University of Arkansas, Fayetteville

ScholarWorks@UARK

Graduate Theses and Dissertations

12-2014

Evaluation of Porcine IPEC-J2 Cell Line Growth Rate and Immune Response to E. coli (0111:B4) LPS

Xiaofan Wang
University of Arkansas, Fayetteville

Follow this and additional works at: https://scholarworks.uark.edu/etd

Part of the Veterinary Microbiology and Immunobiology Commons, Veterinary Preventive Medicine, Epidemiology, and Public Health Commons, and the Zoology Commons

Citation

Wang, X. (2014). Evaluation of Porcine IPEC-J2 Cell Line Growth Rate and Immune Response to E. coli (0111:B4) LPS. *Graduate Theses and Dissertations* Retrieved from https://scholarworks.uark.edu/etd/2053

This Thesis is brought to you for free and open access by ScholarWorks@UARK. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of ScholarWorks@UARK. For more information, please contact scholar@uark.edu.

Evaluation of Porcine IPEC-J2 Cell Line Growth Rate and Immune Response to *E. coli* (0111:B4) LPS

Evaluation of Porcine IPEC-J2 Cell Line Growth Rate and Immune Response to *E. coli* (0111:B4) LPS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Animal Science

by

Xiaofan Wang
Jilin Agricultural University China
Bachelor of Science in Veterinary Medicine, 2008

December 2014
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

____
Dr. Charles Maxwell
Thesis Director

____
Dr. Charles Rosenkrans
Committee Member

____
Dr. Beth Kegley
Committee Member

Abstract

The objective of this study was to evaluate the intestinal porcine epithelial cell-jejunum 2 (IPEC-J2) cell line as a model to study the innate immune function of live pigs. Growth rates of IPEC-J2 cells in T-75 flasks and 96-well plates were evaluated using a hemocytometer and spectrophotometer for cell quantity measurements to determine growth rate and doubling time. Growth rates of IPEC-J2 cells in T-75 flasks and 96-well plates were 0.4016 and 0.2851 times of doubling/day respectively with a doubling time of 1.73 d and 2.43 d, respectively. Confluent IPEC-J2 monolayers were tested at five time intervals (0, 1, 2, 4, and 6 h) and four LPS concentrations (0, 0.1, 1, and 10 μg/mL). Under these treatments, relative gene expression of GM-CSF, IL-1β, TNF-α, IL-6, IL-8, IL-10, TLR1, TLR2, TLR3, TLR4, TLR6, TLR8 and TLR10 were evaluated by quantitative RT-PCR. There were no LPS concentration × culture time interactions observed for any gene ($P > 0.13$). Main effects were analyzed. The GM-CSF, IL-8, and TLR4 were significantly stimulated by LPS challenge at increasing culture time and the expression peaked at 2, 4, and 4 h respectively ($P < 0.05$). Expression of TNF-α tended to increase linearly with increasing LPS concentration and decrease linearly with increasing culture time ($P = 0.10$). TLR2 tended to be up regulated by increasing LPS challenge time (Quadratic effect, $P = 0.08$). Increasing culture time lead to a down regulation of IL-6 expression, and reached a low point at 4 h (Quadratic effect, $P < 0.05$). TLR3 tended to be down regulated with increasing culture time (Quadratic effect, $P = 0.10$). Results of the current study suggest that the IPEC-J2 cell line can be used as a model for evaluating the impact of specific bacteria on immune response in vitro.

Key words: IPEC-J2, growth rate, immune cells gene amplification, LPS-challenge, qRT-PCR.

Acknowledgments

First and foremost, I am grateful for the endless and meticulous support and attention from all my family members both before and after I arrived in the United States, especially my dear mom, dad, grandparents, uncles, aunts, and my beautiful cousin. Thanks also to my parents' friends Uncle Sun and Aunt Pang for the warm reception every time I visit to China. My college friends in China, Yueming Yang, Yao Sun, Xiaofeng Ji, Yihan Qiao, Duo Gao and Yang Gao, thanks a million to you guys for your eternal concerns for me and my life and studies in US.

I thank Dr. Charles Maxwell, my amiable advisor for his patient guidance and encouragement.

Dr. Charles Rosenkrans and Dr. Beth Kegley, for their valuable contributions to help me complete my degree program and for serving on my committee.

Dr. TC Tsai, Dr. HaeJin Kim and Dr. Tes Sales, thanks for their indispensable aid in my research. I am so proud of being tutored by you all.

I also thank my friends Ruiting, Leiying, Jingyu, and Hong; you guys made my days in these two years happy.

Dedication

Assistance from Dr. Charles Maxwell’s lab group.

Love from my family and friends in my lovely motherland.

Companionship from all friends in America.

Table of contents

Chapter I. Introduction 1

Significance of the study 1

Objectives of the study 2

References: 4

Chapter II. Literature Review 5

Immunity in suckling piglets 5

Influence of gut environments on piglets' immunity and nutrition 5

Colibacillosis in nursery pigs 5

Innate immune system in pigs 6

Porcine intestinal cell lines and techniques for maintenance 8

Porcine intestinal epithelium serves as the first immune barrier 8

Three intestinal epithelial cell lines used in pig studies 9

Tissue and cell culture technique and types 12

Gene expression in the immune response to LPS challenge 15

Toll-like receptors and main cytokines involved in innate immune response in pigs 15

Signal transduction pathway 20

Stimulation of cytokines and chemokine through the signal transduction pathway 21

Summary 22

References 24

Chapter III. Evaluation of doubling time and growth rate of IPEC-J2 cell line in 96-well plates or T-75 flasks 33

Abstract 33

Introduction 34
Materials and Methods 35
Growth assay of IPEC-J2 cells in T-75 flask by hemocytometer technique 35
Growth assay of IPEC-J2 cells using the MTT method 36
Statistical analysis 37
T-75 flask-hemocytometer 37
96-well plate-MTT method 37
Results 38
Cell proliferation regression equation, doubling time, and growth rate 38
Discussion 38
Conclusions 40
References 42
Chapter IV. Expression profile of 13 immune-response genes in IPEC-J2 cells stimulated by LPS from *E. coli* (O111:B4) 49
Abstract 49
Introduction 50
Material and methods 51
Cell line and culture condition 51
LPS challenge of IPEC-J2 cell line 52
Total RNA purification, qualification and quantification 52
Reverse Transcription Quantitative-PCR 53
Relative gene expression analysis 54
Statistical analysis 55

Results.....55
Interaction effects between LPS concentration and challenge time.....55
Expression of cytokines in response to increasing levels of LPS.....56
Expression of TLRs in response to increasing levels of LPS.....56
Expression of cytokines in response to increasing challenge time.....56
Expression of TLRs in response to increasing challenge time.....57
Discussion.....57
Conclusions.....65
References:.....66
Chapter V: Conclusions.....88

List of abbreviation

Item	Term
ACTB	Actin, gamma
AP-1	activation protein 1
BLP	bacteria lipopeptide
cDNA	complementary deoxyribonucleic acid
Cq	quantification cycles
Ct	threshold of cycle
DAMPs	damage-associated molecular patterns
DMEM	Dulbecco's modified eagle medium
dsRNA	double strand ribonucleic acid
E-coli	*Escherichia coli*
EGF	epidermal growth factor
ETEC	Enterotoxigenic *Escherichia coli*
FBS	fetal bovine serum
GIT	gastrointestinal tract
GM-CSF	granulocytes-macrophage colony-stimulating factor
GOI	gene of interest
HKG	housekeeping gene
hTERT	human telomerase reverse transcriptase
ICAM-1	intercellular adhesion molecule-1
IEC	intestinal epithelial cells
IFN	type I interferon

IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1	interleukin-1
IL-10	interleukin-10
IL-1β	Interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
IPEC-1	intestinal pig epithelial cell-1
IPEC-J2	intestinal pig epithelial cell-jejunum 2
IRFs	interferon response factors
JNK	c-Jun NH_2-terminal kinase
LBP	LPS-binging proteins
LPS	lipopolysaccharides
LRR	leucine-rich repeat
M cell	membranous cell
MALP-2	macrophage-activating lipopeptide-2
MALP2-SK_4	MALP2 derivative PAM_2C-GNNDESNISFKEKSK_4
MAPK	mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NLRs	NOD-like receptors
PAMPs	pathogen-associated molecular patterns
PGN	peptidoglycan

PIE	porcine intestinal epitheliocyte
Pps	Peyer's Patches
PRR	germ-line-encoded pattern recognition receptors
PSV	sapelovirus
R^2	correlation coefficient
RLR	RIG-like receptor
Rq	relative quantity
SAC	*Staphylococcus aureus Cowan* I bacteria
SC	Choleraesuis
SI	small intestine
ssRNA	single strand ribonucleic acid
ST	*Salmonella enterica* serovars Typhimurium
TB	trypan blue
TEER	trans-epithelial electrical resistance
TIR	Toll/IL-1 receptor
TLR1	toll-like receptor 1
TLR2	toll-like receptor 2
TLR3	toll-like receptor 3
TLR4	toll-like receptor 4
TLR5	toll-like receptor 5
TLR6	toll-like receptor 6
TLR7	toll-like receptor 7
TLR8	toll-like receptor 8

TLR9	toll-like receptor 9
TLR10	toll-like receptor 10
TNF	tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
UBC	Ubiquitin C; Polyubiquitin
ZO-1	Zonus Occludens-1
ZO-2	Zonus Occludens-2

List of Tables

Chapter III

Table 1. LS means of cells numbers in 7 days. 44

Table 2. LS mean of absorptions in 10 day. 45

Chapter IV

Table 1. Primers and probes used for detecting gene expression of the endogenous control, immune related receptors, and cytokines in quantitative-real time polymerase chain reaction. 71

Table 2. LS means of fold change (RQ) of gene expression response to increment levels of LPS challenge in IPEC-J2 cell line. 72

Table 3. LS means of fold change of gene expression response to LPS challenge in 5 increment culture times 73

List of Figures

Chapter III

Figure 1. The proliferation curve of IPEC-J2 cells in T-75 flask with 7 days 46

Figure 2. Proliferation curve of IPEC-J2 cells cultured in 96-well plates 47

Chapter IV

Figure 1. Plate layout for qRT-PCR 74

Figure 2. LS means (± S.E.M) of fold change of TNF-α response to increment levels (0, 0.1, 1, and 10 μg/mL) of LPS challenge in IPEC-J2 cell line. 75

Figure 3. LS means (± S.E.M) of fold change of IL-8 response to increment levels (0, 0.1, 1, and 10 μg/mL) of LPS challenge in IPEC-J2 cell line. 76

Figure 4. LS means (± S.E.M) of fold change of TLR8 response to increment levels (0, 0.1, 1,

and 10 μg/mL) of LPS challenge in IPEC-J2 cell line... 77

Figure 5. LS means (± S.E.M) of fold change of IL-8 response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line... 78

Figure 6. LS means (± S.E.M) of fold change of GM-CSF response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line.. 79

Figure 7. LS means (± S.E.M) of fold change of IL-6 response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line... 80

Figure 8. LS means (± S.E.M) of fold change of TNF-α response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line... 81

Figure 9. LS means (± S.E.M) of fold change of TLR4 response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line... 82

Figure 10. LS means (± S.E.M) of fold change of TLR2 response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line... 83

Figure 11. LS means (± S.E.M) of fold change of TLR6 response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line... 84

Figure 12. LS means (± S.E.M) of fold change of TLR3 response to increment culture time (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line... 85

List of Appendix

Chapter III

Appendix I. Absorbance of color density of reduced MTT in a 10-d culture of IPEC-J2 cells. 48

Chapter IV

Appendix I. Screening of reference genes 86

Appendix II. Primer and probe sequences of seven candidate reference genes 87

Chapter I. Introduction

Significance of the study

The development of the immune system and establishment of microorganisms in the gut begin as early as the piglets' suckling time. After weaning, the pig's solid diet contains a different quantity of microbes and source of nutrients compared with sows' milk, which along with environment changes are the sources of stress in post-weaning pigs. An abrupt increase in microbial uptake can give rise to changes in the intestine, leading to slight or severe diarrhea. A healthy gut and robust immune system eventually benefits the pigs' long-term performance and health.

Current studies have shown that pigs have better growth performance when raised in an outdoor site rather than inside traditional facilities, because the external environment exposes the piglets to microbes, which may promote immune system development in nursery pigs. Rearing environments play a vital role in gut microbiota establishment, which, to some extent, improves gastrointestinal health in the suckling pigs (Kitt et al., 2001). A well-developed intestinal immune system may reduce the incidence of diarrhea in post-weaning pigs and improve weight gain.

Previous studies conducted by University of Arkansas and Agtech Products have shown that outdoor-reared pigs were 2 kg heavier than the indoor-reared ones at the end of the nursery period (Brown et al., 2006). Also, traditionally reared pigs lost 0.09 kg at 24 h after weaning while outdoor-reared ones gained 0.17 in the same period. Besides, serious diarrhea in weaning pigs could lead to at least 10% mortality, and pigs that do survive the infection still suffer a loss of body weight (Shankar et al., 2009). Economic losses attributed to mortality, treatments, and reduced body weights resulting from diarrhea in weaning pigs are enormous (Francis, 1999;

Campbell et al. 2013).

Hence, it has been hypothesized that exposing suckling pigs to outdoor conditions such as the soil environment with more microbes may reduce post-weaning stress by promoting the immune system of nursery pigs, which translates into better growth performance and consequently, increased animal production.

Objectives of the study

In order to study the immune function of the intestine in pigs, cell models originally isolated from the pig are used instead of live animals. Non-transformed continuous intestinal pig epithelial cell-jejunum 2 (IPEC-J2) cells isolated from swine jejunum are currently used in adherence and immunity response studies (Schierack et al. 2006; Berschneider et al. 1989). The cells have been demonstrated to be able to grow and proliferate outside the body when sufficient nutrients are provided and suitable biological environments are met.

Since the lipopolysaccharide (LPS) matrix of *Escherichia coli* (*E. coli*) can stimulate immune response in various cell types, it can serve as a model of *E. coli* bacteria (Geens and Niewold, 2010). Thus, LPS-stimulated IPEC-J2 cells can hypothetically increase expression of cytokines and chemokine as well as modulate immune-related cells to initiate pro-inflammatory functions. Therefore, the objectives of this study are the following:

1) To determine the growth rate and doubling time of healthy IPEC-J2 cells in two different culture environments to verify different growth effects. The measured doubling time can be used as a criterion for cell health and growth.
2) To evaluate whether LPS from *E. coli* can stimulate pro-inflammatory responses in IPEC-J2 cells by determining mRNA levels of immune-related cytokines, which can provide insight into the functions of the innate immune system.

The findings in this study will contribute to a long-term goal of understanding how direct fed microbial or probiotics can impact the immune system and consequently improve performance in the livestock industries.

References:

Berschneider, H. 1989. Development of normal cultured small intestinal epithelial cell lines, which transport Na and Cl. Gastroenterology 96: A41.

Brown, D. C., C. V. Maxwell, G. F. Erf, M. E. Davis, S. Singh, and Z. B. Johnson. 2006. The influence of different management systems and age on intestinal morphology, immune cell numbers and mucin production from goblet cells in post-weaning pigs. Vet. Immunology and Immunopathology 111:187-198.

Campbell, J. M., J. D Crenshaw and J. Polo. 2013. The biological stress of early weaned piglets. Journal of Animal Science and Biotechnology. 4:19.

Francis, D. H. 1999. Colibacillosis in pigs and its diagnosis. Swine Health Prod. 7: 241-244.

Geens, M. M., and T. A. Niewold. 2010. Preliminary Characterization of the Transcriptional Response of the Porcine Intestinal Cell Line IPEC-J2 to Enterotoxigenic Escherichia coli, Escherichia coli, and E. coli Lipopolysaccharide. Comparative and Functional Genomics 2010: 469583.

Kitt, S. J., P. S. Miller, and A. J. Lewis. 2001. Factors affecting small intestine development in weaning pigs. Nebraska Swine Reports. 1-1-2001: 33-35.

Schierack, P, M. Nordhoff, M. Polmann, K. D. Weyrauch, S. Amasheh, U. Lodemann, J. Jores, B. Tachu, S. Kleta, A. Blikslager, K. Tedin, and L. H. Wieler. 2006. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochemistry and Cell Biology 125: 293-305.

Shankar B. P., H. S. Madhusudhan1 and D. B. Harish. 2009. Pre-Weaning Mortality in Pig-Causes and Management. Veterinary World. 2: 236-239.

Chapter II. Literature Review

Immunity in suckling piglets

Influence of gut environments on piglets' immunity and nutrition

Nutrients of newborn piglets are mainly from sow's milk, and pigs also receive passive immunity, such as immunoglobulin A (IgA), IgG and IgM as immune protection from bacterial infection. The gut systems are sterile and not fully developed at birth in the newborn pig and will develop in the first 2 weeks. Along with the growth, the animals ingest bacteria from milk or feed, which stimulates and improves the immune system in the gut. With weaning there are diet and environmental effects, which can potentially make pigs suffer a decrease in feed intake, and daily weight gain (Kyriazakis and Doeschl-Wilson, 2009).

Existing studies on raising nursery pigs in different environments have shown that outdoor-raised-pigs have significantly better growth performance than those raised in traditional indoor facilities, which suggests that outdoor-raised-pigs may alleviate some of the "stress period" effects caused by the transition from sow's milk to a solid diet, which results in changes in the morphology of microvilli and the gastrointestinal tract (Kitt et al., 2001; Campbell, 2013).

Colibacillosis in nursery pigs

The gut environment in pigs at birth are treated as sterile with developed villi and microvilli, but diet, environment and other stressful factors can influence the intestinal environments and, consequently, the animal's growth performance. The gut microbiota, especially some strains of *E. coli* originally carried in the feed to the intestine, can cause diarrhea, when the bacteria start to colonize the microvilli and stimulate excessive excretion of fluid and villi atrophy (Kitt et al., 2001). Diarrhea, which may occur as early as the day of birth to a week after weaning, can cause up to 40% weight loss and impact later growth performance as well, resulting in a big financial loss in pig production (Francis, 1999). Besides the villi damage

caused by the pathogenic bacteria, metabolic and toxic effects from bacterial metabolism and the corresponding immune responses also lead to severe impairments (Thacker, 2004). Although long-time exposure of pre-weaned pigs to bacteria may enhance the development of the immune system and consequently improve growth performance, overstimulation of the immune response is harmful; thus, it is meaningful to understand how the pig's immune system functions and how to optimally stimulate growth during stressful periods. LPS, the primary endotoxin outside the membrane of gram-negative bacteria, is a widely accepted substitute antigen used to challenge pigs or cell lines for in vivo and in vitro studies of immune response (Patience, 2012; Geens and Niewold. 2010).

Innate immune system in pigs

The three major functions of innate immunity are inflammation, antiviral defense, and initiation of the adaptive immune system. The innate immune system consists of skin, epithelial surfaces, and the majority of leukocytes and cytokines (Abbas et al, 2012). As the first line of defense, the skin and epithelium block the majority of microbes. Some invading pathogens are recognized by germline-encoded pattern recognition receptors (PRR) located on the membrane or endosome of leukocytes and epithelial cells and are eliminated by phagocytosis and intracellular chemical substances (Abbas et al, 2012; Philpott et al., 2001). Bacteria or foreign molecules that are ingested by phagocytic cells such as dendritic cells are processed and the fragment peptides are presented on the surface of phagocytic cells, which are further removed by the adaptive immune system (T lymphocytes and B lymphocytes). Unlike the innate immune system, adaptive immune recognition is antigen-specific, more complicated, and needs a longer time to initiate response, but it does have stronger immune defenses against viruses or

pathogenic bacteria. However, the immediate and quick response of innate immunity provides rapid protection from microorganisms (Werling and Jungi, 2003).

Acute inflammation recruits various leukocytes to the infected sites and this process requires a relative shorter time compared to chronic inflammation, which also recruits lymphocytes to the offended place in case of severe invasion (Abbas et al, 2012). Phagocytosis by leukocytes is one of the main strong functions of innate immunity to eliminate pathogens. Recruiting neutrophils, monocytes, or other phagocytes to the infected locus can be greatly improved by expression of high-affinity binding molecules on endothelial or epithelial cells, and cytokines, which enhances the penetration of leukocytes from the blood vessels to injured tissue.

Microbial patterns-ligands

Two main kinds of antigens that can be recognized by the immune system are pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are the components of microbes like the nucleic acids from virus, cell wall proteins and lipids like flagellin, pilin as well as LPS in gram-negative bacteria and lipoteichoic acid in gram-positive bacteria (Hayashi et al., 2001; Spencer et al., 2011). In addition, carbohydrates like mannan and dectin glucans are also the main targets of the PRRs, which can initiate the innate immune system (Abbas et al., 2012). DAMPs refer to any nonfunctional body cells or fragments, which are not only released from dying cells but also healthy ones. The very first step of the stimulation of the innate immunity is the recognition of the PAMPs and DAMPs by the PRRs or NOD-like receptors (NLRs) of endothelial cells or other immune function cell types.

Receptors

Toll-like receptors (TLRs) are type I integral membrane glycoproteins (Netea et al., 2004; Akira, 2011). To date, 10 mammalian TLRs and 12 mice TLRs have been characterized (Guan et

al., 2010; Akira, 2011), and more members of the TLR family are expected to be defined in the future (Takeda et al., 2003). Toll like receptor 1, 2, 4, 5, and 6 are expressed on the cell surface and the remaining receptors are located in the endosome. However, the distribution of each kind of TLR varies with the location of cells, species, as well as environments (Menzies and Ingham, 2006).

