Original Article

Neutrophils inhibit CD8⁺ T cells immune response by arginase-1 signaling in patients with sepsis

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BACKGROUND: Patients with sepsis often exhibit an acute inflammatory response, followed by an immunosuppressive phase with a poor immune response. However, the underlying mechanisms have not been fully elucidated.

METHODS: We sought to comprehensively characterize the transcriptional changes in neutrophils of patients with sepsis by transcriptome sequencing. Additionally, we conducted a series of experiments, including real-time quantitative polymerase chain reaction (RT-qPCR) and flow cytometry to investigate the role of arginase-1 signaling in sepsis.

RESULTS: Through the analysis of gene expression profiles, we identified that the negative regulation of T cell activation signaling was enriched, and the expression of arginase-1 was high in neutrophils from patients with sepsis. Furthermore, we conducted flow cytometry and found that the function of CD8⁺ T cells in septic patients was impaired. Moreover, neutrophils from septic patients inhibited the percentage of polyfunctional effector CD8⁺ T cells through arginase-1. Additionally, the proportions of granzyme B⁺IFN-γ⁺CD8⁺ T and TNF-α⁺IFN-γ⁺CD8⁺ T cells increased after inhibition of arginase-1 signaling.

CONCLUSION: The impaired effector function of CD8⁺ T cells could be restored by blocking arginase-1 signaling in patients with sepsis.

KEYWORDS: Sepsis; Effector CD8⁺ T cells; Neutrophils; Arginase-1; Interferon-γ

INTRODUCTION

Sepsis is a life-threatening organ dysfunction mainly caused by an abnormal infection-induced immune response.[1-4] In the excessive inflammatory response stage of sepsis, the immune response to sepsis may further cause cell and tissue injury or multiple organ dysfunction syndrome (MODS) with high morbidity and mortality.[5-7] In recent years, the incidence rate of sepsis has increased worldwide.[8,9] More attention has been given to the description of unique biological phenotypes in patients with sepsis;[10,11] however, the mechanisms underlying sepsis have not been fully elucidated.

Neutrophils play an important role in the innate immune system.[11] Although neutrophils survive for only several hours, they contribute to eradicating microorganisms and the survival of patients with sepsis. Neutrophils can effectively clear extracellular microorganisms mainly through direct phagocytosis.[12,13] Recent studies suggested that neutrophils can also clear pathogens through neutrophil extracellular traps (NETs).[14,15] In sepsis, the

phenotype and function of circulatory neutrophils can be altered significantly.[16] In early sepsis, neutrophils can migrate from the bone marrow to peripheral blood, and the level of neutrophils can increase by ten-fold within several hours compared to healthy controls.[17]

Sepsis is always accompanied by an impaired immune response mediated by immunosuppressive mechanisms, including the expansion of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), and the production of suppressive factors, including arginase-1 (ARG1).[1,18] Sepsis can expand a CD39+ plasmablast population and promote immunosuppression through adenosine-mediated inhibition of macrophage antimicrobial activity.[19] During sepsis, the level of plasma arginase is elevated. Human neutrophils also express a high level of ARG1, which can deplete arginine from the surrounding medium, suppressing the production of nitric oxide (NO) and impeding the clearance of pathogens.[20,21] In addition, the release of ARG1 from neutrophils has been associated with T cells dysfunction in various pathologies.[20,22]

This study provides insight into the dysfunction of CD8+ T cells in patients with sepsis. Herein, we evaluated the transcriptional profiles and the expression of ARG1 in neutrophils from septic patients and compared them with those of healthy controls. Moreover, we aimed to ascertain whether targeting ARG1 can be a promising approach for restoring the functions of CD8+ T cells in patients with sepsis.

METHODS
Isolation of samples, CD8+ T cells, and neutrophils
Peripheral-blood mononuclear cells (PBMCs) were isolated by ficoll density gradients from patients with sepsis and healthy controls (Table 1 and Supplementary Figure 1), and resuspended in RPMI 1640 complete medium (10% fetal calf serum, and 1% streptomycin/penicillin). CD8+ T cells were purified from PBMCs of healthy controls by a magnetic-activated cell sorter (MACS) kit (Miltenyi Biotec, Germany). CD14- CD15+CD16+ neutrophils were sorted by FACS Aria (BD Bioscience, USA) from PBMCs of patients with sepsis.

