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

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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.

Results

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

AM alter their metabolism in response to Mtb (6). Human macrophages also undergo a rapid increase in ECAR early in response to activation (7) and these pathways can be pharmacologically manipulated (8, 9). The metabolic and functional plasticity of human AM remains unexplored, however recent evidence shows they express both 'M1' and 'M2' markers (10). Murine AM can be reprogrammed through an IFN-γ dependent mechanism (11). We therefore sought to examine whether priming human AM with IFN-γ compared with IL-4 or unprimed AM, could influence their metabolic function and response to bacterial stimuli. We stimulated with whole bacteria; Mtb (iH37Rv) or gram-negative cell wall component; LPS. AM were plated in a Seahorse plate and primed with IFN-γ or IL-4 (both 10 ng/ml) for 24 h or left unprimed. AM ECAR and OCR were recorded for 30 min at baseline. AM were then stimulated in the Seahorse XFe24 Analyzer with medium (control), iH37Rv (MOI; 1-10) or LPS (100 ng/ml) and ECAR (Figure S1A) and OCR (Figure S1B) were continuously monitored.

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

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

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

As per previous observations (7, 12) a sustained increase in ECAR and a transient decrease in OCR occurred in MDM after stimulation (Figure S1E-H). At 150 minutes post stimulation, fold change was calculated compared to unprimed unstimulated MDM (Figure 1E,1F). IFN-γ priming significantly

113 increased the ECAR and OCR of MDM whereas IL-4 priming significantly reduced the ECAR in the
114 absence of stimulation (Figure 1E,1F). Stimulation of human MDM with iH37Rv or LPS significantly
115 increased ECAR in all MDM, however, IL-4 primed MDM stimulated with iH37Rv or LPS have
116 significantly reduced ECAR compared with control or IFN-γ primed MDM (Figure 1E). IFN-γ primed
117 MDM stimulated with iH37Rv have increased ECAR compared with control MDM (Figure 1E).

118 Similar to AM, IFN-γ primed MDM have increased OCR compared with control or IL-4 primed MDM
119 (Figure 1F). In contrast with the AM, stimulation of IFN-γ primed MDM does not further increase OCR
120 however, the elevated OCR in IFN-γ primed MDM remains significantly higher compared to control or
121 IL-4 primed MDM when stimulated with iH37Rv (Figure 1F). The percent change in ECAR upon
122 stimulation (from respective baselines) illustrates that all MDM groups significantly increase ECAR
123 from their own baseline in response to stimulation (Figure 1G). Interestingly, although IL-4 significantly
124 reduced ECAR in iH37Rv and LPS stimulated MDM compared with unprimed stimulated controls
125 (Figure 1E), the IL-4 primed MDM have significantly enhanced capacity to ramp up glycolysis in
126 response to LPS, as evidence by the significantly increased percentage change in LPS stimulated, IL-4
127 primed MDM compared with IFN-γ primed controls (Figure 1G). Control or IFN-γ primed MDM
128 stimulated with either iH37Rv or LPS decreases percentage change in the OCR associated with a
129 stimulation-induced shift to "Warburg"-like metabolism (Figure 1H). This effect is not observed in IL-
130 4 primed MDM, moreover, IL-4 primed MDM stimulated with iH37Rv had significantly elevated
131 percent change in OCR compared with stimulated unprimed or IFN-γ primed MDM (Figure 1H). These
132 data indicate that IL-4 priming prevents human MDM utilising "Warburg"-like metabolism in response
133 to stimulation.

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

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

147

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

150 Having established that energetic responses are plastic in response to IFN-γ in the AM and that post
151 stimulation energetic responses are different in human macrophage types under Th1 or Th2 priming
152 conditions, we next sought to determine the effect on the plasticity of the macrophage phenotype by
153 examining expression of activation markers associated with antigen presentation function. Human AM
154 (Figure 2A,2C,2E) and MDM (Figure 2B, 2D, 2F) were primed with IFN-γ or IL-4 for 24 h or left
155 unprimed. Macrophages were then stimulated with iH37Rv or LPS. After 24 h AM and MDM were

156 analysed by flow cytometry for expression of HLA-DR (Figure 2A,2B,2G), CD40 (Figure 2C,2D,2H) and
157 CD86(Figure 2E,2F,2I). A sample gating strategy for the analysis is provided (Figure S2A).

158 IFN-γ significantly increased the expression of HLA-DR compared with control or IL-4 primed
159 unstimulated AM (Figure 2A). Stimulation with iH37Rv significantly upregulated HLA-DR, but only in
160 unprimed AM (Figure 2A). Similarly, LPS significantly induced HLA-DR in unprimed or IL-4 primed AM
161 but not in IFN-γ primed AM (Figure 2A). IFN-γ also significantly increased the expression of HLA-DR
162 compared with control or IL-4 primed MDM (Figure 2B). Stimulation of IFN-γ primed MDM with iH37Rv
163 or LPS robustly enhanced the expression of HLA-DR (Figure 2B). IFN-γ priming significantly upregulated
164 CD40 expression in unstimulated AM (Figure 2C; right). In addition, CD40 was upregulated following
165 iH37Rv or LPS stimulation of AM in all groups assessed with the exception of IFN-γ primed AM
166 stimulated with iH37Rv (Figure 2C). IFN-γ increased the expression of the co-stimulatory molecule
167 CD40 in unstimulated MDM (Figure 2D). Stimulation of MDM with iH37Rv or LPS significantly
168 increased CD40 expression, with the exception of iH37Rv stimulation in IL-4 primed MDM (Figure 2D).
169 Expression of CD86 in response to stimulation with iH37Rv was only upregulated in IL-4 primed AM,
170 however, LPS induced upregulation of CD86 in all AM, with IFN-γ and IL-4 primed AM exhibiting
171 significantly enhances CD86 expression compared to unprimed control (Figure 2E). CD86 expression
172 induced by MDM in response to iH37Rv or LPS was enhanced by priming with either IFN-γ or IL-4, with
173 IL-4 inducing significantly higher expression compared with unprimed or IFN-γ primed MDM (Figure
174 2F).

