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Human airway macrophages are metabolically reprogrammed by IFN-γ resulting in glycolysis dependent functional plasticity

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Abstract

Airway macrophages (AM) are the predominant immune cell in the lung and play a crucial role in preventing infection, making them a target for host directed therapy. Macrophage effector functions are associated with cellular metabolism. A knowledge gap remains in understanding metabolic reprogramming and functional plasticity of distinct human macrophage subpopulations, especially in lung resident AM. We examined tissue-resident AM and monocyte derived macrophages (MDM; as a model of blood derived macrophages) in their resting state and after priming with IFN-γ or IL-4 to model the Th1/Th2 axis in the lung. Human macrophages, regardless of origin, had a strong induction of glycolysis in response to IFN-γ or upon stimulation. IFN-γ significantly enhanced cellular energetics in both AM and MDM by upregulating both glycolysis and oxidative phosphorylation. Upon stimulation, AM do not decrease oxidative phosphorylation unlike MDM which shift to "Warburg"-like metabolism. IFN-γ priming promoted cytokine secretion in AM. Blocking glycolysis with 2-deoxyglucose significantly reduced IFN-γ driven cytokine production in AM, indicating that IFN-γ induces functional plasticity in human AM, which is mechanistically mediated by glycolysis. Directly comparing responses between macrophages, AM were more responsive to IFN-γ priming and dependent on glycolysis for cytokine secretion than MDM. Interestingly, TNF production was under the control of glycolysis in AM and not in MDM. MDM exhibited glycolysis-dependent upregulation of HLA-DR and CD40, whereas IFN-γ upregulated HLA-DR and CD40 on AM independently of glycolysis. These data indicate that human AM are functionally plastic and respond to IFN-γ in a manner distinct from MDM. These data provide evidence that human AM are a tractable target for inhalable immunomodulatory therapies for respiratory diseases.

eLife assessment

In this **valuable** study, the authors investigate how inflammatory priming and exposure to irradiated Mycobacterium tuberculosis or the bacterial endotoxin LPS impact the metabolism of primary human airway macrophages and monocyte-derived macrophages. The work shows that metabolic plasticity is greater in monocyte-derived macrophages than alveolar macrophages, with **solid** experimental methods and evidence. The work is relevant to the field of immunometabolism.

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Introduction

Airway macrophages (AM) are the sentinels of the lung and the first responders to respiratory insults such as infection. Despite a large body of evidence indicating that these tissue resident AM have a distinct phenotype and function to peripherally derived macrophages, there remains a significant lack of data regarding human AM function and plasticity in response to infection and their ability to change under the influence of Th1 or Th2 environments. Macrophage function exists on a spectrum of activation states based on tissue residency, ontogeny, cytokine milieu and the plasticity of the macrophage in response to environmental factors (1, 2). Much of the research has focused on the contribution of metabolic pathways to polarising macrophages into distinct pro-inflammatory or regulatory phenotypes (3). There is a paucity of data on the role of metabolism in response to Th1 or Th2 microenvironments induced by cytokines-such as IFN-γ or IL-4 respectively, in human macrophages, especially in tissue resident macrophages, such as AM. A knowledge gap remains as to whether the tissue resident AM is metabolically and functionally plastic and therefore capable of mounting effective pro-inflammatory responses despite its homeostatic, regulatory tissue resident phenotype.

Plasticity of macrophage function requires metabolic reprogramming (4, 5). Since AM play a key role in directing and propagating immune responses and inflammation in the lung, we sought to determine the plasticity of AM and monocyte derived macrophages (MDM). Using primary human AM and MDM, we modelled Th1 and Th2 microenvironments with the addition of IFN-γ or IL-4, respectively. To further examine the function of IFN-γ or IL-4 primed macrophages, we stimulated cells with the gram-negative bacterial component, lipopolysaccharide (LPS), or whole bacteria, irradiated *Mycobacterium tuberculosis* (Mtb; iH37Rv). Firstly, we assessed the metabolic phenotype of unprimed human AM, or primed with IFN-γ or IL-4. IFN-γ significantly increased the cellular energetics of both human AM and MDM. Furthermore, subsequent stimulation led to an increase in the extracellular acidification rate (ECAR), a surrogate marker of glycolysis in both macrophages. Therefore, using the glycolytic inhibitor 2-deoxyglucouse (2DG) we then examined the mechanistic role of glycolysis in the phenotypic and functional plasticity of both AM and MDM. Herein the functional plasticity is defined as the ability of primed macrophages to differentially alter cytokine production in response to bacterial stimuli whereas the phenotypic plasticity is defined by alterations in surface expression of activation markers.

These data demonstrate that human AM are functionally plastic and respond to IFN-γ or IL-4 differently than MDM. These novel data demonstrate differential metabolic responses within human macrophage subpopulations that are linked with functionality. Furthermore, these data address a knowledge gap in human respiratory innate immunology and provide evidence that the AM is a tractable target to support human respiratory health.

Methods

Cell Culture

Buffy coats were obtained with consent from healthy donors (aged between 18-69; ethical approval, Trinity College Dublin). Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation over Lymphoprep (StemCell Technologies). Cells were resuspended in RPMI (Gibco) supplemented with 10% AB human serum (Sigma-Aldrich) and plated onto non-treated tissue culture plates (Costar) for 7 days. Non-adherent cells were removed by washing every 2-3 days. Cultures were >90% pure based on co-expression of CD14 and CD68.

Human AM were retrieved at bronchoscopy, (ethical approval, St. James's Hospital) previously as reported (6). Cells were plated in RPMI (Gibco) supplemented with 10% FBS (Gibco), fungizone (2.5 µg/ml; Gibco) and cefotaxime (50 µg/ml; Melford Biolaboratories). Cells were incubated for 24 h before washing to remove non-adherent cells. Adherent cells (predominantly AM) were used for experiments.

BAL Sample Acquisition

All donors were patients undergoing clinically indicated bronchoscopy and written informed consent for retrieving additional bronchial washings for research was obtained prior to the procedure. Patients were not remunerated for participation in this study. Exclusion criteria included age under 18 years, inability to provide written informed consent or a known (or ensuing) diagnosis of malignancy, sarcoidosis, HIV or Hepatitis C. Patients undergoing biopsy as part of bronchoscopy were also excluded.

Sample acquisition during bronchoscopy: Conscious sedation was achieved using intravenous midazolam and lignocaine gel was administered to the nostril. Flexible video-bronchoscope was inserted through the nostril and advanced to the level of the vocal cords by posterior approach. Further lignocaine spray was administered prior to and subsequent to traversing the vocal cords. Following routine bronchoscopy, the bronchoscope was wedged in the right middle lobe bronchus. A total of 180 ml of sterile saline was administered as 60 ml boluses via a connector inserted into the bronchoscope and aspirated within 5–10 s under low suction. The bronchoalveolar lavage fluid (BALF) was then transported directly to the laboratory for AM isolation. Pre- and post-bronchoscopy patient care was not altered by participation in the study. The procedure was prolonged by ~12 min.

Macrophage Stimulation

Macrophages were primed with IFN-γ or IL-4 (both 10 ng/ml) or left unprimed for 24 h. Where indicated, MDM and AM were treated with 2DG (5 mM) for 1 h prior to stimulation with irradiated Mtb strain H37Rv (iH37Rv; MOI 1-10) or LPS (100 ng/ml; Merck). For metabolic flux analysis stimulations were immediately monitored in real-time. All other stimulations were assessed after 24 h.

Metabolic Assays

MDM were placed in ice-cold PBS and incubated at 4°C on ice for 30 minutes, then gently scraped and counted using trypan blue. MDM (1×10^5 cells/well) were re-plated onto Seahorse plates, as previously described (7). AM (1×10^5 cells/well) were directly plated onto Seahorse plates and washed after 24 h. The ECAR and the oxygen consumption rate (OCR), were measured 3 times every 10 minutes to establish baselines. After 30 minutes macrophages were stimulated in-situ and monitored in real-time, with Seahorse medium, iH37Rv or LPS. Post stimulation the ECAR and OCR

were continually sampled at 20-minute intervals for times indicated. Analyses were carried out at approximately 150 minutes as previously described (7). Fold change in ECAR and OCR was then calculated compared to unstimulated unprimed controls. Percent change in ECAR and OCR was also calculated versus the respective primed control to examine the capacity of cells to increase metabolic parameters.

Cytokine assays

IL-1β, IL-10 (BioLegend) and TNF (Invitrogen) concentrations in supernatants were quantified by ELISA, according to manufacturer's protocol.

Flow cytometry

Human AM and MDM were placed in ice-cold PBS and incubated at 4°C on ice for 30 minutes. Cells were removed by gentle scraping, Fc blocked with Human TruStain FcX (BioLegend) and stained with zombie NIR viability dye and fluorochrome-conjugated antibodies for CD14 (FITC), CD68 (PE), CD86 (BV410), CD40 (BV510), and HLA-DR (APC; all BioLegend). For phagocytosis and antigen processing assays, MDM were treated with fluorescent beads (Sigma-Aldrich) or DQ-Ovalbumin (Thermo-fisher) for 30 minutes at 37°C, before scraping as above. DQ-Ovalbumin is fluorescent after proteasomal degradation marking antigen processing. Cells were fixed with 2% PFA and acquired on a BD FACS Canto II. Unstained and FMO controls were used to normalise for background and to set gates. Data were analysed using FlowJo.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10. Statistically significant differences between two or more groups containing more than one variable were determined by two-way ANOVA with Tukey or Bonferroni multiple comparisons tests as stated. P-values of ≤0.05 were considered statistically significant and denoted with an asterisk. Alternatively, P-values of ≤0.05 were denoted with a hashtag where data was analysed in the absence of IFN-γ primed data sets, to analyse statistical differences between no cytokine and IL-4 treated data sets.

Results

IFN-γ induces metabolic reprogramming in both AM and MDM

AM alter their metabolism in response to Mtb (8). Human macrophages also undergo a rapid increase in ECAR early in response to activation (7) and these pathways can be pharmacologically manipulated (9, 10). The metabolic and functional plasticity of human AM remains unexplored, however recent evidence shows they express both 'M1' and 'M2' markers (11). Murine AM can be reprogrammed through an IFN-γ dependent mechanism (12). We therefore sought to examine whether priming human AM with IFN-γ compared with IL-4 or unprimed AM, could influence their metabolic function and response to bacterial stimuli. We stimulated with whole bacteria; Mtb (iH37Rv) or gram-negative cell wall component; LPS. AM were plated in a Seahorse plate and primed with IFN-γ or IL-4 (both 10 ng/ml) for 24 h or left unprimed. AM ECAR and OCR were recorded for 30 min at baseline. AM were then stimulated in the Seahorse XFe24 Analyzer with medium (control), iH37Rv (MOI; 1-10) or LPS (100 ng/ml) and ECAR (Figure S1A) and OCR (Figure S1B) were continuously monitored.

At 150 minutes post stimulation fold change compared to unprimed unstimulated AM was calculated for ECAR (**Figure 1A**) and OCR (**Figure 1B**). IFN-γ priming significantly increased the ECAR and OCR of unstimulated human AM compared with control or IL-4 primed AM (**Figure 1A,B**). Upon stimulation with iH37Rv or LPS, AM significantly increased ECAR compared to their respective unstimulated controls, regardless of cytokine priming (**Figure 1A**). IFN-γ primed and

subsequently stimulated AM exhibited a significantly increased ECAR compared with stimulated control or IL-4 primed AM (**Figure 1A**). IL-4 primed iH37Rv stimulated AM increased ECAR to similar extent as unprimed controls (**Figure 1A**; left). Conversely, IL-4 primed AM stimulated with LPS AM did not increase their ECAR to the same extent as controls (**Figure 1A**; right), suggesting that IL-4 reduces the AM ability to increase ECAR in response to LPS stimulation. IFN-γ significantly increased the OCR of AM in response to stimulation with iH37Rv or LPS, and had enhanced OCR compared with other stimulated controls (**Figure 1B**). These data indicate that priming human AM with IFN-γ increases both glycolytic and oxidative metabolism, which is then further increased upon stimulation.

Since IFN-γ priming increased cellular energetics in the AM at baseline, we calculated percent change in ECAR and OCR from the baseline rate of each group in order to assess if IFN-γ or IL-4 primed AM have altered capacity to change their metabolism in response to stimulation (**Figure 1C,D**). This was carried out to equalise all the primed data sets at baseline before stimulation (Figure S1C, S1D). These data indicate that whilst the peak of glycolysis is elevated in IFN-γ primed AM (Figure S1A), all AM have a similar capacity to increase glycolysis upon stimulation when baseline differences in metabolism were adjusted for the effects of cytokine priming (**Figure 1C**). IFN-γ increased the percent change in OCR of AM in response to both bacterial stimuli compared to the unstimulated IFN-γ primed control (**Figure 1D**). These data indicate that priming AM alters the metabolic baselines of human tissue resident macrophages and not their ability to respond to bacterial stimuli.

In order to compare the metabolic responses of AM with blood derived macrophages, we next assessed MDM. Human MDM were left unprimed or primed with IFN-γ or IL-4 (both 10 ng/ml). 24 h after cytokine priming metabolic flux was monitored by recording ECAR and OCR at baseline for 30 minutes. MDM were then stimulated with medium, iH37Rv or LPS and ECAR and OCR was continuously monitored (Figure S1E-H).

