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The acidic tumor tissue pH: Role in inflammation and metastasis

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Abstract

An acidic tissue pH (acidosis) is found during different pathological conditions, among them being inflammation and cancer. The drop in pH represents a stress situation for the residing cells resulting in changes of cellular phenotype and function. In inflamed tissue acidosis may modulate the immune response by affecting proliferation, differentiation and activation of immune cells, as well as the production and secretion of inflammatory mediators. In solid tumors a reduction in tissue pH is linked to tumor aggressiveness, changes in chemo- and radiotherapy and anti-tumor immunity. Thus in this work the question was addressed whether and how acidosis can affect the inflammatory program of different non-tumor (monocytes/macrophages, fibroblasts, epithelial cells) and tumor (prostate and breast carcinoma) cells. For this, cells were analyzed under control (pH = 7.4) and acidic (pH = 6.6) conditions in concern of the expression of inflammatory mediators IL-1ß, IL-6, TNF-α, SPP1, MCP-1, iNOS and COX-2 *in vitro* but also *in vivo* in experimental tumors, where the complex interaction of the different cell types can be studied best. Tumor tissue pH was reduced *in vivo* by inspiratory hypoxia and blocking of the respiratory chain for 24 h, which forced glycolytic metabolism in experimental tumors of AT-1 prostate and Walker-256 breast carcinoma cells. This induction of acidosis led to a reduced expression of most inflammatory mediators in the tumor tissue. These results were in line with cell culture data, that show no or negative regulation of the inflammatory program in prostate and breast carcinoma cells, as well as NRK-49F fibroblasts and NRK-52E epithelial cells after long-term incubation (24 h). Thus, an acidic tumor tissue pH seems to repress the inflammatory program.

However, *in vitro* data could show that short-term incubation (3-6 h) in an acidic environment induces the expression of some inflammatory mediators. Acidosis-induced up-regulation was found for iNOS, COX-2, IL-6 and TNF-α in a cell line-specific way, that depended on ERK1/2 and p38 activation by acidosis. Fibroblasts had an elevated expression of COX-2, iNOS, IL-6 and TNF-α. In prostate and breast carcinoma cells acidosis raised the level of iNOS and SPP1. IL-6 was only up-regulated in prostate carcinoma cells, while TNF-α induction was only detected in breast carcinoma cells. Also in RAW264.7 and primary human M2 polarized macrophages an increase in COX-2 was observed after short acidic stimulation, together with an elevated phagocytic activity. Therefore, short periods of acidosis might affect tumor growth through the action of IL-6 and TNF-α, tumor progression by the production of prostaglandins and reactive nitrogen species through COX-2 and iNOS, and the recruitment and cytokine secretion of macrophages via SPP1.

Whether short-term acidosis can also affect tumor aggressiveness was studied by priming prostate and breast carcinoma cells in an acidic environment before i.v. injection and subsequent lung metastases formation. Acidic priming strongly fostered lung metastasis. No changes in invasive behavior or adhesion were observed, however the cells displayed elevated migration. Increased migration was even observed when acidosis-primed cells were re-transferred to a normal tissue pH. Thus, the increase in migration by the acidic priming might account for the fostered metastases formation. In summary, a complex interplay between tumor tissue pH, stromal cells and tumor cells exists, which also depends on the duration of acidosis, and has to be taken into account when targeting acidosis for tumor therapy.

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Abbreviations

AE	anion exchanger
ASIC	acid-sensing ion channel
BCECF-AM	2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein, acetoxymethyl ester
CA	carbonic anhydrase
CAFs	cancer-associated fibroblasts
cAMP	3',5'-cyclic adenosine monophosphate
COX-2/PTGS2	cyclooxygenase-2 or prostaglandin-endoperoxide synthase 2
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
FAK/PTK2	focal adhesion kinase or protein tyrosine kinase 2
GPCR	G protein-coupled receptor
HIF	hypoxia-inducible factor
IFNγ	interferon gamma
IL	interleukin
iNOS/NOS2	inducible nitric oxide synthase
MAPK	mitogen-activated protein kinase
MCP-1/CCL2	monocyte chemoattractant protein 1
MCT	monocarboxylate transporter
MDCK-C7	Madin-Darby Canine Kidney-C7 cells
MRP1	multidrug resistance-associated protein 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHE	Na^+/H^+ exchanger
NK cells	natural killer cells
NRK-52E	normal rat kidney epithelial
NRK-49F	normal rat kidney fibroblasts
NO	nitirc oxide
OPN	see SPP1
PGE2	prostaglandin E2
Pgp/MDR1	p-glycoprotein 1 or multidrug resistance protein 1
pH_e	extracellular pH
pH_i	intracellular pH
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PMA/TPA	phorbol 12-myristate 13-acetate
ROS	reactive oxygen species
SPP1	secreted phosphoprotein 1 or osteopontin
TAMs	tumor-associated macrophages
TNF-α	tumor necrosis factor alpha
TRPV	transient receptor potential cation channels subfamily V
UPR	unfolded protein response
V-ATPase	vacuolar-type H^+-ATPases
VEGF	vascular endothelial growth factor

1. Introduction

1.1. Pathophysiology of the tumor microenvironment

Several parameters including blood flow, microcirculation, the supply of oxygen as well as nutrients, the tissue pH, lactate levels and the bioenergetics status differ profoundly when comparing tumor tissue with normal tissue, and define the so-called pathophysiological tumor microenvironment. These factors are closely linked and affect tumor promotion and progression, but also tumor therapy [1,2]. Alterations are mainly due to the aberrant vascular network in solid tumors. The tumor vasculature is often characterized by irregular structure and function and accounts for heterogeneous and often insufficient tumor blood flow, as well as increased microvessel permeability and interstitial pressure [3]. Among the structural and functional alterations are the loss of vessel hierarchy with excessive branching, blind ending vessels and shunts [3,4]. Vasomotion and the regulation of blood flow are frequently missing, as well as functional lymphatic vessels [5]. Additionally, resistance to flow is increased and both the velocity and the direction of flow can vary distinctively [6]. The spatial heterogeneity in the distribution of tumor vessels and the temporal heterogeneity in blood flow result in inadequate supply with oxygen and nutrients, and hampered waste removal [7]. In consequence, a reduction in oxygenation (hypoxia or even anoxia), insufficient nutrients including shortage of glucose and energy depletion, low extracellular pH (acidosis) and high lactate levels among others are ubiquitous features of the tumor microenvironment [6]. Some typical changes of the tumor microenvironment are listed in table 1.

Table 1 Typical pathophysiological changes of key factors of the tumor microenvironment. Modified from [3].

increase in	decrease in
vascular chaos	nutrient supply
perfusion heterogeneities	partial pressure of O_2
permeability of microvessels	waste removal
pressure of interstitial fluid	production of high-energy compounds
partial pressure of CO_2	bicarbonate concentration
production of lactate	pH
production of adenosine	

1.2. The development of extracellular acidosis

Extracellular pH (pH_e) in the microenvironment of solid tumors was found to be lower in comparison to that of normal tissue. Values between pH_e = 6.5 – 7.0 are described in the literature, but pH can also be as low as pH_e = 6.0 [6,8]. These values are mean values and high heterogeneity exists between different tumor entities, as well as within a certain tumor. In contrast to these observation, the intracellular pH (pH_i) in tumors is often neutral or even slightly alkaline [9]. Values between pH_i = 7.0 – 7.2 are typically found in normal tissues, while tumor cells have a pH_i in the range of 7.0 – 7.3 which is optimal for cell proliferation [10]. Not surprisingly, intracellular alkalinization has been linked to cellular transformation, fostered proliferation, local tumor growth, increased motility, metastases formation and resistance to chemotherapy [11]. For normal tissue the intracellular pH is slightly more acidic than the extracellular one (interstitial space pH_e = 7.25 – 7.35), while for tumor cells intracellular pH is much more alkaline than the extracellular space. This so called reversed pH gradient is a typical feature of solid tumors [12].

Different sources have to be taken into account for the increased amount of protons in the extracellular space. In general, changes in tumor cell metabolism leading to an increased proton efflux and the hampered removal of acidic products by the abnormal vasculature are responsible for extracellular acidosis. Metabolic pathways involved in the production of protons are glycolysis and the production of lactic acid, ATP hydrolysis, respiration-derived CO_2 production, glutaminolysis, the oxidative arm of the pentose phosphate pathway, decarboxylation reactions, and amino acid metabolism producing NH_4^+ [3,13]. Increased glucose uptake and glycolysis is typically found in hypoxic tumor areas [14]. However, even in the presence of oxygen, tumor cells switch their metabolism and use glycolysis preferentially (Warburg effect). This phenomenon is found although mitochondria are usually still functional and although the consequence of glycolysis is a reduced efficiency in the production of ATP as well as an increased production of protons [15]. During oxidative phosphorylation up to 36 ATP/Glucose are produced, while aerobic glycolysis yields only 2 ATP/glucose. The benefit of aerobic glycolysis for the tumor cells is still under investigation. Possibly, energy production is switched to glycolysis since it is much faster, or glycolysis can provide the necessary intermediates used for the biosynthesis of nucleotides, amino acids and lipids which are required from the highly proliferative tumor cells [16]. Glycolysis results in an increased production of lactate that is transported out of the cell together with protons by monocarboxylate transporters (MCT), resulting in extracellular acidification and increased lactate concentration. In tumors lactate values of 6 – 15 mM and even up to 40 mM were found, while in normal tissue lactate levels are usually between 1.8 – 2 mM. Interestingly, these high

concentrations of lactate in the tumor microenvironment can affect tumor phenotype independent of acidosis [17].

1.3. Regulation of pH_i

For tumor cell survival the controlling of pH_i is essential. Homeostasis of pH_i is achieved by exporting protons and importing proton acceptors (bicarbonate). Involved in this process are the Na^+/H^+ exchanger (NHE1), Na^+/HCO_3^- co-transporters, Na^+-driven Cl^-/HCO_3^- exchanger, electroneutral anion exchanger (AE1-3), monocarboxylate transporter (MCT1-4), carbonic anhydrase (CA IX, CA XII, CA II) and vacuolar-type H^+-ATPases (V-ATPase) [18–20]. NHE1 is regulating both cell volume and pH homeostasis and is linked to tumor growth and progression [18]. Hyperactive NHE1 leads to cytosolic alkalization which is correlated with malignant transformation, stabilization of spontaneous mutations, uncontrolled proliferation, and augmented DNA synthesis [13]. Also the expression of certain oncogenes as well as enhanced activity of growth factors, the phenomenon of multi drug resistance and the onset of metastases formation, promoted migration and invasiveness are correlated with an alkaline pH_i [13]. Bicarbonate transporters AE1-3 are responsible for an increased pH_i in tumor cells and have been described to interact with carbonic anhydrases (CAs), e.g. AE2 with CA IX in hypoxic tumors [18]. CAs are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and protons. The expression of CA IX is controlled by the hypoxia-inducible factor 1 (HIF-1), thus CA IX is a marker for tumor hypoxia and leads to extracellular acidification. CA IX is involved in pH regulation and signaling in tumor cells, it affects proliferation, survival, migration as well as invasion, and is linked to poor prognosis and resistance to cancer treatment [21,22]. MCTs transport L-lactate, pyruvate and ketone bodies in symport with protons and thus are involved in pH regulation beside their role in cellular metabolism [13]. Interestingly, MCTs are important for the shuttling of lactate produced by hypoxic tumor cells to non-hypoxic cells and are thus gating the oxidative use of lactate [13]. V-ATPases are involved in pH homeostasis, endocytosis, activation of certain proteases, angiogenesis, autophagy and the sensing of amino acids via the interaction with mTOR [13]. Due to the critical role of these transporters for instance in tumor cells they represent promising pharmacological targets [10].

1.4. Consequences of extracellular acidosis

The inefficient vasculature, hypoxic tumor areas as well as changes in metabolism all account for the acidification of the local microenvironment within the tumor (Fig. 1). Acidosis will in consequence lead to distinct changes in tumor phenotype (overview see [23]). Acidosis is involved

in tumor promotion by fostering malignant transformation and clonal selection [24]. Additionally tumor growth and progression is intensified [25,26]. Acidosis can enhance motility, invasiveness and metastasis formation and modulate anti-tumor immunity [27–30] which will be discussed in more detail in chapters 1.5 und 1.6. In addition it has an influence on the efficiency of anti-cancer treatment. Both chemo- and radiotherapy are affected when extracellular pH is lowered [25,31]. Acidosis interferes with the uptake of basic chemotherapeutic drugs such as doxorubicin, daunorubicin or vinblastine [24,25]. Furthermore, drug transporters like multidrug resistance protein 1 (MDR1/Pgp) and multidrug resistance-associated protein 1 (MRP1) are activated by acidosis, leading to reduced intracellular accumulation of chemotherapeutic drugs and increased tumor survival [32–35]. For AT-1 prostate carcinoma cells it was shown that acidosis-induced p38 signaling was critical for MDR1 activation [33]. Beside changes in activity of drug transporters, also their expression or localization may be altered through acidosis leading to the development of a multidrug resistant phenotype. Due to the critical role of pH regulation for tumor growth, metastasis and therapy resistance, attempts are made targeting dysregulated tumor pH for cancer treatment (overview see [11,18,20,26]). Possible therapeutic approaches could address either an increase of pH_e leading to reduced metastasis, or a decrease of pH_i, resulting in reduced proliferation and elevated cell death, or a modulation of both pH_e and pH_i at the same time [26].

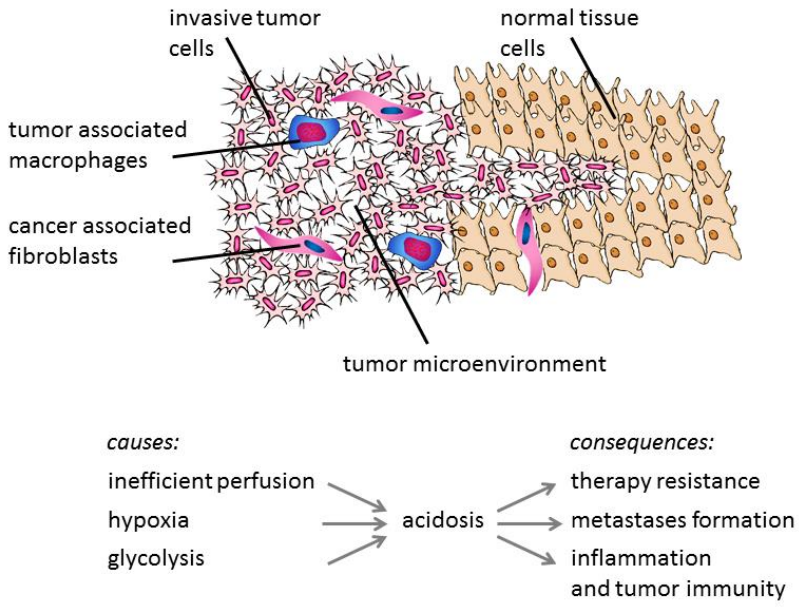

Figure 1 Development and impact of the acidic tumor microenvironment on tumor progression.

As shown in figure 1 the tumor does not solely comprise of tumor cells, but also other cell types like immune cells, fibroblasts, endothelial and epithelial cells are present in the tumor mass. These

stromal cells together with the extracellular matrix are often denominated as tumor microenvironment in the literature, but should not be confused with the metabolic microenvironment described above. Extracellular acidosis thus not only affects the behavior of the tumor cells themselves, but also the phenotype of the stromal cells and their interaction with the tumor cells (and vice versa). By this mechanism stromal cells may affect tumor cell behavior, prognosis, outcome and therapy. Therefore, in this work the impact of acidosis was not only evaluated in tumor cells, but also in fibroblasts, macrophages and epithelial cells (see chapters 2, 3 and 4.3).

1.5. Metastases formation

Metastasis is defined as the spread of tumor cells form the primary neoplasm to distant organs. Although diagnosis, general patient care, and therapy were improving remarkably over the past years, most tumor-associated deaths are still due to the formation of metastases [36]. Metastasis comprises several steps (overview see [36,37]). The tumor cells must detach from the primary tumors and invade in the surrounding tissue after proteolysis of the extracellular matrix and disruption of the basement membrane. For this purpose cell need to loosen their cell-cell-adhesion, reorganize their cytoskeletal structure and increase motility. To acquire such invasive and migratory phenotype, the tumor cells lose their epithelial properties and gain mesenchymal features (epithelial-to-mesenchymal transition, EMT). Additionally, proteases such as matrix-metalloproteinases (MMPs) and cathepsins are secreted. Tumor cells enter the bloodstream as single cells or in cell groups through lymphatics, venules and capillaries. Most circulating tumor cells are destroyed e.g. by protective immune cells or by shear stress. However, those that survive, adhere to the vessel wall, often mediated by leukocytes and platelets, and extravasate typically in small capillaries [38]. The preference of different tumor types to metastasize at certain organs is possibly mediated by the action of chemokines and the type of vasculature present. In the `seed and soil` hypothesis by Stephen Paget the critical role of the microenvironment, in addition to the acquisition of all necessary genetic alterations, for metastases formation at a certain target organ is postulated [36,39]. For the last step of metastasis the tumor cells at the newly formed tumor foci (micrometastases) must survive and proliferate to eventually form clinically detectable macrometastases.

Different models exist for the mechanisms by which tumors cells acquire their full metastatic potential [37]. However, more and more evidence suggests that stromal cells, both in the primary tumor and the pre-metastatic niche, as well as parameters of the microenvironment, which include oxygenation, pH, nutrients, redox state i.a., play a critical role for metastasis, too [36]. As

summarized in figure 2, extracellular acidosis has been shown to modulate metastases formation at the level of all afore-mentioned steps [30,40]. Acidosis may induce clonal selection of tumor cells with a more aggressive phenotype [41]. Some literature suggests that acidosis induce EMT in tumor and non-tumor cells. Incubating mesenchymal stem cells at a low pH led to TGF-ß expression that induced an EMT-like phenotype in melanoma cells [42]. Acidic incubation also induced EMT in renal HK-2 cells [43]. We could show that acidosis reduced E-cadherin expression in kidney epithelial cells as well as in bronchioalveolar carcinoma cells and it increased the level of vimentin in kidney epithelial cells as well as in bronchioalveolar, breast and prostate carcinoma cells [44]. This could lead to changes in cytoskeletal organization, cell-matrix adhesion as well as cell-cell interaction and thus fosters local invasion [10]. Extracellular acidosis can affect directed and random movement of cells by modulating dynamic reorganization of adhesion contacts, the cytoskeleton and extracellular matrix, as well as water and ion transport across the cell membrane [45]. An increase in tumor cell migration by mild acidosis has been described in the literature [41,46–49]. In addition, normal cells also display acidosis-induced modulation of motility, however the effect varies depending on the cell type studied [50–53]. Signaling pathways involved in acidosis-induced cell migration comprise ion channels and transporters, reactive oxygen species (ROS)/redox state, mitogen-activated protein kinase (MAPK), as well as focal adhesion kinase (FAK)-Src pathways [54–56]. Local invasion can be enhanced by acidosis via the expression, release and activation of matrix metalloprotease (MMP-2, MMP-9) and cathepsin (cathepsin B, cathepsin L) family members by the tumor as well as stromal cells (CAFs and TAMs) [10,30,40]. Taking all these mechanism into account, it is not surprising, that the tumor areas with the lowest pH_e display the highest local tumor invasion [57]. Dissemination of tumor cells can also be promoted by acidosis through fostering angiogenesis. Low pH induces angiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) [10,30,40,58]. Additionally, acidosis-induced matrix degradation may result in release of entrapped VEGF in acidic tumor areas [59]. Furthermore, the subsequent steps of metastases formation, including surviving in the circulatory system, extravasation and growth in a new tissue, seem to be susceptible to low pH (Fig. 2). Lung metastases formation of tumor cells injected intravenously was increased, when tumor cells were primed in an acidic environment before [58,60]. And in line with these results, oral bicarbonate administration reduced metastasis by inhibiting extravasation and colonization, while the number of circulating tumor cells was not affected [61]. Interference with tumor acidification through buffering with bicarbonate, imidazoles, Tris, and free base lysine impeded the formation of spontaneous and experimental metastases in animal models and could thus be a promising therapeutic approach [10].

