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

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Fine control of macrophage activation is needed to prevent inflammatory disease, particularly at barrier sites such as the lungs. However, the dominant mechanisms that regulate pulmonary macrophage activation during inflammation are currently poorly understood. Here we found that alveolar macrophages were substantially less able to respond to the canonical type 2 cytokine IL-4, which underpins allergic disease and parasitic worm infections, than lung tissue or peritoneal cavity macrophages. We found that alveolar macrophage hypo-responsiveness to IL-4 was dictated by the lung environment, but was independent of the host microbiota or the lung extracellular-matrix components surfactant protein D or mucin 5b. Alveolar macrophages displayed severely dysregulated metabolism compared to that of cavity macrophages. After being removed from the lung, alveolar macrophages regained responsiveness to IL-4 in a manner dependent on glycolysis. Thus, impaired glycolysis within the pulmonary niche was a central determinant for the regulation of alveolar macrophage responsiveness during type 2 inflammation.
The specialized mucosal environment of the lung is required to enable breathing in the face of continuous exposure to debris and micro-organisms, while also calling for diverse mechanisms to restrict disease caused by over-exuberant inflammatory responses\(^1\). Lung macrophages have been proposed to be central to mediating and regulating type 2 inflammation against allergens and parasitic worms, which together affect billions of people worldwide\(^2\). Against these types of challenge, macrophages can expand \textit{in situ} to type 2 cytokines such as IL-4 that trigger ‘alternative’ (or M(IL-4)) activation, linked to wound repair and type 2 pathology\(^3, 4, 5\). Although pulmonary macrophage sub-populations inhabit dramatically different anatomical sites, such as the airways and tissue parenchyma, it is not yet clear how location influences their ability to respond to type 2 inflammation. In particular, reports of M(IL-4) marker expression on lung macrophages during type 2 inflammation\(^6, 7, 8, 9\) have involved experimental approaches that may not clearly distinguish macrophages from other myeloid cells, raising the possibility that functional differences in key macrophage sub-populations have been inadvertently overlooked.

As the predominant macrophage sub-population in airways, alveolar macrophages (AlvMs) are vital for maintaining lung health and function, having a central role in clearance of debris, surfactant and apoptotic cells\(^10\). In the absence of AlvMs, fluid build-up leads to primary pulmonary alveolar proteinosis, severe lung dysfunction and respiratory failure\(^11\). The majority of AlvMs are thought to be derived from embryonic precursors that seed the lung tissue before birth\(^12\), with recent evidence suggesting that the cytokines GM-CSF and TGF-\(\beta\) induce PPAR-\(\gamma\), a crucial transcription factor for AlvM development\(^11, 13, 14\). During inflammation, AlvMs mediate bacterial clearance and initiate neutrophil recruitment\(^15\), functions that can be regulated by cytokines such as IL-10 or TGF-\(\beta\), and/or the engagement of cell surface receptors such as SIRP\(\alpha\) or CD200\(^16\). Because clear discrimination between AlvMs and other lung macrophage sub-populations is technically challenging\(^17\), far less is known about the function and origin of tissue residing interstitial macrophages (IntMs). Although IntMs may comprise up to three separate sub-populations\(^18\), earlier work may have mistakenly identified them as AlvMs, monocytes or dendritic cells (DCs).

Mucosal environments like the lung play a major role in determining both development and function of macrophages\(^19\), though many of the factors that shape such processes remain unclear, particularly in type 2 inflammation. Lung macrophage upregulation of M(IL-4) markers during parasite-mediated type 2 responses is promoted by environmental factors such as surfactant protein A (SP-A) and engagement of TAM receptors during clearance of apoptotic cells\(^20, 21\). Here we show that lung macrophage subsets, particularly AlvMs, were considerably less responsive to type 2 inflammation than macrophages from other tissues. We demonstrate that this muted phenotype was conferred by the lung environment, and was independent of potential negative regulators such as CD200-CD200R, surfactant protein D (SP-D), mucin 5b (Muc5b) or the host microbiota. Hypo-responsive AlvMs had an altered metabolic profile compared to IL-4-responsive peritoneal exudate cell macrophages (PECMs), and were unable to upregulate glycolysis \textit{in situ}. 
After removal from the lung, AlvMs recovered their IL-4 responsiveness in a glycolysis-dependent manner. Thus, the pulmonary environment controlled AlvM responsiveness during type 2 inflammation via modulation of their metabolic activity.
Results