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

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

184 Since IFN-γ drove glycolysis and the expression of the macrophage activation markers CD40 and HLA-
185 DR in both AM and MDM we wanted to examine if the increased glycolysis was associated with
186 enhanced expression of activation markers expression. Human AM (Figure 3A,3C,3E) and MDM (Figure
187 3B,3D,3F) were primed with IFN-γ or IL-4 for 24 h or left unprimed. Macrophages were then treated
188 with the glycolytic inhibitor, 2DG for 1 h prior to stimulation with iH37Rv or LPS. After 24 h AM and
189 MDM were analysed by flow cytometry for expression of HLA-DR (Figure 3A,3B), CD40 (Figure 3C,3D)
190 and CD86(Figure 3E,3F). 2DG-mediated inhibition of glycolysis following stimulation was confirmed
191 (Figure S2B).

192 Inhibiting glycolysis with 2DG did not alter expression of HLA-DR on AM (Figure 3A). Interestingly, the
193 increased expression of HLA-DR in IFN-γ primed MDM was dependent on glycolysis in unstimulated
194 and iH37Rv stimulated MDM, however, increased expression of HLA-DR by LPS stimulated IFN-γ
195 primed MDM remained elevated in the presence of 2DG (Figure 3B). Expression of CD40 was not
196 affected by 2DG in unstimulated or iH37Rv stimulated AM (Figure 3C). Conversely, LPS induced
197 expression of CD40 was significantly inhibited by 2DG in unprimed and IFN-γ primed AM but not in IL-
198 4 primed AM (Figure 3C). In contrast, enhanced expression of CD40 in IFN-γ primed MDM in
199 unstimulated or iH37Rv stimulated MDM was significantly reduced with the addition of 2DG, with no
200 effect on the expression of CD40 in LPS stimulated human MDM regardless of cytokine priming (Figure

201 3D). 2DG enhanced expression of CD86 in unstimulated IFN-γ or IL-4 primed AM but did not affect
202 expression in any stimulated AM (Figure 3E). Interestingly, when unstimulated AM were examined in
203 the absence of stimulation, IFN-γ priming significantly increased CD86 (Figure 3E). 2DG inhibited the
204 increased expression of CD86 in response to iH37Rv stimulation in IFN-γ or IL-4 primed MDM, but no
205 difference was observed in unstimulated or LPS stimulated MDM (Figure 3F).

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

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

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

223 Changes in macrophage metabolism have been previously associated with altered cytokine
224 production (5, 6, 13). Having established that both IFN-γ and IL-4 can significantly alter metabolism in
225 human macrophages we next sought to examine the ability of AM and MDM to secrete cytokines
226 when primed with IFN-γ or IL-4. Human AM (Figure 4A,4C,4E) and MDM (Figure 4B,4D,4F) were left
227 unprimed or primed with IFN-γ or IL-4 for 24 h. Macrophages were then stimulated with iH37Rv or
228 LPS. Supernatants were harvested 24 h post stimulation and concentrations of IL-1β (Figure 4A,4B),
229 TNF (Figure 4C,4D) and IL-10 (Figure 4E,4F) were quantified by ELISA. While iH37Rv stimulation
230 resulted in IL-1β production in unprimed AM and MDM, IFN-γ only significantly enhanced the
231 production of IL-1β by AM (Figure 4A,4B). IL-4 priming attenuated iH37Rv induced IL-1β in both AM
232 and MDM (Figure 4A,4B). As expected, IL-1β secretion was not induced in response to LPS stimulation
233 however, in the presence of IFN-γ, IL-1β was detectable (Figure 4A, 4B). TNF was significantly induced
234 in unprimed or IFN-γ primed, but not IL-4 primed AM in response to iH37Rv and LPS (Figure 4C). IFN-
235 γ enhanced production of TNF by AM in response to both iH37Rv and LPS. In contrast, IFN-γ enhanced
236 TNF in response to iH37Rv, but not LPS in MDM (Figure 4C,4D). LPS significantly upregulated the
237 production of TNF in all MDM. Notably, IFN-γ priming did not enhance TNF production and IL-4 priming
238 significantly attenuated LPS-induced TNF (Figure 4D). All stimulated AM secreted IL-10 regardless of
239 priming (Figure 4E). IFN-γ significantly enhanced iH37Rv induced IL-10 in AM compared to unprimed
240 or IL-4 primed comparators (Figure 4E). IL-4 priming of human AM significantly reduced IL-10
241 production in response to iH37Rv compared with unprimed AM (Figure 4E). LPS strongly induced IL-
242 10 production in unprimed MDM, which was significantly attenuated by either IFN-γ or IL-4 priming
243 (Figure 4F).

244 These data suggest that the AM has greater functional plasticity in terms of cytokine production in
245 response to IFN-γ than the MDM, as IFN-γ primed AM had enhanced IL-10 and TNF production, in

246 response to Mtb and LPS, respectively. In order to directly compare the human AM and MDM
247 responses, fold change in cytokine production was calculated compared to the average of their
248 respective iH37Rv (Figure 4G) or LPS (Figure 4H) stimulated unprimed control. IFN-γ enhanced human
249 AM ability to secrete IL-1β, TNF and IL-10 in response to iH37Rv compared to MDM (Figure 4G). The
250 IFN-γ primed human AM also has a significantly increased ability to secrete TNF and IL-10 in response
251 to LPS compared to MDM (Figure 4H), however the difference in IL-10 secretion is more associated
252 with MDM decreasing IL-10 when IFN-γ primed.

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

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

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

275

276 **Discussion**

277 AM are the first responders to infections and inflammatory insults in the lung. We and others have
278 reported that lung resident macrophages are dependent on glycolysis to respond to LPS (14) or Mtb
279 (6, 13), however, their metabolic behaviour is distinct from murine AM (15) reinforcing the need to
280 define cellular metabolism and its tractability in human macrophages in order to design effective
281 immunometabolic therapies. We demonstrate here that human AM can be metabolically
282 reprogrammed by IFN-γ which increased both glycolysis and oxidative phosphorylation and is further
283 enhanced by stimulation with Mtb or LPS. This is in contrast with literature showing murine AM do
284 not increase glycolysis in real-time following LPS stimulation (15), although whether IFN-γ can
285 influence this in mice remains unknown. Our previous work shows that upon activation, the human
286 MDM underwent "Warburg"-like metabolism associated with an increase in glycolysis with a
287 concomitant reduction in oxidative phosphorylation (7). We confirmed this observation and this
288 persists when primed with IFN-γ, unlike AM which do not undergo the shift to "Warburg"-like
289 metabolism. IL-4 primed macrophages are associated in the literature with an increased reliance on

290 oxidative phosphorylation (16). Indeed, murine IL-4 primed BMDM have increased ECAR and OCR (17,
291 18). IL-4 did not promote oxidative metabolism in human AM or MDM, even when stimulated. IL-4
292 decreased glycolysis while preventing a decline in oxidative metabolism in MDM, inhibiting their shift
293 to "Warburg"-like metabolism. When directly comparing percentage change in ECAR of unprimed or
294 macrophages primed with IFN-γ or IL-4, they all had similar rates of change despite profound
295 differences in maximal ECAR. These data suggest that the resting state of the macrophage, and the
296 cytokines it has recently been exposed to before activation, may be a key determining factor for the
297 response and outcome to infections.

