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Lactic acid delays the inflammatory response of human monocytes

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ABSTRACT

Lactic acid (LA) accumulates under inflammatory conditions, e.g. in wounds or tumors, and influences local immune cell functions. We previously noted inhibitory effects of LA on glycolysis and TNF secretion of human LPS-stimulated monocytes. Here, we globally analyze the influence of LA on gene expression during monocyte activation. To separate LA-specific from lactate- or pH-effects, monocytes were treated for one or four hours with LPS in the presence of physiological concentrations of LA, sodium lactate (NaL) or acidic pH. Analyses of global gene expression profiles revealed striking effects of LA during the early stimulation phase. Up-regulation of most LPS-induced genes was significantly delayed in the presence of LA, while this inhibitory effect was attenuated in acidified samples and not detected after incubation with NaL. LA targets included genes encoding for important monocyte effector proteins like cytokines (e.g. TNF and IL-23) or chemokines (e.g. CCL2 and CCL7). LA effects were validated for several targets by quantitative RT-PCR and/or ELISA. Further analysis of LPS-signaling pathways revealed that LA delayed the phosphorylation of protein kinase B (AKT) as well as the degradation of IκBα. Consistently, the LPS-induced nuclear accumulation of NFκB was also diminished in response to LA. These results indicate that the broad effect of LA on gene expression and function of human monocytes is at least partially caused by its interference with immediate signal transduction events after activation. This mechanism might contribute to monocyte suppression in the tumor environment.

1. Introduction

Monocytes express a range of pattern-recognition receptors like toll-like receptors (TLRs), which are responsible for the activation of monocyte effector functions. Agonists for TLRs include microbial components, e.g. lipopolysaccharide (LPS) [1], and also endogenous danger signals which can be found amongst others in tumors [2]. Upon ligand binding, signaling cascades are initiated which eventually lead to the degradation of IκBα and to the nuclear transition of the transcription factor nuclear factor κ B (NFκB). In addition, several other signaling cascades such as MAPK signaling pathways and the PI3K/AKT signaling pathway are stimulated by TLRs [1].

Although the effector mechanisms of monocytes provide powerful means to fight tumors, tumor-infiltrating monocytes often exhibit protumoral characteristics including the decreased secretion of TNF or an increased production of IL-10 or IL-23. The altered phenotype of monocytes is thought to result from the presence of soluble factors like hyaluronan or lactic acid (LA) in the tumor milieu [3,4].

Since tumor cells largely limit their energy generation to glycolysis they produce lactate, secrete it together with protons into the microenvironment and acidify their stroma [5]. We demonstrated that LA and in part also the corresponding acidification inhibit TNF secretion and glycolysis of human monocytes [6]. In addition, high lactate concentrations in combination with low pH have been found to reduce the production of TNF and IL-1β by macrophages/monocytes [7]. We and others have reported an inhibition of the differentiation of monocytes to dendritic cells in the presence of LA [8,9]. In addition, we reported LA to strongly inhibit the activation of T cells [10]. However, LA or lactate have also been described to stimulate the expression of IL-23, IL-6 or IL-8 in mononuclear cells [4,11,12]. Furthermore, LA drives the polarization of tumor-associated macrophages [13].

The diverse effects of LA on various immune cells suggest that LA or lactate may influence widely used signaling pathways. Indeed both molecules have been demonstrated to influence several MAP kinases, NFκB signaling or the PI3K/AKT pathway [12,14,15].

Abbreviations: LA, L-lactic acid; NaL, sodium L-lactate; HCl, hydrochloric acid; GO, gene ontology; PTX3, pentraxin 3; GEM, GTP-binding protein overexpressed in skeletal muscle; EGR2, early growth response 2.

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Based on our findings on the inhibition of TNF secretion and glycolysis by LA in human monocytes [6] we now aimed to globally characterize the effect of LA on monocytes. Using whole genome microarray analysis we observed a broad transient negative effect of LA and the associated acidification on the LPS-induced gene expression. Furthermore, the presence of LA delayed LPS-induced signaling pathways. These results indicate that LA profoundly influences the biology of human monocytes which might contribute to immune suppression in tumors.

