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Tumor-Secreted Lactic Acid Promotes IL-23/IL-17 Proinflammatory Pathway[1]

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Tumor-Secreted Lactic Acid Promotes IL-23/IL-17 Proinflammatory Pathway[1]

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IL-23 is a proinflammatory cytokine consisting of a p19 subunit and a p40 subunit that is shared with IL-12. IL-23 is overexpressed in and around tumor tissues, where it induces local inflammation and promotes tumor development. Many tumor cells produce large amounts of lactic acid by altering their glucose metabolism. In this study, we show that lactic acid secreted by tumor cells enhances the transcription of *IL-23p19* and IL-23 production in monocytes/macrophages and in tumor-infiltrating immune cells that are stimulated with TLR2 and 4 ligands. DNA elements responsible for this enhancing activity of lactic acid were detected in a 2.7-kb 5′-flanking region of the human *IL-23p19* gene. The effect of lactic acid was strictly regulated by extracellular pH. Furthermore, by inducing IL-23 overproduction, lactic acid facilitated the Ag-dependent secretion of proinflammatory cytokine IL-17 but not IFN-γ by TLR ligand-stimulated mouse splenocytes. Interestingly, this effect was observed even in the absence of TLR ligand stimulation. These results suggest that rather than just being a terminal metabolite, lactic acid is a proinflammatory mediator that is secreted by tumor cells to activate the IL-23/IL-17 proinflammatory pathway but not the Th1 pathway. Targeting the lactic acid-induced proinflammatory response may be a useful approach for treating cancer. *The Journal of Immunology,* 2008, 180: 7175–7183.

Immune cells often infiltrate in and around many kinds of tumors. Initially, the immune system protects the host from cancer development and, indeed, the infiltration of NK cells in cancers is associated with a favorable prognosis (1). However, the infiltration of innate-immune cells such as macrophages correlates with a poor prognosis, which suggests that these cells may be directly involved in tumor development and metastasis by inducing angiogenesis and tissue remodeling. Moreover, many cancers often arise at sites of chronic inflammation caused by infections with microbes like *Helicobacter pylori* and hepatitis viruses. Noninfectious chronic inflammation such as that caused by asbestos is also associated with tumor development (1–3).

Infectious inflammation is associated with the secretion of several cytokines by innate immune cells in response to pathogen-associated molecular pattern stimuli (4). One of these cytokines is IL-23. IL-23 is a member of the proinflammatory heterodimeric cytokine family and consists of a p19 subunit and a p40 subunit that is shared with IL-12 (5, 6). Whereas IL-12 mainly induces the development of IFN-γ-producing Th1 cells, IL-23 is involved in maintaining the Th17 cells that are generated in response to IL-6 and TGF-β (7–10) and activates memory T cells ($CD44^{high}$ and $CD62L^{low}$) (11). IL-23 also induces the production of the proinflammatory cytokines IL-17 and IL-22 (11, 12). IL-23 is mainly produced by APCs, such as monocytes/macrophages and dendritic cells (DCs)[3] in response to stimulation with TLR2 and 4 ligands, such as peptidoglycan (PGN), LPS, and bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) (13–15).

Recently, the Oft group (16) showed that IL-23 but not IL-12 is overexpressed by macrophages and DCs in human and mouse tumor tissues. They also showed that IL-23 is an important molecule that leads to the up-regulation of IL-17 and the matrix metalloprotease 9, to an increase in angiogenesis, and to a reduction in $CD8^+$ T cell infiltration in the tumor microenvironment (16). Significantly, IL-23p19-deficient mice but not IL-12p35-deficient mice developed chemically induced tumors less frequently than wild-type mice, and tumors transplanted into IL-23 receptor-deficient mice showed reduced growth (16). Furthermore, it was shown that Th17 cells are gradually increased in the tumor microenvironment during tumor development (17) and that IL-17 upregulates the production of a variety of proinflammatory cytokines (18) and proangiogenic factors (19) to promote tumor development (20). Therefore, the activation of the IL-23/IL-17 pathway promotes the incidence and growth of tumors by inducing local inflammatory responses. However, it remains unclear what induces inflammation and IL-23 overproduction in the tumor microenvironment. Notably, peripheral blood cells from patients with lung cancer that had been cultured with TLR stimuli overproduced IL-12/23p40; this response was eliminated in most patients after tumor resection (21). On the basis of these studies, we speculated that the tumor itself produces a factor(s) that promotes IL-23 overproduction. To test our hypothesis, we searched for tumor-secreted

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[3] Abbreviations used in this paper: DC, dendritic cell; PGN, peptidoglycan; BCG, bacillus Calmette-Guérin; CWS, cell wall skeleton; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; siRNA, small interfering RNA; Pam_3CSK_4, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,*S*)propyl]-[*R*]-cysteinyl-[*S*]-seryl-[*S*]-lysl-[*S*]-lysl-[*S*]-lysl-[*S*]-lysine/^{3}HCl; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein/acetoxymethyl ester.

factors that might modulate the production of IL-23 by monocytes/macrophages stimulated with TLR ligands. In this study, we show that lactic acid secreted from tumor cells up-regulates TLR signal-dependent transcription of the IL-23p19 subunit in human and mouse monocytes/macrophages to enhance IL-23 secretion. Therefore, we predict that the lactic acid that is secreted by many tumor cells is a proinflammatory mediator that promotes tumor development.

Materials and Methods

Cell culture

The CADO-LC10 cell line, which was established from a human lung adenocarcinoma (22), was cultured in high glucose DMEM (4.5 mg/ml glucose; Sigma-Aldrich). Human PBMC, human monocytes, mouse splenocytes, the mouse macrophage-like cell line J774.1 (RIKEN cell bank), and the mouse melanoma cell line B16 were cultured in RPMI 1640 (Sigma-Aldrich). All media were supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C under a 5% CO_2 atmosphere.

Reagents and Abs

We purchased the TLR ligands: PGN of *Staphylococcus aureus* and LPS from Sigma-Aldrich and a synthetic tripalmitoylated lipopeptide, Pam_3CSK_4, from InvivoGen. BCG-CWS was prepared as described previously (23, 24). L-Lactic acid was purchased from Sigma-Aldrich and Wako Pure Chemical and sodium lactate was purchased from WAKO. We used anti-mouse IL-23p19 Ab (G23-8; eBioscience) to neutralize IL-23 activity and rat IgG1 Ab (eBRG1; eBioscience) as an isotype control; both Abs were used at a concentration of 10 μg/ml. We purchased recombinant GM-CSF from PeproTech and anti-GM-CSF receptor α-chain Ab (S-20) from Santa Cruz Biotechnology.

Conditioned medium analysis

Conditioned medium was prepared from CADO-LC10 cells that had been cultured for 3 days. The medium was passed through a 0.22-μm pore size filter (Millipore) and stored at −80°C. For some experiments, the conditioned medium was subjected to molecular size fractionation by using Microcon YM-10 centrifugal filter devices, which separates molecules at the nominal 10-kDa molecular mass cutoff (Millipore). The flow-through fraction (<10 kDa) was supplemented with 10% FCS and the retentate (>10 kDa) was diluted with serum-free culture medium to obtain the original volume. In other experiments, the conditioned medium was treated with 50 μg/ml proteinase K at 37°C for 1 h. To remove proteinase K, the medium was passed through a Microcon YM-10 and the flow-through fraction was used for further experimentation. The control media were subjected to the same treatments as the conditioned media.

To examine how glucose concentrations in the culture medium of CADO-LC10 affects the subsequent enhancing activity of the conditioned medium, we cultured confluent CADO-LC10 cells for 3 days in fresh glucose-free DMEM (Invitrogen) supplemented with 1 or 4.5 mg/ml glucose and 10% FCS. In separate experiments, we inhibited lactic acid production by culturing the CADO-LC10 cells in the presence or absence of 20 mM oxamic acid (Sigma-Aldrich) for 2 days. The pH of the conditioned media and the lactic acid-containing media neutralized with NaOH was measured with a pH meter (Beckman Coulter) at 37°C under a 5% CO_2 atmosphere. L-Lactic acid concentrations in the conditioned media were measured by using a Determiner LA Kit (Kyowamedics). In the enhancing activity analysis, the conditioned media of CADO-LC10 cells described above were added to cells with an equal volume of cell culture media (i.e., 50% of the medium consisted of conditioned medium).

