Steroid-induced deficiency of mucosal-associated invariant T cells in the COPD lung: implications for NTHi infection

Timothy S.C. Hinks[1,2,3*], Joshua C. Wallington[1*], Anthony P. Williams[4,5], Ratko Djukanović[1,2], Karl J. Staples[1,5†], Tom M.A. Wilkinson[1,2,5†].

1 Clinical & Experimental Sciences, University of Southampton Faculty of Medicine, Sir Henry Wellcome Laboratories, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

2 Southampton NIHR Respiratory Biomedical Research Unit, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

3 Department for Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Victoria 3000, Australia

4 Cancer Sciences, University of Southampton Faculty of Medicine, Sir Henry Wellcome Laboratories, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

5 Wessex Investigational Sciences Hub, University of Southampton Faculty of Medicine, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

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*, † These authors contributed equally to this work.

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Corresponding author: Dr Karl J. Staples, University of Southampton Faculty of Medicine, Sir Henry Wellcome Laboratories, Mailpoint 850, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK. Tel: +44 23 8079 6397 Fax: +44 23 8070 1771 Email: k.staples@southampton.ac.uk

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At a glance commentary:

Scientific knowledge on the subject:

Inhaled corticosteroids reduce exacerbations in COPD, but increase the risk of developing pneumonia by unknown mechanisms. Non-typeable *Haemophilus influenzae* (NTHi) is the most common airway-colonizing bacterium in COPD, the leading bacterial pathogen during exacerbations and polymicrobial infections, with potential aetiological roles in pneumonia. Mucosal associated invariant T (MAIT) cells are a recently identified, abundant, pro-inflammatory T cell subset with unknown roles in lung immunity.

What this study adds to the field:

MAIT cells can respond to macrophage-presented NTHi antigens by producing the Th1 cytokine IFN-γ. MAIT cells are deficient in the airways of steroid-treated COPD patients and *in vitro* MAIT cell responses are impaired by steroids. Together these findings demonstrate a new role for MAIT cells in human lung antimicrobial defence with implications for a range of airway diseases.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

Rationale:

Mucosal associated invariant T (MAIT) cells are a recently-described, abundant, pro-inflammatory T cell subset with unknown roles in pulmonary immunity. Non-typeable *Haemophilus influenzae* (NTHi) is the leading bacterial pathogen during COPD exacerbations and a plausible target for MAIT cells.

Objectives:

To investigate whether MAIT cells respond to NTHi and the effects of inhaled corticosteroids on their frequency and function in COPD.

Methods:

11 participants with COPD receiving inhaled corticosteroids (ICS), 8 with steroid-naïve COPD and 21 healthy controls underwent phlebotomy, sputum induction, bronchoalveolar-lavage and endobronchial biopsy. Pulmonary and monocyte-derived macrophages were cultured *in vitro* with NTHi.

Measurements: Frequencies of Vα7.2+CD161+ MAIT cells, surface expression of MHC-related protein 1 (MR1) and intracellular IFN-γ expression were measured by flow cytometry.

Main Results:

MAIT cell frequencies were reduced in peripheral blood in ICS-treated COPD (median 0.38% (IQR, 0.25-0.96) compared with health (1.8% (IQR, 1.4-2.5), P=0.001)) or steroid-naïve COPD (1.8% (1.2-2.3), P=0.04). MAIT cells were reduced in bronchial biopsies in steroid-treated COPD (0.73% (0.46-1.3)) compared with health (4.0% (1.6-5.0), P=0.02). Co-culture of live NTHi

increased macrophage surface expression of MR1 and induced IFN-γ from CD4 cells and CD8 cells, but most potently from MAIT cells (median IFN-γ positive frequencies 2.9%, 8.6% and 27.6% respectively). *In vitro* fluticasone and budesonide reduced MR1 surface expression 2-fold and decreased NTHi-induced IFN-γ secretion 8-fold.

Conclusions:

MAIT cells are deficient in blood and bronchial tissue in steroid-treated, but not steroid-naïve COPD. NTHi constitutes a target for pulmonary MAIT cell immune responses, which are significantly impaired by corticosteroids.

Introduction

Exacerbations are major drivers of morbidity, mortality, and health care utilisation in chronic obstructive pulmonary disease (COPD)(1-3). Most exacerbations are triggered by bacterial or viral infections(4), or by acquisition of a new strain of a colonising bacterium(1, 2). Non-encapsulated or non-typeable *Haemophilus influenzae* (NTHi) can be isolated in up to 80-87% of COPD exacerbations(5, 6) and are the most common bacteria colonizing the airways in COPD(6), with colonization correlated with exacerbations, severity of airways inflammation(5), and symptoms(2, 6). NTHi colonization is a major cause of tissue damage and the bacteria can also invade into lung tissue, between epithelial cells and within macrophages(5, 6) potentially facilitating immune evasion and persistence(7, 8). NTHi colonization may induce airways inflammation through specific IgE-mediated hypersensitivity(9), through activation of innate immunity via TLR2/4 leading to increased local production of IL-1β and IL-8 and reactive oxygen species, and through activation of adaptive immunity in which both B and T cell responses are implicated(6). Furthermore NTHi can itself play an aetiological role in pneumonia, either as a single pathogen(10) or in polymicrobial infection with other organisms such as *Streptococcus pneumoniae*(11).

The use of inhaled corticosteroids (ICS) has been shown to improve symptoms and health status and reduce the incidence of exacerbations in COPD(12-15). However, effects of ICS are modest and likely restricted to certain subgroups of this heterogeneous condition(3) whilst evidence is accumulating from well-

designed clinical trials that ICS increase the incidence of community-acquired pneumonia(13-18). The mechanisms underlying this effect remain unknown, and have been highlighted as a research priority(3).

Mucosal associated invariant (MAIT) T cells are a recently-described subset of innate-like T lymphocytes that are abundant in humans, comprising up to 10% of T cells in blood and airway tissue (19-21). Like invariant natural killer T (iNKT) cells, MAIT cells express a semi-invariant T cell receptor, usually comprised of the TCR-α chain TRAV1-2-TRAJ33 (but also sometimes TRAV1-2-TRAJ12 or TRAV1-2-TRAJ20), associated with a limited repertoire of TCR-β chains(21, 22). High surface expression of CD161 allows MAIT cells to be identified as CD3+Vα7.2+CD161+ T cells(23, 24). When activated MAIT cells can rapidly express a range of pro-inflammatory cytokines including TNF-α, IL-17 and IFN-γ(23). The remarkable evolutionary conservation of this system(25) implies an essential role in host defence, which has yet to be defined. It has recently been shown that MAIT cells recognise small molecule derivatives of highly conserved biosynthetic pathways of riboflavin and folic acid metabolism in bacteria, mycobacteria and yeasts(26, 27) presented on the non-polymorphic class 1b antigen presenting molecule MHC-related protein 1 (MR1)(28, 29). Given their propensity for intracellular invasion of epithelia and macrophages(6), and as they express this riboflavin pathway(30), NTHi comprise a likely target of MAIT cell immunity.

We have recently demonstrated a striking deficiency of MAIT cells in blood, sputum and bronchial biopsies in asthma, which was associated with use of ICS(20, 21). Therefore, we hypothesised ICS use in COPD may also be associated with deficiency in MAIT cells in the airways, that NTHi may be a target for MAIT cell activity, and that this activity may be modulated by steroids.

Materials & Methods

Participants

Bronchoscopy cohort

40 participants (18-75 years) were enrolled from the NIHR Southampton Respiratory Biomedical Research Unit and outpatient clinics at University Hospital Southampton: 19 participants with COPD of which 11 were receiving inhaled corticosteroids (ICS) and 8 were not, and 21 healthy non-atopic controls (see Table 1).

Surgical cohort

For the isolation of pulmonary macrophages, lung parenchyma was obtained from additional participants undergoing resection of lung tumors (Supplementary Table E1).

Blood cohort

For the isolation of T cells and monocytes for *in vitro* experiments, blood was obtained from additional healthy volunteers.

The study was approved by the South Central - Hampshire Research Ethics Committees (13/SC/0416, 10/H0504/2, 08/H0502/32). All participants provided written informed consent.

Study procedures

Bronchoscopy participants were assessed by history, examination, skin-prick testing with common aero-allergens, spirometry, carbon monoxide transfer

testing and exhaled nitric oxide (eNO). Lung samples were obtained by sputum induction(31), bronchoalveolar lavage (BAL) and endobronchial biopsy(32, 33). Using flow cytometry, MAIT-cell subsets were characterized by surface expression of TCR-Vα7.2 and CD161(23, 34, 35) as previously described(20).

Blood T cell and Monocyte Isolation & differentiation

CD3+ T cells and monocytes were isolated from human peripheral blood mononuclear cells (PBMC) using MACS technology (Miltenyi Biotec, Bisley, UK) and monocytes differentiated into macrophages (MDM) using 2 ng/ml GM-CSF as previously described(36, 37).

