Short Communication

Unconventional T Cell Immunity in the Lungs of Young Children with Cystic Fibrosis

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Abstract

Background: People with Cystic Fibrosis (CF) develop pulmonary inflammation, chronic infection and structural lung damage early in life, with these manifestations being prevalent among preschool children and infants. While early immune events are believed to play critical roles in shaping the progression, severity and disease burden later in life, T cells and their subsets are poorly studied in the CF lung, particularly during the formative early stages of disease. **Methods**: Using flow cytometry, we analyzed Mucosal Associated Invariant T (MAIT) cells, $\gamma\delta$ T cells, and Natural Killer T (NKT)-like cells in bronchoalveolar lavage (BAL) samples from seventeen children with CF, aged two to six years old. The effect of age, sex and lung infections on the frequencies of these cells in BAL samples was analysed (grouped data were tested for normality and compared by *t*-test or Kruskal-Wallis analysis). **Results**: No difference was noted in the proportions of unconventional T cells related to the sex or age of the children. The frequency of $\gamma\delta$ T cells and MAIT cells appeared unchanged by infection status. However, viral infections were associated with a significant increase in the proportion of NKT-like cells. **Conclusions**: By evaluating T cells in the lungs of children during the early formative stages of CF, this study identified potentially important interactions between these cells and viral pathogens.

Keywords: cystic fibrosis; unconventional T cells; mucosal immunity; host/pathogen

1. Introduction

Although early life immune/inflammatory events are believed to play an important role in the progression and prognosis of cystic fibrosis disease [1], we are only now starting to examine immune cell phenotypes present in the CF lung in early childhood [2]. In healthy individuals, macrophages comprise the predominant population in bronchoalveolar lavage (BAL), with lymphocytes constituting the second most common immune cell population. In CF however, infections and inflammation alter this profile, most markedly by the recruitment of neutrophils, the main immune cell infiltrate associated with CF pathogenesis [3]. Most research on immunity in CF has therefore understandably focused on macrophages and neutrophils.

Individual lymphocyte populations will likely also be altered by infection and inflammation, potentially contributing to CF disease progression. While T cells are present in the CF airway where much of the severe structural damage occurs, the contribution of T cells in CF children are not well studied [4] and it is unclear whether they play protective or pathological roles in CF pathogenesis. Studies that have analyzed T cells in the CF lung have predominantly been performed in adults or adolescents, by which age considerable disease progression has already occurred, and most have only considered conventional T cells.

T cells comprise a complex range of immune cells with a multitude of roles which, depending on the cell type and circumstances, include coordinating and linking different aspects of immunity, regulating inflammation and fighting infection. An improved knowledge of T cell subsets in the lung during the early stages of CF would increase our understanding of the contribution such cells might make to long-term disease progression.

An important group of such cells that have thus far been almost unstudied in the CF lung are the unconventional T cells. Little is known about the roles of unconventional T cells in CF, especially during the formative early years of disease progression. These unconventional cells are important components of the T cell repertoire, providing a large source of inflammatory cytokines, including IFNγ,

TNF and IL-17A, and the ability to kill virally infected cells [5]. Here, we analyze the frequencies of unconventional T cell subsets, specifically Mucosal Associated Invariant T (MAIT) cells, $\gamma\delta$ T cells, and CD3+CD56+ Natural Killer T-like (NKT-like) cells, in bronchoalveolar lavage (BAL) samples from CF children between the ages of 2 and 6. We further assessed the effect of sex, early age differences and infection status on the levels of these cells.

By using FACS analysis to show the presence and frequencies of these cells, we here provide the first identification and analysis of these important immune cells in the lungs of children with CF. Comparing the frequencies of these cells with infection status, we found that the proportion of NKT-like cells increased during viral infections. These findings therefore identify potentially important interactions between mucosal cellular immune responses and pathogenic infection in early CF pathogenesis.

2. Materials and Methods

2.1 Ethics and Sample Collection

Bronchoalveolar lavage (BAL) samples were pooled aliquots of the 2nd and 3rd washings from the right middle lobe, collected from CF patients by bronchoscopy under AREST CF protocols at the Royal Children's Hospital, Melbourne with approval of the hospital Human Research Ethics Committee (HREC #25054). In Australia, antibiotic prophylaxis with amoxicillin-clavulanic acid (15 mg/kg/day) is prescribed during the first two years of life to children with CF. Microbiological clinical status of patients was determined using standard clinical laboratory cultures at the Royal Children's Hospital, Melbourne.

2.2 Flow Cytometric Analysis

BAL samples were passed through a cell strainer to remove debris and any clumps of tissue. After washing, cell pellets were resuspended in 1 mL of RPMI-1640 (Thermo Fisher Scientific) and manually counted using a hemocytometer. Each sample was resuspended in FACS buffer (PBS plus 2% fetal bovine serum (Thermo Fisher Scientific) plus 10% human serum for 20 minutes on ice, followed by staining with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Thermo Fisher) for 15 minutes in the dark to allow the exclusion of dead cells from the analysis. After washing, a cocktail of the antibodies listed below was added and staining completed in the dark for 25 minutes before fixing cells in 2% paraformaldehyde in PBS overnight. Samples were resuspended in FACS buffer, then analyzed on a BD LSRFortessa™ X-20 using BD FACS-Diva (BD Biosciences). Antibody cocktail (all antibodies from BD Biosciences): anti-CD3 UCHT1 BUV737, anti-CD4 SK3 (aka: Leu3a) BV480, anti-CD8 RPA-T8 PE, anti-CD19 HIB19 PerCP-Cy5.5, anti-CD45 HI30 BUV395, anti-CD56 NCAM16.2 BV711, anti-TCRγ/δ-1 11F2 FITC, MR1-5-OP-RU Tetramer (hereafter MR1-tetramer) conjugated to BV421-streptavidin (BD Horizon) was produced as described [6].

