M1-like macrophages are potent producers of anti-viral interferons and M1-associated marker-positive lung macrophages are decreased during rhinovirus-induced asthma exacerbations

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\textbf{ARTICLE INFO}

\textbf{A B S T R A C T}

\textbf{Background:} Macrophages (M\textsubscript{φ}) can be M1/M2 polarized by Th1/2 signals, respectively. M2-like M\textsubscript{φ} are thought to be important in asthma pathogenesis, and M1-like in anti-infective immunity, however their roles in virus-induced asthma exacerbations are unknown. Our objectives were (i) to assess polarised M\textsubscript{φ} phenotype responses to rhinovirus (RV) infection in \textit{vitro} and (ii) to assess M\textsubscript{φ} phenotypes in healthy subjects and people with asthma before and during experimental RV infection in \textit{vivo}.

\textbf{Methods:} We investigated characteristics of polarized/unpolarized human monocyte-derived M\textsubscript{φ} (MDM, from 3–6 independent donors) in \textit{vitro} and evaluated frequencies of M1/M2-like bronchoalveolar lavage (BAL) M\textsubscript{φ} in experimental RV-induced asthma exacerbation in 7 healthy controls and 17 (at baseline) and 18 (at day 4 post infection) people with asthma.

\textbf{Findings:} We observed in vitro: M1-like but not M2-like or unpolarized MDM are potent producers of type I and III interferons in response to RV infection (P<0.0001), and M1-like are more resistant to RV infection (P<0.05); compared to M1-like, M2-like MDM constitutively produced higher levels of CCL22/MDC (P = 0.007) and CCL17/TARC (P<0.0001); RV-infected M1-like MDM were characterized as CD14\textsuperscript+CD80\textsuperscript+CD197\textsuperscript+ (P = 0.002 vs M2-like, P<0.0001 vs unpolarized MDM). In vivo we found reduced percentages of M1-like CD14\textsuperscript+CD80\textsuperscript+CD197\textsuperscript+ BAL M\textsubscript{φ} in asthma during experimental RV16 infection compared to baseline (P = 0.024).

\textbf{Interpretation:} Human M1-like BAL M\textsubscript{φ} are likely important contributors to anti-viral immunity and their numbers are reduced in patients with allergic asthma during RV-induced asthma exacerbations. This mechanism may be one explanation why RV-triggered clinical and pathologic outcomes are more severe in allergic patients than in healthy subjects.

\textbf{Funding:} ERC FP7 Advanced grant 233015, MRC Centre Grant G1000758, Asthma UK grant 08–048, NIHR Biomedical Research Centre funding scheme, NIHR BRC Centre grant P26095, the Predicta FP7 Collaborative Project grant 260895, RSF grant 19-15-00272, Megagrant No 14.W03.31.0024.© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license. (http://creativecommons.org/licenses/by-nc-nd/4.0/)

\section{1. Introduction}

Allergic asthma is characterized by chronic airway inflammation polarized towards the type 2 cytokines interleukin (IL)-4, –5 and –13 \cite{1}. The major morbidity in asthma relates to asthma exacerbations, the great majority caused by respiratory viral infections,
Evidence before this study

Rhinovirus (RV) infection is one of the most common causes of asthma exacerbations. Multiple reports demonstrate impaired anti-viral immune responses to RV infection in asthma. Mechanisms involved are poorly understood. Macrophages (Mφ) are abundant cells in the airways. A current paradigm is that type 1 and type 2 cytokines polarize alveolar Mφ into an M1 (classically activated) and M2 (alternatively activated)-like phenotype, respectively. M2-like Mφ are believed to be important in asthma pathogenesis, and M1-like in anti-infective immunity, however human data are sparse and their roles in asthma exacerbations are unknown.

Added value of this study

In our study we show that polarization of human monocYTE-derived macrophages (MDM) in vitro results in characteristic patterns of cytokine secretion, with robust type I and type III IFN production being characteristic of M1-like, while M2-like MDM had markedly reduced IFN induction and augmented type 2 chemokine production. Moreover, we found that M1-like human MDM expressed higher levels of CD14, CD54, CD80 and CD197 compared with M2-like, thus we identify M1-like as CD14+CD80+CD197+ cells. We found no significant difference in expression of the widely used M2 markers CD206 and HLA-DR on the surface of human M2-like MDM in vitro by IL-4. In vivo we found decreased percentages of CD14+CD80+CD197+ M1-like BAL Mφ in asthma patients during experimental RV infection.

Implications of all the available evidence

Our present findings suggest a novel mechanism behind virus-induced asthma exacerbations because reductions in the M1-like Mφ subpopulation during RV infection may be involved in impaired anti-RV immune responses in atopic asthmatics.

2. Materials and methods

2.1. Viral stocks

RV16 and RV1B serotypes were grown in Ohio HeLa cells and prepared as previously described [24]. Viral stocks were used at 6.3 × 10^6 TCID_{50}/mL and 1.5 × 10^7 for RV16 and RV1B, respectively. Viruses were titrated on Ohio HeLa cells to determine TCID_{50}/mL.

