

Peripheral killer cells do not differentiate between asthma patients with or without fixed airway obstruction

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Abstract

Objective: The three main types of killer cells – CD8⁺ T cells, NK cells and NKT cells – have been linked to asthma and chronic obstructive pulmonary disease (COPD). However, their role in a small subset of asthma patients displaying fixed airway obstruction (FAO), similar to that seen in COPD, has not been explored.

The objective of the present study was to investigate killer cell numbers, phenotype and function in peripheral blood from asthma patients with FAO, asthma patients without FAO, and healthy individuals. *Methods:* Peripheral CD8⁺ T cells (CD8⁺CD3⁺CD56⁻), NK cells (CD56⁺CD3⁻) and NKT-like cells (CD56⁺CD3⁺) of 14 asthma patients with FAO (post-bronchodilator FEV₁/FVC <0.7, despite clinician-optimised treatment), 7 asthma patients without FAO (post-bronchodilator FEV₁/FVC ≥0.7), and 9 healthy individuals were studied.

Results: No significant differences were seen between the number, receptor expression, MAPK signalling molecule expression, cytotoxic mediator expression, and functional cytotoxicity of peripheral killer cells from asthma patients with FAO, asthma patients without FAO and healthy individuals. *Conclusions:* Peripheral killer cell numbers or functions do not differentiate between asthma patients with or without fixed airway obstruction.

Keywords

CD8⁺ T cells; fixed airflow obstruction; natural killer cells; natural killer T cells; protein lysate microarray; asthma.

Introduction

Asthma is a chronic inflammatory disease of the airways, with over 200 million individuals affected worldwide [1]. It is characterised by airway hyper-responsiveness and remodelling resulting in coughing, breathlessness and chest tightness. Asthma encompasses a variety of clinical phenotypes ranging from what was classically defined as extrinsic allergic (IgE-mediated) asthma to intrinsic non-allergic asthma [2]. Recent genomic, transcriptomic and proteomic investigations of asthma, in combination with clinico-pathological parameters, have led to the realisation that clinically-diagnosed asthma encompasses a diverse and complex set of conditions referred to as 'endotypes' [3-5]. These range from classical allergic asthma (Th2-driven, IgE-mediated, eosinophilic), through neutrophilic asthma associated with the activation of innate immunity, to asthma involving little inflammation, but extensive airway remodelling [3-5]. It has also become apparent that the traditional distinction between asthma and chronic obstructive pulmonary disease (COPD) is not always applicable, with some patients having features of both of these conditions [6-8].

The majority of people with asthma display airflow obstruction that is episodic and returns to normal either spontaneously or following appropriate treatment. However, a minority of people with asthma display airflow obstruction that does not respond to treatment, i.e. can be described as 'fixed' ([9-22], reviewed in [23]). This fixed airflow obstruction (FAO) is a feature more commonly associated with COPD [24]. It is not known why some asthma patients develop FAO. It may be a consequence of more severe asthma, duration of asthma, or it may represent a separate disease entirely.

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2
3 In recent years, interest and investigations into the potential role of killer
4 cells in asthma and COPD have steadily increased. CD8 T cells, NK cells and NKT
5 cells are the three main killer cells types; their effector functions include cellular
6 cytotoxicity and immunoregulation [25-36]. Killer cells are thought also to
7 contribute to COPD [37-39]. We have previously shown that, in COPD, the
8 numbers of peripheral blood NK and NK-T cells were reduced and that their
9 cytotoxic function was defective compared to healthy smokers and never-
10 smokers [40].

11
12 Far fewer studies have looked at the role that killer cells play in asthma
13 with FAO. Immunohistochemistry has shown the degree of FAO to be
14 significantly correlated to the number of CD8 T cells in bronchial biopsies [19].
15 In this study, we have investigated the number and function of peripheral human
16 killer cells in asthma with or without FAO to see if these differ, and to determine
17 whether asthma with FAO is more similar to COPD or asthma without FAO in this
18 respect.

19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 **Material and methods**

40 41 42 **Study population and procedures**

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44 The Nottingham Local Research Ethics Committee approved the study protocol
45 (REC reference 10/H0405/2) and informed written consent was taken prior to
46 subjects enrolling on to the study. Thirty individuals were recruited into three
47 participant groups: asthma with fixed airflow obstruction (A+FAO), asthma
48 without FAO (A-FAO), and healthy controls (H). All asthma subjects had a clinical
49 diagnosis. Asthma patients with FAO had a post-bronchodilator FEV/FVC <0.7,
50 despite clinician-optimised treatment [41]; while asthma patients without FAO
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3 had a post-bronchodilator FEV₁/FVC ≥ 0.7 . The bronchodilator (400 μ g
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5 salbutamol) was administered 20 minutes prior to spirometry. All participants
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7 were age-matched, had no co-morbidities, were ex or non-smokers with a pack
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9 history of ≤ 10 years, and had no infections within the previous six weeks. The
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11 demographic and spirometric data of the 30 study participants used to
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13 investigate killer cell number, surface receptor expression and intracellular
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15 cytotoxic mediator expression are shown in Table 1a; details of the 20
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17 participants whose MAPK signalling molecule expression were analysed are
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19 shown in Table 1b; while the details of the 18 participants whose NK cell
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21 cytotoxicity levels were measured are shown in Table 1c.
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31 **Absolute cell counting**

32 To quantify killer cell numbers, fluorescently labelled antibodies specific for
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34 several killer cell markers, shown in Table 2, were added to 100 μ l whole
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36 peripheral blood at half the recommended concentration and left in the dark for
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38 15 minutes. Optilyse C solution (Beckman Coulter, A11895) was used to lyse the
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40 red blood cells and was diluted with an equal volume of phosphate buffered
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42 saline (PBS, Sigma D8537) after 10 minutes. Finally, 100 μ l of flow-count
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44 fluorosphere beads (Beckman Coulter, 7547053) were added to each tube,
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47 before immediate analysis on a Beckman Coulter FC500 flow cytometer.
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53 **Peripheral blood mononuclear cell (PBMC) and killer cell isolation**

