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Time Course Study of Cytokine mRNA Expression in LPS-Stimulated Porcine Alveolar Macrophages

In-Soo Choi, Na-Ri Shin, Sung-Jae Shin, Deog-Yong Lee, Young-Wook Cho and Han-Sang Yoo*

Department of Infectious Diseases, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea

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ABSTRACT

The kinetics of cytokine mRNA expression was studied in porcine alveolar macrophages using an RT-PCR assay. The expression levels of IFN-γ, IL-2, IL-4, IL-6, GM-CSF, IL-12 p35, and IL-12 p40 were examined after 2, 4, 14, 24, 48, and 72 h of incubation in unstimulated control and LPS-stimulated cells. The expression contents of IFN-γ, IL-2, and IL-4 were not detected in both unstimulated and LPS-stimulated cells. On the other hand, the expression levels of IL-6, GM-CSF, and IL-12 in LPS-stimulated cells were almost always higher than those in control cells. Among those cytokines, IL-6 exhibited the predominant expression, and GM-CSF, IL-12 p40, and IL-12 p35 followed in the descending order. The times to reach the peak expression levels for IL-6, and GM-CSF, IL-12 p35, and IL-12 p40 were 14, and 24 h, respectively. After reaching the peak expression point, the expression levels of IL-6, GM-CSF, and IL-12 p40 reduced to the baseline by 72 h after stimulation, however, IL-12 p35 still kept a substantial expression by the same time. This study demonstrates that porcine alveolar macrophages primarily respond to express IL-6, GM-CSF, and IL-12 by LPS-stimulation and have a cytokine-specific expression profile during the stimulation time.

Key words: Kinetics, porcine, cytokine expression, alveolar macrophages, LPS-stimulation.

Introduction

Cytokines are important mediators of immune and inflammatory responses in humans and animals. They regulate immunity at low concentrations and interact with each other to keep the homeostasis in many physiological responses[2]. They are classified into Th1 and Th2 types in the human and mouse immune systems[8]. The Th1 type cytokines, which are represented by IL-2, interferon-γ (IFN-γ), and IL-12, mediate cellular immune responses, whereas the Th2 type cytokines, such as IL-4, IL-5, IL-6, and IL-10 are involved in antibody production and allergic responses[8].

It is generally known that cytokines, especially the inflammatory cytokines, such as IL-1β and TNF-α, are expressed for a short period, and the amounts expressed are very low[1,2,9,17]. In contrast to human and mouse cytokines, only a few reagents for porcine cytokines are available either as proteins or antibodies from commercial companies. For these reasons, several studies were conducted *in vitro* to examine the expression levels or patterns of porcine cytokines in mitogen-stimulated peripheral blood mononuclear cells (PBMC) using a reverse-transcription polymerase chain reaction (RT-PCR) assay[3,13,16]. Reddy *et al*[12] also determined the cytokine expression responses and kinetics in lymphocytes derived from lymph nodes that had been stimulated with mitogens, such as lipopolysaccharide (LPS) and phytohemagglutinin (PHA).

Alveolar macrophages in the lung have important roles in conducting the first defense against invading pathogens[11]. It has been known that they express inflammatory cytokines that respond respiratory diseases[9]. In the present study, we examined the porcine cytokine expression in alveolar macrophages stimulated by LPS to determine the time course patterns and the levels of the mRNA expression.

Materials and Methods

Experimental Animals and Cell Preparations

Alveolar macrophages were obtained from three healthy female pigs by means of lung lavage using sterile 1x PBS as described elsewhere[1]. The cells were washed twice with 1x PBS by centrifugation at 300 x g for 10 min. The cells in 25 cm2 tissue culture flasks were incubated for two hours in a 5% CO2 humidified atmosphere, and unbound cells were removed by washing twice the cells out with 1x PBS.