2. Material and methods

2.1. Chemicals

Unless otherwise noted, chemicals were purchased from Sigma (Deisenhofen, Germany).

2.2. Isolation and culture of monocytes

Monocytes were obtained from healthy donors, as described previously [16], the isolation was approved by the local ethics committee. 1,66 × 10^6 monocytes/ml were cultured 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 Gibco, Karlsruhe, Germany) in the presence of 10 and 20 mM L-lactic acid (LA) or 20 mM sodium L-lactate (NaL). LPS (from *Salmonella abortus equi* S-form, Enzo Life Sciences, Lörrach, Germany) was added to a final concentration of 100 ng/mL. Furthermore, monocytes were incubated with LPS in combination with 1% hydrochloric acid (HCl) to titrate the pH of the medium to ~7.1 or ~6.6, corresponding to the pH of media containing 10 or 20 mM LA, respectively.

2.3. Determination of cytokines

Detection of TNF and CCL2 in culture supernatants was performed using R&D systems (Minneapolis, USA) ELISA kits.

2.4. Preparation of RNA

Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured with ND-1000 NanoDrop Spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany) and quality was controlled on agarose gels or using Agilent Bioanalyzer (Böblingen, Germany).

2.5. Reverse transcription and quantitative real-time PCR (RT-qPCR)

Reverse transcription and RT-qPCR were performed as described previously [6]. Primer sequences (all purchased from Eurofins MWG Operon, Ebersberg, Germany, except primers for TNF (QuantiTect Primer Assay, Quiagen, Hilden, Germany)) are provided in Supplementary Table 1.

2.6. Whole genome expression analysis

Labeling, hybridization and scanning of high quality RNA was conducted as described previously [17]. Microarray analysis and GO term enrichment was performed using GeneSpring 10.2 software (Agilent Technologies, Böblingen, Germany). In case of multiple probes representing one gene the corresponding probes were averaged. Expression data were median-normalized and filtered as follows: genes considered undetectable (fluorescence intensity below 50 in less than three out of 32 samples) were excluded. The resulting gene list was reduced to ~2800 genes showing significantly different expression between relevant conditions (one-way ANOVA with asymptotic P-value calculation and Benjamini–Hochberg correction, 5% FDR).

2.7. Preparation of whole cell lysates and western blotting

Lysate preparation was performed as described previously [18]. In brief, 10 × 10^6 monocytes were washed with phosphate-buffered saline, pelleted, dissolved in 500 μl pre-equilibration buffer [18], centrifuged at 900 × g, dissolved in 150 μl lysis buffer [18] and incubated on ice for 10 min. Lysates were dissolved in 150 μl 2× SDS sample buffer and incubated at 95 °C for 10 min. After blotting membranes were incubated with antibodies against phospho-p38 MAP-Kinase (1:2000), IκBα (1:1000), phospho-AKT (1:1000) (all Cell Signaling, Danvers, USA) or β-actin (1:2000) (Sigma–Aldrich, Munich, Germany).

2.8. Preparation of nuclear lysates and detection of nuclear factor κ B (NFκB)

Nuclear lysates were prepared as described previously [19]. Briefly, 50 × 10^6 monocytes were washed with PBS, dissolved in 400 μl buffer A (Supplementary Table 2), incubated on ice for 3 min, centrifugated (3000 × g), resuspended in buffer A and lysed by passing through a 27G needle. After centrifugation (3000 × g) pelleted nuclei were washed in buffer A, lysed in buffer B (Supplementary Table 2), incubated on ice for 10 min and centrifuged (10,000 × g, 10 min). Protein concentration was determined by Pierce protein assay (Thermo Fisher Scientific, Schwerte, Germany). The amount of NFκB was measured using TransAM NFκB p65 ELISA kit (Active Motif, La Hulpe, Belgium).