Lung macrophage and MDM infection

A clinical isolate of NTHi (ST201) was cultured on chocolate agar plates and grown to mid-log phase in brain-heart infusion medium culture media with 44 mL/L glycerol, 30 mg/L hemin and 10 mg/L nicotinamide adenine dinucleotide and 20% heat inactivated foetal calf serum and stored in aliquots at -80°C until required, as previously described (38). Macrophages were cultured for 2 hours at 37°C with either live or paraformaldehyde-fixed NTHi at multiplicity of infection (MOI)-1 or MOI10 in antibiotic-free medium. Macrophages were washed with complete medium and cultured for a further 22 h before cells were harvested using non-enzymatic cell-dissociation solution (Sigma, Poole, UK). For T cell activation assays 10^6 autologous $CD3^+$ cells were added per well to MDM after the 2 h infection, with monensin (eBioscience, Hatfield, UK) for the final 5 h.

MDM infection with H3N2 X31 strain of influenza was carried out as previously described (36).

MDM stimulation with cytokines and LPS was carried out by adding either TNFα, IL-6 and IFN-γ (10 ng/ml) or LPS (100 ng/ml) to culture wells and incubated for 24 h. To model the effects of steroids in vitro, MDM from healthy volunteers were infected with NTHi as above with the addition of 100 nM fluticasone propionate, 200 nM budesonide or DMSO. Steroid or DMSO were present for both the 2 h infection of MDM, and re-added following washing for the 22h co-culture of NTHi-infected MDM and T cells. For MR1 blocking, the 5 h infected-MDM – T cell co-culture was carried out as above but with the addition of 5 μg/ml IgG2a isotype control or anti-MR1 (26.5, Biolgend, San Diego, USA) as previously described(39).

Flow cytometry analysis

Samples were resuspended in FACS buffer (PBS, 0.5% w/v BSA, 2 mM EDTA) containing 2 mg/ml human IgG before being incubated on ice in the dark for 30 min in the presence of fluorescently-labelled antibodies as previously described(20, 36). Specific median fluorescence intensity (SMFI) was derived by subtracting isotype fluorescence values from MFI. Flow cytometric analysis was performed on a FACSAria using FACSDiva software v5.0.3 (BD Biosciences, Oxford, UK) and FlowJo version 10.1 (FlowJo LLC, Ashland, OR, USA).

RNA Isolation & RT-PCR

RNA was extracted from MDM using TriFast (PeqLab, VWR, Erlangen, Germany). Reverse transcription was carried out using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) with random hexamers carried out according to the manufacturer's protocols. *MR1* 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.

Statistics

Statistical analyses were performed using a Mann-Whitney U test or Wilcoxon's test as appropriate with Bonferroni correction (GraphPad Prism v6.0, GraphPad Software Inc., San Diego, USA). Data are expressed as medians with interquartile ranges (IQR) unless stated otherwise. For clinical characteristics proportions were compared using Fishers exact text where appropriate. Results were considered significant if $p<0.05$. We prospectively estimated 65% power to detect a 70% difference in log-transformed biopsy MAIT cell frequencies with a sample size of 8 / group based on our previous findings in asthma(20).

For additional information on methods see online data supplement.

Results

Participant demographics

The clinical characteristics of participants in the bronchoscopy study are presented in Table 1. Participants with COPD were all ex-smokers and were predominantly GOLD stage 2 with a median FEV_1 of 60 and 61% predicted in steroid-naïve and steroid-treated groups respectively. The steroid-treated group were receiving a median ICS dose of 2000 µg BDP equivalent / day as budesonide (n=4) or fluticasone (n=7). The median age of participants with COPD was 66 years (steroid-naïve) and 69 years (steroid-treated) although healthy controls were significantly younger with a median age of 28 years. Other clinical and demographic characteristics did not differ significantly between steroid-naïve and steroid-treated groups except for the proportion using long acting β agonists (25% v 100% respectively, P=0.001). We observed no significant effect of age on MAIT cell frequencies (See online supplement Figure E1).

MAIT cell frequencies in health and COPD

MAIT cell frequencies were reduced in peripheral blood in COPD amongst participants receiving ICS (median 0.38% (IQR, 0.25-0.96) compared with health (1.8% (IQR, 1.4-2.5), P=0.001)) and compared with participants with COPD not taking ICS (1.8% (1.2-2.3), P=0.04 - Figure 1). MAIT frequencies were also significantly reduced in bronchial biopsies in participants with COPD taking ICS (0.73% (0.46-1.3)) compared with health (4.0% (1.6-5.0), P=0.02), but did not

differ significantly in sputum or bronchoalveolar lavage. No significant differences were observed with conventional T cell frequencies (Supplementary Figure E2).

NTHi stimulates MR1 expression on macrophages

To investigate the potential effects of this MAIT cell deficiency, we interrogated the interactions of MAIT cells and macrophages when stimulated with NTHi. Surface expression of the MAIT cell restriction molecule MR1 and of class II MHC were up-regulated on human pulmonary macrophages by co-culture with live NTHi at MOI1 and MOI10 ($P<0.05$ for all comparisons - Figure 2A & B). Likewise on MDM derived from the blood of healthy volunteers, expression of MR1 and class II MHC molecules were up-regulated by live NTHi at MOI1 ($P=0.002$ and $P=0.007$ respectively - Figure 2C). MHC class I was not affected by NTHi infection (Figure 2 B & C). Paraformaldehyde (PFA)-fixed NTHi induced up-regulation of MR1 ($P=0.02$) but significantly less so than live bacteria ($P=0.05$) and did not induce class II MHC.

To investigate whether the MR1 upregulation was mediated non-specifically by autologous cytokine release or TLR activation we measured surface expression of MR1 on MDM in the presence of pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ), lipopolysaccharide (LPS) or live influenza A (X31), but observed no significant changes in MR1 expression under any condition (Figure 3A-C).

Moreover, although NTHi induced MR1 surface expression on MDM, there was no change in MR1 expression at the mRNA level in response to either live or

dead NTHi (Figure 3D), implying that surface up-regulation occurred at a post-translational level. To ensure that this lack of effect on MR1 gene expression was not due to unresponsive MDM, other genes were also measured in the same samples. HLA-DR gene expression was also unchanged, whereas TLR4 gene expression was decreased. In contrast, the expression of the intracellular pattern-recognition receptors, TLR7 and RIG-1, were found to be significantly upregulated in response to live NTHi (Figures E3).

In vitro modeling of MAIT responses to NTHi

Next we investigated whether NTHi-infected MDM could activate autologous T cell subsets from peripheral blood of healthy volunteers. Co-culture with NTHi-infected MDM induced secretion of IFN-γ from autologous CD4 cells and CD8 cells, but most potently from MAIT cells (median IFN-γ positive frequencies 2.9%, 8.6% and 27.6% respectively, $P<0.0001$ for all comparisons versus unstimulated controls - Figure 4). This effect was most marked with live bacteria, although significant IFN-γ induction was also induced by PFA-fixed NTHi ($P<0.01$ for all comparisons v unstimulated controls). There was a significant correlation between the responsiveness of CD4 and CD8 T cells (r 0.622, $p=0.006$) and CD8 and MAIT (r 0.695, $p=0.002$) from the same donors but not between CD4 and MAIT (r 0.247, $p=0.2$).

IL-17A production was also measured by flow cytometry. No significant increase in MAIT IL-17A expression was observed following co-culture with autologous NTHi-infected-MDM (data not shown). Stimulation of MAIT cells with

PMA/Ionomycin gave only a minor increase of IL-17A (median 0.7% at baseline increasing to 1.2% after PMA/Ionomycin - data not shown).

MR1 is required for MAIT activation

To confirm that the MAIT cell response to NTHi-infected MDM was driven by MR1, co-cultures of NTHi-infected MDM with autologous T cell were repeated in the presence of an MR1 blocking antibody. Previously, Ussher *et al* showed a time-dependant difference in the effect of MR1 blocking so we opted to follow a similar protocol(39). When NTHi-infected MDM were co-cultured with T cells and anti-MR1 for 5 h, IFN-γ production by MAIT cells was significantly inhibited (P=0.03 - Figure 5). No IFN-γ production was observed in the conventional CD4 or CD8 subsets at this time point so blocking with anti-HLA-DR and anti-HLA-ABC respectively was not attempted (data not shown). However, when T cell were co-cultured with NTHi-infected MDM for 22 h, blocking of CD4 and CD8 cells by anti-HLA-DR and anti-HLA-ABC respectively had a significant effect (P=0.004 - supplementary figure E4). Blocking of MR1 at 22 h did not significantly affect IFN-γ production by CD4 or CD8 cells (data not shown)

Effect of steroids on NTHi-induced MAIT activation

Finally we investigated the effect of corticosteroids on healthy volunteer blood MAIT cell activation. The presence of 100 nM fluticasone propionate or 200 nM budesonide significantly impaired surface upregulation of MR1 on NTHi-infected MDM, causing a 2-fold fall in SMFI (Figure 6C & D). Moreover, both steroids

caused a significant decrease in NTHi-induced IFN-γ expression from autologous MAIT cells (Figure 6A & B).