Ex vivo flow cytometry experiments
CD8+ T cells were cocultured with neutrophils and stimulated overnight with or without arginase inhibitor 1 (10 μmol/L; MedChemExpress, USA). Cells were stained with human monoclonal antibodies (as described in Supplementary Table 1). Before staining with antibodies, mouse serum was used to block the binding of nonspecific Fc-receptors. Next, intracellular staining of cytokines was performed with 4 h of stimulation with ionomycin (1 μg/mL; Calbiochem, Germany), phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma, Germany) and monensin (10 μg/mL; Sigma, Germany). Anti-CD107a was added at the same time for 4 h. Cells were then collected and stained for surface or intracellular markers using antibodies (BD Biosciences, USA) according to the manufacturer's instructions. Data were collected using the FCM LSR II flow cytometer (BD Biosciences, USA) and analyzed with FlowJo software (Tree Star, USA).

Transcriptome sequencing and analyses of gene expression
CD14-CD15+CD16+ neutrophils were isolated from PBMCs of patients with sepsis and healthy controls for transcriptome sequencing using the BGISEG platform (Beijing Genomics Institution). The heat map of differentially expressed genes (DEGs) was analyzed with MEV v4.8.1. DEGs were defined as those with a fold change ≥2.0 in the expression between two groups and signal values higher than background signals. An adjusted *P*-value was used to determine DEGs. Next, to obtain insights into the change in phenotype, enrichment analyses of DEGs were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.kegg.jp/) databases and Gene Ontology (GO, www.geneontology.org/).[23] Raw RNA-seq data have been deposited in the Gene Expression Omnibus Repository of the National Center for Biotechnology Information (accession number: GSE186054).

For gene expression analyses, total RNA was isolated using TRIzol reagent (Invitrogen, USA), and cDNA synthesis was performed using M-MLV Reverse Transcriptase (Invitrogen, USA) and random primers according to manufacturer's instructions.[23] qRT-PCR was carried out using SYBR Premix Ex Taq (TaKaRa, Japan). Primers are listed in Supplementary Table 2.

Statistical analysis
GraphPad Prism 8 software were employed to perform statistical analysis. We used two-tailed unpaired or paired Student's *t*-tests between two groups or one-way analysis of variance (ANOVA) across multiple groups. Data were presented as mean ± standard deviation. A *P*-value<0.05 was considered statistically significant.