2.3 Gating Strategy

Single CD45+ leukocyte events were gated on a SSC-A vs FSC-A plot, followed by dead cell exclusion. All subsequent analyses were performed on BAL total T cells gated as CD45+CD3+CD19– cells. Bulk CD4 and CD8 T cells were defined by the expression of CD4+CD8– and CD8+CD4– by BAL total T cells, respectively. $\gamma\delta$ T cells and MAIT cells were identified as CD45+CD3+$\gamma\delta$TCR+ and CD45+CD3+MR1-tetramer+, respectively and were further analyzed for the proportions of cells within these populations that expressed CD4 or CD8. As CD19+ B cells also bind the MR1 tetramer, CD19+ cells were excluded by the initial gating. NKT-like cells were classified as CD45+CD4–CD8–CD3+CD56+ cells. The absence of CD4 and CD8 was used to exclude conventional T cells, which can upregulate Natural Killer cell markers following activation, from the NKT-like cell analysis. This study did not aim to dissect NKT cells in detail and no identification was made of iNKT and non-classical NKT cell populations. The frequency of each T cell subset is reported as a proportion of the total CD3+ cell population or the parent population as specified in the results section. A summary of the gating strategy is shown in **Supplementary Fig. 1**.

2.4 Data Analysis and Statistics

Flow cytometry data were analyzed using FlowJo V10 software (Becton Dickinson, Ashland, USA). Statistical analyses were performed with GraphPad Prism Version 6 (GraphPad Software Inc., California, USA). Data were checked for normality using the Kolmogorov-Smirnov test and normally distributed data analyzed by unpaired t-test. Three groups of data were compared by Kruskal-Wallis test.

3. Results

The aim of this study was to characterize the composition of unconventional T cells within BAL samples of young children with CF. BAL cells were analyzed from 17 children with CF. These comprised 12 females and 5 males aged between 2 and 6 years of age (characteristics and infection status are listed in Table 1).

Routine clinical assessment of BAL cells by light microscopy showed, as usual for such samples, that macrophages were the predominant cell population, with a typical neutrophil infiltration and only a low proportion of lymphocytes (0–1.7%) (**Supplementary Fig. 2**). T cells within the BAL fluid from young children with CF were identified by flow cytometric analysis as CD45+CD3+CD19– cells (gating strategy, **Supplementary Fig. 2**), and contained a mix of CD4+ and CD8+ T cells (**Supplementary Fig. 3**). These contained a higher proportion of CD8+ T cells (median 34% to 44% respectively) which is consistent with previous studies in healthy children, and children with CF [2,7].
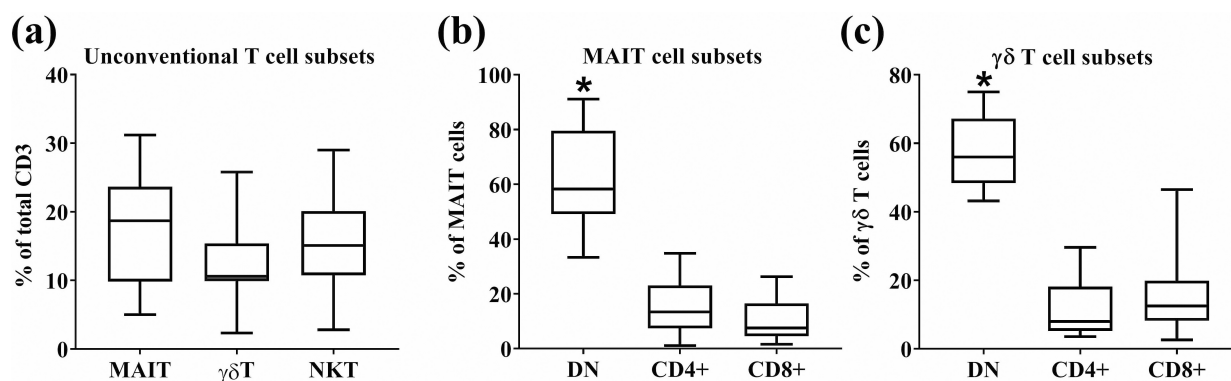
Fig. 1. Flow cytometric phenotyping of unconventional T cells in the BAL of young children with CF. (a) The relative proportions of MAIT cells (CD45+CD3+CD19−MR1-tetramer+), $\gamma\delta$ T cells (CD45+CD3+$\gamma\delta$TCR+) and NKT-like cells (CD45+CD3+CD56+) as a percentage of total T cells were analysed by flow cytometry, in BAL samples from young children with CF (n = 17). CD4+, CD8+ and CD4 and CD8 double negative (DN) subsets of (b) MAIT cell and (c) $\gamma\delta$ T cell sub populations. *Significantly greater than the CD4+ and CD8+ subsets (*$p < 0.0001$ Kruskal-Wallis). Data are single values pooled from children analyzed individually over an 18-month period. Boxplots present the middle 50% of values (box), the median (line) and range (error bars).

