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*J Immunol* 2012; 188:1847-1855; Prepublished online 6 January 2012;
doi: 10.4049/jimmunol.1004085

http://www.jimmunol.org/content/188/4/1847
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NKG2D ligand surface expression is important for immune recognition of stressed and neotransformed cells. In this study, we show that surface expression of MICA/B and other NKG2D ligands is dependent on N-linked glycosylation. The inhibitor of glycolysis and N-linked glycosylation, 2-deoxy-D-glucose (2DG), potently inhibited surface expression of MICA/B after histone deacetylase inhibitor treatment; the inhibition occurred posttranscriptionally without affecting MICA promoter activity. Transient overexpression of MICA surface expression was also inhibited by 2DG. 2DG blocks N-linked glycosylation of MICA/B by a reversible mechanism that can be alleviated by addition of D-mannose; this does not, however, affect the inhibition of glycolysis. Addition of D-mannose restored MICA/B surface expression after 2DG treatment. In addition, specific pharmacological or small interfering RNA-mediated targeting of glycolytic enzymes did not affect MICA/B surface expression, strongly suggesting that N-linked glycosylation, and not glycolysis, is essential for MICA/B surface expression. Corroborating this, tunicamycin, a selective inhibitor of N-linked glycosylation, abolished MICA/B surface expression without compromising activation of MICA promoter activity. NK cell-mediated killing assay and staining with a recombinant NKG2D–Fc fusion protein showed that all functional NKG2D ligands induced by histone deacetylase inhibitor treatment were abolished by 2DG treatment and fully reconstituted by further addition of D-mannose. Our data suggest that posttranslational N-linked glycosylation is strictly required for NKG2D ligand surface expression. Cancer and infection often result in aberrant glycosylation, which could likely be involved in modulation of NKG2D ligand expression. Our data further imply that chemotherapeutic use of 2DG may restrict NKG2D ligand surface expression and inhibit secretion of immunoinhibitory soluble NKG2D ligands.


The generation of adaptive immunity is dependent on activation of professional APCs, primarily dendritic cells. Dendritic cells can sense foreign pathogenic molecules through Toll receptor interaction with pathogen-associated molecules or by inflammatory cytokines produced during the infection or inflammation (1). Malfunctioning or stressed cells from the body itself cannot be sensed by the Toll receptor system; self-cells can alert the immune system through expression of stress molecules that can be recognized by immune effector cells (2, 3). The NKG2D/NKG2D ligand system is well recognized for its importance in immune recognition of unhealthy cells caused by infection or cancer transformation (4–6). Surface expression of calreticulin, DNAM-1 ligands, and Hsp70 has also been shown to be involved in immune recognition of cancer cells (7–9).

The stimulatory receptor NKG2D is expressed by NK cells, CD8 T cells, and subsets of CD4 and γ/δ T cells (5). Healthy cells have low surface expression of NKG2D ligands, which is, however, induced by cell stress, infection, and transformation (10). Several different molecules can act as NKG2D ligands; in humans, NKG2D ligands are comprised of the glycoproteins MICA, MICB, ULBP1–4, RAET1L, and RAET1G (11). Different types of pathologies circumvent immune activation by affecting NKG2D ligand expression. Cancer cells can escape immune recognition by cleaving NKG2D ligands from their cell surfaces (12, 13) or by limiting surface expression of NKG2D ligands (14).

Recently, attention has focused on the posttranscriptional regulation of NKG2D ligand surface expression. Melanoma cancer cells can restrict MICA cell-surface expression despite possessing significant intracellular levels of MICA protein (15). The murine NKG2D ligand MULT1 can be restricted from surface expression by the MARCH4 and MARCH9 E3 ubiquitin ligases, an effect that can be reversed by heat shock treatment (16). CMV infection can also hamper NKG2D ligand surface expression through specific retention of NKG2D ligands in the endoplasmic reticulum (ER)/Golgi system by the CMV-encoded proteins UL16 and UL142 (17–19).

Cancer cells preferentially use aerobic glycolysis for energy production, a phenomenon termed the Warburg effect. Glucose deprivation by use of the analog 2-deoxy d-glucose (2DG) is widely used for inducing cytotoxicity in transformed cells, and evidence suggests that generation of metabolic oxidative stress is a critical part of the cytotoxic mechanism (20–22). Recent data, however, suggest a more complicated way of action. 2DG also inhibits N-linked glycosylation of proteins in the ER, which can be alleviated by D-mannose supplementation without affecting 2DG.
inhibition of glycolysis. Kurtogi et al. (23) showed that a significant portion of the 2DG-mediated cell death in different cancer cells can be rescued by D-mannose, strongly implying that changes in N-linked glycosylation is involved in the chemotherapeutic effect of 2DG.