Discussion

We describe a deficiency of TCR-Vα7.2+CD161+ MAIT cells in blood and bronchial tissue in steroid-treated COPD. Furthermore we show for the first time that live NTHi infection can induce surface expression of the MAIT cell restriction molecule MR1 on pulmonary macrophages and a potent IFN-γ response from MAIT cells: providing strong evidence that that *H. influenzae* is a target of MAIT cell immunity. Significantly both NTHi-induced surface expression of MR1 and IFN-γ responses were significantly impaired by the presence of steroids. Thus ICS may impair both the frequency and the function of MAIT cells. The consequences of this in the airways may be complex. ICS improve symptoms and reduce the incidence of exacerbations in COPD(12-15), which is likely due to their efficacy in decreasing the inflammatory response to bacteria and viruses(6). Steroid suppression of inflammatory responses is also likely to explain the reduction in time to clinical stability achieved with oral prednisolone in community-acquired pneumonia(40). Given their abundance(20) and pro-inflammatory phenotype(23), MAIT cells are likely to be significant contributors to immune pathology in both these situations.

To our knowledge this is the first description of MAIT cells and their deficiency in the COPD lung. We have previously described a similar deficiency in blood, sputum and bronchial biopsies in severe, steroid-treated, but not mild, steroid-naïve asthma(20, 21). In that study we observed a strong relationship between ICS dose and MAIT cell frequencies in peripheral blood and induced sputum(20).

Furthermore in two open-label studies with inhaled and oral corticosteroids we found a specific decrease in peripheral blood MAIT cell frequencies after a short course of oral corticosteroids, but not of low dose ICS(20). Our findings extend the recent report of reduced MAIT cells in peripheral blood in COPD(41). The effect of steroid use was not examined in that study, but 76% of their participants were receiving ICS and 20% oral corticosteroids. Furthermore MAIT cell deficiency was observed in GOLD stages 2-4, but not GOLD stage 1; the group likely to have least steroid exposure(41). Our data suggest the explanation for these findings is that chronic use of higher doses of inhaled corticosteroids can reduce MAIT cell frequency and inflammatory cytokine production in patients with COPD.

A deficiency of MAIT cells in bronchial biopsy tissue may arise from corticosteroids suppressing the local expansion of pulmonary MAIT cells which has been shown to occur in response to microbial or ligand-induced MAIT cell stimulation ((42) and unpublished observations). In addition, MAIT cells are likely to be sensitive to corticosteroid-induced apoptosis due to their high expression of caspase 3(43). Furthermore we have shown previously that MAIT cell frequencies in peripheral blood are very sensitive to serum concentrations of vitamin D_3: a structurally-related steroid. Given these properties of MAIT cells, and the significant systemic bioavailability of inhaled corticosteroids(44) the observed deficiencies of MAIT cells in peripheral blood might be expected. An alternative interpretation of our data is that corticosteroids may exert their effect

in vivo through effects on anatomic localisation. However we did not observe a significant deficiency of MAIT cells in lavage and sputum, and it is possible that this lack of effect may result from phenotypic differences between MAIT cells in tissue and those that migrate into the lumen where their survival may be reduced(43).

Although the nature of the ligands recognised by MAIT cells has been recently described as microbially-derived riboflavin intermediates – which trigger rapid secretion of effector cytokines from MAIT cells, but not conventional T cells(21, 23) – the function of MAIT cells in health and disease is still unknown. It has been shown that MAIT cells can be activated by and kill intestinal epithelial cells infected with the invasive bacteria *Shigella flexneri,* but MAIT cells are equally abundant in the human airways – average mucosal MAIT cell frequencies are 4.0% in healthy human bronchi(20) compared with 1.5-4.9% in human intestine(23, 45, 46) – and to date it is not known which respiratory pathogens induce a strong MAIT cell response. MAIT cells are unlikely to be important for immunity to *Streptococcus pneumonia* as they do not induce MAIT cell accumulation in murine *S. pneumoniae* infection (Zhenjun Chen, personal communication) and in critically ill patients with severe bacterial infections changes in peripheral MAIT cell frequency were observed only in non-streptococcal infections(47). However several factors implicate *H. influenzae* as a likely target of MAIT cell responses. *H. influenzae* expresses the riboflavin biosynthetic pathway from which the MAIT cell ligands are derived(30). The co-

stimulatory molecule CD161 is a key expression marker for MAIT cells, which can recognise the ligand lectin-like transcript 1 (LLT1) expressed by respiratory epithelial cells in response to infection and proinflammatory cytokines(48). *H. influenzae* can invade into lung tissue(49, 50) and enter respiratory epithelial cells and macrophages in chronic bronchitis(6-8, 51). Indeed exacerbations of chronic bronchitis are associated with an increase in the presence of NTHi bacteria inside epithelial cells from 33% to 87% of biopsies(6, 8). As *H. influenzae* is a facultative anaerobe this may allow the bacterium to survive and persist intracellularly and evade humoral immunity(6, 7). T cell immunity would be essential for eradication of such bacilli; indeed the importance of T cells is underlined by the association of HIV with an increased risk of pneumonia, bronchiectasis and acute lower respiratory tract infections, particularly in children (6), in which an important causative pathogen is *H. influenzae*(5). Furthermore, we have recently described a defect in CD8+ T cell immunity in COPD(52) and an additional MAIT deficiency could compound this impaired cellular immune response. Severe or recurrent infections with *H. influenzae* are also a feature of XLP-2, a rare genetic immunodeficiency with pleiotropic effects including a 10-fold decrease in MAIT cell frequencies(53), although hypogammaglobulinaemia may also be a contributing factor.

Despite the potentially beneficial anti-inflammatory effects, the suppression of MAIT cell responses we describe is likely to occur at a cost. Whilst MAIT cells may not be required for defence against mono-microbial infections with *S.*

pneumoniae a deficiency of MAIT cells in non-streptococcal bacterial sepsis has been described in the intensive care setting, and is associated with increased acquisition of nosocomial infections(47). Thus our findings may begin to provide an explanation for the increased risk of pneumonia associated with ICS use in COPD(13-18). In addition, whilst NTHi is a commensal of the upper respiratory tract, it is not present as a persistent, distinct microbial community in the lower respiratory tract in health. It is not known by what mechanisms it establishes a niche in the lower respiratory tract, but once colonization is established NTHi can become pathogenic itself, or contribute to the pathology of other bacteria(10), or of viral infections which lead to increased bacterial biomass(54) and disrupt epithelial tight junctions allowing bacterial invasion(55). It is possible that steroid-suppression of MAIT cells could also contribute to this initial colonization step in COPD and other airways diseases.

Glucocorticoids have long been known to inhibit T cell proliferation and induce apoptosis(56), as well as inhibiting cytokine expression. NF-κB is involved in the expression of IFN-γ(57) and fluticasone reduces activation of this transcription factor(58). The reduction of MAIT cell frequency in response to steroids in COPD patients was not recapitulated *in vitro* (data not shown) even though we observed reduced IFN-γ expression. Our *in vitro* investigations were limited to 24 h cultures and thus culture times may not have been long enough to observe an effect of steroids on either T cell proliferation or apoptosis. Regarding the action of steroids on the macrophages, our data suggest that MR1 may be

regulated by post-transcriptional mechanisms, similarly to a previous study demonstrating post-transcriptional inhibition of MHC I expression by dexamethasone(59).

There are direct clinical implications to our findings. We have demonstrated a dose-response relationship between MAIT cell frequencies and ICS (20, 21) that underlines the importance of titrating ICS dose carefully to the indication, avoiding injudicious use of highly potent ICS at high doses unless supported by empiric data of efficacy. Given the heterogeneous clinical spectrum of COPD(4) there should be a priority for careful clinical phenotyping and targeted use of inhaled or oral steroids only in subgroups likely to respond(60). The possibility of steroids facilitating initial lower airway colonization with NTHi should be further explored epidemiologically and in model systems.

Our study has several limitations: firstly that healthy control subjects were younger than those with COPD. MAIT cell frequencies may decline in the elderly(61), although we observed no age-related difference between healthy individuals in their 3rd and 6th decades (Figure E1, online supplement). Moreover, the two COPD cohorts were well matched to each other in age, but the MAIT cell deficiencies were observed only in the steroid-treated groups, implying this is related to steroids rather than age. Furthermore, even if increasing age were a contributory factor to MAIT cell decline in COPD, this would only serve to further emphasize the importance of minimizing additional iatrogenic suppression in

such individuals. A second limitation is that confounding by the indication for steroid treatment cannot be excluded due to the cross-sectional design. A third limitation is that, due to low cell numbers from bronchoscopy samples, it was not possible to investigate whether airway MAIT cells differed in responsiveness to NTHi infection from participants either receiving or not receiving ICS, but this could be pursued in further studies using resected tissues. The study is also not powered to detect whether a previously reported sex difference in MAIT frequency(62) may have contributed to the observed deficiency in COPD participants receiving ICS, and as the bronchoscopy data are exploratory in nature, findings will need further replication in a larger cohort.

