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

Donal J Cox, Sarah A Connolly, Cilian Ó Maoldomhnaigh, Aenea AI Brugman, Olivia Sandby Thomas, Emily Duffin, Finbarr O'Connell, James J Phelan, Karl M Gogan, Laura E Gleeson, Sharee A Basdeo[1]*, Joseph Keane[1]

Trinity Translational Medicine Institute, St James's Hospital, Trinity College Dublin, The University of Dublin, Dublin 8, Ireland.

[1]These authors share senior authorship.

*Correspondence to Dr Sharee Basdeo: basdeos@tcd.ie

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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 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, CD40 and CD86, 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.

Key words: Human alveolar macrophage, Glycolysis, Oxidative Phosphorylation, Immunometabolism, Infection, Polarization.

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). 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 downstream macrophage functions.

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 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 ($1x10^5$ cells/well) were re-plated onto Seahorse plates, as previously described (7). AM ($1x10^5$ 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. The ECAR and OCR were continually sampled 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 multiple comparisons tests. P-values of ≤0.05 were considered statistically significant and denoted with an asterisk or hashtag where data was reanalysed in the absence of IFN-γ.

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. 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). 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). 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 their baseline rates, to assess if IFN-γ or IL-4 primed AM have altered capacity to upregulate their metabolism in response to stimulation (Figure 1C,D). These data indicate that whilst the peak of glycolysis is elevated in IFN-γ primed AM (Figure S1A), they have a similar capacity to increase glycolysis when stimulated compared with control and IL-4 primed AM (Figure 1C). IFN-γ increased the percent change in OCR of AM in response to both bacterial stimuli (Figure 1D).

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 unstimulated and LPS stimulated MDM compared with controls, 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 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 OCR compared with stimulated unprimed or IFN-γ primed MDM (Figure 1H). These data indicate that IL-4 priming prevents human MDM utilising Warburg 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 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 the Warburg effect. IL-4 reduced glycolysis inAM stimulated with LPS.

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).

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 dependent on glycolysis for upregulation of CD40 and HLA-DR, and antigen processing but 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). 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 antigen processing and presentation 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.

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, however IFN-γ enhanced iH37Rv induced IL-10 in AM (Figure 4E) whereas IL-4 priming of human AM significantly reduced IL-10 production in response to iH37Rv compared with unprimed or IFN-γ primed 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 than the MDM, particularly in response to IFN-γ. 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).

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 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" 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" 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 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 (25) 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, 26, 27). 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, 28). 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 (29). 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. 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). Interestingly, IFN-γ primed AM had increased TNF in response to LPS but not IL-10. 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 (30-33). 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 have an increased ability to produce all cytokines assayed in response to Mtb stimulation. 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 (34, 35). 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 (36). 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 reducedIL-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 (26, 27, 37-39). Both IFN-γ and IL-4 can induce trained immunity in murine and human macrophages (12, 19, 40, 41). 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 better target for innate training, as increased IL-1β is associated with optimal training (37, 41, 42). 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, 26). We have previously demonstrated that an adenovirus vectored vaccine induces trained immunity in human monocytes and postulated that this may be IFN-γ dependent (43). 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, 44-47). Clinical trials have indicated that nebulized IFN-γ is a viable HDT to help combat Mtb (48, 49) 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 (50), 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.

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. Our data supports the hypothesis that there may be distinct roles for AM and infiltrating MDM during infection since IFN-γ driven 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:

DJC conception and design, data acquisition, analysis and interpretation, original draft of the article, critical revision of the article.

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

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

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

AIB data acquisition, analysis and interpretation.

OST data acquisition.

ED data acquisition.

KMG data acquisition.

FOC data acquisition.

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.

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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.

1. Huang L, Nazarova EV, Tan S, Liu Y, Russell DG. Growth of Mycobacterium tuberculosis in vivo segregates with host macrophage metabolism and ontogeny. *The Journal of experimental medicine* 2018; 215: 1135.
2. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 2010; 330: 841-845.
3. Wang F, Zhang S, Jeon R, Vuckovic I, Jiang X, Lerman A, Folmes CD, Dzeja PD, Herrmann J. Interferon Gamma Induces Reversible Metabolic Reprogramming of M1 Macrophages to Sustain Cell Viability and Pro-Inflammatory Activity. *EBioMedicine* 2018; 30: 303-316.
4. Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, Foresti R, Immenschuh S. Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A divergent role for glycolysis. *Redox biology* 2019; 22: 101147.
5. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, Frezza C, Bernard NJ, Kelly B, Foley NH, Zheng L, Gardet A, Tong Z, Jany SS, Corr SC, Haneklaus M, Caffrey BE, Pierce K, Walmsley S, Beasley FC, Cummins E, Nizet V, Whyte M, Taylor CT, Lin H, Masters SL, Gottlieb E, Kelly VP, Clish C, Auron PE, Xavier RJ, O'Neill LA. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature* 2013; 496: 238-242.
6. O'Leary SM, Coleman MM, Chew WM, Morrow C, McLaughlin AM, Gleeson LE, O'Sullivan MP, Keane J. Cigarette smoking impairs human pulmonary immunity to Mycobacterium tuberculosis. *American journal of respiratory and critical care medicine* 2014; 190: 1430-1436.
7. Ó Maoldomhnaigh C, Cox DJ, Phelan JJ, Malone FD, Keane J, Basdeo SA. The Warburg Effect Occurs Rapidly in Stimulated Human Adult but Not Umbilical Cord Blood Derived Macrophages. *Frontiers in immunology* 2021; 12.
8. Gleeson LE, Sheedy FJ, Palsson-McDermott EM, Triglia D, O'Leary SM, O'Sullivan MP, O'Neill LA, Keane J. Cutting Edge: Mycobacterium tuberculosis Induces Aerobic Glycolysis in Human Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication. *Journal of immunology (Baltimore, Md : 1950)* 2016; 196: 2444-2449.
9. Cox DJ, Coleman AM, Gogan KM, Phelan JJ, Ó Maoldomhnaigh C, Dunne PJ, Basdeo SA, Keane J. Inhibiting Histone Deacetylases in Human Macrophages Promotes Glycolysis, IL-1β, and T

