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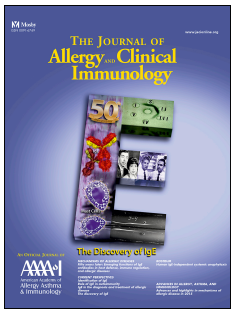

IL-12 and IL-7 synergise to control MAIT cell cytotoxic responses to bacterial infection

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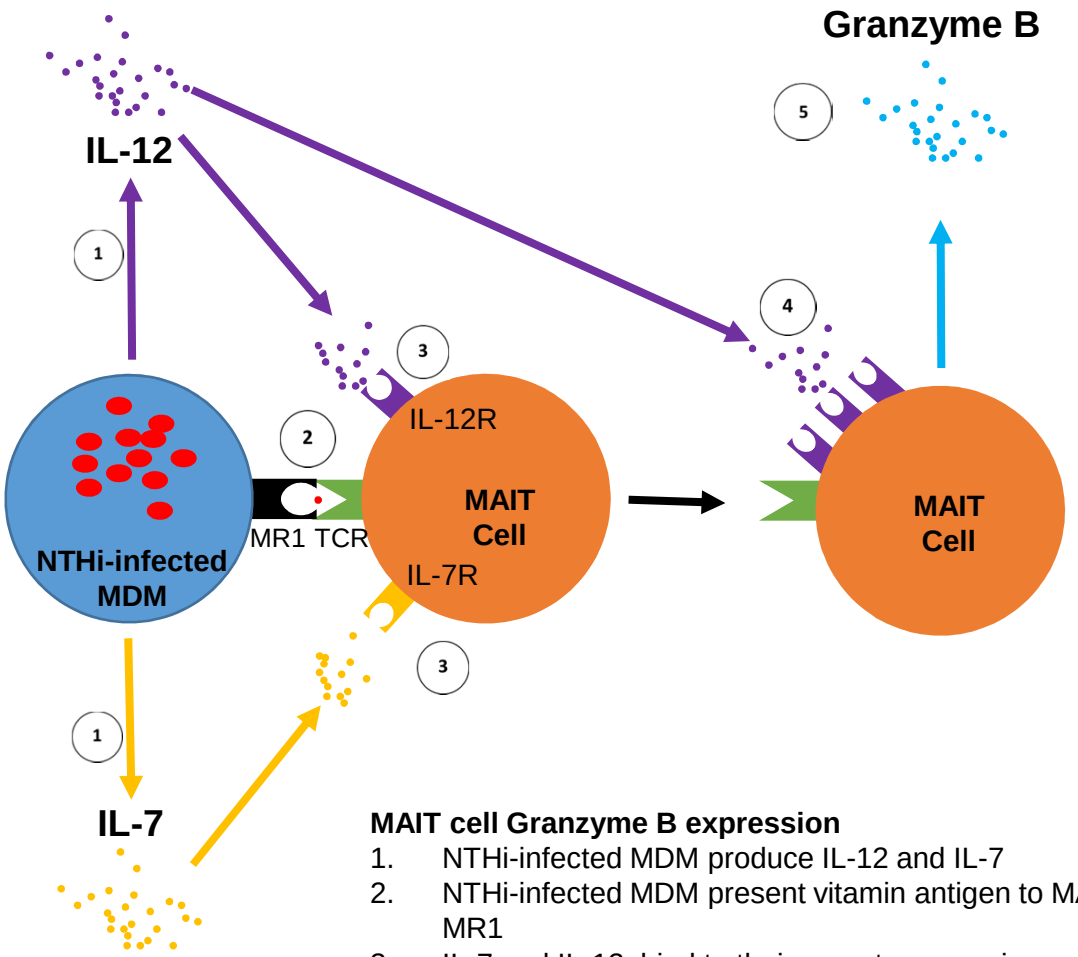

MAIT cell Granzyme B expression

1. NTHi-infected MDM produce IL-12 and IL-7
2. NTHi-infected MDM present vitamin antigen to MAIT TCR via MR1
3. IL-7 and IL-12 bind to their receptors, causing upregulation of IL-12R and downregulation of IL-7R
4. IL-12 (+ MR1 signal) can then cause granzyme B upregulation
5. Granzyme B is released

IL-12 and IL-7 synergise to control MAIT cell cytotoxic responses to bacterial infection

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Short running title: MAIT cell cytotoxic responses to NTHi

Abstract

Background

Bacterial respiratory tract infections and exacerbations of chronic lung diseases are commonly caused by nontypeable *Haemophilus influenzae* (NTHi). Cell-mediated cytotoxicity may be key to controlling infection, but the responses of NTHi-specific T cell populations are not well understood. Mucosal-associated invariant T (MAIT) cells are a recently-discovered, innate-like subset of T cells with cytotoxic function, whose role in lung immunity is unclear.

Objective

The aim of this study was to determine the mechanisms behind conventional T and MAIT cell cytotoxic responses to NTHi.

Methods

Human *ex vivo* lung explants were infected with a clinical strain of NTHi. Monocyte-derived macrophages were also infected with NTHi *in vitro* and co-cultured with autologous T cells. Cytotoxic responses of T cell subsets were measured by flow cytometry.

Results

We found significant upregulation of the cytotoxic markers, CD107a and granzyme B, in lung CD4+, CD8+ and MAIT cell populations. We show that MAIT cell cytotoxic responses were upregulated by a combination of both time-dependent antigen presentation and through a novel mechanism by which IL-12 and IL-7 synergistically control granzyme B through upregulation of the IL-12 receptor.

Conclusions

Overall our data provide evidence for a cytotoxic role of MAIT cells in the lung and highlight important differences in the control of adaptive and innate-like T cell responses.

Understanding these mechanisms may lead to new therapeutic opportunities to modulate the anti-bacterial response and improve clinical outcome.

Key Messages

- Lung MAIT cells upregulate markers of cytotoxicity in response to NTHi bacterial infection.
- These responses are controlled by both antigen-presentation and by synergistic IL-12 and IL-7 signalling.
- Together, these findings indicate a role for MAIT cells in human lung infections.

Capsule Summary

The mechanisms controlling the cytotoxic response of MAIT cells to NTHi infection are unknown. Our observations provide a new understanding of protective immune mechanisms and also potential new therapeutic opportunities to improve clinical outcome.

Keywords: T cells; MAIT cells; bacterial infection; cytotoxicity

Abbreviations:

APC: Antigen presenting cell

COPD: Chronic obstructive pulmonary disease

MAIT cell: Mucosal-associated invariant T cell

MDM: Monocyte-derived macrophage

MOI: Multiplicity of infection

MR1: MHC-related protein 1

NTHi: Non-typeable *Haemophilus influenzae*

PFA: Paraformaldehyde

PBMC: Peripheral blood mononuclear cells

SMFI: Specific mean fluorescence intensity

TCR: T cell receptor

Introduction

The human lungs are colonised by a wide variety of commensal bacterial species, although much is still unknown about the role of the microbiome in the lung in health and disease [1][2][3][4][5][6][7]. In particular, it is unknown how microbiome interactions with the local immune system modulate the balance between protective immune response and inflammation [8][9].

The Gram-negative bacterium non-typeable *Haemophilus influenzae* (NTHi) is a highly diverse commensal microbe which can become an opportunistic intracellular pathogen [10][11][12]. This obligate human pathogen is a leading cause of respiratory infections in the very young and elderly and is associated with a range of clinically important infections including acute otitis media, pneumonia and bacteraemia [13][14]. NTHi is also commonly associated with lung diseases such as chronic obstructive pulmonary disease (COPD), which is an increasingly prevalent condition and is predicted to be the 3rd leading cause of death by 2020 [15][16][17]. Microbial dysbiosis in COPD is dominated by a loss of microbial diversity and overgrowth of key species, with NTHi-induced exacerbations of COPD being the main drivers of disease progression and mortality [7][11][18][19].

In the lung, NTHi appears capable of intracellular survival within macrophages [20] and this niche may explain the persistence of NTHi in the airway. Cell-mediated immune control of the airway microbiome is not well described and in particular the responses of T cells to NTHi are not well understood. Interestingly, an increase in CD8+ T cells is found in the COPD airway, but this does not appear to confer increased immunity to bacterial pathogens [21][22]. Recently, innate-like mucosal-associated invariant T (MAIT) cells have been identified and these recognise antigens derived from bacterial riboflavin (vitamin B2) synthesis, which are presented by the non-polymorphic MHC-related protein 1 (MR1) [23]. Upon activation, MAIT cells rapidly express effector cytokines such as TNFα, IFNγ and IL-17[24][25] and are also able to inhibit intracellular bacterial growth within infected macrophages [26]. Whilst conventional T cells largely depend on antigen presentation for

activation, MAIT cells can produce IFNγ in response to T cell receptor (TCR)-independent, IL-12 and IL-18 stimulation [27] [26][28][29].