In summary we have demonstrated a numerical and functional deficiency of MAIT cells in the airways in COPD that is related to therapeutic corticosteroids. We provided evidence of a role for this enigmatic new T cell subset in host defence against a leading respiratory pathogen. Our findings will have implications for a range of airways diseases in which acute and chronic NTHi infections contribute to immune pathology including severe asthma, cystic fibrosis, bronchiectasis and COPD.

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Figure Legends

Figure 1.

MAIT cells (Vα7.2+CD161+) as proportions of CD3+ T cells in (A) blood, (B) sputum, (C) bronchoalveolar lavage fluid, and (D) endobronchial biopsy specimens in healthy subjects and participants with COPD. Horizontal lines show medians. Mann-Whitney tests were performed on untransformed data with Bonferonni correction for multiple comparisons. * $P < 0.05$ and ** $P < 0.01$.

Figure 2.

A) Cytometric gating strategy. Lung macrophages were identified as positive for CD45-PE-CF594, HLA-DR-APC-Cy7 and Lin1-FITC. B) Specific mean fluorescence intensity (SMFI) of surface MR1, HLA-DR and HLA-ABC on macrophages from resected human lung either uninfected (NI) or infected with live NTHi at MOI 1 and MOI 10. *n*=6 or 4. C) SMFI of surface MR1, HLA-DR and HLA-ABC on MDM from peripheral blood of healthy volunteers either uninfected (NI), infected with live NTHi at MOI 1 or with PFA-NTHi at MOI 1. *n*=9, 10 and 7 respectively. Bars indicate medians. P values represent Wilcoxon signed-rank tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 3.

Specific median fluorescence intensity (SMFI) of MR1 on monocyte-derived macrophages (MDM) from peripheral blood either not treated (NT) or treated with

A) TNF-α, IL-6, IFN-γ (10ng/ml), *n*=6, B) LPS (100ng/ml), *n*=6 or C) infected with influenza virus X31, *n*=3. D) MR1 mRNA from MDM either uninfected (NI), infected with live NTHi at MOI 1 or with paraformaldehyde (PFA) fixed-NTHi at MOI 1. RNA expression is expressed as $\Delta\Delta C_t$ normalised to β_2M. Bars indicate medians. P values represent Wilcoxon signed-rank tests. * P < 0.05, ** P < 0.01, and *** P < 0.001.

Figure 4.

Representative cytometry plots and graphs showing percentage of peripheral, healthy blood CD4+ T cells, CD8+ T cells and Vα7.2+CD161+ MAIT cells expressing IFN-γ in response to monocyte-derived macrophages infected with live NTHi or paraformaldehyde (PFA) fixed-NTHi at MOI 10. Representative of at least 12 independent experiments. Bars indicate medians. P values represent Wilcoxon signed-rank tests. * P < 0.05, ** P < 0.01, and *** P < 0.001.

Figure 5

Blocking of MR1. Percentage of peripheral blood MAIT cells producing IFN-γ in response to autologous monocyte-derived macrophages (MDM) infected with NTHi at MOI 10, in the presence of 5 μg/ml anti-MR1 (clone 26.5) or IgG2a isotype control, *n*=5. Infected-MDM and autologous T cells were co-cultured for 5 h. MDM and MAIT cells were obtained from the blood of healthy volunteers. Bars indicate medians. P values represent Wilcoxon signed-rank tests. * P < 0.05.

Figure 6.

A&B) Percentage of MAIT cells producing IFN-γ in response to autologous monocyte-derived macrophages (MDM) infected with live NTHi at MOI 10, *n*=7 and 5 respectively. C&D) SMFI of MR1 on MDM infected with live NTHi at MOI 1, *n*= 12 and 5 respectively. MDM and MAIT cells were obtained from the blood of healthy volunteers. In both experiments, either DMSO, 100 nM fluticasone proprionate (A&C) or 200 nM budesonide (B&D) were present throughout the experiment. Bars indicate medians. P values represent Wilcoxon signed-rank tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 1: Demographic and clinical characteristics of the participants.

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF THE PARTICIPANTS

	Healthy control subjects	COPD not receiving ICS	COPD receiving ICS	p value COPD ICS v health	p value COPD ICS v COPD no ICS
n	21	8	11		
Demographics					
Sex (M/F)	13 / 8	3 / 5	9 / 2	0.07	0.4
Age (median [range], years)	28 (24-35)	66 (63-69)	69 (65-73)	**<.0001**	0.3
Pulmonary function					
FEV_1 (% predicted)	108 (105-112)	61 (55-64)	60 (45-65)	**<.0001**	0.4
FEV_1 reversibility (%)	2.9 (1.8-6.1)	11 (4.5-19)	10 (5-14)	**0.03**	1
FEV1/FVC ratio	83 (79-88)	58 (54-62)	49 (40-59)	**<.0001**	0.2
TLCO (%pred)	ND	74 (66-90)	73 (55-83)	-	0.7
VA (%Pred)	ND	93 (86-96)	85 (79-94)	-	0.2
KCO (%Pred)	ND	81 (74-94)	82 (69-112)	-	0.8
Exhaled nitric oxide (ppb, at 50 L/s)	16 (11-21)	31 (22-56)	24 (13-30)	0.2	0.09
Clinical					
Duration of COPD	N/A	4 (3-5)	5 (3.5-7)	-	0.8
GOLD stage	N/A	2 (2-2)	2 (2-3)	-	0.2
Frequent exacerbator phenotype (≥2/yr)	N/A	1 / 7	3 / 8	-	0.6
MRC dyspnoea score	0 (0-0)	2 (1.8-2)	1 (1-3)	**0.001**	0.8
Atopy (Skin test positive, Y/N)	0 / 21	2 / 6	5 / 6	**0.002**	0.6
No. of skin test allergens positive	0 (0-0)	2 (1-2)	4 (2-7)	-	0.06
Peripheral eosinophil count (10^9/L)	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.2 (0.1-0.3)	0.3	0.5
Body mass index (kg/m^2)	25.3 (23.3-28.1)	28.6 (25.7-32.2)	28.3 (28.0-31.9)	**0.01**	0.6
Smoking status	0 /4 / 17	0 / 8/ 0	0 /11 / 0		
Never smoker, no. (%)	17 (81)	0 (-)	0 (-)	-	-
Former smoker, no. (% [mean pack-years])	4 (19 [3.5])	8 (100 [36])	11 (100 [61])	**0.0001**	1
Current smoker, no. (% [mean pack-years])	0 (-)	0 (-)	0 (-)	-	-
Treatment					
Inhaled steroids	No	No	Yes	-	-
Dose (equivalent mcg BDP)	N/A	N/A	2000 (800-2000)	-	-
Maintenance oral corticosteroids (Y/N)	No	No	No	-	-
Short-acting b-agonist (Y/N), no. (%)	No	5 (62)/ 3 (38)	2 (18)/ 9 (82)	-	0.07
Long acting β agonist (Y/N), no. (%)	No	2 (25)/ 6 (75)	11 (100)/ 0 (0)	-	**0.001**
Long acting muscarinic agonist (Y/N), no. (%	No	1 (13)/ 7 (87)	7 (64)/ 4 (36)	-	0.06
Relevant comorbidities (n, %)					
Hypertension	1 (5)	1 (13)	3 (27)	-	-
Cardiac disease	0 (0)	2 (25)	3 (27)	-	-
Vascular disease	0 (0)	0 (0)	3 (27)	-	-
Inflammatory bowel disease	0 (0)	1 (13)	1 (9)	-	-
Other (n=1 each)	Eczema	Hepatitis B	Spinal muscular atrophy, pernicious anaemia, pleural plaques,	-	-

Values are medians with interquartile ranges, unless stated otherwise. Percentages are of those with valid data. BDP, beclometasone dipropionate; COPD, chronic obstructive pulmonary disease; FEV1, pre-bronchodilator forced expiratory volume in 1 second; FVC, forced vital capacity; GOLD, Global initiative for chronic Obstructive Lung Disease; KCO, carbon monoxide transfer coefficient; mcg, micrograms; ppb, parts per billion; TLCO, transfer factor of the lung for carbon monoxide; VA, alveolar volume.

