CHARACTERIZATION OF EQUINE ALVEOLAR MACROPHAGE PHENOTYPES IN RECURRENT AIRWAY OBSTRUCTION

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ABSTRACT

CHARACTERIZATION OF EQUINE ALVEOLAR MACROPHAGE PHENOTYPES IN RECURRENT AIRWAY OBSTRUCTION

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A central feature of recurrent airway obstruction (RAO) is an enhanced sensitivity to hay dust (HD) compared to control horses, but the cellular and molecular mechanisms accounting for this differential airway sensitivity are unknown. The microbial components of HD can stimulate pathogen recognition receptors (PRRs), which are abundant on alveolar macrophages (AM). Thus inhaled HD would be expected to evoke an inflammatory response from AM, however, work in other species indicates that the nature of the response depends on the macrophage phenotype. Two major polarized phenotypes, classified by their distinct gene expression and functions, have been described. The classically-activated M1 exerts a robust pro-inflammatory response and the alternatively-activated M2 produces anti-inflammatory cytokines, and little in the way of pro-inflammatory cytokines. Thus, our overarching hypothesis was that the HD hypersensitivity observed in RAO may be mediated by the presence of pro-inflammatory (M1) or the absence of immune-regulatory (M2) phenotypes.

There are limited data characterizing equine M1 and M2 phenotypic markers, thus to address this, equine AM were cultured in IFNγ+LPS or IL-4 generating equine M1 and M2 phenotypes respectively, and the gene expression of predicted surrogate M1/M2 genes and the effect of polarization on the response to pro-inflammatory agonists was evaluated. Equine M1s were characterized by increased expression of pro-inflammatory genes (TNFα,

IL1β, IL-12p40, IL-8, CD80), regulatory IL-10 and a potentiated pro-inflammatory gene expression response when stimulated. Equine M2s were characterized by elevated scavenger receptor CD206, low expression of M1 associated genes and potently suppressed pro-inflammatory cytokines and IL-10 when stimulated. Further, this study determined that canonical murine macrophage markers iNOS and arginase were not M1 or M2 associated in the horse.

Next the expression of equine M1 and M2 transcriptional markers and the cytokine response to HD components was assessed in AM from RAO-susceptible and control horses prior to (at baseline) and following exposure to hay. These results determined that different AM phenotypes exist in RAO-susceptible and control horses at baseline: RAO-susceptible horses had greater gene expression of IL-10 and enhanced responsiveness to LPS stimulation suggesting an M2-like, immune regulatory phenotype that maintains LPS responsiveness. Further, exposure to hay induced different phenotypes in both groups: AM from control horses expressed elevated IL-1β, IL-8, IL-10, CD206, and developed enhanced responsiveness to LPS indicating a mixed M1/M2 phenotype. In RAO-susceptible horses, hay exposure only increased the expression of CD206, an M2 marker.

In summary, these data provide the first characterization of the transcriptional signature of equine AM M1 and M2 phenotypes that will assist in studying their role in equine pulmonary disease. Furthermore, these data demonstrate that divergent AM exist in RAO-susceptible and control horses, indicating that the AM plays a role in RAO-immunopathology.

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KEY TO ABBREVIATIONS

ARG 1/2	arginase isoform 1/2
AMcase	acidic mammalian chitinase
αVβ6	integrin (alpha v beta 6)
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
βGR	β-glucan receptor
BGUS	β-glucuronidase
BL	baseline
B2M	β-2 microglobulin
CCL2	chemokine (c-c) motif ligand 2
c/EBP	CCAAT/enhancer-binding protein
CD-	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
Cdyn	dynamic compliance
CHIT-1	chitotriosidase
CHI3L3	chitinase 3-like 3
CHI3L4	chitinase 3-like 4
CLEC7A	C-type lectin domain family 7, member A
CT	cross threshold point
CTLD	C-type lectin-like carbohydrate recognition domain
CR	cysteine rich domain

CXCL2	chemokine (c-x) motif ligand 2
ΔCT	delta cross threshold
ΔPplmax	delta trans-pulmonary pressure (maximum)
EDTA	ethylenediaminetetraacetic acid
ELF1α	elongation factor 1α
FIZZ1	found in inflammatory zone protein 1
FN	fibronectin domain
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HD	hay dust
HPRT	hypoxanthine phosphoribosyltransferase
IFNγ	interferon-gamma
IgE	immunoglobulin type E
IL-	interleukin
IL-4Rα	interleuking-4 receptor alpha
iNOS	inducible nitric oxide synthase
IRF-	interferon regulatory factor -
ISRE	interferon sequence response elements
JAK	janus kinase
LPS	lipopolysaccharide
MACS	magnetic-activated cell sorting
MMP-	matrix metalloproteinase
MIP2	macrophage inflammatory protein 2
M1	classically activated macrophage

M2	alternatively activated macrophage
MyD88	myeloid differentiation primary response gene (88)
NFκB	nuclear factor-κB
PAMP	pathogen associated molecular pattern
Pep	peptidoglycan
PI3K	phosphoinositide-binding protein
PPRγ	peroxisome proliferator activated receptor gamma
PRR	pathogen recognition receptor
qRT-PCR	quantitative real time polymerase chain reaction
RAO	Recurrent airway obstruction
RELMα/β	resistin like molecule alpha/beta
RIN	RNA integrity number
RL	pulmonary resistance
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDHA	succinate dehydrogenase complex
SOCS-	suppressor of cytokine signaling-
STAT	signal transducer and activator of transcription
Th-	T- helper lymphocyte
TLR	toll-like receptor
TNFα	tumor necrosis factor- alpha

TGFβ	transforming growth factor beta
UCSC	University of California Santa Cruz
Zym	zymosan

Chapter 1.

Literature Review

Overview

A fundamental characteristic of equine recurrent airway obstruction (RAO) is an exaggerated inflammatory response to hay dust (HD). However, the exact mechanisms that elicit this uncontrolled inflammatory response remain uncertain. Hay dust contains a medley of microbial components containing conserved pathogen associated molecular patterns (PAMPs) that can stimulate innate immune cells through pathogen recognition receptors (PRRs). Alveolar macrophages are equipped with abundant PRRs and inhaled HD would be expected to evoke an inflammatory response. However, it is clear from work in other species that the nature of the response depends on the macrophage phenotype, which is itself governed by the microenvironment in which the macrophage resides. Two main phenotypes, classified by their distinct gene expression and functions have been described. The M1 phenotype is particularly sensitive to PRR stimulation and exerts a robust pro-inflammatory response. In contrast the M2 phenotype produces anti-inflammatory cytokines, little in the way of pro-inflammatory cytokines, and can function in tissue repair.

An imbalance of macrophage phenotypes can be associated with chronic inflammatory disease, however, it is unknown if an imbalance of macrophage phenotypes contributes to the enhanced sensitivity to HD that is observed in RAO. This lead me to generate the *overarching hypothesis* that the enhanced sensitivity to HD observed in RAO-susceptible horses could be mediated by the presence of pro-inflammatory (M1) phenotypes or the absence of anti-inflammatory (M2) phenotypes. To address this overarching hypothesis I first characterized the gene expression patterns of in-vitro induced M1 and M2 equine alveolar macrophage phenotypes. Then, to investigate the role of M1/M2 phenotypes in RAO immune-pathology I next evaluated the M1/M2 expression patterns in alveolar macrophages from RAO-susceptible horses during disease remission and after HD exposure (disease exacerbation), and compared the alveolar macrophage gene expression patterns with that in control horses.

Section 1. Recurrent Airway Obstruction

The following section provides background detailing the inflammatory response that occurs during RAO exacerbation and discusses the proposed mechanisms that mediate this hypersensitivity response. Finally the role of the alveolar macrophage in RAO is discussed.

The Impact of RAO

Recurrent airway obstruction is a prevalent, chronic, inflammatory lung disease that affects adult horses and is characterized as a hypersensitivity to inhaled HD. Following inhalation of HD, RAO-susceptible horses develop clinical disease (airway neutrophilic inflammation and bronchoconstriction) which culminates in varying degrees of respiratory distress and impaired performance.[1] These clinical signs resolve when the stimulus (HD) is removed and susceptible horses return to a subclinical (remission) state.

The prevalence of RAO may vary by region,[2] but in Great Britain it has been estimated to affect a substantial 14% of the general horse population.[3] There is no cure for RAO and susceptible horses require life long management, which fundamentally requires permanently eliminating exposure to hay.[4,5] However, indoor housing and feeding hay are common equine husbandry practices and owner compliance to implement and maintain environmental modifications is poor[6] resulting in recurrent episodes of clinical disease.[7] Although the monetary cost of the disease to the equine industry is not known, RAO presents a significant welfare issue as affected horses suffer reduced quality of life due to recurrent bouts of respiratory distress and chronic coughing. Further, the resulting impaired athletic performance results in early athletic retirement putting horses at increased risk for surrender or premature euthanasia.[2] *Critically, despite the prevalence and* *impact of RAO there remains a gap in our understanding of the immunologic mechanisms that underlie this disease and if novel therapeutic targets are to be sought we must first understand the basic disease mechanisms.*

The Inflammatory Response of RAO

Inhalation of HD triggers an influx in non-septic neutrophils and increased mucus secretion into the airways. This inflammatory response is accompanied by bronchoconstriction and a non-specific airway hyperresponsiveness. Consequently, affected horses have increased coughing and visibly labored breathing pattern (expiratory dyspnea). Pulmonary inflammation and dysfunction gradually resolve with avoidance of HD and during remission bronchoalveolar lavage (BAL) cytology and pulmonary function may be indistinguishable from normal horses.

Challenge with HD in RAO induces neutrophil movement into the airways within 4 hours.[8] These neutrophils are primed[9] and produce oxidative products[10] which can promote tissue damage and oxidative stress.[11] Further, neutrophils can produce pro-inflammatory cytokines TNFα, IL-1β and potent neutrophilic chemokines IL-8 and CXCL2 (MIP-2), that may further amplify inflammation.[12]

A number of chemokines (IL-8, IL-17 and CXCL2) are elevated during chronic RAO.[13–16] Increased expression of IL-8 and CXCL2 are detected in BAL cells and respiratory epithelium.[13–15,17,18] The cellular source of IL-17 is unclear and it may originate from Th17 lymphocytes cells or neutrophils.[13] Importantly, elevations of IL-8 and IL-17 ensue after the initial neutrophil influx into the airway[13,18] and the exact mediators/cytokines that stimulate the early recruitment of neutrophils following HD exposure remain unclear.

However, bronchial epithelial cell cultures from RAO-susceptible horses display enhanced up-regulation of CXCL2 expression following in-vitro HD stimulation, which may account for the early influx of neutrophils, however, this has yet to be verified in-vivo.[19] Exposure to hay (RAO-exacerbation) induces increased expression of IL-1β and TNFα [13,15,20] IL-10[18] and TGFβ[16] but not IL-6[15,16,20] in BAL cells. The exact source of these cytokines is not well established, as BAL is comprised of a mixture of macrophages, lymphocytes and neutrophils. A number of other inflammatory mediators are elevated during RAO-exacerbation including matrix metalloproteinase-9 (MMP-),[21] leukotriene-B4,[22] and arachidonic acid metabolites 15-hydroxyeicosatetraenoic[23] and thromboxane.[24] Importantly, the role of interferon-gamma (IFNγ) or IL-4 and IL-13, (Th1 and Th2 cytokines respectively), in the pathogenesis of RAO is unclear as there are mixed findings among studies (discussed below).

The Role of an Antigen-Specific Response in RAO

Recurrent airway obstruction-susceptible horses are hypersensitive to HD compared to unaffected horses.[25] Over the last 30 years, RAO research has focused on identifying specific causal agents to which RAO-susceptible horses are hypersensitive and identifying mechanisms that mediate this hypersensitivity. The association of disease exacerbation and moldy hay[26,27] are suggestive that RAO could be a hypersensitivity to specific fungal or actinomycete allergens, as these pathogens are abundant in poorly cured (moldy) hay. Thus, it has been proposed that, akin to atopic asthma, a type 1 hypersensitivity response to specific fungal elements underlies the RAO immunopathology, i.e *RAO is an allergen specific response*. However, certain features of the RAO response differ from the typical atopic response.

Allergen provocation in human asthmatics induces a biphasic response; an acute phase/immediate response and a late phase/delayed response, which are precipitated by different immune mechanisms. Atopic asthma is characterized by elevated levels of allergen-specific IgE antibodies that bind to high affinity receptors on mast cells and basophils within the airway. Inhalation of allergen crosslinks IgE and activates degranulation of mast cells and basophils releasing a variety of inflammatory mediators including histamine, leukotrienes, prostaglandins, platelet activating factor and cytokines (e.g. IL-4). These inflammatory mediators contribute to the immediate allergic inflammatory response characterized by bronchoconstriction, vasodilation and airway edema, a response that occurs within minutes of allergen exposure.

Allergen also activates allergen-specific CD4-Th2 cells that are critical in orchestrating the delayed allergic response that develops within hours after exposure. Allergen activated CD4-Th2 cells proliferate and express effector cytokines IL-5, IL-13 and IL-4 that contribute to pathology; IL-5 stimulates airway eosinophilia, IL-13 promotes mucus hypersecretion and airway hyperresponsiveness and IL-4 promotes IgE isotype class switching and promotes further Th2 cell differentiation.

The diagnosis of allergic asthma in humans is principally dependent on detection of systemically elevated allergen-specific IgE (i.e elevated sera antibody) or a positive intradermal skin test indicative of allergen specific sensitized mast cells. A number of studies have failed to detect elevated allergen-specific IgE (serum) in RAO-affected horses[28–31] and immediate skin reactions (wheal formation) during intradermal skin testing is not associated with the RAO-phenotype.[30,32] Taken together, this suggests that systemic production of IgE and mast cell sensitization does not occur in RAO. In contrast, two early studies demonstrated elevated allergen-specific IgE (to *Micropolyspora faeni* and *Aspergillus fumigatus*) in BALF[28,29] which could suggest that local production of IgE could contribute to RAO. However, these studies utilized in-vitro allergen assays with low specificity for equine allergen-specific IgE and specific-allergen epitopes, which may have resulted in an increased rate of false positives.[33] The performance of in-vitro allergen assays has been improved by using pure allergen extracts and monoclonal antibodies[34] but the measurement of allergen-specific IgE in BALF of RAO horses has not been repeated. However, RAO-affected horses do not possess more IgE positive cells in BALF[35] or lung tissue samples[36] and following exposure to organic dust, RAO horses do not develop immediate changes in pulmonary function.[37] Taken together these data suggest that unlike allergic asthma, allergen-specific mast cell sensitization and degranulation are not features of RAO and exacerbations of RAO develop independent of IgE-mediated mechanisms.

Following exposure to HD, inflammation and pulmonary dysfunction develop within hours; however, it remains unclear if CD4-Th2 cells drive this delayed pulmonary response in RAO (as described above). Again, the horse exhibits some distinct differences from typical atopic response. Primarily, RAO exacerbation is associated with neutrophil recruitment (and not eosinophils) into the airway lumen.[8] Further, no studies have detected underlying Th2 cell polarization during remission and Th2 cytokines following allergen exposure are not consistently detected. Two studies demonstrated that increased IL-4 and IL-5 expression develops in RAO-susceptible horses within the first 24 hours of hay exposure.[38,39] Further, horses with chronic inflammation (≥9 days hay exposure) also exhibited elevated IL-4/IL-5 and reduced IFNγ expression[38,39] suggestive of a polarized Th2 cytokine profile similar to human allergic airway disease.[40,41] In contrast, short exposure (24-48 hours) to hay had no effect on IFNγ, IL-4 or IL-5 in BAL cells or isolated BAL lymphocytes.[42–44] Therefore, it is unclear if polarized allergen-specific Th2 cells orchestrate the initial neutrophilic influx. Further, chronic exacerbations may be characterized by elevated IFNγ expression (Th1 polarized) [16,20,42] elevated IL-13 and IFNγ expression (mixed Th1/Th2)[45] or reduced Th2 cytokine, IL-13.[42,46] Thus the role of Th2 and Th1 cytokines in disease pathogenesis remains unclear.

It is likely that, similar to asthma, sub-phenotypes of RAO exist with heterogeneous immune mechanisms that could account for these varied results.[47–49] Indeed, one group of investigators demonstrated that the RAO-affected horses from two different lineages show associations with two different chromosome locations (genetic heterogeneity). This suggests that between different families, different genes/immune mechanisms mediate the disease but ultimately produce an identical clinical phenotype (neutrophilic inflammation, coughing and bronchoconstriction).[48,50,51] This genetic heterogeneity may have significant impact on the study of the disease as Lanz et al[52] demonstrated that peripheral blood mononuclear cells from the two horse families respond differently to in-vitro stimulation with HD.

Furthermore, it remains to be determined if the lymphocytic bronchiolitis that develops during RAO is composed of polarized Th2 lymphocytes (as apposed to the luminal lymphocytes). It has been suggested that RAO is similar to "intrinsic asthma"[53] which differs from atopic asthma (extrinsic) due to the absence of peripheral eosinophilia or allergen-specific IgE. Affected patients do not show sensitivity to commonly tested allergens (i.e. negative skin prick test) thus the causative agent remains unspecified. However, it has been suggested that the allergen-specific-IgE and -CD4-Th2 cells remain localized within the lung as bronchial biopsies from extrinsic and atopic asthmatics show similar bronchial mucosal cellular infiltrate[54] which would indicate that the mechanism of intrinsic asthma resembles extrinsic asthma.

Taken together, an allergen-specific IgE-mediated mechanism is not a component of RAO immunopathology and evidence for allergen-specific Th2 lymphocytes as mediators of the HD hypersensitivity is conflicting. Thus, it remains unclear if an allergen specific immunopathology is responsible for the enhanced sensitivity to HD.

The Non-Specific Response

Clinical signs of RAO can be induced by exposure to individual allergens that are present in HD (e.g. *Aspergillus fumigatus*, and *Micropolyspora faenia* now renamed as *Saccharopolyspora rectivirgula*),[26,27,55] but these experimental inhalational studies do not completely reproduce the severity of disease and development of pulmonary dysfunction is inconsistent. While this could be attributed to the experimental designs (inadequate quantity of antigen delivered, inadequate frequency of exposures or failure to identify and deliver the correct antigen) it is also suggestive that naturally induced RAO exacerbations require activation of additional immune mechanisms. Indeed, both the adaptive and the innate immune system are activated in atopic asthma as protein allergens (e.g HD mite and cat dander) are associated with non-allergenic ligands such as fungi and bacteria which engage the innate immune system.[56] HD contains a mixture of plant stems, pollens, many different species of fungi, mite feces and exoskeleton and bacteria.[57,58] Thus, although molds may act as allergens, the HD mixture is abundant with PAMPs that can stimulate the PRRs of the innate immune system.

The bacterial ligand lipopolysaccharide (LPS) is present in HD and stimulates the innate immune system through ligation of Toll-like receptor 4 (in cooperation with CD14 and MD-2). LPS on its own (at concentrations equivalent to those present in HD) are insufficient to induce an inflammatory response. However, the presence of LPS on fungal extracts[21,59] and HD particles[60] enhances their inflammatory potential as depletion of LPS significantly reduces neutrophilic inflammation and the activity of MMP-9 in RAO-susceptible horses.