As per previous observations (7, 13) a sustained increase in ECAR and a transient decrease in OCR occurred in MDM after stimulation (Figure S1E-H). At 150 minutes post stimulation, fold change was calculated compared to unprimed unstimulated MDM (**Figure 1E,1F**). IFN-γ priming significantly increased the ECAR and OCR of MDM whereas IL-4 priming significantly reduced the ECAR in the absence of stimulation (**Figure 1E,1F**). Stimulation of human MDM with iH37Rv or LPS significantly increased ECAR in all MDM, however, IL-4 primed MDM stimulated with iH37Rv or LPS have significantly reduced ECAR compared with control or IFN-γ primed MDM (**Figure 1E**). IFN-γ primed MDM stimulated with iH37Rv have increased ECAR compared with control MDM (**Figure 1E**).

Similar to AM, IFN-γ primed MDM have increased OCR compared with control or IL-4 primed MDM (**Figure 1F**). In contrast with the AM, stimulation of IFN-γ primed MDM does not further increase OCR however, the elevated OCR in IFN-γ primed MDM remains significantly higher compared to control or IL-4 primed MDM when stimulated with iH37Rv (**Figure 1F**). The percent change in ECAR upon stimulation (from respective baselines) illustrates that all MDM groups significantly increase ECAR from their own baseline in response to stimulation (**Figure 1G**). Interestingly, although IL-4 significantly reduced ECAR in iH37Rv and LPS stimulated MDM compared with unprimed stimulated controls (**Figure 1E**), the IL-4 primed MDM have significantly enhanced capacity to ramp up glycolysis in response to LPS, as evidence by the significantly increased percentage change in LPS stimulated, IL-4 primed MDM compared with IFN-γ primed controls (**Figure 1G**). Control or IFN-γ primed MDM stimulated with either iH37Rv or LPS decreases percentage change in the OCR associated with a stimulation-induced shift to “Warburg”-like metabolism (**Figure 1H**). This effect is not observed in IL-4 primed MDM, moreover, IL-4 primed MDM stimulated with iH37Rv had significantly elevated percent change in

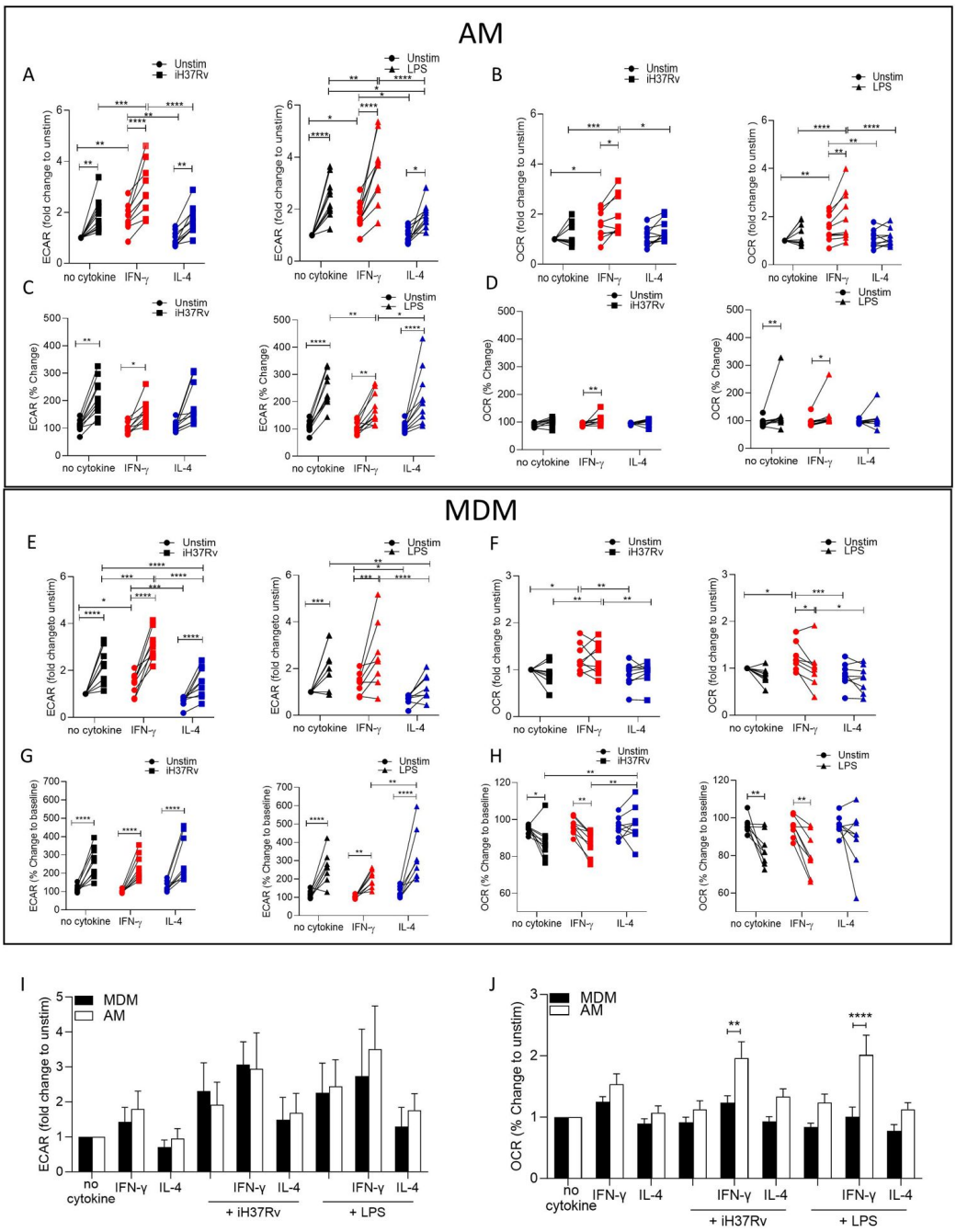

Figure 1.

IFN-γ increases energetic metabolism in the AM but enhances “Warburg”-like metabolism in MDM in response to inflammatory stimuli. Human AM (A-D) were isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and MDM (E-H) were differentiated and adherence purified for 7 days in 10% human serum. Cells were left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. Baseline measurements of the Extracellular Acidification Rate (ECAR) and the Oxygen Consumption Rate (OCR) were established before AM or MDM were stimulated with medium (circle), irradiated Mtb H37Rv (iH37Rv; MOI 1-10; square) or LPS (100 ng/ml; triangle), in the Seahorse XFe24 Analyzer, then monitored at 20-minute intervals. At 150 minutes, post stimulation fold change in ECAR (A, E, I) and OCR (B, F, J) was analysed, and percentage change (from baseline of the respective treatment group) was also calculated for ECAR (C, G) and OCR (D, H) at 150 minutes. A direct comparison of AM and MDM was also assessed at 150 minutes (I, J). Each linked data point represents the average of technical duplicates for one individual biological donor (MDM; n=8-9, AM; n=9-10). Statistically significant differences were determined using two-way ANOVA with a Tukey (A-H) or Bonferroni post-test (I-J); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.001.

OCR compared with stimulated unprimed or IFN-γ primed MDM (**Figure 1H**). These data indicate that IL-4 priming prevents human MDM utilising "Warburg"-like metabolism in response to stimulation.

Since AM and MDM had distinct responses to priming and stimulation, we next directly compared the metabolic responses of AM and MDM. AM and MDM had similar levels of ECAR relative to their own unprimed controls, which were both enhanced upon stimulation (**Figure 1I**). The OCR is elevated in the AM compared with the MDM; IFN-γ primed AM exhibit significantly increased OCR compared with MDM in response to stimulation with iH37Rv or LPS (**Figure 1J**).

In summary, human AM upregulate glycolysis early in response to stimulation. IFN-γ significantly promoted cellular energetics (both ECAR and OCR) in unstimulated AM which was further enhanced by stimulation. IFN-γ promotes increased cellular energetics in stimulated human MDM by promoting both glycolysis and oxidative phosphorylation, whilst maintaining the capacity for the cells to shift to "Warburg"-like metabolism in response to stimulation. IL-4 priming significantly reduced the cellular energetics compared with control or IFN-γ primed MDM. Importantly, IL-4 prevents the drop in OCR occurring in stimulated MDM thereby inhibiting "Warburg"-like metabolism. IL-4 primed AM had reduced fold change in glycolysis upon stimulation with LPS compared with controls.

IFN-γ promotes HLA-DR and CD40 markedly more on human MDM than AM whereas IL-4 promoted CD86

Having established that energetic responses are plastic in response to IFN-γ in the AM and that post stimulation energetic responses are different in human macrophage types under Th1 or Th2 priming conditions, we next sought to determine the effect on the plasticity of the macrophage phenotype by examining expression of activation markers associated with antigen presentation function. Human AM (**Figure 2A,2C,2E**) and MDM (**Figure 2B**, 2D, 2F) were primed with IFN-γ or IL-4 for 24 h or left unprimed. Macrophages were then stimulated with iH37Rv or LPS. After 24 h AM and MDM were analysed by flow cytometry for expression of HLA-DR (**Figure 2A,2B,2G**), CD40 (**Figure 2C,2D,2H**) and CD86(**Figure 2E,2F,2I**). A sample gating strategy for the analysis is provided (Figure S2A).

IFN-γ significantly increased the expression of HLA-DR compared with control or IL-4 primed unstimulated AM (**Figure 2A**). Stimulation with iH37Rv significantly upregulated HLA-DR, but only in unprimed AM (**Figure 2A**). Similarly, LPS significantly induced HLA-DR in unprimed or IL-4 primed AM but not in IFN-γ primed AM (**Figure 2A**). IFN-γ also significantly increased the expression of HLA-DR compared with control or IL-4 primed MDM (**Figure 2B**). Stimulation of IFN-γ primed MDM with iH37Rv or LPS robustly enhanced the expression of HLA-DR (**Figure 2B**). IFN-γ priming significantly upregulated CD40 expression in unstimulated AM (**Figure 2C**; right). In addition, CD40 was upregulated following iH37Rv or LPS stimulation of AM in all groups assessed with the exception of IFN-γ primed AM stimulated with iH37Rv (**Figure 2C**). IFN-γ increased the expression of the co-stimulatory molecule CD40 in unstimulated MDM (**Figure 2D**). Stimulation of MDM with iH37Rv or LPS significantly increased CD40 expression, with the exception of iH37Rv stimulation in IL-4 primed MDM (**Figure 2D**). Expression of CD86 in response to stimulation with iH37Rv was only upregulated in IL-4 primed AM, however, LPS induced upregulation of CD86 in all AM, with IFN-γ and IL-4 primed AM exhibiting significantly enhances CD86 expression compared to unprimed control (**Figure 2E**). CD86 expression induced by MDM in response to iH37Rv or LPS was enhanced by priming with either IFN-γ or IL-4, with IL-4 inducing significantly higher expression compared with unprimed or IFN-γ primed MDM (**Figure 2F**).

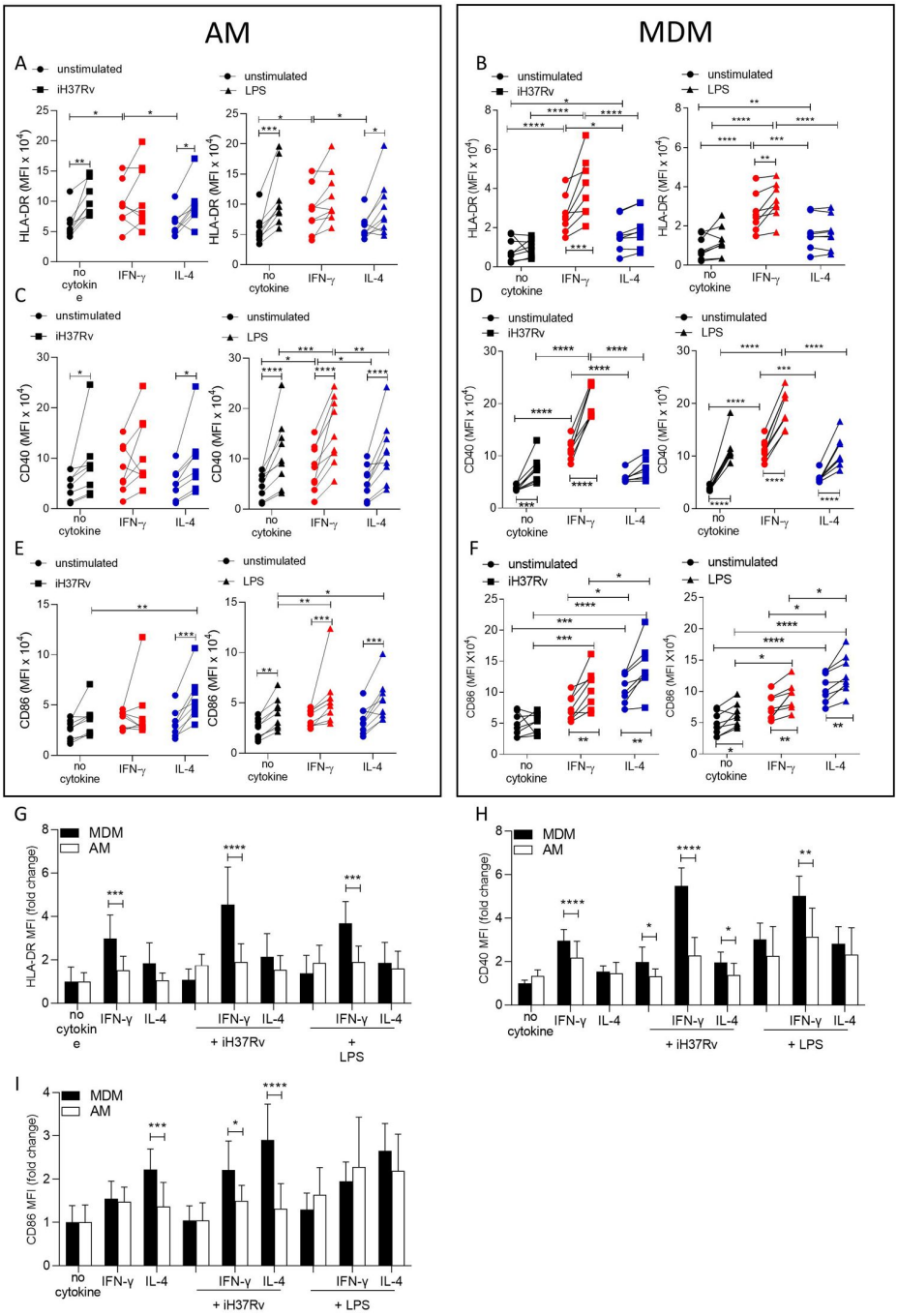

Figure 2.