Table 1. Children with CF cohort characteristics. URTF (upper respiratory tract flora).

Child number	Age (Years)	Sex	Infection status
1	2.9	F	*Haemophilus parainfluenzae* (not type B), URTF
2	5.2	F	URTF, *Respiratory syncytial virus*
3	4.6	F	*H. parainfluenzae*, URTF
4	4.9	F	URTF, *fungus*
5	5.0	M	*H. parainfluenzae*, URTF, *Haemophilus influenzae* (not type B)
6	5.9	F	URTF
7	6.0	F	*S. aureus, Stenotrophomonas maltophilia, Escherichia coli, H. influenzae* (not type B), URTF, *Parainfluenza virus*
8	6.0	F	*Pseudomonas aeruginosa* (rough), URTF, *Influenza virus*
9	3.0	F	*H. influenzae, Methicillin resistant Staphylococcus aureus*, URTF
10	6.1	F	*H. influenzae* (not type B), *S. aureus*, URTF
11	3.0	M	URTF
12	3.0	M	*Haemophilus haemolyticus, S. aureus*, URTF
13	3.0	F	URTF, *H. parainfluenzae, parainfluenza virus*
14	5.9	F	URTF
15	5.1	F	*Respiratory syncytial virus, Moraxella catarrhalis, H. parainfluenzae*
16	2.9	M	*Respiratory syncytial virus*, URTF, *Haemophilus species*
17	3.9	M	*H. parainfluenzae, Gram negative bacilli, H. influenzae* (not type B), URTF

3.1 Frequency of Unconventional T Cells

Further analyses quantified the proportions of the unconventional T cell populations, specifically NKT-like cells, $\gamma\delta$ T cells and MAIT cells, within the BAL from young children with CF. While this revealed considerable variability in the proportions of these unconventional cells between individuals, as a population overall there were similar levels of MAIT cells (median 18.7%, interquartile range (IQR) 9.8–23.7%) and NKT-like cells (median 15.1%; IQR 10.8–20.1%) with slightly fewer $\gamma\delta$ T cells (median 10.6%; IQR 9.9–15.4) (Fig. 1a).

MAIT cells and $\gamma\delta$ T cells were further analyzed for their relative proportions of CD4+, CD8+ and double negative (DN) subsets. For both immune cell types, the DN sub-

set was the predominant population, with smaller but similar sized subsets that were single positive for either CD4 or CD8 (Fig. 1b and c).

3.2 Changes during Early Development

To see if age-related changes in unconventional T cell frequencies were detectable in BAL during very early progression in CF, the frequencies of T cell subsets were compared with the age of the children. Correlating age against the frequencies of the unconventional T cell subtypes analyzed provided no evidence of any change in the proportions of these cells, as children with CF progressed from 2 to 6 years of age (Fig. 2a). Similarly, no significant difference was detected in the frequencies of unconventional T
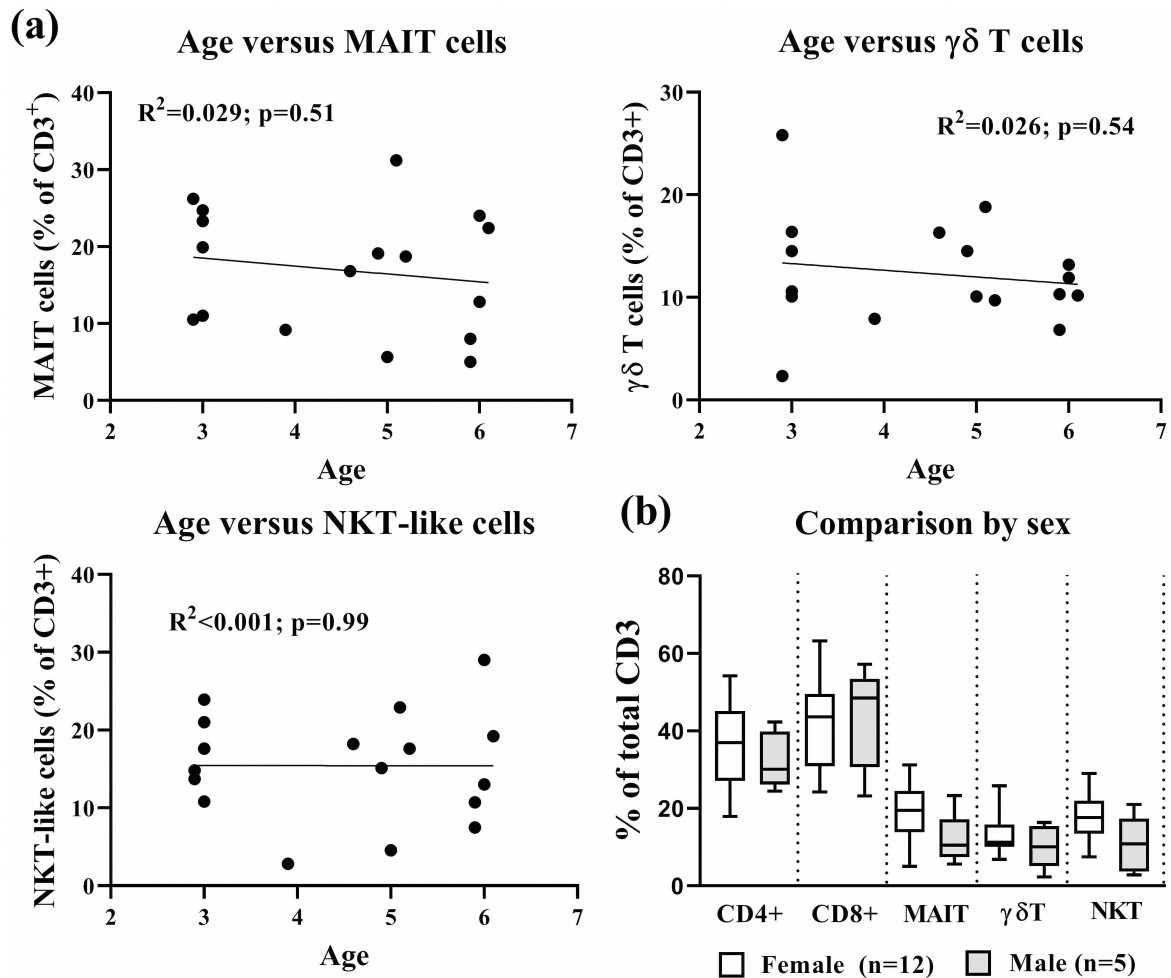
(a)