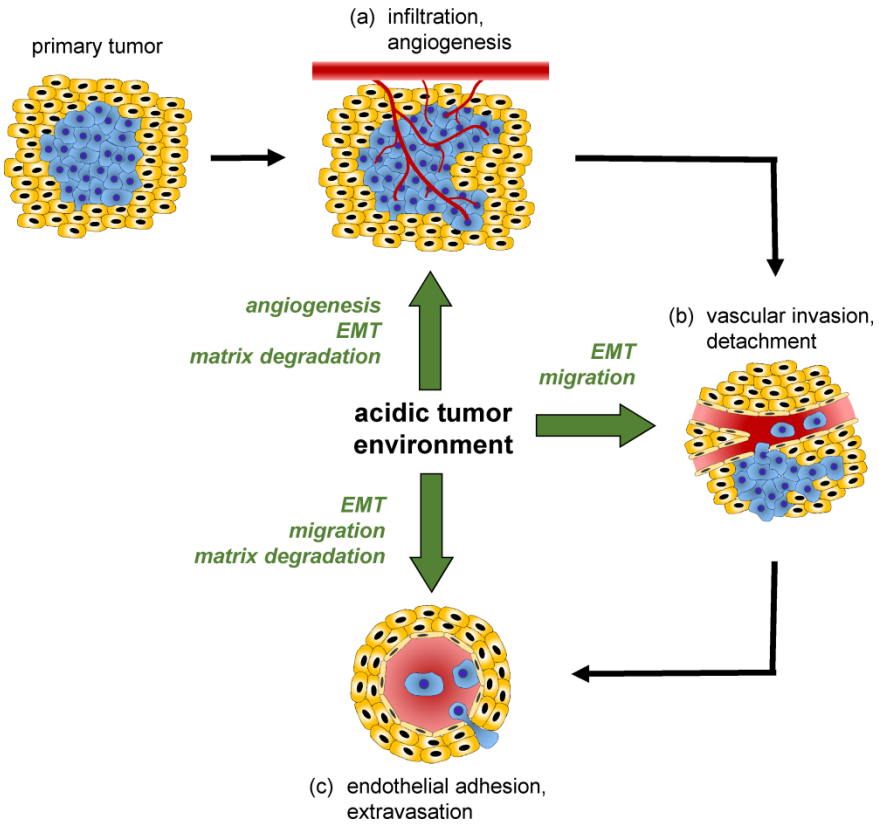

Figure 2 Impact of low pH on the steps of metastases formation, including (a) local invasion and angiogenesis, (b) intravasation of tumor cells into the circulatory system and (c) adhesion to the endothelium and extravasation into distant secondary sites. [30]

1.6. Inflammation and tumor immunity

Inflammation and tumor promotion as well as progression are often linked [62]. Solid tumors seem to trigger the inflammatory response and remodel their microenvironment through the recruitment of leukocytes, including macrophages, neutrophils, dendritic cells, eosinophils, mast cells and lymphocytes [62]. In such an environment sustained proliferation and fostered survival and migration is found [63]. Leukocytes produce chemokines and cytokines, angiogenic growth factors, proteases, prostaglandins and reactive oxygen species (ROS) as well as reactive nitrogen species [62,63]. For instance M2 macrophages can promote tumor growth and are involved in angiogenesis, invasion as well as metastasis, and correlate with poor prognosis. Also dendritic cells and T-regulatory lymphocytes can promote tumor growth. T cells can have both a tumor-suppressive and a tumor-promoting role [62]. The outcome, that means either promotion of tumor growth and metastases formation or suppression of tumor proliferation, depends on the abundance of anti-inflammatory as well as pro-inflammatory cytokines [63].

Extracellular acidification of a microenvironment can modulate the immune response by different mechanisms. Its functional impact depends on the cell type studied. The function of neutrophils is

inhibited by acidosis including reduced production of superoxide, decreased respiratory burst as well as phagocytosis [64–66]. Also macrophages displayed inhibition of superoxide production and phagocytosis, as well as suppressed cytokine production and cytotoxic activity, but increased NOS activity [64]. However, other works suggest that high concentrations of protons can be recognized by innate immune cells as a danger-associated molecular pattern (DAMP), leading to differentiation/maturation and increased cytokine production of neutrophils, macrophages and dendritic cells [66]. Chemotaxis of polymorphonuclear leukocytes was reduced at low pH, which was even further aggravated by hypoxia [64]. Lymphocytes seem to have an increased motility but diminished proliferation, natural killer cells (NK cells) were less active and cytotoxic T cells less capable of tumor cell lysis at low pH [64]. Hence, acidosis seems to have an overall immunosuppressive effect and might also blunt the effectiveness of antitumor immunity by reducing T cells activation and secretion of interferon gamma (IFNγ), tumor necrosis factor α (TNF-α) and interleukin-2 (IL-2) [27]. In clinical studies an impairment of immune function by acidosis, including reduced proliferation, chemotaxis and antibody production, is described, too [64]. Additional evidence comes from studies showing that high lactate concentrations can affect inflammation and angiogenesis. Lactate can block the differentiation and activation of monocytes and T cells, VEGF secretion as well as M2 macrophage polarization and has immunosuppressive effects on tumor-infiltrating T and NK cells [67]. Whether these effects depend on lactate alone, or rather on the combination with acidosis, has to be clarified. But putting together the data from the literature shows that extracellular acidification affects chemotaxis of leukocytes, ROS production (respiratory burst), phagocytosis and apoptosis, as well as complement activation [65,68]. Preventing the acidification might improve antitumor response to immunotherapy [27]. However, it is important to note that extracellular acidosis is not limited to the tumor microenvironment of solid tumors. It can be present in other pathological situations, including ischemia, acid-base-imbalance during sepsis, trauma or acute respiratory distress, leukemia and in inflammation in general [66]. The aforementioned implications of acidosis apply in all of these different pathological alterations. For instance, ischemia-driven tissue acidification during wound healing stimulates production of inflammatory cytokines by endothelium and induces neoangiogenesis [69]. Metabolic acidosis in patients suffering from critical illness leads to changes in the expression of interleukin-6 (IL-6), interleukin-10 (IL-10), TNF-α and inducible nitric oxide synthase (iNOS/NOS2) by circulating leukocytes [65]. In the present studies a set of different inflammatory mediators was analyzed including interleukin-1ß (IL-1ß), IL-6, TNF-α, osteopontin (OPN/SPP1), monocyte chemoattractant protein 1 (MCP-1/CCL2), iNOS and cyclooxygenase-2 (COX-2/PTGS2).

Several cytokines found in the tumor microenvironment can influence tumor growth, tissue remodeling, angiogenesis, metastasis and immunosuppression [70]. Also circulating cytokines in

the blood stream seem to be of prognostic value, especially during metastasis, during which increased levels of IL-1, IL-6 and TNF-α can be found [63,70]. IL-1ß can affect the innate and the adaptive immune response and thus plays an important role in inflammation (overview see [71]). A possible link to acidosis is its involvement in the cellular response to tissue damage and cell death. Besides it activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and MAPK signaling, the latter being described to be induced by acidosis, too [33]. Production of IL-6 occurs in response to tissue injury or infections, and it can shift the balance between tissue repair and carcinogenesis. Overexpression of IL-6 is found in colorectal, breast and prostate cancer and it is involved in tumor growth, angiogenesis, activation of stromal cells and immune modulation (overview see [72]). Hence it is discussed as marker for cancer diagnosis, prognosis and even target for therapy [70,73]. IL-6 is involved in several chronic inflammatory processes and can affect immunity by regulating migration of monocytes, differentiation of B- and T-cells among others [74,75]. TNF-α is one of the most important pro-inflammatory cytokines regulating cell proliferation, death, differentiation, but most importantly innate and adaptive immune cells [76]. MCP-1 plays a role in recruitment and activation of immune cells. It can be expressed by a variety of different cell types either constitutively or after induction by growth factors, cytokines and oxidative stress [77]. Osteopontin is a secreted matricellular protein that can modulated tissue repair, inflammation and tumor progression [78]. It is involved in the recruitment of monocytes and macrophages, as well as the secretion of cytokines by immune cells [79]. COX-2 and iNOS can contribute to inflammation by the production of prostaglandins and reactive nitrogen species, respectively. COX-2 acts pro-tumorigenic by fostering proliferation, inflammation, metastasis and therapy resistance and is thus discussed as target for tumor therapy [80]. Additionally, COX-2 can contribute to immune evasion and resistance to cancer immunotherapy [81]. The expression of iNOS is often elevated in tumors, but its role is still matter of discussion. It can be pro-tumorigenic, augmenting proliferation, angiogenesis as well as metastasis or anti-tumorigenic, inducing apoptosis or necrosis [82]. Importantly, not only immune cells, but also tumor cells and activated fibroblasts are sources of cytokines.

1.7. Stress-induced signaling

Cells need to respond to changes of their microenvironment and adapt to ensure cell survival. Modulation of the cellular response to hypoxia is well characterized and involves signaling via hypoxia-inducible factor 1 (HIF-1) [83]. However, for acidosis data in the literature is sparse and no generally valid mechanism is known so far. Lowering of extracellular pH can regulate cellular signaling by numerous ways. It can modulate the cytosolic and membrane-associated enzyme

activity, the ion transport activity, protein as well as DNA synthesis, the levels of cAMP and Ca^{2+}, the activity of hydrolytic enzymes and much more [64]. The response to acidosis may involve unfolded protein response (UPR) or MAPK signaling as described for different situations of cellular stress [84]. UPR inhibits general protein translation and up-regulates the functional capacity of the endoplasmatic reticulum to maintain cellular homeostasis and ensure survival [84]. MAPK signaling leads to the phosphorylation of cytoplasmatic as well as nuclear substrates. One example is the regulation of the expression of inflammatory cytokines and chemokines like IL-6 and MCP-1 by MAPK signaling by coordinately modulating transcription, mRNA stability as well as translation [84]. Studies from our group have shown a critical role for MAPK signaling during extracellular acidosis, preventing necrotic cell death at low pH and increasing chemoresistance of prostate tumor cells *in vitro* and *in vivo* [33,85]. MAPK activation by acidosis has also been observed in other tumor and non-tumor cell lines [85,86]. Involved in the activation of acidosis-induced signaling are proton transporters like NHE1 [87], that are described in more detail in chapter 1.3, and pH sensors like proton-sensing G protein-coupled receptors (GPCRs) or transient receptor potential cation channels subfamily V (TRPVs) [88].

1.8. Objective of the presented work

In the presented work the impact of the acidic tissue pH on inflammation and tumor metastasis was analyzed. The expression of inflammatory mediators IL-1ß, IL-6, TNF-α, MCP-1, COX-2, iNOS and osteopontin was analyzed in fibroblasts and macrophages, since the stromal cells play a critical role for tumor growth, angiogenesis, metastasis and evasion of immunosurveillance [89]. Beside the role of these cells in tumor metastasis and inflammation, the results from fibroblasts and macrophages can be transferred to other pathological situations, which are also characterized by an acidic microenvironment, like inflammation or ischemia. Changes in the expression of the inflammatory mediators mentioned above were analyzed *in vitro* and *in vivo* in two tumor cell lines originating from prostate and breast tissue. Differences between acidosis-induced and hypoxia-induced changes are evaluated. Additionally, the results were compared to epithelial cells derived from normal tissue. The impact of acidosis on the metastatic potential, including cell adhesion, migration and invasion *in vitro* and metastasis formation by breast and prostate carcinoma cells *in vivo* was analyzed. Finally, signaling induced by lowering of the tissue pH is discussed. The role of MAPK activation in the stromal and in the tumor cells is discussed. The effect of MAPK on the expression of inflammatory mediators both under physiological and under acidic conditions was studied. Possible mechanisms for pH sensing and the role of the modulation of intracellular pH and

how this might result in MAPK activation are discussed. In addition, the role of MAPK signaling on tumor cell migration was studied.

2. Acidic environment activates inflammatory programs in fibroblasts

Acidosis affects mRNA expression of inflammatory mediators

Extracellular acidosis, that is present in varying pathological conditions such as inflammation, ischemia and in solid tumors, can modulate the inflammatory response in neutrophils, eosinophils, monocytes, macrophages, dendritic cells and endothelial cells as well as platelets [64,69,90–93]. However, the effect of extracellular acidosis on the inflammatory program of fibroblasts that are omnipresent in the tissue is less well understood. Therefore, pH-dependent changes in the expression of IL-1ß, IL-6, TNF-α, MCP-1, COX-2, iNOS and osteopontin (SPP1) were analyzed in normal rat kidney fibroblasts (NRK-49F). A pH value of 6.6 was chosen since it reflects well the situation found in solid tumors (pH_e 6.5 – 6.8) and inflammation (pH_e 6.0 – 7.0) [6,69]. Expression of IL-1ß was not detected in NRK-49F cells under the experimental conditions and was thus excluded [94].

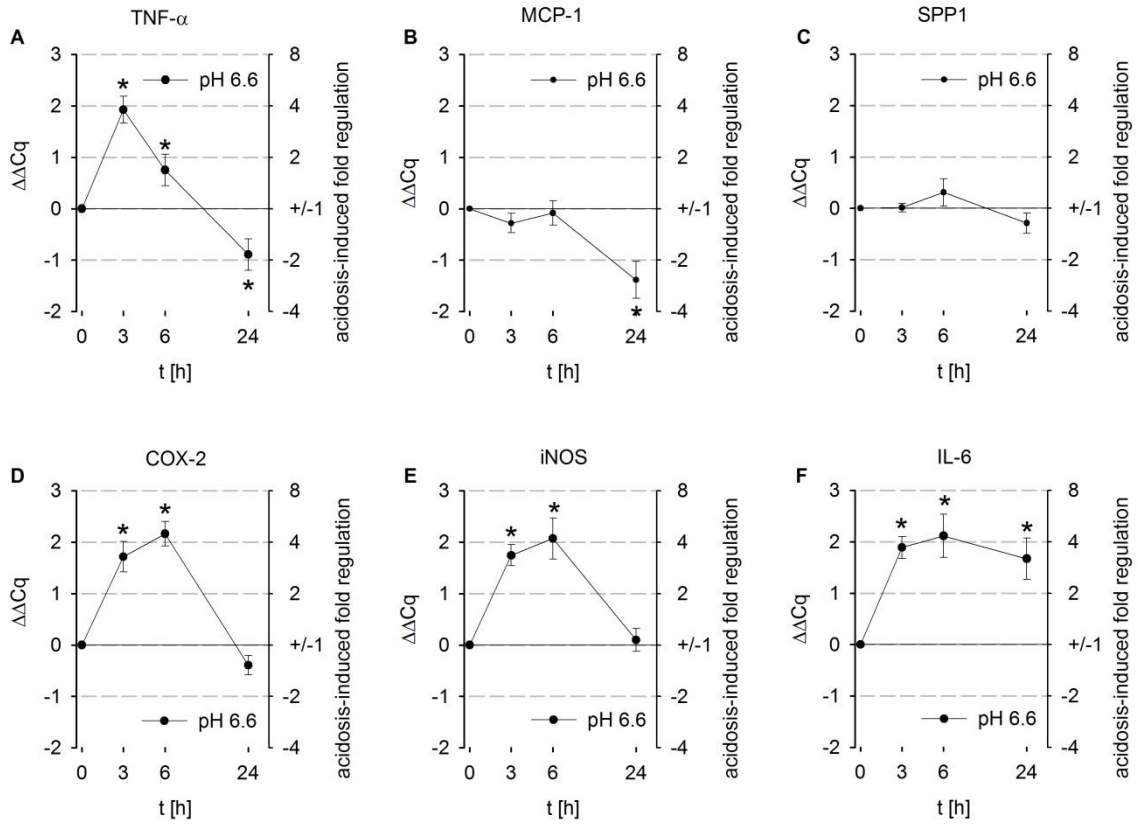

Figure 3 Time course of changes in mRNA expression of (**A**) TNF-α, (**B**) MCP-1, (**C**) Osteopontin (SPP1), (**D**) COX-2, (**E**) iNOS and (**F**) IL-6 induced by extracellular acidosis in NRK-49F fibroblasts. Fold regulation as well as ΔΔCq values of pH 6.6 compared to pH 7.4 at 3 h, 6 h and 24 h are displayed, n = 7-19 (triplicates each). (*) $p < 0.05$; modified from [94,95], see chapter 8.1 and 8.3

Short-term acidosis (3 h and 6 h) elevated the expression of TNF-α, COX-2, iNOS and IL-6, but had practically no impact on the expression of MCP-1 and SPP1 (Fig. 3). After 24 h of acidosis a weak decrease in TNF-α and MCP-1 mRNA was observed, while COX-2, iNOS as well as SPP1 were not regulated by long-term acidosis. Interestingly, only the level of IL-6 up-regulation remained stable over the whole time period of 3 h to 24 h at pH 6.6 (Fig. 3 F).

2.2. Acidosis affects protein expression of TNF-α, COX-2 and iNOS

The up-regulation of TNF-α, COX-2 and iNOS mRNA in NRK-49F fibroblasts after 3 h and 6 h of acidosis was validated by analyzing functional protein. Acidosis increased total TNF-α protein after 3 h and total COX-2 protein after 3 h as well as 6 h (Fig. 4). The increase in iNOS expression also seemed to be translated into more iNOS protein, since the production of nitrate and nitrite, which is a functional measurement for iNOS activity, was elevated after 3 h and -even more pronounced- after 6 h of acidosis (Fig. 4 C). These protein data correspond well with regulation found on mRNA level.

