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Studies of Molecular Pathways Associated with Blood Neutrophil Corticosteroid Insensitivity in Equine Asthma

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Résumé

L'asthme équin est caractérisé par une hyperactivité bronchique, une inflammation neutrophilique, et des altérations structurelles des voies aériennes. Chez les patients asthmatiques présentant une inflammation neutrophilique, les corticostéroïdes sont peu efficaces pour contrôler les signes cliniques. L'hypothèse de cette étude est que l'hypoxie et/ou le stress oxydatif dans le microenvironnement des poumons peuvent contribuer à une insensibilité des neutrophiles aux corticostéroïdes dans l'asthme. Les chevaux sont les meilleurs modèles animaux naturellement affectés par l'asthme neutrophilique et partagent plusieurs caractéristiques physiopathologiques avec l'humain. La viabilité, l'apoptose, l'expression génique d'IL-1β, TNF-α et IL-8 ont été mesurées dans des neutrophiles isolés à partir de sang périphérique de chevaux asthmatiques sévères (N=8) et de chevaux sains (N=8) en condition de culture stimulant l'hypoxie et le stress oxydatif, en présence ou non de corticostéroïdes (Dexamethason (Dex)). L'IL-1β et le TNF-α, mais pas l'IL-8, ont été régulés négativement en présence de dexaméthasone dans des conditions de stress oxydatif induites par la pyocyanin. Bien que l'IL-17 et le LPS aient stimulé les neutrophiles équins, l'expression génique pro-inflammatoire n'a pas diminué après l'administration de Dex. En condition d'hypoxie induite par $CoCl_2$, seulement l'expression génique de TNF-α a considérablement été diminuée par la Dex, mais ceci a aussi été vrai en condition sans hypoxie. En conclusion, le stress oxydatif, mais pas l'hypoxie, entraîne une insensibilité aux corticostéroïdes, qui constitue une voie de régulation génique sélective (celle de l'IL-8). Les deux groupes de

chevaux ont démontré une réponse similaire indiquant une réaction à la dexaméthasone et non liée à une inflammation asthmatique.

Les mots clés : Asthme, Corticostéroïde, Équin, Hypoxie, Neutrophile, Stress oxydatif

Abstract

Equine asthma is characterized by bronchial hyperactivity, neutrophilic inflammation, and structural alterations of the airways. Asthmatic patients with neutrophilic inflammation, are insensitive to corticosteroids. The hypothesis of this study is that hypoxia and /or oxidative stress in the microenvironment of the asthmatic lungs may contribute to this insensitivity of neutrophils to corticosteroids. Horses are unique animal models naturally affected by neutrophilic asthma and share several pathophysiological features with humans. Viability, apoptosis, IL-1β, TNF-α and IL-8 gene expression were measured in neutrophils isolated from peripheral blood of severe asthmatic horses (N = 8) and healthy horses (N = 8) in culture conditions stimulating hypoxia and oxidative stress, in the presence or absence of corticosteroids (Dexamethasone (Dex)). IL-1β and TNF-α but not IL-8 were downregulated in the presence of Dex under pyocyanin-induced oxidative stress conditions. Also, in IL-17 and LPS stimulated equine neutrophils, pro-inflammatory gene expression did not decrease after Dex administration. In hypoxemic conditions induced by $CoCl_2$, gene expression of TNF-α was significantly reduced by Dex, this was also true in conditions without hypoxia. In conclusion, oxidative stress, but not hypoxia, leads to insensitivity to corticosteroids, which presents a selective gene regulatory pathway (that of IL-8). Both groups of horses demonstrated a similar response indicating a reaction to dexamethasone and not related to asthmatic inflammation.

Keywords: Asthma, Corticosteroid, Equine, Hypoxia, Neutrophil, Oxidative stress

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List of abbreviations

ASM: Airway smooth muscle

ATP: Adenosine triphosphate

BAL: Broncho-alveolar lavage

BALF: Broncho alveolar lavage fluids

C: Degree Celsius

CCL2: chemokine (C-C motif) ligand 2

cDNA: Complementary deoxyribonucleic acid

CS: Corticosteroid

COPD: Chronic Obstructive Pulmonary Disease

DEX: Dexamethasone

dNTP: Deoxy-nucleotide triphosphate

DNA: Deoxyribonucleic acid

EDTA: Ethylene diamine tetra acetate

EIPH: Exercise-induced pulmonary hemorrhage

GC: Glucocorticoid

GR: Glucocorticoid receptor

GRE: Glucocorticoid responsive element

HAT: Histone acetyltransferase

hGR: Human GR

H_2O_2: Hydrogen peroxide

HIF-1α: Hypoxia-inducible factor 1-alpha

HDAC: Histone deacetylase

HSP: Heat shock protein

IAD: Inflammatory airway disease

ICS: Inhaled corticosteroids

IL-1β: Interleukin-1β

IL-5: Interleukin-5

IL-8: Interleukin-8

ILC: Innate lymphoid cell

LTB4: leukotriene B4

Ig: Immunoglobulins

IFN-γ: Interferon gamma

JNK: c-Jun N-terminal kinases

kDA: Kilo Dalton (s)

Kg: Kilogram (s)

LABA: Long-acting β2 adrenergic receptor agonists

LBD: Ligand-binding domain

LPS: lipopolysaccharide

mg: Milligram (s)

mL: Milliliter (s)

MMP-9: Matrix metalloproteinase 9

MLKL: Mixed lineage kinase domain like pseudokinase

MPO: Myeloperoxidase

NK-Cells: Natural killer cells

NF-κB: Nuclear factor-κB

NET: Neutrophil extra-cellular trap

NADPH: Nicotinamide adenine dinucleotide phosphate

NO: Nitric oxide

NO_2: Nitrogen dioxide

NOX: NADPH oxidase

NTD: N-terminal domain

$O_2^{\bullet -}$: Superoxide anion radicals

$OH^{\bullet}$: Hydroxyl radical

PCR: Polymerase chain reaction

PMNs: Polymorpho nuclear cell family

PMA: Phorbol esters

RAO: Recurrent airway obstruction

PAF: Platelet activation factor

RIPK1: Receptor-interacting serine/threonine-protein kinase 1

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

SABA: Short-acting beta-agonists

SEM: Standard error of the mean

TNF-α: Tumor necrosis factor-α

U: unit (s)

µg: Microgram (s)

µl: Microliter (s)

WBC: White blood cell count

To everyone I love

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Introduction

The neutrophil is a fascinating cell to study. Since its "discovery" by Paul Ehrilch (1854-1915), the neutrophil has been associated with various pathologies such as parasitic infections, cancers, inflammatory diseases, and allergic diseases such as asthma. The neutrophil is a characteristic cell of the mucous membranes (1), especially of the digestive tract and the skin, but it is also found in the thymus, the mammary glands and the uterus during pregnancy. Despite an abundant scientific literature regarding neutrophils (nearly 16,000 articles in "PubMed" to date), the precise role of this cell in maintaining homeostasis of the immune system remains to be clarified.

Severe equine asthma is an inflammatory disease that affects almost 15% of the horse population in Canada, and its incidence seems to have increased in recent decades. The presence of neutrophils is a feature of various chronic diseases and inflammatory conditions such as asthma. Many studies have related the presence of neutrophils to the severity of the disease. However, there is no consensus on the role of this cell in the pathophysiology of asthma. The study of neutrophil biology is therefore topical and new knowledge may one day contribute to improving the quality of life of humans and animals suffering from asthma and even prevent its occurrence.

Several molecular mechanisms have now been identified for CS insensitivity in patients with severe neutrophilic asthma. In inflammatory conditions, CS bind to their receptors and contribute to the activity of HDAC2 which attenuates the inflammatory responses by blocking pro-inflammatory gene expression. Our laboratory has previously demonstrated that human

and equine blood neutrophils from healthy individuals are as responsive as other leukocytes to CS in vitro (2). These results suggest that the lung microenvironment is responsible for the insensitivity of pulmonary neutrophils to CS in asthma. Oxidative stress and hypoxia, two conditions present in the lungs of severe asthmatic patients, have an indirect inhibitory effect on HDAC activity, allowing the induction of cytokine production. The aim of this study was therefore to further investigate equine neutrophil behavior by creating conditions of hypoxia and/or oxidative stress in vitro. We also compared the response of blood neutrophils from severely asthmatic horses and healthy controls in the presence or absence of CS.

Literature review

1.1 Asthma

1.1.1 Human asthma

Definitions of asthma in humans have emphasized the characteristic fluctuations of symptoms over time. The 2019 revision of the GINA Guidelines[1] proposes defining asthma as an illness which, "causes symptoms such as wheezing, shortness of breath, chest tightness and cough that vary gradually in their occurrence, frequency and intensity. These symptoms are associated with variable expiratory airflow, i.e., difficulty breathing air out of the lungs due to bronchoconstriction (airway narrowing), airway wall thickening, and increased mucus." These features are common, but variable. The airflow obstruction within the lung is often reversible, either spontaneously or with treatment.

In humans with severe asthma, there is persistent immune activation that is represented by pulmonary leukocyte infiltration (often eosinophils, but also neutrophils). Equine asthma is one of the rare conditions affecting domestic mammals that has characteristics in common with human asthma, making it a model of choice for the study of this disease.

Asthma is a heterogeneous disease, characterized by chronic inflammation. Asthma has two key features. The first is a history of respiratory symptoms, such as wheezing, shortness

[1] Global Strategy for the Diagnosis, Management, and Prevention of chronic Obstructive Pulmonary Disease. Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2019; Available from: URL: www.goldcopd.org

of breath, chest tightness and cough that vary over time and intensity. The second is variable expiratory airflow limitations. Based on GINA, human asthma could be divided in three groups:

Uncontrolled asthma: Patients with frequent symptoms and/or flare-ups (exacerbations). Many of these patients may have mild asthma, i.e., their asthma could be well controlled with low-dose inhaled corticosteroides (ICS) if taken regularly.

Difficult-to-treat asthma: Asthma uncontrolled despite prescribing high dose of preventative treatment. Contributory factors may include incorrect diagnosis, incorrect inhaler technique, poor adherence, and comorbidities.

Severe asthma: "Severe asthma" has had many different meanings (3, 4). It is now defined as asthma that is uncontrolled despite maximal optimized therapy and treatment of contributory factors, or that worsens when treatment dose is decreased (5). It is relatively refractory to corticosteroids (CS). This is a retrospective definition, dependent on how thoroughly contributory factors are excluded. Based on data from a Dutch population survey, 3.7% of people over the age of 18 years have severe asthma. Type 2 inflammation is found in 50% of people with severe asthma. It is characterized by cytokines such as interleukin (IL)-4, IL-5, and IL-13 which are produced by the adaptive immune system in recognition of allergens. Increased eosinophils which may be accompanied by atopy, is another feature in severely asthmatic patients. In moderate asthma, inflammation rapidly improves when inhaled CS are regularly taken – whereas in severe asthma, the inflammation may be relatively resistance to high doses of inhaled CS.

The presence of distinct inflammatory cell types such as eosinophils or neutrophils in the lung tissue or bronchoalveolar lavage (BAL) of asthmatic patients is one of the criteria for

distinguishing two distinct asthmatic phenotypes: eosinophilic and neutrophilic asthma. It should be noted that neutrophilic airways are one of the subsets of severe asthma, which is based on the altered level of neutrophils in the airways and the presence of asthma symptoms (6).

IL-8 (a strong neutrophilic chemoattractant (7)), IL-17 and IFN-γ are the principal cytokines in airways of patients with neutrophilic human asthma (8). Stimulation of IL-8 production is through activation of nuclear factor (NF-κB) which is a protein complex that controls the transcription of DNA, cytokine production and cell survival. In contrast, eosinophilic asthma demonstrated elevated levels of eosinophilic inflammation despite high doses of CS. Severe eosinophilic asthma has a significantly higher number of TGF-β positive cells in the airway submucosa, and higher levels of the mediator in mast cells (β-Tryptase) in the bronchoalveolar lavage fluids (BALF). Although IL-8 is produced by many cells, neutrophils are the main airway cells containing IL-8, suggesting its secretion in asthma. On the other hand, levels of IL-8 have been observed to be also increased in non-eosinophilic asthma. Higher concentrations of IL-8 (9) and neutrophils in blood and BALF are associated with severe asthma (10, 11).

1.1.2 Equine asthma

There are numerous similarities between human asthma and equine heaves (recurrent airway obstruction, RAO) and inflammatory airway disease (IAD) (12). Therefore, the term "equine asthma" was introduced to encompass the different severity of these conditions (13). The clinical and diagnostic features of equine asthma are presented in Table 1-1 (14).

In equine asthma, common clinical features of inflammatory mechanisms result from intricate interactions between genetics and the environment (15). It has been shown that exposure to antigens such as air pollution, ozone, viral infections, and bacterial endotoxins, as well as exercise and stress, will induce an obstruction of airway smooth muscle (ASM) (12).

There are two classifications for equine asthma: mild-moderate and severe asthma. Symptoms intensify moving from the mildest type of disease to the severe form. Moreover, higher numbers of mast cells, basophils, and eosinophils is associated with mild and moderate asthma and not with severe equine asthma. According to the ACVIM[2] consensus statement in 2007, 'Although neutrophilic inflammation is commonly observed in BALF from horses with severe equine asthma, summer pasture severe equine asthma, and mild and moderate asthma, the neutrophilia is usually less pronounced with mild and moderate asthma (i.e., 20%)' (16). Airway neutrophilic inflammation is usually 20 % neutrophils or more in BALF cytology of severe asthmatic horses (17-19).

Increased respiratory rate and abdominal contraction of expiration is noted in severe asthma. Exacerbation of equine asthma is usually described as the presence of severe airflow obstruction, and increased airway smooth muscle mass, particularly in the peripheral (20-22).

[2] American college of veterinary internal medicine. http://www.acvim.org

Table 1.1 Typical features of equine asthma syndrome (16). Copyright permission obtained.

		Equine Asthma Syndrome	
	Characteristics	IAD (Mild – Moderate Equine Asthma)	RAO or SPRAO (Severe Equine Asthma)
Clinical presentation	Age of onset	Usually young to middle age but can be observed at any age	Usually older than 7 years
	Clinical signs	Occasional coughing, poor performance, no increased respiratory efforts at rest Signs are chronic (at least 4 weeks in duration)	Regular to frequent coughing, exercise intolerance, increased respiratory efforts at rest Signs and severity may vary over time, often limiting activity
	Time course	Often improve spontaneously or with treatment. Risk of recurrence low	Typically last for weeks to months before diagnosis. Usually improves with strict environmental control or treatment. The disease cannot be cured but signs can be controlled
	History	Exposure to stable environment. Genetic susceptibility has not been investigated	Exposure to dust or allergen in stable or at pasture. Some may have a familial history of equine asthma. Clinical signs may be seasonal
Diagnostic confirmation	Airway endoscopy (resting or dynamic)	Excess mucus in tracheobronchial tree (score >1 for racehorses and >2 for sports/pleasure horses). Rule out other differentials	Excess mucus in tracheobronchial tree Rule out other differentials
	Airway cytology	Mild increase in BALF neutrophils, eosinophils, and/or metachromatic cells	Moderate to severe increase in neutrophils
	Lung function	No evidence of airflow limitation based on esophageal balloon catheter technique (DPmax <10 cm H_2O) Airflow limitation detected using sensitive methods Airway hyperresponsiveness	Moderate to severe airflow limitation during disease exacerbation based on esophageal balloon catheter technique (DPmax >15 cm H_2O) Reversible with bronchodilator or environmental change Airway hyperresponsiveness

1.1.3 Asthma and the inflammation of the respiratory tract

1.1.3.1 Chronic Obstructive Pulmonary Disease (COPD)

Until recently, definitions of 'chronic obstructive pulmonary disease' (COPD) in humans included the terms "chronic bronchitis" and "emphysema." The GOLD Guidelines, first published in 2002 (23) and revised in 2019[3], the American Thoracic Society/European Respiratory Society (ATS–ERS) Guidelines issued in 2004 (24), and the NICE Guidelines[4] (25) reported in 2004, deliberately omitted these terms and used only the umbrella term COPD.

[3] Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GOLD), 2019; Available from: URL: www.ginasthma.org

[4] National Institute for Clinical Excellence (NICE) Guideline available at URL: www.nice.org.uk/CG012niceguideline

The 2006 revision of the GOLD guidelines[2] defines COPD as: “a preventable and treatable disease with some significant extra-pulmonary effects that may contribute to the severity of individual patients”.

