

THE INFLUENCE OF AGE AND RHODOCOCCLUS EQUI INFECTION ON CD1  
EXPRESSION BY EQUINE ANTIGEN PRESENTING CELLS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of INDIRA SONJA PARGASS find it satisfactory and recommend that it be accepted.

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There is a distinct age-associated susceptibility of horses to *Rhodococcus equi* infection, as only foals less than 6 months of age are typically affected. *R. equi* is closely related and structurally similar to *Mycobacterium tuberculosis* and causes similar pathologic lesions. Protective immune responses to *M. tuberculosis* involve classical MHC-restricted T cells as well as MHC-independent T cells that recognize mycobacterial lipid antigen presented by CD1 molecules. Given the structural similarity between these two pathogens and our previous observations regarding *R. equi*-specific, MHC-unrestricted cytotoxic T lymphocytes (CTL), we developed 3 related hypotheses: (1) CD1 molecules are expressed on equine antigen presenting cells (APC) (2) CD1 expression on APC is less in foals compared to adults and (3) Infection with live virulent *R. equi* induces upregulation of CD1 on both adult and perinatal APC. CD1 expression was examined by flow cytometric analysis using a panel of monoclonal CD1 antibodies with different species and isoform specificities.

Results: Three CD1 antibodies specific for CD1b showed consistent cross-reactivity with both foal and adult PBAC. CD1b and MHC class II expression were significantly higher in

adult PBAC compared with foals, suggesting that there are age-related differences in expression of these molecules. *R. equi* infected PBAC showed significantly lower expression of CD1b. Dual antibody staining of PBMC revealed that 45 -71% of the monocyte population stained positive for CD1b, and that the majority of these also co-expressed MHC II molecules, indicating that they were antigen presenting cells. The anti-CD1 antibodies showed no binding or minimal binding to BAL derived macrophages.

Conclusion: The CD1b isoform is evolutionarily conserved, and is present on equine PBAC and circulating blood monocytes. The unique susceptibility of foals to *R. equi* infection may be due in part to lower expression of CD1 and MHC class II observed in this study. The data also suggests that infection with *R. equi* induces down-regulation of CD1b on equine PBAC. This may represent a novel mechanism by *R. equi* to avoid detection and killing of infected cells by the immune system, similar to that observed when human APC are infected with *Mycobacterium tuberculosis*.

## ABSTRACT

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# CHAPTER ONE

## INTRODUCTION

### *Rhodococcus equi*

*Rhodococcus equi* is a facultative intracellular gram positive coccobacillus and an important cause of subacute or chronic abscessating bronchopneumonia in foals up to 3-5 months of age (13,14). Infection in adult horses and other species is rare, and is usually associated with immunosuppression (13). *R. equi* is also recognized as an opportunistic pathogen in immunosuppressed humans, notably those with HIV-AIDS, where it causes severe pyogranulomatous pneumonia that mimics tuberculosis (21). Both *R. equi* and *Mycobacterium tuberculosis* are nocardioform actinomycetes and produce similar pyogranulomatous lung lesions. These lesions reflect the ability of these organisms to survive and persist within the phagosomes of macrophages (13, 14, 26), which are the primary targets for infection. Both organisms belong to the Mycolata family, which is a group of G+C gram positive bacteria that have a unique cell wall envelope consisting of mycolic acids linked to arabinogalactan wall polysaccharides and glycolipids.

### **CD1**

CD1 molecules are a unique family of antigen presenting molecules that bind bacterial and autologous lipid antigens via specialized hydrophobic ligand binding grooves (29, 33, 38). These molecules enable T cell recognition of fatty acids, glycolipids and lipopeptide antigens of foreign or self origin (32, 51). Certain lipids function as antigens and are recognized by CD1-restricted T cells, whereas others function as adjuvants by activating antigen presenting cells via Toll-like

receptors (51). Mycobacterial cell wall lipid antigens found to be presented by CD1 include mycolates, glycosphingolipids, phospholipids such as lipoarabinomannan, sulfoglycolipids, and lipopeptides such as didehydromycobactin. Self lipids presented by CD1 include gangliosides, sulfatides and phospholipids such as phosphatidylinositols and phosphatidylcholines (27,30,35)

CD1 is a MHC class I homologue encoded outside the MHC by a non-polymorphic gene family. The family is comprised of up to 5 distinct genes that appear to be conserved in most mammalian species. Based on sequence homology, these genes are divided into three groups: a) Group 1, which consists of CD1a, CD1b, CD1c; b) Group 2, consisting of only CD1d, is present in most mammalian species studied so far (27, 31, 30, 33,38); c) Group 3, currently consisting only of CD1e. Most mammalian species studied so far have multiple CD1 group 1 isoforms, although there are some striking species differences.

Group 1 CD1 proteins are expressed mainly on hemopoietic cells, primarily antigen presenting cells such as activated Langerhans cells, dendritic cells, macrophages and B cells (37). CD1d proteins are more widely expressed, and in addition to antigen presenting cells (APC), can be found on thymocytes and a wide variety of epithelial cells (27). CD1d is known to present antigens mostly to natural killer T cells (NKT), a unique T cell population that carries a semi-variant T cell receptor and possesses considerable immunoregulatory functions (8, 20). Antigens presented by CD1d include glycolipids from marine sponges, bacterial glycolipids, normal endogenous lipids, and tumor derived phospholipids and glycolipids. CD1d is likely involved in important immunoregulatory functions, such as aiding in the immune response against malaria, enhancing clearance of certain viral infections, and enhancement or suppression of immune responses against tumors and autoimmune diseases (52,53,54). New studies have

shown that the CD1e isoform functions within endosomes as lipid transfer proteins that assist in antigen loading and does not participate directly in antigen presentation (52, 56).

### **Immunity to *R. equi***

*R. equi* is considered ubiquitous in the equine environment, where it is found in surface soil and manure. Inhalation is believed to be the primary route of exposure (14). All horses are likely exposed to *R. equi* in the first days to few weeks of life, which is when initial infection is thought to occur. There is distinct age-associated susceptibility, as only foals less than 4-6 months of age typically develop rhodococcal pneumonia. Most foals ultimately develop protective immune responses and subsequent life long acquired immunity. As a result, adult horses are resistant to *R. equi* infection and do not develop clinical signs of disease unless immunocompromised.

The immunity of adult horses to *R. equi* reflects acquired immune responses and the ability to mount effective type 1 responses (2,3). Clearance of virulent *R. equi* from the lung in adults is associated with an increase in CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes (2,3,10), IFN- $\gamma$  production and lymphoproliferative responses at the site of challenge (10,16,17). These observations support previous studies that utilized mouse models. In mice, modulation of the immune response to *R. equi* using monoclonal antibodies that favor Th1 or Th2 responses resulted in immune clearance or characteristic lesions, respectively (24). Additionally, adoptive transfer studies in mice show that CD4<sup>+</sup> Th1 lymphocytes are essential and sufficient to effect pulmonary clearance of virulent *R. equi*, whereas induction of a Th2 response results in characteristic lung lesions (25). The role of CD4<sup>+</sup> T cells is also supported by the failure of transgenic class II deficient mice, which lack functional CD4<sup>+</sup> T cells, to

effectively clear *R. equi* infection in the lungs (19, 25). These observations suggest that the life threatening pyogranulomatous bronchopneumonia which develops in affected foals reflects an inability to mount the responses necessary to effect clearance of the organism from the lungs (2, 3, 23). The exact mechanisms behind this age predisposition are unclear, but are thought to be related to immaturity of the immune system in individual foals and their failure or decreased ability to mount potent Th1 responses (10, 23, 25,61).

In addition to CD4+ Th1 lymphocytes, antigen-specific CD8+ T lymphocytes also contribute significantly to the protective immune response against *R. equi* (2, 3,19). Just as immune clearance in adult horses is associated with increased numbers of CD8+ T lymphocytes in the lung, transgenic mice that have CD8+ T lymphocytes but lack MHC class II-restricted CD4+ T lymphocytes are able to significantly reduce *R. equi* numbers in the lungs (19). This likely reflects the ability of CD8+ CTL to recognize and lyse infected cells, although their ability to produce IFN- $\gamma$  may also play a role.

Importantly, immune adult horses invariably have CD8+ CTL which lyse *R. equi* infected cells ex vivo, but do not kill uninfected versions of the same target cell. These CTL are MHC unrestricted, having the equivalent ability to lyse infected cells from other (mismatched) horses who express different MHC class I haplotypes. This is suggestive of a nonclassical method of antigen presentation. Foals less than 3 weeks of age appear to lack this *R. equi* specific CTL activity although CTL do begin to appear subsequently and are present at 8 weeks of age. Given that CTL contribute to immunity to *M. tuberculosis* (see below), are present in all immune adult horses, and develop in foals during the first 8 weeks or so following birth, the lack of CTL activity in perinatal foals as well as the Th2- biased immune

response of neonates also likely play a role in the distinct age-associated susceptibility of foals to rhodococcal pneumonia.

### **Immunity to *M. tuberculosis***

A similar type of cell mediated Type 1 immune response involving both CD4<sup>+</sup> Th1 cells and CD8<sup>+</sup> cytotoxic T lymphocytes is required for successful elimination of the related organism *M. tuberculosis* in humans and animal models (12,27,57,76). The production of IFN- $\gamma$  by CD4<sup>+</sup> T lymphocytes is considered the best immunologic correlate for effective immune clearance (76). However, in addition to classical MHC class I and class II restricted immune responses, there is a growing body of evidence which suggests that MHC-unrestricted recognition of lipid antigens may be important, particularly as microbial lipids are different from those in mammalian cells and are less susceptible to genetic alterations due to their important structural functions in microbial cell walls (26,31). A substantial fraction of CD4<sup>+</sup> T cells and a portion of the CD8<sup>+</sup> T cells from peripheral blood of asymptomatic *M. tuberculosis* infected humans showed significant proliferation and IFN- $\gamma$  production upon re-exposure to mycobacterial lipid antigens. These T cell responses were diminished in CD1 antibody blocking experiments, confirming that a substantial portion of the *M. tuberculosis*-reactive T cells were CD1-restricted and recognized antigens presented by cell surface molecules other than MHC class I and II (27). Porcelli et al 1992 also demonstrated the existence of CD1-restricted CD4<sup>-</sup> CD8<sup>-</sup>  $\alpha\beta$  T lymphocytes cells specific for mycobacterial antigens presented by CD1b. Additionally, guinea pigs vaccinated with mycobacterial lipids are able to mount CD1-restricted T cell responses, and show decreased bacterial burden and lung pathology upon subsequent challenge with virulent *M. tuberculosis* (40)

CD1-restricted T cells which recognize unique mycobacterial lipids appear to contribute to protective immunity by the production of high levels of Th1 cytokines such as IFN- $\gamma$  and TNF $\alpha$ , and are able to lyse macrophages infected with virulent *M. tuberculosis* (12, 48, 27). Given the structural similarity between *R. equi* and *M. tuberculosis* and the fact that similar immune responses are necessary for clearance of infections by both organisms, it seems likely that CD1 mediated antigen presentation also plays a part in the protective immune response to *R. equi*. A likely explanation for the MHC-unrestricted CTL activity against *R. equi* infected cells present in immune adult horses is recognition of infected cells by CD1-restricted T lymphocytes that bear T cell receptors for lipid antigen.

