Macrophages are metabolically heterogeneous within the tumor microenvironment

Graphical abstract

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In brief

Metabolic characteristics of *ex vivo* tumor-associated macrophages (TAMs) are an understudied topic in immunometabolism. Geeraerts et al. unravel the metabolism of TAM subsets co-existing within mouse lung carcinoma. Lactate differentially impacts these TAM subsets by harming the metabolism of anti-tumoral TAMs and promoting pro-tumoral TAMs' suppressive capacity and metabolic activity.

Highlights

- TAM subsets are transcriptionally and metabolically distinct
- TAM subsets differentially use lactate as a carbon source to fuel the TCA cycle
- Lactate differentially affects TAM subset metabolism, derogating MHC-II^{hi} TAMs
- Lactate affects the MHC-II^{lo} TAM transcriptome, stimulating T cell suppression

Article

Macrophages are metabolically heterogeneous within the tumor microenvironment

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SUMMARY

Macrophages are often prominently present in the tumor microenvironment, where distinct macrophage populations can differentially affect tumor progression. Although metabolism influences macrophage function, studies on the metabolic characteristics of *ex vivo* tumor-associated macrophage (TAM) subsets are rather limited. Using transcriptomic and metabolic analyses, we now reveal that pro-inflammatory major histocompatibility complex (MHC)-II^{hi} TAMs display a hampered tricarboxylic acid (TCA) cycle, while reparative MHC-II^{lo} TAMs show higher oxidative and glycolytic metabolism. Although both TAM subsets rapidly exchange lactate in high-lactate conditions, only MHC-II^{lo} TAMs use lactate as an additional carbon source. Accordingly, lactate supports the oxidative metabolism in MHC-II^{lo} TAMs, while it decreases the metabolic activity of MHC-II^{hi} TAMs. Lactate subtly affects the transcriptome of MHC-II^{lo} TAMs, increases L-arginine metabolism, and enhances the T cell suppressive capacity of these TAMs. Overall, our data uncover the metabolic intricacies of distinct TAM subsets and identify lactate as a carbon source and metabolic and functional regulator of TAMs.

INTRODUCTION

Tumors of various histological origins show abundant infiltration of macrophages from the early stages of disease progression. These cells have a profound impact on anti-tumor immunity

and influence fundamental processes that underlie malignancy, including neoangiogenesis, cancer cell proliferation, metastasis, and therapy resistance (Lahmar et al., 2016; Qian and Pollard, 2010; Ruffell and Coussens, 2015). For these reasons, the development of therapeutic approaches to deplete or reprogram

macrophages in cancer is an emerging field of interest (Cassetta and Pollard, 2018; Mantovani et al., 2017).

Interestingly, different tumor-associated macrophage (TAM) subsets residing in different tumor locations and performing specialized functions have been identified (Bolli et al., 2017; Laoui et al., 2014; Movahedi et al., 2010). Yet, there is little information on how this TAM heterogeneity is established and maintained. In this respect, the macrophage metabolic profile may be important, since precisely oriented metabolic changes occur in distinctly activated macrophage subsets, which drive their effector mechanisms and function (Geeraerts et al., 2017; O'Neill et al., 2016). *In vitro* studies showed that lipopolysaccharide (LPS)/interferon (IFN)- γ -stimulated macrophages are characterized by enhanced glycolysis and reduced oxidative phosphorylation (OXPHOS), while IL-4-stimulated macrophages are characterized by an oxidative metabolism (Jha et al., 2015; Van den Bossche et al., 2016). Nevertheless, under *in vivo* situations, such sharply defined phenotypes are often more obscure, with macrophages representing a broad spectrum of activation states (Murray et al., 2014; Xue et al., 2014). Moreover, macrophages are able to move along this continuum, thanks to their high plasticity, in order to adapt to a changing microenvironment (Locati et al., 2019). While crucial to regulate macrophage functions, the metabolic characteristics and needs of macrophages *in vivo* remain largely unexplored (Van den Bossche et al., 2017; Van den Bossche and Saraber, 2018).

This is especially relevant in the metabolically challenging environment of a tumor. Environmental factors such as hypoxia (Casazza et al., 2013; Laoui et al., 2014), restricted nutrient availability (Chang et al., 2015), low pH (Bohn et al., 2018), and the presence of oncometabolites (Bunse et al., 2018; Carmona-Fonseca et al., 2017; Wu et al., 2020), including high concentrations of lactate (up to 40 μ mol/g tumor tissue) (Walenta et al., 2000), are main physiological features of the tumor microenvironment (TME), which could interfere with TAM metabolism and function. As these factors are not evenly distributed within the tumor, TAM subsets may be differentially affected by them. High lactate levels have been correlated with poor prognosis and metastasis (Rizwan et al., 2013; Walenta et al., 2000; Xie et al., 2014), linked to the inhibition of tumor immunosurveillance and the increased immunosuppressive character of tumor-infiltrating immune cells (Angelin et al., 2017; Brand et al., 2016; Brown et al., 2020; Dietl et al., 2010; Fischer et al., 2007; Watson et al., 2021), including macrophages (Colegio et al., 2014; Errea et al., 2016; Mu et al., 2018; Ohashi et al., 2017; Zhao et al., 2015). The G-protein-coupled-receptor 132 (Gpr132) was identified as a sensor for lactate on the macrophage membrane that promotes the alternative activation of TAMs (Chen et al., 2017). However, whether lactate or other environmental cues differentially affect distinct TAM subsets is largely unknown.

In this study, we define the metabolic differences between TAM subsets that co-exist within mouse lung carcinoma tumors and extend the concept of TAM metabolic heterogeneity to human non-small-cell lung carcinoma (NSCLC) tumors. Moreover, we reveal lactate as an additional carbon source that fuels the tricarboxylic acid (TCA) cycle in pro-tumoral TAMs. Further, we provide direct evidence that lactate can differentially affect the

metabolism and functional properties of distinct TAM subsets independent from the Gpr132 sensor.

RESULTS

A reduced carbon flow through the TCA cycle defines the metabolic phenotype of MHC-II^{hi} TAMs

In vitro polarized macrophages harbor metabolic features that are strongly related to their function (Jha et al., 2015; Liu et al., 2017a, 2017b; O'Neill and Artyomov, 2019; Williams and O'Neill, 2018). To assess whether metabolic differences exist between TAM subsets residing within the same tumor, we investigated 3LL-R Lewis lung carcinoma tumors (a mouse model for NSCLC) that are known to be highly infiltrated by two phenotypically diverse TAM subsets, termed MHC-II^{hi} and MHC-II^{lo} TAMs (Laoui et al., 2014; Movahedi et al., 2010) (Figure S1A). These subsets are known to reside in different intratumoral locations, with MHC-II^{lo} TAMs being enriched in hypoxic tumor areas (Laoui et al., 2014). In 3LL-R tumors, both TAM subsets became more abundant over time, reaching almost 40% of the tumor immune infiltrate at the end stage (Figure S1B). MHC-II^{lo} TAMs were always the dominant subset (MHC-II^{lo}/MHC-II^{hi} TAM ratio >1) and became even more prominent in late-stage tumors (Figure S1C). Notably, the greater abundance of MHC-II^{lo} TAMs was not due to a selective proliferation of these cells, as they lacked the proliferation marker Ki67 (Figure S1D) and presented low staining upon *in vivo* BrdU pulse labeling (<0.1% bromodeoxyuridine [BrdU]⁺; Figure S1E).

We next isolated MHC-II^{hi} and MHC-II^{lo} TAMs from 3LL-R tumors and immediately subjected them to RNA sequencing. In total, 1,000 genes were differentially expressed (DE; $\log_2(\text{FC}) > 1$ or < -1 and corrected p value < 0.05) between these TAM populations (Figure 1A). Metascape analysis associated Gene Ontology (GO) functions such as "Regulation of immune response," "T cell selection," "T cell co-stimulation," "Cytokine-cytokine receptor interaction," and "Cytokine secretion" with MHC-II^{hi} TAMs (Figure 1B), whereas MHC-II^{lo} TAMs were associated with "Vasculature development," "Collagen degradation," and "Tissue remodelling" (Figure 1C). This illustrates the more immunostimulatory M1-like and more wound-healing M2-like features of MHC-II^{hi} and MHC-II^{lo} TAMs, respectively. Many of the genes responsible for these distinct GO functions have indeed been associated with a M1/M2 phenotype (Ghassabeh et al., 2006; Jablonski et al., 2015; Mantovani et al., 2004). While MHC-II^{lo} TAMs showed higher expression levels of typical M2 genes (*Cd163*, *Stab1*, *Lyve1*, *Tmem26*, *Folr2*, *Mmp9*, *Clec10a*, *Il4Ra*, and *Itgb3*), MHC-II^{hi} TAMs demonstrated an upregulation of M1-associated genes (*Il12b*, *Ccr4*, *Il1b*, *Trem1*, *F11r*, *Fgr*, and *Cd80*) (Figure S1F). Of note, MHC-II^{hi} TAMs also expressed some M2-associated genes (*Ccl17*, *Cldn1*, *Cdh1*, *Mgl2*, and *Retnla*), which have previously been shown to be upregulated in this TAM subset under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Van Overmeire et al., 2016). Hence, TAMs only partly resemble the *in vitro*-determined M1/M2-associated expression patterns. Focusing on the gene coding for enzymes involved in core metabolic pathways (glycolysis, pentose phosphate pathway, TCA cycle,

Figure 1. TAM subsets differ at the transcriptomic level, including metabolic genes related to glycolysis and TCA cycle

(A) Volcano plot showing DE genes between MHC-II^{hi}/MHC-II^{lo} TAMs. Significantly upregulated and downregulated genes are indicated in red and blue, respectively. Vertical green lines: $\log_2(\text{FC}) > 1$ and < -1 ; horizontal green line: corrected p values < 0.05 ($n = 5$).

(B and C) Metascape bar graph with the top 20 GO enriched terms for genes upregulated in MHC-II^{hi} (B) and MHC-II^{lo} TAMs (C) and are colored by p values ($n = 5$).

(D) Heatmap of expression of metabolic genes that significantly differ comparing MHC-II^{hi} and MHC-II^{lo} TAMs (corrected p value < 0.05). Columns represent biological replicates for MHC-II^{hi}/MHC-II^{lo} TAMs ($n = 5$). Rows represent metabolic genes and are colored by metabolic pathway according to the key and ordered according to $\log_2(\text{FC})$ values. A relative scale is used per gene row, with blue/red indicating replicates with low/high expression (compared with the mean expression), respectively.

(E) Pathway diagram of glycolysis/gluconeogenesis and TCA cycle. Genes that significantly differ between both TAM subsets are colored according to the key ($n = 5$).

See also Table S1. Statistical analysis by likelihood-ratio test. p values corrected for multiple testing by the Benjamini–Hochberg method. Corrected p values < 0.05 are statistically significant.

OXPHOS, fatty acid metabolism), 38 genes were found to be significantly different (corrected p value < 0.05) between TAM subsets (Figure 1D). Since several of these genes were linked

to glycolysis/gluconeogenesis and the TCA cycle (Figure 1E; Table S1), we further zoomed in on these pathways at the metabolite level.

Figure 2. The TCA cycle of MHC-II^{hi} TAMs is hampered at the citrate-to- α KG conversion

(A) Schematic overview of the TCA cycle with relative intracellular abundances of the indicated metabolites in MHC-II^{hi}/MHC-II^{lo} TAMs (orange boxes) and LPS/IFN- γ - and IL-4-stimulated BMDMs (black boxes). Metabolite levels normalized to glutamate and protein content. Significant differences are indicated by a tinted background. n = 3–7 (TAMs); n = 5 (BMDMs).

(B) Ratio of intracellular citrate-to- α KG. n $\geq$ 5.

(C) Ratio of intracellular α KG-to-succinate. n $\geq$ 3.

