The Immune Mechanisms of Severe Equine Asthma

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Severe equine asthma is a chronic respiratory disease of adult horses, occurring when genetically susceptible individuals are exposed to environmental respirable particles. This results in airway inflammation, mucus accumulation and bronchial constriction. Affected horses present with cough, nasal discharge and increased respiratory effort at rest. Although a complex diversity of genetic and immunological pathways contribute to the disease, these remain to be fully understood.

severe equine asthma immunology

1. Introduction

Severe equine asthma (sEA) is a naturally occurring chronic respiratory disease [1], affecting up to 20% of adult horses in the Northern hemisphere [2]. Disease develops upon exposure of genetically susceptible individuals to environments with high concentrations of airborne respirable particles, capable of inducing airway inflammation [3]. A vast array of antigens have been implicated in the etiology of sEA and it is thought that airway inflammation results from the synergistic effect of multiple allergens [4], to which individuals are susceptible in unique ways. This disease has been mostly associated with hay feeding and stabling, being termed as stable-associated sEA, but summer pasture-associated sEA also occurs [1][5][6]. Fungal spores, bacterial endotoxins, forage and storage mites, microbial toxins, peptidoglycans, proteases, pollen and plant debris, as well as inorganic particles trigger clinical signs of disease [5][6][7][8][9][10]. Several fungi (>50 species), especially *Aspergillus fumigatus*, have been widely recognised as significant risk factors for sEA [11][12][13]. Recent research by White and colleagues has uncovered the potential role of novel allergens including new species of fungi, mites, pollen, and arthropods, but also that of latex proteins [5], which hadn't yet been clearly associated with the disease.

During disease exacerbation affected horses develop cough, nasal discharge and increased respiratory effort at rest [1][14][15], due to neutrophil recruitment, mucus plugging, bronchospasm and airway remodeling [16][17].

Severely asthmatic horses are usually managed through antigen avoidance and the use of corticosteroids and bronchodilators to reduce airway inflammation, bronchoconstriction and improve lung function [18]. However, some horses are unresponsive to corticosteroid treatment posing a challenge to clinicians. Thus, the identification of causal antigens and the development of antigen screening tests is fundamental and will enable a personalized treatment approach using specific immunotherapy [5][19].

Disease diagnosis is mostly based on history, clinical signs, and bronchoalveolar lavage fluid (BALF) differential cytology. Although lung function testing can accurately detect sEA, such equipment is unavailable to most field practitioners [1][14][20]. The genetic and immunological mechanisms associated with this disease are complex and heterogenous, implicating the activation of different inflammatory pathways [21][22][23]. Currently there is a need to better characterize the immune events leading to the occurrence and persistence of airway inflammation as this will help clinicians in determining the best treatment approach and in providing an accurate prognosis. Moreover, the development of novel ancillary diagnostic tests and therapeutic targets are required for early diagnosis of sEA and total resolution of airway inflammation in refractory cases.

Because sEA shares many similarities to its human counterpart, the horse is considered a good model for the study of the non-allergic and late on-set asthma phenotypes, since disease occurs naturally and sample collection can be easily performed [24]. Thus, further contributions to the disease's characterization will benefit both horses and humans alike.

2. Genetic Background

Although sEA's heritability has been shown in several horse breeds, and a familial aggregation has long been ascertain, external factors, such as environment, increase the likelihood of expressing the disease [3][21][22].

The chromosome region ECA13 has been associated with sEA in one family of Swiss Warmbloods, while region ECA15 has been implicated in a different family of the same breed. The inheritance mode differed between both families, being autosomal recessive in the first family and autosomal dominant in the second [25]. Additionally, in the first family of horses the Interleukin 4 receptor (IL-4R) gene and its neighboring regions in ECA13 appeared to contribute to disease in some individuals [26][27][28]. In humans, polymorphic differences in the Interleukin 4 receptor α chain (IL4Rα) gene play an important role in the development of asthma, since they induce the isotopic switch to immunoglobulin E (IgE) and the differentiation of T-helper type 2 (Th2) lymphocytes [29][30].

