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Cytotoxic Non-T_H2 CD4+ T Cells are associated with Asthma Severity

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Abstract

Patients with severe uncontrolled asthma represent a distinct endotype with persistent airway inflammation and remodeling that is refractory to corticosteroid treatment. CD4+ T_H2 cells play a central role in regulating asthma pathogenesis, and biologic therapies targeting their cytokine pathways have had promising outcomes. However, not all patients respond well to such treatment, and their effects are not always durable nor reverse airway remodeling. This observation raises the possibility that other CD4+ T cell subsets and their effector molecules may drive airway inflammation and remodeling. We performed single-cell transcriptome analysis of >50,000 airway CD4+ T cells isolated from Bronchoalveolar Lavage (BAL) samples from 30 patients with mild and severe asthma. We observed striking heterogeneity in the nature of CD4+ T cells present in asthmatics' airways with Tissue-Resident Memory (T_{RM}) cells making a dominant contribution. Notably, in severe asthmatics, a subset of CD4+ T_{RM} cells (CD103-expressing) was significantly increased, comprising nearly 65% of all CD4+ T cells in the airways of male patients with severe asthma when compared to mild asthma (13%). This subset was enriched for transcripts linked to T Cell Receptor (TCR) activation (*HLA-DRB1*, *HLA-DPA1*) and cytotoxicity (*GZMB, GZMA*) and, following stimulation, expressed high levels of transcripts encoding for pro-inflammatory non-T_H2 cytokines (CCL3, CCL4, CCL5, TNF, LIGHT) that could fuel persistent airway inflammation and remodeling. Our findings indicate the need to look beyond the traditional T2 model of severe asthma to better understand the heterogeneity of this disease.

Keywords: CD4+ tissue-resident memory T cells • Airway T cells • Asthma • Cytotoxic CD4+ T cells • Severe asthma • Single-cell RNA sequencing • Translation to patients

Introduction

Asthma is one of the most common chronic inflammatory diseases affecting both children and adults which is characterized by coughing, wheezing, shortness of breath, and chest tightness [1,2]. These classical symptoms are triggered by the inflammation of the airways which results in increased mucus production, remodeling of the airways, bronchial hyperresponsiveness, and airway obstruction [1]. This airway inflammation has been previously associated with CD4+ T Helper 2 (T_H2) cell responses and their cytokines (interleukin [IL]-4, IL-5, IL-9, and IL-13) which are involved in the recruitment and activation of high number of innate immune cells such as eosinophils, basophils, and mast cells [1,3-5]. While current treatments aiming to suppress airway inflammation focus on the use of corticosteroids or/ and, more recently, biological agents blocking type 2 cytokines or Immunoglobulin E (Ig E), many patients with severe forms of asthma fail to respond to these treatments and suffer from persistent symptoms and recurrent life-threatening exacerbations [3,6-11]. Recent studies performed in airway specimens from asthmatic patients have also implicated other effector CD4+ T cell subsets with asthma pathogenesis [1,12-20]. However, due to the difficulty in sample collection from severe asthmatics and limitations in the number of cells available for research, unbiased studies providing a precise characterization of CD4+ T cell subsets associated with asthma severity are limited.

Literature Review

In our study (Herrera-De La Mata et al., Med 2023), we specifically focused on identifying differences in the properties of CD4+ T cells

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present in the airways of patients with severe asthma. Therefore, we collected Bronchoalveolar Lavage (BAL) samples from severe asthmatic patients (n=16, 50% male) from the Wessex AsThma CoHort of difficult asthma (WATCH) study [21], which has extensively characterized patients with severe asthma requiring 'high dose' and/or 'continuous or frequent use of oral corticosteroid', in accordance with Global Initiative of Asthma (GINA) management steps 4 and 5 [22,23]. We also collected BAL samples from mild asthmatic patients (n=14, 64% male) as controls.

Using an oil-droplet-based single-cell RNA-sequencing platform (10 x Genomics), we performed an unbiased clustering analysis of airway $CD4^+$ T cells (n=27,771), which revealed eight transcriptionally distinct subsets. Among this heterogeneity, we identified two clusters that accounted for the majority (71%) of $CD4^+$ T cells present in the airways of these asthmatic patients and that were significantly enriched for Tissue-Resident Memory (T_{RM}) signature genes [20,24,25], and thus were defined as T_{RM} cells. Even though both T_{RM} clusters expressed high levels of the T_{RM} marker gene *CD69* [25], they differed in the expression of another T_{RM} marker gene, *ITGAE*, encoding for the alpha chain of the integrin CD103, a transmembrane protein which is required for the adhesion of T cells to E-cadherin expressed by epithelial cells [24,26,27]. Hence, based on the expression of these T_{RM} markers, we referred to these clusters as $CD103^+$ T_{RM} and $CD103^-$ T_{RM}.

