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Lactate dehydrogenase inhibitors can reverse inflammation induced changes in colon cancer cells

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Abstract

The inflammatory microenvironment is an essential component of neoplastic lesions and can significantly impact on tumor progression. Besides facilitating invasive growth, inflammatory cytokines were also found to reprogram cancer cell metabolism and to induce aerobic glycolysis.

Previous studies did not consider the possible contribution played in these changes by lactate dehydrogenase (LDH). The A isoform of LDH (LDH-A) is the master regulator of aerobic glycolysis; it actively reduces pyruvate and causes enhanced lactate levels in tumor tissues. In cancer cells, lactate was recently found to directly increase migration ability; moreover, when released in the microenvironment, it can facilitate matrix remodeling. In this paper, we illustrate that treatment of human colon adenocarcinoma cells with TNF-α and IL-17, two pro-inflammatory cytokines, modifies LDH activity, causing a shift towards the A isoform which results in increased lactate production. At the same time, the two cytokines appeared to induce features of epithelial-mesenchymal transition in the treated cells, such as reduction of E-cadherin levels and increased secretion of metalloproteinases. Noteworthy, oxamate and galloflavin, two inhibitors of LDH activity which reduce lactate production in cells, were found to relieve the inflammation-induced effects.

These results suggest LDH-A and/or lactate as common elements at the cross-road between cancer cell metabolism, tumor progression and inflammation. At present, LDH inhibitors suitable for clinical use are actively searched as possible anti-proliferative agents; our data lead to hypothesize for these compounds a wider potential in anticancer treatment.

Keywords: Cancer cell metabolism; Epithelial-mesenchymal transition; Inflammation; Lactate dehydrogenase.

1. Introduction

Inflammation plays a critical role in neoplastic change and was found to impact on several steps of tumorigenesis, from initiation to metastatic diffusion. Colon cancer, which is a major worldwide health concern, can be considered a model disease to examine the links between chronic inflammation and neoplasia. It often arises in areas of inflamed mucosa and even when it can be mainly imputed to the influence of life-style factors, it is commonly characterized by active inflammation and immune cell infiltration (Terzić et al., 2010). Although infiltrating immune-inflammatory cells should help tumor clearance, they paradoxically facilitate tumor progression, because of their capacity to activate extracellular matrix breakdown and tissue remodeling (Grivennikov et al., 2010). The most frequent tumour infiltrating immune cells are macrophages and T lymphocytes (Grivennikov et al., 2010). Among T lymphocytes, in patients with colorectal cancer a critical role seems to be played by the Th17 cell subset, which was found to be associated with shortened disease-free survival (De Simone et al., 2013). The tumor-promoting effect of both macrophages and Th17 cells are mediated by NF-kB induced cytokine production (Grivennikov et al., 2010), mainly TNF-α and IL-17. TNF-α is one of the most important inflammatory factors in the pathogenesis of inflammatory bowel disease and in colitis-associated cancer (Terzić et al., 2010). IL-17 is frequently present in acute and chronic inflammation and, as stated above, was found to be relevantly linked to colon cancer (De Simone et al., 2013).

Tumor associated inflammatory states were also found to be characterized by a feed-forward loop: cytokines released by macrophages and lymphocytes activate NF-kB in cancer cells, leading to chemokine production and recruitment of additional inflammatory cells (Grivennikov et al., 2010). In cancer cells, NF-kB signaling cascade also activates the program of epithelial-mesenchymal transition (EMT) and facilitates tumor invasion and

metastasis by up-regulation of Snail and repression of E-cadherin synthesis (Min et al., 2008). As well known, NF-kB is also a key regulator of energy homeostasis; it increases glucose metabolism and induces aerobic glycolysis (AG) (Moretti et al., 2012; Neriah and Karin, 2011).

AG (the so-called Warburg effect) is one of the hallmarks of cancer and is now considered a possible target for the development of innovative anticancer treatments (Fiume et al., 2014). Several reports have documented increased glycolysis and lactate production in cancer cells exposed to inflammatory cytokines (Kumar et al., 2014; Strauss, 2013; Vaughan et al., 2013), and the inhibition of inflammatory cascade was found to reduce the Warburg effect (Leidgens et al., 2015; Vaughan et al., 2013). However, to our knowledge these studies did not consider the role played by lactate dehydrogenase (LDH) in inflammation-induced metabolic changes. LDH is a key enzyme in glucose metabolism and is the master regulator of the Warburg effect (Fiume et al., 2014). We hypothesized that in these previous studies the involvement of LDH in inflammation-induced metabolic changes could be disguised since none of the Authors considered the differences between the two enzymatic isoforms, LDH-A and LDH-B. These two enzyme isoforms catalyse the same reaction, the conversion of pyruvate to lactate at the end of glycolysis. However, their relevance in neoplastic change is quite different: LDH-A is constantly up-regulated in cancer cells, while the role of LDH-B is still controversial (Fiume et al., 2014).

In this paper, we treated cultured colon cancer cells with TNF-α and IL-17 and studied the effect of these inflammatory signals on LDH-A and -B gene transcription and on LDH enzymatic activity. The adopted cell model was the CaCo-2 line. This well-studied colon carcinoma was found to reversibly express differentiation markers according to the time-course of culture (Sambui et al., 2005). In the treated CaCo-2 cells, we evidenced features of

EMT. Furthermore, the above described connections between inflammation, tumor progression and cell metabolism, prompted us to study the effects of LDH inhibitors on the cell alterations induced by TNF-α and IL-17. Because of LDH position in the glycolytic pathway, inhibition of LDH activity allows to hinder AG of cancer cells without affecting the glucose metabolism of normal cells (Fiume et al., 2014).

2. Materials and Methods

2.1 Cell culture and reagents

CaCo-2 cells (ATCC) were grown in DMEM containing 10% FBS, 100 U/ml penicillin/streptomycin, 4 mM glutamine. They were routinely tested for Mycoplasma contamination and found to be free. Before each experiment, cells were let to reach the confluence, then harvested and seeded at a density of 50000 cells/cm^2. This procedure was adopted for all experiments, since it was found to be important for evaluating the expression of some of the studied proteins. Cell viability was measured by using the Neutral Red assay, following the procedure described by Farabegoli et al. (2012).

TNF-α and IL-17 were from R&D Systems and were used at a concentration of 25 and 50 ng/ml, respectively.

Galloflavin (GF) was synthesized according to the procedure described in (Manerba et al., 2012). It was added to the culture media in the presence of 0.6% DMSO for all the experiments. Equivalent amounts of DMSO were also added to the control (untreated) cultures. Oxamate (OXA) and all other compounds and reagents were purchased from Sigma-Aldrich, unless otherwise indicated.

2.2 Real time PCR

LDH-A, LDH-B and H2B histone expressions were evaluated by measuring their mRNA levels, using β-tubulin and HPRT as internal controls of the reaction. RNA was extracted according to the procedure described by Chomczynski and Sacchi (1987), and was quantified spectrophotometrically. Retro-transcription to cDNA was performed by using the Revert Aid TM First Strand cDNA Synthesis Kit, in different steps: 5 min denaturation at 65°C, 5 min annealing at 25°C, 1 h retro-transcription at 42°C and 5 min denaturation at 70°C.