Cytotoxic T cells are vital in the control and clearance of intracellular bacterial pathogens by degranulation of granzymes and perforin [30][31] [32][33]. However, little is known about the cytotoxic responses of MAIT cells. Recent work has shown that MAIT cells upregulate cytotoxic mediators such as granzyme B, but this has not been shown in the lung nor in response to respiratory pathogens [34][35][36]. The mechanisms controlling MAIT cell cytotoxicity is not fully understood, neither is it known how these mechanisms and responses differ from that of conventional T cells.

As MAIT cells are antibacterial cells resident in the lung [37][38], it seems likely that they are playing a role in host defence to lung pathogens and in the potential surveillance and control of the lung microbiome. Given that there is no current vaccine for NTHi, understanding the mechanisms behind the NTHi-induced responses of both conventional and innate-like T cells is important in order to guide future vaccine development [39].

Here we found that both conventional lung T cells and, more potently, MAIT cells exhibit cytotoxic responses to NTHi infection. We further demonstrate the mechanisms controlling these responses and suggest that innate cytokine signalling controls MAIT granzyme B expression through apparent regulation of the IL-12 receptor.

Methods

Patient Recruitment

Lung tissue distal from tumour sites was obtained from lobectomy patients. For the isolation of T cells and monocytes, blood was obtained from healthy human volunteers. All studies were approved by Southampton and South West Hampshire Research Ethics Committees (13/SC/0416, 09/H0504/109). All participants provided written informed consent.

Preparation of lung tissue and explant infection

NTHi was prepared as previously described [40][41]. Lung tissue explants were prepared as previously described [42]. Tissue explants were infected with $5x10^6$ CFU NTHi for 24 hours, after which T cells were dispersed from tissue by 0.5 mg/ml collagenase digestion.

Blood T cell and Monocyte Isolation & differentiation

CD3+ T cells and CD14+ monocytes were isolated from human peripheral blood mononuclear cells (PBMC) obtained from the blood of healthy volunteers using MACS (Miltenyi Biotec, Bisley, UK) and monocytes differentiated into macrophages (MDM) over 12 days using 2 ng/ml GM-CSF as previously described [40].

Macrophage infection

MDM infection was performed as previously described [40]. Briefly, MDM were cultured for 2 h at 37℃ with NTHi. After 2 h, MDM were washed and then incubated either alone or co-cultured with autologous T cells for a further 22 h unless otherwise indicated. Viability of cells was confirmed by trypan blue staining.

MAIT Killing assay

MAIT cells were FACS-sorted (purity >93%) from autologous T cells and co-cultured with MDM for 48 h at a ratio of 2:1. Killing of MDM by MAIT cells was confirmed by viability staining.

Flow cytometry analysis

Flow cytometry was performed as previously described [40]. Cells were first stained for surface markers or appropriate isotype controls. Cells were then resuspended in Cytofix/Cytoperm (BD Biosciences, Oxford, UK) before staining for intracellular markers.Flow cytometric analysis was performed on a FACSAria using FACSDiva software v5.0.3 (BD Biosciences). The gating strategy is outlined in Figure 1.

RNA Isolation & RT-PCR

RNA was extracted from MDM and reverse transcribed to cDNA as previously described [40]. *IL-12p35, IL-12p40* and *IL-7* gene expression was analysed using TaqMan universal PCR master mix, No AmpErase® UNG in a 7900HT fast real-time PCR system instrument (all Life Technologies Loughborough, UK). Gene expression was normalized to β_2-microglobulin gene expression and quantified using the $\Delta\Delta C_T$ method.

ELISA

IL-12p70 ELISA MAX (Biolegend), and granzyme B duoset ELISA (R&D Systems) were all carried out according to the manufacturers' protocol.

Statistics

Statistical analyses were performed using either a Wilcoxon's matched-pairs signed-rank test or Friedman test with Dunn's multiple comparison testing (GraphPad Prism v6.0, GraphPad Software Inc., San Diego, USA). Data are expressed as medians. Results were considered significant if $P<0.05$.

For additional information on methods, please see the online supplement

Results

Conventional lung T cells and MAIT cells upregulate cytotoxic markers in response to NTHi

To first determine the cytotoxic responses of conventional lung T cells and MAIT cells to NTHi, human lung tissue explants were infected with NTHi and cytotoxic responses were measured by flow cytometry. Gating strategies are outlined in figure 1.

Significant ($P<0.01$) upregulation of granzyme B was detected in lung CD8+ T cells in response to NTHi infection (figure 2A). CD4+ T cells, which are classically associated with cytokine production, also upregulated both granzyme B expression and CD107a (both $P<0.01$; figure 2B) following infection. Similarly, a significant ($P<0.01$) upregulation of both cytotoxic markers was measured in MAIT cells in response to NTHi (figure 2C). Analysis of lung explant supernatant showed that NTHi infection caused a 2-fold increase in granzyme B release ($P<0.01$; supplementary figure 1).

Overall, these data show that both conventional T and MAIT cells in the human lung manifest cytotoxic responses to NTHi infection, which may be important in controlling bacterial respiratory infections and modulating the microbiome. Due to the nature of human tissue samples, the yields of cells derived from lung explants were often very limited. Therefore in order to perform experiments to define function, we used a previously established autologous *in vitro* human model to further investigate both the cytotoxic responses of T cell and MAIT cells to NTHi infection and the mechanisms behind their activation [40].

Conventional T and MAIT cells upregulate cytotoxic markers following co-culture with NTHi-infected macrophages

Monocyte-derived macrophages (MDM) were infected with NTHi and then co-cultured with autologous peripheral T cells for 22h. CD8+ T cells had a significant upregulation of both cytotoxic markers (figure 3A); granzyme B (uninfected 21.8% vs NTHi 40.6%, P=0.0005), CD107a (uninfected 2.8% vs NTHi 4.2%, P=0.002). There was no significant increase in either cytotoxic marker when MDM were infected with paraformaldehyde-killed-NTHi (PFA-NTHi), indicating that the cytotoxic response of T cells in this model requires live NTHi infection.

Similarly to lung T cells, the upregulation of granzyme B and CD107a was not restricted to blood CD8+ T cells. CD4+ T cells produced a minor but statistically significant increase in both granzyme B (uninfected 4% vs NTHi 8%, P=0.0005) and CD107a (uninfected 1.7% vs NTHi 2.5%, P=0.0015) following co-culture with NTHi-infected MDM (figure 3B). Significant upregulation of both granzyme B (uninfected 27% vs NTHi 65%; P=0.0005) and CD107a (uninfected 8% vs NTHi 20%; P=0.001), were also detected in MAIT cells in response to NTHi infection (figure 3C). PFA-NTHi did not cause upregulation of the cytotoxic markers in either CD4+ T or MAIT cell subset. Analysis of co-culture supernatant showed that NTHi infection caused a 5-fold increase in granzyme B release (P=0.01; supplementary figure 1).

Both CD4+ and CD8+ T cells had a minor but significant (both P<0.05) upregulation in perforin in response to NTHi infection (supplementary figure 2). However, MAIT cells expressed high perforin levels at baseline, which were not upregulated further. For this reason, we decided to concentrate our further studies on CD107a and granzyme B.

Conventional T cell cytotoxic responses to bacterial infection are completely inhibited by blocking antigen presentation

To determine whether the NTHi-induced cytotoxic responses of CD8+ and CD4+ T cells were antigen-dependent, co-cultures were repeated with the addition of MHC-blocking antibodies. Anti-HLA-ABC and anti-HLA-DR antibodies respectively significantly reduced

CD8+ and CD4+ T cell granzyme B and CD107a expression to baseline (all P<0.01 IgG2a isotype vs blocking antibody) (figure 4A). The decrease to baseline suggests that other factors such as cytokine activation are less involved in the conventional T cells cytotoxic response. For this reason, conventional T cells were not investigated further in this study.

MAIT cell cytotoxic responses are dependent on MR1 in a time-dependent manner

For MAIT cell cytotoxic responses, blocking of MR1 was performed in both a 5 h and 22 h co-culture model, in line with our previous study [40]. At 5h, blocking of MR1 significantly (P=0.03) reduced CD107a upregulation back to baseline (IgG2a isotype 4.1% vs anti-MR1 2.6%), but MR1 blocking in the 22 h co-culture did not affect CD107a expression (figure 4B). Conversely, granzyme B upregulation was not blocked at 5 h, but there was a significant (P=0.01) inhibition after the 22 h co-culture (IgG2a isotype 74% to anti-MR1 67%) (Figure 4B). The modest effect of MR1 blocking could not reduce expression of granzyme B back to uninfected baseline. Blocking of HLA-DR had no effect on MAIT cell granzyme B but blocking of HLA-ABC only caused a minor inhibition (supplementary figure 3A). We reasoned that the requirement for MR1 in activating MAIT cells may be time-dependent and that MR1-independent factors such as cytokines were also responsible for controlling MAIT cell cytotoxic responses.