RESULTS

Transcriptional profiles of neutrophils from septic patients

When diagnosed with sepsis, the number of neutrophils was significantly increased in blood (Table 1). Neutrophils can inhibit the effector function and activation of T cells.[14,24,25] Insufficient T cells activation was associated with an inability to recognize pathogens properly. To gain further mechanistic insights, we isolated neutrophils from patients with sepsis and healthy controls for whole-genome transcriptome analyses by microarrays (Figure 1A). The transcriptional profiles of neutrophils from patients with sepsis differed from those of healthy controls. A total of 2,365 genes exhibited upregulated expression, and 1,354 genes showed downregulated expression in septic patients compared with healthy controls (Figure 1B). Enrichment analyses using the GO database revealed varying immunological processes, including negative regulation of T cell activation (Figure 1C). By comparing septic patients with healthy controls, we found that acute inflammatory response signaling and negative regulation of T cell
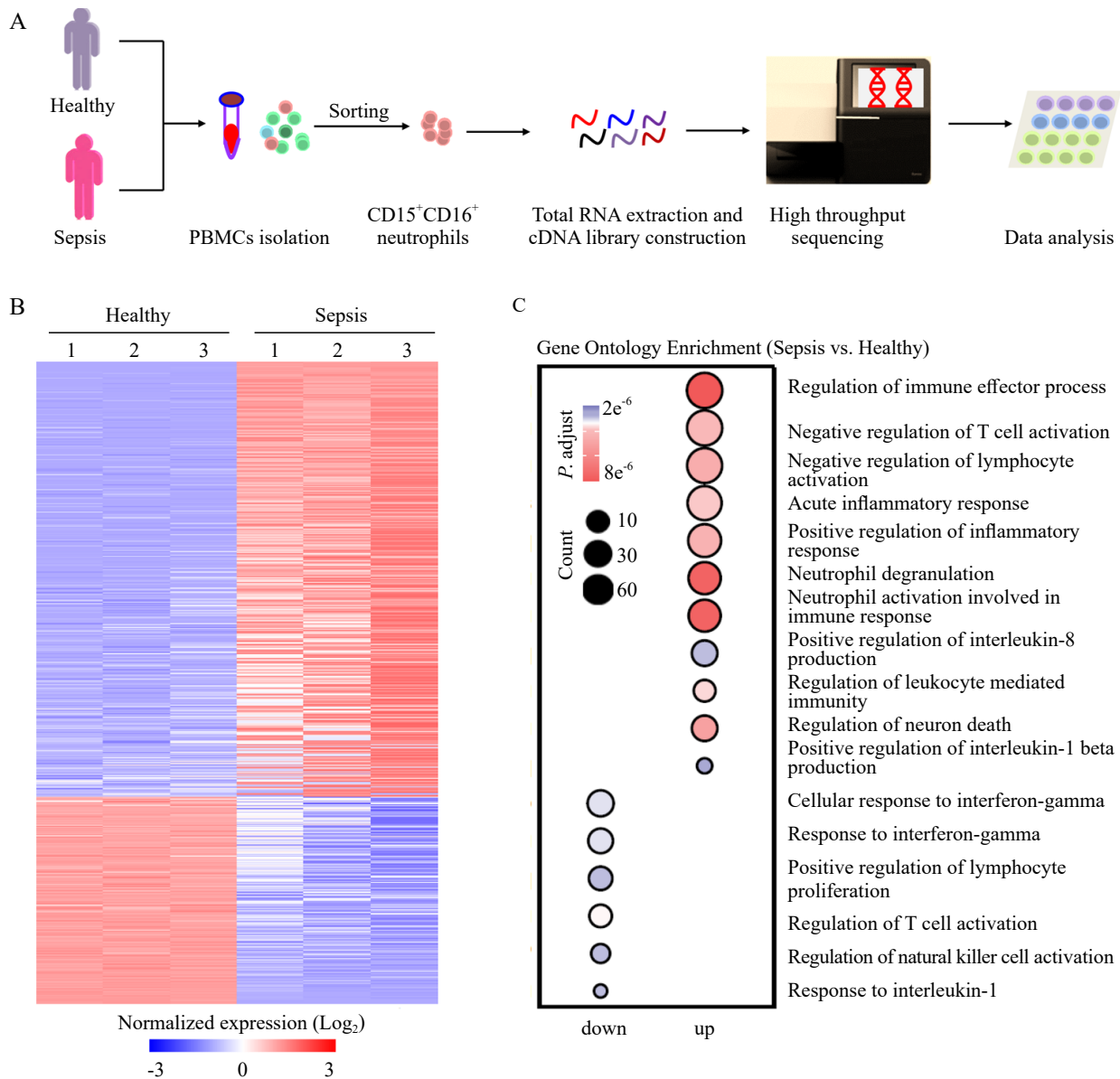
Figure 1. The transcriptional profiles of neutrophils from patients with sepsis differed from those of healthy controls. A: the protocol of isolation of neutrophils from healthy controls (*n*=3) and patients with sepsis (*n*=3); B: the top-200 genes with differential expression (fold change greater than two-fold) in neutrophils from patients with sepsis compared with those in healthy controls were selected for heat map analyses; each column depicts one sample; C: enrichment analyses of differentially expressed genes were performed (using the Gene Ontology database) to evaluate enriched biological processes between neutrophils from patients with sepsis and healthy controls; enrichment of regulation of negative regulation of T cell activation-related biological processes was included.

Table 1. Characteristics of sepsis patients and healthy controls

Characteristic	Healthy controls (*n*=10)	Sepsis patients (*n*=10)
Gender (M/F)	5/5	6/4
Age, years	27 (19–43)	31 (23–42)
White blood cell count, ×10^9/L	5.05 (4.07–7.46)	9.46 (6.78–21.30)
C-reactive protein, mg/L	2.07 (1.44–3.56)	98.30 (70.70–133.50)
Procalcitonin, ng/mL	0.11 (0.03–0.25)	46.60 (21.70–54.50)
Neutrophil count, ×10^9/L	3.64 (1.25–7.11)	23.74 (15.60–36.70)
Lymphocyte count, ×10^9/L	1.84 (0.76–2.60)	0.53 (0.32–1.51)
Monocyte count, ×10^9/L	0.48 (0.38–0.71)	0.66 (0.39–1.21)
SOFA score	0	6.35 (3.00–9.00)
Infection with *Klebsiella pneumoniae*	8	8
Infection with *Staphylococcus aureus*	2	2