Age versus MAIT cells

$R^2=0.029$; p=0.51

Age versus $\gamma\delta$ T cells

$R^2=0.026$; p=0.54

Age versus NKT-like cells

$R^2<0.001$; p=0.99

(b) Comparison by sex

☐ Female (n=12) ☐ Male (n=5)

Fig. 2. Effect of age and sex on unconventional T cells subsets in BAL from young children with CF. (a) Correlation of age versus frequencies of unconventional T cells in children with CF. (b) Comparison analysis of different T cells subtypes between female (n = 12) and male (n = 5) CF patients. There were no statistically significant differences between the two sexes (unpaired *t* test) or any correlation of cell frequency with age (linear regression analysis). Data are single values pooled from children analyzed individually over an 18-month period (a) presented as individual patients or (b) as groups in boxplots, which present the middle 50% of values (box), the median (line) and range (error bars).

cell populations in BAL cells collected from young male (n = 5) and female (n = 12) children with CF (Fig. 2b).

3.3 Association between Unconventional T Cell Proportions and Clinical Infection Status

To determine if the frequencies of T cell subsets present in the lungs of children with CF might be influenced by their infection status, we analyzed the proportions of BAL T cell subsets, in relation to clinically prevalent CF bacterial and viral pathogenic infections present in BAL from our cohort of CF children. As the majority of children with CF in the present cohort had multiple infections (Table 1), T cell subset proportions were compared in terms of commonly presented CF viral and bacterial pathogens (Fig. 3).

Infection with *Haemophilus* species bacteria (including both *H. influenzae* and *H. parainfluenzae*), while highly prevalent in the patients studied (11 out of 17 children), had no significant effect on the proportions of MAIT cells, $\gamma\delta$ T cells or NKT-like cells, relative to samples from children with CF not infected with this pathogen (Fig. 3a). Similarly, no differences were observed for unconventional T cell populations for samples obtained from children with CF infected with *S. aureus* (Fig. 3b).

Some children included in this study were infected with respiratory syncytial virus (n = 3), parainfluenza (n = 2) and influenza (n = 1). As there were insufficient cases of any virus to analyze individually, viral infections were studied as a group. While the proportions of most T cell subsets were similar in all children regardless of viral infection status, there was a significant increase in the proportion of NKT-like cells in BAL cells from young children with CF who were infected with a virus (Fig. 3c).
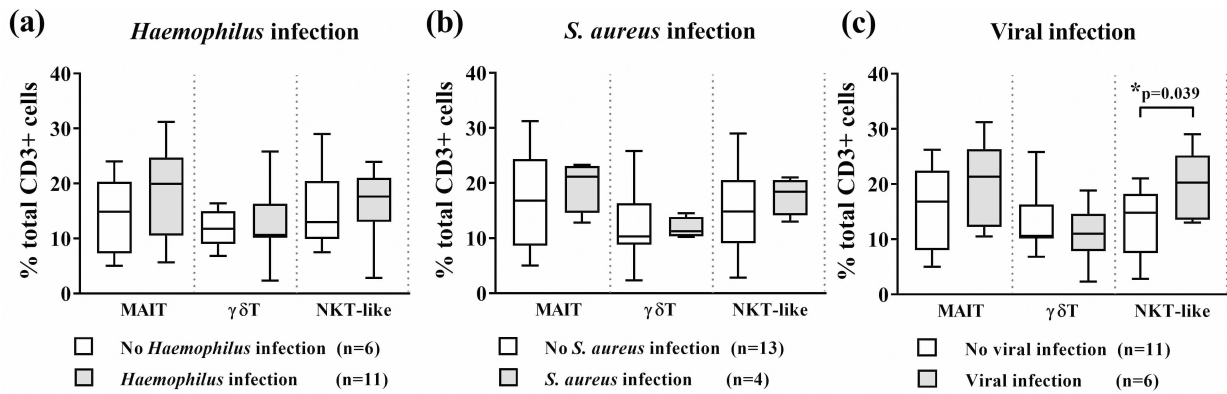
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(a) *Haemophilus* infection

