

1 **The lung environment controls alveolar macrophage metabolism and responses in type 2**
2 **inflammation**

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43 **Fine control of macrophage activation is needed to prevent inflammatory disease,**
44 **particularly at barrier sites such as the lungs. However, the dominant mechanisms that**
45 **regulate pulmonary macrophage activation during inflammation are currently poorly**
46 **understood. Here we found that alveolar macrophages were substantially less able to**
47 **respond to the canonical type 2 cytokine IL-4, which underpins allergic disease and**
48 **parasitic worm infections, than lung tissue or peritoneal cavity macrophages. We found that**
49 **alveolar macrophage hypo-responsiveness to IL-4 was dictated by the lung environment,**
50 **but was independent of the host microbiota or the lung extracellular-matrix components**
51 **surfactant protein D or mucin 5b. Alveolar macrophages displayed severely dysregulated**
52 **metabolism compared to that of cavity macrophages. After being removed from the lung,**
53 **alveolar macrophages regained responsiveness to IL-4 in a manner dependent on**
54 **glycolysis. Thus, impaired glycolysis within the pulmonary niche was a central determinant**
55 **for the regulation of alveolar macrophage responsiveness during type 2 inflammation.**

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61 The specialized mucosal environment of the lung is required to enable breathing in the face
62 of continuous exposure to debris and micro-organisms, while also calling for diverse mechanisms
63 to restrict disease caused by over-exuberant inflammatory responses¹. Lung macrophages have
64 been proposed to be central to mediating and regulating type 2 inflammation against allergens and
65 parasitic worms, which together affect billions of people worldwide². Against these types of
66 challenge, macrophages can expand *in situ* to type 2 cytokines such as IL-4 that trigger
67 'alternative' (or M(IL-4)) activation, linked to wound repair and type 2 pathology^{3, 4, 5}. Although
68 pulmonary macrophage sub-populations inhabit dramatically different anatomical sites, such as the
69 airways and tissue parenchyma, it is not yet clear how location influences their ability to respond to
70 type 2 inflammation. In particular, reports of M(IL-4) marker expression on lung macrophages
71 during type 2 inflammation^{6, 7, 8, 9} have involved experimental approaches that may not clearly
72 distinguish macrophages from other myeloid cells, raising the possibility that functional differences
73 in key macrophage sub-populations have been inadvertently overlooked.

74 As the predominant macrophage sub-population in airways, alveolar macrophages (AlvMs)
75 are vital for maintaining lung health and function, having a central role in clearance of debris,
76 surfactant and apoptotic cells¹⁰. In the absence of AlvMs, fluid build-up leads to primary pulmonary
77 alveolar proteinosis, severe lung dysfunction and respiratory failure¹¹. The majority of AlvMs are
78 thought to be derived from embryonic precursors that seed the lung tissue before birth¹², with
79 recent evidence suggesting that the cytokines GM-CSF and TGF- β induce PPAR- γ , a crucial
80 transcription factor for AlvM development^{11, 13, 14}. During inflammation, AlvMs mediate bacterial
81 clearance and initiate neutrophil recruitment¹⁵, functions that can be regulated by cytokines such
82 as IL-10 or TGF- β , and/or the engagement of cell surface receptors such as SIRP α or CD200¹⁶.
83 Because clear discrimination between AlvMs and other lung macrophage sub-populations is
84 technically challenging¹⁷, far less is known about the function and origin of tissue residing
85 interstitial macrophages (IntMs). Although IntMs may comprise up to three separate sub-
86 populations¹⁸, earlier work may have mistakenly identified them as AlvMs, monocytes or dendritic
87 cells (DCs).

88 Mucosal environments like the lung play a major role in determining both development and
89 function of macrophages¹⁹, though many of the factors that shape such processes remain unclear,
90 particularly in type 2 inflammation. Lung macrophage upregulation of M(IL-4) markers during
91 parasite-mediated type 2 responses is promoted by environmental factors such as surfactant
92 protein A (SP-A) and engagement of TAM receptors during clearance of apoptotic cells^{20, 21}. Here
93 we show that lung macrophage subsets, particularly AlvMs, were considerably less responsive to
94 type 2 inflammation than macrophages from other tissues. We demonstrate that this muted
95 phenotype was conferred by the lung environment, and was independent of potential negative
96 regulators such as CD200-CD200R, surfactant protein D (SP-D), mucin 5b (Muc5b) or the host
97 microbiota. Hypo-responsive AlvMs had an altered metabolic profile compared to IL-4-responsive
98 peritoneal exudate cell macrophages (PECMs), and were unable to upregulate glycolysis *in situ*.

99 After removal from the lung, AlVMs recovered their IL-4 responsiveness in a glycolysis-dependent
100 manner. Thus, the pulmonary environment controlled AlVM responsiveness during type 2
101 inflammation via modulation of their metabolic activity.
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104 **Results**

105 **AlvMs are unresponsive to IL-4 *in vivo***

106 To better understand how pulmonary macrophages respond during type 2 inflammation, we
107 utilized MerTK, CD64, Siglec-F and CD11b as markers that distinguish AlvMs from IntMs^{11, 17, 18}.
108 The majority of lung tissue and bronchoalveolar lavage (BAL) macrophages were
109 MerTK⁺CD64⁺CD11b⁻Siglec-F⁺ AlvMs (>89%), alongside a smaller population (<10%)
110 of MerTK⁺CD64⁺CD11b⁺Siglec-F⁻ IntMs (Fig. 1a,b and Supplementary Fig. 1a). Analysis of
111 additional macrophage markers showed that, while both AlvMs and IntMs expressed F4/80 and
112 CD11c, AlvMs were also Ym1^{hi}, a feature of M(IL-4) (Fig. 1c). Further, only IntMs expressed
113 CX3CR1 (Fig. 1c), supporting the idea that IntMs are derived from monocytes, while AlvMs at
114 steady-state are resident cells^{12, 18}. To verify that AlvMs reside in airways and IntMs in lung tissue,
115 we administered CD45-PE antibodies intranasally (i.n.) and CD45-FITC antibodies intravenously
116 (i.v.) prior to lung processing, to discriminate CD45-PE⁺ airway macrophages from CD45-FITC⁺
117 blood monocytes and tissue CD45-PE-FITC⁻ tissue macrophages²². This approach indicated that
118 AlvMs (defined throughout this study as MerTK⁺CD64⁺CD11b⁻Siglec-F⁺) were predominantly found
119 in the airways, and IntMs (defined throughout this study as MerTK⁺CD64⁺CD11b⁺Siglec-F⁻) within
120 the lung tissue (Fig. 1d), demonstrating that refined flow cytometry could discriminate between
121 AlvM and IntM subsets.

