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Lactic Acid and Acidification Inhibit TNF Secretion and Glycolysis of Human Monocytes

Katrin Dietl,† Katrin Renner,‡ Katja Dettmer,† Birgit Timischl,† Karin Eberhart,‡ Christoph Dorn,‡ Claus Hellerbrand,‡ Michael Kastenberger,‡ Leoni A. Kunz-Schughart,§ Peter J. Oefner,† Reinhard Andreesen,§ Eva Gottfried,† and Marina P. Kreutz,†,*

High concentrations of lactic acid (LA) are found under various pathophysiological conditions and are accompanied by an acidification of the environment. To study the impact of LA on TNF secretion, human LPS-stimulated monocytes were cultured with or without LA or the corresponding pH control. TNF secretion was significantly suppressed by low concentrations of LA (≤10 mM), whereas only strong acidification had a similar effect. This result was confirmed in a coculture model of human monocytes with multicellular tumor spheroids. Blocking synthesis of tumor-derived lactate by oxamic acid, an inhibitor of lactate dehydrogenase, reversed the suppression of TNF secretion in this coculture model. We then investigated possible mechanisms underlying the suppression. Uptake of [3-13C2]lactate by monocytes was shown by hyphenated mass spectrometry. As lactate might interfere with glycolysis, the glycolytic flux of monocytes was determined. We added [1,2-13C2]glucose to the culture medium and measured glucose uptake and conversion into [2,3-13C2]lactate. Activation of monocytes increased the glycolytic flux and the secretion of lactate, whereas oxygen consumption was decreased. Addition of unlabeled LA resulted in a highly significant decrease in [2,3-13C2]lactate secretion, whereas a mere corresponding decrease in pH exerted a less pronounced effect. Both treatments increased intracellular [2,3-13C2]lactate levels. Blocking of glycolysis by 2-deoxyglucose strongly inhibited TNF secretion, whereas suppression of oxidative phosphorylation by rotenone had little effect. These results support the hypothesis that TNF secretion by human monocytes depends on glycolysis and suggest that LA and acidification may be involved in the suppression of TNF secretion in the tumor environment. The Journal of Immunology, 2010, 184: 1200–1209.

Monocytes are essential cellular components of the innate immune system. They originate from the bone marrow and circulate in the blood for several days before they extravasate into (inflammatory) tissues or tumor tissues. Recruitment is thought to be regulated by chemotactic factors like MCP-1/CCL-2 or M-CSF (1, 2). Besides phagocytosis and Ag presentation, a major function of monocytes is the secretion of anti- and proinflammatory cytokines (e.g., IL-1, IL-6, and TNF). The secretion of cytokines is induced after monocyte activation with LPS, lipopeptide, flagellin, or tumor cell membranes (3, 4).

Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; 2-DG, 2-deoxyglucose; 2-DG, 2-deoxyglucose; GC-MS, gas chromatography-mass spectrometry; HCl, hydrochloric acid; HIF-1α, Hypoxia-inducible factor-1α; LA, lactic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MCT, monocarboxylate transporter; MCTS, multicellular tumor spheroid; NaL, sodium L-lactate; PFK-1, 6-phosphofructo-1-kinase; ROS, reactive oxygen species.

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inhibit macrophage/monocyte TNF and β form of pro-IL-1 release. In addition, we and others have shown that tumor-derived LA strongly inhibits both the differentiation of monocytes to dendritic cells (23, 24) and the activation of T cells (25). These data demonstrate that lactate can have opposing effects on activation in different types of immune cells.

In this study, we examined the effects of tumor-derived LA and concomitant acidification on human monocytes and investigated the mechanism underlying the suppression of monocyte activation. We found that monocytes take up extracellular lactate, which results in a decreased glycolytic flux and an inhibition of TNF release. Our data suggest that impaired glycolysis may contribute to the immunosuppressive effects of LA in wounds and tumors.

Materials and Methods

Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany). Methanol (liquid chromatography-mass spectrometry) was from Fisher Scientific (Ulm, Germany). Sodium-DL-[2,3-13C2]glucose, [U-13C]pyruvate, [2,3,3,4,4-2H5]glutamic acid, [1,2,3,4-13C4]glucose, and sodium-[3-13C]lactate were from Euriso-top (Saint-Aubin Cedex, France).