Bacterial activation of MAIT cells in a non-contact model shows that soluble factors are capable of activating MAIT cells independently of MR1

To further explore whether factors independent of antigen presentation could activate MAIT cells, the co-culture model was adapted to incorporate 0.4 µm transwells which prevented physical contact of T cells with NTHi-infected MDM. MAIT cells in transwells still significantly upregulated granzyme B expression (P=0.03; figure 5), although expression did not

approach the maximal response as seen when NTHi-infected MDM and T cells were in direct contact (figure 3C). Minor upregulation of MAIT CD107a was also observed (figure 5). To see if contact-dependency was required for other MAIT responses, IFNγ was also measured and was found to be upregulated in the transwell model, but again to a lesser extent compared to direct contact (supplementary figure 3B). In combination with the MR1 blocking data, we demonstrate that MAIT cells can become activated in the absence of physical contact and antigen presentation, indicating that soluble factors such as cytokines may play an important role in MAIT activation during infections.

MDM produce IL-12 in response to NTHi infection

Previous reports have shown that IL-12 is capable of driving MAIT cell activation in *E.coli* infection [26][28], therefore we investigated if MR1-independent MAIT activation in our model was driven by IL-12.

NTHi infection lead to a significant (P=0.01) 1200-fold induction of IL-12p40 mRNA but did not affect IL-12p35 gene expression by MDM (figure 6A). IL-12p70 protein levels were significantly (P=0.01) increased from 0 to 290 pg/ml at 22 h post-NTHi infection, but little IL-12p70 protein was detected when MDM supernatants were harvested 5 h after NTHi infection (figure 6B). No IL-12p70 protein was detected in supernatants from MDM infected with PFA-NTHi, indicating that non-viable bacteria are not sufficient to induce IL-12 protein production. To confirm that IL-12 was relevant to the lung, supernatants from NTHi-infected lung explants were analysed for IL-12 protein. In agreement with our MDM data, infected explants released a small, but significant (P<0.01) amount of IL-12p70 (supplementary figure 5A).

Blocking of IL-12 inhibits granzyme B but does not affect CD107a upregulation of NTHi-activated MAIT cells

Once we had established that NTHi-infected MDM release IL-12, MDM-T cell co-cultures were repeated in the presence of an anti-IL-12p40 antibody. A previous report by Kurioka et al showed that IL-12 played a role in modulating MAIT granzyme B [34]. In agreement with this report, blocking of IL-12p40 in our model significantly (P=0.01) impaired granzyme B upregulation (figure 6D) but had no effect on the upregulation of CD107a (figure 6C). Inhibition of granzyme B was further reduced to baseline when both MR1 and IL-12 blocking antibodies were present (figure 6E, P=0.03). Together with the MR1 blocking, our data show that the mechanisms of MAIT activation in response to NTHi are time dependent, with both MR1-dependent and IL-12-dependent responses.

IL-12 stimulation can induce MAIT cell granzyme B expression in the absence of antigen presentation

We next sought to determine whether IL-12 alone was sufficient to induce MAIT cell granzyme B activation to the same extent as NTHi-infected MDM. We found that stimulation of T cells alone with IL-12 did cause a minor increase in MAIT cell granzyme B (P=0.004; supplementary figure 4). However, when T cells were co-cultured with autologous MDM and IL-12 combined, a far greater upregulation of granzyme B was detected in MAIT cells (P=0.03, figure 6F).

IL-7 is expressed by macrophages and contributes towards granzyme B upregulation

To understand why the IL-12-induced granzyme B upregulation was greater in the presence of MDM, we reasoned that MDM may produce other cytokines which synergised with IL-12 to induce granzyme B expression.

A previous report by Leeansyah et al showed that stimulation of MAIT cells with IL-7 caused upregulation of granzyme B [43]. We therefore hypothesised that IL-7 was also involved in the upregulation of granzyme B in our model. Quantitative-PCR indicated that MDM express IL-7 mRNA in response to NTHi infection (figure 7A). NTHi-infected MDM were then stained to detect the presence of intracellular IL-7 protein by flow cytometry. MDM appeared to express IL-7 at baseline and this was upregulated following NTHi infection ($P=0.03$; figure 7A).

T cells alone were then stimulated with IL-12 and/or IL-7 and granzyme B expression was measured by flow cytometry. Individually IL-7 and IL-12 caused a minor upregulation in MAIT cell granzyme B. However, combined IL-12 and IL-7 stimulation synergistically caused greater granzyme B expression ($P<0.001$; figure 7B), similar to the levels observed with NTHi infection. Consistent with this, blocking of IL-7 in the NTHi-infected co-culture model lead to a significant decrease in MAIT granzyme B expression ($P<0.05$; figure 7C). Taken together, our data show that granzyme B expression in MAIT cells can be activated by combined IL-12 and IL-7 signalling.

The IL-12 receptor is upregulated on MAIT cells following NTHi infection in a mechanism dependent on IL-12 and IL-7

We hypothesised that granzyme B upregulation could be controlled by expression of either the IL-12 or IL-7 receptor, so the expression of both receptors was first measured on MAIT cells in the NTHi-infected co-culture. NTHi infection upregulated both IL-12Rβ1 and IL-12Rβ2 on MAIT cells ($P<0.05$; figure 7D). In contrast, expression of IL-7Rα expression on MAIT cells was significantly decreased following infection ($P<0.05$; figure 7D). IL-12Rβ2 was also significantly ($P<0.05$) upregulated on lung MAIT cells from NTHi-infected explants (supplementary figure 5B).

Finally, to deduce how IL-12 and IL-7 synergistically combine to control MAIT granzyme B expression, the IL-12 and IL-7 receptors were measured following stimulation of T cells alone with IL-12 and/or IL-7. IL-12Rβ1 expression was unaffected by stimulation with either cytokine, whereas IL-7 stimulation lead to a significant decrease in IL-7Rα ($P<0.001$; figure 7E). However, MAIT cell IL-12Rβ2 expression was upregulated in the presence of both cytokines ($P<0.01$; figure 7E). Taken together, these data show that IL-7 in combination with IL-12 synergistically upregulates MAIT cell IL-12Rβ2 expression, which may allow greater IL-12 signalling and resulting in upregulation of MAIT cell granzyme B expression.

Killing of NTHi-infected MDM by MAIT cells

To confirm that MAIT cells were capable of actually killing NTHi-infected MDM, the co-culture model was adapted so that MDM were co-cultured with pure FACS-sorted MAIT cells and death of macrophages was assessed by viability staining. At 24 h no increase in MAIT cell-induced death of MDM from MDM-MAIT co-culture was detected (data not shown). However, at 48 h, there was a significant ($P=0.03$) increase in MAIT cell-induced death of NTHi-infected MDM compared to uninfected MDM, indicating that MAIT cells are capable of killing NTHi-infected macrophages (figure 8A&B). Consistent with this, an increase in LDH was detected in NTHi-infected MDM-MAIT co-cultures (data not shown). MAIT cell granzyme B was also significantly ($P<0.05$) upregulated in response to NTHi infection in this killing assay at 48 h (figure 8C). Overall, we show that MAIT cells are cytotoxic cells that respond to NTHi infection and kill infected macrophages.

Discussion

MAIT cells are rapidly gaining recognition as important effector cells with potential roles in a variety of diseases. Here we show for the first time that human lung MAIT cells exhibit cytotoxic responses to the respiratory pathogen, NTHi. In combination with our previous study [40], these observations provide further evidence that NTHi represents a target of lung MAIT cell immunity.

Little is known about the mechanisms behind MAIT cell cytotoxic responses, nor how they differ from that of conventional T cells. Here, conventional CD4+ and CD8+ T cell granzyme B responses were dependent exclusively on antigen presentation. In contrast, MAIT cell expression of granzyme B was regulated by a combination of both MR1-antigen presentation and through IL-12 and IL-7 stimulation. The ability of MAIT cells to upregulate granzyme B in response to cytokines may represent a mechanism which allows MAIT cells to become primed and rapidly respond to infection.

Previous studies have also reported that IL-12 and IL-7 are involved in MR1-independent MAIT cell IFNγ and cytotoxic responses [26][28][43]. However, this is the first study to our knowledge which links IL-12 and IL-7 stimulation together in a novel mechanism whereby both cytokines synergise to positively regulate the IL-12 receptor, allowing increased IL-12 signalling and leading to greater granzyme B upregulation.

Since IL-7, -12, -15 and -18 have all been reported to activate MAIT cells, further work is needed to explore the full interactions and combined effects of these cytokines on MAIT cell activation[44][45]. The ability of MAIT cells to respond to cytokine stimulation alone expands their definition as anti-bacterial cells and indicates a role for bystander activation in infections with non-riboflavin synthesising bacteria and viruses [46][45][47]. Diseases characterised by an increased inflammatory environment could also result in bystander activation of MAIT cells, furthering inflammation. Indeed, elevated levels of IL-12 have been found in COPD patients and inversely correlates with lung function [48]. Whether increased IL-12 mediates

lung function decline by activating MAIT cell cytotoxicity is a question that remains to be addressed.

In addition to upregulation of cytotoxic markers, we show that MAIT cells are able to kill NTHi-infected MDM, but, due to technical limitations we were unable to demonstrate that IL-12/IL-7 blocking actually impaired the killing ability of MAIT cells. However, increases in granzyme B correlated with increases in killing, suggesting a role for IL-12/IL-7 in this killing mechanism. Further work will be required to fully address this.