The present study investigated the expression of CD1 on equine APC in horses. I also sought to determine whether differences in CD1 expression between adults versus foals could explain the age-associated susceptibility to rhodococcal pneumonia. I hypothesized that (1) Antigen presenting CD1 molecules recognized by previously described monoclonal antibodies are expressed on equine APC (2) CD1 expression on APC is less in foals compared to adults and (3) In response to *R. equi* infection, APC from both adult and perinatal horses upregulate expression of CD1 – thereby increasing the ability of infected cells to be recognized by CD1 restricted T lymphocytes.

## **CHAPTER TWO**

### **RESEARCH DESIGN AND METHODOLOGY**

#### **Blood collection**

The use of animals in this study for blood and BAL-cell collection was approved by IACUC. Six healthy 7 to 12 day old foals, and eight healthy 2 to 15 year old adult horses of various breeds, currently maintained in the Washington State University Teaching Hospital, were randomly selected. Venous blood was collected from the jugular vein of each horse using 200 and 500 ml evacuated containers (Baxter, Deerfield) containing anticoagulant acid citrate dextrose (ACD) at approximately 2 mls ACD per 10 mls blood. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by using a Ficoll-Hypaque technique (1, 2, 3). Cytospin preparations of the PBMC followed by a nucleated cell differential count were performed to determine the percentages of the different nucleated present before plating. The PBMC were then plated as described below to obtain peripheral blood adherent cells (PBAC), which were regarded as monocyte-derived macrophages/antigen presenting cells (APC).

#### **Peripheral Blood Adherent Cell (PBAC) preparation**

PBAC were harvested from PBMC and used as antigen presenting cells as previously described (1, 2, 3). 150cm<sup>2</sup> petri dishes were coated with 2% sterile bovine collagen (6 ml per plate) by incubating the dishes for 2 h at 37°C with 5% CO<sub>2</sub>. The collagen solution was then removed and the dishes air dried for 30 minutes in a sterile hood incubator. The dishes were coated with 6 ml of NHS (normal horse serum) by incubation for 30 minutes at 37°C with 5% CO<sub>2</sub>. The serum was removed and PBMC were plated at a density 1.5x10<sup>8</sup> cells/dish in 20 ml of

antibiotic-free complete medium. Cells were incubated for 12 to 15 hours at 37°C with 5% CO<sub>2</sub>. Following incubation, non-adherent cells were resuspended in the culture medium by repeated pipetting ten times and then removed by washing each plate twice with 10 ml of HBSS (Hanks Balanced Saline Solution) by repeated pipetting (ten times). Twenty mls of antibiotic free-complete medium were added to the remaining adherent cells which were used as APC (also referred to as PBAC or monocyte-derived macrophages) for infection with *R. equi*.

### **Bronchoalveolar lavage (BAL) and cell preparation**

Three healthy adult horses were sedated with xylazine at 0.06 mg/kg of body weight. A Bivona BAL tube was inserted through one nostril into the trachea and wedged into place. To collect BAL cells from the horses, three separate aliquots of 180 ml of 0.9% NaCl containing 0.84% sodium bicarbonate (BAL wash) were instilled into the lung. Following each aliquot, fluid was aspirated. Fluid was placed into 50 ml conical tubes and centrifuged for 8 minutes at 600 X g at 4° C. Pellets were consolidated and washed three times with complete medium containing decreasing amounts of antibiotic/antimycotic (wash 1, 1 µg of amphotericin/ml [Gibco]; wash 2, 2 U of penicillin/ml, 2 U streptomycin/ml, and 0.1 µg of amphotericin/ml [Gibco]; wash 3, antibiotic and antimycotic free). Complete medium consisted of RMPI supplemented with 2 mM L-glutamine, 0.05 µM 2-mercaptoethanol, 6.25mM HEPES and 10% heat inactivated normal horse serum (NHS; Gibco).

A cyto-spin preparation of the washed cells followed by a nucleated cell differential count was performed to determine the different proportions of nucleated cells present before plating. The cells were plated at  $2 \times 10^6$  cells/ml on 75-cm<sup>2</sup> collagen/serum-coated petri dishes and incubated for 1 h at 37°C with 5% CO<sub>2</sub> as previously described (2,3). Following incubation, non-



adherent cells were removed by vigorously pipetting of the supernatant 10 times and washing again with HBSS via pipetting of 10 ml of HBSS over the plate 10 times. The remaining adherent cells were used as APC for infection with *R. equi*.

## **Bacteria**

The plasmid bearing (PL+) virulent *R. equi* strain ATCC 33701, which contains the 80.6-kb virulence-associated plasmid Vap A, was used in all experiments. The bacteria were stored at -80°C as a frozen stabulate and reconstituted for the experiments. Bacteria were initially grown on brain heart infusion (BHI) agar and then cultivated overnight in BHI broth at 37°C in a shaking incubator at 200 rpm. After washing twice in sterile phosphate buffered saline (PBS), the bacteria were adjusted to a concentration of  $1.5 \times 10^8$  bacteria/ml in PBS by obtaining a corresponding optical density of 0.05 by spectrophotometry ( $OD_{600}=0.05$ ) as previously described by Patton et al (2, 3). The adjusted concentration was confirmed by plating serial dilutions on BHI plates and calculating the number of CFU per milliliter (2, 3).

## **Infection of APC**

The percentage of monocytes and macrophages present in the PBMC suspension and BAL fluid respectively was determined by performing a nucleated cell differential count before plating of the cells. The total number of APC (adherent cells) present in each plate was determined by multiplying these percentages by the total number of cells initially plated ( $1.5 \times 10^8$  cells/dish for PBMC and  $2 \times 10^6$  cells/ml for BAL cells). The adherent cells were then infected with 2.5 MOI of live *R. equi* bacteria and incubated for 8 hours at 37°C with 5% CO<sub>2</sub> alongside a negative control

(uninfected) culture. One hour post infection, gentamycin (50 mg/ml) was added to each culture at a rate of 1 µl/ml of antibiotic-free complete medium to kill any extracellular bacteria.

### **Elution of adherent cells**

PBAC: Following the 8 hour incubation period, adherent cells were eluted by gently removing the culture medium and then adding 20 mls of antibiotic free complete-medium containing 20% normal horse serum (NHS) mixed 1:1 with 10 mM EDTA [0.5 M EDTA, GIBCO] for 15 min at 37°C with 5% CO<sub>2</sub>. The cells were collected by vigorous pipetting of the eluting fluid 10 times followed by washing each dish twice with 10 ml HBSS. Eluted cells were combined and centrifuged at 1150 rpm for 15 min at 4°C. The cells were then washed with antibiotic free-complete medium, resuspended in 2 mls of first wash buffer (450 ml 1X PBS, 50 ml of ACD, 2.25 ml of 20% NaN<sub>3</sub>, 10 ml NHS, 5 mls phenol red solution), and counted with trypan blue to assess viability and total cell number. The cell concentration was adjusted to 2x10<sup>7</sup> cells/ml in first wash buffer (FWB) for flow cytometric analysis.

BAL cells: The adherent BAL cells were removed by covering the plates with 10 ml of Accutase (Sigma) and incubating for 10 minutes at 37°C with 5% CO<sub>2</sub>. The cells were collected by repeated pipetting of the suspension 10 times followed by washing with 10 mls of HBSS 10 times. Cells were consolidated via centrifugation at 600 X g at 4°C for 15 minutes, and washed once with antibiotic-free complete medium containing no NHS. The cell pellet was resuspended in 2 mls of FWB, assessed with trypan blue for viability, counted, and adjusted to a concentration of 2x10<sup>7</sup> cells/ml in FWB for flow cytometric analysis.

Approximately 100 µl of the eluted cells from each horse was used to make a cytopsin preparation. The cytopsin slide was stained with Wright's Giemsa stain, and examined by light

microscopy. The percentage of infected cells was determined by examining 200 PBAC or BAL cells on the slide, and counting the number that contained intracellular bacteria. The number of contaminating cells, predominantly lymphocytes, was also estimated by microscopy.