Isolation and culture of monocytes

Monocytes were isolated by leukapheresis from healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque and separation by countercurrent centrifugation (J6M-E centrifuge; Beckmann, Munich, Germany) as described previously (26). Monocyte purity was ≥85% as determined by CD14 expression. Isolated monocytes were cultured for 4–20 h in RPMI 1640 supplemented with 2% human AB serum (PAN Bio tech, Aidenbach, Germany), L-glutamine (2 mmol/L), 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies, Karlsruhe, Germany). To minimize effects from donor variation, all experiments were performed with monocytes from at least three different healthy donors.

Activation of monocytes

Monocytes were incubated at a density of 0.5 × 106 cells/ml in the presence of 2, 5, 10, and 20 mM l-LA or sodium l-lactate (NaL). LPS (kindly provided by Prof. M. Freudenberg, Max Planck Institute of Immunobiology, Freiburg, Germany) was added at a final concentration of 100 ng/ml. Control samples were cultured with LPS with or without 1% hydrochloric acid (HCl) to determine the pH of the medium to ~7.1 and ~6.6, corresponding to the pH of media containing 10 and 20 mM L-lactate, respectively. 2-Deoxyglucose (2-DG), a competitive inhibitor of hexokinase, was added to monocyte cultures at concentrations of 1 and 10 mM. Rotenone, an inhibitor of respiratory chain complex I, was added at concentrations of 0.1 and 1 mM.

Determination of cytokines

For the determination of extracellular cytokine concentrations, cell culture supernatants were harvested after 18–20 h of incubation, filtered, and stored at −20°C prior to analysis of cytokines (TNF, IL-6) by means of commercially available ELISAs (R&D Systems, Minneapolis, MN).

Reverse transcription-quantitative real-time PCR

Total RNA was isolated from monocytes using RNeasy Spin Columns from Qiagen (Hilden, Germany). Reverse transcription was performed with 500 ng RNA in a total volume of 20 μl using an M-MLV Reverse Transcriptase from Promega (Mannheim, Germany). To quantify TNF expression, the Mastercycler Ep Realplex (Eppendorf, Hamburg, Germany) was used. For reverse transcription-quantitative real-time PCR, 1 μl cDNA, 1 μl of TNF QuantiTec Primer Assay (Qiagen) or 0.5 μl of 18S primers (10 μM), and 5 μl QuantiFast SYBR Green PCR Kit (Qiagen) in a total of 10 μl were applied. The sequences of the 18S primers were as follows: 18S forward 5′-ACGATTTGGTAGTTAGGAG-3′ and 18S reverse 5′-CCTACG-GAAACTTTGTGGAC-3′.

Analysis of cell viability by Annexin-V-FITC/7-aminoactinomycin D staining

For analysis of cell viability, monocytes were seeded at a concentration of 0.5 × 106 cells/ml in hydrophobic Teflon bags with or without 100 ng/ml LPS and 20 mM LA. After 18–20 h, cells were harvested, washed with PBS, counted, and stained with Annexin-V-FITC and 7-aminoactinomycin D (7-AAD) (both from BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. Flow cytometric analyses were performed on a FACS Calibur (BD Biosciences) using BD CellQuestPro for data acquisition and analysis.

Sample preparation for intra- and extracellular metabolite determination by mass spectrometry

A total of 1 × 106 monocytes were incubated in 6 ml medium in six-well plates for 18 h with or without LPS (100 ng/ml) in the presence of unlabeled 20 mM NaL or 5–20 mM LA. For pH control, culture medium was adjusted to pH 6.6 or 7.1 with HCl. For determination of glycolytic flux, a glucose-free medium supplemented with 2 mM l-Lactate was used. For determination of lactate uptake, [3,4-13C2]lactate was used. In the lactate uptake experiments, the pH was adjusted to ~7.1 with HCl. After incubation cells were scraped off the plate, counted (CASY, Schärfe System, Reutlingen, Germany), washed three times with PBS, again counted, then flash frozen and stored at −80°C. Cell pellets were homogenized by three freeze and thaw cycles and ultrasonification. To calculate total protein amount in the sample, the volume of the cell pellet was determined. A subsample for protein determination (Coomassie, Pierce, Bonn, Germany) was removed and a protease inhibitor mixture was added.

