Interleukin-4, interleukin-5, and interleukin-13 gene expression in cultured mononuclear cells from porcine circovirus type 2–vaccinated pigs after cells were challenged with porcine circovirus type 2 open reading frame 2 antigen

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Objective—To characterize the kinetics of interleukin (IL)-4, IL-5, and IL-13 secretion in peripheral blood and lymph node mononuclear cells isolated from porcine circovirus type 2 (PCV2)–vaccinated pigs after cells were challenged with PCV2 open reading frame 2 antigen.

Animals—10 pigs.

Procedures—5 pigs were vaccinated with a PCV2 vaccine and received a booster dose 3 weeks later. They were kept together with a similar group of 5 nonvaccinated pigs that served as controls. One week after the second vaccination, peripheral blood mononuclear cells (PBMCs) and excised retropharyngeal lymph node mononuclear cells (LNMCs) were isolated and cultured. Cells were then challenged by exposure to PCV2 open reading frame 2 and evaluated at 2, 12, 24, and 48 hours to determine the expression of IL-4, IL-5, and IL-13 via quantitative PCR assay. Changes in gene expression were analyzed relative to the results from analysis of the sample at 0 hours (calibrator).

Results—All ILs were upregulated differently in LNMCs and PBMCs from vaccinated pigs. Lymph node mononuclear cells from vaccinated animals produced significantly more IL-4 mRNA than did PBMCs at 2, 12, and 48 hours (relative change: 2.8 vs –3.6, 13.0 vs 3.6, and 9.8 vs 1.8, respectively) and more IL-5 mRNA at 2, 12, 24, and 48 hours (relative change: 1.2 vs –4.8, 2.2 vs 0.2, 3.2 vs –1.9, and 4.0 vs –3.6, respectively). Interleukin-13 mRNA reached its highest concentration at 24 hours but was 11.9-fold higher in PBMCs than in LNMCs.

Conclusions and Clinical Relevance—Results supported the importance of IL-4, IL-5, and IL-13 in pigs, suggesting that PBMCs and LNMCs express cytokines in a tissue-specific manner. (*Am J Vet Res* 2013;74:110–114)

The T-helper type 1–T-helper type 2 paradigm is based on cytokine secretion patterns in activated T-helper cells. The T-helper type 2 response primarily stimulates B-cell proliferation and specific antibody formation.[1] This humoral immune response is largely regulated by the secretion of IL-4, IL-5, IL-10, and IL-13 by T-helper type 2 cells, but it is unclear which of these are most important in the swine immune response. The mechanisms in pigs that regulate cytokine profiles at the time of T-cell stimulation, which are largely responsible for T-helper differentiation, seem to be similar to those described in species such as mice and humans.[1–3]

Abbreviations

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL	Interleukin
LNMC	Lymph node mononuclear cell
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PCV	Porcine circovirus
qPCR	Quantitative PCR

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Whereas IL-4 is a major T-helper type 2 cytokine in mice and humans, it is rarely expressed in PBMCs of swine,[1,4] and some authors even describe a different role for this IL.[5] In contrast, IL-13 is readily produced by porcine PBMCs,[6] and some authors suggest that it may have a similar role to IL-4 in other species.[7] Studies[1,3] on the effect of vaccines on specific T-helper type 2–mediated responses in pigs are limited.

The inadequate sensitivity of ELISAs to detect low concentrations of ILs in the supernatants of biological samples, coupled with the low presence of IL-producing cells in peripheral blood, likely explains the difficulties in detecting cytokines by this technique.[8,9] In contrast, real-time qPCR assay has proved to be a specific, sensitive, and accessible technology to quantify gene expression at the mRNA level.[10]

Porcine circovirus type 2 (PCV2) infection is ubiquitous in swine populations and the cause of postweaning multisystemic wasting syndrome, a disease considered a major economic problem for the swine industry worldwide.[11] Piglets with high antibody titers to PCV2 are well protected from a severe PCV2 challenge.[12] Advances in the understanding of the immunologic response against PCV2 infection in pigs could help devise strategies to enhance the design of therapeutic and prophylactic interventions. Peripheral blood mononuclear cell analysis is easy because blood samples can be easily obtained, but the immune response takes place in lymph nodes, and these can have a different T-helper type 2 response, compared with PBMCs.[13] This makes it necessary to use cells from blood and lymphoid organs to evaluate immunocompetence against pathogens.

