Immunophenotypic Characteristics of Equine Monocytes and Alveolar Macrophages

by

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Hematopoietic cells of the myelomonocytic lineage play a central role in orchestrating both innate and adaptive immunity. They are important in the control of infectious agents and in the pathogenesis of diseases characterized by dysregulated immune response. Like allergic asthma in human patients, recurrent airway obstruction (RAO) of horses is a disease exemplified by chronic airway inflammation in the absence of infectious agents. However, unlike allergic asthma, RAO is marked by preponderance of neutrophils rather than eosinophils in the airways. Attempts to understand the immunological basis of RAO by studying lymphocytes produced equivocal results. This thesis examined the possible role of alveolar macrophages (AM) recovered from bronchoalveolar lavage fluid (BALF) in RAO. Since macrophages are predominantly derived from circulating monocytes, the thesis investigated first the phenotypic characteristics of circulating monocytes, second those of macrophages in vitro derived from monocytes, and finally attributes of AM derived in vivo.

Flow cytometric analysis following antibody staining of monocytes from 61 horses showed that the clustering pattern of human leukocytes may not always be extrapolated to horses when using this technique since clusters of granulocytes often spill over into the
monocyte population. The study showed that DH24A, a monoclonal antibody directed against CD90, which recognizes T cells in other species, will specifically recognize granulocytes in horses and was therefore used to separate neutrophils from monocytes during analysis. In addition, investigation of circulating monocytes showed that expression of the hemoglobin-haptoglobin receptor CD163 on circulating monocytes is significantly increased in horses with systemic inflammation when compared with healthy horses. Evaluating cytokine and chemokine production by macrophages, it was demonstrated that CD163⁺ macrophages preferentially expressed IL10 while CD163⁻ macrophages showed predominant expression of CCL17. It was, therefore, concluded that CD163⁺ IL10-producing macrophages of horses are homologues of the alternatively activated anti-inflammatory macrophage subset of humans. Finally, probing of alveolar macrophages for CD163 and CD206 expression showed a significant reduction in the proportion of CD163⁺ macrophages in horses with RAO. These findings suggest that RAO is associated with a reduction in anti-inflammatory macrophages, an observation that may in part explain the chronic airway inflammation associated with this disease.
I wish to thank my supervisor, Dr. Dorothee Bienzle for her support, and for giving me a fresh training not only in the science of clinical pathology but also in the art of mentoring and exemplary leadership. I also wish to thank the members of my committee, Drs Viel and Caswell for their support, encouragement and timely critique at each stage of this work. I count it a unique opportunity to have these proven experts in the field of respiratory diseases on my committee.

The time in Guelph will always remain memorable because of the friendship cultivated in the Bienzle lab: Mary Ellen, who was always ready to help and ensure the lab did not become a zoo; Janet Beeler-Marfisi, who took on the horses to make sure I did not die of anaphylactic shock; Melanie, my nocturnal co-conspirator in the lab; Hakimeh; and Olivier Cote will always occupy a happy place on my heart.

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Chapter 1

Literature Review

*The Mononuclear Phagocyte System and Recurrent Airway Obstruction of Horses*

The mononuclear phagocyte system (MPS) is a network of cells that include committed bone marrow precursors, circulating blood monocytes and tissue macrophages, and dendritic cells (DCs) found in different body organs (1). This family of cells is characterized by their ability to non-specifically phagocytose particles such as latex and colloidal carbon, and to specifically uptake immunoglobulin-coated particles through their surface Fc receptors (2). It is traditionally believed that bone marrow precursors proliferate and enter the circulation as monocytes before migrating into tissues, where they mature into macrophages and DCs (3). However, the unity of the MPS has been challenged by evidence for the existence of a separate embryonic lineage of phagocytic cells (4). Evidence that cells of the MPS can transdifferentiate into cells of other lineages (5-7), *in situ* differentiation and renewal of macrophages, and the recognition of DCs not derived from monocytes (8), the so-called plasmacytoid DCs, have changed the view of MPS cells as a homogenous population of cells (2, 8). Thus, differences in the origin and sequence of maturation of cells of the MPS may confer specific phenotypic and functional differences to tissue macrophages found in different parts of the body.

**Origin, maturation and tissue distribution of monocytes and macrophages**

The use of bone marrow reconstitution experiments in recent years has led to the development of methods that more faithfully replicate haematopoiesis *in vivo*. In such studies, highly-radiosensitive bone marrow stem cells are destroyed by gamma irradiation (9). Subsequent reconstitution of bone marrow with hematopoietic stem cells could then be used to trace the kinetics of appearance of cells bearing the markers of specific leukocyte lineages. Using this method, a distinct population of bone marrow cells which do not express any lineage-specific marker, lineage negative cells (Lin-), but rather express the Sca1 protein (spinal cerebellar ataxia 1) and c-kit (the receptor for stem cell factor) was recognized (10, 11). Inoculation of these Lin-, Sca1+, c-kit+ (LSK) cells was shown to completely reconstitute all hematopoietic cells in fully irradiated mice. However, it was recognized that, depending on whether these cells express Flt3 (Flt3+) or not (Flt3-), the ability of the LSK cells to reconstitute
hematopoietic cells was indefinite (Flt3\(^-\)) or definite (Flt3\(^+\)). This led to the identification of Flt3\(^+\) LSK cells as the earliest common progenitor of all hematopoietic cells including erythrocytes/megakaryocytes, lymphocytes, granulocytes and monocytes (12).

The Flt3\(^+\) LSK cells split into 3 distinct lineages: megakaryocyte/erythroid progenitor (MEP), Common Lymphoid Progenitor (CLP) and Common Myeloid Progenitor (CMP). These cells give rise to megakaryocytes/erythrocytes (MEP), lymphocytes (CLP), and monocyte/granulocytes (CMP). The expression of the IL-7R\(\alpha\)-chain by CLP makes this cell population easily separated from CMP which does not express this marker (13). The progression of CMP to either the granulocytic or monocytic lineage depends on the expression of specific transcription factors and surface receptors for specific cytokines and growth factors. In mice, the bone marrow cells that eventually become blood monocytes and tissue macrophages/DCs, variably called the Granulocyte Macrophage Progenitor (GMP) or Macrophage/Dendritic cell Progenitor (MDP), were found to be those that express CX3CR1, CD115, CD34 and CD16 (13-15).

The type of monocytes that migrates out of the bone marrow into the blood, and their subsequent fate, depend on the condition existing in peripheral tissue during development and maturation in the bone marrow. Detailed studies in the mouse have revealed that, when there is ongoing inflammation in peripheral tissue, monocytes emerging from the bone marrow are very high in the expression of Ly6C (Ly6C\(^++\) monocytes). Under homeostatic condition, however, monocytes leaving the bone marrow express this marker at a very low level (Ly6C\(^-\) monocytes). It has been shown that Ly6C\(^++\) monocytes must return to the bone marrow before they could become Ly6C\(^-\) and can subsequently return to the circulation. Studies using CCR2\(^-/-\) mice demonstrated that the emigration of specific monocyte subtype from the bone marrow may depend on chemokine signals produced in infected tissues (16).

Monocytes are found in the bone marrow, blood and in the vascular bed of the spleen and do not proliferate under homeostatic conditions (17). However, they have the capacity to function as immune effector cells and possess chemokine receptors that allow them to migrate to specific tissues and sites of inflammation. Under homeostatic conditions, monocytes migrate to tissues and differentiate into macrophages, which are often called different names depending on the organ, e.g. osteoclasts of bone, Kupffer cells in the liver, microglial cells in the nervous system, and alveolar macrophages in the lung. In response to inflammation, they
can also migrate into tissues where they differentiate into macrophages or inflammatory dendritic cells (iDCs), depending on the prevailing inflammatory milieu and specific pathogen-associated molecular patterns (PAMP) initiating the inflammatory response (9). In general, macrophages are believed to arise from circulating blood monocytes. According to this model, tissue macrophages lack the capacity to divide (18). However, using genetically-marked bone marrow from donor organs it was recently shown that this dogma may not be completely true (19, 20). This possibility was recently confirmed by the identification of a population of self-renewing stromal cells with a distinct capacity to produce monocytes and macrophages; these cells lack all currently known characteristics of stem cells (8).

**Monocyte-macrophage subsets and the regulation of adaptive immunity**

In humans, like mice, circulating blood monocytes have been divided into two subsets on the basis of expression of CD14, the co-receptor for LPS, and CD16, the FcγRIII receptor (9). In addition to these two receptors, monocyte subsets were found to express a distinct combination of chemokine, immunoglobulin and scavenger receptors (21). The CD14$^{++}$CD16$^{-}$ monocytes are large (~18 µm) and represent approximately 80-90% of circulating monocytes in human blood(21). Conversely, there is a dramatic increase in the proportion of the second subset, CD14$^{low}$CD16$^{++}$ monocytes, during infection (22). Indeed, there are distinct differences in the chemokine signals that lead to the recruitment of CD14$^{++}$CD16$^{-}$ versus CD14$^{low}$CD16$^{++}$ monocytes: while CD14$^{++}$CD16$^{-}$ monocytes express high levels of CCR1, CCR2 and CXCR2 and low levels of CX3CR1, CD14$^{low}$CD16$^{++}$ monocytes express a high level of CX3CR1 and low levels of CCR2 (23, 24). Thus, a proinflammatory subtype (CD14$^{low}$CD16$^{++}$) and an anti-inflammatory subtype (CD14$^{++}$CD16$^{-}$) of human monocytes were recognized (Table 1.1). Comparison of human monocyte subsets with those of mice produced a rather confusing result: the Ly6C$^{+}$ monocytes, released into circulation in response to inflammation in the mouse, expressed chemokine patterns similar to the anti-inflammatory human monocytes, while the Ly6C$^{-}$ monocytes of mice turned out to be similar to the proinflammatory human monocytes (25). Nonetheless, further characterization of monocyte subsets in mice and human showed that, on the basis of characteristics other than chemokine receptor expression, proinflammatory human monocytes are indeed similar to Ly6C$^{++}$ monocytes in the mouse (9).
Table 1.1  Monocyte/Macrophage subsets in humans and mice

<table>
<thead>
<tr>
<th>Marker</th>
<th>M1</th>
<th>M2</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Scavenger receptors:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD163</td>
<td>+</td>
<td>+++</td>
<td>CD163 = haptoglobin-hemoglobin receptor</td>
</tr>
<tr>
<td>CD206</td>
<td>+</td>
<td>+++</td>
<td>CD206 = mannose receptor</td>
</tr>
<tr>
<td>Ag Presentation:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD80,86, MHC II</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Chemokines:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL17 (TARC, ligand =</td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>CCR4)</td>
<td></td>
<td>??</td>
<td>TIE-2 is pro-angiogenic</td>
</tr>
<tr>
<td>TIE-2 (ligand = angiopoietin)</td>
<td></td>
<td>??</td>
<td></td>
</tr>
<tr>
<td>Cytokines:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ, TNF, IL-12, IL-1</td>
<td>+</td>
<td>+++</td>
<td>IL-12 is a heterodimer of p35 and p40</td>
</tr>
<tr>
<td>IL-4, TGF-β, IL-10, IL-13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolic Enzymes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginase</td>
<td>-</td>
<td>+++</td>
<td>Expression of iNOS and arginase varies across species</td>
</tr>
<tr>
<td>Inducible nitric oxide (iNOS)</td>
<td>+++</td>
<td>-</td>
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The importance of monocyte subtypes in the adaptive immune response became apparent once it was demonstrated that the responses of proinflammatory and anti-inflammatory monocytes to pathogens were different (1). In addition, the proinflammatory and anti-inflammatory phenotypes were found to be maintained even when monocytes differentiated into macrophages in tissue (18). Thus, proinflammatory monocytes were found to be similar to proinflammatory (M1) macrophages while anti-inflammatory monocytes were found to be similar to anti-inflammatory (M2) macrophages (24). Like T cells, production of specific cytokines and surface receptors identifies the two phenotypes: proinflammatory monocytes (M1) produce pro-inflammatory cytokines, while anti-inflammatory monocytes (M2) produce low levels of IL-1β, IL-12 and IL-23 but high levels of IL-10 (26). In addition, there is a marked difference in nitrogen metabolism between the two phenotypes: M1 produce a high amount of NO and upregulate nitric-oxide synthase 2 (NOS2), while M2 upregulate arginase-1 (Arg-1) leading to the catabolism of L-arginine to proline, polyamines and urea (27). Recent studies in rodents and human asthmatics have also associated the induction of the chitinolytic enzymes, chitotriosidase (ChT) and acidic mammalian chitinase (AMCase), in alveolar macrophages (AMs) with Th2 polarization in the allergic airway (28-30). Further studies identified these AMs as belonging to the M2 phenotype (30). Several studies have associated the preponderance of M1 or M2 monocyte-macrophages with diseases where Th1 or Th2 cells predominate, respectively (31-34). To date, M1 and M2 monocyte-macrophages have only been well characterized in human, mice, rat, cow and pigs (35). There is currently a paucity of information on monocyte-macrophage subsets in horses, including their possible role in disease.

**Diseases of the Equine Lower Respiratory Airway**

Many diseases of the lower respiratory airway of horses are non-infectious in nature. A potential complication of trauma, pneumonia and lung biopsy is pneumothorax and/or hemothorax; which may also occur spontaneously in horses after maximal exertion (36-38). Similarly, acute respiratory distress syndrome/acute lung injury (ARDS) often results from lesions that directly affect the lung parenchyma or secondary to vascular lesions causing capillary leakage. The condition is characterized by a normal pulmonary wedge pressure, decreased pulmonary compliance, hypoxemia caused by diffusion impairment and/or
ventilation-perfusion mismatch, and radiographic appearance of diffuse lung infiltrate. ARDS is distinguished from the surfactant-associated respiratory distress syndrome (RDS) seen in premature foals. To date, ARDS has only been reported in foals but not in adult horses (39). However, an ARDS-like syndrome has reportedly been produced in horses by inoculation with *Escherichia coli* or sublethal doses of endotoxin (38). It has been shown that the high sensitivity of the equine lung to endotoxin depends on the proinflammatory activities of pulmonary intravascular macrophages, suggesting that inflammatory response is tightly controlled in the equine airway. It is, therefore, not surprising that the most frequently reported diseases of the equine lung and lower respiratory airway are inflammatory in nature, often in the absence of any recognizable infectious agent.

**Inflammatory Airway Disease of Horses**

A study examining horses monthly in seven British flat race yards over a three-year period estimated a prevalence rate of 13.8% and incidence of 8.9 cases/100 horses/month for Inflammatory Airway Disease (IAD) in young horses (40). This disease is generally regarded as a mild inflammatory airway disease of performance horses younger than 7 years old (41). Investigation of IAD has been hampered by inconsistency of clinical case definition in the veterinary literature. In 2007, a consensus panel of the American College of Veterinary Internal Medicine (ACVIM) issued specific criteria to define IAD of horses. The inclusion criteria were: poor performance, exercise intolerance, or coughing, with or without excess tracheal mucus in a horse of any age; and non-septic inflammation detected by cytologic examination of bronchoalveolar lavage fluid (BALF) or pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange at rest or during exercise. The exclusion criteria were: evidence of systemic signs of infection (fever, hematologic abnormalities compatible with infection); and increased respiratory efforts at rest (42).