(legend continued on next page)

We quantified metabolites in isolated MHC-II^{hi} and MHC-II^{lo} TAMs and, for comparison, also in LPS/IFN- γ -(polarized M1) and IL-4-stimulated (polarized M2) bone marrow-derived macrophages (BMDMs). Interestingly, intracellular citrate levels were significantly higher in MHC-II^{hi} TAMs as compared with MHC-II^{lo} TAMs (Figure 2A). Similar changes were observed between LPS/IFN- γ - and IL-4-stimulated BMDMs (Figure 2A). An accumulation of citrate has been reported to result from a break in the TCA cycle at the (iso)citrate-to- α -ketoglutarate (α KG) conversion in LPS/IFN- γ -stimulated macrophages (Jha et al., 2015). A similar TCA cycle breakpoint in MHC-II^{hi} TAMs was suggested by the high intracellular citrate levels (0.65 ± 0.14) and the low α KG pool (0.017 ± 0.012), resulting in a high citrate/ α KG ratio. This ratio was more than 2-fold higher in MHC-II^{hi} TAMs, suggesting a flow constriction through the TCA cycle (Figure 2B). Conversely, the α KG/succinate ratio was significantly higher in MHC-II^{lo} TAMs compared with MHC-II^{hi} TAMs (Figure 2C). A higher α KG/succinate ratio was reported to mediate the M2-like orientation of macrophages (Liu et al., 2017a, 2017b), which is consistent with the more M2-like activation state of MHC-II^{lo} TAMs.

To further verify the diminished glucose oxidation in MHC-II^{hi} TAMs, we followed the incorporation of ¹³C₆-D-glucose-derived carbon into the TCA cycle upon 24 h labeling (Figure S2). Considering the low α KG pool in MHC-II^{hi} TAMs, we used the labeling pattern of glutamate (which is interchangeable with α KG) as a surrogate for α KG. In MHC-II^{lo} TAMs, citrate and glutamate contained $29.51\% \pm 2.90\%$ and $14.41\% \pm 4.16\%$ glucose-derived carbon, respectively (2-fold decrease of glutamate labeling versus citrate labeling) (Figure 2D), while only $24.43\% \pm 0.88\%$ labeling of citrate and $7.97\% \pm 1.09\%$ labeling of glutamate (3-fold decrease) was detected in MHC-II^{hi} TAMs, indicating that a smaller fraction of glucose-derived citrate was oxidized to α KG (and, accordingly, glutamate) in MHC-II^{hi} TAMs. This experiment also highlighted a larger fraction of glucose-derived M+3-labeled TCA cycle intermediates in MHC-II^{lo} TAMs (Figure S2). The presence of M+3-labeled TCA cycle intermediates suggests pyruvate anaplerosis in MHC-II^{lo} TAMs, leading to the direct conversion of pyruvate into oxaloacetate via pyruvate carboxylase (Buescher et al., 2015). In line with this, MHC-II^{lo} TAMs showed a significantly enhanced gene expression of *Pcx* (Figure 2E).

Additionally, we performed ¹³C₅-L-glutamine tracing experiments, which clearly indicated the incorporation of glutamine-

derived carbon into glutamate, metabolites of the TCA cycle, and their derivatives aspartate and itaconate (Figure S2). For these metabolites, the total carbon fraction derived from labeled glutamine did not differ between TAM subsets, suggesting that the total glutamine contribution is similar (Figure 2F). Nevertheless, we observed small but significant differences in the partial labeling patterns of TCA cycle intermediates when comparing both TAM subsets (Figure S2). The M+3 fraction in glutamate was slightly smaller in MHC-II^{hi} TAMs, while the M+4 fraction in citrate was larger in these cells. This resulted in a 5.6-fold decrease of M+4-labeled citrate to M+3-labeled glutamate in MHC-II^{hi} TAMs (Figure 2G), while only a 3.6-fold decrease was observed in MHC-II^{lo} TAMs. This enhanced drop in the partial labeling in MHC-II^{hi} TAMs demonstrates again that the conversion of citrate into α KG/glutamate is less fluent in these cells, providing further proof for a reduced oxidation in the TCA cycle of MHC-II^{hi} TAMs. Of note, MHC-II^{hi} TAMs showed a significantly reduced *Idh2* gene expression, which converts (iso)citrate into α KG in the mitochondria (Figures 1D, 1E, and 2H), while the cytosolic *Idh1* was comparably expressed in both TAM subsets (Figures 1E and 2H). Transcriptomics data were confirmed by western blotting, which showed diminished IDH2 levels in MHC-II^{hi} TAMs and no differences for IDH1 (Figure 2I). This resulted in an overall reduced IDH enzymatic activity in MHC-II^{hi} TAMs (Figure 2J), explaining the lower conversion of citrate in these cells.

The accumulation of succinate and itaconate is another feature of inflammatory macrophages (Cordes et al., 2016; Jha et al., 2015; Lampropoulou et al., 2016; Michelucci et al., 2013; Tannahill et al., 2013). While we could recapitulate these findings in LPS/IFN- γ BMDMs, MHC-II^{hi} TAMs did not show this second TCA cycle break, as demonstrated by similar succinate and itaconate levels (Figure 2A), a similar succinate/fumarate ratio (Figure 2K), and comparable glutamine labeling (Figure 2F) in both TAM subsets.

Overall, our data demonstrated that macrophage populations derived from the same tumor are metabolically distinct and that the TCA cycle in MHC-II^{hi} TAMs, but not MHC-II^{lo} TAMs, is hampered at the citrate-to- α KG conversion.

The higher metabolic activity of MHC-II^{lo} TAMs is strongly driven by an oxidative metabolism

To assess the energy metabolism of MHC-II^{hi} and MHC-II^{lo} TAMs in more detail, we performed quantitative measurements

(D) Total fraction of labeled citrate and glutamate upon 24 h incubation with ¹³C₆-D-glucose. The ratio between the fraction of labeled citrate to labeled glutamate is indicated as fold decrease (FD). n = 5.

(E) Gene expression of *Pcx*. Datapoints represent fragments per kilobase of transcript per million mapped reads (FPKM) values. n = 5.

(F) Total fraction of the indicated metabolites labeled by ¹³C₅-L-glutamine upon 24 h incubation. n = 3.

(G) Fraction of M+4-labeled citrate and M+3-labeled glutamate upon 24 h incubation with ¹³C₅-L-glutamine. The ratio between the fraction of M+4-labeled citrate to M+3-labeled glutamate is indicated as FD. n = 3.

(H) Gene expression of *Idh2* and *Idh1*. Datapoints represent FPKM values. n = 5.

(I–K) Western blots and quantification of IDH2 and IDH1 protein, normalized to loading control vinculin. n = 3.

(J) IDH1 and IDH2 combined activity. n = 4.

(K) Ratio of intracellular succinate-to-fumarate. n $\geq$ 4.

See also Figure S2. Bar graphs with error bar represent mean $\pm$ SEM; dots represent individual values. Statistical analyses by: unpaired two-tailed t test (with Welch's correction being performed when F-test indicated significantly different variances) (A); unpaired two-tailed t test on log-transformed data (B, C, and K); likelihood-ratio test with p values corrected for multiple testing by the Benjamini–Hochberg method (E and H); unpaired two-tailed t test with p values corrected for multiple testing using the Bonferroni–Dunn method (F); and unpaired two-tailed t test (I and J). For statistically significant differences, the (corrected) p value is indicated as follows: *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, ****p $\leq$ 0.0001.

Figure 3. Oxidative metabolism as a characteristic of metabolically highly active MHC-II^{lo} TAMs

(A and B) OCRs (A) and ECARs (B) of MHC-II^{lo} and MHC-II^{hi} TAMs monitored under basal conditions and upon sequential injection of oligomycin (OM), FCCP, and rotenone (ROT) plus anti-mycin A (AA) plus 2-deoxyglucose (2-DG). $n \geq 4$ (replicates of one representative experiment).

(C and D) Changes in OCRs and ECARs upon injection of OM, FCCP, and ROT/AA/2-DG allowed to calculate the indicated respiratory (C) and glycolytic (D) parameters. $n \geq 4$ (replicates of one representative experiment).

(E) Mitotracker Green labeling was used to quantify mitochondrial mass. A representative histogram and corresponding quantification of surface expression ($\Delta\text{MFI} = [\text{median fluorescence intensity}]_{\text{positive staining}} - [\text{median fluorescence intensity}]_{\text{unstained}}$) are shown. $n = 4$.

(F) Percentages of mitochondria with different morphologies in MHC-II^{hi}/MHC-II^{lo} TAMs. 178 and 193 mitochondria were blindly scored in MHC-II^{hi} and MHC-II^{lo} TAMs, respectively. Stacked bar plot is representative for two independent experiments. Representative transmission electron micrographs are shown of MHC-II^{hi} and MHC-II^{lo} TAMs with mitochondrial morphological diversity varying from normal mitochondria (regular shaped, numerous transversely oriented cristae, intact outer membrane) and minor defective mitochondria (enlarged intermembrane space in cristae, fewer/disarrayed/fading cristae) to severe defective mitochondria (total absence of cristae, mitochondrial remnants in autolysosomes, mitochondria in autophagy surrounded by isolation membrane [mitophagy]). Arrows point to mitochondria that belong to these classes. Scale bars: 500 nm. Mitochondrial morphology of 371 mitochondria from 30 randomly selected cells of each subtype was scored blindly.

(G) Schematic overview of the flux from glucose and lactate upon 12 h incubation with $^{13}\text{C}_6$ -D-glucose. Bar plots represent the net glucose uptake rate and the net lactate secretion rate. Pie charts represent incorporation of ^{13}C -label into lactate (^{12}C : grey; ^{13}C : orange). Arrows represent metabolic pathway connections known from literature. $n = 4$.

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of their oxygen consumption rate (OCR) (Figure 3A) and their extracellular acidification rate (ECAR) (Figure 3B) immediately following isolation, again using LPS/IFN- γ and IL-4 BMDMs as reference populations. Basal OCRs and ECARs were used as measurements for basal respiration and basal glycolysis (Figure S3). Interestingly, MHC-II^{lo} TAMs combined high basal OCR (Figure 3C) and high basal ECAR levels (Figure 3D), suggesting that these cells were metabolically highly active, in contrast to the lower overall metabolic activity (low basal OCR and ECAR levels) of MHC-II^{hi} TAMs. Nevertheless, a notable amount of mitochondrial respiration remained intact in MHC-II^{hi} TAMs. These findings distinguish TAMs from polarized BMDMs, where IL-4 BMDMs also show a higher basal OCR but a lower basal ECAR as compared with LPS/IFN- γ BMDMs (Figures S4A–S4D).

Both maximal respiration and spare respiratory capacity (SRC; calculated as detailed in Figure S3) were significantly higher in MHC-II^{lo} compared with MHC-II^{hi} TAMs (Figure 3C), as well as in IL-4- compared to LPS/IFN- γ -activated BMDMs (Figure S4C). Since SRC has been linked to mitochondrial mass (van der Windt et al., 2012), we performed MitoTracker Green staining (Figure 3E). The mitochondrial mass in MHC-II^{lo} TAMs was significantly higher, likely explaining their stronger oxidative metabolism. Moreover, electron microscopy showed significant differences in the mitochondrial morphology of sorted TAM subsets (Figure 3F). MHC-II^{hi} TAMs contained the highest percentage of mitochondria with severe defects, and MHC-II^{lo} TAMs contained more normal mitochondria. The larger fraction of high-quality mitochondria in MHC-II^{lo} TAMs is in line with their enhanced respiratory capacity. Further, a higher glycolytic reserve and maximal glycolytic capacity (calculated as detailed in Figure S3) was observed in MHC-II^{lo} TAMs (Figure 3D). Conversely, glycolytic capacity and glycolytic reserve were more prominent in LPS/IFN- γ BMDMs in comparison with IL-4 BMDMs, supporting the strong reliance of LPS/IFN- γ BMDMs on glycolysis (Figure S4D). Taken together, these results indicate a higher overall metabolic activity of MHC-II^{lo} TAMs as compared with MHC-II^{hi} TAMs.