Figures

Figure 1

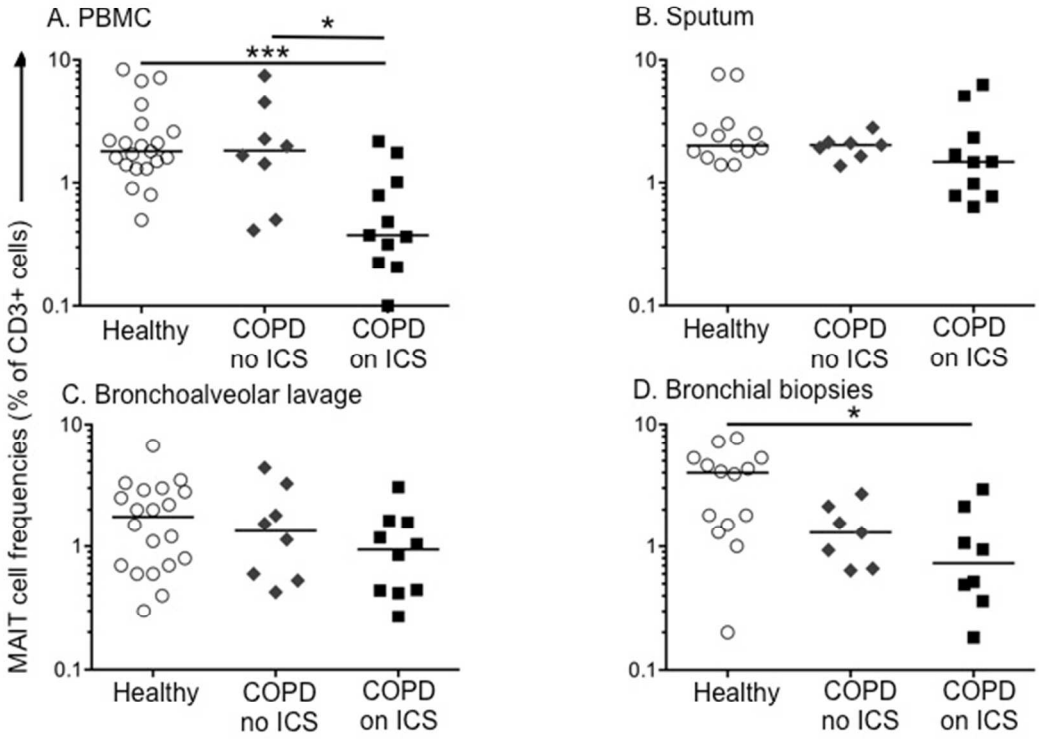


Figure 2

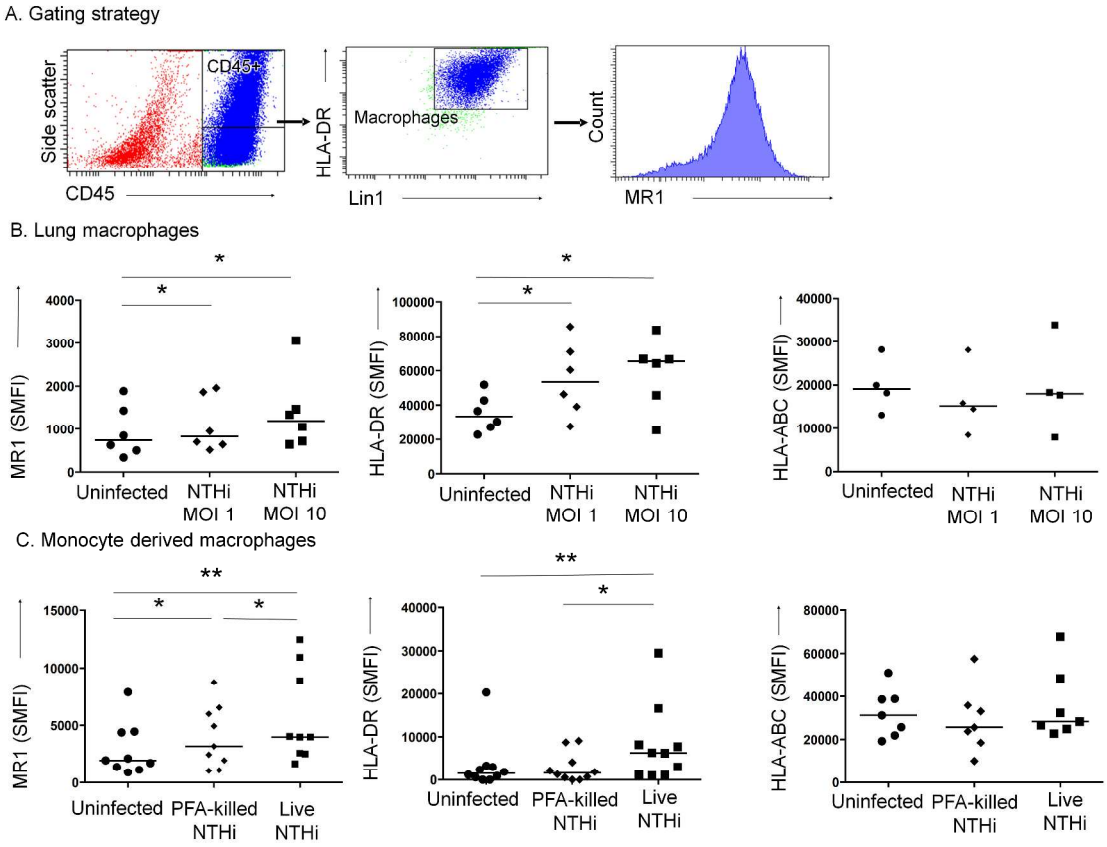

A. Gating strategy
Side scatter
CD45+
CD45
HLA-DR
Macrophages
Lin1
Count
MR1
B. Lung macrophages
MR1 (SMFI)
HLA-DR (SMFI)
HLA-ABC (SMFI)
Uninfected
NTHi MOI 1
NTHi MOI 10
C. Monocyte derived macrophages
MR1 (SMFI)
HLA-DR (SMFI)
HLA-ABC (SMFI)
Uninfected
PFA-killed NTHi
Live NTHi

Figure 3

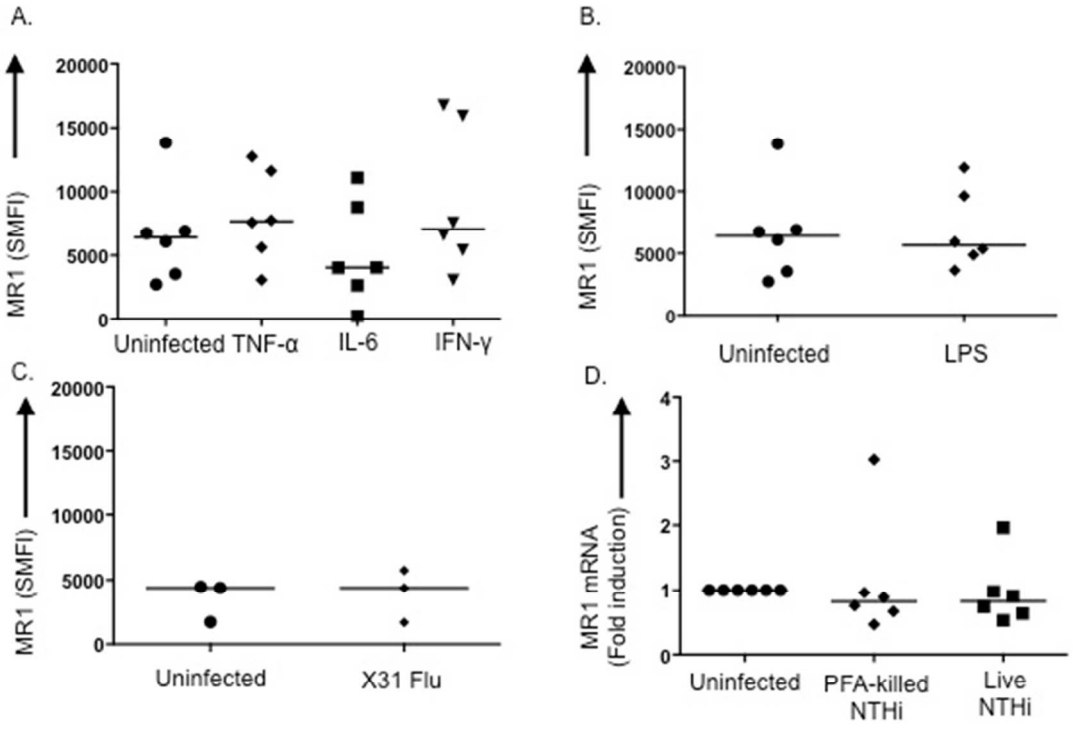

A.
MR1 (SMFI)
20000
15000
10000
5000
0
Uninfected
TNF-α
IL-6
IFN-γ
B.
MR1 (SMFI)
20000
15000
10000
5000
0
Uninfected
LPS
C.
MR1 (SMFI)
20000
15000
10000
5000
0
Uninfected
X31 Flu
D.
MR1 mRNA
(Fold induction)
4
3
2
1
0
Uninfected
PFA-killed
NTHi
Live
NTHi

