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

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Abstract

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

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 calculated compared to unstimulated
114 unprimed controls at 150 minutes, where unstimulated unprimed macrophages were set to 1. This
115 allows for analysis of the effects of both priming and subsequent stimulation for and accounts for the
116 variation in the raw ECAR and OCR reading between runs thereby making each donor its own control.

117 Percent change in ECAR and OCR was also calculated to equalise groups to the same point prior to
118 stimulation. Each condition was compared to its own respective primed or unprimed baseline at 30
119 minutes and this was set to 100%, prior to stimulation, this was carried to examine the capacity of
120 cells to increase metabolic parameters in response to stimulation. Post stimulation percent change
121 data was then extracted and analysed at 150 minutes. This controls for the priming effect and enables
122 the analysis of metabolic capacity in each dataset.

123 **Cytokine assays**

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

126 **Flow cytometry**

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

136

137 **Statistical analysis**

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

144

145 **Results**

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

147 AM alter their metabolism in response to Mtb (8). Human macrophages also undergo a rapid increase
148 in ECAR early in response to activation (7) and these pathways can be pharmacologically manipulated
149 (9, 10). The metabolic and functional plasticity of human AM remains unexplored, however recent
150 evidence shows they express both 'M1' and 'M2' markers (11). Murine AM can be reprogrammed
151 through an IFN-γ dependent mechanism (12). We therefore sought to examine whether priming

152 human AM with IFN-γ compared with IL-4 or unprimed AM, could influence their metabolic function
153 and response to bacterial stimuli. We stimulated with whole bacteria; Mtb (iH37Rv) or gram-negative
154 cell wall component; LPS. AM were plated in a Seahorse plate and primed with IFN-γ or IL-4 (both 10
155 ng/ml) for 24 h or left unprimed. AM ECAR and OCR were recorded for 30 min at baseline. AM were
156 then stimulated in the Seahorse XFe24 Analyzer with medium (control), iH37Rv (MOI; 1-10) or LPS
157 (100 ng/ml) and ECAR (Figure S1A) and OCR (Figure S1B) were continuously monitored.

158 At 150 minutes post stimulation fold change compared to unprimed unstimulated AM was calculated
159 for ECAR (Figure 1A) and OCR (Figure 1B). IFN-γ priming significantly increased the ECAR and OCR of
160 unstimulated human AM compared with control or IL-4 primed AM (Figure 1A,B). Upon stimulation
161 with iH37Rv or LPS, AM significantly increased ECAR compared to their respective unstimulated
162 controls, regardless of cytokine priming (Figure 1A). IFN-γ primed and subsequently stimulated AM
163 exhibited a significantly increased ECAR compared with stimulated control or IL-4 primed AM (Figure
164 1A). IL-4 primed iH37Rv stimulated AM increased ECAR to similar extent as unprimed controls (Figure
165 1A; left). Conversely, IL-4 primed AM stimulated with LPS AM did not increase their ECAR to the same
166 extent as controls (Figure 1A; right), suggesting that IL-4 reduces the AM ability to increase ECAR in
167 response to LPS stimulation. IFN-γ significantly increased the OCR of AM in response to stimulation
168 with iH37Rv or LPS, and had enhanced OCR compared with other stimulated controls (Figure 1B).
169 These data indicate that priming human AM with IFN-γ increases both glycolytic and oxidative
170 metabolism, which is then further increased upon stimulation.

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

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

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

194 Similar to AM, IFN-γ primed MDM have increased OCR compared with control or IL-4 primed MDM
195 (Figure 1F). In contrast with the AM, stimulation of IFN-γ primed MDM does not further increase OCR
196 however, the elevated OCR in IFN-γ primed MDM remains significantly higher compared to control or

197 IL-4 primed MDM when stimulated with iH37Rv (Figure 1F). The percent change in ECAR upon
198 stimulation (from respective baselines) illustrates that all MDM groups significantly increase ECAR
199 from their own baseline in response to stimulation (Figure 1G). Interestingly, although IL-4 significantly
200 reduced ECAR in iH37Rv and LPS stimulated MDM compared with unprimed stimulated controls
201 (Figure 1E), the IL-4 primed MDM have significantly enhanced capacity to ramp up glycolysis in
202 response to LPS, as evidence by the significantly increased percentage change in LPS stimulated, IL-4
203 primed MDM compared with IFN-γ primed controls (Figure 1G). Control or IFN-γ primed MDM
204 stimulated with either iH37Rv or LPS decreases percentage change in the OCR associated with a
205 stimulation-induced shift to "Warburg"-like metabolism (Figure 1H). This effect is not observed in IL-
206 4 primed MDM, moreover, IL-4 primed MDM stimulated with iH37Rv had significantly elevated
207 percent change in OCR compared with stimulated unprimed or IFN-γ primed MDM (Figure 1H). These
208 data indicate that IL-4 priming prevents human MDM utilising "Warburg"-like metabolism in response
209 to stimulation.

