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, Karl M Gogan, Oisin Ó Gallchobhair, Dearbhla M Murphy, Sinead A O'Rourke, Finbarr O'Connell, Parthiban Nadarajan, James J Phelan, 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

Declarations of interest: none

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.

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). 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 ($1x10^5$ 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,

111 with Seahorse medium, iH37Rv or LPS. Post stimulation the ECAR and OCR were continually sampled
112 at 20-minute intervals for times indicated. Analyses were carried out at approximately 150 minutes as
113 previously described (7). Fold change in ECAR and OCR was then calculated compared to unstimulated
114 unprimed controls. Percent change in ECAR and OCR was also calculated versus the respective primed
115 control to examine the capacity of cells to increase metabolic parameters.

116 **Cytokine assays**

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

119 **Flow cytometry**

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

129

130 **Statistical analysis**

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

137

138 **Results**

139 **IFN-γ induces metabolic reprogramming in both AM and MDM.**

140 AM alter their metabolism in response to Mtb (8). Human macrophages also undergo a rapid increase
141 in ECAR early in response to activation (7) and these pathways can be pharmacologically manipulated
142 (9, 10). The metabolic and functional plasticity of human AM remains unexplored, however recent
143 evidence shows they express both 'M1' and 'M2' markers (11). Murine AM can be reprogrammed
144 through an IFN-γ dependent mechanism (12). We therefore sought to examine whether priming
145 human AM with IFN-γ compared with IL-4 or unprimed AM, could influence their metabolic function
146 and response to bacterial stimuli. We stimulated with whole bacteria; Mtb (iH37Rv) or gram-negative
147 cell wall component; LPS. AM were plated in a Seahorse plate and primed with IFN-γ or IL-4 (both 10
148 ng/ml) for 24 h or left unprimed. AM ECAR and OCR were recorded for 30 min at baseline. AM were
149 then stimulated in the Seahorse XFe24 Analyzer with medium (control), iH37Rv (MOI; 1-10) or LPS
150 (100 ng/ml) and ECAR (Figure S1A) and OCR (Figure S1B) were continuously monitored.

151 At 150 minutes post stimulation fold change compared to unprimed unstimulated AM was calculated
152 for ECAR (Figure 1A) and OCR (Figure 1B). IFN-γ priming significantly increased the ECAR and OCR of
153 unstimulated human AM compared with control or IL-4 primed AM (Figure 1A,B). Upon stimulation
154 with iH37Rv or LPS, AM significantly increased ECAR compared to their respective unstimulated
155 controls, regardless of cytokine priming (Figure 1A). IFN-γ primed and subsequently stimulated AM
156 exhibited a significantly increased ECAR compared with stimulated control or IL-4 primed AM (Figure
157 1A). IL-4 primed iH37Rv stimulated AM increased ECAR to similar extent as unprimed controls (Figure
158 1A; left). Conversely, IL-4 primed AM stimulated with LPS AM did not increase their ECAR to the same
159 extent as controls (Figure 1A; right), suggesting that IL-4 reduces the AM ability to increase ECAR in
160 response to LPS stimulation. IFN-γ significantly increased the OCR of AM in response to stimulation
161 with iH37Rv or LPS, and had enhanced OCR compared with other stimulated controls (Figure 1B).
162 These data indicate that priming human AM with IFN-γ increases both glycolytic and oxidative
163 metabolism, which is then further increased upon stimulation.

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

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

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

187 Similar to AM, IFN-γ primed MDM have increased OCR compared with control or IL-4 primed MDM
188 (Figure 1F). In contrast with the AM, stimulation of IFN-γ primed MDM does not further increase OCR
189 however, the elevated OCR in IFN-γ primed MDM remains significantly higher compared to control or
190 IL-4 primed MDM when stimulated with iH37Rv (Figure 1F). The percent change in ECAR upon
191 stimulation (from respective baselines) illustrates that all MDM groups significantly increase ECAR
192 from their own baseline in response to stimulation (Figure 1G). Interestingly, although IL-4 significantly
193 reduced ECAR in iH37Rv and LPS stimulated MDM compared with unprimed stimulated controls
194 (Figure 1E), the IL-4 primed MDM have significantly enhanced capacity to ramp up glycolysis in
195 response to LPS, as evidence by the significantly increased percentage change in LPS stimulated, IL-4
196 primed MDM compared with IFN-γ primed controls (Figure 1G). Control or IFN-γ primed MDM

197 stimulated with either iH37Rv or LPS decreases percentage change in the OCR associated with a
198 stimulation-induced shift to "Warburg"-like metabolism (Figure 1H). This effect is not observed in IL-
199 4 primed MDM, moreover, IL-4 primed MDM stimulated with iH37Rv had significantly elevated
200 percent change in OCR compared with stimulated unprimed or IFN-γ primed MDM (Figure 1H). These
201 data indicate that IL-4 priming prevents human MDM utilising "Warburg"-like metabolism in response
202 to stimulation.

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

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

216

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

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

227 IFN-γ significantly increased the expression of HLA-DR compared with control or IL-4 primed
228 unstimulated AM (Figure 2A). Stimulation with iH37Rv significantly upregulated HLA-DR, but only in
229 unprimed AM (Figure 2A). Similarly, LPS significantly induced HLA-DR in unprimed or IL-4 primed AM
230 but not in IFN-γ primed AM (Figure 2A). IFN-γ also significantly increased the expression of HLA-DR
231 compared with control or IL-4 primed MDM (Figure 2B). Stimulation of IFN-γ primed MDM with iH37Rv
232 or LPS robustly enhanced the expression of HLA-DR (Figure 2B). IFN-γ priming significantly upregulated
233 CD40 expression in unstimulated AM (Figure 2C; right). In addition, CD40 was upregulated following
234 iH37Rv or LPS stimulation of AM in all groups assessed with the exception of IFN-γ primed AM
235 stimulated with iH37Rv (Figure 2C). IFN-γ increased the expression of the co-stimulatory molecule
236 CD40 in unstimulated MDM (Figure 2D). Stimulation of MDM with iH37Rv or LPS significantly
237 increased CD40 expression, with the exception of iH37Rv stimulation in IL-4 primed MDM (Figure 2D).
238 Expression of CD86 in response to stimulation with iH37Rv was only upregulated in IL-4 primed AM,
239 however, LPS induced upregulation of CD86 in all AM, with IFN-γ and IL-4 primed AM exhibiting
240 significantly enhances CD86 expression compared to unprimed control (Figure 2E). CD86 expression

241 induced by MDM in response to iH37Rv or LPS was enhanced by priming with either IFN-γ or IL-4, with
242 IL-4 inducing significantly higher expression compared with unprimed or IFN-γ primed MDM (Figure
243 2F).

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

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

253 Since IFN-γ drove glycolysis and the expression of the macrophage activation markers CD40 and HLA-
254 DR in both AM and MDM we wanted to examine if the increased glycolysis was associated with
255 enhanced expression of activation markers expression. Human AM (Figure 3A,3C,3E) and MDM (Figure
256 3B,3D,3F) were primed with IFN-γ or IL-4 for 24 h or left unprimed. Macrophages were then treated
257 with the glycolytic inhibitor, 2DG for 1 h prior to stimulation with iH37Rv or LPS. After 24 h AM and
258 MDM were analysed by flow cytometry for expression of HLA-DR (Figure 3A,3B), CD40 (Figure 3C,3D)
259 and CD86(Figure 3E,3F). 2DG-mediated inhibition of glycolysis following stimulation was confirmed
260 (Figure S2B).