To correct for loss of metabolites during extraction and evaporation, stable isotope-labeled [U-13C]pyruvate was added to the cell pellet before extraction of intracellular metabolites with methanol. The initial extraction was performed with 80% (v/v) aqueous methanol (Merck, Darmstadt, Germany), the second extraction with pure methanol. Extracts were combined, dried by evaporation (Hettich Combi-Dancer, Zinsser Analytic, Frankfurt, Germany), and reconstituted as described below.

For the analysis of excreted metabolites, cells were pelleted by centrifugation, and 2 ml supernatant was removed and stored at −80°C. Prior to analysis by the different hyphenated mass spectrometric methods (i.e., gas chromatography-mass spectrometry [GC-MS] and liquid chromatography-tandem mass spectrometry [LC-MS/MS]), proteins in the supernatants were removed by filtration through 5 kDa cutoff filter tubes (Vivaspin 4, Sartorius, Goettingen, Germany), and internal standards were added as described above.

Analysis of glucose, lactate, and amino acid levels in supernatants by gas chromatography-Ms

Analysis of amino acids was performed as previously described (27). An aliquot of 20 μl cell culture medium was analyzed. To calculate glucose uptake, glucose concentrations in the cell culture medium were analyzed by GC-MS. In addition, lactate levels in the supernatants were determined.

For GC-MS, we used an Agilent model 6890 GC (Agilent, Palo Alto, CA) equipped with a Mass Selective Detector model 5975 Inert XL and an Auto Liquid Injector model 7683B. Separation was carried out on an RXI-5MS column, 30 m × 0.25 mm inner diameter × 0.25 μm film thickness (Restek, Bad Homburg, Germany). Sampling injection was performed in splitless mode at 280°C using an injection volume of 1 μl. The initial oven temperature was set at 50°C, ramped at 8°C/min to 300°C, and held for 10 min. Helium was used as carrier gas at a flow-rate of 0.6 ml/min. The transfer line to the mass spectrometer was kept at 310°C. The mass spectrometer was operated in full scan mode from 50–600 m/z with a scan time of 0.5 s. A 10-μl aliquot of the cell culture medium was spiked with 10 μl of an internal standard solution containing [U-13C5]glucose and [1-13C]lactate (1 mM each). The samples were dried using a vacuum evaporator and derivatized prior to injection. For derivatization, 50 μl of 10 mg/ml methoxyamine hydrochloride in pyridine were added and incubated at 60°C for 60 min, followed by 50 μl of N-methyl-N-(trimethylsilyl)tri- fluoroacetamide for 60 min at 60°C. Quantification was performed using calibration curves corrected with the internal standard. The fragment ion 319 m/z was employed for the area calculation, which provides total glucose in solution, and the fragment ion 219 m/z was used to quantify unlabeled lactate in the solution.

Hyphenated MS analysis of lactate isotope flux for analysis

Intra- and extracellular lactate levels were measured by LC-MS/MS. For determination of relative glucose flux through glycolysis and the pentose-phosphate pathway, the conversion of [1,2-13C2]glucose to [2,3-13C2]lactate, derived from glycolysis, and to [3,4-13C2]lactate, derived from the pentose-phosphate pathway, was analyzed by on-pair LC-MS/MS using sodium-DL-[2,3,3,4,4-2H5]glucose as internal standard. The described method (28) was adapted as follows: LC was performed on an Agilent 1200 SL HPLC system (Agilent) employing a Synergi Hydro
RP column 150 mm × 2.0 mm inner diameter, 4 μm particles, 80 Å pore size. Eluent A was an aqueous solution of 10 mM tributylamine and 15 mM acetic acid (pH 4.95), whereas eluent B was pure methanol. The flow rate was set to 350 μl/min, the column was kept at 50°C. The optimized gradient conditions were as follows: 0–7 min from 0–90% B and hold for 3 min at 90% B. Prior to each run, the column was equilibrated at 100% A for 6 min. The HPLC system was coupled to a 4000 Q TRAP triple-quadrupole ion-trap mass spectrometer (Applied Biosystems/MDI SCIEX, Concord, Ontario, Canada) operated in negative ion mode with selected reaction monitoring. The following selected reaction monitoring transitions were monitored to determine the [13C]label distribution in lactate: 89 > 43, unlabeled lactate; 90 > 43, [1-13C1]lactate; 90 > 44, [2-13C1]lactate and [3-13C1]lactate; 91 > 44, [1,2-13C2]lactate and [1,3-13C2]lactate; 91 > 45, [1,2,3-13C3]lactate.

