Early immune responses to an adjuvant (AbISCO®-100) tested in porcine peripheral blood mononuclear cells

By Anna Wallenbeck Department of Animal Breeding and Genetics, SLU.

Annika Bolind Bågenholm

Caroline Fossum
Department of Biomedical Sciences and Veterinary Public Health
Division of Immunology

Exam project, Biology – 15p
SLU, Uppsala 2009

Abstract

Immune stimulating complex (iscom) matrix is a formulation that is used as an adjuvant within research. The capability of the iscom matrix to elicit an immune response when not present together with an antigen has not been extensively investigated. One way to evaluate the status of the immune system is to measure the amount of cytokines that usually are produced by various cell types during an immune response. The aim of the present study was to evaluate the early immune responses in porcine peripheral blood mononuclear cells (PBMC) after stimulation *in vitro* with the iscom matrix, AbISCO®-100. The immune response was evaluated by measuring the mRNA expression for various cytokines, a chemokine receptor (CXCR4) and regulator of G-protein signaling (RGS16) in porcine PBMC after stimulation with AbISCO®-100 or other inducers like a lipopolysaccharide (LPS) and a CpG oligodeoxynucleotide (ODN 2216) used as controls. Quantitative real-time PCR analysis showed low increase ($p \leq 0.05$) in mRNA expression of the pro-inflammatory cytokine TNF-α after 6 hours of stimulation with AbISCO®-100. This result indicates that AbISCO®-100 may have the capacity to evoke a cytokine response by itself. In addition, low but detectable increases in expression of IL-10, CXCR4 and RGS16 were observed in one of five pigs after stimulation with AbISCO®-100. In comparison, mRNA for IL-1β, IL-6, IL-10 ($p \leq 0.001$), IL-12p40 and TNF-α ($p \leq 0.01$) was induced after 6 hours exposure of the PBMC to LPS and for IFN-α ($p \leq 0.01$) after 6 h in the presence of ODN 2216. Proliferation and viability test performed, demonstrated that AbISCO®-100 was not mitogenic and just had a small lytic effect on the PBMC . Thus, the iscom matrix, AbISCO®-100, does not appear to be toxic or induce any harmful early inflammatory responses in porcine PBMC. An extended period of incubation and use of a lager number of pigs are needed to better examine the effects of AbISCO®-100.

Sammanfattning

Immun-stimulerande komplex (iscom) matrix är en formulering, vilken i forskningssammanhang används, som adjuvant. Stimulering av immunaktivering i porcina PBMC med enbart iscom matrix (utan närvaro av ett antigen) är sparsamt redovisat i litteraturen. Ett sätt att utvärdera den immunreglerande effekten är att mäta mängden cytokiner som normalt produceras av olika celltyper under ett immunsvar. Syftet med denna studie var att utvärdera den tidiga immunaktiveringen i porcina PBMC efter stimulering *in vitro* med iscom matrix, AbISCO®-100. Immunaktiveringen utvärderades genom mätning av mRNA-uttryck för olika cytokiner, en kemokin receptor (CXCR4) och en regulator av G-protein signalering (RGS16) i porcina PBMC efter stimulering med iscom matrix AbISCO®-100 eller andra inducerare, som en lipopolysaccharid (LPS) och en CpG oligodeoxynukleotid (ODN 2216), vilka användes som kontroller. Kvantitativa real-tids PCR-analyser visade en liten höjning i mRNA uttryck ($p \leq 0.05$) av det proinflammatoriska cytokinet TNF-α efter 6 timmars stimulering med AbISCO®-100. Det resultatet indikerar att AbISCO®-100 kan ha förmåga att väcka ett cytokinsvar. Låga detekterbara ökningar i uttryck av IL-10, CXCR4 och RGS16 kunde observeras i en av fem grisar efter stimulering med AbISCO®-100. I jämförelse hade mRNA för IL-1β, IL-6, IL-10 ($p \leq 0.001$), IL-12p40 och TNF-α ($p \leq 0.01$) inducerats efter 6 timmars exponering med LPS och för IFN-α ($p \leq 0.01$) efter 6 timmars induktion med ODN 2216. Proliferations- och viabilitetstester, visade att AbISCO®-100 ej var mitogent och bara hade en liten lytisk effekt på PBMC. Följaktligen verkar inte iscom matrix, AbISCO®-100, vara toxisk eller inducera några skadliga tidiga inflammatoriska reaktionen i porcina PBMC. En längre inkubationstid och användning av ett större antal grisar behövs för att bättre kunna undersöka effekterna av AbISCO®-100.

Introduction

An adjuvant is a substance that has the capacity to enhance the immune response when it is presented together with an antigen. AbISCO®-100 is an adjuvant formulation that corresponds to the structural components (the matrix) of immune stimulating complexes (iscoms). Iscoms formed by incorporation of defined antigen in their matrix have a well-documented capacity to induce protective immunity to a vast number of pathogens in several species.

The iscom was first described 25 years ago by Morein et al. (1984) as a new particulate structure for antigen presentation. After that the iscom was tested in different animal models with the intention to develop safe and effective vaccines. The iscoms showed to be efficient inducers of both humoral and cellular immune responses with minimal side effects (Pearse & Drane, 2005). However the traditional iscom-based vaccines were limited to only presenting antigen consisting of hydrophobic parts of viral membrane proteins. Furthermore the antigen needed to be prepared together with the iscom structure and could not spontaneously be incorporated afterwards. New findings that enabled incorporation of a broader range of antigens have increased the usefulness of iscoms. To overcome difficulties in the manufacturing processes, the possibility to mix antigens with preformed iscom matrixes has been evaluated for use as vaccines (Sanders et al., 2005).