Real Time PCR analysis of cDNA was performed using SYBR Green (SSO Advanced, BioRad), with the following primers: LDH-A, Forw: 5'-GACCTACGTGGCTTGGAAGA-3', Rev: 5'-TCCATACAGGCACACTGGAA-3'; LDH-B, Forw: 5'-CCAACCCAGTGGACATTCTT-3', Rev: 5'-AAACACCTGCCACATTCACA-3'; H2B histone, Forw: 5'-CAGTGCTATGCCAGAGCCAGCGAA-3', Rev: 5'-CTGTTTACTTAGCGCTGGTGTACTTGGTGA-3'. Annealing temperature was 60 °C.

All samples were run in triplicate, in 10 µl reaction volume containing 100 ng of cDNA. The thermal cycler (CFX96 TM Real Time System, BioRad) was programmed as follows: 30 sec at 95°C; 40 cycles of 15 sec at 95°C; 30 sec at 60°C.

2.3 LDH and lactate assays

For LDH assay, control and cytokines treated cells (2×10^6) were harvested, pelleted and suspended in 100 µl of PBS. The suspension was lysed by sonication and centrifuged (1600 g, 30 min at 4°C) to discard the cell debris. Protein content of the supernatant was measured according to the method of Bradford. This cell extract (5-10 µl amounts) was used to measure LDH activity and composition (% of A and B isoform), following the procedure

originally set up by Goldman et al. (1964). This method is based on the comparison of enzymatic activity measured in a reaction mixture containing 220 μM reduced nicotinamide hypoxanthine dinucleotide (a NADH analogue) and low pyruvate concentration (330 μM) with that measured in the presence of 130 μM NADH and high pyruvate concentration (10 mM). The detailed procedure is reported in (Vettraino et al., 2013). LDH activity was expressed as mU / mg cell proteins.

Lactate production was measured after 6 h incubation in the presence of scalar amounts of OXA (0-60 mM) or GF (0-200 μM). Determinations were performed on samples of 5×10^5 cells, according to the method of Barker and Summerson (1941) and following the procedure described by Farabegoli et al. (2012).

2.4 Glucose uptake assay

CaCo-2 cells were seeded into 96-well black plates at 20000 cells/well and let to adhere overnight. They were then treated with TNF-α and IL-17 for 24 h, after which they were maintained for 20 min in the presence of 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), 100 μM in glucose free medium. Medium was removed and cells were washed with PBS. Fluorescence (λexc 485; λem 530) was measured by using the Fluoroskan Ascent FL reader (Labsystems).

2.5 Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was measured on cell homogenate using p-nitrophenyl phosphate (p-NPP) as substrate and measuring the release of p-nitrophenol (p-NP), detected at 405 nm in a microplate multifunctional reader. The procedure described by

Ferruzza et al. (2012) was followed. ALP activity in each sample was calculated from a p-NP standard curve.

2.6 Immunoblotting

Cell cultures were exposed to cytokines treatment (4 or 24 h). After incubation they were lysed in 100 µl RIPA buffer containing protease inhibitors. The cell homogenates were left 30 min on ice and then centrifuged 15 min at 10000 g. 40 or 100 µg proteins of the supernatants (measured according to Bradford) were loaded onto 10% or 8% polyacrylamide gels for electrophoresis. The separated proteins were blotted on a low fluorescent PVDF membrane (GE Lifescience) using a standard apparatus for wet transfer with an electrical field of 80 mA. The blotted membrane was blocked with 5% BSA (or casein) in TBS-Tween and probed with the primary antibody. The used antibodies were: mouse anti-E-cadherin (R&D Systems), rabbit anti-Snail [C15D3] (Cell Signaling), rabbit anti-Notch1 [D1E11] (Cell Signaling), rabbit anti-MYC [Y69] (Abcam), rabbit anti-Actin (Sigma Aldrich), rabbit anti-LDHA (Cell Signaling), rabbit anti-Oct4 (Cell Signaling). Binding was revealed by a Cy5-labelled secondary antibody (anti rabbit-IgG, GE Lifescience; anti mouse-IgG, Jackson Immuno-Research). All incubation steps were performed according to the manufacturer's instructions. Fluorescence of the blots was assayed with the Pharos FX scanner (BioRad) at a resolution of 100 µm, using the Quantity One software (BioRad).

2.7 Gelatin zymography

Matrix metalloproteinases (MMP) secretion and activity was assessed using gelatin zymography (Heussen and Dowdle, 1980). Cells were grown at density of 50000 cells/cm², treated for 24 h with TNF-α and IL-17 and then maintained in serum-depleted media for 18

hours. Conditioned media were collected by spinning each sample at 1700 rpm for 5 min, to pellet cell debris. Supernatants (3 ml) were then concentrated using Amicon Ultra-centrifuge filters (Millipore), according to the manufacturer's directions. Protein concentration was determined using the Lowry method. Equal amounts of samples (30 μg) were then loaded with sample buffer (1 M Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol) onto a 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, SDS was removed from the gel by washing twice with 2.5% Triton X-100 for 1 h. After a brief rinse, gel was incubated at 37°C for 18 h in a zymography buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, 20 mM NaCl, pH 7.6). Gel was stained with 1% Coomassie Brilliant Blue R250 for 30 min and then treated with a de-staining solution (40% methanol, 10% acetic acid, 50% distilled water). The MMP activities, indicated by clear bands of gelatin digestion on a blue background, were quantified by using an image analysis software (Kodak 1D Image Analysis Software).

2.8 Statistical analyses

All statistical analyses were performed using the software GraphPad Prism 5. Each experiment was repeated two or three times, with at least triplicate samples per treatment group. Results are expressed as mean ± SE of replicate values. Analyses were performed by ANOVA or Student's t test. P values < 0.05 were considered statistically significant.

3. Results

3.1 Inflammatory cytokines shift LDH activity toward the -A isoform

Actively growing (50000 cells/cm^2) CaCo-2 cells were treated with TNF-α and IL-17 for 4-72 h. At different time intervals, LDH-A and -B mRNAs were measured by Real Time PCR. Results are shown in Fig. 1A and 1B. No significant changes in LDH-A mRNA were observed;

on the contrary, LDH-B mRNA appeared to significantly decrease ($p < 0.01$, as evaluated by ANOVA) from 4 h onward. A constant 30% decrease was measured; as a consequence, cytokines treatment changed the predominant enzyme isoform in CaCo-2 cells from LDH-B to -A (Fig. 1C). Fig. 1D shows that the anti-inflammatory drug dexamethasone increased both LDH-A and -B expression in CaCo-2 cells and, when added to the cultures treated with TNF-α and IL-17 (24 h), it abolished the cytokines induced effect and restored LDH-B as the prevalent enzyme isoform in cells.