122 Next, we investigated whether lung AlvMs and IntMs were functionally similar to
123 macrophages in other tissues following systemic (intraperitoneal, i.p.) administration of
124 recombinant IL-4 complexed with mAb to IL-4 (IL-4c), which extends the bioactive half-life of the
125 cytokine and induces type 2 inflammation in C57BL/6 and BALB/c mice^{4, 5}. PECMs underwent
126 rapid expansion by day 4 after i.p. IL-4c injection on day 0 and day 2 (Fig. 1e)^{4, 5}, while AlvMs and
127 IntMs were markedly less responsive to IL-4c, with no measurable increase in numbers of either
128 population (Fig. 1e). Additionally, PECMs from IL-4c injected mice had elevated expression of
129 markers of M(IL-4) activation (RELM α) and proliferation (Ki67 and EdU) (Fig. 1f and
130 Supplementary Fig. 1b)⁴. AlvMs did not upregulate RELM α , Ki67 or EdU in response to IL-4c,
131 whilst IntMs expressed intermediate levels of RELM α and Ki67 in comparison to PECMs (Fig. 1f
132 and Supplementary Fig. 1b). Similar observations were made in BALB/c mice (data not shown).
133 AlvMs and IntMs had lower responsiveness to systemic IL-4c compared to MerTK⁺CD64⁺CD11b⁺
134 liver, colon or pleural cavity (PLEC) macrophages, which responded similarly to PECMs (Fig. 1g
135 and Supplementary Fig. 1c). Together, this indicated that hypo-responsiveness to IL-4c was a
136 feature of lung AlvMs and IntMs, and was particularly evident in AlvMs.

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138 **AlvMs express functional IL-4 receptor**

139 Next we assessed whether AlvMs had reduced expression of IL-4 receptor (IL-4R)
140 compared to IntMs and PECMs⁴. AlvM IL-4R α expression was similar to that of IntMs and PECMs,
141 and was not significantly affected by i.p. IL-4c (Fig. 2a). In addition, IL-4 was detected in BAL fluid

142 with similar dynamics as in peritoneal washes (Fig. 2b), indicating that i.p. injected IL-4c could
143 reach the airways. To further address whether AlVM responsiveness to IL-4c depended on route of
144 administration, we administered a range of concentrations of IL-4c i.n. (0.05 µg, 0.5 µg or 5 µg),
145 with the lowest dose typical of that detected in airways during type 2 inflammation. Although IntMs
146 significantly upregulated RELM α in response to i.n. IL-4c compared to PBS (Fig. 2c), AlVMs did not
147 do so, even at the highest IL-4c dose (Fig. 2c). These observations indicated that lack of M(IL-4)
148 activation was a characteristic feature of AlVMs, irrespective of IL-4c delivery route.

149 To investigate whether the lack of IL-4c responsiveness in AlVMs was due to impaired
150 signalling, we measured expression of phosphorylated STAT6 (p-STAT6), a key transcription
151 factor downstream of IL-4R α engagement². Both AlVMs after IL-4 i.n., and PECMs after IL-4 i.p.,
152 had increased p-STAT6 expression compared to PBS controls which was not evident in *I4ra*^{-/-}
153 mice (Fig. 2d). In addition, AlVMs had high basal expression of p-STAT6, Ym1, pAkt T308
154 (mTORC1) and pAkt S473 (mTORC2) compared to PECMs, which was also evident in *I4ra*^{-/-} mice
155 (Fig. 2d and Supplementary Fig. 2). This showed that AlVMs displayed IL-4R α -independent 'tonic'
156 STAT6 and mTORC signalling in the steady state, and could respond to i.n. IL-4 through STAT6
157 phosphorylation.

158

159 **AlVMs show limited M(IL-4) activation during helminth infection**

160 To assess whether differential activation of AlVMs and IntMs was apparent in settings other
161 than IL-4c injection, we infected C57BL/6 mice s.c. with the parasite *Nippostrongylus brasiliensis*,
162 against which a type 2 response is essential for tissue repair as larvae migrate through the lung,
163 and for clearance of adult worms from the intestines²³. As expected, a type 2 response, with
164 eosinophilia and increased levels of RELM α in BAL fluid, was detected after infection, compared to
165 naïve mice (Fig. 3a,b). As infection progressed from day 2 to day 7, IntMs increased in numbers
166 (Fig. 3c,d) and upregulated the M(IL-4) markers RELM α , Arginase-1 and Ym1 markedly more than
167 AlVMs (Fig. 3e and Supplementary Fig. 3b,c). Further, IntMs expressed higher levels of Ki67 than
168 AlVMs by day 7 post-infection (Fig. 3e and Supplementary Fig. 3b), indicating that AlVMs did not
169 acquire a clear M(IL-4) phenotype during infection. These observations contradict previous reports
170 of AlVM M(IL-4) activation during type-2 inflammation^{6, 7, 8, 9, 23}. However, these previous studies
171 have generally defined AlVMs as CD11c⁺Siglec-F⁺ (Supplementary Fig. 4a). Reliance on CD11c
172 and Siglec-F to identify AlVMs could result in the inclusion of RELM α ⁺ IntMs and eosinophils,
173 particularly in inflamed mice (Supplementary Fig. 4a-d). Furthermore, use of scatter parameters in
174 flow cytometry to exclude eosinophils could remove macrophages with similar granularity and
175 SiglecF, CD11b or CD11c expression (Supplementary Fig. 4e,f). Using refined flow cytometry, we
176 have demonstrated that M(IL-4) activation of AlVMs was impaired in comparison to IntMs during *N.*
177 *brasiliensis* infection.

178

179 **The pulmonary niche regulates AlVM responsiveness to IL-4**

180 The lung environment is a unique site that shapes macrophage development²⁴, with
181 environmental signals vital for directing this process¹⁹. Further, upon removal from tissues,
182 macrophages in culture display fundamentally altered gene expression^{25,26}. Consistent with reports
183 that AlVMs from mice and humans can respond to IL-4 *in vitro*², AlVMs isolated from the lungs of
184 C57BL/6 mice significantly up-regulated expression of *Retnla* (encoding RELM α) and *Arg-1* after
185 48h in culture with IL-4, while expression of *Chil3* (encoding Ym1) was elevated after 12h
186 compared to PBS controls (Fig. 4a). We next addressed whether the airway environment limited
187 the ability of AlVMs to undergo M(IL-4) polarization. We transferred CD45.2⁺ PECMs, which
188 responded strongly to IL-4 *in vivo* (Fig. 1), i.n. into naïve CD45.1⁺ mice, followed by administration
189 of IL-4c i.p. Donor CD45.2⁺ PECMs were detected in the lungs of recipient mice at day 5 post-
190 transfer (Fig. 4b). However, PECMs transferred i.n. displayed an activation profile similar to that of
191 resident AlVMs, failing to up-regulate RELM α and Ki67 in response to i.p. IL-4c administration
192 compared to recipient PECMs (Fig. 4c). IL-4R α expression on transferred CD45.2⁺ PECMs was
193 similar to recipient PECMs (Fig. 4d), suggesting that the impaired response of i.n. PECMs to IL-4
194 was not due to altered IL-4R α expression.