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55 To obtain PBMCs, peripheral whole blood was diluted to a ratio of 2:1 with
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57 RPMI-1640 media (Sigma, R8758), layered over histopaque (Sigma, H8889) and
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3 centrifugated at 800g for 23 minutes. The resulting PBMC layer was harvested.
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5 CD8 T cells, NK cells and NKT-like cells were isolated from PBMCs via
6
7 immunomagnetic separation using a CD56 multisort kit (Miltenyi Biotec, 130-
8
9 055-401) and CD3 and CD8 microbeads (Miltenyi Biotec, 130-050-101 and 130-
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11 045-201, respectively) according to the supplied protocols. Briefly, PBMCs were
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13 incubated with CD56 microbeads, washed, and passed through an MS column
14
15 situated within a MACS magnet. After microbead release, using the supplied
16
17 reagent, and another wash, the CD56⁺ fraction was incubated with CD3
18
19 microbeads; while the CD56⁻ fraction was incubated with CD8 microbeads. Both
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21 fractions were run through a fresh MS column, which yielded a CD56⁺CD3⁻
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23 fraction (NK cells), a CD56⁺CD3⁺ fraction (NKT-like cells), and a CD56⁻CD8⁺
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25 fraction (CD8 T cells). All reagents and incubation steps were used and
26
27 performed at 4°C. A portion of each fraction was removed, stained extracellularly
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29 and analysed by flow cytometry to ascertain purity. The median CD8 T cell purity
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31 was above 92%, the median NK cell purity was above 97%, and the median NKT
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33 cell purity was above 74%.
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42 **Flow cytometry**

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44 Killer cell purity, surface receptor expression, and intracellular cytotoxic
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46 mediator expression were all analysed by flow cytometry. Briefly, cells were
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48 quick-fixed using a 3% formaldehyde fix solution. Those stained extracellularly
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50 were washed in phosphate buffered albumin (PBA- PBS (Oxoid BR0014G) with
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52 1% BSA (Sigma A7284) and 0.1% NaN₃ (Sigma S8032)), incubated with
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54 fluorescently-conjugated antibodies (see Table 2) for 30 minutes in the dark, and
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56 washed again. Those stained intracellularly were washed with 0.04% PBA-
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3 saponin (Sigma, S4521) followed by PBA-saponin with 10% FCS, before a two-
4
5 hour incubation and a final wash in PBA-saponin. All cells were stored in a 0.5%
6
7 formaldehyde fix solution until analysis. Fluorescence-minus-one controls,
8
9 containing all the antibodies bar one, were also included. Flow cytometry
10
11 analysis was performed on a Beckman Coulter FC500 flow cytometer, with 50
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13 000 events collected for each sample. Dead cells were excluded based on their
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15 forward (size) and side scatter (granularity).
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20 21 **Reverse-phase protein microarray (RPPA) analysis**

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23 An in-house cell lysate microarray system was used to quantify killer cell
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25 cytotoxic signalling molecule levels [42]. Briefly, samples were printed onto 16
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27 pad nitrocellulose slides (Grace BioLabs) using a Microgrid II arraying robot
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29 (Digilab Inc., Marlborough, MA, USA). Slides were blocked, washed and probed
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31 with marker-specific rabbit antibodies (Cell Signaling Technology) and mouse
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33 anti-human β -actin control antibody (Sigma Aldrich, UK). Antibody binding was
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35 visualized by incubating with goat anti-mouse IR680 and goat anti-rabbit IR00
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37 infrared secondary antibodies (Li-Cor BioSciences) diluted 1:5000 in 0.1% TBS-
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39 Tween for 30 minutes in the dark. Having been washed, rinsed with ultrapure
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41 water, and dried; the slides were scanned at 700nm and 800nm wavelengths on
42
43 a Li-Cor Odyssey scanner (LI-COR Biosciences). Fluorescent signal for each
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45 feature (spot) was determined using Axon GenePix Pro software (Axon
46
47 Instruments Inc.) Local background was subtracted and signals were
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49 normalized to β actin levels using the R statistical language and the RPPAnalyser
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Cytotoxicity assay

NK cell cytotoxicity was quantified using a non-radioactive LDH release assay kit (Promega, G1780) and the K562 human myelogenous leukaemia cell line (ECACC). The cell line was cultured in a 37°C incubator using RPMI-1640 media (Sigma, R8758) with 10% FCS (HyClone, CH30160.03).

Kit instructions were followed. Briefly, the K562 and NK cells were washed twice in RPMI-1640 media without phenol red (Invitrogen, 11835063), and placed at an effector:target ratio of 10:1 in a U-bottomed 96 well tissue culture plate (Nunc, 163320), in quadruplicate. Following a four-hour incubation at 37°C and subsequent centrifugation, 50µl of supernatant from each well was transferred to a flat-bottomed 96 well tissue culture plate (Nunc, 167008) and mixed with an equal volume of substrate mix (supplied with kit) to quantify LDH levels. After 30 minutes in the dark, the reaction was stopped and the absorbance read at 492nm on a BMG Labtech FLUOstar Optima spectrophotometer. Cytotoxicity was calculated as a percentage using the following equation:

$$\text{Cytotoxicity (\%)} = \left[\frac{(\text{effector/target mix} - \text{spontaneous effector LDH release} - \text{spontaneous target LDH release})}{(\text{maximum target LDH release} - \text{spontaneous target LDH release})} \right] \times 100$$

Statistical analysis

Flow cytometry data were analysed using Weasel v2.7 software. The spot intensity of the microarray samples was measured using Genepix software, before RPPanalyzer software corrected for background, normalised to the actin signal of the same spot, and amplified the intensity of each spot. Subsequent

