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Propionic Acid Secreted from Propionibacteria Induces NKG2D Ligand Expression on Human-Activated T Lymphocytes and Cancer Cells

Lars Andresen,2* Karen Aagaard Hansen,2* Helle Jensen,* Stine Falsig Pedersen,† Peter Stougaard,‡ Helle Rüsz Hansen,§ Jesper Jurlander,¶ and Søren Skov3* we have previously shown that intracellular calcium, GSK-3 activity, and Sp1 binding to the promoter region is involved in regulation of MICA expression after exposure to propionate and CMV. In contrast, compounds with histone deacetylase-inhibitory activity such as butyrate and FR901228 stimulated MICA/B expression through a pathway that was not affected by inhibition of glycolysis, clearly suggesting that MICA/B is regulated through different molecular mechanisms. We propose that propionate, produced either by bacteria or during cellular metabolism, has significant immunoregulatory function and may be cancer prophylactic. The Journal of Immunology, 2009, 183: 897–906.

The ability of the immune system to recognize infected or transformed cells is dependent on the induction of immunostimulatory molecules by distressed cells. One of the principal sensing mechanisms is the NKG2D/NKG2D ligand system.

NKG2D ligands are induced on the cell surface of numerous stressed, transformed, and infected cells, while the expression on healthy human cells is low. The immune system recognizes NKG2D ligand-positive cells through the NKG2D receptor, a major activating receptor on CD8 T lymphocytes, NK cells, and some γ/δ T lymphocytes and activated CD4 T lymphocytes (1, 2).

A central step in activation of the NKG2D/NKG2D ligand pathway is the induction of NKG2D ligands on stressed cells. Several different proteins act as NKG2D ligands: MHC class I polypeptide-related sequence A (MICA) and MICB belong to the MIC gene family, and UL16 binding protein 1–4 (ULBP1–4) and retinoic acid early transcript 1G (RAET1G) belong to the RAET1 gene family (1, 3, 4). The plethora of NKG2D ligands most likely reflects an evolutionary protection against pathogen-inflicted inhibition of NKG2D ligand expression. Several different forms of cellular stress have been implicated in increased NKG2D ligand expression, including heat shock, virus infection, inflammatory cytokines, histone deacetylase (HDAC) inhibitors, retinoic acid, proteasome inhibitors, TLR signaling, and DNA damage response (3, 5–13).

We have previously shown that intracellular calcium, GSK-3 activity, and Sp1 binding to the promoter region is involved in regulation of MICA expression after exposure to propionate and CMV. In contrast, compounds with histone deacetylase-inhibitory activity such as butyrate and FR901228 stimulate MICA/B expression through a pathway that was not affected by inhibition of glycolysis, clearly suggesting that MICA/B is regulated through different molecular mechanisms. We propose that propionate, produced either by bacteria or during cellular metabolism, has significant immunoregulatory function and may be cancer prophylactic. The Journal of Immunology, 2009, 183: 897–906.

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treatment with Propionibacterium granulosum increased survival of patients with colorectal carcinoma (17, 18).

Certain propionibacteria are pathogenic. The acne-causing Propionibacterium acne stimulates macrophages through TLR2 and primes them for proinflammatory cytokine production (19, 20).

Propionate can also be produced during metabolism and can be generated from propionyl-CoA, an end product in the metabolism of the amino acids valine, isoleucine, threonine, and methionine, as well as odd-chain fatty acid oxidation and degradation of cholesterol. Propionyl-CoA is metabolized to succinyl-CoA and is thereby feed into the TCA cycle. Defects in propionyl-CoA metabolism, mainly observed in patients with propionyl-CoA carboxylase gene defects (21), leads to increased propionate levels in the blood (propionic acidemia). Continuous propionic acidemia leads to neuroregeneration, mental retardation, cardiomyopathy, and high frequency of infections (22, 23).

Here we show that propionibacteria or purified SCFA can induce gene activation and surface expression of the NKG2D ligands MICA/B. Of interest, we could separate the various inducers of MICA/B by their dependency on cellular glycolysis, suggesting that different signaling pathways regulate MICA/B expression.