Clinical signs associated with IAD in horses include chronic, intermittent cough, increased mucoid airway secretions, and decreased performance. Cough is either exercise-induced or could occur at rest, although some horses with IAD do not cough (42). In addition, it is very rare to see horses with IAD without endoscopic evidence of excessive airway mucus; this is particularly true in yearlings and 2-year-old horses. Although horses
with IAD may have increased respiratory rate, no significant changes are observed when pleural pressure is measured at expiration. A marked worsening of exercise-induced hypoxemia has been consistently associated with IAD in racehorses (43).

The diagnosis of IAD involves cytologic evaluation of BALF and pulmonary function testing. The BALF of horses with IAD is characterized by a mixed inflammatory response with an increase in total nucleated cell count and mild neutrophilia, lymphocytosis and monocytosis (44). An increase in the proportion of mast cells (>2%) and eosinophils (>0.1%) has also been consistently associated with IAD. Pulmonary function testing does not show a marked deviation from normal values in horses with IAD. However, the use of rebreathing techniques along with forced expiration and forced oscillation techniques has been shown to induce changes consistent with airway obstruction in horses with IAD (45). In addition, horses with elevated proportions of mast cells and eosinophils show airway hyperresponsiveness.

The pathogenesis of IAD is currently not fully understood. However, it has been suggested that bacteria and viruses may play a role in the pathogenesis of this condition. The presence of an increased proportion of eosinophils and mast cells, and the exacerbation of symptoms following stabling of affected horses have led to the suggestion that there is an allergic component to IAD (46).

**Recurrent Airway Obstruction**

Equine recurrent airway obstruction (RAO), also called heaves, is a chronic disease of the lower airways of stabled horses characterized by bronchospasm, excessive mucus production, increased respiratory efforts, and exercise intolerance. It is one of the most common diseases of mature stabled horses. Unlike IAD, the extensive obstruction of the lower airways in horses leads to the presence of clinical signs even at rest. The clinical signs associated with RAO may be seasonal and exacerbated when horses are housed indoors and exposed to moldy hay. Clinical signs often resolve within a few days after horses are placed on pasture. This condition must be differentiated from summer-associated pulmonary disease reported in horses in southern United States; SAOPD is specifically associated with allergic reaction to pollens found in southern USA. Like IAD, horses with RAO often present with complaints of chronic cough, exercise intolerance, and mucopurulent nasal discharge. However, unlike IAD
where hypoxemia follows exercise, gas exchange impairment could be detected in resting horses with IAD.

**Epidemiology of RAO**

A detailed analysis of risk factors for RAO in 1,444 horses examined over a 10-year period gave the best indication of the epidemiological factors associated with this disease. The study showed that horses ≥7 years old were 6-7 times more likely to have RAO than those <4 years old, confirming that age is a major risk factor for this disease (47). The study also showed that Thoroughbreds and American Trotters were more likely to be diagnosed with RAO than ponies. This finding on breed association was different from the results obtained previously by Bracher et al in Swiss horses where ponies were found to be more susceptible to RAO than other horses (48). There is currently no consensus on the impact of breed on the susceptibility to RAO. However, other studies investigating full and half-sibling groups of Warmblood and Lipizzaner horses showed an increased risk in offsprings with one or two affected parents (49). In this group, RAO was demonstrated to be a polygenic disease rather than a condition inherited in a simple Mendelian fashion (50). This study used segregation analysis to show that RAO is controlled by an autosomal dominant gene in some horses while it is controlled by an autosomal recessive gene in others (50). A more recent study used a genome-wide scanning approach to identify regions on chromosomes 13 and 15 that were significantly associated with RAO in Warmblood horses (51, 52).

In addition to age and genetic factors, the incidence of RAO has consistently been shown to depend on climate and weather conditions. It has long been noted that the incidence of RAO is high in Switzerland and Great Britain, representing cold and wet climate, while it is very low in Australia, representing a warm and dry climate (53). In the study of Ward et al., a significant association was found between the occurrence of warm and wet weather 3 months before an increase in the incidence of hospital admission for RAO in the United States (54). The development of RAO was also associated with stables where straw beddings and stored hay were used (55). These findings were supported by the report of Ward and Couëtil showing a strong association between the development of RAO and elevated environmental
pollen and mould counts in areas where horses were stabled (54). In general, respirable particles have a median aerodynamic diameter of <5um. The study of Wood et al demonstrated that at least 17% of total dust particles suspended in the air of horse stables were respirable (56). A recent study by Fleming et al showed that the type of beddings and management practices around horse stables determines the concentration of respirable particles present in the air (57). An association was also found between the elevation of respirable particles or increase in the concentration of beta-glucans in the ambient air of horse stables and neutrophilic airway inflammation characteristic of RAO (58).

A major environmental factor that has been consistently associated with the development of RAO in horses is the feeding of poorly preserved and moldy hay (59). Although more than 50 species of mold have been identified in hay dust, only Feani rectivirgula, Thermoactinomyces vulgaris and Aspergillus fumigatus are thought to be important in the induction of RAO (60). Models of RAO based on the challenge of horses with spores of Aspergillus fumigatus and other fungi have been used to induce clinical disease in RAO horses (61). Nonetheless, the full clinical disease with all the associated pathophysiological changes can only be reproduced in conventional stable environments, suggesting that other components of hay dust, probably endotoxin, are required for the development of this disease in horses (62-64). These findings have led to the conclusion that RAO is a result of an allergic response to fungal allergens found in moldy hay. Although this concept is still debated, the contribution of moldy hay to the epidemiology of RAO in horses has been reproducibly demonstrated in both natural disease and experimental models.

**Pathogenesis of Recurrent Airway Obstruction**

Studies demonstrating a greater propensity of mast cells from horses with RAO to degranulate when exposed to fungal antigens initially led to the belief that this disease is a typical mast cell-mediated type I hypersensitivity reaction (65). In type I hypersensitivity reactions, crosslinking of allergen-specific IgE on mast cells by environmental allergens leads to mast cell degranulation and histamine release. The potential importance of this mechanism in RAO was demonstrated by studies that showed a higher prevalence of mold allergen-specific IgE in the BALF of horses with RAO compared to healthy horses (66-68).
Nonetheless, other studies could not ascribe a role to IgE-mediated mechanisms in the pathogenesis of RAO (69-71).

Similarly, intradermal injection of antigens prepared from *Aspergillus fumigatus* showed an evidence of a delayed-type hypersensitivity reaction in RAO but not healthy horses (72). In this study, histopathologic examination of skin biopsies from wheals formed 24 hours after the administration of fungal antigens showed the presence of macrophages, lymphocytes, neutrophils and eosinophils; these were not observed in samples from healthy horses. These findings were similar to the histopathologic picture of RAO in vivo. Although the analysis of BALF consistently characterizes the inflammation of RAO as a non-septic neutrophilic inflammation, submucosal infiltrates consist of lymphocytes, mast cells, plasma cells and, occasionally eosinophils. In severe inflammation, lymphoid aggregates are also found, suggesting that the immunopathologic changes observed in RAO are lymphocyte-mediated. In addition, development of severe obstructive disease is associated with marked mucous cell metaplasia. Although inconsistent, marked fibroplasia and fibrosis is also observed in the submucosa. In horses showing signs of bronchoconstrictive disease, a marked smooth muscle hyperplasia is often demonstrable. Thus, RAO appears to be an allergen-induced lymphocyte-mediated chronic neutrophilic inflammatory airway disease characterized by mucous cell metaplasia, smooth muscle hyperplasia and submucosal fibrosis.

Further investigation of peribronchiolar lymphocytes in RAO horses showed that they are immunoglobulin-producing (B) cells and CD4+ (helper) T lymphocytes. Several studies have characterized these T cells as type 2 helper T cells (Th2) which produce interleukin (IL)-4, IL-8 and IL-13. These cytokines are very important in the recruitment of neutrophils (IL-8) and induction of IgE production by B lymphocytes (IL-4 and IL-13) (73).

**Clinical Signs of RAO**

Overt clinical signs may not be observed early in the course of RAO. Early clinical signs are occasional exercise-associated cough or coughing during eating when stabled (74). As the disease progresses the episode of coughing increases and develops into paroxysmal cough even at rest (74). During periods of exacerbation, nostril flare, tachypnea and double expiratory effort become more frequent. If left untreated, the horse may lose weight and develops hypertrophic external abdominal oblique muscles leading to the observation of a
distinct “heaves line” (75). At this point, the horse alternates between periods of exacerbation and remission, during which clinical signs may not be apparent. Thoracic auscultation may not be rewarding; however, the use of rebreathing methods often allows the detection of wheezes throughout all lung fields, and a tracheal rattle resulting from excessive mucus production may also be heard (76).

Diagnosis of RAO

Severe cases of RAO are quite easy to diagnose from clinical signs and thorough physical examination. The absence of pyrexia with a complete blood count that does not show any evidence of infectious disease differentiates RAO from infectious diseases of the lower respiratory tract including bacterial and viral pneumonia (74).

In less severe cases, BALF cytology is the mainstay of RAO diagnosis in horses where this disease cannot be easily diagnosed by any other means (42). Bronchoalveolar lavage (BAL), a method aimed at recovering the fluid lining the peripheral airways and alveolar space, has been used extensively to characterize the airway inflammation in different diseases of the lower respiratory tract of horses (45, 77, 78). BALF differential cell count has been shown to correlate very well with exercise-induced hypoxemia/lactic acidosis (45, 79), airway obstruction (45), and airway hyperresponsiveness in horses (80). Although the BAL procedure is generally believed to have no effect on subsequent BAL cytology, a procedure-induced neutrophilia has been reported when BAL sampling was done within 48 hours apart (81, 82).

The cytologic composition of BALF from clinically normal horses consists predominantly of macrophages (65-80%), lymphocytes (8-10%), neutrophils (1-<5%), mast cells (<2%) and eosinophils (<0.1%) (78, 83, 84). In RAO, a BALF differential cell count consistent with a moderate to severe non-septic neutrophilic (15-85%) inflammation is usually observed. This is in contrast to the mild to moderate neutrophilia (5-15% neutrophils in the BALF) often seen in IAD (46). The presence of Curschmann’s spirals, which are mucous casts of small bronchioles, is often an evidence of a chronic airway disease (74). Nonetheless, several studies have shown that BALF cytology, although a great diagnostic tool, could not consistently be used to assess the severity of RAO in horses. The collection of BALF is often done along with endoscopic examination of the airway, which reveals a
hyperemic trachea with excessive mucoid exudates and increased sensitivity evidenced by
coughing during the procedure (74).

Arterial blood gas analysis is a method that is also used to reveal a compromise in
blood-gas exchange associated with RAO in horses. A reduction in the partial pressure of
oxygen in the arterial blood (P_{a}O_2) to 50-80 mmHg has been reported in moderate to severe
disease (74).

In addition, pulmonary function testing is available as a diagnostic tool for RAO in
research centres. The most widely available pulmonary function testing method utilizes an
eosophageal catheter to transduce changes in intrathoracic oesophageal pressure to a
physiologic recorder. Although this is a highly reproducible method that provides very
useful data to assess pulmonary function, it is not sensitive enough to detect mild changes
associated with less severe disease. The sensitivity of this method in mild RAO can be
improved by using the same set-up to detect intrathoracic pressure following histamine
provocation challenge, which evaluates the airway smooth muscle hyperreactivity often
associated with RAO (85).

Apart from detecting disease, a major goal in the diagnosis of RAO is to provide
prognostic information related to the level of pathophysiological changes observed in the
disease. This is usually done through histological examination of biopsy samples.
Histological changes detected in lung biopsies correlate well with disease severity (86).
However, although rare, transthoracic biopsies may produce life-threatening complications
like hemothorax and pneumothorax (87). Biopsies could also be taken via bronchoscopy but
bronchoscopic biopsies do not offer as valuable histologic specimen as as transthoracic
biopsies since only the mucosal layer of the airway is sampled. Pulmonary function test
carried out before and after the administration of \( \beta_2 \)-adrenergic agonists (albuterol or
fenoterol) are used to evaluate reversibility of bronchospasm.

**Treatment of RAO**

Therefore, the goals in RAO are mainly to reduce of airway inflammation as well as abate
bronchospasm. In untreated horses, clinical signs may be reversible within weeks of
removing affected horses from the source of allergen triggers. Controlling exposure to
allergen-rich environments, modifications of dusty hay through wetting, and provision of
good ventilation to reduce the amount of dust in the stable environment, have all been found to benefit horses with RAO. It has been shown consistently that limiting source exposure of horses in confinement and regular watering of stable aisles and riding surfaces are the best methods for reducing exacerbation of RAO during winter months. However, horses in remission are known to maintain low-grade pulmonary inflammation, meaning that environmental intervention alone may not completely eliminate the clinical signs of RAO in affected horses.

The use of corticosteroids has been the mainstay of anti-inflammatory therapy in RAO. Clinical signs are dramatically improved following systemic administration of corticosteroids. Triamcinolone acetonide (0.09 mg/kg administered intramuscularly) as a single dose has been shown to moderately ameliorate signs of airway obstruction for up to 4 weeks. However, triamcinolone has also been shown to induce adrenal suppression that can last up to 4 weeks in treated horses. Dexamethasone also provides significant benefits, even if it is only for 7 days. Adrenal suppression by dexamethasone usually lasts less than one week. Alternatively, inhaled corticosteroids (most often fluticasone) have been shown to reduce pulmonary inflammation and improve pulmonary function. Corticosteroid therapy is most effective in horses with RAO of mild to moderate severity (45).

In horses with moderate to severe RAO, the use of bronchodilators is required for immediate relief of airway smooth muscle constriction (rescue therapy). In this case, aerosolized β2-adrenergic agonists are used. Such treatments are often followed by reduced pulmonary resistance, increased pulmonary compliance and decreased maximal change in pleural pressure. Even in dust-free environments, RAO affected horses still benefit from bronchodilator therapy, suggesting that environmental management alone cannot completely resolve airway bronchoconstriction in horses with RAO. For bronchodilator therapy, short acting β2-adrenergic agonists like albuterol, pirbuterol and fenoterol are often used. Long-acting β2-adrenergic agonists are often not helpful in horses with severe impairment of pulmonary function because of their usually long lag period.

**Comparative Aspects of Recurrent Airway Obstruction with Human Asthma**

RAO in horses has often been regarded as similar to allergic asthma in human because of clinical signs and pathophysiologic changes common to both conditions (88). Like RAO,
allergic asthma is an episodic obstructive pulmonary disease characterized by chronic airway inflammation, airway smooth muscle hyperresponsiveness to cholinergic drugs, and airflow obstruction that can be reversed through the administration of $\beta_2$-adrenergic agonists (89). The histopathological changes associated with asthma also include airway smooth muscle hyperplasia, submucosal and mucus cell hyperplasia, and allergen deposition. Like RAO, allergic asthma has also been associated with exposure to house dust mite and fungal allergens in the environment; spores of *Aspergillus fumigatus*, *Alternaria alternate*, *Cladosporium herbarum*, and *Penicillium notatum* have all been associated with disease exacerbations in human allergic asthmatics (90). The management of allergic asthma, like RAO, also involves allergen avoidance, reduction of airway inflammation through the use of inhaled and systemic corticosteroids, and the use of $\beta_2$-adrenergic agonists as bronchodilators (91).