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3 analysis of all data was performed with Prism 4.0 software. For visibly skewed
4 data, the values were first log transformed, before the normality of the data was
5 tested using the D'Agostino and Pearson omnibus normality test. Differences
6 between parametric data were explored using the one-way ANOVA test, while
7 differences between non-parametric data were explored using the Kruskal
8 Wallis test. Any pairs of groups holding statistical significance ($p < 0.05$) following
9 the ANOVA test were identified using the Tukey's multiple comparison post-hoc
10 test.
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23 Results

24 Peripheral killer cell numbers

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26 Peripheral CD8 T cell ($CD8^+CD3^+CD56^-$), NK cell ($CD56^+CD3^-$) and NKT-like cell
27 ($CD56^+CD3^+$) numbers were quantified using absolute cell counting (Fig. 1).
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29 There were no statistically significant differences between the numbers or
30 proportions of each killer cell type across the three participant groups (asthma
31 patients with or without FAO and healthy controls).
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40 Subsets of the killer cell types were also quantified using this method
41 (data not shown). These subsets included: naïve ($CD45RA^+CCR7^+$), central
42 memory (CM, $CD45RA^-CCR7^+$), effector memory (EM, $CD45RA^-CCR7^-$) and
43 effector T (Eff T, $CD45RA^+CCR7^-$) CD8 T cells [43]; $CD56^{dim}$ ($CD56^+/CD16^+$) and
44 $CD56^{bright}$ ($CD56^{++}/CD16^-$) NK cells [44]; and $CD4^-CD8^-$, $CD4^+CD8^-$, $CD4^-CD8^+$ and
45 $CD4^+CD8^+$ NKT-like cells [45]. Again, no significant differences were seen
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Peripheral killer cell NKG2D and KIR3DL1 receptor expression

Peripheral killer cell *ex vivo* expression of the activatory NKG2D and inhibitory KIR3DL1 receptors was quantified using flow cytometry (Fig. 2). No statistically significant differences were present between the participant groups for NKG2D or KIR3DL1 expression for any of the killer cell types.

The killer cell expression of a further four receptors (activatory KLRC2 and KIR2DS4, and inhibitory KLRD1 and KIR2DL2) was analysed by RPMA (data not shown). There were no significant differences in the expression of these four receptors between the participant groups for any of the killer cell types.

Peripheral killer cell cytotoxic signalling molecule expression

Peripheral CD8 T cells, NK cells and NKT-like cells were isolated (to purities of >92%, >97% and >74%, respectively), before their expression of total and phosphorylated ERK1/2, total and phosphorylated SAPK/JNK and total p38 MAPK was measured by RPMA and compared between asthma patients with and without FAO and healthy individuals (Fig. 3). There were no significant differences in the expression of the aforementioned signalling molecules in the three isolated killer cell types.

Peripheral killer cell cytotoxic function

The peripheral killer cell *ex vivo* intracellular expression of the cytotoxic mediators perforin and granzyme B was analysed by flow cytometry and compared across the three participant groups (Fig. 4). There were no significant differences in the expression of either of these cytotoxic mediators between the participant groups for any of the three killer cell types. While most CD8 T cells

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3 expressed neither perforin nor granzyme B (Fig. 4a); the majority of NK cells
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5 expressed both cytotoxic mediators (Fig. 4f).
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8 Peripheral NK cells were isolated to a purity of over 97%, before their
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10 cytotoxicity levels were quantified using the K562 cell line and a non-radioactive
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12 LDH release assay (Fig. 5). There were no statistically significant differences in
13
14 NK cell cytotoxicity levels between the three participant groups.
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17 18 19 **Discussion**

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21 The work presented here shows that the number and function of killer cells in a
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23 small subset of asthma patients who display FAO are similar to those in asthma
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25 patients without FAO and in healthy controls. This is distinctly different from
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27 our previous findings concerning peripheral killer cells in COPD [40, 46].
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31 CD8 T cell subsets can have opposing effects in asthma: Tc1 CD8 T cells
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33 protect against asthma, by mediating IL-12 and IL-18 Th1 cytokine production
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35 from dendritic cells [26]; whereas Tc2 CD8 T cells are thought to contribute to
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37 the inflammatory profile seen in asthma patients [26]. NK cell subsets have also
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39 been shown to have opposing effects in asthma [27, 31, 35]. NKT cells have been
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41 implicated in asthma [25, 30] as they produce Th2 cytokines, such as IL-4, IL-5
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43 and IL-13, which recruit eosinophils and promote B cell class switching [32-34].
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45 However, NKT cells are also capable of cytotoxicity and producing Th1 cytokines;
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47 thereby inhibiting the actions of the Th2 cytokine-producing NKT cells involved
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49 in the pathogenesis of asthma [28, 29, 36].
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53 No significant differences were observed in peripheral NK or NKT-like cell
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55 numbers or proportions between the three participant groups in the present
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57 study (healthy controls, asthma patients without FAO and asthma patients with
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3 FAO), whereas COPD patients were found to have significantly lower proportions
4 of peripheral NK and NK-T cells than healthy controls (both never-smokers and
5 smokers) [40]. A point of caution in drawing firm conclusions from the present
6 results is the relatively small numbers of subjects in the healthy control, asthma
7 without FAO and asthma with FAO groups, which limits the power of the study;
8 although, having said that, similar numbers of subjects were used in our previous
9 study that did show significant differences between peripheral killer cells in
10 COPD patients and healthy controls [40]. However, it is clear that any
11 differences in the number or proportions of killer cells between the healthy
12 control, asthma without FAO and asthma with FAO groups are marginal, since
13 power calculations showed that the numbers of subjects in each group required
14 for 80% power would need to be 121 for the proportion of CD8 T cells, 468 for
15 NK cell proportions and 84 for NKT-like cell proportions. Furthermore,
16 modelling of the data in figure 1 with ten times more data points (but with the
17 same distribution of data) still did not predict there to be any significant
18 differences in killer cell numbers/proportions between the asthma patients
19 without or with FAO.