**Materials and Methods**

**Cells and reagents**

Jurkat E6-1 (J6E-1) T cells were from American Type Culture Collection; Jurkat T cells stably transfected with large T Ag from SV40 virus (J TAG-9) were provided by C. Geisler (University of Copenhagen), J TAG-9 cells were primarily used for transient transfection studies. Adh77 (human placenta cell leukemia), DOHH-2 (human B cell lymphoma), Com (human T cell leukemia), Granu (human B cell lymphoma), Aml193 (human acute myeloid leukemia), and Jurkat T cells were grown in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, 2 mM glutamine, 2 mM L-glutamic acid, and streptomycin. HT-29 cells (human colon adenocarcinoma) were provided by B. Deihl from Herlev Hospital, Herlev, Denmark. HT-29 cells were grown in McCoy’s 5A medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM glutamine, 2 mM L-glutamic acid, and streptomycin. Buffy coats from healthy human volunteer donors were obtained from The State Hospital, Copenhagen. PBMC were obtained using Lymphoprep (Axis-Shield) according to the description of the manufacturer. Cells isolated from human blood were grown in RPMI 1640 medium supplemented with 150 U/ml IL-2 (Boehringer Mannheim). Polyclonal T cells were expanded according to the protocol from Dyna beads CD3/CD28 T cell expander (Invitrogen); cells were used 3 days after restimulation. The HDAC inhibitor FR901228 was provided by the National Cancer Institute (Bethesda, MD). 2-Fluoro-2-deoxy-γ-glucose, ammonium acetate, sodium propionate (≥99%), sodium butyrate (98%), Sodium i-n-lactate (≥99%), BAPTA-AM, EGTA, iodoacetic acid (IAA), capric acid, sodium succinate, and 6-amaminonucleotidase (6-AN) were from Sigma-Aldrich. Capric acid was neutralized with NaOH before use. Recombinant human NKG2D-Fc chimera was from BD Biosciences. Ammonium formate (≥98%) was acquired from Riedel-de Haën, and methanol was from BDH Chemicals.

**Bacterial cultures**

Three propionibacterium strains, Propionibacterium freudenreichii subsp. freudenreichii (F20721), P. freudenreichii subsp. shermanii (SY902), and Propionibacterium acidipropionicum (A20272) were collected from Deutsche Sammlung von Mikroorganismen und Zellkulturen. The propionibacteria were grown under anaerobic conditions in Luria-Bertani media at 24°C. Cultivation was conducted in anaerobic jars (AnaeroCult; Merck) with Anaerotest strips (Merck) to control the anaerobic environment. Pseudomonas aeruginosa (PA01) bacteria was provided by M. Kolpen (University of Copenhagen) and grown in ox bouillon (Statens Serum Institut, Copenhagen Denmark). Escherichia coli OneShot OmniMAX 2-T,4 cells (Invitrogen) were grown in Luria-Bertani media at 37°C.

**Flow cytometry**

For surface staining, cells were washed twice in cold PBS and stained with PE-coupled MICA/B Ab (558352; BD Biosciences) at a dilution of 1/100 for 30 min at 4°C, washed, resuspended, and analyzed in PBS. For GFP expression, cells were washed, resuspended, and analyzed in PBS. Data acquisition and flow cytometric analysis were done on a FACS Calibur using CellQuest software (BD Biosciences). Results are always shown as forward scatter/side scatter on a linear scale and fluorescence on a log10 scale. JE6-1 cells were stained with recombinant human NKG2D-Fc chimera at a dilution of 1/100 for 30 min at 4°C. Secondary staining was made with FITC-coupled Ab against human IgGI (Dako). For annexin V staining the cells were stained with annexin V-FITC (BD Biosciences, catalog no. 556420) in combination with either control-IgG-PE (BD Biosciences, catalog no. 555574) or MICA/B-PE Ab in annexin V staining buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2) for 30 min at 4°C.

**Cytolytic assay**

The functional cytolytic assay was performed as described by Kane et al. (24). In brief, the target cells were incubated with 0.6 μM 3,3′-dilinoleoyl-1,2-dimyristoyl-sn-glycerol perchlorate (DiO; Invitrogen) and allowed to rest in the incubator for 6 h. The cells were then split in two and treated with or without 10 mM sodium propionate. Twelve hours later the cells were mixed with effecter cells. As effector cells we used PBMCs obtained from healthy blood donors and cultured them at a concentration of 5 × 10^5/ml in RPMI 1640 supplemented with 10% human serum (Lonza) and 20 ng/ml IL-15 (R&D Systems) for 3 days. Effector cells and target cells were mixed at a ratio of 10:1 in 200 μl of media with 10% human serum in a round-bottom microplate. For blocking experiments a monoclonal NKG2D blocking Ab clone 148910 (R&D Systems) was added at a final concentration of 2.5 μg/ml. After 4 h in the incubator the cells were stained with propidium iodide (PI) and immediately analyzed by flow cytometry. To measure cytolytic activity we gated on DiO-positive cells and recorded the percentage of PI-positive cells.

**Transient transfections**

JTAG-9 cells were transiently transfected with the Nucleofector kit (Amaxa) according to the protocol of the manufacturer. In brief, 2 × 10^5 cells were resuspended in 1 μl of Cell Line Nucleofector Solution V, mixed with 2.4 μg of plasmid or 0.8 nmol of small interfering RNA (siRNA) and pulsed using the Nucleofector program G10. The siRNA sequences were: small interfering triosephosphate isomerase (siTPI)266: CCUCGCAUG AUCAAAGACU and siTPI1188: CCACCAUGUGAGGGAUAU. Control was described previously (25).

