Transcriptional immune response in mesenteric lymph nodes in pigs with different levels of resistance to *Ascaris suum*

Skallerup, Per; Nejsum, Peter; Cirera, Susanna; Skovgaard, Kerstin; Pipper, Christian B.; Fredholm, Merete; Jørgensen, Claus B; Thamsborg, Stig M.

Published in:
Acta Parasitologica

Link to article, DOI:
[10.1515/ap-2017-0017](https://doi.org/10.1515/ap-2017-0017)

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Skallerup, P., Nejsum, P., Cirera, S., Skovgaard, K., Pipper, C. B., Fredholm, M., Jørgensen, C. B., & Thamsborg, S. M. (2017). Transcriptional immune response in mesenteric lymph nodes in pigs with different levels of resistance to *Ascaris suum*. *Acta Parasitologica*, 62(1), 141-153. <https://doi.org/10.1515/ap-2017-0017>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Transcriptional immune response in mesenteric lymph nodes in pigs with different levels of resistance to *Ascaris suum*

Per Skallerup^{1,2}, Peter Nejsum^{1,2}, Susanna Cirera², Kerstin Skovgaard³, Christian B. Pipper⁴, Merete Fredholm², Claus B. Jørgensen² and Stig M. Thamsborg^{1*}

¹Parasitology and Aquatic Diseases, Department of Veterinary Disease Biology, University of Copenhagen, Dyrlægevej 100, DK-1870 Frederiksberg C, Denmark; ²Animal Genetics, Bioinformatics and Breeding, Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, Grønnegårdsvej 3, DK-1870 Frederiksberg C, Denmark; ³Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark; ⁴Department of Public Health, University of Copenhagen, Øster Farimagsgade 5, DK-1014 Copenhagen K, Denmark

Abstract

A single nucleotide polymorphism on chromosome 4 (SNP TXNIP) has been reported to be associated with roundworm (*Ascaris suum*) burden in pigs. The objective of the present study was to analyse the immune response to *A. suum* mounted by pigs with genotype AA (n = 24) and AB (n = 23) at the TXNIP locus. The pigs were repeatedly infected with *A. suum* from eight weeks of age until necropsy eight weeks later. An uninfected control group (AA; n = 5 and AB; n = 5) was also included. At post mortem, we collected mesenteric lymph nodes and measured the expression of 28 selected immune-related genes. Recordings of worm burdens confirmed our previous results that pigs of the AA genotype were more resistant to infection than AB pigs. We estimated the genotype difference in relative expression levels in infected and uninfected animals. No significant change in expression levels between the two genotypes due to infection was observed for any of the genes, although IL-13 approached significance ($P = 0.08$; $P_{\text{unadjusted}} = 0.003$). Furthermore, statistical analysis testing for the effect of infection separately in each genotype showed significant up-regulation of IL-13 ($P < 0.05$) and CCL17 ($P < 0.05$) following *A. suum* infection in the ‘resistant’ AA genotype and not in the ‘susceptible’ AB genotype. Pigs of genotype AB had higher expression of the high-affinity IgG receptor (FCGR1A) than AA pigs in both infected and non-infected animals ($P = 1.85 \times 10^{-11}$).

Keywords

Ascaris suum, pig, single nucleotide polymorphism, gene expression, qPCR; RT-qPCR, cytokine, TXNIP, immunity

Introduction

The large roundworm of pigs, *Ascaris suum*, has a global distribution with high prevalence in both extensive and intensive production systems (Vlaminck and Geldhof 2013). Infections may cause decreased weight gain and reduced productivity, and may further negatively interfere with vaccinations (Steenhard *et al.* 2009; Thamsborg *et al.* 2013). Although *A. suum* and its sibling species *A. lumbricoides* – which infects humans – may both be treated efficiently with anthelmintics (Keiser and Utzinger, 2008), drug resistance has been reported in another ascarid, *Parascaris equorum* (Reinemeyer 2012) and there are sporadic reports that *A. lumbricoides* does not re-

spond well to treatment (Adugna *et al.* 2007). In the light of these challenges, an improved understanding of the host response to parasitic infections is crucial for development of new and more sustainable control strategies.

Helminth infections typically elicit a Th2-type response characterized by the production of the cytokines IL-4, IL-5 and IL-13. These cytokines and other signalling 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 (T_{regs}) and suppressive cytokines like TGF- β and IL-10 (Taylor *et al.* 2012). Resistance, i.e., the

*Corresponding author: smt@sund.ku.dk

ability to suppress establishment and/or subsequent development of infection (Albers *et al.* 1987), is under genetic control by the host (Wakelin 1975). There are probably a number of molecular mechanisms involved in such a complex process. Studies in susceptible and resistant mouse strains have shown that resistance to helminth infection is mediated by a Th2-type response whereas susceptibility is associated with the induction of a Th1-type response (Else *et al.* 1992). A similar pattern has been found in sheep (Pernthaner *et al.* 2005; Gossner *et al.* 2013) and cattle (Zaros *et al.* 2010) though not always as pronounced as in murine models. Indeed, the use of new molecular tools which allow the expression of a high number of genes and proteins to be profiled simultaneously (e.g., microarray, microfluidic dynamic arrays, protein arrays) has revealed a much more complex picture than previously expected (Ingham *et al.* 2008; Araujo *et al.* 2009; Andronicos *et al.* 2010).

There is evidence that pigs mount a Th2-type response following helminth infection (e.g., Dawson *et al.* 2005); however, these studies did not compare genetically susceptible and resistant animals. Regulation of *A. suum* infections has been demonstrated to be under genetic control with a heritability of 0.45 for worm burdens (Nejsum *et al.* 2009). Our group has identified a single nucleotide polymorphism (SNP TXNIP) on porcine chromosome 4 which is associated with the burden of *A. suum* in crossbred Duroc/Danish Landrace/Yorkshire (DLY) pigs (Skallerup *et al.* 2012). Hence, pigs with the 'susceptible' AB genotype at the TXNIP locus had a 2.5 fold higher total worm burden than the 'resistant' AA genotype (Skallerup *et al.* 2012).

The objective of the present study was to characterize the transcriptional immune response mounted by an independent group of crossbred DLY pigs with these two different TXNIP genotypes after repeated (trickle) infections with *A. suum* for eight weeks. Their parasitological traits have been described elsewhere (Skallerup *et al.* 2014). Briefly, at the end of the study pigs of the AA genotype had lower mean macroscopic worm burden than pigs of the AB genotype (2.4 vs. 19.3; $P = 0.06$) and lower mean total worm burden (26.5 vs. 70.1; $P = 0.09$). In the present study we measured the expression of different genes in mesenteric lymph nodes sampled from infected pigs and uninfected controls. We analysed candidate genes in linkage disequilibrium (LD) with the TXNIP marker (Skallerup *et al.* 2012) as well as different markers of immune activation that would allow us to analyse the immune response (i.e., markers indicative of 'innate'/Th1/Th2/Th17/T_{reg} response).