298 Directly comparing the AM with the MDM demonstrates that human AM are more reliant on oxidative
299 metabolism upon IFN-γ priming and stimulation. Previous observations had identified a high baseline
300 of lactate in supernatants of human AM compared to MDM (6). Interestingly, IFN-γ primed AM and
301 MDM had significantly reduced capacity to increase ECAR (percentage change) in response to LPS
302 stimulation, suggesting that there may be a point of maximal glycolysis. This ability to induce max
303 glycolysis may be advantageous during infection as lactate, a breakdown product of glycolysis, has
304 been shown to have anti-microbial functions against Mtb (12, 19) . Moreover, pathogens such as Mtb
305 can downregulate metabolic pathways after infection (20, 21) and IFN-γ is crucial for control of Mtb
306 via glycolysis *in vivo* (22). Based on our current data, and as recently suggested by others (23), we
307 speculate that control of Mtb in humans may be dependent on IFN-γ regulating glycolysis and not
308 "Warburg"-like metabolism.

309 To our knowledge, we are the first to demonstrate that IFN-γ alone is sufficient to cause metabolic
310 reprogramming of both lung resident AM and peripherally derived MDM. While other studies have
311 demonstrated a role for IFN-γ inducing metabolic alterations in macrophages these studies have
312 focused on murine macrophages (3, 22). In contrast evidence in murine BMDM indicates that alone
313 IFN-γ does not increase glycolysis but that LPS was required (24). In addition, the use of LPS in
314 combination with IFN-γ to polarise macrophages toward the an inflammatory phenotype is not a
315 model easily translatable to humans, which are strikingly more sensitive to LPS than mice (25) and the
316 'M1' macrophage elicited cannot be subsequently challenged with infectious agents, as the response
317 is confounded by the initial LPS stimulation. Moreover, the use of LPS in addition to IFN-γ to polarise
318 the macrophage towards the 'M1' phenotype is arguably not comparable with a macrophage that is
319 polarised with IL-4 (or IL-10) in the absence of TLR stimuli. We wanted to assess the ability of IFN-γ
320 alone to affect the function of human macrophages, to enable direct comparisons of macrophage
321 subpopulations in order to fully assess the functional differences elicited in response to subsequent
322 stimulation, in keeping with other human models (23).

323 AM expression of activation markers was more limited compared to MDM, even when primed and
324 stimulated. AM upregulated only HLA-DR consistently in response to IFN-γ, broadly in keeping with
325 murine AM (11, 26, 27). In contrast, MDM have a greater capacity to increase all activation markers in
326 response to IFN-γ and stimulation. The response of IFN-γ primed MDM to Mtb was dependent on
327 glycolysis for optimal activation marker expression, while LPS upregulated these markers
328 independently of glycolysis, irrespective of cytokine priming. In contrast the AM was dependent on
329 glycolysis for upregulation of these markers in response to LPS and not Mtb. These data once again
330 suggest a differential role of glycolysis within human macrophages.

331 Glycolytic metabolism in macrophages has been intrinsically linked to cytokine production, particularly
332 IL-1β (3, 5, 6, 9, 28). We have demonstrated that increased ECAR early in MDM activation is associated
333 with increased secretion of IL-1β in both MDM and AM (8). Moreover IFN-γ can promote IL-1β by
334 inhibiting miR-21, a negative regulator of glycolysis in both human MDM and murine BMDM (29). Our
335 data builds on the observation that IFN-γ upregulates pro-IL-1β by a glycolytic dependent mechanism

336 in murine BMDM (3), by demonstrating the increased secretion of mature IL-1β by IFN-γ primed
337 human macrophages. Moreover, we have confirmed that IL-1β secretion is dependent on glycolysis in
338 IFN-γ primed human AM and MDM, which is line with data from murine BMDM (22). In the current
339 study we also demonstrate that 2DG can reduce both IL-1β and IL-10 secretion by IFN-γ primed AM
340 and MDM in response to Mtb. Moreover, 2DG inhibited LPS induced IL-10 in AM and unprimed MDM.
341 2DG has previously been shown to inhibit IL-10 production by LPS stimulated human MDM (4),
342 however, restricting glycolysis using glucose free medium inhibited IL-1β but promoted IL-10 secretion
343 (6). Furthermore, IL-10 production is inhibited by IFN-γ in human MDM, which is similar to previous
344 data in IFN-γ primed murine BMDM where IL-10 secretion was also inhibited (30). However, our data
345 also demonstrates that IFN-γ does not inhibit or promote IL-10 in human AM stimulated with LPS even
346 though interestingly, IFN-γ primed AM had increased TNF in response to LPS. This suggests there may
347 be additional pathways involved in IL-10 secretion by human macrophages, which is supported by
348 reductions in IL-10 secretion by IL-4 primed AM, which are not metabolically altered.

349 TNF is crucial to control infections such as Mtb (31-34). We demonstrate that IFN-γ enhances TNF
350 production in response to Mtb stimulation in human MDM and AM, however AM have a much greater
351 ability to increase TNF. Moreover, IFN-γ primed AM stimulated with Mtb have significantly more
352 production of IL-1β, TNF and IL-10 compared with unprimed controls. This is in contrast with IFN-γ
353 primed MDM which only upregulate TNF compared to their unprimed controls. These data indicate
354 that effective immune responses to Mtb in the lung may require AM to be primed with IFN-γ and may
355 in part explain why patients deficient in IFN-γ or associated signalling have increased risk of TB (35,
356 36). IFN-γ driven production of TNF is dependent on glycolysis in AM. MDM secretion of TNF is
357 independent of glycolysis and conversely, inhibition of glycolysis in IL-4 primed Mtb stimulated MDM
358 enhanced TNF production. Previous studies have demonstrated that glycolysis was required by murine
359 BMDM but not AM to secrete TNF and IL-6 (15). Conversely, our data demonstrates that human AM
360 need glycolysis for optimal TNF production, especially in the presence of IFN-γ, whereas MDM do not.
361 Once again, we highlight that there is variation in the metabolic requirements within human
362 macrophage subpopulations, and importantly, that the AM is metabolically tractable to modulate its
363 function.