Measurement of cytokines

Human PBMC were isolated from healthy volunteers by using Ficoll-Paque Plus (GE Healthcare Bio-Sciences). Human monocytes were purified from the PBMC by using the MACS system (Miltenyi Biotec) and monocyte isolation kit II (Miltenyi Biotec). To measure human IL-23 production, 1.5×10^5 monocytes were cultured in 96-well tissue culture plates in the presence or absence of lactic acid for 24 h and then treated with 10 μg/ml PGN for 24 h. The IL-23, IL-12/23p40, and IL-6 levels in the culture supernatants were measured by using human IL-23 (Bender MedSystems), human IL-12p40, and human IL-6 (BioSource International) ELISA kits, respectively. To measure mouse IL-23 production, 1.0×10^5 J744.1 cells were stimulated with lactic acid and 10 μg/ml PGN. The measurements of mouse IL-23 production were performed by using a mouse IL-23 ELISA kit (eBioscience).

Mouse splenocytes isolated from an OVA-specific, MHC class II-restricted $\alpha\beta$ TCR-transgenic mouse, OT-II (25), were cultured at 5×10^5 cells/well in 96-well tissue culture plates with 0.2 μg/ml $OVA_{323-339}$ peptides (Biosynth International) in the presence or absence of TLR ligands and lactic acid. After 4 days of incubation, the cytokines in the culture supernatants were measured by using IL-17A (R&D Systems) and IFN-γ (BioSource International) ELISA kits. These experiments using animals were conducted according to our institutional guidelines.

Real-time RT-PCR

Total RNA was isolated from cells by using the SV96 Total RNA Isolation System (Promega) according to the manufacturer's instructions, after which it was treated with RNase-free DNase I. cDNA was synthesized at 42°C for 50 min by using oligo$(dT)_{12-18}$ primers and SuperScript III reverse transcriptase in the presence of RNase inhibitor (Invitrogen). Diluted cDNA samples were mixed with a pair of primers derived from human *IL-23p19* or *β-actin* cDNA sequences and PCR was performed by using SYBR Green PCR master mix (Applied Biosystems) and an Applied Biosystems 7500HT sequence detection system. The following PCR primers were designed: for human *IL-23p19,* forward primer, 5′-AGTGTGGAG ATGGCTGTGACC-3′ and reverse primer, 5′-GCTGGGACTGAGGCT TGGAATCTG-3′; for human *IL-12/23p40*, forward primer, 5′-ATGCCGT TCACAAGCTCAAGTATG-3′ and reverse primer, 5′-GAACGCAGAAT GTCAGGGAGAAGT-3′; and for human *β-actin*, forward primer, 5′-TCA CCCACACTGTGCCCATCTACGA-3′ and reverse primer, 5′-CAGCGG AACCGCTCATTGCCAATGG-3′. Copy numbers were calculated from the amount of cDNA cloned into the pGEM-T easy vector (Promega) and normalized to *β-actin*. PCR for mouse *IL-23p19* and *β-actin* was performed by using the TaqMan PCR Core reagent kit, TaqMan probes, and primer sets of the TaqMan Gene Expression assay system (for *IL-23p19*, Mm00518984_m1 and for *β-actin*, Mm00607939_s1; Applied Biosystems). The relative expression of *IL-23p19* was normalized to that of *β-actin* and calculated by using the ΔΔCt method (16, 26).

Luciferase assay

A luciferase reporter plasmid for monitoring *IL-23p19* transcription was constructed as follows. A 6.7-kb fragment of the human *IL-23p19* gene from the *Sac*I site (−6654 bp) to the ATG initiation site that contained the 5′-flanking region and 5′-untranslated region of the gene was amplified by PCR using the RP11-348M3 clone (Research Genetics) as the template. The fragment was then subcloned between the *Sac*I and *Nco*I sites of the pGL3 promoter vector (Promega) to generate the p19–5′ luc vector. We also constructed a reporter gene plasmid containing the 2.7-kb 5′-flanking region of the human *IL-23p19* gene as follows. The p19–5′ luc plasmid was digested with *Sac*I and *Xma*I, treated with T4 DNA polymerase (Takara Bio), and self-ligated. We then inserted the *Xho*I and *Bam*H I fragments of the PGK promoter-driven neomycin resistance gene from pGKEM7-neoW into the *Sal*I and *Bam*H I sites of the plasmid to generate the p19–5′ 2.7k luc neo vector. The luciferase reporter plasmid for monitoring NF-κB activity was constructed as follows. The following synthetic oligonucleotides were annealed: 5′-TCGAGAAATGGGGACTTTCCGC TGGGGACTTTCCGCAAACCGC-3′ and 5′-GGTTTGCGGAAAGTCC CCAGCGGAAAGTCCCCATTTC-3′ (the underlined sequences indicate canonical NF-κB binding sites). The annealed oligonucleotides were then subcloned together with a *Sac*II/*Nco*I fragment of the minimal promoter sequence of the pNF-κB-Luc vector (Stratagene) between *Xho*I and *Nco*I sites of the pGL3 promoter vector containing the PGK promoter-driven neomycin resistance gene to generate the pGL3–2κB luc neo vector. J774.1 cells were then transfected with the p19-5′ luc vector and pGKEM7-neoW, the p19-5′ 2.7k luc neo vector, or pGL3–2κB luc neo vector by using FuGENE 6 (Roche) according to the manufacturer's instructions. The transfected cells were selected with 200 μg/ml G418. The cells were seeded in 96-well tissue culture plates at 1×10^5 cells/well and incubated with stimulants for 24 h as described above. After incubation, the cells were lysed with GloLysis buffer (Promega) and the luciferase activity was measured by using the Bright-Glo luciferase assay system (Promega) and a Mithras LB940 multimode reader (Berthold Technologies).

Small interfering RNA (siRNA)

We purchased *SMART*pool siRNA reagents for the human *LDHA* gene from Dharmacon and the Allstars negative control siRNA rhodamine from Qiagen. CADO-LC10 cells were transfected with siRNA (73 nM) by using XtremeGENE siRNA Transfection Reagent (Roche) according to the manufacturer's instructions. This transfection procedure was repeated on the second day to increase the RNA interference efficiency. On the fourth day,