IFN-γ boosts activation marker expression on MDM to a greater extent than AM.

Human AM (A, C, E) isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and MDM (B, D, F) were differentiated and adherence purified for 7 days in 10% human serum. Cells were left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. AM or MDM were left unstimulated (circle) or stimulated with iH37Rv (MOI 1-10; square) or LPS (100 ng/ml; triangle). After 24 h cells were detached from the plates by cooling and gentle scraping and stained for HLAR-DR (A, B), CD40 (C, D), CD86 (E, F) and analysed by flow cytometry. Fold change of HLA-DR (G), CD40 (H) and CD86 (I) was calculated for AM (white bar) and MDM (black bar) based on the average of their respective no cytokine controls. Each linked data point represents the average of technical duplicates for one individual biological donor (n=8-9). Statistically significant differences were determined using two-way ANOVA with a Tukey (A-F) or Bonferroni post-test (G-I) (A-F); *P≤0.05, **P≤0.01, P***≤0.001, ****P≤0.001.

In order to directly compare human AM and MDM responses to IFN-γ and IL-4, fold change in HLA-DR, CD40 and CD86 was calculated compared to the average of respective unstimulated unprimed controls (**Figure 2G-I**). The human MDM has increased HLA-DR and CD40 expression in response to IFN-γ compared to the human AM (**Figure 2G,2H**). This increased expression of HLA-DR and CD40 by MDM, becomes even more profound after stimulation. MDM also have greater expression of CD86 when primed with IL-4 compared to AM, which was again enhanced by stimulation (**Figure 2I**). IFN-γ primed MDM stimulated with iH37Rv also increased expression of CD86 compared to AM (**Figure 2I**).

MDM are more dependent on glycolysis for the IFN-γ driven upregulation of HLA-DR and CD40 while AM are not

Since IFN-γ drove glycolysis and the expression of the macrophage activation markers CD40 and HLA-DR in both AM and MDM we wanted to examine if the increased glycolysis was associated with enhanced expression of activation markers expression. Human AM (**Figure 3A,3C,3E**) and MDM (**Figure 3B,3D,3F**) were primed with IFN-γ or IL-4 for 24 h or left unprimed. Macrophages were then treated with the glycolytic inhibitor, 2DG for 1 h prior to stimulation with iH37Rv or LPS. After 24 h AM and MDM were analysed by flow cytometry for expression of HLA-DR (**Figure 3A,3B**), CD40 (**Figure 3C,3D**) and CD86(**Figure 3E,3F**). 2DG-mediated inhibition of glycolysis following stimulation was confirmed (Figure S2B).

Inhibiting glycolysis with 2DG did not alter expression of HLA-DR on AM (**Figure 3A**). Interestingly, the increased expression of HLA-DR in IFN-γ primed MDM was dependent on glycolysis in unstimulated and iH37Rv stimulated MDM, however, increased expression of HLA-DR by LPS stimulated IFN-γ primed MDM remained elevated in the presence of 2DG (**Figure 3B**). Expression of CD40 was not affected by 2DG in unstimulated or iH37Rv stimulated AM (**Figure 3C**). Conversely, LPS induced expression of CD40 was significantly inhibited by 2DG in unprimed and IFN-γ primed AM but not in IL-4 primed AM (**Figure 3C**). In contrast, enhanced expression of CD40 in IFN-γ primed MDM in unstimulated or iH37Rv stimulated MDM was significantly reduced with the addition of 2DG, with no effect on the expression of CD40 in LPS stimulated human MDM regardless of cytokine priming (**Figure 3D**). 2DG enhanced expression of CD86 in unstimulated IFN-γ or IL-4 primed AM but did not affect expression in any stimulated AM (**Figure 3E**). Interestingly, when unstimulated AM were examined in the absence of stimulation, IFN-γ priming significantly increased CD86 (**Figure 3E**). 2DG inhibited the increased expression of CD86 in response to iH37Rv stimulation in IFN-γ or IL-4 primed MDM, but no difference was observed in unstimulated or LPS stimulated MDM (**Figure 3F**).

Cumulatively, these data indicate that IFN-γ upregulates the expression of activation markers more effectively in human MDM than AM. Since these markers are associated with activating T cells during presenting antigen, the ability of IFN-γ or IL-4 primed MDM to process antigen was next assessed, along with the dependency on glycolysis. MDM were primed with IFN-γ or IL-4 for 24 h or left unprimed. MDM were then treated with 2DG for 1 h prior to stimulation with DQ-Ovalbumin (500 ng/ml) for 30 min. IL-4 primed MDM had significantly reduced ability to process DQ-Ovalbumin compared with control or IFN-γ primed MDM (Figure S2C). Treatment of MDM with 2DG significantly reduced antigen processing in all groups, however IFN-γ primed MDM retained enhanced abilities to process antigen (Figure S2C). The reduced capacity of MDM to process antigen was not due to a deficiency in phagocytosis, as measured by both bead or bacterial uptake or due to increased cell death (Figure S2D-F).

Overall, these data suggest that IFN-γ promotes expression of activation markers via increased glycolysis in human MDM, whereas AM are not as phenotypically plastic in response to cytokine priming and subsequent stimulation. Moreover, AM upregulation of cell surface markers (with the exception of CD40) in response to priming or stimulation is not associated with glycolysis, in contrast to the MDM.

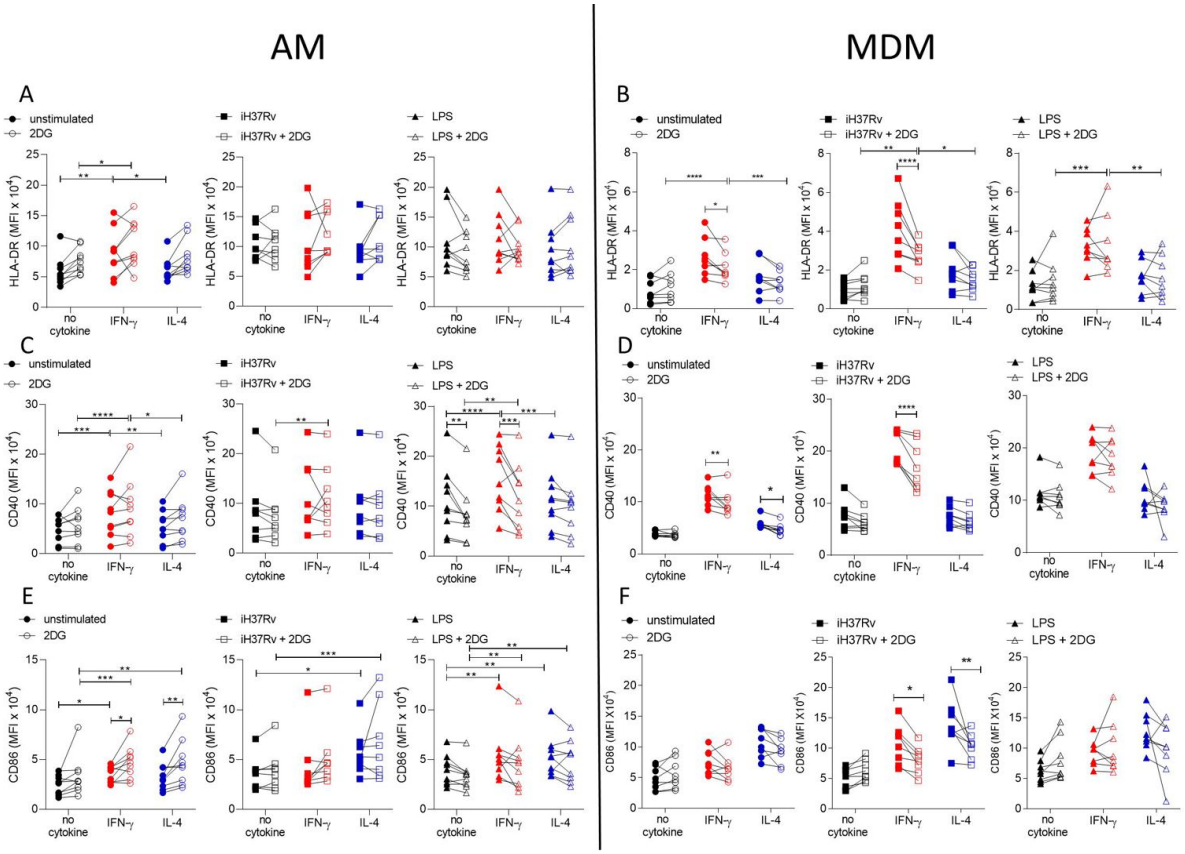

Figure 3.

Glycolysis is required for IFN-γ induced expression of activation markers by MDM and not AM.

Human AM (A, C, E) isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and MDM (B, D, F) were differentiated and adherence purified for 7 days in 10% human serum. Cells were left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. Cells were left untreated (solid) or treated with 2DG (5 mM; empty) 1 h prior to stimulation with iH37Rv (MOI 1-10; square) or LPS (100 ng/ml; triangle) or left unstimulated (circle). After 24 h cells were detached from the plates by cooling and gentle scraping and stained for HLAR-DR (A, B), CD40 (C, D), CD86 (E, F) and analysed by flow cytometry. Each linked data point represents the average of technical duplicates for one individual biological donor (n=8-9). Statistically significant differences were determined using two-way ANOVA with a Tukey post-test (A-F); *P≤0.05, **P≤0.01, P***≤0.001, ****P≤0.001.

IFN-γ enhances cytokine production in human AM more than MDM

Changes in macrophage metabolism have been previously associated with altered cytokine production (5, 8, 14). Having established that both IFN-γ and IL-4 can significantly alter metabolism in human macrophages we next sought to examine the ability of AM and MDM to secrete cytokines when primed with IFN-γ or IL-4. Human AM (**Figure 4A,4C,4E**) and MDM (**Figure 4B,4D,4F**) were left unprimed or primed with IFN-γ or IL-4 for 24 h. Macrophages were then stimulated with iH37Rv or LPS. Supernatants were harvested 24 h post stimulation and concentrations of IL-1β (**Figure 4A,4B**), TNF (**Figure 4C,4D**) and IL-10 (**Figure 4E,4F**) were quantified by ELISA. While iH37Rv stimulation resulted in IL-1β production in unprimed AM and MDM, IFN-γ only significantly enhanced the production of IL-1β by AM (**Figure 4A,4B**). IL-4 priming attenuated iH37Rv induced IL-1β in both AM and MDM (**Figure 4A,4B**). As expected, IL-1β secretion was not induced in response to LPS stimulation however, in the presence of IFN-γ, IL-1β was detectable (**Figure 4A**, 4B). TNF was significantly induced in unprimed or IFN-γ primed, but not IL-4 primed AM in response to iH37Rv and LPS (**Figure 4C**). IFN-γ enhanced production of TNF by AM in response to both iH37Rv and LPS. In contrast, IFN-γ enhanced TNF in response to iH37Rv, but not LPS in MDM (**Figure 4C,4D**). LPS significantly upregulated the production of TNF in all MDM. Notably, IFN-γ priming did not enhance TNF production and IL-4 priming significantly attenuated LPS-induced TNF (**Figure 4D**). All stimulated AM secreted IL-10 regardless of priming (**Figure 4E**). IFN-γ significantly enhanced iH37Rv induced IL-10 in AM compared to unprimed or IL-4 primed comparators (**Figure 4E**). IL-4 priming of human AM significantly reduced IL-10 production in response to iH37Rv compared with unprimed AM (**Figure 4E**). LPS strongly induced IL-10 production in unprimed MDM, which was significantly attenuated by either IFN-γ or IL-4 priming (**Figure 4F**).

These data suggest that the AM has greater functional plasticity in terms of cytokine production in response to IFN-γ than the MDM, as IFN-γ primed AM had enhanced IL-10 and TNF production, in response to Mtb and LPS, respectively. In order to directly compare the human AM and MDM responses, fold change in cytokine production was calculated compared to the average of their respective iH37Rv (**Figure 4G**) or LPS (**Figure 4H**) stimulated unprimed control. IFN-γ enhanced human AM ability to secrete IL-1β, TNF and IL-10 in response to iH37Rv compared to MDM (**Figure 4G**). The IFN-γ primed human AM also has a significantly increased ability to secrete TNF and IL-10 in response to LPS compared to MDM (**Figure 4H**), however the difference in IL-10 secretion is more associated with MDM decreasing IL-10 when IFN-γ primed.