As MHC-II^{lo} TAMs demonstrated high basal ECAR levels (Figure 3D), we examined whether this could be indicative of their reliance on aerobic glycolysis, which is characterized by the conversion of glucose into lactate. Therefore, we assessed the net glucose uptake rate and the net lactate secretion rate in both TAM subsets incubated over a 12 h time period in RPMI with ¹³C₆-D-glucose. Both TAM subsets consumed glucose, with MHC-II^{lo} TAMs showing a slightly higher glucose uptake rate (Figure 3G). Moreover, secretion of lactate was observed in both TAM subsets (rate < 0) and was slightly higher in MHC-II^{lo} TAMs, as demonstrated by an enhanced net lactate secretion rate. Interestingly, the high fraction of ¹³C-labeling of lactate in both TAM subsets indicates that most secreted lactate is glucose-derived (pie charts, Figure 3G). Next, we assessed

which fraction of glucose is fueled into lactate. Knowing that 1 mol of glucose results in the production of 2 moles of lactate, an absolute ratio of 2 calculated as the net lactate secretion rate (from glucose) over the net glucose uptake rate indicates that all glucose is converted into lactate. Notably, for this calculation, the net lactate secretion rate (from glucose) was determined based on ¹³C₃-lactate concentrations in the media, excluding any other nutrient sources than ¹³C₆-D-glucose from the calculation. Since the ratio of both TAM subsets inclines to 2 (Figure 3H), both fuel the biggest fraction of glucose towards lactate, while a minor fraction is directed elsewhere, for example, into glycolytic side pathways or the TCA cycle. Taken together, these data confirm the importance of glucose-fueled glycolysis in both TAM subsets.

MHC-II^{lo} TAMs adapt to the TME by using lactate as a carbon source to fuel the TCA cycle

We next questioned whether other carbon sources, besides glucose and glutamine, could be used by MHC-II^{lo} and MHC-II^{hi} TAMs. In this respect, lactate is highly produced by 3LL-R cells under aerobic culture conditions, reaching a concentration of 26 mM in the culture medium at confluency (Figure 4A). Consistently, the lactate concentration in the interstitial fluid of 1,046 $\pm$ 55mm³-sized 3LL-R tumors ranged between 27 and 43 mM (Figure 4B). To assess whether lactate could serve as an alternative carbon source for TAMs, these cells were incubated for 12 h in RPMI with ¹³C₆-D-glucose but were now supplemented with 20 mM sodium L-lactate (Figure 4C). While the net glucose uptake rate remained unaltered in the presence of extracellular lactate in MHC-II^{hi} TAMs (Figure 4D), it significantly dropped in MHC-II^{lo} TAMs (Figure 4E). Strikingly, MHC-II^{lo} TAMs no longer secreted lactate but showed a net uptake of this metabolite (rate > 0) in the presence of extracellular lactate (Figures 4C and 4G). This shows that MHC-II^{lo} TAMs use both glucose and lactate as carbon sources. To assess which carbon source is the preferred one, we calculated the carbon flux from glucose (6 carbons) and lactate (3 carbons) in MHC-II^{lo} TAMs. The absolute carbon flux from glucose exceeds the one from lactate by 23% (Figure 4H), indicating that glucose remains the preferential carbon source. The fact that MHC-II^{lo} TAMs consume both glucose and lactate in the presence of extracellular lactate implies an enhanced carbon uptake. Interestingly, carbon uptake via glutamine was significantly decreased in MHC-II^{lo} TAMs in the presence of lactate (Figure 4I). In contrast, MHC-II^{hi} TAMs still show a net secretion of lactate (rate < 0) in the presence of extracellular lactate (Figures 4C and 4F). Taken together, these data show that the presence of extracellular lactate differentially affects the metabolism of TAM subsets, as it diminishes aerobic glycolysis in MHC-II^{hi} TAMs and turns MHC-II^{lo} TAMs from a lactate-secreting into a lactate-consuming cell type.

As an alternative approach, TAM subsets were incubated in the presence of 20 mM ¹³C₃-sodium-L-lactate. Notably, a

(H) Ratio of net lactate secretion rate from glucose over net glucose uptake rate. $n = 4$.

See also Figures S3 and S4. (A–D) Line and bar graphs with error bar represent mean $\pm$ SD. (E and G–H) Bar graphs with error bar represent mean $\pm$ SEM; dots represent individual values. Statistical analyses by: unpaired two-tailed t test with p values corrected for multiple testing using the Bonferroni-Dunn method (C and D), unpaired two-tailed t test (E and G), chi-squared test (F), and unpaired two-tailed t test on log-transformed data (H). For statistically significant differences, the (corrected) p value is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 4. MHC-II^{lo} TAMs can use lactate as a carbon source to fuel the TCA cycle

(A) Lactate concentration in tumor-conditioned medium of the 3LL-R cell line collected upon confluency. $n = 3$.
 (B) Concentration of intratumoral lactate in interstitial fluid (IF) of 3LL-R tumors. $n = 4$.
 (C) Schematic overview of the flux from glucose and lactate in MHC-II^{hi} and MHC-II^{lo} TAMs upon 12 h incubation with $^{13}\text{C}_6$ -D-glucose + 20 mM sodium L-lactate. Bar plots represent the net glucose uptake rate and net lactate uptake/secretion rate in both TAM subsets. Pie charts represent incorporation of ^{13}C -label into lactate (^{12}C : grey; ^{13}C : orange). Arrows represent metabolic pathway connections known from literature. $n \geq 3$.
 (D and E) Net glucose uptake rate in MHC-II^{hi} (D) and MHC-II^{lo} (E) TAMs upon 12 h incubation in the absence (–LAC) and the presence (+LAC) of 20 mM sodium L-lactate. $n = 4$.
 (F and G) Net lactate uptake/secretion rate in MHC-II^{hi} (F) and MHC-II^{lo} (G) TAMs upon 12 h incubation in the absence/presence of 20 mM sodium L-lactate. $n \geq 3$.
 (H) Carbon flux from glucose and lactate in MHC-II^{lo} TAMs. $n \geq 3$.
 (I) Net glutamine uptake rate in MHC-II^{lo} TAMs upon 12 h incubation in the absence/presence of 20 mM sodium L-lactate. $n \geq 3$.
 (J) Total fraction of the indicated metabolites labeled by $^{13}\text{C}_3$ -sodium-L-lactate upon 24 h incubation of MHC-II^{hi} and MHC-II^{lo} TAMs. $n \geq 5$.
 See also [Figures S5](#) and [S6](#). Bar graphs with error bar represent mean $\pm$ SEM; dots represent individual values. Statistical analyses by: unpaired two-tailed t test (C–I) and unpaired two-tailed t test with p values corrected for multiple testing using the Bonferroni-Dunn method (J). For statistically significant differences, the (corrected) p value is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

significant exchange of lactate between intra- and extracellular pools can highly influence intracellular labeling patterns (Quek et al., 2016). This likely explains why lactate-derived carbon labeling was observed in intracellular lactate, pyruvate, and TCA cycle intermediates of both TAM subsets, despite the fact that only MHC-II^{lo} TAMs display a net lactate uptake (Figure S5). Nevertheless, the total fractional labeling (i.e., the percentage of a certain metabolite that is—partially or fully— ^{13}C -labeled) of glutamate, succinate, malate, and aspartate was significantly higher in MHC-II^{lo} TAMs, while

the lactate-derived labeling of citrate remained similar in both TAM subsets (Figure 4J). Considering our evidence for reduced glucose oxidation in MHC-II^{hi} TAMs, we attribute the lower ^{13}C -incorporation in glutamate, succinate, malate, and aspartate in MHC-II^{hi} TAMs to the hampered citrate-to- α KG conversion rather than to an inherent difference in the capacity of TAM subsets to use lactate for the generation of TCA cycle intermediates. Together, these results indicate that 3LL-R tumors strongly rely on Warburg metabolism, leading to high intratumoral lactate levels, which could serve as a carbon

Figure 5. Long-term treatment with lactate impairs the metabolic characteristics of MHC-II^{hi} TAMs, while short-term lactate injection promotes oxidative metabolism of TAMs

(A and B) Percentual change in OCRs (A) and ECARs (B) upon injection of 20 mM sodium L-lactate. $n \geq 4$ (replicates of one representative experiment).

(C and E) OCRs (C) and ECARs (E) upon long exposure to lactate or full absence of lactate. OCRs and ECARs were monitored under basal conditions and upon sequential injection of OM, FCCP, and ROT/AA/2-DG. $n \geq 4$ (replicates of one representative experiment).

(D and F) Changes in OCRs and ECARs upon injection of OM, FCCP, and ROT/AA/2-DG allowed to calculate the indicated respiratory (D) and glycolytic (F) parameters in both TAM subsets upon long exposure to lactate or full absence of lactate. $n \geq 4$ (replicates of one representative experiment). Line and bar graphs with error bars represent mean $\pm$ SD; dots represent individual values. Significance was evaluated by: two-way ANOVA and Bonferroni's multiple comparisons test (A and B) or two-way ANOVA and Bonferroni's multiple comparisons test within each parameter (D and F). For statistically significant differences, the (corrected) p value is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

source for MHC-II^{lo} TAMs and lead to the excessive exchange of lactate in both TAM subsets.

Gpr132 was reported as a lactate sensor on TAMs (Chen et al., 2017). To assess its potential role, 3LL-R tumors were grown in Gpr132-knockout (KO) mice and wild-type (WT) littermate controls, and $^{13}\text{C}_3$ -sodium-L-lactate incorporation in TAMs was analyzed. Gpr132-KO TAM subsets incorporated $^{13}\text{C}_3$ -sodium-L-lactate-derived carbon in all TCA cycle intermediates (Figure S6C), similar to the observation in WT TAMs. Again, the total fractional labeling of glutamate, succinate, and aspartate, but also citrate, was higher in MHC-II^{lo} TAMs. Moreover, no differences in 3LL-R tumor weight were observed between Gpr132-KO and control mice (Figure S6A), nor were there major shifts in the myeloid or lymphoid immune composition, including MHC-II^{lo} and MHC-II^{hi} TAMs, except for small changes in intra-tumoral CD4⁺ T cells (Figure S6B). Hence, Gpr132 does not impact the lactate-derived carbon flow in TAM subsets, and its absence does not affect tumor growth nor the composition of the immune infiltrate.

Lactate impairs the metabolic features of MHC-II^{hi} TAMs

We next assessed whether lactate is able to fuel respiration in TAM subsets. In the first instance, we used an extracellular flux analysis to measure the change in ECAR and OCR levels of freshly sorted MHC-II^{hi} and MHC-II^{lo} TAMs upon acute exposure to 20 mM sodium L-lactate. Interestingly, the addition of lactate immediately increased OCR and simultaneously decreased ECAR in both TAM subsets (Figures 5A and 5B), suggesting that lactate is able to fuel OXPHOS. To assess the effect of a more continuous lactate presence, sorted TAMs were exposed to an extracellular flux analysis, with all procedures performed

either in the continuous absence or presence of 20 mM sodium L-lactate (Figures 5C and 5E). The incubation of MHC-II^{lo} TAMs with lactate slightly enhanced their basal OCR and lowered their maximal respiration and SRC (Figure 5D) while enhancing glycolytic capacity and the glycolytic reserve (Figure 5F). Lactate had a more pronounced impact on MHC-II^{hi} TAMs, with a further reduction of OXPHOS, ECAR, and all respiratory/glycolytic parameters (Figures 5C–5F).

Overall, these data suggest that lactate acutely increases respiration in both MHC-II^{lo} and MHC-II^{hi} TAMs, but in the longer term, lactate reduces the metabolic activity of MHC-II^{hi} TAMs, suggesting that they do not tolerate lactate as well as MHC-II^{lo} TAMs.

