) were found to trigger phagocytosis significantly compared to the untreated control (Fig. 3.3).

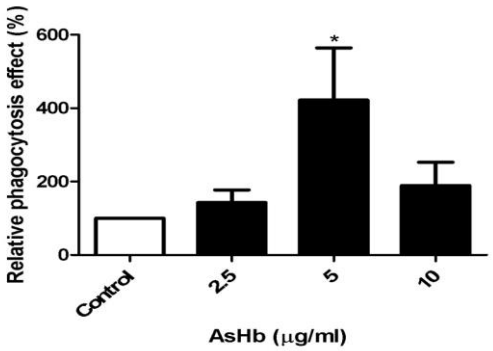

Fig. 3.3 AsHb induced phagocytosis by peripheral blood monocyte culture of LR pigs. The phagocytic capacity of this population is represented as percentage. The result is a combination of 5 replicates in each treatment group. The value is expressed as mean ± SEM. * indicates $p<0.05$ for comparison between control vs treated group.

Influence of breed on AsHb treated phagocytes

The functionality of monocytes was found to be affected by host genetic factors and hence, phagocytic potentiality may vary between hosts with different genetic background. Relative phagocytic ability of AsHb (2.5 µg/ml) treated monocytes from PBMCs of LR and Pi origin were compared, where the control were their own untreated monocytes. Variation ($p<0.05$) in the phagocytosis rate was observed where monocytes derived from PBMCs of LR pigs showed higher ability for phagocytosis compared to that of Pi (fig. 3.4).

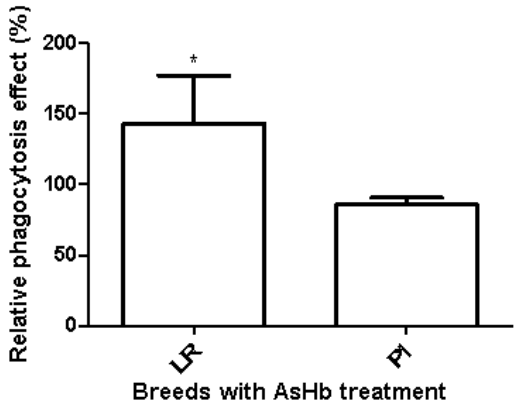

Fig 3.4 Variation in phagocytosis effect resulting from breed difference in AsHb treated monocyte culture. Monocytes isolated from PBMCs of LR and Pi pigs were treated with AsHb and incubated for 24 h and then evaluated for phagocytosis. Comparison between breed was made using GraphPad Prism plotting relative phagocytosis effect against breed. The values are expressed as mean ± SEM. * indicates $p<0.05$. Pi: Pietrain, LR: German Landrace.

mRNA expression of cytokines in naïve PBMCs treated with AsHb

Relative cytokine expression was measured in PBMCs from LR and Pi piglets challenged with AsHb (2.5 µg/ml) in relation to untreated control PBMCs. A steady and progressive rise of IL-10 mRNA was observed in AsHb sensitized PBMCs of LR origin throughout the duration of cultivation and the upregulation significantly differed between breed at all three time points (Fig. 3.5A). For both breeds, the peak of IL-10 surge was observed at 72 h of culture. But in case of Pi, the responsiveness was not significantly evident until 72 h of incubation. Quantitative and qualitative kinetics of IL-2 expression was similar between LR and Pi, where the peak of IL-2 expression was noticed at 48 h of culture and the effect of treatment was significant ($p<0.05$) (Fig. 3.5B) .The relative expression of IL-6 was upregulated across the experimental period in LR group (Fig. 3.5C). But in Pi, the upregulation was more pronounced at 24 and 72 h post treatment. Significant breed and AsHb effect was evident only at 48 h of post treatment. The relative IFN-γ expression was slightly elevated 48 h after culture, but overall the effect of breed or treatment was not statistically significant (Fig. 3.5D). In both LR and Pi group, there was a tendency of downregulation of TGF-β1 followed by an upregulation at 72 h in the AsHb treated group (Fig. 3.5E).

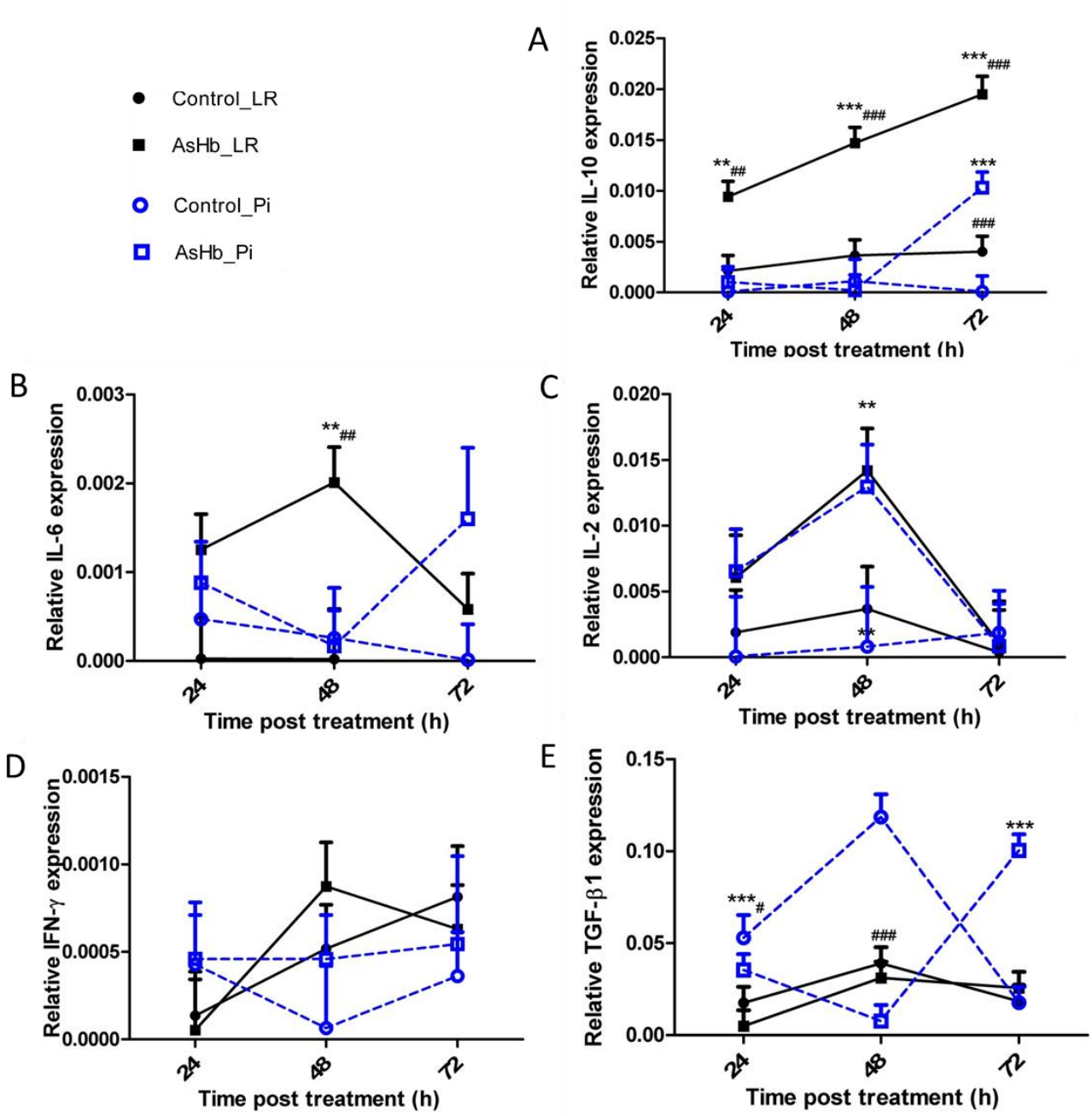

Fig. 3.5 (A-E) Expression dynamics of innate immune cytokines A. IL-10, B. IL-6, C. IL-2, D. IFN-γ and E. TGF-β1 in AsHb treated PBMCs. The results were combined from four independent animal experiments. The values show the differences between non-treated controls versus AsHb treated PBMCs. The data are presented as the least square mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared within breed between control vs. treated group at same point of time and # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ when compared between breed with same treatment at same point of time. Pi: Pietrain, LR: German Landrace.