Variables are expressed as mdian (interquartile range) or number. M: male; F: female;SOFA: sequential organ failure assessment.

activation signaling were enriched in neutrophils from patients with sepsis (Figure 1C). In general, our data demonstrated differences in transcriptional profiles in neutrophils from patients with sepsis and healthy controls. Specifically, neutrophils from septic patients mainly showed the characteristics of negative regulation of T cell activation.

Increased expression of *ARG1* in neutrophils

Human neutrophils express high amounts of *ARG1*, which could significantly downregulate T-cell activation.[18] To determine whether there was an increase in the expression of *ARG1* in the neutrophils of patients with sepsis, we first performed DEGs analysis and gene set enrichment analysis (GSEA). Meanwhile, we found an increase in the expression of DEGs related to the negative regulation of T cells activation in neutrophils of patients with sepsis compared with that in neutrophils of healthy controls (Figure 2). Of these, there was an 82-fold increase in ARG1 expression (Figure 2A), implying its significant role in regulating the activation of T cells. These results demonstrated the impaired activation of T cells in the sepsis microenvironment. To determine the expression of ARG1 and some other immunosuppressive-related genes such as *IL-10*, which has long been described as one of the mediators of sepsis-induced immunosuppression,[26-29] we purified neutrophils from patients with sepsis and healthy controls to carry out PCR. Notably, the expression of *ARG1* was increased in the neutrophils of septic patients (Figures 2A and 3A-D). The expression of *ARG1* in neutrophils from healthy controls was low (Figures

2A and 3A-D). This difference in expression was also confirmed using flow cytometry (Figure 3E). These data suggested that in neutrophils from patients with sepsis, *ARG1* expression was significantly upregulated.

Neutrophils inhibited CD8⁺ T cells immune response by ARG1 signaling *ex vivo*

To further demonstrate that inhibition of ARG1 signaling could restore the effector function of CD8⁺ T cells of septic patients, we first processed the neutrophils from septic patients with arginase inhibitor 1. The expression of ARG1 was decreased (Figure 4A). Next, we purified CD8⁺ T cells from PBMCs of healthy controls and neutrophils from patients with sepsis (Figure 4B). After CD8⁺ T cells were cocultured with
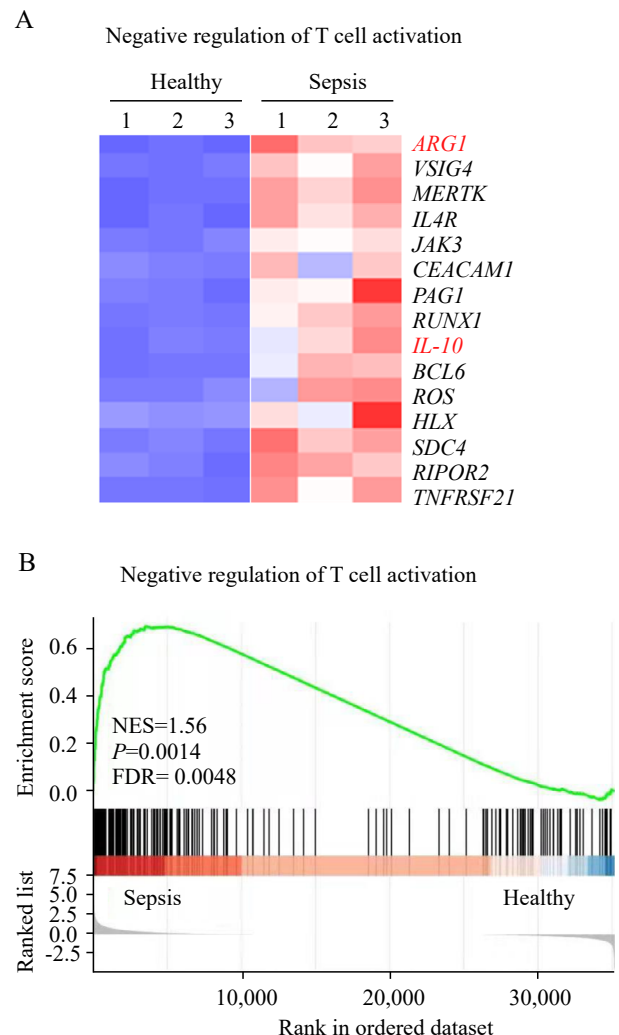
Figure 2. Differentially expressed genes (DEGs) analysis and gene set enrichment analysis (GSEA) of neutrophils. A: heat map of negative regulation of T cell activation-related genes with differential expression in neutrophils from patients with sepsis compared with those from healthy controls; B: GSEA revealed an increase in negative regulation of T cell activation processes in neutrophils from patients with sepsis compared with that in healthy controls. NES: normalized enrichment score; FDR: false discovery rate.