The preferred method to assess LDH protein level in the treated cells was the assay of enzymatic activity in cell cytoplasm. Moreover, we adopted the procedure described by Goldman et al. (1964), which allows to discriminate between the two enzyme forms. Evaluation by immunoblotting was considered inaccurate, since we found that the commonly available anti-LDH antibodies show cross-reactivity for LDH-A and -B. This problem was also observed by other Authors (Allison et al., 2014). The results are shown in Table 1A. At 24 h, TNF-α and IL-17 caused a moderate (≈ 30%), but statistically significant increase of total LDH activity in cell cytoplasm and, as already observed by PCR, a reduction of the enzymatic activity imputable to the -B isoform. After 24 h exposure to TNF-α and IL-17, an indicative evaluation of LDH-A level was also performed by immunoblot and is reported in Fig. 2; the densitometric analysis of the protein band is in line with the results obtained in the enzymatic assay. Taken together, these data suggest that the enhanced LDH activity measured in treated cells can be ascribed to the enzyme A isoform, but cannot be imputed to increased mRNA transcription. Post-translational changes increasing LDH-A activity have been reported in literature (Fan et al., 2011; Zaho et al., 2013). The enhanced enzymatic activity measured in the treated cells in spite of the observed reduction of LDH-B mRNA could also be explained by the kinetic properties of the A isoform, which has a higher Vmax compared to LDH-B. A

further explanation could also be hypothesized from the data reported in Fig. 1E. Besides participating in glycolysis, LDH-A is a nuclear protein and was found to be necessary for H2B histone gene transcription (Dai et al., 2008). Treatment with TNF-α and IL-17 markedly reduced the expression of H2B histone, suggesting that inflammation can preferentially direct LDH-A function to cell cytoplasm.

Inflammatory cytokines were also found to enhance glucose uptake in the treated cells (Kumar et al., 2014; Strauss, 2013; Vaughan et al., 2013); as shown in Table 1A, this finding was also confirmed by our experiments and, together with the observation of the increased LDH-A activity, justifies the raised lactate production measured in treated CaCo-2 cells (> 50 % per hour, see Table 1A).

The above described results are in line with data of previous studies, which evaluated LDH isoenzyme composition in normal colonic mucosa, in polyps and adenocarcinomas. LDH-A, which is absent in normal epithelium, was found to increase concomitantly with the manifestation of neoplastic change; an opposite pattern was instead documented for LDH-B (Carda-Abella et al., 1982). In treated CaCo-2 cultures, we also detected signs of de-differentiation, as evidenced by alkaline phosphatase (ALP) activity, which dropped to undetectable levels after 24 h exposure to TNF-α and IL-17 (Table 1A). ALP is a protein located in the apical membrane of enterocytes, involved in the maintenance of gut homeostasis; it is one of the markers of mature intestinal cells (Ferruzza et al., 2012).

3.2 Inflammation induces EMT signatures in proliferating cells

Increased lactate production by LDH-A can play an active role in tumor progression and was found to increase cancer cell migration (Baumann et al., 2009). Following the above reported results, in CaCo-2 cells treated with TNF-α and IL-17 we assessed the expression of

proteins indicative of increased proliferative or metastatic potential and known markers of EMT. As a first step, the study was attempted in both confluent and actively growing (50000 cells / cm^2) cells, in which the levels of Myc and HIF-1α proteins were measured after 4 h exposure to the inflammatory cytokines (Fig. 2). Myc was previously found to be markedly induced by TNF-α (Liu et al., 2015); HIF-1α plays a major role in inflammation and is critical for the maintenance of cancer stem cells (Peng and Liu, 2015). Confluent and proliferating CaCo-2 cultures showed a different response to inflammatory cytokines: in confluent cells, Myc is not expressed and was not induced by TNF-α and IL-17 treatment (see insert of Fig. 2). In proliferating cells, the 4 h treatment markedly increased the level of Myc protein; a 1.6-fold increase in HIF-1α level was also detected, which, however, did not result statistically significant. This finding suggested that the growth rate of tumor cells could significantly impact on their response to inflammatory microenvironment. In proliferating cells, the observation was extended to 24 h (Fig. 2); at this time, the effect of TNF-α and IL-17 on Myc protein levels appeared in gradual depletion, while upregulation of Notch (cleaved form), Oct-4 and Snail was detected. Notch is a known regulator of cell fate, controlling proliferation and apoptosis; it has recently been implicated in the induction of the glycolytic switch associated with tumor progression (Basak et al., 2014). Oct-4 protein enhances cell self-renewal ability and inhibit the genes that start differentiation; it was found to be dependent on LDH-A expression (Zhang et al., 2012) and to predict disease recurrence in patients with rectal cancer (Saigusa et al., 2009). The best known function of Snail is the induction of the phenotypic changes associated with EMT (Barrallo-Gimeno et al., 2005; Min et al., 2008). Notch and Snail levels appeared significantly raised by TNF-α and IL-17 treatment; the change measured in Oct-4 protein (a mean 4.5-fold increase) was scarcely reproducible in the replicated experiments and did not result statistically significant.

3.3 LDH inhibitors can reverse the phenotype induced by inflammation in CaCo-2 cells

The overall picture emerging from the above reported experiments substantially confirmed the notion of the tumor promoting effects exerted by inflammatory cytokines. The development of these effects was previously shown to be controlled by commonly used anti-inflammatory agents (Usman et al., 2015). The recent rediscovery of inflammation as a direct inducer of AG (Moretti et al., 2012; Neriah and Karin, 2011), and the evidenced correlations between the metabolic alterations of cancer cells and their metastatic potential, led us to investigate whether the tumor promoting effects caused by inflammation could also be controlled by a metabolic reprogramming of cancer cells, mediated by LDH inhibition. Inhibition of LDH was obtained by using OXA and GF. OXA is a pyruvate analogue hindering LDH activity by competition with the enzyme natural substrate (Papacostantinou and Colowick, 1961). It is a well-studied and specific inhibitor, active in cultured cells in the millimolar range. GF is polyphenolic compound endowed with LDH inhibitory activity. It was identified by our research group during a virtual screening campaign and is active on cultured cells in the micromolar range (Manerba et al., 2012). As a first step of the study, we probed CaCo-2 cell cultures with scalar amounts of OXA or GF, in order to identify doses of compounds significantly affecting LDH activity, without causing heavy cell death. Inhibition of LDH was assessed by measuring the level of lactate in cell cytoplasm and culture medium. The selected doses of compounds, together with their effect on both lactate production and cell viability, are shown in Table 1B. We tested the effect caused by these OXA and GF doses on the levels of proteins previously found to be significantly modified by TNF-α and IL-

17 treatment. The results are shown in Fig. 3, which also reports an immunoblotting evaluation of E-cadherin in the treated cells. Reduction of E-cadherin expression is the major phenotypic change associated with EMT (Farahani et al., 2014). E-cadherin is down-regulated by the transcription factor Snail; its reduction is very frequently observed during the progression of malignant epithelial tumors, and is responsible for the loss of intercellular adhesion during invasion (Perl et al., 1998).

LDH inhibitors, added to the culture medium of cytokines exposed cells, were found to significantly mitigate all the inflammation induced phenotypic changes; the major effects were observed with OXA, the specific enzyme inhibitor. In particular, Snail levels appeared reduced to the levels measured in untreated cells, while Myc expression was significantly downregulated, compared to control cultures. This last finding can be explained considering the effect caused by OXA and GF on cell viability (see Table 1B).

