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doi: 10.1074/jbc.M114.591537 originally published online September 25, 2014

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The Selenium Metabolite Methylselenol Regulates the Expression of Ligands That Trigger Immune Activation through the Lymphocyte Receptor NKG2D*

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Background: Immune activation through a balanced cell surface expression of human NKG2D ligands is crucial for the elimination of diseased cells.

Results: Methylselenol induces the expression of the NKG2D ligands MICA/B but specifically inhibits ULBP2 protein expression.

Conclusion: Methylselenol regulates NKG2D ligand expression on transcriptional and posttranscriptional levels.

Significance: Methylselenol is the first identified metabolite that diversely regulates NKG2D ligands, and, therefore, its implementation could improve NKG2D-based immune therapy.

For decades, selenium research has been focused on the identification of active metabolites, which are crucial for selenium chemoprevention of cancer. In this context, the metabolite methylselenol (CH₃SeH) is known for its action to selectively kill transformed cells through mechanisms that include increased formation of reactive oxygen species, induction of DNA damage, triggering of apoptosis, and inhibition of angiogenesis. Here we reveal that CH₃SeH modulates the cell surface expression of NKG2D ligands. The expression of NKG2D ligands is induced by stress-associated pathways that occur early during malignant transformation and enable the recognition and elimination of tumors by activating the lymphocyte receptor NKG2D. CH₃SeH regulated NKG2D ligands both on the transcriptional and the posttranscriptional levels. CH₃SeH induced the transcription of MHC class I polypeptide-related sequence MICA/B and ULBP2 mRNA. However, the induction of cell surface expression was restricted to the ligands MICA/B. Remarkably, our studies showed that CH₃SeH inhibited ULBP2 surface transport through inhibition of the autophagic transport pathway. Finally, we identified extracellular calcium as being essential for CH₃SeH regulation of NKG2D ligands. A balanced cell surface expression of NKG2D ligands is considered to be an innate barrier against tumor development. Therefore, our work indicates that the application of selenium compounds that are metabolized to CH₃SeH could improve NKG2D-based immune therapy.

NKG2D ligands are induced on the cell surface of a variety of stressed, transformed, and infected cells, whereas the expression on healthy human cells is low. The immune system recognizes NKG2D ligand-positive cells through the NKG2D receptor, a major activating receptor expressed on natural killer cells, NKT cells, CD8⁺ T cells, γδ T cells, and some activated CD4⁺ T cells (1–4). There are eight different human NKG2D ligands described, belonging to the MIC (MICA and MICB) and UL16-binding protein (ULBP1–6) families (5). All ligands are MHC class I-related glycoproteins (6). Different forms of cellular stress result in increased NKG2D ligand surface expression, including heat shock, virus infection, inflammatory cytokines, histone deacetylase (HDAC) inhibitors, propionic acid, retinoic acid, proteasome inhibitors, Toll-like receptor (TLR) signaling, and DNA damage response (7–17). Moreover, surface expression of NKG2D ligands on a variety of tumors derived from different origins present an attractive target for anticancer therapy (18–20).

Selenium is a fundamental nutrient in the human diet. Uptake of 50–60 μg of the trace element per day is recommended for healthy adults (21). In the body, ingested selenium is metabolized to a variety of low molecular weight compounds and selenoproteins. In the latter case, selenium is incorporated as selenocysteine. The low molecular weight compounds are divided into organic and inorganic forms, and both can be used as nutritional and supplemental sources. Inorganic selenium is mainly represented by selenate and selenite, whereas the selenoamino acids selenomethionine (SeMet)³ and selenium methylselenocysteine (MSC) are members of organic selenium forms and can be identified in vegetables such as garlic and onions (22, 23). Methylselenic acid (MSA) is a synthetic sele-
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Selenium compounds are divided into organic and inorganic forms. The metabolism of these organic and inorganic selenium compounds is complex and closely regulated, with two key metabolites: Selenide (H₂Se) and methylselenol (CH₃SeH). On the basis of the current understanding of the selenium metabolism, NKG2D ligands are only regulated by CH₃SeH-generating selenium compounds.

