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CD39 pathway inhibits Th1 cell function in tuberculosis

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Abstract

The role of CD39 pathway in Th1 cell function in tuberculosis (TB) is rarely elucidated. The present study aims to investigate the modulating mechanism of CD39 pathway during *Mycobacterium tuberculosis* (MTB) infection. CD39 expression was examined on host immune cells among patients with TB. The relationship between CD39 expression and Th1 cell function was analysed. Patients with TB displayed dramatically higher CD39 expression on Th1 cells than healthy controls, and a significantly increased expression of surface markers, including activation, exhaustion and apoptosis markers, were noted in $CD39^+$ Th1 cells in comparison with $CD39^-$ Th1 cells. Conversely, CD39 expression on Th1 cells was associated with diminished number of polyfunctional cells producing Th1-type cytokines, and $CD39^+$ Th1 cells showed obviously lower proliferation potential. Notably, tetramer analysis demonstrated a predominant CD39 expression on TB-specific $CD4^+$ cells, which was associated with higher apoptosis and lower cytokine-producing ability. Transcriptome sequencing identified 27 genes that were differentially expressed between $CD39^+$ and $CD39^-$ Th1 cells, such as *IL32*, *DUSP4* and *RGS1*. Inhibition of CD39 pathway could enhance the activation, proliferation and cytokine-producing ability of Th1 cells. Furthermore, there was a significantly negative correlation between CD39 expression on Th1 cells and nutritional status indicators such as lymphocyte count and albumin levels, and we observed a significant decline in CD39 expression on Th1 cells after anti-TB treatment. CD39 is predominantly expressed on TB-specific Th1 cells and correlated with their exhausted function, which suggests that CD39 could serve as a prominent target for TB therapy.

KEYWORDS
CD39, inhibitory role, Th1 cells, tuberculosis

INTRODUCTION

Tuberculosis (TB) is a major public health problem caused by *Mycobacterium tuberculosis* (MTB) infection [1]. Approximately 10.0 million new cases occurred and 1.4 million people died from TB in 2019 [2]. Accurate diagnosis and effective treatment are essential for TB control globally. However, current therapy for TB is associated with long duration and toxicity [3]. Besides, the increasing spread of multidrug-resistant TB brings severe

challenges to the success of treatment [4]. As a result, further understanding the pathogenesis of the disease is needed and provides opportunities for the discovery of new therapeutic targets.

Cellular immune responses, which are mediated mainly by T cells, play a central role in protective immunity against MTB infection. Among them, CD4$^+$ Th1 cells are critical for macrophage activation and bacterial clearance, based on the secretion of IFN-γ, TNF-α and IL-2 [5–7]. Nevertheless, T cell dysfunction known as exhaustion is commonly noted in patients with MTB infection [8–11], which is characterized as the loss of effector function including cytokine secretion ability under antigen stimulation [12]. Emerging inhibitory receptors and molecules, such as PD-1, Tim-3 and CTLA-4, are reported to mediate Th1 cell dysfunction during MTB infection [13, 14]. Inhibition of these checkpoint receptors has displayed the potential to reverse T cell dysfunction and boost anti-pathogen immunity [12, 15]. Consistent with this notion, previous studies have showed that PD-1/PD-L1 pathway inhibits both adaptive and innate immunity [14, 16], and blocking PD-1/PD-L1 interaction can augment the production of IFN-γ by T cells in patients with TB [17]. Paradoxically, other studies have observed aggravated MTB infection and a more serious disease after PD-1 blockade [18, 19]. Discrepancy in the two different outcomes may be due to the critical role of PD-1 in preventing lethal immune-mediated pathology during MTB infection [20]. These data indicate that the underlying mechanisms of T cell exhaustion in patients with TB remain to be uncovered.

CD39, originally known as ectoNTPDase-1 (ENTPD1), acts by degrading adenosine triphosphate (ATP) into adenosine (ADO) [21, 22]. Increasing evidences support the notion that CD39 is a new inhibitory receptor and highly expressed on exhausted tumour-specific CD8$^+$ T cells [23]. Accordingly, blockade of the CD39 pathway can partly restore the function of CD8$^+$ T cells in tumour microenvironment [24]. However, the role of CD39 pathway in regulating MTB infection remains rarely investigated. Limited studies found that CD39 expression was increased on regulatory T cells (Treg) and involved in mediating suppression of them [25]. Currently, little is known about the role of CD39 in regulating Th1 cell function, particularly in patients with TB. Here, we describe that CD39 was predominantly expressed on MTB-specific CD4$^+$ T cells and involved in the inhibitory regulation of Th1 cell function in patients with TB. Inhibiting CD39 pathway could partially restore the function of Th1 cells. But interestingly, the expression of PD-1 on CD39$^+$ Th1 cells could be used as an indicator for predicting the effect of PD-1 blockade.

METHODS AND MATERIALS

Study design

Active patients with TB were recruited from Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology between January 2019 to January 2022. The diagnosis of TB was established based on clinical and radiological data together with the identification of MTB in sputum or bronchoalveolar lavage fluid using mycobacterial culture or GeneXpert MTB/RIF. For the collection of bronchoalveolar lavage fluid, 2% lidocaine was injected into the segment of the lung for local anaesthesia. A total of 150 ml sterile saline was instilled into the right middle lobe or the left lingual segment of the lung. Bronchoalveolar lavage fluid was retrieved by gentle syringe suction and put into sterile containers. The healthy controls (HCs) were recruited among individuals with negative T-SPOT.TB results and without any symptoms or signs suspected as active diseases. Participants in HC group were matched to patients with TB according to sex and age (Table S1). Anti-TB treatment was performed with isoniazid, rifampicin, pyrazinamide and ethambutol. Patients were given standard anti-TB treatment consisting of 2 months of rifampicin, isoniazid, ethambutol and pyrazinamide and 4 months of rifampicin and isoniazid after active TB diagnosis. After treatment, the negative GeneXpert MTB/RIF results and relief of the patient's symptoms were considered signs of effective treatment. This study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-IRB20190421). Written informed consent was obtained from all the participants.

Cell isolation

Peripheral blood (PB) samples were collected in heparinized tubes from all individuals. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll–Hypaque gradient centrifugation and suspended in complete RPMI-1640. Enriched CD4$^+$ cells were obtained by positive selection using magnetic beads (Miltenyi Biotec). The purity of the cell fractions was evaluated by flow cytometry (>95% for CD4$^+$ T cells).

Flow cytometry

Polychromatic flow cytometry was used to detect the expressions of surface markers and intracellular cytokines. For surface marker staining, fluorescence-labelled

monoclonal antibodies were added to cell suspensions and incubated for 30 min at room temperature in the dark. In some experiments, the cells were fixed and permeabilized after surface staining, then stained with anti-IFN-γ, anti-TNF-α, anti-IL-2. Isotype controls with irrelevant specificities were included as negative controls. Fixable Viability Stain 700 was used to exclude dead cells from analysis. After washings, the cells were resuspended in 300 μl staining buffer, followed by analysis with FACSCanto II flow cytometer (BD Biosciences). Data were subsequently analysed with FlowJo software (Tree Star Inc.). The used antibodies in the present study were summarized in Table S2.

Tetramer analysis

MHC peptide tetramer was used to evaluate MTB-specific $CD4^+$ T cells in patients with TB. Briefly, DNA was extracted from PBMCs to determine high-resolution human leukocyte antigen (HLA) class II genotypes by polymerase chain reaction using sequence-specific primers. HLA allele ambiguities were resolved by allele-specific DNA sequencing. For MTB-specific MHC peptide tetramer staining, 2 mg/ml MHC class II tetramers loaded with MTB ESAT-6 (ProImmune) was added to PBMC samples and incubated for 1 h at 37°C. Cells were then washed and stained with surface marker antibodies. Appropriate isotype-matched control was used to determine the background level of staining. After washings, the cells were resuspended in 300 μl staining buffer, followed by analysis with flow cytometer.

T-cell proliferation

T-cell proliferation assays were performed as previously described [26]. Briefly, sorted $CD4^+$ T cells were labelled with 2.5 μM carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) (BD Horizon) for 10 min at 37°C. After that, the cells were stimulated with 2.5 μg/ml anti-CD28, 2.5 μg/ml anti-CD3 and 20 ng/ml IL-2 and cultured in 5% CO_2 at 37°C. After 4 days of culture, cells were harvested and analysed for CFSE intensities via flow cytometry.

Transcriptome sequencing of $CD39^+$ and $CD39^-$ Th1 cells

To investigate the transcriptome of $CD39^+$ Th1 cells, single-cell RNA (scRNA) sequencing was performed on PBMCs isolated from six patients with TB. Briefly, single-cell capture was achieved by random distribution of a single-cell suspension across >200 000 microwells through a limited dilution approach. Beads with oligonucleotide barcodes were added to saturation so that a bead was paired with a cell in a microwell. Cell-lysis buffer was added so that poly-adenylated RNA molecules hybridized into the beads. Beads were collected into a single tube for reverse transcription. On cDNA synthesis, each cDNA molecule was tagged on the 5′ end (that is, the 3′ end of a mRNA transcript) with a molecular index and cell label indicating its cell of origin. Whole transcriptome libraries were prepared using the BD Resolve single-cell whole-transcriptome amplification workflow. In brief, the second-strand cDNA was synthesized, followed by ligation of the adaptor for universal amplification. Eighteen cycles of PCR were used to amplify the adaptor-ligated cDNA products. Sequencing libraries were prepared using random priming PCR of the whole-transcriptome amplification products to enrich the 3′ end of the transcripts linked with the cell label and molecular indices. Sequencing was performed with Illumina (NovaSeq) according to the manufacturer's instructions (Illumina). Th1 cells were determined based on canonical markers including *IFNG, NKG7, CCL4, CCL5* [27]. $CD39^+$ and $CD39^-$ cells were differentiated by the staining of AbSeq antibody-oligo-conjugates targeting CD39 [28]. Raw sequencing data are available at the NCBI Sequence Read Archive (SRA) under the accession numbers SRR19547952-SRR19547962.