The traditional iscom matrix consists of three components; cholesterol, saponin and phopholipid that form a 40 nm in diameter cage-like structure. Thus, the iscom matrix has the same composition and shape as the iscom but has no antigen incorporated (Sanders et al., 2005). The cholesterol functions as a ligand that binds to the saponin to form 12 nm rings, where the phospholipid glues the rings together, forming the cage-like structure (Morein et al., 2004). Both the cholesterol and the phospholipids can be produced synthetically, whereas the saponins are derived from the *Quillaja saponaria* Molina tree bark (Sanders et al., 2005). Free saponins are by themselves potent inducer of immune responses but may have negative side effects e.g. lytic properties. An advantage of using an iscom matrix as adjuvant (instead of free saponins) is that the haemolytic activity of the saponins in an iscom matrix is very much reduced (Morein et al., 2004).

AbISCO®-100 is a commercially available iscom matrix formulation aimed to be used in research. AbISCO®-100 consists of cholesterol, phospholipid and a combined structure of two saponin fractions, fraction A and fraction C. The cholesterol is produced from lanolin (from wool) and the phospholipid is a phosphatidyl choline obtained from fresh egg yolk. It has been tested in several animal species including mice, pigs, monkeys, non-human primates and horses but is particularly designed for use in mice (www.isconova.se). In the present study effects of AbISCO®-100 on porcine pheripheral blood mononuclear cells were tested *in vitro*.

In the experiments the levels of mRNA expression of various cytokines, a chemokine receptor (CXCR4) and a regulator of G-protein signaling (RGS16) was measured after treatment with AbISCO®-100. Two additional stimulators, lipopolysaccharide (LPS) and a CpG oligodeoxynucleotide (ODN 2216) were included as positive controls for certain cytokines. The LPS forms part of gram-negative bacterial cell walls and has the capability to elicit an inflammatory response. ODN 2216 is synthetically made and contains "CpG"-motifs that mimic immunostimulatory DNA released by intracellular microbes.

The mRNA expression was determined by quantitative real-time PCR analyze of the cytokines: IFN-α, IFN-γ, IL-6, IL-10, IL-12p40, IL-1β, TNF-α and TGF-β. Pro-inflammatory cytokines like IL-1, IL-12 and TNF-α are elicited in the early stage of an immune response. The release of these cytokines facilitates production of the same cytokines or various others. For example, IL-12 activates cells to release IFN-γ whereas TNF-α stimulates the secretion of IL-6. There is a large number of cytokines that activate different cells of the immune system. However there are also inhibitory cytokines to counter balance these effects to keep the homeostatic level, for example, when a response is no longer needed. Two examples of cytokines with anti-inflammatory properties are IL-10 and TGF-β. Thus, cytokines are the principal mediators of communication between cells in the immune system and consequently the ability of an adjuvant to induce cytokine production is important for its mode of immune regulation.

Materials and methods

Inducers used for cytokine gene expression

The inducers used were AbISCO®-100 (Isconova AB, Uppsala, Sweden), LPS (Sigma Aldrich, Steinheim, Germany) and ODN 2216 (Cybergene AB, Huddinge, Sweden). The inducers were diluted in RPMI 1640 medium (BioWhittaker, Cambrex Bioscience, Verviers, Belgium) supplemented as described below but without fetal calf serum. The inducers were used at a final concentration of AbISCO®-100 (1 μg/ml), LPS (10 μg/ml) or ODN 2216 (5 μg/ml) in the cultures.

Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were collected in heparinized, 10-ml evacuated test tubes (BD Vacutainer System, Plymouth, UK) from five pigs conventionally reared at the University Research Station, Funbo Lövsta, Uppsala, Sweden. PBMC were separated from plasma, neutrophils and erythrocytes by density gradient centrifugation on Ficoll-pague (Amersham Pharmacia Biotech, Uppsala, Sweden) for 45 minutes at 1800 rpm (without brake). The band containing PBMC was collected and transferred to new test tubes. Phosphate buffered saline (PBS) was added to the PBMC and the PBMC were washed three times by centrifugation for 10 minutes at 1000 rpm (with brake). A final wash in PBS to remove any thrombocytes was performed by centrifugation for 5 min at 1500 rpm. Cells were resuspended in complete medium (RPMI 1640 supplemented with 20 mM HEPES buffer, 2 mM L-glutamine, 200 IU penicillin/ml, 100 μg/ml streptomycin 0.5 μM 2-mercaptoethanol and 5% fetal calf serum (Invitrogen, Life Technologies, Carlsbad, CA, USA). Ten μl was used to determine the total amount of PBMC by counting in Türks solution that lyses red blood cells and thereby facilitates the estimation of the number of the PBMC. The concentration of PBMC was adjusted to 10×10^6 cells per ml culture medium.

Cell cultures for analysis of cytokine, CXCR4 and RGS16 mRNA expression were established in 25 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at a volume of 1.5 ml/flask. Then an equal volume of the inducers: AbISCO®-100, LPS, ODN 2216 or plain medium was added to a final volume of 3 ml/flask.
The cell cultures were incubated for 6 hours at 37°C, 7% CO_2 and thereafter transferred to 10 ml tubes and centrifuged for 5 min at 1500 rpm. A part of the supernatants (1 ml) was collected and stored at -20°C for conceivable further analysis.

In parallel, cell cultures for proliferation and viability tests were established in round bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 2 x 10^5 cells per well (100 µl/well). AbISCO®-100, LPS, ODN 2216 or medium was added to a final volume of 200 µl/well.

The final concentration of the cells in the tissue culture flasks was 5 x 10^6 cells per ml. Final concentration of the cells in the wells in the microtiter plate was 1 x 10^6cells per ml.