Helper Cell Responses to Mycobacterium tuberculosis. *Frontiers in immunology* 2020; 11: 1-15.
10. Phelan JJ, McQuaid K, Kenny C, Gogan KM, Cox DJ, Basdeo SA, O'Leary S, Tazoll SC, Ó Maoldomhnaigh C, O'Sullivan MP, O'Neill LA, O'Sullivan MJ, Keane J. Desferrioxamine Supports Metabolic Function in Primary Human Macrophages Infected With Mycobacterium tuberculosis. *Frontiers in immunology* 2020; 11.
11. Mitsi E, Kamng'ona R, Rylance J, Solórzano C, Jesus Reiné J, Mwandumba HC, Ferreira DM, Jambo KC. Human alveolar macrophages predominately express combined classical M1 and M2 surface markers in steady state. *Respiratory Research* 2018; 19: 66.
12. Yao Y, Jeyanathan M, Haddadi S, Barra NG, Vaseghi-Shanjani M, Damjanovic D, Lai R, Afkhami S, Chen Y, Dvorkin-Gheva A, Robbins CS, Schertzer JD, Xing Z. Induction of Autonomous Memory Alveolar Macrophages Requires T Cell Help and Is Critical to Trained Immunity. *Cell* 2018; 175: 1634-1650.
13. Ó Maoldomhnaigh C, Cox DJ, Phelan JJ, Mitermite M, Murphy DM, Leisching G, Thong L, O'Leary SM, Gogan KM, McQuaid K, Coleman AM, Gordon SV, Basdeo SA, Keane J. Lactate Alters Metabolism in Human Macrophages and Improves Their Ability to Kill Mycobacterium tuberculosis. *Frontiers in immunology* 2021; 12.
14. Gleeson LE, O'Leary SM, Ryan D, McLaughlin AM, Sheedy FJ, Keane J. Cigarette Smoking Impairs the Bioenergetic Immune Response to Mycobacterium tuberculosis Infection. *American journal of respiratory cell and molecular biology* 2018; 59: 572-579.
15. Lavrich KS, Speen AM, Ghio AJ, Bromberg PA, Samet JM, Alexis NE. Macrophages from the upper and lower human respiratory tract are metabolically distinct. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2018; 315: L752-L764.
16. Woods PS, Kimmig LM, Meliton AY, Sun KA, Tian Y, O'Leary EM, Gökalp GA, Hamanaka RB, Mutlu GM. Tissue-Resident Alveolar Macrophages Do Not Rely on Glycolysis for LPS-induced Inflammation. *American journal of respiratory cell and molecular biology* 2020; 62: 243-255.
17. Van den Bossche J, Saraber DL. Metabolic regulation of macrophages in tissues. *Cellular Immunology* 2018; 330: 54-59.
18. Huang SC, Smith AM, Everts B, Colonna M, Pearce EL, Schilling JD, Pearce EJ. Metabolic Reprogramming Mediated by the mTORC2-IRF4 Signaling Axis Is Essential for Macrophage Alternative Activation. *Immunity* 2016; 45: 817-830.
19. Lundahl MLE, Mitermite M, Ryan DG, Case S, Williams NC, Yang M, Lynch RI, Lagan E, Lebre FM, Gorman AL, Stojkovic B, Bracken AP, Frezza C, Sheedy FJ, Scanlan EM, O'Neill LAJ, Gordon SV, Lavelle EC. Macrophage innate training induced by IL-4 and IL-13 activation enhances OXPHOS driven anti-mycobacterial responses. *eLife* 2022; 11: e74690.
20. Krishnamoorthy G, Kaiser P, Abu Abed U, Weiner J, 3rd, Moura-Alves P, Brinkmann V, Kaufmann SHE. FX11 limits Mycobacterium tuberculosis growth and potentiates bactericidal activity of isoniazid through host-directed activity. *Disease models & mechanisms* 2020; 13.
21. Cumming BM, Addicott KW, Adamson JH, Steyn AJ. Mycobacterium tuberculosis induces decelerated bioenergetic metabolism in human macrophages. *eLife* 2018; 7.
22. Mendonca LE, Pernet E, Khan N, Sanz J, Kaufmann E, Downey J, Grant A, Orlova M, Schurr E, Krawczyk C, Jones RG, Barreiro LB, Divangahi M. Human alveolar macrophage metabolism is compromised during Mycobacterium tuberculosis infection. *Frontiers in immunology* 2022; 13: 1044592.
23. Braverman J, Sogi KM, Benjamin D, Nomura DK, Stanley SA. HIF-1α Is an Essential Mediator of IFN-γ-Dependent Immunity to Mycobacterium tuberculosis. *Journal of immunology (Baltimore, Md : 1950)* 2016; 197: 1287-1297.
24. Cumming BM, Pacl HT, Steyn AJC. Relevance of the Warburg Effect in Tuberculosis for Host-Directed Therapy. *Frontiers in Cellular and Infection Microbiology* 2020; 10.
25. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *Journal of immunology (Baltimore, Md : 1950)* 2004; 172: 2731-2738.