Bioinformatic analysis of scRNA sequencing

The BD Resolve analysis pipeline was used to process sequencing data. The R package "Seurat" was used to analyse the matrix obtained from BD pipeline, normalize data, dimensionality reduction, clustering and differential expression. we used Seurat alignment method canonical correlation analysis [29] for integrated analysis of datasets. For clustering, highly variable genes were selected, and the principal components based on those genes were used to build a graph, which was segmented with a resolution of 0.6. Based on filtered gene expression matrix by Seurat, samples differential expression analysis was carried out using the edgeR package [30] to obtain zone-specific marker genes. we used Benjamini–Hochberg correction to adjust the *p* values. The R package "clusterProfiler" was used to perform gene set enrichment analysis (GSEA).

Inhibition of CD39, CD73 or $A_{2A}R$

For the inhibition of CD39, freshly isolated cells were firstly preincubated with 10 μM sodium metatungstate (POM-1) (Selleck) for 2 h. Next, 100 μM ATP (Selleck)
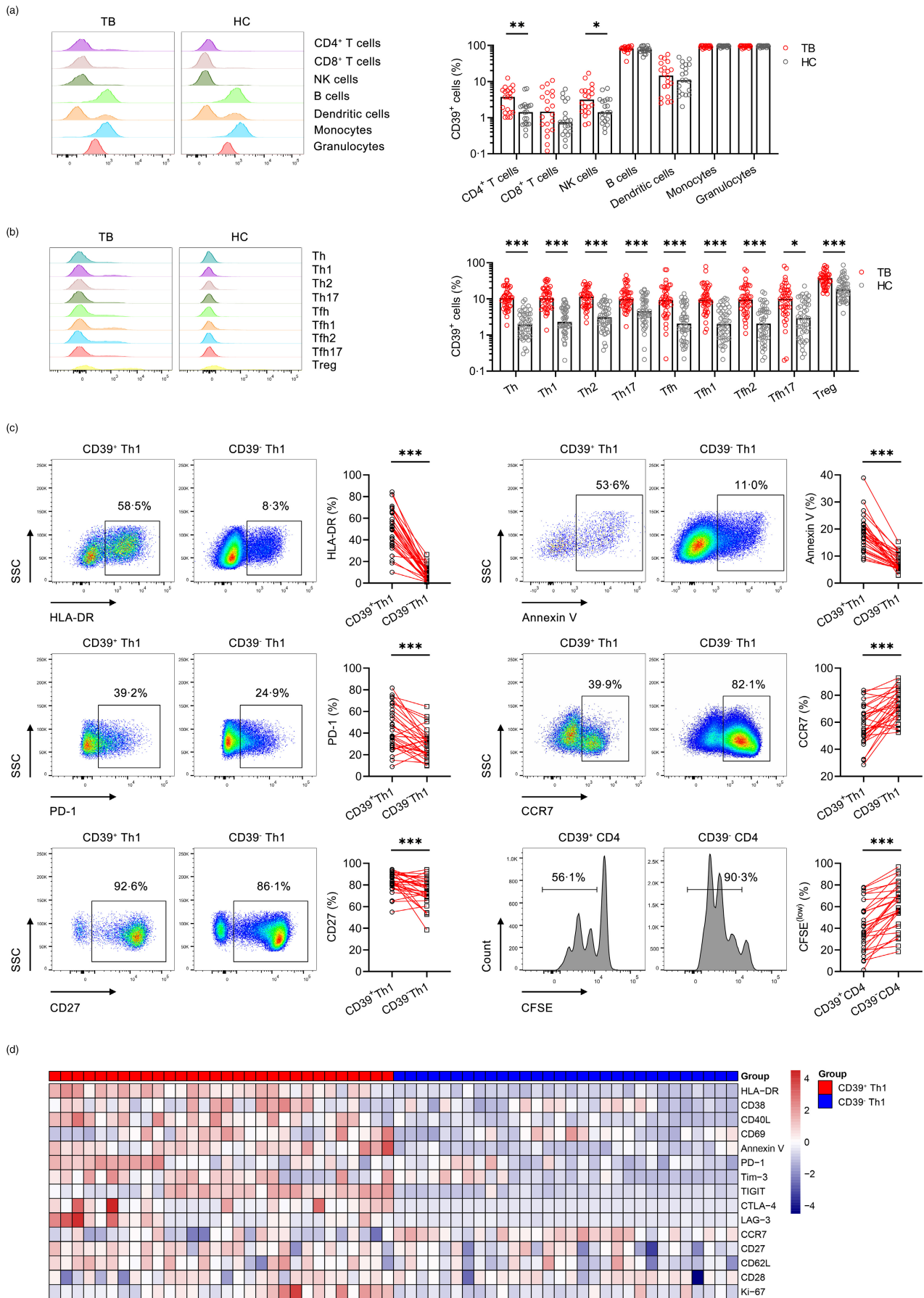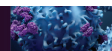
FIGURE 1 Legend on next page.

was added to the culture system, and the cells were incubated for 24 h. For the inhibition of CD73, freshly isolated cells were firstly preincubated with 1 μM LY-3475070 (Selleck) for 2 h. Next, 100 μM adenosine monophosphate (AMP) (Selleck) was added to the culture system, and the cells were incubated for 24 h. For the inhibition of A_2AR, freshly isolated cells were firstly preincubated with ZM241385 (1 μM) (TargetMol) or Istradefylline (1 μM) (Selleck) for 2 h. Next, 5 μM ADO (Selleck) was added to the culture system, and the cells were incubated for 24 h. A total of 1 million cells were added to each well. When it is difficult to meet the cell number requirement, we would make the number of cells in the experimental group as the same as that in the control group.

Statistical analysis

Comparisons between groups were conducted using Mann–Whitney U test or Wilcoxon test. Significance was determined when $p < 0.05$. Pearson correlation analysis was used to evaluate the relationship between CD39 expression and clinical indicators. Results were graphed and analysed using SPSS 25.0 (SPSS Inc.), GraphPad Prism 8.0 (GraphPad Software, Inc.), MedCalc version 11.6 (MedCalc) and R 4.0.2 program (R Core Team).

RESULTS

Th1 cells highly expressed CD39 in TB

The expression of CD39 on various immune cells, including CD4$^+$ T cells, CD8$^+$ T cells, NK cells, B cells, dendritic cells, monocytes and granulocytes (gating strategies were shown in Figure S1a,b), was compared between patients with TB and HC. Although CD39 was highly expressed on B cells, dendritic cells, monocytes and granulocytes, no significant difference was observed between patients with TB and HC (Figure 1a). Notably, patients with TB displayed dramatically higher CD39 expression on CD4$^+$ T cells ($p = 0.0057$) and NK cells ($p = 0.031$), but not on CD8$^+$ T cells, compared with HC (Figure 1a). More specifically, the expression of CD39 on different subsets of CD4$^+$ T cells, including regulatory T cells (Treg), T-helper cells (Th) and follicular T-follicular-helper cells (Tfh), was examined by using surface marker staining (CD25, CD127, CD45RA, CXCR5, CXCR3 and CCR6) (gating strategies were shown in Figure S1c). Expectedly, a dramatically increased expression of CD39 on different subsets of CD4$^+$ T cells, including Treg ($p < 0.001$), Th ($p < 0.001$), Th1 ($p < 0.001$), T-helper 2 (Th2) ($p < 0.001$), T-helper 17 (Th17) ($p < 0.001$), Tfh ($p < 0.001$), T-follicular-helper 1 (Tfh1) ($p < 0.001$), T-follicular-helper 1 (Tfh2) ($p < 0.001$) and T-follicular-helper 17 (Tfh17) ($p = 0.035$), were noted in patients with TB in comparison with HC (Figure 1b).

CD39$^+$ Th1 cells display activation, effector memory and exhaustion phenotype in TB

Because Th1 cells play central roles in protective immunity against MTB infection, we, thus, focused on the role of CD39 in regulating Th1 cells in patients with TB. We observed a significantly higher expression of activation markers (HLA-DR ($p < 0.001$), CD38 ($p = 0.012$), CD69 ($p < 0.001$) and CD40L ($p < 0.001$)) and exhaustion markers (PD-1, Tim-3, TIGIT, LAG-3 and CTLA-4) ($p < 0.001$), but lower expression of naïve markers (CCR7) on CD39$^+$ Th1 cells compared with CD39$^-$ Th1 cells ($p < 0.001$) (Figure 1c,d, Figure S2). CD39$^+$ Th1 cells expressed higher levels of the cell cycle protein Ki-67 ($p < 0.001$) and apoptosis marker Annexin V ($p < 0.001$) than CD39$^-$ Th1 cells, supporting a more differentiated status of CD39-expressing Th1 cells (Figure 1c,d, Figure S2). Consistent with this notion, it was found that the proliferation of CD39$^+$CD4$^+$ T cells was significantly lower than that of CD39$^-$CD4$^+$ T cells, on CD3/CD28 antibodies and IL-2 stimulation ($p < 0.001$) (Figure 1c). These data suggest that the functional potential of CD39$^+$ Th1 cells was impaired in patients with TB.