In a series of papers, Pirie et al[61,62] determined that both LPS and HD induce pulmonary neutrophilia in a dose dependent fashion. However, in comparison to control horses, RAO-susceptible horses develop robust pulmonary neutrophilia at lower concentrations of these agonists. Yet, delivery of very high agonist concentrations can induce an equivalent neutrophilic response in control horses. Thus, RAO-affected and control horses have a difference in sensitivity but both groups can respond in a similar manner (using pulmonary neutrophilia as an end point) *suggesting that a shared non-allergen-specific mechanism may play an important role.*

Indeed, a number of studies suggest that exposure to hay can induce similar inflammatory responses in control and RAO-susceptible horses, but that the responses often differ in magnitude or duration. As in RAO-susceptible horses, HD induces a lower degree of pulmonary neutrophilia[63,64] associated with elevated IL-8 expression[65] in control horses. Further, initial exposure induces a transient cough rather than a chronic cough.[63] Interestingly, RAO-susceptible and control horses exposed to 5 days of moldy hay showed similar degrees of bronchiolitis and histopathological scores, further suggesting a common response to hay exposure.[66] Further, in comparison to controls, RAO-susceptible horses, exhibit similar but greater increases in systemic acute phase proteins.[67] Also, HD induces similar increases of IL-8 and TNFα expression in peripheral blood neutrophils from RAO-susceptible and control horses. However, following natural challenge, IL-4 induced IL-8 is enhanced in RAO-affected horses compared to controls.[68]

Overall, this suggests that HD is a noxious stimulant with the capacity to induce a non-specific inflammatory response in all horses. However, compared to control horses, the inflammatory response that occurs in RAO-susceptible horses is unregulated and greater in magnitude, suggesting that differential sensitivity could occur at the level of the innate immune system. The mechanisms that underlie the increased sensitivity to HD or LPS are unknown. With regard to LPS responsiveness, there is no evidence that RAO-susceptible horses (in remission) express greater levels of TLR-4 in BALF cells[13] or respiratory epithelium,[69] although elevations are associated with exposure to hay.[13,16,69] Asthmatics may have higher levels of airway CD14 which may contribute to LPS sensitivity[70] but expression of CD14 has not been investigated in the horse airways. Furthermore, polymorphisms of TLR4 have also been associated with asthma and may play a role in LPS sensitivity.[71] Within the healthy horse population, inter-horse LPS responsiveness varies greatly. Variability is not attributed to TLR4 polymorphisms,[72] however, it remains to be determined if RAO-susceptible horses possess TLR4 polymorphisms that enhance LPS responsiveness.

It is also unknown if RAO-susceptible horses exhibit increased sensitivity to other PAMPs, such as β-glucan and peptidoglycan, that are present in HD. However, the potency of HD (assessed by degree of neutrophilia and active MMP9 levels) is correlated with the content of the fungal cell wall component β-glucan.[25,73] β-glucan stimulates the PRR the β-glucan receptor and in a model of *Aspergillus fumigatus* allergic disease, stimulation of the β-glucan receptor contributes to development of airway hypersensitivity, airway neutrophilia, pro-inflammatory and pro-allergic cytokines indicating that this receptor plays an important role in fungal allergy.[74] Despite its potential importance there have been no studies evaluating the expression of this receptor in RAO-susceptible horses and it is unknown if RAO-susceptible horses would also display hypersensitivity to β-glucan exposure.

In addition to LPS, which originates from gram-negative bacteria, HD (and other agricultural organic dusts) contain gram-positive bacteria.[75,76] Exposure of mice to a swine-barn-dust induces significant neutrophilic pulmonary inflammation and the presence of high quantities of peptidoglycan (a component of gram-positive bacterial cell walls) plays a significant role. Peptidoglycan stimulate TLR-2, and TLR-2 knock out mice display a significantly attenuated inflammatory response on exposure to swine dust extract.[77] The response to exposure of TLR-2 agonists (such as peptidoglycan) has not been investigated in RAO-affected horses. However, investigations of the response to *Sacchropolyspora rectivirgula* inhalation (formerly *Micropolyspora faeni*, a thermophilic, gram positive, actinomycete prevalent in moldy hay) suggest that RAO-susceptible horses have increased sensitivity to this agent: RAO-susceptible horses developed a greater influx of airway neutrophils compared to control horses.[27] Further, in a separate study, RAO-susceptible and control horses both developed similar degrees of airway neutrophils but only RAO horses developed pulmonary dysfunction.[78] *Sacchropolyspora rectivirgula* is a known allergen in people and is associated with hypersensitivity pneumonitis (HP) an allergic alveolitis mediated by allergen-specific Th1 cells.[79] However, certain clinical and pathologic features of HP differ from RAO: HP is associated with systemic signs of fever and malaise and histopathology demonstrates alveolitis, lymphocytic interstitial infiltration and fibrosis, and the presence of non-necrotizing granulomas.[80] It is unknown if the peptidoglycan content of *Sacchropolyspora rectivirgula* could contribute to this apparent sensitivity via engagement of the innate immune system. Previous studies have demonstrated that RAO is not associated with increased elevations in TLR-2 expression of bronchial epithelial cells,[65,81] but expression of TLR-2 in alveolar macrophages has not been investigated.

The current evidence indicates that HD can incite an inflammatory response in both non-RAO-susceptible horses and RAO-susceptible horses suggesting activation of common innate pathways. However, in contrast to control horses that regulate the inflammatory response, RAO-susceptible horses develop uncontrolled inflammation. The exact mechanisms through which HD elicits this uncontrolled inflammatory reaction remains uncertain but it is possible the differential sensitivity lies at the level of the innate immune system such as the alveolar macrophage.

The Alveolar Macrophage in RAO

There are little data focusing on the contribution of the alveolar macrophage in RAO pathogenesis. In RAO-susceptible horses, HD exposure is associated with an influx of neutrophils, but, in contrast, the absolute numbers of macrophages do not change[43,59,60] or are significantly decreased[27,38,62,82,83] and the macrophage cytology percentage is frequently reduced.[35,38,42,84] In control horses, exposure to HD generally does not alter macrophage absolute number.[27,38,42,62] The reduced macrophage numbers could be explained by increased apoptosis or migration from the airways. However, there is little difference in the proportion of apoptotic alveolar macrophages (<3% difference between groups) between control and RAO-susceptible horses following exposure to hay,[85] thus it is unlikely that this contributes to the reductions in macrophage numbers. It is also possible that low macrophage yield could be a consequence of reduced lavage recovery due to bronchoconstriction and mucus plugging in the diseased lung[86] and may not accurately reflect changes in macrophage numbers in the RAO-affected lung. It is also possible that reduced numbers of alveolar macrophages could delay resolution of inflammation as the alveolar macrophage clears airway neutrophils via phagocytosis.[9]

It is unclear if the alveolar macrophage contributes to the inflammatory response during RAO-exacerbation. Following acute exposure to HD, alveolar macrophages from RAO affected horses express higher levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-8) compared to normal horses[87] suggesting that a pro-inflammatory macrophage phenotype exists in RAO-susceptible horses. However, in contrast, Joubert et al[88] found no difference in the pro-inflammatory response of alveolar macrophages from RAO-affected and normal horses exposed to natural challenge.

A potential mechanism for the enhanced sensitivity and uncontrolled inflammation in RAO is the presence of divergent alveolar macrophage phenotypes. Macrophages are positioned at the host environment interface, and possess a vast array of receptors enabling them to respond to pathogens. Importantly, they can develop into distinct functional phenotypes that alter their sensitivity to stimulation. Macrophages can polarize into pro-inflammatory or anti-inflammatory/regulatory phenotypes and 2 basic macrophage phenotypes are described with counter active functions. The M1 phenotype is considered pro-inflammatory, and functions to augment microbiocidal effector functions required for successful host defense against pathogens. These functions include enhanced respiratory burst capacity, antigen presentation and production of pro-inflammatory cytokines in response to subsequent microbial stimulation. In contrast, M2 macrophages are considered anti-inflammatory, have poor microbiocidal mechanisms and oppose and regulate inflammation and promote tissue repair or specialize in host defense against parasites. *Thus, divergent alveolar macrophage phenotypes could contribute to the enhanced sensitivity and uncontrolled inflammation observed in RAO. This formed the basis for my overarching hypothesis, that the enhanced sensitivity to hay dust observed in RAO-susceptible horses may be mediated by the presence of pro-inflammatory M1 phenotypes or the absence of anti-inflammatory M2 phenotypes.*

Section 2. The Biology of Alveolar Macrophages

The following section provides background detailing the critical importance of alveolar macrophages within the healthy lung, their functional plasticity in response to changing environments and development of M1 and M2 phenotypes. Further, this section discusses alveolar macrophage phenotypes in chronic airway disease with the focus on asthma.

Origins of Alveolar Macrophages

Until recently the classical theory was that alveolar macrophages (and other tissue macrophages) are primarily derived from circulating monocytes produced by common myeloid progenitor cells in the bone marrow. Further, interstitial macrophages could serve as an intermediary step between blood monocytes and alveolar macrophages.[89] However, it is now acknowledged that resident alveolar macrophages are derived from embryonic yolk sac progenitor cells that are maintained through out life by local self-renewal.[90] Alveolar macrophages are long-lived cells (at least 4 months) and maintainance of alveolar macrophage cell numbers under steady state conditions can be accomplished by local proliferation of the embryonically derived tissue alveolar macrophages rather than constitutive replenishment by infiltrating monocytes.[91,92] However, during inflammation, peripheral monocytes are recruited to the lung, attracted by the release of chemotactic stimuli (CC-Chemokine ligand 2 [CCL2]).[93] These infiltrating monocytes (phenotypically characterized as “inflammatory” monocytes) then differentiate into macrophages (infiltrating macrophages). It is unclear if these infiltrating macrophages then persist long term to become resident macrophages. It has been demonstrated that weeks after an inflammatory insult, resident alveolar macrophages are once again predominately derived from the embryonically derived resident population, indicating local proliferation and re-establishment of numbers.[92] Thus airway macrophages may be derived from blood or local proliferation, but regardless of their origin, once in the airway, exposure to the distinctive pulmonary environment will impact their phenotype.

The Role of the Alveolar Macrophage in the Healthy Lung

Tissue macrophages exist in every organ and have an integral role in maintaining tissue homeostasis. However, within each organ, resident macrophages are highly adapted to perform the unique functions that are required for each specific environment.[94] Thus, the environment induces functional heterogeneity.

The lungs are continuously exposed to aeroallergens and PAMPs and it is a constant challenge for the pulmonary immune system to differentiate between innocuous and harmful antigens and respond appropriately. Unnecessary activation of the immune system will result in costly damage to the delicate lung architecture, but on the other hand a sluggish response to inhaled pathogens could result in severe microbial infections. Thus the pulmonary immune system must monitor the challenge material carefully and regulate an appropriate response.

In the healthy human lung, the alveolar macrophage is the most abundant leukocyte lining the airways and alveoli and constitutes 90% of the cells of the bronchoalveolar lavage.[95] By contrast, in the healthy equine lung, the macrophage remains a dominant cell type but constitutes between 40-70% of the cells of the BAL, with the remainder of cells being mostly lymphocytes (30-60%) with a few neutrophils (<5%).[96] In the healthy lung, the resident alveolar macrophage plays a key role in maintaining pulmonary homeostasis by performing a janitorial role engaged in phagocytosis of aeroallergens, cellular debris such as apoptotic or necrotic cells, and pulmonary surfactant.[97] However, the alveolar macrophage provides first line defense against inhaled pathogens and is equipped with a vast array of PRRs that recognize PAMPs. Stimulation of these PRRs can incite an inflammatory response.

However, alveolar macrophages also express a variety of receptors that block the inflammatory response when engaged (negative regulators). The unique environment of the healthy lung expresses ligands that engage these negative regulators maintaining the resident alveolar macrophage in a quiescent state suppressing their inflammatory potential by blocking inflammatory pathways. Cross talk between bronchiolar and alveolar epithelial cells down-regulates the alveolar macrophage. The regulatory protein CD200 expressed by respiratory epithelial cells and apoptotic cells binds to the alveolar macrophages CD200 receptor (CD200R), inhibiting pro-inflammatory signaling pathways.[98] Further, TGFβ is produced in healthy lungs but is secreted in an inactive (latent) form. Bronchial and alveolar epithelium express the αVβ6 integrin which activates latent TGFβ,[99] and αVβ6 tethered TGFβ further suppresses the induction of inflammatory cytokines in the alveolar macrophage.

Other soluble factors that are present in high quantities in the lung contribute to basal suppression of the alveolar macrophage. Surfactant proteins (SPA and SPD) bind to the alveolar macrophage receptor SIRPα (signal inhibitory regulatory protein-α) and inhibit $NF_{K}B$ activation.[100] IL-10 is constituently expressed by alveolar epithelial cells[101] and binding to the IL-10 receptor of alveolar macrophages reduces inflammatory cytokine production. Further, prostaglandin-E, produced by bronchial epithelium, suppresses microbicidal activity.[102] Gap-junction channels allow direct communication between a population of alveolar macrophages and alveolar epithelium so that waves of calcium can provide an immunosuppressive signal to the alveolar macrophage.[103] Thus, in many ways, the unique lung environment exerts a regulatory influence on the alveolar function maintaining a basal quiescent state. Furthermore, adoptive transfer of other tissue macrophages into the lung allows them to "take on" these alveolar macrophage characteristics.[104]

Resting alveolar macrophages also actively suppress neighboring dendritic cells and T-cells further contributing to the tonic immune suppression in the healthy lung.[97] However, the need to suppress the inflammatory potential of the alveolar macrophage must be balanced with the need to protect the lung from inhaled pathogens and stimulation with PAMPs can shift the balance from the immunosuppressive state to a pro-inflammatory response. Through the previously mentioned mechanisms the lung establishes an elevated threshold, which must first be overcome to ensure that a pro-inflammatory response is only reached when absolutely necessary.

Thus the alveolar macrophage has a dual function in the lung: to maintain homeostasis and to initiate host defense. The macrophage is well equipped to respond to a wide variety of presented pathogens but this pro-inflammatory potential must be curtailed to ensure that delicate lung tissue is not unnecessarily damaged. This is possible because the alveolar macrophage possesses a plasticity that allows it to respond to signals in the environment and adapt appropriately.

M1 and M2 Phenotypes

Macrophages are equipped with a vast array of receptors that impart extensive monitoring capabilities enabling macrophages to detect a variety of alterations in their environment including exogenous pathogenic molecules, endogenous alarmins released during tissue damage, and locally released cytokines. The macrophages integrate this information and respond accordingly. Thus alveolar macrophages are sentinel cells that play a critical role in initiating inflammatory reactions and becoming effector cells - protecting the host from pathogenic challenge. However, uncontrolled inflammation is detrimental and macrophages are also key in regulating and resolving the inflammatory reaction and promoting tissue repair. Macrophages can achieve this by developing into functionally distinct populations. The prototypical example of this functional plasticity is the development of the functional polar extremes: pro-inflammatory (M1) and an anti-inflammatory (M2).

The M1 macrophage is a specialized microbicidal effector cell and achieves this through enhanced phagocytosis, production of reactive oxygen species, and release of pro-inflammatory mediators and cytokines. This pro-inflammatory phenotype is often referred to as "classical activation" and can be induced in response to pathogen recognition receptor stimulation e.g in response to bacterial challenge.[105] Stimulation by IFNγ also activates an M1 phenotype and in-vivo, this may originate from innate cells (natural killer cells) or antigen-specific Th1 cells which are also part of the host response to intracellular pathogens.

In vitro, M1 macrophages can be activated by simultaneous stimulation with IFNγ and TNFα. Stimulation with LPS also can be an activator because LPS induces macrophage production of TNFα that then acts synergistically with IFNγ to promote an M1 phenotype.[105] Murine M1 macrophages are typified by high expression of inducible nitric oxide synthase (iNOS2) that converts L-arginine into nitric oxide. Nitric oxide can combine with hydrogen peroxide or superoxide radicals within phagolysomes to produce anti-microbial peroxynitrite radicals. M1 macrophages also release pro-inflammatory cytokines (including TNFα, IL-1β, IL-6, IL-12) and chemokines (IL-8) that function to stimulate and amplify inflammatory responses. Further, cytokines secreted during M1 activation may influence T-lymphocyte differentiation (Th1 via secretion of IL-12, Th17 via secretion of IL-23)[106] augmenting the host defense against pathogens. However, chronic activation of this pro-inflammatory phenotype can lead to tissue damage and organ dysfunction. The M1 phenotype is also associated with increased expression of MHC class II and of co-stimulator molecules (CD80 and CD86) resulting in increased antigen-presenting activity. M1 macrophages are significant pathologic effector cells in inflammatory bowel disease,[107] hepatotoxicity[108] and obesity related insulin resistance.[106]

In contrast to the classical pro-inflammatory M1 phenotype, alternative macrophage phenotypes exist that mediate tissue repair or regulate inflammation. These phenotypes are collectively called alternatively activated macrophages (M2) and they evolve in response to a range of stimuli including IL-4, IL-10 and glucocorticoids although it is now clear that the different activating stimuli promote differentiation of phenotypes with different characteristics.[109,110]

Interleukin-4 activated M2 macrophages have an important role in tissue repair and are associated with host defense against helminths and some fungi. The IL-4 may be derived from innate cells (basophils and mast cells) or antigen specific Th2 cells.[111] In contrast to the M1 phenotype, these M2 macrophages have poor microbiocidal mechanisms, produce minimal pro-inflammatory cytokines and promote tissue repair. M2 macrophages are associated with a number of signature proteins (induced by IL-4) that include arginase-1 (Arg-1), mannose receptor (CD206), β -glucan receptor, YM1 and FIZZ1 (found in inflammatory zone 1). IL-4 activated M2 macrophages express elevated Arg-1, which competes with iNOS for the substrate L-arginine and converts it to L-ornithine. L-ornithine is a substrate for both L-proline, which is an essential substrate for collagen, and polyamines, which regulate cell proliferation and differentiation indicating a role in tissue repair.

M2 macrophages also display increased expression of a number of phagocytic receptors including the members of the C-type lectin family; CD206[112] and β-glucan receptor (Dectin -1).[113] The CD206 receptor is the canonical M2 associated receptor.[112] It has three different binding domains (cysteine rich domain (CR), fibronectin domain (FN) and C-type lectin-like carbohydrate recognition domain (CTLD)) that bestow a capacity to bind a variety of endogenous and exogenous material. It is predominately expressed intra-cellularly within endosomes, and samples material within the phagosome.[114] The CTLD recognizes mannose on many bacteria and fungi and can induce production of pro- or anti-inflammatory cytokines depending on the stimulant[115,116] or modulate the inflammatory cascade induced by some TLR.[117] The receptor also plays an important role in tissue homeostasis and the CR domain recognizes a variety of endogenous materials such as thyrotropin and chondroitin sulphate.[115] Further, CD206 contributes to the resolution of inflammation and repair through its capacity to endocytose myeloperoxidase[118] and degrade collagen.[119]

The β-glucan receptor (Dectin-1) recognizes β-1,3 and β-1,6 linked glucans (carbohydrate polymers), which are a major structural component of fungal cells. Recognition of fungi by the β-glucan receptor induces a pro-inflammatory response and is important for protection against fungal infection.[120–122] Stimulation with zymosan (a β-glucan rich yeast particle) can stimulate a proinflammatory response via a number of intracellular pathways. Binding of β-glucan induces phosphorylation of the tyrosine kinase Syk which leads to activation of transcription factors nuclear factor-kappa-B (NFκB) and nuclear factor of activated T cells (NFAT)[123] and the inflammasone NLPR3.[124] M2 macrophages are typically considered an anti-inflammatory phenotype (because they produce low quantities of pro-inflammatory cytokines in response to subsequent stimulation). However IL-4 induced M2 macrophages exhibit an enhanced pro-inflammatory response to zymosan stimulation which is mediated by the β-glucan receptor,[113] suggesting that (IL-4 induced) M2 macrophages can promote a pro-inflammatory response to fungi.