195 Interaction between the inhibitory receptor CD200R and its ligand CD200 has been
196 described as a dominant negative regulator of AlVM activation in non-type 2 settings¹⁶. Although
197 expression of CD200R was highest on AlVMs compared to IntMs (Supplementary Fig. 5a), we
198 observed no significant difference in numbers of AlVMs or IntMs, or their expression of RELM α or
199 Ki67, following i.p. IL-4c injection of *Cd200r1*^{-/-} mice (Supplementary Fig. 5b), indicating that AlVM
200 hypo-responsiveness to IL-4 was independent of regulatory CD200-CD200R interactions.

201 In addition to immune mechanisms, macrophage responses at barrier sites may be
202 modulated by airway components such as surfactant or mucus. SP-A and SP-D are abundant in
203 the lower airways²⁷ and have been implicated in promotion of type 2 inflammation and M(IL-4)
204 activation of AlVMs during helminth infection^{21,28}, while mucus is a major regulator of responses in
205 lung and airway macrophages²⁹. IL-4c increased expression of the dominant pulmonary mucin
206 Muc5b in airway epithelial cells compared to PBS-treated mice (Supplementary Fig. 5c). However,
207 IntMs and AlVMs in *Muc5b*^{-/-} and *Sfpta*^{-/-} mice responded to IL-4c similarly to wild-type mice
208 (Supplementary Fig. 5d,e), indicating that neither Muc5b nor SP-D were dominant factors in
209 limiting the ability of lung AlVMs to undergo M(IL-4) polarization.

210 The airways host a wide diversity of commensals that could influence macrophage
211 responses and are proposed to be key in regulating pulmonary allergic inflammation³⁰. Further, gut
212 microbe-derived short chain fatty acids are able to systemically regulate type 2 responses in the
213 lung³¹. To test the involvement of commensals in regulating IL-4 responsiveness of AlVMs, we
214 compared expression of RELM α and Ki67 in gnotobiotic (germ free (GF)) mice and conventionally-
215 housed (specific pathogen free (SPF)) mice following i.p. IL-4c administration. RELM α and Ki67
216 expression on AlVMs, IntMs or PECMs was similar in IL-4c-treated GF and SPF mice (Fig. 4e),
217 indicating that neither commensals nor their metabolites were involved in regulation of IL-4

218 responsiveness in any of these macrophage types. Together, these data indicated that the lung
219 environment controlled AlvM responsiveness to IL-4, but this was independent of the microbiota,
220 Muc5b or SP-D.

221

222 **AlvMs and PECMs have distinct metabolic gene profiles**

223 To address which factors might determine the lack of AlvM responsiveness to IL-4 *in vivo*,
224 we next performed genome-wide mRNA profiling of AlvMs, IntMs and PECMs isolated from
225 C57BL/6 mice injected i.p. with IL-4c or PBS. IL-4c induced a marked alteration of PECM gene
226 expression, with 2074 transcripts significantly up- or down-regulated compared to PECMs from
227 PBS-injected mice, including up-regulation of core M(IL-4) genes such as *Chil3*, *Retnla*, *Arg1* and
228 *Mrc1* (encoding mannose receptor) (Fig. 5a,b and Supplementary Tables 1,2). IntMs in IL-4c-
229 treated mice significantly up- or down-regulated 107 transcripts relative to IntMs from PBS-injected
230 mice, including up-regulation of *Chil3* and *Retnla*, but not *Arg1* or *Mrc1* (Fig. 5a,b and
231 Supplementary Tables 3,4), while IL-4c did not significantly up-regulate any of the core transcripts
232 previously associated with M(IL-4) responsiveness in AlvMs, having almost no measurable impact
233 on mRNA expression, with only 2 genes significantly down-regulated compared to AlvMs from
234 PBS-treated mice: *Mipol1*, a putative tumor suppressor³² and *Gnpat*, which is involved in lipid
235 metabolism³³ (Fig. 5a,b and Supplementary Table 5). This indicated that AlvMs were broadly
236 unresponsive to IL-4 *in vivo*. Further, AlvMs in PBS-treated mice had high basal expression of
237 *Chil3* and *Mrc1* mRNA (Fig. 5b), consistent with high expression of Ym1 protein in steady-state
238 AlvMs (Fig. 1c) and indicating that these markers are not suitable for M(IL-4) assessment in AlvMs.
239 Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that the major pathways
240 altered in PECMs by IL-4c included those involved in proliferation and metabolic processes
241 (Supplementary Fig. 6a). However, few of these pathways were altered in response to IL-4c in
242 AlvMs or IntMs (Supplementary Fig. 6b,c).

243 Next, we directly compared transcript expression in AlvMs and PECMs from IL-4c-treated
244 mice. This analysis indicated substantially different gene expression profiles between the two
245 macrophage populations (Fig. 5c and Supplementary Tables 6,7). In particular, pathways
246 associated with glycolysis were impaired, while those associated with lipid metabolism and
247 differentiation, such as PPAR and TGF- β , were elevated in AlvMs compared to PECMs (Fig. 5d,e
248 and Supplementary Fig. 6d). These observations suggested that AlvM hypo-responsiveness to IL-
249 4 may be due to impaired glycolysis, and confirmed previous reports that lung macrophages have
250 a distinctive metabolic state compared to macrophages in other tissues¹³.

251

252 **Impaired glycolysis limits AlvM IL-4 responsiveness *in vivo***

253 To investigate whether AlvMs had reduced glycolytic ability compared to PECMs we
254 analyzed changes in extracellular acidification rates (ECAR), a measure of glycolytic activity
255 through detection of lactic acid as an end product of glucose metabolism³⁴, in AlvMs and PECMs

256 from naïve C57BL/6 mice. AlVMs exhibited significantly impaired glycolysis (reduced ECAR
257 following glucose addition) and glycolytic reserve and capacity (defined as the ability to upregulate
258 aerobic glycolysis) compared to PECMs (Fig. 6a). Analysis of oxygen consumption rates (OCR)
259 showed that AlVMs also displayed reduced respiratory capacity (oxidative phosphorylation
260 (OXPHOS)) compared to PECMs (Supplementary Fig. 7a). Culture of freshly isolated AlVMs or
261 PECMs with the glucose analogue 2-NBDG, to measure uptake potential and glycolytic activity,
262 showed that AlVMs acquired less 2-NBDG than PECMs, even when co-cultured at a 1:1 ratio with
263 PECMs (Fig. 6b). Further, both CD45.2⁺ PECMs transferred i.n. into CD45.1⁺ mice and resident
264 CD45.1⁺ AlVMs had a reduced ability to acquire 2-NBDG *in vivo* following i.p. IL-4c, when
265 compared to resident CD45.1⁺ PECMs (Fig. 6c). Together, these observations indicated that the
266 lung environment impaired the ability of AlVMs to both take up and utilize glucose.