FIGURE 1 Phenotype of CD39$^+$ Th1 cells in patients with TB. (a) Wave chart showing the representatives of CD39 expression on various immune cells in patients with TB and HC. Scatter plot showing the expression of CD39 on various immune cells in patients with TB and HC. Horizontal lines indicate the medians. *$p < 0.05$, **$p < 0.01$ (Mann–Whitney U test). (b) Wave chart showing the representatives of CD39 expression on various subsets of CD4$^+$ T cells in patients with TB and HC. Scatter plot showing the expression of CD39 on various subsets of CD4$^+$ T cells in TB and HC. Horizontal lines indicate the medians. ***$p < 0.001$ (Mann–Whitney U test). (c) Representative FACS plots showing the phenotype of CD39$^+$ and CD39$^-$ Th1 cells in patients with TB. Line graphs showing the comparison of the phenotype between CD39$^+$ and CD39$^-$ Th1 cells in patients with TB. ***$p < 0.001$ (Wilcoxon test). (d) Heatmap showing the phenotype of CD39$^+$ and CD39$^-$ Th1 cells in patients with TB. Each rectangle indicates a result of a subject. TB, tuberculosis; HC, healthy controls
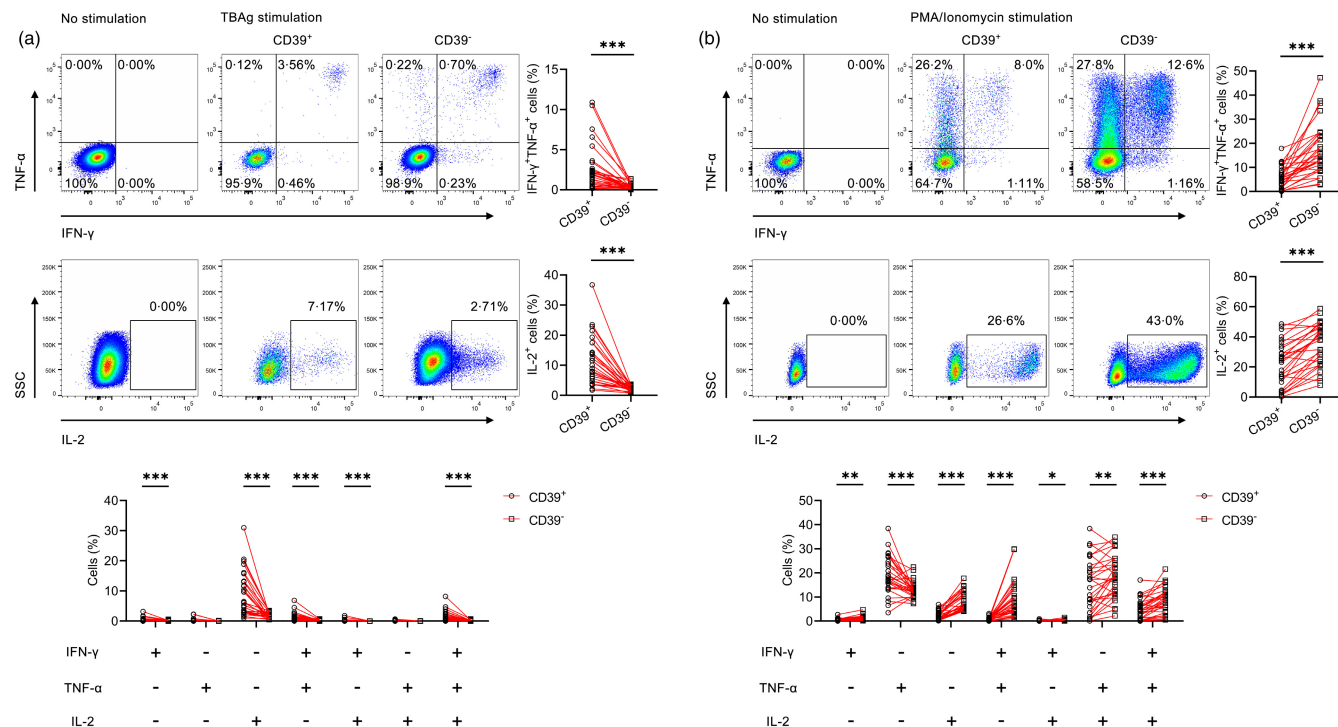
FIGURE 2 Production of Th1-type cytokine of CD39$^+$ and CD39$^-$ CD4$^+$ T cells. (a) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD39$^+$ and CD39$^-$ CD4$^+$ T cells under TBAg stimulation in patients with TB. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 between CD39$^+$ and CD39$^-$ CD4$^+$ T cells under TBAg stimulation in patients with TB. ***$p < 0.001$ (Wilcoxon test). (b) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD39$^+$ and CD39$^-$ CD4$^+$ T cells under PMA/ionomycin stimulation in patients with TB. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 between CD39$^+$ and CD39$^-$ CD4$^+$ T cells under PMA/ionomycin stimulation in patients with TB. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon test). TBAg, tuberculosis-specific antigens; PMA, Phorbol 12-Myristate 13-acetate
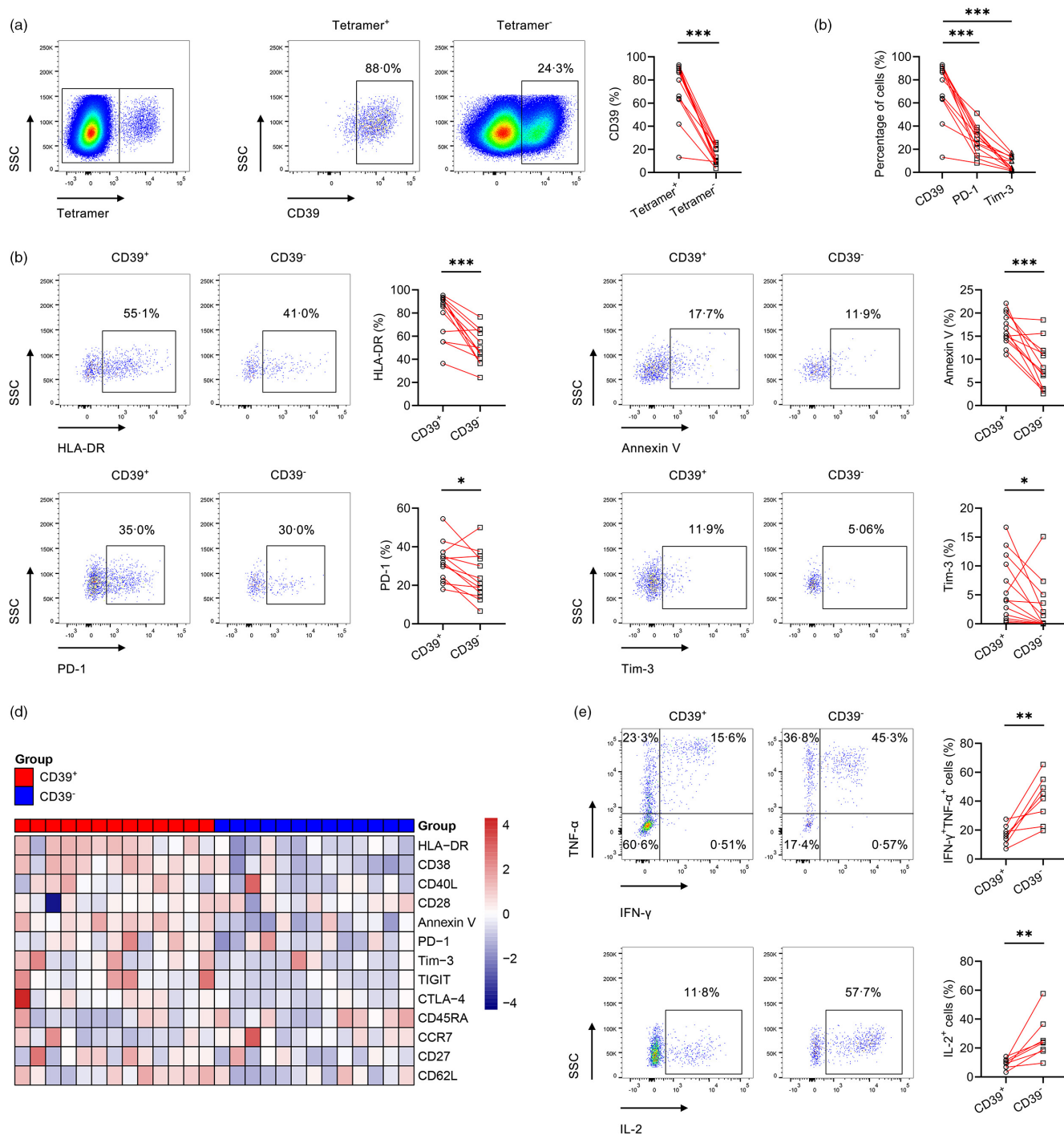
CD39$^+$CD4$^+$ T cells produce less Th1 cytokines in TB

Th1 cells protects against MTB infection mainly based on the production of Th1 cytokines including IFN-γ, TNF-α and IL-2. The cytokine production characteristics of CD39$^+$CD4$^+$ T cells was further determined on different stimulations. On TB-specific antigens (TBAg) (early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) complex) stimulation, CD39$^+$CD4$^+$ T cells showed significantly higher secretion of IFN-γ, TNF-α and IL-2 compared with CD39$^-$CD4$^+$ T cells ($p < 0.001$), supporting the ideal that CD39 was mainly expressed on MTB-specific CD4$^+$ T cells (Figure 2a). Conversely, on non-specific stimulation (Phorbol 12-Myristate 13-Acetate/ionomycin [PMA/ionomycin]), CD39$^+$CD4$^+$ T cells demonstrated significantly decreased expression of cytokines including IFN-γ, TNF-α and IL-2 compared with CD39$^-$CD4$^+$ T cells (Figure 2b). More specifically, the proportions of IFN-γ$^-$TNF-α$^-$IL-2$^+$, IFN-γ$^+$TNF-α$^+$IL-2$^-$ and IFN-γ$^+$TNF-α$^+$IL-2$^+$ cells were all significantly lower in CD39$^+$CD4$^+$ T cells than CD39$^-$CD4$^+$ T cells ($p < 0.001$) (Figure 2b). However, the proportion of IFN-γ$^-$TNF-

α$^+$IL-2$^-$ cells was significantly higher in CD39$^+$CD4$^+$ T cells than CD39$^-$CD4$^+$ T cells on PMA/ionomycin stimulation ($p < 0.001$) (Figure 2b).