Effect of AsHb on cytokine mRNA expression in co-stimulated PBMCs

Cytokine mRNAs level was measured in PBMCs from LR and Pi treated with AsHb followed by LPS plus PHA costimulation (Fig. 3.6A-E). Cells treated with only LPS+PHA were considered as control for respective timepoints.

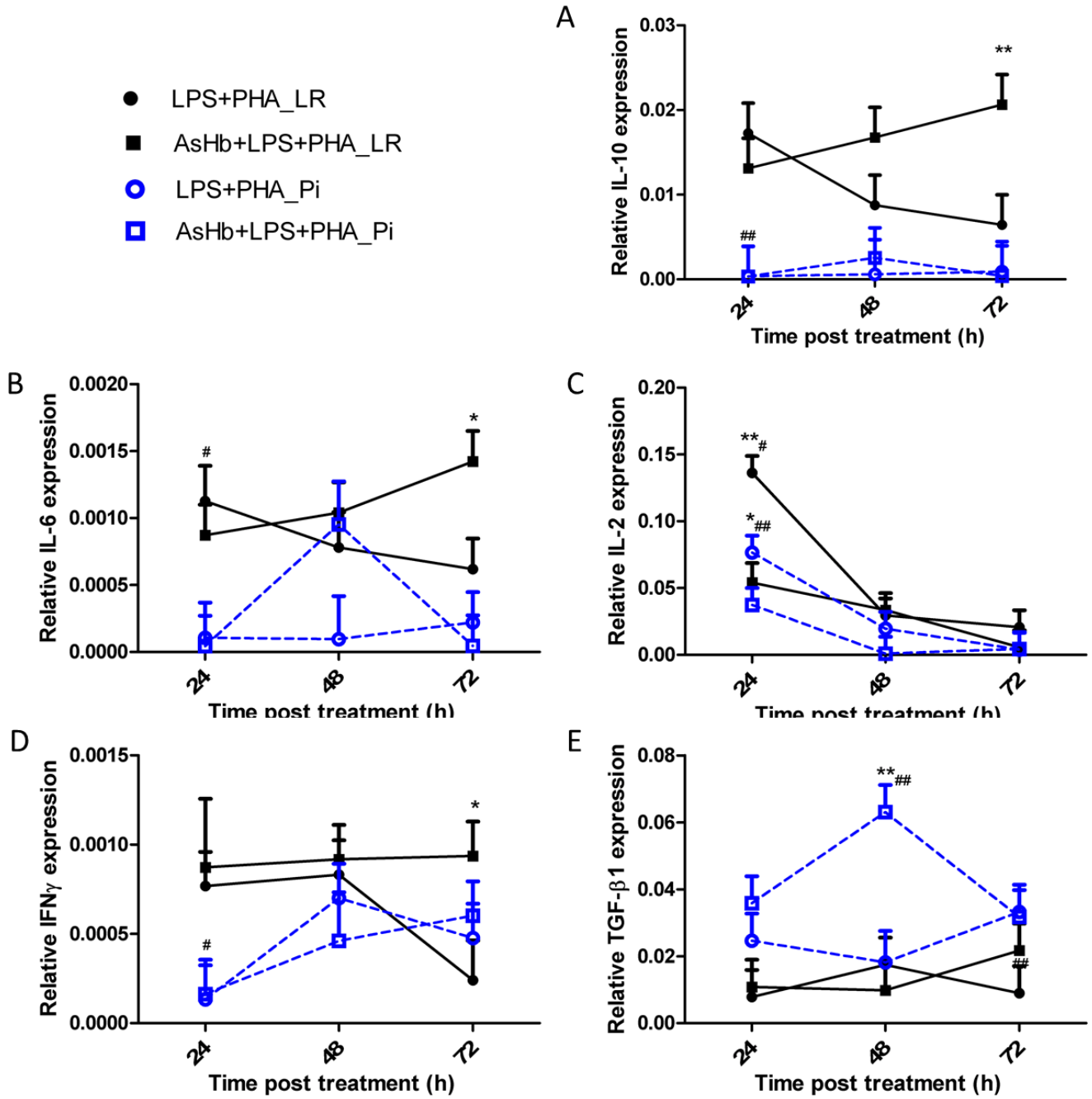

Fig. 3.6 (A-E). The mRNA expression of pro- and anti-inflammatory and regulatory cytokines, IL-10, IL-6, IL-2, IFN-γ and TGF-β1 in AsHb treated PBMCs. The results were combined with four individual animal replicates. The data are represented as the least square mean ± SEM. Asterisks (***p<0.0001, **p<0.01 and *p<0.05) indicated comparison within breed at different treatment at same timepoint and hashes (###p<0.0001, ##p<0.01 and #p<0.05) indicate comparison between breed with the same treatment at same timepoint. Pi: Pietrain, LR: German Landrace.

Addition of AsHb in the culture resulted in similar trend of kinetic change for IL-10 and IL-6 in LR PBMCs, where at 24 h AsHb induced mRNAs was suppressed followed by progressive upregulation until 72 h of the culture (Fig. 3.6A-B). On the contrary, in Pi, no significant variation from AsHb exposure between co-stimulated cultures was noticed except at 48 h, where there was a dramatic rise of IL-6 which again dropped back to control level after 24h. The pattern of IL-2

production was similar in both breeds and significant level of suppression was noticed at 24 h time point resulting from breed and treatment factors (Fig. 3.6C). The influence of breed and treatment was found inconsistent and had less specific effect in the expression dynamics of TGF-β1 and IFN-γ within this 72 h of experimental duration (Fig. 3.6.D-E). TGF-β1 expression in Pi at 48 h had a significant ($p<0.01$) rise compared to its own control as well as LR counterparts. IFN-γ level only significantly changed from treatment at 72 h in PBMCs of LR pigs.