**Plasmids, generation of promoter constructs, and mutagenesis**

The plasmids pEQ336 (encoding the CMV IE promoter, but without any IE sequences) and pEQ326 (encoding the CMV IE2) were provided by A. Geballe (Fred Hutchinson Cancer Research Center, Seattle, WA). The pEQ326 was used as a template for PCR amplification of the p3.2k wild-type (WT)-GFP plasmid, the p3.2k GC-mut-GFP plasmid, and the p3.2k GC-mut-GFP plasmid, each containing a point mutation at position 2.4
g/ml. After4hi nt h eincubator the cells were stained with propidium iodide (PI) and immediately analyzed by flow cytometry. To measure cytolytic activity we gated on DiO-positive cells and recorded the percentage of PI-positive cells.

**Real time RT-PCR analysis**

**Measurement of intracellular pH**

JE6-1 cells were resuspended in RPMI 1640 media at 2 × 10^6 cells/ml, and measurements of intracellular pH (pH_i) were performed spectrophotometrically using the pH-sensitive probe 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM), essentially as described previously (26). An aliquot (5 ml) of cells was incubated with BCECF-AM (3.2 mM) for 30 min at 37°C. The samples were centrifuged for 30 s at 700 × g, washed once in RPMI 1640 medium, and resuspended in 1.25 ml of RPMI 1640 medium. The samples were placed in a magnetically stirred cuvette in a fluorescence spectrophotometer model PTI RatioMaster (Photon Technology International).
BCECF emission was detected at 525 nm after excitation at 445 nm and 500 nm. Background fluorescence was subtracted before calculation of the 445 nm/500 nm fluorescence ratio, and a three-point, linear calibration of BCECF ratio to pH was performed using the high K+/nigericin technique as described previously (26).

**FIGURE 1.** MICA/B expression by Jurkat T cells after exposure to supernatant from anaerobic propionibacteria. A. Three propionibacteria, *P. freudenreichii* subsp. *freudenreichii* (F20271), *P. freudenreichii* subsp. *shermanii* (S4902), and *P. acidipropionici* (A20272), were grown under anaerobic conditions. *E. coli* and *P. aeruginosa* (PAO1) were grown under aerobic conditions. JE6-1 cells were incubated with a 1/10 dilution of the collected supernatant and incubated for 18 h. As a positive control we used JE6-1 cells stimulated with 20 ng/ml HDAC inhibitor FR901228 for 18 h. Cells were then stained with anti-MICA/B Ab and analyzed by flow cytometry. Data shown are representative of two independent experiments. The bar graph shows mean ± SD from two independent experiments. Selected data are also shown as dot plots. B. JE6-1 cells were treated with different concentrations of propionate or 20 ng/ml FR901228 for 18 h. The cells were stained with anti-MICA/B Ab and analyzed by flow cytometry. The bar graph shows means ± SD from two independent experiments. Selected data are also shown as dot plots. C. JE6-1 cells were treated for the indicated time with 10 mM propionate, stained with anti-MICA/B Ab, and analyzed by flow cytometry. D. JE6-1 cells were incubated with propionate as indicated for 18 h and then stained with annexin V-FITC in combination with either control IgG-PE or MICA/B-PE Abs followed by PI staining. Stained cells were analyzed by flow cytometry. The dot plots show PI-negative cells with annexin V-FITC fluorescence on the y-axis and MICA/B-PE fluorescence on the x-axis. Numbers indicate percentage gated cells in each quadrant. The figure shown is representative of three independent experiments.

E. Granta cells were labeled with 0.6 µM DiO for 6 h and divided into a control group or treated with 10 mM propionate. After another 12 h the cells were mixed with PBMCs at an effector/target ratio of 10:1 and incubated for 4 h in the incubator. For the blocking experiment anti-NKG2D Ab was added to the wells at a final concentration of 2.5 µg/ml. The cells were then stained with PI and analyzed by flow cytometry. The figure consists of four dot plots showing DiO-positive cells with PI fluorescence on the y-axis and DiO fluorescence on the x-axis. The two upper plots show the experiment without blocking Ab (−ab) and the two lower plots show an experiment with blocking Ab (+ab). The numbers indicate percentage gated cells in each quadrant. The figure shown is representative of three independent experiments.
Measurement of intracellular propionate by HPLC-electrospray mass spectrometry

JE6-1 cells ($2 \times 10^6$ cells/ml) were grown in RPMI 1640 media without FBS and stimulated with 20 ng/ml FR901228 or 10 mM propionate for 0, 2, 4, and 18 h. The cells were pelleted and resuspended in 1/10 methanol/water. The liquid chromatographic (LC) system used for separation of organic acids consisted of an Agilent 1100 series HPLC pump system (Agilent Technologies) and a Hamilton PRP-X100 analytical column (150 × 4.1 mm, × 3 μm). The mobile phase was a mixture of 50 mM ammonium formate and 10% methanol and its flow rate was 0.7 ml/min. The injection volume was 50 μl and biological samples were filtered through a 0.45-μm nylon filter before injection. HPLC-electrospray mass spectrometry measurements were performed on a Hewlett-Packard series 1100 MSD single quadropole mass spectrometer with an electrospray ionization interface operating in the negative ionization mode. Nitrogen was used as nebulizing gas at a pressure of 40 psi at 12 L/min; the temperature was 350°C and the capillary voltage 4000 V. The instrument was operated at cone voltage of 50 V in either the full scan mode or in the selected ion monitoring mode (m/z of 73 and m/z of 89 for detecting propionic and lactic acid, respectively) (for further details, see Ref. 27). The system was optimized on 0.5 mM propionic and L-lactic acid.