Figure 4

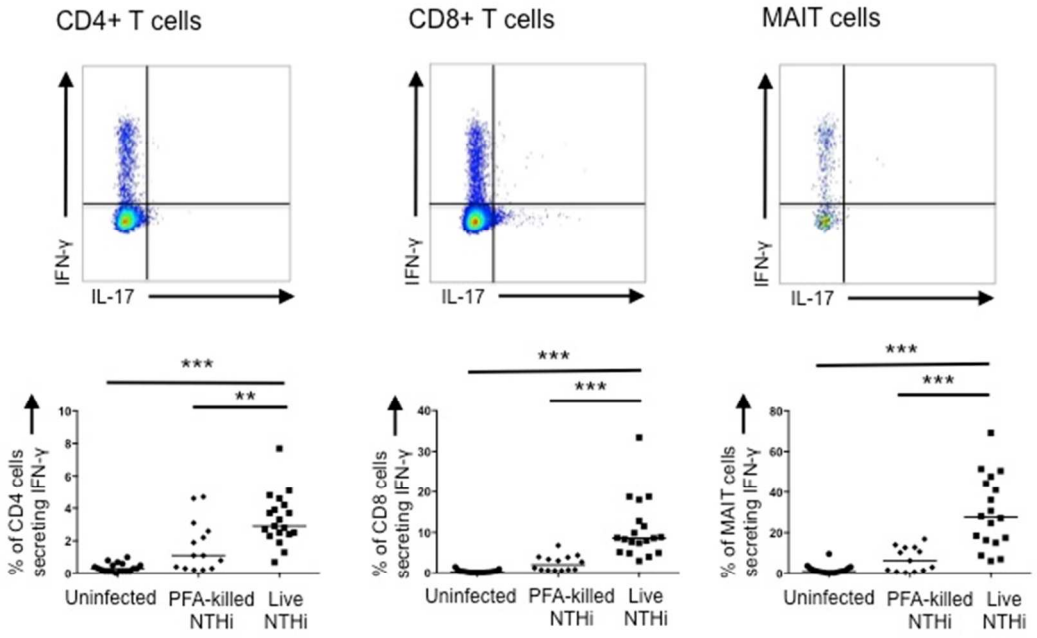

CD4+ T cells
CD8+ T cells
MAIT cells
IFN-γ
IL-17
% of CD4 cells secreting IFN-γ
% of CD8 cells secreting IFN-γ
% of MAIT cells secreting IFN-γ
***
**
Uninfected
PFA-killed NTHi
Live NTHi

Figure 5

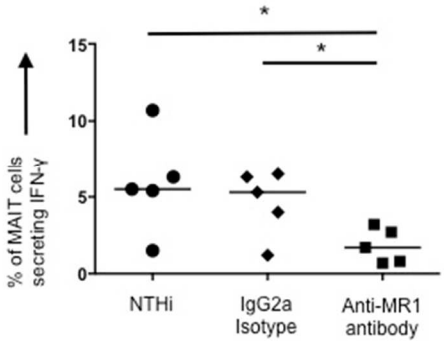
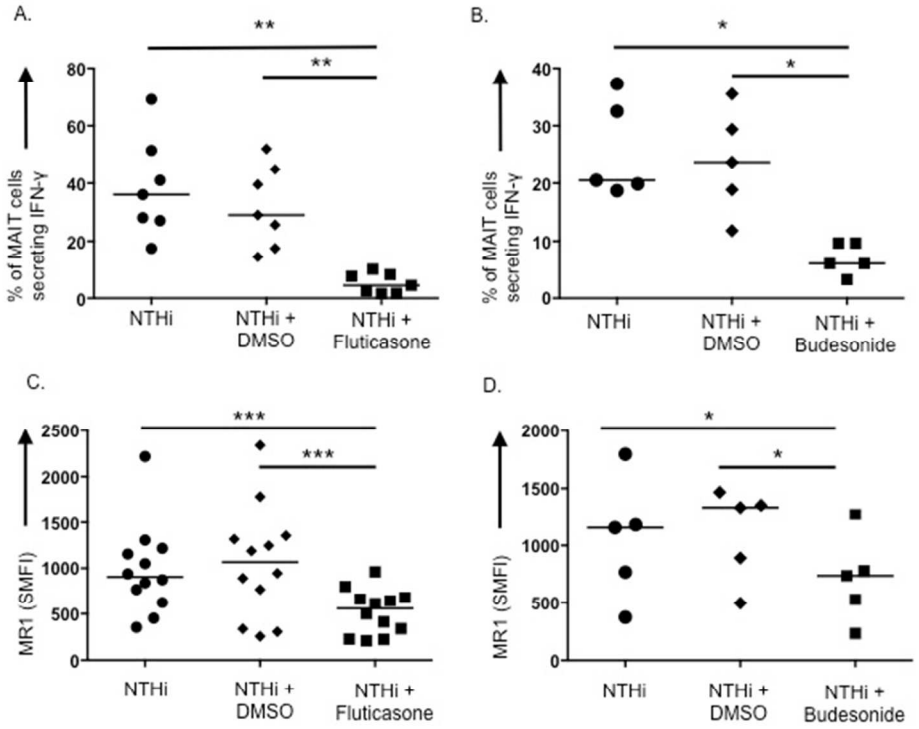

*
*
15
10
5
0
% of MAIT cells secreting IFN-γ
NTHi
IgG2a Isotype
Anti-MR1 antibody

Figure 6


A.
**
**
% of MAIT cells secreting IFN-γ
80
60
40
20
0
NTHi
NTHi + DMSO
NTHi + Fluticasone
B.
*
*
% of MAIT cells secreting IFN-γ
40
30
20
10
0
NTHi
NTHi + DMSO
NTHi + Budesonide
C.
***
***
MR1 (SMFI)
2500
2000
1500
1000
500
0
NTHi
NTHi + DMSO
NTHi + Fluticasone
D.
*
*
MR1 (SMFI)
2000
1500
1000
500
0
NTHi
NTHi + DMSO
NTHi + Budesonide

Steroid-induced deficiency of Mucosal-Associated Invariant T cells in the COPD lung: implications for NTHi infection

Timothy S.C. Hinks[1,2,3*], Joshua C. Wallington[1,4*], Anthony P. Williams[4,5], Ratko Djukanović[1,2], Karl J. Staples[1,5†], Tom M.A. Wilkinson[1,2,5†].

*, † These authors contributed equally to this work.

Online data supplement

Additional Methods

Sputum and bronchoalveolar lavage cytospin analysis

Cytospins for differential cell counts using the rapid Romanowsky stain were performed on all sputum and bronchoalveolar lavage samples: samples with high (>30%) squamous cell contamination or blood contamination were excluded.

Monocyte isolation and maturation into macrophages

Monocytes were cultured in 2ng/ml GM-CSF over 12 d to differentiate into monocytes-derived macrophages (MDM) and $CD3^+$ cells were frozen at -80°C in 10% dimethyl sulfoxide (DMSO) in foetal calf serum (FCS) until needed(1, 2).

Isolation of pulmonary macrophages

Lung tissue obtained from patients (see Table E1) undergoing lung resection was taken from sites distal to any tumours present, as judged by the surgical and pathology team involved. Tissue was transported in phosphate buffered saline (PBS), mechanically disrupted using a scalpel into $1mm^3$ cubes, and rested in PBS for 1 h, then washed through 0.70 μm filters and centrifuged at 400 g for 5 min. Cell pellets were resuspended in 20 ml PBS, mononuclear cells were isolated by differential density centrifugation using Ficoll Paque-Plus (GE Healthcare) at 800 g, 20°C for 30 min and washed again in PBS before trypan blue cell viability estimation and 2 hour rest to sterilise tissue in Roswell Park Memorial institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 2 mg/ml L-glutamine, 0.05 U/ml penicillin, 50 μg/ml streptomycin and 0.5 μg/ml

amphotericin B (all from Invitrogen) at $5x10^5$ cells/well in 48-well culture plates. Media were then replaced with complete RPMI plus 10% FCS and cells rested overnight at 37°C, 5% CO_2(1, 2).

NTHi infection of Lung macrophages and MDM

Macrophages were washed in culture plates with phosphate buffered saline (PBS) followed by addition of either live or fixed NTHi at MOI1 or MOI10 resuspended in 0.1% FCS-RPMI without penicillin/streptomycin and incubated for 2 h at 37°C. Wells were then washed with basal RPMI, followed by addition of 0.1% FCS-RPMI with penicillin/streptomycin, incubated at 37°C for a further 22 h. Cells were then washed with PBS, before addition of 200 µl non-enzymatic cell-dissociation media incubated for 15 min, 37°C. Cells were then removed from wells and stained for flow cytometry.

For T cell activation assays 10^6 autologous $CD3^+$ cells were added per well to MDM after the 2 h infection, co-cultured for a further 22 h, with monensin (eBioscience, Hatfield, UK) for the final 5 h.

MDM stimulation with cytokines and LPS was carried out by adding either TNFα, IL-6 and IFNγ (10 ng/ml) or LPS (100 ng/ml) to culture wells and incubated for 24 h. To model the effects of steroids in vitro, MDM from healthy volunteers were infected with NTHi as above with the addition of 100 nM fluticasone propionate, 200 nM budesonide or DMSO. Steroid or DMSO was present for both the 2 h

infection of MDM, and re-added following washing for the 22h co-culture of NTHi-infected MDM and T cells. For MR1 blocking, the 5 h infected-MDM – T cell co-culture was carried out as above but with the addition of 5 µg/ml IgG2a isotype control or anti-MR1 (26.5, Biolgend, San Diego, USA) as previously described(3). For MHC blocking at 22 h, anti-HLADR (L243), anti-HLAABC (W6/32 - both Biolegend) were used at 10 µg/ml.

