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

Katrin Dietl, Kathrin 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

J Immunol 2010; 184:1200-1209; Prepublished online 21 December 2009; doi: 10.4049/jimmunol.0902584
http://www.jimmunol.org/content/184/3/1200

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/12/22/jimmunol.0902584.DC1

References
This article cites 43 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/184/3/1200.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Lactic Acid and Acidification Inhibit TNF Secretion and Glycolysis of Human Monocytes

Katrin Dietl,*† Kathrin 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 [3-13C]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; GC-MS, gas chromatography-mass spectrometry; HCl, hydrochloric acid; HIF-1α, Hypoxia-inducible factor-1α (HIF-1α) appears to be essential for the regulation of glycolysis in myeloid cells and controls the inflammatory response by switching the cell metabolism to glycolysis (8).

The function of tumor-infiltrating monocytes seems to be depressed (9), and intratumoral monocytes show an altered cytokine profile (e.g., suppression of TNF and an increase in IL-10) (10). Several tumor-derived factors have been implicated as potential modulators of monocyte activation including PGE2, gangliosides, and lactic acid (LA) (11–14).

Lactate accumulates in the tumor microenvironment due to the accelerated glycolysis of tumor cells, a phenomenon known as aerobic glycolysis, or Warburg effect (15). In addition, high lactate levels are found under inflammatory conditions (16). Glycolytic enzymes such as lactate dehydrogenase and glucose transporters are upregulated by hypoxia/HIF-1α or oncogenic transformation (17, 18). As a consequence, tumor cells produce lactate and secrete lactate together with protons, which in turn lowers the pH of the tumor environment (19). It has been reported that extracellular lactate in wounds stimulates macrophages to secrete vascular endothelial growth factor and TGF-β, both known to be immunosuppressive factors (16, 20). In addition, data from Shimé et al. (14) demonstrated that LA upregulates transcription and secretion of IL-23, a tumor-promoting cytokine, in human monocytes. Sauvè et al. (21) have shown recently that preincubation of myeloid U937 and monocyte-derived macrophages with lactate increases the expression of inflammatory cytokines (e.g., IL-6 and IL-8). However, Douvdéan et al. (22) have described that a low pH and high concentration of lactate in peritoneal dialysis fluids...
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 impairment of 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-[2,3-3H]gluconate, [U-13C]pyruvate, [2,3,3,4,4-2H5]glutamic acid, [1,2-13C2]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; Beckman, 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 Biotech, 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 × 10^6 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) added to titrate the pH of the medium to ~7.1 and ~6.6, corresponding to the pH of media containing 10 and 20 mM LA, 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 µM.

**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 RNaseasy 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 eDNA, 1 µl of TNF QuantiTect 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′-ACGATTGTCAGGGTTTGGAG-3′ and 18S reverse 5′-CCTAGGCAGAACACTGGTGTCGAGC-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 × 10^6 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 FACSCalibur (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 × 10^6 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, and additionally a medium supplemented with 2 mMol/l [1,2-13C2]glucose was used. For determination of lactate uptake, [3-13C]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 above.

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). Sample injection was performed in splitless mode at 280°C with 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 [1,2-13C2]glucose and [1,3-13C]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) trifluoroacetamide for 60 min at 60°C. Quantification was performed using calibration curves corrected with the internal standard. The fragment ion 319 m/z was used for quantification. The fragment ion 219 m/z was used to quantify unlabeled lactate in the sample.

**Hyphenated MS analysis of lactate isotopes for flux 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 [3-13C]lactate or [1,3-13C]lactate, derived from the pentose-phosphate pathway, was analyzed by on-line LC-MS/MS using sodium-DL-[2,3,3,4-3H5]glutamic acid 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-
RPL column 150 mm × 2.0 mm inner diameter, 4 μm particle 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/MDS 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 > 44, [1-13C1]lactate; 93 > 44, [1,2-13C2]lactate; 91 > 44, [1,2-13C2]lactate and [1,3-13C2]lactate; 91 > 45, [2,3-13C2]lactate; and 92 > 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 Luminescence 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 × 10^5 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 determine the amount of ROS, monocytes alone showed only background levels of [3H]thymidine incorporation (677 ± 6 cpm). Monitoring of tumor cell cytostasis

For determination of the cytostatic activity of monocytes, 30,000 MelIm melanoma cells/well and 100,000 monocytes/well were co-cultured in 96-well microtiter plates in the presence of 2.5, and 10 mM LA, respectively, as well as in medium titrated with HCl to pH ~7.1 (corresponding to the pH of the medium supplemented with 10 mM LA) in a final volume of 200 μL/well. Monocytes were stimulated with 100 ng/mL LPS. After 30 min, cells were pulsed with 0.0185 MBq [methyl-3H]thymidine/well (Amer sham Pharmacia, Piscataway, NJ) and harvested after 18 h onto UniFilter plates using a Wallac harvester (PerkinElmer, Gaithersburg, MD). Incorporation of [3H]thymidine was determined by means of a Wallac Betaplate counter (PerkinElmer). Monocytes alone showed only background levels of [3H]thymidine incorporation (677 ± 355 cpm).