N-linked and O-linked glycosylation of proteins have multiple effects, ranging from intracellular sorting to regulation of n1/2 and bioactivity (24, 25). However, many proteins that are normally glycosylated show no loss of function when glycosylation is prevented (24), suggesting that additional processes can act in parallel. Cancer and infection often result in altered glycosylation of cellular proteins (26–28). Although the purpose may be numerous, it is likely that changes in glycosylation participate in escaping immune recognition.

In this study, we demonstrate that 2DG regulates NKG2D ligand surface expression through modulation of N-linked glycosylation.

Materials and Methods

Cells

For this study, we used Jurkat E6-1 and Jurkat Tag-9 (T-ALL), HEK293 Phoenix (human embryonic kidney-derived), Cem (T-ALL), Granta (mantle cell lymphoma), Ramos (Burkitt B cell lymphoma), PC3 (prostate cancer), and HaCaT (human keratinocyte-derived) cells. Suspension cells were cultured as previously described (29). The HEK293 Phoenix cell line was kindly provided by Dr. M. T. Petersen (Biotech Research and Innovation Centre, University of Copenhagen) (30). The GFP-MICA*018 plasmid that contained the full-length MICA cDNA from Jurkat cells was a gift from Dr. K. Helin (Biotech Research and Innovation Centre, University of Copenhagen) (31). The pMyc-MICA plasmid was made by cloning the MICA coding sequence into pENTR-D-TOPO vector (Invitrogen) and then recombining by Dr. M. Wills (University of Cambridge, Cambridge, U.K.) (31). The pmax-GFP plasmid was provided in the Amaxa Nucleofector kit (Lonza), and the MICA promoter plasmid, p3.2k-WT-GFP, was previously described (29). Promoter activity was calculated as previously described (29). The pCR containing the MICA coding sequence into pELEN vector (Invitrogen) and then recombining by Dr. M. Wills (University of Cambridge, Cambridge, U.K.) (31).

Flow cytometry

Cell-surface staining was done as previously described (29), using the PE-conjugated anti-human MICA/B clone 6D4, anti-human MHC class I clone W6/32 (sc-32235; Santa Cruz Biotechnology), and anti-human ICAM-1 (C170; Leinco Technologies). For intracellular staining, we used the BD Cytofix/Cytoperm kit (555028; BD Biosciences) following the manufacturer’s protocol. The staining was performed with anti-Myc tag, clone 4A6, and Alexa Fluor 488 conjugate (16-224; Millipore).

Transient transfections

Transient transfections of Jurkat cells were done using an Amaca-Nucleofector kit as previously described (Lonza) (29). Standard Calcium phosphate transfection method was used for transfection of HEK293 Phoenix cells. The pmax-GFP plasmid was provided in the Amaca-Nucleofector kit (Lonza), and the MICA promoter plasmid, p3.2k-WT-GFP, was previously described (29). Promoter activity was calculated as previously described (29). The pMyc-MICA plasmid was made by cloning the MICA coding sequence into pENTR-D-TOPO vector (Invitrogen) and then recombining this vector with pCMV-Myc using standard Gateway technology (Invitrogen). The Gateway compatible pCMV-Myc vector was kindly provided by Dr. K. Helin (Biotech Research and Innovation Centre, University of Copenhagen) (30). The GFP-MICA*018 plasmid that contained the full-length allele MICA*018 downstream of a generic leader, a GFP cassette, and a myc tag was kindly provided by Dr. M. Wills (University of Cambridge, Cambridge, U.K.) (31).

Reagents

The histone deacetylase (HDAC) inhibitor FR901228 (romidepsin, FK228, or depsipeptide) was provided by the National Cancer Institute (Bethesda, MD). Tunicamycin (T7765), 2-deoxy-D-glucose (D6134), 2-fluoro-2-deoxy-D-glucose (FDG; PS006), nanomannose (M0020), t-mannose (M1134), and iodoacetic acid (IAA; 57840) were all from Sigma-Aldrich.

Small interfering RNA sequences

Small interfering RNAs (siRNAs) were purchased at Eurofins MWG Operon. The sequences used in this study were siControl: 5'-UUA UGU AUU GGA ACG CAU ATT-3'; siGPI_117: 5'-UGG UAC CGG GAC GAG CCG CCG UTT-3'; and siGPI_1520: 5'-CCA ACC AAC UCU AUU GUG UTT-3'.

\(\text{\textsuperscript{13}}\)-Lactate measurements

For determination of 13-lactate in the media, we used the Accutrend Lactate device (Roche) and corresponding BM-lactate test strips (Roche).