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**SCHEME 1. Regulation of NKG2D ligands by different selenium compounds.** Selenium compounds are divided into organic and inorganic forms. The metabolism of these organic and inorganic selenium compounds is complex and closely regulated, with two key metabolites: Selenide (H₂Se) and methylselenol (CH₃SeH). On the basis of the current understanding of the selenium metabolism, NKG2D ligands are only regulated by CH₃SeH-generating selenium compounds.

with H₂Se (36). Selenium intervenes with two hallmarks of cancer: apoptosis and angiogenesis. Studies have shown that selenium compounds, most likely CH₃SeH, can induce caspase-mediated apoptosis in cancer cells (37–39). Angiogenesis is affected by CH₃SeH precursors because of its inhibition of VEGF from several cancer cell lines (40). Other studies have shown that metabolites of MSC and SeMet inhibit HDAC activity (41, 42) and that CH₃SeH is able to inhibit PKC activity by redox modifications of cysteines (43, 44). More recently, selenium compounds have also been applied in cancer treatment (33). An anticancer effect was restricted to selenium compounds that could generate active metabolites during in vivo metabolism. In this context, preclinical as well as clinical trials showed that SeMet (45), MSA (46), and selenite (47, 48) mediated tumor suppression. Moreover, adjunct selenium therapy in addition to chemotherapy caused a synergistic effect regarding the induction of apoptosis and improvement of the overall clinical outcome of cancer patients (49).

Autophagy is an evolutionary ancient pathway that ensures that cells can maintain their cell-autonomous homeostasis through the removal of intracellular material by lysosomal degradation (50, 51). Moreover, autophagy is utilized by infected cells to eliminate intracellular pathogens and likely serves as one of the earliest forms of eukaryotic defense against intracellular pathogens (52). Autophagy is characterized by the translocation of microtubule-associated protein 1 light chain 3 (LC3) from the cytoplasm to the autophagosome, where it is targeted to the lysosome for degradation (53).

We have shown previously that the synthetic selenium compound MSA modulates NKG2D ligands (54). In this study, we investigated the effect of different selenium compounds, metabolites, or intermediates with regard to expression of NKG2D ligands, and we identified CH₃SeH as a key metabolite involved in the regulation of NKG2D ligands.

**EXPERIMENTAL PROCEDURES**

**Cells**—Two Jurkat T cell lines were used in this study. Jurkat E6-1 was purchased from the ATCC, and Jurkat Tag-9 was provided by Dr. Carsten Geisler (Department of International Health, Immunology, and Microbiology, University of Copenhagen, Denmark). Jurkat Tag-9 cells are stably transfected with the large T antigen from SV40, and they were used primarily for transient transfection studies. Jurkat cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM glutamine, and 2 mM penicillin and streptomycin. U2OS cells (human osteosarcoma) were purchased from Millipore as part of the FlowCellekt™ GFP-LC3 reporter autophagy assay kit (catalog no. CF200096). U2OS cells were cultured in 10% DMEM + GlutaMAX with 2 mM penicillin and streptomycin, 1× non-essential amino acids (from 100× stock), 10 mM HEPES, and 250 mg/ml geneticin. All cells were incubated at 37 °C and 5% CO₂.

**Reagents**—FR901228 was provided by the NCI, National Institutes of Health (Bethesda, MD). L-selenomethionine (SeMet) (catalog no. 561505) was from Calbiochem. Sodium selenite (selenite) and sodium selenate (selenate) (catalog no. SS261), SeCys₂ (catalog no. M6680), MSC (catalog no. M6680), dimethyl diselenide (DMDSe) (catalog no. 328502), MSA (cat-
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alog no. 541281), EGTA (catalog no. E8389), wortmannin (catalog no. W1628), 3-methyladenine (catalog no. M9281), MG132 (catalog no. M7449), and propidium iodine (PI) (catalog no. P4170) were from Sigma-Aldrich. l-methionine γ-lyase (catalog no. 42616-25-1) was from Wako Pure Chemical Industries. TRizol reagent (catalog no. 15596-026) was from Invitrogen.

