Tumor-Derived Lactate Modifies Antitumor Immune Response: Effect on Myeloid-Derived Suppressor Cells and NK Cells

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Tumor-Derived Lactate Modifies Antitumor Immune Response: Effect on Myeloid-Derived Suppressor Cells and NK Cells

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In this study, we explore the hypothesis that enhanced production of lactate by tumor cells, because of high glycolytic activity, results in inhibition of host immune response to tumor cells. Lactate dehydrogenase-A (LDH-A), responsible for conversion of pyruvate to lactate, is highly expressed in tumor cells. Lentiviral vector–mediated LDH-A short hairpin RNA knockdown Pan02 pancreatic cancer cells injected in C57BL/6 mice developed smaller tumors than mice injected with Pan02 cells. A decrease occurred in the frequency of myeloid-derived suppressor cells (MDSCs) in the spleens of mice carrying LDH-A–depleted tumors. NK cells from LDH-A–depleted tumors had improved cytolytic function. Exogenous lactate increased the frequency of MDSCs generated from mouse bone marrow cells with GM-CSF and IL-6 in vitro. Lactate pretreatment of NK cells in vitro inhibited cytolytic function of both human and mouse NK cells. This reduction of NK cytotoxic activity was accompanied by lower expression of perforin and granzyme in NK cells. The expression of Nkp46 was decreased in lactate-treated NK cells. These studies strongly suggest that tumor-derived lactate inhibits NK cell function via direct inhibition of cytotoxic function as well as indirectly by increasing the numbers of MDSCs that inhibit NK cytotoxicity. Depletion of glucose levels using a ketogenic diet to lower lactate production by glycolytic tumors resulted in smaller tumors, decreased MDSC frequency, and improved antitumor immune response. These studies provide evidence for an immunosuppressive role of tumor-derived lactate in inhibiting innate immune response against developing tumors via regulation of MDSC and NK cell activity. The Journal of Immunology, 2013, 191: 1486–1495.

There is increasing evidence that a defect in immune surveillance results in tumor growth (1, 2). Growing tumor cells develop strategies to overcome host immune surveillance. One of the strategies used by tumor cells is to change their microenvironment by the release of lactate, an important side product of tumor metabolism (3–5). Well-characterized immunosuppressive mechanisms include suppressor cells (regulatory T cells [Tregs], myeloid-derived suppressor cells [MDSCs], and M2-polarized macrophages) that infiltrate the tumor environment as well as numerous cytokines such as TGF-β, and IL-10 among others (6).

Bone marrow–derived suppressor cells were described in cancer > 25 years ago (7). MDSCs are a heterogeneous population of myeloid cells made up of monocytes/macrophages, granulocytes, and dendritic cells that are dramatically increased in the blood of cancer patients and in tumor-bearing mice (8–10). In mice, MDSCs are commonly characterized as CD11b+Gr-1+. Human MDSCs have an immature phenotype and are CD11b+ CD33+, and HLA-DR− (11). Importantly, MDSCs are present at the tumor site (or in preneoplastic lesions) and in spleens before appearance of full-blown cancer in genetic models and in transplanted syngeneic mouse tumors and well before the appearance of Tregs (12).

Developing tumors secrete a wide variety of factors including vascular endothelial growth factor, TGF-β, GM-CSF, IL-10, and PGE2 that promote accumulation of MDSCs (13, 14). MDSCs mediate immunosuppression by using two enzymes involved in arginine metabolism (ARG and NOS) and also through the enzyme IDO that is involved in tryptophan metabolism. MDSCs also suppress immune effector function by generation of Tregs (11). Modification of the behavior of immune cells such as T cells, NK cells, and myeloid cells in the tumor microenvironment may play a key role in tumor development (8–11, 25).

Interactions between cancer metabolism and immunity have begun to be investigated recently. The role of metabolism in cancer has received attention ever since the initial observations of Otto Warburg on increased glucose utilization by cancer cells (16–18). In contrast, there is only limited information on immunometabolic interactions other than in the context of nutritional metabolism and immunity (19–21). Recent studies (15) support the fact that tumor-derived lactate or low extracellular pH (as a consequence of the “Warburg effect”) affects dendritic cell maturation and results in arrested differentiation of APCs from myeloid progenitors. Other studies suggest that investigations of the effect of tumor metabolism on the adaptive immune response may generate potential therapeutic targeting strategies (22). In this study, we further define the impact of lactate on host immune responses to tumor cells in a syngeneic Pan02 murine pancreatic cancer model.