Extraction of RNA and cDNA synthesis

Total RNA was isolated by combining the recommended protocols for Trizol® Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) and E.Z.N.A. ® total RNA Kit I (Omega Bio-tek, Norcross, GA). In brief, the cell pellets (containing approximately 15 x 10^6 cells) were resuspended in Trizol® (2 ml/flask), mixed and incubated for 5 minutes. The samples were aliquoted to two eppendorf tubes (1 ml/tube) and stored at -80°C. The concentration and purity of the RNA was measured (OD ratio 260/280) by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Montchamin, DE.). The isolated RNA was treated with Dnase (Rnase-free Dnase Kit, Promega, Madison, WI) accordning to manufactures instructions, but with an extended incubation time of 40 minutes to eliminate any residual DNA in the samples. The synthesis of complementary DNA (cDNA) was performed according to Superscript II Reverse Transcriptase kit protocol (Invitrogen, Carlsbad, CA). The first cDNA strand was synthesized using 2 µg of RNA as template. In addition to the regular cDNA synthesis, a mixture without Superscript II reverse transcriptase was made and run in parallel to those with the enzyme added. This was used as a control

enabling the detection of genomic DNA as the gene encoding IFN-α lacks introns. The cDNAs were stored at -20°C.

Proliferation and viability tests of PBMC

Proliferation and viability tests were performed on PBMC obtained from three of the pigs. The proliferation of the PBMC was tested after 48 hours of incubation with the various stimulators. Cells stimulated with AbISCO®-100, LPS, ODN 2216 or plain medium were cultured in 96-well micro titer plates in triplicates for 24 hours at 37°C, 7% CO_2. Thereafter 25 µl of ^{3}H-thymidine (0.5µCi ^{3}H-thymidine specific activity 5 Ci/mmol; Amersham, England) was added to two wells in each triplicate. After 24 hours in the presence of ^{3}H-thymidine (total incubation time with the stimulators, 48 hours), the cells were harvested onto nitrocellulose filters and the amount of incorporated ^{3}H-thymidine (radioactivity, counts per minute) was measured in a liquid scintillation β-counter (Betaplate; LKB Wallac, Turku, Finland). The relative proliferation expressed as stimulation index (SI) was calculated by dividing the cpm values of the stimulated cultures with cpm values of unstimulated cultures.

The viability test was performed on cells in one well from each triplicate, after 24 hours of incubation. The cell cultures were stained with Trypan Blue to distinguish viable from non-viable cells. The proportion of dead cells was determined by counting in a Bright-line hematocytometer metalized counting chamber (Becton Dickinson).

Quantification of cytokine, CXCR4 and RGS16 mRNA expression in PBMC

The level of cytokine, CXCR4 and RGS16 mRNA expression in porcine PBMC was measured after various stimulations (AbISCO®-100, LPS, ODN 2216 or medium) using Quantitative Taqman real-time PCR (qRT-PCR). The qRT-PCR was performed for IFN-α, IFN-γ, IL-6, IL-10, IL-12p40, IL-1β, TNF-α, TGF-β, CXCR4, RGS16 and two reference genes, Cyclophilin A (CyA) and Hypoxanthine-guanine phosphoribosyl transferase (HPRT), using previously published primers and probes (Table 1). As a control the IFN-α analysis was also run on cDNA prepared in the absence of reverse transcriptase. The cDNA was diluted 5 times in sterile water and added in 1.1 µl volumes in triplicate 25 µl reactions, containing 1x ABsolute™ QPCR Mix (ABgene, Epsome, UK), 0.4 µM forward primer, 0.4 µM reverse primer and 0.2

μM 5´-6-carboxyfluorescein (FAM) and 3´-Black Hole Quencer 1 (BHQ1) labeled probe. The samples were amplified and analyzed in an IQ5 Real Time PCR machine (Biorad, Hercules, CA) using an initial 15 min at 95°C followed by 40 cycles of 15 s at 95°C and 60°C for 30 s. The FAM fluorescence signal was measured at the 60°C step for each cycle.

Relative mRNA expression levels of cytokines, CXCR4 and RGS16 in comparison to the reference genes in the different samples were calculated by using the following formula:

$$R = 1 \div (x/y)$$

Ct	= Ct mean value of triplicate
Stimulation	= AbISCO®-100, LPS or ODN 2216
Target	= 8 various cytokines, CXCR4 or RGS16
Ref1 and Ref2	= CyA and HPRT
Medium	= Treatment of PBMC with plain medium
R	= Relative mRNA expression

Where x and y is equal to the following formulas:

$$x = Ct_{Stimulation,Target} / ((Ct_{Stimulation,Ref1} + Ct_{Stimulation,Ref2})/2)$$

$$y = Ct_{Medium,Target} / ((Ct_{Medium,Ref1} + Ct_{Medium,Ref2})/2)$$

The formula used for calculation of the relative mRNA expression (R) is composed by x and y and then inverted.

In brief, the relative mRNA expression of the target genes (eight selected cytokines, CXCR4 and RGS16) in comparison to the reference genes (Cy A = Ref1 and HPRT = Ref2) was estimated by comparing the Ct mean values (mean values of triplicates). First the target gene expression was divided with the mean value of expression of the two reference genes of the stimulated PBMC. This value was then related to the target gene expression of PBMC cultured in plain growth medium divided with the expression of the two reference genes in PBMC cultured in plain growth medium. The results were inverted in relation to each other, by taking 1 divided with the result.

Statistical analysis

To analyze whether the different treatments (AbISCO®-100, LPS and ODN 2216, respectively) differed in their cytokine inducing capacity the paired t-test (Student's t-test) was used. The t-test was performed by comparing the values generated from the formula for calculation of the relative mRNA expression, which is given above. P values less than 0.05 were considered statistically significant.