(b) *S. aureus* infection

(c) Viral infection

No *Haemophilus* infection (n=6)
Haemophilus infection (n=11)

No *S. aureus* infection (n=13)
S. aureus infection (n=4)

No viral infection (n=11)
Viral infection (n=6)

Fig. 3. Effect of infections on unconventional T cells subsets in BAL from young children with CF. Flow cytometric comparisons of MAIT cells (CD45+CD3+CD19–MR1-tetramer+), $\gamma\delta$ T cells (CD45+CD3+$\gamma\delta$TCR+) and NKT-like cells (CD45+CD3+CD56+) as a percentage of total CD3+ T cells in BAL cells from CF children (n = 17) with or without (a) viral infection, (b) *Haemophilus* infection or (c) *S. aureus* infection. *Unpaired *t*-test. Data are single values pooled from children analyzed individually over an 18-month period. Boxplots present the middle 50% of values (box), the median (line) and range (error bars).

4. Discussion

The principal cause of death in people with CF remains loss of lung function due to pulmonary disease arising from chronic infection, with sustained inflammation and interspersed acute exacerbations. As for any infection-driven inflammation, T cells would be expected to play vital roles in the CF lung in fighting infections and modulating inflammation. There are two key ways that T cells could impact and interact with CF disease. First, T cell populations already present in the lung or recruited by infection could either increase or decrease the severity of the resulting inflammation and thus enhance disease progression. In addition, T cells themselves express CFTR which, when functionally impaired, can exhibit reduced ion channel activity with effects on the secretion of cytokines including the anti-inflammatory IL-10 [8]. Little is known about the precise role of T cell immunity in CF, particularly in early childhood, although regarding conventional T cell responses, increases in Th17 and Th2 responses in the adult CF lung have been identified, with potential negative effects on disease progression [9,10].

Importantly, T cells are comprised of heterogeneous cell populations, each with specific and varied functions, and the potential of either reducing or exacerbating the pathological process in chronic disease. With the paucity of information available regarding many important cell types in early CF, this study set out to perform the first analysis of unconventional T cell composition in the BAL of young children with this disease. Unconventional T cells comprising MAIT cells, NKT cells and $\gamma\delta$ T cells are found both resident within lung tissues and in fluids lining the lung epithelium, as accessed by bronchoalveolar lavage. The role of these unconventional T cells and any potential impact on disease remains uncertain, although it is thought they might contribute to early defence against pathogenic infection, as well as potentially exacerbating some chronic in-

flammatory lung conditions [11]. Such cells appear to be located constitutively in the lung fluid lining the epithelium, as they are present there in healthy individuals and it has even been reported that unconventional T cell numbers in BAL can reduce during lung inflammatory pathologies, including chronic obstructive pulmonary disease and asthma [12].

MAIT cells are a subset of unconventional T cells, often highly abundant in humans, which due to their responsiveness to riboflavin-derived antigens, respond to a range of yeast and bacterial pathogens, including at mucosal surfaces such as the lung [13,14]. Recent studies in mice indicated a role for these cells as early innate immune responders in lung defense against an array of pathogens, including *Mycobacterium bovis* bacillus Calmette-Guérin, *Legionella longbeachae* and influenza viruses [15–17]. MAIT cells have also been implicated in the pathogenesis of non-infectious chronic pulmonary disorders such as asthma [18]. Despite the potential importance of these cells, little is known about their frequency and potential role in the CF lung.

In the current study, we observed that MAIT cells comprised the largest proportion of the unconventional T cells in BAL fluid and that their frequency was relatively independent of the infection status. A previous study which examined MAIT cells in the peripheral blood of adults with CF with lung bacterial infections (particularly *P. aeruginosa*) found the frequency of these cells to be significantly lower in CF blood as compared to healthy controls and noted that reduced circulating MAIT cell numbers positively correlated with the severity of the lung disease [19]. Adults with CF typically have a more advanced disease state as well as different pathogen infection profiles compared to children with CF, and so it is unknown whether this inverse association would also be present during childhood. It also remains to be determined if this reflects a causative

link between MAIT cells and pathogenesis or simply that the chronic inflammation in CF might deplete these cells in the circulation. However, it is interesting to note the report of one individual with CF who succumbed to bacterial infections at only 22 years of age and who was found to have a profound deficiency in circulating MAIT cells [20].

Human MAIT cells are heterogeneous, with the most commonly described subpopulations being CD4–CD8– (DN) or CD4–CD8+, which are developmentally related but functionally distinct subtypes [21,22]. Our analyses found that DN MAIT cells were the dominant subpopulation in the CF lung, with smaller but fairly equivalent levels of CD4+ and CD8+ subsets. This distribution is different from human blood, where the majority of MAIT cells are CD4–CD8+ [13]. Potentially explaining this difference, DN MAITs from the peripheral blood have been suggested to be functionally more mature, expressing increased levels of cytokine receptors yet have also been shown to have a generally reduced proinflammatory cytokine response to bacterial (*Escherichia coli*) stimulation [22]. The increased proportions of DN MAIT cells in these BAL samples might therefore represent an increased activation or maturation state caused by the chronic inflammatory environment of the CF lung. Another important point is that the frequency of blood MAIT cells increases from childhood into adulthood, peaking at about 30 years of age before declining [23,24]. As this study analyzed BAL samples from young children, it is possible the results for MAIT cells from older people with CF might be different, not only due to changes as the disease progresses, but also due to age-related increases in MAIT cells.