Materials and Methods

Experimental design

The study was designed to demonstrate a significant difference in macroscopic *A. suum* worm burden between experi-

mentally infected pigs with genotypes AA and genotype AB (SNP TXNIP). A statistical power analysis showed that 24 pigs of each genotype would be sufficient to achieve significance (Skallerup *et al.* 2014). Pigs (crossbred Duroc/Danish Landrace/Yorkshire) were purchased from a commercial specific pathogen-free farm. In order to identify pigs with the genotypes needed for the study (AA, AB), 112 piglets from this herd were genotyped prior to inclusion as described previously (Skallerup *et al.* 2014). Based on genotype, pigs from 10 different litters were allocated to trickle-infected groups ($n_{AA} = 27$; $n_{AB} = 25$) or uninfected groups ($n_{AA} = 5$; $n_{AB} = 5$). The farmer used mixed semen to produce the littermates which were thus full-sibs or half-sibs; males were castrated.

The experimental protocol used to generate *A. suum* infected pigs as well as their phenotypic traits has been described elsewhere (Skallerup *et al.* 2014). Briefly, upon arrival at seven weeks of age the pigs were allocated into three pens with concrete floors ensuring an equal distribution of genotype, litter of origin, sex and weight in each pen; a fourth pen was used for the uninfected controls. Embryonated eggs used for trickle infections were prepared from uteri of female *A. suum* worms collected at a Danish slaughterhouse (Oksanen *et al.* 1990). After one week of acclimatization, pigs were infected with *A. suum* eggs (25 eggs/kg/day) twice per week. The pens were littered with wood shavings on a daily basis, and water was provided *ad libitum*. The animals were fed a diet consisting of ground barley supplemented with proteins and minerals. Staff used separate boots and protective clothing to avoid accidental infections of the control group. One pig of each genotype was sacrificed on day 13 post first infection (PI) to test the infectivity of the *A. suum* egg batch and these two animals were excluded from the gene expression analysis.

The pigs were euthanized using a captive bolt pistol followed by exsanguination on days 55–59 PI. From each pig, a mesenteric lymph node was sampled from the central part of jejunum because by week 8 PI most of the worms are located here (Roepstorff *et al.* 1997). The samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. The small intestine was opened longitudinally and any macroscopic *A. suum* worms (large juveniles and adults) were removed and counted. Larvae were isolated from the intestinal contents by the agar-gel method (Slotved *et al.* 1997). The study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark (Ref. 2010/561-1914). Care and maintenance of all animals were in accordance with applicable Danish and European guidelines.

Primer design and optimization (Fluidigm and MxPro platforms)

Primers were designed using Primer3 (<http://primer3.wi.mit.edu>) (Rozen and Skaletsky, 2000). Primers were designed to span an intron, if possible, and to target most or all splice variants of the gene of interest, if applicable. We performed a BLAST

search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of each primer sequence to confirm binding at the intended locus. Furthermore, primer specificity was confirmed by visual inspection of amplicons on agarose gels and by inspection of melting curves. For the Fluidigm array, three separate pools of equal amounts of pre-amplified cDNA from all samples were used to prepare three standard curves (dilutions: 1:2, 1:10, 1:50, 1:250) from which PCR amplification efficiencies were calculated. Primer sequences, amplicon lengths, and primer PCR efficiencies are shown in a separate file (Supplementary Table S1). With the objective of characterizing the immune response of the two SNP TXNIP genotypes, we included a panel of genes involved in the innate and adaptive branches of the immune system, including a Th1-type and Th2-type immune response and regulatory T cells. We also included four candidate genes in LD with SNP TXNIP (TNFAIP8L2, PIAS3, RFX5, RBM8A) (Skallerup *et al.* 2012) as well as an additional candidate gene within our QTL (FCGR1A). In addition, we tested the expression of TNFSF13B, a candidate gene for *A. lumbricoides* burden (Williams-Blangero *et al.* 2002; 2008). A functional grouping of the genes is provided in Supplementary Table S1.

RNA extraction

Total RNA was extracted from lymph nodes using Tri Reagent® (Molecular Research Center, Inc., USA) according to the manufacturer's instructions. Briefly, 100 mg of tissue were homogenized in 1 ml Tri Reagent on a gentleMACS™ Octo Dissociator (Miltenyi Biotec, Germany); after addition of 100 µl bromochloropropane, RNA was precipitated with isopropanol, washed in 75% ethanol and dissolved in 200 µl RNAase-free water. RNA concentration and purity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, USA). Total RNA quality was evaluated by visual inspection of 28S/18S rRNA bands on agarose gels and subsequently by assessment of RNA integrity data (Experion™ machine, BioRad Laboratories, USA) using RNA Std-Sens Analysis Kit (BioRad Laboratories, USA). RNA quality indicator (RQI) values above 6 were accepted. RNA was kept at -80°C until cDNA synthesis.

Reverse transcription of mRNA into cDNA

Genomic DNA removal and reverse transcription of total RNA was performed using QuantiTect Reverse Transcription Kit (Qiagen) following the instructions of the manufacturer with minor modifications. In brief, 500 ng of total RNA was DNAase treated in 96-well PCR plates by incubating (2 min, 42°C) in a DNA Engine® Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., USA). Next, reverse transcriptase enzyme and a mix of random primers and dNTPs (1:4) were added and samples were incubated for 15 min at 42°C; enzymes were denatured for 3 min at 95°C and cDNA cooled down to 4°C. For each sample two cDNA replicates were prepared. We also

included non-reverse transcriptase controls. Samples were diluted 1:8 or 1:10 in low EDTA TE-buffer (VWR-Bie & Berntsen, Denmark) prior to pre-amplification (Fluidigm array) or qPCR (MxPro), respectively.

Pre-amplification and exonuclease treatment (Fluidigm array)

The pre-amplification was performed as described previously (Skovgaard *et al.* 2013). Briefly, five µL of TaqMan PreAmp Master Mix (Applied Biosystems), 2.5 µL of primer pair mix (a 200 nM pool of all primer pairs used in the present study) and 2.5 µL diluted cDNA were mixed and incubated at 95°C for 10 min followed by 15 cycles at 95°C for 15 sec and 60°C for 4 min. Next, pre-amplified cDNA was incubated with 4 µL of 4U/µL exonuclease (30 min at 37°C, followed by 15 min at 80°C). Then samples were diluted 1:8 in low EDTA TE-buffer before quantitative real-time PCR (qPCR).