The immune functional molecules including leukocytes and epithelial cells, consistently express receptors on the cell surface or endosomes. Recognition of the microbes by innate immune molecules is not as broad as adaptive immunity molecules, and the receptors can only discriminate the whole or parts of microbes, which are not present in mammals (Abbas et al, 2012), and also a small part of the DAMPs. Receptors include not only membrane embedded proteins like the TLR family, but also cytosolic soluble compounds including NLRs, RIG-like receptors (RLR), N-formylmethionyl receptors), and carbohydrate-specific receptors such as the mannose receptor (dectin). Innate immune functional molecules are not able to differentiate from various bacterial species because the receptors bind the ligands shared by most bacteria, owning identical structure and character (Abbas et al., 2012). For instance, TLR4 is able to recognize general bacteria LPS, while C-type lectin-like receptors can identify mannose on bacteria's surface membrane. Furthermore, different membrane or cytosolic receptor proteins have distinct functions, and most of them are involved in signal activation and transmission to elicit biological functions (Abbas et al, 2012).

Porcine intestinal cell lines and techniques for maintenance

Porcine intestinal epithelium serves as the first immune barrier

The intestinal epithelial surface, overlaid by only one layer of epithelial cells in the gastrointestinal tract (Montilla et al., 2004), plays a role in absorption of nutrients and exchanges

of water. It also serves as one of the initial barriers between the body and environment to prevent the invasion of foreign microbes in humans and animals (Oswald, 2006; Meurens et al., 2009; Fairbrother et al., 2005; Brosnahan and Brown, 2012) due to its high frequency exposure to microorganisms from the diet (Mariani et al., 2009; Pitman and Blumberg, 2000). Confronted with more than 10^{14} microbes with a diversity of around 400 bacterial species, the mucosa in the gastrointestinal tract (GIT) has at least 10^{12} lymphocytes and a large quantity of antibodies, which is much greater than other locations in the entire body to fight against pathogenic microbes (Burkey et al., 2009). At the same time, the monolayer of epithelial surface is important in withstanding pathogens. The penetration of bacteria into the epithelial cells results in initiating the innate immune response, which is the first line of defense in the immune system to defend against the invasion of viruses and bacteria, while severe infection can subsequently activate the adaptive immune response (Arce et al., 2010; Philpott et al., 2001; Veldhuizen et al., 2006).

Three intestinal epithelial cell lines used in pig studies

Recently, several porcine intestinal cell lines were established as better models and are replacing the human small intestine cell lines in some swine studies. The three overwhelmingly used intestinal epithelial cells in vitro are the intestinal pig epithelial cell-1 (IPEC-1), IPEC-J2 and IPI-2I, all isolated from swine small intestine epithelium. After isolation from the ileum of an adult male pig, the IPI-2I cell line was permanently genetically transformed, so some of the physiological natures, such as phenotype, were changed (Mariani et al., 2009). Another intestine-derived cell line, IPEC-J2 (Berschneider, 1989), from the mid-jejunum of a newborn pig (< 12 hours old), is viewed as a better model representing the intestinal epithelial cells because it is a

non-transformed cell line (Lu et al, 2010). The IPEC-1 cell line, derived from swine jejunum and ileum, is more efficiently used in studying the synthesis and secretion of lipoprotein and apolipoprotein because the low-lipid transport efficiency makes a more apparent observation of the change (Liu et al., 2006; Koh et al., 2008; Gonzalez-Vallina et al., 1996).

Generally, all three cell lines are used as a pathogen infection model in exploring the porcine immune system (Koh et al., 2008; Arce et al., 2010). IPEC-J2 and IPI-2I have been widely co-cultured with common gut microbes like rotavirus, *E-coli*, *Salmonella enterica* erovars Typhimurium (ST) and Choleraesuis (SC), sapelovirus (PSV) and *Entamoeba histolytica* in order to achieve a better understanding of the adherence between bacteria and epithelial cells; regulation of gene expression; signal pathway in immune response; and the interaction between innate and adaptive immune system (Skjolaas et al., 2006; Burkey et al., 2009; Johnson et al., 2009; Mariani et al., 2009: Pasare and Medzhitov, 2004).

IPEC-J2 cell lines in adhesion studies

The characteristic of IPEC-J2 cells to easily adhere to the bottom of a flask and form a monolayer (Freshney, 2005) makes this cell line the ideal tool for adhesion studies. In vivo, the intercellular adhesion feature is made by three connections in the apical region: tight junctions, adherens junctions, and the cell-cell adhesion (desmosomes; Gumbiner et al., 1996).

Tight junctions are produced by occludin, claudin, Zonus Occludens-1 (ZO-1), ZO-2 and cingulin, and these belt-like structural proteins connect two adjoining epithelial cells to make up the barrier. Adhesion junction (zonula adheren) is regulated by single membrane-spanning proteins, which can stabilize cell-cell adhesion as well as regulate the intracellular signal (Hartsock and Nelson, 2008). In vitro, the adhesion complex can easily integrate free intestinal epithelial cells to form a monolayer adhering to the bottom of the flask. This function can be

influenced by nutrient composition, hormones, proteins, foreign bacterial, and secreted toxins (Oswald, 2006). The trans-epithelial electrical resistance (TEER) technique can measure the adhesion power of the junction in the monolayer and the confluence level (Schierack et al., 2006). Finally, desmosomes regulate cell-cell adhesion by the mediation of multiple membrane-spanning proteins (Schierack et al., 2006). After the cells reach confluence, desmosomes keep growing and then the morphological organization is formed, which results in a tough adhesion, usually at an approximately 80% confluence of the subculture (Ashkenazi et al., 2014).

In addition to these connections between intestinal cells, microvilli and glycocalyx-bound mucins, secreted by goblet cells, not only contribute to the adhesion of commensal bacteria but also to the defense mechanisms and nutrient absorption of microbes (Johnson et al., 2009). Pathogenic bacteria, expressing fimbriae and flagella on their membrane, are able to adhere to the intestine epithelium (colonization) in order to invade the host (Schierack et al., 2013; Thacker, 2004). Usually, ingested bacteria only locate on the vacant niche, which has not been occupied by microbes. However, the bacteria with firmer adhesion ability can outcompete the preferentially colonized individuals. Considering this point, more research groups are interested in whether a microbial infection can be reduced by non-pathogenic or commensal bacterial competing for the niche (Schierack et al., 2013). All in all, the study of adhesion between epithelial cells and bacteria can provide insights into the interrelationship between epithelial cells and bacteria, and how that affects the defense functions of the gastrointestinal immune system. Koh et al. (2008) investigated two swine adherence models: IPEC-1 and IPEC-J2 using several *E. coli* strains, and concluded that both cell lines are ideal for adherence studies, and different adhesive affinities were shown between various *E. coli* strains.

IPEC-J2 cell lines in studies of the innate immune system and gene expression

Since the GIT is one of the main places confronting food-derived pathogens, numerous diseases caused by viral infection are initiated here and some severe infections can lead to severe mortality (Moue et al., 2008; Schierack et al., 2013). Investigating the mechanism of the porcine GIT immune mechanism is important for the swine industry in order to reduce nutrient expenditure due to bacterial infections.

Unfortunately, the porcine innate immune system has not been as completely characterized as the human or murine systems because appropriate cell lines have been lacking (Schierack et al. 2006). The IPEC-J2, IPEC-1 and IPI-2I cell lines isolated from the swine small intestine have been utilized for characterizing the porcine innate immune system through assessments of gene expression levels, particularly of cytokines, chemokines, and pathogen recognition receptors (Skjolaas et al., 2007; Liu et al., 2010). These functional proteins may provide clues to how innate immunity eliminates pathogenic microbes.

Tissue and cell culture technique and types

Cultures, composed of either organ tissues or cells, are techniques used to propagate organ tissue or cells isolated from live animals, plants or humans to investigate biological functions, which as has been suggested, can largely reduce the use of live animals (Thorpe, 2007; Leland and Ginocchio, 2007). Cell culture is widely used not only to study intracellular processes at the protein, gene or signal transduction level, but also in the medical field for studying drug metabolism. Primary and continuous cultures are two main types of cultures used in animal tissue or cell culture procedures (Freshney, 2005).

Primary culture derived from the original tissue, which is often a disaggregated cell line. It keeps most of the original characteristics of cells as in live animals, but is sometimes

comprised of multiple cell types. Continuous cell lines, however, made up of the single cells (Stacey, 2005), are naturally occurring cancer cells or intensively induced mutations of the original tissue that can keep dividing without meeting cellular senescence (immortal). Continuous cell lines are usually transformed, so they are more advantageous: they have an infinite lifespan and superior plating features (good adherence to culture vessel, shorter doubling time, less serum needed. Typically, cells are classified as fibroblastic, epithelial-like, and lymphoblast-like depending on their morphology. The first two cell types can easily adhere to the artificial substrate, whereas lymphoblast-like cells are cultured in a suspension-type environment. Due to these physical or biological characters, as well as origin, the cell culture environment (media and incubator) differs from cell to cell.

Culture environment

Although culture conditions vary among cell cultures for different species, aseptic standards are uniform across all labs, which include lab management, personal hygiene, equipment sterilization, and quality control (Coté, 2001). For instance, prior to initiation of a culture assay, all glassware (beakers, media suppliers, transfer tips, culture vessels and etc.) must be autoclaved. The hood and incubator need be sterilized with 70% alcohol (or other disinfectant solution) and exposed to a germicidal lamp with ultraviolet ray radiation for 30 minutes. A good aseptic practice to a large extent can reduce the incidence of contamination.

In addition to the lab environment, the preparation of medium is much more complex because it provides an indispensable nourishment source for cells. Maintenance of appropriate carbon dioxide and oxygen tension, temperature, pH, as well as humidity can help approach the best physicochemical growth condition for most cells types. Carbon dioxide is usually controlled at a range of 4% to 10%, whereas pH is kept consistently at 7.4 in most mammalian cell lines

with a few exceptions (insect cell line pH 7.0-7.4; fibroblast pH 7.4-7.7; Invitrogen Inc, 2002; Froehlich and Anastassiades, 2005). Depending on different incubator types, humidity is maintained by a water supply in the culture incubator. For those incubators devoid of a water supply, the culture vessel needs to be tightly closed, but that may limit air exchange. Appropriate temperature should be kept constant in order to maximize the activities of enzymes, which further benefits cell growth.

Meanwhile, the basal medium provides the nutrient supply, as well as serum, antibiotics, hormones (insulin), growth factors, and other specific needed components, depending upon the cell types. The basal medium nutrients include amino acids, vitamins, salts, glucose, and other balance chemicals (e.g. HEPES) and a pH indicator (e.g. phenol red; Arora, 2013). Serum is rich in adhesion factors, growth factors, hormones, minerals, lipid, and micronutrients (Brunner et al., 2010). However, because of the high cost, low production rate, and difficulty in standardization of base contents of serum, many research groups use an alternative nutrient complex with specific formulated composition according to the requirement of cells (Monhamed et al., 2014).

Maintenance of cell culture

Generally, culture begins with thawing frozen cells from liquid nitrogen storage, and cells then develop to confluence after a few days' culture. A standard growth pattern versus time consumed can be represented by a semi-logarithmic plot: low amplification rates both at the beginning (lag phase) and at confluence (stationary phase; Jennie and Penelope, 1998). A subculture (also called passage) is necessary to give cells more rooms to amplify when a 70-80% confluence is achieved (Canfield, 2011). Under that condition, cells are at a fast growth rate and high growth inhibitors are secreted in the medium. Over culturing, however, can lead to a harder detach due to a thick matrix. The cells that need to be used for future cultures can be returned to

liquid nitrogen storage (Jennie and Penelope, 1998).

Gene expression in the immune response to LPS challenge

The inflammatory response is initiated and enhanced by the increasing expression of immune-related receptors and cytokines in many cell types like plasma membranes and endosomal membranes of dendritic cells, B cells, and epithelial cells. Once the pathogen patterns are conjugated with these receptors outside or inside the cells, the immune system is capable of distinguishing self from non-self cells, and then initiates the signal transmission resulting in the up or down regulation of transcription levels of immune functional receptors or cytokines so as to further control innate immunity. Moreover, bacterial infections can also induce adaptive immunity, which gives a more specific protection than the innate immune system. Pathogen levels and types also influence the immune response. However, the whole blueprint of the immune response in swine is not thoroughly understood so far. Signals are only transduced when the corresponding pathogen ligand is conjugated to the receptors, when the receptor is expressed on the cell surface. The transcriptional profile of immune related cytokines and molecules in response to pathogens, to some extent, can provide insight into how the innate immune system functions (Philpot et al., 2001).

Toll-like receptors and main cytokines involved in innate immune response in pigs

The expression of TLRs has been reported to be indicative of the ability of the responsiveness of the immune system (Menzies and Ingham, 2006; Takeda and Akira, 2005), which stimulates gene expression and sequentially triggers the host defense. The characterization of TLRs in various species developed differently in terms of molecular and functional aspects. More studies are needed to understand whether all TLRs are expressed in domestic animals. In

addition, the type of leukocytes and the maturity status in the same species impact expression of distinct TLR profiles (Matsushima et al., 2004; Menzies and Ingham, 2006).

TLR 1, 2, and 6

Werling and Jungi (2003) have characterized the ligands of nine mammalian TLRs derived from PAMPs or DAMPs. As a co-receptor with TLR2, TLR1 associates in binding lipoproteins from mycobacteria (Hajjar et al., 2001), gram-negative bacteria, and *Borrelia burgdorferi,* which (Poltorak et al., 1998; Brightbill et al., 1999; Schwandner et al., 1999; Underhill et al., 1999; Bulut et al., 2001; Alexopoulou et al., 2002). Secondly, TLR2 recognizes a vast variety species of bacteria: lipoproteins/lipopeptides from gram-positive bacteria or mycoplasmas; mannuronic acid polymers from *Pseudomonas aeruginosa*; zymosan from yeast; and LPS from gram-negative bacteria Spirochetae (Takeuchi et al., 1999; Takeuchi et al., 2001; Buwitt-Beckmann et al., 2006; Hajjar et al., 2001; Netea et al., 2004; Cario et al., 2007). Meanwhile, TLR2-knock out mice failed to respond to peptidoglycan (PGN; Takeuchi et al., 2000), so TLR2 is also capable of stimulating the signal pathway in the case of PGN invasion. It is suggested that more TLR2 ligands may be discovered in the future because TLR2 performs the recognition by forming co-receptors with many other structure or non-structure related components (Akira, 2011), such as dectin-1, which is derived from the lectin receptor family (Takeda and Akira, 2005). Finally, TLR6 is reported to recognize gram-positive bacterial ligands like modulin, soluble tuberculosis, and also the lipoproteins' cell surface membrane (Bulut et al., 2001).

The most consistent finding was that TLR2 functions in association with TLR1 or TLR6 as heterodimers in recognizing or discriminating subtle differences in the structure of the fatty terminals on the bacterial cell wall (Takeuchi et al., 2001; Takeuchi et al., 2002; Akira, 2011).

Almost all bacteria lipoproteins have triacylated N-terminus cysteine residues except mycoplasma macrophage-activating lipopeptide-2 (MALP-2), which is diacylated (Takeuchi et al., 2001). Takeuchi et al., (2001) reported that the heterodimer TLR2/TLR6 recognizes the diacylated MALP-2, whereas TLR2 and TLR1 co-receptors stimulate responses from binding of triacylated bacteria lipopeptide (BLP; Akira, 2011).

TLR3

The TLR3 cytosolic receptor is expressed on small intestinal epithelia and mature dendritic cells (Cario and Podolsky, 2000; Muzio et al., 2000; McAllister et al., 2013), and has the ability to recognize virus double strand ribonucleic acid (dsRNA). TLR3-induced immune response can weaken or suppress the growth of virus in the GIT (Takeda and Akira, 2005). The TLR3-virus dsRNA complex initiates the immune defense by activation of TLR3-TRIF-caspase 8 and TLR3-caspase 3 signal pathway. Once the TLR 3 pathway is activated, the dsRNA virus may not be able to seriously destroy the integrity of the small intestinal mucosa and subsequently undergoes elimination by immune cells. Instead, the corresponding protective immune activity induces the intestinal villus epithelia apoptosis following an incidence of diarrhea, allowing the epithelium to recover in a short period of time (McAllister et al., 2013).

TLR4

The TLR4 is a transmembrane TLR with the ability to regulate the gram-negative bacteria derivative LPS response (Hoshino et al., 1999; Cario and Podolsky, 2000). Basically, LPS has a high affinity to bind serum LPS-binding proteins (LBP). The LPS-LBP complex further associates with the serum soluble or membrane-anchored CD14, which is a homing receptor for CD14 (Maliszewski, 1991), and then the ligation sequentially transfers the signal to

TLR4/MD-2 signaling complexes (Borzecka et al., 2013), which in turn elicits innate and adaptive immune responses (Shimazu et al., 1999; Ohashi et al., 2000; Philpott et al., 2001; Flo et al., 2002; Moue et al., 2008). The MD-2 is a molecule located on the cell membrane surface conjugated with TLR4 and functions as the signal transduction between TLR4 and LPS (Shimazu et al., 1999; Cario et al., 2000; Visintin et al., 2001). CD14 protein is not expressed on the intestinal epithelial cells in vitro, but exists as a soluble glycoprotein in serum or membrane-anchored molecule in vivo (Cario et al., 2000; Philpott et al., 2001). The LPS-LBS complex can either directly bind with CD14 to activate the mitogen-activated protein kinase (MAPK), or alternatively stimulate the NF-κB pathway (Cario et al., 2000).

TLR5

The immune-related function of TLR5 is recognizing flagellin on both gram-positive and gram-negative bacteria. Evolutionary conserved TLR5 is exclusively expressed on the basolateral side of myelomonocytic cells, like immature dendritic cells and epithelial cells (Hayashi et al., 2001). Thus, only virus and bacteria with flagellin can be bound to the basolateral sides of the sensor cells. Hence, the immune system may not respond when the epithelial cells are confronted with flagellated bacteria extracellularly (Gewirtz et al., 2001).

TLR7 and TLR8

Both TLR 7 and TLR 8 are collectively expressed on endosome receptors with their toll domains swaying in cytoplasm detecting pathogenic double strand ribonucleic acid (ssRNA; Takeda and Akira, 2005). Because of innate immune tolerance, TLR7 and TLR8 can only confer an inflammatory response by the ligation of pathogenic ssRNA but not the numerous host ssRNA. Interestingly, the two receptors respond to the shared ligands independently. In the

murine, however, only TLR7 can recognize synthetic compounds to perform its anti-viral or anti-tumor functions. Moreover, some anti-viral and anti-tumor mediators are found to be ligands for murine TLR7 (Takeda and Akira, 2005).

TLR9

Endosome-expressed TLR9 has been shown to have the ability to recognize bacterial CpG DNA for the presence of unmethylated CpG dinucleotides, and this is distinguished from mammalian methylated dsDNA (Hemmi et al., 2000). Hence, mammalian CpG DNA is not an immune-stimulatory antigen. However, bacterial CpG DNA is able to stimulate inflammatory cytokine expression, and also triggers the adaptive immune responses such as activation of the T helper-type-1 cells. Moreover, cells derived from autoimmune disorder disease can be internalized by B cell with the IgG2a receptors, and subsequently provide the chance of engagement of the chromatin or CpG and TLR9 in B cells. This ligation in turn induces further immune activation (Takeda and Akira, 2005). In swine, TLR9 is expressed not only in immune cells but also in follicle-associated epithelial cells in Peyer's Patches (Pps), like membranous (M) cells (Shimosato et al., 2005). Distribution of TLR9 is high in Pps cells and lymph nodes, but appears to be expressed less in the porcine spleen.

TLR10

First cloned in 2001, TLR10 has not been clearly characterized so far in all species. In cases where it was characterized, TLR10 has been shown to be highly homologous with TLR1 and TLR6, as well as sharing similar agonists with TLR1 (Guan et al., 2010). Screening pathogenic ligands by TLR10 requires the participation of TLR2 to activate the NF-κB pathway. TLR10 is highly expressed on intestinal epithelial cells, T and B cells (Hornung et al. 2002,

Guan et al, 2010), and other immune tissues such as spleen (Regan et al., 2013). Interestingly, TLR10 is expressed in the intracellular epithelium, but it needs the cooperation of membrane TLR2 to recognize PAMPs, especially those from heat-killed *Listeria monocytogenes*, *Staphylococcus aureus Cowan* I bacteria (SAC), and triacylated lipopeptides (Bourke et al., 2003; Guan et al., 2010).

Signal transduction pathway

The primary molecule involved in mediating antiviral activity are type I interferon (IFN)-α/β. The expression of these molecules requires the participation of transcription factors (NF-κB), activation protein 1 (AP-1) and interferon response factors (IRFs), and only functions after being activated. Inactive transcription factors presented in the cytoplasm can be stimulated by an upstream signal: TLRs-PAMPs ligation, then transferred to the nucleus to a binding gene locus (Takeuchi et al., 2000), where relative genes are expressed. However, IFN regulatory molecule activation requires the recruitment of adaptor proteins by the receptor-ligands compounds. Among the most common ten TLRs, TLR 3, 7, 8, and 9 are able to distinguish pathogenic nucleic acid in the nucleus from self, and all of them as well as TLR4 can migrate to nucleus to active the IRF3 and IRF7 to drive the type I IFNs expression, but this pathway is activated by different adaptor proteins. Both TLR7 and 9 promote the transcription factors via engagement of adaptors MyD88 but not TIR-domain-containing adapter-inducing interferon-β (TRIF), Whereas TLR3 needs TRIF to further motivate the expression of type I IFNs. Molecular surface receptors TLR 1, 2, 5 and 6 signal MyD88 directly to promote the transcription of immune functional proteins. Meanwhile, TRIF facilitates the synthesis of type I IFNs independently. The LPS-bound TLR4 can precede both immune signal pathways with both transcription mediators

(Abbas et al., 2012).