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

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

223

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

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

234 IFN-γ significantly increased the expression of HLA-DR compared with control or IL-4 primed
235 unstimulated AM (Figure 2A). Stimulation with iH37Rv significantly upregulated HLA-DR, but only in
236 unprimed AM (Figure 2A). Similarly, LPS significantly induced HLA-DR in unprimed or IL-4 primed AM
237 but not in IFN-γ primed AM (Figure 2A). IFN-γ also significantly increased the expression of HLA-DR
238 compared with control or IL-4 primed MDM (Figure 2B). Stimulation of IFN-γ primed MDM with iH37Rv
239 or LPS robustly enhanced the expression of HLA-DR (Figure 2B). IFN-γ priming significantly upregulated
240 CD40 expression in unstimulated AM (Figure 2C; right). In addition, CD40 was upregulated following

241 iH37Rv or LPS stimulation of AM in all groups assessed with the exception of IFN-γ primed AM
242 stimulated with iH37Rv (Figure 2C). IFN-γ increased the expression of the co-stimulatory molecule
243 CD40 in unstimulated MDM (Figure 2D). Stimulation of MDM with iH37Rv or LPS significantly
244 increased CD40 expression, with the exception of iH37Rv stimulation in IL-4 primed MDM (Figure 2D).
245 Expression of CD86 in response to stimulation with iH37Rv was only upregulated in IL-4 primed AM,
246 however, LPS induced upregulation of CD86 in all AM, with IFN-γ and IL-4 primed AM exhibiting
247 significantly enhances CD86 expression compared to unprimed control (Figure 2E). CD86 expression
248 induced by MDM in response to iH37Rv or LPS was enhanced by priming with either IFN-γ or IL-4, with
249 IL-4 inducing significantly higher expression compared with unprimed or IFN-γ primed MDM (Figure
250 2F).

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

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

260 Since IFN-γ drove glycolysis and the expression of the macrophage activation markers CD40 and HLA-
261 DR in both AM and MDM we wanted to examine if the increased glycolysis was associated with
262 enhanced expression of activation markers expression. Human AM (Figure 3A,3C,3E) and MDM (Figure
263 3B,3D,3F) were primed with IFN-γ or IL-4 for 24 h or left unprimed. Macrophages were then treated
264 with the glycolytic inhibitor, 2DG for 1 h prior to stimulation with iH37Rv or LPS. After 24 h AM and
265 MDM were analysed by flow cytometry for expression of HLA-DR (Figure 3A,3B), CD40 (Figure 3C,3D)
266 and CD86(Figure 3E,3F). 2DG-mediated inhibition of glycolysis following stimulation was confirmed
267 (Figure S2B).

268 Inhibiting glycolysis with 2DG did not alter expression of HLA-DR on AM (Figure 3A). Interestingly, the
269 increased expression of HLA-DR in IFN-γ primed MDM was dependent on glycolysis in unstimulated
270 and iH37Rv stimulated MDM, however, increased expression of HLA-DR by LPS stimulated IFN-γ
271 primed MDM remained elevated in the presence of 2DG (Figure 3B). Expression of CD40 was not
272 affected by 2DG in unstimulated or iH37Rv stimulated AM (Figure 3C). Conversely, LPS induced
273 expression of CD40 was significantly inhibited by 2DG in unprimed and IFN-γ primed AM but not in IL-
274 4 primed AM (Figure 3C). In contrast, enhanced expression of CD40 in IFN-γ primed MDM in
275 unstimulated or iH37Rv stimulated MDM was significantly reduced with the addition of 2DG, with no
276 effect on the expression of CD40 in LPS stimulated human MDM regardless of cytokine priming (Figure
277 3D). 2DG enhanced expression of CD86 in unstimulated IFN-γ or IL-4 primed AM but did not affect
278 expression in any stimulated AM (Figure 3E). Interestingly, when unstimulated AM were examined in
279 the absence of stimulation, IFN-γ priming significantly increased CD86 (Figure 3E). 2DG inhibited the
280 increased expression of CD86 in response to iH37Rv stimulation in IFN-γ or IL-4 primed MDM, but no
281 difference was observed in unstimulated or LPS stimulated MDM (Figure 3F).

282 Cumulatively, these data indicate that IFN-γ upregulates the expression of activation markers more
283 effectively in human MDM than AM. Since these markers are associated with activating T cells during
284 presenting antigen, the ability of IFN-γ or IL-4 primed MDM to process antigen was next assessed,
285 along with the dependency on glycolysis. MDM were primed with IFN-γ or IL-4 for 24 h or left

286 unprimed. MDM were then treated with 2DG for 1 h prior to stimulation with DQ-Ovalbumin (500
287 ng/ml) for 30 min. IL-4 primed MDM had significantly reduced ability to process DQ-Ovalbumin
288 compared with control or IFN-γ primed MDM (Figure S2C). Treatment of MDM with 2DG significantly
289 reduced antigen processing in all groups, however IFN-γ primed MDM retained enhanced abilities to
290 process antigen (Figure S2C). The reduced capacity of MDM to process antigen was not due to a
291 deficiency in phagocytosis, as measured by both bead or bacterial uptake or due to increased cell
292 death (Figure S2D-F).