Quantitative real-time PCR (qPCR)

Fluidigm array

The qPCR was performed in 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, CA, USA). This platform allows the simultaneous analysis of 48 primers in 48 cDNA samples, i.e., a total of 2,304 individual qPCR reactions arranged in a grid as described elsewhere (Skovgaard *et al.* 2013). In brief, for each of the 48 cDNA sample lanes on the chip, we prepared a 'sample mix' consisting of 3 µL of ABI TaqMan Gene Expression Master Mix (Applied Biosystems), 0.3 µL of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.3 µL 20X EvaGreen (Biotium, VWR-Bie & Berntsen), 0.9 µL low EDTA TE-buffer and 1.5 µL of pre-amplified exonuclease-treated cDNA. Next, for each of the 48 primer set lanes on the chip, we prepared a 'primer mix' consisting of 2.5 µL of 2X Assay Loading Reagent (Fluidigm), 0.25 µL low EDTA TE-buffer and 2.3 µL of 20 µM forward and reverse primer (Supplementary Table S1). After priming the 48.48 Dynamic Array chip in the IFC controller (Fluidigm), it was loaded with cDNA samples and primers and again placed in the IFC controller to ensure the solution in each inlet was equally distributed across all 48 reactions in a lane, thus combining the 48 primer sets with the 48 cDNA samples.

The qPCR was performed on a BioMark HD Reader (Fluidigm) under the following conditions: 2 min at 50°C, 10 min at 95°C followed by 35 rounds of 15 s at 95°C and 1 min at 60°C. After each run, melting curves were generated to confirm primer specificity (from 60°C to 95°C, increasing 1°C/3 s). Reactions were run in duplicate (two cDNA replicates). The three cDNA pools (each diluted 1:10) described above were used as interplate calibrators. Data were retrieved and inspected using Fluidigm's Real-Time PCR Analysis software, version 3.0.2.

MxPro

Fluidigm qPCR data for the gene encoding the high affinity receptor for Fc fragment of IgG (FCGR1A) were excluded because the variation between cDNA replicates was too large (see below). Hence, expression of this gene was measured on a Stratagene Mx3000P™ (Agilent) using the QuantiFast™ SYBR Green PCR Kit (Qiagen) according to the manufacturer's recommendations. The qPCR reaction was run under the following conditions: 5 min at 95°C followed by 40 rounds of 10 s at 95°C and 30 s at 62°C (FCGR1A) or 60°C (GAPDH, TBP, HPRT1). After each run, melting curves were generated to confirm primer specificity (from 55°C to 95°C). For each transcript, a calibration curve was made using serial dilutions of pre-amplified PCR product. We used the baseline-corrected normalized fluorescence method of the Stratagene Mx3000P™ software (Agilent) to determine the Cq in each reaction.

Data processing and statistical analysis

Data processing was made in GenEx version 5.4.0 (MultiD, Sweden) and included the following steps (in sequential order): Interplate calibration, amplification efficiency correction (calculated separately for each primer pair), normalization to several reference genes, calculation of average of the two technical cDNA replicates, calculation of relative expression values, and log2 transformation. Careful quality checks were performed after each step. We used NormFinder (Andersen *et al.* 2004) to determine the optimal number of reference genes. For the Fluidigm array the following six reference genes were tested: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), peptidylprolyl isomerase A (PPIA), TATA box binding protein (TBP), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (YWHAB). For the MxPro platform we tested GAPDH, TBP, and HPRT1. Accounting for intra- and intergroup expression, the NormFinder algorithm ranks reference genes according to stability values. The following reference genes were selected for normalization based on their stability value: TBP, HPRT1, GAPDH, YWHAB and PPIA (Fluidigm array) and TBP and HPRT1 (MxPro) (Supplementary Table S1).

To visualize the variation in expression levels of each gene, Cq values were transformed to relative gene expression levels by setting the maximum Cq value (the lowest expressed sample) in each the primer-specific dataset to one. Relative quantities were log2-transformed prior to statistical analysis. Primer pairs with too low efficiencies (<0.69), with too many missing values, or with too many cDNA replicates varying more than ± 1.5 Cq (cutoff: more than 14% of samples out of range) were excluded from the analysis of Fluidigm array data. Samples for which there were no data for neither of the cDNA replicates (Fluidigm array only) were assigned a con-

servative Cq value by adding one to the highest Cq value in the primer-specific dataset (20 out of 3078 cDNA samples). On the MxPro platform, the expression of FCGR1A in 6 out of 57 samples could not be detected reliably and these samples were excluded from the analysis. Data from a total of 28 non-reference genes were included in the analyses (Supplementary Table S1). Experimental practice and reporting was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009).

Log2-transformed relative gene expression profiles (Fluidigm array) were analysed in R version 3.0.0 (R Core Team 2013) by a mixed linear model. We fitted gender, starting weight, and a gene-specific combined effect of SNP TXNIP genotype (AA, AB) and infection (*A. suum*-infected, uninfected) as fixed effects (Barger 1993). Pig, litter of origin, pen, and necropsy day were fitted as random effects. Model assumptions in the initial model were checked graphically by means of normal quantile-quantile plots and residual plots.

For each gene, the difference between the genotypes in relative expression level was estimated for infected and uninfected pigs and compared by the model described above; hence, we tested if there was a modification of expression levels in pigs with the two marker genotypes due to infection. To test the effect of infection, we evaluated the difference in expression level between infected and uninfected pigs for each genotype using the above model. REML estimation was used to compute estimates in the model and comparisons were adjusted for multiple testing by means of the single step procedure (Hothorn *et al.* 2008).

Since the expression of the FCGR1A gene was measured on a different platform (MxPro platform), these data were analysed separately; we used a mixed linear model which included a systematic combined effect of genotype and infection (infected, uninfected) adjusted for gender and starting weight as well as random effects of litter of origin, pen, and necropsy day. The effect of genotype and infection was assessed by backwards elimination based on likelihood ratio tests.

Results

Two of the infected pigs (both genotype AA) died during the experiment for reasons not related to the study treatment. Tissue samples were taken from all but one pig (infected, genotype AB) and the final dataset comprised 57 pigs (trickle-infected pigs: $n_{AA} = 24$; $n_{AB} = 23$; uninfected pigs: $n_{AA} = 5$; $n_{AB} = 5$).