1.1.3.2 Similarities and differences between human asthma and COPD

The concept of asthma and COPD belongs to a spectrum of diseases that cause airflow obstruction, and both are inflammatory diseases with important similarities and differences. The 2019 GOLD guidelines describes both diseases as an inflammatory response that causes airflow limitations, through gene–environment interactions, cell populations, and mediators. According to GOLD guidelines, “the airflow limitation ranges from completely reversible (the asthma end of the spectrum) to completely irreversible (the COPD end of the spectrum). Both diseases are characterized by airflow obstruction except in the early or mild stages”. COPD, in contrast to asthma, is defined by irreversible airflow limitations, and this becomes progressively more severe as the disease advances. In many (but not all) long-standing asthma patients, there is also an appreciable component of chronic irreversible airflow obstruction with reduced lung function. As GINA guidelines recommend, reversibility could be defined as a 20% post-bronchodilator improvement in lung function. Therefore, irreversibility refers to the incomplete response to short-acting bronchodilators, or to oral or inhaled CS.

Asthma and COPD may co-exist or overlap (asthma-COPD overlap), particularly in smokers and the elderly. Asthma-COPD overlap has more severe features than asthma or COPD alone, which is likely caused by several mechanisms. However, given the risks associated with treating with bronchodilators alone in patients with asthma, patients with COPD should

be treated with at least low dose inhaled CS if there is any history of asthma or diagnosis of asthma.

The definition of asthma shows exacerbations as a characteristic of the disease. Thus, it could highlight the fluctuations of the disease. The definition of COPD excludes any mention of exacerbations (23-25). The major differences in pulmonary inflammation between asthma and COPD are described in Table 1.2 from the 2006 GOLD report.

Table 1.2. Principal differences in pulmonary inflammation between asthma and COPD. Copyright permission obtained.

	COPD	Asthma	Severe Asthma
Cells	Neutrophils ++ Macrophages +++ $CD8^+$ T-cells (Tc1)	Eosinophils ++ Macrophages + CD4 + T-cells (Th2)	Neutrophils +, Eosinophils, Macrophages CD4 + T-cells (Th2), CD8+ T-cells (Tc1)
Key mediators	IL-8 TNF-α, IL-1β, IL-6 NO +	Eotaxin IL-4, IL-5, IL-13 NO +++	IL-8 IL-5, IL-13 NO++
Oxidative Stress	+++	+	+++
Site of disease	Peripheral airway Lung parenchyma	Proximal airways	Proximal airways Peripheral airways
Consequences	Mucous cell metaplasia	Fragile epithelium	
	Small airway fibrosis	Mucous metaplasia	
	Parenchymal destruction	Increased thickness of basement membrane	
	Pulmonary vascular remodeling	Bronchoconstriction	
Response to therapy	Poor response to steroids	Good response to steroids	Reduced response to steroids

1.1.3.3 Limitations of the definitions

Definitions for both asthma and COPD have limitations since they can reflect only our current understanding of the diseases, which is incomplete. Both syndromes will continue to be redefined as our understanding of them deepens, and as new effective preventive

strategies and treatments become available. In order to better understand the mechanism of a disease and the treatments, investigation of different cellular interactions could be an asset.

1.2 Leucocytes

The leucocytes (known as white blood cells) are the rounded, colorless cells circulating in the blood and lymphatic system fluids. They can be classified either by structure (granulocytes and agranulocytes) or by cell lineage (myeloid cells and lymphoid cells). All leukocytes are derived from hematopoietic stem cells (multipotent cells in the bone marrow), differentiating into five broad categories: neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Further subtypes can be classified; for example, among lymphocytes, there are B cells, T cells, innate lymphoid cells (ILCs), and natural killer cells (NK-Cells).

Increased numbers of leukocytes in the blood is often an indicator of disease, and thus the white blood cell count is an important subset of the complete blood count. Increased white blood cell count (WBC), termed leukocytosis, can be a result of bacterial or viral infection, stress, trauma, drug administration or immune-mediated disease. Numerous situations, such as overwhelming bacterial or viral infection, bone-marrow disease or endotoxemia, lead to a decrease in the total numbers of circulating white blood cells (leukopenia). A total absence of neutrophils or a significant decrease in their number leads to death or severe immunodeficiency, respectively (reviewed in (26)).

1.3 Granulocytes

The granulocytes (known as polymorphonuclear leukocytes) are groups of white blood cells characterized by the presence of secretory granules in their cytoplasm (27). They are of four types: neutrophils, basophils, eosinophils, and mast cells. They form the polymorphonuclear cell family (PMNs) (28, 29).

1.4 Mast cells

Mast cells (also known as mastocytes) contain granules rich in histamine and heparin. They are characterized by their morphology and histologic staining (30). Mast cells have an essential role in acute allergic responses, development of chronic allergic inflammation, and importantly in immune tolerance, wound healing, angiogenesis, and fighting against infective agents.

Mast cells may be involved in autoimmune and inflammatory disorders. They release pro-inflammatory mediators, chemo-attractive and immunomodulatory cytokines (31). Consequently, they are involved in the recruitment of inflammatory cells to the site of inflammation and they will contribute to the development inflammatory disease (such as asthma) (32) through the triggering airway hyper responsiveness and increase eosinophil infiltration. Tissues enclosing blood vessels and nerves are rich in mast cells (33). This includes, the skin, mucosa of the lungs, digestive tract, mouth, nose, and conjunctiva; in other words, the first lines of immune defense in the body.

1.5 Neutrophils

Blood neutrophils are round cells of 12-15 micrometers in diameter, with a segmented nucleus. A mature neutrophil has 3–5 segments. They account for approximately 50-70 % of all leukocytes in most mammals, around 50% in horses, and about 20-30 % in farm animals. Neutrophils are phagocytes normally found in the bloodstream. During inflammation, bacterial infection, viral infections, environmental exposure (34), and some cancers (35, 36), neutrophils are one of the first lines of defense which will migrate towards the site of inflammation (29).

Upon activation, they exit the bone marrow and enter circulation, a process called transcellular migration. Neutrophils released from the bone marrow have a bloodstream half-life of approximately 6.6 ± 1.4 hours in humans (37), 11.4 hours in mice (38), and 10.5 hours in horses (39). Neutrophils will migrate into tissues, where they survive for 1–2 days (40), or even, based on studies on human neutrophils, up to 4-5 days (reviewed in (26)). Neutrophil life span may be modulated by soluble signals; when exposed to stimuli such as TNF-α, neutrophils undergo apoptosis or programmed cell death.

Increased neutrophil lifespan is observed under inflammatory conditions and has been attributed to both direct interaction with microbes and the release of cytokines from other immune cells (41, 42). Interestingly, studies in mice have shown that the half-life of neutrophils in the bone marrow is significantly longer because of the expression of the survival factor in immature neutrophils, suggesting a mechanism to maintain a neutrophil marrow reserve (43).

Changes in neutrophil morphology is an indicator of neutrophil differentiation and maturation. This includes staining of distinct granules, and changes in nuclear shape and chromatin patterns (Fig. 1.1). Immature (band) neutrophils are an intermediary step to the

complete differentiation of segmented neutrophils. Neutrophil differentiation happens gradually in bone marrow. Mature neutrophils have the ability to leave the bone marrow and move through the circulatory system. An increase in band neutrophils typically means that the bone marrow has been signaled to release more WBCs and increase production of neutrophils, also known as a "left shift." Most often, this is due to infection or inflammation. Neutrophils cannot divide because of their compacted chromatin. However, band neutrophils nuclei may be subdivided into segmented neutrophils, leading to a mature granulocyte. Mature neutrophils have a small Golgi apparatus, mitochondria, and ribosomes or rough endoplasmic reticulum. Neutrophils can be found in the bone marrow, spleen, liver, and lungs. The lung seems to be rich in matured neutrophils.

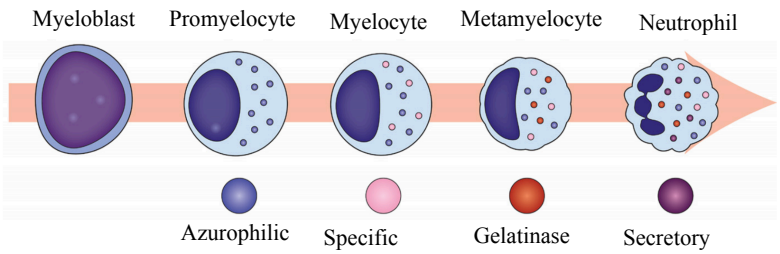

Fig. 1.1. The progression of neutrophil differentiation. Changing in neutrophil shape and morphology during the maturation process could be seen. Mature neutrophils have the complete granules to battle against infection (44). Copyright permission obtained.

1.5.1 Netosis

Neutrophil activation may cause the release of web-like filaments composed of chromatin and serine proteases (45) in order to trap and contain bacteria. This may play an important role in inflammatory diseases. Brinkmann *et al*. (2004) have, for the first time, described neutrophil extra-cellular trap (NET) formation induced by phorbol esters (PMA),

lipopolysaccharides (LPS), platelet activation factors (PAF), and interleukin-8 (IL-8) (46). As NETs contain neutrophil elastase (NE), myeloperoxidase, DNA and histones, they can also potentiate tissue damage, in part through cytotoxic effects on epithelial and endothelial cells (47). These NETs can be stimulated by bacteria, fungi and protozoa. Because of their physical and antimicrobial properties, they may serve as a barrier to prevent further spread of pathogens (Fig. 1.2).

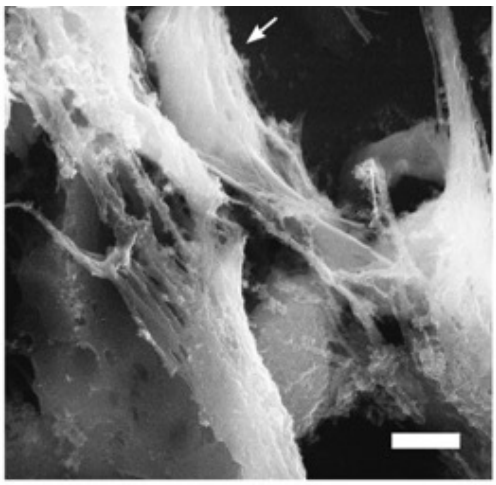

Fig. 1.2. Electron microscopy showing NETs covering fungal cells (*Candida albicans*) in an infected mouse lung (arrow) (45). Copyright permission obtained.

1.5.2 Key role of neutrophils in asthma and respiratory tract inflammation

Neutrophils play an important role in the innate immune system. During early stages of asthma development, neutrophils release many inflammatory mediators (48). During inflammation, neutrophils migrate to the airways of the circulatory system. In the process of migration, upregulation of adhesion molecules, including CD11b and CD18 (Mac-1 integrin), occurs. Their migration to the airways depends on chemo-attractive signals, and the

chemotactic activity of neutrophils is increased as asthma develops. This phenomenon is explained by an increase in IL-8 and TNF-α concentrations in the circulatory systems of patients suffering from asthma, both being chemotactic cytokines released from macrophages, epithelial cells and neutrophils (49-51). Furthermore, IL-8 triggers degranulation of granulocytes and release of myeloperoxidase, elastase, leukotriene B4 (LTB4) and reactive oxygen species (ROS) (50). Release of ROS during the mechanism of oxidative burst is one of the major roles of neutrophils. The granulocytes may release ROS after bacterial stimulation (e.g., fMLP (N-formylmethionyl-leucyl-phenylalanine) or LPS) or under stimulation by immunoglobulins (e.g., IgE) or plant-derived stimulants (e.g., PMA).

In mild asthma, the number of neutrophils in the airways is similar to that of healthy controls (52). However, in severe neutrophilic asthma, the number of neutrophils in bronchi is elevated compared to healthy subjects (53, 54). In severely asthmatic horses, as our group previously reported, levels of circulating low-density neutrophil-like granulocytes increased, showing an increased response to stimulation in vitro in terms of NET formation (55). Neutrophils are regulatory cells in asthma, since they infiltrate airways and release an array of inflammatory mediators triggering asthma development (48).

1.5.3 Neutrophil-mediated immune responses

The immune response is the activation of defense processes of the immune system in recognition of the molecules of foreign bodies then transmitting it to the defense cells to activate effectors to remove these foreign bodies. The immune response is divided into innate and adaptive immune responses. The latter is further classified in humoral response

(production of specific antibodies) and cell-mediated response, both of which are associated with immune memory. Finally, the immune response is a complex process orchestrated through several cell types, the ultimate goal being to eliminate as quickly and as effectively as possible potential threats to ensure maximum protection of the body. The types of cytokines produced influence the types of cell lines that can be activated. For example, neutrophils will be activated and will migrate through the blood vessels, by chemical signals such as IL-8, C5a, fMLP, LTB4 and hydrogen peroxide (H_2O_2) (56). In condition of inflammation, neutrophils capture and destroy foreign particles through phagocytosis. After they are encapsulated in phagosomes, the cells kill the pathogens using NADPH oxygenase-dependent mechanisms (reactive oxygen species) or antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme) (57, 58). These proteins are released from the neutrophil granules either into phagosomes or into the extracellular milieu. Although phagocytosis is a continuous process, it can be divided into four discrete stages: chemotaxis, adherence, ingestion, and digestion (Fig. 1.3).

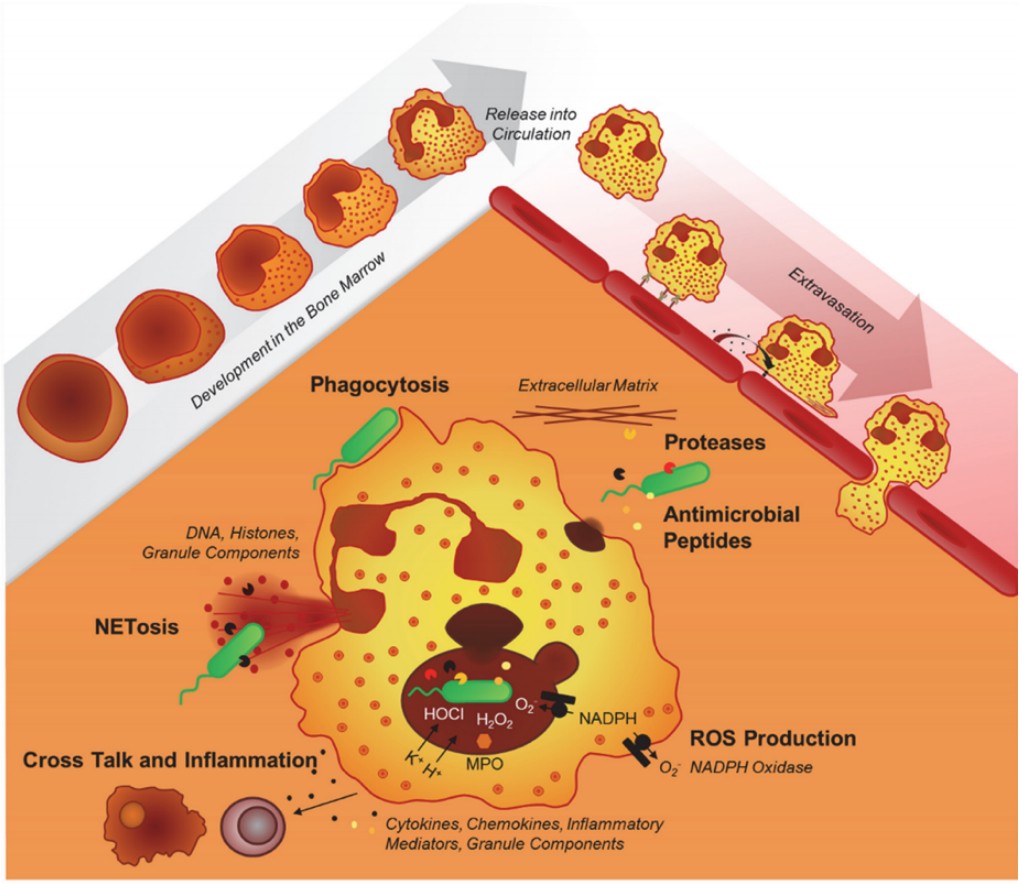

Fig. 1.3. Sequential stages of neutrophil recruitment from blood to tissue and neutrophil phagocytosis (59). Copyright permission obtained.

1.5.4 Production of cytokines and chemokines by neutrophils

Cells of the immune system secrete a variety of glycoproteins in response to the inductive intercellular signals that regulate immune responses. The generic term for these regulatory proteins is 'cytokines'. These "biological response modifiers" have autocrine effects, as they may bind to the receptor of the cell of origin, as well as paracrine and endocrine effects, as they can also spread throughout the body and affect cells in distant locations.