3. Results

3.1. LA delays the LPS-induced transcriptional response

To analyze the impact of LA on activated monocytes we performed whole genome microarray expression analyses with monocytes incubated for 1 or 4 h with LPS in the presence or absence of 10 or 20 mM LA, NaL or in culture medium with pH 7.1 or 6.6 (corresponding to pH of 10 or 20 mM LA, respectively). As controls we used monocytes without incubation or monocytes incubated without LPS. To isolate LA-specific effects (relative to acidification), we focused on genes that were significantly regulated at least two-fold by LPS and at least two-fold different between the conditions 20 mM LA/LPS and LPS as well as 20 mM LA/LPS and pH 6.6/LPS. After 1 h incubation we found a predominantly negative impact of 20 mM LA and to a lesser extent of the corresponding acidification which had mostly disappeared after 4 h (Fig. 1 and Supplementary Fig. 1) indicating that 20 mM LA delayed LPS-induced gene activation. The incubation of monocytes with LPS and 20 mM NaL, 10 mM LA or medium with pH 7.1 only slightly affected the expression of few genes. In line with the focus on LPS-regulated genes, a number of gene ontology (GO) terms significantly enriched (FDR 5%) in the cluster were associated with an ongoing immune response (Supplementary Table 3).

3.2. LA affects gene expression with different pH-dependency and duration

For validation of array data we concentrated on genes showing interesting regulation patterns and/or coding for proteins with immunological function. To analyze the persistence of the LA effect we compared mRNA expression between monocytes incubated for 1 h and 17 h.

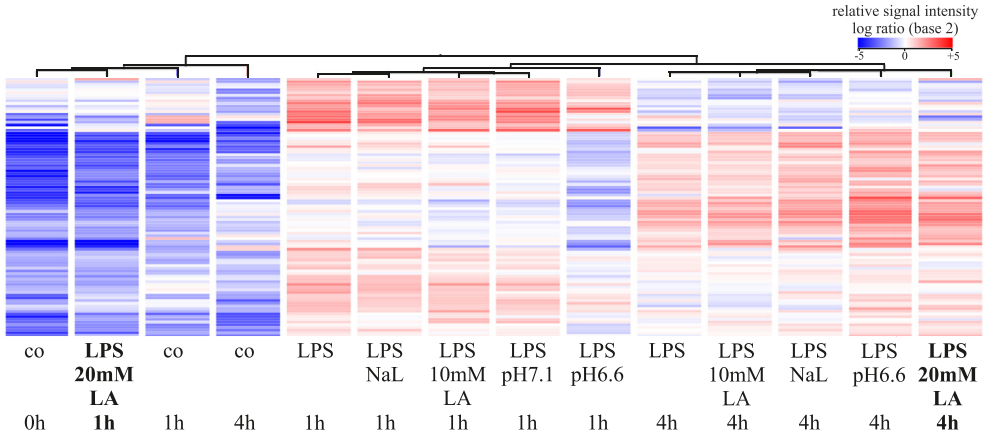

Fig. 1. mRNA expression profiles of genes significantly influenced by lactic acid (LA). Freshly isolated monocytes were incubated for 1 or 4 h with LPS with or without 10 or 20 mM LA, 20 mM sodium lactate (NaL) or acidification to pH 7.1 or pH 6.6 (corresponding to pH of 10 (only 1 h) or 20 mM LA, respectively). As controls freshly isolated monocytes (co 0 h) or monocytes incubated for 1 h (co 1 h) or 4 h (co 4 h) without LPS were used. The heat map displays data of 4 independent donors (co 0 h n = 4; 1 h n = 2; 4 h n = 2). Blue and red represent at least five-fold down- or up-regulation, respectively.

