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Single-cell profiling of bronchoalveolar cells reveals a Th17 signature in neutrophilic severe equine asthma

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Abstract

Severe equine asthma (SEA) is a complex respiratory condition characterized by chronic airway inflammation. It shares many clinical and pathological features with human neutrophilic asthma, making it a valuable model for studying this condition. However, the immune mechanisms driving SEA have remained elusive. Although SEA has been primarily associated with a Th2 response, there have also been reports of Th1, Th17, or mixed-mediated responses. To uncover the elusive immune mechanisms driving SEA, we performed single-cell mRNA sequencing (scRNA-seq) on cryopreserved bronchoalveolar cells from 11 Warmblood horses, 5 controls and 6 with SEA. We identified six major cell types, including B cells, T cells, monocytes–macrophages, dendritic cells, neutrophils, and mast cells. All cell types exhibited significant heterogeneity, with previously identified and novel cell subtypes. Notably, we observed monocyte–lymphocyte complexes and detected a robust Th17 signature in SEA, with CXCL13 upregulation in intermediate monocytes. Asthmatic horses exhibited expansion of the B-cell population, Th17 polarization of the T-cell populations, and dysregulation of genes associated with T-cell function. Neutrophils demonstrated enhanced migratory capacity and heightened aptitude for neutrophil extracellular trap formation. These findings provide compelling evidence for a predominant Th17 immune response in neutrophilic SEA, driven by dysregulation of monocyte and T-cell genes. The dysregulated genes identified through scRNA-seq have potential as biomarkers and therapeutic targets for SEA and provide insights into human neutrophilic asthma.

KEYWORDS
asthma, horse, inflammation, lung, Th17

INTRODUCTION

Equine asthma is a common respiratory disease of the horse characterized by bronchoconstriction, mucus production and bronchospasm [1]. Its severe form, severe equine asthma (SEA), presents with frequent coughing, increased breathing effort at rest, airway remodelling and

Abbreviations: MO, monocyte; MΘ, alveolar macrophage; NETs, neutrophil extracellular traps; NEU, neutrophil; SEA, severe equine asthma.

Vidhya Jagannathan and Vinzenz Gerber contributed equally to this work.

in most cases, airway neutrophilia [1]. In clinical practice, the diagnosis of SEA is typically based on the history, the clinical examination, and the response to therapy. The Horse Owner Assessed Respiratory Signs Index (HOARSI) questionnaire can be used to identify cases based on their respiratory sign history [2, 3]. Diagnosis confirmation is typically achieved through cytological examination of the bronchoalveolar lavage fluid (BALF). Equine asthma is triggered by an exaggerated immune response to respirable particulates found in the environment and particularly in hay dust. These entails organic and inorganic elements such as endotoxins, fungal elements, mites, or ammonia [1]. The reason for the development of SEA in some horses and not others, despite being exposed to the same environment, remains incompletely understood. Individual susceptibility appears to be influenced, at least in part, by genetic factors [4, 5].

In humans, asthma is considered an umbrella diagnosis encompassing a plethora of diseases with distinct pathophysiologic cellular and molecular mechanisms (so-called endotypes). More than half of asthmatic patients suffer from the Th2-type (allergic) form of the disease. SEA shares clinical and pathological features with several human asthma endotypes, including allergic, non-allergic, and late-onset asthma [6]. Specifically, horses with SEA exhibit striking similarity to humans with severe neutrophilic asthma [7]. As horses are exposed to high levels of dust in stables, they also represent an ideal model for organic dust-induced asthma of agricultural workers [8]. In contrast to murine models with experimentally induced airway inflammation, horses develop asthma under natural conditions. Although promising asthma drugs have been identified based on murine studies, their limited clinical efficacy when applied to humans [9] may be attributed to disparities in the underlying pathophysiological mechanisms between experimentally induced and naturally occurring diseases. The longer lifespan of horses (more than 20 years) enables the study of disease progression, particularly airway remodelling. Furthermore, their size facilitates the collection of lower airway samples. For instance, collection of BALF is a routine procedure in horses, in contrast to humans and conventional laboratory animal models. Investigating the immunological mechanism behind SEA may contribute to a better understanding of the pathophysiology of severe neutrophilic asthma and organic dust-induced asthma in humans.

Research into the primary immune pathway in SEA has generated conflicting findings. The disease has been linked alternatively to a Th2-type, Th1-type, Th17-type, or mixed immune response through mRNA or cytokine analysis of peripheral blood or BALF in affected horses [10]. The prevailing perception of SEA is that of a Th2-type disease, supported by the observation that affected horses may also experience hypersensitivities like insect bite hypersensitivity or urticaria [11, 12]. However, recent studies have increasingly pointed to the involvement of the Th17 pathway.

The complexity of the disease and limitations of experimental techniques may have contributed to these inconsistent findings. To address this knowledge gap, we leveraged the emerging single-cell mRNA sequencing (scRNA-seq) technology to dissect the immune mechanisms of SEA at the single-cell level. In a previous experiment, we demonstrated that scRNA-seq can be successfully applied to fresh frozen equine BALF cells [19]. Here, we employed the scRNA-seq technology to characterize BALF cells from six horses with SEA and five control horses.

MATERIALS AND METHODS

In this observational case–control study, we recruited SEA-affected horses and controls based on their medical history. We selected six asthmatic and six control horses, using BALF quality, history of respiratory signs, and BALF neutrophilia as inclusion criteria. We performed 10× Genomics 3′-end scRNA-seq on ∼6000 cryopreserved bronchoalveolar cells per horse (Table S1). One control horse was excluded due to low cell number and quality, leaving 11 horses for the data analysis. Our objectives were to assess the effect of SEA on (i) the distribution of cell types and cell subtypes in the BALF and (ii) the differential gene expression (DGE) within each of the cell types/subtypes identified (refer to Data S1 and S2 for details).

RESULTS

Single-cell landscape of BALF from asthmatic and control horses

We analysed the scRNA-seq data obtained from the BALF cells collected from six asthmatic and five control horses. Characteristics of the study population are listed in Table 1.

Unsupervised clustering identified 19 distinct cell clusters (Figure 1a). Through automated annotation using the top 10 differentially expressed genes (DEGs) derived from major cell types identified in our pilot study [19], we successfully predicted the identity of 99.6% of the cells. Cell cluster identities were validated using the expression of known canonical markers and the top DEGs specific to each cell group (Data S3). Subsequently, the cell clusters were consolidated into six major cells

TABLE 1 Study population characteristics.

Variable	Control ($N = 5$)[a]	Asthmatic ($N = 6$)[a]	*p*-Value[b]
Sex			0.6
Mare	3 (60%)	2 (33%)	
Gelding	2 (40%)	4 (67%)	
Age (years)	12 (11, 12)	12 (7, 19)	>0.9
HOARSI score	1 (1, 1)	4 (3, 4)	**<0.01**
Weight (kg)	594 (582, 613)	578 (548, 609)	>0.9
Clinical score (/23)	1 (0, 1)	6 (5, 7)	**<0.01**
Tracheal mucous score (/5)	1 (1, 2)	3 (3, 4)	**<0.05**
BALF yield (%)	48 (48, 48)	52 (45, 56)	0.7
BALF macrophages (%)	51 (48, 56)	51 (38, 55)	0.5
BALF lymphocytes (%)	40 (36, 47)	36 (22, 40)	0.4
BALF neutrophils (%)	4 (2, 7)	18 (12, 25)	**<0.01**
BALF mast cells (%)	1 (0, 1)	1 (1, 2)	0.5
BALF eosinophils (%)	0 (0, 0)	0 (0, 0)	>0.9

Abbreviations: BALF, bronchoalveolar lavage fluid; HOARSI: Horse Owner Assessed Respiratory Signs.

[a]Median (first quartile, third quartile).