The EMT signatures were not only measured at the biochemical level, after 24 h of TNF-α and IL-17 treatment; they also become evident at the morphological level when the treatment was extended to 72 h. Fig. 4A shows the growth pattern of CaCo-2 cells maintained in culture medium: they are characterized by a regular, polygonal shape and show a growth front with a well-defined rim. On the contrary, TNF-α and IL-17 exposed cells (shown in Fig. 4B) appear irregularly shaped, especially in proximity of the growth front, and form tissue islets with undefined margins. This growth pattern is indicative of increased invasive properties. The effects caused by both OXA and GF on cell viability did not allow the evaluation of the treated cells at 72 h. However, a further proof of the potential of LDH inhibitors in controlling cell invasive behavior was obtained by assaying in the treated cells the release of proteinases in culture medium at 24h. This was assessed by gelatin zymography, which allows a semi-quantitative measure of the matrix metalloproteinases -2 and -9 (MMP-2

and MMP-9) (Heussen and Dowdle, 1980). The obtained results are reported in Fig. 4C. In TNF-α and IL-17 treated cells, zymography showed a major band of degraded gelatin with a molecular weight of about 76 kDa, compatible with activated MMP-2. LDH inhibitors were found to significantly reduce gelatin degradation; similar to previous experiments, the best evidenced results were observed with OXA.

4. Discussion

AG is characterized by enhanced conversion of pyruvate to lactate even in the presence of adequate oxygen levels. Similar to other traits characterizing cancer cells, it has been linked to altered oncogenes and tumor suppressor genes, which besides regulating the proliferation of cancer cells, also increase LDH-A expression and modify their metabolism (Iurlaro et al., 2014). Currently, AG is widely accepted as a proper metabolic pathway to maximize the synthesis of “building blocks” (nucleotides, amino acids, fatty acids) needed to sustain the anabolic process of cancer cell proliferation. Otto Warburg, who discovered AG of cancer cells in the first half of the past century, was also the first scientist to observe that this metabolic pathway is not exclusive of cancer cells, since it can be induced in leukocytes by inflammation (Warburg et al., 1958).

The relationship between cancer and inflammation has long been investigated and was confirmed by epidemiological studies. With regard to colorectal tumors, patients suffering from persistent inflammatory bowel disease are among those at highest risk of neoplastic disease in the general population (Kraus and Arber, 2009); furthermore, the regular administration of nonsteroidal anti-inflammatory drugs was found to lower mortality in colorectal cancer patients and to cause regression of adenomas in individuals with familiar adenomatous polyposis (Guadagni et al., 2007). Multiple factors could contribute to the increased risk of neoplastic

change related to inflammation. Chronic inflammation is characterized by tissue destruction and oxidative stress, which stimulate cell proliferation and angiogenesis, but also facilitate gene mutations.

Inflammation-driven effects are usually orchestrated by extracellular signals diffusing in tissue microenvironment; among these, cytokines have the widest distribution and can mediate effects on both immune and tissue-resident epithelial cells. As explained in the Introduction, the cytokines used in our study (IL-17 and TNF-α) were found to be relevantly linked to colon cancer. Their intracellular signaling is activated by the transcription factor NF-kB. By increasing the expression of apoptotic inhibitors and of genes promoting cell growth, NF-kB provides a mechanistic link between inflammation and neoplastic change and can be considered the most important component of the tumor-promoting machinery activated by inflammation. It should also be noticed that, although most sporadic colon cancers do not seem to arise on an inflammatory background, in established tumors inflammation is observed to invariably proceed with mass growth, becoming an essential component of the lesion in advanced stages.

The inflammatory microenvironment was also found to have a direct effect on cancer cell metabolism. By increasing the transcription of HIF-1α and Myc, the macrophagic cytokine TNF-α positively regulates some genes of the glycolytic pathway (Strauss, 2013); IL-17 was found to cooperate with TNF-α by stabilizing the mRNA of the induced genes (Lee et al., 2008). These findings suggest that inflammatory cytokines can operate in concert with oncogene mutations in altering normal cell biology and enforce the notion that inflammation can contribute in affecting different stages of neoplastic change, from initiation to progression.

The capacity of local invasion, the most critical feature of cancer lesions, is also relevantly affected by the inflammatory microenvironment. Invasive growth requires

remodeling of the adjacent tissue and generation of new blood vessels; this process involves the same signaling pathways activated by inflammation in physiological wound healing, with the difference that, contrary to normal tissues, neoplastic growth is not self-limiting. As well known, this understanding led to define tumors as “wounds that do not heal” (Dworak, 1986).

Lactate, the end-product of LDH reaction, is one of the molecular signals involved in wound healing, when vascular injury reduces oxygen tension in tissues. Lactate response elements were identified in genes involved in tissue remodeling (Formby and Stern, 2003) and this metabolite was found to directly stimulate VEGF production by endothelial cells (Beckert et al., 2006), leading to neo-angiogenesis. In neoplastic tissues, enhanced lactate levels are basically linked to the increased LDH-A expression. By promoting TGF-β mediated regulation of matrix metalloproteinase-2 (MMP-2), lactate was found to increase cancer cell migration (Baumann et al., 2009). Moreover, the metabolite diffusing form malignant cells was found to stimulate hyaluronan synthesis in surrounding fibroblasts, causing a rearrangement of extra-cellular matrix, which facilitates cell invasive growth (Walenta and Mueller-Klieser, 2004). By modifying cell metabolism, the inflammatory microenvironment surrounding tumor tissue can further enhance the levels of lactate, as also confirmed by our experiments.

The inflammation induced proteins examined in this paper, and their role in tumor progression are summarized in Fig. 5.

Taking these data together, LDH-A and/or lactate could be assumed as common elements coordinating neoplastic proliferation, invasive growth and inflammation. The results of our experiments are in support of this hypothesis; OXA and GF, two inhibitors of LDH activity, reduced lactate levels in cultured CaCo-2 cells, and appeared to mitigate all the effects caused by TNF-α and IL-17.

At present, molecules with drug-like properties hindering LDH activity are actively searched, to test the validity of AG inhibition as a possible strategy to control cancer cell proliferation. Unfortunately, because of the characteristics of the LDH active site (Fiume et al., 2014), this search is proving to be more complex than expected, in spite of important research investments. Our results can contribute to highlight the involvement of LDH protein in crucial signaling pathways for cancer cell survival and spread, and suggest for LDH inhibitors wider potential in anticancer treatment.