A major difference between asthma and RAO is the heterogeneity of cytological pictures of the BALF samples collected from human asthmatic patients. Unlike horses, where RAO is consistently described as a non-septic neutrophilic inflammation of the airway, there are at least four known inflammatory phenotypes of human asthma (92). Recognized cytological phenotypes in human asthma include eosinophilic, mixed granulocytic, neutrophilic and paucigranulocytic (92, 93). Additionally, while eosinophilic asthma like RAO is very responsive to corticosteroids, non-eosinophilic asthma phenotypes are often poorly responsive to corticosteroid therapy (94, 95). Recent studies have attempted to correlate the inflammatory phenotype of human asthma with specific pathophysiological and clinical subtypes (96). The heterogeneity of airway inflammation in human asthmatics has led to the notion that different pathogenetic mechanisms drive this condition in different patient groups.

Several studies in human asthmatics, and using small animal and primate models, have allowed a reproducible delineation of the pathogenetic mechanisms behind eosinophilic (allergic) asthma (97, 98). It is now generally accepted that allergic asthma is an immune-mediated disease driven by allergen-specific CD4+ T cells (helper, Th) that promote eosinophilic airway inflammation and maturation of IgE-producing B cells. In general, Th cells are classified into at least two subtypes, Th1 and Th2, depending on the type of cytokines secreted following activation (99). Following activation, antigen-specific Th1 cells
secrete cytokines such as tumor necrosis factor (TNF), interferon-gamma (IFN\(\gamma\)) and interleukin (IL)2, while Th2 cells preferentially produce IL4, IL5, and IL13 (100). A balance of Th1 and Th2 activity is required for an appropriate immune response to pathogens. In human allergic asthmatics, however, there is a profound deviation of the immune response towards Th2, leading to an exuberant production of IL5 that promotes maturation of eosinophils in the bone marrow, IL4 that promotes immunoglobulin isotype switching in B cells towards the production of IgE, and IL13 that has been shown to promote hyperreactivity of airway smooth muscles(101-103).

Results of some studies in horses with RAO have suggested that a Th2 deviation present in allergic asthma drives chronic neutrophilic airway inflammation in RAO horses. Giguere et al. using reverse transcriptase (RT)-PCR detected an increase in both IL4 and IFN\(\gamma\) without detecting IL2, IL5 and IL10 mRNA in the BAL of horses with RAO during different stages of the disease (104). Similarly, Cordeau et al, using in situ hybridization detected IL4, IL5 and IFN\(\gamma\) and documented a significant increase in IL4 and IL5 mRNA in RAO horses compared to control, suggesting a Th2 response and a role for IL4 (105, 106). Conversely, Ainsworth et al. using quantitative RT-PCR could detect neither IL4 nor IL5 but reported a significantly higher level of IFN\(\gamma\) mRNA in RAO than control horses, suggesting the absence of a Th2 response in heaves (107). Thus, unlike in human allergic asthmatics, measuring cytokine expression in BAL fluid and lymphocytes could not establish a clear immunological basis for RAO in horses.

Development of CD4+ lymphocytes into either Th1 or Th2 is initiated by interaction of naïve T cells with antigen presenting cells (APCs) under different cytokine milieu in the airway; environments rich in IL12 promote Th1 while those with IL4 support the development of Th2 (108). Measurement of cytokines in BALF samples from human allergic asthmatics showed the presence of cytokines that promote Th2 but not Th1 cells (109). This favors eosinophil maturation in the bone marrow via the action of IL5 and infiltration into the lungs through the action of eotaxin (99). Conversely, despite the detection of significantly higher IL4 and IL5 mRNA in the BAL of horses with heaves compared to healthy controls, RAO is characterized by a profound neutrophilic inflammation (105, 110).

It is now known that the humoral environment at the site of T cell maturation is not the primary factor responsible for development into Th1 to Th2. Studies investigating antigen
presenting cells (APCs) have demonstrated that these cells play a central role in immune regulation (111). Indeed, the effect of cytokine mix in an inflammatory milieu is primarily exerted through their action on the phagocytic activity, maturation and expression of specific co-stimulatory molecules on APCs (111). The major APCs in the lung and airways are dendritic cells (DCs) and macrophages (112). Although DCs are the major APCs in the airways, the use of dichlornate to deplete alveolar macrophages (AM) in the airway was shown to downregulate the local population of pulmonary antigen-specific T cells and inhibited DC maturation (113, 114). Using a similar model, Tang et al showed that depletion of AM breaks immune tolerance and leads to a default Th2 response in the airway, reversed by the adoptive transfer of IFN-γ-activated AM (115). Thus, like T cells, cells of the mononuclear phagocyte system may play a central role in orchestrating the airway inflammation characteristic of both allergic asthma in human and RAO in horses.

Alveolar Macrophages in the Regulation of RAO

Exposure of equine AMs to hay dust suspension and Aspergillus fumigatus resulted in a copious production of TNF and IL1β, suggesting that these AMs, that are potentially M1 in phenotype based on cytokine secretion pattern, may play a role in T cell polarization in the equine airway through cytokine secretion (116). Indeed, the secretion of mediators by AMs was shown to precede neutrophil infiltration of the airway following exposure of normal and RAO horses to moldy hay (117). In a similar study, AM from RAO horses were shown to produce a higher amount of TNF, IL-1 and IL-8 than those from non-RAO horses while the anti-inflammatory cytokine IL-6 was higher in non-RAO than RAO horses, suggesting a predominance of M1 macrophages in RAO compared to control horses (60). These studies suggested that, like in human asthma (118), failure of the mechanisms whereby macrophages mediate tolerance to environmental allergens in the respiratory airway may be an important component of RAO pathophysiology in horses. However, unlike human and rodent allergic asthmatics, AM from RAO horses were not found to express a significantly higher level of chitinase than normal horses, suggesting that regulation of tolerance may be different in horses and human subjects (119).
Hypothesis

It is hypothesized that in RAO the phenotype of monocytes and alveolar macrophages reflects a polarized immune response that promotes chronic neutrophilic inflammation rather than tolerance to environmental allergen.

Objectives: The general objective of the studies proposed herein is to determine the phenotypes of monocytes and AM in healthy horses and horses with RAO and thus provide a basis for future studies to delineate the role of AM in the induction and maintenance of airway inflammation in this condition.

III. Experimental Goals

The objectives of this study will be accomplished by carrying out the following specific experiments:

1. Probing blood monocytes for the expression of CD163 as a marker of M2 polarization in horses

2. Determining the expression of cytokines and chemokines associated with M1 and M2 phenotypes by CD163+ and CD163- monocytes and macrophages in normal horses.

3. Determining the expression of CD163 on macrophages from healthy and RAO horses.
References


Horses have multiple subsets of monocytes, and systemic inflammation is associated with upregulation of the haptoglobin-hemoglobin receptor CD163 on monocytes
**Introduction**

Blood monocytes are members of the mononuclear phagocyte system (MPS) involved in innate immune response (1, 2). However, it has been recognized that monocytes are functionally heterogeneous and play a central role in the orchestration of both innate and adaptive responses (3, 4). Initial studies using mouse models of peritonitis classified monocytes into at least two functionally distinct phenotypes based on cell size and expression of surface markers such as F4/80, Ly6C (recognized by the Gr-1 antibody), CD11b, CD43, and the chemokine receptor CX3CR1(5). In this model, monocytes that expressed high levels of Ly6C (Ly6C\text{hi}) but low levels of CX3CR1 (CX3CR1\text{low}) preferentially migrated to the peritoneal cavity during inflammation. Conversely, Ly6C\text{hi}CX3CR1\text{high} monocytes predominated under non-inflammatory condition and migrated to different organs, where they differentiated into resident tissue macrophages. Other studies showed that the propensity of Ly6C\text{hi}CX3CR1\text{low} monocytes to extravasate into inflammatory environments was related to the selective expression of the chemokine CCR2 and the adhesion molecule L-selectin (6).

Thus, expression of Ly6C, CX3CR1, CCR2 and L-selectin was used to separate classical (Ly6C\text{hi}CCR2+CD62L+CX3CR1\text{low}) from non-classical (Ly6C\text{low}CCR2-CD62L-X3CR1\text{high}) monocytes in the mouse. These two classes of monocyte were subsequently shown to give rise to different subsets of resident mononuclear phagocyte populations, including macrophages and dendritic cells in tissues during inflammation (proinflammatory or M1) and in the absence of inflammatory stimuli (anti-inflammatory or M2) and (7).

In humans, using flow cytometry to analyze light scatter properties and expression of CD14 and CD16, circulating blood monocytes were also classified into two subsets. A population similar to the classical Ly6C\text{hi}CX3CR1\text{low} monocytes (M1) of the mouse expresses high levels of CD16 but no or low levels of CD14 (CD14+/CD16++) and were found to expand under inflammatory conditions. A second subset, similar to the non-classical subtype (M2) in the mouse, expressed CD14 but not CD16 (CD14++/CD16−) (8, 9). More recent studies using microarray technology have confirmed these subsets in human monocytes, established concordance with the subsets in the mouse, and demonstrated distinct functions related to each monocyte subset (3, 10-12).

Additional studies identified CD163 as another subset-differentiating marker that is highly expressed on human monocytes. This protein is a cysteine-rich scavenger receptor that

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specifically binds hemoglobin-haptoglobin complexes to promote uptake by monocytes and macrophages. It was also recently recognized as an anti-inflammatory scavenger receptor for tumour necrosis factor-like inducer of apoptosis (TWEAK), a survival-promoting receptor found on erythroblasts, and as a pattern-recognition receptor for both Gram-positive and Gram-negative bacteria (9, 10). In humans, expression of CD163 has been associated with non-classical (M2; CD14⁺CD16⁺⁺) monocytes (13-16). In addition, macrophages that express CD163 (CD14⁺CD16⁺CD163⁺) were identified as those involved in the resolution of inflammation. Indeed, changes in the expression of CD163 on monocytes and macrophages have been implicated in the immunopathogenesis of diseases such as asthma, cancer, ulcerative colitis, type 2 diabetes, and HIV/AIDS (15, 17-19). The expression of CD163 on monocytes and macrophages is increased following treatment with corticosteroids, and incubation with M-CSF, IL-6 and IL-10 in vitro, but decreased following incubation with GM-CSF, IL-4, IFNγ and TNFα (14, 20). The specific expression of CD163 on monocytes and macrophages and its involvement in well-characterized diseases have informed its proposed use as a target for immunotherapy in many of these conditions in humans (21).

Several diseases of domestic animals are characterized by derangement of immune homeostasis. Recurrent airway obstruction (RAO) in horses is a chronic non-septic neutrophilic inflammation that is responsive to treatment with corticosteroids and characterized by airflow obstruction reversible with beta-2 agonists; hyperreactivity of airway smooth muscle; and airway remodeling (22). The apparent clinical and pathophysiologic similarities between RAO in horses and allergic asthma in humans have led to attempts to define it as a disease driven by lymphocytes polarized towards the Th2 phenotype. However, studies investigating lymphocyte polarization in RAO have produced equivocal results (23, 24). Severe asthma in human is associated with a significant increase in the proportion of CD163-expressing monocytes (18). There is paucity of information in the literature on the expression of CD163 on equine monocytes and the association of equine monocyte subsets with RAO or other diseases. Therefore, this study was designed to investigate the expression of CD163 on equine monocytes in healthy horses versus horses with systemic inflammation. In addition, we examined the expression of this marker on blood monocytes during experimental RAO of horses.
Materials and Methods

**Horses, housing, handling, and sampling**

All the procedures described were approved under AUP R06-067 by the University of Guelph Animal Care committee, in accordance with the guidelines of the Canadian Council on Animal Care. Research horses were kept on pasture with indoor access, fed grass hay, and received routine vaccinations and anthelminthics. For the induction of experimental RAO, horses were housed in separate, enclosed, air-conditioned stalls, bedded on damp wood shavings, fed non-dusty extruded feeds, and given free access to water. No hay was fed. Pulmonary function testing (PFT), endoscopic examination of the upper and lower portions of the respiratory tract, and bronchoalveolar lavage fluid were carried out on day 1, and on the day after the last nebulization with a mixture of fungal spores-lipopolysaccharide-microsphere suspension (FLS) in sterile saline solution as previously described (25). Horses classified as having RAO had been diagnosed with RAO by clinical signs and PFT for >4 years; however, no abnormalities were detected during current physical examination, complete blood count and serum biochemistry profile of the horses before disease exacerbation (25). In addition, bronchoscopy showed no evidence of edema or inflammation, and the proportion of neutrophils in the pre-challenge BAL fluid was less than 5%. These horses were therefore considered to be in remission. Control horses had no history of lung disease, and showed normal airway bronchoscopic and pulmonary function findings. Baseline CBC and serum biochemical parameters were within normal ranges for both RAO and control horses. All the procedures for induction of RAO were previously described (25).

**Blood samples**

Samples analysed (Appendix 1) were excess blood samples from client horses visiting the Ontario Veterinary College Teaching Hospital (OVCTH), Guelph. Approximately 10mL of blood were collected into EDTA tubes and submitted for complete blood cell (CBC) count at the Animal Health Laboratory. The same samples were prepared and stained with antibodies for flow cytometry within 24 hours. The blood samples analyzed were selected randomly from horses visiting the OVCTH between June 2009 and August 2011. In addition, blood samples from the University of Guelph’s research herd were used in this study. Current and
historical clinical information for each horse was extracted from the medical records in the Laboratory and Medical Information Management Systems of the OVCTH.