Materials and Methods

**Media and reagents**

Cells were cultured in RPMI 1640 medium supplemented with 100 μg/ml penicillin and streptomycin, 1% sodium pyruvate, 1% sodium glutamate, 50 μM 2-ME, 50 μM HEPES, and 10% heat-inactivated FBS, all pur-
chased from Life Technologies (Carlsbad, CA). Collagenase (C5138), DNase (D5025), hyaluronidase (H3884), and lactate were purchased from Sigma-Aldrich. The tumor cell line YAC-1 was purchased from the American Type Culture Collection (Manassas, VA). To study the effect of lactic acid on NK activity, we used human NKL cell line provided by Dr. J. Ritz (Dana Farber Cancer Institute, Boston, MA). Fluorochrome-labeled mAbs anti-NKp30, anti-NKp44, anti-NKp46, anti-NKG2D, anti-perforin, or anti-granzyme were purchased from eBioscience (San Diego, CA). Sheep anti-LDH-A polyclonal Ab and anti-LDH-B mouse monoclonal were obtained from Abcam (Cambridge, MA). Appropriately labeled isotype-specific Abs were used as control. PBS containing 1% FBS (FACS staining medium) was used in staining of cells with mAbs.

**Generation of LDH-A–deficient Pan02 cell line**

Mouse pancreatic cancer cell line Pan02 is a well described tumor cell line known to induce tumors when injected in syngeneic C57BL/6 mice. To develop a LDH-A–negative tumor cell line, LDH gene expression was knocked down in the cell line Pan02 as follows: Pan02 cells were infected with an empty short hairpin RNA (shRNA) vector control and three different LDH-A shRNAs lentiviruses as described previously. Briefly, recombinant lentiviruses were produced by transient transfection of 293 T cells according to a standard protocol (4). Pan02 cells were treated with the above cell culture supernatant containing lentiviral particles for 24 h. These cells were then selected in puromycin to generate stable cell lines encoding empty vector shRNA and LDH-A shRNA. The selected cell lines were validated for diminished LDH-A expression by Western blot analysis. Total cellular proteins were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes and immunoblotted with anti-LDH-A sheep polyclonal Ab (Abcam) or anti-LDH-B mouse mAb overnight at 4˚C. After washing with TBS-T, the membrane was incubated with anti-sheep IgG. Protein bands were detected using SuperSignal West Pico Chemiluminescent substrate (Pierce).

**FIGURE 1.** (A) Western blot analysis of control and mLDH-A–deficient Pan02 cells with two independent mLDH-A–shRNA over 48 h (control [left lane], clone 31 [middle lane], and clone 32 [right lane]). (B) Tumor volume for Pan02 tumors expressing control or LDH-A–shRNA after 21 d (n = 5). (C) Graphical format of tumor volume, LDH-A–shRNA Pan02 tumors were significantly smaller (p = 0.03). (D) Cytotoxic activity of NK cells against YAC targets from C57BL/6 mice that were injected s.c. with Pan02 cells expressing GFP or LDH-A–shRNA after 21 d. Error bars, SD. (E) NK cytotoxicity/tumor volume. Increase in cytotoxicity was significant (p = 0.001). (F) MDSC staining of splenic CD11b+Gr-1+ cells from Pan02 control or LDH-A–shRNA tumors. (G) Effect of MDSCs from LDH-A knockdown tumor-bearing mice on NK cell cytotoxicity. MDSCs were isolated from spleens of animals transplanted with either Pan02 or LDH-A–shRNA Pan02 cells and incubated for 24 h with NK cells isolated from healthy mice (1:1). Cytotoxicity was tested against YAC-1 cells. Error bars represent SD. Results are average of three independent experiments.
**Mice and in vivo studies**

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in the Animal Facility of the Beth Israel Deaconess Medical Center. Six- to 8-week-old female C57BL/6 mice weighing ~17 g were maintained for 1 wk before use. Mice were housed five per cage in a limited access area at a mean room temperature of 20 ± 1 °C and a humidity of 50 ± 10% with free access to food and water. All animal experiments were approved by the Institutional Review Board. Mice were inoculated s.c. with either Pan02 cells (5 × 10⁵ cells/mice) that had been infected with an empty shRNA vector control or three different LDH-A shRNA lentiviruses that had LDH-A GFP. Tumor volume was determined by measuring two perpendicular diameters (diam1 and diam2) of the tumor mass every 3 d using a caliper. Tumor volume was calculated using the formula V = 0.523 (diam1 × diam2)³ (mm³). The effect of in vivo deletion of LDH-A on tumor growth was calculated as: T/C (%) = (mean value of tumor size of Pan02-LDH-A/mean value of tumor size of Pan02) × 100.