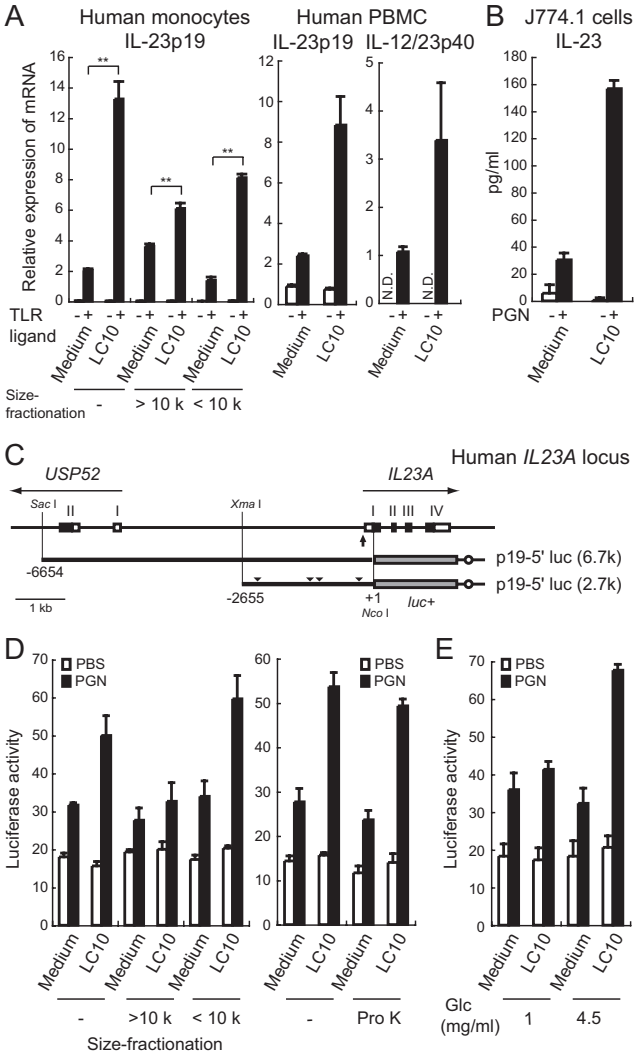

FIGURE 1. Tumor cell-conditioned medium enhances IL-23 expression in TLR ligand-stimulated monocytes/macrophages. *A*, Conditioned medium of the lung adenocarcinoma cell line CADO-LC10 enhances the TLR ligand-induced *IL-23p19* mRNA expression in human monocytes/macrophages (*left*). Human monocytes were stimulated with 10 μg/ml PGN (■) or PBS (□) in the presence (LC10) or absence (Medium) of 50% of the conditioned medium for 4 h. The conditioned medium was also fractionated according to molecular size, with the cutoff being at the nominal 10-kDa molecular mass (*left*). Human PBMC were stimulated with 5 μg/ml BCG-CWS in the presence or absence of the conditioned medium (*middle* and *right*). The relative expression of *IL-23p19* (*left* and *middle*) and *IL-12/23p40* (*right*) mRNA was measured by real-time RT-PCR and normalized to β-*actin* expression. N.D., Not detected. *B*, J774.1 cells were stimulated with 10 μg/ml PGN for 24 h in the presence or absence of the conditioned medium and the IL-23 that was secreted was measured by ELISA. *C*, Schematic representation of the human *IL23A* gene locus and two luciferase reporter genes carrying the 5′-flanking regions of the *IL-23p19* gene. The position from the translation start site (+1) is indicated in each construct. Shown are the untranslated regions (□), the coding region of *IL-23p19* and the *USP52* exon (■), the *luciferase* gene (▨), the SV40 late poly(A) signal (○), and the TATA box sequence (arrow). The arrowheads indicate putative NF-κB binding sites (–2311 to –2302, −1261 to –1252, −1049 to –1040, and –256 to –245) in the 2.7-kb 5′-flanking region. *D*, J774-p19-5′ luc cells were stimulated with 10 μg/ml PGN in the presence of the whole conditioned medium or the size-fractionated conditioned medium (*left*) or conditioned medium that had been treated with 50 μg/ml proteinase K (Pro K) for 1 h (*right*). Luciferase activity was then measured. *E*, Conditioned media were prepared from CADO-LC10 cells cultured in medium containing 1 or 4.5 mg/ml glucose (Glc). The control media (Medium) were subjected to the same treatments as the conditioned media (LC10). These media were added at 50% to determine the enhancing activity. The data represent mean values ± SD ($n = 3$). **, $p < 0.01$.

the cells were collected and plated at 1.4×10^6 cells/well in 12-well plates. After incubation for 24 h, we collected the conditioned medium and incubated the cells further for 24 h. The collected media were combined and used for further analysis. The expression of LDHA in siRNA-transfected CADO-LC10 cells was evaluated by performing semiquantitative RT-PCR and Western blotting. The following PCR primers were used: for human *LDHA,* forward primer, 5′-GCACGTCAGCAAGAGGGAGAAAG-3′ and reverse primer, 5′-AGGTAACGGAATCGGGCTGAATC-3′ and for human β-*actin*, forward primer, 5′-GCGGGAAATCGTGCGTGACATT-3′ and reverse primer, 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′. Anti-LDHA (N-14; Santa Cruz Biotechnology) and anti-β-tubulin (TUB 2.1; Sigma-Aldrich) Abs were used for Western blotting. The expression levels of proteins were calculated by using Image J software (http://rsb.info.nih.gov/ij/) and were normalized to those of β-tubulin expression.

Measurement of intracellular pH

Intracellular pH was measured by using BCECF/AM (Invitrogen) (27). Fluorescence at 535 nm with excitation at 485 and 388 nm was measured for 0.1 s every 30 s after adding lactic acid or hydrochloric acid (WAKO) by using an ARVO MX 1420 multilabel counter (PerkinElmer).

Purification of tumor-infiltrating immune cells

Tumor-infiltrating immune cells were purified from tumors formed by B16 melanoma cells in C57BL/6 mice by using CD45 MACS MicroBeads (Miltenyi Biotec) as described previously (28). The purified cells were stained with FITC-conjugated anti-CD45.2 (104; eBioscience), FITC-conjugated anti-CD11b (M1/70; eBioscience), and PE-conjugated anti-CD11c (HL3; BD Biosciences) and examined by using the FACSCalibur system (BD Biosciences).

Statistical analyses

In measurement of cytokines, real-time PCR, and luciferase assay, data represent mean values ± SD of triplicate stimulations. Differences between groups were analyzed for statistical significance by the Student *t* test. Representative data from at least two independent experiments are shown in the figures.

Results

Tumor cell-conditioned medium enhances TLR ligand-induced IL-23p19 *expression in monocytes/macrophages*

To determine whether tumor-secreted factors might modulate the TLR ligand-stimulated production of IL-23 by monocytes/macrophages, we first generated medium conditioned by the lung adenocarcinoma cell line CADO-LC10. Monocytes isolated from normal human PBMC were then stimulated with PGN in the presence (LC10) or absence (medium) of the conditioned medium. PGN induced *IL-23p19* transcription in human monocytes/macrophages and the transcription was significantly increased by the presence of the conditioned medium (Fig. 1*A*, *left*). The conditioned medium alone did not induce *IL-23p19* transcription in unstimulated monocytes/macrophages (Fig. 1*A*, *left*). Similar results were obtained for PBMC stimulated with BCG-CWS (Fig. 1*A*, *middle*).

We then size-fractionated the conditioned medium and the control medium into two fractions bearing the >10-kDa or <10-kDa molecules and performed the same experiment described above. The PGN-stimulated expression of *IL-23p19* in the monocytes/macrophages was more strongly enhanced by the lower molecular mass fraction (5.9-fold) than by the higher molecular mass fraction (1.7-fold) (Fig. 1*A*, *left*). Thus, it appears that the tumor cells secrete a small molecule that augments TLR ligand-induced IL-23p19 expression in monocytes/macrophages.

We also examined the effect of the conditioned medium on the transcriptional expression of *IL-12/23p40* in human PBMC. Although the unfractionated medium and the higher molecular mass

fraction clearly enhanced the transcription of *IL-12/23p40*, the lower molecular mass fraction did not (Fig. 1*A*, *right*, and data not shown). The enhancement of IL-12/23p40 expression was significantly inhibited by the anti-GM-CSF receptor α-chain Ab, suggesting that GM-CSF in the conditioned medium mainly enhanced IL-12/23p40 expression (data not shown).

The conditioned medium also enhanced the PGN-induced secretion of IL-23 by the mouse macrophage-like cell line J774.1 (Fig. 1*B*).

The conditioned medium enhances IL-23p19 *promoter activity*

To examine the effect of the conditioned medium on *IL-23p19* gene promoter activity, we performed a luciferase reporter assay using p19-5′ luc, which is a luciferase reporter plasmid containing the 6.7-kb 5′-flanking DNA region of the human *IL-23p19* gene (Fig. 1*C*). We first established several stable J774.1 cell lines that contained the reporter plasmid p19-5′ luc (J774-p19–5′ luc cells). When these cell lines were stimulated with PGN, *IL-23p19* promoter activity was increased (Fig. 1*D*, *left*). A further increase was observed when the cells were treated with PGN in the presence of the conditioned medium (Fig. 1*D*, *left*). Here again, the lower molecular mass fraction of the conditioned medium was proficient in stimulating *IL-23p19* promoter activity (Fig. 1*D*, *left*). We also generated additional luciferase reporter cells (J774-p19-5′ 2.7k luc) from p19-5′ 2.7k luc that contained only the 2.7-kb 5′-flanking region of the human *IL-23p19* gene (Fig. 1*C*). The reporter activity of this plasmid in J774.1 cells was increased by TLR stimuli (BCG-CWS and PGN) and this effect was further increased by the conditioned medium (data not shown).

Thus, the conditioned medium augments the stimulatory effect of TLR ligands on *IL-23p19* promoter activity.