IL-4 induced M2 macrophages also express members of the chitinase family. Chitin is a polysaccharide present in cell walls of fungi, plants and parasites. YM1/YM2 (CHI3I3/CHI3I4) are expressed in murine M2 and can bind chitin but lack enzymatic function (chitinase-like proteins)[125] and their exact function is unclear.[126] The chitinases acidic mammalian chitinase (AMCase) and chitotriosidase possess chitinolytic activity and are important for host defense against fungi and parasites. Resistin-like-α (RELMα) also known as FIZZ1 contributes to extracellular matrix dynamics by inducing myofibroblast differentiation and survival.[127]

Alternatively activated macrophages also express increased anti-inflammatory cytokines TGFβ and IL-10. Interleukin-10 is classically considered immunosuppressive and anti-inflammatory and can exert effects on a range of cells including granulocytes, monocytes, T-cells and B-cells. It can function to decrease oxidative burst, the expression of pro-inflammatory cytokines and co-stimulatory molecules (CD80, CD86) in monocytes/macrophages and dendritic cells. Reduction of co-stimulatory molecules impairs T-cell differentiation, effector cytokine production and induces anergic T cells.[128,129] The anti-inflammatory response is mediated by signaling through the IL-10 receptor (IL-10R) which activates Janus kinase (JAK) 1-STAT3 pathway.[130] Induction of the transcription factor STAT3 selectively reduces the transcriptional rate of a subset of LPS induced pro-inflammatory genes.[131]

TGFβ plays an important role in wound healing where it induces a broad spectrum of effects including leukocyte recruitment, angiogenesis and collagen synthesis.[132] Further, it contributes to resolution of inflammation and regulates many immune cells including inhibition of T-cell differentiation and proliferation, B-cell proliferation and down-regulation of the macrophage inflammatory response to IFNγ or LPS. TGFβ also promotes differentiation of T-regulatory cells.[133] The TGFβ protein is synthesized and secreted as an inactive (latent) precursor and activation can be performed by a variety of factors including reactive oxygen species, matrix metalloproteinases, and epithelial integrin αVβ6. Binding to the TGFβ receptor activates the family of Smad proteins that regulate gene transcription.[133]

The term alternatively activated macrophage refers to a spectrum of macrophage phenotypes that are broadly associated with immune regulation and repair.[106] In addition to the IL-4 stimulated M2 phenotype, another sub-phenotype called a regulatory macrophage (M-reg) has been described. The M-reg is phenotypically and functionally distinct from either the classically activated M1 or IL-4 induced M2 as described above.[110,134] Regulatory macrophages can develop subsequent to stimulation with a variety of factors including, stimulation with macrophage-colony stimulating factor and IFNγ,[134] immune complexes[106] efferocytosis,[135] glucocorticoids[136] and adenosine.[137] Further, TLR stimulated (pro-inflammatory) macrophages initiate an intrinsic mechanism that induces a transition into a M-reg phenotype.[138] Functionally, the M-reg modulates the inflammatory response.[139] They can inhibit mitogen stimulated T-cell proliferation and are less responsive to LPS stimulation.[134] Furthermore, M-regs can preferentially remove allogenic T-cells by phagocytosis and delivery of M-regs can prolong organ transplant survival.[134] Indeed, delivery of regulatory macrophages as an adjunct immunosuppressive therapy in two people receiving kidney transplants has shown some promise.[140]

Likely, the repertoire of cytokines and receptors expressed by M-regs may vary depending on the exact stimulating conditions, however, the hallmark of regulatory macrophages are elevated expression of IL-10 which usually coincides with reduced expression of IL-12. Like M1, murine M-reg express iNOS but have low expression of pro-inflammatory cytokines such as TNFα, IL-6 and IL-12 or co-stimulatory molecule CD80 when compared to M1 phenotypes. Furthermore, M-regs express low levels of CD206 (in comparison to IL-4-M2 phenotype) but display elevated levels of scavenger receptors β-glucan receptor and macrophage galactose-type c-type lectin 1 receptor.[134]

As a group, the alternatively activated macrophages have been associated with host defense against parasites, down-regulation of inflammation and wound healing. However, the exact contribution of alternatively activated macrophages is context specific. In some instances alternatively activated macrophages are beneficial e.g defense against certain parasites while in others they may promote pathology e.g M2 presence in tumors.[141] Importantly, macrophage polarization states are dynamic and individual cells can shift between phenotypes depending on external environment,[142–146] once more highlighting the dynamic flexibility of these cells.

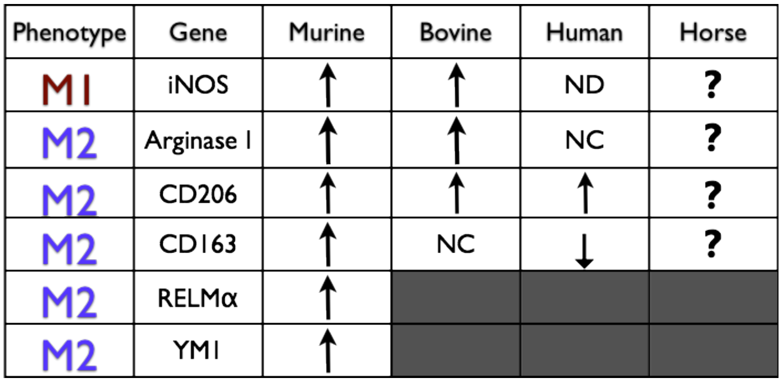
Utilizing Gene Expression Signatures to Identify Macrophage Phenotypes

Distinct gene signatures that reflect the opposing functions of macrophages are used to identify M1 and M2 phenotypes. The genetic signatures of murine macrophage phenotypes are well established, however, data from human macrophage phenotypes indicates substantial variation in the "genetic signature" despite preservation of general phenotypic function (see Figure 1).

Elevated expression of inducible nitric oxide synthase (iNOS) or arginase 1 provides well-established markers for murine M1 and M2 phenotypes, respectively. However, neither iNOS nor arginase expression is significantly induced in polarized human macrophage phenotypes.[147] Stimulation with IL-4 fails to induce expression of arginase in equine neutrophils suggesting that gene expression signatures in equine M2 macrophages may similarly differ from mice.

Further, YM1 (chitinase-like protein) and RELMα are murine restricted as humans (and horses) lack these genetic orthologs.[126] Thus, this has necessitated investigation of different gene family members in such as chitotriosidase (CHIT 1)[148] and AMCase (chitinase), or YKL-40 (human chitinase-like protein)[149] or RELMβ in human macrophages. Definitive M2 markers have been difficult to establish due to inconsistent findings. The receptor CD163 is commonly used as a human M2 marker however, Th2 cytokines reduced expression of CD163 in human monocyte derived macrophages.[150] The lack of specific gene markers for M1 or M2 phenotypes in humans creates a challenge in accurately categorizing macrophage phenotypes as M1 or M2 and limits the ability to infer how these macrophage phenotypes are induced.

Table 1. Species Specific Differences in M1/M2 Polarized Phenotypes



Phenotype	Gene	Murine	Bovine	Human	Horse
M1	iNOS	↑	↑	ND	?
M2	Arginase 1	↑	↑	NC	?
M2	CD206	↑	↑	↑	?
M2	CD163	↑	NC	↓	?
M2	RELMα	↑	▨	▨	▨
M2	YM1	↑	▨	▨	▨

Illustrates expression of select genes in murine, bovine and human M1 and M2 macrophage phenotypes. Arrows indicate increased or decreased expression. Grey boxes indicate no genetic ortholog. ND-not detected, NC-no change, question mark indicates not yet determined.

There are no data comparing equine macrophage M1 and M2 phenotypes and cross species variations limit direct translation of genetic markers. Thus, there is a fundamental need for in vitro characterization to establish reliable genetic markers in equine alveolar macrophage *phenotypes. Establishing these gene expression templates will enhance our ability to understand alveolar macrophage biology in-vivo.*

Activation Pathways of M1 and M2 Macrophages

M1 and M2 activating stimuli achieve polarized alveolar macrophage phenotypes by initiating distinct signaling pathways. The latter alter transcriptional responses consequently controlling the expression pattern of cytokines and surface receptors resulting in the functional phenotype.

Activation of M1 macrophages occurs through stimulation with IFNγ and LPS. Activation of the IFNγ receptor recruits Janus kinase adaptors that then activate transcription factors STAT1 (signal transducers and activators of transcription) and interferon regulatory factors (IRF-5).[151] STAT1 binds to interferon-sequence response elements (ISRE) in the promoter region of M1 signature genes such as iNOS, CD80 and IL-12. Stimulation of TLR by pathogens also stimulates a pro-inflammatory M1 phenotype. LPS stimulates TLR4 and through induction of MyD88 and NFκβ induces pro-inflammatory cytokines. Many genes contain promoter regions that contain sequences that can be regulated by both IFNγ and LPS and consequently similar gene expression patterns are seen and LPS stimulated macrophages are considered M1-like phenotypes.[152]
IL-4 and IL-13 can induce induction of M2 phenotypes by binding to IL-4Rα. Induction of M2 associated genes in alternative macrophages involves a number of transcription factors, including STAT6, PPRγ and C/EBP but stimulation with IL-4 specifically induces the JAK-STAT6 pathway and STAT6^{-}/$^{-}$ mice have impaired M2 polarization. Interleukin-4 also

activates PI3K (phosphoinositide 3-kinase) which augments STAT6 transcription and selectively activates certain M2 associated markers (e.g Arginase 1, YM1, FIZZ1).[153] Maintenance of polarization states is complex and in addition to receptor-mediated activation a number of other regulatory proteins play critical roles. Maintenance of M1/M2 phenotypes are also influenced by of the balance of SOCS1 (suppressor of cytokine signaling-) and SOCS3 proteins. IL-4 induces SOCS1 (with concomitant suppression of SOCS3), which inhibits M1 activating pathways (e.g JAK/STAT1, and NFκβ). In contrast, IFNγ/LPS stimulation induces SOCS3 which inhibits pathways leading to M2 polarization pathways (e.g IL-4 and TGFβ receptor activated pathways).[154,155] However, SOCS3 is also induced by IL-10 stimulated macrophages (induced by STAT3)[130] and can mediate early inhibition of LPS induced inflammation.[156] Furthermore, the phosphatase SHIP (src homology 2-domain containing inositol -5'-phosphatase) is elevated in M1 activation states and represses the M2 signaling proteins PI3K.[153,157]

Polarized lymphocyte phenotypes (e.g Th1, Th2) are fixed by chromatin modifications,[158] however, dynamic chromatin remodeling (epigenetic regulation) occurs in alternatively activated macrophages. Induction of STAT6 (by IL-4) results in lysine demethylation (Histone-3, Lysine-27) at the promoter sites of M2 marker genes, leading to a M2 gene expression pattern. However, the demethylated state is dynamic, and removal of IL-4 results in a return to repression of M2 marker genes.[159]

Thus there are many factors involved in the control of macrophage activation states that allow an ability to fine tune function. While there is cross regulation between heterologous phenotypes (e.g M2 associated proteins suppressing M1 signaling pathways) complex environments that contain both M1 and M2 activating stimuli can result in concurrent expression of both M1 and M2 markers.[144] This suggests that an infinite spectrum of macrophage phenotypes can be induced in complex physiologic systems.[106,145]

The Inflammatory Response of M1/ M2 to Subsequent PAMP Stimulation

M1 and M2 polarization states should be considered "primed states" that can influence their response to subsequent stimulation.[106] The M1 phenotype exhibits a potentiated inflammatory response when subsequently stimulated with LPS. IL-4 priming may suppress respiratory burst and production of pro-inflammatory cytokines when subsequently stimulated with microbial agonists such as LPS and zymosan.[110,160–162] However, other data also suggests that IL-4 priming is not anti-inflammatory and actually enhances the pro-inflammatory response to microbial stimulation.[152,163] *Critically, it is unknown how equine alveolar macrophage M1 and M2 phenotypes will respond to stimuli that are relevant to RAO.*

Macrophage Phenotypes and Airway Disease

M1 and M2 phenotypes prototypically play critical roles in host defense against intracellular bacteria and helminths respectively. However, M1 and M2 phenotypes are also associated with chronic airway disease such as asthma. In murine models of allergic asthma Th2 cytokines are crutial[164] and alveolar macrophages derived from this IL-4/IL-13 enriched condition exhibit prototypical M2 (IL-4 primed) polarization characteristics (e.g. YM1, FIZZ, ARG-1, CD206).[165–168] Studies of murine allergic airway disease have provided evidence that the M2 phenotype promotes the inflammatory response and airway hyperresponsiveness.[167,169,170] The M2 phenotype can increase Th2 cytokine production via stimulation of CD4$^+$ lymphocytes or can directly contribute to Th2 effector cytokines (increased production of IL-13).[171,172] Further, M2 polarization is enhanced by IL-33 (overexpressed in asthmatics[173]) and the depletion of alveolar macrophages reduces IL-33 driven airway inflammation.[174] Allergen-induced acute exacerbations are also associated with elevated production TNF, IL-6, and IL-1β by murine alveolar macrophages.[171] Additionally, the M2 associated production of pro-fibrotic mediators (TGFβ, L-proline and YM1) contribute to the airway remodeling that characterizes persistent allergic airway disease.[170,175,176] Thus, M2 macrophages can promote the allergic inflammatory response through a variety of mechanisms.

Allergic airway disease is heterogenous however, and is not always characterized by Th2 polarization.[49,177,178] Consequently, other macrophage phenotypes can be associated with allergic airway disease. Indeed, murine neutrophilic-allergic-asthma is mediated by Th1 and Th17 lymphocytes and isolated alveolar macrophages displayed an M1-like phenotype (elevated expression of TNFα, IL-12p40 and IFNγ) when compared to murine eosinophilic-allergic-asthma.[179] Thus, alveolar macrophages may enhance the allergic response however, in contrast, some murine studies suggest that alveolar macrophages may attenuate airway inflammation and airway hyperresponsiveness by antagonizing production of Th2 cytokines during asthma.[180] Further, adoptive transfer of alveolar macrophages from allergy resistant rats modulates the airway hyperresponsiveness that develops in allergy sensitive rats suggesting that a protective role for alveolar macrophages may be influenced by genetic background.[181,182] Furthermore, the alternative alveolar macrophage phenotype plays an important role in healing the damaged lung and resolving inflammation.[175]

Thus a number of alveolar macrophage phenotypes have been detected in rodent models of allergic airway disease and similarly, a number of different alveolar macrophage phenotypes have been detected in human asthma. Alternatively activated macrophages can be detected in bronchoalveolar lavage[172] and lung tissue of asthmatics[183] and analogous to murine studies, can promote allergic inflammation by stimulating production of Th2 cytokines,[184] direct production of Th2 cytokines[185] or promoting Th2 cell recruitment.[150] However, the M2 signature can differ among studies and alveolar macrophages may express partial M2 expression profiles (expression of select IL-4-assocaited genes).[150] Other studies have detected M1 macrophages in asthmatics,[186] and allergen challenge induced an M1 phenotype in patients with eosinophilic-asthma.[187] Further, patients with corticosteroid resistant asthma exhibit classically activated alveolar macrophages which may be a consequence of increased environmental endotoxin exposure.[188] However, opinion is still widely divided as a number of studies have found no evidence for different alveolar macrophage phenotypes between normal or asthmatic patients.[47,189]

Overall, many different alveolar macrophage phenotypes have been associated with chronic inflammatory airway disease and these phenotypes may perform a variety of functions including potentiation, suppression or resolution of disease.[169] *This heterogeneity of phenotype may be a consequence of a number of factors including the disease (or disease model), the stage of disease (chronic versus exacerbation) or the genetic background and reflects the integration of the complexity of disease and the dynamic plasticity of macrophages.*

Section 3. Conclusion and Hypotheses

RAO is a complicated hypersensitivity disease that differs significantly from many animal models of allergic airway disease and atopic human asthma. Principally, RAO does not appear to be mediated by allergen-specific IgE, infiltrating cells are neutrophils (rather than eosinophils) and it is not clearly associated with Th2 or Th1 lymphocytes. Thus the relative importance of an allergen-specific response is unclear, and the immune mechanisms that result in disease pathogenesis are unknown. The stimulus for RAO-exacerbation, HD, contains a multitude of PAMPs. Exposure to HD induces pulmonary neutrophilia, and elevations in systemic inflammation in both control horses and RAO-susceptible horses suggesting that HD incites an inflammatory response via stimulation of the innate immune system. However, as the inflammatory response observed in RAO-susceptible horses is greatly enhanced compared to control horses, it is possible that the increased responsiveness to HD could be mediated by alveolar macrophage phenotypes that possess differential sensitivity.

Macrophage M1 and M2 phenotypes represent extremes of macrophage function with M1 cells performing a pro-inflammatory function and M2 cells performing anti-inflammatory or regulatory functions. Macrophage M1 and M2 phenotypes are characterized by distinct transcriptional expression patterns, however, the transcriptional expression patterns are species specific and there are no data evaluating transcriptional signatures in equine alveolar macrophage M1/M2 phenotypes. Further, murine studies have produced conflicting evidence relating to the suppressive effects of IL-4 primed M2 macrophages upon subsequent microbial stimulation. To address this gap in knowledge the first hypothesis was generated:

Hypothesis 1: *The gene expression markers upregulated in equine M1 and M2 alveolar macrophages will differ from those established in other species but equine M1 and M2 will maintain pro-inflammatory and anti-inflammatory function respectively.*

Specific Aim 1: *To investigate this hypothesis, commonly used markers will be evaluated in equine M1 and M2 alveolar macrophages. The function of the M1 and M2 phenotypes will be determined based on the pro- and anti-inflammatory gene expression response following stimulation with HD and its individual components.*

The results of this investigation are presented in chapter 2.

It is possible that control horses regulate the inflammatory response to HD via development of an immune-regulatory M2 phenotype, while RAO-exacerbation is associated with development of an M1 phenotype or an absence of an immune regulatory M2 phenotype. However a comprehensive evaluation of alveolar macrophage M1 and M2 phenotypes in response to HD has not been performed in horses. To address this gap in knowledge two hypotheses were generated:

Hypothesis 2: *After inhalation of HD, alveolar macrophages from RAO-susceptible horses will exhibit an M1 and control horses will exhibit an M2 phenotype.*

Specific Aim 2: *To investigate this hypothesis, the phenotype of alveolar macrophages isolated from RAO-susceptible and control horses before and after inhalation of HD will be characterized based on the expression of M1 and M2 associated genes.*

Hypothesis 3: *After inhalation of HD, alveolar macrophages from RAO-susceptible horses will exhibit a pro-inflammatory function and control horses will exhibit an anti-inflammatory function when stimulated with individual components of HD in vitro.*

Specific Aim 3: *To investigate this hypothesis, alveolar macrophages isolated from RAO-susceptible and control horses before and after inhalation of HD will be stimulated with individual components of HD in vitro and the gene expression of pro-inflammatory and anti-inflammatory cytokines will be evaluated.*

The results of these investigations are presented in chapter 3.

Chapter 2.

Polarized Equine Alveolar Macrophages Have a Species Specific Gene Expression Profile

Abstract

Background: Polarized murine macrophage phenotypes M1 and M2 express distinct transcriptional signatures. M1 (classically-activated) is anti-microbial and expresses nitric oxide synthase, pro-inflammatory cytokines and co-stimulatory molecules (TNFα, IL-12, CD80) while M2 (alternatively-activated) is immune-regulatory and expresses the regulatory cytokines (IL-10, TGFβ), arginase, and scavenger receptors. It is unclear if equine alveolar macrophages (AM) develop similar phenotype-specific transcriptional signatures.

Hypothesis: Gene expression markers upregulated in equine M1 and M2 alveolar macrophages will differ from those established in other species but equine M1 and M2 will maintain pro-inflammatory and anti-inflammatory function respectively.

Methods: Equine AMs were cultured in IFNγ+LPS or IL-4 generating M1 and M2 phenotypes respectively. The gene expression of predicted surrogate M1/M2 genes and the effect of polarization on the response to LPS, peptidoglycan, zymosan and hay dust suspension was evaluated.

Results: Equine M1s were characterized by increased expression of pro-inflammatory cytokines (TNFα, IL-1β, IL-12p40), the chemokine IL-8, co-stimulatory molecule CD80 and elevated regulatory cytokine IL-10. However, unlike murine phenotypes, equine M1s did not express iNOS. Equine M2s were characterized by elevated scavenger receptor CD206 and low expression of M1 associated genes. However, dissimilar to murine or human M2s neither arginase nor β-glucan receptors were M2 associated. Further, compared to the equine M1 that potentiated pro-inflammatory gene expression when stimulated, the M2 potently suppressed pro-inflammatory cytokines and IL-10.

Conclusions/clinical importance: The transcriptional profile of equine M1/M2 AMs is species-specific. This first systematic comparison of the transcriptional signature of equine AM M1 and M2 phenotypes will assist in studying their role in equine pulmonary disease.

Introduction

Macrophages can respond to changes in their microenvironment (e.g. the presence of host-derived factors and pathogen associated molecular patterns (PAMP)) by adapting into functionally distinct phenotypes.[106,142] Though a spectrum of phenotypes exists, two major phenotypes have been well categorized in the mouse.[106] The classically-activated phenotype (M1) develops under pro-inflammatory stimuli such as IFNγ or bacterial pathogens. In contrast, IL-4 (derived from Th2 polarized inflammation) fosters development of so-called alternatively-activated macrophages (M2) with anthelmintic and immune-regulatory activities.[190] Accordingly, these primed phenotypes have divergent contributions to the course and resolution of inflammation.