364 Whether the AM can respond to IL-4 has been debated (37). Here we demonstrate that the human
365 AM can respond to IL-4 with evidence that IL-4 reduced glycolysis in response to LPS stimulation. In
366 addition, AM were functionally altered by IL-4 resulting in reduced IL-1β and IL-10 production and
367 upregulated CD86. These data provide evidence that the human AM is capable of responding to IL-4
368 which may inform type 2 lung immunity, and susceptibility to infection in patients with asthma, for
369 example.

370 Trained immunity improves innate responses to infection and is emerging as a key component of host
371 directed therapies (HDT) and strategies to improve vaccine efficacy and the design of respiratory
372 mucosal vaccines (26, 27, 38-40). Both IFN-γ and IL-4 can induce trained immunity in murine and
373 human macrophages (11, 18, 41, 42). MDM trained with IFN-γ and LPS, and stimulated with Mtb had
374 increased TNF in the acute activation phase of trained immunity (18), which we observed in IFN-γ
375 primed MDM subsequently stimulated with Mtb. We also observed an increase in TNF, IL-1β and IL-
376 10 in AM potentially suggesting that AM will be a target for innate training, as increased IL-1β is
377 associated with optimal training (38, 42, 43). AM from mice that received an inhaled adenovirus
378 vectored vaccine undergo trained immunity mediated by IFN-γ resulting in elevated MHC-II
379 expression, enhanced cytokine production, and protection against specific and non-specific infection
380 challenges (11, 26). We have previously demonstrated that an adenovirus vectored vaccine induces
381 trained immunity in human monocytes and postulated that this may be IFN-γ dependent (44). The

382 current study provides evidence that IFN-γ can metabolically reprogramme the human AM, resulting
383 in enhanced HLA-DR expression and cytokine production in response to subsequent stimulation.
384 Cumulatively this highlights the importance of ascertaining whether IFN-γ can induce trained
385 immunity in the human AM, which may enhance the design of respiratory mucosal vaccines.

386 Immune augmentation therapies delivered directly to the lung are necessary to help combat the
387 growing threat of drug resistant pathogens, including Mtb. We have demonstrated such approaches
388 both *in vitro* and *in vivo* (8, 9, 45-48). Clinical trials have indicated that nebulized IFN-γ is a viable HDT
389 to help combat Mtb (49, 50) and is in clinical trials for sepsis
390 (https://clinicaltrials.gov/study/NCT04990232). Our data supports the use of inhalable IFN-γ as an
391 immuno-supportive therapy which modulates metabolic responses. Moreover, our data indicates that
392 IFN-γ affects metabolism and cytokine secretion in AM significantly more than MDM which lends
393 support for the therapeutic strategy of delivering IFN-γ to the lung, by targeting the macrophage
394 population that most need immune augmentation (1) and limiting potential side effects.

395 **Study limitations**

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

403 The inhibition of glycolysis with 2DG cannot definitively link all observations solely to glycolysis, as
404 limiting glycolysis will ultimately limit oxidative phosphorylation. Blocking oxidative phosphorylation
405 with oligomycin reduced LPS induced cytokine secretion in the human AM and not MDM (51), both
406 glycolysis and oxidative phosphorylation may therefore be needed for optimal AM function. The
407 concentration of 2DG used only partially inhibited glycolysis, however ablation of glycolysis induces
408 significant cytotoxicity and confounds assay outcomes. Therefore, where 2DG had no effect, a role for
409 glycolysis cannot be definitively excluded. Furthermore, only one dose of IFN-γ was utilised due to
410 limitations in AM yield, however, recently both low and high doses of IFN-γ have been shown to have
411 similar effects on AM *in vitro* (52).

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

418 **Conclusion**

419 Human AM and infiltrating MDM both increase glycolysis and oxidative phosphorylation in response
420 to IFN-γ, and stimulation results in a further increase in glycolysis. Cumulatively, the data presented
421 herein suggests that the MDM maybe more phenotypically plastic than the AM, while the AM have
422 enhanced functional plasticity in their ability to produce cytokine after exposure Th1 and Th2
423 cytokines. Our data supports the hypothesis that there may be distinct roles for AM and infiltrating
424 MDM during infection since IFN-γ increased metabolic responses are mechanistically associated with
425 different cellular functions. Our data demonstrates that cytokine production in human AM can be

426 promoted by supporting cellular metabolism, thus providing evidence that human tissue resident AM
427 are a tractable target for host-directed immuno-supportive adjunctive therapies.

428 **Materials and methods**

429 **Cell Culture**

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

437 Human AM were retrieved with informed consent from patients (aged between 42-72, 50% Female)
438 undergoing bronchoscopy, (ethical approval, St. James' Hospital (SJH) / Tallaght University Hospital
439 (TUH) Joint Research Ethics Committee) previously as reported (53) and outlined below. Cells were
440 plated in RPMI (Gibco) supplemented with 10% FBS (Gibco), fungizone (2.5 µg/ml; Gibco) and
441 cefotaxime (50 µg/ml; Melford Biolaboratories). Cells were incubated for 24 h before washing to
442 remove non-adherent cells. Adherent cells (predominantly AM) were used for experiments**.**

443

444 **BAL Sample Acquisition**

445 All donors were patients undergoing clinically indicated bronchoscopy and written informed consent
446 for retrieving additional bronchial washings for research was obtained prior to the procedure. Patients
447 were not remunerated for participation in this study. Patients were informed and consented that
448 collective results arising from samples given would be published in academic journals. Exclusion
449 criteria included age under 18 years, inability to provide written informed consent or a known (or
450 ensuing) diagnosis of malignancy, sarcoidosis, HIV or Hepatitis C. Patients undergoing biopsy as part
451 of bronchoscopy were also excluded.