The objective of the study reported here was to evaluate the kinetics of porcine IL-4, IL-5, and IL-13 in PBMCs and LNMCs obtained from PCV2-vaccinated pigs after cells were exposed in vitro to the PCV2 ORF2 antigen.

Materials and Methods

The University of Murcia Animal Care Committee reviewed and approved the experimental protocols. The experiment was performed in accordance with the guidelines of the European Union for animal experimentation (Directive 86/609/EEC).

Experimental animals and vaccination schedule—Ten healthy 10-week-old specific-pathogen–free Large White pigs were randomly allocated to 2 experimental groups. The pigs from the first group (n = 5) were vaccinated IM in the cervical region with 2 mL of PCV2 vaccine[a] and received a booster dose 3 weeks later. The pigs from the second group (n = 5) remained unvaccinated and served as controls. Both groups were kept together in the same pen and were clearly marked by ear tags. On day 7 after the second vaccination, all pigs were sedated with tiletamine-zolazepam[b] and then euthanized by a lethal dose of 5% sodium thiopental.[c] Samples of blood and retropharyngeal lymph nodes were obtained from each pig and processed immediately.

Preparation of PBMCs and LNMCs—Immediately after death, retropharyngeal lymph nodes were excised and fat was removed. They were then transported in sterile plastic bags and homogenized by a laboratory blender[d] in 7 mL of sterile PBS solution. Tissue homogenates were transferred to 15-mL tubes and centrifuged at 400 × *g* at 20°C for 10 minutes. Both PBMCs and LNMCs were isolated by density gradient centrifugation with a separating agent.[e] The isolated cells were washed 3 times with PBS solution. Peripheral blood mononuclear cells and LNMCs obtained from each pig were adjusted to 2.5×10^6 cells/mL and cultured in complete RPMI 1640 medium (penicillin [50 U/mL], streptomycin [50 µg/mL], 0.01% β-mercaptoethanol, and 10% fetal calf serum), which was distributed in 24-well plates in 1-mL aliquots. Peripheral blood mononuclear cells and LNMCs from each pig were divided into 10 equal aliquots; a series of 5 aliquots was used for stimulation with PCV2 ORF2 antigenic peptides at a final concentration of 1 µg/mL, and the other 5 aliquots were used for PBS solution (control) treatment. Porcine circovirus type 2 ORF2 antigenic peptides were synthetized and purified as reported.[14] The entire contents of each well were aspirated and centrifuged (200 × *g* for 10 minutes at 20°C) to form a cell pellet. Total RNA from PBMC and LNMC was extracted at 0, 2, 12, 24, and 48 hours after PCV2 ORF2 stimulation or control treatment.

RNA extraction and cDNA synthesis—The RNA was extracted from controls and PCV2 ORF2–stimulated PBMCs and LNMCs with an RNA purification kit[f] according to the manufacturer's instructions. Total RNA quantity and purity were measured with a spectrophotometer.[g] The DNA was eliminated from all RNA samples with DNase reagent.[h] Reverse transcription of RNA into cDNA was performed with a reverse transcription kit.[i]