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22 In addition, no significant differences were observed in the proportion of
23 peripheral CD8 T cells or NKT-like cells expressing both perforin and granzyme
24 B between the three participant groups in the present study, whereas these
25 proportions were found to be significantly decreased in COPD patients compared
26 to healthy controls (both never-smokers and smokers) [40]. Again, despite the
27 relatively small size of the healthy control, asthma without FAO and asthma with
28 FAO groups in the present study, the lack of significant differences in the
29 proportions of perforin⁺/granzyme-B⁺ cells is supported by power calculations
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3 indicating that the number of subjects required for 80% power would be 2113
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5 for CD8⁺ T cells, 201 for NK cells and 1875 for NKT-like cells; and that modelling
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7 of the data with ten time more data points and the same distributions still
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9 predicts there to be no significant differences.
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12 Furthermore, no significant differences were observed in the cytotoxic
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14 activity of peripheral NK cells between the three participant groups in the
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16 present study, whereas these cells were found to have significantly lower
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18 cytotoxic activity in COPD patients than in healthy controls (both never-smokers
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20 and smokers) [40]. However, the relatively small group sizes require there to be
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22 cautious interpretation of the data.
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26 Overall, with respect to these observations concerning peripheral killer
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28 cells, asthma with FAO is unlike COPD and is more akin to asthma without FAO.
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30 This is despite FAO being a feature more usually associated with COPD than with
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32 asthma. In otherwords, asthma with FAO displays a combination of features that
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34 distinguish it both from asthma without FAO and from COPD.
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38 Expression of KIR3DL1 (also known as CD158e1) by peripheral killer
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40 cells does not distinguish asthma with or without FAO from COPD, since this is
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42 unchanged in all three of these disease states compared to healthy (never-
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44 smoker) controls [46]; indeed, smoking *per se* (rather than COPD) leads to
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46 reduced KIR3DL1/CD158e1 expression by peripheral killer cells [46]. NKG2D
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48 expression by peripheral killer cells is also normal in asthma with or without
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50 FAO and in COPD [46].
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54 With regards to the signalling molecule expression results, no significant
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56 differences were observed between healthy controls and asthma patients with or
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58 without FAO. It is, however, interesting to note the similarities between the NK
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3 cell profiles of phosphorylated (activated) ERK1/2 and cellular cytotoxicity. In
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5 both, the levels in healthy individuals and asthma patients with FAO were
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7 similar, while those of asthma patients without FAO were slightly elevated
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9 (although not significantly – possibly due to insufficient numbers). ERK1/2 is
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11 necessary for NK cell cytotoxicity [47, 48] and has been shown to be elevated in
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13 asthma patients without FAO compared to healthy individuals [49]. This is the
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15 first time it has been specifically investigated in asthma patients with FAO.
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19 A key difference in the pathology of COPD compared to asthma with FAO
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21 is the association of chronic bronchitis and/or emphysema with COPD. Our
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23 findings in relation to killer cells in COPD and, reported here, for asthma with or
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25 without FAO, may help to explain these differences. In particular, the reduced
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27 numbers and effector function of peripheral killer cells in COPD [40] was found
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29 to correlate inversely with increased numbers, activation state and effector
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31 function of lung-associated killer cells in the induced sputum of COPD patients
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33 [37, 46]. Thus, the recruitment to pulmonary tissues, and activation, of killer
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35 cells in COPD may contribute to the inflammation and tissue destruction leading
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37 to the development of chronic bronchitis and/or emphysema. Conversely, the
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39 relatively normal numbers and function of peripheral killer cells in asthma with
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41 or without FAO may be indicative of their lack of recruitment to the lungs and
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43 the absence of chronic bronchitis and emphysema. It is thus apparent that a
44
45 main limitation of the present study is that only peripheral killer cells were
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47 investigated. Although inflammatory cells are recruited from the periphery to
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49 the lung, both the origin of systemic inflammation and its relationship to the
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51 pulmonary inflammation of asthma remain unclear [50]. Therefore, although
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53 these results are clinically relevant, further study using material derived from
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3 the lung would be required to show the recruitment, and the potential role, of
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5 killer cells in the pathogenesis of asthma with FAO.
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8 **Although the present study does not elucidate the basis for the different**
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10 **features of asthma without or with FAO, it does imply that this is not a**
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12 **consequence of differences in killer cell numbers or activity. Further studies are**
13
14 **therefore required to understand these differences.**
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17 Regarding our definition of FAO (post-bronchodilator FEV/FVC <0.7,
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19 despite clinician-optimised treatment [41]), we believe the length of disease
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21 duration of the asthma patients with FAO (a median of 45 years) allowed us to
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23 classify this airflow obstruction as fixed; although repeated spirometry
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25 measurements would have been desirable. Furthermore, an oral corticosteroid
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27 trial could be considered necessary to fully confirm fixed airflow obstruction but,
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29 as all patients were on inhaled steroids, this was not deemed ethically
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31 acceptable.
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35 In summary, the three main types of killer cells – CD8 T cells, NK cells and
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37 NKT-like cells – are similar in peripheral blood in asthma patients and healthy
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39 controls. The observation that peripheral killer cells are similar in asthma with
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41 FAO and in asthma without FAO indicates that peripheral killer cell phenotype
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43 and function is not linked to the differences in lung function associated with
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45 these clinically distinct endotypes of asthma. Furthermore, these findings are
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47 different from our previous observations concerning peripheral killer cells in
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49 COPD [40], indicating that asthma with FAO and COPD are distinct phenotypes.
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Figure legends

Figure 1. Peripheral killer cell numbers in asthma patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H). Whole blood was mixed with flow-count fluorosphere beads, incubated with fluorescently-conjugated antibodies and analysed by flow cytometry. Lymphocytes (L) and flow-count fluorosphere beads (B) were identified based on forward and side scatter (**a**). CD8 T cells (CD8+CD3+CD56-, **b-e**), NK cells (CD56+CD3-, **f-i**) and NKT-like cells (CD56+CD3+, **j-l**) were identified from the lymphocyte population (representative plots shown) and displayed as cells per μl (**d, h, k**) and as a proportion of the total killer cell number (**e, i, l**). There were no statistically significant differences in killer cell number, or the proportion of the killer cell total this value represented, between the three participant groups for any of the three killer cell types. (h) and (k) log transformed. All data analysed by ANOVA.