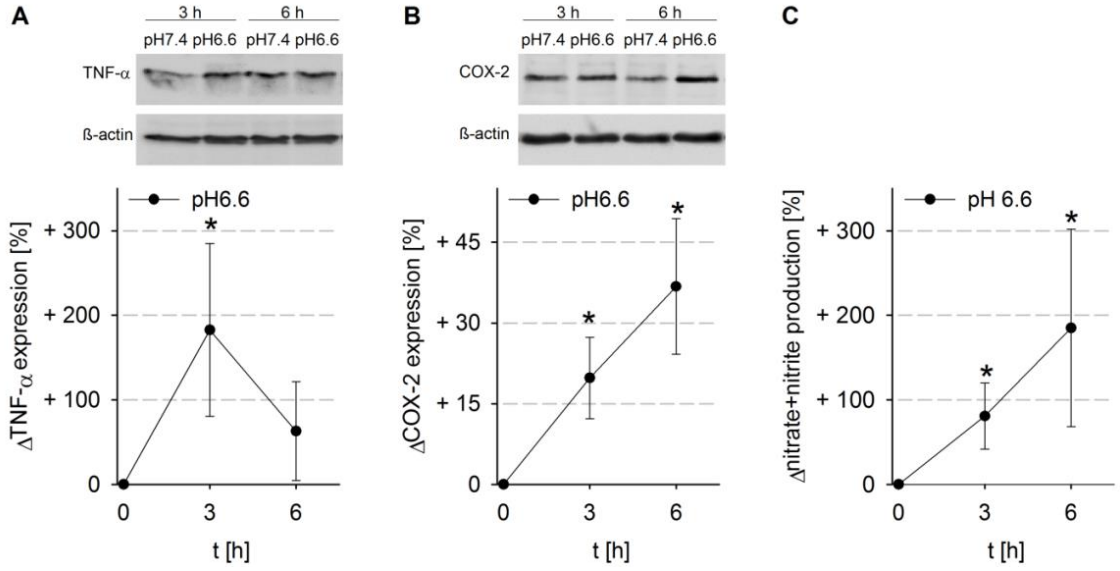

Figure 4 Acidosis-induced regulation in (**A**) total TNF-α protein, (**B**) total COX-2 protein and (**C**) formation of nitrate and nitrite in NRK-49F fibroblasts. Values at pH 6.6 are compared to the respective values at pH 7.4 at the indicated time points (3 h and 6 h), n = 4-15. In addition, representative Western blots for TNF-α, COX-2 and ß-actin are shown. (*) $p < 0.05$ [94], see chapter 8.1

2.3. Differential regulation by hypoxia and acidosis

In the pathological tissue microenvironment not only pH, but also oxygen partial pressure (pO_2) is lowered. Therefore, it was studied whether and how hypoxia (pO_2 = 1.5 mmHg) would modulate the inflammatory program in fibroblast, especially in comparison to acidosis (pH 6.6). In addtion the combination of hypoxia and acidosis was analyzed to evaluate whether the sperate effects are additive of can be explained by one predominant mechanism. In figure 5 the regulation of MCP-1, IL-6, TNF-α, iNOS, SPP1 and COX-2 are shown for acidosis, hypoxia and the combination of both. Interestingly, hypoxia had only a minor impact on the inflammatory mediators. MCP-1, IL-6, iNOS and SPP1 were not regulated by hypoxia. Only COX-2 was weakly up-regulated and TNF-α was weakly down-regulated. The combination of acidosis and hypoxia together mostly reflected the impact of acidosis alone. Osteopontin (SPP1) was neither regulated by acidosis nor by hypoxia in NRK-49F fibroblasts.

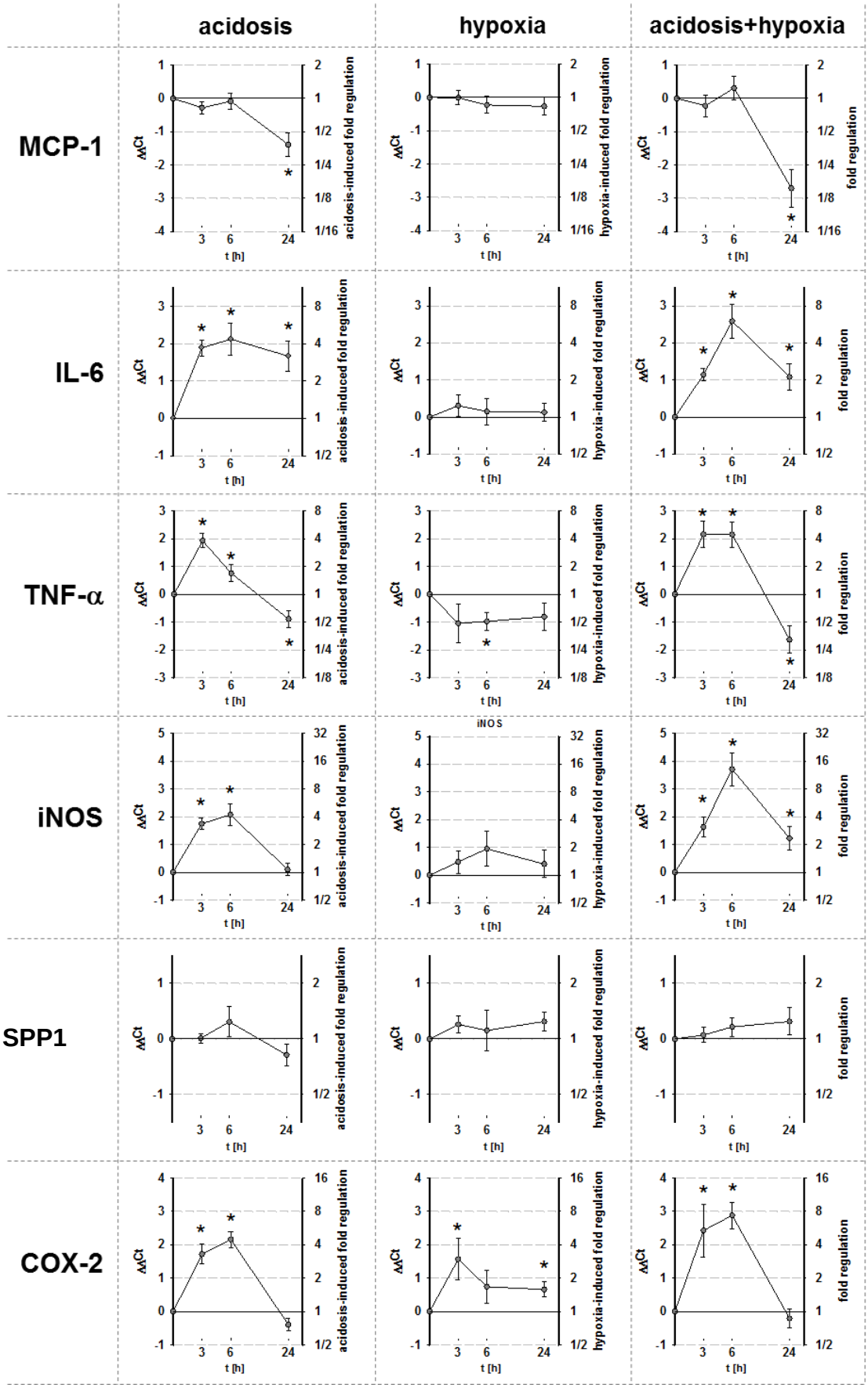

Figure 5 Changes of the expression of inflammatory mediators in NRKF fibroblasts during extracellular hypoxia (pO_2=1.5 mmHg), acidosis (pH 6.6), or combined acidosis and hypoxia; n=5-33; (*) $p<0.05$ vs. control [95], see chapter 8.3

2.4. Summary of pH-dependent changes in the inflammatory program of fibroblasts

In summary, acidosis modulated the inflammatory program in fibroblasts markedly. Short-term acidosis possibly fosters the inflammatory program in fibroblasts via TNF-α, COX-2, iNOS and IL-6. TNF-α, COX-2 and iNOS were elevated at the mRNA (Fig. 3) as well as functional protein level (Fig. 4). Therefore, inflammation might be fostered by secretion of TNF-α and elevated production of reactive nitrogen species and prostaglandins, respectively. However, this effect was only seen after short-time exposure to an acidic pH (3-6 h). Long-term acidosis (24 h) is possibly limiting the inflammatory program. COX-2 and iNOS were not regulated, while TNF-α as well as MCP-1 were even down-regulated. Reduction of MCP-1 might decrease the infiltration of immune cells. In contrast to acidosis, hypoxia had almost no effect on the inflammatory programm in fibroblasts and did not modulated the acidosis-induced regulation. In the tumor microenvironment, cancer-associated fibroblasts (CAFs) form a highly heterogeneous group of cells that affect tumor progression, metastasis formation as well as tumor immunity [96]. CAFs primarily foster the immunosuppressive tumor microenvironment, for example by remodelling of the extracellular matrix, angiogenesis as well as immune cell recruitment and activation due to secreted factors. The secreted immunomodulatory mediators comprise IL-6, TNF-α, MCP-1, prostaglandin E2 (PGE2) and NO, all of them regulated in this study by acidosis. For instance IL-6 secretion from fibroblasts has been shown to modulate monocyte differentiation and fosters tumor progression [97]. However, because of the complex interaction between the different cell types (fibroblasts, immune cells, tumor cells etc.) in the tumor tissue the overall effect of acidosis on tumor immunity and progression needs further investigations.

3. Acidosis differently modulates the inflammatory program in monocytes and macrophages

3.1. Impact of acidosis on inflammatory marker expression in immortalized monocytic and macrophage cell lines

In the literature, the impact of extracellular acidosis on immune cell function is described to be mostly inhibitory, however data on macrophages is scarce [64]. Processes of cellular immunity affected by low pH include proliferation, cell survival, migration, phagocytosis, cytotoxicity, respiratory burst, as well as antibody production. Since macrophages and their precursors are exposed to acidosis in pathological conditions such as inflammation, ischemia and in solid tumors, the effect of acidosis on the expression of different inflammatory markers, as well as on cellular function (phagocytosis, migration) was analyzed [98]. The set of inflammatory markers studied included TNF-α, IL-1ß, IL-6, MCP-1, SPP1 (osteopontin), COX-2 and iNOS. In figure 6 the acidosis-induced mRNA regulation in the human monocytic cell line Mono-Mac-6 (Fig. 6 A) and THP-1 (Fig. 6 C) is shown. COX-2, IL-6, MCP-1, SPP1 and TNF-α were downregulated in both cell lines by acidosis. By this mechanism, acidosis might reduce immune cell recruitment and inflammatory behavior. A discrepancy between the monocytic cell lines was only seen concerning iNOS (not detected in Mono-Mac-6, up-regulated in THP-1) and IL-1ß (downregulated in Mono-Mac-6, not regulated in THP-1) expression. This variance might reflect the differences in monocytic differentiation of these cell lines. Mono-Mac-6 cells constitutively express markers of mature monocytes, that are absent in THP-1 cells [99]. In murine macrophages (RAW264.7, Fig. 6 B) the regulation of inflammatory markers by acidosis differed profoundly when compared to the monocytic cell lines. In RAW264.7 cells the expression of COX-2, iNOS and IL-1ß was up-regulated, while that of SPP1 and TNF-α was not altered. The difference in the regulation of inflammatory markers between monocytic cells and macrophages was also seen when comparing unstimulated THP-1 cells to phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells which are known to show a macrophage-like phenotype [100]. While most of the inflammatory markers were down-regulated in unstimulated THP-1 monocytes, they were not regulated in PMA-differenciated THP-1 macrophages by acidosis (Fig. 6 D). However, PMA stimulation itself can influence cytokine expression [101] and might thus mask pH-dependent changes in the PMA-stimulated THP-1 macrophages. The elevated expression of COX-2, IL-1ß and iNOS in RAW264.7 macrophages could possibly foster inflammation by an increase in prostaglandins, secreted IL-1ß and reactive nitrogen species. An incubation time of 6 h at pH 6.6 led to a significant increase in COX-2 protein expression

(+99 ± 30 %, n = 7), in IL-1ß secretion (+580 ± 249 %, n = 6) and formation of nitrate and nitrite (+306 ± 145 %, n = 6) in RAW264.7 macrophages [98].

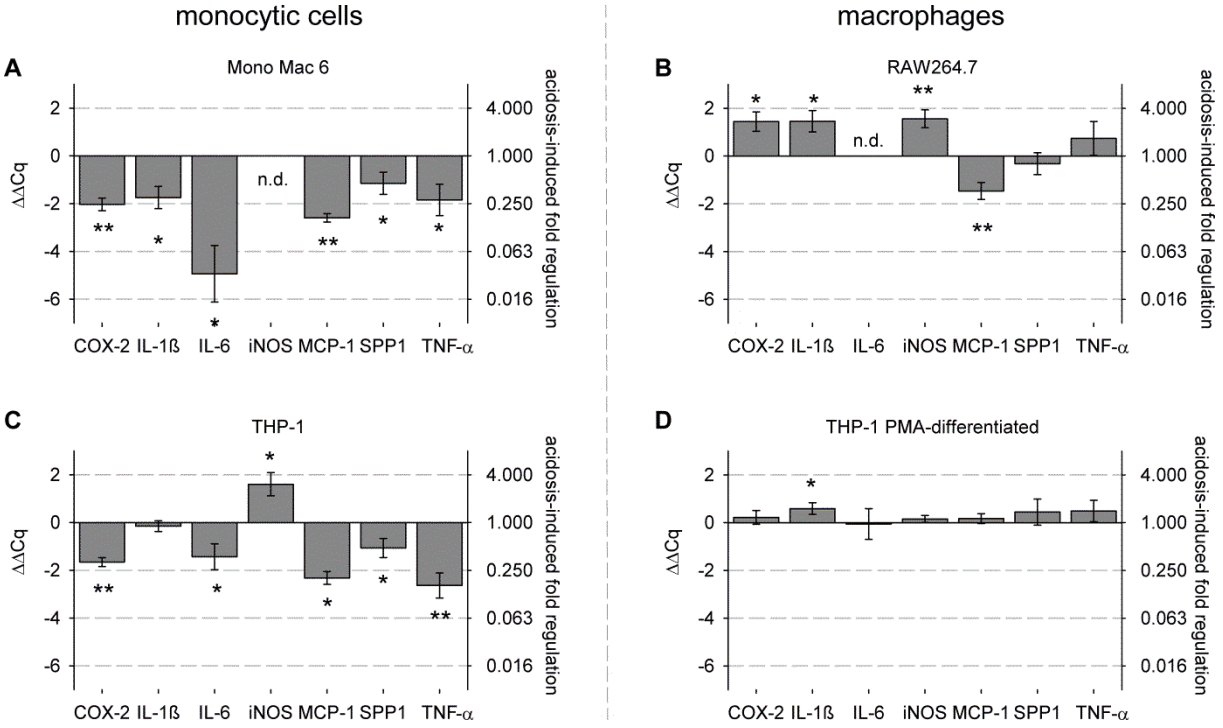

Figure 6 Acidosis affects the mRNA expression of inflammatory markers in monocytic cell lines (**A**) Mono-Mac-6 and (**C**) THP-1, as well as in macrophages (**B**) RAW264.7, and (**D**) PMA-differentiated THP-1. Acidosis-induced changes in the expression of COX-2, IL-1ß, IL-6, iNOS, MCP-1, SPP1 and TNF-α are shown as fold regulation and ΔΔCq (normalized values at pH 7.4 compared to normalized values pH 6.6) after an incubation time of 3 h, n = 6-12 (triplicates each). (*) $p < 0.05$, (**) $p < 0.01$ vs pH 7.4, (n.d.) not detectable [98], see chapter 8.2

3.2. Acidosis affects the inflammatory marker expression in primary human monocytic cells and macrophages

The results in monocytic and macrophage-like cell lines were compared with human primary monocytes and macrophages isolated from peripheral blood (Fig. 7). When challenging freshly isolated human monocytes with pH 6.6 for an incubation time of 3 h, the expression of COX-2 and IL-6 was significantly increased, but decreased for MCP-1 and TNF-α (Fig. 7 A). The impact of acidosis on unpolarized (M0) and M2 polarized macrophages was similar to that seen in the RAW264.7 macrophage cell line (Fig. 7 B+D). In M0 macrophages acidosis reduced the expression of IL-1ß, MCP-1, SPP1 and TNF-α. COX-2 showed a trend of elevated expression (p=0.06). Expression of iNOS was very low or even not detectable in some samples analyzed and thus statistical testing was not possible. M2 polarization was induced by stimulation with IL-4 and IL-10 (20ng/ml each) and macrophages reacted on acidosis by reducing MCP-1 and TNF-α, but strongly up-regulating COX-2

expression. M1 polarization was induced by LPS (50 ng/ml) and IFNγ (20 ng/ml) stimulation. M1 polarized macrophages displayed reduced expression of COX-2, IL-1ß, MCP-1 and TNF-α when subjected to acidosis (Fig. 7 B). Interestingly, a decrease in the expression of MCP-1 and TNF-α at pH 6.6 was seen in all primary monocytes/macrophages. Thus, acidosis might result in a reduced recruitment of immune cells in an acidic tissue that is irrespective of the differentiation state of primary human monocytes/macrophages. An overall inhibitory effect of acidosis on the inflammatory program was also seen in M1 polarized macrophages as well as in the monocytic cell lines Mono-Mac-6 and THP-1. On the other hand, the acidosis-induced increase in the expression of COX-2 and partly iNOS in RAW264.7 macrophages as well as primary M2 polarized macrophages might foster inflammation (Fig. 9).

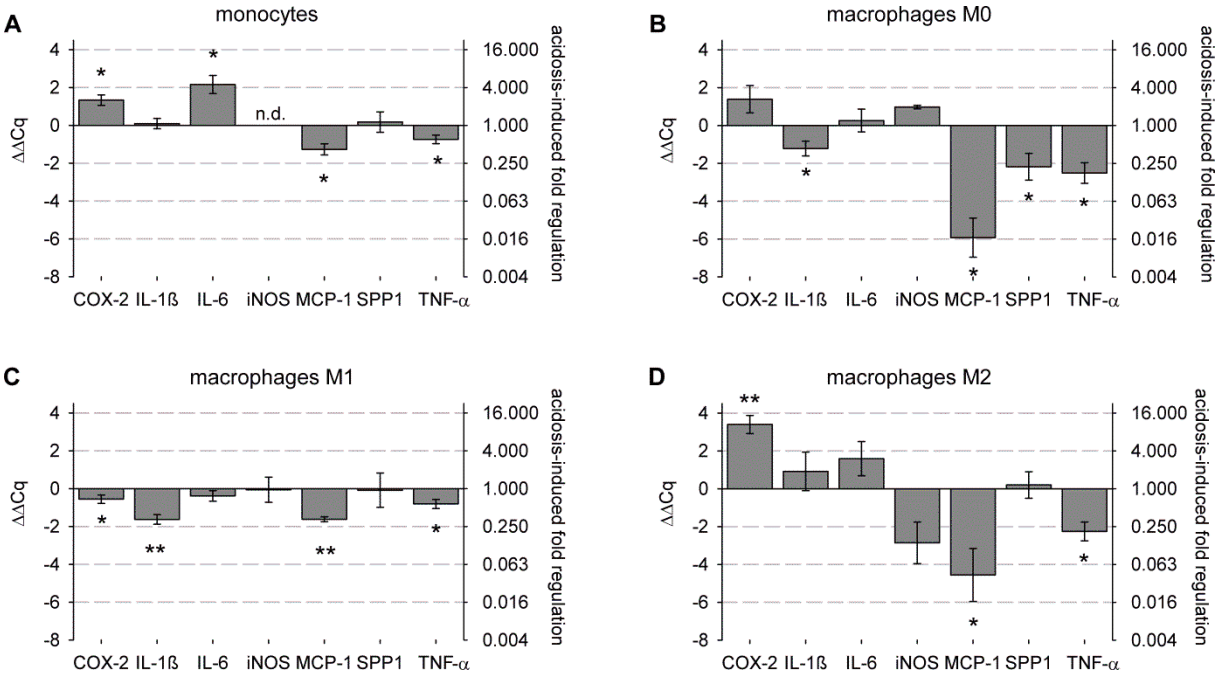

Figure 7 Impact of acidosis on mRNA expression of inflammatory markers in (**A**) primary human monocytes, (**B**) unpolarized primary macrophages and (**C**) M1 or (**D**) M2 polarized primary human cells. Fold regulation and ΔΔCq values of pH 6.6 compared to pH 7.4 after an incubation period of 3 h are shown, n = 4-5 (except for iNOS: n = 2; triplicates each). (*) p<0.05, (**) p<0.01 vs. pH 7.4, (n.d.) not detectable [98], see chapter 8.2

3.3. Effect of extracellular acidosis on immune cell function

Several studies have focused on the impact of acidosis on immune cell behavior, especially on T-cell function or antibody synthesis. To elucidate the impact of extracellular acidosis on macrophage function (cell migration, phagocytosis) RAW264.7 cells were used that showed similar regulation of inflammatory mediators by acidosis as primary M0 and M2 macrophages. Migratory activity of RAW264.7 macrophages was assayed using time lapse microscopy. Acidosis did not affect migratory

speed (random movement) of RAW264.7 cells (Fig. 8 C). Additionally, Mono-Mac-6 and THP-1 were analyzed concerning random migration under acidic conditions. Like the murine macrophages no change in migratory speed was seen (Fig. 8 A+B). This is in good accordance with the literature for random leukocyte movement [102]. Since the expression of chemokines is reduced by acidosis (Chapter 3.1), directed migration might even be reduced in an acidic tumor tissue. Phagocytosis was studied with flow cytometry by measuring the uptake of IgG FITC-coated latex beads. Extracellular acidosis elevated phagocytic activity in a time-dependent manner in PMA-differentiated THP-1 and RAW264.7 macrophages (Fig. 8 D+E). The increase in phagocytosis was most prominent after 24 h at pH 6.6. Thus, extracellular acidosis did not affect random migration, but fostered phagocytosis of macrophages.