MTB-specific CD4$^+$ T cells highly express CD39

Next, tetramer analysis was used to determine CD39 expression on MTB-specific CD4$^+$ T cells. Notably, the frequency of CD39$^+$ cells in the MTB-specific CD4$^+$ T cells (tetramer$^+$) was significantly higher than in non-MTB-specific CD4$^+$ T cells ($p < 0.001$) (Figure 3a). Meanwhile, the expression of CD39 on MTB-specific CD4$^+$ T cells was obviously higher than that of PD-1 ($p < 0.001$) or Tim-3 ($p < 0.001$) (Figure 3b). Similarly, CD39$^+$ MTB-specific CD4$^+$ T cells also displayed a significantly higher expression of activation (HLA-DR and CD38) ($p < 0.001$), exhaustion (PD-1 ($p = 0.0105$), Tim-3 ($p = 0.0134$), CTLA-4 ($p < 0.001$) and TIGIT ($p < 0.001$)) and apoptosis (Annexin V) ($p < 0.001$) markers than CD39$^-$ MTB-specific CD4$^+$ T cells (Figure 3c,d, Figure S3). Conversely, the expression of naïve marker CCR7 was significantly lower on
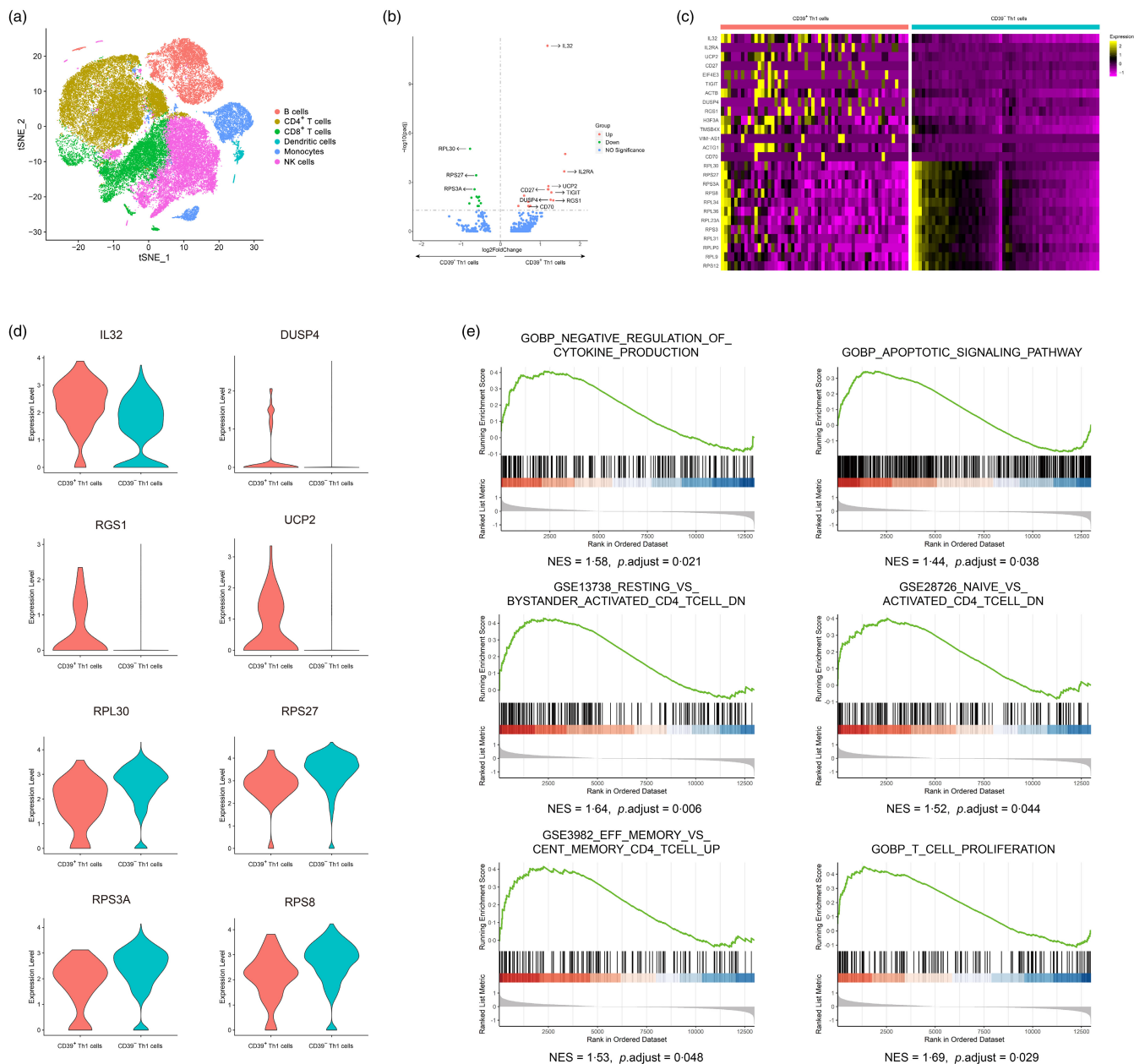
FIGURE 3 Phenotype of MTB-specific CD39^{+} cells. (a) Representative FACS plots showing the expression of CD39 on tetramer^{+} and tetramer^{−} CD4^{+} T cells in patients with TB. Line graphs showing the comparison of CD39 expression between tetramer^{+} and tetramer^{−} CD4^{+} T cells in patients with TB. ***$p < 0.001$ (Wilcoxon test). (b) Line graphs showing the expression of CD39, PD-1, Tim-3 on tetramer^{+}CD4^{+} T cells in patients with TB. ***$p < 0.001$ (Wilcoxon test). (c) Representative FACS plots showing the phenotype of CD39^{+} and CD39^{−} tetramer^{+}CD4^{+} T cells in patients with TB. Line graphs showing the comparison of phenotype between CD39^{+} and CD39^{−} tetramer^{+}CD4^{+} T cells in patients with TB. *$p < 0.05$, ***$p < 0.001$ (Wilcoxon test). (d) Heatmap showing the phenotype of CD39^{+} and CD39^{−} tetramer^{+}CD4^{+} T cells in patients with TB. Each rectangle indicates a result of a subject. (e) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD39^{+} and CD39^{−} tetramer^{+}CD4^{+} T cells under PMA/ionomycin stimulation in patients with TB. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 between CD39^{+} and CD39^{−} tetramer^{+}CD4^{+} T cells under PMA/ionomycin stimulation in patients with TB. **$p < 0.01$ (Wilcoxon test). MTB, *mycobacterium tuberculosis*; PMA, Phorbol 12-Myristate 13-acetate

FIGURE 4 Transcriptional analysis of CD39$^+$ and CD39$^-$ Th1 cells in patients with TB. (a) T-SNE plot depicting clustering of all captured PBMCs. (b) Volcano plots depict the differential expression of the assessed genes between CD39$^+$ and CD39$^-$ Th1 cells. Red dots: Up-regulated genes; green dots: Down-regulated genes. A-axis: log2 fold change; y-axis: -log10(padj). (c) Heatmap displaying the differentially expressed genes between CD39$^+$ and CD39$^-$ Th1 cells. (d) Violin chart showing the differential gene expression between CD39$^+$ and CD39$^-$ Th1 cells. (e) GSEA showing the significantly enriched pathways of expressed genes in CD39$^+$ Th1 cells. T-SNE, T-distributed stochastic neighbour embedding; GSEA, gene set enrichment analysis

CD39$^+$ MTB-specific CD4$^+$ T cells compared with CD39$^-$ MTB-specific CD4$^+$ T cells ($p = 0.0061$) (Figure 3d, Figure S3). Notably, on PMA/ionomycin stimulation, the frequencies of multifunctional IFN-γ^+TNF-α^+ ($p = 0.0078$) and IL-2$^+$ ($p = 0.0078$) cells were significantly decreased in CD39$^+$ MTB-specific CD4$^+$ T cells compared with CD39$^-$ MTB-specific CD4$^+$ T cells (Figure 3e). These data confirmed that CD39 was predominantly expressed on MTB-specific CD4$^+$ T cells and associated with the dysfunction of them.

