



Mass cytometry analysis identifies T cell immune signature of aplastic anemia and predicts the response to cyclosporine

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Abstract

Aplastic anemia (AA) is an auto-activated T cell-mediated bone marrow failure. Cyclosporine is often used to treat non-severe AA, which demonstrates a more heterogeneous condition than severe AA. The response rate to cyclosporine is only around 50% in non-severe AA. To better predict response to cyclosporine and pinpoint who is the appropriate candidate for cyclosporine, we performed phenotypic and functional T cell immune signature at single cell level by mass cytometry from 30 patients with non-severe AA. Unexpectedly, non-significant differences of T cell subsets were observed between AA and healthy control or cyclosporine-responder and non-responders. Interestingly, when screening the expression of co-inhibitory molecules, T cell trafficking mediators, and cytokines, we found an increase of cytotoxic T lymphocyte antigen 4 (CTLA-4) on T cells in response to cyclosporine and a lower level of CTLA-4 on CD8⁺ T cells was correlated to hematologic response. Moreover, a decreased expression of sphingosine-1-phosphate receptor 1 (S1P₁) on naive T cells and a lower level of interleukin-9 (IL-9) on T helpers also predicted a better response to cyclosporine, respectively. Therefore, the T cell immune signature, especially in CTLA-4, S1P₁, and IL-9, has a predictive value for response to cyclosporine. Collectively, our study implies that immune signature analysis of T cell by mass cytometry is a useful tool to make a strategic decision on cyclosporine treatment of AA.

Keywords Aplastic anemia · Mass cytometry · CTLA-4 · S1P₁ · IL-9 · Cyclosporine

Introduction

Aplastic anemia (AA) is a bone marrow failure syndrome attacked by auto-activated T cell on autologous bone marrow hematopoietic progenitors, which is characterized by pancytopenia [1–3]. Non-severe AA (non-SAA) with a variably clinical feature accounts for 80% of AA [4, 5]. Up to two-thirds of patients would finally progress to transfusion dependence or severe AA (SAA) without intervention [6–8]. Although cyclosporine, which has effect on reducing T cell proliferation and activation, can reverse pancytopenia and alleviate transfusion requirements, there still exist 50% of patients who fail to respond and 35% relapse after responding [9–11]. Additional novel therapeutic approaches are required for AA patients who fail to achieve response, and along with this, more robust diagnostic examinations that predict response to cyclosporine are necessary.

Mechanisms of T cell-mediated bone marrow failure include excessive production of inhibitory cytokines conferred by Th1 polarization, Th17 immune response,

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and direct toxicity to hematopoietic cells [12–14]. Previous researches also have revealed a decreased number and impaired function of regulatory T cells (Tregs) in AA [15–17]. Furthermore, a wide variety T cell immune checkpoint molecules have been identified to regulate T cell activation, including increased expression of T cell immunoglobulin mucin-3 (TIM-3) and programmed death-1 (PD-1) in AA [18–20]. Moreover, the sphingosine-1-phosphate receptor (S1PR) signaling is related to various physiological processes including T lymphocyte recirculation and their biology [21, 22]. Cytokines also play a crucial role in driving immune responses and HSPC growth inhibition and apoptosis [23]. Interleukin-9 (IL-9) is a pleiotropic cytokine secreted by T helpers. This growth factor not only is associated with the pathogenesis of autoimmune diseases but also regulates hematopoiesis [24–26]. Altogether, T cell function and activation are critical to regulate the pathophysiology of AA [27, 28]. Thus, we hypothesize that defining T cell immune signature will facilitate the development of predicting cyclosporine response and exploring novel immune-based therapeutics.

In this study, a panel of surface and intracellular markers associated with T cell phenotype and function was designed, and the complexity of T cell subsets was examined by mass cytometry. This study enabled us to identify the T cell signature in AA patients and explore immune biomarkers for predicting the response to cyclosporine treatment.

Materials and methods

Patients' and healthy donors' samples

A total of thirty patients with acquired non-SAA from Institute of Hematology and Blood Diseases Hospital were enrolled in the study. Inclusion criteria were predefined as follows: (1) meet the international study group criteria for AA [29]; (2) did not meet the criteria of SAA; and (3) obtained consent to cyclosporine. Exclusion criteria were defined as follows: (1) inherent bone marrow failure; (2) suffer from other hematological disorders or autoimmune diseases; (3) received an immunosuppressive agent before; and (4) suffer fever or infection within 4 weeks before diagnosis. Based on disease severity [30, 31], patients were classified into transfusion dependent (defined as red blood cell or platelet transfusion dependence or neutrophil count $< 0.5 \times 10^9/L$) and transfusion independent.

Each patient received oral cyclosporine at 3–5 mg/kg daily. Dose adjustment was based on the serum level of cyclosporine which was maintained between 100 and 200 ng/mL. According to established criteria [29], response to cyclosporine was evaluated in the first 6 months after starting therapy. Partial response (PR) included transfusion

independent (if previously dependent on transfusion), or at least one cell lineage doubling or reach to the normal level, or increase of hemoglobin concentration above 30 g/L (if initially less than 60 g/L), absolute neutrophil counts $> 0.5 \times 10^9/L$ (if initially less than $0.5 \times 10^9/L$), and platelets $> 20 \times 10^9/L$ (if initially less than $20 \times 10^9/L$). Complete response (CR) was defined as hemoglobin concentration recovered to be normal, neutrophil count $> 1.5 \times 10^9/L$, and platelet count $> 100 \times 10^9/L$. Patients with no increase of blood cell or still transfusion dependent or progressed to SAA were defined as no response (NR).