**Isolation of NK cells**

Spleens were harvested from age-matched normal or tumor-bearing C57BL/6 mice, and single-cell suspensions were made by passing the spleens through a nylon mesh screen. Erythrocytes were depleted using RBC lysis buffer (Sigma-Aldrich), and splenocytes were washed in MACS buffer (1× PBS supplemented with 2 mM EDTA and 0.5% BSA). NK cells were isolated using MACS microbeads (NK Cell Isolation kit; Miltenyi Biotec, Auburn, CA). Total number of live cells was determined by trypan blue exclusion method. Human NK cells were isolated from PBLs (obtained after Ficoll–Hypaque centrifugation) and negatively selected using an NK cell isolation kit (StemCell Technologies, Vancouver, BC, Canada). The purity of cells after separation ranged between 90 and 94%.

**NK cytotoxicity assay**

Lactate-treated and control NK cells were tested for cytotoxic activity against K562 (for human) and YAC-1 (for mouse) target cells using a standard 4-h chromium release assay. For some experiments, NK cytotoxicity was determined by Cytotox 96 Nonradioactive Cytotoxicity Assay Kit (Celltox assay; Cell Technology, Mountain View, CA). Briefly, NK cells were incubated with target cells at different E:T ratios at 37 °C for 4 h, and targeted cell lysis was calculated according to the manufacturer’s instructions.

**MDSCs in LDH-A–deficient Pan02 tumors**

MDSCs were isolated from spleens of mice. Briefly, the MDSC isolation kit was used to isolate CD11b+Gr-1+ cells from erythrocyte-depleted splenocytes following the manufacturer’s instructions (Miltenyi Biotec). The purity of the total MDSC population or the MDSC subfractions was typically higher than 90%.

Tumors were dissected and chopped into small pieces using a razor blade before incubation (1 g/10 ml) at 37 °C for 1 h with a mixture of enzymes dissolved in HBSS: 0.05 mg/ml collagenase (Sigma-Aldrich), 0.025 mg/ml hyaluronidase (Sigma-Aldrich), 0.01 mg/ml DNase I (Sigma-Aldrich). Red cells were lysed by ammonium chloride–potassium lysis buffer, and dead cells were removed by centrifugation with Lympholyte M. MDSC were isolated by using anti-Gr-1 Ab and with MiniMACS columns (Miltenyi Biotec).

**MDSC suppression assay**

MDSC suppression of T cells was carried out against splenic T cells isolated from C57BL/6 mice without tumors. T cells were isolated using T cell–enrichment column (R&D Systems, Minneapolis, MN). Isolated T cells (2 × 10⁶), at different ratios, were activated with anti-CD3 and anti-CD28 cultured with irradiated MDSCs (2500 rad; 5 × 10⁴). During the last 10 h of the 72-h incubation, [³H]thymidine was added, and radioactivity was measured to determine T cell proliferation. In studies demonstrating suppression of NK cytotoxicity, MDSCs were incubated with NK cells for 24 h prior to using for cytotoxicity studies against target cells. Although we have determined that MDSCs alone did not kill target cells to control for any MDSC effects, we used blank (no NK cell):target cells as additional controls.

**Bone marrow–derived MDSCs**

Bone marrow cells were obtained from the femurs of wild-type (C57BL/6) mice. Single-cell suspensions were prepared following red cell lysis in RPMI 1640 medium. Bone marrow cells (1 × 10⁶) were cultured in complete RPMI 1640 medium in the presence of GM-CSF (10 ng/ml) and IL-6 (10 ng/ml) at 37 °C for 4–6 d. Medium was replaced on day 3. Cells were harvested and stained for CD11b+Gr1+ as described previously.