Their binding to receptors leads to a variety of cell signaling events. This can contribute to the development of cellular and humoral immune responses, the regulation of hematopoiesis, the control of cellular proliferation and differentiation, wound healing, and the synthesis of a variety of receptors or other proteins. Cytokines are secreted mainly by lymphocytes and macrophages, but also by granulocytes, endothelial cells, fibroblasts, and by most structural cells. Human endothelial cells are capable of expressing a broad spectrum of pro- and anti-inflammatory cytokines, including IL-1 (interleukin-1), IL-5, IL-6, IL-8, CCL2 (chemokine (C-C motif) ligand 2) and CSF (colony-stimulating factors). Inflammation itself, and hypoxia via hypoxia-inducible factor 1-alpha (HIF-1α), promote the secretion of chemokines that attract neutrophils and monocytes (60).

Considering the predominance of neutrophils under certain inflammatory conditions, their level of cytokine production may be considerable (61, 62). Thus, they can contribute to regulation of the inflammatory response by influencing the behavior of resident or recruited cells at the site of inflammation, in both autocrine and paracrine manners (63). It has been demonstrated that neutrophils can even modulate their cytokine production based on the

types of antigens they encounter, in a manner dependent on neutrophil type, with blood and pulmonary neutrophils showing specific responses. The vast number of cytokines produced by human neutrophils (listed in Table 1.3) supports the idea that the immune system is mediated by a complex pathway of cytokine signaling.

Table 1.3. Cytokines and chemokines that neutrophils can potentially express and/or produce (Influenced by (64)).

Pro-inflammatory Cytokines	IL-1 α, IL-1β, IL-6, IL-7, IL-18, IL-22, G-CSF, MIF.
Anti-inflammatory Cytokines	IL-1ra, TGF-β1, TGF-β2, IL-4
Immuno-regulatory Cytokines	IL-10, IL-12, P40, IL-21, IL-22, IL-23, IFN-γ, IFN-β
TNF family members	APRIL, BAFF, CD 3OL, CD 4OL, FasL, RANKL, Lymphotoxin-β, TNF-α.
C-C chemokines	CCL2, CCL3, CCL4, CCL17, CCL18, CCL19, CCL20, CCL22, CCL23
C-X-C Chemokines	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10, CXCL11.
Angiogenic and fibrogenic factors	Angiopoietin1, Bv8, FGF2, HB-EGF, HGF, TGF-α, VEGF.
Other Cytokines	Activing A, amphiregulin, endothelin, midkine, oncostatin-M, PBEF.

1.6 Asthma therapies

Asthma is the consequence of the interplay of genetics and environmental factors. Therefore, there is a possibility that adequate control of symptoms cannot be achieved for some patients (65), and inter-individual differences in response to asthma treatment and evolution is expected. Nowadays, the standard therapy for asthmatic patients is inhaled CS alone or a combined with long-acting β2 adrenergic receptor agonists. It has been reported that the response of patients to asthma therapy, or the success in controlling the disease, can be affected by a number of criteria, such as obesity (66, 67). In addition, genetic variations not related to drug metabolism or action may modify the clinical manifestations of asthma (68). Since cigarette smoking would be an environmental factor, that may influence asthma

treatments by impairing the response to CS (both inhaled and oral) (69), and perpetuating symptoms despite treatment (70).

The treatment of inflammatory diseases of the airways differs, based on different stages or diseases. For instance, neutrophilic asthma and COPD share some common features in the lower airways, including inflammation (neutrophilia with increased IL-8, Matrix metalloproteinase 9 (MMP-9), and neutrophil elastase) (71). Thus, the common principles of therapeutic approaches are the same. Treatments are used to control exacerbation, and to intercept accelerated functional decline of the lungs. The endpoint for treatment is a resolution of symptoms and a return to baseline.

In human asthma, two primary levels of treatment are recommended. One is termed 'population-level recommendations', which are preferred asthma treatments for the majority of patients in a population. On the other hand, patient-level treatment decisions are based on individual characteristics, risk factors, comorbidities or phenotypes that predict the patient's likely response to treatment in terms of their symptoms and exacerbation risk, together with their personal goals and practical issues such as inhaler technique, adherence, and affordability (GINA 2019).

Drugs used for asthma treatment are classified into two main groups. The first are controller medications, which are used for symptom control, reducing airway inflammation, and preventing complications such as lung function deterioration or symptom exacerbations. The second group are relievers or rescue drugs, which are used for immediate symptom control when exacerbations or aggravation of asthma occur (72).

1.6.1 Treatment with inhaled corticosteroids (ICS)

Inhaled corticosteroids (ICS) constitute the main anti-inflammatory drug therapy used to treat asthma. ICS reduce ongoing inflammation (73-75). It has been demonstrated that ICS have several benefits: they markedly reduce the frequency and severity of asthma symptoms and the risk of flare-ups, as well as improving lung function, airway responsiveness, and quality of life (74, 76). ICS also have many beneficial effects on the airway epithelium (77-79).

1.7 Glucocorticoids

1.7.1 Glucocorticoids: explanations and functions

Glucocorticoids (GC) regulate a variety of physiological functions. They are also widely prescribed as treatments for inflammation, autoimmunity, and lymphoproliferative disorders (80). The mode of action of GC is via trans-activation of anti-inflammatory responses, as well as via trans-repression of pro-inflammatory mediators (81).

1.7.2 Glucocorticoid receptor

1.7.2.1 Genomic point of view

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily and is encoded by the gene nr3c1 on human chromosome 5 (region 5q31p). The human GR (hGR) gene has nine exons; results in two highly homologous protein forms: α and β. GRα functions as a ligand-dependent transcription factor when activated and is expressed in most tissues. GRβ does not bind GC agonists, and is not as ubiquitously expressed as GRα. GRβ acts in a dominant-negative manner, suppressing GRα's activity (82). Several studies have shown that

GRβ regulates gene transcription of non-GRα target genes, in a GRα- and GC-independent manner (83). Table 1.4 demonstrates the up-regulatory and down-regulatory effects of CS on gene expression.

All splicing isoforms show diminished activity compared to GRα (84-86). Besides splicing, GR mRNA is further regulated post-transcriptionally. The GR protein variants all have a similar GC and glucocorticoid responsive element (GRE) binding affinity, but they differ in the length of their N-termini and their transcriptional activity. They show different subcellular localization, regulate distinct sets of genes, and their relative levels vary between and within cells.

1.7.2.2 Protein (non-genomic) point of view

The GR *N*-terminal domain (NTD) contains the major transactivation domain, which is ligand-independent. This domain is involved in the initiation of transcription. GR DNA-binding domain (DBD) is next to the NTD; it has two zinc finger motifs that bind to specific DNA sequences known as GREs. The ligand-binding domain (LBD), which is the site of GC binding, plays a critical role in ligand-induced activation of the receptor and is involved in dimerization, nuclear translocation, binding to heat shock proteins, and interaction with coactivators (82).

Research data shows a potential mechanism through which glucocorticoid binding to the GR may indirectly exert a non-genomic effect on inhibition of MAPKs (by specific inhibitors of JNK and p38 MAPKs) and thereby inhibit the synthesis of ROS (87).

1.7.3 Glucocorticoids activation and activity

In the inactivated state, GR exists predominantly within the cytoplasm, bound to a chaperone complex which includes heat shock proteins (HSPs) 90, 70, and 50, and immunophilins. HSP90 regulates ligand binding and retains GR in the cytoplasm. Upon binding ligands (GC), GR undergoes conformational changes that release it from chaperone proteins, inducing rapid transit into the nucleus (88). Although uninduced GR is predominantly cytoplasmic and activated GR is predominantly nuclear, both activated and inactivated GR forms have been shown to shuttle between the nucleus and cytoplasm (82).

Once in the nucleus, GR binding to GRE increases transcription of target genes via *cis*-activation (89). However, binding to negative GRE sites, through *cis*-repression reduces transcription of certain genes (83, 90). In addition to interaction with DNA directly, activated GR is also known to interact with other transcription factors through protein-protein interactions.

1.7.4 The anti-inflammatory effects of glucocorticoids

Many inflammatory genes are transcriptionally repressed by GC (86). Dexamethasone, like other steroid-derived lipophilic ligands, diffuses through the cell membrane into the cytoplasm, where it binds and activates its receptor (Fig. 1.4) (91). After GR is transported to the nucleus, it will inhibit histone acetylation by reducing histone acetyltransferase (HAT) activity. Additionally, GC administration increases histone deacetylase 2 (HDAC2) expression (82). HDAC2 targets NF-κB complexes, which is associated with halted transcription of NF-κB-dependent genes (Fig. 1.5) (84, 85).

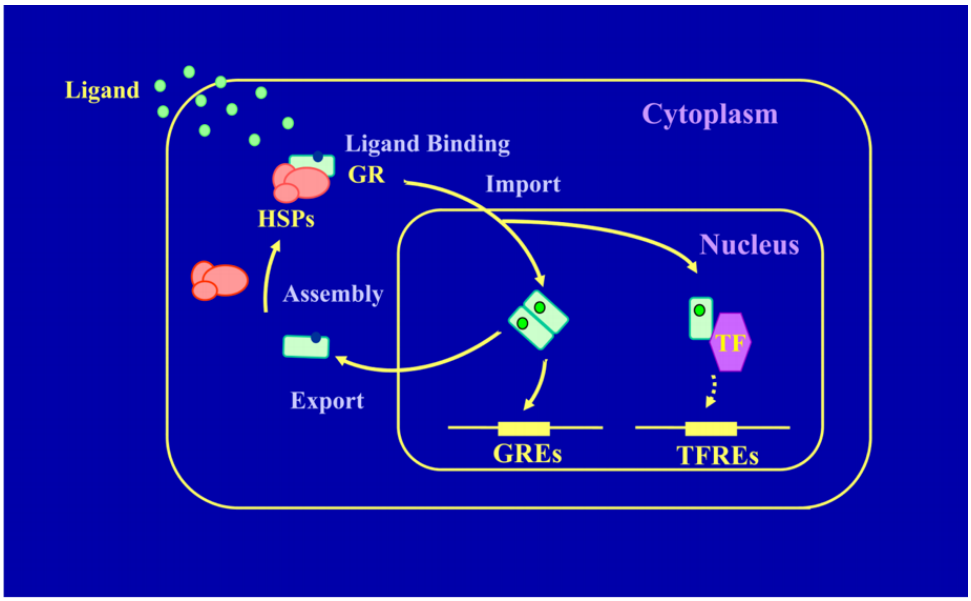

Fig. 1.4. Nucleocytoplasmic shuttling of the glucocorticoid receptor. Upon binding to the ligand, the activated GRα dissociates from HSPs and translocate into the nucleus, where it homodimerizes and binds to GRE in the promoter region of target genes (91). Copyright permission obtained.

Table 1.4. Effects of corticosteroids on gene expression. Copyright permission obtained.

Increased Transcription	Decreased Transcription
• Anti-inflammatory or inhibitory cytokines • IL-10, IL-12, IL-1 receptor antagonist • Mitogen-activated protein kinase phosphatase-1 (MKP-1, Inhibits MAP kinase pathway) • Secretory leukocyte inhibitory protein • 2-adrenergic receptors • Iκβ-α (inhibitory of NF-κB)	• Inflammatory cytokines (IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, IL-15, TNF-α, GM-CSF, SCF) • Chemokines (IL-8, RANTES, MIP-1α, eotaxin) • Inflammatory enzymes (inducible nitric oxide synthase (iNOS), inducible cycloxygenase (COX-2) inducible phospholipase A 2 (cPLA2) • Inflammatory peptides (Endothelin-1) • Mediator receptors (Neurokinin (NK 1), bradykinin (B2)-receptors) • Adhesion molecules (ICAM-1, VCAM-1)

NF-κB remains in an inactive state by being bound to IκB alpha. In response to stimulation, these two molecules dissociate, the IκB alpha is rapidly degraded, and the active NF-κB is free to act. Glucocorticoids block all NF-κB-mediated processes, including cytokine synthesis. They also have effects on leukocyte circulation, blocking the emigration of leukocytes from the capillaries by inhibiting vascular permeability and vasodilation.

Glucocorticoids modulate the activities of inflammatory mediators and modify protein, carbohydrate, and fat metabolism. At the same time, CS impair antigen processing by macrophages. ICS restricts many activated inflammatory genes in airway epithelial cells (Fig. 1.6). In the later stages of inflammation, CS inhibit capillary and fibroblast proliferation and enhance collagen breakdown. Consequentially, they may delay wound and fracture healing, allowing prolonged/increased damage to cells and tissues.

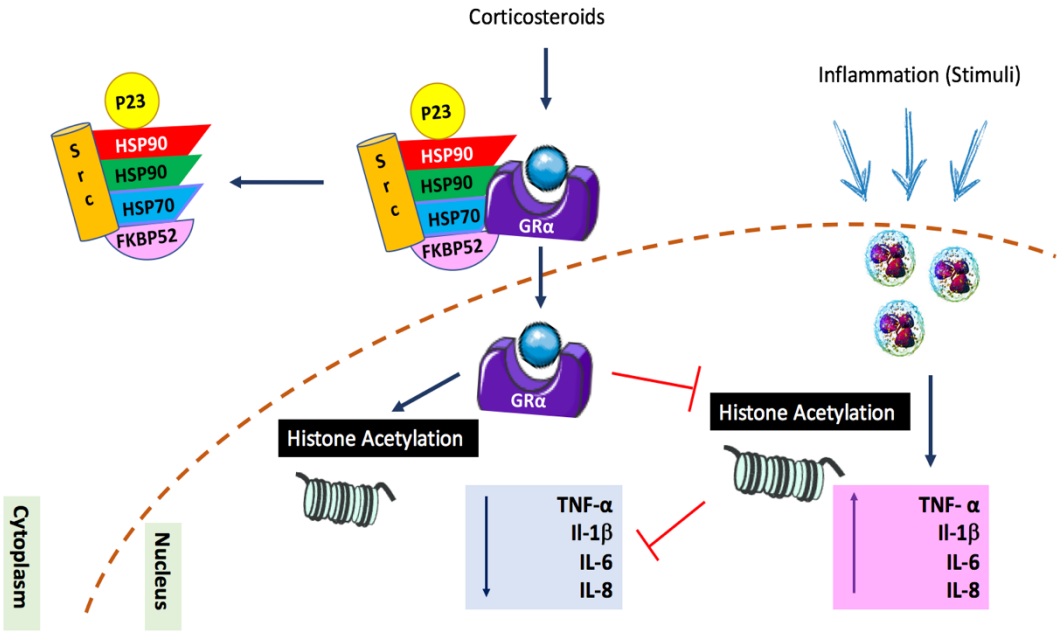

Fig. 1.5. Inflammatory gene suppression after glucocorticoid treatment. Inflammatory stimuli will activate nuclear factor and other transcription factors to switch on histone acetylation, and subsequently the transcription of genes encoding inflammatory proteins. Glucocorticoids will bind to their receptors to mediate anti-inflammatory responses. This binding will cause dissociation of GR from its chaperone protein complex, and induces translocation into the nucleus and will activate HDAC. This will contribute to switches off the activated inflammatory genes and decrease production of pro-inflammatory genes (Influenced by (92)).