Four healthy males were enrolled, with the median age of 44.5 years old (range from 16 to 50 years old). Routine blood tests were done before collecting bone marrow samples, and all of them had normal levels of hemoglobin (range from 145 to 160 g/L), platelets (range from 161 to $244 \times 10^9/L$), and absolute neutrophil counts (range from 2.1 to $2.75 \times 10^9/L$).

Bone marrow specimens were collected from participants including AA patients and healthy volunteers. Bone marrow mononuclear cells were isolated by Ficoll-Hypaque (1.077 g/mL) density gradient centrifugation, frozen in cryoprotectant, and stored at liquid nitrogen until laboratory analysis.

Mass cytometry staining and acquisition

A panel of antibodies based on surface and intracellular markers was designed (see supplemental Table 1). Each antibody was labeled with a rare metal isotope. The CyTOF mass cytometer (Fluidigm) was used for data acquisition. Forty-one bone marrow samples were tested, including thirty of patients without any treatment, seven of post cyclosporine, and four healthy individuals.

After 72-h stimulation with phorbol myristate acetate (50 ng/mL) and ionomycin (1 $\mu\text{g/mL}$), cells were collected and stained with metal-tagged antibodies. Samples were processed by six batches separately; the same protocol and culture conditions were done to minimize the variety from stimulation and staining. The protocol was referred to previous report, detailed steps are listed in supplementary methods. Cisplatin solution staining was used for identifying dead cells. After staining with metal-coupled surface antibody cocktail, nuclear antigen staining perm working solution was added for fixation and permeabilization. Then, samples were stained with antibody cocktail of cytokines and transcription factors. Next, 1.6% paraformaldehyde was used to fix cells, and 1 mL of intercalation solution was added for each sample by adding cell-ID Intercalator-Ir into fixation and permeabilization buffer to a final concentration of 125 nM and vortex to mix. To prepare cells for acquisition, cells were adjusted to $0.5\text{--}2 \times 10^6/mL$ in double-distilled H_2O and filtered by a 35–70- μm mesh. EQ beads were added prior to data acquisition by using a CyTOF mass cytometer. Data was exported in a format of flow cytometry file for further analysis.

Referred to previous report [32], CD45RA⁺CCR7⁺ defined naive T, CD45RA⁻CCR7⁺ defined central memory T, CD45RA⁻CCR7⁻ defined effector memory T, CD45RA⁺CCR7⁻ defined effector T, CD38⁺HLA-DR⁺ defined activated T, CD4⁺CXCR3⁺CCR6⁻ defined Th1, CD4⁺CXCR3⁻CCR6⁺ defined Th17, CD4⁺CXCR3⁻CCR6⁻ defined Th2, and CD4⁺CCR4⁺CD25⁺Foxp3⁺ defined Tregs.

Statistical analysis

All analyses were performed using Cytobank software and Prism 8.0.1. Data were presented as median or mean \pm standard error mean (SEM). Mann–Whitney test and *t* test were used for assessing the significance of differences. It was considered to be statistically significant with the value of $p < 0.05$.

Study approval

Prior to inclusion in the study, informed consent was provided by all participants. Biological specimen collection protocols were complied with local guidelines and approved by the ethics committee of the Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences.

Results

T cell subsets and key transcription factor analyses in AA patients

Demographic information of 30 non-SAA patients was obtained from medical records. The cohort demonstrated a median age of 32 years old, and 56.7% was male. Intervals from onset to diagnosis differed largely and the median interval was 8 months (ranging from 1 month to 10 years). Twenty percent of patients were dependent on red blood cell or platelet transfusion; the median concentration of hemoglobin and platelet was 92.5 g/L (ranging from 47 to 149 g/L) and 25×10^9 /L (ranging from 13 to 69×10^9 /L) respectively. Fifteen patients (50%) respond to 6-month oral cyclosporine treatment (Table 1).

We compared the frequency of sixteen T cell subsets between AA patients and healthy individuals (Supplementary Fig. 1). Non-significant increased ratio of effector or memory CD8⁺ cytotoxic T cells was observed between patients and healthy individuals (Fig. 1A). Furthermore, the ratio of T helpers between patients and healthy donors was similar (Fig. 1B). Previous studies revealed that the frequency of Tregs in patients with AA was lower than that in healthy controls [15, 17]. Here, we also observed

Table 1 Clinical characteristics of AA patients

Clinical characteristics	Non-SAA patients <i>N</i> = 30
Male (%)	56.7
Median age (y) (range)	32 (10–70)
Intervals from onset to diagnosis (m) (median, range)	8 (1–120)
Transfusion dependent (%)	20
HGB (g/L) (median, range)	92.5 (47–149)
ANC ($\times 10^9$ /L) (median, range)	1.42 (0.55–1.95)
PLT ($\times 10^9$ /L) (median, range)	25 (13–69)
ARC ($\times 10^9$ /L) (median, range)	54.5 (27.2–154.3)
PNH clone at time of diagnosis (%)	16.7
Size of PNH clone, median (%) (range)	
Red cells	4.8 (3.1–27.5)
Granulocytes	19.3 (0.8–57.6)
Monocytes	14.7 (1.1–55.8)
Bone marrow cellularity (%)	10 (5–30)
Numbers of megakaryocyte	6 (0–48)
Response to cyclosporine (%)	50

a relatively lower but not statistically significant level of Tregs in AA patients than healthy controls ($2.30 \pm 1.03\%$ vs. $3.42 \pm 1.13\%$, $p = 0.056$). Considering that T-box expressed in T cells (T-bet) and Eomesodermin (Eomes) participate in regulating T cell differentiation and phenotype shift [33, 34], we further analyzed the expression of T-bet and Eomes between patients and healthy individuals but found no significant differences due to the variation between individuals (Fig. 1C).