Racine and colleagues described an interaction between IL-4R and products of the SOCS5 gene, which may influence the molecular cascades involving nuclear factor (NF)-κB [31]. The gene coding for SOCS5 is located in the ECA15 chromosome region, and it is predominantly expressed by Th1 cells while further inhibiting Th2 differentiation. The inhibitory effect of SOCS5 on IL-4 signaling contributes to the non-Th2 cytokine profile observed in human non-allergic asthma [32], and may explain further similarities between both species.

In a genome wide association study (GWAS), the gene responsible for the TXNDC11 protein, also located in the ECA13 region, has been linked to sEA [33]. In humans, TXNDC11 controls the production of hydrogen peroxide in the respiratory epithelium [34], as well as the expression of MUC5AC mucin, which has been shown to play a significant role on airway hyperreactivity in mice [35]. In sEA-affected horses MUC5AC is upregulated, thus contributing to the mucus plugging observed in the disease [36].

The analysis of genomic copy number variants did not reveal any relevant variant regions which could be associated with the sEA, although a copy number loss was reported on chromosome 5 involving the gene NME7 [37]. The expression of this gene is necessary for ciliary function in the lungs and may be involved in sEA, since in knockout mice it induces primary ciliary dyskinesia [38]. Also, using RNA sequencing technique, a single point substitution was detected in the PACRG and RTTN genes in asthmatic horses, predictively altering their proteins, which are related to ciliary function [39].

In a gene set enrichment analysis of the bronchial epithelium after hay dust exposure, asthmatic horses presented upregulated genes of the E2F transcription factor family, which contribute to cell cycle regulation. Thus, asthmatic horses may suffer from impaired bronchial epithelial regeneration associated to subepithelial remodeling [40].

These recent studies have shown that the respiratory epithelium contributes to the immunological response observed in severely asthmatic horses.

Furthermore, an analysis of expression quantitative trait loci (eQTLs) allied with GWAS did not find a significant association between observed genetic variants and sEA, except for a disease-genetic variant in CLEC16A gene, which regulates gene expression of dexamethasone-induced protein (DEXI) [41]. This is of special importance in comparative pathology, as DEXI has also been reported in human asthma [42], although in horses it appears to not be a reliable indicator of sEA [41].

The identification and differential expression analysis of microRNAs (miRNAs) present in the serum of sEA-affected horses, showed a downregulation of miR-128 and miR-744. These findings suggest that a Th2/Th17 immunological response may characterize sEA [10][43].

Additionally, a recent work on Polish Konik horses aimed to detect the effects of inbreeding on sEA, however no effects were observed at the individual level [44].

Although most of these findings relate to certain families of Swiss Warmblood horses, they illustrate the complex genetic heterogeneity of sEA, which most likely results from the interaction of different genes. However, the use of such specific horse families and the likelihood of high variety of genetic background mechanisms contributing to the disease limits the application of these findings to the general equine population.

3. Immunological Phenotypes and Endotypes

Phenotype is the term used to describe the observable clinical characteristic of a disease, whereas endotype, a subclass of phenotype, refers to its molecular and genetic mechanism or treatment response [45].

As stated in the 2016 consensus, equine asthma (EA) is currently defined by two major phenotypes, which differ according to disease onset, clinical presentation and its severity—mild/moderate EA (mEA) and the already described sEA, which is the focus of this review [1]. However, phenotypes are insufficient when deciding upon the

appropriate therapeutic management or determining the prognosis, which mainly depend on the immunological mechanisms of the disease.