Transient Transfections and Constructs—Jurkat Tag-9 cells were transiently transfected using Nucleofector technology (Lonza). In brief, Jurkat Tag-9 cells were resuspended in 100 μl of Ingenio electroporation solution (Mirus Bio LLC), mixed with 1 μg of plasmid/1 × 10⁶ cells, and pulsed using the Nucleofector program G-010. The MICA promoter plasmid p3.2k-WT-GFP and the +2 control plasmid have been described previously (55). Promoter activity was calculated by multiplying the percentage of GFP-expressing cells with the mean fluorescence of these cells. The plasmids Ub-M-GFP (catalog no. 11938), Ub-R-GFP, catalog no. 11939), and Ub-G76-GFP (catalog no. 11941) used to study the proteasome inhibition were purchased from Addgene.

Flow Cytometry—Cells were washed twice in cold PBS and stained with the specific antibodies at a dilution of 1:100 for 30 min at 4 °C. Following antibody incubation, the cells were washed, resuspended, and analyzed in PBS. For staining of dead cells, the cells were incubated with 1 μg/ml PI for at least 5 min at room temperature prior to analysis. Staining of apoptotic cells by Annexin V was carried out by washing and staining the cells in buffer containing 10 mM Hepes (pH 7.4), 0.14 M NaCl, and 2.5 mM CaCl₂. Lysosome accumulation was investigated by staining the cells with 4-nitro-7-(1-piperazinyl)-2,1,3-benzoiazadizole according to the protocol of the manufacturer (Cayman Chemical Co., catalog no. 600310). In brief, 4-nitro-7-(1-piperazinyl)-2,1,3-benzoiazadizole (1:1000)-stained cells were incubated for 10 min at 37 °C, centrifuged, and resuspended in cell-based assay buffer. The flow cytometry data acquisition was performed on a BD Accuri C6 flow cytometer and CFlow software, and the analysis of the collected data was carried out using FlowJo software, version 3.4 (PerkinElmer Life Sciences), and the chromatographic data were handled by TotalChrom software (PerkinElmer Life Sciences).

Proteasome Sensor Vector Kit Assay—The induction of proteasome inhibition after MG132, FR901228, and MSA treatment was investigated by using the proteasome-sensitive fluorescent reporter ZsProSensor-1 according to the protocol of the manufacturer (Clontech, catalog no. 632425). In brief, 3 × 10⁶ Jurkat Tag-9 cells were transfected as described above and incubated with 0.2 μM MG132, 20 ng/ml FR901228, and 5 μM MSA for 18 h. The cells emitted green fluorescence when there was a drop in proteasome activity, which was analyzed by flow cytometry.

GFP-LC3 Reporter Autophagy Assay—The induction of autophagy after FR901228 and MSA treatment was investigated by using a FlowCelllect™ GFP-LC3 reporter autophagy assay kit (Millipore, catalog no. FCCH100181) according to the protocol of the manufacturer. In brief, 30,000 U2OS cells were seeded into a 96-well plate. Duplicates of adhered cells were treated with either 20 ng/ml FR901228 or 5 μM MSA for 18 h or were left untreated. After the treatment, medium was aspirated, and cells were washed once with 1 × Hanks’ balanced salt solution. One copy of FR901228, MSA, or untreated cells was incubated for 2 h with 200 μl of plating medium containing autophagy reagent A (100 μM), and the second half was kept in culture medium. Post-incubation, the plating medium was aspirated, and cells were washed once with 200 μl of 1 × Hanks’ balanced salt solution. Cells were detached using versene, resuspended in 200 μl of fresh culture medium, and transferred to a new 96-well (U-bottom) plate. Cells were pelleted by cen-
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RESULTS