267 However, AlVMs isolated from the lung and cultured for 48h *in vitro* showed increased
268 expression of *Slc2a6* and *Eno1*, genes involved in glucose uptake and glycolysis (Fig. 6d),
269 indicating that *ex vivo* culture of AlVMs enhanced their glycolytic ability. Next, we addressed
270 whether glucose or fatty acid utilization was required for AlVMs to regain IL-4 responsiveness *in*
271 *vitro*. The ability of cultured AlVMs to upregulate *Retnla*, *Arg1* and *Chil3* *in vitro* in response to IL-4
272 was markedly inhibited by 2-deoxyglucose (2-DG), a competitive glucose inhibitor, compared to
273 culture with IL-4 alone (Fig. 6e), but not significantly affected by addition of etomoxir, an inhibitor of
274 fatty acid oxidation (FAO) (Supplementary Fig 7b). Similarly, even though AlVMs had high
275 expression of genes associated with the TGF- β pathway (Fig. 5d), and addition of TGF- β reduced
276 expression of IL-4-induced *Retnla* in cultured AlVMs (Supplementary Fig. 7c), it had no significant
277 effect on *Chil3* expression, and increased *Arg-1* expression, suggesting that TGF- β was not a vital
278 factor in limiting AlVM IL-4 responsiveness. Together, these data indicated that the lung
279 environment regulated AlVM M(IL-4) activation through modulation of their metabolism.

280

281 **Discussion**

282 Here we have shown that AlVMs were hypo-responsive to type 2 inflammation mediated by
283 IL-4c injection or helminth infection. This lack of responsiveness was conferred by the lung
284 environment and impacted AlVM metabolic activity and ability to both take up and metabolize
285 glucose. Removal of AlVMs from the lung reversed this metabolic constraint, enabling their M(IL-4)
286 activation.

287 Although numerous studies have reported that pulmonary macrophages upregulate M(IL-4)
288 markers, they either did not unequivocally distinguish between AlVMs and IntMs in their analyses,
289 or relied on IL-4 stimulation of macrophages *ex vivo*, or used M(IL-4) markers that are already
290 highly expressed by AlVMs at steady-state^{6, 7, 8, 9, 20, 21}. Our results suggest that such work may
291 require re-assessment to precisely identify which macrophage populations respond to IL-4 *in vivo*.
292 Our data indicate that IntMs will be the major macrophage sub-population to respond in pulmonary
293 type 2 inflammatory settings. This distinction is likely to be important for accurate understanding of
294 the pathogenesis of pulmonary type 2 disease, given that M(IL-4) macrophages have been
295 implicated in wound repair during type 2 inflammation^{23, 35}. Thus, we would speculate that IntMs
296 will play a more important role than AlVMs in processes such as resolving tissue damage in the
297 lung, due to their greater ability to respond to IL-4.

298 Although negative regulation of macrophage activation is a well described feature of the
299 lung, and is thought to be vital to restrict over-exuberant responses against inhaled material, viral
300 or bacterial infection¹, how pulmonary M(IL-4) responses are regulated is currently poorly
301 understood. While we have not identified which specific components of the pulmonary environment
302 restricted AlVM activation by IL-4, we have shown that this was independent of Muc5b, SP-D and
303 commensals or their metabolite products, all of which are features of the lung that have previously
304 been implicated in modulating pulmonary macrophage responses to bacteria, helminth infection
305 and allergic airway inflammation^{28, 29, 31}.

306 Metabolism is a key determinant of immune cell function and is central in governing how
307 macrophages respond to a variety of signals, including type 1 and type 2 cytokines^{34, 36}. The
308 majority of studies so far have profiled metabolic responses in bone marrow-derived macrophages
309 *in vitro*³⁷, and have not addressed how tissue environments alter macrophage metabolism and
310 function *in vivo*. From such work, it has been proposed that type 2 cytokines promote amino acid
311 and lipid metabolism (including FAO) feeding into OXPHOS, whereas glycolysis is more
312 associated with type 1 macrophage polarization^{34, 37, 38, 39}. We found that AlVMs had a distinctive
313 metabolic state compared to PECMs, with elevated expression of genes associated with PPAR-γ
314 and lipid metabolism, a profile that would be expected to enhance FAO, OXPHOS and M(IL-4)
315 activation^{34, 37, 38}. However, defective glycolytic ability rendered AlVMs hypo-responsive to IL-4,
316 consistent with recent observations that glycolysis can mediate macrophage responses to IL-4^{40, 41},
317 and with studies linking altered metabolic state with AlVM ability to respond to *Mycobacterium*
318 *tuberculosis*⁴². Our demonstration that the lung environment controls macrophage metabolism

319 during type 2 inflammation, together with recent evidence that metabolism also regulates DC
320 control of allergic airway inflammation⁴³, suggests caution in interpreting metabolic data generated
321 from model macrophages or DCs in culture. Our data also imply that the distinctive metabolic
322 profile of AlVMs may be directly linked to negative regulation of their activation and function at
323 steady-state and during inflammation¹.

324 One factor to consider in how the lung may affect AlVM activation is amounts of metabolic
325 substrates, including glucose, present in airways. Glucose levels in air surface liquid, which covers
326 the airway epithelium, are 12.5-fold lower than in blood⁴⁴. Such low glucose concentrations,
327 maintained through highly-effective epithelial cell glucose transport⁴⁵, appear vital to prevent
328 bacterial outgrowth in airways^{44, 46}. Elevated glucose is found in patient sputum during chronic
329 obstructive pulmonary disease⁴⁷, while glucose levels and glucose metabolism rise in the lung
330 during asthma^{48, 49}. Together with our data, this leads to the intriguing hypothesis that glucose
331 availability and/or utilization could be exploited to therapeutically modify pulmonary disease.

332 We showed that AlVMs removed from the airways regained ability to respond to IL-4 *in vitro*, while PECMs transferred into the airways lost IL-4 responsiveness. The stark difference
333 between AlVM ability to respond to IL-4 *in vitro* and *in vivo* resonates with the reported
334 transformation of microglial transcriptional identity when removed from the brain^{25, 26}, and cautions
335 against reliance on AlVMs *in vitro* for functional studies. This may be particularly relevant for
336 human AlVMs, given current experimental dependence on their culture *ex vivo*, and highlights the
337 need for development of innovative approaches to better assess human AlVM function *in vitro*.
338 Similarly, identification of markers for human macrophage subpopulations and their M(IL-4)
339 activation is urgently needed. The current revolution in single cell sequencing for definition of
340 cellular networks suggests that this approach applied to human AlVMs should be illuminating. In
341 both human and murine type 2 inflammation, it will also be important to understand how
342 monocytes recruited to the lung differentiate and influence airway or tissue macrophages, as
343 resident AlVMs can be replaced by regulatory monocytes during viral infection⁵⁰. Our data
344 suggests that the airway environment will play a key role in influencing activation and function of
345 AlVMs during type 2 inflammation, irrespective of their origin.