Transcriptomic characteristics of CD39$^+$ Th1 cells in TB

The global gene expression profiles of CD39-expressing Th1 cells were analysed for further characterizing CD39$^+$ Th1 cells in TB. We used unbiased clustering approaches to identify whether CD39$^+$ and CD39$^-$ Th1 cells showed distinct patterns of gene expression. When genes with adjusted p value <0.05 were considered, 27 genes were
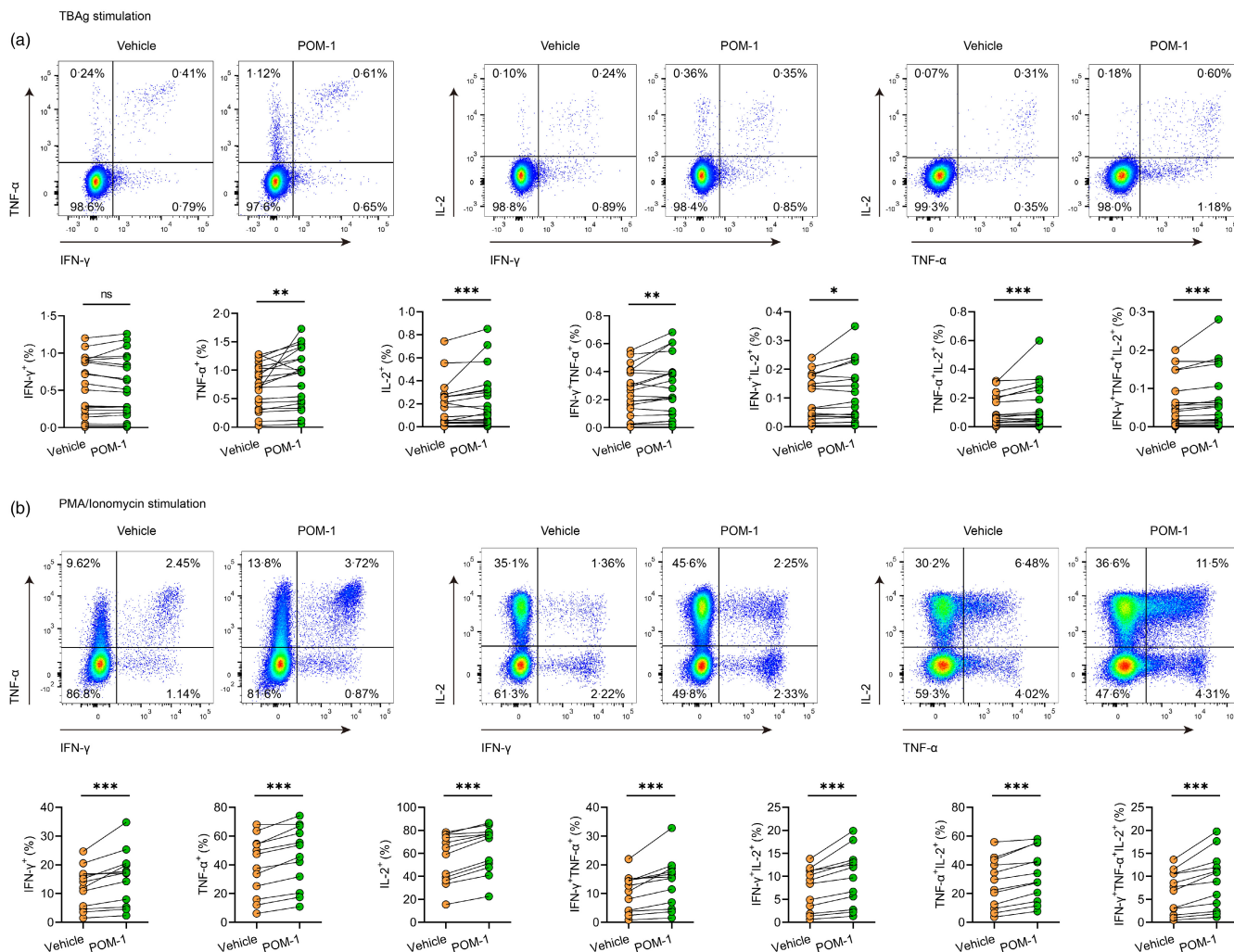
FIGURE 5 The effect of the inhibition targeting CD39 for Th1 cytokine secretion on CD4^{+} T cells from patients with TB. (a) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD4^{+} T cells before and after POM-1 inhibition under TBAg stimulation. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of CD4^{+} T cells before and after POM-1 inhibition under TBAg stimulation. Ns, no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon test). (b) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD4^{+} T cells before and after POM-1 inhibition under PMA/ionomycin stimulation. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of CD4^{+} T cells before and after POM-1 inhibition under PMA/ionomycin stimulation. ***$p < 0.001$ (Wilcoxon test). TB, tuberculosis; TBAg, tuberculosis-specific antigens; PMA, Phorbol 12-Myristate 13-acetate

found to be differentially expressed between CD39^{+} and CD39^{−} Th1 cells; 15 (*IL-32, DUSP4, TIGIT, UCP2* and so on) were up-regulated and 12 (*RPL30, RPS27, RPS3A, RPS8* and so on) were down-regulated (Figure 4a–d). *IL32*, a marker of T-cell activation, was the gene with highest fold difference between the two groups (adjusted p value = 6.85E-16). Inspection of the list of differentially expressed genes revealed many with known roles in Th1 cell biology such as increased expression of the inhibitory receptor gene *TIGIT* and negative modulation of proliferation gene *DUSP4* in CD39^{+} Th1 cells. Subsequently, GSEA showed that the expressed genes in CD39^{+} Th1 cells were significantly enriched in pathways associated with the negative regulation of cytokine production, apoptosis, activation and proliferation (Figure 4e).

Inhibition of CD39 pathway restores Th1 cell function in TB

CD4^{+} T-cell exhaustion has been identified as an important cause for TB occurrence. We, thus, assessed whether inhibiting CD39 pathway could restore Th1 cell function in patients with TB by using CD39 inhibitor POM-1. Expectedly, inhibiting CD39 pathway could significantly enhance the frequency of Th1-cytokine producing CD4^{+} T cells, especially multifunctional CD4^{+} T cells, under no matter TBAg or PMA/ionomycin stimulation (Figure 5a,b). Besides, we observed a significantly increased expression of HLA-DR on CD4^{+} T cells after CD39 inhibition under PMA/ionomycin stimulation ($p < 0.001$) (Figure S4a). However, we failed to find any difference in CD38 expression
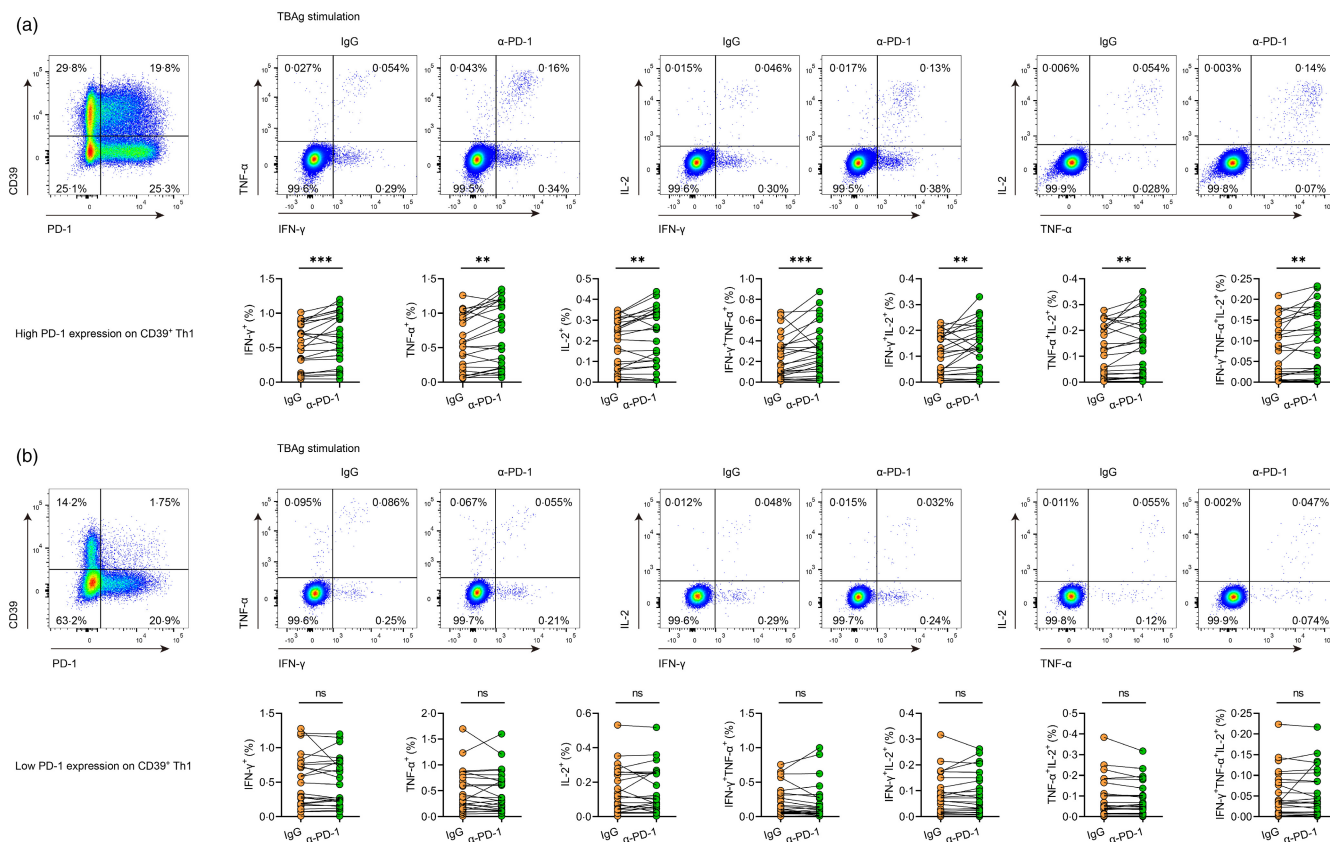
FIGURE 6 The relationship between PD-1 expression on CD39$^+$ Th1 cells and the effect of α-PD-1 blockade for Th1 cytokine secretion under TBAg stimulation. (a) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation in patients with high PD-1 expression on CD39$^+$ Th1 cells (≥14%). Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation in patients with high PD-1 expression on CD39$^+$ Th1 cells (≥14%). **$p < 0.01$, ***$p < 0.001$ (Wilcoxon test). (b) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation in patients with low PD-1 expression on CD39$^+$ Th1 cells (<14%). Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation in patients with low PD-1 expression on CD39$^+$ Th1 cells (< 14%). Ns, no significance (Wilcoxon test). TBAg, tuberculosis-specific antigens

before and after CD39 inhibition (Figure S4a). Meanwhile, inhibiting CD39 pathway had no effect on the frequency of Annexin V$^+$ apoptotic cells (Figure S4b). Notably, inhibiting CD39 pathway could increase the proliferation of CD4$^+$ T cells from patients with TB ($p = 0.0094$) (Figure S4c).