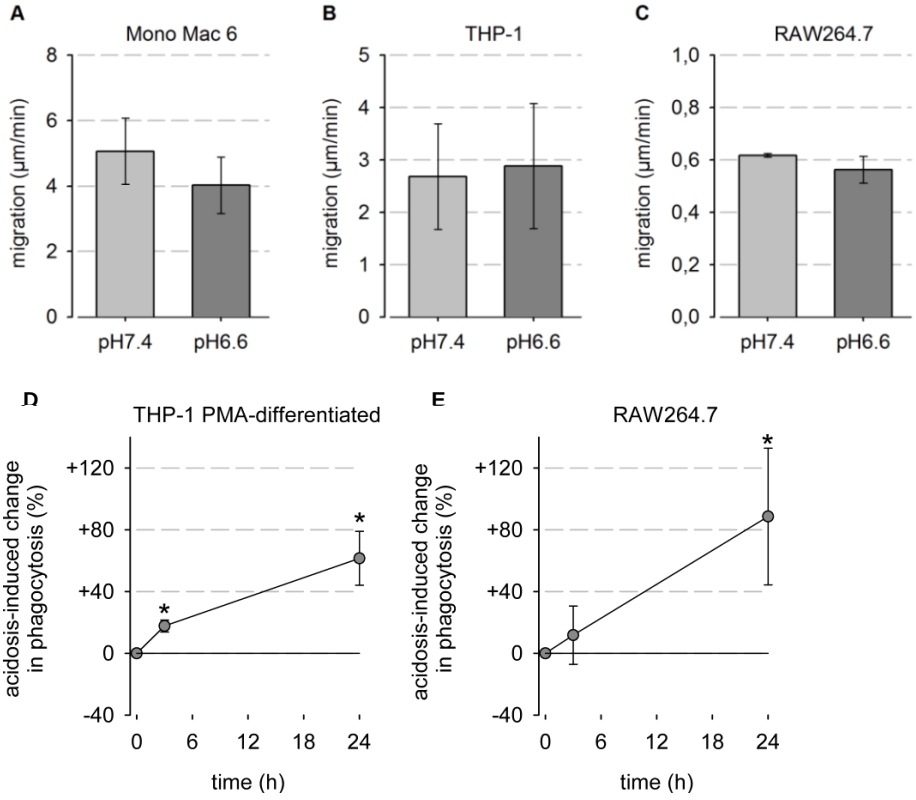

Figure 8 Impact of extracellular acidosis on cell migration in (**A**) Mono-Mac-6, (**B**) THP-1 and (**C**) RAW264.7 cells. Migratory speed in µm/min is shown at pH 7.4 and pH 6.6, respectively, n = 4-5 (minimum 10 cells per cell passage). Acidosis-mediated changes of phagocytic activity of (**D**) PMA-differentiated THP-1 and (**E**) RAW264.7 macrophages. The time course shows relative changes in phagocytosis comparing pH 6.6 to pH 7.4, n = 4-7. (*) $p < 0.05$ vs. pH 7.4; modified from [98], see chapter 8.2

3.4. Summary of acidosis-induced regulation of the inflammatory program immune cells

Taken together, our results indicate that the impact of acidosis, that is present in different pathological conditions such as acute and chronic inflammation, ischemia or in solid tumors,

depends on the differentiation state, as well as the activation state of immune cells. In monocytic immortalized human cell lines (Mono-Mac-6, THP-1) acidosis restricted inflammation by reducing the expression of inflammatory mediators COX-2, IL-6, MCP-1, SPP1 and TNF-α. Therefore, acidosis possibly results in diminished recruitment of additional immune cells to the acidic tissue, reduced secretion of cytokines and decreased production of prostaglandins. These data was only partially validated with primary human monocytes. Acidosis reduced the expression of MCP-1 and TNF-α. This might limit inflammation by a reduced chemoattraction of immune cells and by reduced levels of pro-inflammatory TNF-α. COX-2 and IL-6 expression was increased, and SPP1 not regulated at all. Thus, data obtained from immortalized cell lines with leukemic background cannot always be transferred to the human situation. In macrophages the effects of acidosis on immune cell function was more complex. On the one hand acidosis reduced MCP-1 expression in murine macrophage cell line RAW264.7 as well as in primary human macrophages (M0, M1 and M2), and might thus attenuate inflammation by reducing the recruitment of immune cells. Additionally, TNF-α expression was reduced in primary human macrophages (M0, M1 and M2 polarized). Beside the reduction of MCP-1 and TNF-α, the expression of COX-2 and IL-1ß was decreased by acidosis in M1 polarized primary human macrophages. Thus, acidosis seems to be mainly inhibitory on immune cell function, when analyzing classically activated M1 macrophages, that are described as pro-inflammatory, immunostimulatory and tumor suppressing [103].

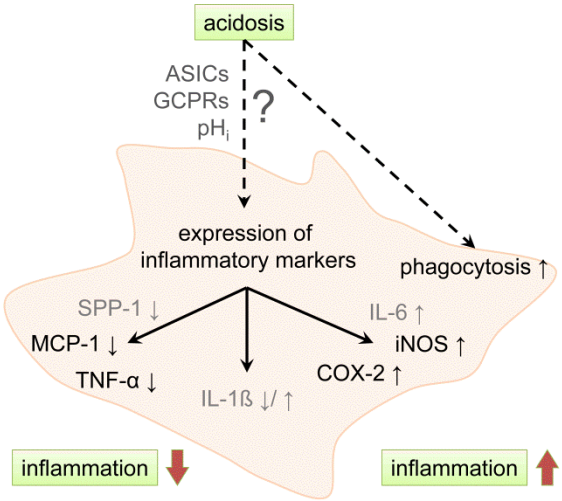

Figure 9 Summary of the impact of acidosis on macrophage activity in inflammation. Extracellular acidosis modulates the expression of inflammatory markers and the phagocytic activity possibly mediated by acid sensitive ion channels (ASICs), proton-sensing G protein-coupled receptors (GCPRs) or changes in intracellular pH (pH_i). [98], see chapter 8.2

On the other hand, acidosis could foster inflammation by increased phagocytic activity, as well as prostaglandin and reactive nitrogen production due to elevated COX-2 and iNOS expression. These

data were observed in RAW264.7 murine macrophages, but also partially in unpolarized M0 and M2-polarized primary human macrophages. The modulation of the inflammatory profile by extracellular acidosis might affect immune response in tumors and thus the prognosis of cancer patients. An elevated expression of COX-2 in tumor-associated macrophages was shown to be correlated with poor prognosis of tumor patients [104]. Prostaglandin E_2 can cause tumor progression by regulating the cytokine expression of the tumor stromal cells and inducing a stem cell-like state in normal as well as neoplastic epithelial cells [105]. Additionally, RAW264.7 macrophages displayed increased IL-1ß expression and secretion that might promote tumor formation and the switch to a stem cell-like phenotype [105,106]. Taking all the results into account, the impact of acidosis on immune response is somehow Janus-faced. It displays rather inhibitory actions in monocytic cell lines and M1 polarized primary human macrophages, but both anti- and pro-inflammatory effects in macrophage-like cell line RAW264.7, in primary human monocytes and in unpolarized as well as M2 polarized primary human macrophages (summarized in figure 9).

4. Impact of the acidic tumor microenvironment on cancer cells

4.1. Impact of acidosis on the inflammatory program of tumor cell lines (*in vitro* situation)

Acidosis as well as hypoxia are common characteristics of the microenvironment of solid tumors. The impact of both on the expression of the inflammatory programm of tumors cells was studied in rat prostate carcinoma (AT-1) as well as rat breast carcinoma (Walker-256) cells since the inflammatory programm can affect tumor promotion as well as tumor progression. Different steps of the malignant behavior, among them proliferation, angiogenesis, invasion and metastasis formation, have been shown in the literature to be modulated by the inflammatory tumor microenvironment [62,63,107]. In figure 10 the regulation of MCP-1, IL-6 and TNF-α by acidosis, hypoxia or the combination of both is shown. This regulation depended strongly on the cell type studied (AT-1 or Walker-256) as well as on the microenvironmental parameter (acidosis or hypoxia). MCP-1 for instance was not regulated by acidosis, but down-regulated by hypoxia in prostate carcinoma cells (AT-1). In mammary carcinoma cells (Walker-256) short-term incubation (3 h to 6 h) with acidosis led to an increase in MCP-1 expression, while hypoxia reduced the expression. Long-term incubation (24 h) reduced MCP-1 expression by acidosis, but increased it severely by hypoxia. The latter pattern was also seen when looking at the regulation of IL-6 and TNF-α in Walker-256 cells and of iNOS in both tumor cell lines (Fig. 10 and Fig. 11). Osteopontin (OPN/SPP1) expression was elevated by acidosis, but lowered by hypoxia (Fig. 11). This was also true for IL-6 expression in AT-1 prostate carcinoma cells (Fig. 10). The effect of combined acidosis and hypoxia mainly resembles the additive effects of acidosis and hypoxia alone. Thus, regulation of the inflammatory program by these microenvironmental parameters seems to be independent of each other. The expression of iNOS for instance was increased by acidosis after 3 h and 6 h, but not regulated after 24 h (Fig. 11). Hypoxia on the other hand, had no or only mild effects after 3 h or 6 h, but induced a pronounced increase of iNOS expression after 24 h. The impact of acidosis and hypoxia together is a general augmentation of iNOS expression over 3 h to 24 h. However, separating acidic and hypoxic conditions has shown that the initial increase is due to acidosis, while long-term effects are due to hypoxia. COX-2 expression was not detected in AT-1 prostate or Walker-256 breast carcinoma cells.

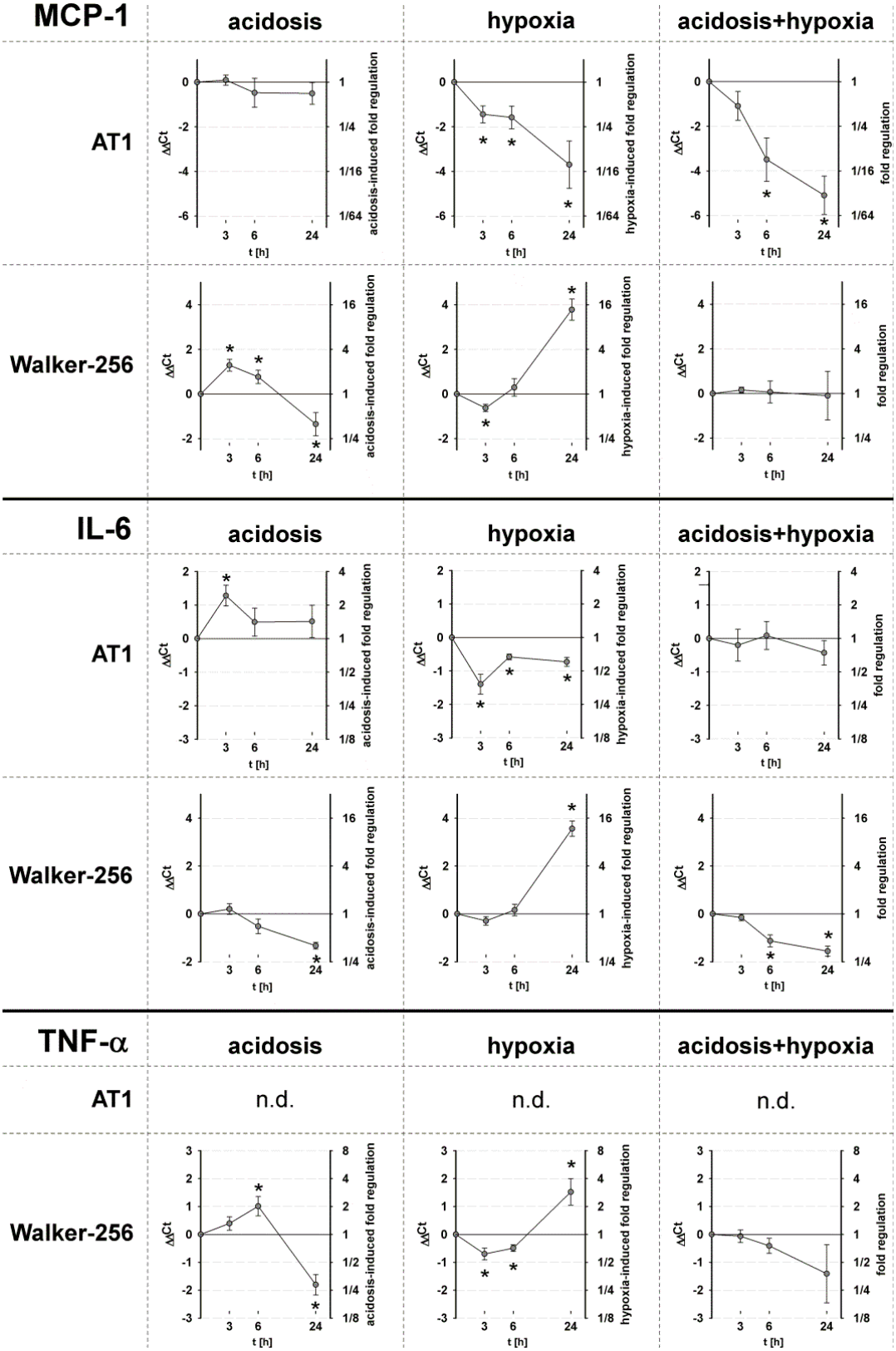

Figure 10 Regulation of MCP-1, IL-6 and TNF-α expression in AT-1 and Walker-256 tumor cells during extracellular acidosis (pH 6.6), hypoxia (pO_2 = 1.5 mmHg) or combined acidosis and hypoxia. TNF-α was not detectable in AT-1 cells. n = 5-17 for AT-1 and n = 4-11 for Walker-256. (*) $p < 0.05$ [95], see chapter 8.3 and 8.7

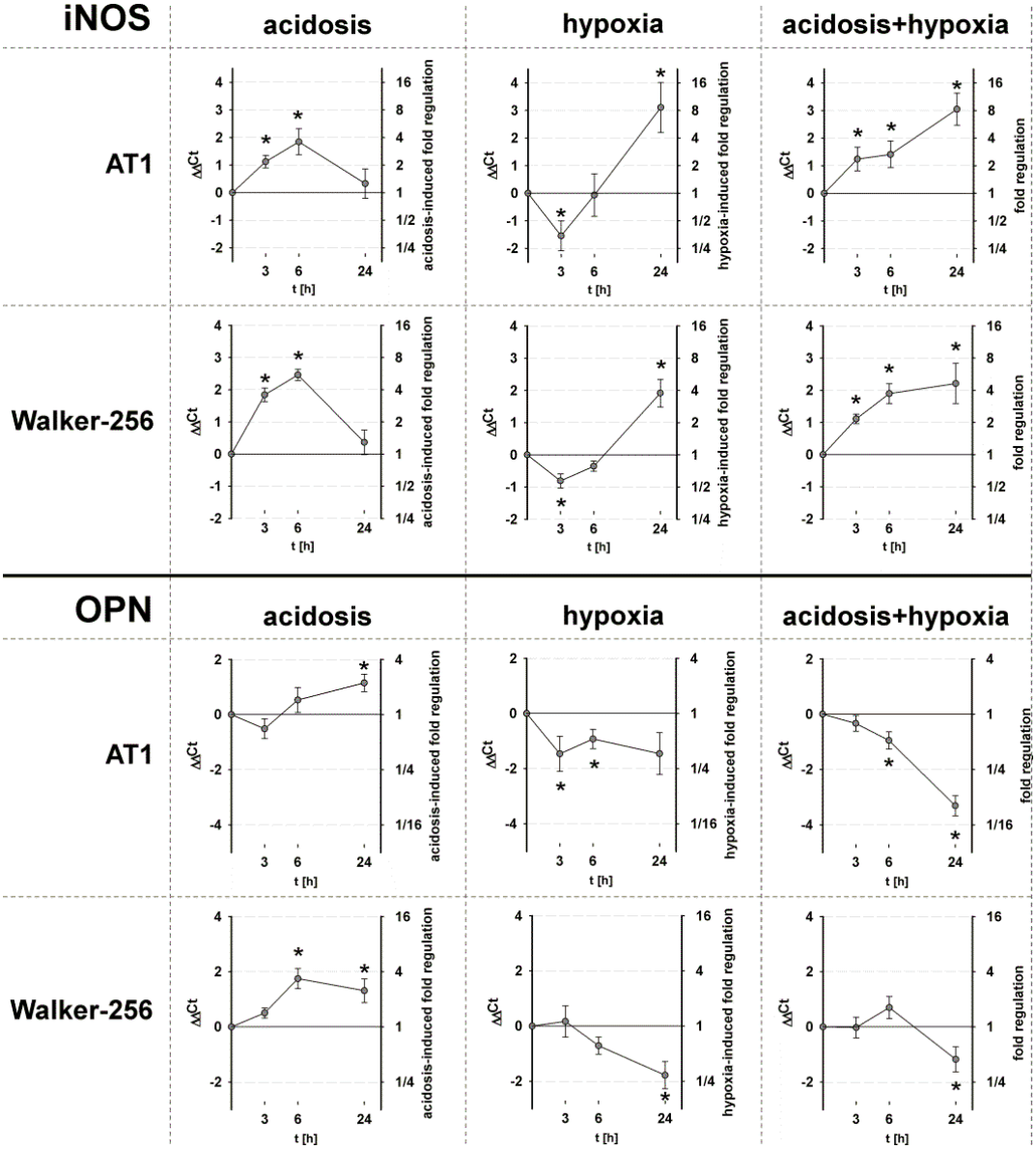

Figure 11 Changes in iNOS and SPP1 (OPN, osteopontin) mRNA expression in AT-1 and Walker-256 tumor cells during extracellular acidosis (pH 6.6), hypoxia (pO_2 = 1.5 mmHg) or the combination of both; n = 4-17 for AT-1, n = 5-13 for Walker-256. (*) $p < 0.05$ [95], see chapter 8.3 and 8.7

4.2. Relevance of the tumor microenvironment in the animal model (experimental tumors *in vivo*)

To analyze whether the results obtained in cell culture also hold true for the *in vivo* situation, the expression of inflammatory markers MCP-1, IL-6, TNF-α, iNOS, COX-2 and osteopontin (SPP1) was analyzed in experimental animal tumors of AT-1 prostate and Walker-256 breast carcinoma cells. To foster acidosis in these solid tumors artificially, animal were exposed to inspiratory hypoxia (8% O_2) in combination with an inhibitor of the respiratory chain (metaiodobenzylguanidin MIBG) for 24 h, thus forcing glycolytic metabolism [33]. This acidosis treatment led to a reduction of mean tumor pH (AT-1 control: pH 7.02 ± 0.04, acidosis treatment: pH 6.48 ± 0.08, N = 4 and Walker-256

control: pH 7.16 ± 0.03, acidosis treatment: pH 6.65 ± 0.07, N = 4-6) [108] and pO_2 value of about 1 to 3 mmHg [109]. In experimental tumors of AT-1 prostate carcinoma cells the expression of MCP-1, iNOS and COX-2 was reduced (Fig. 12 A).