Real-time qPCR assay—The oligonucleotide primer and probe sequences used for the detection of IL-4, IL-5, IL-13, B-actin, GAPDH, and cyclophilin gene expression by the qPCR assay have been described.[15–17] Primers were designed in the exon-exon junction to avoid genomic DNA amplification. All of the probes were labeled with 6-carboxyfluorescein. All of the oligonucleotides were commercially synthesized.[j] Real-time qPCR reactions were performed with gene expression master mix[k] and a sequence detection system.[l] The amplification conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Fluorescence signals were measured at the end of each cycle. All reactions were performed in duplicate. Interleukin-4, IL-5, and IL-13 gene expression were standardized to the geometric mean expression of B-actin, GAPDH, and cyclophilin (as housekeeping genes). Data were normalized with the Pfaffl method.[18] The PCR efficiencies were calculated with the following formula[18]:

$$10^{-1/\text{slope}} - 1$$

with the slope displayed by software.[m] To determine the effect of stimulation by PCV2 ORF2 antigen on T-helper type 2 cytokine expression in PBMCs and LNMCs, the B-actin, GAPDH, and cyclophilin genes were used as endogenous control genes and samples at 0 hours for each pig were used as calibrators.

Statistical analysis—A Kolmogorov-Smirnov test was used to determine whether IL-4, IL-5, and IL-13 gene expression followed a normal distribution. To evaluate the effect of the stimulation with PCV2 ORF2 on IL production, a Mann-Whitney *U* test and Pearson correlation were used. Values of $P < 0.05$ were considered significant. Analyses were performed with statistical software.[n]

Results

Kinetic analysis of IL gene expression in PBMCs and LNMCs—Polymerase chain reaction assay efficiencies were 98% for IL-4, 99% for IL-5, 92% for IL-13, 99% for B-actin, and 100% for GAPDH and cyclophilin. The kinetics of IL-4, IL-5, and IL-13 gene expression that occurred in PCV2 ORF2 antigen–exposed LNMCs and PBMCs from vaccinated pigs were determined. Little variation in IL-4, IL-5, and IL-13 gene expression among individuals within groups was detected (**Table 1**). Quantitative PCR assays revealed a downregulation of IL-4, IL-5, and IL-13 gene expression at 2 hours in PBMCs. Interleukin-4 mRNA gene expression increased during the first 12 hours in the PBMCs and LNMCs from vaccinated animals. Interleukin-5 gene expression was increased at 12 hours in the LNMC from vaccinated animals. Interleukin-13 mRNA gene expression was increased at 24 and 48 hours in the PBMCs and LNMCs from vaccinated animals. Porcine circovirus type 2 ORF2 exposure did not cause any changes in the expression of any of the IL genes at any time in PBMCs and LNMCs from control pigs. No IL gene expression change was detected for any of the ILs tested in any PBS solution–treated sample derived from vaccinated or control animals.

Lymph node mononuclear cells isolated from PCV2-vaccinated pigs had greater IL-4 gene expression than did PBMCs at 2 (2.8 vs –3.6; $P = 0.008$), 12 (13.0 vs 3.6; $P = 0.047$), and 48 (9.8 vs 1.8; $P = 0.032$) hours, with the highest gene expression at 12 hours. The gene expression of IL-5 mRNA was significantly higher in LNMCs than in PBMCs at 2 (1.2 vs –4.8; $P = 0.009$), 12 (2.2 vs 0.2; $P = 0.008$), 24 (3.2 vs –1.9; $P = 0.008$), and 48 (4.0 vs –3.6; $P = 0.008$) hours, with the highest gene expression at 48 hours. Interleukin-13 mRNA gene expression reached its highest value at 24 hours for PBMCs (11.9 times the expression at 0 hours) and at 48 hours for LNMC (5.9), with significant ($P = 0.008$) differences at 24 hours (0.0 for LNMCs and 11.9 for PBMCs; Table 1). Taken together, the results indicated that LNMCs obtained from PCV2-vaccinated pigs and exposed to PCV2 ORF2 in culture had significantly more IL-4 and IL-5 mRNA gene expression than did PBMCs, whereas IL-13 mRNA gene expression was higher in PBMCs than in LNMCs.