Detailed parasitological data from infected pigs such as worm counts, liver white spots and serum IgG antibody titres are presented elsewhere (Skallerup *et al.* 2014). Briefly, pigs of the AA genotype had lower mean macroscopic worm burden (2.4 vs. 19.3; $P = 0.06$), lower mean total worm burden (26.5 vs. 70.1; $P = 0.09$) and excreted fewer *A. suum* eggs at week 8 PI (mean number of eggs/g faeces: 238 vs. 1259;

Fig. 1. Relative estimated gene expression levels in mesenteric lymph nodes. The pigs were either homozygous or heterozygous at the single nucleotide polymorphism (SNP) 0_TXNIP_DS087128.1_2_2 (TXNIP) locus. Lymph nodes were sampled from uninfected pigs ($n_{AA} = 5$; $n_{AB} = 5$) and pigs trickle-infected with *Ascaris suum* ($n_{AA} = 24$; $n_{AB} = 23$) at 8 weeks post first infection. Cq values were transformed to relative gene expression levels by setting the maximum Cq value in each primer-specific dataset to one and then log2-transformed. Gene expression levels were estimated according to a statistical model in which gender, starting weight, and a gene-specific combined effect of SNP TXNIP genotype (AA, AB) and infection (*A. suum*-infected, uninfected) were fitted as fixed effects. Pig, litter of origin, pen, and necropsy day were fitted as random effects

$P = 0.14$) than pigs of the AB genotype. No differences in number of liver white spots were observed between the two genotypes. None of the pigs in the control groups had any worms at necropsy.

A total of 27 genes (Fluidigm array) passed quality testing and were included in the analysis. The relative expression levels of the 27 genes stratified by infection (infected, uninfected) and TXNIP genotype (AA, AB) have been plotted in Figure 1. For each gene, we estimated the genotype difference (AA vs. AB) in relative expression level (infected, uninfected) and a comparison between infected and uninfected pigs was then made to test if there was a modification of expression levels in pigs with the two marker genotypes due to infection. We did not observe a significant change in expression between the two genotypes due to infection for any of the genes. However, for IL-13 the difference approached significance ($P = 0.077$; $P_{\text{unadjusted}} = 0.00313$; Figures 1 and 2).

We next sought to determine the effect of *A. suum* infection on relative expression levels. For each gene and each SNP TXNIP genotype, we estimated the difference in relative expression in infected vs. uninfected pigs. We found a significant up-regulation of IL-4 in infected pigs of genotype AA ($P < 0.05$) and genotype AB ($P < 0.05$). In addition, a significant up-regulation of CCL17 ($P < 0.05$) and IL-13 ($P < 0.05$) was observed in infected pigs of genotype AA only (Figures 1 and 2).

The expression of the FCGR1A gene was evaluated using another platform and the statistical analysis showed that there was no modification of expression levels in pigs with the two TXNIP genotypes due to infection. Interestingly, we found that genotype had a highly significant effect on the expression of this receptor ($P = 1.85 \times 10^{-11}$) with higher expression levels in pigs with the AB genotype than pigs with AA genotype whereas there was no effect of infection ($P = 0.59$) (Figure 2). Gender also had a significant effect on expression of this gene ($P < 0.05$).

Fig. 2. Relative gene expression levels for interleukin (IL)-13, IL-4, chemokine ligand 17 (CCL17) and the high-affinity IgG receptor (FCGR1A) in mesenteric lymph nodes. Lymph nodes were sampled from uninfected pigs ($n_{AA} = 5$; $n_{AB} = 5$) and pigs trickle-infected with *Ascaris suum* ($n_{AA} = 24$; $n_{AB} = 23$) at 8 weeks post first infection. Error bars are 95% confidence intervals. For further details, see Fig. 1

Discussion

The host response to several pathogens of livestock, humans and mouse models has been dissected using gene expression analysis and proteomics (Dawson *et al.* 2005; Noyes *et al.* 2011). As part of these efforts, studies in murine models and ruminants have compared strains, breeds or individuals with predicted variation in resistance/susceptibility to various helminths (Else *et al.* 1992; Pernthaner *et al.* 2005; Ingham *et al.* 2008; Gossner *et al.* 2013). However, such studies have not been conducted in pigs.

In the present study we compared trickle-infected pigs with two genotypes (AA, AB) which we hypothesized had divergent resistance to the large roundworm *A. suum* based on prior association (Skallerup *et al.* 2012). Generally, a low establishment of adult worms was observed but most pigs harboured intestinal larvae at necropsy eight weeks PI. Pigs of genotype AA had, as expected, lower mean worm counts and lower faecal egg counts than AB pigs, although the statistical analyses showed only borderline significance (Skallerup *et al.*

2014). Despite this limitation of the study it was relevant to investigate if the transcriptional immune response of selected genes might be involved in the observed differences.

Applying a statistical analysis which adjusted for multiplicity and which took into account the various layers of variation in the data, we were not able to detect any significant modification of gene expression levels in the two genotypes following *A. suum* infection. A main explanation could be the low establishment of adult worms in both groups as discussed above but it is also possible that samples were taken too late during trickle infection. Research undertaken in sheep has suggested that the difference between resistant and susceptible animals is due to the rate at which protective immunity develops, rather than gene expression levels *per se* (Ingham *et al.* 2008; Andronicos *et al.* 2010; Hassan *et al.* 2011b). It is a limitation to the present study that we only had one end-point, i.e., when a mature immune response was expected. Further work should focus on profiling the response of the two phenotypes at earlier stages of *A. suum* infection.

We hypothesize that the most important site of immunity to *A. suum* is the small intestine. Lymph nodes draining the small intestine were expected to contain regulatory T cells and cytokine-secreting T cells (e.g., Th1, Th2, Th17, follicular helper T cells) which had been activated by antigen-presenting cells (Zhu *et al.* 2010; Taylor *et al.* 2012). Such antigen-presenting cells would present *A. suum* antigens produced in the small intestine. An additional argument for choosing mesenteric lymph nodes was that the samples were taken after long-term trickle infection (at a 'chronic' stage of infection), as opposed to the strong immune response observed around week 3 PI (Roepstorff *et al.* 1997); we hypothesized a higher immune cell activity in mesenteric lymph nodes, which drain from the entire small intestine, rather than in localized samples of intestinal mucosa.

Although several QTLs for parasite resistance have been identified (Hanotte *et al.* 2003; Gutiérrez-Gil *et al.* 2009), few of these QTLs have been replicated or the underlying genetic markers explored in functional studies (Matika *et al.* 2011). Hassan *et al.* (2011a, b) produced carrier and non-carrier lambs of the DRB1*1101 allele (MHC locus) which were experimentally infected with *Teladorsagia circumcincta* and subsequently necropsied at various time-points post infection. While such studies – as well as studies comparing 'resistant' and 'susceptible' animals identified by genotyping a single SNP – are scarce, there is extensive research carried out using animals that are genetically more divergent. Gene expression studies in phenotypes that are resistant and susceptible to helminth infection have primarily been conducted in sheep, cattle, and rodent models; however, the results are difficult to compare for a number of reasons, e.g., different tissues examined (mucosa, lymph nodes), infection with different parasite species, use of different gene expression platforms (microarray, qPCR), or use of host animals that cannot easily be compared between studies. As an example, some use different breeds or rodent strains whereas others compare resistant and susceptible lines from resource flocks developed by selection and assortative mating for several generations (Pernthaner *et al.* 2005; Ingham *et al.* 2008), or measure gene expression in the most resistant and susceptible animals in a flock after trickle or natural infection (Araujo *et al.* 2009; Zaros *et al.* 2010; Gossner *et al.* 2012; 2013). Nevertheless, ovine studies using lymph nodes or lymph cells have found an increased expression of IL-13 but not IL-4 in resistant animals compared to susceptible animals (Pernthaner *et al.* 2005; Gossner *et al.* 2013). Although increased, we did not find a significant change of expression levels of IL-13 and cannot conclude that this cytokine is a key player in the 'resistant' AA genotype in pigs.