IFN-γ enhanced cytokine production is markedly more reliant on glycolysis in AM compared with MDM

Since IFN-γ drove glycolysis in both AM and MDM, we next sought to examine if cytokine production was associated with enhanced glycolysis. Human AM (**Figure 5A,5C,5E**) and MDM (**Figure 5B,5D,5F**) were primed with IFN-γ or IL-4 for 24 h or left unprimed. Macrophages were treated with 2DG (5 mM) for 1 h prior to stimulation with iH37Rv or LPS. Supernatants were harvested 24 h post stimulation and concentrations of IL-1β (**Figure 5A,5B**), TNF (**Figure 5C,5D**) and IL-10 (**Figure 5E,5F**) were quantified. 2DG significantly abrogated production of IL-1β in both IFN-γ primed AM and MDM stimulated with iH37Rv (**Figure 5A,5B**). Moreover, 2DG significantly reduced TNF production driven by IFN-γ in the AM, and significantly reduced TNF production in unprimed AM stimulated with iH37Rv (**Figure 5C**). Unlike IL-1β, TNF production was not affected by 2DG in unprimed or IFN-γ primed MDM. Conversely, IL-4 primed MDM exhibited increased TNF production in the presence of 2DG (**Figure 5D**). IL-10 production was significantly inhibited by 2DG in AM, irrespective of priming or stimulation (**Figure 5E**). MDM production of IL-10 in response to LPS or iH37Rv was inhibited with 2DG (**Figure 5F**).

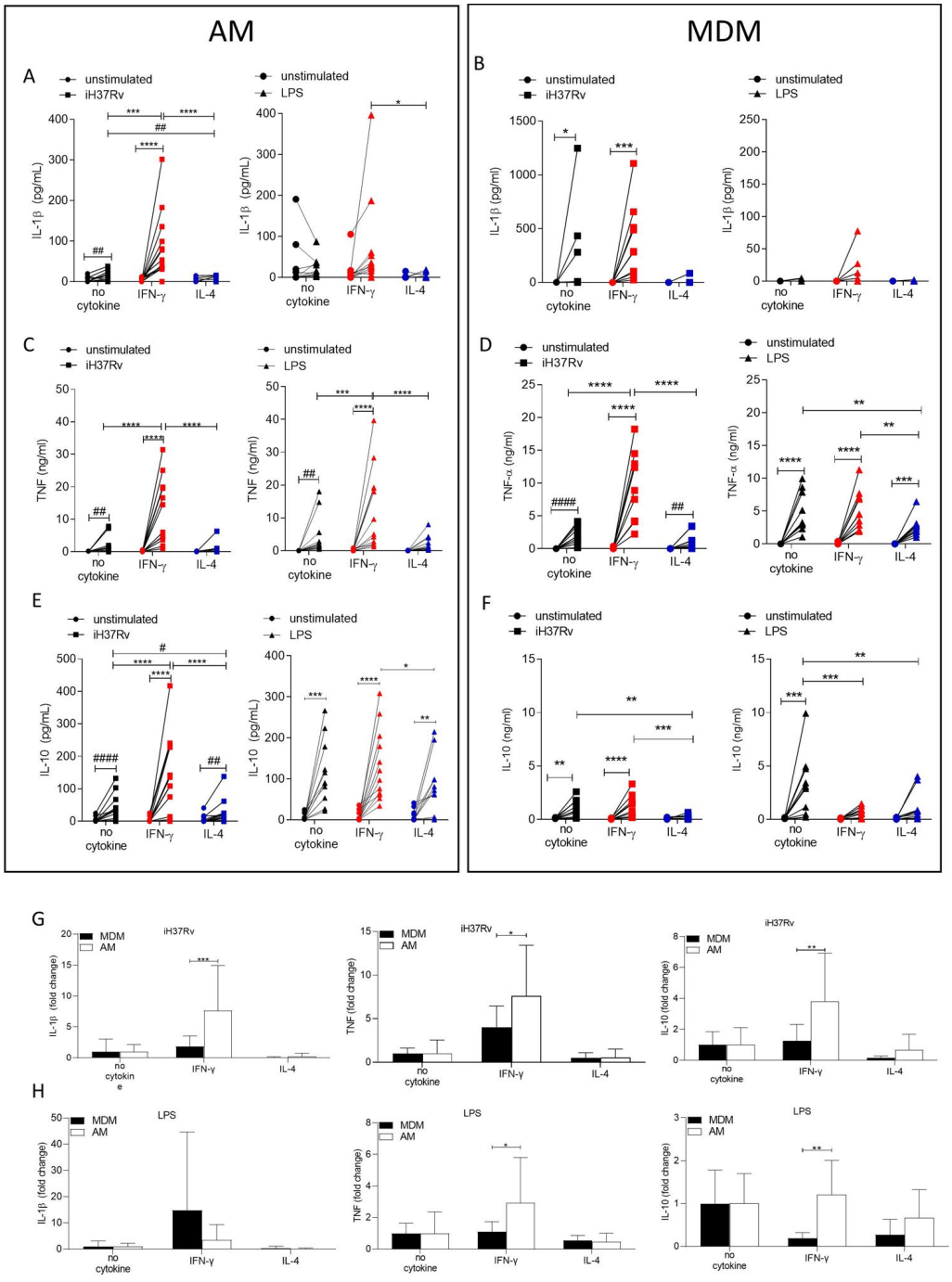

Figure 4.

IFN-γ enhances cytokine production more in AM compared with MDM. Human AM (A, C, E) isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and MDM (B, D, F) were differentiated and adherence purified for 7 days in 10% human serum. Cells were left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. AM or MDM were left unstimulated (circle) or stimulated iH37Rv (MOI 1-10; square) or LPS (100 ng/ml; triangle). Supernatants were harvested 24 h after stimulation and concentrations of IL-1β (A, B), TNF (C, D) and IL-10(E, F) were quantified by ELISA. Fold change in IL-1β, TNF and IL-10 was calculated for AM and MDM based on the average of respective no cytokine controls for iH37Rv (G) and LPS (H). Each linked data point represents the average of technical duplicates for one individual biological donor (AM; n=12-13, MDM; n=8-10). Statistically significant differences were determined using two-way ANOVA with a Tukey (A-F) or Bonferroni post-test (G-H); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 or #P≤0.05, ##P≤0.01, ####P≤0.0001 (where IFN-γ treated data sets were excluded for post-test analysis to analyse statistical differences between no cytokine and IL-4 treated data sets).

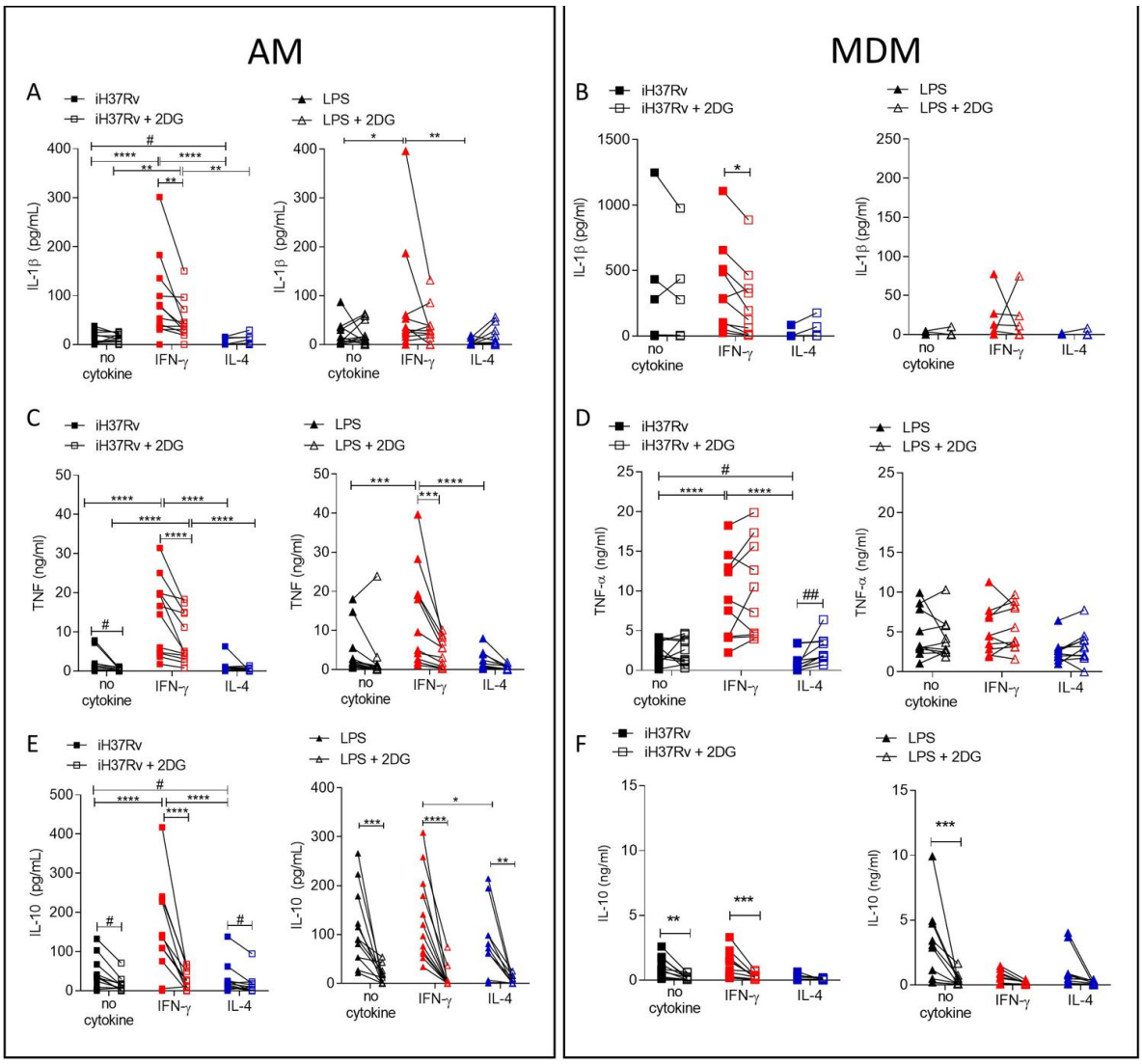

Figure 5.

Cytokine secretion by AM is more reliant on glycolysis than MDM.

Human AM (A, C, E) isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and MDM (B, D, F) were differentiated and adherence purified for 7 days in 10% human serum. Cells were left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. Cells were left untreated (solid) treated with 2DG (5 mM; empty) for 1 h prior to stimulation with iH37Rv (MOI 1-10; square) or LPS (100 ng/ml; triangle) or left unstimulated (circle). Supernatants were harvested 24 h after stimulation and concentrations of IL-1β (A, B), TNF (C, D) and IL-10(E, F) were quantified by ELISA. Each linked data point represents the average of technical duplicates for one individual biological donor (AM; n=12-13, MDM; n=8-10). Statistically significant differences were determined using two-way ANOVA with a Tukey post-test (A-D); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 or #P≤0.05, ##P≤0.01 (where IFN-γ primed data sets were excluded for post-test analysis to analyse statistical differences between no cytokine and IL-4 treated data sets).

In summary, IL-1β is under the control of glycolysis in IFN-γ primed AM and MDM. TNF production is strongly under the control of glycolysis in AM but not in MDM. Cumulatively these data indicate that IFN-γ promotes cytokine production in the AM via a process that is dependent on glycolysis. Consistent with the observation that IL-4 attenuated ECAR in LPS stimulated AM, IL-4 reduced cytokine production in the AM. Furthermore, while IL-10 was not associated with differential energetic profiles, its production is significantly attenuated by 2DG in both human macrophage populations, irrespective of priming. These data indicate that IFN-γ priming has a profound effect on AM function which is mediated, at least in part, by metabolic reprogramming.

Discussion

AM are the first responders to infections and inflammatory insults in the lung. We and others have reported that lung resident macrophages are dependent on glycolysis to respond to LPS (15) or Mtb (8, 14), however, their metabolic behaviour is distinct from murine AM (16) reinforcing the need to define cellular metabolism and its tractability in human macrophages in order to design effective immunometabolic therapies. We demonstrate here that human AM can be metabolically reprogrammed by IFN-γ which increased both glycolysis and oxidative phosphorylation and is further enhanced by stimulation with Mtb or LPS. This is in contrast with literature showing murine AM do not increase glycolysis in real-time following LPS stimulation (16), although whether IFN-γ can influence this in mice remains unknown. Our previous work shows that upon activation, the human MDM underwent “Warburg”-like metabolism associated with an increase in glycolysis with a concomitant reduction in oxidative phosphorylation (7). We confirmed this observation and this persists when primed with IFN-γ, unlike AM which do not undergo the shift to “Warburg”-like metabolism. IL-4 primed macrophages are associated in the literature with an increased reliance on oxidative phosphorylation (17). Indeed, murine IL-4 primed BMDM have increased ECAR and OCR (18, 19). IL-4 did not promote oxidative metabolism in human AM or MDM, even when stimulated. IL-4 decreased glycolysis while preventing a decline in oxidative metabolism in MDM, inhibiting their shift to “Warburg”-like metabolism. When directly comparing percentage change in ECAR of unprimed or macrophages primed with IFN-γ or IL-4, they all had similar rates of change despite profound differences in maximal ECAR. These data suggest that the resting state of the macrophage, and the cytokines it has recently been exposed to before activation, may be a key determining factor for the response and outcome to infections.

Directly comparing the AM with the MDM demonstrates that human AM are more reliant on oxidative metabolism upon IFN-γ priming and stimulation. Previous observations had identified a high baseline of lactate in supernatants of human AM compared to MDM (8). Interestingly, IFN-γ primed AM and MDM had significantly reduced capacity to increase ECAR (percentage change) in response to LPS stimulation, suggesting that there may be a point of maximal glycolysis. This ability to induce max glycolysis may be advantageous during infection as lactate, a breakdown product of glycolysis, has been shown to have anti-microbial functions against Mtb (13, 20). Moreover, pathogens such as Mtb can downregulate metabolic pathways after infection (21, 22) and IFN-γ is crucial for control of Mtb via glycolysis *in vivo* (23). Based on our current data, and as recently suggested by others (24), we speculate that control of Mtb in humans may be dependent on IFN-γ regulating glycolysis and not “Warburg”-like metabolism.