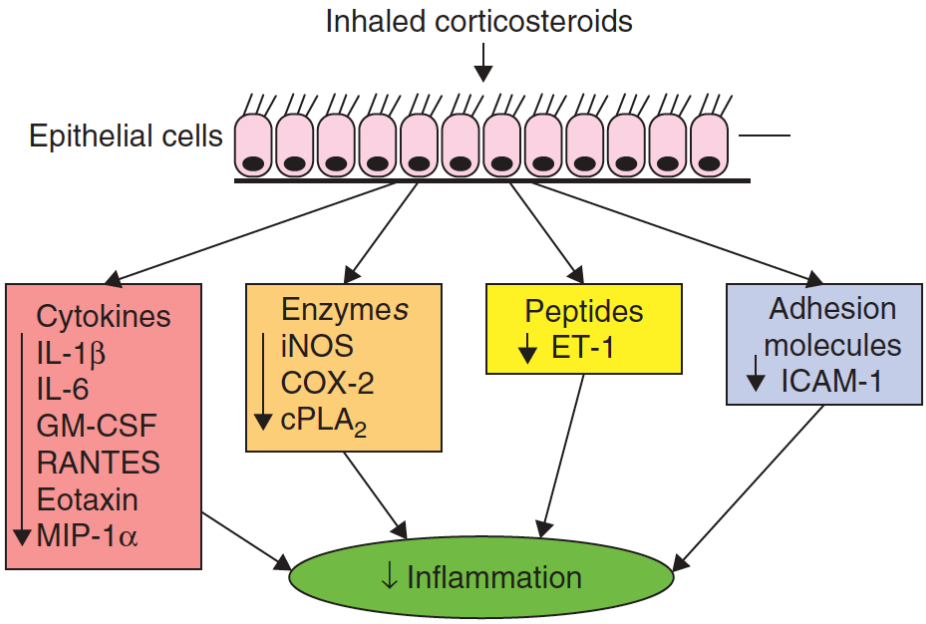

Fig. 1.6. Inhaled corticosteroids inhibit the transcription of several inflammatory genes in airway epithelial cells. NF-κB: nuclear factor κB; AP-1: activator protein-1; GM-CSF: granulocyte-macrophage colony-stimulating factor; IL-1: interleukin-1; iNOS: inducible nitric oxide synthase; NO: nitric oxide; COX-2: inducible cyclooxygenase; cPLA2: cytoplasmic phospholipase A2; PG: prostaglandin; ET: endothelin; ICAM: intercellular adhesion molecule (93). Copyright permission obtained.

1.7.5 Resistance to the administration of CS

The effects of CS on the various blood cell populations are diverse and often conflicting. In horses and cattle, the numbers of circulating eosinophils, basophils, and lymphocytes decline in a dose dependent manner when CS are administered. The number of neutrophils,

on the other hand, increases as a result of a decrease in adherence to vascular endothelium and reduced emigration into inflamed tissues. CS inhibits the chemotaxis of immune cells such as neutrophils, monocytes and eosinophils, but neutrophil migration is enhanced in the presence of CS (94). Dexamethasone is widely prescribed for inflammatory diseases like asthma. However, long-term use is associated with various side effects and insensitivity to CS (95). By definition, severe asthmatic individuals show a poor response to CS, which requires higher doses of medication. Only a few asthma patients shows CS resistance. All patients with COPD show some degree of CS resistance. Asthmatics who smoke are also relatively CS-resistant and require increasing doses of CS for asthma control (96).

Resistance to CS treatment, exacerbations of the disease and occupational forms of asthma could be all associated with increased neutrophil counts in the peripheral blood and BALF (97). Neutrophilic mediators cause remodeling of bronchial walls, which leads to local fibrotic changes (48). A similar mechanism occurs in certain autoimmune conditions (98). Remodeling of large central bronchi could be at least partly responsible for resistance to therapy with bronchodilators. CS improve clinical signs and airway obstruction in severe asthmatic horses, while airway neutrophilia persists (20, 99), and it has a similar effect on human asthma too. Therefore, it should be the focus of further studies to investigate how neutrophils might be linked to asthma pathophysiology, which could provide a common target for future therapeutic interventions.

Several molecular mechanisms have now been identified to account for CS insensitivity in human patients with severe asthma and COPD [18]. In patients with COPD, smoking asthmatics and severe asthmatics, there is a reduction in HDAC2 activity and expression, which

prevents CS from switching off the activated inflammatory genes (Fig. 1.7) (92, 100, 101). In steroid-resistant asthma, other mechanisms may also contribute to CS insensitivity, including reduced translocation of GR due to phosphorylation by p38 MAP kinase (102) and abnormal histone acetylation patterns (103). Increase in GRβ prevents GR binding to DNA, but there is little evidence that this would be sufficient to account for CS insensitivity, as the amount of GRβ is very low (104). These results likely reflect the resistance of pulmonary inflammation to CS in COPD patients as a result of the reduction in HDAC (92). HDAC is an enzyme that prevents the transcription of pro-inflammatory genes by blocking NF-κB pathways as well as other transcription factors, such as TNF-α, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF). In an inflammatory condition, CS bind to their receptors and contribute to the activity of HDAC. Consequently, HDAC will block the inflammatory responses. Oxidative stress and hypoxemic conditions have an indirect inhibitory effect on HDAC activity, allowing the induction of the NF-κB pathway and cytokine production with higher intensity. Hypoxia also influences neutrophil function. Neutrophils alter the mRNA expression profiles of epithelial cells as they migrate across the epithelium, which consequently upregulates genes responding to hypoxia. Working together, they will cause hypoxia-associated inhibition of neutrophil apoptosis, contribute to neutrophil survival (105), increase cytokine and chemokine production (106), and increase neutrophil migration (106).

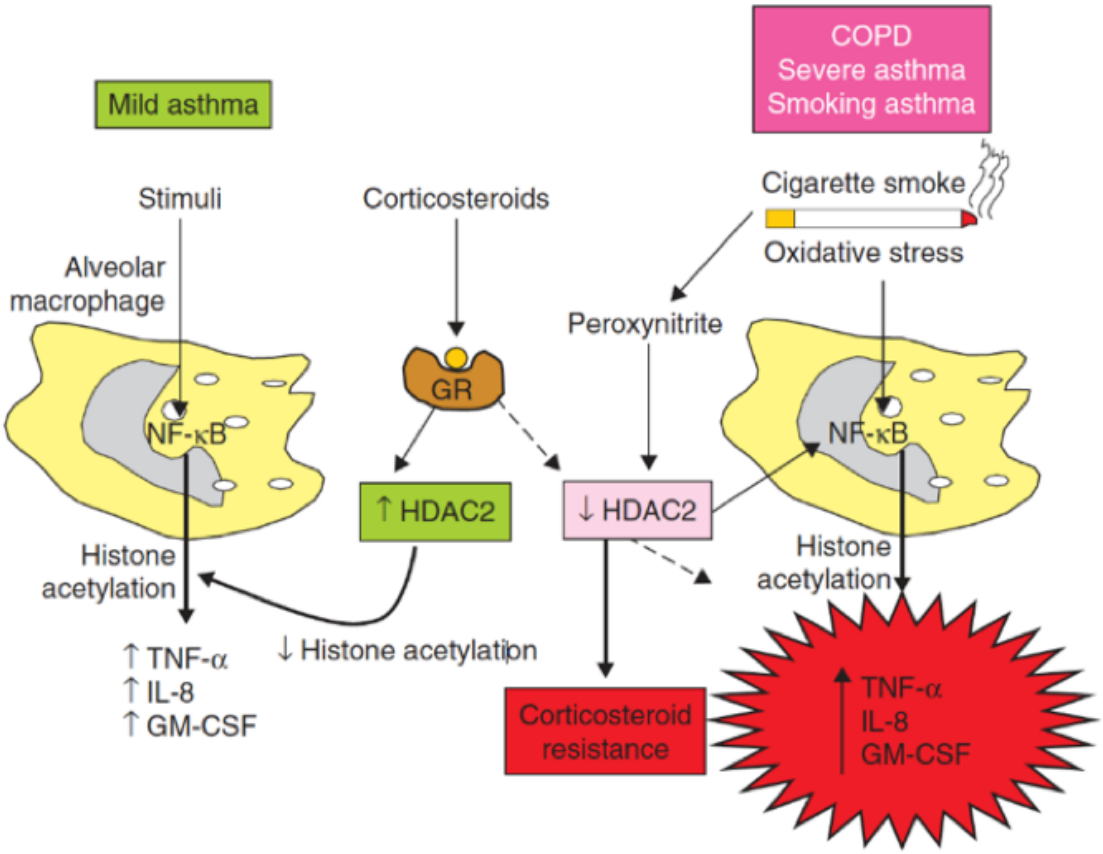

Fig. 1.7. Histone acetylation activity will be impaired and will prevent the GC function and will lead to increase production of pro-inflammatory genes. In an inflammatory condition, CS bind to their receptors and contribute to the activity of HDAC which is an enzyme that prevents the transcription of pro-inflammatory genes. The NF-κB pathway is therefore blocked and leads to the pro-inflammatory gene suppression. Oxidative stress and hypoxia are the conditions that have an indirect inhibitory effect on HDAC allowing the induction of the NF-κB pathway and cytokine production (93). Copyright permission obtained.

1.8 Oxidative stress

1.8.1 Definition

Oxidative stress has been defined as a disturbance in the balance between the production of ROS (or free radicals) and antioxidant defenses. Oxidative stress was initially defined by Sies (1985, 1986) as “a disturbance in the oxidant–antioxidant balance in favor of

the former, leading to potential damage." Balanced formation of ROS and induction of the antioxidant network is essential for biological processes and cell regulations (107). However, oxidative stress is explained as uncontrolled formation of ROS. Antioxidant defenses are leading to tissue injury, and to damage to a variety of cellular structures such as DNA, proteins and lipids (107). High levels of ROS are thought to be linked to a number of chronic diseases such as liver disease, hepatocellular carcinoma, hepatitis B and C or aflatoxin-B1 (108), severe asthma, and COPD.

1.8.2 ROS production

Neutrophils, eosinophils, alveolar macrophages, bronchial epithelial cells, and endothelial cells are the first line of defense in case of inflammation. They are the main cellular sources of ROS in the lung (109-111).

Production of ROS was first reported in 1968 by Paul and Sbarra, who showed that hydrogen peroxide was produced by stimulated phagocytes (112). In 1973, Babior and co-workers established that superoxide was a major product of the oxidase enzyme (113). It is now accepted that NADPH oxidase (NOX) is a key enzyme driving ROS production in activated PMNs. Exposure of a phagocyte to pro-inflammatory cytokines, such as TNF-α, IFN-γ, and/or IL-1β, induces the NADPH oxidase complex and thus increases ROS levels within the cell. NADPH is a membrane-bound enzyme which converts oxygen to its one electron-reduced product, superoxide, by the following reaction:

$$NADPH + H^{(+)} + 2\ O_2 \rightarrow NADP^{(+)} + 2\ H^{(+)} + 2\ O^{(-)}$$

Hydrogen peroxide arises from subsequent dismutation of the superoxide. The oxygen molecules needed for this chemical reaction derive from extracellular sources of oxygen species, produced by the action of NADPH oxidase or the mitochondrial respiratory chain. Dismutation occurs as follows:

$2O_2^{(-)} + 2\ H^{(+)} \rightarrow H_2O_2 + O_2$

ROS can also be derived from either mitochondrial (RIPK1/3, MLKL-dependent) or non-mitochondrial (NOX1 complex or RIPK1-dependent) sources (114). ROS production during oxidative bursts is non-mitochondrial and results from the regulated activation of NOX proteins. In contrast, non-phagocytic cells and phagocytes such as granulocytes, such as neutrophils, monocytes and macrophages generate ROS by the use of an analogous NADPH oxidase complex, which is considered a mitochondrial source of ROS (115, 116). However, exogenous sources of ROS, such as environmental agents, can excessively elevate ROS levels and induce chronic oxidative stress in hepatocytes (117).

1.8.3 Different forms of reactive oxygen species (ROS)

ROS such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2) affect cells differently, react with diverse antioxidant enzymes, and have different preferred cellular targets. They can be derived from many sources including mitochondria, uncoupled nitric oxide synthases and NADPH oxidase (118).

ROS have a close relationship with the oxygen content of cells and with numerous biological processes. It is clear that ROS mediate inflammatory damage and control apoptosis and cell proliferation (119, 120). ROS generation is itself induced by cytokines, and it can in turn stimulate pro-inflammatory cytokine production by activating NF-κB. Previous studies have indicated that TNF-α, ROS, and NF-κB are inextricably tied together in immunity, inflammation, and cancer. ROS production is believed to be responsible for chronic inflammation and its associated tissue damage in inflammatory diseases.

Among the different species of reactive oxygen, H_2O_2 has been shown to penetrate the phagocyte membrane and promote pathogen phagocytosis (115, 121). It might also act as a second messenger: H_2O_2 can affect pH and ion flux, trigger proteolytic enzyme release, and function as a myeloperoxidase (MPO) substrate. H_2O_2 also triggers TNF expression via activation of the P38 and JNK pathways (122), and can activate MAPKs by catalyzing the MAPK-inactivating phosphatase cysteine (123).

Other relevant forms of reactive species exist, such as reactive nitrogen species (RNS) nitric oxide (NO) and nitrogen dioxide (NO_2). Like ROS, RNS-signaling influences innate immune cells under inflammatory conditions. RNS are signaling molecules with antiviral and antibacterial properties, and can inhibit DNA synthesis, cell proliferation and collagen production (124).

NO is generated within the human lung and is measurable in exhaled breath (125). Interestingly, whereas NO levels are decreased in nasal polyposis (124), asthmatics exhibit increased levels of NO (126). A higher level of exhaled level of NO is seen during disease

exacerbation in severe asthmatic patients, especially ones who also exhibit a higher eosinophil percentage in BALF (127).

1.8.4 Mitochondria, a source of reactive oxygen species

High levels of mitochondrial ROS production can be a consequence of a chronic cellular stress, which can alter pro-inflammatory responses, leading to a translation of certain proteins, and consequently lipid and DNA damage. The neutrophils in patient with chronic granulomatous disease (CGD) phagocytose normally, but the respiratory burst is absent and NADPH oxidase activity (and radical production) is undetectable. This indicates that the oxidase has an important mitochondrial function. Cytokines mediate the ROS production pathway by participating in signal transduction pathways (involving kinases and/or phosphate activities) that control HIF-1α translocation and activation. HIF-1α is the transcriptional regulator of cellular responses to hypoxia. Accordingly, ROS generated in the mitochondrial respiratory chain cause HIF-1α stabilization (128, 129). High levels of ROS and mitochondrial dysfunction could result in changed or compromised cellular function.

1.8.5 Oxidative stress and consequence in neutrophils

ROS have essential roles in the innate immune response against microbial pathogens. Neutrophils and macrophages mediate effective innate immune responses through oxidative burst. This immune pathway can control the rapid production of higher levels of intracellular ROS and the activation of proteases that degrade phagocytosed microbes (130).

Respiratory burst is the rapid release of ROS from different types of cells. This happens during phagocytosis to degrade internalized particles and bacteria. It was thought to result

from stimulation of mitochondrial respiration in order to provide energy for phagocytosis. However, neutrophils and mononuclear cells produce most of their ATP through glycolysis, rather than oxidative metabolism. An extreme environment arises from the combination of low oxygen levels, low glucose levels, high concentrations of inflammatory mediators, and high levels of ROS and nitrogen species which characterize the microenvironment of inflammatory sites, such as asthma. This could enhance the subcellular fractionation of NADPH oxidase activity in neutrophils. NADPH activation is associated with the plasma membrane fraction, which could induce respiratory burst in the whole cell. The activation of NADPH oxidase takes place in granule-depleted neutrophil cytoplasts.

1.8.6 Oxidative stress and DNA damage

Nucleic acids also exist in different compartments, and the oxidative environment is different between transcribing and non-transcribing nuclear DNA, as well as between mitochrondrial DNA and different types of RNA. Increased ROS levels contribute to oxidative DNA damage and may lead to mutations. DNA damage can be detected and repaired by several mechanisms or, when not repaired, can induce apoptosis to avoid the passing on of DNA mutations to progeny cells. This apoptotic pathway is activated by NF-κB. NF-κB is also involved in the activation of other antioxidant genes (131). ROS may also interfere with hydroxylase activity. It has been demonstrated that the macrophage-secreted cytokines interl IL-1 and TNF-α promote the expression and DNA binding of HIF-1, via MAPK and PI3 pathways. In addition to ROS, reactive nitrogen species such as NO may be converted into more reactive derivatives, such as NO_2, N_2O_3, and N_2O_4 and $ONOO^-$ (peroxynitrite). Nitration of tyrosine in

cells exposed to reactive nitrogen species derived from cigarette smoke can also lead to DNA damage (132, 133). One of the primary and most crucial steps in the defense against ROS includes the expression of nuclear factor (erythroid-derived 2)-like 2 (NRF2) which promotes expression of antioxidant scavengers, e.g. superoxide dismutase and glutathione (GSH), which act by binding to antioxidant response elements (ARE) (134).

Recent studies in horses have shown nuclear and mitochondrial DNA damage in response to elevated levels of ROS (135). Furthermore, low ROS levels act as second messengers to promote the expression of defense mechanisms against oxidative stress-induced injury to cells. Another related study showed that endogenous oxidative DNA damage is increased in the peripheral blood mononuclear cells of old horses, relative to to healthy adult horses (136).