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

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

299 Changes in macrophage metabolism have been previously associated with altered cytokine
300 production (5, 8, 14). Having established that both IFN-γ and IL-4 can significantly alter metabolism in
301 human macrophages we next sought to examine the ability of AM and MDM to secrete cytokines
302 when primed with IFN-γ or IL-4. Human AM (Figure 4A,4C,4E) and MDM (Figure 4B,4D,4F) were left
303 unprimed or primed with IFN-γ or IL-4 for 24 h. Macrophages were then stimulated with iH37Rv or
304 LPS. Supernatants were harvested 24 h post stimulation and concentrations of IL-1β (Figure 4A,4B),
305 TNF (Figure 4C,4D) and IL-10 (Figure 4E,4F) were quantified by ELISA. While iH37Rv stimulation
306 resulted in IL-1β production in unprimed AM and MDM, IFN-γ only significantly enhanced the
307 production of IL-1β by AM (Figure 4A,4B). IL-4 priming attenuated iH37Rv induced IL-1β in both AM
308 and MDM (Figure 4A,4B). As expected, IL-1β secretion was not induced in response to LPS stimulation
309 however, in the presence of IFN-γ, IL-1β was detectable (Figure 4A, 4B). TNF was significantly induced
310 in unprimed or IFN-γ primed, but not IL-4 primed AM in response to iH37Rv and LPS (Figure 4C). IFN-
311 γ enhanced production of TNF by AM in response to both iH37Rv and LPS. In contrast, IFN-γ enhanced
312 TNF in response to iH37Rv, but not LPS in MDM (Figure 4C,4D). LPS significantly upregulated the
313 production of TNF in all MDM. Notably, IFN-γ priming did not enhance TNF production and IL-4 priming
314 significantly attenuated LPS-induced TNF (Figure 4D). All stimulated AM secreted IL-10 regardless of
315 priming (Figure 4E). IFN-γ significantly enhanced iH37Rv induced IL-10 in AM compared to unprimed
316 or IL-4 primed comparators (Figure 4E). IL-4 priming of human AM significantly reduced IL-10
317 production in response to iH37Rv compared with unprimed AM (Figure 4E). LPS strongly induced IL-
318 10 production in unprimed MDM, which was significantly attenuated by either IFN-γ or IL-4 priming
319 (Figure 4F).

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

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

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

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

351

352 **Discussion**

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

374 Directly comparing the AM with the MDM demonstrates that human AM are more reliant on oxidative
375 metabolism upon IFN-γ priming and stimulation. Previous observations had identified a high baseline

376 of lactate in supernatants of human AM compared to MDM (8). Interestingly, IFN-γ primed AM and
377 MDM had significantly reduced capacity to increase ECAR (percentage change) in response to LPS
378 stimulation, suggesting that there may be a point of maximal glycolysis. This ability to induce max
379 glycolysis may be advantageous during infection as lactate, a breakdown product of glycolysis, has
380 been shown to have anti-microbial functions against Mtb (13, 20) . Moreover, pathogens such as Mtb
381 can downregulate metabolic pathways after infection (21, 22) and IFN-γ is crucial for control of Mtb
382 via glycolysis *in vivo* (23). Based on our current data, and as recently suggested by others (24), we
383 speculate that control of Mtb in humans may be dependent on IFN-γ regulating glycolysis and not
384 "Warburg"-like metabolism.

385 To our knowledge, we are the first to demonstrate that IFN-γ alone is sufficient to cause metabolic
386 reprogramming of both lung resident AM and peripherally derived MDM. While other studies have
387 demonstrated a role for IFN-γ inducing metabolic alterations in macrophages these studies have
388 focused on murine macrophages (3, 23). In contrast evidence in murine BMDM indicates that alone
389 IFN-γ does not increase glycolysis but that LPS was required (25). In addition, the use of LPS in
390 combination with IFN-γ to polarise macrophages toward the an inflammatory phenotype is not a
391 model easily translatable to humans, which are strikingly more sensitive to LPS than mice (26) and the
392 'M1' macrophage elicited cannot be subsequently challenged with infectious agents, as the response
393 is confounded by the initial LPS stimulation. Moreover, the use of LPS in addition to IFN-γ to polarise
394 the macrophage towards the 'M1' phenotype is arguably not comparable with a macrophage that is
395 polarised with IL-4 (or IL-10) in the absence of TLR stimuli. We wanted to assess the ability of IFN-γ
396 alone to affect the function of human macrophages, to enable direct comparisons of macrophage
397 subpopulations in order to fully assess the functional differences elicited in response to subsequent
398 stimulation, in keeping with other human models (23).

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

407 Glycolytic metabolism in macrophages has been intrinsically linked to cytokine production, particularly
408 IL-1β (3, 5, 8, 10, 29). We have demonstrated that increased ECAR early in MDM activation is
409 associated with increased secretion of IL-1β in both MDM and AM (9). Moreover IFN-γ can promote
410 IL-1β by inhibiting miR-21, a negative regulator of glycolysis in both human MDM and murine BMDM
411 (30). Our data builds on the observation that IFN-γ upregulates pro-IL-1β by a glycolytic dependent
412 mechanism in murine BMDM (3), by demonstrating the increased secretion of mature IL-1β by IFN-γ
413 primed human macrophages. Moreover, we have confirmed that IL-1β secretion is dependent on
414 glycolysis in IFN-γ primed human AM and MDM, which is line with data from murine BMDM (23). In
415 the current study we also demonstrate that 2DG can reduce both IL-1β and IL-10 secretion by IFN-γ
416 primed AM and MDM in response to Mtb. Moreover, 2DG inhibited LPS induced IL-10 in AM and
417 unprimed MDM. 2DG has previously been shown to inhibit IL-10 production by LPS stimulated human
418 MDM (4), however, restricting glycolysis using glucose free medium inhibited IL-1β but promoted IL-
419 10 secretion (8). Furthermore, IL-10 production is inhibited by IFN-γ in human MDM, which is similar
420 to previous data in IFN-γ primed murine BMDM where IL-10 secretion was also inhibited (31).
421 However, our data also demonstrates that IFN-γ does not inhibit or promote IL-10 in human AM