PD-1 expression on CD39$^+$ Th1 cells decides the effect of anti-PD-1

We further determined whether there is a synergistic effect between CD39 and PD-1 blockade, as blockade of PD-1 signalling is considered to be the most promising strategy for restoring exhausted T cells in chronic infection and tumour. We found that the combination of CD39 and PD-1 blockade displayed a synergistic effect on the production of IL-2 by CD4$^+$ T cells on TBAg stimulation ($p < 0.001$) (Figure S5).

But importantly, we observed that the effect of PD-1 blockade could be stratified by the expression level of PD-1 on CD39$^+$ Th1 cells in TB (Figure 6). More specifically, under MTB-antigen stimulation, using anti-PD-1 antibody demonstrated a significantly increased proportion of IFN-γ$^+$, TNF-α$^+$ and IL-2$^+$ CD4$^+$ T cells in patients with TB with high PD-1 expression (≥14%) on CD39$^+$ Th1 cells, compared with using control IgG (Figure 6a). However, no differences were detected in Th1 cytokine production of CD4$^+$ T cells in patients with TB with low PD-1 expression (<14%) on CD39$^+$ Th1 cells between anti-PD-1 and control groups (Figure 6b). But it is noteworthy that, if we classified patients according to PD-1 expression on total Th1 cells but not on CD39$^+$ Th1 cells, no obvious association was observed between PD-1 expression on Th1 cells and the effect of PD-1 blockade on the production of Th1 cytokine (Figure S6). We also compared the change of Th1-cytokine production after anti-PD-1 treatment between CD39$^+$ and CD39$^-$ CD4$^+$
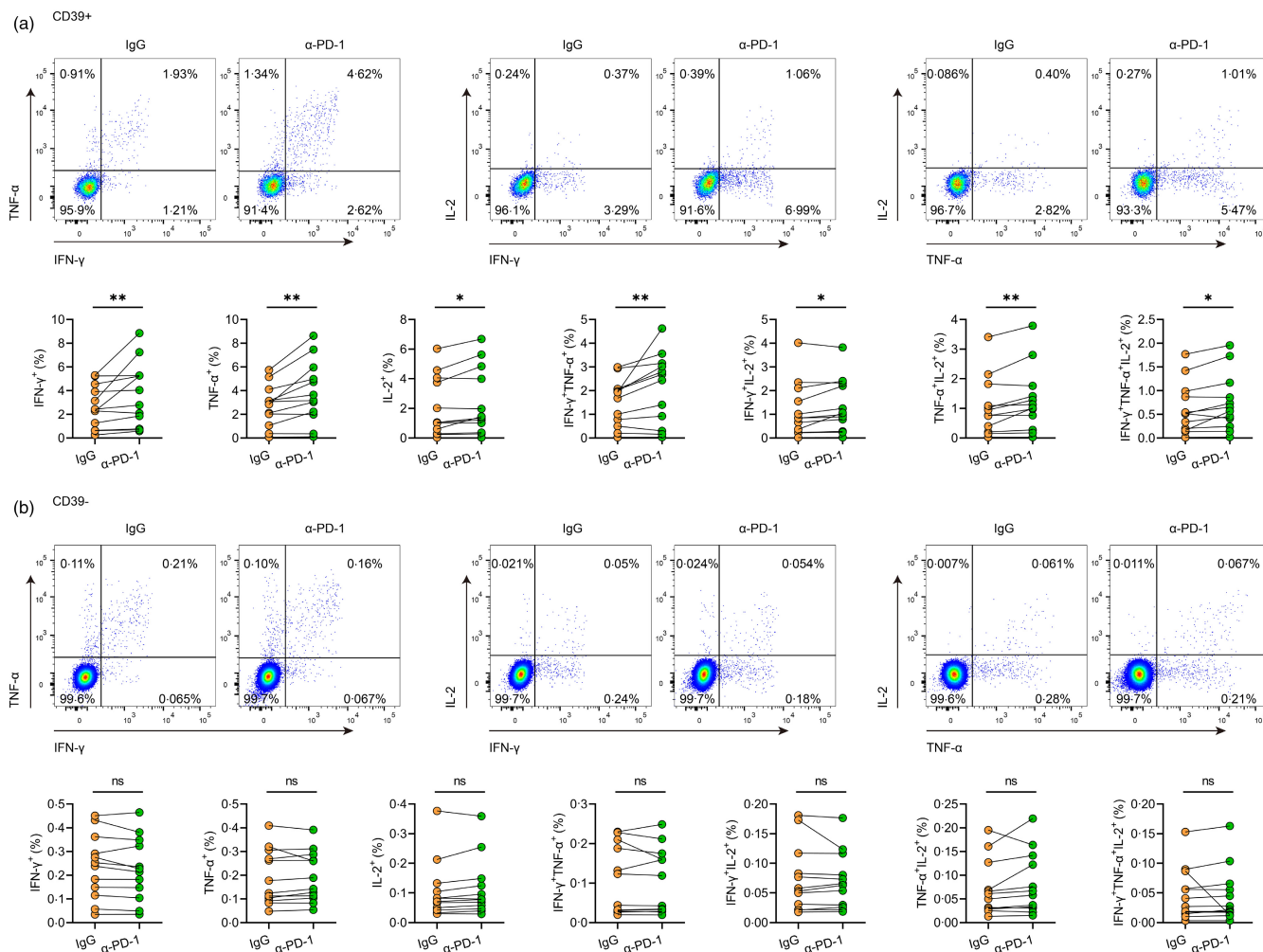
FIGURE 7 The effect of α-PD-1 blockade on CD39$^+$ and CD39$^-$ CD4$^+$ T cells in patients with TB with high PD-1 expression under TBAg stimulation. (a) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD39$^+$CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of CD39$^+$CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation. *$p < 0.05$, **$p < 0.01$ (Wilcoxon test). (b) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of CD39$^-$CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of CD39$^-$CD4$^+$ T cells before and after α-PD-1 blockade under TBAg stimulation. Ns, no significance (Wilcoxon test). TB, tuberculosis; TBAg, tuberculosis-specific antigens

T cells in patients with TB with high PD-1 expression (≥14%). It was observed that CD39$^+$ T cells showed obvious increase in the production of IFN-γ, TNF-α and IL-2 after anti-PD-1 treatment, while there was no statistical difference in the production of IFN-γ, TNF-α and IL-2 on CD39$^-$ T cells before and after anti-PD-1 treatment (Figure 7). These data emphasized that the expression level of PD-1 on CD39$^+$ MTB-specific Th1 cells decides the effect of anti-PD-1.

Inhibition of A$_{2A}$R pathway restores Th1 cell function in TB

Through CD39 and CD73, ATP can be eventually converted to ADO. ADO can bind to T cell surface receptors such as A$_{2A}$R for subsequent intracellular regulation. Therefore, we