**Human MDSC generation from PBMCs**

PBMCs isolated from whole blood using Ficoll–Paque Plus (GE Healthcare) density gradient separation were cultured in RPMI 1640 medium

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**FIGURE 2. Intratumoral MDSC (CD11b+Gr-1+) cells in Pan02-LDH-A knockdown mice.** Tumors were isolated from mice 3 wk following injection s.c. with either Pan02 cells or Pan02–LDH-A KD cells. Following enzymatic digestion of tumors, isolated cells were stained with CD11b–FITC and Gr-1–PE and subjected to FACS analysis. (A) Gating strategy. (B) Isotype control. (C) CD11b+Gr-1+ cells isolated from Pan02 wild-type tumors. (D) CD11b+Gr-1+ cells isolated from LDH-A KD tumors. Representative data from two separate experiments shown. Each group included five animals.
(RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with GM-CSF (10 ng/ml; R&D Systems) and IL-6 (10 ng/ml; R&D Systems) for 4–6 d. All cells were collected and used to isolate CD33+ cells using anti-CD33 microbeads and LS columns (Miltenyi Biotec) following the manufacturer’s instructions. Cells were stained for HLA-DR, CD80, CD86, and reactive oxygen species production.

The suppressive function of myeloid cells was measured by their ability to inhibit the proliferation of autologous T cells. CD4+ and CD8+ cells isolated from PBMCs by microbeads and magnetic column separation (Miltenyi Biotec). CD4+ were labeled with 3 μM CFSE (Molecular Probes, Eugene, OR) and seeded in 96-well plates with MDSC at 2 × 10^5 cells/well 2:1 ratio (or 1:1 in some cases) of CD4:MDSCs. T cell proliferation was induced with beads coated with anti-CD3/anti-CD28 Abs (Miltenyi Biotec) in the presence of 500 IU/ml IL-2 (PeproTech, Rocky Hill, NJ). Cells were analyzed by flow cytometry for T cell proliferation after 5 d. Parallel experiments were set up to stain CD8+ T cells for IFN-γ levels by intracellular staining.

Monoclonal Abs and flow cytometry

To determine the frequency of NK1.1+ cells in spleens, isolated cells were stained with NK1.1-specific mAb and analyzed using flow cytometry. Lactate-treated and untreated (control) NK cells were incubated with appropriate Abs in 100 μl FACS staining medium and incubated for 30 min on ice, washed three times, and finally resuspended in 1 ml FACS medium. Perforin and granzyme B was determined using intracellular staining procedure. Mouse splenocytes were used for staining CD4, CD8 T cells, and Tregs. Analysis was performed using FACSCalibur flow cytometer, and data analysis was performed using FlowJo software (FlowJo, Ashland, OR).

Ketogenic diet

Mice received a ketogenic diet (KD) (Bio-Serv, Frenchtown, NJ) as part of the dietary regimen of the study. This regimen contained a balance of mouse nutritional ingredients (supplemented as AIN-78; Bio-Serv) and contained 0.76% carbohydrate as compared with 44.2% in standard diet. Mice were placed in individual cages 1 wk before tumor implantation. Animals used for tumor studies were injected with 5 × 10^5 Pan02 or Pan02-LDHKD cells. All tumor-bearing mice were fed ad libitum for the first 48 h after tumor implantation and were then randomly assigned to one of two diet groups that received rodent chow as either normal diet (ND; n = 5) or KD (n = 10). The two groups were matched for body weight prior to the initiation of KD treatments. The study was continued for 21 d after tumor implantation until the tumor-bearing mice started decreasing their body weight and food intake.

Statistical analyses

Statistical analyses were performed using the Student t test. Statistical significance was determined for p values < 0.05.

Results

LDH-A–deficient Pan02 cells result in smaller tumor size

In the first series of experiments, we tested the in vivo effect of LDH knockdown on tumor growth, on innate immune function (by monitoring NK function), and on immune suppression (by monitoring MDSC frequency) in spleens and tumors of mice injected with pancreatic cell line Pan02 and LDH-A knocked-down Pan02 cells.