## Antibodies and Flow Cytometric Analysis

**Table 1- List of antibodies used**

Antibody	Specificity	Isotype	Working concentration	Source
BCD1b3	Human CD1b	IgG1	15 µg/ml	Steve Porcelli (2)
CD1F2/1b12.1	GP CD1b	IgG2a	15 µg/ml	Steve Porcelli (2)
CD1F2/5E3	GP CD1b,c	IgG1	1: 100 dilution	Steve Porcelli (2,8)
CD1F2/6B5.1	GP CD1b,c	IgG1	1:100 dilution	Steve Porcelli (2,8)
20.27	Sheep CD1b	IgG1	1:5 dilution	U of Melbourne, Australia (2,8)
TH97	Bovine CD1b	IgG2a	15 µg/ml	VMRD Inc, Pullman WA (2)
7C4	Human CD1b	IgG1	15 µg/ml	Otto Majdic (5)
OKT6	Human CD1a	IgG1	15 µg/ml	Steve Porcelli (63)
CBT6a	Human CD1a	IgG1	15 µg/ml	Fabio Malavasi (11)
B-B5	Human CD1b/c	IgG1	1:5 dilution	Biosource International Inc, Camarillo, CA
WM25	Human CD1b	IgG1	15 µg/ml	Chemicon International Inc, Hampshire, UK (8)
MT101	Human CD1b	IgG1	15 µg/ml	Serotec Ltd, Oxford, UK (64)
NA1/34-HLK	Human CD1a	IgG2a	15 µg/ml	Serotec Ltd, Oxford, UK
DH59B	Equine CD172a (monocytes/macrophages /granulocytes)	IgG1	15 µg/ml	VMRD Inc, Pullman WA
TH81A	MHC II	IgG2a	15 µg/ml	VMRD Inc, Pullman WA
HB19	Equine CD5	IgG2a	15 µg/ml	VMRD Inc, Pullman, WA
HB88	Equine CD2	IgG1	15 µg/ml	VMRD Inc, Pullman WA
B29	Equine B cells	IgG2a	15 µg/ml	VMRD Inc, Pullman WA
ECOLIS69A	Control monoclonal antibody	IgG1	15 µg/ml	VMRD Inc, Pullman WA

GP- Guinea pig

A panel of 13 CD1 antibodies with different species and isoform specificities was used to investigate CD1 expression (**Table 1**). In addition, the monoclonal antibodies DH59A (monocyte/macrophage marker), TH81A (MHC class II marker), HB19 or HB88 (CD5 or CD2 T cell markers respectively), and B29 (B cell marker), were also used to define the cellular composition of the PBMC (**Table 1**). The CD1 antibodies were tested at various concentrations with PBMC and PBAC in flow cytometry to obtain optimal working dilutions and to rule out that a suboptimal antibody concentration was not the reason for an apparent lack of cross reactivity with equine cells. PBMC from three horses were analyzed by two-color flow cytometry for expression of CD1 and other cell surface molecules, whilst infected and non-infected PBAC and BAL derived macrophages were examined by one color flow cytometric analysis.

The appropriately diluted primary antibodies were distributed at 50µl/well into a 96 well V-bottom assay plate on ice. One million cells per well suspended in 50 µl of FWB were added, and the plate was incubated for 15 minutes on ice. The cells in the plate were then centrifuged at 2000 rpm, 4°C for 15 minutes, and washed three times with 200 µl FWB. Appropriately diluted second step reagent (secondary antibody) in FWB was added to each well followed by incubation on ice for 15 minutes. For single color fluorescence labeling, 100 µl of the second step reagent Caltag goat anti-mouse IgG + IgM (H+L) human adsorbed FITC conjugate, diluted 1:200 was used. For two-color fluorescence labeling, 50 µl of the second step reagents goat anti-mouse IgG2a-RPE, human adsorbed, 1:600 dilution, and Caltag goat anti-mouse IgG1 FITC conjugate, 1:100 dilution, were used. The cells were then washed twice in second wash buffer (same as first wash buffer but without horse serum), and suspended in 200 µl of 2% formaldehyde in PBS. Labeled cells were analyzed with a FACscan equipped with a Macintosh computer and Cell Quest software (Becton Dickinson Immunocytometry Systems). Data was

collected on 5000 cells from each sample. Macrophages (BAL and peripheral blood monocyte-derived cells) and lymphocytes were gated according to their characteristic forward and side scatter patterns. Data were analyzed using repeated measures ANOVA (NCSS). Age was a between factor and infection was a within factor. Interpretation of the statistical analysis was based on the main effects of age and infection, because there were no significant interactions between these two factors.  $P < 0.05$  were considered significant.

## CHAPTER THREE

### RESULTS

#### Identification of *R. equi* infected cells

To visually confirm that the majority of nucleated cells were monocyte-derived macrophages, and that a MOI of 2.5 was sufficient to infect the majority of these cells without significantly affecting cell viability, Wright's Giemsa stained cytopsin slides of the PBAC (8 hours post-infection) from each horse were prepared and examined. The percentages of *R. equi* infected cells and contaminating lymphocytes were calculated (**Table 2**). The appearance of uninfected PBAC and *R. equi*-infected PBAC is shown in **Figure 1**.

**Table 2- Composition of cytopsin preparations of PBAC and BAL cells**

	Mean % of <i>R. equi</i> -infected cells at MOI of 2.5, 8 hours post infection		Mean % of monocyte-derived macrophages or BAL macrophages		Mean % of lymphocytes	
	Adults	Foals	Adults	Foals	Adults	Foals
<b>PBAC preparation<sup>1</sup></b>	93	92	94	98	6	2
<b>BAL preparation<sup>2</sup></b>	81	ND	93	ND	7	ND

ND- Not done

1: n = 8 (adults); n = 6 (foals)

2: n = 3 adult horses

The results in **Table 2** indicated that the predominant cell obtained from overnight culture of PBMC on gelatin were monocyte derived macrophages (PBAC). These results correlated with the findings on flow cytometry, in which over 90% of the PBAC from both foals and adults were positive for CD172a (DH59). The low number of lymphocytes in the cytologic preparations also correlates with the findings on flow cytometry, in which less than 6% of the cells stained positive

for CD5 (HB19). Similarly, cells with macrophage morphology constituted >80% of the total cell population present in cytopsin slides of cells obtained by bronchoalveolar lavage from adult horses. This percentage also correlated with the findings on flow cytometry, in which over 95% of the cells stained positive for CD172a.

Preliminary experiments to determine the effect of differing concentrations of *R. equi* on PBAC were also performed. The optimum MOI (multiplicity of infection) at which there was a high infection rate and minimal cell lysis was 2.5. At MOI's of 5 and 10, over 98% of the cells were infected, but the degree of cell lysis was marked. At a MOI of 1, the infection rate was only 50-60% and cell lysis was minimal.

### Flow Cytometry Results

**Table 3- SUMMARY: Influence of age and *R. equi* infection (8 hours post infection) on the mean percentage of positive PBAC**

<b>Antibody</b>	<b>Significant difference in cell marker expression between foals and adults, p&lt;0.05</b>	<b>Significant difference in cell marker expression between infected and uninfected cells, p&lt;0.05</b>
TH81 (MHC II)	Yes – Adults higher than foals	No
BCD1b3 (CD1b)	Yes – Adults higher than foals	Yes – Infected cells have lower expression
MT101 (CD1b)	No	No
CD1F2/1B12.1 (CD1b)	No	Yes – Infected cells have lower expression

For the remaining antibodies listed in Table 1, there were no significant differences in the percentage of positive PBAC between foals and adults, or between infected and un-infected cells. n=8 for adults; n=6 for foals

**Table 4- SUMMARY: Influence of age and *R. equi* infection (8 hours post infection) on the mean fluorescence intensity from PBAC**

<b>Antibody</b>	<b>Significant difference in mean fluorescence expression between foals and adults, p&lt;0.05</b>	<b>Significant difference in mean fluorescence expression between infected and uninfected cells, p&lt;0.05</b>
TH81 (MHC II)	No	No
BCD1b3 (CD1b)	Yes- Adults higher than foals	Yes- Infected cells have lower expression
MT101 (CD1b)	Yes- Adults higher than foals	No
CD1F2/1B12.1 (CD1b)	No	No

For the remaining antibodies listed in Table 1, there were no significant differences in the mean fluorescence expression on PBAC from foals and adults, or between infected and un-infected cells. n=8 for adults; n=6 for foals

**Cross reactivity of monoclonal antibodies with equine cells, assessed by the mean percentage of positive cells**

PBAC

To investigate our first hypothesis that antigen presenting CD1 molecules, reported to be cross reactive with previously described antibodies, are present on equine antigen presenting cells, a panel of CD1 monoclonal antibodies from various sources were tested with adult PBAC (n=8) and BAL-derived macrophages (n=3) using flow cytometry. The antibodies showing significant binding were chosen for subsequent experiments (see below).

The results in **Figure 2** show significant interspecies cross reactivity, primarily by the anti-CD1b antibodies BCD1b3, CD1F2/1B12.1, and MT101. Approximately 20-80% of the PBAC stained positive with these three antibodies. Specifically, the human anti-CD1b antibody BCD1b3 showed the highest and most consistent binding to PBAC (mean % positive cells =



82%). Antibodies against human CD1a (OKT6, CBT6a, NA 1/34-HLK) as well as the remaining antibodies against CD1b (**Table 1**), showed minimal binding (< 5%).

The monocyte/macrophage marker DH59 (CD172a) stained greater than 95% of adult PBAC, confirming that the majority of the cells were monocyte derived macrophages and therefore suitable targets for *R. equi* infection.

### BAL-derived macrophages

The majority of the adherent BAL cells consistently stained positive with DH59 (97% ± 2%), confirming that they were pulmonary macrophages and therefore suitable targets for *R. equi* infection. No CD1 antibodies bound to more than 6% of BAL-derived cells from adult horses (**Figure 2**). This was not considered significantly different from the monoclonal control antibody, ECOLIS 69A, which bound to 3% of the cells. Pulmonary macrophages also had lower expression of MHC class II (17% ± 2.8), as assessed by TH81, when compared to PBAC from adult horses (77%± 15).

### **Comparison of cell surface marker expression by foal and adult PBAC, assessed by the percentage of positive cells and mean fluorescence intensity**

The CD1b and CD1b/c antibodies BCD1b3 (human CD1b), MT101 (human CD1b), CD1F2/1B12.1 (guinea pig CD1b), and CD1F2/5E3 (guinea pig CD1b/c), were selected from the initial panel of CD1 antibodies and tested against foal PBAC (n=6). These antibodies were chosen as they showed consistent binding to adult PBAC and stained greater than 5% of the adult cells.

### Mean percentage of positive cells

Similar to adult PBAC, greater than 95% of foal PBAC stained positive for CD172a (DH59), confirming that the majority of cells were monocyte derived macrophages (**Figure 3a**). No significant differences in CD172a expression were observed between the two age groups.

A significant age-related difference in MHC II expression (assessed by antibody TH81) was observed ( $p=0.006$ ), with a greater number of adult PBAC ( $77\% \pm 15\%$ ) expressing MHC II compared with foals ( $42\% \pm 28\%$ ).