To explore the relationship between T cell subsets and responsiveness to cyclosporine treatment, the frequency of T cell subpopulations between responders and non-responders was analyzed. However, no significant differences were observed, suggesting that the frequency of T cell subpopulations was not correlated with patients' sensitivity to cyclosporine (Supplementary Fig. 2A–B). Furthermore, to investigate whether calcineurin/nuclear factor of activated T cell (NFAT) pathways are related to cyclosporine response, we further detected the expression levels of NFAT2, NFAT4, and calcineurin on T cells. Unexpectedly, no differences were observed between responders and non-responders (Supplementary Fig. 3).

Taken together, our data suggest that the frequency of T cell subpopulations and the expression level of T cell functional regulatory transcript factors are unrelated to the occurrence and development or the cyclosporine sensitivity of non-SAA which might be due to the heterogeneity of non-SAA. Additionally, the expression level of calcineurin/NFAT pathway could not discriminate the response to cyclosporine.

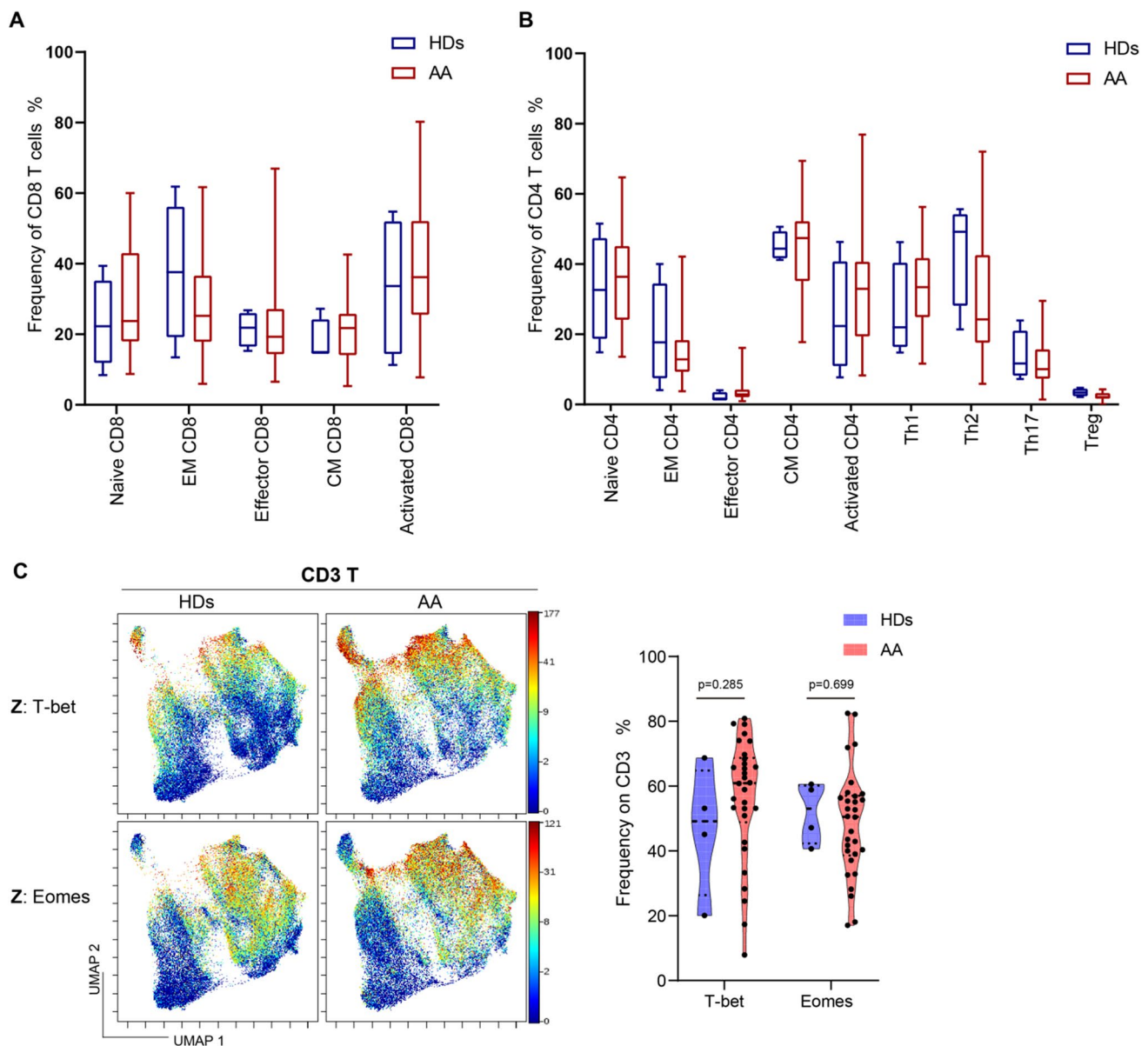


Fig. 1 No difference on T cell subset proportion and T-box transcript factor expression between non-SAA patients and healthy donors. **A, B** Frequencies of T cell subpopulations expressed as frequency of CD8⁺ T cells (**A**) and of CD4⁺ T cells (**B**). Data are shown as box plots extending from the 25th to 75th percentiles and the whiskers from the minimum to the maximum point; middle line represents the median.