**Immunofluorescent antibody staining of horse leukocytes**

To stain specific surface markers with antigen-specific antibodies, 100µL of blood sample was added to a flow cytometry tube (Fisher Scientific, Mississauga, Canada). Following titration to determine the optimum concentration of each antibody to use as shown in Table 2.1, 10 µL of appropriately diluted fluorochrome-conjugated primary antibodies (CD163-FITC, CD16-PE, CD206-PE) were added to the blood samples and incubated on ice for 15 minutes. When the primary antibody was not conjugated to a fluorochrome (CD14, MHC II, CD5, CD90, CD163), 10µL of appropriately-diluted antibody was first added to the blood sample and incubated for 15 minutes on ice. After washing in 3 mL of staining buffer (phosphate-buffered saline containing 10% acid citrate dextrose, 2% gamma globulin-free horse serum, 10mM EDTA, and 0.2% sodium azide), 10µL of appropriately-diluted secondary antibodies as shown in Table 2.1 (goat anti-mouse-IgG-Alexa 488, Invitrogen; goat anti-mouse-IgG-Qdot655, Invitrogen, Mississauga, Ontario, Canada) were added, incubated again for 15 minutes on ice. To stain with two or more antibodies, unconjugated primary antibodies were first applied, followed by secondary antibodies, before directly-conjugated primary antibodies were added. Following a final wash step in 3 mL of wash buffer, the sample was resuspended in 400µL of lysis buffer (0.16M NH₄Cl, 10.0mM KHCO₃, 0.1mM Na₂-EDTA) (26), gently mixed and incubated at room temperature for 10 minutes. The leukocytes were washed in 3 mL of staining buffer by centrifuging at 300g for 5 minutes. Pellets were then resuspended in 350-400 µL staining buffer and analyzed. The primary and secondary antibodies used in the present study are summarized in Table 2.1.
Table 2.1: List of primary antibodies used for labeling leukocytes for flow cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD5</td>
<td>CVS5</td>
<td>Serotec</td>
<td>Lymphocytes (~50%)</td>
</tr>
<tr>
<td>Dilution: 15µL per 100µL staining volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD14</td>
<td>Big 10</td>
<td>Cell Sciences</td>
<td>Monocytes and NK cells</td>
</tr>
<tr>
<td>Dilution: 20ng/100µL staining volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-equine MHC class II</td>
<td>CVS-20</td>
<td>Serotec</td>
<td>Lymphocytes, macrophages, dendritic cells</td>
</tr>
<tr>
<td>Dilution: 5µL per 100µL staining volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD16-PE</td>
<td>MEM-154</td>
<td>Novus Biologicals</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Dilution: 20µL per 100µL staining volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD163-FITC</td>
<td>Ber-Mac3</td>
<td>MBL</td>
<td>Monocytes, macrophages</td>
</tr>
<tr>
<td>Dilution: 5µL per 100µL staining volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD206-PE</td>
<td>3.29B1</td>
<td>Beckman Coulter</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Dilution: 10µL per 100µL staining volume</td>
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<td></td>
</tr>
</tbody>
</table>
Whole blood

Depletion of RBC by dextrose sedimentation

Wash leukocytes

Immunomagnetic depletion of neutrophils (CD90) and lymphocytes (CD5)

Remaining cells = CD90⁻ and CD5⁻ leukocytes

Incubation with CD163 antibody

Negative selection: CD163⁻ leukocytes (monocytes)

Positive immunomagnetic selection: CD163⁺ leukocytes (monocytes)

Culture for 4 hours

Removal of non-adherent cells

Overnight culture of adherent cells (macrophages)

Flow cytometry for CD14, CD90, CD163, CD206

Figure 2.1: Schematic for the isolation of equine monocytes
Flow cytometry

Samples from 61 horses (37 healthy, 24 with systemic inflammation) (Appendix 1) were analyzed with a FACScan flow cytometer (BD Biosciences) using Cell Quest Pro acquisition software (BD Biosciences, Mississauga, Ontario). The forward and side scatter thresholds on linear scales were first adjusted using unstained cells, without gating out populations with very low side and forward scatter properties; such as membranes of lysed erythrocytes and platelets. Fluorescence from the green channel (FL1), yellow channel (FL2) and red channel (FL3) was set using cells stained with secondary antibodies alone (anti-mouse-FITC, anti-mouse-PE, anti-mouse Alexa488 or anti-mouse-Qdot655). The photomultiplier tube (PMT) voltage and fluorescence gain on each channel was adjusted such that these negatively-stained cells were within $10^0$ to $10^1$ fluorescent units. For multiparameter acquisition where fluorescence values from the three channels were combined, spectral overlap was minimized by adopting the fluorescence-minus-one strategy (27). Briefly, when FITC/Alexa488-, PE- and Qdot655-conjugated antibodies were used together, compensation for FITC/Alexa488 was adjusted using tubes staining positive for antibodies conjugated to PE and Qdot655; PE-conjugated antibodies were compensated using tubes containing cells staining positive for antibodies conjugated to Alexa488/FITC and Qdot655; while Qdot was compensated using positive samples stained with antibodies conjugated with FITC/Alexa488 and PE. The validity of the compensation settings during acquisition was subsequently verified using post-acquisition compensation with Flow Jo software (Tree Star Software Inc., Ashland, Oregon). After drawing gates to identify the population of interest, the flow cytometer was set to acquire a minimum of 10,000 events from the monocyte gate.

Enrichment of monocytes from peripheral blood

Monocytes were enriched from peripheral blood by both negative (bulk monocytes) and positive selections (CD163$^+$ monocytes) using an immunomagnetic separation method with anti-mouse IgG microbeads (Miltenyi biotec, Auburn, CA, USA). For negative selection, total blood leukocytes were first obtained after mixing whole blood with 6% dextrose in phosphate-buffered saline (Sigma-Aldrich, Mississauga, Canada) at a ratio of 1:10 to increase erythrocyte sedimentation rate. The mixture was incubated at room temperature for 45 minutes, after which the leukocyte-rich supernatant was carefully separated from the
erythrocyte sediment. Leukocytes were washed in phosphate-buffered-saline (PBS\(^+\) contains 1% autologous horse serum and 1mM EDTA) three times and counted. The cells were then labelled with an antibody cocktail containing mouse anti-equine CD5 and anti-CD90 (about 50% of circulating equine lymphocytes are CD5\(^+\) while all granulocytes are CD90\(^+\)) (11939315) for 10 minutes. After washing with PBS\(^+\), the resuspended cells were labelled with anti-mouse-IgG microbeads and incubated on ice for a further 10 minutes. After another round of washing in PBS\(^+\), the cells were resuspended in 500µL of PBS\(^+\) and passed through an LS column (positive selection) or LD column (negative selection). For negative selection, cells flowing through the column under a strong magnetic field created by a MidiMACS Separator (Miltenyi Biotec.) were collected in a 15 mL tube. Positively selected (antibody-labeled) cells were collected by allowing buffer to flow through the column after removing it from the magnetic field. The isolated cells were counted and cytospin preparations were made. Wright-Giemsa staining was used to identify and determine the purity of the isolated cells. While positive selection delivered cells with up to 95% purity, negative selection enriched the leukocyte preparation such that approximately 60-75% of leukocytes were monocytes. The cells were washed and resuspended in growth medium (RPMI-1640 with 20% autologous horse serum; 100 I.U./mL penicillin; 100 µg/mL streptomycin).

**Differentiation of monocytes to macrophages**

The monocytes obtained via negative or positive (CD163\(^+\) cells) selection were diluted to contain 1x10\(^6\) cells/mL. From these, 200µL were plated into 12-well tissue-culture-grade plates (Fisher Scientific). After incubating the plates in a humidified cell culture incubator (37\(^\circ\)C) for 4 hours, non-adherent cells (other cells apart from monocytes) were vigorously washed away from each well with warm (37\(^\circ\)C) medium. Medium was then replaced, followed by incubation of the plates overnight. To determine differentiation to macrophages, adherent cells were detached from the plates after incubating with 5mM EDTA for 5 minutes at 37\(^\circ\)C. The cells were transferred to flow cytometry tubes, washed in 3mL of staining buffer and stained with CD163-FITC and CD206-PE on ice for 15 minutes. The cells were passed through the flow cytometer after washing with PBS\(^+\).
**Data analysis**

Analysis of flow cytometry data was carried out using Flow Jo software. Initial analysis to determine a measure of marker expression showed perfect concordance between percentage of marker positive cells and marker density as determined using Median Fluorescence Intensity (MFI). Statistical analysis was done using Fisher’s Exact test as executed in GraphPad Prism (Graphpad software Inc., La Jolla, CA, USA).

**Results**

**Light scatter characteristics of equine leukocytes**

In flow cytometry, the forward (low-angle) light scatter characteristics, a correlate of cell size, is often used in combination with the side (wide-angle) light scatter properties, a rough indicator of the granularity of cells, to identify blood cell types. Using this method with human blood, lymphocytes are usually identified as cells with low forward and side scatters (Region 1 = R1), monocytes have high forward scatter but medium side scatter (Region 2 = R2) while granulocytes have high side and forward scatters (Region 3 = R3). Previous studies had applied these scatter patterns to identify horse leukocytes by flow cytometry (28). Using forward and side scatter properties of human leukocytes as reference points; three regions were also identifiable in horse samples (Figure 2.2a & b). However, we found this pattern inconsistent when several horses were examined, even when optimal photomultiplier voltage settings were applied for each sample. A major reason for this observation was the apparent higher side scatter (granularity) of equine blood lymphocytes compared to human lymphocytes, making the separation of monocytes from granulocytes and lymphocytes less distinct than what is observed with human leukocytes (Figure 2.2c & d). Thus, based on light scatter properties alone, it was difficult to probe equine monocytes since some (up to 80% in some horses) did cluster with granulocytes.
CD90 is a marker of equine neutrophils

To classify monocytes into subsets as described in human and mice, we employed anti-CD16 antibody (mAb MEM-154) to probe the expression of this molecule as a marker of non-classical monocytes as previously described in human. Unlike human neutrophils and monocytes; however, neither monocytes nor neutrophils from 20 horses tested during preliminary analysis reacted with this antibody (Figure 2.3 A-D). We, therefore, employed an anti-CD90 antibody (clone DH24A from VMRD, Seattle, Washington, USA) previously shown to react differentially with leukocytes from different species as a potential marker on neutrophils and monocytes. Interestingly, this anti-CD90 antibody reacted only with equine granulocytes but not monocytes or lymphocytes. When CD90 was applied, along with CD5, it was possible to separate monocytes (CD5<sup>-</sup>CD90<sup>-</sup>) from other leukocytes for further analysis (Figure 2.4 A-D). Thus, we employed CD5 and CD90 to identify undesired cell populations (approximately 50% lymphocytes are CD5<sup>+</sup>; all neutrophils are CD90<sup>+</sup>) and create an exclusion channel as previously described (29). When double labeling was done with Alexa488/FITC and PE, a Qdot655-conjugated secondary antibody that fluoresces in the red channel (FL3) was used to label CD5 and CD90 so that these could be eliminated from analysis. When double labeling was done on FL1 and FL3, PE-conjugated antibodies were used in the exclusion channel to eliminate CD5<sup>+</sup> and CD90<sup>-</sup> cells. However, in 20 horses initially tested and all 61 horses investigated in this study, the use of CD90 in conjunction with forward and side scatter properties was enough to eliminate both granulocytes and lymphocytes when analyzing monocytes.
Figure 2.2: Forward and side scatter characteristics of human versus equine leukocytes. Monocytes are cells with medium side scatter and high forward scatter in regions R2 in human (A) and horses (B). The lower granularity (side scatter) of human (C) compared to equine (D) monocytes facilitates separation of human but not equine monocytes on the basis of light scatter characteristics.
Figure 2.3: Reactivity of human but not equine neutrophils with anti-CD16 antibody (clone MEM-154). Leukocytes from human (A) and horse (B) did not react with isotype control (IgG-PE). Human granulocytes (C) with high side scatter (R3, reactivity is indicated by a shift to the right in the direction of the red arrow) but not equine granulocytes (D) reacted with anti-CD16 antibody (MEM-154). Thus, this antibody could not be used to classify equine monocytes on the basis of lack of CD16 expression. Note that, in addition to neutrophils, a proportion of human monocytes and lymphocytes are positive for CD16. These CD16⁺ monocytes would be classified as non-classical (M2) in human.
**Figure 2.4:** Specific staining of equine granulocytes by anti-CD90 antibody (clone DH24A). Using anti-CD5 antibody, equine leukocytes (A) could be separated into lymphocytes (R1) and granulocytes/monocytes (R2/R3) as shown in Figure 3B. The use of anti-CD90 antibody specifically separated granulocytes (R3) from other leukocytes (C). Thus, a combination of anti-CD90 and anti-CD5 antibodies allowed separation of monocytes from other leukocytes for further analysis (D). Note that only about half of the lymphocyte population is CD5+, but lymphocyte contamination of monocytes was much reduced using this method.
Expression of CD14 and CD163 on equine monocytes

In addition to CD16, the expression of CD14 and CD163 is used to classify monocytes into subsets in both human and mouse. We therefore investigated the expression of these markers on equine monocytes after eliminating neutrophils and lymphocytes as described above. The Big10 monoclonal anti-CD14 antibody (Cell Sciences, Sharon, MA) was titrated on human and equine monocytes; CD14 was still detectable on human monocytes after diluting the antibody up to 1:4000 but could not be detected on equine monocytes at any dilution lower than 1:500 (Figure 2.5 A-D). Following double labeling, we determined the co-expression of CD14 and CD163 (MBL International, Woburn, MA) on equine monocytes. In general, almost all CD14-expressing monocytes co-expressed CD163. However, a small percentage of CD163-expressing cells did not express CD14. In contrast to the relatively dim expression of CD14, we noted bright expression of CD163 on monocytes (Figure 2.6A and B) and the brightness of CD163 expression appeared to vary among horses. We therefore investigated CD163 expression on 61 horse samples to determine the factors that might be associated with the intensity of CD163 expression on equine monocytes. The horses were divided into two groups depending on the results of CBC, history and serum biochemistry analysis. Horses with three or more of the following were classified as belonging to the “systemic inflammation” group: leukocytosis or leukopenia, elevated fibrinogen concentration or elevated serum amyloid A concentration, history of acute colitis, or cytologic evidence of neutrophil toxicity. “Healthy” horses had complete blood count and serum biochemical values within reference intervals. The median fluorescence intensity (MFI) of CD163 expression for each sample was computed and normalized to the MFI of a corresponding sample of unstained cells. Using the Mann-Whitney test, a significant difference (p <0.0001, two-tailed) was found in CD163 expression between the two groups of horses (Figure 2.6C). Thus, we concluded that CD163-expression on monocytes is increased during inflammatory conditions in horses.

RAO is not associated with a change in CD163 expression on monocytes

To further investigate increased CD163 expression on monocytes with systemic inflammation, we asked whether neutrophilic airway inflammation was also associated with an increased proportion of circulating CD163⁺ monocytes. Blood samples (remission) were
collected from four horses with RAO during remission and stained for CD163 expression. Bronchoscopy was carried out, followed by collection of bronchoalveolar lavage (BAL) fluid. Cytospin preparations of BAL fluid were made and the degree of airway inflammation was assessed. After resting the horses for three days, RAO was induced through repeated nebulization of a mixture of fungal spores, lipopolysaccharide (LPS) and 1µm microspheres, as previously described by Beeler-Marfisi et al. (25). Nebulization was stopped after clinical signs, bronchoscopy and cytospin analysis confirmed exacerbated RAO (25). Blood samples (exacerbation) were then collected, and analyzed for CD163 expression on monocytes. Horses in RAO exacerbation had CBC values within reference interval, suggesting that respiratory signs were not associated with systemic inflammation. Our results showed no significant differences (p = 0.8857) in the expression of CD163 on monocytes during the period of remission versus exacerbation of RAO (Figure 2.7).
Figure 2.5: Reactivity of equine monocytes with anti-human CD14 (Clone Big10). Using 20ng of clone Big10 antibody in a 100µL staining reaction, over 75% of human (A) monocytes were positive for CD14 while only 40% of equine monocytes (B) were positive for this monocyte marker. However, at a concentration as low as 2.5ng (C) human monocytes still reacted with this antibody while equine monocytes showed no detectable reactivity even at a concentration of 10ng (D). No increase in reactivity to CD14 was detected when this antibody was used at a concentration higher than 20ng per 100µL staining volume in equine monocytes.
Figure 2.6: Expression of CD163 on equine monocytes with systemic inflammation. Blood samples were collected from healthy horses and horses with evidence of systemic inflammation. Anti-CD90 antibody staining (A), anti-CD163 antibody staining (B), and the percentage of cells expressing CD163 was determined. Ratio of the median fluorescence intensity (MFI) of stained cells (in region Q1) to unstained cells (in region Q4) was calculated for each sample to generate “Change in MFI” values. Comparison of “Change in MFI”. Comparison of “Change in MFI” between healthy horses and those with systemic inflammation showed a higher expression of CD163 on monocytes from horses with systemic inflammation (C). The box and whisker plots show median, range, interquartile range, and mode.
Figure 2.7: Expression of CD163 on equine monocytes during remission and exacerbation of recurrent airway obstruction. Blood samples were collected from horses in remission and expression of CD163 on monocytes was determined. Following induction of exacerbation, horses were again sampled and monocytes were probed for CD163 expression. No significant differences in the fluorescence intensity of CD163 expression by monocytes were noted. Thus, unlike systemic inflammation, which was characterized by an increased density of CD163 expression on monocytes, induction of local inflammation in the airway did not induce an increase in CD163 expression by blood monocytes.
Differentiation of equine monocytes to macrophages is associated with increased expression of CD206 but not CD163