Stimulation of cytokines and chemokine through the signal transduction pathway

Signals of TLRs binding pathogens induce innate and adaptive immunity in animals and humans. Signal transduction is followed by the expression of transcription factors, which further perform the inflammatory and antiviral responses. Activated macrophages and mast cells produce cytokines that recruit and differentiate cells, but other cell types like endothelial and epithelial cells as well as neutrophils are also good sources (Takeda and Akira, 2005).

The term "interleukins" was first coined in 1979 to describe a group of immune-related functional proteins secreted by leukocytes, and works on the immune response through the whole process (Brocker et al., 2010). Interleukin-1β (IL-1β) can be secreted by many activated cell types including monocytes, macrophages, endothelial cells, epithelial cells, fibroblasts and neutrophils. It can initiate hundreds of downstream effector proteins to regulate immune function. Interleukin (IL)-6 is another cytokine besides IL-1β that induces the acute phase response and activates the immune system against antigen infection (Zhou et al., 1998). It is secreted not only by macrophages but also by other cell types including epithelial cells (Hedges et al., 1992). IL-8 is neutrophil chemotactic factor that stimulates the expression of adhesion molecules on the epithelial cells, such as Intercellular Adhesion Molecule 1 (ICAM-1), in order to recruit more neutrophils to the infected places (Scharek and Tedin, 2007; Devrient et al., 2010). IL-10, an anti-inflammatory cytokine mainly secreted by activated T and B lymphocytes, has the opposite immune function and can compromise the immune response.

The cytokine tumor necrosis factor-α (TNF-α) can induce apoptosis of tumor cells as well as being a pro-inflammatory protein that can initiate the innate immune system, and works

similarly to IL-1β and IL-6. TNF-α can be produced and secreted by a wide range of cell types and induced the pro-inflammatory response to further activates the acute phase response.

The granulocytes-macrophage colony-stimulating factor (GM-CSF) is an important leukocyte growth modulator produced in stimuli-activated cells, such as T cells, epithelial cells, macrophage and fibroblasts, and works on the leukocytes on their recruitments to the infected tissues (Shi et al., 2006). It also stimulates stem cells to differentiate into multiple immune functional cells like neutrophils, basophils, eosinophils and monocytes. Furthermore, GM-CSF drives the exit of monocytes from the blood vessels, into tissues where they are converted to macrophages, which can engulf and present the pathogens for adaptive immunity.

Although GM-CSF exists at very low levels in blood circulation, the amount of cells rises dramatically with immune stimulation (Shi et al., 2006). One of the most potent stimuli is LPS, which exerts the signal after binding with TLR4, and subsequently produces cytokines such as IL-1, IL-6 and TNF-α, which then induce GM-CSF expression (Megyeri et al., 1990; Goletti et al., 1996). Therefore, LPS induces the NF-κB signal pathway through TLR4 to produce cytokines, which then can stimulate the expression of GM-CSF to promote the differentiation or amplification of other critical immune functional cells (Parajuli et al., 2012).

Summary

The intestinal system is essential for nutrient exchange and immune function development and function. The suckling period is important for pigs because the gut, initially undeveloped, grow rapidly in the first two weeks. During this period, piglets ingest pathogenic bacteria from milk and the environment, which stimulate immune system development. Rearing pigs in outdoor sites are reported to produce better growth performance in the long run than traditional facilities because exposure to high bacteria in suckling pigs may improve the immune

function and reduce the weaning stress. Hence, artificially adding bacteria in the diet may help to develop the immune system in the gut.

In previous studies, non-transformed IPEC-J2 cell line, derived from newborn porcine jejunum epithelial cells, was used as a model of live pigs for monitoring immune function in vitro. LPS was used as the stimuli to induce an immune response. The stimulated immune system leads to an increasing expression of cytokines and PRRs in order to mediate the defensive activities. The regulated gene expression can indicate how the immune function works and then can help to direct the screening of bacteria suitable for consideration as potential direct fed microbes.

References

Abbas, A. K., A. H. Lichtman and S. Pillai. 2012. Cellular and molecular immunology. David L. Baker, MA and Alexandra Baker, MS, CMI. DNA Ilustrations, Inc.

Akira, S. 2011. Innate immunity and adjuvants. Philosophical transactions of the Royal Society of London. Series B, Biological Sciences 366: 2748-2755.

Alexopoulou, L., V. Thomas, M. Schnare, Y. Lobet, J. Anguita, R. T. Schoen, R. Medzhitov, E. Fikrig, and R. A. Flavell. 2002. Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. Nature Medicine 8: 878-884.

Arce, C., M. Ramirez-Boo, C. Lucena, and J. J. Garrido. 2010. Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from Salmonella typhimurium. Comparative Immunology, Microbiology and Infectious Diseases. 33: 161-174.

Arora, M. 2013. Cell Culture Media: A Review. Mater Methods 3:175.

Ashkenazi, A., J. A. Wells and J. Yuan. 2014. Regulated Cell Death Part B — Necroptotic, Autophagic and other Non-apoptotic Mechanisms. Methods in Enzymology. 545: 2-337.

Berschneider, H. 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Gastroenterology 96: A41.

Borzecka, K., A. Plociennikowska, H. Bjorkelund, A. Sobota, and K. Kwiatkowska. 2013. CD14 Mediates Binding of High Doses of LPS but Is Dispensable for TNF- alpha Production. Mediators of inflammation 2013: 824919.

Bourke, E., D. Bosisio, J. Golay, N. Polentarutti, and A. Mantovani. 2003. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 102: 956-963.

Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R.B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M.V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science (New York, N.Y.) 285: 732-736.

Brocker, C., D. Thompson, A. Matsumoto, D. W. Nebert and V. Vasiliou. 2010. Evolutionary divergence and functions of the human interleukin (IL) gene family. HUMAN GENOMICS. 5: 30–55.

Brosnahan, A. J., and D. R. Brown. 2012. Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. Veterinary Microbiology 156: 229-237.

Brown, D. C., C. V. Maxwell, G. F. Erf, M. E. Davis, S. Singh, and Z. B. Johnson. 2006. The

influence of different management systems and age on intestinal morphology, immune cell numbers and mucin production from goblet cells in post-weaning pigs. Vet. Immunology and Immunopathology 111:187-198.

Brunner, D., J. Frank, H. Appl, H. Schöffl, W. Pfaller, and G. Gstraunthaler. 2010. Serum-free cell culture: The serum-free media interactive online database. ALTEX 27: 1-10.

Bulut, Y., E. Faure, L. Thomas, O. Equils, and M. Arditi. 2001. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. Journal of Immunology 167: 987-994.

Burkey, T. E., K. A. Skjolaas, S. S. Dritz, and J. E. Minton. 2009. Expression of porcine Toll-like receptor 2, 4 and 9 gene transcripts in the presence of lipopolysaccharide and Salmonella enterica serovars Typhimurium and Choleraesuis. Veterinary Immunology and Immunopathology 130: 96-101.

Buwitt-Beckmann, U., H. Heine, K. H. Wiesmüller, G. Jung, R. Brock, S. Akira, and A. J. Ulmer. 2006. TLR1- and TLR6-independent recognition of bacterial lipopeptides. The Journal of Biological Chemistry 281: 9049-9057.

Campbell, J. M., J. D. Crenshaw and J. Polo. 2013. The biological stress of early weaned piglets. Journal of Animal Science and Biotechnology 4:19.

Canfield, T. 2011. Propagation of Mouse NIH-3T3 Embryonic Fibroblasts. ATCC Inc.

Cario, E., and D. K. Podolsky. 2000. Differential Alteration in Intestinal Epithelial Cell Expression of Toll-Like Receptor 3 (TLR3) and TLR4 in Inflammatory Bowel Disease. Infection and Immunity 68: 7010-7017.

Cario, E., G. Gerken, and D. K. Podolsky. 2007. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology 132: 1359-1374.

Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. Journal of Immunology 164: 966-972.

Coté, R. J. 2001. Aseptic technique for cell culture. Curr. Protoc. Cell Biol. 2001 May; Chapter 1:Unit 1.3.

Devriendt, B., E. Stuyven, F. Verdonck, B. M. Goddeeris, and E. Cox. 2010. Enterotoxigenic Escherichia coli (K88) induce proinflammatory responses in porcine intestinal epithelial cells. Developmental and Comparative Immunology. 34. 1175-1182.

Fairbrother J. M., E. Nadeau, and C. L. Gyles. 2005. *Escherichia coli* in postweaning diarrhea in

pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim Health Res Rev 6: 17–39.

Flo, T. H., L. Ryan, E. Latz, O. Takeuchi, B. G. Monks, E. Lien, Ø Halaas, S. Akira, G. Skjåk-Braek, D. T. Golenbock, and T. Espevik. 2002. Involvement of toll-like receptor (TLR) 2 and TLR4 in cell activation by mannuronic acid polymers. The Journal of Biological Chemistry 277: 35489-35495.

Francis, D. H. 1999. Colibacillosis in pigs and its diagnosis. Swine Health Prod.7: 241-244.

Freshney, R. I. 2005. Culture of animal cells. A John Wiley & Sons, Inc., Publication.

Froehlich, J. E., and T. P. Anastassiades. 2005. Role of pH in fibroblast proliferation. Journal of Cellular Physiology. 84: 253-259.

Geens, M. M., and T. A. Niewold. 2010. Preliminary Characterization of the Transcriptional Response of the Porcine Intestinal Cell Line IPEC-J2 to Enterotoxigenic Escherichia coli, Escherichia coli, and E. coli Lipopolysaccharide. Comparative and Functional Genomics 2010: 469583.

Gewirtz, A. T., T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. Journal of Immunology. 167: 1882-1885.

Goletti, D., A. L. Kinter, E. C. Hardy, G. Poli, and A. S. Fauci. 1996. Modulation of endogenous IL-1 beta and IL-1 receptor antagonist results in opposing effects on HIV expression in chronically infected monocytic cells. Journal of Immunology. 156: 3501-3508.

Gonzalez-Vallina, R., H. Wang, R. Zhan, H. M. Berschneider, R. M. Lee, N. O. Davidson, and D. D. Black. 1996. Lipoprotein and apolipoprotein secretion by a newborn piglet intestinal cell line (IPEC-1). The American Journal of Physiology 271: 249-259.

Guan, Y. D. R. Ranoa, S. Jiang, S. K. Mutha, X. Li, J. Baudry, and R. I. Tapping. 2010. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. Journal of Immunology 184: 5094-5103.

Gumbiner, B. M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84: 345-357.

Hajjar, A. M., D. S. O'Mahony, A. Ozinsky, D. M. Underhill, A. Aderem, S. J. Klebanoff, and C. B. Wilson. 2001. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. Journal of Immunology 166: 15-19.

Hartsock, A., and W. J. Nelson. 2008. Adherens and Tight Junctions: Structure, Function and Connections to the Actin Cytoskeleton. Biochim Biophys Acta. 1778: 660–669.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410: 1099-1103.

Hedges, S., M. Svensson, and C. Svanborg. 1992. Interleukin-6 Response of Epithelial Cell Lines to Bacterial Stimulation In Vitro Infection and Immunity. 60:1295-1301.

Hemmi, H. O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akrira. 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408: 740-745.

Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitiative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168. 4531-4537.

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. Journal of immunology 162: 3749-3752.

Invitrogen. 2002. http://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics.html

Jennie, P. M. and E. R. Penelope. 1998. Introduction to Cell and Tissue Culture: theory and technique. New York. Genentech Inc.

Johnson, A. M., R. S. Kaushik, D. H. Francis, J. M. Fleckenstein, and P. R. Hardwidge. 2009. Heat-labile enterotoxin promotes Escherichia coli adherence to intestinal epithelial cells. Journal of Bacteriology 191: 178-186.

Jurk, M. F. Heil, J. Vollmer, C. Schetter, A. M. Krieg, H. Wagner, G. Lipford, and S. Bauer. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nature Immunology 3: 499.

Kitt, S. J., P. S. Miller, and A. J. Lewis. 2001. Factors affecting small intestine development in weaning pigs. Nebraska Swine Reports 1: 33-35.

Koh, S., Y. S. George, V. Brözel, R. Moxley, D. Francis, and R. S. Kaushik. 2008. Porcine intestinal epithelial cell lines as a new in vitro model for studying adherence and pathogenesis of enterotoxigenic Escherichia coli. Veterinary Microbiology 130: 191-197.

Kyriazakis, I. and A. Doeschl-Wilson, 2009. Anorexia during infection in mammals: variation and its sources in Voluntary Feed Intake in Pigs. D. Torrallardona and E. Roura, eds. Wageningen Academic Publishers. Wageningen. Netherlands.

Leland, D. S. and C. C. Ginocchio. 2007. Role of cell culture for virus detection in the age of technology. Clinical Microbiology Reviews 20: 49-78.

Liu F. N., G. Li, K. Wen, T. Bui, D. Cao, Y. Zhang, and L. Yuan. 2010. Porcine Small Intestinal Epithelial Cell Line (IPEC-J2) of Rotavirus Infection As a New Model for the Study of Innate Immune Responses to Rotaviruses and Probiotics. Viral Immunology 23: 135-149.

Lu, S., Y. Yao, X. Cheng, S. Mitchell, S. Leng, S. Meng, J. W. Gallagher, G. S. Shelness, G. S. Morris, J. Mahan, S. Frase, C. M. Mansbach, R. B. Weinberg, and D. D. Black. 2006. Overexpression of apolipoprotein A-IV enhances lipid secretion in IPEC-1 cells by increasing chylomicron size. The Journal of Biological Chemistry 281: 3473-3483.

Maliszewski, C. R. 1991. CD14 and immune response to lipopolysaccharide. Science (New York, N.Y.) 252: 1321-1322.

Mariani, V., S. Palermo, S. Fiorentini, A. Lanubile, and E. Giuffra. 2009. Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. Veterinary Immunology and Immunopathology 131: 278-284.

Matsushima, H., N. Yamada, H. Matsue, and S. Shimada. 2004. TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. Journal of Immunology 173: 531-541.

McAllister, C. S., O. Lakhdari, G. Pineton de Chambrum, M. G. Gareau, A. Broquet, G. H. Lee, S. Shenouda, L. Eckmann, and M. F. Kagnoff. 2013. TLR3, TRIF, and caspase 8 determine double-stranded RNA-induced epithelial cell death and survival in vivo. Journal of Immunology 190: 418-427.

Megyeri, P., J. Sadowska, T. B. Issekutz, and A. C. Issekutz. 1990. Endotoxin-stimulated human Mariani, 2009s produce a factor that induces polymorphonuclear leucocyte infiltration and is distinct from interleukin-1, tumour necrosis factor alpha and chemotactic factors. Immunology 69: 155-161.

Menzies, M., and A. Ingham. 2006. Identification and expression of Toll-like receptors 1-10 in selected bovine and ovine tissues. Veterinary Immunology and Immunopathology 109: 23-30.

Meurens, F., F. Girard-Misguich, S. Melo, A. Grave, H. Salmon, and N. Guillén. 2009. Broad early immune response of porcine epithelial jejunal IPI-2I cells to Entamoeba histolytica. Molecular Immunology 46: 927-936.

Monhamed, M. S., Y. Chen, and C. L. Yao. 2014. A serum-free medium developed for expansion of murine intestinal stem cells in vitro. Biotechnol J. DOI: 10.1002/biot.201400016.

Montilla, N. A., M. López Santalla, and J. M. Martín Villa. 2004. Mucosal immune system: A brief review. Infection and Immunity 23: 207-216.

Moue, M., M. Tohno, T. Shimazu, T. Kido, H. Aso, T. Saito, and H. Kitazawa. 2008. Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. Biochimica et Biophysica Acta 1780: 134-144.

Muzio, M., D. Bosisio, N. Polentarutti, G. D'amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Pento-Rol, L. P. Ruco, P. Allavena, and A. Mantovani. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. Journal of Immunology 164: 5998-6004.

Netea, M. G., J. W. Van der Meer, and B. J. Kullberg. 2004. Toll-like receptors as an escape mechanism from the host defense. Trends in Microbiology 12: 484-488.

Ohashi, K., V. Burkart, S. Flohe, and H. Kolb. 2000. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. Journal of Immunology 164: 558-561.

Oswald, I. P. 2006. Role of intestinal epithelial cells in the innate immune defence of the pig intestine. Veterinary Research 37: 359-368.

Parajuli, B., Y. Sonobe, J. Kawanokuchi, Y. Doi, M. Noda, H. Takeuchi, T. Mizuno, and A. Suzumura. 2012. Journal of Neuroinflammation. 9:268.

Pasare, C., and R. Medzhitov. 2004. Toll-like receptors: linking innate and adaptive immunity. Microbes and Infection / Institut Pasteur 6: 1382-1387.

Patience, J. F. 2012. Feed Efficiency in Swine. John F. Patience. eds. Wageningen Academic Publishers. Wageningen.

Philpott, D. J., S. E. Girardin, and P. J. Sansonetti. 2001. Innate immune responses of epithelial cells following infection with bacterial pathogens. Current Opinion in Immunology 13: 410-416.

Pitman, R. S., and R. S. Blumberg. 2000. First line of defense: the role of the intestinal epithelium as an active component of the mucosal immune system. Journal of Gastroenterology 35: 805-814.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science (New York, N.Y.) 282: 2085-2088.

Regan, T., K. Nally, R. Carmody, A. Houston, F. Shanahan, J. Macsharry, and E. Brint. 2013.

Identification of TLR10 as a key mediator of the inflammatory response to Listeria monocytogenes in intestinal epithelial cells and macrophages. Journal of Immunology 191: 6084-6092.

Scharek, L. and K. Tedin. 2007. The porcine immune system – differences compared to man and mouse and possible consequences for infections by *Salmonella* serovars. Berl Munch Tierarztl Wochenschr. 120: 347-54.

Schierack, P., M. Nordhoff, M. Polmann, K. D. Weyrauch, S. Amasheh, U. Lodemann, J. Jores, B. Tachu, S. Kleta, A. Blikslager, K. Tedin, and L. H. Wieler. 2006. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochemistry and Cell Biology 125: 293-305.

Schierack, P., S. Rödiger, C. Kuhl, R. Hiemann, D. Roggenbuck, G. Li, J. Weinreich, E. Berger, L. K. Nolan, B. Nicholson, A. Römer, U. Frömmel, L. H. Wieler, and C. Schröder. 2013. Porcine E. coli: virulence-associated genes, resistance genes and adhesion and probiotic activity tested by a new screening method. PloS one 8: e59242.

Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. The Journal of Biological Chemistry 274: 17406-17409.

Shankar B. P., H. S. Madhusudhan1 and D. B. Harish. 2009. Pre-Weaning Mortality in Pig-Causes and Management. Veterinary World. 2: 236-239.

Shi, Y., C. H. Liu, A. I. Roberts, J. Das, G. Xu, G. Ren, Y. Zhang, L. Zhang, Z. R. Yuan, H. S. W. Tan, G. Das, and S. Devadas. 2006. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T- cell responses: what we do and don't know. Cell Research16:126-133.

Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. The Journal of Experimental Medicine 189: 1777-1782.

Shimosato, T. M. Tohno, H. Kitazawa, S. Katoh, K. Watanabe, Y. Kawai, H. Aso, T. Yamaguchi, and T. Saito. 2005. Toll-like receptor 9 is expressed on follicle-associated epithelia containing M cells in swine Peyer's patches. Immunology Letters 98: 83-89.

Skjolaas, K. A., T. E. Burkey, S. S. Dritz, and J. E. Minton. 2006. Effects of Salmonella enterica serovars Typhimurium (ST) and Choleraesuis (SC) on chemokine and cytokine expression in swine ileum and jejunal epithelial cells. Veterinary Immunology and Immunopathology 111: 199-209.

Skjolaas, K. A., T. E. Burkey, S. S. Dritz, and J. E. Minton. 2007. Effects of Salmonella enterica serovar Typhimurium, or serovar Choleraesuis, Lactobacillus reuteri and Bacillus licheniformis on chemokine and cytokine expression in the swine jejunal epithelial cell line, IPEC-J2. Veterinary Immunology and Immunopathology 115: 299-308.

Spencer, S. J., M. A. Galic, and Q. J. Pittman. 2011. Neonatal programming of innate immune function. American journal of physiology. Endocrinology and Metabolism 300: E11-18.

Stacey, G. 2005. Primary cell cultures and immortal cell lines. Encyclopedia of Life Sciences. doi: 10.1038/npg.els.0003960

Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. International Immunology 17: 1-14.

Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. Annual Review of Immunology 21: 335-376.

Takeuchi, O. A. Kaufmann, K. Grote, T. Kawai, K. Hoshino, M. Morr, P. F. Mühlradt, and S. Akira. 2000. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. Journal of Immunology 164: 554-557.

Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential Roles of TLR2 and TLR4 in Recognition of gram-Negative and gram-Positive Bacterial Cell Wall Components. 11: 443-445.

Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin, and S. Akira. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. Journal of Immunology 169: 10-14.

Takeuchi, O., T. Kawai, P. F. Mühlradt, M. Morr, J. D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. International Immunology 13: 933-940.