Human asthma is usually considered to be a type 1 hypersensitivity, due to increased levels of IgE associated with a Th2 response, resulting in the recruitment of eosinophils into the airways [46]. However, an endotype which does not appear to be associated to a Th2 response has also been identified. As such, human asthma is divided into two major endotypes according to cytokine profile: Th2 and non-Th2 type asthma [47]. The Th2 type asthma is considered an allergic phenotype with the aforementioned eosinophil involvement and because its cytokine profile has been thoroughly described, several biomarkers are available for characterizing the disease and will be addressed bellow.

However, sEA is typically characterized by a neutrophilic response [1][48], and appears to not have the typical presentation of a type 1 hypersensitivity [49]. Although a Th2 cytokine profile has been described in sEA-affected horses, these animals do not display an early phase response [50][51]. However, a late phase response leading to neutrophilic bronchiolitis, associated with an increase in CD4+ T cells in the bronchoalveolar lavage fluid (BALF), has been described [50][52][53]. These features have led to the hypothesis that a type 3 hypersensitivity response, resulting in antibody-antigen complexes and activation of complement cascade, were involved in the disease's immunology [54]. However, because sEA does not possess most of the features described in type 3 hypersensitivities, it is unlikely that this type of response accounts for the main immunological features of the disease [55].

Still the precise cytokine profile of sEA remains unclear, with a multitude of reports pointing to either a Th1, a Th2, a Th17 or a mixed mediated response. **Table 1** illustrates the cytokines reported in sEA-affected horses.

Table 1. Cytokines reported in sEA-affected horses according to T helper subtype [10][43][49][52][56][57][58][59][60][61][62].

Th2	Th17	Th1/Th2	Th1/Th17	Th2/Th17	Undefined
↑ IL-4 [1(r)]	↑ CXCL13 [2(r)]	↑ IL-4 [1(r)]	↑ IL-1β[1(r)]	↓ miR-197 [2(r)]	↓ IFN-γ [1(r)]
↑ IL-5 [1(r)]		↑ IFN-γ [1(r)]	↑ IL-8 [1(r); 3(r); 3(p)]	↑ miR-744 [2(r)]	↓ IL-4 [1(r)]
↓ IFN-γ [1(r)]			↑ IFN-γ [1(r)]	↓ miR-26a [4(r)]	↓ IL-5 [1(r)]
			↑ TNF-α [1(r)]	↑ miR-31 [4(r)]	↓ IL-13 [1(r)]
			↑ IL-17 [1(r)]	↓ TNF-α [4(r)]	
				↑ IL-4R [4(r)]	

[1]—BALF recovered cells; [2]—Peripheral blood; [3]—bronchial epithelial biopsy; [4]—Lung tissue (post-mortem). r—RNA detected; p—protein detected. ↑—increased expression; ↓—decreased expression. IL—interleukin; IL-4R—interleukin 4 receptor; IFN-γ—gamma-interferon; TNF-α—tumor necrosis factor-α; miR—microRNA.

References

Using immunohistochemistry and in situ hybridization, the expression of IL-4 and IL-5 was observed in the BALF lymphocytes of sEA-affected horses [49][53]. However, the Th2 cytokine profile of these animals was accompanied by airway neutrophilia, but not eosinophilia.

Other authors have reported an increased expression of IL-1β, IL-8, gamma-interferon (IFN-γ), tumor necrosis factor (TNF)-α, and IL-17, mainly suggesting a Th1 and/or Th17 mediated response [58][60][61].

Gene expression analysis of BALF cells and bronchial epithelium of severely asthmatic horses, using reverse transcription polymerase chain reaction (RT-PCR), revealed that the expression of IL-1β, IL-8, NF-κB and toll-like receptor (TLR)4 was upregulated in these animals. Furthermore, authors reported that these findings correlated with the neutrophil percentage detected in the BALF [59].

Ainsworth and colleagues reported that during remission severely asthmatic horses exhibited an increased expression of IL-13 and despite BALF neutrophilia no differences in cytokine expression were observed 24 h after environmental challenge. However, after 5 weeks of chronic exposure to aeroallergens asthmatic horses presented increased IFN-γ and IL-8 gene expression [60].