[b]Fisher's exact test; Wilcoxon rank sum test. *P*-values < 0.05 are indicated in bold.

groups: B cells, dendritic cells (DCs), mast cells, monocytes–macrophages (Mo/Ma), neutrophils, and T cells. To explore the diversity of each major cell type, we re-analysed them independently. We identified three distinct B-cell clusters (Figure 2a), three neutrophil clusters (Figure 3a), seven T-cell clusters (Figure 4a), six Mo/Ma clusters (Figure 5a), and four DC clusters (Figure 6a). The mast cell population remained homogenous, without convincing sub-clustering. Cell clusters were annotated based on the calculated marker genes (S5-S11/Tables S2–S8) and the expression of cell-type marker genes. Data S3 provide supporting evidence for the annotation.

The BALF of asthmatic horses is enriched in B cells but specifically depleted in activated plasma cells

As expected, asthmatic horses showed a significantly higher proportion of neutrophils compared with the control horses (Table 2 and Figure 1b,c). A novel finding was the B-cell enrichment in the BALF of asthmatic horses (Table 2 and Figure 1b,c). Concurrently, asthmatic horses exhibited approximately three times fewer activated plasma cells (B2 cluster) than control horses (Table 3 and Figure 2b,c). This suggests that expansion of the naïve B cells and non-switched plasma cells primarily contributed to the increased B-cell proportion in asthmatic horses. No significant differences were observed between asthmatic and control groups for other major cell types or subtypes (Table 2 and Figures 1b, 2b, 3b, 4b, 5b, and 6b). The distribution of major cell types and subtypes for each horse is depicted in Figures 1c, 2c, 3c, 4c, 5c, and 6c.

Gene expression profile of neutrophils indicates altered NETosis and migratory function in SEA

Using a mixed model approach, we compared gene expression between asthmatic and control horses within each cell type and subtype. S12 (Table S9) and S13/Table S10 provide the results of the DGE analysis for the major cell types and the neutrophil subtypes, respectively. A positive log fold change indicates upregulation in the SEA group.

Neutrophils exhibited an 'asthma signature' characterized by upregulation of *CHI3L1* and *MAPK13*, known markers of neutrophilic asthma in humans [20–22], and downregulation of *SLC7A11*, an indicator of ferroptosis reduced in neutrophilic mice asthma [23]. Apoptotic neutrophils (Neu0) had upregulated *S100A9* and *RETN*, both involved in NETosis function [24, 25]. Pro-inflammatory neutrophils (Neu1) had downregulated *KLF2*. Reduced KLF2 levels can promote neutrophil migration [26] and exacerbate NET-induced transfusion-related acute lung injury [27]. In the ISG^{high} neutrophils (Neu2), we observed upregulation of *ADGRE5*, also known as *CD97*, which may promote migration of ISG^{high} neutrophils to the lungs [28].

Gene expression features with a potential protective effect on the lower airways were also identified. The antileukoproteinase gene *SLPI* was upregulated in asthmatic horses, which has an anti-inflammatory role by inhibiting the NFκB pathway and preventing excess NET formation [29]. *NFKB1* was indeed downregulated in neutrophils from asthmatic horses. Downregulation of *IL17RC* suggested a reduced capacity to respond to the Th17 cytokine IL17. The predominant contributor of protective features was the apoptotic neutrophil subtype, with upregulation of *SLPI* and downregulation of *CCL20* and *NR4A3*. The Th17-associated cytokine CCL20 is a potent chemotactic factor for lymphocytes and DCs. The downregulation of *CCL20* could thus have an overall

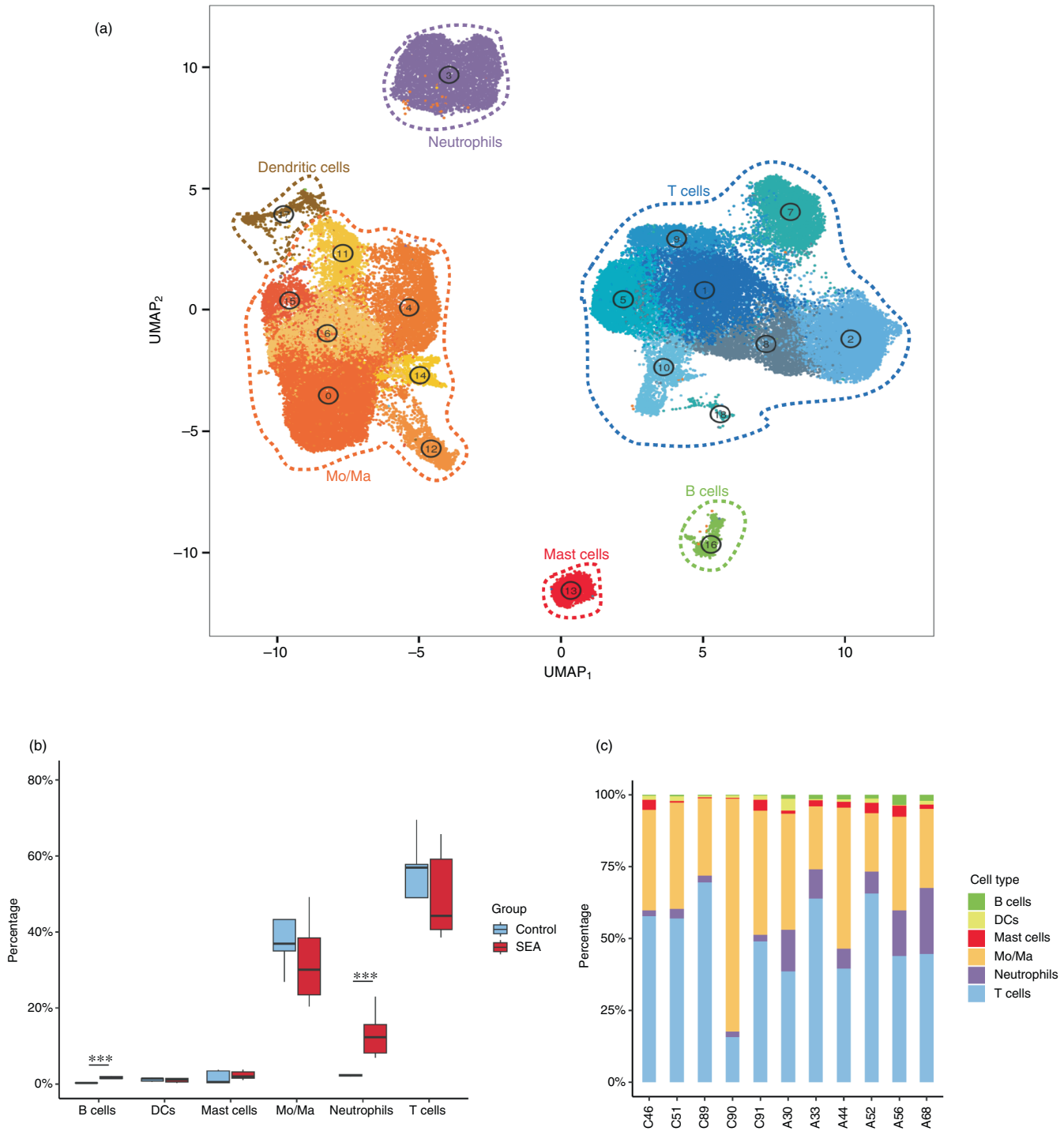

FIGURE 1 Major cell types identified in the bronchoalveolar lavage fluid of asthmatic and control horses using single-cell mRNA sequencing. (a) UMAP representation of the 19 clusters identified as six major cell types. Mo/Ma, monocyte–macrophage. (b) Distribution of the six major cell types in the asthmatic and control groups. SEA, severe equine asthma. ***p-value <0.001. (c) Distribution of the six major cell types for each horse. A, asthmatic; C, control; DCs, dendritic cells.

anti-inflammatory effect, with reduced chemotaxis of immune cells and reduced Th17-signalling. *NR4A3* positively regulates neutrophil survival [30]. Hence, its down-regulation may mitigate neutrophil persistence in the lungs in SEA.