26. D'Agostino MR, Lai R, Afkhami S, Khera A, Yao Y, Vaseghi-Shanjani M, Zganiacz A, Jeyanathan M, Xing Z. Airway Macrophages Mediate Mucosal Vaccine–Induced Trained Innate Immunity against Mycobacterium tuberculosis in Early Stages of Infection. *The Journal of Immunology* 2020; 205: 2750-2762.
27. Afkhami S, D'Agostino MR, Zhang A, Stacey HD, Marzok A, Kang A, Singh R, Bavananthasivam J, Ye G, Luo X, Wang F, Ang JC, Zganiacz A, Sankar U, Kazhdan N, Koenig JFE, Phelps A, Gameiro SF, Tang S, Jordana M, Wan Y, Mossman KL, Jeyanathan M, Gillgrass A, Medina MFC, Smaill F, Lichty BD, Miller MS, Xing Z. Respiratory mucosal delivery of next-generation COVID-19 vaccine provides robust protection against both ancestral and variant strains of SARS-CoV-2. *Cell* 2022; 185: 896-915.e819.
28. Lachmandas E, Boutens L, Ratter JM, Hijmans A, Hooiveld GJ, Joosten LAB, Rodenburg RJ, Fransen JAM, Houtkooper RH, van Crevel R, Netea MG, Stienstra R. Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. *Nature microbiology* 2016; 2: 16246.
29. Hackett EE, Charles-Messance H, O'Leary SM, Gleeson LE, Muñoz-Wolf N, Case S, Wedderburn A, Johnston DGW, Williams MA, Smyth A, Ouimet M, Moore KJ, Lavelle EC, Corr SC, Gordon SV, Keane J, Sheedy FJ. Mycobacterium tuberculosis Limits Host Glycolysis and IL-1β by Restriction of PFK-M via MicroRNA-21. *Cell reports* 2020; 30: 124-136.e124.
30. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *The New England journal of medicine* 2001; 345: 1098-1104.
31. Harris J, Hope JC, Keane J. Tumor Necrosis Factor Blockers Influence Macrophage Responses to Mycobacterium tuberculosis. *The Journal of infectious diseases* 2008; 198: 1842-1850.
32. Harris J, Keane J. How tumour necrosis factor blockers interfere with tuberculosis immunity. *Clinical and experimental immunology* 2010; 161: 1-9.
33. Bourigault ML, Segueni N, Rose S, Court N, Vacher R, Vasseur V, Erard F, Le Bert M, Garcia I, Iwakura Y, Jacobs M, Ryffel B, Quesniaux VF. Relative contribution of IL-1alpha, IL-1beta and TNF to the host response to Mycobacterium tuberculosis and attenuated M. bovis BCG. *Immunity, inflammation and disease* 2013; 1: 47-62.
34. Ni Cheallaigh C, Sheedy FJ, Harris J, Munoz-Wolf N, Lee J, West K, McDermott EP, Smyth A, Gleeson LE, Coleman M, Martinez N, Hearnden CH, Tynan GA, Carroll EC, Jones SA, Corr SC, Bernard NJ, Hughes MM, Corcoran SE, O'Sullivan M, Fallon CM, Kornfeld H, Golenbock D, Gordon SV, O'Neill LA, Lavelle EC, Keane J. A Common Variant in the Adaptor Mal Regulates Interferon Gamma Signaling. *Immunity* 2016; 44: 368-379.
35. Remus N, Reichenbach J, Picard C, Rietschel C, Wood P, Lammas D, Kumararatne DS, Casanova J-L. Impaired Interferon Gamma-Mediated Immunity and Susceptibility to Mycobacterial Infection in Childhood. *Pediatric Research* 2001; 50: 8-13.
36. Kulikauskaite J, Wack A. Teaching Old Dogs New Tricks? The Plasticity of Lung Alveolar Macrophage Subsets. *Trends in immunology* 2020; 41: 864-877.
37. Moorlag SJCFM, Khan N, Novakovic B, Kaufmann E, Jansen T, van Crevel R, Divangahi M, Netea MG. β-Glucan Induces Protective Trained Immunity against Mycobacterium tuberculosis Infection: A Key Role for IL-1. *Cell reports* 2020; 31: 107634-107634.
38. Netea MG, Giamarellos-Bourboulis EJ, Domínguez-Andrés J, Curtis N, van Crevel R, van de Veerdonk FL, Bonten M. Trained Immunity: a Tool for Reducing Susceptibility to and the Severity of SARS-CoV-2 Infection. *Cell* 2020; 181: 969-977.
39. Zhou J, Lv J, Carlson C, Liu H, Wang H, Xu T, Wu F, Song C, Wang X, Wang T, Qian Z. Trained immunity contributes to the prevention of Mycobacterium tuberculosis infection, a novel role of autophagy. *Emerging microbes & infections* 2021; 10: 578-588.
40. Schrijver DP, Röring RJ, Deckers J, de Dreu A, Toner YC, Prevot G, Priem B, Munitz J, Nugraha EG, van Elsas Y, Azzun A, Anbergen T, Groh LA, Becker AMD, Pérez-Medina C, Oosterwijk RS, Novakovic B, Moorlag SJCFM, Jansen A, Pickkers P, Kox M, Beldman TJ, Kluza E, van Leent

