

Original research

# Altered distribution, activation and increased IL-17 production of mucosal-associated invariant T cells in patients with acute respiratory distress syndrome

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## ABSTRACT

**Objective** Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T cells that are engaged in a number of diseases, but their roles in acute respiratory distress syndrome (ARDS) are not fully examined yet. This study aimed to examine levels and functions of MAIT cells in patients with ARDS.

**Methods** Peripheral blood samples from patients with ARDS (n=50) and healthy controls (HCs, n=50) were collected. Levels of MAIT cells, cytokines, CD69, programmed cell death-1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) were measured by flow cytometry.

**Results** Circulating MAIT cell levels were significantly reduced in patients with ARDS than in HCs. MAIT cell levels were inversely correlated with disease severity and mortality. Cytokine production profiles in MAIT cells showed that percentages of interleukin (IL)-17 producing MAIT cell were significantly higher in patients with ARDS than in HCs. Patients with ARDS exhibited higher expression levels of CD69, PD-1 and LAG-3 in circulating MAIT cells. Moreover, levels of MAIT cells and expression levels of CD69, PD-1 and IL-17 in MAIT cells were higher in bronchoalveolar lavage fluid samples than in peripheral blood samples. Our *in vitro* experiments showed that MAIT cells triggered macrophages to produce proinflammatory cytokines such as tumour necrosis factor- $\alpha$ , IL-1 $\beta$  and IL-8.

**Conclusions** This study demonstrates that circulating MAIT cells are numerically deficient in patients with ARDS. In addition, MAIT cells were found to be activated, migrate into lung, secrete IL-17 and then stimulate macrophages. These findings suggest that MAIT cells contribute to the worsening of inflammation in the lung of patients with ARDS.

## INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a medical condition that exhibits a rapid impairment of gas exchange leading to hypoxaemic respiratory failure.<sup>1</sup> The incidence of ARDS is estimated from 10 to 86 cases per 100 000,<sup>2,3</sup> and approximately 10% of patients in intensive care unit are associated with this syndrome.<sup>4</sup> It is caused mostly by pneumonia, sepsis or non-respiratory aetiologies such as burns, trauma and pancreatitis.<sup>1,5</sup> Once damaged by those insults, the lung rapidly responds by activating the innate immune system, a stage referred to

## Key messages

### What is the key question?

⇒ What are the numbers and functions of mucosal-associated invariant T (MAIT) cells in patients with acute respiratory distress syndrome (ARDS), and do these cells reflect the ARDS disease activity?

### What is the bottom line?

⇒ Circulating MAIT cells are depleted in patients with ARDS, and this tendency reflects disease severity. The MAIT cells were activated, accumulated in bronchoalveolar lavage fluid, secreted IL-17 and then stimulated macrophages.

### Why read on?

⇒ MAIT cells may play an immune-propagatory role in the acute exudative phase of ARDS and they can be a therapeutic target of ARDS.

as the exudative phase.<sup>2</sup> In this phase, protein-rich oedema fluid is filled within interstitial and alveolar space through injured epithelial barriers and various proinflammatory cytokines and mediators are secreted by alveolar macrophages, resulting in the recruitment of neutrophil and macrophage that subsequently perpetuate inflammatory reactions.<sup>2,6</sup> Recently, other immune cells such as innate lymphoid cells (ILCs) and innate-like T cells are identified as lung tissue-resident lymphocyte and considered to be important players in mediating pulmonary immune response.<sup>5,7,8</sup>

Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T cells that express invariant T cell receptor (TCR)  $\alpha$ -chain (V $\alpha$ 7.2-J $\alpha$ 33/12/20 in humans) with paring limited set of V $\beta$  chains and recognise microbial riboflavin (vitamin B2) ligand bound on MHC class 1b-like related protein (MR1).<sup>9</sup> Human MAIT cells comprise up to 10% of CD3<sup>+</sup> cells, which is 10-fold to 1000-fold larger than the population of invariant natural killer T (iNKT) cells, another subset of innate-like T cells.<sup>10</sup> On activation via their MR1-recognising TCRs and/or cytokine receptors, MAIT cells release various cytokines and molecules to kill infected host cells<sup>11</sup> as well as recruit and activate other immune cells.<sup>12</sup>

Recent studies suggest its involvement in metabolic, malignant and chronic inflammatory diseases including a range of respiratory disorders.<sup>13</sup> In COPD, for example, the MAIT cell levels in blood were reduced and that deficiency was related to disease activity.<sup>14</sup> In a study on asthmatic patients, decreased MAIT cell levels were observed in blood, sputum and lung tissue, which was correlated with disease severity.<sup>15</sup>

Several mouse models are describing the relevance of ILCs and innate-like T cells to ARDS. In a recombinase-activating gene 2 (RAG2) KO mouse model, pulmonary group three innate lymphoid cells (ILC3s) secretes interleukin (IL)-17A, one of the key mediators in recruiting neutrophils.<sup>16</sup> Another mouse model in which mice were presensitised with  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) followed by inhalation of lipopolysaccharide showed activation of iNKT cells that leads to produce interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$  and exacerbate respiratory failure.<sup>17</sup> However, MAIT cell levels and functions have yet to be investigated in ARDS. Besides, the relevance of MAIT cells to other innate immune cells has not been determined. Accordingly, this study aims to examine the levels and functions of MAIT cells in ARDS, evaluate the clinical relevance of MAIT cell levels, and investigate its association within the innate immune system.

## MATERIALS AND METHODS

### Monoclonal antibodies and flow cytometry

Detailed information is available in online supplemental methods.