Of the four CD1 antibodies tested (**Figure 3a, Table 3**), the guinea pig-specific antibody BCD1b3 (CD1b) showed the most consistent binding and stained the highest proportion of PBAC from foals and adults. A statistically greater number of adult uninfected PBAC stained positive for CD1b (BCD1b3) compared with those of foals (adults  $82\% \pm 14\%$  and foals  $69\% \pm 14\%$ ,  $p=0.045$ ). Although additional antibodies against CD1b (MT101 and CD1F2/1B12.1) also stained a greater number of adult PBAC compared with foal PBAC, the differences were not statistically significant. When assessed by CD1F2/1B12.1, almost four times as many adult PBAC ( $51\% \pm 18\%$ ) appear to express CD1b compared with foal PBAC ( $15\% \pm 3.32$ ). Likewise, when assessed by MT101, the difference in CD1b expression between foals and adult cells approached statistical significance (adults  $20\% \pm 16\%$  and foals  $6\% \pm 3\%$ ,  $p=0.051$ ). Overall, the results with these CD1b antibodies show that foal PBAC express less CD1b molecules compared with those of adults.

### Mean fluorescence intensity

Adult PBAC assessed by BCD1b3 (CD1b) also showed significantly higher mean fluorescence intensity compared with foals, as  $p=0.014$  (**Figure 3b, 3c, Table 4**). This is

expected, as a higher percentage of adult cells expressed CD1 when assessed by this antibody. Adult cells also had significantly greater fluorescence than foal cells ( $p=0.015$ ) when assessed by another anti-CD1b antibody, MT101 (**Figure 3b, 3c**). This second observation suggests that, although foal and adult PBAC appear to have no differences in the total number of cells expressing CD1b when assessed by MT101, there are more CD1b molecules present on adult PBAC, resulting in a higher mean fluorescence. This is a good example of how measurement of the mean fluorescence intensity is useful in determining if differences in molecular expression of cell surface molecules exist when no differences in the total percentage of positive cells are observed. Overall, these results are consistent with differences in CD1b expression observed when foal and adult PBAC were assessed by the measuring the mean percentage of positive cells.

Although not statistically significant ( $p=0.059$ ), adult PBAC also showed almost three times as much mean fluorescence intensity of MHC II as foal PBAC when assessed by TH81 (**Figure 3b, 3c**). This is consistent with the significantly higher MHC II expression observed when adult PBAC were assessed by measuring the mean percentage of positive cells.

### **Modulation of CD1 expression in response to *R. equi* infection**

#### Mean percentage of positive cells

No significant changes in MHC II expression were observed between uninfected and infected PBAC (from both foals and adults), suggesting that *R. equi* infection has no effect on the level of expression of this antigen presenting molecule, regardless of age (**Figures 4a, 4b, Table 3**). This differs from the decreased MHC II expression that is observed on *M. tuberculosis* infected macrophages in patients with granulomatous tuberculosis (57).

In both age groups (n=6 for foals, n=8 for adults), the number of *R. equi*-infected PBAC expressing CD1b was significantly less compared with uninfected cells ( $p < 0.05$ ), as assessed by antibodies BCD1b3 (CD1b) and CD1F2/1B12.1 (CD1b) (**Fig 4a, 4b, Table 3**). Changes in CD1 expression due to infection were similar for both age groups ( $p = 0.138$ ), suggesting that infection with *R. equi* affects CD1 expression in a similar manner on both foals and adult APC. These results suggest that infection of equine PBAC with *R. equi* leads to down-regulation of CD1b.

#### Mean fluorescence intensity

Both foal and adult PBAC infected with *R. equi* showed similar but significant decreases in the mean fluorescence intensity when assessed by antibody BCD1b3 (CD1b) (**Fig 4c, 4d, Table 4**). This is expected, as there was a decrease in the total number of infected PBAC expressing CD1b which would affect the overall mean fluorescence from the cells.

#### Pulmonary macrophages (results not shown)

No significant changes in CD1 expression or other cell surface marker expression such as MHC II were observed when pulmonary macrophages obtained by bronchoalveolar lavage from adult horses (n=3) were infected with *R. equi*.

### **Characterization of CD1 positive cells from peripheral blood by two color fluorescence**

The monocyte population in PBMC was gated on according to their characteristic forward and side scatter pattern. Dual staining for MHC II (TH81) and CD172a (DH59) showed that 83% to 85% of the monocyte population expressed MHC II and were putative antigen presenting cells (**Figure 5, column 3**). Approximately 51% to 81% of the cells expressing MHC II were also positive for CD172a, indicating that a high proportion of the cell population were of monocytic origin. It is likely that not all the monocytes express the peptide antigen presenting molecule MHC class II, as an additional 9-11% of the cells stained positive for CD172a but were negative for MHC class II. Additionally, a small proportion of the cells gated on as the monocyte population may consist of large B and T lymphocytes which also express MHC II (73, 74).

Dual staining of the cells for CD1b (BCD1b3) and MHC II (TH81) revealed that 45% to 71% of the monocyte population stained positive for CD1b (assessed by BCD1b3) (**Figure 5, column 2**). Approximately 96% to 98% of the cells expressing CD1b were also positive for MHC II, indicating that the majority of cells expressing CD1b are putative antigen presenting cells. Staining of the cells to examine co-expression of CD172a and CD1b was not performed.

## CHAPTER FOUR

### DISCUSSION

There is growing evidence that CD1-mediated lipid antigen presentation contributes to the adaptive immune response against mycobacteria and possibly other related organisms. As *R. equi* is closely related to *M. tuberculosis*, and possesses similar cell wall lipids, observations regarding host immune responses to mycobacterial antigens may be also pertinent to immune responses against *R. equi*.

The majority of lipids discovered so far that can be presented by CD1 to T cells have been derived from *Mycobacterium tuberculosis*, *M. leprae* and related mycobacterial species. CD1b is the isoform most commonly involved in the presentation of mycobacterial lipids such as phosphatidylinositol mannoside (PIM), mycolic acid, lipoarabinomannan, sulfolipids, and glucose monomycolate, all of which are abundant in the cell wall of mycobacteria (40,70). CD1a and CD1c are also involved in antigen presentation of mycobacterial lipids such as didehydroxymycobactin to clonal populations of CD1a and CD1c- restricted T cells (30).

Our study demonstrated that CD1b and possibly CD1c molecules are constitutively expressed on unstimulated peripheral blood mononuclear cells from adult horses at higher levels than reported for other species. These CD1 isoforms were expressed on up to 34% of PBMC and up to 72% of monocytes within the PBMC population in our study. CD1b and possibly CD1c may also be present on low numbers of peripheral blood large B-lymphocytes that may have been included in the gated monocyte population, but there are currently no reliable equine B cell markers available to accurately assess this. Previous studies indicate CD1 is generally expressed at very low levels (less than 5-10 %) on unstimulated human monocytes, and that culturing in the

presence of GM-CSF is needed to induce CD1a, CD1b and CD1c expression at significant levels (51). Porcine monocytes also show minimal expression of CD1 (49). In contrast, CD1d is constitutively expressed on most circulating human monocytes and B lymphocytes prior to antigenic stimulation. Expression of the CD1d isoform was not investigated in this study as previous *M. tuberculosis* research indicates that the immune response to mycobacterial antigens involves primarily group 1 CD1 molecules (4,5).

Four anti-CD1 antibodies (BCD1b3, MT101, CD1F2.1B12.1 and CD1F2/5E3) were selected from a panel of CD1 antibodies and used for further testing with foal APC, as these had displayed greater than 5% binding to adult horse monocyte-derived macrophages in preliminary experiments. BCD1b3 is highly specific for human CD1b and is known to cross react with guinea pig (GP) CD1b isoforms, specifically GP CD1b2,-b3, -c3, -b4. MT101 is also specific for human CD1b, although cross reactivity in other species has not been assessed. CD1F2/1B12.1 is specific for GP CD1b and is cross reactive with human CD1b, whilst CD1F2/5E3 is able to recognize both GP CD1b and CD1c isoforms and is cross reactive with the human CD1b and CD1c (8).

There was a consistent proportion of equine PBAC staining positive with the CD1b antibodies (BCD1b3, MT101, CD1F2/1B12.1), indicating that CD1b is expressed on foal and adult APC. The antibody BCD1b3 (human CD1b) showed the highest and most consistent binding to equine cells. The other CD1b antibodies used in this study (MT101, CD1F2/5E3 and CD1F2/1B12.1) also bound to cells within the monocyte population, but to a lesser degree than the antibody BCD1b3, indicating that MT101 and CD1F2/5E3 were less specific for equine CD1b. These observations are similar to previous studies which demonstrate that there is a high degree of interspecies cross reactivity of CD1 antibodies among mammals such as sheep, cattle,

guinea pigs and humans (7). Our research provides evidence that horses can now be included in the list of species known to express CD1. At least one of the CD1 isoforms, CD1b, is highly expressed on equine APC and is recognized by human and guinea pig specific anti-CD1b antibodies. This finding suggests that guinea pig, human and equine CD1b are homologous. Supporting evidence for the homology between equine and human CD1b is derived from the partial sequencing and cloning of equine CD1a and CD1b, which shows 75% and 82% nucleotide homology respectively to their respective human sequences (75). The variability in binding among the CD1b antibodies used in this study likely reflects antigenic differences in CD1b structure among species. Studies investigating the total number of CD1 genes, and diversity and structure of the various CD1 isoforms present in horses are yet to be performed.

The anti-CD1a antibodies used in our study showed minimal cross reactivity with the PBMC, PBAC and BAL derived macrophages, suggesting that CD1a is not expressed or is expressed at very low levels on equine APC. Alternatively, the epitopes on equine CD1a molecules may be structurally different from those of human CD1a and are therefore not recognized by the human anti-CD1a antibodies used in this study.

It is possible that certain CD1 isoforms may be absent on equine antigen presenting cells, as considerable variation in number of CD1 genes and isoforms exists among mammalian species. Humans, for example, possess one copy of each of the 5 classes of CD1. Pigs also possess all 5 classes of CD1 genes which are highly homologous to human CD1 genes. Mice and rats express CD1d but lack the group 1 CD1 genes classes. Guinea pigs express multiple CD1b and CD1c genes but have lost their CD1a and CD1d genes (49, 51). Bovine express functional CD1a, CD1e, and multiple CD1b molecules but possess no CD1c gene, and lack intact CD1d genes (56). The immune response to various pathogens is likely unaffected by the variation in CD1



isoform expression observed among mammals because there is overlap of the lipids recognized by the various isoforms (69, 70).