Results

Proliferation analyses and viability test of PBMC

The viability test of PBMC from three of the pigs, showed up to 20% of dead cells after 24 hours of incubation in the presence of 1 μg AbISCO®-100 per ml. The viability of the cells incubated for 24 hours with ODN 2216 or LPS were similar or occasionally lower than for cells cultured in plain medium. The death of non-treated cells (cultured in plain growth medium) was approximately 6 % (Table 2).

The proliferative capacity of PBMC obtained from three of the pigs was tested on cells which had been stimulated with AbISCO®-100, LPS, ODN 2216 or cultured in plain growth medium for 48 h (Figure 1). The spontaneous proliferation (cells cultured in plain growth medium) was approximately two times higher for PBMC isolated from pig no. 4 than for PBMC isolated from pig no. 3 and no. 5. For all three pigs, PBMC stimulated with ODN 2216 showed the highest proliferation, samples stimulated with LPS had a moderate proliferation whereas the samples stimulated with AbISCO®-100 had the lowest proliferation.

Expression of mRNA for selected cytokines, CXCR4 or RGS16 after 6 h of stimulation with the inducers

In the first series of experiments, where the mRNA expressions from pig no. 1 and no. 2 were examined, the mRNA expressions were related to each reference gene separately as well as to the mean value of the two reference genes. No difference in the mRNA expression levels of the examined genes was observed after they had been related to each reference gene separately or to a mean value of both reference genes. Therefore following calculations were performed using only the mean value of mRNA expression of the two reference genes. In general, the results obtained from the five pigs were in good agreement as illustrated by the small SD values (Figure 2). The compiled results are given in Table 3.

The result of PBMC from the five pigs stimulated with AbISCO®-100 showed no significant increase in mRNA expression of the different target genes examined except for TNF-α (Table 3). The increase in TNF-α mRNA expression was low and had a p value ≤ 0.05. In PBMC from one of the pigs (pig no. 1) a small increase in the

relative mRNA expression of IL-10 (R=1.0365), CXCR4 (R=1.0432) and RGS16 (R=1.0808) was seen after stimulation with AbISCO®-100.

The porcine PBMC stimulated with LPS showed significant increases in mRNA expressions with the highest levels of expression of IL-1β, IL-6, IL-10 ($p \leq 0.001$) and lower levels of expression of TNF-α and IL-12p40 ($p \leq 0.01$). Low but significant decreases in mRNA expression after stimulation with LPS was detected for IFN-α and TGF-β ($p \leq 0.05$).

The porcine PBMC stimulated with ODN 2216 showed a significant increase in mRNA expression with high levels of expression for IFN-α ($p \leq 0.01$) and IL-12p40 ($p \leq 0.05$) and lower levels of IL-6, TNF-α, IFN-γ and IL-10 ($p \leq 0.05$). A low but significant decrease in mRNA expression after stimulation with ODN 2216 was detected for CXCR4 ($p \leq 0.01$).

The LPS-induced expression of IL-1β, IL-6, IL-10, IL-12p40 and TNF-α in porcine PBMC was expected as well as the IFN-α, IL-12p40, IL-6 and IFN-γ induction by ODN 2216. Taken together these positive controls prove that the methodology used is applicable and that the present qRT-PCR is useful for detection of these target genes. The methods used thus appear to be appropriate for examination of responses elicited after stimulation of porcine PBMC with AbISCO®-100.

Discussion

In vitro exposure of porcine PBMC to AbISCO®-100 for 6 hours had no profound effect on the mRNA expression of a number of cytokine genes. Of the eight cytokines analyzed, only a slight increase in the expression of mRNA for TNF-α was detected and neither the expression of the chemokine receptor CXCR4 or its regulator RGS16 was altered. These results are in agreement with the absence of fever and local reactions in toxicity tests performed by injection of seven days old piglets with varying doses (up to 150 μg) of iscom matrix (McIntosh et al., manuscript).

To study effects of AbISCO®-100 on the viability of porcine PBMC the cells were incubated in the presence of AbISCO®-100 for 24 hours. Parallel incubations were established with LPS and ODN 2216. The higher proportion of dead cells among PBMC cultured in presence of AbISCO®-100 compared to the other inducers indicates that *in vitro* exposure of porcine PBMC to AbISCO®-100 (1 μg/ml, 24 hours) affects their viability. The fraction-C saponin that is present in combination with the fraction-A saponin in the formulation AbISCO®-100 has a strong lytic effect on PBMC itself. If AbISCO®-100 has been taken up by the cells and then been degraded, the content of Fraction-C saponin could theoretically exert a lytic effect.

The proliferation test performed showed that non-stimulated PBMC (grown in plain medium) had a low spontaneous proliferation. The spontaneous proliferation was slightly higher for PBMC isolated from pig no. 4 than from the other two pigs tested which could reflect an *in vivo* activation prior to the collection of blood samples. Regardless of the initial status, the proliferative response of the PBMC was consistently lower after stimulation with AbISCO®-100 than after stimulation with LPS or ODN 2216, indicating that AbISCO®-100 is not mitogenic for porcine PBMC. However the cell division, determined by incorporation of ^{3}H thymidine during the last 24 of the 48 hours of culture, confirmed that a considerable proportion of the PBMC were still alive and dividing despite the slight decrease in viability observed after 24 hours.

A small increase in expression of TNF-α was observed for PBMC stimulated with AbISCO®-100 from four of the five pigs. This indicates that AbISCO®-100 may have the property to induce an inflammatory response. Indeed, analyses of mRNA collected from two of the pigs after 20 hours of incubation showed an increased expression of mRNA for IL-1β at that time (results not shown). After 6 hours of incubation with AbISCO®-100 a small increase in the expression of IL-10, CXCR4

and RGS16 was found in PBMC from one of the pigs when compared to non-stimulated PBMC. A previous study by Morein et al. (2004) has shown that one specific feature of iscom matrix is the capacity to simultaneously induce both a Th1 and Th2 type of immune response. The production of IL-10 is usually up regulated during a Th2 response. The results from the PBMC from pig no. 1 therefore indicate that AbISCO®-100 at certain conditions can induce cytokines associated with a Th2 type of response in the pig.