Although upregulation of granzyme B is controlled by IL-12 and IL-7, degranulation appeared to be largely dependent on cell contact and antigen presentation. This would indicate that whilst MAIT cells can substantially accumulate cytotoxic mediators intracellularly in response to cytokine stimulation, cytotoxic release is mainly controlled by cell contact. However, as the requirement for MR1 in degranulation appeared to be time-dependent and as minor CD107a upregulation was also seen in the transwell setup, other non-contact factors may contribute to degranulation. Overall, it seems likely that MR1 is required to initiate MAIT degranulation but is not required in the beginning to induce granzyme B expression. Initial degranulation may release other granzymes, such as granzyme A and granzyme K, whilst granzyme B release occurs later [34].

The type of pathogen, intracellular location of pathogen, inflammatory environment and type of infected cell may all be factors in determining the extent to which MAIT activation is MR1-dependent or –independent. Indeed, the responses in our transwell model were surprising at first as a previous report using transwells showed that MAIT cells did not activate in the absence of cell-to-cell contact, possibly due to lack of expression of IL-12 or IL-7 in that system [49]. Anatomical location may also be important, as a recent study found that granzyme B production by MAIT cells from tuberculosis pleural effusions was not inhibited by IL-12 blocking [50]. The time-course of activation could also be a key factor in determining which pathway predominates for MAIT activation. Ussher et al found that short

incubations of infected APCs caused IFNγ upregulation by MAIT cells in an MR1-mediated manner, but longer incubations meant that MAIT activation was dependent on both MR1 and IL-12 [28].

Although conventional cytotoxic T cells are typically thought of as just CD8+, CD4+ T cells have also been found to express CD107a following viral challenge [51] and are known to express granzyme B [52], but have not been investigated in the context of NTHi infection. Wilkinson et al also found an inverse correlation between CD4+ cells and patient symptoms following viral challenge [51], suggesting an important role of CD4+ cytotoxic activity in response to influenza infection. The data presented here may also suggest a role for cytotoxic CD4+ cells in responding to NTHi infection.

The mechanisms involved in NTHi colonising the lower airways and becoming pathogenic are not well understood, but could potentially be due to impaired T cell responses, as a result of cigarette-smoke induced damage [55][56][57]. We have previously shown that cytotoxic T cells are dysregulated in the COPD lung by aberrant regulation of the programmed cell death protein (PD) -1 signalling pathway and this may be a mechanism by which T cells contribute to excessive inflammation in response to respiratory pathogens (McKendry et al. 2016). Future work is needed in order to understand the mechanisms controlling T cell activation and how these become impaired in diseases associated with chronic NTHi colonisation.

In summary, we show here that both conventional T cells and, more potently, MAIT cells demonstrate cytotoxic responses to NTHi infection. We have also highlighted important differences in the mechanisms of innate and adaptive T cell cytotoxic responses. Further work is needed to understand the mechanisms behind innate and adaptive T cell responses to bacterial pathogens in airways diseases. More importantly, with their ability to respond to cytokine stimulation, MAIT cells could be an important source of bystander inflammation and cytotoxic-mediated damage in a variety of lung diseases and infections. This observation

therefore provides both new understanding of protective immune mechanisms but also potential new therapeutic opportunities to modulate this response and improve clinical outcome.

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613 10

614

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Figure Legends

Figure 1

Gating strategies for T cells. A) Gating for lung CD4+ and CD8+ T cells and MAIT cells obtained from lung explants. B) Gating for CD4+ and CD8+ T cells and MAIT cells from CD3+ cells that were MACS sorted from peripheral blood mononuclear cells. C) Representative plots for CD107a and granzyme B expression. DN= double negative populations DP= double positive populations.

Figure 2

Cytotoxic responses of lung T cells in response to NTHi infection. CD107a and granzyme B expression in A) CD8+ B) CD4+ and C) MAIT cells from lung explants. For all graphs, median values are shown; data are from different donors (N=8-9) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01.

Figure 3

Cytotoxic responses of peripheral T cells in response to NTHi-infected MDM. CD107a and granzyme B expression in A) CD8+ B) CD4+ and C) MAIT cells from autologous MDM-T cell co-cultures. For all graphs, median values are shown; data are from different donors (N=8-11) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01, and ***P<0.001

Figure 4

Blocking of antigen presentation impairs cytotoxicity. CD107a and granzyme B expression in A) CD8+ and CD4+ cells or B) MAIT cells from autologous MDM-T cell co-cultures in the

presence of either 10 µg/ml anti-HLA-ABC (W6/32), 10 µg/ml anti-HLA-DR (L243), 5 µg/ml anti-MR1 (26.5) respectively or IgG2a isotype control. For all graphs, median values are shown; data are from different donors (N=5-12) and are paired. Red line indicates uninfected baseline expression. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05 and **P<0.01.

Figure 5

Antigen-independent factors affect cytotoxic responses of T cells. CD107a and granzyme B expression in MAIT cells from autologous MDM-T cell co-cultures incorporating 0.4 µm transwells. For all graphs, median values are shown; data are from different donors (N=5) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

Figure 6

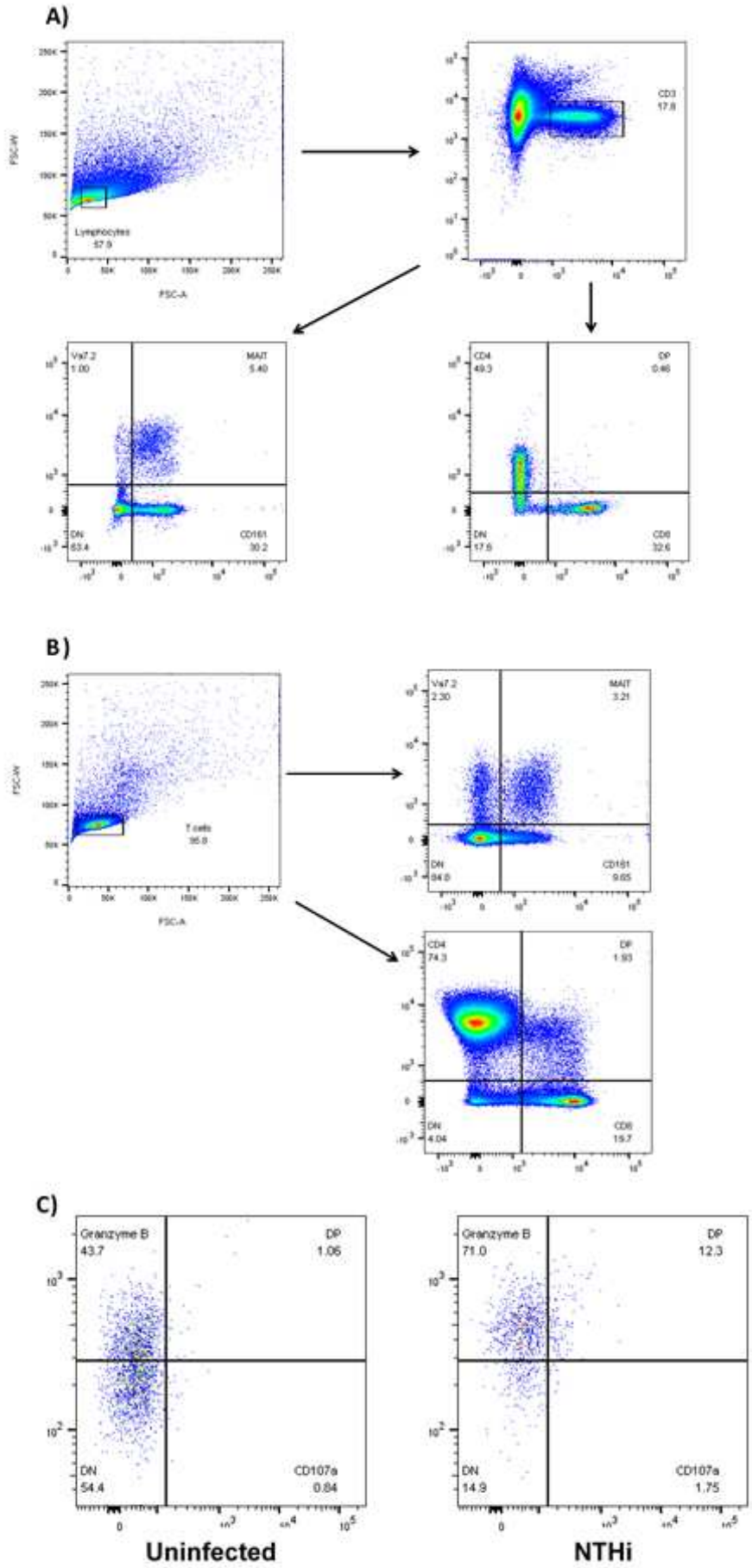
Macrophages express IL-12 in response to NTHi and IL-12 contributes to granzyme B expression. A) Gene expression of *IL-12a (p35)* and *IL-12b (p40)* expressed as $\Delta\Delta C_t$ normalised to β_2M. B) IL-12 p70 expression measured by ELISA. C) CD107a and D&E) Granzyme B expression in MAIT cells from autologous MDM-T cell co-cultures in the presence of either 10 µg/ml anti-IL-12 (C8.6), IgG1 isotype control or 5 µg/ml anti-MR1 (26.5). Red line indicates uninfected baseline expression. F) Granzyme B expression in MAIT cells from IL-12-simulated autologous MDM-T cell co-cultures. For all graphs, median values are shown; data are from different donors (N=5-7) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05.