Distinct gene expression signatures that reflect their opposing functions are used as surrogates to identify the presence M1 and M2 phenotypes. Murine M1 macrophages exhibit high expression of antimicrobial nitric oxide synthase (iNOS), pro-inflammatory cytokines and co-stimulatory molecules (TNFα^{hi}, IL-6^{hi}, IL-12^{hi} CD80^{hi}). In contrast, murine M2 macrophages exhibit elevated regulatory cytokines (IL-10^{hi}, TGFβ^{hi}), low expression of pro-inflammatory cytokines, simultaneously elevated arginase 1 (Arg1) expression, and a range of scavenger receptors including mannose receptor 1 (CD206) and β-glucan receptor.[106126]

The M1 and M2 phenotypes should be considered “primed” states that can influence the response to subsequent stimulation with pathogen associated molecular patterns[190] and while M1 macrophages typically potentiate the pro-inflammatory response, M2 macrophages typically suppress TLR induced inflammation. However, when stimulated with very potent microbial ligands (e.g LPS), initial M2-priming may have little influence and or may potentiate the release of certain pro-inflammatory/M1 cytokines (such as IL-6, IL-12a),[152] underscoring the complexity of macrophage responsiveness to a broad range of stimuli.

The alveolar macrophage plays an essential role in pulmonary homeostasis, defense against inhaled substances and resolution of inflammation. Indeed, changes in M1/M2 alveolar macrophage phenotype status are thought to play an important role in pathogenesis of many pulmonary diseases including infectious,[191] parasitic,[192] allergic,[167] and occupational disease.[193] These pulmonary ailments also occur in horses, however, little is known about equine M1/M2 transcriptional signatures or if analogous phenotypes occur in equine lungs during disease. Thus, the aim of this study was to characterize the transcriptional signature of equine alveolar macrophage M1 and M2 phenotypes.

Conventional equine husbandry (stabled and fed hay) makes exposure to hay dust ubiquitous even though inhalation has been associated with both development of lower airway inflammation[194] and recurrent airway obstruction (RAO).[1] As M1 and M2 phenotypes from other species typically respond to inflammatory stimuli with divergent inflammatory responses, we further sought to characterize the effect of M1 and M2 phenotypes on elements present in hay dust and select constituents.

Using primary equine alveolar macrophages cultured in M1 (IFNγ+LPS activation) or alternatively activated M2 (IL-4 activation) polarizing conditions, we evaluated the expression of surrogate M1 and M2 genes and analyzed the response of polarized equine macrophages to subsequent stimulation with HD and individual constituents. This is the first comprehensive analysis of equine alveolar macrophage polarization responses to a group of relevant inflammatory stimuli.

Materials and Methods

Animals

Alveolar macrophages were isolated from the bronchoalveolar lavage fluid (BALF) of eight clinically healthy horses (mean age 13.4 years, range 4-22, mixed light breeds: TB, STB, QH, Arabian, Grade). Horses were selected based on clinical history and physical exam, and had remained free from obstructive airway disease when challenged with hay straw. All horses were maintained at pasture for at least 1 month before cell collection and were fed a supplemental complete pelleted feed. The Michigan State University Institutional Animal Care and Use Committee approved all procedures.

Collection of Hay Dust

The hay dust (HD) was collected as previously described[62] from hay with proven ability to induce pulmonary inflammation in RAO-susceptible horses. The same batch of HD was used for all parts of the investigation. Dry dust was further size fractionated using a series of stacked metal sieves (USA Standard Testing Metal Sieves) and the smallest fraction of HD (< 43 micron diameter) was collected. Analysis of aerodynamic properties (Aerodynamic Particle Size Spectrometer, TSI 3322) indicated a median aerodynamic diameter of 1.5um indicating that the particles could be deposited into the alveoli when inhaled. The HD was suspended in PBS at a concentration of 1mg/ml, vortexed for 5 minutes, placed in aliquots and stored at -80F.

Isolation of Alveolar Macrophages

Horses were sedated with detomidine hydrochloride (10ug/kg IV) (Zoetis) and butorphanol tartrate (0.02mg/kg, IV) (Zoetis) and bronchoalveolar lavage (BAL) was performed using a sterilized video endoscope passed intra-nasally and wedged in a peripheral bronchus. A total of 500ml of sterile saline was infused in 200ml aliquots and lavage fluid was retrieved using gentle manual suction using a 60ml syringe. The BAL fluid (BALF) was immediately placed on ice and processed within 30 minutes.

The BALF was passed through an 80µm sterile filter and centrifuged (250xg, 4°C, 10 min). Cell pellets were re-suspended in sterile medium (RPMI+ L-glutamine supplemented with antibiotic/antimycotic, 5% heat inactivated equine serum, 2mM EDTA) and washed twice. Cell number and viability were assessed using a hemocytometer and trypan blue exclusion respectively.

Alveolar macrophages were isolated from the mixed cell population by magnetically-activated cell sorting (MACS) using negative selection. Briefly, cells were resuspended in medium (2x10^7 cells/ml) and incubated with antibody against lymphocytes (mouse monoclonal IgG, HB88) at 4°C for 30 minutes. After washing, cells were incubated with secondary antibodies conjugated to metal beads (anti-mouse IgG polyclonal) at 4°C for 20 minutes and washed before passing through magnetic columns to remove labeled lymphocytes. The eluted cells (enriched macrophage population, 87%±8: mean±sd) were collected and re-suspended (6.25x10^5 cells/ml) in sterile medium (minus EDTA). A small aliquot was collected for cell cytology (cytospin preparation) and to assess viability by use of light microscopy (400 cell count of Diff Quik stained slides) and trypan blue exclusion, respectively.

Cell Culture

The alveolar macrophages were plated into sterile cell culture dishes (12-well at 5.x10^5 cells/well) and incubated (5% CO2, 37°C) for 2 hours to allow adherence. After a medium change, adherent cells were incubated for 20 hours with either medium (non-polarized control), recombinant equine IFNγ (20ng/ml) +LPS (1ng/ml) or recombinant equine IL-4 (20ng/ml). Cells were then harvested and RNA was extracted and stored (-80°C) until gene expression was evaluated.

In a separate experiment, alveolar macrophages were stimulated either with recombinant equine IFNγ (20ng/ml) +LPS (1ng/ml) or IL-4 (20ng/ml) for 24 hours and then incubated for 16 hours with medium alone or, medium plus LPS (100ng/ml), HD (0.02ug/ul), peptidoglycan (1ug/ml) or zymosan (10ug/ml).

RNA Extraction, Reverse Transcription and Quantitative Real Time PCR

Cells were harvested by adding RLT-lysis buffer plus (Quiagen) and homogenized using Qiagen QIAshredder™ spin columns. Total RNA was extracted and purified using Qiagen

RNeasy® Plus Micro Kit which included a step for genomic DNA removal. RNA concentration was measured using Qubit® 2.0 fluorometer and integrity of RNA (RIN) was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) and a RIN score of >6.5 was considered acceptable for qPCR.[195] Equal concentrations of RNA were reverse transcribed (High Capacity cDNA Reverse Transcription kit, Applied biosciences) to create cDNA. Six μl of cDNA was then pre-amplified using TaqMan® PreAmp Master Mix kit (according to manufacturer's instructions) and amplified cDNA was stored at -80°C until further analysis. Amplification uniformity was assured for all gene assays tested. Quantitative PCR was performed using predesigned TaqMan® gene expression assays (Life Technologies) and TaqMan® Gene Expression Master Mix. When predesigned assays were unavailable, primers and probes were designed using the Custom TaqMan® Assay Design Tool or Custom TaqMan® (Plus) Assay Design Tool (Table 2). All samples were run at once, in triplicate on 384 well optical plates ABI 7900HT real time PCR machine (Applied Biosciences) using standard conditions (50°C (2min), 95°C (10 min), 40 cycles (95°C/15 seconds, 60°C/1min). The average of two stable endogenous genes, hypoxanthine ribosyltransferase (HPRT) and elongation factor 1α (ELF1) were used to normalize each sample (See Appendix 1).

Table 2. Life Technologies Assay ID for Proprietary (A) and Custom Designed (B) Taqman Gene Assays

A.

Gene	**Life Technologies™ Assay ID**	**Gene**	**Life Technologies™ Assay ID**
HPRT	Ec03470222_m1	**TLR2**	Ec03818334_s1
TNFα	Ec03467871_m1	**TLR4**	Ec03468994_m1
IL1β	Ec04260296_g1	**Arg-2**	Ec03470258_m1
IL-12p40	Ec03468777_m1	**TGFβ**	Ec03468030_m1
iNOS	Ec03467519_m1	**IL-10**	Ec03468647_m1
IL-8	Ec03468860_m1	**Chit**	Ec03818149_m1
IL-6	Ec03468678_m1		

B.

Gene	**Life Technologies™ Assay ID**	**NCBI Gene Reference**	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')
EF1	AIXOZ4N	AY237113.1	CCACCAACTCGTCCAACTGATAAG	GACAGTACCGATACCACCAATTTTG	CCCTTGCGTCTGCCCC
CD206	AIGJQ7N	XM_005606899.1	CGCCAGGAATAGTGGAAGTAGAC	TGTGCCCAATCAAATAGCAGTAGAA	CCAGCCCTTCCGGCAGC
CD80	AII1NJ3	XM_005601958.1	ACCTGACTTCCGTGATGTTATTGG	ATGGATTTCCAACTTCAGCTATGGT	TCAGAGCCAACTTTCC
Relmβ*	AJAAZEG	XM_001503230.1			
B-glucan*	AJ89J89	XM_001499567.3			
Arg -1*	AJCSVPA	XM_001503285.2			

Assay ID and NCBI gene reference provided for all genes. Primer and probe sequences are provided when available. *Primer and probe sequences designed using Custom TaqMan® (Plus) Assay Design Tool are proprietary and withheld by Life Technologies™ (Table 2B).

Data Analysis

Normality of errors of each variable was assessed using visual inspection of error histogram, probability plots, and normality testing using Shapiro-Wilk. Normally distributed data were analyzed using an ANOVA with the fixed effect of treatment and the random effect of horse (SAS Proc Mixed). Errors that were not normally distributed were log transformed (IL-10, β-glucan receptor-inflammatory response of polarized cells) and normality of transformed data was assessed as described. Non-parametric variables (TLR2, TLR4, β-glucan receptor -gene expression of polarized cells) were analyzed using the paired Wilcoxon signed-rank test. Significance was set at ($P<0.05$). Results are expressed as fold change (using $2^{-\Delta\Delta CT}$) compared to untreated, non-polarized cells. In the case of IL-12p40, expression was undetectable in control and IL-4 treated cells and an empirically CT value of 35 was used to allow approximation of fold change in IFNγ /LPS treated or agonist stimulated cells.

Results

Gene Expression of Polarized Phenotypes

Our first goal was to determine the effect of M1 polarizing conditions on the gene expression profile in equine alveolar macrophages. Incubation of alveolar macrophages with IFNγ +LPS induced significant up-regulation of the pro-inflammatory cytokines (TNFα, IL-8, IL-12p40, IL-6) that are typically associated with the M1 phenotype in other animal species[158,109] (Table 3A). Furthermore, we observed robust up-regulation of CD80 co-stimulatory molecule, a functional hallmark of M1 macrophage polarization. Expression of the major M1 marker for rodent species, the inducible nitric oxide synthase (iNOS), was not detected in any equine alveolar macrophage samples. However, iNOS expression was detected in separate samples of equine lung tissue (data not shown), indicating that our methodology was appropriate for the detection of equine iNOS mRNA.

Similarly to non-equine species putative M2 marker genes arginases, mannose receptor-CD206, and chitotriosidase were not significantly induced by M1 polarizing conditions, however the latter resulted in a highly variable up and down regulation of β-glucan receptor (Table 3B). Finally, stimulation with IFNγ +LPS had also no significant effect on other pathogen recognition receptors (TLR2 and TLR4) mRNA levels but did induce significant expression of the regulatory cytokine, IL-10.

Our second goal was to determine the effect of M2 polarizing conditions on gene expression profile in equine alveolar macrophages. In contrast, with IFNγ +LPS, the IL-4 had no significant effect on the expression of M1/pro-inflammatory cytokines TNFα and IL-8 while IL-12p40 was undetectable, similar to non-polarized control cells (Table 3A). Of note, although not significant, IL-6 expression was elevated in 3 of 4 samples and when AM were incubated for a longer duration (40 hours) this elevation reached significance (data not shown). IL-4 treatment also significantly suppressed expression of IL-10. Finally, IL-4 treatment had no affected on the expression of CD80 and the iNOS remained undetectable in IL-4 stimulated cells. The major M2 macrophage marker of rodent species, arginase-I was not detected in any alveolar macrophage samples but was detected in equine liver (data not shown). Rather, the alternative isoform, arginase-II, was detectable but interestingly, significantly suppressed by IL-4 (compared to control alveolar macrophages). Furthermore, arginase II expression level post-IL4 stimulation was not significantly different from IFNγ +LPS stimulated cells. IL-4 treatment significantly up-regulated the mannose receptor CD206 but had no effect on the β-glucan receptor, which showed a large degree of variability between horses. Further, there was no significant effect on gene expression of TGFβ or chitotriosidase expression. Relmβ could not be detected in any alveolar macrophage samples.

Table 3. Candidate Genes for M1 (A) and M2 (B) Macrophage Phenotypes

A.

Gene	**IFNγ/LPS**	**IL-4**
TNF	48±23 *‡	1.42±1.9
IL-8	17.19±14.29 *‡	-2.7±0.92
IL-12p40	305±55.82 #	ND
IL-6	38.68±28.47 *	12.8±12
CD80	50±65 *‡	-6±6.37
iNOS	ND	ND
TLR2	1±0.67	-1.05±1.52
TLR4	1.0±3.36	2.13±0.73

B.

Gene	**IFNγ/LPS**	**IL-4**
Arg 2	-1.04±3.04	-11.46±7.29 *
CD206	1.6±1.07	13.72±2.9 *‡
B-glucan R	25.07±45.91	-28±48
IL-10	46.16±45.22 *	-9.4±7.2 *‡
TGFβ	5.77±7.2	-1.36±2.7
Chitotriosidase	-1.3±1.94	-1.29±0.168
Relmβ	ND	ND

Table 3 (cont'd)

The effect of IFNγ +LPS and IL-4 stimulation on the gene expression of M1 and M2 anticipated genes in equine alveolar macrophages (Tables A and B respectively). Gene expression is presented as fold change (mean±sd) compared to untreated control cells. *$p \leq 0.05$, compared to non-polarized (control) alveolar macrophages, ‡ $p \leq 0.05$ comparing M1 and M2 alveolar macrophages. ND, not detectable. # note fold change calculated using empirical CT value in non-polarized control cells (see methods) (n=4)

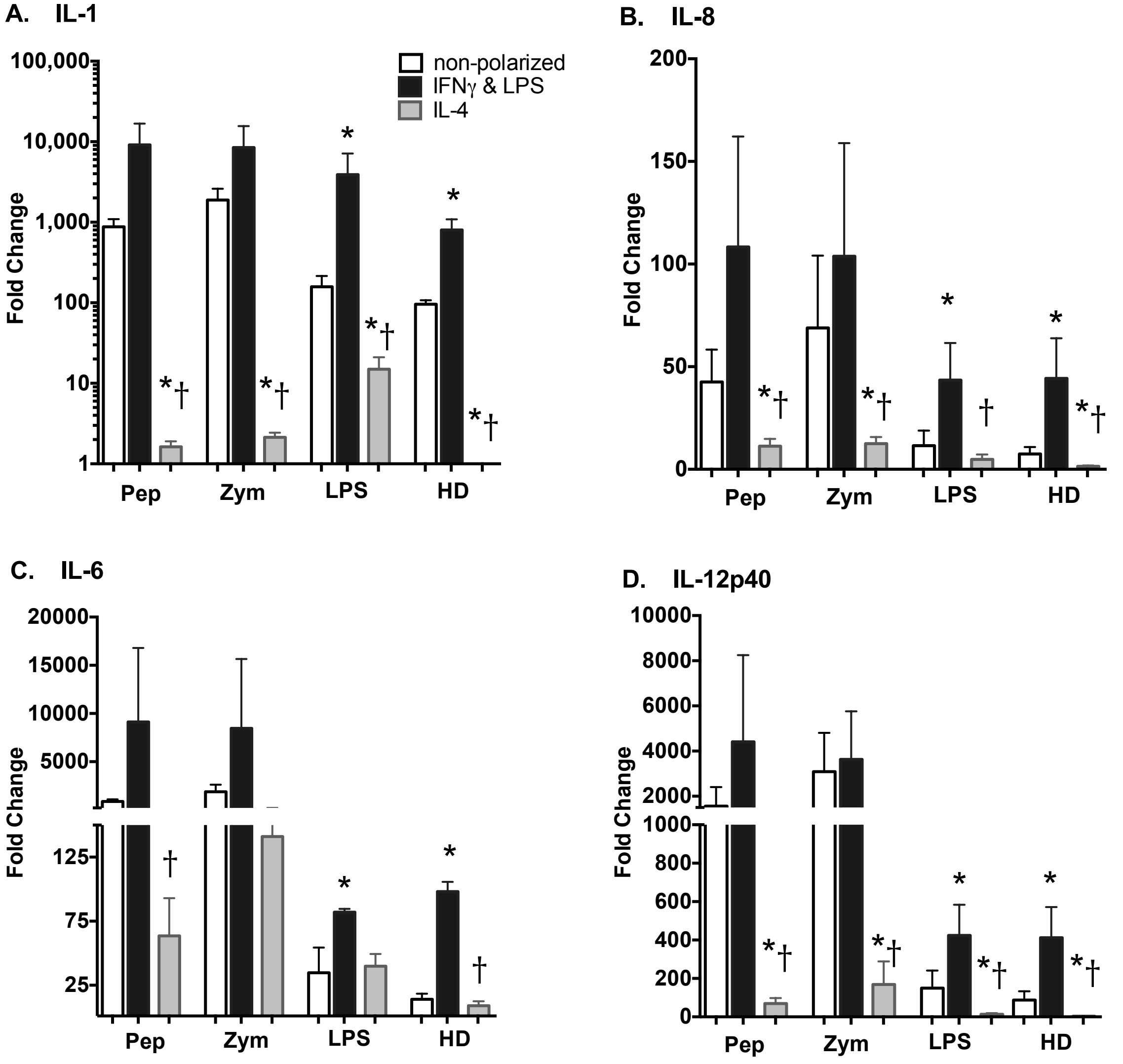
PAMP Stimulation of Polarized Alveolar Macrophage Phenotypes

Inflammatory Cytokines

We next determined how AM polarization state would affect gene expression of inflammatory cytokines when stimulated with peptidoglycan, zymosan, LPS and HD. Compared to non-polarized cells, IFNγ+LPS polarization tended to enhance the expression of pro-inflammatory cytokines IL-1β, IL-8, IL-6 and IL-12p40 (Figure 1A-D). This potentiation was significant with LPS and HD stimulation but not peptidoglycan and zymosan where a large degree in variability was observed.

In contrast, the M2 polarization state resulted in significant down-regulation of the expression of pro-inflammatory IL-1β and IL-8 and IL-12p40 (compared to non-polarized control cells) although the difference was not significant in IL-8 expression in LPS stimulated cells. However, this suppressive effect did not extend to expression of IL-6, which was expressed similarly in M2 and non-polarized cells (Figure 1C).