In summary, neutrophils from asthmatic horses exhibited DGE patterns indicative of asthma, including known markers of human asthma. Moreover, these neutrophils displayed an expression profile consistent with increased migratory capacity and the potential for NET formation.

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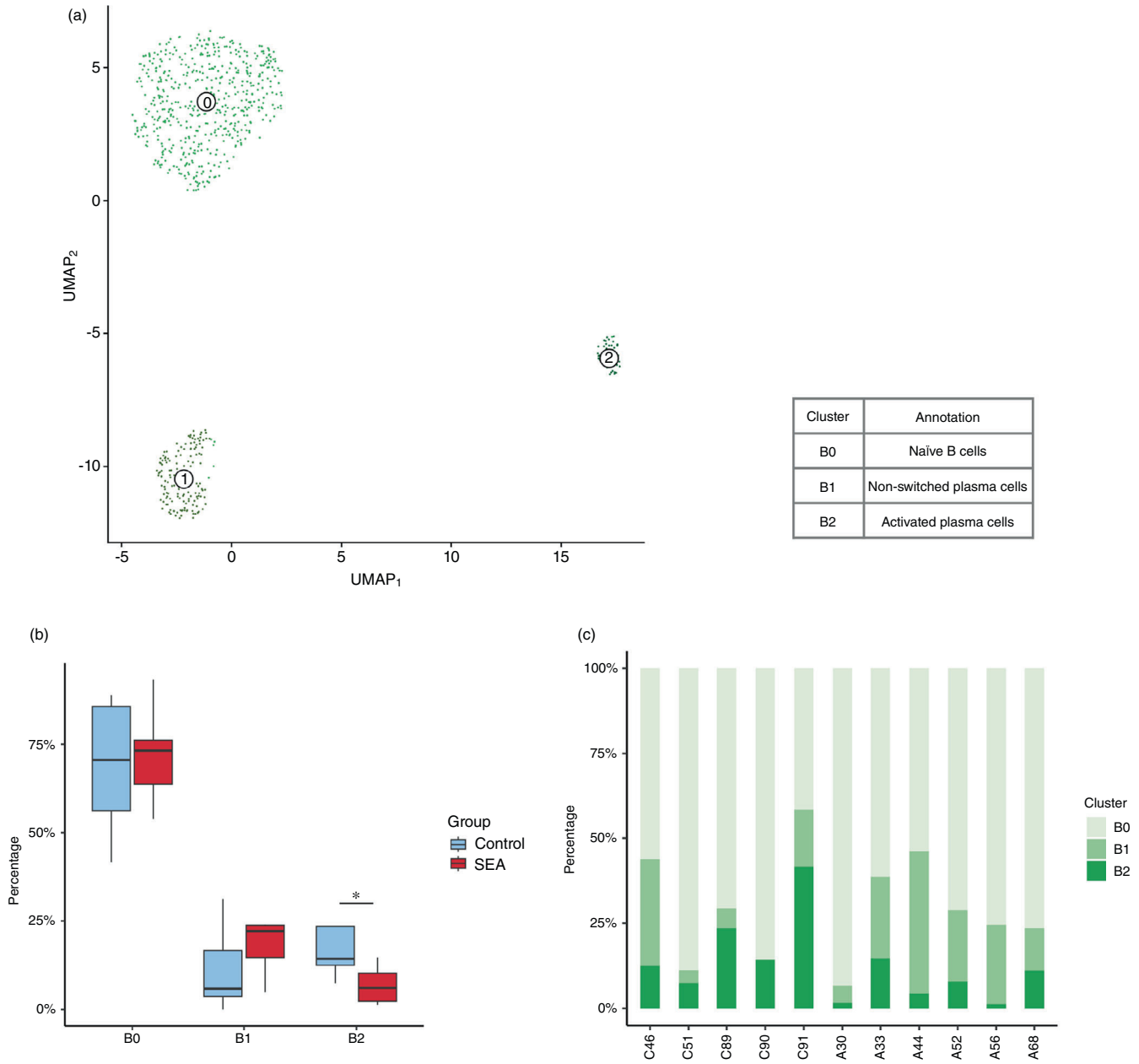


FIGURE 2 B-cell subtypes identified in the bronchoalveolar lavage fluid of asthmatic and control horses using single-cell mRNA sequencing. (a) UMAP representation of the three clusters identified. (b) Distribution of the B cell subtypes types in the asthmatic and control groups. SEA, severe equine asthma. *p-value <0.05. (c) Distribution of the B-cell subtypes for each horse. A, asthmatic; C, control.

The simultaneous expression of genes with a protective action suggests a dual pro- and anti-inflammatory role of neutrophils in SEA.

Gene expression patterns of T cells support a Th17-oriented immune response in SEA

S12 (Table S9) and S14/Table S11 provide the results of the DGE analysis for the major cell types and the T-cell subtypes, respectively. In the T cells of asthmatic horses, two known markers of human asthma, *IL26* [31] and *OLFM4* [32], were upregulated. As in neutrophils, the acute asthma marker *RETN* [33] was upregulated in cytotoxic T cells (T0). Moreover, T cells of asthmatic horses exhibited a robust Th17 signature, characterized by the upregulation of *IL17A*, *IL17F*, *IL21*, and *CCL20* (Figure 4d).

Naïve *CD4*$^+$ T cells (T4) showed a simultaneous upregulation of Th17-associated genes (*IL17A*, *IL1B*, *CCL20*, and *NFKBID*) and *FOXP3*. This supported the hypothesis that naïve *CD4*$^+$ T cells adopt a Th17 pathway during differentiation in SEA, as *FOXP3* expression is transiently present during Th17 cell development [34].