Monocytes and naïve B and T lymphocytes normally express the chemokine receptor CXCR4, which facilitate the migration of the cells to lymphoid organs. CXCR4 is a G protein coupled receptor, which can be down modulated by binding to its ligand, stromal-derived factor-1 (SDF-1) (Cole SW et al. 1999 and Signoret N et al.1997). The increased expression of CXCR4 is probably just a normal feature in the migration of naive lymphocytes to the lymphoid organs. An up-regulation of RGS16, as indicated in pig no. 1, could aim to counteract the increased expression of CXCR4. Such an inhibition of CXCR4 due to an increased expression of RGS16 appears logical as RGS16 down-regulates CXCR4 in megakaryocytes (Berthebaud et al., 2005). However this result was only observed in one of the five pigs, which emphasizes the need to perform the tests on PBMC collected from a larger number of pigs and to include additional cytokines representing a Th2 type of response.

Stimulation of the PBMC with LPS for 6 hours was included as a positive control for induction of mRNA expression for IL-1β, IL-12p40, IL-10 and TNF-α since an increase of these cytokines is known to accompany the early response to LPS (Boeuf et al., 2005). An increased expression of the pro-inflammatory cytokines IL-1β, IL-12 and TNF-α was expected since they are released by activated macrophages and dendritic cells during the early stage of an immune response e.g. against a bacterial infection. Also an increase in the expression of IL-6 was expected since this cytokine is generally produced in response to LPS stimulation. Macrophages and endothelial cells stimulated by IL-1 and TNF-α, elicits IL-6, which promotes the differentiation of T and B cells and stimulates an acute-phase response. In accordance, the expression of mRNA for IL-1β, IL-6, IL-10 and TNF-α was increased in porcine cells collected from the mammary gland 24 h after inoculation with *Escherichia coli*. This expression

of genes for pro-inflammatory cytokines was correlated with fever and other clinical symptoms of mastitis (Zhu et al. 2008). Thus, LPS seemed to be a proper control for induction of a cytokine profile associated with the later development of a Th2 type of immune response.

Indeed, an increased expression of mRNA for IL-1β, IL-12p40, IL-10 and TNF-α was detected in the present study for PBMC stimulated with LPS. In addition, a slight decrease in expression (lower level of expression than in medium) was found for IFN-α and TGF-β. The decrease in expression of IFN-α after stimulation with LPS may be due to that this cytokine is needed mainly against intracellular infectious agents (e.g. viruses) and is not essential during an extracellular bacterial infection. Thus it is more efficient for the immune system to favour the important pro-inflammatory cytokines released during an early immune response towards a bacterial infection, for example via LPS. The decrease in expression of TGF-β could be explained by its immunosuppressive properties; it is usually secreted in the late stages of an immune response to inhibit proliferation of both T and B lymphocytes. TGF-β also activates macrophages in order to suppress the immune activities when its no longer needed.

Stimulation of the PBMC with ODN 2216 for 6 hours was used as a positive control for the expression of IFN-α, IL-12p40, IL-6 and IFN-γ because expression of these cytokine genes has been observed after *in vitro* exposure of porcine PBMC to this CpG ODN (Andersson et al. 2007). The cytokine IL-12 has a central role for activation of various cell types in the immune system and also for further cytokine secretions. For example, IL-12 has the capability to activate macrophages and natural killer cells and stimulate the secretion of IL-6 and IFN-γ. The antiviral cytokine IFN-α is produced during early stages of a viral infection and is usually induced by intracellular signalling from endosomal TLR9 (Jiang et al. 2005). Therefore, ODN 2216 that activates cytokine production via TLR9 was used to mimic a virus-induced cytokine response that subsequently commonly leads to a Th1 type of immune response. In the present study the mRNA expressions of IFN-α, IL-12p40, IL-6 and IFN-γ of the PBMC induced by ODN 2216 also agreed well with the predicted response. In addition, increased expression of mRNA for IL-10 and TNF-α were observed. In general, IL-12 induces differentiation of Th1 cells resulting in a Th1 immune response i.e. production of IL-2 and IFN-γ. Th2 cells normally produce IL-10 to inhibit a Th1 response to maintain the homeostatic state of innate and cell-mediated

immune reactions. A low increase in expression of IL-10 may therefore be due to Th2 cells secreting IL-10 to inhibit the elicited Th1 type of response. The decrease in the expression of CXCR4 due to the stimulation with ODN 2216 can be explained by that the chemokine receptor is usually down-regulated during cellular activation (Signoret et al.1997).

Because of the short period of incubation the majority of cytokines induced by the *in vitro* stimulators are most likely to be produced by cells of the innate immunity such as dendritic cells and monocyte/macrophages. In the present study however the PBMC were obtained from conventionally reared pigs that are likely to be "primed" by previous exposure to a number of microorganisms. Thus, the detection of mRNA for some T-cell derived cytokines that are elicited by the *in vitro* "re-stimulation" can be expected. From a practical point of view this is the likely situation when vaccinating animals and therefore the modest detection of T-cell cytokines observed during the present conditions are promising.