Characterization of the small molecule in the conditioned medium responsible for the increase in TLR-stimulated IL-23p19 *promoter activity*

To identify the small molecule in the conditioned medium, we subjected the conditioned medium to further molecular size fractionation that separated the molecules at the nominal molecular mass of 500 Da. The <500-Da fraction, but not the >500-Da fraction, increased the *IL-23p19* promoter activity in TLR ligand-stimulated J774-p19-5′ luc (data not shown). The enhancing activity of the entire conditioned medium was not diminished by treatment with proteinase K (Fig. 1*D*, *right*) or heat treatment at 90°C for 10 min (data not shown). Thus, it appeared that the tumor-secreted small molecule we were interested in would not be a protein or peptide.

Interestingly, we found that the enhancing activity of the conditioned medium varied depending on whether the medium was obtained from tumor cell cultures in DMEM (2.4 ± 0.21), RPMI 1640 (1.6 ± 0.06), or MEM (1.0 ± 0.07). DMEM was better than RPMI 1640 and MEM had no enhancing effect at all. DMEM, RPMI 1640, and MEM contain different concentrations of glucose, namely, 4.5, 2, and 1 mg/ml, respectively. To investigate whether the glucose concentration does indeed affect the subsequent enhancing activity of the conditioned medium, we generated conditioned media from CADO-LC10 cells cultured for 3 days in DMEM supplemented with low (1 mg/ml) or high (4.5 mg/ml) concentrations of glucose. The conditioned medium prepared in high glucose DMEM enhanced the TLR ligand-stimulated *IL-23p19* promoter activity in J774-p19-5′ luc cells, unlike the conditioned medium prepared in low glucose DMEM (Fig. 1*E*). A high concentration of glucose alone had no effect (Fig. 1*E*). Glucose was metabolized to pyruvic acid, which is catalyzed by lactate dehydrogenase (LDH) to generate lactic acid. Because tumor cells show up-regulated glycolysis, even under aerobic conditions, they generally secrete large amounts of lactic acid into the culture medium (29–31). Indeed, the conditioned media of CADO-LC10 cells cultured in 4.5 or 1 mg/ml glucose were found to contain 26.4 and 7.8 mM lactic acid, respectively (final concentrations of 13.2 and 3.9 mM in our assay). Therefore, we hypothesized that lactic acid may be the most likely candidate tumor cell-secreted factor that enhances TLR ligand-stimulated *IL-23p19* transcription.

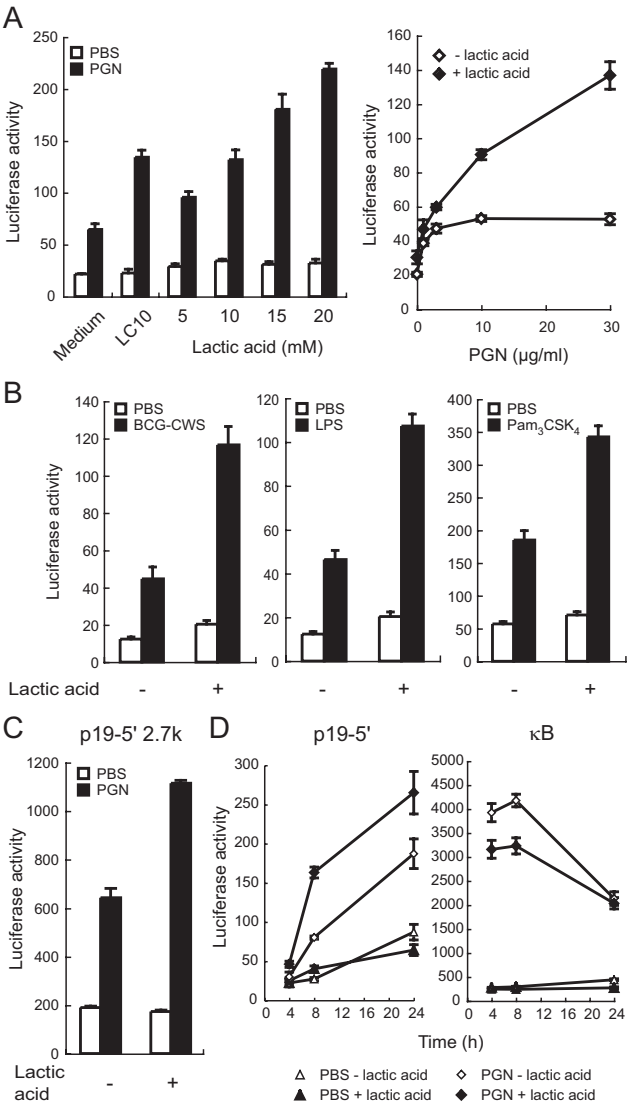

FIGURE 2. Lactic acid enhances TLR ligand-induced human *IL-23p19* promoter activity. *A*, J774-p19-5′ luc cells were stimulated with 10 μg/ml PGN in the presence of 0 (Medium), 5, 10, 15, or 20 mM lactic acid (*left*) or with 0, 2.5, 5, 10, or 30 μg/ml PGN in the presence of 15 mM lactic acid (*right*). *B*, J774-p19-5′ luc cells were stimulated with 10 μg/ml BCG-CWS (*left*), 100 ng/ml LPS (*middle*), or 100 ng/ml Pam_3CSK_4 (*right*) in the presence or absence of 15 mM lactic acid. *C*, J774-p19-5′ 2.7k luc cells were stimulated with 10 μg/ml PGN in the presence or absence of 15 mM lactic acid. *D*, J774-p19-5′ luc cells (*left*) and J774-2κB luc cells (*right*) were stimulated with 10 μg/ml PGN in the presence or absence of 15 mM lactic acid. After incubation for 4, 8, and 24 h, the cells were lysed and their luciferase activities were measured. The data represent mean values ± SD ($n = 3$).

Lactic acid in the conditioned medium enhances the IL-23p19 *gene expression induced by TLR ligands*

To examine whether lactic acid indeed enhances the *IL-23p19* gene expression induced by TLR ligands, J774-p19-5′ luc cells were stimulated with PGN in the presence of lactic acid. The luciferase activity in J774-p19-5′ luc cells was increased by lactic acid in a dose-dependent manner (Fig. 2*A*, *left*) and this enhancing

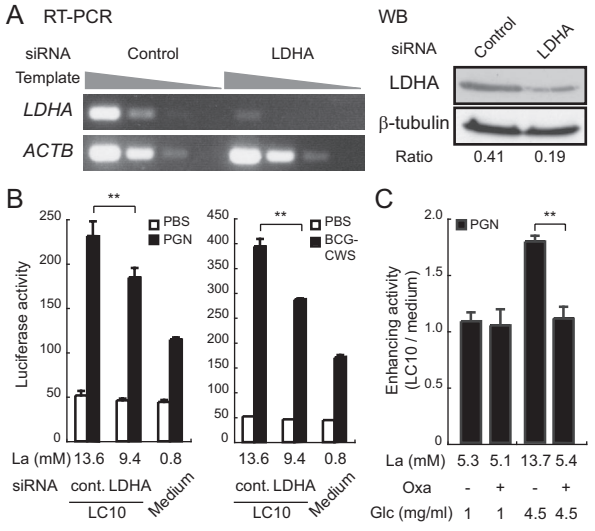

FIGURE 3. Inhibiting the production of lactic acid blocks the enhancing activity of the conditioned medium. *A*, CADO-LC10 cells were transfected with siRNA for the human *LDHA* gene (LDHA) or negative control siRNA (Control). The inhibitory effect of *LDHA* siRNA was assessed by semi-quantitative RT-PCR (*left*) and Western blotting (WB; *right*). cDNA templates prepared from siRNA-transfected CADO-LC10 cells were serially diluted (1, 0.1, 0.01, and 0.001) and amplified with *LDHA* and *β-actin* (*ACTB*) primers. Cell lysates of siRNA-transfected CADO-LC10 cells were immunoblotted with anti-LDHA or anti-β-tubulin Abs. The normalized protein expression levels of LDHA are shown below the picture. *B*, J774-p19-5′ luc cells were cultured with the conditioned media prepared from siRNA-transfected CADO-LC10 cells in the presence of 10 μg/ml PGN (*left*) or 10 μg/ml BCG-CWS (*right*) for 24 h. *C*, Alternatively, CADO-LC10 cells were incubated in low or high glucose DMEM with 20 mM oxamic acid (Oxa), an inhibitor of LDH. J774-p19-5′ luc cells were cultured with these conditioned media in the presence of 10 μg/ml PGN for 24 h. The data indicate the relative enhancing activity of CADO-LC10 conditioned medium over that of the average of control medium. The concentration of lactic acid (La) in each conditioned medium was measured in *B* and *C*. The data represent mean values ± SD ($n = 3$). **, $p < 0.01$.