Infection with *Ascaris* in both humans and pigs has been shown to be associated with a Th2-type response characterized by elevated expression of IL-4, IL-5 and IL-13 (Dawson *et al.* 2005; Cooper and Figueredo, 2013; Masure *et al.* 2013) and our results are in agreement with these findings. Hence, an analysis specifically testing for the effect of infection (each genotype at a time) showed the expression of IL-4, the signa-

ture cytokine of a Th2-type response, was significantly elevated in *A. suum*-infected pigs (both genotypes) compared with the uninfected controls (Fig. 2). Likewise, pigs of genotype AA significantly increased IL-13 expression following *A. suum* infection. Both IL-4 and IL-13 bind to the type II IL-4 receptor complex expressed by a variety of cells types (LaPorte *et al.* 2008); activation of the receptor complex initiates different parasite expulsion mechanisms, e.g., elevated mucus and RETNLB (RELM β) production by intestinal goblet cells, increased smooth muscle contractility and increased intestinal permeability (Anthony *et al.* 2007). In addition, IL-4 induces alternatively activated macrophages which may impair health and mobility of tissue-dwelling helminths, including *Ascaris* larvae migrating through the liver (Anthony *et al.* 2006).

Infection with *A. suum* also led to a significant up-regulation of chemokine ligand 17 (CCL17; also known as TARC) in pigs of genotype AA. CCL17 is a chemokine produced by epithelial cells and dendritic cells which binds to Th2 cells (Hartl *et al.* 2009). Dawson *et al.* (2009) reported significant higher CCL17 expression on day 7 PI in liver tissue of pigs given retinoic acid and infected with *A. suum* than in uninfected controls; however, to our knowledge, a significant up-regulation due to *A. suum* infection alone has not previously been demonstrated for this chemokine. In agreement with our results, Geiger *et al.* (2013) found infection with helminths was associated with higher CCL17 concentration in human serum and Burke *et al.* (2011) reported higher CCL17 expression in lungs of *Schistosoma japonicum*-infected mice compared with uninfected controls.

The expression of the high-affinity IgG receptor (FCGR1A; also known as CD64), a candidate gene within our QTL, was strongly dependent on genotype but not on infection with *A. suum* (Fig. 2). This receptor is expressed on most myeloid cells, including macrophages, dendritic cells and granulocytes. Although its role in immunity is not fully elucidated, it has been proposed to help scavenging extracellular antigen bound by IgG (van der Poel *et al.* 2011). In mast cells, activation of this receptor leads to the release of mediators which then act on different cells. Whilst expression of FCGR1A in lymph nodes does not seem to be crucial in determining the phenotypes in pigs at 8 weeks PI, it could be involved at an earlier time-point or be differentially expressed in other tissues, e.g., intestinal mucosa. FCGR1A expression levels in the two genotypes were significantly different, with pigs of the AB genotype having a higher expression level than the AA genotype. This suggests that there are two variants of this gene in LD with SNP TXNIP. The observed differences in FCGR1A expression could be due to variation in for instance a regulatory region; it is also possible that our marker is linked with a copy number variant of the FCGR1A gene (Hindorff *et al.* 2009). In humans, there is a segmental duplication region on chromosome 1q23 which encompasses several low-affinity Fc gamma receptors, e.g., FCGR2A, FCGR2B, and FCGR3A (Schaschl *et al.* 2009). This locus is located about 10 Mb from the human FCGR1A gene. In assembly Sscrofa10.2 of the

pig genome (Groenen *et al.* 2012), these low-affinity receptors are poorly annotated. Since no polymorphisms associated with FCGR1A receptor affinity or function have been found to date (van der Poel *et al.* 2011), it would be interesting to search for mutation(s) or copy number variants underlying the different expression in pigs with the two SNP TXNIP genotypes to assess their importance in the pig's response to various pathogens.

In addition to FCGR1A, we measured the expression of three candidate genes within our QTL (TNFAIP8L2, PIAS3, and RBM8A) as well as TNFSF13B, a candidate gene for *A. lumbricoides* burden (Williams-Blangero *et al.* 2002; 2008) located on porcine chromosome 11. We did not observe a significant change of expression levels in the two genotypes due to *A. suum* infection for any of these genes. Likewise, infection status did not have an effect on their expression levels. As discussed above, these genes could be differentially expressed at an earlier time-point or in other tissues. It would be interesting to measure gene expression in mucosa samples from the small intestine which we have but do not have the resources to analyse at the moment.

In conclusion, we set out to decipher gene expression patterns in mesenteric lymph nodes sampled from trickle-infected pigs with two genotypes. We hypothesized that changes in gene expression levels, in response to infection, were different in the two genotypes. Such a significant modification of gene expression depending on genotype was not observed for any of the genes. However, significant up-regulation of IL-13 and CCL17 following *A. suum* infection was observed in the 'resistant' genotype AA and not in the 'susceptible' genotype. Future studies are needed to characterize the differential effector mechanisms in the two genotypes. No differences in expression levels due to infection were observed for any of the five candidate genes tested in this study and they still remain to be functionally validated. However, we demonstrate that the two genotypes expressed different levels of the high-affinity IgG receptor and further work should focus on deciphering the mechanisms underlying this difference.

Acknowledgements. The authors wish to thank all those who contributed to the research project which forms the basis of this paper: Anne Strandsby, Tina Neergaard Mahler and Karin Tarp provided excellent technical assistance. We would also like to acknowledge Dries Masure for providing input to the selection of genes. P.S. is supported by a University of Copenhagen (Denmark) Ph.D. grant. This work was supported by a Danish Agency for Science, Technology and Innovation grant awarded to P.N.