In the present study quantitative real-time PCR was used to determine the mRNA expression of cytokines, CXCR4 and RGS16. Quantitative real-time PCR is a rapid, and sensitive technique commonly used for detection of the mRNA expression levels in different cells and tissues (Giulietti et al. 2001). The relative mRNA expression of the various cytokines, CXCR4 and RGS16 was used to evaluate the expression of the genes. The relative expression was calculated by comparing the mRNA expression of one gene to a mean value of expression of two reference genes. The reference genes should be constantly expressed in the samples regardless of treatment. There where two reference genes used in this experiment: one with a high expression level, cyclophilin A and one with a lower expression level, HPRT. The relative expression of the cytokines, CXCR4 and RGS16, after various stimulation were also compared to the mRNA expression level in samples treated with plain growth medium. The level of mRNA expression of some cytokines was below the detection limit (40 cycles) for the qRT-PCR assay. Expression levels below this limit was therefore given a Ct value of 40 cycles in the mathematical calculations. Furthermore, the present study was conducted on samples from only five pigs. Such a population is quite restricted and may not give representative results and to get a more reliable result the amount of animals tested should be expanded. Another suggestion for further studies is to use a

well-known method for estimation of the relative mRNA expression e.g. using the geometric mean method according to Vandesompele et al. (2002), thereby enable comparison to previously published results. Nevertheless, the immune stimulating complex matrix, AbISCO®-100, does not seem to elicit any massive and uncontrolled immune activation by itself and it does not have a strong lytic effect on porcine PBMC. It may have a small immune stimulating capacity by itself but not notably high at this concentration and time of stimulation. Thus, further studies are needed with PBMC obtained from a greater number of pigs and using a wider range of matrix concentrations and periods of incubation. The measurements should also be expanded to include more cytokines/chemokines and their receptors.

References

Thesis

Hasslung Wikström, F. (2008). *Interaction between porcine circovirus type 2 and the immune system of the pig*. Diss. Uppsala: doctoral thesis no. 2008:29 faculty of veterinary medicine and animal science, Swedish University of Agricultural science

Journals

Andersson M, Berg M, Fossum C, Jensen-Waern M (2007). Development of a Microarray for Studying Porcine Cytokine Production in Blood Mononuclear Cells and Intestinal Biopsies
J Vet Med A Physiol Pathol Clin Med. 54(3):161-8

Berthebaud M, Rivière C, Jarrier P, Foudi A, Zhang Y, Compagno D, Galy A, Vainchenker W, Louache F. (2005). RGS16 is a negative regulator of SDF-1-CXCR4 signaling in megakaryocytes.
Blood. 1;106(9):2962-8.

Boeuf P, Vigan-Womas I, Jublot D, Loizon S, Barale J-C, Akanmori B D, Mercereau-Puijalon O, Behr C (2005). CyProQuant-PCR: a real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use
BMC Immunology, 6(1):5

Cole SW, Jamieson BD, Zack JA (1999). cAMP up-regulates cell surface expression of lymphocyte CXCR4: implications for chemotaxis and HIV-1 infection.
Immunol. 1;162(3):1392-400.

Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression.
Methods. 25(4):386-401.

McIntosh KA, Parker SE, Andreoni C, Harding JHS, Charreyre C, Fossum C, Lövgren K and Ellis JA.Vaccination with the matrix of immunostimulating complexes (ISCOMs) mixed with porcine circovirus type 2 (PCV2) virus-like particles (VLP) in a porcine circovirus disease (PCVD)-affected commercial swine herd (manuscript in preparation)

Morein B, Hu KF, Abusugra I (2004). Current status and potential application of ISCOMs in veterinary medicine.
Avd Drug Deliv Rev. 23;56(10):1367-82.

Morein B, Sundquist B, Höglund S, Dalsgaard K, Osterhaus A. (1984). Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses.
Nature. 4;308(5958):457-60.

Pearse MJ, Drane D. (2005). ISCOMATRIX adjuvant for antigen delivery.
Adv Drug Deliv Rev. 10;57(3):465-74. Review.

Sanders MT, Brown LE, Deliyannis G, Pearse MJ. (2005). ISCOM-based vaccines: the second decade.
Immunol Cell Biol. 83(2):119-28.

Signoret N, Oldridge J, Pelchen-Matthews A, Klasse PJ, Tran T, Brass LF, Rosenkilde MM, Schwartz TW, Holmes W, Dallas W, Luther MA, Wells TN, Hoxie JA, Marsh M. (1997). Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4.
J Cell Biol. 3;139(3):651-64

Timmusk S, Merlot E, Lövgren T, Järvekulg L, Berg M, Fossum C. Regulator of G protein signaling 16 (RGS16) is a target for a porcine circovirus type-2 protein. Submitted for publication.

Jiang W, Lederman M.M., Salkowitz J.R., Rodriguez B., Harding C.V., Sieg S.F. (2005). Impaired Monocyte Maturation in Response to CpG Oligodeoxynucleotide Is Related to Viral RNA Levels in Human Immunodeficiency Virus Disease and Is at Least Partially Mediated by Deficiencies in Alpha/Beta Interferon Responsiveness and Production
JOURNAL OF VIROLOGY, p. 4109–4119

Zhu Y, Magnusson U, Fossum C, Berg M. (2008). Escherichia coli inoculation of porcine mammary glands affects local mRNA expression of Toll-like receptors and regulatory cytokines.
Vet Immunol Immunopathol. 15;125(1-2):182-9. Epub 2008 May 8.