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Conflict of interest

The Authors have no conflict of interest to declare

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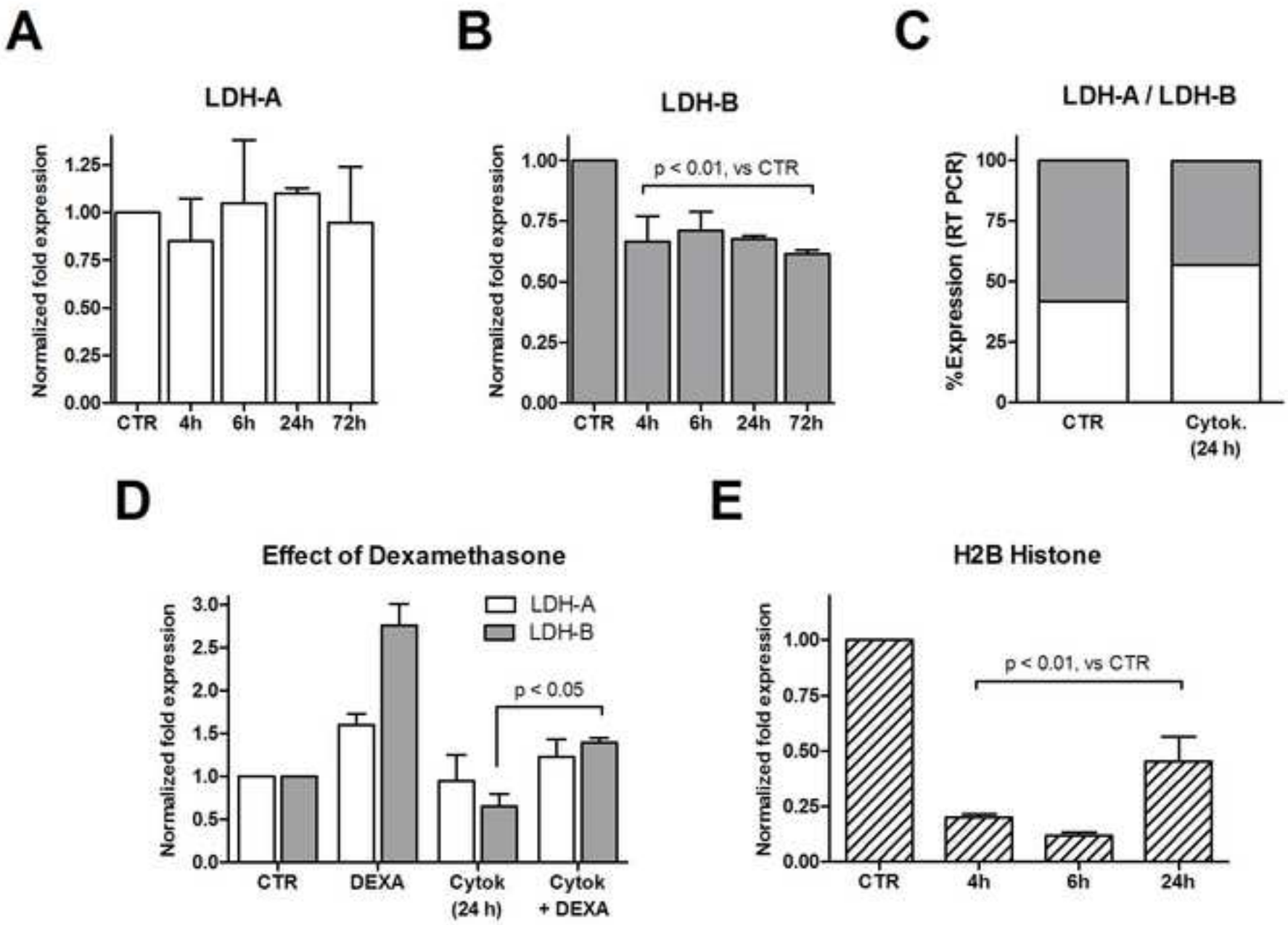
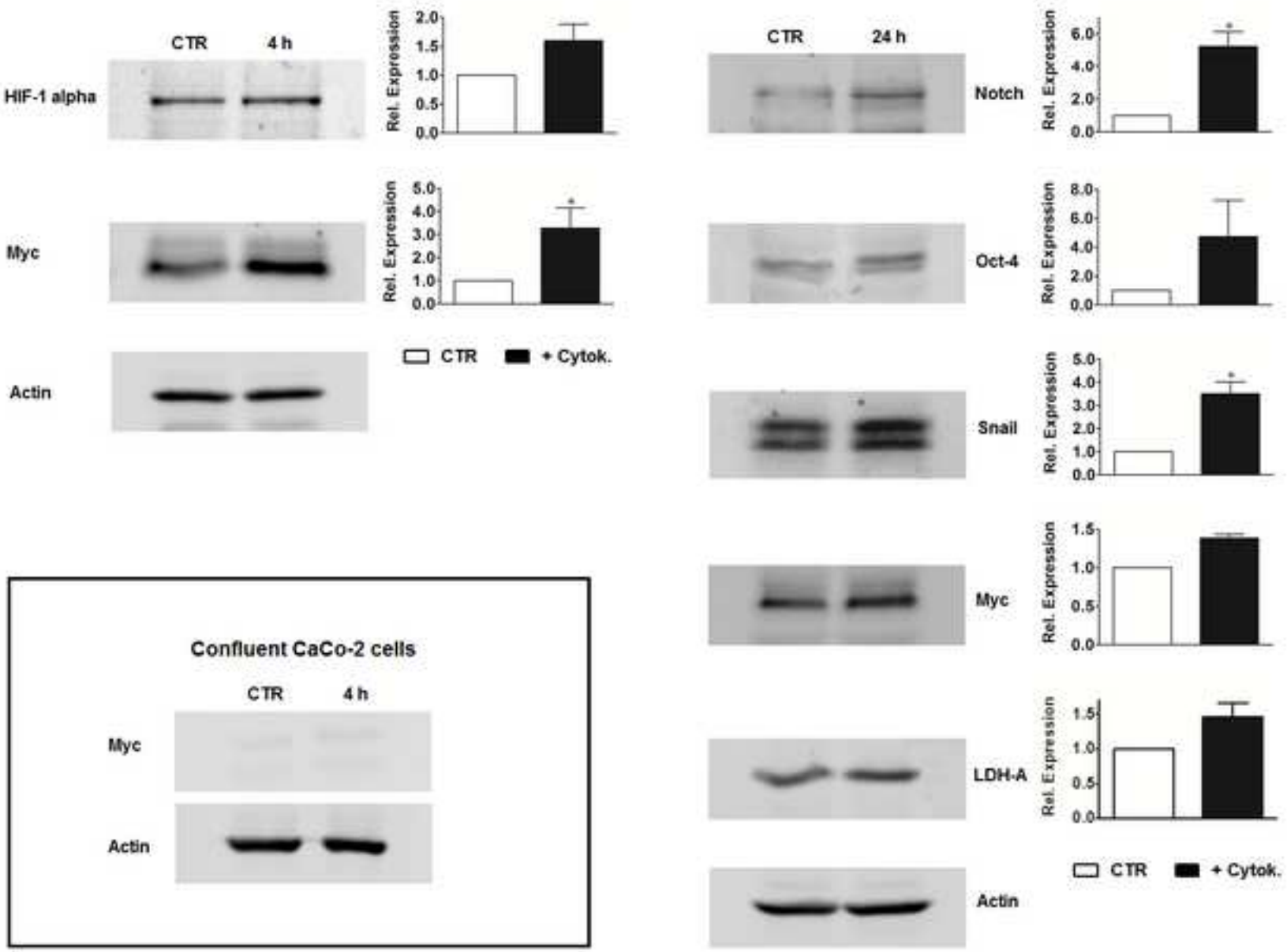
Figure captions