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

461

462 **Macrophage Stimulation**

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

468 **Metabolic Assays**

469 MDM were placed in ice-cold PBS and incubated at 4°C on ice for 30 minutes, then gently scraped and
470 counted using trypan blue. MDM ($1x10^5$ cells/well) were re-plated onto Seahorse plates, as previously
471 described (7). AM ($1x10^5$ cells/well) were directly plated onto Seahorse plates and washed after 24 h.
472 The ECAR and the oxygen consumption rate (OCR), were measured 3 times every 10 minutes to
473 establish baselines. After 30 minutes macrophages were stimulated in-situ and monitored in real-time,
474 with Seahorse medium, iH37Rv or LPS. Post stimulation the ECAR and OCR were continually sampled
475 at 20-minute intervals for times indicated. Analyses were carried out at approximately 150 minutes as
476 previously described (7). Fold change in ECAR and OCR was calculated compared with unstimulated
477 unprimed controls at 150 minutes, where unstimulated unprimed macrophages were set to 1. This
478 allows for analysis of the effects of both priming and subsequent stimulation for and accounts for the
479 variation in the raw ECAR and OCR reading between runs thereby making each donor its own control.

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

486 **Cytokine assays**

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

489 **Flow cytometry**

490 Human AM and MDM were placed in ice-cold PBS and incubated at 4°C on ice for 30 minutes. Cells
491 were removed by gentle scraping, Fc blocked with Human TruStain FcX (BioLegend) and stained with
492 zombie NIR viability dye and fluorochrome-conjugated antibodies for CD14 (Alexa488, Cat#325610),
493 CD68 (PE, Cat#333808), CD86 (BV421, Cat#305426), CD40 (BV510, Cat#334330), and HLA-DR (APC,
494 Cat#307610; all BioLegend). For phagocytosis and antigen processing assays, MDM were treated with
495 fluorescent beads (Sigma-Aldrich) or DQ-Ovalbumin (Thermo-fisher) for 30 minutes at 37°C, before
496 scraping as above. DQ-Ovalbumin is fluorescent after proteasomal degradation marking antigen
497 processing. Cells were fixed with 2% PFA and acquired on a BD FACS Canto II. Unstained and FMO
498 controls were used to normalise for background and to set gates. Data were analysed using FlowJo.

499

500 **Statistical analysis**

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

507

508 **Ethics statement**

All research herein was carried out in accordance with the Declaration of Helsinki and ethically approved, as outlined in the materials and methods section by the School of Medicine Research Ethics Committee, Trinity College Dublin and the SJH/TUH Joint Research Ethics Committee. All individuals provided written informed consent and were made aware that collective results arising from samples given would be published in academic journals.

Acknowledgements

We would like to acknowledge the Irish Blood Transfusion Service for supporting our research by approving us to use anonymised un-transfused blood components for our research. We gratefully acknowledge all people undergoing bronchoscopy at St. James's Hospital Dublin who consented to 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.

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

Datasets are available on Dryad (https://doi.org/10.5061/dryad.98sf7m0t5). No data has been omitted from this manuscript. Not all samples were used for every assay/stimulation due to limitations in cellular yield or due failure of positive or negative controls.

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714

715 **Figure Legends**

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

730

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

743 **Figure 3. Glycolysis is required for IFN-γ induced expression of activation markers by MDM and not**
744 **AM.** Human AM (A, C, E) isolated from bronchoalveolar lavage fluid. PBMC were isolated from buffy
745 coats and MDM (B, D, F) were differentiated and adherence purified for 7 days in 10% human serum.
746 Cells were left unprimed (black) or primed with IFN-γ (red) or IL-4 (blue) (both 10 ng/ml) for 24 h. Cells
747 were left untreated (solid) or treated with 2DG (5 mM; empty) 1 h prior to stimulation with iH37Rv
748 (MOI 1-10; square) or LPS (100 ng/ml; triangle) or left unstimulated (circle). After 24 h cells were
749 detached from the plates by cooling and gentle scraping and stained for HLAR-DR (A, B), CD40 (C, D),
750 CD86 (E, F) and analysed by flow cytometry. Each linked data point represents the average of technical

751 duplicates for one individual biological donor (n=8-9). Statistically significant differences were
752 determined using two-way ANOVA with a Tukey post-test (A-F); *P≤0.05, **P≤0.01, P***≤0.001,
753 ****P≤0.001.