References

Adugna S., Kebede Y., Moges F., Tiruneh M. 2007. Efficacy of mebendazole and albendazole for *Ascaris lumbricoides* and hookworm infections in an area with long time exposure for antihelminthes, Northwest Ethiopia. *Ethiopian Medical Journal*, 45, 301–306

- Albers G.A., Gray G.D., Piper L.R., Barker J.S., Le Jambre L.F., Barger I.A. 1987. The genetics of resistance and resilience to *Haemonchus contortus* infection in young Merino sheep. *International Journal for Parasitology*, 17, 1355–1363
- Andersen C.L., Jensen J.L., Orntoft T.F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*, 64, 5245–5250
- Andronikos N., Hunt P., Windon R. 2010. Expression of genes in gastrointestinal and lymphatic tissues during parasite infection in sheep genetically resistant or susceptible to *Trichostrongylus colubriformis* and *Haemonchus contortus*. *International Journal for Parasitology*, 40, 417–429. DOI: 10.1016/j.ijpara.2009.09.007
- Anthony R.M., Rutitzky L.I., Urban J.F., Jr., Stadecker M.J., Gause W.C. 2007. Protective immune mechanisms in helminth infection. *Nature Reviews Immunology*, 7, 975–987
- Anthony R.M., Urban J.F., Jr., Alem F., Hamed H.A., Roza C.T., Boucher J.L., *et al.* 2006. Memory T_H2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nature Medicine*, 12, 955–960
- Araujo R.N., Padilha T., Zarlinga D., Sonstegard T., Connor E.E., Van Tassel C., *et al.* 2009. Use of a candidate gene array to delineate gene expression patterns in cattle selected for resistance or susceptibility to intestinal nematodes. *Veterinary Parasitology*, 162, 106–115. DOI: 10.1016/j.vetpar.2008.12.017
- Barger I.A. 1993. Influence of sex and reproductive status on susceptibility of ruminants to nematode parasitism. *International Journal for Parasitology*, 23, 463–469
- Burke M.L., McGarvey L., McSorley H.J., Bielefeldt-Ohmann H., McManus D.P., Gobert G.N. 2011. Migrating *Schistosoma japonicum* schistosomula induce an innate immune response and wound healing in the murine lung. *Molecular Immunology*, 49, 191–200. DOI: 10.1016/j.molimm.2011.08.014
- Bustin S.A., Benes V., Garson J.A., Hellemans J., Huggett J., Kubista M., *et al.* 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR 10.1373/clinchem.2008.112797
- Cooper P.J., Figueiredo C.A. 2013. Immunology of *Ascaris* and immunomodulation. In: (Ed. C. V. Holland) *Ascaris: The neglected parasite*. Elsevier, London, 3–19
- Dawson H., Solano-Aguilar G., Beal M., Beshah E., Vangimalla V., Jones E., *et al.* 2009. Localized Th1-, Th2, T regulatory cell-, and inflammation-associated hepatic and pulmonary immune responses in *Ascaris suum*-infected swine are increased by retinoic acid. *Infection and Immunity*, 77, 2576–2587. DOI: 10.1128/IAI.00827-07
- Dawson H.D., Beshah E., Nishi S., Solano-Aguilar G., Morimoto M., Zhao A., *et al.* 2005. Localized multigene expression patterns support an evolving Th1/Th2-like paradigm in response to infections with *Toxoplasma gondii* and *Ascaris suum*. *Infection and Immunity*, 73, 1116–1128
- Else K.J., Hultner L., Grencis R.K. 1992. Cellular immune responses to the murine nematode parasite *Trichuris muris*. II. Differential induction of T_H-cell subsets in resistant versus susceptible mice. *Immunology*, 75, 232–237
- Geiger S.M., Jardim-Botelho A., Williams W., Alexander N., Diemert D.J., Bethony J.M. 2013. Serum CCL11 (eotaxin-1) and CCL17 (TARC) are serological indicators of multiple helminth infections and are driven by *Schistosoma mansoni* infection in humans. *Tropical Medicine and International Health*, 18, 750–760. DOI: 10.1111/tmi.12095
- Gossner A., Wilkie H., Joshi A., Hopkins J. 2013. Exploring the abomasal lymph node transcriptome for genes associated with resistance to the sheep nematode *Teladorsagia circum-*