Enzymatic determination of lactate

Lactate levels in cell culture supernatants were measured by means of an ADVIA1650 (Bayer, Tarrytown, NY) analyzer using reagents from Roche (Mannheim, Germany). These measurements were performed at the Department of Clinical Chemistry at the University of Regensburg, Regensburg, Germany.

Determination of intracellular ATP levels

After incubation, intracellular ATP levels were determined in viable cells immediately after scraping them off the plate and counting by a luciferase-based assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega) according to the manufacturer’s protocol. Standard dilutions of ATP were prepared freshly each time to avoid instability.

Determination of reactive oxygen species

Reactive oxygen species (ROS) were determined with a chemiluminescence assay using a luminometer (Luminometer Sirius, Berthold Detection Systems, Pforzheim, Germany). A total of 1 × 105 monocytes/100 μl were incubated in RPMI 1640 without phenol red (PAN Biotech) supplemented with 2% human AB serum in the presence of 0.1 mg/ml lucigenin. To incubate in RPMI 1640 without phenol red (PAN Biotech) supplemented with 0.1 mg/ml lucigenin. To demonstrate the effect of LA on monocytes in a more in vivo-like model, we used MCTSs generated from the melanoma cell line MelIm that secretes lactate (24). We blocked lactate production in tumor cells with oxamic acid, an inhibitor of lactate dehydrogenase. After 5 d, half of the medium was replaced by a monocyte suspension. In MCTS cocultures, monocytes secreted ∼65% of the TNF as compared with cells cultured without tumor cells (Fig. 1D). Oxamic acid led to a decrease in the lactate content of the coculture from ∼3 mM to ∼0.5 mM and reversed the suppressive effect of the MCTSs on TNF secretion (i.e., the TNF concentration in the coculture supernatant was comparable to the control culture without tumor cell contact). These results prove that tumor-derived LA is a significant inhibitor of monocyte TNF secretion. In contrast, IL-6 secretion was not altered in the MCTS coculture (data not shown).

To clarify whether LA also alters other monocyte functions, we analyzed the impact of LA on the ability of monocytes to inhibit proliferation of MelIm melanoma cells and their capacity to produce ROS. The cytostatic effect of monocytes after LPS activation was weakly affected by LA (Supplemental Fig. 1), whereas LA and acidification strongly suppressed ROS production (Supplemental Fig. 2).

LA and acidification have no effect on cell viability

To demonstrate that the observed inhibitory effect of LA on monocytes did not result from cell death, monocytes were cultured for 18–20 h with or without 20 mM LA in the absence or presence of LPS. After washing and staining with Annexin-V-FITC and 7-AAD, flow cytometric analysis was performed. No differences in the number of viable cells were detected after incubation of monocytes with LA compared with monocytes cultured without LA. These results confirm that the reduced levels of TNF and IL-6 in the culture supernatants were not a result of increased cell death (Fig. 2).

The administration of 10 mM and 20 mM LA decreased the pH of the cell culture medium from 7.5 to 7.1 and 6.6, respectively. To study the effect of acidification on monocyte survival, we decreased the pH of the culture medium by the addition of HCl. Cell survival of either stimulated or unstimulated monocytes was altered by acidification (Fig. 2).
The effect of LA is partially pH dependent