MMT, Teunissen AJP, van der Meel R, Fayad ZA, Joosten LAB, Fisher EA, Merkx M, Netea MG, Mulder WJM. Resolving sepsis-induced immunoparalysis via trained immunity by targeting interleukin-4 to myeloid cells. *Nature Biomedical Engineering* 2023.
41. Li W, Moorlag S, Koeken V, Röring RJ, de Bree LCJ, Mourits VP, Gupta MK, Zhang B, Fu J, Zhang Z, Grondman I, van Meijgaarden KE, Zhou L, Alaswad A, Joosten LAB, van Crevel R, Xu CJ, Netea MG, Li Y. A single-cell view on host immune transcriptional response to in vivo BCG-induced trained immunity. *Cell reports* 2023; 42: 112487.
42. Teufel LU, Arts RJW, Netea MG, Dinarello CA, Joosten LAB. IL-1 family cytokines as drivers and inhibitors of trained immunity. *Cytokine* 2022; 150: 155773.
43. Murphy DM, Cox DJ, Connolly SA, Breen EP, Brugman AAI, Phelan JJ, Keane J, Basdeo SA. Trained immunity is induced in humans after immunization with an adenoviral vector COVID-19 vaccine. *The Journal of clinical investigation* 2023; 133.
44. Cahill C, O'Connell F, Gogan KM, Cox DJ, Basdeo SA, O'Sullivan J, Gordon SV, Keane J, Phelan JJ. The Iron Chelator Desferrioxamine Increases the Efficacy of Bedaquiline in Primary Human Macrophages Infected with BCG. *International Journal of Molecular Sciences* 2021; 22: 2938.
45. Coleman MM, Basdeo SA, Coleman AM, Ni Cheallaigh C, Peral de Castro C, McLaughlin AM, Dunne PJ, Harris J, Keane J. All-trans Retinoic Acid Augments Autophagy during Intracellular Bacterial Infection. *American journal of respiratory cell and molecular biology* 2018.
46. O'Connor G, Krishnan N, Fagan-Murphy A, Cassidy J, O'Leary S, Robertson BD, Keane J, O'Sullivan MP, Cryan S-A. 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* 2019; 134: 153-165.
47. Lawlor C, O'Connor G, O'Leary S, Gallagher PJ, Cryan S-A, Keane J, O'Sullivan MP. Treatment of Mycobacterium tuberculosis-Infected Macrophages with Poly(Lactic-Co-Glycolic Acid) Microparticles Drives NFκB and Autophagy Dependent Bacillary Killing. *PLoS One* 2016; 11: e0149167-e0149167.
48. Dawson R, Condos R, Tse D, Huie ML, Ress S, Tseng CH, Brauns C, Weiden M, Hoshino Y, Bateman E, Rom WN. Immunomodulation with recombinant interferon-gamma1b in pulmonary tuberculosis. *PLoS One* 2009; 4: e6984.
49. Bharti R, Roy T, Verma S, Reddy DVS, Shafi H, Verma K, Raman SK, Pal S, Azmi L, Singh AK, Ray L, Mugale MN, Misra A. Transient, inhaled gene therapy with gamma interferon mitigates pathology induced by host response in a mouse model of tuberculosis. *Tuberculosis (Edinburgh, Scotland)* 2022; 134: 102198.
50. Pereverzeva L, van Linge CCA, Schuurman AR, Klarenbeek AM, Ramirez Moral I, Otto NA, Peters-Sengers H, Butler JM, Schomakers BV, van Weeghel M, Houtkooper RH, Wiersinga WJ, Bonta PI, Annema JT, de Vos AF, van der Poll T. Human alveolar macrophages do not rely on glucose metabolism upon activation by lipopolysaccharide. *Biochim Biophys Acta Mol Basis Dis* 2022; 1868: 166488.

Figure 1. IFN-γ increases energetic metabolism in the AM but enhances Warburg 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 primed with IFN-γ or IL-4 (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, irradiated Mtb H37Rv (iH37Rv; MOI 1-10) or LPS (100 ng/ml), 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). Each linked data point represents one individual donor (MDM; n=8-9, AM; n=9-10). Statistically significant differences were determined using two-way ANOVA (A-J); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.001.

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 primed with IFN-γ or IL-4 (both 10 ng/ml) for 24 h. AM or MDM were left unstimulated or stimulated with iH37Rv (MOI 1-10) or LPS (100 ng/ml). 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 and MDM based on the average of their respective no cytokine controls. Each linked data point represents one individual donor (n=8-9). Statistically significant differences were determined using two-way ANOVA (A-F); *P≤0.05, **P≤0.01, P***≤0.001, ****P≤0.001.

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 primed with IFN-γ or IL-4 (both 10 ng/ml) for 24 h. Cells were treated with 2DG (5mM) 1 h prior to stimulation with iH37Rv (MOI 1-10) or LPS (100 ng/ml). 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 one individual donor (n=8-9). Statistically significant differences were determined using two-way ANOVA (A-F); *P≤0.05, **P≤0.01, P***≤0.001, ****P≤0.001.

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 primed with IFN-γ or IL-4 (both 10 ng/ml) for 24 h. AM or MDM were left unstimulated or stimulated iH37Rv (MOI 1-10) or LPS (100 ng/ml). 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 (A) and LPS (B). Each linked data point represents one individual donor (AM; n=12-13, MDM; n=8-10). Statistically significant differences were determined using two-way ANOVA (A-D); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 or #P≤0.05, ##P≤0.01, ####P≤0.0001 (IFN-γ excluded for analysis).

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 primed with IFN-γ or IL-4 (both 10 ng/ml) for 24 h. Cells were treated with 2DG (5mM) for 1 h prior to stimulation with iH37Rv (MOI 1-10) or LPS (100 ng/ml). 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 one individual donor (AM; n=12-13, MDM; n=8-10). Statistically significant differences were determined using two-way ANOVA (A-D); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 or #P≤0.05, ##P≤0.01 (IFN-γ excluded for analysis).