Methylselenol-generating Selenium Compounds Induce MICA/B Surface Expression—The monomethylated, synthetic selenium compound MSA is directly reduced to CH$_3$SeH (26) and induces the expression of the NKG2D ligands MICA/B (54). To investigate the role of CH$_3$SeH in relation to NKG2D ligand regulation, different selenium compounds were tested for their ability to modulate surface expression of the NKG2D ligands. The tested compounds included MSC, which is converted to CH$_3$SeH by the enzyme β-lyase; DMDSe, which is converted directly to CH$_3$SeH; SeMet and SeCys$_2$, which are primarily converted to H$_2$Se or directly converted to CH$_3$SeH by γ-lyase activity (SeMet + γ-lyase); as well as selenite and selenate, which are precursors of H$_2$Se (Scheme 1). Jurkat E6-1 cells were incubated with the different selenium compounds for 18 h, and surface expression of MICA/B and ULBP2 was analyzed by flow cytometry. Jurkat T cells display a low basal level of MICA/B and a high basal level of ULBP2 cell surface expression. A clear trend emerged when the different selenium compounds were tested. All selenium compounds metabolized into CH$_3$SeH, MSA, MSC, and DMDSe induced MICA/B but not ULBP2 surface expression (Fig. 1A). MSA induced maximal MICA/B expression at a concentration of 500 μM, which was ~100 times higher compared with DMDSe and MSA (5 μM).
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This is most likely due to limited expression of the β-lyase needed for the conversion into CH$_3$SeH (56). The precursors of H$_2$Se, selenite, selenate, SeCys$_2$, and SeMet had no effect on MICA/B or ULBP2 surface expression (Fig. 1B). In these experiments, the HDAC inhibitor FR901228 (20 ng/ml) and MSA (5 μg/ml) were used as positive controls. The different concentrations of each selenium compound were on the basis of our previous studies (57) as well as on work performed by other researchers in the field (26). Treatment of Jurkat T cells with the highest concentration of the selenium compounds MSA, MSC, selenite, and selenate induced cell death in 10–20% of the cells (Fig. 1C). Notably, all tested selenium compounds caused less cell death compared with the HDAC inhibitor FR901228, which is well known to regulate NKG2D ligands (16). To further assure that selenium regulation of NKG2D ligands occurs in living cells, we additionally show that MICA/B up-regulation after MSA treatment only occurs in viable, non-apoptotic cells, whereas apoptotic cells (Annexin V$^+$) did not up-regulate MICA/B (Fig. 1D, left and center panels). In previous experiments we have shown that FR901228 only increases NKG2D ligand surface expression of non-apoptotic cells (58, 59). Therefore, treatment with FR901228 was used as a control (Fig. 1D, right panel). To strengthen our hypothesis that CH$_3$SeH is required for MICA/B surface expression, we tested whether the combined treatment of SeMet and γ-lyase induced the surface expression of MICA/B in Jurkat E6-1 cells. We performed this experiment because SeMet is converted to CH$_3$SeH in the presence of γ-lyase activity. As shown in Fig. 1E, left panel, the combined treatment of 5 μM SeMet and 0.02–0.04 U γ-lyase, but not SeMet alone, resulted in MICA/B surface expression. Again, no change in ULBP2 surface expression was observed (Fig. 1E, right panel). Our results strongly suggest that the generation of CH$_3$SeH during the treatment with selenium compounds is required for cell surface induction of the NKG2D ligands MICA/B.

Methylselenol Activates the Transcription of the NKG2D Ligands MICA and ULBP2—We and others have shown that NKG2D ligands can be regulated on the transcriptional level (55, 60, 61). To investigate whether CH$_3$SeH also causes this transcriptional activation of NKG2D ligands, Jurkat Tag-9 cells were transiently transfected with a MICA promoter containing plasmid as well as a promoterless (+2) variant. Post-transfection, the cells were treated with MSC, selenite, 20 ng/ml FR901228 (control), and 5 μM MSA (control) for 18 h. The promoter activity was calculated by multiplying the percentage of GFP-expressing cells with the mean fluorescence of these cells (55). The CH$_3$SeH-generating compound MSC directly stimulated the transiently transfected MICA promoter construct, whereas selenite had no effect (Fig. 2A). Non-transfected and selenite-treated cells showed a slight increase in fluorescence intensity compared with the other untransfected cells. This might be due to increased autofluorescence, a recognized effect of selenite treatment (62). However, this effect is likely quenched by the strong GFP signal during the transfection experiments. To elaborate these findings, we tested NKG2D ligand mRNA induction after the treatment with CH$_3$SeH, and non-CH$_3$SeH-generating selenium compounds. For this experiment, Jurkat E6-1 cells were treated with (10 μM) selenite, 20 ng/ml FR901228 (control), and 5 μM MSA (control) for 4 h. RNA was isolated and reverse-transcribed into cDNA, followed by real-time quantitative PCR analysis. The level of MICA and ULBP2 mRNA was measured using the housekeeping gene RPLP0, and displayed as the fold change relative to the control. Data (mean ± S.D.) are representative of at least three separate experiments.