2.2. Isolation of human monocytes, in vitro differentiation into MDM and infection with RV

PBMCs were isolated from the Component Donation Leucocyte cones of healthy donors [25] by Ficoll-Hypaque density gradient centrifugation. The cells were washed, resuspended in Macrophage Serum Free media (MSFM, Invitrogen) and seeded into either 6-wells plates (NUNC) at 3 × 10^6 cells/well or PrimariaTM Tissue Culture Dishes (Falcon®) at 30 × 10^6 cells/dish. Non-adherent cells were removed after 2 h of incubation at 37 °C in a humidified atmosphere containing 5% CO₂. An equal volume of fresh MSFM containing 10 ng/mL of human granulocyte-macrophage colony stimulating factor (GM-CSF, Invitrogen) and penicillin/streptomycin mixture (Invitrogen) were then added to the cells. The cells were differentiated for 7 days. Media was replaced on day 3 and day 6. The mature MDM were then stimulated overnight with either 2 ng/mL of tumour necrosis factor (TNF) plus 20 ng/mL of IFN-γ (both from R&D Systems) or with 20 ng/mL of IL-4 (Invitrogen) to obtain MDM/IFN/IL4 and MDM/IL4 cells. Un-polarized control MDM were maintained in culture overnight in MSFM alone. Polarized or un-polarized MDM were then treated with live or UV-inactivated RV16 or RV1B MOI of 0.1, 0.5, 1, 2 for 1 h at room temperature. Cells were washed and any non-adherent virus was removed and re-suspended in the fresh media. Cell supernatants and RNA lysates were harvested at times indicated and stored at −80 °C (the 0 h time point means the time immediately after the 1 h incubation with RV, washing of non-adherent virus and adding fresh media).

2.3. Experimental RV-induced asthma exacerbation study - subjects and clinical assessments

The samples used for these analyses came from a previously reported study focusing on type-2 responses during rhinovirus induced asthma exacerbations. As part of this study, RV16 experimental infections were successfully induced in RV16 neutralizing M2-like and MDM for unpolarized cells, and we will specify the surface markers used for in vivo characterization. Identification of M1- and M2-like Mφ relies on a combination of membrane receptors, cytokines, chemokines and effector mediators [16,20], based mostly on in vitro or animal studies. The roles of the different phenotypes of Mφ in vivo in man is poorly understood as human studies are sparse, and in asthma controversial, as frequencies of Mφ expressing the M2-like marker CD206 are reported increased in stable asthma in one study [21], but not in another [22]. The role of M1 and M2-like Mφ in virus-induced asthma exacerbations is completely unknown.

We therefore studied cytokine secretion properties and surface marker characteristics of in vitro MDM/TNF/IFNg and MDM/IL4 polarized human MDMs to investigate their properties in the absence of infection. As we have reported that human MDMs produce type I IFNs in response to RV infection, though the phenotype of these MDMs was not studied [23], we also investigated cytokine and IFN secretion properties and surface marker characteristics following in vitro RV infection of MDM/TNF/IFNg and MDM/IL4, as well as their anti-rhinoviral activities. To investigate the role of M1- and M2-like Mφ in stable asthma, and in virus-induced asthma exacerbations, we then evaluated bronchoalveolar lavage (BAL) Mφ and peripheral blood monocyte subpopulations before and during experimental RV-induced asthma exacerbations.
antibody seronegative subjects with moderate \((n = 17)\) and mild \((n = 11)\) atopic asthma and 11 non-atopic age-matched healthy control subjects as previously described \([7]\). Ethics approval 09/H0712/59 was obtained from the local research ethics committee and informed consent was obtained from all subjects. Successful experimental RV16 infection was confirmed by detection of RV16 viral RNA in BAL fluid or nasal lavage and/or seroconversion as previously reported \([7]\). Asthma was categorized as mild or moderate according to Global Initiative for Asthma (GINA) criteria \([26]\).

Daily lower respiratory symptom scores were calculated from diary card records of symptom scores (cough on waking; wheeze on waking; daytime cough; daytime wheeze; daytime chest tightness; daytime shortness of breath; nocturnal cough, wheeze or shortness of breath), graded 0–3 (absent, mild, moderate, severe) as previously reported \([7]\). Total lower respiratory scores were calculated by summing the daily scores for the 2-week post-infection period as previously reported \([7]\).

The same diary cards recorded home spirometry (Piko-1; nSpire Health, CO) on waking each morning and the maximum \( (%) \) fall from baseline for PEF and FEV1 for each subject during the 2 week post-infection period was calculated as previously reported \([7]\).

Of these successfully infected subjects, BAL cells were obtained for the present studies at baseline from 7 subjects with mild and 10 subjects with moderate asthma and from 7 healthy control subjects and at day 4 after infection with RV16 from 7, 11 and 7 subjects respectively. Additionally, PBMCs were obtained at baseline from 8, 13 and 7 subjects, and at day 4 after infection from 8, 13 and 10 subjects respectively. These BAL cells and PBMCs were analysed by flow cytometry as described below.

2.4. Flow cytometry analysis

PBMC and BAL cells from clinical samples and in vitro polarized MDM were used for flow cytometry analysis. Adherent, in vitro polarized MDM were detached by incubation for 20 min with 1:1 mixture of 10 mM EDTA in PBS and RPMI 1640 media (PAA) at 37 °C. Cells were incubated with “The LIVE/DEAD® Fixable Dead Cell Stain Kit” (Invitrogen) to exclude dead cells from the analysis. Nonspecific binding of antibodies (Abs) was eliminated by pre-incubating the cells in medium containing 10% normal human serum for 15 min at 4 °C. Cells were stained in a solution containing 10% normal human serum for 15 min at 4 °C. We used the following Abs for our surface staining: anti-CD14-PacificBlue™ (BD Bioscien Cat# 557682, RRID:AB_396765); anti-CD36-FITC (BD Biosciences Cat# 557648, RRID:AB_10374200); anti-CD206-APC/Cy7 (BioLegend Cat# 251120, RRID:AB_2144930); anti-CD197-PE-Cy7™ (BioLegend Cat# 557648, RRID:AB_396765); anti-CD36-FITC (BD Biosciences Cat# 561820, RRID:AB_10896285); anti-CD80-PE (BD Biosciences Cat# 557227, RRID:AB_396606); anti-CD54-APC (BD Biosciences Cat# 559771, RRID:AB_398667). After incubation with Abs cells were washed and fixed with 2% paraformaldehyde. Flow cytometry was performed using a BD LSR II instrument (BD Biosciences). Unstained cells and single fluorochrome stained BD™Comp beads CompensationParticles (BD Biosciences) were used to set up the machine. Compensation was set in BD FACs Diva Software (BD Biosciences). Fifty thousand MDM and BAL cells and thirty thousand PBMC were acquired compensated. The results were analysed with Flowjo software version 7.6.5 (Tree Star). Results are reported as the percentage of positive cells or as mean fluorescence intensity (MFI), the latter being used to describe the level of expression on a population of positive cells. The fluorescence-minus-one (FMO) controls were used for gating of positive cells. BAL Mφ and MDC were identified as CD206+ cells (Fig S1) \([27]\).