AsHb induced overall mRNAs expression in naïve and costimulated PBMCs

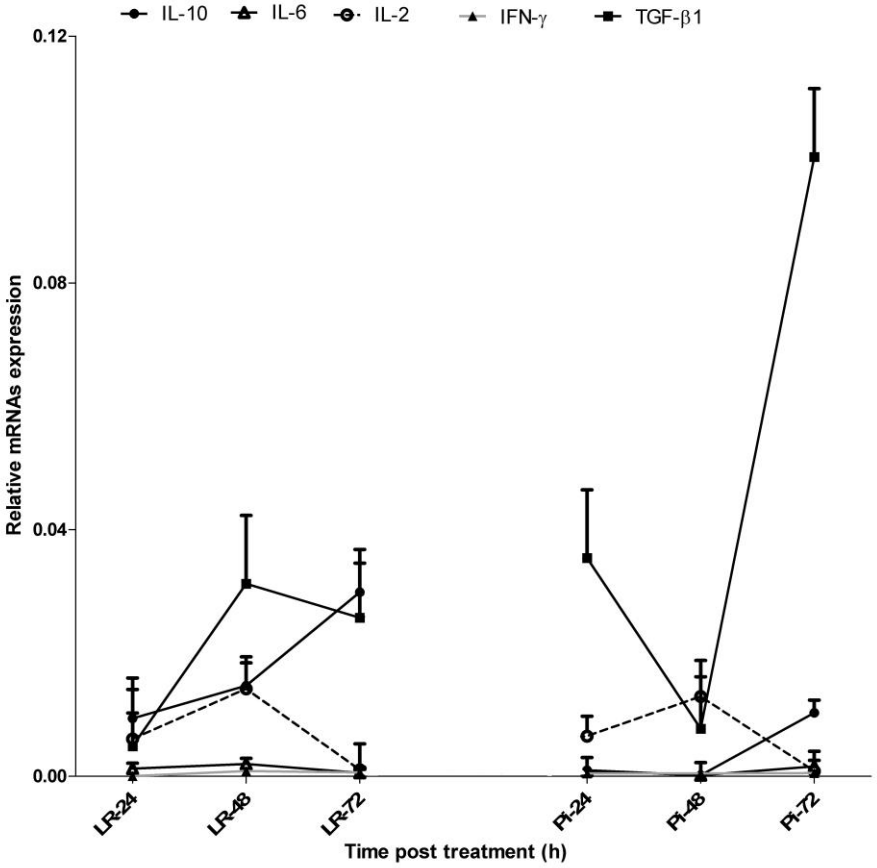

Fig. 3.7 Overall dynamics of AsHb induced cytokine expression in LR and Pi in AsHb treated PBMCs cultures. In both breeds, the microenvironment of each time point of AsHb treated PBMC culture was dominated by the mRNA expression level of TGF-β1 and IL-10 compared to other cytokines. In the LR group, a relatively elevated level of IL-10 and IL-6 at all three time points was observed. IFN-γ and IL-2 expression in LR and Pi was in accordance to each other. IL-10 and IL-6 were in a harmony in at all the time points in LR. This result suggested the role of individual microenvironment also being crucial in determining the ultimate outcome of antigenic challenge. Pi: Pietrain, LR: German Landrace.

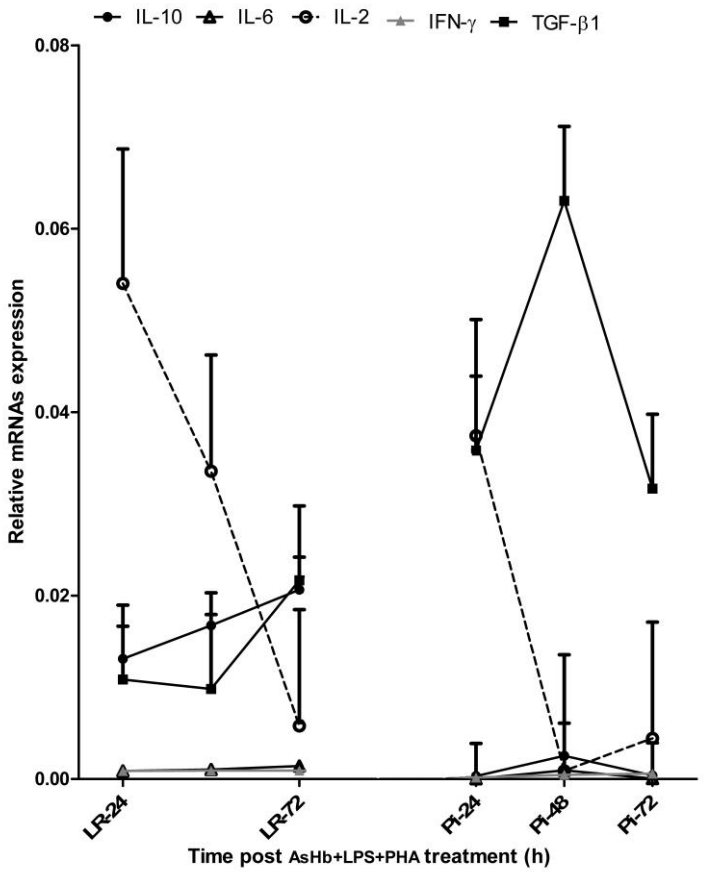

Fig. 3.8 Overall dynamics of AsHb induced cytokine expression in costimulated PBMCs from LR and Pi pigs. The local microenvironment in LR had a dominance of IL-2 until 48 h, whereas in Pi, there was equilibrium at 24 h between IL-2 and TGF-β1 followed by dominance of TGF-β1 until 72 h. In LR, TGF-β1 and IL-10 was progressively increasing towards 72 h, but in Pi, the elevation of IL-10 was not distinct from the respective control. This suggested that the individual microenvironment is affected by the duration of exposure as well by the host genetics. Pi: Pietrain, LR: German Landrace.

Overall expression of tested cytokines in AsHb treated naïve PBMCs (Fig. 3.7) and costimulated PBMCs (Fig. 3.8) revealed that there was difference in the cytokine dominance in the microenvironment created by AsHb treatment between breeds and between with or without costimulated PBMCs. In naïve PBMCs, there was dominance of TGF-β1 in both LR and Pi pigs, but in LR, it was accompanied with high level of IL-10. In costimulated groups, the LR groups were dominated by IL-2 followed by IL-10 and TGF-β1, while in Pi groups, the predominant cytokine was TGF-β1 followed by IL-2 and IL-10.

3.5 Discussion

Biologically active excretory-secretory proteins from helminthes with zoonotic implication is continuously receiving great interest not only as a prospective vaccine candidate, but also as therapeutic agents for several human immune-related disorders like allergy, asthma, autoimmunity, malignancies. The excretory-secretory products (Andrade et al. 2005) and lipid products (Kean et al. 2006) of

A. suum have been shown to trigger in vitro nitric oxide from macrophages. While the pseudocoelomic fluid (McConchie et al. 2006) and high molecular weight components (Silva et al. 2006) derived from *A. suum* can suppress dendritic cell response to secondary antigens. The *A. suum* antigens possess immunomodulatory properties and are able to downregulate the expression level of co-stimulatory molecules on dendritic cells both in vivo and in vitro studies (Boesen et al. 2006). Also various *A. suum* products can alter expression of allergic diseases (Itami et al. 2005, Araujo et al. 2008). Suppression of innate immunity is thought to be a mechanism responsible for decreased allergic inflammation in pseudocoelomic fluid treated animals. However, porcine ascariasis is reported to sensitize lymphocytes and suppresses their responsiveness to phytomitogens (Barta et al. 1986). Hence, characterization of the role of individual molecules of *A. suum* in the regulation of immune cells as well as the identification of mechanisms involved needs to be investigated. The understanding of innate immune interaction of *A. suum* on porcine PBMCs is limited, particularly in relation to the haemoglobin antigen and variation in innate immune responses among different breeds are not well addressed.

High molecular weight components of A. suum are known to suppress costimulatory molecules responsible for immune signal transduction (Silva et al. 2006). Findings from our cell viability assay suggested that AsHb had suppressive effect on the viability of PBMCs in culture in a dose-dependent manner (Fig. 3.1A and B). A similar finding was reported by Deehan et al. (2002) who demonstrated that LPS or antibody induced proliferation of splenic B cells was significantly reduced in presence of *A. suum* derived phosphocholine containing glycosphingolipids. In addition, actively secreted protein of nematodes are known to suppress the proliferation and cytokine production of antigen specific T cells by inducing cell death in case of *Onchocerca volvulus* (Hartmann et al. 2013). Further validation of this AsHb mediated lympho-depletion in PBMC culture might be useful to exclude any other possible contributing factors.

Breed specific differences in cell viability were assessed in AsHb treated naïve (Fig. 3.2A) and co-stimulated culture (Fig. 3.2B). Significant variation in breed specific viability was noticed only in co-stimulated groups after 72 h of cultivation. The background behind the distinct variation being dominant only at 72 h was not clearly understood. One reason might be the general trend of slower development of immunity against parasitic infection (Yazdanbakhsh and Sacks 2010).