Figure 1. The Effect of Polarization on PAMP Stimulation of Inflammatory Cytokines

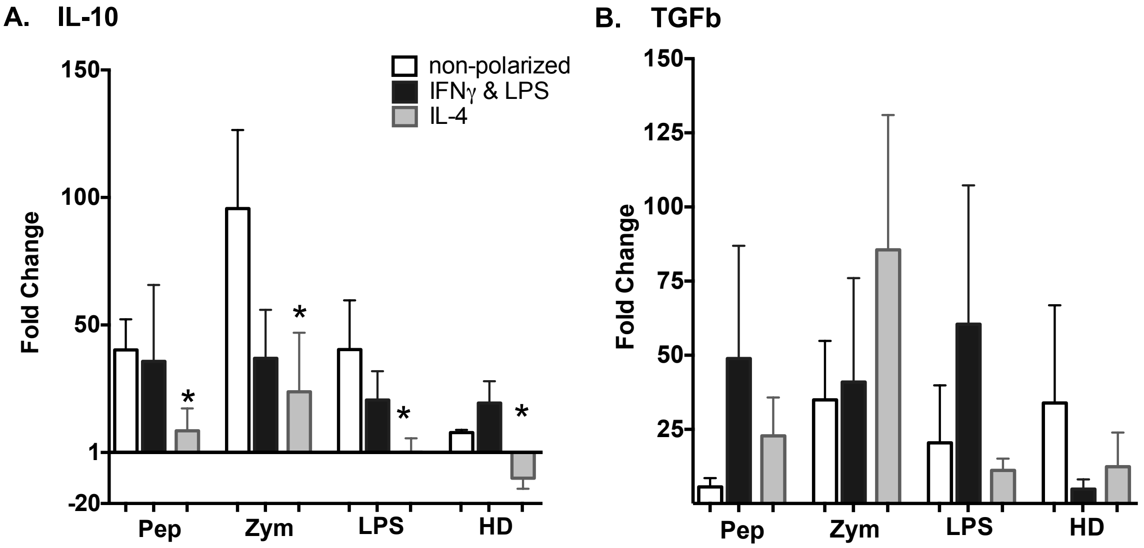


Gene expression of inflammatory cytokines in non-polarized controls (white) and IFNγ/LPS (black) or IL-4 (grey) stimulated macrophages following stimulation with peptidoglycan (Pep), Zymosan (Zym) lipopolysaccharide (LPS) or hay dust (HD, n=3) for 16 hours. Data are expressed as fold change compared to non-polarized-non-stimulated control (mean ±sem). $*p \leq 0.05$, compared to non-polarized control, $\dagger p \leq 0.05$ compared to IFNγ/LPS treatment. Note differing y-axis scale among graphs.

Regulatory Cytokines

Expression of IL-10 was similarly elevated in both M1 and non-polarized cells (Figure 2A). However, the M2 phenotype significantly suppressed IL-10 expression compared to non-polarized controls. Furthermore, a large degree of variability was observed in TGFβ expression and there was no significant difference effect of either polarization state (Figure 2B).

Figure 2. The Effect of Polarization on PAMP Stimulation of Regulatory Cytokines

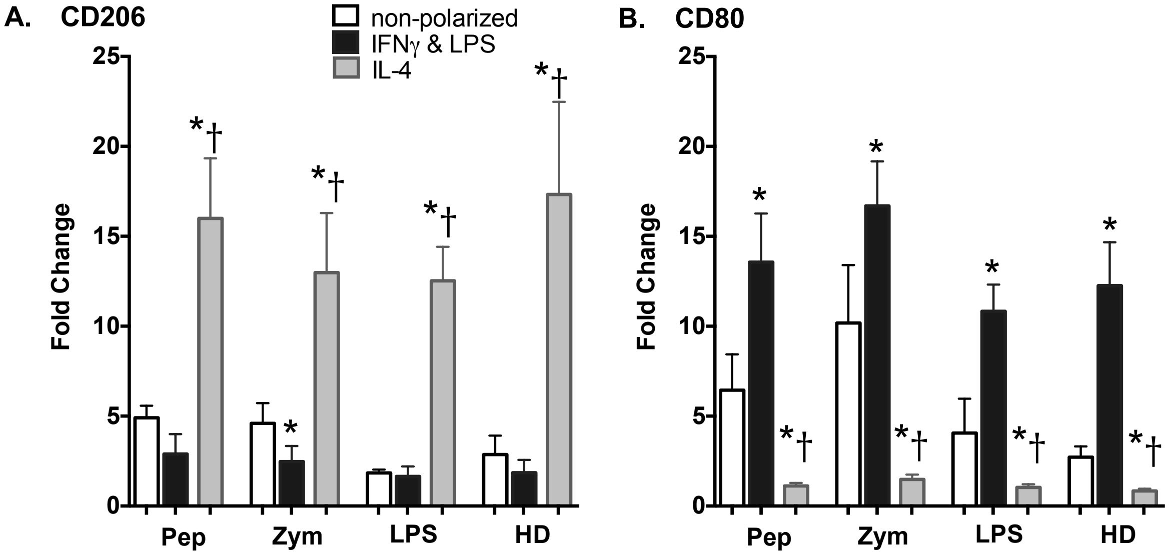


Gene expression of regulatory cytokines in non-polarized controls (white) and IFNγ/LPS (black) or IL-4 (grey) stimulated macrophages following stimulation with peptidoglycan (Pep), Zymosan (Zym) lipopolysaccharide (LPS) or hay dust (HD, n=3) for 16 hours. Data are expressed as fold change compared to non-polarized-non-stimulated control (mean ±sem). $*p \leq 0.05$, compared to non-polarized control.

Surface Receptors

As demonstrated above (Table 3B) the M1 phenotype had no effect on expression of CD206 (Figure 3A) except for zymosan stimulated cells in which CD206 was slightly but significantly suppressed. In contrast, M2 polarization induced significantly greater expression of CD206 across all stimulants (Figure 3A). Polarization states had opposite effects on CD80 expression, which was significantly enhanced by M1 but significantly suppressed by M2 polarized macrophages in the presence of all stimuli (Figure 3B).

Figure 3. The Effect Of Polarization on PAMP Stimulation of Surface Receptors



Gene expression of surface receptors in non-polarized controls (white) and IFNγ/LPS (black) or IL-4 (grey) stimulated macrophages following stimulation with peptidoglycan (Pep), Zymosan (Zym) lipopolysaccharide (LPS) or hay dust (HD, n=3) for 16 hours. Data are expressed as fold change compared to non-polarized-non-stimulated

Figure 3 (cont'd).

control (mean ±sem). *p≤0.05, compared to non-polarized control. †p≤0.05 compared to IFNγ/LPS treatment.

Discussion

M1 and M2 macrophage phenotypes have been associated with human chronic inflammatory pulmonary disease and animal models of pulmonary disease.[168] However, there is little information on transcriptional signatures of equine M1 and M2 phenotypes. This limits the ability to detect or accurately categorize macrophage phenotypes isolated from diseased equine lung. Our goal was to characterize equine M1 and M2 phenotypes in the context of respiratory stimuli relevant to equine respiratory diseases, such as RAO, which in future could be applicable to study alveolar macrophages from lungs of horses affected with pulmonary disorders. With this in mind, we specifically studied polarization of the alveolar macrophage (rather than blood derived macrophages) and used pro-inflammatory agonists that represented stimuli to which horses are routinely exposed through inhalation of stable dust. Phenotypic characterization was comprised of 1) defining the transcriptional signature under polarizing stimuli and 2) determining how these polarization states might influence the response to the pro-inflammatory stimuli.

Our data demonstrate that, similar to other species, equine alveolar macrophages can also develop into distinct M1 and M2 polar phenotypes and present the distinct transcriptional signatures. However, we identify some key species-specific differences in the transcriptional profile between equine and murine polarized macrophages. Further, we demonstrate that M1 and M2 states significantly impact the magnitude of gene expression of pro-inflammatory/regulatory cytokines and surface receptors when stimulated with stimuli that are abundant in the stable environment.

The transcriptional signature of the equine alveolar M1 (IFNγ+LPS activated) was characterized by an overall enhanced pro-inflammatory profile (increased expression of TNFα, IL-8, IL-12p40, CD80), which is similar to that of other species. However, unexpectedly, this phenotype also demonstrated elevated expression of regulatory cytokine IL-10, indicating that, in addition to their dominant pro-inflammatory profile equine M1s are capable of inducing immune-regulatory signals.

In contrast, the equine M2 (IL-4 activated) could be differentiated from the equine M1 based on the combination of no/low induction of pro-inflammatory genes and simultaneous high expression of CD206. This is consistent with M2 characteristics found in other species. However, we also found important differences. In mice M2 prototypical markers resistin-like molecule (Relm)α/FIZZ1 and Ym1 are specifically induced by IL-4 exposure (and STAT6 activation) and their expression marks a predominant Th2/M2 response. However, expression of YM1 (a member of the chitinase family) and FIZZ1 (member of resistin family)[196] are restricted to mice.[126,125] Thus, in this study we explored expression of alternative equine homologues within the same gene families: chitotriosidase and Relm β, however, neither was upregulated with the equine M2 polarization.

A prototypical feature of murine M1 and M2 macrophages phenotypes is the induction of iNOS or arginase-I (respectively) reflecting opposing pathways of L-arginine metabolism, however, induction of these genes was not associated with equine M1 or M2 phenotypes. Interestingly, neither iNOS nor arginase expression are significantly induced in polarized human macrophage phenotypes.[147] and our data show that in this respect equine and human macrophages are alike. The absence of iNOS expression in stimulated equine macrophages is in keeping with recent findings.[197] In contrast to rodents, it is arginase-II isoform that is predominantly expressed in human alveolar macrophages,[198] demonstrating another parallel with human macrophage phenotypes. Unlike arginase-I, arginase-II appears to be associated with the pro-inflammatory M1 phenotype.[199] In this study, arginase-II expression was not induced by the equine M1, but rather polarization with IL-4 induced a slight but significant suppression of arginase-II, consistent with reinforcement of general anti-inflammatory profile of M2 macrophage in equine lungs. Another important difference between equine M2 and those of human and mouse is the differential induction of the β-glucan receptor. Induction of this receptor was not a feature of equine M2, indicating that while the transcriptional signature of polarized equine macrophages appears to share key similarities with polarized human macrophages some differences remain.

M2 macrophages are generally considered immune-regulatory by virtue of low production of pro-inflammatory cytokines and increased expression of regulatory cytokines (such as TGFβ and IL-10). In this study the equine M2 produced low levels of pro-inflammatory cytokines but also suppressed expression of IL-10. Despite this, overall the M2 macrophage possessed an immune-regulatory function as pro-inflammatory cytokines IL-1β, IL-8, IL-12p40 and the co-stimulatory receptor CD80 were significantly suppressed when stimulated with HD or constituent elements. This suppressive effect is in agreement with the findings of Jackson et al[161] who previously demonstrated immunosuppressive effects of IL-4 on LPS induced IL-8 and IL-1 β in equine AM. In the present investigation however, the suppressive effect was not universal, as IL-6 expression remained unaffected. IL-6 has both pro and anti-inflammatory properties[200] but is considered an M1 associated gene. However, IL-4 has been shown to induce and potentiate IL-6 expression.[152] Thus in the horse, expression of IL-6 alone cannot differentiate between M1 and M2 polarized states.

The agonists selected for this study were based on their presence in HD, which has been associated with inciting pulmonary inflammation: namely induction of the hypersensitivity disease RAO but also low-grade pulmonary inflammation in otherwise healthy horses.[64,194,201] Hay dust is a complex mixture containing among other things, fungi and gram negative and positive bacteria[58] thus we investigated zymosan, LPS and peptidoglycan to respectively represent these components. Our data indicate that both non-polarized cells (controls) and M1 polarized cells respond to agonist stimulation with similar gene expression patterns (including a modest increase in expression of CD206) that differed only in magnitude. This is not unexpected as stimulation with either IFNγ or microbial products can induce M1 phenotypes though different signaling pathways mediate their development.[105]

In contrast, regardless of the stimuli, the M2 phenotype has a potent immune-regulatory effect. While the M2 phenotype was still capable of responding to the inflammatory stimuli, the suppression of gene expression of pro-inflammatory IL-1β, IL-8, IL-12p40 and CD80 was considerable. Further, the surface receptor CD206 remained significantly elevated under these conditions. The marked difference in the response of M1 and M2 macrophages to these pathogen associated ligands could certainly contribute to differential outcomes of inflammatory response that follow natural hay-dust challenges in normal horses and those susceptible to recurrent airway diseases.

In the context of identifying M1 and M2 phenotypes as potential factors in respiratory disease, the data in the present paper provide an equine specific, transcriptional expression profile that can guide interpretation of alveolar macrophages harvested from pulmonary diseases. Expression of certain murine genes (e.g YM1 or FIZZ) are considered pathognomonic for IL-4 activated M2 phenotype but a unique equine M2 marker was not identified in this study. Thus, M1/M2 categorization is dependent on assessing the pattern of the gene expression profile in relation to the relative magnitude and the relative response to stimuli. Our data indicate that evaluating the pro-inflammatory cytokines TNFα, IL-1β, IL-12p40, IL-8, regulatory IL-10 and surface receptors CD80 and CD206 can be used to differentiate between equine M1 and M2 macrophages. These data further illustrate that in the absence of M2 polarizing conditions, stimulation with HD or its components has the capacity to induce an M1 transcriptional profile. This should be considered in clinical studies of equine pulmonary disease and prior exposure to HD should be tightly controlled when assessing macrophage phenotype.

In conclusion, equine M1 phenotypes are characterized by a general expression of pro-inflammatory cytokines profile with the exception of induction of regulatory IL-10. When stimulated M1 macrophages predominately enhanced the pro-inflammatory response. In contrast the equine M2 is characterized by increased expression of CD206, in combination with low expression of pro-inflammatory cytokines (e.g TNFα, IL-1β, IL-8, IL-12p40, CD80) and low expression of regulatory IL-10, a profile that is maintained following subsequent stimulation with HD and its components. The data presented here expands current knowledge of the equine alveolar macrophage, and paves the road for future studies regarding the role of macrophage polarization in equine pulmonary diseases.

Chapter 3.

RAO-Susceptible and Control Horses Possess Different Alveolar Macrophage Phenotypes

Abstract

Background: A central feature of recurrent airway obstruction (RAO) is a greatly enhanced sensitivity to hay dust compared to non-RAO-susceptible control horses. The cellular and molecular mechanisms accounting for this differential airway sensitivity are unknown. The presence of divergent alveolar macrophage (AM) phenotypes with differential sensitivity to microbial hay dust components could play a contributing role. To investigate this two hypothesis were generated:

Hypothesis 2: After inhalation of HD, alveolar macrophages from RAO-susceptible horses will exhibit an M1 and control horses will exhibit an M2 phenotype.

Hypothesis 3: After inhalation of HD, alveolar macrophages from RAO-susceptible horses will exhibit a pro-inflammatory function and control horses will exhibit an anti-inflammatory function when stimulated with individual components of HD in vitro.

Methods: To test these hypotheses RT-qPCR was used to evaluate the expression of equine M1 and M2 transcriptional markers (hypothesis 2) and the AM cytokine response to HD components (lipopolysaccharide (LPS), peptidoglycan (Pep) and zymosan (Zym)) (hypothesis 3) in RAO-susceptible (n=6) and control horses (n=5) at baseline and following exposure to hay (natural challenge).

Results: At baseline, compared to control horses, AM of RAO-susceptible horses had greater gene expression of IL-10 and enhanced expression of IL-10 and IL-8 when stimulated with LPS. Further, in RAO-susceptible horses, natural challenge increased the expression of

CD206, but had no significant effect on the cytokine response to hay dust components. In contrast, natural challenge induced increased expression of IL-1β, IL-8, IL-10 and CD206 in control horses and altered the AM cytokine response to LPS, enhancing the expression of IL-1β, IL-10 and CD206 when compared to baseline.

Conclusions/clinical importance: RAO-susceptible and control horses possess divergent AM phenotypes. Natural challenge induces an M1-like phenotype in control horses while RAO-susceptible horses maintain an IL-10 producing macrophage phenotype. These results suggest that unknown host factors present in RAO-susceptible horse lungs promote an IL-10-producing macrophage phenotype.

Introduction

Organic dust contains a medley of microbial components that can stimulate innate immune cells[202] and inhalation of organic dust (e.g hay dust) induces a pulmonary inflammatory response in horses. A central feature of recurrent airway obstruction (RAO) is a greatly enhanced sensitivity to hay dust compared to non-RAO-susceptible, control horses.[62] RAO-susceptible horses develop prominent pulmonary neutrophilia, excess mucus cell production, bronchiolitis and bronchoconstriction, and display pronounced clinical signs. Control horses develop low-grade pulmonary neutrophilia but show no clinical signs. The exact mechanisms through which hay dust elicits this unregulated inflammatory response in RAO and what cell types contribute to this differential sensitivity remain uncertain.[203]

Alveolar macrophages can be activated to exhibit distinct phenotypes that have polarized functions and distinct transcriptional expression profiles. Classical (M1) activated macrophages (stimulated by IFNγ and LPS) are characterized by high levels of pro-inflammatory cytokines and exhibit strong microbicidal activity. In contrast, alternative (M2) activated macrophages (stimulated by IL-4) are considered immuno-regulatory as a result of their reduced production of pro-inflammatory cytokines and increased production of regulatory cytokines.[106] Importantly, the activation status influences the magnitude of response to subsequent stimulation with pathogen-derived stimulants and M1 activated macrophages possess greater magnitude of pro-inflammatory response compared to M2 activated macrophages. Previously we characterized the polarization of equine AM, M1 and M2 phenotypes, identifying the distinct transcriptional expression profiles and response to agonist stimulation (Chapter 2).

Macrophage M1 and M2 phenotypes have been associated with allergic airway disease.[204,205] However, there are no data systematically evaluating alveolar macrophage phenotype markers in RAO-susceptible horses. Thus to gain a greater understanding of the role of the AM in disease and in response to hay dust we compared the gene expression profile of M1 and M2 associated genes in RAO-susceptible and control horses at pasture and following chronic exposure to hay/straw. As RAO-susceptible horses have a hypersensitive response to organic dust compared to control horses, we hypothesized that after inhalation of HD, alveolar macrophages from RAO-susceptible horses will exhibit an M1 and control horses will exhibit an M2 phenotype (hypothesis 2) and further, after inhalation of HD, alveolar macrophages from RAO-susceptible horses will exhibit a pro-inflammatory function and control horses will exhibit an anti-inflammatory function when stimulated with individual components of HD in vitro (hypothesis 3).

Materials and Methods

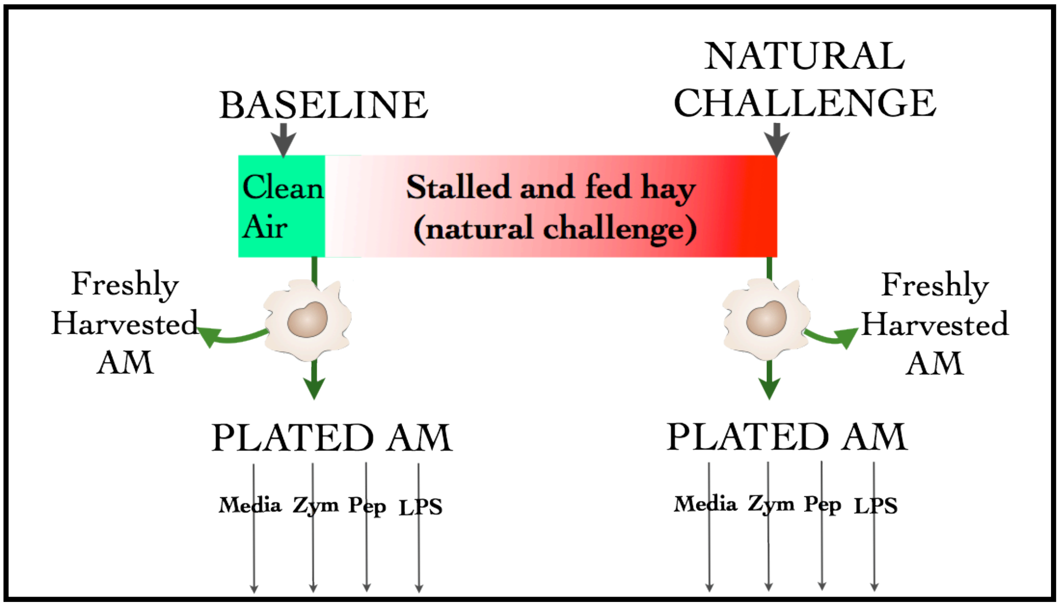
Animals

The investigation, which used six RAO-susceptible and five control horses, was approved by Michigan State University's Institutional Animal Care and Use Committee. Horses were classified as RAO-susceptible if exposure to dusty hay and straw (natural challenge) induced airway obstruction [determined by measurement of maximal change in pleural pressure during tidal breathing (ΔPplmax)] that was significantly ameliorated by anti-cholinergic treatment and was reversed when horses were removed from exposure[1]. Horses that did not develop airway obstruction under natural challenge qualified as controls. Except during natural challenge, all horses were maintained on pasture and supplemented with complete pelleted feed. RAO-susceptible horses were in remission (ΔPplmax $<10 cmH_20$) at the start of the protocol.

