University of Kentucky
UKnowledge

Theses and Dissertations--Veterinary Science | Veterinary Science

2012

RHODOCOCCUS EQUI INFECTION AND INTERFERON-GAMMA REGULATION IN FOALS

Lingshuang Sun
University of Kentucky, sunlingshuang@yahoo.com.cn

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Recommended Citation
Sun, Lingshuang, "RHODOCOCCUS EQUI INFECTION AND INTERFERON-GAMMA REGULATION IN FOALS" (2012). *Theses and Dissertations--Veterinary Science*. 7.
https://uknowledge.uky.edu/gluck_etds/7

This Doctoral Dissertation is brought to you for free and open access by the Veterinary Science at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Veterinary Science by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained and attached hereto needed written permission statements(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine).

I hereby grant to The University of Kentucky and its agents the non-exclusive license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless a preapproved embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's dissertation including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Lingshuang Sun, Student

Dr. David W. Horohov, Major Professor

Dr. Daniel K. Howe, Director of Graduate Studies

RHODOCOCCUS EQUI INFECTION AND INTERFERON-GAMMA REGULATION IN FOALS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By

Lingshuang Sun

Lexington, Kentucky

Director: Dr. David W. Horohov, Professor of Immunology

Lexington, Kentucky

2012

Copyright © Lingshuang Sun 2012

ABSTRACT OF DISSERTATION

RHODOCOCCUS EQUI INFECTION AND INTERFERON-GAMMA REGULATION IN FOALS

Rhodococcus equi (*R. equi*) is one of the most serious causes of pneumonia in young foals. The clinical disease is of great concern to breeding farms worldwide due to the impact of mortality on economic losses. While adult horses are resistant to *R. equi*, foals exhibit a distinct age-associated susceptibility. The mechanism underlying this susceptibility in foals is not well understood. Interferon-gamma (IFNγ) plays an important role in the clearance of *R. equi*, but its expression is impaired in neonatal foals. Moreover, the regulation of this age-related IFNγ expression in foals remains unknown. In humans, IFNγ expression has been shown to be regulated by DNA methylation, lymphoproliferation, and influenced by environmental exposure. Therefore, we hypothesized that environmental exposure promotes IFNγ expression through regulation of DNA methylation and lymphoproliferation. The objectives were: (1) to estimate the relevance of IFN-γ production and *R. equi* infection in foals; (2) investigate the role of lymphoproliferation and DNA methylation in the regulation of IFN-γ expression in foals; (3) to evaluate the effect of environmental exposure on IFN-γ expression by housing foals in a barn environment verses pasture.; (4) to investigate the effect of environment exposure on antigen-presenting cells (APC), which sensor the environmental antigens and modulate IFN-γ production by T cells. The results demonstrated that the IFN-γ expression was inversely correlated with the age-related susceptibility to *R. equi* infection. lymphoproliferation promoted IFN-γ expression in foals, whereas, DNA methylation repressed IFN-γ expression. The IFN-γ expression was augmented in foals exposed to the barn air which contained higher numbers of aerosol miroorganisms. DNA on the IFN-γ promoter was demethylated and the lymphoproliferative activity was elevated in foals with barn-air exposure. The barn-air exposure also promoted the maturation and activation of APC to prime IFN-γ expression by T cells in foals. Overall, this body of work demenstrated a relationship between IFN-γ expression and *R. equi* infection, provided novel information on mechanisms that regulate IFN-γ expression, and identified the effect of environment on mechanisms responsible for IFN-γ expression.

KEY WORDS: Foal, Interferon-gamma, Regulation, *Rhodococcus equi*, Environment

Lingshuang Sun

December 12, 2012

RHODOCOCCUS EQUI INFECTION AND INTERFERON-GAMMA REGULATION IN FOALS

By

Lingshuang Sun

David W. Horohov
Director of Dissertation

Daniel Howe
Director of Graduate Studies

December 12, 2012

This doctoral dissertation is dedicated to my beloved family members:

Father Chengsong Sun

Mother Shaohua Xu

Sister Lingyun Sun & Yuanyuan Sun

Brother Yunce Sun

ACKNOWLEDGMENTS

The completion of my doctorate would not have been possible without the support, patience and guidance of many people that I have met and worked with during my graduate studies. It is a pleasure to convey my gratitude to everyone in my humble acknowledgment.

I owe my deepest gratefulness to Dr. Dvid W. Horohov for his advice, direction, and generous support throughout my studies. He opened the doors for me to join the program and care for me the entire time. His way of scientific thinking and teaching inspired me and enriched my growth as a student, a researcher and a scientist. I am indebted for all his contributions of time and ideas which helped to make my graduate experience productive and stimulating. I sincerely appreciate the years spent under his guidance.

I would like to convey my sincere gratitude to my other Ph.D dissertation committee members, Drs. Frank Cook, Thomas Chambers, Don Cohen, and the outside examiner, Dr. James Bryson, for their time, insightful questions and valuable discussions during my exam as well as the committee meetings. Without the support of Dr. Cohen, the APC projects would not have been completed.

Thank you to all my collaborators: Dr. Alan T. Loynachan (LDDC, University of Kentucky) for all the pathological work on *R. euqi* study, Zhangbin Gong (Shanghai University of Traditional Chinese Medicine) for contribution to the DNA methylation project.

I also appreciate the support received from all of our lab members. Particularly, I am indebted to Dr. Amanda A. Adams for her guidance and scientific contribution. Without her valuable organization, I could not have completed the environmental project.

I would also like to thank Drs. Catherine Merant, Tracy Sturgill, and Cormac Breathnach for their leading and training. The appreciation would also address to Dr. Allen Page for his work on almost all the BAL. I am grateful to our technicians, especially Alejandra Betancourt. I am indebt to her for her always willing to help and wonderful management of the lab. I would also like to thank the great help I got from our undergraduate student, especially Eric J. Oberst and John Craig Stewart. It was an honor to work with Macarina Sanz and Chong Liu. Everyone was great!

I gratefully appreciate the farm crew for their great job on taking care of the foals. In particular, I would like to thank Lynn and Kevin for their organization and management of the farm crew. I would also appreciate John Allen and Millard Stamper for their taking care of the pony foals, without them the environmental study wouldn't complete. The appreciation is also sent to all the foals for their contribution to the studies.

I would like to thank Dr. Daniel Howe for his support as the Director of Graduate Studies. I appreciate all the faculty and staff in the department, particularly to the members of Drs. Macleod, Chambers, Timoney, Balasuriya, and Troedesson's laboratories for allowing me use their equipment and instructing me on how to operate everything. I would like to extend my appreciation to all the graduate students in Gluck for their help and company. Special appreciation would like to address to Stevephen, Debi Even, Elizabeth Woodward and Ablesh Gautam for their work as a representative of our department and their organization of graduate activities. All this made my life colorful.

I would also like to thank all of the administrative staff in the Department of Veterinary Science, especially Diane Furry, Patsy Garrett, Roy Leach, Debbie Mollet,

and Gail Watkins who were always ready to help and made being a graduate student in the department pleasant.

I gratefully acknowledge the funding sources that made my Ph.D. work possible: Bioniche Life Science Inc.,William Robert Mills Endowment and United States Department of Agriculture. The Wright Markey Trust Fund is greatly acknowledged for providing my personal financial stipend.

Last, but not the least, I would like to thank all of my family members for their unconditional love, trust, and encouragement. Thanks to my dad, Chengsong Sun; my mom, Shaohua Xu; my sister Lingyun Sun and Yunyuan Sun; and my brother Yunce Sun, for all the support and love since I was little.

TABLE OF CONTENTS

Aknowledgements iii
Table of Contents vi
List of Tables viii
List of Figures ix
Chapter One - Introduction & Literature review 1

Introduction 1
Literature review 5
Immunity development in foals 5
Respiratory immunity and *R. equi* infection in foals 17
Regulation of IFN-γ expression 37

Research Objectives 53

Chapter Two - Age-associated susceptibility to *R. equi* infection in foals 54

Summary 54
Introduction 56
Materials and methods 58
Results 65
Discussions and conclusions 74

Chapter Three - The role of proliferation in the regulation of interferon gamma (IFN-γ) expression in foals 80

Summary 80
Introduction 81
Materials and methods 82
Results 85
Discussions 97

Chapter Four - The hypermethylation of IFN-γ gene promoter is correlated with IFN-γ expression in the neonatal foals 101

Summary 101
Introduction 102
Materials and methods 103
Results 109
Discussions 116

Chapter Five - The effect of environment on interferon-gamma production in neonatal foals 120

Summary 120
Introduction 121
Materials and methods 122
Results and discussions 125

Chapter Six - The effect of environment on lung antigen presenting cells in foals 135

Summary 135
Introduction 136
Materials and methods 138
Results 145
Discussions and conclusions 158

Chapter Seven - Conclusions and Directions 166
References 169
Vita 187

LIST OF TABLES

Table 1.1. Immunoglobulin in foal serum 9
Table 2.1. Primers and probes sequence 63
Table 2.2. Age-associated susceptibility to *R. equi* challenge in foals 66
Table 2.3. The correlation between age of foal and its susceptibility to *R. equi* infection 66
Table 2.4. The correlation of basal gene mRNA expression with the age of foals at challenge and the susceptibility to *R. equi* infection.....69
Table 2.5. The correlation of *ex vivo R. equi*-stimulated gene mRNA expression with age of foals at challenge and the susceptibility to *R. equi* infection 72
Table 3.1. Proliferative index by lymphocyte subsets for foals, adult and old horses88
Table 4.1. MSP primer sequences.....107
Table 5.1. BAL cell recoveries from foals.....127
Table 6.1. Primers and probes sequence 141

LIST OF FIGURES

Figure 1.1. The strategy of intracellular bacteria to escape the cellular killing.....23
Figure 1.2. Immunity to intracellular bacterial infection.....29
Figure 1.3. Production of RNI and ROI.....32
Figure 1.4. Functions of IFN-γ.....38
Figure 1.5. Model of transcription regulation of IFN-γ gene expression.....40
Figure 2.1. The same level of specific antibody to VapA was observed post *R. equi* challenge in the younger and the older foals. 67
Figure 2.2. The basal cytokine gene expression *in vivo* in peripheral blood from foals 69
Figure 2.3. The *ex vivo R. equi*-stimulated peripheral blood gene expression in the foals 71
Figure 2.4. *In vitro* PMA- or *R. equi*-stimulated IFN-γ expression by lymphocytes in BAL or PBMC from foals.....73
Figure 2.5. Correlation of age and *in vitro* PMA- or *R. equi*-stimulated IFN-γ expression by lymphocytes in BAL and PBMC from foals 74
Figure 3.1. The IFN-γ expression by ConA-stimulated PBMC from foals and adult horses 86
Figure 3.2. Titration of ConA on PBMC proliferation.....88
Figure 3.3. Proliferative Indices of lymphocytes from foals, adult and old horses.....90
Figure 3.4. The percentage of IFN-γ^+ cells in each generation of dividing $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes 92
Figure 3.5. The effect of hydroxyurea (HU) on proliferation (A, B) and IFN-γ production (C, D).....95
Figure 3.6. IFN-γ production by HU-inhibited lymphocytes from foals increases over time 96
Figure 4.1. Sorting $CD4^+$ cells 104
Figure 4.2. Verification of specificity of MSP primers.....110

Figure 4.3. DNA hypermethylation in the promoter region of IFN-γ in neonatal foals and its correlation with mRNA expression and age.....112
Figure 4.4. The activity of total DNMTs was higher in foals 113
Figure 4.5. Environment effect on lymphoproliferation and IFN-γ expression in foals 115
Figure 4.6. Environment effect on DNA methylation in *Ifng* promoter.....116
Figure 5.1. Culturable microorganism in the air samples from barn and pasture 126
Figure 5.2. IFN-γ production by BAL cells. 129
Figure 5.3. Percentage of IFN-γ^+ cells in PBMC 132
Figure 6.1. Identification of lung DC and Mϕ from BAL.....140
Figure 6.2. Environment effect on surface molecules of lung Mϕ.....147
Figure 6.3. Elevated percentage of MHC II^+ Mϕ in Poly (I:C) - stimulated BAL cells from foals with barn-air exposure.....148
Figure 6.4. Elevated frequency of DC and frequency of $CD1b^+$ and $CD86^+$ DC in BAL cells form foals placed in the barn 150
Figure 6.5. Elevated frequency of $CD83^+$ DCs in PAMP - stimulated BAL cells from barn-air exposed.....152
Figure 6.6. Mϕ and DC favor Th1-priming in foals with barn-air exposure.....154
Figure 6.7. Elevated chemokine gene mRNA expression by BAL cells from barn-air exposed foals.....155
Figure 6.8. Elevated Th1, Th2 and Th17 cytokine mRNA expression in foals with barn-air exposure 156
Figure 6.9. Elevated Th1, Th2 and Th17 cytokine mRNA expression in foals with barn-air exposure 157
Figure 6.10. Elevated Th1, Th2 and Th17 cytokine mRNA expression in foals with barn-air exposure 158
Figure 6.11. The effect of environment on APC in the lung.....164

CHAPTER ONE

Introduction & Literature Review

INTRODUCTION

The fetal immune system develops in a sterile and protected environment, and therefore lacks antigenic experience. Soon after birth, the newborn is exposed to the "hostile world" of bacteria, viruses, fungi, and parasites. The exposure to these microbes, on the one hand, challenges the health of the neonates which requires the body to immediately defend itself; on the other hand, sharpens the maturity of the immune response in neonates (Ygberg and Nilsson, 2012). In humans, the immunologic competence of the neonate progresses rapidly in the first three months of life (PrabhuDas et al., 2011). Likewise, the immunity is immature in neonatal foals and the immunity develops over time (Giguere and Polkes, 2005a). However, the effect of environment on the maturation of the immunity in foals is completely unknown.

The immaturity of the immune response in foals is considered to be responsible for their susceptibility to bacterial and viral infection, such as *Rhodococcus equi* (*R. equi*). *R. equi*, an intracellular bacteria, is one of the most important causes of high morbidity respiratory disease in foals, causing great concern to breeding farms (Machangu and Prescott, 1991). It inflicts a significant economic impact on the horse industry, both in the Americas and worldwide (Prescott, 1991). The costs of treatment and foal losses from infection on farms with endemic disease can be substantial. Thus, it is very important to understand what part of the naivety of the neonatal immune response is responsible for this susceptibility.

While both humoral and cell-mediated immune response play an important role in the defense against *R. equi* infection, the cellular immune response is pivotal in clearance of intracellular pathogens. Interferon-gamma (IFN-γ) is a critical cytokine for innate and adaptive cell-mediated immunity against intracellular bacterial infections. It promotes T and B cell differentiation, activates cytotoxic T cell activities, and enhances microbicidal function of macrophage (Mϕ). However, IFN-γ expression is impaired in neonates of most species (Vuillermin et al., 2009a), including foals (Breathnach et al., 2006b). This reduced expression is associated with an increased risk for intracellular bacterial infections, such as those caused by *R. equi*. However, the underlying regulatory mechanism of IFN-γ expression in foals remained to be investigated.

The expression of IFN-γ mRNA correlates with the protein production in foals, indicating that its expression is regulated at the transcriptional level (Breathnach et al., 2006b). The mechanism underling this impaired IFN-γ expression is unknown in foals. Gene transcription begins with the transcription factors (TF) binding to the elements on the promoter region, followed by recruitment of RNA polymerase and initiation of transcription. Studies in humans and mice have shown that transcription regulation of IFN-γ gene is controlled at two levels: the availability of TFs, and the accessibility of the elements in the promoter region (Jones, 2012; Wilson and Merkenschlager, 2006). The DNA methylation suppressively regulates gene expression which creates the "closed" structure of chromatin and prevents the TF accessibility (Wilson and Merkenschlager, 2006). On the contrary, the "open" structure and demethylation of DNA indicates an active transcription of the gene, which can be induced through lymphocyte proliferation and differentiation (Wilson and Merkenschlager, 2006). These inheritable changes of

gene expression is derived from DNA modification or chromatin remodeling rather than changes in the DNA sequence, termed epigenetic regulation.

The epigenetic regulation of IFN-γ expression is postulated to be induced by environmental exposure (Kuriakose and Miller; Miller and Ho, 2008; Vuillermin et al., 2009a). IFN-γ expression in the early life of infants is influenced by exposure to environmental microbes (Gereda et al., 2000b; Roponen et al., 2005a). However, the underlying mechanisms leading to this effect remain to be unveiled. The specific antigen-presenting cells (APC) recognize environmental antigens and present these to T cells which primes and modulates the IFN-γ expression by the T cells (Paul, 2003). It was recently found that exposure to various environmental antigens promoted the function of APC in mice and rats (Debarry et al., 2007; Peters et al.; Vogel et al., 2008b). And the *in vitro* stimulation with environmental microbial components appeared to activate and promote the maturation of APC in foals (Flaminio et al., 2007; Merant et al., 2009b). However, the effect of environment on the maturation of APC in foals is unknown.

This chapter provides basic background describing the development of immunocytes and respiratory immunity to *R. equi* infection in foals, and the regulation of IFN-γ expression. The overall hypothesis of the following chapters is that environmental exposure promotes IFN-γ expression through regulation by DNA methylation and lymphoproliferation. The specific aims of the following chapters were to:

1) Evaluate the relevance of IFN-γ expression and the age-associated susceptibility to *R. equi* in foals (Chapter 2).

2) Characterize the role of proliferation on the regulation of IFN-γ expression in foals (Chapter 3).
3) Identify the role of DNA methylation on the regulation of IFN-γ expression in foals (Chapter 4)
4) Estimate the effect of environment exposure on IFN-γ production (Chapter 5).
5) Investigate the environment effect on APC phenotype and function (Chapter 6).
6) Conclusions and Future Directions (Chapter 7).

LITERATURE REVIEW

Immunity development in foals

The immune response of the horse is composed of both innate and adaptive systems to defend against infections. However, the immune response of neonatal foals is born immature compared with that of adult horses, like in other species (Tizard, 2009). The inability to mount an adult-like immune response is responsible for the susceptibility to many pathogen infections in neonates. Studies in humans indicate that the immunity of infants undergo a gradual progression toward maturation. Likewise, the foal immune system gradually undergoes maturation with exposure to an abundant and diverse population of microbial components in the environment. These maturation events include the expansion of immunocytes such as lymphocytes; and a massive increase of immunological molecules produced by those cells, such as immunoglobulins and cytokines. To promote the maturation procedure, as well as to promote IFN-γ expression, the postnatal development of the immunocytes in foals needs to be illustrated.

A wide range of distinct cell types comprise the immune system, each of which plays an important role. The lymphocytes engage in a central role in adaptive immunity since they are the cells that determine the specificity of immunity-specific antibody production, cytokine secretion, and cytotoxicity. It is their response that directs the effector limbs of the immune response, humoral immune response and cell-mediated immune response. Other types of cells interact with lymphocytes either in the way of antigen presentation or mediation of immunologic functions. These cells include: cells that eliminate invaders and initiate adaptive immunity during innate immunity, such as

granulocytes (neutrophils, eosinophils, and basophils); and cells that present antigens to lymphocytes, such as dendritic cells (DC) and monocytes (Mo)/macrophages (Mϕ).

Lymphocytes

Previous studies in the foal suggest an age-related maturation of lymphocytes (Flaminio et al., 2000b). The appearance of lymphocytes in the peripheral blood of the fetus begins at about 90 days, presumably circulating to populate the various lymphoid tissues from early developed thymus (Mackenzie, 1975). The majority of lymphocytes are present in peripheral blood by day 120, and their proliferative response to Pokeweed Mitogen (PWM) is low at this time. The significant immune response of B cells to PWM can be obtained by day 140 (Perryman et al., 1980).

Foals are born with comparable lymphocyte counts to that of adults in peripheral blood (Flaminio et al., 2000b; Smith et al., 2002). The absolute number of lymphocytes increase significantly over time after birth, with an increase of 2.5 times by the third month of age (Flaminio et al., 2000b). The changes in total lymphocyte populations reflect an antigenic stimulation of lymphocyte proliferation and differentiation following environmental exposure (Berek and Ziegner, 1993). The age-related development of lymphocytes in fetus and foals are reviewed as follows, including B and T cells.

B cells and immunoglobulin

B cells are a pivotal type of cell for humoral immune response in term of immunoglobulin (Ig) secretion, including IgA, IgG, IgM, IgD and IgE. In adult horses, IgG is the principal plasma Ig, representing 80% of the total Ig repertoire. Four

subclasses of IgG have been identified by this point, IgGa (IgG1), IgGb (IgG4/7), IgGT (IgG3/5) and IgGc (IgG6) (Wagner, 2006).

During equine fetal development, the B cells delvelope to become immunologically competent. B cells progress from the liver to the bone marrow, and onto the spleen by 90-120 days of gestation (Butler et al., 2009; Sinkora et al., 1998; Tallmadge et al., 2009). Among all types of Ig, only IgM can be detected in the fetal spleen around 90-120 days of gestation (Tallmadge et al., 2009) by which point it obtains the ability of class switching at this time. Specific responses to utero vaccination have been detected in equine fetuses around 180-200 days of gestation (Martin and Larson, 1973; Mock et al., 1978; Morgan et al., 1975). Meanwhile, the memory of B cells are possibly developed (Tallmadge et al., 2009). Therefore, the capacity of B cells to respond to stimulation is considered to be competent at birth (Giguere and Polkes, 2005a).

After birth, the B cell counts in the blood and the proportion in peripheral blood mononuclear cells (PBMC) increase significantly until 3 months of age (Cebulj-Kadunc et al., 2003; Flaminio et al., 2000b; Smith et al., 2002). The numerical increase of peripheral B cells reflects a B cell expansion as a response to environmental exposure (Butler and Sinkora, 2007). The distribution of subtypes of B cells also exhibits an age-association. Foals are born with a majority of IgM^+ B cells and limited IgD^+ and IgA^+ B cells in the spleen (Sheoran et al., 2000; Tallmadge et al., 2009). Until 1-3 months of age, B cells in germinal centers present large numbers of IgM^+, $IgGa^+$, $IgGb^+$, $IgGT^+$ B cells and very rare IgA^+ B cells, whereas in adult horses all IgG types of B cells and IgA^+ B cells are abundant in germinal centers (Tallmadge et al., 2009). Similarly, in lymph nodes, there are a majority of IgM^+ B cells in neonatal foals, some $IgGa^+$ and $IgGb^+$ B cells and

very few $IgGT^+$ and IgA^+ B cells, whereas adult horses have an abundance of all types of B cells (Tallmadge et al., 2009). In addition, IgE^+ B cells appear in peripheral blood only in foals 2.5-5 months old and they present in lung and skin tissue at this time point (Marti et al., 2009; Wagner, 2006, 2009).

The capacity of endogenous Ig generation by B cells also exhibited an age-dependant phenomenon. In the serum of pre-suckle neonatal foals, IgM concentration is 3 times lower, total IgG concentration is 300 times lower, IgGa concentrations are 70 times lower and IgGb concentration 120 times lower, compared to adult horses (Tallmadge et al., 2009). The onset of endogenous Ig is also delayed and exhibits an age-related increase phenomenon. In addition, the endogenous serum IgE is detectable until 5-6 months in foal peripheral blood (Marti et al., 2009; Wagner, 2006, 2009).

While B cells are immunologically competent, their production of endogenous Ig or antibodies is defective in neonatal foals, though increasing gradually over time. Thus the passive immunity transferred from the mare is critical for foals to conquer most infections. Given the epitheliochorial placentation of mares, maternal Ig is rarely transferred to the fetus. The transfer of passive immunity is mainly through colostrum. The concentrations of Ig in colostrum rank as IgG (IgGb> IgGa> IgGT)>IgA(negligible within 12 to 24hrs) > IgM (Sheoran et al., 2000). Neonatal absorption of immunoglobulin occurs within 2 hrs aftrer birth by unselective pinocytosis through specialized enterocytes at birth rapidly. The maternal immunoglobulins can be detected within 4-6 hrs. The IgGa peaks at 18 -24 hrs in the foals. Thereafter, the maternal immunoglobulins decrease gradually in the foals, and disappear after 1 month for IgA and IgM, and 6 month for IgGa, IgGb and IgGT (Table 1.1).

With the decrease of immunoglobulin from passive transfer and the gradual increase of endogenous immunoglobulin over time in foals, the serum IgM production reaches the adult level within 1 month of age; the IgG, IgGT, and IgA catch the adult level at age 3 months, followed by adult-level of IgE in foals of 8 months old. However, the production of IgGb by B cells in foals catches up with adult level by 1 year of age (Table 1.1).

Table 1.1 Immunoglobulin in foal serum

Immunoglobulin	IgG			IgA	IgM	IgE
	IgGa	IgGb	IgGT			
Half-life (days)	18	32	21	3-5	3-5	?
Disappearance of maternal antibodies (months)	6	6	6	1	1	0
Reach adult level at age (months)	3	12	3	3	1	8

Reference: (Wagner et al., 2006); (Giguere and Polkes, 2005a; Sheoran et al., 2000; Tallmadge et al., 2009)

Antibodies play a critical role in resistance to bacterial infection by neutralization and opsonization; the period of lacking the protection provided by maternal and endogenous antibodies may be, in part, responsible for the increased susceptibility of foals to infections. The phenomenon that the nadir of serum IgG concentration in foals from age 2-3 months precedes a period of increased incidence of bacterial respiratory infections (Hoffman et al., 1993) indicates a lack of serum IgG and may contribute in part to the susceptibility to infection because the serum IgG typically increases during this period (Astier et al., 1999). This inability of B cells to produce these Ig, even though B cells were born with immunocompetent, is proposed to be caused by the incompetence of

another type of lymphocyte, T cells, which is required for maturation of B cells and differentiation of plasma cells (Giguere and Polkes, 2005a).

T cells

T cells are an essential component of the cell-mediated adaptive immune response. The helper and cytotoxic T cells are the two main types of effector T cells protecting against infections by means by cytokine secretion and lysis of infected cells. They are characterized of specific antigen recognization by TCR and CD4 or CD8. The CD4 T cells are responsible for coordinating the immune response to intracellular pathogens and for providing help to B cells, whereas CD8 T cells lyse the pathogen infected cells (Paul, 2003).

The functional T lymphocytes are present in equine fetus by day 100 of gestation and significant responses can be obtained by day 140, represented by proliferation in response to phytohaemagglutinin (PHA) stimulation (Perryman et al., 1980). After birth, with environmental exposure, T cells undergo a cell expansion as that in B cells. The absolute number of T cells in peripheral blood from foals increases with age (Flaminio et al., 1999; Smith et al., 2002), although the proportion of T cells in PBMCs stays consistent in foals over time (Flaminio et al., 1999). The numerical increase of the T cells may be mainly due to the increase of CD8 T cells, since the proportion of CD8 T cells increases nearly 5-fold by the fourth month of age, whereas CD4 T cells remain constant with age (Flaminio et al., 2000b). The significantly higher proliferative activity of the peripheral T cells in response to concanavalin A (ConA) in the neonatal foals compared to the adults (Baker et al., 2011; Flaminio et al., 2000b; Sun et al., 2012) also reflects a possible T cell expansion in foals in response to environmental microbes.

The function of T cells can also be evaluated over time by their cytokine production. Cytokines are potent regulators of innate and adaptive immunity. The expression of IFN-γ, IL-4, IL17 and IL-10 represent the function of the type 1 helper T (Th1), type 2 helper T (Th2) and type 17 helper T (Th17) cells, and regulatory T cells (Treg), respectively (Paul, 2003). The impaired expression of all these cytokines by peripheral lymphocytes in response to mitogen in neonatal foals (Breathnach et al., 2006a; Nerren et al., 2009a; Wagner et al., 2010) compared to adults indicates a universally impaired helper T cell function in foals.

Among all the cytokines, the production of IFN-γ is thought to be an indicator of cell mediated immune response. The level of IFN-γ in serum from neonatal foals is extremely low compared with that of adults (Rizos et al., 2007). The mitogen-stimulated IFN-γ expression in peripheral blood lymphocytes also appears defective in neonatal foals, (Breathnach et al., 2006a). Moreover, the specific IFN-γ expression is not detectable in PBMCs with *in vitro* pathogen stimulation in neonatal foals. The *in vitro* IFN-γ expression is also negligible in neonatal foals with pathogen challenge compared to adult horses (Butler et al., 2006; Paillot et al., 2005; Paillot et al., 2007). Similar age-related limited IFN-γ expression is also found in humans (Gasparoni et al., 2003), bovines (Horiuchi et al., 2007) and pigs (Butler and Sinkora, 2007). This impaired IFN-γ expression is considered to be responsible for foals' susceptibility to viral and intracellular bacterial infection (Breathnach et al., 2006a).

The IFN-γ expression by mitogen-stimulated cytotoxic T lymphocytes (CTLs) also shows an impaired function in neonatal foals compared to adults (Wagner et al.,

2010). In addition, the specific IFN-γ expression by CD8 T cells in response to pathogen stimulation is reduced in neonatal foals. The reduced level of specific IFN-γ expression suggests an impaired cytotoxicity of CD8 T cells (Paillot et al., 2005). The *in vitro* stimulated cytotoxicity of CD8 T cells is not detectable in neonatal foals (Patton et al., 2005). This cytotoxic T-lymphocyte mediated lysis (CTL) starts to appear in foals at 3 weeks; it increases but still remains deficient in 6-week-old foals; the significant CTL activity appears in 8-week-old foals (Patton et al., 2005). The impaired cytotoxicity of CD8 T cells in foals is also indicated by the reduced cytotoxicity of lymphokine-activated killer (LAK) cells in yearlings compared with adult horses (Liu et al., 2011a). The lack of LAK cytotoxicity in foals is also reflected by the impaired expression of Granzyme B (GrzB), which induces apoptosis of infected cells (Liu et al., 2011a).

There are other subtypes of T cells, such as Natural Killer T (NKT) cells and γδT cells. NK cells, like NKT cells, are capable of rapid induction and secretion of IFN-γ upon infection as effector cells of innate immunity (Schoenborn et al., 2007). The γδT cells are the first line of defense and bridge between innate and adaptive responses (Holtmeier and Kabelitz, 2005). However, the information on development of these cells in foals is limited. The proportion of γδT cells in peripheral blood of cows and pigs is extremely low in neonates compared with adults. The numbers of these cells in horses may likewise be limited in foals.

Antigen-presenting cells (APC)

The activation of lymphocytes is primed and modulated by APC, which function as a bridge linking innate and adaptive immune response. The APC process and present antigens from the environment to T cells, which then direct the pattern of adaptive

immune response toward either Th1, Th2, Th17 or Treg by communication with T cells via surface molecules and production of cytokines (Paul, 2008).

There are two types of specific APCs, including Mϕ (in tissue) and DCs which are derived from blood Mo moving into tissues. The DC is a critical specific APC, playing a key role in the innate as well as specific immune response. It acts as a sensor for potentially dangerous microbes, either by directly recognizing microbial components or by receiving signals formulated by the innate immune system that is exposed to microbes. DCs decode and integrate such signals and ferry this information to adaptive immune cells. Thus, the type of adaptive immune responses is highly dependent on the nature of the activating stimuli that DCs receive from the innate immune system (Paul, 2003). To elicit anti-microbial immunity, DCs undergo a complex process of maturation, from an antigen-capturing phenotype to an antigen-presenting phenotype, such as the loss of endocytic/phagocytic receptors; up regulation of costimulatory molecules, such as CD40, CD80, and CD86; translocation of MHC class II (MHC II) compartments to the cell surface; and secretion of cytokines that differentiate and polarize the attracted immune effectors (Paul, 2008). While the DC play the key role in induction and modulation of adaptive immune response, the phenotype of neonatal DCs is different from adults and the function of antigen presentation is immature in neonatal humans and mice, such as lower expression of costimulatory molecules on DC surface and lower cytokine expression in response to stimulation (Willems et al., 2009b). However, the analysis of neonatal DCs in horses was limited by the antibodies availability. The similar immaturity and phenotype difference was also observed by study of monocyte derived DCs (MoDCs) (Merant et al., 2009b), although the MoDCs in foals express similar levels of

costimulatory molecules on the cell surface, such as CD40 (Flaminio et al., 2009), and comparable cytokine expression such as IL-12, IL-15 and IL-18 (Flaminio et al., 2007; Flaminio et al., 2009; Merant et al., 2009b). It was observed that in foals versus adult horses: (1) the antigen-presenting associated molecules, such as MHC II, CD1b and $CD1b^{+}/CD86^{+}$ on MoDCs were lower (Flaminio et al., 2009; Merant et al., 2009b); (2) the cytokine production was reduced, such as TNFα (Merant et al., 2009b); (3) responses to multiple stimuli, such as LPS and CpG-ODN, is impaired as no IL-12 and IFN-α is produced (Flaminio et al., 2007). Additionally, the blood Mo in neonatal foals exhibits a biased IL-10 expression in response to IFN-γ and LPS stimulation compared to that of adults (Sponseller et al., 2009a).

Mϕ, derived from Mo is an important antigen scavenger in tissue. Aside from sustained and repeated phagocytic activity, compared with neutrophils, Mϕ can process and present the antigens to the lymphocytes (Paul, 2003). The effective killing of bacteria, fungi, protozoa, some helminthes and tumor cells by phagocytosis of Mϕ is dependent on sustained production of nitric oxide (NO) by synthesizing inducible nitric oxide synthase (iNOS) and respiratory burst (Paul, 2003). The development of Mϕ in the fetus has not been studied in the horse. In pigs, the Mo and Mϕ are developed during the second stage of gestation in the liver, and they are phagocytic functionally competent prenatally (Rehakova et al., 1998). In contrast, the phagocytosis and chemotactic migration of Mϕ are markedly impaired in neonatal foals compared with that of adults. MHC II and CD1b expression on the neonatal Mo derived Mϕ is extremely low though they have comparable CD86 expression compared with that of adult horses (Flaminio et al., 2007; Pargass et al., 2009). The Mϕ in neonatal foals cannot respond to CpG-ODN

stimulation in terms of IL-12 and IFN-α production though they have the same level of TLR9 expression compared to adults (Flaminio et al., 2007).

Granulocytes

There are three types of granulocytes in the blood. Neutrophil is the majority, composing about 95% of all the granulocytes in the blood (Tizard, 2009). It can migrate to the infection tissue, and be activated by cytokines and/or chemokines. It kills pathogens through degranulation and phagocytosis via oxidative burst and release of chemokines and lysozyme (McTaggart et al., 2001). The cytokine and chemokine production in response to stimulation also contributes to the induction or promotion of cascade inflammation and adaptive immune response. Cytokine production by neutrophils in neonatal foals is different from adult horse, with lower IL-23, IL-12 and IL-18; and higher IL-6 and IL-8 with *R. equi* stimulation (Liu et al., 2009a; Nerren et al., 2009a). However, migration (Morris et al., 1987; Wichtel et al., 1991) and oxidative burst (McTaggart et al., 2001) responses of neonatal neutrophils are similar to adult horses. While the phagocytosis capacity of neutrophils in neonatal foals is mostly likely to be competent at birth (Morris et al., 1987), their *in vivo* opsonic phagocytosis is lower (Wichtel et al., 1991). The deficiency is caused by reduced serum opsonic capacity (Flaminio et al., 2000b; Grondahl et al., 1999; Hietala and Ardans, 1987b) due to reduced neonatal complement activity (Bernoco et al., 1994; Lavoie et al., 1989). The higher expression of complement receptor (CD18) on neutrophils may be a means of compensating for lower levels of neonatal serum opsonins (Grondahl et al., 1999).

In basophils, IgE expression is very low even in 6 month old foals (Wagner et al., 2003). Its production of IL-4 is also significantly lower than that of adult horses (Wagner

et al., 2010). Little is known about the eosinophils and mast cells in the foal compared with that of the adult horse.

Respiratory immunity and *R. equi* infection in foals

Respiratory immunity in foals

All newborns are challenged by the environment after birth. The respiratory tract is the second largest front-line exposure of the body to environment (Tizard, 2009). In horses, the respiratory tract is particularly at risk for infection, because large air volumes pass through the respiratory tract of the horse (100,000 L per 24 hrs in an adult horse). However, the immune system of the respiratory tract in foals is functionally immature and mostly develops after birth (Mair et al., 1988b). This may contribute to a high rate of mortality in foals due to respiratory disease, especially diseases caused by intracellular pathogenic infections, such as *R. equi*. The function of respiratory immunity is based on prevention of inhaled particle penetration via the epithelial barrier; elimination of particles by innate immune responses, such as Mϕ, and induction of adaptive immune responses to pathogenic particles by lymphocytes in lymphoid tissues. The large particles inhaled (>10 micron) are mostly trapped by the nasel turbinates, which act as a first line of defense for the lungs (Tizard, 2009). The rest of large and some smaller particles (>5 micron) are actively moved by the cilia and mucus that lines the airways from terminal bronchioles back to trachea. Most of the microorganisms are killed by the antimicrobial molecules in the mucus, including lysozyme, surfactant proteins, and lactoferrin (Paul, 2008). Very small particles (<5 micron) that reach the alveoli are ingested by alveolar Mϕ, which kill the pathogenic particles and move them back to the bronchoalveolar junction and for clearance by the flow of mucus. The particles caught by alveolar DC stimulate the DC, which then migrate to lymph nodes and initiate or activate the adaptive immune response, including antibody-mediated immune response by

activation and differentiation of B cells, and cell-mediated immune responses by activation of T cells (Paul, 2008; Tizard, 2009). IgA is mainly secreted in the upper regions of the respiratory tract probably protects by means of immune exclusion, whereas IgG, secreted in bronchioles and alveoli, act by means of immune elimination (Tizard, 2009). Small particles may also penetrate alveoli, which are then cleared to the draining lymphoid tissue where the adaptive immune response may also be induced (Tizard, 2009).

While the mucosa-associated lymphoid tissue (MALT) plays an important role in the protection of the respiratory tract and in the expression of local lung immunity (Mair et al., 1987a, 1988a; Mair et al., 1987b, 1988c), the MALT in the respiratory tract is incomplete in foals (Mair et al., 1988a). The number and competence of immune cells is impaired in foals compared with that of adult horses. The development of the local MALT in the respiratory tract and the immunocytes in foals' lung are reviewed as follows.

MALT in respiratory tract

In adult horses, MALT in the respiratory tract is composed of nasal-associated lymphoid tissue (NALT), pharyngeal tonsils, larynx (LALT), trachea-associated lymphoid tissue (TALT), and bronchus-associated-lymphoid tissue (BALT) (Liebler-Tenorio and Pabst, 2006). The appearance of the MALT begins in the fetus and gradually develops until 2 years of age, as determined by an acetic acid fixation method (Mair et al., 1987a, 1988a; Mair et al., 1987b, 1988c). The first appearance of single isolated lymphoid nodules in fetuses occurs as early as 9 months gestation at the vestibule, nasal cavity, nasophaynx, and LALT (Mair et al., 1988a). The number of nodules shows a marked increase after birth and reaches the adult level at 2 years old. In

addition, the nasopharyngeal tonsil forms the largest single mass of lymphoid tissue in the respiratory tract at all ages. However, BALT is not present in fetuses and neonatal foals, and is only found in 2-year-old foals (Blunden and Gower, 1999; Mair et al., 1988a). In adult horses, a few organized lymphoid nodules and predominately unorganized infiltrates of closely packed lymphocytes are seen in small intrapulmonary bronchi (Mair et al., 1987a). In thoroughbred horses, organized lymphoid tissues and infiltrates of lymphocytes are also virtually absent in the lung of the neonates by an histological study (Blunden and Gower, 1999). In addition, the lymphoid follicles are seen in bronchi and bronchioles in foals aged 8-22 weeks (Blunden and Gower, 1999). This age-associated distribution of mucosal lymphoid nodules reflects a gradual maturation of the respiratory immunity in foals. The occurrence of the nodules at specific sites within the tract and the areas where inhaled antigens accumulate (Mair et al., 1988a) suggests an influence of environmental exposure on this development. This age-related and exposure-associated development of respiratory immunity is also found in rabbits (Bienenst.J et al., 1973).

Immunocytes in the lung

Not only dose the distribution of nodules in the foals exhibit an age-related development, but also the maturity of the immunocytes in the lung appears to exhibit an age-dependent development. Besides being located in MALT, lymphocytes are distributed diffusely throughout the lung: in the walls of the airways, the mucus, parenchymal tissues and alveoli. Current understanding of foals' lymphocytes in the lung is mostly based on a technique called bronchial alveolar lavage (BAL). Alveolar lymphocytes compise about 40% of the total BAL cells in adult horses (Tizard, 2009),

compared to about 4-6% in neonatal foals (Balson et al., 1997b). Both the absolute number and the frequency of lymphocytes in BAL fluid has been well documented to increase over time but remains low in foals at 10 weeks (Balson et al., 1997b; Flaminio et al., 2000b; Zink and Johnson, 1984), as in the blood (Flaminio et al., 2000b). The plasma cells are absent in the walls of small bronchi and bronchioles (Blunden and Gower, 1999). IgG-, IgM- and IgA-producing plasma cells appear one week after birth and the numbers of the cells reached an adult level in foals at 8 to 12 weeks (Blunden and Gower, 1999).

The paucity of T cells is also found in neonatal BAL fluid. Both lower numbers and lower proportions of T cells in lymphocyte gate are seen in foals less than 6 weeks old (Flaminio et al., 2000a). This reduced number of lymphocytes present in alveoli and the lack of available lymphocytes to participate in the immune response in foals less than 6 weeks old may have relevance for foals pulmonary disease susceptibility (Balson et al., 1997b). Memory T cells may also be absent in BAL cells from neonatal foals because fewer MHC II^+ cells are found in foals than in adult horses (Balson et al., 1997b). An impaired function of lung T cells in neonatal foals is shown as low expression of cytokines in response to mitogen stimulation (Breathnach et al., 2006a).

The BAL Mϕ play an important role in the horse defense mechanism in the lung. Mϕ comprise of about 60% of all BAL cells in an adult horse (Tizard, 2009), compared to over 80% in foals (Flaminio et al., 2000b). Like lymphocytes, foal BAL Mϕ also exhibit an impaired chemotactic function (Fogarty and Leadon, 1987), and a lack of phagocytosis and intracellular killing of bacteria (Liu et al., 1987).

Alveolar DCs compise a very small portion of BAL cells (less than 1% in humans) (Tsoumakidou et al., 2006). No other studies of the alveolar DCs in neonatal

humans, mice, or foals have been reported. The studies of DCs in the respiratory tract of neonatal rat lungs demonstrate a low density of MHC II expression compared with that of adults. On the contrary, the pulmonary DCs from digested lung tissues of neonatal sheep do not exhibit an intrinsic functional defect that would impair their ability to take up antigen and stimulate naïve T cells (Fach et al., 2006). Whether there are phenotypical differences and/or functional immaturity in neonatal lung DC in foals is unknown.

***R. equi* infection**

R. equi was first identified in 1923. It is a facultative, intracellular, gram positive pathogen (Prescott, 1991). The infection manifests as subacute or chronic pyogranulomatous bronchopneumonia with abscessation and may present numerous extra-pulmonary disorders, such as pyogranulomatous enterotyphylocolitis and polysynovitis (Giguere et al., 2011a). The disease ranks second, behind injury, as a leading cause of death in foals less than six months old. It inflicts a significant economic impact on the horse industry both in the USA and worldwide (Prescott, 1991). The costs of treatment and foal losses from infection on farms can be substantial. Thus, understanding the susceptibility mechanism and estimation of the risk of foals to *R. equi* infection is of great importance in reducing foal wastage.

It is well accepted that all disease development is controlled by the interaction between virulence of the pathogen and the susceptibility of the host. On one hand, intracellular bacteria, such as *R. equi*, have multiple ways to escape the defense mechanism of the body (Figure 1.1). The pathogenesis of the bacteria depends on *R. equi* resistance to intracellular killing by Mϕ phagocytosis. On the other hand, horses exhibit an age-associated susceptibility, though all horses are exposed to the ubiquitous *R. equi*

in an equine environment. The pneumonia is observed almost exclusively within foals less than 6 months old, not in adult horses, even in the same farm with epidemic *R. equi* (Giguere et al., 2011b; Prescott, 1991). In foals, *R. equi* pneumonia cases occur between 3 and 24 weeks of age (Giguere et al., 2011a), occurring in most clinical cases by 2 months of age (Prescott, 1991). The naivety of the immune response is believed to be the contributor, as in other neonate-susceptible diseases reviewed above. The immature innate, humoral and cellular immune responses are responsible for foals' susceptibility to this intracellular pathogen infection (Figure 1.1). The following section will reviews the clinical signs, etiology of *R. equi* infection, and pathogenesis and virulence of *R. equi*. The following section will also demonstrate recent understanding of the immunity to *R. equi* infection and the possible factors in the immunity that may be responsible for foals' susceptibility to the disease.

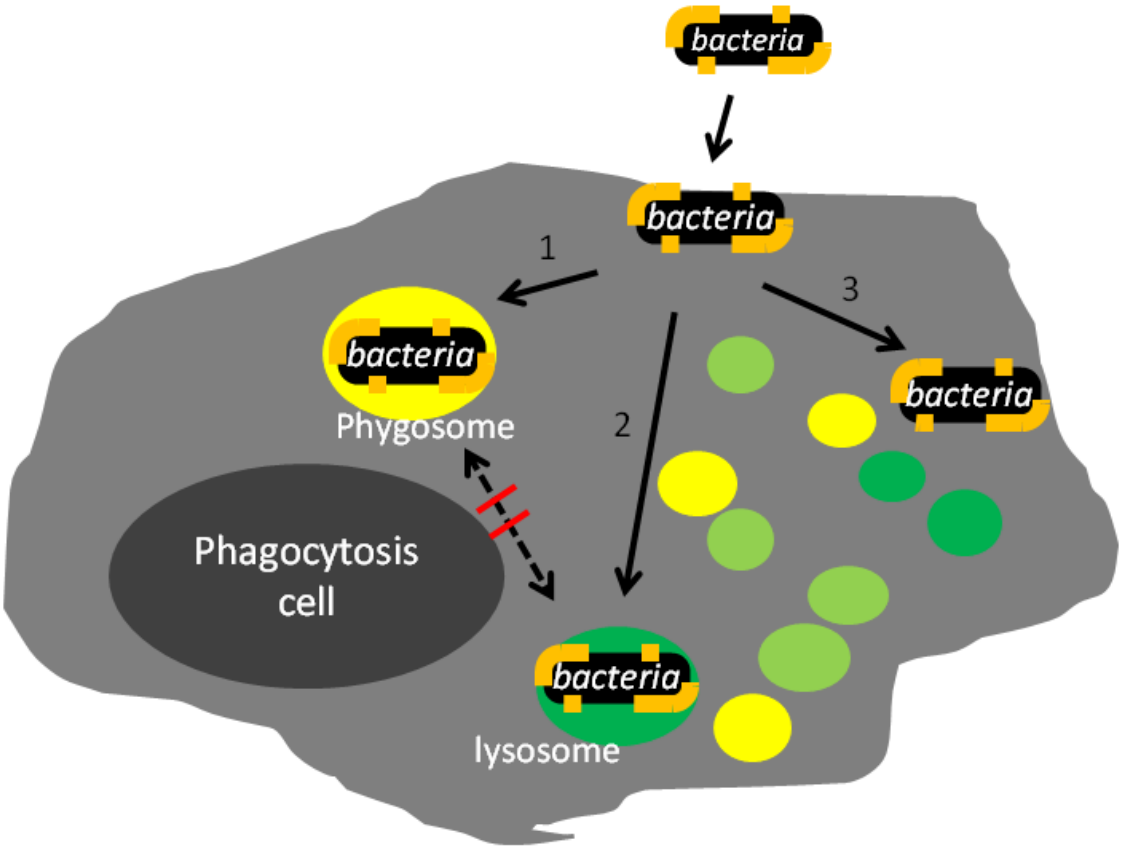


Figure 1.1. The strategy of intracellular bacteria to escape the cellular killing. 1, Prevention of lysosome-phagosome fusion; 2, Resistance to lysosomal enzymes; 3, Escape into the cytoplasm.

Clinical signs

Nowadays, the standard case of *R. equi* infection is a subclinical infection with mild neutrophilic leucocytosis and hyperfibrinogenaemia, which can be associated with abscessation or pulmonary changes (Muscatello et al., 2007; Vazquez Boland et al., 2009), possibly due to a better clinical monitoring of foals and more effective antibiotic treatment regimens. The severe variant of *R. equi* pneumonia is characterized by massive abscessation of the lung with fever, neutrophilia, mucopurulent respiratory discharge, and cough (Yager, 1987) and is rarely observed anymore (Muscatello et al., 2007). Inhaled *R.*

equi are phagocytosed by alveolar Mϕ but may not be killed (Hietala & Ardans, 1987; Zink et al., 1987). In the early stage of the disease, pulmonary lesions develop and alveoli fill with neutrophils, Mϕ and giant cells. Many of these cells contain intracellular *R. equi* (Johnson et al., 1983a). As the disease progresses, the lung parenchyma becomes necrotic (Johnson et al., 1983a; Zink et al., 1986), and bronchial and mesenteric lymph nodes are affected (Zink et al., 1986; Yager, 1987). In many cases, granulomatous foci in the lung open up, and *R. equi* spreads through the body and affects other organs (Prescott, 1991). Severe diarrhea with an ulcerative enteritis and mucosal invasion of *R. equi* is observed frequently, particularly in chronic disease (Cimprich & Rooney, 1977; Zink et al., 1986). This might be due to the ingestion of sputum containing large numbers of bacteria. Accordingly, intestinal lesions can be experimentally induced by oral infection of foals over a prolonged period of time (Johnson et al., 1983b).

Epidemiology

The virulent *R. equi* is a facultative pathogen that usually induces bronchopneumonia in foals and occasionally induces chronic cervical lymphoadenitis of pigs, tuberculosis-like lesions in the lymph nodes of cattle and in the liver of young goats. While *R. equi* causal diseases occur in multiple animals, rhodococcal bronchopneumonia is the most enzootic, occurs worldwide and leads to significant economic losses. Foals less than 6 months of age are susceptible to development of rhodococcal bronchopneumonia with a majority of cases occurring in foals less than 3 months old. The mortality ratio of foals with *R. equi* pneumonia nearly reached 80% before rifampin/erythromycin therapy was launched, but the mortality rate was reduced to 12% after utilization of these antibiotics in treatment (Hillidge, 1987). Nowadays, in Australia,

1-10% of foals are affected with a mortality rate, usually <1% due to antibiotic treatment, yet in some farms, it may be as high as 20% or more (Muscatello et al., 2006). The prevalence of *R. equi* pneumonia was 47% of 138 cases of pneumonia in non-randomized samples from American horse-breeding farms, with 13% of all foals on affected farms being infected and a mortality rate of 8%.