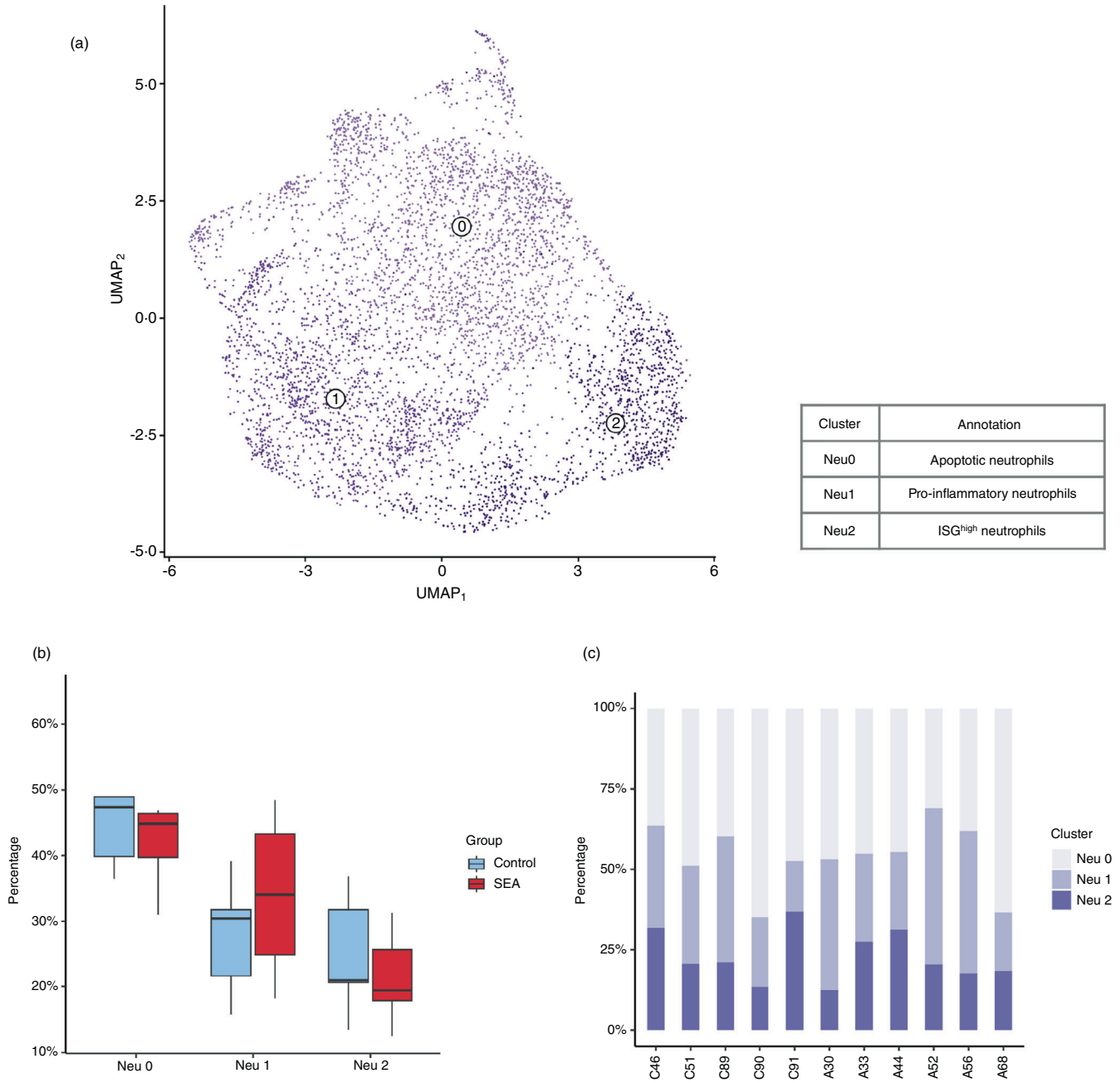


FIGURE 3 Neutrophils subtypes identified in the bronchoalveolar lavage fluid of asthmatic and control horses using single-cell mRNA sequencing. (a) UMAP representation of the three clusters identified. ISG, interferon-stimulated genes. (b) Distribution of the neutrophil subtypes in the asthmatic and control groups. SEA, severe equine asthma. (c) Distribution of the neutrophil subtypes for each horse. A, asthmatic; C, Control.

Furthermore, Treg cells (T1) displayed a Th17-oriented profile, with upregulation of *IL21* and *IL17A* and downregulation of *EOMES*, a known suppressor of Th17 differentiation in human Treg cells [35].

The γδ T (T3) cells conjointly upregulated *IL17A* and *IL1R*, consistent with a γδ17 phenotype [36]. In mice, γδ T cells possess an intrinsic capacity for IL17 production, which is directly induced by IL23 and IL1 [37]. Notably, γδ17 T cells are implicated in various human inflammatory diseases [37], and increased *IL1R* expression has been associated with neutrophilic asthma and reduced pulmonary function in humans [36].

Genes associated with T-cell function are dysregulated in SEA

Several genes involved in T-cell function were differentially expressed in asthmatic horses. Specifically, the marker of T-cell exhaustion, *TOX2* [38], was upregulated,

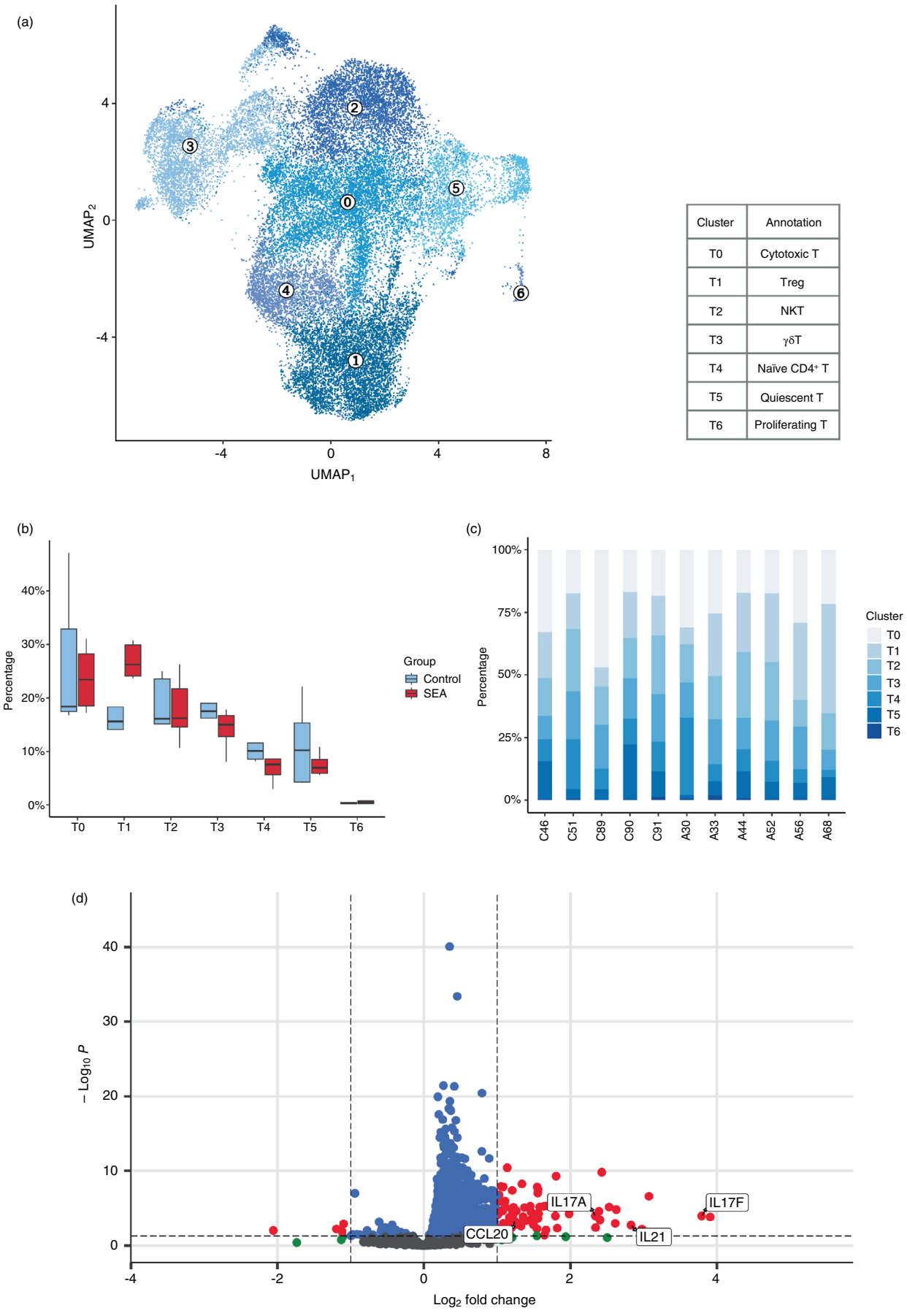


FIGURE 4 Legend on next page.

along with *S1PR5*, whose expression is induced by antigen exposure [39]. Cytotoxic T cells (T0) downregulated *GZMB*, a gene associated with lymphocytic inflammation in the lungs [40]. The downregulation of *IL18R1* and *XCL1* supported Treg cell dysfunction. Indeed, downregulation of the IL18 receptor is associated with unresponsiveness of exhausted $CD8^+$ T cells [41]. Furthermore, dysfunctional Treg cells in individuals with allergic asthma have been shown to downregulate *XCL1* [42]. Among the T-cell subtypes, NKT cells (T2) upregulated *NPY*, a gene associated with reduced NK function [43].

Monocytes and alveolar macrophages display a Th17 signature in SEA

S12 (Table S9) S15/Table S12 provide the results of the DGE analysis for the major cell types and the Mo/Ma subtypes, respectively. Among the upregulated genes in Mo/Ma were *CXCL13*, a B-cell chemoattractant, *OLFM4*, associated with severe lung disease in humans [44, 45], and *CHI3L1*, a marker of neutrophilic asthma (Figure 5d) [21]. *S100A8*, known for its increased expression in individuals with steroid-resistant neutrophilic asthma [46], was upregulated, and so was *TLR1*, recently identified as a potential therapeutic target for asthma in humans (Figure 5d) [47].