Online measurement of oxygen concentration in cell culture

The SDR SensorDish Reader (PreSens Precision Sensing, Regensburg, Germany) is a 24-channel oxygen and pH meter. The optical oxygen (OxoDish) sensor is integrated at the bottom of each well of a 24-well multidish. The sensors are luminescence dyes embedded in an analyte-sensitive polymer. The luminescence lifetime of these dyes depends on the amount of analyte. The sensors are read out noninvasively through the bottom of the multidish by the SensorDish Reader. The resulting signal is converted automatically to the respective parameter (dissolved oxygen) using calibration parameters stored in the software. A total of 5 × 10^6 monocytes were incubated in 1 mL medium. The SensorDish Reader was used in the incubator for the whole duration of the 16-h cultivation period, and measurements were performed in 30 s intervals.

Results

TNF production of monocytes is inhibited by LA

To investigate the influence of LA on TNF production by human monocytes, we stimulated monocytes with LPS for 18–20 h in the absence or presence of LA. A significant effect of LA on TNF secretion was detected at a concentration as low as 5 mM (Student t test, p < 0.05; Fig. 1A). At the transcriptional level, quantitative RT-PCR revealed that 10 mM LA significantly (Student t test, p = 0.01) decreased the amount of TNF mRNA after 4 h of incubation compared with the LPS control (Fig. 1B). In contrast, 20 mM LA was needed to strongly suppress IL-6 compared with the LPS control (Fig. 1C; Student t test, p < 0.005).

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 confirmed 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 neither stimulated nor 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), whereas pH 6.6 decreased TNF levels significantly (Fig. 3B; Student t test, p = 0.01). The sodium salt of LA, NaL, did not change the pH of the culture medium, but it decreased the release of TNF in three out of four experiments. However, this effect was not statistically significant (Fig. 3A, 3B). When we combined both treatments and added NaL under low pH conditions, TNF secretion was inhibited. Titration of the culture medium back to pH 7.6 reversed the effect. These findings demonstrate that LA and, to a lesser extent, NaL inhibit the secretion of the proinflammatory cytokine TNF by monocytes. At low LA concentrations, both lactate and mild acidification are necessary for the inhibition of TNF secretion; at higher LA concentrations, the acidification seems to be the dominant factor.

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-[3-13C]lactate in the absence or presence of mere acidification by HCl and LPS. Control cells were cultured without sodium-[3-13C]lactate. Quantification of intracellular [3-13C]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 [3-13C]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).

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 with or without LPS stimulation. Fig. 5A shows that glucose uptake was not significantly increased after LPS activation; however, extracellular lactate levels were significantly elevated (Fig. 5A; p = 0.0001). It appeared that endogenous LA could also modulate TNF secretion. To that end, we determined the secretion of TNF and lactate after LPS stimulation in a time-course experiment. TNF secretion was detected early after LPS stimulation with a maximum at ~4 h. Lactate showed slower kinetics. After 17 h, lactate levels had strongly increased, and TNF production had decreased (Supplemental Fig. 3). These data suggest that endogenous lactate production might indeed be involved in a negative feedback regulation.
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 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 alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients, we determined the amount of glutamine in the culture medium but found no change in the level of glutamine or alternative nutrients.

Additionally, we analyzed whether the glycolytic flux in monocytes was influenced by LA. To address this issue, we incubated monocytes with [1,2-13C]glucose. We determined by LC-MS/MS the conversion of [1,2,3-13C3]glucose into [2,3-13C2]lactate, which originates from glycolysis, and into [1,3-13C2]lactate and [3,3-13C2]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 [2,3-13C2]lactate is shown in Fig. 6A and 6B. The addition of extracellular LA dose-dependently resulted in lower extracellular levels of [2,3-13C2]lactate, whereas the level of intracellular [2,3,3-13C3]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 [2,3,3-13C3]lactate and increased intracellular [2,3,3-13C3]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 [2,3,3-13C3]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...
monocytes with or without LPS and LA and determined ATP with a bioluminescent assay after 18 h. Interestingly, the ATP level of nonstimulated and stimulated monocytes did not significantly differ from each other, but 20 mM LA diminished intracellular ATP levels (Fig. 7A). Acidification lowered the ATP level to a similar extent compared with LA. However, this effect most likely depended more on the decreased respiration rate and to a lesser extent on the diminished glycolysis, as LA decreased glycolysis more efficiently (in terms of glucose uptake and extracellular lactate). A summary of the presented data and a comparison between the effects of LA and acidification is shown in Table I. We conclude 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 where monocytes/macrophages are exposed to lactate for a prolonged time period and become adapted to the 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 Annexin/V-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.