261 Inhibiting glycolysis with 2DG did not alter expression of HLA-DR on AM (Figure 3A). Interestingly, the
262 increased expression of HLA-DR in IFN-γ primed MDM was dependent on glycolysis in unstimulated
263 and iH37Rv stimulated MDM, however, increased expression of HLA-DR by LPS stimulated IFN-γ
264 primed MDM remained elevated in the presence of 2DG (Figure 3B). Expression of CD40 was not
265 affected by 2DG in unstimulated or iH37Rv stimulated AM (Figure 3C). Conversely, LPS induced
266 expression of CD40 was significantly inhibited by 2DG in unprimed and IFN-γ primed AM but not in IL-
267 4 primed AM (Figure 3C). In contrast, enhanced expression of CD40 in IFN-γ primed MDM in
268 unstimulated or iH37Rv stimulated MDM was significantly reduced with the addition of 2DG, with no
269 effect on the expression of CD40 in LPS stimulated human MDM regardless of cytokine priming (Figure
270 3D). 2DG enhanced expression of CD86 in unstimulated IFN-γ or IL-4 primed AM but did not affect
271 expression in any stimulated AM (Figure 3E). Interestingly, when unstimulated AM were examined in
272 the absence of stimulation, IFN-γ priming significantly increased CD86 (Figure 3E). 2DG inhibited the
273 increased expression of CD86 in response to iH37Rv stimulation in IFN-γ or IL-4 primed MDM, but no
274 difference was observed in unstimulated or LPS stimulated MDM (Figure 3F).

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

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

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

292 Changes in macrophage metabolism have been previously associated with altered cytokine
293 production (5, 8, 14). Having established that both IFN-γ and IL-4 can significantly alter metabolism in
294 human macrophages we next sought to examine the ability of AM and MDM to secrete cytokines
295 when primed with IFN-γ or IL-4. Human AM (Figure 4A,4C,4E) and MDM (Figure 4B,4D,4F) were left
296 unprimed or primed with IFN-γ or IL-4 for 24 h. Macrophages were then stimulated with iH37Rv or
297 LPS. Supernatants were harvested 24 h post stimulation and concentrations of IL-1β (Figure 4A,4B),
298 TNF (Figure 4C,4D) and IL-10 (Figure 4E,4F) were quantified by ELISA. While iH37Rv stimulation
299 resulted in IL-1β production in unprimed AM and MDM, IFN-γ only significantly enhanced the
300 production of IL-1β by AM (Figure 4A,4B). IL-4 priming attenuated iH37Rv induced IL-1β in both AM
301 and MDM (Figure 4A,4B). As expected, IL-1β secretion was not induced in response to LPS stimulation
302 however, in the presence of IFN-γ, IL-1β was detectable (Figure 4A, 4B). TNF was significantly induced
303 in unprimed or IFN-γ primed, but not IL-4 primed AM in response to iH37Rv and LPS (Figure 4C). IFN-
304 γ enhanced production of TNF by AM in response to both iH37Rv and LPS. In contrast, IFN-γ enhanced
305 TNF in response to iH37Rv, but not LPS in MDM (Figure 4C,4D). LPS significantly upregulated the
306 production of TNF in all MDM. Notably, IFN-γ priming did not enhance TNF production and IL-4 priming
307 significantly attenuated LPS-induced TNF (Figure 4D). All stimulated AM secreted IL-10 regardless of
308 priming (Figure 4E). IFN-γ significantly enhanced iH37Rv induced IL-10 in AM compared to unprimed
309 or IL-4 primed comparators (Figure 4E). IL-4 priming of human AM significantly reduced IL-10
310 production in response to iH37Rv compared with unprimed AM (Figure 4E). LPS strongly induced IL-
311 10 production in unprimed MDM, which was significantly attenuated by either IFN-γ or IL-4 priming
312 (Figure 4F).

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

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

324 Since IFN-γ drove glycolysis in both AM and MDM, we next sought to examine if cytokine production
325 was associated with enhanced glycolysis. Human AM (Figure 5A,5C,5E) and MDM (Figure 5B,5D,5F)
326 were primed with IFN-γ or IL-4 for 24 h or left unprimed. Macrophages were treated with 2DG (5 mM)
327 for 1 h prior to stimulation with iH37Rv or LPS. Supernatants were harvested 24 h post stimulation
328 and concentrations of IL-1β (Figure 5A,5B), TNF (Figure 5C,5D) and IL-10 (Figure 5E,5F) were
329 quantified. 2DG significantly abrogated production of IL-1β in both IFN-γ primed AM and MDM
330 stimulated with iH37Rv (Figure 5A,5B). Moreover, 2DG significantly reduced TNF production driven by

331 IFN-γ in the AM, and significantly reduced TNF production in unprimed AM stimulated with iH37Rv
332 (Figure 5C). Unlike IL-1β, TNF production was not affected by 2DG in unprimed or IFN-γ primed MDM.
333 Conversely, IL-4 primed MDM exhibited increased TNF production in the presence of 2DG (Figure 5D).
334 IL-10 production was significantly inhibited by 2DG in AM, irrespective of priming or stimulation
335 (Figure 5E). MDM production of IL-10 in response to LPS or iH37Rv was inhibited with 2DG (Figure 5F).

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

344

345 **Discussion**

346 AM are the first responders to infections and inflammatory insults in the lung. We and others have
347 reported that lung resident macrophages are dependent on glycolysis to respond to LPS (15) or Mtb
348 (8, 14), however, their metabolic behaviour is distinct from murine AM (16) reinforcing the need to
349 define cellular metabolism and its tractability in human macrophages in order to design effective
350 immunometabolic therapies. We demonstrate here that human AM can be metabolically
351 reprogrammed by IFN-γ which increased both glycolysis and oxidative phosphorylation and is further
352 enhanced by stimulation with Mtb or LPS. This is in contrast with literature showing murine AM do
353 not increase glycolysis in real-time following LPS stimulation (16), although whether IFN-γ can
354 influence this in mice remains unknown. Our previous work shows that upon activation, the human
355 MDM underwent "Warburg"-like metabolism associated with an increase in glycolysis with a
356 concomitant reduction in oxidative phosphorylation (7). We confirmed this observation and this
357 persists when primed with IFN-γ, unlike AM which do not undergo the shift to "Warburg"-like
358 metabolism. IL-4 primed macrophages are associated in the literature with an increased reliance on
359 oxidative phosphorylation (17). Indeed, murine IL-4 primed BMDM have increased ECAR and OCR (18,
360 19). IL-4 did not promote oxidative metabolism in human AM or MDM, even when stimulated. IL-4
361 decreased glycolysis while preventing a decline in oxidative metabolism in MDM, inhibiting their shift
362 to "Warburg"-like metabolism. When directly comparing percentage change in ECAR of unprimed or
363 macrophages primed with IFN-γ or IL-4, they all had similar rates of change despite profound
364 differences in maximal ECAR. These data suggest that the resting state of the macrophage, and the
365 cytokines it has recently been exposed to before activation, may be a key determining factor for the
366 response and outcome to infections.

367 Directly comparing the AM with the MDM demonstrates that human AM are more reliant on oxidative
368 metabolism upon IFN-γ priming and stimulation. Previous observations had identified a high baseline
369 of lactate in supernatants of human AM compared to MDM (8). Interestingly, IFN-γ primed AM and
370 MDM had significantly reduced capacity to increase ECAR (percentage change) in response to LPS
371 stimulation, suggesting that there may be a point of maximal glycolysis. This ability to induce max
372 glycolysis may be advantageous during infection as lactate, a breakdown product of glycolysis, has
373 been shown to have anti-microbial functions against Mtb (13, 20) . Moreover, pathogens such as Mtb
374 can downregulate metabolic pathways after infection (21, 22) and IFN-γ is crucial for control of Mtb
375 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

422 production of IL-1β, TNF and IL-10 compared with unprimed controls. This is in contrast with IFN-γ
423 primed MDM which only upregulate TNF compared to their unprimed controls. These data indicate
424 that effective immune responses to Mtb in the lung may require AM to be primed with IFN-γ and may
425 in part explain why patients deficient in IFN-γ or associated signalling have increased risk of TB (36,
426 37). IFN-γ driven production of TNF is dependent on glycolysis in AM. MDM secretion of TNF is
427 independent of glycolysis and conversely, inhibition of glycolysis in IL-4 primed Mtb stimulated MDM
428 enhanced TNF production. Previous studies have demonstrated that glycolysis was required by murine
429 BMDM but not AM to secrete TNF and IL-6 (16). Conversely, our data demonstrates that human AM
430 need glycolysis for optimal TNF production, especially in the presence of IFN-γ, whereas MDM do not.
431 Once again, we highlight that there is variation in the metabolic requirements within human
432 macrophage subpopulations, and importantly, that the AM is metabolically tractable to modulate its
433 function.