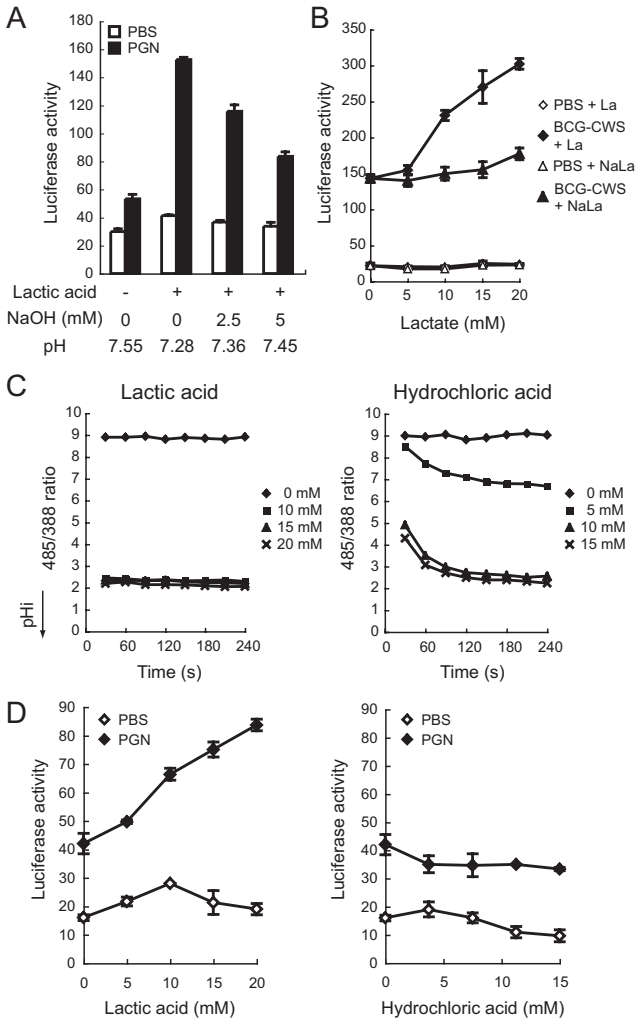

FIGURE 4. The enhancing activity of lactic acid is dependent on the pH of the medium. *A*, pH of the medium containing 15 mM lactic acid (pH 7.28) was adjusted to 7.36 and 7.45 with 2.5 and 5 mM NaOH, respectively. J774-p19-5′ luc cells were stimulated with 10 μg/ml PGN in the presence of these media. *B*, J774-p19-5′ luc cells were stimulated with 10 μg/ml BCG-CWS in the presence of 0, 5, 10, 15, or 20 mM lactic acid (La) or sodium lactate (NaLa). *C*, BCECF-loaded J774-p19-5′ luc cells were incubated with 0, 10, 15, or 20 mM lactic acid (*left*) or 0, 5, 10, or 15 mM hydrochloric acid (*right*). Intracellular pH (pHi) was detected by examining the fluorescent pH dye BCECF every 30 s as described in *Materials and Methods*. The ratio of fluorescence at 535 nm after excitation at 485 nm/388 nm was calculated. *D*, J774-p19-5′ luc cells were stimulated with 10 μg/ml PGN in the presence of various concentrations of lactic acid (*left*) or hydrochloric acid (*right*). After a 24-h incubation, the cells were lysed and the luciferase activity was measured. The data represent mean values ± SD ($n = 3$).

effect was observed at all concentrations of PGN (Fig. 2*A*, *right*). However, lactic acid alone had no detectable effect in this assay (Fig. 2*A*, *left*, □). Lactic acid at 10 mM was as effective as the conditioned medium, which contained 27.3 ± 1.09 mM lactic acid (final concentration of 13.7 ± 0.55 mM in our assay; Fig. 2*A*, *left*). The enhancing activity of lactic acid was also observed with cells stimulated with not only PGN but also other TLR ligands, namely, BCG-CWS, LPS, and Pam_3CSK_4 (Fig. 2*B*).

We next examined the region in the *IL-23p19* promoter that was responsive to lactic acid by using J774-p19-5′ 2.7k luc cells. DNA elements responsible for this enhancing activity of lactic acid like the conditioned medium were detected in a 2.7-kb 5′-flanking region of the human *IL-23p19* gene (Fig. 2*C*). Searching of a TRANSFAC database (32) with the TFSEARCH program version 1.3 (http://www.rwcp.or.jp/papia/) revealed four predicted NF-κB binding sites in a 2.7-kb 5′-flanking region (Fig. 1*B*, arrowheads). However, when we constructed J774.1 cells transfected with a luciferase reporter construct carrying canonical NF-κB binding sites and tested their responses to TLR ligands in the presence or absence of lactic acid, we did not observe any enhancing activity (Fig. 2*D*).

To test whether the lactic acid secreted from the tumor cells is indeed responsible for augmenting the TLR ligand-induced *IL-23p19* promoter activity, we inhibited the production of lactic acid from CADO-LC10 cells with *LDHA*-specific siRNA. The expression of *LDHA* mRNA (Fig. 3*A*, *left*) and protein (Fig. 3*A*, *right*) in *LDHA* siRNA-transfected cells was reduced to <10 and 50% of that of control siRNA-transfected cells, respectively. We examined enhancing activity using these conditioned media (Fig. 3*B*). Alternatively, we inhibited the LDH activity by adding oxamic acid, an inhibitor of LDH (Fig. 3*C*). Both treatments significantly reduced the concentration of lactic acid in the conditioned medium and this was matched with a decreased ability of the conditioned medium to enhance TLR ligand-stimulated *IL-23p19* promoter activity (Fig. 3, *B* and *C*). Thus, the lactic acid secreted by the tumor cells was largely responsible for the enhancing effect of the conditioned medium.

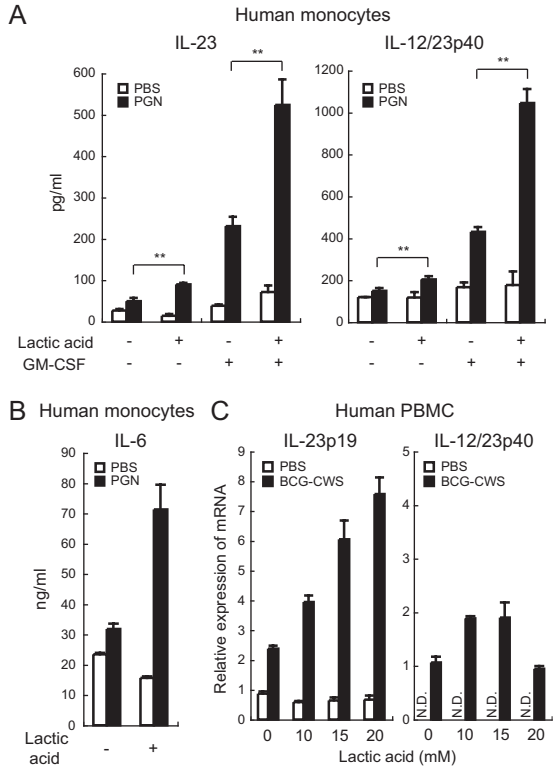

FIGURE 5. Lactic acid enhances proinflammatory cytokine production by TLR ligand-stimulated human primary monocytes/macrophages. *A*, Human monocytes were cultured for 24 h in the presence or absence of 15 mM lactic acid with or without 5 unit/ml GM-CSF. The cells were then stimulated with 10 μg/ml PGN for 24 h and the supernatants were collected. The IL-23 heterodimer and IL-12/23p40 concentrations were measured by ELISA. *B*, Human monocytes were cultured for 24 h in the presence or absence of 15 mM lactic acid. The cells were then stimulated with 10 μg/ml PGN for 24 h and the IL-6 concentrations of the supernatants were measured by ELISA. *C*, Human PBMC were stimulated with 5 μg/ml BCG-CWS in the presence of 0, 10, 15, or 20 mM lactic acid. After 4 h, *IL-23p19* (*left*) and *IL-12/23p40* (*right*) transcripts were measured by real-time RT-PCR. Relative expression of these transcripts was normalized to *β-actin* expression. N.D., Not detected. The data represent mean values ± SD ($n = 3$). **, $p < 0.01$.