Figure 1

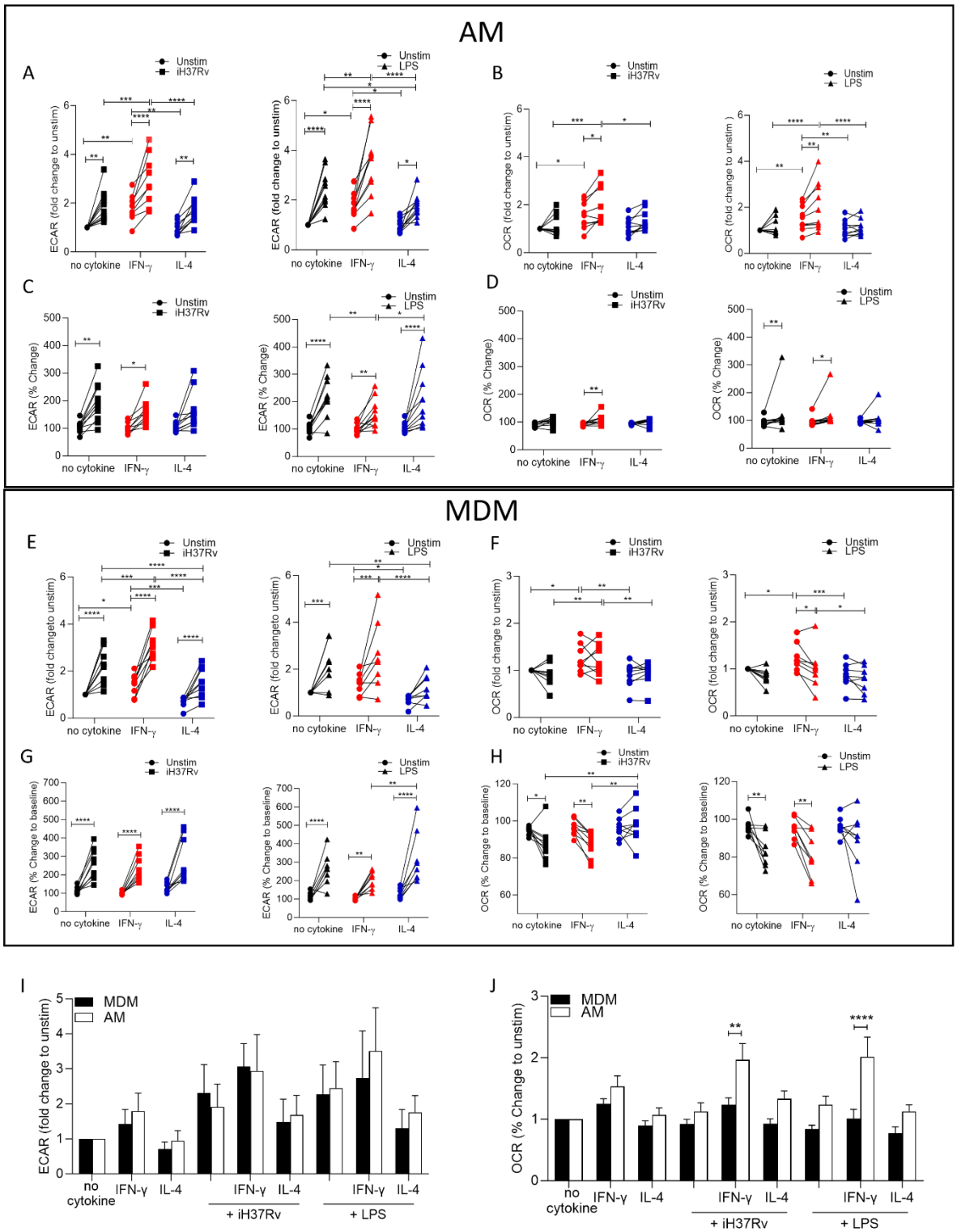
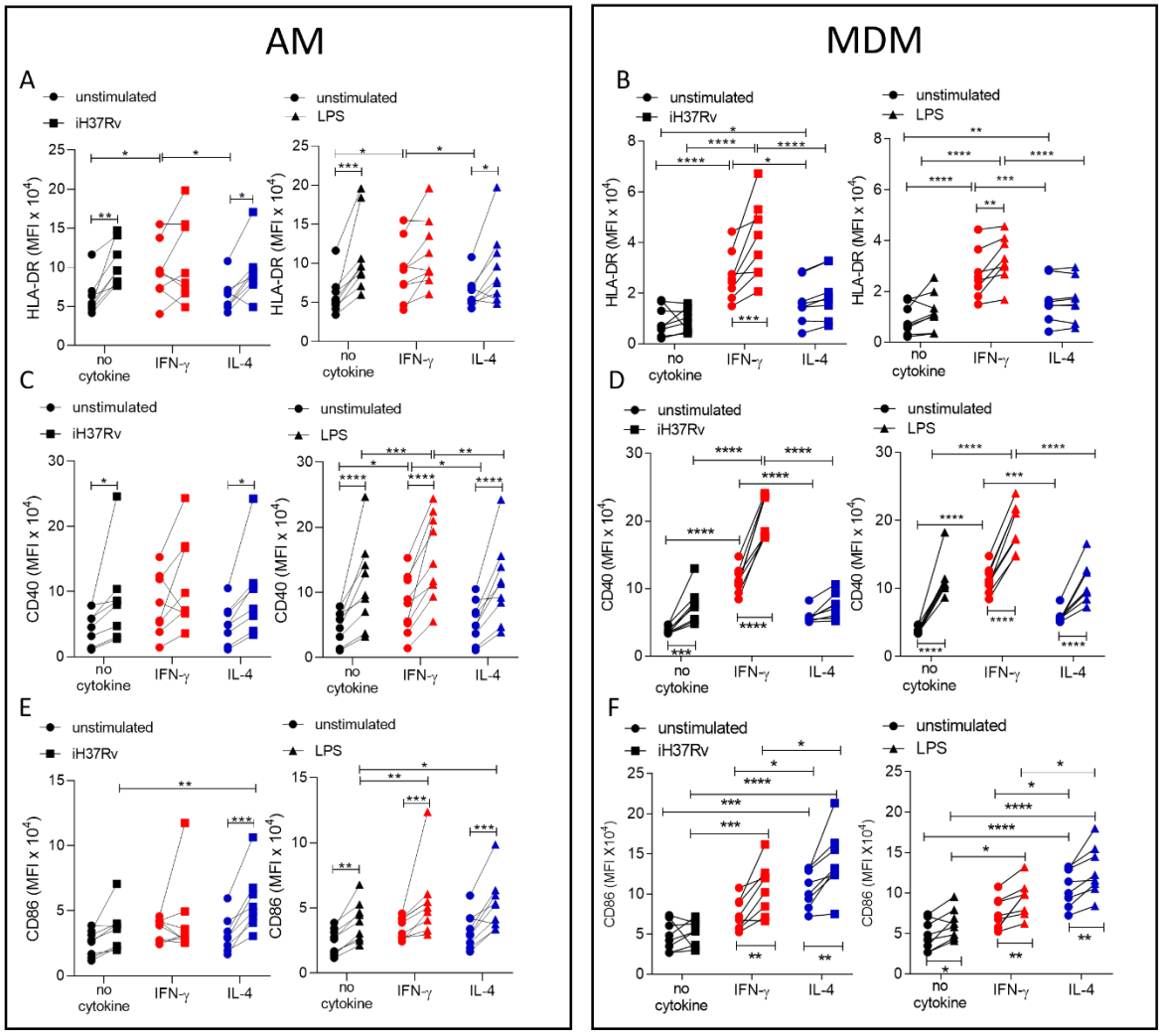
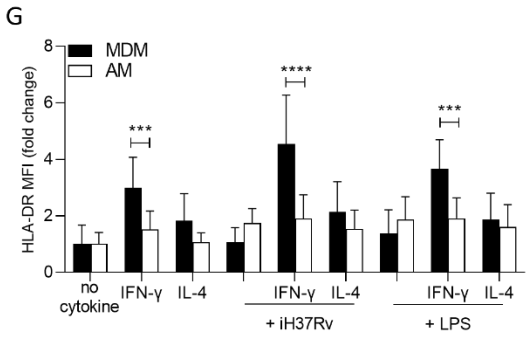
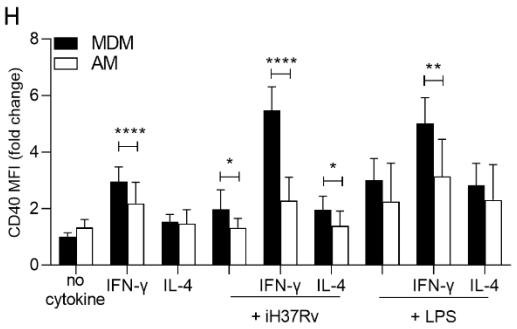