Correlations of IL gene expression in PBMCs and LNMCs—A positive correlation was found between mRNA gene expression for IL-4 and IL-5 ($r = 0.93$; $P = 0.024$), IL-4 and IL-13 ($r = 0.91$; $P = 0.031$), and IL-5 and IL-13 ($r = 0.99$; $P = 0.001$) at 24 hours after stimulation in PBMCs. Interleukin-4 and IL-5 mRNA gene expression was also correlated at 48 hours in PBMCs ($r = 0.88$; $P = 0.049$). For LNMCs, no correlation was found for mRNA gene expression among any of the ILs at any time point.

Discussion

Cytokines play an important role in the immune response signaling cascade, and the balance of different cytokines determines the outcome of infectious challenges.[19] The T-helper type 1–T-helper type 2 paradigm discovered in mice has provided a framework for vaccine development in swine, but some authors describe substantial differences in cytokine function between pigs and mice at the cellular and molecular level.[5] Therefore, some controversy exists regarding the importance of IL-4, IL-5, and IL-13 in the T-helper type 2 immune response in pigs.[5]

This study aimed to characterize the pattern of IL gene expression in mononuclear cells isolated from

Table 1—Kinetics of mRNA expression of IL-4, IL-5, and IL-13 in PBMCs and LNMCs obtained from PCV type 2 (PCV2)–vaccinated pigs and control pigs at 2, 12, 24, and 48 hours after PCV2 stimulation or PBS solution (control) treatment.

				IL-4					IL-5					IL-13				
Group	**Cell type**	**Treatment**		**0**	**2**	**12**	**24**	**48**	**0**	**2**	**12**	**24**	**48**	**0**	**2**	**12**	**24**	**48**
PCV2-vaccinated pigs	PBMC	PCV2 stimulation	Mean	1.0	–3.6	3.6	4.2	1.8	1.0	–4.8	0.2	–1.9	–3.6	1.0	–11.6	3.5	11.9	9.4
			SD	0.0	1.4	1.1	2.4	0.7	0.0	0.6	1.3	0.3	1.2	0.0	9.6	4.2	9.4	7.0
		Control	Mean	1.0	–4.0	0.3	0.8	0.4	1.0	–4.9	–1.2	–1.6	–3.0	1.0	–12.5	0.5	0.6	–0.1
			SD	0.0	2.6	0.3	0.7	0.9	0.0	0.8	0.1	0.2	1.0	0.0	11.7	1.3	1.2	1.8
	LNMC	PCV2 stimulation	Mean	1.0	2.8	13.0	11.2	9.8	1.0	1.2	2.2	3.2	4.0	1.0	–4	–0.7	0.05	5.18
			SD	0.0	2.1	11.3	8.2	4.8	0.0	0.2	0.5	0.6	2.2	0.0	2.3	2.8	1.7	3.6
		Control	Mean	1.0	0.94	1.04	0.96	0.86	1.0	1.0	0.8	0.8	0.8	1.0	–3.8	–2.4	–1.1	0.4
			SD	0.0	0.2	0.1	0.2	0.1	0.0	0.1	0.2	0.3	0.2	0.0	2.4	1.3	1.4	0.2
Nonvaccinated pigs	PBMC	PCV2 stimulation	Mean	1.0	–3.8	1.1	1.1	0.9	1.0	–3.7	–3.3	–3.5	–3.7	1.0	–11.6	1.0	1.0	1.1
			SD	0.0	1.0	0.2	0.4	0.4	0.0	1.0	1.1	0.9	0.7	0.0	6.2	0.3	0.4	0.2
		Control	Mean	1.0	–4.4	0.3	0.9	1.0	1.0	–4.3	–3.9	–4.0	–4.5	1.0	–9.5	0.8	1.0	1.0
			SD	0.0	1.7	1.1	0.3	0.2	0.0	1.2	1.1	1.4	1.2	0.0	4.9	0.3	0.3	0.2
	LNMC	PCV2 stimulation	Mean	1.0	1.1	1.1	1.1	0.9	1.0	0.9	0.9	0.8	0.8	1	–4.3	–1.7	0.2	0.6
			SD	0.0	0.3	0.2	0.2	0.3	0.0	0.3	0.3	0.4	0.3	0.0	2.4	1.0	0.3	0.2
		Control	Mean	1.0	0.8	0.9	0.9	1.0	1.0	0.5	0.7	0.8	0.6	1.0	–6.0	–2.1	0.5	0.5
			SD	0.0	0.1	0.3	0.1	0.2	0.0	0.0	0.2	0.3	0.1	0.0	2.0	1.0	0.3	0.2

Changes in gene expression were expressed relative to the results from analysis of the sample at 0 hours (calibrator).