*Corresponding author: Han Sang Yoo
Department of Infectious Diseases, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea
Tel: 82-31-290-2737, E-mail: yoohs@plaza.snu.ac.kr
Fax: 82-31-290-2737

L-glutamine-containing RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin was added to the cells. Adherent cell populations were >95% macrophages and >97% viable as determined by nonspecific esterase staining and trypan blue dye exclusion, respectively. In this study, the cells were classified into two groups, unstimulated control and LPS (1 μg/ml)-stimulated cells. The cells were cultured for 2, 4, 14, 24, 48, and 72 h, and total RNA was prepared from the cells of the two groups. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by centrifugation at 900 x g for 30 min in the presence of Histopaque 1077 (Sigma, St. Louis, MO). PBMC were washed twice with 1x PBS and then stimulated with phytohemagglutinin (PHA) (2% v/v) for 1 day at 37℃ in a 5% CO2 humidified atmosphere.

Preparation of RNA

Total RNA was extracted from PBMC and alveolar macrophages at the end of each incubation time using Trizol reagent (Gibco BRL) by following the manufacturer's instruction. The RNA was precipitated with isopropanol (Sigma, St. Louis, MO) and washed with 70% ethanol. The RNA was then dissolved in 100 μl of DEPC-treated water and treated with RNase-free DNase (Promega, Madison, WI) for 30 min at 37℃. The DNase-treated RNA was again purified with Trizol, and the purified RNA was dissolved in 50 μl of DEPC-treated water. The RNA extracted from PBMC was used as the positive control sample for cytokine gene amplification. The RNAs obtained from three pigs were pooled into a single tube prior to the synthesis of cDNA. The concentration of the combined RNA was determined by measuring the optical density at 260 nm with a GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, England).

Synthesis of cDNA

Synthesis of single-stranded cDNA was performed using the Superscript Preamplification System for First Strand cDNA Synthesis (Gibco BRL) according to the manufacturer's instructions. Briefly, 5 μg of total RNA was used as a template, and other components, such as 0.5 μg of oligo(dT)12-18, 2 μl of 10x PCR buffer, 2 μl of 25 mM MgCl2, 1 μl of 10 mM dNTP mix, 2 μl of 0.1 M DTT, and 200 units of Superscript II RT were added to the reaction tube containing the RNA. cDNA synthesis was conducted at 42℃ for 50 min, and the reaction was terminated by incubating the reaction tube for 15 min at 70℃. The residual RNA was digested by adding 2 units of RNase H and incubating the reaction tube for 20 min at 37℃. The cDNA was stored at -20℃ and used as a template for amplification of the cytokine genes by PCR.

Amplification of Cytokine Genes by PCR

Cyclophilin A and cytokine-specific primer sets (Table 1) were designed and synthesized based on the cDNA sequences obtained from the GenBank database. The cyclophilin A gene was amplified under the following PCR conditions for 30 cycles; 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s. All of the cytokine genes examined in this study were amplified for 40 cycles by PCR. The IFN-γ gene was amplified under the following PCR conditions; 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 45 s. The IL-2 gene was amplified under the following PCR conditions; 94℃ for 30 s,

Table 1. Primer sets used to amplify cytokine genes, their oligonucleotide sequences, the sizes of PCR products, and GenBank accession numbers

Primer sets	Primer sequences	Sizes of PCR products(bp)	GenBank accession
IFN-γ	5'-ATGAGTTATACAACTTATTTCTTAG-3' 5'-TTATTTTGATGCTCTCTGGCC-3'	501	S63967
IL-2	5'-ATGTATAAGATGCAGCTCTTG-3' 5'-TCAAGTCAGTGTTGAGTAGATG-3'	465	X56750
IL-4	5'-ATGGGTCTCACCTCCCAACTG-3' 5'-TCAACACTTTGAGTATTTCTCCTTC-3'	402	X68330
IL-6	5'-ATGAACTCCCTCTCCACAAGC-3' 5'-CTACATTATCCGAATGGCCCTC-3'	639	M80258
GM-CSF	5'-ATGTGGCTGCAGAACCTGC-3' 5'-TTACTTTTTGACTGGCCCCCAG-3'	435	U67175
IL-12 p35	5'-ATGTGTCCGCTGCGCAAC-3' 5'-TTAGGAAGAATTCAGATAGCTC-3'	669	L35765
IL-12 p40	5'-ATGCACCTTCAGCAGCTGGTTG-3' 5'-CTAATTGCAGGACACAGATGC-3'	975	U08317
Cyclophilin A	5'-ATGGTTAACCCCACCGTCTTC-3' 5'-GTTTGCCATCCAACCACTCAG-3'	376	F14571