neutrophils from septic patients, the percentage of IFN-γ and TNF-α were coexpression. In addition, granzyme B and CD107a were coexpression, as well as IFN-γ and granzyme B coexpression by CD8^{+} T cells were significantly decreased. However, after being treated with arginase inhibitor 1, the percentage of IFN-γ and TNF-α coexpression and IFN-γ and granzyme B coexpression by CD8^{+} T cells were significantly restored (Supplementary Figure 2; Figure 4C and D). Importantly, we found that the polyfunctional effector CD8^{+} T cells were significantly decreased in septic patients compared with that of healthy controls (Figures 4C and D). In general, these findings suggested that the immune response of CD8^{+} T cells from septic patients was impaired, and the inhibition of ARG1 signaling may be related to the recovery of effector function of CD8^{+} T cells.

DISCUSSION

Our current study provided direct evidence that the expression of ARG1 in the neutrophils of septic patients was high. Moreover, we demonstrated that ARG1 inhibition significantly restored the effector function of CD8^{+} T cells. In summary, our results provided three main insights into the underlying mechanisms and potential for sepsis therapy.

First, to elucidate the immunological characteristics of septic patients, we performed routine laboratory tests. Consistent with the results of other studies, we found that the number of neutrophils was significantly increased,[16,30-32] while the lymphocytes were decreased in septic patients. To investigate the difference in neutrophils between septic patients and healthy controls, we performed RNA-seq analysis and found that the transcriptional profiles of the neutrophils differed between the two groups. Additionally, we verified that negative regulation of T cell activation signaling was enriched in neutrophils from patients with sepsis. Furthermore, we observed high expression of several immunosuppressive indicators, such as IL-10 and ARG1 in neutrophils from septic patients.