2.5. RNA extraction, reverse transcription and Taqman® real-time PCR

RNA was extracted from cells using the RNeasy method (RNeasy Mini Kit; Qiagen) following the manufacturer’s instructions, including DNase digestion (Dnase (Rnase free Dnase); Qiagen). cDNA was synthesized using Omniscript RT and components as directed by the manufacturer (Qiagen). Sequences, concentrations of primers and probes for the detection of human IFN-α,1/2,3 detecting IFN-α subtypes 1, 2 and 6 and 2, 5, 8, 10, 14, 17 and 21, IFNB, IL28A/B, IL29, 18S and conditions of RT-qPCR reaction were used as previously described \([28]\). Relative quantification of RT-qPCR was used to detect changes in expression of the interferon genes relative to a reference gene, the housekeeping human 18S gene. Quantitative PCR results for mRNA expression were presented as ΔCt values, calculated by the formula: ratio (reference/target)=2^[(Ct (reference)-Ct (target)]. The number of copies for viral RNA quantification was calculated using a standard curve generated by amplification of plasmid DNA.

2.6. Enzyme-linked immunosorubant assay to evaluate IFN-α, IFN-β, IFN-λ, CCL22/MDC, CCL17/TARC, CCL18/PARC and IL-10 levels in culture supernatants

IFN-α, IFN-β, IFN-λ3, CCL22/MDC, CCL17/TARC, CCL18/PARC and IL-10 proteins were quantified by enzyme-linked immunosorubant assay (ELISA) in supernatants collected and stored at –80 °C using commercially available paired antibodies and standards, following the manufacturer’s instructions using the IFN-α human VeriKine™ ELISA kit (PBL Assay Science Inc.) detecting all human IFN-α subtypes except IFN-αF encoded by gene IFNA21, human IFN-β ELISA kit (FUJIREBIO INC.), human IL-10 ELISA Ready-Set-Go! (ebioscience), human CCL22/MDC, human IL-29/IL-28B (IFN-λ3), human CCL17/TARC and human CCL18/PARC DuoSets ELISA kits (R&D Systems). The detection limits for described assays are 156 pg/mL for IFN-α; 2.5 IU/mL for IFN-β; 2 pg/mL for IL-10; 7.8 pg/mL for CCL22/MDC, CCL18/PARC and CCL17/TARC; 62.5 pg/mL for IFN-λ3.

2.7. Statistical analysis

The Shapiro-Wilk test for normality was performed. Data were presented as means ± s.e.m for parametric analyses and as medians ± interquartile range for nonparametric analyses, as indicated in the figure legends. Statistical analyses were performed using Prism 8 (GraphPad Software). For normally distributed data we performed ordinary one-way ANOVA or one-way repeated measures ANOVA with (if the ANOVA was significant) Tukey’s post hoc tests for multiple comparisons to determine differences between groups, or if not normally distributed the Kruskal–Wallis test followed by post hoc testing (if the Kruskal–Wallis was significant) using un-paired Mann-Whitney U tests was performed, as mentioned in the respective figure legends. The Spearman correlation test was used for Fig. 8. Data were accepted as significantly different when \( P<0.05\).

For in vitro studies, we compared differences between infected and uninfected MDM, MDM/TNF/IFNγ and MDM/IL4 within the group (infected versus uninfected) and between the groups (infected/uninfected MDM/TNF/IFNγ versus infected/uninfected MDM or MDM/IL4) at the indicated time points. For clinical study we compared differences between healthy subjects and asthma group as whole or with mild and moderate asthma subgroups at the indicated time points or within the group (at baseline versus day 4 post infection).

3. Results

3.1. MDM/TNF/IFNγ but not MDM/IL4 or MDM are potent producers of rhinovirus-induced type I and III IFNs and are more resistant to RV infection

In order to evaluate antiviral capacity of different Mφ phenotypes we measured type I and III IFN induction in response to RV infection. Gene expression of IFN-α, IFN-β, IFN-λ1 and IFN-λ2/3 were significantly induced in MDM/TNF/IFNγ infected with RV16 compared to...
MDM/TNF/IFNg treated with media, and compared to both MDM/IL4 and MDM treated with RV16 at 8 h (all $P<0.0001$, Fig. 1a and c-e). Gene induction peaked at 8 h for all IFN genes and all had returned to baseline levels by 48 h, while some remained weakly induced at 24 h (Fig. S2a-e). In addition, we assessed the effects of UV-inactivated RV16 to determine whether type I and III IFN induction was replication dependent. We found no significant induction of any type I IFN or type III IFN mRNA in any cells treated with UV-inactivated virus ($P$=not significant, Fig. S3a-e).