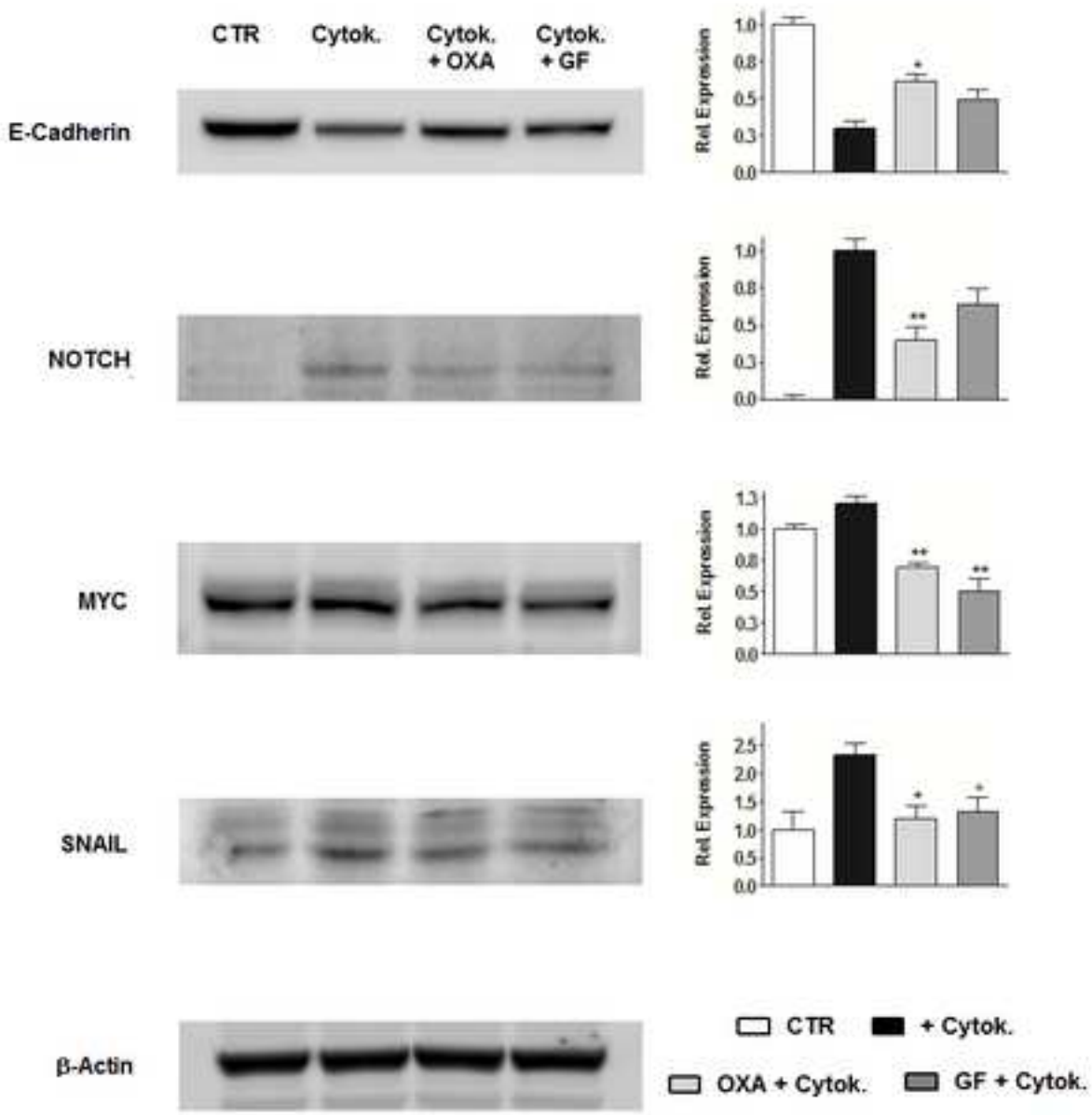
1. **Real Time PCR evaluating mRNA expression of LDH-A (A), LDH- B (B) and H2B histone (E) at different times after exposure to TNF-α and IL-17.** Data were analyzed by ANOVA followed by Dunnet's post-test. In experiments (B) and (E) a statistically significant difference between controls and all treated samples was found. (C): a 24 h treatment with the two cytokines changes the relative ratio between LDH-A and -B mRNAs. (D): dexamethasone restores LDH-B as the prevalent mRNA in cytokines treated cells (24 h). A statistically significant difference in LDH-B expression was observed between cells exposed to DEXA + cytokines and those treated with cytokines alone.
2. **Effects of inflammatory cytokines are dependent on the proliferative status of CaCo-2 cells.** Confluent cultures (figure insert): no effect was observed; as an example, the immunoblotting evaluation of Myc expression after a 4 h treatment is reported. Proliferating cells: a 4 h treatment causes a statistically significant increase of Myc protein, which is followed by the appearance of EMT signatures at 24 h. Asterisk indicates a statistically significant difference with untreated cultures, with $p < 0.05$.
3. **LDH inhibitors reverse the inflammation-induced effects.** OXA (40 mM) and GF (200 μM) were added to the CaCo-2 culture medium during the 24 h treatment with TNF-α and IL-17. Asterisks indicate a statistically significant difference of protein expression in comparison with cytokines treated cells. *, $p < 0.05$; **, $p < 0.01$.
4. **Morphology of treated cells and zimography evaluation.** Untreated (A) and cytokines-exposed CaCo-2 cells (B) were maintained in culture for 72 h and then photographed under a reverse microscope at a global magnification of 200x. The pictures evidence the differences in cell shape and growth pattern. (C) zimography

evaluation of MMP secretion in culture medium after a 24 h treatment in the presence of cytokines and LDH inhibitors. In cells exposed to cytokines, a single band of degraded gelatin with a molecular weight of 76 kDa was observed, which appeared reduced by LDH inhibitors. Asterisks indicate a statistically significant difference in comparison with cytokines treated cells. *, $p < 0.05$; **, $p < 0.01$.