Lactate affects the transcriptome of TAM subsets with implications for their T cell suppressive capacity

Next, we investigated to what extent the TAM subset phenotype is altered by lactate. First, sorted MHC-II^{hi} and MHC-II^{lo} TAMs were incubated for 24 h in absence or presence of 20 mM sodium L-lactate, and gene expression was analyzed via RNA sequencing. Notably, 1,260 genes were DE ($\log_2(\text{FC}) > 1$ or < -1 and corrected p value < 0.05) between both TAM subsets upon 24 h culture (Figure 6A), whereas only 54 and 248 genes were altered by lactate in MHC-II^{hi} and MHC-II^{lo} TAMs, respectively (Figure 6B) (Table S4). Of these, only 15 genes were commonly regulated by lactate in both TAM subsets (Figure 6B) (Table S4), suggesting that lactate exerts rather subtle and distinct effects in the different subsets. Genes that have previously been associated with MHC-II^{hi} and MHC-II^{lo} TAMs (Movahedi et al., 2010; Van Overmeire et al., 2016) were not noticeably changed upon 24 h *in vitro* culture of TAM subsets in the presence of lactate (Figure 6C). At the

Figure 6. Lactate affects TAM subsets at transcriptomic and functional levels

(A and B) Volcano plot showing DE genes between MHC-II^{hi} versus MHC-II^{lo} TAMs upon 24 h incubation (A) and MHC-II^{hi} and MHC-II^{lo} TAMs incubated for 24 h in the presence versus the absence of 20 mM sodium L-lactate (B). Significantly upregulated and downregulated genes are indicated in red and blue, respectively.

(legend continued on next page)

metabolic level, the majority of genes encoding glycolytic enzymes was significantly more highly expressed in MHC-II^{hi} TAMs compared to MHC-II^{lo} TAMs, while several TCA-cycle-associated genes (including *Idh1* and *Idh2*) were lower in the former upon 24 h incubation (Table S5). Also these differences were maintained in the presence of lactate (Table S5). In MHC-II^{lo} TAMs, lactate upregulates GO biological processes such as “Cytokine-cytokine receptor interaction,” “Cytokine production,” “Reactive oxygen species metabolic process,” and “Positive regulation of cell death” (Figure 6D), while it downregulates processes such as “Leukocyte differentiation,” “Inflammatory process,” and “Cell-cell adhesion” (Figure 6E). Due to the relatively low number of DE genes in lactate-treated MHC-II^{hi} TAMs, the identification of lactate-regulated GO biological processes is less reliable in these cells.

Arg1 expression was significantly enhanced in freshly sorted MHC-II^{lo} TAMs (Figure 6F), and also *Nos2* expression tended to be higher in this subset (Figure 6G). *Arg1* was previously highlighted as a prototypical lactate-regulated gene in macrophages (Colegio et al., 2014). We now confirm that lactate upregulates *Arg1* expression in MHC-II^{lo} TAMs (fold increase 2.33 ± 1.01) but not MHC-II^{hi} TAMs (fold increase 1.06 ± 0.41), although both TAM subsets demonstrated an increased arginase enzymatic activity in the presence of lactate (Figure 6H). To get a broader view on the L-arginine metabolism, we also assessed *Nos2* gene expression, which was more extensively upregulated in MHC-II^{lo} TAMs (fold increase 2.87 ± 1.75) compared with MHC-II^{hi} TAMs (fold increase 1.65 ± 0.74). Moreover, nitric oxide (NO) secretion was significantly enhanced by lactate in MHC-II^{lo} TAMs and tended to be higher in MHC-II^{hi} TAMs (Figure 6I). Since L-arginine metabolism is known to affect the T cell-suppressive capacity of TAMs (Liu et al., 2003; Movahedi et al., 2010), these cells' impact on polyclonal T cell-stimulation was tested. Interestingly, MHC-II^{lo} TAMs displayed an enhanced T cell-suppressive capacity in the presence of lactate, which was abolished by the *Nos2*-inhibitor L-NMMA, and also tended to be lower in the presence of the arginase inhibitor NorNOHA (Figure 6J). However, lactate did not significantly enhance the suppressive capacity of MHC-II^{hi} TAMs (Figure 6J). Hence, lactate influences the MHC-II^{lo} TAM phenotype and may stimulate its tumor-promoting activities.

Metabolic heterogeneity of TAMs in mouse and human NSCLC tumors is observed on the single-cell level

To assess whether further phenotypic and metabolic heterogeneity can be observed beyond the MHC-II^{hi} and MHC-II^{lo} TAM subsets from 3LL-R tumors, we employed mass-cytometry-based single-cell metabolic profiling (scMEP) (Hartmann et al., 2020). Using FlowSOM-based clustering of pooled samples from 16 independent 3LL-R tumors, we grouped the mononuclear phagocytes (MPs; defined as CD45⁺CD90⁺B220⁺Ter119⁺CD11b⁺Ly6G⁺SiglecF⁺Ly6C^{lo-hi}CD64^{lo-hi}F480^{lo-hi}CD11c^{lo-hi}MHC-II^{lo-hi}, as detailed in Figures S7A and S7B) and defined 10 distinct subclusters (Figures 7A–7C), including 1 monocyte cluster (cluster 1; Ly6C⁺MHC-II⁺), 1 immature TAM cluster (cluster 9; Ly6C⁺MHC-II⁺), and 1 tumor-associated dendritic cell (TADC) cluster (cluster 8; Ly6C⁺F4/80⁺CD64⁺CD206⁺SRA⁺CD86⁺PD-L1⁺MHC-II⁺CD11c⁺). Mature TAMs (clusters 2–7 and 10) were defined based on the absence of Ly6C expression and an enhanced macrophage marker expression (CD64, F4/80, CD206, SRA) (Gautier et al., 2012; Zilionis et al., 2019) (Figures 7B and 7C). Interestingly, a high MHC-II expression level separates the closely associated clusters 3 and 5 (i.e., MHC-II^{hi} TAM) from the MHC-II^{lo} clusters 2, 4, 6, 7, and 10 (Figures 7B and 7C). Hence, although both TAM subsets can be further split up in additional phenotypic/metabolic clusters that differentially express metabolic markers (Figure 7D), MHC-II remains a valid marker to roughly distinguish two major metabolic TAM subsets in mouse 3LL-R tumors. Cluster 4 dominates the MHC-II^{lo} TAM pool and expresses the highest levels of the M2 markers CD206 and SRA and of IDH2, while CD206^{lo}SRA^{lo}IDH2^{lo} cluster 3 is dominant within the MHC-II^{hi} TAM pool, largely explaining the overall higher M2 profile and the higher IDH2 expression within the pool of MHC-II^{lo} TAM clusters (Figure 7E).

Finally, we relied on scMEP to assess the metabolic heterogeneity of TAMs from NSCLC patients. FlowSOM-based clustering of pooled samples from 5 independent subjects grouped the MPs (defined as CD45⁺CD3⁺CD57⁺CD19⁺CK_Bcat⁺CD14⁺CD64⁺CD11c⁺HLA-DR^{lo-hi}, as detailed in Figures S7A and S7B) into 10 distinct subclusters (Figure 7A), 4 of which represented CD68-expressing macrophage clusters (subclusters 3, 5, 8, and 10) (Figures 7B and 7C). Interestingly, subsets 8 and

Vertical green lines: $\log_2(\text{FC}) > 1$ and < -1 ; horizontal green line: corrected p values < 0.05 . Names of genes that are commonly DE in both TAM subsets upon incubation for 24 h in the presence of 20 mM sodium L-lactate are indicated. $n = 5$.

(C) Heatmap of M1/M2-associated gene expression by MHC-II^{hi} and MHC-II^{lo} TAMs upon incubation for 24 h in the presence (+LAC) or the absence (–LAC) of 20 mM sodium L-lactate. Columns represent biological replicates ($n = 5$). A relative scale is used per gene row, with blue/red indicating replicates with low/high expression (compared to the mean expression), respectively.

(D and E) Metascape bar graph with the top 20 GO enriched terms for genes upregulated (D) and downregulated (E) in MHC-II^{lo} TAMs upon 24 h incubation in the presence of 20 mM sodium L-lactate, colored by p values. $n = 5$.

(F and G) Expression of *Arg1* (F) and *Nos2* (G) genes in freshly sorted MHC-II^{lo} and MHC-II^{hi} TAMs. The expression levels were normalized based on the S12 housekeeping gene. $n \geq 3$.

(H) Arginase enzymatic activity was measured in lysates of 3×10^5 MHC-II^{hi} and MHC-II^{lo} TAMs upon 48 h *in vitro* incubation in the absence/presence of 20 mM sodium L-lactate. $n = 3$.

(I) NO secretion by 2×10^5 MHC-II^{hi} and MHC-II^{lo} TAMs upon 24 h *in vitro* incubation in the absence/presence of 20 mM sodium L-lactate. $n = 8$.

(J) The relative percentage of suppression of anti-CD3/anti-CD28 induced T cell proliferation by MHC-II^{hi} and MHC-II^{lo} TAMs (1:2 ratio) upon co-culture with/without 20 mM sodium L-lactate (LAC), the iNOS inhibitor L-NMMA, and the arginase inhibitor NorNOHA. $n \geq 7$.

See also Tables S4 and S5. Bar graphs with error bars represent mean $\pm$ SEM; dots represent individual values. Statistical analyses by: likelihood-ratio test with p values corrected for multiple testing by the Benjamini–Hochberg method (A–E), unpaired two-tailed t test (F and G), paired two-tailed t test (H and I), and mixed effects analysis with pairwise comparison and Bonferroni's multiple comparisons test on log-transformed data (J). For statistically significant differences, the (corrected) p value is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 7. scMEP unravels the metabolic heterogeneity of TAMs in mouse and human lung tumors

(A) Uniform manifold approximation and projection (UMAP) dimensionality reduction plot showing 10 subclusters defined within the mononuclear phagocytes (MPs) population in mouse ($n = 16$) and human ($n = 5$) lung tumors. The dashed pink line indicates all TAM clusters.

(B) UMAP visualization of subclusters shown in (A) representing the normalized expression value of Ly6C, F4/80, CD64, MHC-II, CD11c, CD206, SRA, CD86, and PDL1 (mouse) and CD68, CD163, and HLA-DR (human).

(C) Heatmap showing the enrichment (purple) or depletion (yellow) of phenotypic surface markers across subclusters shown in (A)

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10 expressed relatively higher levels of HLA-DR and lower levels of CD163 (reminiscent of mouse MHC-II^{hi} TAMs), while the opposite is true for subsets 3 and 5 (reminiscent of mouse MHC-II^{lo} TAMs) (Figures 7B and 7C). Similar to mouse tumors, human TAM subsets displayed metabolic heterogeneity, as exemplified by the differential expression of multiple metabolic markers (Figure 7D). Hence, while it is difficult to directly relate macrophage heterogeneity in human and mouse lung tumors (Zilionis et al., 2019), our single-cell metabolic analysis supports the notion that TAM subsets show high phenotypic and metabolic heterogeneity in both species.