### Isolation of peripheral blood mononuclear cells and identification of MAIT cells

Detailed information is available in online supplemental methods.

### Functional MAIT cell assay

Expression levels of IFN- $\gamma$ , IL-17 and TNF- $\alpha$  in MAIT cells were detected by intracellular cytokine flow cytometry. Detailed information is available in online supplemental methods.

### Coculture system for human macrophage activity assay

Detailed information is available in online supplemental methods.

### Statistical analysis

Detailed information is available in online supplemental methods.

## RESULTS

### Patient characteristics

The study cohort included 50 patients with ARDS (11 women and 39 men, mean age $\pm$ SD: 63.8 $\pm$ 18.9 years) and 50 healthy controls (HCs, 23 women and 27 men; mean age $\pm$ SD: 65.8 $\pm$ 8.79 years), indicating that there was a higher proportion of men in the ARDS group compared with HCs. Clinical and laboratory characteristics of patients are summarised in table 1. The severity of ARDS can be classified into the following three grades according to PaO<sub>2</sub>/FiO<sub>2</sub> ratio<sup>18</sup>: mild grade (200 mm Hg < PaO<sub>2</sub>/FiO<sub>2</sub> ratio  $\leq$  300 mm Hg); moderate grade (100 mm Hg < PaO<sub>2</sub>/FiO<sub>2</sub> ratio  $\leq$  200 mm Hg) and severe grade (PaO<sub>2</sub>/FiO<sub>2</sub> ratio  $\leq$  100 mm Hg). Among a total of 50 patients, 2 (4%) had mild disease; 31 (62%) had moderate disease; and 17 (34%) had severe disease.

**Table 1** Clinical and laboratory characteristics of the patients with ARDS

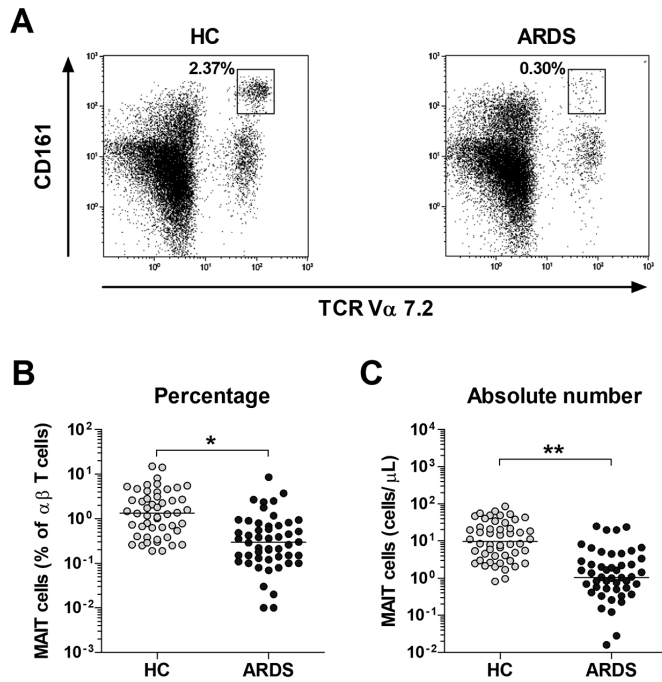
Characteristic	ARDS
Total no.	50
Sex (no. male/no. female)	39/11
Age (years), median (IQR)	69.5 (52.8–77.0)
Clinical variables	
Cause of disease, n (%)	
Bacterial pneumonia	31/50 (62)
Viral pneumonia	3/50 (6)
Non-pulmonary sepsis	6/50 (24)
Aspiration	7/50 (14)
Others*	3/50 (6)
Disease severity, n (%)	
Mild	2/50 (4)
Moderate	31/50 (62)
Severe	17/50 (34)
Mortality, n (%)	
Mild	0/2 (0)
Moderate	12/31 (38.7)
Severe	13/17 (76.5)
PEEP (cmH <sub>2</sub> O), median (IQR)	12.0 (10.0–13.0)
Plateau pressure (cmH <sub>2</sub> O), median (IQR)	28.0 (24.5–31.5)
ECMO (%)	9/50 (18)
Laboratory variables, median (IQR)	
Leucocyte count (cells/ $\mu$ L)	12 300 (7900–15 225)
Lymphocyte count (cells/ $\mu$ L)	620 (402.5–1073)
Monocyte count (cells/ $\mu$ L)	485 (205.0–927.5)
Neutrophil count (cells/ $\mu$ L)	10 110 (6163–13 813)
Haemoglobin level (g/dL)	9.8 (8.7–11.0)
Platelet count ( $\times 10^3$ cells/ $\mu$ L)	173.0 (86.8–242.3)
PaCO <sub>2</sub> (mm Hg)	42.0 (36.0–48.0)
PaO <sub>2</sub> (mm Hg)	77.5 (65.6–86.7)
FiO <sub>2</sub> (mm Hg)	0.6 (0.55–0.80)
PaO <sub>2</sub> /FiO <sub>2</sub> ratio	119.0 (83.8–141.0)
Total protein level (g/dL)	5.5 (4.8–6.1)
Albumin (g/dL)	2.6 (2.3–2.9)
AST (U/L)	42.0 (22.0–78.0)
ALT (U/L)	23.0 (13.5–60.5)
BUN (mg/dL)	29.8 (20.9–50.4)
Creatinine (mg/dL)	1.0 (0.7–1.69)
CRP level (mg/dL)	17.2 (9.1–27.2)

\*included trauma, pancreatitis, and hepatitis.