To investigate the effects of diminished LDH-A expression in Pan02 cells, we generated LDH-A–deficient stable Pan02 cell lines. Pan02 cells were infected with an empty shRNA vector control and three different LDH-A shRNA lentiviruses as described previously (4). Western blot analysis with anti–LDH-A Ab (Fig. 1A) demonstrated that cell lines 31 and 32, representing two separate shRNAs.
targeting two different sequences within the LDH-A mRNA have diminished LDH-A protein expression compared with the control cell line. Moreover, these LDH-A–deficient Pan02 cells also secrete lower levels of lactate in cell culture (Supplemental Fig. 3). We chose to carry out all subsequent LDH-A depletion studies using cell line 31.

To investigate the effect of lactate on tumor growth, we compared tumor growth in mice injected with the wild-type and LDH-A–deficient Pan02 cells. Pan02 and LDH-A–deficient Pan02 tumor cells were injected into C57BL/6 mice s.c. Measurement of tumor volume shows that LDH-A–deficient tumors were significantly smaller compared with tumors in control mice injected with Pan02 with empty shRNA (Fig. 1B, 1C).

Cytotoxic activity of NK cells from LDH-A–deficient Pan02 cells injected mice was significantly higher

To study the role of lactate on innate immune function, we investigated NK cell function during tumor growth in C57BL/6 mice challenged with s.c. injection with Pan02 cells as well as with Pan02–LDH-A shNA cells. Cytotoxic activity of spleen NK cells was determined against YAC target cells at E:T ratios of 1:1, 11:1, and 33:1. NK cells activity in spleens of LDH-A–deficient tumor was significantly higher against YAC-1 cell killing and was almost similar to wild-type C57BL/6 spleen NK cells in their ability to kill target cells (Fig. 1D). Although we did not determine NK cell function in the tumors, increased NK activity (Fig. 1E) makes it tempting to speculate that reduction in tumor size in LDH-A–deficient tumors could be due to increased NK cell function on a per cell basis.

LDH-A–deficient Pan02 cell–injected mice demonstrate lower MDSC frequency

To further explore the impact on the immune system of LDH-A knockdown in the tumor cell compartment, we hypothesized that MDSCs present in growing tumors are diminished by LDH-A knockdown in tumor cells. We carried out analysis of MDSC frequencies in LDH-A shRNA and control shRNA tumor–bearing mice. A significant reduction in CD11b+Gr-1+ cells was observed in the spleens of mouse transplanted with shRNA for LDH-A (Fig. 1F) as well as in tumors (Fig. 2). Importantly, MDSCs isolated from LDH-A–depleted tumor–bearing mice had lower suppressive effect on NK cell killing of YAC-1 target cells (Fig. 1G). For these experiments, MDSCs isolated from LDH-A shRNA and control shRNA Pan02 tumor–bearing mice were incubated with NK cells isolated from healthy animal spleens for 24 h. Results show that although MDSCs isolated from Pan02 tumors were suppressive of NK cytotoxicity against target cells, MDSCs from LDH-A–depleted tumors were much less suppressive.

Lactate inhibits cytotoxic activity of NK cells in vitro

Because MDSCs may influence NK cell number and activity, our data on the effect of lactate on MDSC help explain our findings of
impaired NK function in the tumor microenvironment. To determine the effect of lactate on NK cytolytic activity in vitro, we incubated purified human NK cells in the presence of lactate at different concentrations for 72 h and tested for cytotoxic activity against K562 cells. As shown in Fig. 3A–C, we observed a significant decrease in cytotoxic activity of NK cells treated with lactate. Similar data were observed with lactate treatment of mouse NK cells (data not shown). We also tested whether the decrease in cytotoxic activity was due to low pH of the culture medium or due to the presence of lactate by varying pH using HCl or lactate. Mouse spleen NK cells incubated in the pH range of 5–7 for 72 h were used for cytotoxicity determination against target cells. We observed decreased cytotoxic activity of NK cells both at HCl adjusted lower pH and at high lactate concentrations in vitro (Fig. 3D). A marked decrease in the killing of target cells was observed at two E:T ratios (10:1 and 50:1). There was a 20.8% decrease (E:T = 50) in killing of YAC-1 cells when the pH was lowered from 7.0 to 6.5, whereas a steep (67%) drop was observed when the pH was lowered from 6.8 to 6.0 (at E:T = 50:1). At lactate concentrations of 10 and 15 mM, a similar drop in cytotoxic activity was observed.