Flow Cytometry

For staining of macrophages, $5x10^5$ MDM were resuspended in 100 µl FACS buffer (2 mM EDTA, 0.5% BSA in PBS) with 2 mg/ml purified IgG from human serum (Sigma-Aldrich)per tube. MDM were then stained with the following antibodies αHLA-DR-APC-CY7 (BD-Bioscience), αMR1-APC (Biolegend,), αHLA-ABC-PE (eBioscience) or appropriate isotype controls. After incubation on ice for 30 min, cells were resuspended in 200 µl FACSfix (2% Paraformaldehyde in PBS), incubated on ice for 20 min, washed and then resuspended in 350 µl MACS buffer.

Staining of lung macrophages followed a similar protocol as above. Cells were instead stained with CD45-PE-CF594, HLA-DR-APC-CY7 and Lineage 1-FITC (all BD-Biosciences) and MR1-PE (Biolegend) or appropriate isotype controls. All staining was analysed by 9-colour FACSAria (BD)

For $CD3^+$ intracellular cytokine staining (ICS), 10^6 $CD3^+$ cells were resuspended in 100 µl FACS buffer with 2 mg/ml purified IgG from human serum (Sigma-Aldrich) per tube. Cells were then stained with the following antibodies; αCD3-

PE-CY7, αCD4-PERCP-CY5.5, αCD8-APC-CY7, αCD161-APC (BD-Bioscience) and αVα7.2-PE (Biolegend). After incubation on ice for 30mins, cells were washed, resuspended in 200 µl Cytofix/Cytoperm (BD Biosciences) and incubated for 20 min on ice before washing with 1 x Perm/Wash (BD Biosciences) and resuspending in 100 µl 1 x Perm/Wash buffer with αIFNγ-FITC or αIL17A-BV421 (Biolegend). After incubation on ice for 30 min, MDM were washed again resuspended in 350 µl FACS buffer. All staining was then analysed by 9-colour FACSAria (BD Biosciences).

Additional results

Effect of age on MAIT cell frequencies

Although our two COPD cohorts were matched for age, our healthy control subjects were younger. We investigated the possible impact of this on our key findings. Firstly it should be noted that as the two COPD cohorts were matched for age, an effect of age will not explain the discordant findings between COPD patients with and without steroids: a significant MAIT cell deficiency was noted only in those on steroid therapy. Secondly, we conducted a comparison of two groups of healthy individuals of differing ages: the first, younger group comprised the healthy control participants described in the main study, whilst the second, older group comprised an additional 12 healthy, non-atopic volunteers (5 male, 7 female) separately recruited via the Southampton Biomedical Research Unit specifically for this project. We found no difference in peripheral blood MAIT cell frequencies between individuals who were older (median age of 53 years, IQR 48-57, *n*=12) or younger (median age 27, IQR 24-34, *n*=15, P=0.4)(Figure E1).

Conventional T cell frequencies

Differences in MAIT cell frequencies in ICS-treated COPD were specific to the Vα7.2+CD161+ (MAIT) cell subset as they were not observed with Vα7.2+CD161- conventional T cells (Figure E2, P>0.2 for all comparisons).

Results of routine microbiological culture

Results of routine microbiological culture of the bronchoalveolar lavage were available in 15/19 COPD patients. Amongst COPD subjects on ICS *H. influenzae* was cultured in 2/9 samples, whilst 1 further sample produced a scanty growth of typical mixed upper respiratory tract flora with some gram positive cocci seen. Amongst patients not receiving ICS a scanty growth of mixed upper respiratory tract flora were cultured in 2/6 samples, and a scanty growth of coliforms cultured in 1/6 samples. No organisms were cultured in the remaining subjects, although it should be noted that microbiological culture is an insensitive method for analysing the complex microbiota within the COPD airways, in which 70% of species are not culturable (16), and which are known to have different microbial assemblages within different micro-anatomic regions of the same lung (17). Furthermore, a somewhat low *Haemophilus* carriage may be expected in our study due selection of patients with less severe disease considered safe to undergo research bronchoscopy.

N	6
Age (years)	71.5 (68.75 – 78.75)
Gender, M/F	3 / 3
Ex-smoker, N	6
Pack Years	35.00 (14.50 – 40.00)
FEV_1%	83.00 (60.25 – 93.00)

Supplementary Table E1: Clinical characteristics of lung resection patients. Data are presented as median and IQR. Ex-smokers were defined as individuals who had stopped smoking for > 6 months. Pack year data was only available from 5 patients.

Figure legends

Figure E1. Peripheral blood MAIT cell frequencies in younger and older healthy participants.

Peripheral blood frequencies of Vα 7.2+CD161+ MAIT cells did not differ between younger healthy participants (median age=27 years (IQR 24-34), *n*=15), older healthy participants (53 years (48-57), *n*=12, P=0.4). HC, healthy control.

Figure E2. Peripheral blood MAIT cell frequencies in younger and older healthy participants.

Conventional T cells (Vα7.2+CD161-) as proportions of CD3+ T cells in (A) blood, (B) sputum, (C) bronchoalveolar lavage fluid, and (D) endobronchial biopsy specimens in healthy subjects and participants with COPD. Horizontal lines show medians. Mann-Whitney tests were performed on untransformed data with Bonferonni correction for multiple comparisons. No differences were statistically significant.

Figure E3. MDM gene expression in response to NTHi

HLA-DR, RIG-I, TLR4 and TLR7 mRNA expression from healthy-blood derived MDM either uninfected (NI), infected with live NTHi at MOI 1 or with paraformaldehyde (PFA) fixed-NTHi at MOI 1. RNA expression is expressed as ΔΔCt normalised to β2M. Graphs show medians. P values represent Wilcoxon signed-rank tests. * $P < 0.05$

Fig E4. Blocking of CD4 and CD8 NTHi responses

Percentage of peripheral blood CD4 and CD8 T cells producing IFN-γ in response to autologous monocyte-derived macrophages (MDM) infected with NTHi at MOI 10, in the presence of 10 μg/ml anti-HLA-DR, anti-HLA-ABC or IgG2a isotype control, *n*=8. Infected-MDM and autologous T cells were co-cultured for 22 h. MDM and MAIT cells were obtained from the blood of healthy volunteers. Graphs show medians. P values represent Wilcoxon signed-rank tests. ** $P < 0.01$.

Fig E5. IFNγ MFI data for CD4, CD8 and MAIT cells

MFI of peripheral blood CD4+ T cells, CD8+ T cells and Vα7.2+CD161+ MAIT cells producing IFN-γ in response to monocyte-derived macrophages infected with live NTHi or paraformaldehyde (PFA) fixed-NTHi at MOI 10. *n*=18. Bars indicate median values. P values represent Wilcoxon signed-rank tests. **** $P < 0.0001$.

References

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2. Staples KJ, Nicholas B, McKendry RT, Spalluto CM, Wallington JC, Bragg CW, Robinson EC, Martin K, Djukanovic R, Wilkinson TM. Viral infection of human lung macrophages increases PDL1 expression via IFNbeta. *PLoS One* 2015; 10: e0121527.

3. Ussher JE, Bilton M, Attwod E, Shadwell J, Richardson R, de Lara C, Mettke E, Kurioka A, Hansen TH, Klenerman P, Willberg CB. CD161++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur J Immunol* 2014; 44: 195-203.