The genes coding for the chemokines *CCL2* (Fig. 2A) and *CCL7* (Fig. 2B) were markedly suppressed by LA and also by acidification. This effect was still detected after 17 h. Both 20 mM LA and acidification to pH 6.6 also strongly suppressed the CCL2 secretion (Supplementary Fig. 2A). The expression of *PTX3* (Fig. 2C), *EGR2* (Fig. 2D) and *GEM* (Fig. 2E) was also suppressed by LA in a long and pronounced manner and -with the exception of GEM-also slightly by acidification.

In accordance with our earlier study [6] 20 mM LA but not the corresponding acidification transiently down-regulated the LPS-induced *TNF* mRNA level (Fig. 3A). The presence of 20 mM LA also reduced the TNF level in the supernatants (Supplementary Fig. 2B). In contrast to the mRNA data, the corresponding acidification to pH 6.6 also significantly diminished the level of TNF in the supernatant. *IL-8* showed a similar gene expression pattern as *TNF* (Fig. 3B).

In contrast to these down-regulated genes, 20 mM LA strongly increased the LPS-induced expression of *LIF* after 17 h (Fig. 3C). The corresponding acidification increased the expression of *LIF* at all analyzed time points, indicating a pH-dependent effect. Also the gene *IL-23A* showed an up-regulated expression in the presence of 20 mM LA and the corresponding acidification after 17 h (Fig. 3D).

3.3. LA delays LPS-induced signaling pathways

Since the array analysis suggested a LA-induced delay of the LPS response we hypothesized that this might result from an intervention of LA with TLR signaling. Therefore, we performed western blot analysis with lysates of monocytes incubated for 5, 15 or 30 min with or without LPS and the combinations of LPS with 20 mM LA, 20 mM NaL or culture medium with pH 6.6. Furthermore freshly isolated monocytes without incubation were used.

The LPS-induced phosphorylation of MAP kinase p38 was neither affected by LA, nor by the corresponding acidification or NaL (Fig. 4A). As expected, LPS led to phosphorylation of AKT after 5 min which was stable up to 30 min (Fig. 4B). 20 mM LA and slightly also acidification to pH 6.6 decreased the LPS-induced phosphorylation of AKT at all time points, however, the effect was less pronounced after 30 min.

Monocytes incubated for 5 and even 15 min with LPS and 20 mM LA contained a higher amount of IκBα compared to LPS-stimulated cells (Fig. 4C). Neither the corresponding acidification nor NaL affected the amount of IκBα. After 30 min the IκBα level was lower in monocytes incubated in the presence of LA compared to the LPS control. These data indicate that incubation with 20 mM LA delays the LPS-induced degradation of IκBα and the phosphorylation of AKT. However, the effect on AKT phosphorylation is at least partially pH-dependent.

To determine nuclear accumulation of NFκB we incubated monocytes for 15 min with LPS in the presence of 20 mM LA or the corresponding acidification to pH 6.6. As controls we used monocytes without incubation or monocytes incubated without LPS. In accordance with the delayed degradation of IκBα LA significantly reduced the LPS-induced amount of nuclear NFκB after 15 min (Fig. 4D). Our results show that LA and in part also the corresponding acidification interfere with TLR signaling. This effect might at least in part be causative for the profound negative effect of LA on the LPS-induced gene expression in monocytes.

4. Discussion

We and others have observed that LA affects various immune cell types [8,10,14,20] and inhibits TNF secretion and glycolysis in human monocytes [6]. In the present study we globally elucidated the effect of physiological LA concentrations on monocytes. LA significantly delayed up-regulation of the majority of LPS-induced genes. Acidification often had similar but mostly less pronounced effects whereas NaL only slightly affected gene expression. This finding is in accordance with Chen et al. who performed microarray analysis with human mammary epithelial cells incubated with 25 mM LA and also found LA and acidosis to trigger similar transcriptional responses, which were distinct from the response to lactate [15]. In contrast to our data they detected the LA-induced up-regulation of a number of genes. This discrepancy is most likely caused by different culture conditions and cell types: We performed short-term cultures with LPS to study the effect of LA in the context of TLR stimulation since TLR stimulation is also observed in tumors [2]. Chen et al. incubated human mammary epithelial cells without further activation [15]. They also observed a negative effect of LA on expression of genes coding for glycolytic enzymes and reported LA to inhibit the serum-induced activation of the PI3K/AKT pathway in a prostate cancer cell line. AKT is involved in the regulation of glycolytic proteins such as hexokinase and glucose transporters [21]. Therefore, glycolysis seems to be regulated by LA via transcriptional and posttranscriptional pathways. In line with this we previously had detected a strong impact of LA on the LPS-induced glycolytic flux of monocytes [6]. However,