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

440 Trained immunity improves innate responses to infection and is emerging as a key component of host
441 directed therapies (HDT) and strategies to improve vaccine efficacy and the design of respiratory
442 mucosal vaccines (27, 28, 39-41). Both IFN-γ and IL-4 can induce trained immunity in murine and
443 human macrophages (12, 19, 42, 43). MDM trained with IFN-γ and LPS, and stimulated with Mtb had
444 increased TNF in the acute activation phase of trained immunity (19), which we observed in IFN-γ
445 primed MDM subsequently stimulated with Mtb. We also observed an increase in TNF, IL-1β and IL-
446 10 in AM potentially suggesting that AM will be a target for innate training, as increased IL-1β is
447 associated with optimal training (39, 43, 44). AM from mice that received an inhaled adenovirus
448 vectored vaccine undergo trained immunity mediated by IFN-γ resulting in elevated MHC-II
449 expression, enhanced cytokine production, and protection against specific and non-specific infection
450 challenges (12, 27). We have previously demonstrated that an adenovirus vectored vaccine induces
451 trained immunity in human monocytes and postulated that this may be IFN-γ dependent (45). The
452 current study provides evidence that IFN-γ can metabolically reprogramme the human AM, resulting
453 in enhanced HLA-DR expression and cytokine production in response to subsequent stimulation.
454 Cumulatively this highlights the importance of ascertaining whether IFN-γ can induce trained
455 immunity in the human AM, which may enhance the design of respiratory mucosal vaccines.

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

465 **Study limitations**

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

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

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

488 **Conclusion**

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

498

499 **Ethics statement**

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

502 **Acknowledgements**

503 We would like to acknowledge the Irish Blood Transfusion Service for supporting our research by
504 approving us to use anonymised un-transfused blood components for our research. We gratefully
505 acknowledge all people undergoing bronchoscopy at St. James's Hospital Dublin who consented to
506 take part in our research. We acknowledge the key contributions of the Clinical Research Facility at St.

James's Hospital and the bronchoscopy suite, and the core facilities at the Trinity Translational Medicine Institute. The following reagent was obtained through BEI Resources, NIAID, NIH: Mycobacterium tuberculosis, Strain H37Rv, Gamma-Irradiated Whole Cells, NR-49098.

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.

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.

Funding

This work was supported by The Royal City of Dublin Hospital Trust (RCDH app 185, awarded to JK), The National Children's Research Centre (D/18/1 awarded to COM) and The Health Research Board (EIA-2019-010 awarded to SAB).

Funders had no role in the study design, collection, analysis or interpretation of the data nor in the writing or submission of the article for publication.

Data availability

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

540 **References**

541 1. Huang L, Nazarova EV, Tan S, Liu Y, Russell DG. Growth of Mycobacterium tuberculosis in vivo
542 segregates with host macrophage metabolism and ontogeny. *The Journal of Experimental*
543 *Medicine* 2018; 215: 1135.
544 2. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley
545 ER, Samokhvalov IM, Merad M. Fate mapping analysis reveals that adult microglia derive from
546 primitive macrophages. *Science (New York, NY)* 2010; 330: 841-845.
547 3. Wang F, Zhang S, Jeon R, Vuckovic I, Jiang X, Lerman A, Folmes CD, Dzeja PD, Herrmann J. Interferon
548 Gamma Induces Reversible Metabolic Reprogramming of M1 Macrophages to Sustain Cell
549 Viability and Pro-Inflammatory Activity. *EBioMedicine* 2018; 30: 303-316.
550 4. Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, Foresti R, Immenschuh S. Human
551 and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A
552 divergent role for glycolysis. *Redox biology* 2019; 22: 101147.
553 5. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, Frezza C,
554 Bernard NJ, Kelly B, Foley NH, Zheng L, Gardet A, Tong Z, Jany SS, Corr SC, Haneklaus M, Caffrey
555 BE, Pierce K, Walmsley S, Beasley FC, Cummins E, Nizet V, Whyte M, Taylor CT, Lin H, Masters
556 SL, Gottlieb E, Kelly VP, Clish C, Auron PE, Xavier RJ, O'Neill LA. Succinate is an inflammatory
557 signal that induces IL-1beta through HIF-1alpha. *Nature* 2013; 496: 238-242.
558 6. O'Leary SM, Coleman MM, Chew WM, Morrow C, McLaughlin AM, Gleeson LE, O'Sullivan MP, Keane
559 J. Cigarette smoking impairs human pulmonary immunity to Mycobacterium tuberculosis.
560 *American journal of respiratory and critical care medicine* 2014; 190: 1430-1436.
561 7. Ó Maoldomhnaigh C, Cox DJ, Phelan JJ, Malone FD, Keane J, Basdeo SA. The Warburg Effect Occurs
562 Rapidly in Stimulated Human Adult but Not Umbilical Cord Blood Derived Macrophages.
563 *Frontiers in immunology* 2021; 12.
564 8. Gleeson LE, Sheedy FJ, Palsson-McDermott EM, Triglia D, O'Leary SM, O'Sullivan MP, O'Neill LA,
565 Keane J. Cutting Edge: Mycobacterium tuberculosis Induces Aerobic Glycolysis in Human
566 Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication.
567 *Journal of immunology (Baltimore, Md : 1950)* 2016; 196: 2444-2449.
568 9. Cox DJ, Coleman AM, Gogan KM, Phelan JJ, Ó Maoldomhnaigh C, Dunne PJ, Basdeo SA, Keane J.
569 Inhibiting Histone Deacetylases in Human Macrophages Promotes Glycolysis, IL-1β, and T
570 Helper Cell Responses to Mycobacterium tuberculosis. *Frontiers in immunology* 2020; 11: 1-
571 15.
572 10. Phelan JJ, McQuaid K, Kenny C, Gogan KM, Cox DJ, Basdeo SA, O'Leary S, Tazoll SC, Ó
573 Maoldomhnaigh C, O'Sullivan MP, O'Neill LA, O'Sullivan MJ, Keane J. Desferrioxamine
574 Supports Metabolic Function in Primary Human Macrophages Infected With Mycobacterium
575 tuberculosis. *Frontiers in immunology* 2020; 11.
576 11. Mitsi E, Kamng'ona R, Rylance J, Solórzano C, Jesus Reiné J, Mwandumba HC, Ferreira DM, Jambo
577 KC. Human alveolar macrophages predominately express combined classical M1 and M2
578 surface markers in steady state. *Respiratory Research* 2018; 19: 66.
579 12. Yao Y, Jeyanathan M, Haddadi S, Barra NG, Vaseghi-Shanjani M, Damjanovic D, Lai R, Afkhami S,
580 Chen Y, Dvorkin-Gheva A, Robbins CS, Schertzer JD, Xing Z. Induction of Autonomous Memory
581 Alveolar Macrophages Requires T Cell Help and Is Critical to Trained Immunity. *Cell* 2018; 175:
582 1634-1650.
583 13. Ó Maoldomhnaigh C, Cox DJ, Phelan JJ, Mitermite M, Murphy DM, Leisching G, Thong L, O'Leary
584 SM, Gogan KM, McQuaid K, Coleman AM, Gordon SV, Basdeo SA, Keane J. Lactate Alters
585 Metabolism in Human Macrophages and Improves Their Ability to Kill Mycobacterium
586 tuberculosis. *Frontiers in immunology* 2021; 12.
587 14. Gleeson LE, O'Leary SM, Ryan D, McLaughlin AM, Sheedy FJ, Keane J. Cigarette Smoking Impairs
588 the Bioenergetic Immune Response to Mycobacterium tuberculosis Infection. *American*
589 *journal of respiratory cell and molecular biology* 2018; 59: 572-579.