Next, to confirm that ARG1 expression was high in neutrophils from septic patients, we performed qRT-PCR and flow cytometry. The expression of ARG1 was significantly increased in neutrophils from patients with sepsis. CD8^{+} T cells play important roles in the host
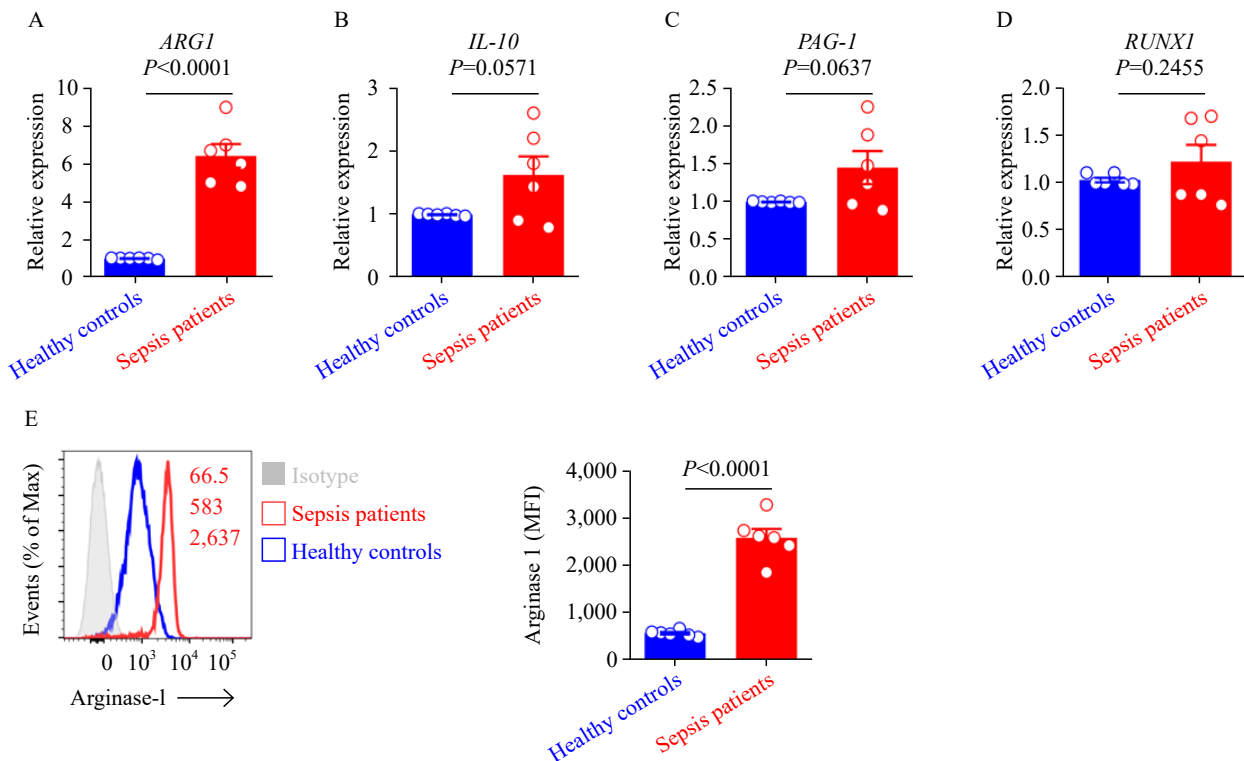
Figure 3. Neutrophils expression of arginase-1 in patients with sepsis. A-D: *ARG1*, *IL-10*, *PAG1*, and *RUNX1* expression in neutrophils from patients with sepsis and healthy controls, as assessed by qRT-PCR. Data were representative of six experiments; E: representative histograms (left) and percentage statistics (right) calculated for the expression of arginase-1 in neutrophils of patients with sepsis (red; *n* = 6) and healthy controls (blue; *n* = 6). Data were analyzed by two-tailed unpaired Student's *t*-test; data were presented as mean ± standard deviation.
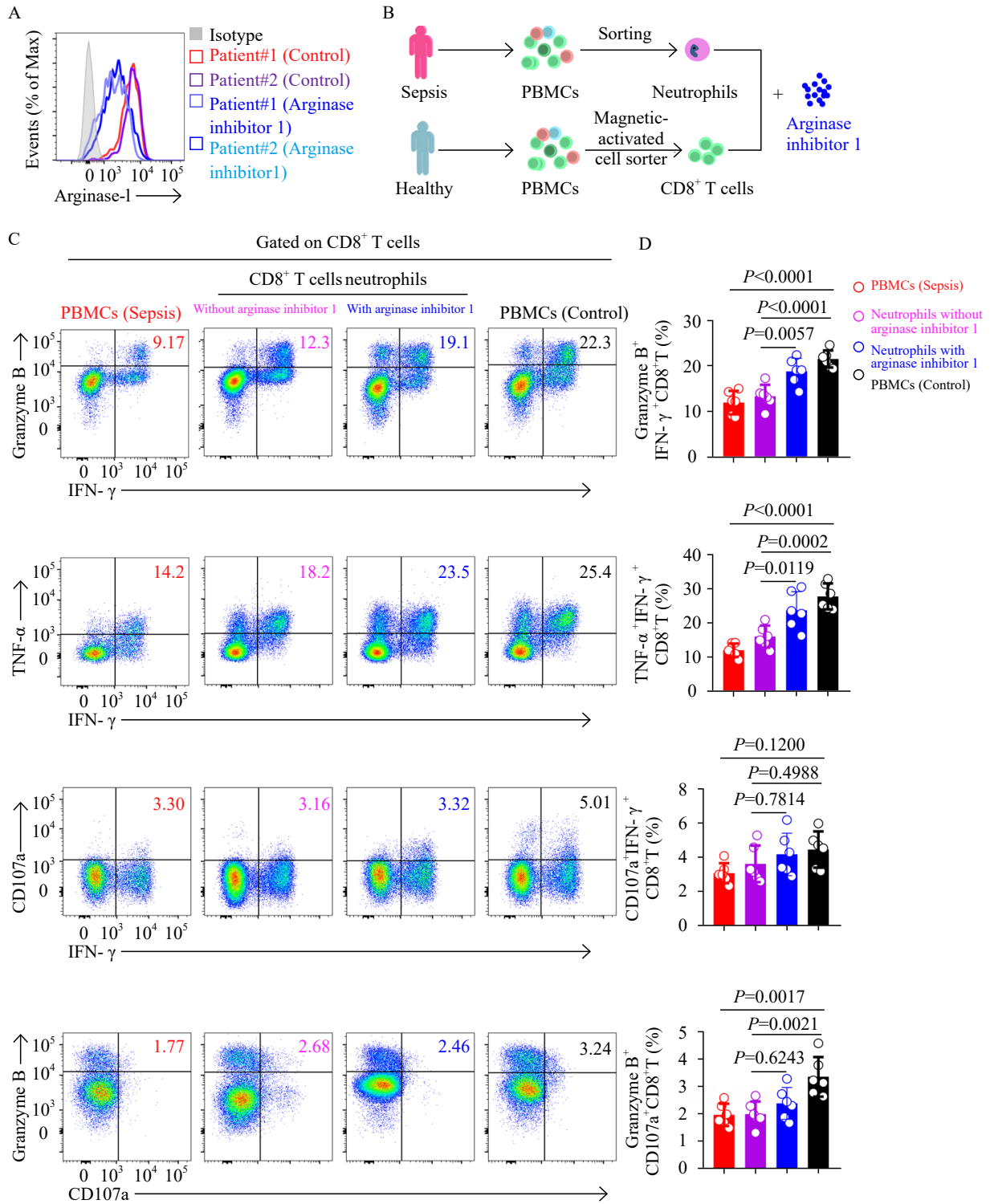
Figure 4. Inhibition of arginase-1 signaling elevates the percentage of polyfunctional effector CD8+ T cells. A: representative histograms showing the analysis of arginase-1 expression in neutrophils of two patients with sepsis after being treated with arginase inhibitor 1; B: the protocol of the isolation of CD8+ T cells from patients with sepsis and healthy controls; and CD14-CD15+CD16+ neutrophils from patients with sepsis. Purified CD8+ T cells from healthy controls were cocultured with neutrophils (ratio = 3:1), treated with or without arginase inhibitor 1 (10 μmol/L); C and D: PBMCs of septic patients (red), or CD8+ T cells purified from healthy controls and cocultured with neutrophils, and treated with (blue) or without (purple) arginase inhibitor 1 (10 μmol/L) overnight, or PBMCs of healthy