Thacker, E. 2004. The battle between the immune system and pathogens. The Pig Journal 54: 55-69.

Thorpe, T. A. 2007. History of plant tissue culture. Mol Biotechnol. 37:169-180.

Underhill, D., M. A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 401: 811-815.

Veldhuizen, E. J., H. G. Hendriks, A. Hogenkamp, A. van Dijk, W. Gaastra, P. C. Tooten, and H. P, Haagsman. 2006. Differential regulation of porcine beta-defensins 1 and 2 upon Salmonella infection in the intestinal epithelial cell line IPI-2I. Veterinary Immunology and Immunopathology 114: 94-102.

Visintin, A., A. Mazzoni, J. H. Spitzer, D. H. Wyllie, S. K. Dower, and D. M. Segal. 2001.

Regulation of Toll-like receptors in human monocytes and dendritic cells. Journal of Immunology 166: 249-255.

Werling, D., and T. W. Jungi. 2003. Toll-like receptors linking innate and adaptive immune response. Veterinary Immunology and Immunopathology 91: 1-12.

Zhou X., J. Gauldie, G. Cox, H. Baumann, M. Jordana, X. Lei, and M. K. Achong. 1998. Il-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. J. Clin. Invest. 101: 311–320.

Chapter III. Evaluation of doubling time and growth rate of IPEC-J2 cell line in 96-well plates or T-75 flasks

Abstract

The IPEC-J2 cell line has been utilized in a variety of fields as a model for live animals, and its culture condition and maintenance has been characterized. However, the growth rate and doubling time have been rarely investigated and evaluated. Growth curves of IPEC-J2 cells were investigated when cultured in T-75 flasks or 96-well plates using two methods of cell quantity measurements: hemocytometer and spectrophotometer [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT)]. Growth rate and doubling time were calculated from the cell counts and absorptions. Growth rate and doubling time of IPEC-J2 cells cultured in 96-well plates were 0.2851 times of doubling/d and 2.43 d, respectively. Cells grown in T-75 flasks resulted in a growth rate with 0.4016 times of doubling/d and a doubling time with 1.73 d. The IPEC-J2 cell line can be cultured in both 96-well plates and T-75 flasks. The MTT method is applicable in measuring growth condition of IPEC-J2 cells.

Key words: culture, growth rate, doubling time, MTT, hemocytometer, flask, IPEC-J2 cells

Introduction

The untransformed continuous IPEC-J2 cell line isolated from porcine mid-jejunum epithelium in a less than 12-h-old piglet has proven to be a good model for gene expression studies (Paszti-Gere et al., 2012b; Sun et al. 2012; Berschneider, 1989). In vitro, cells are provided with sufficient nutrient additives in the culture medium to fulfill the requirements for growth and maintenance, but nutrient formulation for a specific cell line varies slightly from one lab to another. Therefore, the growth rate of cells may be not consistent between labs, and should be demonstrated in each lab.

Growth kinetics of IPEC-J2 cells is also important for the routine maintenance. IPEC-J2 cells normally develop to confluence in 7-9 d (Geens and Niewold, 2011), and then the culture vessel is not an optimal environment due to high density of cells, fast exhaustion of medium, contact inhibition between cells and toxin production. A 70-80% confluence is recommended in subcultures, where the cells are in a "log" phase. During a subculture, reseeded cells can enter a "lag" phase to recover from trypsinization, but it usually takes no more than 48 h to rebuild the matrix and form a new monolayer (Iloki Assanga et al., 2013).

Knowledge of the growth kinetics of cells can guide scheduling of cell culture maintenance (such as determination of seeding density, culture vessels, and subculture), which further reduces the expense of time and material inputs such as flasks, plates, pipette tips, electricity, etc. In addition, a standard growth pattern provides the general amount of cells in the culture vessel at a determined time. Moreover, growth rate is a good criterion for evaluating cell health: a low amplification level implies a depressed health condition. Together with TEER, proliferation rate of cells is also regarded as a response variable when verifying the optimal culture environment (Geens and Niewold, 2011).

Materials and Methods

Growth assay of IPEC-J2 cells in T-75 flask by hemocytometer technique

Culture and maintenance of IPEC-J2 cell line

IPEC-J2 cells were stored in liquid nitrogen, and culture of IPEC-J2 cells started by thawing out cell suspensions. A total of three vials containing 10^6 cells per vial were thawed and transferred to 3 T-75 flasks, each with 20 mL pre-warmed full medium: Dulbecco's modified eagle medium (50% DMEM: 50% F-12; American Type Culture Collection, VA; ATCC® 30-2006™) supplemented with 1% insulin (Sigma, cat: 11884-1VL), 10% fetal bovine serum (Hyclone, UT, Cat. No. SH 30070.02), 5ng/mL recombinant human epidermal growth factor (EGF) (Sigma, Cat. No. 13247-051), and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, Cat. No.15140-122). The complete medium was adjusted to pH 7.2-7.4 using HCl or NaOH solutions, and cells were cultured in a humidified atmosphere of 5% CO_2 at 38°C. The culture medium was refreshed twice a week.

After a 9-d culture, the cell monolayers were rinsed with 5 mL 0.05% Trypsin-EDTA (Ethylenediaminetetraacetic acid) solution for 30 s and removed. Then the cell monolayer was digested using 3 mL of 0.05% trypsin-EDTA solution (Invitrogen Life Technologies, Carlsbad, CA, cat: 25300) for 15 min. Once IPEC-J2 cells were detached from the bottom of the flask and separated into individual cells, cells from three flasks were combined and the hemocytometer technique was used to examine cell density. Cells were then re-suspended to 9×10^5 cells/mL with pre-warmed medium. The re-suspended cell pellets were then seeded into 14 flasks and each flask filled with 19 mL pre-warmed complete medium.

Cell count using hemocytometer technique

After seeding, two flasks were randomly selected for cell count every 24 h. On each day,

before trypsinization, monolayers were rinsed with 5 mL of pre-warmed PBS for 30 s and 3 mL of trypsin for 30 s due to different cell densities each day. Cell disaggregation was achieved by a 15-min treatment with 1 mL trypsin. Cell pellets were gently aspirated up and down against the corners of the flasks to sufficiently disperse cells. Fresh complete medium was added at appropriate levels to inactivate trypsin. A 200-µL volume of well-mixed cell suspension was pipetted to a centrifuge tube and mixed with 200 µL of 0.04% trypan blue (TB). The cell-TB mixtures were vortexed for 30 s and then 10 µL of the mix was loaded into the hemocytometer chamber, which was then placed under a 10x ocular and 40x objective of an inverted microscope (CX41RF, Olympus). Cells in five small squares were distinguished and counted by phase contrast.

Cell concentration was calculated using the formula:

$$\text{Cell/cm}^{-3} = \frac{(\text{cell counted in n squares}) \times (10^4) \times 25 \times \text{dilution factor}}{\text{n squares}}$$

Doubling time and growth rate were calculated using the formula cited in the Roth V. 2006 website (http://www.doubling-time.com/compute.php).

Growth assay of IPEC-J2 cells using the MTT method

The MTT is a yellow water-soluble tetrazolium salt, which can be reduced to a blue insoluble dye called formazan by mitochondrial enzymes in live cells (Twentyman and Luscombe, 1987). Formazan is solubilized by MTT solvents such as isopropyl alcohol, and the color density, which is linearly relative to cell numbers, is determined using a spectrophotometer.

Culture and maintenance of IPEC-J2 cell line

Culture conditions were the same as described earlier for the hemocytometer technique.

However, after evaluating cell densities with the hemocytometer, cells were resuspended with phenol red-free medium for a final concentration of 7,000 cells/mL. Cell suspensions were seeded into six 96-well plates, with 0.5 mL cell suspension/well in 36 wells/plate. The plates were cultured for 10 d in a humidified atmosphere of 5% CO_2 at 38°C.

Evaluation of growth using MTT method

On each day, one plate was randomly selected and 18 wells were treated with 20 µL MTT solution. Untreated wells were covered by a film to protect cells from MTT-generated fumes. The plate was cultured in the incubator for 3.5 h after which the MTT solution and media were gently aspirated. A 150µL MTT solvent was added and cells were agitated by orbital shaker for 15 min. Wells were read at a wavelength of 590 nm.

Doubling time and growth rate were calculated using the formula cited in the Roth V. 2006 website (http://www.doubling-time.com/compute.php).

Statistical analysis

T-75 flask-hemocytometer

The experiments were arranged in a completely randomized design. Cell number data were analyzed using the PROC GLM of SAS (SAS Institute, Inc., Cary, NC) with day as the main fixed effect. Flask served as the experimental unit for ANOVA. Orthogonal contrasts were used to determine linear, quadratic, cubic and quartic response. The growth curve and regression equations were generated in MSExcel.

96-well plate-MTT method

Growth was determined within a 10-d culture period, using a completely randomized design with well as the experimental unit, day as treatment, and dye absorbance as variable.

Absorptions were analyzed using the PROC GLM of SAS with well as the experimental unit. Orthogonal contrasts were used to determine linear, quadratic, cubic and quartic response. Growth curve and regression equations were generated in MSExcel.

Results

Cell proliferation regression equation, doubling time, and growth rate

Cell numbers generated using the hemocytometer technique are presented in Figure 1. Least square means of cell numbers are shown in Table 1. IPEC-J2 cells amplified in a cubic ($P = 0.05$) polynomial trend in T-75 flasks (Table 1). The fitted regression equation generated in MSExcel is $y = -58368x^3 + 902250x^2 - 2E+06x + 2E+06$ with $R^2 = 0.99834$. The doubling time in the exponential period (days 3-7) of IPEC-J2 cells growing in a T-75 flask is 1.73 d with a growth rate of 0.4016 times of doubling/day.

The absorbance of the color density in each well during a 10-d culture in 96-well plates is shown in Table 1. L.S. means of absorbance are shown in Table 2. The color density and the culture time of IPEC-J2 cells show a linear relationship ($P < 0.0001$) with $R^2 = 0.89974$, and it tended to fit a quadratic regression model ($P = 0.09$) with $R^2 = 0.96506$ (Figure 2 and Table 2). Doubling time for IPEC-J2 cell line cultured in a 96-well plate is 2.43 with a growth rate of 0.2851 times of doubling/day (Roth, 2006).

Discussion

So far, few studies of the growth rate or doubling time of the IPEC-J2 cell line have been reported in the literature although it has been well characterized in morphology of the monolayer, culture condition, gene expressions and adherence studies (Schierack, et al., 2006; Zakrzewski, et al., 2013; Hartsock and Nelson. 2008; Johnson et al., 2009). It was only reported by Diesing in 2011 that it took 4 d to reach confluence when seeded 5×10^4 IPEC-J2 cells in a 75 mL plastic

flask. The DSMZ company (a member from Leibniz Institution) stated the doubling time of IPEC-J2 cell line is 72-90 h. From our studies, the doubling times of IPEC-J2 cell line in T-75 flasks and 96-well plates were not equivalent (1.73 d and 2.43 d), and the monolayers reached confluence at 6 and 7 d respectively. In subculture, the trypsinized cells grew in the "lag" phase because it took 1-2 days for reproducing the outside adhesion and junction proteins (Iloki Assanga et al., 2013). Doubling time was calculated during the "log" phase of the cells, which was determined from the growth curve.

As can be seen from both growth curves in two experiments, cells in T-75 flasks experienced around 3 d to reach to the exponential proliferation phase, while, in 96-well plates, it took more than 4 d. A long "lag" phase always is followed by a long confluence day, which stands for a low cell production.

Culturing cells in T-75 flasks had higher growth rate 0.4016 time of doubling/d, which is twice the growth rate in 96-well plates. The initial seeding density of IPEC-J2 cells in 96-well plates (3500 cells/well) was lower than used in other studies, and the 6400 cells recommended by ATCC cell culture guide, which also contributed to a long "lag" phase and confluence time. According to the growth trend, a subculture was needed at the 5th or 6th d because cells were still in the exponential phase and cells were close to the confluence.

As a relatively new method of quantification with a low standard deviation (Iloki Assanga et al. 2013), the MTT assay has been widely used on various cell lines such as mouse tumor cell and lymphocytes to evaluate the viability of cells when infected with antigens and chemicals as well as in measuring the proliferation rate (Denizot and Lang, 1986; Twentyman and Luscombe, 1987; Schierack et al. 2013). In terms of the IPEC-J2 cell line, so far the MTT method was successfully used in a pro-inflammatory study such as deoxynivalenol, mycotoxin

infection for measuring variability (Diesing et al., 2011; Wan et al., 2013). However, our study focused on the growth trend of healthy IPEC-J2 cells without comparing with antigen-infected counterparts, which took a longer time culturing in 96-well plates. As discovered from the culture experience, the healthy status of IPEC-J2 cells in 96-well plates were not constant: longer culture may lead to higher cell mortality in the marginal wells. Besides, the intestinal cells are anaerobic, keeping cells under 5% CO_2 is very important and this can be improved by covering the plates with plastic film and less movement (data not shown).

In addition, the MTT assay can be applied to a wider range of cell amounts when compared to the TB method. In the T-75 flask, an IPEC-J2 cell concentration lower than 160,000 cells/mL (cell count on the first day) made the cell count very difficult, while the spectrophotometer was able to measure the color density of MTT formazan at low cells numbers (3,500 cells were seeded per well). However, the MTT method verifies the cell density by measuring the absorption of the color and cannot give exact cell numbers. Meanwhile, contamination and cell morphology cannot be observed by the optical measurements (Iloki Assanga et al. 2013). Hence, hemocytometer is still a vital method for the cell count for the IPEC-J2 cell line.

Conclusions

Both T-75 flasks and 96-well plates are good vessels for IPEC-J2 cells due to high growth rates. However, as a purpose of cell production, the T-75 flasks are the better choice than 96-well plates because of higher growth rates and consistent growth can be achieved. As a conclusion on the culture of IPEC-J2 cells in T-75 flasks, 1-2 days are needed for cells to recover from the trypsin digestion, and the growth enter an exponential growth pattern at the 3rd day. This condition may vary with different cell seeding number.

The MTT method is applicable in evaluating the growth pattern of IPEC-J2 cells but exact cell numbers are hard to obtain. However, this optical measurement can be widely used in measuring the cell variability under medical or bacterial challenge study.

References:

Berschneider, H. 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Gastroenterology 96: A41.

Denizot, F. and R. Lang. 1986. Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods 89: 271-277.

Diesinga, A., C. Nossola, P. Panthera, N. Walka, A. Posta, J. Kluessa, P. Kreutzmannb, S. Dänickec, H. Rothköttera, and S. Kahlert. 2011. Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicology Letters 200: 8–18.

Freshney, R. Ian. 2005. Culture of animal cells. A John Wiley & Sons, Inc., Publication.

Geens, M. M., and T. A. Niewold. 2011. Optimizing culture conditions of a porcine epithelial cell line IPEC-J2 through a histological and physiological characterization. Cytotechnology 63: 415-423.

Hartsock, A. and W. J. Nelson. 2008. Adherens and Tight Junctions: Structure, Function and Connections to the Actin Cytoskeleton. Biochim Biophys Acta. 1778: 660–669.

Iloki Assanga, S. B., A. A. Gil-Salido, L. M. Lewis Luján, A. Rosas-Durazo, A. L. Acosta-Silva, E. G. Rivera-Castañeda and J. L. Rubio-Pino. 2013. Cell growth curves for different cell lines and their relationship with biological activities. Int J. of Biotechnol. Mol Biol. Res: 4: 60-70.

Johnson, A. M., R. S. Kaushik, D. H. Francis, J. M. Fleckenstein, and P. R. Hardwidge. 2009. Heat-labile enterotoxin promotes Escherichia coli adherence to intestinal epithelial cells. Journal of Bacteriology 191: 178-186.

Paszti-Gere, E., K. Szeker, E. Csibrik-Nemeth, R. Csizinszky, A. Marosi, O. Palocz, O. Farkas, and P. Galfi. 2012b. Metabolites of Lactobacillus plantarum 2142 prevent oxidative stress induced overexpression of proinflammatory cytokines in IPEC-J2 cell line. Inflammation 35:1487–1499.

Roth V. 2006 http://www.doubling-time.com/compute.php.

Schierack, P., M. Nordhoff, M. Pollmann, K. D. Weyrauch, S. Amasheh, U. Lodemann, J. Jores, B. T. S. Kleta, A. Blikslager, K. Tedin, and L. H. Wieler. 2006. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem. Cell Biol. 125: 293-305.

Schierack, P., S. Rödiger, C. Kuhl, R. Hiemann, D. Roggenbuck, G. Li, J. Weinreich, E. Berger, L. K. Nolan, B Nicholson, A. Römer, U. Frömmel, L. H. Wieler, and C. Schröder. 2013. Porcine E. coli: virulence-associated genes, resistance genes and adhesion and probiotic

activity tested by a new screening method. PloS one 8: e59242.

Sun, Z., V. C. Huber, K. McCormik, R. S. Kaushik, A. C. M. Boon, L. Zhu, B. Hause, R. J. Webby, and Y. Fang. 2012. Characterization of a porcine intestinal cell line for influenza virus production. J. Gen. Virol. 93: 9.

Twentyman, P. R. and M. Luscombe. 1987. A study of some variable in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br. J. Cancer 56: 279-285.

Wan, L. M., C. J. Woo, P. C. Turner, J. M. Wan, and H. El-Nezami. 2013. Individual and combined effects of Fusarium toxins on the mRNA expression of pro-inflammatory cytokines in swine jejunal epithelial cells. Toxicology Letters 220: 238-246.

Zakrzewski S. S., J. F. Richter, S. M. Krug, B. Jebautzke, I. M. Lee, J. Rieger, M. Sachtleben, A. Bondzio, J. D. Schulzke, M. Fromm, and D. Günzel. 2013. Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. PLoS ONE 8(11): e79643. Doi: 10.1371/journal.pone.0079643

Table 1. LS means of cells numbers in 7 days.

Day	Cells LSMEAN	Standard Error Mean	P - value
1	415000.00	351561	Day: < 0.01
2	650000.00		Cubic: 0.04
3	1226000.00		
4	3340750.00		
5	5600000.00		
6	7706250.00		
7	9744000.00		

The cells were determined in a completely randomized design with flask as the experimental unit, day as treatment, and cell numbers in each flask as the variable. Cell number data were analyzed using the PROC GLM of SAS (SAS Institute, Inc., Cary, NC) with day as the main fixed effect, with flask as experimental unit. Orthogonal contrast was used to determine linear, quadratic, cubic and quartic response.

Table 2. LS mean of absorbance in 10 day.

Day	LSMEAN	Standard Error Mean	*P* - value
1	0.07213	0.03	Day: 0.01
2	0.1292		Linear: < 0.01
3	0.1639		Quad: 0.09
4	0.2061		
5	0.2828		
6	0.3797		
7	0.4646		
8	0.8882		
9	0.9297		
10	1.0688		

The growth was determined within a 10-d culture period, using a completely randomized design with well as the experimental unit, day as treatment, and dye absorbance as variable. Absorbance was analyzed using the PROC GLM of SAS (SAS Institute, Inc., Cary, NC). Orthogonal contrasts were used to determine linear, quadratic, cubic and quartic response.

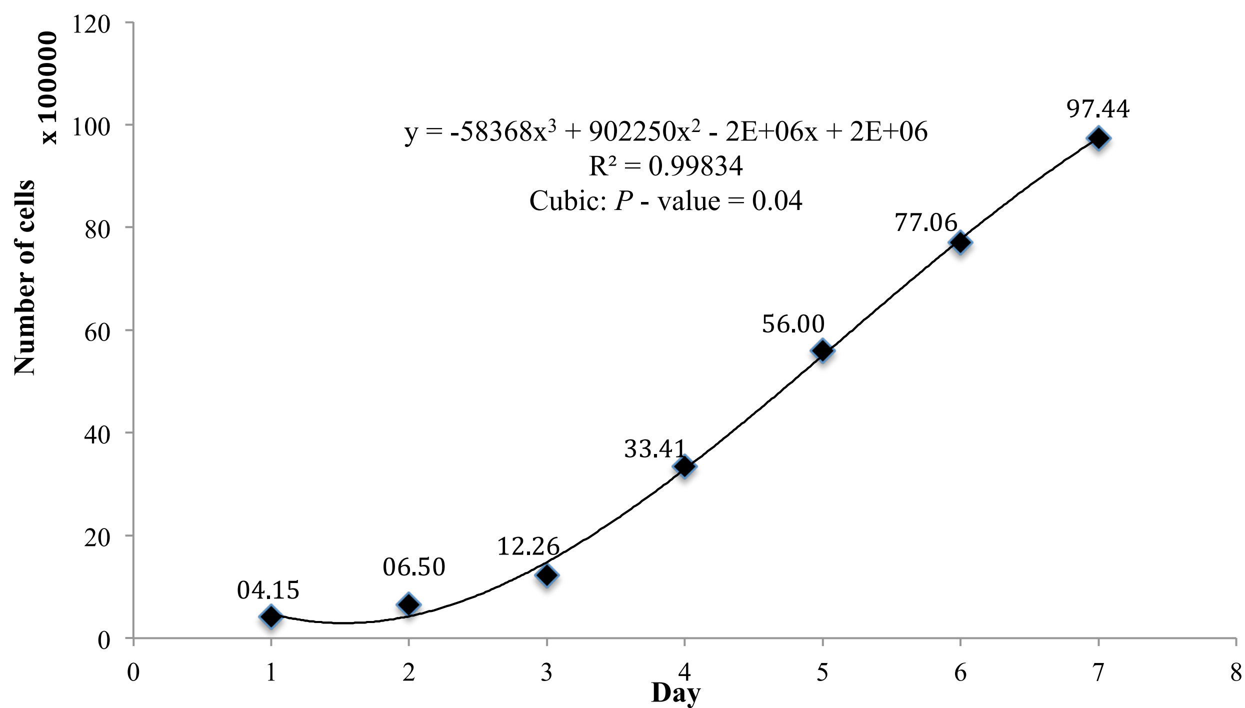

Figure 1. The proliferation curve of IPEC-J2 cells in T-75 flask (7 days). Cell numbers were analyzed by PROC GLM of SAS with flask as the experimental unit. Orthogonal contrasts were used to determine linear, quadratic, cubic and quartic response. Cell numbers on each day represent least square means of two replicates. Regression curve and equation were generated in MS Excel.