In the $FCN1^{high}$ AMs (Mo/Ma0), upregulated genes included *PGLYRP1*, *PTX3*, and *CCL20*. In mice, *Pglyrp1* promotes pro-asthmatic Th2 and Th17 responses [48], while *PTX3* is a marker of non-eosinophilic asthma in humans [49]. In horses, BALF *PTX3* expression increases in acute asthmatic crisis, particularly in dust-activated foamy macrophages [50]. The simultaneous upregulation of the Th17-associated cytokine *CCL20* and downregulation of the Th1-cytokine *CCL11* supported a Th17 polarization of $FCN1^{high}$ AMs. Moreover, in the ISG^{high} AMs (Mo/Ma2), genes encoding *CCL20* and its receptor *CCR6* were upregulated, further advocating for a Th17 phenotype.

The B-cell chemoattractant *CXCL13* was upregulated in intermediate monocytes (Mo/Ma3), and putative monocyte–lymphocyte complexes (Mo/Ma5). Furthermore, intermediate monocytes demonstrated upregulation of *S100A9*, *S100A12*, *CCL17*, and *S1PR5*. *S100A9* and *S100A12* serve as biomarkers for neutrophilic asthma [51, 52]. *CCL17* is associated with asthma and may contribute to airway remodelling through fibroblast activation via the CCR4–CCL17 axis [53, 54]. *S1PR5* regulates monocyte trafficking [55], suggesting intermediate monocytes from asthmatic horses may possess a higher migratory capacity.

Th17 activation may result from a crosstalk between monocytes and lymphocytes

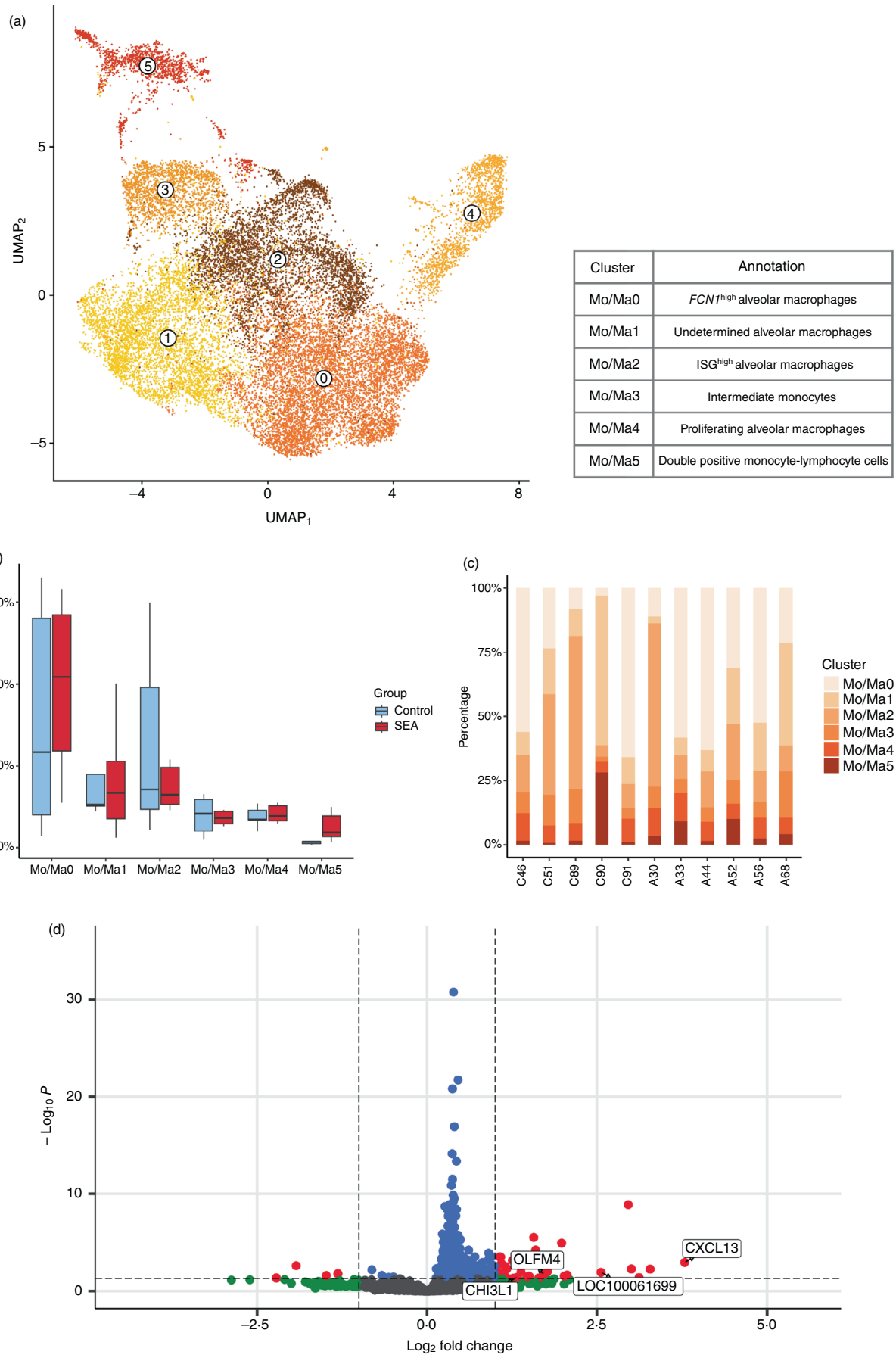
The presence of multiple cell types within the Mo/Ma5 cluster was supported by the high number of DEGs identified. This cluster exhibited simultaneous upregulation of *CXCL13* and *IL17A*, both associated with the Th17 pathway. Interestingly, while several T-cell clusters in the dataset upregulated *IL17A*, none of the Mo/Ma clusters, except Mo/Ma5, showed this upregulation. Conversely, *CXCL13* upregulation was exclusive to Mo/Ma5 and not observed in any T-cell clusters. This led us to conclude that the co-upregulation of *IL17A* and *CXCL13* originated from the dual nature of Mo/Ma5 as monocyte–lymphocyte complexes. Downregulation of the Th1-associated gene *CD27* and granzyme B-like genes further suggested a Th17 polarization within the cells composing the complexes [56]. Additionally, inflammasome-related genes (*SIGLEC14*, *KCNK13*, and *PELI2*) [57] were upregulated.

Gene expression patterns of DCs suggest enhanced migratory capacity and non-Th2 response in SEA

Table S9 (S12) and Table S13 (S16) provide the results of the DGE analysis for the major cell types and the DC subtypes, respectively. The gene *MARCO* was downregulated in asthmatic DCs. In a murine OVA-asthma model, *Marco*-deficient mice showed increased eosinophilic airway inflammation and airway hyper-responsiveness, accompanied by enhanced migration of lung DCs to draining lymph nodes [58]. Consequently, reduced *MARCO* expression in equine lung DCs may enhance their migration to lymph nodes, leading to an amplified immune response against aeroallergens.

Further analysis of DC subtypes yielded significant results for DC0 (annotated as cDC2s), with the notable

FIGURE 4 T-cell subtypes identified in the bronchoalveolar lavage fluid of asthmatic and control horses using single-cell mRNA sequencing. (a) UMAP representation of the seven clusters identified. NKT, natural killer T cell; Treg, regulatory T cell. (b) Distribution of the T-cell subtypes in the asthmatic and control groups. SEA, severe equine asthma. (c) Distribution of the T-cell subtypes for each horse. A, asthmatic; C, control. (d) Volcano plot illustrating the differential expression of genes between groups. Red dots represent DEGs with a *p*-value <0.05 and a log2 fold change >1. Dots positioned to the right of the plot represent genes that are upregulated in asthmatic horses.