Phagocytosis is one of the vital events of innate immune function. *Ascaris suum* infection was reported to reduce phagocytic efficiency of LPS activated alveolar macrophages from experimentally infected pigs (Keane-Myers et al. 2008). Repeated infection with *A. suum* in pig was reported to be

associated with increased eosinophilia, reduced phagocytosis and a decrease in intracellular tyrosin phosphorylation. Results from our phagocytosis test (Fig. 3.3) showed that AsHb at a dose of 2.5 was not able to significantly increase or decrease phagocytosis rate and the effect was in a dose-dependent manner. The reason behind might be the variation in the subsets of monocytes and DCs, as the examined duration of antigen pulsed monocyte culture was only 24 h post cultivation. The discrepancy between our study and previous reports might have resulted from the use of different culture model with different activation state of cells and from difference in the animal age. Keane-Myers et al. (2008) used LPS treated alveolar macrophages of experimentally infected pigs whereas our model was monocytes of PBMC origin from clinically healthy piglets. Findings from our study further suggested that there was significantly higher phagocytic efficiency of AsHb treated monocytes of LR pigs compared to Pi (Fig. 3.4). Besides, the observed priming for phagocytosis by AsHb may serve to enhance the response to secondary parasite challenge or alternatively support scavenger-like functions such as the removal of damaged or dead cells either from host or parasite as noticed in *A. suum* body fluid mediated response in mouse granulocyte model (Falcone et al. 2001).

The typical feature of anti-helminth immune response is Th2 cell type response accompanied by eosinophilia and mucosal mastocytosis (Jungersen 2002). IL-10 has been reported to be responsible for immunesuppression by high molecular weight proteins of *A. suum* in mouse models (Souza et al. 2004). In our study, AsHb treatment was found to be associated with elevation of IL-10 expression at all three time points in PBMCs from LR piglets (Fig. 3.5A). This increased level of IL-10 during helminth antigen exposure could contribute to the immune homeostasis by raising the threshold for the induction of effector inflammatory response to the challenge. On the other hand, the response was a bit slower and no distinct response was detected in antigen treated PBMCs of Pi origin until 72 h of incubation. The reason for this delayed response is not clearly understood. Ait-Ali et al. (2011) also noticed this breed dependent variation in the timing of immune activation in LR and Pi pigs where in vitro macrophages were challenged with Porcine Reproductive and Respiratory Syndrome virus. However, in general, development of helminth immunity in the host is relatively slower compared to other microbial unicellular pathogens.

Although IL-6 is one of the key cytokine in health and disease, the role of IL-6 in case of porcine ascariasis is not well-delineated. In the current study, AsHb induced progressive elevation of IL-6 in both PBMCs with or without co-stimulation and this was in coordination with IL-10 with an exception at 72 h in mitogen treated PBMCs (Fig. 3.5B). In naïve T cells, IL-6 trigger Th17 immunity in

presence of TGF-β, while in contrast, it also restricts TGF-β-induced Treg differentiation (Kimura and Kishimoto 2010, Fernando et al. 2014).

The modified polarization dominated by IL-10, IL-6 might be a tolerant phenotype that is different from classical IL-4 induced Th-2 immunity that is considered to drive allergic inflammation. Such modified Th2/Treg like response representing a regulated Th2 response may be an important feature of balanced parasitism that ensures parasite survival but also protect the host from Th2 induced pathology. In human ascariasis, parasitism is linked with a regulatory set of cytokines, as IL-10 and TGF-β are significantly linked with hyporesponsiveness and susceptibility (Reina Ortiz et al. 2011). In mice, repeated infection with *A. suum* was found to induce a higher level of circulating inflammatory cells and increased production of systemic cytokines, mainly, IL-6, including the rise of IL-5, IL-4, IL-10, IL-17A and TNF-α compared to single infected one (Nogueira et al. 2016). From our study, IFN-γ was found to show no significant alteration from treatment in both breeds LR and Pi which was consistent with the findings of Dawson et al. (2005) who reported no variation between experimentally infected and control pigs.

Mitogen induced IL-2 production has been reported as a promising marker for immune responsiveness in pig PBMCs and associated with genetic variability (Mach et al. 2013). Activated T cells can elicit IL-2 which can serve in manipulating non-specific and specific immunity. Cell mediated immune response evident from antibody production, cytotoxic T cell activity and prolonged proliferation of activated T-cell clones are all IL-2 dependent. In our study, effect of breed and AsHb mediated suppression of IL-2 expression was clearly evident at mitogen activated PBMCs at 24 h stimulation (Fig. 3.6C). This difference was no further evident later. It might be because of the concentration of antigenic dose used along with the possibility that the effect of mitogen has overcome the effect of AsHb mediated suppression. This is further supported by the fact that the LPS plus PHA mediated cell growth reaches its peak at 72 h of exposure (data not shown).

In our study, we found that in both naïve and costimulated PBMCs from Pi were dominated by TGF-β1 (Fig. 3.7 and 3.8). TGF- β is also known as immunosuppressive cytokine. TGF-β has been reported to be associated with age-dependent resistance development in pig ascarisis (Helmby 2015). We can not associate our findings with this statement as we used animals of a specific age group. Epidemiological data suggests that the prevalence of infection was higher in young growing pigs (i.e. weaners and fatterners) compared to sows or boars (Roepstorff and Nansen 1998). So the choice of post-weaned piglets for this study was rational. The in vitro model has some advantages over in vivo in case of *A. suum* because experimental infections are characterized by a high degree

of unpredictability with regard to the outcome of a patent infection. Typically the population is over dispersed with only a few pigs harboring the majority of worms, some having only light worm loads and most pigs have no infection as reviewed by Jungersen (2002). Moreover, in vitro situation provides a better opportunity for control and excludes the exogenous environmental influence on animal health and immunity.

Since, AsHb causes very fast activation of DCs to produce IL-10 hours after the activation and then act via a negative feedback mechanism to stop an ongoing effector response, so it can be a potent adjuvant. In essence, whether AsHb has a stimulating or suppressive effect depends on the microenvironment of the cells or culture (Keane-Mayers et al. 2008). Since in our study, ficoll-gradient centrifugation method was used to isolate the PBMCs, it was not possible to exclude other cell types e.g. platelets besides T cells. We also did not rule out the contaminating endotoxin or toxic low molecular contaminants as suppressive elements in the complete media prepared. And these might also have effect on our experimental results.

3.6 Conclusions

In conclusion, preliminary evaluation of effect of AsHb revealed that the breed associated variation in the innate immune responsiveness in pigs exists between LR and Pi pigs in a time-course dependent manner. Further studies are needed to better understand the immune mechanisms that induced this variation even if there were some similarities in some specific immune cytokine expression. This study also suggested that AsHb could result Th2/Treg response in PBMCs culture due to the evaluation of a limited number of cytokines. In addition, it can be concluded that in-vitro PBMCs is a potent model for host immunogenetic study for parasitic cases.

Acknowledgements

The authors are thankful to Prof. Dr. Peter Geldhof and Dr. Johnny Vlaminck, Ghent University, Belgium, for their kind support with *Ascaris suum* antigen and helpful suggestions. The author would like to acknowledge staffs of research station at Frankenfrost for the animal husbandry support and Ms. Nadine Leyer for technical assistance. The author is also thankful to German Academic Exchange Service (DAAD) for providing the scholarship support.

Author's contribution

SAR, MAI, CN and KS conceived and designed the experiments; CN arranged the kits, chemicals and supervised the works; SAR and MAI performed the blood sampling and cell culture works; SAR analyzed and interpreted the data; SAR wrote the first draft of manuscript. MAI, CN and KS critically reviewed the manuscript.

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Chapter 4: General discussion and conclusions

4.1 General discussion

Helminths are master as host immunomodulators through a variety of debilitating mechanisms using their antigenic molecules. The possibility of molecular crosstalk between helminth antigen and the mammalian immune system, and the progressive understanding that helminths impair the host innate ability in generating immune reaction also against other antigens or pathogens make the immunology of helminth infection an complex, crucial, fascinating and challenging choice for investigation (MacDonald et al. 2002).