Lipids, proteins, and carbohydrates are further examples of molecules that can be modified by excessive ROS in vivo. In asthmatic horses, 8-epi-PGF2 alpha, a marker of oxidative stress, is increased in the lungs (137). ROS can be measured directly (e.g., by electron spin resonance or various trapping methods), or indirectly (by examining end products of their reaction with biomolecules, i.e. oxidative damage).

1.8.7 Study the pathway of oxidative stress in equine asthma

Horses suffer from diseases caused by pulmonary and systemic oxidative stress, such as equine asthma, exercise-induced pulmonary hemorrhage (EIPH), and respiratory infection. Equine asthma results in hyper responsiveness, airflow obstruction, mucus hyper-production, and airway wall remodeling (138). Oxidative stress can be detected in both the lungs and

peripheral blood of horses that suffer from severe asthma. Besides, it has been shown that exercise induces pulmonary oxidative stress in both equine and human athletes and can increase pulmonary oxidants/antioxidant disequilibrium (139).

Both eosinophils and neutrophils participate in the innate immune response, and produce ROS and RNS in response to different stimuli (140). The severity of oxidative stress appears to be mild during the early stages of an inflammatory response in severe equine asthma, despite a high number of neutrophils in the lung (141). The effects of neutrophilic airway inflammation on the pulmonary antioxidant status and on the induction of oxidative stress are dependent upon the duration of inflammation and the magnitude of the inflammatory response, suggesting that inflammatory bursts contribute to higher ROS production (139, 142, 143).

Increased levels of biomarkers for oxidative stress, such as higher endogenous oxidative DNA damage, have been determined in peripheral blood mononuclear cells derived from severe asthmatic horses in clinical remission. Moreover, increases in IL-1β, IL-6, TNF-α, IL-8, oxidized glutathione (GSSG), 8-isoprostane and MPO, as well as decreased levels of ascorbic acid in pulmonary epithelial lining fluid (PELF), and increased levels of GSH and GSSG in erythrocytes, are other indicators of oxidative stress in asthmatic horses (139, 142, 143). The elevated levels of cytokines can activate the innate immune system, potentiating ROS production. This autoregulation may result in oxidative damage to cellular macromolecules, and consequently in dysfunctional enzymatic activity and the induction of early apoptosis (144, 145).

1.9 The pathway of cellular hypoxia

1.9.1 General definition

All cellular functions require a constant supply of oxygen; therefore, changes in oxygen tension can have a profound impact on a cell. It has been found that specialized proteins in the cells are able to catalyze the combustion of oxygen at body temperature. These enzymes bind to oxygen so these chemical reactions can occur. The liberated energy from this reaction can be stored as ATP for later use in the cell. Because of the importance of this process, eukaryotic organisms have developed a near-instantaneous mechanism for the critical task of regulating oxygen homeostasis. In 1995, a hypoxia-responsive transcription factor was described and termed hypoxia-inducible factor-1 (HIF-1) was introduced. The inception of HIF-1 signaling depends on the absence of a molecule rather than the presence of one. Since that discovery, a substantial number of studies have been performed to elucidate HIF-related signaling pathways.

1.9.2 HIF-1: central regulator of hypoxia

HIF-1 is a master regulator of oxygen homeostasis. HIF-1 is comprised of two subunits: one α and one β subunit (146). While the β subunit is constitutively present within cells, the α subunit is highly susceptible to oxygen and becomes immediately hydroxylated at specific prolyl and asparaginyl residues in the presence of oxygen, which keeps the subunit in an inactive state (147-149). Under low oxygen concentrations (less than 6%), hydroxylation reactions are impaired, which allows the HIF-1α subunit to be stabilized, permitting translocation of the protein to the nucleus where it dimerizes with HIF-1β.

HIF-1α is of crucial importance for cellular adaptation to hypoxia (150, 151). About 100 target genes of the oxygen-sensing pathway are known. HIF-pathway activation alters transcription of a wide array of genes, often antagonizing in function - including those involved in apoptosis, cell survival, glucose metabolism, and angiogenesis (152).

1.9.3 Physiology and pathology of hypoxia

Hypoxia describes the diminished availability of oxygen in body tissues. Lack of oxygen is a hallmark of a multitude of acute and chronic diseases (153). Hypoxia inducible factors can transcriptionally activate a diverse group of genes, including those related to cell metabolism and differentiation. This occurs under man physiological conditions, such as embryonic development, cell differentiation, and wound healing. Hypoxia may also occurs as the consequences of several pathologies including neoplasia, interstitial lung diseases, COPD, arteriosclerosis and asthma (154-156). HIF-1α, when activated, regulates mitochondrial metabolism by decreasing reactive oxygen species using various mechanisms (157). Furthermore, HIF-1α has been shown to regulate metabolic shifts by increasing utilization of glycolysis in hypertensive pulmonary endothelial cells (158).

1.9.4 Dexamethasone functioning in hypoxic conditions

Hypoxia can significantly influence inflammatory diseases. Hypoxia influences various aspects of neutrophil biology, and these cells display distinct properties when exposed to hypoxic conditions. Respiratory burst represents an essential antimicrobial pathway of neutrophils. During inflammation, neutrophils activate the assembly of an electron transport

chain involving NADPH oxidase, which shuttles electrons across the membrane to molecular oxygen, generating hypochlorous acid (HClO) and ROS which are then used to lyse the microbes (159). This process increases consumption of molecular oxygen (160) and could contribute to a hypoxic condition. Under hypoxia, neutrophil activities will change. They could alter tissue microenvironment by depleting the local oxygen molecules. Neutrophils could alter the mRNA expression of certain pro-inflammatory cytokines like TNF-α and IL-1β in airway epithelial cells (161). Consequentially, these changes stabilize HIF-1 gene upregulation in a positive feedback manner (162-165).

In this study, we aimed to investigate whether hypoxia and oxidative stress, present in the lung microenvironment of asthmatic horses, contribute to the neutrophil insensitivity to CS observed in these animals. We designed an *in vitro* experiment to study the effects of inflammatory conditions by mimicking these conditions in cell culture.

Hypothesis and objectives

Hypothesis

Microenvironmental driven bio-activators in airway inflammation may contribute to a corticosteroid insensitivity of blood neutrophils in asthmatic horses

Objectives

Objective 1 : To identify culture conditions capable of inducing resistance of blood neutrophils to corticosteroids.

Objective 2: To identify the signaling pathways of corticosteroid-mediated responses and resistance (comparing neutrophil glucoresistance in healthy and asthmatic horses).

Chapter 2: Article

Studies of Molecular Pathways Associated with Blood Neutrophil Corticosteroid Insensitivity in Equine Asthma

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Keywords: Asthma, Corticosteroid, Equine, Hypoxia, Neutrophil, Oxidative stress

- All authors contributed to the design of the study. SPD performed the expriments. SPD and J-P L contributed to the analysis and the interpretion of the data. All the authors prepared the manuscript and gave their final approval.

1. Abstract

Equine asthma is characterized by airway hyperactivity, neutrophilic inflammation and structural alterations of the airways. In patients with neutrophilic inflammation, there may be an insensitivity to corticosteroid action characterized by a poor clinical response and a persistence of neutrophils within the airways. We hypothesized that hypoxia and/or oxidative stress in the microenvironment of the lungs contributes to the insensitivity of neutrophils to corticosteroids in asthmatic horses. Horses are the best animal models naturally affected by neutrophilic asthma and share several pathophysiological features with human. Blood neutrophils isolated from severe asthmatic horses (N=8) and healthy horses (N-8) were incubated under different cell culture in the presence of dexamethasone. Neutrophil function studied by comparing pro-inflammatory gene and protein expression of neutrophils under culture conditions simulating hypoxemia and oxidative stress. Oxidative stress increased the mRNA expression of IL-8, IL-1β, and TNF-α in equine neutrophils from both groups. IL-1β, and TNF-α but not IL-8 was downregulated with dexamethasone in pyocyanin-induced oxidative stress condition. Although, IL-17 and LPS stimulated equine neutrophils, increased pro-inflammatory gene expression was unaffected after dexamethasone administration. In hypoxemia mimicked by cobalt chloride ($CoCl_2$), dexamethasone significantly down regulated gene expression. Greater IL-8 production in neutrophils from severe asthmatic horses compared with healthy subjects were seen, though neutrophils from both groups shown the similar response. Oxidative stress but not hypoxemia results in corticosteroid insensitivity

which has a selective gene regulation pathways. Equine neutrophils response similar in both groups, indicating that neutrophils have a response to dexamethasone and not related to asthmatic inflammation.

Keywords: Asthma, Corticosteroid, Equine, Hypoxia, Neutrophil, Oxidative stress

2. Introduction

Equine asthma is characterized by airway hypersensitivity, neutrophilic inflammation and structural alterations of the respiratory tract. The inflammatory process leads to tissue injury and remodeling of the airways (61). Corticosteroids (CS) are widely used to treat various inflammatory and immune diseases, including equine and human asthma. Although they are the most effective drugs for the treatment of asthma, some patients, especially those with neutrophilic inflammation, are insensitive to their action. Furthermore, in both human and equine asthma, the neutrophilic inflammation persists in the airways of individuals treated with these drugs. Our laboratory has previously demonstrated that human and equine blood neutrophils from healthy individuals are as responsive as other leukocytes to CS *in vitro*. These results suggest that the lung microenvironment is responsible for the insensitivity of pulmonary neutrophils to CS in asthma.

CS treatment is effective in reducing the expression of cytokines, chemokines and adhesion molecules involved in the recruitment and activation of inflammatory cells (166), yet it fails to do the same in severely asthmatic or chronic obstructive pulmonary disease (COPD) patients. Airway neutrophilia (167, 168), a characteristic feature of COPD, is also present in a subset of human patients with severe neutrophilic asthma, and both conditions are relatively insensitive to CS. As neutrophils migrate through lung tissues, they release proteinases and reactive oxygen species (ROS) (169). Unbalance between ROS production and antioxidant defenses leads to oxidative stress, and consequently to tissue injury and damage to cell structures (107). Moreover, severe neutrophilic asthma results in the destruction of lung

tissue, which will restrict oxygen flow throughout the body. Therefore, oxidative stress and hypoxia are two conditions present in lungs with severe asthma (154, 170).

Several molecular mechanisms have now been identified to account for CS insensitivity in human patients with severe neutrophilic asthma. In these patients, there is a reduction in histone deacetylase 2 (HDAC2) activity and expression, which prevents CS from switching off the activated inflammatory genes (101). These results are likely to reflect the resistance of pulmonary inflammation to CS in severe asthmatic patients as a result of the reduction in HDAC2 (171, 172). HDAC2 is an enzyme that prevents the transcription of pro-inflammatory genes by blocking nuclear factor-κB (NF-κB) pathways and inhibiting the transcription of other factors, such as tumor necrosis factor-α (TNF-α), IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF). In an inflammatory condition, CS bind to their receptors and contribute to the activity of HDAC2. Consequently, it will block the inflammatory responses. Conditions of oxidative stress and hypoxia have an indirect inhibitory effect on HDAC activity, allowing the induction of the NF-κB pathway and cytokine production at even higher levels. Severe asthmatic patients suffer from both oxidative stress and hypoxia, because of limited oxygen supply to the lungs. Hypoxia also has an influence on neutrophil function. Neutrophils change the mRNA expression profile of epithelial cells as they migrate across the epithelium, resulting in the upregulation of genes responding to hypoxia.

Upon activation, blood neutrophils synthesize proteins, including a variety of chemokines, that are implicated in the recruitment of distinct leukocytes. They can release mediators such as elastase, as well as free oxygen radicals, which lead to tissue damage. Human neutrophils are known to express more than 20 cytokines and chemokines which could

be an indicator of the severity of disease (173, 174). Similarly, equine neutrophils have the capacity to regulate their cytokine and chemokine production in response to stimuli and inflammation (62). Cytokine production (such as. IL-8, IL-1β , and TNF-α) will increase the degree of inflammation, as is the case in severely asthmatic humans and horses (62, 175, 176). Studies have described pro-inflammatory gene regulation in neutrophil activation and recruitment into the airway in human asthma (177, 178), and insensitivity of human neutrophils to CS (179-182).

Neutrophils could be predisposed to cytokine production associated with Th2 type receptors, in order to respond to inflammation (183-185). This activation is thought to contribute to the relationship between cytokine production and severity in equine asthma (176, 186). However, the molecular pathway of CS insensitivity in severely asthmatic horses and of the consequent cytokine production in neutrophils remains unclear. Previous *in vitro* works on severe equine asthma with CS insensitivity showed that blood neutrophils respond normally to CS (2). Moreover, neutrophil extracellular traps (NET) formation was controlled by CS in an equine *in vivo* and *in vitro* study while the pulmonary neutrophilia did not improve with the treatment (187). The aim of this study was therefore to further investigate equine blood neutrophil behavior by creating conditions of hypoxia and/or oxidative stress *in vitro*. We also compared the response of neutrophils from severely asthmatic horses and healthy controls in the presence or absence of CS.

3. Methods

2.1 Ethics statement

Eight mixed breeds healthy mares and eight mixed breeds asthmatic horses, including four mares and four castrated males were studied (mean age 11.6 years (range 9–15); mean weight 527 kg (ranges 435–604 kg). The asthmatic horses were diagnosed with severe asthma based on previous results of pulmonary function. These horses had a transpulmonary pressure (PL) change above 15 cm of H2O when stabled. Horses were part of the research and teaching herds at the Faculty of Veterinary Medicine of the Université de Montréal. All animal experimental procedures were performed following the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal (Rech-1716).

2.2 Neutrophil isolation

Venous blood was collected from a jugular vein into sterile glass tubes containing 7.5% EDTA liquid (Tyco healthcare, Pointe-Claire, QC, CA). The plasma rich layer was recovered after 30 minutes of sedimentation at room temperature using a density gradient method of separation Ficoll-PaqueTM Premium 1084 (Fisher Scientific, Ottawa, ON, CA). The leukocyte polymorphonuclear-depleted and polymorphonuclear-rich cell layers were both harvested, and the remaining erythrocytes were lysed by hypotonic treatment using ultra-pure water (Life technologies, Burlington, ON, CA). Cells were washed and suspended in a buffer solution containing PBS 1X, EDTA 0.5 mM (Life technologies, Burlington, ON, CA), and BSA 0.2% (Sigma-Aldrich, St Louis, MO, USA). Purity was evaluated in cytospin stained slide for differential

counting. Neutrophils with a mean purity of 99.5% and viability of 99% were used for the studies.

2.3 Cell culture

Freshly isolated peripheral blood neutrophils were suspended at 5×10^6/ml and cultured in 5 ml tubes (Corning Incorporated, Corning, NY, USA) in RPMI medium supplemented with L-glutamine 200 mM, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% of low-endotoxin, heat-inactivated fetal bovine serum (Life Technologies, Burlington, ON, CA). For gene expression, cells were cultured for 3.5 hours in the presence or absence of dexamethasone (Dex) at 10^{-6} M (Sigma-Aldrich), 100 ng/mL LPS and/or equine recombinant protein IL-17 (eIL-17) (Cederlane, Burlington, ON, CA) at 10 ng/mL, and/or $CoCl_2$ (Sigma Aldrich, 15,862, Poole, UK) at 300 μM, and/or pyocyanin (Sigma, P0046, Copenhagen, Denmark) at 0.06 mM and 0.02 mM. Cells were incubated at 37 ºC in 5% CO_2. Neutrophils were then recovered and lysed in Trizol (Thermo fisher Science, Ottawa, ON, Canada) for mRNA and protein analysis.

2.4 RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

Total RNA extraction from the neutrophils in Trizol reagent was performed following the manufacturer's instructions (Invitrogene, Carlsbad, USA). The concentration and purity of RNA were evaluated with Nanodrop ND1000 by measuring the absorbance at 260/280 nm (Fisher Scientific, Waltham, MA, USA). The cDNA was generated by reverse transcription of

3.0 µg of total RNA with 100 nM of dNTPs, 50 pM of primer Oligo (dT) 15, 200 U of superscript III reverse transcriptase, 5x × RT buffers and 2.5 ml of 0.1 M DTT for 1h at 55°C. mRNA expression of the pro-inflammatory cytokines (IL-8, TNF-α, IL-1β) and HIF-1α as our index of hypoxia were measured with qPCR. The reactions were performed in a total volume of 20 µl containing 10 µl Quanti Tect SYBR Green PCR Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions with the Rotor-Gene RG3000 (Corbett Research, Sydney, AS) for each gene. Briefly, 1 µl of cDNA was used in 20 µl of final volume qPCR reaction, containing 0.5 µM each sense and antisense primers. Primers were designed to span exon—intron boundaries to prevent amplification of genomic DNA (Table 1). Samples were run in duplicate with an appropriate negative control. The mRNA levels were normalized to GAPDH and the relative changes of target gene expression in different groups were measured.