To provide a better understanding of the potential kinetics of CD163 expression when blood monocytes mature into macrophages in tissues, we investigated changes in CD163 expression during *in vitro* maturation of monocytes. After separating monocytes into CD163− and CD163+ fractions using immunomagnetic beads, we allowed the cells to attach to sterile tissue culture wells for 4 hours. Non-adherent cells were washed off and the adherent cells were cultured for 24 hours. Initial experiments showed rapid upregulation of CD206 expression as early as 2 hours after attachment to tissue culture plates. At 24 hours, almost all adherent cells expressed CD206. However, the proportion of CD163+ and CD163− cells did not change; CD163− monocytes remained negative for this marker after becoming macrophages, and CD163+ monocytes also retained this marker when they became macrophages (Figure 2.8 A-C). Thus, maturation of monocytes to macrophages *in vitro* does not result in the induction of CD163 expression. However, it is difficult to extrapolate this result to *in vivo* conditions since plastic adherence may represent a specific stimulus that may not be present *in vivo*. 
Figure 2.8: Differentiation of monocytes to macrophages is not associated with a change in CD163 phenotype. Using immunomagnetic beads, monocytes were separated into CD163^+ (Q1) and CD163^- (Q4) fractions (Figure 7A; represents pre-sort cells). Following differentiation to macrophages, each fraction was probed for expression of CD163 and CD206. Following differentiation to macrophages, CD163^+ monocytes differentiated into CD163^+CD206^+ macrophages (B) while CD163^- monocytes differentiated into CD163^- CD206^+ macrophages (C). These findings suggest that macrophages maintain the CD163 phenotype of monocytes from which they originate. This figure is a representative plot of monocyte to macrophage differentiation in 15 different horses.
**Discussion**

Monocytes are bone marrow derived mononuclear cells that enter the circulation as non-dividing cells before entering tissues. Although *in vitro* studies have consistently demonstrated that monocytes could readily differentiate into macrophages and dendritic cells, the fate of circulating monocytes *in vivo* is still poorly understood. However, analyses of tissue-dwelling macrophages *in vivo*, along with macrophages generated *in vitro*, have identified two functionally distinct phenotypes; the classical (M1) and non-classical (M2) macrophages. Subsequent analysis of circulating monocytes showed that these circulating mononuclear cells could also be divided into classical and non-classical subsets that express markers homologous to those found in the respective differentiated macrophage subsets. Other studies have associated each monocyte-macrophage subset with specific diseases in human and mouse.

In an attempt to classify equine monocytes into functional subsets in this study, we have examined the light scatter properties and the staining characteristics of equine monocytes with antibodies to CD14, CD16, CD163 and CD206. Previous studies examining equine leukocytes using flow cytometry were predominantly aimed at determining the cross-reactivity of a wide array of anti-human antibodies with horse leukocytes. Such studies, employing samples from a limited number of horses, have successfully laid the groundwork for the application of flow cytometry in equine medicine (30). The present study, using clinical samples, has further expanded the use of flow cytometry and validated more antibodies that may help in the integration of flow cytometry as a routine procedure in the diagnosis of equine diseases in the future.

The reactivity of the monoclonal anti-CD90 antibody DH24A with horse leukocytes in the present study is different from the pattern previously found in the dog where this mAb specifically reacted with CD90 on lymphocytes (31). In our hands, DH24A showed no reactivity with horse lymphocytes. It has previously been suggested that DH24A does not stain equine lymphocytes (30). However, a strong and consistent reactivity of this mAb with granulocytes was demonstrated. The present study has confirmed that DH24A is a specific marker of granulocytes in horses. The distribution of CD90 expression in horses is currently unknown; however, CD90 is expected to be expressed on lymphocytes and their precursors. It is possible that the epitope detected on CD90 of other species by DH24A is present on
equine neutrophils but not lymphocytes. It is also possible that the conformation of a granulocyte-specific antigen of horses is similar to that recognized by the DH24A antibody on CD90 protein of other species; this might explain the cross-reactivity noted in this study. Nonetheless the use of this antibody to separate granulocytes from monocytes became a convenient method to identify monocytes for further characterization in the present study. This finding facilitated avoidance of potential problems associated with inconsistent light scatter properties of equine monocytes that, unlike human monocytes, tend to cluster very tightly with granulocytes. This may be related to a higher granularity of equine monocytes when compared to human monocytes.

The differential expression of CD14 and CD16 on human monocytes has been used for classification into classical and non-classical subsets (32). The use of a mAb specific for human CD16 (MEM154) did not detect this surface marker on equine neutrophils in the present study. Previous attempts by Ibrahim et al to identify equine neutrophils using other anti-CD16 antibodies also produced negative or equivocal results (30). Combined with our findings, these results suggest: (i) CD16 is not expressed on equine leukocytes; (ii) CD16 from horses is antigenically distinct from human CD16; or (iii) the epitope recognized by MEM154 on human neutrophils is not present on equine neutrophils. The use of CD16 to classify monocytes into two subsets was therefore not possible. Nonetheless, the Big 10 monoclonal antibody directed against human CD14 showed good reactivity with equine monocytes, as previously reported (33, 34). Conversely, a recently-described monoclonal antibody to equine CD14 was shown to also detect CD14-expressing lymphoid cells, presumably NK cells (28). Using backgating strategies and immunocytochemistry, the Big 10 mAb used in our study did not stain cells found within the lymphocyte or neutrophil gate, suggesting that this antibody is specific for equine monocytes. This mAb showed that 3-45% of circulating blood monocytes in horses are CD14+. This wide variation may be related to inducible expression of CD14 in equine monocytes. The expression of this marker is in contrast to the pattern of expression in circulating blood monocytes in human, where approximately 95% are known to express CD14 without CD16 (CD14++CD16-) and are therefore classified as classical monocytes (32, 35, 36). Thus, if CD14 is indeed a marker of classical monocytes in horses as it is in human, our study suggests that the proportion of classical monocytes in horses is much less than what has been found in humans.
In contrast to CD14, strong and variable expression of CD163, the marker of non-classical human monocytes, was observed on equine monocytes. The use of mAb Ber-Mac3 to detect this marker, along with Big 10 for CD14, identified four distinct groups of circulating monocytes in horses; CD14⁺CD163⁺, CD14⁻CD163⁺, CD14⁺CD163⁻, CD14⁻CD163⁺ (Figure 5A). In general, in the horses examined in this study, almost all (over 98%) CD14⁺ monocytes were CD163⁺, whereas a small percentage of monocytes were CD163⁻ but CD14⁺. This pattern of CD163 expression is consistent with what was previously observed on human CD14⁺ monocytes but in contrast to the predominance of CD163 expression on CD14⁻CD16⁺ monocytes of pigs (9, 37-39). Indeed, recent studies have confirmed that almost all CD14⁺ human monocytes also express CD163 (40). Thus, in the horse, CD14⁺CD163⁺ monocytes may be a homologue of the CD14⁺CD16⁻ classical monocytes. In pigs, Chamoro et al interpreted the four groups as different stages of monocyte maturation because of differences in their ability to produce tumor necrosis factor (39). The CD163⁺CD14⁻ subsets were regarded as the most mature subset. We did not investigate differences in the maturation of the four groups of monocytes in the present study.

In addition to surface markers, differences in the kinetics of monocyte subsets in circulation have been used to identify classical (pro-inflammatory) and non-classical (anti-inflammatory) monocytes in human blood. We, therefore, examined the proportion and characteristics of CD163⁻ versus CD163⁺ monocytes in equine blood during systemic inflammation. We demonstrated a marked increase in the density of expression of CD163 on monocytes during systemic inflammation. This observation, therefore, suggests that CD14⁺CD163⁺ monocytes are associated with inflammation in equine blood, as also previously reported for human monocytes. Interestingly, however, an increase in circulating CD163⁺ monocytes was not found during RAO. We hypothesize that either increased CD163⁺ monocytes were not recruited from the bone marrow or that CD163⁺ cells were preferentially recruited to the airway, where they mature into CD163⁺ macrophages. To further investigate CD163 phenotypic fidelity during maturation, we determined the maturation of CD163⁺ and CD163⁻ monocytes to macrophages in vitro. We observed no change in CD163 expression following differentiation of monocytes to macrophages. Thus, we concluded that, if in vitro findings predict in vivo conditions, then migration of monocytes to tissues, followed by differentiation into macrophages, might not result in changes in
CD163 expression. It would, therefore, be conceivable that expression of CD163 on monocytes is pre-determined as they mature and exit the bone marrow. This scenario then suggests that the specific local and systemic cytokine milieu will play important roles in the induction of CD163 expression during development of monocytes from bone marrow precursors. Indeed, the association of increased circulating CD163\(^+\) monocytes with specific diseases may be a consequence of local and systemic changes in the expression of cytokines, chemokines, and metabolic products that favour the development of CD163\(^+\) monocytes and their release into the circulation. It is, therefore, not surprising that sepsis, with accompanying cytokine storm, and treatment with glucocorticoids, induced an increase in the proportion of CD163\(^+\) monocytes in both mouse and human. *In vitro*, CD163 is upregulated by glucocorticoids and anti-inflammatory cytokine IL-10 and IL-6 but not by IL4 or IL-13 (41). Binding of haptoglobin-hemoglobin complexes is also recognized to increase CD163 expression through positive feedback triggered by IL-10 secretion. Conversely, CD163 is downregulated by pro-inflammatory factors including tumour necrosis factor, interferon gamma and the chemokine CXCL4. Thus, all the mechanisms regulating CD163 expression point to its role as a marker of cells with anti-inflammatory functions.

In conclusion, we have shown that a CD90-specific antibody specifically recognizes equine granulocytes and allows their separation from monocytes. Focusing on monocytes, we showed that equine monocytes express CD14 and are therefore similar to the human CD14\(^+\) classically activated monocytes. Unlike human classical monocytes, however, equine monocytes express abundant CD163, which is a marker of alternatively activated human monocytes. We could not use CD16 expression to resolve this discrepancy due to lack of cross-reactive antibodies. We investigated the kinetics of CD163 expression during inflammation and showed a marked increase in CD163-expressing monocytes in horses with systemic inflammation compared to healthy horses. This observation suggests that CD14\(^+\)CD163\(^+\) monocytes of horses are similar to pro-inflammatory monocytes in human and mouse. This conclusion was supported by finding that RAO, a non-septic inflammatory process confined to the respiratory tract, was not accompanied by a rise in circulating CD163\(^+\) monocytes, as observed in systemic inflammation.
References


Chapter 3

Cytokine Expression Patterns in Classical and Non-classical Equine Macrophages
Introduction

Macrophages are tissue-dwelling bone-marrow-derived cells that remain quiescent in tissues and exhibit minimal oxygen consumption, a low rate of protein synthesis, and little to moderate cytokine production (1, 2). They are efficient phagocytes that remove apoptotic cells under homeostatic conditions. However, following tissue invasion by pathogens or marked increase in cell death through apoptosis or necrosis, macrophages become activated and begin to produce increased cytokines and metabolic products (3). This ability to elaborate specific cytokines and metabolic factors following interaction with apoptotic cells or pathogens allows them to orchestrate the inflow of other cellular and humoral elements of the innate immune response and regulate the local tissue microenvironment, including the differentiation of incoming monocytes to macrophages and dendritic cells (4). In addition, the expression of co-stimulatory molecules allows them to interact with lymphocytes and other cells of the adaptive immune response and thus function as immunoregulatory cells in adaptive immunity (5). This heterogeneity of macrophage function has led to specializations that can already be recognized in monocytes, the precursors of macrophages. Thus, early studies showed that human alveolar, intestinal, and adipose tissue macrophages exhibit marked immunoregulatory function while macrophages found in the liver (Kupffer cells), peritoneum and lymphoid organs were shown to be more efficient at killing microorganisms (6, 7).

The concept of functional heterogeneity in macrophages is frequently related to the well known polarization of lymphocytes into Th1 and Th2 cells; with each cell type being characterized by the expression of specific cytokines and transcription factors (8). In the same way, macrophages are now classified as M1 (pro-inflammatory/classically activated) and M2 (anti-inflammatory/alternatively activated) subsets; interestingly, the cytokines produced by Th1 cells (e.g. IFN-γ) are known to induce macrophage polarization into M1 while Th2 cytokines (e.g. IL-4 and IL-13) direct the differentiation of macrophages into the M2 phenotype. Like Th1 and Th2 cells, M1 and M2 macrophages are classified according to the expression of specific surface receptors and elaboration of specific combinations of cytokines and metabolic products (9). Classical activation of macrophages leads to a preponderance of cells that produce a large amount of IL-12 and IL-23 but low quantities of IL-10. This subset of macrophages produces copious amounts of reactive oxygen and
nitrogen species, and inflammatory cytokines like IL-1beta, TNF, and IL-6. These macrophages, therefore, act as effector cells in Th1 responses and function in the elimination of tumor cells, other antigenically altered cells, and intracellular pathogens. Thus, the presence of M1 macrophages in tissues may be a surrogate indicator of a prevailing Th1 response (10).

In contrast to M1 macrophages, M2 macrophages produce only a little IL-12 and IL-23 but abundant IL-10. In addition, while M1 macrophages produce nitric oxide synthase to generate reactive nitrogen species, M2 macrophages synthesize arginase that inhibits nitric oxide production and allows macrophages to generate ornithine, leading to the production of hydroxyproline and polyamines, a process evidenced by the release of urea (11). M2 macrophages are primarily prominent in the context of tissue repair and remodeling, processes that are crucial for the downmodulation of immune response and restoration of homeostasis. This group of macrophages has also been recognized to participate in Th2-type responses and often contributes to the elimination of parasites. However, M2 macrophages have been associated with the altered immune response that leads to the development of atopy, an essential step in the immunopathogenesis of allergic asthma. Tumour-associated macrophages have often been characterized as M2; this subset of macrophages has therefore been implicated in the orchestration of the immunoregulatory changes that promote tumor progression (12, 13). In human and mouse, in addition to the expression of type 2 cytokines, M2 macrophages have been shown to express high levels of chitinase and chitinase-like genes (e.g. Ym-1). Indeed, elevation of chitinase activity in bronchoalveolar lavage fluid has been shown to be an important marker of allergic-type responses in the respiratory airway (14, 15).

Recurrent airway obstruction (RAO) of horses is a chronic non-septic neutrophilic airway inflammation characterized by pathophysiological changes similar to allergic asthma of humans (16). The similarities between RAO and allergic asthma informed several studies that investigated potential common pathways in the immunopathology of these diseases. Such immunological similarities would allow the equine patient to benefit from the substantial immunotherapeutic potentials generated from studies of allergic asthmatic humans, where a Th2 environment predominates. However, results of studies to ascertain the predominant cytokine environment in the equine airway were contradictory. Giguere et al., using RT-PCR, detected an increase in both IL-4 and IFN-γ without detecting IL-2, IL-5 and IL-10
mRNA, the signature cytokines of a Th2 environment, in the BAL of horses with RAO during different stages of exacerbation and remission (17). Similarly, Cordeau et al., using in situ hybridization detected IL-4, IL-5 and IFN-γ and documented a significant increase in IL-4 and IL-5 mRNA in RAO horses compared to control, suggesting a Th2 response and a role for IL-4 as seen in human asthmatics (18, 19). Conversely, Ainsworth et al., using real-time RT-PCR, detected neither IL-4 nor IL-5 but reported a significantly higher level of IFN-γ mRNA in RAO than control horses, suggesting the absence of a Th2 response in heaves (20). Thus, measuring cytokine expression in BAL fluid and lymphocytes could not establish a clear immunological basis for RAO in horses.