Table 1. Primers and probes used for detection of mRNA expression by Quantitative Taqman real-time PCR

Gene		Oligonucleotide sequences (5'-3')
IFN-α[1]	FW	AGC CTC CTG CAC CAG TTC TG
	RV	TCA CAG CCA GGA TGG AGT CC
	Pr	5AG GCT TCC AGG TCC CTG AGC TGC TX
IFN-γ[1]	FW	TGG TAG CTC TGG GAA ACT GAA TG
	RV	GGC TTT GCG CTG GAT CTG
	Pr	5CT TCG AAA AGC TGA TTA AAA TTC CGG TAG ATA ATC TGC X
IL-6[1]	FW	CTG GCA GAA AAC AAC CTG AAC C
	RV	TGA TTC TCA TCA AGC AGG TCT CC
	Pr	5TT GAA CCC AGA TTG GAA GCA TCC GTC TTT TX
IL-10[1]	FW	CGG CGC TGT CAT CAA TTT CTG
	RV	CCC CTC TCT TGG AGC TTG CTA
	Pr	5AG GCA CTC TTC ACC TCC TCC ACG GCX
IL-1β[1]	FW	GTG ATG GCT AAC TAC GGT GAC AA
	RV	CTC CCA TTT CTC AGA GAA CCA AG
	Pr	5AA TAA TGA CCT GTT ATT TGA GGC TGA TGG CX
TNF-α[1]	FW	AGC CTC TTC TCC TTC CTC CTG
	RV	GAG ACG ATG ATC TGA GTC CTT GG
	Pr	5AG GAG CCA CCA CGC TCT TCT GCC TX
TGF-β[1]	FW	TAC GCC AAG GAG GTC ACC C
	RV	CAG CTC TGC CCG AGA GAG C
	Pr	5CT AAT GGT GGA AAG C G GC AAC CAA ATC TAX
IL-12p40[1]	FW	TCT TGG GAG GGT CTG GGT TG
	RV	AAG CTG TTC ACA AGC TCA AGT ATG A
	Pr	5AC CAG CAG CTT CTT CAT CAG GGA CAT CAX
HPRT[1]	FW	GTG ATA GAT CCA TTC CTA TGA CTG TAG A
	RV	TGA GAG ATC ATC TCC ACC AAT TAC TT
	Pr	5AT CGC CCG TTG ACT GGT CAT TAC AGT AGC TX
CyA[1]	FW	TGC TTT CAC AGA ATA ATT CCA GGA TTT A
	RV	GAC TTG CCA CCA GTG CCA ATT A
	Pr	5TG CCA GGG TGG TGA CTT CAC ACG CCX
CXCR4[2]	FW	CAT CTT CTT AAC TGG CAT AGT GGG TAA
	RV	CCG TCA TGC TTC TCA GTT TCT TC
	Pr	5CT GGT AAC CCA TGA CCA GGA TGA CCA ATC X
RGS16[2]	FW	TCC GAT CCC GCG AGT GT
	RV	CAC TCC ATT TTT ACT GCT CAG CAA
	Pr	5CT TTG CTG TGT TTA CTG CCC CAC TCG AX

[1]From Hasslung Wikström, F. 2008, [2] Timmusk et al. manuscript

Table 2. Viability of PBMC from three of the pigs, cultured in the presence of AbISCO®-100 (1 μg/ml), LPS (10 μg/ml), ODN 2216 (5 μg/ml) or medium for 24 h

	Proportion (%) dead cells		
Inducer	Pig no. 3	Pig no. 4	Pig no. 5
AbISCO®-100	20.3 %	7.1 %	11.1 %
LPS	8.6 %	3.6 %	8.7 %
ODN 2216	6 %	5.1 %	2 %
Medium	6 %	6.6 %	5.3 %

Table 3. Significance of increase/decrease in relative mRNA expression (R) after different treatments of porcine PBMC compared to cultured in plain growth medium calculated as described in material and method. NS = Not significant, *p≤0.05, ** p≤0.01, *** p≤0.001; Student's paired t-test, (n=5).

Target gene	AbISCO®-100 vs. medium p value	LPS vs. medium p value	ODN 2216 vs. medium p value
IFN-α	NS	*	**
IFN-γ	NS	NS	*
IL-6	NS	***	*
IL-10	NS	***	*
IL-1β	NS	***	NS
TNF-α	*	**	*
TGF-β	NS	*	NS
IL-12p40	NS	**	*
CXCR4	NS	NS	**
RGS16	NS	NS	NS

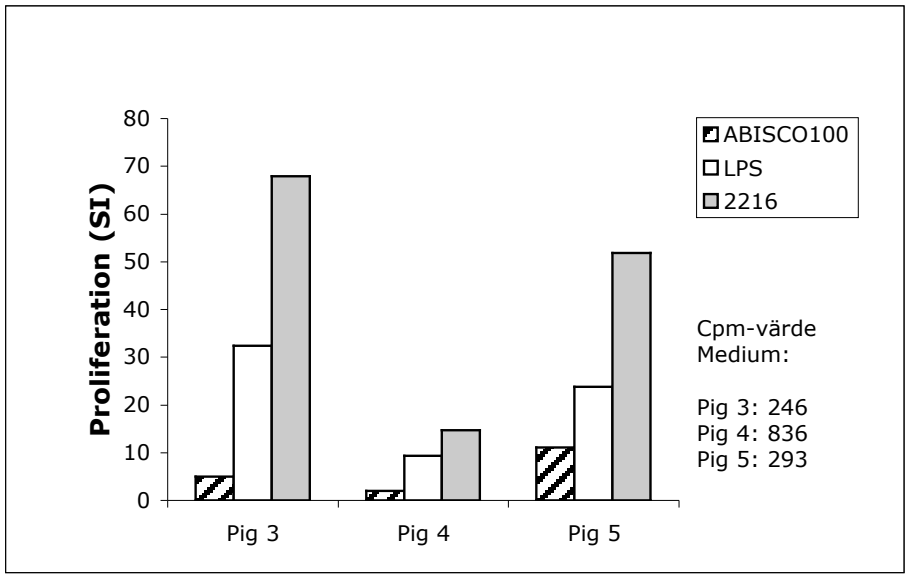
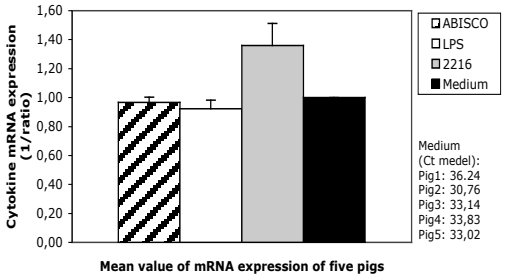
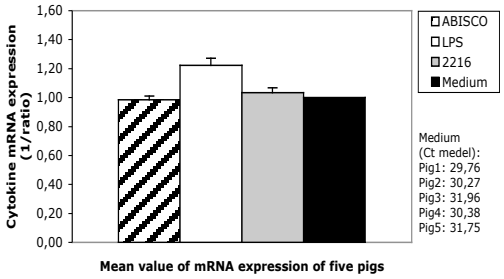