assessed whether inhibiting A$_{2A}$R could restore Th1 cell function in patients with TB by using A$_{2A}$R inhibitor such as ZM241385 and Istradefylline. We found that inhibiting A$_{2A}$R could significantly enhance the frequency of IL-2$^+$, IFN-γ$^+$TNF-α$^+$ and TNF-α$^+$IL-2$^+$ CD4$^+$ T cells under TBAg stimulation (Figure 8a). Meanwhile, inhibiting A$_{2A}$R could also significantly enhance the frequency of Th1-cytokine producing CD4$^+$ T cells under PMA/ionomycin stimulation (Figure 8b). Besides, we observed a significantly elevated expression of HLA-DR on CD4$^+$ T cells after using ZM241385 under TBAg or PMA/ionomycin stimulation (Figure S7a). However, no significant difference was observed in CD38 expression before and after A$_{2A}$R inhibition (Figure S7a,d). Besides, inhibiting A$_{2A}$R had no effect on the apoptosis (Figure S7b,e). Notably, inhibiting A$_{2A}$R could increase the proliferation of CD4$^+$ T cells from
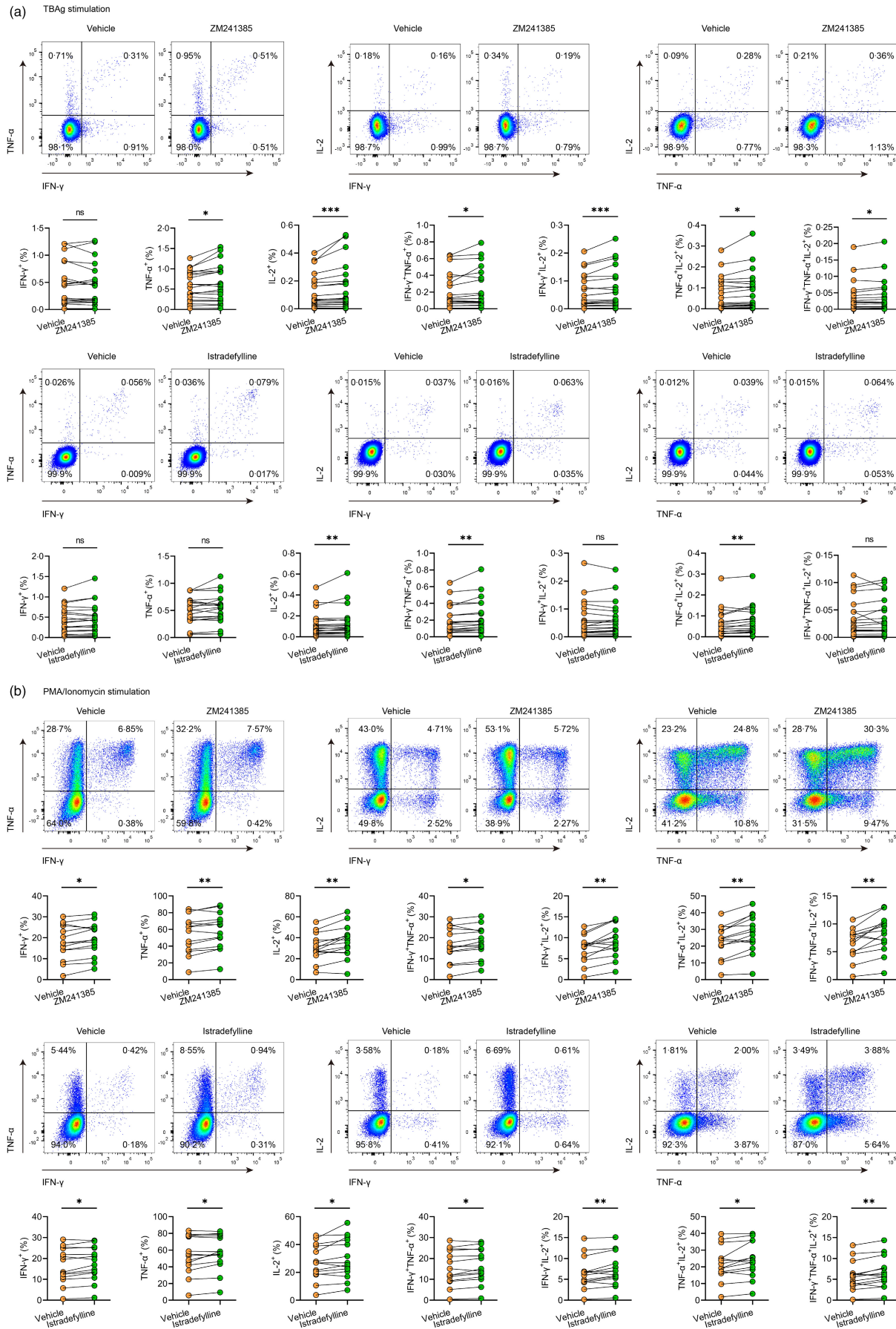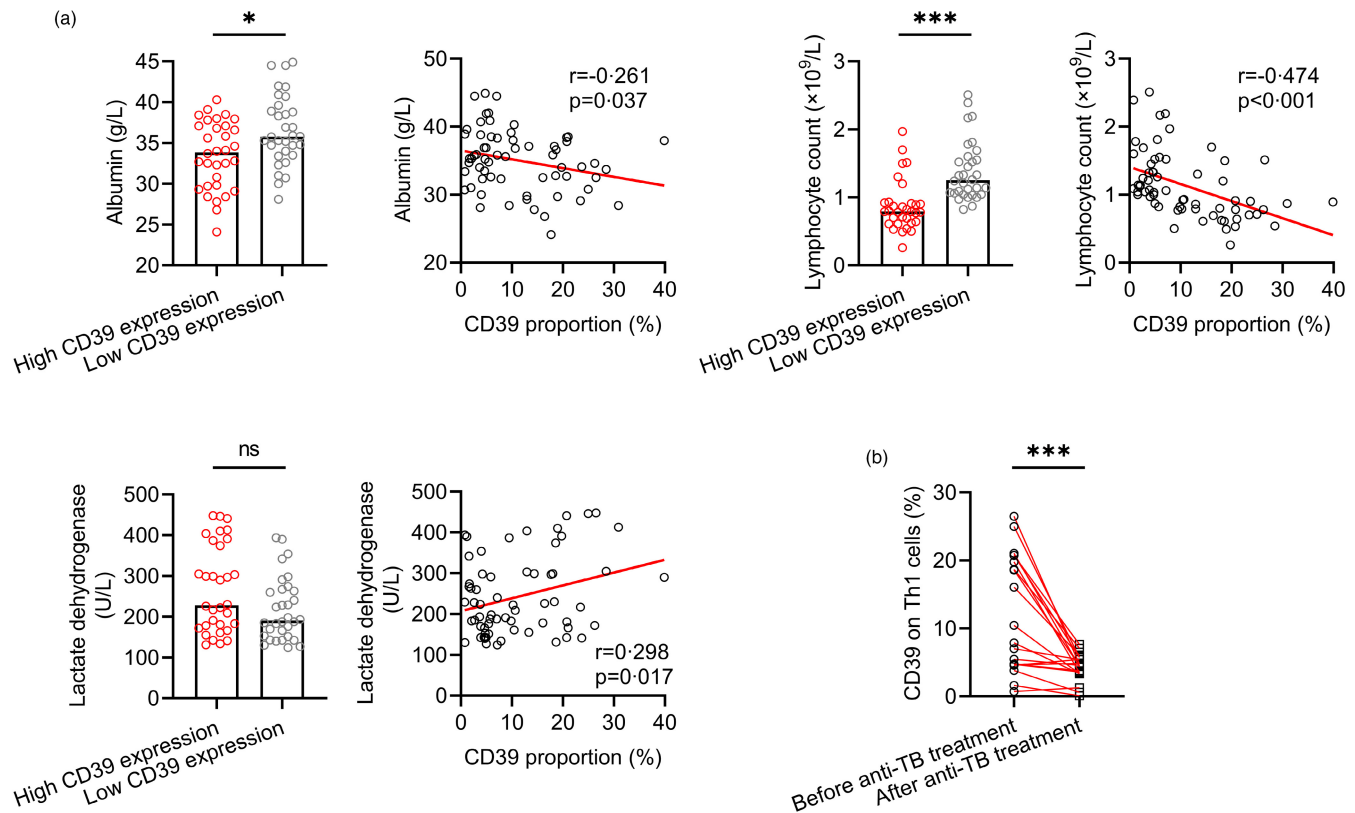
FIGURE 8 Legend on next page.

FIGURE 9 The relationship between clinical indicators and CD39 expression on Th1 cells. (a) Column diagram showing the results of laboratory indicators in patients with TB with high (>7.5%) and low (<7.5%) CD39 expression on Th1 cells. Horizontal lines indicate the medians. Ns, no significance, *$p < 0.05$, ***$p < 0.001$ (Mann–Whitney U test). Scatter plot showing the correlation between CD39 expression on Th1 cells and laboratory indicators. (b) Line graphs showing the comparison of the CD39 expression on Th1 cells before and after anti-TB treatment. ***$p < 0.001$ (Wilcoxon test). TB, tuberculosis

patients with TB (Figure S7c,f). We further evaluated the effect of the inhibition for CD39 and $A_{2A}R$ on the intracellular cyclic adenosine monophosphate (cAMP). It was observed that the inhibition for both CD39 and $A_{2A}R$ could reduce the concentration of intracellular cAMP of $CD4^+$ T cells from patients with TB (Figure S8).

High CD39 expression on Th1 cells indicates poor prognosis in patients with TB

We finally assessed the relationship between CD39 expression on Th1 cells and the clinical characteristics of patients

with TB. It was found that patients with TB with high CD39 expression displayed significantly lower nutritional status indicators such as albumin level ($p = 0.0137$) and lymphocyte count ($p < 0.001$) than those with low CD39 expression (Figure 9a). Furthermore, these nutritional status indicators, especially the lymphocyte count, were dramatically negatively correlated with the frequency of $CD39^+$ Th1 cells ($p = 0.037$) (Figure 9a). Conversely, there was a significant positive correlation between host cellular damage marker lactate dehydrogenase and CD39 expression on Th1 cells ($p = 0.017$) (Figure 9a). Surprisingly, we failed to find any difference in inflammatory indicators including hypersensitive C-reactive protein, erythrocyte sedimentation rate and procalcitonin, between patients with high and low CD39

FIGURE 8 The effect of the inhibition targeting $A_{2A}R$ for Th1 cytokine secretion on $CD4^+$ T cells from patients with TB. (a) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of $CD4^+$ T cells before and after ZM241385 or Istradefylline inhibition under TBAg stimulation. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of $CD4^+$ T cells before and after ZM241385 or Istradefylline inhibition under TBAg stimulation. Ns, no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon test). (b) Representative FACS plots showing the secretion of IFN-γ, TNF-α and IL-2 of $CD4^+$ T cells before and after ZM241385 or Istradefylline inhibition under PMA/ionomycin stimulation. Line graphs showing the comparison of the secretion of IFN-γ, TNF-α and IL-2 of $CD4^+$ T cells before and after ZM241385 or Istradefylline inhibition under PMA/ionomycin stimulation. *$p < 0.05$, **$p < 0.01$ (Wilcoxon test). TB, tuberculosis; TBAg, tuberculosis-specific antigens; PMA, Phorbol 12-Myristate 13-acetate

expression on Th1 cells (Figure S9). We also repeatedly tested CD39 expression on Th1 cells in patients with TB before and after successful anti-TB treatment. Strikingly, the proportion of CD39$^+$ cells within Th1 cells was significantly decreased after successful anti-TB treatment ($p < 0.001$) (Figure 9b).