To investigate whether acidification of the medium, lactate, or a combination of both is responsible for the observed reduction in TNF secretion, we studied all conditions in parallel. Acidification to pH 7.1 had only a minimal effect on the TNF secretion (Fig. 3A; Student t test, \( p < 0.05 \); \( **p < 0.005 \) compared with the LPS control). The relative amount of TNF mRNA was determined after 4 h. Only 10 mM LA caused a statistically significant inhibition of the TNF mRNA expression after 4 h (B; Student \( t \) test, \( p = 0.01 \); control, LPS, LPS + 10 mM LA, \( n = 4 \), mean \( \pm \) SEM; LPS + 5 mM LA, \( n = 2 \)). In addition, IL-6 was measured by ELISA in the monocyte supernatants. A total of 20 mM LA was necessary to significantly inhibit IL-6 secretion compared with the LPS control. Data represent means \( \pm \) SEM (\( n = 7 \)) (C; Student \( t \) test, \( **p < 0.005 \)). MCTS of the melanoma cell line MelIm were generated in the presence or absence of 30 and 90 mM oxamic acid. On day 5, half of the medium was replaced by a monocyte suspension, and the coculture was stimulated with 100 ng/ml LPS. Control monocytes were cultured without tumor cells in the presence of LPS. TNF levels were significantly lower in the coculture compared with the monocyte control culture (D; Student \( t \) test, \(* *p < 0.005 \)). Addition of oxamic acid increased TNF levels back to the control level. Lactate levels were analyzed with a clinical routine procedure. Data represent means \( \pm \) SEM (\( n \geq 5 \)).

Monocyte activation alters energy metabolism

It is known that the activation of immune cells such as macrophages or dendritic cells depends on glycolysis (32, 33). Therefore, we hypothesized that monocyte activation may also depend on both glucose uptake and glycolysis. We analyzed glucose metabolism and acidification resulted in a significantly (Student \( t \) test, \( p = 0.034 \)) increased intracellular level of \([3-^{13}C]\)lactate.

These results demonstrate the uptake of extracellular lactate by monocytes. Uptake was further increased in the presence of protons. This suggests that lactate was at least partly transported into monocytes by monocarboxylate transporters (MCTs), which co-transport protons and lactate anions through the plasma membrane depending on the concentration gradient (30, 31). It is known that MCTs are expressed in human monocytes, and we confirmed the expression of MCT-1 and MCT-4 by RT-PCR. The expression was unaffected by extracellular LA (data not shown).

Uptake of lactate by monocytes

In an effort to elucidate the molecular mechanisms underlying the inhibitory effect of LA on monocytes, we investigated the uptake of lactate by means of LC-MS/MS. Cells were incubated for 18 h with sodium-[\(^{13}C\)]lactate in the absence or presence of mere acidification by HCl and LPS. Control cells were cultured without sodium-[\(^{13}C\)]lactate. Quantification of intracellular [\(^{13}C\)]lactate levels revealed that lactate was taken up by monocytes constitutively without stimulation (Fig. 4). Mild acidification (pH 7.1) of the culture medium as well as LPS stimulation alone had only a slight effect on the uptake. However, the combined administration of LPS and acidification resulted in a significantly (Student \( t \) test, \( p = 0.034 \)) increased intracellular level of [\(^{3-^{13}}\)C]lactate.
High extracellular LA levels inhibit glycolysis in monocytes

To clarify whether addition of extracellular LA interferes with monocyte energy metabolism, we investigated the glucose uptake by activated monocytes. Monocytes were incubated with LPS for 18 h in the absence or presence of LA or NaL. Extracellular LA reduced the glucose uptake of stimulated monocytes in a dose-dependent manner (Fig. 5B), whereas lowering the medium pH to 6.6 was less pronounced.

To assess whether reduced glucose uptake enhanced the uptake of alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or its metabolite glutamate after monocyte stimulation or incubation with LA (Fig. 5C).

Additionally, we analyzed whether the glycolytic flux in monocytes was influenced by LA. To address this issue, we incubated monocytes with [1,2-13C2]glucose. We determined by LC-MS/MS the conversion of [1,2-13C2]glucose into [2,3,13C3]lactate, which originates from glycolysis, and into [1,3,13C3]lactate and [3,13C3]lactate, both of which originate from the pentose-phosphate pathway. Because the levels of lactate produced by the pentose phosphate pathway were ~2–5% of the level originating from glycolysis, only the [2,3,13C3]lactate is shown in Fig. 6A and 6B. The addition of extracellular LA dose-dependently resulted in lower extracellular levels of [2,3,13C3]lactate, whereas the level of intracellular [2,3,13C3]lactate increased (Fig. 6A, 6B). Furthermore, the addition of 5–20 mM LA increased the amount of unlabeled intracellular [13C]lactate, suggesting an influx of LA into monocytes. Lowering the pH of the culture medium to 6.6 also resulted in a decrease in extracellular levels of [2,3,13C3]lactate and increased intracellular [2,3,13C3]lactate levels, albeit to a lesser extent compared with 20 mM LA. These results can be explained by the findings of Poole and Halestrap (30), showing that lactate transport depends on MCTs. These transporters cotransport protons and lactate anions through the plasma membrane depending on the concentration gradient (31). Accordingly, high levels of extracellular LA invert the lactate gradient between extracellular milieu and cytoplasm and in turn lead to an influx of lactate.