Primarily, the access and use of a relatively easily reproducible model system is needed for experimentation. In this perspective, porcine PBMCs is already well-established model for studying pathogenesis and immunogenetics (Islam et al. 2016, Uddin et al. 2012, Wilkinson et al. 2012, Fairbarin et al. 2011, Gao et al. 2010). Moreover, the in vitro PBMCs model is a good choice for immunogenetic studies for two reasons. One, being a primary cell culture model, PBMCs might have more resemblance to the in vivo situation compared to other secondary cell culture for studying disease-specific immunogenetics and excludes the possibilities of external environmental or heath condition change related effect on the output (Kapetanovic et al. 2013). Secondly, respective control without stimulus for each biological replicate is possible throughout the duration of the in vitro experiment (Kapetanovic et al. 2013). Most importantly, in vivo experimental infection with porcine ascariasis and trichinellosis results in a high degree of unpredictability in the outcome of patent infection (Skallerp et al. 2017, Jongersen et al. 2002, Lunney et al. 1988). It is because of the fact that in both cases, pigs, even from the same line, show a varying degree of resistance in harboring the infection. Moreover, PBMCs are the easily accessible tissue sample without doing significant harm to the donor. In addition, cell to cell interaction can be considered with possible exclusion of the presence of RBCs, serum, plasma, platelets. Antigenic response specific production of cytokines can be measured easily in supernatant as well as on cell lysate.

Next to this model issue, we should consider that the use of live or whole worms outside of their natural host habitat is hard and most of the excretory-secretory (ES) preparations are a heterogenous mixture of molecules. Therefore, it is difficult to identify signaling molecules to pathogen recognition receptors to ensure subsequent transduction pathways. Purified native antigens from helminthes or recombinant antigens provide a promising solution to this problem. Hence, native antigens obtained from the parasite or commercially synthesized antigens were used to mimic primary infection conditions. In this study, *A. suum* haemoglobin and synthetic homolog of *T. spiralis* derived tyvelose were used. AsHb is the highly produced, one of the major ES antigen of L3, L4 and adult ascaris worm (Keane-Myers et al. 2008) and it is easy to produce, inexpensive,

highly reactive against the hepatic migratory phases of parasites, which is the most crucial point of economic loss in porcine ascariasis. Besides, tyvelose is highly immunodominant epitope of *T. spiralis* muscle larvae stage which is the infective stage and very efficient host immune modulator encouraging cyst survival and persistence (Beiting et al. 2004). It is commercially available stable and relatively cheap antigen. Both AsHb and tyvelose are two recognized serodiagnostic antigens (Vlaminck et al. 2012, Wisnewsky et al. 1993). Therefore it might be expected that these antigens will interact with the host at the level of systemic immunity irrespective of host breed or geographic location.

In this study, the immunogenetic variation was studied in the piglets from German Landrace and Pietrain pigs, because of the their better production performance as breeding animal and as meat producer respectively compared to indigenous breeds of helminth endemic regions. Many developing countries with tropical and subtropical climate and high frequency of helminth infection are interested and some are still practicing exotic breeds in breeding as well as to enhance farm production. But one major constrain with these exotic breeds appears to be the disease resistance in addition to the environment stress. Breeds with high productivity traits as well as relatively high resistance to local infections might be a good choice for profitable farming. While investigating possible differences among the breeding lines used in this study, various environmental factors with a potential influence on susceptibility, such as origin of pigs, age, sex, vaccination and feeding were standardized. Post-weaned piglets appear to be relatively more vulnerable for ascariasis according to the epidemiological data experienced (Roepstorff and Nansen 1994, Boes et al. 2010).

Generally, upon exposure to pathogen, a couple of days is required to develop antigen-specific Th cell responses. Therefore, innate immune responses are very crucial for restricting the early establishment and expanding actions of invading pathogens or foreign antigens (Koyasu and Moro 2013). Moreover, innate immunity in parasitic cases is less investigated so far. Next to this, the question of which helminth parasites to study arises. We selected *A. suum* and *T. spiralis* because of their significant role in farm productivity and profitability and for their role as zoonoses. Furthermore they represent intracellular and extracellular gastrointestinal nematode group and current knowledge on the effect of selected antigens in pig PBMCs in terms of innate immunity and host breed variation have not been previously investigated.

In this dissertation, the immunogenetic variation in susceptibility to *A. suum* and *T. spiralis* between two established pure breeds, German Landrace and Pietrain pigs was investigated using an in vitro PBMC model. Pigs naturally exposed to helminth infection, have been shown to harbor the

infection into chronic state and elicit pronounced anti-inflammatory cytokine profiles. Unfortunately, as helminthes are known to induce immunity slowly and are masters of host-immune regulation, most of the studies previously emphasized the adaptive immune response without focusing on innate immunity using whole worm or crude extracts or heterogenous mixture of excretory secretory antigens. Moreover, most of these studies used relatively high doses of antigens or infective helminths (Eriksen et al. 1980, Roepstorff and Murrell 1997, Miquel et al. 2005), which were mostly inconsistent with the exposure of pigs to helminths under natural conditions. In addition to this, as most of the gastrointestinal helminthes local niche is the intestine, most of these research highlighted intestinal immunity. As intestinal effector immunity is mostly regulated by granulocytes, specifically eosinophils, mast cells, basophils are associated with the effector mediated expulsion of infection (Allen and Sutherland 2014), little attention was paid to peripheral mononuclear cells, more specifically to T cell mediated innate immunity. With the advancement of knowledge, the contribution of innate immunity comes forward especially while a broad spectrum of disease resistance is considered more important to emphasize. Peripheral T cells are considered as crucial for dictating the nature of innate immunity (Luckheeram et al. 2012). This innate immunity further determines the development of adaptive immunity in the context of host-parasite interaction. Additionally, helminth ES antigens are found therapeutically promising to treat several immune mediated disorders (Helmby 2015); hence more interest has grown on the individual role of antigenic molecules of helminth origin. In this study, we considered these gaps and exploited the role of helminth antigens to elucidate the immune variation resulting from pig breed differences.

Several reports have already reported the huge variability in the relative disease resistance or susceptibility of swine populations and breeds against several viruses, bacteria and parasites even at similar performance levels (Reiner 2009, Reiner et al. 2010). Hence the influence of host genetics on the interaction between host and parasites is now subject of both epidemiological and experimental animal studies (as reviewed by Mangano and Modiano 2014). Many of these studies have shown how the immune response is influenced substantially by the host genetics during helminth infections. One fundamental constraint in the assessment of genetic susceptibility or resistance is the complex interaction of several genes and their mask by heterogeneous non-systematic environmental factors. It is now a question of identifying the genes by reducing environmental effects and applying modern approaches of genome analysis. Significant number of evidence for host genetic variation in resistance was determined from available published data, including breed comparison, heritability studies, quantitative trait loci (QTL) studies, evidence of candidate genes with significant effects, data on pathogen sequence and on host gene expression analyses (Davies et al. 2009). The efficiency of the innate immunity relies on the host genetic

susceptibility level, age and sex as well as the genotype of parasites. These factors mean different levels of hormones in the body which are expected to affect the parasite. It is also possible for the parasite to synchronize their reproductive cycle with the host to maximize their evasion abilities. Innate immunity is of immense importance as an index of host immunocompetence (Flori et al. 2011).

Systemic parasite specific cytokine profiles become Treg/Th-2 polarized relative to naïve PBMC controls; but infection significantly altered immune response to helminth antigens in both breeds. Landrace pigs expressed substantially less IL-8R beta (CXCR2) than the other breeds Duroc, Hampshire and Pietrain (Kapetanovic et al. 2013). Windisch et al. (2000) reported elevated blood plasma level of IGF-1while studying protein metabolism in Pi pigs compared to LR pigs. The IGF-1 is reported to be associated with Treg stimulation and monocyte suppression, thereby suppression of inflammatory response (Bilbao et al. 2014, Ge et al. 2015).