To address whether this decrease in cytotoxic activity was due to the decreased expression of perforin and granzyme B or due to the reduced release of lytic granules, we tested perforin and granzyme B expression in NK cells by intracellular FACS analysis of lactate-treated NK cells. FACS analysis demonstrated that lactate-treated cells expressed lower level of perforin and granzyme B compared with the untreated NK cells (Fig. 4). This was further confirmed by analysis of perforin and granzyme gene expression. RT-PCR anal-

![Image](http://www.jimmunol.org/)
ysis of lactate-treated NK cells showed that the perforin mRNA expression was drastically reduced in NK cells treated with 15 mM lactate for 72 h (Fig. 4), and there was also a decrease in granzyme B expression level. These findings suggest that the observed low NK cell activity is engendered by the lactate and the low pH of tumor microenvironment resulting in the reduced expression of perforin and granzyme B gene expression.

**Lactate inhibits the expression of activating receptor NKp46 in NK cells**

NK cells express activation receptors, such as NKp30, Nkp44, NKp46, and NKG2D, which participate in killing of tumor cells by recognition of receptor specific ligands. We asked whether lactate induced inhibition of NK cell activity is mediated by reduction in the expression of certain activation receptors. NK cells were stained with monoclonal Abs specific for natural cytotoxicity receptors anti-NKp30, Nkp44, NKp46, and NKG2D.

Lactate-treated NK cells demonstrated a significant downmodulation of the expression level of NKp46 compared with the untreated cells (Fig. 5). There was no significant change in the level of NKp30, Nkp44, and NKG2D. This reduced expression level of NKp46 suggested that the inhibitory effect of lactate on natural cytotoxicity receptors expression is gene-specific.

**Impact of lactate on immunosuppressive mouse bone marrow–MDSCs and MDSCs generated from human PBMCs in vitro**

We also carried out in vitro studies on the generation of MDSCs from mouse bone marrow cells using GM-CSF and IL-6 (BM-MDSCs) (Fig. 6) (23). Our results demonstrate that BM-MDSCs can be generated using these two factors, and these cells are immunosuppressive as determined by suppression of CD4 cell proliferation and CD8 cytotoxicity (Fig. 6B–D). In the presence of MDSCs (1:1 coculture of MDSCs and NK cells), NK cell function is reduced considerably (Fig. 6E).

Addition of lactate to the culture (GM-CSF, IL-6, and lactate) significantly increases the frequency of MDSCs in culture as determined by staining of cells with anti-CD11b and anti–Gr-1 Abs (Fig. 7, top panel). Moreover, these cells have significantly higher NK suppressive activity against target cells (Fig. 7, bottom panel).

**Treatment of human PBMCs with GM-CSF and IL-6 also results in the expansion of a population of cells that express low HLA-DR and are immunosuppressive (Supplemental Figs. 1, 2). These cells are mostly positive for the early myeloid marker CD33. The immunosuppressive capability of CD33^H-LA-DR^lo MDSCs was demonstrated by their inhibition of CD4^+ proliferation, decreased in CD8^+//IFN-γ—positive cells, and lowered lytic activity of NK cells following in vitro cocultures (Supplemental Fig. 2). Addition of lactate (15 mM) to PBMC cultures with GM-CSF and IL-6 results in an increase in the population of CD33^H-LA-DR^lo cells (Supplemental Fig. 1).**

**Effect of KD on Pan02 transplanted tumors**

We used a KD formulation (0.76% total carbohydrate) from BioServ. Mice fed KD had lower blood glucose levels (~15%, over a 1-mo observation period) (Fig. 7) and had smaller tumors than ND-fed mice (35.1% smaller). shRNA LDH-A Pan02 tumors were significantly smaller in size as compared with wild-type tumors whether on a ND or a KD without a change in body weight.
(results not shown) (Fig. 8). A decrease in tumor volume was also observed in GFP tumors in KD (35.1% decrease), although a more significant decrease was noted with LDH-A depletion alone or the combination of KD and LDH-A depletion (87.8% decrease), perhaps because KD is unlikely to impact lactate production as much as LDH-A knockdown would do. In contrast, the KD may exert nonlactate mediated antitumor effects.