ALT, alanine transaminase; ARDS, acute respiratory distress syndrome; AST, aspartate transaminase; BUN, blood urea nitrogen; CRP, C reactive protein; ECMO, extracorporeal membrane oxygenation; FiO<sub>2</sub>, fraction of inspired oxygen; PaCO<sub>2</sub>, partial pressure of arterial carbon dioxide; PaO<sub>2</sub>, partial pressure of arterial oxygen; PaO<sub>2</sub>/FiO<sub>2</sub>, ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen; PEEP, positive end expiratory pressure.

### Reduced numbers of circulating MAIT cells in patients with ARDS

The percentages and absolute numbers of MAIT cells in the peripheral blood samples from 50 patients with ARDS and



**Figure 1** Decreased circulating MAIT cell numbers in the peripheral blood of patients with ARDS. Freshly isolated peripheral blood mononuclear cells from 50 HCs and 50 patients with ARDS were stained with APC-Cy7-conjugated anti-CD3, FITC-conjugated anti-TCR  $\gamma\delta$ , APC-conjugated anti-TCR V $\alpha$ 7.2 and PE-Cy5-conjugated anti-CD161 monoclonal antibodies and then analysed by flow cytometry. Percentages of MAIT cells were calculated within a  $\alpha\beta$  T cell gate. (A) Representative MAIT cell percentages determined by flow cytometry. (B) MAIT cell percentages among peripheral blood  $\alpha\beta$  T cells. (C) Absolute MAIT cell numbers (per microlitre of blood). Symbols represent individual subjects and horizontal lines are median values. \* $p < 0.005$ , \*\* $p < 0.0005$  by the analysis of covariance (ANCOVA) test. ARDS, acute respiratory distress syndrome; HC, healthy control; MAIT, mucosal-associated invariant T.

50 HCs were determined by flow cytometry. All comparisons of the percentages and absolute numbers of MAIT cells were performed by analysis of covariance after adjusting for age and sex using the Bonferroni correction for multiple comparisons, as described in the Materials and methods section. MAIT cells were defined as CD3<sup>+</sup> $\gamma\delta$ <sup>-</sup> T cells expressing TCR V $\alpha$ 7.2 and CD161<sup>high</sup> (figure 1A; isotype control shown in online supplemental figure S1A). Percentages of MAIT cells were significantly lower in patients with ARDS than in HCs (median (IQR) 0.30 (0.13–0.73)% vs 1.35 (0.51–3.32)%,  $p < 0.005$ ; figure 1B). Patients with ARDS had significantly lower absolute numbers of MAIT cells as compared with HCs (median (IQR) 1.05 (0.40–3.00) cells/ $\mu$ L vs 9.74 (3.72–25.4) cells/ $\mu$ L,  $p < 0.0005$ ; figure 1C). Furthermore, our additional analysis based on the stratification of ARDS according to aetiology showed that there were no significant differences in MAIT cell percentages among ARDS subgroups (online supplemental figure S2). To clarify the gender effect of ARDS on MAIT cell levels, percentages and absolute numbers of circulating MAIT cells were further analysed according to gender. No significant differences were observed in the percentages and absolute numbers of MAIT cells between male or female subgroups of patients with ARDS and HCs, indicating no gender-differences in the circulating MAIT cell levels (online supplemental figure S3).

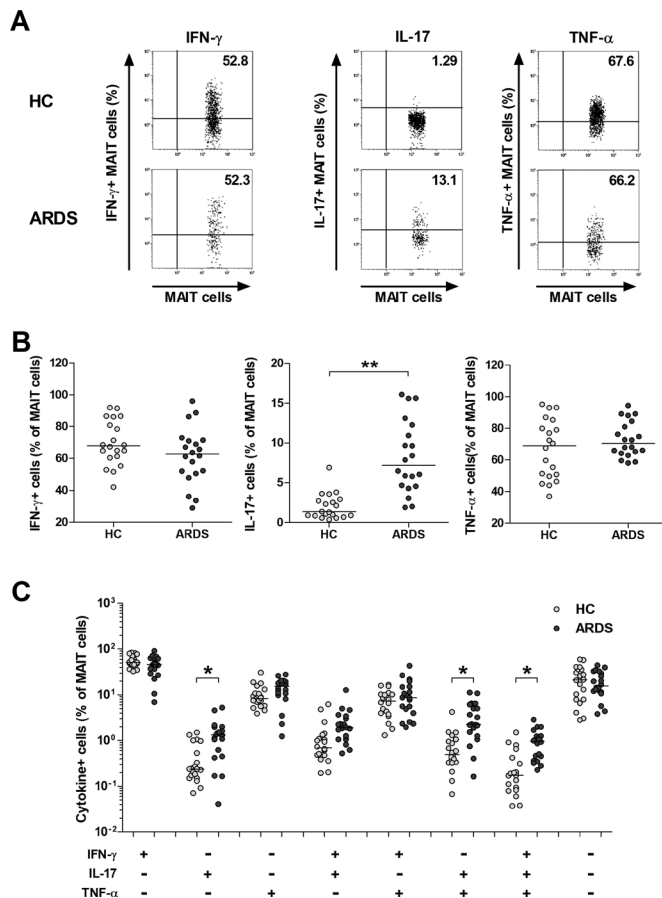
**Table 2** Regression coefficients for log-transformed absolute MAIT cell numbers with respect to clinical and laboratory findings in patients with ARDS