To our knowledge, we are the first to demonstrate that IFN-γ alone is sufficient to cause metabolic reprogramming of both lung resident AM and peripherally derived MDM. While other studies have demonstrated a role for IFN-γ inducing metabolic alterations in macrophages these studies have focused on murine macrophages (3, 23). In contrast evidence in murine BMDM indicates that alone IFN-γ does not increase glycolysis but that LPS was required (25). In addition, the use of LPS in combination with IFN-γ to polarise macrophages toward the an inflammatory phenotype is not a model easily translatable to humans, which are strikingly more

sensitive to LPS than mice (26) and the 'M1' macrophage elicited cannot be subsequently challenged with infectious agents, as the response is confounded by the initial LPS stimulation. Moreover, the use of LPS in addition to IFN-γ to polarise the macrophage towards the 'M1' phenotype is arguably not comparable with a macrophage that is polarised with IL-4 (or IL-10) in the absence of TLR stimuli. We wanted to assess the ability of IFN-γ alone to affect the function of human macrophages, to enable direct comparisons of macrophage subpopulations in order to fully assess the functional differences elicited in response to subsequent stimulation, in keeping with other human models (23).

AM expression of activation markers was more limited compared to MDM, even when primed and stimulated. AM upregulated only HLA-DR consistently in response to IFN-γ, broadly in keeping with murine AM (12, 27, 28). In contrast, MDM have a greater capacity to increase all activation markers in response to IFN-γ and stimulation. The response of IFN-γ primed MDM to Mtb was dependent on glycolysis for optimal activation marker expression, while LPS upregulated these markers independently of glycolysis, irrespective of cytokine priming. In contrast the AM was dependent on glycolysis for upregulation of these markers in response to LPS and not Mtb. These data once again suggest a differential role of glycolysis within human macrophages.

Glycolytic metabolism in macrophages has been intrinsically linked to cytokine production, particularly IL-1β (3, 5, 8, 10, 29). We have demonstrated that increased ECAR early in MDM activation is associated with increased secretion of IL-1β in both MDM and AM (9). Moreover IFN-γ can promote IL-1β by inhibiting miR-21, a negative regulator of glycolysis in both human MDM and murine BMDM (30). Our data builds on the observation that IFN-γ upregulates pro-IL-1β by a glycolytic dependent mechanism in murine BMDM (3), by demonstrating the increased secretion of mature IL-1β by IFN-γ primed human macrophages. Moreover, we have confirmed that IL-1β secretion is dependent on glycolysis in IFN-γ primed human AM and MDM, which is line with data from murine BMDM (23). In the current study we also demonstrate that 2DG can reduce both IL-1β and IL-10 secretion by IFN-γ primed AM and MDM in response to Mtb. Moreover, 2DG inhibited LPS induced IL-10 in AM and unprimed MDM. 2DG has previously been shown to inhibit IL-10 production by LPS stimulated human MDM (4), however, restricting glycolysis using glucose free medium inhibited IL-1β but promoted IL-10 secretion (8). Furthermore, IL-10 production is inhibited by IFN-γ in human MDM, which is similar to previous data in IFN-γ primed murine BMDM where IL-10 secretion was also inhibited (31). However, our data also demonstrates that IFN-γ does not inhibit or promote IL-10 in human AM stimulated with LPS even though interestingly, IFN-γ primed AM had increased TNF in response to LPS. This suggests there may be additional pathways involved in IL-10 secretion by human macrophages, which is supported by reductions in IL-10 secretion by IL-4 primed AM, which are not metabolically altered.

TNF is crucial to control infections such as Mtb (32–35). We demonstrate that IFN-γ enhances TNF production in response to Mtb stimulation in human MDM and AM, however AM have a much greater ability to increase TNF. Moreover, IFN-γ primed AM stimulated with Mtb have significantly more production of IL-1β, TNF and IL-10 compared with unprimed controls. This is in contrast with IFN-γ primed MDM which only upregulate TNF compared to their unprimed controls. These data indicate that effective immune responses to Mtb in the lung may require AM to be primed with IFN-γ and may in part explain why patients deficient in IFN-γ or associated signalling have increased risk of TB (36, 37). IFN-γ driven production of TNF is dependent on glycolysis in AM. MDM secretion of TNF is independent of glycolysis and conversely, inhibition of glycolysis in IL-4 primed Mtb stimulated MDM enhanced TNF production. Previous studies have demonstrated that glycolysis was required by murine BMDM but not AM to secrete TNF and IL-6 (16). Conversely, our data demonstrates that human AM need glycolysis for optimal TNF production, especially in the presence of IFN-γ, whereas MDM do not. Once again, we highlight that there is variation in the metabolic requirements within human macrophage subpopulations, and importantly, that the AM is metabolically tractable to modulate its function.

Whether the AM can respond to IL-4 has been debated (38). Here we demonstrate that the human AM can respond to IL-4 with evidence that IL-4 reduced glycolysis in response to LPS stimulation. In addition, AM were functionally altered by IL-4 resulting in reduced IL-1β and IL-10 production and upregulated CD86. These data provide evidence that the human AM is capable of responding to IL-4 which may inform type 2 lung immunity, and susceptibility to infection in patients with asthma, for example.

Trained immunity improves innate responses to infection and is emerging as a key component of host directed therapies (HDT) and strategies to improve vaccine efficacy and the design of respiratory mucosal vaccines (27, 28, 39–41). Both IFN-γ and IL-4 can induce trained immunity in murine and human macrophages (12, 19, 42, 43). MDM trained with IFN-γ and LPS, and stimulated with Mtb had increased TNF in the acute activation phase of trained immunity (19), which we observed in IFN-γ primed MDM subsequently stimulated with Mtb. We also observed an increase in TNF, IL-1β and IL-10 in AM potentially suggesting that AM will be a target for innate training, as increased IL-1β is associated with optimal training (39, 43, 44). AM from mice that received an inhaled adenovirus vectored vaccine undergo trained immunity mediated by IFN-γ resulting in elevated MHC-II expression, enhanced cytokine production, and protection against specific and non-specific infection challenges (12, 27). We have previously demonstrated that an adenovirus vectored vaccine induces trained immunity in human monocytes and postulated that this may be IFN-γ dependent (45). The current study provides evidence that IFN-γ can metabolically reprogramme the human AM, resulting in enhanced HLA-DR expression and cytokine production in response to subsequent stimulation. Cumulatively this highlights the importance of ascertaining whether IFN-γ can induce trained immunity in the human AM, which may enhance the design of respiratory mucosal vaccines.

Immune augmentation therapies delivered directly to the lung are necessary to help combat the growing threat of drug resistant pathogens, including Mtb. We have demonstrated such approaches both *in vitro* and *in vivo* (9, 10, 46–49). Clinical trials have indicated that nebulized IFN-γ is a viable HDT to help combat Mtb (50, 51) and is in clinical trials for sepsis (*https://clinicaltrials.gov/study/NCT04990232*). Our data supports the use of inhalable IFN-γ as an immuno-supportive therapy which modulates metabolic responses. Moreover, our data indicates that IFN-γ affects metabolism and cytokine secretion in AM significantly more than MDM which lends support for the therapeutic strategy of delivering IFN-γ to the lung, by targeting the macrophage population that most need immune augmentation (1) and limiting potential side effects.

Study limitations

We acknowledge that our *in vitro* model is simplified and may not fully reflect macrophages *in vivo*. Nevertheless, these data address knowledge gaps in human macrophage biology and are required to aid the translation of immunometabolism into clinical benefits in respiratory medicine. We used LPS and irradiated Mtb to model successful macrophage responses to infection. Future experiments should examine how virulent respiratory pathogens such as gram-negative *Pseudomonas aeruginosa, Klebsiella pneumoniae* and Mtb effect human AM in Th1 or Th2 environments, to determine infection-specific effects.

The inhibition of glycolysis with 2DG cannot definitively link all observations solely to glycolysis, as limiting glycolysis will ultimately limit oxidative phosphorylation. Blocking oxidative phosphorylation with oligomycin reduced LPS induced cytokine secretion in the human AM and not MDM (52), both glycolysis and oxidative phosphorylation may therefore be needed for optimal AM function. The concentration of 2DG used only partially inhibited glycolysis, however ablation of glycolysis induces significant cytotoxicity and confounds assay outcomes. Therefore, where 2DG had no effect, a role for glycolysis cannot be definitively excluded. Furthermore, only one dose of IFN-γ was utilised due to limitations in AM yield, however, recently both low and high doses of IFN-γ have been shown to have similar effects on AM *in vitro* (53).

Establishing the immunometabolic and functional outputs of human macrophages will aid in future work examining the plasticity of the human AM. While we have established herein that the human AM is plastic in response to IFN-γ, since the AM is yolk-sac derived and long-lived, this raises the question of whether the plasticity of the AM can allow multiple sequential changes to respond and adapt to changing microenvironments in the lung. Another question raised is whether other tissue resident macrophages behave similarly to AM or whether they have unique responses.

Conclusion

Human AM and infiltrating MDM both increase glycolysis and oxidative phosphorylation in response to IFN-γ, and stimulation results in a further increase in glycolysis. Cumulatively, the data presented herein suggests that the MDM maybe more phenotypically plastic than the AM, while the AM have enhanced functional plasticity in their ability to produce cytokine after exposure Th1 and Th2 cytokines. Our data supports the hypothesis that there may be distinct roles for AM and infiltrating MDM during infection since IFN-γ increased metabolic responses are mechanistically associated with different cellular functions. Our data demonstrates that cytokine production in human AM can be promoted by supporting cellular metabolism, thus providing evidence that human tissue resident AM are a tractable target for host-directed immuno-supportive adjunctive therapies.

Ethics statement

All research herein was carried out in accordance with the Declaration of Helsinki and ethically approved, as outlined in the materials and methods section.

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Author contributions

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SAC data acquisition, analysis and interpretation, critical revision of the article.

COM data acquisition, analysis and interpretation, critical revision of the article. AIB data acquisition, analysis and interpretation.

OST data acquisition. ED data acquisition. KMG data acquisition. OOG data acquisition. DMM data acquisition. SAOR data acquisition. FOC data acquisition. PN data acquisition.

JJP conception and design, analysis and interpretation, critical revision of the article. LEG analysis and interpretation, critical revision of the article.

SAB conception and design, data acquisition, analysis and interpretation, original draft of the article, critical revision of the article, funding acquisition, final approval of the article.

JK conception and design, funding acquisition, final approval of the article.

All authors have approved the final version of this article.

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Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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48. O'Connor G, Krishnan N, Fagan-Murphy A, Cassidy J, O'Leary S, Robertson BD, Keane J, O'Sullivan MP, Cryan S-A. (2019) **Inhalable poly(lactic-co-glycolic acid) (PLGA) microparticles encapsulating all-trans-Retinoic acid (ATRA) as a host-directed, adjunctive treatment for Mycobacterium tuberculosis infection** *European Journal of Pharmaceutics and Biopharmaceutics* **134**:153–165

49. Lawlor C, O'Connor G, O'Leary S, Gallagher PJ, Cryan S-A, Keane J, O'Sullivan MP. (2016) **Treatment of Mycobacterium tuberculosis-Infected Macrophages with Poly(Lactic-Co-Glycolic Acid) Microparticles Drives NFκB and Autophagy Dependent Bacillary Killing** *PLoS One* **11**:e0149167–e0149167

50. Dawson R *et al.* (2009) **Immunomodulation with recombinant interferon-gamma1b in pulmonary tuberculosis** *PLoS One* **4**

51. Bharti R *et al.* (2022) **Transient, inhaled gene therapy with gamma interferon mitigates pathology induced by host response in a mouse model of tuberculosis** *Tuberculosis (Edinburgh)* **134**

52. Pereverzeva L *et al.* (2022) **Human alveolar macrophages do not rely on glucose metabolism upon activation by lipopolysaccharide** *Biochim Biophys Acta Mol Basis Dis* **1868**

53. Thiel BA *et al.* (2024) **Human alveolar macrophages display marked hypo-responsiveness to IFN-γ in both proteomic and gene expression analysis** *PLoS One* **19**

Editors

Reviewer #1 (Public review):

Summary:

The researchers demonstrated that when cytokine priming is combined with exposure to pathogens or pathogen-associated molecular patterns, human alveolar macrophages and

monocyte-derived macrophages undergo metabolic adaptations, becoming more glycolytic while reducing oxidative phosphorylation. This metabolic plasticity is more in monocyte derived macrophages as compared to alveolar macrophages.

Strengths:

This study presents evidence of metabolic reprogramming in human macrophages, which significantly contributes to our existing understanding of this field primarily derived from murine models.

https://doi.org/10.7554/eLife.98449.2.sa3

Reviewer #2 (Public review):

Summary:

The current study is presented to assess the shift in metabolism (Glycolysis and Oxidative phosphorylation) of differently primed human Alveolar macrophages and Monocyte derived macrophages in response to TLR4 activating signals (such as LPS and dead Mtb bacteria). They conducted this macrophage characterization in response to type II interferon and IL-4 priming signals, followed by different stimuli of irradiated Mycobacterium tuberculosis and LPS.

Strengths:

(1) The study employs thorough measurement of metabolic shift in metabolism by assessing extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of differentially polarized primary human macrophages using the Seahorse XFe24 Analyzer.
(2) The effect of differential metabolic shift on the expression of different surface markers for macrophage activation is evaluated through immunofluorescence flow cytometry and cytokine measurement via ELISA.