The induction of IFN mRNA expression in MDM/TNF/IFNg was accompanied by significant induction of type I and III IFN at 24 h and 72 h, respectively (Fig. S2a-e). In addition, we assessed the effects of UV-inactivated RV16 to determine whether type I and III IFN induction was replication dependent. We found no significant induction of any type I IFN or type III IFN mRNA in any cells treated with UV-inactivated virus ($P$=not significant, Fig. S3a-e).

4.2. MDM/TNF/IFNg control RV replication better than MDM/IL4

To determine how different phenotypes of Mφ support virus replication, supernatants of RV16 and RV1B infected MDM/TNF/IFNg, MDM/IL4 and MDM were titrated on Ohio HeLa cells to determine their 50% tissue culture infective dose TCID$_{50}$/mL. We found that MDM/IL4 were characterised by limited RV16 virus replication peaking at 24 h ($P<0.05$, Fig. 2a and S5a), while an increase in virus load was not observed in MDM/TNF/IFNg, where levels of live virus simply decayed (Fig. S5a), with greater levels of virus load observed in MDM/
IL-10 is often tied to M2 macrophages [29]. However, in our experiments we did not find significant differences between MDM subtypes in production of IL-10 in either virus-infected or media control conditions (Fig. 3a and S6a,b).

The type 2 chemokines CCL22/MDC and CCL17/TARC were produced constitutively by MDM/IL4 (Fig. S6c-f). At 24 and 72 h/24 and 48 h (for CCL22/MDC and CCL17/TARC, respectively) time points the levels of both chemokines were significantly higher in non-infected MDM/IL4 compared with non-infected MDM and MDM/TNF/IFNg (P < 0.05, P < 0.001, P < 0.0001, Fig. 3b, c, Fig. S6c, e). While in RV16-infected MDM/IL4 production of CCL22/MDC and CCL17/TARC was significantly induced at 8, 24, 48, 72 h/24, 48, 72 h, respectively if compared with infected MDM and MDM/TNF/IFNg (P < 0.05, P < 0.001, Fig. 3b, c, Fig. S6d, f). We found no difference in CCL18/PARC production between different MDM groups in any condition (Fig. S6g, h).

3.4. Surface molecule expression of in vitro differentiated human MDM for the identification of subtype-specific markers

M1-like Mφ are prototypically induced by IFN-γ, TNF or bacterial components like lipopolysaccharide (LPS), while M2-like Mφ are induced by IL-4 or IL-13 [16]. First, we analyzed each of M1- and M2-associated marker separately and express data as MFI for all of them except CD197 (as% of positive cells). In our experiments, CD14 was significantly higher in MDM and MDM/TNF/IFNg, compared to MDM/IL4 (P < 0.001, P < 0.0001, Fig. 4a), while polarization of Mφ towards M1-like (MDM/TNF/IFNg) in the absence of RV infection induced significant up-regulation of CD80, CD54 and CD197, compared to both MDM and MDM/IL4 polarized Mφ (Fig. 4b-d).

The overall patterns of expression of M1 associated surface markers (CD14, CD54, CD80 and CD197) were not greatly altered in infected cells compared to uninfected cells (Fig. 4a-d).

According to this data we identify M1-like MDM/TNF/IFNg as CD14+CD80+CD197+ cells and analyzed different subsets of Mφ in this manner. We found that infected and uninfected MDM/TNF/IFNg were characterized by high percentage (10.93 ± 2.17% [media] and 14.29 ± 2.61% [RV16]) of CD14+CD80+CD197+ cells compared to both uninfected MDM and MDM/IL4 (3.82 ± 0.88%, 5.298 ± 1.018%, respectively) and infected MDM or MDM/IL4 (4.270 ± 1.082, 4.858 ± 1.081%, respectively) (P = 0.041 to P < 0.0001, see Figure for exact P values, Fig. 4e).

We next assessed levels of expression of surface molecules reported in other studies to be markers of M2-like Mφs (CD36 [30], CD206 [21,31] and HLA-DR [32]) in the different subsets of MDMs to determine whether we could identify surface markers specific for M2-like Mφs in human cells. However, there were no differences in the expression of CD36, CD206 and HLA-DR between MDM, MDM/TNF/IFNg and MDM/IL4 in either uninfected or infected MDMs (Fig. 4f-h).

3.5. Asthma triggered by RV infection is more severe in asthma patients than in healthy subjects

The baseline demographic and clinical characteristics of the healthy volunteers and those for the subjects with asthma, both as a single group of 28 subjects, and separated according to asthma severity into mild and moderate asthma groups have been reported previously [7,33], and are presented again in Table 1 for clarity.

The clinical outcomes during infection for the healthy volunteers and for the asthma subjects as a single group of 28 subjects have

![Figure 2](image-url)
been reported previously [6], but are reported in Fig. 5 for asthma subjects separated according to asthma severity into mild and moderate asthma groups. Compared to healthy subjects, daily lower respiratory symptom scores were significantly greater on days 3–6 for the subjects with mild asthma and on days 3–13 for the subjects with moderate asthma and daily lower respiratory symptom scores were significantly greater for the subjects with moderate asthma compared to the subjects with mild asthma on days 8, 10, 11 and 13 (Fig. 5a).

Rhinovirus-induced falls in lung function were also more severe according to asthma severity as the greatest fall from baseline in morning peak expiratory flow (PEF) in the group with moderate asthma occurred on day 5 and this was significantly greater than the fall in subjects with mild asthma on that day (17.4 ± 3.1% vs 7.2 ± 3.5%, \( P = 0.045 \)). The maximal fall in forced expiratory volume in one second (FEV\(_1\)) during the 14 day post-infection period for the group with moderate asthma (20.8 ± 2.04%) was also greater compared to both mild asthma (20.3 ± 4.9, \( P = 0.004 \)) and also significantly greater in subjects with mild asthma vs healthy subjects (\( P = 0.005 \), Fig. 5c).