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

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

Figure 1

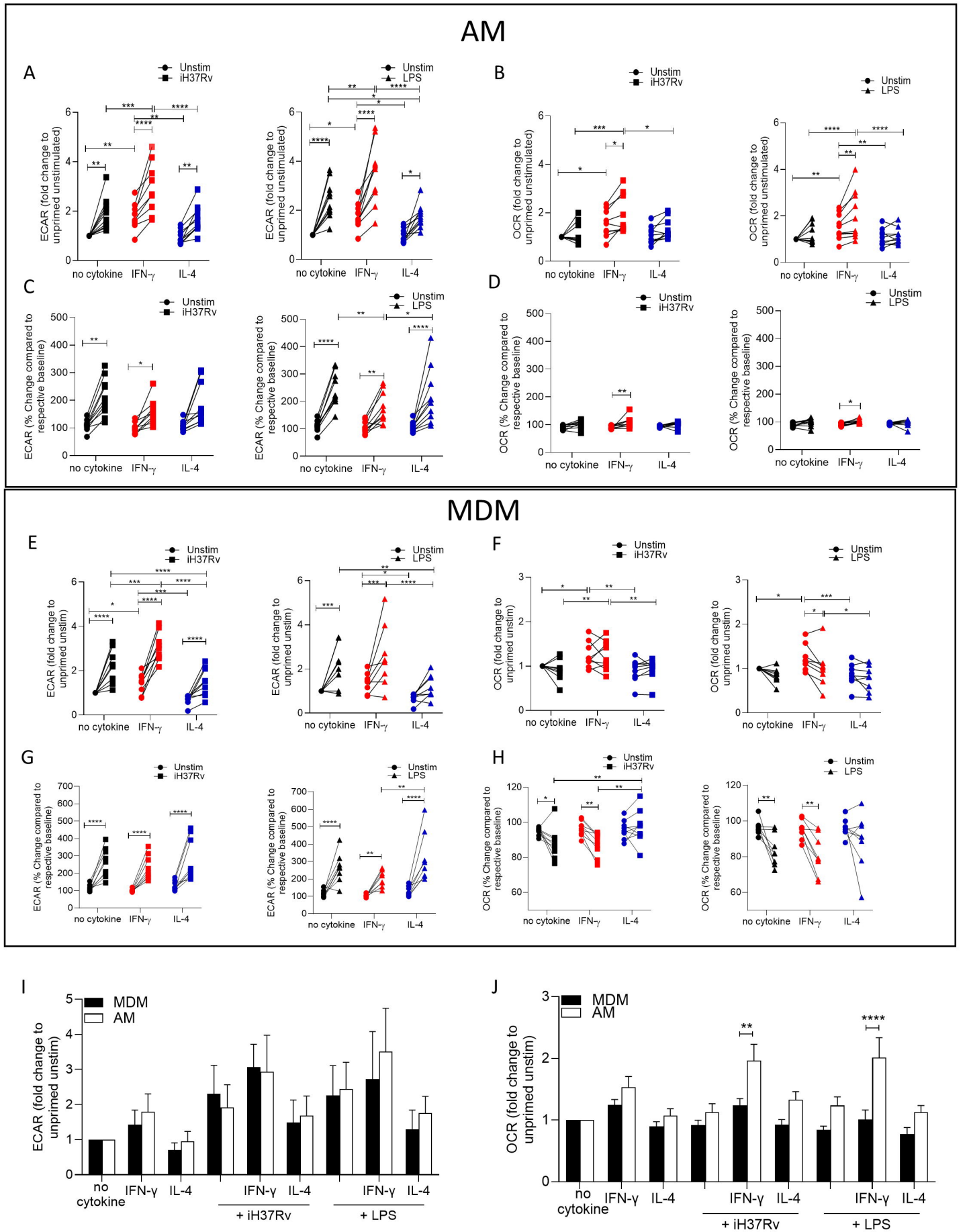

AM
A
B
C
D
MDM
E
F
G
H
I
J
Unstim
iH37Rv
LPS
ECAR (fold change to unprimed unstimulated)
OCR (fold change to unprimed unstimulated)
ECAR (% Change compared to respective baseline)
OCR (% Change compared to respective baseline)
ECAR (fold change to unprimed unstim)
OCR (fold change to unprimed unstim)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

Figure 2

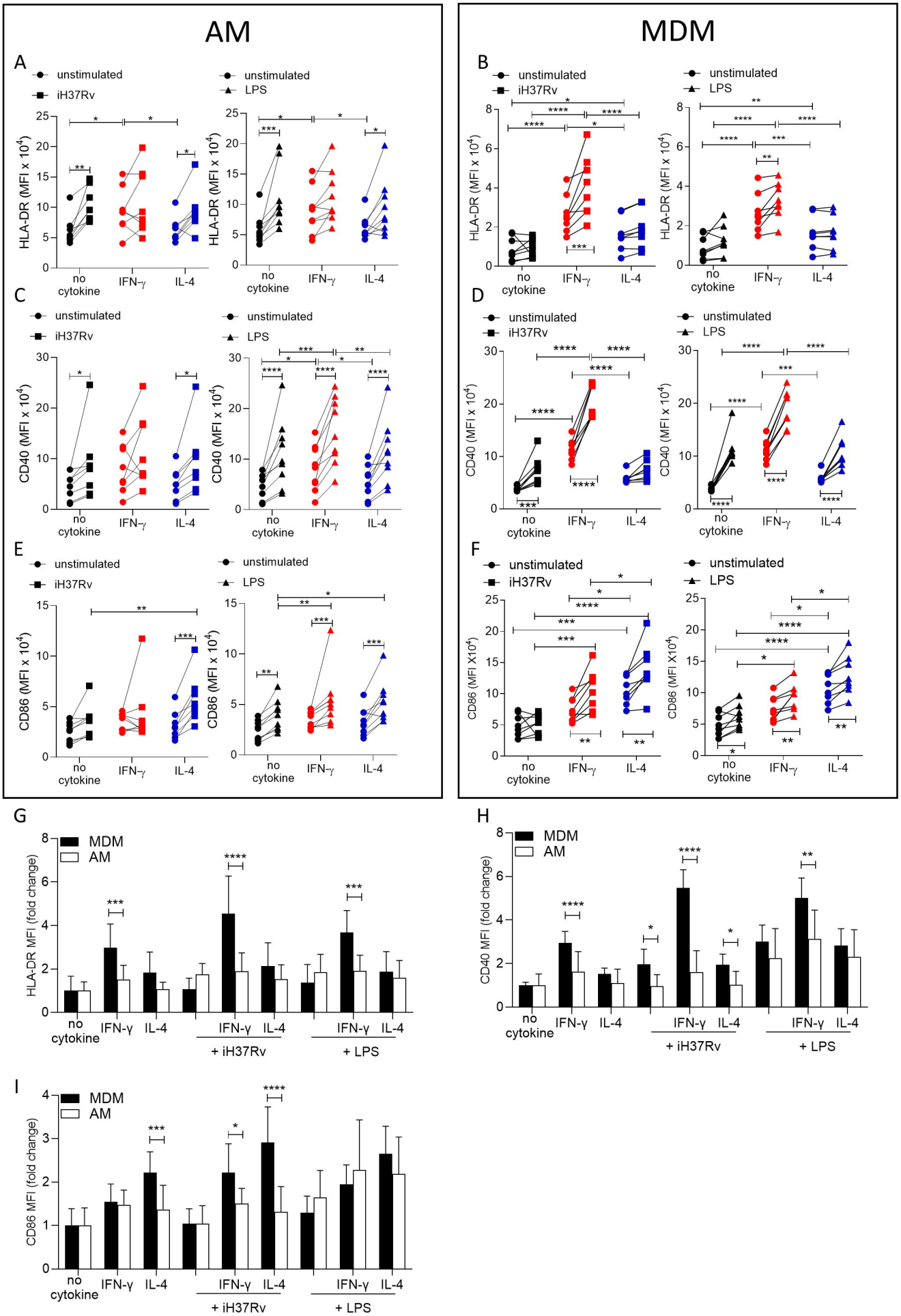

AM
MDM
A
B
C
D
E
F
G
H
I
unstimulated
iH37Rv
LPS
HLA-DR (MFI x 10^4)
CD40 (MFI x 10^4)
CD86 (MFI x 10^4)
CD86 (MFI X10^4)
no cytokine
IFN-γ
IL-4
HLA-DR MFI (fold change)
CD40 MFI (fold change)
CD86 MFI (fold change)
MDM
AM
+ iH37Rv
+ LPS