Study Design

Horses were transported from the clean air environment (pasture) to the stable and were bedded on straw and fed hay (natural challenge). In RAO-susceptible horses, natural challenge continued until clinical signs of RAO (labored breathing) were induced and ΔPplmax was greater than or equal to 15 cmH_2O. As the time to induce this ΔPplmax varied between RAO-susceptible horses, the control horse was paired with an RAO-susceptible horse and each received the same duration of hay/straw exposure. For logistical reasons, the two horses in each pair could not be sampled on the same day, so the two were consecutively exposed with overlapping periods in the same air space when possible. Measurements of lung function were made and bronchoalveolar lavage fluid (BALF) was harvested at two time points; baseline (BL: immediately after being brought to the laboratory from pasture) and at the end of natural challenge (Figure 4).

Figure 4. Natural Challenge Study Design



Pulmonary Function Tests

Pulmonary function was assessed by measurement of ΔPplmax, pulmonary resistance (RL) and dynamic compliance (Cdyn) as previously described[206].

Isolation of Alveolar Macrophages

Horses were sedated with detomidine hydrochloride (10ug/kg IV) (Zoetis) and butorphanol tartrate (0.02mg/kg, IV) (Zoetis) and bronchoalveolar lavage (BAL) was performed as previously described (Chapter 2). The BAL fluid (BALF) was immediately placed on ice and processed within 30 minutes.

The BALF was passed through an 80μm sterile filter and centrifuged (250xg, 4°C, 10 min). Cell pellets were re-suspended in sterile medium (RPMI+ L-glutamine supplemented with antibiotic/antimycotic, 5% heat inactivated equine serum, 2mM EDTA) and washed twice. Cell number and viability were assessed using a hemocytometer and trypan blue exclusion respectively.

Alveolar macrophages were isolated from the mixed cell population by magnetically-activated cell sorting (MACS) using negative selection. Briefly, BALF cells were resuspended in medium (2x10^7 cells/ml) and incubated with antibodies against lymphocytes and neutrophils (mouse monoclonal IgG, HB88, mouse monoclonal IgM DH24 respectively {Monoclonal Antibody Center, Washington State University}) at 4°C for 30min. After washing, BALF cells were incubated with secondary antibodies conjugated to metal beads (anti-mouse IgG polyclonal and anti-mouse IgM) at 4°C for 20 min and washed before passing through magnetic columns to remove labeled cells. The eluted cells (enriched macrophage population) were collected and re-suspended (6.25x10^5 cells/ml) in sterile medium (minus EDTA). An aliquot of cells was collected for RNA analysis -"freshly harvested alveolar macrophages,"(Figure 4) (n=6 RAO-susceptible horses, n=5 control horses). A small aliquot was collected for cell cytology (cytospin preparation) and to assess viability by use of light microscopy (400 cell count of Diff Quik stained slides) and trypan blue exclusion, respectively.

Cell Culture

The remaining cells were plated into sterile tissue culture dishes (12-well at 5.x10^5 cells/well) and incubated (5% CO2, 37°C) for 2 hours to allow adherence. After a medium change, adherent cells were incubated for 16 hours with medium alone (control) or, medium plus lipopolysaccharide (LPS, 10ng/ml), peptidoglycan (Pep, 1ug/ml) or zymosan (Zym, 10ug/ml). Cells were then harvested -"plated AM" (Figure 4) (n=5 RAO-susceptible, n=5 control horses) and RNA was extracted and stored (-80°C) until gene expression was evaluated.

RNA Extraction, Reverse Transcription and Quantitative Real Time PCR

Cells were harvested by adding RLT-Lysis Buffer (Qiagen) and homogenized using Qiagen QIAshredder™ spin columns. Total RNA was extracted and purified using Qiagen RNeasy® Mini Kit (freshly harvested AM) or Qiagen RNeasy® Plus Micro Kit (plated AM) which include steps for genomic DNA removal. RNA concentration was measured using Qubit® 2.0 fluorometer and integrity of RNA (RIN) was assessed using the Agilent 2100 Bioanalyzer® (Agilent Technologies) and a RIN score of >6.5 was considered acceptable for qPCR.[195] Equal concentrations of RNA were reverse transcribed (High Capacity cDNA Reverse Transcription Kit®, Applied Biosystems) to create cDNA. Six µl of cDNA was then pre-amplified (TaqMan® PreAmp Master Mix Kit, Life Technologies) according to manufacturer's instructions) and amplified cDNA was stored at -80°C until further analysis. Amplification uniformity was assured for all gene assays tested.

Quantitative real time PCR was performed using predesigned TaqMan® Gene Expression Assays (Life Technologies) and TaqMan® Gene Expression Master Mix (Life Technologies).

When predesigned assays were unavailable, primers and probes were designed using the Custom TaqMan® Assay Design Tool or Custom TaqMan® (Plus) Assay Design Tool (Life Technologies). For each experiment, all samples were run at once, in triplicate on 384 well optical plates in the ABI 7900HT Real-Time PCR System® (Applied Biosystems). The average of two stable endogenous genes, hypoxanthine ribosyltransferase (HPRT) and elongation factor 1α (ELF1) were used to calculate the deltaCT of each sample.

Data Analysis

Normality of errors of each variable was assessed using visual inspection of error histogram, probability plots, and normality testing using SAS-Proc Univariate procedure. Normally distributed data were analyzed using an ANOVA with the fixed effect of group and time and the random effect of horse (cytology, pulmonary function, freshly harvested AM gene expression). Variables from "stimulated alveolar macrophages" were analyzed with the fixed effect of group, time and treatment and the random effect of horse (SAS Proc Mixed). Errors that were not normally distributed were log transformed (IL-1β-plated alveolar macrophages) and normality of transformed data was assessed as described. Data from "plated alveolar macrophages" was corrected for multiple treatment comparisons using Bonnferroni correction. Results were considered significant if ΔCT between comparisons was ≥1 (2 fold difference) and $p \leq 0.05$. All statistical analyses were run on SAS 9.4® (SAS Institute Inc., SAS Campus Drive, Cary, NC).

Results

BALF Cytology and Evaluation of Pulmonary Function

The median duration of natural challenge was 9 days (range 7-21). At baseline, there were no significant differences in macrophage or neutrophil percentage between the RAO-susceptible and control horses (Table 4) but lymphocyte percentage was significantly greater in RAO-susceptible horses compared to control horses. Natural challenge induced a significant increase in airway neutrophil percentage in both control and RAO-susceptible horses although the magnitude of airway neutrophils was significantly greater in RAO-susceptible. The increase in airway neutrophils was accompanied by a significant decrease in lymphocyte percentage in RAO-susceptible population only. In keeping with RAO phenotype, RAO-susceptible horses but not controls develop impairments in pulmonary function following natural challenge (significant elevations in ΔPpl, and RL and significantly reduced Cdyn).

Table 4. Effect of Natural Challenge on BALF Cytology and Pulmonary Function

	Baseline		Natural Challenge	
	Control	RAO-susceptible	Control	RAO-susceptible
Macrophage (%)	69.5±9.2	54.6±12.2	54.3±18.94	42.5±13.13
Lymphocyte (%)	29.6±9	41.3±11.84†	28.9±8.8	24.67±4.3*
Neutrophil (%)	0.7±0.5	3.23±2.6	16.1±11.9*	31.83±12.59*†
Eosinophils (%)	0.18±0.36	0	0.17±0.3	0.067±0.1
DpPl max (mmHg)	3.9±0.67	5.19±0.85	4.32±0.46	29.42±13.5*†
Resistance ($cmH_2O/l/s$)	0.48±0.14	0.58±0.24	0.78±0.34	2.5±0.89*†
Dynamic Compliance (cmH_2O/l)	1.27±0.46	1.41±0.36	0.9±0.29	0.27±0.19*†

Data presented as (mean±sd), *p≤0.05 compared to pasture, † p≤0.05 compared to control horses.

Freshly Harvested Alveolar Macrophages

Macrophage purity was not significantly different between groups or time points (85%±8.9, mean±sd) and contaminating cells were lymphocytes. We first compared gene expression between RAO-susceptible and control horses at baseline and following natural challenge (Table 5). There were no significant differences in gene expression of M1 or M2 regulated genes or pathogen receptors at either time point. However, at baseline, RAO-susceptible horses expressed greater quantities of the immune-regulatory IL-10 mRNA transcript compared to control horses but this difference was not present following natural challenge.

We next evaluated the effect of natural challenge within each group (Table 6). Within the control group, natural challenge had no significant effect on M1 or M2 regulated genes or pathogen receptors but IL-10 expression was significantly increased. Within the RAO-susceptible horses there was a modest but significant reduction in IL-12p40 expression. There was no effect on any other genes tested.

Table 5. Gene Expression in Freshly Harvested Alveolar Macrophages in RAO-Susceptible Horses *Relative to* Control Horses at Pasture and Following Natural Challenge

Gene Name	**Baseline**	**P value**	**Natural Challenge**	**P value**
Genes regulated in Equine M1				
TNFα	1.26 ± 0.94	NS	1.06 ± 0.37	NS
IL-1β	1.334 ± 0.40	NS	1.79 ± 2.16	NS
IL-12 p40	1.38 ± 0.83	NS	0.35 ± 0.18	NS(p<0.08)
CD80	1.03 ± 0.33	NS	0.98 ± 0.37	NS
IL-8	1.07 ± 0.83	NS	0.84 ± 0.47	NS
IL-6	1.70 ± 1.75	NS	1.40 ± 1.69	NS
Genes regulated in Equine M2				
ARG 2	1.24 ± 0.61	NS	0.82 ± 0.14	NS
CD206	1 ± 0.34	NS	1.1 ± 0.51	NS
Pathogen Recognition Receptors				
β-Glucan Receptor	0.76 ± 0.17	NS	0.89 ± 0.16	NS
TLR4	0.87 ± 0.35	NS	0.91 ± 0.11	NS
TLR2	1.27 ± 0.35	NS	0.86 0.45	NS
Regulatory Cytokines				
TGFβ	1.13 ± 0.38	NS	0.92 ± 0.22	NS
IL-10	4.35 ± 2.32	0.006	0.8 ± 0.7	NS

Data are presented as relative fold difference (mean ±sd) with 1 being equivalent expression >1 greater expression and <1 lower expression. NS not significant.

Table 6. The Effect of Natural Challenge on Gene Expression of Freshly Harvested Alveolar Macrophages in RAO-Susceptible and Control Horses

Gene Name	**Control**	**P value**	**RAO-susceptible**	**P value**
Genes regulated in Equine M1				
TNFα	0.98 ±0.25	NS	1.27 ±0.74	NS
IL-1β	2.89 ±3.04	NS	2.21 ±1.2	NS
IL-12 p40	1.79 ±1.24	NS	0.59 ±0.47	0.027
CD80	0.70 ±0.16	NS	0.70 ±0.65	NS
IL-8	1 ±0.2	NS	1 ±0.47	NS
IL-6	1.71 ±0.9	NS	1.47 ±1.1	NS
Genes regulated in Equine M2				
ARG 2	1.42 ±0.39	NS	0.97 ±0.28	NS
CD206	1.02 ±0.16	NS	1.15 ±0.34	NS
Pathogen Recognition Receptors				
β-Glucan Receptor	0.81 ±0.14	NS	0.99 ±0.07	NS
TLR4	0.74 ±0.24	NS	0.99 ±0.39	NS
TLR2	1 ±0.09	NS	0.98 ±0.35	NS
Regulatory Cytokines				
TGFβ	1.04 ±0.29	NS	0.89 ±0.4	NS
IL-10	15.59 ±13.24	0.006	1.76 ±0.77	NS

Data presented as fold change (mean ±sd) at post natural challenge relative to baseline with 1 being equivalent expression >1 greater expression and <1 lower expression. NS not significant.

Phenotype of Plated Alveolar Macrophages

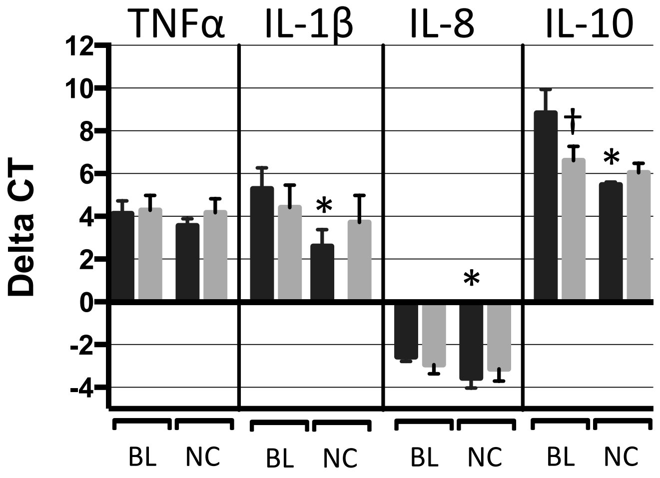
We next evaluated the response of the alveolar macrophages to incubation with pathogen-derived stimuli (LPS, Pep and Zym) for 16 hours. At baseline, in keeping with freshly harvested cells, the unstimulated (media control) alveolar macrophages of RAO-susceptible horses had significantly greater expression of IL-10 than those from control horses (Figure 5A). Further, natural challenge was associated with significantly increased expression of IL-1β, IL-8, IL-10 in the control horses only. Both RAO-susceptible and control horses developed increased gene expression of CD206 following natural challenge (Figure 5B).

In comparing the response of stimulated alveolar macrophages from RAO-susceptible and control horses at baseline (Figure 6), there were no significant differences in expression TNFα, IL-1β, CD80 or CD206 following stimulation with LPS, Pep or Zym (Fig. 6A-C). However, compared to control horses, RAO horses demonstrated significantly greater expression of both IL-10 and IL-8 when stimulated with LPS (Fig 6A). Following natural challenge, there were no significant differences between RAO-susceptible and control horses in gene expression of TNF, IL-1, IL-8, IL-10, CD80 and CD206 by LPS, Pep and Zym stimulated alveolar macrophages.

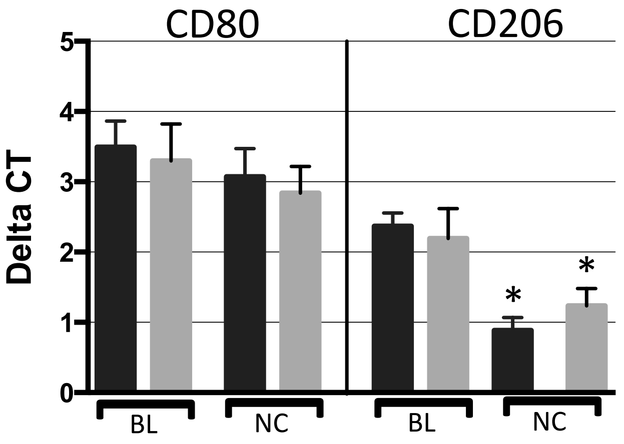
Within the control group there was a significant effect of natural challenge on the response to LPS stimulation: following natural challenge, LPS stimulated alveolar macrophages expressed significantly more IL-1β and IL-10 than at baseline (Fig. 6A). However, gene expression of IL-1β and IL-10 was not significantly different from the unstimulated cells at the natural challenge time point, although IL-1β did approach significance (Fig.7). By contrast, there was no effect of natural challenge on TNFα, IL-1β, or IL-10 in RAO-susceptible horses and gene expression was equivalent for all stimulants at baseline and after natural challenge. However, natural challenge induced elevations in CD206 of LPS stimulated alveolar macrophages in both control horses and RAO-susceptible horses and in zymosan stimulated alveolar macrophages in RAO-susceptible horses. Further, there were also no changes (between groups or over natural challenge) in pro-inflammatory IL-6, regulatory TGFβ (data not shown).

Figure 5. Alveolar Macrophages From RAO-Susceptible and Control Horses Differ in Gene Expression of Pro-Inflammatory, Regulatory and Surface Receptors at Baseline and Following Natural Challenge

A. Pro-inflammatory and regulatory cytokines



B. Surface Receptors



Gene expression (deltaCT, mean ± sem) of unstimulated (media control) alveolar macrophages from control (dark bars, n=5) and RAO-susceptible (light bars, n=5) horses at baseline (BL) and following natural challenge (NC). (A) Pro-inflammatory and regulatory cytokines (B) Surface receptors. *Note, a lower deltaCT indicates greater mRNA expression.* * $p \leq 0.05$ compared to baseline † $p \leq 0.05$ compared to control group.

Figure 6. Alveolar Macrophages From RAO-Susceptible Horses Exhibit Enhanced LPS Responsiveness at Baseline but Not After Natural Challenge

A.

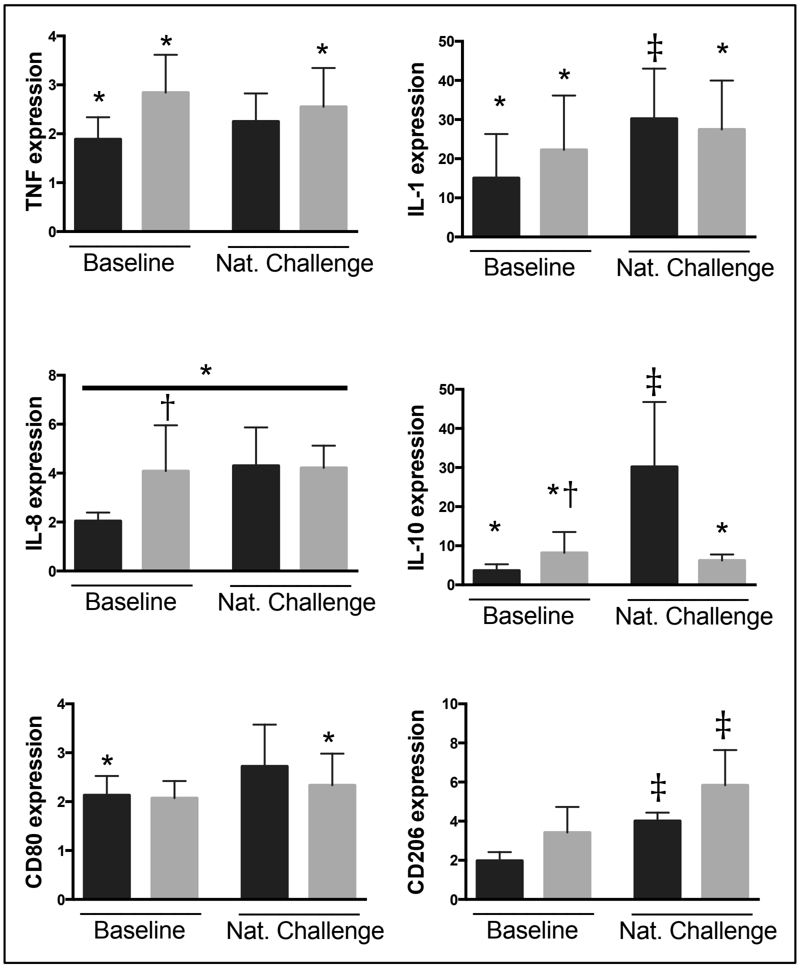


Figure 6 (cont'd).