DISCUSSION

Most metabolism studies have focused on *in vitro* polarized macrophages, which consider so-called M1/M2 macrophages as the two extremes of a spectrum of activation states (Jha et al., 2015; Liu et al., 2017a, 2017b; Mills et al., 2000; O'Neill et al., 2016; O'Neill and Artyomov, 2019; Seim et al., 2019; Williams and O'Neill, 2018). However, the complex microenvironment, such as the one present in tumors, harbors *in vivo* stimuli that drive the co-existence of macrophage subsets with specific characteristics (Xue et al., 2014; Carmona-Fonseca et al., 2017). Previous work showed the simultaneous presence of anti-tumoral MHC-II^{hi} TAMs and pro-tumoral MHC-II^{lo} TAMs in mouse subcutaneous and orthotopic lung tumors (Laoui et al., 2014; Movahedi et al., 2010). Moreover, it was shown that MHC-II^{hi} and MHC-II^{lo} TAMs share at least some phenotypic characteristics of *in vitro*-generated M1/M2 macrophages, respectively. Here, we demonstrate such common characteristics at the metabolic level. Indeed, an accumulation of citrate in combination with a low α KG pool is detected in MHC-II^{hi} TAMs. This reduced glucose oxidation from citrate to α KG (or glutamate) is corroborated by ¹³C-labeled glucose and glutamine tracer experiments. This was previously reported as a metabolic feature of LPS/IFN- γ -stimulated macrophages as a consequence of flux discontinuity at the IDH level (Jha et al., 2015). This observation was related to a nitrosation-dependent reduction in IDH activity but not to reduced IDH protein levels (Bailey et al., 2019). We now demonstrated that MHC-II^{hi} TAMs showed a diminished expression of the mitochondrial Idh2 at both the transcriptional and protein levels, leading to reduced IDH activity. However, in contrast to LPS/IFN- γ -stimulated macrophages, a small percentage of glucose-derived labeling remained in glutamate in MHC-II^{hi} TAMs, suggesting residual TCA cycle activity in these cells. Other differences with polarized LPS/IFN- γ -macrophages are the lack of itaconate (Michelucci et al., 2013; Mills et al., 2018) and succinate (Mills et al., 2016; Tannahill et al., 2013) accumulation in MHC-II^{hi} TAMs. These two phenomena are linked since itaconate can inhibit

SDH and enhance succinate accumulation (Cordes et al., 2016; Lampropoulou et al., 2016). As citrate in MHC-II^{hi} TAMs is not redirected into itaconate, the accumulated citrate could be converted into acetyl-coenzyme A (CoA) or could contribute to NADPH production and, as such, be involved in mechanisms that have been described to shape a pro-inflammatory macrophage phenotype, such as histone acetylation, fatty acid synthesis, or production of the inflammatory mediators NO and reactive oxygen species (ROS) (Geeraerts et al., 2017). Overall, MHC-II^{hi} TAMs only partially recapitulate the metabolic features of polarized M1 macrophages, likely reflecting the absence of high IFN- γ levels and TLR ligands in tumors. Notably, GM-CSF was reported to shape this TAM subset (Van Overmeire et al., 2016), so further research is warranted to assess whether this cytokine directly affects TCA cycle activity.

The flux discontinuity at the IDH level undermines TCA cycle activity and supports the idea of a less oxidative metabolism in MHC-II^{hi} TAMs, which is further exemplified by a reduced basal OCR in these cells. Conversely, MHC-II^{lo} TAMs seemed to strongly rely on an oxidative metabolism with a high basal respiration rate but also a high basal glycolysis. A similar energetic metabolic phenotype has been observed in adipose-tissue macrophages from obese mice under the influence of oxidized phospholipids (Serbulea et al., 2018) and was recently referred to as a hypermetabolic state (Di Gioia et al., 2020). Interestingly, enhanced lipid uptake and metabolism has also been reported for TAMs (Su et al., 2020), but it needs to be examined whether this is mostly restricted to MHC-II^{lo} TAMs. Interestingly, the few studies that examined the metabolism of TAM-like macrophages *in vitro* suggested a glycolytic profile of TAMs (Liu et al., 2017a, 2017b; Penny et al., 2016), whereas Wenes et al. showed that hypoxic TAMs strongly upregulated REDD1, which negatively regulated mTORC and braked their glycolytic metabolism (Wenes et al., 2016). These data again highlight that TAMs confronted with different microenvironments (oxygen availability, lipid availability, etc.) adopt different metabolic characteristics. MHC-II^{lo} TAMs also displayed high mitochondrial respiration, which could be linked to their enhanced mitochondrial mass, as was previously reported for macrophages (Baardman et al., 2018). Interestingly, high SRC has been linked to enhanced cell survival (Nickens et al., 2013; Pfleger et al., 2015), possibly conveying a first survival benefit on MHC-II^{lo} TAMs in a challenging TME. This TAM subset indeed accumulates during the course of tumor growth (Movahedi et al., 2010). Of note, single-cell technologies are in the process of further fine-tuning TAM heterogeneity in both humans and mice (Antunes et al., 2021; Qian et al., 2020; Zilionis et al., 2019) beyond the two main subsets we characterized here. To relate current insights obtained by bulk analyses to human

(D) Heatmaps showing the enrichment (purple) or depletion (yellow) of metabolic markers in all TAM clusters (2, 3, 4, 5, 6, 7, and 10) (mouse) and CD68⁺ clusters 3, 5, 8, and 10.

(E) Mean expression of the indicated phenotypic and metabolic markers in the different mouse tumor samples in the MHC-II^{hi} TAM cluster pool (clusters 3 + 5) and MHC-II^{lo} TAM cluster pool (clusters 2 + 4 + 6 + 7 + 10).

Statistical analysis by Welch two sample t test (two-sided). The p values are indicated in the graphs. p values ≤ 0.05 are considered as statistically significant. See also Figure S7 and Tables S2, S3, and S6.

pathology, future single-cell approaches could help characterize the phenotypic, metabolic, and functional differences within the macrophage compartment in relation to treatment and disease outcome. As a proof of concept, we performed mass-cytometry-based scMEP of human and mouse NSCLC. While it is hard to directly compare mouse and human TAM subsets (Zilionis et al., 2019), our analysis confirmed clear phenotypic and metabolic heterogeneity of TAMs in both species. In mouse, our data showed that MHC-II is a valid marker to roughly divide TAM subsets into two pools, despite how further metabolic and phenotypic heterogeneity exists beyond the two major TAM subsets we investigated. Also, in human NSCLC, HLA-DR^{hi} and HLA-DR^{lo} TAM subsets can be found, which can be further separated in additional metabolic clusters. Metabolic differences are also observed within HLA-DR^{hi} TAMs (i.e., between subsets 8 and 10) and HLA-DR^{lo} TAMs (between subsets 3 and 5). For example, subsets 3 and 10 share a high expression of LAT1/CD98 and glutaminase (GLS), suggesting the utilization of amino acids such as glutamine as nutrients, and an expression of the redox regulator glutathione synthetase (GSS), the mitochondrial regulator TOMM20, and phosphorylation of the ribosomal protein S6 (S6_p). Subset 10 showed a particularly high expression of markers related to the TCA cycle and electron transport chain (ETC), including CS, SDHA, OGDH, ATP5A, and CytC. Conversely, subsets 5 and 8 showed the opposite expression pattern for several metabolic markers in comparison with 3 and 10.

Recent studies demonstrated that lactate should not only be considered as a waste product of aerobic glycolysis but that it can also serve as a prominent carbon source that fuels oxidative metabolism in different tissues and tumors (Chen et al., 2016; Faubert et al., 2017; Hui et al., 2017). We found that in the presence of extracellular lactate, MHC-II^{lo} TAMs switch from aerobic glycolysis (and the consequent secretion of lactate) to a cell type that uses lactate as an additional carbon source besides glucose. This excess of carbon is at least partly compensated by the reduced uptake of glutamine and enhanced TCA cycle-mediated respiration. In line with the latter, lactate was found to promote respiration and related parameters in MHC-II^{lo} TAMs, suggesting a role for lactate as a fuel for the TCA cycle. Additional pathways that may capture the excess carbon include the pentose phosphate pathway (PPP), amino acid synthesis, fatty acid synthesis, histone acetylation (Lauterbach et al., 2019), or glycolysation, which has been linked to enhanced GlcNAc synthesis in IL-4-mediated M2 macrophages (Jha et al., 2015). The fact that MHC-II^{lo} TAMs can use lactate as an additional carbon source and fuel it into the TCA cycle may eventually provide them with an advantage in the lactate-enriched environment of a tumor. In contrast, MHC-II^{hi} TAMs stick to net lactate secretion in the presence of extracellular lactate, with this metabolite ultimately lowering the metabolic activity of these cells. Possibly, these cells have difficulties in regenerating NAD⁺ due to the combination of a disturbed TCA cycle and diminished NAD⁺ regeneration through aerobic glycolysis, but this needs further investigation.

Gpr132 was shown to mediate the M2-like phenotype in macrophages instructed by lactate (Chen et al., 2017), but it

is not involved in mediating the lactate exchange flux in TAMs, nor did it affect 3LL-R tumor growth or the composition of its immune compartment. Hence, different molecules regulate lactate uptake and lactate-dependent gene expression. Notably, the *ex vivo* treatment of TAM subsets with lactate resulted in relatively minor changes in gene expression, possibly because these cells had been preconditioned with lactate *in vivo*. Nevertheless, the enzyme activity of arginase-1, similar to previous findings (Chen et al., 2017; Colegio et al., 2014), but also Nos2 was consistently upregulated in both TAM subsets. The combined induction of these L-arginine-catabolizing enzymes has been linked to an increased immunosuppressive capacity of monocytes/macrophages (Bronte et al., 2003), a feature which we recapitulated here for MHC-II^{lo} TAMs. Hence, lactate promotes the immunosuppressive capacity of pro-tumoral TAMs.

Interestingly, similar mechanisms have been proposed recently for regulatory T cells (T_{regs}), which maintain their immunosuppressive function under the high-lactate environment of the tumor (Angelin et al., 2017; Watson et al., 2021), while lactate limits the proliferation of effector T cells (Quinn et al., 2020). The fact that several immune cells with the ability to metabolize lactate share a pro-tumoral activity (T_{reg}, MHC-II^{lo} TAM), while anti-tumoral immune cells (T cells, MHC-II^{hi} TAM) suffer from high lactate concentrations, makes lactate metabolism an interesting target for cancer therapy.

Limitations of the study

Current approaches do not allow an in-depth metabolic characterization of the wide variety of TAM subsets that we observed in mouse and human NSCLC at the single-cell level. Not only the need for relatively high cell numbers to perform metabolomics and extracellular flux analysis but also the lack of specific surface markers required to sort the variety of TAM subsets currently limit a metabolic screening of low-abundant TAM subsets (Artyomov and Van den Bossche, 2020). It should be realized that scMEP only provides an indirect estimate of cellular metabolism (based on the protein expression of a limited set of metabolic enzymes but not their activity), still necessitating bulk metabolic analyses such as metabolomics, ¹³C-label-tracing, and extracellular flux analysis to provide the full picture. Considering these practical limitations, we had to limit our metabolic characterization to two major TAM subsets (MHC-II^{lo} and MHC-II^{hi} TAMs), which show clear phenotypical, transcriptional, functional, and localization differences (Laoui et al., 2014; Movahedi et al., 2010). Thus, we assessed the metabolic profile of two major and distinct macrophage subsets that co-exist in mouse lung carcinomas. Additional smaller subsets may have different metabolic phenotypes and may be further studied by emerging techniques like imaging mass-spectrometry-based single-cell metabolomics (Artyomov and Van den Bossche, 2020). Moreover, our manuscript describes the distinct effects of lactate on the TAM subsets' metabolic, transcriptomic, and functional profiles. Further *in vivo* studies are needed to address the therapeutic implications of our finding that major macrophage subtypes display a differential lactate uptake. Therefore, strategies that are able to affect lactate metabolism in a TAM-specific way will be inevitable.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2021.110171>.