The factors that seem to be associated with increased incidence of *R. equi* infection include a high density and population size of foals, a large farm size, and high numbers of airborne virulent *R. equi*, which correlates with low soil moisture, high temperatures and a poor pasture grass cover (Muscatello et al., 2006). The virulent *R. equi* is prevalent in soil located in association with horses since their growth can be enhanced by the fatty acids found in horse manure and they can survive in extreme conditions. Therefore, bacteria are found in greater numbers where horses are present, with the numbers increasing with the concentration of horses, and a progressive build-up of infection on horse farms that have been used for rearing foals for a prolonged period. For instance, *R. equi* were isolated from most of the soil samples in horse breeding farms (24 out of 31) in a prevalence survey in Japan, at numbers of 10^2 to 10^5 colony forming units (CFU) per gram of soil. In accordance with the prevalence of virulent *R. equi* in soil, the aerosol dust particles contain a higher number of culturable colonies per cubic meter in air from stalls with condensed horses than that of paddocks (Kuskie et al., 2011). This indicates that foals on *R. equi* pneumonia enzootic farms are frequently exposed to high numbers of virulent bacteria. Yet, the actual proportion of virulent strains in the soil is no indication for prevalence of *R. equi* pneumonia, and the relative proportion of virulent *R. equi* in dams' feces is not indicative of the development of *R. equi* pneumonia

in their foals, although a recent study on the prevalence of aerosolized virulent *R. equi* observed a correlation between the number of *R. equi* in the air and the natural infection rate of foals with *R. equi* (Kuskie et al., 2011).

The inhalation of aerosolized dust contaminated with the bacteria is believed to be the route of *R. equi* infection and it is important for the induction of bronchopneumonia (Muscatello et al., 2009), however, intestinal infection in foals through a fecal-oral route may also occur since *R. equi* can replicate in intestines of foals up to 3 month of age (Takai, 1997). Transmission of *R. equi* pneumonia from foal to foal has not been documented, though the bacteria can be found in the breath of sick foals (Muscatello et al., 2009), which leaves a possibility of a contagiousness.

Aetiology

As one of the *Rhodococcus* species, *R. equi* is a Gram-positive, obligate aerobic bacterium. The shape of *R. equi* varies from bacillary to coccoid, depending on the growth conditions. With the optimal growth temperature between 30 and 37ºC (Prescott, 1991), the bacteria are rod shaped after 4 hours of growth in culture broth, whereas they become coccoid after 24 hours growth in liquid media or on agar (Fuhrmann and Lammler, 1997). The nutrient requirement for the organism is simple, and they grow well on normal non-selective media, such as tryptic soy broth or agar. The irregular, smooth and mucoid colonies can be achieved usually after 48 hours of culture at 37ºC on agar plate, although characteristic colonial variants occur. Salmon-pink or rosy red colonies may develop after 4 days or longer of incubation, or during storage (Prescott, 1991). These colonial and microscopic appearances are utilized for clinical laboratory identification and detection for *R. equi* infection.

R. equi can deal with some extreme environmental conditions such as low pH and oxidative stress (Benoit et al., 2002), which is due to their complex hydrophobic cell wall. The *R. equi* cell envelope is comprised of mycolic acid-containing lipids and lipoglycans such as lipoarabinomannan (ReqLAM) (Garton et al., 2002; Sutcliffe, 1998). The thick and lamellar polysaccharide capsule surrounding the *R. equi* cell wall also contributes to the survival in tough environmental conditions (Prescott, 1991). The virulence factors of *R. equi* include an extra-chromosomal plasmid of 80 to 90 kb, which encodes the production of a series of virulence-associated proteins (Vap), such as Vap A, VapB, VapC ect. Chromatin virulent gene also encoded some proteins that contribute to the virulence of *R. equi*, such as capsular polysaccharide, cell wall mycolic acid, and lipid metabolic associated enzyme.

Pathogenesis

R. equi is a facultative intracellular pathogen. Its ability to persist in, multiply and eventually destroy alveolar Mϕ is its fundamental characteristic of pathogenicity. There are three ways of *R. equi* entry into the Mϕ: via ReqLAM binding to mannose receptor on the Mϕ, via binding complement and complement receptors (CR) , such as CR3, on the Mϕ; and via binding antibodies and Fc receptors (FcR) on the Mϕ (Giguere et al., 2011a). However, the mechanism that contributes to the survival and even replication of *R. equi* in the endosomal vacuole after phagocytosis is poorly understood.

In the normal phagocytosis procedure, foreign particles are wrapped into the phagocyte membrane to form a new organelle, called the phagosome. The phagosome undergoes a fusion/fission event with vesicles from the endocytic pathway. The complex

phagosome maturation process occurs by interaction with the endosomal system via movement of phagosome on microtubules and change of the molecules on its surface. The phagosome matures into an early phagosome by fusion with an early/sorting endosome, obtaining a pH between 6.0 and 6.5. The early phagosome then fuses with the late endosome, which leads to the acidification of the late phagosome (pH 5.0-6.0). And the maturation of the phagosome is achieved by finally fusing with the lysosome. The gradual acidification drives the whole process of phagolysosome maturation. The killing of the particle occurs by the combined action of a low pH (4.0-5.0) and many hydrolytic enzymes (protease, lipase, DNAase, RNAse and more). Besides the lysosomal degradation, bacteria can also be killed by the production of reactive oxygen metabolites, such as superoxide radicals, hydrogen peroxide and peroxynitrite or nitrite oxide, by Mϕ through the respiratory burst phagocyte oxidase and inducible NO synthase (iNOS). For the bacteria that do not interfere with the process, the formation of phagosome from phagocytosis takes about 3-10 minutes, while the normal formation of phagolysosome takes about 15-60 minutes varying from bacteria to bacteria. Most phagosomes containing virulent *R. equi* do not progress into a late endocytic organelle, instead forming a *R. equi* containing vacuole (RCV). Intracellular bacteria have developed various strategies to avoid phagolysosome formation and killing. So, this brings up the question of what strategy is used by *R. equi*.

To arrest phagosome maturation at a stage between the early and late endosomes is the most important strategy used by *R. equi* to escape the killing system of phagocytosis (Fernandez-Mora et al., 2005). The successful establishment of the RCV depends on the virulence plasmid, even for formalin-killed *R. equi* in Mϕ. The RCV

form a loose membrane in Mϕ and multiply after 2 hours of initial lag-phase and the membrane remains morphologically intact and nothing escapes into the cytoplasm. The cytotoxicity of *R. equi* for Mϕ is regulated by the virulence plasmid by inducing necrosis, rather than apoptosis, compared with the avirulent *R. equi* (Luhrmann et al., 2004). This degradation of host cells becomes detectable at about 8 hours post infection (Luhrmann et al., 2004).

Immunity to *R. equi* infection

The immune response to *R. equi* includes innate, antibody-mediated and cell-mediated immune responses as represented in the graphic (Figure 1.2).

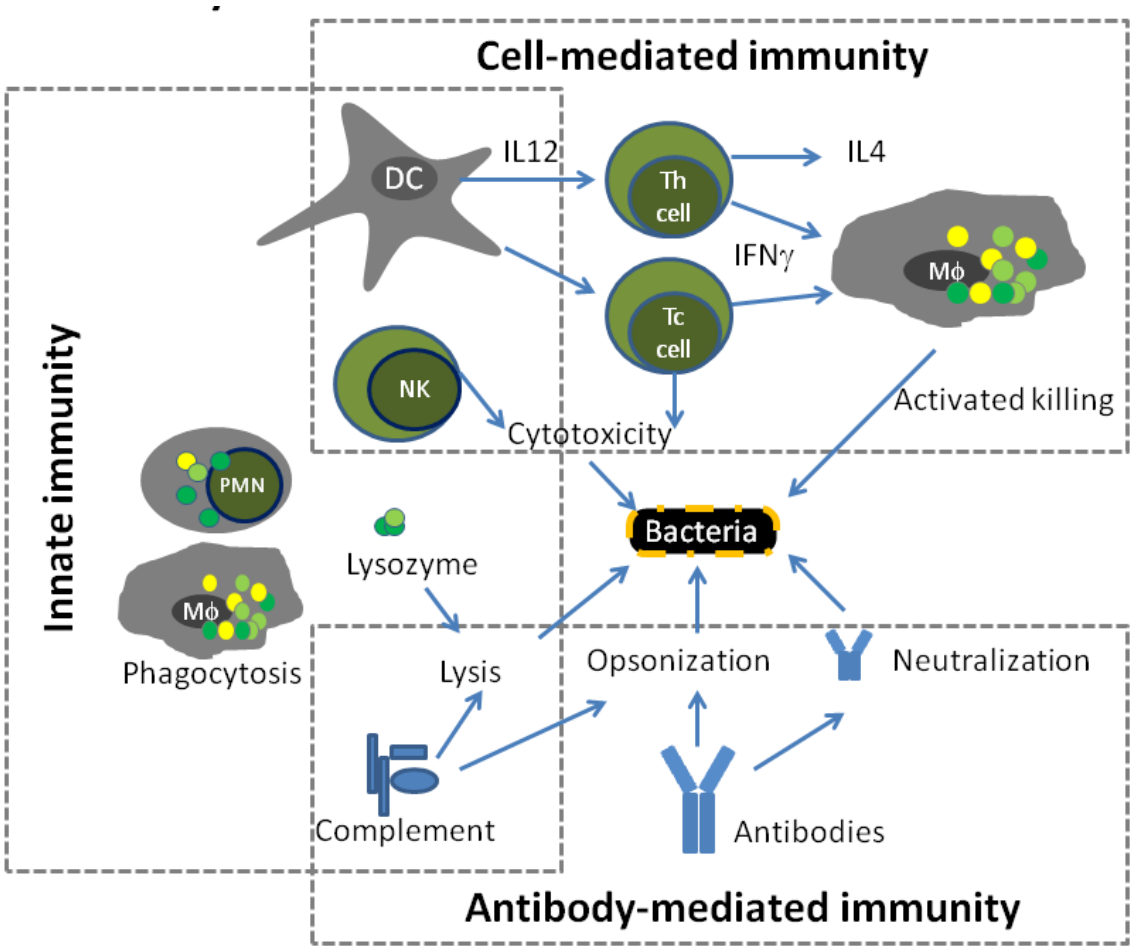


Figure 1.2. Immunity to intracellular bacterial infection. The immune response of host bodies to intracellular bacterial infection is comprised of: innate immunity, including phagocytosis of Mϕ and neutrophil, complement and lysozyme lysis, antigen recognition by APC (DCs and Mϕ), and cytotoxicity by NK cells; antibody-mediated immunity, including opsonization and neutralization by antibodies; cell-mediated immunity, such as activated Mϕ killing, cytokine secretion by helper T cells and lysis of the infected cells by cytotoxic T cells.

Innate immunity

Immunity to *R. equi* infection begins with the innate immune response. The innate immune response to intracellular bacterial infection is composed of humoral components such as complement, and cellular components, such as neutrophils, Mϕ and NK cells.

There are two ways of complement killing of bacteria: complement lysis and opsonization. However, the polysaccharide capsule surrounding *R. equi* and the thick peptidylglycan on the *R. equi* cell wall (Prescott, 1991) provides resistance against complement lysis (Rautemaa and Meri, 1999), as in *Mycobacterium tuberculosis*. Therefore, the complement-induced killing of *R. equi* relies on opsonization to facilitate phagocytosis by neutrophils through the complement receptor. However, the phagocytosis induced by the complement receptors is not competent. Instead, efficiently phagocytes ingest of CR3-opsonized targets only occure when cells are activated both *in vivo* and *in vitro* by cytokines, such as IL1 and TNFα. Thus, the capacity of these receptors for ingestion is not constitutive but depends on many other cellular events.

Neutrophil killing of bacteria includes: phagocytosis, degranulation and neutrophil extracellular traps. The phagocytosis killing function of neutrophils to control primary infections is widely documented in immunity to *R. equi* infection. Neutrophils exhibit a substantial protective role by reduction of *R. equi* burden in the lung (Martens et al., 2005). They also produce the cytokines to promote the later immune responses, such as TNFα, IL-12 and IL-6, in response to *R. equi* stimulation (Liu et al., 2009a; Nerren et al., 2009a). While the neutrophil function is impaired in foals as no association of reviewed previously, its relevance to *R. equi* susceptibility is not clear, as no age-associated cytokine expression is found in *R. equi*-stimulated neutrophils (Liu et al., 2009b; Nerren et al., 2009b).

However, the killing of intracellular bacteria is primarily accomplished by professional phagocytotic cells, Mϕ, by highly reactive toxic molecules, particularly reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). The production of ROI is through respiratory burst, another way of bacterial killing within the phagosome (Figure 1.3). The procedure consumes oxygen and generates reactive oxygen species. The ROI production is initiated by a membrane bound NADPH oxidase, which is activated by IFN-γ and by IgG-FcR binding. It functions by destroying and S-nitrolylating bacterial proteins, damaging DNA and membrane lipids, and generating highly reactive antibacterial peroxynitrite ($ONOO^-$) with $NO^\bullet$ (Figure 1.3). Both $ONOO^-$ and $NO^\bullet$ appear to have central roles in antibacterial defense against *M. tuberculosis* in mice. $NO^\bullet$ is exclusively derived from the terminal guanidine-nitrogen atom of L-arginine. This reaction is catalyzed by iNOS, which lead to the formation of L-citrulline and $NO^\bullet$. $NO^\bullet$ not only destroys the protein, DNA, and membrane lipids, but also inactivates the

ion-sulfur enzyme of the bacteria. While the production of NO is NADPH dependent, it requires iNOS as catalyst (Figure 1.3). There are three types of NOS isoenzymes. The two constitutive NOS (cNOS) exist in various host cells and account for basal NO synthesis, whereas iNOS is primarily found in professional phagocytes and is responsible for microbial killing. Its induction is controlled by exogenous stimuli such as IFN-γ. This iNOS stimulation results in a burst of high RNI concentrations required for microbial killing, whereas the low NO levels produced by cNOS perform physiologic functions.

$$O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} NADP + O_2^- + H^+$$

$$\text{L-Arginine} \xrightarrow{\text{iNOS}} NO^\bullet + \text{L-Citrulline}$$

$$O_2^- + NO^\bullet \rightarrow ONOO^-$$

Figure 1.3. Production of RNI and ROI. The generation of ROI, such as O_2^-, occurs during respiratory burst by NADPH oxidase catalysis. The generation of RNI, such as $NO^\bullet$ is induced by iNOS catalyst. The peroxynitrite is generate by $NO^\bullet$ and O_2^- reaction.

The Mϕ, as a professional phagocytotic cell, plays a central role in eliminating *R. equi*. It serves not only as the habitat but also as the effector cells for *R. equi* killing. However, the phagocytic killing by infected Mϕ is blocked by *R. equi* via inhibition of phagosome and lysosome fusion or phagolysosome maturation. The inhibition was found in CR3 and mannose receptor mediated Mϕ phagocytosis. However, IFN-γ stimulation can activate Mϕ, resulting in transition of Mϕ from habitat-supporting microbial

replicator cells into immune effector cells capable of terminating, or at least restricting, microbial survival. IFN-γ can activate NADPH oxidase and iNOS, which ultimately generate the ROI and RNI. Activated Mϕ have increased phagocytosis, elevated CR, reduced FcR expression and higher overall metabolic rates.

Antibody-mediated immunity

As described above, specific antibodies neutralize and facilitate opsonization of *R. equi*. The specific antibody-induced opsonization is important for killing of *R. equi* by Mϕ, as it promotes phagosome-lysosome fusion (Cauchard et al., 2004). The evidence for the protective role of antibodies against *R. equi* is that passive transfer of anti- *R. equi* hyperimmune equine plasma protects foals against *R. equi* infection. The purified anti-VapA and anti-VapC antibodies exhibit the same protective effect as the hyperimmune plasma in foals against experimental pneumonic infection (Hooper-McGrevy and Prescott, 2001). Therefore, foals' lack of the *R. equi*-specific antibodies because of the reduced absolute number of B cells and the impaired endogenous IgA, IgG and IgM generation in the lung may contribute to their susceptibility to *R. equi* infection.

In addition, subisotypes of IgG have different functions: IgGa and IgGb opsonize microbes and fix complement, whereas IgGT may competitively inhibit the fixation and opsonization of IgGa and IgGb, and produces weak leukocyte respiratory burst (Lewis et al., 2008) (Banks and McGuire, 1975; McGuire and Bariso, 1972). Because of this, IgGa and IgGb are considered the most important antibodies against *R. equi* (Lewis et al., 2008; Taouji et al., 2004). VapA specific IgGa and IgGb are effectively generated by foals with experimental *R. equi* challenge (Hooper-McGrevy et al., 2005; Jacks et al., 2007b). The

IgG subisotypes reflect T-cell responses which influence class switching in B cells, with IgGb and IgGT associated with a Th2 (IL-4) response and IgGa associated with a Th1 (IFN-γ) response (Hooper-McGrevy et al., 2003; Jacks et al., 2007b).

Cell-mediated immunity

The immunity of adult horses to *R. equi* reflects an acquired immune response and the ability to mount effective Th1 responses. Clearance of virulent *R. equi* from the lung of adult horses is associated with an increase in CD4 and CD8 T lymphocytes and lymphoproliferative responses at the site of challenge (Hines et al., 2001). Adoptive transfer studies in mice showed that CD4 Th1 lymphocytes are essential and sufficient for effective pulmonary clearance of virulent *R. equi*, whereas induction of a Th2 response results in characteristic lung lesions (Kanaly et al., 1996a). CD8 T lymphocytes are also involved in the clearance of *R. equi* as CD8 CTL in adult horses lyse *R. equi* infected cells *ex vivo* but not kill uninfected versions of the same target cell (Kanaly et al., 1993). This CTL presents a MHC I unrestricted and bacteria lipid recognization format. These studies strongly suggest that induction of a Th1 immune response involving both CD4 and CD8 T lymphocytes and characterized by IFN-γ and CTL activity is essential for pulmonary clearance and protective immunity against *R. equi* in adult horses. The life-threatening pyogranulomatous bronchopneumonia in foals likely reflects a decreased ability to mount the responses necessary for effective clearance of the organism from the lung. The mechanisms that underlies the age-related predisposition are unknown, but it is thought to be related to immaturity of the immune system in foals and their impaired potential to mount potent Th1 immune response and CTL activity early in life.

In foals, the reduced number of lymphocytes present in alveoli and the lack of available lymphocytes to participate in the immune response in foals less than 6 weeks old may have relevance for susceptibility to *R. equi* infection (Balson et al., 1997b). The impaired IFN-γ expression by both CD4 and CD8 T cells is considered to be associated with foals' susceptibility to *R. equi* infection. Additionally, other impaired cytokine expression, such as IL-12 and IL-17, in neonatal foals may also play a role (Liu et al., 2011b; Liu et al., 2009c). Foals less than 3 weeks of age lack the *R. equi*-specific CTL activity although the CTL do begin to appear subsequently and are present at 8 weeks of age (Patton et al., 2005). This age-associated CTL response may also contribute to the susceptibility.

The primary activation of naive lymphocytes in response to *R. equi* depends on antigen presentation of peptides and strong co-stimulation from professional APCs. Therefore, the impaired APC function of neonatal foal DC may also contribute to the susceptibility to *R. equi* infection, such as low levels of essential surface molecules (MHC II and CD86) and cytokine (TNFα) expressions as reviewed before. In addition, a source of lung APC exhibits an IL-10 production bias in Mo stimulated with LPS and IFN-γ in neonatal foals; this may also play a role (Sponseller et al., 2009a). In practice, the ineffective up regulation of MHC II and CD1b in response to *R. equi* stimulation may also contribute to the susceptibility (Flaminio et al., 2009; Pargass et al., 2009).

In summary, besides 'naivety' of the neonatal immune system because of lack of exposure to microbial antigens before birth, there may be age-related impairments both in important antigen-presenting molecules and in cytokines important for effective cell-mediated immune responses, which synergize with the pathogen in driving an ineffective

immune response, thus allowing the successful continued replication of *R. equi* in Mϕ and its spread from the lungs back into the environment.

The susceptibility of *R. equi* infection and IFN-γ production in foals

R. equi successfully infects certain host animals and cell types with high specificity, strongly suggesting that not only bacterial virulence factors, but also particular host (cell) factors determine success or failure of *R. equi* invasion. Most of the foals on endemic farms do not develop disease, reflecting a predisposition of only a certain subpopulation of foals to *R. equi* infection. The factors that account for the particular susceptibility of foals to *R. equi* infection are not well known. Given that IFN-γ expression activates the Mϕ clearance of *R. equi* and there is limited IFN-γ expression in foals, the extremely low expression of IFN-γ in foals contributes partly to its susceptibility to *R. equi* infection. Therefore, it is very important to know how IFN-γ expression is regulated in foals and how to promote the expression.

Regulation of IFN-γ expression

IFN-γ as a canonical Th1 cytokine, is critical for innate and adaptive immunity against viral and intracellular bacterial infections. The importance of IFN-γ in the immune response derives from its immunostimulatory and immunomodulatory effects and in part derives from its ability to inhibit viral replication directly. IFN-γ promotes naïve T cells differentiation into Th1 effector cells, which mediate cellular immunity against viral and intracellular bacterial infections; it contributes to Mϕ activation by increasing phagocytosis and priming the production of proinflammatory cytokines and potent antimicrobials, including superoxide radicals, nitric oxide, and hydrogen peroxide (Boehm et al., 1997); it increases NK cell activity in killing of infected cells; it activates cytotoxic T cells; and it up regulates both MHC I and MHC II antigen presentation by increasing expression of subunits of MHC I and II molecules, TAP1/2, invariant chain, and the expression and activity of the proteasome (Schoenborn et al., 2007) , as shown in Figure 1.4.

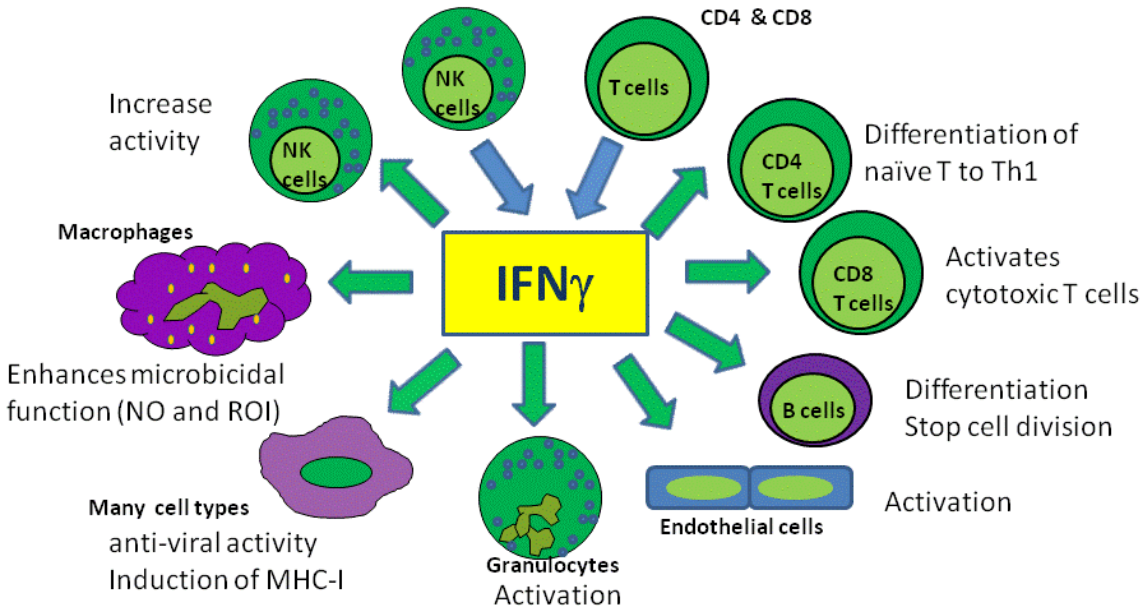


Figure 1.4. Functions of IFN-γ. IFN-γ is produced by CD4, CD8 and NK cells. It promotes T and B cell differentiation, contributes to Mϕ activation, increases NK cells activity, activates cytotoxic T cells and granulocytes, and up regulates both MHC I and MHC II in many cell types.

In humans, decreased IFN-γ induction or signaling is associated with strikingly increased susceptibility to mycobacterial infections (Filipe-Santos et al., 2006). Systemic infections with *Salmonella* are also more common (de Jong et al., 1998) in those with defective IFN-γ production or defective signaling regulation of IFN-γ production (MacLennan et al., 2004). In mice, the clearance of *R. equi* depends on IFN-γ production by $CD4^+$ T cells (Kanaly et al., 1995b, 1996b). In adult horses, clearance of virulent strains of *R. equi* from the lungs is also associated with the production of IFN-γ by T cells (Hines et al., 2003b; Patton et al., 2004b) as reviewed above. However, IFN-γ production is deficient in foals (Boyd et al., 2003; Breathnach et al., 2006a) and this deficiency is thought to be responsible for their susceptibility to *R. equi* infection (Marodi,

2006b). Similarly, reduced IFN- γ expression has also been observed in humans and murine neonates (Lewis et al., 1986a; Lewis et al., 1991; Wilson et al., 1986a). IFN- γ is necessary to prevent various infections (Gasparoni et al., 2003; Schoenborn and Wilson, 2007), yet little is known regarding the underlying mechanism regulating its expression in horses.

The primary sources of IFN-γ are CD8 and CD4 Th1 effector T cells of the adaptive immune system, and NK cells and NKT cells of the innate immune response. In contrast to rapid induction of IFN-γ in NK and NKT cells, naive CD4 and CD8 T cells produce little IFN-γ immediately following their initial activation (Schoenborn et al., 2007). However, naive CD4 and CD8 T cells can gain the ability to efficiently transcribe the gene encoding IFN-γ in a process which is dependent on their proliferation, differentiation, upregulation of IFN-γ-promoting transcription factors (TF), and remodeling of chromatin within the *Ifng* locus (Schoenborn et al., 2007). The differentiation into IFN-γ- producing CD4 Th1 effector T cells, and to a lesser extent into CD8 effector T cells, is dependent on the nature of the infecting pathogen and the cytokine milieu emanating from the innate immunity (Schoenborn et al., 2007).

Consistent with transcriptional up-regulation of IFN-γ expression in differentiated CD4 and CD8 effector cells in humans, the IFN-γ expression in foals is regulated at the transcriptional level as indicated by correlated protein and mRNA expression of IFN-γ gene in stimulated PBMCs (Breathnach et al., 2006a). The transcription of IFN-γ gene starts with the regulatory TF binding to elements, recruitment of RNA polymerase and initiation of IFN-γ gene transcription. The transcription regulation operates in three

aspects: the availability, magnitude and types of TFs; the accessibility of the elements controlled by DNA methylation and histone modificational of chromatin structure which is named epigenetic regulation; and the cell proliferation which opens a window for modulation of chromatin structure, DNA methylation and TF production, as shown in the model (Figure 1.5).

In the following section, we will review function of TF and epigenetic (DNA methylation and histone modification) mechanisms on regulation of IFN-γ gene transcription, and the signaling pathways that control IFN-γ gene transcription in CD4 and CD8 T cells.

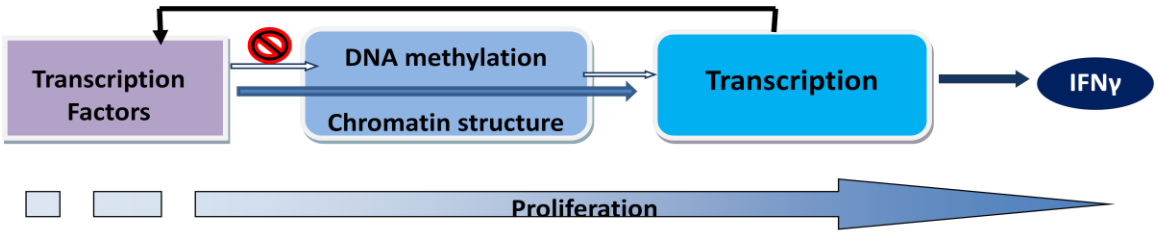


Figure 1.5. Model of transcription regulation of IFN-γ gene expression. Transcription factors initiate *Ifng* transcription by binding to the regulatory elements on *Ifng* promoter. However, the transcription is repressed when the DNA is methylation and/or chromatin exhibit a compact structure around the *Ifng* promoter. Nevertheless, the repression can be overcome by lymphoproliferation which "open" a window for the DNA demethylation, the chromatin remodeling and the regulation of transcription factor production.

Regulation signals for IFN-γ gene transcription

The regulation signals for IFN-γ gene transcription are antigen recognization, T cell receptor (TCR) signaling from antigen presentation by MHC, cytokine induced JAK-

STAT and SMAD signaling, and Notch signaling from APC. The four signaling pathways are described as follows.

Regulation of IFN-γ gene transcription through TCR signaling

The efficient IFN-γ secreting cells differentiated from naive CD4 and CD8 T cells require several rounds of proliferation induced by TCR signals. During proliferation, environmental signals influence the expression and activation status of specific receptors, downstream signaling molecules, and transcription factors. These, in turn, allow these T cells to express IFN-γ and commit to the Th1 CD4, or CD8 CTL effector lineage (Murphy and Reiner, 2002a; Reiner, 2001). For naive CD8 T cells, signals delivered from the TCR and costimulatory molecules induce differentiation into a fully committed CTL that is capable of IFN-γ secretion, which is thought to be the result of the constitutive expression of the T-bet paralog Eomesodermin (Eomes) (Pearce et al., 2003a). On the contrary, naïve CD4 T cells stimulated whereby TCR and co-stimulatory molecules signaling produce small amounts of IL-2, IFN-γ, and IL-4 via rapidly activated transcription factors nuclear factor of activated T cells (NFATs), nuclear factor (NFκB), and activator protein-1 (AP-1). The fate of the IFN-γ producing Th1 cells is predominantly governed by the cytokine milieu produced by APCs, and also regulated by the nature of the APC and the magnitude of stimulation, with stronger and longer signaling generally favoring Th1 development (Pulendran and Ahmed, 2006).

Cytokine up-regulation of IFN-γ gene transcription through STAT signaling

Cytokines, such as IL-12, IL-18, IFN-γ, Type I IFN-s, IL-23, and IL-27, play an important roles in induction and maintenance of IFN-γ gene transcription as reviewed below.

IL-12 binding with its receptor activates Jak2/Tyk2 and induces signals via STAT4 (Trinchieri et al., 2003), which in turn induce expression of the IL-18 receptor. The combined signals from IL-12, IL-18, IFN-γ, maximize expansion and optimal activation of Th1 T cells (Murphy and Reiner, 2002a).

IFN-γ acts in a positive feedback loop to facilitate its own expression by T cells via focused recruitment of IFN-γ receptors to the immunological synapse, whereby IFN-γ secretion results in an autocrine response (Maldonado et al., 2004). IFN-γ binding to receptor on CD4 T cells leads to STAT1 phosphorylation and translocation into nucleus which induces Tbet (Lighvani et al., 2001).

Type I IFNs induce IFN-γ transcription via activation of STAT4 in human CD4 T cells (Tyler et al., 2007). The signals by IFN-α/IFN-β combined with IL-18 stimulation drive acute IFN-γ secretion (Matikainen et al., 2001). IFN-α can also up-regulate MyD88 and synergize with IL-12 to augment the IL-18 stimulating signals by elevating IL-18 receptor expression (Sareneva et al., 2000) in CD4 T cells. In CD8 T cells, IFN-α appears to have a sufficient effect on inducing IFN-γ production though not as efficient as the IL-12 (Curtsinger et al., 2005).

IL-23, expressed by activated DC (Trinchieri et al., 2003), regulates the maintenance of IFN-γ transcription in CD4 T cells via activation of STAT3/STAT4 (Oppmann et al., 2000), although it may also affect the survival and expansion of Th17 cells (Weaver et al., 2006). IL-27 is expressed by virus-infected cells, activated Mϕ, and DCs. It activates resting CD4 T cells via both STAT1 and STAT3. The activation of STAT1 induces Tbet expression, and in turn up-regulates IFN-γ transcription (Pflanz et al., 2002). The synergization induction of IFN-γ transcription with IL-12 and IL-18 is also observed (Pflanz et al., 2002).

Cytokine down-regulation of IFN-γ gene transcription

In addition, some cytokines inhibit IFN-γ transcription, such as TGF-β and IL-6. TGF-β suppress IFN-γ transcription directly and indirectly by inhibiting expression of Tbet, STAT4 IL-12Rb2 and itself, which signals through SMAD3/4 heterodimers activated by TGF- βR (Schoenborn et al., 2007). IL-6 inhibits IFN-γ transcription by blocking STAT1 activation induced by IFN-γR through potentiating expression of the suppressor of cytokine signaling-1 (SOCS-1) (Diehl et al., 2000).

Notch signaling pathway regulats oIFN-γ gene transcription

There are four members of Notch, namely Notch 1 to 4. Five identified Notch ligands are expressed on DCs in mammals, namely Jagged 1 and 2, Delta 1, 3 and 4. Notch plays a role in regulating differentiation of naïve CD4 T cells in to distinct Th cells. Notch 3 - Delta 1 interaction results in Th1 differentiation (Maekawa et al., 2003). The promotion of Th1 differentiation of naïve T cells is found upon Notch ligand interaction with Delta 4 via an IL-12 independent way (Skokos and Nussenzweig, 2007). By contrast,

Th2 differentiation is induced by the interaction of Notch- Jagged 1 via activation of GATA3 and IL-4 transcription (Amsen et al., 2004). The activation of IFN-γ transcription by Notch signaling is based on the cascade cooperating NFκB binding on the IFN-γ gene (Minter et al., 2005). It also controls the activation of T cells by promoting CD4 and CD8 T cell proliferation (Adler et al., 2003).

TF regulation of IFN-γ gene transcription

Regulation for IFN-γ gene expression is signaled from the receptors on the plasma membrane through the cytoplasm and into the nucleus by induction of TF expression, posttranslational modification, and/or nunclear translocation. Because TFs that are downstream of TCR and costimulatory signaling, such as NFAT, NFκB and AP-1, are ubiquitously expressed and responsible for regulating multiples genes (Szabo et al., 2003). These TFs are not described in the review. The STATs and the "master" TFs, such as Tbet and GATA3, which specificly regulate IFN-γ gene transcription, are reviewed below.

STATs play critical roles in the initiation of IFN-γ transcription in naïve T cells via differentiation to and expansion of Th1 cells. Of the six STATs in the family, STAT1 and STAT4 facilitate IFN-γ transcription. STAT1 plays an important role in indirectly influencing IFN-γ transcription by potentiating Tbet expression. Although this can be activated by multiple cytokines, such as IFN-γ, IL-27 and Type I IFNs, only STAT1 transduction of the signaling from IFN-γ activation is sufficient to induce sustained expression of Tbet (Lighvani et al., 2001). In contrast to STAT1, STAT4 activation by IL-12 facilitates IFN-γ transcription mainly by promoting the commitment to Th1 lineage

and sustained Th1 response in CD4 T cells (Mullen et al., 2002). In collaboration with NFκB activated by IL-18, IL-12-activated STAT4 also induces IFN-γ production in an antigen-independent manner in both CD4 and CD8 T cells (Carter and Murphy, 1999; Mullen et al., 2002). Other STATs, such as STAT3 and STAT5, play an important role in regulation of IFN-γ transcription in NK and NKT cells (Fujii et al., 1998; Hibbert et al., 2003).

T-box family members of TFs are critical for induction of IFN-γ transcription. Tbet, encoded by Tbx21 gene, is a "master regulator" for inducing IFN-γ transcription and Th1 cell differentiation (Szabo et al., 2000). It acts directly on the *Ifng* gene to facilitate its transcription by binding to multiple sites within the *Ifng* promoter (Hatton et al., 2006). Indirectly, Tbet up-regulates IL-12Rβ2 expression and inhibits GATA3 expression (Mullen et al., 2001; Usui et al., 2006). In effector CD8 T cells, Tbet functions to sustain *Ifng* transcription (Sullivan et al., 2003). The induction of *Ifng* transcription by Tbet can be synergized by STAT4 as a collaborative effect which also induces IL-18R1 and IL-12Rβ2 expression (Thieu et al., 2008). Tbet also directly induces TFs, such as Runx and Hlx, which work in concert with Tbet in promoting *Ifng* transcription and reinforcing Th1 commitment (Singh and Pongubala, 2006). Runx3, expressed by Th1 cells and CD8 T cells, interacts with Tbet and binds cooperatively with it to *Ifng* promoter which in turn activates its transcription (Djuretic et al., 2007). Like Runx3, Hlx, expressed by Th1 cells, also increases expression of IFNγ by synergism with Tbet, also enhancing the expression of Tbet and IL-12Rβ2 (Mullen et al., 2002). In addition, Tbet collaborately works with another T-box family TF, Eomes, to assure proper differentiation of naive CD8 T cells (Pearce et al., 2003a). The function of Eomes on

upregulation of *Ifng* transcription is independent of Tbet but plays an overlapping role with Tbet (Szabo et al., 2002).

GATA3 is a pivotal TF for induction of IL-4 transcription and differentiation of naïve T cells into Th2 cells. Ectopic GATA3 expression in developing Th1 cells inhibits IFN-γ production while inducing IL-4. Although IL-4 is known to inhibit IFN-γ production, inhibition of IFN-γ production by GATA3 is not dependent on IL-4, as previous thought (Yagi et al., 2011). GATA3 suppresses IFN-γ production by indirectly blocking IL-12Rβ2 and STAT4 up-regulation, neutralizing the capacity of Runx3 to induce IFN-γ transcription. It may also play a role in silencing Tbet and Runx3 gene expression through chromatin remodeling after direct binding to their genes (Yagi et al., 2011). It functions in regulation of IFN-γ expression indirectly via promote the commitment of naive T cells to Th2 lineage whereby compete Th1 lineage. It also indirectly repress *Ifng* transcription by binding to TF and may block *Ifng* transcription or recruit other repressors to *Ifng* (Yagi et al., 2011).

Epigenetic regulation of IFN-γ gene transcription

In principle, TFs regulate *Ifng* transcription at a number of levels, but in practice the fundamental effects of all TFs rely on the capacity to bind to regulatory elements in DNA. Facing the challenge of fitting a genome consisting of several billion nucleotides into a nucleus of a micron diameter, while maintaining spatial organization and accessibility to TFs that govern the transcription, the eukaryotic cells associate DNA with proteins in chromatin. The basic unit of chromatin is the nucleosome, consisting of an octamer of histone protein with two copies of H2A, H2B, H3 and H4, around which

about 150bp of DNA is wrapped (Felsenfeld and Groudine, 2003). The control of chromatin structure on gene transcription is regulated by DNA methylation and histone modification, which encode information without affecting the DNA sequence, termed epigenetic regulation.

The epigenetic regulation of gene transcription is an important mechanism that controls transcriptional activation or repression of a gene locus (Spilianakis and Flavell, 2007), including DNA methylation and histone modifications which result in chromatin remodeling (Zhu et al., 2010). In the following, the mechanism of DNA methylation and histone modification is reviewed and their roles in regulation of *Ifng* are described.

DNA methylation

DNA methylation refers to the transfer of a methyl group, S-adenosylmethionine (SAM), to the 5 position of the cytosine (C) to form 5-methylcytosine (5meC) by DNA methyltransferase (DNMT) (Ansel et al., 2003; Holliday, 2006). The DNA methylation suppresses gene transcription either directly by inhibiting TF binding or by allowing methyl-CpG-binding proteins (MeCP) to bind to the methylated DNA which ultimately "closes" chromatin structure (He et al., 2011; Jones, 2012). The DNA methylation is restricted to the symmetric CG context, although non-CG methylation is prevalent in embryonic stem cells (Ramsahoye et al., 2000). While the DNA methylation pattern is set up during embryo development, regulating tissue-specific gene transcription throughout the genome, the DNA methylation at specific sites is plastic and regulates specific gene transcription as a result of a dynamic process involving both *de novo* DNA methylation and demethylation (He et al., 2011). The mechanism of specific site DNA methylation and its role in regulation is reviewed below.

DNA methylation is induced by three types of DNMTs: DNMT1, DNMT3A, and DNMT3B. The existing DNA methylation is maintained by DNMT1 at hemi-methylated DNA during DNA replication and cell division, whereas establishment of *de novo* DNA methylation is generated by DNMT3A and DNMT3B (Chen and Li, 2004). The *de novo* DNA methylation is facilitated by DNMT3-like (DNMT3L), a third member of the DNMT3 family, which has no catalytic activity (Bourc'his et al., 2001).

While DNA methylation can be established and maintained, DNA demethylation also occurs through passive and active DNA methylation (He et al., 2011). Inhibition of DNMT1 during DNA replication in cell proliferation leads to passive DNA methylation (Szyf, 2005). However, in other cases, the DNA methylation is removed in active demethylation. There is evidence for the existence of DNA demethylase to demethylate methyl CpG dinucleotides by directly replacing 5meC with C independent of DNA replication (Gjerset and Martin, 1982; Hamm et al., 2008; Hendrich and Bird, 1998). Recently, the DNA demethylation mechanism was also postulated to be associated with a methylated cytokine removal by DNA repair system (Wu and Zhang, 2010).

Histone modification-induced chromatin changes in regulation of gene transcription

Chromatin is not only a DNA scaffold but also regulates the transcription of a gene by structural changes in response to external cues. Modification of histones, principal component of chromatin, plays a key role in this regulation. There are mainly four types of histone modification: acetylation, methylation, phosphorylation and ubiquitylation. Among them, histone acetylation and methylation, have critical influences on chromatin structure and specific gene transcription, which differ depending on the type and location of the modification.

The N-terminus of a histone (H2A, H2B, H3 and H4) tail is acetylated by histone acetyltransferases (HATs) transfering an acetyl group to a lysine side chain. The acetylation of the histone neutralizes the positive charge of the lysine with the negatively charged phosphate groups of DNA as reviewed. As a consequence, the condensed chromatin is transformed into a more relaxed structure that is associated with greater levels of gene transcription. Relaxed, transcriptionally active DNA is referred to as euchromatin. More condensed (tightly packed) DNA is referred to as heterochromatin. The lysine acetylation can be reversed by a histone deacetylase (HDAC), which opposes the effects of HATs. This action potentially stabilizes the local chromatin architecture and is consistent with suppression of gene transcription. The HDAC is considered a repressor for gene transcription not only based on its opposite effect of HATs but also on induction of final DNA methylation by recruitment of DNMT3 (Feldman et al., 2006).

Histone methylation mainly occurs on the side chains of lysines and arginines. The methylation is catalyzed by histone lysine methyltransferase (HKMT) transfer of a methyl group from SAM to a lysine, and the lysine is finally mono-, di- or tri-methylated. Whereas, arginine methylation is induced by arginine methyltransferase transferring a methyl group to arginine from a variety of substrates to form a mono-, symmetrically or asymmetrically di-methylated arginine. The opposed demethylation of histone which occurs depending on the position and the degree of the methylation. In contrast to the constant gene transcription activation of histone acetylation, the histone methylation acts by both repressing and activating gene transcription based on the type and position of histone methylation. In general, H3K4 methylation is associated with active gene transcription (Benevolenskaya, 2007; Koch et al., 2007), whereas H3K9 and H3K27

methylation is associated with repressive gene transcription (Barski et al., 2007). The level of methylation also influences gene transcription, such as mono-methylation of H3K9 and H3K27, inducing active gene transcription. In contrast, di- and tri-methylation of these histone residues suppresses gene transcription (Barski et al., 2007). Unlike acetylation, the demethylation and methylation of histones turns the gene transcription "on" and "off", not relying on the alternating charge of histone protein. It alters the chromatin structure by induction of heterochromatin or euchromatin via interaction with heterochromatin proteins whereby represses or facilitates gene transcription. For instance, H3K9 methylation serves as a binding site for the chromodomain protein heterochromatin protein 1 which recruits HDAC and DNMT3, thus generating a form of local heterochromatin (Bannister and Kouzarides, 2011). The histone methylation also functions in concert with DNA methylation. For instance, the histone demethylase-induced unmethylated H3K4 promotes *de novo* DNA methylation by interaction with DNMT3L which facilitates the DNA methylation by DNMT3A and DNMT3B (Cedar and Bergman, 2009a; He et al., 2011). Mutually, DNA methylation also influences histone acetylation and methylation (Bannister and Kouzarides; Cedar and Bergman, 2009b). Unmethylated DNA is largely associated with nucleotides that wrap on the acetylated histone, and methylated DNA is associated with unacetylated H3 and H4 histone. This interaction is likely induced by the methyl DNA binding protein via recruitment of HDAC (Cedar and Bergman, 2009b). The DNA methylation also induces H3K4 demethylation and H3K9 dimethylation (Cedar and Bergman, 2009b).

DNA methylation and histone modification on regulation of Ifng transcription

Regulation of DNA methylation at specific sites has been reported to be involved in regulation of *Ifng* transcription. In naïve CD4 T cells and Th2 cells most CpG dinucleotides at regulatory elements in *Ifng* are methylated, whereas most of the methyl groups on CpG dinucleotides are removed in Th1 cells (Chang and Aune, 2007). Likewise, there are more methylated DNA in *Ifng* of naïve CD8 T cells compared with effector and memory CD8 T cells (Kersh et al., 2006a). In addition, no methylation of DNA in the *Ifng* locus has been reported in fresh NK cells which express IFN-γ immediately upon stimulation (Tato et al., 2004). In accordance, NK cells express 200 times and memory CD8 T cells expression 20 times more IFN-γ than naive T cells (Kersh et al., 2006a; Matsuda et al., 2003). The importance of DNA methylation in regulation of IFN-γ transcription exists not only in different types of lymphocytes but also exists in age-related IFN-γ transcription. The promoter of the *Ifng* locus in naïve CD4 T cells is hypermethylated in neonatal humans compared to adults (White et al., 2002b).

Histone modification has also been documented in regulation of *Ifng* transcription. Most of the epigenetic regulation of IFN-γ transcription occurs at distal transcriptional regulatory elements, about 90kb up- and downstream of *Ifng*. In extremely low IFN-γ–expressing naïve CD4 T cells, there are lower levels of permissive dimethylated H3K4 ($H3K4^{me2}$) upstream of *Ifng* and small amounts of repressive dimethylated H3K27 ($H3K27^{me2}$) downstream of *Ifng* (Schoenborn et al., 2007). The *Ifng* locus of dominant IFN-γ expressing Th1 cells gains $H3K4^{me2}$ and loses of $H3K27^{me2}$ including the loci around most enhancers and some insulators (Schoenborn et al., 2007). In contrast, the

Ifng locus of Th2 cells obtains the repressive H3K27 ^{me2} throughout the locus, including loci around promoter and enhancers, except for histone insulator elements (Schoenborn et al., 2007).

The regulation of IFN-γ transcription in naïve T cell differentiation by alteration of DNA methylation, histone acetylation and histone methylation may result from the signals induced by TCR and/or cytokine stimulation, and the cascade of TFs binding to enhancer or insulator elements.

RESEARCH OBJECTIVES

The following studies were designed based on previous knowledge from other species and preliminary studies in equine neonates which have been reviewed in this body of work to investigate the effect of environment on IFNγ expression and the underlying regulatory mechanism. While IFN-γ plays an important role in defense against *R. equi* infection, the relationship between the IFNγ expression and the *R. equi* infection in foals remained to be clarified. Therefore, the relevance of the age-related IFN-γ expression and the age-related susceptibility to *R. equi* infection in foals was estimated. Regulation of IFN-γ expression has been reported to be controlled by lymphoproliferation and DNA methylation in humans and mice, but it is completely unknown in the horse. Thus, the role of lymphoproliferation and DNA methylation in regulation of IFN-γ expression was investigated. The environment has been observed to have an effect on IFN-γ expression in human neonates, but the effect of environment on IFN-γ expression and the underlying mechanisms remain unknown in foals. Therefore, the effect of environment on IFN-γ expression was evaluated and the underlying mechanisms were characterized regarding to lymphoproliferation and DNA methylation. Furthermore, the environmental effect on the maturation of lung APC phenotype in the foals was investigated.

Copyright © Lingshuang Sun 2012

CHAPTER TWO

Age-associated susceptibility to *R equi* infection in foals

SUMMARY

Rhodococcus equi (*R. equi*) pneumonia inflicts a significant economic impact on the horse industry worldwide and it is of great concern to equine farms. While adult horses are resistant to *R. equi*, foals exhibit a distinct age-associated susceptibility. However, the reason for this unique susceptibility remains unknown. Humoral and cell-mediated immunity play an important role in the defense against *R. equi* infection. Antibody production and Th1 immune response is impaired in foals. Therefore, we hypothesize that the susceptibility of foals to *R. equi* infection is age related and it correlates with the ability of specific antibody production post infection and induction of basal and *R. equi*-inducible Th1 cytokines at challenge. Younger (3 weeks) and older (6 weeks) foals were challenged with virulent *R. equi*. The severity of pneumonia on necropsy was scored and the culturable *R. equi* per gram of lung tissue was calculated. The conversion of antibodies was determined by VapA-ELISA, basal and *R. equi*-inducible Th1 and Th2 cytokine expression in peripheral blood were determined using RT-PCR. The 6-week-old foals were found to be not susceptible to *R. equi* infection in contrast with the 3-week-old foals which were 100% susceptible. Age was inversely correlated with the pneumonia score and the bacteria burden in the lung. The age-related susceptibility to *R. equi* infection correlated with the impaired basal and *R. equi*-inducible Th1 cytokine expression. This is likely due to an age-associated change in transcription factor expression, although both groups showed the same level of specific antibody production post infection. The basal expression of blood IFN-γ, IL-12 and IL-18, and *R.*

equi-stimulated IL-12 and GrzB may be useful as indicators for foals' risk of *R. equi* infection. In conclusion, the age-related foals' susceptibility to *R. equi* infection is likely due to an impaired basal and inducible Th1 immunity at challenge. Future studies on this age-related susceptibility in foals less than 3 weeks old are needed.

INTRODUCTION

Rhodococcus equi (*R. equi*), an intracellular, gram positive pathogen (Prescott, 1991), is one of the most important causes of respiratory disease in foals and it is of great concern to breeding farms worldwide (Machangu and Prescott, 1991). The disease ranks second behind injury as a leading cause of death in foals less than six months old (Giguere et al., 2011a; Prescott, 1991). The infection manifests as subacute or chronic pyogranulomatous bronchopneumonia with abscessation and may present associated to numerous extrapulmonary disorders, such as pyogranulomatous enterotyphylocolitis and polysynovitis (Giguere et al., 2011a). The costs of treatment and foal losses from infection on farms with endemic disease can be substantial (Giguere et al., 2011a; Giguere et al., 2011b; Prescott, 1991).