Western blotting

Western blotting was done as previously described (29). The Abs used in this study were anti-c-myc (9E10) (sc-40; Santa Cruz Biotechology), anti-GAPDH (TRK54A-6C5) from Research Diagnostics, anti-MICA (AP8626c) from Abgent, anti-ULBP-2 (BAF1298) from R&D Systems, and anti-GFP (3999-100) from Biovision.

PNGase F treatment

Treatment of cell lysates with PNGase F (P7367; Sigma-Aldrich) was performed as recommended by the manufacturer. Briefly, we denatured the cell lysates by heating to 100°C for 10 min. Following cooling of the samples, 0.5 U PNGase F was added to 50 μl denatured cell lysate, and the samples were incubated at 37°C for 2 h.

Trichloroacetic acid precipitation

Protein precipitation using TCA was done as described by Chevallet et al. (32). The cells in this experiment was incubated in RPMI 1640 media with 0.5% FBS. The experiment was terminated by centrifuging the cells at 90 × g for 10 min. The supernatant was transferred to a new tube, and deoxycholate was added to a final concentration of 0.01%. After mixing, TCA was added to a final 7.5% concentration, and the solution was precipitated for 2 h on ice. The precipitate was pelleted at 10,000 × g for 10 min at 4°C and washed twice with 500 μl cold tetradecahydrofuran. Finally, the pellet was redissolved in 100 μl lysis buffer.

Cytolytic assay

The cytolytic assay was performed as previously described (33). As target cells, either Granta B cells induced to express MICA by treatment with FR901228 or TTag9 cells transfected with GFP-MICA*018 was used. In brief, 3 × 10⁶ TTag9 cells were transfected with 1 μg GFP-MICA*018 construct by nucleofection and allowed to recover for 2 h. The cells were then split in two and treated with 20 mM 2DG and 0.5 μM dilmofelyxycarbocyanine (DIO; Invitrogen) for 20 h. Granta B cells were treated with 20 ng/ml FR901228, 20 mM 2DG, and 1 mM D-mannose, and 0.5 μM DIO was added for 12 h. After the treatments, the target cells were mixed with effector cells. As effector cells, we used PBMCs obtained from healthy blood donors and cultured at a concentration of 5 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% human serum (Lonza) and 20 ng/ml IL-15 (R&D Systems) for 3 d. Effector and target cells were mixed at a ratio of 20:1 in 1 ml medium with 10% human serum in a round-bottom microplate. For blocking experiments, a monoclonal NKG2D-blocking Ab (clone 149810; R&D Systems) was added at a final concentration of 2.5 μg/ml. After 4 h incubation, the cells were stained with propidium iodide and immediately analyzed by flow cytometry. To measure cytolytic activity, we gated on DIO-positive cells and recorded the percentage of propidium iodide-positive cells. The NKG2D-specific cytolytic activity was found as the cytolytic activity of a given sample subtracted the cytolytic activity of an equivalent sample containing blocking NKG2D Ab.

Results

2DG inhibits MICA/B expression on the cell surface

HDAC inhibitors induce NKG2D ligands on cancer cells and activated T cells, but not on resting nondividing cells (14). Proliferating cells are characterized by increased glycolysis, and we therefore hypothesized this could be important for NKG2D ligand surface expression. To test this idea, we measured the effect of the glycolytic inhibitor 2DG, a competitive inhibitor of hexokinase, on HDAC inhibitor-mediated expression of the NKG2D ligands MICA/B.

As illustrated in Fig. 1A, 2DG inhibited MICA/B surface expression on Jurkat T cells after treatment with the HDAC inhibitor FR901228 in a dose-dependent manner. As expected, 2DG inhibited cellular glycolysis as measured by the concentration of 1-lactate in the culture medium (Fig. 1B).

To corroborate our findings, we used Jurkat T cells transiently transfected with a MICA promoter construct (29). To our surprise, even high concentrations (20 mM) of 2DG did not affect
FR901228-induced MICA promoter activity (Fig. 1C), strongly suggesting that 2DG affects MICA expression at a point after transcriptional initiation. To test whether 2DG treatment would affect the cell-surface expression of other membrane proteins, we measured the expression of MHC-1 and ICAM-1 on Jurkat T cells after 2DG treatment. As shown in Fig. 1D, 2DG treatment did not affect cell-surface expression of these proteins, suggesting that the downregulation of MICA/B was not due to a general toxic effect of 2DG.