Figure 2. NKG2D and KIR3DL1 receptor expression in killer cells from asthma patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H). Separated PBMC were stained with fluorescently-conjugated antibodies and analysed by flow cytometry. CD8 T cells (**a-c**), NK cells (**d-f**) and NKT-like cells (**g-i**) were identified (representative plots shown), and the NKG2D and KIR3DL1 expression of each was displayed as mean fluorescence intensity (MFI). There were no statistically significant differences in NKG2D or KIR3DL1 expression between the three participant groups for any of the killer cell types. (i) Log transformed. All data analysed by ANOVA.

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3 **Figure 3.** MAPK signalling molecule expression in killer cells from asthma
4 patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H).
5 Levels of total (T) and phosphorylated (P) ERK1/2, SAPK/JNK and p38 MAPK
6 were measured in isolated CD8 T cells (a-e), NK cells (f-j) and NKT-like cells (k-
7 o), by reverse phase protein lysate microarray (RPMA). Background values were
8 subtracted from the signal intensities, before normalisation to actin expression.
9 There were no statistically significant differences in the expression of any of the
10 signalling molecules between the three participant groups for any of the three
11 killer cell types. Values are expressed in relative fluorescent units (RFU). (b), (e),
12 (k), (l) and (o) Log transformed. All data analysed by ANOVA, except (j)-
13 analysed by Kruskal Wallis.
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30 **Figure 4.** Perforin and granzyme B expression in killer cells from asthma
31 patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H).
32 PBMC were isolated and stained with fluorescently-conjugated antibodies before
33 analysis by flow cytometry. CD8 T cells (a-d), NK cells (e-h) and NKT-like cells (i-
34 l) were identified and the perforin and/or granzyme B expression of each was
35 quantified. There were no statistically significant differences in the expression of
36 either of the two cytotoxic mediators between the three participant groups for
37 any of the killer cell types. (c), (d), (h), (k) and (l) Log transformed. All data
38 analysed by ANOVA, except (j)- analysed by Kruskal Wallis.
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52 **Figure 5.** NK cell cytotoxicity in asthma patients with and without FAO (A+FAO,
53 A-FAO) and healthy individuals (H). Isolated peripheral NK cells were incubated
54 with target K562 cells for 4 hours, and lactate dehydrogenase (LDH) levels used
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to determine cytotoxicity levels. There were no statistically significant differences in NK cell cytotoxicity levels between the three participant groups. Data analysed by ANOVA.

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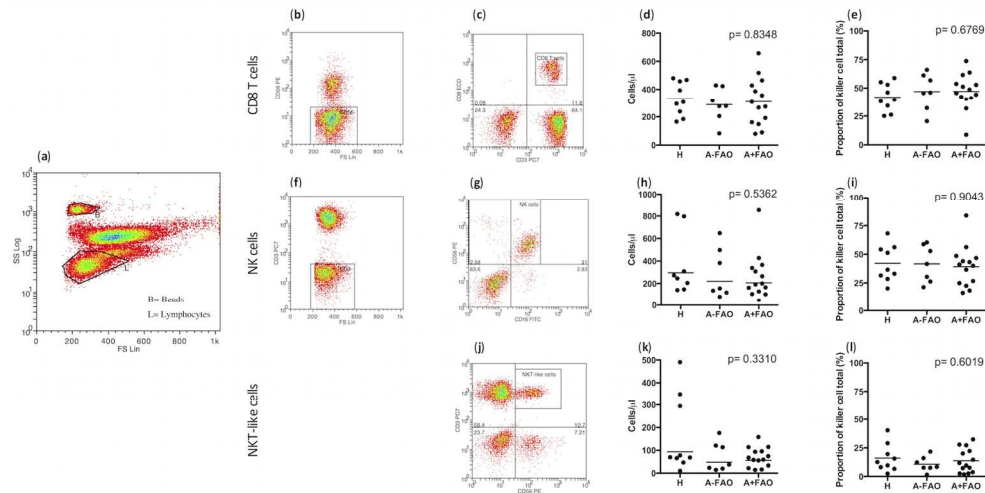


Figure 1. Peripheral killer cell numbers in asthma patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H). Whole blood was mixed with flow-count fluorosphere beads, incubated with fluorescently-conjugated antibodies and analysed by flow cytometry. Lymphocytes (L) and flow-count fluorosphere beads (B) were identified based on forward and side scatter (a). CD8 T cells (CD8+CD3+CD56-, b-e), NK cells (CD56+CD3-, f-i) and NKT-like cells (CD56+CD3+, j-l) were identified from the lymphocyte population (representative plots shown) and displayed as cells per μl (d, h, k) and as a proportion of the total killer cell number (e, i, l). There were no statistically significant differences in killer cell number, or the proportion of the killer cell total this value represented, between the three participant groups for any of the three killer cell types. (h) and (k) log transformed. All data analysed by ANOVA.