Variables	$\beta$	95% CI		P value
		Lower	Upper	
Sex (male)	-0.579	-1.254	0.095	0.091
Age (years)	-0.007	-0.022	0.009	0.393
Disease severity	-0.746	-1.235	-0.257	0.004
Mortality	-0.622	-1.169	-0.075	0.027
PEEP (cmH <sub>2</sub> O)	-0.022	-0.132	0.087	0.680
Plateau pressure (cmH <sub>2</sub> O)	0.026	-0.066	0.119	0.557
ECMO	0.178	-0.580	0.936	0.638
Leucocyte count (cells/ $\mu$ L)	0.0001	0.0001	0.001	0.076
Lymphocyte count (cells/ $\mu$ L)	0.0001	0.0001	0.001	0.012
Monocyte count (cells/ $\mu$ L)	0.0001	0.0001	0.001	0.440
Neutrophil count (cells/ $\mu$ L)	0.0001	0.0001	0.0001	0.146
Haemoglobin (g/dL)	0.114	-0.073	0.302	0.227
Platelet count (10 <sup>3</sup> cells/ $\mu$ L)	0.001	-0.002	0.003	0.629
PaCO <sub>2</sub> (mm Hg)	0.009	-0.016	0.034	0.485
PaO <sub>2</sub> (mm Hg)	0.004	-0.007	0.015	0.503
FiO <sub>2</sub> (mm Hg)	-1.827	-3.284	-0.370	0.015
PaO <sub>2</sub> /FiO <sub>2</sub> ratio	0.010	0.003	0.016	0.004
Total protein (g/dL)	-0.237	-0.550	0.075	0.133
Albumin (g/dL)	0.526	-0.164	1.216	0.132
AST (U/L)	0.0001	-0.001	0.0001	0.647
ALT (U/L)	0.0001	-0.002	0.002	0.806
BUN (mg/dL)	-0.001	-0.016	0.015	0.948
Creatinine (mg/dL)	0.063	-0.168	0.293	0.588
CRP (mg/dL)	-0.002	-0.031	0.027	0.885

ALT, alanine transaminase; ARDS, Acute respiratory distress syndrome; AST, aspartate transaminase; BUN, blood urea nitrogen; CRP, C reactive protein; ECMO, extracorporeal membrane oxygenation; FiO<sub>2</sub>, fraction of inspired oxygen; MAIT, mucosal-associated invariant T; PaCO<sub>2</sub>, partial pressure of arterial carbon dioxide; PaO<sub>2</sub>, partial pressure of arterial oxygen; PaO<sub>2</sub>/FiO<sub>2</sub>, ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen; PEEP, positive end expiratory pressure;  $\beta$ , regression coefficient.

### Relationship between circulating MAIT cell levels and clinical parameters in patients with ARDS

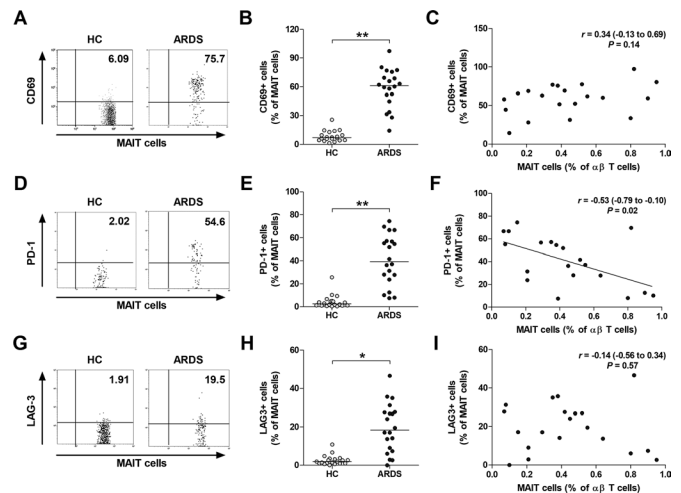
To evaluate the clinical relevance of MAIT cell in patients with ARDS, we investigated the correlation between absolute MAIT cell numbers in peripheral blood and clinical parameters using regression analysis (table 2). Because the distributions were skewed, the absolute numbers of MAIT cells were log-transformed for the analysis. Linear regression analysis showed that log-transformed absolute numbers of MAIT cells were significantly correlated with disease severity, mortality, lymphocyte count, FiO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub> ratio ( $p = 0.004$ ,  $p = 0.027$ ,  $p = 0.012$ ,  $p = 0.015$  and  $p = 0.004$ , respectively). However, no significant correlations were observed between MAIT cell numbers and gender, age, PaCO<sub>2</sub>, PaO<sub>2</sub>, PEEP, plateau pressure, ECMO, leucocyte count, monocyte count, neutrophil count, haemoglobin level, platelet count, total protein level, albumin level, aspartate aminotransferase level, alanine aminotransferase level, blood urea nitrogen level, creatinine level or C-reactive protein level (table 2).



**Figure 2** Increased expression of IL-17 in circulating MAIT cells of patients with ARDS. Freshly isolated peripheral blood mononuclear cells ( $1 \times 10^6$ /well) from 20 HCs and 20 patients with ARDS were incubated for 1 hour in the presence of PMA (100 ng/mL) and IM (1  $\mu$ M). (A) Representative IFN- $\gamma$ , IL-17 and TNF- $\alpha$  expression in the MAIT cell population was determined by intracellular flow cytometry after stimulation with PMA and IM. (B) Percentages of IFN- $\gamma$ , IL-17 or TNF- $\alpha$  producing MAIT cells. (C) Proportions of single-cytokine, double-cytokine and triple-cytokine producing MAIT cells. Symbols in (B) and (C) represent individual subjects and horizontal lines are median values. \* $p < 0.01$ , \*\* $p < 0.0005$  by the ANCOVA test. ARDS, acute respiratory distress syndrome; HC, healthy control; IFN, interferon; IL, interleukin; MAIT, mucosal-associated invariant T; TNF, tumour necrosis factor.