While adult horses are resistant to *R. equi*, foals exhibit a distinct age-associated susceptibility to *R. equi* infection. Although all foals are exposed to ubiquitous *R. equi* in the environment, pneumonia is observed almost exclusively in foals less than 6 months old, even in farms where *R. equi* is endemic (Giguere et al., 2011b; Prescott, 1991). Studies in adult horses showed that both humoral- and cell-mediated immune responses are important to protect against *R. equi* infection (Giguere et al., 2011a). Type 1 cellular immune response is sufficient to induce clearance of *R. equi* by IFN-γ mediated enhancement of the killing mechanisms of the infected macrophage, as it increases iNOS expression (Dawson et al., 2010). In addition, the cytotoxic T cell response is key to protect against infection by killing infected cells via granzyme B (GrzB) or IFN-γ secretion (Patton et al., 2004a). The antibody-mediated opsonization also significantly enhances *R. equi* killing by alveolar macrophages (Hietala and Ardans, 1987a).

Immunoglobulin G (IgG) promotes *R. equi* phagocytosis and participates in down-regulation of intracellular bacterial growth by enhancing phagosome-lysosome fusion improving bacterial killing (Cauchard et al., 2004).

While in adult horses the protective immunity against *R. equi* infection seems to rely on both humoral and cellular immune response, the mechanism of age-associated susceptibility in foals remains unknown. The naivety of the immune response is proposed to be a contributor (Dawson et al., 2010). Foals have delayed endogenous synthesis of IgG and IFN-γ production is significantly reduced for the first 12 weeks of age (Breathnach et al., 2006a; Holznagel et al., 2003). These differences between adult and foal immune responses may account for the difference to *R equi* susceptibility. While most studies focus on the immune response post challenge in foals, no studies have estimated the basal and *R. equi*-inducible immune response before challenge and the relevance between those immune factors and the foals' susceptibility to *R. equi* infection.

The purposes of this study are to evaluate the relationship between the susceptibility to *R. equi* infection and the age of foals; to estimate the relationship between the susceptibility to *R. equi* infection and the inducible antibody production post challenge; and to evaluate the relationship between the susceptibility to *R. equi* infection and basal and stimulated cytokine expression in foals at challenge. These parameters were used to estimate the risk of *R. equi* infection in foals. Therefore, foals 3 and 6 weeks of age were challenged with virulent *R. equi* and the severity of pneumonia was evaluated. Serum specific antibodies were determined post challenge, and basal and *ex vivo R. equi*-stimulated Th1 and Th2 cytokine gene expression was determined at challenge, as well as GrzB expression. The relevance of these factors to foals' susceptibility to *R. equi* infection and severity of pneumonia was evaluated.

MATERIALS AND METHODS

R. equi and culture

***R. equi* and culture.** The *R. equi* 103+ strain obtained from a frozen stock in 20% glycerol was streaked onto a Tryptic Soy Agar Yeast Extract (TSAYE). A single mucoid, creamy colony was selected and used to inoculate 4 ml of Tryptic Soy Broth Yeast Extract (TSBYE) and the bacterial broth was then incubated at 37°C for 48 hrs. An aliquot of this sample was sent to Kentucky Veterinary Diagnostic laboratory (UKVDL) for bacterial identification and PCR test for the presence of VAP. The optical density of the incubated broth was determined by a spectrophotometer at wavelength 540 nm with a 1/10, dilution of the sample. This information was used to dilute the culture media into PBS to a total volume of 25ml with a concentration of ~4×10^3 cfu/ml (total challenge dose ~1×10^5 cfu). At the same time, a serial dilution of the culture media was plated and the exact concentration of cfu/ml was calculated.

Foals and *R. equi* challenge

A total of 10 foals were included in the study. The mares and foals belonged to the Department of Veterinary Science's North Farm at University of Kentucky, Lexington, Kentucky. All research procedures were approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee and Guide for the Care and Use of Agricultural Animals in Research and Teaching. Foals were included if they had a normal complete physical examination (normal thoracic auscultation, normal rectal temperature), complete blood count, plasma fibrinogen concentration and thoracic ultrasonography, all of which were performed within 48h of birth. The foals were moved

to individual stalls after birth and transfer of maternal antibodies was determined using a SNAP® test (foals were included if IgG > 800mg/dl). None of the foals received any type of plasma or other treatment during the study.

Foals were challenged at 3 (18 to 24 days, n=3) or 6 weeks (39 to 54 days, n=7) of age using 10^5 cfu. *R. equi* per horse via intrabronchial instillation, as previously described (Horohov et al., 2011). Briefly, the foals were sedated and 25 ml of the bacteria in sterile PBS were delivered into both main bronchi. Foals were monitored daily for lethargy, inappetence, recumbency, cough, respiratory distress and extrapulmonary pathology. Physical examination and CBC were performed before and weekly after challenge for the duration of the study.

Gross pathology and bacteriology

The foals challenged at age of 3 and 6 weeks were euthanized and necropsied at 21 days and 59-63 days post infection (PI) respectively by a board-certified veterinary anatomic pathologist. Complete necropsies of all foals were performed at the University of Kentucky's Veterinary Diagnostic Laboratory. The lungs were weighed and the texture was scored as either normal, firm and/or consolidated. The pneumonia score was calculated as: Score=100×(percentage of firmed lung+percentage of consolidated lung).

Sterile samples were collected from the cranial apical, middle diaphragmatic, and dorsal diaphragmatic regions of the lung. The tissues were homogenized with sterile saline and serial dilutions of 0.025 ml were transferred onto TSAYE plates. Colonies were assessed by their characteristic morphology as gram positive coccobacillus bacterial colonies were observed and the culturable *R. equi* per gram of the lung tissue was calculated.

ELISA test for serum antibodies against *R. equi* VAP A

Serum antibody titers were determined in all foals before (<7 days of age) and post challenge using a slightly modified ELISA technique (Prescott et al., 1996). A total of 0.2ug/well of recombinant VAP A protein (courtesy Drs. Sturgill and Giguere, University of Georgia) mixed in carbonate bicarbonate buffer (C3041, Sigma) was plated on a 96 well ELISA plate (Immulon, 14-245-78, Thermo Scientific Inc., Rochester, NY) overnight at 4ºC. The plate was then blocked with 200µl of 0.5% polyvinylalkohol (PVA, Moviol® 6-98, Sigma) - PBS at room temperature (RT) for 1 hr. To evaluate total IgG, a 1:100 dilution of the serum was incubated at 37ºC for 1 hr followed by incubation with peroxidase-conjugated goat anti-horse IgG (Jackson ImmunoResearch, West Grove, PA) at 37ºC for 1 hr. For IgG subisotype evaluation a 1:25 dilution of the serum was used and incubated at 37ºC for 1 hr, then incubated with antibodies against horse IgGa (CVS48), IgGb (CVS39) or IgGT (CVS40) followed by incubation with horseradish peroxidase (HRP) conjugated goat anti mouse IgG (Bethyl Laboratories. Inc., Montgomery, TX). The plate was washed 3 times with 300µl of 0.05% Tween 20-PBS (pH=7.2) after each incubation reaction. The substrate development was performed by a Sur Blue™ TMB microwell Peroxidase substrate (52-00-02, KPL, Gaithersburg, MD) at RT for 5 min and the reaction was stopped by a 3,3',5,5'-Tetramethylbenzidine (TMB) stopping solution (50-85-05). Double absorbance was measured at A_{450} and A_{630}.

Isolation and stimulation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation, as previously described (Breathnach et al., 2006b), and cultured in RPMI 1640 media (Gibco, Grand Island, NY), supplemented with 2.5% fetal equine serum (FES; Sigma, St. Louis, MO), 2 mM glutamine (Sigma), 100 U/ml

penicillin/streptomycin (Sigma) and 55 mM 2-mercaptoethanol (GIBCO). 3×10^6 PBMC were pulsed with phorbol 12-myristate 13-acetate (PMA; 25 µg/ml; Sigma, p8139) /ionomycin (iono; 1 µM; Sigma) and brefeldin A (BFA; 10 µg/ml; Sigma) for 4 hrs.

Isolation and stimulation of BAL cells

Foals were sedated using xylazine (0.15 mg/kg; Butler Co., Dublin, OH), acepromazine (0.01 mg/kgH; Butler Co., Dublin, OH) and butorphanol tartrate (0.01 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). A sterile BAL tube was passed through the nasal passage, the trachea, into the distal airway until gently seated. Approximately 60 mls of saline (0.9% NaCl; Abbott Laboratories, Chicago, IL) were slowly instilled into the lung, then immediately withdrawn and the BAL fluid transferred to a sterile flask. This was repeated until a volume of 150 mls of BAL fluid was collected. The fluid was kept on ice until it was processed. The BAL fluid was centrifuged at 400g for 10 min and washed twice with PBS. The cell pellet was re-suspended in 10 mls of PBS prior to counting (Vi Cell XR, Beckman Coulter, Brea, CA) and the absolute number of viable cells was determined. 4×10^6 BAL cells were stimulated for IFN-γ production, as described above for PBMC.

Intracellular staining

Intracellular staining for IFN-γ was performed, as previously described (Adams et al., 2008; Breathnach et al., 2006b). Briefly, ~4×10^5 cells were fixed overnight with 2%-paraformaldehyde before being permeabilized with saponin buffer (0.1% saponin, 1% FBS, 0.1% NaN3). The cells were stained for IFN-γ by adding 100µl of CC302 (FITC conjugated mouse anti-bovine IFN-γ; Serotec, Raleigh, NC) or an isotype control antibody (FITC conjugated mouse IgG1, Serotec). Stained cells were acquired and analyzed using a FACS Calibur (BD, Franklin Lakes, NJ).

Blood stimulation and mRNA isolation

Blood (3ml) was collected by venipuncture into two green-top tubes (lithium heparin, BD) and a PAXGENE blood RNA tubes (PreAnalytiX, Valencia, CA) from all groups of foals at the day before challenge. 180 μl of killed *R. equi* (1×10^9CFU/ml) was aseptically added to one of the green-top tubes and the other was used as control. Both tubes were incubated in a humidified incubator for 24 hrs. The blood was then transferred to a PAXGENE tube. All PAXGENE tubes were processed and total mRNA was isolated using a manual protocol.

RT-PCR

Reverse transcription for total mRNA isolated from the PAXGENE tube was performed as described previously (Breathnach et al., 2006a). The resultant cDNA was diluted 1:1 with RNAse-free water. Gene expression was quantified by an Applied Biosystems 7900HP Sequence Detection System (Applied Biosystems, Foster City, CA). Intron-spanning equine specific Hlx, Eomes, IFN-γ, IL-1, IL-2, IL-6, IL-10, IL-12, IL-18, TNFα, GrzB, GATA3, FoxP3, and Tbet primer/probe sets were designed (Assays-by-Design, Applied Biosystems). The primer and probe sequences are shown in table 2.1. The selected primers and probes failed to amplify genomic DNA and reverse transcription-negative RNA samples and their efficiencies were greater than 95% as tested by LinRegPCR (Ramakers et al., 2003). PCR reactions were performed in duplicate wells per sample, as described previously (Breathnach et al., 2006a). The efficiency of the amplifications was tested by LinRegPCR and the reactions with efficiencies lower than 90% were omitted. Beta-glucuronidase (b-Gus) was used as a

house-keeping gene and the relative quantification (RQ) method for mRNA expression was used (Breathnach et al., 2006a).

Table 2.1 Primer and probe sequences

Target gene		Sequence or reference	Reference
IFN-γ	Fwd	AGCAGCACCAGCAAGCT	
	Rev	TTTGCGCTGGACCTTCAGA	
	Probe	ATTCAGATTCCGGTAAATGA	
IL4	Fwd	TGACTGTAGCGGATGCCTTTG	
	Rev	GCCCTGCAGATTTCCTTTCCAT	
	Probe	CTGGCCCGAAGAAC	
IL10	Fwd	AGGACCAGCTGGACAACATG	(Merant et al.,
	Rev	GGTAAAACTGGATCATCTCCGACAA	2009b)
	Probe	CCAGGTAACCCTTAAAGTC	
Tbet	Fwd	CGGGAAACTAAAACTCACAAACAACA	
	Rev	GCTCTCCATCATTTATCTCCACAATGT	
	Probe	ATGTGACCCAGATGATCG	
GATA3	Fwd	GCCTGCGGGCTGTACTAC	
	Rev	TGGATCCCTTCCTTCTTCATAGTCA	
	Probe	AAGCTGCACAATATTAAC	
FoxP3	Fwd	GGCAGCCACGGAAACAG	
	Rev	GCATGTTGTGGAACTTGAAGTAGTC	
	Probe	ACATTCCCAGAGTTCTTC	

Table 2.1 (continued)

GrzB	Fwd	GGACCCGAAGGAAAAGAAGTCTT	
	Rev	CCTGGATCACGTTCTTACACACAAG	
	Probe	CCGGAGTCCCCCTTAAA	
IL-12	Fwd	CTACACCAGCGGCTTCTTCAT	
	Rev	GCTTCAGCTGCAGGTTCTTG	
	Probe	CAGGGACATCATCAAACC	
IL-18	Fwd	CCTGTGTTTGAGGATATGCCTGATT	
	Rev	GCTAGACCTCTAGTGAGGCTATCTT	
	Probe	ATTGTACAGACAACGCACCC	
IL-6	Fwd	GGATGCTTCCAATCTGGGTTCAAT	(Merant et al.,
	Rev	CCGAAAGACCAGTGGTGATTTT	2009b)
	Probe	ATCAGGCAGGTCTCCTG	
IL-13	Fwd	CCTGGAGTCCCTGAGCAA	
	Rev	CATCTTCCGCGTGTTTTGGAT	
	Probe	TCTCCACCTGCAGTGCC	
TNFα	Fwd	TTACCGAATGCCTTCCAGTCAAT	(Quinlivan et al.,
	Rev	GGGCTACAGGCTTGTCACTT	2007)
	Probe	CCAGACACTCAGATCAT	
β-	Fwd	GCTCATCTGGAACTTTGCTGATTTT	(Quinlivan et al.,
glucuronidase	Rev	CTGACGAGTGAAGATCCCCTTT	2007)
(b-Gus)	Probe	CTCTCTGCGGTGACTGG	

Statistical analysis

All data were analyzed using a commercially available statistical software package (Sigma Stat version 10.0; Systat, San Jose, CA). A Two-way ANOVA was used to test the statistical significance for serum antibodies with groups and age of foals as two parameters. Some of the data were log-transformed or rank-transformed to meet the assumptions of normality and equal variance. A student's t-test was used for statistical analysis between two age groups of foals for other assays and the differences were considered significant at $p < 0.05$. A Mann-Whitney Rank sum test was used if the normality test failed to meet the assumption of t-test. The linear regression was used for the analysis of the correlation association, and $p<0.05$ was considered to be significantly correlated.

RESULTS

Age-related *R equi* infection in foals

The challenge dosage for the 3-week-old foals was 3×10^5 and for the 6-week-old foals was $1.8\pm1.3\times10^5$ cfu per horse. There was no difference in the challenge dose between the two groups. While none of the foals developed clinical signs of disease, *R. equi* was isolated from the lungs of all the foals challenged at 3 weeks old (100%), and from one of the foals challenged at 6 weeks of age (14.29%). The younger foals had significantly higher numbers of culturable *R. equi* ($4.1\pm6.9\times10^8$ cfu per gram of lung tissue) than older foals (140 cfu/gram in older foals) ($p=0.038$, Table 2.2). In addition, all the younger foals showed pulmonary lesions (100%), whereas only 57.14% of the older foals had lung lesions. Three-week-old foals had higher pulmonary lesion scores

(p=0.013) when compared with the 6-week-old foals (Table 2.2). The percentage of consolidated lung in *R. equi* challenged foals correlated with the age at challenge (p=0.0461, Table 2.3). A positive correlation between the age at challenge and the number of culturable *R. equi* per gram of lung tissue (p<0.001, Table 2.3) and the pulmonary lesion score (p=0.089, Table 2.3) was observed.

Table 2.2. Age-associated susceptibility to *R. equi* challenge in foals

Age of foals (weeks)	Dosage of challenge ($\times 10^5$cfu)	Infection Rate (%)	Lung bacterial burden (cfu/g lung)	Percentage of foals with pulmonary lesion	Pulmonary lesion score
3	3.0	100	$4.1 \pm 6.9 \times 10^8$*	100	5.1±0.46*
6	1.8±1.3	14.29	140	57.14	2.9±4.07

The asterisk indicated a significant difference between the younger and older foals, p<0.04.

Table 2.3. The correlation between age of foal and its susceptibility to *R. equi* infection.

Correlation with age	Bacteria burden in the lung (log (CFU/g lung))	Pulmonary texture			Pneumonia Score
		Normal (%)	Firm (%)	Consolidation (%)	
R^2	-0.906	0.586	-0.536	-0.640	-0.552
p	<0.001	0.075	0.111	0.0461	0.089

Note: The bacteria burden in the lung (log (cfu/g lung)) was given 0 if there were no *R. equi* identified since log(0) is negatively infinite and cannot be compared.

Capacity of *R. equi* specific antibody conversion in response to challenge was not age correlated

The capacity of sera-conversion (total IgG and its subisotypes IgGa, IgGb and IgGT) of foals challenged with *R. equi* at 3 and 6 weeks of age was determined and compared. While no Vap A-specific antibody was observed in either group of foals at birth and before challenge (Figure 2.1), a significant increase of total *R. equi*-specific IgG was observed post infection in both groups ($p<0.04$, Figure 2.1). There was no difference in the antibody production post challenge between the two groups (Figure 2.1). In addition, no difference was observed for specific IgGa and IgGb before or after challenge between the two groups (Figure 2.1), though they were significantly elevated post challenge. There was no difference in *R. equi*-specific IgGT between the two groups of foals post challenge or between before and after challenge. Moreover, no significant correlation was found between the serum antibody level and the lung lesion score (data not shown).

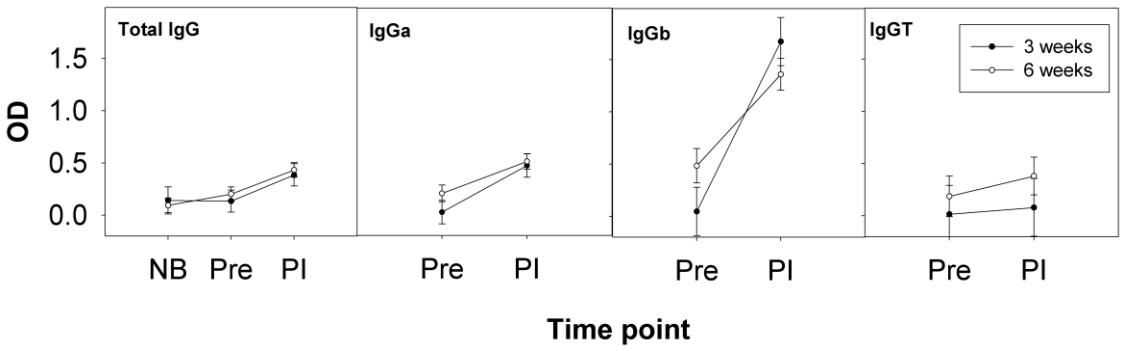


Figure 2.1. The same level of specific antibody to VapA was observed post *R. equi* challenge in the younger and the older foals. The blood was drawn from both 3-week-old and 6-week-old foals at birth (NB), before challenge (Pre) and 21 days post infection (PI).

The sera were collected and the specific total IgG and subisotype IgGa, IgGb, and IgGT against VapA were measured by the ELISA with serum dilution of 1:100 for the total IgG test and 1:25 for the IgG subisotype test.

The *in vivo* basal Th1 cytokine gene expression in foals

The *in vivo* basal mRNA expression of Th1, Th2 and Treg cytokines and their representative regulating transcription factors (TF) were determined at the day of challenge by PCR and the relationships of the gene expression with the number of isolated *R. equi* in the lung tissue and the pulmonary lesion score were analyzed. There was no difference between the two age groups of foals in basal Th1 (IFN-γ, IL-12 and IL18), Th2 (IL-4, IL-13) and Treg (IL-10) cytokine mRNA expression (Figure 2.2). Th1 cytokines IFN-γ (p=0.003), IL-12 (p=0.038) and IL-18 (p=0.022) as well as the Treg cytokine IL-10 (p=0.011) exhibited a significant negative correlation with the lung lesion score (Table 2.4). However, no significant association was found between the Th2 (IL-4 and IL-13) cytokine and age or lung lesion score (data not shown). There was a significant positive correlation between mRNA expression of GATA3 and FoxP3 (p=0.003, and p=0.02, respectively) with the pneumonia score and a negative correlation (p=0.003 and p=0.005, respectively) with the age of foals (Table 2.4). No correlation was observed in Tbet mRNA expression and the age of foals or the pneumonia score (data not shown) even though the expression was higher (p=0.017) in the 3 week old foals when compared to the 6-week-old foals (Figure 2.2). In addition, there was no correlation observed between the pneumonia score (data not show), and the gene expression of any inflammatory cytokines (data not shown), although TNFα (p=0.011) and IL-6 (p= 0.008) exhibited higher at levels in the 3-week-old than in the 6-week-old foals (Figure 2.2).

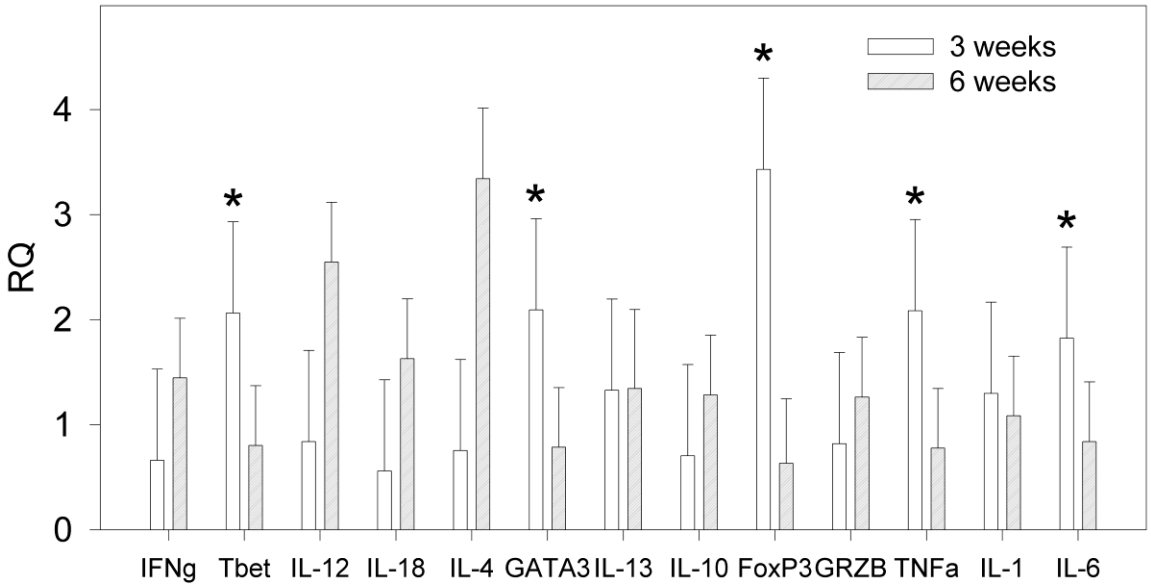


Figure 2.2. The basal cytokine gene expression *in vivo* in peripheral blood from foals. The blood was drawn from both 3- and 6- week old foals into a Paxgene tube. The total mRNA was isolated following reverse transcription and real time quantitative RT-PCT. The t-test or Mann-Whitney Rank sum test was used for statistical analysis though the graph was drew after two-way ANOVA analysis. *The mean RQ of the mRNA was significantly different between two groups of foals $p<0.03$.

Table 2.4. The correlation of basal gene mRNA expression with the age of foals at challenge and the susceptibility to *R. equi* infection.

Correlation		**IFN-γ**	**IL-12**	**IL18**	**GATA3**	**IL-10**	**FoxP3**
Age	R^2	No	No	0.450	-0.832	No	-0.834
	P	No	No	0.192	0.003	No	0.005
Pneumonia	R^2	-0.803	-0.652	-0.696	0.809	-0.747	0.741
Lesion Score	P	0.003	0.038	0.022	0.003	0.011	0.020

The *ex vivo R. equi*-stimulated cytokine expression in foals

To evaluate the inducible cellular immune response to *R. equi* at challenge, the Th1, Th2 and Treg cytokine and associated TF mRNA expression by killed *R. equi*-stimulated whole blood were determined and their correlation with age and *R. equi* infection was analyzed. At challenge time point, the stimulated mRNA expression of IFN-γ (p=0.024), IL-12 (p=0.022) and GrzB (p=0.047) was significantly higher in older foals when compared to younger foals, though no difference was found in IL-18 and Tbet mRNA expression (Figure 2.3). In addition, the inducible IL-12 (p=0.048, p=0.088, respectively) and GrzB (association with age, p=0.053; association with pneumonia score, p=0.098) expression in response to *R. equi* stimulation before challenge demonstrated a positive correlation with the age and a negative correlation with the developed pneumonia score post challenge (Table 2.5). A significant negative correlation was also observed for *R. equi*-inducible mRNA expression of Tbet and pneumonia score (p=0.039). On the contrary, gene expression of cytokine IL-10 (p=0.060) and its representative TF, FoxP3 (p=0.033) decreased with age in response to *R. equi* stimulation (Figure 2.3). FoxP3 appeared a negative correlation with age and positive correlation with the pneumonia score (p=0.003, Table 2.5). No difference was found in GATA3 and TNFα expression between the two groups and no correlation observed between them and age or pneumonia score (Figure 2.3 and Table 2.5). In addition, the mRNA for IL-4 and Il-13 was undetected in the younger and un-stimulated whole blood of the older foals.

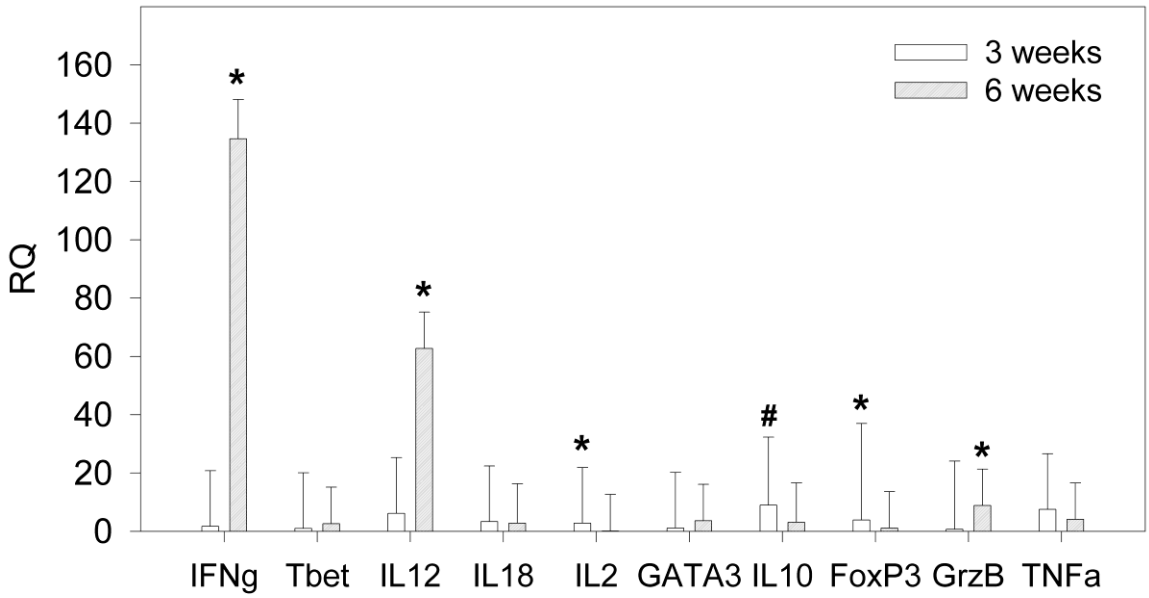


Figure 2.3. The *ex vivo R. equi*-stimulated peripheral blood gene expression in the foals. The blood was drawn from foals of 3 and 6 weeks age into pre-heparinized green top tubes. The blood was either stimulated with killed R. equi or nothing (control) for 24 hrs then transferred to the Paxgene tubes. The total mRNA was isolated from the tubes followed by reverse transcription and RT-PCR. The RQ was calculated with the control as the calibrator. The t-test or Mann-Whitney Rank sum test was used for statistical analysis while the graph was drawn after two-way ANOVA analysis since the variable of RQ and gene was significant differently (p=0.001). *The mean RQ of the mRNA was significantly different between two groups of foals, $p < 0.05$. #Means RQ of mRNA differ between the two groups of foals, p=0.06.

Table 2.5. The correlation of *ex vivo R. equi*-stimulated gene mRNA expression with age of foals at challenge and the susceptibility to *R. equi* infection.

Correlation		**IFN-γ**	**Tbet**	**IL-12**	**GrzB**	**IL-10**	**FoxP3**
Age	R^2	No	No	0.636	0.661	-0.777	-0.727
	p	No	No	0.048	0.053	0.023	0.017
Pneumonia	R^2	No	-0.656	-0.567	-0.586	No	0.836
Lesion Score	p	No	0.039	0.088	0.098	No	0.003

Note: the "No" means no correlation was detected with p>0.2.

The *in vitro R equi*-stimulated IFN-γ expression by the lung lymphocytes was elevated in older foals

The mean fluorescent intensity (MFI) of IFN-γ-producing BAL lymphocytes in response to killed *R. equi* stimulation from 6-weeks-old foals was significantly higher than that of 3-week-old foals (p=0.041, Figure 2.4, upper left), whereas there was no difference in MFI of IFN-γ^+ lymphocytes with PMA stimulation (Figure 2.4, upper right). In addition, the MFI of IFN-γ^+ BAL lymphocytes in response to killed *R. equi* stimulation demonstrated a positive correlation with the age of foals (p=0.028, Figure 2.5, upper left). No difference was observed between the two groups in the percentage of IFN-γ^+ BAL lymphocytes with either killed *R. equi*- or PMA- stimulation (data not shown), and no correlation was observed between the percentage of IFN-γ^+ cells and the age. Compared with BAL lymphocytes, the IFN-γ production by peripheral blood lymphocytes exhibited no difference between the two groups of foals in term of frequency (data not shown) and MFI of IFN-γ^+ cells neither in response to *R. equi* or PMA stimulation (Figure 2.4, lower

panel). There was no correlation observed between the production of IFN-γ and the age of foals (Figure 2.5, lower panel).

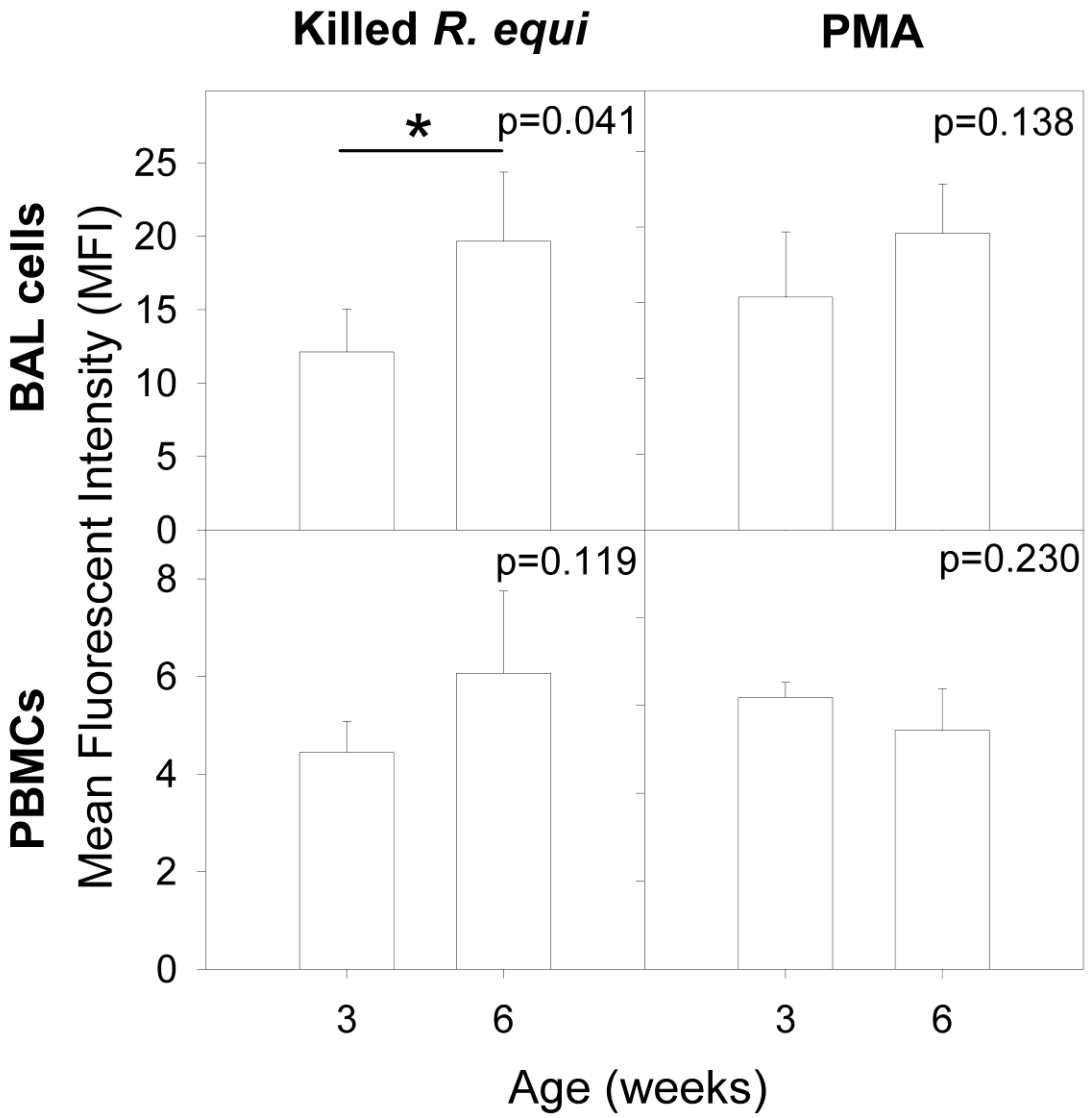


Figure 2.4. *In vitro* PMA- or *R. equi*-stimulated IFN-γ expression by lymphocytes in BAL or PBMC from foals. The PBMCs and the BAL cells were isolated from foals of 3- and 6- weeks- age. Both cells were stimulated either with PMA/iono for 4 hrs or with killed *R. equi* for 24 hrs. The IFN-γ expression was determined with intracellular

staining. The cells were acquired by FACSCalibur and the MFI of IFN-γ^+ cells were then analyzed. * The Mean MFI are significantly different between two groups of foals.

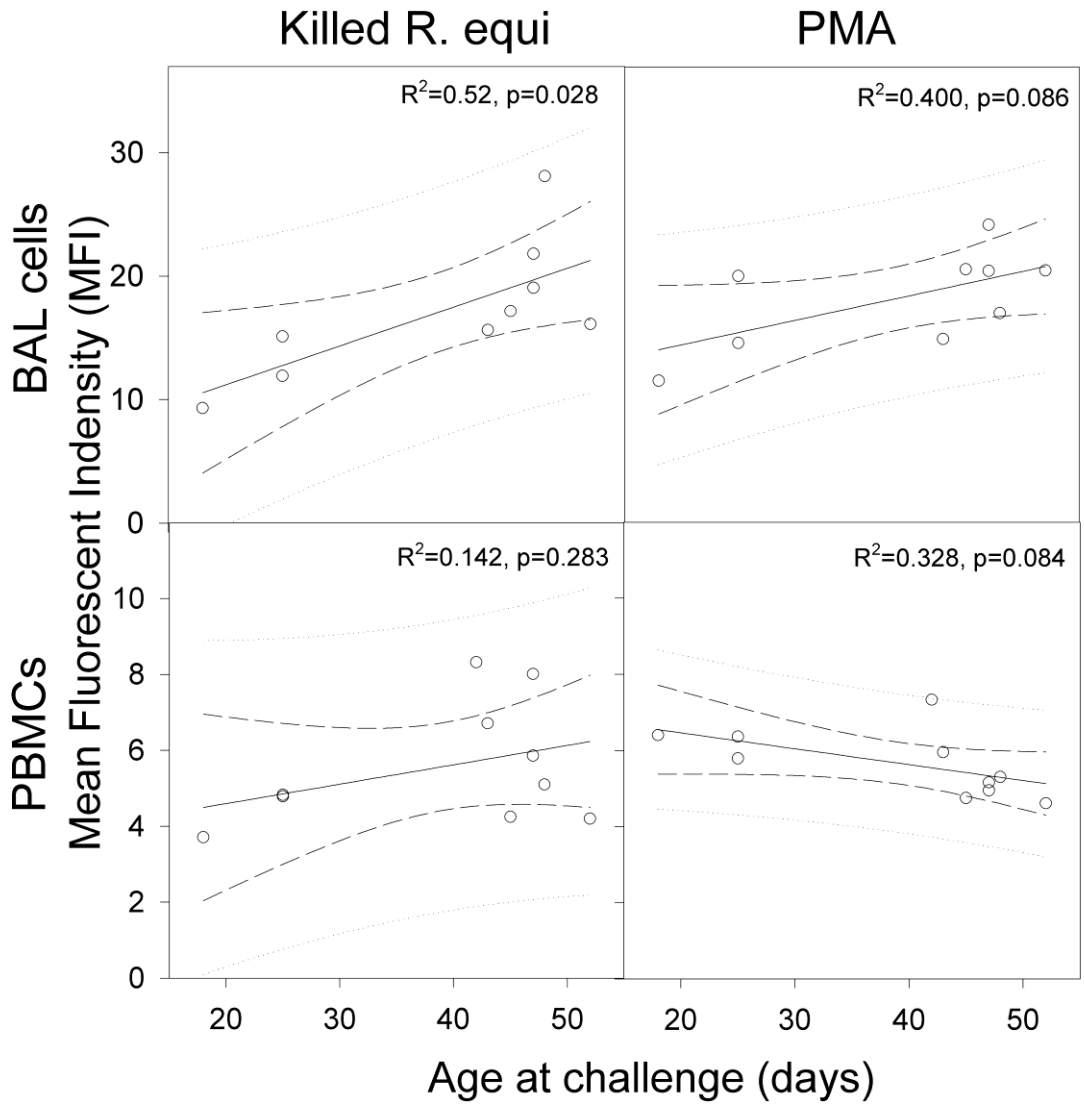


Figure 2.5. Correlation of age and *in vitro* PMA- or *R. equi*-stimulated IFN-γ expression by lymphocytes in BAL and PBMC from foals.

DISCUSSION AND CONCLUSIONS

While adult horses are resistant to *R. equi*, foals have a documented distinct susceptibility to *R. equi* infection (Meijer and Prescott, 2004). An age-related

susceptibility to *R. equi* infection has been reported in foals via epidemiological survey based on naturally infected cases (Meijer and Prescott, 2004). In our study using an experimental challenge model, 6 week old foals were almost not susceptible to *R. equi* as indicated by the lower rate of isolated *R. equi* from the lungs, in contrast to the 3-week-old foals which were all positive to *R. equi* culture. It could be argued that the lower rate of *R. equi* isolation observed in the older foals may reflect clearance of bacteria that was possible because they were euthanized at a later time than were the 3-week-old foals. Resistance is also supported by the fact that the older foals had a lower rate of lung lesions that were present in all the younger foals. Moreover, the pneumonia score was inversely correlated with the age of the foals. Thus, these findings provides for the first time direct experimental evidence to support the theory that foals' susceptibility to *R. equi* infection is age-dependent.

This susceptibility is proposed to be associated with the naivety of foals' immunity (Breathnach et al., 2006a; Harris et al.; Merant et al., 2009b; Pargass et al., 2009). While B cells are immunologically competent, their endogenous synthesis and production of specific antibodies assisted by helper T cells is impaired in neonatal foals (Giguere and Polkes, 2005b). The specific antibody-induced opsonization is important for killing of *R. equi* by macrophages as it promotes phagosome-lysosome fusion (Cauchard et al., 2004). In our study, the same level of *R. equi* specific IgG was observed in both groups of foals after their first encounter with *R. equi*. *R. equi*-specific antibody induction was also found in neonatal foals after both experimental challenges and natural infection (Hooper-McGrevy et al., 2003; Jacks et al., 2007b). As no *R. equi*-specific antibodies were detected at birth or before challenge, it can be concluded that the

capacity of sera-conversion to *R. equi* is competent in young foals; hence it may not be responsible for foals' age-dependent susceptibility to *R. equi* infection.

The subisotypes of IgG have different functions: IgGa and IgGb opsonize microbes and fix complement, whereas IgGT may competitively inhibit the fixation and opsonization of IgGa and IgGb, and has weak leukocyte respiratory burst (Lewis et al., 2008) (Banks and McGuire, 1975; McGuire and Bariso, 1972). Because of this, IgGa and IgGb are considered the most important antibodies against *R. equi* (Lewis et al., 2008; Taouji et al., 2004). In our study, both groups of foals produced VapA-specific IgGa and IgGb antibodies but no IgGT antibodies, similar to what has been reported (Hooper-McGrevy et al., 2005; Jacks et al., 2007b). No difference in IgGa and IgGb induction between susceptible and non-susceptible foals was observed in our study. This indicates that the age-related susceptibility to *R. equi* is neither due to the inability to produce the protective subisotypes of specific antibodies (IgGa and IgGb) nor due to the overwhelming induction of detrimental specific antibodies (IgGT). Additionally, IgG subisotypes reflect T-cell responses which influence class switching in B cells, with IgGb and IgGT associated with a Th2 (IL-4) response and IgGa associated with a Th1 (IFN-γ) response (Hooper-McGrevy et al., 2003; Jacks et al., 2007b). In our study, both Th1 and Th2 subisotypes of IgG were induced properly in 3-week old susceptible foals suggesting that an improper humoral immune response to *R. equi* infection in term of B cell class switching and humoral immune response is not responsible for foals' age-dependent susceptibility to *R. equi* infection.

While humoral immune response to *R. equi* challenge appeared appropriate, the cell-mediated immune response was impaired as both basal *in vivo* and *ex vivo R. equi*-

stimulated cytokine expression was impaired in younger susceptible foals. Both Type 1 and Type 2 cell mediated immunity are immature in foals, represented by impaired Th1 (IFN-γ) and Th2 (IL-4) cytokine expression (Breathnach et al., 2006a). Expression of basal IFN-γ is reduced in foals and in other neonatal mammals (Hines et al., 2003a; Kanaly et al., 1996a; Lopez et al., 2002). Our study shows, for the first time, an inverse correlation between basal IFN-γ mRNA expression in blood and the severity of *R. equi* pneumonia in foals. Thus, the basal *in vivo* IFN-γ mRNA in blood may be used in the future as a risk indicator of *R. equi* infection. In addition, *R. equi*-stimulated IFN-γ mRNA expression in blood was significantly impaired in susceptible foals at challenge when compared to older foals. Similar impaired IFN-γ mRNA expression was also observed in *R. equi*-stimulated PBMCs from neonatal foals (Liu et al., 2011b; Liu et al., 2009c) and in foals post virulent *R. equi* challenge (Giguere et al., 1999). In addition, IFN-γ production by *R. equi*-stimulated BAL lymphocytes in susceptible foals was also impaired compared to non-susceptible foals. The age-correlated IFN-γ production by BAL lymphocytes but not peripheral lymphocytes suggests that the *R. equi*-stimulated IFN-γ production by BAL lymphocytes is a possible indicator for foals' risk to *R. equi* infection. The inability to mount a Th1 cytokine response at first encounter with *R. equi* may be due to a higher basal expression of IFN-γ-suppressive TF, such as GATA3 and FoxP3, and inability to elevate Tbet expression in response to *R. equi* infection as observed in our study.

In our study the basal expression of other Th1 cytokines, such as IL-12 and IL-18 mRNA expression were also impaired in the young foals and were negatively correlated with foals' susceptibility to *R. equi* infection. The impaired production of IL-12 and IL-

18 was reported in human neonates and it was thought to be the basis of the deficiency in neonatal innate immunity (Levy, 2007; Marodi, 2006a). A similar impaired basal IL-12 and IL-18 expression was also shown in neonatal foals (Levy, 2007; Marodi, 2006a). In addition, *R. equi*-stimulated IL-12 expression was impaired in the younger *R. equi* susceptible foals compared to the older ones, and had a negative correlation with the foals' susceptibility to *R equi* infection. Similar finding was reported when neonatal foals were compared with 8-week-old foals (Liu et al., 2009c). While Th1 cytokines promote macrophages killing of *R. equi* via induced IFN-γ expression, cytotoxic T cells lyse *R. equi*-infected cells by secreting GrzB (Hines et al., 2001; Hines et al., 2003a; Patton et al., 2004a). *R. equi* specific cytotoxic T cells are absent in 3 week-old foals and appear only after 6 weeks of age.(Patton et al., 2005) Our results support this finding as the *R. equi*-stimulated expression of GrzB was observed impaired in susceptible 3-week-old foals. In addition, we found that the inability of GrzB expression on respond to *R. equi* stimulation was correlated with the severity of *R. equi* pneumonia. Our findings indicate that basal expression of blood IL-12 and IL-18, and *R. equi*-stimulated IL-12 and GrzB may be possible indicators of the risk of *R. equi* infection.

In contrast to the beneficial effect of the type 1 cellular immune response to *R. equi*, the type 2 immune response was found to be detrimental in mice (Kanaly et al., 1995a, 1996a) and it was assumed to be detrimental in foals (Dawson et al., 2010). A higher magnitude of IL-10 was observed in foals challenged with a virulent strain of *R. equi* when compared with those infected with a non-virulent strain, hence IL-10 was considered to be immunopathogenic.(Giguere et al., 1999) And the elevation of IL-10 is observed in *R. equi* infected foals but not in adult horses. A similar trend of increase in *R.*

equi-stimulated IL-10 expression was also observed in this study. However, IL-10 expression after *R. equi* stimulation was not correlated with the severity of *R. equi* pneumonia in contrast to basal IL-10 expression which was negatively correlated. There was no correlation between basal IL-4 expression and severity of pneumonia. This indicates that the age-associated severity of *R. equi* pneumonia is not likely due to the elevated basal or inducible elevated Th-2 cytokine expression.

This study is limited by the age-range of the foals. Studies in younger foals are needed to further unveil the mechanism for foals' susceptibility to *R. equi* infection. A dose that mimics natural infection is needed to further assess the role of Th1 cytokines as an indicator in the age-associated susceptibility to *R. equi* in younger foals.

Despite these limitations, this is the first estimation of statistical correlation between age of foal susceptibility to *R. equi* challenge and the basal and inducible cytokine expression at challenge. Six-week old foals were seldom susceptible to *R. equi* infection when the experimental model described in this study was used, in contrast to that observed in 3-week-old foals. Based on our findings, this age-correlated susceptibility to *R. equi* is probably due to an impaired basal and *R. equi*-inducible Th1 cytokine response rather than overwhelming Th2 cytokine expression, and the IFN-γ expression is suggested as an indicator for the risk estimation of *R. equi* infection in foals. This is likely due to the age-related regulation of transcription factors.

Copyright © Lingshuang Sun 2012

CHAPTER THREE

The role of proliferation in the regulation of interferon gamma (IFN-γ) expression in foals

Dev Comp Immunol. 2012 Mar; 36(3):534-9.
Reprinted with permission

SUMMARY

Interferon-gamma (IFN-γ) plays an important role against viral and intracellular bacterial infections and its production is deficient in foals. Cellular proliferation provides an opportunity for *de novo* gene expression, though little is known about its role in regulating IFN-γ expression in foals. While stimulation of foal peripheral blood mononuclear cells (PBMC) with concanavalin A (Con A) increased the frequency of IFN-γ^+ cells, the overall percentage of IFN-γ^+ cells remained below that of adults. By contrast, the proliferative response of foal PBMC was significantly greater than that of the adults. In foals, IFN-γ production was predominantly associated with those T cells that underwent proliferation, whereas in adults non-dividing cells also produced IFN-γ. While treatment with hydroxyurea inhibited cellular division, it failed to completely block IFN-γ production. This residual IFN-γ production likely represented memory cells as the proportion of these proliferation-independent IFN-γ^+ cells increased with foal age. However, memory cells may not account for all of the IFN-γ production as Con A stimulation likely provided additional signals that can control IFN-γ expression.

INTRODUCTION

Interferon gamma (IFN-γ) plays an important role against viral and intracellular bacterial infections by enhancing the microbicidal function of macrophages, increasing the cytotoxic activity of NK cells, driving the differentiation of naïve T cells into Th1 cells, increasing the activity of cytotoxic T cells and inducing MHC I and MHC II expression on infected cells (Lewis and Wilson, 1990). In mice, the resistance to *Rhodococcus equi* (*R. equi*) depends on IFN-γ production by $CD4^+$ T cells (Kanaly et al., 1995b, 1996b). In adult horses, clearance of virulent strains of *R. equi* from the lungs is also associated with the production of IFN-γ by T cells (Hines et al., 2003b; Patton et al., 2004b). However, IFN-γ production is deficient in foals (Boyd et al., 2003; Breathnach et al., 2006b) and this deficiency is thought to contribute to their susceptibility to *R. equi* infection (Marodi, 2006b). Reduced IFN-γ expression has also been observed in human and murine neonates (Lewis et al., 1991; Wilson et al., 1986b). In human neonates, this deficiency increases the risk for infection with *Mycobacterium tuberculosis* (Aubert-Pivert et al., 2000). Thus, IFN-γ is necessary for the prevention of various infections (Gasparoni et al., 2003; Schoenborn and Wilson, 2007), yet little is known regarding its regulation early in life.

While the underlying mechanism responsible for this deficiency in IFN-γ production remains unknown, the immunological naivety of neonates likely plays a role since naive T lymphocytes produce IFN-γ only after undergoing multiple rounds of cellular division (Gett and Hodgkin, 1998; Gudmundsdottir et al., 1999). This is due to the fact that the DNA structure in naïve lymphocytes is less accessible for *Ifng*

transcription (Avni and Rao, 2000). Stimulation of the immune system leads to cellular proliferation which provides the opportunity for *de novo* gene expression (Bird et al., 1998). However the role of cellular proliferation in regulating *Ifng* expression in foals is unknown. Therefore, we examined the relationship between cellular proliferation and IFN-γ expression by foal and adult T lymphocytes in response to mitogen stimulation.