- cincta*. *Veterinary Research*, 44, 68. DOI: 10.1186/1297-9716-44-68
- Gossner A.G., Venturina V.M., Shaw D.J., Pemberton J.M., Hopkins, J. 2012. Relationship between susceptibility of blackface sheep to *Teladorsagia circumcincta* infection and an inflammatory mucosal T cell response. *Veterinary Research*, 43, 26. DOI: 10.1186/129797164326
- Groenen M.A., Archibald A.L., Uenishi H., Tuggle C.K., Takeuchi Y., Rothschild M.F., *et al.* 2012. Analyses of pig genomes provide insight into porcine demography and evolution. *Nature* 491, 393–398. DOI: 10.1038/nature11622
- Gutiérrez-Gil B., Perez J., Alvarez L., Martínez-Valladares M., de la Fuente L.F., Bayon Y., *et al.* 2009. Quantitative trait loci for resistance to trichostrongylid infection in Spanish Churra sheep. *Genetics Selection Evolution*, 41, 46. DOI: 10.1186/1297-9686-41-46
- Hanotte O., Ronin Y., Agaba M., Nilsson P., Gelhaus A., Horstmann R., *et al.* 2003. Mapping of quantitative trait loci controlling trypanotolerance in a cross of tolerant West African N'Dama and susceptible East African Boran cattle. *Proceedings of the National Academy of Sciences U. S. A.*, 100, 7443–7448
- Hartl D., Lee C.G., Da Silva C.A., Chupp G.L., Elias J.A. 2009. Novel biomarkers in asthma: Chemokines and chitinase-like proteins. *Current Opinion in Allergy and Clinical Immunology*, 9, 60–66. DOI: 10.1097/ACI.0b013e32831f8ee0
- Hassan M., Good B., Hanrahan J.P., Campion D., Sayers G., Mulcahy G., Sweeney T. 2011a. The dynamic influence of the DRB1*1101 allele on the resistance of sheep to experimental *Teladorsagia circumcincta* infection. *Veterinary Research*, 42, 46. DOI: 10.1186/1297-9716-42-46
- Hassan M., Hanrahan J.P., Good B., Mulcahy G., Sweeney T. 2011b. A differential interplay between the expression of Th1/Th2/Treg related cytokine genes in *Teladorsagia circumcincta* infected DRB1*1101 carrier lambs. *Veterinary Research*, 42, 45. DOI: 10.1186/1297-9716-42-45
- Hindorff L.A., Sethupathy P., Junkins H.A., Ramos E.M., Mehta J.P., Collins F.S., Manolio T.A. 2009. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proceedings of the National Academy of Sciences U. S. A.*, 106, 9362–9367. DOI: 10.1073/pnas.0903103106
- Hothorn T., Bretz F., Westfall P. 2008. Simultaneous inference in general parametric models. *Biometrical Journal*, 50, 346–363. DOI: 10.1002/bimj.200810425
- Ingham A., Reverter A., Windon R., Hunt P., Menzies M. 2008. Gastrointestinal nematode challenge induces some conserved gene expression changes in the gut mucosa of genetically resistant sheep. *International Journal for Parasitology*, 38, 431–442
- Keiser J., Utzinger J. 2008. Efficacy of current drugs against soil-transmitted helminth infections: Systematic review and meta-analysis. *Journal of the American Medical Association*, 299, 1937–1948. DOI: 10.1001/jama.299.16.1937
- LaPorte S.L., Juo Z.S., Vaclavikova J., Colf L.A., Qi X., Heller N.M., Keegan A.D., Garcia K.C. 2008. Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell*, 132, 259–272. DOI: 10.1016/j.cell.2007.12.030
- Masure D., Vlamincck J., Wang T., Chiers K., Van den Broeck W., Vercruysse J., Geldhof P. 2013. A role for eosinophils in the intestinal immunity against infective *Ascaris suum* larvae. *PLoS Neglected Tropical Diseases*, 7, e2138
- Matika O., Pong-Wong R., Woolliams J.A., Bishop S.C. 2011. Confirmation of two quantitative trait loci regions for nematode resistance in commercial British terminal sire breeds. *Animal*, 5, 1149–1156. DOI: 10.1017/S175173111100022X
- Nejsum P., Roepstorff A., Jorgensen C.B., Fredholm M., Göring H.H., Anderson T.J., Thamsborg S.M. 2009. High heritability for *Ascaris* and *Trichuris* infection levels in pigs. *Heredity*, 102, 357–364. DOI: 10.1038/hdy.2008.131
- Noyes H., Brass A., Obara I., Anderson S., Archibald A.L., Bradley D.G., *et al.* 2011. Genetic and expression analysis of cattle identifies candidate genes in pathways responding to *Trypanosoma congolense* infection. *Proceedings of the National Academy of Sciences U. S. A.*, 108, 9304–9309. DOI: 10.1073/pnas.1013486108
- Oksanen A., Eriksen L., Roepstorff A., Ilsoe B., Nansen P., Lind P. 1990. Embryonation and infectivity of *Ascaris suum* eggs. A comparison of eggs collected from worm uteri with eggs isolated from pig faeces. *Acta Veterinaria Scandinavica*, 31, 393–398
- Pernthaner A., Cole S.A., Morrison L., Hein W.R. 2005. Increased expression of interleukin-5 (IL-5), IL-13, and tumor necrosis factor alpha genes in intestinal lymph cells of sheep selected for enhanced resistance to nematodes during infection with *Trichostrongylus colubriformis*. *Infection and Immunity*, 73, 2175–2183
- R Core Team 2013. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria
- Reinemeyer C.R. 2012. Anthelmintic resistance in non-strongylid parasites of horses. *Veterinary Parasitology*, 185, 9–15. DOI: 10.1016/j.vetpar.2011.10.009
- Roepstorff A., Eriksen L., Slotved H.C., Nansen, P. 1997. Experimental *Ascaris suum* infection in the pig: Worm population kinetics following single inoculations with three doses of infective eggs. *Parasitology*, 115, 443–452
- Rozen S., Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology*, 132, 365–386
- Schaschl H., Aitman T.J., Vyse T.J. 2009. Copy number variation in the human genome and its implication in autoimmunity. *Clinical and Experimental Immunology*, 156, 12–16. DOI: 10.1111/j.1365-2249.2008.03865.x
- Skallerup P., Nejsum P., Jorgensen C.B., Göring H.H., Karlskov-Mortensen P., Archibald A.L., *et al.* 2012. Detection of a quantitative trait locus associated with resistance to *Ascaris suum* infection in pigs. *International Journal for Parasitology*, 42, 383–391. DOI: 10.1016/j.ijpara.2012.02.010
- Skallerup P., Thamsborg S.M., Jorgensen C.B., Enemark H.L., Yoshida A., Göring H.H., *et al.* 2014. Functional study of a genetic marker allele associated with resistance to *Ascaris suum* in pigs. *Parasitology*, 141, 777–787. DOI: 10.1017/S0031182013002175
- Skovgaard K., Cirera S., Vasby D., Podolska A., Breum S.O., Durrwald R., *et al.* 2013. Expression of innate immune genes, proteins and microRNAs in lung tissue of pigs infected experimentally with influenza virus (H1N2). *Innate Immunity*, 19, 531–544. DOI: 10.1177/1753425912473668
- Slotved H.C., Barnes E.H., Eriksen L., Roepstorff A., Nansen P., Bjorn H. 1997. Use of an agar-gel technique for large scale application to recover *Ascaris suum* larvae from intestinal contents of pigs. *Acta Veterinaria Scandinavica*, 38, 207–212.
- Steenhard N.R., Jungersen G., Kokotovic B., Beshah E., Dawson H.D., Urban J.F., Jr, *et al.* 2009. *Ascaris suum* infection negatively affects the response to a *Mycoplasma hyopneumoniae* vaccination and subsequent challenge infection in pigs. *Vaccine* 27, 5161–5169. DOI: 10.1016/j.vaccine.2009.05.075
- Taylor M.D., van der Werf N., Maizels R.M. 2012. T cells in helminth infection: The regulators and the regulated. *Trends in Immunology*, 33, 181–189. DOI: 10.1016/j.it.2012.01.001
- Thamsborg S.M., Nejsum P., Mejer H. 2013. Impact of *Ascaris suum* in livestock. In: (Ed. C. V. Holland) *Ascaris: The neglected parasite*. Elsevier, London, 363–381

- van der Poel C.E., Spaapen R.M., van de Winkel J.G., Leusen J.H. 2011. Functional characteristics of the high affinity IgG receptor, FcγRI. *Journal of Immunology*, 186, 2699–2704. DOI: 10.4049/jimmunol.1003526
- Vlaminck J., Geldhof P. 2013. Diagnosis and control of ascariasis in pigs. In: (Ed. C. V. Holland) *Ascaris: The neglected parasite*. Elsevier, London, 395–425
- Wakelin D. 1975. Genetic control of immune responses to parasites: Immunity to *Trichuris muris* in inbred and random-bred strains of mice. *Parasitology* 71, 51–60
- Williams-Blangero S., VandeBerg J.L., Subedi J., Aivaliotis M.J., Rai D.R., Upadhyay R.P., *et al.* 2002. Genes on chromosomes 1 and 13 have significant effects on *Ascaris* infection. *Proceedings of the National Academy of Sciences U. S. A.*, 99, 5533–5538
- Williams-Blangero S., VandeBerg J.L., Subedi J., Jha B., Corrêa-Oliveira R., Blangero J. 2008. Localization of multiple quantitative trait loci influencing susceptibility to infection with *Ascaris lumbricoides*. *The Journal of Infectious Diseases*, 197, 66–71. DOI: 10.1086/524060
- Zaros L.G., Bricarello P.A., Amarante A.F., Rocha R.A., Kooyman F.N., De Vries E., Coutinho L.L. 2010. Cytokine gene expression in response to *Haemonchus placei* infections in Nelore cattle. *Veterinary Parasitology*, 171, 68–73. DOI: 10.1016/j.vetpar.2010.03.020
- Zhu J., Yamane H., Paul W.E. 2010. Differentiation of effector CD4 T cell populations. *Annual Review of Immunology*, 28, 445–489. DOI: 10.1146/annurev-immunol-030409-101212