### Increased production of IL-17 in circulating MAIT cells of patients with ARDS

To examine the cytokine production profiles in MAIT cells, peripheral blood mononuclear cells from 20 patients with ARDS and 20 HCs were incubated with PMA and IM for 1 hour and then the expression levels of IFN- $\gamma$ , IL-17 and TNF- $\alpha$  in the MAIT cell population was examined at the single-cell level by triple-cytokine intracellular staining flow cytometry (figure 2A; isotype controls shown in online supplemental figure S1B). Percentages of IL-17 producing MAIT cell were found to be significantly higher in patients with ARDS than in HCs (median (IQR) 7.18 (4.58–12.0)% vs 1.34 (0.87–2.88)%,  $p < 0.0005$ ; figure 2B). However, IFN- $\gamma$  and TNF- $\alpha$  producing MAIT cell levels were comparable between the patients and HCs. Moreover, percentages of single-producing (IL-17), dual-producing (IL-17/TNF- $\alpha$ ) and triple-producing (IFN- $\gamma$ /IL-17/TNF- $\alpha$ ) MAIT cells were found to be significantly higher in patients with ARDS than in HCs (median (IQR) 1.33 (0.49–1.87)% vs 0.24 (0.16–0.87)%,

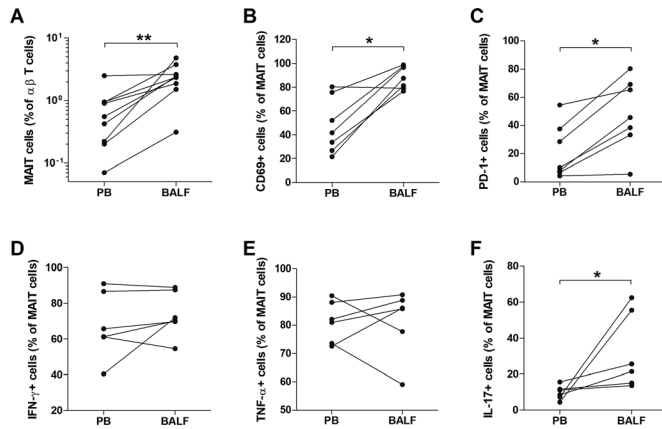


**Figure 3** Expression of CD69, PD-1 and LAG-3 in circulating MAIT cells of patients with ARDS. Freshly isolated peripheral blood mononuclear cells were stained with FITC-conjugated anti-CD3, APC-conjugated anti-TCR V $\alpha$ 7.2, PE-conjugated anti-CD3, PE-conjugated anti-CD69, PE-conjugated anti-PD-1, PE-conjugated anti-LAG-3 and PE-Cy5-conjugated anti-CD161 monoclonal antibodies, and then analysed by flow cytometry. Representative percentages of CD69-expressing cells (A), PD-1-expressing cells (D) and LAG-3-expressing cells (G) among the MAIT cell population. Data in (B), (E) and (H) were obtained from 20 HCs and 20 patients with ARDS. Symbols represent individual subjects and horizontal lines are median values. \* $p < 0.005$ , \*\* $p < 0.0001$  by the ANCOVA test. Relationship between MAIT cell percentages among the  $\alpha\beta$  T cell population and CD69+ MAIT cells (C), PD-1+ MAIT cells (F) and LAG-3+ MAIT cells (I) in patients with ARDS were determined using Spearman  $r$  (95% CI) correlation analysis. ARDS, acute respiratory distress syndrome; HC, healthy control; MAIT, mucosal-associated invariant T.

$p < 0.01$ , for IL-17; median (IQR) 2.23 (1.32–5.86)% vs 0.49 (0.33–1.03)%,  $p < 0.01$ , for IL-17/TNF- $\alpha$ ; median (IQR) 0.96 (0.39–1.25)% vs 0.17 (0.08–0.39)%,  $p < 0.01$ , for IFN- $\gamma$ /IL-17/TNF- $\alpha$ , respectively; figure 2C, online supplemental figure S4). However, no significant differences in the other single or dual cytokine producers were observed between the patients and controls.

### Activation of MAIT cells in patients with ARDS

CD69 is known to be early activation marker, whereas programmed cell death-1 (PD-1) is considered as relative late activation marker. Following TCR activation, CD69 is upregulated within ~4 hours,<sup>19</sup> whereas coinhibitory receptors such as PD-1 and lymphocyte-activation gene-3 (LAG-3) are upregulated within 24–72 hours.<sup>20,21</sup> These coinhibitory receptors are also known to be synergistically involved in negative regulation of T cell over-activation.<sup>22–24</sup> Moreover, coexpression of these multiple coinhibitory receptors has been reported to have a close association with anergy or exhaustion.<sup>25</sup> To determine whether MAIT cells might be activated or exhausted in ARDS, expression levels of CD69, PD-1 and LAG-3 in circulating MAIT cells from 20 patients with ARDS, and 20 HCs were determined by flow cytometry (figure 3A, D and G; isotype controls shown in online supplemental figure S1C). Percentages of CD69+ MAIT cells were significantly higher in patients with ARDS compared with HCs (median (IQR) 61.2 (46.5–74.1)% vs 6.92 (4.31–12.0)%,  $p < 0.0001$ ; figure 3B). We next examined expression levels of PD-1 and LAG-3 in patients with ARDS. Percentages of PD-1+