Weaknesses:

(1) Prior studies with human macrophages have shown a glycolytic shift with similar signals, including live Mycobacterium tuberculosis infection.
(2) Results are often described with detailed methodology for each experiment, and data are replotted and presented in duplicates for cross-analyses which can be confusing.
(3) The data presented shows a distinct functional profile of airway macrophages (AMs) compared to monocyte (blood)-derived macrophages (MDMs) in response to the same priming signals. However, the study does not attempt to explore the underlying mechanisms for this difference.

Appraisal:

(1) The authors have achieved their aim of preliminarily characterizing the glycolysis-dependent cytokine profile and activation marker expression of IFN-g and IL-4 primed primary human macrophages.
(2) The results of the study support its conclusion of glycolysis-dependent phenotypical differences in cytokine secretion and activation marker expression of AMs and MDMs.
(3) However, the study is descriptive in nature, and the results validate IFN-g-mediated glycolytic reprogramming in primary human macrophages without providing mechanistic insights.

Impact:

The study provides evidence of metabolic reprogramming in human primary macrophages and their dependence on glycolysis for downstream secretion of cytokines and expression of activation markers.

Additional comments:

The results of this study are generated from a very large experiment with different treatments and phenotypic characterization. The data is plotted and analyzed in different figures to aid the reader.

https://doi.org/10.7554/eLife.98449.2.sa2

Reviewer #3 (Public review):

Summary:

In this manuscript the authors explore the contribution of metabolism to the response of two subpopulations of macrophages to bacterial pathogens commonly encountered in the human lung, as well as the influence of priming signals typically produced at a site of inflammation. The two subpopulations are resident airway macrophages (AM) isolated via bronchoalveolar lavage and monocyte-derived macrophages (MDM) isolated from human blood and differentiated using human serum. The two cell types were primed using IFNγ and Il-4, which are produced at sites of inflammation as part of initiation and resolution of inflammation respectively, followed by stimulation with either heat-killed tuberculosis (Mtb) or LPS to simulate interaction with a bacterial pathogen that is either gram-negative in the case of Mtb or gram-positive in the case of LPS. The authors use human cells for this work, which makes use of widely reported and thoroughly described priming signals, as well as model antigens. This makes the observations on the functional response of these two subpopulations relevant to human health and disease to a greater extent that the mouse models typically used to interrogate theses interactions. To examine the relationship between metabolism and functional response, the authors measure rates of oxidative phosphorylation and glycolysis under baseline conditions, primed using IFNγ or IL-4, and primed and stimulated with Mtb or LPS.

The authors addressed most of the initial critiques. The dose of IFNγ used was justified, figure legends were harmonized, a contextual definition was provided for the term "functional plasticity," and the airway macrophage population was partially characterized by flow cytometry. However, some concerns remain relating to the clarity of methods and use of statistics. The authors have not adequately explained how % change was calculated in Figure 1, in either the figure legend or the methods section. Additionally, the use of multiple statistical analyses on the same data set in figures 4 and 5, with data exclusion resulting in lower p values, is not satisfactorily justified.

Strengths:

• The data indicate that both populations of macrophages increase metabolic rates when primed, but MDMs decrease their rates of oxidative phosphorylation after IL-4 priming and bacterial exposure while AMs do not.

• It is demonstrated that glycolysis rates are directly linked to the expression of surface molecules involved in T-cell stimulation and while secretion of TNFα in AM is dependent on glycolysis, in MDM this is not the case. IL-10 secretion does not appear regulated by glycolysis in either population. It is also demonstrated that Mtb and LPS stimulation produces responses that are not metabolically consistent across the two macrophage populations. The Mtb-induced response in MDMs differed from the LPS response, in that it relies on glycolysis,

while this relationship is reversed in AMs. The difference in metabolic contributions to functional outcomes between these two macrophage populations is significant, despite acknowledgement of the reductive nature of the system by the authors.

• The observations that AM and MDM rely on glycolysis for production of cytokines during a response to bacterial pathogens in the lung, but that only AM shift to Warburg Metabolism following exposure to IL-4, are supported by the data and a significant contribution the study of the innate immune response.

Weaknesses:

Critiques:

• It is still difficult to interpret the metabolism data due to inconsistent normalization. It appears that in the case of rate measurement the data is normalized to unstimulated macrophages where values are set to one, but in the case of % change the values from unstimulated cells are not set to 100% and the methods say that values were calculated using primed controls, which is ambiguous. It is therefore unclear how exactly the % change values were determined. This makes it difficult to conclude whether the changes in glycolysis and oxidative phosphorylation in primed cells after stimulation are proportional to changes in unprimed cells. This would suggest that the majority of the observed effect on metabolism comes from priming itself and not from the subsequent stimulation as the authors claim.

• The use of repeated statistical analyses with different comparison groups in the same figure/data set (e.g., in Fig.4) is still not justified. The current approach, using two-way ANOVA, removing a third of the dataset, and then applying another two-way ANOVA, produces the desired p values, but is not appropriate.

Conclusion:

Overall, this study reveals how inflammatory and anti-inflammatory cytokine priming contributes to the metabolic reprogramming of AM and MDM populations. Their conclusions regarding the relationship between cytokine secretion and inflammatory molecule expression in response to bacterial stimuli are supported by the data. The involvement of metabolism in innate immune cell function is relevant when devising treatment strategies that target the innate immune response during infection. The data presented in this paper further our understanding of that relationship and advance the field of innate immune cell biology.

https://doi.org/10.7554/eLife.98449.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The researchers demonstrated that when cytokine priming is combined with exposure to pathogens or pathogen-associated molecular patterns, human alveolar macrophages and monocyte-derived macrophages undergo metabolic adaptations, becoming more glycolytic while reducing oxidative phosphorylation. This metabolic plasticity is greater in monocyte-derived macrophages than in alveolar macrophages.*

> *Strengths:*
>
> *This study presents evidence of metabolic reprogramming in human macrophages, which significantly contributes to our existing understanding of this field primarily derived from murine models.*
>
> *Weaknesses:*
>
> *The study has limited conceptual novelty.*

We acknowledge that the study has limited conceptual novelty, however, the current manuscript provides the field with evidence of the changes in the phenotype and functions of human macrophages in response to IFN-γ or IL-4 which is currently lacking in the literature. Moreover, our data shows for the first time that human airway macrophages change their function in response to IFN-γ.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The authors aimed to functionally characterize primary human airway macrophages and monocytederived macrophages, correlating their glycolytic shift in metabolism. They conducted this macrophage characterization in response to type II interferon and IL-4 priming signals, followed by different stimuli of irradiated Mycobacterium tuberculosis and LPS.*
>
> *Strengths:*
>
> *(1) The study employs a thorough measurement of metabolic shift in metabolism by assessing extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of differentially polarized primary human macrophages using the Seahorse XFe24 Analyzer.*
>
> *(2) The effect of differential metabolic shift on the expression of different surface markers for macrophage activation is evaluated through immunofluorescence flow cytometry and cytokine measurement via ELISA.*
>
> *(3) The authors have achieved their aim of preliminarily characterizing the glycolysis-dependent cytokine profile and activation marker expression of IFN-g and IL-4 primed primary human macrophages.*
>
> *(4) The results of the study support its conclusion of glycolysis-dependent phenotypical differences in cytokine secretion and activation marker expression of Ams and MDMs.*
>
> *Weaknesses:*
>
> *(1) The data are presented in duplicates for cross-analyses.*
>
> *(2) The data presented supports a distinct functional profile of airway macrophages (Ams) compared to monocyte (blood)-derived macrophages (MDMs) in response to the same priming signals. However, the study does not attempt to explore the underlying mechanism for this difference.*
>
> *(3) The study is descriptive in nature, and the results validate IFN-g-mediated glycolytic reprogramming in primary human macrophages without providing mechanistic insights.*

(1) We acknowledge the data is presented in duplicate for cross-analyses. This duplication allowed us to examine both (A) the effect of IFN-γ or IL-4 on primary human airway and monocyte derived macrophages in the presence or absence of distinct stimulations and (B) to

directly compare the fold change in function occurring in the AM with the changes in the MDM.

(2 & 3) We acknowledge that our study is descriptive however, by inhibiting glycolysis using 2DG we have demonstrated that increased flux through glycolysis is mechanistically required to mediate enhanced cytokine responses in both primary human AM and MDM primed with IFN-γ. However, we acknowledge that we have not determined the differential molecular mechanisms downstream of IFNγ in the AM versus the MDM. IFN-γ promotes both pro- and anti-inflammatory cytokines in AM and this was reduced by inhibiting glycolysis with 2DG. This identifies glycolysis as a key mechanistic pathway which can be therapeutically targeted in AM to modulate inflammation. Mechanistic studies on human AM are limited due to low number of AM retrieved from BAL samples. Nevertheless, the differences between AM and MDM identified in the current study indicate that future mechanistic studies are warranted to identify why IFN-γ promotes IL-10 in AM and not MDM, and, why TNF is differentially regulated by glycolysis in the two macrophage subpopulations, for example.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In this manuscript, the authors explore the contribution of metabolism to the response of two subpopulations of macrophages to bacterial pathogens commonly encountered in the human lung, as well as the influence of priming signals typically produced at a site of inflammation. The two subpopulations are resident airway macrophages (AM) isolated via bronchoalveolar lavage and monocyte-derived macrophages (MDM) isolated from human blood and differentiated using human serum. The two cell types were primed using IFNγ and Il-4, which are produced at sites of inflammation as part of initiation and resolution of inflammation respectively, followed by stimulation with either irradiated Mycobacterium tuberculosis (Mtb) or LPS to simulate interaction with a bacterial pathogen. The authors use human cells for this work, which makes use of widely reported and thoroughly described priming signals, as well as model antigens. This makes the observations on the functional response of these two subpopulations relevant to human health and disease. To examine the relationship between metabolism and functional response, the authors measure rates of oxidative phosphorylation and glycolysis under baseline conditions, primed using IFNγ or IL-4, and primed and stimulated with Mtb or LPS.*
>
> *Strengths:*
>
> *• The data indicate that both populations of macrophages increase metabolic rates when primed, but MDMs decrease their rates of oxidative phosphorylation after IL-4 priming and bacterial exposure while AMs do not.*
>
> *• It is demonstrated that glycolysis rates are directly linked to the expression of surface molecules involved in T-cell stimulation and while secretion of TNFα in AM is dependent on glycolysis, in MDM this is not the case. IL-1β is regulated by glycolysis only after IFN-γ priming in both MDM and AM populations. It is also demonstrated that Mtb and LPS stimulation produces responses that are not metabolically consistent across the two macrophage populations. The Mtb-induced response in MDMs differed from the LPS response, in that it relies on glycolysis, while this relationship is reversed in AMs. The difference in metabolic contributions to functional outcomes between these two macrophage populations is significant, despite acknowledgement of the reductive nature of the system by the authors.*
>
> *• The observations that AM and MDM rely on glycolysis for the production of cytokines during a response to bacterial pathogens in the lung, but that only MDM shift to*

> *Warburg Metabolism, though this shift is blocked following exposure to IL-4, are supported by the data and a significant contribution the study of the innate immune response.*
>
> *Weaknesses:*
>
> *• It is unclear whether changes in glycolysis and oxidative phosphorylation in primed cells are due to priming or subsequent treatments. ECAR and OCR analyses were therefore difficult to interpret.*

All data sets have been presented and analysed relative to both unprimed unstimulated to show both the effect of priming and subsequent stimulation. A second analysis was subsequently conducted where each data set was normalised to its own baseline in terms of percentage change. Therefore, each of unprimed, IFN-γ and IL-4 primed cells were set to 100% in order to assess the effect of stimulation independent of the baseline priming effect. For clarity we have removed the following line:

“Percentage change for ECAR and OCR was calculated from the respective baseline of each data set to visualise the differential ability of IFN-γ, IL-4 primed or unprimed AM to respond to stimulation (Figure S1C,D).”

We have amended the text in the manuscript (lines 164-173) to “Since IFN-γ priming increased cellular energetics in the AM at baseline, we calculated percent change in ECAR and OCR from the baseline rate of each group in order to assess if IFN-γ or IL-4 primed AM have altered capacity to change their metabolism in response to stimulation (Figure 1C,D). This was carried out to equalise all the primed data sets at baseline before stimulation (Figure S1C, S1D). These data indicate that whilst the peak of glycolysis is elevated in IFN-γ primed AM (Figure 1A), all AM have a similar capacity to increase glycolysis upon stimulation when baseline differences in metabolism were adjusted for the effects of cytokine priming (Figure 1C). IFN-γ increased the percent change in OCR of AM in response to both bacterial stimuli compared to the unstimulated IFN-γ primed control (Figure 1D). These data indicate that priming AM alters the metabolic baselines of human tissue resident macrophages and not their ability to respond to bacterial stimuli.”

> *• The data may not support a claim that AM has greater "functional plasticity" without a direct comparison of antigen presentation. Moreover, MDM secrete more IL-1β than AM. The claim that AM "have increased ability to produce all cytokines assayed in response to Mtb stimulation" does not appear to be supported by the data.*

Our data suggests that the MDM are more phenotypically plastic (in terms of their ability to alter expression of cell surface markers in response to cytokine cues), whereas AM have a greater ability to alter cytokine production, our measure of functional plasticity. We have now defined the use of the terms ‘functional plasticity’ and ‘phenotypic plasticity’ in the context of our paper in lines 6063. To consider different culture and plating requirements of MDM versus AM, cytokine production was analysed relative to the average of the unprimed Mtb or LPS control of the respective MDM or AM. This allowed us to draw more accurate comparisons between the two macrophage populations by examining their relative ability to increase their cytokine production (expressed as fold change) rather than defining this functional plasticity only in terms of concentrations of cytokine produced in culture.