![Figure 2. Methylselenol activates the transcription of the NKG2D ligands MICA and ULBP2](http://www.jbc.org/content/289/45/31580.full.pdf)

**A** Jurkat Tag-9 cells were transfected with either the 3.2-kb wild-type MICA promoter construct or the +2 control construct. After 24 h, cells were treated with 20 ng/ml FR901228, 5 μM MSA, 500 μM MSC, and 10 μM selenite for 18 h. Cells were analyzed for their expression of GFP by flow cytometry. **B** Jurkat E6-1 cells were left untreated or treated with 20 ng/ml FR901228, 5 μM MSA, or 10 μM selenite. After 4 h, total RNA was extracted and used for quantitative real-time PCR analysis. MICA and ULBP2 mRNA expression was normalized to the housekeeping gene RPLP0 and displayed as the fold change relative to the control. Data (mean ± S.D.) are representative of at least three separate experiments.

Methylselenol-generating Selenium Compounds Inhibit ULBP2 Surface Expression after Treatment with the HDAC Inhibitor FR901228—The results stated above indicate that CH$_3$SeH has no effect on the surface expression of ULBP2. Interestingly, the induction of surface-expressed ULBP2 after HDAC inhibitor treatment was inhibited when treated in combination with MSA (54). Therefore, we tested whether this inhibition is caused by CH$_3$SeH-generating selenium compounds.
To this end, Jurkat E6-1 cells were treated for 18 h with MSC, DMDSe, selenite, and SeMet in combination with FR901228 (20 ng/ml). The cell surface expression of NKG2D ligands was analyzed by flow cytometry. As seen in Fig. 3C, left panel, selenite did not affect the expression of NKG2D ligands, whereas the combination of FR901228 and MSC or DMDSe strongly inhibited the cell surface expression of ULBP2 (Fig. 3B). Here it should be noted that SeMet exhibited a dose-dependent inhibition of both FR901228-induced MICA/B and ULBP2 (Fig. 3C, right panel). This is likely due to an increasing number of cells going into apoptosis. These results further imply that only CH$_3$SeH-generating selenium compounds can regulate the expression of NKG2D ligands.

The Induction of the NKG2D Ligands MICA/B Is Not Caused by CH$_3$SeH Generated from HDAC Inhibitors—CH$_3$SeH can be generated in cells through metabolism (33). Given the similarities between the effect of both HDAC inhibitors and CH$_3$SeH on the induction of the NKG2D ligands MICA/B, we wanted to investigate whether HDAC inhibitors in general regulate NKG2D ligands through the generation of CH$_3$SeH. This was,
however, not the case. As shown in Fig. 4A, no detection of selenium-metabolized products was observed in the FR901228-treated samples, arguing against the generation of CH$_3$SeH during FR901228 metabolism.

The NKG2D Ligand-regulating Effect of CH$_3$SeH and an HDAC Inhibitor Is Stable in Aqueous Solution—Preclinical studies in nonhuman primates as well as reports from a phase I trial in adults describe the FR901228 terminal half-life to be limited to a maximal 8 h (63, 64). Furthermore, studies suggest that infusions of FR901228 over a time period of 1–4 h are most effective in terms of cytotoxicity toward multiple tumor cell lines but at the same time are least toxic (65–67). The compounds formed after MSA treatment, CH$_3$SeH, dimethylselenide, and DMDSe, are considered to be highly volatile and difficult to detect (68). Therefore, we examined whether MSA or FR901228 diluted in different aqueous solutions for several days would alter their regulation of NKG2D ligands compared with freshly prepared solutions. As seen in Fig. 4B, 4 days of preincubation of 20 ng/ml FR901228 or 5 μM MSA in culture medium with 10% FBS or H$_2$O did not affect their ability to induce NKG2D ligands when compared with freshly prepared MSA or FR901228. Similar results were observed with the compounds diluted in PBS or culture medium without FBS (data not shown). Therefore, the cellular effects of MSA and FR901228 are stable for 4 days in serum-containing media, which is of significant interest for therapeutic applications. The double peaks seen in the histograms could be due to the fact that a fraction of the cells will undergo apoptosis caused by the different treatments, as shown in Fig. 1D.