Figure 1. The proliferative capacity of PBMC obtained from three experimental pigs after stimulation with AbISCO®-100 (1 μg/ml), LPS (10 μg/ml), ODN 2216 (5 μg/ml) or plain medium for 48 h. The bars show proliferation after different stimulations compared to the spontaneous proliferation (proliferation in medium). Striped bars represent stimulation with AbISCO®-100, white bars LPS and grey bars ODN 2216.

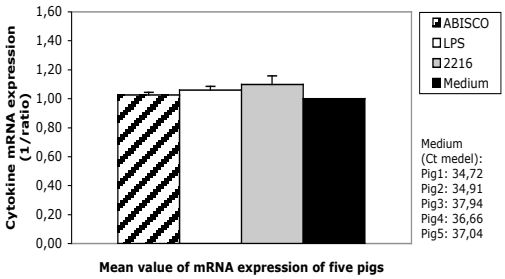
IFN-α



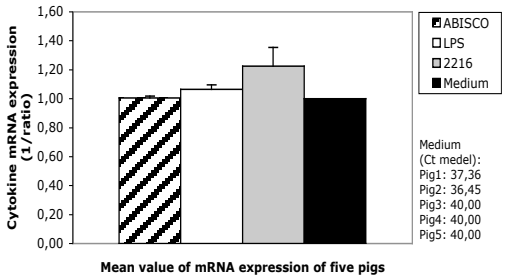
IL-1β



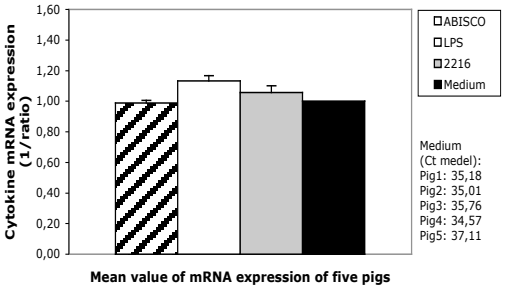
TNF-α



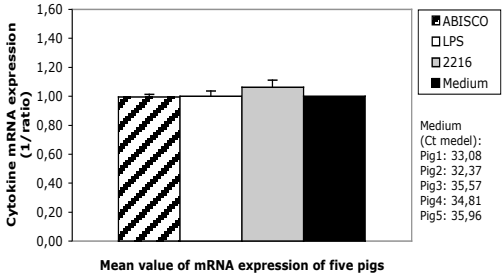
IL-12



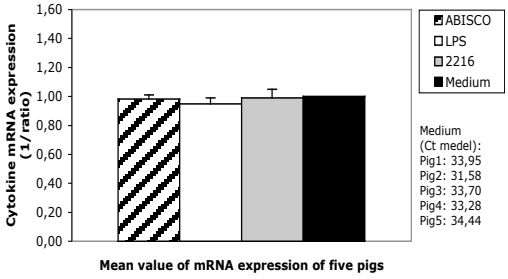
IL-6



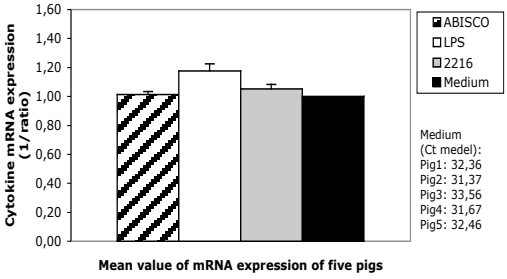
IFN-γ



TGF-β



IL-10



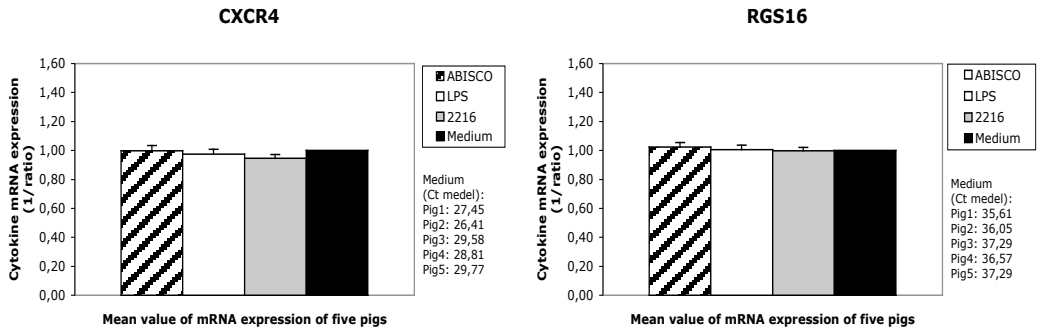


Figure 2. Total RNA was extracted, cDNA synthesized and quantified by qRT-PCR to determine cytokine, CXCR4 and RGS16 mRNA expression levels as described in material and methods. The bar graphs displays the mRNA expressions of IFN-α, IFN-γ, IL-6, IL-10, IL-12p40, IL-1β, TNF-α, TGF-β, CXCR4, RGS16 normalized to two reference genes (cyclophilin A and HPRT) and the mean±SD within the five pigs.