590 15. Lavrich KS, Speen AM, Ghio AJ, Bromberg PA, Samet JM, Alexis NE. Macrophages from the upper
591 and lower human respiratory tract are metabolically distinct. *American Journal of Physiology-*
592 *Lung Cellular and Molecular Physiology* 2018; 315: L752-L764.
593 16. Woods PS, Kimmig LM, Meliton AY, Sun KA, Tian Y, O'Leary EM, Gökalp GA, Hamanaka RB, Mutlu
594 GM. Tissue-Resident Alveolar Macrophages Do Not Rely on Glycolysis for LPS-induced
595 Inflammation. *American journal of respiratory cell and molecular biology* 2020; 62: 243-255.
596 17. Van den Bossche J, Saraber DL. Metabolic regulation of macrophages in tissues. *Cellular*
597 *Immunology* 2018; 330: 54-59.
598 18. Huang SC, Smith AM, Everts B, Colonna M, Pearce EL, Schilling JD, Pearce EJ. Metabolic
599 Reprogramming Mediated by the mTORC2-IRF4 Signaling Axis Is Essential for Macrophage
600 Alternative Activation. *Immunity* 2016; 45: 817-830.
601 19. Lundahl MLE, Mitermite M, Ryan DG, Case S, Williams NC, Yang M, Lynch RI, Lagan E, Lebre FM,
602 Gorman AL, Stojkovic B, Bracken AP, Frezza C, Sheedy FJ, Scanlan EM, O'Neill LAJ, Gordon SV,
603 Lavelle EC. Macrophage innate training induced by IL-4 and IL-13 activation enhances OXPHOS
604 driven anti-mycobacterial responses. *eLife* 2022; 11: e74690.
605 20. Krishnamoorthy G, Kaiser P, Abu Abed U, Weiner J, 3rd, Moura-Alves P, Brinkmann V, Kaufmann
606 SHE. FX11 limits Mycobacterium tuberculosis growth and potentiates bactericidal activity of
607 isoniazid through host-directed activity. *Disease models & mechanisms* 2020; 13.
608 21. Cumming BM, Addicott KW, Adamson JH, Steyn AJ. Mycobacterium tuberculosis induces
609 decelerated bioenergetic metabolism in human macrophages. *Elife* 2018; 7.
610 22. Mendonca LE, Pernet E, Khan N, Sanz J, Kaufmann E, Downey J, Grant A, Orlova M, Schurr E,
611 Krawczyk C, Jones RG, Barreiro LB, Divangahi M. Human alveolar macrophage metabolism is
612 compromised during Mycobacterium tuberculosis infection. *Frontiers in immunology* 2022;
613 13: 1044592.
614 23. Braverman J, Sogi KM, Benjamin D, Nomura DK, Stanley SA. HIF-1α Is an Essential Mediator of IFN-
615 γ-Dependent Immunity to Mycobacterium tuberculosis. *Journal of immunology (Baltimore,*
616 *Md : 1950)* 2016; 197: 1287-1297.
617 24. Cumming BM, Pacl HT, Steyn AJC. Relevance of the Warburg Effect in Tuberculosis for Host-
618 Directed Therapy. *Frontiers in Cellular and Infection Microbiology* 2020; 10.
619 25. Van den Bossche J, Baardman J, de Winther MP. Metabolic Characterization of Polarized M1 and
620 M2 Bone Marrow-derived Macrophages Using Real-time Extracellular Flux Analysis. *Journal of*
621 *visualized experiments : JoVE* 2015.
622 26. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology.
623 *Journal of immunology (Baltimore, Md : 1950)* 2004; 172: 2731-2738.
624 27. D'Agostino MR, Lai R, Afkhami S, Khera A, Yao Y, Vaseghi-Shanjani M, Zganiacz A, Jeyanathan M,
625 Xing Z. Airway Macrophages Mediate Mucosal Vaccine–Induced Trained Innate Immunity
626 against Mycobacterium tuberculosis in Early Stages of Infection. *The Journal of Immunology*
627 2020; 205: 2750-2762.
628 28. Afkhami S, D'Agostino MR, Zhang A, Stacey HD, Marzok A, Kang A, Singh R, Bavananthasivam J, Ye
629 G, Luo X, Wang F, Ang JC, Zganiacz A, Sankar U, Kazhdan N, Koenig JFE, Phelps A, Gameiro SF,
630 Tang S, Jordana M, Wan Y, Mossman KL, Jeyanathan M, Gillgrass A, Medina MFC, Smaill F,
631 Lichty BD, Miller MS, Xing Z. Respiratory mucosal delivery of next-generation COVID-19
632 vaccine provides robust protection against both ancestral and variant strains of SARS-CoV-2.
633 *Cell* 2022; 185: 896-915.e819.
634 29. Lachmandas E, Boutens L, Ratter JM, Hijmans A, Hooiveld GJ, Joosten LAB, Rodenburg RJ, Fransen
635 JAM, Houtkooper RH, van Crevel R, Netea MG, Stienstra R. Microbial stimulation of different
636 Toll-like receptor signalling pathways induces diverse metabolic programmes in human
637 monocytes. *Nature Microbiology* 2016; 2: 16246.
638 30. Hackett EE, Charles-Messance H, O'Leary SM, Gleeson LE, Muñoz-Wolf N, Case S, Wedderburn A,
639 Johnston DGW, Williams MA, Smyth A, Ouimet M, Moore KJ, Lavelle EC, Corr SC, Gordon SV,