MATERIALS AND METHODS

Animals

A group of 12 foals (2 to 16 weeks old), 10 adult horses (10 years old) and 5 old horses (21 to 26 years old) were used in this study. The horses were housed at the Department of Veterinary Science's equine facility in Lexington, Kentucky. All horses were kept on pasture with *ad libitum* access to water and forage and handled in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching. All research procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

Peripheral blood mononuclear cells (PBMC)

Heparinized blood was collected from each horse by aseptic jugular venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation, as previously described (Breathnach et al., 2006b), and cultured in RPMI 1640 media (Gibco, Grand Island, NY), supplemented with 2.5% fetal equine serum (FES; Sigma, St. Louis, MO), 2 mM glutamine (Sigma), 100 U/ml penicillin/streptomycin (Sigma), 55 mM 2-mercaptoethanol (GIBCO).

Proliferation assays

Proliferation was assessed using flow cytometry, as previously described (Adams et al., 2008), with minor changes. Briefly, 1×10^7 PBMC were suspended in 1ml PBS (Sigma,) and stained with 1 ml of carboxyfluorescein succinimidyl ester (CFSE; 5µM/ml, Sigma) for 8 min. The reaction was quenched by adding 2 mls of FBS (Sigma), followed by two washes with PBS supplemented with 10 % FBS. The cell pellet was then re-suspended in 5 ml of cRPMI media (final concentration of 2×10^6 cells / ml). The CFSE – labeled PBMC were transferred to a 24-well-plate at 1 ml/well. The cells were then stimulated with 3 µg/ml concanavalin A (ConA; Sigma) or incubated with medium alone for 4 days. Afterwards, the cells were washed with FACS Flow and analyzed with a FACSCalibur (BD, Franklin Lakes, NJ) flow cytometer. The Proliferation Index (PI) was calculated using ModFit LT™ (Version 3.0, Verity Software House, Inc., Topsham, ME). To inhibit proliferation, CFSE-labeled PBMC were incubated with1mM hydroxyurea (HU; Sigma, H8627) for 1 hr at 37 ºC prior to stimulation with ConA, as above.

Surface and Intracellular staining

Surface staining was performed, as previously described (Merant et al., 2009a). Cells were labeled with primary antibodies to CD3 (F6G.3), CD4 (CVS4), CD8 (F18H.2) or an IgG1 isotype control (BD Pharmingen™, Sparks, MD) followed by fluorescent labeling with secondary antibodies (PE/Cy5.5 conjugated goat Fab' anti-mouse IgG [Southern Biotechnology Associates, Birmingham, AL]). The PI of lymphocyte subtypes was analyzed by gating on the $CD3^+$, $CD4^+$ or $CD8^+$ CFSE-labeled cells within the lymphocyte gate during acquisition and analyzing the resulting data using ModFit LT™.

For IFN-γ analysis, ConA-stimulated PBMC were pulsed with phorbol 12-myristate 13-acetate (PMA; 25 µg/ml; Sigma, p8139) /ionomyocin (iono; 1 µM; Sigma) and brefeldin A (BFA; 10 µg/ml; Sigma) during the last 4 hrs of incubation. Intracellular staining for IFN-γ was performed, as previously described (Adams et al., 2008; Breathnach et al., 2006b) with minor modifications. Briefly, ~4×10^5 cells were fixed overnight with 2%-paraformaldehyde before being permeabilized with saponin buffer (0.1% saponin, 1% FBS, 0.1% NaN3). The cells were stained for IFN-γ by adding 100µl of CC302 (PE conjugated mouse anti-bovine IFN-γ; Serotec, Raleigh, NC) or an isotype control antibody (PE conjugated mouse IgG1, Serotec). Stained cells were acquired and analyzed using a FACSCalibur (BD, Franklin Lakes, NJ). For IFN-γ staining of T cell subsets, surface labeling was performed prior to intracellular staining. IFN-γ expression by lymphocytes subtypes was analyzed by gating on the $CD3^+$, $CD4^+$ or $CD8^+$ FITC-labeled cells within the lymphocyte gate during acquisition and then assessing PE staining for IFN-γ.

Statistical analysis

All data were analyzed using a commercially available statistical software package (Sigma Stat version 10.0; Systat, San Jose, CA). Some of the data were log-transformed or rank-transformed to meet the assumptions of the ANOVA. Either two-way ANOVA or two-way ANOVA with repeated measures was used to test for statistical significance. Differences were considered significant at $p < 0.05$.

RESULTS

ConA stimulation increase IFN-γ expression by PBMC in foals

Since entry into the cell cycle provides an opportunity for gene expression (Bird et al., 1998), stimulating PBMC to proliferate has the potential to promote IFN-γ expression (Gudmundsdottir et al., 1999). Stimulation of adult PBMC with ConA significantly increased the percentage of IFN-γ^+ lymphocytes (Figure 3.1). While stimulation of foal ($\leq$ 6 weeks of age) PBMC with ConA also increased the percentage of IFN-γ^+ lymphocytes over time, the overall percentage of foal cells producing IFN-γ remained less than that of the adults (Figure 3.1).

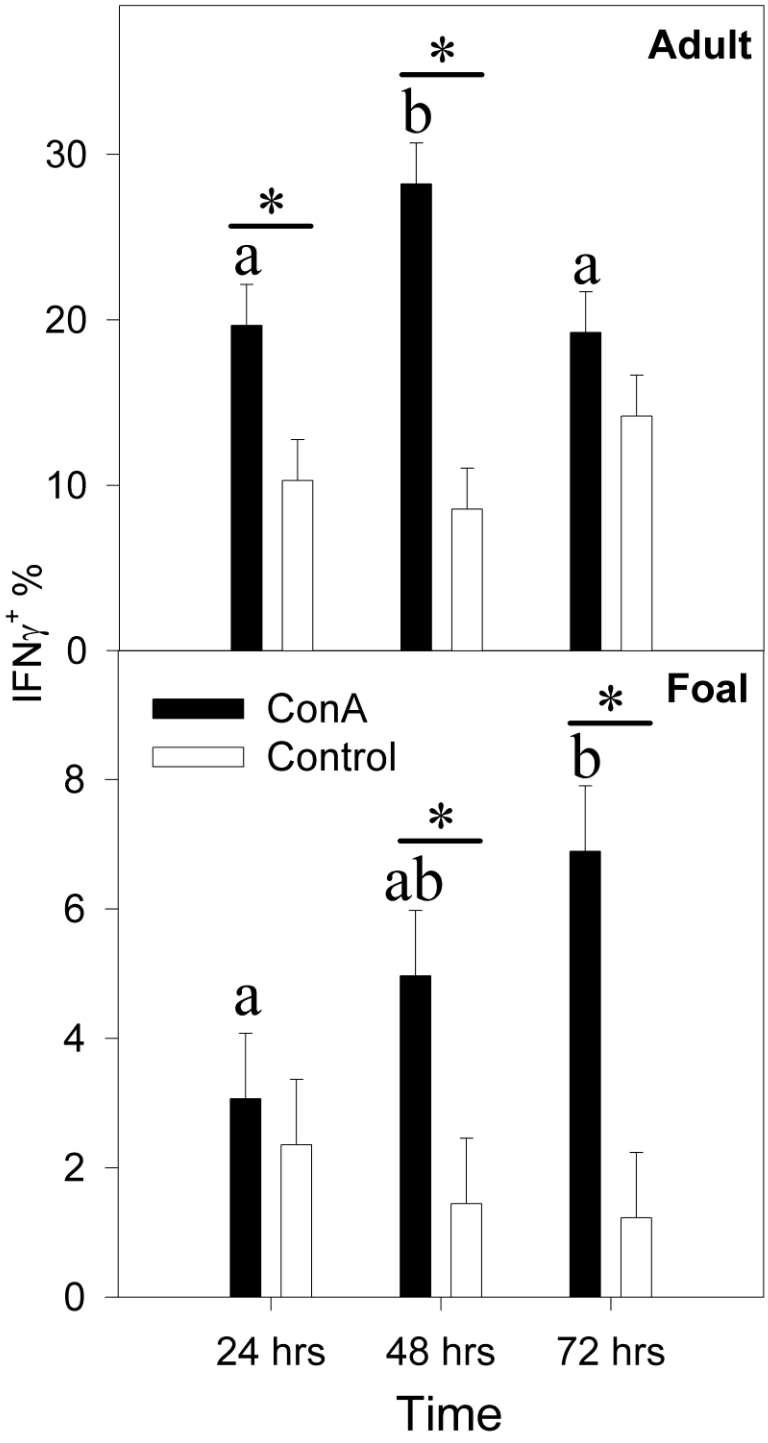


Figure 3.1. IFN-γ expression by ConA-stimulated PBMC from foals and adult horses. PBMC from 5 foals (≤ 6 weeks , n=4) and adult horses (10yrs, n=4) were

stimulated with ConA or medium (Control) for 24, 48 and 72 hrs then pulsed with PMA/iono and BFA for the last 4 hrs before intracellular staining for IFN-γ. While ConA stimulation increased IFN-γ expression in the adult and foal PBMC cultures, there were significantly more IFN-γ producing cells in the PBMC cultures from adult horses compared to those from foals. *Means differ significantly between ConA and Control at $p<0.03$. [a,b] Different letters indicate significant effect of time at $p<0.02$.

Proliferative response of lymphocytes changes with foal age

To determine if the failure of ConA to induce adult levels of IFN-γ production in foals was the result of a reduced proliferative capacity, the proliferation index (PI) of foal lymphocytes was compared to that of adult and old horses using flow cytometry. The PI of lymphocytes from the foals were higher than that of adult horses ($p<0.05$), which were higher than the old horses ($p<0.005$) (Table 3.1). This enhanced proliferative activity of the foals' cells peaked at 8 weeks of age ($p<0.001$). This same pattern was seen with both $CD3^+$ and $CD4^+$ lymphocytes, though proliferation of $CD8^+$ cells was not always different between adults and foals. This difference in proliferative capacity was not due to a differential dose response, as titration of the mitogen yielded similar results for each age group (Figure 3.2).

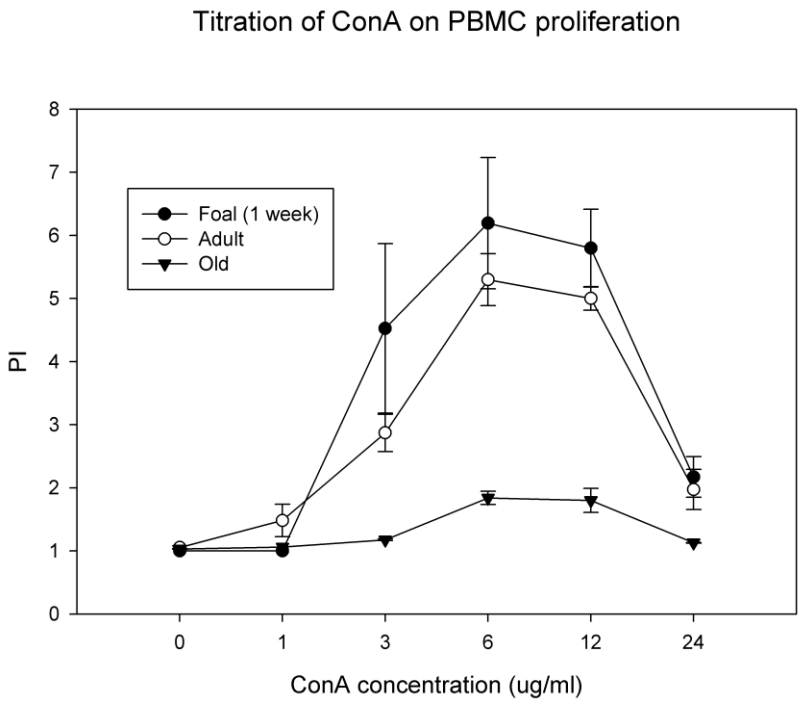


Figure 3.2. Titration of ConA on PBMC proliferation.

Table 3.1. Proliferative index by lymphocyte subsets for foals, adult and old horses

Age Group	Lymphocytes	CD3	CD4	CD8
2 weeks foals	$5.23\pm0.84^{a,1}$	$6.58\pm0.64^{a,1}$	$6.51\pm0.64^{a,1}$	$5.43\pm0.64^{a,1}$
4 weeks foals	$7.09\pm1.47^{a,2}$	$8.69\pm0.68^{a,2}$	$8.57\pm0.68^{a,1}$	$6.64\pm0.71^{a,1}$
8 weeks foals	$10.05\pm1.77^{a,3}$	$11.78\pm0.71^{a,3}$	$10.85\pm0.71^{a,2}$	$9.44\pm0.76^{a.2}$
12 weeks foals	$8.75\pm1.28^{a,3,4}$	$10.37\pm0.76^{a,3,4}$	$10.26\pm0.76^{a,2}$	$8.24\pm0.76^{a,2}$
16 weeks foals	$6.74\pm1.74^{a,4}$	$7.41\pm0.71^{a,4}$	7.59 ± 0.71^{a1}	$4.15\pm0.71^{b1,3}$
10 yrs horses	$3.41\pm0.26^{a,5}$	$4.06\pm0.87^{a,5}$	$4.10\pm0.87^{a,3}$	$3.69\pm0.87^{a,3}$
>20 yrs horses	$1.95\pm0.19^{e,6}$	$2.10\pm0.87^{a,6}$	$1.97\pm0.87^{a,4}$	$1.66\pm0.87^{a,4}$

Means of PI within an age group that are not significantly different share the same letter superscript. Means of PI that are not significantly different within cell populations share the same numerical superscript.

Characterization of the divisional history of cells is another method for assessing cellular proliferation (Adams et al., 2008). When the divisional histories of PBMC from foals, adults and aged horses were compared, the percentage of parental generation or non-dividing PBMC of foals were lower than the adult ($p<0.04$) and old horses ($p<0.001$) (Figure 3.3). Aged horses had the greatest percentage of non-dividing cells ($p<0.001$). Within foals, the 3rd generation of cells was the most prevalent in 2 week old foals, whereas in older foals the 4th generation of cells were in greater abundance along with the 3rd (4, 12 and 16 weeks) or 5th (8weeks) generations. In adult horses, there was no clear peak generation and in old horses the non-dividing cells represented the majority of the cells.

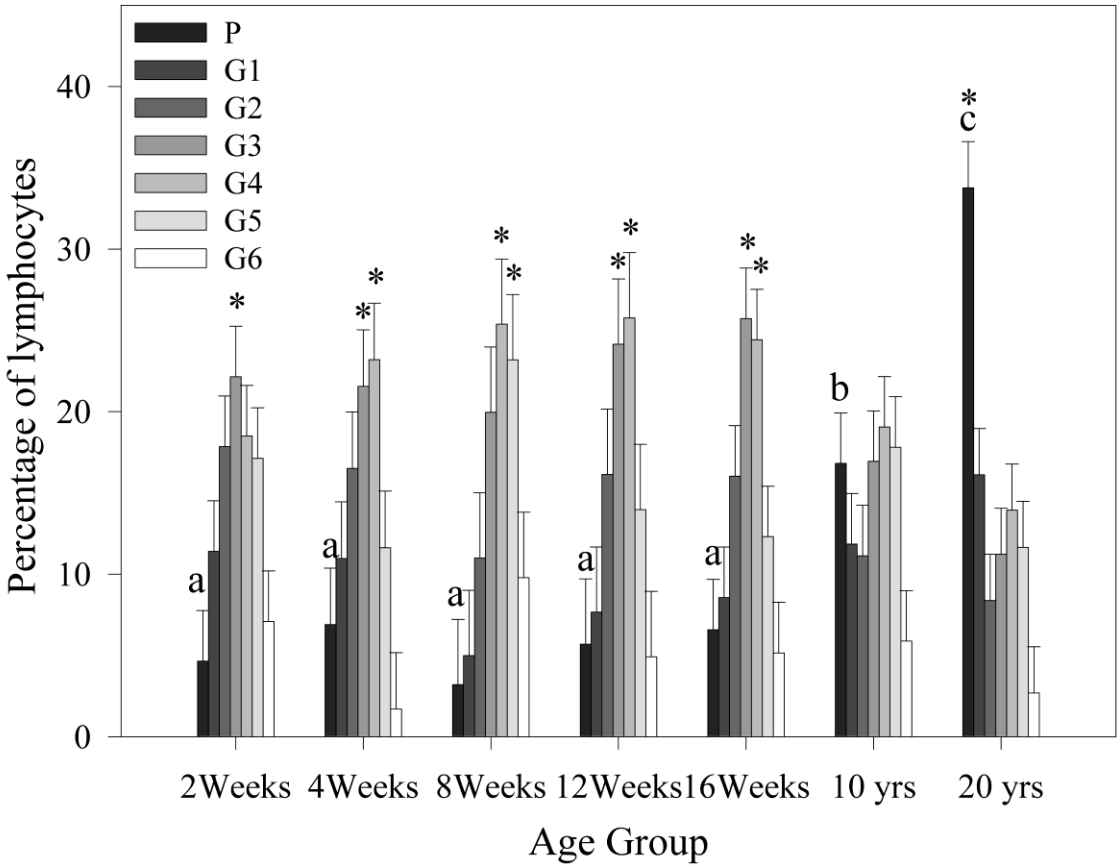


Figure 3.3. Proliferative Indices of lymphocytes from foals, adult and old horses. PBMC from foals at 2, 4, 8, 12, 16 weeks old (n=5), adult horses (10yrs; n=5) and old horses (>20yrs; n=5) were pre-loaded with CFSE, stimulated with ConA for 4 days and then analyzed by flow cytometry. There was a significantly greater frequency of non-dividing cells (P) in the PBMC cultures from 10 and 20 year old horses compared to the foals ($p<0.02$) and the non-dividing cells represented the majority of cells in the 20 year old horses' cultures. In foals, those cells undergoing 3 or more divisions represented the greatest proportion of cells in the PBMC cultures ($p<0.02$).

Proliferating T lymphocytes produce IFN-γ in foals

To further characterize the relationship between proliferation and IFN-γ expression, IFN-γ production by each generation of cells within the $CD3^+$, $CD4^+$ or $CD8^+$ T cell subsets was determined (Figure 3.4). For each of the lymphocyte subsets, the frequency of non-dividing IFN-γ^+ cells was lowest in foals, regardless of age, when compared to adult ($p<0.04$) and aged horses ($P<0.01$). Each of the foals' lymphocyte subsets acquired the ability to produce IFN-γ only after undergoing cellular division with the maximum percentage of IFN-γ^+ cells being obtained after 3 or more generations. By contrast, adult horses' IFN-γ -producing cells were distributed amongst all generations and the parental generation contained the majority of the IFN-γ-producing cells in the old horses.

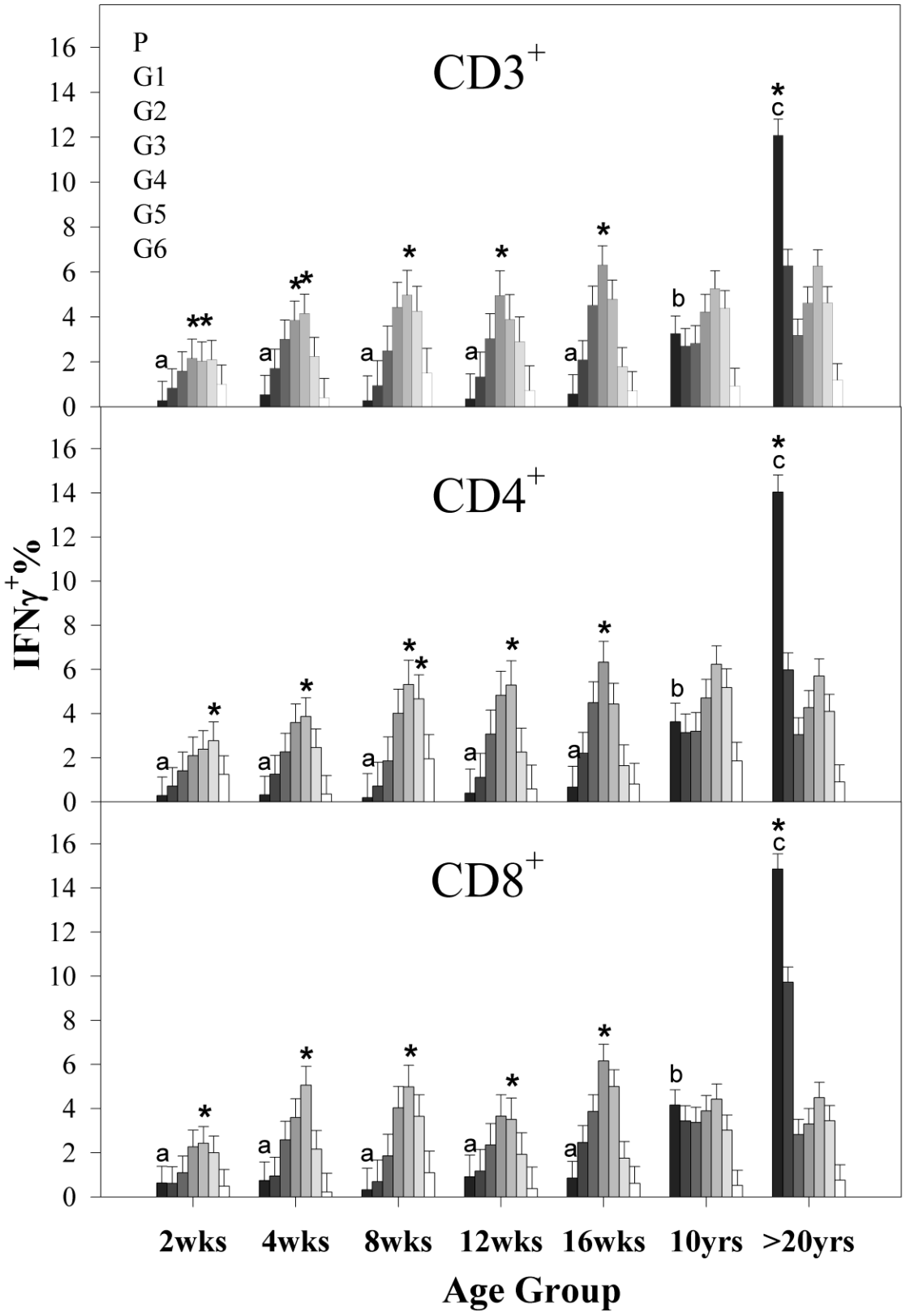

P
G1
G2
G3
G4
G5
G6
CD3+
CD4+
CD8+
IFNγ+%
2wks
4wks
8wks
12wks
16wks
10yrs
>20yrs
Age Group

Figure 3.4. The percentage of IFN-γ^+ cells in each generation of dividing $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes. PBMC from foals (2, 4, 8, 12, 16 weeks old; n=5/age group), adult horses (10 yrs, n=5) and old horses (>20yrs, n=5) were stimulated with ConA for 4 days followed by surface staining prior to intracellular staining for IFN-γ. There was a significantly greater frequency IFN-γ producing cells of each subset amongst the non-dividing cells (P) in the PBMC cultures from 10 and 20 year old horses compared to the foals ($p<0.05$) with the non-dividing representing the majority of IFN-γ^+ cells in the 20 year old horses' cultures. In foals, those cells undergoing 3 or more divisions represented the greatest proportion of cells producing IFN-γ for each subset ($p<0.05$). The percentage of non-dividing (P) IFN-γ^+ cells labeled with different lower case letters are significantly different. An"*" indicates the generation with significantly greatest number of IFN-γ^+ lymphocytes, $p<0.05$.

IFN-γ expression by proliferation-inhibited lymphocytes

In order to determine if IFN-γ expression was dependent on DNA synthesis and cellular division, hydroxyurea (HU) was used to inhibit proliferation. HU exerts an anti-proliferative effect on T cells without impacting cellular activation (Benito et al., 2007; Lori et al., 2005) by inhibiting ribonucleoside diphosphater reductase which converts ribonucleotides to deoxyribonucleotides (Lori and Lisziewicz, 1998). Pretreatment of PBMC with HU prevented cellular proliferation in response to Con A such that less than 5% of the cells underwent cellular division (Figure 3.5). Nevertheless, the percentage of IFN-γ producing cells in these cultures increased significantly from day 1 to day 2 and remained elevated through day 4, though the difference between adult horses and foals remained (Figure 3.5). However, proliferation-independent IFN-γ production by T cell

subsets did increase with foal age (Figure 3.6). Thus, the percentage of $CD3^+$ IFN-γ^+ cells from foals significantly increased from 2 weeks to 16 weeks ($p<0.04$) (Figure 3.6), though the percentage of IFN-γ– producing $CD3^+$ cells at 16 weeks remained lower than the older horses ($p<0.03$). A similar pattern of age-related increase in IFN-γ production was found in proliferation-inhibited $CD4^+$ cells. In $CD8^+$ cells, IFN-γ production by proliferation-inhibited cells from foals increased significantly from 2 weeks to 8 weeks ($p<0.04$), peaking at 16 weeks ($p<0.05$) where it reached the level of both adult and old horses.

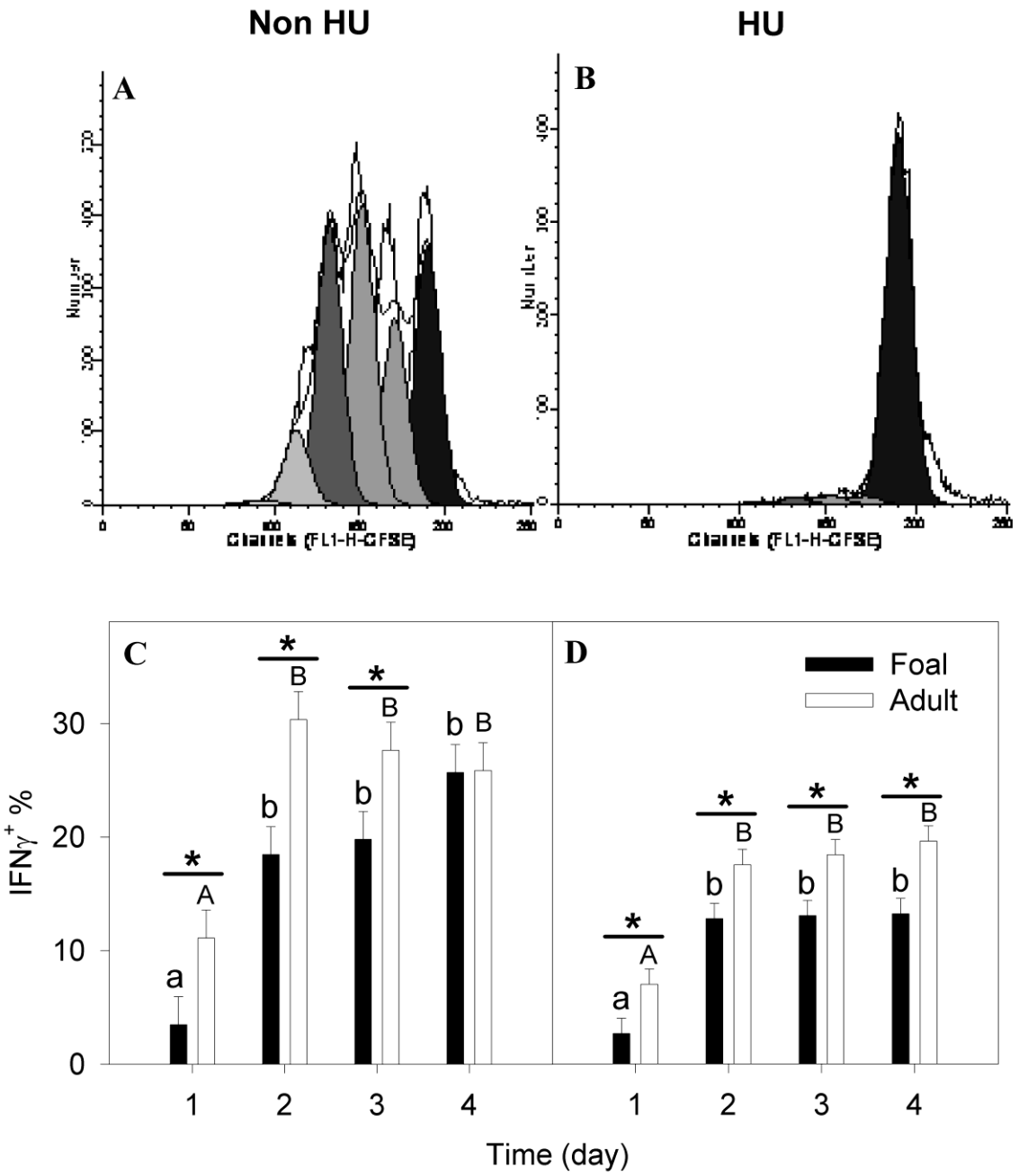


Figure 3.5. The effect of hydroxyurea (HU) on proliferation (A,B) and IFN-γ production (C,D). PBMC were isolated from foals (3 months, n=4) and adult horses (10 yrs, n=4) were pre-labeled with CFSE and incubated without or with HU for 1hour at 37ºC, then cultured with ConA for 4 days followed by PMA/ionomycin and BFA during the last 4

hrs prior to intracellular staining for IFN-γ. While ConA-stimulated lymphoyctes (A) proliferated, HU treatment (B) abrogated this response. Likewise, two days of ConA stimulation significantly increased the percentage of IFN-γ^+ cells in both adults and foals (C) and HU treatment reduced the frequency of IFN-γ^+ lymphocytes in both the adult and foals (D). An "*" indicates significantly different percentage of IFN-γ^+ lymphocytes between foals and adult horses, $p<0.05$. Percentages of IFN-γ^+ cells at each time point labeled with different lower case letters are significantly different for foals. Percentages of IFN-γ^+ cells at each time point labeled with different upper case letters are significantly different for adult horses.

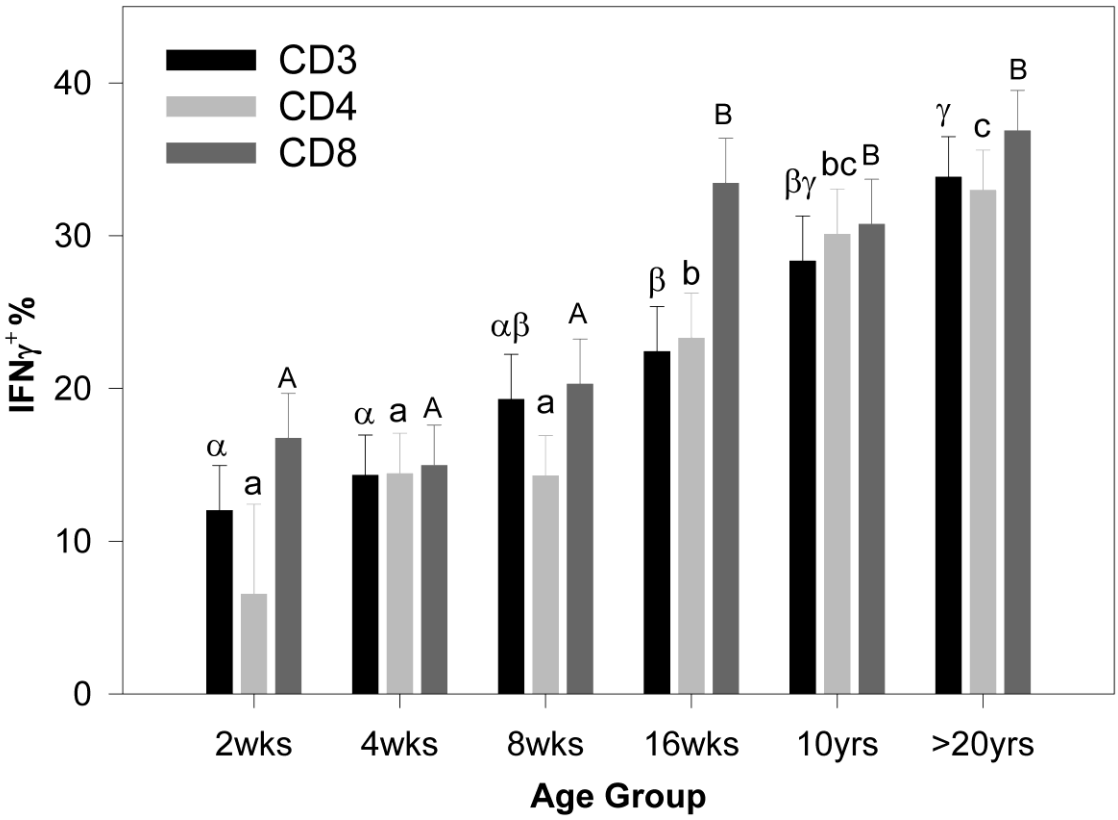


Figure 3.6. IFN-γ production by HU-inhibited lymphocytes from foals increases over time. PBMC were isolated from foals (2, 4, 8, 16 weeks old; n=5), adult horses (10 yrs;

n=5) and old horses (>20yrs; n=5) and incubated with HU at 37ºC for 1hr and stimulated with ConA for 2 days, then pulsed with PMA/iono and BFA for the last 4 hrs. The cells were then stained with antibodies against CD3, CD4 and CD8, separately, before intracellular staining with anti-IFN-γ antibodies. While the youngest foals had fewer IFN-γ^+ cells amongst the three subsets, older foals had a significantly higher percentages of IFN-γ^+ cells in the $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes and were not different from the older horses by 16 weeks of age. The mean percentages of IFN-γ^+ lymphocytes within each subset labeled with different letters (lower case, upper case or Greek) are significantly different ($p<0.05$).

DISCUSSIONS AND CONCLUSIONS

The production of IFN-γ by neonates of various species is deficient compared to adults (Boyd et al., 2003; Breathnach et al., 2006b; Lewis et al., 1986b; Wilson et al., 1986b). This failure to produce IFN-γ is associated with an increased susceptibility to intracellular infections (Gasparoni et al., 2003; Schoenborn and Wilson, 2007). Cellular proliferation provides the opportunity for gene expression by altering DNA structure (Bird et al., 1998). While we observed elevated IFN-γ expression by ConA-stimulated lymphocytes in both foals and adults, the overall foal response remained lower. One possibility for this reduced response could have been decreased proliferation by the foals' PBMC. Human neonatal lymphoyctes have reduced proliferative responses to ConA stimulation compared to those of older children and adults (Gasparoni et al., 2003). This same pattern of reduced proliferative activity was also reported for neonatal guinea pig lymphocytes (Jones et al., 1996). However, a higher proliferative response has been

reported for bronchial lymph node cells and PBMC of foals (Flaminio et al., 2000a; Jacks et al., 2007a). Here, we likewise found that PBMC from foals exhibited an enhanced proliferative response to ConA when compared to older horses. By characterizing the divisional history of the proliferating cells we also determined that most of the foal PBMC proliferated in response to the mitogen, whereas in adults a significant portion of the cells remained in the parental, non-dividing population. This was not the result of differential responsiveness to the mitogen as the dose response curves were identical. The reasons for this enhanced proliferative response of foal PBMC to mitogens are unknown and could reflect species' differences in the regulatory mechanisms that control lymphocyte proliferation in neonates. Human neonate lymphocytes produce elevated levels of IL-10 which could account for their reduced proliferative activity (Belderbos et al., 2009b). While it was initially reported that PBMC from foals produced elevated levels of IL-10 mRNA (Sponseller et al., 2009b), another report indicated reduced expression of this cytokine (Wagner et al., 2010). The role of IL-10 and other factors in the regulation of the lymphoproliferative activity of foal PBMC remains to be determined.

While a proportion of the adult T cells failed to proliferate in response to Con A stimulation, they were capable of producing IFN-γ. This was particularly true for those T cells collected from aged horses, as previously described (Adams et al., 2008), and likely reflects a memory T cell population which does not require proliferation for IFN-γ production (Kersh et al., 2006b). In foals, the relatively few cells that failed to divide also did not produce IFN-γ, whereas those lymphocytes undergoing multiple cellular divisions did produce this cytokine. This probably reflects there being mostly naïve T cells in the foal's circulation and naive T lymphocytes produce IFN-γ only after

undergoing multiple rounds of cellular division (Gett and Hodgkin, 1998; Gudmundsdottir et al., 1999).

The memory T cells in the adult and older horses likely accounted for the proliferation-independent IFN-γ production seen in the HU-treated cultures. While HU-treatment significantly reduced IFN-γ production in the foals' PBMC cultures, the percentage of cells making this cytokine did increase over time. This proliferation-independent IFN-γ production might also be attributed to memory T cells, as these cells would be expected to increase with foal age. Indeed, as the foals aged there was an overall increase in this activity.

Memory cells may not account for all of the IFN-γ production as inhibition of cellular division does not always prevent cytokine production in naïve T cells (Ben-Sasson et al., 2001). Since HU inhibits cellular proliferation without impacting cellular activation (Benito et al., 2007; Lori et al., 2005; Lova et al., 2005), Con A stimulation of HU-treated cells likely provided additional signals that control IFN-γ expression. For example, transcription factors (TFs) play an important role in the regulation of *Ifng* expression with GATA3 decreasing and Tbet promoting the transcription of IFN-γ (Grogan and Locksley, 2002). The inability to up- regulate Tbet and down-regulate GATA3 is associated with lower IFN-γ expression (Cheng et al., 2009; Yu et al., 2003). Thus, it is possible that the reduced IFN-γ expression by proliferating foal lymphocytes may be due to higher expression of GATA3 and/or lower expression of Tbet in response to ConA stimulation. Differences in transcription factor expression could also account for the increased IFN-γ expression in the $CD8^+$ T cells from the youngest foals. Naïve

$CD8^+$ T cells express eomesodermin, a TF that induces IFN-γ mRNA synthesis in the absence of cellular division (Araki et al., 2008; Pearce et al., 2003b; Takemoto et al., 2006). Also, $CD8^+$ T cells do not require Tbet for IFN-γ expression as do $CD4^+$ T cells (Szabo et al., 2002). Differences in DNA structure of $CD4^+$ and $CD8^+$ naïve T cells has been reported (White et al., 2002a) and this could limit accessibility of TF to their binding sites in the *Ifng* locus. Thus, the control of IFN-γ expression by $CD8^+$ T cells appears less rigorous and cell division less essential when compared to $CD4^+$ T cells. Our results are consistent with this differential mechanism for the regulation of IFN-γ expression in foal $CD8^+$ T cells.

In conclusion, while PBMC from foals exhibit higher proliferative responses to ConA, their overall IFN-γ expression nevertheless remained lower than that of adult horses. This is likely due to fewer memory T cells in foals, limited accessibility to regulatory elements on *Ifng* loci, or reduced availability of TFs. Future studies on epigenetic regulation and TFs expression are needed to better understand the mechanism underlying IFN-γ deficiency in foals.

Copyright © Lingshuang Sun 2012

CHAPTER FOUR

The hypermethylation of IFN-γ gene promoter is correlated with IFN-γ expression in the neonatal foals

Dev Comp Immunol. 2012 Oct 11

Reprinted with permission

SUMMARY

While born with a limited production, foals' interferon-gamma (IFN-γ) expression increases after birth. The underlying mechanisms remain unknown. DNA methylation is considered to be involved in. Therefore, the DNA methylation status of the *Ifng* promoter in $CD4^+$ cells from neonatal foal was determined using a methylation-specific PCR (MSP), and its relevance to IFN-γ mRNA expression was estimated. The effect of environment on the DNA methylation was also evaluated by comparing ponies that were kept in a barn versus those on pasture. The DNA in the *Ifng* promoter was hypermethylated and its demethylation was correlated with an increase in IFN-γ mRNA expression and age. This age-associated demethylation was accelerated by barn-air exposure. In conclusion, IFN-γ expression in foals appears to be controlled by DNA methylation in promoter of *Ifng*. The age-associated demethylation of the DNA in foals may be induced by exposure to environmental antigens and their effect on lymphoproliferation.

INTRODUCTION

Interferon-gamma (IFN-γ) expression is reduced in neonates of most species (Vuillermin et al., 2009a), including foals (Breathnach et al., 2006b). This reduced expression is associated with an increased risk for intracellular bacterial infections, such as those caused by *Rhodococcus equi* (*R. equi*). This depressed IFN-γ expression in the neonate is likely the consequence of a protective strategy against fetal loss caused by IFN-γ production at the fetal/maternal interface (Murphy et al., 2009). The expression of IFN-γ mRNA correlates with protein production in foals indicating its expression is regulated at the transcriptional level (Breathnach et al., 2006b). However, the underlying regulatory mechanisms are unknown. DNA methylation, induced by DNA methyltransferase (DNMT), is widely accepted as a primary mechanism for regulating gene transcription (Ansel et al., 2003; Holliday, 2006). Methylation inhibits gene transcription either directly by inhibiting transcription factor (TF) binding or by allowing a methyl-binding protein to bind to the methylated DNA which ultimately "closes" chromatin structure (Spilianakis and Flavell, 2007). In the case of humans and mice, the degree of methylation of the promoter determines the level of IFN-γ expression (Spilianakis and Flavell, 2007; Wilson et al., 2009). Thus, hypermethylation of the CpG motif in the *Ifng* promoter region results in limited IFN-γ expression in human neonates (White et al., 2002b). Reduced IFN-γ expression in neonatal foals may likewise be the result of DNA hypermethylation.

In foals, as in other neonates, IFN-γ expression increases rapidly after birth (Breathnach et al., 2006b). This temporal increase in IFN-γ expression is accelerated by

exposure to environments containing high levels of bacterial and fungal antigens (Sun et al., 2011). While it is postulated that DNA methylation of *Ifng* may be altered by environmental exposure to microbial antigens, there is little evidence available to support this hypothesis (Vuillermin et al., 2009a). Therefore, we examined the methylation status of the promoter region of *Ifng* in the foal in order to correlate methylation status with *Ifng* expression. We also determined the effect of the environmental exposure to microbial antigens on the methylation status of the foals' *Ifng* promoter. Lastly, we determined the effect of the environment on lymphoproliferation since proliferation is reported to regulate DNA demethylation (Wilson et al., 2009).

MATERIALS AND METHODS

Horses

A group of 12 horse foals (2 to 16 weeks old) and four adult horses (6 to10 years old) were used to determine DNA methylation level in the equine IFN-γ promoter. The horses were housed at the Department of Veterinary Science's equine facility in Lexington, Kentucky. Another group of 10 pony foals were used for the study of environmental effects on the DNA methylation, along with 2 adult ponies (6 and 8 years old). The foals were maintained on the University of Kentucky's Department of Veterinary Science's farm in Versailles, Kentucky. Five of the pony foals were chosen randomly at birth to spend 4 hours a day for 3 days (MWF) per week in individual stalls with their mares. This barn exposure started when the foals were less than 1 week old and stopped after they reached 2 months of age. When not in the barn, the foals were kept on pasture with their mares. Throughout the study period, the ponies, as well as the horses

above, had *ad libitum* access to water and forage in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research. All research procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

Lymphocytes isolation and cell sorting

Heparinized blood was collected from each horse by aseptic jugular venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation, as previously described (Breathnach et al., 2006b). The cells were resuspended in cRPMI 1640 media (Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (FES; Sigma, St. Louis, MO), 2 mM glutamine (Sigma), 100 U/ml penicillin/streptomycin (Sigma), 55 mM 2-mercaptoethanol (GIBCO) and surface stained for CD4, as described (Merant et al., 2009b). The cells were labeled with primary antibodies to CD4 (CVS4) or an IgG1 isotype control (BD Pharmingen™, Sparks, MD) followed by fluorescent labeling with secondary antibodies (PE conjugated goat Fab' anti-mouse IgG [Southern Biotechnology Associates, Birmingham, AL]). The $CD4^+$ cells were then sorted by a Cytomation MoFlo® high-speed cytometer cell sorter and analyzer from Cytomation (Fort Collins, CO) as gated (Figure 4.1).

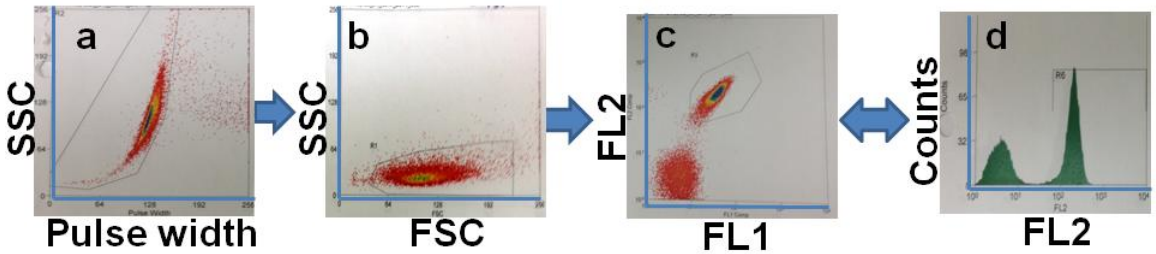


Figure 4.1. Sorting $CD4^+$ cells. The PBMCs were isolated and surface stained for CD4 molecules. The stained cells were analyzed and sorted with Cytomation MoFlo® high-

speed cytometer. The single cells, gated by pulse width (a); with few granularity, gated by lower SSC (b); and RPE fluorescent, gated by FL2 positive (c and d), were sorted as $CD4^+$ lymphocytes.

Quantitation of cellular proliferation and IFN-γ expression

Proliferation was assessed using flow cytometry, as previously described (Sun et al., 2012). Briefly, 1×10^7 PBMC were suspended in 1ml PBS (Sigma) and stained with 1 ml of carboxyfluorescein succinimidyl ester (CFSE; 5μM/ml, Sigma) for 8 min. The reaction was quenched by adding 2 mls of FBS (Sigma), followed by two washes with PBS supplemented with 10 % FBS. The cell pellet was then resuspended in 5 ml of cRPMI media (final concentration of 2×10^6 cells/ml). The CFSE – labeled PBMC were transferred to a 24-well-plate at 1 ml/well. The cells were then stimulated with 3 μg/ml concanavalin A (ConA; Sigma) for 4 days. Afterwards, the cells were washed with FACS Flow and analyzed with a FACSCalibur (BD, Franklin Lakes, NJ) flow cytometer. The Proliferation Index (PI) was calculated using ModFit LT™ (Version 3.0, Verity Software House, Inc., Topsham, ME).

For IFN-γ analysis, ConA-stimulated PBMC were pulsed with phorbol 12-myristate 13-acetate (PMA; 25 μg/ml; Sigma, p8139) /ionomycin (iono; 1 μM; Sigma) and brefeldin A (BFA; 10 μg/ml; Sigma) during the last 4 hrs of incubation. Intracellular staining for IFN-γ was performed, as previously described (Sun et al., 2012).

RT-PCR

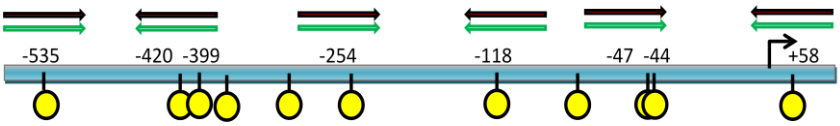
Total cellular RNA was isolated from 1×10^6 cells preserved in RNA-STAT 60 (Tel-Test, Inc. Friendswood, TX) according to the manufacturer's protocol. Reverse

transcription was performed as described previously (Breathnach et al., 2006a). The resultant cDNA was diluted 1:1 with RNAse-free water. The gene expression was quantitated using an Applied Biosystems PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Intron-spanning equine IFN-γ primers and probe failed to amplify genomic DNA and reverse transcription negative RNA samples (Breathnach et al., 2006b) and their amplification efficiencies were greater than 95% , as determined using LinRegPCR (Ramakers et al., 2003). PCR reactions were performed in duplicate wells per sample, as described previously (Breathnach et al., 2006a). Beta-glucuronidase (b-Gus) was used as the housekeeping gene and the relative quantification (RQ) method for mRNA expression was used (Breathnach et al., 2006a).

Methylation specific PCR (MSP)

The likely promoter region of equine *Ifng* was predicted using promoter 2.0 prediction server (http://www.cbs.dtu.dk/services/Promoter/). Based on the CpG sites, the three sets of MSP primers were designed by Methprimer (http://www.urogene.org/methprimer/index1.html) and synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA). The primers sequences are listed in Table 4.1.

Table 4.1. MSP primer sequences[a]

Specificity	Primers	Set 1	Set 2	Set 3
				
^{m}CpG	Fwd	ATGAAGAATTTTTTTATTATATCGG	GTATAATGGGTTTGTTTTATCGT	GTAGTATATTTTTTTGATCGTCGG
	Rev	CGTTAATCATTTATTTATAATCGTA	ATTAAAATCTCATCAAAATTACGTA	AATAAAACTTATATAATTCATTATTTCGAA
	Product	160 bp	179 bp	151 bp
^{u}CpG	Fwd	ATGAAGAATTTTTTTATTATATTGG	GGTATAATGGGTTTGTTTTATTGT	GGTAGTATATTTTTTTGATTGTTGG
	Rev	CACATTAATCATTTATTTATAATCATA	ATTAAAATCTCATCAAAATTACATA	AATAAAACTTATATAATTCATTATTTCAAA
	Product	162 bp	180 bp	152 bp

[a]Methylated- and unmethylated-specific primers were designed for three regions within the *Ifng* promoter predicted to be involved in the epigenetic regulation of this gene. Predicted amplicon size for each product is also indicated for each primer set.

Genomic DNA was isolated using Gentra Puregene Cell Kit (QIAGEN, Valencia, CA) and 400 ng of the DNA was bisulfite treated using EZ DNA Methylation-Gold™ Kit (D5005, Zymo Research Corporation, Irvine, CA). The real time PCR was performed in duplicate for each sample with 2 mM of specific primers for methylated CpG (^{m}CpG) or un-methylated CpG (^{u}CpG), 30 ng of bisulfite converted genomic DNA as template, and 10 μl of PCR reaction mix (QuantiTec ® SYBR® Green PCR Kit, QIAGEN), in a total of 20 μls. The PCR reaction conditions were as follows: pre-heat at 95ºC for 15 min; 10 cycles of touch-down PCR with 95 ºC 30 s, 60 ºC 30 s (-0.5 ºC per cycle) and 72 ºC 30s; 40 cycles of amplification with 95 ºC 30 s, 55 ºC 30 s 72 ºC 30s, 60 ºC 45s (signaling); 72ºC 10 min for final elongation followed by determination of dissociation curve. Completely methylated genomic DNA generated by methyltransferase (M0226, New England Biolabs, Ipswich, MA) from genomic DNA was used as a positive control, and the completely un-methylated genomic DNA generated using GenomiPhi DNA Amplification Kit (25-6600-30, GE healthcare, Piscataway, NJ) was used as a negative control. The Ct values were corrected using LinRegPCR, with efficiencies below 90% excluded. The percentage of CpG methylation for each region of *Ifng* promoter was calculated as ^{m}CpG%= $(2^{-CtmCpG} / (2^{-CtmCpG} + 2^{-CtuCpG})) \times 100\%$.