Figure 3

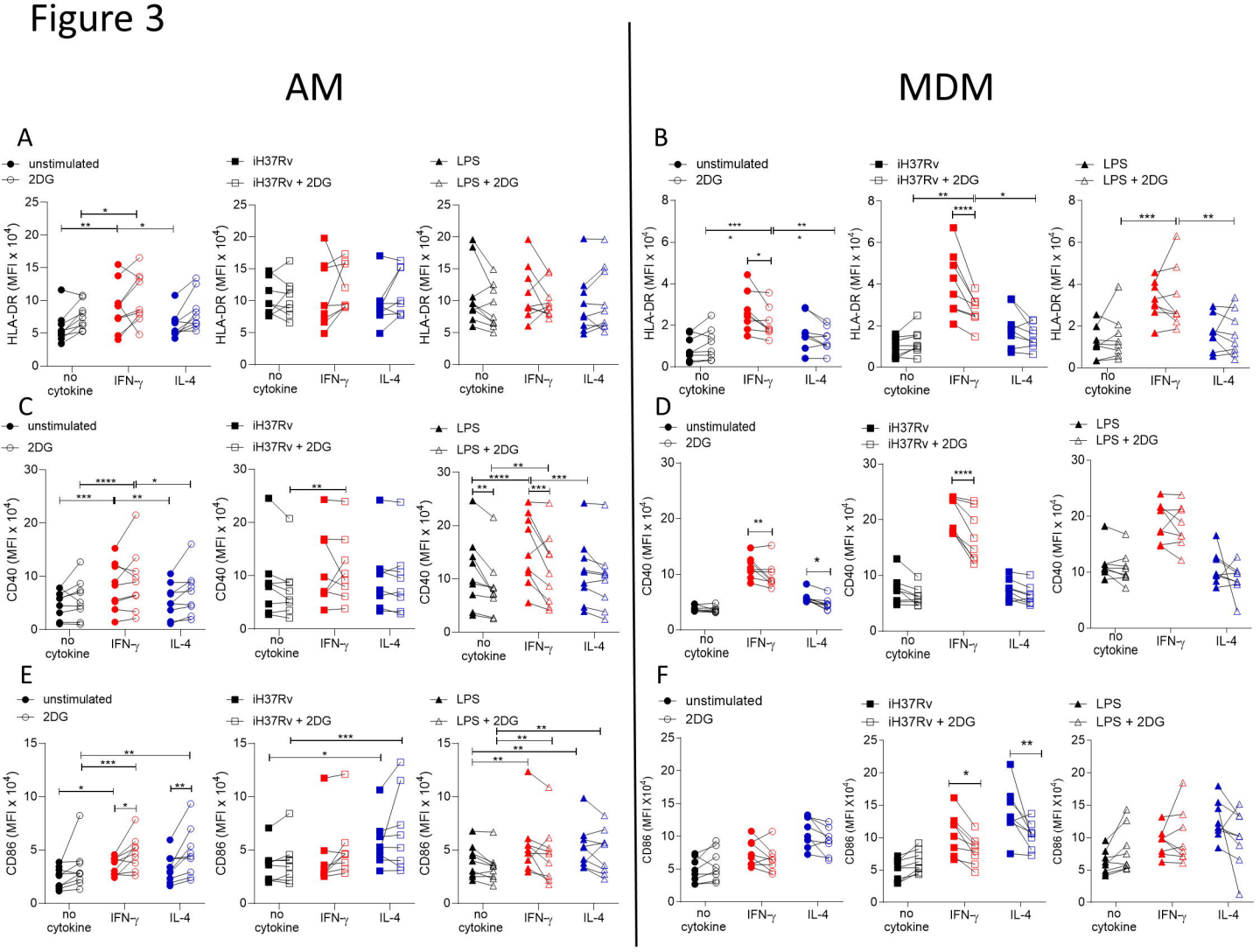

AM
MDM
A
B
C
D
E
F
unstimulated
2DG
iH37Rv
iH37Rv + 2DG
LPS
LPS + 2DG
HLA-DR (MFI x 10⁴)
CD40 (MFI x 10⁴)
CD86 (MFI x 10⁴)
CD86 (MFI X10⁴)
no cytokine
IFN-γ
IL-4