Potential genetic influence on variation of innate and adaptive immune traits in pigs has been reported (Flori et al. 2011). For instance, differences in innate immune traits, i.e. NK cells, monocytes, IFNα production or phagocytosis are heritable (Edfors-Lilja et al. 1994, Clapperton et al. 2008). High heritability is also reported for total WBCs, $CD4^+$ T lymphocytes, $CD8\alpha^+$ T lymphocyte and B lymphocyte subset, delayed type hypersensitivity reaction, lymphoproliferation, lymphocyte mediated IL-2 production and level of acute phase protein (Edfors-Lilja et al. 1994, Clapperton et al. 2009). Monocytes are crucial player in innate immunity and serve also as antigen presenting cells (APCs) essential for developing adaptive immunity. The traditional concept that $CD4^+\alpha\beta$ T subsets differentiate into Th1 or Th2 lineages expressing specific cytokines is now controverted. Rather, current expansion of knowledge explored that cytokine production by the different $CD4^+$ T cell subsets (Th1, Th2, Th, Th17 and iTreg) is highly flexible. This gave us new insight into the Th cell plasticity (O'Shea and Murray 2008, O'Shea and Paul 2010).

4.2 Phenotypic response in terms of cell viability and phagocytosis towards AsHb and TY

Gastrointestinal nematodes coordinate with host immune responses for its own benefit and can adopt diversied mechanisms to interfere with antigen processing and presentation, to modulate macrophage and antigen-presenting cell function, to interrupt the cytokine signaling besides induction of immunoregulatory condition (Maizels and Yazdanbakhsh 2003). In many cases, immunosuppressive activity has been attributed to molecules that are excreted or secreted by the nematodes (Hewitson et al. 2009, Grainger et al. 2010). Mechanistically, helminth manipulation of the host immune system is most likely to be activated via the release of soluble mediators which

ligate, degrade or otherwise interact with host immune cells and molecules. Studies on the bovine parasite *Ostertagia ostertagi* and ovine parasite *Teladorsagia circumcincta* showed that soluble somatic extracts or larval excretory-secretory products were capable of suppressing mitogen induced bovine lymphocyte proliferation in vitro (Gomez-Munoz et al. 2004) and suggested that the parasite may actively induce regulatory T cell responses during infection (McNeilly et al. 2013). There are several studies reporting the down regulation of immune response at murine macrophage level (Bai et al. 2012, 2016) and depletion of swine lympho-proliferation by *Trichinella* ES (excretory-secretory antigens) (Gerencer et al. 1992). From our study, the lymphoproliferative role of tyvelose of *Trichinella sp*. and lymphosuppressive role of *Ascaris sp*., in naïve PBMC culture indicates that these two parasites modulate the host immunity in a different manner. This finding is also in coordination with the findings from Frontera et al. (2007) who demonstrated that *Trichinella spiralis* has an antagonistic relation with the concurrent infection with *Ascaris suum* in pigs. There was also a varying level of differences in the cell viability between breeds (LR and Pi) in both AsHb and Tyvelose treated and mitogen activated PBMCs.

Cell survivability and proliferation, when challenged with antigenic materials, are indicators of innate immune response. In many parasitic infections, the initial stages of the disease can be characterized by the induction of a non-specific lymphoproliferation, which is believed to disrupt antigen recognition and interfere with protective immune responses. On the contrary, in many cases, helminth or helminth product mediated immunosuppression also can be seen. The hyporesponsiveness to antigen-specific and mitogenic stimuli in chronic helminth infections could be associated with immunosuppressive cytokines (i.e., IL-10 and TGF-β) secreted by APCs and regulatory T cells (Treg cells). A growing list of parasite derived molecules is leading such a polarized cytokine secretion. Both AsHb and TY have shown to be potential immunomodulator in our in vitro PBMC model. Although the effect of AsHb was highly influential in lymphocyte depletion, but the effect was not the same on co-stimulated PBMCs.

Phagocytic efficiency significantly differed between breeds. Relatively higher phagocytosis ability was noticed in monocytes of LR piglets compared to Pi from both AsHb and tyvelose treatment. Besides host genetics, the discrepancies may have resulted from mature monocyte composition difference or the level of anergic cell population. The SLA complex consists of many important immune-related genes and has been repeatedly associated with variations in immunological and physiological performances in Yorkshire and Landrace pigs (Gao et al. 2017). Therefore, it is essential to define the SLA allelles and haplotypes of these pigs, to clarify the molecular mechanisms responsible for host immune responses.

Discrimination of helminth pathogens in the expression of innate phenotypes could be based on the recognition of parasite glycoconjugates, which contain unusual sugars and that have been implicated in Th2 response. Loss of T-lymphocyte proliferation concomitant with the development of Th2 response is characteristic for infections with other parasitic nematodes (King et al. 1992, Allen and MacDonald 1998).

4.3 Dynamics of cytokine mRNAs in PBMCs resulting from AsHb and TY exposure

Typically, helminths elicit IL-4, IL -5 and IL-13 dominated modified Th2 immune response. These cytokines and other signaling molecules activate a number of other cells, e.g. eosinophils, mast cells, basophils, epithelial cells and smooth muscle cells (Anthony et al. 2007). More recent studies have shown that the immune response during helminth infection is regulated by a network of immunosuppressive regulatory T cells and suppressive cytokines like IL-10 and TGF-β (Taylor et al. 2012). Techniques for prolonging helminth survival and persistence might be the rise in immunoregulatory cells, arresting of Th1 or Th2 cells, targeting pattern recognition receptors and lowering the immune cells quantity via enforcing apoptosis. Helminths and their products have demonstrated the potential to trigger apoptosis and anergy of host immune cells especially T lymphocytes (Chow et al. 2000, O'Connor et al. 2003, Smith et al. 2004), antigen presenting cells like APCs, NKs and eosinophils (Moreau and Chauvin 2010, Babu and Nutman 2012). The mechanisms by which they elicit such response are not fully understood. At this stage, while a lot is known about how innate immunity works in bacterial or viral infection, relatively little is known about helminth immunity that activates, polarizes and makes the immune response functional. One possible justification for this fact could be that helminthes are multicellular organisms and they have parasitic and non-parasitic stages. Helminths are very diverse in their intra and extracellular architecture including several life stages and may have several habitats within the same host at the same time by different developmental stages. Multiple approaches should be utilized to handle these issues.

The most striking findings from this study indicated that both AsHb and TY can activate naïve PBMC culture in a host, time and stimulus dependent-manner. Both AsHb and tyvelose could elevate overall IL-10, IL-6 expression in the PBMC culture in German Landrace pigs earlier than Pietrain pigs. Similar response was also reported in another study (Ait-Ali et al. 2011) devoted for innate immunity in Porcine Reproductive and Respiratory Syndrome Virus challenged macrophages of German Landrace and Pietrain pigs. The IL-10 and IL-6 response from their study also varied between host breed and in a time course manner. IL-10 is considered as the mastermind of

immuneregulation and IL-6 is considered as bridge between innate and adaptive immunity. Low parasite specific IL-6 and TGF- β concentrations are associated with development of severe liver pathology (Wilson and Maizels 2006). Therefore, IL-6 feedback regulation and synergy with anti-inflammatory responses may be required to limit immunopathology.

Breed variation between German Landrace and Pietrain was also clearly evident in the level of IL-2 mRNA expression in mitogen-activated group at the earliest time point we tested, i.e. 24 h post exposure. Both tyvelose and AsHb had downregulated the IL-2 level in both breeds. Mitogen induced IL-2 production is already proved as a prospective candidate gene for swine PBMC mediated immune responsiveness and associated with host genetic factors (Mach et al. 2013). Susceptibility level to porcine circovirus infection of German Landrace and Pietrain was also different (Opriessnig et al. 2009). Inherent genetic variation has been reported between LR and Pi pigs in terms of nutrient utilization and metabolic functions (Ponsuksili et al. 2007), which might indicate that immune system of LR and Pi pigs react differently to pathogens. In our recent study, whole transcriptome profiles of PBMCs obtained from PRRSV vaccinated LR and Pi pigs revealed distinct gene expression pattern (Islam et al. 2017)

Polarization of PBMCs was not clear-cut as three timepoints were considered and there was shift in changes in cytokine level from time to time. Moreover, large numbers of cytokines indicative of typical Th2 (IL-4, IL-5, IL-13) or Th17 (IL-17) were beyond the scope of our study. Furthermore, typical cytokine pairs, IFN-γ and IL-2, and IL-10 and TFG-β which were expected to be correlated in expression, did not show any significant similarity trend. In addition to this, the expression dynamics was also influenced by the host genetics. Therefore, the tested cytokine level varied between LR and Pi piglets.