**Inhibition of glycolytic enzymes does not downregulate MICA/B expression**

The metabolism and conversion of glucose to pyruvate/lactate involves several enzymes. We inhibited glycolysis further downstream in the pathway with IAA, a widely used glycolytic inhibitor that inhibits the enzyme GAPDH. As illustrated in Fig. 2A, IAA was not able to inhibit FR901228-induced MICA/B surface expression; in fact, we repeatedly observed a slight increase in the number of MICA/B-positive cells. Measurement of l-lactate production showed that the results were not due to inefficient inhibition of glycolysis (data not shown).
The most straightforward explanation is that an intact glucose metabolism is not important for HDAC inhibitor-induced MICA/B expression. To delineate the mechanism required for MICA/B expression, we targeted glycolysis further upstream by using siRNA against the enzyme GPI that is responsible for the second step in glycolysis converting glucose-6-phosphate to fructose-6-phosphate. Two siRNAs were constructed, siGPI_147 and siGPI_1520, but only siGPI_1520 was able to downregulate GPI as judged by Western blot analysis (Fig. 2C). In line with the IAA experiments, downregulation of GPI had no effect on HDAC inhibitor-mediated MICA/B expression (Fig. 2B).

Hexokinase can also be inhibited by 2FDG, a more potent and specific inhibitor of hexokinase than 2DG (23). 2FDG was not able to inhibit MICA/B expression caused by FR901228 (Fig. 2D), although the inhibition of glycolysis was comparable to 2DG treatment, as judged by the inhibition of l-lactate production (Fig. 2E).

These observations clearly demonstrate that intact glycolysis is not required for HDAC inhibitor-mediated MICA/B surface expression and furthermore suggest that the inhibition of MICA/B expression is not caused by blocking of hexokinase activity.

Inhibition of N-linked glycosylation is responsible for inhibition of MICA/B expression after 2DG treatment

It has recently been shown that 2DG can also interfere with N-linked glycosylation (23), a process that is much less affected by 2FDG. To determine how 2DG prevents MICA/B expression, we took advantage of the findings that inhibition of N-linked glycosylation by 2DG can be effectively reversed by addition of exogenous d-mannose (23).

Jurkat T cells were incubated with 20 mM 2DG in combination with 1 mM d-mannose or 1 mM l-mannose, a biologically inactive stereoisomer of d-mannose. The cells were then treated with FR901228 to induce MICA/B expression. As illustrated in Fig. 3A, the addition of 1 mM d-mannose to 2DG-treated cells completely restored MICA/B expression, whereas addition of 1 mM l-mannose had no effect. Moreover, the ability of 2DG to inhibit glycolysis was not affected by d-mannose as measured by l-lactate production. In conclusion, our results suggest that 2DG blocks HDAC inhibitor-mediated MICA/B surface expression through inhibition of N-linked glycosylation.

**FIGURE 3.** d-Mannose can restore 2DG-inhibited MICA/B expression. A, Jurkat cells were incubated in fresh medium with 20 ng/ml FR901228, 20 mM 2DG, 1 mM d-mannose, and 1 mM l-mannose as indicated (+). Eighteen hours later, the l-lactate concentration in the medium was measured, and the cells were stained with anti-MICA/B-FITC and analyzed by flow cytometry. The bar graph shows mean + SD from three independent experiments. B, HEK293 Phoenix cells were transfected with pMyc-MICA or pmaxGFP. Approximately 1 h later, 2DG and d-mannose were added to the cells at the indicated concentrations. The next day, pMyc-MICA–transfected cells were divided in two and either stained for myc level on the cell surface (1–3) or for intracellular presence of myc (4–6) and analyzed by flow cytometry. C, Jurkat Tag-9 cells were transfected with GFP-MICA*018, and 2 h posttransfection, 10 mM 2DG was added to the cells in combination with 0, 0.01, 0.1, or 1 mM d-mannose. The next day, the cells were lysed, and a fraction was treated with PNGase F as described in Materials and Methods. The samples were then used for Western blotting using anti-MICA Ab diluted 1:1000. D, Cells from C were stained with an anti-myc tag 4A6 Ab and analyzed by flow cytometry. Dot plots show representative results from two independent experiments. E, Jurkat Tag-9 cells were incubated with or without 10 mM 2DG for 24 h in culture medium containing 0.5% FBS. The cells were gently collected by centrifugation and lysed in lysis buffer. The proteins in the media fraction were precipitated by TCA as described in Materials and Methods. Western blotting was performed using the media fractions and cell lysates. The figure shows the blots obtained using anti-MICA Ab diluted 1:250 and anti–ULBP-2 Ab diluted 1:200.
N-linked glycosylation in the ER is frequent, and more than half of the membrane proteins in a cell have this modification (24). Many glycoproteins, however, show no apparent loss of function when glycosylation is prevented. When an effect of glycosylation has been ascribed, it is often associated with protein folding or degradation in the ER (24).