AlvMs are unresponsive to IL-4 in vivo

To better understand how pulmonary macrophages respond during type 2 inflammation, we utilized MerTK, CD64, Siglec-F and CD11b as markers that distinguish AlvMs from IntM$^{11,17,18}$. The majority of lung tissue and bronchoalveolar lavage (BAL) macrophages were MerTK$^+$CD64$^+$CD11b$^+$Siglec-F$^+$ AlvMs (>89%), alongside a smaller population (<10%) of MerTK$^+$CD64$^+$CD11b$^+$Siglec-F$^+$ IntMs (Fig. 1a,b and Supplementary Fig. 1a). Analysis of additional macrophage markers showed that, while both AlvMs and IntMs expressed F4/80 and CD11c, AlvMs were also Ym1$^{11}$, a feature of M(IL-4) (Fig. 1c). Further, only IntMs expressed CX3CR1 (Fig. 1c), supporting the idea that IntMs are derived from monocytes, while AlvMs at steady-state are resident cells$^{12,18}$. To verify that AlvMs reside in airways and IntMs in lung tissue, we administered CD45-PE antibodies intranasally (i.n.) and CD45-FITC antibodies intravenously (i.v.) prior to lung processing, to discriminate CD45-PE$^+$ airway macrophages from CD45-FITC$^+$ blood monocytes and tissue CD45-PE-FITC$^-$ tissue macrophages$^{22}$. This approach indicated that AlvMs (defined throughout this study as MerTK$^+$CD64$^+$CD11b$^+$Siglec-F$^+$) were predominantly found in the airways, and IntMs (defined throughout this study as MerTK$^+$CD64$^+$CD11b$^+$Siglec-F$^+$) within the lung tissue (Fig. 1d), demonstrating that refined flow cytometry could discriminate between AlvM and IntM subsets.

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

AlvMs express functional IL-4 receptor

Next we assessed whether AlvMs had reduced expression of IL-4 receptor (IL-4R) compared to IntMs and PECMs$^4$. AlvM IL-4R$\alpha$ expression was similar to that of IntMs and PECMs, and was not significantly affected by i.p. IL-4c (Fig. 2a). In addition, IL-4 was detected in BAL fluid
with similar dynamics as in peritoneal washes (Fig. 2b), indicating that i.p. injected IL-4c could reach the airways. To further address whether AlvM responsiveness to IL-4c depended on route of administration, we administered a range of concentrations of IL-4c i.n. (0.05 μg, 0.5 μg or 5 μg), with the lowest dose typical of that detected in airways during type 2 inflammation. Although IntMs significantly upregulated RELMα in response to i.n. IL-4c compared to PBS (Fig. 2c), AlvMs did not do so, even at the highest IL-4c dose (Fig. 2c). These observations indicated that lack of M(IL-4) activation was a characteristic feature of AlvMs, irrespective of IL-4c delivery route.

To investigate whether the lack of IL-4c responsiveness in AlvMs was due to impaired signalling, we measured expression of phosphorylated STAT6 (p-STAT6), a key transcription factor downstream of IL-4Rα engagement. Both AlvMs after IL-4 i.n., and PECMs after IL-4 i.p., had increased p-STAT6 expression compared to PBS controls which was not evident in Il4ra−/− mice (Fig. 2d). In addition, AlvMs had high basal expression of p-STAT6, Ym1, pAkt T308 (mTORC1) and pAkt S473 (mTORC2) compared to PECMs, which was also evident in Il4ra−/− mice (Fig. 2d and Supplementary Fig. 2). This showed that AlvMs displayed IL-4Rα-independent ‘tonic’ STAT6 and mTORC signalling in the steady state, and could respond to i.n. IL-4 through STAT6 phosphorylation.