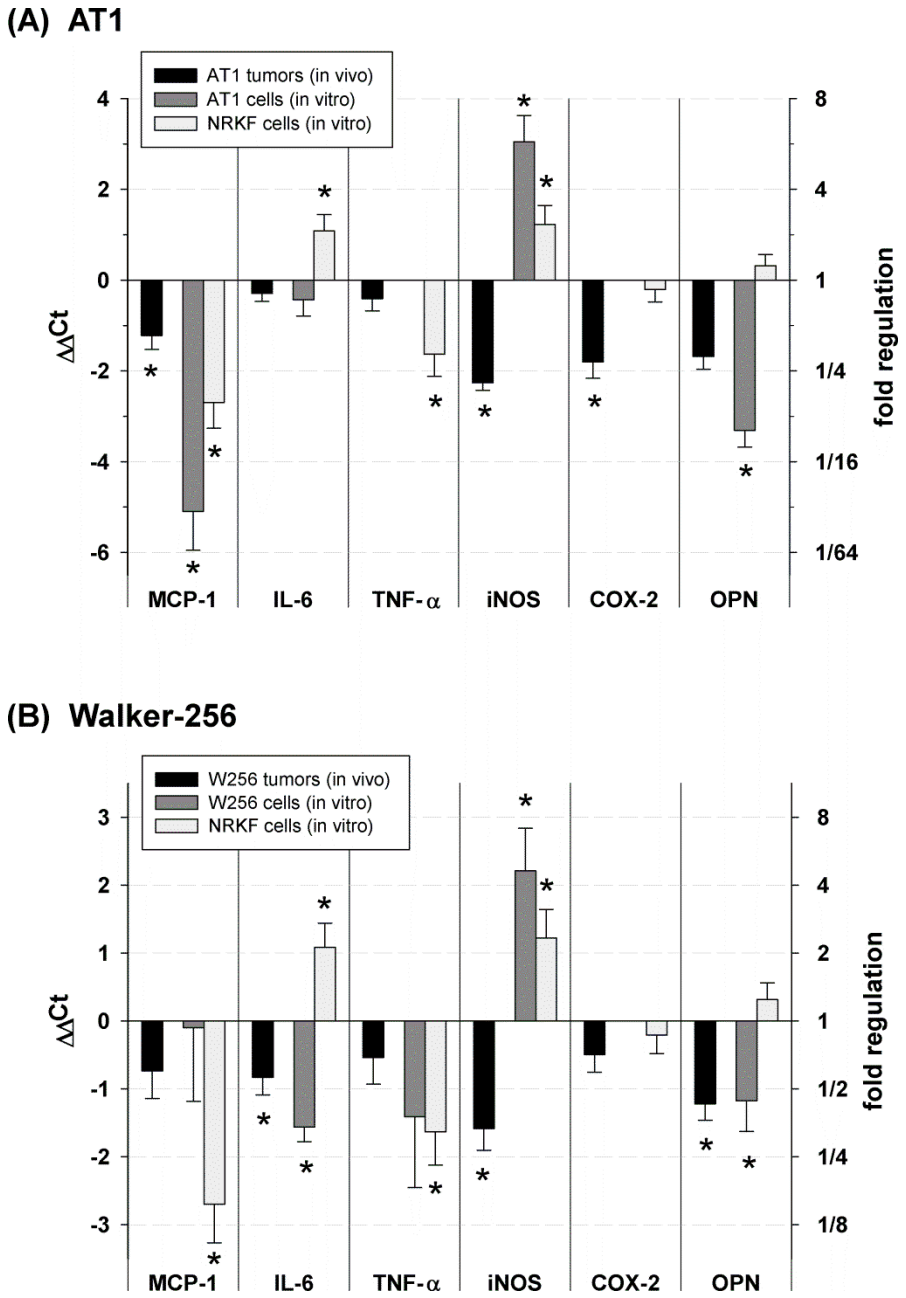

Figure 12 Acidosis in combination with hypoxia for 24 h modulates the expression of inflammatory mediators MCP-1, IL-6, TNF-α, iNOS, COX-2 and SPP1 (OPN, osteopontin) in (**A**) AT-1 prostate and (**B**) Walker-256 mammary carcinoma experimental tumors *in vivo* (black bars). For comparison the results of the respective carcinoma cells *in vitro* (dark grey bars) and normal fibroblasts (NRK-49F, light grey bars) are shown, n = 5-6 (*) $p < 0.05$ [95], see chapter 8.3

No regulation was found in IL-6 and TNF-α expression, while there was a trend in SPP1 down-regulation that was, however, expressed on a very low level or not detected at. These results are in good agreement with the cell data of acidosis in combination with hypoxia for 24 h. Only the regulation of iNOS differed to the *in vitro* situation. Walker-256 tumors displayed a decline in the expression of IL-6, iNOS and SPP1 (Fig. 12 B) which is again in good accordance with the cell experiments for IL-6 and SPP1, but not for iNOS. Possibly, iNOS expression in solid tumors depends more on the action of immune cells. Macrophages have been shown to express large amounts of iNOS [110]. Additionally, the interaction of the stromal cells with the tumors cells might lead to the discrepancy seen in iNOS expression. MCP-1, TNF-α and COX-2 were not regulated *in vivo* (Fig. 12 B). Therefore, the results indicate that the impact of hypoxia and acidosis on the inflammatory program in solid tumors is mainly due to changes in the expression in tumors cells themselves (Fig. 12, dark grey bars). It is not depending on expression changes in fibroblasts (Fig. 12, light grey bars), although fibroblasts are a relevant part of the tumor mass, too. Most of the inflammatory mediators (MCP-1, IL-6, iNOS, COX-2, SPP1) were downregulated *in vivo*. Since they are predominantly involved in triggering the immune response, for instance by attracting and activating immune cells, fostering phagocytic activity or producing other pro-inflammatory mediators, hypoxic and acidic conditions seem to limit the physiological immune response against the tumor. Furthermore, given that the efficacy of therapeutic immune modulation in cancers depends on an adequate cytokine production [111], tumor microenvironmental hypoxia plus acidosis might restrict immune therapy by reducing cytokine secretion. On the other hand, tumor progression can rely on the action of several inflammatory mediators that foster tumor cell proliferation, survival, migration and metastasis [63]. A reduced expression of pro-inflammatory mediators by acidosis plus hypoxia might thus have a beneficial influence on tumor control. The net effect of the tumor microenvironment on tumor growth is difficult to predict because of the opposing implications in tumor immunity and tumor progression.

4.3. Differences of pH-dependent changes in the inflammatory response between tumor and epithelial cells

Since the carcinoma cell lines used are derived from epithelial tissues the acidosis-induced modulation of the inflammatory program in tumor cells was compared to non-tumor, epithelial cells (normal rat kidney epithelial cells, NRK-52E). Short-term incubation (3-6 h pH 6.6) reduced the expression of MCP-1 and iNOS (Fig. 13) which is opposite to that found in tumor cells. TNF-α was strongly upregulated which, in principal, was similarly seen in Walker-256 tumor cells. No or only minor changes were observed in osteopontin (SPP1), IL-6 and COX-2. Long-term acidosis (24 h)

reduced TNF-α and MCP-1 expression, while IL-6, SPP1, iNOS and COX-2 were not modulated. The impact of acidosis on the expression of MCP-1, IL-6, SPP1 and iNOS in non-tumor epithelial NRK-52E cells was fundamentally different to that seen in prostate and breast carcinoma cells (see Fig. 10 and Fig. 11 for comparison). In NRK-52E cells most of the inflammatory mediators were down-regulated and might thus limit the immune response and protect the tissue. This is partly in contradiction to data from the literature, where extracellular acidosis has been described to foster inflammation in kidney tubular epithelial cells [112]. Only TNF-α regulation by acidosis was consistent in non-tumor and tumor cells and might thus reflect a general mechanism in the cellular response to both short- and long-term acidosis.

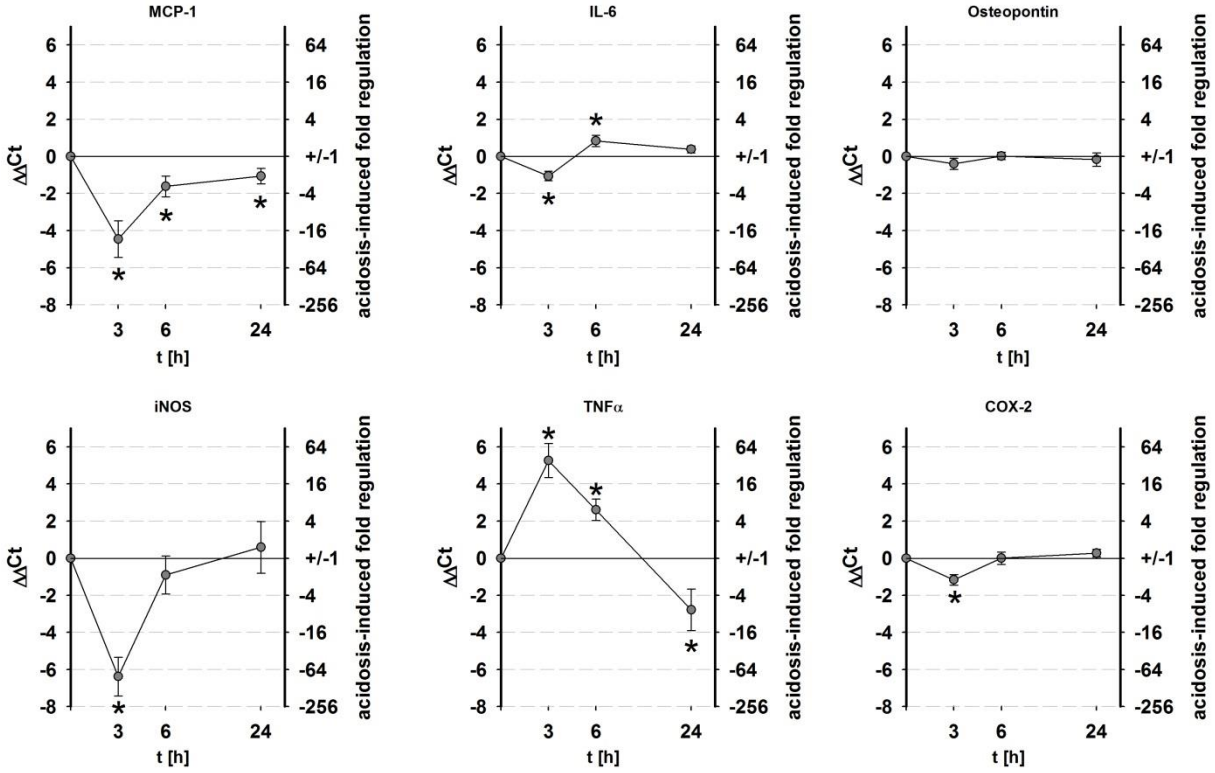

Figure 13 Acidosis-induced modulation of the mRNA expression of inflammatory mediators in NRK-52E epithelial cells after different intervals at pH 6.6 (3-24 h). The values are normalized to control conditions at pH 7.4, n = 8-20. *p < 0.05 [113], see chapter 8.5

4.4. Functional impact of acidosis on migration and metastasis formation

Not only the genetic background but also the tumor microenvironment affects tumor phenotype. Since metastases formation is still the leading cause of cancer morbidity and mortality, the impact of microenvironmental parameters on metastasis is of special interest. Metastases formation involves changes in migratory behavior of the tumor cell, detachment, local invasion through the

degradation of the extracellular matrix, intravasation, circulation in the blood stream, extravasation and finally growth at a distant side [36]. Acidosis has been shown to affect metastasis at the level of local invasion through increased activity of matrix metalloproteinases and cathepsins, as well as through the destruction of the surrounding tissue by the low pH itself [114,115]. Additionally, low pH can foster local invasion through changes in gene expression, cytoskeletal reorganization and modulation in cell motility [28]. In this study acidic priming of fluorescence labeled prostate and breast tumor cells increased lung metastasis formation significantly (Fig. 14). Comparable results in melanoma cells were described in the literature [41,58]. Since primed cells were injected intravenously to study subsequent lung metastases formation, an impact of acidosis on tumor cell detachment and intravasation cannot be responsible for the observed effects. This is in line with a study of Robey and colleges showing that alkalizing of the tumor tissue diminished metastasis without affecting the number of circulating tumor cells [61]. The increase in metastasis formation in our study was probably not due to an increase in local invasion, because acidic priming did not foster the invasive capacity monitored by the disruption of an intact epithelial layer (without direct cell to cell contact) by transepithelial electrical resistance measurements *in vitro* [116]. Additionally, acidic pre-incubation did not enhance the invasion through basement membrane resembled by Matrigel-coated invasion chambers. Directed migration through non-coated chambers was also unaffected, as well as cell proliferation in general. Furthermore, acidic priming did not modulate cellular adhesion to plastic Petri dishes or to endothelial cell monolayers. However, acidic priming enhanced random migration of tumor cells significantly by a factor of +32 ± 4 % (n = 37). This increase in motility was persistent when transfering the cells back to normal pH for 3 h (+32 ± 8 %; n = 4; p = 0.035). Thus, acidois triggers an enduring increase in random migration ("cellular memory") that could be involved in the increased metastasis formation after acidic priming of the cells. However, when the cell were transferred back to normal pH and measured after a time span as long as 24 h, the increase in migration was lost (+7 ± 8 %; n = 7; p = 0.442) and is therefore reversible. When analyzing epithelial cells (NRK-52E) or fibroblasts (NRK-49F) no impact of acidosis on random migration was found, thus the impact of an acidic microenvironment on cell motility obviously depends on the cell type studied. The steps of tumor migration that are pH-dependend comprise coordinated release and formation of adhesion contacts, dynamic cytoskeletal re-organization, as well as local ion and water transport across the cell membrane [45]. The pH-dependent activation of ion chanels might be crucial for the observed changes in migration. Several potassium channels can interact with integrins or induce signaling pathways involved in migration [54]. Ca^{2+} currents can be crucial for cell migration [117] and were demonstrated to be modulated by acidosis in the prostate carcinoma cell line [32]. Additionally, sodium channels are directly

involved in cell migration [118]. Further studies are needed to unravel the mechanism by which acidosis affects tumor cell migration.

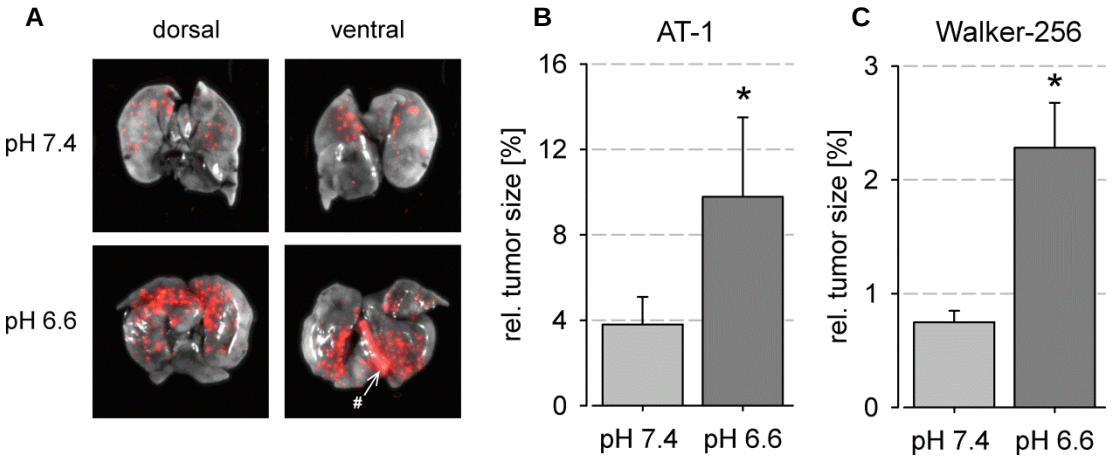

Figure 14 Impact of extracellular pH on metastasis formation. Tumor cells were pre-incubated for 6 h at pH 7.4 or pH 6.6 and formation of lung metastases after was analyzed after i.v. injection of tumor cells. (**A**) Representative overlays of bright field and fluorescence images of lungs after AT-1 cell injection and (**B**) and relative tumor size of lung metastases at day 4; n = 4-5. (#) marks a blood vessel which was excluded from further analysis (**C**) Relative tumor size of Walker-256 lung metastases at day 21; n = 3. [116], see chapter 8.4 and 8.6

4.5. Summary of the role of acidosis in the modulation of the tumor phenotype

An acidic tumor microenvironment can affect the biological behavior of tumor cells in different ways. On the level of single tumor cells, acidosis affects gene expression to modulate the inflammatory program. In breast and prostate tumor cells iNOS was up-regulated by acidosis alone and even more pronounced by acidosis in combination with hypoxia. Induction of iNOS and increased production of NO in consequence can foster tumor cell survival and proliferation. Hypoxia-driven NO production, involved in tumor perfusion and angiogenesis, has been shown in the literature [119]. These effects might also be triggered by extracellular acidosis. The mechanism behind the modulation of the expression of iNOS (and other inflammatory markers) is, however, different for acidosis and for hypoxia. This can clearly be distinguished when looking at the time course of iNOS induction (Fig. 11). Acidosis affects iNOS expression only after short-term incubation (3 h and 6 h), while hypoxia exclusively elevates the expression after long-term incubation (24 h). Acidosis also raised osteopontin/SPP1 expression in breast and prostate carcinoma cells. Osteopontin is a biomarker for tumor progression and is involved in cell survival and proliferation, angiogenesis, chemoresistance, invasiveness and metastasis formation [120]. Thus, acidosis might lead to tumor progression via osteopontin induction. However, acidosis in combination with hypoxia -which is the condition typically found in the tumor microenvironment *in vivo*- reduced

osteopontin expression. This shows that the overall impact of the tumor microenvironment is difficult to predict. Partially it also depends on the cancer cell type studied, as seen for the regulation of MCP-1 or IL-6. Acidosis also modulates the behavior of tumor cells on the level of metastases formation, which is of particular importance for patient outcome, as well as tumor therapy. An acidic pre-incubation of prostate and breast cancer cells fostered lung metastasis formation *in vivo*. This was not due to changes in proliferation, adhesion or invasion. However, random motility was increased after acidic pre-incubation and transiently stayed increased even when switching to a more physiological pH. Thus, acidosis-induced increase in tumor cell motility might contribute to elevated metastasis. Additionally, the above mentioned changes in gene expression of the tumor cells could modulate the tissue microenvironment at the metastatic site, facilitating tumor cell survival and growth.