A trivial explanation for the cell-surface downregulation of MICA/B could be that the anti-MICA/B Ab cannot detect the nonglycosylated MICA/B protein, which could still be expressed on the cell surface. To address this issue, we constructed an N-terminal myc-tagged MICA construct under the control of a constitutive CMV promoter; the myc-tag is not glycosylated and can be recognized by Abs regardless of the glycosylation status. This construct, termed pMyc-MICA, was transfected into HEK293 Phoenix cells, and the cells were subsequently treated with 20 mM 2DG or 20 mM 2DG together with 10 mM D-mannose. After 18 h, the cells were split and stained for the presence of surface or intracellular myc-MICA using an anti-myc Ab. As a control, we used a GFP construct (pmaxGFP) for transfection.

We observed a robust expression of myc-MICA fusion protein on the surface of the HEK293 Phoenix cells (Fig. 3B); a similar staining pattern was observed with the anti-MICA/B Ab, verifying that the fusion protein contained intact MICA (data not shown). The constitutive expression of myc-MICA was downregulated from the cell surface after treatment with 20 mM 2DG, and the expression was restored after further addition of D-mannose (Fig. 3B, 3). These results clearly suggest that MICA surface expression is stalled if N-linked glycosylation is inhibited; this effect is quite potent, as it can prevent surface expression caused by overexpression of a MICA construct.

We then speculated whether treatment with 2DG would lead to intracellular accumulation of MICA. To address this question, we performed intracellular staining of pMyc-MICA–transfected HEK293 Phoenix cells. The staining showed that the intracellular expression of myc-MICA was regulated essentially as the cell surface expression, when treated with 2DG and D-mannose (Fig. 3B–D). An obvious explanation for these findings is that inhibition of N-linked glycosylation leads to degradation of MICA or to retention of MICA in a cellular compartment inaccessible to the Ab-staining. 2DG could also inhibit protein production through cellular energy depletion after inhibition of glycolysis; this is most likely not the case as 2DG still blocks glycosylation when D-mannose rescues MICA surface expression (Fig. 3A). We got a very robust GFP expression after transfection with a constitutive GFP plasmid, pmaxGFP (Fig. 3B), and there was a minor decrease in GFP expression of 2DG-treated cells (Fig. 3B). Inhibition of glycosylation may thus to some extent reduce expression of very highly expressed molecules, but cannot account for the nearly complete block of MICA expression. As illustrated in Fig. 3B (9), D-mannose had no effect on expression of GFP (GFP is not N-linked glycosylated).

To further investigate MICA protein production in cells treated with 2DG, we transfected Jurkat cells with a GFP and myc-tagged MICA construct (GFP-MICA*018) and treated these cells with 2DG and increasing amounts of D-mannose (0 to 1 mM). The next day, a small fraction of cells were reserved for FACS analysis (Fig. 3D), and the remaining cells were lysed and subjected to Western blotting using anti-GFP Ab. As illustrated in Fig. 3C, 2DG treatment leads to the production of a faster migrating GFP-MICA protein when compared with the sample without 2DG. Addition of D-mannose to the media makes the slower form reappear. We speculated that the faster migrating band represents a deglycosylated form of MICA, and therefore, we treated the cell lysates from Fig. 3C, lanes 2 and 3, with PNGase F, an enzyme that removes N-linked glycans, and did another anti-GFP Western blot on these samples. As illustrated in the left panel of Fig. 3C, the GFP-MICA band from 2DG-treated cells migrates together with the PNGase F-treated sample, suggesting that the low m.w. form indeed represents a less N-linked glycosylated form of MICA.

Another mechanism to the observed downregulation of MICA from the cell surface of cells treated with 2DG may be due to shedding of MICA into the media. To test for this, we incubated Jurkat cells for 24 h with or without 2DG and precipitated the proteins in the media using TCA, and cells were lysed in standard lysis buffer. A Western blot on the media fractions and the corresponding cell lysates was done using anti-MICA and anti–ULBP-2 Abs, and as shown in Fig. 3E, we could not detect any shedding of MICA in both untreated and 2DG-treated media fractions, whereas some shedding of ULBP-2 was observed. The shedding of ULBP-2 was, however, decreased from cells treated with 2DG. This suggests that 2DG-mediated downregulation of MICA from the cell surface is not due to increased shedding.

N-linked glycosylation regulates MICA/B expression on the cell surface

To further substantiate our findings, we examined the effect of tunicamycin, an inhibitor of N-acetylglucosamine transferases. As illustrated in Fig. 4A, tunicamycin completely inhibited MICA/B surface expression after FR901228 treatment, and, as expected, this inhibition could not be reverted by D-mannose (D-mannose does not reverse the action of tunicamycin). Tunicamycin did not affect transient GFP expression in Jurkat T cells, showing that the effect is not caused by gross toxicity or loss of cellular integrity (Fig. 4B).