The addition of 10 mM 2-DG, a competitive inhibitor of hexokinase, the enzyme catalyzing the first and rate-limiting step of glycolysis, also led to a decrease in extracellular levels of [2,3-13C2]lactate, whereas intracellular lactate levels remained unchanged (Fig. 6A, 6B).

Apart from energy production via glycolysis, ATP is produced through oxidative phosphorylation. Therefore, we analyzed oxygen consumption of monocytes with or without stimulation. LPS stimulation resulted in a decreased oxygen consumption, indicating a shift from respiration to anaerobic glycolysis during monocyte activation (Fig. 6C). The reduced glycolytic flux in response to the addition of LA was paralleled by an induction of respiration during the first 2–3 h. In contrast to LA, acidification of the culture medium suppressed the respiration of monocytes.

In line with these results, 2-DG decreased the glycolytic flux and in parallel increased oxygen consumption (Fig. 6D). These data suggest that an inhibition of glycolysis during monocyte activation leads to an increased oxidative phosphorylation as monocytes try to rebalance their disturbed energy supply via increased respiration.

To investigate whether the described effect of LA on glucose metabolism also affects intracellular ATP levels, we incubated...
To further investigate the impact of impaired glycolysis on monocyte TNF secretion, we incubated monocytes with 2-DG and LPS. The addition of 2-DG led to a dose-dependent inhibition of TNF secretion and diminished ATP levels (Fig. 7C).

Inhibition of glycolysis by 2-DG inhibits monocyte TNF secretion

To examine the importance of oxidative phosphorylation for TNF production, we incubated monocytes with rotenone, an inhibitor of the respiratory chain complex I. Rotenone decreased the oxygen consumption (Fig. 6D) and lowered the ATP level (Fig. 7C) of human monocytes but had only a marginal effect on TNF secretion (Fig. 7B).

Our results show that the disturbance of the glycolytic flux but not an interruption of the respiratory chain results in an impaired monocyte TNF secretion. This demonstrates that the TNF secretion of monocytes strongly depends on glycolysis.

Discussion

Low pH and lactate accumulation are characteristic features of the tumor microenvironment and inflammatory conditions in wounds. Little is known about the biological effects of pH and lactate on infiltrating monocytes, neutrophils, and T cells. In this report, we investigated the effect of LA and pH variation on human monocytes and analyzed the underlying mechanisms. LA decreased in a dose-dependent manner the capacity of monocytes to produce TNF. A low pH (<6.5) in the absence of LA also inhibited the secretion of TNF. Accordingly, paracrine lactate secretion in a tumor spheroid coculture model led to an inhibition of TNF secretion and this effect was reversed by oxamic acid, an inhibitor of lactate dehydrogenase. However, the inhibition of IL-6 was only found when we added high LA concentrations and was not detected in the coculture model with tumor spheroids. Intratumoral analyses revealed that LA concentrations can reach values up to 40 mM (34). However, in our spheroid cultures, LA levels normally did not exceed 10 mM. As TNF seems to be more sensitive to the suppression compared with IL-6, LA may reduce TNF but not IL-6 secretion in vivo under pathophysiological conditions. Similar results for TNF secretion were obtained by Douvdevani et al. (22), who studied the influence of peritoneal dialysis fluid on human macrophages and monocytes and suggested that the inhibitory effect of the dialysate is due to the low pH and high lactate content. Bidani et al. (35) found that a pH ≤7 suppressed the TNF release by rabbit alveolar macrophages. In contrast, Bellocq et al. (36) and Jensen et al. (37) reported that pH 7.0 and LA elevated TNF in rat macrophages. Shime et al. (14) recently demonstrated that LA enhanced the transcription and production of IL-23 in a pH-dependent manner. In addition, a recent paper by Samuvel et al. (21) reported that preincubation of U937 or macrophages with sodium lactate resulted in an increase in the secretion of inflammatory cytokines like IL-6 and IL-8. However, culture conditions and cells used for analysis in both studies differed from our experimental setting. Therefore, one may speculate that the observed discrepancies are due to species variations, different cell types used for the analysis, or differences in the culture conditions. In our tumor spheroid model, we investigated the effect of LA on freshly infiltrating blood monocytes during short-time exposure to LA, whereas the data by Samuvel et al. (21) may reflect a situation in a spheroid model, we investigated the effect of LA and pH variation on human monocytes and the effect of lactate in the tumor environment.