DISCUSSION

More and more studies have concluded that patients with immunosuppression status, such as AIDS, transplant recipients, and those undergoing immunosuppressant treatment, have increased risk of active TB, which highlights the importance of host immunity in protecting against MTB infection [31, 32]. Accordingly, the exhaustion of T cells, especially MTB-specific CD4$^+$ T cells, is considered to be the most important cause of TB occurrence. Although extensive research has focused on this area, the mechanism of CD4$^+$ T cell exhaustion in patients with TB remains obscure. In this study, we first demonstrated that the new inhibitory receptor CD39 was predominantly expressed on MTB-specific CD4$^+$ T cells and associated with the dysfunction of them. Inhibition of CD39 pathway could restore MTB-specific CD4$^+$ T cell function. Meanwhile, high PD-1 expression on CD39$^+$ Th1 cells predicted good effect of anti-PD-1. The present study put forwards that CD39 is a prominent marker of exhausted MTB-specific CD4$^+$ Th1 cells and could serve as a therapeutic target in TB.

Although many studies have focused on the pathogenesis of TB, the mechanism of T cell exhaustion, especially the dysfunction of MTB-specific CD4$^+$ T cells, was still largely unknown. PD-1, the most widely investigated checkpoints to date, has been extensively as negative regulators of T cell activation. However, the effect of the intervention on PD-1 differed among different studies [33]. PD-1, that was previously shown to be expressed on tumour-specific lymphocytes, were also expressed on many tumour infiltrating lymphocytes (TILs), which may be one of the most important reasons to cause such disparity. That is to say, PD-1 is a widely expressed but non-specific inhibitory receptor, and restoration of non-MTB-specific CD4$^+$ T cell function through blocking PD-1 pathway would have a minor effect on the control of MTB infection. Thus, the new inhibitory receptors, which are highly expressed on antigen-specific CD4$^+$ T cells, are promising targets for restoring the function of MTB-specific CD4$^+$ T cells.

In addition to PD-1, the expression levels of many other inhibitory receptors, such as CTLA-4, Tim-3 and LAG3, are increased on activation following TCR stimulation. However, all these inhibitory receptors were widely expressed and had no characteristics of antigen specificity. Unlike these molecules, CD39 is a new inhibitory receptor that is preferentially expressed on disease-related lymphocytes. The most important evidence to support this idea is that CD39 was highly expressed by tumour-specific CD8$^+$ TILs but a striking lack of CD39 expression was observed in non-tumour-specific bystander CD8$^+$ TILs [23]. Moreover, expression of CD39 defines a population of highly exhausted CD8$^+$ T cells, whereas the absence of CD39 in CD8$^+$ TILs define a population whose phenotype is inconsistent with chronic antigen stimulation at the tumour site, consistent with a bystander role [23]. These data suggest that CD39 could serve as a prominent disease-related inhibitory receptor. Similar to tumour microenvironment, persistent MTB antigen exposure induces the exhaustion of MTB-specific CD4$^+$ T cells, which is characterized by impaired functional capacity such as reduced proliferation and cytokine production, and increased co-expression of inhibitory receptors. Thus, it is possible that CD39 may be the key regulator of MTB-specific CD4$^+$ T cell exhaustion. Consistent with this notion, the most important findings of the study were that the inhibitory receptor CD39 was predominantly expressed on MTB-specific CD4$^+$ T cells and that CD39 expression level on MTB-specific CD4$^+$ T cells was remarkably higher than other inhibitory receptors such as PD-1 and Tim-3.

Since CD4$^+$ Th1 cells are the central mediator of anti-TB immune response, the present study focused on the role of CD39 pathway in regulating CD4$^+$ Th1 cell function. As expected, CD39 expression on CD4$^+$ T cells, but not on CD8$^+$ T cells, were significantly higher in patients with TB than healthy individuals. Overall, the increase of CD39 expression in CD127^{high}CD25^{low}CD45RA$^-$CCR7$^-$CXCR5$^-$ effector CD4$^+$ Th cells was more pronounced compared with total CD4$^+$ T cells, supporting an important role of CD39 in regulating Th1 cell function. Given that CD39 was predominantly expressed on MTB-specific CD4$^+$ T cells and blockade of PD-1 signalling was considered to be the most effective strategy for restoring exhausted CD4$^+$ T cells, we hypothesized that the effect of blocking PD-1 was depending on the expression level of PD-1 on CD39$^+$ MTB-specific CD4$^+$ T cells. Interestingly, in the presence of MTB antigen stimulation, the same PD-1 blocking strategy could just significantly increase the production of cytokine in high-PD-1-expression CD39$^+$ Th1 cell group, but not in low-PD-1-expression CD39$^+$ Th1 cell group. These findings are of important value for understanding why the limit value of PD-1 blockade was noted in some conditions. Thus, our results emphasized that the effect of PD-1 blockade may be depending on the expression level of PD-1 on CD39$^+$ antigen-specific T cells.

One meaningful finding of the study was that CD39 could serve as a marker of PB MTB-specific CD4$^+$ Th1 cells and associated with the dysfunction of them. Thus, high CD39 expression on Th1 cells indicated severe exhaustion of MTB-specific CD4$^+$ Th1 cells, which was supported by our clinical data that high CD39 expression on Th1 cells indicates poor prognosis in patients with TB. To further ascertain the role of CD39 in the exhaustion of CD4$^+$ Th1 cells, transcriptome analysis was conducted between CD39$^+$ and CD39$^-$ Th1 cells by using RNA-Seq technology. In line with previous reports, differential analysis showed that CD39$^+$ Th1 cells expressed activation marker (*IL2RA*) and exhaustion marker (*TIGIT*) [34]. Regarding the intracellular signalling of CD39, several negative modulation genes such as *DUSP4* and *RGS1*, which associated with inhibition of T cell proliferation and differentiation through negatively regulate mitogen-activated protein (MAP) kinase and G-protein-coupled receptors, were significantly increased in CD39$^+$ Th1 cells compared with CD39$^-$ Th1 cells. Additionally, *UCP2*, the gene of an inner mitochondrial membrane protein that is thought to regulate energy metabolism by separating oxidative phosphorylation from ATP synthesis and reduce reactive oxygen species generation, was also increased in CD39-expressing Th1 cells. These findings confirmed that the inhibitory CD39 negatively regulate Th1 cell function by a variety of mechanisms, such as by down-regulation of signalling involving proliferation and by inducing cell apoptosis through reducing the mitochondrial membrane potential. Given the existence of CD39-CD73-A$_{2A}$R axis, we further investigated the effect of inhibiting A$_{2A}$R on the function of CD4$^+$ T cells from patients with TB. As we expected, the inhibition of A$_{2A}$R could also restore the CD4$^+$ T cells' function including Th1 cytokine secretion, activation and proliferation. Meanwhile, the ELISA assay confirmed the reduction effect of inhibiting CD39 or A$_{2A}$R on intracellular cAMP accumulation. These evidences indicated that attenuating the inhibitory effect of ADO on T cells could restore T cell function. Another question is that why the proportion of CD39$^+$CD4$^+$ T cells is elevated in patients with TB. We thought that the increase of CD39$^+$CD4$^+$ T cells in patients with TB may be caused by various factors including TCR engagement and cytokine stimulation (IL-2, TGF-β, IL-27 and IL-35) [35–38]. Some other studies found that CD39 was also regulated by genetic factors such as SNP at the position rs10748643 [39, 40]. However, our findings indicated that CD39 was highly expressed on MTB-specific cells. This phenomenon denotes that sustained MTB antigen exposure may be an important cause of elevated CD39 expression. Under the constant stimulation of the MTB antigen, the T cells gradually lose function and tend to be exhausted, with the elevated CD39 expression. Thus, the strategy targeting reducing MTB burden could be applied to reduce CD39 expression.

Several points should be mentioned. First, since both innate and acquired immunity play critical role in controlling MTB infection. The fact that CD39 expression on both NK cells and CD4$^+$ T cells was increased in patients with TB also indicated that CD39 may participant these two immune responses. To fully exploit the potential of CD39 pathway during MTB infection, more understanding of the role of CD39 pathway in regulating other immune cells is required, or the overall effect of anti-CD39 therapy in patients with TB could not be predicted. Second, CD73 is another important intermediate regulatory molecule which could convert AMP to ADO during ATP metabolism. Several studies have investigated the role of combination of anti-CD39 and anti-CD73 blockade in tumour [24]. We also investigated the effect of intervening CD73 on the function of Th1 cells. It was observed that the inhibition of CD73 could also enhance the frequency of Th1-cytokine producing CD4$^+$ T cells (Figure S10). These evidences indicated that CD73 could also serve as a target for restoring the function of Th1 cells in TB. Third, since highly expressed FcrRs on myeloid cells might affect the staining results, we compared the staining results with using Fc blocking buffer verse without using Fc blocking buffer. It was observed that the adding or not of Fc blocking buffer did not obviously influence the staining results on T cells, B cells, dendritic cells and monocytes (Figure S11). Therefore, the staining results in the present study is reliable. Fourth, because the present study was conducted on patients with TB from one hospital located in China, the conclusions drawn from our findings should be further validated in other areas. Finally, the validation of the effect of anti-CD39 on the prognosis of MTB-infected animal models is lacking in the present study, and further study should be conducted to verify this.

Collectively, our study provides evidence that CD39 is predominantly expressed on MTB-specific Th1 cells and correlated with their exhausted function. This notion is strongly supported by the fact that blockade of CD39 pathway effectively restores the function of MTB-specific Th1 cells and that high CD39 expression on Th1 cells indicates poor prognosis in patients with TB, which suggests that CD39 could serve as a prominent target for TB therapy.

AUTHOR CONTRIBUTIONS

CONFLICT OF INTEREST

The authors have no competing conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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