5. Signaling pathways involved in the cellular response to acidosis

5.1. Effect of an acidic microenvironment on MAPK activity

The question arises how an acidic microenvironment can affect cellular function, for instance transcriptional programs, migration, phagocytosis etc. While general mechanisms have been described for hypoxia, like the action of hypoxia-inducible factors (HIFs), no such universal mechanism is known for acidosis so far. In our studies, mitogen-activated protein kinase (MAPK) signaling was sensitive to extracellular pH. MAPKs modulate a variety of cellular processes determining cell fate, including cell proliferation, differentiation and death and thus could contribute to the effects of acidosis on cellular function. Studies of our working group showed that in AT-1 prostate carcinoma cells MAPKs ERK1/2 and p38, but not JNK, were activated by extracellular acidosis [33]. When analyzing two additional carcinoma cell lines (human lung carcinoma NCI-H358, human colorectal carcinoma LS513) and three non-tumor cell lines (Madin-Darby canine kidney MDCK-C7, Chinese hamster ovary CHO, opossum kidney OK cells), p38 activation by acidosis was observed in all cell lines, while ERK1/2 activation was cell line-specific [85]. Thus, p38 might be a general mechanism of cellular response to extracellular acidification. In the literature an impact of the acidic microenvironment on MAPK activity has also been described for p38 in cardiomyocytes during hypoxic injury [121], in astrocytes during brain ischemia [122] or in infiltrating neutrophils [123]. ERK1/2 activation was also found in renal proximal tubule cells during acid load [124] or in acidic medulloblastoma tissue [125] and also in infiltrating neutrophils [123]. In this study we could detect ERK1/2 activation by acidosis in NRK-49F fibroblasts, NRK-52E epithelial cells and in Walker-256 breast carcinoma cells (see also Tab. 3A). Activation of p38 was found in NRK-49F fibroblasts, NRK-52E epithelial cells and RAW264.7 macrophages. In contrast to our previous results no p38 activation was found in Walker-256 breast carcinoma cells (see also Tab. 3B). Thus, MAPK activity seems to be of importance for the cellular response to acidosis, but is highly cell-specific.

5.2. MAPK activation through pH-sensitive structures

The question yet remained to be answered is how acidosis leads to the activation of MAPK signaling. An importance of acid sensing receptors is more and more appreciated in the literature and could possibly lead to the observed changes in signaling. Sensing and transduction of acidic pH can be facilitated by proton-sensing G protein-coupled receptors (GPCRs) GPR4, GPR65 (TDAG8), GPR68 (OGR1), GPR132 (G2A) or non-GPCRs like acid-sensing ion channels (ASICs) and transient receptor potential cation channels subfamily V (TRPVs) [88]. These pH-sensitive structures affect the

inflammatory program via cAMP, Ca^{2+} and MAPKs (see [126] for overview). G-coupled receptors have been shown to activate ERK1/2 or p38 in carcinomas, smooth muscle and immune cells [125–129].

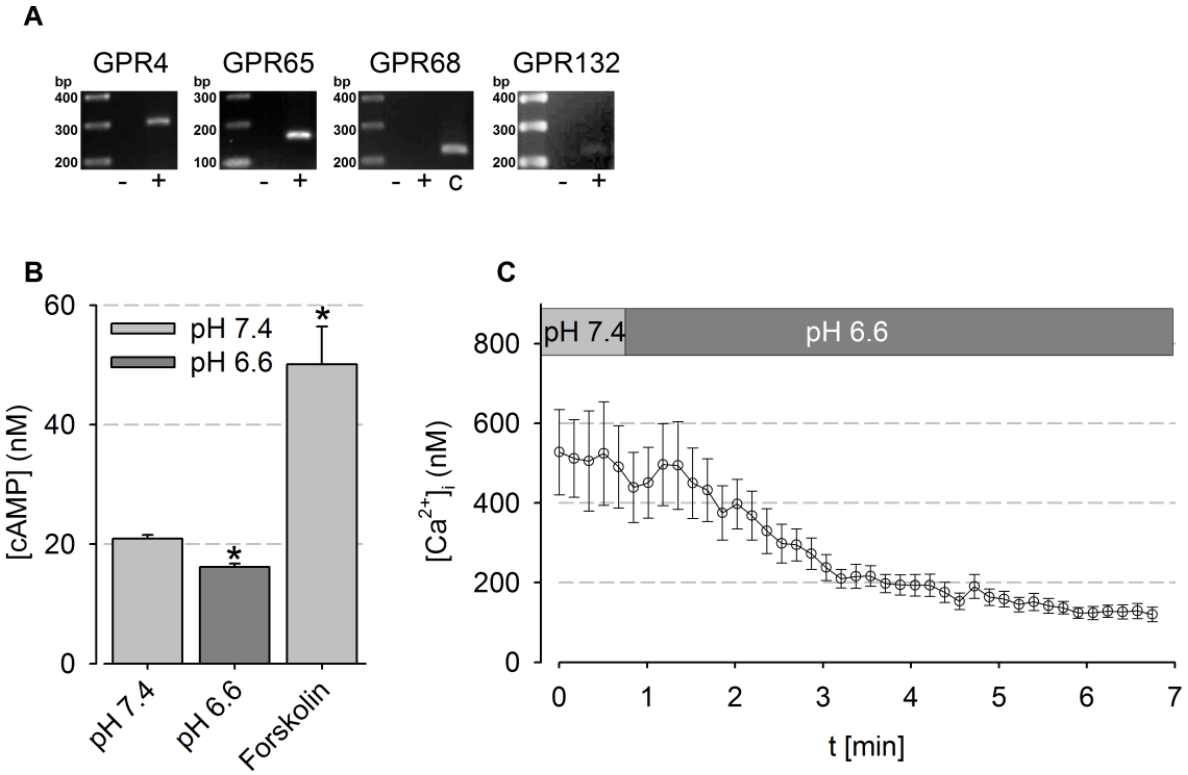

Figure 15 Involvement of GPCRs in acidosis-induced signaling. (**A**) GPCR expression in NRK-49F fibroblasts (c positive control, rat lung, spleen and thymus; - negative control, no reverse transcriptase added to reverse transcription; + cDNA, reverse transcriptase added to reverse transcription) (**B**) Effect of 15 min acidosis on cellular cAMP content in NRK-49F quantified with a colorimetric cAMP ELISA, n = 10 (Forskolin = positive control) (**C**) Dynamic change in intracellular free Ca^{2+} concentration monitored with Fura-2, n = 12 [94], see chapter 8.1

In NRK-49F cells GPR4, GPR65 and GPR135 were expressed, while GPR68 was missing (Fig. 15A). In AT-1 prostate carcinoma cells only GPR68 [85], GPR4 and GPR132 (data not published) were found. These GPRs could sense extracellular acidosis and subsequently modulate cell signaling. Thus, an impact of GPRs on MAPK was analyzed in more detail. However, in our study unspecific blocking of GPCR activity with micromolar concentrations of Cu^{2+} [130] did not abrogate MAPK activation by acidosis in NRK-49F fibroblasts [94] or AT-1 prostate carcinoma cells [85]. In addition, there was no increase in intracellular Ca^{2+} and no elevated cAMP level in NRK-49F cells (Fig. 15B + C), all of which would have been expected if GPCRs were involved. The same was true for AT-1 cells, that displayed no increase in intracellular Ca^{2+} [32] or cAMP (data not published). These findings also render

unlikely an involvement of ASICs as pH-sensitive structures activating MAPK, since the actions of ASICs have been described to depend on intracellular Ca^{2+} accumulation [47,88].

5.3. Activation of MAPK through changes in intracellular pH (pH_i)

Another possible way of MAPK activation is via intracellular acidification. Previous studies have shown that extracellular acidosis leads to a drop in intracellular pH in tumor and non-tumor cells *in vitro* which varied between ΔpH = -0.15 and -0.58 [85]. Intracellular acidosis could lead to changes in protein structure, dynamics and interactions of intracellular pH sensors that will transduce small changes in pH_i to the appropriate signaling pathways and by this modulate proliferation, cell death, migration and differentiation [131]. Intracellular acidosis induced p38 signaling in cardiomyocytes [121] and ERK1/2 signaling in neuronal cells [132].

Table 2 The impact of intracellular acidosis on pH_i and MAPK activity in prostate carcinoma cells and fibroblasts. PH_i was measured using pH-sensitive dye BCECF-AM. Intracellular acidosis was induced by adding 40 mM lactic acid or propionic acid and simultaneously blocking bicarbonate transport by 200 µM 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (in addition to 10 µM 5-(N-Ethyl-N-isopropyl)-amiloride in fibroblasts) , N = 4. For comparison pH_i under control conditions (pH 7.4) and extracellular acidosis (pH 6.6) are given. Activation of MAPKs ERK1/1 and p38 was analyzed by Western Blot using phosphorylation-specific antibodies, n = 3-7. Modified from [85,86,94], see chapters 8.1 and 8.8

cell type	pH_i			MAPK activation by intracellular acidosis	
	control (pH_e = 7.4)	extracellular acidosis (pH_e = 6.6)	intracellular acidosis (pH_e = 7.4)	ERK1/2	p38
prostate carcinoma (AT-1)	7.22 ± 0.08	6.75 ± 0.09	6.96 ± 0.01	no	yes
fibroblast (NRK-49F)	6.84 ± 0.01	6.5 ± 0.02	6.65 ± 0.02	no	no

In table 2 pH_i under control conditions, as well as extra- and intracellular acidosis, is shown for prostate tumor cells and fibroblasts. While during extracellular acidosis pH_e and pH_i were modulated, intracellular acidosis selectively reduced pH_i, although weaker than extracellular acidosis. In contrast to extracellular acidosis, intracellular acidosis was not sufficient to induce MAPK signaling, except for p38 in prostate carcinoma cells. This is in line with previous findings that could show that the magnitude of an acidosis-induced drop in pH_i did not correlate with the degree of MAPK activation [85]. Additionally, it is of importance to note that pronounced reduction of pH_i is only found in cell culture settings, with the large extracellular space in combination with the high

buffer capacity of the media used, but not in the *in vivo* situation, where pH_i is tightly regulated to normal or even slightly alkaline values [40].

5.4. Other molecular mechanisms involved in MAPK activation during acidosis

Other signaling pathways could be involved in the activation of p38 and ERK1/2 including epidermal growth factor receptor (EGFR), protein kinase C (PKC), phosphatidylinositol 3-kinases (PI3K), and Src family. However, none of those seems to play a significant role in MAPK activation by extracellular acidosis in AT-1 prostate carcinoma cells or in NRK-49F fibroblasts [85,94]. In fibroblasts cAMP level was reduced (Fig. 15). Thus, in further experiments its role in the activation of p38 and ERK1/2 signaling was analyzed. Antagonizing cAMP-mediated signaling under control conditions (pH 7.4) increased p38 as well as ERK1/2 activity, which in turn reduced the effect of acidosis (pH 6.6) on MAPK activation (Fig. 16 A).

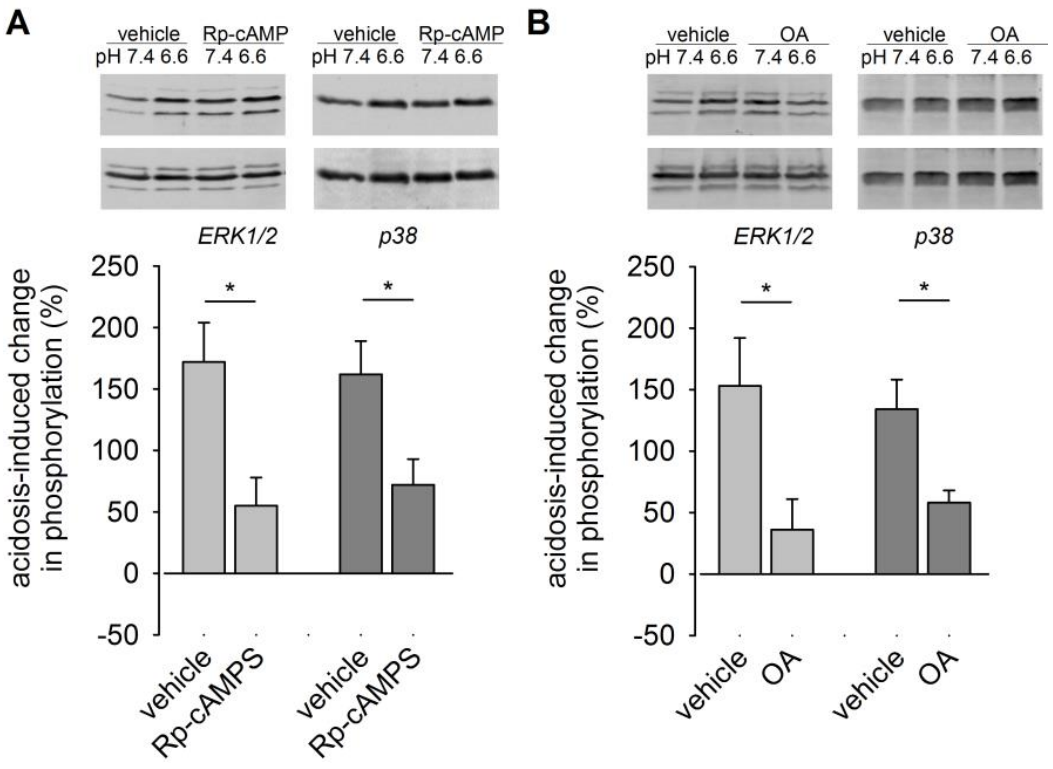

Figure 16 Involvement of cAMP signaling and serine/threonine phosphatases on acidosis-induced ERK1/2 and p38 activation in NRK-49F fibroblasts. Semi-quantitative analysis as well as representative Western blots are shown for (**A**) cAMP antagonist Rp-cAMPS (50 µM) and (**B**) Ser/Thr phosphatase inhibitor okadaic acid (100 nM), n = 4-5. [94], see chapter 8.1

Therefore, signaling via cAMP had an inhibitory effect on MAPK activity. Reduction of the cellular cAMP content by extracellular acidosis elevated the activity of ERK1/2 and p38. Comparable results concerning cAMP-mediated effects of acidosis on MAPK activity, have been found for ERK1/2 in AT-1 prostate carcinoma cells, where addition of cAMP or stimulation of cAMP formation (by forskolin) abrogated ERK1/2 activation by acidosis [85]. In fibroblasts cAMP signaling could not completely account for activation of ERK1/2 and p38 since acidosis-induced phosphorylation was not completely abrogated (Fig. 16 A). In addition to reduced cAMP signaling an inhibition of protein serine/threonine phosphatases, but not tyrosine phosphatases by acidosis was involved in MAPK activation (Fig 16 B).

5.5. Impact of ERK1/2 and p38 in cytokine expression

After activation, the MAPK phosphorylate their respective targets including transcription factors, nuclear pore proteins, membrane transporters, cytoskeletal elements and other protein kinases. Through these mechanisms acidosis-induced ERK1/2 activation can foster processes such as cell growth, survival, differentiation and development [133]. MAPK p38 has been described to be activated under various stress conditions [133]. Through acidosis-induced p38 activation inflammation, apoptosis, as well as cell growth and differentiation might be modulated. Our results show a strong correlation between activation of ERK1/2 or p38 and the regulation of the inflammatory program in all cell lines studied (Tab. 3). MCP-1 expression under control conditions (pH 7.4) depended on ERK1/2 signaling in all cell lines. An importance of ERK1/2 signaling for MCP-1 expression in RAW264.7 cells (that was not analyzed in our study) was shown in the literature [53]. The role of ERK1/2 and p38 for IL-1ß, IL-6, TNF-α and iNOS was cell type-specific. For instance, TNF-α expression under control conditions (pH 7.4) depended on ERK1/2 signaling in normal kidney fibroblasts and epithelial cells, but not in breast carcinoma cells (Tab. 3A). The impact of ERK1/2 or p38 signaling on the regulation of the inflammatory program under acidic conditions (pH 6.6) differed profoundly between the cell lines. In the breast carcinoma cell line, as well as the epithelial cell line modulation of inflammatory mediators did not depend on neither ERK1/2 nor p38. Acidosis-induced activation of p38 was on the other hand critical for the induction of IL-1ß, iNOS and COX-2 in the murine macrophage cell line (Tab. 3B). In fibroblasts iNOS and COX-2 also depended on p38 activity. ERK1/2 signaling was critical for the induction of IL-6 in fibroblasts and in the prostate carcinoma cell line. Similar results can also be found in osteocytes during bone remodeling [134].

Table 3 Impact of acidosis on (**A**) ERK1/2 and (**B**) p38 signaling and subsequent regulation of cytokine expression. Activation ("yes") and no regulation ("no") of MAPK activity (phosphorylation after 3 h) is shown for tumor and non-tumor cell lines; n = 6-118. Influence of MAPK signaling on cytokine expression is shown for basal conditions (pH 7.4) and for acidosis (pH 6.6); n = 5-15. [86,94,95,98,113,135], see chapters 8.1 - 8.3, 8.5, 8.7

A) ERK1/2 signaling:

cell type	acidosis induction	cytokine regulation pH 7.4	cytokine regulation pH 6.6
prostate carcinoma (AT-1)	yes	yes (MCP-1)	yes (IL-6, MCP-1)
mammary carcinoma (Walker-256)	yes	yes (MCP-1)	no
epithelial (NRK-52E)	yes	yes (MCP-1, TNF-α, iNOS)	no
fibroblast (NRK-49F)	yes	yes (MCP-1, TNF-α)	yes (IL-6)
macrophages (RAW264.7)	no	(no data)	no

B) p38 signaling:

cell type	acidosis induction	cytokine regulation pH 7.4	cytokine regulation pH 6.6
prostate carcinoma (AT-1)	yes	yes (IL-6)	no
mammary carcinoma (Walker-256)	no	(no data)	no
epithelial (NRK-52E)	yes	yes (MCP-1, iNOS)	no
fibroblast (NRK-49F)	yes	no	yes (iNOS, COX-2)
macrophages (RAW264.7)	yes	yes (MCP-1, IL-1ß)	yes (iNOS, COX-2, IL-1ß)

5.6. Impact of ERK1/2 and p38 on tumor cell migration

Since MAPK are involved in cell migration (see [56] for overview), it is tempting to speculate that acidosis exerts its effect on cell migration via activation of p38 and ERK1/2. When ERK1/2 or p38 signaling was blocked under control conditions (pH 7.4), random migration of AT-1 prostate carcinoma cells was reduced. Blocking both pathways simultaneously led to a significant decrease in migration (- 24 ± 4 %, n = 5-15). However, the acidosis-induced increase in migration was not prevented by blocking MAPK signaling simultaneously. Thus, although ERK1/2 and p38 seem to be involved in cell migration and although they were activated in an acidic microenvironment in prostate carcinoma cells, they were not responsible for the observed increase in tumor cell migration by acidosis. Another important signaling pathway involved in cell migration is via Src kinase family of non-receptor tyrosine kinases, that were shown to be critical for acidosis-induced migration and invasion of hepatocellular carcinoma cells [46]. However, enhanced migration of AT-1 prostate carcinoma cells at a low pH in the present experiments was not affected when blocking Src signaling. Previous studies could show that acidosis increased the level of ROS in prostate carcinoma cells and those ROS are involved in the activation of cellular signaling and subsequent changes in the transcriptional program [85]. In the presence of ROS scavengers the acidosis-induced regulation of cell migration was abolished in AT-1 prostate carcinoma cells. Therefore, an acidic

microenvironment could foster tumor cell migration by elevating ROS level, which subsequently aggravate metastases formation and worsen long-term prognosis of tumor patients. The impact of an acidic microenvironment on signaling and the possible effect on inflammation and metastases formation is shown schematically in figure 17.