Cluster	Annotation
Mo/Ma0	*FCN1*^{high} alveolar macrophages
Mo/Ma1	Undetermined alveolar macrophages
Mo/Ma2	ISG^{high} alveolar macrophages
Mo/Ma3	Intermediate monocytes
Mo/Ma4	Proliferating alveolar macrophages
Mo/Ma5	Double positive monocyte-lymphocyte cells

FIGURE 5 Legend on next page.

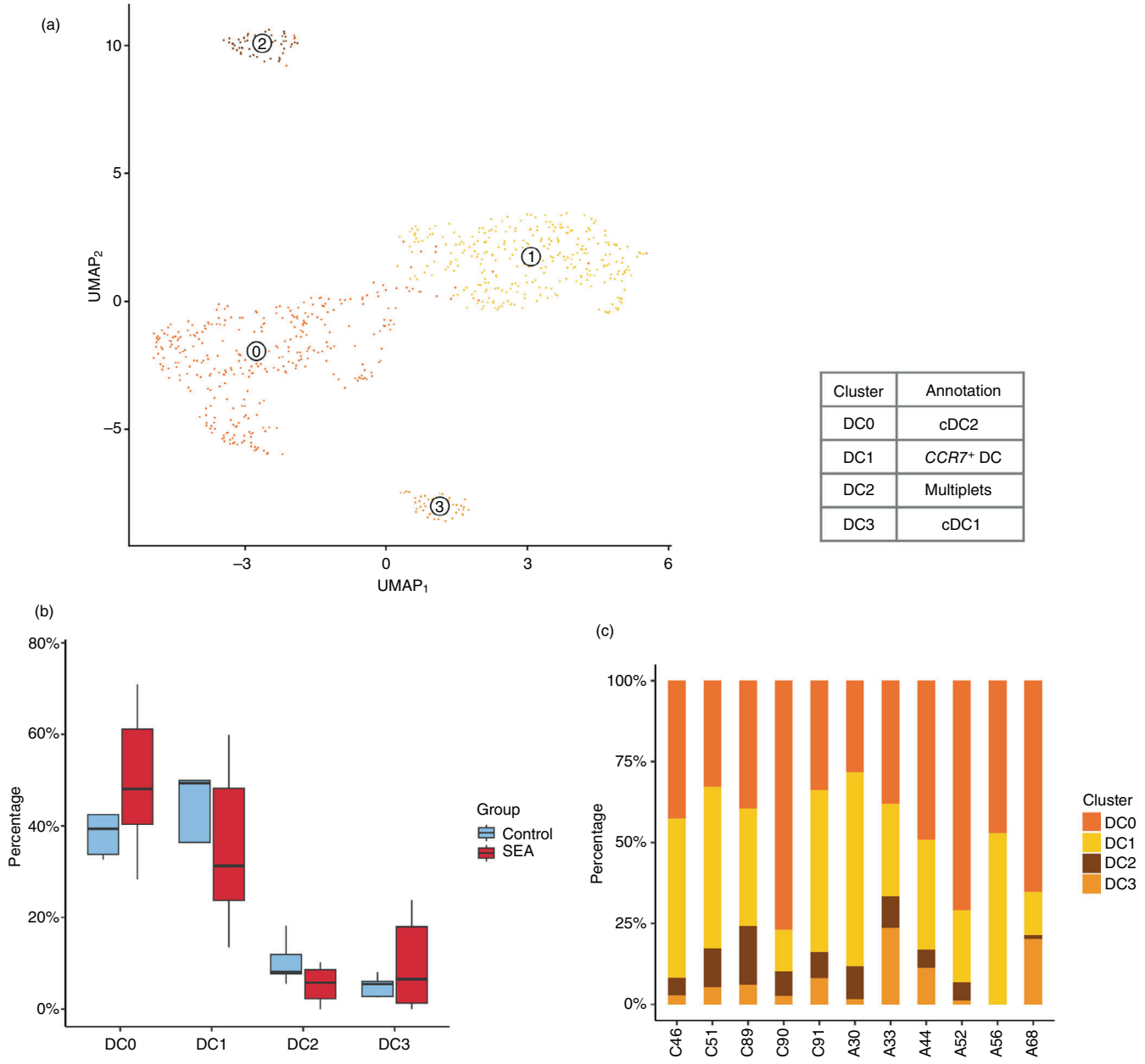


FIGURE 6 Dendritic cell (DC) subtypes identified in the bronchoalveolar lavage fluid of asthmatic and control horses using single-cell mRNA sequencing. (a) UMAP representation of the four clusters identified. cDC, conventional dendritic cell. (b) Distribution of the DC subtypes in the asthmatic and control groups. SEA, severe equine asthma. (c) Distribution of the DC subtypes for each horse. A, asthmatic; C, control.

upregulation of *GLRX2* and downregulation of *CCL8*. Administration of GLRX2 has been shown to reduce airway inflammation in an OVA-asthma model [59], indicating its potential protective function. CCL8 is responsible for the recruitment of basophils, eosinophils, and mast cells in allergic processes and contributes to

FIGURE 5 Monocytes–macrophages (Mo/Ma) subtypes identified in the bronchoalveolar lavage fluid of asthmatic and control horses using single-cell mRNA sequencing. (a) UMAP representation of the six clusters identified. ISG, interfero-n-stimulated genes. (b) Distribution of the Mo/Ma subtypes in the asthmatic and control groups. SEA, severe equine asthma. (c) Distribution of the Mo/Ma subtypes for each horse. A, asthmatic; C, control. (d) Volcano plot illustrating the differential expression of genes between groups. Red dots represent DEGs with a p-value <0.05 and a log2 fold change >1. Dots positioned to the right of the plot represent genes that are upregulated in asthmatic horses. NCBI 103 annotation for LOC100061699: S100A8.

TABLE 2 Proportions of the major cell types determined with scRNA-seq and compared between asthmatic (SEA) and control (CTL) groups.

Cell type	Number[a] ($N = 11$)	Mean % ($N = 11$)	Mean % (CTL, $N = 5$)	Mean % (SEA, $N = 6$)	Ratio SEA/CTL	*p*-Value	FDR
B cells	756	1.3	0.3	1.9	5.9	**<0.001**	**<0.001**
Neutrophils	5145	8.5	2.4	13	5.3	**<0.001**	**<0.001**
Mast cells	1232	2.0	1.7	2.3	1.4	0.13	0.25
Mo/Ma	22 370	37.1	44.6	32.0	0.7	0.18	0.27
Dendritic cells	754	1.3	1.2	1.4	1.2	0.80	0.93
T cells	30 005	50.0	50.0	49.4	1.0	0.93	0.93

Abbreviation: scRNA-seq, single-cell mRNA sequencing. *P*-values < 0.05 are indicated in bold.
[a]Post-quality filtering.

TABLE 3 Proportions of the B-cell subtypes identified with scRNA-seq and compared between asthmatic (SEA) and control (CTL) groups.

B-cell cluster	Mean % ($N = 11$)	Mean % (CTL, $N = 5$)	Mean % (SEA, $N = 6$)	Ratio SEA/CTL	*p*-value	FDR
B0	72.2	68.6	71.9	1.0	0.62	0.62
B1	20.5	11.5	21.3	1.8	0.18	0.26
B2	7.3	19.9	6.8	0.3	**0.02**	**<0.05**

Abbreviation: scRNA-seq, single-cell mRNA sequencing. *P*-values < 0.05 are indicated in bold.

airway allergic inflammation by promoting a Th2 immune response [60]. Hence, *CCL8* downregulation in cDC2s argues against a Th2 response in SEA.