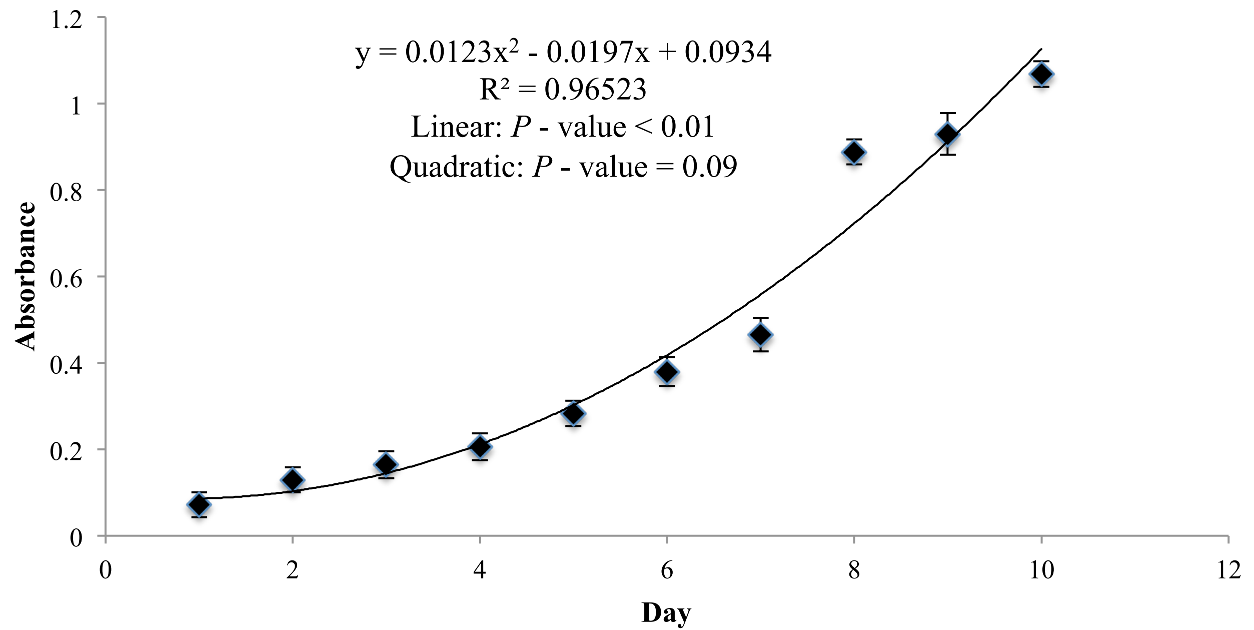

Figure 2. Proliferation curve of IPEC-J2 cells cultured in 96-well plates. The absorbance readings from all wells were analyzed by PROC GLM of SAS, with well as experimental unit. Orthogonal contrasts were used to determine linear, quadratic, cubic and quartic response. Regression curve and equation were generated in MS Excel.

Appendix I. Absorbance of color density of reduced MTT in a 10-d culture of IPEC-J2 cells.

Absorbance									
d1	d2	d3	d4	d5	d6	d7	d8	d9	d10
0.065	0.097	0.209	0.106	0.238	0.435	0.369	0.626	0.938	1.169
0.089	0.161	0.153	0.215	0.378	0.198	0.623	0.732	0.724	1.148
0.092	0.148	0.18	0.166	0.312	0.311	0.316	1.163	1.035	1.119
0.07	0.156	0.134	0.244	0.27	0.308	0.536	1.092	1.016	1.024
0.044	0.105	0.163	0.23	0.264	0.346	0.404	0.988	1.04	1.392
0.054	0.129	0.201	0.252	0.405	0.325	0.557	1.207	0.825	0.912
0.078	0.108	0.176	0.267	0.305	0.31	0.524	0.506		0.929
0.082	0.123	0.208	0.292	0.231	0.358	0.399	0.589		0.785
0.065	0.129	0.152	0.145	0.206	0.461	0.453	1.064		1.086
0.076	0.104	0.153	0.198	0.333	0.649		1.048		0.787
0.099	0.121	0.145	0.188	0.331	0.409		0.98		1.382
0.049	0.108	0.123	0.193	0.299	0.446		1.115		1.205
0.043	0.13	0.144	0.158	0.248			0.825		1.074
0.101	0.109	0.153	0.232	0.273			0.615		0.931
0.083	0.113			0.273			0.956		1.089
0.064	0.226			0.158			0.705		

Each day, one plate was randomly chosen for absorbance evaluation. Medium was removed and MTT reagent was applied to wells followed by 3.5-h incubation under 38°C. MTT reagent was removed, and MTT solvent was added before absorbance was read at a wavelength of 590 nm in a spectrophotometer. The missing data are from wells excluded from evaluation due to unhealthy/abnormal growth, which cells were observed to be dead or didn’t grow during the culture.

Chapter IV. Expression profile of 13 immune-response genes in IPEC-J2 cells stimulated by LPS from *E. coli* (O111:B4)

Abstract

The objective of the current study was to investigate the expression of 13 immune functional genes IPEC-J2 cells challenged with *E. coli.* LPS. Fixed amount of IPEC-J2 cells were seeded into 24 well plates. Four center wells of a plate were administrated with one of four concentrations of LPS (0, 0.1, 1, and 10 μg/mL). Plates, were then randomly assigned to one of five culture times (0, 1, 2, 4, and 6 h) to evaluate the expression levels of GM-CSF, IL-1β, TNF-α, IL-6, IL-8, IL-10, TLR1, TLR2, TLR3, TLR4, TLR6, and TLR8 in confluent IPEC-J2 monolayers amplified by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). There was no interaction between LPS concentrations and culture times among genes ($P > 0.10$). However, GM-CSF, IL-8, and TLR4 were significantly up regulated by increasing LPS challenge time and peaked at 2, 4, and 4 h ($P < 0.05$) respectively. Expression of TNF-α tended to be linearly up regulated by increasing LPS concentration (Linear effect, $P = 0.1$) and tended to be linearly down regulated by increasing culture time (Linear effect, $P = 0.1$). Similarly, TLR2 tended to be up regulated to by increasing LPS challenge time (Quadratic effect, $P = 0.1$), whereas IL-6 was down regulated by LPS challenge (quadratic effect, $P < 0.05$). TLR3 tended to be down regulated after LPS challenge (Quadratic effect, $P = 0.1$). The IPEC-J2 cell line and LPS can be used as a model for evaluation of potential direct fed microbial candidates.

Key words: IPEC-J2, LPS, RT-PCR, gene expression, time course

Introduction

Outside-reared piglets were observed to have improved growth performance compared to pigs grown in traditional facilities. One possible explanation is that early and frequent exposure of pre-weaned pigs to bacteria in soil could enhance the development of the immune system. This exposure might provide a mechanism for enhancing the development of innate immune system in the swine intestinal epithelium, which could reduce the stress of diet and environment changes post-weaning.

The IPEC-J2 cell line was first isolated in 1989 for use in nutrition studies, and subsequently be used in the investigations of immunity. The primary purpose of this study was to determine whether the IPEC-J2 cell line stimulated by *E. coli* LPS is a good model to investigate innate immunity in live pigs. Based on previous knowledge, epithelial cells can secrete immune-related cytokines such as IL-1β, TNFα, IL-6, and IL-8 in response to antigen challenge. In addition, the expressions of TLRs in response to LPS were investigated. The transcriptional profile of immune-related cytokines and TLRs in response to pathogens, can provide insight into how the innate immune system functions. Interaction effects between stimulation time and LPS concentrations were investigated in the IPEC-J2 cell line to determine the optimum combination that produces the strongest immune response.

Material and methods

Cell line and culture condition

The non-transformed continuous IPEC-J2 cell line, which was isolated from mid-jejunum intestinal epithelial cells in a neonatal, unsuckled piglet, was a gift from Dr. Tom Burkey of the University of Nebraska-Lincoln. The IPEC-J2 cells were stored in liquid nitrogen and a new cell culture was started from thawed cell suspensions with a density of 10^6 cells/mL. Resurgent cells were maintained in Dulbecco's modified eagle medium (50% DMEM-50% F-12; American Type Culture Collection, Manassas, VA; ATCC® 30-2006™) supplemented with 1% insulin (Sigma, St. Louis, MO, Cat. No. 11884-1VL), 10% fetal bovine serum (Hyclone, Logan, UT, Cat. No. SH 30070.02), 5ng/mL recombinant human EGF (Sigma, St. Louis, MO, Cat. No. 13247-051), and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, Cat. No. 15140-122). The complete medium was adjusted to pH 7.4 with HCl or NaOH solution. Cells were incubated at 38 °C in a humidified atmosphere with 5% CO_2.

Thawed cell suspensions of passage 42 containing approximately 10^6 cells were transferred to a T-75 flask (Corning Life Sciences, Sunnyvale, CA 94086) mixed with pre-loaded 19 mL pre-warmed full medium. Medium was changed every three days. A confluent cell monolayer was formed at the bottom of the flask after 8 d of culture. A subculture was performed to improve the cell production and to obtain the next generation - passage 43. The IPEC-J2 cells from both passage 42 and 43 were equally separated into two batches and were challenged by LPS on different days, which were used as biological replicates. The monolayer was rinsed with 5 mL trypsin-EDTA solution for 15-30 sec to remove trypsin inhibitors, and then trypsinized with 3 mL of the same solution for 15 min until cells detached from the flask. Once IPEC-J2 cells were detached from the bottom of the flask and separated into individual cells, the hemocytometer technique was used to determine cell density and cell suspensions

were diluted to a final concentration of 3×10^5 cells/mL with antibiotic-free culture medium.

LPS challenge of IPEC-J2 cell line

Cell suspensions of two biological replicates from both passages 43 and 44 containing 3×10^5 IPEC-J2 cells/mL were placed in the center 16 wells of 24-well culture plates at 1 mL per well. Cells were cultured for a week to form monolayers with antibiotic-free medium changed every 3 days. On the day of LPS challenge, the monolayer was rinsed three times with 1 mL of warm PBS before adding 2 mL of antibiotic-free medium. Wells on the same plate were assigned to one of four LPS concentration treatments: 0, 0.1, 1, and 10 µg/mL. Treated wells of IPEC-J2 cells were added corresponding LPS concentrations at 5 µL per well, whereas the same volume of medium was added to the untreated wells (control). Each plate, then was randomly selected to be incubated at one of five culture times (0, 1, 2, 4, and 6 h). During the LPS challenge period, plates were maintained at 38°C in a 5% CO_2 incubator. After each culture period, medium was removed and the cell monolayers were washed twice with 1 mL of warm PBS. A 380-µL volume of TRIzol Reagent (Invitrogen, Carlsbad, CA) was added to the washed cells for total RNA extraction following manufacturer's protocol. Extracted RNA from the same treatment within plates was merged and then transferred to 1.5 mL micro-centrifuge tubes, which were stored at -80°C for further analysis.

Total RNA purification, qualification and quantification

Total RNA samples were thawed from -80˚C freezer and purified by the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer's handbook. The quality of twelve randomly selected RNA samples were evaluated using the Experion™ RNA

StdSens Starter Kit Automated Electrophoresis Station (Bio-Rad, Hercules, CA). The samples for qualification were mRNA from 0/0, 0/10, 1/1, 1/10, 2/1, 2/10 h/μg/mL from both passage 42 and 43. Total RNA concentration from each sample was measured using the Qubit® RNA HS Assay Kit and Qubit® 2.0 Fluorometer (Invitrogen, Grand Island, NY). Concentrations of all total RNA samples were normalized by diluting each sample with nuclease-free water (Qiagen, Valencia, CA) to the concentration of the most dilute sample (42 ng/uL).

Reverse Transcription Quantitative-PCR

The two-step reverse transcription quantitative-qPCR technique was used to detect gene expression by combining reverse transcription of total RNA to its complementary deoxyribonucleic acid (cDNA) and amplification of these cDNA transcripts by PCR. For the first step, total RNA samples were reverse transcribed to cDNA by reverse-transcription PCR in a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Foster City, CA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Foster City, CA). Thermal profile for the RT-PCR was set up as following: initial incubation at 25°C for 10 min, reverse transcription at 37°C for 120 min, inactivation of the reverse transcriptase at 85°C for 5 min, followed by a holding temperature of 4°C.

The resulting cDNA was then amplified in the second step, which is the quantitative real-time PCR (qPCR), performed in the same StepOnePlus™ Real-Time PCR machine, after adding each sample to qPCR assay plates. The qPCR assay plates were 96-well plates containing the primers and fluorescent probes specific for the 13 genes of interest (GOI) and two normalization or reference genes, also known as housekeeping genes (HKG) which have been previously screened from a panel of seven candidate genes (See Appendix I and II). The HKG selected had

consistent and similar quantification cycles (Cq) across treatments, which are required since reference genes serve to correct for differences in RNA sampling. The qPCR assay plates were prepared at the Diet, Genomics and Immunology Lab of the USDA-ARS in Maryland (see Figure 1 for plate layout). Nucleotide sequences of the primers and probes are listed in Table 1. The 20-uL qPCR mix added to each well in the assay plate consisted of 10 µL of iTaq Universal Probes Surpermix (Bio-Rad, Hercules. CA, cat: 172-5134), 9 µL of nuclease-free water, and 1 µL of cDNA sample. HKG and GOI were replicated three times in each plate. The qPCR thermal profile consisted of an initial denaturation of 95 °C for 10 minutes, and 40 cycles of denaturation at 95°C for 15 s followed by annealing and elongation at 60°C for 1 min.

Relative gene expression analysis

Outliers among three technical replicates were removed when calculating the mean Cq. Gene expression level of each target gene was expressed as a relative quantity (RQ), calculated using the formulas modified from Livak (2001) with terminology (e.g. C_q for C_T) standardized according to the MIQE guidelines (Derveaux et al. 2010), as follow:

$$\Delta C_q = C_{q(GOI)} - C_{q(ref)} \quad (1)$$

$$\Delta\Delta C_q = \Delta C_{q(unkn)} - \Delta C_{q(cal)} \quad (2)$$

$$RQ = 2^{-\Delta\Delta Cq} \quad (3)$$

where:

ΔC_q = C_q for each GOI (Cq_{GOI}) normalized to the geometric mean of the two reference genes (Cq_{ref})

$\Delta\Delta C_q$ = ΔC_q of treatment samples (unkn) normalized to the untreated control (0 h culture time and 0 µg/mL LPS).

Statistical analysis

The calculated RQ data were analyzed using a complete randomly block design with cell passage and replication as blocks, and individual assay plate well as the experimental unit. Treatments were a 4×5 factorial arrangement with 4 levels of LPS concentration (0, 0.1, 1, and 10 µg/mL), and 5 incubation times (0, 1, 2, 4, and 6 h). The analysis of variance was determined using the mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with LPS level, incubation time, and LPS level x incubation time interaction as fixed effects, whereas cell passage and replicate from each passage served as random effects in the model. Least square means were separated using the PDIFF option. Due to the uneven intervals in the LPS levels and incubation times, PROC IML of SAS was used to generate the coefficients for polynomial contrast (linear and quadratic). In addition, simple contrasts were generated to compare the impact of genes with or without LPS challenge.

Results

Two genes Actin, gamma (ACTB) and Ubiquitin C (UBC) were chosen as normalization or reference genes, also known as endogenous controls, because their Cq were most similar and had the lowest standard deviations (data not shown), which indicate that they are consistently expressed and were not affected by the treatment conditions (Pfaffl et al., 2004).

Interaction effects between LPS concentration and challenge time

There were no LPS × time interaction effects to the expression of any of the 12 genes (2 reference genes and TLR10 were not analyzed) in LPS challenged IPEC-J2 cells ($P > 0.13$); therefore, only main effects are presented. There was no positive PCR response in TLR10, so

data are not shown. Expression of TNF-α and IL-8 tended to be greater (Table 2; $P < 0.10$) in all LPS-challenged IPEC-J2 cells (average of all treatments of 0.1, 1, 10 µg/mL at all culture times) than non-LPS challenged cells (0 µg/mL at all culture times).

Expression of cytokines in response to increasing levels of LPS

Increasing LPS concentration (0, 0.1, 1, 10 µg/mL) had no effect (Table 2; $P > 0.22$) on gene expressions of GM-CSF, IL-1β, IL-6, and IL-10 in IPEC-J2 cells. However, TNFα tended to be linearly affected by increasing LPS concentration ($P = 0.10$; Figure 2), and IL-8 tended to show a quadratic response (quadratic effect: $P < 0.10$) and gain the greatest expression level when challenged with 1 µg/mL of LPS (Figure 3).

Expression of TLRs in response to increasing levels of LPS

The expression levels of TLR1, TLR2, TLR3, and TLR4 were not affected ($P > 0.22$) by the various LPS concentrations in stimulated IPEC-J2 cells. Expression of TLR8, however, increased linearly ($P < 0.05$) with increasing LPS treatments (Figure 4), and TLR6 tended to increase linearly (Table 2, $P = 0.11$).

Expression of cytokines in response to increasing challenge time

Expression of IL-8 increased quadratically ($P < 0.05$) with challenge time (Table 3 and Figure 5). Expression of GM-CSF (Figure 6) in LPS-stimulated IPEC-J2 cells increased following longer incubation time with the highest amplification at 2 h, and decline with further incubation (Quadratic, $P < 0.01$). Unlike GM-CSF, IL-6 expression was decreased with incremental incubation time with the lowest level at 4 h (Figure 7), and expression gradually

increased backed to a higher level of expression at 6 h (Quadratic, $P < 0.05$). As challenge time increased, TNF-α expression tended to decrease linearly (Figure 8; $P = 0.10$).

Expression of TLRs in response to increasing challenge time

Expression of TLR4 (Figure 9; $P < 0.05$), was affected by challenge time, and tended to show a quadratic response ($P = 0.09$), which peaked at 4 h. Both TLR2 ($P < 0.1$) and TLR6 ($P = 0.10$) tended to be upregulated by increasing challenge time and peaked at 4 and 2 h respectively (Figure 10), and 2 h (Figure 11), respectively. Expression of TLR3 tended to be down regulated post LPS challenge and gained the lowest level at 2 h (Figure 12; Quadratic effect, $P = 0.10$). The RQs of all genes are depicted in Table 3.

Discussion

The IPEC-J2 cells, treated as a model of porcine intestine epithelial cells, have been used in a variety of bacterial infection studies, especially for the purpose of looking for a direct-fed microbial to reduce the usage of antibiotics (Skjolaas et al., 2007; Brown et al., 2004). In order to investigate the metabolism of the bacteria and the interaction, previous studies challenged the IPEC-J2 cells line with pathogenic bacteria including ST and *E. coli* to gain insight on how the pro-inflammatory response, which is mediated by cytokines; and other factors such as the interaction between bacteria density and challenge time have been studied as well (Geens and Niewold, 2010; Burkey et al., 2009).

Cytokines IL-8 and TNF-α can be used to indicate the innate immune response. IL-8, an early produced *neutrophil chemotactic factor,* is responsible for recruiting neutrophils and assisting the inflammatory processes in porcine cells (Scharek and Tedin, 2007). Similar to IL-8 is TNF-α, which can stimulate the expression of receptors on the surface of the epithelium (Stahl

et al., 2003), which attract neutrophils to penetrate into the infected tissue (Scharek and Tedin. 2007). In our study, IL-8 was up regulated in the post-challenge of *E. coli* LPS challenge in the IPEC-J2 cell line, which is consistent with previously reported results (Devrient et al., 2010; Arce et al., 2010; Hermes et al., 2011). In Arce et al. (2010), IPEC-J2 cells were cultured with 1 μg/mL of LPS from ST, and IL-8 peaked at 2 h post-challenge. By using a different species derived LPS, our study showed an increased trend in IL-8 mRNA expression up to 4 h and then started to decrease at 6 h. Another small intestine segment perfusion study described different peak times (2 or 4 hours) of IL-8 gene expression among three isogenic pigs when the jejunum loops were surgically perfused with ST (Hulst et al., 2013).

Expression of IL-8 in IPEC-J2 cells were challenged by increasing LPS level and tended to show a quadratic response with the highest expression when LPS concentration was 1 μg/mL (P = 0.10), as observed in a similar study (Arce et al., 2010). The concentration of *E. coli* LPS used in the current study to challenge IPEC-J2 cells was based on studies testing both human and porcine intestinal epithelial cells, which is supposed to exert a strong response (Geens and Niewold, 2010; Bandyopadhaya et al., 2007). However, a higher level of antigen is expected to induce stronger immune response, and this has been supported by a recent study. Razzuoli et al., (2013) reported that when IPEC-J2 cell line was challenged with LPS at concentrations of 1, 2, and 4 μg/mL for 18 h, an apparent increase in IL-8 production as LPS concentration increased was observed, whereas TNF-α was not significantly different regardless of the LPS concentration. Moreover, both IL-8 and TNF-α mRNA levels were stimulated after culture with LPS for 18 h. Similarly, Skjolaas (2007) reported significant stimulation of IL-8 at the protein level when IPEC-J2 cells were cultured with increasing ST numbers from 10^6 to 10^8 CFU/well, but the antigen was neither LPS nor derived from *E. coli*.