**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 (<6.6) 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 Douvddevani 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 where monocytes/macrophages are exposed to lactate for a prolonged time period and become adapted to the lactate in the tumor environment.

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.

**FIGURE 5.** Metabolic changes during monocyte activation and inhibition of glucose uptake by LA. Monocytes were incubated with or without LPS for 18 h. Glucose and lactate levels in the supernatants were determined by GC-MS. Glucose uptake was calculated relative to the medium control without monocytes. Data are means ± SD, n = 3; Student t test. The influence of extracellular LA (5–20 mM), 20 mM NaL, or pH 6.6 on glucose uptake was analyzed by GC-MS and calculated relative to the medium control (B; mean ± SD, n = 3; Student t test, *p < 0.05, **p < 0.005). Molar levels of glutamine and its metabolite glutamate in the supernatants were analyzed by GC-MS (C; mean ± SD, n = 3; no significant difference between LPS and LPS + LA was detected).
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 $[3-^{13}\text{C}]$ 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.

**FIGURE 6.** Inhibition of the glycolytic flux and modulation of oxygen consumption by LA, 2-DG, and rotenone. The effect of extracellular LA on the glycolytic flux was measured by incubating monocytes in glucose-free medium supplemented with $[1,2-^{13}\text{C}]$glucose in the presence or absence of 100 ng/ml LPS and LA, NaL, or 2-DG (10 mM), an inhibitor of glycolysis. The secretion of $[2,3-^{13}\text{C}]$lactate into the culture supernatant (A) and the level of accumulated intracellular $[2,3-^{13}\text{C}]$lactate (B) resulting from $[1,2-^{13}\text{C}]$glucose metabolism were determined by LC-MS/MS. Monocytes were cultured in the presence or absence of LPS and/or 5–20 mM LA. Extracellular lactate was significantly diminished for 10 mM LA, 20 mM LA, pH 6.6, NaL, and 2-DG compared with the LPS control (A; mean $\pm$ SD, $n$ = 3; Student $t$ test, $p < 0.05$; $**p < 0.005$; $***p < 0.0005$). Intracellular lactate was significantly elevated for 5–20 mM LA and pH 6.6 compared with the control (B; mean $\pm$ SD, $n$ = 3; Student $t$ test, $p < 0.05$; $**p < 0.005$; $***p < 0.0005$). To analyze the effect of LA and pH 6.6 on oxygen consumption, monocytes were incubated in RPMI medium for 16 h. Oxygen consumption was analyzed in 24-well multidishes (C; OxoDish). One representative experiment out of four is shown. In addition, the effect of 10 mM 2-DG and 0.1 $\mu$M rotenone, an inhibitor of the respiratory chain complex I, on LPS-stimulated monocytes was studied. As a control, monocytes without LPS stimulation were used (D). One representative experiment out of three is shown.
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 (mean ± SEM, n = 6; 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 0.1 and 1 mM NaL differ significantly from the LPS control (*p < 0.05). To analyze a possible correlation between intracellular ATP and TNF secretion, and the possible impact of intracellular lactate on ATP levels in monocytes, we measured intracellular ATP levels in 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).

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 activation but also differentiation of monocytes into macrophages is associated with enhanced glycolysis.

In our hands, glucose uptake was reduced in response to extracellular LA 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.
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. Inactivation of monocyes with rotenone, an inhibitor of the oxidative phosphorylation, exerted little effect on LPS-stimulated TNF secretion. 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.

Acknowledgments
We thank Alice Peuker, Gabi HartmannsgruBer, Monika Wehrstein, Alexandra Müller, Ireen Ritter, Jan Linnemann, and Nadine Nünberger for excellent technical support.

Disclosures
The authors have no financial conflicts of interest.

References
separated human granulocytes, lymphocytes, and monocytes. *J. Histochem.
Cytochem.* 52: 1483–1493.


slc5a12 as a sodium-coupled low-affinity transporter for monocarboxylates

41. Roiniotis, J., H. Dinh, P. Masendycz, A. Turner, C. L. Elsegood, G. M. Scholz,
and J. A. Hamilton. 2009. Hypoxia prolongs monocyte/macrophage survival and
enhanced glycolysis is associated with their maturation under aerobic conditions.

42. Costa Leite, T., D. Da Silva, R. Guimarães Coelho, P. Zancan, and M. Sola-Penna.
2007. Lactate favours the dissociation of skeletal muscle 6-phosphofructo-1-kinase
tetramers down-regulating the enzyme and muscle glycolysis. *Biochem. J.* 408:
123–130.

43. Murray, C. M., R. Hutchinson, J. R. Bantick, G. P. Belfield, A. D. Benjamin,
Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nat.