Samples from 30 non-SAA patients and 4 healthy donors were tested. **C** The UMAP plots showed the expression of T-bet and Eomes on CD3 T cells in patients (left). The violin plots showed frequencies of T-bet and Eomes on CD3 T cells (right). T-bet T-box expressed in T cells, Eomes Eomesodermin, HDs healthy donors. Data was shown as mean \pm SEM; $p < 0.05$

Cyclosporine treatment restores the expression of immune checkpoint molecule in responder patients

Immune checkpoint molecules were considered to play a critical role in the immune response of AA patients [18, 19, 35]. Thus, we analyzed the expression of these inhibitory molecules on T cell subsets. As compared to healthy individuals, higher frequency of CTLA-4⁺ CD8⁺ T cells was found

in AA ($22.63 \pm 2.52\%$ vs. $11.7 \pm 2.11\%$, $p < 0.05$, Fig. 2A). Furthermore, increased level of CTLA-4 was displayed on naive CD8⁺ T cells ($25.55 \pm 2.92\%$ vs. $11.86 \pm 2.28\%$, $p < 0.05$) and effector memory CD8⁺ T cells ($19.19 \pm 2.02\%$ vs. $10.26 \pm 1.63\%$, $p < 0.05$, Fig. 2B). But no significant difference of CTLA-4 on effector CD8 was observed between healthy controls and AA patients ($28.66 \pm 2.77\%$ vs. $17.38 \pm 2.04\%$, $p = 0.105$). Unexpectedly, no statistically significant difference on PD-1⁺, LAG-3⁺, TIM3⁺, or TIGIT⁺

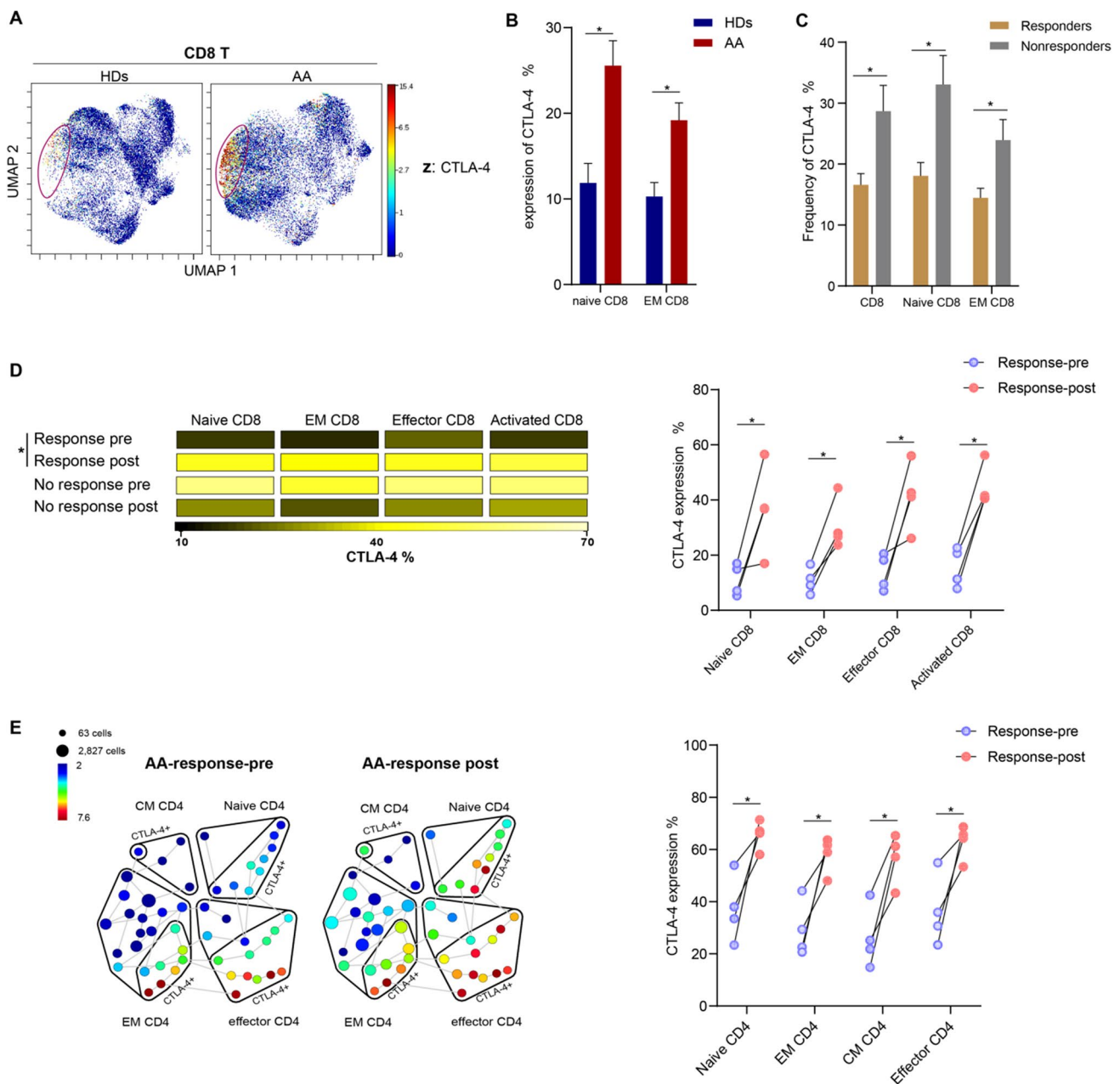


Fig. 2 Expression of CTLA-4 is altered upon cyclosporine treatment. **A** The UMAP figure showed the expression of CTLA-4 on CD8 T cells in patients at diagnosis. Samples from 30 non-SAA patients and 4 healthy donors were tested. **B** Box plots showed the expression difference of CTLA-4 on naive CD8 and EM CD8 in patients at diagnosis. **C** The percentage of CTLA-4 on CD8, naive CD8, and EM CD8 was significantly lower in responders. Samples were collected from 15 responders and 15 non-responders. **D** The heatmap showed increased expression of CTLA-4 on CD8 subsets following