Total DNMT activity test

Nuclear protein was extracted from $CD4^{+}$ T cells using EpiQuik™ Nuclear Extraction Kit I (P-0002, Epigentek Group Inc., Farmingdale, NY). The protein was aliquoted and stored at -80°C. The concentration of the nuclear protein was determined using a BCA protein Assay Kit (23225, Fisher Thermo Scientific Inc., IL) and the total DNMT activity of the protein was determined using EpiQuik™ DNA Methyltransferase

Activity/Inhibition Assay Kit (P-3001, Epigentek Group Inc.). The assay determines the quantity of ^{m}CpG transferred by the DNMTs using an indirect ELISA with ^{m}CpG specific antibodies. The DNMT activity was calculated using the formula: Total DNMT Activity (OD/h/mg) = (OD_{sample} - OD_{blank})/ (protein (μg) ×incubation time (hour)) ×100%

Statistic analysis

All data were analyzed using a commercially available statistical software package (Sigma Stat version 10.0; Systat, San Jose, CA). Student's t-test was used to test the significance between foals and adult horse samples. Linear Regression was used to determine the correlation between DNA methylation, age and IFN-γ expression. A Two Way ANOVA was used to test for statistical significance with age and environment as the two factors. Some of the data were log-transformed or rank-transformed to meet the assumptions of normality and equal variance. Differences were considered significant at $p < 0.05$.

RESULTS

Optimization of MSP reaction condition

Using our optimized touch-down PCR reaction conditions, the three sets of ^{m}CpG specific primers only amplified methylated the but not unmethylated genomic DNA (Figure 4.2 a, b and c), while, the ^{u}CpG specific primers only amplified the unmethylated genomic DNA (Figure 4.2 d, e and f) with only a single amplification product generated for each primer pair (Figure 4.2 g, h and i).

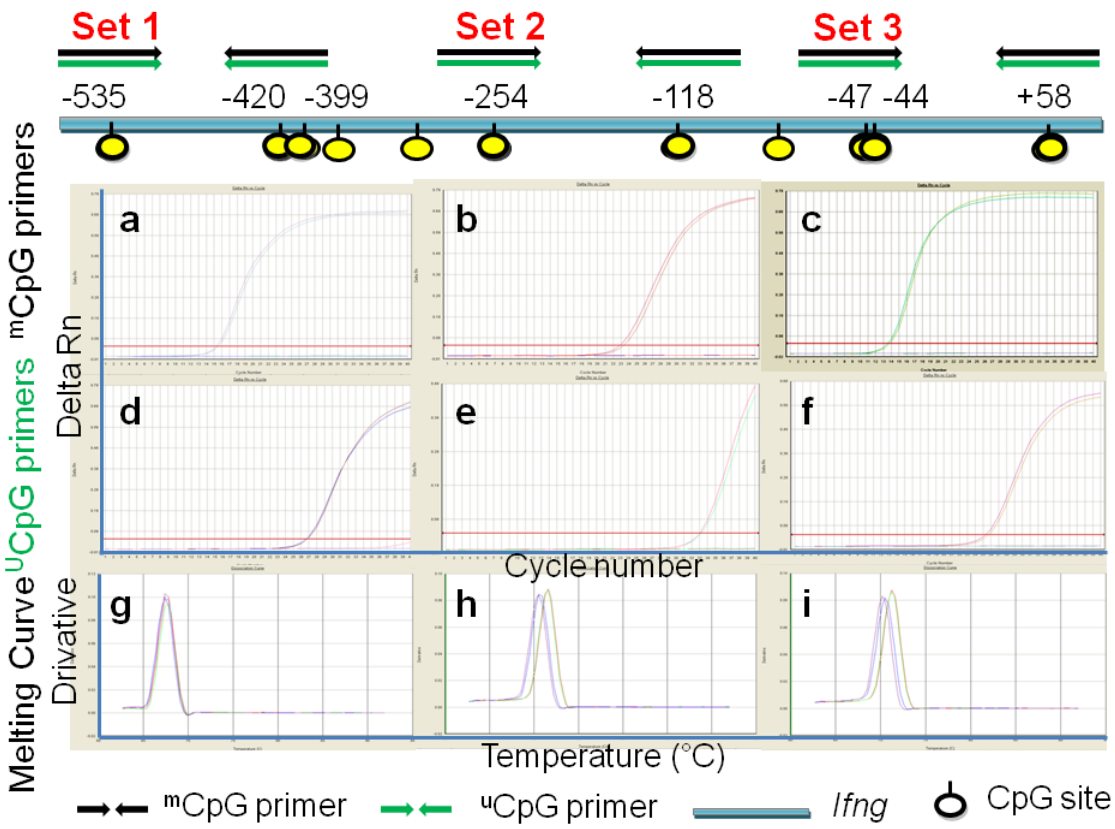


Figure 4.2. Verification of specificity of MSP primers. The genome wide methylated and the unmethylated genomic DNA were used as the templates for MSP to test the specificity of ^{m}CpG (upper panel) or ^{u}CpG (middle panel) specific primers in three region of *Ifng* promoter, region 1 (left panel), region 2 (middle panel) and region 3 (right panel). Three ^{m}CpG specific primers, set 1(a), set 2 (b) and set 3 (c), only amplified methylated genomic DNA but not unmethylated genomic DNA. Whereas, ^{u}CpG specific primers, set 1 (d), set 2 (e) and set 3 (f), only amplified unmethylated genomic DNA. A single melting curve was obtained for each primer pairs and MSP products (g, h and i).

DNA hypermethylation in the promoter region of IFN-γ is correlated with age and IFN-γ expression

DNA methylation at three locations within the IFN-γ promoter region was determined. Region one (-539, -420 and -399), region two (-254 and -118), and region three (-47, -44 and +58) were analyzed using the three sets of methylated and unmethylated DNA specific primers. While methylation at IFN-γ promoter region one in neonatal foals was over 99.9%, it was not significantly higher than that of adult horses (p=076, Figure 4.3 a). DNA demethylation in region one was not correlated with age (p=0.244, Figure 4.3 b). While there was a trend for demethylation in region one to correlate with IFN-γ expression, this was not statistically significant (p=0.058, Figure 4.3 c). Region two of the *Ifng* promoter was also found to be more methylated in foals than that of adult horses (p=0.007, Figure 4.3 d), but the demethylation of the DNA was not associated with IFN-γ expression (p=0.064, Figure 4.3 f) though it was inversely correlated with horses' age (p=0.003, Figure 4.3 e). Similarly, DNA methylation was significantly increased in the third region in neonatal foals when compared to adult horses (p=0.004, Figure 4.3 g). Nevertheless, DNA demethylation in this region was not only inversely correlated with age (p<0.001, Figure 4.3 h) but also with IFN-γ expression (p=0.042, Figure 4 i). These results indicate that this region of the *Ifng* promoter plays an important role in the regulation of IFN-γ expression in horses.

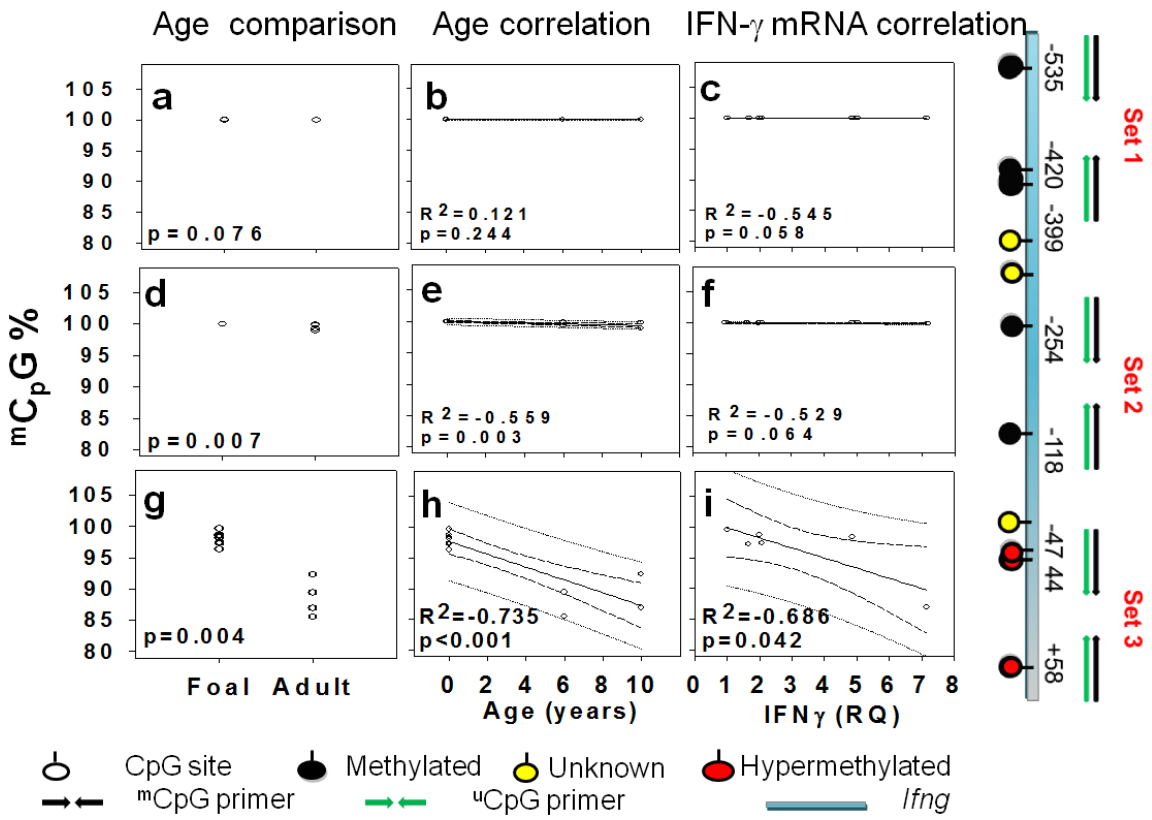


Figure 4.3. DNA hypermethylation in the promoter region of IFN-γ in neonatal foals and its correlation with mRNA expression and age. The DNA methylation in *Ifng* promoter of $CD4^+$ cells from foals and adult horses was compared (a, d, and g) and its relationship with age of horses (b, e, and h) and IFN-γ mRNA expression (c, f, and i) was estimated.

DNA methylation of the Ifng promoter is not associated with total DNMT activity

Since DNA methylation involves the transfer of a methyl group to CpG by DNMTs, total DNMT activity was determined in $CD4^+$ T cells from foals and adult horses. DNMT activity was found to be lower in foals $CD4^+$ cells, higher in those from adult horses (Figure 4.4, left panel), and correlated with horse age (Figure 4.4, right panel). However, there was no association found between the DNMT activity and the degree of DNA methylation in the three regions of the *Ifng* promoter (data not shown).

There was also no relationship between DNMT activity and IFN-γ mRNA expression (data not shown).

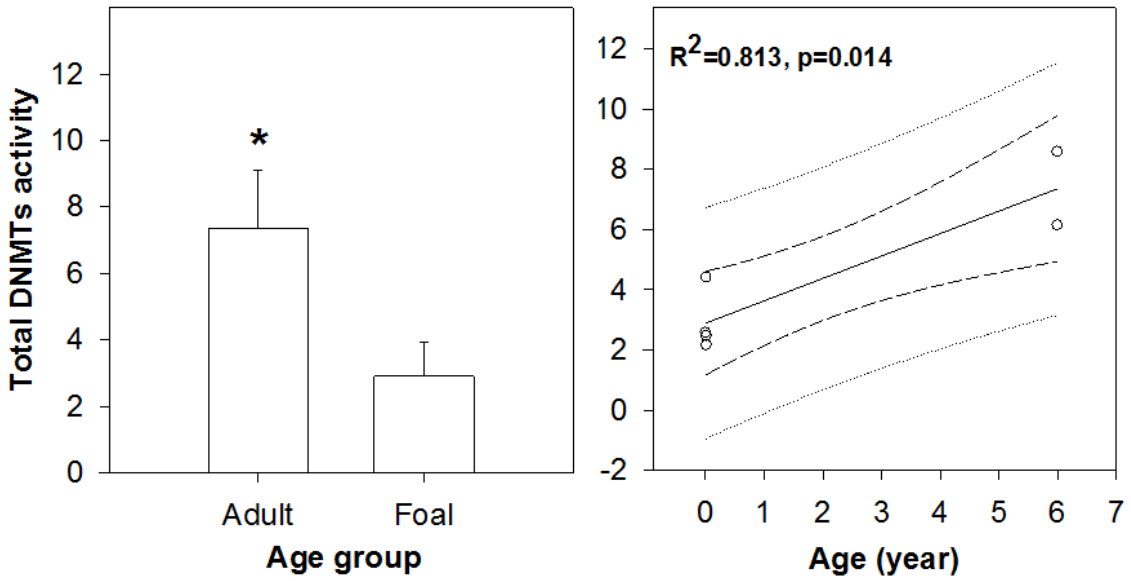


Figure 4.4. The activity of total DNMTs was higher in foals. The nuclear protein was extracted from $CD4^+$ T cells in both foal and adult horses and the DNMT activity was then measured and compared. The activity of total DNMTs was significantly higher in foals than in adult horses (left panel), and the activity was significantly correlated with age (right panel). *Mean of DNMT activity was significantly different between the two groups of horses, p=0.014.

The effect of environment on DNA demethylation, lymphoproliferative activity and IFN-γ expression by PBMC

Since lymphoproliferation provides the opportunity for DNA demethylation, we determined the effect of environment on lymphoproliferation, IFN-γ expression and promoter methylation. The proliferative response to ConA was significantly increased for those foals exposed to barn air for 8 weeks when compared to foals kept on pasture

(Figure 4.5, upper panel). A similar pattern was seen regarding IFN-γ expression (Figure 4.5, lower panel). The percentage of IFN-γ^+ lymphocytes was significantly increased in foals with 8 weeks of barn air exposure when compared to that of foals with pasture only exposure ($p=0.029$, Figure 4.5, lower panel). The effect of foal environment on DNA methylation was also determined for foals exposed to barn air versus those on pasture. The DNA in *Ifng* promoter region (-47bp, -44bp and +58bp) was hypermethylated in both groups of foals compared with adult ponies ($p<0.001$, Figure 4.6 right panel). However, those foals exposed to barn air reduced DNA methylation compared with those on pasture at 12 weeks of age (Figure 4.6 right panel). No difference in DNA methylation was found between the two groups of foals at the other two promoter regions (Figure 4.6, left and middle panel).

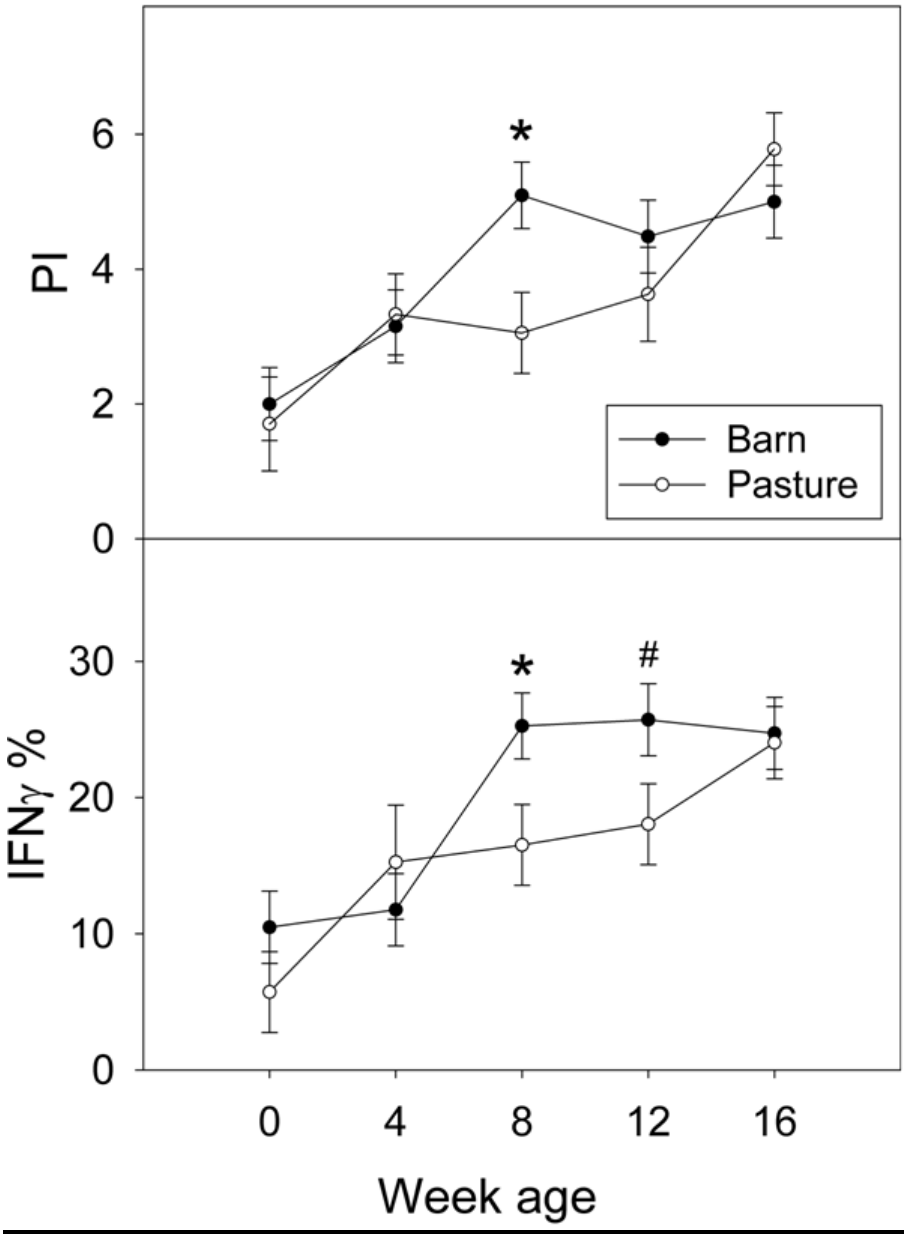


Figure 4.5. Environment effect on lymphoproliferation and IFN-γ expression in foals. Both PI (upper panel) and the percentage of IFN-γ^+ cells (lower panel) were increased in the foals with barn-air exposure. *Means of PI or percentage of IFN-γ^+ cells was significantly different between two groups of foals $p<0.03$; #Means of percentage of IFN-γ^+ cells was different between two groups of foals $p=0.062$.

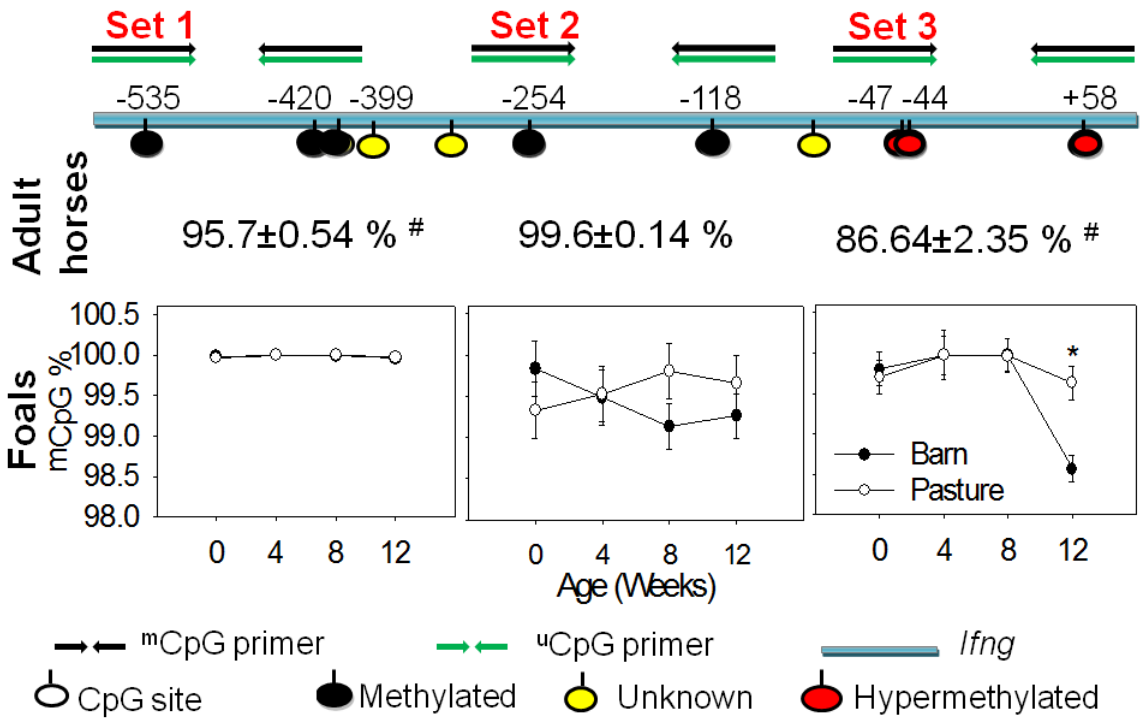


Figure 4.6. Environment effect on DNA methylation in *Ifng* promoter. The DNA methylation level at three regions in *Ifng* promoter was determined and compared between barn-air and pasture-air exposed foals and versus adult horses. DNA in region 1 and 3 in *Ifng* promoter exhibited a higher level of methylation in both groups of foals compared with adult horses. The DNA in region 3 was demethylated in foals with barn-air exposure compared with foals with pasture-air exposure (right panel), while no difference in DNA methylation in region 1 (left panel) and 2 (middle panel) was observed. *Means of percentage of ^{m}CpG was different between two groups of foals $p<0.001$. $^{\#}$Means of percentage of ^{m}CpG differed between adult horses and foals, $p<0.001$.

DISCUSSIONS

Gene expression is negatively correlated with the level of DNA methylation in the promoter region of many genes (Law and Jacobsen, 2010). The methylation of CpG

either directly blocks TF binding or recruits methyl-binding proteins that indirectly interfere with TF binding thereby preventing initiation of gene expression. Accumulating evidence has implicated CpG methylation in the IFN-γ promoter region as an important negative transcriptional regulator of IFN-γ gene expression in humans and mice (Spilianakis and Flavell, 2007; Wilson et al., 2009) Here, we report for the first time that the degree of DNA methylation in the equine IFN-γ gene promoter region (around -47, -44 and +58) affects mRNA expression of IFN-γ in young horses. Similarly, DNA methylation at -54 CpG site in the human *Ifng* promoter region controls mRNA expression. As in humans, the CpG site at -47 and -44bp on *Ifng* in horses encompasses a possible transcription initiation site around a TATA box. Thus, this region likely plays a key role in the regulation of IFN-γ expression in horses.

While DNA in this proximal promoter region is hypermethylated in neonatal foals, it is less so in adult horses. These findings are in concurrence with data indicating that the DNA in the proximal IFN-γ promoter is hyper methylated in neonatal humans while hypomethylated in adults (White et al., 2002b). What induces this age related demethylation of DNA is unknown. Since methylation of DNA involves DNMTs transfer of a methyl group to CpG during DNA replication, decreased DNMT activity could be associated with reduced methylation. However, the total DNMT activity was found not to be correlated with either the degree of DNA methylation of the *Ifng* promoter or IFN-γ mRNA expression. This indicated that DNA methylation of the *Ifng* was likely not regulated by total DNMT activity, though we cannot exclude the possibility of an effect on a specific DNMT (Pradhan and Esteve, 2003; Svedruzic, 2008; Ting et al., 2006).

The external mechanism driving the age-associated DNA demethylation of the *Ifng* promoter was also examined. Here, we observed increased DNA demethylation in the IFN-γ loci of foals with barn air exposure when compared with similar aged foals kept on pasture. We have previously reported that exposure to barn air enhanced IFN-γ expression (Sun et al., 2011). Since there are significantly higher number of culturable bacteria and fungi in barn air versus pasture (Sun et al., 2011), exposure to these microbial components could lead to the demethylation of the *Ifng* promoter . Infection with viral, bacterial and parasitic agents is known to induce DNA demethylation (Vuillermin et al., 2009a). While the mechanisms involved in environmental regulation of DNA methylation remains unknown, it is known that proliferation of naïve T cells is required for the initial transcription of the *Ifng* locus (Ansel et al., 2003; Bird et al., 1998; Murphy and Reiner, 2002a). In this study, the proliferative response to ConA was significantly increased for those foals exposed to barn air and this paralleled the increase in IFN-γ expression and demethylation in the proximal promoter region. Together these results are consistent with the hypothesis that environmental exposure increased lymphoproliferation leading to DNA demethylation in the IFN-γ promoter region.

To conclude, DNA methylation in the proximal promoter region of *Ifng* regulates IFN-γ gene expression in horses and the hypermethylation in this region contributes to the reduced IFN-γ expression in neonatal foals. The age-associated demethylation of the *Ifng* promoter loci is likely due to environmental microbial exposure via the induction of lymphoproliferation leading to increased gene transcription. Future studies on how demethylation was induced via lymphoproliferation could identify novel targets for new adjuvants or immunostimulators.

Copyright © Lingshuang Sun 2012

CHAPTER FIVE

The effect of environment on interferon-gamma production in neonatal foals

Vet Immunol Immunopathol. 2011 Sep 15;143(1-2):170-5.
Reprinted with permission

SUMMARY

While interferon-gamma (IFN-γ) plays an important role in protection against viral and intracellular bacterial infections, its production in neonates is deficient. Exposure to environmental antigens can promote the maturation of the immune system of neonatal humans and mice. We hypothesize that exposure to high level of microbial components would increase the production of IFN-γ in neonatal foals. To test this hypothesis, one group of foals was placed into stalls three times a week for 8 weeks. A second group of foals remained on pasture. Air samples were collected from the barn and pasture for microbial culture. There were more bacteria and fungi in the air samples collected from the barn compared with those from the pasture. Bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMC) were collected from both groups of foals at various times to assess IFN-γ production. The frequency of IFN-γ^+ lymphocytes in BAL cells and PBMC was higher for foals kept in the stalls.

INTRODUCTION

Interferon-gamma (IFN-γ) plays an important role in innate and adaptive immune responses (Rowell and Wilson, 2009). However, in neonatal foals (Boyd et al., 2003; Breathnach et al., 2006b), like neonatal humans and mice (Lewis et al., 1986b; Lewis et al., 1991), IFN-γ production is deficient. In human neonates, IFN-γ-deficiency is associated with an increased risk for developing recurrent wheezing (Guerra et al., 2004) and atopic dermatitis (Herberth et al.). In foals, this deficiency in IFN-γ production likely contributes to their susceptibility to infections with *Rhodococcus equi* and other pathogens (Marodi, 2006a).

Exposure to environmental microbial antigens has been shown to promote IFN-γ production in neonatal humans (Gereda et al., 2000a; Roponen et al., 2005a). The underlying theory that environmental exposures to microbial antigens affects the development of the immune system, referred to as the hygiene hypothesis, was first introduced about two decades ago (Strachan, 1989). This hypothesis postulated that exposure to environmental microbial components promotes a Th1 immune response, which reduces the risk of allergic disease caused by the Th2 cells (Strachan, 1989). This theory has been widely supported by epidemiological studies and laboratory research (Belderbos et al., 2009a; Garn and Renz, 2007). However, little is known about the underlying mechanism involved in this process. Even less is known about the effect of environmental microbial exposure on immune development in neonatal foals. Here, we compared IFN-γ expression by both peripheral blood mononuclear cells (PBMC) and

bronchoalveolar lavage (BAL) cells from neonatal pony foals kept in environments with different levels of microbial antigen exposure.

MATERIAL and METHODS

Animals

Air samples were collected from both the barn and the pasture at the beginning and end of the study period. At each time, air samples from 4 individual stalls and 4 spots randomly chosen on the pasture were collected using a MAS-100 eco (MBV AG, CH-8712, Stäfa) air sampler set at a sampling rate of 100 liters/min. For each pasture (500 liters of air) and barn (100 liters of air) sampling, four samples were collected onto different petri dishes. Selective media were used for bacteriologic cultures to identify total (Trypticase soy agar, TSA), gram^{+} (Columbia CNA Agar with 5% horse blood, C-CNA), and gram^{-} (MacConkey agar, MAC) bacteria. The petri dishes for bacteriologic culture were incubated at 37ºC overnight. Fungi were cultured on sabouraud agar (SAB) at room temperature for 2 days. The number of colonies on the petri dishes was counted and CFU per m^{3} of air calculated. Fungal identification was performed by Aerobiology Laboratory Associates, Inc. (Atlanta, GA).

Air sampling

Air samples were collected from both the barn and the pasture at the beginning and end of the study period. At each time, air samples from 4 individual stalls and 4 spots randomly chosen on the pasture were collected using a MAS-100 eco (MBV AG, CH-8712, Stäfa) air sampler set at a sampling rate of 100 liters/min. For each pasture (500 liters of air) and barn (100 liters of air) sampling, four samples were collected onto

different petri dishes. Selective media were used for bacteriologic cultures to identify total (Trypticase soy agar, TSA), gram$^+$ (Columbia CNA Agar with 5% horse blood, C-CNA), and gram$^-$ (MacConkey agar, MAC) bacteria. The petri dishes for bacteriologic culture were incubated at 37ºC overnight. Fungi were cultured on sabouraud agar (SAB) at room temperature for 2 days. The number of colonies on the petri dishes was counted and CFU per m^3 of air calculated. Fungal identification was performed by Aerobiology Laboratory Associates, Inc. (Atlanta, GA).

Isolation and stimulation of PBMC

Heparinized blood was collected from each pony by aseptic jugular venipuncture. The PBMC were isolated and cultured in cRPMI, as previously described (Breathnach et al., 2006b), 3×10^6 PBMC from each foal were placed into duplicate wells of a 24-well plate in 1ml of medium alone, or in 1 ml of medium containing phorbol 12-myristate 13-acetate (PMA; 25 ng/ml; Sigma) and ionomycin (1 mM; Sigma). Brefeldin A (BFA, 10 mg/ml) was also added to both cultures at the onset to block the export of synthesized proteins. Plates were incubated for a total of 4 h at 37 ºC in 5% CO_2 atmosphere.

Isolation and stimulation of BAL cells

The BAL fluid was collected as previously described (Breathnach et al., 2006b). Approximately 150 mls of BAL fluid was collected from each foal. The BAL fluid was centrifuged at 400g for 10 min and washed twice with PBS. Fresh BAL cells were analyzed by flow cytometry (FacsCalibur, Becton Dickinson, San Jose, CA) using forward and side scatter parameters. Additionally, 4×10^6 BAL cells were stimulated for IFN-γ production, as described above for PBMC.

Surface staining of PBMC

Surface staining were performed using anti-CD3 (F6G.3), anti-CD4 (CVS4) and anti-CD8β (F18H.2) and anti-CD8α (CVS8) monoclonal antibodies (see supplementary e-file), as previously described (Merant et al., 2009a) . An IgG1 (BD Pharmingen™, Sparks, MD) was used as an isotype control. The secondary antibody was PE conjugated Goat F(ab')2 anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL).

Intracellular staining of IFN-γ

The PBMC (0.6×10^6) and BAL (0.8×10^6) cells were stained intracellularly with anti- IFN-γ antibody, as previously described (Breathnach et al., 2006b) using FITC conjugated mouse anti-bovine IFN-γ (Serotec, Raleigh, NC) and an isotype control antibody (FITC conjugated mouse IgG1, Serotec). For the PBMC, the frequency of IFN-γ^+ cells was determined for $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes. For the BAL cells, the percentage of IFN-γ^+ cells were analyzed using forward and side scatter characteristics to define the populations.

Statistical analysis

All data were analyzed using a commercially available statistics package (Sigma Stat version 10.0; Systat, San Jose, CA). Some of the data were log-transformed to meet the assumptions of the ANOVA. Two-way ANOVA was used to test for statistical significance. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

There were significantly more culturable bacterial colonies, both gram$^+$ and gram$^-$, collected in the air samples from the barn (Figure 5.1). There were also more fungi collected from the barn samples. The predominant fungal species identified in the pasture samples was *Cladosporium sp.* (~99%), whereas *Penicillium sp*. (83%) were predominant in the barn with *Cladosporium* also being present (~17%). There was no difference in the number of colonies detected at the two sampling times at either location. A number of studies have reported bacterial components including endotoxin (lipopolysachride, LPS) and peptidoglycans, as well as fungal extracellular polysaccharides in air samples collected from equine barns (Nardoni et al., 2005; Woods et al., 1993). A more recent study reported that culturable bacterial levels in equine barns averaged 3.1×10^3 CFU/m^3 and ranged from 67 to 1.9×10^4 CFU/ m^3 (Samadi et al., 2009). Culturable bacteria in the air samples from our barn averaged $5.1 \pm 1.7 \times 10^3$ CFU/m^3. Mold concentrations in the previous study ranged from 74 to 2.4×10^4 CFU m^3 (Samadi et al., 2009), whereas we observed $2.7 \pm 1.1 \times 10^3$ CFU/m^3. As such, the levels of microbial exposure our foals experienced can be considered typical for equine barn environments.

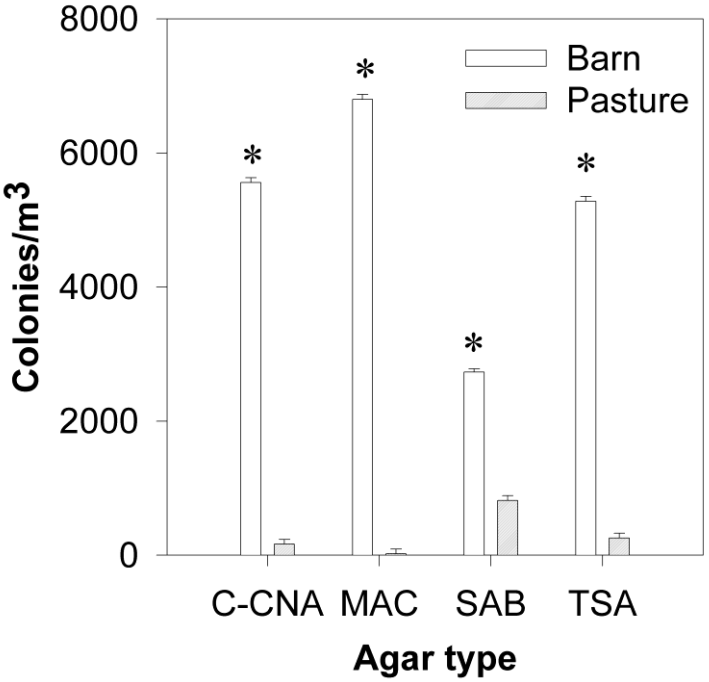


Figure 5.1. Culturable microorganism in the air samples from barn and pasture. Air samples were collected from 4 individual stalls and 4 spots on pasture by MAS-100 eco. 4 types of media were used to culture the microorganism in the air. C-CAN, MAC and TSA plates were incubated at 37ºC for overnight and SAB were incubated at room temperature for 2 days. The number of colonies was then counted and calculated. *Means of colonies/m^3 differ between air samples from barn and pasture ($p<0.02$).

Microbial exposure in the lung can lead to inflammatory responses resulting in increased numbers of cells in the airway (Iwamura and Nakayama, 2008). As there was no difference in the total number of cells in the BAL fluid in barn and pasture groups of foals (Table 5.1), this indicated that no additional cells were being recruited to the lung. This may reflect the fact that the level of exposure to bacterial antigens required to induce an inflammatory response needs to exceed a certain threshold. Such a threshold is known to exist for inhaled LPS in humans (Loh et al., 2006) and horses (Simonen-Jokinen et al.,

2005). This lack of induced cellular infiltration into the lungs of the foals is also supported by the fact that similar numbers of cells in BAL samples from neonatal foals have been reported in other studies (Balson et al., 1997a; Flaminio et al., 2000a).

Table 5.1. BAL cell recoveries from foals

	BAL cells/ml (mean ± SEM × 10^3)	
Week of Age	Barn	Pasture
2	363 ± 133	223 ± 81
4	425 ± 29	582 ± 25
8	344 ± 66	392 ± 50
12	609 ± 56	415 ± 116

Note: Bronchoalveolar lavage cells (BAL) were collected from foals at the indicated weeks of age. There was no significant difference in cell recoveries between Barn and Pasture foals at any of the sample times.

While there was a significant ($p<0.005$) increase in the number of Gate 1 cells (lymphocytes) and a corresponding decrease in the number of Gate 2 cells (macrophages) over time (Figure 5.2 C and D), there was no difference between the two groups of foals in this regard. The majority of cells in BAL fluid of young foals are macrophages and lymphocytes, representing 85 - 90% and 5 - 20% of the BAL population, respectively. Similar changes in the proportion of macrophages and lymphocytes over time have been reported elsewhere (Giguere and Polkes, 2005b). However, there was no dramatic increase in total cell numbers, as reported in other studies (Balson et al., 1997a; Flaminio et al., 2000a). This could reflect differences in how the foals were maintained during the study periods, the methods used, and the frequency of sampling (Sweeney et al., 1992).

While exposure to microorganisms in the air of the barn did not induce recruitment of more lymphocytes into the lungs of the foals, exposure did increase the frequency of IFN-γ^+ lymphocytes amongst BAL cells (Figure 5.2 E and F). Overall, the frequency of IFN-γ^+ cells in the lymphocyte gate was significantly (p=.0486) higher for the foals exposed to barn air compared to those left on the pasture. There was also a significant (p=0.045) difference between the two groups of foals at the 12 week sampling. In Gate 2, percentage of IFN-γ^+ cells was significantly (p=0.001) different between the two groups of foals at 2 weeks, as well as there being an overall significant (p=0.0133) difference between the treatment groups. There was no difference in mean fluorescence intensity (MFI) at any of the time points or due to treatment (data not shown). Similarly, inhalation of *Bacillus licheniformis*, a bacterial spore identified in farm dust, induced higher IFN-γ concentrations in BAL fluids from exposed mice (Vogel et al., 2008a). By contrast, IFN-γ concentrations in BAL fluids of neonatal mice exposed to corn dust alone was no different than that of control mice (George et al., 2006a). The effect of barn air on IFN-γ production likely reflects the multiple and various microbial components it contains.

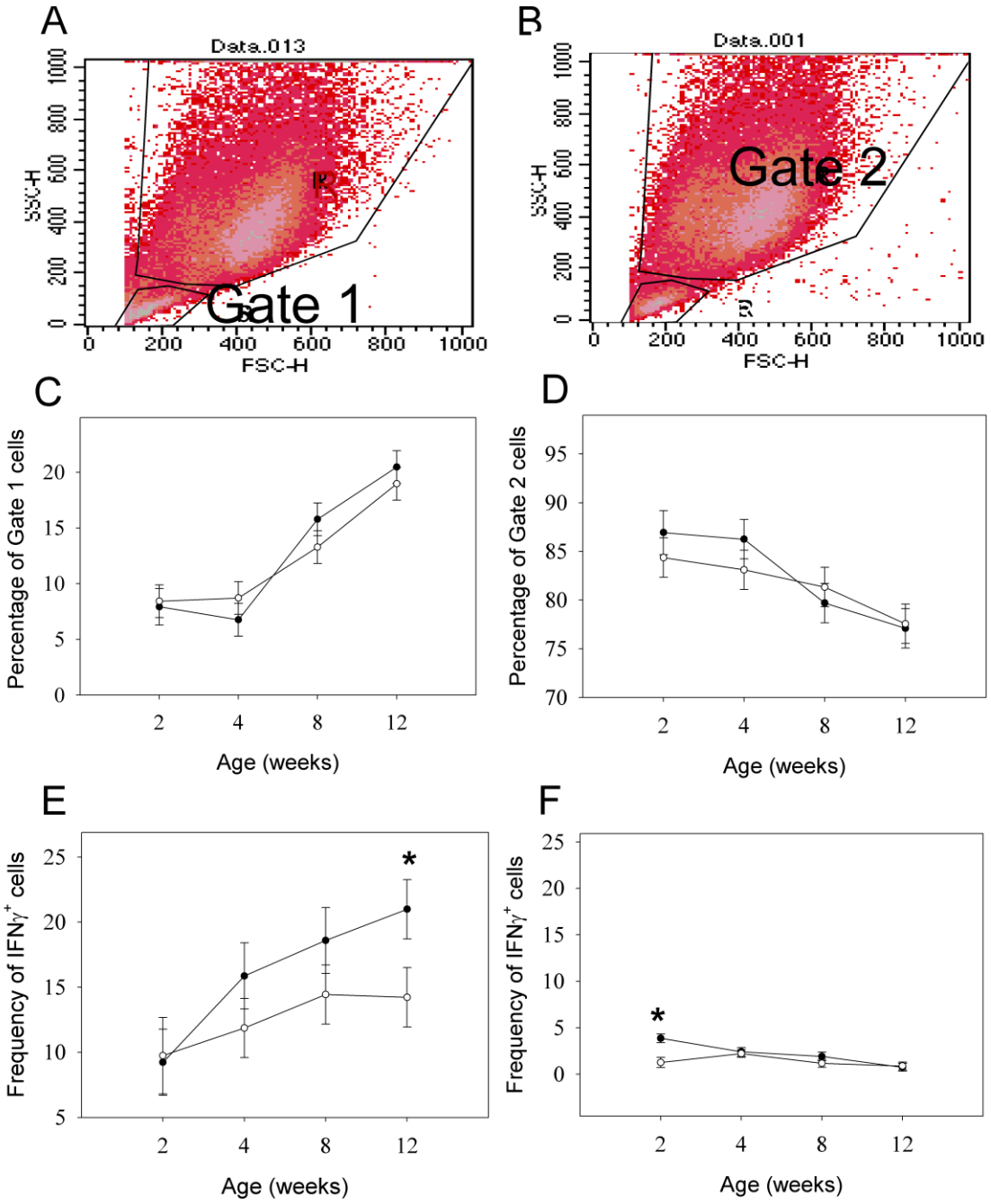


Figure 5.2. IFN-γ production by BAL cells. BAL fluid was collected from both barn (n=10) and pasture (n=10) groups of pony foals at 2, 4, 8 and 12 weeks of age (5 ponies per time points for each group). The cells from the fluid were washed with PBS twice and counted. The fresh cells were acquired and analyzed with FACSCalibur. The cells were gated into Gate 1and Gate 2, based on the morphology (A and B representing two 8

weeks old foals from barn and from pasture respectively). The frequency of cells in Gate 1 (C) and Gate 2 (D) was analyzed. The cells were stimulated with or without PMA/iono and BFA for 4 hrs followed by IFN-γ staining and acquired by FACSCalibur. Percentage of IFN-γ^+ cells within Gate 1(E) and gate 2 (F) were analyzed. * Means of percentage significantly differ between two groups ($p<0.05$).

The effect of environment on IFN-γ production was also observed in peripheral blood. It was found that the overall percentage of IFN-γ^+ lymphocytes in the PBMC was significantly ($p=0.0029$) higher in those foals exposed to barn air (Figure 5.3). This effect lasted after the foals were returned to the pasture at the end of the exposure period with $p<0.001$ at 12 weeks (Figure 5.3), though eventually the pasture group of foals caught up to the level of IFN-γ production as those foals kept in the barn at 16 weeks. Similarly, IFN-γ production by whole blood cells increased more rapidly in infants that were exposed to higher environmental concentrations of endotoxin (Gereda et al., 2000a; Roponen et al., 2005a).

While the frequency of IFN-γ^+ $CD3^-$ cells in the PBMC (Figure 5.3) was statistically higher in those foals exposed to barn air ($p=0.065$), and specifically elevated at 6 and 12 weeks-old foals ($p=0.014$ and $p=0.082$, respectively), these represented relatively few cells overall. By contrast, the frequency of IFN-γ^+ cells in $CD3^+$ cells significantly ($p=0.043$) increased over time, with significantly higher frequencies of IFN-γ^+ $CD3^+$ cells in those foals exposed to barn air at 6 ($p=0.046$) and 12 ($p=0.044$) weeks (Figure 5.3). The overall percentage of IFN-γ-producing cells in the $CD4^+$ population was also significantly ($p=0.0104$) higher for those foals exposed to the barn air (Figure 5.3).

In detail, $CD4^+$ IFN-γ production was increased in the foals exposed to the barn air at 4 weeks (p=0.063) and significantly (p=0.008) elevated at12 weeks. A similar increase in IFN-γ - producing $CD4^+$ cells was also seen in 9-24 month –old infants with high endotoxin exposure (Gereda et al., 2000a). While there was no difference in IFN-γ production by $CD8\alpha^+$ cells between the two groups of foals (Figure 5.3), the percentage of IFN-γ^+ cells in $CD8\beta^+$ cells was found significantly (p=0.0102) higher at 16 weeks for those foals exposed to barn air (Figure 5.3). These particular cells (IFN-γ^+CD8αβ) compromise only a small percentage of the total CD8 (which includes both CD8α/α and CD8α/β) population and likely represent $CD8^+$ memory T cells since prolonged nonspecific stimulation of these cells induces IFN-γ production (Noble, 2009).

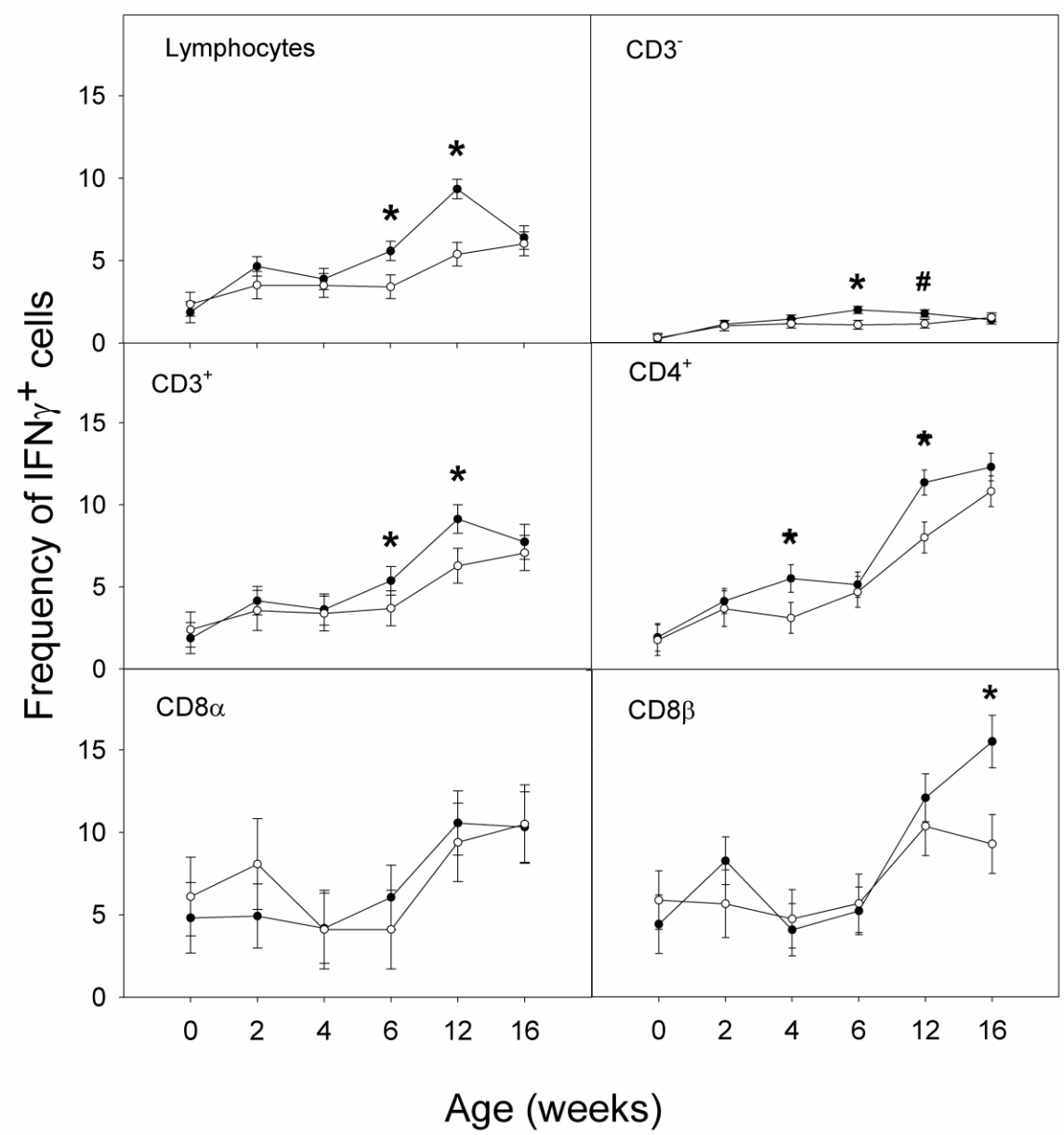


Figure 5.3. Percentage of IFN-γ^+ cells in PBMC. PBMC were isolated from blood in barn (•) and pasture (O) groups of ponies (n=6/group). The cells were stimulated with PMA and surface stained with different anti or isotype control antibodies then fixed and followed by intracellular staining of IFN-γ. Percentage of IFN-γ^+ cells within each subtype of lymphocytes was analyzed within each cell types. * Means of percentage

significant differ between two groups of foals ($p<0.05$). [#] Means of percentage differ between two groups ($0.05<p<0.19$).

The mechanism whereby exposure to barn air promotes an increase in IFN-γ expression is unknown. One possibility is that environmental effects on gene expression occur via epigenetic regulation as infection with viral, bacterial and parasitic agents induces DNA demethylation (Vuillermin et al., 2009a). The *Ifng* locus is highly methylated in neonatal CD4 T cells (White et al., 2002a). Therefore, increased demethylation of *Ifng* may be the mechanism behind the increased IFN-γ production following exposure to environmental microbial components within the barn. Other factors in epigenetic regulation may also contribute to the mechanism, such as regulation of chromatin structure and transcription factors (Vuillermin et al., 2009a). Future studies will need to focus on these possible mechanisms.

While it was initially reported that neonatal T cells appear to be heavily biased toward Th2 responses both *in vitro* and *in vivo* (Adkins and Du, 1998; Delespesse et al., 1998), this more likely reflects an inability to generate a Th1 (IFN-γ) response due to insufficient accessory signaling by antigen presenting cells (Upham et al., 2002; Zaghouani et al., 2009). Exposure to microbial antigens appears to facilitate dendritic cell maturation in neonates leading to improved Th1 signaling (Upham et al., 2002; Willems et al., 2009a). Foals exhibit similar age-related deficiencies in dendritic cell function (Flaminio et al., 2007; Merant et al., 2009a). Whether exposure to environmental antigens increases the Th1-promoting function of dendritic cells in foals remains to be determined.