Inhibition of N-linked glycosylation downmodulates MICA/B surface expression in different cancer cells

To see if 2DG generally downmodulates MICA/B surface expression, we tested a variety of different cell types. Fig. 5A shows that 2DG effectively downmodulated FR901228-mediated MICA/B expression in Granta (mantle cell lymphoma), PC3 (prostate cancer), and, to a lesser extent, CEM (T-ALL) and HaCaT (human keratinocyte-derived) cells. D-mannose potently alleviated the reduced MICA/B expression in all of the cells lines mentioned. Addition of D-mannose instead of D-mannose did not affect the 2DG downmodulation, demonstrating integrity of the assay. Neither FR901228 nor 2DG affected MICA/B expression in Ramos cells (Burkitt B cell lymphoma).

2DG inhibited glycolysis (measured by lactate production) in all the cells tested, with Ramos cells being the striking exception. As expected, D- or L-mannose did not affect 2DG inhibition of lactate production (Fig. 5B).

To test the effect of 2DG on cancer cells showing high basal expression of MICA/B, two melanoma cell lines were treated with 2DG and D-mannose. The data presented in Fig. 5C show that 2DG also was able to downmodulate basal MICA/B expression in both FM78 and FM86 cells and that D-mannose potently alleviated this effect.

These data suggest that 2DG generally inhibits MICA/B expression in several types of cancer through inhibition of N-linked glycosylation.

Surface expression of NKG2D ligands is dependent on N-linked glycosylation

To verify that N-linked glycosylation is important for surface expression of MICA/B and potentially other NKG2D ligands, we...
used the native receptor NKG2D coupled to the Fc portion of IgG (NKG2D-Fc) for surface staining.

As previously demonstrated (14, 34, 35), HDAC inhibitors induce surface expression of the NKG2D ligands MICA, MICB, and ULBP2. Fig. 6A show that FR901228 induce a robust surface expression of NKG2D ligands on Jurkat T cells, as measured by the interaction with the NKG2D-Fc receptor. NKG2D ligand expression was fully inhibited by 2DG and further inclusion of α-mannose prevented this downregulation.

This clearly indicates that the HDAC inhibitor-induced NKG2D ligands are dependent on N-linked glycosylation for transport to the cell surface and further demonstrate that no functional (less glycosylated) NKG2D ligands can be detected on the cell surface.

To corroborate the functional significance of these findings, we performed a cytolytic assay with both Granta-519 and Jurkat cells. On Granta-519 cells, MICA expression was induced by FR901228 treatment, and Jurkat cells were transfected with a MICA plasmid. Subsequently, the cells were treated with 2DG. It was found that 2DG treatment of target cells increased NK cell-dependent cytolysis.

Therefore, to calculate the NKG2D-specific cytolytic activity, we measured the overall activity and subtracted the activity remaining after addition of an NKG2D-blocking Ab. Fig. 6B illustrates that treatment of Granta cells (left panel) and Jurkat cells (right panel) with 2DG prior to mixing with effector cells potently inhibited the NKG2D-induced cell death.

These experiments show that inhibition of N-linked glycosylation results in a decreased cell-surface expression of functional NKG2D ligands, which prevents NKG2D-specific cell death.

**Discussion**

Induction of stress-regulated NKG2D ligands on the cell surface postinfection or post-tumor transformation is critical for the initial recognition and activation of the immune system (5). To escape immune recognition, foreign cell invaders or precancer cells can either actively suppress or evolve toward a phenotype leading to diminished function of the NKG2D/NKG2D ligand system. In contrast, certain overreactions of the immune system (e.g., during rheumatoid arthritis) (36) are characterized by increased NKG2D ligand expression. It is therefore of particular interest to understand how homeostasis of the NKG2D/NKG2D ligand system is maintained and elucidate the interfering pathological mechanisms, a knowledge that is essential for future therapeutic targeting.

Cancer cells and activated T cells are characterized by a high level of glycolysis. Initially, we wanted to elucidate the role of enhanced glycolysis for NKG2D ligand expression. The glucose mimetic 2DG potently blocked HDAC inhibitor-mediated MICA/B surface expression; however, in addition to glycolysis, 2DG can also inhibit N-linked glycosylation (23). The inhibition of N-linked glycosylation can specifically be alleviated by coadministration of α-mannose. It is therefore simple to distinguish between the two scenarios. Our data strongly suggest that blocking of N-linked glycosylation is responsible for hindering MICA/B surface expression after 2DG treatment, a finding corroborated by experiments with the specific inhibitor of N-linked glycosylation, tunicamycin. In line with these data, siRNA targeting of the glycolytic enzyme GAP and pharmacologic inhibition of glycolysis by IAA did not affect MICA surface expression. Our data, therefore, firmly establish that N-linked glycosylation, and not glycolysis, is important for NKG2D ligand surface expression. Different cell types were tested, and 2DG inhibited constitutive or HDAC inhibitor-induced MICA/B surface expression, suggesting that the effect is not specific for Jurkat T cells. Functional NK cell killing assay and labeling with recombinant NKG2D–Fc verified that 2DG, as expected, inhibited functional NKG2D ligand expression.