**FIGURE 2.** Activated T cells and various cancer cells express MICA/B after propionate treatment. A, The indicated cells were treated with and without 10 mM propionate for 18 h and stained with anti-MICA/B; the gate is set arbitrarily to enlighten the difference in staining intensity. B, PBMCs were stimulated with or without CD3/CD28 beads and IL-2 as described in Materials and Methods. The activated and naive PBMCs were exposed to 10 mM propionate or 20 ng/ml FR901228 for 18 h, stained with anti-MICA/B Ab, and analyzed by flow cytometry.

**FIGURE 3.** Propionate activates MICA promoter activity. A, JE6-1 cells were treated with 20 ng/ml FR901228 or 10 mM propionate for 4 h. Total RNA was then extracted and used for quantitative RT-PCR. MICA mRNA expression was normalized to the expression of the housekeeping gene RPLP0 and displayed as fold expression relative to control. B, JTag-9 cells were transfected with 5' deletions of the MICA promoter construct as indicated. After 24 h, the cells were either left untreated (control) or treated with 10 mM propionate for 18 h. The cells were analyzed for GFP fluorescence by flow cytometry. The bar graph shows means ± SD from two independent experiments. C, JTag-9 cells were transfected with p-115-GFP and p+z-GFP and incubated for 24 h. The cells were stimulated with vehicle control, 10 mM propionate, 20 ng/ml FR901228, or 1,1:10 supernatant from F20271, A20272, S4902, E. coli, or PA01 for 18 h. The next day, cells were analyzed for GFP fluorescence by flow cytometry. The bar graph shows means ± SD from two independent experiments. D, JTag-9 cells were either left untreated (control) or treated with 20 ng/ml FR901228, 10 mM propionate, and 10 mM butyrate for 18 h. The cells were analyzed for GFP fluorescence by flow cytometry. The bar graph shows means ± SD from two independent experiments.

**TPI assay**

The activity of TPI was measured by adding 5 μl of cell lysate to 85 μl of reaction mixture consisting of 0.025 M triethanolamine (pH 7.9), 0.1 mM NADH (Sigma-Aldrich, catalog no. N6660), and 1 U α-glycerophosphate dehydrogenase (Sigma-Aldrich, catalog no. G6880). The mixture was incubated 10 min at room temperature before the addition of 30 μl of 15 mM DL-glyceraldehyde 3-phosphate solution (Sigma-Aldrich, catalog no. G5251), which starts the reaction. The decrease in A340 nm was monitored in 10-s intervals, and specific activity in mU/mg was calculated as A340 nm/volume/20/(6.3 × protein concentration).

**GAPDH assay**

GAPDH activity was determined using a KDalet GAPDH assay kit from Ambion. Briefly Jurkat E6-1 cells were lysed with KDalet lysis buffer. Lysate (20 μl) was added to 180 μl of reaction mix in a microplate and incubated at room temperature for 15 min. A standard curve was also included. The OD630 was measured in a microplate reader and the activity in the samples was calculated.

**Immunoblotting**

Immunoblotting was done as previously described (25). The TPI Ab was from Santa Cruz.
Results

Propionic acid from propionibacteria regulates MICA/B expression

We were interested in identifying bacterial components that could modulate the NKG2D/NKG2D ligand axis of immunoactivation. Various bacteria and their growth supernatants were screened for the ability to induce MICA/B surface expression on Jurkat T cells; we have previously shown that Jurkat T cells can be induced to express MICA/B after different kinds of cell stress (12, 25). Fig. 1A shows surface MICA/B expression after 18 h of cocultivation of Jurkat T cells with supernatants from E. coli, P. aeruginosa, and three different strains of propionibacteria: P. freudenreichii subsp. freudenreichii (F20271), P. freudenreichii subsp. shermanii (S4902), and P. acidipropionici (A20272). Growth supernatants from the three strains of propionibacteria induced strong MICA/B expression, whereas E. coli and P. aeruginosa had no effect. The propionibacterial supernatants were potent; a 100-fold dilution still induced MICA/B expression (data not shown).