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DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Antibodies for flow cytometry	This paper (Table S7)	N/A
Purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™) Clone 2.4G2	BD Biosciences	Cat#553142; RRID:AB_394657
IDH1 Rabbit Polyclonal Antibody	Cell Signaling Technology	Cat#3997; RRID:AB_1904011
IDH2 (D8E3B) Rabbit mAb	Cell Signaling Technology	Cat#56439; RRID:AB_2799511
Vinculin Antibody	Cell Signaling Technology	Cat#4650; RRID:AB_10559207
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	Cat#7074; RRID:AB_2099233
Heavy-metal conjugated antibodies for scMEP	This paper (Tables S2 and S3)	N/A
Purified Hamster Anti-Mouse CD3e Clone 145-2C11	BD Biosciences	Cat#550275; RRID:AB_393572)
CD28 Monoclonal Antibody (37.51)	eBioscience	Cat#16-0281-82; RRID:AB_468921
Biological samples		
Lung cancer patient samples	Cliniques universitaires Saint-Luc (CUSL), Belgium	https://www.saintluc.be/
Chemicals, peptides, and recombinant proteins		
L-glutamine	Sigma-Aldrich	Cat#G8540
Penicillin-Streptomycin	ThermoFisher Scientific	Cat#15140122
Sodium L-lactate	Sigma-Aldrich	Cat#7022
Collagenase, Type 1, Filtered	Worthington	Cat#LS004214
Collagenase, Type 4, Filtered	Worthington	Cat#LS004209
Deoxyribonuclease I, Filtered	Worthington	Cat#LS002060
Lymphoprep	Axis-Shield	Cat#1114547
LPS, E.Coli 0111:B4	Sigma-Aldrich	Cat#LPS25
Recombinant Mouse IFN- γ	BD Biosciences	Cat#554587
Recombinant Mouse Interleukin-4	Sigma-Aldrich	Cat#IL016
D-Glucose (U- $^{13}\text{C}_6$, 99%)	Cambridge Isotope Laboratories	Cat#CLM-1396
L-Glutamine- $^{13}\text{C}_5$	Sigma-Aldrich	Cat#605166
Sodium L-lactate-(13-C)3, 45%-55% aqueous solution	Santa Cruz Biotechnology	Cat#sc-251030
Methanol Chromasolv™ LC-MS	Honeywell Fluka	Cat#34966
Chloroform, anhydrous, $\geq 99\%$	Sigma-Aldrich	Cat#288306
L-Norvaline	Sigma-Aldrich	Cat#N7627
Glutaric acid, 99%	Sigma-Aldrich	Cat#G3407
O-Methoxyamine hydrochloride	Supelco	Cat#33045-U
N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchlorosilane	Sigma-Aldrich	Cat#375934
Pyridine	Sigma-Aldrich	Cat#270970
Formaldehyde 16%, Sol, EM grade	Electron Microscopy Sciences	Cat#15710
Dimethyl sulfoxide	Merck	Cat#102931
Oligomycin	Sigma-Aldrich	Cat#O4876
2-deoxy-D-glucose	Sigma-Aldrich	Cat#D6134

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone	Sigma-Aldrich	Cat#C2920
Rotenone	Sigma-Aldrich	Cat#R8875
Antimycin A	Sigma-Aldrich	Cat#A8674
Formaldehyde 10%, sol, EM grade	Electron Microscopy Sciences	Cat#15712
Glutaraldehyde 25%, EM grade	Electron Microscopy Sciences	Cat#16220
Sodium Cacodylate buffer 0.2 M	Electron Microscopy Sciences	Cat#11652
Osmium tetroxide 4%, Sol	Electron Microscopy Sciences	Cat#19150
Potassium ferricyanide (III)	Sigma-Aldrich	Cat#702587
Uranyl acetate dehydrate	Honeywell Fluka	Cat#94260
Sulfanilamide	Sigma-Aldrich	Cat#S9251
N-(1-Naphthyl)ethylenediamine dihydrochloride	Sigma-Aldrich	Cat#222488
Phosphoric acid	Supelco	Cat#100573
Triton™ X-100	Sigma-Aldrich	Cat#X100
Manganese (II) chloride tetrahydrate	Sigma-Aldrich	Cat#M3634
L-Arginine	Sigma-Aldrich	Cat#A8094
H ₂ SO ₄ (96%) sulfuric acid	Merck	Cat#1.00317.1000
H ₃ PO ₄ (85%) phosphoric acid	Merck	Cat#1.00573.1000
Alpha-isonitropropiphenone	Sigma-Aldrich	Cat#I3502
Urea	Sigma-Aldrich	Cat#U0631
N ω -Hydroxy-nor-L-arginine, Diacetate Salt (NorNoha)	Calbiochem	Cat#399275
N ^G -Methyl-L-arginine acetate salt (L-NMMA)	Sigma-Aldrich	Cat#M7033

Critical commercial assays

FITC brdU Flow Kit	BD Biosciences	Cat#559619
Pierce™ BCA Protein Assay Kit	ThermoFisher Scientific	Cat#23227
RNeasy Plus Micro Kit	Qiagen	Cat#74034
Isocitrate Dehydrogenase Assay Kit (Colorimetric)	Abcam	Cat#ab102528
Low viscosity embedding kit (Spurr's resin)	Electron Microscopy Sciences	Cat#14300
Lactate Colorimetric Assay Kit II	BioVision	Cat#K627

Deposited data

RNA sequencing data	This paper.	GEO: GSE188549
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Experimental models: Cell lines

3LL-R	Remels and de Baetselier (1987)	NA
NCTC clone 929 [L cell, L-929, derivative of Strain L]	ATCC	Cat#CCL-1; RRID:CVCL_0462

Experimental models: Organisms/strains

Mouse: C57BL/6Rj	Janvier Labs	SC-CJ-6S-F
Mouse: GPR132 ^{KO} : B6.129X1(C)-Gpr132 ^{tm1Witt/J}	Le et al. (2001)	NA

Oligonucleotides

Primer: Nos2 Forward: 5'-TCCACCAGGAGATGTTGAAC-3'	This paper	NA
Primer: Nos2 Reverse: 5'-TGGAGCCAAGGCCAACACAG-3'	This paper	NA

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: <i>Arg1</i> Forward: 5'-TCACCTGAGCTTTGATGTCG-3'	This paper	NA
Primer: <i>Arg1</i> Reverse: 5'-TTATGGTTACCCTCCCGTTG-3'	This paper	NA
Primer: <i>Mrps12</i> Forward: 5'-GGAAGGCATAGCTGCTGGAGGTGT-3'	This paper	NA
Primer: <i>Mrps12</i> Reverse: 5'-CCTCGATGACATCCTTGGCCTGAG-3'	This paper	NA

Software and algorithms

FlowJo 10.7.1	TreeStar	https://www.flowjo.com
Matlab R2018b	MathWorks	https://nl.mathworks.com/products/matlab.html
MSD Chem Station Data Acquisition/Analysis (vE.02.02.1431)	Agilent	https://www.agilent.com/cs/library/usermanuals/Public/G1701-90055.pdf
MassHunter Software	Agilent	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software
R 3.6.1	R	https://www.r-project.org
Rstudio 1.4.1103	RStudio	https://www.rstudio.com
Metascape	Zhou et al. (2019)	https://metascape.org/gp/index.html#/main/step1
Seahorse Wave	Agilent	https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
QuPath_0.2.3	Bankhead et al. (2017)	https://qupath.readthedocs.io/en/latest/
GraphPad Prism 8.2.1	GraphPad Software	https://www.graphpad.com
El-Maven v0.12.0	Elucidata	https://resources.elucidata.io/elmaven
Image Studio™ Lite	Licor	https://www.licor.com/bio/image-studio-lite/download
Package: EDASeq	Risso et al. (2011) ; Robinson and Oshlack (2010)	https://bioconductor.org/packages/release/bioc/html/EDASeq.html
Package: edgeR 3.20.9	Robinson et al. (2010)	https://bioconductor.org/packages/release/bioc/html/edgeR.html
Package: ggplot2	Wickham (2009)	https://cran.r-project.org/web/packages/ggplot2/index.html
Package: pheatmap	Kolde (2019)	https://cran.r-project.org/web/packages/pheatmap/index.html
Package: FlowSOM	Van Gassen et al. (2015)	https://bioconductor.org/packages/release/bioc/html/FlowSOM.html
Package: uMAP	McInnes et al. (2018)	https://github.com/jlmelville/uwot
Package: MEM	Diggins et al. (2017)	https://github.com/JonathanIrish/MEMv3

Other

RPML-1640 medium	ThermoFisher Scientific	Cat#52400041
RPML-1640 medium, no glucose	ThermoFisher Scientific	Cat#11879020
RPML-1640 medium, no glutamine	ThermoFisher Scientific	Cat#31870025
DMEM, high glucose	ThermoFisher Scientific	Cat#41965062
Fetal Bovine Serum	Capricorn Scientific	Cat#FBS-12A
7-AAD	Biolegend	Cat#420404
MitoTracker™ Green FM	ThermoFisher Scientific	Cat#M7514

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cisplatin-198Pt	Fluidigm	Cat#201198
Cell-ID™ Intercalator-Rh-500μM	Fluidigm	Cat#201103A
RIPA Lysis Buffer	VWR	Cat#N653
cOmplete™ Protease Inhibitor Cocktail	Roche	Cat#11697498001
PhosSTOP	Roche	Cat#04906837001
NuPAGE™ 4 to 12%, Bis-Tris, 1.5 mm, Mini Protein Gel, 15-well	ThermoFisher Scientific	Cat#NP0336BOX
iBlot™ Transfer Stack, nitrocellulose, mini	ThermoFisher Scientific	Cat#IB301002
Pierce™ ECL Western Blotting Substrate	ThermoFisher Scientific	Cat#33209
SYBR Green Supermix	Bio-Rad	Cat#1725274
Oligo(dT)	Invitrogen	Cat#18-418-012
SuperScript™ II Reverse Transcriptase	Invitrogen	Cat#18-064-071

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jo A. Van Ginderachter (jo.van.ginderachter@vub.be).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The RNA sequencing data generated during this study are available at the GEO repository [GEO: GSE188549]. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse strains

Female C57Bl/6J mice of 6–8 weeks old were purchased from Janvier. Gpr132KO mice (female, 6–8 weeks) (Le et al., 2001) were provided by Yihong Wan (University of Texas Southwestern Medical Center, Dallas, USA). Littermates of the same sex and age were randomly assigned to experimental groups and housed in Type III open cages from UNO without filtertop in groups of 6 or 7. All procedures followed the guidelines of the Belgian Council for Laboratory Animal Science and were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (licenses 15-220-8, 18-220-18 and 20-220-36).

Cell lines and tumor models

3LL-R Lewis Lung carcinoma (Remels and de Baetselier, 1987) and L929 cells were cultured in RPMI-1640 and DMEM medium (Sigma-Aldrich), respectively, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Capricorn Scientific), 300 μg/ml L-glutamine (Sigma-Aldrich), 100 mg/ml streptomycin (ThermoFisher Scientific) and 100 units/ml penicillin. All cell lines were tested for the absence of mycoplasma. Cell lines were cultured at 37°C in a humidified tissue culture incubator with 5% CO₂.

For tumor implantation, 3×10^6 3LL-R cells were injected subcutaneously into the right flank of 6- to 8-week-old mice. Tumor volumes were assessed by caliper measurements and calculated using the formula: $V = \pi \times (d^2 \times D)/6$, where d is the minor and D is the major tumor axis. Tumor-bearing mice were killed when tumor volumes ranged between 910 and 1,158 mm³.

Lung cancer patient samples

Fresh tumor tissue was obtained from 5 non-small-cell lung carcinoma (NSCLC) patients undergoing surgical resection at Cliniques universitaires Saint-Luc (CUSL), Belgium. Clinical patient information is provided in Table S6. The study of human samples was approved by the local ethical committees of the Vrije Universiteit Brussel and Saint-Luc-UCLouvain. All patients provided informed consent after the nature and possible consequences of the study had been explained. Upon resection, tumor samples were instantly transported on ice to the research facility at the Vrije Universiteit Brussel for preparation of a tumor single-cell suspension.

METHOD DETAILS

Tumor single-cell suspension preparation

Tumor single-cell suspensions of both mouse and human tumors were prepared as described previously (Movahedi et al., 2010) using RPMI-based complete medium whether or not supplemented with 20mM sodium L-lactate (Sigma-Aldrich). In short, excised tumors were cut in small pieces, enzymatically digested with 10 units/ml collagenase I, 400 units/ml collagenase IV and 30 units/ml deoxyribonuclease (DNase)-I (Worthington) for 25min at 37°C, squashed and filtered. Single-cell suspensions were treated with erythrocyte lysis buffer.

Flow cytometry

Single-cell suspensions were pre-incubated with rat anti-mouse CD16/CD32 (2.4G2, BD Biosciences) and subjected to cell surface staining with fluorescently labeled antibodies (Table S7). Flow cytometry data were acquired with BD FACSCanto II (BD Biosciences) and analyzed using FlowJo (Tree Star).

Cell viability was determined by 7-amino-actinomycin D (7AAD) (Biolegend) staining. Intracellular staining of Ki67 was performed following the manufacturer's protocol (BD Biosciences). Mitochondrial mass was assessed by incubating tumor single cell suspension for 30min at 37°C with 50 nM MitoTracker Green (ThermoFisher Scientific) staining. Unlabelled cells were used as background level. Delta-median fluorescence intensity was calculated as $\Delta\text{MFI} = (\text{Median fluorescence intensity})_{\text{MitoTracker}} - (\text{Median fluorescence intensity})_{\text{background}}$.