Methylselenol Affects the Expression of NKG2D Ligands by Regulating Autophagy—The results stated above highlight the regulatory potential of CH$_3$SeH on the induction as well as inhibition of NKG2D ligands. This regulatory pathway is clearly distinct from the HDAC inhibitor FR901228. We then focused our attention on the mechanism used by CH$_3$SeH to modulate NKG2D ligands. Because the stable, monomethylated compound MSA was developed specifically to generate CH$_3$SeH just by reduction (26), all further CH$_3$SeH-requiring experiments were performed using MSA. CH$_3$SeH is described to act both as an oxidant and antioxidant as well as an inducer of
endoplasmic reticulum stress. Therefore, compounds such as N-acetyl-cysteine (NAC) and vitamin E (antioxidants); buthionine sulfoximine (BSO) (oxidant); and thapsigargin, aristolochic acid, arsenic trioxide, and DTTC (endoplasmic reticulum stressors) were tested for their ability to regulate the cell surface expression of NKG2D ligands. None of the compounds caused an effect similar to CH$_3$SeH (data not shown), suggesting that direct regulation of oxidative stress or endoplasmic reticulum stress in general is not essential for CH$_3$SeH regulation of NKG2D ligands. Next we focused on posttranslational pathways important for NKG2D ligand regulation. It has been recognized that proteasomal inhibition is important for the regulation of NKG2D ligands, specifically for the induction of cell surface-expressed ULBP2 (72, 73). There is interest because FR901228-mediated accumulation of LC3 (Fig. 5D) suggests that autophagy specifically regulates ULBP2 surface expression. This is interesting because CH$_3$SeH also inhibited ULBP2 surface expression (Fig. 3). To investigate whether CH$_3$SeH regulation of ULBP2 involved the autophagic transport pathway, we used a GFP-LC3 reporter assay to monitor the autophagic flux after the treatment with FR901228 and MSA-generated CH$_3$SeH. Monitoring of the LC3 flux through the autophagy pathway is the gold standard for measuring autophagy activity and is widely applied in the field (76). During autophagy, the protein LC3 is translocated from a soluble cytoplasmic form and bound to autophagosomes. Fixation of LC3 during autophagy can be examined in U2OS cells stably expressing LC3-GFP, where vesicle-bound LC3-GFP is retained after washing out free LC3-GFP using a weak detergent solution. As seen in Fig. 5D (top row), LC3-GFP accumulated after treatment with FR901228 and also accumulated to a lower extent after CH$_3$SeH treatment. On the basis of these experiments it was not possible to distinguish whether accumulation of LC3 occurred as a result of activation of the autophagic transport pathway (flux) or because of an inhibition downstream of LC3. The inclusion of a lysosomal inhibitor, however, made it possible to distinguish whether LC3 accumulation occurred through an increased autophagic flux or as a result of buildup of LC3 because of autophagy inhibition. LC3 accumulation after FR901228 treatment was further increased after lysosomal inhibition (Fig. 5D, bottom row). This indicates that FR901228 enhances autophagic flux. Strikingly, CH$_3$SeH blocked the accumulation of LC3 after lysosomal inhibition as well as FR901228-mediated accumulation of LC3 (Fig. 5D, bottom row), suggesting that CH$_3$SeH directly inhibits the autophagic flux. Inhibition of lysosomal activity was measured by 4-nitro-7-(1-piperazinyl)-2,1,3-benzoxadiazole accumulation in lysosomes, a well-described method for measuring lysosomal activity in live cells (77, 78). As expected, treatments of cells with the lysosomal inhibitor chloroquine (control) lead to a robust lysosomal accumulation. The CH$_3$SeH-generating compounds MSC and MSA also affected lysosomal activity, although not to the extent observed with chloroquine (Fig. 5F). Because the two different autophagy inhibitors 3-methyladenine and wortmannin specifically inhibited the cell surface expression of ULBP2 upon HDAC-inhibitor treatment, as did CH$_3$SeH, our data suggest that stimulation of the autophagic transport pathway is crucial for cell surface transport of ULBP2. This hypothesis is in line with our data reported previously showing that ULBP2 traffics over an endosomal/lysosomal pathway to the cell surface (54).