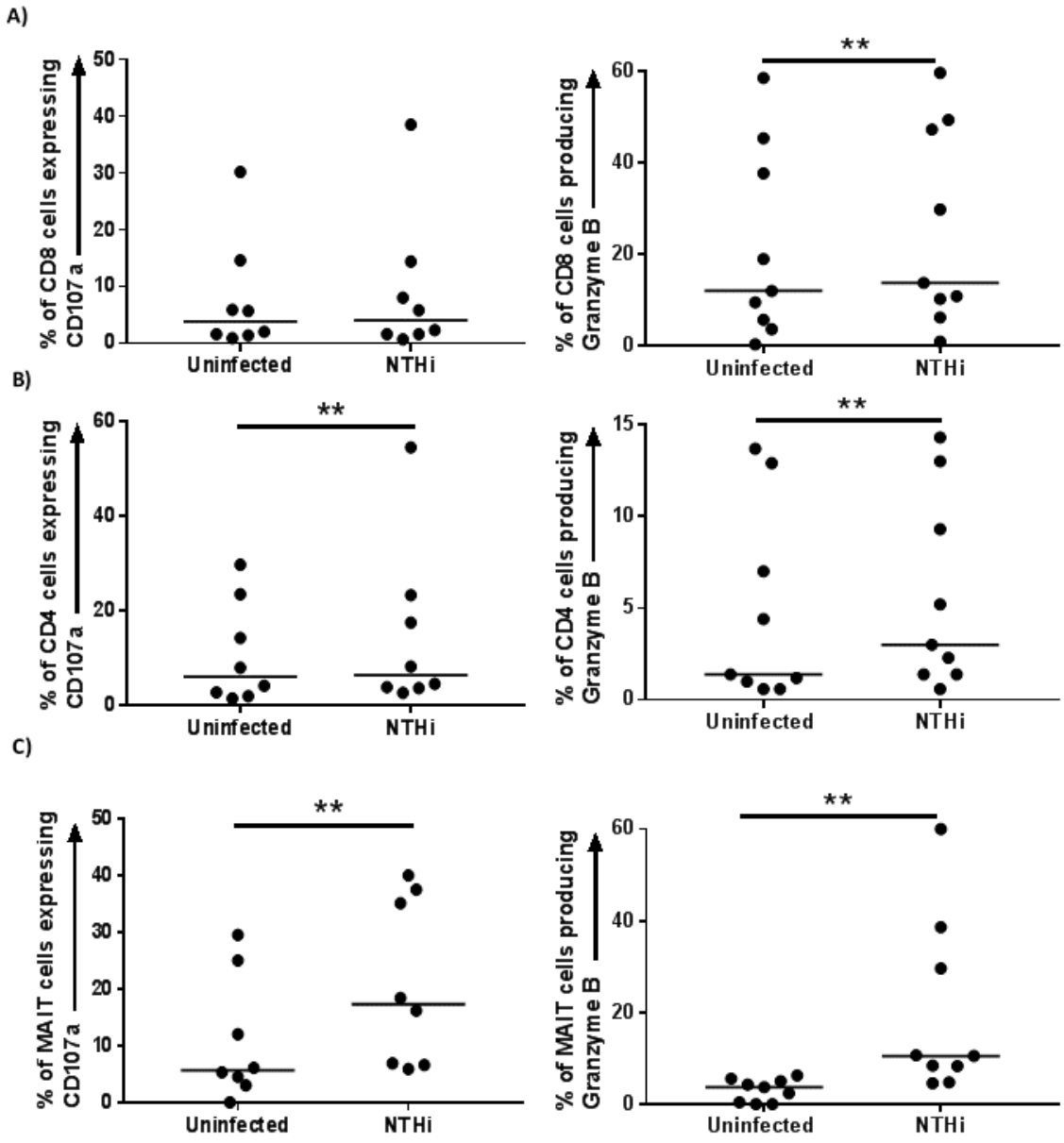
Figure 7

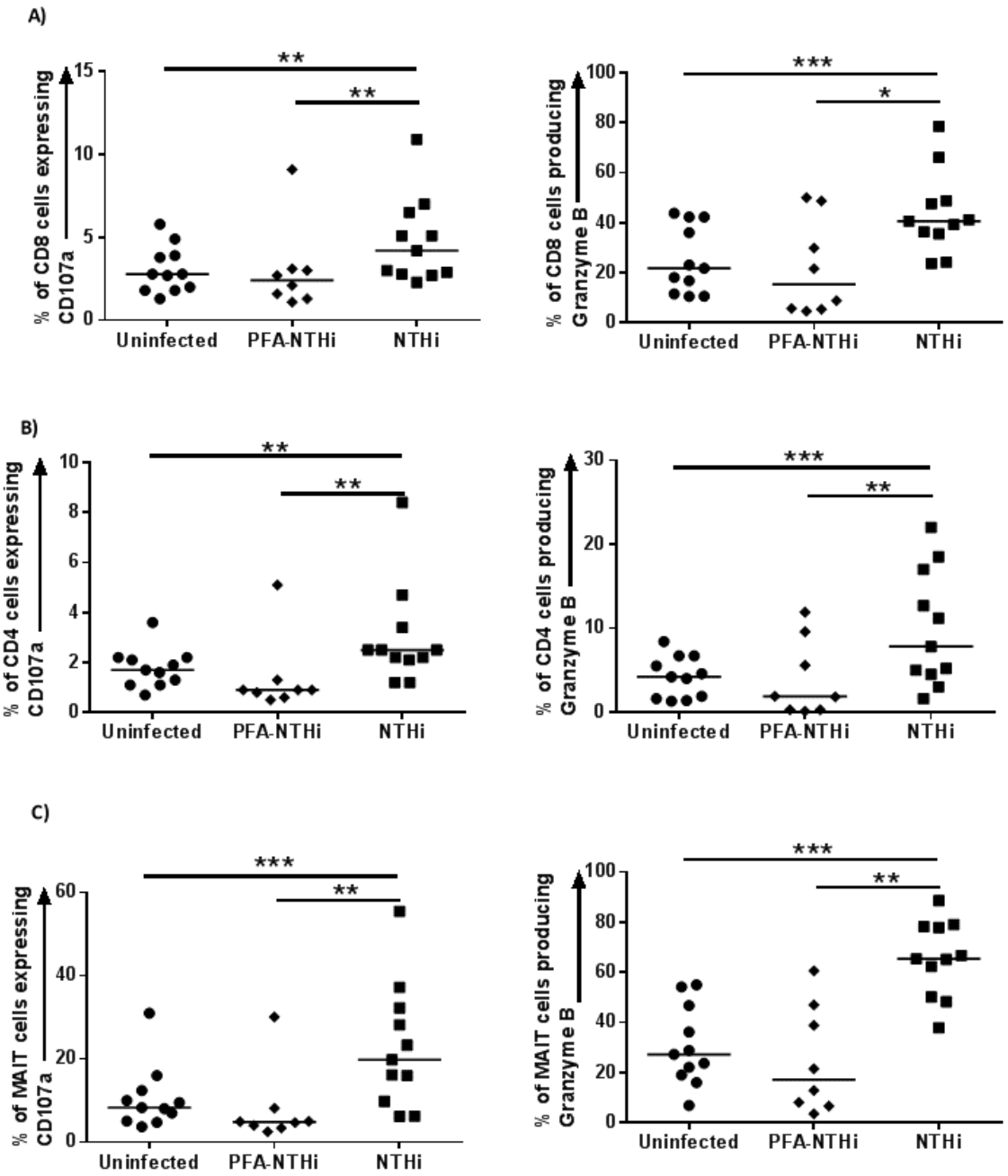
IL-7 and IL-12 control MAIT cell granzyme B via IL-12 receptor expression. A) *IL-7* gene expression as $\Delta\Delta C_t$ normalised to β_2M and IL-7 protein expression expressed as specific mean fluorescence intensity (SMFI) determined by intracellular flow cytometry. B) Granzyme B expression in MAIT cells from IL-12 and/or IL-7 stimulated T cells. C) Granzyme B expression in MAIT cells from autologous MDM-T cell co-cultures in the presence of either 10 µg/ml anti-IL-7 (BVD10-40F6) or IgG1 isotype control. Red line indicates uninfected baseline expression. D) IL-12Rβ1 and β2 and IL-7Rα expression in MAIT cells from autologous MDM-T cell co-cultures. E) IL-12Rβ1 and β2 and IL-7Rα expression in MAIT cells from IL-12 and/or IL-7 stimulated T cells. For all graphs, median values are shown; data are from different donors (N=5-6) and are paired. Statistical significance was determined by Wilcoxon signed-rank test or Friedman test with Dunn's multiple comparison. *P<0.05 **P<0.01 ***P<0.001.