Bromodeoxyuridine labeling

Tumor-bearing mice were injected intraperitoneally with 1mg bromodeoxyuridine (BrdU) (BD Biosciences) 4h prior to tumor collection. Intracellular staining of BrdU was performed following the manufacturer's protocol (BD Biosciences) and analyzed using flow cytometry. Detection of BrdU-positive cells was based on staining of control mice which did not receive BrdU but are injected with HBSS (100μl).

TAM subset isolation and lactate treatment

For sorting of TAM subsets, single-cell suspensions from a pool of 4–8 tumors were submitted to a lymphoprep density gradient (Axis-Shield). Subsequently, live, single CD11b⁺Ly6G[−]Ly6C^{low}MHC-II^{low} (MHC-II^{lo} TAMs) and CD11b⁺Ly6G[−]Ly6C^{low}MHC-II^{high} (MHC-II^{hi} TAMs) cells were sorted using a BD FACS Aria II (BD Biosciences) and collected into RPMI-based complete medium whether or not supplemented with 20mM sodium L-lactate (Sigma-Aldrich). For further *ex vivo* sodium L-lactate treatment experiments, sorted cells were seeded for 24h at a concentration of 10⁶ cells/ml in RPMI-based complete medium with/without 20mM sodium L-lactate.

Generation and polarization of mouse BMDMs

Bone marrow cells were isolated from tibia and femur, and cultured for 13 days in RPMI-1640 medium supplemented with 300 μg/ml L-glutamine (Sigma-Aldrich), 100 mg/ml streptomycin (ThermoFisher Scientific), 100 units/ml penicillin, 20% (v/v) heat-inactivated FBS (Capricorn Scientific) and 30% L929-conditioned medium. Subsequently, BMDMs were harvested and plated at a concentration of 10⁶ cells/ml in RPMI-based complete medium. Next, BMDMs were stimulated with either 10 ng/ml LPS (Sigma-Aldrich) and 100 units/ml IFN-γ (BD Biosciences) or 20ng/ml IL-4 (Sigma-Aldrich). Macrophage polarization was assessed by ELISA and flow cytometry as described before (Ying et al., 2013).

Ex vivo ¹³C isotope labeling experiments

For ¹³C-labeling experiments, sorted TAMs were cultured first for 1h in complete medium to allow re-adherence of the cells and subsequently for 24h in complete medium, in which glucose, glutamine or lactate were replaced with the following ¹³C tracers: 10.8 mM ¹³C₆-D-glucose (Cambridge Isotope Laboratories), 4 mM ¹³C₅-L-glutamine (Sigma-Aldrich), 20mM ¹³C₃-sodium-L-lactate (Santa Cruz Biotechnology). In tracer experiments, 10% dialyzed fetal bovine serum was used instead of normal serum, to avoid dilution of the ¹³C labeled tracer as well as specialized RPMI media without glucose or glutamine (ThermoFisher Scientific) (Zhang et al., 2014).

Quenching and metabolite extraction

To extract metabolites, media supernatants from 3h cultured TAMs or polarized BMDMs were aspirated, plates were washed with 0.9% saline and quenched on liquid nitrogen. Intracellular metabolites were extracted by scraping the cells using a precooled methanol-water (5:3) (62.5%) buffer, including glutaric acid (Sigma-Aldrich) as an internal standard, and a precooled chloroform extraction buffer, as previously described (Altea-Manzano et al., 2020). After vortexing (10min, 4°C) and centrifugation (10min, 4°C, 14,000 g), a three-phase separation was achieved. The upper methanol/water phase, containing polar metabolites, and the intermediate protein layer, were separated and dried by vacuum centrifugation (8 h at 4°C and 1h at 20°C).

Gas chromatography-mass spectrometry analysis

Polar metabolite derivatization was performed as previously described (Altea-Manzano et al., 2020). In brief, polar metabolites were derivatized with 20mg/ml methoxyamine (Supelco) in pyridine (Sigma-Aldrich) (90min, 37°C), and subsequently with N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide with 1% tert-butyldimethylchlorosilane (Sigma-Aldrich) (60min, 60°C).

Metabolite levels and isotopologue distributions were measured with a 7890A GC system coupled to a 5975C Inert MS System (Agilent Technologies) with previously described settings (Altea-Manzano et al., 2020). For each derivatized sample, 1 μ l was injected into a DB35MS column (Agilent Technologies) in splitless mode, with an inlet temperature of 270°C. Helium was used as carrier gas, at a flow rate of 1 ml/min. The GC oven temperature was initially held at 100°C for 3min, and upon sample injection increased to 105°C with a gradient of 2.5°C/min, subsequently increased to 240°C with a gradient of 3.5°C/min, and finally ramped up to 320°C with a gradient of 22°C/min. A 2min post-run was performed at 320°C. The MS system was operated under electron impact ionization at 70 eV, and a mass range of 100–650 a.m.u. was scanned.

Identification of the metabolites was based on exact mass-to-charge ratio (m/z) and retention times. Ion counts for each metabolite isotopologue and isotopologue distributions for each metabolite were calculated from the raw chromatograms using MSD Chem Station Data Acquisition and Analysis software followed by a home-made Matlab script, applying a consistent integration range and baseline determination for all isotopologues of a given metabolite. To calculate total metabolite levels, the sum of the ion counts for all isotopologues corresponding to the metabolite of interest was normalized to sample protein content and internal standard level (glutaric acid) (Altea-Manzano et al., 2020). The total fractional contribution of a tracer to the production of a metabolite of interest was calculated using the formula: $\sum_{i=0}^n i \times X_i / n$, where n is the number of carbon atoms in the metabolite of interest, i represents the different isotopologues, and X_i is the fractional abundance of a particular isotopologue (corrected for natural abundance) (Fernández-García et al., 2020). The method of Fernandez et al. was used to correct for natural abundance (Fernandez et al., 1996).

The dried protein pellet was resuspended in 100 μ l 0.2 M NaOH and incubated at 95°C for 20min. Protein content was quantified using PierceTM BCA protein assay kit (ThermoFisher Scientific).

Quantification of glucose and lactate in supernatant by liquid chromatography-mass spectrometry

To quantify glucose and lactate in medium supernatant, sorted cells were seeded at a concentration of 10^6 cells/ml in RPMI-based complete medium with/without 20mM sodium L-lactate. Upon 12h incubation, medium supernatant was collected and stored at –80°C upon extraction. Polar metabolites were extracted using 190 μ l precooled methanol, including norvaline (Sigma-Aldrich) as an internal standard, from 10 μ l medium supernatant or external standards for each metabolite of interest dissolved at specified concentrations. Metabolite levels were measured with an Infinity II 1290 LC system coupled to a 6546 Q-TOF System (Agilent Technologies). A volume of 1 μ l sample was injected into an Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 mm $\times$ 150 mm, 2.7 μ m poroshell column (Agilent Technologies) in negative mode, with an oven temperature of 50°C. Identification of the metabolites was based on exact mass-to-charge ratio (m/z) and retention times. The data analysis was performed using MassHunter software (Agilent). Lactate and glucose concentrations were quantified by external standard calibration.

Bulk RNA sequencing

2.5×10^5 sorted MHC-II^{hi} and MHC-II^{lo} TAMs (5 independent sorts) were cultured for 24h in 200 μ l RPMI-based complete medium with/without 20mM sodium L-lactate. RNA was isolated using the RNeasy Plus Micro Kit (Qiagen) and RNA sequencing was performed using a NextSeq sequencer (Illumina). The preprocessing of the RNA sequencing data was done by Trimmomatic. Adapters were cut off, and reads were trimmed when the quality dropped below 20. Reads with a length <35 were discarded. All samples passed quality control based on the results of FastQC. Reads were mapped to the mouse reference genome (Mus musculus.Ensembl.GRCm38.82) via STAR and counted via FeatureCounts. We aggregated the runs by summing the counts per gene and per sample. We removed 32,825 genes for which all samples have less than 1 counts-per-million, as such we continued with 13,778 genes (Robinson and Smyth, 2007). We corrected for GC-content, library size and RNA composition using the EDASeq package from Bioconductor (Risso et al., 2011; Robinson and Oshlack, 2010). The edgeR 3.20.9 package of Bioconductor (Robinson et al., 2010) was used to calculate the differentially expressed (DE) genes (GLM likelihood ratio test). Benjamini-Hochberg was used to adjust the p values for multiple testing (Benjamini and Hochberg, 1995). The DE genes were defined based on a log₂(fold change) > 1 or < –1 and corrected p value < 0.05. The heatmaps display the relative expression per gene based on FPKM (Fragments Per Kilobase Million). This is determined by calculating the mean expression value per gene over all cell types in the heatmap and then subtracting this mean from each cell type's particular gene expression value. Volcano plots and heatmaps were determined in a R environment using the ggplot2 and pheatmap packages respectively (Kolde, 2019; Wickham, 2009).

Gene ontology enrichment analysis

Gene ontology analysis was performed to assess biological functions based on differential gene expression. Hereto, differentially expressed genes (log₂(fold change) > 1 or < –1 and corrected p value < 0.05) were used as input for the online tool “Metascape” (Zhou et al., 2019), using default parameters.

Single-cell metabolic profiling of human and mouse lung tumors

Single-cell metabolic profiling was performed according to a previously described method (Hartmann et al., 2020). This approach combines antibodies against immune activation markers and key metabolic regulators and thus allows to simultaneously characterize cellular phenotypes and their metabolic configuration. The list of metabolic and phenotypic markers used for scMEP in mouse and human tumor samples are listed in Tables S2 and S3 respectively. Single cell suspensions of five independent human NSCLC samples and 16 independent mouse 3LLR tumor samples were incubated for 5min at room temperature (RT) with 25 μ M cisplatin-198Pt (Fluidigm) for live/dead cell discrimination. Upon washing with RPMI-based complete medium, cells were fixed for 10min at RT with 1.6% paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS. After washing, the fixed cells were stored at -80°C in RPMI-based complete medium supplemented with 10% dimethyl sulfoxide (DMSO) (Merck). Individual tumor samples were palladium barcoded and subsequently merged to a composite sample for each species. Next, samples were stained with heavy metal-conjugated antibodies for 30min at RT. In case of intracellular staining, cells were permeabilized by incubation with 1ml of pre-cooled methanol for 10min on ice and stained with antibodies for 1h at RT. After washing, cells were resuspended for 20min at RT in intercalation solution (1.6% PFA + 0.5 μ M rhodium intercalator (Fluidigm)). Next, cells were washed twice in ddH₂O, passed through a cell strainer and acquired on a CyTOF mass cytometer (Fluidigm). Mass cytometry data were bead normalized whereafter live, single cells were gated. For both species, cells were clustered based on both immune and metabolic markers (listed in Tables S2 and S3) using the FlowSOM algorithm and annotated manually based on immune marker profiles. In both mouse and human, one cluster demonstrated high expression of genes specific to mononuclear phagocytes and were selected for an unsupervised clustering based on all relevant immune and metabolic markers using the metacustering function of the FlowSOM package (Van Gassen et al., 2015). UMAP dimensionality reduction was with the following parameters ($n_neighbors = 15$, $min_dist = 0.02$) and the indicated input channels (McInnes et al., 2018). Heatmaps represent the result of marker enrichment modeling (MEM) (Diggins et al., 2017) with depletion or enrichment of protein expression visualized as yellow and purple respectively.

Extracellular flux analysis

BMDMs (5×10^4) were seeded into XF-96-well culture plates (Seahorse Bioscience), and incubated for 1h at 37°C , 5% CO₂. Next, BMDMs were stimulated for 24h to generate M1 or M2 BMDMs. After 24h, the cell culture medium was replaced by seahorse medium (XF base medium, 10 mM D-glucose, 2 mM L-glutamine, 5 mM HEPES, pH 7.4). Sorted TAMs (5×10^4) were seeded into XF-96-cell culture plates in seahorse medium whether or not supplemented with/without sodium L-lactate (20mM final concentration).