53℃ for 30 s, and 72℃ for 45 s. The IL-4 gene was amplified under the following PCR conditions; 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 40 s. The IL-6 gene was amplified in the following PCR conditions; 94℃ for 30 s, 53℃ for 30 s, and 72℃ for 45 s. The GM-CSF gene was amplified under the following PCR conditions; 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 40 s. The IL-12 p35 gene was amplified under the following PCR conditions; 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 45 s. The IL-12 p40 gene was amplified under the following PCR conditions; 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 60 s. The PCR products were analyzed by electrophoresis in 1.5% agarose gels. The gene-specific DNA bands were detected under ultraviolet light following the staining of the gels with ethidium bromide. The densities of DNA bands were measured using the Gel Documentation System (Bio-Rad, Hercules, CA).

Normalization of Cytokine Gene Expression

Because cyclophilin A was used as the house-keeping gene in this study, the expression level of each cytokine gene was normalized against that of the cyclophilin A gene. The final expression of each cytokine gene was determined by subtracting the expression level of a cytokine in control cells from that of it in LPS-stimulated cells.

Results

Specificity of PCR Reactions

PCR reactions were performed under each gene-specific condition to amplify 7 kinds of cytokines, IFN-γ, IL-2, IL-4, IL-6, GM-CSF, IL-12 p35, and IL-12 p40, and cyclophilin A with cDNA prepared from PHA-stimulated PBMC. The electrophoresed PCR products showed the correct sizes of the amplified gene-specific DNA, including IFN-γ (501 bp), IL-2 (465 bp), IL-4 (402 bp), IL-6 (639 bp), GM-CSF (435 bp), IL-12 p35 (669 bp), IL-12 p40 (975 bp), and cyclophilin A (376 bp) (Fig. 1). This result demonstrated that the PCR conditions used in this study were suitable for the amplification of cytokines and cyclophilin A genes.

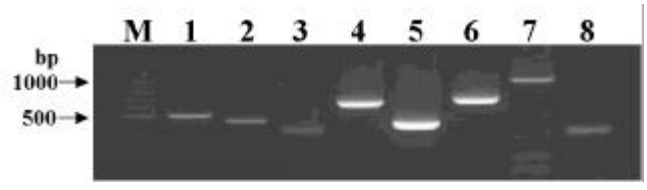

fig. 1. Specificity of PCR. Gene specific DNA bands were identified by analyzing the PCR products in 1.5% agarose gel. M, DNA marker; Lane 1, IFN-γ (501 bp); Lane 2, IL-2 (465 bp); Lane 3, IL-4 (402 bp); Lane 4, IL-6 (639 bp); Lane 5, GM-CSF (435 bp); Lane 6, IL-12 p35 (669 bp); Lane 7, IL-12 p40 (975 bp); Lane 8, cyclophilin A (376 bp).

Expression Profile of IFN-γ, IL-2, and IL-4

Although IFN-γ, IL-2, and IL-4 expression was evident in PHA-stimulated PBMC as shown in fig. 1, their expression was not detected in LPS-stimulated alveolar macrophages during the entire stimulation period (data not shown). The result obtained in this study may be reasonable, because IFN-γ, IL-2, and IL-4 are typically produced from T lymphocytes.

Kinetics of IL-6, GM-CSF, and IL-12 Expression

The expression levels of all cytokines examined in this study were determined in three different stages, such as the early (2 and 4 h), the intermediate (14 and 24 h), and the late (48 and 72 h) periods after LPS-stimulation. There was a slight increase of IL-6 expression in the early stage (2 and 4 h) of LPS-stimulation (fig. 2A). After the early period of induction, IL-6 expression was so dramatically increased in the intermediate stage of stimulation that it reached a peak point at 14 h. A substantially high expression of IL-6 was still observed until 24 h of stimulation. However, IL-6 expression was sharply decreased almost to the base level in the late period (48 and 72 h) of stimulation (fig. 2A).

A little increase of GM-CSF expression was observed in the early period, and then the expression was descended to the base line at 14 h of LPS-stimulation (fig. 2B). The highest expression of GM-CSF was detected at 24 h of stimulation. In the late period of stimulation, GM-CSF expression was continually reduced and reached the base line at 72 h (fig. 2B). Compared to the expression period of IL-6, a high expression of GM-CSF occurred for a short period.