Studies examining alveolar macrophages (AMs) in RAO have provided more consistent results. Exposure of equine AMs to hay dust suspension and *Aspergillus fumigatus* resulted in copious production of TNF and IL-1β, suggesting that AMs may play a role in T cell polarization in the equine airway through cytokine secretion (21). Indeed, the secretion of mediators by AMs was shown to precede neutrophil infiltration of the airway following exposure of normal and RAO horses to moldy hay (22). In a similar study, AMs from RAO horses were shown to produce a higher amount of TNF, IL-1β and IL-8 than those from non-susceptible horses while the anti-inflammatory cytokine IL-6 was higher in non-susceptible than RAO horses (23). These studies suggested that failure of macrophage-mediated mechanisms, rather than, or in addition to, T-cell-related mechanisms, in the respiratory airway is an important component of RAO immunopathology in horses. However, unlike in human and rodent allergic asthmatics, AMs from RAO horses were not found to express a significantly higher level of chitinase than those from normal horses, suggesting that the mechanisms through which macrophages regulate the pulmonary immune responses may be different in horses and human subjects (24).

It is currently unknown whether macrophages from horses display the same type of functional heterogeneity reported in humans. Hitherto, there has been no attempt to classify equine macrophages into M1 or M2 subsets. However, we have already shown that, like human monocytes, CD14^+^CD163^+^ monocytes are increased in circulation during systemic inflammation, suggesting that macrophage precursors (monocytes) in horses may behave like those of humans during inflammatory conditions. We have, therefore, investigated the expression of signature cytokines, cytokine receptors, and metabolic enzymes in macrophages.
generated *in vitro* and those recovered from bronchoalveolar lavage fluid of RAO horses. Our results suggest that, like human and mouse, equine macrophages display functional dichotomy that may be important in their role in RAO.

**Materials and Methods**

**Horses, housing, handling, and sampling**

All animal procedures described were approved under AUP 10R030 by the University of Guelph Animal Care committee, in accordance with the guidelines of the Canadian Council on Animal Care. Research horses were kept on pasture with indoor access, fed hay, and received routine vaccinations and anthelmintics. Blood samples (100mL) were collected from 5 healthy research horses for the isolation of monocytes, which were then cultured and differentiated to macrophages *in vitro*. In addition, alveolar macrophages (representing *in vivo* differentiated macrophages) were cultured from bronchoalveolar lavage fluid (BALF) samples obtained from the same horses previously characterized as being affected with RAO in phase of remission and/or exacerbation (25).

**Isolation of monocytes from peripheral blood**

Following collection into heparinized 50mL syringes, 100mL of blood was divided into four 50mL tubes, mixed with 5mL 6% dextrose (in phosphate-buffered-saline; PBS) and allowed to stand at room temperature for 45 minutes. The supernatant was removed and carefully layered on 12mL of Ficoll-paque PLUS (GE Biosciences, Mississauga, Canada) in a 50mL tube. The tube was centrifuged at 300xg for 30 minutes with “no brake” and “ambient temperature” settings. Mononuclear cells, consisting of two visible white rings below the plasma layer, were carefully removed and transferred to a 15mL tube. The cells were counted and washed three times in sterile PBS+ and resuspended at 10⁶ cells/500µL.

To isolate CD163⁺ and CD163⁻ monocytes, mononuclear cells were incubated with anti-CD163 antibody (Clone Ber-Mac3; MBL Inc., Japan) for 15 minutes. This was followed by a single wash with 3mL of PBS+ and incubation with anti-mouse IgG immunomagnetic beads. After another round of washing in PBS+ (PBS containing 1% autologous horse serum and 1mM EDTA), the cells were resuspended in 500µL of PBS⁺ and passed through an LS column (positive selection) under a magnetic field created by a MidiMACS Separator (both
from Miltenyi Biotech, Auburn, California). The flow-through (negatively-selected cells) was collected and washed three times; this fraction contained CD163\(^{-}\) cells, including lymphocytes. Antibody-labelled cells were recovered by allowing buffer to flow through the column after removing it from the magnetic field; this sample contained CD163\(^{+}\) monocytes (see Figure 2.1). Positively-selected cells (CD163\(^{+}\)) were washed three times in growth medium and counted. The cells were then resuspended to contain 1x10\(^6\) cells/mL. Both CD163\(^{+}\) and CD163\(^{-}\) (500µL/well) cells were allowed to attach to tissue culture plates for 4 hours at 37\(^\circ\)C, after which any remaining non-adherent cells were removed by vigorously washing each well with warm growth medium. The adherent cells were incubated at 37\(^\circ\)C for 16 hours, washed with PBS and harvested by lysis in Cell Lysis Buffer from QIAPrep RNA isolation kit (Qiagen) for RNA extraction.

**Differentiation of monocytes to macrophages under M1- and M2-promoting conditions**
Following isolation of monocytes from 15 horses, cells were resuspended in growth medium (RPMI-1640 containing 20% autologous serum, 200U/mL penicillin, and 200µg/mL streptomycin) to contain 10\(^6\) cells per mL. The cells were then distributed into 12-well tissue culture grade plates (200µL) and cultured for one week at 37\(^\circ\)C after adding human GM-CSF to a concentration of 200U/mL to differentiate them into macrophages as previously described (26). In preliminary experiments, macrophages were activated with LPS and harvested after 4, 8, 16, 32 and 64 hours to determine the optimal time for RNA isolation and quantification of gene expression. In subsequent experiments, phenotypic polarization of fully-differentiated macrophages was initiated by addition of either with LPS (100ng/mL) and IFN-\(\gamma\) (20ng/mL) or IL-4 (20ng/mL) for 16 hours (2). Supernatants were collected for a Griess assay to determine the activity of nitric oxide synthase as described below. The adherent macrophages were lysed directly in the tissue culture plate for RNA extraction using the Qiagen RNA isolation kit (Invitrogen, Mississauga, Canada). Treatments were carried out in duplicates for each sample.

**Culture of macrophages from bronchoalveolar lavage fluid samples**
To isolate macrophages, 300mL of bronchoalveolar lavage fluid (BALF) was filtered through a double layer of sterile gauze to remove mucus. The cells in the fluid were counted and
cytospins were prepared to estimate the percentage of macrophages. The fluid was centrifuged at 300xg for 10 minutes at 4°C. The pellet was resuspended in 3mL of sterile PBS+ and washed three times. This step was followed by another 3 rounds of washing using growth medium (RPMI 1640 supplemented with 10% autologous horse serum, penicillin, streptomycin, and L-glutamine). After the final wash, cells were resuspended in growth medium to contain 1x10^6 macrophages/mL; 250µL of this suspension was seeded into a 6-well tissue culture plate. The culture plate was incubated at 37°C for 2 hours. Non-adherent cells were removed through vigorous washing with growth medium, followed by warm (37°C) PBS+. The adherent cells (macrophages) were then lysed in lysis buffer (Qiagen) and total RNA was extracted, measured and stored at -80°C. To avoid contamination with chromosomal DNA, an in-column DNA digestion step using the RNase-free DNase set (Qiagen) was carried out. In other experiments, the macrophages were not lysed; rather, they were cultured for 16 hours in the presence of 10ng/mL LPS before lysis and RNA extraction.

**Griess assay**

The activity of nitric oxide synthase (Nos) was determined by measuring the accumulation of inorganic nitrite (NO_2^-), a stable oxidative end-product of the antimicrobial effector molecule nitric oxide (27) in the culture medium of LPS or cytokine-stimulated macrophages. The method described by Classen et al. was used (28). This method is based on the oxidation of L-arginine to nitric oxide to yield citrulline, nitrites and nitrates. The specificity of the reaction was confirmed by using an analog of L-arginine, NG-monomethyl-L-arginine (L-NAME), as an inhibitor of nitric oxide synthase activity by macrophages. The Griess reagent was then used to measure nitrites in the culture medium. Briefly, 5 x 10^5 cells/well alveolar macrophages (AMs) or monocytes were seeded into 24-well tissue culture plates and allowed to attach for 2 hours (AMs) or 4 hours (monocytes). The cells were then treated as follows: (i) no treatment; (ii) IFNγ; (iii) IFNγ and LPS; (iv) IFNγ, LPS and L-NAME; (v) IL4; (vi) IL4 and LPS; (vii) IL4, LPS and L-NAME; and (viii) LPS alone. The concentrations of cytokines, chemicals and LPS were as follows: IFN-γ (100ng/mL; R&D Systems); LPS (100ng/mL, Sigma-Aldrich); L-NAME (10µM, Sigma-Aldrich); IL4 (20U/mL; R&D Systems). Cells were cultured for 16 hours at 37°C, at which time aliquots of supernatant (5µL) were collected and transferred to a 96-well plate. Serial dilutions of sodium nitrite (125
nM to 1µM) were then prepared. This was then followed by the addition of 50µL each of Griess reagent solutions (1% w/v sulfanilamide; and 0.1% w/v naphthylethylenediamine dihydrochloride; all were dissolved separately in 2.5% phosphoric acid, Sigma-Aldrich). Absorbance was measured at 550 nm in a Titertek microplate reader (Huntsville, AL, USA). The remaining adherent cells were washed in PBS+, and lysed in lysis buffer (Qiagen) before the isolation of RNA using the RNeasy kit (Qiagen). For PCR, samples with L-NAME were not analyzed.

**Primer design for RT-PCR**

Intron-spanning primers targeting equine-specific genes coding for mRNA of IL12 (p35 gene) and tumor necrosis factor (TNF) to typify M1 cytokines, and mRNA of CCL17, IL-10, and TIE-2 to typify M2 cytokines were designed using the primer tools at the National Center for Bioinformatics Institute (www.ncbi.nlm.nih.gov/tools/primer-blast/). For this purpose, the mRNA sequence for the human analogue of each target was retrieved from GenBank. A BLAST search was then carried out against the equine genome to identify homologous areas for each target on the horse genome database. Following alignment against all available equine sequences, conserved areas were identified. PCR primers were anchored at these consensus areas. When no consensus could be reached, the primers were made degenerate at positions of nucleotide disagreement to accommodate presently known single nucleotide polymorphisms at such locations. Primers for housekeeping genes (18S rRNA, equine glyceraldehyde phosphate dehydrogenase, and ubiquitin C) were also synthesized. The primers used in this study, including their positions on the equine genome are shown in Table 3.1.

**Polymerase chain reaction**

RNA obtained from macrophage culture was reverse-transcribed using the Superscript III First-Strand Synthesis System (Invitrogen). Cycling conditions were optimized in an Eppendorf thermal cycler for annealing temperature and magnesium concentration using cDNA obtained from lymphocytes activated with concanavalin A. The optimized cycling conditions were as follows: Step 1, 94°C for 4 minutes; Step 2, 94°C for 15 s; Step 3, 69°C for 30 s; Step 4, 72°C for 30 s; Step 5, return to Step 2 and repeat steps 2-4 35 times; Step 6,
extend at 72°C for 10 minutes. PCR products were analyzed using agarose gel electrophoresis and purified from the gel using the QIAquick Gel Extraction Kit (Qiagen). The concentration of purified product was measured and adjusted to 1µg/mL. For quantitative PCR, amplification was performed in a 25µL reaction volume and 96-well plate format using the SYBR Green PCR Master Mix and a LightCycler 480 (Roche Applied Science, Mannheim, Germany). The linear range for each primer pair and target was established by making eight 10-fold dilutions of the purified PCR product. The same dilutions were used to generate a standard curve after carrying out real-time PCR at each dilution in quintuplicate for each target within the linear range. After preliminary experiments established that the amount of equine GAPDH (eGAPDH) and 18s rRNA did not change between treated and untreated samples, eGAPDH was chosen as the housekeeping gene to normalize the cytokine amplification to result for each target.

**Data analysis**

Results were expressed as the ratio of target concentration (ng/mL) to eGAPDH concentration. Comparison of ratios was carried out using Mann Withney non-parametric and Student t-test executed in GraphPad Prism software. A p-value less than 0.05 was regarded as statistically significant and led to the rejection of null hypothesis of no difference between groups.
Table 3.1: List of Primers for RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIE-2</td>
<td>For 5’-ATGGACTCTTTTAGCCGGCTTA-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CCTTATAGCCTGCTCTCGAA-3’</td>
</tr>
<tr>
<td>CCL17</td>
<td>For 5’-GrGAcCyMCTCCTGGRCTCCWGW-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-GAACAGAYGGMCCTGCCCWWG-3’</td>
</tr>
<tr>
<td>IL10</td>
<td>For 5’-ACAGCTGCACCCAATCTCCA-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CCAGGGAGTTCACTGACRTGCKCC-3’</td>
</tr>
<tr>
<td>Equine GAP</td>
<td>For 5’-GTTTGTGATGGCGTGTAACC-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-TTGGCAGCACTGAGTAAGGC-3’</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>For 5’-ATGCGGCGGCGTTATCC-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-GCTATCAATCTGCAATCTGCTCC-3’</td>
</tr>
<tr>
<td>P35</td>
<td>For 5’-CTAATGGGAGTTGCTGRCYCC-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-TGCGCRGATTCTGAAGGCATG-3’</td>
</tr>
<tr>
<td>TNF</td>
<td>For 5’-TGGGGTGATCGGCCCCCAGAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CCCAGGAGGGCATKTGCACGCC-3’</td>
</tr>
</tbody>
</table>

*Primers were designed after obtaining analogous human gene sequences and comparing them with the equine genome. Sequences retrieved from available equine entries in GenBank were aligned to generate consensus sequences as targets for the primers. When there was no consensus, degenerate primers were designed to accommodate single nucleotide polymorphisms. Nucleotides at degenerate positions are shown in red (International Union of Biochemistry nucleotide codes at degenerate positions: R=A/G; Y=C/T/U; M=A/C; W=A/T/U; and K=G/T/U)
Results

PCR for M1 and M2 markers

Previous studies in humans established IL-12 (p35) and TNF as markers of M1 macrophages while IL-10 and the regulatory T-cell (Treg) chemokine CCL17 were shown to be expressed by M2 macrophages (29). In addition, TIE-2-expressing monocytes are pro-angiogenic and tend to upregulate M2 markers following differentiation into macrophages in tissue (30).

Using our set of primer pairs, we successfully amplified these targets and generated a single PCR band. The specificity of each amplicon was determined by sequencing and BLAST search of the results to determine similarity with equine sequences. All our targets were confirmed by BLAST search to amplify the desired sequences. Specificity of real-time PCR amplification was confirmed using melt-curve analyses and showed a single peak. An amplification efficiency of 1.80 – 1.85 was noted for all the primer pairs, allowing us to compare the results. To evaluate analytical accuracy, 5 different samples were repeated over 20 different runs; and coefficients of variation of 3.2 to 8.5% were calculated. Any run where the result showed a variation in the ratios of target to eGAPDH greater than 10% in these samples was rejected and repeated. Sample melt and amplification curves are shown in Appendix 2.