We have therefore added the following sentence into the conclusion of the manuscript. “Cumulatively, the data presented herein suggests that the MDM maybe more phenotypically plastic than the AM, while the AM have enhanced functional plasticity in their ability to modulate cytokine production after exposure Th1 and Th2 cytokines.”

We have edited the discussion (lines 421-423) to clarify the following "have increased ability to produce all cytokines assayed in response to Mtb stimulation" and changed it to “stimulated with Mtb have significantly more production of IL-1β, TNF and IL-10 compared with unprimed controls. This is in contrast with IFN-γ primed MDM which only upregulate TNF compared to their unprimed controls.”

> *• The claim that AM are better for "innate training" via IFNγ may not be consistent with increased IL1β and a later claim that MDM have increased production and are "associated with optimal training."*

We have removed the word “better” and now simply state that AM are a tractable target to induce innate training in the human lung.

> *• Statistical analyses may not appropriately support some of the conclusions.*

We have consulted with a statistician. Please see response to reviewer 3 recommendations for authors point 1 below.

> *• AM populations would benefit from further definition-presumably this is a heterogenous, mixed population.*

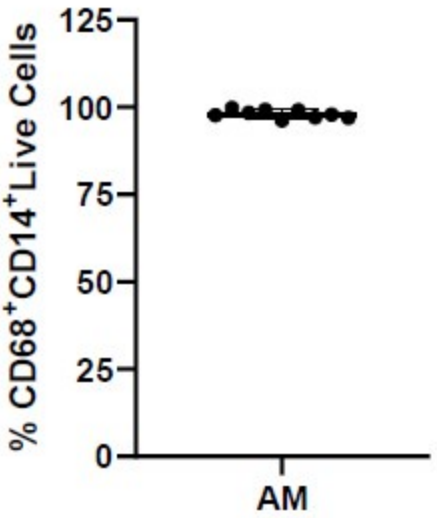
AM are routinely >97% CD68+CD14+ used in the current study (Author response image 1). However, we acknowledge that tissue resident macrophages represent a spectrum of phenotypes. Given limitations in cell numbers from primary human AM derived from BALF, we have not attempted to define the function of discreet subpopulations of AM.

> *• The term "functional plasticity" could also be more stringently defined for the purposes of this study.*

We are terming functional plasticity to be the macrophages’ ability to alter their production of cytokines in response to external cues like IFN-γ and IL-4 whereas phenotypic plasticity is measured based on ability to alter the cell surface expression of activation markers. We have now defined this in the manuscript (lines 60-63).

Author response image 1.

Expression of macrophage markers on AM.



> *Conclusion:*
>
> *Overall, the authors succeed in their goals of investigating how inflammatory and anti-inflammatory cytokine priming contributes to the metabolic reprogramming of AM and MDM populations. Their conclusions regarding the relationship between cytokine secretion and inflammatory molecule expression in response to bacterial stimuli are supported by the data. The involvement of metabolism in innate immune cell function is relevant when devising treatment strategies that target the innate immune response during infection. The data presented in this paper further our understanding of that relationship and advance the field of innate immune cell biology.*
>
> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) Authors are suggested to provide rationale for their choice of cytokines as IFN-gamma and IL-4. This will be useful for the readers.*

We have updated the following sentence (line 44-46) in the manuscript to add more rationale for the choice of IFN-γ and IL-4. "There is a paucity of data on the role of metabolism in response to Th1 or Th2 microenvironments induced by cytokines-such as IFN-γ or IL-4 respectively, in human macrophages, especially in tissue resident macrophages, such as AM."

> *(2) Authors have shown the final outcome of metabolic reprogramming in terms of expression of HLADR and CD-40, and cytokine release. What pathways/receptors are activated or associated with IL-4 and IFN-gamma priming as a first line of response?*

The relationship between IFN-γ or IL-4 induced expression of CD40 is established in haematological cell lines and fibroblasts as well as APC, with roles for the JAK/STAT pathways and upregulation of IRFs defined (1-3). Similarly, the relationship between exogenous IFN-γ and upregulation of HLA-DR expression on human monocytes or endothelial cells is established (4, 5). Whist our work does not outline the signalling pathways downstream of Th1 or Th2 cytokine priming, we have shown for the first time that glycolysis mechanistically underpins the shift in phenotype and function observed in human macrophages upon priming with IFN-γ or IL-4.

> *(3) What are the intracellular signals leading to glycolytic shift?*

One of the most likely mechanisms that under pin the shift to glycolytic metabolism is the stabilisation of HIF-1α mediated by activation of mTOR (see response below and rebuttal figure 2).

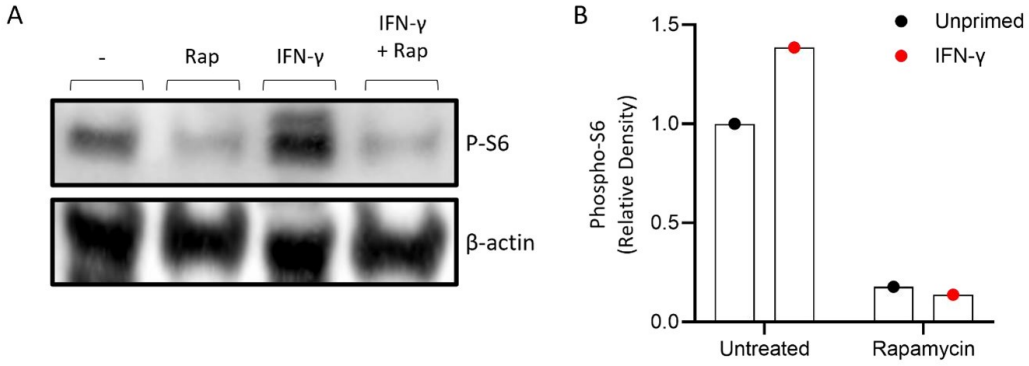
> *(4) Additional evidence is required to show Warburg effect such as stabilization and activation of HIF1alpha.*

We acknowledge that we have not shown the activation and stabilisation of HIF-1α, however, we have provided functional evidence of increased glycolysis with concomitant decreased oxidative phosphorylation indicative of Warburg metabolism.

In order to address this gap in evidence we have reworded the manuscript to describe this functional change to "Warburg-like metabolism" throughout the manuscript. In addition, we have undertaken Western Blotting to provide evidence of mTOR activation when cells are primed with IFN-γ (Author response image 2).

Author response image 2.

IFN-γ activates mTOR in primary human monocytes. Monocytes were isolated from healthy donor PBMC using magnetic separation. Monocytes were left untreated (-), stimulated with rapamycin as a negative control (Rap; 50 nM), IFN-γ (10 ng/ml) or IFN-γ and rapamycin simultaneously (IFN-γ + Rap) for 15 minutes. Phosphorylation of S6 was used as a readout of mTOR activation and measured by western blot using β-actin as a control with a blot (A) and (b) densitometry results are shown as the relative expression of pS6: β-actin from. Graphs show data of n=1 of unprimed (black dot) vs IFN-γ primed (red) with and without rapamycin. ImageLab (Bio-Rad) software was used to perform densitometric analysis.



> *(5) What is the importance of showing percentage change vs fold change in figure 1 (1C vs 1A)?*

All data sets have been presented and analysed relative to both unprimed unstimulated to show the effect of first priming and subsequent stimulation (Figure 1A). A second analysis was subsequently conducted where each data set was normalised to its own baseline in terms of percentage change (Figure 1C). Therefore, each of unprimed, IFN-γ or IL-4 primed cells were set to 100% to assess the effect of stimulation independent of the pre-existing effect of priming on the baseline metabolism. For clarity we have removed the following line:

"Percentage change for ECAR and OCR was calculated from the respective baseline of each data set to visualise the differential ability of IFN-γ, IL-4 primed or unprimed AM to respond to stimulation (Figure S1C,D)."

We have amended the text (lines 164-173) in the manuscript to "Since IFN-γ priming increased cellular energetics in the AM at baseline, we calculated percent change in ECAR and OCR from the baseline rate of each group in order to assess if IFN-γ or IL-4 primed AM have altered capacity to change their metabolism in response to stimulation (Figure 1C,D). This was carried out to equalise all the primed data sets at baseline before stimulation (Figure S1C, S1D). These data indicate that whilst the peak of glycolysis is elevated in IFN-γ primed AM (Figure S1A), all AM have a similar capacity to increase glycolysis upon stimulation when baseline differences in metabolism were adjusted for the effects of cytokine priming (Figure 1C). IFN-γ increased the percent change in OCR of AM in response to both bacterial stimuli compared to the unstimulated IFN-γ primed control (Figure 1D). These data indicate that priming AM alters the metabolic baselines of human tissue resident macrophages and not their ability to respond to bacterial stimuli."

> *(6) Why IL-4 primed cells have lower glycolysis than unprimed control cells even in absence of pathogen in Figure 1A?*

IL-4 primed AM do not have statistically significant changes in glycolysis compared with unprimed control cells in the absence of stimulation.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *The manuscript entitled "Human airway macrophages are metabolically reprogrammed by IFN-γ resulting in glycolysis dependent functional plasticity" by Cox et al., characterizes glycolytic-linked cytokine secretion and surface receptor expression of primary human airway macrophages (AM) and monocyte-derived macrophages (MDM). The authors primed the primary macrophages with type II interferon (IFN-γ) or interleukin-4 (IL-4) into Th1 and Th2 polarized states. This was followed by measurement of the shift in macrophage metabolism to glycolysis (ECAR measurement) and/or oxidative phosphorylation (OCR measurement) in response to lipopolysaccharide and irradiated Mycobacterium tuberculosis. The authors then utilize 2-DG (an inhibitor of glycolysis) to show the reliance of glycolytic shift in metabolism to drive the expression of different macrophage activation markers in MDMs and cytokine secretion in AMs.*
>
> *Significance:*
>
> *The study provides important validation of IFN-γ-mediated glycolytic shift and its correlated functionalities in primary human macrophage populations.*
>
> *Highlights: The study characterizes glycolytic-linked cytokine secretion and expression of macrophage activation markers in primary human resident (lung) and monocyte (blood)-derived macrophages. The study also shows data in support of IFN-γ alone in mediating glycolytic reprogramming of human primary macrophages.*
>
> *Limitations:*
>
> *The study lacks novelty and does not provide any new or different information in relation to IFN-γmediated glycolytic shift in the metabolism of human macrophages.*
>
> *Major comments:*
>
> *(1) The authors have relied on irradiated Mycobacterium tuberculosis (Mtb) and LPS stimulation to measure different correlates of macrophage functions. Additionally, the*

> *authors have discussed their results with irradiated Mtb with that of infection with live Mtb. There are also recent reports that show Mtb infection limiting glycolytic reprogramming in murine and human macrophages (PMID: 31914380) in contrast to their observation with irradiated Mtb. The authors should also include live Mtb infection or other replicative live bacterium for the induction of surface activation markers and cytokine release in their setup.*

We thank the reviewer for this suggestion; however, this is beyond the scope of the current study which was to assess AM and MDM in the context of immune stimulation in a reductive manner using TLR4 ligand LPS and a more complete whole bacteria stimulation. The selected bacterial ligands were employed in the study to allow us to model an optimal macrophage host response. This minimises the confounding variable of live bacteria which can perturb cellular metabolism and immune responses, which we have highlighted in the discussion. Since both LPS and irradiated Mtb induced similar metabolic and phenotypic profiles, it is likely that the effects of priming are maintained with diverse stimuli.

> *(2) The authors should add a quantitative measure (like extracellular lactate secretion or ECAR level) for the extent of glycolytic inhibition by the use of 5 mM 2-DG in their setup.*

We would like to draw the attention of the reviewer to the data represented in supplementary figure 2B, demonstrating that 2DG lowers ECAR at 5mM at both 1 and 24 h post stimulation with iH37Rv by an average of approximately 40%. In addition, we have acknowledged that inhibition with 5 mM 2DG does not fully inhibit glycolysis as outlined in the study limitations (lines 477-480).

> *(3) Percent change and fold change have been used to show the same or similar result in Fig. 1 and 2. Whereas, supplementary Fig. 1 shows absolute ECAR/OCR values in addition to fold change. The authors can plot either fold change or percent change in different measurements to avoid confusion. For example, do ECAR changes upon LPS stimulation in Fig. 1A and 1C come from the same dataset? One of the data points in percent change shows a decrease in percent ECAR change under no cytokine control, whereas all the data points in fold change show an increase.*

We have addressed this comment above in response to reviewer 1 point 5 (recommendations for the authors).

We thank the reviewer for highlighting this single error in the data points for percent change. We have fixed this data point which was a result of a calculation error. All data throughout the manuscript has now been rechecked.

> *Minor comments:*
>
> *(1) The manuscript for review should be line-marked for referencing and commenting during review.*

We have now included line-marking on the manuscript.

> *(2) The authors can depict marker legends differently for all figures. In all figures, circles to squares or triangles represent treatment/stimulation with iH37Rv or LPS. The authors can depict this as circles to squares/triangles in contrast to different legends.*

We have changed the legend to include a more detailed description of data represented inserting additional information regarding the colours and symbols represented in the figures.

> *(3) Describe bars in supplementary figure 1A - 1H in its legend?*

We thank the reviewer for highlighting this oversight, we have amended the legend to state "error bars represent standard deviation"

> *(4) Discuss the significant increase in CD86 expression in IFN-γ and IL-4 primed unstimulated AMs in Fig. 3E.*

We have updated the results section to state that IFN-γ increased the expression of CD86 when isolated in the absence of bacterial stimulations in Fig. 3E (lines 271-272). There is no significant increase in CD86 by IL-4 primed unstimulated AM. IL-4 primed human AM only upregulated CD86 when treated with 2DG or in the presence of stimulation.