In conclusion, exposure to microbial components in barn air promoted an increase in IFN-γ production by both BAL cells in the lung and lymphocytes in the circulating blood, without the additional recruitment of cells to the lung. Which specific bacterial and/or fungal component contributed to this promotion of IFN-γ production remains unknown. Likewise, the mechanism responsible for this increased production is also unknown, though epigenetic modification of the *Ifng* locus in these foals seems likely. Since antigen presenting cells contribute to the increased expression of IFN-γ, further studies on the effect of environment on their function are also needed.

Copyright © Lingshuang Sun 2012

CHAPTER SIX

The effect of environment on lung antigen presenting cells (APC) in foals

SUMMARY

While environmental microbial component exposure promotes the maturity development of Th1 immunity in foals and infants, the underlying mechanism is unknown. Antigen-presenting cells (APC), macrophages (Mϕ) and dendritic cells (DC), sample and process antigens from the environment and present then to T cells which prime and drive the direction of the immune response toward Th1 or Th2, including in the lung. Therefore, we hypothesize that environmental exposure to aerosol microbes promotes the Th1-polarized maturation and activation of alveolar Mϕ and DC in foals. The environmental effect on the frequency, maturity and activation of the DC and Mϕ from bronchial alveolar lavage (BAL) was evaluated and compared in foals placed in the barn versus those kept on pasture via estimation of the cell surface molecules using flow cytometry, and determination of their mRNA expression of representative Th1 and Th2 genes by RT-PCR. The barn-air exposure promoted DC localization into the lung and elevated the antigen-presenting and co-stimulatory molecule expression on DC and Mϕ, and their Th1-priming (Delta 4 and iNOS) and Th1 cytokine gene expression were also augmented. In conclusion, the barn-air exposure promoted Th1- biased activation of alveolar Mϕ and DC.

INTRODUCTION

All newborns are challenged by the environment after birth. While the respiratory tract is the second large front-line exposure of the body, the respiratory immunity in foals is functionally immature and is mostly developed after birth (Mair et al., 1988b). This contributes to a high rate of mortality in foals due to respiratory disease, especially diseases caused by intracellular pathogenic infections such as *Rhodococcus equi* (*R. equi*) and Equine Herpes Virus (EHV). Although environmental microbes challenge the health of the neonates, they are also believed to promote the maturity of the neonatal immunity (Vuillermin et al., 2009b). In particular, bacterial and fungal exposure elevates interferon-gamma (IFN-γ) expression by lung lymphocytes in foals (Sun et al., 2011). This maturity development of Th1 immunity also appears in humans and mice. These findings are consistent with the hygiene hypothesis, which states that exposure to a higher load of microbial components during the very early life promotes the Th1-polarized immunity, represented by IFN-γ expression, thereby inhibiting the Th2 immune response. However, the underlying mechanisms of this biased immunity maturation are unknown.

The specific APC, dendritic cells (DC) and a majority of Macrophages (Mϕ), sample, processe and present antigens to T cells, which then prime and modulate the immune response toward either Th1 or Th2 by communication with T cells via surface molecules and production of cytokines (Paul, 2003). The APC are activated upon recognization of environmental pathogens through a group of pathogen-associated molecular patterns (PAMPs). The type of PAMPs and the method or type of infection determine the presenting molecules of APC and types of priming cytokines. For instance, exogenous antigens are presented by MHC II, endogenous antigens are presented by

MHC I, and lipid molecules are presented by CD1 (Paul, 2003). While the co-stimulatory molecules (CD86 and CD83) provide co-stimulatory signals that are necessary for T cell activation and survival, cytokines direct the differentiation of naïve T cells into either Th1 or Th2 T cells (Paul, 2003). For example, IL-12 and TNFα favor the priming of Th1, whereas IL-4 drives Th2 T cells (Lee et al., 2006; Murphy and Reiner, 2002b; Zhu et al.). However, DC in neonatal foals have low levels of CD86 and CD1b, and IL-12 and TNFα expression in response to stimulation (Flaminio et al., 2007; Merant et al., 2009b), as in humans (Giguere and Polkes, 2005b; Willems et al., 2009b). Similarly, low expression of antigen-presenting molecules in Mϕ in foals, such as MHC II and CD1b, has also been observed (Flaminio et al., 2007; Fogarty and Leadon, 1987). The mechanism responsible for the promotion of this age-associated Th1-polarized maturity development of DC and Mϕ in foals is unknown.

It was recently found that exposure to various environmental antigens promoted the function of APC in mice and rats (Debarry et al., 2007; Peters et al.; Vogel et al., 2008b). The *in vitro* multiple PAMPs stimulation appeared to activate and promote the maturity of DCs and Mϕ (Flaminio et al., 2007; Merant et al., 2009b). Therefore, we hypothesized that the environmental exposure promoted the Th1 poliarized maturation of alveolar DC and Mϕ. To test this hypothesis, we determined the effect of barn-air exposure versus pasture-air exposure on the frequency of surface molecules on the DCs and Mϕ in the bronchial alveolar lavage (BAL) from the foals, as well as the corresponding Th1- and Th2- priming gene expression by the cells. Additionally, we estimated the maturity level of DC and Mϕ from both groups of foals in terms of their

response to stimulation of Th1- and/or Th2-promoting PAMPs. The environmental effect on surface molecules and phagocytosis of MoDC was used as a reference.

MATERIALS AND METHODS

Animals

A total of 20 healthy pony foals was used for this study. The foals were maintained on the University of Kentucky's Department of Veterinary Science's farm in Versailles, Kentucky. Ten of the foals were chosen randomly at birth to spend 4 hours a day for 3 days (MWF) of each week in individual stalls with their mares. The stalls were not cleaned during the study period. This barn exposure started when the foals were less than 1 week old and stopped after they reached 2 months of age. When not in the barn, the foals were kept on pasture with ten other foals and their mares. Throughout the study period, the ponies had *ad libitum* access to water and forage in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research. All research procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

Isolation and stimulation of BAL cells

The BAL fluid was collected as previously described (Breathnach et al., 2006b). Approximately 150 mls of BAL fluid was collected from each foal. The BAL fluid was centrifuged at 400 g for 10 min and washed with PBS twice. 4×10^6 BAL cells were stimulated with various type of PAMPs for 24 hours: lipopolysaccharides (LPS, 2 μg/ml, Sigma, St Louis, MO, L2630) for TLR4, peptidoglycan (PGN, 20 μg/ml, InvivoGen, San

Diego, CA, tlrl-pgnsa) for TLR2, Poly (I:C) (25 μg/ml, Sigma) for TLR3, Pam_3CSK_4 (PAM, 50 ng/ml, IMGENEX, San Diego, CA, IMG-2201) for TLR1/TLR2, or R848 (2μg/ml, InvivoGen, tlrl-r848-5) for TLR7/TLR8 activation.

Surface staining of BAL cells

The fresh and stimulated BAL cells, 0.5×10^6, were surface stained with single or double antibodies using mouse anti-CD14 (Big 10, biometec, Greifswald, Germany), mouse PE anti-bovine CD1w2 (CC20, Serotec, Raleigh, NC), mouse anti-bovine CD1w2 (Serotec), mouse PE anti-CD206 (3.29B1.10, Beckman coulter, Brea, CA), mouse PE anti-CD83 (B15a, Beckman coulter, Brea, CA), mouse PE/Cy5 anti-CD86 (IT2.2, BioLegend, San Diego, CA), mouse PE anti-CD86 (BioLegend) or mouse anti-MHC II (CVS10), as previously described (Merant et al., 2009b). The mouse IgG1 (BD Pharmingen™, Sparks, MD), mouse PE IgG2a (Serotec), mouse PE IgG2b (Beckman coulter), mouse IgG2a (Serotec) and mouse PE/Cy5 (Biolegend) were used as isotype controls. The secondary antibody PE/Cy5 conjugated Goat anti-mouse IgG1 (Invitrogen, Camarillo, CA), PE/Cy5 Goat $F(ab')_2$ anti-mouse IgG (H+L) (Southern Biotechnology Associates, Birmingham, AL) were used when primary antibody was not fluorescent labeled. The cells were fixed with 2% paraformaldehyde and acquired by FACSCalibur (BD, Franklin Lakes, NJ) the second day. The data was analyzed as shown in Figure 6.1 based on a method referenced in Van Haarst et al. (van Haarst et al., 1994).

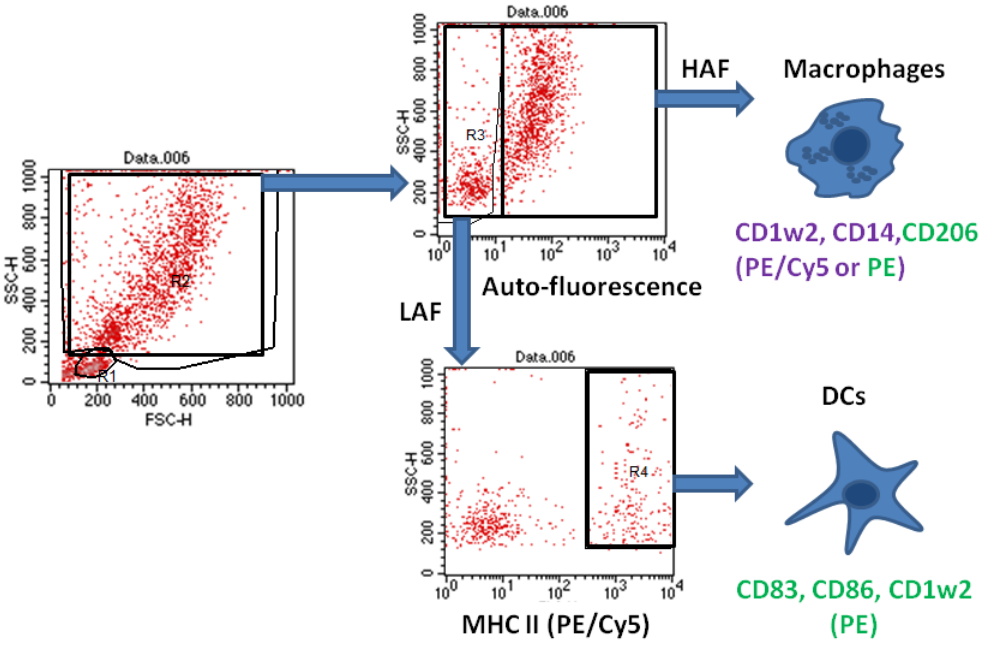


Figure 6.1. Identification of lung DC and Mϕ from BAL. The BAL cells were isolated and surface stained for CD4 molecules. Large granulocytes, identified high SSC and FSC, with high autofluorescent (HAF) at FL1 was identified as macrophages, and Mϕ was analyzed based on PE/Cy5 staining of CD1w2 and CD14, and PE staining of CD206. The large granulocytes with low autofluorescent (LAF) and MHC II^{high} were identified as DCs, and the DCs were analyzed by CD1w2, CD83 and CD86 staining.

RT-PCR

Total cellular RNA was isolated from 2×10^6 fresh or stimulated BAL cells preserved in RNA-STAT 60 (Tel-Test) through manual protocol. Reverse transcription was performed as described previously (Breathnach et al., 2006a). The resultant cDNA

was diluted 1:1 with RNAse-free water. Cytokine gene expression was measured by an Applied Biosystems 7900HP Sequence Detection System (Applied Biosystems, Foster City, CA). Intron-spanning equine specific primer/probe sets were designed (Assays-by-Design, Applied Biosystems) for the following genes: Delta 4, Jagged 1, arginase, TLSP, iNOS, CD14, MCP-1, and GM-CSF. The sequences are shown in Table 6.1. The selected primers and probes failed to amplify genomic DNA and reverse transcription-negative RNA samples and their efficiencies were greater than 95% as tested by LinRegPCR (Ramakers et al., 2003). PCR reactions were performed in duplicate wells per sample (Breathnach et al., 2006a). The amplification efficiencies were tested by LinRegPCR and the reactions with efficiencies lower than 90 % were omitted. B2M and b-Gus were used as reference genes as determined by the geNorm reference gene application (Vandesompele et al., 2002). Changes in cytokine gene expression were calculated as relative expression ratio (rER) based on the method used in Bustin (Bustin et al., 2009).

Table 6.1. Primers and probes sequence

Target gene		Sequence or reference	Reference
IL-12	Fwd	CTACACCAGCGGCTTCTTCAT	
	Rev	GCTTCAGCTGCAGGTTCTTG	
	Probe	CAGGGACATCATCAAACC	
IL-6	Fwd	GGATGCTTCCAATCTGGGTTCAAT	(Merant et
	Rev	CCGAAAGACCAGTGGTGATTTT	al., 2009b)
	Probe	ATCAGGCAGGTCTCCTG	
IL-10	Fwd	AGGACCAGCTGGACAACATG	(Merant et
	Rev	GGTAAAACTGGATCATCTCCGACAACC	al., 2009b)
	Probe	AGGTAACCCTTAAAGTC	

Table 6.1 (continued)

CD3ζ	Fwd	TCTGCTGTTTCCCTCCCATCT	
	Rev	ATGACGGCATCGCGGATA	
	Probe	CGGGTCCCGGGCTCGTCTC	
TNFα	Fwd	TTACCGAATGCCTTCCAGTCAAT	(Quinlivan
	Rev	GGGCTACAGGCTTGTCACTT	et al.,
	Probe	CCAGACACTCAGATCAT	2007)
CD11a	Fwd	GGCTGAGGGTCAAGTGATCATG	
	Rev	GAGAAGGACAGCAGAGTGATGAC	
	Probe	CTGAGCTCGCGGCCTG	
MCP-1	Fwd	GCGGCCGCCTTCAG	(Merant et
	Rev	CAGCAGGTGACTGGAGAATTAATTGCA	al., 2009b)
	Probe	GGTGCTGGCTCAGC	
CD163	Fwd	GGCAGTGTCAACACCATGAATG	
	Rev	CCTCCACCTATAAGTCTCAGTTCCATCC	
	Probe	ATCAGAACATGTCACCC	
Delta 4	Fwd	GTGGACAGGTGTACCAGCAA	
	Rev	CGGCACATTCGGGTTGGA	
	Probe	CTGGCCCCCATTGGCA	
Jagged 1	Fwd	CTCCCCTTCTGCAAACAATGAAAT	
	Rev	GGTTTCCATCGTCCCGTATGTC	
	Probe	TTCAGCAGAAATGGCC	
iNOS	Fwd	GCGTTACTCCACCAACAATGG	(Merant et
	Rev	CCAGATCCGGAAGTCATGCTTTC	al., 2009b)
	Probe	ATGGCCGACCTGATGTT	
Arginase	Fwd	CAGAAGGTCATGGAACAGACATTTGTC	
	Rev	AAAACTCAGATGGATTGGCCTTTCAAA	
	Probe	ACTCAGATGGATTGGCCTTT	
TGFβ	Fwd	CCCTGCCCCTACATTTGGA	(Merant et
	Rev	TGTACAGGGCCAGGACCTT	al., 2009b)
	Probe	CCTGGACACGCAGTACAG	

Table 6.1 (continued)

IL-1β	Fwd	CCGACACCAGTGACATGATGA	(Quinlivan et al., 2007)
	Rev	ATCCTCCTCAAAGAACAGGTCATTCATT	
	Probe	GCCGCTGCAGTAAG	
IL-6	Fwd	GGATGCTTCCAATCTGGGTTCAAT	(Quinlivan et al., 2007)
	Rev	TCCGAAAGACCAGTGGTGATTTT	
	Probe	ATCAGGCAGGTCTCCTG	
IL-4	Fwd	TGACTGTAGCGGATGCCTTTG	
	Rev	GCCCTGCAGATTTCCTTTCCAT	
	Probe	CTGGCCCGAAGAAC	
IL-13	Fwd	CCTGGAGTCCCTGAGCAA	
	Rev	CATCTTCCGCGTGTTTTGGAT	
	Probe	TCTCCACCTGCAGTGCC	
TSLP	Fwd	AATGTCATTTTTCTAGCCCTGAAGGAGC	
	Rev	CGGTCTCCACAGTAGA	
	Probe	ATGAATGGAATCAAAAGTACCC	
IL-2	Fwd	CCCAAACTCTCCAAGATTCTCACATCA	
	Rev	GAGGTTTGAGTTCTTCTTCTAGACAATG	
	Probe	CCCAAGAAGGCCAC	
IL-18	Fwd	CCTGTGTTTGAGGATATGCCTGATTGCT	
	Rev	AGACCTCTAGTGAGGCTATCTTATTGTA	
	Probe	CAGACAACGCACCC	
IL-17c	Fwd	AAGGGCCTCAGATTACCACAAC	
	Rev	TCGCCTCCCAGATCACAGA	
	Probe	TTGCGGTGGAGATTC	
IL-23p19	Fwd	GCTGTGATCCTGAAGGACTCA	
	Rev	CCCTGGTGGATCCTTTGCA	
	Probe	CAGGGCTGACTGTTGTC	
GM-CSF	Fwd	GGCCAGCCACTACAAGCA	
	Rev	GAAGGTGATCATCTGGGTTGCA	
	Probe	CACCCTGGAAACTTC	

Table 6.1 (continued)

IFN-γ	Fwd	AGCAGCACCAGCAAGCT	
	Rev	TTTGCGCTGGACCTTCAGA	
	Probe	ATTCAGATTCCGGTAAATGA	
Beta-2-microglobulin (B2M)	Fwd	CGGGCTACTCTCCCTGACT	
	Rev	GTGACGTGAGTAAACCTGAACCTT	
	Probe	CCGTCCCGCGTGTTC	
β-glucuronidase (b-Gus)	Fwd	GCTCATCTGGAACTTTGCTGATTTT	(Quinlivan et al., 2007)
	Rev	CTGACGAGTGAAGATCCCCTTT	
	Probe	CTCTCTGCGGTGACTGG	

Preparation and assessment of MoDC

Heparinized blood was collected from both groups of foals by aseptic jugular venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated as previously described (Breathnach et al., 2006b; Merant et al., 2009b). The cells (7.5 to 15×10^6 cells/ml) were resuspended in cRPMI 1640 media (Gibco, Grand Island, NY) supplemented with 10% autologous serum, 2 mM glutamine (Sigma), 100 U/ml penicillin/streptomycin (Sigma), 55 mM 2-mercaptoethanol (GIBCO), and 0.1% amphotericin B (Sigma) in tissue culture grade Petri dishes as described (Merant et al., 2009b). The non-adherent cells were removed 4 hrs latter, and recombinant equine IL-4 (1809EL, R&D Systems, Inc. Minneapolis, MN) and GM-CSF was added at 10% for 4 days. The non-adherent MoDC were then purified by centrifugation on Nycoprep 1.068 (Axis Shield, Greiner BioOne), and their number and viability were determined by an automated cell counter (Beckman Coulter).

A million MoDC were stimulated with LPS (1μg/ml, Sigma) for 24 hrs. Both the fresh and the stimulated MoDC were surface stained with antibodies against: CD86 (PE-Cy5-labeled IT2.2, Biolegend), CD1w2 (PE-labeled-CC20), CD83, MHC II (CVS10), CD206 and the CD14 as described as described previously (Merant et al., 2009b). The endocytosis capacity of MoDC with/without LPS stimulation was measured by DQ^{TM} Ovalbumin (Molecular Probes, Inc., Eugene, OR) as product protocol.

Statistical analysis

All data were analyzed by a commercially available statistics package (Sigma Stat version 10.0; Systat, San Jose, CA). Some of the data were log-transformed or rank-transformed to meet the assumptions of the normality or equivalence variation. Two-way ANOVA was used to test statistical significance. Differences are considered significant at $p < 0.05$.

RESULTS

Environmental effect on Mϕ

Since alveolar macrophages comprise the majority of cells in BAL and function as antigen surveillance, the effect of environment on Mϕ was estimated from foals. There was no difference between the frequencies of Mϕ in BAL cells from foals placed in the barn compared with that kept on pasture (data not shown). However, the frequency of overall CD86^{+} (p=0.011) and MHC II^{+} (p=0.0087) Mϕ was observed to be significantly elevated in foals with barn air exposure, especially at 2 weeks of age (p<0.001, Figure 6.2). There was also a trend of elevated proportion of CD1b^{+} Mϕ (p=0.061) in BAL cells from foals. The elevation was observed after 4 weeks of barn-air

exposure and the higher level maintained until the end of the study, with $p=0.077$ and $p=0.074$ at 8- and 12- weeks, respectively (Figure 6.2). In addition, the frequency of $CD14^+$ Mϕ in BAL from foals was also elevated after 2 weeks of barn-air exposure ($p< 0.001$, Figure 6.2). There was no difference in the frequencies of $CD206^+$ and $CD83^+$ Mϕ in BAL cells between two groups although proportion of $CD206^+$ Mϕ increase in both groups over time (data not shown).

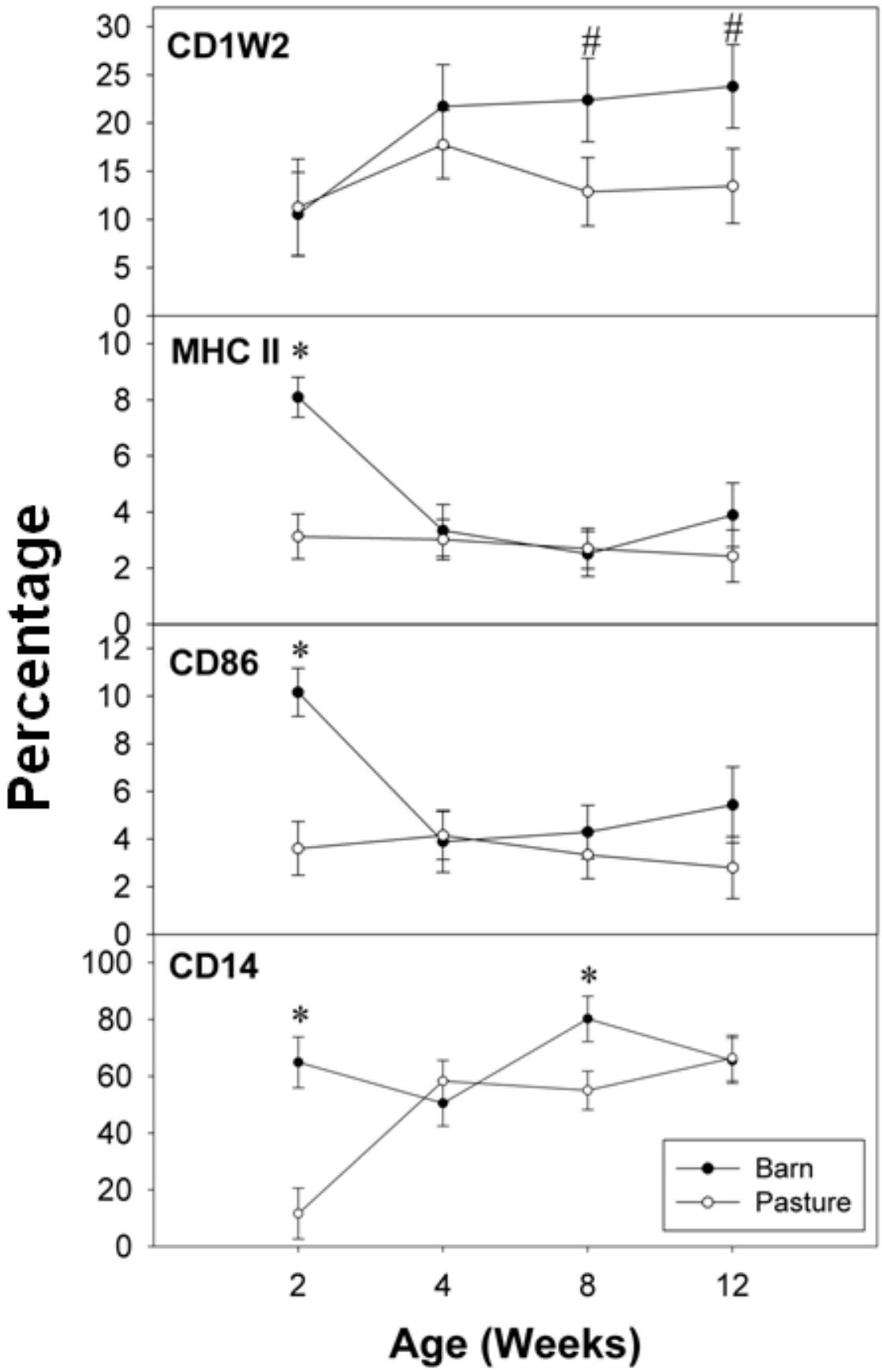

CD1W2
#
#
MHC II *
CD86 *
CD14
*
*
Barn
Pasture
Percentage
Age (Weeks)
2
4
8
12

Figure 6.2. Environment effect on surface molecules of lung Mϕ. Cells from BAL fluid were isolated and surface stained. The Mϕ were identified and the frequency of the CD1b, MHC II and CD86 positive Mϕ were analyzed. *Mean of frequency was significantly different between the two groups of foals, $p<0.05$.

The environment effect on the activation of alveolar Mϕ was also estimated by determining surface molecules on Mϕ with *in vitro* stimulation of Th1/Th2-, Th1- or Th2-promoting PAMPs, LPS, Poly (I:C), or PGN. With Poly (I:C) stimulation, the overall percentage of MHC II $^{+}$ Mϕ was significantly ($p=0.015$) elevated in foals with barn-air exposure (Figure 6.3). No significant difference was found in the frequencies of $CD14^{+}$, $CD206^{+}$, $CD1w2^{+}$, $CD83^{+}$ and $CD86^{+}$ Mϕ with various PAMPs stimulations, between the two groups of foals (data not shown).

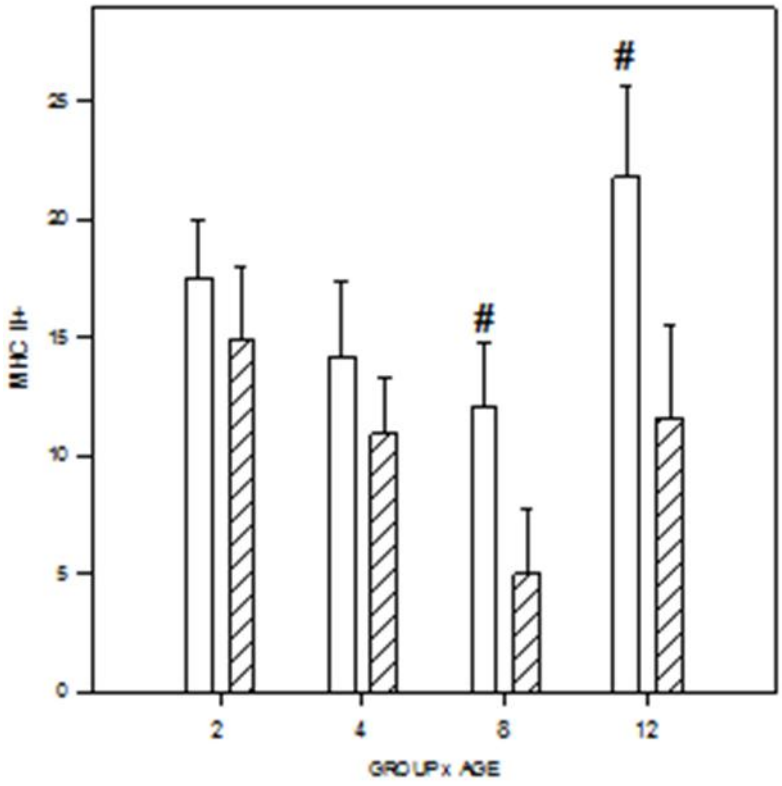


Figure 6.3. Elevated percentage of MHC II^+ Mϕ in Poly (I:C) - stimulated BAL cells from foals with barn-air exposure. The isolated BAL cells were stimulated with Poly (I:C), then surface stained for MHC II. [#]Means of MHC II^+ percentage were significantly different between foals placed in the barn (hatched bar) and foals kept on pasture (open bar), $p<0.08$.

Environment effect on alveolar DCs and MoDC

Since DCs prime and direct the immune response, the proportion of DCs and the frequencies of the antigen-presenting associated and co-stimulatory molecules were estimated. The percentage of DCs was augmented from foals after two weeks placed in the stall compared with foals kept on the pasture ($p=0.04$, Figure 6.4). Overall, the DC frequency in foals increased significantly between birth and age 12 weeks ($p <0.01$). At age 2 weeks, the proportion was significantly higher in the foals with barn-air exposure than the foals kept on pasture ($p<0.006$, Figure 6.4). However, there was a rapid increase in the pasture-exposed group from 2 weeks to 8 weeks ($p=0.002$), reaching the level of the barn-exposed group at age 4 weeks (Figure 6.4). In addition, the percentage of overall $CD1b^+$ ($p=0.0235$) and $CD86^+$ ($p< 0.001$) DCs was significantly higher in foals placed in the stall than that of foals kept on pasture, with percentage of $CD86^+$ DCs significantly higher at 2 weeks ($p<0.001$) and 8 weeks ($p=0.042$, Figure 6.4).

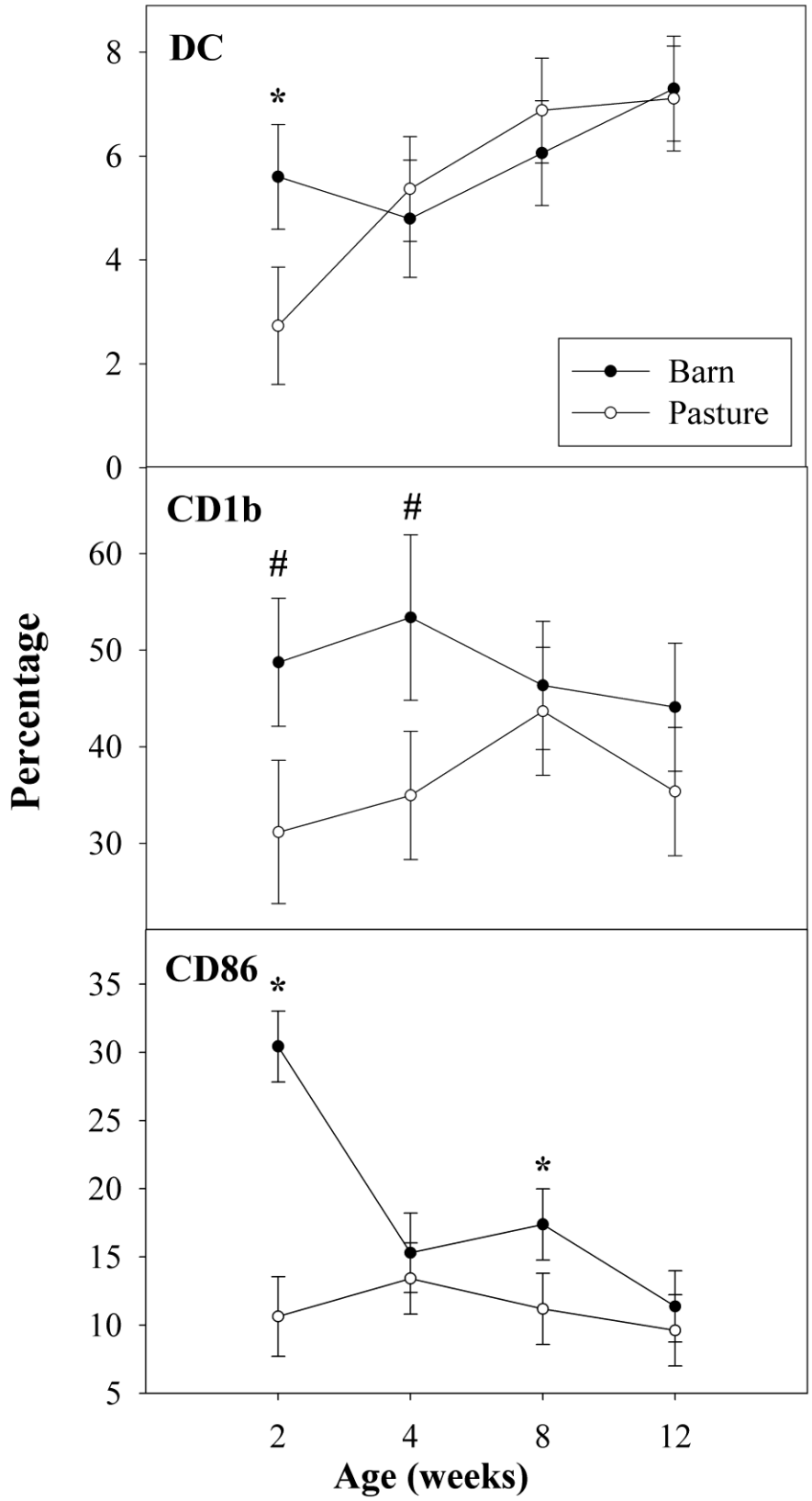

DC
CD1b
CD86
Barn
Pasture
Percentage
Age (weeks)
*
#
#
*
*
0
2
4
6
8
30
40
50
60
5
10
15
20
25
30
35
2
4
8
12

Figure 6.4. Elevated frequency of DC and frequency of $CD1b^+$ and $CD86^+$ DC in BAL cells from foals placed in the barn. The DCs were identified as described and the frequency of DC in large granulocytes with LAF BAL cells was analyzed. The proportion of CD86 and CD1b positive cells of the DC were analyzed. *Mean of frequency was significantly different between two groups ($p<0.05$). #Mean of proportion differs significantly between two groups of foals ($p<0.09$).

To further characterize the effect of the environment on DCs, the activation of Th1- and Th2- promoting DCs was assessed via evaluation of their surface molecule expression in response to LPS, Poly (I:C) and PGN stimulation, since different types of PAMPs direct the DC development into either Th1- or Th2- maturation (Lebre et al., 2005). Overall, the percentage of $CD83^+$ DC was significantly higher in BAL cells from the barn-exposed foals in response to LPS ($p=0.01$) and Poly (I:C) ($p<0.001$) stimulation (Figure 6.5). No significant difference was found in the frequency of $CD14^+$, $CD206^+$, $CD1w2^+$, MHC II^+ and $CD86^+$ DC with various PAMPs stimulation between the two groups of foals (data not shown).

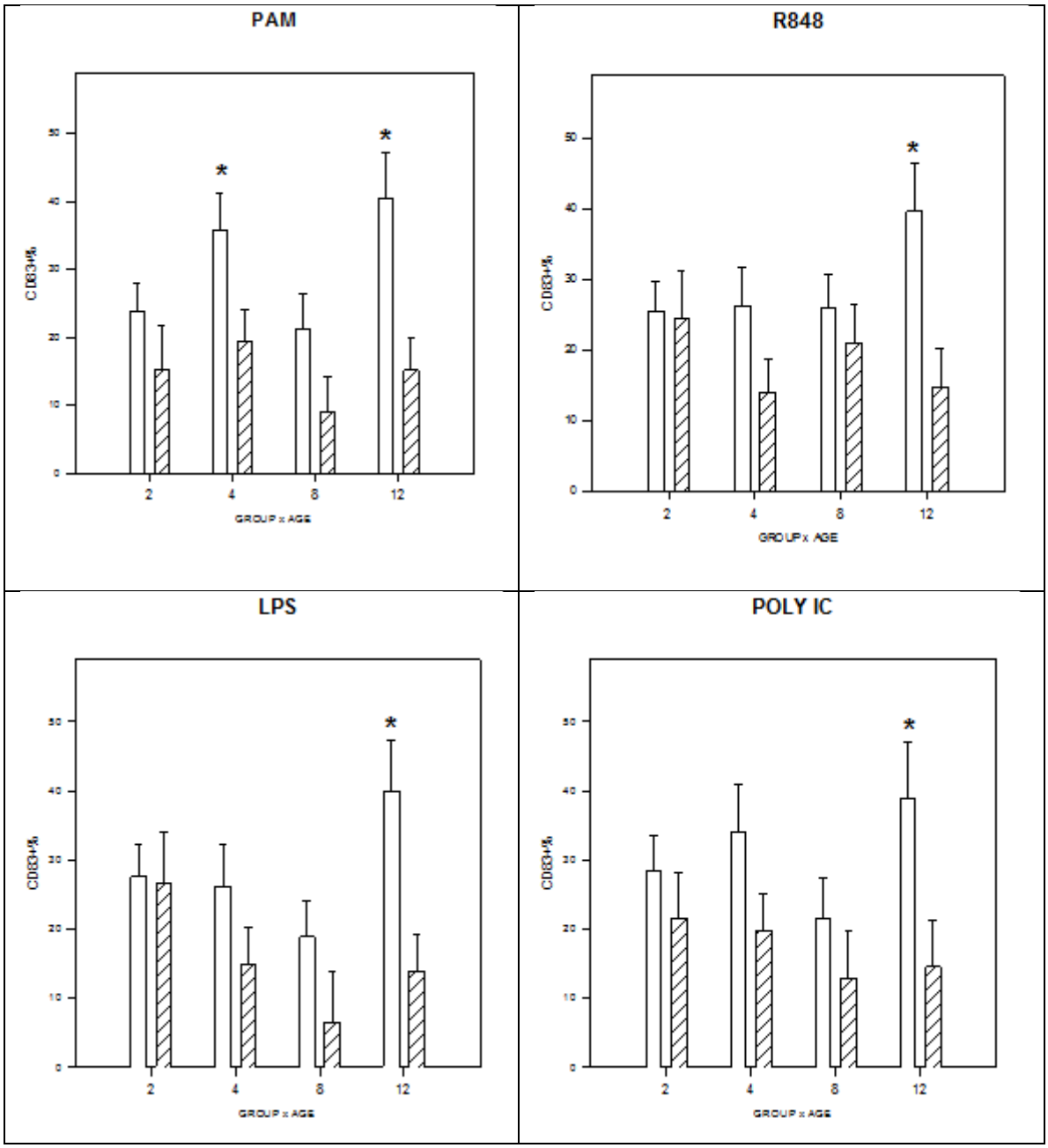


Figure 6.5. Elevated frequency of CD83^{+} DCs in PAMP - stimulated BAL cells from barn-air exposed. The BAL cells were stimulated with different PAMPs and the CD83 were stained and analyzed in identified DCs. *Mean of frequency in DCs differ between foals placed in the barn (hatched bar) and foals kept on pasture (open bar), $p<0.05$

The effect of environment on MoDC was also estimated as a reference DCs to alveolar DC, since there is no report on alveolar DCs in horses. However, no difference was observed in either surface molecules or endocytosis of MoDC between the two groups of foals at any time point, though $CD86^+$ and $CD1b^+$ DC increased with foal over time in both groups (data not shown).

Mϕ and DC favor Th1-priming in foals with barn-air exposure

Since the expression of Delta 4 and Jagged 1 in DC is considered a marker for Th1- and Th2- priming respectively (Amsen et al., 2004; Debarry et al., 2007; Maekawa et al., 2003), the mRNA levels of these two genes were used to represent the driving force of DCs on priming immune response. The mRNA level of Delta 4 was observed to be significantly elevated and maintained at a high level in BAL cells from the barn-exposed foals compared with the pasture-exposed group (Figure 6.6), with no difference in the mRNA level of Jagged 1. In addition, the environment effect on Mϕ in regarding Th1- and Th2- priming was also evaluated by determining mRNA expression of representative molecules-iNOS and arginase, respectively. Likewise, the mRNA of iNOS was significantly and consistently enhanced in the BAL cells from barn-exposed foals compared with pasture-exposed group, and there was no difference between the mRNA levels of arginase in the two groups of foals (Figure 6.6).

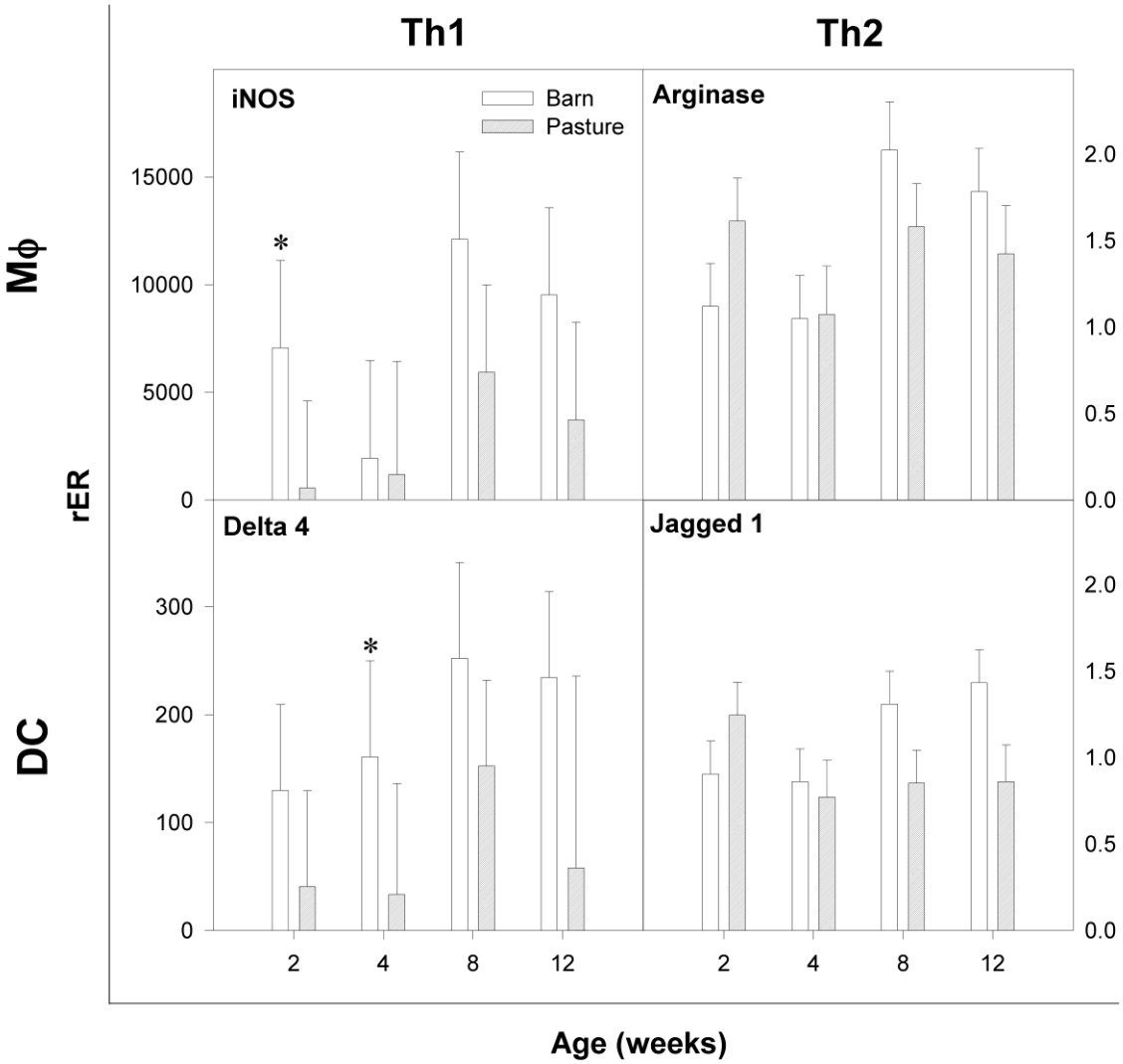


Figure 6.6. Mϕ and DC favor Th1-priming in foals with barn-air exposure. The mRNA expression of BAL cells were determined by RT-PCR. *Mean of rER was different between two groups of foals, $p<0.05$.

Chemokine and cytokine gene expression by BAL cells were altered with barn air exposure

Chemokine and cytokine production is a way by which APC modulate immune response. The barn air exposure promoted chemokine mRNA expression, such as GM-CSF ($p=0.04$) and MCP-1 ($p=0.073$) overall, to enhance the recruitment of monocytes to

the lung and their differentiation into APC (Figure 6.7). Similarly, the Th1 cytokine mRNA expression, such as IFN-γ, IL-18, and IL-12, was augmented in BAL cells from the barn-exposed foals (p=0.046, Figure 6.8; p=0.0109, Figure 6.8; p=0.073, Figure 6.9; p=0.074, Figure 6.9, respectively). An increase of Th2 cytokines, such as IL-13 (p=0.002), was also observed, though a trend of lower IL-4 mRNA levels was seen in BAL from barn-exposed foals, (Figure 6.8). Additionally, a Th17-promoting cytokine gene expression, IL-17, was also found elevated in the BAL cells from the barn-exposed foals (p=0.003), as well as IL-23 (p=0.013, Figure 6.8). However, no difference was observed between the two groups in regards to the cytokine associated with regulatory function of T cells (IL-10, TGF-β and TLSP) (Data not shown).

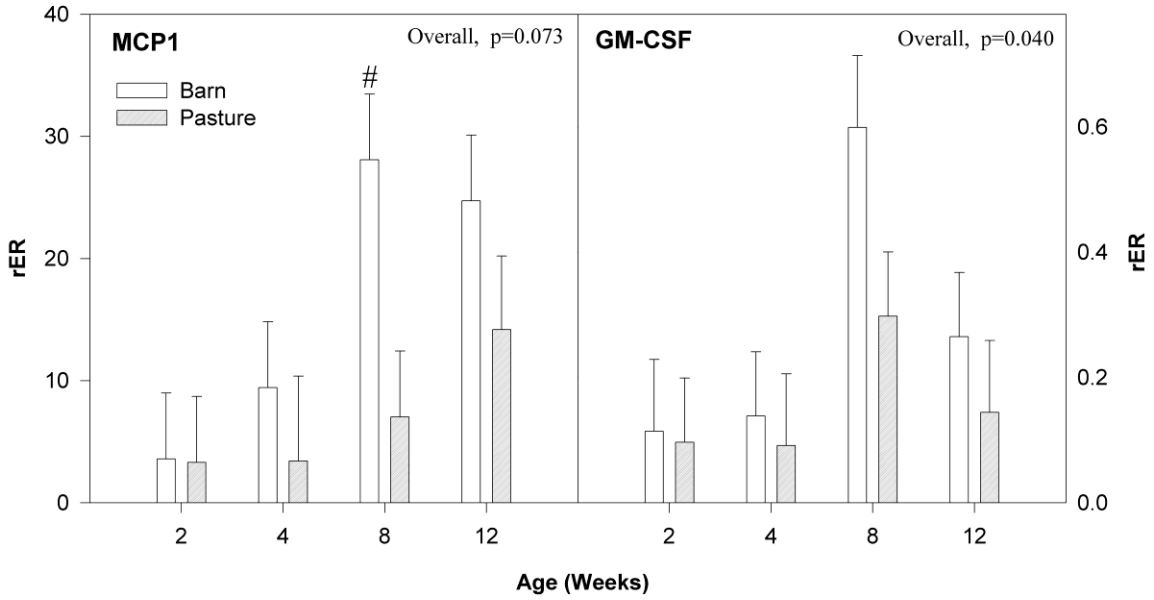


Figure 6.7. Elevated chemokine gene mRNA expression by BAL cells from barn-air exposed foals. The mRNA expression of BAL cells were determined by RT-PCR. [#]Mean of rER was different between two groups of foals, p<0.07.

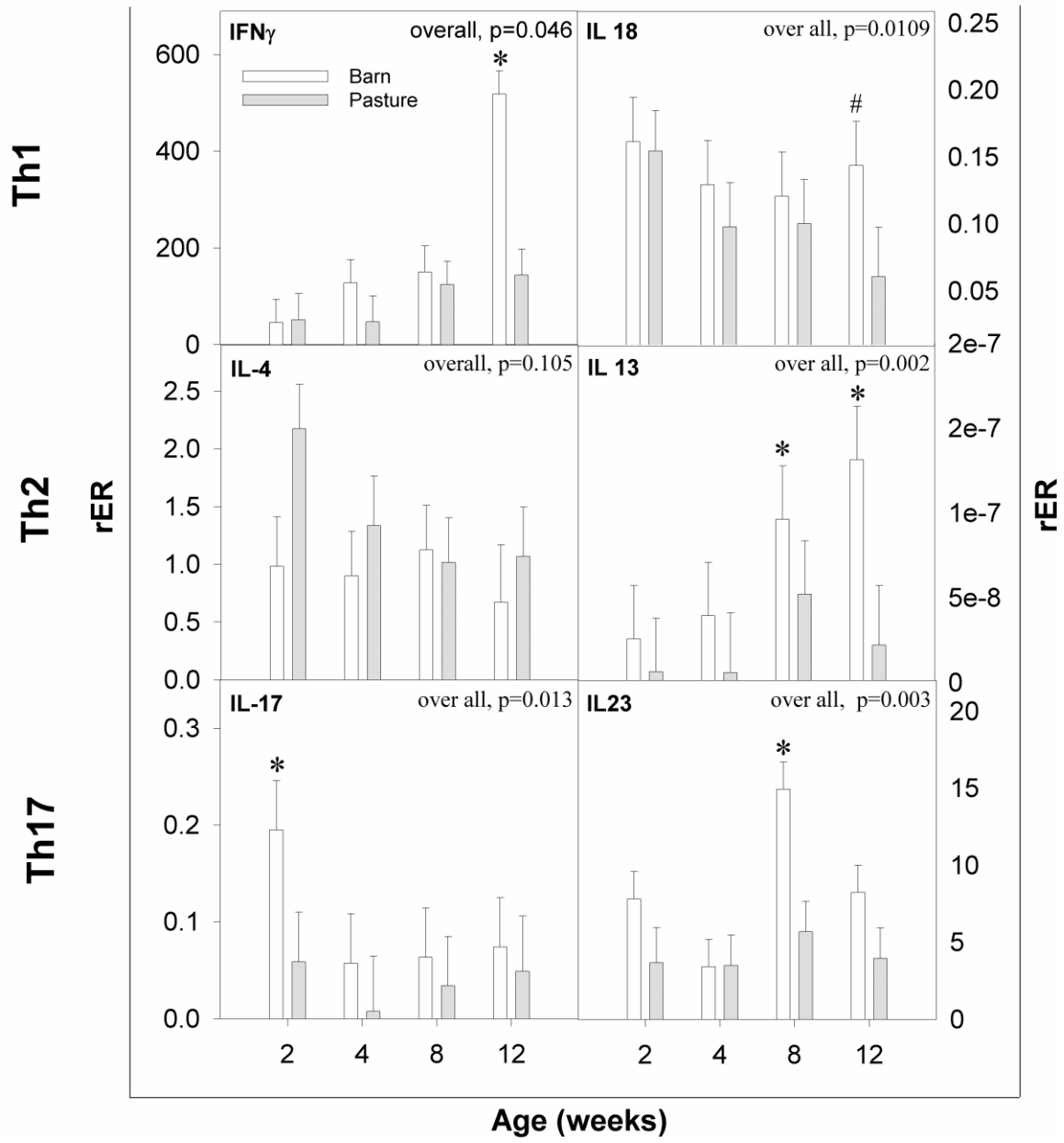


Figure 6.8. Elevated Th1, Th2 and Th17 cytokine mRNA expression in foals with barn-air exposure. The mRNA expression of BAL cells were determined by RT-PCR. The mRNA expression of BAL cells were determined by RT-PCR. *Mean of rER was different between two groups of foals, $p<0.04$. #Mean of rER was different between two groups of foals, $p<0.07$.