346 More broadly, this work illustrates the pivotal role of the tissue environment in the regulation
347 of metabolic activity and ability to respond to type 2 cytokines in AlVMs, a principle that will likely be
348 relevant in diverse tissue and disease settings. Local differences in substrate availability, and
349 alterations of such during inflammation, may provide an elegant metabolic mechanism to modulate
350 the activation and function of macrophages and other immune cells in a tissue-specific manner in
351 health and in disease.

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357 **Acknowledgements**

358 We thank members of the MCCIR and MacDonald laboratory (University of Manchester) for
359 scientific discussions and some experimental assistance, J. Allen (University of Manchester) for
360 critical reading of the manuscript, the University of Manchester Single Cell Facility for flow
361 cytometry, cell sorting and ImageStream, K. Couper and J. Grainger (University of Manchester) for
362 provision of Pep3 and CX3CR1eGFP mice and M. Travis for providing recombinant TGF- β . This
363 research was supported by a MCCIR PhD studentship (F.R.S.), the Medical Research Council
364 (MR/P026907/1, H.C. and J.M.) the National Institutes of Health (HL080396 and HL130938,
365 C.M.E.), the Wellcome Trust Institutional Strategic Support Fund [105610] (R.K.G., D.J.T. and
366 M.Z.K.), Medical Research Foundation UK joint funding with Asthma UK (MRFAUK-2015-302,
367 T.E.S.), BBSRC studentship (C.S.), a University of Manchester Dean's Prize Early Career
368 Research Fellowship (P.C.C.), Springboard Award (Academy of Medical Sciences, SBF002/1076,
369 P.C.C.) and MCCIR core funding (A.S.M. and T.H.). This work was also made possible through
370 use of the Manchester Gnotobiotic Facility that was established with the support of the Wellcome
371 Trust [097820/Z/11/B], using founder mice obtained from the Clean Mouse Facility, University of
372 Bern, Switzerland. The Bioimaging Facility microscopes used in this study were purchased with
373 grants from BBSRC, Wellcome Trust and the University of Manchester Strategic Fund.

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375 **Authors contributions**

376 Conceptualization, P.C.C, A.S.M.; Methodology, F.R.S., S.L.B., M.Z.K., L.C., M.C., G.H., A.C.I.,
377 P.C.C.; Investigation, F.R.S., S.L.B., M.Z.K., L.C., C.S., M.C., G.H., T.E.S., A.C.I., P.C.C.;
378 Resources, H.C., J.M., C.M.E., T.E.S., D.J.T., R.K.G., D.M.C., T.H., A.S.M.; Writing – Original
379 Draft, F.R.S. P.C.C., A.S.M.; Writing – Review & Editing, F.R.S., S.L.B., C.M.E., A.C.I., D.J.T.,
380 R.K.G., D.M.C., T.H., P.C.C., A.S.M.; Funding Acquisition, D.J.T, R.K.G., D.M.C., T.H., P.C.C.,
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382

383 **Competing interests**

384 The authors declare no competing interests.

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389 **Figure legends**

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392 **Fig 1. Alveolar macrophages are unresponsive to IL-4.** **a**, Flow cytometry plots identifying
393 AlVMs and IntMs from BAL fluid or lung tissue of naïve mice. Data representative of 8 independent
394 experiments. **b**, Imaging cytometry of AlVMs and IntMs from lung tissue of naïve mice (scale bar:
395 10µm). **c**, Histograms of expression of F4/80, CD11c, Ym1 and eGFP by *Cx3cr1*^{eGFP/+} mice by
396 IntMs and AlVMs from lung tissue of naïve mice. **d**, Flow cytometry plots of AlVMs, IntMs and
397 monocytes from lung tissue of naïve mice following CD45 i.n. and i.v. administration. b-d,
398 representative data from 3 independent experiments. **e**, Numbers of lung tissue AlVMs and IntMs,
399 or PECMs, on d4 following i.p. PBS or IL-4c administration on d0 and d2. Data representative of 7-
400 9 experiments, n=26 (AlvM PBS, AlvM IL-4c, IntM PBS, IntM IL-4c), n=18 (PECM PBS), n=13
401 (PECM IL-4c) mice per group. **f**, RELMα and Ki67 expression, or EdU incorporation, by lung tissue
402 AlVMs and IntMs, or PECMs, on d4 following i.p. PBS or IL-4c administration on d0 and d2, and
403 EdU injection i.p. 3h before tissue collection. Graphs show individual replicate mice, data pooled
404 from 5-9 independent experiments, RELMα: n=29 (AlvM PBS, AlvM IL-4c, IntM PBS, IntM IL-4c),
405 n=24 (PECM PBS), n=23 (PECM IL-4c) mice per group. Ki67: n=24 (AlvM PBS, IntM PBS), n=23
406 (AlvM IL-4c, IntM IL-4c), n=20 (PECM PBS, PECM IL-4c) mice per group. EdU: n=22 (AlvM PBS,
407 AlvM IL-4c, IntM PBS), n=23 (IntM IL-4c), n=14 (PECM PBS), n=17 (PECM IL-4c) mice per group.
408 **g**, Percentage of RELMα⁺ PECMs, PLECMs, Kupffer cells, IntMs and AlVMs on d4 following i.p.
409 PBS or IL-4c administration on d0 and d2. Data representative of 2-5 independent experiments,
410 n=5 (PBS PECMs, Kupffer cells, IntMs and AlVMs), n=4 (IL-4c PLECMs), n=3 (PBS PLECMs,
411 colon Ms and IL-4c PECMs, Kupffer cells, colon Ms, IntMs, AlVMs) mice per group. e-g, data
412 analysed by two-way analysis of variance (ANOVA) with Tukey's post-test for multiple
413 comparisons, displayed as mean ± SEM, **P*<0.05, ****P*<0.001 and *****P*<0.0001.

413

414 **Fig. 2. Alveolar macrophages are less responsive than interstitial macrophages to IL-4c**
415 **administered directly into the airways.** **a**, IL-4Rα expression by AlVMs, IntMs or PECMs from
416 mice injected with PBS or IL-4c i.p. on d0 and d2, and lung tissue and PEC collected on d4.
417 Histograms representative of 2 independent experiments. **b**, ELISA of IL-4 levels in BAL or PEC
418 fluids 6h, 12h, 24h or 48h after i.p. injection of PBS or IL-4c. Data representative of 2 independent
419 experiments, n=2 (PBS, 24h and 48h), n=3 (6h and 12h) mice per group. **c**, Flow cytometry plots
420 of RELMα expression in lung tissue AlVMs and IntMs on d4 following i.n. PBS or IL-4c
421 administration on d0 and d2 (left) and quantification of the percentage of RELMα⁺ cells (right). Data
422 representative of 3 independent experiments, n=2 (5µg AlvM), n=3 (AlvM: PBS, 0.05 & 0.5µg.
423 IntM: PBS, 0.05, 0.5 & 5µg) mice per group. **d**, Histograms of pSTAT6 levels in lung tissue AlVMs
424 or PECMs 15 min after PBS or rIL-4 administered i.n. or i.p. to WT or *Il4ra*^{-/-} mice (left), and
425 quantification of AlvM and PECM pSTAT6 expression (right). Data representative of 2
426 independent experiments, n=3 mice per group. Data analysed by one-way analysis of variance

427 (ANOVA) with Tukey's post-test for multiple comparisons, displayed as mean \pm SEM, * P <0.05,
428 *** P <0.001 and **** P <0.0001.