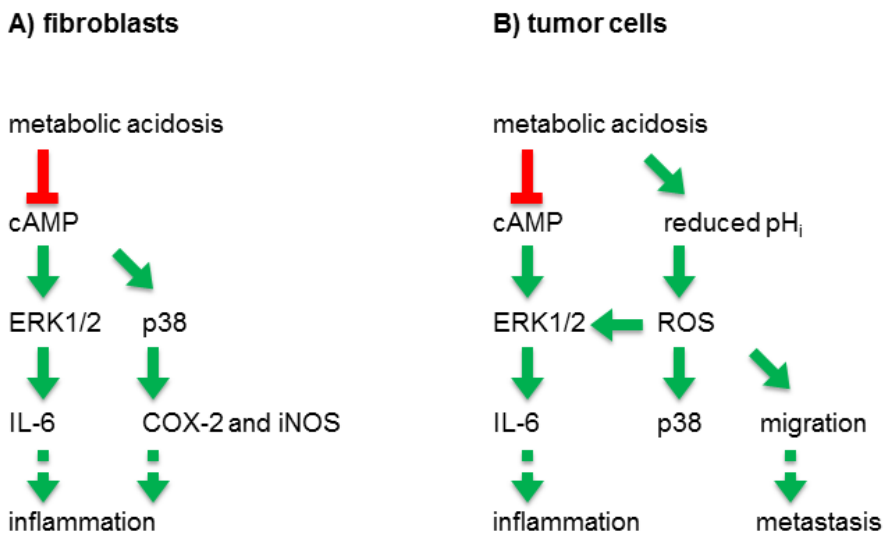

Figure 17 Summary of signaling pathways involved in the cellular response to metabolic acidosis of (**A**) NRK-49F fibroblasts and (**B**) AT-1 prostate tumor cells. Acidosis (pH 6.6) reduced cAMP-mediated signaling which led to the activation of ERK1/2 and p38 and subsequent changes in the expression of different cytokines that could foster inflammation. In AT-1 prostate tumor cells additionally an increase in ROS was critical for ERK1/2 and p38 activation and the increase in migration that might facilitate metastasis. [85,86,94,116,136], see also chapters 8.1, 8.4, 8.6 - 8.8

6. Conclusion and outlook

Extracellular acidosis which is present in the tumor microenvironment, but can also be found during ischemia or inflammation, modulates the expression of inflammatory mediators (Fig. 18). MCP-1 expression was reduced by acidosis in all of the non-tumor cells studied *in vitro*, including fibroblasts, epithelial cells and monocytic cells/macrophages, as well as in prostate and breast tumors *in vivo*. By this, acidosis could limit the recruitment and the activation of immune cells, which might in consequence reduce anti-tumor immunity. On the other hand, MCP-1 is normally strongly expressed in tumors and is correlated with poor prognosis, since it is involved in the recruitment of TAMs that will foster tumor progression [137]. A correlation to poor prognosis has also been described for osteopontin [78] and also for iNOS since low levels seem to stimulate the migratory and pro-angiogenic properties of tumor cells [138]. In the rat breast and prostate carcinoma cell line (Walker-256, AT-1) iNOS and osteopontin were up-regulated. Fibroblasts (NRK-49F), that probably provide the majority of stromal cells [89], showed an increased expression of iNOS, COX-2 and IL-6, which might foster inflammation. COX-2 is linked to more aggressive tumors and poor survival [139] and also IL-6 is pro-tumorigen, increasing the expression of multiple oncogenes [140]. Interestingly, in non-transformed epithelial cells (NRK-52E) iNOS, COX-2 and IL-6 were diminished at low pH. In monocytic cells/macrophages the impact of acidosis varied strongly between cell lines and primary cells, and between differently polarized cells (M0, M1 or M2). In monocytic cell lines (Mono-Mac-6 and THP-1) acidosis attenuated inflammation by reducing the expression of IL-6, TNF-α, COX-2 and osteopontin. In classically activated M1 macrophages TNF-α, COX-2 and IL-1ß were also reduced. Thus, acidosis found in inflamed tissue would limit the inflammatory program of M1 polarized macrophages. TNF-α expression decreased in primary M0 and M2 polarized macrophages in an acidic environment, too. Since TNF-α promotes tumor growth, angiogenesis and metastatic potential, acidosis might attenuate tumor aggressiveness via reduced TNF-α expression. However, since high levels of TNF-α could also lead to tumor cell killing [141] acidosis might also protect tumor cells from cell death. In contrast to monocytic cells, the macrophage cell line RAW264.7 had elevated levels of pro-inflammatory IL-1ß, COX-2 and iNOS in an acidic environment. Beside transcriptional activation they displayed an increased phagocytic activity when subjected to acidosis. Also in M2 polarized macrophages, which usually resemble the tumor associated macrophages, COX-2 expression was increased. Hence, acidosis might foster tumor growth by increasing COX-2-mediated production of prostaglandins by macrophages and by fibroblasts. The situation in patients is further complicated because different cancer-immune phenotypes seem to exist, including immune-desert, immune-excluded and inflamed tumors [142], that display differences in the presence of immune cells as well as cytokines. Hence, beside

parameters of the microenvironment, also the immune profile of a patient is critical for the evaluation of the impact of stromal cells on tumor growth and response to e.g. immunotherapy.

When looking at the time course of acidosis-induced regulation in most of the cell lines studied, up-regulation of inflammatory markers was usually found after short incubation times at low pH (3-6 h), while long-time incubation (24 h) had no effect or repressed the expression. This repression in the expression was also seen in experimental tumors *in vivo*. Future experiments have to clarify whether shorter acidification periods (3-6 h) of those tumors would increase the level of inflammatory markers or whether the *in vivo* situation differs profoundly due to the interaction of the tumor cells with the tumor stroma. Since short-term incubation also induced ERK1/2 and p38 signaling, which profoundly modulated the expression of inflammatory mediators under control conditions (pH 7.4), the observed changes might be transduced by MAPK activity. The acidosis-induced increase in IL-6 was mediated through ERK1/2 activation. For the increase in COX-2 and IL-1ß expression at low pH, p38 activation by acidosis was critical. Additionally, p38 activation was needed for the elevated level of iNOS in macrophages and fibroblasts. In the prostate and breast carcinoma cell lines the underlying mechanism for iNOS up-regulation differed and did not depend on MAPK activation.

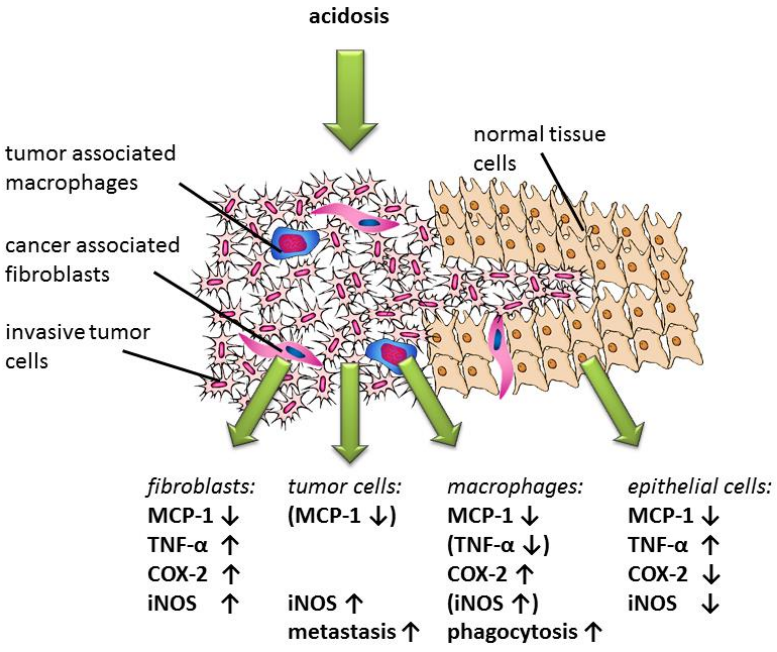

Figure 18 Summary of acidosis-induced changes in the expression of inflammatory mediators as well as functional changes in different cell types of the tumor mass. Cytokines that varied in their regulation between the different cell lines are given in brackets. See chapter 8.1 -8.7

Other possible signaling pathways involved in transcriptional regulation include CREB signaling which was up-regulated in AT-1 prostate cancer cells [85] or post-transcriptional regulation by microRNAs. For instance microRNAs miR-7a-5p, miR-183-5p, miR-203a-3p and miR-215 were shown to be regulated by acidosis in prostate and breast carcinoma cells [108,143]. MicroRNAs play an important role in inflammation and immune response by modulating immune cell development and differentiation or by regulating Toll-like receptor and NF-κB signaling, or antigen presentation [144,145]. Knockdown of miR-183 has been shown to increase phagocytosis and intracellular bacterial killing capacity in macrophages and neutrophils in parallel to the regulation of IL-1ß, MCP-1 and TNF-α expression [146]. Also miR-203 was described as a modulator of pro-inflammatory cytokines among them IL-6 and TNF-α [147,148]. In addition, a diminished level of miR-203 fosters migration and metastasis [149,150]. This might also account for the increased metastatic potential due to acidosis observed in our *in vivo* models. However, a main drawback of the work is the lack of a major pH sensor that transduces the signal acidosis from the extracellular space to intracellular signaling cascades like ERK1/2 or p38. For hypoxia HIF-1 has been shown to play a critical role. However, for acidosis no such major player is known or could be identified in the presented work. Structures sensing pH like GPCRs and non-GPCRs seem to be involved in acidosis-induced signaling and gene expression. However, whether there is a critical component in acidosis stress signaling comparable to HIF-1 in hypoxia, has to be addressed in future studies.

The impact of acidosis on the expression of inflammatory mediators differed profoundly to that of hypoxia. Regulation was often in the opposite direction. Also the time course differed, with hypoxia usually displaying the strongest regulation of the expression of inflammatory mediators after 24 h. This is in line with data from the literature showing that a minimum of 12 h is needed for hypoxia-induced gene expression changes [151]. Different signaling pathways seem to be involved in the response to acidosis and to hypoxia. Hypoxia-induced changes in transcription are probably mediated through HIF-1α, while for acidosis an important role for ERK1/2 and p38 signaling was found. Interestingly, although hypoxia had a strong impact on inflammatory mediator expression in the breast and prostate carcinoma cells, it had almost no impact in fibroblasts. Thus in an hypoxic and acidic tumor microenvironment the interaction of fibroblasts with the tumor cells might solely be modulated by acidosis but not by hypoxia, while tumor cells themselves on the other hand are affected by both factors.

Finally, acidosis did not only modulate the expression of inflammatory mediators but also affected cellular function (Fig. 18). In prostate carcinoma cells migration was elevated by acidosis *in vitro*. This increase in motility was even sustained if cells were transferred back to normal pH, which is the situation when tumors cell disseminate from the primary tumor site. By this mechanism acidosis

could foster metastasis. In line with these results metastases formation in the animal model was strongly increased when tumor cells were primed in an acidic environment prior to injection. The fostered metastases formation might partly depend on the raised motility of the tumor cells, but also other mechanism could account for the observed effects, like increased survival in the blood stream, adhesion to the endothelial lining or invasive capability. However, adhesion and invasion was not affected by acidosis in our tumor model. In addition, an increase in circulating tumor cells was not found in the literature [61]. The question arises whether targeting tumor acidosis might suppress metastasis formation. In the literature acidification of the tumor tissue was neutralized by systemic buffering with bicarbonate, imidazoles, Tris, or free base lysine, which resulted in a reduced metastases formation [10]. Interestingly, targeting tumor acidosis was also shown to improve antitumor response to immunotherapy [10]. For instance acidosis-induced impairment of cytolytic activity and cytokine secretion of T cells was reversed by buffering tumor pH with proton pump inhibitors [152]. Future studies have to clarify whether such therapeutic approach can counteract the acidosis-induced increase in the formation of lung metastases in our system of experimental prostate and breast carcinoma cells. Additionally, it would be important to analyze how the expression of inflammatory mediators and activity of monocytes/macrophages in the tumor tissue is affected by systemic buffering.

In conclusion, acidosis affects both the expression of inflammatory mediators in tumor and stromal cells which could influence tumor growth, progression, anti-tumor immunity and finally therapy, and acidosis elevates the metastatic potential of the tumor cells itself. Thus, targeting the acidic tumor tissue might have a beneficial effect on tumor therapy and patient prognosis, but further studies are needed to unravel the mechanisms of acidosis-induced stress signaling and the complex interaction of tumor and stromal cells modulated by extracellular acidosis.

7. References

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8. Published articles relevant for this work

8.1 Acidic Environment Activates Inflammatory Programs in Fibroblasts via a cAMP-MAPK Pathway. A. Riemann, A. Ihling, J. Thomas, B. Schneider, O. Thews, and M. Gekle; 2015; Biochimica et Biophysica Acta 1853 (2): 299–307. https://doi.org/10.1016/j.bbamcr.2014.11.022.

Chapter: 2.1 - 2.4, 5.1 - 5.5

8.2 Acidosis Differently Modulates the Inflammatory Program in Monocytes and Macrophages. A. Riemann, H. Wußling, H. Loppnow, H. Fu, S. Reime, and O. Thews; 2016; Biochimica et Biophysica Acta - Molecular Basis of Disease 1862 (1): 72–81. https://doi.org/10.1016/j.bbadis.2015.10.017.

Chapter: 3.1 - 3.4, 5.1, 5.5

8.3 Tumor Acidosis and Hypoxia Differently Modulate the Inflammatory Program: Measurements *In Vitro* and *In Vivo*. A. Riemann, S. Reime, and O. Thews; 2017; Neoplasia (United States) 19 (12): 1033–42. https://doi.org/10.1016/j.neo.2017.09.005.

Chapter: 2.1, 2.3, 4.1, 5.1, 5.3, 5.5

8.4 Acidic Priming Enhances Metastatic Potential of Cancer Cells. A. Riemann, B. Schneider, D. Gündel, C. Stock, O. Thews, and M. Gekle; 2014; Pflügers Archiv : European Journal of Physiology 466 (11): 2127–38. https://doi.org/10.1007/s00424-014-1458-6.

Chapter: 4.4, 5.6

8.5 Extracellular Acidosis Regulates the Expression of Inflammatory Mediators in Rat Epithelial Cells. A. Riemann, S. Reime, M. Gießelmann, and O. Thews; 2019; Advances in Experimental Medicine and Biology (in press).

Chapter: 4.3, 5.1, 5.5

8.6 Acidosis Promotes Metastasis Formation by Enhancing Tumor Cell Motility.
A. Riemann, B. Schneider, D. Gündel, C. Stock, M. Gekle, and O. Thews; 2016; Advances in Experimental Medicine and Biology 876: 215–20.
https://doi.org/10.1007/978-1-4939-3023-4_27.

Chapter: 4.4, 5.6

8.7 Impact of the Tumor Microenvironment on the Expression of Inflammatory Mediators in Cancer Cells. A. Riemann, A. Ihling, S. Reime, M. Gekle, and O. Thews; 2016; Advances in Experimental Medicine and Biology, 923:105–11.
https://doi.org/10.1007/978-3-319-38810-6_14.

Chapter: 4.1, 5.1, 5.5

8.8 Impact of Extracellular Acidosis on Intracellular pH Control and Cell Signaling in Tumor Cells. A. Riemann, Anne, A. Ihling, B. Schneider, M. Gekle, and O. Thews; 2013; Advances in Experimental Medicine and Biology 789: 221–28.
https://doi.org/10.1007/978-1-4614-7411-1-30.

Chapter: 5.1, 5.3

* All manuscripts can be found in the appendix of this work or can be downloaded from PubMed.

9. Theses

1. An acidic tumor tissue pH (acidosis) can modulate inflammation by affecting the expression of inflammatory mediators in different non-tumor (fibroblasts, monocytes/macrophages, epithelial cells) and tumor (prostate and breast carcinoma) cells.

2. In fibroblasts acidosis increases the expression of pro-inflammatory mediators tumor necrosis factor alpha (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Hypoxia in contrast leads to no changes (iNOS) or reduced expression (TNF-α).

3. Acidosis reduces the expression of inflammatory mediators in monocytic immune cells, while cell lines with macrophage-like properties are activated by acidosis displaying elevated expression of IL-1ß, COX-2 and iNOS, as well as fostered phagocytic activity. However, random migratory behavior of these immune cells was not affected by acidosis.

4. Primary human monocytes and macrophages express less monocyte chemoattractant protein 1 (MCP-1) and TNF-α in an acidic environment. In M1 polarized macrophages, that have pro-inflammatory function, also IL-1ß and COX-2 are reduced. Tumor-associated M2 macrophages on the contrary have a higher COX-2 expression that might support tumor growth.

5. Acidosis reduces the expression of pro-inflammatory mediators MCP-1 and iNOS, but up-regulated TNF-α expression in epithelial cells. Thus, the overall impact of acidosis on the role of epithelial cells in inflammation is hard to predict.

6. An acidic tumor microenvironment increases the expression of iNOS and SPP1 in prostate and breast carcinoma cells, while hypoxia reduced SPP1 and iNOS expression. Thus, different signaling pathways seem to be involved in acidosis- and hypoxia-mediated transcriptional regulation of inflammatory mediators.

7. While transcriptional modulation by hypoxia is mainly mediated by hypoxia-inducible factor 1 (HIF-1), for acidosis no major player in transcriptional regulation is known so far. Acidosis-induced activation of ERK1/2 was critical for IL-6 expression, while activation of p38 led to up-regulation of iNOS, COX-2 and IL-1ß. Future studies need to unravel the complete signal transduction leading from acidosis to ERK1/2 and p38 activation.

8. In ectopic experimental tumors of prostate and breast cancer cells chronic acidosis can be induced by inspiratory hypoxia plus inhibition of the respiratory chain. This leads to reduced expression of inflammatory mediators. Thus, in the *in vivo* situation, where a complex interaction exists between tumor and non-tumor cells, inflammation might be limited by acidosis.

9. Metastases formation is increased when tumor cells are primed in an acidic environment. This might partly be due to an increase in tumor cell migration. Targeting acidosis, for instance by systemic buffering with bicarbonate, might thus improve the clinical outcome of patients.

10. Supplement

10.1. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die hier vorliegende Habilitationsschrift selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Ein Habilitationsverfahren wurde an keiner anderen Universität eröffnet oder beantragt. Frühere Habilitationsversuche sind nicht unternommen worden.

Halle (Saale), den 16.08.2019

gez. Dr. rer. nat. Anne Riemann

10.2. Curriculum vitae

Name	Anne Riemann
Date of birth	04.10.1982
Birthplace	Halle (Saale), Germany

Vita

09.1993 – 06.2002	**Thomas-Müntzer-Gymnasium Halle/Saale (Germany)** university-entrance diploma (Abitur)
09.1999 – 06.2000	**Simon Langton Grammar School, Canterbury (England)** exchange student with the following subjects: Chemistry, History, French, English Literature and General Education
10.2002 – 09.2007	**Study of Biochemistry at the Martin Luther University Halle Wittenberg (Germany)** academical grade: „Diplom-Biochemikerin"; grade point average: 1,6
10.2005 – 11.2005	**Laboratory internship at Max Planck Research Unit for Enzymology of Protein Folding, Halle (Germany)** theme: Establishment of Flp recombinase systems in eukaryotic cells
11.2006 – 08.2007	**Diploma thesis at University Hospital Halle (Germany)** **Section Molecular Gastroenterologic Oncology** theme: Analysing SUMO modification of the transcription factor Prospero-homeobox related 1 (PROX1); final grade: 1,4
09.2007 – 12.2007	**University Copenhagen (Denmark)** **Department of Cellular and Molecular Medicine** Leonardo da Vinci-internship for studying fluorescence- and electron microscopy, as well as isolation of lipid microdomains
04.2008 – 11.2012	**Dissertation at Martin Luther University Halle-Wittenberg** **Julius Bernstein Institute of Physiology (Germany)** theme: Acidosis-induced changes in signal transduction and invasiveness in rat prostate carcinoma cells (AT-1): mechanisms and significance; academic grade: Dr. rer. nat.; final mark: magna cum laude
since 12.2012	**Research fellow at Martin Luther University Halle-Wittenberg** **Julius Bernstein Institute of Physiology (Germany)** theme: Impact of the tumor microenvironment on tumor and stromal cells (fibroblasts, immune cells, amongst others)

Further functions:

Member of the International Society on Oxygen Transport to Tissue (ISOTT)

Third-party funding acquired

Martin Luther University, Wilhelm Roux program (Nachwuchsförderung), FKZ 29/12: „Tumor-Azidose und miRNAs: Bedeutung für Tumorgenese und -Progression“

Ten most relevant publications:

- **Riemann A**, Rauschner M, Gießelmann M, Reime S, Haupt V, Thews O. Extracellular Acidosis Modulates the Expression of Epithelial-Mesenchymal Transition (EMT) Markers and Adhesion of Epithelial and Tumor Cells. Neoplasia 2019; 21:450–458.