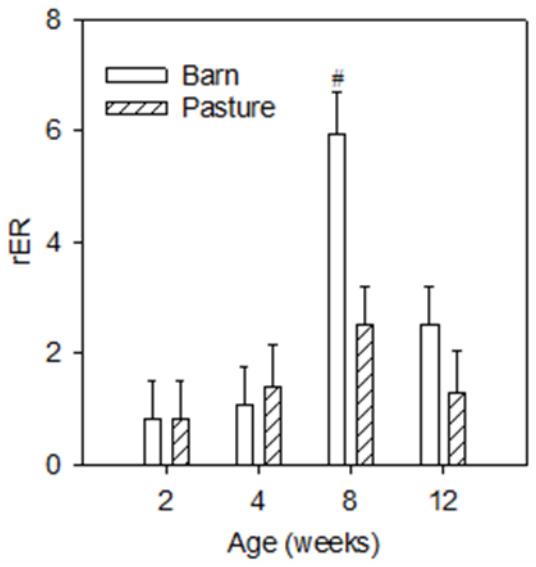


Figure 6.9. Elevated IL-12 mRNA expression in foals with barn-air exposure. The mRNA expression of BAL cells were determined by RT-PCR. The mRNA expression of BAL cells were determined by RT-PCR. [#]Mean of rER was different between two groups of foals, p=0.074.

Effect of barn-air exposure on inflammation

Inflammation response has been reported to be associated with exposure to microbial components. Therefore, we estimated the influence of the barn air exposure on the induction of inflammation by observing patterns of cells recruited to the lung and patterns of cytokines expressed by the BAL cells. Cell types were characterized by testing the mRNA level of cellular markers. No difference was observed between the gene expression of CD11α (a neutrophil marker), CD3ζ (a lymphocyte marker) and CD163 (an inflammation-associated Mϕ marker) between the two groups through the study (Data not shown). Despite a lack of inflammatory cells in the lung, the mRNA level of TNFα was increased in BAL cells from barn-exposed foal versus pasture-

exposed group overall (p=0.0339) and specifically at 8 weeks (p=0.035, Figure 6.10). However, there was no difference in IL-1 and IL-6 mRNA expression by BAL cells from the two groups (data not shown).

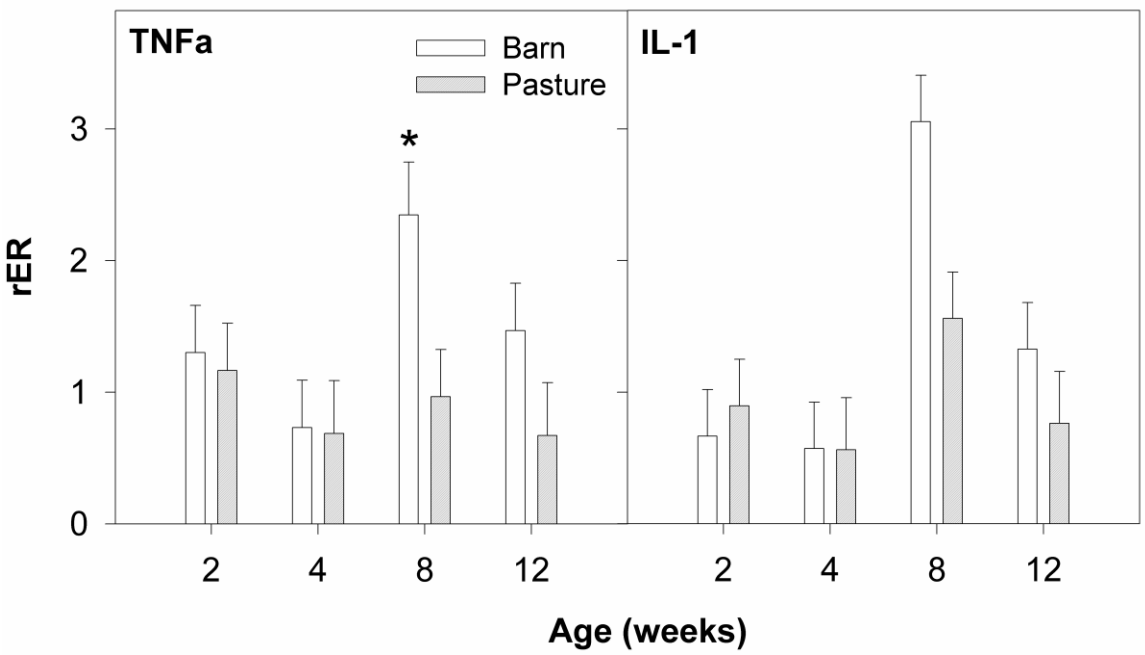


Figure 6.10. Elevated inflammatory cytokine mRNA expression. The mRNA expression of BAL cells were determined by RT-PCR. The mRNA expression of BAL cells were determined by RT-PCR. *Mean of rER was different between two groups of foals, p=0.035.

DISCUSSIONS AND CONCLUSIONS

Recent studies found that exposure to higher loads of microbial components in early life result in the Th1-polarized immunity, such as IFN-γ expression, in humans (Roponen et al., 2005b), which provides the evidence for the hygiene hypothesis. The same elevated IFN-γ production in foals exposed to higher level of aerosol bacteria and fungi has also been observed (Sun et al., 2011). However, the underlying mechanisms for

this age-associated Th1-biased immunity development are unknown. APC play a pivotal role in sampling antigens from the environments and presenting them to drive the immunity to Th1 or Th2 immune response (Paul, 2003). The *in vitro* studies showed that the direction induced by the activated APC depends on the type of PAMP. For instance, LPS activates APC toward Th1 and Th2 priming, the Poly IC activates APC toward Th1-priming, and PGN activates APC toward Th2-priming immunity. *In vivo*, the environmental PAMP activated DC move to nearest lymphoid tissue where the prime occurs (Hume, 2008). With consistent immature DC and Mϕ recruitment into the lung when exposed to non-pathogenic ubiquitous environmental aerosol microbial components exposure, the turn-over rate of lung DC exhibits a steady-state phenomenon (Holt et al., 1994).

In this study, an increase of DC localization into the lung in foals after birth with environmental exposure was observed. Similarly, the number of DC in the respiratory tract was increased in rats after birth (Holt, 2000; Nelson et al., 1994). The recruitment of DC into the lung was proposed to be the effect of the exposure of environmental microbial components (Holt, 2000; Nelson et al., 1994). A wave of incoming DCs has been widely observed recruited in to the lung as a response to LPS, bacterial and viral stimulation in adult rats (Holt et al., 1994; McWilliam et al., 1996). However, the rate of recruitment of DC into the lung is defective in neonatal rats in response to microbial stimuli (Nelson and Holt, 1995). The seeding of DC into the lung in neonates may likewise be deficient, since the mucosal-associated immune tissue in respiratory tract is absent in neonatal foals and the tissue developed after birth correlates with distance to ambient air (Mair et al., 1988b). In addition, the localization of DC was observed significantly elevated in foals placed in the barn, which was reported to contain higher

levels of culturable bacteria and fungi (Sun et al., 2011). Thus, the age-associated increase of the DC seeding into the lung is likely due to cumulative effect of ubiquitous non-pathogenic antigens in the air. This may be induced by chemokines produced in response to microbes in the aerosol since GM-CSF and MCP-1, which promote monocyte recruitment and differentiation into DC, were found elevated in the foals with barn-air exposure.

The effect of barn-air exposure was not only on promoting the localization of alveolar DCs in the foals but also on stimulating the maturation and/or activation of DC, regarding of up-regulation of CD83 and CD1b expression on the DC surface. This may be due to the up-regulated GM-CSF and TNFα observed elevated in foals placed in the barn, since the maturation and activation of the DC in the lung is induced by GM-CSF and TNFα cooperation (Colsman et al., 2006). Since no previous studies have been performed on alveolar DC in the foals, we resorted to the use of MoDCs. While a similar increase of antigen-presenting associated surface molecules on MoDC, such as CD86 and CD1b, was observed in this study as in previous study (Merant et al., 2009b), no differences in those molecules were influenced by the effect of environment. Similarly, the phenotype and function of lung DC was also observed different from MoDC in mice, rats and humans. Thus, it is better to use lung DC instead of MoDC in horses for future study on *in vivo* effect of microbes. The effect of barn-air exposure on the surface molecules of lung Mϕ was also observed. The promoted CD1b and MHC II expression on Mϕ with barn-air exposure may increase the resistance of foals to respiratory infections, such as *R. equi*, because the age-limited expression of CD1b and MHC II on Mϕ was observed in foals, and it was considered to be responsible for foals'

susceptibility to *R. equi* infection (Flaminio et al., 2007; Pargass et al., 2009). In addition, the elevation of CD14 on Mϕ indicates a promotion of the capacity and efficacy of antigen surveillance with barn air exposure, since the level of CD14 binding to LPS represents the ability of Mϕ to capture antigens. In agreement, children with farm life experience were observed to have significantly higher expression of CD14 mRNA in blood, which correlated with skewed Th1 immune maturation (Ege et al., 2007).

The favored Th1-priming Mϕ in foals with barn-air exposure was observed, which supports the hygiene hypothesis. In agreement with elevated iNOS expression in foals with barn-air exposure, the augmented iNOS activity in the lung from mice exposed to LPS was observed (Stumbles et al., 2003; Tulic et al., 2001). The biased Th1-priming Mϕ from foals placed in the barn was further evidenced by the significantly higher response of Mϕ to Th1-promoting PAMP (Poly (I:C)) with no difference in response of Mϕ to Th2-priming PAMP. The Th1-biased activation of alveolar DCs was also observed in foals with barn-air exposure. Without stimulation, the immature alveolar DC primes for a Th2 immunity (Stumbles et al., 1998; Stumbles et al., 2003), due to the suppressive effect from adjacent alveolar Mϕ (von Garnier et al., 2005). The environmental effects on promoting a Th1 immunity are considered to be conducted by a Notch signaling pathway via cross-talk between DC and naïve T cells (Amsen et al., 2004; Maekawa et al., 2003). A high burden of microbial components elevates Delta 4 expression, which induces and elicits naïve T cells to differentiate into Th1 cells, however, a low burden of microbial components maintains the Jagged 1 expression on suppressed DC and conducts the differentiation of naïve T cells into Th2 cells (Amsen et al., 2004; Debarry et al., 2007; Maekawa et al., 2003). Given a higher level of microbial

components in the air from the barn (Sun et al., 2011), our observation of high Delta 4 expression in barn-exposed foals correlates with the above theory. In addition, Th1-promoting DC were more mature in barn-air exposed foals than those kept on pasture, since Poly I:C stimulated alveolar DC elicited higher expression of CD83, while no PGN stimulated Th2-promoting DC (Lebre et al., 2005) were found different between two groups of foals.

The priming preference of lung APC in Th1 response also appeared in the elevated Th1 cytokine gene expression in BAL cells from foals placed in the barn. The increased IL-12, IL-18 and TNFα mRNA expression in foals with barn-air exposure likely contributes to an increased IFN-γ production which reported in our previous study (Sun et al., 2011). The elevated IL-12 and TNFα expression may be due to the modulating effect of airway epithelial cells on DC differentiation to the Th1-promoting type in response to ubiquitous non-pathogenic antigen in the air via GM-CSF production (Rate et al., 2009) which was observed elevated in foals placed in the barn. Consistent with the augmented Th1 cytokines, a Th2 cytokines, IL-13, was also found elevated in foals with barn-air exposure, though the pivotal Th2 cytokine (IL-4) expression was not. The augment of both Th1 and Th2 cytokine expression may due to relatively unchanged IL-10 and TGF-β expression. Similarly, *in vitro* IL-10 expression was observed unchanged in microbial exposed animals (George et al., 2006b; Peters et al.; Vogel et al., 2008b), though its production increased in response to various aerosol or cowshed microbial stimuli *in vitro* and lung explants *ex vivo* (Peters et al.; Rate et al., 2009; Vogel et al., 2008b). In addition, the function of barn-air exposure elevated IL-17 and IL-23 cytokines is unknown. They are critical for host defense against bacterial, fungal, and

viral infections at mucosal surfaces (Khader et al., 2009), although they are also report to be associated with autoimmunity. Given the premise of inflammation on autoimmune induction, the barn air exposure was unlikely to induce any autoimmune diseases, since no significant inflammation was observed in the foals kept in the barn versus pasture. This suggests that it is highly likely that the barn-air exposure enhances the general immunity without increasing the potential of autoimmunity. The observation of no inflammation induced by barn-air exposure may due to the effect of increased IL-12 on reduction of airway hyperreactivity (Debarry et al., 2007). The same observation of no inflammation was reported in neonatal mice with corn dust exposure represented by comparable neutrophils in BAL fluid to control mice (George et al., 2006a).

Lungs exposed to different or combined microbial components induce various and complicated immune responses. Although the polarization property of PAMPs has been studied in vitro, the results of single PAMP in vivo in promotion of the APC immunity development varies: the effect depends on the type and consistency concentration of the microbes (Debarry et al., 2007; Peters et al.; Vogel et al., 2008b). A combination of multiple PAMP or microbes may responsible for the Th1-polarized immunity development of DC and APC in the lung. Further investigation into identification and evaluation of the microbes in the barn air must be undertaken to further understand its effect.

To conclude, a model is drawn based on the finding. The environmental microbe exposure: promotes the DC localization in the lung; enhances antigen-presenting associate molecule (CD1b, CD86, MHC II and CD14) expression on DC and Mϕ; promote Th1-priming activation of DC (Delta 4) and Mϕ (iNOS); and augments Th1

cytokine (IL-12, TNFα and IL-18) and chemokine (GM-CSF, MCP-1) expression (Figure 8). This is the first observation that evaluates the effect of barn-air exposure on lung APC in foals. The ambient finding was that barn-air exposure favors the Th1-priming activation of lung DC and Mϕ in foals. Identification and evaluation of the barn air microbial components is necessary for understanding the underlying mechanisms and ultimate novel immunomodulator development.

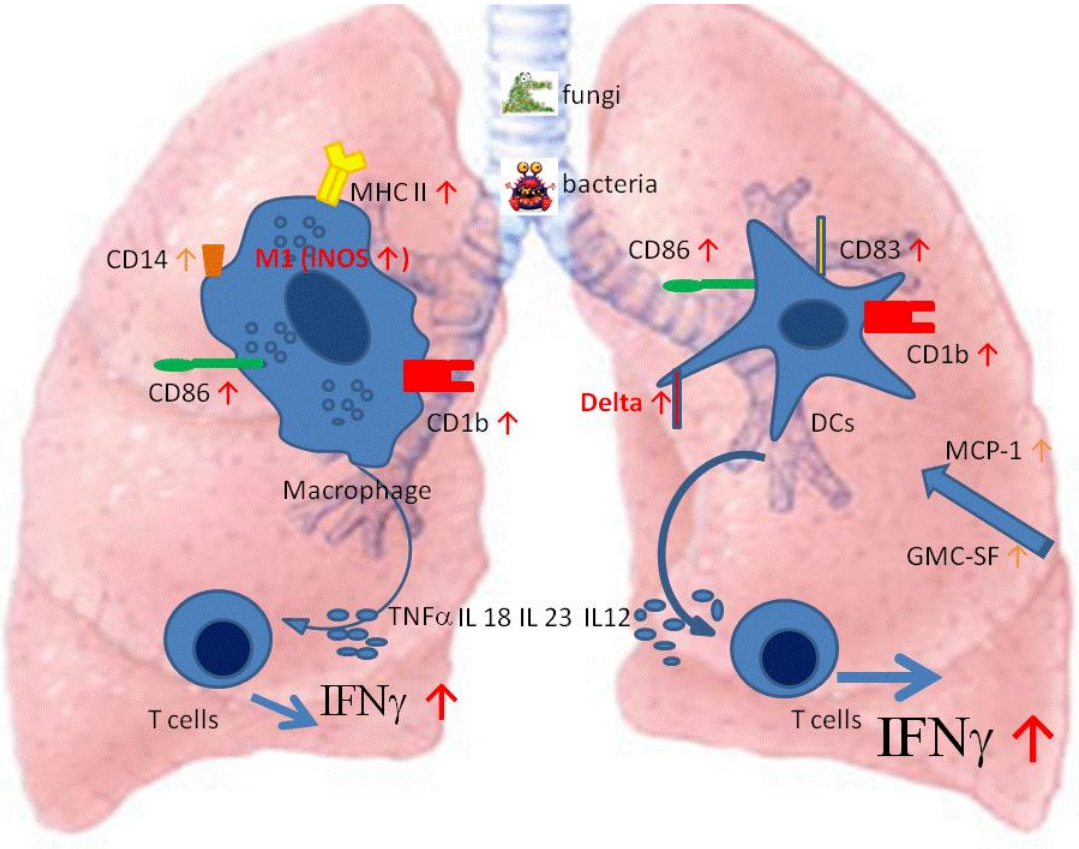


Figure 6.11. Graphic summarization of the effect of environment on APC in the lung. Microbial components in the environment enhance alveolar DCs localization; promote antigen-presenting associated and costimulatory molecules expression on DC, such as CD1b and CD86; up regulate antigen-presenting associated and costimulatory molecules expression on Mϕ, such as MHC II, CD1b and CD14; activate the Th1-priming by both

DC and Mf represented by elevated Delta and iNOS expression in them respectively; and augment Th1 cytokine expression, such as TNFα, IL-12 and IL-18.

Copyright © Lingshuang Sun 2012

CHAPTER SEVEN

Conclusions & Future Directions

While IFN-γ plays an important role in the protection against intracellular bacterial and viral infection, such as *R. equi*, its expression is reduced in foals. However, the underlying mechanism for this decreased IFN-γ expression remains unknown. Understanding the regulatory mechanisms of IFN-γ expression and the environmental effect on its expression is necessary for our ultimate goal of promoting foals' protection against various infections via modulation of IFN-γ expression in foals. The body of the research contained in this dissertation provides novel information characterizing the relevance of IFN-γ expression to *R. equi* infection, and the mechanisms that control the impaired IFN-γ expression in neonatal foals and the environmental effect on this expression.

IFN-γ production correlates with age-related susceptibility to *R. equi* infection in foals

The results of the *R. equi* infection study in chapter two found that the age-related susceptibility to *R. equi* infection in foals was correlated with the IFN-γ expression prior to the challenge infection. Characterizing this relevance provides potential indicators (IFN-γ) for foals' susceptibility risk to *R. equi* infection. Perhaps, the levels of IFN-γ could be used as a diagnostic tool for the prevention of *R. equi* infection in individual foals. Future examination of this age-related susceptibility in younger foals, less than 3 weeks old, is important because these neonatal foals are exposed to *R. equi* immediately

after birth and have an even lesser mature immune system when compared to foals of 3 weeks of age and older.

Lymphoproliferative activity and DNA methylation regulate IFN-γ expression in foals

IFN-γ expression is found to be promoted by lymphoproliferation and repressed by DNA methylation in neonatal foals. The age-related increase of IFN-γ expression is associated with an increase in lymphoproliferative activity and correlated with DNA demethylation. Understanding the regulation mechanisms for IFN-γ expression provides the fundamental theory for us to find potential means to enhance IFN-γ expression. Additional mechanisms by which the IFN-γ gene is transcriptionally regulated in foals would be interesting to discover, such as chromatin remodeling and histone modification. These mechanisms have been reported to synergistically work together with DNA methylation on regulation of IFN-γ gene transcription.

Effect of environment on IFN-γ expression in foals

This study found, for the first time, that exposure to a high load of microbial components in the air promotes the increase of IFN-γ production by lymphocytes. This is likely due to the age-associated demethylation of the *Ifng* promoter region via the induction of lymphoproliferation, leading to increased gene transcription. These novel findings suggest a way of modulating IFN-γ production, hence preventing pathogen infection. Investigations of which specific bacterial and/or fungal components in the air contribute to this augment of IFN-γ production would shed light on the development of novel adjuvants and immunostimulators. It would also be of interest to understand how demethylation is induced by lymphoproliferation in foals.

The effect of environment on maturation of APC in foals

The results of the environment effect on APC provide us with an understanding of how the IFN-γ production is increased with barn-air exposure. We found that the barn-air exposure promotes the maturation and Th1-polarization of the lung DC and Mϕ in foals. Identification and evaluation of the barn air microbial components is necessary for understanding the underlying mechanisms of this process. Understanding how this promotion of APC maturation is induced requires further investigations, which ultimately will provide ways to promote the immunity maturation of foals, hence enhancing their protection against various infections.

Overall, this body of work provides novel information characterizing the regulation mechanisms of IFN-γ expression in foals. These findings pave the way for future investigations of novel immunomodulators and adjuvants for vaccine development, and will allow strategies for promoting the maturation of the immune response, in particular, IFN-γ expression, thereby protecting foals from infection.

Copyright © Lingshuang Sun 2012

References

Adams, A.A., Breathnach, C.C., Katepalli, M.P., Kohler, K., Horohov, D.W., 2008. Advanced age in horses affects divisional history of T cells and inflammatory cytokine production. Mech Ageing Dev 129, 656-664.

Adkins, B., Du, R.-Q., 1998. Newborn Mice Develop Balanced Th1/Th2 Primary Effector Responses In Vivo But Are Biased to Th2 Secondary Responses. J Immunol 160, 4217-4224.

Adler, S.H., Chiffoleau, E., Xu, L.W., Dalton, N.M., Burg, J.M., Wells, A.D., Wolfe, M.S., Turka, L.A., Pear, W.S., 2003. Notch signaling augments T cell responsiveness by enhancing CD25 expression. Journal of Immunology 171, 2896-2903.

Amsen, D., Blander, J.M., Lee, G.R., Tanigaki, K., Honjo, T., Flavell, R.A., 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell 117, 515-526.

Ansel, K.M., Lee, D.U., Rao, A., 2003. An epigenetic view of helper T cell differentiation. Nature Immunology 4, 616-623.

Araki, Y., Fann, M., Wersto, R., Weng, N.P., 2008. Histone acetylation facilitates rapid and robust memory CD8 T cell response through differential expression of effector molecules (eomesodermin and its targets: perforin and granzyme B). J Immunol 180, 8102-8108.

Astier, P., Autiero, D., Baldisseri, A., Baldo-Ceolin, M., Ballocchi, G., Banner, M., Bassompierre, G., Benslama, K., Besson, N., Bird, I., Blumenfeld, B., Bobisut, F., Bouchez, J., Boyd, S., Bueno, A., Bunyatov, S., Camilleri, L., Cardini, A., Cattaneo, P.W., Cavasinni, V., Cervera-Villanueva, A., Collazuol, G., Conforto, G., Conta, C., Contalbrigo, M., Cousins, R., Daniels, D., Degaudenzi, H., Del Prete, T., De Santo, A., Dignan, T., Di Lella, L., Silva, E.D.E., Donnelly, I.J., Dumarchez, J., Ellis, M., Fazio, T., Feldman, G.J., Ferrari, R., Ferrere, D., Flaminio, V., Fraternali, M., Gaillard, J.M., Gandolfo, A., Gangler, E., Geiser, A., Geppert, D., Gibin, D., Gninenko, S., Godley, A., Gomez-Cadenas, J.J., Gosset, J., Gossling, C., Gouanere, M., Grant, A., Graziani, G., Guglielmi, A., Hagner, C., Hernando, J., Hubbard, D., Hurst, P., Hyett, N., Iacopini, E., Joseph, C., Juget, F., Kirsanov, M., Klimov, O., Kokkonen, J., Kovzelev, A., Krasnoperov, A., Kuznetsov, V.E., Lacaprara, S., Lachaud, C., Lakic, B., Lanza, A., La Rotonda, L., Laveder, M., Letessier-Selvon, A., Levy, J.M., Linssen, L., Ljubicic, A., Long, J., Lupi, A., Manola-Poggioli, E., Marchionni, A., Martelli, F., Mechain, X., Mendiburu, J.P., Meyer, J.P., Mezzetto, M., Mishra, S.R., Moorhead, G.F., Mossuz, L., Nedelec, P., Nefedov, Y., Nguyen-Mau, C., Orestano, D., Pastore, F., Peak, L.S., Pennacchio, E., Pessard, H., Petti, R., Placci, A., Pluquet, A., Polesello, G., Pollmann, D., Popov, B., Poulsen, C., Rathouit, P., Reno, R., Renzoni, G., Rico, J., Riemann, P., Roda, C., Rubbia, A., Salvatore, F., Schahmaneche, K., Schmidt, B., Schmidt, T., Segneri, G., Sevior, M., Sillou, D., Soler, F.J.P., Sozzi, G., Steele, D., Steininger, M., Stiegler, U., Stipcevic, M., Stolarczyk, T., Tareb-Reyes, M., Taylor, G.N., Tereshchenko, V., Toropin, A., Touchard, A.M., Tovey, S.N., Tran, M.T., Tsesmelis, E., Ulrichs, J., Vacavant, L., Valdata-Nappi, M., Valuev, V., Vannucci, F., Varvell, K.E., Veltri, M., Vercesi, V., Verkindt, D., Vieira, J.M., Vinogradova, T., Vo, M.K., Volkov, S., Weber, F.V., Weisse, T., Wilson, F.F., Winton, L.J., Yabsley, B.D., Zaccone, H., Zuber, K., Zuccon, P., Collaboration, N., 1999. A more sensitive search for nu(mu) -> nu(tau) oscillations in NOMAD. Physics Letters B 453, 169-186.

Aubert-Pivert, E.M., Chedevergne, F.M., Lopez-Ramirez, G.M., Colle, J.H., Scheinmann, P.L., Gicquel, B.M., de Blic, J.M., 2000. Cytokine transcripts in pediatric tuberculosis: a study with bronchoalveolar cells. Tuber Lung Dis 80, 249-258.

Avni, O., Rao, A., 2000. T cell differentiation: a mechanistic view. Curr Opin Immunol 12, 654-659.

Baker, A.M., Cox, T.R., Bird, D., Lang, G., Murray, G.I., Sun, X.F., Southall, S.M., Wilson, J.R., Erler, J.T., 2011. The Role of Lysyl Oxidase in SRC-Dependent Proliferation and Metastasis of Colorectal Cancer. Journal of the National Cancer Institute 103, 407-424.

Balson, G.A., Smith, G.D., Yager, J.A., 1997a. Immunophenotypic analysis of foal bronchoalveolar lavage lymphocytes. Vet Microbiol 56, 237-246.

Balson, G.A., Smith, G.D., Yager, J.A., 1997b. Immunophenotypic analysis of foal bronchoalveolar lavage lymphocytes. Veterinary Microbiology 56, 237-246.

Banks, K.L., McGuire, T.C., 1975. Surface receptors on neutrophils and monocytes from immunodeficient and normal horses. Immunology 28, 581-588.

Bannister, A.J., Kouzarides, T., Regulation of chromatin by histone modifications. Cell Res 21, 381-395.

Bannister, A.J., Kouzarides, T., 2011. Regulation of chromatin by histone modifications. Cell Research 21, 381-395.

Barski, A., Cuddapah, S., Cui, K.R., Roh, T.Y., Schones, D.E., Wang, Z.B., Wei, G., Chepelev, I., Zhao, K.J., 2007. High-resolution profiling of histone methylations in the human genome. Cell 129, 823-837.

Belderbos, M., Levy, O., Bont, L., 2009a. Neonatal innate immunity in allergy development. Curr Opin Pediatr 21, 762-769.

Belderbos, M.E., van Bleek, G.M., Levy, O., Blanken, M.O., Houben, M.L., Schuijff, L., Kimpen, J.L., Bont, L., 2009b. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clin Immunol 133, 228-237.

Ben-Sasson, S.Z., Gerstel, R., Hu-Li, J., Paul, W.E., 2001. Cell division is not a "clock" measuring acquisition of competence to produce IFN-gamma or IL-4. Journal of Immunology 166, 112-120.

Benevolenskaya, E.V., 2007. Histone H3K4 demethylases are essential in development and differentiation. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire 85, 435-443.

Benito, J.M., Lopez, M., Lozano, S., Ballesteros, C., Gonzalez-Lahoz, J., Soriano, V., 2007. Hydroxyurea exerts an anti-proliferative effect on T cells but has no direct impact on cellular activation. Clinical and Experimental Immunology 149, 171-177.

Benoit, S., Benachour, A., Taouji, S., Auffray, Y., Hartke, A., 2002. H2O2, which causes macrophage-related stress, triggers induction of expression of virulence-associated plasmid determinants in Rhodococcus equi. Infection and Immunity 70, 3768-3776.

Berek, C., Ziegner, M., 1993. The Maturation of the Immune-Response (Vol 14, Pg 400, 1993). Immunology Today 14, 479-479.

Bernoco, M.M., Liu, I.K.M., Willits, N.H., 1994. Hemolytic Complement Activity and Concentrations of Its 3rd Component during Maturation of the Immune-Response in Colostrum-Deprived Foals. American Journal of Veterinary Research 55, 928-933.

Bienenst.J, Johnston, N., Perey, D.Y.E., 1973. Bronchial Lymphoid-Tissue .1. Morphologic Characteristics. Laboratory Investigation 28, 686-692.

Bird, J.J., Brown, D.R., Mullen, A.C., Moskowitz, N.H., Mahowald, M.A., Sider, J.R., Gajewski, T.F., Wang, C.R., Reiner, S.L., 1998. Helper T cell differentiation is controlled by the cell cycle. Immunity 9, 229-237.

Blunden, A.S., Gower, S.M., 1999. A histological and immunohistochemical study of the humoral immune system of the lungs in young thoroughbred horses. Journal of Comparative Pathology 120, 347-356.

Boehm, U., Klamp, T., Groot, M., Howard, J.C., 1997. Cellular responses to interferon-gamma. Annual Review of Immunology 15, 749-795.

Bourc'his, D., Xu, G.L., Lin, C.S., Bollman, B., Bestor, T.H., 2001. Dnmt3L and the establishment of maternal genomic imprints. Science 294, 2536-2539.

Boyd, N.K., Cohen, N.D., Lim, W.S., Martens, R.J., Chaffin, M.K., Ball, J.M., 2003. Temporal changes in cytokine expression of foals during the first month of life. Vet Immunol Immunopathol 92, 75-85.

Breathnach, C.C., Sturgill-Wright, T., Stiltner, J.L., Adams, A.A., Lunn, D.P., Horohov, D.W., 2006a. Foals are interferon gamma-deficient at birth. Veterinary Immunology and Immunopathology 112, 199-209.

Breathnach, C.C., Sturgill-Wright, T., Stiltner, J.L., Adams, A.A., Lunn, D.P., Horohov, D.W., 2006b. Foals are interferon gamma-deficient at birth. Vet Immunol Immunopathol 112, 199-209.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611-622.

Butler, J.E., Lager, K.M., Splichal, I., Francis, D., Kacskovics, I., Sinkora, M., Wertz, N., Sun, J., Zhao, Y., Brown, W.R., DeWald, R., Dierks, S., Muyidermans, S., Lunney, J.K., McCray, P.B., Rogers, C.S., Welsh, M.J., Navarro, P., Klobasa, F., Habe, F., Ramsoondar, J., 2009. The piglet as a model for B cell and immune system development. Veterinary Immunology and Immunopathology 128, 147-170.

Butler, J.E., Sinkora, M., 2007. The isolator piglet: a model for studying the development of adaptive immunity. Immunologic Research 39, 33-51.

Butler, J.E., Sun, J.S., Wertz, N., Sinkora, M., 2006. Antibody repertoire development in swine. Developmental and Comparative Immunology 30, 199-221.

Carter, L.L., Murphy, K.M., 1999. Lineage-specific requirement for signal transducer and activator of transcription (Stat)4 in interferon gamma production from CD4(+) versus CD8(+) T cells. Journal of Experimental Medicine 189, 1355-1360.

Cauchard, J., Sevin, C., Ballet, J.J., Taouji, S., 2004. Foal IgG and opsonizing anti-Rhodococcus equi antibodies after immunization of pregnant mares with a protective VapA candidate vaccine. Veterinary Microbiology 104, 73-81.

Cebulj-Kadunc, N., Kosec, M., Cestnik, V., 2003. The variations of white blood cell count in Lipizzan horses. Journal of Veterinary Medicine Series a-Physiology Pathology Clinical Medicine 50, 251-253.

Cedar, H., Bergman, Y., 2009a. Linking DNA methylation and histone modification: patterns and paradigms. Nature Reviews Genetics 10, 295-304.

Cedar, H., Bergman, Y., 2009b. Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 10, 295-304.

Chang, S., Aune, T.M., 2007. Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-gamma during the differentiation of T helper type 2 cells. Nature Immunology 8, 723-731.

Chen, T.P., Li, E., 2004. Structure and function of eukaryotic DNA methyltransferases, in: Schatten, G.P. (Ed.), Stem Cells in Development and Disease. Elsevier Academic Press Inc, San Diego, pp. 55-89.

Cheng, Y., Zhang, J., Hou, W., Wang, D., Li, F., Zhang, Y., Yuan, F., 2009. Immunoregulatory effects of sinomenine on the T-bet/GATA-3 ratio and Th1/Th2 cytokine balance in the treatment of mesangial proliferative nephritis. Int Immunopharmacol 9, 894-899.

Colsman, A., Sticherling, M., StÃ¶pel, C., Emmrich, F., 2006. Computer-assisted learning in medicine. Archives of Dermatological Research 298, 1-6.

Curtsinger, J.M., Valenzuela, J.O., Agarwal, P., Lins, D., Mescher, M.F., 2005. Cutting edge: Type IIFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. Journal of Immunology 174, 4465-4469.

Dawson, T.R.M.Y., Horohov, D.W., Meijer, W.G., Muscatello, G., 2010. Current understanding of the equine immune response to Rhodococcus equi. An immunological review of R. equi pneumonia. Veterinary Immunology and Immunopathology 135, 1-11.

de Jong, R., Altare, F., Haagen, I.A., Elferink, D.G., de Boer, T., Vriesman, P.J.C.V., Kabel, P.J., Draaisma, J.M.T., van Dissel, J.T., Kroon, F.P., Casanova, J.L., Ottenhoff, T.H.M., 1998. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. Science 280, 1435-1438.

Debarry, J., Garn, H., Hanuszkiewicz, A., Dickgreber, N., Blumer, N., von Mutius, E., Bufe, A., Gatermann, S., Renz, H., Holst, O., Heine, H., 2007. Acinetobacter lwoffli and Lactococcus lactis strains isolated from farm cowsheds possess strong allergy-protective properties. Journal of Allergy and Clinical Immunology 119, 1514-1521.

Delespesse, G., Yang, L.P., Ohshima, Y., Demeure, C., Shu, U., Byun, D.G., Sarfati, M., 1998. Maturation of human neonatal CD4+ and CD8+ T lymphocytes into Th1/Th2 effectors. Vaccine 16, 1415-1419.

Diehl, S., Anguita, J., Hoffmeyer, A., Zapton, T., Ihle, J.N., Fikrig, E., Rincon, M., 2000. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. Immunity 13, 805-815.

Djuretic, I.M., Levanon, D., Negreanu, V., Groner, Y., Rao, A., Ansel, K.M., 2007. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. Nature Immunology 8, 145-153.

Ege, M.J., Frei, R., Bieli, C., Schram-Bijkerk, D., Waser, M., Benz, M.R., Weiss, G., Nyberg, F., van Hage, M., Pershagen, G., Brunekreef, B., Riedler, J., Lauener, R., Braun-Fahrlander, C., von Mutius, E., team, P.S., 2007. Not all farming environments protect against the development of asthma and wheeze in children. Journal of Allergy and Clinical Immunology 119, 1140-1147.

Fach, S.J., Brockmeier, S.L., Hobbs, L.A., Lehmkuhl, H.D., Sacco, R.E., 2006. Pulmonary dendritic cells isolated from neonatal and adult ovine lung tissue. Vet Immunol Immunopathol 112, 171-182.

Feldman, N., Gerson, A., Fang, J., Li, E., Zhang, Y., Shinkai, Y., Cedar, H., Bergman, Y., 2006. G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. Nat Cell Biol 8, 188-194.

Felsenfeld, G., Groudine, M., 2003. Controlling the double helix. Nature 421, 448-453.

Fernandez-Mora, E., Polidori, M., Luhrmann, A., Schaible, U.E., Haas, A., 2005. Maturation of Rhodococcus equi-containing vacuoles is arrested after completion of the early endosome stage. Traffic 6, 635-653.

Filipe-Santos, O., Bustamante, J., Chapgier, A., Vogt, G., de Beaucoudrey, L., Feinberg, J., Jouanguy, E., Boisson-Dupuis, S., Fieschi, C., Picard, C., Casanova, J.L., 2006. Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: molecular, cellular, and clinical features. Semin Immunol 18, 347-361.

Flaminio, M.J., Borges, A.S., Nydam, D.V., Horohov, D.W., Hecker, R., Matychak, M.B., 2007. The effect of CpG-ODN on antigen presenting cells of the foal. J Immune Based Ther Vaccines 5, 1.

Flaminio, M.J., Nydam, D.V., Marquis, H., Matychak, M.B., Giguere, S., 2009. Foal monocyte-derived dendritic cells become activated upon Rhodococcus equi infection. Clin Vaccine Immunol 16, 176-183.

Flaminio, M.J., Rush, B.R., Davis, E.G., Hennessy, K., Shuman, W., Wilkerson, M.J., 2000a. Characterization of peripheral blood and pulmonary leukocyte function in healthy foals. Vet Immunol Immunopathol 73, 267-285.

Flaminio, M.J.B.F., Rush, B.R., Davis, E.G., Hennessy, K., Shuman, W., Wilkerson, M.J., 2000b. Characterization of peripheral blood and pulmonary leukocyte function in healthy foals. Veterinary Immunology and Immunopathology 73, 267-285.

Flaminio, M.J.F.B., Rush, B.R., Shuman, W., 1999. Peripheral blood lymphocyte subpopulations and immunoglobulin concentrations in healthy foals with Rhodococcus equi pneumonia. (vol 13, pg 206, 1999). Journal of Veterinary Internal Medicine 13, 504-504.

Fogarty, U., Leadon, D.P., 1987. Comparison of systemic and local respiratory tract cellular immunity in the neonatal foal. J Reprod Fertil Suppl 35, 593-598.

Fuhrmann, C., Lammler, C., 1997. Characterization of Rhodococcus equi-isolates from horse and man. Berliner Und Munchener Tierarztliche Wochenschrift 110, 54-59.

Fujii, H., Ogasawara, K., Otsuka, H., Suzuki, M., Yamamura, K., Yokochi, T., Miyazaki, T., Suzuki, H., Mak, T.W., Taki, S., Taniguchi, T., 1998. Functional dissection of the cytoplasmic subregions of the IL-2 receptor beta c chain in primary lymphocyte populations. Embo Journal 17, 6551-6557.

Garn, H., Renz, H., 2007. Epidemiological and immunological evidence for the hygiene hypothesis. Immunobiology 212, 441-452.

Garton, N.J., Gilleron, M., Brando, T., Dan, H.H., Giguere, S., Puzo, G., Prescott, J.F., Sutcliffe, I.C., 2002. A novel lipoarabinomannan from the equine pathogen Rhodococcus equi - Structure and effect on marcophage cytokine production. Journal of Biological Chemistry 277, 31722-31733.

Gasparoni, A., Ciardelli, L., Avanzini, A., Castellazzi, A.M., Carini, R., Rondini, G., Chirico, G., 2003. Age-related changes in intracellular Th1/Th2 cytokine production, immunoproliferative T lymphocyte response and natural killer cell activity in newborns, children and adults. Biology of the Neonate 84, 297-303.

George, C.L., White, M.L., Kulhankova, K., Mahajan, A., Thorne, P.S., Snyder, J.M., Kline, J.N., 2006a. Early exposure to a nonhygienic environment alters pulmonary immunity and allergic responses. Am J Physiol Lung Cell Mol Physiol 291, L512-522.

George, C.L.S., White, M.L., Kulhankova, K., Mahajan, A., Thorne, P.S., Snyder, J.M., Kline, J.N., 2006b. Early exposure to a nonhygienic environment alters pulmonary immunity and allergic responses. Am. J. Physiol.-Lung Cell. Mol. Physiol. 291, L512-L522.

Gereda, J.E., Leung, D.Y., Thatayatikom, A., Streib, J.E., Price, M.R., Klinnert, M.D., Liu, A.H., 2000a. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 355, 1680-1683.

Gereda, J.E., Leung, D.Y.M., Thatayatikom, A., Streib, J.E., Price, M.R., Klinnert, M.D., Liu, A.H., 2000b. Relation between house-dust endotoxin exposure, type 1 T cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 355, 1680-1683.

Gett, A.V., Hodgkin, P.D., 1998. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. Proceedings of the National Academy of Sciences of the United States of America 95, 9488-9493.

Giguere, S., Cohen, N.D., Chaffin, M.K., Hines, S.A., Hondalus, M.K., Prescott, J.F., Slovis, N.M., 2011a. Rhodococcus equi: Clinical Manifestations, Virulence, and Immunity. Journal of Veterinary Internal Medicine 25, 1221-1230.

Giguere, S., Cohen, N.D., Chaffin, M.K., Slovis, N.M., Hondalus, M.K., Hines, S.A., Prescott, J.F., 2011b. Diagnosis, Treatment, Control, and Prevention of Infections Caused by Rhodococcus equi in Foals. Journal of Veterinary Internal Medicine 25, 1209-1220.

Giguere, S., Polkes, A.C., 2005a. Immunologic disorders in neonatal foals. Veterinary Clinics of North America-Equine Practice 21, 241-+.
Giguere, S., Polkes, A.C., 2005b. Immunologic disorders in neonatal foals. Vet Clin North Am Equine Pract 21, 241-272, v.
Giguere, S., Wilkie, B.N., Prescott, J.F., 1999. Modulation of cytokine response of pneumonic foals by virulent Rhodococcus equi. Infect Immun 67, 5041-5047.
Gjerset, R.A., Martin, D.W., 1982. Presence of a DNA Demethylating Activity in the Nucleus of Murine Erythroleukemic Cells. Journal of Biological Chemistry 257, 8581-8583.
Grogan, J.L., Locksley, R.M., 2002. T helper cell differentiation: on again, off again. Current Opinion in Immunology 14, 366-372.
Grondahl, G., Johannisson, A., Demmers, S., Waern, M.J., 1999. Influence of age and plasma treatment on neutrophil phagocytosis and CD18 expression in foals. Veterinary Microbiology 65, 241-254.
Gudmundsdottir, H., Wells, A.D., Walsh, M., Turka, L.A., 1999. Examining individual T cells during in vivo responses: What regulates proliferation and cytokine production? Transplantation 67, 885.
Guerra, S., Lohman, I.C., Halonen, M., Martinez, F.D., Wright, A.L., 2004. Reduced interferon gamma production and soluble CD14 levels in early life predict recurrent wheezing by 1 year of age. Am J Respir Crit Care Med 169, 70-76.
Hamm, S., Just, G., Lacoste, N., Moitessier, N., Szyf, M., Mamer, O., 2008. On the mechanism of demethylation of 5-methylcytosine in DNA. Bioorg Med Chem Lett 18, 1046-1049.
Harris, S.P., Hines, M.T., Mealey, R.H., Alperin, D.C., Hines, S.A., Early development of cytotoxic T lymphocytes in neonatal foals following oral inoculation with Rhodococcus equi. Veterinary Immunology and Immunopathology 141, 312-316.
Hatton, R.D., Harrington, L.E., Luther, R.J., Wakefield, T., Janowski, K.M., Oliver, J.R., Lallone, R.L., Murphy, K.M., Weaver, C.T., 2006. A distal conserved sequence element controls Ifng gene expression by T cells and NK cells. Immunity 25, 717-729.
He, X.J., Chen, T.P., Zhu, J.K., 2011. Regulation and function of DNA methylation in plants and animals. Cell Research 21, 442-465.
Hendrich, B., Bird, A., 1998. Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol 18, 6538-6547.
Herberth, G., Heinrich, J., Roder, S., Figl, A., Weiss, M., Diez, U., Borte, M., Herbarth, O., Lehmann, I., 2010. Reduced IFN-gamma- and enhanced IL-4-producing CD4+ cord blood T cells are associated with a higher risk for atopic dermatitis during the first 2 yr of life. Pediatr Allergy Immunol 21, 5-13.
Hibbert, L., Pflanz, S., Malefyt, R.D., Kastelein, R.A., 2003. IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12R beta 2 in naive T cells. Journal of Interferon and Cytokine Research 23, 513-522.
Hietala, S.K., Ardans, A.A., 1987a. Interaction of Rhodococcus-Equi with Phagocytic-Cells from R-Equi-Exposed and Non-Exposed Foals. Veterinary Microbiology 14, 307-320.
Hietala, S.K., Ardans, A.A., 1987b. NEUTROPHIL PHAGOCYTIC AND SERUM OPSONIC RESPONSE OF THE FOAL TO CORYNEBACTERIUM-EQUI. Veterinary Immunology and Immunopathology 14, 279-294.
Hillidge, C.J., 1987. Use of Erythromycin Rifampin Combination in Treatment of Rhodococcus-Equi Pneumonia. Veterinary Microbiology 14, 337-342.
Hines, M.T., Paasch, K.M., Alperin, D.C., Palmer, G.H., Westhoff, N.C., Hines, S.A., 2001. Immunity to Rhodococcus equi: antigen-specific recall responses in the lungs of adult horses. Vet Immunol Immunopathol 79, 101-114.

Hines, S.A., Stone, D.M., Hines, M.T., Alperin, D.C., Knowles, D.P., Norton, L.K., Hamilton, M.J., Davis, W.C., McGuire, T.C., 2003a. Clearance of virulent but not avirulent Rhodococcus equi from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4(+) and CD8(+) T lymphocytes. Clinical and Diagnostic Laboratory Immunology 10, 208-215.

Hines, S.A., Stone, D.M., Hines, M.T., Alperin, D.C., Knowles, D.P., Norton, L.K., Hamilton, M.J., Davis, W.C., McGuire, T.C., 2003b. Clearance of virulent but not avirulent Rhodococcus equi from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4+ and CD8+ T lymphocytes. Clin Diagn Lab Immunol 10, 208-215.

Hoffman, A.M., Viel, L., Prescott, J.F., 1993. Microbiologic Changes during Antimicrobial Treatment and Rate of Relapse of Distal Respiratory-Tract Infections in Foals. American Journal of Veterinary Research 54, 1608-1614.

Holliday, R., 2006. Epigenetics A Historical Overview. Epigenetics 1, 76-80.

Holt, P.G., 2000. Antigen presentation in the lung. Am. J. Respir. Crit. Care Med. 162, S151-S156.

Holt, P.G., Haining, S., Nelson, D.J., Sedgwick, J.D., 1994. ORIGIN AND STEADY-STATE TURNOVER OF CLASS-II MHC-BEARING DENDRITIC CELLS IN THE EPITHELIUM OF THE CONDUCTING AIRWAYS. Journal of Immunology 153, 256-261.

Holtmeier, W., Kabelitz, D., 2005. gammadelta T cells link innate and adaptive immune responses. Chem Immunol Allergy 86, 151-183.

Holznagel, D.L., Hussey, S., Mihalyi, J.E., Wilson, W.D., Lunn, D.P., 2003. Onset of immunoglobulin production in foals. Equine Vet J 35, 620-622.

Hooper-McGrevy, K., Prescott, J.F., 2001. Editorial: Is fatal Rhodococcus equi pneumonia of foals only an infection acquired by the perinate? Journal of Veterinary Internal Medicine 15, 169-170.

Hooper-McGrevy, K.E., Wilkie, B.N., Prescott, J.F., 2003. Immunoglobulin G subisotype responses of pneumonic and healthy, exposed foals and adult horses to Rhodococcus equi virulence-associated proteins. Clin Diagn Lab Immunol 10, 345-351.

Hooper-McGrevy, K.E., Wilkie, B.N., Prescott, J.F., 2005. Virulence-associated protein-specific serum immunoglobulin G-isotype expression in young foals protected against Rhodococcus equi pneumonia by oral immunization with virulent R-equi. Vaccine 23, 5760-5767.

Horiuchi, Y., Nakajima, Y., Nariai, Y., Asanuma, H., Kuwabara, M., Yukawa, M., 2007. Th1/Th2 balance in canine peripheral blood lymphocytes - A flow cytometric study. Veterinary Immunology and Immunopathology 118, 179-185.

Horohov, D.W., Loynachan, A.T., Page, A.E., Hughes, K., Timoney, J.F., Fettinger, M., Hatch, T., Spaulding, J.G., McMichael, J., 2011. The use of streptolysin O (SLO) as an adjunct therapy for Rhodococcus equi pneumonia in foals. Veterinary Microbiology 154, 156-162.

Hume, D.A., 2008. Macrophages as APC and the Dendritic Cell Myth. Journal of Immunology 181, 5829-5835.

Iwamura, C., Nakayama, T., 2008. Toll-like receptors in the respiratory system: their roles in inflammation. Curr Allergy Asthma Rep 8, 7-13.

Jacks, S., Giguere, S., Crawford, P.C., Castleman, W.L., 2007a. Experimental infection of neonatal foals with Rhodococcus equi triggers adult-like gamma interferon induction. Clin Vaccine Immunol 14, 669-677.

Jacks, S., Giguere, S., Preseott, J.F., 2007b. In vivo expression of and cell-mediated immune responses to the plasmid-encoded virulence-associated proteins of Rhodococcus equi in foals. Clinical and Vaccine Immunology 14, 369-374.

Jones, A.C., Burdge, G.C., Warner, J.A., Postle, A.D., Warner, J.O., 1996. Ontogeny of circulating leucocytes in the fetal guinea pig. Biology of the Neonate 70, 108-115.

Jones, P.A., 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nature Reviews Genetics 13, 484-492.

Kanaly, S.T., Hines, S.A., Palmer, G.H., 1993. Failure of pulmonary clearance of Rhodococcus equi infection in CD4+ T-lymphocyte-deficient transgenic mice. Infect Immun 61, 4929-4932.