We found an average 18% of BAL T cells from children with CF aged 2–6 years of age were MAIT cells (Fig. 1), which is considerably higher than recently reported in control children aged 0–8 years of age, where MAIT cells comprised an average of only 1% of CD3+ BAL T cells [25]. In this latter study, the proportion of MAIT cells in BAL increased to an average of 3% in age and sex matched children with community acquired pneumonia [25]. Interestingly, MAIT cell populations in the lungs of mice dramatically expand in infection models, leading to the suggestion that laboratory mice have markedly lower levels of MAIT cells than humans due to their lack of exposure to infections [13,17,26]. The high levels of MAIT cells observed in the lungs of children with CF in this study is therefore likely to be at least partially the effect of their chronic and commonly multiple pathogenic infections.

This study also examined $\gamma\delta$ T cells, which are virtually unstudied in the CF lung, although their presence has been indicated in tissues from at least some samples from CF patients with end stage disease [27]. This cell type is known to be relatively abundant in mucosal sites including the lung [28], and consistent with this we also found $\gamma\delta$ T cells to be relative abundant in the CF lung. We performed a sub-analysis to evaluate if sex or childhood age (which

in CF can translate to disease progression) might affect $\gamma\delta$ T cell frequency, and no effect was observed. However, it should be recognized that this analysis did involve relatively small group sizes due to limited sample availability. Lung infection status was also not found to affect $\gamma\delta$ T cell frequency in the BAL samples analyzed.

As for other unconventional T cells, there are several $\gamma\delta$ T cell subpopulations. Although we were unable to extensively phenotype the BAL $\gamma\delta$ T cells due to the limited numbers of BAL cells available for analysis, we did note that $\gamma\delta$ T cells in the BAL of children with CF were predominantly CD4–CD8–, consistent with the limited information available from lungs of normal mice [29,30]. $\gamma\delta$ T cells have been proposed to defend against a range of lung infections including influenza, *M. tuberculosis* and *Streptococcus pneumoniae* [31–33], and so could potentially play an important role in combatting infection in CF. Stimulation with the CF pathogen *P. aeruginosa* has been shown to induce more secretion of the pro-inflammatory TNF and IFNγ by peripheral blood $\gamma\delta$ T cells than conventional $\alpha\beta$ T cells [34], suggesting these cells could be an important source of proinflammatory cytokines in the infected CF lung. However, no difference was observed between cells isolated from people with CF or non-CF controls, indicating the *CFTR* mutation did not modify this response [34].

Finally, we evaluated CD56+ NKT-like cells. NKT cells have multiple anti-microbial effector functions and are capable of cytotoxic T cell activation [35,36]. As such they potentially provide an important innate defensive role in combatting infection in CF, especially against viruses. Consistent with this, it is interesting that we observed a significant increase in the frequencies of NKT-like cells in samples from virally infected children with CF. The anti-viral activity of NKT cells is well established, so this increase in NKT-like cell frequency supports an important role for these cells in fighting viral infection in the lungs of children with CF.

While the status of NKT cells in CF is largely unknown, mutations in the *Cftr* gene significantly increased invariant NKT (iNKT) cell accumulation in the lungs of mice in a model of ceramide-induced autoimmune disease, with the presence of these cells associated with increased chronic inflammation in CF lungs [37]. While only limited data are available on the frequency of NKT cells in BAL from healthy children, comparing our results with those available suggests the frequency of NKT-like cells in children with CF might be increased. For example, Hodge *et al.* [38] reported that NKT-like cells constituted a median average of <8% of T cells in healthy control Australian children (mean age 40 months), while our study of Australian CF children (mean age 53 months) found NKT-like cells comprised a median 15% of T cells. This difference is likely indicative of a response to chronic infection, in particular to the presence of pulmonary viral infections.

An important limitation of this study was that, as for $\gamma\delta$ T cells, cell availability and flow cytometer panel size limits meant we were unable to include additional NKT cell markers that would allow accurate enumeration of NKT cell subpopulations such as the iNKT, of which only some express CD56. Given the findings linking NKT-like cells and viral infection reported in this study, a further expanded evaluation of these NKT cells will be an important feature of future investigations, especially as iNKT cells have been shown to help recruit neutrophils into airways via the production of IL-17, at least in mice [39]. A further limitation was the low number of samples available for analysis, with seventeen samples analysed over an 18 month period. This low sample size means it is possible some positive associations might not have been identified due to insufficient power of analysis.

5. Conclusions

This study provides the first identification and evaluation of unconventional T cells in the BAL of children with CF. This provides valuable insights into the potential roles these cells might play during the critical early formative years of this disease. Significantly, this study identified changes in the frequency of one T cell subtype that was linked to viral infections, indicating potentially important interactions between these cells and individual pathogens. Improving our understanding of these T cell subsets during the early stages of the disease process is essential for determining whether the role of these cells in CF is only to combat infection, or if they also contribute to the pathogenic process, and thereby provide new therapeutic targets for immunomodulatory approaches aimed at regulating inflammation and improving disease prognosis.