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

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

**The pulmonary niche regulates AlvM responsiveness to IL-4**
The lung environment is a unique site that shapes macrophage development\textsuperscript{24}, with environmental signals vital for directing this process\textsuperscript{19}. Further, upon removal from tissues, macrophages in culture display fundamentally altered gene expression\textsuperscript{25, 26}. Consistent with reports that AlvMs from mice and humans can respond to IL-4 \textit{in vitro}\textsuperscript{2}, AlvMs isolated from the lungs of C57BL/6 mice significantly up-regulated expression of \textit{Retnla} (encoding RELM\textsubscript{a}) and Arg-1 after 48h in culture with IL-4, while expression of \textit{Chil3} (encoding Ym1) was elevated after 12h compared to PBS controls (Fig. 4a). We next addressed whether the airway environment limited the ability of AlvMs to undergo M(IL-4) polarization. We transferred CD45.2\textsuperscript{+} PECMs, which responded strongly to IL-4 \textit{in vivo} (Fig. 1), i.n. into naïve CD45.1\textsuperscript{+} mice, followed by administration of IL-4c i.p. Donor CD45.2\textsuperscript{+} PECMs were detected in the lungs of recipient mice at day 5 post-transfer (Fig. 4b). However, PECMs transferred i.n. displayed an activation profile similar to that of resident AlvMs, failing to up-regulate RELM\textsubscript{a} and Ki67 in response to i.p. IL-4c administration compared to recipient PECMs (Fig. 4c). IL-4R\textsubscript{a} expression on transferred CD45.2\textsuperscript{+} PECMs was similar to recipient PECMs (Fig. 4d), suggesting that the impaired response of i.n. PECMs to IL-4 was not due to altered IL-4R\textsubscript{a} expression.

Interaction between the inhibitory receptor CD200R and its ligand CD200 has been described as a dominant negative regulator of AlvM activation in non-type 2 settings\textsuperscript{16}. Although expression of CD200R was highest on AlvMs compared to IntMs (Supplementary Fig. 5a), we observed no significant difference in numbers of AlvMs or IntMs, or their expression of RELM\textsubscript{a} or Ki67, following i.p. IL-4c injection of \textit{Cd200r1}\textsuperscript{-/-} mice (Supplementary Fig. 5b), indicating that AlvM hypo-responsiveness to IL-4 was independent of regulatory CD200-CD200R interactions.

In addition to immune mechanisms, macrophage responses at barrier sites may be modulated by airway components such as surfactant or mucus. SP-A and SP-D are abundant in the lower airways\textsuperscript{27} and have been implicated in promotion of type 2 inflammation and M(IL-4) activation of AlvMs during helminth infection\textsuperscript{21, 28}, while mucus is a major regulator of responses in lung and airway macrophages\textsuperscript{29}. IL-4c increased expression of the dominant pulmonary mucin Muc5b in airway epithelial cells compared to PBS-treated mice (Supplementary Fig. 5c). However, IntMs and AlvMs in \textit{Muc5b}\textsuperscript{-/-} and \textit{Sfptd}\textsuperscript{-/-} mice responded to IL-4c similarly to wild-type mice (Supplementary Fig. 5d,e), indicating that neither Muc5b nor SP-D were dominant factors in limiting the ability of lung AlvMs to undergo M(IL-4) polarization.

The airways host a wide diversity of commensals that could influence macrophage responses and are proposed to be key in regulating pulmonary allergic inflammation\textsuperscript{30}. Further, gut microbe-derived short chain fatty acids are able to systemically regulate type 2 responses in the lung\textsuperscript{31}. To test the involvement of commensals in regulating IL-4 responsiveness of AlvMs, we compared expression of RELM\textsubscript{a} and Ki67 in gnotobiotic (germ free (GF)) mice and conventionally-housed (specific pathogen free (SPF)) mice following i.p. IL-4c administration. RELM\textsubscript{a} and Ki67 expression on AlvMs, IntMs or PECMs was similar in IL-4c-treated GF and SPF mice (Fig. 4e), indicating that neither commensals nor their metabolites were involved in regulation of IL-4
responsiveness in any of these macrophage types. Together, these data indicated that the lung environment controlled AlvM responsiveness to IL-4, but this was independent of the microbiota, Muc5b or SP-D.