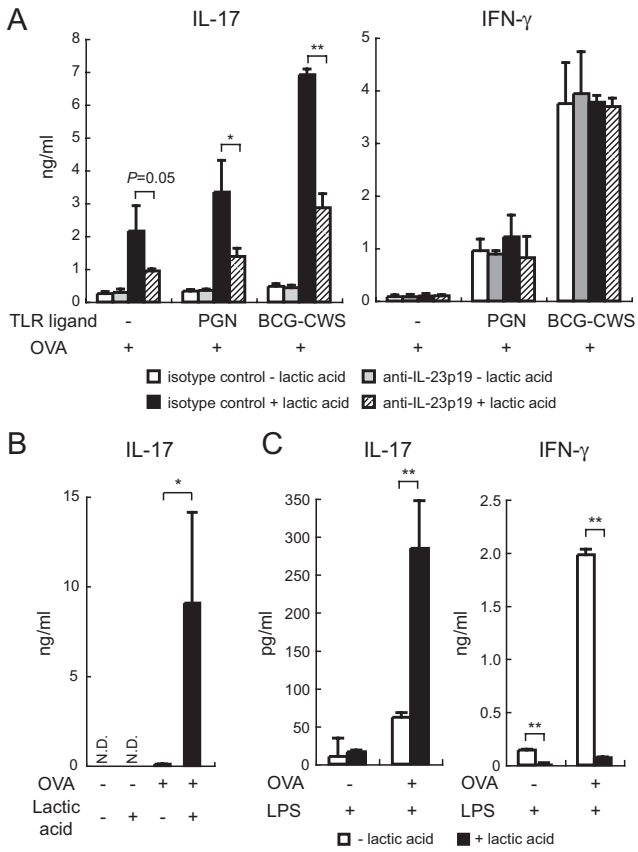

FIGURE 6. The IL-17 production by Ag-stimulated mouse splenocytes is augmented by lactic acid. *A*, Splenocytes from OT-II mice were stimulated with (■ and ▨) or without (□ and ▩) 15 mM lactic acid and PBS, 10 μg/ml PGN, or 1 μg/ml BCG-CWS. Anti-IL-23p19 Ab (▩ and ▨) or isotype control rat IgG1 (□ and ■) was also present, along with 0.2 μg/ml $OVA_{323–339}$ peptide. *B*, Splenocytes from OT-II mice were stimulated with or without 0.2 μg/ml OVA peptide and 15 mM lactic acid. *C*, Splenocytes from OT-II mice were stimulated with 100 ng/ml LPS and 0.2 μg/ml OVA peptide in the presence (■) or absence (□) of 15 mM lactic acid. The supernatants were collected after 4 days and the IL-17 and IFN-γ concentrations were measured by ELISA. N.D., Not detected. The data represent mean values ± SD ($n = 3$). *, $p < 0.05$ and **, $p < 0.01$.

Pathway involved in the enhancing activity of lactic acid

Lactate anions are transported together with protons into cells by monocarboxylate transporters (MCTs) in a pH-dependent manner (33). Therefore, to examine whether the enhancing activity of lactic acid depends on the pH of the medium, we incubated J774-p19-5′ luc cells with 15 mM lactic acid in the presence of NaOH, which altered the pH of the medium (Fig. 4*A*). The enhancing activity of lactic acid was decreased in a NaOH dose-dependent manner. Furthermore, sodium lactate did not show any enhancing activity (Fig. 4*B*). The intracellular pH of the cells decreased rapidly upon incubation with lactic acid (Fig. 4*C*, *left*), suggesting that protons were transported into the cells along with lactic acid. However, in contrast to lactic acid, hydrochloric acid, which also decreased the intracellular pH, had no enhancing effect (Fig. 4, *C*, *right*, and *D*). These results suggest that only the lactate anion in its transportable state, but not the neutralized lactate anion or proton, was responsible for the enhancing activity.

Lactic acid enhances secretion of proinflammatory cytokines by human monocytes/macrophages

The IL-23p19 subunit is covalently linked to the IL-12/23p40 subunit to form an IL-23 heterodimer. The heterodimer is secreted by monocytes/macrophages/DCs stimulated with TLR ligands (5, 34). To investigate whether lactic acid enhances IL-23 secretion, human monocytes were stimulated with PGN in the presence of lactic acid for 24 h (Fig. 5*A*, *left*). The secretion of IL-23 was enhanced 1.8-fold by lactic acid. When GM-CSF was only present, IL-23 secretion was elevated 4.7-fold. When lactic acid and GM-CSF were both present, IL-23 secretion was enhanced 10.6-fold. Similarly, the PGN-induced secretion of IL-12/23p40 was slightly increased by lactic acid but synergistically enhanced by the further addition of GM-CSF (Fig. 5*A*, *right*). These results indicate that lactic acid and GM-CSF cooperate to stimulate TLR ligand-induced IL-23 and IL-12/23p40 production by human monocytes/macrophages. Furthermore, lactic acid also enhanced the secretion of another proinflammatory cytokine, IL-6, from human monocytes/macrophages stimulated with the TLR ligand (Fig. 5*B*).

We also observed that BCG-CWS increased *IL-23p19* transcription in PBMC, and like the conditioned medium (Fig. 1*A*, *middle*), lactic acid significantly enhanced this response in a dose-dependent manner (Fig. 5*C*, *left*). Conversely, lactic acid did not increase *IL-12/23p40* transcription (Fig. 5*C*, *right*), suggesting that lactic acid specifically acts on *IL-23p19* transcription.

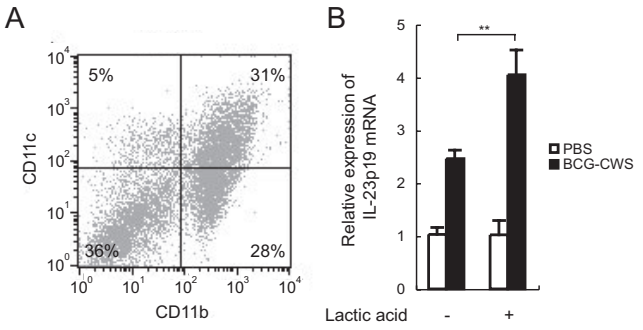

FIGURE 7. Lactic acid enhances the expression of *IL-23p19* mRNA in tumor-infiltrating immune cells. *A*, Tumor-infiltrating CD45-positive cells were purified from tumors formed by B16 mouse melanoma cells and stained for CD11b and CD11c. The stained cells were analyzed by FACS. *B*, One hundred thousand tumor-infiltrating immune cells (CD45-positive cells, 86%) were stimulated with 10 μg/ml BCG-CWS in the presence or absence of 15 mM lactic acid. After 4 h, the expression of *IL-23p19* and *β-actin* transcripts was measured by TaqMan RT-PCR. Relative expression was normalized to that of *β-actin*. The data represent mean values ± SD ($n = 3$). **, $p < 0.01$.