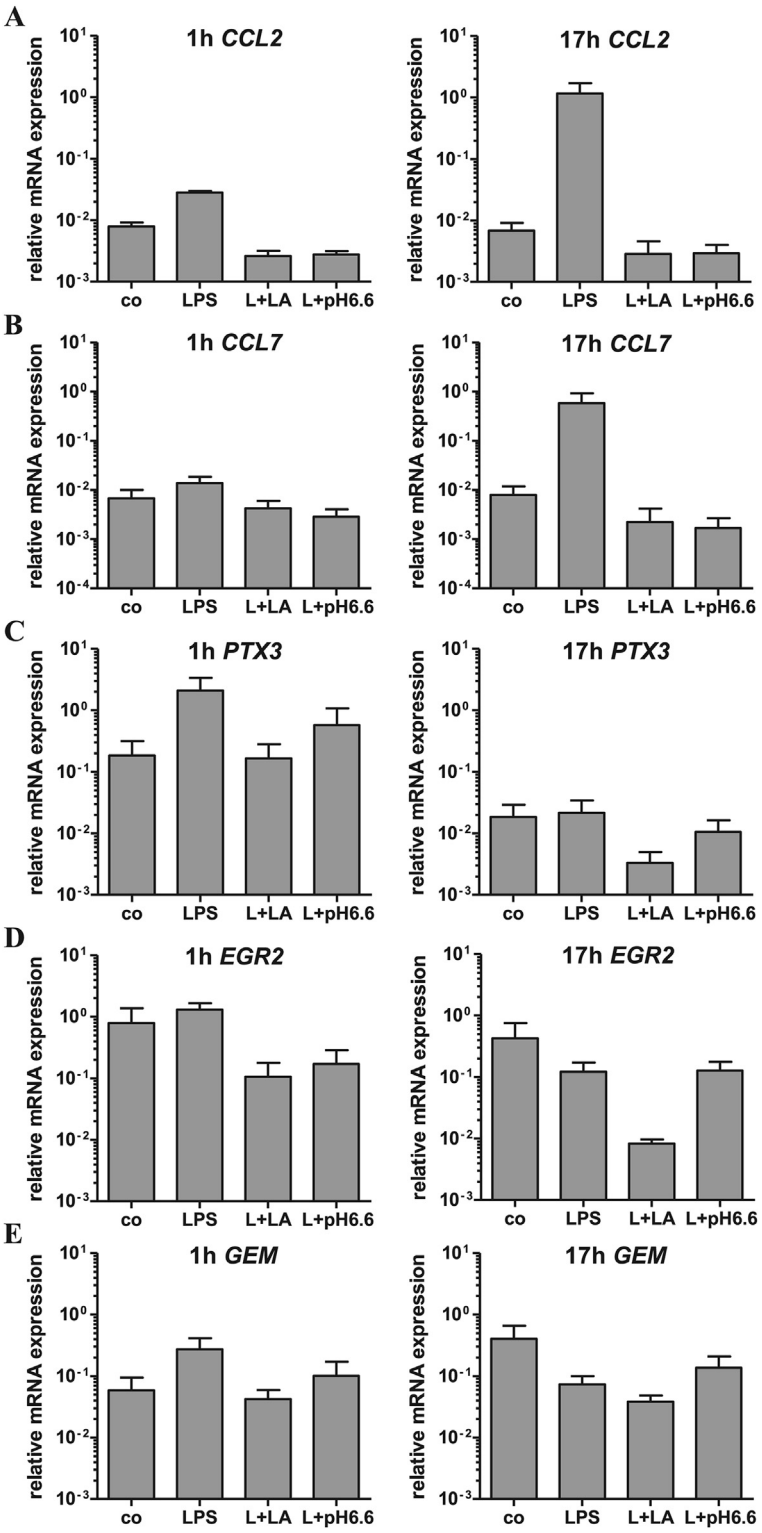

Fig. 2. RT-qPCR analysis of genes with prolonged expression change in response to lactic acid (LA). Freshly isolated monocytes were incubated for 1 or 17 h in the absence of LPS (co) or with LPS with or without 20 mM LA (L + LA) or acidification to pH 6.6 (L + pH6.6). mRNA expression mRNA expression of *CCL2* (A), *CCL7* (B), *PTX3* (C), *EGR2* (D), *GEM* (E) relative to *18S* rRNA expression was determined by RT-qPCR. Data represent mean values of 3 independent experiments ± SEM.

in the current analysis we did not observe an effect of LA on genes associated with glycolysis but also observed inhibition of AKT phosphorylation. This underlines the importance of post-transcriptional mechanisms involved in the inhibition of monocyte glycolysis by LA or acidification.

Among the significantly enriched GO terms a number of terms associated with immune activation like “chemokine”, “cytokine” or “immune response” were detected. This indicates that LA might influence a broad range of factors secreted by activated monocytes which is in line with reports about an altered phenotype of tumor-