Abbreviations

CF, Cystic Fibrosis; MAIT, Mucosal Associated Invariant T; NKT, Natural Killer T; BAL, Bronchoalveolar Lavage; FACS, Fluorescence Activated Cell Sorting.

Author Contributions

PS designed the research study. RM and GAT performed the research. CMH, AJC and DGP provided help and advice on flow cytometric analysis of immune cells. RC and SR provided samples, help and advice relating to cystic fibrosis. RM, GAT and PS analyzed the data. RM, GAT and PS wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Human Research Ethics Committee of The Royal Children's Hospital Melbourne (protocol code HREC #25054).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2705149.

References

[1] Pittman JE, Noah H, Calloway HE, Davis SD, Leigh MW, Drumm M, *et al*. Early childhood lung function is a stronger predictor of adolescent lung function in cystic fibrosis than early *Pseudomonas aeruginosa* infection. PLoS ONE. 2017; 12: e0177215.

[2] Shanthikumar S, Burton M, Saffery R, Ranganathan SC, Neeland MR. Single-Cell Flow Cytometry Profiling of BAL in Children. American Journal of Respiratory Cell and Molecular Biology. 2020; 63: 152–159.

[3] Regamey N, Tsartsali L, Hilliard TN, Fuchs O, Tan H, Zhu J, *et al*. Distinct patterns of inflammation in the airway lumen and bronchial mucosa of children with cystic fibrosis. Thorax. 2012; 67: 164–170.

[4] Giacalone VD, Dobosh BS, Gaggar A, Tirouvanziam R, Margaroli C. Immunomodulation in Cystic Fibrosis: Why and How? International Journal of Molecular Sciences. 2020; 21: 3331.

[5] Pellicci DG, Koay HF, Berzins SP. Thymic development of unconventional T cells: how NKT cells, MAIT cells and $\gamma\delta$ T cells emerge. Nature Reviews Immunology. 2020; 20: 756–770.

[6] Corbett AJ, Eckle SBG, Birkinshaw RW, Liu L, Patel O, Mahony J, *et al*. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. Nature. 2014; 509: 361–365.

[7] de Mendonca Picinin IF, Camargos P, Mascarenhas RF, Santos SME, Marguet C. Cell count and lymphocyte immunophenotyping of bronchoalveolar lavage fluid in healthy Brazilian children. European Respiratory Journal. 2011; 38: 738–739.

[8] Moss RB, Bocian RC, Hsu Y, Dong Y, Kemna M, Wei T, *et al*. Reduced IL-10 secretion by CD4+ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR). Clinical and Experimental Immunology. 1996; 106: 374–388.

[9] Tan H, Regamey N, Brown S, Bush A, Lloyd CM, Davies JC. The Th17 Pathway in Cystic Fibrosis Lung Disease. American Journal of Respiratory and Critical Care Medicine. 2011; 184: 252–258.

[10] Hartl D, Griese M, Kappler M, Zissel G, Reinhardt D, Rebhan C, *et al*. Pulmonary TH2 response in *Pseudomonas aeruginosa*–infected patients with cystic fibrosis. Journal of Allergy and Clinical Immunology. 2006; 117: 204–211.

[11] Borger JG, Lau M, Hibbs ML. The Influence of Innate Lymphoid Cells and Unconventional T Cells in Chronic Inflammatory Lung Disease. Frontiers in Immunology. 2019; 10: 1597.

[12] Urboniene D, Babusyte A, Lotvall J, Sakalauskas R, Sitkauskiene B. Distribution of gammadelta and other T-lymphocyte subsets in patients with chronic obstructive pulmonary disease and asthma. Respiratory Medicine. 2013; 107: 413–423.

[13] Godfrey DI, Koay H, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. Nature Immunology. 2019; 20: 1110–1128.

[14] Le Bourhis L, Martin E, Péguillet I, Guihot A, Froux N, Coré M, et al. Antimicrobial activity of mucosal-associated invariant T cells. Nature Immunology. 2010; 11: 701–708.

[15] Chua W, Truscott SM, Eickhoff CS, Blazevic A, Hoft DF, Hansen TH. Polyclonal Mucosa-Associated Invariant T Cells have Unique Innate Functions in Bacterial Infection. Infection and Immunity. 2012; 80: 3256–3267.

[16] van Wilgenburg B, Loh L, Chen Z, Pediongco TJ, Wang H, Shi M, et al. MAIT cells contribute to protection against lethal influenza infection in vivo. Nature Communications. 2018; 9: 4706.

[17] Wang H, D'Souza C, Lim XY, Kostenko L, Pediongco TJ, Eckle SBG, et al. MAIT cells protect against pulmonary Legionella longbeachae infection. Nature Communications. 2018; 9: 3350.

[18] Lambrecht BN, Hammad H. The immunology of asthma. Nature Immunology. 2015; 16: 45–56.

[19] Smith DJ, Hill GR, Bell SC, Reid DW. Reduced mucosal associated invariant T-cells are associated with increased disease severity and Pseudomonas aeruginosa infection in cystic fibrosis. PLoS ONE. 2014; 9: e109891.