Expression of M1 and M2 markers by activated in vitro derived macrophages is time-dependent

Following activation of macrophages derived in vitro with LPS, we determined the optimum time of activation for evaluating changes in cytokine expression. Surprisingly, there was a bimodal pattern of gene expression by activated macrophages (Figure 3.1). We therefore chose 16h following activation as the optimal time-point to evaluate differences in target gene expression. This pattern was confirmed in alveolar macrophages (regarded as in vivo-derived) from healthy horses.
Figure 3.1: Time-dependent expression of cytokine mRNA in equine macrophages activated with lipopolysaccharide (n=5). Plots show mean and standard errors of the mean.
**Differential expression of M1 and M2 markers on CD163⁺ and CD163⁻ monocytes:**

Since we showed in Chapter 2 that CD163⁺ and CD163⁻ monocytes do not change their phenotypes after maturing to macrophages, we sought to determine the profiles of cytokine and chemokine expression in these two subsets of equine monocytes following adherence to plastic and limited culture *in vitro* (16h). Significantly higher expression of TNF (p = 0.032; n = 16; unpaired t-test with Welch correction) and IL-10 (p = 0.049) was found in CD163⁺ cells when compared to CD163⁻ monocytes. Conversely, CD163⁻ monocytes expressed a significantly higher amount of CCL17 (p = 0.017) than CD163⁺ monocytes (Figure 3.2). However, there were no significant differences in the expression of Tie-2 and IL-12 by both subsets of monocytes.

**Expression of M1 and M2 markers on alveolar macrophages during remission and exacerbation of RAO:**

Finally, we investigated the response to LPS activation of alveolar macrophages isolated from BALF before and after induction of RAO. Our results showed that while pre-challenge alveolar macrophages responded to LPS by increasing the expression of TNF, IL12, and IL-10 (Figures 3.3), cells isolated from post-challenge BALF were refractory to LPS stimulation except IL-10, where LPS treatment led to a marked reduction in mRNA levels (Figures 4).

**Equine macrophages express no detectable inducible nitric oxide activity:**

Colorimetric measurement of nitrates in the supernatant of cultured macrophages as an indicator of nitric oxide synthase activity, whether generated *in vitro* or recovered from BALF, yielded no detectable nitric oxide.
Figure 3.2: Expression of cytokines CD163\(^+\) and CD163\(^-\) monocytes.
Figure 3.3: Response of alveolar macrophages from horses in remission (Pre-RAO) and RAO exacerbation (Post-RAO) to stimulation by lipopolysacharide
Discussion

Expression of cytokines and metabolites in macrophages is often determined at a single time point. However, such approach may fail to capture the dynamics of marker expression in cells of the immune system. The expression of these regulatory proteins is tightly controlled through specific signaling pathways and intracellular mRNA induction via distinct transcription factors that show time-dependent expression (31). In the present study, our first goal was to capture the kinetics of TNF, IL-10, IL-12, CCL-17 and TIE-2 mRNA expression and to determine the best time-point to evaluate differences in expression among treatment groups. We found a bimodal expression pattern where, following activation with LPS, there was low expression at 8h, high expression at 16h, followed by low expression at 32h, and higher expression at 64h. This observation is evidence of the tightly controlled expression of cytokines, at least at the transcriptional level, and the limitations inherent in measuring cytokine mRNA rather than protein. Using an in vitro model of macrophage activation, Bastonini et al. demonstrated that TNF expression is induced as early as 10 minutes following activation (32). However, like our results, these authors demonstrated a bimodal pattern of mRNA expression whereby TNF mRNA was high after 10 minutes, lower at 1 h and elevated again at 3 h. These findings illustrate the danger in extrapolating data obtained by collecting a single time-point sample from patients, measuring cytokine expression using mRNA level and concluding on the prevailing milieu in the respiratory tract. This notion is further accentuated by findings of so-called polarization of macrophages, which unlike T-cell polarization, is reversible following removal of the polarizing environment (10, 33-35). A major limitation of our study is that cytokine expression was analysed following adherence of macrophages to plastic. This may not fully replicate the dynamics of mRNA expression by macrophages in vivo. The use of flow cytometry, a technique that allows immediate evaluation of marker expression shortly after sample collection, may be a better assay for characterizing the in vivo microenvironment from which macrophages are obtained. However, for this technique to be useful, specific markers measured using flow cytometry must be reliably correlated with the phenotype of macrophages.

Cells of the myelomonocytic lineage are known to show a high degree of plasticity, and are capable of adaptive differentiation mainly on the basis of environmental signals (36, 37). The functional properties of these cells are constantly reprogrammed as they respond to
signals from damaged tissues, microbes and activated and naïve lymphocytes (37). Thus, differentiation and polarization of cells of this lineage could be used as indicators of the prevailing environment during chronic inflammatory diseases like RAO. In this study, our first goal was to determine whether there were differences in the profile of M1 and M2 marker expression in CD163\(^+\) and CD163\(^-\) non-differentiated monocytes. Using monocytes cultured for a short time (16 hours) \textit{in vitro}, we showed significant differences in the expression of CCL17 and IL-10 in CD163\(^+\) versus CD163\(^-\) monocytes. While CD163\(^+\) cells tended to express a significantly higher amount of IL10 mRNA, CD163\(^-\) cells expressed a significantly greater amount of CCL17 mRNA than CD163\(^+\) cells. Since both subsets were isolated from the same donors and treated similarly, these findings suggest that even by the time they appear in circulation, monocytes have already been polarized on the basis of their potential to express specific cytokines. These results are in agreement with what we found in Chapter 2, where CD163\(^+\) and CD163\(^-\) monocytes were shown to maintain their phenotypes following differentiation to macrophages \textit{in vitro}. Our findings are also consistent with studies showing the specific recruitment of CD163\(^+\) cells into circulation during cardiopulmonary bypass, HIV infection and systemic sclerosis in human (38-40). However, in humans and mice, both CCL17 and IL10 are considered to be effectors of M2 macrophages. Thus, while our findings support the conclusion that CD163\(^+\) monocytes are alternatively-activated (M2) cells, the higher expression of CCL17 by CD163\(^-\) monocytes suggests that these cells are more like classically-activated monocytes in other species. This discrepancy may be explained by the recent finding that even M1 cells are subject to the regulatory effects of CCL17 (41, 42). Increased production of CCL17 was shown to lead to inhibition of M1 cells and polarization to M2. In addition, a recent systematic evaluation of markers associated with alternative polarization of macrophages confirmed that IL-10 expression is specifically associated with M2 macrophages in human (43). Thus, on this basis, we conclude that IL-10-rich CD163\(^+\) macrophages in horses are similar to M2 macrophages of human. Nonetheless, these results are interpreted with caution since in vitro culture conditions may not replicate \textit{in vivo} conditions, especially in a cell type that constantly changes its phenotype in response to environment cues.

In addition, we evaluated the response of alveolar macrophages to LPS during remission and following induction of acute exacerbation of RAO. Interestingly, while
macrophages from horses in remission generally responded to LPS by upregulation of cytokine expression, cells from RAO horses in exacerbation appeared refractory to LPS activation. This finding underscores the importance of LPS in the immunopathogenesis of RAO. The phenomenon of endotoxin tolerance in macrophages has been described in sepsis (44). It was also recently shown that endotoxin tolerance concurs with M2 polarization of macrophages (45). This might explain the increased expression of IL-10 mRNA but not other cytokines in alveolar macrophages from horses with exacerbated RAO.

In conclusion, we have shown that, when examining cytokine mRNA expression in both in vitro and in vivo derived equine macrophages, the time of sample collection is crucial since expression levels vary with time. In addition, we demonstrated that CD163⁺ macrophages produce significantly more IL10 and, as such, are equivalent to alternatively activated human macrophages. These findings should provide a basis for using this marker as an indicator of M2 polarization in horses.
References


Chapter 4

Heterogeneity of Equine Alveolar Macrophages in Recurrent Airway Obstruction
Introduction

Recurrent airway obstruction is a respiratory disease of horses characterized by a chronic non-septic neutrophilic inflammation and airway smooth muscle hyperreactivity to cholinergic agents reversible by beta-adrenergic agonists. The similarities in the pathophysiology of this disease and allergic asthma in humans has led to the conclusion that they share common immunopathologic mechanisms. Unlike RAO, however, allergic asthma in humans is characterized by chronic eosinophilic airway inflammation resulting from a polarized immune response to innocuous environmental allergens. Polarized Th2 cells secrete IL-4, IL-5 and IL-13, among other type 2 cytokines, and chemokines. Production of IL-5 by lymphocytes is a crucial step in the maturation of eosinophils in the bone marrow, and their recruitment and survival in the respiratory tract (1). Conversely, the immunological basis of the marked neutrophil infiltration into the airway of horses with RAO is poorly understood since polarized lymphocytes have not been demonstrated to play a prominent role. However, studies examining macrophages from RAO horses have suggested that this cell type may be very important in this condition (2).

Resident macrophages and dendritic cells play a crucial role during acute inflammation in any tissue. The activities of these cells lead to the rapid recruitment of granulocytes that eventually assume the primary role in the initial attempts to eliminate invading pathogens (2). Thus, it is conceivable that, in the absence of an ongoing septic process during RAO, resident airway macrophages may play an important role in the deranged immunological response that leads to chronic infiltration of the airway and lung tissue by neutrophils. However, the role of alveolar macrophages in airway inflammation has not been extensively studied.

The bronchoalveolar lavage fluid (BALF) provides a unique window into the prevailing cellular and humoral environment of the lung and can be used to evaluate the physiology and pathology of the equine lung (3, 4). It is extensively used in the evaluation of lung disease in human patients (5), and since the first use of this technique in horses in the 1970s, it is now widely recognized that BALF analysis is more sensitive than radiography, endoscopy or tracheal wash for the diagnosis of inflammatory disease of the airway in horses (5). The BALF analysis has been used by several workers to estimate the prevalence of inflammatory airway disease, evaluate therapies and measure inflammatory mediators in the
airway of horses (6-10). Although differential cell counts in BALF are routinely used to evaluate the ongoing inflammatory process in the lungs, it has only recently become apparent that cell distribution may not provide adequate information on pulmonary disease processes. Thus, the recent trend is to exploit both the phenotypic characteristics of the cells and the proteomic and genomic profiles of the fluid component of the BALF to provide unprecedented and valuable information on the pathogenesis of lung diseases like RAO (11). We have shown in Chapters 2 and 3 that equine monocytes and macrophages can be classified into two subsets on the basis of CD163 expression. Cells that express CD163 were shown to predominantly produce IL-10 following activation with LPS, suggesting that they are similar to alternatively-activated (M2) macrophages of human. In the present study, we used flow cytometry to characterize the phenotypes of macrophages in BALF and to interrogate the expression of CD163 and CD206, markers of M2 macrophages in human.

Materials and Methods

Antibodies:
The primary antibodies used in this study were FITC-conjugated anti-human CD163 (clone Ber-Mac3; MBL, Nagoya, Japan), anti-human CD14 (clone biG 10; Cell Sciences Canton, MA, USA), PE-conjugated anti-human CD206 (Clone 3.29B1.10; Beckman Coulter), anti-horse MHC Class II (Clone CVS20; Serotec), anti-horse CD5 (clone CVS5; Serotec), and anti-CD90 (clone DH2A; VRMD, Seattle, WA, USA). Secondary antibodies consisted of Alexa-fluor-488-conjugated rabbit anti-mouse IgG (Invitrogen, Mississauga), Q-dot655-conjugated rabbit anti-mouse IgG (Invitrogen). Purified mouse IgG (Invitrogen) was used as a control to ensure specificity of antibody binding.

Horses and Sampling:
Samples (Appendix 3) were collected from 16 horses without RAO belonging to the University of Guelph (Healthy). Horses were kept on pasture with indoor access, fed hay, and received routine vaccinations and anthelmintics. Horses with RAO (n=16) consisted of client horses visiting the OVCTH because of exacerbated clinical signs of previously diagnosed RAO, which was confirmed using respiratory examination, endoscopy and evaluation of
bronchoalveolar lavage fluid (BALF). Bronchoalveolar lavage was performed by an experienced clinician under standing sedation as previously described (12). Aliquots of BALF samples were placed on ice and immediately transported to the laboratory for flow cytometry. All samples were analyzed within 3 to 4 hours of collection. Data from samples that could not be processed within 4 hours of collection were excluded from analysis.

**Sample preparation:**

BALF samples (Appendix 3) were first filtered through sterile gauze to remove visible mucus plugs followed by centrifugation at 300g for 10 minutes at 4°C. The supernatant was discarded and pellet was washed three times in staining buffer (phosphate-buffered saline containing 10% acid citrate dextrose, 2% gamma globulin-free horse serum, 10mM EDTA, and 0.2% sodium azide). The cells were then resuspended in 3mL of staining buffer, counted and diluted to contain $10^6$ cells/mL. Aliquots (100µL/$10^5$) were distributed into flow cytometry tubes and placed on ice. For staining, 10µL of pre-titrated directly-conjugated primary antibodies (CD163-FITC; CD206-PE) (final concentrations are given on Table 2.1) was added and the cells were incubated for 15 minutes on ice. In some experiments, the primary antibody was conjugated (CD14, CD90, MHC II) to FITC before staining. The only unconjugated primary antibodies used in this study were anti-CD5 and anti-CD163 (same clone as CD163-FITC but used when it was convenient to examine CD163 reactivity on a channel different from the green channel, FL1). When these antibodies were used, they were first added to the staining reaction, followed by a round of washing and incubation with Alexa-488 or Q-dot-conjugated anti-mouse IgG as the secondary reagent. The stained cells were washed in 3 mL of staining buffer by centrifuging at 300g for 5 minutes. Pellets were then resuspended in 350-400µL staining buffer and analyzed in a FACSscan (BD Biosciences, Mississauga, Canada). Preliminary experiments showed that stained cells could be fixed in 5% paraformaldehyde and kept in the refrigerator (4°C) overnight without any loss of fluorescence intensity.
Flow cytometry:
Stained cells were analyzed with a FACScan flow cytometer (BD Biosciences) using the Cell Quest Pro acquisition software. The forward and side scatter thresholds on logarithmic scales were first adjusted using unstained cells, without gating out populations with very low side and forward scatter properties. Fluorescence from the green channel (FL1), yellow channel (FL2) and red channel (FL3) was set using cells stained with secondary antibodies alone (anti-mouse-FITC, -PE, Alexa488 or -Qdot655). The photomultiplier tube (PMT) voltage and fluorescence gain on each channel was adjusted such that the cells with only secondary antibodies were within $10^0$ to $10^1$ fluorescent units. However, high autofluorescence was noted on a proportion of cells. These cells were therefore first gated on the basis of autofluorescence in the unused channel (generally FL3) before data acquisition. Since lymphocytes are generally of much lower autofluorescence, it was sometimes necessary to carry out a second data acquisition using lymphocytes for the settings. For multiparameter acquisition, where fluorescence values from the three channels were combined, spectral overlap was minimized by adopting the fluorescence-minus-one strategy (13). However, this method did not work well for BALF cells and it was difficult to set up compensations for three different antibodies; therefore, most of the work in this study was done with double rather than triple antibody staining. Briefly, when FITC/Alexa488-, PE- and Qdot655-conjugated antibodies were used together, compensation for FITC/Alexa488 was adjusted using tubes staining positive for antibodies conjugated to PE and Qdot655; PE-conjugated antibodies were compensated using tubes containing cells staining positive for antibodies conjugated to Alexa488/FITC and Qdot655; while Qdot was compensated using positive samples stained with antibodies conjugated with FITC/Alexa488 and PE. The validity of the compensation settings during acquisition was subsequently checked using post-acquisition compensation with FlowJo software. After drawing gates to identify the population of interest, the flow cytometer was set to acquire a minimum of 10,000 events from the macrophage gate.