640 Keane J, Sheedy FJ. Mycobacterium tuberculosis Limits Host Glycolysis and IL-1β by Restriction
641 of PFK-M via MicroRNA-21. *Cell reports* 2020; 30: 124-136.e124.
642 31. Müller E, Christopoulos PF, Halder S, Lunde A, Beraki K, Speth M, Øynebråten I, Corthay A. Toll-
643 Like Receptor Ligands and Interferon-γ Synergize for Induction of Antitumor M1 Macrophages.
644 *Frontiers in immunology* 2017; 8: 1383.
645 32. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun
646 MM. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent.
647 *N Engl J Med* 2001; 345: 1098-1104.
648 33. Harris J, Hope JC, Keane J. Tumor Necrosis Factor Blockers Influence Macrophage Responses to
649 Mycobacterium tuberculosis. *The Journal of Infectious Diseases* 2008; 198: 1842-1850.
650 34. Harris J, Keane J. How tumour necrosis factor blockers interfere with tuberculosis immunity.
651 *Clinical and experimental immunology* 2010; 161: 1-9.
652 35. Bourigault ML, Segueni N, Rose S, Court N, Vacher R, Vasseur V, Erard F, Le Bert M, Garcia I, Iwakura
653 Y, Jacobs M, Ryffel B, Quesniaux VF. Relative contribution of IL-1alpha, IL-1beta and TNF to
654 the host response to Mycobacterium tuberculosis and attenuated M. bovis BCG. *Immunity,*
655 *inflammation and disease* 2013; 1: 47-62.
656 36. Ni Cheallaigh C, Sheedy FJ, Harris J, Munoz-Wolf N, Lee J, West K, McDermott EP, Smyth A, Gleeson
657 LE, Coleman M, Martinez N, Hearnden CH, Tynan GA, Carroll EC, Jones SA, Corr SC, Bernard
658 NJ, Hughes MM, Corcoran SE, O'Sullivan M, Fallon CM, Kornfeld H, Golenbock D, Gordon SV,
659 O'Neill LA, Lavelle EC, Keane J. A Common Variant in the Adaptor Mal Regulates Interferon
660 Gamma Signaling. *Immunity* 2016; 44: 368-379.
661 37. Remus N, Reichenbach J, Picard C, Rietschel C, Wood P, Lammas D, Kumararatne DS, Casanova J-
662 L. Impaired Interferon Gamma-Mediated Immunity and Susceptibility to Mycobacterial
663 Infection in Childhood. *Pediatric Research* 2001; 50: 8-13.
664 38. Kulikauskaite J, Wack A. Teaching Old Dogs New Tricks? The Plasticity of Lung Alveolar Macrophage
665 Subsets. *Trends in immunology* 2020; 41: 864-877.
666 39. Moorlag SJCFM, Khan N, Novakovic B, Kaufmann E, Jansen T, van Crevel R, Divangahi M, Netea
667 MG. β-Glucan Induces Protective Trained Immunity against Mycobacterium tuberculosis
668 Infection: A Key Role for IL-1. *Cell reports* 2020; 31: 107634-107634.
669 40. Netea MG, Giamarellos-Bourboulis EJ, Domínguez-Andrés J, Curtis N, van Crevel R, van de
670 Veerdonk FL, Bonten M. Trained Immunity: a Tool for Reducing Susceptibility to and the
671 Severity of SARS-CoV-2 Infection. *Cell* 2020; 181: 969-977.
672 41. Zhou J, Lv J, Carlson C, Liu H, Wang H, Xu T, Wu F, Song C, Wang X, Wang T, Qian Z. Trained immunity
673 contributes to the prevention of Mycobacterium tuberculosis infection, a novel role of
674 autophagy. *Emerging microbes & infections* 2021; 10: 578-588.
675 42. Schrijver DP, Röring RJ, Deckers J, de Dreu A, Toner YC, Prevot G, Priem B, Munitz J, Nugraha EG,
676 van Elsas Y, Azzun A, Anbergen T, Groh LA, Becker AMD, Pérez-Medina C, Oosterwijk RS,
677 Novakovic B, Moorlag SJCFM, Jansen A, Pickkers P, Kox M, Beldman TJ, Kluza E, van Leent
678 MMT, Teunissen AJP, van der Meel R, Fayad ZA, Joosten LAB, Fisher EA, Merkx M, Netea MG,
679 Mulder WJM. Resolving sepsis-induced immunoparalysis via trained immunity by targeting
680 interleukin-4 to myeloid cells. *Nature Biomedical Engineering* 2023.
681 43. Li W, Moorlag S, Koeken V, Röring RJ, de Bree LCJ, Mourits VP, Gupta MK, Zhang B, Fu J, Zhang Z,
682 Grondman I, van Meijgaarden KE, Zhou L, Alaswad A, Joosten LAB, van Crevel R, Xu CJ, Netea
683 MG, Li Y. A single-cell view on host immune transcriptional response to in vivo BCG-induced
684 trained immunity. *Cell reports* 2023; 42: 112487.
685 44. Teufel LU, Arts RJW, Netea MG, Dinarello CA, Joosten LAB. IL-1 family cytokines as drivers and
686 inhibitors of trained immunity. *Cytokine* 2022; 150: 155773.
687 45. Murphy DM, Cox DJ, Connolly SA, Breen EP, Brugman AAI, Phelan JJ, Keane J, Basdeo SA. Trained
688 immunity is induced in humans after immunization with an adenoviral vector COVID-19
689 vaccine. *The Journal of Clinical Investigation* 2023; 133.

690 46. Cahill C, O'Connell F, Gogan KM, Cox DJ, Basdeo SA, O'Sullivan J, Gordon SV, Keane J, Phelan JJ.
691 The Iron Chelator Desferrioxamine Increases the Efficacy of Bedaquiline in Primary Human
692 Macrophages Infected with BCG. *International Journal of Molecular Sciences* 2021; 22: 2938.
693 47. Coleman MM, Basdeo SA, Coleman AM, Ni Cheallaigh C, Peral de Castro C, McLaughlin AM, Dunne
694 PJ, Harris J, Keane J. All-trans Retinoic Acid Augments Autophagy during Intracellular Bacterial
695 Infection. *American journal of respiratory cell and molecular biology* 2018.
696 48. O'Connor G, Krishnan N, Fagan-Murphy A, Cassidy J, O'Leary S, Robertson BD, Keane J, O'Sullivan
697 MP, Cryan S-A. Inhalable poly(lactic-co-glycolic acid) (PLGA) microparticles encapsulating all-
698 trans-Retinoic acid (ATRA) as a host-directed, adjunctive treatment for Mycobacterium
699 tuberculosis infection. *European Journal of Pharmaceutics and Biopharmaceutics* 2019; 134:
700 153-165.
701 49. Lawlor C, O'Connor G, O'Leary S, Gallagher PJ, Cryan S-A, Keane J, O'Sullivan MP. Treatment of
702 Mycobacterium tuberculosis-Infected Macrophages with Poly(Lactic-Co-Glycolic Acid)
703 Microparticles Drives NFκB and Autophagy Dependent Bacillary Killing. *PLoS One* 2016; 11:
704 e0149167-e0149167.
705 50. Dawson R, Condos R, Tse D, Huie ML, Ress S, Tseng CH, Brauns C, Weiden M, Hoshino Y, Bateman
706 E, Rom WN. Immunomodulation with recombinant interferon-gamma1b in pulmonary
707 tuberculosis. *PLoS One* 2009; 4: e6984.
708 51. Bharti R, Roy T, Verma S, Reddy DVS, Shafi H, Verma K, Raman SK, Pal S, Azmi L, Singh AK, Ray L,
709 Mugale MN, Misra A. Transient, inhaled gene therapy with gamma interferon mitigates
710 pathology induced by host response in a mouse model of tuberculosis. *Tuberculosis*
711 *(Edinburgh, Scotland)* 2022; 134: 102198.
712 52. Pereverzeva L, van Linge CCA, Schuurman AR, Klarenbeek AM, Ramirez Moral I, Otto NA, Peters-
713 Sengers H, Butler JM, Schomakers BV, van Weeghel M, Houtkooper RH, Wiersinga WJ, Bonta
714 PI, Annema JT, de Vos AF, van der Poll T. Human alveolar macrophages do not rely on glucose
715 metabolism upon activation by lipopolysaccharide. *Biochim Biophys Acta Mol Basis Dis* 2022;
716 1868: 166488.
717 53. Thiel BA, Lundberg KC, Schlatzer D, Jarvela J, Li Q, Shaw R, Reba SM, Fletcher S, Beckloff SE, Chance
718 MR, Boom WH, Silver RF, Bebek G. Human alveolar macrophages display marked hypo-
719 responsiveness to IFN-γ in both proteomic and gene expression analysis. *PLoS One* 2024; 19:
720 e0295312.

721

722 **Figure Legends**

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

737

738 **Figure 2. IFN-γ boosts activation marker expression on MDM to a greater extent than AM.** Human
739 AM (A, C, E) isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and
740 MDM (B, D, F) were differentiated and adherence purified for 7 days in 10% human serum. Cells were
741 left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. AM or MDM
742 were left unstimulated (circle) or stimulated with iH37Rv (MOI 1-10; square) or LPS (100 ng/ml;
743 triangle). After 24 h cells were detached from the plates by cooling and gentle scraping and stained
744 for HLAR-DR (A, B), CD40 (C, D), CD86 (E, F) and analysed by flow cytometry. Fold change of HLA-DR
745 (G), CD40 (H) and CD86 (I) was calculated for AM (white bar) and MDM (black bar) based on the
746 average of their respective no cytokine controls. Each linked data point represents the average of
747 technical duplicates for one individual biological donor (n=8-9). Statistically significant differences
748 were determined using two-way ANOVA with a Tukey (A-F) or Bonferroni post-test (G-I) (A-F); *P≤0.05,
749 **P≤0.01, P***≤0.001, ****P≤0.001.