- Thews O, **Riemann A.** Tumor pH and metastasis: a malignant process beyond hypoxia. Cancer Metastasis Rev 38 (2019) 113–129.

- Güttler A, Theuerkorn K, **Riemann A**, Wichmann H, Kessler J, Thews O, Bache M, Vordermark D. Cellular and radiobiological effects of carbonic anhydrase IX in human breast cancer cells. Oncol Rep 2019; 41:2585–2594.

- **Riemann A**, Reime S, Thews O. Tumor Acidosis and Hypoxia Differently Modulate the Inflammatory Program: Measurements *In Vitro* and *In Vivo*. Neoplasia 2017;19(12):1033–42.

- **Riemann A**, Güttler A, Haupt V, Wichmann H, Reime S, Bache M, D. Vordermark, O. Thews. Inhibition of Carbonic Anhydrase IX by Ureidosulfonamide Inhibitor U104 Reduces Prostate Cancer Cell Growth, But Does Not Modulate Daunorubicin or Cisplatin Cytotoxicity. Oncol Res. 2018 Mar 5;26(2):191-200.

- **Riemann A**, Ihling A, Thomas J, Schneider B, Thews O, Gekle M. Acidic environment activates inflammatory programs in fibroblasts via a cAMP-MAPK pathway. Biochim Biophys Acta 2015, 1853:299–307.

- **Riemann A**, Wußling H, Loppnow H, Fu H, Reime S, Thews O. Acidosis differently modulates the inflammatory program in monocytes and macrophages. Biochim Biophys Acta 2015, 1862(1):72-81

- **Riemann A**, Schneider B, Gündel D, Stock C, Thews O, Gekle M. Acidic priming enhances metastatic potential of cancer cells. Pflugers Arch Eur J Physiol 2014, 466:1–12.

- **Riemann A**, Schneider B, Ihling A, Nowak M, Sauvant C, Thews O, Gekle, M. Acidic Environment Leads to ROS-Induced MAPK Signaling in Cancer Cells. PLoS One 2011, 6, e22445

- Sauvant C, Nowak M, Wirth C, Schneider B, **Riemann A**, Gekle M, Thews O. Acidosis induces multi-drug resistance in rat prostate cancer cells (AT1) in vitro and in vivo by increasing the activity of the p-glycoprotein via activation of p38. Int J Cancer 2008, 123:2532–2542.

Selected talks and posters:

- The acidic tumor microenvironment affects epithelial-mesenchymal transition markers as well as adhesion of NCI-H358 lung cancer cells; **Riemann A**, Rauschner M, Gießelmann M, Reime S, Thews O
  47th Annual Meeting of the International Society on Oxygen Transport to Tissue, Albuquerque, USA, 2019 ***speaker***

- Tumor microenvironmental acidosis and hypoxia differentially regulate the expression of tumor-related microRNAs; **Riemann A**, Reime S, Thews O
  Europhysiology 2018, London, UK, 2018 ***speaker***

- Extracellular acidosis regulates the expression of inflammatory mediators in rat epithelial cells; **Riemann A**, Reime S, Wollny P, Thews O
  46th Annual Meeting of the International Society on Oxygen Transport to Tissue, Seoul, Korea, 2018; Adv Exp Med Biol (in press) ***speaker***

- Hypoxia-related tumor acidosis affects the miRNA expression pattern in prostate and breast tumor cells; **Riemann A**, Reime S, Thews O
  44th Annual Meeting of the International Society on Oxygen Transport to Tissue, Chicago IL, USA, 2016 ***speaker***

- Hypoxia-related acidosis promotes metastasis formation by enhancing tumor cell motility; **Riemann A**, Schneider B, Gündel D, Stock C, Gekle M, Thews O.
  42nd Annual Meeting of the International Society on Oxygen Transport to Tissue, London, UK 2014 Adv Exp Med Biol 2014, 812:51–58. ***speaker***

- Acidic priming enhances metastatic potential of cancer cells *in vivo* and *in vitro*; **Riemann A**, Schneider B, Gündel D, Stock C, Thews O, Gekle M.
  Jahrestagung der ISPDC (International society of proton dynamics in cancer), München 2013
  Front. Pharmacol. Conference Abstract: 4th Annual Meeting of the International Society of Proton Dynamics in Cancer. (2013) ***invited speaker***

- An acidic environment activates inflammatory programs in fibroblasts; **Riemann A**, Ihling A, Thomas J, Schneider B, Thews O, Gekle M.
  92. Jahrestagung der Deutschen Physiologischen Gesellschaft, Heidelberg 2013
  Acta Physiologica 207 (Suppl. 694): P177 (2013) ***poster***

- Acidosis-induced activation of ERK1/2 and p38 kinases in rat prostate cancer cells (AT1); **Riemann A**, Schneider B, Sauvant C, Thews O, Gekle M.
  International Symposium Signal Transduction and Disease, Aachen 2009 ***poster***

10.3. Danksagung

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10.4. Original articles

Acidic Environment Activates Inflammatory Programs in Fibroblasts via a cAMP-MAPK Pathway.
A. Riemann, A. Ihling, J. Thomas, B. Schneider, O. Thews, and M. Gekle; 2015; Biochimica et Biophysica Acta 1853 (2): 299–307.
https://doi.org/10.1016/j.bbamcr.2014.11.022.

Abstract: The tissue micromilieu in disorders (inflammation, ischemia, tumor) often shows pronounced metabolic acidosis that may alter signaling and transcriptional activity in resident cells which can be of special importance for omnipresent fibroblasts. In the present study we investigated the impact of metabolic acidosis on rat fibroblasts with special emphasis on their role in inflammation by regulation of TNF-α, MCP-1, COX-2 and iNOS expression and the signaling pathways involved. Extracellular acidosis led to an enhanced expression of TNF-α, COX-2 and iNOS in parallel to an activation of p38 and ERK1/2 kinases that was not observed by sole intracellular acidosis. Accordingly, the protein amounts of TNF-α and COX-2 as well as the production of nitrate and nitrite were elevated. Acidosis-induced expression of COX-2 and iNOS depended on p38 kinase, but not on ERK1/2. In contrast acidosis-induced TNF-α expression was independent of both kinases. Although GPR4, GPR68 and GPR132 are expressed in fibroblasts, the involvement of these potential candidate pH sensors could be ruled out since no acidosis-induced elevation in intracellular cAMP or free calcium content was observed. Furthermore our data show that MAPK activation by an acidic micromilieu depends on Ser/Thr phosphatase activity, but not on the production of reactive oxygen species and is sensitive to cAMP antagonism by Rp-cAMPS. In conclusion, our results show that an acidic microenvironment induces a differential transcriptional program of pathological relevant genes in fibroblasts via the cAMP-phosphatase-MAPK pathway and thereby generates a parainflammatory situation that can result in tissue remodeling.

Acidosis Differently Modulates the Inflammatory Program in Monocytes and Macrophages.
A. Riemann, H. Wußling, H. Loppnow, H. Fu, S. Reime, and O. Thews; 2016; Biochimica et Biophysica Acta - Molecular Basis of Disease 1862 (1): 72–81.
https://doi.org/10.1016/j.bbadis.2015.10.017.

Abstract: Inflammation, ischemia or the microenvironment of solid tumors is often accompanied by a reduction of extracellular pH (acidosis) that stresses the cells and acts on cellular signaling and transcription. The effect of acidosis on the expression of various inflammatory markers, on functional parameters (migration, phagocytic activity) and on signaling pathways involved was studied in monocytic cells and macrophages. In monocytic cell lines acidosis led to a reduction in expression of most of the inflammatory mediators, namely IL-1ß, IL-6, TNF-α, MCP-1, COX-2 and osteopontin. In primary human monocytes MCP-1 and TNF-α were reduced but COX-2 and IL-6 were increased. In RAW264.7 macrophage cell line IL-1ß, COX-2 and iNOS expression was increased, whereas MCP-1 was reduced similar to the effect in monocytic cells. For primary human monocyte-derived macrophages the regulation of inflammatory markers by acidosis depended on activation state, except for the acidosis-induced downregulation of MCP-1 and TNF-α. Acidosis affected functional immune cell behavior when looking at phagocytic activity which was increased in a time-dependent manner, but cellular motility was not changed. Neither ERK1/2 nor CREB signaling was stimulated by the reduction of extracellular pH. However, p38 was activated by acidosis in RAW264.7 cells and this activation was critical for the induction of IL-1ß, COX-2 and iNOS expression. In conclusion, acidosis may impede the recruitment of immune cells, but fosters

inflammation when macrophages are present by increasing the level of COX-2 and iNOS and by functionally forcing up the phagocytic activity.

Tumor Acidosis and Hypoxia Differently Modulate the Inflammatory Program: Measurements *In Vitro* and *In Vivo*. A. Riemann, S. Reime, and O. Thews; 2017; Neoplasia (United States) 19 (12): 1033–42.
https://doi.org/10.1016/j.neo.2017.09.005.

Abstract: Inflammatory mediators produced by the tumor cells are of importance for immune response but also for malignant progression. The aim of the study was to analyze the expression of monocyte chemoattractant protein-1, interleukin-6 (IL-6), tumor necrosis factor-α, inducible isoform of nitric oxide synthase (iNOS), cyclooxygenase-2, and osteopontin in vitro in two different tumor cell lines under hypoxia (pO_2≈1.5 mmHg) and/or acidosis (pH=6.6) for up to 24 hours since hypoxia and acidosis are common characteristics of solid tumors. Additionally, the same tumor cell lines implanted in vivo were made hypoxic and acidotic artificially for 24 hours, after which the cytokine expression was measured. Finally, the activation of ERK1/2 and p38 by acidosis/hypoxia and their impact on cytokine expression were studied. The results indicate that acidosis and hypoxia have fundamentally different (often opposing) effects on cytokine expression. In addition, these effects were tumor cell line specific. When combining hypoxia and acidosis, the overall changes reflect an additive effect of both conditions alone, indicating that hypoxia and acidosis act by independent mechanisms. The in vivo changes corresponded well with the results obtained in the isolated tumor cells. Only iNOS expression was downregulated in vivo but increased in cell culture. For IL-6 expression, the acidosis-induced changes were dependent on ERK1/2 activation. In conclusion, it was demonstrated that the environmental pO_2 and pH strongly affect the expression of inflammatory mediators in tumor cells. In vivo, most of the inflammatory mediators were downregulated, which could limit the activation of immune cells and by this foster the immune escape of tumors.

Acidic Priming Enhances Metastatic Potential of Cancer Cells.
A. Riemann, B. Schneider, D. Gündel, C. Stock, O. Thews, and M. Gekle; 2014; Pflügers Archiv : European Journal of Physiology 466 (11): 2127–38.
https://doi.org/10.1007/s00424-014-1458-6.

Abstract: Metabolic acidosis is a common feature of tumor microenvironment and may affect the phenotype of tumor cells, including invasive capacity and formation of metastases. We tested whether previous exposure to an acidic environment alters metastatic potential of two rat carcinoma cell lines in the animal model. In addition, we determined the effect of an acidic environment on motility and invasive capacity of AT-1 prostate carcinoma cells in culture. Exposure of tumor cells to an acidic environment (pH 6.6, 5 % CO2, 6 h) prior to tail vein injection in rats enhanced formation of lung metastases significantly. In culture, acidosis increased cellular motility of AT-1 cells. When the tumor cells were transferred back to pH 7.4, enhanced motility persisted for at least 3 h but vanished after longer periods (24 h), therefore presenting a "short-term memory effect." Although acidosis augmented phosphorylation of ERK1/2 and p38, and inhibition of ERK1/2 phosphorylation or of p38 kinase activity reduced basal motility at pH 7.4, acidosis-induced increase in motility was not dependent on ERK1/2 or p38 kinase. Src family kinases were not involved either. By contrast, scavenging reactive oxygen species (ROS), known to be increased in AT-1 cells under acidic conditions, blunted acidosis-induced motility increase. Our data indicate that tumor cells may acquire enhanced motility in an acidic micromilieu, at least in part due to enhanced ROS formation. Because enhanced motility persists for at least 3 h after leaving the acidic environment, this may promote metastasis formation, as observed in our in vivo model.

Extracellular Acidosis Regulates the Expression of Inflammatory Mediators in Rat Epithelial Cells. A. Riemann, S. Reime, M. Gießelmann, and O. Thews; 2019; Adv Exp Med Biol. 2020;1232:277-282.
https://doi.org/10.1007/978-3-030-34461-0_35

Abstract: Acidification of the cellular microenvironment is found in different pathological states such as inflammation, ischemia and in solid tumors. It can affect cell function and phenotype, and by this aggravate the pathological process. Epithelial cells are a relevant functional part in several normal organs as well as in tumors and will thus be challenged by the acidic extracellular pH (acidosis). Therefore, the impact of acidosis on the expression of different inflammatory mediators (MCP-1, IL-6, osteopontin, iNOS, TNF-α, and COX-2), as well as the role of different signaling pathways regulating the expression, was studied in epithelial normal rat kidney cells (NRK-52E). Acidosis led to an increase in TNF-α expression but a down-regulation of MCP-1, iNOS and COX-2. Expression of IL-6 was only slightly modulated, while osteopontin was not regulated at all. Since acidosis activates ERK1/2 and p38 signaling in NRK-52E cells, the impact of MAP kinase signaling pathways on the expression of the inflammatory markers was analyzed. At normal pH, blocking ERK1/2 or p38 decreased the level of MCP-1, iNOS and partly TNF-α. However, the effect of acidosis on the expression of inflammatory mediators was not affected by inhibition of the MAP kinase pathways. In conclusion, our results show that an acidic microenvironment affects the transcriptional program of epithelial cells. Low pH mostly reduced the expression of pathological relevant genes and might thus repress inflammatory processes induced by epithelial cells.

Acidosis Promotes Metastasis Formation by Enhancing Tumor Cell Motility.
A. Riemann, B. Schneider, D. Gündel, C. Stock, M. Gekle, and O. Thews; 2016; Advances in Experimental Medicine and Biology 876: 215–20.
https://doi.org/10.1007/978-1-4939-3023-4_27.

Abstract: The tumor microenvironment is characterized by hypoxia, acidosis as well as other metabolic and biochemical alterations. Its role in cancer progression is increasingly appreciated especially on invasive capacity and the formation of metastasis. The effect of acidosis on metastasis formation of two rat carcinoma cell lines was studied in the animal model. In order to analyze the pH dependency of different steps of metastasis formation, invasiveness, cell adhesion and migration of AT-1 prostate cancer cells as well as possible underlying cell signaling pathways were studied in vitro. Acidosis significantly increased the formation of lung metastases of both tumor cell lines in vivo. In vitro, extracellular acidosis neither enhanced invasiveness nor affected cell adhesion to a plastic or to an endothelial layer. However, cellular motility was markedly elevated at pH 6.6 and this effect was sustained even when extracellular pH was switched back to pH 7.4. When analyzing the underlying mechanism, a prominent role of ROS in the induction of migration was observed. Signaling through the MAP kinases ERK1/2 and p38 as well as Src family kinases was not involved. Thus, cancer cells in an acidic microenvironment can acquire enhanced motility, which is sustained even if the tumor cells leave their acidic microenvironment e.g. by entering the blood stream. This increase depended on elevated ROS production and may contribute to the augmented formation of metastases of acidosis-primed tumor cells in vivo.

Impact of the Tumor Microenvironment on the Expression of Inflammatory Mediators in Cancer Cells. A. Riemann, A. Ihling, S. Reime, M. Gekle, and O. Thews; 2016; Advances in Experimental Medicine and Biology, 923:105–11.
https://doi.org/10.1007/978-3-319-38810-6_14.

Abstract: Hypoxia and extracellular acidosis are common features of solid malignant tumors. The aim of the study was to analyze whether these pathophysiological parameters affect the expression of inflammatory mediators in tumor cells. Therefore the mRNA expression of MCP-1 (monocyte chemotactic protein 1), iNOS and osteopontin was measured under hypoxic (pO2 1 mmHg) and acidotic (pH 6.6) conditions by qPCR in AT1 R-3327 prostate cancer cells. In addition, the underlying signaling cascades were analyzed by using inhibitors of the p38 and ERK1/2 MAP kinase pathways.Hypoxia led to a significant decrease of the expression of MCP-1 and osteopontin over the complete observation period of 24 h, whereas the iNOS expression after an initial reduction slightly increased. Acidotic conditions for up to 6 h increased the iNOS expression significantly which was functional as indicated by an elevated level of nitrate/nitrite formation by 30 %. Acidosis had almost no impact on the MCP-1 expression of tumor cells, whereas the osteopontin level tended to increase leading to a significantly elevated level after 24 h at pH 6.6. Inhibiting the p38 and ERK1/2 under control conditions revealed that the MAPKs play a significant role for the regulation of the expression of inflammatory mediators. MCP-1 expression could be lowered by inhibiting ERK1/2 whereas iNOS expression was dependent on both p38 and ERK1/2 MAPK. These results indicate that the adverse tumor microenvironment affects the expression of inflammatory mediators by tumors cells and may therefore modulate the immune response within the tumor tissue.

Impact of Extracellular Acidosis on Intracellular pH Control and Cell Signaling in Tumor Cells.
A. Riemann, Anne, A. Ihling, B. Schneider, M. Gekle, and O. Thews; 2013; Advances in Experimental Medicine and Biology 789: 221–28.
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Abstract: Cells in solid tumors generate an extracellular acidosis due to the Warburg effect and tissue hypoxia. Acidosis can affect the functional behavior of tumor cells, causing, e.g., multidrug resistance. In this process ERK1/2 and p38 mitogen-activated protein kinases (MAPK) seem to play a key role. However, the underlying mechanism of MAPK activation by extracellular acidosis remains unclear. Experiments were performed in three tumor and three normal tissue cell lines in which the cells were exposed to an extracellular pH of 6.6 for 3 h. Intracellular pH (pHi), protein expression and activation, acidosis-induced transactivation, and reactive oxygen species (ROS) formation were measured. Extracellular acidosis resulted in a rapid and sustained decrease of pHi leading to a reversal of the extra-/intracellular pH gradient. Extracellular acidosis led to p38 phosphorylation in all cell types and to ERK1/2 phosphorylation in three of six cell lines. Furthermore, p38 phosphorylation was also observed during sole intracellular lactacidosis at normal pHe. Acidosis-enhanced formation of ROS, probably originating from mitochondria, seems to trigger MAPK phosphorylation. Finally, acidosis increased phosphorylation of the transcription factor CREB and resulted in increased transcriptional activity. Thus, an acidic tumor microenvironment can induce a longer-lasting p38 CREB-mediated change in the transcriptional program.