Since IL-12 is a heterodimer cytokine composed of two subunits of p35 and p40, we separately examined the expression kinetics of two subunits. The whole expression levels of both IL-12 p35 and IL-12 p40 were lower than those of IL-6 and GM-CSF. Both IL-12 subunits demonstrated the peak expression levels at 24 h (fig. 2C and D). However, the expression patterns of both subunits were different. In the case of IL-12 p35 subunit, the expression contents in the late period (48 and 72 h) of stimulation were almost the same as the maximum level determined at 24 h (fig. 2C). In contrast, the expression of IL-12 p40 subunit was rapidly decreased after 24 h of stimulation, and lower than in control cells at 72 h (fig. 2D).

Discussion

Cytokines have the ability to regulate a broad range of immune and inflammatory responses, including humoral and cell-mediated immune reactions[15]. It has been suggested that the onset of the respiratory diseases or endotoxemia caused by Gram-negative bacterial infection is induced by the expression of proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6[6,9,10]. Although several studies have been conducted to examine the patterns or kinetics of porcine cytokine gene expression in PBMC or leukocytes[3,12,13,16], there is presently no study

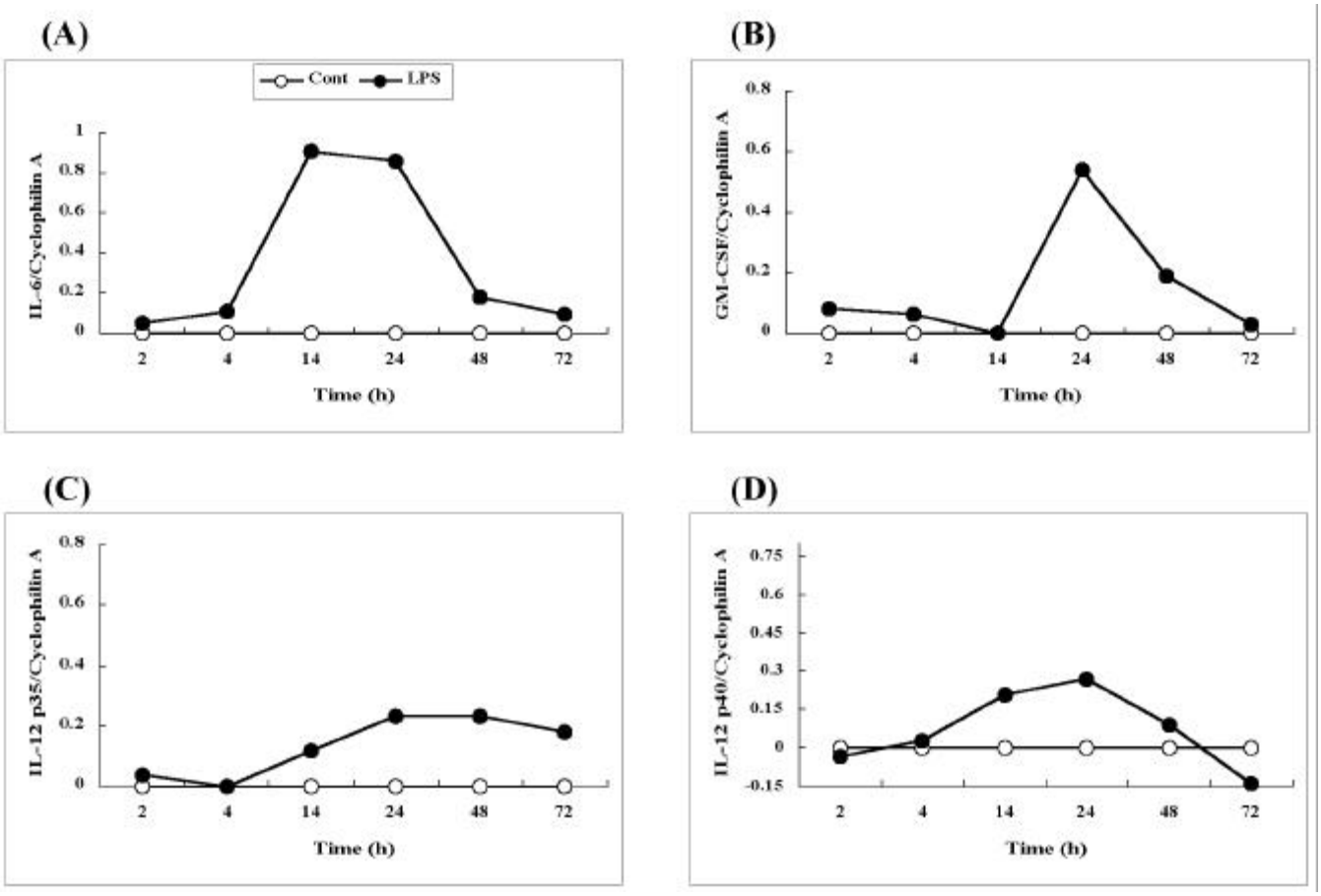

fig. 2. Kinetics of IL-6 (A), GM-CSF (B), IL-12 p35 (C), and IL-12 p40 (D) mRNA expression. Cytokine gene expression levels were determined in LPS-stimulated alveolar macrophages after 2, 4, 14, 24, 48, and 72 h of incubation. To normalize the expression levels of each cytokine, the ratios of each cytokine/cyclophilin A were calculated, and the ratios of cytokine/cyclophilin A in the control were subtracted from those in LPS-stimulated cells. The subtracted ratios were plotted against the stimulation times.

of the kinetics of porcine cytokine mRNA expression in alveolar macrophages. In this study, we examined seven porcine cytokine expression levels over a time course in LPS- stimulated alveolar macrophages.