To exclude the possibility that the inhibition of cytokine secretion by LA is based on the induction of apoptosis, we performed AnnexinV/7-AAD staining. In our hands, pH values in the range of 6.5–7.0 and concentrations of LA up to 20 mM had no effect on cell viability. Accordingly, Jensen et al. (37) demonstrated that lactic acidosis at 15 mM did not reduce cell viability, whereas Bidani et al. (35) showed that the viability of macrophages declined only at pH values ≤6.0.
Next, we investigated the mechanism underlying the inhibitory effect and analyzed whether monocytes take up lactate. At physiological pH, LA almost entirely dissociates to the lactate anion, which cannot cross the plasma membrane by free diffusion but requires a transport system. MCTs cotransport protons and lactate and other monocarboxylates dependent on the gradient between the extracellular and intracellular milieu (30, 31). The expression of MCTs has been demonstrated in several immune cells including monocytes (38).

Without stimulation, we found a constitutive uptake of [3-13C]lactate, which was further enhanced by the combined treatment with LPS and mild acidification of the medium. This indicates the involvement of MCTs in the uptake of lactate by activated monocytes. A pH-dependent uptake of lactate was also demonstrated by Loike.
nrotenone significantly decreased ATP levels in monocytes (cell lysates after 18 h and found that 10 mM 2-DG as well as 0.1 and 1 mM rotenone caused a significant decrease in TNF levels; Student’s test, p < 0.05). Monocytes were incubated with 100 ng/ml LPS for 18–20 h in the absence or presence of 1 and 10 mM 2-DG, an inhibitor of glycolysis, or 0.1 and 1 mM rotenone, an inhibitor of respiratory chain complex I. TNF was measured after 18–20 h in the supernatant by ELISA; 10 mM 2-DG but not rotenone significantly decreased ATP levels in monocytes (mean ± SEM, n = 6; Student t test, *p < 0.05; **p < 0.005; ***p < 0.0005).

We hypothesized that the uptake and accumulation of lactate in monocytes would suppress the glycolytic flux and thereby impair monocyte TNF secretion. Monocyte, macrophage, and dendritic cell activation is energy-dependent and involves stimulation of glycolysis (5, 32, 33). LPS induces 6-phosphofructo-2-kinase, which in turn synthesizes fructose 2,6-bisphosphate, the most potent stimulator of 6-phosphofructo-1-kinase (PFK-1) (5). Elevated activity of PFK-1 was also observed in activated rat macrophages together with an increased lactate release (32). Therefore, PFK-1 expression and regulation seem to be important for monocyte activation, and monocytes may upregulate glycolysis via regulatory enzymes such as PFK-1.

Next, we analyzed uptake and metabolism of glucose and its importance for monocyte TNF secretion. After stimulation with LPS, we found no significant increase in glucose uptake, but an increased conversion of glucose into lactate. These findings suggest an accelerated glycolysis to meet the increased energy demand after LPS stimulation. In line with this hypothesis, it is known that monocyte activation requires stimulation of glycolysis via HIF-1α (5, 8). In addition, data by Roiniotis et al. (41) show that not only monocyte activation but also differentiation of monocytes into macrophages is associated with enhanced glycolysis.

In our hands, glucose uptake was reduced in response to extracellular lactate and also resulted in a decreased secretion of [2,3-13C2]lactate produced from [1,2-13C2]glucose. Acidification in the range of pH 6.6 had a moderate effect on both parameters. The reduced glucose uptake by monocytes and the lowered levels of extracellular [2,3-13C2]lactate indicate that LA and concomitant acidification inhibit the glycolytic flux.