750 **Figure 3. Glycolysis is required for IFN-γ induced expression of activation markers by MDM and not**
751 **AM.** Human AM (A, C, E) isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy
752 coats and MDM (B, D, F) were differentiated and adherence purified for 7 days in 10% human serum.
753 Cells were left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. Cells
754 were left untreated (solid) or treated with 2DG (5 mM; empty) 1 h prior to stimulation with iH37Rv
755 (MOI 1-10; square) or LPS (100 ng/ml; triangle) or left unstimulated (circle). After 24 h cells were
756 detached from the plates by cooling and gentle scraping and stained for HLAR-DR (A, B), CD40 (C, D),
757 CD86 (E, F) and analysed by flow cytometry. Each linked data point represents the average of technical
758 duplicates for one individual biological donor (n=8-9). Statistically significant differences were
759 determined using two-way ANOVA with a Tukey post-test (A-F); *P≤0.05, **P≤0.01, P***≤0.001,
760 ****P≤0.001.

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

774 **Figure 5. Cytokine secretion by AM is more reliant on glycolysis than MDM.** Human AM (A, C, E)
775 isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and MDM (B, D, F)
776 were differentiated and adherence purified for 7 days in 10% human serum. Cells were left unprimed
777 (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. Cells were left untreated
778 (solid) treated with 2DG (5 mM; empty) for 1 h prior to stimulation with iH37Rv (MOI 1-10; square) or
779 LPS (100 ng/ml; triangle) or left unstimulated (circle). Supernatants were harvested 24 h after
780 stimulation and concentrations of IL-1β (A, B), TNF (C, D) and IL-10(E, F) were quantified by ELISA. Each
781 linked data point represents the average of technical duplicates for one individual biological donor
782 (AM; n=12-13, MDM; n=8-10). Statistically significant differences were determined using two-way
783 ANOVA with a Tukey post-test (A-D); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 or #P≤0.05,

784 ##P≤0.01 (where IFN-γ primed data sets were excluded for post-test analysis to analyse statistical
785 differences between no cytokine and IL-4 treated data sets).

786

Figure 1

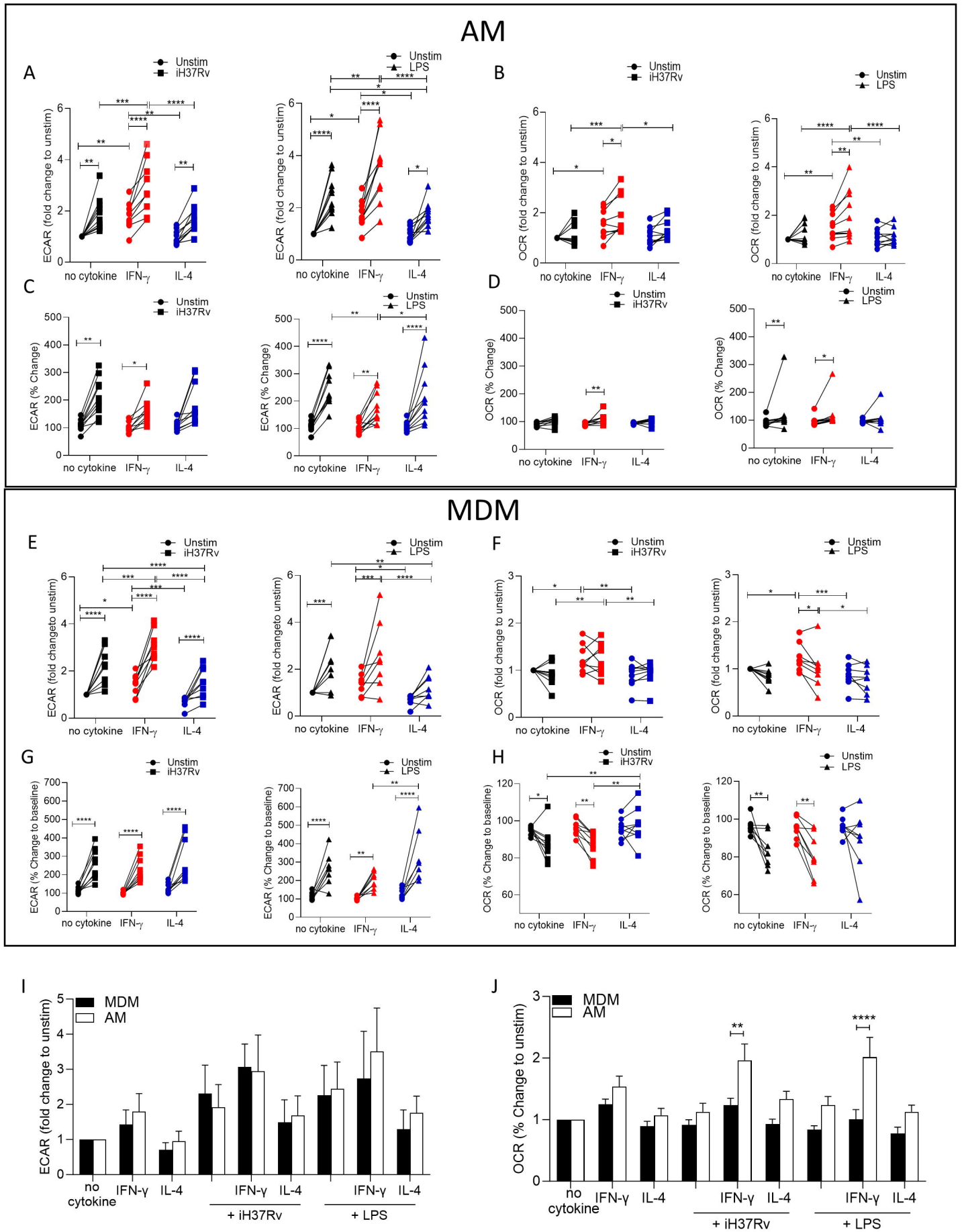

AM
A
Unstim
iH37Rv
ECAR (fold change to unstim)
no cytokine
IFN-γ
IL-4
Unstim
LPS
B
OCR (fold change to unstim)
C
ECAR (% Change)
D
OCR (% Change)
MDM
E
ECAR (fold changeto unstim)
F
G
ECAR (% Change to baseline)
H
OCR (% Change to baseline)
I
MDM
AM
ECAR (fold change to unstim)
+ iH37Rv
+ LPS
J
OCR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