cyclosporine treatment in responders (left, $n=3$). The alteration was shown in dot plots (right, $n=3$). **E** SPADE clustering of CD4⁺ T cells showed significant difference of CTLA-4 on CD4 subsets after cyclosporine treatment in responders (left), and the alteration was also shown in dot plots (right). Prior and post treatment samples were collected from 3 responders. HDs healthy donors, EM CD8 effector memory CD8, CM CD4 central memory CD4, EM CD4 effector memory CD4. Data was shown as mean \pm SEM; * $p < 0.05$

T cells was observed between the AA cohort and healthy volunteers (Supplementary Fig. 4).

As shown in Fig. 2C, responders displayed a lower frequency of CTLA-4⁺ CD8⁺ at diagnosis, compared to non-responders ($16.59 \pm 1.83\%$ vs. $28.67 \pm 4.22\%$, $p < 0.05$).

Decreased expression of CTLA-4 on naive CD8⁺ T cells was also observed in responders ($18.05 \pm 2.18\%$ vs. $33.05 \pm 4.75\%$ $p < 0.01$). Additionally, effector memory CD8⁺ T cells expressed a lower level of CTLA-4 in responders ($14.46 \pm 1.55\%$ vs. $23.93 \pm 3.36\%$, $p < 0.05$).

Following cyclosporine treatment, the expression of CTLA-4 was significantly increased in responders, but the alteration was not observed in non-responders. As shown in Fig. 2D, the frequency of CTLA-4⁺ CD8⁺ T cells was significantly increased upon treatment ($11.28 \pm 2.73\%$ vs. $33.94 \pm 6.38\%$, $p < 0.05$) in responders. The increased expression of CTLA-4 on effector CD8⁺ T cells ($13.80 \pm 3.28\%$ vs. $41.46 \pm 6.11\%$, $p < 0.05$) and activated CD8⁺ T cells ($15.63 \pm 3.57\%$ vs. $44.76 \pm 3.82\%$, $p < 0.01$) was also observed after cyclosporine (Fig. 2D). Furthermore, the expression of CTLA-4 on CD4⁺ T subsets in responders, including naive CD4⁺ T cells ($37.20 \pm 6.37\%$ vs. $65.70 \pm 2.76\%$, $p < 0.05$), CD4⁺ effector memory ($29.20 \pm 5.31\%$ vs. $56.78 \pm 4.77\%$, $p < 0.05$), CD4⁺ central memory ($26.09 \pm 5.87\%$ vs. $56.74 \pm 9.54\%$, $p < 0.05$), and CD4⁺ effector ($36.24 \pm 6.74\%$ vs. $63.0 \pm 3.34\%$, $p < 0.05$), was also significantly increased after the intervention of cyclosporine (Fig. 2E).

Collectively, our data demonstrates that the frequency of CTLA-4 on T cells is altered upon cyclosporine in responders, and an increased expression is correlated to cyclosporine response.

T cell trafficking mediator is linked to cyclosporine response in AA

S1P₁ receptor signaling is correlated with various physiological processes including lymphocyte recirculation, their biology, and phenotype determination [21]. To explore whether the expression of S1P₁ has influence on immune function of T cells in AA, we compared the expression of S1P₁ on T cell subsets between healthy donors and AA. As expected, increased expression of S1P₁ on naive CD4⁺ T cells ($27.02 \pm 2.35\%$ vs. $10.48 \pm 6.24\%$, $p < 0.05$) and naive CD8⁺ T cells ($27.02 \pm 2.35\%$ vs. $10.48 \pm 6.24\%$, $p < 0.05$) was observed in AA (Fig. 3A). Central memory CD8⁺ T cells also showed an increased level of S1P₁ in AA ($24.71 \pm 1.96\%$ vs. $10.61 \pm 5.55\%$, $p < 0.05$). Furthermore, expression difference of S1P₁ on T helpers was also observed, and Th2 subset demonstrated a higher level of S1P₁ ($18.81 \pm 1.78\%$ vs. $7.85 \pm 3.82\%$, $p < 0.05$).

We also found that patients with a lower level of S1P₁ on CD4⁺ T subsets at diagnosis seem to be more prone to achieve response after cyclosporine (Fig. 3B). As compared to non-responder patients, a significantly decreased level of S1P₁ on naive CD4 was observed in responders ($21.86 \pm 2.67\%$ vs. $32.18 \pm 3.46\%$, $p < 0.05$). Furthermore, a significantly decreased level of S1P₁ on CD4⁺ effector ($20.03 \pm 2.40\%$ vs. $28.10 \pm 3.23\%$, $p < 0.05$) and activated CD4⁺ T cells ($23.70 \pm 2.49\%$ vs. $34.36 \pm 3.49\%$, $p < 0.05$) was observed in responder patients. No significant difference of S1P₁ expression on CD8 subsets was observed between two groups.