Table 2.1. Primers used in the study.

Gene	**Forward 5'—3'**	**Reverse 3'—5'**
IL-8	CAAGCTGGCTGTTGCTCTCTTG	CTCAGTCCTCTTTAGAAACGCC
IL-1β	AGTGGTGTTCTGCATGAGCTTTG	GTATTGGGGTCTACTGTCTCCA
TNF-α	GACTGGAAGGCATTCGGTACT	ATCCGAGATGTGGAGCTGC
HIF-1α	CAGCAATGACACAGACACTG	CTGGGACTGTTAGGCTCTG
GAPDH	CACCCAGAAGACCGTGGATG	TGCCAGTGAGCTTCCCATTC

2.5 Protein isolation and Western blot analysis

Following treatments, cultured neutrophils were rinsed with ice-cold PBS, and the total proteins were extracted using M-per protein extraction kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Protein suspensions were stored at -80ºC until protein assays. Equal amounts of protein (30 µg per lane) were separated based on their size by SDS—PAGE 12% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories, Mississauga, ON, CA) then they were blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, USA). We detected HIF-1 α protein using a Rabbit Polyclonal antibody (20960-1-AP, HIF1 α Rabbit Poly Ab, Cederlane, Burlington, ON, CA) with the starting dilution ratio of 1:300 in blocking solutions that binds Goat Anti-Rabbit IgG (Jackson Immuno Research, West Grove, Pennsylvania) with the dilution of 1:2000. Signal was enhanced by Super Signal West Dura (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer's instructions. Signals were detected by chemiluminescence with Fusion FX Vilber Lourmat (Montreal Biotech Inc., Dorval, QC, Canada).

2.7 Quantification of hydrogen peroxide production

Generation of extracellular superoxide was assessed to measure ROS production in cultured neutrophils. We used My Qubit Amplex™ Red Peroxide Assay to detect hydrogen peroxide (H_2O_2) or peroxidase activity in culture media (cat. number A22188). This method enables the Qubit™ Fluorometer to calculate and display the H_2O_2 concentration of samples based on an optimized standard curve algorithm.

Neutrophils were incubated with pyocyanin, IL-17, and LPS for 3.5 hours as described above. The medium was then removed and 100 µM Amplex Red reagents, in combination with 0.2 U/ml horseradish peroxidase (HRP) was added and incubated at 37°C for 15 minutes. In the presence of peroxidase, the Amplex. Red reagent reacts with H_2O_2 to produce the red-fluorescent oxidation product. Fluorometrically or spectrophotometrically assay has been used to detect H_2O_2 concentration. The Qubit™ Fluorometer gives values for My Qubit Amplex™ Red Peroxide assay in µM. This value corresponds to the concentration after samples were diluted into the assay tube. To calculate the concentration of samples, we used the following equation:

$$\text{Concentration of samples} = \text{QF value} \times \left(\frac{240}{\text{X}}\right)$$

2.8 Statistical analysis

Repeated measures linear mixed model and a priori multiple comparisons were used to evaluate cytokine mRNA expression of neutrophils using SAS statistical software, and $p < 0.05$ was considered significant. Results are expressed as the mean ± S.E.M. The raw data were used to make the graphs using GraphPad Prism 7. The gene expression analysis was done using the log transformed data.

4. Results

3.1 Pyocyanin mimicked the condition of oxidative stress in neutrophils.

Oxidative stress refers to elevated levels of reactive oxygen species (ROS). We measured H_2O_2 production level (as ROS) in equine blood neutrophils primed by IL-17 and/or LPS, and pyocyain (0.02, and 0.06 mM) in healthy and severe asthmatic horses. Pyocyanin induced a significant increase in H_2O_2 production at both concentrations. IL-17 and LPS did not alter H_2O_2 levels (Fig. 2.1).

3.2 Pyocyanin induced pro-inflammatory gene expression by equine neutrophils which caused the corticosteroid insensitivity in blood neutrophils through the IL-8 pathway.

Compared to unstimulated neutrophils, pyocyanin induced an increase in the mRNA expression of IL-8 ($P<0.0001$) and IL-1β ($P=0.04$, and $P=0.03$, respectively in healthy and asthmatic horses, not significant after adjustement), but not in TNF-α ($p=0.14$ and $p=0.22$, respectively in healthy and asthmatic horses) (Fig. 2.2). This upregulation of IL-8 mRNA by pyocyanin at both low (lPCN) and high (hPCN) concentrations ($P<0.0001$) was neither attenuated by Dex in healthy horses nor in asthmatic horses ($P=0.33$, and $P=0.16$, respectively in lPCN, and $P=0.12$, and $P=0.12$, respectively in hPCN) (Fig. 2.2 A).

3.3 IL-17 and LPS induced pro-inflammatory gene expression by equine neutrophils.

We compared the gene expression of neutrophils from healthy and severely asthmatic horses stimulated by the combination of IL-17 and LPS after 3.5 hours of cell culture. Compared

to unstimulated neutrophils, IL-8 (P<0.0001) and IL-1β (P<0.0001) mRNA expression was significantly upregulated in both healthy and severely asthmatic horses (Fig. 2.3 A, B). This response was not attenuated by Dex in either of the groups of horses (IL-8, P=0.47, and P=0.49, respectively; IL-1β P=0.76, and P=0.32) (Fig. 2.3 A, B). TNF-α mRNA expression was not altered by IL-17 and LPS stimulation in both groups of horses (P=0.16 and P=0.14, respectively) (Fig. 2.3 C).

3.4 Cobalt chloride, a hypoxia-mimicking agent in equine neutrophils.

Equine neutrophils expressed HIF-1α at protein levels (Fig. 2.4) when incubated with $CoCl_2$ (at 150 μM and 300 μM). This response was unaffected by Dex (3.5 hours).

3.5 Hypoxia blocked the pro-inflammatory gene expression pathways of cultured neutrophils of asthmatic and healthy horses.

The mRNA expression of IL-8, IL-1β, and TNF-α (Fig. 2.5) were unaffected by hypoxia conditions induced by $CoCl_2$. Although, Dex downregulated the expression of IL-1β, this improvement was not significantly different between healthy and asthmatic horses (P=0.15, and P=0.014 (which was not significant after adjustement, respectively) (Fig. 2.5 B). Similarly, Dex blocked the gene expression of TNF-α in both healthy and asthmatic horses but this downregulation was significant in both healthy and asthmatic horses (P=0.0001, and P<0.0001, respectively) (Fig. 2.5 C).

5. Discussion

Previous studies have indicated that airway neutrophils are insensitive to CS administration. After one to two weeks of treatment with CS, the neutrophilia persists in the respiratory tract of the

asthmatic horses (187). Conversely, Hirsch *et al*. showed that Dex strongly reduces the activation of pro-inflammatory genes of blood neutrophils and it has a similar effect on blood neutrophils as on other blood leukocytes (2). Taken together, these findings suggest that the bioactivators present in the lung microenvironment contribute to the CS insensitivity of neutrophils in the airways of CS-treated equine and human patients after their recruitment from blood into the airway.

In human patients suffering from severe asthma, the presence of neutrophils may be associated with increased severity of disease (188, 189). Because of limited supply of oxygen to the lungs, severe neutrophilic asthma patients suffer from hypoxia. Moreover, as neutrophils are potential promoters of ROS production, and they are considered as one of the major sources of oxidants (190), neutrophilic inflammation could contribute to oxidative stress, particularly in neutrophilic severe asthma which has been also reported in asthmatic horses with neutrophilic inflammation (191, 192).

The lung environment can elicit biological responses such as oxidative stress and/or hypoxia as a consequence of physiological changes. Responding to this pathologic stress in the lung microenvironment, neutrophils can modulate the gene expression of the epithelium as they migrate across this layer. Hypoxia and/or oxidative stress can also influence neutrophils functions. In this study, we sought to determine whether oxidative stress or hypoxia activates and induces a relative insensitivity of neutrophils to CS, by investigating the ability of neutrophils to regulate pro-inflammatory gene transcription. We studied asthmatic horses as these animals develop severe neutrophilic inflammation and arepoorly responsive to CS (187, 193), as seen in humans (179-182). We first demonstrated that oxidative stress and hypoxia were induced in our

cell cultures. We then observed that equine neutrophils have selective gene regulatory pathways contributing to CS insensitivity. Indeed, CS insensibility is gene-dependent as shown by the preferentially regulation of IL-8 in response to the oxidative stress. Moreover, similar effects on neutrophilic activity were observed in both healthy and asthmatic horses, indicating that the response of these leukocytes is not influenced by the asthmatic status. Taken together, these findings indicate a possible contribution of IL-8 to the persistence of neutrophils in the airways of CS-treated samples as a key chemokine regulating neutrophil recruitment in the inflamed airways.

PCN participates in neutrophilic inflammation by increasing IL-8 (a neutrophil chemoattractant) gene expression in the human airway (194, 195). Similarly, here we found that PCN-induced oxidative stress increases the mRNA expression of IL-8 in neutrophils derived from healthy and asthmatic horses. Based on our results of pro-inflammatory gene expression, unlike IL-1β and TNF-α, IL-8 appears to be the core factor of CS insensitivity in neutrophils, as its expression was not attenuated by Dex administration. Human studies revealed that PCN enhances neutrophil extracellular trap (NET) formation in neutrophils (196). As our and other group previously reported, Netosis is increased in the lungs of asthmatic horses (187, 197). Considering this, these findings suggest that Netosis is associated with CS insensitivity in severe neutrophilic asthma (198). Therefore, one possible explanation for this insensitivity to CS could be the effect of PCN on Netosis, which would be in agreement with human studies which reported that low concentration of PCN can rapidly lead to NET formation (196). According to Narasaraju *et al.*, IL-8 could prompt NET formation of human neutrophils (199), leading to CS insensitivity of neutrophils. Our results confirmed that equine neutrophils can be activated by

PCN. On the other hand, this finding supports that IL-8 can contribute to continued neutrophil recruitment to the inflamed site, and therefore, to the persistence of neutrophils within the airways of CS treated patients.

In hypoxic conditions, induced *in vitro* by stimulation with $CoCl_2$, pro-inflammatory gene expression was not triggered. There was a slight increase in IL-8 gene expression which was not statistically significant. Previous studies revealed that hypoxia selectively inhibits respiratory burst activity of human neutrophils. In these studies, IL-8 gene expression was unaffected by hypoxia even after 2 h to 20 h of cell culture (200). Considering the increased chemokine secretion in response to $CoCl_2$ observed in previous studies, it is possible that in our study, despite the use of higher concentrations of $CoCl_2$, neutrophils adjusted their inflammatory response to the hypoxia condition in order to adapt to the systematic stress. It has been reported that HIF-1α enhances NET formation in human and bovine neutrophils in a ROS-dependent manner (201). Considering the key roles of HIF-1α in Netosis, HIF-1α pathway could be an important mechanism in neutrophil functional response to treatment during the inflammation.

Our results demonstrate that Dex did not decreases the mRNA expression of IL-1β and TNF-α only in IL-17+LPS stimulated neutrophils, but that IL-8 expression was unaffected under all culture conditions. Therefore, neutrophils have selective activation in the presence of Dex based on the culture condition. In agreement with these findings, IL-17 alone increased production of these cytokines, which are hallmarks of acute inflammatory processes, in horse (193) and in human cells (202-204). Our results are in concordance with published studies which report that cytokines and chemokines have distinct kinetic characteristics in response to different stimuli (205, 206). In agreement with previous equine studies, IL-17 alone activates neutrophils and

importantly, this effect is not inhibited by CS (193). IL-17 has been reported to be necessary for LPS-induced airway neutrophilia in mice (207). At the cellular level, higher levels of IL-17 are detected in asthmatic horses (186, 208), and in asthmatic patients with steroid insensitivity (209-211). It is believed that IL-17, as a pro-inflammatory cytokine, serves a crucial role in triggering inflammatory responses in the equine (187, 193) and human asthma (212). Evidence also shows that IL-17 might promote the secretion of inflammatory mediators by epithelial cells, which in turn, recruit neutrophils to the airways of asthmatic horses (213), and human patients (212, 214). In agreement with previous studies, IL-17 mRNA upregulation in BALF cells of asthmatic horses was unaffected by CS (187) and accelerated inflammation and oxidative stress could be induced by LPS stimulation (215). However, we were unable to stimulate this condition by using IL-17/LPS. This study adds to the current knowledge by showing that LPS does not enhance the IL-17-induced ROS production, although they could imitate the condition in which equine neutrophils will be insensitive to CS which is not due to th condition of oxydative stress.

6. Conclusion

In our study, greater IL-8 production was observed in untreated neutrophils from horses with severe asthma compared with that of healthy subjects, although both healthy and asthmatic neutrophils were similarly affected by the condition of oxidative stress and hypoxia. Our study showed that PCN, IL-17+LPS and, to a laisser extend, $Cocl_2$ increase IL-8 mRNA expression of neutrophils, and this activation is not attenuated by Dex. Beside the function of Netosis in CS insensitivity of neutrophils, all these stimulators have an effect on NET formation of neutrophils. Taken together, these findings, suggest the association between IL-8 as a preferable marker to

identify asthma status and the insensitivity to treatments. This raises the possibility of developing new agents for IL-8 inhibition and helps provide more precise asthma care.

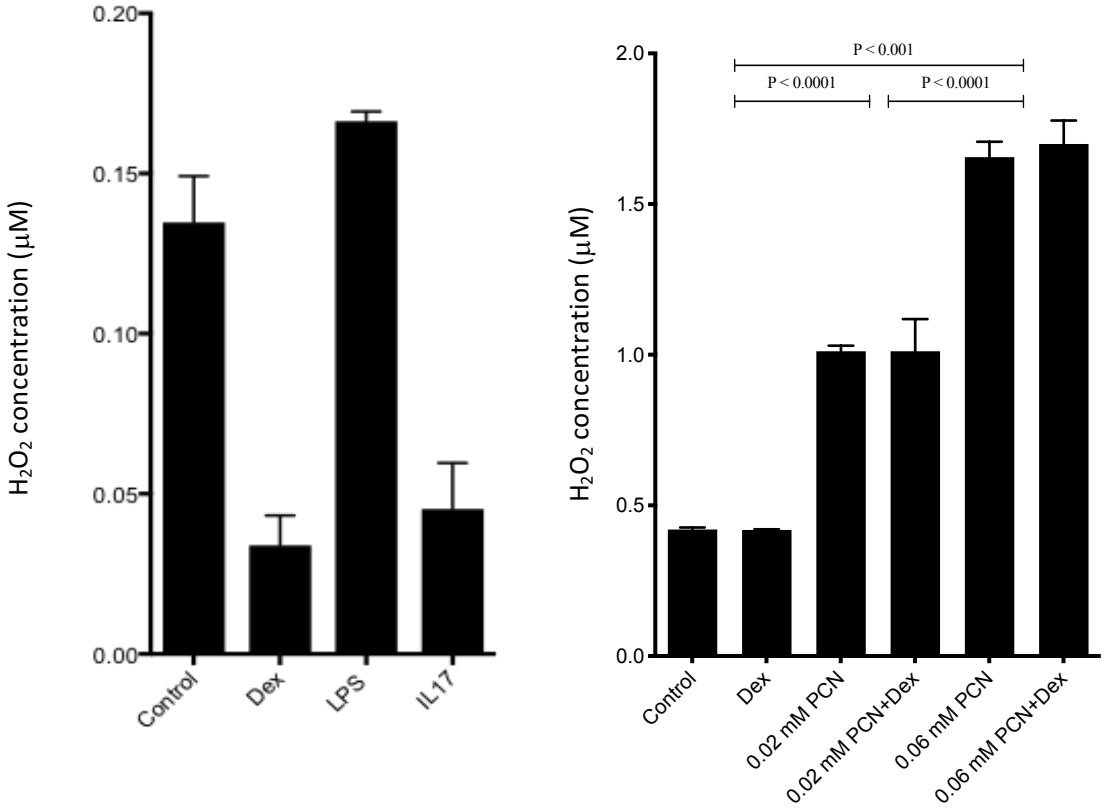

Fig. 2.1 H_2O_2 concentration in culture media after stimulation with IL-17, LPS, and pyocyanin in healthy horses.