Propionibacteria produces propionate and other SCFAs during their anaerobic growth. To determine whether propionate could be a causative factor in MICA/B expression, we exposed Jurkat T cells to purified propionate. There was a robust induction of MICA/B expression by 10 – 20 mM propionate and less but distinct expression down to 1 mM (Fig. 1B). Labeling of Jurkat T cells with an isotype-matched control Ab was not affected by the propionibacteria supernatants or propionate (data not shown). A kinetic analysis using 10 mM propionate showed that MICA/B expression could be detected 8 h after propionate exposure, with maximal expression within 14 to 24 h (Fig. 1C). The propionate-mediated MICA/B expression was comparable to the induction mediated by HDAC inhibitors (12), with the general distinction that the intensity (mean fluorescence intensity) of MICA/B staining was higher after treatment with the HDAC inhibitor FR901228, and the percentage of MICA/B-positive cells was higher after propionate treatment (Fig. 1B).
In some tumor cell lines propionic acid has been shown to induce apoptosis at concentrations ranging from 10 to 40 mM (15). To reveal whether MICA/B-expressing cells were apoptotic cells, we double stained propionate-treated Jurkat cells with annexin V-FITC and MICA/B-PE Ab. As shown in Fig. 1D, the apoptotic annexin V-positive cells did not express MICA/B, and vice versa, suggesting that the underlying pathway from propionate to MICA/B expression does not connect with the apoptotic pathway.

NKG2D ligand expression induced by propionibacteria growth supernatant or propionate was also detected with the native purified receptor NKG2D-Fc (Fig. 1E), demonstrating that propionate-induced NKG2D ligand expression can interact with the native receptor NKG2D. Coincubation with anti-MICA/B Ab blocked NKG2D-Fc binding to propionate-induced NKG2D ligands by 45% (data not shown). Ab blocking is often incomplete, suggesting that most propionate-induced NKG2D ligands stem from MICA/B. To demonstrate the functional ability of propionate-induced NKG2D ligands, we made a cytotoxic assay with purified blood lymphocytes activated for 3 days with IL-15 to enhance NKG2D-mediated killing by NK cells. We have previously shown that Granta cells are efficient target cells for monitoring NKG2D ligands, we made a cytotoxic assay with purified MICA/B. To demonstrate the functional ability of propionate-induced NKG2D ligands, we made a cytotoxic assay with purified blood lymphocytes activated for 3 days with IL-15 to enhance NKG2D-mediated killing by NK cells. We have previously shown that Granta cells are efficient target cells for monitoring NKG2D ligand-dependent NK cell activity (12). Fig. 1F shows that Granta cells exposed to 10 mM propionate for 12 h, in contrast to controls, were potently killed by IL-15-activated PBLs. Inclusion of a blocking NKG2D Ab strongly inhibited killing of these cells. This suggests that propionate-induced NKG2D ligands are able to functionally activate NK cells.

Next we wanted to see if propionate also regulated MICA/B expression in other cancer cell types. Fig. 2A shows that propionate clearly induced MICA/B expression in HT-29 (colon cancer), Granta (mantle cell lymphoma), Arh77 (multiple myeloma), DOHH2 (malignant non-Hodgkin’s lymphoma), Aml193 (acute myelogenous leukemia), and Cem (T cell acute lymphoblastic leukemia) cells. We have previously shown that activated, but not resting, CD4 T lymphocytes can be induced to express MICA/B (12). In line with this, propionate enhanced MICA/B surface expression on CD4 T lymphocytes previously stimulated with beads coupled to anti-CD3/CD28 Abs. Propionate did not affect MICA/B expression on resting CD4 T lymphocytes (Fig. 2B).

Molecular regulation of MICA/B by propionate

Real-time PCR analysis demonstrated that propionate leads to an increase in MICA mRNA level (Fig. 3A), suggesting that propionate either stabilizes MICA mRNA or directly stimulates MICA promoter activity.

Using MICA reporter constructs, consisting of 3.2 kb or subsequent deletions of the proximal MICA promoter in front of GFP (25), we tested the ability of propionate to activate the MICA promoter. As shown in Fig. 3B, propionate stimulated MICA promoter activity in transiently transfected Jurkat T cells. Supernatants from the different propionibacteria were also tested and, as expected, they induced a robust MICA promoter activity (Fig. 3C). These different promoter activations were comparable to, or higher than, those observed after optimal HDAC inhibitor treatment, which has previously been shown by us (25). Promoter deletion analysis showed that the region from −115 to −97, relative to the transcription start site, was important for propionate-mediated promoter activity (Fig. 3B). The interesting region from −115 to −97 contains a GC-box motif and has earlier been shown to interact with the transcription factor Sp1, which is important for MICA promoter activity after heat shock and HDAC inhibitor exposure (14, 25). In agreement, mutation of the GC-box motif from −113 to −105 by shifting CCC to AAA in position −107 to −105 strongly inhibited propionate-mediated MICA promoter activity (Fig. 3D). These results strongly suggest that propionate directly stimulates MICA promoter activity through involvement of the proximal GC-box motif.