Prior to the assay run, the cells were incubated for 1h at 37°C in absence of CO₂. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured by the extracellular flux analyzer (XF-96 flux analyzer, Seahorse Bioscience), as previously described (Van den Bossche et al., 2015). Changes in ECAR and OCR in response to sequential injection of 1.5 μ M oligomycin (OM) (Sigma-Aldrich), 1.5 μ M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich) and 1.25 μ M rotenone (ROT)/2.5 μ M antimycin A (AA)/1 M 2-deoxyglucose (2-DG) (Sigma-Aldrich) allow to calculate glycolytic and respiratory parameters, as detailed in Figure S3.

Isocitrate dehydrogenase colorimetric assay

Isocitrate dehydrogenase (IDH) activity was assessed for the NADP⁺ dependent isoforms (IDH1 and IDH2) using a colorimetric assay kit (Abcam). Briefly, 10^6 sorted TAM subsets were resuspended in 200 μ l ice-cold supplied assay buffer. NADPH formation was determined by measuring absorbance at 450 nm at 37°C in a kinetic manner in 50 μ l cell lysates and NADH standards upon incubation with NADP⁺, isocitrate substrate and developer. IDH activity was calculated after 20min using the formula: IDH activity = (Amount of NADPH/(Reaction time x Sample volume)) x Dilution factor. One unit IDH represent the amount of enzyme generating 1.0 μ mol NADPH per minute at pH 8 at 37°C .

Western blotting

Cell lysates were prepared by homogenizing sorted TAMs in ice-cold RIPA lysis buffer (VWR) containing protease (Roche) and phosphatase (PhosSTOP) (Roche) inhibitors. Upon an on-ice incubation for 20min, lysates were centrifuged at max speed for 10min at 4°C and the exact protein content was quantified using PierceTM BCA protein assay kit (ThermoFisher Scientific). After denaturation at 95°C for 5min, 30 μ g protein (IDH1) and 20 μ g protein (IDH2) was loaded on a NuPAGE 4–12% Bis-Tris gel (ThermoFisher Scientific). Upon transferring to a nitrocellulose membrane using the dry transfer iBlot2 system (ThermoFisher Scientific), the membrane was blocked with 5% milk for an hour and subsequently incubated with the primary antibodies against IDH1 (Cell Signaling Technology, #3997, 1:500 dilution), IDH2 (Cell Signaling Technology, #56439, 1:1,000 dilution) or Vinculin (Cell Signaling Technology, #4650S, 1:1,000 dilution) in 5% BSA overnight at 4°C . The next day, the blots were washed three times with TBS-T before being incubated with a secondary anti-IgG rabbit antibody (Cell Signaling, #7074S, 1:5,000 dilution) in 5% milk for 1h at RT. Detected proteins were visualized using Pierce ECL reagent (ThermoFisher Scientific). The signals in the Western blots were quantified using Image Studio Lite software.

Electron microscopy

For EM analysis, 1.8×10^6 sorted MHC-II^{hi} and MHC-II^{lo} TAMs were cultured for 3h in RPMI-based complete medium in 12 well plates containing a glass cover slip. Cells were prepared for transmission electron microscopy as previously described

(Fraire et al., 2020). Briefly, the cells were gradually fixed using a primary fixative solution (4% formaldehyde (EM-grade) (Electron Microscopy Sciences), 2.5% glutaraldehyde (EM grade) (Electron Microscopy Sciences) in 0.1 M cacodylate buffer (Electron Microscopy Sciences)) at RT followed by fixation overnight at 4°C. After washing three times for 20min with buffer solution, cells were post-fixed in 1% osmium tetroxide (OsO₄) (Electron Microscopy Sciences) with 1.5% potassium ferricyanide (K₃Fe(CN)₆) (Sigma-Aldrich) in 0.1 M sodium cacodylate buffer for 1h at RT. After washing in ddH₂O, cells were subsequently dehydrated through a graded ethanol series, including a bulk staining with 1% uranyl acetate (Honeywell Fluka) at the 50% ethanol step followed by embedding in Spurr's resin (Electron Microscopy Sciences). Ultrathin sections of a gold interference color were cut using an ultra-microtome (Leica EM UC7), followed by a post-staining in a Leica EM AC20 for 40min in uranyl acetate at 20°C and for 10min in lead stain at 20°C. Sections were collected on formvar-coated copper slot grids. Grids were viewed with a transmission electron microscope JeolJEM1400plus (Jeol, Tokyo, Japan) operating at 80 kV. Mitochondrial morphology of 371 mitochondria from 30 randomly selected cells of each subtype was scored blindly by a trained TEM expert. Similarly to what has been previously described (Xiong et al., 2016), mitochondria were classified as normal, minor defective or severe defective based on several criteria, including mitochondrial shape, intactness of the outer membrane, orientation/organization/width/vagueness/absence of cristae, presence of mitochondrial remnants in autolysosomes and mitophagy. Image analysis was carried out using QuPath_0.2.3 software (Bankhead et al., 2017).

Lactate colorimetric assay

Lactate concentrations were assessed using a lactate colorimetric assay kit (Biovision) in 3LLR culture medium upon confluency.

Interstitial fluid collection from tumors

Tumors were harvested for collection of interstitial fluid by an established centrifugation method (Christen et al., 2016; Nagahashi et al., 2016) with small modifications. Briefly, upon harvest, tumor tissue was washed in 0.9% saline, dried in a gauze from excess liquid and stored on ice. After sectioning the tumor in small pieces, samples up to 200mg were transferred to an insert with a nylon mesh (20 μm opening, 55 μm thickness, Spectrum Labs) placed on top of a RNeasy mini spin column (Qiagen) after removing the original bottom filter. After centrifuging the tubes for 15min at 400g at 4°C, the insert was removed whereafter the tubes were centrifuged for another 5min at 400g at 4°C. After separating the IF from the cell pellet, the IF from 3 samples derived from the same initial tumor was pooled and spun for 10min at 4000g at 4°C to remove remaining cell content. 5 μl IF was transferred to a new Eppendorf and stored at −80°C upon metabolite extraction.

Quantification of lactate in interstitial fluid by liquid chromatography-mass spectrometry

Polar metabolites were extracted from 8 μl IF using a methanol-water (80%) buffer in a 1:10 ratio. Following extraction, precipitated proteins and insolubilities were removed by centrifugation at 20,000 g for 20min at 4°C. The supernatant was transferred to the appropriate mass spectrometer vials. Measurements were performed using a Vanquish LC System (Thermo Scientific) in-line connected to a Lumos Orbitrap mass spectrometer (Thermo Scientific). 5 μl sample was injected onto a Agilent Hilic column (InfinityLab Poroshell 120 HILIC-Z PEEK lined for NP/SFC). A linear gradient was carried out starting with 90% solvent A (LC-MS grade acetonitrile) and 10% solvent B (10 mM ammoniumacetate pH 9.3). From 2 to 20min the gradient changed to 80% B and was kept at 80% until 23min. Next a decrease to 40% B was carried out to 25min, further decreasing to 10% B at 27min. Finally 10% B was maintained until 35min. The solvent was used at a flow rate of 200ul/min, the columns temperature was kept constant at 25 degrees Celsius. The mass spectrometer operated in negative ion mode, settings of the HESI probe were as follows: sheath gas flow rate at 35, auxiliary gas flow rate at 14. Spray voltage was set at 2.9 kV, temperature of the capillary at 300 degrees Celsius and S-lens RF level at 50. A full scan (resolution of 240,000 and scan range of m/z 70–750) was applied. The data analysis was performed using El-Maven v0.12.0 (Elucidata). Absolute quantification was calculated using a reference solution of lactate.

RNA extraction, cDNA preparation and quantitative real-time PCR

RNA was extracted from 1 × 10⁶ sorted MHC-II^{hi} and MHC-II^{lo} TAMs using the RNeasy Plus Micro Kit (Qiagen). RNA was reverse-transcribed using oligo(dT) (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using a CFX Connect Real-Time PCR Detection System (BioRad), with SYBR Green Supermix (Bio-Rad). The following PCR cycling parameters were used: 30sec at 94°C, 30sec at 54°C and 45sec at 72°C. Gene expression levels were normalized to the housekeeping gene ribosomal protein S12 (*Mrps12*). The following primers were used to determine the relative gene expression: *Nos2*: FP, 5'-TCCACCAGGAGATGTTGAAC-3'; RP, 5'-TGGAGCCAAGGCCAACACAG-3'. *Arg1*: FP, 5'-TCACCTGAGCTTTGATGTCG-3'; RP, 5'-TTATGGTTACCCTCCCGTTG-3'. *Mrps12*: FP, 5'-GGAAGGCATAGCTGCTGGAGGTGT-3'; RP, 5'-CC TCGATGACATCCTTGGCCTGAG-3'.

NO assay

NO concentration was measured respectively in the supernatant of sorted TAM subsets as described before (Liu et al., 2003). NO was quantified as NO₂[−] by adding 50 μl supernatant to 50 μl of the Griess reagent (0.5% sulfanilamide (Sigma-Aldrich); 0.05% N-(1-Naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich); 2.5% phosphoric acid (Supelco)). NO₂[−] concentration was measured at 550 nm using a Elx 808 IU Ultra Microplate Reader (BioTek Instrument) and calculated based on the standard curve.

Arginase assay

Arginase activity was measured in the lysate of sorted TAM subsets as described before (Liu et al., 2003). Cell pellets were resuspended in 25 μ l of 0.1% Triton X-100 (Sigma-Aldrich) and lysed upon shaking for 30min. 3.5 μ l 10 mM manganese chloride (MnCl_2) (Sigma-Aldrich) was added to 10 μ l lysate and incubated for 10min at 56°C. After, 10 μ l 0.5 M L-arginine (Sigma-Aldrich) was added and incubated at 37°C for 60min. After the reaction was stopped by adding 90 μ l acid mix (H_2SO_4 (96%)/ H_3PO_4 (85%)/ H_2O (1v/3v/7v) (Merck)), 4 μ l isonitrosopropiophenone (ISPP) (9%) (Sigma-Aldrich) was added, incubated for 30min at 95°C and subsequently cooled down to RT for 10min. Urea concentration was measured at 540 nm by Elx 808 IU Ultra Microplate Reader (BioTek Instrument) and calculated based on the standard curve. One unit of enzymatic activity is defined as the amount of enzyme that catalyzes the conversion of 1 μ mol urea per min.

T cell suppression assay

10^5 sorted TAMs were co-cultured in RPMI-based complete medium for 24h with 2×10^5 naive C57BL/6J splenocytes stimulated with 1 μ g/ml anti-CD3 (BD Biosciences) and 2 μ g/ml anti-CD28 (eBioscience). L-NMMA (0.5 mmol/l, Sigma-Aldrich) and/or NorNOHA (0.5 mmol/l, Calbiochem) were added to inhibit iNOS and arginase activity, respectively. Subsequently, cultures were pulsed with [^3H] thymidine, incubated for another 18h and measured using a Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter. Relative percentage suppression of T cell proliferation was calculated as follows: $[(\text{T cell proliferation with or without lactate} / \text{T cell proliferation in presence of TAMs with or without lactate and with or without inhibitor}) / (\text{T cell proliferation in presence of TAMs without lactate} / \text{T cell proliferation without lactate})] \times 100$.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean $\pm$ standard error of the mean (SEM) or mean $\pm$ standard deviation (SD). Data were analysed with Graph-Pad Prism 8.2.1 software and tests. The statistical tests that were used to assess significance are indicated in the figure legends. For data defined by 2 groups, significance was determined by a two-tailed t test, and corrected for multiple testing using the Bonferroni-Dunn method, where necessary. For data defined by 2 group and 2 factors, significance was determined by two-way ANOVA followed by the Bonferroni's multiple comparisons test. Bulk RNA sequencing data were tested using a Likelihood-ratio test, corrected for multiple testing by the Benjamini-Hochberg method. T cell suppression data were tested using a mixed effects analysis with pairwise comparison followed by the Bonferroni's multiple comparisons test. For statistically significant differences, the (corrected) p value is indicated in graphs as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Comparisons found to be non-significant are not shown.