To establish if N-linked glycosylation is important for MICA gene activation or posttranscriptional modulation, we transiently transfected Jurkat T cells with a MICA promoter construct and showed that HDAC inhibitor-induced MICA promoter activity was not affected by either 2DG or tunicamycin. This suggests that the inhibition of MICA/B surface expression occurred at a posttranscriptional level.

Measurement of secreted soluble MICA and ULBP-2 showed that 2DG treatment did not lead to increased shedding, suggesting that 2DG-mediated decrease in NKG2D ligand surface expression is due to intracellular sequestration and not increased shedding.

We have previously shown that propionibacteria induce MICA/B surface expression through production of propionate (33). Intriguingly, propionate-mediated MICA/B surface expression is dependent on hexokinase activity and the pentose phosphate pathway, which is in contrast to HDAC inhibitor-mediated MICA/B expression (33). This suggests that different molecular pathways can regulate MICA/B expression.

Besides HDAC inhibitors and propionate, a variety of other signals lead to MICA/B expression, including DNA damage, viral infection, and heat shock (37–39). To get an overall assessment of the involvement of N-linked glycosylation in MICA surface ex-
pression, we made a construct encoding MICA controlled by the constitutive CMV promoter. Transient transfection with this construct made cells MICA surface positive without any additional stress or stimulation. Despite the constitutive promoter, 2DG readily inhibited MICA surface expression on HEK293 Phoenix cells and Jurkat T cells by a mechanism alleviated by D-mannose, again supporting that N-linked glycosylation is critically involved in the posttranscriptional transport of MICA/B to the cell surface. The direct N-linked glycosylation of MICA was further verified by experiments with the endoglycosidase PNGase F that specifically targets N-linked glycoproteins. Treatment with 2DG of transiently transfected MICA altered migration in SDS-PAGE similar to PNGase F treatment. Importantly, 2DG in combination with PNGase F did not further affect the size of MICA. This strongly suggests that MICA is N-linked glycosylated through a mechanism that can be inhibited by 2DG.

Our experiments had the potential caveat that the Ab used to detect MICA/B molecules on the cell surface might only detect the fully glycosylated MICA/B protein, meaning that an altered or deglycosylated form of MICA/B could be expressed on the cell surface despite inhibition of N-linked glycosylation. This is, however, most likely not the case because: 1) the native receptor NKG2D–Fc fusion protein did not bind to HDAC inhibitor-treated cells after 2DG treatment; binding was re-established after further...
and according to the crystal structure, all are accessible for glycosylation. FR901228, 20 mM 2DG, and 1 mM D-mannose as indicated for 18 h. A linked glycosylation. 2DG and 1 mM D-mannose. Granta-519 cells were treated for 12 h, treated with 20 ng/ml FR901228 to induce MICA/B expression or Jurkat experiments. Selected results are shown as dot plots. The cells were stained with human NKG2D–Fc and analyzed by flow cytometry. The bar graph shows mean + SD from two independent experiments. Selected results are shown as dot plots. B, Granta-519 cells treated with 20 ng/ml FR901228 to induce MICA/B expression or Jurkat Tag9 cells transfected with GFP-MICA*018 were treated with 20 mM 2DG and 1 mM D-mannose. Granta-519 cells were treated for 12 h, whereas transfected Jurkat tag9 cells were treated for 20 h. These cells were then used as target cells in a cytolytic assay with PBMCs as effector cells. The bar graph in the left panel shows the mean + SD of NKG2D-specific cytolytic activity toward Granta-519 cells run in duplicate. The bar graph in the right panel shows the NKG2D-specific cytolytic activity (mean + SD) toward GFP-MICA*018–transfected Jurkat Tag-9 cells run in duplicate. The NKG2D-specific cytolytic activity was calculated as described in Materials and Methods. For Jurkat cells, similar results were obtained using pMyc-MICA transfactions.

addition of D-mannose, clearly demonstrating that 2DG blocks functional NKG2D ligand expression; and 2) transiently expressed Myc-tagged MICA led to robust surface expression of MICA that could be inhibited by 2DG. Inhibition was also observed when MICA was detected using a Myc-specific Ab. Because the Myc tag is not glycosylated, this strongly suggests that the tagged MICA construct is not transported to the cell surface after inhibition of N-linked glycosylation. Likewise, Spreu et al. (40) have previously demonstrated that cell-surface expression of transiently expressed MICB in COS-7 cells is reduced by tunicamycin treatment.