> *(5) Contrary to Fig. 2, the data points of unstimulated cells in Fig. 4 vary for different treatment conditions (no cytokine, IFN-γ, and IL-4) for each cytokine measurement. What is the difference between unstimulated cells in Fig. 4 (for each cytokine) from that of Fig. 2 (for each receptor MFI)?*

Unstimulated cells change their surface activation markers and phenotype in response to IFN-γ and IL-4 in Fig. 2. For Fig. 4, IFN-γ and IL-4 are not sufficient to induce cytokine secretion in the absence of stimulation with bacterial ligands.

> *(6) The methodology for seeding and treatment of cells is reemphasized for almost all results. Defining macrophage priming and stimulation of macrophages in the method section and once at the start of results should be fine.*

Plating happens differently for Seahorse compared to the flow cytometric phenotyping and ELISA for cytokine production. For clarity we have stated and reemphasized the seeding and treatment of cells throughout the results section.

> *(7) Clarify "IL-4 reduced glycolysis in response to LPS stimulation" in relation to the results depicted in Fig. 1A and 1C. Similarly, clarify "IL-4 resulting in reduced IL-1β and IL-10 production" in relation to Fig. 4E.*

For clarity we have added the following lines (157-160, 164-170) to the manuscript:

"IL-4 primed iH37Rv stimulated AM increased ECAR to similar extent as unprimed controls (Figure 1A; left). Conversely, IL-4 primed AM stimulated with LPS AM did not increase their ECAR to the same extent as controls (Figure 1A; right), suggesting that IL-4 reduces the AM ability to increase ECAR in response to LPS stimulation."

"Since IFN-γ priming increased cellular energetics in the AM at baseline, we calculated percent change in ECAR and OCR from the baseline rate of each group in order to assess if IFN-γ or IL-4 primed AM have altered capacity to change their metabolism in response to stimulation (Figure 1C,D). This was carried out to equalise all the primed data sets at baseline before stimulation (Figure S1C, S1D). These data indicate that whilst the peak of glycolysis is elevated in IFN-γ primed AM (Figure S1A), all AM have a similar capacity to increase glycolysis upon stimulation when baseline differences in metabolism were adjusted for the effects of cytokine priming (Figure 1C)."

For clarity we have amended the sentence the reviewer has highlighted (lines 214-215): "IL-4 primed AM had reduced fold change in glycolysis upon stimulation with LPS compared with controls".

Since IFN-γ priming induced large effect sizes, we statistically analysed the IL-4 primed and unprimed data sets in the absence of the IFN-γ primed data sets to determine how IL-4 influenced macrophage function. The only data where this resulted in any statistical significance was in response to cytokine production. We have now clarified this in the methods and relevant figure legends by stating, “Statistically significant differences were determined using two-way ANOVA with a Tukey post-test (AD); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 or #P≤0.05, ##P≤0.01 (where IFN-γ primed data sets were excluded for post-test analysis to analyse statistical differences between no cytokine and IL4 treated data sets).

To further clarify this, we have amended the text of the manuscript (lines 307-310) to reflect this. “All stimulated AM secreted IL-10 regardless of priming (Figure 4E). IFN-γ significantly enhanced iH37Rv induced IL-10 in AM compared to unprimed or IL-4 primed comparators (Figure 4E). IL-4 priming of human AM significantly reduced IL-10 production in response to iH37Rv compared with unprimed AM (Figure 4E). LPS strongly induced IL-10 production in unprimed MDM, which was significantly attenuated by either IFN-γ or IL-4 priming (Figure 4F).”

> *(8) Clarify whether data points in unstimulated, iH37Rv stimulated, and LPS-stimulated control cells in Fig. 3A - 3F are from independent experiments from those in Fig. 2A - 2F? The distribution of data points of control (no 2-DG treatment) in Fig. 3 is highly similar to the corresponding data points in Fig. 2. Similarly, provide clarification for similarity in Fig. 5A - 5F and Fig. 4A - 4F.*

The data illustrated in figure 2 and 3 are from one very large dataset, as are the data in figures 4 and 5. This large experiment was designed to test the effect of priming macrophages with IFN- or IL-4 (in the presence or absence of stimulation), and also to determine if the differential responses elicited due to priming were dependent on glycolysis (by inhibiting with 2DG). For clarity and transparency, the same stimulated dataset is repeated in both figures. Given the size and complexity of the experiment, we chose to present the data this way to aid the reader.

> *(9) Clarify the statement "where data was reanalyzed in the absence of IFN-γ" in the section pertaining to Statistical analysis. The authors should clearly mention nature of biological and technical replicates for each experiment in its figure legend. The authors should also confirm multiple comparison correction in all 2-way ANOVA tests done in each figure legend."*

We have amended the text (lines 133-136) to clarify this point “P-values of ≤0.05 were considered statistically significant and denoted with an asterisk. Alternatively, P-values of ≤0.05 were denoted with a hashtag where data was analysed in the absence of IFN-γ primed data sets, to analyse statistical differences between no cytokine and IL-4 treated data sets.”

Figures represent biological replicates (which are the average of technical replicates, presented as a single data point). This is indicated by the following sentence in each figure legend: “Each linked data point represents the average of technical duplicates for one individual biological donor”.

Each legend has been amended to include the multiple comparison post-test applied.

> *(10) Discuss the differences and similarities of IFN-γ driven metabolic reprogramming of primary murine macrophages with the results of this study relative to cytokine secretion and activation marker expression.*

We have added additional discussion and detail comparing human and murine macrophages in lines 381-382, 403, 407 and 412-415 of the manuscript.

> *(11) The repetitive data plots of similar results can be significantly reduced to improve the interpretation of the results.*

The benefit of the plotting the data in this way is for a clearer understanding and representation of the data. The repetitive data plots allow the benefit of being able to first delineate the effect of priming and priming plus stimulation and then, separately, to further examine the differences in AM versus MDM. The repetition of the primed data points then allows of the reader to determine the effect of inhibiting glycolysis with 2DG on unprimed and primed macrophages (with and without stimulation).

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *The methods used and data reported in this manuscript contribute to our understanding of the role of metabolism in programming of macrophages during priming. Suggestions for improving the presentation and interpretation of results include:*
>
> *• Consult with a statistician regarding analyses of the multiple conditions used during these assays. The use of repeated statistical analyses with different comparison groups in the same figure/data set seems atypical and should either be amended or fully justified in the text. Also, use of two-way vs. one-way ANOVA should be evaluated and clarified.*

We have now consulted a statistician. We have amended the text (lines 133-136) to clarify this point "P-values of ≤0.05 were considered statistically significant and denoted with an asterisk. Alternatively, P-values of ≤0.05 were denoted with a hashtag where data was analysed in the absence of IFN-γ primed data sets, to analyse statistical differences between no cytokine and IL-4 treated groups."

There are two variables in the data sets; cytokine priming as well as stimulation status therefore we opted for a two-way ANOVA rather than a One-way ANOVA. There are three stimulation groups: unstimulated, Mtb-stimulated and LPS-stimulated. Cytokine priming also has three groups: no cytokine, IFN-γ, or IL-4. There are two variables (priming and stimulation), each with 3 groups i.e., six treatment conditions in total, therefore two-way AVOVA with multiple comparisons tests help pinpoint exactly which groups (e.g., the 6 different levels of the 'stimulation' and 'cytokine' treatments) are significantly different from each other. This was important for understanding the specific effects of our treatments. The reader can therefore also deduce how these six treatment conditions compare to each other.

In contrast, performing multiple single comparisons independently of the rest of the dataset (e.g. t tests), increases the risk of false positives (type 1 error). Multiple comparisons ANOVA with post-tests adjust for this, helping to reduce the likelihood of a type 1 error. These stats are more stringent, and it is therefore harder to get P values <0.05. Hence, if we compared all six treatment groups without adjustment, you increase the chance of finding false positives due to the sheer number of comparisons, leading to biased and incorrect conclusions.

In our case, multiple comparisons tests were essential after the two-way ANOVA because they helped to objectively identify specific treatment group differences and control the overall error rate when we were extracting our conclusions, thereby reducing any risk of biases in our conclusions.

A one-way ANOVA is used to test the effect of a single variable with more than two groups contained in the dataset. For example, in our case if you only want to test how different

'stimulation' groups affect ECAR or OCR, only in unprimed macrophages, a one-way ANOVA would be used.

The current study used two-way ANOVA to test the effects of two variables (priming and stimulation, or in some cases priming and inhibition) each containing 3 groups, and see if there is any interaction between the two factors. For example, in our case this allowed us to examine how the 'stimulation' and the 'cytokine' priming affect ECAR/OCR levels and to determine if the effect of 'stimulation' depends on the 'cytokine' priming.

> *• More justification could be given for the dose of IFNγ used for priming. Inflammatory priming is typically performed with a "low-dose" treatment (e.g., ~1 ng/ml), whereas the authors use 10 ng/ml, which would be considered a high dose. It would be useful to repeat select experiments with a more standard low-dose treatment of IFNg to demonstrate that this is also sufficient to induce the observed metabolic changes.*

Previous work has identified little difference in the response of AM and peripheral monocytes to low versus high doses of IFN-γ (6). We have inserted the following into the study limitations (lines 479-481).

“Furthermore, only one dose of IFN-γ was utilised due to limitations in AM yield, however, recently both low and high doses of IFN-γ have been shown to have similar effects on AM *in vitro* (6).”

> *• Check for accuracy of the Fig.4 legend. Also check that 4G and 4B math is consistent.*

The legend for Figure 4 has been amended for incorrect A,B to state G,H. The math has been double checked for accuracy and is correct. 3 out of 10 MDM donors produced IL-1β in the absence of IFN-γ in Figure 4B, therefore the average used to calculate the data represented in Figure 4G was brought down markedly by donors who produced little or no IL-1β.

> *• Functional plasticity is a vague term and difficult to interpret in this context. It is stated that AM have greater functional plasticity, but MDMs appear to have greater capacity to secrete IL-1β and respond more robustly to IL-4 in terms of T cell stimulation. On that note, the claims regarding antigen presentation would be more impactful if a direct comparison of antigen presentation capacity was made between AM and MDM.*

Our data suggests that AM have a greater ability to alter cytokine production, such as IL1β. To consider different culture and plating requirements of MDM v AM cytokine concentration was normalised and expressed in terms of fold change. This gives a more controlled and accurate comparison of the ability of IFN-γ or IL-4 to modulate cytokine production in AM compared with MDM.

The terms ‘functional plasticity’ and phenotypic plasticity’ have now been defined in the manuscript in lines 60-63.

We have therefore added the following sentence into the conclusion of the manuscript (lines 490-493). “Cumulatively, the data presented herein suggests that the MDM maybe more phenotypically plastic than the AM, while the AM have enhanced functional plasticity in their ability to produce cytokine after exposure Th1 and Th2 cytokines.”

However, we acknowledge that the MDM may be regarded as more plastic because of their ability to respond robustly to IL-4, whereas the phenotypic and functional changes in the AM in response to IL4 are more limited. Whilst the focus of our work was to determine if AM are a tractable target to promote immunity in the lungs through upregulation of pro-inflammatory effector function, their ability to downregulated inflammation in response to IL-4 is comparatively less profound compared with MDM.

We acknowledge the shortcomings of our work which did not allow us to directly measure antigen processing in the AM, due to limitations in the cellular yield from BALF. We have edited the text (lines 251-252 and 286) to clarify this for the reader.

> *• Inconsistent normalization complicates interpretation of metabolic data. For example, it is unclear, for example, whether changes in glycolysis and oxidative phosphorylation in primed cells are due to priming or subsequent treatments. Check harmony of methods for analysis of "metabolic assays" with Fig.1 data, axis, and legend.*

We have addressed this comment, which is similar to points made by the other reviewers and amended the manuscript to increase clarity. These changes are outlined in the response to reviewer 1, point 5 (recommendations for the author). In addition, we have amended the metabolic assay method (lines 111-112) to state that “Post stimulation the ECAR and OCR were continually sampled at 20-minute intervals for times indicated.”

> *• A direct comparison of cytokine production after priming and stimulation with Mtb or LPS is limited by inconsistent axes. The data may not support a claim that AM has greater "functional plasticity" without a direct comparison of antigen presentation. Moreover, MDM secrete more IL-1β than AM. The claim that that AM "have increased ability to produce all cytokines assayed in response to Mtb stimulation" does not appear to be supported by the data.*

We have amended the text to clarify this issue (lines 313-315). “These data suggest that the AM have greater functional plasticity in terms of their ability to upregulate cytokine production in response to IFN-γ, compared with the MDM. IFN-γ primed AM have enhanced IL-10 and TNF production in response to Mtb and LPS, respectively.”

We have amended the manuscript and have replaced “IFN-γ primed AM have increased ability to produce all cytokines assayed in response to Mtb stimulation” with the following (lines 421-423) “IFNγ primed AM stimulated with Mtb have significantly more production of IL-1β, TNF and IL-10 compared with unprimed controls. This is in contrast with IFN-γ primed MDM which only upregulate TNF compared to their unprimed controls.”

> *• AM populations could be defined experimentally.*

Airway macrophages were adherence purified from bronchoalveolar lavage fluid defined as CD68+CD14+ as per rebuttal figure 1. The purpose of this study was to examine if human peripherally derived or lung resident macrophages were plastic in response to the classical polarising cytokines IFNγ and IL-4. We have identified that the AM and MDM do indeed have different functional and metabolic responses to these cytokines. However, determining functional differences within the AM subpopulations is beyond the scope of the current study and hampered by low cell numbers in human BALF.

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