The Regulation of NKG2D Ligands by CH$_3$SeH Is Dependent on Extracellular Calcium — The level of intracellular calcium is crucial for the regulation of MICA/B and ULBP2 cell surface expression upon HDAC inhibitor treatment (58). To examine the calcium dependence of CH$_3$SeH-regulated MICA/B and ULBP2 surface expression, Jurkat E6-1 cells were incubated with the extracellular calcium chelator EGTA before treat-
ment with CH₃SeH generated by MSA. Cells treated in combination with EGTA and FR901228 were used as a control. As shown in Fig. 5F, treatment with EGTA decreased the CH₃SeH-induced cell surface expression of MICA/B in a dose-dependent manner similar to control cells. These results suggest that extracellular calcium is involved in regulating the expression of NKG2D ligands by CH₃SeH.

**DISCUSSION**

Selenium compounds have been highly discussed as chemopreventive agents but also as potential drugs or adjuvants in cancer therapy. Especially CH₃SeH has been suggested as a key metabolite in cancer prevention. Studies have shown that selenium precursors metabolized into CH₃SeH are more potent tumor inhibitors than compounds derived from H₂Se (26, 79).
At the same time, the induction of NKG2D ligands by traditional drugs or tolerable, chemical compounds has been investigated for many years, and more knowledge is required to further improve NKG2D-based therapy. In this study, we discovered that monomethylated selenium compounds induced surface expression of the NKG2D ligands MICA/B on viable and non-apoptotic cells. Precursors of H2Se had no effect on the expression of NKG2D ligands, implying that the generation of CH3SeH is crucial for NKG2D ligand regulation. In alignment with this hypothesis, we show that treatment with SeMet in combination with γ-lyase induced surface expression of MICA/B, similar to the treatment with monomethylated selenium compounds. As noted previously, SeMet can be metabolized into CH3SeH in the presence of γ-lyase (29). Our cell culture data further demonstrated that there was a hierarchy of efficiency in inducing the surface expression of MICA/B in the order MSA ≥ DMDSe ≥ SetMet + 0.04 units γ-lyase > MSC. In general, MSA induced the surface expression of MICA/B at 1/100 the concentration of MSC. A difference in response between the selenium compounds in regard to tumor inhibition has been described previously (26). In vivo, however, the difference disappeared, and the effects caused by MSA and MSC were found to be comparable (26). The authors suggested that the high presence of β-lyase, the enzyme required to produce CH3SeH from MSC, in vivo could be responsible for this compensation. Whether this also applies to the regulation of NKG2D ligands needs to be tested in further experiments in vivo. Additionally, a continuous generation of CH3SeH might be of importance for successful modulation of NKG2D ligands in vivo to counteract the naturally occurring metabolism of CH3SeH into dimethylselanide, trimethylselenonium, or H2Se. Notably, the tested Jurkat T cell line, but also other lymphoma cell lines, can generate the volatile selenium metabolites CH3SeH, DMDSe, and dimethylselanide in vitro because of the presence of the required and functional enzymes (26, 68, 80). When taken up by cells, MSA can also be reduced to CH3SeH via a nonenzymatic process (81). For this, an excess of thiol, e.g. GSH, is required. In our study, we did not distinguish whether CH3SeH generation by enzymatic or nonenzymatic processes regulates the expression of NKG2D ligands. We did try to inhibit the thioredoxin system by treating our cellular systems with auranofin, a potent inhibitor of the seleno-enzyme thioredoxin reductase (82). The treatment did not affect the cell surface expression of NKG2D ligands, implying that this type of cell stress is not responsible for the regulation of NKG2D ligands in our current setting.