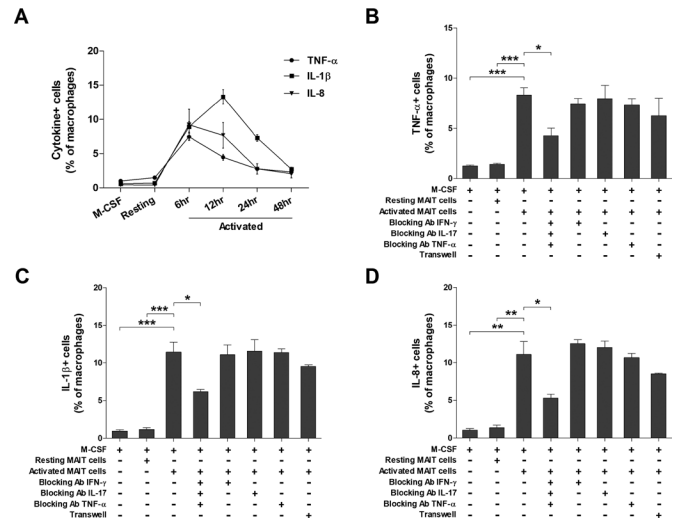


**Figure 4** Increased MAIT cell numbers in BAL fluid from patients with ARDS. Paired samples of peripheral blood and BAL fluid were obtained from patients with ARDS, and then the percentages of total MAIT cells (A), CD69+MAIT cells (B) and PD-1+MAIT cells (C) were determined by flow cytometry. Data in (A) and (B, C) were obtained from 9 and 7 patients with ARDS, respectively. (D–F) Expression of IFN- $\gamma$ , TNF- $\alpha$  and IL-17 in the MAIT cell population determined by intracellular flow cytometry after stimulation with PMA and IM. Data in (D–F) were obtained from 6 patients with ARDS. Symbols represent individual patients. \* $p$ <0.05, \*\* $p$ <0.005 by Wilcoxon matched-pairs signed rank test. ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; HC, healthy control; IFN, interferon; IL, interleukin; MAIT, mucosal-associated invariant T; TNF, tumour necrosis factor.

and LAG-3<sup>+</sup> MAIT cells were significantly higher in patients with ARDS than those in HCs (median (IQR) 39.4 (24.7–57.0)% vs 2.49 (1.00–5.00)%,  $p$ <0.0001, for PD-1<sup>+</sup> cells; median (IQR) 18.3 (7.83–27.7)% vs 1.93 (1.01–3.44)%,  $p$ <0.0001, for LAG-3<sup>+</sup> cells, respectively; [figure 3E,H](#)). In particular, the PD-1 expression of MAIT cells was inversely correlated with the frequency of MAIT cells in patients with ARDS ( $\gamma$ =−0.53, 95% CI −0.79 to −0.10,  $p$ <0.05; [figure 3F](#)). However, no significant correlation was observed between CD69<sup>+</sup> or LAG-3<sup>+</sup> MAIT cell levels and MAIT cell percentages ([figure 3C,I](#)).

### Increased MAIT cell numbers in bronchoalveolar lavage fluid from patients with ARDS

To examine whether circulating MAIT cell deficiency in patients with ARDS might be associated with the accumulation of MAIT cells in bronchoalveolar lavage (BAL) fluid, we obtained paired samples of peripheral blood and BAL fluid from nine patients. The percentages of MAIT cells were significantly higher in BAL fluid than in peripheral blood (median (IQR) 2.36 (1.69–3.20)% vs 0.55 (0.21–0.95)%,  $p$ <0.005; [figure 4A](#)). Next, we analysed the percentages of CD69 and PD-1 expressing MAIT cells in paired peripheral blood and BAL fluid samples from seven patients. The percentages of both CD69<sup>+</sup> and PD-1<sup>+</sup> MAIT cells were significantly higher in BAL fluid compared with peripheral blood (median (IQR) 87.5 (79.1–97.6)% vs 41.7 (26.7–75.7)%,  $p$ <0.05, 45.7 (33.3–69.2)% vs 10.1 (6.67–37.5)%,  $p$ <0.05, respectively; [figure 4B,C](#)). To investigate whether the production of proinflammatory cytokines by MAIT cells is different between peripheral blood and BAL fluid, we measured IFN- $\gamma$ , TNF- $\alpha$  and IL-17 in paired samples of peripheral blood and BAL fluid from six patients. The production of IL-17 was significantly higher in BAL fluid compared with peripheral blood (median (IQR) 23.5 (14.7–57.3)% vs 9.76 (6.52–12.6)%,  $p$ <0.05; [figure 4F](#)), but



**Figure 5** Expression of proinflammatory cytokines in human macrophage by activated MAIT cells. Purified monocytes ( $2.5 \times 10^6$  cells/well) were cultured for 7 days in the presence of M-CSF (100 ng/mL). Freshly isolated MAIT cells were cultured and activated in the presence of PMA (100 ng/mL) and IM (1  $\mu$ M) for 16 hours before cocultured with macrophages. Macrophages were cultured with resting MAIT cells ( $4 \times 10^5$  cells/well), or activated MAIT cells ( $4 \times 10^5$  cells/well) in the presence or absence of individual cytokine inhibitors and their combination (ie, blocking antibodies against a cocktail of IFN- $\gamma$ , IL-17 and TNF- $\alpha$ ). In the transwell systems, the activated MAIT cells and macrophages were seeded respectively into the upper and lower compartment of the transwell with 0.4  $\mu$ m pores in the presence of M-CSF. (A) Time course of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production in macrophages. (B–D) Expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in macrophage determined by intracellular flow cytometry. Data in (B–D) were three independent experiments. Values are expressed as the mean  $\pm$  SEM. \* $p$ <0.05, \*\* $p$ <0.001, \*\*\* $p$ <0.0001 by unpaired t-test. ARDS, acute respiratory distress syndrome; IFN, interferon; IL, interleukin; MAIT, mucosal-associated invariant T; TNF, tumour necrosis factor.

the production of IFN- $\gamma$  and TNF- $\alpha$  by MAIT cells was comparable between peripheral blood and BAL fluid ([figure 4D,E](#)).