Propionate regulation of MICA/B through calcium and pH

Intracellular calcium has been implicated in HDAC inhibitor- and CMV-mediated MICA/B expression (25). To assess the role of calcium in propionate-induced MICA/B expression, we loaded Jurkat T cells with BAPTA-AM (an intracellular calcium chelator) or EGTA (an extracellular calcium chelator). Cells were analyzed for MICA/B surface expression after treatment with propionate. As shown in Fig. 4, A and B, depletion of calcium either by BAPTA-AM or EGTA abrogated MICA/B expression in a dose-dependent manner. BAPTA-AM also inhibited propionate-mediated MICA mRNA production measured by quantitative PCR (data not shown). Both BAPTA-AM and EGTA blocked the propionate-induced promoter activity, measured using the p3.2k WT-GFP and 173-MICA-GFP promoter constructs (Fig. 4C), indicating that intracellular calcium affects transcription downstream from bp −173 relative to the MICA transcription start site. These results demonstrate that intracellular calcium is a critical component in the propionate-induced signaling pathway leading to MICA gene activation and surface expression. The blocking by EGTA further suggests that the rise in intracellular calcium is dependent on extracellular influx.

Propionate causes rapid intracellular acidification, and the decrease in pH can, in some circumstances, be regulated by intracellular calcium (28–30). To determine whether intracellular acidification generally was implicated in MICA/B expression, we recorded pH, changes after exposure to propionate, butyrate, and
Jurkat T cells were loaded with the pH-sensitive BCECF-AM and the pH was recorded to ~7.6 (Fig. 4, D and E). As expected, addition of either 10 mM propionate or 10 mM butyrate caused a rapid intracellular acidification followed by a gradual recovery. However, we did not observe any immediate changes in pH after treatment with FR901228 (Fig. 4F). Likewise, prolonged exposure to FR901228 (up to 4 h) did not result in pH changes (data not shown). These results suggest that intracellular acidification is not essential for MICA/B expression.

Propionate can be generated by cell metabolism, mainly through propionyl-CoA generation after catabolism of the amino acids valine, isoleucine, threonine, and methionine, as well as odd-chain fatty acid oxidation and degradation of cholesterol. We initially hypothesized that the various signals that lead to MICA/B expression might all interfere with metabolism and cause high levels of intracellular propionate, meaning that propionate would be a general causative factor regulating MICA/B expression.
Intracellular propionate was measured by mass spectrometry and, as shown in Fig. 4G, treatment with FR901228 did not result in increased intracellular levels of propionate in Jurkat T cells. The assay was fully functional as intracellular propionate was readily observed after treatment with propionate, and increased propionate was also detected in the FR901228-treated cells pulsed with propionate before the assay (Fig. 4G). These results strongly suggest that metabolic buildup of propionate is not an essential integrating component shared by other MICA/B stimulators.

**Different SCFAs induce MICA/B expression**

Propionibacteria can produce different kinds of SCFAs in addition to propionate, especially acetate, butyrate, and lactate. It is obvious that the induction of MICA/B by propionibacteria may not be solely related to propionate; it is more likely a combined effect of several different compounds, given the potent effect of the propionibacteria supernatant (see Fig. 1A).

Jurkat T cells were treated with different concentrations of acetate, lactate, and butyrate for 18 h and analyzed for MICA/B surface expression (Fig. 5A–C). Acetate and lactate induced a robust increase in MICA/B expression, although the effect was most pronounced at relatively high concentrations from 30 to 50 mM. These concentrations are, however, achievable during bacterial metabolism in the intestine (16, 28, 30). The effect of butyrate was more prominent, and sizable MICA/B expression could be detected down to 1 mM. Butyrate is a direct HDAC inhibitor (31), so the observed effect on MICA/B is not surprising. To test whether the structure of the SCFA had any importance for MICA/B induction we tested succinate, which is a four-carbon SCFA-like butyrate but with two carboxylic acids instead of one, and caproate, which is a six-carbon SCFA. As shown in Fig. 5D, succinate could not induce MICA/B expression and caproate was approximately half as potent as propionate to induce MICA/B expression, indicating that optimal MICA/B expression is induced by SCFAs with three- to four-carbon length and only one carboxylic acid group.

**HDAC inhibitors differ from propionate and CMV with regard to glycolytic dependency of MICA/B expression**

MICA/B is primarily expressed in activated/proliferating cells. Activated T lymphocytes and cancer cells are characterized by a high level of metabolism, especially glycolysis, when compared with naive cells (32, 33). Based on these considerations, we wanted to elucidate the importance of glycolysis for MICA/B expression.

Glycolysis was inhibited using the specific inhibitor 2-fluoro-2-deoxy-D-glucose (2fDG), which is a nonmetabolizing mimetic of glucose that is more specific than the more widely used 2-deoxy-D-glucose (34). We noted an interesting difference in MICA/B regulation when glycolysis was blocked; propionate-induced MICA/B surface expression was strongly inhibited by 2fDG in a dose-dependent manner, which on the other hand did not affect MICA/B expression after FR901228 treatment (Fig. 6A). The lack of effect by 2fDG on FR901228-mediated MICA/B expression excludes that the inhibition of propionate-mediated MICA/B is caused by severe toxicity or depletion of cellular energy. This is further substantiated by Jurkat T cells transiently expressing GFP; here, 2fDG did not affect GFP expression within 24 h (Fig. 6A, insert). However, as expected, 2fDG inhibited glycolysis as measured by decreased cellular lactate production (data not shown).