Figure 4

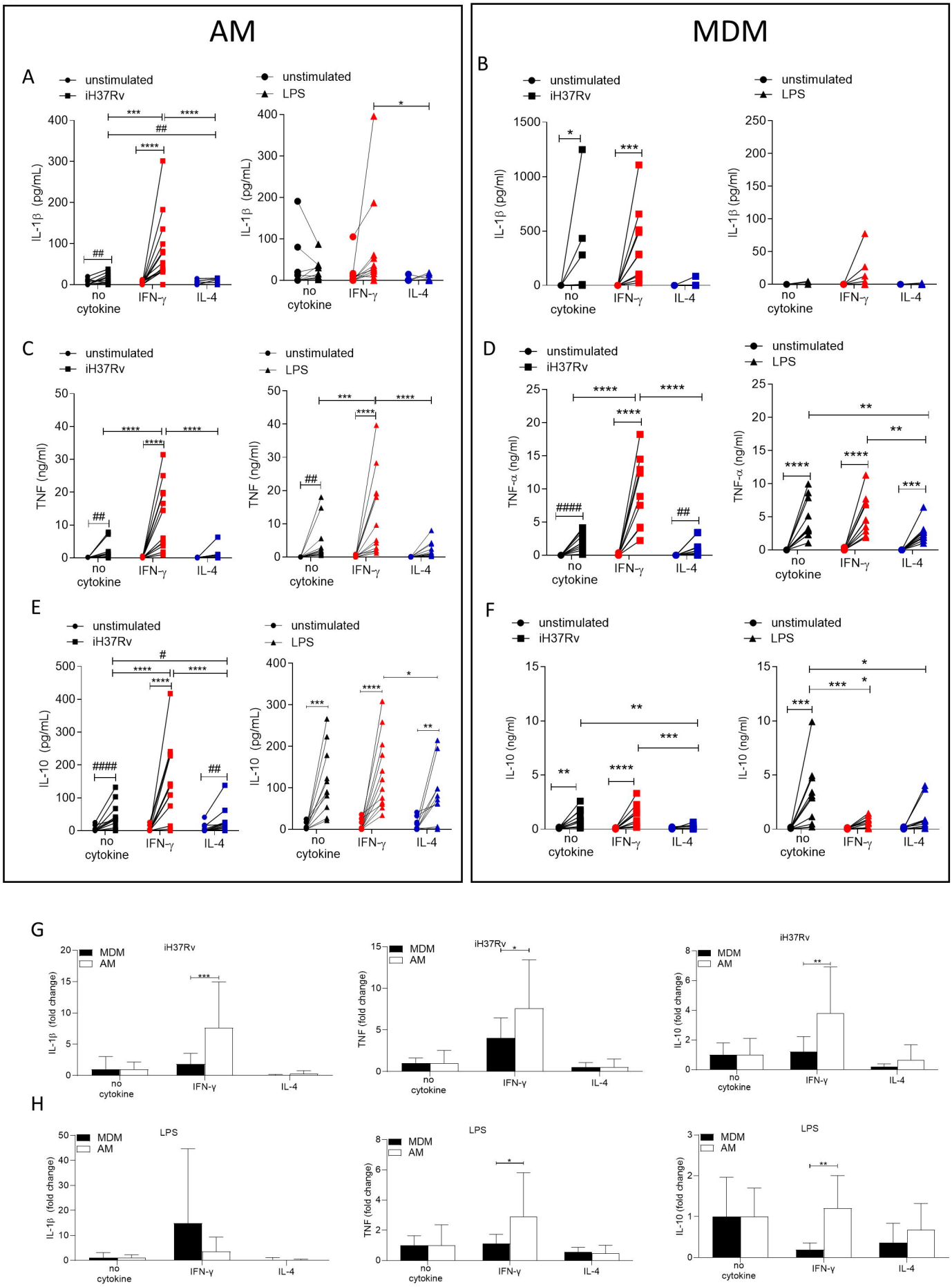
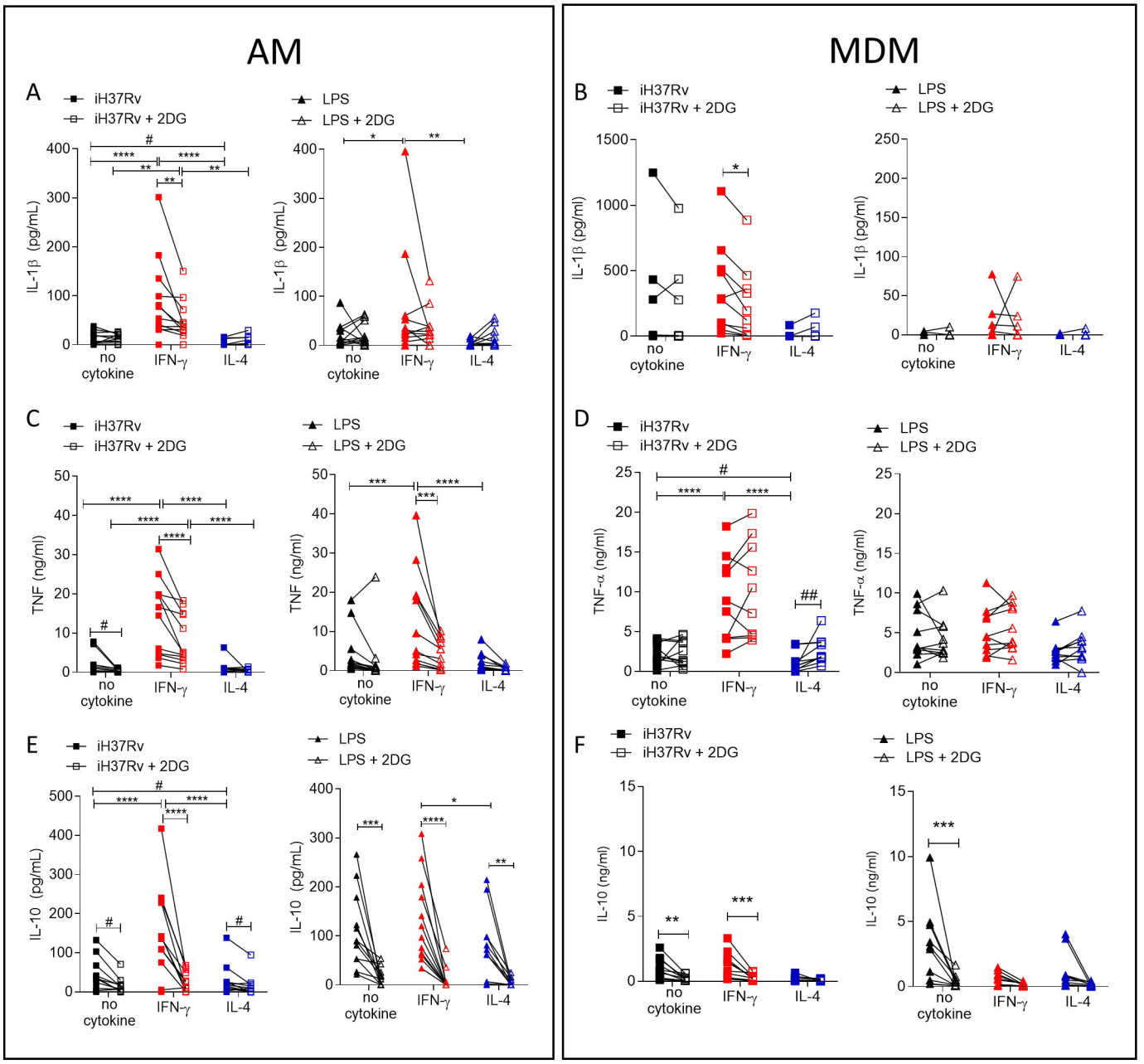

AM
MDM
A
B
C
D
E
F
G
H
unstimulated
iH37Rv
LPS
IL-1β (pg/mL)
IL-1β (pg/ml)
TNF (ng/ml)
TNF-α (ng/ml)
IL-10 (pg/mL)
IL-10 (ng/ml)
no cytokine
IFN-γ
IL-4
MDM
AM
iH37Rv
LPS
IL-1β (fold change)
TNF (fold change)
IL-10 (fold change)

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