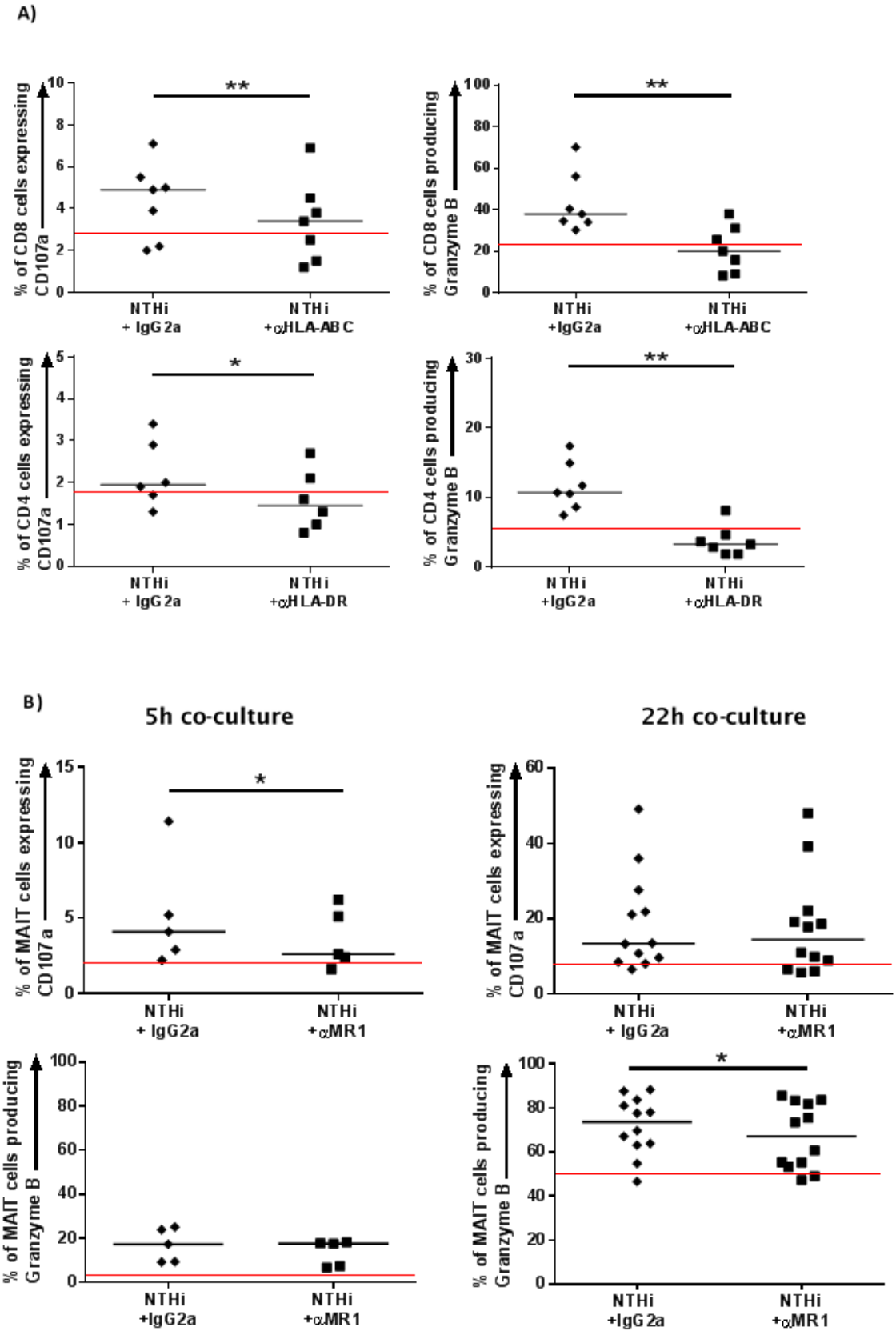
Figure 8

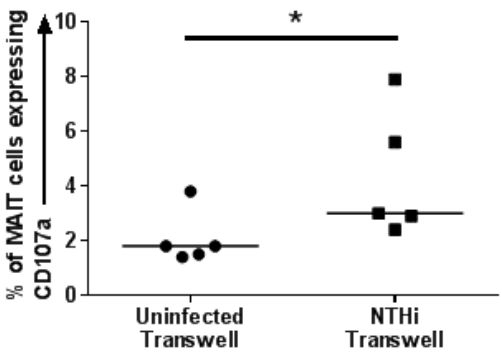
Killing of NTHi-infected MDM by MAIT cells. A) MDM were first gated on size, then on HLA-DR-APC-CY7 and Zombie Violet Fixable Viability dye. B) MDM were co-cultured with FACS-sorted autologous MAIT cells for 48 h and killing of MDM by MAIT cells was assessed by viability staining measured by flow cytometry. C) Granzyme B expression in MAIT cells from autologous MDM-MAIT cell co-cultures. For all graphs, median values are shown; data are from different donors (N=5) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

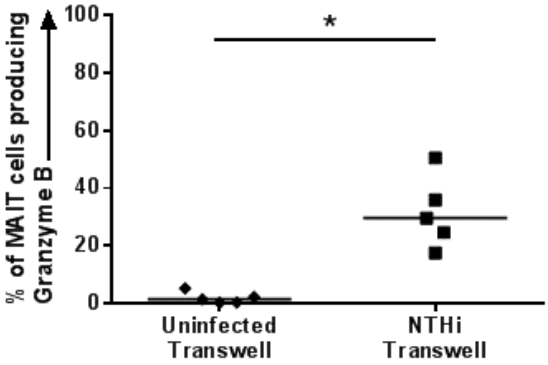

A)
Lymphocytes
57.9
FSC-A
CD3
17.8
Vα7.2
1.00
MAIT
5.40
DN
63.4
CD161
30.2
CD4
49.3
DP
0.46
DN
17.6
CD8
32.6
B)
T cells
95.8
FSC-A
Vα7.2
2.30
MAIT
3.21
DN
84.0
CD161
9.65
CD4
74.3
DP
1.93
DN
4.04
CD8
19.7
C)
Granzyme B
43.7
DP
1.06
DN
54.4
CD107a
0.84
Granzyme B
71.0
DP
12.3
DN
14.9
CD107a
1.75
Uninfected
NTHi