Total lower respiratory symptoms scores (sum of daily scores over the 14 day post-infection period) were significantly greater in subjects with moderate asthma (45.2 ± 6.2) than in both healthy subjects (1.45 ± 3.5, \( P < 0.001 \)) and subjects with mild asthma (20.3 ± 4.9, \( P = 0.004 \)) and also significantly greater in subjects with mild asthma vs healthy subjects (\( P = 0.005 \), Fig. 5c).

These data highlight significantly worse clinical outcomes following RV16 infection in subjects with mild asthma, compared to healthy control subjects, as previously reported [5], and for the first time demonstrate that subjects with moderate asthma have a much more prolonged and severe exacerbation than the subjects with mild asthma.

We performed screening of freshly isolated BAL cells and PBMCs, using flow cytometry and measured the individual expression of M1- (CD14, CD80, CD197) and M2- (CD36, CD206, HLA-DR) associated surface markers at baseline before infection, and on day 4 following in vivo infection (d4pi) with RV16 in healthy and asthmatic subjects. We did not find any significant differences in expression of these M1-associated markers on BAL Mφ between asthma patients and healthy subjects at either baseline or day 4 post infection (Fig. 6a-c,e). However, we found a significant decline of CD80 expression on the surface of BAL Mφ on day 4 post infection compared to baseline in the moderate asthma group (\( P = 0.0127 \), Fig. 6c).

In addition, we found a significantly lower expression of CD14 on PBMCs of asthma patients compared to normal subjects and between normal subjects and moderate asthma group at day 4 post infection (\( P = 0.0132 \), \( P = 0.0214 \), respectively Fig. 7a). Moreover, CD80 expression on PBMCs was significantly lower in all asthma patients and the mild asthma groups compared to normal subjects at day 4 post infection (\( P = 0.0458 \), 0.0435, respectively Fig. 7c).

We then investigated changes in M1- and M2-associated marker expression between baseline and day 4 post infection in those subjects who had paired successful bronchoalveolar lavages with sufficient cell numbers to perform these analyses at both baseline and day 4 (\( N = 7 \) healthy, \( N = 12 \) asthma: 4 mild, 8 moderate). We found no significant changes in expression of either CD14 or CD197, but did find significantly greater down-regulation of CD80 expression on BAL Mφ during experimental RV infection in all asthma patients and in moderate asthma patients, compared to normal subjects (\( P = 0.0098 \) and \( P = 0.0059 \), respectively, Fig. 6d). Additionally, we found significant correlations between the change in CD80 expression from baseline to infection and important markers of clinical illness severity, the maximum reduction in lung function (both PEF and FEV\(_1\)) from baseline to infection (Fig. 8a,b) (\( r = 0.49 \), \( P = 0.035 \) and \( r = 0.55 \), \( P = 0.017 \), respectively) and a similar trend for a relationship between degree of change in CD80 expression and lower respiratory symptom severity (total chest score, \( r = -0.45 \), \( P = 0.058 \)) (Fig. 8c).
Next, we analysed BAL Mφ to evaluate the CD14⁺CD80⁺CD197⁺ M1-like population (Fig. S1). As a result, we found a tendency to increased frequencies of M1-like Mφ in healthy subjects on day 4 post infection compared to baseline (4.531±1.413, 2.236±0.036%, respectively; \( P = 0.0655 \), Fig. 9a) and a significant decrease of this cell type in the asthmatic group on day 4 post infection compared to baseline (3.561±1.589 and 7.396±2.327% respectively, \( P = 0.0242 \)) and in the moderate asthmatic group on day 4 post infection compared to baseline (1.867±0.7313 and 5.528±1.099% respectively, \( P = 0.0157 \) (Fig. 9a). Furthermore, we assessed changes between baseline and day 4 post experimental infection and found a tendency to decreased frequencies of the CD14⁺CD80⁺CD197⁺ cells in all asthma group –3.977±4.464% (\( P = 0.0556 \)) and significant decrease of this cell type in the moderate asthma group –3.612±1.788% (\( P = 0.0205 \)) compared to healthy controls 2.729±1.832% (Fig. 9b).

We found no significant difference in the expression of M2-associated surface markers (CD36, CD206, HLA-DR) on either the BAL Mφ or PBMCs between different groups of patients at either baseline or day 4 post infection (Fig. S7 and S8).

4. Discussion

Macrophages are key cells in the immune response to infectious agents. Following interaction with various bacterial and viral pathogens, they become activated and secrete a wide range of antiviral, pro-inflammatory and/or immunomodulatory cytokines [34]. We have reported that limited replication of RV occurs in MDM, resulting in induction of both IFN-α and IFN-β [23]. We have also reported RV-induction of IFNs-α, β and ω occurs in BAL cells (which are mostly macrophages) ex vivo [8,12]. It was therefore tempting to speculate that airway Mφ are an important source of RV-induced type I and type III IFNs in vivo. Relatively few studies have investigated interactions between different phenotypes of Mφ and viral infections [35,36] and none have investigated the role of different Mφ phenotypes during virus-induced asthma exacerbations.