429

430 **Fig. 3. Alveolar macrophages are less responsive than interstitial macrophages during**
431 **helminth infection. a**, Eosinophil numbers from lung tissue of naïve mice or on d2, d4 and d7
432 following infection s.c. with 500 L3 *N. brasiliensis* larvae. Graphs show individual replicate mice,
433 data pooled from 4 independent experiments, n=18 (naïve), n=17 (d2), n=9 (d4), n=5 (d7) mice per
434 group. **b**, ELISA of RELM α levels in BAL fluid from naïve or infected mice. Data pooled from 2
435 independent experiments, n=8 (naïve), n=7 (d2), n=4 (d4), n=3 (d7) mice per group. **c**, Numbers of
436 lung tissue AlvMs and IntMs from naïve or infected mice. Data pooled from 4 independent
437 experiments, n=18 (naïve), n=17 (d2), n=9 (d4), n=8 (d7) mice per group. **d**, Flow cytometry plots
438 identifying lung tissue AlvMs and IntMs from naïve or infected mice. Data representative of 4
439 independent experiments. **e**, Quantification of the percentage of RELM α ⁺ and Ki67⁺ lung tissue
440 AlvMs and IntMs from naïve or infected mice. Data pooled from 4 independent experiments, n=18
441 (Naïve RELM α), n=17 (d2 RELM α), n=9 (d4 RELM α), n=8 (d7 RELM α), n=13 (Naïve Ki67), n=12
442 (d2 Ki67), n=5 (d4 Ki67), n=3 (d7 Ki67) mice per group. Data analysed by one-way analysis of
443 variance (ANOVA) with Tukey's post-test for multiple comparisons, displayed as mean \pm SEM,
444 * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001.

445

446 **Fig. 4. The pulmonary niche regulates alveolar macrophage responsiveness to IL-4**
447 **independently of host commensals. a**, mRNA expression by qPCR of lung tissue AlvMs from
448 naïve mice following culture for 12h, 24h or 48h in media alone or with rIL-4 (20 ng/ml). A.U.
449 arbitrary units. Data representative of 4 independent experiments, n=2 (media), n=3 (rIL-4) wells
450 per group, each group pooled cells from 8 mice. **b - e**, Donor (CD45.2⁺) and host (CD45.1⁺)
451 macrophage populations identified by flow cytometry in lung tissue or PEC from host mice on d5
452 after i.n. PBS or donor PECM transfer on d0, then injection with IL-4c i.p. on d1 and d3. **b**, Flow
453 cytometry plots identifying donor PECMs in host lung tissue. Data representative of 4 independent
454 experiments. **c**, Quantification of the percentage of RELM α ⁺ and Ki67⁺ host and donor
455 macrophages isolated from host lung tissue or PEC. Data representative of 4 independent
456 experiments, n=6 mice per group. **d**, IL-4R α expression by donor PECMs isolated from host lung
457 tissue. Histogram representative of 2 independent experiments. **e**, RELM α and Ki67 expression by
458 lung tissue AlvMs and IntMs, or PECMs, from specific pathogen free (SPF) or Germ Free (GF)
459 mice on d4 following i.p. PBS or IL-4c administration on d0 and d2. Data representative of 3
460 independent experiments, n=3 mice per group. Data analysed by one-way analysis of variance
461 (ANOVA) with Tukey's post-test for multiple comparisons, displayed as mean \pm SEM, ** P <0.01,
462 *** P <0.001 and **** P <0.0001.

463

464 **Fig. 5. Alveolar and peritoneal macrophages display dramatically different metabolic gene**
465 **profiles. a,** mRNA expression profiles (volcano plots) as determined by RNA-seq of PECMs, IntMs
466 or AlvMs isolated from lung tissue or PEC by flow cytometry on d4 following i.p. PBS or IL-4c
467 administration on d0 and d2. Dashed lines represent $P < 0.01$, and ± 2 -fold change, IL-4c relative to
468 PBS. **b,** Heatmaps of selected mRNA transcripts of genes that have been previously described as
469 M(IL-4) markers, *indicates significance between IL-4c vs. PBS of at least $p < 0.01$. **c,** mRNA
470 expression profile (volcano plot) of AlvMs vs. PECMs isolated from IL-4c injected mice. Dashed
471 lines represent $P < 0.01$, and ± 2 -fold change. **d,** Selected pathways from KEGG analysis
472 (Supplementary Fig. 6) of significantly altered mRNA transcripts from (c), black lines represent
473 $P < 0.05$. **e,** Relative transcript expression by AlvMs vs. PECMs from IL-4c injected mice that were
474 significantly altered ($P < 0.01$, log₂ normalized intensity), as identified from the glycolysis pathway
475 by network analysis (several genes displayed more than one altered transcript variant), n=2
476 (PECM PBS, PECM IL-4c, AlvM PBS, IntM PBS, IntM IL-4c), n=3 (AlvM IL-4c) separate biological
477 replicates, each replicate pooled cells from 3-5 mice.

478

479 **Fig. 6. Impaired uptake and utilization of glucose renders alveolar macrophages**
480 **unresponsive to IL-4.**

481 **a,** ECAR of AlvMs and PECMs isolated from lung tissue or PEC of naïve mice by flow cytometry,
482 at baseline and after sequential treatment (vertical lines) with glucose, oligomycin (Oligo) or 2-
483 Deoxy-D-glucose (2-DG) to measure glycolysis, glycolytic reserve and glycolytic capacity. Data
484 representative of 4 independent experiments, n=6 (AlvM), n=10 (PECM) glycolytic stress test
485 profile, n=6 (AlvM) glycolysis, glycolytic capacity and glycolytic reserve, n=10 (PECM) glycolysis,
486 glycolytic capacity, n=9 (PECM) glycolytic reserve, wells per group, each group pooled cells from 8
487 mice. **b,** Flow cytometry plots of 2-NBDG uptake *in vitro* by BAL AlvMs or PECMs from naïve mice
488 cultured separately or at a 50:50 mix for 20 min with fluorescently labelled 2-NBDG. 2-NBDG
489 uptake *in vivo* by donor (CD45.2⁺) and host (CD45.1⁺) macrophage populations identified by flow
490 cytometry in lung tissue or PEC from host mice on d5 after i.n. PBS or donor PECM transfer on d0,
491 administration of IL-4c i.p. on d1 and d3, and i.p. injection of fluorescently labelled 2-NBDG 20 min
492 prior to lung tissue and PEC collection. Data representative of 2 independent experiments, n=6
493 mice per group. **d,** mRNA expression by qPCR of lung tissue AlvMs from naïve mice cultured for
494 12h, 24h or 48h in media alone. A.U. arbitrary units. Data representative of 6 (Eno1) or 5 (Slc2a6)
495 independent experiments, n=3 (Eno1), n=2 (Slc2a6) wells per group, each group pooled cells from
496 6-8 mice. **e,** mRNA expression by qPCR of lung tissue AlvMs from naïve mice cultured for 48h in
497 media alone, or with rIL-4 \pm 2-DG. Data representative of 3 independent experiments, n=2 (media),
498 n=3 (rIL-4), n=3 (rIL-4 + 2-DG) wells per group, each group pooled cells from 6-8 mice. Data
499 analysed using unpaired *t* test (a) or a one way analysis of variance (ANOVA) with Tukey's post-
500 test for multiple comparisons as indicated (b, c & e) or compared to 0h (d), displayed as mean \pm
501 SEM, * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$.