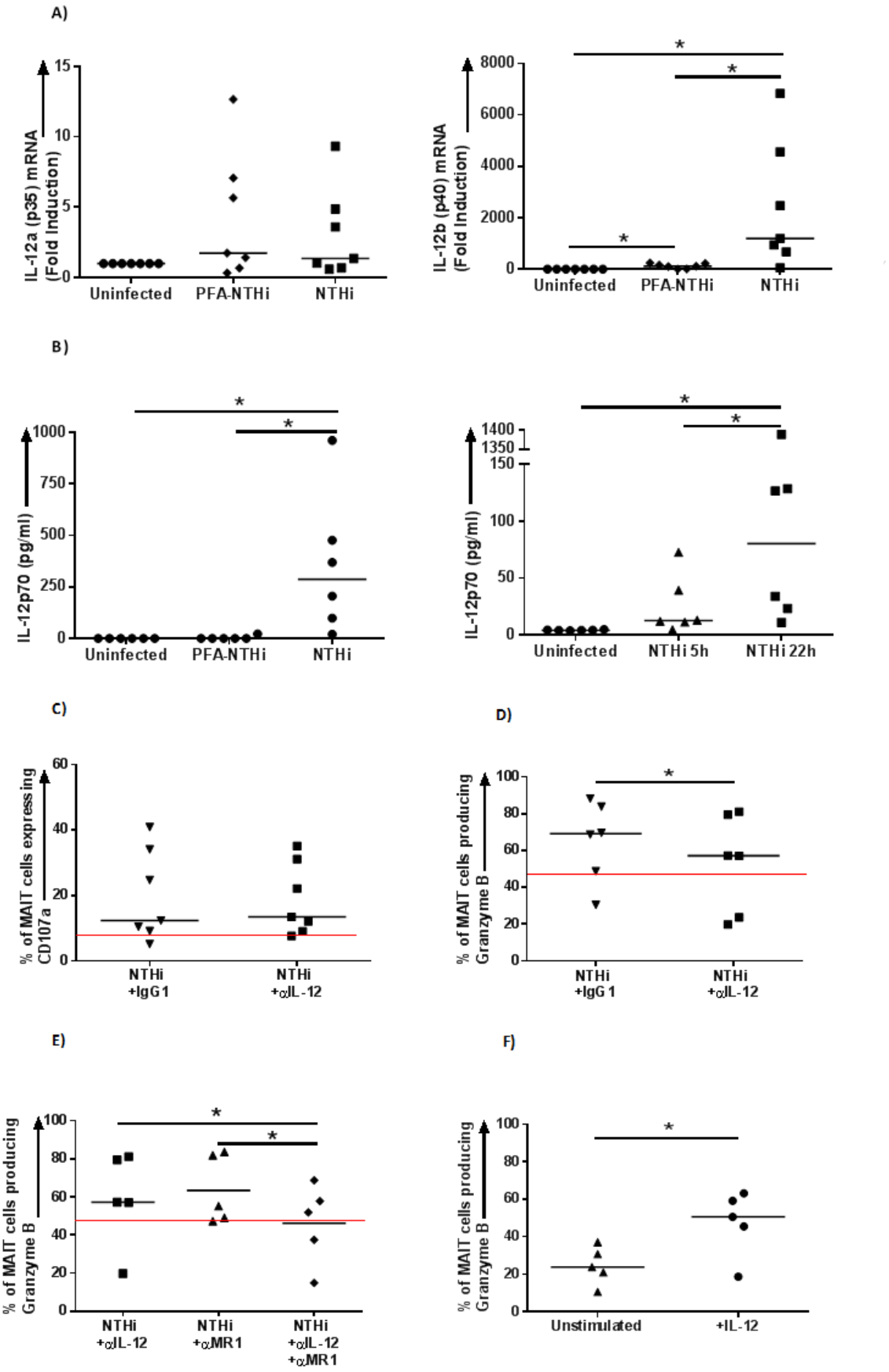

A)
% of CD8 cells expressing CD107a
50
40
30
20
10
0
Uninfected
NTHi
% of CD8 cells producing Granzyme B
**
60
40
20
0
Uninfected
NTHi
B)
% of CD4 cells expressing CD107a
**
60
40
20
0
Uninfected
NTHi
% of CD4 cells producing Granzyme B
**
15
10
5
0
Uninfected
NTHi
C)
% of MAIT cells expressing CD107a
**
50
40
30
20
10
0
Uninfected
NTHi
% of MAIT cells producing Granzyme B
**
60
40
20
0
Uninfected
NTHi

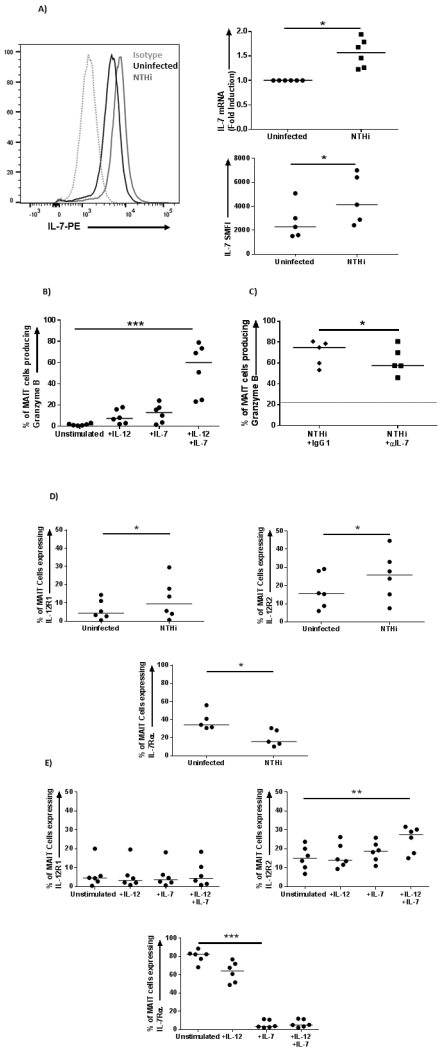

A)
% of CD8 cells expressing CD107a
% of CD8 cells producing Granzyme B
B)
% of CD4 cells expressing CD107a
% of CD4 cells producing Granzyme B
C)
% of MAIT cells expressing CD107a
% of MAIT cells producing Granzyme B
Uninfected
PFA-NTHi
NTHi


A)
% of CD8 cells expressing CD107a
**
NTHi + IgG2a
NTHi +αHLA-ABC
% of CD8 cells producing Granzyme B
**
NTHi +IgG2a
NTHi +αHLA-ABC
% of CD4 cells expressing CD107a
*
NTHi + IgG2a
NTHi +αHLA-DR
% of CD4 cells producing Granzyme B
**
NTHi +IgG2a
NTHi +αHLA-DR
B)
5h co-culture
22h co-culture
% of MAIT cells expressing CD107a
*
NTHi + IgG2a
NTHi +αMR1
% of MAIT cells expressing CD107a
NTHi + IgG2a
NTHi +αMR1
% of MAIT cells producing Granzyme B
NTHi +IgG2a
NTHi +αMR1
% of MAIT cells producing Granzyme B
*
NTHi +IgG2a
NTHi +αMR1

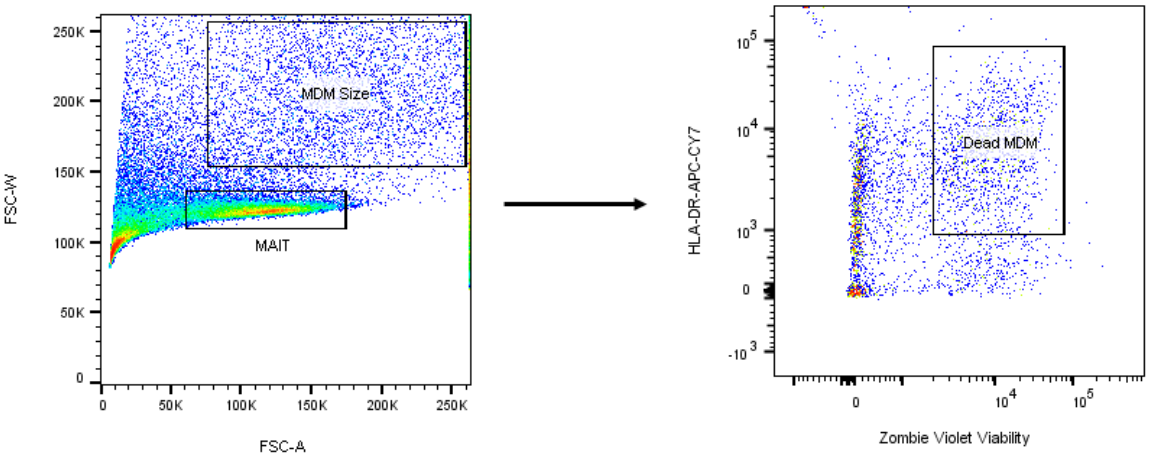


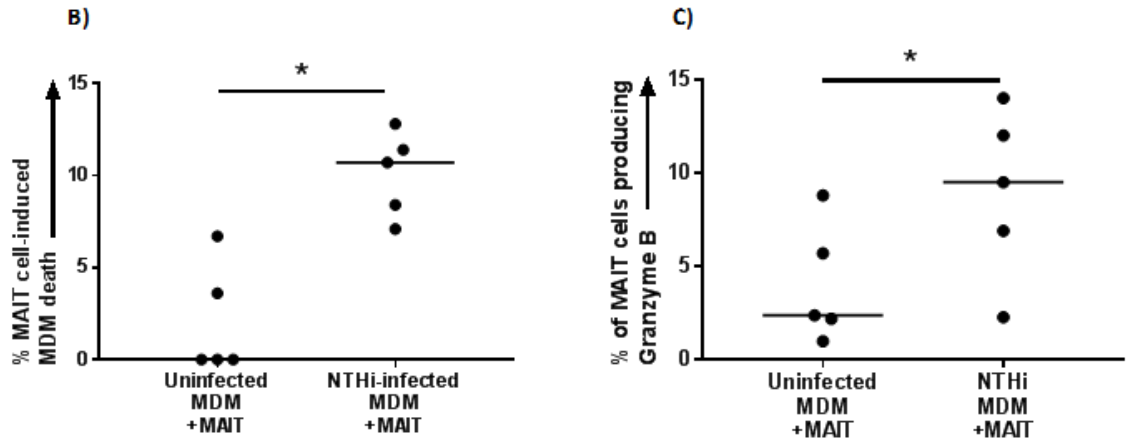



A)
IL-12a (p35) mRNA (Fold Induction)
15
10
5
0
Uninfected
PFA-NTHi
NTHi
IL-12b (p40) mRNA (Fold Induction)
8000
6000
4000
2000
0
Uninfected
PFA-NTHi
NTHi
B)
IL-12p70 (pg/ml)
1000
750
500
250
0
Uninfected
PFA-NTHi
NTHi
IL-12p70 (pg/ml)
1400
1350
150
100
50
0
Uninfected
NTHi 5h
NTHi 22h
C)
% of MAIT cells expressing CD107a
60
40
20
0
NTHi +IgG1
NTHi +αIL-12
D)
% of MAIT cells producing Granzyme B
100
80
60
40
20
0
NTHi +IgG1
NTHi +αIL-12
E)
% of MAIT cells producing Granzyme B
100
80
60
40
20
0
NTHi +αIL-12
NTHi +αMR1
NTHi +αIL-12 +αMR1
F)
% of MAIT cells producing Granzyme B
100
80
60
40
20
0
Unstimulated
+IL-12


A)
Isotype
Uninfected
NTHi
IL-7-PE
IL-7 mRNA (Fold Induction)
Uninfected
NTHi
IL-7 SMFI
B)
% of MAIT cells producing Granzyme B
Unstimulated
+IL-12
+IL-7
+IL-12 +IL-7
C)
% of MAIT cells producing Granzyme B
NTHi +IgG1
NTHi +αIL-7
D)
% of MAIT Cells expressing IL-12R1
% of MAIT Cells expressing IL-2Rβ
% of MAIT Cells expressing IL-7Rα
E)
% of MAIT Cells expressing IL-12R1
% of MAIT Cells expressing IL-2Rβ
% of MAIT cells expressing IL-7Rα

A)





Supplementary

Supplementary Methods

Preparation of NTHi

A strain of NTHi cultured from the sputum of a Southampton COPD patient was used as previously described [40]. Briefly, NTHi was cultured on chocolate agar plates and colonies were used to inoculate brain-heart infusion media supplemented with 10 mg/L nicotinamide adenine dinucleotide, 30 mg/L hemin and 44 mL/L glycerol. Bacterial suspension was grown to mid-log phase and heat-inactivated foetal calf serum (FCS) was added at a final concentration of 20%. 1 ml aliquots were stored at -80℃ as described [41].