Strong overexpression of MICA in both HEK293 and Jurkat cells overruled the 2DG inhibitory effect of MICA cell-surface expression (data not shown). We believe that this is due to overloading of the inhibitory system that otherwise blocks the cell-surface transport of deglycosylated MICA.

Involvement of glycosylation in intracellular protein trafficking is firmly established. The importance of glycosylation is, however, often less clear, as many normally glycosylated proteins show no apparent loss of function when glycosylation is prevented (24). MICA has eight different potential N-linked glycosylation sites, and according to the crystal structure, all are accessible for glycosylation (41). The molecular mechanism behind 2DG inhibition of MICA remains to be established, and it is important to note that our current data cannot reveal how N-linked glycosylation regulates MICA surface expression; it could be through glycosylation of MICA itself or through an unidentified protein likely residing in the ER.

It could be speculated that 2DG inhibition of MICA expression is due to other and more trivial effects than decreased N-linked glycosylation. 2DG (e.g., leads to growth inhibition and ER stress); however, ER stress and growth inhibition are also caused by HDAC inhibitors, which opposite to 2DG directly induce MICA surface expression (14). It is thus unlikely that there is a simple correlation between general ER stress or growth inhibition and decreased MICA surface expression after 2DG treatment.

HDAC inhibitors, which have shown striking effects as anticancer therapeutics (42), induce NKG2D ligand expression in various cancer cells (14, 34, 35). It is tempting to speculate that the clinical efficacy of HDAC inhibitors in part stems from their potent induction of NKG2D ligands. 2DG is currently in clinical trials for use as a chemotherapeutic agent. Several studies have furthermore shown a synergistic effect of 2DG in combination with other cancer therapeutics, including HDAC inhibitors (43, 44). Therapeutic synergy has been shown between a novel HDAC inhibitor LAQ824 (that can pass the blood–brain barrier) and 2DG in treatment of glioblastoma (44). We would predict that this regimen will limit the NKG2D ligand surface expression on glioblastoma cells; however, the potential implication of this needs to be established. Our current study advise some caution with the use of 2DG, especially if it turns out that the specific therapeutic setting is dependent on NKG2D ligand expression; alternatively, inclusion of D-mannose with 2DG may be an option. Lack of NKG2D ligand induction may hamper an otherwise beneficial immune attack and immunological memory against the cancer.

In contrast, several forms of cancer, including some prostate cancers, B-CLL, and melanomas, secrete large amounts of soluble NKG2D ligands—in particular, ULBP-2 (45–47). The soluble ULBP-2 paralyzes the NK and CD8 cell-dependent activation by downmodulating expression of NKG2D, and it is well established that soluble ULBP-2 is associated with poor prognosis (45, 46). In these particular circumstances, it may be beneficial that 2DG inhibits ULBP-2 expression and its secretion (Fig. 3E). It is furthermore interesting that we observe increased NK cell killing of 2DG-treated Granta and Jurkat cells through an NKG2D ligand–independent pathway. The nature of this 2DG immunostimulatory pathway is currently elusive and under examination in our laboratory.

Both cancer and infection can lead to altered glycosylation of cellular proteins (26–28). There are undoubtedly several explanations for this; it is likely that a part of the changes in cellular glycosylation patterns may be associated with altered expression of NKG2D ligands, possibly caused by an evolutionary pressure to limit immune recognition. A recent study by Fuertes et al. (15) has shown that some melanoma cells have low MICA surface expression despite possessing MICA transcripts. These melanomas were positive for intracellular MICA, suggesting that surface expression was hindered by a posttranslational mechanism. Although the molecular mechanism behind this observation needs to be established, Fuertes et al. (15) interestingly found that intracellular MICA in the affected melanomas was more sensitive to endoglycosidase H. This enzyme primarily deglycosylate proteins with a less mature N-glycosylation pattern (high mannose form), implying that the melanomas might affect MICA surface expression by interfering with N-linked glycosylation.

In contrast to stressed or transformed cells, healthy cells have very low NKG2D ligand promoter activity. If stressed cells avoid NKG2D
ligand surface expression by altering glycosylation, it may open up for selective therapeutic intervention. Correcting or forcing a glycosylation pattern that allows surface expression of NKG2D ligand surface will, quite specifically, result in recognition of stressed cells that have otherwise evolved to be invisible to the immune system.

Acknowledgments

We thank Prof. K. Helin, University of Copenhagen, for the pCMV-Myc plasmid, Dr. M. Wills, University of Cambridge, for the GFP-MICA*018 plasmid, and Dr. Per thor Straten, University Hospital Herlev, for melanoma cell lines.

Disclosures

The authors have no financial conflicts of interest.

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