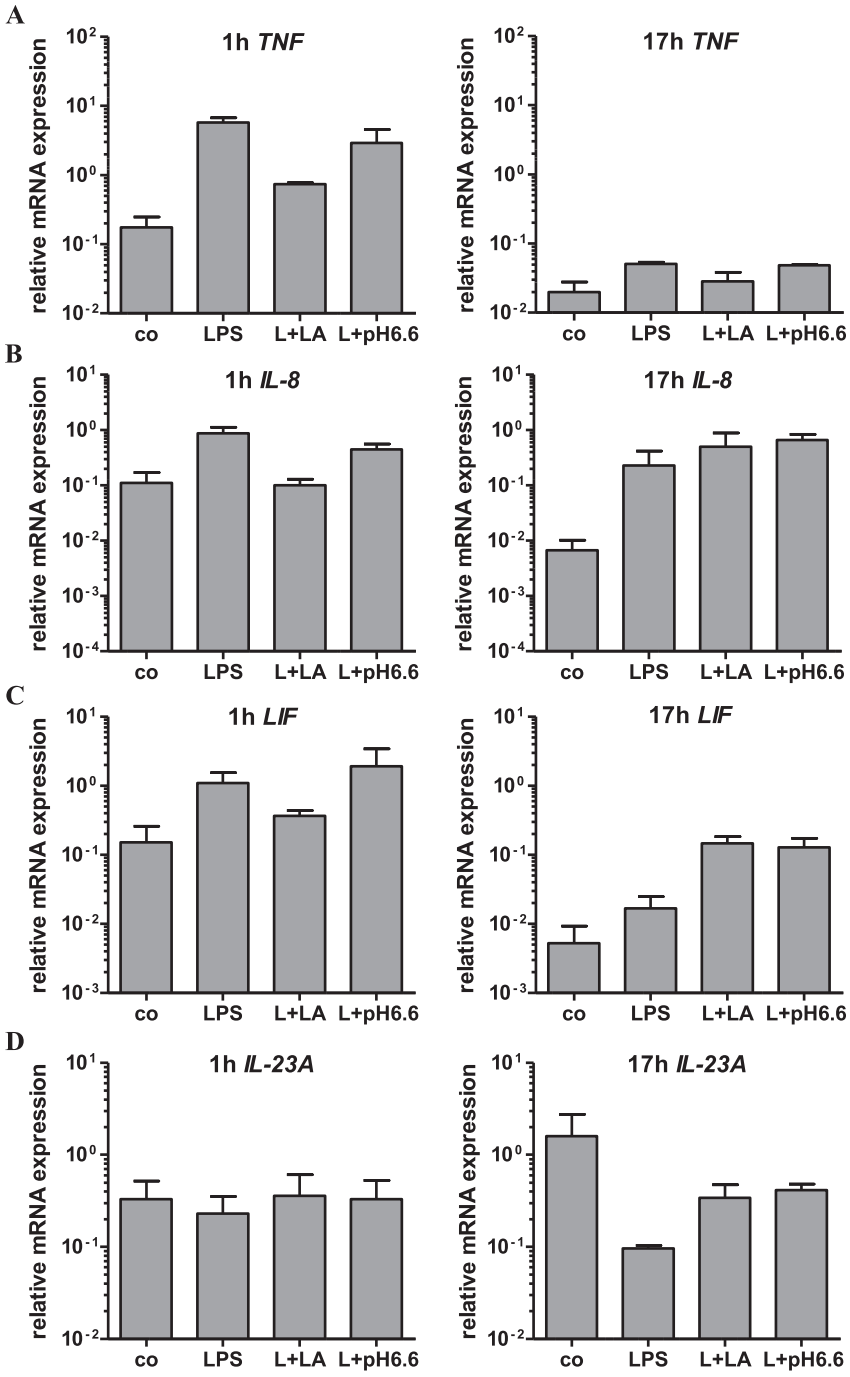

Fig. 3. RT-qPCR analysis of genes with transient expression change (A, B) or up-regulated expression (C, D) in response to lactic acid (LA). Freshly isolated monocytes were incubated for 1 or 17 h in the absence of LPS (co) or with LPS with or without 20 mM LA (L + LA) or acidification to pH 6.6 (L + pH6.6). mRNA expression of *TNF* (A), *IL-8* (B), *LIF* (C) or *IL-23A* (D) relative to *18S* rRNA expression was determined by RT-qPCR. Data represent mean values of 3 (17 h control *LIF*: n = 2) independent experiments ± SEM.

associated monocytes [3,4]. Consistently, LA inhibited TNF and stimulated *IL-23A* expression of monocytes. Furthermore, LA or acidification stimulated *LIF*, but reduced the majority of LPS-regulated genes, e.g. *CCL7*, *PTX3*, and *CCL2*, which play an important role in tumors [22–24]. In contrast, other authors mainly described positive effects of lactate or LA on cytokine secretion of macrophages [4,11,12]. However, these authors preincubated cells with lactate or LA. This experimental setting reflects a physiological situation where immune cells are exposed to high lactate levels in the tumor environment followed by sudden TLR stimulation. In contrast, our approach is based on the hypothesis that in the tumor environment high lactate levels are often paralleled by chronic inflammation. As both physiological situations are likely to occur in tumors both aspects may be important for tumor development.

Furthermore, LA and acidification increased the expression of *LIF*. Since LIF has been described to drive the differentiation of IL-10^{high} and IL-12^{low} tumor-associated macrophages (TAM) from monocytes [25] LA might trigger this development. In line with this, Colegio et al. recently described the LA-induced polarization of TAM [13]. As we and others have demonstrated that LA inhibits the