The TNF-α tended to increase linearly when LPS concentration increased in our study. A similar response of TNF-α transcription to increasing LPS concentration from *E. coli* was reported by Borzęchka (2013): LPS at a concentration of 1 μg/mL had the most significant response when compared to the ones challenged with lower concentrations. However, 1 μg/mL of LPS is the highest concentration that was tested in that study, so there is no reference to be consulted for higher LPS response. Similar effects of various LPS concentrations on TNF-α protein secretion were observed in LPS-challenged murine peritoneal macrophages: as LPS density increased from 0 to 100 μg/mL, the expression of TNF-α rose as well (Hoshino et al., 1999). When porcine intestinal epitheliocyte (PIE) cells were challenged with Enterotoxigenic *Escherichia coli* (ETEC)-derived LPS, TNF-α mRNA was not affected, (Moue et al., 2008). However, 1 μg/mL of LPS is the highest concentration that was tested in that study, so there is no reference to be consulted for higher LPS response.

Results of the current study indicated that GM-CSF was up regulated and peaked 2 h after LPS application, which might suggested that LPS is a factor, which stimulates GM-CSF. GM-CSF was constitutionally expressed in IPEC-J2 cells (Mariani et al., 2009; Oswald, 2006; Schierack et al. 2006). One of the most potent stimuli of GM-CSF is LPS, which exerts the signal after binding with TLR4, and subsequently produces cytokines such as IL-1, IL-6 and TNF-α, which then induces GM-CSF expression (Megyeri et al., 1990; Goletti et al., 1996; Parajuli et al., 2012; Hallsworth et al., 1994). Upon oral LPS challenge, GM-CSF protein peaked at 20 h in peripheral blood mononucleated cells (Eggesbø et al., 1995). Increased GM-CSF secretion enhances LPS binding ability through increasing expression of TLR4 and CD14 (Parajuli et al., 2012; Lendemans et al. 2006).

The stimulated expression of IL-8 is also a response to accelerated gene expression of

TNF-α and IL-1β (Lang et al., 2004). Hence, the responses of TNF-α and IL-1β were expected to start earlier than IL-8. This assumption was supported by Arce et al. (2010). Transcripts of TNF-α and IL-1β peaked at 2 h and 1 h, whereas IL-8 showed a linear increase and peaked at 4 h when IPEC-J2 cells were challenged with LPS from ST. In our study, expression of TNF-α mRNA tended to be down regulated after LPS challenge. Despite an unexpected high level of TNF-α in 0 h treatments, TNF-α level peaked at 2 h, and subsequently IL-8 peaked 4 h later, which agreed with previous studies. However, when epithelium of lung tissue was exposed to LPS, TNF-α kept increasing till 24 hours, which was the longest time point evaluated (Elizur et al., 2008).

The IL-6 was observed to be down-regulated in IPEC-J2 cells under LPS challenge in the current study, whereas Parajuli et al. (2012) reported that IL-6 in murine microglia (macrophage-like cells) increased at both the gene and protein levels in response to LPS challenge, a response which had been improved by the additive effect of GM-CSF expression. IL-6 showed a significantly increased expression in PIE cells at 3 h after 5×10^7 cells/mL of ETEC challenge (Moue et al., 2008). However, whole ETEC challenge on epithelial cells includes LPS and flagellin, which initiate both TLR4- and TLR5-dependent immune responses (Hayashi et al., 2001; Hermes et al., 2011; Arce et al., 2010; Devriendt et al., 2010). Purified flagellin derived from *E. coli* can also stimulate the expression of IL-6 and IL-8 in IPEC-J2 cells through TLR5 (Devriendt et al., 2010). In another related study, both IL-6 and IL-1β were down regulated by LPS challenge in IPEC-J2 cells at 18 hours, and IL-8 and TNF-α were still at a high expression level (Razzuoli et al., 2013). IL-6, produced by activated T cells, macrophages, and endothelial cells can promote the differentiation of stem cells to transfer to neutrophils (Abbas et al., 2012; Shi et al., 2006; Khajah et al., 2011). It can be induced by both IL-1 and TNF (Abbas et al.,

2012). In the current study, there was no significant stimulations in either IL-1β and TNF-α by LPS challenge in IPEC-J2 cells, hence, this may be the reason why expression of IL-6 was not up regulated.

Both LPS concentration and culture time did not affect IL-1β, and this was not consistent with the findings of related research (Arce et al., 2010), which used LPS derived from ST rather than *E. coli*. Studies on IL-8 expression showed IL-1β to be the main stimulus for IL-8 as well as TNF-α (Saperstein et al., 2009; Stahl et al., 2003). Other than IL-8 and TNF-α, IL-1β can also stimulate the expression of adhesion molecules on epithelial cells such as ICAM-1 (Stahl et al., 2003). Hence, IL-1β was supposed to be up regulated by LPS challenge. However, from the results, expression of IL-1β numerically increased until 4 h after a slight decline at 1 h, which implied that IL-1β might respond to the LPS but was decreased by the high level of mRNA IL-1β in the 0 h treatment. Previous studies (Schierack et al., 2006; Mariani et al., 2009) have shown that IL-1β and IL-10 were not expressed in IPEC-J2 and IPI-2I cells, which contradicted others (Arce et al., 2010; Zhou et al., 2014).

We failed to observe a significant response of IL-10 in IPEC-J2 cells after LPS exposure. The IL-10 is an anti-inflammatory cytokine that can suppress immune function and is expressed by activated macrophages and dendritic cells. The function is also called homeostasis (Abbas et al., 2012; Crimeen-Irwin et al., 2005; Moore et al., 2001). It is expected to respond to infection later than other cytokines and chemokines such as IL-6, IL-8, IL-1β and TNF-α because overexpression of cytokines, also called cytokine storm or hypercytokinemia, can potentially damage tissues and organs (Murphy et al., 2007; Abbas et al., 2012). IL-10 was found to be slightly down-regulated by *E. coli* exposure on IPEC-J2 cell line (Hermes et al., 2011). Generally, expression of TNF-α, IL-1β, IL-8, and immune-inhibitive cytokines such as IL-10 are

always inversely regulated (Zhou et al., 2014; Hermes et al., 2011).

So far, the results of bacterial challenge on IPEC-J2 cell lines are consistent with other studies in the cytokine production (IL-1β, IL-6, IL-8 and TNF-α), but not TLRs. As current knowledge on the receptors expressed on molecules and their corresponding ligands, most observations of previous studies gained the expected response and can be explained reasonably, however, some studies still exhibit the response from other unrelated TLRs (Wang et al., 2014; Arce et al., 2010).

The TLR4 was up regulated by LPS challenge in this study. LPS binds the TLR4 receptor on the cell membrane to initiate the transcription of immune-related genes, which further stimulates the immune response to protect the cells. Among most bacterial or antigen challenge, this combination achieves one of the strongest responses (Arce et al., 2010). After LPS challenge in IPEC-J2 cells, expression of TLR4 has been reported to significantly increase (Hermes et al., 2011; Arce et al., 2010), and the results from our study support these findings. Transcription of TLR4 increased and peaked at 4 h after LPS challenge, followed by an obvious decline. This expression pattern was similar to the findings of Arce (2010) that mRNA of TLR4 slightly increased and started to be suppressed after 4 h. The same trend was observed in other challenge studies on the epithelial cell lines where Abreu et al. (2001) and Hermes et al. (2011) observed that TLR4 was mildly stimulated by LPS exposure. However, in Hermes's study, the whole bacteria E. coli was used and Abreu challenged the colon epithelial cells instead of the small intestinal cell line.

Expression of TLR2 is responsible for binding a variety of PAMPs including lipoproteins/lipopeptides from gram-positive bacteria or mycoplasmae, mannuronic acid polymers from *Pseudomonas aeruginosa*, zymosan from yeast, and LPS from gram-negative

bacteria Spirochetae (Takeuchi et al., 1999). In both the Arce et al. (2010) and the current study, TLR2 showed a quadratic response and peaked at 3 h and 4 h respectively after LPS challenge, which was not theoretically expected. This circumstance has also been observed in Kaushik's unpublished IPEC-J2 challenge report in 2010. However, it is known that TLR2 does response to gram-negative bacterial (Pridmore et al. 2001). TLR2 expression can be enhanced by increased GM-CSF in neutrophils (Kurt-Jones, et al., 2002), hence another assumption is that TLR2 expression may be stimulated by increased GM-CSF, which peaked at 2 h in epithelial cells (Kurt-Jones et al., 2002).

The TLR3 also tended to be slightly down regulated by LPS challenge up to 4 hours, which is not consistent with Arce et al. (2010) because virus dsRNA is the ligand of TLR3. However, it is depicted that GM-CSF can stimulate the expression of TLR3 in mast cells and epithelial cells (Schaefer et al., 2005), hence we expected the similar mechanism may occurs in IPEC-J2 cell lines. TLR6 had a quadratic response in IPEC-J2 cells and peaked at 2 h following LPS challenge, which is consistent with the recently published literature that TLR6 was stimulated by LPS (ST) challenge on a more stable porcine small intestinal cell line ZYM-SIEC02 (Wang et al., 2014). This cell line was recently designed by introducing human telomerase reverse transcriptase (hTERT) into cells to improve the immortalization and tumorigenes, but still maintained as a non-transformed cell line (Wang et al., 2014). The reason of up-regulation of TLR6 response to LPS challenge has not been fully explained in the literature, but it is undergoing investigation currently (Wang et al., 2014). TLR 8 mRNA was linearly up regulated by LPS challenge in the current study, which is consistent with the study conducted by Arce et al. (2010) that both TLR 4 and TLR 8 responded to LPS challenge in IPEC-J2 cells. ST derived LPS was used in that study. TLR 8 has been described to be important

in resistance to viral infection, but it has been up regulated by gram-negative bacteria challenge in a human monocytic cell line THP-1 (Zarember and Godowsky, 2002).

For those genes (TNF-α, IL-1β, IL-6 etc.) in the current study with amplification levels that did not agree with other findings, one speculation is that the inclusion of fetal bovine serum (FBS) media in LPS challenge (Mariani et al., 2009) altered the response. Serum and antibiotic are the two compounds usually used in the culture medium to moderate the stress of the cells, and serum contains undetermined inhibitory compounds, which may influence basal gene transcription. In the general cell culture of our work, antibiotic and FBS were supplemented but antibiotic was removed from the media in the culture one week before the challenge. However, the same media types were successfully used in previous challenge studies (Burkey et al., 2009; Devriend et al., 2010). Mariani and colleagues (2009) reported that TNF-α, IL-6, and IL-8 had enormous reductions on the gene expression levels in the IPI-2I cell line when cultured in media with FBS compared with an FBS-free medium. However, GM-CSF in IPI-2I was not affected significantly by FBS. Furthermore, there were no obvious FBS effects on gene expression in the IPEC-J2 cell line either (Mariani et al., 2009). Both IPEC-J2 and IPI-2I cell lines were from porcine small intestine, specifically the jejunum and ileum, respectively, but IPEC-J2 cells were isolated from a neonatal pig, whereas IPI-2I is a transformed cell type originating from an adult boar.

Besides swine, LPS can stimulate the synthesis of adaptive-immune-inducing cytokines including IL-1α, -1β, -6, -8, and TNF-α in humans and mice. Several gut sections have been investigated such as colon (Caco-2, T-84, HT-29 human cells), small intestine (IEC-6 murine cells), and small intestinal biopsies (Moue et al., 2008, Kuntz et al., 2009). These cell lines were studied as models for pigs initially to study the secretion patterns due to the absence of a porcine

cell line (Geens and Niewold, 2010). However, gene expression is species-specific although the innate response is to some extent similar and comparable. Because of a shortage of investigations on the porcine innate immune system, and inconsistent results from different research groups, more studies are necessary for exploring the impact of LPS on swine immune function.

Conclusions

The mRNA levels of both cytokines (IL-6, IL-8, GM-CSF, and TNF-α) and TLRs (TLR2, 3, 4, 6, and 8) in IPEC-J2 cells were down- or up-regulated by LPS challenge. The stimulated IL-8, GM-CSF and TLR4 indicated that LPS can elicit an immune response in the IPEC-J2 cell line. Most results in our study are consistent with other studies (except IL-6 and IL-1β), and differences may be due to the LPS concentrations and the LPS challenge times investigated.

Expression at the mRNA level largely affect the protein translation level, which makes the two products comparable, although longer times are expected for protein generation. Moreover, some species including human, swine, and mouse, share a similar innate signal transduction pathway, and this allows comparative studies at various levels. Overall, the IPEC-J2 cell line can be used as a model of live pigs for the bacterial challenge studies, but more investigations are necessary in terms of functions of TLRs and cytokines, both at the mRNA and protein levels.

References:

Abbas, A. K., A. H. Lichtman and S. Pillai. 2012. Cellular and molecular immunology. David L. Baker, MA and Alexandra Baker, MS, CMI. DNA Ilustrations, Inc.

Abreu, M. T., P. Vora, E. Faure, L.S. Thomas, E.T. Arnold, and M. Arditi. 2001. Decreased expression of toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. Journal of Immunol. 167: 1609–1616.

Arce, C., M. Ramirez-Boo, C. Lucena, and J. J. Garrido. 2010. Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from Salmonella typhimurium. Comparative Immunology, Microbiology and Infectious Diseases 33: 161-174.

Bandyopadhaya, A., M. Sarkar, and K. Chaudhuri. 2007. Transcriptional up regulation of inflammatory cytokines in human intestinal epithelial cells following Vibrio cholera infection. FEBS Journal 274: 4631-4642.

Borzecka, K., A. Plociennikowska, H. Bjorkelund, A. Sobota, and K. Kwiatkowska. 2013. CD14 Mediates Binding of High Doses of LPS but Is Dispensable for TNF- alpha Production. Mediators of Inflammation 2013: 824919.

Brown, D. C., M. S. Dirain, M. E. Davis, C. V. Maxwell, Z. B. Johnson, and T. Rehberger. 2004. Feeding Direct-Fed Microbials to Piglets from Lactation Through the Nursery Phase on Nursery Pig Performance. AAES Research Series 535: 11-14.

Burkey, T. E., K. A. Skjolaas, S. S. Dritz, and J. E. Minton. 2009. Expression of porcine Toll-like receptor 2, 4 and 9 gene transcripts in the presence of lipopolysaccharide and Salmonella enterica serovars Typhimurium and Choleraesuis. Veterinary Immunology and Immunopathology 130: 96-101.

Crimeen-Irwin, B., K. Scalzo, S. Gloster, P. L. Mottram and M. Plebanski. 2005. Failure of immune homeostasis – the consequences of under and over reactivity. Current Drug Targets - Immune, Endocrine and Metabolic Disorders. 5: 413-22.

Derveaux S, J. Vandesompele, and J. Hellemans. 2010. How to do successful gene expression analysis using real-time PCR. Methods 50:227–230.

Devriendt, B., E. Stuyven, F. Verdonck, B. M. Goddeeris, and E. Cox. 2010. Enterotoxigenic Escherichia coli (K88) induce proinflammatory responses in porcine intestinal epithelial cells. Developmental and Comparative Immunology 34:1175-1182.

Eggesbø, J. B., I. Hjermann, G. B. Joø, R. Øvstebø, and P. Kierulf. 1995. LPS-induced release of EGF, GM-CSF, GROα, LIF, MIP-1α and PDGF-AB in PBMC from persons with high or low levels of HDL lipoprotein. Cytokine 7: 562–567.

Elizur A., T. L. Adair-Kirk, D. G. Kelley, G. L. Griffin, D. E. deMello, and R. M. Senior. 2008. Tumor Necrosis Factor-alpha from Macrophages Enhances LPS-Induced Clara Cell Expression of Keratinocyte-Derived Chemokine. Am. J. Respir. Cell Mol. Biol. 38: 8–15.

Geens, M. M., and T. A. Niewold. 2010. Preliminary Characterization of the Transcriptional Response of the Porcine Intestinal Cell Line IPEC-J2 to Enterotoxigenic Escherichia coli, Escherichia coli, and E. coli Lipopolysaccharide. Comparative and Functional Genomics 2010: 469583.

Goletti, D., A. L. Kinter, E. C. Hardy, G. Poli, and A. S. Fauci. 1996. Modulation of endogenous IL-1 beta and IL-1 receptor antagonist results in opposing effects on HIV expression in chronically infected monocytic cells. Journal of Immunol 156: 3501-3508.

Hallsworth, M. P., C.P.C. Soh, S.J. Lane, J.P. Arm, and T.H. Lee. 1994. Selective enhancement of GM-CSF, TNF-α, IL-1β and IL-8 production by monocytes and macrophages of asthmatic subjects Eur. Respir. J. 7: 1096–1102.

Hayashi, F., K. D, Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, and D. M. Underhill, A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410: 1099-1103.

Hermes, R. G., E. G. Manzanillaa, S. M. Martín-Orúea, J. F. Péreza, and K. C. Klasing. 2011. Influence of dietary ingredients on in vitro inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic Escherichia coli (K88) Comparative Immunology, Microbiology and Infectious Diseases 34: 479– 488.

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda and S. Akira. 1999. Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Lipopolysaccharide: Evidence for TLR4 as Hyporesponsive to the Lps Gene Product. Journal of Immunol 162: 3749-3752.

Hulst, M., M. Smits, S. Vastenhouw, A. Wit, T. Niewold, and J. Meulen. 2013. Transcription networks responsible for early regulation of Salmonella-induced inflammation in the jejunum of pigs. Journal of Immunol 10: 18.

Khajah, M., B. Millen, D. C. Cara, C. Waterhouse, and D. M. McCafferty. 2011. Granulocyte-macrophage colonystimulating factor (GM-CSF): a chemoattractive agent for murine leukocytes in vivo. Journal of Leukocyte Biology 89: 945-953.

Kuntz, S., S. Rudloff, J. Ehl, R. G. Bretzel, and C. Kunz. 2009. Fodd derived carbonyl compounds affect basal and stimulated secretion of interleukin-6 and -8 in Caco-2 cells. European Journal of Nurtrition 48: 499-503.

Kurt-Jones. E. A., L. Mandell, C. Whitney, A. Padgett, K. Gosselin, P. E. Newburger, and R. W. Finberg. 2002. Role of Toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils.

Blood.100: 1860-1868.

Lang, A., M. Lahav, E. Sakhnini, I. Barshack, H. H. Fidder, B. Avidan, E. Bardan, R. Hershkoviz, S. Bar-Meir, and Y. Chowers. 2004. Allicin inhibits spontaneous and TNF-α induced secretion of proinflammatory cytokines and chemokines from intestinal epitheilial cells. Clinical Nutrition 23: 1199-1208.

Lendemans, S., M. Rani, C. Selbach, E. Kreuzfelder, F. U. Schade, and S. Flohé. 2006. GM-CSF priming of human monocytes is dependent on ERK1/2 activation. J Endotoxin Res. 1: 10-20.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using Real-time Quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods 25: 402-408.

Mariani, V., S. Palermo, S. Fiorentini, A. Lanubile, and E. Giuffra. 2009. Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. Veterinary Immunology and Immunopathology 131: 278-284.

Megyeri, P., J. Sadowska, T. B. Issekutz, and A. C. Issekutz. 1990. Endotoxin-stimulated human produce a factor that induces polymorphonuclear leucocyte infiltration and is distinct from interleukin-1, tumour necrosis factor alpha and chemotactic factors. Immunology 69: 155-161.

Moore K. W., M. R. de Waal, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Journal of Immunol 19: 683-765.

Moue, M. M. Tohno, T. Shimazu, T. Kido, H. Aso, T. Saito, and H. Kitazawa. 2008. Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. Biochimica et Biophysica Acta 1780: 134-144.

Murphy, K., P. Travers, and M. Walport. 2007. Signaling Through Immune System Receptors. Janeway's Immunobiology. (7th ed.). London: Garland. ISBN 0-8153-4123-7.

Oswald, I. P. 2006. Role of intestinal epithelial cells in the innate immune defence of the pig intestine. Veterinary Research 37: 359-368.

Parajuli, B., Y. Sonobe, J. Kawanokuchi, Y. Doi, M. Noda, H. Takeuchi, T. Mizuno, and A. Suzumura. 2012. GM-CSF increases LPS-induced production of proinflammatory mediators via upregulation of TLR4 and CD14 in murine microglila. Journal of Neuroinflammation 9:268.

Pfaffl, M. W., A. Tichopad, C. Prgomet, and T. P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: *BestKeeper* – Excel-based tool using pair-wise correlations. Biotechnology Letters 26: 509-515.

Pridmore, A. C., D. H. Wyllie, F. Abdillahi, L. Steeghs, P. van der Ley, S.K. Dower, and R. C. Read. 2001. A lipopolysaccharide-deficient mutant of Neisseria meningitidis elicits attenuated cytokine release by human macrophages and signals via toll-like receptor (TLR) 2 but not via TLR4/MD2. J Infect Dis. 183: 89–96.

Ruzzouli, E., R. Villa, and M. Amadori. 2013. IPEC-J2 cells as reporter system of the inflammatory control actions of interferon-alpha. Journal of Interferon and Cytokine Research. 33: 597-605.