After antigen challenge, the BALF cells of sEA-affected horses showed elevated gene expression of IL-17, IL-8 and TLR4. Gene expression of IL-8 was also increased in the bronchial epithelium, and using immunohistochemistry was tracked to the ciliated epithelium of affected horses. Additionally, stimulated peripheral blood neutrophils of asthmatic horses incubated with lipopolysaccharide (LPS) and formyl-methionyl-leucine phenylalanine (fMLP), two potent pro-inflammatory agents associated with sEA, revealed upregulated gene expression of IL-17 and TLR4 [61].

The presence of a mixed Th1/Th2 cytokine profile, involving mediators such as IL-4 and IFN-γ, has also been reported [57][58]. Disease exacerbation, post-antigen challenge, was also accompanied by elevated expression of IL-10, TNF-α, IL-8 and IFN-γ, and treatment with fluticasone decreased mRNA expression of TNF-α [57]. Similarly, horses diagnosed with summer pasture-associated sEA developed disease exacerbation during the summer months, with increased expression of IL-13 and IFN-γ by BALF lymphocytes and CD4+ lymphocytes from peripheral blood. Furthermore, during disease remission, in the winter, these animals exhibited increased IL-4 mRNA expression [58].

The possibility of a mixed Th2/Th17 response has also been postulated [43], associated with a dysregulated Th17 cell differentiation pathway [62]. Eleven differentially expressed miRNAs (DEmiRs) were reported in the serum of asthmatic horses, compared to healthy individuals. Also, a shift towards the maturation of Th2 cells was proposed, supported by decreased levels of miR-128, which in association with decreased miR-197 and increased levels of miR-744 affects the maturation of Th cells towards a Th17 profile [43].

The analysis of the miRNAs and mRNA found in the lung tissue of sEA-affected horses supports the hypothesis of a Th17 mediated response, but also of a Th2 immune response [62]. Additionally, the upregulated miRNAs miR-142-3p and miR-223 found in asthmatic horses are also associated with severe neutrophilic asthma in humans,

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and with increased expression of IL-13, IL-6 and IL-8 [63] cytokines, some of which have been associated with sEA [57][58].

Contrarily, Kleiber and colleagues reported neither a specific Th1 nor a Th2 specific response, but a downregulation of expressed cytokines (IL-4, IL-5, IL-13 and IFN-γ) in the CD4 and CD8 populations of the peripheral blood and BALF of sEA-affected horses [56], which could implicate the involvement of other pathways in the disease.

Thus, the reported results may reflect the heterogeneity of the cytokine profile involved in sEA and may imply the existence of different disease endotypes. However the interpretation of these results must necessarily take into consideration the described methodologies of the above-mentioned studies. For example, cytokine expression was investigated using distinct samples, namely BALF, bronchial and lung tissue, as well as peripheral blood. As such, results may not only reflect the inflammatory response of the examined cells, but also differences between local and systemic inflammatory responses.

With the development of transcriptomics, novel techniques for assessing the existence and relative prevalence of several RNA species have been introduced to the scientific community. This is portrayed in the reported methodologies of the aforementioned studies, where recent experiments sequence mRNA and miRNA, contrasting with the less comprehensive/detailed methods, such as traditional targeted immunohistochemistry, in situ hybridization, and RT-PCR.

Additionally, the experimental design of most studies involved the exacerbation of the disease by exposing the asthmatic horse to an intense pro-inflammatory environment, using hay dust and/or by stabling the affected horses. It cannot be excluded that the experimental induction of airway inflammation may interfere to some extent with the expressed cytokine profile, especially considering individual susceptibilities to specific allergens. Therefore, this factor also needs to be taken into account when interpreting reported results.