Lactic acid enhances the IL-23/IL-17 pathway

Because lactic acid further enhanced TLR-stimulated IL-23 production, we predicted that lactic acid should enhance IL-23 production from APCs stimulated with TLR ligand, leading to increased IL-17 production by IL-17-producing T cells. We then used OT-II-transgenic mice, which have $OVA_{323–339}$ peptide-specific T cells (25). In the presence of the OVA peptide, we examined the effect of lactic acid on the IL-17 production of OT-II mouse splenocytes that had been stimulated with the TLR ligand (PGN or BCG-CWS) for 4 days. Lactic acid remarkably enhanced the secretion of IL-17 induced by PGN and BCG-CWS (Fig. 6*A*, *left*, □ and ■). Anti-IL-23p19 Ab significantly inhibited the lactic acid-induced enhancement of IL-17 production (Fig. 6*A*, *left*, ■ and ▨). This result indicates that lactic acid indeed stimulates APCs to produce IL-23, which then drives peptide-activated T cells to produce IL-17. Interestingly, in the presence of the peptide, lactic acid induced IL-17 production even without the TLR ligand and this effect was inhibited by the anti-IL-23p19 Ab (Fig. 6, *A*, *left*, and *B*). The peptide or lactic acid alone rarely induced IL-17 production by splenocytes (Fig. 6*B*).

We also examined the effect of lactic acid on the secretion of IFN-γ (Fig. 6*A*, *right*). When the TLR ligand was present along with the peptide, the splenocytes produced IFN-γ. However, lactic acid did not potentiate IFN-γ production under these conditions (Fig. 6*A*, *right*, □ and ■). In contrast to IL-17 production, lactic acid did not induce IFN-γ production when the TLR ligand was absent (Fig. 6*A*, *right*). Notably, the effect of lactic acid on IFN-γ production was different when the TLR ligand was replaced by LPS. Although lactic acid potentiated IL-17 production by splenocytes irrespective of the ligand used for stimulation (Fig. 6*C*, *left*), it severely inhibited IFN-γ production (Fig. 6*C*, *right*).

Lactic acid enhances the expression of IL-23p19 *mRNA in tumor-infiltrating immune cells*

Many immune cells infiltrate the tumor microenvironment and induce local inflammation in and around the tumor. We examined whether tumor-infiltrating immune cells actually have the ability to express a high amount of IL-23 in response to lactic acid. We purified CD45-positive cells (CD11b- and/or CD11c-positive cells, 64%) that had infiltrated the tumors formed by B16 melanoma cells (Fig. 7*A*). These cells were stimulated with BCG-CWS and lactic acid for 4 h. The level of *IL-23p19* transcripts significantly increased in the presence of lactic acid (Fig. 7*B*). This result suggests that lactic acid also enhances the production of IL-23 in tumor-infiltrating immune cells, probably in macrophages and/or DCs.

Discussion

Tumor-secreted lactic acid enhances TLR ligand-dependent IL-23 expression in monocytes/macrophages

In this study, we sought to elucidate the mechanisms that induce immune cells in the tumor microenvironment to produce proinflammatory cytokines such as IL-23. We also asked, how does the tumor induce chronic inflammation without being attacked by the immune cells? We found that many tumor cell lines secrete factors that enhance TLR ligand-stimulated *IL-23p19* transcription and IL-23 secretion by human and mouse monocytes/macrophages (Fig. 1*A* and data not shown). We clarified that the main factor responsible for this effect is a molecule smaller than 500 Da that is protease resistant, heat stable, and only secreted from tumor cell lines when they are cultured in high glucose medium (Fig. 1, *D* and *E*, and data not shown). We then discovered that this molecule is lactic acid (Figs. 2–5). Lactic acid increased the production of proinflammatory cytokines, IL-23, and IL-6 from human monocytes (Fig. 5).

GM-CSF also enhanced TLR ligand-induced IL-23 and IL-12/23p40 production (Fig. 5). GM-CSF is secreted by many tumor cells as well as CADO-LC10 cells (35) and increases the expression of TLR2 (36). We suggest that tumor-secreted GM-CSF and lactic acid cooperate to increase IL-23 production following TLR2 ligand stimulation.

Predicted lactic acid signaling pathway

Although lactic acid is completely ionized, even under neutralized conditions (37), the neutralization of lactic acid suppressed its ability to enhance *IL-23p19* promoter activity in a pH-dependent manner (Fig. 4*A*). Moreover, neither sodium lactate nor acidification with hydrochloric acid enhanced *IL-23p19* transcription (Fig. 4, *B* and *D*). Lactate anions are transported together with protons through MCTs and a pH gradient is necessary for effective transport of lactic acid into cells (33). Therefore, we predict that lactic acid transported into monocytes/macrophages by MCTs may be recognized by an intracellular sensor that, along with the TLR signal, activates the *IL-23p19* promoter.

We found that DNA elements responsible for the enhancing activity of lactic acid are localized in the 2.7-kb 5′-flanking region of the human *IL-23p19* gene (Fig. 2*C*). We found four predicted NF-κB binding sites in this region (Fig. 1*C*). Although NF-κB signaling plays an important role in the TLR signaling pathway (4), lactic acid did not enhance the luciferase activity of a reporter vector carrying canonical NF-κB-binding elements (Fig. 2*D*). Therefore, the lactic acid signal pathway may be independent of the NF-κB pathway.

Lactic acid is not only a terminal metabolite of glycolysis but also a proinflammatory mediator

In most normal mammalian cells, the metabolism from glucose to lactate is inhibited by the presence of oxygen, which leads to the oxidization of pyruvate to CO_2 and H_2O in the mitochondria. However, in cancer cells, glycolysis is up-regulated, even in aerobic conditions, a phenomenon known as the "Warburg effect" or "aerobic glycolysis" (29, 30). This results in the production of large amounts of lactic acid and the accumulation of lactic acid in the microenvironment of many cancer cell types (31). High concentrations of lactate in solid tumors such as cervical carcinoma

(10.0 ± 2.9 μmol/g or >8.3 μmol/g) and head and neck cancer (>7.1 μmol/g) are associated with higher frequencies of distant metastasis (31, 38–40). Moreover, low lactate tumors in primary lesions are associated with longer disease-free survival than high lactate tumors. In addition, when the LDHA activity that induces the metabolism of pyruvate to lactate is suppressed, the tumorigenicity is severely diminished (41).

In this study, we showed that lactic acid enhances the expression of IL-23p19 in tumor-infiltrating immune cells activated by TLR stimuli (Fig. 7) and in splenocytes in the presence of Ag stimulus (data not shown) and induces the Ag- and IL-23-dependent secretion of IL-17 from splenocytes (Fig. 6). Significantly, we detected this IL-23-dependent enhancing activity even when TLR ligands were absent. Since lactic acid alone did not induce IL-23 production in the absence of Ag stimulation, we predicted that an interaction between APCs and T cells mediated by Ag and lactic acid causes IL-23-dependent IL-17 production in the absence of the TLR ligand. We observed that lactic acid severely inhibited IFN-γ production by Ag-stimulated splenocytes treated with LPS (Fig. 6*C*, *right*). The Kreutz group (42) reported that lactic acid represses the secretion of IL-12p70 by LPS-stimulated DCs. Therefore, lactic acid may inhibit IFN-γ production by suppressing the IL-12p70 production of monocytes/macrophages. In contrast, lactic acid did not affect IFN-γ production of Ag-stimulated splenocytes treated with PGN or BCG-CWS (Fig. 6*A*, *right*), because it is assumed that PGN and BCG-CWS do not stimulate IL-12p70 production by monocytes/macrophages (14). These results suggest that upon antigenic stimulation of T cells, lactic acid mediates the activation of the IL-23/IL-17 pathway rather than the induction of IFN-γ-producing Th1 cells. We predict that lactic acid derived from tumor cells may induce inflammation but not the infiltration of CTLs in the tumor microenvironment, even in the absence of TLR ligand stimuli from microbial infections, and that inflammation promotes angiogenesis and tumor development.

The Kreutz group (42, 43) also reported that lactic acid downregulates the cytokine production and proliferation of CTLs. Furthermore, the Murray group (44) showed that MCT1 inhibitors, which inhibit the transport of lactic acid, suppress T lymphocyte proliferation. These groups suggested that a high concentration of extracellular lactic acid or inhibition of lactic acid excretion might cause intracellular accumulation of lactic acid with consequent disturbance of T cell metabolism and function. In contrast, our results indicate that lactic acid up-regulates *IL-23p19* transcription in monocytes/macrophages.

In conclusion, our results show that lactic acid acts as a novel tumor-derived factor that strongly induces the IL-23/IL-17 proinflammatory pathway without inducing Th1 responses. Thus, the production and excretion of lactic acid appears to be not only essential for the proliferation of tumor cells with up-regulated glycolysis, but also for the induction of inflammation in the tumor microenvironment, which promotes tumor progression. Therefore, lactic acid and the lactic acid/IL-23 signal pathway may be an attractive target for treating tumors.