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Methods

Experimental animals

Cx3cr1^{1eGFP/+}, *Cd200r1^{-/-}*, *CD45.1⁺*, *Il4ra^{-/-}*, *Muc5b^{-/-}* and *Sfptd^{-/-}* were generated as described previously^{16, 28, 29}. All were on a C57BL/6 background except *Il4ra^{-/-}* which were BALB/c. C57BL/6 or BALB/c mice were purchased from Envigo. Mice were bred and maintained under specific pathogen free conditions at The University of Manchester. Germ free mice were from the University of Manchester Gnotobiotic Facility. All experiments were approved under a project license granted by the Home Office U.K., and by the University of Manchester Animal Welfare and Ethical Review Body, and performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

In vivo mouse models

IL-4 complex delivery in vivo: Long acting IL-4 complexes (IL-4c: IL-4/anti IL-4mAb) were prepared and used as previously described^{4, 5}. Recombinant murine IL-4 (BioLegend) was combined with rat IgG1 anti-IL-4 mAb 11B11 (BioXcell) at a 1:5 molecular weight ratio⁵¹. Mice were injected i.p. with 5 µg of IL-4 (complexed to 11B11) or Dulbeccos PBS (PBS, Sigma) on d0 and d2. Alternatively 50 µl of PBS or varying doses of IL-4c (5 – 0.05 µg) was administered directly i.n. on d0 and d2. Tissues were collected on d4 post initial injection.

N. brasiliensis infection: WT mice were infected s.c. with 500 *N. brasiliensis* third-stage larvae and tissues collected d2, d4 and d7 post infection.

In both models, to assess cell proliferation mice were injected i.p. with 0.5mg 5-ethynyl-2'-deoxyuridine (EdU) (ThermoFisher) in 200 µl PBS 3h prior to harvest to label cells in S-phase of cell cycle as has previously been described³. This short window was chosen to provide an accurate readout of *in situ* cell proliferation at the tissue of interest, and avoid detection of cells that had recently proliferated elsewhere prior to recruitment.

Isolation of immune cells from the peritoneal cavity, bronchoalveolar lavage, lung, intestine and liver.

Following sacrifice, PEC or BAL cells were obtained by washing of the peritoneal cavity or lungs with PBS containing 2% FBS and 2mM EDTA (Sigma). Lungs were processed as previously described⁵², incubated at 37°C for 40 min with 0.8 U/ml Liberase TL and 80 U/ml DNase I type VI in HBSS (all Sigma). The digestion was stopped with PBS containing 2% FBS (Sigma) and 2 mM EDTA (Sigma), with the resulting suspension then passed through a 70 µm cell strainer. In some cases, prior to collection, i.v. or i.n. instillation of fluorescently labeled anti-CD45 (clone: 30-F11) was used to distinguish between blood circulating (i.v. CD45 FITC⁺), airway resident (i.n. CD45 PE⁺) and tissue resident leukocytes (CD45 FITC⁻PE⁻), as described previously^{22, 53}. Mononuclear cells from the intestine and liver were isolated as previously described^{54, 55}. Erythrocytes were lysed using RBC lysis buffer (Sigma) and cells counted and processed for flow cytometry.

Flow cytometry and cell sorting

Equal numbers of cells were stained for each sample, washed with ice-cold PBS and stained with Zombie UV dye (@1:2000, BioLegend) for 10 min at room temperature. All samples were then blocked with 5 µg/ml αCD16/CD32 (2.4G2; BioLegend) in FACS buffer (PBS containing 2% FBS and 2mM EDTA) before staining for specified surface markers at 4°C for 25 minutes. For detection of intracellular molecules, following surface staining cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, and permeabilized with the Transcription factor staining kit (eBioscience) then stained with the relevant antibodies. If mice had been treated with EdU, cells were stained using the Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Molecular Probes) using an adapted protocol for a final staining volume of 50µl. Samples were acquired using a 5 laser Fortessa with BD FACSDiva software and analyzed with FlowJo software (v9 and v10, Tree Star). Sorting of macrophage populations from the PEC (based on DAPI⁺F4/80⁺) and lung (DAPI⁺, CD45⁺MerTK⁺CD64⁺ and CD11b⁺ IntMs or Siglec-F⁺ AlvMs) was performed using an Influx (BD Biosciences) using the 140 µm nozzle and 7.5 psi pressure, to a purity of ~95-99%. In some cases, myeloid cells were enriched prior to sorting by removal of lymphoid cells using Dynabeads (ThermoFisher) (biotinylated anti-CD3, CD19, B220, Ly6G, NK1.1, Ter119 and streptavidin-Dynabeads) according to the manufacturers instructions.

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pSTAT6 and pAkt intracellular staining

To assess pSTAT6 and pAkt activation, 5µg rIL-4 was administered i.n. or i.p. 15 min prior to tissue collection. Cells from PEC or BAL washes were directly incubated with an equal volume of formalin (final concentration 2% formalin) for at least 10 mins at room temperature, resuspended in 500µl ice cold methanol at 4°C for 10 min, washed twice with FACS buffer, then stained and acquired (as described above).

Intranasal transfer of PEC macrophages into the airways

PECMs were sorted as described above from CD45.2 mice. PBS, or 1 x 10⁶ donor PECMs in PBS, were instilled into the airways of CD45.1 recipient mice via i.n. transfer. Mice were treated with IL-4c (as described above) and cells from the lungs were isolated and processed as described above. In some experiments, 100 µg FITC labelled 2-NBDG (Sigma), internalization of which measures glucose uptake potential and glycolytic activity⁵⁶, was injected (i.p.) 20 min prior to tissue collection.