Picherot et al. (2007) also found that swine intestinal mucosa as well as splenic cells was unable to induce distinct Th1 or Th2 response to *T. spiralis* where dominance of Th1 cytokine, IFN-γ and Th2 cytokine, IL-10 was found accompanied by predominant IgG1 and IgA (Type 2). Increased expression of IL-10 in absence of IL-4 at the mucosal level triggered TGF-β mRNA production (Picherot et al. 2007). TGF-β was linked to a tolerogenic profile in the intestine (McGuirk et al. 2002). The absence of TGF-β mRNA modulation suggests that the anti-*T. spiralis* mucosal response was not directed toward a tolerogenic feature (Picherot et al. 2007). In similar instances, the sensitivity profile of the assay in evaluating mRNA modulation could also be a limiting factor in analysis, providing an explanation for difficulties in detecting IL-4 and IL-12 in a heterogenous model such as PBMCs.

The backyard and free-ranged pigs are at highest risk for *Trichinella sp*. as well as *Ascaris sp*. infection. Although commercial pig farming is based on highly intensive management system in the developed countries, the backyard and free-ranged pig farming are still common in developing countries (Gottstein et al. 2009). Moreover, consumption of animal foods from green or organic farming is becoming popular day by day in concern with animal welfare issues. The routine meat inspection is primarily aimed to prevent the human consumption of infected pork and little efforts on preventing animals to be infected. The pigs reared in free range system, often fed food scraps or other forms of meat-containing waste and have ready access to rodents, which facilitate the trichinella infection. Persons working in *Ascaris sp*. infected farm were also found in risk of zoonotic transmission of ascaris worms. Taken together, both of these infection appeared to be a serious concern for domestic and international trading of the safe hygienic meat and meat products and needs to be prevented feasibly and sustainably through proper selection of animals according to the farming style, geographic location and other relevant issues.

4.4 Conclusions

The results of the current thesis clearly indicate there is substantial variation in the innate immune responsiveness to two helminth antigens tested between German Landrace and Pietrain pigs. Our study on the swine immune response to tyvelose and AsHb in PBMC cultures showed that both the phenotypes and genotypes of tested immune traits were of different patterns in two breeds depending on stimulus, host genetics and time-course. It also suggested that in-vitro PBMCs can be a useful model for immunogenetic study of other parasitic helminthes or pathogens. These findings will contribute on understanding the host immune response to helminthes parasites, both for a feasible and sustainable advancement of porcine health and welfare. Information on relatively higher resistance or susceptibility in a specified pig breed would facilitate the implementation strategy to control infection, enhance performance and quality. These will ultimately reduce the use of medicaments, would improve consumer health and animal welfare. This study also highlights the importance of considering the potential for the variation in immune responsiveness in terms of pig health. In addition to these, because of the physiological resemblance between pigs and human, findings from this study may be translated to human studies for host-parasite interaction and allergic immunity.

4.5 Future perspectives

According to the spectrum of this immunogenetic study, of which chapter 2 and chapter 3 discussed two individual parasites, there are few issues needed to be properly addressed, considered and

validated further.

Firstly, the validation of disease specific marker gene expression would be highly suggested. For instances, thioredoxin-interacting protein (TXNIP) single nucleotide polymorphisms was linked with resistance in porcine *Ascaris suum* infection (Skallerp et al. 2012); Matrix metalloproteinase (MMP)-2 and MMP-9 have been found to be associated with inflammation level as marker in *Trichinella spiralis* in mice (Bruschi et al. 2014) and associated with immune traits in pig (Huang et al. 2009). MMP-9 was revealed as a reliable marker for inflammation in early human trichinellosis (Bruschi et al. 2016).

Secondly, evaluation of a larger set of cytokines, related cell surface markers, activation markers, co-stimulatory molecules, transcription factors, marker genes for cell proliferation, ROS production and apoptosis might be useful to correlate the phenotypic and genotypic expression and to come to a more concrete conclusion. Of special interest can be IL-4, IL-5, CD4, CD8, MHCII, FOXP3, T-bet1, GATA3, ROR-γδ., iNOS etc.

Thirdly, assessment of protein expression in the supernatant and cell lysate will provide more details understanding of the pathogenesis in vivo. Age-structured evaluation will also be helpful to check if this variation persists over the lifespan of these breeds. Because, innate immunity is reported to vary from age, sex dependent manner and our study focused animals only of a special age group, 5-6 weeks old. To ensure the breed variation, local tissue based evaluation of the effect of these antigens is further suggested as differences in the systemic and local inflammatory response were evident from previous study (Picherot et al. 2007).

4.6 References

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5. Appendices

List of chemicals and reagents used for this experiment

Chemical	Cat.no.	Manufacturer/Supplier
Meliseptol®	1110493	Labomedic GmbH, Bonn, Germany
NaCl	P029.2	Carl Roth GmbH+ Co. KG, Karlsruhe, Germany
Na2HPO4.2H2O	T877.1	Carl Roth GmbH+ Co. KG, Karlsruhe, Germany
KCl	678.1	Carl Roth GmbH+ Co. KG, Karlsruhe, Germany
KH2PO4	3904.1	Carl Roth GmbH+ Co. KG, Karlsruhe, Germany
Fungizone® antimycotic	15290-026	Life Technologices GmbH, Darmstadt, Germany
Penicillin-streptomycin (10,000 u/ml)	15140-122	Life Technologies GmbH, Darmstadt, Germany
NH4Cl	K298.2	Carl Roth GmbH+Co. KG, Karlsruhe, Germany
KHCO3	P748.2	Carl Roth GmbH+Co. KG, Karlsruhe, Germany
EDTA	8043.2	Carl Roth GmbH+Co. KG, Karlsruhe, Germany
Histopaque-1077	10771	Sigma-Aldrich, Munich, Germany
RPMI 1640 medium	61870-044	Life Technologies GmbH, Darmstadt, Germany
FBS	10270-106	Life Technologies GmbH, Darmstadt, Germany
Trypan blue stain (4%)	93595	Sigma-Aldrich (Fluka), St. Louis, USA
Ascaris suum haemoglobin		A kind gift from Prof. Dr. Peter Geldhof, Gent University, Belgium
Tyvelose (Tyvelose®)	CAS-5658-12-8	Santa Cruz Biotechnology, Canada
Lipopolysaccharide (LPS)	L2880	Sigma Aldrich, Germany
Phytohaemagglutinin-M (PHA-M)	10576015	ThermoFisher Scientific, Germany
CCK-8 Cell Viability Assay Kit	CK04-10	Dojindo Molecular Technologies Inc., EU GmbH, Germany
VybrantTM Phagocytosis Assay Kit	V-6694	Molecular Probes, Germany
miRNeasy mini kit	217004	Qiagen, GmbH, Hilden
First Strand cDNA Synthesis	K1681	ThermoPhisher, Co., Germany
iTaqTM Universal SYBR® Green Supermix	172-5120	Bio-Rad Laboratories GmbH, München, Germany
Ethanol (≥99.8%)	9065.2	Carl Roth GmbH+Co. KG, Germany
RNase-free DNase set	79254	Qiagen, GmbH, Hilden
Agarose	N3101-0500	STARLAB GmbH, Ahrensbur, Germany
Ethidium bromide (1%)	2218.1	Carl Roth GmbH+Co. KG, Germany
Agar	2266.2	Carl Roth GmbH+Co. KG, Germany
Taq DNA polymerase	786-447	G-Biosciences, St Louis, USA