In summary, these results demonstrate that T cells, especially naive T cells, in AA had an increased level of S1P₁, which is linked to the response to cyclosporine.

Expression of IL-9 is correlated to cyclosporine response in AA

IL-9/IL-9 receptor plays a dual effect on inflammation and autoimmune disease, including both pro-inflammatory and protective roles in occurrence of disease [36, 37]. We explored whether there was difference on the expression of IL-9 between healthy controls and AA patients. We found that patients displayed a significant higher level of IL-9 on naive CD4⁺ T cells ($18.68 \pm 2.62\%$ vs. $7.73 \pm 2.41\%$, $p < 0.05$, Fig. 4A). Furthermore, an increased frequency of IL-9 on Th2 cells was also found in AA patients ($18.78 \pm 2.36\%$ vs. $7.64 \pm 2.78\%$, $p < 0.05$, Fig. 4B). These results indicate that IL-9 may participate in the pathophysiology of AA.

To investigate the correlation between IL-9 expression prior treatment and cyclosporine response, we further analyzed the characteristics of IL-9 on naive T cells and T helper subsets. In responders, naive CD4⁺ T cells showed a significantly lower expression of IL-9 as compared to non-responder patients ($24.52 \pm 2.97\%$ vs. $40.42 \pm 4.80\%$, $p < 0.05$). Of note, the percentage of IL-9⁺ Th1 cells and IL-9⁺ Th2 cells in responders was $25.64 \pm 2.67\%$ and $13.55 \pm 1.64\%$, respectively, which was significantly lower compared with non-responders ($39.35 \pm 4.55\%$ and $24.00 \pm 4.07\%$, $p < 0.05$). Moreover, Th17 clusters expressed lower expression of IL-9 in responders ($17.30 \pm 2.18\%$ vs. $29.13 \pm 3.88\%$, $p < 0.05$, Fig. 4C). This encouraging finding suggests a significant association between IL-9 expression and clinical response to cyclosporine.

Discussion

Abnormal T cell subsets and function play a critical role on the occurrence and progression of AA. Kordasti et al. tested 63 patients with AA and have shown that patients had an increased frequency of Th1 and Th2 compared to healthy donors. They also found that the absolute counts of Th2 and Th17 were higher in SAA [16]. Shi et al. detected Tregs in 55 AA patients, which included 27 of SAA, and demonstrated a decreased number and impaired functions of Tregs [17]. It has been reported that frequencies of some T cell subsets were age-related and the disease severity of AA might have a different pathogenesis with different immunological signatures, where very severe or long-lasting diseases might have a Th17 signature while severe or new-onset AA a Th1 profile [23, 38]. Therefore, due to the heterogeneity of group and disease severity of non-SAA, our study did not demonstrate significant difference on T cell subpopulations