Figure E1

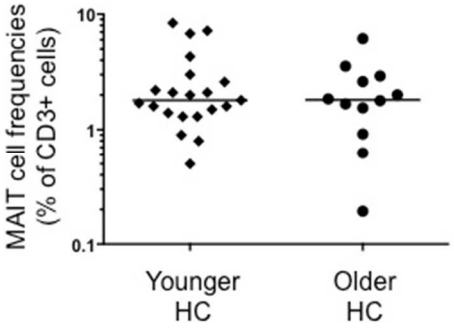

MAIT cell frequencies
(% of CD3+ cells)
10
1
0.1
Younger
HC
Older
HC

Figure E2

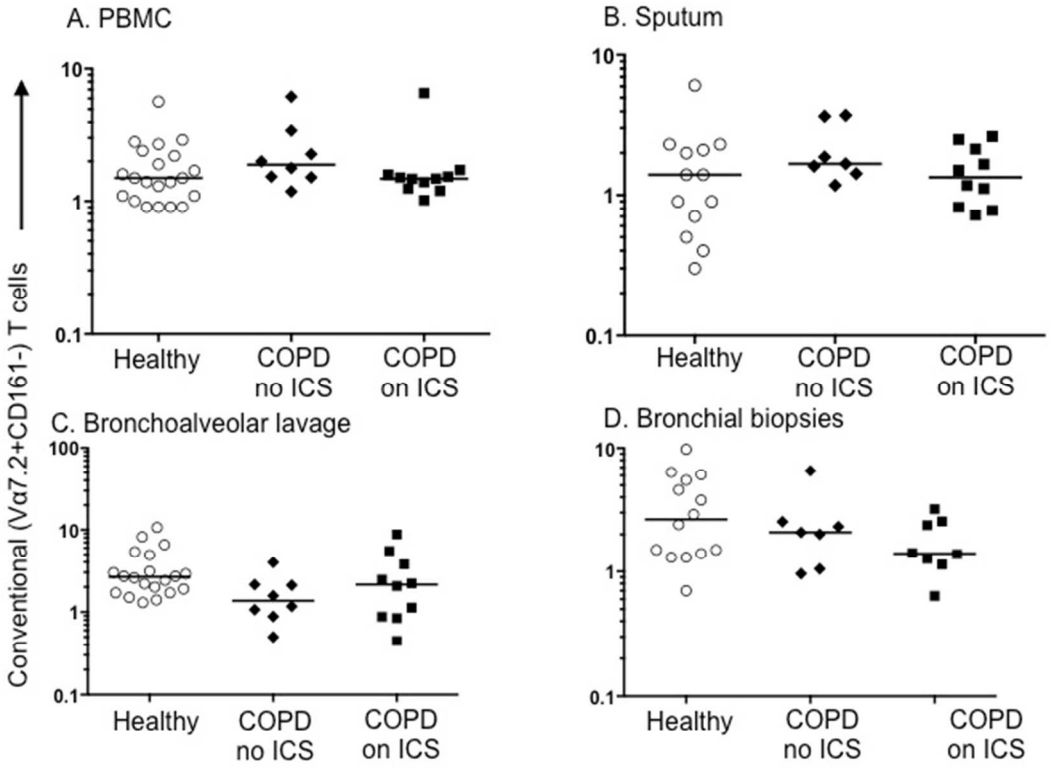

A. PBMC
B. Sputum
C. Bronchoalveolar lavage
D. Bronchial biopsies
Conventional (Vα7.2+CD161-) T cells
10
1
0.1
100
Healthy
COPD no ICS
COPD on ICS

Figure E3

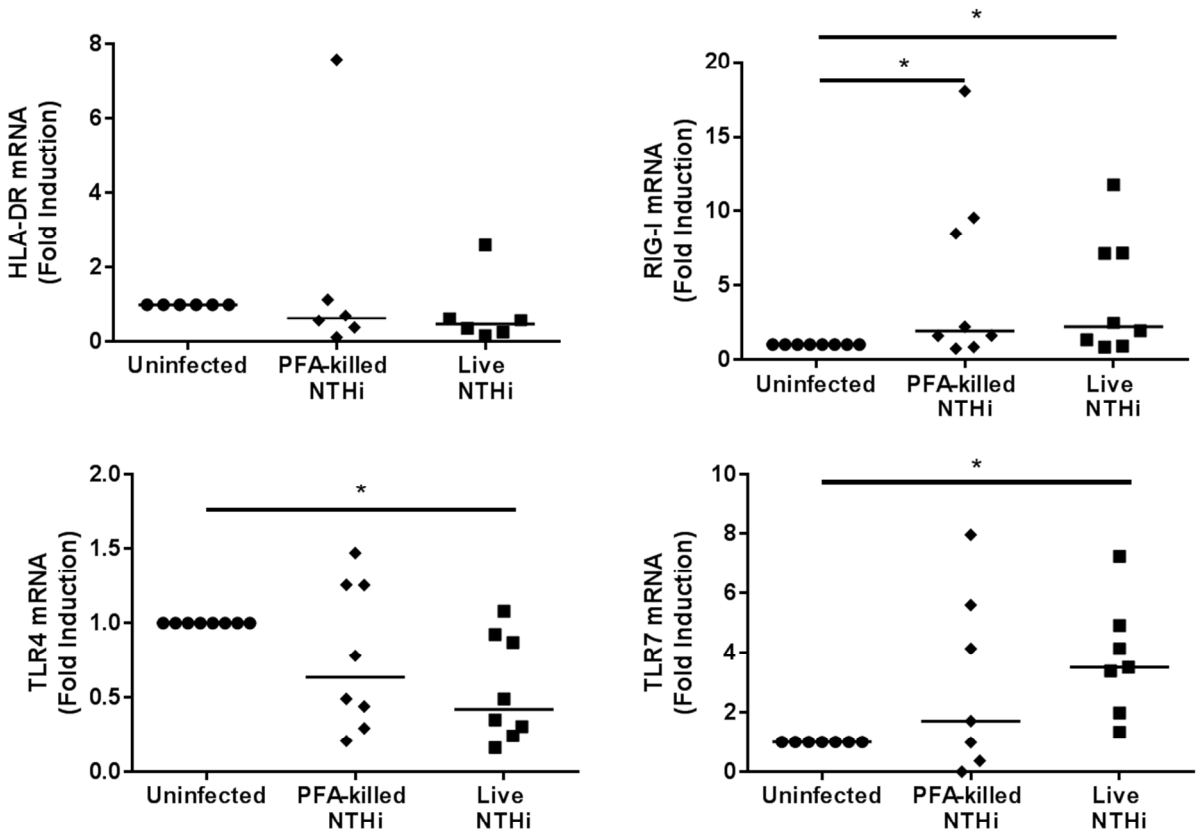

HLA-DR mRNA (Fold Induction)
8
6
4
2
0
Uninfected
PFA-killed NTHi
Live NTHi
RIG-I mRNA (Fold Induction)
20
15
10
5
0
*
*
Uninfected
PFA-killed NTHi
Live NTHi
TLR4 mRNA (Fold Induction)
2.0
1.5
1.0
0.5
0.0
*
Uninfected
PFA-killed NTHi
Live NTHi
TLR7 mRNA (Fold Induction)
10
8
6
4
2
0
*
Uninfected
PFA-killed NTHi
Live NTHi

Fig E4.

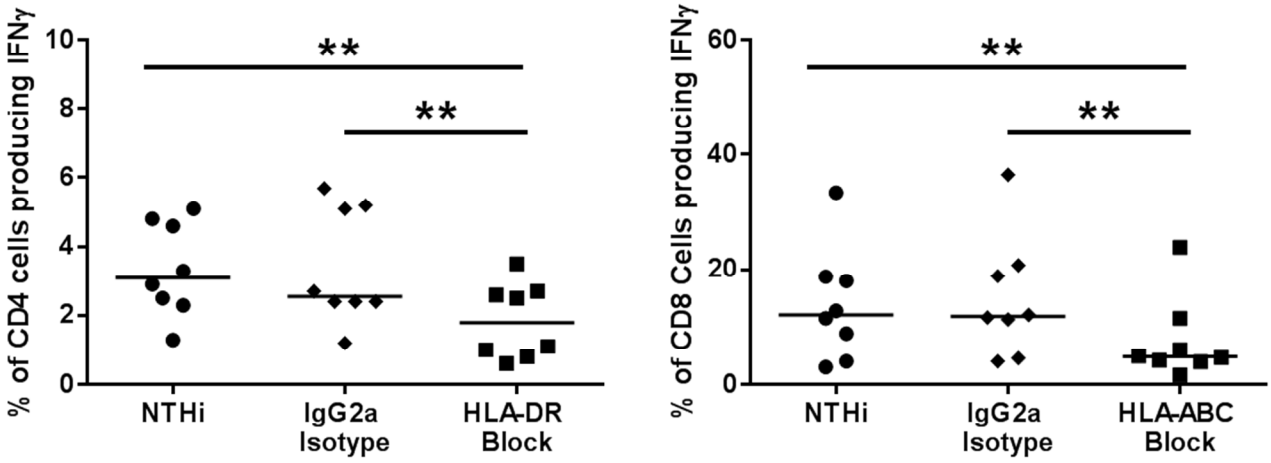
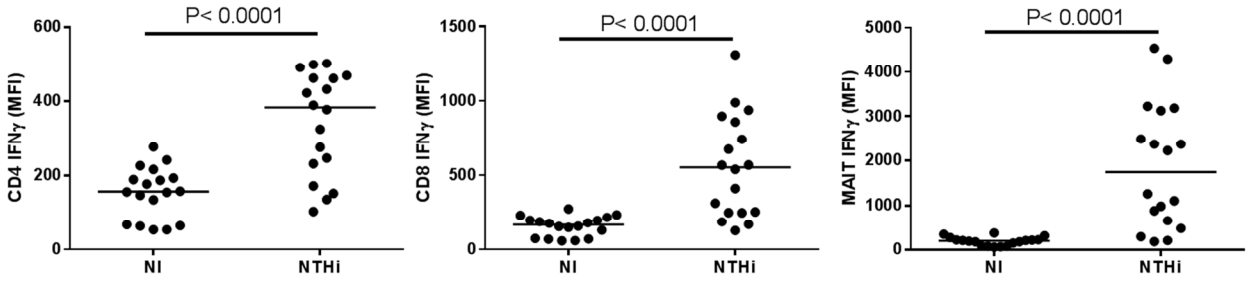

% of CD4 cells producing IFNγ
10
8
6
4
2
0
**
**
NTHi
IgG2a Isotype
HLA-DR Block
% of CD8 Cells producing IFNγ
60
40
20
0
**
**
NTHi
IgG2a Isotype
HLA-ABC Block

Fig E5


P< 0.0001
CD4 IFNγ (MFI)
600
400
200
0
NI
NTHi
P< 0.0001
CD8 IFNγ (MFI)
1500
1000
500
0
NI
NTHi
P< 0.0001
MAIT IFNγ (MFI)
5000
4000
3000
2000
1000
0
NI
NTHi