Buffer, media and gel preparation

PBMC washing media, 1X PBS (without Ca2+ and Mg2+, pH 7.4)	(g/L)
NaCl Na2PO4.2H2O KCl KH2PO4	8 1.44 0.20 0.24
1X RBC Lysis Buffer	(g/L) or (ml/L)
NH4Cl KHCO3 5% EDTA ddH2O added to	8.30 g/L 1g/L 1.80 ml/L 1L
PBMCs culture medium	Percentage, %
RPMI-1640 medium FBS Pen-Strep (100Xconcentrate) Fungizone® antimycotic	88 10 1 1
PBMCs counting	µL
4% Trypan blue stain medium PBMC suspension PBMCs culture media	10 50 40
Tris-acetate-EDTA (TAE) (10X)	Ml/L or g/L
Acetic acid (100%) Tris base EDTA ddH2O added to	571 ml 242 g 100 ml 1L
Agarose gel (2%)	g or ml or µl
Agarose TAE (1X) Ethidium bromide	4g 200 ml 9 µl
dNTP solution	400 ml
dATP (100 mM) dGTP (100 mM) dTTP (100 mM) ddH2O added to	10 µl 10 µl 10 µl 400 ml

Equipment and consumbles

Equipment and consumables	Manufacturer/Supplier
Centrifuge tubes (15 ml, 50 ml) Centrifuge (5810R, 5424, 5416, 5415R) Haemocytometer Microscope (ECLIPSE TS100)	SARSTEDT AG & Co., Nümbrecht Eppendorf AG, Hamburg Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen Nikon GmbH, Düsseldorf
Digital camera system for microscopy digital sight series (DS-Fi1)	Nikon GmbH, Düsseldorf
Memmert CO2 incubator	Fisher Scientific UK Ltd, Loughborough
6 – well cell culture plates	STARLAB GmbH, Hamburg
96 – well cell culture plates	STARLAB GmbH, Hamburg
Serological pipettes (1, 2, 5, 10, 25 ml)	Greiner Bio-One GmbH, Frickenhausen
Aspirating pipette	Greiner Bio-One GmbH, Frickenhausen
Cell scrapers	Greiner Bio-One GmbH, Frickenhausen
Plate reader synergyTM 2	Bio Tek Instruments GmbH, Bad Friedrichshall
StepOnePlusTM real time PCR system	Life Technologices GmbH, Darmstadt (Applied Biosystems®)
MicroAmp® fast optical 96-well reaction plate with barcode, 0.1 ml	Life Technologices GmbH, Darmstadt (Applied Biosystems®)
Water-bath 1083	GFL Gesellschaft für Labortechnik mbH, Burgwedel
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific Biosciences GmbH, St. Leon-Roth
Universal High speed centrifugation (Z300 K, Z300, Z200 M/H, Z233 MK, Z323 K)	HERMLE Labortechnik GmbH, Wehingen
ELV fully automatic autoclave (3870)	Tuttnauer Europe B.V., Netherlands
Multi®- ultra tubes 0.65 ml	Carl Roth GmbH + Co. KG, Karlsruhe
SafeSeal® tubes 1.5 ml	Carl Roth GmbH + Co. KG, Karlsruhe
SafeSeal® tubes 2.0 ml	Carl Roth GmbH + Co. KG, Karlsruhe
PCR® strip tubes	VWR International GmbH, Darmstadt (Axygen®)
Pipette tips (10 µl, 200 µl, 1000 µl)	Labomedic GmbH, Bonn
Pipettes (0.5-10, 2-20, 20-200, 100-1000 µl)	Eppendorf AG, Hamburg

Software programs and statistical packages

Software	Use	Source
Primer 3	qRT-PCR primer design	http://simgene.com/Primer3
BLAST4	Check alignment specificity	http://blast.ncbi.nlm.nih.gov/Blast.cgi
GraphPad Prism	Analysis of phenotypic data and plotting results	Proprietory, Builtin citreon server
SAS	Analysis of qRT-PCR data	Proprietory, Builtin citreon server
Citavi	Reference management	Open sources, Builtin citreon server

6. Acknowledgements

All praises go to Almighty who enables us to be here. From my experience, PhD is actually a combined effort of so many people helping one particular person from so many aspects to achieve a particular goal. With the opportunity of my PhD thesis completion, I would also like to thank all those people who contributed to my current status.

First and foremost, I would like to express my sincere gratitude to my mentor, Dr. Karl Schellander, Professor, Institute of Animal Science (ITW), University of Bonn, Germany. He dealt with all of my states of panic. His well-balanced and optimistic attitude about life and success had encouraged me to go through this phase. This thesis would not have existed without his guidance. For me, he is the best role model as mentor and as a person as well. I wish I could be a human being like prof. Schellander.

I would like to thank Prof. Dr. agr. Helga Sauerwein for her kindness to accept the request for being my second supervisor. I sincerely acknowledge PD. Dr. Michael Holker for being the scientific committee member and investing his precious time in scientific discussion during doctoral meetings.

My sincere thanks to Dr. Christiane Neuhoff for her intellectual guidance, invaluable comments and patience in correcting my manuscripts. Without her support, it would have been quite impossible to at this level of writing the last few lines of my thesis. She was always there for me whenever I needed help and of course for German translation of my thesis abstract.

I am extremely grateful to prof. Dr. Peter Geldhof and Dr. Johnny Vlaminck, Laboratory of Parasitology, Ghent University, Belgium, for donating *Ascaris suum* haemoglobin antigen.

My special thanks go to Dr. Md. Aminul Islam for his scientific contribution in this thesis, both inside and outside the laboratory. I am extremely thankful to Dr. Christine Grosse-Brinkhaus for her scholastic supervision and guidelines in statistical part of my thesis. Dr. Maren Julia Pröll deserves a very special thank for critically reviewing and carefully pointing out the crucial parts of my manuscript My thank you note extends to Dr. Dawit Tesfaye, Dr. Ernst Tholen for their great assistance, valuable discussion, and contribution in my experiments.

I want to acknowledge Mr. Buschen for his help in animal care; Ms. Nadine Leyer, Ms. Birgit Koch-Fabritius and azubis, especially Kilian, Laura, Julia for their enormous help in laboratory

works. Thanks also extend to Mr. Thomas Blees for helping me with fluorescent reader at ITW, Physiology and Hygiene, University of Bonn.
I would like to thank all administrative members of the ITW, particularly Ms. Bianca Peters, Mr. Peter Müller and Mr. Stephan Knauf for their assistance in office management. Many thanks go to all technical assistant Ms. Helga Brodeßer, Ms. Stephanie Fuchs for their technical help and to Ms. Renate Kicker for her smile at every meeting and small talks.

I sincerely acknowledge the German Academic Exchange Service (DAAD) for financial support through Scholarship Programme to pursue my PhD study abroad.

I am grateful to all the present and former members of ITWfor creating a pleasant atmosphere at work, Dr. Bazlur Rahman, Dr. Dessie Salilew-Wondim, Dr. Samuel Etay, Dr. Eryck Andreas and sweet lady, Dr. Qin Yang. I should never forget the contribution of my current colleagues Mr. Mohammed Zidane, Mr. Hari Om Pandey, Mr. Omar Khadrawy, Mr. Mohammed Taqi, Ms. Tsige Hagos, Ms. Hoda Samir, Mr. Mikhael Poirier and Mr. Teshome Alemu.

Further, I would like to thank all my Bangladeshi community, especially Ms. Bazlur Rahman for her invaluable family-friendly support. I am sincerely grateful to my late parents, my elder sisters and brothers, my uncle and mother-in-law for their great contribution in my personal and professional life.

Last but not least, my deepest thanks to my beloved family, my husband and our lovely kids, Saif and Taif for their endless sacrifice during the whole study period. May Almighty grant our good deeds and bless us with His mercy.