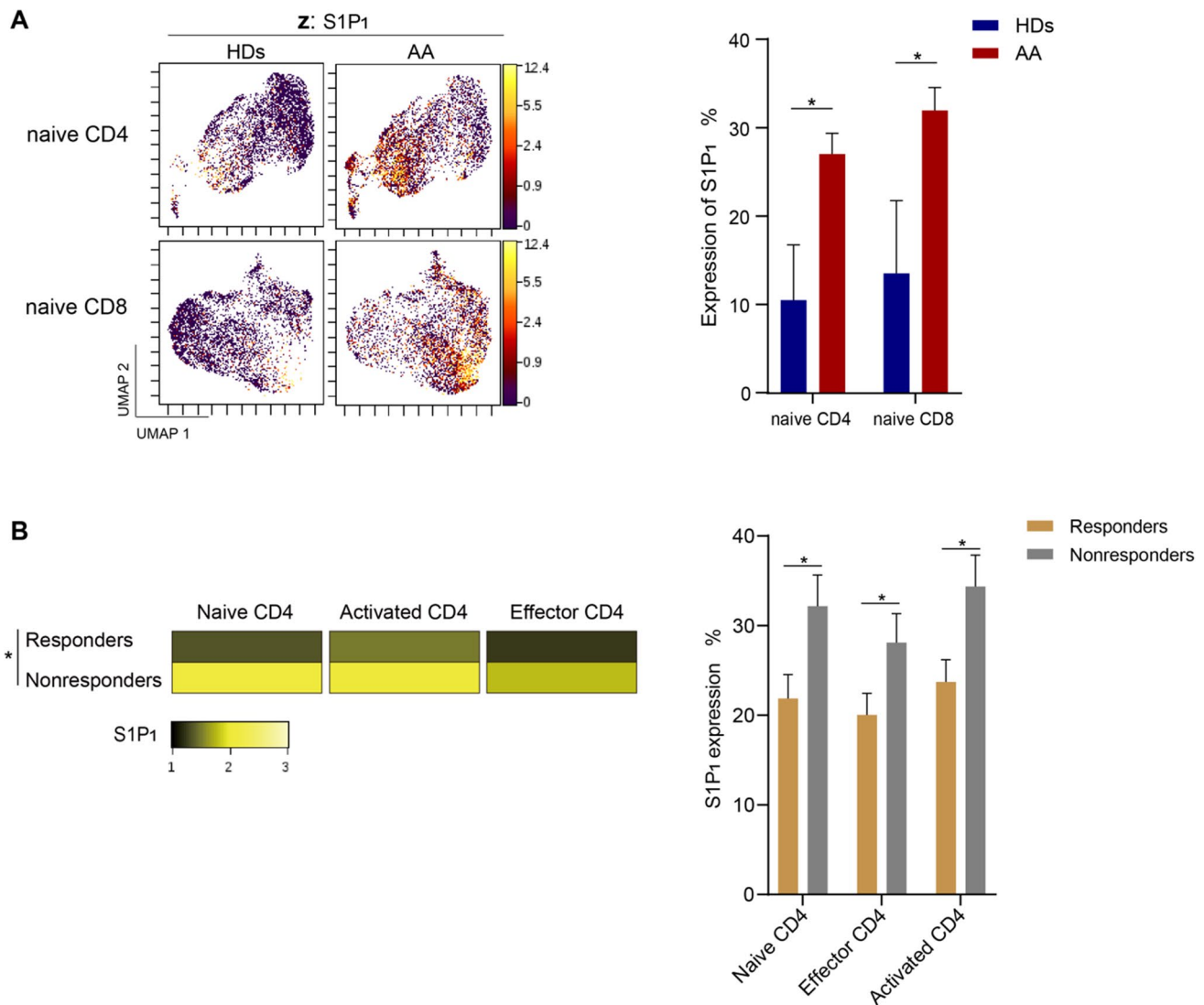


Fig. 3 Expression of S1P₁ is correlated to clinical response. **A** The UMAP plots showed higher expression of sphingosine-1-phosphate-receptor 1 (S1P₁) on naive T cells (left) and the higher percentage of S1P₁ on naive CD4 and naive CD8 (right). Samples from 30 non-SAA patients and 4 healthy donors were tested. **B** Heatmap showed

the difference of S1P₁ on CD4 subsets between responders and non-responders (left); the expression of S1P₁ was significantly lower in responders (right). Samples were collected from 15 responders and 15 non-responders. Data was shown as mean \pm SEM; * p < 0.05

between patients and healthy individuals. But the frequency of Tregs is inclined to be decreased in non-SAA. This result provided new evidence that the reduction of Tregs plays a critical role in the pathogenic mechanism of AA.

A variety of T cell immune checkpoint molecules have been identified to regulate T cell activation; increased TIM-3 and PD-1 have been observed in AA [18–20]. Although no obvious differences on TIM3 and PD-1 were found, an increased expression of CTLA-4 was shown. CTLA-4 belongs to the CD28 family; the expression and function of this molecule are intrinsically correlated with T cell activation. In autoimmunity, it can be targeted to curb the over-exuberant immune response [20, 39]. It has also been

reported that the expression of CTLA-4 on Tregs in SAA was higher than that in healthy controls [40–42], but its expression on effector T cells is still unclear. We demonstrated that patients with AA had an increased expression of CTLA-4 on naive CD8⁺ T and central memory CD8⁺ T cells, whereas no significant difference was observed on effector T cells. This result indicates that CD8⁺ T cells in patients were undergoing an abnormal effector function, which may aggravate injury to hematopoietic stem progenitor cells. Furthermore, a higher level of CTLA-4 predicted a worse response to cyclosporine, and responders displayed significant increment in CTLA-4 expression following cyclosporine. Thus, we conclude that patients with lower

