

Mass cytometry-based identification of a unique T-cell signature in childhood allergic asthma

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July 27, 2021

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Short title: T-cell signature in paediatric AA

Key words:

allergic asthma, paediatric, clinical disease parameters, CyTOF, T-cell subpopulations

To the Editor,

Allergic asthma in childhood is characterized by a dominance of type 2 immunity driven by CD4⁺ T helper 2 (Th2) cells expressing the transcription factor (TF) GATA3 and inefficient counter-regulation by Tregs among other mechanisms.¹ However, a detailed analysis of T-cells associated with paediatric AA is still needed.

To explore T-cell phenotypes associating with paediatric AA, we applied a 42-antibody mass cytometry panel in combination with unsupervised computational analyses in a cohort of well-characterized 14 treatment-naïve AA and 9 healthy children (HC) from the CLARA/CLAUS study (Table S1,S2 Figure S1A).² Integrating information from 12 lineage-markers we identified seven major T- and NK-cell populations within peripheral blood mononuclear cells (PBMC) (Figure S1B,C) of which CD8⁺ T-cell abundance was reduced with underrepresented memory compartment in AA vs HC (Figure 1A,S1D,E).

Accordingly, the CD4⁺/CD8⁺ T-cell ratio was elevated in AA vs HC and correlated with blood eosinophil frequencies, a determinant of AA severity³, in AA but not in HC (Figure 1B,C,S1F). To address potential disease-associated changes within the CD4⁺ T-cell compartment, we selected 30 markers for subsequent clustering using FlowSOM algorithm. Two clusters, cluster_c6 and cluster_c30, were expanded in AA vs HC (Figure 1D,E,S1G,H). Cluster CD4_c6 represented Th2 cells (Figure 1F), since it expressed the Th2-specific TF GATA3 and chemokine receptors CRTH2 and CCR4.⁴ It uniquely co-expressed TIGIT and ICOS, which was not reported in AA before and could be specific for childhood, since *TIGIT* hypomethylation has been described only in paediatric AA.⁵ Manual gating confirmed higher abundance of ICOS⁺TIGIT⁺Th2-cells in AA vs HC (Figure S2A-C) and revealed increased CD161 expression characterising Th2-cells restricted to atopic adults,⁶ thereby underpinning their pro-allergic nature. The frequency of the TIGIT⁺ICOS⁺Th2-cluster, correlated with eosinophilia in AA with allergic comorbidities and also with CD4/CD8 T-cell ratio in

asthmatics having intermittent disease symptoms, while inversely in stable disease (Figure 1G,H), suggesting its association with more symptomatic disease and with allergic comorbidity linked to eosinophilia.

Cluster CD4_c30 expressing markers characterizing naïve/resting Tregs (Figure S2D) matched the previously described CD45RA⁺FOXP3^{low} Treg fraction(Fr)-I,⁷ while effector (e)Tregs represented Fr-II (CD45RA⁺FOXP3^{high}) and Fr-III (CD45RA⁺FOXP3^{low}) (Figure 1I). Accordingly, the frequencies of Fr-I were enriched in AA vs HC, while Fr-II and Fr-III were similar (Figure S2E). Cluster CD4_c30 abundance tended to correlate inversely with lung function, and significantly with memory CD8⁺ T-cell frequencies (Figure 1J,S2F), indicating its partial connection to lung function and CD8⁺ T-cell alterations. The abundances of Fr-I vs Fr-II correlated inversely (Figure 1K), consistent with the linear developmental model⁷, suggesting a differentiation-block from Fr-I towards Fr-II, and a possible altered eTreg compartment.

Therefore, we next analyzed manually gated eTregs (Figure S1B), by FlowSOM clustering, which revealed an underrepresented cluster_c2 and partially cluster_c10 in AA (Figure 2A,B,S2G). Considering a possible eTregs impairment and an overrepresentation of TIGIT⁺ICOS⁺Th2-cells (CD4_c6), we asked if the two phenomena are connected. Indeed, the TIGIT⁺ICOS⁺ Th2/eTregs ratio tended to be higher in children with AA versus HC, but failed to associate with eosinophilia in AA and in HC, whereby eosinophilia was markedly lower in HC vs AA (Figure 2C,D,S2H). In contrast, TIGIT⁺ICOS⁺ Th2-cell ratio to eTreg_c2 and eTreg_c10 correlated significantly in AA but not in HC, suggesting a specific relation between eTreg_c2 and eTreg_c10 underrepresentation and TIGIT⁺ICOS⁺Th2-associated eosinophilia in paediatric AA. Thus, eTregs (eTreg_c2 and eTreg_c10) and naïve/resting Tregs (CD4_c30) are linked to two different pathological features of AA, to the eosinophilia and in part to the lung function, respectively.