### Expression of proinflammatory cytokines in human macrophage by activated MAIT cells

To determine whether activated MAIT cells could activate macrophage to produce proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-8, purified monocytes were cultured with M-CSF for 7 days to differentiate into macrophage and freshly isolated MAIT cells were stimulated with PMA and IM for 16 hours. The macrophages were cocultured with resting MAIT cells or activated MAIT cells in the presence of M-CSF for 48 hours to assess the peak time point and measure the amount of proinflammatory cytokine expression. The absolute numbers of both TNF- $\alpha$  and IL-8 producing macrophages peaked at 6 hours and IL-1 $\beta$  producing macrophages at 12 hours, respectively ([figure 5A](#)).

Next, we activated macrophages with the following conditions: M-CSF only, resting MAIT cells with M-CSF, activated MAIT cells with M-CSF, activated MAIT cells with M-CSF and a cocktail of all blocking antibodies against IFN- $\gamma$ , IL-17 and TNF- $\alpha$ , and activated MAIT cells with M-CSF and each blocking antibody. All percentages of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 expressing macrophages were significantly higher in activated MAIT cells with M-CSF than either M-CSF only (mean  $\pm$  SEM,

8.31%±0.72% vs 1.25%±0.10%,  $p<0.0001$ , for TNF- $\alpha$ ; 11.4%±1.33% vs 0.95%±0.18%,  $p<0.0001$ , for IL-1 $\beta$ ; 10.9%±1.86% vs 1.04%±0.22%,  $p<0.001$ , for IL-8) or resting MAIT cells with M-CSF (mean±SEM, 8.31%±0.72% vs 1.42%±0.13%,  $p<0.0001$ , for TNF- $\alpha$ ; 11.4%±1.33% vs 1.16%±0.22%,  $p<0.0001$ , for IL-1 $\beta$ ; 10.9%±1.86% vs 1.34%±0.36%,  $p<0.001$ , for IL-8, respectively; figure 5B–D). Furthermore, when a cocktail of all blocking antibodies was added together with activated MAIT cells, each cytokine production of macrophages was significantly reduced compared with activated MAIT cells with M-CSF (mean±SEM, 4.27%±0.77% vs 8.31%±0.72%,  $p<0.05$ , for TNF- $\alpha$ ; 6.18%±0.31% vs 11.4%±1.33%,  $p<0.05$ , for IL-1 $\beta$ ; 5.28%±0.52% vs 10.9%±1.86%,  $p<0.05$ , for IL-8; figure 5B–D). When each of individual blocking antibodies was added, however, there was no effect on the percentages of cytokine production in macrophages. Finally, to determine whether the cytokine production of macrophages by activated MAIT cells is contact-dependent, a Transwell separation assay was performed. The percentage of cytokine-secreting macrophages tended to decrease in the Transwell experiment but did not reach statistical significance (figure 5B–D).

## DISCUSSION

To the best of our knowledge, this is the first study to examine the levels and functions of MAIT cells in non-COVID-19 induced ARDS, evaluate the clinical relevance of MAIT cell levels, and investigate its association within macrophages, which are also essential for the process of the inflammatory response in acute lung injury and its severe form, ARDS. We found that the percentages and absolute numbers of circulating MAIT cells capable of producing Th1 cytokines were deficient in patients with ARDS. However, the IL-17 producing MAIT cells were found to be increased. We observed that patients with ARDS exhibited higher expression of CD69, PD-1 and LAG-3 by circulating MAIT cells. Of these surface activation markers, in particular, only PD-1 expression by MAIT cells was inversely correlated with their frequencies in patients with ARDS. We further observed that the expression of CD69, PD-1 and IL-17 by MAIT cells was higher in BAL fluid compared with peripheral blood from patients with ARDS. Notably, activated MAIT cells triggered the activation of macrophage that secreted proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-8. Overall, these findings indicate that MAIT cells might be a crucial immune modulator in the propagation of inflammation in the lung of patients with ARDS.

Our data showed that patients with ARDS displayed a decrease in circulating MAIT cells. These findings are consistent with previous studies of COVID-19-driven ARDS showing the reduction of peripheral blood MAIT cell levels in COVID-19 associated patients with ARDS.<sup>26–28</sup> Unlike those studies, ours included only non-COVID-19 causes such as bacterial or viral infection, trauma, sepsis, interstitial lung disease and operation and so on. Most (about 60%) of the causes were bacterial pneumonia. However, there were no differences in the MAIT cell levels regarding the causes (infection vs non-infection; bacterial vs viral other than COVID-19), suggesting that MAIT cell decline is not specific for the causes of ARDS. This MAIT cell deficiency was also commonly observed in patients with infections and immune-mediated respiratory diseases.<sup>15 29–31</sup> Of note, previous studies demonstrated that the drop was more prominent in patients with sepsis and severe asthma.<sup>15 31</sup> which is consistent with our data revealing that the MAIT cell levels were inversely

correlated with disease severity and mortality in patients with ARDS. Collectively, these results indicate that the reduction of MAIT cells could reflect the worsening of the lung inflammation that leads to the impaired oxygenation and subsequently affect mortality in patients with ARDS.