controls (black), were stimulated with PMA for 4 h. CD8+ T cells were gated for analysis. Representative flow cytometry plots (C) and pool data (D) of the proportion of IFN-γ and granzyme B coexpression, IFN-γ and TNF-α coexpression, IFN-γ and CD107a coexpression, as well as CD107a and granzyme B coexpression by CD8+ T cells. n 6. For (D), data were analyzed by one-way ANOVA with Tukey's multiple comparisons test; Data are presented as mean ± standard deviation.

defense against infections.[33-36] However, in patients with sepsis, the immune response of CD8^{+} T cells was impaired. As ARG1 was reported to be associated with T-cell dysfunction in several pathologies,[18,20,22] we considered whether inhibition of ARG1 would contribute to restoring the function of CD8^{+} T cells.

Third, consistent with expectations, we demonstrated that the effector function of CD8^{+} T cells was impaired in septic patients. Furthermore, after coculturing with neutrophils from septic patients, the immune response of CD8^{+} T cells from healthy controls decreased significantly. Additionally, arginase inhibitor 1 led to the marked restoration of immune function of CD8^{+} T cells *ex vivo*. However, several important questions remain to be answered, including the challenges of developing and using new model systems. Furthermore, we did not clarify other mechanisms that could lead to sepsis and the dysfunction of CD8^{+} T cells. We also did not explain how ARG1 obstructs the function of CD8^{+} T cells in sepsis.

CONCLUSION

Our results reveal the transcriptional profile features of patients with sepsis and indicate that inhibition of ARG1 might help relieve sepsis.

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