422 stimulated with LPS even though interestingly, IFN-γ primed AM had increased TNF in response to LPS.
423 This suggests there may be additional pathways involved in IL-10 secretion by human macrophages,
424 which is supported by reductions in IL-10 secretion by IL-4 primed AM, which are not metabolically
425 altered.

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

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

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

463 Immune augmentation therapies delivered directly to the lung are necessary to help combat the
464 growing threat of drug resistant pathogens, including Mtb. We have demonstrated such approaches
465 both *in vitro* and *in vivo* (9, 10, 46-49). Clinical trials have indicated that nebulized IFN-γ is a viable HDT
466 to help combat Mtb (50, 51) and is in clinical trials for sepsis
467 (https://clinicaltrials.gov/study/NCT04990232). Our data supports the use of inhalable IFN-γ as an

468 immuno-supportive therapy which modulates metabolic responses. Moreover, our data indicates that
469 IFN-γ affects metabolism and cytokine secretion in AM significantly more than MDM which lends
470 support for the therapeutic strategy of delivering IFN-γ to the lung, by targeting the macrophage
471 population that most need immune augmentation (1) and limiting potential side effects.

472 **Study limitations**

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

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

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

495 **Conclusion**

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

505

506 **Ethics statement**

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

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

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

AIB data acquisition, analysis and interpretation.

OST data acquisition.

ED data acquisition.

KMG data acquisition.

OOG data acquisition.

DMM data acquisition.

SAOR data acquisition.

FOC data acquisition.

PN data acquisition.

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

LEG analysis and interpretation, critical revision of the article.

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

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

All authors have approved the final version of this article.

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Data availability

544 The raw data supporting the conclusions of this article will be made available by the authors, without
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546

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728

729 **Figure Legends**

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

742 donor (MDM; n=8-9, AM; n=9-10). Statistically significant differences were determined using two-way
743 ANOVA with a Tukey (A-H) or Bonferroni post-test (I-J); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.001.

744

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

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

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

781 **Figure 5. Cytokine secretion by AM is more reliant on glycolysis than MDM.** Human AM (A, C, E)
782 isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy coats and MDM (B, D, F)
783 were differentiated and adherence purified for 7 days in 10% human serum. Cells were left unprimed
784 (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. Cells were left untreated
785 (solid) treated with 2DG (5 mM; empty) for 1 h prior to stimulation with iH37Rv (MOI 1-10; square) or
786 LPS (100 ng/ml; triangle) or left unstimulated (circle). Supernatants were harvested 24 h after
787 stimulation and concentrations of IL-1β (A, B), TNF (C, D) and IL-10(E, F) were quantified by ELISA. Each

788 linked data point represents the average of technical duplicates for one individual biological donor
789 (AM; n=12-13, MDM; n=8-10). Statistically significant differences were determined using two-way
790 ANOVA with a Tukey post-test (A-D); *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 or #P≤0.05,
791 ##P≤0.01 (where IFN-γ primed data sets were excluded for post-test analysis to analyse statistical
792 differences between no cytokine and IL-4 treated data sets).