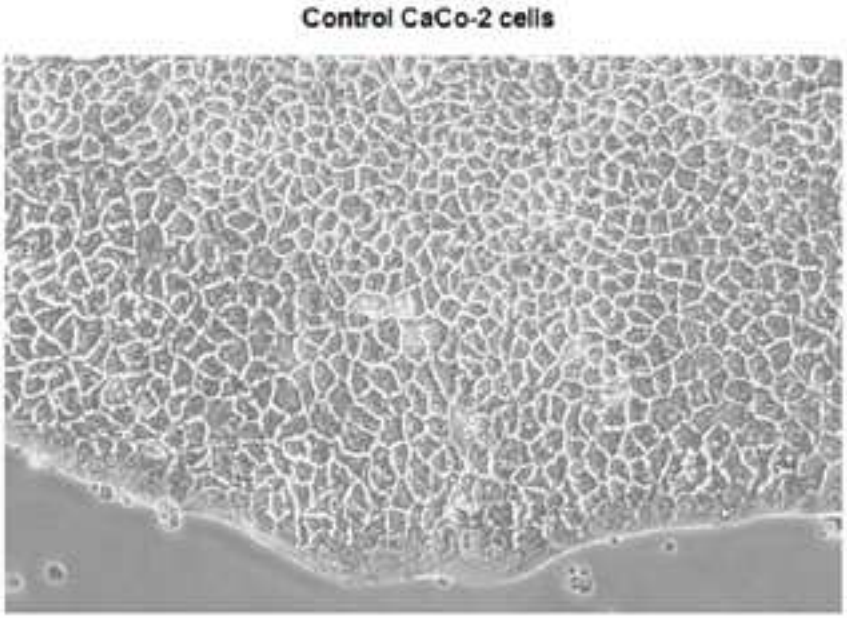
5. **Proteins induced in colon cancer cells by TNF-α and IL-17 and their role in tumor progression.**


A
LDH-A
Normalized fold expression
1.25
1.00
0.75
0.50
0.25
0.00
CTR
4h
6h
24h
72h
B
LDH-B
Normalized fold expression
1.00
0.75
0.50
0.25
0.00
p < 0.01, vs CTR
CTR
4h
6h
24h
72h
C
LDH-A / LDH-B
%Expression (RT PCR)
100
75
50
25
0
CTR
Cytok.
(24 h)
D
Effect of Dexamethasone
Normalized fold expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
LDH-A
LDH-B
p < 0.05
CTR
DEXA
Cytok
(24 h)
Cytok
+ DEXA
E
H2B Histone
Normalized fold expression
1.00
0.75
0.50
0.25
0.00
p < 0.01, vs CTR
CTR
4h
6h
24h

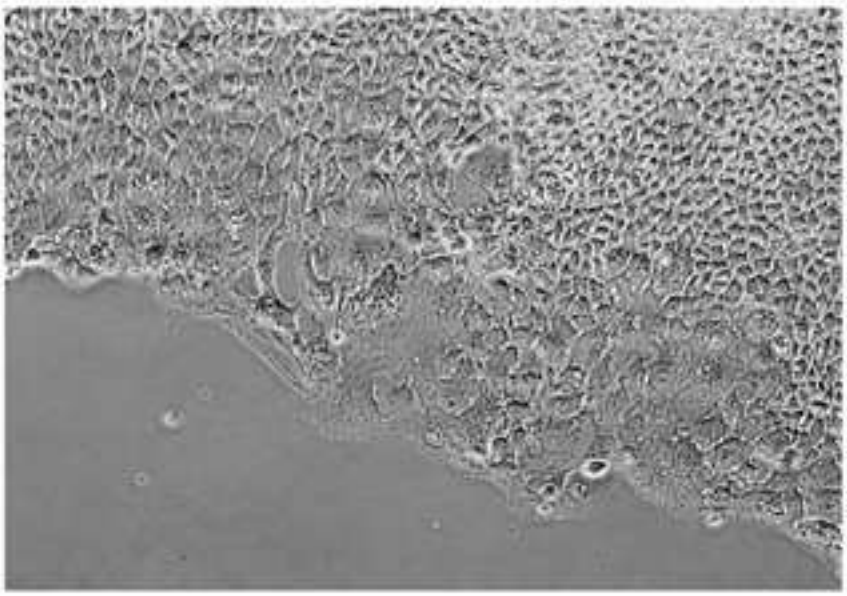

Proliferating CaCo-2 cells exposed to TNF-alpha and IL-17
CTR
4 h
HIF-1 alpha
Rel. Expression
2.0
1.5
1.0
0.5
0.0
Myc
5.0
4.0
3.0
2.0
1.0
0.0
CTR
+ Cytok.
Actin
CTR
24 h
Notch
6.0
4.0
2.0
0.0
Oct-4
8.0
6.0
4.0
2.0
0.0
Snail
5.0
4.0
3.0
2.0
1.0
0.0
Myc
1.5
1.0
0.5
0.0
LDH-A
1.5
1.0
0.5
0.0
Actin
CTR
+ Cytok.
Confluent CaCo-2 cells
CTR
4 h
Myc
Actin


CTR
Cytok.
Cytok. + OXA
Cytok. + GF
E-Cadherin
NOTCH
MYC
SNAIL
β-Actin
Rel. Expression
CTR
+ Cytok.
OXA + Cytok.
GF + Cytok.

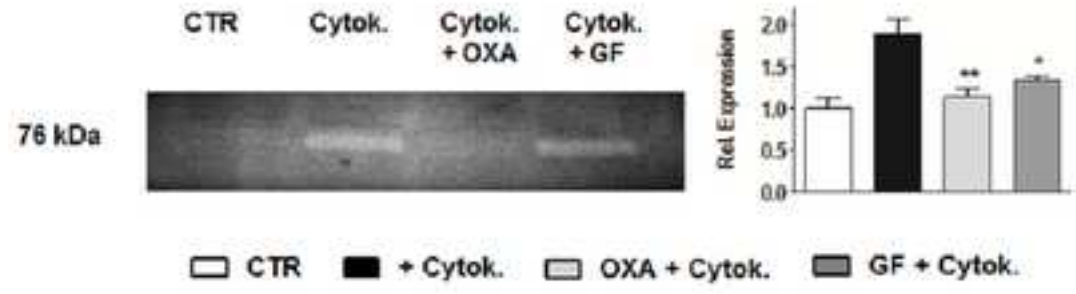
A


Control CaCo-2 cells

B


CaCo-2 cells exposed to cytokines (72h)

C


CTR
Cytok.
Cytok. + OXA
Cytok. + GF
76 kDa
Rel Expression
2.0
1.5
1.0
0.5
0.0
**
*
CTR
+ Cytok.
OXA + Cytok.
GF + Cytok.