Saperstein, S., L. Chen, D. Oakes, G. Pryhuber, and J. Finkelstein. 2009. IL-1β Augments TNF-α–Mediated Inflammatory Responses from Lung Epithelial Cells. Journal of Interferon and Cytokine Research. 29: 273-284.

Schaefer, T. M., J. V. Fahey, J. A. Wright, and C. R. Wira. 2005. Innate Immunity in the Human Female Reproductive Tract: Antiviral Response of Uterine Epithelial Cells to the TLR3 Agonist Poly(I:C). The Journal of Immunology 174: 992-1002.

Scharek, L. and K. Tedin. 2007. The porcine immune system – differences compared to man and mouse and possible consequences for infections by *Salmonella* serovars. Berl Munch Tierarztl Wochenschr. 120: 347-54.

Schierack, P., M. Nordhoff, M. Polmann, K. D. Weyrauch, S. Amasheh, U. Lodemann, J. Jores, B. Tachu, S. Kleta, A. Blikslager, K. Tedin, and L. H. Wieler. 2006. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochemistry and Cell Biology 125: 293-305.

Shi, Y., C. H. Liu, A. I. Roberts, J. Das, G. Xu, G. Ren, Y. Zhang, L. Zhang, Z. R. Yuan, H. S. W. Tan, G. Das, and S. Devadas. 2006. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T- cell responses: what we do and don't know. Cell Research 16: 126-133.

Skjolaas, K. A., T. E. Burkey, S. S. Dritz, and J. E. Minton. 2007. Effects of Salmonella enterica serovar Typhimurium, or serovar Choleraesuis, Lactobacillus reuteri and Bacillus licheniformis on chemokine and cytokine expression in the swine jejunal epithelial cell line, IPECJ2. Vet. Immunol. Immunopathol. 115: 299-308.

Stahl, J. L., E. B. Cook, F. M. Graziano, and N. P. Barney. 2003. Differential and Cooperative Effects of TNFα, IL-1β, and IFN on Human Conjunctival Epithelial Cell Receptor Expression and Chemokine Release. IOVS. 44: 2001-2015.

Takeda, K. and S. Akira. 2005. Toll-like receptors in innate immunity. Int. Immunol. 17: 1-14.

Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential Roles of TLR2 and TLR4 in Recognition of gram-Negative and gram-Positive Bacterial Cell Wall Components. Journal of Immunol. 11: 443-45.

Wang, J., G. Hu, Z. Lin, L. He, L. Xu, and Y. Zhang. 2014 Characteristic and Functional Analysis of a Newly Established Porcine Small Intestinal Epithelial Cell Line. PLoS ONE 9(10): e110916. doi:10.1371/journal.pone.0110916.

Zakrzewski, S. S., J. F. Richter, S. M. Krug, B. Jebautzke, I. M. Lee, J. Rieger, M. Sachtleben, A. Bondzio, J. D. Schulzke, M. Fromm, and D. Günzel. 2013. Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. PLos ONE 8(11):e79643. doi:10.1371/journal.pone.0079643.

Zarember K. A, and P. J. Godowsky. 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. Journal of Immunol 168: 554–561.

Zhou, M., J. Zhu, H. Yu, X. Yin, P. M. Sabour, L. Zhao, W. Chen, and J. Gong. 2014. Investigation into in vitro and in vivo models using intestinal epithelial IPEC-J2 cells and Caenorhabditis elegans for selecting probiotic candidates to control porcine enterotoxigenic Escherichia coli. J. Appl. Microbiol 117: 217-26.

Table 1. Primers and probes used for detecting gene expression of the endogenous control, immune related receptors, and cytokines in quantitative-real time polymerase chain reaction.

Gene	GenBank ID	Forward, reverse primers and probes
ACTB	397653	F: GAGATCGTGCGGGACATCA R: GCCGTGGCCATCTCCTG Probe: AGCTCTGCTACGTCGCCCTGGACTTC
UBC	XM_003132870.2	F: GCGCACCCTGTCTGACTACA R: AGATCTGCATCCCACCTCTGA Probe: AGTCCACCCTGCACCTGGTCCTCC
CSF2 (GM-CSF)	NM_214118.1	F: AGCGGCTGTGATGAATGAAAC R: CGCAGGCCCTGCTTGTAC Probe: TGACCCCCAGGAGCCGACATG
TNFα	NM_214022	F: TGGCCCCTTGAGCATCA R: CGGGCTTATCTGAGGTTTGAGA Probe: CCCTCTGGCCCAAGGACTCAGATCA
IL-1B	NM_214055	F: TTGAATTCGAGTCTGCCCTGT R: CCCAGGAAGACGGGCTTT Probe: CAACTGGTACATCAGCACCTCTCAAGCAGAA
IL-6	NM_214399.1	F: AATGTCGAGGCTGTGCAGATT R: TGGTGGCTTTGTCTGGATTCT Probe: AGCACTGATCCAGACCCTGAGGCAAA
IL-8	NM_213867.1	F: CCGTGTCAACATGACTTCCAA R: GCCTCACAGAGAGCTGCAGAA Probe: CTGTTGCCTTCTTGGCAGTTTTCCTGC
IL-10	NM_214041.1	F: TGAGAACAGCTGCATCCACTTC R: TCTGGTCCTTCGTTTGAAAGAAA Probe: CAACCAGCCTGCCCCACATGC
TLR1	NM_001031775.1	F: CATTAAATGAGAGGTCCAAGTGCTT R: AGTGTTTTCAAATTCAACCAGGAA Probe: CCAACTTGTTGTGGGACAAATCCAAGTATTC
TLR2	NM_213761.1	F: TATCCAGCACGAGAATACACAGTTTAA R: CGAGTTGAGATTGTTATTGCTAATATCTAAAA Probe: CATTGGCTTCCCCAGACCCTGGA
TLR3	NM_001097444.1	F: AAAATCTCCAAGAGCTTCTATTAGCAA R: TTGTATTTGATTTGATGACAACTCTAATCTTT Probe: CGTGAAGAACTTGATTTCCTTGGCAATTCTTC
TLR4	NM_001113039.1	F: TGGCAGTTTCTGAGGAGTCATG R: CCGCAGCAGGGACTTCTC Probe: CGGCATCATCTTCATCGTCCTGCAG
TLR6	NM_213760.1	F: GGGCCAACCTTACTAAATTTTACG R: TGCTTTCAACTATTGTTAAATTGTAAATACTG Probe: CCATGTGGAAACAACTTGGAAATGTTTGG
TLR8	NM_214187.1	F: GATACCATTGCGGCGATAATATG R: TTTACCTTGGCTAAGCACACATG Probe: ATGTTGGCTGCCCTGGCTCACC
TLR9	NM_213958.1	F: GAGACCCTGCTGTTGTCCTACAA R: AGTTCCCCCCCACATCAAG Probe: CGCCTGAGGACCTGGCCAATCTG
TLR10	NM_001030534.1	F: CCAGGTATCCTGCACTGAAAGC R: AACATGAATAGCAGCTCTAAGGTTTG Probe: ACGCCTATCCTTGGGCCATTCCA

The sequences of the primers and probes are referenced from gene bank of United States Department of Agriculture. http://www.ars.usda.gov/main/main.htm.

Table 2. LS means of fold change (RQ) of gene expression response to increment levels of LPS challenge in IPEC-J2 cell line.

Genes RQ	LPS				*P* – Value				
	0 µg/mL	0.1 µg/mL	1 µg/mL	10 µg/mL	SEM	LPS	Linear	Quadratic	Non-LPS vs. LPS
GM-CSF	4.93	5.82	6.29	6.77	4.47	0.92	0.60	0.72	0.55
TNFα	0.82	1.66	2.12	2.70	1.20	0.22	0.10	0.31	0.08
IL-1β	0.68	0.90	0.89	0.84	0.22	0.82	0.90	0.60	0.36
IL6	0.42	0.34	0.47	0.40	0.13	0.56	0.98	0.32	0.78
IL8	3.15	4.18	5.82	5.39	2.00	0.25	0.32	0.10	0.10
IL10	0.47	0.38	0.57	0.44	0.12	0.69	0.86	0.34	0.93
TLR1	0.56	0.48	0.70	0.58	0.11	0.53	0.91	0.19	0.82
TLR2	1.07	0.81	1.10	0.69	0.27	0.36	0.19	0.50	0.34
TLR3	0.48	0.35	0.48	0.45	0.06	0.40	0.88	0.48	0.44
TLR4	0.64	0.43	0.71	0.68	0.12	0.31	0.47	0.31	0.77
TLR6	0.89	0.65	0.73	1.16	0.31	0.35	0.11	0.71	0.87
TLR8	0.61	0.65	0.74	0.94	0.28	0.23	0.05	0.55	0.21

IPEC-J2 cells were challenged by LPS at 4 (0, 0.1, 1, and 10 µg/mL of LPS concentrations) × 5 (0, 1, 2, 4, and 6 hours of culture time) treatments. LS means of C_T of each gene was normalized to housekeeping genes ACTB and UBC to calculate the ΔC_T. ΔC_T of all treatments were normalized to the control (0 µg/mL LPS). PROC IML of SAS was used to generate the coefficient for polynomial contrast (linear and quadratic). Simple contrast was generated to compare the impact of genes with or without LPS challenge. Since no significant LPS level by time interaction was observed, treatment interactions are not reported.

Table 3. LS means of fold change of gene expression response to LPS challenge in 5 increment culture times.

	Time					*P* – Value			
Gene RQ	0 hour	1 hour	2 hour	4 hour	6 hour	SEM	Time	Linear	Quadratic
GM-CSF	0.91^{a}	7.78^{bc}	13.12^{c}	4.45^{ab}	3.52^{ab}	3.10	< 0.01	0.67	< 0.01
TNF-α	2.67	1.65	2.54	1.18	1.07	1.24	0.39	0.10	0.92
IL1β	0.81	0.71	0.75	1.28	0.58	0.29	0.16	0.95	0.15
IL6	0.60^{b}	0.55^{b}	0.32^{a}	0.26^{a}	0.32^{a}	0.10	< 0.01	< 0.01	0.03
IL8	0.60^{a}	3.71^{ab}	7.03^{c}	7.07^{c}	4.77^{bc}	1.60	< 0.01	0.01	< 0.01
IL10	0.63	0.32	0.43	0.67	0.27	0.17	0.12	0.40	0.54
TLR1	0.63	0.44	0.53	0.84	0.44	0.29	0.12	0.99	0.29
TLR2	0.65	1.00	1.03	1.13	0.79	0.10	0.46	0.72	0.08
TLR3	0.60	0.40	0.34	0.46	0.41	0.18	0.09	0.27	0.10
TLR4	0.52^{a}	0.43^{a}	0.61^{a}	0.95^{b}	0.58^{a}	0.34	0.05	0.14	0.09
TLR6	0.71	0.74	1.11	1.08	0.64	0.20	0.57	0.99	0.10
TLR8	0.78	0.60	0.66	0.88	0.77	0.18	0.62	0.47	0.88

IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/m) × 5 culture times (0, 1, 2, 4, and 6 hours). LS means of C_T of each gene was normalized to housekeeping genes ACTB and UBC to calculate the ΔC_T. ΔC_T of all treatments were normalized to the control (0 hour). PROC IML of SAS was used to generate the coefficient for polynomial contrast (linear and quadratic). [a.b.c.] Means in the same row without a common superscript differ ($P < 0.05$).

	1	2	3	4	5	6	7	8	9	10	11	12
A	GM-CSF	TNF-α	IL-1β	IL6	IL8	TLR1	TLR2	TLR3	TLR4	TLR6	TLR8	IL-10
B	GM-CSF	TNF-α	IL-1β	IL6	IL8	TLR1	TLR2	TLR3	TLR4	TLR6	TLR8	IL-10
C	GM-CSF	TNF-α	IL-1β	IL6	IL8	TLR1	TLR2	TLR3	TLR4	TLR6	TLR8	IL-10
D	GM-CSF	TNF-α	IL-1β	IL6	IL8	TLR1	TLR2	TLR3	TLR4	TLR6	TLR8	IL-10
E	GM-CSF	TNF-α	IL-1β	IL6	IL8	TLR1	TLR2	TLR3	TLR4	TLR6	TLR8	IL-10
F	GM-CSF	TNF-α	IL-1β	IL6	IL8	TLR1	TLR2	TLR3	TLR4	TLR6	TLR8	IL-10
G	TLR10	TLR10	TLR10	ACTB	ACTB	ACTB	UBC	UBC	UBC	ACTB -ntc	ACTB -ntc	ACTB -ntc
H	TLR10	TLR10	TLR10	ACTB	ACTB	ACTB	UBC	UBC	UBC	UBC -ntc	UBC -ntc	UBC -ntc

Figure 1. Plate layout for qRT-PCR. Two samples were randomly selected to run on the same PCR plate. Triplicates per sample/gene were conducted as technical replicates. ntc: non template control.

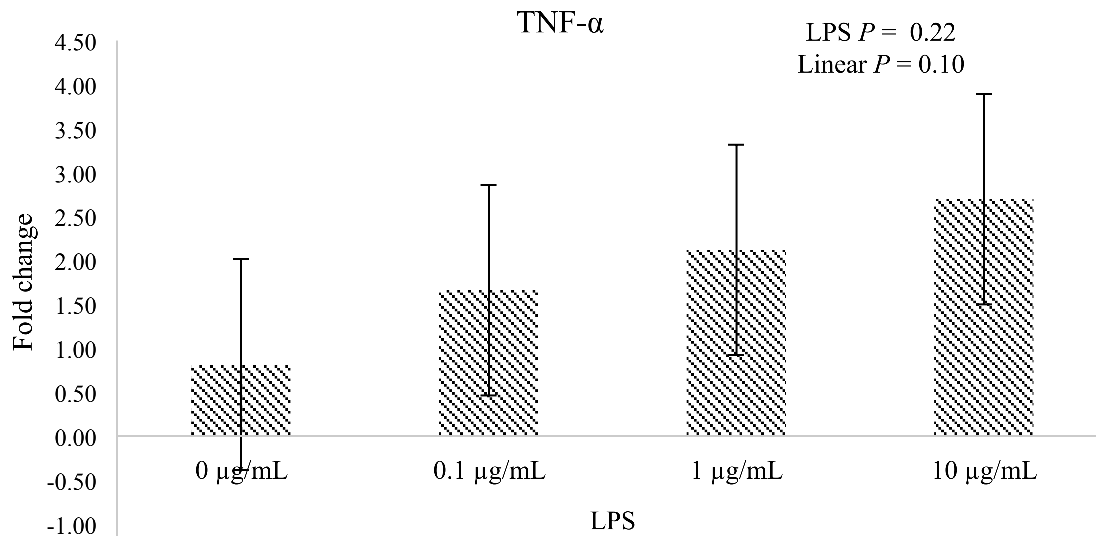

Figure 2. LS means (± S.E.M) of fold change of TNF-α response to incremental levels (0, 0.1, 1, and 10 μg/mL) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/mL) × 5 culture times (0, 1, 2, 4, and 6). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 μg/mL LPS). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with LPS level as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model.

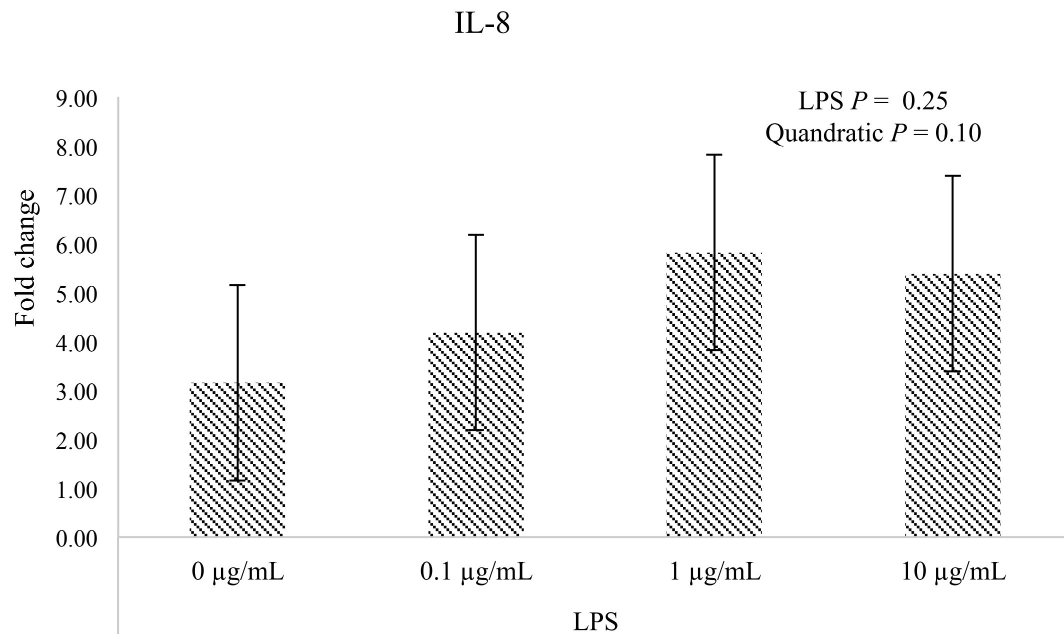

Figure 3. LS means (± S.E.M) of fold change of IL-8 response to incremental levels (0, 0.1, 1, and 10 µg/mL) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 µg/mL) × 5 culture times (0, 1, 2, 4, and 6). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 µg/mL LPS). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with LPS level as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model.

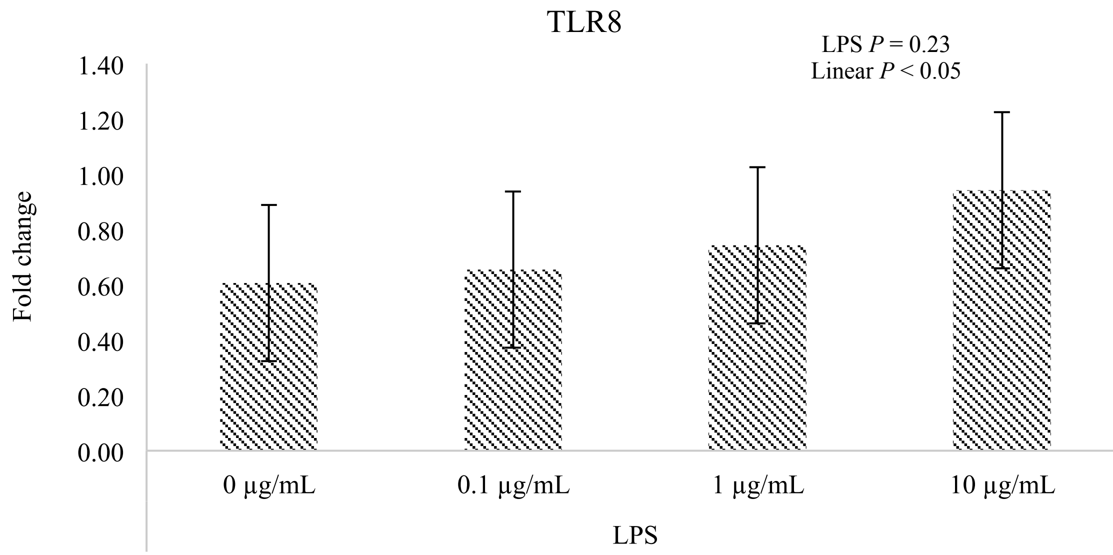

Figure 4. LS means (± S.E.M) of fold change of TLR8 response to incremental levels (0, 0.1, 1, and 10 µg/mL) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 µg/mL) × 5 culture times (0, 1, 2, 4, and 6). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 µg/mL LPS). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with LPS level as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model.

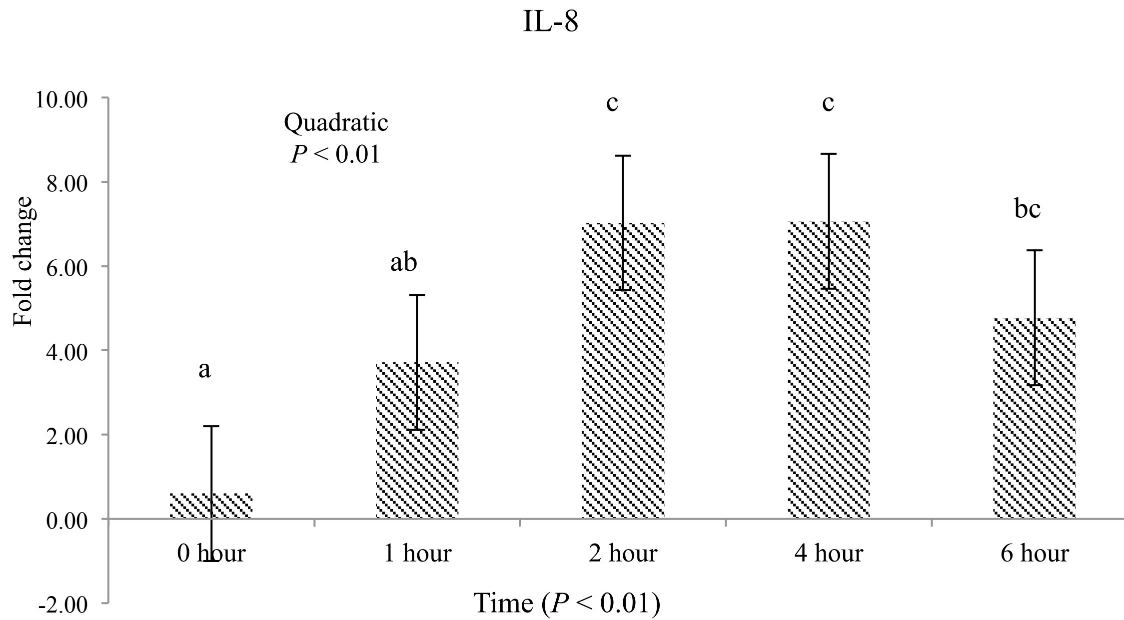

Figure 5. LS means (± S.E.M) of fold change of IL-8 response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model. [a.b.c.] Means without a common superscript differ ($P < 0.05$).