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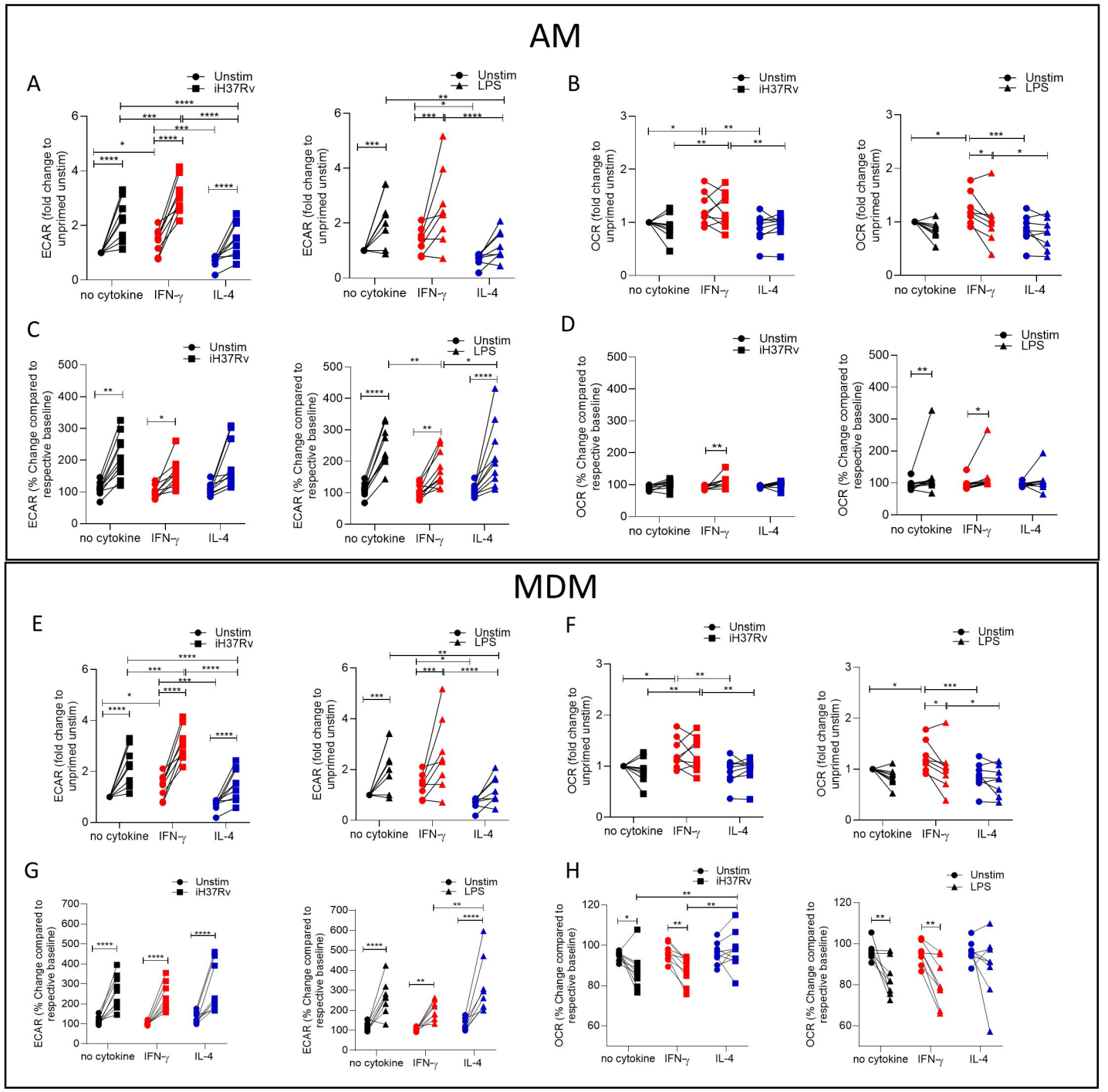
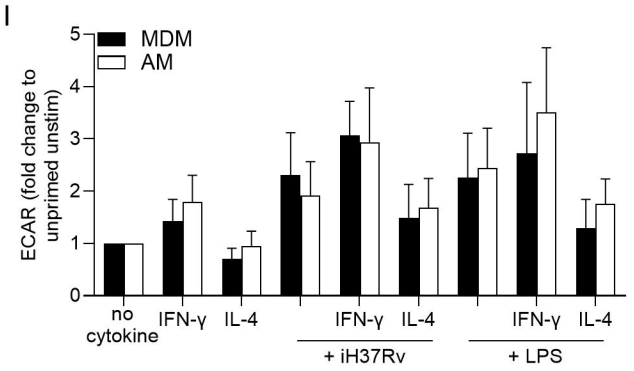
Figure 1


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


I
MDM
AM
ECAR (fold change to unprimed unstim)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS

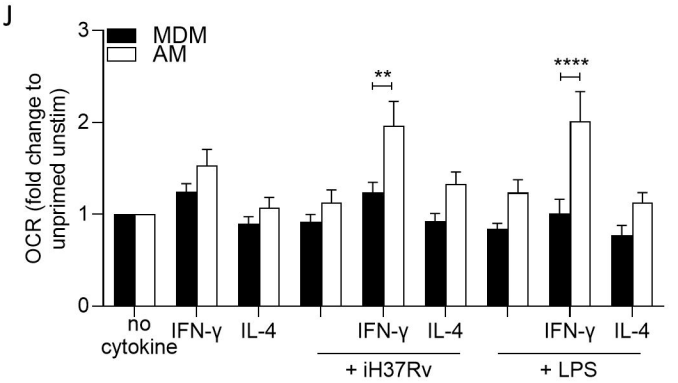

J
MDM
AM
OCR (fold change to unprimed unstim)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS

Figure 2

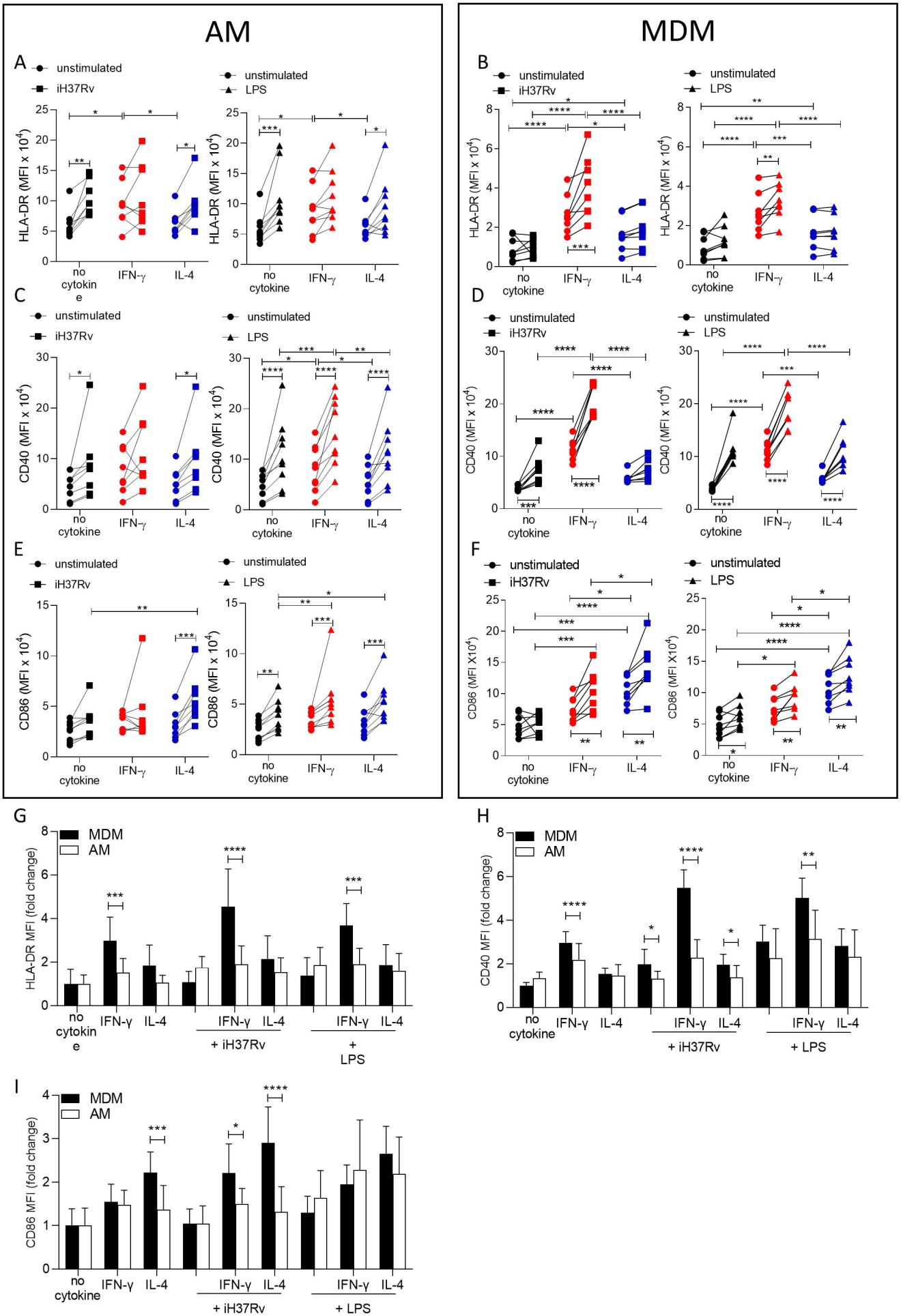

AM
MDM
A
B
C
D
E
F
G
H
I
unstimulated
iH37Rv
LPS
HLA-DR (MFI x 10^4)
CD40 (MFI x 10^4)
CD86 (MFI x 10^4)
CD86 (MFI X10^4)
no cytokine
IFN-γ
IL-4
+ iH37Rv
+ LPS
HLA-DR MFI (fold change)
CD40 MFI (fold change)
CD86 MFI (fold change)
MDM
AM