Patient Recruitment

For *ex vivo* human explant experiments, lung tissue distal from tumour sites was obtained from patients undergoing lung resection surgery performed at Southampton General Hospital. For the isolation of T cells and monocytes for *in vitro* experiments, blood was obtained from healthy volunteers. All studies were approved by Southampton and South West Hampshire Research Ethics Committees (13/SC/0416, 09/H0504/109). All participants provided written informed consent.

Preparation of lung tissue and explant infection

Lung tissue explants were prepared as previously described [42]. Briefly, lung tissue obtained from patients undergoing lung re-sectioning was taken from sites distal to any tumours present. Lung tissue was cut into 3-5mm^3 cubes and washed extensively before being place in RPMI (Sigma-Aldrich, Poole, UK) supplemented with 2 mg/ml L-glutamine, 1% gentamicin and 0.5 µg/ml amphotericin B (all from Invitrogen, Paisley, UK) and

incubated overnight 37℃, 5% CO_2. Tissue explants were then infected with $5x10^6$ CFU NTHi for 24 hours, after which T cells were dispersed from tissue by 0.5 mg/ml collagenase digestion. T cells were then stained for flow cytometry.

Blood T cell and Monocyte Isolation & differentiation

CD3+ T cells and CD14+ monocytes were isolated from human peripheral blood mononuclear cells (PBMC) obtained from the blood of healthy volunteers using MACS (Miltenyi Biotec, Bisley, UK) and monocytes differentiated into macrophages (MDM) over 12 d using 2 ng/ml GM-CSF as previously described [40]. $CD3^+$ cells were frozen at -80℃ in 10% dimethyl sulfoxide (DMSO) in FCS until needed. CD3+ T cells were chosen so as to not exclude double negative (CD4-CD8-) MAIT cells.

Macrophage infection

MDM infection was performed as previously described [40]. Briefly, MDM were cultured for 2 hours at 37℃ with either live or paraformaldehyde- fixed (PFA-) NTHi at multiplicity of infection (MOI)-10 in antibiotic-free 0.1% FCS-RPMI. After 2 h, MDM were washed and then incubated either alone or with autologous T cells in 0.1% FCS-RPMI with antibiotics for a further 22 h unless otherwise indicated. Where specified, co-cultures were carried out with the addition of either anti-MR1 (26.5), -HLA-DR (L243), -HLA-ABC (W6/32), -IL-12 (C8.6) or -IL-7 (BVD10-40F6) or appropriate isotype controls at 5 or 10 µg/ml as indicated (all Biolegend, San Diego, USA). For cytokine stimulations, cells were incubated with 10 ng/ml recombinant IL-12 or IL-7 as indicated (both Biolegend). To measure intracellular IL-7, monensin was added to the final 5 h of culture. Viability of cells was confirmed by trypan blue staining.

MAIT Killing Assay

MDM were infected as described above and were co-cultured with autologous FACS-sorted MAIT cells to create a pure MDM-MAIT cell co-culture (>93% purity). MDM and MAIT cells were co-cultured for 48 h, after which cells were harvested. MDM were stained for HLA-DR-APC-CY7 (BD Biosciences, Oxford, UK) and for viability with Zombie Violet Fixable Viability Kit (Biolegend). To determine specific MAIT cell-induced killing of NTHi-infected MDM, % death of MDM alone (not co-cultured with MAIT cells) was deducted from % death of MDM from MDM-MAIT co-culture.

Flow cytometry analysis

All cells were resuspended in 100 µl FACS buffer (2 mM EDTA, 0.5% w/v BSA in PBS) with 2 mg/ml purified IgG from human serum (Sigma-Aldrich) per tube. Cells were stained for surface markers or appropriate isotype controls for 30 min on ice. Cells were then washed and were resuspended in 200 µl Cytofix/Cytoperm (BD Biosciences) and incubated on ice for 20 min. Following fixation, cells were washed in 1x Perm/Wash, resuspended in 100 µl 1 x Perm/Wash buffer and stained for intracellular markers on ice for 30 min. Cells were then washed again and resuspended in FACS buffer. T cells were stained for surface markers: CD4-PERCP-CY5.5, CD8-APC-CY7, CD161-APC and CD107a-BV510 (all BD Bioscience); Vα7.2-PE and IL-7Rα-BV510 (both Biolegend); and IL-12Rβ1-FITC and IL-12Rβ2-AF488 (R&D Systems, Abingdon, United Kingdom). T cells were stained intracellularly for: IFNγ-FITC, Perforin-FITC (Biolegend) and Granzyme B-FITC (BD-Bioscience). Staining for lung T cells was identical to above, with the addition of CD3-PE-CY7 (BD Bioscience). For MDM-IL-7 staining, MDM were first fixed and permeablised as above and then stained intracellularly for purified human anti-IL-7 (BVD10-40F6) and then anti-rat IgG1-PE (MRG1-58) (both Biolegend).

Specific mean fluorescence intensity (SMFI) was derived by subtracting isotype fluorescence values from each sample's MFI.

Flow cytometric analysis was performed on a FACSAria using FACSDiva software v5.0.3 (BD Biosciences). The gating strategy is outlined in Figure 1.

RNA Isolation & RT-PCR

RNA was extracted from MDM using TriFast (PeqLab, VWR, Erlangen, Germany). Reverse transcription was performed according to the manufacturer's protocols using a High Capacity cDNA Reverse Transcription Kit (Life Technologies Loughborough, UK) with random hexamers. *IL-12p35, IL-12p40* and *IL-7* gene expression was analysed using TaqMan universal PCR master mix, No AmpErase® UNG in a 7900HT fast real-time PCR system instrument (all Life Technologies). Gene expression was normalized to β_2-microglobulin gene expression and quantified using the $\Delta\Delta$ C_T method.

ELISA

IL-12p70 ELISA MAX (Biolegend), and granzyme B duoset ELISA (R&D Systems) were all carried out according to the manufactures' protocol.

Statistics

Statistical analyses were performed using either a Wilcoxon's matched pairs signed rank test or Friedman test with Dunn's multiple comparison testing (GraphPad Prism v6.0, GraphPad Software Inc., San Diego, USA). Data are expressed as medians. Results were considered significant if $P<0.05$.

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Supplementary Figure Legends

Supplementary Figure 1

Granzyme B is produced in response to NTHi infection. Supernatants from lung explants or autologous MDM-T cell co-cultures were measured for granzyme B by ELISA. Median values are shown; data are from different donors (N=6-7) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01.

Supplementary Figure 2

Further cytotoxic responses of peripheral T cells in response to NTHi-infected MDM. Perforin expression in CD8+, CD4+ and MAIT cells from autologous MDM-T cell co-cultures. For all graphs, median values are shown; data are from different donors (N=6) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05.

Supplementary Figure 3

Further effects of inhibiting antigen presentation. A) Impact of blocking HLA-DR and HLA-ABC on MAIT cell granzyme B. B) MAIT cells produce IFNγ in response to antigen-independent factors. IFNγ expression in MAIT cells from autologous MDM-T cell co-cultures incorporating 0.4 µm transwell inserts. For all graphs, median values are shown; data are from different donors (N=5-7) and are paired. Statistical significance was determined by Friedman test with Dunn's multiple comparison or Wilcoxon signed-rank test. *P<0.05.

Supplementary Figure 4

MAIT cells in the absence of MDM can still produce minor granzyme B in response to IL-12 stimulation. Granzyme B expression in MAIT cells from IL-12 stimulated T cells. Median

values are shown; data are from different donors (N=9) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. **P<0.01.

Supplementary Figure 5

IL-12 in the lung. A) Supernatants from lung explants were measured for IL-12p70 by ELISA. B) IL-12Rβ2 expression on lung MAIT cells. Median values are shown; data are from different donors (N=5-7) and are paired. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01.