Gene expression patterns of B cells and mast cells of asthmatic horses points to airway remodelling

S12/Table S9 provides the results of the DGE analysis for the major cell types, including B cells. The B cells of asthmatic horses upregulated *POU2AF1*, whose elevated expression has been associated with interstitial pulmonary fibrosis [61] and chronic obstructive pulmonary disease [62] in humans. Its expression negatively correlates with lung function [62]. Additionally, *YBX3* was downregulated in the mast cells of asthmatic horses. Reduced circulating *YBX3* mRNA is a sensitive predictor of idiopathic pulmonary fibrosis in humans [63].

DISCUSSION

SEA is characterized by neutrophilic inflammation in the lower airways, resembling a subset of non-Th2 human asthma. We utilized scRNA-seq to investigate the immune mechanisms underlying SEA. Among the six major cell types identified, B cells and neutrophils were more abundant in asthmatic horses. Notably, the fraction of activated (switched) plasma cells was decreased, indicating a non-Th2 response. Both T cells and Mo/Ma displayed a strong Th17 signature, including upregulation of *CXCL13* by intermediate monocytes. Furthermore, a subset of cells exhibited an expression profile indicative of monocyte–lymphocyte complexes potentially contributing to Th17 activation. Neutrophils showed an increase in NETosis function and reduced capacity to respond to Th17 signals. These findings support a primary Th17-mediated immune response in neutrophilic SEA, probably initiated through monocyte-T-cell crosstalk (Figure 7).

Similar Th17-associated responses have been observed in non-Th2 asthma in humans, including organic dust-induced asthma and a subset of non-Th2 asthma patients [8, 64]. Although SEA is traditionally considered a Th2-mediated disease, an increasing body of evidence suggests the involvement of Th17 inflammation in the pathological process. Increased levels of *IL17* mRNA have been observed in the BALF of horses with SEA following antigen challenge [13]. Dysregulation of miRNA in the serum of asthmatic horses supports the existence of a mixed Th2/Th17 response [15]. Furthermore, a comprehensive miRNA-mRNA study in equine lung tissues suggests a predominant Th17 pathway, along

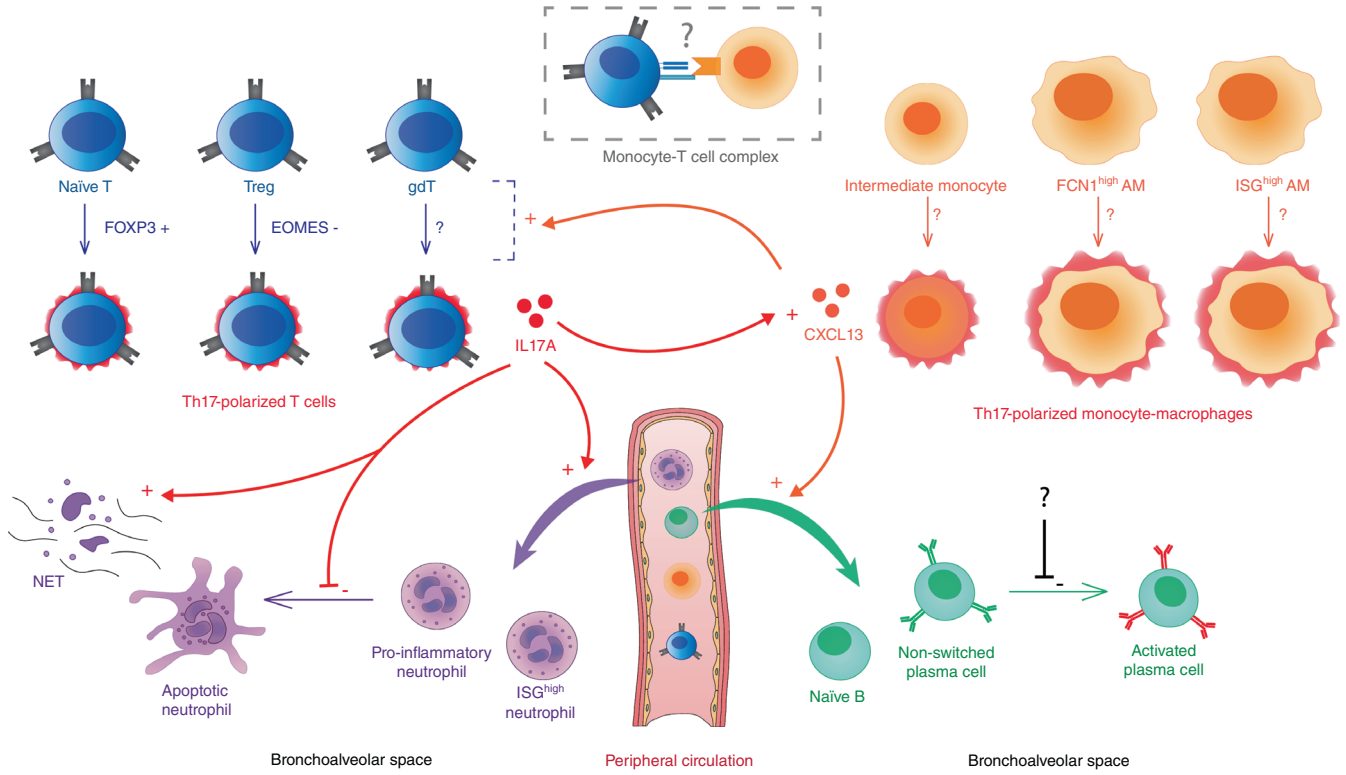


FIGURE 7 Th17-polarization of pulmonary immune response in severe equine asthma (SEA)-suggested pathways based on single-cell mRNA sequencing analysis of bronchoalveolar cells. Th17-polarized T cells and monocyte–macrophages fuel an inflammation loop where activated monocytes release CXCL13, promoting Th17 polarization of T cells. IL17A, released by activated T cells, further induces CXCL13 release. Reciprocal activation of T cells and monocytes may also occur via direct cell–cell contact (monocyte–T cell complexes). IL17A and CXCL13 recruit B cells and neutrophils, respectively, from peripheral blood. IL17A influences neutrophils by decreasing apoptosis and enhancing their capacity for NETosis. In SEA, there is a reduced activation of non-switched plasma cells producing IgM antibodies, resulting in a decreased pool of activated plasma cells necessary for a Th2 response. AM, alveolar macrophages; ISG, interferon interferon-stimulated genes; NET, neutrophil extracellular trap.

with some indications of a parallel Th2-type response [16]. Transcriptomics, proteomics, and tissue staining analyses of mediastinal lymph nodes in horses further support a predominant Th17 response in SEA [14]. Recently, T cells isolated from the BALF of horses with SEA demonstrated a Th17 polarization, as evidenced by an elevated frequency of IL17A-secreting lymphocytes following in vitro stimulation [18].

While studies on asthma have mainly focused on T cells [64], our study demonstrated the involvement of both T cells and Mo/Ma populations in driving Th17 inflammation in SEA. Importantly, this resulted from alterations in gene expression patterns rather than expansion of these cell populations. The upregulation of key Th17 cytokines such as *IL17A*, *IL21*, and *CCL20* was observed in T-cell clusters, suggesting their engagement in a Th17 differentiation pathway. Alveolar macrophages and intermediate monocytes also exhibited a strong Th17 signature, including *CXCL13* upregulation. CXCL13 cytokine levels are elevated in the serum and the BALF of asthmatic humans [65, 66]. An anti-CXCL13 antibody reduced inflammation in an asthma mouse model, highlighting CXCL13 as a promising therapeutic target [65]. This B-cell chemoattractant predominantly produced by Mo/Ma and Th17-derived cells [67] is also upregulated in hay dust extract-stimulated PBMCs of asthmatic horses [17]. Because the latter study was performed on a cell mixture, the cellular origin of the increased *CXCL13* expression could not be ascertained. Our single-cell data indicated activated monocytes as the main source of *CXCL13*, most likely induced by IL17 released from T cells [68]. Activated monocytes could in turn induce Th17 differentiation of T cells [69–71]. Collectively, our results support a crosstalk between *IL17A*-producing T cells and *CXCL13*-producing monocytes in the context of a Th17-mediated immune response in SEA (Figure 7).