Data analysis analysis
Normal distribution was not assumed in the data set generated in this study. Therefore, Mann-Whitney test with a significance level of 5% was used to compare populations in Graphpad Prism (Graphpad, La Jolla, CA, USA).
Results

*Forward and side scatter properties of BALF cells:*

In healthy horses, two distinct populations of cells were identifiable on the basis of forward and side scatter: a population with low forward and side scatter (R1) and a population with medium to high side scatter (R2) (Figure 4.1A). In horses with exacerbated RAO, a third population with high forward scatter and medium to high side scatter (R3) was prominent (Figure 4.1B). When population R2 was further analyzed on the basis of autofluorescence in the red (FL3) channel, two populations became apparent: A1 contained cells with low autofluorescence and low forward scatter, A2 contained cells with medium autofluorescence and low to medium forward scatter. Exacerbated RAO was associated with an increase in the A1 population (Figure 4.1C & D).

*Gating Strategies:*

Using antibodies against CD5 and MHC II, cells in population R1 were identified as lymphocytes (CD5⁺MHCII⁺) (Figure 4.2A – B). No CD5⁺ cells were found within the R2 and R3 populations (Figure 2C - D). Since we had previously shown that CD90 is a specific marker of granulocytes in horses, we used anti-CD90 antibody to probe cell populations in R2 and R3 and showed that neutrophils (CD90⁺ cells) were present within the A1 subpopulation of R3, while the A2 region did not contain neutrophils (Figure 3A-D). Similarly, having previously shown that monocytes express CD163, and that the differentiation of monocytes to macrophages in horses is accompanied by CD206 expression, we used anti-CD163 and anti-CD206 antibodies to probe R2 and R3 for macrophages. Our results showed that CD163⁺CD206⁺ cells are present in both regions R2 and R3 (Figure A - B). Thus, we concluded that the distribution of cells in BALF is as follows: (i) R1 contains lymphocytes; (ii) R2 contains macrophages and neutrophils; (iii) R3 contains macrophages without neutrophils. Thus, two populations of macrophages are present in BALF; (a) small macrophages that cluster with neutrophils but have high autofluorescence; and (b) large macrophages with high forward and side scatter, and high autofluorescence. This finding was confirmed when BALF from horses with different proportions of neutrophils were examined since a proportionate increase in the density of the R2 population correlated with an increase in the proportion of neutrophils determined from differential counts of cytopsin preparations.
Cell-specificity of flow cytometric detection of CD90, CD163 and CD206 expression was confirmed using immunocytochemistry (Figure 4A - C).

**RAO is associated with a decrease in the proportion of CD163+ cells:**
Since we established that CD163+ cells might be phenotypically different from other macrophages, we sought to investigate the expression of this marker on non-RAO (n=15) versus RAO horses in exacerbation (n=15) using flow cytometry. To achieve this aim, cells were stained with antibodies against CD90, CD206 and CD163. After gating on forward and side scatter, followed by CD90 expression, neutrophils were excluded as cells that expressed CD90. The remaining CD90-CD206+ cells in the R2 and R3 gates were examined. CD163 fluorescence was expressed as fold change in median fluorescence intensity when stained cells were compared with unstained cells. Our results showed that BALF from healthy horses contained a significantly higher proportion of CD163+ macrophages than BAL from horses with exacerbated RAO (p<0.05) (Figure 4.5).
Figure 4.1: Forward and side scatter characteristics of BALF cells. Flow cytometric analysis of BALF cells from a healthy horse showed two distinct populations (Figure 1a); R1 consists of cells with low side scatter (granularity) and low forward scatter (cellular size), R2 is made up of cells with medium to high side scatter and low to high forward scatter. Additional population (R3) with medium side scatter and low forward scatter appears in horses with exacerbated RAO (Figure 1b). Further analysis of BALF cells from healthy horses (Figure 1c), showed that cells in the R2 region have different autofluorescent properties; cells with low autofluorescence (A1) and high autofluorescence (A2) in the red channel. Population A1 is increased in exacerbated RAO while there is a reduction in the proportion of population A2 (Figure 4.1d)
Figure 4.2: Expression of lymphocyte and macrophage markers on BALF cell populations. Following staining with antibodies to CD5 (lymphocyte marker), cells in the R1 but not R populations were identified as lymphocytes (Figures 4.2a&b) while staining with antibodies against macrophages (CD163 and CD206) showed that the R2 but not R1 populations contain macrophages (Figures 4.2c &d).
Figure 4.3: Distribution of macrophages and neutrophils in equine BALF cells. Following staining with antibodies to neutrophils (CD90) and macrophages (CD163 and CD206), macrophages are present in both populations A1 and A2 (Figure 4.3a & b) while neutrophils are present in population A1 (Figure 3c) but not A2 (Figure 3d), which contains only macrophages.
**Figure 4.4:** Immunocytochemical staining of BALF cells. (A) Neutrophils but not macrophages express CD90. (B) Macrophages but not lymphocytes express CD163 and CD206 (C).
Figure 4.5: Reduced proportion of CD163-expressing alveolar macrophages during RAO exacerbation (Comparison of mean percentages using unpaired t-test with Welch’s correction n=32; healthy =16, RAO= 16; p=0.0009; Comparison of variance using F-test, p=0.003)
Discussion:
We used flow cytometry to interrogate the cellular components of BALF of horses. Like McGorum et al (1993) we found three distinct cell populations in BALF (14). However, the cytometer settings used by these authors did not allow obtaining consistent population clusters, leading to the exclusion of most of the samples collected and inability to analyze macrophages in their study. Nonetheless, by focusing on lymphocytes, they were able to demonstrate a significant increase in B lymphocytes in BAL of horses with RAO and thus confirm, for the first time, the utility of flow cytometry in understanding this disease. We addressed this challenge of distinguishing macrophages with marked variation in size and complexity by using logarithmic rather than linear settings for flow cytometry. We also took advantage of autofluorescence to separate cells into distinct populations that could be more readily analysed.

A previous study in pigs showed that alveolar macrophages exist as a heterogeneous population when recovered from BALF (15). Similar to our findings, these authors were able to identify three distinct non-lymphoid populations which they classified as large high autofluorescent cells (LHC), small high autofluorescent cells (SHC), and small low autofluorescent cells (SLC) (17). They interpreted the LHC and SHC as macrophages and SLC as granulocytes. In a similar study using flow cytometry to examine BALF from human patients with chronic obstructive pulmonary disease (COPD), Hodge et al. used specific macrophage, granulocyte and dendritic cell markers to identify three distinct populations of cells within the population identified as R2 in the present study (16). In our study, the equivalent SLC population was shown to consist of CD90$^+$ cells, equine neutrophils. Both LHC and SHC populations were comprised of CD206$^+$ cells, confirming that they were indeed macrophages. Thus, our study indicated that the distribution of BALF macrophages is conserved across species and suggests that flow cytometric analysis of BALF cells is a suitable method to evaluate the intraluminal cellular component of the lung.

The study of Berndt and Muller demonstrated a significant increase in the SLC population following experimental infection of pigs with Pasteurella multocida, an indication of neutrophil infiltration (15). Similarly, we showed a consistent increase in the population of CD90$^+$ neutrophils within this population in horses with exacerbated RAO. The use of CD90 to separate neutrophils from macrophages in equine BALF also allowed us to investigate the
heterogeneity of macrophage phenotypes in the horse. Changes in the phenotypic characteristics of macrophage populations have been used to monitor disease progression in a mouse model of pulmonary tuberculosis (17). Using antibodies specific for macrophages and dendritic cells, the authors were able to differentiate between resident and infiltrating macrophages and dendritic cells during pulmonary tuberculosis. Reproducible changes were also found in the expression of activation markers on these cells during acute and chronic diseases. A recent study in human patients with COPD further investigated the possible roles of different macrophage populations in BALF (18). In their study, Frankenberger et al. demonstrated that the small macrophages (the equivalent of the small R2 macrophage population in our study) accounted for over 85% of the R2 population, compared to just 12% in control subjects. In addition, they identified this population of macrophages as the major producers of chemokines and TNF, and thus as the orchestrators of airway inflammation in this disease (18).

In our study, we neither attempted to separate macrophages from dendritic cells nor to distinguish small from large macrophages. However, having used CD90 to separate macrophages from neutrophils, we found a significant reduction in the proportion of CD163-expressing macrophages in horses with RAO compared with healthy or non-RAO controls. This finding is very interesting since it suggests a fundamental difference in the prevailing immunological environment in horses with RAO compared to healthy horses or horses with other inflammatory conditions. We have previously shown that CD163+ macrophages are characterized by expression of IL-10 mRNA, a well known anti-inflammatory cytokine produced by M2 macrophages (19). Our study, therefore, suggests that, at the minimum, the inflammation of RAO is potentially related to a macrophage-directed pro-inflammatory environment also deficient in anti-inflammatory cell types. Although this is a very interesting proposition, further studies are needed to characterize the two populations of macrophages identified in this study and to determine whether they are indeed active participants in the orchestration of the neutrophilic inflammation of RAO. A clearer understanding of this basic immunological phenomenon could lay the foundation for potential immunotherapeutic strategies that manipulate macrophage populations in the lungs.
References


Chapter 5

General Discussion
The work presented in this thesis has underscored the importance of alveolar macrophages in the pathogenesis of pulmonary diseases, evaluated the phenotypes of monocytes and macrophages and demonstrated that flow cytometry could be a useful tool in probing the local immune response in the lung.

In Chapter 2, the phenotypic characteristics of blood monocytes were evaluated in healthy horses and RAO horses with airway inflammation. Antibody staining of samples from 61 horses, followed by flow cytometry, showed that the clustering pattern of human leukocytes can not always be extrapolated to horses when using this technique. The spillover of granulocytes and NK cells has long been recognized as a problem in the phenotyping of monocytes, even in human subjects where there is a relatively good separation of monocytes from other cells on the basis of forward and side scatter characteristics (1). We demonstrated that an antibody directed against CD90, which recognizes T cells in other species, will specifically recognize granulocytes in horses. This antibody became a very important tool for separating granulocytes from monocytes when studying the expression of surface markers on equine blood monocytes. Monocytes were characterized by probing with antibodies to CD14 and CD163 to classify them into M1 and M2 subsets. Although anti-human CCR2 was tested as a possible marker of M1 monocytes as reported in human and mice (2), equine monocytes showed no reactivity with this antibody. A major limitation of this part of the thesis is the restriction of monocyte characterization to just 2 antibodies; the extensive use of other antibodies, e.g. antibodies against MHC class II (used only on limited sample numbers in this study), CD80 and CD86, could have provided important data on other characteristics of CD163+ versus CD163- cells. Nonetheless, the study showed that CD163 expression is associated with systemic inflammation in horses. A limitation of this finding is the failure to measure soluble CD163 in the plasma since this would have indicated whether there was truly an increase in CD163 expression or whether changes in expression were due to shedding of surface CD163, as previously reported in humans (1). However, it is known that the density of CD163 detected by monoclonal antibodies to this protein could vary as a result of the location of the epitopes recognized by the antibodies. Antibodies recognizing epitopes located on the transmembrane portion of CD163 do not show variations in staining density that have previously been interpreted as indicators of shedding (3).
In Chapter 3, we attempted to associate cytokine and chemokine mRNA expression profiles with specific subsets of equine macrophages. We showed that CD163+ and CD163− macrophages showed significant differences in their expression of CCL17 and IL10. The preferential expression of IL10, an anti-inflammatory cytokine, in CD163+ macrophages allowed us to conclude that these are alternatively activated (M2) as previously reported in human, mice, rats and other species (4, 5). Using activation with LPS, we showed that alveolar macrophages from horses in RAO exacerbation were unresponsive to activation. We interpreted this observation as consistent with the well-known phenomenon of endotoxin tolerance (6-8). A limitation of this part of the study was the failure to correlate mRNA expression with protein expression. The regulation of mRNA and protein stability may be different; this means that the kinetics of mRNA expression may not always be the same as protein expression. However, the bimodal pattern of mRNA expression that we observed in our study is consistent with other findings (9), where it was also demonstrated that sampling time may be a crucial factor in determining what kind of cytokines are expressed in the airways whether by the use of PCR or protein measurement.

Finally, in Chapter 4, we showed that flow cytometry can be conveniently used to characterize the cellular components of bronchoalveolar lavage fluid samples. This possibility opens new opportunities for the use of this method as equine diagnostic tool. A similar study using samples from human patients with chronic obstructive pulmonary disease (COPD) showed a comparable distribution of inflammatory cells and demonstrated that manual differential counts may not be highly reliable (10). Unfortunately the reliability of manual differential counts compared to counts obtained using flow cytometry was not evaluated in this study. In addition, we found a reduction in the proportion of CD163+ macrophages in horses with RAO exacerbation when compared with healthy horses. Whether this observation was a cause or effect of RAO is unknown. Similarly, the presence of small and large-sized macrophages, as demonstrated in our study, has been shown to be very important in human cystic fibrosis (11). Wright et al. demonstrated that CD68low sputum small macrophages were associated with cystic fibrosis and characterized them as immature macrophages originating from recently-recruited monocytes in the airways. We did not carry out further characterization of small versus large macrophages in this study. Nonetheless, these findings provide important preliminary data that could form the basis of future studies.
of the immunology of RAO using cells obtained from bronchoalveolar lavage fluid samples. Studies in mouse models and human patients have suggested that the phenotypes of lung macrophages could be used to guide stratification and treatment of patients with airway disease (12).

**Future Directions:** The studies reported in this thesis have provided the groundwork and preliminary data that could be used to justify further investigation of alveolar macrophages in recurrent airway obstruction. Such studies should attempt to answer the following questions:

1. What are the different populations of non-lymphoid cells in healthy and RAO horses? This question will be best addressed by using a greater panel of differentiation markers to identify small and large macrophages, including dendritic cells. Studies in human asthmatics and using mouse models have shown that dendritic cells play a central role in the pathogenesis of allergic asthma (13). Indeed, therapeutic strategies that removed dendritic cells during asthma exacerbation were found to reduce the severity of disease (13, 14). Reprogramming of macrophages has also been touted as a possible therapy in asthma (15). The ability to recover a large number of cells from the equine bronchoalveolar lavage fluid makes dendritic cell based therapy a more practical alternative in horses than any other species.

2. What are the functional properties of the different populations of macrophages recovered from the equine bronchoalveolar lavage fluid? It would be interesting to understand the specific functional profiles of the different populations of macrophages in the BAL of RAO and healthy horses.
References


### Appendix 1: Horses samples used for characterization of CD163 expression in blood monocytes

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*Mean fluorescence index; b QH=quarterhorse; STB=standardbred; TB=thoroughbred; WB=warmblood; MH=miniature horse; c Not available; **samples were analyzed three times to investigate the variability of results between analyses.
Appendix 2: Sample Amplification Curves and Melt Curve Analyses of PCR products
Melt curve: 18s

Melt curve: CCL17
### Appendix 3: Summary of BAL samples

**BAL differential cell count**

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*a* Ly=lymphocyte, Neut=neutrophil, Mac=macrophage, Eo=eosinophil, Mast=mast cell

*b* diagnosis;  
*c* recurrent airway obstruction;  
*d* inflammatory airway disease;  
*e* exercise-induced pulmonary hemorrhage