***In vitro* culture of macrophages**

AlvMs or PECMs FACS isolated from naïve mice (as described above) were cultured in RPMI 1640 (containing 10% FBS, 1% PenStrep, 1% L-glutamine, all Sigma) for up to 48h at 37°C. In some experiments, they were incubated with 50 µg/ml FITC labeled 2-NBDG (Sigma) for 20 min (either separately or a 50:50 mix of the two), or in the presence of rIL-4 (20 ng/ml) ± 1 mM 2-DG (Sigma), 200 µM etomoxir (Sigma) or recombinant human TGF-β (10 ng/ml) (Peprotech).

Imaging cytometry

Cells were stained and fixed (as described above) in ImageStream buffer (PBS containing 1% FBS and 2 mM EDTA). Data acquisition was performed on ImageStreamX (Amnis/EMD Millipore, Seattle, WA) equipped with 405, 488, 561, and 642 nm lasers. Single cells were discriminated from cell aggregates based on area and aspect ratio. In focus cells were selected based on high gradient RMS of the bright field image. Images of cells were acquired with a ×40 objective including bright field images (Channels 1 & 9; 420–480nm and 570-595nm), CD11b (Channel 2; 480-560nm), MerTK (Channel 3; 560–595nm), Siglec-F (Channel 4; 595-660nm), CD64 (Channel 6; 740-800nm), Zombie UV (Channel 7; 420-505nm) and CD45 (Channel 8; 505-570nm). All data analysis was performed using the IDEAS® software version 6.

Histology

Histological sections were prepared from lungs perfused with freshly prepared metha-carnoy's solution (60% absolute methanol, 30% chloroform, 10% acetic acid) and embedded in paraffin. 5 µm sections were subjected to immunohistochemical analysis for Muc5b (custom polyclonal antisera)⁵⁷. Bound primary antibody was detected with goat anti-rabbit Alexa fluor 488. Images were captured using an Olympus BX51 upright microscope using a 20x /0.5 EC Plan-neofluar objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Images were then processed and analyzed using ImageJ⁵⁸.

Enzyme linked immunosorbent assay (ELISA)

ELISAs to detect RELMα (PeproTech) and IL-4 (BioLegend) were performed on BAL or PEC fluid, as per manufacturers instructions.

RNA isolation, library construction and analysis

To generate RNA libraries of sorted macrophage populations, mice were exposed to PBS or IL-4c and two separate pooled biological replicates were generated for PECM PBS, PECM IL-4c, AlvM PBS, IntM PBS and IntM IL-4c groups while three separate pooled replicates were collected for the AlvM IL-4c group. Each pooled biological replicate was generated from cells isolated from 3 – 5 mice. After FACS sorting, each sample was lysed with RLT buffer (Qiagen) and RNA isolated with RNeasy microkits (Qiagen) according to the manufacturers instructions. Sample RNA integrity was confirmed using TapeStation (Agilent), with all samples showing RNA integrity numbers of ~8.8-10. RNA quality was assessed by Fragment Analyzer (Advanced Analytical Technologies), and 20 ng total RNA was used for each library. RNA samples were processed with an Illumina TruSeq RNA

733 Access Library prep kit, following the manufacturers instructions. Libraries were quantified with
734 Qubit HS (ThermoFisher) and Fragment Analyzer (Advanced Analytical Technologies). Indexed
735 libraries were pooled and sequenced on an Illumina NextSeq 500 using paired-end chemistry with
736 75 bp read length.

737

738 For analysis, the raw RNA sequences were quality assessed using FASTQC and no further
739 trimming was performed. The latest mouse transcript set (release 87, "REL87") was obtained by
740 ftp from ensembl (ftp://ftp.ensembl.org/pub/release-87/fasta/mus_musculus/), and annotation
741 acquired using BioMart. Transcripts for both cDNA and ncRNA were used. Alignments (--end-to-
742 end, --very-sensitive -p 30 --no-unal --no-discordant settings) to the REL87 reference set were
743 performed using bowtie2 (version 2.2.7). Alignments were stored in indexed BAM files. Normalized
744 data provided the input for statistical hypothesis testing, in which we sought to identify loci that
745 were statistically significantly different between sample groups. We were also interested in the
746 degree of difference, i.e. the fold-change. In the outputs, the fold-changes (logFC) are given as
747 log2 values, with a positive logFC representing up-regulation, and a negative logFC indicating
748 down-regulation. For each comparison, the first group (A) is the numerator, while the second group
749 (B) is the denominator. Thus, a positive logFC for the comparison 'A-B' indicates up-regulation in A
750 relative to B. Comparisons, manually chosen to explore the data, were undertaken using linear
751 modelling. Subsequently, empirical Bayesian analysis was applied (including vertical (within a
752 given comparison) *P* value adjustment for multiple testing, which controls for false discovery rate).
753 For each comparison, the null hypothesis was that there was no difference between the groups
754 being compared. The Bioconductor package limma was used and an overview of the underlying
755 biological changes occurring within each comparison obtained by functional enrichment analysis
756 from KEGG pathway membership. The significance threshold for functional analysis was manually
757 chosen to be $p < 0.01$

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759 **Quantitative PCR**

760 Post-culture macrophages were lysed in the plate using RLT lysis buffer and RNA was isolated
761 with RNeasy microkits (Qiagen) according to the manufacturers instructions. cDNA was generated
762 from extracted RNA using SuperScript-III and Oligo-dT (ThermoFisher). Relative quantification of
763 genes of interest was performed by qPCR analysis using QuantStudio 12K Flex system and SYBR
764 Green master mix (ThermoFisher), compared with a serially diluted standard of pooled cDNA.
765 Expression was normalized to β -actin (primers as in Table S8).

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767 **Seahorse extracellular flux analysis**

768 FACS isolated PECMs or AlVMs from a pool of 8 mice were plated at 150,000 cells per well and
769 allowed to adhere for at least 1h. ECAR and OCR were measured in XF media (modified DMEM
770 containing 2 mM L-glutamine) under basal conditions, in response to 25 mM glucose, 20 μ M
771 oligomycin, 100 mM 2-DG (ECAR) or 20 μ M Oligomycin, 15 μ M FCCP, 10 μ M Antimycin A, 1 μ M
772 Rotenone (OCR) (Sigma) using a 96-well extracellular flux analyzer XFe-96 (Seahorse
773 Bioscience).

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775 **Statistical analysis**

776 Data are shown as mean values \pm S.E.M. Where applicable, data were analyzed by unpaired *t*
777 test, one-way or two-way ANOVA with Tukey's post-test as appropriate. Significant differences
778 were defined at $P < 0.05$. Statistical analysis was performed using GraphPad PRISM version 7.

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780 **Reporting Summary**

781 Further information on research design and reagents is available in the Life Sciences Reporting
782 Summary linked to this article.

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784 **Code availability statement**

785 Bioinformatics analyses was performed with publicly available code from bioconductor.org.

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787 **Data Availability**

788 The data that support the findings of this study are available from the corresponding author upon
789 request. RNA-seq data were deposited at Gene Expression Omnibus, with the following accession
790 code: GSE126309.

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793 **Method References**

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