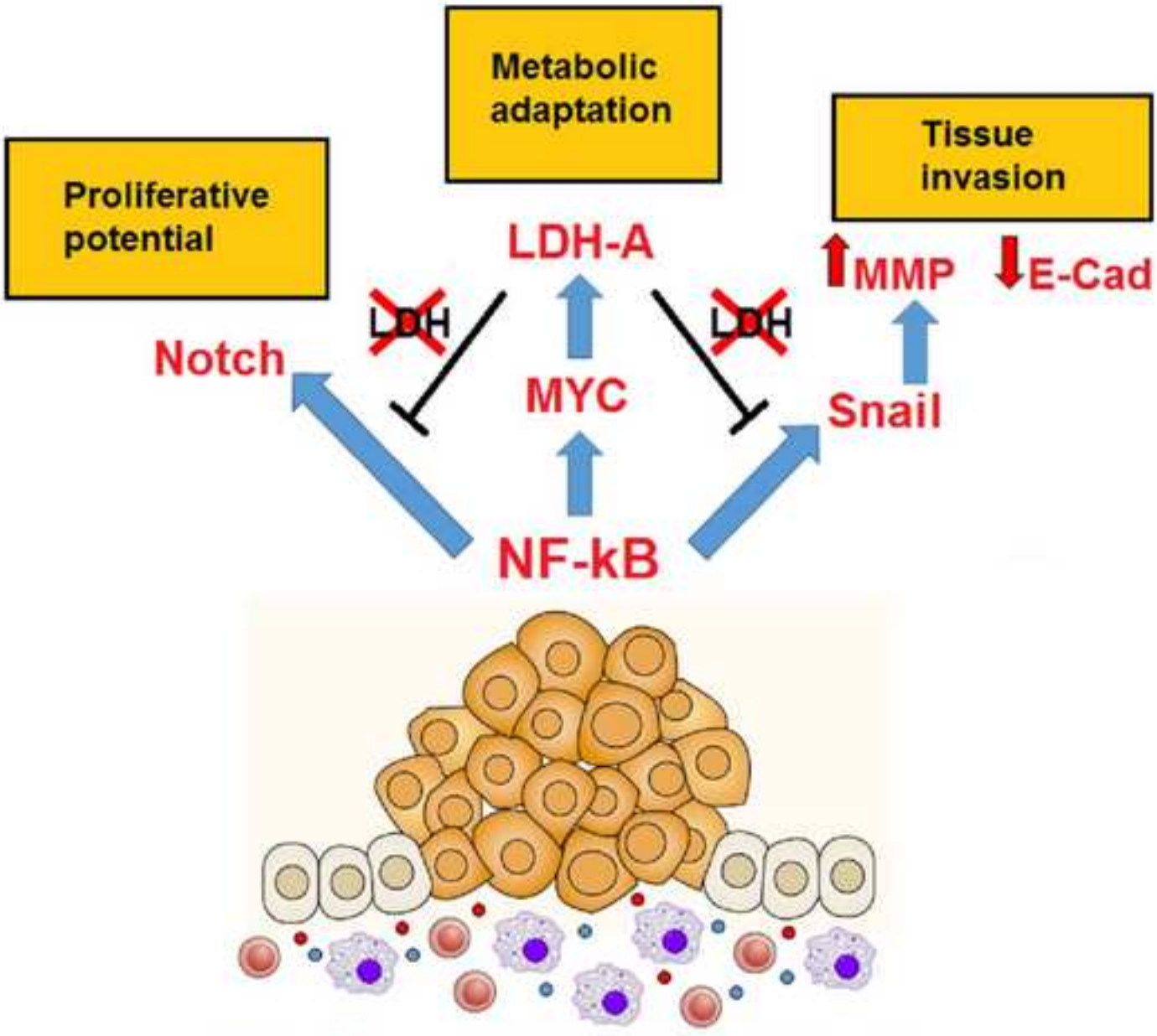

Metabolic adaptation
Proliferative potential
Tissue invasion
LDH-A
LDH
LDH
MMP
E-Cad
Notch
MYC
Snail
NF-kB
TNF-alpha and IL-17 producing cells

TABLE 1

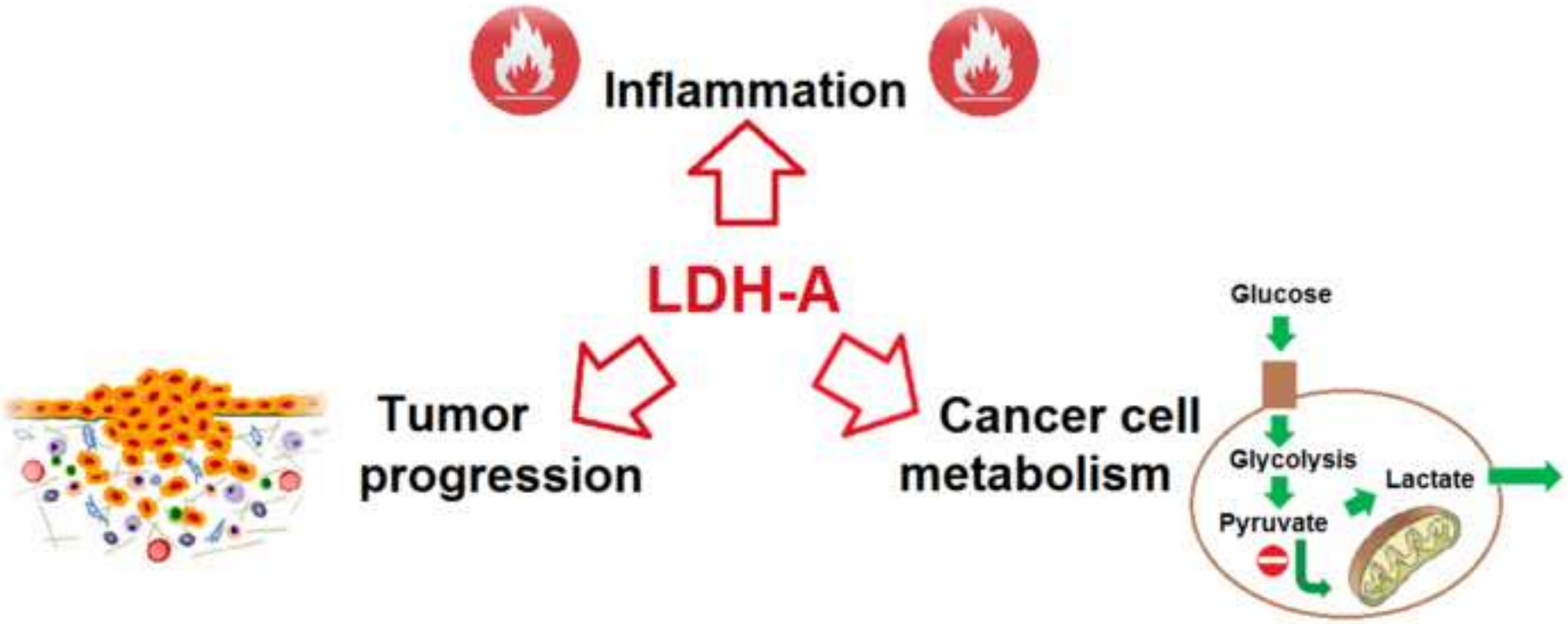
A - Effects of inflammatory cytokines on CaCo-2 cells

	Control CaCo-2 cells	**Treated CaCo-2 cells**
LDH activity (mU/mg cell proteins)	699 ± 30	890 ± 60 *
% LDH composition (**LDH-A**/LDH-B) (assayed by enzyme activity)	**42 ± 12** / 58 ± 12	**57 ± 20** / 43 ± 20
Glucose uptake (relative to controls)	1.00	1.25 ± 0.05 *
Lactate production (μg / 10^6 cells / 1h)	15.7 ± 1.1	24.8 ± 2.0 *
ALP activity (nmoles p-NP/min/mg cell proteins)	8.3 ± 0.9	Undetectable

B - Effects of the used LDH inhibitors on CaCo-2 cells

	OXA (40 mM)	**GF (200 μM)**
Lactate production (% reduction at 6h)	43 ± 5 *	48 ± 7 *
Cell growth (% reduction at 24h)	32 ± 6 *	46 ± 5 *

* $p < 0.05$, as evaluated by Student's t-test


Inflammation
LDH-A
Tumor progression
Cancer cell metabolism
Glucose
Glycolysis
Lactate
Pyruvate