PCV2-vaccinated pigs and challenged with an exposure to a PCV2 antigen. Production of IL-4, IL-5, and IL-13 protein in PCV2 ORF2–exposed cultured mononuclear cells occurs at low concentrations and is difficult to detect by standard ELISA techniques. Although changes in mRNA gene expression do not necessarily reflect alterations of the translated proteins, a good correlation between mRNA gene expression and secreted protein concentrations exists for these ILs in humans.[20,21] In the present study, a qPCR assay was used in an effort to enhance the quantitation of cytokines that are poorly detected by ELISA. Interleukin-13 had a low efficiency of amplification (92%). Although different concentrations of primers and amplification conditions were tested to increase IL-13 amplification efficiency, 92% was the highest efficiency obtained. Differences in the efficiency of amplification must be taken into account for proper calculation of initial target gene expression. To address the differences in the efficiency between the genes analyzed, a quantification model with kinetic PCR efficiency correction was used.[18]

There was little variation in IL gene expression among pigs of the same group. The initial downregulation of IL-4, IL-5, and IL-13 gene expression at 2 hours might have been due to the adaptation of cells to culture conditions, which are different from those existing in vivo. Not surprisingly, PBMCs and LNMCs from PCV2-vaccinated pigs had much greater gene expression for IL-4, IL-5, and IL-13 mRNA than did those from nonvaccinated pigs, which was indicative of an anamnestic T-cell response. The synthesis of these ILs was consistent with their role in the orchestration of a protective T-helper type 2 immune response to PCV2 challenge in vaccinated pigs.

The results indicated that classical T-helper type 2–secreted cytokines were upregulated differently in LNMCs and PBMCs. In vaccinated pigs, IL-5 mRNA upregulation was only detected in LNMCs, whereas IL-4 and IL-13 transcripts were expressed in both PBMCs and LNMCs. For IL-4, mRNA synthesis was greater in LNMCs, and for IL-13, the response was faster and greater in PBMCs. The results corroborate, with a different experimental system (ex vivo cell culture system to evaluate PCV2-specific T-helper type 2 immune response), those of another study[6] that lymph node cultures produce high concentrations of IL-4 protein. The low concentrations of IL-4 mRNA in PBMCs were consistent with observations of Díaz and Mateu,[4] who related low or even undetectable IL-4 concentrations in culture supernatants of phytohemagglutinin-stimulated PBMCs analyzed by use of ELISA. Raymond and Wilkie[1] detected a large amount of IL-13 transcripts but not IL-4 mRNA in blood T cells cultured with hen egg white lysozyme–treated monocyte-derived dendritic cells. It is necessary to consider that differences observed in the literature about IL-4, IL-5, and IL-13 synthesis by PBMCs could be explained because the peripheral response would depend on the relative quantity of different cell populations in the peripheral blood at sampling time.[19]