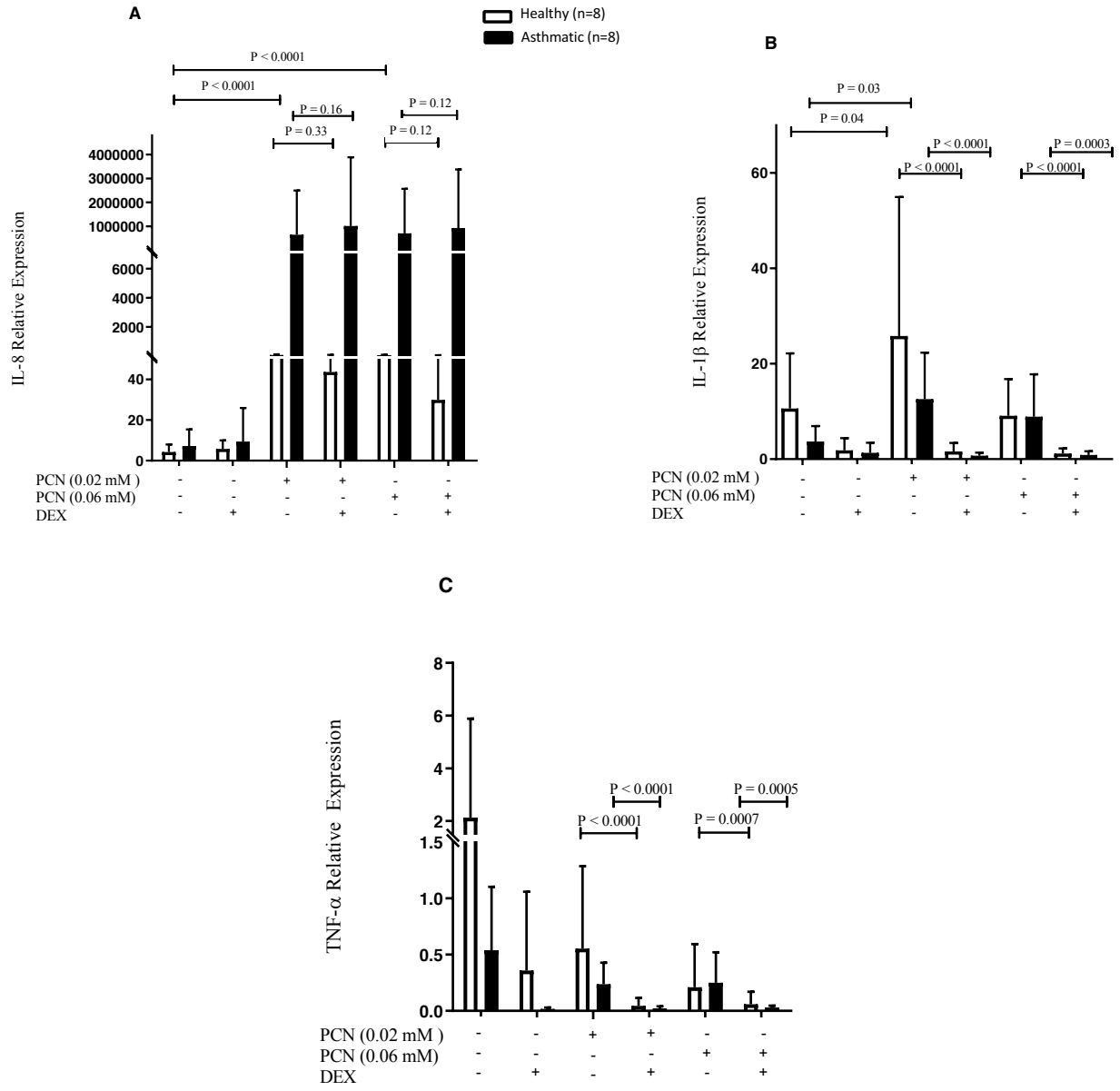

Fig. 2.2 Gene expression in blood neutrophils (n = 8 asthmatic horses in exacerbation, n=8 healthy horses). Blood neutrophils from either asthmatic horse in exacerbation (n = 8) or healthy horses (n = 8) were incubated with pyocyanin. After 3.5 h, pro-inflammatory gene expression was measured by qPCR. Blood neutrophils activation with pyocyanin (0.02, and 0.06 mM) in the presence or absence of dexamethasone (10^{-6} M). A. IL-8, B. IL-1β and C. TNF-α mRNA expression.

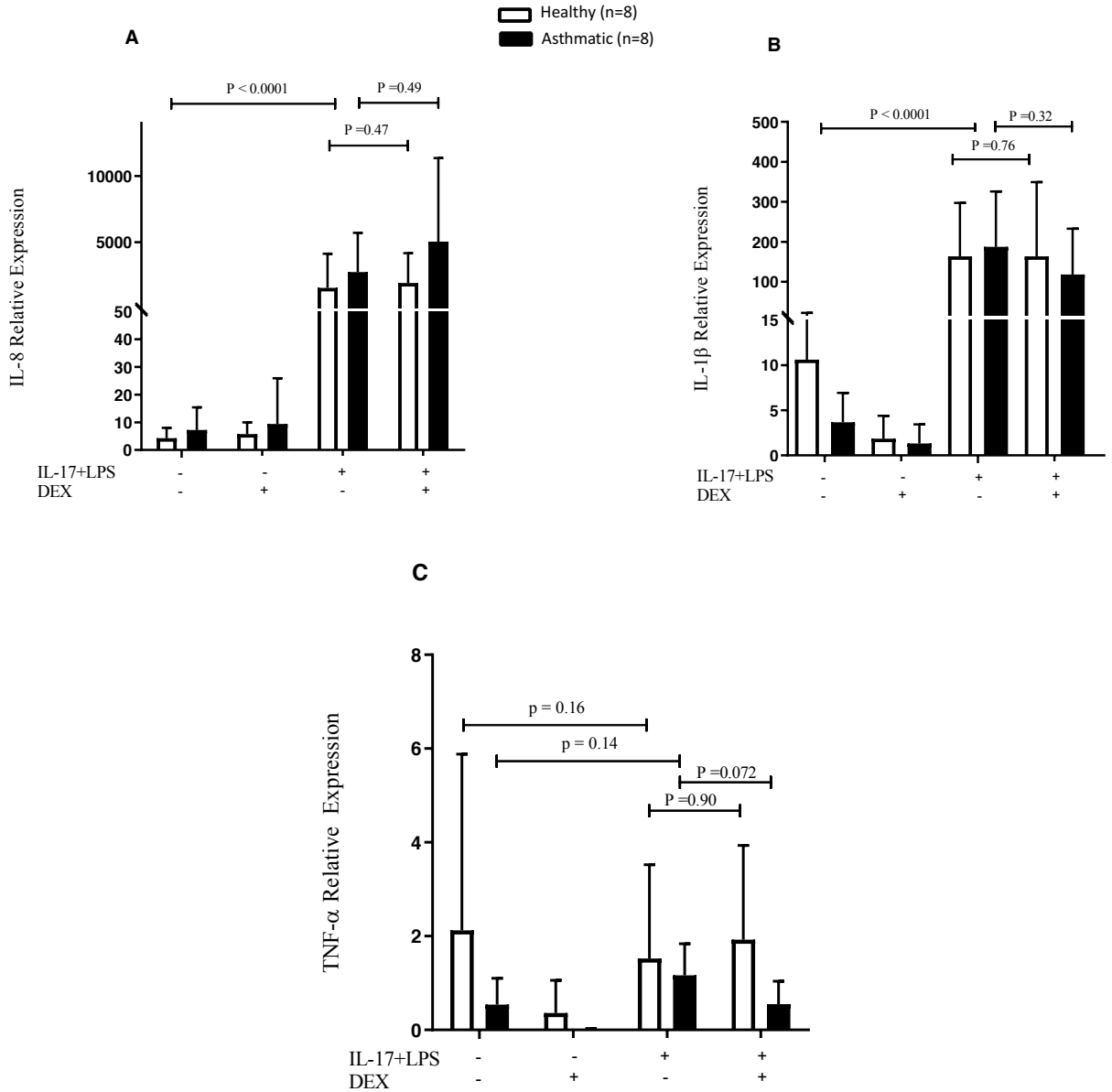

Fig. 2.3 Gene expression in blood neutrophils (n = 8 asthmatic horses in exacerbation, n=8 healthy horses). Blood neutrophils from asthmatic horses in exacerbation (n = 8) or healthy horses (n = 8) were incubated with IL-17 and LPS. After 3.5 h, pro-inflammatory gene expression was measured by qPCR. Blood neutrophils activation with IL-17 (10 ng/ml) and LPS (100 mM) in the presence or absence of dexamethasone (10^{-6} M). A. IL-8, B. IL-1β and C. TNF-α mRNA expression.

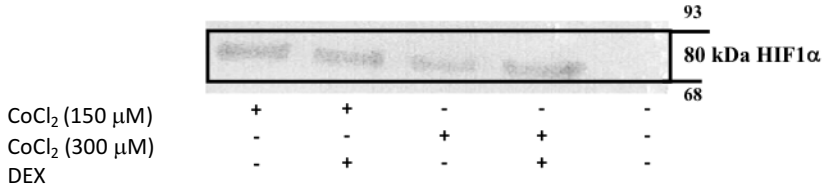

Fig. 2.4 Protein expression of HIF-1α (the index for the condition of hypoxia) in healthy horses.

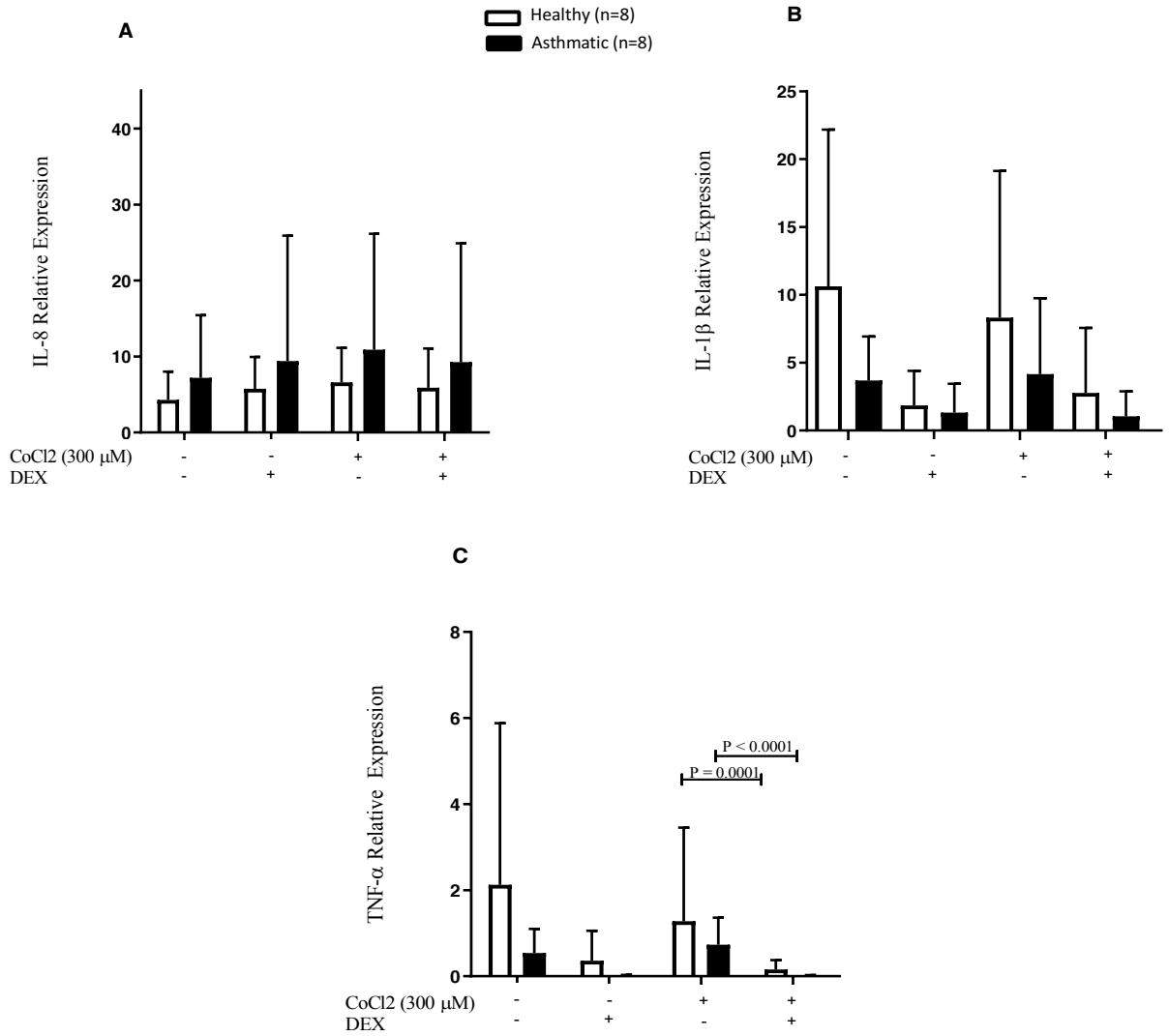

Fig. 2.5 Gene expression in blood neutrophils (n = 8 asthmatic horses in exacerbation, n=8 healthy horses). Blood neutrophils from asthmatic horses in exacerbation (n = 8) or healthy horses (n = 8) were incubated with $CoCl_2$. After 3.5 h, pro-inflammatory gene expression was measured by qPCR. Blood neutrophils activation with $CoCl_2$ (300 μM) in the presence or absence of dexamethasone (10^{-6} M). A. IL-8, B. IL-1β and C. TNF-α mRNA expression.

Chapter 3: General Discussion

Our results demonstrate that equine neutrophils have selective gene regulatory pathways contributing to corticosteroid (CS) insensitivity. Moreover, similar responses in the activities of neutrophils were observed in both healthy and asthmatic horses, indicating that neutrophils have a response to CS that is not affected by the asthmatic inflammation. Further, in human patients suffering from severe asthma, the presence of neutrophils may be associated with increased severity of disease (188, 189). Because of limited supply of oxygen to the lungs, severe asthma patients suffer from hypoxia. Moreover, as neutrophils are potential promoters of ROS production, and they are considered as one of the major sources of oxidants in lungs (190), oxidative stress may contribute to asthmatic inflammation, particularly in neutrophilic severe asthma. Combined, we hypothesized that oxidative stress and hypoxia are contributing to the insensitivity of neutrophils to corticosteroids in severely asthmatic horses.

3.1 Oxidative stress and effects on pro-inflammatory gene expression and CS insensitivity

Our result confirmed that oxidative stress could participate to the CS insensibility of equine blood neutrophils. In order to mimic oxidative stress conditions, we used pyocyanin (PCN) in our culture. PCN-mediated induction of oxidative stress is due to its ability to increase intracellular ROS levels, especially of hydrogen peroxide (H_2O_2) (216). PCN, secreted by the gram-negative bacterium *Pseudomonas aeruginosa*, contributes to the inflammatory response in patients with cystic fibrosis (217, 218). PCN has been used as an inducer of oxidative stress in biological studies (219-221). Similarly, in our model, PCN led to increased levels of H_2O_2 production by equine neutrophils and consequently, it induced oxidative stress in cell culture

media. PCN function has mainly been studied in epithelial cells and neutrophils (194, 196, 222, 223). Our study is the first to demonstrate that PCN stimulates an inflammatory response in asthma. PCN participates in neutrophilic inflammation by increasing IL-8 (a neutrophil chemoattractant) gene expression in the human airways (194, 195). Similarly, herein we found that PCN-induced oxidative stress increases the mRNA expression of IL-8, but not of IL-1β and TNF-α in neutrophils derived from healthy and asthmatic horses. IL-8 upregulation was unaffected after dexamethasone (Dex) treatment in PCN-induced oxidative stress conditions in vitro. These results contrast with previous studies which demonstrated IL-8 suppression in bronchial epithelial cells after CS administration (224-226). In the study of Pan and coworkers, Dex was the most effective CS for attenuating the IL-8 release in cultured human bronchial epithelia cells stimulated by PCN (224). Differences between our study and previous reports could be attributed to cell type studied and the culture conditions, as we used equine neutrophils from healthy and severely asthmatic horses.