Besides the lowered glycolytic flux, high extracellular LA concentrations led to a significant accumulation of intracellular lactate. The accumulation of intracellular lactate was most likely caused by an influx of extracellular lactate and a block in the efflux of [2,3-13C2]lactate produced from [1,2-13C2]glucose during glycolysis. MCTs transport lactate between the extracellular and intracellular milieu, and this transport is crucially dependent on the presence of protons (30, 31). Accordingly, the addition of extracellular LA and acidification did not only lead to a block in the export of [2,3-13C2]lactate by MCTs, but also to an influx of lactate into the cell.

It is known that lactate inhibits PFK-1, a key regulatory enzyme of glycolysis, and favors the dissociation of active PFK-1 tetramers into less active dimers (42). In the light of our findings, intracellular lactate accumulation could suppress PFK-1 and thereby reduce the glycolytic flux in monocytes.

### Table I. Summary of the presented data concerning the suppression of 20 mM LA and acidification (pH 6.6) relative to the LPS control

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATP levels (%)</th>
<th>TNF in supernatant (%)</th>
<th>Glucose uptake (%)</th>
<th>Extracellular lactate (%)</th>
<th>Intracellular ATP (%)</th>
<th>O2 consumption (%)</th>
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### Figure 7

**Figure 7.** Inhibition of glycolysis by 2-DG but not suppression of respiration by rotenone disturbs monocyte TNF secretion and the possible impact of intracellular ATP. To determine ATP levels, freshly isolated human monocytes were stimulated with 100 ng/ml LPS for 18–20 h in the absence or presence of 5–20 mM LA or 20 mM NaL. In addition, the medium pH was lowered to the respective pH (pH 7.1 or 6.6). Intracellular ATP levels were measured in cell lysates. Data represent the mean of five independent experiments ± SEM. Data for 20 mM LA, pH 6.6, and 20 mM NaL differ significantly from the LPS control (A; Student t test, *p < 0.05). Monocytes were incubated with 100 ng/ml LPS in the absence or presence of 1 and 10 mM 2-DG, an inhibitor of glycolysis, or 0.1 and 1 mM rotenone, an inhibitor of respiratory chain complex I. TNF was measured after 18–20 h in the supernatant by ELISA; 10 mM 2-DG but not rotenone caused a significant decrease in TNF levels (B; mean ± SEM, n = 6; Student t test, *p < 0.05). To analyze a possible correlation between intracellular ATP levels and TNF, we measured intracellular ATP levels in cell lysates after 18 h and found that 10 mM 2-DG as well as 0.1 and 1 mM rotenone significantly decreased ATP levels in monocytes (C; mean ± SEM, n = 4; Student t test, *p < 0.05; **p < 0.005).

et al. (39) in murine macrophages, and they also described that the lactate uptake was greater in elicited than in resident macrophages, suggesting a role of activation in the regulation of the transporters. Beside MCTs, other transporters might also be involved in lactate uptake and export in a proton-independent manner (e.g., via the sodium coupled low-affinity transporter for monocarboxylates) (40).
Based on the hypothesis that glycolysis is essential for immune cell activation, MCTs also play a crucial role because lactate has to be exported from the cell to allow an undisturbed glycolytic flux. Accordingly, blocking of MCT-1 by pharmacological intervention suppressed the glycolytic flux in T cells and resulted in a decreased T cell proliferation and activation (43).

Inhibition of hexokinase activity by the addition of 2-DG suppressed glycolysis and TNF release by human monocytes. Inclusion of monocytogen with rotenone, an inhibitor of the oxidative phosphorylation, exerted little effect on LPS-stimulated TNF secretion (8). Similar results were obtained by Jantsch et al. (33), who blocked dendritic cell activation by the addition of 2-DG and demonstrated that activation crucially depends on glycolysis even in the presence of oxygen. Furthermore, data by Cramer et al. (8) suggest a basic role of glycolysis for the inflammatory response of myeloid cells as functional inactivation of HIF-1α significantly reduced lactate production and TNF secretion.

In summary, our results show that TNF secretion of monocytes depends both on glycolysis and export of its end product lactate. Tumor-derived LA and concomitant acidification could modulate and disturb monocyte cytokine production by blocking lactate export and glycolytic flux. Together with other observations on the effects of LA on T cells, these results suggest that selective targeting of glycolysis in tumor cells could restore immune cell activation and immune response against tumor cells.

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Disclosures

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References