Kanaly, S.T., Hines, S.A., Palmer, G.H., 1995a. Cytokine Modulation Alters Pulmonary Clearance of Rhodococcus-Equi and Development of Granulomatous Pneumonia. Infection and Immunity 63, 3037-3041.

Kanaly, S.T., Hines, S.A., Palmer, G.H., 1995b. Cytokine modulation alters pulmonary clearance of Rhodococcus equi and development of granulomatous pneumonia. Infect Immun 63, 3037-3041.

Kanaly, S.T., Hines, S.A., Palmer, G.H., 1996a. Transfer of a CD4+ Th1 cell line to nude mice effects clearance of Rhodococcus equi from the lung. Infection and Immunity 64, 1126-1132.

Kanaly, S.T., Hines, S.A., Palmer, G.H., 1996b. Transfer of a CD4+ Th1 cell line to nude mice effects clearance of Rhodococcus equi from the lung. Infect Immun 64, 1126-1132.

Kersh, E.N., Fitzpatrick, D.R., Murali-Krishna, K., Shires, J., Speck, S.H., Boss, J.M., Ahmed, R., 2006a. Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. Journal of Immunology 176, 4083-4093.

Kersh, E.N., Fitzpatrick, D.R., Murali-Krishna, K., Shires, J., Speck, S.H., Boss, J.M., Ahmed, R., 2006b. Rapid Demethylation of the IFN-Î³ Gene Occurs in Memory but Not Naive CD8 T Cells. The Journal of Immunology 176, 4083-4093.

Khader, S.A., Gaffen, S.L., Kolls, J.K., 2009. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. Mucosal Immunol. 2, 403-411.

Koch, C.M., Andrews, R.M., Flicek, P., Dillon, S.C., Karaoz, U., Clelland, G.K., Wilcox, S., Beare, D.M., Fowler, J.C., Couttet, P., James, K.D., Lefebvre, G.C., Bruce, A.W., Dovey, O.M., Ellis, P.D., Dhami, P., Langford, C.F., Weng, Z.P., Birney, E., Carter, N.P., Vetrie, D., Dunham, I., 2007. The landscape of histone modifications across 1% of the human genome in five human cell lines. Genome Research 17, 691-707.

Kuriakose, J.S., Miller, R.L., Environmental epigenetics and allergic diseases: recent advances. Clin Exp Allergy 40, 1602-1610.

Kuskie, K.R., Smith, J.L., Sinha, S., Carter, C.N., Chaffin, M.K., Slovis, N.M., Brown, S.E., Stepusin, R.S., Takai, S., Cohen, N.D., 2011. Associations between the Exposure to Airborne Virulent Rhodococcus equi and the Incidence of R equi Pneumonia among Individual Foals. Journal of Equine Veterinary Science 31, 463-469.

Lavoie, J.P., Spensley, M.S., Smith, B.P., Bowling, A.T., Morse, S., 1989. Complement Activity and Selected Hematologic Variables in Newborn Foals Fed Bovine Colostrum. American Journal of Veterinary Research 50, 1532-1536.

Law, J.A., Jacobsen, S.E., 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nature Reviews Genetics 11, 204-220.

Lebre, M.C., Burwell, T., Vieira, P.L., Lora, J., Coyle, A.J., Kapsenberg, M.L., Clausen, B.E., De Jong, E.C., 2005. Differential expression of inflammatory chemokines by Th1- and Th2-cell promoting dendritic cells: A role for different mature dendritic cell populations in attracting appropriate effector cells to peripheral sites of inflammation. Immunology and Cell Biology 83, 525-535.

Lee, G.R., Kim, S.T., Spilianakis, C.G., Fields, P.E., Flavell, R.A., 2006. T helper cell differentiation: regulation by cis elements and epigenetics. Immunity 24, 369-379.

Levy, O., 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol 7, 379-390.

Lewis, D.B., Larsen, A., Wilson, C.B., 1986a. REDUCED INTERFERON-GAMMA MESSENGER-RNA LEVELS IN HUMAN NEONATES - EVIDENCE FOR AN INTRINSIC T-CELL DEFICIENCY INDEPENDENT OF OTHER GENES INVOLVED IN T-CELL ACTIVATION. Journal of Experimental Medicine 163, 1018-1023.

Lewis, D.B., Larsen, A., Wilson, C.B., 1986b. Reduced interferon-gamma mRNA levels in human neonates. Evidence for an intrinsic T cell deficiency independent of other genes involved in T cell activation. J Exp Med 163, 1018-1023.

Lewis, D.B., Wilson, C.B., 1990. Gamma-interferon: an immunoregulatory lymphokine with immunotherapeutic potential. Pediatr Infect Dis J 9, 642-651.

Lewis, D.B., Yu, C.C., Meyer, J., English, B.K., Kahn, S.J., Wilson, C.B., 1991. Cellular and molecular mechanisms for reduced interleukin 4 and interferon-gamma production by neonatal T cells. J Clin Invest 87, 194-202.

Lewis, M.J., Wagner, B., Woof, J.M., 2008. The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Mol Immunol 45, 818-827.

Liebler-Tenorio, E.M., Pabst, R., 2006. MALT structure and function in farm animals. Veterinary Research 37, 257-280.

Lighvani, A.A., Frucht, D.M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B.D., Nguyen, B.V., Gadina, M., Sher, A., Paul, W.E., O'Shea, J.J., 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proceedings of the National Academy of Sciences of the United States of America 98, 15137-15142.

Liu, C., Betancourt, A., Cohen, D.A., Adams, A.A., Sun, L., Horohov, D.W., 2011a. Granzyme B-mRNA expression by equine lymphokine activated killer (LAK) cells is associated with the induction of apoptosis in target cells. Veterinary Immunology and Immunopathology 143, 108-115.

Liu, I.K., Walsh, E.M., Bernoco, M., Cheung, A.T., 1987. Bronchoalveolar lavage in the newborn foal. J Reprod Fertil Suppl 35, 587-592.

Liu, M., Bordin, A., Liu, T., Russell, K., Cohen, N., 2011b. Gene expression of innate Th1-, Th2-, and Th17-type cytokines during early life of neonatal foals in response to Rhodococcus equi. Cytokine 56, 356-364.

Liu, M., Liu, T., Bordin, A., Nerren, J., Cohen, N., 2009a. Activation of foal neutrophils at different ages by CpG oligodeoxynucleotides and Rhodococcus equi. Cytokine 48, 280-289.

Liu, M., Liu, T., Bordin, A., Nerren, J., Cohen, N., 2009b. Activation of foal neutrophils at different ages by CpG oligodeoxynucleotides and Rhodococcus equi. Cytokine 48, 280-289.

Liu, T., Nerren, J., Liu, M., Martens, R., Cohen, N., 2009c. Basal and stimulus-induced cytokine expression is selectively impaired in peripheral blood mononuclear cells of newborn foals. Vaccine 27, 674-683.

Loh, L.C., Vyas, B., Kanabar, V., Kemeny, D.M., O'Connor, B.J., 2006. Inhaled endotoxin in healthy human subjects: a dose-related study on systemic effects and peripheral CD4+ and CD8+ T cells. Respir Med 100, 519-528.

Lopez, A.M., Hines, M.T., Palmer, G.H., Alperin, D.C., Hines, S.A., 2002. Identification of pulmonary T-lymphocyte and serum antibody isotype responses associated with protection against Rhodococcus equi. Clinical and Diagnostic Laboratory Immunology 9, 1270-1276.

Lori, F., Lisziewicz, J., 1998. Hydroxyurea: mechanisms of HIV-1 inhibition. Antiviral Therapy 3, 25-33.

Lori, F., Pollard, R.B., Whitman, L., Bakare, N., Blick, G., Shalit, P., Foli, A., Peterson, D., Tennenberg, A., Schrader, S., Rashbaum, B., Farthing, C., Herman, D., Norris, D., Greiger, P., Frank, I., Groff, A., Lova, L., Asmuth, D., Lisziewicz, J., 2005. Lowering the dose of hydroxyurea minimizes toxicity and maximizes anti-HIV potency. AIDS Res Hum Retroviruses 21, 263-272.

Lova, L., Groff, A., Ravot, E., Comolli, G., Xu, J.Q., Whitman, L., Lewis, M., Foli, A., Lisziewicz, J., Lori, F., 2005. Hydroxyurea exerts a cytostatic but not immunosuppressive effect on T lymphocytes. Aids 19, 137-144.

Luhrmann, A., Mauder, N., Sydor, T., Fernandez-Mora, E., Schulze-Luehrmann, J., Takai, S., Haas, A., 2004. Necrotic death of Rhodococcus equi-infected macrophages is regulated by virulence-associated plasmids. Infect Immun 72, 853-862.

Lunn, D.P., Schram, B.R., Vagnoni, K.E., Schobert, C.S., Horohov, D.W., Ginther, O.J., 1996. Positive selection of EqCD8(+) precursors increases equine lymphokine-activated killing. Veterinary Immunology and Immunopathology 53, 1-13.

Machangu, R.S., Prescott, J.F., 1991. Role of Antibody to Extracellular Proteins of Rhodococcus-Equi in Protection against R-Equi Pneumonia in Foals. Veterinary Microbiology 26, 323-333.

Mackenzie, C.D., 1975. Histological development of the thymic and intestinal lymphoid tissue of the horse. J S Afr Vet Assoc 46, 47-55.

MacLennan, C., Fieschi, C., Lammas, D.A., Picard, C., Dorman, S.E., Sanal, O., MacLennan, J.M., Holland, S.M., Ottenhoff, T.H.M., Casanova, J.L., Kumararatne, D.S., 2004. Interleukin (IL)-12 and IL-23 are key cytokines for immunity against salmonella in humans. Journal of Infectious Diseases 190, 1755-1757.

Maekawa, Y., Tsukumo, S., Chiba, S., Hirai, H., Hayashi, Y., Okada, H., Kishihara, K., Yasutomo, K., 2003. Delta1-Notch3 interactions bias the functional differentiation of activated CD4(+) T cells. Immunity 19, 549-559.

Mair, T.S., Batten, E.H., Stokes, C.R., Bourne, F.J., 1987a. The Histological Features of the Immune-System of the Equine Respiratory-Tract. Journal of Comparative Pathology 97, 575-586.

Mair, T.S., Batten, E.H., Stokes, C.R., Bourne, F.J., 1988a. The Distribution of Mucosal Lymphoid Nodules in the Equine Respiratory-Tract. Journal of Comparative Pathology 99, 159-168.

Mair, T.S., Batten, E.H., Stokes, C.R., Bourne, F.J., 1988b. The distribution of mucosal lymphoid nodules in the equine respiratory tract. J Comp Pathol 99, 159-168.

Mair, T.S., Stokes, C.R., Bourne, F.J., 1987b. Cellular Content of Secretions Obtained by Lavage from Different Levels of the Equine Respiratory-Tract. Equine Vet J 19, 458-462.

Mair, T.S., Stokes, C.R., Bourne, F.J., 1988c. Distribution and Ultrastructure of Mast-Cells in the Equine Respiratory-Tract. Equine Vet J 20, 54-58.

Maldonado, R.A., Irvine, D.J., Schreiber, R., Glimcher, L.H., 2004. A role for the immunological synapse in lineage commitment of CD4 lymphocytes. Nature 431, 527-532.

Marodi, L., 2006a. Innate cellular immune responses in newborns. Clin Immunol 118, 137-144.

Marodi, L., 2006b. Neonatal innate immunity to infectious agents. Infection and Immunity 74, 1999-2006.

Martens, R.J., Cohen, N.D., Jones, S.L., Moore, T.A., Edwards, J.F., 2005. Protective role of neutrophils in mice experimentally infected with Rhodococcus equi. Infect Immun 73, 7040-7042.

Marti, E., Ehrensperger, F., Burger, D., Ousey, J., Day, M.J., Wilson, A.D., 2009. Maternal transfer of IgE and subsequent development of IgE responses in the horse (Equus callabus). Veterinary Immunology and Immunopathology 127, 203-211.

Martin, B.R., Larson, K.A., 1973. Immune-Response of Equine Fetus to Coliphage-T2. American Journal of Veterinary Research 34, 1363-1364.
Matikainen, S., Paananen, A., Miettinen, M., Kurimoto, M., Timonen, T., Julkunen, I., Sareneva, T., 2001. IFN-alpha and IL-18 synergistically enhance IFN-gamma production in human NK cells: differential regulation of Stat4 activation and IFN-gamma gene expression by IFN-alpha and IL-12. European Journal of Immunology 31, 2236-2245.
Matsuda, J.L., Gapin, L., Baron, J.L., Sidobre, S., Stetson, D.B., Mohrs, M., Locksley, R.M., Kronenberg, M., 2003. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. Proceedings of the National Academy of Sciences of the United States of America 100, 8395-8400.
McGuire, J.L., Bariso, C.D., 1972. Isolation and preliminary characterization of a progestogen specific binding macromolecule from the 273,000 g supernatant of rat and rabbit uteri. Endocrinology 90, 496-506.
McTaggart, C., Yovich, J.V., Penhale, J., Raidal, S.L., 2001. A comparison of foal and adult horse neutrophil function using flow cytometric techniques. Research in Veterinary Science 71, 73-79.
McWilliam, A.S., Napoli, S., Marsh, A.M., Pemper, F.L., Nelson, D.J., Pimm, C.L., Stumbles, P.A., Wells, T.N., Holt, P.G., 1996. Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. J Exp Med 184, 2429-2432.
Meijer, W.G., Prescott, J.F., 2004. Rhodococcus equi. Vet Res 35, 383-396.
Merant, C., Breathnach, C.C., Kohler, K., Rashid, C., Van Meter, P., Horohov, D.W., 2009a. Young foal and adult horse monocyte-derived dendritic cells differ by their degree of phenotypic maturity. Vet Immunol Immunopathol 131, 1-8.
Merant, C., Breathnach, C.C., Kohler, K., Rashid, C., Van Meter, P., Horohov, D.W., 2009b. Young foal and adult horse monocyte-derived dendritic cells differ by their degree of phenotypic maturity. Veterinary Immunology and Immunopathology 131, 1-8.
Miller, R.L., Ho, S.M., 2008. Environmental epigenetics and asthma: current concepts and call for studies. Am J Respir Crit Care Med 177, 567-573.
Minter, L.M., Turley, D.M., Das, P., Shin, H.M., Joshi, I., Lawlor, R.G., Cho, O.H., Palaga, T., Gottipati, S., Telfer, J.C., Kostura, L., Fauq, A.H., Simpson, K., Such, K.A., Miele, L., Golde, T.E., Miller, S.D., Osborne, B.A., 2005. Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. Nature Immunology 6, 680-688.
Mock, R.E., Morgan, D.O., Jochim, M.M., Lock, T.F., 1978. Antibody response of the fetus and adult equine to Venezuelan equine encephalomyelitis virus (VEE-TC-84): immunoglobulins Ga&b, M, and T. Equine infectious diseases IV, 209-219.
Morgan, D.O., Bryans, J.T., Mock, R.E., 1975. Immunoglobulins Produced by Antigenized Equine Fetus. Journal of Reproduction and Fertility, 735-738.
Morris, D.D., Gaulin, G., Strzemienski, P.J., Spencer, P., 1987. Assessment of neutrophil migration, phagocytosis and bactericidal capacity in neonatal foals. Vet Immunol Immunopathol 16, 173-184.
Mullen, A.C., High, F.A., Hutchins, A.S., Lee, H.W., Villarino, A.V., Livingston, D.M., Kung, A.L., Cereb, N., Yao, T.P., Yang, S.Y., Reiner, S.L., 2001. Role of T-bet in commitment of T(H)1 cells before IL-12-dependent selection. Science 292, 1907-1910.
Mullen, A.C., Hutchins, A.S., High, F.A., Lee, H.W., Sykes, K.J., Chodosh, L.A., Reiner, S.L., 2002. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. Nature Immunology 3, 652-658.

Murphy, K.M., Reiner, S.L., 2002a. The lineage decisions of helper T cells. Nature Reviews Immunology 2, 933-944.
Murphy, K.M., Reiner, S.L., 2002b. The lineage decisions of helper T cells. Nat Rev Immunol 2, 933-944.
Murphy, S.P., Tayade, C., Ashkar, A.A., Hatta, K., Zhang, J.H., Croy, B.A., 2009. Interferon Gamma in Successful Pregnancies. Biology of Reproduction 80, 848-859.
Muscatello, G., Anderson, G.A., Gilkerson, J.R., Browning, G.F., 2006. Associations between the ecology of virulent Rhodococcus equi and the epidemiology of R-equi pneumonia on Australian thoroughbred farms. Applied and Environmental Microbiology 72, 6152-6160.
Muscatello, G., Gilkerson, J.R., Browning, G.F., 2009. Detection of Virulent Rhodococcus equi in Exhaled Air Samples from Naturally Infected Foals. Journal of Clinical Microbiology 47, 734-737.
Nardoni, S., Mancianti, F., Sgorbini, M., Taccini, F., Corazza, M., 2005. Identification and seasonal distribution of airborne fungi in three horse stables in Italy. Mycopathologia 160, 29-34.
Nelson, D.J., Holt, P.G., 1995. Defective regional immunity in the respiratory tract of neonates is attributable to hyporesponsiveness of local dendritic cells to activation signals. J Immunol 155, 3517-3524.
Nelson, D.J., McMenamin, C., McWilliam, A.S., Brenan, M., Holt, P.G., 1994. DEVELOPMENT OF THE AIRWAY INTRAEPITHELIAL DENDRITIC CELL NETWORK IN THE RAT FROM CLASS-II MAJOR HISTOCOMPATIBILITY (IA)-NEGATIVE PRECURSORS - DIFFERENTIAL REGULATION OF IA EXPRESSION AT DIFFERENT LEVELS OF THE RESPIRATORY-TRACT. Journal of Experimental Medicine 179, 203-212.
Nerren, J.R., Martens, R.J., Payne, S., Murrell, J., Butler, J.L., Cohen, N.D., 2009a. Age-related changes in cytokine expression by neutrophils of foals stimulated with virulent Rhodococcus equi in vitro. Veterinary Immunology and Immunopathology 127, 212-219.
Nerren, J.R., Martens, R.J., Payne, S., Murrell, J., Butler, J.L., Cohen, N.D., 2009b. Age-related changes in cytokine expression by neutrophils of foals stimulated with virulent Rhodococcus equi in vitro. Vet Immunol Immunopathol 127, 212-219.
Noble, A., 2009. Do we have memory of danger as well as antigen? Trends in Immunology 30, 150-156.
Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y.M., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M.R., Gorman, D., Wagner, J., Zurawski, S., Liu, Y.J., Abrams, J.S., Moore, K.W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J.F., Kastelein, R.A., 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13, 715-725.
Paillot, R., Daly, J.M., Juillard, V., Minke, J.M., Hannant, D., Kydd, J.H., 2005. Equine interferon gamma synthesis in lymphocytes after in vivo infection and in vitro stimulation with EHV-1. Vaccine 23, 4541-4551.
Paillot, R., Daly, J.M., Luce, R., Montesso, F., Davis-Poynter, N., Hannant, D., Kydd, J.H., 2007. Frequency and phenotype of EHV-1 specific, IFN-gamma synthesising lymphocytes in ponies: The effects of age, pregnancy and infection. Developmental and Comparative Immunology 31, 202-214.
Pargass, I.S., Wills, T.B., Davis, W.C., Wardrop, K.J., Alperin, D.C., Hines, S.A., 2009. The influence of age and Rhodococcus equi infection on CD1 expression by equine antigen presenting cells. Veterinary Immunology and Immunopathology 130, 197-209.

Patton, K.M., McGuire, T.C., Fraser, D.G., Hines, S.A., 2004a. Rhodococcus equi-infected macrophages are recognized and killed by CD8(+) T lymphocytes in a major histocompatibility complex class I-unrestricted fashion. Infection and Immunity 72, 7073-7083.

Patton, K.M., McGuire, T.C., Fraser, D.G., Hines, S.A., 2004b. Rhodococcus equi-infected macrophages are recognized and killed by CD8+ T lymphocytes in a major histocompatibility complex class I-unrestricted fashion. Infect Immun 72, 7073-7083.

Patton, K.M., McGuire, T.C., Hines, M.T., Mealey, R.H., Hines, S.A., 2005. Rhodococcus equi-specific cytotoxic T lymphocytes in immune horses and development in asymptomatic foals. Infection and Immunity 73, 2083-2093.

Paul, W.E., 2003. Fundamental immunology, 5th ed. Lippincott Williams & Wilkins, Philadelphia.

Paul, W.E., 2008. Fundamental immunology, 6th ed. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia.

Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C., Shen, H., Cereb, N., Yang, S.Y., Lindsten, T., Rossant, J., Hunter, C.A., Reiner, S.L., 2003a. Control of effector CD8(+) T cell function by the transcription factor Eomesodermin. Science 302, 1041-1043.

Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C.A., Shen, H., Cereb, N., Yang, S.Y., Lindsten, T., Rossant, J., Hunter, C.A., Reiner, S.L., 2003b. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science 302, 1041-1043.

Perryman, L.E., Mcguire, T.C., Torbeck, R.L., 1980. Ontogeny of Lymphocyte Function in the Equine Fetus. American Journal of Veterinary Research 41, 1197-1200.

Peters, M., Kauth, M., Scherner, O., Gehlhar, K., Steffen, I., Wentker, P., von Mutius, E., Holst, O., Bufe, A., Arabinogalactan isolated from cowshed dust extract protects mice from allergic airway inflammation and sensitization. J Allergy Clin Immunol 126, 648-656 e641-644.

Pflanz, S., Timans, J.C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W.M., Mattson, J.D., Wagner, J.L., To, W., Zurawski, S., McClanahan, T.K., Gorman, D.M., Bazan, J.F., Malefyt, R.D., Rennick, D., Kastelein, R.A., 2002. IL-27, a heterodimeric cytokine composed of EB13 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity 16, 779-790.

PrabhuDas, M., Adkins, B., Gans, H., King, C., Levy, O., Ramilo, O., Siegrist, C.A., 2011. Challenges in infant immunity: implications for responses to infection and vaccines. Nature Immunology 12, 189-194.

Pradhan, S., Esteve, P.O., 2003. Allosteric activator domain of maintenance human DNA (cytosine-5) methyltransferase and its role in methylation spreading. Biochemistry 42, 5321-5332.

Prescott, J.F., 1991. Rhodococcus-Equi - an Animal and Human Pathogen. Clinical Microbiology Reviews 4, 20-34.

Prescott, J.F., Fernandez, A.S., Nicholson, V.M., Patterson, M.C., Yager, J.A., Viel, L., Perkins, G., 1996. Use of a virulence-associated protein based enzyme-linked immunosorbent assay for Rhodococcus equi serology in horses. Equine Vet J 28, 344-349.

Pulendran, B., Ahmed, R., 2006. Translating innate immunity into immunological memory: Implications for vaccine development. Cell 124, 849-863.

Quinlivan, M., Nelly, M., Prendergast, M., Breathnach, C., Horohov, D., Arkins, S., Chiang, Y.W., Chu, H.J., Ng, T., Cullinane, A., 2007. Pro-inflammatory and antiviral cytokine expression in vaccinated and unvaccinated horses exposed to equine influenza virus. Vaccine 25, 7056-7064.

Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Letters 339, 62-66.

Ramsahoye, B.H., Biniszkiewicz, D., Lyko, F., Clark, V., Bird, A.P., Jaenisch, R., 2000. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci U S A 97, 5237-5242.

Rate, A., Upham, J.W., Bosco, A., McKenna, K.L., Holt, P.G., 2009. Airway epithelial cells regulate the functional phenotype of locally differentiating dendritic cells: implications for the pathogenesis of infectious and allergic airway disease. J Immunol 182, 72-83.

Rautemaa, R., Meri, S., 1999. Complement-resistance mechanisms of bacteria. Microbes and Infection 1, 785-794.

Rehakova, Z., Trebichavsky, I., Sinkora, J., Splichal, I., Sinkora, M., 1998. Early ontogeny of monocytes and macrophages in the pig. Physiol Res 47, 357-363.

Reiner, S.L., 2001. Helper T cell differentiation, inside and out. Current Opinion in Immunology 13, 351-355.

Rizos, D., Protonotariou, E., Malamitsi-Puchner, A., Sarandakou, A., Trakakis, E., Salamalekis, E., 2007. Cytokine concentrations during the first days of life. European Journal of Obstetrics Gynecology and Reproductive Biology 131, 32-35.

Roponen, M., Hyvarinen, A., Hirvonen, M.R., Keski-Nisula, L., Pekkanen, J., 2005a. Change in IFN-gamma-producing capacity in early life and exposure to environmental microbes. J Allergy Clin Immunol 116, 1048-1052.

Roponen, M., Hyvarinen, A., Hirvonen, M.R., Keski-Nisula, L., Pekkanen, J., 2005b. Change in IFN-gamma-producing capacity in early life and exposure to environmental microbes. Journal of Allergy and Clinical Immunology 116, 1048-1052.

Rowell, E., Wilson, C.B., 2009. Programming perpetual T helper cell plasticity. Immunity 30, 7-9.

Samadi, S., Wouters, I.M., Houben, R., Jamshidifard, A.-R., Van Eerdenburg, F., Heederik, D.J.J., 2009. Exposure to Inhalable Dust, Endotoxins, β(1→3)-Glucans, and Airborne Microorganisms in Horse Stables. Annals of Occupational Hygiene 53, 595-603.

Sareneva, T., Julkunen, I., Matikainen, S., 2000. IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. Journal of Immunology 165, 1933-1938.

Schoenborn, J.R., Dorschner, M.O., Sekimata, M., Santer, D.M., Shnyreva, M., Fitzpatrick, D.R., Stamatoyonnapoulos, J.A., Wilson, C.B., 2007. Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. Nature Immunology 8, 732-742.

Schoenborn, J.R., Wilson, C.B., 2007. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol 96, 41-101.

Sheoran, A.S., Timoney, J.F., Holmes, M.A., Karzenski, S.S., Crisman, M.V., 2000. Immunoglobulin isotypes in sera and nasal mucosal secretions and their neonatal transfer and distribution in horses. American Journal of Veterinary Research 61, 1099-1105.

Simonen-Jokinen, T., Pirie, R.S., McGorum, B., Maisi, P., 2005. Dose responses to inhalation of endotoxin, hay dust suspension and Aspergillus fumigatus extract in horses as measured by levels and activation of matrix metalloproteinase-9. Equine Vet J 37, 155-160.

Singh, H., Pongubala, J.M.R., 2006. Gene regulatory networks and the determination of lymphoid cell fates. Current Opinion in Immunology 18, 116-120.

Sinkora, M., Sinkora, J., Rehakova, Z., Splichal, I., Yang, H., Parkhouse, R.M.E., Trebichavsky, I., 1998. Prenatal ontogeny of lymphocyte subpopulations in pigs. Immunology 95, 595-603.

Skokos, D., Nussenzweig, M.C., 2007. CD8(-) DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. Journal of Experimental Medicine 204, 1525-1531.

Smith, R., Chaffin, M.K., Cohen, N.D., Martens, R.J., 2002. Age-related changes in lymphocyte subsets of Quarter Horse foals. American Journal of Veterinary Research 63, 531-537.

Spilianakis, C.G., Flavell, R.A., 2007. Epigenetic regulation of Ifng expression (vol 8, pg 681, 2007). Nature Immunology 8, 893-893.

Sponseller, B.A., de Macedo, M.M., Clark, S.K., Gallup, J.M., Jones, D.E., 2009a. Activation of peripheral blood monocytes results in more robust production of IL-10 in neonatal foals compared to adult horses. Vet Immunol Immunopathol 127, 167-173.

Sponseller, B.A., de Macedo, M.M.A., Clark, S.K., Gallup, J.M., Jones, D.E., 2009b. Activation of peripheral blood monocytes results in more robust production of IL-10 in neonatal foals compared to adult horses. Veterinary Immunology and Immunopathology 127, 167-173.

Strachan, D.P., 1989. Hay fever, hygiene, and household size. Bmj 299, 1259-1260.

Stumbles, P.A., Thomas, J.A., Pimm, C.L., Lee, P.T., Venaille, T.J., Proksch, S., Holt, P.G., 1998. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. J Exp Med 188, 2019-2031.

Stumbles, P.A., Upham, J.W., Holt, P.G., 2003. Airway dendritic cells: Co-ordinators of immunological homeostasis and immunity in the respiratory tract. Apmis 111, 741-755.

Sullivan, B.M., Juedes, A., Szabo, S.J., von Herrath, M., Glimcher, L.H., 2003. Antigen-driven effector CD8 T cell function regulated by T-bet. Proceedings of the National Academy of Sciences of the United States of America 100, 15818-15823.

Sun, L.S., Adams, A.A., Betancourt, A., Stewart, J.C., Liu, C., Horohov, D.W., 2012. The role of proliferation in the regulation of interferon gamma (IFN gamma) expression in foals. Developmental and comparative immunology 36, 534-539.

Sun, L.S., Adams, A.A., Page, A.E., Betancourt, A., Horohov, D.W., 2011. The effect of environment on interferon-gamma production in neonatal foals. Veterinary Immunology and Immunopathology 143, 170-175.

Sutcliffe, I.C., 1998. Cell envelope composition and organisation in the genus Rhodococcus. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 74, 49-58.

Svedruzic, Z.M., 2008. Mammalian cytosine DNA methyltransferase Dnmt1: enzymatic mechanism, novel mechanism-based inhibitors, and RNA-directed DNA methylation. Curr Med Chem 15, 92-106.

Sweeney, C.R., Rossier, Y., Ziemer, E.L., Lindborg, S., 1992. Effects of lung site and fluid volume on results of bronchoalveolar lavage fluid analysis in horses. Am J Vet Res 53, 1376-1379.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X.K., Fathman, C.G., Glimcher, L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100, 655-669.

Szabo, S.J., Sullivan, B.M., Peng, S.L., Glimcher, L.H., 2003. Molecular mechanisms regulating Th1 immune responses. Annual Review of Immunology 21, 713-758.

Szabo, S.J., Sullivan, B.M., Stemmann, C., Satoskar, A.R., Sleckman, B.P., Glimcher, L.H., 2002. Distinct effects of T-bet in T(H)1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science 295, 338-342.

Szyf, M., 2005. DNA methylation and demethylation as targets for anticancer therapy. Biochemistry (Mosc) 70, 533-549.

Takai, S., 1997. Epidemiology of Rhodococcus equi infections: A review. Veterinary Microbiology 56, 167-176.

Takemoto, N., Intlekofer, A.M., Northrup, J.T., Wherry, E.J., Reiner, S.L., 2006. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. J Immunol 177, 7515-7519.

Tallmadge, R.L., McLaughlin, K., Secor, E., Ruano, D., Matychak, M.B., Flaminio, M.J.B.F., 2009. Expression of essential B cell genes and immunoglobulin isotypes suggests active development and gene recombination during equine gestation. Developmental and Comparative Immunology 33, 1027-1038.

Taouji, S., Nomura, I., Giguere, S., Tomomitsu, S., Kakuda, T., Ganne, V., Takai, S., 2004. Immunogenecity of synthetic peptides representing linear B-cell epitopes of VapA of Rhodococcus equi. Vaccine 22, 1114-1123.

Tato, C., Martins, G.A., High, F.A., DiCioccio, C.B., Reiner, S.L., Hunter, C.A., 2004. Cutting edge: Innate production of IFN-gamma by NK cells is independent of epigenetic modification of the IFN-gamma promoter. Journal of Immunology 173, 1514-1517.

Thieu, V.T., Yu, Q., Chang, H.-C., Yeh, N., Nguyen, E.T., Sehra, S., Kaplan, M.H., 2008. Signal Transducer and Activator of Transcription 4 Is Required for the Transcription Factor T-bet to Promote T Helper 1 Cell-Fate Determination. Immunity 29, 679-690.

Ting, A.H., Jair, K.W., Schuebel, K.E., Baylin, S.B., 2006. Differential requirement for DNA methyltransferase 1 in maintaining human cancer cell gene promoter hypermethylation. Cancer Res 66, 729-735.

Tizard, I.R., 2009. Veterinary immunology : an introduction, 8th ed. Saunders, St. Louis, Mo.

Trinchieri, G., Pflanz, S., Kastelein, R.A., 2003. The IL-12 family of heterodimeric cytokines: New players in the regulation of T cell responses. Immunity 19, 641-644.

Tsoumakidou, M., Tzanaki, N., Papadaki, H., Koutala, H., Siafakas, N.M., 2006. Isolation of myeloid and plasmacytoid dendritic cells from human bronchoalveolar lavage fluid. Immunology and Cell Biology 84, 267-273.

Tulic, M.K., Knight, D.A., Holt, P.G., Sly, P.D., 2001. Lipopolysaccharide inhibits the late-phase response to allergen by altering nitric oxide synthase activity and interleukin-10. Am J Respir Cell Mol Biol 24, 640-646.

Tyler, D.R., Persky, M.E., Matthews, L.A., Chan, S., Farrar, J.D., 2007. Pre-assembly of STAT4 with the human IFN-alpha-/beta receptor-2 subunit is mediated by the STAT4 N-domain. Molecular Immunology 44, 1864-1872.

Upham, J.W., Lee, P.T., Holt, B.J., Heaton, T., Prescott, S.L., Sharp, M.J., Sly, P.D., Holt, P.G., 2002. Development of interleukin-12-producing capacity throughout childhood. Infect Immun 70, 6583-6588.

Usui, T., Preiss, J.C., Kanno, Y., Yao, Z.J., Bream, J.H., O'Shea, J.J., Strober, W., 2006. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. Journal of Experimental Medicine 203, 755-766.

van Haarst, J.M., de Wit, H.J., Drexhage, H.A., Hoogsteden, H.C., 1994. Distribution and immunophenotype of mononuclear phagocytes and dendritic cells in the human lung. Am J Respir Cell Mol Biol 10, 487-492.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3.

Viveiros, M.M., Antczak, D.F., 1999. Characterization of equine natural killer and IL-2 stimulated lymphokine activated killer cell populations. Developmental and Comparative Immunology 23, 521-532.

Vogel, K., Blumer, N., Korthals, M., Mittelstadt, J., Garn, H., Ege, M., von Mutius, E., Gatermann, S., Bufe, A., Goldmann, T., Schwaiger, K., Renz, H., Brandau, S., Bauer, J., Heine, H.,

Holst, O., 2008a. Animal shed Bacillus licheniformis spores possess allergy-protective as well as inflammatory properties. J Allergy Clin Immunol 122, 307-312, 312 e301-308.

Vogel, K., Blumer, N., Korthals, M., Mittelstadt, J., Garn, H., Ege, M., von Mutius, E., Gatermann, S., Bufe, A., Goldmann, T., Schwaiger, K., Renz, H., Brandau, S., Bauer, J., Heine, H., Holst, O., 2008b. Animal shed Bacillus licheniformis spores possess allergy-protective as well as inflammatory properties. Journal of Allergy and Clinical Immunology 122, 307-312.

von Garnier, C., Filgueira, L., Wikstrom, M., Smith, M., Thomas, J.A., Strickland, D.H., Holt, P.G., Stumbles, P.A., 2005. Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. Journal of Immunology 175, 1609-1618.

Vuillermin, P.J., Ponsonby, A.L., Saffery, R., Tang, M.L., Ellis, J.A., Sly, P., Holt, P., 2009a. Microbial exposure, interferon gamma gene demethylation in naive T-cells, and the risk of allergic disease. Allergy 64, 348-353.

Vuillermin, P.J., Ponsonby, A.L., Saffery, R., Tang, M.L., Ellis, J.A., Sly, P., Holt, P., 2009b. Microbial exposure, interferon gamma gene demethylation in naive T-cells, and the risk of allergic disease. Allergy 64, 348-353.

Wagner, B., 2006. Immunoglobulins and immunoglobulin genes of the horse. Dev Comp Immunol 30, 155-164.

Wagner, B., 2009. IgE in horses: Occurrence in health and disease. Veterinary Immunology and Immunopathology 132, 21-30.

Wagner, B., Burton, A., Ainsworth, D., 2010. Interferon-gamma, interleukin-4 and interleukin-10 production by T helper cells reveals intact Th1 and regulatory T(R)1 cell activation and a delay of the Th2 cell response in equine neonates and foals. Veterinary Research 41.

Wagner, B., Flaminio, J.B.F., Hillegas, J., Leibold, W., Erb, H.N., Antczak, D.F., 2006. Occurrence of IgE in foals: Evidence for transfer of maternal IgE by the colostrum and late onset of endogenous IgE production in the horse. Veterinary Immunology and Immunopathology 110, 269-278.

Wagner, B., Radbruch, A., Rohwer, J., Leibold, W., 2003. Monoclonal anti-equine IgE antibodies with specificity for different epitopes on the immunoglobulin heavy chain of native IgE. Veterinary Immunology and Immunopathology 92, 45-60.

Weaver, C.T., Harrington, L.E., Mangan, P.R., Gavrieli, M., Murphy, K.M., 2006. Th17: An effector CD4 T cell lineage with regulatory T cell ties. Immunity 24, 677-688.

White, G.P., Watt, P.M., Holt, B.J., Holt, P.G., 2002a. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. J Immunol 168, 2820-2827.

White, G.P., Watt, P.M., Holt, B.J., Holt, P.G., 2002b. Differential patterns of methylation of the IFN-gamma promoter at CpG and Non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO(-) T cells. Journal of Immunology 168, 2820-2827.

Wichtel, M.G., Anderson, K.L., Johnson, T.V., Nathan, U., Smith, L., 1991. INFLUENCE OF AGE ON NEUTROPHIL FUNCTION IN FOALS. Equine Vet J 23, 466-469.

Willems, F., Vollstedt, S., Suter, M., 2009a. Phenotype and function of neonatal DC. Eur J Immunol 39, 26-35.

Willems, F., Vollstedt, S., Suter, M., 2009b. Phenotype and function of neonatal DC. European Journal of Immunology 39, 26-35.

Wilson, C.B., Merkenschlager, M., 2006. Chromatin structure and gene regulation in T cell development and function. Current Opinion in Immunology 18, 143-151.

Wilson, C.B., Rowell, E., Sekimata, M., 2009. Epigenetic control of T-helper-cell differentiation. Nature Reviews Immunology 9, 91-105.

Wilson, C.B., Westall, J., Johnston, L., Lewis, D.B., Dower, S.K., Alpert, A.R., 1986a. DECREASED PRODUCTION OF INTERFERON-GAMMA BY HUMAN NEONATAL CELLS - INTRINSIC AND REGULATORY DEFICIENCIES. Journal of Clinical Investigation 77, 860-867.

Wilson, C.B., Westall, J., Johnston, L., Lewis, D.B., Dower, S.K., Alpert, A.R., 1986b. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. J Clin Invest 77, 860-867.

Woods, P.S., Robinson, N.E., Swanson, M.C., Reed, C.E., Broadstone, R.V., Derksen, F.J., 1993. Airborne dust and aeroallergen concentration in a horse stable under two different management systems. Equine Vet J 25, 208-213.

Wu, S.C., Zhang, Y., 2010. Active DNA demethylation: many roads lead to Rome (vol 11, pg 607, 2010). Nature Reviews Molecular Cell Biology 11.

Yagi, R., Zhu, J.F., Paul, W.E., 2011. An updated view on transcription factor GATA3-mediated regulation of T(h)1 and T(h)2 cell differentiation. International Immunology 23, 415-420.

Ygberg, S., Nilsson, A., 2012. The developing immune system - from foetus to toddler. Acta Paediatrica 101, 120-127.

Yu, H.R., Chang, J.C., Chen, R.F., Chuang, H., Hong, K.C., Wang, L., Yang, K.D., 2003. Different antigens trigger different Th1/Th2 reactions in neonatal mononuclear cells (MNCs) relating to T-bet/GATA-3 expression. Journal of Leukocyte Biology 74, 952-958.

Zaghouani, H., Hoeman, C.M., Adkins, B., 2009. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. Trends in immunology 30, 585-591.

Zhu, J., Yamane, H., Paul, W.E., Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol 28, 445-489.

Zhu, J.F., Yamane, H., Paul, W.E., 2010. Differentiation of Effector CD4 T Cell Populations. Annu Rev Immunol 28, 445-489.

Zink, M.C., Johnson, J.A., 1984. CELLULAR-CONSTITUENTS OF CLINICALLY NORMAL FOAL BRONCHOALVEOLAR LAVAGE FLUID DURING POSTNATAL MATURATION. American Journal of Veterinary Research 45, 893-897.

Copyright © Lingshuang Sun 2012

VITA

Lingshuang Sun

PERSONAL INFORMATION:

Date of Birth: Feburary 11, 1980 (Mudanjiang, Heilongjiang, People's Republic of China)

ACADEMIC DEGREES:

MPVM, 2005 Master of Preventive Veterinary Medicine, Department of Preventive Veterianry Medicine, College of Veterinary Medicine, Northeast Agriculture University, Heilongjiang, China (Major in Veterinary Animal Diseases and Immunology)

BASc., 2002 College of Veterinary Medicine, Northeast Agriculture University, Heilongjiang, China

PROFESSIONAL EXPERIENCE:

2007 - 2012 Graduate Research Assistant, Department of Veterinary Science, Maxwell H. Gluck Equine Research Center, University of Kentucky

2003 - 2006 Graduate Research Assistant, Department of Preventive Veterianry Medicine, College of Veterinary Medicine, Northeast Agriculture University, Heilongjiang, China

2004 Spring Research Assistant, China Highly Pathogenic Avian Influenza Prevention and Control Headquarters, Branch of Science and Technological in Heilongjiang province, China.

TEACHING EXPERIENCE:

2011Spring Guest Lecturer, Topics in the Equine Industry (EQS309), 2010 Fall Midway Collage, Midway, KY 4034Guest Lecturer

2011Summer Guest Lecturer, Introductory anatomy, physiology and animal hygiene (VS350), Department of Veterinary Science, University of Kentucky 2011 Summer Teaching Assistant: Introductory anatomy, physiology and animal hygiene (VS350), 2011 Summer. Department of Veterinary Science, University of Kentucky

2004 - 2005 Graduate Student Teaching Instructor, College of Veterinary Medicine, Northeast Agriculture University, Heilongjiang, China

HONORS/AWARDS AND FELLOWSHIPS:

1. Wright Markey Trust Fund, University of Kentucky (2007-Present)
2. Conference/Research Student Support Funding, the Graduate School, University of Kentucky (2008, 2009, 2001, 2011)
3. First Grade Scholarship of Northeast Agriculture University, Northeast Agriculture

University,China, (2001,2002)

4. Da Bei Nong Scholarship (certificate No. DBND2000-009), China, 2000

PEER-REVIEWED PUBLICATIONS:

1. * **Lingshuang Sun**, Amanda A. Adams, Allen E. Page, Alejandra Betancourt, David W. Horohov.The effect of environment on interferon-gamma production in neonatal foals. Veterinary immunology and immunopathology 2011 Sep 15;143(1-2):170-5.

2. * **Lingshuang Sun**, Amanda A. Adams, Alejandra Betancourt, John C Stewart, Chong Liu, David W. Horohov. The role of proliferation in the regulation of IFNγ expression by foals. Development and Comparative Immunology. 2011 Nov 4.

3. * **Sun L**, Gong Z, Oberst EJ, Betancourt A, Adams AA, Horohov DW. The promoter region of interferon-gamma is hypermethylated in neonatal foals and its demethylation is associated with increased gene expression. Dev Comp Immunol. 2012 Oct 11.

4. Hansen S, **Sun L**, Baptiste KE, Fjeldborg J, Horohov DW. Age-related changes in intracellular expression of IFN-γ and TNF-α in equine lymphocytes measured in bronchoalveolar lavage and peripheral blood. Dev Comp Immunol. 2012 Dec 5.

5. Chong Liu, Alex Betancourt, Cohen DA, Amanda A. Adams, **Lingshuang Sun,** David W. Horohov, Granzyme B-mRNA expression by equine lymphokine activated killer (LAK) cells is associated with the induction of apoptosis in target cells. Vet Immunol Immunopathol. 2011 Sep 15;143(1-2):108-15. Epub 2011 Jul 13.

6. **Sun Lingshuang**, Qi yan. Duck and Goose Immunoglobulins Purification from Egg Yolk and Their Comparison. (2007) 01-0010-02, Chinese Journal of Veterinary Science and Technology.

7. **Sun Lingshuang**; Qi Yan; Shi Nan; Wang Junwei. Purification and Comparison of Duck and Goose Immunoglobulins from Yolk. 2006, v19, Chinease Poultry.

8. Qi Yan, **Sun Lingshuang**, Zhang Henan, Wang Bin, Qu Zhehui, Shi Nan, Shi Dongfang, Wang Junwei. Preliminary comparative analysis on HA and HA1 expressed in Sf-9 insect cells of H5 subtype AIV as detective antigen. 2008 39(11). Journal of northeast agricultural university.

9. Jia Shanshan, **Sun Lingshuang**, Qi Yan. Expression of HA1 gene of avian influenza virus H7 subtype in recombinant baculovirus and analysis of antigenicity of the expressed HA1 protein. v. 37 (3) p. 232 - 235, 2007, Veterinary Science in China.

10. Qi Yan, **Sun Ling-shuang**, Wang Bin, Sun Jin-hua, Wang Jun-wei, Shi Dong-fang. Eukaryotic expression of HA1 gene of H5 avian influenza virus in insect cells and the antigenicity of the expressed protein. 2006 36(5). Veterinary science in China.

11. Wang Bin, Tang Zhi-fen, Qi Yan, **Sun Ling-shuang**, Wang Jun-wei. Recombinant baculovirus expression of HA2 gene of avian influenza virus H9N2 subtype. 2006 36(5). Veterinary science in China.

**Publications from PhD dissertation research project*

PUBLICATIONS IN PREPARATION:

1. * **Lingshuang Sun**, Eric J. Oberst, Alejandra Betancourt, Don Cohen, Frank Cook, Tom Chambers Chong Liu, David W. Horohov. The effect of environment on lung antigen presenting cells (APC) in foals. (in preparation)

2. * **Lingshuang Sun**, Whitney Zoll, Alan T. Loynachan, Allen Page, Michael Fettinger, Alejandra Betancourt, John C. Stewart, David W. Horohov. Age-associated susceptibility to R equi infection in foals (in preparation)

3. Zhangbin Gong, **Lingshuang Sun,** David W. Horohov. Specific CpG sites methylation status in the IFNgamma gene promoter region of aged equine.

4. Adams, AA, A. Betancourt, M. Brummer, **L. Sun**, P. Lawless and D.W. Horohov. In Vitro and In Vivo Immunomodulatory Effects of Resveratrol on Immune Function in Aged Horses. Submitted to Mechanisms of Ageing and Development.

**Publications from PhD dissertation research project*

ABSTRACTS/PRESENTATIONS:

1. Lingshuang Sun, A.A. Adams, D.W. Horohov. Regulation of interferon-gamma (IFNγ) gene expression in foals by proliferation and DNA methylation. Poster presentation, *89th Conference of Research Worker in Animal Diseases, Chicago, Illinois, 2008.*

2. **Lingshuang Sun**, Amanda A. Adams, Eric J. Oberst, Daisy M. Ramos, David W. Horohov. The effect of environment on interferon-gamma (IFNγ) production in neonatal foals. Oral presentation, *90th Conference of Research Worker in Animal Diseases, Chicago, Illinois, 2009.*

3. **Lingshuang Sun**, Alejandra Betancourt, Allen Page, Eric J. Oberst, Amanda A. Adams, David W. Horohov. Environment affects interferon-gamma (IFNγ) production in neonatal foals through possible effects on dendritic cells. Oral

presentation, *91st Conference of Research Worker in Animal Diseases, Chicago, Illinois, 2010*.

4. **Lingshuang Sun**, Zhangbin Gong, David W. Horohov. The DNA promoter of the interferon gamma gene (Ifng) is hypermethylated in neonatal foals. Oral presentation. *92nd Conference of Research Worker in Animal Diseases, Chicago, Illinois, 2011*.

5. **L. Sun**, Z. Gong, D. W. Horohov, The methylation level of the IFNγ gene promoter is correlated with IFNγ expression in foals. Will be oral present in *International Conference on Equine Infectious Diseases IX, Oct. 21-26 in Lexington, KY*.

6. **Lingshuang Sun**, Zhangbin Gong, Eric J. Oberst, David W. Horohov. The hypermethylation of IFNγ gene promoter is correlated with IFNγ deficiency in the neonatal foals and its demethylation with aging is associated with environment. *Havemeyer Workshop on Rhodococcus equi, Deauville, France, 9-12th of July, 2012.*

7. **Lingshuang Sun**, Macarena G. Sanz, Alan T. Loynachan, Whitney Zoll, Allen Page, David W. Horohov. Age-related susceptibility to R equi infection in foals. Oral presentation at *93rd Conference of Research Worker in Animal Diseases, Chicago, Illinois, 2012.*

8.David W. Horohov, **Lingshuang Sun**, Age-dependent susceptibility of young foals to Rhodococcus equi. *5th Havemeyer Workshop on Rhodococcus equi, Deauville, France, 9- 12th of July, 2012.*

9. Zhangbin Gong, **Lingshuang Sun**, David W. Horohov. A specific CpG site demethylation in the IFNgamma gene promoter region of different aged equine. Presented during *92nd Conference of Research Worker in Animal Diseases, Chicago, Illinois, 2011*.

10. D.W. Horohov, M. Sanz, **L. Sun**, A. Betancourt, A. Page. Reasons for the age-dependent susceptibility of young foals to Rhodococcus equi. *International Conference on Equine Infectious Diseases IX, Oct. 21-26 in Lexington, KY*.

11. Hansen S, Baptiste KE, Fjeldborg J, **Sun L** and Horohov D. Comparison of intracellular expression of IFN-γ and TNF-α in equine lymphocytes measured in bronchoalveolar lavage (BAL) and peripheral blood. *30th Annual VCRS Symposium, October 2012, Columbia, Missouri.*

12. Adams AA, Betancourt A, Brummer M, **Sun L**, Lawless P and Horohov DW. In Vitro and In Vivo Immunomodulatory Effects of Resveratrol on Immune Function in Aged Horses. Poster presentation during the *90th Conference of Research Workers in Animal Disease, Chicago, Illinois, 2009.*

13. Adams AA, Betancourt A, Brummer M, **Sun L**, Lawless P and Horohov DW. In Vitro and In Vivo Immunomodulatory Effects of Resveratrol on Immune Function in

Aged Horses. Poster presentation. *1st International Workshop on the Geriatric Horse-Havemeyer Foundation, Boston, Massachusetts, 2010.*

Copyright © Lingshuang Sun 2012