Equine pulmonary macrophages appear to have no or minimal CD1 expression, based on the data from our study. In contrast, CD1 expression on uninfected PBAC from adult horses ranged from 20-82% when assessed by the same CD1 antibodies (BCD1b3, CD1F2/1B12.1, and MT101). In a study by Patton et al, 2005(2), CD1 expression in equine pulmonary macrophages was also found to be very low, with only 3-5% of cells staining positive with antibodies BCD1b3 (CD1b) and CD1F2/1b/12.1 (CD1b). Despite the apparently minimal CD1 expression, MHC class I- independent lysis of *R. equi* infected BAL macrophages by cytotoxic T lymphocytes (CTL) was observed, suggesting antigen presentation by non-classical MHC molecules. Some considerations for the lack of CD1 expression on these cells is that pulmonary macrophages require a high threshold of activation, initiated by interaction with T lymphocytes and exposure to cytokines such as IFN- $\gamma$ , in order to upregulate and express CD1. Pulmonary macrophages may also differ from monocyte-derived macrophages and express modified versions of the CD1b isoforms, or preferentially express certain CD1 isoforms not recognized by the anti-CD1 antibodies used in our experiments. In addition, our experiments did not assess mature dendritic cells, which are more efficient at antigen presentation, and may express CD1 at levels higher than on pulmonary macrophages.

In this study, we show that *R. equi* infection causes a reduction in CD1b expression on monocyte-derived macrophages that is of similar magnitude in both foals and adult horses. This may represent a strategy to subvert the immune response to *R. equi*, as a similar effect on CD1b expression has been observed in APC infected with the related organism *M. tuberculosis*. Human APC infected with *M. bovis* bacillus Calmette-Guérin (BCG) and then stimulated with GM-CSF

show decreased cell surface expression of CD1b compared with uninfected cells, indicating that BCG appears to inhibit the stimulating effects of GM-CSF on CD1 expression. Northern blot analysis revealed that the mechanism involved was likely either decreased CD1b transcription or accelerated degradation of transcription products (12). Active *M. tuberculosis* infection in humans is associated the increased production of immunosuppressive cytokines such as IL-10 and TGF- $\beta$  that can alter interaction of APC with T cells and decrease expression of MHC II, CD1, and co-stimulatory molecules on APC (71). These observations may all represent evasive strategies to suppress the host immune system and diminish or limit protective T cell immune responses against *M. tuberculosis* (48, 57, 71). Further studies analyzing the effects of *R. equi* infection on CD1 expression are needed to determine if similar mechanisms are involved.

There is increasing evidence that other organisms besides mycobacteria also possess mechanisms to avoid detection and surveillance by the CD1 system. Human monocyte-derived dendritic cells infected with *Leishmania donovani* or *L. major* exhibit decreased expression of both group 1 and 2 CD1 molecules (63). Certain viral organisms such as vesicular stomatitis virus, human immunodeficiency virus (HIV), herpes simplex virus-1 (HSV-1), and Kaposi sarcoma associated- herpes virus have all been shown to impair CD1d expression, which in turn affects natural killer T cell (NKT) activation. NKT cell responses to virally infected cells include cytolysis, modulation of the innate immune response via activation of dendritic cells and NK cells, and modulation of the adaptive immune response by rapid cytokine release (67, 69). Human cytomegalovirus possesses a gene, CMVIL10 that is responsible for downregulation of CD1a expression on infected dendritic cells (68).

In adult horses, clearance of *R. equi* is associated with proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> bronchoalveolar lavage fluid lymphocytes and induction of a type 1 immune response (2, 3, 16,

17, 68). There is also evidence suggesting that innate immune responses involving Toll-like receptor-2 (TLR-2) signaling and resultant expression of co-stimulatory molecules and production of TNF, NO and IL-12 by *R. equi* infected macrophages contribute to the resistance of adult horses to challenge with virulent *R. equi* (71). Some neonatal foals appear unable or less able to mount these protective immune responses against *R. equi* and succumb to life threatening infection. The mechanisms responsible for this are complex and poorly understood, and are currently being investigated. Based on previous studies, the susceptibility of neonates to pathogens has been attributed to diminished CD4<sup>+</sup> T helper cell responses, suboptimal production of IFN- $\gamma$ , and an inherent bias towards a T<sub>h</sub>2 immune response. It is assumed that young foals are similarly immunodeficient, and that failure to mount an effective cell mediated immune response is responsible for the vulnerability to rhodococcal pneumonia. Breathnach et al (65) reported that blood and pulmonary T lymphocytes of newborn foals have decreased ability to express the IFN- $\gamma$  gene and produce substantial levels of IFN- $\gamma$ , and as a result, are likely biased towards developing T<sub>h</sub>2 responses upon exposure to pathogens. The low IFN- $\gamma$  level was most pronounced in foals less than 3 months of age, following which an age-dependant increase in IFN- $\gamma$  production towards adult levels occurred up to 6 months of age.

Recent research, however, disputes these findings. In a study by Jacks et al (61), *R. equi*-stimulated bronchial lymph node lymphocytes from 7-10 day old foals showed significant IFN- $\gamma$  expression and lymphoproliferative recall responses similar to adult horses, suggesting that foals are immunocompetent and capable of mounting T<sub>h</sub>1 responses to virulence associated proteins (Vap) of *R. equi* that are of similar magnitude and cytokine phenotype as those of adult horses (61). Additionally, despite the endemic nature of *R. equi* in the equine environment, only a small percentage of foals on farms develop subclinical disease with eventual clearance of the infection,

or rhodococcal pneumonia, suggesting that most foals are capable of mounting effective cell-mediated immune responses against *R. equi*. Previous research also indicates that neonatal T cells are capable of producing IFN- $\gamma$  when stimulated with strong T<sub>h</sub>1 polarizing antigens or adjuvants (65). Collectively, these studies indicate that although neonates of different species have a naïve immune system, they are capable of mounting T<sub>h</sub>1 responses to antigen under appropriate conditions of stimulation. Other factors such as an immunocompromised state in individual foals, decreased CD8<sup>+</sup> CTL activity in neonatal foals (2), impaired maturation and activation of APC, decreased production of cytokines or expression of TLRs by APC (62, 71) or decreased expression of important peptide or lipid presenting molecules on APC such as MHC or CD1 (as observed in this study), may therefore play a role in the age-dependant susceptibility to *R. equi* infection.

A higher proportion of adult APC expressed CD1b compared with those of the perinatal foals when assessed by three anti-CD1b antibodies in this experiment, suggesting that an age-related limitation in CD1b expression does exist. If in fact the CD1 system plays a role in protective immunity against *R. equi*, as is observed with the related organism *M. tuberculosis*, then the observed lower CD1b expression in foals may contribute to their unique susceptibility to rhodococcal pneumonia, possibly via decreased antigen presentation and activation of CD-1 restricted T cells. This is supported by the finding that lysis of *R. equi* infected APC by CD8<sup>+</sup> CTL is MHC-unrestricted, suggesting a non-classical method of antigen presentation such as by CD1, and by the observation that CTL activity is deficient in perinatal foals less than 3 weeks of age (2,3). This is the same age range as the foals used in our experiment, and coincides with the time period that foals are first exposed to and infected with *R. equi*. Given that CD8<sup>+</sup> T cells play an important role in immune clearance of and protective immunity against *R. equi*, impaired

antigen presentation due to decreased CD1b expression by foal APC may be partly responsible for the decreased CTL activity in perinatal foals and therefore vulnerability to *R. equi* infection.

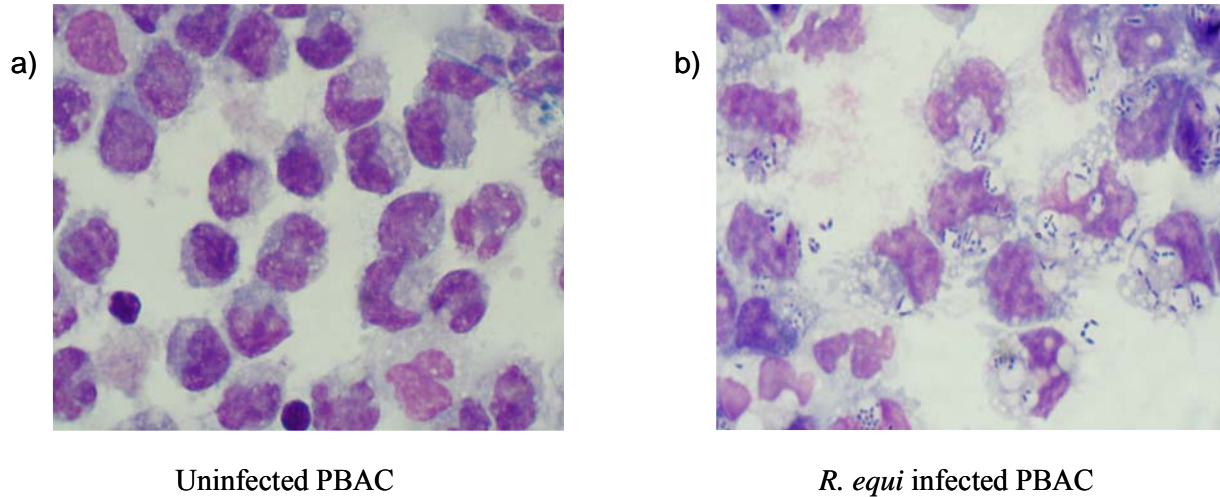
Our research also indicates that MHC II expression is lower in foal PBAC compared with those of adults. Adult PBAC expressed significantly more MHC molecules (1.8 times), and had almost three times more mean fluorescence of MHC II molecules compared with foal cells. The latter was not statistically significant, but is still noteworthy, as the p value did approach significance (p=0.059). Flamino et al (62) also demonstrated that there was significantly less median fluorescence of MHC II molecules on unstimulated macrophages and dendritic cells from foals less than 3 months of age compared with adult cells (62). These findings are similar to the observations in our study and suggest that foals have limited expression of MHC II, which may play a role in foal susceptibility to infections. Many other studies also provide supporting evidence that MHC II expression is decreased on neonatal APC, but it is unclear as to how this decreased expression correlates with APC functions such as antigen processing and presentation. It is assumed however, that decreased MHC II expression affects antigen processing and presentation and in turn alters the priming of T cells and initiation of CD4 T helper type I responses.

The data shows that *R. equi* infection does not alter MHC II expression on infected APC. In contrast, APC from the lungs of humans with tuberculosis express lower levels of MHC II and the co-stimulatory molecule CD86 that may limit cell mediated immune responses and favor persistence and progression the disease (71). The lack of change in MHC II expression on *R. equi* infected cells is surprising, given the many similarities between these two organisms.