AM
A
B
C
D
MDM
E
F
G
H
Unstim
iH37Rv
LPS
ECAR (fold change to unstim)
OCR (fold change to unstim)
ECAR (% Change)
OCR (% Change)
ECAR (fold changeto unstim)
ECAR (% Change to baseline)
OCR (% Change to baseline)
no cytokine
IFN-γ
IL-4
I
J
MDM
AM
ECAR (fold change to unstim)
OCR (% Change to unstim)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS

Figure 2


AM
MDM
A
B
C
D
E
F
unstimulated
iH37Rv
LPS
HLA-DR (MFI x 10⁴)
CD40 (MFI x 10⁴)
CD86 (MFI x 10⁴)
CD86 (MFI X10⁴)
no cytokine
IFN-γ
IL-4


G
MDM
AM
HLA-DR MFI (fold change)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS


H
MDM
AM
CD40 MFI (fold change)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS

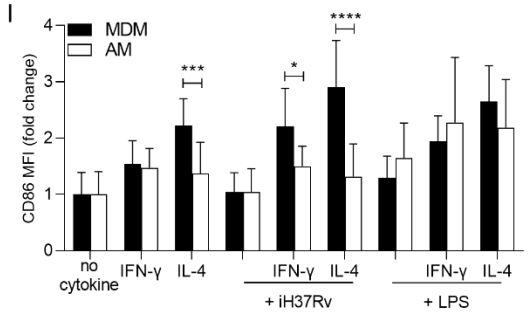

I
MDM
AM
CD86 MFI (fold change)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS

Figure 3

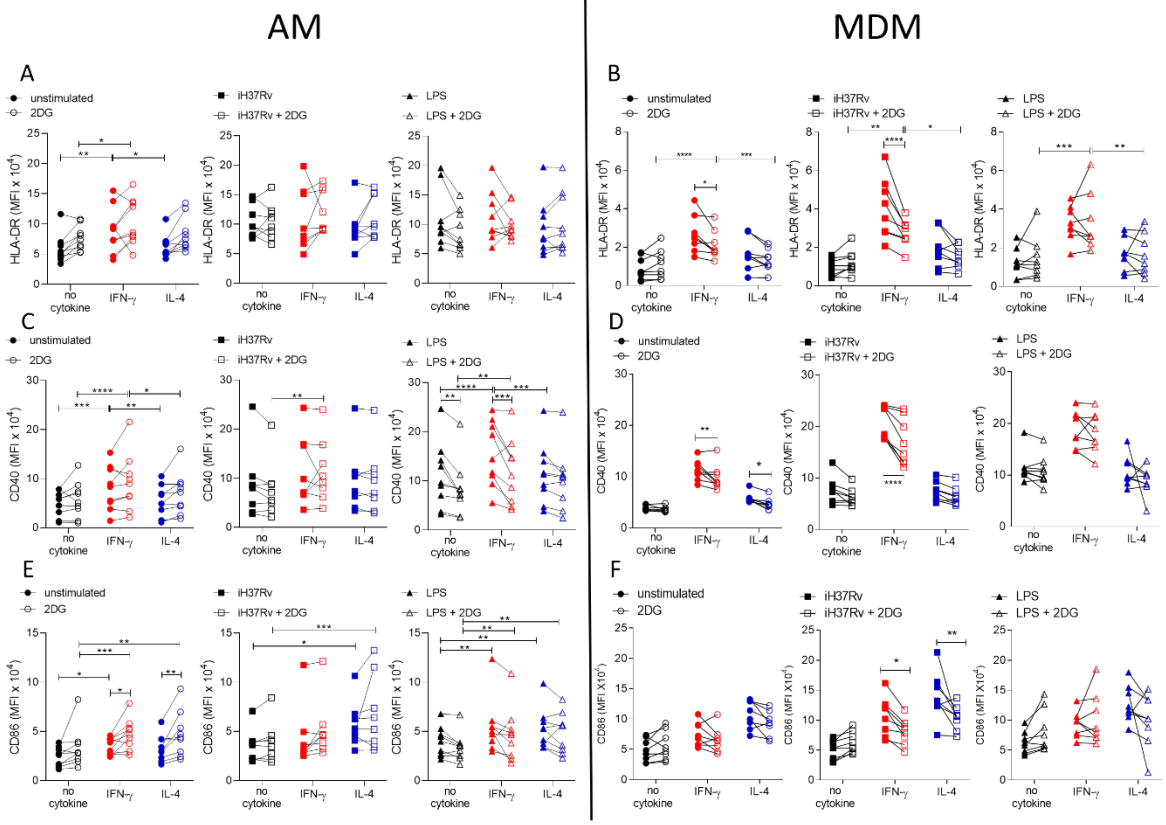
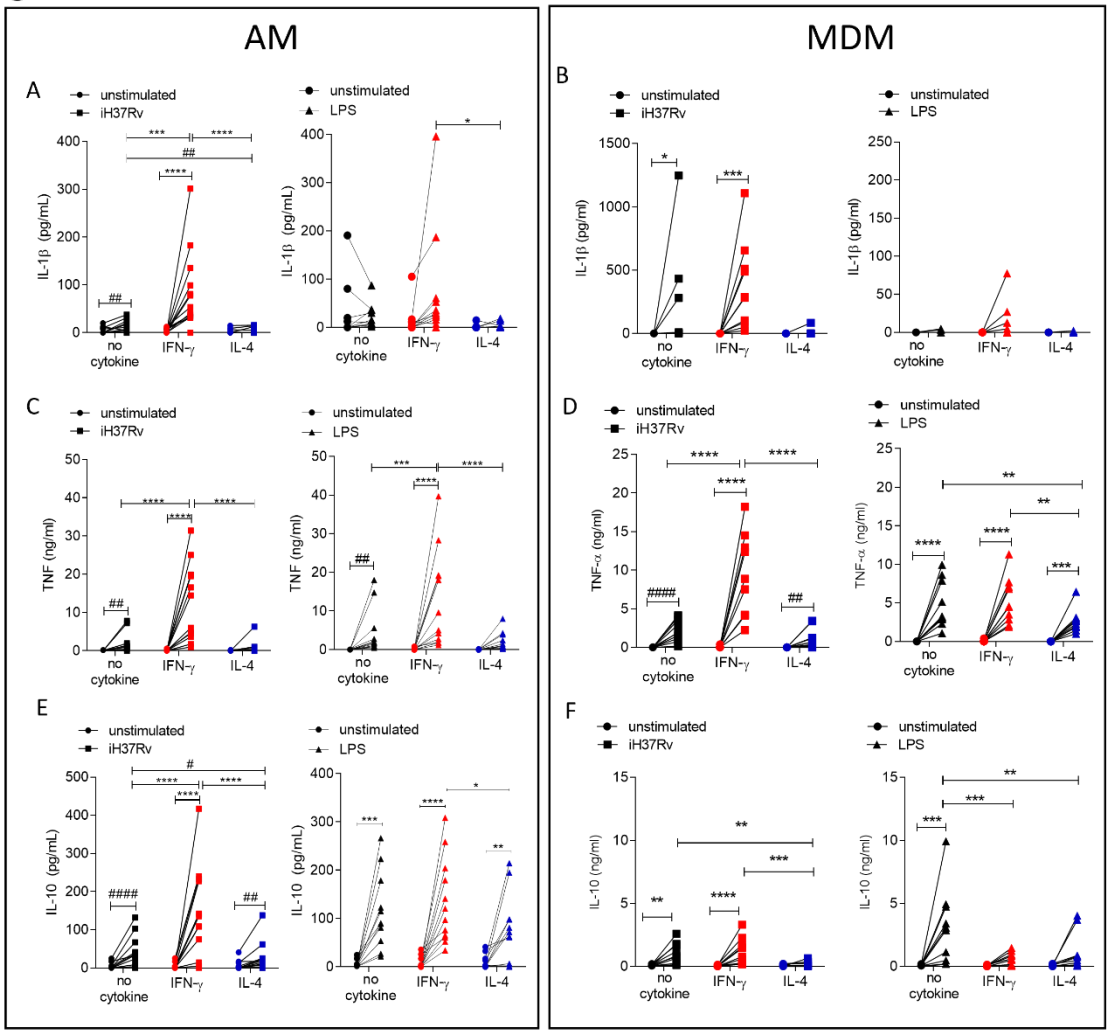

AM
MDM
A
B
C
D
E
F
unstimulated
2DG
iH37Rv
iH37Rv + 2DG
LPS
LPS + 2DG
HLA-DR (MFI x 10⁴)
CD40 (MFI x 10⁴)
CD86 (MFI x 10⁴)
no cytokine
IFN-γ
IL-4

Figure 4


AM
MDM
A
B
C
D
E
F
unstimulated
iH37Rv
LPS
IL-1β (pg/mL)
IL-1β (pg/ml)
TNF (ng/ml)
TNF-α (ng/ml)
IL-10 (pg/mL)
IL-10 (ng/ml)
no cytokine
IFN-γ
IL-4