As in human asthma, it is highly likely that sEA possesses multiple endotypes, and considering the neutrophil recruitment observed in affected horses, a Th17 mediated response is probably part of the inflammatory pathways involved in this disease. Nevertheless, more encompassing studies involving genomics, transcriptomics and proteomics are required to better define the cytokine profile of sEA and to determine therapeutic targets in affected horses, and, although further confirmation is required, the reported DEmiRs may constitute novel therapeutic targets for sEA.

Severely asthmatic horses may also present an altered response to allergens, since ex vivo and in vivo hay dust stimulation revealed upregulation of several genes participating in the inflammation [10][64]. Pacholewska and colleagues reported an increased expression of CXCL13 chemokine [10] which may indicate a Th17 mediated response [65], but no evidence of a Th1 nor a Th2 response was found. Additionally, in a murine model of allergic airway inflammation increased expression of CXCL13 has been reported and its neutralization reduced allergic inflammation by decreasing lymphocytes, eosinophils, as well as the recruitment of CXCR5-bearing cells [66].

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Accordingly, in humans, IL-17 expression has been associated with severe neutrophilic asthma and in horses this cytokine is responsible for the activation and persistence of neutrophils in the airways. Also, IL-17 was shown to be associated with poor response to corticosteroids, with post-treatment persistence of IL-8 [67][68].

The described heterogeneity also occurs in human asthma, where one could consider the existence of three distinct phenotypes: allergic asthma, non-allergic asthma and late-onset asthma [22]. These phenotypes may also be applicable when describing sEA. In this sense the allergic asthma phenotype would be characterized by a Th2 mediated response and usually associated with other allergic diseases. In general, horses affected with sEA may also suffer from other allergic diseases, such as insect bite hypersensitivity [69][70]. Interestingly, Lo Feudo and colleagues have reported the presence of a type 1 hypersensitivity in sEA-affected horses in response to intradermal allergen test, which may further support the hypothesis of an allergic phenotype [71]. Additionally, the use of skin prick tests has previously been used to identify allergic sensitization in severely asthmatic horses [19]. Similarly, evidence of a type 1 hypersensitivity to different allergens has also been described in severely asthmatic horses using allergen inhalation [72].

The non-allergic phenotype, in humans is usually associated with the presence of neutrophils in the airways, a hallmark of sEA and phenotypically reflects the involvement of a Th1 and/or a Th17 response [62], which has also been described as contributing to the immune response in asthmatic horses.

Finally, the late-onset asthma is age-associated and also occurs in sEA where affected individuals are mature adults [2][73]. This age association is thought to be the consequence of immunosenescence and inflammaging, which describe the immune and inflammatory modifications observed in geriatric patients [74][75][76]. Immunosenescence, a subject extensively reviewed by Bulmer et al. [75], is essentially a dysfunction of the immune system. In horses it is usually characterized by a dysregulation of adaptive immunity associated with a lower proliferative response of T lymphocytes [77], and a decrease in mean percentage of regulatory T cells [78].

On the other hand, the term inflammaging defines the chronic inflammatory state observed in older individuals accompanied by an increased expression of inflammatory cytokines [74]. A correlation between age and IL-6 has also been described in healthy geriatric horses [79]. Also, compared to young adults, geriatric horses with colitis had higher levels of IL-6 and TNF-α [80]. It has also been described that older horses exhibit increased expression of IL-1β, IL-15, IL-18, IFN-γ and TNF-α mRNA [77][81][82]. These studies confirm that inflammaging and immunosenescence occur in geriatric horses both systemically and locally [74], and are most likely involved in the immunology of sEA, although further research is needed to clarify the age-associated changes and how they affect airway inflammatory response.

The reported differences in the immunological pathways contributing to sEA illustrate the complexity of this disease and suggest the existence of several endotypes, which converge into the same clinical phenotype. One must also consider that the methodological differences of the above-mentioned studies, such as time of sample collection, natural vs. stimulated inflammatory response and duration of the disease, may have contributed to the reported variations. It is therefore fundamental that holistic studies, encompassing more exhaustive and complementary

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