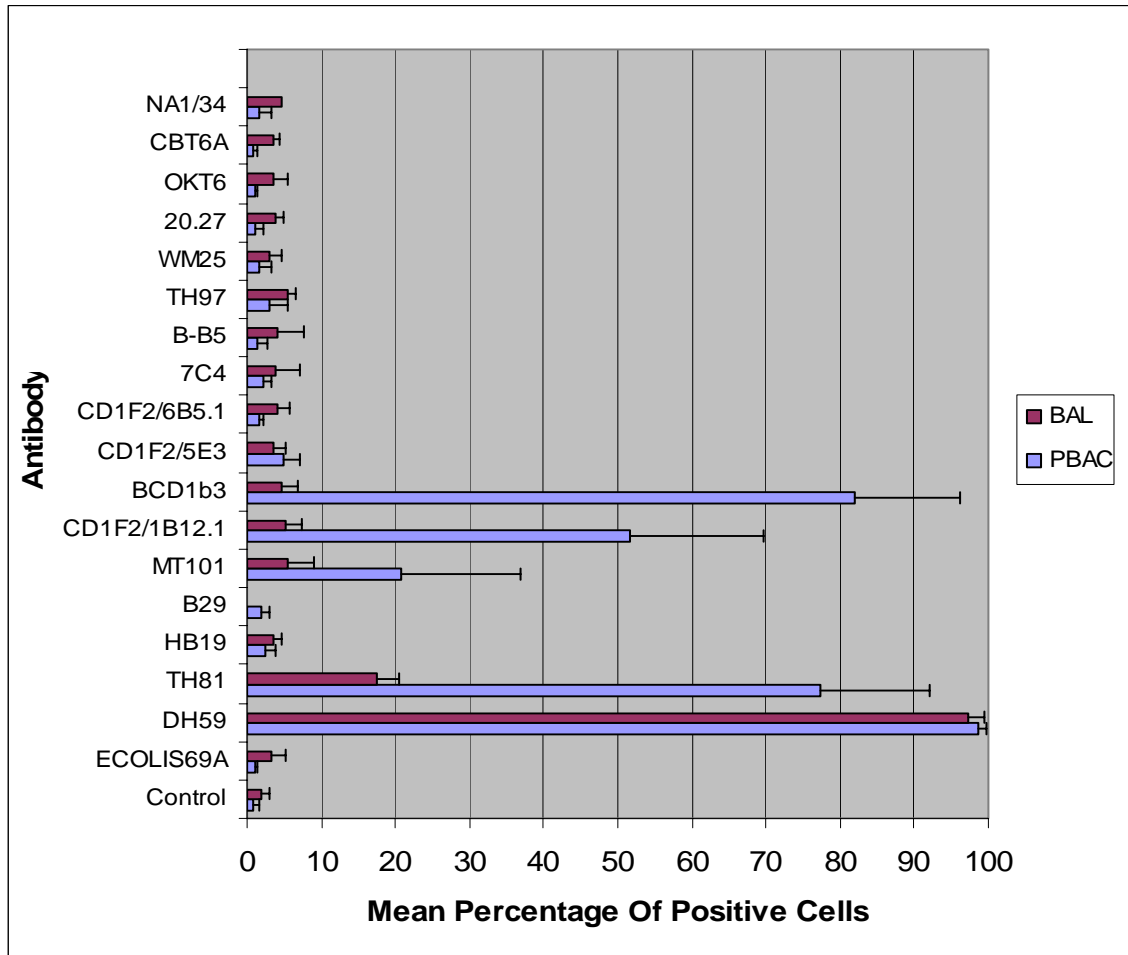
In conclusion, this study demonstrates that CD1b is expressed on equine APC, and is likely similar in structure to human and guinea pig CD1b molecules. Given the similarities in structure

and host response to infection by *R. equi* and *M. tuberculosis*, the growing evidence that lipid-specific T cell responses contribute to immune responses against microbes, and our previous findings regarding *R. equi*-specific MHC-unrestricted CTL in foals and adult horses, the observation that CD1 is expressed on equine cells suggests that CD1-mediated lipid antigen presentation plays a role in supplementing the classical MHC-restricted CD4+ and CD8+ T cell responses that are critical for protective immunity and clearance of *R. equi*. This is further supported by the observation that *R. equi* infected APC expressed lower levels of CD1b compared with uninfected controls, suggesting that there is downregulation of this lipid-antigen presenting in an attempt to avoid surveillance and detection by the CD1 system. This research also revealed that perinatal foal APC have lower cell surface expression of CD1b, which may result in sub-optimal lipid antigen presentation and T cell activation and contribute to the vulnerability of neonatal foals to *R. equi* infection. There are several issues to be investigated in the future. These include confirming the presence of CD1b on equine APC using molecular techniques and equine monoclonal anti-CD1 antibodies, determining whether other CD1 isoforms besides CD1b are expressed on equine cells, determining if lipid antigens derived from *R. equi* are immunogenic and are able to activate CD1-restricted T cells, and if so, the degree to which CD-1 restricted responses contribute to the protective immune response in adult horses. The discovery of the CD1 antigen presenting system in horses has implications for the development of effective vaccination strategies against *R. equi*, as inclusion of lipids and glycolipids in vaccines may augment T cell responses and lead to improved protective immune responses against rhodococcal pneumonia.

## FIGURES



**Figure 1:** Cytologic appearance of *R. equi*-infected PBAC. Slide preparation of *R. equi*-infected adult PBAC (2.5 MOI, 8 hours post-infection, Wright's Giemsa stain) and an uninfected control, examined under X100 magnification. The majority of cells in (b) contain *R. equi* organisms.

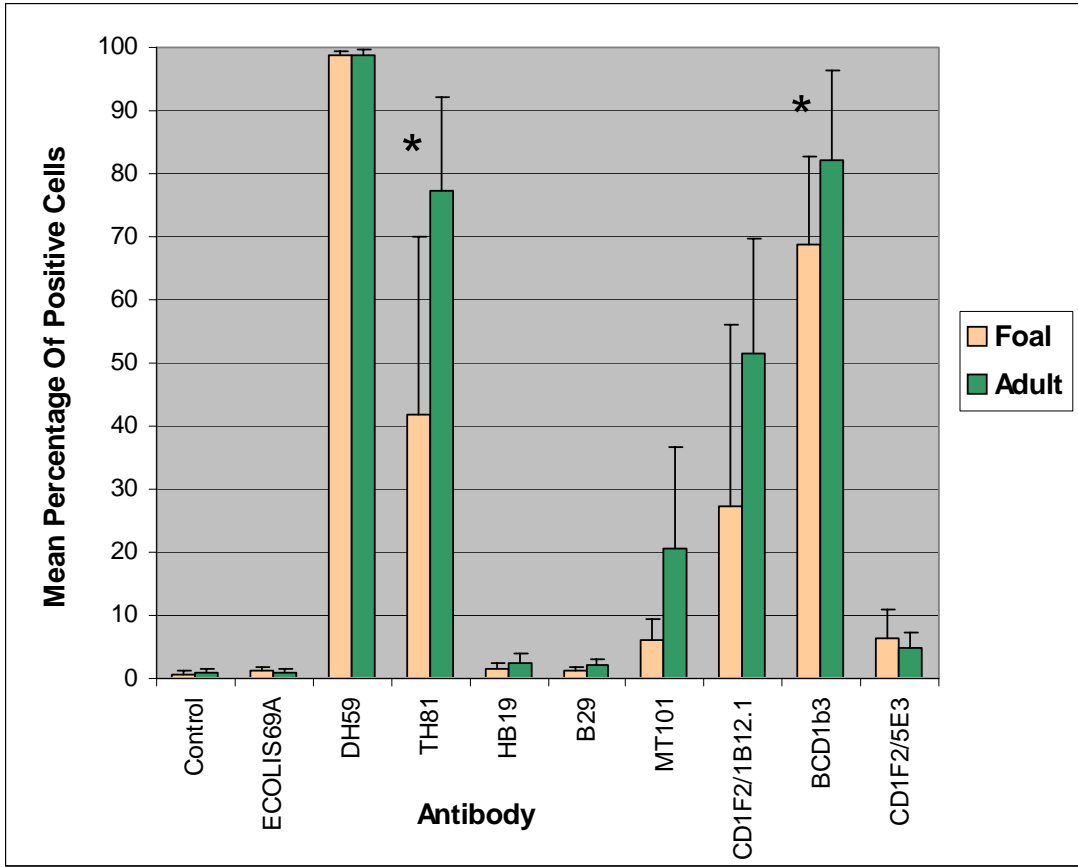


**Figure 2: Cross reactivity of anti-CD1 antibodies with equine cells.**

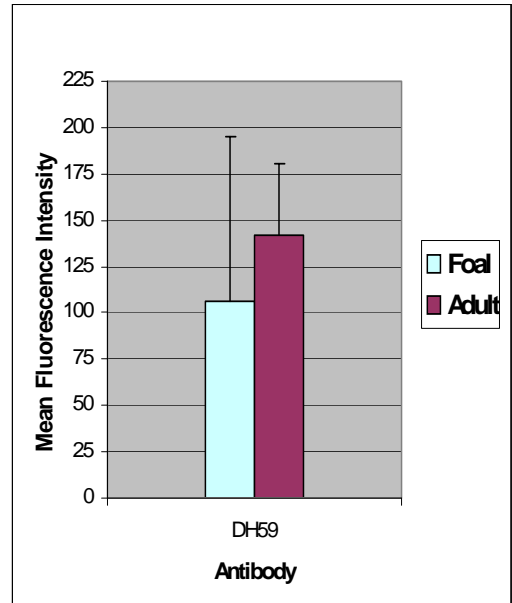
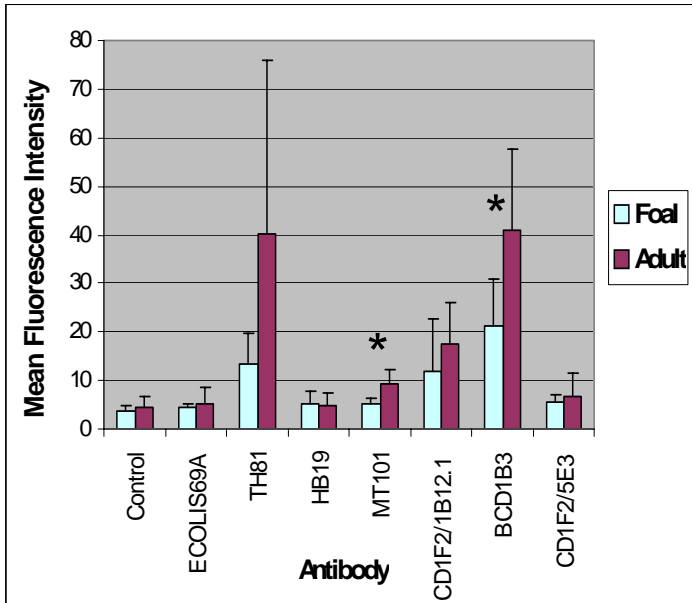
The data represent the mean percentage of cells positive for each indicated cell surface markers. In addition to anti-CD1 antibodies, antibodies to CD172a, MHC class II, CD5-T cell marker, and B cells (DH59, TH81, HB19, and B29 respectively) were used to assess the types of nucleated cells present in the PBAC and BAL cell preparations. Error bars represent the standard deviation (n = 8 adult horses for PBAC; n=3 adult horses for BAL macrophages). ECOLIS69A was used as a monoclonal antibody control (isotype = IgG1). “Control” represents equine cells incubated with secondary antibody alone.