In horses, CS are commonly used for the treatment of severe equine asthma (12). Airway neutrophilia, a characteristic of this condition, persists after CS administration, even though a significant improvement in clinical signs and airway obstruction are seen (12, 227, 228). Moreover, human patients with severe neutrophilic asthma are insensitive to CS. However, previous discoveries confirmed that CS inhibit pro-inflammatory gene expression in neutrophils from different species. The resistance to Dex treatment observed in IL-8 expression is related to the condition of oxidative stress induced by PCN.

Human studies revealed that PCN enhances NET formation in neutrophils (196). As our and other groups previously reported, Netosis is increased in the lungs of asthmatic horses (187,

197). Considering this, these findings suggest that Netosis could contribute to CS insensitivity in severe neutrophilic asthma (198). Therefore, one possible explanation for this insensitivity to CS could be the effect of PCN on neutrophil Netosis in equine neutrophils, which is in agreement with human studies which reported that PCN at low concentration can rapidly lead to NET formation (196). Based on our results of pro-inflammatory gene expression, unlike IL-1β and TNF-α, IL-8 appears to be the core factor of CS insensitivity in neutrophils, as it was not attenuated by Dex administration. According to Narasaraju and colleagues, IL-8 could prompt NET formation of human neutrophils (199), which is in concurrence with the possible CS insensitivity of neutrophils because of neutrophil Netosis induced by PCN. Our results confirmed that equine neutrophils are able to be activated directly by PCN.

3.2 Hypoxia and effects on pro-inflammatory gene expression and CS insensitivity

Cobalt chloride ($CoCl_2$) is a heavy metal which has been reported to inhibit PHD enzymes (oxygen sensors) and induce a hypoxia condition chemically (229). Results of a HIF-1α protein assay confirmed that $CoCl_2$ induces hypoxic conditions in cultured equine neutrophils.

Hypoxia-inducible factor-1 alpha (HIF-1α) is a master regulator of oxygen homeostasis. HIF-1α is of crucial importance for cellular adaptation to hypoxia (150, 151). $CoCl_2$ was shown to stabilize HIF-1α thus preventing its degradation (230).

In hypoxic conditions, induced in vitro by stimulation with $CoCl_2$, pro-inflammatory gene expression was not triggered. Previous studies revealed that hypoxia selectively inhibits respiratory burst activity of human neutrophils (231). In their study, IL-8 gene expression was unaffected by hypoxia even after 2 h to 20 h of cell culture. It has been shown that pro-

inflammatory gene expression in macrophages can be upregulated via HIF-1α stabilization (200). Deletion of HIF-1α caused a profound depletion of ATP in these cells compared to neutrophils (165). This suggest that energy stores are more intimately related to the levels of the transcription factor in mononuclear cells compared to neutrophils.

Equine blood neutrophils may need more exposure time to the condition of hypoxia in order to show significantly higher level of HIF-1α in comparison to untreated neutrophils. Although in vitro detection of many inflammatory mediators was not significantly altered by either hypoxia or LPS stimulation in neutrophils (231), others have shown an increase in IL-8 gene and protein expression in $CoCl_2$-induced hypoxia conditions in human endothelial cells (232). In contrast to our results, it has been reported that $CoCl_2$ increases the release of inflammatory cytokines through TLR4 pathways in immune cells like monocytes and neutrophils (233, 234). Also, it has been demonstrated that $CoCl_2$ triggers the release of neutrophil extracellular traps (235). HIF-1α enhances NET formation in human and bovine neutrophils in a ROS-dependent manner (201). Althought, the HIF-1α pathway could be an important mechanism in neutrophil responses to treatment during inflammation.

3.3 Effect of IL-17 and LPS on activation, corticosteroid insensitivity, viability and apoptosis of equine neutrophils

Studies from our group have suggested that IL-17 could contribute to neutrophilic inflammation by upregulating IL-8 gene expression (61). Additionally, Murcia *et al*. demonstrated that IL-17 may directly activate and recruit neutrophils to the airways by the mechanism not attenuated by Dex (193). We first attempted to stimulate oxidative stress using the combination

of IL-17 and LPS. Bacterial lipopolysaccharide (LPS) is a potent inducer of inflammation in many cell types (236). LPS-induced lung injury is frequently used in animal models. Moreover, LPS can activate neutrophil recruitment, and following this, increase the levels of pro-inflammatory cytokines (212, 236). Previous studies have also shown that the LPS-induced oxidative stress pathway is activated in mouse lung injury models where it causes increased ROS production (215, 237). In our study, we used 100 ng/ml LPS to prime equine neutrophils, as LPS has been shown to induce mild ROS production in this cell type (238). Therefore, we hypothesized that activating neutrophils with IL-17 would enhance LPS-induced ROS production. This assumption was supported by a study showing that IL-17 induces the NAD(P)H-oxidase dependent generation of superoxide and hydrogen peroxide, which is involved in the release of pro-inflammatory cytokines in human vascular smooth muscle cells (239). Also, 10 ng/ml of IL-17, the same concentration used in our study, increases markers of oxidative stress in cultured human ASM (240). Nevertheless, using the two agonists failed to successfully prime neutrophils to release ROS in the current study.

However, we showed that neutrophil stimulation with IL-17+LPS increases the mRNA expression of the pro-inflammatory genes IL-8, IL-1β and TNF-α. In agreement with these findings, IL-17 alone increased production of these cytokines, which are hallmarks of acute inflammatory processes, in horse (193) and in human cells (202-204). Our results demonstrate that, the mRNA expression of IL-1β and TNF-α similar to that of IL-8 in IL-17+LPS stimulated neutrophils, were unaffected after Dex treatement. LPS induced the release of IL-8, TNF-α, IL-6, IL-10, and MIP-1β, but this was not modified by hypoxia (231). Therefore, neutrophils had selective activation. Our results are in agreement with published studies which report that

cytokines and chemokines have distinct kinetic characteristics in response to different stimuli (205, 206). In our study, greater IL-8 production was observed in untreated neutrophils from horses with severe asthma compared with that of healthy subjects, although both healthy and asthmatic neutrophils were similarly affected by the stimulation. IL-8 and IL-1β had considerably higher expression than TNF-α in both groups. In concordance with previous human and equine studies, IL-17 activates neutrophils, and importantly, this effect is not inhibited by CS (193). IL-17 has been reported to be necessary for LPS-induced airway neutrophilia (207). At the cellular level, higher levels of IL-17 are detected in asthmatic horses (186, 208), and in asthmatic patients with steroid insensitivity (209-211). It is believed that IL-17, as a pro-inflammatory cytokine, serves a crucial role in triggering inflammatory responses in equine (187, 193), and human asthma (212). Evidence also shows that IL-17 may promote epithelial cells to secrete mediators which, in turn, recruit neutrophils to the airways of asthmatic horses (213) and human patients (212, 214). IL-17 mRNA upregulation in BAL cells of asthmatic horses was unaffected by CS (187), and accelerated inflammation and oxidative stress could be induced by LPS stimulation (215). Thus, we were unable to stimulate this condition by using IL-17/LPS. This study adds to the current knowledge by showing that LPS could not enhance the IL-17-induced ROS production, although they could imitate the condition in which equine neutrophils will be insensitive to CS.

3.4 Choosing the agonists for equine neutrophils (Pilot study)

We performed several pilot studies in order to identify agonists that would best prime equine neutrophils. First, we studied DMNQ which is a non-alkylating redox cycling quinone, an agent for studying oxidant species and oxidative stress. Our tests for this study have shown that

DMNQ does not prime equine neutrophils. furthermore, at high concentrations, DMNQ was cytotoxic.

In order to induce oxidative stress, IL-17 and/or LPS, and PCN were tested. Provisional dose and time titration studies were done in order to achieve the most effective time of exposure and product concentrations (data not shown).

3.5 Using blood neutrophils vs pulmonary neutrophils

Under inflammatory conditions, neutrophils comprise the majority of cells recruited into the lung. This recruitment into tissues is favored by a variety of inflammatory mediators such as IL-1B, IL-6, TNF-α, IL-8, GM-CSF and G-CSF released by structural cells, including epithelial cells, and endothelial. Studies in human and mice with lung injuries have shown that neutrophils varied their chemokine expression due to the tissue location (241-243). GM-CSF is a key myeloid growth factor protein, which has important functional effects on mature cells, including neutrophils, monocytes, and eosinophils. It has been reported that GM-CSFRβ expression was also lower in BALF neutrophils compared to blood neutrophils in the equine model study (244). However, isolation of blood neutrophils is easier to conduct than for BALF neutrophils. This is in part due to this fact, that neutrophil counts in BALF from healthy horses are very low. Further limitations include the PMNs isolation from BALF, which typically involves a low purity due to contamination with lymphocytes and macrophages. Magnetic antibody cell separation (MACS) method could be used to purify cell types (245).

3.6 Assessment of apoptosis

Assessment of neutrophil viability and apoptosis were made after incubation of cells for each culture. In order to validate the incubation time for equine neutrophil flow cytometric assay was done after 3 h, 6 h, 12 h, 18 h and 24 h. APC Annexin V staining was performed following the manufacturer's instructions. Briefly, one hundred microliters of the cell suspension was incubated with 5 µl of APC Annexin V, an apoptosis cell marker, and 5 µl of 7-Aminoactinomycin D (7-AAD), a necrosis cell marker (BD Pharmingen, San Diego, CA, USA). Cells were analyzed using Cell Quest Pro software on a FACSCalibur instrument (BD Biosciences, Mississauga, ON, CA). Data were collected from 10,000 events gated on granulocytes; analyzed unstained cells and single marker-stained cells were used to set photomultipliers voltage and compensation parameters for fluorescence detection in FL-3 and FL-4 channels. APC Annexin V-negative and 7-AAD-negative cells were considered viable.

Our results shows the reduction of neutrophil apoptosis process starting after 18 h of incubation.

3.7 Limitations encountered

As previously mentioned, we have investigated the gene expression of HIF-1α to ensure that hypoxia was induced in cultured equine neutrophils. However, the gene expression of HIF-1α was unchanged after incubation with $CoCl_2$. It has been reported that $CoCl_2$ can increase HIF-1α expression at the protein level in human neutrophils, without seeing any significance difference in its mRNA expression (232). Similarly, our results show that equine neutrophils expressed HIF-1α at protein level, which is evidence for the existence of hypoxia conditions in the cell culture. Furthermore, based on the results, IL-8 and IL-1β were more expressed than TNF-

α in both groups under all experimental conditions. In vitro studies showed that TNF-α is a critical mediator of inflammatory conditions, although corticosteroid treatments could reduce the inhibitory effect of the medication by TNF-α production (246).

Samples size were not large enough to generate sufficient statistical power for PCN-stimulated oxidative stress on IL-1β mRNA . In our study, using 13 horses in each experimental groups would have been required to obtain a significant effect.

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Annex

Protocol #1

Isolation des neutrophiles équins par centrifugation sur gradient Ficoll

sous la hotte biologique en conditions stériles.

Matériels :

- Les Solutions A et B

Pour préparer la solution A et la solution B :

Stock solution A		**Conc. (g/l)**
Anhydrous D-glucose	0.1%	1.0
$CaCl_2 \times 2H_2O$	5.0×10^{-5} M	0.0074
$MgCl_2 \times 6H_2O$	9.8×10^{-4} M	0.1992
KCl	5.4×10^{-3} M	0.4026
Tris	0.145 M	17.565
Conc. HCl	10 N	to pH 7.6
Distilled water[1]		to 1000 ml

[1] Dissolve in approximately 950 ml distilled water and add 10 N HCl until pH is 7.6 before adjusting the volume to 1 l.

Stock solution B		**Conc. (g/l)**
NaCl	0.14 M	8.19

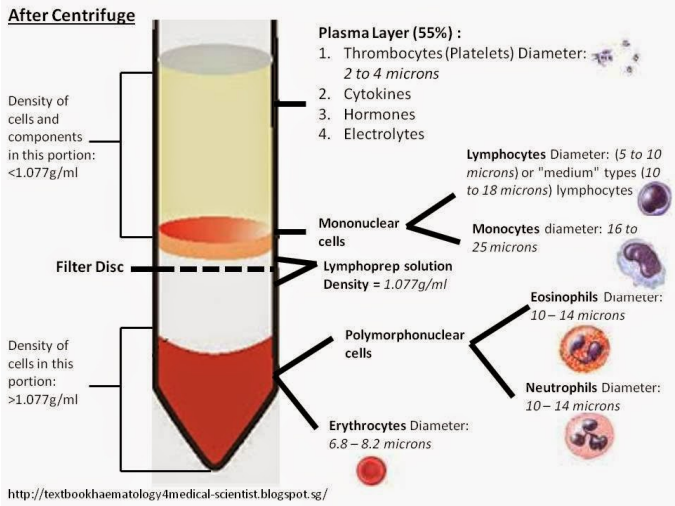
- Ficoll-Paque PREMIUM 1.084, GE heath life sciences.
- L'eau sterile
- PBS-BSA Solution (PBS 1X + BSA 0,5% + EDTA 2mM)

(Par exemples: 500 ml PBS 1x + 2.5 g BSA + 2 ml EDTA)

Manipulations :

- Prélever le sang dans des tubes EDTA ou HÉPARINE.
- Laisser sédimenter 20 à 40 minutes environ.
- Agiter le Ficoll vigoureusement avant de l'utiliser. Préparer des tubes 50mL avec 10 mL de Ficoll (ÉTEINDRE LA LUMIÈRE, Ficoll est très sensible à lumière).
- Préparer la solution isotonique A/B avec un ratio 1 : 9.
- Récupérer le plus de plasma possible et le déposer dans un bécher/cheval.
- Mélanger le plasma avec la solution A/B avec un ratio 1 : 1 (6.5 ml : 6.5 ml).
- Déposer très délicatement le plasma sur le Ficoll avec une pipette pasteur. Faire des chemins dans le tube pour que les gouttes se dépose doucement. (Éteindre la lumière).
- Centrifuger 40 min à 400 g (1300 rpm sur la centrifugeuse Beckman) à la température de la pièce. Mettre le frein à NON.
- S'il reste des tubes de Ficoll inutilisés, les envelopper dans de l'aluminium et les remettre dans l'armoire (Ficoll coûte très cher, alors on ne le jette pas).
- Préparer de l'eau stérile et une solution PBS 1X/BSA 0,5%/EDTA 2mM stérile ainsi qu'une glacière.

- Après la centrifugation, on doit obtenir ceci :



- Retirer les couches supérieures avec le vacuum et laisser le culot de neutrophiles.
- Bien nettoyer les bords des tubes, car cela affectera la qualité des neutrophiles. Laisser les tubes sur la glace.
- Pooler les tubes pour un même cheval (+5 ml de PBS.BSA) et Centrifuger à 2000 rpm pour 5 minutes et à 4 °C.
- Retirer le surnageant avec le vacuum. Ne pas toucher le culot.
- Faire une lyse des érythrocytes en les resuspendant dans environ 1mL d'eau stérile, pendant 30 Secondes (Maximum).
- Stopper la lyse avec 15-20 mL de PBS-BSA-EDTA par tube.
- Centrifuger à 2000 rpm- 5minutes à 4 °C.

- Retirer le surnageant et resuspendre les neutrophiles dans un volume de PBS-BSA-EDTA en fonction de la grosseur du culot (ex. 10ml). (Répéter la lyse s'il reste des érythrocytes).
- Prendre un aliquot pour faire des cytospin (10% et 20%).

Par exemple, diluer 20 µl de suspension cellulaire dans 180 µl de PBS (5 min a 1000 rpm). Faire une différentielle sur 400 cellules.

- Compter les cellules avec le compteur (avec la solution T et N par ADAM).

Protocol #2

- Mix vigorously Cyber green Qiagen #cat 204143.
- Leave the samples and the PCR reactive to be defreeze on the ice. Do the Vortex and quick spin.
- Prepare the master mix with the following components (for 1 reaction of 20 μL)

REACTIVES	VOLUME	CONCENTRATION
CYBR green	10 μL	1X
Forward Primer	2 μL	5X
Reverse Primer	2 μL	5X
H_2O nuclease free	4 μL	-
cDNA	2 μL	300 ng/μL
Total volume	20 μL	-

- Distribute 18 μL of master Mix and add 2 μL of cDNA (RT) per each reaction.
- Start the PCR reaction using following program:

(Hold)	50°C - 2 minutes	
(Activation of the enzyme)	95°C - 10 minutes	
(Denaturation)	95°C - 15 seconds	} x 35-45
*(annealing /Extension)	60°C - 60 seconds	

- Using the RotorGene qPCR machine, choose the channel FAM for fluorescence acquisition during the annealing / extension step (choose the CYB G channel).

* Change the annealing temperature based on the primers has been used.