Next we inhibited glycolysis farther downstream (see Fig. 6B for illustration). The enzyme TPI, which catalyzes the interconversion between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (Gly-3P), was targeted by siRNA-mediated knockdown. Interestingly, MICA/B expression was actually superinduced after both propionate and FR901228 exposure (Fig. 6C). The siRNA targeting of TPI was effective as shown by Western blot analysis and measurement of TPI activity (Fig. 6D); moreover, transiently expressed GFP was not affected by the TPI knockdown (data not shown), excluding gross cell toxicity. To substantiate these data we blocked GAPDH by the potent, although more unspecific, inhibitor IAA (Fig. 6F); again, there was no decrease of either propionate- or FR901228-mediated MICA/B expression (Fig. 6E).

A possible explanation for these findings could be that MICA/B expression is dependent on a signaling pathway linked to proximal glycolytic products. The pentose phosphate pathway (PPP) is derived from glucose-6-phosphate (Glu-6P) by the enzyme glucose-6-phosphate dehydrogenase (G6PDH) (see Fig. 6B), and the oxidative PPP can be very specifically inhibited by 6-AN (35, 36). In agreement with this, pretreatment with 6-AN completely inhibited propionate-induced MICA/B expression without affecting expression caused by FR901228 exposure (Fig. 6G).

When combining these results with the siTPI/IAA data, our findings suggest that proximal glycolysis through the oxidative PPP is essential for propionate-mediated MICA/B surface expression. The HDAC inhibitor FR901228 can bypass this requirement through an unknown mechanism.

We decided to look at the glycolytic dependency of a variety of stimuli that lead to MICA/B activity. An interesting pattern emerged: all HDAC inhibitors tested (trichostatin A, FR901228, and butyrate) were not dependent on proximal glycolysis for MICA/B expression. This was in contrast to acetate, lactate, propionate, and CMV, which could not induce MICA/B expression in the presence of 2fDG (Fig. 6H and data not shown).

**Discussion**

In this report we describe that propionic acid and other SCFAs, such as acetate, butyrate, and lactate, secreted from propionibacteria induce surface expression of the NKG2D ligands MICA/B on several cancer cells and activated T lymphocytes, while leaving naive blood cells unaffected. NKG2D ligands induced by propionibacteria bound specifically to the chimeric NKG2D-Fc receptor. Approximately half of the propionate-mediated NKG2D-Fc binding was blocked by incubation with a MICA/B Ab, suggesting that MICA/B are the main NKG2D ligands expressed. Moreover, we observed a strong NKG2D-dependent NK cell killing of propionate-exposed cancer cells. We anticipate that other ligands are likely also induced; in this regard, Vales-Gomez et al. have recently shown that HDAC inhibitor exposure leads to ULBP2 surface expression, in addition to MICA/B (11).

The molecular signaling pathway that mediated propionate-induced MICA/B expression was shown to be dependent on intracellular calcium. Chelating calcium with BAPTA-AM or EGTA strongly suppressed propionate-mediated MICA/B surface expression and MICA promoter activity. Additionally, deletion and point mutation of the MICA promoter construct showed that the GC-box between −113 to −105 relative to the transcription start site is important for propionate-induced MICA promotor activity. Heat shock and HDAC inhibitors induce MICA expression through the same GC-box (14, 25), clearly suggesting that they are generally important for regulation of MICA expression.

Propionibacteria mainly reside in the intestine where they produce SCFAs during fermentation of plant-derived dietary fiber. SCFAs are considered protective against colon carcinogenesis (16), one of the leading causes of cancer deaths in the Western part of the world. Propionate derived from different stains of propionibacteria can directly kill different human colorectal carcinoma...
cell lines (15). Preoperative treatment with different strains of propionibacteria caused beneficial immunostimulation and increased survival of patients with colorectal carcinoma (17, 18). It is tempting to speculate that a part of these effects is mediated by increased MICA/B expression. In this regard it is interesting that the gastrointestinal epithelium, which is in direct contact with intraepithelial lamina propria lymphocytes, is one of the only sites in the body with continuous elevated MICA/B expression (5, 37).

Propionyl-CoA, which is readily transformed to propionate, is produced in most cells by catabolism of the amino acids valine, isoleucine, methionine, and threonine, odd-chain fatty acids, and cholesterol. Patients with defects in the catabolism of propionyl-CoA have a severe and harmful buildup of propionic acid in the blood, a state termed propionic acidemia (22). From our studies it is evident that patients with propionic acidemia can suffer from increased MICA/B expression. In this regard, note that these patients often have recurrent infections (23), although this is likely also influenced by other factors than MICA/B.

During propionic acidemia the accumulated propionyl-CoA can combine with oxaloacetate leading to methylcitric acid, which can affect cellular metabolism (38). However, addition of purified 2-methylcitric acid did not affect MICA/B induction by propionate or FR901228 (data not shown), suggesting that propionate-induced MICA/B expression is not caused by methylcitric acid production.