The present study showed that patients with ARDS display an increased secretion of IL-17 by circulating MAIT cells. Accumulating evidence suggests that human MAIT cells produce proinflammatory cytokines in a mixed Th1/Th17 pattern: most blood MAIT cells secrete Th1 type cytokines such as IFN- $\gamma$  and TNF- $\alpha$  under TCR-mediated activation, while a small population of MAIT cells tends to produce Th17 type cytokines, including IL-17 and IL-22, in a TCR-independent manner.<sup>32–34</sup> In agreement with our data, previous studies have shown that circulating MAIT cells displayed enhanced production of IL-17 in inflammatory bowel disease, asthma, ankylosing spondylitis and multiple sclerosis.<sup>35–39</sup> Interestingly, circulating IL-17-producing MAIT cells are known to be associated with symptoms, including the number of severe exacerbations and asthma control test score, in children with asthma, indicating the involvement of IL-17-producing MAIT cells in asthma pathogenesis. Likewise, these findings suggest that the IL-17-producing MAIT cells potentially contribute to the pathogenesis of ARDS.

In the present study, the circulating MAIT cell depletion was accompanied by the upregulation of CD69, a kind of early activation markers, and PD-1 and LAG-3, which was implicated in the late activation or anergy/exhaustion of MAIT cells, respectively.<sup>19 23 40</sup> These results are consistent with previous studies on infectious, inflammatory and metabolic diseases.<sup>34 41–44</sup> Interestingly, ex vivo blockade of PD-1/PD-L pathway resulted in reduction in TGF- $\beta$ 1 and IL-17A expression from CD4<sup>+</sup> T cells, indicating that PD-1 upregulation on CD4<sup>+</sup> T cells is directly associated with IL-17A and TGF- $\beta$ 1 production.<sup>45</sup> The association between PD-1 and IL-17 has been also observed in CD8<sup>+</sup> T cells from lung cancer and breast cancer.<sup>46 47</sup> Collectively, these findings suggest that PD-1 upregulation may be associated with IL-17 upregulation in MAIT cells from patients with ARDS.

In this study, patients with ARDS displayed higher frequencies of MAIT cells in BAL fluid compared with peripheral blood, in line with several previous studies in pulmonary tuberculosis and community-acquired pneumonia.<sup>48 49</sup> Notably, these MAIT cells showed a higher expression of both CD69 and PD-1 in BAL fluid than in peripheral blood. These findings led us to speculate that the activated MAIT cells might either undergo cell death or predominantly move into BAL fluid, resulting in the reduction of circulating MAIT cells. This speculation is partly explained by our data revealing that there was a significant negative correlation between the circulating MAIT cell level and their PD-1 expression. However, there is another possibility that explain this tendency such as cellular expansion of the resident MAIT cell population in BAL fluid. To clarify this, tracing labelled MAIT cells or compare the MAIT cell frequencies in BAL fluid between HC and patients with ARDS should be conducted, which is not possible in human studies. Additional experiments on animal models could illuminate these possibilities. Furthermore, these activated MAIT cells in BAL fluid secreted more IL-17 than those in peripheral blood. It is known that IL-17 recruits neutrophils and increases the permeability of alveolar endothelium as well as triggers the augmentation of other cytokines and inflammatory mediators, a critical pathological process involved in asthma, pseudomonas infection, and early exudative phase of ARDS.<sup>6 16 50–53</sup> Collectively, our findings suggest that the activated MAIT cells could migrate into the lung, leading to the increased production of IL-17 that

subsequently contributes to the worsening of inflammation in the lung of ARDS.

It is widely known that macrophages are one of the main components involved in the pathophysiology of the early phase of ARDS.<sup>54–56</sup> Under various stimuli, both tissue-resident alveolar macrophages and recruited monocyte-derived macrophages are differentiated into M1 macrophages.<sup>57–58</sup> These M1 macrophages act as an initiator of immune responses by releasing several proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-8 that lead to trigger various immune cells.<sup>6–53–56</sup> Our data showed that the activated MAIT cells induced the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 by monocyte-derived macrophages. The cytokine production was normalised after treatment with a cocktail of all blocking antibodies against IFN- $\gamma$ , IL-17 and TNF- $\alpha$ , produced by activated MAIT cells. Additional experiments using the Transwell system showed that the percentage of cytokine-secreting macrophages tended to decrease but did not reach statistical significance. These results suggest that activated MAIT cells may act on the macrophages in a cytokine-dependent manner rather than in contact-dependent manner. Furthermore, the single use of individual blocking antibodies showed no significant effect on the percentages of cytokine production in macrophages, implying that the combination of IFN- $\gamma$ , IL-17 and TNF- $\alpha$  produced by MAIT cells but not alone may contribute to the activation of macrophages.

In conclusion, this study first demonstrates that circulating MAIT cells are numerically deficient in ARDS, and this MAIT cell deficiency reflects disease severity. In addition, we report a novel finding that MAIT cells are activated, migrate into lung, secrete IL-17 and then stimulate macrophages. These findings show the potential role of MAIT cells in the pathogenesis of ARDS and that MAIT cells can be a therapeutic target of ARDS.

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