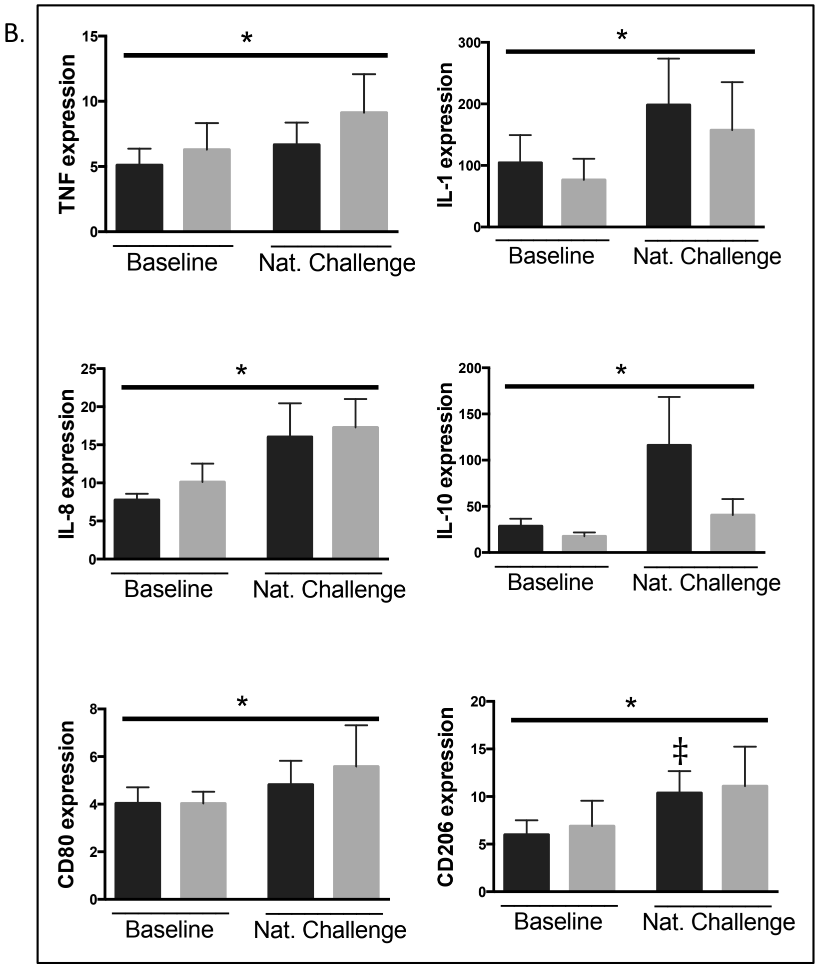
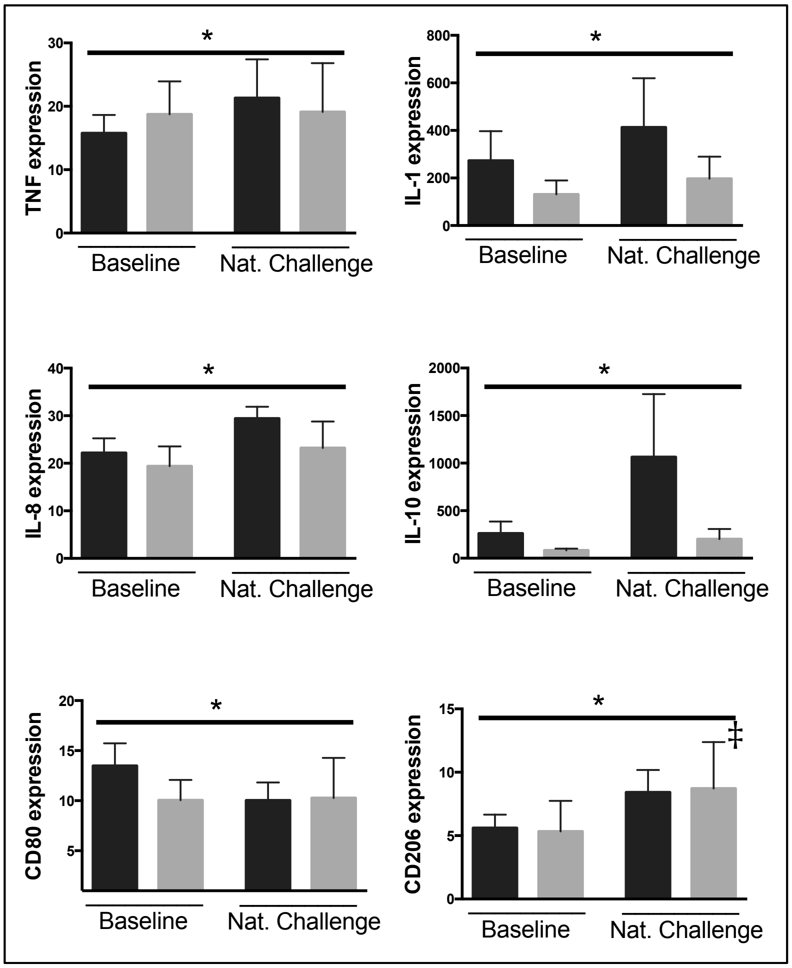


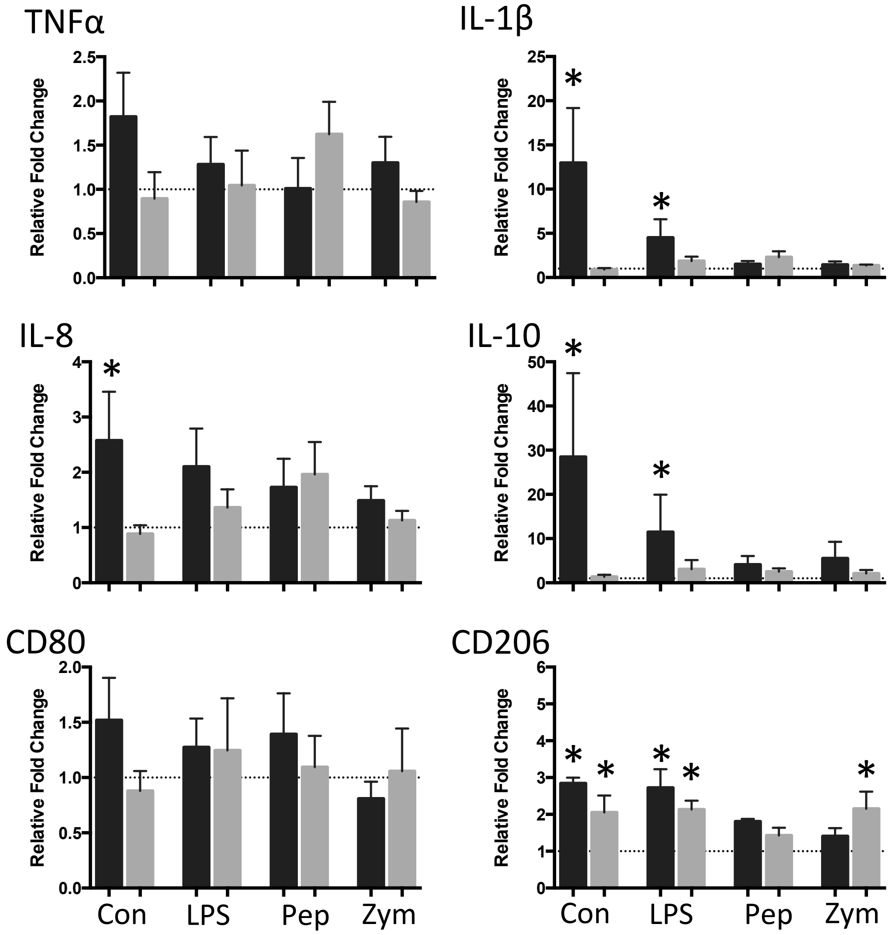
Figure 6 (cont'd).

C.



Graphs represent the gene expression response of AM from control horses (dark) and RAO-susceptible (light) horses when stimulated with LPS (A), peptidoglycan (B), or zymosan (C) at baseline and following natural challenge (Nat. Challenge). Data represents gene expression relative to media treated cells at baseline (fold change, mean ± sem). * $p \leq 0.05$ compared to media treated cells within same time point, ‡ $p \leq 0.05$ compared to same stimulation conditions at baseline, † $p \leq 0.05$ compared to non-RAO susceptible group.

Figure 7. Natural Challenge Differentially Alters the Phenotype of Alveolar Macrophages From RAO-Susceptible and Control Horses



Graphs represent AM gene expression in control horses (black bars) and RAO-susceptible (light bars) following incubation with media control (Con), lipopolysaccharide (LPS), peptidoglycan (Pep), or zymosan (Zym). Data from figure 2 are presented as the fold change (mean ± sem) following natural challenge relative to its expression at baseline. $^*p<0.05$ significant effect of natural challenge.

Discussion

Our findings indicate that the phenotype of alveolar macrophages from RAO-susceptible horses at baseline (in remission) differs from that of control horses in that the former have greater gene expression of IL-10, and display an enhanced responsiveness to LPS stimulation (increased expression of IL-8 and IL-10).

The term "alternatively activated macrophages" (M2 phenotype) refers to a spectrum of phenotypes that are broadly associated with immune regulation, resolution of inflammation and wound repair. The term “alternatively activated” refers to the fact that it differs from the classical, microbial/IFNγ-activated cell.[207] In addition to IL-4, other mediators can induce different M2 sub-phenotypes, including regulatory macrophages (M-reg). The M-reg phenotype predominately expresses IL-10 and can be induced in response to stimulation with immune complexes[208], certain TLR ligands[209] corticosteroids,[136] adenosine[137,138] and phagocytosis of apoptotic cells.[135] Additionally, activated M1 macrophages can transition to become M-regs as a means of controlling and resolving inflammation.[138] The IL-10 producing alveolar macrophage phenotype detected in RAO-susceptible horses is suggestive of an M-reg phenotype. Typically, M-reg also display significantly suppressed IL-12 expression. In the present study, baseline expression of IL-12p40 was similar in RAO-susceptible horses and control horses. However, in support of the M-reg phenotype, natural challenge induced a modest but significant decrease (mean -1.7 fold difference) in RAO-susceptible horses (Table 6) and there was a trend for reduced IL-12p40 expression in RAO-susceptible horses relative to controls (mean -2.8 fold difference (p=0.08)(Table 5).

Interleukin-10 is a pleotropic cytokine that can induce tolerance in T-cells, inhibit T-cell proliferation and Th1/Th2 cytokines and decrease the pro-inflammatory response of monocytes/macrophages, thus it is classically considered immunosuppressive and anti-inflammatory.[128,129] In RAO, it is possible that alveolar macrophage derived IL-10 contributes to the regulation of the subclinical pulmonary inflammation that remains during remission.[10,11,67,210–212] Furthermore, it may contribute to the down-regulation of Th1/Th2 cytokines in BAL lymphocytes that has been documented during RAO-remission.[43]

Increased numbers of IL-10 producing macrophages have been detected in atopic asthmatics [213–216] however, there are opposing accounts on the exact role that IL-10 plays in the context of chronic pulmonary inflammatory disease. Interleukin-10 has been associated with reduced allergic airway inflammation and airway hyperresponsiveness.[217,218] Yet, IL-10 can also potentiate pulmonary pathology by promoting Th2 effector cytokines,[213,219] airway hypersensitivity,[220] mucus metaplasia, and airway remodeling.[221] There are opposing data on the contribution of Th2 cytokines to RAO pathogenesis[38,39,42,43,46] thus it is unclear if alveolar macrophage derived IL-10 could promote disease pathogenesis by augmenting the Th2 pathway.

Generally, exogenous IL-10 significantly reduces the pro-inflammatory response of LPS stimulated macrophages.[131,222] Thus it was somewhat surprising that the alveolar macrophage from RAO-susceptible horses maintained responsiveness to agonist stimulation and in fact, expressed elevated neutrophilic chemokine IL-8 when stimulated with LPS. However, stimulation of TLR-4 or TLR-2 can modify IL-10-receptor function in alveolar macrophages, which could contribute to the lack of autocrine immunosuppression observed.[101] Further, while M2 macrophages are typically considered to exhibit a diminished pro-inflammatory response to microbial stimuli, M2 macrophages induced in murine allergic asthma[223] or in presence of in-vitro IL-33[174,224] (associated with Th2 polarized pathology[225]) possess enhanced expression of pro-inflammatory genes when stimulated with LPS. In the present study, enhanced response to LPS was not mediated by differences in expression of TLR-4 (Table 5) and the molecular mechanisms that mediate this sensitivity requires further investigation.

Furthermore, stimulation of isolated alveolar macrophages with LPS generally induces expression of pro-inflammatory TNFα, IL-1β, IL-8, and anti-inflammatory IL-10, thus it is interesting that only IL-8 and IL-10 were enhanced by LPS stimulation at baseline (Figure 6). This could suggest that there is differential regulation of IL-8 and IL-10 within the RAO-susceptible alveolar macrophage. One possible explanation could be exposure to adenosine which is elevated in horses with lower airway inflammation.[226] Adenosine can induce regulatory macrophages and activation of adenosine receptors can modulate the inflammatory response of equine monocytes to LPS, differentially enhancing production of IL-10 and IL-8.[227]

In vivo, RAO-susceptible horses are more sensitive to LPS than control horses, developing enhanced neutrophilic inflammation at lower LPS doses.[61] It is possible that enhanced IL-8 production by alveolar macrophage could contribute to the early neutrophil recruitment. In this respect, our data are in keeping with Laan et al[87] who also reported that AMs from RAO-susceptible horses display increased sensitivity to nebulized LPS and hay dust suspension. On the other hand, in the present study, IL-10 is similarly enhanced by LPS stimulation, (Figure 6A) and as IL-10 can reduce neutrophil recruitment to the lung[228,229] it is possible that elevated IL-10 could counteract the effects of IL-8.

In contrast to our study, Laan et al[87] failed to detect increased expression of IL-10 from alveolar macrophages from RAO-susceptible horses. It is possible that differences in macrophage isolation technique and culture conditions could account for disparity amongst investigators. Polarized M2 phenotypes may be less adherent,[161,222,230,231] and isolation of alveolar macrophages by adhesion (as used by Laan et al.) could select against this phenotype. Interestingly, in the present study we did not detect an enhanced response to agonists peptidoglycan and zymosan. It is possible that alveolar macrophages specifically develop sensitivity to LPS but not to other microbial stimuli, but as the concentrations of peptidoglycan and zymosan induced a much greater inflammatory response compared to LPS stimulation, it is certainly possible that this masked any subtle differences in macrophage sensitivity.

We also compared the phenotypic response of both groups to hay and interestingly, alveolar macrophages from each group responded differently to natural challenge. Control horses developed an M1-like phenotype (i.e. mixed expression of pro-inflammatory IL-1β, IL-8 and immune-regulatory IL-10, and enhanced response to LPS) in conjunction with increased CD206 expression (M2 marker). In contrast, the phenotype in RAO-susceptible horses remained relatively stable only showing increased CD206 expression. CD206 is the canonical M2 associated receptor induced by IL-4.[112] As other characteristics of IL-4 activated equine M2 macrophages were not observed in either group (Chapter 2) it is unlikely that a pulmonary environment dominated by IL-4 induced the CD206 expression observed. CD206 is a multifunctional phagocytic receptor and may have a number of functions in the context of chronic pulmonary inflammatory disease. Recognition of bacteria and fungi can induce production of pro- or anti-inflammatory cytokines depending on the stimulant.[115] The CD206 receptor can also phagocytose a variety of allergens (e.g hay dust mite, dog and cat allergens) and promote Th2 polarization.[232] Furthermore, CD206 may contribute to the resolution of inflammation through its capacity to phagocytose myeloperoxidase.[118]

The present data demonstrates that organic dust is a noxious stimulant that elicits an M1-like phenotype in control horses and suggests that the alveolar macrophage participates in the normal inflammatory response to hay dust.[64,194,201,233] In partial agreement, Joubert et al[88] reported that 24 hours of natural challenge induced increased expression of neutrophil chemokines (IL-8 and MIP2) in equine alveolar macrophage from control horses, but in contrast to our findings RAO-susceptible horses had an equivalent response. However, Joubert et al also suggested that variations in natural challenge had a greater influence on the inflammatory response as opposed to disease state. Thus presumably, variations in composition or concentrations of organic dust could influence the response on the alveolar macrophage, and account for differences between studies.

In vitro activated M1 and M2 phenotypes typically have distinct gene expression patterns and functions. It is interesting that complex macrophage phenotypes are present in both populations. At baseline the RAO-susceptible expresses an immune-regulatory M2-like phenotype (producing IL-10) that surprisingly displays enhanced LPS responsiveness. Further, following natural challenge control horses display mixed pro-inflammatory M1 markers, the M2 marker CD206 and enhanced LPS responsiveness. These phenotypes are likely a consequence of the complex physiological environment of the inflamed lung.

Macrophage activation states are dynamic and can change with the course of inflammation[138,143] and dual up-regulation of M1 and M2 phenotypic markers can result from represent exposure to a mixture of M1 and M2 activating stimuli[144] or could be indicative of dynamic transition between activation states.[138]

In summary, RAO-susceptible horses in remission possess a divergent alveolar macrophage phenotype that responds differently to natural challenge. Overall, this suggests that the alveolar macrophage plays a prominent role in RAO immunopathology. However, exactly how this phenotype affects other cells of the innate and adaptive immune system in this complex inflammatory environment remains to be determined.

Chapter 4.

Concluding Discussion

A central feature of RAO is a greatly enhanced inflammatory response to HD compared to control horses.[62] However, the underlying immune mechanisms that result in this differential inflammatory response are unknown. It is clear, however, that HD induces an inflammatory response from the innate immune system and that RAO-susceptible horses have a hyperresponsive innate immune system.[61] However, which immune cells or exact mechanisms that underlie this are unknown. The work presented here addressed the overarching hypothesis that the differential response to HD would be associated with differential alveolar macrophage phenotypes.

The equine specific, transcriptional, gene expression markers of alveolar M1 and M2 phenotypes have been presented. Data from other species indicate that gene expression profiles can differ between monocyte/macrophage subpopulations[234] and macrophages from different sources exhibit differences in M2 gene expression profiles and function.[235] The data presented in Chapter 2 is thus particularly relevant for the study of equine pulmonary disease as alveolar macrophages (and not monocyte-derived-macrophages) were used to investigate M1/M2 phenotypes and the agonists used to study the inflammatory response were selected due to their presence in hay dust. These investigations could be extended in the future to include gene expression characterization of additional in-vitro derived alternative macrophages.[110] The characterization of equine macrophage phenotypes provides novel and important information about equine alveolar macrophage biology and adds to a growing body of evidence of unique equine immune mechanisms.

The data presented in Chapter 3 demonstrates that RAO-susceptible horses in remission possess a divergent alveolar macrophage phenotype compared to control horses. Based on the increased expression of IL-10, this could be considered an M2-immune regulatory phenotype that retains LPS responsiveness. The presence of different macrophage phenotypes suggests that an M2-immune regulatory macrophage plays a role in RAO immunopathology. However, further studies are required to determine what factors induce this phenotype and how this phenotype contributes to individual pulmonary responses. It will be important to next investigate interactions with adjacent cells in the lung environment to explore if this phenotype promotes or attenuates the inflammatory response. Although IL-10 is an immune regulatory cytokine it is possible that this phenotype promotes neutrophilic inflammation. Macrophage phagocytosis of apoptotic neutrophils is an important mechanism for resolution of airway neutrophilia,[9] however, persistent neutrophilia is common weeks after antigen avoidance.[20] Alternatively-activated macrophages show impaired phagocytosis of bacteria and zymosan,[163] thus it is possible that phagocytosis of apoptotic neutrophils is similarly impaired. Further, impaired phagocytosis of hay dust components could promote persistence of inflammation as it could prolong the presence of these stimulatory molecules within the airways. As a counterbalance, IL-10 can reduce neutrophil pro-inflammatory cytokine production[236] thus it would also be interesting to investigate the effects of alveolar macrophage products on the neutrophil inflammatory response.

In summary, these data provide novel information that contributes to our knowledge of equine alveolar macrophage biology and RAO immunopathology and supports that continued investigations of the alveolar macrophage in RAO disease pathogenesis are warranted.

APPENDICES

Appendix 1.