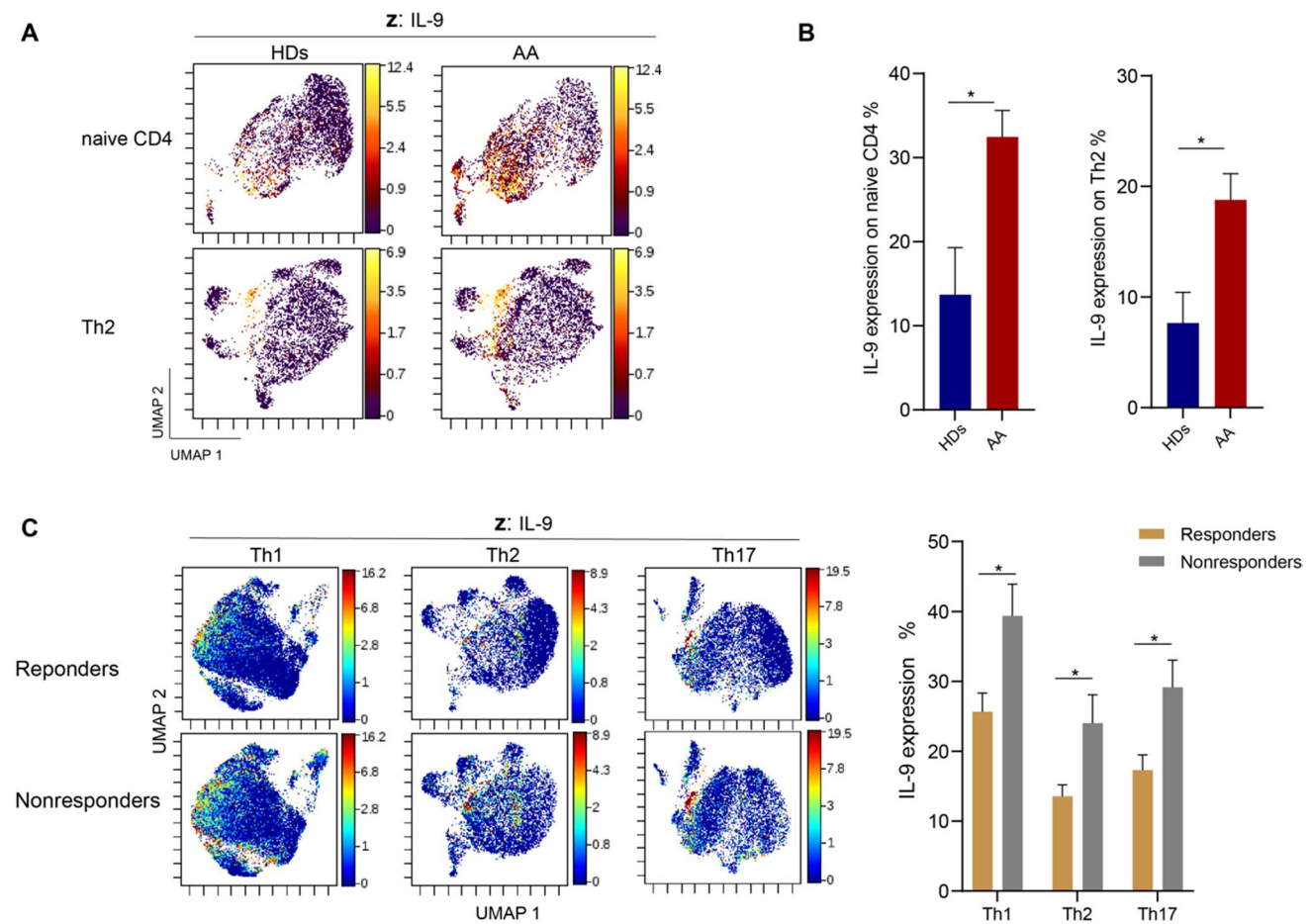


Fig. 4 Non-SAA has a higher level of IL-9 at diagnosis and non-responders have even higher IL-9 level. The UMAP plots showed higher frequency of interleukin-9 (IL-9) on naive CD4⁺ T and Th2 in patients. Samples from 30 non-SAA patients and 4 healthy donors were tested. **B** The percentage of IL-9 on naive CD4⁺ and Th2 was

significantly higher in patients than HDs. **C** The UMAP plots showed the difference of IL-9 on Th1, Th2, and Th17 between responders and non-responders (left); columns showed a lower expression of IL-9 in responders (right). Samples were collected from 15 responders and 15 non-responders. Data was shown as mean \pm SEM; * p < 0.05

expression of CTLA-4 are more sensitive to immunosuppression and increased CTLA-4 may play a synergistic effect to inhibitor dysfunctional T cells.

T cell circulation among secondary lymphoid organs was guided by the chemoattractant S1P. The S1P₁ receptor regulates the emigration of T cells out of the thymus and lymphoid organs [43]. Furthermore, the S1P signaling pathway maintains the mitochondrial content of naive T cells, supplying cells with energy for their constant egress [44]. We discovered that the expression of S1P₁ on naive T cells was higher in AA. It may be related to disrupted immune homeostasis, which would aggravate hematopoiesis injury. Moreover, patients with higher expression of S1P₁ on effector and activated CD4⁺ T cells was resistant to achieve response, which may be due to hyperimmune condition. It has been reported that S1P1 antagonist could inhibit progress of chronic colitis [45]. Considering that, the inhibition

of S1P₁ may also be considered a promising choice for AA patients.

As the expression of IL-9 was connected with the expansion of Th2 subsets, it was originally identified as a Th2-type cytokine. In recent years, Th9 has been verified to be an important population of Th cells to produce IL-9. This special population is considered to be less exhausted, fully cytolytic, and hyperproliferative [46, 47]. Recent studies have shown that Th9 population and IL-9 are closely correlated with several autoimmune disorders, including systemic sclerosis, inflammatory bowel diseases, and experimental autoimmune encephalitis [25, 48–50]. In addition to Th9 and Th2 cells, other cell types are also able to produce IL-9, such as Th17, natural killer T cells, and innate lymphoid cell type 2 [51]. Under the conditions of Th9 differentiation, cytotoxic CD8⁺ T cells can also polarize into IL-9-producing cytotoxic CD8⁺ T cells [48]. We found a significantly higher level of IL-9 in AA than in healthy individuals, which

indicates that IL-9 may play an important role in T cell dysfunction. Although it has been reported that IL-9 could activate STAT3 and STAT5 to promote Treg survival and enhance Treg-mediated suppression [52], no significantly higher expression of IL-9 on Tregs was shown in patients, which may be related to impaired function of Tregs in AA. Furthermore, in our study, patients with a lower level of IL-9 showed better response to cyclosporine, suggesting that IL-9 expression could act as a predictor for overall response. Evidence from animal studies had shown that IL-9 deficiency or inhibition of IL-9 was correlated with decreased inflammatory cytokines and attenuated inflammatory condition [53, 54]. Thus, IL-9 may be a potential therapeutic target for braking T cell dysfunction in AA.

In summary, we showed the differences on T cell immune signature between AA and healthy individuals. More importantly, we observed that cyclosporine restored the expression of CTLA-4 on T cells in responders. A lower level of S1P₁ and IL-9 predicts a better response. Evaluation of targeting these T cell markers should be widely taken into consideration for the optimal management of AA taken into the future. Further studies focusing on immune checkpoint molecules, T cell trafficking, and secretion of IL-9 could provide us more information to do decision-making of cyclosporine treatment strategy.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1007/s00277-023-05097-6>.

Author contribution LZ and JM performed the study and wrote the paper; JS and WY designed the study; YL, YC, and WL operated bone marrow samples; QL, HP, ZG, LF, and JZ collected essential clinical data. JS gave an approval of the final manuscript.

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Data availability The data that support the findings of this study are available on request from the corresponding author [Jun Shi].

Declarations

Conflict of interest The authors declare no competing interests.

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