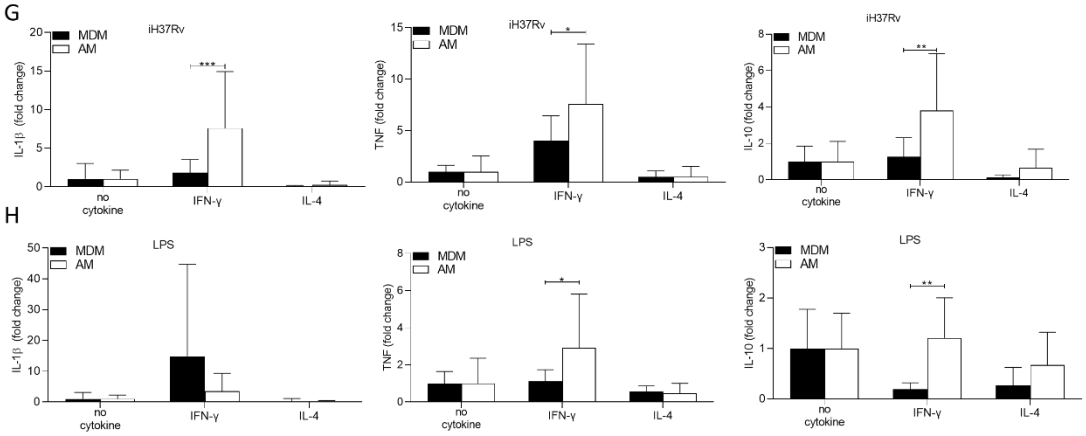
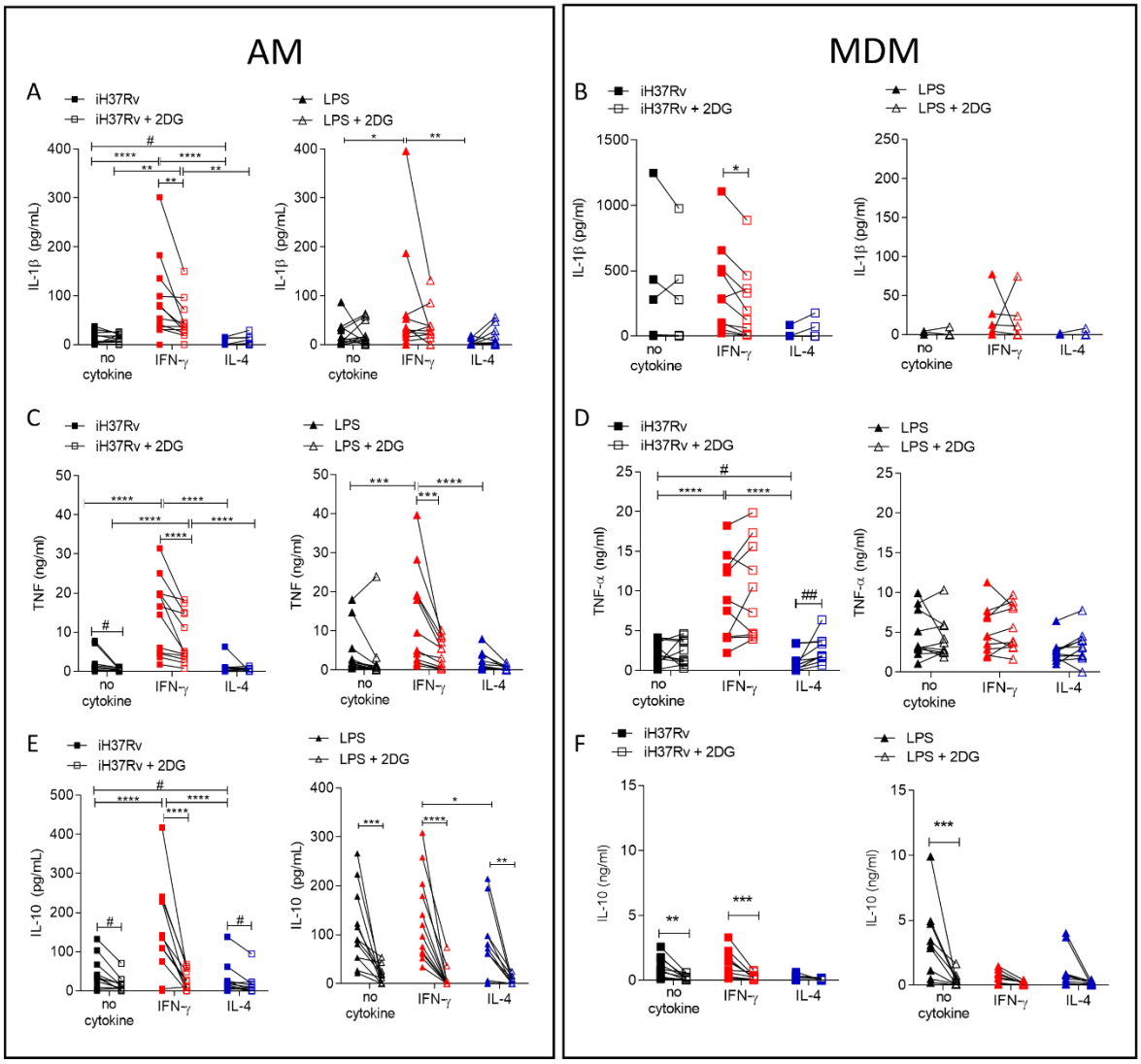

G
H
MDM
AM
iH37Rv
LPS
IL-1β (fold change)
TNF (fold change)
IL-10 (fold change)
no cytokine
IFN-γ
IL-4

Figure 5


AM
MDM
A
B
C
D
E
F
iH37Rv
iH37Rv + 2DG
LPS
LPS + 2DG
IL-1β (pg/mL)
IL-1β (pg/ml)
TNF (ng/ml)
TNF-α (ng/ml)
IL-10 (pg/mL)
IL-10 (ng/ml)
no cytokine
IFN-γ
IL-4