Received: December 18, 2015

Revised: September 19, 2016

Accepted for publication: October 14, 2016

Appendix A. Supplementary data

Supplementary Table S1

Genes analyzed by quantitative real-time PCR (qPCR), including primer sequences, product size and PCR efficiency. See text for further details

Gene symbol	Gene name	Functional grouping	Primer sequence (5'-3')	Product size (bp)	PCR efficiency (%)
GAPDH ¹	glyceraldehyde-3-phosphate dehydrogenase	Reference gene	F: ACCCAGAAAGACTGTGGATGG R: AAGCAGGGATGATGTTCTGG	79	78.9
HPRT ^{1,2}	hypoxanthine phosphoribosyltransferase 1	Reference gene	F: AACTGGCAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71	84.8 ¹ / 102.1 ²
PPIA ¹	peptidylprolyl isomerase A	Reference gene	F: CAAGACTGAGTGGTTGGATGG R: TGTCCACAGTCAGCAATGGT	138	74.2
TBP ^{1,2}	TATA box binding protein	Reference gene	F: ACGTTCGGTTTAGGTTGCAG R: CAGGAACGCTCTGGAGTTCT	96	99.3 ¹ / 98.7 ²
YWHA ¹	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	Reference gene	F: GCTGCTGGTGTATGATAAGAAGG R: AGTTAAGGGCCAGACCCCAAT	124	82.4
C3	Complement component 3	Innate	F: ATCAAAATCAGGCTCCGATGA R: GGGCTTCTCTGCAATTTGATG	76	94.5
CCL2	chemokine (C-C motif) ligand 2	Innate, Th17	F: GCAAAGTGTCTTAAAGAAGCAGTG R: TCCAGGTGGCTTATGGAGTC	103	80.7
CCL3	chemokine (C-C motif) ligand 3	Innate	F: CCAGGTCTTCTCTGCACCCAC R: GCTACGAATTTGCGAGGAAG	90	96.5
CCL5	chemokine (C-C motif) ligand 5	Innate	F: CTCCATGGCAGCAGTCGT R: AAGGCTTCCCTCCATCCTAGC	121	89.2
CCL17 (TARC)	chemokine (C-C motif) ligand 17	Innate, Th2-associated	F: GGGTGGTACCAGACCTCAGA R: GTCCTTGGGGTCAGAACAGA	90	102.7
CSF2	colony stimulating factor 2	Th1	F: CCGAGGAAACTTCCCTGTGAA R: GCAGTCAAAGGGGATGGTAA	92	97.6
FOXP3	forkhead box P3	Treg	F: GAAAGGACAGCACCCCTTTCAA R: AGGAAGTCCCTCTGGCTCCTC	111	115.4
GZMB	granzyme B	Apoptosis	F: CCAGGACCAGGATAATCGAA R: GGGTGACGTTGATTGAGCTT	101	87.6

IFNG	interferon, gamma	Th1	F: CCATTCAAAGGAGCATGGAT R: TTCAGTTTCCCAGAGCTACCA	76	73.5
IL1A	interleukin 1, alpha	Innate, Th17	F: GACGAAACCCGTGTGCTG R: CCATATTGCCATGCTTTTC	97	70.0
IL4	Interleukin 4	Th2	F: TCGGCACATCTACAGACACC R: CTTCTTGGCTTCATGCACAG	109	107.2
IL5	Interleukin 5	Th2	F: TGCCTACGTTAGTGCCATTG R: TCGATGAATGGAGAGCAGTG	82	114.0
IL10	Interleukin 10	Treg, Th2, Th17	F: TACAACAGGGGCTTGCTCTT R: GCCAGGAAGATCAGGCAATA	110	104.0
IL12B	interleukin 12B	Th1, Th17	F: GTGCTGGAAGCTGTTCACAA R: TGGTTTGATGATGTCCTGA	75	69.4
IL13	Interleukin 13	Th2, Th17	F: CCAAGCGAGCAAGTTCCCTG R: AACTACCCGTGGCGAAAAAT	109	87.0
IL13RA2	interleukin 13 receptor, alpha 2	Th2	F: TGAAAGCTGGAAAGACGATCA R: GCCCTGGCAGAAAGTGATGT	101	73.5
IL33	Interleukin 33	Th2	F: AGGCATTCAACCAACAAAAGG R: ACAGACCGTTCAAAGGTGCC	92	79.7
NOS2	nitric oxide synthase 2, inducible	Innate	F: GCAGCTACTGGGTCAAAGGAC R: GCTGTTGGTGAACTTCCACTT	200	77.0
PIAS3	protein inhibitor of activated STAT, 3	Candidate gene	F: AGATGAACAGGATGCCCTTG R: GGTGAGAGCTCCCCAGTGT	95	89.6
RBM8A	RNA binding motif protein 8A	Candidate gene	F: ACATCCACCTCAACCTGGAC R: TGGGCTCCTTGATGTTTC	82	92.0
STAT6	signal transducer and activator of transcription 6	Th2	F: TCCCAGATGATCCACCACA R: ATCTGCAGGTGAGGTTCTCTG	107	86.3
TGFB1	transforming growth factor, beta 1	Treg	F: GCAAAGTCTCTGGCTCTGTA R: TAGTACACGATGGCAGTGG	108	73.7
TLR1	toll-like receptor 1	Innate	F: CCTTCAAGACCTTAACACACAGAG R: CAGATTACTGCGGTGCTGA	100	96.0
TNF	tumor necrosis factor	Innate	F: CACGTTGTAGCCAATGTCAAAG R: GAGGTACAGCCCATCTGTCG	129	93.4

TNFAIP3	tumor necrosis factor, alpha-induced protein 3	Innate	F: CCCAGCTTTTCTCTCAATGGAC R: TTGGTTCTTCTGCCGTCTCT	113	93.8
TNFAIP8L2 (TIPE2)	tumor necrosis factor, alpha-induced protein 8-like 2	Candidate gene	F: TCTATGGGCCTGACTTCACC R: CAGAGTTTCCCCCTCATCCAG	79	111.2
TNFSF13B (BAFF)	tumor necrosis factor (ligand) superfamily, member 13b	Candidate gene	F: CATTGCAAAAGCTGGAGGAA R: AAAAATGTGCCGCTCCATC	90	99.6
FCGR1A (CD64)	Fc fragment of IgG, high affinity Ia, receptor	Candidate gene	F: AGTGGTGAATACAGGTGCCA R: TCGAGACCTGGAGTAGTAGC	95	99.7

¹Reference genes used for normalization²Reference genes used for normalization of FCGR1A