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Disclosures

The authors have no financial conflict of interest.

References

1. de Visser, K. E., A. Eichten, and L. M. Coussens. 2006. Paradoxical roles of the immune system during cancer development. *Nat. Rev. Cancer* 6: 24–37.
2. Karin, M., T. Lawrence, and V. Nizet. 2006. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 124: 823–835.
3. Balkwill, F., K. A. Charles, and A. Mantovani. 2005. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 7: 211–217.
4. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783–801.
5. Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, et al. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715–725.
6. Hunter, C. A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat. Rev. Immunol.* 5: 521–531.
7. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGF-β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.
8. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
9. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-β induces development of the T_H17 lineage. *Nature* 441: 231–234.
10. Weaver, C. T., L. E. Harrington, P. R. Mangan, M. Gavrieli, and K. M. Murphy. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 24: 677–688.
11. Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278: 1910–1914.
12. Zheng, Y., D. M. Danilenko, P. Valdez, I. Kasman, J. Eastham-Anderson, J. Wu, and W. Ouyang. 2007. Interleukin-22, a T_H17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 445: 648–651.
13. Begum, N. A., K. Ishii, M. Kurita-Taniguchi, M. Tanabe, M. Kobayashi, Y. Moriwaki, M. Matsumoto, Y. Fukumori, I. Azuma, K. Toyoshima, and T. Seya. 2004. *Mycobacterium bovis* BCG cell wall-specific differentially expressed genes identified by differential display and cDNA subtraction in human macrophages. *Infect. Immun.* 72: 937–948.
14. McKenzie, B. S., R. A. Kastelein, and D. J. Cua. 2006. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol.* 27: 17–23.
15. Tsuji, S., M. Matsumoto, O. Takeuchi, S. Akira, I. Azuma, A. Hayashi, K. Toyoshima, and T. Seya. 2000. Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guérin: involvement of toll-like receptors. *Infect. Immun.* 68: 6883–6890.
16. Langowski, J. L., X. Zhang, L. Wu, J. D. Mattson, T. Chen, K. Smith, B. Basham, T. McClanahan, R. A. Kastelein, and M. Oft. 2006. IL-23 promotes tumour incidence and growth. *Nature* 442: 461–465.
17. Kryczek, I., S. Wei, L. Zou, S. Altuwaijri, W. Szeliga, J. Kolls, A. Chang, and W. Zou. 2007. Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J. Immunol.* 178: 6730–6733.
18. Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* 21: 467–476.
19. Takahashi, H., M. Numasaki, M. T. Lotze, and H. Sasaki. 2005. Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells. *Immunol. Lett.* 98: 189–193.
20. Numasaki, M., J. Fukushi, M. Ono, S. K. Narula, P. J. Zavodny, T. Kudo, P. D. Robbins, H. Tahara, and M. T. Lotze. 2003. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 101: 2620–2627.
21. Matsumoto, M., T. Seya, S. Kikkawa, S. Tsuji, K. Shida, M. Nomura, M. Kurita-Taniguchi, H. Ohigashi, H. Yokouchi, K. Takami, et al. 2001. Interferon γ-producing ability in blood lymphocytes of patients with lung cancer through activation of the innate immune system by BCG cell wall skeleton. *Int. Immunopharmacol.* 1: 1559–1569.
22. Sakuma, T., K. Kodama, T. Hara, Y. Eshita, N. Shibata, M. Matsumoto, T. Seya, and Y. Mori. 1993. Levels of complement regulatory molecules in lung cancer: disappearance of the D17 epitope of CD55 in small-cell carcinoma. *Jpn. J. Cancer Res.* 84: 753–759.
23. Akazawa, T., H. Masuda, Y. Saeki, M. Matsumoto, K. Takeda, K. Tsujimura, K. Kuzushima, T. Takahashi, I. Azuma, S. Akira, et al. 2004. Adjuvant-mediated tumor regression and tumor-specific cytotoxic response are impaired in MyD88-deficient mice. *Cancer Res.* 64: 757–764.
24. Azuma, I., E. E. Ribi, T. J. Meyer, and B. Zbar. 1974. Biologically active components from mycobacterial cell walls: I. Isolation and composition of cell wall skeleton and component P3. *J. Natl. Cancer Inst.* 52: 95–101.
25. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
26. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta CT}$ method. *Methods* 25: 402–408.

27. Wilson, M. C., V. N. Jackson, C. Heddle, N. T. Price, H. Pilegaard, C. Juel, A. Bonen, I. Montgomery, O. F. Hutter, and A. P. Halestrap. 1998. Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. *J. Biol. Chem.* 273: 15920–15926.
28. Saio, M., S. Radoja, M. Marino, and A. B. Frey. 2001. Tumor-infiltrating macrophages induce apoptosis in activated $CD8^+$ T cells by a mechanism requiring cell contact and mediated by both the cell-associated form of TNF and nitric oxide. *J. Immunol.* 167: 5583–5593.
29. Kim, J. W., and C. V. Dang. 2006. Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res.* 66: 8927–8930.
30. Gatenby, R. A., and R. J. Gillies. 2004. Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer* 4: 891–899.
31. Walenta, S., and W. F. Mueller-Klieser. 2004. Lactate: mirror and motor of tumor malignancy. *Semin. Radiat. Oncol.* 14: 267–274.
32. Heinemeyer, T., E. Wingender, I. Reuter, H. Hermjakob, A. E. Kel, O. V. Kel, E. V. Ignatieva, E. A. Ananko, O. A. Podkolodnaya, F. A. Kolpakov, et al. 1998. Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL. *Nucleic Acids Res.* 26: 362–367.
33. Halestrap, A. P., and N. T. Price. 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function, and regulation. *Biochem. J.* 343: 281–299.
34. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3: 133–146.
35. Bronte, V., D. B. Chappell, E. Apolloni, A. Cabrelle, M. Wang, P. Hwu, and N. P. Restifo. 1999. Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits $CD8^+$ T cell responses by dysregulating antigen-presenting cell maturation. *J. Immunol.* 162: 5728–5737.
36. Flo, T. H., O. Halaas, S. Torp, L. Ryan, E. Lien, B. Dybdahl, A. Sundan, and T. Espevik. 2001. Differential expression of Toll-like receptor 2 in human cells. *J. Leukocyte Biol.* 69: 474–481.
37. Gladden, L. B. 2004. Lactate metabolism: a new paradigm for the third millennium. *J. Physiol.* 558: 5–30.
38. Walenta, S., M. Wetterling, M. Lehrke, G. Schwickert, K. Sundfor, E. K. Rofstad, and W. Mueller-Klieser. 2000. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res.* 60: 916–921.
39. Schwickert, G., S. Walenta, K. Sundfor, E. K. Rofstad, and W. Mueller-Klieser. 1995. Correlation of high lactate levels in human cervical cancer with incidence of metastasis. *Cancer Res.* 55: 4757–4759.
40. Walenta, S., A. Salameh, H. Lyng, J. F. Evensen, M. Mitze, E. K. Rofstad, and W. Mueller-Klieser. 1997. Correlation of high lactate levels in head and neck tumors with incidence of metastasis. *Am. J. Pathol.* 150: 409–415.
41. Fantin, V. R., J. St.-Pierre, and P. Leder. 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* 9: 425–434.
42. Gottfried, E., L. A. Kunz-Schughart, S. Ebner, W. Mueller-Klieser, S. Hoves, R. Andreesen, A. Mackensen, and M. Kreutz. 2006. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* 107: 2013–2021.
43. Fischer, K., P. Hoffmann, S. Voelkl, N. Meidenbauer, J. Ammer, M. Edinger, E. Gottfried, S. Schwarz, G. Rothe, S. Hoves, et al. 2007. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* 109: 3812–3819.
44. Murray, C. M., R. Hutchinson, J. R. Bantick, G. P. Belfield, A. D. Benjamin, D. Brazma, R. V. Bundick, I. D. Cook, R. I. Craggs, S. Edwards, et al. 2005. Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nat. Chem. Biol.* 1: 371–376.