Interestingly, when we looked at the quantitative differences of these subsets between disease severity, we observed that the proportions of the $CD103^+$ T_{RM} cluster were significantly increased in the airways of patients with severe asthma compared to mild asthma (46% *versus* 21%), while the proportions of the $CD103^-$ T_{RM} subset were significantly decreased (22% *versus* 44%). Moreover, we observed that there was an influence of biological sex on the composition of airway $CD4^+$ T cells in severe asthma, as the increase in the proportions of the $CD103^+$ T_{RM} cluster was significant only when comparing male patients with severe and mild asthma (64% *versus* 13%), while the proportions of the $CD103^-$ T_{RM} cluster were significantly lower in males with severe asthma. An unbiased Spearman correlation analysis also determined a positive correlation in male but not female asthmatics between the proportions of the $CD103^+$ T_{RM} cluster and several clinical and physiological parameters related to asthma severity, such as a composite asthma severity score adapted from the Asthma Severity Scoring System (ASSESS) [28] ranging from 0-20 from the Severe Asthma Research Program (SARP) [29] (r_S=0.8 and P<0.01), and severity of airflow obstruction (using post-bronchodilator FEV_1 and FEV_1/FVC) (r_S=0.7 and P<0.01).

Next, to determine the differences among the molecular properties between both T_{RM} subsets, we performed a Differential Gene Expression Analysis (DGEA) between cells in the $CD103^+$ T_{RM} cluster and cells in the $CD103^-$ T_{RM} cluster across all patient groups and using sex as a covariate. The $CD103^+$ T_{RM} cluster showed a significant enrichment of genes involved in cytotoxic function (*GZMB, GZMA, GZMH, FASLG*) [30] together with an increased expression of transcripts encoding for transcription factors linked to cytotoxicity in T cells: HOBIT (*ZNF683*), which is linked to T_{RM} differentiation and persistence of cytotoxic effector T cells, [24,31,32] and *HOPX*, known to regulate *GZMB* expression and to increase *in vivo* persistence of T_H1 cells [33,34]. We also observed a significant upregulation of genes associated with T Cell Receptor (TCR) signaling (*HLA-DRB1, HLA-DRB5, HLA-DRA*), suggesting that the $CD103^+$ T_{RM} subset may be enriched for T cells that were recently activated in the asthmatic airways due to the association of HLA-DR expression with T cell activation following *in vivo* antigen-specific TCR engagement [35,36]. A number of transcripts encoding for several cytokines (IFN-γ, TNF, LIGHT/TNFSF14) and chemokines (CCL4/MIP-1β, CCL5/RANTES) linked to airway inflammation and remodeling [37-39] were also significantly expressed in the $CD103^+$ T_{RM} subset. Furthermore, when we performed a DGEA of $CD4^+$ T cells from severe *versus* mild asthmatics for each sex and per cluster, we observed that the most differentially expressed genes increased in severe asthma were significantly enriched in male patients, especially those linked to cytotoxicity (*GZMB, GZMH, ZNF683, HOPX, CCL4*) which were differentially expressed only in the $CD103^+$ T_{RM} subset.

However, the $CD103^-$ T_{RM} cluster showed a significant enrichment of several transcripts encoding for molecules known to dampen TCR signaling and effector functions in T cells (CREM, DUSP1, DUSP2, DUSP4, TNFAIP3) [40-43]. One of the most upregulated differentially expressed genes was *CREM* (Cyclic AMP Responsive Element Modulatory), which encodes for a transcription factor known to repress promoters of inflammatory cytokine genes like *IL2*, *IL3*, *IL4* and to dampen type 2 inflammation [41].

Finally, we stimulated *ex vivo* a fraction of BAL cells with Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin for 2 hours, and performed single-cell RNA-sequencing on sorted $CD4^+$ T cells to assess the effector potential of airway $CD4^+$ T cells from patients with mild and severe asthma. We observed that transcripts encoding for chemokines known to be released by cytotoxic $CD4^+$ T cells like CCL3, CCL4, and CCL5 that play key roles in the recruitment of several immune cell types, [30,44-46] and therefore have the potential to drive airway inflammation and remodeling, were significantly expressed in stimulated airway $CD4^+$ T cells from patients with severe asthma compared to mild asthma. In addition, transcripts associated with T_H1 effector (*IFNG*, *TNF*, *FASLG*, *XCL1*, *XCL2*), and pro-fibrotic (*LIGHT*, *AREG*, *TGFB1*) properties were also upregulated by stimulation. Notably, we observed that several of these pro-inflammatory cytokines and chemokines contributing to airway inflammation, fibrosis, and remodeling co-expressed with canonical cytotoxicity-associated maker genes such as *GZMB*. However, only a relatively small fraction of cells expressed T_H2 and T_H17 cytokine transcripts and they were significantly reduced in patients with severe asthma compared to mild asthma. Overall, these findings supported the existence of a cytotoxic non-T_H2 $CD4^+$ T_{RM} population with effector potential present in the airways of severe asthmatics.

Conclusion

The findings from our study highlight the heterogeneity of the disease and the importance to look beyond the T2-paradigm when categorizing patients purely through a T2 High/Low lens to better stratify severe asthma diversity. The identification of airway cytotoxic $CD4^+$ T cells as a potentially important driver of asthma pathogenesis in a subgroup of severe asthmatic patients also supports the need to explore other asthma biomarkers different than the current ones to develop effective therapies, mostly for patients that are unresponsive to the current available asthma treatments.

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