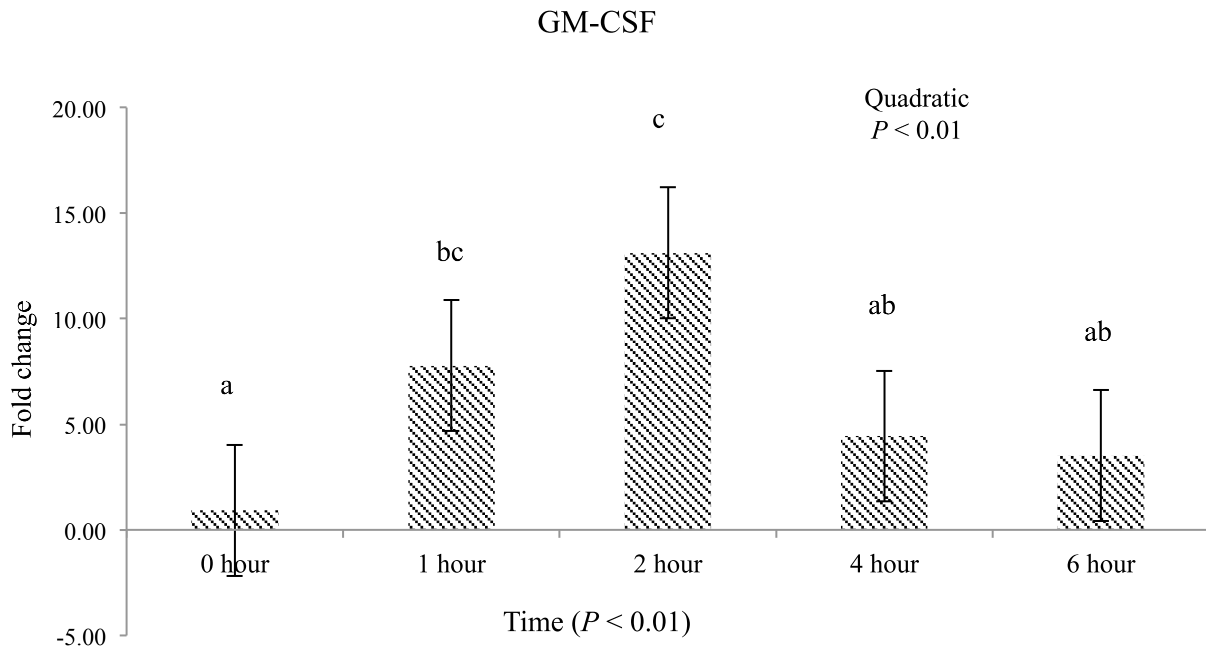

Figure 6. LS means (± S.E.M) of fold change of GM-CSF response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 µg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model. [a.b.c.] Means without a common superscript differ ($P < 0.05$).

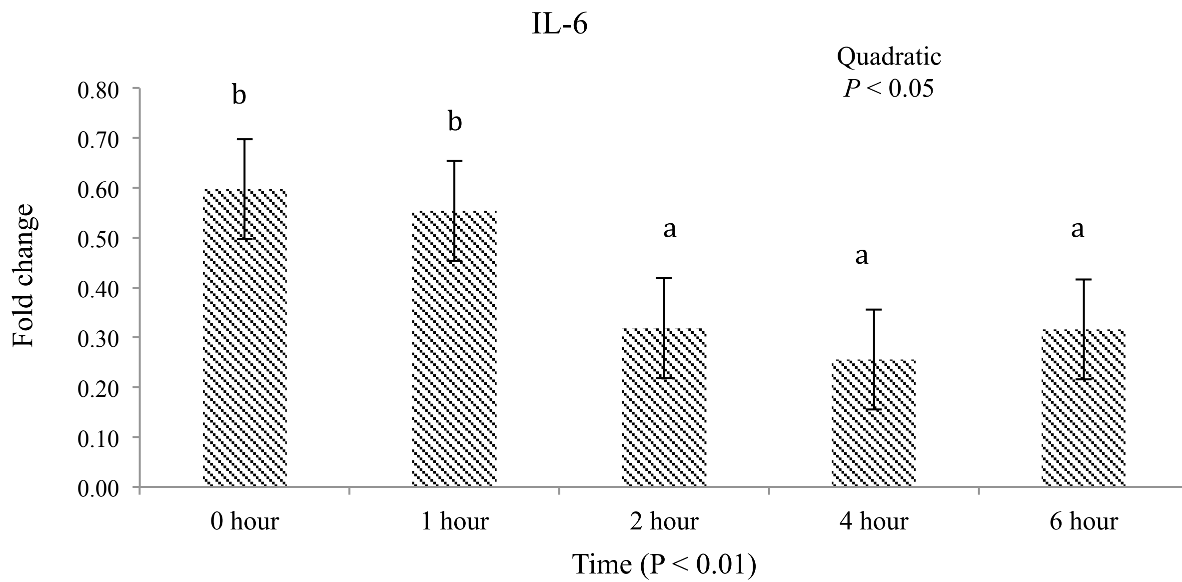

Figure 7. LS means (± S.E.M) of fold change of IL-6 response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model. [a.b.c.] Means without a common superscript differ ($P < 0.05$).

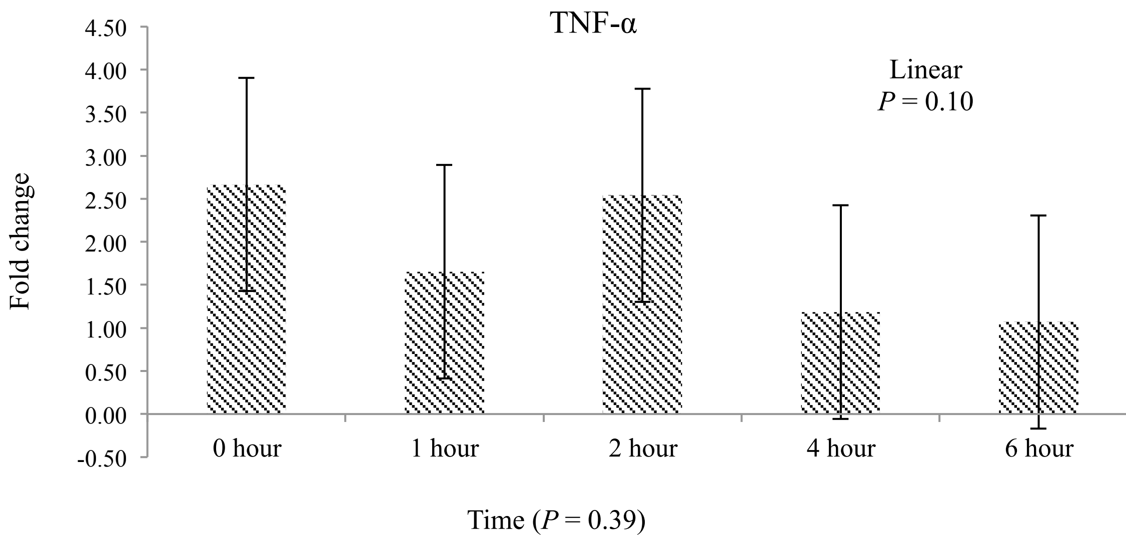

Figure 8. LS means (± S.E.M) of fold change of TNF-α response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model.

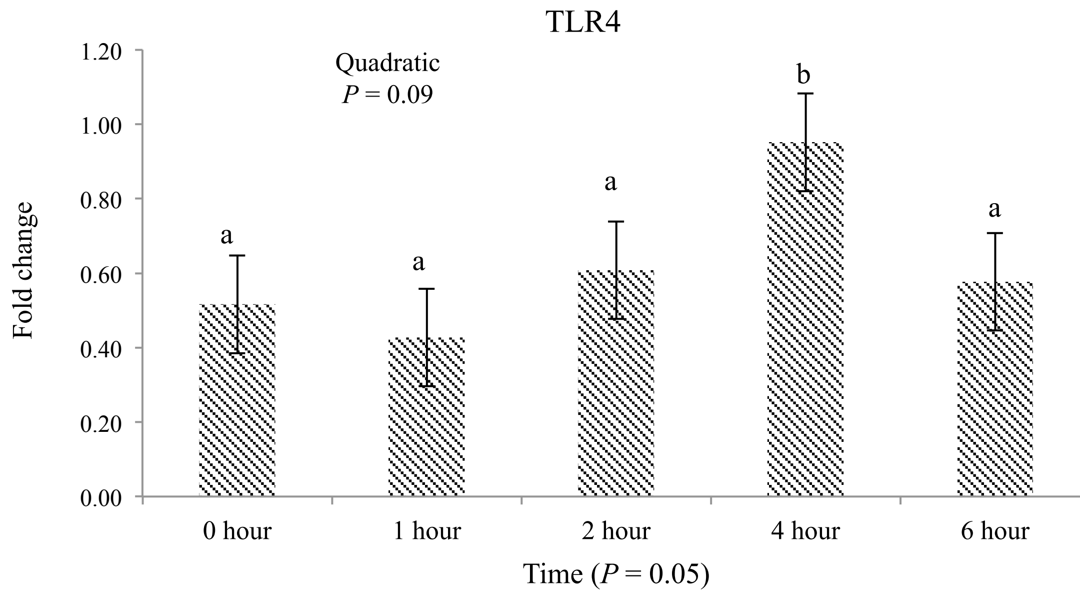

Figure 9. LS means (± S.E.M) of fold change of TLR4 response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model. [a.b.c.] Means without a common superscript differ ($P < 0.05$).

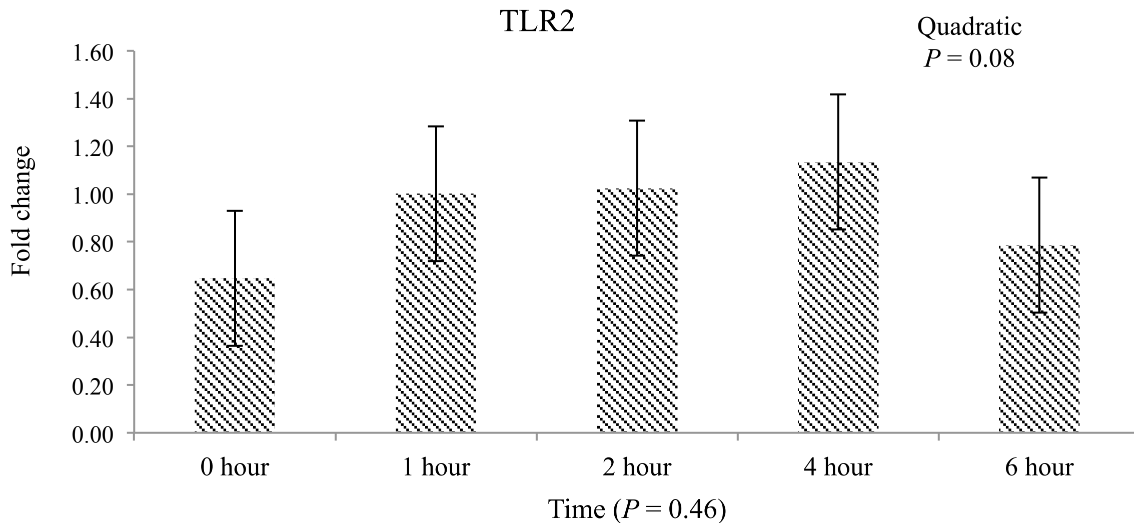

Figure 10. LS means (± S.E.M) of fold change of TLR2 response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model.

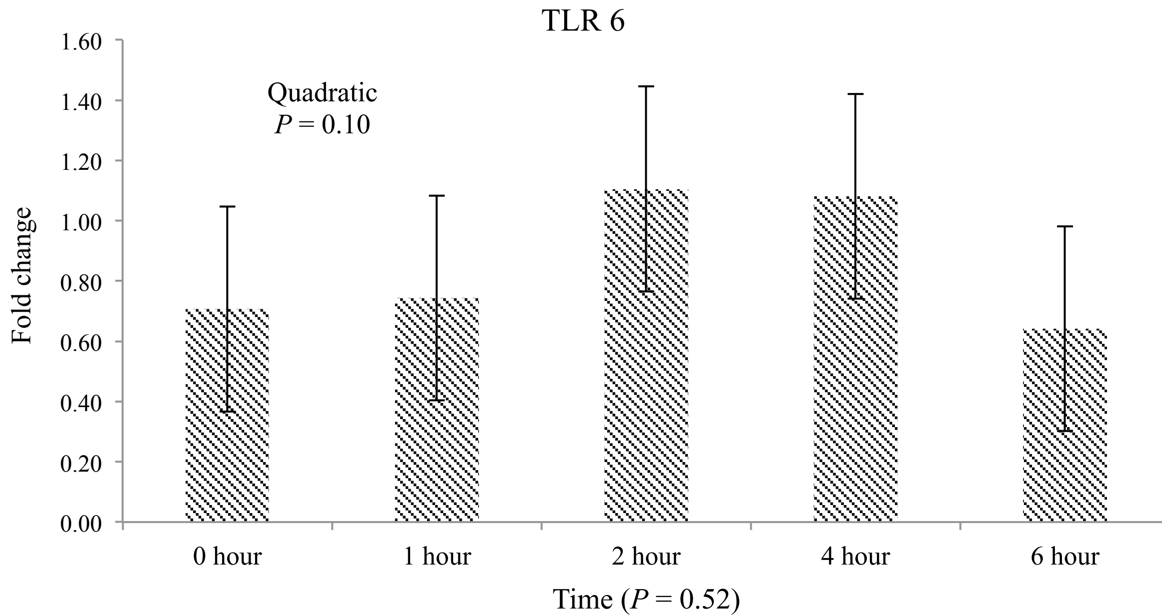

Figure 11. LS means (± S.E.M) of fold change of TLR6 response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 μg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model.

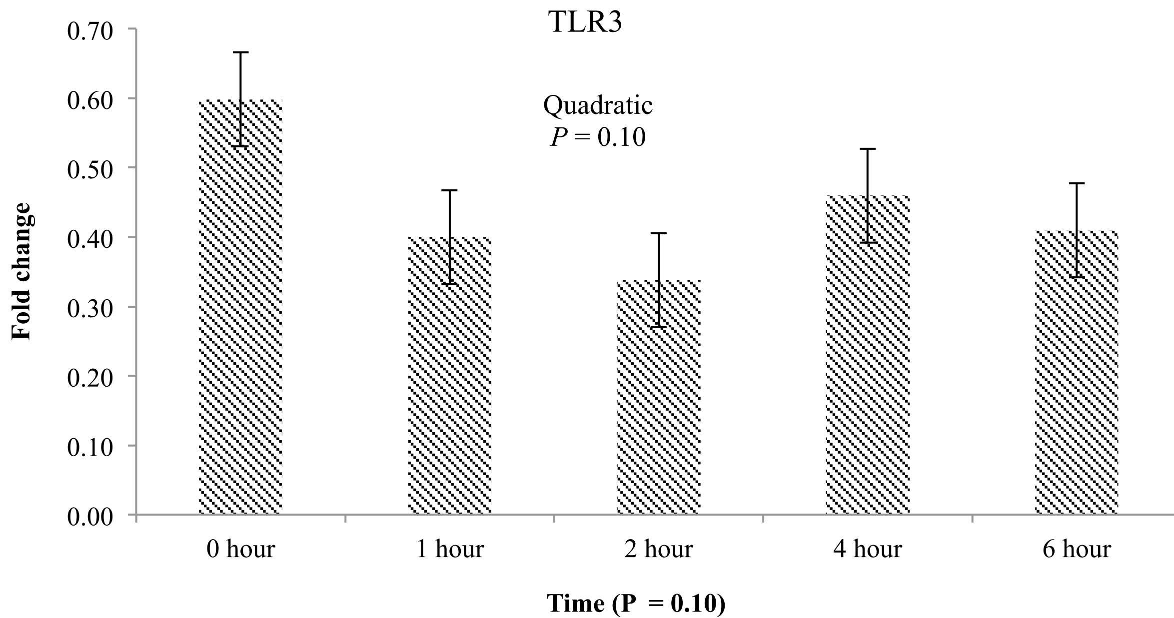

Figure 12. LS means (± S.E.M) of fold change of TLR3 response to incremental culture times (0, 1, 2, 4, and 6 hours) of LPS challenge in IPEC-J2 cell line. IPEC-J2 cells were challenged by LPS at 4 concentrations (0, 0.1, 1, and 10 µg/mL) × 5 culture times (0, 1, 2, 4, and 6 hours). C_T of each gene/treatment/replicate was normalized to geometric mean of two housekeeping genes ACTB and UBC to calculate the ΔC_T. The LS mean ΔC_T for all four biological replicates from all treatments were normalized to the control (0 hour). The analysis of variance was generated with mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with time course as fixed effects, while four biological replicates from two passages (42 and 43) served as random effects in the model.

Appendix I. Screening of reference genes.

Ten cDNA samples at two different concentrations (1:2 and 1:4 dilution) were randomly selected as template in the selection of two reference genes from seven porcine genes: RPL32, ACTB, GUSB, RPL13, UBC, PPIA, and RPLPO. The sequences of the primers and probes are listed in Appendix II. The qPCR assay plates for the screening were prepared at the Diet, Genomics and Immunology Lab of the USDA-ARS in Maryland. The two reference genes were selected based on consistent and similar quantification cycles (Cq) as analyzed using the *Bestkeeper* software.

Appendix II. Primer and probe sequences of seven candidate reference genes

No.	Gene	GenBank ID	Sequences
1	RPL32 Ribosomal protein L32	NM_001001636.1	Primer Forward: p-L32-90F: TGGAAGAGACGTTGTGAGCAA Primer Reverse: p-L32-183R: CGGAAGTTTCTGGTACACAATGTAA Probe: p-L32-126T: ATTTGTTGCACATTAGCAGCACTTCAAGCTC
2	ACTG1 (ACTB) Actin, gamma	397653	Primer Forward:p-ACTG1-3F: GAGATCGTGCGGGACATCA Primer Reverse: p-ACTG1-73R: GCCGTGGCCATCTCCTG Probe: p-ACTG1-28T: AGCTCTGCTACGTCGCCCTGGACTTC
3	GUSB Glucuronidase, beta	NM_001123121	Primer Forward:p-GUS-58F: GCTGCTTACTACTTCAAGATGCTGAT Primer Reverse: p-GUS-140R: CTGGAGCTGGTCACGAAGGT Probe: p-GUS-98T: CCTTGGACCCCTCCCGGCC
4	RPL13A Ribosomal protein L13a	NM_001244068.1	Primer Forward:p-RPL13-78F: GTGGCCAAGCAGGTACTTCTG Primer Reverse: p-RPL13-144R: CAGAAATGTTGATGCCCTCACA Probe: p-RPL13-101T: CCGGAAGGTGGTGGTTGTGCG
5	UBC Ubiquitin C	XM_003132870.2	Primer Forward:p/h-UBC-47F: GCGCACCCTGTCTGACTACA Primer Reverse: p-UBC -126R: AGATCTGCATCCCACCTCTGA Probe: p-UBC-79T: AGTCCACCCTGCACCTGGTCCTCC
6	PPIA Peptidylprolyl isomerase A (cyclophilin A)	NM_214353.1	Primer Forward:p/h-PPIA-463F: GCCATGGAGCGCTTTGG Primer Reverse: p-PPIA-540R: TTATTAGATTTGTCCACAGTCAGCAAT Probe: p/h-PPIA-511 revT: TGATCTTCTTGCTGGTCTTGCCATTCCT
7	RPLP0 Ribosomal protein, large, P0, 60S acidic ribosomal protein P0	NM_001098598	Primer Forward:p-RPLP0-1153F: AAGCCACGTTGCTGAACATGT Primer Reverse: p-RPLP0-1221R: CAAACACCTGCTGGATGATCA Probe: p/h-RPLP0-1177T: ACATCTCCCCCTTCTCCTTTGGGCT

Chapter V: Conclusions

1. IPEC-J2 cells grew faster in T-75 flasks than in 96-well plates, and gained a shorter doubling time, which implies that they need a shorter time to reach confluence.

2. The MTT method is applicable in evaluating the growth pattern of IPEC-J2 cells but exact cell numbers cannot be provided, hence, the traditional method and MTT-optical evaluation can be used in combination in further research.

3. Both cytokines (IL-6, IL-8, GM-CSF, and TNF-α) and TLRs (TLR2, 3, 4, 6, and 8) in IPEC-J2 respond to LPS challenge, as demonstrated by the significantly up-regulation of IL-8, GM-CSF and TLR4. Most results in our study are consistent with other researchers (except IL-6 and IL-1β) although slightly different LPS concentrations and the times of mRNA extraction varied from the LPS challenge.

4. No interactions between LPS concentrations and challenge times were observed for the investigated genes. Various LPS concentrations did not significantly affect the expressions of the investigated genes in our study.

5. Overall, the IPEC-J2 cell line can be used as a model of live pigs for evaluation of potential bacterial direct fed microbial, but more investigations are necessary in terms of functions of TLRs, cytokines and production on both mRNAs and proteins.