Swine monocyte–derived dendritic cells can be generated in vitro by culturing cells in IL-4 and granulocyte- and macrophage colony–stimulating factor.[22] Bautista et al[7] described the generation of porcine monocyte–derived dendritic cells in vitro with porcine IL-13, which were phenotypically and functionally undistinguishable from those induced with IL-4. The successful use of IL-4 and IL-13 to generate monocyte-derived dendritic cells from pigs in vitro was attributable to the existence of a common receptor chain for IL-13 and IL-4.[22] This common receptor can explain the observation that IL-13 and IL-4 share many structural and functional properties.[23,24] Therefore, it is possible that IL-13 is synergistic with IL-4 in regulating the immune response in pigs. The immune response is induced by multiple cytokines and chemokines in a complex manner. The amount of expression of each IL and the interaction between multiple cytokines are not the only important factors because the effects of each IL may differ depending on their anatomic localization.[25] Similarly to our results, Levast et al[26] identified 2 expression patterns of T-helper type 2 cytokines in ileal and jejunal Peyer's patches. Ileal Peyer's patches were characterized by a higher expression of IL-5 mRNA, whereas jejunal Peyer's patches had higher expression of IL-4 mRNA. There are at least 2 possibilities to explain the differential expression pattern of ILs observed in the present study between PBMCs and LNMCs. First, IL gene expression patterns could be differentially regulated depending on the tissue-specific environment. Second, the differential expression of ILs observed could reflect the existence of different populations depending on their anatomic localization[27] (although phenotypic populations in blood and lymph nodes were not characterized in the study reported here).

Cytokines IL-4, IL-5, and IL-13 are all transcribed from a single gene cluster in pigs on chromosome 2, presenting coordinated regulators and independent transcriptional mechanisms.[28,29] This coordinated regulation system could explain the positive correlations found between IL-4 and IL-5, IL-4 and IL-13, and IL-5 and IL-13 gene expression at 24 hours after stimulation in PBMCs. On the basis of our results and the literature, we suggest that IL-4, IL-5, and IL-13 are all important in swine, depending on the tissue analyzed, and that these T-helper type 2 ILs could act in synergy in the T-helper type 2 porcine immune response because of their transcription profile in blood and lymph node tissues.

The investigation of PBMCs is advantageous because blood samples are readily accessible, and this facilitates longitudinal studies. Conversely, PBMC analysis includes multiple cell types and includes both autocrine and paracrine regulations.[9] Immune response development takes place in lymph nodes, which can have a different T-helper type 2 pattern, compared with PBMCs.[13] Results of the study reported here indicated that analysis of both PBMCs and LNMCs in immunologic studies gives a better insight into the workings of the porcine immune response because of the differences detected in the cytokine profile of each cell population.

The major distinctions of the experimental approach in the present study, compared with others, were that an ex vivo cell culture system (not artificial in vitro data) was evaluated to study porcine T-helper type 2 immune response initiation at the level of PCV2 antigen–PBMC and PCV2 antigen–LNMC interactions.

Taken together, the results supported the importance of IL-4, IL-5, and IL-13 in pigs, suggesting that blood and lymph nodes expressed a different cytokine mRNA pattern, which may be due to differential regulation depending on the tissue-specific environment or to the existence of different lymphoid populations in both organs. Results could provide useful information for evaluation of vaccine efficacy measured as the porcine T-helper type 2 immune response generated after application of the immunostimulant. Moreover, the data obtained in the present study could help researchers select which T-helper type 2 cytokine to evaluate depending on the porcine tissue to be studied.

a. Circovac, Merial Ltd, Lyon, France.
b. Zoletil, Virbac, Madrid, Spain.
c. Thiovet, Vet Ltd, London, England.
d. Stomacker Lab-Blender, PBI International, Baranzate, Italy.
e. Pancoll, density 1.077 g/mL, PAN Biotech Gmbh, Aidenbach, Germany.
f. RNeasy Mini Kit, Qiagen, Hilden, Germany.
g. Nanodrop spectrophotometer, Thermo Scientific, Waltham, Mass.
h. Turbo DNA Free, Ambion Inc, Austin, Tex.
i. Quantitect reverse transcription kit, Qiagen, Hilden, Germany.
j. Oligonucleotides, TIB-MOLBIOL GmBH, Berlin, Germany.
k. Taqman gene expression master mix, Applied Biosystems Inc, Foster City, Calif.
l. ABI Prism 7500 Sequence Detection System Inc, Applied Biosystems Inc, Foster City, Calif.
m. 7500 system software, Applied Biosystems Inc, Foster City, Calif.
n. SPSS, version 15.0, SPSS Inc, Chicago, Ill.

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