Figure 3

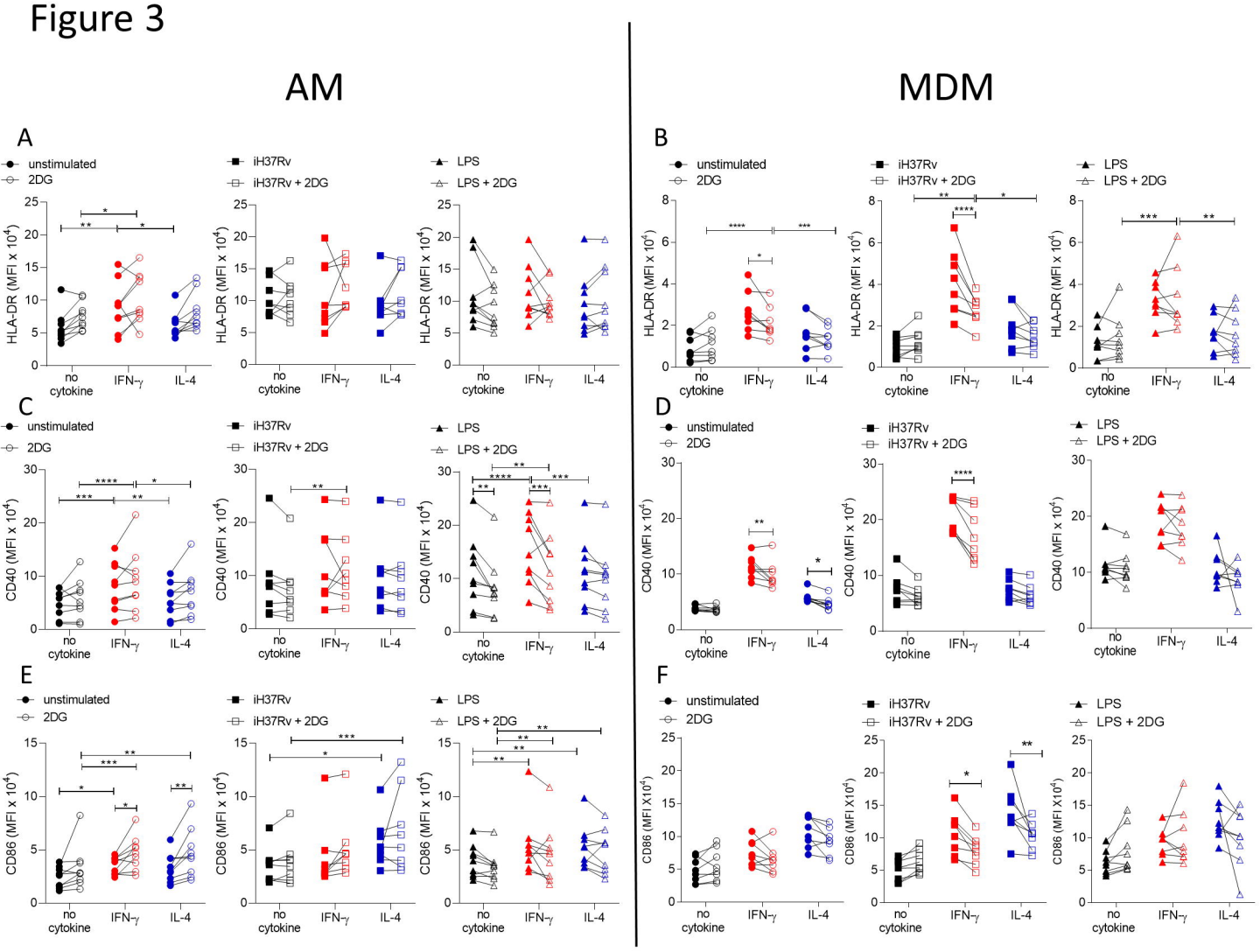

AM
MDM
A
B
C
D
E
F
unstimulated
2DG
iH37Rv
iH37Rv + 2DG
LPS
LPS + 2DG
HLA-DR (MFI x 10⁴)
CD40 (MFI x 10⁴)
CD86 (MFI x 10⁴)
CD86 (MFI X10⁴)
no cytokine
IFN-γ
IL-4