Of particular interest was the cluster Mo/Ma5 expressing both lymphocytes and monocytes markers, a transcriptomic profile previously observed in other equine BALF scRNA-seq studies [19, 72]. The presence of monocyte–T cell interactions has been reported in

human blood, with the frequency and phenotype of these cell–cell complexes varying depending on the immune response polarization [73]. Considering that the crosstalk between monocytes and T cells plays a key role in the development of various human inflammatory diseases [69–71], the potential presence of bona fide monocyte–lymphocyte complexes in the lower airway compartment is particularly intriguing. The reciprocal activation of monocytes and lymphocytes may occur through direct cellular contact rather than solely through endocrine or paracrine mechanisms.

In contrast to previous reports (reviewed in Refs. [6, 10, 74]), we did not detect a Th2 or Th1 signature in the cells from asthmatic horses. Notably, we did not observe upregulation of characteristic Th2 and Th1 cytokines such as *IL4*, *IL13*, or *IFNγ*, which aligns with the results of an equine BALF flow cytometric study [18]. Consistent with our findings, Th2 and Th17-associated gene expression seems to be regulated in opposite direction in the human airways [75]. In SEA-affected horses, downregulation of *IL4* correlates with increased IL17 staining intensity in the mediastinal lymph nodes [14]. Moreover, Th1- and Th2-associated genes are downregulated in antigen-challenged PBMCs from asthmatic horses [17]. The reduced fraction of activated plasma cells in our study population further argued against a Th2 response. Upon antigen stimulation, non-switched IgM-producing plasma cells become activated and produce immunoglobulins of other classes, a prerequisite for Th2 responses. While switched plasma cells were less frequent in asthmatic horses, the proportion of total B and plasma cells was significantly higher, likely due to *CXCL13* signalling [68]. Consequently, asthmatic horses have a larger pool of B cells, which can potentially differentiate into plasma cells and be activated. This could explain the increased susceptibility of asthmatic horses to certain Th2-associated diseases, such as insect bite hypersensitivity and urticaria [11]. Overall, our findings indicate that SEA is driven primarily by a Th17-mediated immune response characterized by an *IL17*-induced *CXCL13*-mediated recruitment of B cells into the lower airways (Figure 7), potentially predisposing asthmatic horses to secondary Th2-type responses.

The transcriptomic profile of T cells suggested alterations in T-cell function, including T-cell exhaustion, unresponsiveness of Treg cells, and reduced cytotoxicity in NKT cells. It remains unclear whether these dysregulations are associated with the Th17 polarization of the T-cell population, or if they represent independent mechanisms. Nevertheless, these alterations in T-cell function may potentiate the abnormal immune response observed in SEA.

Neutrophils are short-lived cells that persist in the lower airways of asthmatic horses due to IL17-induced influx and reduced apoptosis [76]. Neutrophil apoptosis is sometimes accompanied by the formation of NETs, which can trigger tissue damage and sustain chronic inflammation [76]. The observed dysregulation of NETosis-associated genes conforms to the previous observations of excessive NETosis in the lungs of severely asthmatic horses [77]. Conversely, several DEGs indicated an anti-inflammatory phenotype, particularly in the apoptotic neutrophil subtype, potentially representing a protective mechanism against excessive inflammation. In summary, our findings confirm that BALF neutrophils from SEA-affected horses have a significant pro-inflammatory effect through increased neutrophil persistence and facilitated NET formation in the lungs. The concomitant anti-Th17 transcriptomic profile observed in apoptotic neutrophils suggests a parallel attempt to mitigate lung inflammation. Hence, neutrophils seem to act as effectors rather than primary instigators of asthmatic lung inflammation. Targeting treatment specifically towards the pro-inflammatory neutrophil subtype could disrupt the self-perpetuating inflammatory circle while preserving the antimicrobial functions of other neutrophil subtypes.

Employing scRNA-seq on equine BALF cells elucidated important underlying immune mechanisms of SEA, yet this study had limitations. One significant challenge when studying horses is the inadequate quality of the current reference annotation, necessitating manual annotation of the cell clusters, particularly for poorly defined cell subtypes. Nonetheless, the detection of previously identified cell types and subtypes in equine BALF [19, 72] supports the reproducibility of our annotation. Some clusters, such as the ‘undetermined AMs’ cluster, could not be confidently annotated. Further scRNA-seq studies and complementary techniques are required for conclusive insights.

ScRNA-seq is a relatively new technology that comes with computational challenges. One such challenge is the ability to detect and filter technical multiplets without removing biologically significant signals representing cell–cell complexes or new cell types with a dual lineage signature. In this study, we hypothesized that cluster Mo/Ma5 represented bona fide monocyte–lymphocyte complexes, supported by the presence of a similar transcriptomic signature in equine BALF cells [19, 72] and human PBMCs [73, 78]. Although the existence of cellular complexes was confirmed in human PBMCs using imaging flow cytometry [73, 78], validation in horses has yet to be performed. Another potential limitation associated with the 10× Genomics droplet-based technique is its low sensitivity for genes with a low average expression, which could explain

the discrepancies with previous bulk RNA or proteomics studies, such as the absence of upregulated Th1 and Th2-associated cytokines.

As this study focused on neutrophilic SEA, results may not apply to other asthma subtypes. This is exemplified by a previous scRNA-seq study on BALF cells from horses with mastocytic asthma [72], which exhibited a different transcriptomic signature. For example, *FKBP5* was significantly upregulated in mast cells, a gene that we did not detect in our dataset. Moreover, a recent flow cytometric analysis of BALF with functional assays on T cells provided further evidence that distinct mechanisms exist among the various forms of equine asthma [18]. Therefore, studying different endotypes separately is crucial to obtain meaningful results.

CONCLUSION

The presented scRNA-seq analysis of equine bronchoalveolar cells provides insights into the major immune mechanisms underlying SEA. The use of scRNA-seq allowed us to overcome the influence of varying cell type distribution associated with the disease and to gain unprecedented resolution into the pathophysiology of SEA. This represents a significant breakthrough, challenging the prevailing perception of SEA as a Th2-associated disease. We identified the crucial role of monocytes in initiating the Th17 response in the lungs. The upregulation of *CXCL13* in lung and blood monocytes suggests its potential as a SEA biomarker and therapeutic target. Our findings indicate that monocyte activation may occur through direct cell–cell contact, a hypothesis that should be tested using imaging flow cytometry. This could reshape our understanding of immunotherapy approaches. Therapies targeting Th17-associated cytokines have proven ineffective in reducing symptoms in human asthma [64]. One possible approach could be to prevent monocyte activation by targeting monocyte-T-cell synapses. Our results demonstrate several parallels with previous studies on non-Th2 neutrophilic asthma in humans, further validating the horse as a valuable model for studying human asthma.

AUTHOR CONTRIBUTIONS

Sophie E. Sage, Tosso Leeb, and Vinzenz Gerber designed the study. Funding was acquired by Vinzenz Gerber. Sophie E. Sage collected and processed the samples. Sophie E. Sage and Vidhya Jagannathan performed the computational analysis. Sophie E. Sage wrote the original draft. All authors reviewed, edited, and approved the final version of the article.

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CONFLICT OF INTEREST STATEMENT

Authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available in the European Nucleotide Archive (ENA) repository https://www.ebi.ac.uk, under the accession number PRJEB51962. The R code used for data analysis can be found at https://github.com/vetsuisse-unibe/ScRNA-seq_BALF_SEA.

ETHICS STATEMENT

All animal experiments were performed according to the local regulations and with the consent of horse owners. This study was approved by the Animal Experimentation Committee of the Canton of Bern, Switzerland (BE4/20+).

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SUPPORTING INFORMATION

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