[20] Pincikova T, Paquin-Proulx D, Moll M, Flodström-Tullberg M, Hjelte L, Sandberg JK. Severely Impaired Control of Bacterial Infections in a Patient with Cystic Fibrosis Defective in Mucosal-Associated Invariant T Cells. Chest. 2018; 153: e93–e96.

[21] Chiba A, Murayama G, Miyake S. Mucosal-Associated Invariant T Cells in Autoimmune Diseases. Frontiers in Immunology. 2018; 9: 1333.

[22] Dias J, Boulouis C, Gorin JB, van den Biggelaar R, Lal KG, Gibbs A, et al. The CD4(-)CD8(-) MAIT cell subpopulation is a functionally distinct subset developmentally related to the main CD8(+) MAIT cell pool. Proceedings of the National Academy of Sciences of the United States of America. 2018; 115: E11513–E11522.

[23] Walker LJ, Tharmalingam H, Klenerman P. The rise and fall of MAIT cells with age. Scandinavian Journal of Immunology. 2014; 80: 462–463.

[24] Gherardin NA, Souter MN, Koay H, Mangas KM, Seemann T, Stinear TP, et al. Human blood MAIT cell subsets defined using MR1 tetramers. Immunology & Cell Biology. 2018; 96: 507–525.

[25] Lu B, Liu M, Wang J, Fan H, Yang D, Zhang L, et al. IL-17 production by tissue-resident MAIT cells is locally induced in children with pneumonia. Mucosal Immunology. 2020; 13: 824–835.

[26] Chen Z, Wang H, D'Souza C, Sun S, Kostenko L, Eckle SBG, et al. Mucosal-associated invariant T-cell activation and accumulation after in vivo infection depends on microbial riboflavin synthesis and co-stimulatory signals. Mucosal Immunology. 2017; 10: 58–68.

[27] Hagner M, Albrecht M, Guerra M, Braubach P, Halle O, Zhou-Suckow Z, et al. IL-17A from innate and adaptive lymphocytes contributes to inflammation and damage in cystic fibrosis lung disease. The European respiratory journal. 2021; 57: 1900716.

[28] Cheng M, Hu S. Lung-resident $\gamma\delta$ T cells and their roles in lung diseases. Immunology. 2017; 151: 375–384.

[29] Augustin A, Kubo RT, Sim GK. Resident pulmonary lymphocytes expressing the gamma/delta T-cell receptor. Nature. 1989; 340: 239–241.

[30] Pinheiro MB, Antonelli LR, Sathler-Avelar R, Vitelli-Avelar DM, Spindola-de-Miranda S, Guimarães TM, et al. CD4−CD8−$\alpha\beta$ and $\gamma\delta$ T cells display inflammatory and regulatory potentials during human tuberculosis. PLoS ONE. 2012; 7: e50923.

[31] Kirby AC, Newton DJ, Carding SR, Kaye PM. Evidence for the involvement of lung-specific gammadelta T cell subsets in local responses to Streptococcus pneumoniae infection. European Journal of Immunology. 2007; 37: 3404–3413.

[32] El Daker S, Sacchi A, Montesano C, Altieri AM, Galluccio G, Colizzi V, et al. An abnormal phenotype of lung Vγ9Vδ2 T cells impairs their responsiveness in tuberculosis patients. Cellular Immunology. 2013; 282: 106–112.

[33] Li H, Xiang Z, Feng T, Li J, Liu Y, Fan Y, et al. Human Vγ9Vδ2-T cells efficiently kill influenza virus-infected lung alveolar epithelial cells. Cellular & Molecular Immunology. 2013; 10: 159–164.

[34] Raga S, Julià MR, Crespí C, Figuerola J, Martínez N, Milà J, et al. Gammadelta T lymphocytes from cystic fibrosis patients and healthy donors are high TNF-alpha and IFN-gamma-producers in response to Pseudomonas aeruginosa. Respiratory Research. 2003; 4: 9.

[35] Ihara F, Sakurai D, Takami M, Kamata T, Kunii N, Yamasaki K, et al. Regulatory T cells induce CD4− NKT cell anergy and suppress NKT cell cytotoxic function. Cancer Immunology, Immunotherapy. 2019; 68: 1935–1947.

[36] Gansert JL, Kieβler V, Engele M, Wittke F, Röllinghoff M, Krensky AM, et al. Human NKT Cells Express Granulysin and Exhibit Antimycobacterial Activity. The Journal of Immunology. 2003; 170: 3154–3161.

[37] Siegmann N, Worbs D, Effinger F, Bormann T, Gebhardt M, Ulrich M, et al. Invariant Natural Killer T (iNKT) Cells Prevent Autoimmunity, but Induce Pulmonary Inflammation in Cystic Fibrosis. Cellular Physiology and Biochemistry. 2014; 34: 56–70.

[38] Hodge G, Upham JW, Chang AB, Baines KJ, Yerkovich ST, Pizzutto SJ, et al. Increased peripheral blood pro-inflammatory/cytotoxic lymphocytes in children with bronchiectasis. PLoS ONE. 2015; 10: e0133695.

[39] Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. The Journal of Experimental Medicine. 2007; 204: 995–1001.