AlvMs and PECMs have distinct metabolic gene profiles

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

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

Impaired glycolysis limits AlvM IL-4 responsiveness in vivo

To investigate whether AlvMs had reduced glycolytic ability compared to PECMs we analyzed changes in extracellular acidification rates (ECAR), a measure of glycolytic activity through detection of lactic acid as an end product of glucose metabolism34, in AlvMs and PECMs...
from naïve C57BL/6 mice. AlvMs exhibited significantly impaired glycolysis (reduced ECAR following glucose addition) and glycolytic reserve and capacity (defined as the ability to upregulate aerobic glycolysis) compared to PECMs (Fig. 6a). Analysis of oxygen consumption rates (OCR) showed that AlvMs also displayed reduced respiratory capacity (oxidative phosphorylation (OXPHOS)) compared to PECMs (Supplementary Fig. 7a). Culture of freshly isolated AlvMs or PECMs with the glucose analogue 2-NBDG, to measure uptake potential and glycolytic activity, showed that AlvMs acquired less 2-NBDG than PECMs, even when co-cultured at a 1:1 ratio with PECMs (Fig. 6b). Further, both CD45.2+ PECMs transferred i.n. into CD45.1+ mice and resident CD45.1+ AlvMs had a reduced ability to acquire 2-NBDG in vivo following i.p. IL-4c, when compared to resident CD45.1+ PECMs (Fig. 6c). Together, these observations indicated that the lung environment impaired the ability of AlvMs to both take up and utilize glucose.

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

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

Although numerous studies have reported that pulmonary macrophages upregulate M(IL-4) markers, they either did not unequivocally distinguish between AlvMs and IntMs in their analyses, or relied on IL-4 stimulation of macrophages ex vivo, or used M(IL-4) markers that are already highly expressed by AlvMs at steady-state. Our results suggest that such work may require re-assessment to precisely identify which macrophage populations respond to IL-4 in vivo.

Our data indicate that IntMs will be the major macrophage sub-population to respond in pulmonary type 2 inflammatory settings. This distinction is likely to be important for accurate understanding of the pathogenesis of pulmonary type 2 disease, given that M(IL-4) macrophages have been implicated in wound repair during type 2 inflammation. Thus, we would speculate that IntMs will play a more important role than AlvMs in processes such as resolving tissue damage in the lung, due to their greater ability to respond to IL-4.

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

Metabolism is a key determinant of immune cell function and is central in governing how macrophages respond to a variety of signals, including type 1 and type 2 cytokines. The majority of studies so far have profiled metabolic responses in bone marrow-derived macrophages in vitro, and have not addressed how tissue environments alter macrophage metabolism and function in vivo. From such work, it has been proposed that type 2 cytokines promote amino acid and lipid metabolism (including FAO) feeding into OXPHOS, whereas glycolysis is more associated with type 1 macrophage polarization. We found that AlvMs had a distinctive metabolic state compared to PECMs, with elevated expression of genes associated with PPAR-γ and lipid metabolism, a profile that would be expected to enhance FAO, OXPHOS and M(IL-4) activation. However, defective glycolytic ability rendered AlvMs hypo-responsive to IL-4, consistent with recent observations that glycolysis can mediate macrophage responses to IL-4, and with studies linking altered metabolic state with AlvM ability to respond to Mycobacterium tuberculosis. Our demonstration that the lung environment controls macrophage metabolism
during type 2 inflammation, together with recent evidence that metabolism also regulates DC
control of allergic airway inflammation⁴³, suggests caution in interpreting metabolic data generated
from model macrophages or DCs in culture. Our data also imply that the distinctive metabolic
profile of AlvMs may be directly linked to negative regulation of their activation and function at
steady-state and during inflammation¹.

One factor to consider in how the lung may affect AlvM activation is amounts of metabolic
substrates, including glucose, present in airways. Glucose levels in air surface liquid, which covers
the airway epithelium, are 12.5-fold lower than in blood⁴⁴. Such low glucose concentrations,
maintained through highly-effective epithelial cell glucose transport⁴⁵, appear vital to prevent
bacterial outgrowth in airways⁴⁴, ⁴⁶. Elevated glucose is found in patient sputum during chronic
obstructive pulmonary disease⁴⁷, while glucose levels and glucose metabolism rise in the lung
during asthma⁴⁸, ⁴⁹. Together with our data, this leads to the intriguing hypothesis that glucose
availability and/or utilization could be exploited to therapeutically modify pulmonary disease.