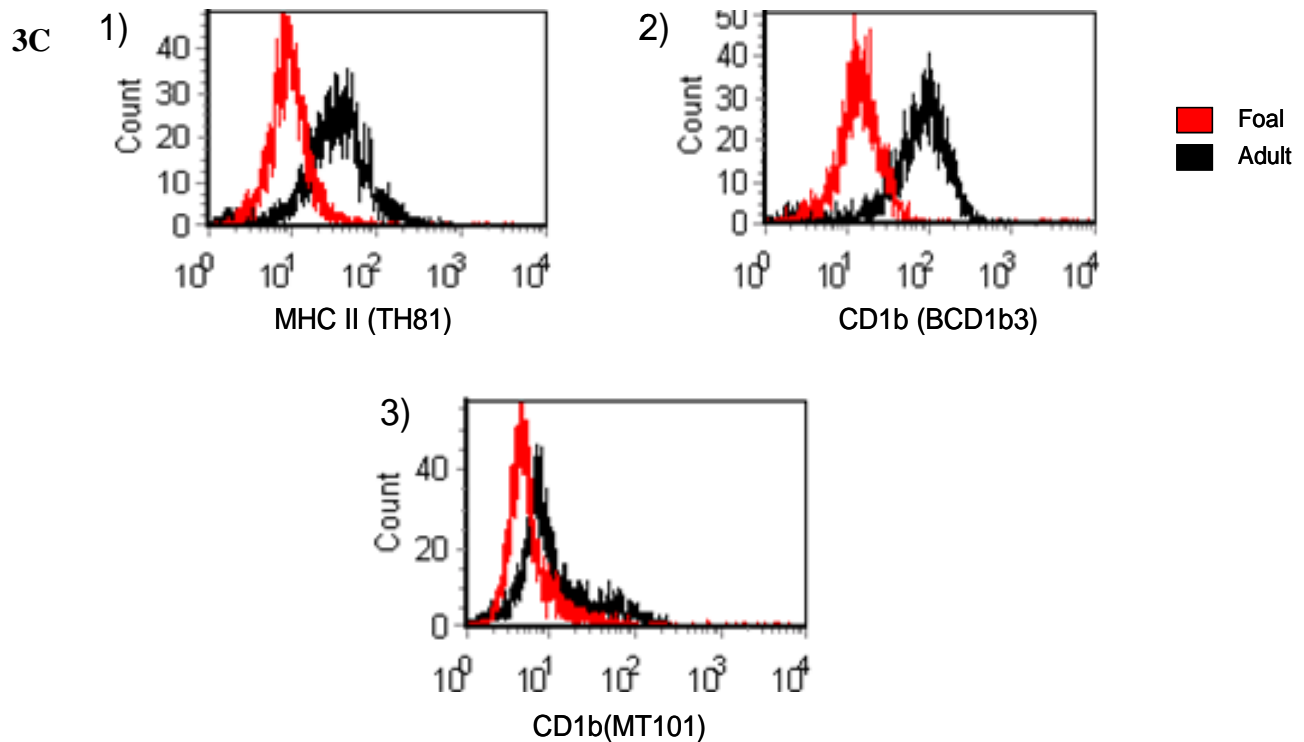


3A



3B

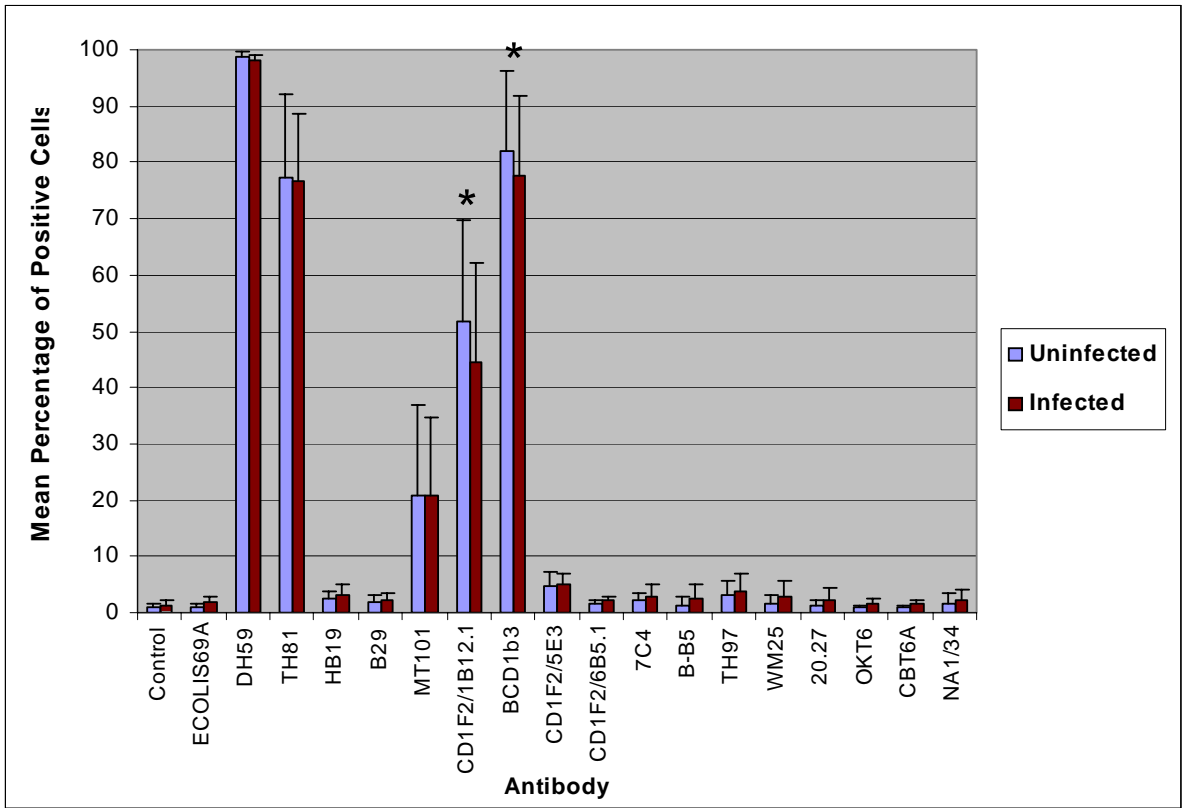




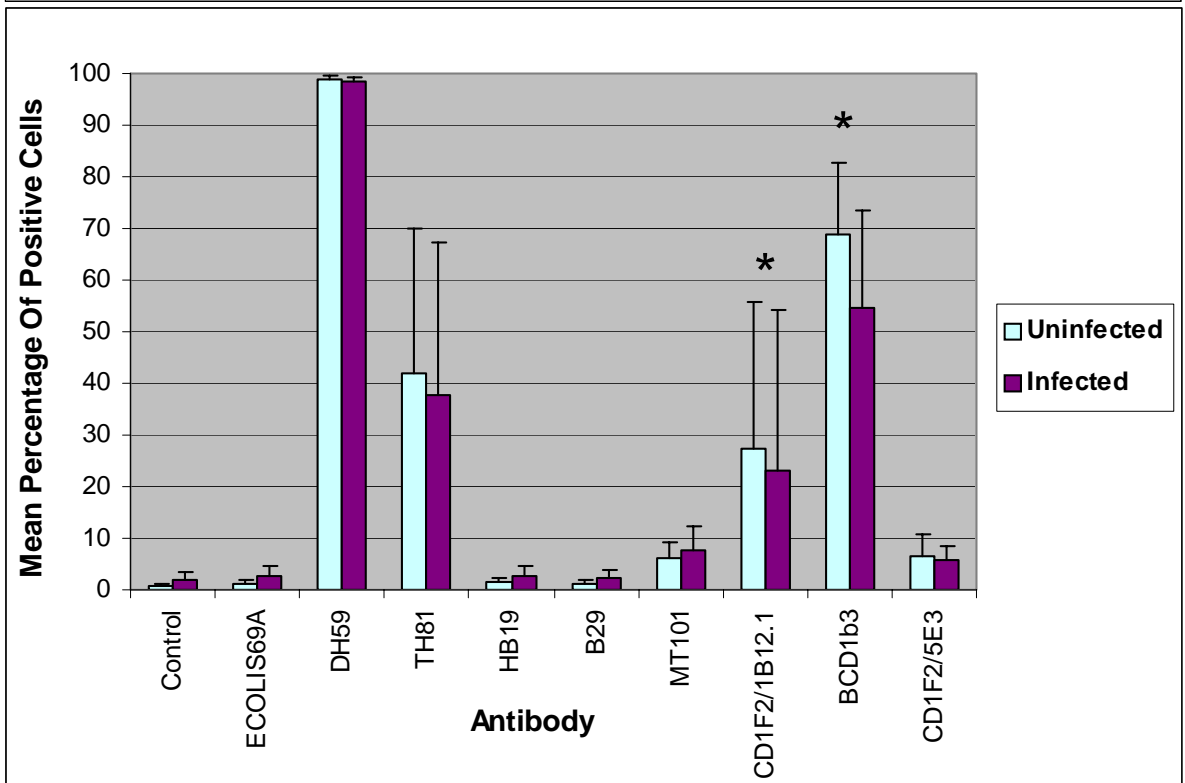
**Figure 3: Expression of CD1 and other cell surface markers on equine PBAC.**