The biology of macrophages is a subject of extensive research, but findings in man are mostly extrapolated from cells derived from peripheral blood monocytes (MDMs). MDMs can be derived from PBMCs in a variety of ways. Although GM-CSF polarizes macrophages towards a pro-inflammatory M1-like phenotype (but not to the fully activated state) [37] we used GM-CSF to generate MDMs in our in vitro experiments. It has been shown that freshly isolated BAL Mφ exhibit increased expression of genes associated with pro-inflammatory responses [38]. Moreover, the study of Akagawa et al. demonstrated that the phenotype of MDM generated with GM-CSF but not with M-CSF closely resembles that of human BAL Mφ [39]. In addition, Shibata et al. showed that GM-CSF stimulates terminal differentiation of AMs through PU1 [40]. The ontogeny of AMφ is a subject of controversy. Our understanding of resident macrophage sources is largely based on mouse models and these mouse models support the hypothesis that tissue resident Mφ are mainly embryonic in origin [41,42]. However, it has been shown recently in elegant studies using transplanted lungs that the majority of human BAL Mφ are derived from circulating monocytes [43]. Thus, in order to obtain cells with characteristics closer to human BAL Mφ we used MDM differentiated with GM-CSF in vitro. Differentiated MDM then were treated with IL-4 or TNF/IFN-γ to generate M1-like or M2-like MDM. Unpolarized GM-CSF treated MDM were used as control.

In this study, we demonstrated that unpolarized MDM and MDM/IL4 (M2-like) have increased susceptibility to RV replication compared to MDM/TNF/IFNg (M1-like). This is likely due to the dramatically decreased ability of these subsets of Mφ to produce type I and III IFN in response to viral infection. In contrast, MDM/TNF/IFNg were characterized by replication- and dose-dependant and receptor-independent robust up-regulation of type I and III IFN gene expression and protein release. According to previous studies Mφ polarization to M1-like cells was associated with a dramatic change in the transcriptome, in particular, M1 polarization induced the activation of interferon regulatory factors (IRF)–1, IRF-7 [44] and IRF-5 [45]. Activation of interferon stimulated genes could explain the capacity for more effective virus clearance by M1-like cells via type I and III IFN production. In study by Rajput et al. gene expression profiles of unpolarized RV-infected Mφ showed significant overrepresentation of genes involved in IFN-α/β signalling and M1-like polarization increased the RV response of IFIH1, which regulates MDA5 expression and augments the immune response to RV via IFN production.
We previously reported that RV-induction of IFN-λ in BAL cells is deficient in asthma and deficiency was related to clinical illness severity and virus load upon subsequent RV infection in vivo [8]. We have more recently also reported delayed and deficient IFN-α and IFN-β production in response to RV infection by BAL cells from asthma subjects [12]. Thus, deficiency in both type I and III IFN induction by RVs can be observed in BAL M\(\text{M}\)L from asthma subjects. Since we now report similar profound deficiency in RV-induction of each of IFN-α, -β and -λ in MDM and MDM/IL4, with very robust induction of each IFN in MDM/TNF/IFNg in the present studies, it seems likely that IFN deficiency in asthma may be at least in part a consequence of insufficient numbers of M1-like M\(\text{M}\)L being present in patients with allergic asthma.

Expression of some chemokines (CCL7/TARC, CCL18/PARC and CCL22/MDC) [31] is reported to be a marker of M2-like polarization. Our data from in vitro studies are consistent with this as both chemokines were induced in M2-like MDM/IL4 constitutively, with neither being induced by RV-infection. We also measured CCL7/TARC and CCL22/MDC in the nasal and bronchial mucosal lining fluid samples from the present experimental RV-16 infection study, and found nasal levels of CCL7/TARC and CCL22/MDC were both induced during infection in both subject groups, but to a greater degree in asthma than in healthy...
controls [52]. These data suggest that both constitutive M2-like polarization and increased CCL17/TARC and CCL22/MDC production during infection, possibly from another cell type, such as BECs, may be important in virus-induced asthma exacerbation pathogenesis and further work in this context is warranted.

CCL18/PARC has been reported to be a marker of M2-like polarization [31]. However, production of CCL18/PARC was similar in both uninfected and RV-infected MDM, MDM/TNF/IFNg and MDM/IL4. These data suggest that CCL18/PARC is not a marker of M2-like MDM polarized to an M2 phenotype with IL-4. The same tendency was observed for IL-10. Despite IL-10 being a possible indicator for M2-like M0 [17] we did not detect statistically significant IL-10 induction in MDM/IL4, possibly because we used GM-CSF to generate these MDM. The same effect was observed in the study of Akagawa et al. [39]. However, it is also possible that significant induction of IL-10 may have been observed if we had studied greater numbers of subjects.

Thus our first set of data demonstrate that polarization of human MDM in vitro results in characteristic patterns of cytokine secretion, with very robust type I and type III IFN production being characteristic of M1-like MDM/TNF/IFNg, while M2-like MDM/IL4 had markedly reduced IFN induction and augmented type 2 chemokine production.

In order to investigate polarization of M1 and M2-like M0 in RV-induced exacerbations of asthma in vivo, we wished to identify surface markers specific for each M0 phenotype to enable their assessment by flow cytometry in subjects during experimental RV infection. A controversial aspect of M0 biology concerns perceived differences between rodent and human M0. We therefore next investigated the expression of cell surface molecules on in vitro differentiated human MDM in order to identify reliable markers for characterization of polarized human M0 in vivo. We chose several widely reported M1 (CD14, CD80, CD54 and CD197) and M2 markers (CD36, CD206 and HLA-DR) [16,53] and quantified their expression in polarized MDM/TNF/IFNg and MDM/IL4 by flow cytometry before and after infection with RV16. We found that MDM/TNF/IFNg had higher levels of expression of CD14, CD54, CD80 and CD197 compared with MDM/IL4 and RV infection of MDM/TNF/IFNg did not significantly change their surface levels. Expression of CD54, CD80 and CD197 were similar in both infected and uninfected MDM compared with MDM/IL4, thus these markers were specific for M1-like MDM/TNF/IFNg, however CD14 expression was up-regulated in both infected and uninfected MDM compared with M2-like MDM/IL4 and therefore CD14 was not specific for MDM/TNF/IFNg.