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
between AlvM ability to respond to IL-4 in vitro and in vivo resonates with the reported
transformation of microglial transcriptional identity when removed from the brain²⁵, ²⁶, and cautions
against reliance on AlvMs in vitro for functional studies. This may be particularly relevant for
human AlvMs, given current experimental dependence on their culture ex vivo, and highlights the
need for development of innovative approaches to better assess human AlvM function in vitro.
Similarly, identification of markers for human macrophage subpopulations and their M(IL-4)
activation is urgently needed. The current revolution in single cell sequencing for definition of
cellular networks suggests that this approach applied to human AlvMs should be illuminating. In
both human and murine type 2 inflammation, it will also be important to understand how
monocytes recruited to the lung differentiate and influence airway or tissue macrophages, as
resident AlvMs can be replaced by regulatory monocytes during viral infection⁵⁰. Our data
suggests that the airway environment will play a key role in influencing activation and function of
AlvMs during type 2 inflammation, irrespective of their origin.

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

Competing interests
The authors declare no competing interests.
Figure legends

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

Fig. 2. Alveolar macrophages are less responsive than interstitial macrophages to IL-4c administered directly into the airways. a, IL-4Ra expression by AlvMs, IntMs or PECMs from mice injected with PBS or IL-4c i.p. on d0 and d2, and lung tissue and PEC collected on d4. Histograms representative of 2 independent experiments. b, ELISA of IL-4 levels in BAL or PEC fluids 6h, 12h, 24h or 48h after i.p. injection of PBS or IL-4c. Data representative of 2 independent experiments, n=2 (PBS, 24h and 48h), n=3 (6h and 12h) mice per group. c, Flow cytometry plots of RELMα expression in lung tissue AlvMs and IntMs on d4 following i.n. PBS or IL-4c administration on d0 and d2 (left) and quantification of the percentage of RELMα<sup>+</sup> cells (right). Data representative of 3 independent experiments, n=2 (5μg AlvM), n=3 (AlvM: PBS, 0.05 & 0.5μg, IntM: PBS, 0.05, 0.5 & 5μg) mice per group. d, Histograms of pSTAT6 levels in lung tissue AlvMs or PECMs 15 min after PBS or rIL-4 administered i.n. or i.p. to WT or Il4ra<sup>−/−</sup> mice (left), and quantification of AlvM and PECM pSTAT6 expression (right). Data representative of 2 independent experiments, n=3 mice per group. Data analysed by one-way analysis of variance
(ANOVA) with Tukey’s post-test for multiple comparisons, displayed as mean ± SEM, *P<0.05, ***P<0.001 and ****P<0.0001.

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

Fig. 4. The pulmonary niche regulates alveolar macrophage responsiveness to IL-4 independently of host commensals. a, mRNA expression by qPCR of lung tissue AlvMs from naïve mice following culture for 12h, 24h or 48h in media alone or with rIL-4 (20 ng/ml). A.U. arbitrary units. Data representative of 4 independent experiments, n=2 (media), n=3 (rIL-4) wells per group, each group pooled cells from 8 mice. b - e, Donor (CD45.2+) and host (CD45.1+) macrophage populations identified by flow cytometry in lung tissue or PEC from host mice on d5 after i.n. PBS or donor PECM transfer on d0, then injection with IL-4c i.p. on d1 and d3. b, Flow cytometry plots identifying donor PECMs in host lung tissue. Data representative of 4 independent experiments. c, Quantification of the percentage of RELMα+ and Ki67+ host and donor macrophages isolated from host lung tissue or PEC. Data representative of 4 independent experiments, n=6 mice per group. d, IL-4Rα expression by donor PECMs isolated from host lung tissue. Histogram representative of 2 independent experiments. e, RELMα and Ki67 expression by lung tissue AlvMs and IntMs, or PECMs, from specific pathogen free (SPF) or Germ Free (GF) mice on d4 following i.p. PBS or IL-4c administration on d0 and d2. Data representative of 3 independent experiments, n=3 mice per group. Data analysed by one-way analysis of variance (ANOVA) with Tukey’s post-test for multiple comparisons, displayed as mean ± SEM, **P<0.01, ***P<0.001 and ****P<0.0001.
Fig. 5. Alveolar and peritoneal macrophages display dramatically different metabolic gene profiles. a, mRNA expression profiles (volcano plots) as determined by RNA-seq of PECMs, IntMs or AlvMs isolated from lung tissue or PEC by flow cytometry on d4 following i.p. PBS or IL-4c administration on d0 and d2. Dashed lines represent $P<0.01$, and ± 2-fold change, IL-4c relative to PBS. b, Heatmaps of selected mRNA transcripts of genes that have been previously described as M(IL-4) markers, *indicates significance between IL-4c vs. PBS of at least $p<0.01$. c, mRNA expression profile (volcano plot) of AlvMs vs. PECMs isolated from IL-4c injected mice. Dashed lines represent $P<0.01$, and ± 2-fold change. d, Selected pathways from KEGG analysis (Supplementary Fig. 6) of significantly altered mRNA transcripts from (c), black lines represent $P<0.05$. e, Relative transcript expression by AlvMs vs. PECMs from IL-4c injected mice that were significantly altered ($P<0.01$, log2 normalized intensity), as identified from the glycolysis pathway by network analysis (several genes displayed more than one altered transcript variant), n=2 (PECM PBS, PECM IL-4c, AlvM PBS, IntM PBS, IntM IL-4c), n=3 (AlvM IL-4c) separate biological replicates, each replicate pooled cells from 3-5 mice.