Figure 2

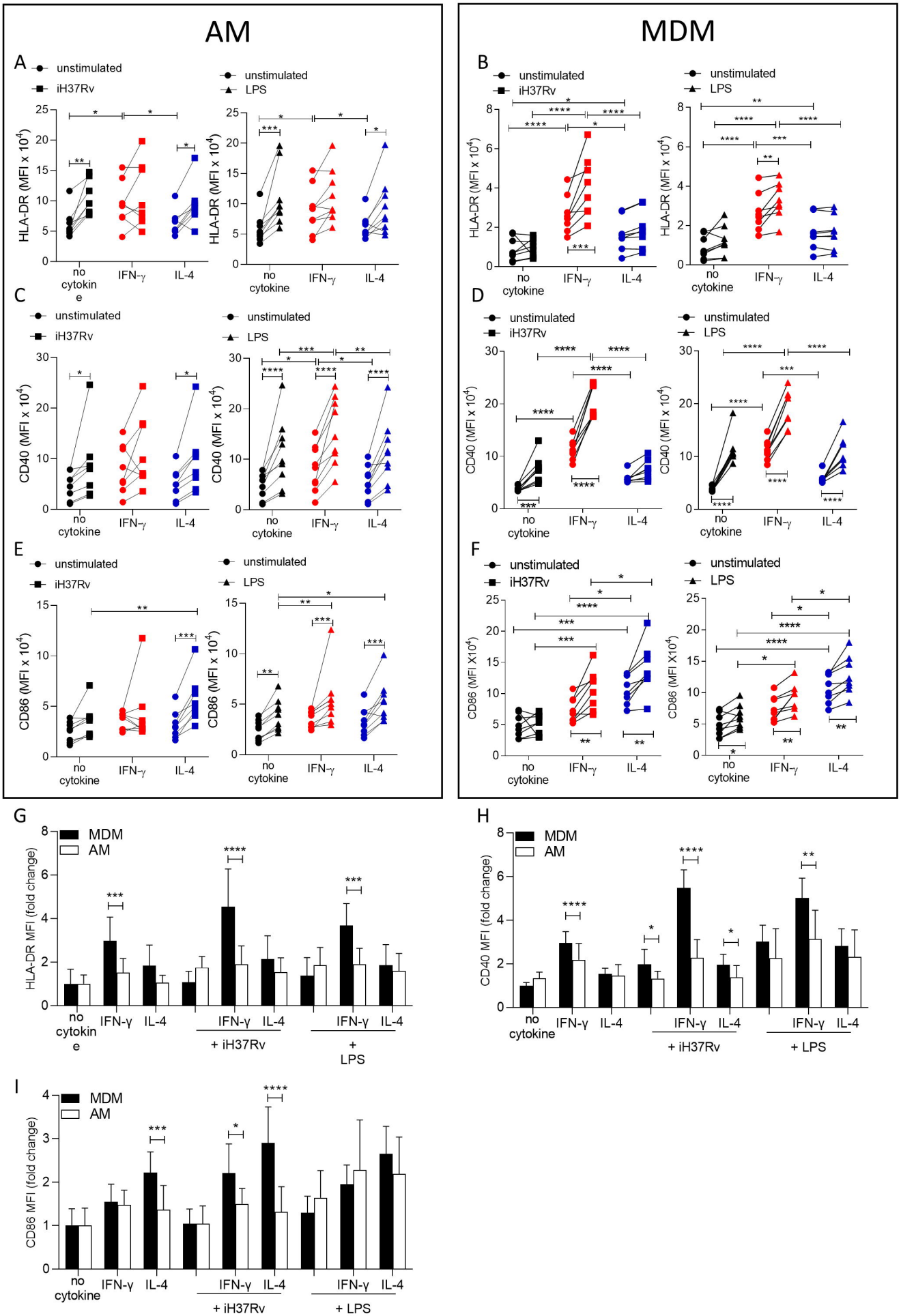

AM
MDM
A
B
C
D
E
F
G
H
I
unstimulated
iH37Rv
LPS
HLA-DR (MFI x 10^4)
CD40 (MFI x 10^4)
CD86 (MFI x 10^4)
CD86 (MFI X10^4)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS
HLA-DR MFI (fold change)
CD40 MFI (fold change)
CD86 MFI (fold change)
MDM
AM

Figure 3

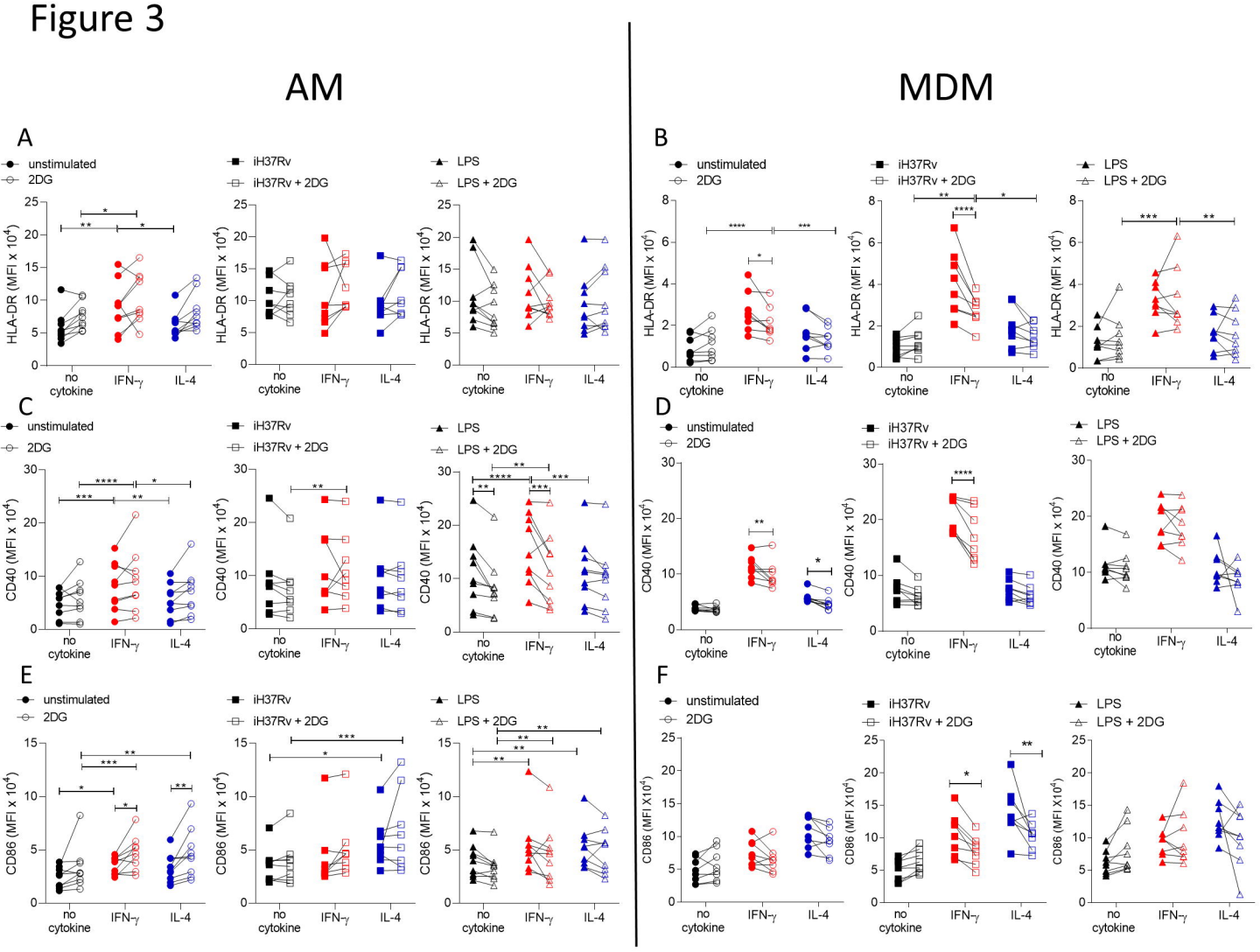

AM
MDM
A
B
C
D
E
F
unstimulated
2DG
iH37Rv
iH37Rv + 2DG
LPS
LPS + 2DG
HLA-DR (MFI x 10⁴)
CD40 (MFI x 10⁴)
CD86 (MFI x 10⁴)
CD86 (MFI X10⁴)
no cytokine
IFN-γ
IL-4