CD14 acts as a co-receptor for LPS [54], CD80 (B7.1) provides a co-stimulatory signal for T-cell activation [55], CD197 (CCR7) controls migration of immune cells [56] and CD54 (ICAM-1) interacts with the $\beta_2$ integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) and regulates leucocyte migration to sites of inflammation [57]. Collectively, these data support the hypothesis that MDM/IL4 and MDM are deficient in recognition of and responses to pathogens compared to MDM/TNF/IFNg.

We found no significant difference in expression of the widely used M2 markers CD206, CD36 and HLA-DR on the surface of human MDM differentiated with GM-CSF and polarized by IL-4 in vitro. These findings suggest that CD206, CD36 and HLA-DR are not specific markers for human MDM/IL4. Moreover, we used CD206 to gate our BAL M0 as suggested by Yu, et al. [27].

Our next important goal was to use our results from the in vitro experiments to analyses the levels of expression of M1- and M2-associated markers on the surface of PBMCs and BAL M0 of asthma patients and healthy control subjects at baseline and during an experimental infection with RV16. As the only surface markers that were specific for M0 polarization were CD54, CD80 and CD197 (specific for M1-like M0) and CD14 (reduced in M2-like compared to MDM and M1-like M0) we focused our attention on these markers. As numbers of BAL and PBMCs samples with sufficient cell numbers available for investigation of expression of CD54 were too small, we have not included analysis of CD54 in the study results.

First, we analyzed BAL and PBMC samples for individual expression of listed markers. No significant differences were observed in CD14 expression in BAL M0 between asthma and control subjects in our study, in accordance with previous reports [58,59]. However, CD14 expression in PBMCs of asthma subjects as a whole, and of moderate asthma as a subgroup was downregulated during viral infection compared to healthy controls. Moreover, CD80 expression in PBMCs of whole asthma group and mild asthma subgroup was down-regulated during viral infection. In addition, we found reduced expression of CD80 in BAL M0 of moderate asthma group on day 4 post infection compared to baseline.

Then we compared the change in CD80 expression in BAL cells between baseline and day 4 during infection we found that CD80 in the asthma group as a whole, and in the moderate asthma group alone, was significantly down-regulated during viral infection compared to normal subjects. Moreover, greater reductions from baseline in BAL cell CD80 expression during infection were significantly related to the severity of reductions in lung function (both PEF and FEV1) during infection, with a trend for greater reductions in CD80 being related to greater lower respiratory symptom severity.

Fig. 5. Clinical outcomes of normal control subjects and subjects with mild/moderate asthma during experimental RV16 infection. Daily lower respiratory symptom scores in healthy and asthma subjects defined by baseline asthma severity $P<0.05$; $**P<0.01$; $***P<0.001$ asthma vs healthy subjects; $+P<0.05$ moderate asthma vs mild asthma. (b) Maximal fall in morning FEV1 from baseline during the 14 day period following rhinovirus inoculation in subjects with asthma defined by baseline asthma severity $P<0.05$; $**P<0.01$; $***P<0.001$. (c) Total lower respiratory symptom scores (sum of daily scores over the 14 day post-infection period) in healthy subjects and subjects with asthma defined by baseline asthma severity $+P<0.01$; $**P<0.001$. 

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Fig. 6. Expression of CD14, CD80 and CD197 on the surface of BAL macrophages during experimental rhinovirus infection. Expression of CD14 (a), CD80 (c) (mean fluorescence intensity) and CD197 (e) (percentage positive) were evaluated by flow cytometry in BAL macrophages at baseline (healthy $N = 7$, asthma $N = 17$, 7 mild and 10 moderate) and on day 4 post experimental RV16 infection (healthy $N = 7$, asthma $N = 18$, 7 mild and 11 moderate). Change in expression of CD80 (b), CD14 (d) and CD197 (f) (day 4 post infection minus baseline) on the surface of BAL macrophages during experimental RV16 infection was measured by flow cytometry ($N = 7$ for healthy, $N = 12$ for asthma, 4 mild and 8 moderate). Differences between multiple groups were estimated using a Kruskal–Wallis test followed by post-hoc testing (if the Kruskal–Wallis was significant) using un-paired Mann-Whitney U tests. Data show medians + interquartile range. P-values from Mann-Whitney U tests, where significant are shown on the graphs. NS = Kruskal–Wallis not significant.
Fig. 7. Expression of CD14, CD80 and CD197 on the surface of human peripheral blood monocytes during experimental rhinovirus infection. Expression of CD14 (a), CD80 (c) (mean fluorescence intensity) and CD197 (percentage positive) (e) were evaluated by flow cytometry in human PBMC at baseline (healthy $N=7$, asthma $N=21$, 8 mild and 13 moderate) and day 4 post experimental RV16 infection (healthy $N=10$, asthma $N=21$, 8 mild and 13 moderate). Change in expression of CD14 (b), CD80 (d) and CD197 (f) (day 4 post infection minus baseline) on the surface of human PBMC during experimental RV16 infection was measured by flow cytometry (healthy $N=7$, asthma $N=21$, 8 mild and 13 moderate). Differences between multiple groups were estimated using a Kruskal–Wallis test followed by post-hoc testing (if the Kruskal–Wallis was significant) using un-paired Mann-Whitney U tests. Data show medians + interquartile range. P-values from Mann-Whitney U tests, where significant are shown on the graphs. NS = Kruskal–Wallis not significant.
Then we analyzed BAL cells to identify CD14+CD80+CD197+ positive cells as an M1-like population. We found reductions in numbers of CD14+CD80+CD197+ M1-like in asthma patients during virus induced exacerbation compared to baseline. This data suggest that M1 polarization is a dynamic process dependent on lung microenvironment and rhinovirus-induced asthma exacerbations in allergic patients is characterized by an amplified Th2 immune response. This interpretation is strongly supported by the robust type I and type III IFN production by MDM/TNF/IFNg but not MDM/IL4 observed in our in vitro studies and suggests that imbalance away from M1-like and towards M2-like Mø phenotypes is likely implicated in virus-induced asthma exacerbation pathogenesis. Further support for this interpretation is provided by a study that showed the same results for CD80 expression on monocytes and NK cells in children during virus-induced asthma exacerbations [61].