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


5. Jenkins, S.J. et al. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. The Journal of experimental medicine 210, 2477-2491 (2013).


Methods

Experimental animals

cx3cr1^{eGFP/+}, cd200r1^{-/-}, Il4ra^{-/-}, Muc5b^{-/-} and Sfptd^{-/-} were generated as described previously. 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. Recombinant murine IL-4 (BioLegend) was combined with rat IgG1 anti-IL-4 mAb 11B11 (BioXcell) at a 1:5 molecular weight ratio. Mice were injected i.p. with 5 μg of IL-4 (complexed to 11B11) or Dulbecos 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 described. 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 described, 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. Mononuclear cells from the intestine and liver were isolated as previously described. 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 FACS Diva 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*Merk*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.
pSTAT6 and pAkt intracellular staining
To assess pSTAT6 and pAkt activation, 5 μg 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 500 μl 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 10^6 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 activity, 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 x40 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; 420-505 nm) 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-carnoys solution (60% absolute methanol, 30% choroform, 10% acetic acid) and embedded in paraffin. 5 μM sections were subjected to immunohistochemical analysis for Muc5b (custom polyclonal antisera). 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 ImageJ.

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

For analysis, the raw RNA sequences were quality assessed using FASTQC and no further trimming was performed. The latest mouse transcript set (release 87, "REL87") was obtained by ftp from ensembl (ftp://ftp.ensembl.org/pub/release-87/fasta/mus_musculus/), and annotation acquired using BioMart. Transcripts for both cDNA and ncRNA were used. Alignments (--end-to-end, --very-sensitive -p 30 --no-unal --no-discordant settings) to the REL87 reference set were performed using bowtie2 (version 2.2.7). Alignments were stored in indexed BAM files. Normalized data provided the input for statistical hypothesis testing, in which we sought to identify loci that were statistically significantly different between sample groups. We were also interested in the degree of difference, i.e. the fold-change. In the outputs, the fold-changes (logFC) are given as log2 values, with a positive logFC representing up-regulation, and a negative logFC indicating down-regulation. For each comparison, the first group (A) is the numerator, while the second group (B) is the denominator. Thus, a positive logFC for the comparison 'A-B' indicates up-regulation in A relative to B. Comparisons, manually chosen to explore the data, were undertaken using linear modelling. Subsequently, empirical Bayesian analysis was applied (including vertical (within a given comparison) P value adjustment for multiple testing, which controls for false discovery rate).

For each comparison, the null hypothesis was that there was no difference between the groups being compared. The Bioconductor package limma was used and an overview of the underlying biological changes occurring within each comparison obtained by functional enrichment analysis from KEGG pathway membership. The significance threshold for functional analysis was manually chosen to be p<0.01

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

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

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

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

Code availability statement
Bioinformatics analyses was performed with publicly available code from bioconductor.org.

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

Method References