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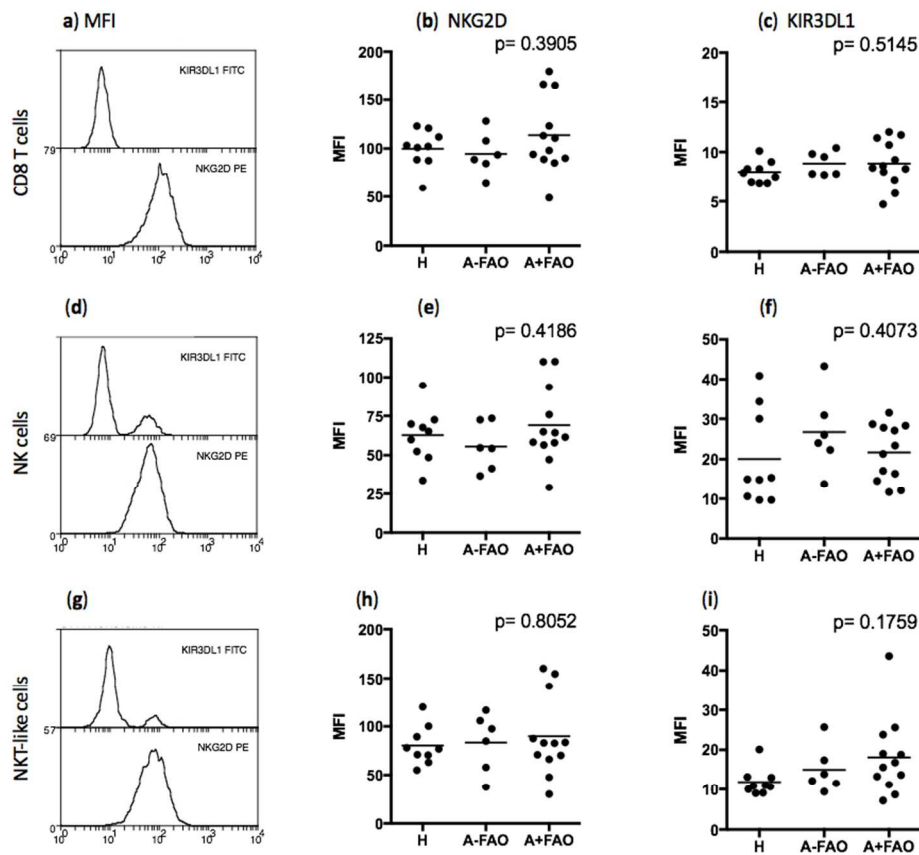


Figure 2. NKG2D and KIR3DL1 receptor expression in killer cells from asthma patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H). Separated PBMC were stained with fluorescently-conjugated antibodies and analysed by flow cytometry. CD8 T cells (a-c), NK cells (d-f) and NKT-like cells (g-i) were identified (representative plots shown), and the NKG2D and KIR3DL1 expression of each was displayed as mean fluorescence intensity (MFI). There were no statistically significant differences in NKG2D or KIR3DL1 expression between the three participant groups for any of the killer cell types. (i) Log transformed. All data analysed by ANOVA.

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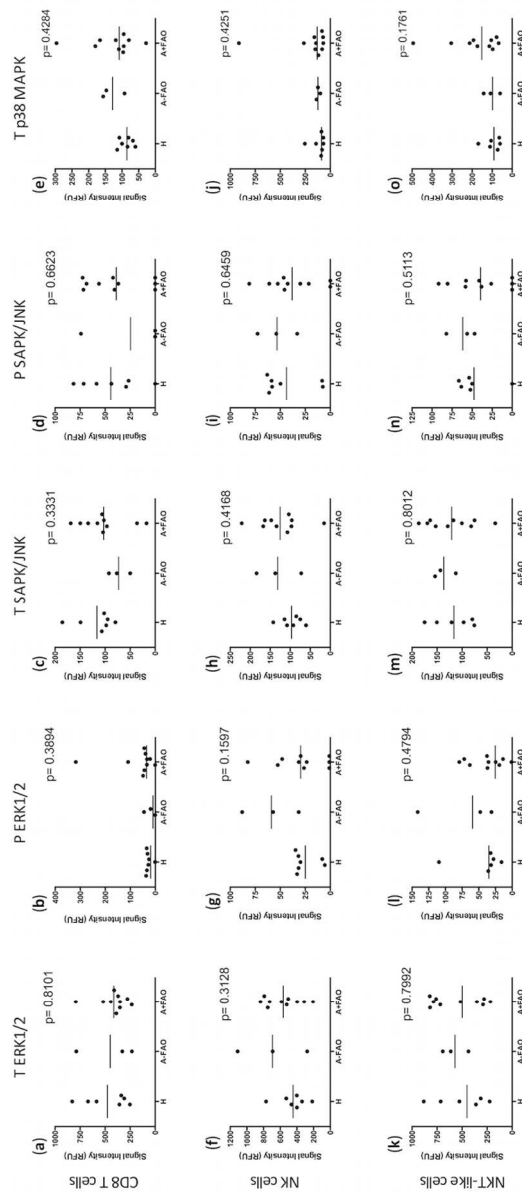


Figure 3. MAPK signalling molecule expression in killer cells from asthma patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H). Levels of total (T) and phosphorylated (P) ERK1/2, SAPK/JNK and p38 MAPK were measured in isolated CD8 T cells (a-e), NK cells (f-j) and NKT-like cells (k-o), by reverse phase protein lysate microarray (RPMA). Background values were subtracted from the signal intensities, before normalisation to actin expression. There were no statistically significant differences in the expression of any of the signalling molecules between the three participant groups for any of the three killer cell types. Values are expressed in relative fluorescent units (RFU). (b), (e), (k), (l) and (o) Log transformed. All data analysed by ANOVA, except (j)- analysed by Kruskal Wallis.