Selection of Reference Genes for Quantitative Real Time PCR

Introduction

In the reported studies, the alterations of alveolar macrophage phenotype are evaluated by measuring gene expression. These changes were measured using relative quantification; which standardizes the target gene expression against that of a stable reference gene ($\Delta CT = CT_{\text{target gene}} - CT_{\text{reference gene}}$). This allows correction for differences in sample quantity and quality so that changes in target gene expression reflect gene-specific variation. A variety of reference genes are commonly employed to perform this function, however, the stability of reference genes can vary between biological sample types (e.g macrophage versus neutrophil) and experimental methodology. It is now recommended that the reference gene stability be validated for each tissue type or experimental methodology, and it is also suggested that an average of 3 or more endogenous genes be used.[237] Thus to study equine alveolar macrophage phenotype characterization using qRT-PCR, it was first important to establish the most appropriate reference genes. The stability of 9 candidate reference genes was evaluated (Table 7). These were tested in alveolar macrophages enriched from 6 control horses (using adhesion technique) that were untreated or stimulated with 1ug/ml LPS (n=15 samples total). The stability of the endogenous genes was then evaluated using Best Keeper analysis.[238]

Table 7. Panel of Candidate Reference Genes

Reference Gene	Abbreviation
β-actin	ACTIN
Ubiquitin	UBIQ
β-2 microglobulin	B2M
Elongation factor 1α	ELF1
18s ribosomal RNA	18S
Succinate dehydrogenase complex	SDHA
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Hypoxanthine phosphoribosyltransferase	HPRT
β glucuronidase	BGUS

Results and Discussion

Alveolar macrophages were isolated from six control horses. Bronchoalveolar lavage was performed as standard and BAL fluid (BALF) was immediately placed on ice. BALF was then transferred to sterile 50ml tubes and centrifuged at 400xg for 8 min. Supernatant was discarded and cells were washed twice more then counted and resuspended at a concentration of 1x10^6 cells/ml in RPMI containing 10% heat inactivated equine serum, antimycotic and antibiotic. Cells were then transferred onto 6 well culture dishes (3mls), allowed to adhere for 2 hours at 37°C, 5% $CO_{2,}$ then non adherent cells were washed off. Cells were then bathed in equine media alone (control) or equine media containing LPS (1μg/ml or ml) and incubated for 1hr (n=8), or 9 hours (n=7) after which cells were rinsed once with HBSS, harvested by adding RLT-Lysis Buffer (Qiagen) and homogenized using Qiagen QIAshredder™ spin columns. Total RNA was extracted and purified using Qiagen RNeasy® Mini Kit and then frozen at -80°C until RNA extraction. RNA concentration was measured using a spectrophotometer and integrity of

RNA (RIN) was assessed using the Agilent 2100 Bioanalyzer® (Agilent Technologies) and a RIN score of >6.5 was considered acceptable.

Equal concentrations of RNA were reverse transcribed (High Capacity cDNA Reverse Transcription Kit®, Applied Biosystems) to create cDNA. The cDNA was then pre-amplified (TaqMan® PreAmp Master Mix Kit, Life Technologies) according to manufacturer's instructions). Quantitative PCR was performed using TaqMan® Gene Expression Master Mix (Life Technologies) and TaqMan® Expression Assays. PCR was performed in triplicate on ABI 7900HT Real-Time PCR System® (Applied Biosystems).

The descriptive statistics for each candidate reference gene are shown (Table 8). Based on the standard deviation (SD), the genes were ranked 1-9 with 1 being the lowest SD. Genes with SD>1 was considered too variable and excluded from further analysis in BestKeeper.

Using the CT values, BestKeeper analysis creates a "BestKeeper Index" for each sample and then performs repeated pair-wise correlation analysis creating correlation coefficient (r) and p-value (p) for each gene (Table 9). The genes with the highest correlation coefficient and lowest p-values were β-actin, ELF1 and HPRT. Analysis of the average CT of ELF1 and HPRT together slightly improved the correlation coefficient and p-value. The average of β-actin, ELF1 and HPRT was almost identical to the combination ELF1 and HPRT. Thus based on this data ELF1 and HPRT were selected for as the most appropriate reference genes. The alveolar macrophages used for this validation experiment were isolated from healthy control horses and did not include RAO-susceptible horses. However, there was no difference in reference gene values (average of ELF/HPRT) between groups (RAO-susceptible, 15.844±0.40, control horses, 15.873±0.419 (mean CT ±sd, chapter 1) indicating that these genes were appropriate for use.

Table 8. Descriptive Statistics of Candidate Reference Genes Based on the Cross Threshold Point (CT)

	ACTIN	UBIQ	B2M	ELF1	18S	SDHA	GAPDH	HPRT	BGUS
geo Mean [CT]	8.84	23.46	6.61	7.36	12.34	15.47	25.81	15.92	16.33
ar Mean [CT]	8.86	23.50	6.62	7.37	12.35	15.49	25.99	15.94	16.38
min [CT]	8.28	20.38	6.24	6.73	11.62	14.38	19.48	14.74	14.70
max [CT]	10.04	26.50	7.20	8.24	13.99	17.20	30.75	17.47	18.61
std dev [± CT]	0.48	*1.06	0.20	0.28	0.43	0.58	*2.23	0.55	*1.06
CV [% CT]	5.40	4.51	3.09	3.83	3.44	3.72	8.58	3.46	6.52
ranking	4	7	1	2	3	6	9	5	8

Data represents descriptive statistics (CT) for each candidate reference gene from 15 samples. *Indicates genes excluded from analysis due to standard deviation >1

Table 9. BestKeeper Analysis Showing Correlation Coefficient (r) and P-Value (p) From Candidate Reference Genes

	ACTIN	B2M	ELF	18S	SDHA	HPRT	HPRT/ELF	ACTIN/ELF/ HPRT
Correlation coefficient (r)	0.866	0.301	0.93	0.68	0.36	0.85	0.94	0.958
p-value (p)	0.001	0.275	0.001	0.005	0.1	0.001	0.001	0.001

Appendix 2.

Characterization of β-Glucan Receptor Isoforms in the Horse

Introduction

Equine recurrent airway obstruction (RAO) is a chronic inflammatory disease that is triggered with the inhalation of hay dust. The exact immunopathology of RAO is unclear, and while an allergic etiology has been proposed the data supporting this is conflicting. However, in addition to containing potential allergens, hay dust contains a complex mixture of microbial cell wall components[202] which can stimulate the innate immune system. The presence of fungal elements in hay dust is particularly significant as both moldy hay[27] (overgrowth of fungal species) and the β-glucan content of hay dust[62] are potent triggers for inducing exacerbation of RAO.

Fungal cell walls contain the complex carbohydrate β-glucan,[239] which binds to the innate pathogen recognition receptor, the β-glucan receptor ((βGR), Dectin 1 Dendritic cell associated C-type lectin)). This receptor is expressed by many innate immune cells including neutrophils and macrophages, and stimulation of this receptor is important in fungal immunity.[116] The βGR is a C-type lectin transmembrane receptor and is composed of a glycosylated extracellular C-type lectin binding domain, a short extra-cellular stalk, a transmembrane domain and a cytoplasmic tail that contains a signaling domain. In other species a number of splice variants of the βGR gene (*CLEC7A*) are expressed.[240–244] Principally, 2 major splice isoforms are expressed that differ in their presence of the extracellular stalk. The full length (βGRA) or stalkless isoforms (βGRB) are both functional in recognizing β-glucan.[240,241] Additionally, 6 less abundant splice variants (minor variants) have been described in humans and 1 additional minor variant has been described in pigs.[242] In humans, these minor variants have no β-glucan binding capacity but it is proposed that their expression could serve a regulatory role.[241]

Thus, despite β-glucan being a major component of hay dust there are no investigations characterizing the equine βGR. Thus our objective was to determine if equine splice isoforms of the βGR are expressed in pulmonary cells.

Materials and Methods

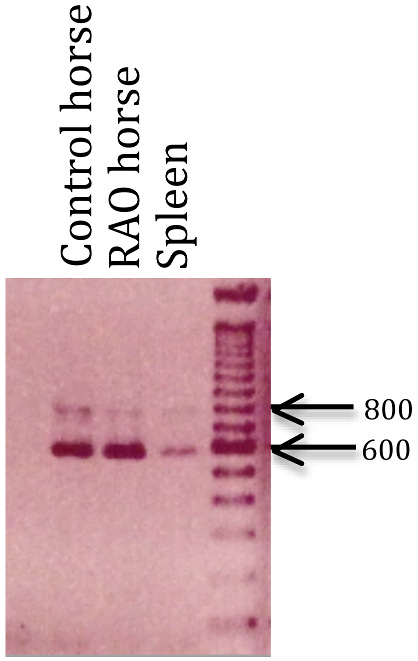
To clone the β-glucan receptor the human and equine *CLEC7A* gene sequences were aligned (UCSC Genome Browser/EquCab2.0). Primers were designed using Primer3 software to amplify the full-length coding sequence (forward primer 5'-3' TCA AAC GCT ATG TCA ATT CAG G, reverse primer 5'-3' TGG TCG TAA ATG ATT GAT AGG TG). Equine pulmonary cells were obtained from bronchoalveolar lavage (as previously described[245]) from RAO-susceptible and control horses. Some samples were collected from RAO-susceptible and control horses prior to and following >1week exposure to hay and straw. Tissues (spleen, respiratory mucosa) were collected post euthanasia from healthy horses and immediately snap frozen using liquid nitrogen and the stored at -80°C until RNA isolation. BAL cells were lysed using RLT-Lysis Buffer (Qiagen) and homogenized using a Qiagen QIAshredder™ spin columns. Tissue samples were disrupted using a mortar and pestle and RLT-Lysis Buffer and homogenized using Qiagen QIAshredder™. RNA was isolated and purified from all samples using Qiagen RNeasy Mini®Kit which involves a DNAase digest step. RNA concentration was measured using Qubit® 2.0 fluorometer and RNA and was reverse transcribed (High Capacity cDNA Reverse Transcription Kit, Applied

Biosystems) to create cDNA. PCR was performed (4 minutes at 94°C, 40 cycles of 1 minute 94°C, 1 minute 60°C, 1 minute 72°C) using *Taq* DNA Polymerase (Life Technologies™) and PCR products were visualized using gel electrophoresis using ethidium bromide stained 2% agarose gel. Amplified PCR products from 1 RAO-susceptible and 1 control horse were mixed together and cloned using Zero Blunt® TOPO® PCR Cloning Kit (Life Technologies™). Transformed bacteria were plated overnight on selective media (LB with 50ug/ml kanamycin) after which positive transformants were transferred onto a reference agar plate (plate with labeled grid lines). For each positive transformant, PCR and 2% agarose gel electrophoresis was used to assess the cloned product size. A selection of clones with variable product sizes were sequenced using ABI-3730 genetic analyzer. The resulting sequences from each sample were aligned next to the human and equine *CLEC7A* gene sequences using the UCSC Genome Browser. Using the sequences derived from cloning, primers were designed to specifically amplify the splice variants and the expression of the splice variants was investigated in a variety of tissues using RT-PCR and gel electrophoresis as above.

Results and Discussion

RT-PCR and gel electrophoresis indicated two main variants with a large isoform being approximately 800 base pair (bp) in size and a smaller isoform being approximately 600 bp (Figure 8). These bands approximately corresponded to the full-length isoform (βGRA) (comprised of exons 1-6, 744 base pairs) and the stalkless isoform (βGRB, spliced exon 3, 606 base pairs). Sequencing of the cloned PCR products confirmed that these isoform variants corresponded to human βGRA and βGRB respectively (Figure 9). Thus, similar to other species,[240–244] the two major isoforms of the βGR (βGRA and βGRB) are expressed in the horse. The intensity of the lower band was greater suggesting that in BAL cells and equine spleen the βGRB isoform is the predominantly expressed. This is similar to the expression pattern of human monocytes, however, expression patterns of βGRA and βGRB can differ amongst cell types suggesting cell specific regulation.[241] Regulation of isoform expression could impact the inflammatory response, as zymosan stimulated βGRA and βGRB produce significantly different quantities of TNFα.[246]

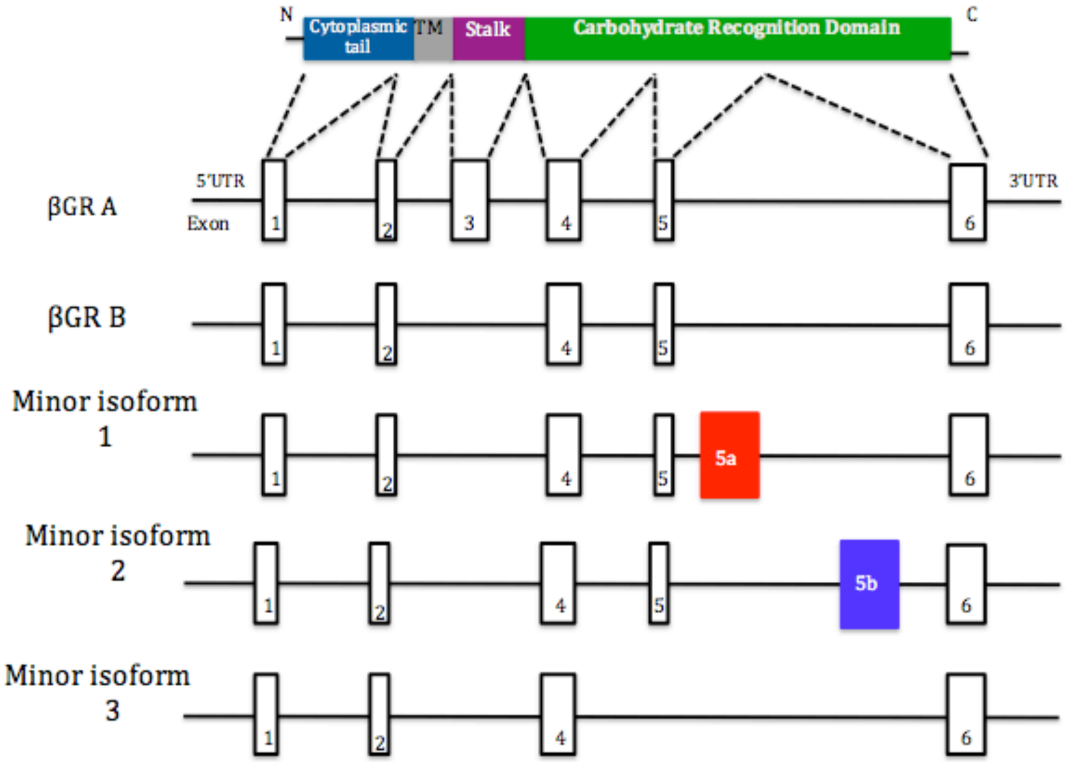
Figure 8. Expression of β-Glucan Receptor Splice Isoforms Using RT-PCR



PCR amplified cDNA from equine bronchoalveolar lavage cells (taken from a normal healthy horse and a RAO-susceptible horse) and spleen was analyzed for the presence of the β-glucan receptor splice variants. The two major bands at 800 and 600bp corresponding to βGRA and βGRB respectively are indicated by arrows.

In addition, sequencing also identified 3 further isoforms, which were variations of βGRA (Figure 9). Two of these isoforms (minor isoform 1 and 2) contained small insertions (between exons 5 and 6) and do not correspond to any human isoform variants. Exons 3 and 5 were deleted from minor isoform 3 and this configuration corresponds with human βGRD.[241] Minor isoforms 1,2, and 3 were 720, 663 and 471 nucleotides long respectively. Exons 1 and 2 code for the cytoplasmic tail and transmembrane domain, exon 3 codes for the extracellular stalk and exons 4-6 code for the carbohydrate recognition domain (CRD). As each of the 3 minor variants detected had sequence alterations that affected the CRD these variants would be predicted to have impaired βGR function and indeed, human minor variants βGRC-H do not have zymosan binding capacity.[241] These minor isoforms were not observed as distinct bands when amplified PCR products (primers amplified full length coding sequence) were analyzed using ethidium bromide agarose gel electrophoresis (Figure 8), thus it is likely that the level of expression of these variants is minor relative to βGRA and βGRB isoforms and below the level of detection using this method.

Figure 9. Equine β-Glucan Receptor Splice Isoforms Determined by Cloning and Sequencing



The cDNA from 1 RAO-susceptible and 1 control horse were amplified with primers designed to include the entire coding region of the *CLEC7A* gene. PCR product was then cloned in *E.coli* and positive transformants were selected for sequencing and Figure 9 illustrates the splice variants that were detected. The colored bar illustrates the regions of the receptor that each exon encodes (based on human β-glucan receptor). The red (5a) and blue (5b) boxes represent exon insertions. All minor isoform variants possess alterations in the carbohydrate recognition domain.

β-glucan expression on myeloid cells is well described. However, βGR is also expressed on airway epithelium.[247] Using primers designed to specifically amplify individual isoforms (Table 10), βGRA and βGRB were found to be expressed in respiratory mucosa and lung in addition to non-respiratory tissues liver and spleen (Table 11). However, the relative quantities the isoforms were not compared. Further, minor isoform 3 was expressed in all tissues tested but minor isoforms 1 and 2 were not consistently expressed, suggesting a degree of tissue specificity for isoform expression.

Table 10. Location of Primers Used to Identify β-Glucan Splice Isoforms

Splice Variant	**Forward Primer**	**Reverse Primer**
βGRA	Exon 4-3 junction	Exon 3
βGRB	Exon 4	Exon 4-2 junction
Minor isoform 1	Exon 6-5a junction	Exon 5a
Minor isoform 2	Exon 5b-5 junction	Exon 5
Minor isoform 3	Exon 6-4 junction	Exon 4

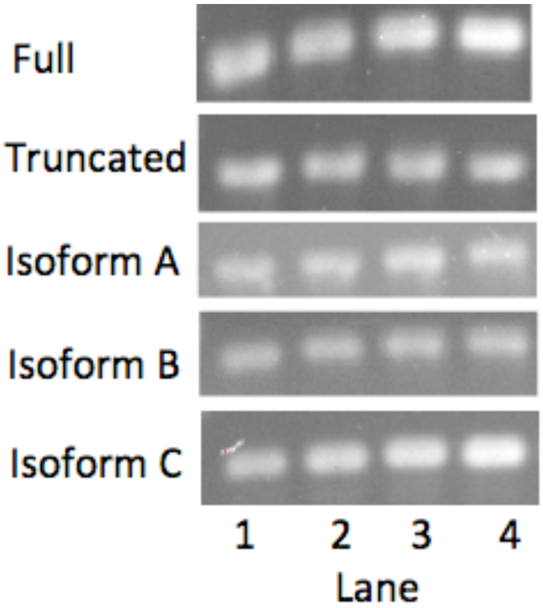
Table 11. Tissue Specific Expression of β-Glucan Receptor Splice Isoforms

Equine Splice isoform	**Nasal Mucosa**	**Tracheal mucosa**	**Lung**	**Liver**	**Spleen**
βGRA	2/2	2/3	2/2	1/2	2/2
βGRB	2/2	2/3	2/2	2/2	2/2
Minor isoform 1	0/1	1/2	1/2	0/1	1/1
Minor isoform 2	2/2 (weak)	0/3	1/3 (weak)	1/2 (weak)	0/2
Minor isoform 3	2/2	3/3	2/2	2/2	2/2

For each isoform the numbers indicate the frequency positive samples / the total number of samples tested in each tissue e.g 2 of 3 (2/3) tracheal mucosal samples expressed βGRA. Minor isoform 2 was only weakly expresses in certain tissues as indicated.

Similarly, expression of isoform variants was evaluated in alveolar macrophages isolated from normal and RAO-susceptible horses preceding and following exposure to hay. Both groups of horses expressed each of the 5 isoform variants both preceding and following exposure to hay/straw (Figure 10), suggesting that major differences in alveolar macrophage regulation of βGR isoform variants was not associated with exposure to hay/straw in either group.

Figure 10. Expression Profile of β-Glucan Receptor Splice Isoforms in Alveolar Macrophages From RAO-Susceptible and Control Horses



RT-PCR showing the β-glucan receptor splice variants detected in alveolar macrophages. Lanes 1& 2 are representative of alveolar macrophages from RAO-susceptible horses (n=3) before and after hay exposure respectively. Lanes 3 & 4 are

Figure 10 (cont'd)

representative of alveolar macrophages from normal horses (n=3) before and after hay exposure respectively. All isoforms were detected in all samples

The functional consequence of alternative splicing is not always apparent. It is suggested that alternative splicing is often a non-functional stochastic event,[248] however, alternative splicing may also serve as a means of gene regulation by generating splice isoforms that are subjected to nonsense mediated decay or generating proteins with different functions.[249,250] It is not known if changes in the expression of βGR isoforms could contribute to regulation the inflammatory response to fungi.

In conclusion, βGRA and βGRB are expressed in horses in addition to 3 minor isoforms. Expression is present in white blood cells but also respiratory epithelium. This is the first study to characterize the β-glucan receptor in horses and will provide a fundamental platform for future studies investigation interaction of β-glucan and the equine innate immune system.

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