To relate tumor growth to immune response, we evaluated several immune parameters in KD-fed mice carrying wild-type LDH-A and LDH-A knockdown Pan02 tumors. Our results (Fig. 9) clearly demonstrate that increased effector functions (as measured by increased frequencies of CD4 and CD8 T cells) as well as reduced suppressor function (as measured by Tregs and MDSC frequencies) accompanied KD and LDH-A knockdown. This suggests that lowered glucose levels combined with reduced lactate production (by lowering LDH-A gene expression) improved antitumor immune response, potentially lowering tumor burden.

Discussion
One of the almost universal characteristics of tumor cells is the acquisition of an altered metabolic phenotype known as the Warburg effect (16). The altered metabolic phenotype includes activation of aerobic glycolytic pathway, increased LDH activity, and increased lactate production (3). Tumor secreted lactate accumulates in the extracellular space. The increased production of lactate has been correlated to increased metastatic potential (24–26). In terms of lactate’s impact on immune cells, recent studies have shown that lactate suppresses the function of tumor-specific T cells, inhibits tumor specific cytotoxic T cell activity (27), and induces defect in signal transduction (28). Lactate/low pH also alters the Ag-presenting ability of dendritic cells (29), inhibits cytotoxic activity (30), and promotes IL-23/IL-17 proinflammatory pathway (31). These studies have suggested a role for lactate/extracellular pH on tumor growth through inhibition of immune surveillance. Our study extends these observations to MDSCs and NK cells, and to a more limited extent to Tregs, and provides further evidence that lactate acts to provide a more tolerant immune environment, thereby facilitating tumor growth. We also show that low pH in the tumor microenvironment may synergize with increased lactate to amplify lactate’s role in this process.

There is increasing evidence that a defect in immunosurveillance results in development and survival of tumor cells. It is now well established that tumor cells acquire altered metabolic phenotype that results in increased LDH activity, and increased lactate production results in lower pH of the tumor microenvironment (15). In this study, using the Pan02 pancreatic cancer model, we provide evidence that lactate from cancer cells inhibits NK cell activity in vivo and increased the size of the tumors. Furthermore, we observed that lactate inhibits the cytotoxic activity of NK cells by downmodulating the expression of NKP46 and also by interfering in the secretory pathways of NK-mediated cytotoxic activity. The suppressive effect of lactate is further amplified in a low pH tumor microenvironment to which NK cells are exposed.

We examined the role of lactate on NK cells in vivo by comparing NK activity of LDH-A–positive Pan02 tumor–bearing mice with that of the LDH-A–negative cancer cells in tumor-bearing mice. We observed a negative association between tumor growth and NK activity. NK activity was significantly reduced in LDH-A–positive tumor bearing mice and was high in mice with LDH-A–negative tumors. That the Pan02 tumor cells secreted lactate but not the LDH-A–negative tumors suggests that NK cell activity was affected by the lactate released by tumor cells.

A characteristic feature of the tumor microenvironment is acidosis that is attributed to the local decrease in pH. The increasing secretion increases the local concentration of lactate acidification of the region and reduces the pH (32). In several studies, the effects of decreased pH on the modulation of immune function have been reported. Our results show that lowering pH from pH 6.8 to 6.0 resulted in a significant decrease of NK cell activity. Lactate treatment of NK cells demonstrated that the production of perforin/granzyme B was significantly inhibited as observed by intracellular staining and expression studies. Because NK cells kill tumor cells via the secretory perforin/granzyme B pathway (33), it suggests that the lactate exerts its influence by interfering with the secretory pathway.

Although the fundamental assertion of MDSC accumulation during tumor progression is now well established, the molecular mechanism of MDSC recruitment, expansion, and activation are still not defined. A “two-signal” model (34) suggests that two separate signaling pathways regulate MDSC accumulation: a first step that is promoted by chronic inflammation involves factors including GM-CSF and IL-6 and signals via STAT3 and STAT5 and promotes proliferation of immature myeloid cells. The second step triggered by molecules such as IFN-γ, IL-1β, and TLR ligands uses STAT1 and NF-kB transcription factors and upregulates arginase and NO. Separately, Bronte and colleagues (35) have suggested that the family of C/EBP transcription factors along with STAT proteins regulate shift from normal to aberrant hematopoiesis, allowing differentiation into MDSCs, as well as their recruitment and activation. In proposing a novel mechanism of increased MDSC numbers, Ostrand-Rosenberg et al. (36) suggested that MDSC levels in developing tumors is maintained by resistance to apoptosis by FasL+ expressing activated T cells. This was mediated by inflammation and could result from factors like TGF-β secreted by tumors. We wished to extend these observa-
tions by studying the impact of tumor derived lactate on MDSC first in vivo and then in vitro.