Two distinctive expression patterns of the porcine cytokines were observed in alveolar macrophages. First, the expression of porcine IFN-γ, IL-2, and IL-4 was not detected in either LPS-stimulated or control cells. In Con A-stimulated porcine PBMC, the mRNA expression levels of IFN-γ, IL-2, and IL-4 peaked after 24 h of stimulation[3]. However, our study using alveolar macrophages that were stimulated by LPS demonstrated no expression of them. These differences in cytokine gene expression are attributable to the difference of cell types. Second, IL-6, GM-CSF, IL-12 p35, and IL-12 p40 expression levels were almost always higher in LPS-stimulated alveolar macrophages than in unstimulated controls. Among these cytokines, IL-6 showed the highest expression. Therefore, it is confirmed that bacterial endotoxin is an effective stimulator for production of these inflammatory cytokines in alveolar macrophages.

The facts showing the definite augmentation of IL-6 and IL-12 expression demonstrated in this study were similar to other data observed in LPS-stimulated porcine or human alveolar macrophages[4,5,7,14]. It is also known that IL-6 level is increased in the sera of pigs that are infected with *Actinobacillus pleuropneumoniae*, one of the most potent respiratory disease-inducing Gram-negative bacteria of pigs[6]. The highest expression of IL-6 in the LPS-stimulated porcine alveolar macrophages was demonstrated after 6 h of stimulation[14]. In our study, however, the peak expression of IL-6 occurred at 14 h of incubation. On the other hand, in kinetics studies of IL-12 p35 and IL-12 p40 expression in LPS-stimulated porcine alveolar macrophages, the highest IL-12 p35 and IL-12 p40 expression levels were detected after 2 and 18 h of stimulation, respectively[4]. However, our study showed the highest expression of both IL-12 p35 and IL-12 p40 after 24 h of stimulation. The possible reason for these differences even using the same cells and stimulating

reagent would be because of the different experimental methods employed, such as a northern blotting and an RT-PCR.

In conclusion, the results obtained in this study indicate that each porcine cytokine has its own expression pattern in alveolar macrophages depending upon the stimulator and the length of stimulation. The increased expression of IL-6 and IL-12 in LPS-treated alveolar macrophages especially suggests that the respiratory disease-inducing Gram-negative bacterial infections stimulate expression of both cytokines *in vivo*. Taken together, the mRNA expression profiles of IL-6, IL-12, and other cytokines examined in this study would be useful references for the diagnosis of bacterial respiratory diseases in pigs.

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