We wondered if other activators of MICA/B expression could function through a metabolic buildup of propionic acid. This did not seem to be the case, as cellular levels of propionic acid was not changed by the HDAC inhibitor FR901228 as judged by mass spectrometry analysis. This suggests that increased levels of propionate are not a universal factor used by other stimuli that regulate MICA/B expression.

It is well described that propionate leads to a decrease in pH, (29), which in some cases can be linked to increases in intracellular calcium concentration (28, 30). Since we have found that calcium is involved in MICA/B expression after propionate exposure (Fig. 4, A and B), which is similar to our previous results with HDAC inhibitors and CMV IE2 transfection (25), we hypothesized that a decrease in pH could be generally involved in MICA/B expression. This was however not the case: FR901228 treatment did not affect pH, whereas both propionate and butyrate led to an immediate pH drop. The metabolic difference (described below), where the HDAC inhibitors butyrate and FR901228, in contrast to propionate, induced MICA/B independently of glycolysis, further implies that a decrease in pH is not generally coupled to MICA/B regulation; however, we cannot completely rule out that the decrease in pH affects MICA/B expression after propionate or butyrate exposure.

Cancer cells have a high degree of aerobic glycolysis, named the Warburg effect (33), and enhanced glycolysis is also seen in activated T cells (32). Thus, we wondered if glycolysis was linked to the ability to express MICA/B. The specific inhibitor of glycolysis, 2DG, had a striking impact: propionate-induced MICA/B expression was nearly completely blocked, whereas the HDAC inhibitor FR901228 led to a normal MICA/B increase. We also tried to block glycolysis farther downstream by siRNA-mediated knockdown of TPI or inhibition of GAPDH with IAA. Interestingly, this did not inhibit MICA/B expression after propionate exposure; in fact, both propionate and FR901228 actually superinduced MICA/B after TPI knockdown. These results suggest that only the beginning of the glycolytic pathway is critical for MICA/B expression. Since the PPP diverges early from the glycolytic pathway from glucose-6-phosphate, we tried to target this step. Indeed blocking the oxidative PPP with the specific inhibitor 6-AN potently inhibited propionate-mediated MICA/B expression without affecting the expression caused by FR901228. These results clearly suggest that there are specific differences in the regulation of MICA/B by propionate and the HDAC inhibitor FR901228.

When broadening our studies to other stimuli causing MICA/B expression, an interesting pattern emerged: lactate and CMV exposure behaved like propionate, whereas established HDAC inhibitors such as trichostatin A and butyrate behaved like FR901228.

Propionate can also lead to HDAC inhibitory activity (39); however, it is unclear if propionate functions as a direct HDAC inhibitor or whether a secondary effect causes elevated cellular HDAC inhibitory activity. Cousens et al. have shown that propionate leads to HDAC inhibitory activity, although the direct in vitro HDAC inhibitory activity was rather weak compared with butyrate (31). This could imply the rather trivial explanation that propionate merely functions as a weak HDAC inhibitor. Several of our findings argue against this explanation: 1) propionate and butyrate induce MICA/B expression in similar concentrations, which is not consistent with butyrate being a superior HDAC inhibitor; 2) acetate and lactate also lead to MICA/B induction; however, these compounds possess limited, if any, direct HDAC inhibitory activity (31); and 3) there is a clear metabolic difference between established HDAC inhibitors (FR901228, trichostatin A, and butyrate) and propionate with regard to MICA/B induction.

What is the basis for the dissimilar MICA/B regulation mediated by direct enzymatic HDAC inhibitors and other inducers? We have a number of hypothesis and speculations that may enlighten this area. SCFA could cause cellular HDAC inhibitory activity through proximal glycolysis and the PPP. HDAC inhibitors can directly target HDAC enzymes and would therefore not be dependent on intact metabolism. However, butyrate and propionate induce MICA/B expression with similar concentration kinetics (compare Figs. 1 and 5), which is not consistent with butyrate causing superior overall HDAC inhibition. This suggests that propionate can also regulate MICA/B through a pathway not shared by HDAC inhibitors. Eventual different signaling pathways may, however, converge at a distal point, as there is no additive effect on MICA/B expression by combined exposure to FR901228 and propionate (data not shown). Future studies will hopefully provide more conclusive and general understanding of the complex MICA/B regulation.

MICA is strongly expressed at the surface of intestinal epithelial cells from patients with autoimmune celiac disease, and this activates intraepithelial T lymphocytes through NKG2D, leading to enterocyte damage (40, 41). Celiac disease can result in bacterial overgrowth of the small intestine (42), and we think that it is an interesting hypothesis that the increased MICA expression can be caused by bacterial-produced SCFAs. If this is the case, artificial lowering of SCFA production may be useful in the future treatment of celiac disease.

Disclosures
The authors have no financial conflicts of interest.

References

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