The effect of both FR901228 and MSA to regulate the expression of NKG2D ligands was stable for at least 4 days when diluted in H2O, PBS, or culture medium. Even the removal of FBS, a product discussed to help drug stability and cellular uptake (83, 84), did not lead to a change of effectiveness. This is restricted to our in vitro experiments and might be different in vivo, where clearance by organs will occur. In the course of these experiments, we also observed that untreated cells, cultured in the same plates as cells treated with SeMet + γ-lyase, had induced surface expression of MICA/B compared with cells cultured in separate plates (data not shown). This implies that, during the treatment process, reactive selenium gases were developed, most likely CH3SeH, that modulated the expression of NKG2D ligands in untreated cells.

Our studies revealed that only CH3SeH-generating selenium compounds regulate the expression of NKG2D ligands. Here we recognized that CH3SeH induces gene activation and up-regulation of MICA/B surface expression. In contrast, the CH3SeH-induced ULBP2 gene activation was combined with a dominant suppression of ULBP2 surface expression. This is an important regulatory difference compared with HDAC-inhibitor activity known to induce surface expression of both ligand families (8, 16), and it strongly suggests that inhibition of ULBP2 expression occurs posttranscriptionally. Therefore, we attempted to study the parallels and differences of posttranscriptional actions upon treatment with HDAC inhibitors (in our experiments, FR901228) and monomethylated selenium compounds (MSA) to regulate the surface expression of NKG2D ligands. Interestingly, monitoring of the autophagic flux by measuring the accumulation of LC3 revealed that CH3SeH blocks the autophagic transport pathway, possibly through its potential to inhibit lysosomal activity. In addition, these data suggest that autophagic stress can facilitate ULBP2 surface transport. Here the induction of ULBP2 mRNA by CH3SeH and FR901228 implies that a common stress pathway exists but that the dominating posttranscriptional effect of CH3SeH prevents ULBP2 transport to the cell surface. This hypothesis is supported by the fact that autophagy is highly evolutionarily conserved and that autophagy has been adapted for more diverse uses than clearance of pathogens and maintenance of homeostasis, e.g. fusion of autophagosomes with endosomes or MHC class II loading compartments (85). In any case, the activity of CH3SeH described here provides mechanistic insights into the initiation of endosomal/lysosomal-dependent ULBP2 cell surface transport (54).

Summarizing our data, it is highly interesting that monomethylated selenium compounds, which are fundamental nutrients and easily accessible through our diet, can modulate the expression of NKG2D ligands in cancer cells, thereby enhancing their recognition and elimination by NKG2D-expressing immune effector cells. The fact that modulation of NKG2D ligands is restricted to CH3SeH-generating compounds is especially interesting for treatment approaches because H2Se metabolites are associated with genotoxic effects in cells (86, 87). Therefore, this so far unrecognized immune regulatory effect caused by CH3SeH-generating compounds should be added to the list of chemopreventive potential mediated by selenium compounds, and in particular considered for implementation in the treatment of NKG2D ligand-expressing tumors or adjuvant therapy in general. Certain tumors, such as melanoma and prostate and ovarian cancer (19, 20, 88), secrete large amounts of soluble ULBP2. Aberrant soluble ULBP2 is immunosuppressive because of constant immune activation and subsequent down-modulation of NKG2D (89). Therefore, CH3SeH-generating compounds are potentially ideal for the treatment of ULBP2-overexpressing cancer types because they can block the immunosuppressive soluble ULBP2 and, on the other hand, induce immune activation through the induction of MICA/B.
Methylselenol Regulates NKG2D Ligands MICA/B and ULBP2

Acknowledgments—We thank Dr. Carsten Geisler (Department of International Health, Immunology, and Microbiology, University of Copenhagen, Denmark) for providing the TTag9 cell line, Prof. K. Helin (University of Copenhagen) for the pCMV-Myc plasmid, and Dr. M. Wills (University of Cambridge) for the GFP-MICA’018 plasmid.

REFERENCES

9. Cosman, D., Müller, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N. J. (2001) ULBP ligands, novel MHC class I-related molecules bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14, 123–133
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compounds. Biochem. Pharmacol. 53, 921–926