Figure 4

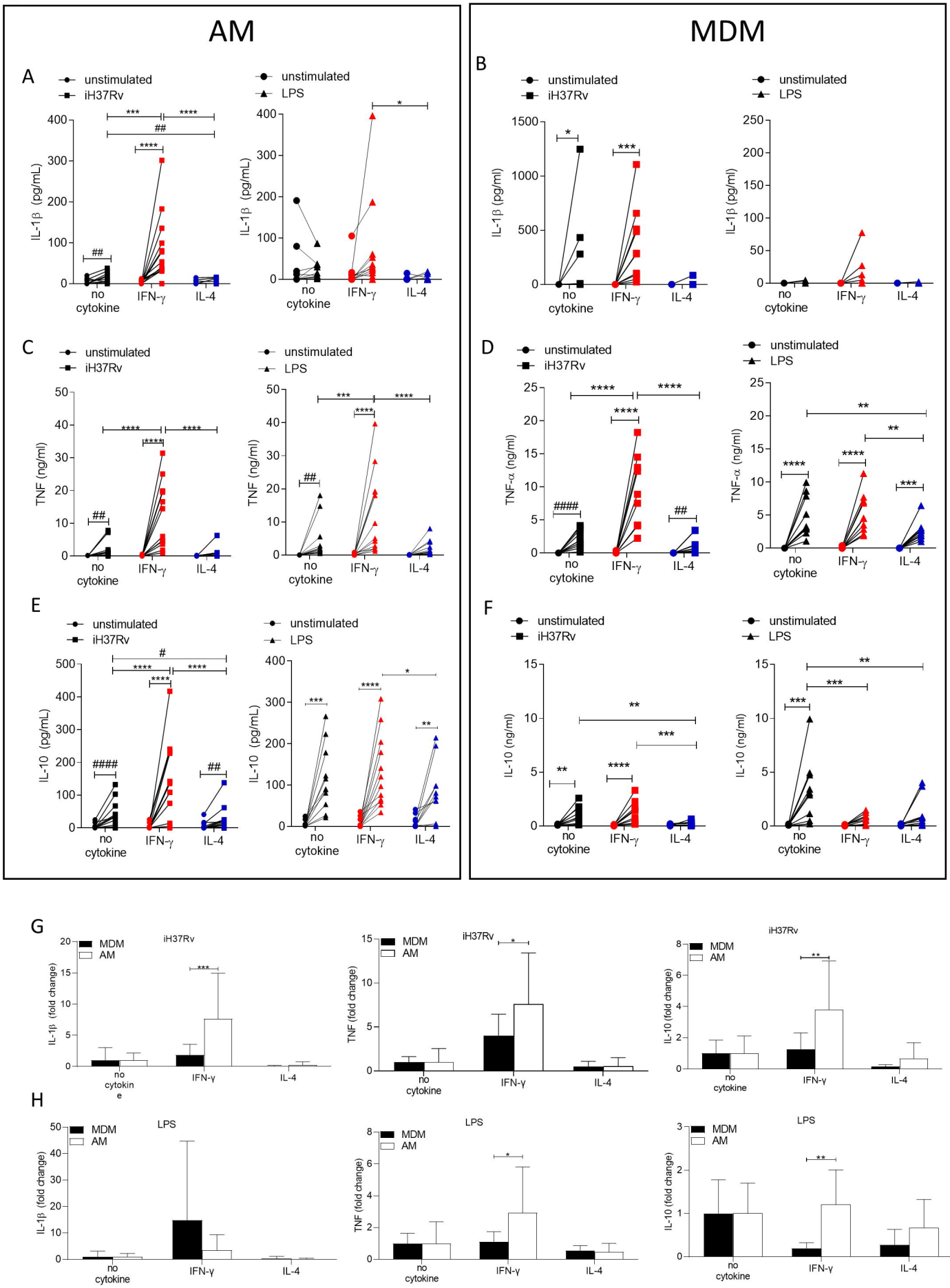
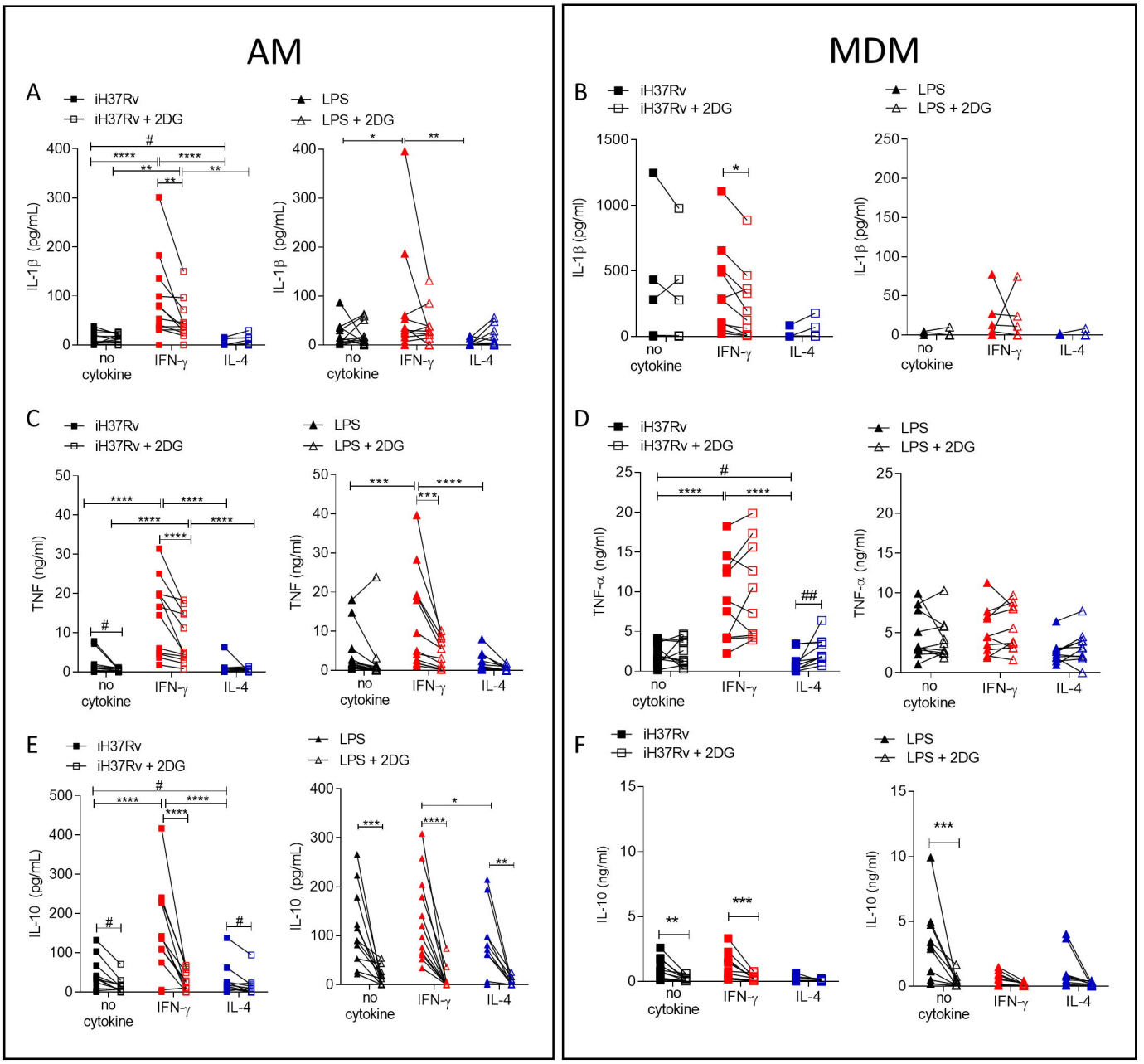

AM
MDM
A
B
C
D
E
F
G
H
unstimulated
iH37Rv
LPS
IL-1β (pg/mL)
IL-1β (pg/ml)
TNF (ng/ml)
TNF-α (ng/ml)
IL-10 (pg/mL)
IL-10 (ng/ml)
no cytokine
IFN-γ
IL-4
MDM
AM
iH37Rv
LPS
IL-1β (fold change)
TNF (fold change)
IL-10 (fold change)

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