Figure 4

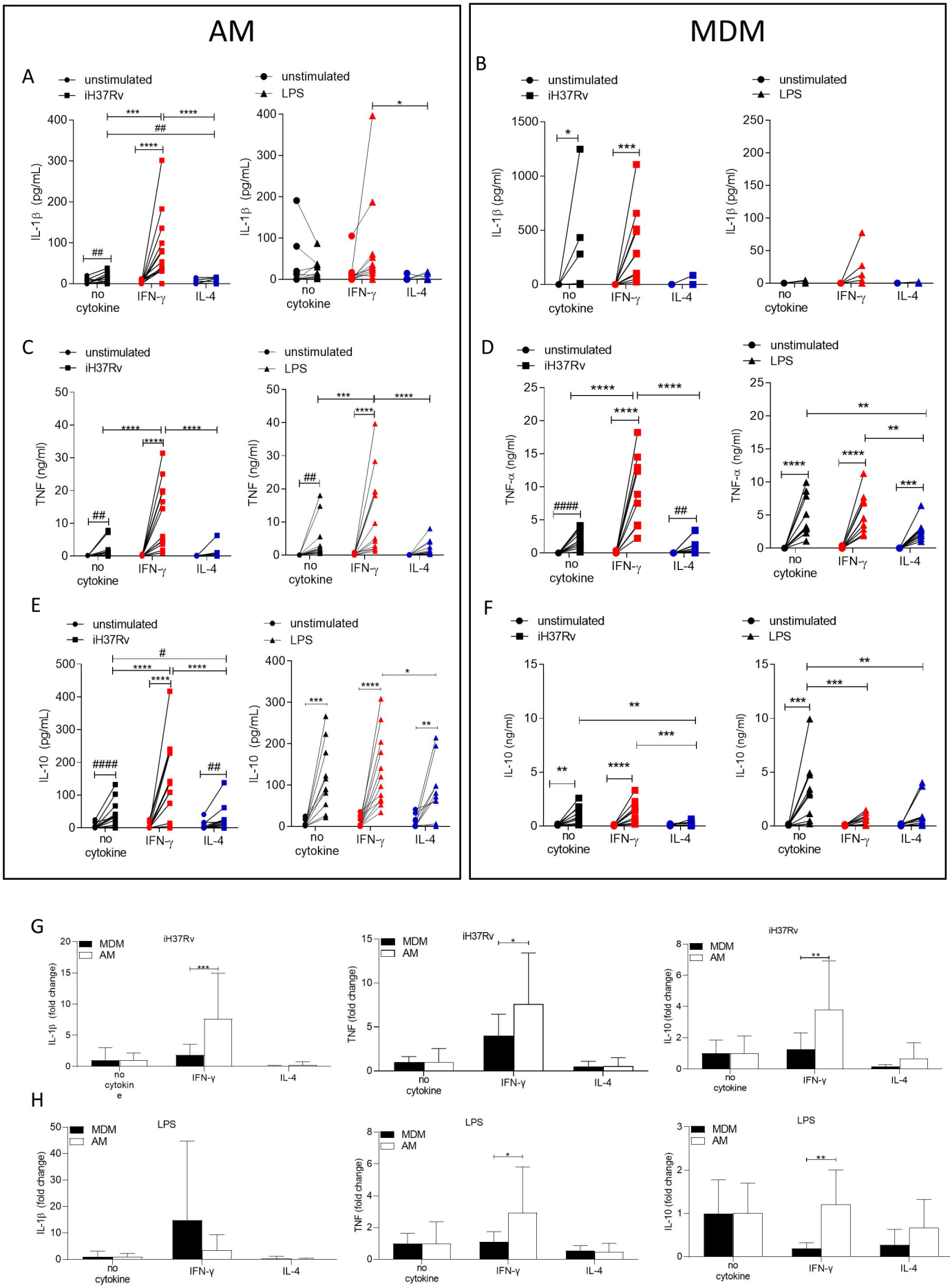
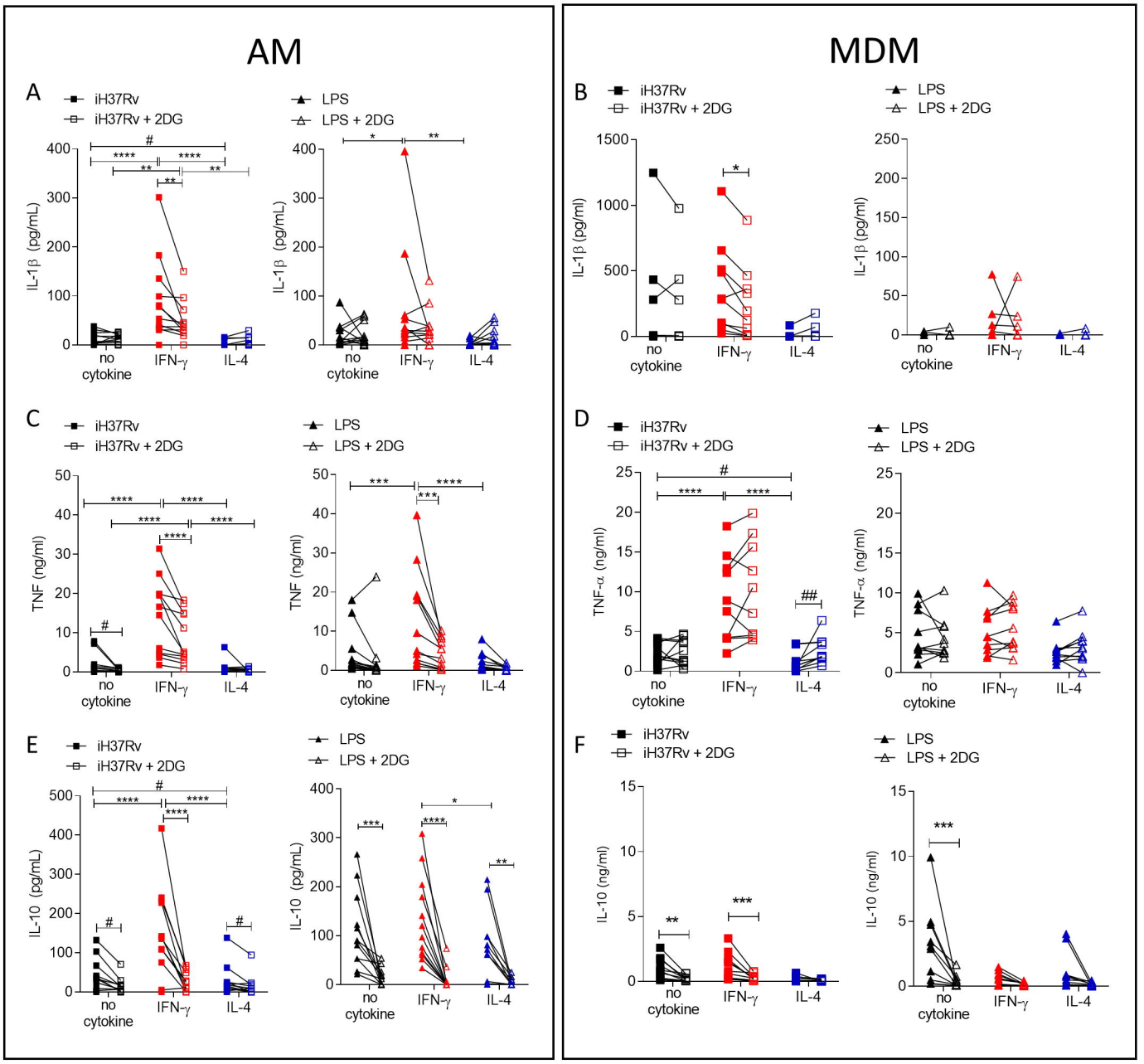

AM
MDM
A
B
C
D
E
F
G
H
unstimulated
iH37Rv
LPS
IL-1β (pg/mL)
IL-1β (pg/ml)
TNF (ng/ml)
TNF-α (ng/ml)
IL-10 (pg/mL)
IL-10 (ng/ml)
no cytokine
IFN-γ
IL-4
MDM
AM
iH37Rv
LPS
IL-1β (fold change)
TNF (fold change)
IL-10 (fold change)

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