We found that both the frequency of MDSC generation and the immunosuppressive capability of MDSCs on a per cell basis were markedly impacted in animals bearing LDH-A knockdown tumor cells versus wild-type controls. Recognizing that we could not strictly ascribe our in vivo results to diminished lactate production by tumor cells, we wondered if lactate could directly impact the generation of MDSCs in vitro. For this purpose, we used an in vitro system in which a cytokine mixture drives the generation of MDSC from mouse bone marrow cells. The results were clear-cut: lactate significantly increased MDSC frequency in this model and per cell NK suppressive activity against target cells (although intratumoral NK lytic function was not measured in this study). Similarly, addition of lactate to human PBMC cultures along with a cytokine mixture resulted in increased MDSCs.

Thus, our study introduces lactate as a tumor-derived factor that also promotes survival and proliferation of MDSCs in both mouse models and in human GM-CSF/IL-6 generated MDSCs. Although we have made some extrapolations from our in vitro studies, the fact that publications have demonstrated increased Warburg effect in glycolytic tumors and decreased immune response warrants a better understanding of this connection. We have also assumed that in vitro lowered levels of lactate by LDH-A KD reflects intratumoral lowering of lactate levels—an assumption that remains to be validated. As strategies designed to lower MDSC levels are being developed, more attention is being focused on potential MDSC targets to aim for. Ultimately, lowering lactate levels may help in more than one-way: lactate depletion will decrease immune suppressor function (by reducing frequency of MDSCs) and increase innate immune function (by protecting NK cytolytic function).

How can one decrease the concentration of extracellular lactate in the tumor microenvironment? There are no drugs currently available that inhibit LDH-A and thereby control lactate production. Drugs to block lactate export from tumor cells through monocarboxylic acid transporters are also not available. Alternatively, “manipulations” that decrease blood glucose will also affect lactate production. Dietary carbohydrate restriction, as enacted by a KD, is one approach to reducing circulating glucose, serum insulin and insulino-

**FIGURE 9.** Effect of KD and in combination with LDH-A depletion on immune parameters. Animals were injected with Pan02 cells expressing GFP or LDH-A–shRNA (5 x 10⁵) and fed either normal or ketogenic diet for 1 mo. Splenocytes isolated from mice belonging to each group were stained with various Abs (as described in Materials and Methods) and analyzed by FACS. Data represents one of two separate experiments.
like growth factor-I levels, and lactate production. Although such approaches do not reflect direct manipulation of lactate levels, they still provide therapeutic alternatives in the absence of a currently approved LDH-A inhibitor. Interestingly, such a diet has been found to reduce prostate tumor (37) and brain cancer (38) growth. In fact, a commercially available KD for children with epilepsy significantly reduces orthotopic growth, prolongs survival, and reduces vacuolization of a mouse astrocytoma (CT-2A) and a human glioma (U87) line (39). This has been generally attributed to lack of metabolic flexibility of the brain and the general dependence of brain tumor cells on glucose metabolism (38). Our results suggest an additional mechanism of improved tumor outcome with a KD in that it overcomes immune suppression and innate immunity. Animals fed with a KD alone or when combined with lactate depletion in tumors resulted in increased CD4 and CD8 counts and decreased Tregs and MDSC frequencies (Fig. 9).

Recently, Michalek et al. (40) showed distinct glycolytic and lipid oxidative metabolic programs regulate CD4 T cells such that effector T cells are susceptible to high lactate, but Tregs are insensitive. Our results showing improved T cell frequencies in LDH-A-depleted animals on a KD suggest possible improved survival of T cells was associated with lowered lactate. However, we observed drastic reduction in Tregs in both KD as well as LDH-A-depleted tumor-bearing mice. It can be speculated that lowered Treg frequencies may have resulted indirectly from improved NK function and/or lowered MDSC levels that have been shown to promote Tregs in animal models (41, 42).

In summary, our studies point to a major role for tumor-derived lactate in modulating MDSCs, NK cells, and Tregs.

Disclosures
The authors have no financial conflicts of interest.

References