Next, we performed principal component analysis (PCA) based on significantly changed ratio and subset-frequencies, which separated AA from HC children at the first PC, indicating that the detected dysbalanced T-cell composition allows a discrimination between these two groups (Figure 2E). Additionally, ROC analyses revealed a relation of resting/naïve Tregs (CD4_c30), eTreg_c2 and eTreg_c10 to the paediatric AA phenotype (sensitivity, true positive rate) (Figure 2F), further supporting the relevant involvement of the Treg dysbalance in childhood AA.

Summarizing, our approach identifies a unique T-cell signature of childhood AA and provides insights for pathophysiological involvement of dysbalanced Tregs, TIGIT⁺ICOS⁺ Th2 and memory CD8⁺ T-cells. This can be useful for immunomonitoring, immunomodulation and for confirmatory larger studies in childhood AA.

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Acknowledgments

This work was generously supported by the Else Kröner-Fresenius-Stiftung (Research group: “Functional, therapy-aimed clustering of T-cell sub-phenotypes across plasticity”). MH is also supported by the Deutsche Forschungsgemeinschaft (DFG) (HU-1824/9-1, HU-1824/7-1, HU-1824/5-1). BS is supported by grants from BMBF 01GL1742A, DFG (DFG-SCHA 997/3-1, DFG-SCHA 997/7-1, DFG-SCHA 997/8-1), from SFB TR22, grants Comprehensive Pneumology Center. JT is supported by grants from the DFG (DFG-SCHA 997/7-1). AB is supported by grants from BMBF (01GL1742A).

Conflict of interest

HR, ARS, JT, AJRO, AB, WB, BTS, HRC, ML, HDC, BS, HEM and MH have nothing to disclose.

Figure Legends

Figure 1. Dysbalanced CD4⁺ and CD8⁺ T-cell compartment in paediatric AA. **A.** T- and NK-cell frequency based on manually gated t-SNE plot (S1B, gates 1-8). **B.** CD4/CD8 T-cell ratio. **C, G, H, J, K** Linear regression analysis in AA children of: (C, G) blood eosinophil frequency versus (C) CD4/CD8 T-cell ratio, (G) cluster_c6 frequency with/without comorbidity, (H) CD4/CD8 T-cell ratio versus c6(TIGT⁺ICOS⁺) with intermittent symptoms or stable disease, (J) cluster_c30 frequency versus percent-predicted FEV1/FVC, (K) Fraction(Fr)-I versus Fr-II frequency, which were manually gated (I). **D.** tSNE-visualisation of 30 CD4⁺ T-cell clusters. **E.** Cluster_c6 and cluster_c30 frequency. **F.** Cluster_c6 mean marker expression. **I.** Dot-plot displaying CD4⁺ T-cell manual gating for Treg-Fr-I-III with cluster_c30 and manually gated eTregs (S1B, gate 8) overlay. A, P -value by one-way ANOVA with Benjamini-Hochberg adjustment. B, E. P -value by Mann-Whitney test.

Figure 2. Integrated T-cell signature distinguishes children with AA from HC. **A.** eTregs tSNE-visualization with cluster_c2 and cluster_c10. **B.** eTregs cluster_c2 and cluster_c10 frequency. **C.** Cluster_c6TIGT⁺ICOS⁺/eTreg ratio. **D.** Linear regression analysis of the respective ratios versus blood eosinophil

frequency in AA children. **E.** Principal component analysis of the AA and HC samples based on the significantly regulated features (CD8⁺ T-cells, CM CD8⁺ T-cells, CD4⁺ T-cell_c6, CD4⁺ T-cell_c30, eTreg_c2, eTreg_c10 and CD4/CD8 T-cell ratio). **F.** Receiver operating characteristic (ROC) curves were calculated for CD4⁺ T-cell cluster_c30, eTreg cluster_c2 and eTreg_c10. B,C (*P* value by Mann-Whitney test).