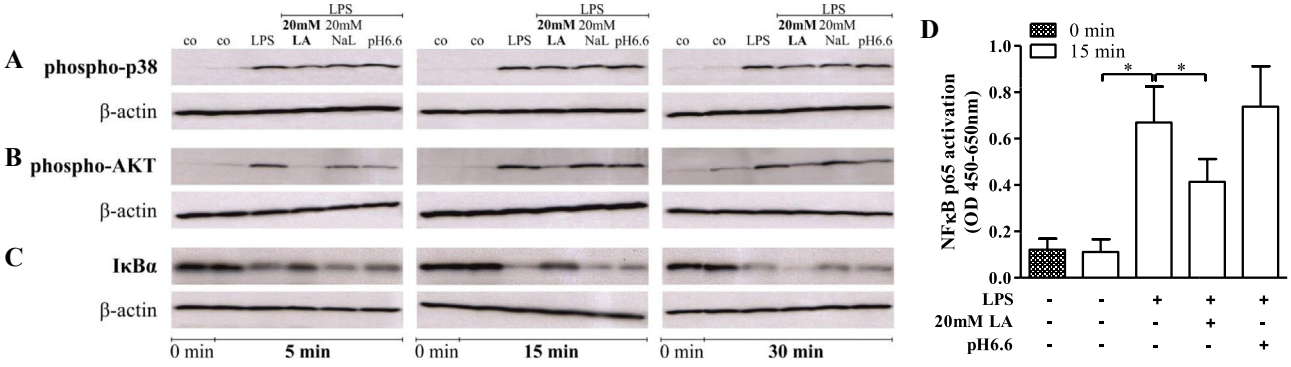

Fig. 4. Lactic acid (LA) delays LPS-induced signaling pathways. Freshly isolated monocytes were incubated with LPS in the presence or absence of 20 mM LA, acidification to pH 6.6 or 20 mM sodium lactate (NaL; only A–C). As controls (co) freshly isolated monocytes (0 min) or monocytes incubated in the absence of LPS were used. (A–C) After 5, 15 or 30 min incubation western blot analysis was performed using antibodies against phospho-p38 MAP-Kinase (A), phospho-AKT (B) or IκBα (C) as well as β-actin (A, B, C). Shown are representative blots from 1 of 3 independent donors (LPS + NaL 1 donor). (D) The amount of nuclear NFκB was determined by ELISA after 15 min incubation. Data are means of 3 (LPS + pH6.6 n = 2) independent experiments ± SEM (Student t test, *$p < 0.05$ compared with LPS-treated control).

activation and differentiation of human dendritic cells and suppresses IL-12 production [8,9], LIF may contribute to this effect in the tumor environment. Since the *EGR2* gene codes for a transcription factor which has been discussed to be involved in monocyte to macrophage differentiation [26,27] also the negative effect of LA on *EGR2* expression might contribute to the modulation of monocyte differentiation. In light of our data on the up-regulation of GEM expression upon differentiation of human monocytes to macrophages [17] the down-regulation of *GEM* expression in response to LA might represent a further mechanism how LA might affect monocyte differentiation.

In accordance with the predominant early effects of LA on the LPS-induced gene expression we found that LA and slightly also the corresponding acidification delayed the LPS-induced phosphorylation of AKT and the degradation of IκBα. In addition, LA reduced the LPS-induced amount of nuclear NFκB in monocytes. In line with our results Kellum et al. found a reduced NFκB DNA binding in response to LA in the LPS-stimulated murine macrophage cell line RAW 264.7 [28]. Watanabe et al. observed a reduced degradation of IκBα in the presence of LPS and LA in the human monocytic cell line THP-1 [29]. In contrast, in T cells LA reduced the phosphorylation of p38 but not the phosphorylation of AKT [14] indicating a cell type-specific involvement of particular signaling pathways. The delayed nuclear transition of NFκB might be responsible for the effects of LA on NFκB-controlled genes like *TNF* [1]. However, since we also detected long-lasting suppression as well as induction of gene expression in response to LA the delay of signaling pathways is unlikely to be responsible for these effects. One possible mechanism for long-term suppression of gene expression might be a longer-lasting effect of LA on genes coding for transcription factors like EGR2 which we found to be reduced by LA on the mRNA level over the whole analyzed time period.

With this study we demonstrate how globally the phenotype of a single immune cell type is altered by the tumor-derived metabolite LA and emphasize the glycolytic phenotype of tumors as a promising target to restore the immune response against tumors.

Conflict of interest

None.

Acknowledgments

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Transparency document

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Appendix A. Supplementary data

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