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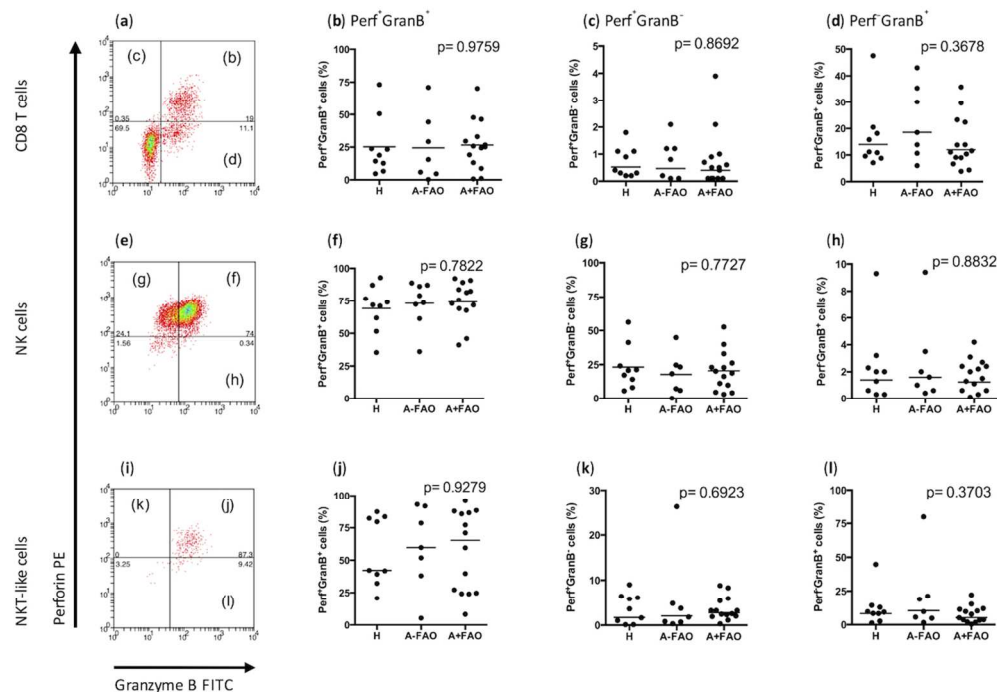


Figure 4. Perforin and granzyme B expression in killer cells from asthma patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H). PBMC were isolated and stained with fluorescently-conjugated antibodies before analysis by flow cytometry. CD8 T cells (a-d), NK cells (e-h) and NKT-like cells (i-l) were identified and the perforin and/or granzyme B expression of each was quantified. There were no statistically significant differences in the expression of either of the two cytotoxic mediators between the three participant groups for any of the killer cell types. (c), (d), (h), (k) and (l) Log transformed. All data analysed by ANOVA, except (j)- analysed by Kruskal Wallis.

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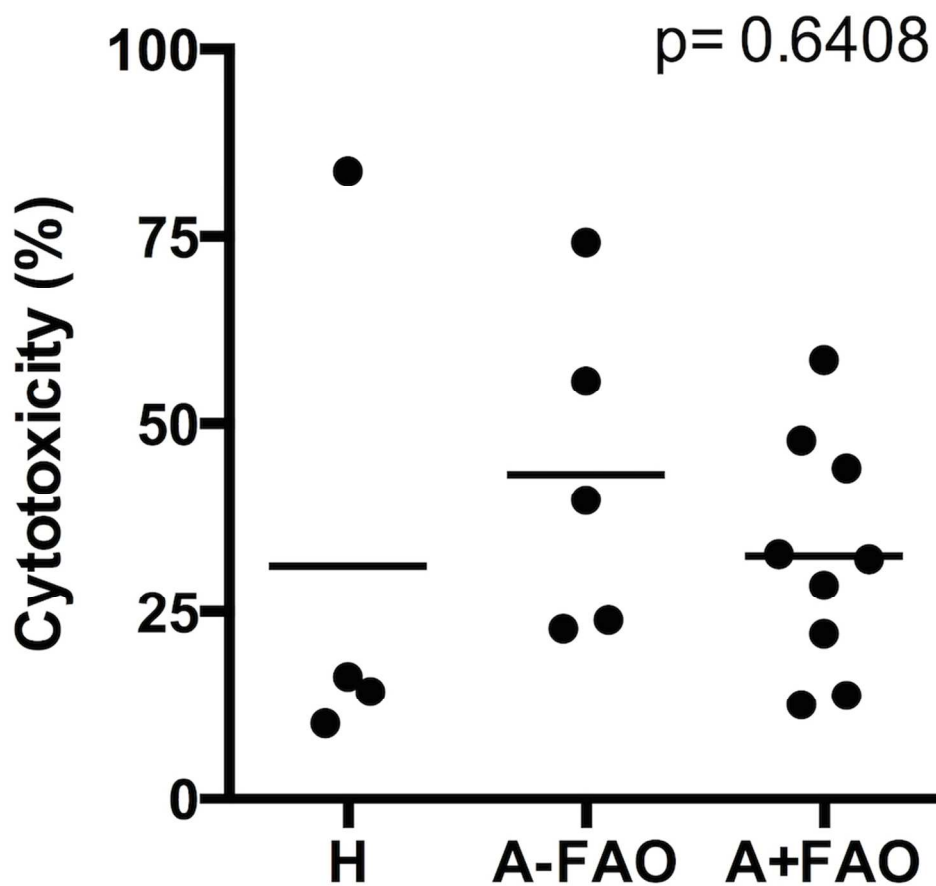


Figure 5. NK cell cytotoxicity in asthma patients with and without FAO (A+FAO, A-FAO) and healthy individuals (H). Isolated peripheral NK cells were incubated with target K562 cells for 4 hours, and lactate dehydrogenase (LDH) levels used to determine cytotoxicity levels. There were no statistically significant differences in NK cell cytotoxicity levels between the three participant groups. Data analysed by ANOVA.

360x333mm (72 x 72 DPI)