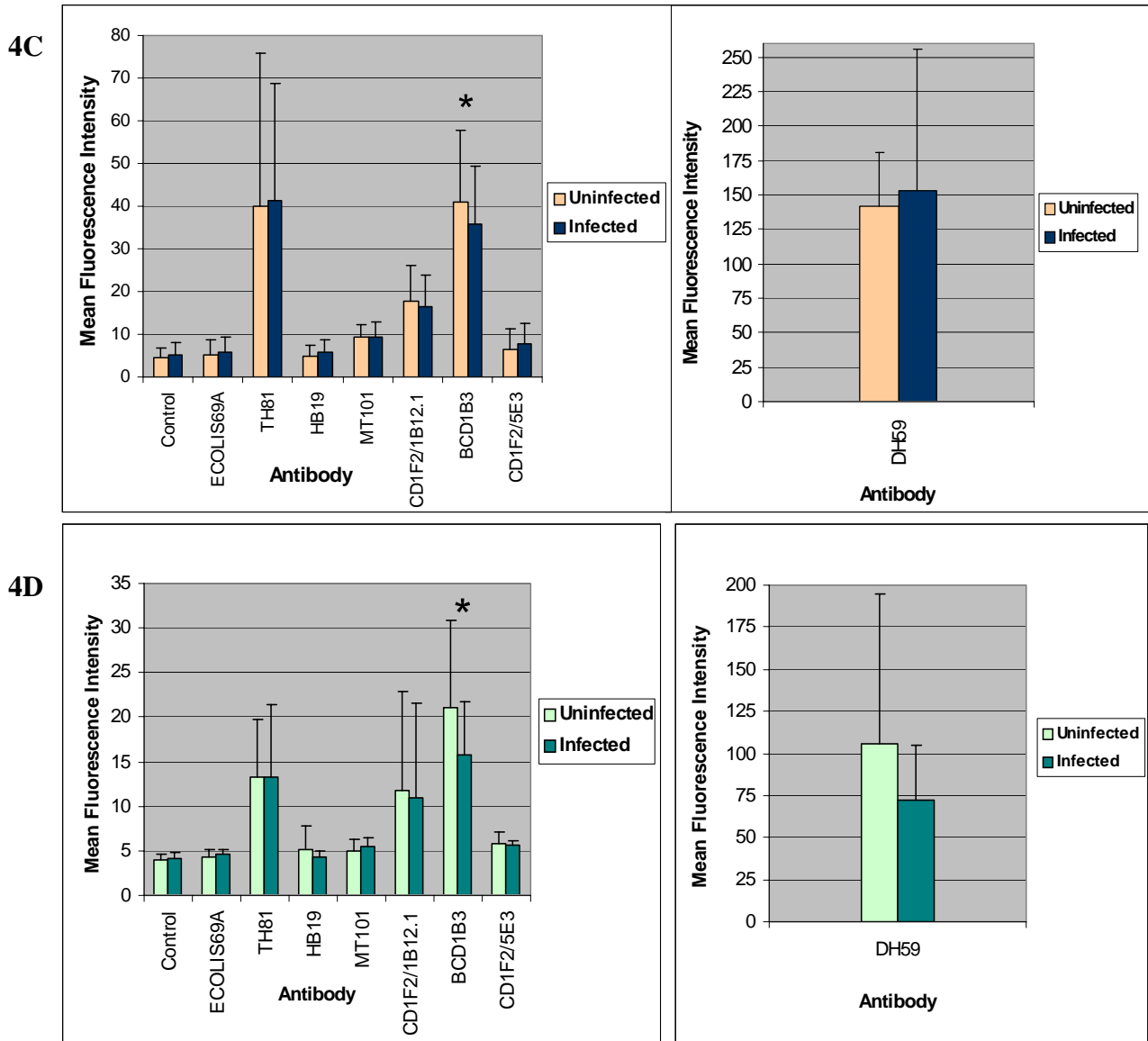
Uninfected PBAC from foals and adult horses were assessed for various cell surface molecules. ECOLIS69A was used as a monoclonal antibody control (isotype = IgG1). “Control” represents equine cells incubated with secondary antibody alone. Error bars represent the standard deviation (n = 6 foals and 8 adult horses). An asterisk indicates a statistically significant difference (p < 0.05) between foals and adult horses. **A)** Mean percentage of cells positive for each indicated cell surface marker **B)** Mean fluorescence intensity, assessed by measuring the geometric mean fluorescence intensity (histogram data) from PBAC from all 8 adult horses and 6 foals **C)** Example of mean fluorescence intensity on equine PBAC – Histogram of foal A2226 is compared to adult horse 241. Adult PBAC have a higher mean fluorescence of MHC II and CD1b molecules

4A



4B

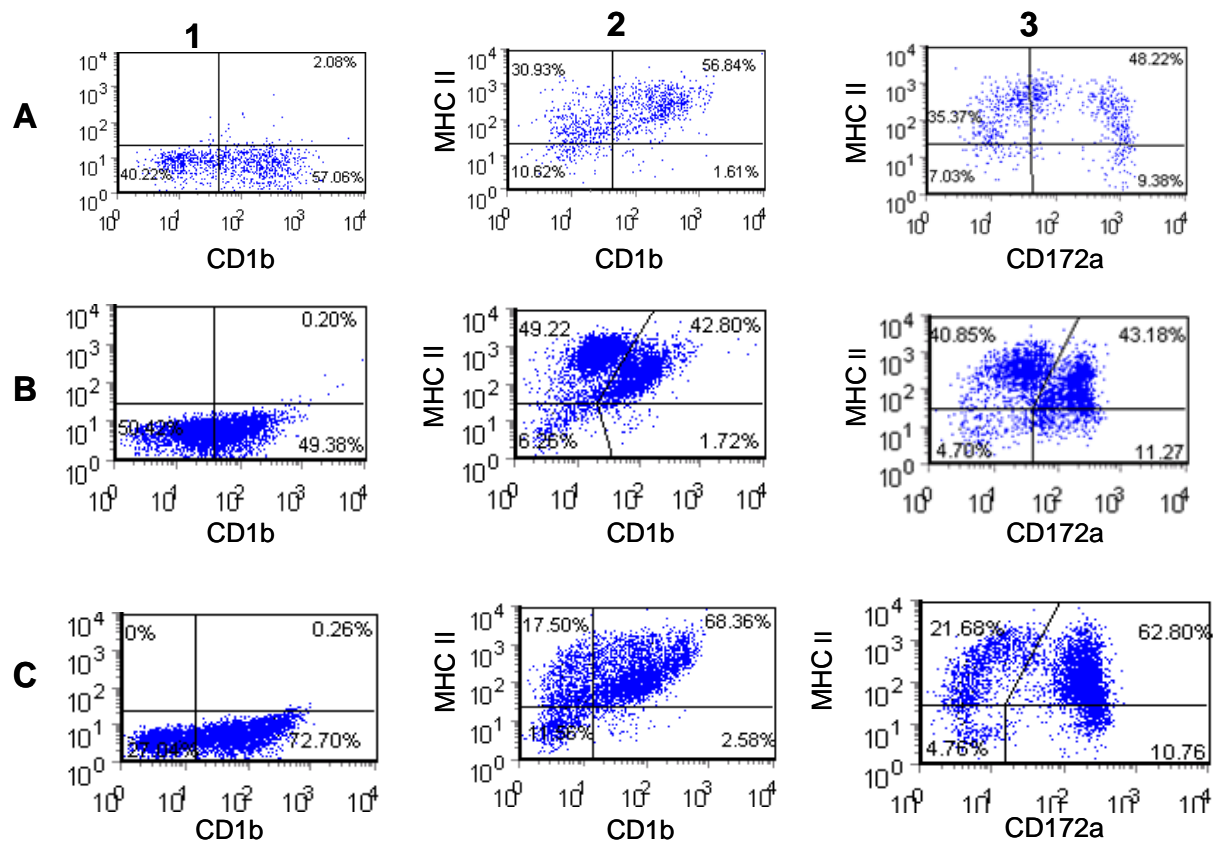




**Figure 4: Modulation of CD1 expression on equine APC following *R. equi* infection.** Error bars represent the standard deviation (n = 8 adult horses, n = 6 foals). ECOLIS69A was used as a monoclonal antibody control (isotype = IgG1). The indicated “control” represents equine cells incubated with secondary antibody alone. An asterisk indicates a statistically significant difference (p < 0.05) between uninfected and *R. equi* infected cells.

**A and B:** Adult horses and foals, respectively. Mean percentage of cells positive for each indicated cell surface marker.

**C and D:** Adult horses and foals, respectively. Mean fluorescence intensity, assessed by measuring the geometric mean fluorescence intensity (histogram data) from uninfected and *R. equi* -infected PBAC.



**Figure 5: Example of two color flow cytometry on PBMC from 3 adult horses.**

The monocyte population was gated according to characteristic forward and side scatter parameters, and assessed for various cell surface markers including CD1, MHC II (TH81) and CD172a (DH59). **A-** Horse 1; **B-** Horse 2; **C-**Horse 3.

**Column 1** show dot plots of monocytes assessed for only CD1b expression using the anti-CD1 antibody, BCD1b3. The percentage in the lower right quadrant represents the percentage of cells positive for CD1b.

**Column 2** shows dual staining of monocytes for CD1b (BCD1b3) and MHC II (TH81) expression. The percentage in the upper right quadrant represents the percentage of cells positive for both CD1b and MHC II.

**Column 3** shows dual staining of monocytes for MHC II (TH81) and CD172a (DH59). The percentage in the upper right quadrant represents the percentage of cells positive for both MHC II and CD172a.

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## APPENDIX

### List of commonly used abbreviations

<b>APC</b>	<b>Antigen Presenting Cells</b>
<b>BAL</b>	<b>Bronchoalveolar Lavage</b>
<b>CTL</b>	<b>Cytotoxic T Lymphocytes</b>
<b>FBW</b>	<b>First Wash Buffer</b>
<b>MHC</b>	<b>Major Histocompatibility Complex</b>
<b>PBMC</b>	<b>Peripheral Blood Mononuclear Cells</b>
<b>PBAC</b>	<b>Peripheral Blood Adherent Cells</b>