Several studies have shown higher levels of M2-like Mø in BAL from people with atopic asthma [21,62,63]. Although the enhanced presence of M2-like Mø in asthma intuitively makes sense given the type 2 cytokine enriched lung environment, growing evidence supports the parallel development and involvement of both subsets of macrophages in lung disease. For instance, Draijer et al. showed more IRF5+ M1-like-polarized macrophages as well as more CD206+ M2-
like Mφ in asthma patients compared to healthy controls [64]. Furthermore, according to Byrne et al. in *in vivo* mouse pulmonary Mφs express both M2 markers and IRF5 and are capable of developing mixed phenotypes during pulmonary inflammatory disease [65]. Normal human lungs are also characterized by presence of different Mφ subsets (M0-74%, M1-26%, M2-7%) with percentages of both M1 and M2 marker-positive Mφs increasing significantly with smoking and COPD severity [66]. This study also reported dual positivity for M1 and M2 markers in the same human AMφ. None of the above mentioned studies evaluated changes in Mφ populations during RV infection which itself can amplify both Th1 and Th2 responses in asthma [67]. In our study we did not find increased frequencies of M2-like Mφ populations in asthma, either at baseline, or during infection. We used the M2-like markers CD36, CD206 and HLA-DR based on published observations [16,53]. It is possible that differences may have been observed if we had studied other markers of M2-like activity as discussed by Murray et al [19].

Thus, despite our negative findings in respect of the M2 markers we studied, impaired interferon production by RV-infected human BAL cells from asthma patients was confirmed by several studies [8,12,68], supporting the hypothesis that M2-like BAL Mφs are likely predominant in asthma during RV infection. To the best of our knowledge, this is the first report on Mφ populations during experimental RV infection in healthy control subjects and people with asthma. Our findings suggest that the deficiency of antiviral IFNs observed in asthma and strongly implicated in asthma exacerbation pathogenesis, may be at least in part a consequence of insufficient numbers of M1-like Mφs being present during virus-induced exacerbation in patients with allergic asthma. Our results have translational significance because the management of patients with virus-induced asthma exacerbation remains a challenge. Further studies investigating new immunotherapeutic strategies that promote M1-like macrophage polarization, for instance, through TLR4 activation [69], prophylactic type I or type III IFN administration [70], or use of mebendazole may identify novel therapeutic strategies for this unmet medical need [71].

Taken together, our work indicates that M1-like MDM/TNF/IFNγ are potent producers of type I and III IFNs and suggests reductions in the CD14^+CD80^+CD197^+ M1-like BAL Mφ subpopulation during infection may be involved in asthma exacerbation pathogenesis. The mechanisms of deficient M1 responses in virus-induced asthma in allergic patients now require additional clinical study with increased number of subjects and careful exploration.

### Declaration of Competing Interest

Dr. Johnston reports personal fees from Therapeutic Frontiers, personal fees from Virtus Respiratory Research, personal fees from Myelo Therapeutics GmbH, personal fees from Concert Pharmaceuticals, personal fees from Bayer, personal fees from Synairgen, personal fees from Novartis, personal fees from Boehringer Ingelheim, personal fees from Chiesi, personal fees from Gerson Lehrman Group, personal fees from restORbio, personal fees from Bioforce, personal fees from Materia Medical Holdings, personal fees from PrepBio Pharma, personal fees from Pulmotect, personal fees from Virion Health, personal fees from Lallemand Pharma, personal fees from AstraZeneca, outside the submitted work; In addition, Dr. Johnston has a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-virus therapy for respiratory diseases. UK patent application No. GB 0405634.7, 12 March 2004. with royalties paid, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Interferon-Beta for Anti-Virus Therapy for Respiratory Diseases. International Patent Application No. PCT/GB05/50031, 12 March 2004. with royalties paid, and a patent Davies DE, Wark PA, Holgate ST, Johnston SL. Interferon Lambda therapy for the treatment of respiratory disease. UK patent application No. 6779645.9, granted 15th August 2012. Licensed. Rudolf Valetna has received research grants from the Austrian Science Fund (FWF) and Viravaxx, Vienna, Austria and serves as a consultant for Viravaxx. The other authors do not have any conflict of interest.

### Acknowledgements

SLJ is supported by a Chair from Asthma UK (CH115S). This work was supported by ERC FP7 Advanced grant 233015 (to SLJ), MRC Centre Grant G1000758, Asthma UK grant 08–048, the National Institute of Health Research (NIHR) Biomedical Research Centre funding scheme, NIHR BRC Centre grant P26095, the Predicta FP7 Collaborative Project grant 260895. Results of Sections 4, 5 has been obtained under support of the RSF grant No. 19–15–00272. SLJ is an NIH Senior Investigator. RV is recipient of a Megagrant of the Government of the Russian Federation, grant No 14.W03.31.0024. The funders had no role in study design, data collection, data analysis, interpretation, writing of the report.

### Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102734

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