Table 1. Subject demographic and spirometric data

	<i>(a) Full cohort- cell numbers, receptor expression and cytotoxic mediator expression</i>			<i>(b) MAPK signalling pathways</i>			<i>(c) NK cell cytotoxicity</i>		
	H	A-FAO	A+FAO	H	A-FAO	A+FAO	H	A-FAO	A+FAO
Subjects	9	7	14	7	3	10	4	5	9
Age (years)	57 (52-62)	66 (60-74)	67 (58-71)	55 (49-57)	64 (60-74)	68 (58-73)	56 (51-59)	73 (58-75)	65 (56-68)
Gender (M/F)	6/3	1/6	9/5	5/2	0/3	8/2	2/2	0/5	7/2
Post-branch FEV ₁ (L)	3.14 (2.92-3.96)	2.05 (1.62-2.78)	1.73 (1.47-2.10)	3.14 (3.02-4.14)	2.02 (1.62-2.18)	1.65 (1.55-1.80)	2.98 (2.81-3.89)	2.05 (1.58-2.52)	1.69 (1.43-1.80)
Post-branch FEV ₁ (%pred)	112 (101-117)	103 (97-110)	64 (55-76)	109 (95-113)	98 (97-116)	64 (55-79)	114 (108-121)	103 (95-113)	59 (46-70)
Post-branch FVC (L)	4.05 (3.71-4.78)	2.73 (2.22-3.75)	2.89 (2.39-4.02)	4.05 (3.68-4.98)	2.73 (2.22-2.89)	3.07 (2.50-4.02)	3.82 (3.46-4.79)	2.40 (2.07-3.24)	3.08 (2.36-4.17)
Post-branch FVC (%pred)	110 (102-121)	116 (100-121)	88 (76-109)	106 (100-118)	116 (110-121)	84 (76-105)	115 (108-132)	110 (97-122)	90 (76-101)
Post-branch FEV ₁ /FVC	0.81 (0.78-0.83)	0.76 (0.73-0.80)	0.59 (0.50-0.67)	0.82 (0.79-0.83)	0.73 (0.70-0.80)	0.59 (0.51-0.67)	0.78 (0.76-0.82)	0.80 (0.75-0.83)	0.55 (0.46-0.59)
Airflow obstruction severity (mild/moderate/severe/ very severe)*	n/a	n/a	3/8/2/1	n/a	n/a	2/6/2/0	n/a	n/a	1/5/2/1
Age of asthma onset (years)	n/a	46 (39-54)	8 (6-51)	n/a	50 (42-53)	8 (6-42)	n/a	50 (41-55)	10 (6-56)
Disease duration (years)	n/a	22 (15-30)	45 (18-59)	n/a	24 (6-30)	53 (20-63)	n/a	20 (12-27)	47 (11-57)

Asthma severity (GINA treatment step 1/2/3/4/5)**	n/a	0/3/3/1/0	0/1/7/6/0	n/a	0/1/1/1/0	0/1/4/4/0	n/a	0/2/2/1/0	0/1/3/5/0
Inhaled corticosteroids (ICS) (Y/N)	n/a	7/0	14/0	n/a	3/0	10/0	n/a	5/0	9/0
Long-acting beta agonists (LABAs) (Y/N)	n/a	2/5	9/5	n/a	0/3	6/4	n/a	2/3	7/2
Short-acting beta agonists (SABAs) (Y/N)	n/a	7/0	12/2	n/a	3/0	10/0	n/a	5/0	7/2
Leukotriene modifiers (Y/N)	n/a	0/7	1/13	n/a	0/3	1/2	n/a	0/5	1/8
Atopic (Y/N)	2/7	6/1	9/5	1/6	3/0	7/3	0/4	5/0	6/3
Smoking status (current/ex/never)	0/1/8	0/4/3	0/2/12	0/1/6	0/2/1	0/1/9	0/0/4	0/2/3	0/0/9
Pack years	0 (0-0)	1 (0-2)	0 (0-0)	0 (0-1.5)	1.5 (0-2)	0 (0-0)	0 (0-0)	0 (0-1.5)	0 (0-0)
CD8 T cell purity (%)				93.6 (90.9-95.0)	92.9 (83.9-95)	91.9 (88.4-94.2)			
NK cell purity (%)				97.5 (95.7-98.3)	97.3 (96.5-98.1)	97 (93.6-98.8)	97.9 (96.2-98.4)	97.6 (96.3-98.1)	97.6 (93.3-98.3)
NKT-like cell purity (%)				82.5 (71.2-90.0)	75.7 (55.4-92.4)	73.9 (58.1-92.6)			

Results expressed as the median with the interquartile range in brackets. A+/-FAO, asthma patients with/without fixed airflow obstruction; H, healthy individuals.

*Based on post-bronchodilator FEV₁ (%pred): >80% mild, 50-79% moderate, 30-49% severe, and <30% very severe. **Based on drug treatment [51].

Table 2. Antibodies used in flow cytometry and reverse phase protein lysate microarray (RPMA)

Target	Conjugate	Clone	Company	Catalogue number	Lot number
<i>Flow cytometry</i>					
CD3	ECD	UCHT1	Beckman Coulter	A07748	30
CD3	PC7	UCHT1	Beckman Coulter	737657	7973014
CD8	ECD	SFCI21Thy2D3	Beckman Coulter	737659	7974018
CD8	PC5	HIT8a	BD Pharmingen	555636	98195
CD56	PE	N901 (NHK-1)	Beckman Coulter	A07788	13
CD56	PC5	N901 (NHK-1)	Beckman Coulter	A07789	15
Granzyme B	FITC	GB11	BD Pharmingen	560211	37817
KIR3DL1	FITC	DX9	BioLegend	312706	B110125
NKG2D	PE	ON72	Beckman Coulter	A08934	13
Perforin	PE	δG9	BD Pharmingen	556437	46510
<i>Reverse phase protein lysate microarray (RPMA)</i>					
T ERK1/2	-	-	Cell Signalling	4695	13
T p38MAPK	-	-	Cell Signalling	9212	13
T SAPK/JNK	-	-	Cell Signalling	9258	9
P ERK1/2	-	-	Cell Signalling	9101	26
P SAPK/JNK	-	-	Cell Signalling	4668	7

CD, cluster of differentiation; ECD, phycoerythrin-Texas Red; ERK1/2, extracellular signal-regulated kinase 1 and 2; FITC, fluorescein isothiocyanate; PC5, phycoerythrin cyanin 5; KIRx, killer cell immunoglobulin-like receptor x; KLRx, killer cell lectin-like receptor x; P, phosphorylated; p38 MAPK, p38 mitogen-activated protein kinase; PC7, phycoerythrin cyanin 7; PE, phycoerythrin. SAPK/JNK, stress-activated protein kinase and c-Jun N-terminal kinase; T, total.