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Selenite Induces Posttranscriptional Blockade of HLA-E Expression and Sensitizes Tumor Cells to CD94/NKG2A-Positive NK Cells

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CD94/NKG2A is an inhibitory receptor that controls the activity of a large proportion of human NK cells following interactions with the nonclassical HLA class Ib molecule HLA-E expressed on target cells. In this study, we show that selenite (SeO_3^{2-}), an inorganic selenium compound, induces an almost complete loss of cell surface expression of HLA-E on tumor cells of various origins. Selenite abrogated the HLA-E expression at a posttranscriptional level, since selenite exposure led to a dose-dependent decrease in cellular HLA-E protein expression whereas the mRNA levels remained intact. The loss of HLA-E expression following selenite treatment was associated with decreased levels of intracellular free thiols in the tumor cells, suggesting that the reduced HLA-E protein synthesis was caused by oxidative stress. Indeed, HLA-E expression and the level of free thiols remained intact following treatment with selenomethionine, a selenium compound that does not generate oxidative stress. Loss of HLA-E expression, but not of total HLA class I expression, on tumor cells resulted in increased susceptibility to CD94/NK group 2A-positive NK cells. Our results suggest that selenite may be used to potentiate the anti-tumor cytotoxicity in settings of NK cell-based immunotherapies. ***The Journal of Immunology*****, 2011, 187: 3546–3554.**

Natural killer cell function is regulated through the integrated signaling of multiple inhibitory and activating cell surface receptors that interact with ligands expressed on target cells (1). The inhibitory signals are mainly mediated by HLA class I-binding receptors, including the leukocyte Ig-like receptor B1, killer cell Ig-like receptors (KIRs), and CD94/NK group (NKG) 2A. The latter two are also involved in the functional maturation process of NK cells termed education or licensing, where NK cells that express CD94/NKG2A (2, 3) or at least one KIR specific for self-HLA class I molecules (4) becomes responsive to stimulation by HLA class I-negative target cells (5, 6). Thus, the clonal distribution of inhibitory CD94/NKG2A and/or KIRs in the NK cell repertoire secures self-tolerance and allows NK cells to recognize and kill cells that lose expression of single HLA class I alleles due to viral infection or tumor transformation (7), a phenomenon called "missing-self" recognition (8). Activation of NK cells by cellular targets involves cooperative triggering of two or more activating receptors, including NKG2D, DNAX accessory molecule-1 (DNAM-1), natural cytotoxicity receptors, CD94/NKG2C, and KIRs with activating intracellular domains (1).

The CD94/NKG2A/B, -C, and -E/H receptors, where A/B and E/H are splice variants, bind to the nonclassical HLA class Ib molecule HLA-E (9–11). Although members of the CD94/NKG2 family bind to the same ligand, they can mediate different functions depending on their intracellular domain. CD94/NKG2A has two inhibitory ITIM motifs and functions as an inhibitory receptor whereas CD94/NKG2C associates with the DAP12 adapting signaling molecule and transmits activating signals (12, 13). The CD94/NKG2A receptor appears early during NK cell differentiation and is expressed on 20–70% of human NK cells at steady-state (14). The expression of CD94/NKG2A on NK cells is largely complementary to the expression of both CD94/NKG2C (15, 16) and inhibitory KIRs and is therefore the dominating inhibitory receptor in individuals who have low frequencies of KIR-expressing cells (17–19).

HLA-E is ubiquitously expressed by nearly all cells in the body and primarily presents peptides derived from the conserved leader sequences of classical HLA class I and HLA-G molecules (9–11). HLA-E is overexpressed by several tumor types, including lymphomas (20), ovarian carcinomas (21), gliomas (22), colon carcinomas (23), acute myeloid leukemias (AML) (24), and melanomas (25) and is likely to protect from NK cell lysis through inhibition via interactions with the CD94/NKG2A receptor.

Selenium is a trace element that is essential for the synthesis of selenoproteins (26). Important selenoproteins, such as glutathione

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Abbreviations used in this article: AML, acute myeloid leukemia; DNAM-1, DNAX accessory molecule-1; KIR, killer cell Ig-like receptor; MIC, MHC class I chain; NKG, NK group; SeCys, selenocysteine; SeMet, selenomethionine; TCEP, tris(2-carboxyethyl)phosphine; ULBP, UL16 binding protein.

peroxidases and thioredoxin reductases, are central in redox regulation and ribonucleotide synthesis in the cell (27, 28). Low dietary intake of selenium has been associated with an increased incidence of prostate cancer (29), whereas supplementation has been linked to reduced risk for cancers such as skin and liver cancer (30–32). Interestingly, the inorganic selenium compound selenite (SeO_3^{2-}) has been shown to induce apoptosis in a wide range of solid tumor cells (33–36) and various hematological malignancies (37, 38), with a selective cytotoxic effect on malignant cells compared with normal cells (39–41). Selenite can also hit drug-resistant tumor cells (33, 42) and potentiate the effect of some chemotherapeutic drugs (35, 43). Based on these data, selenite is currently being evaluated in a phase I clinical trial as a single agent in the treatment of lung cancer (M. Björnstedt, personal communication). Hence, selenium might be important in both the prevention of and therapy for cancer.

Several strategies to enhance NK cell recognition of tumors have been proposed, including those aimed at using cytokines to stimulate endogenous NK cells and those that act by sensitizing target cells to the effector mechanisms of NK cells (44). The synergies between several types of anti-cancer therapies and immune-mediated tumor rejection hold promise for the induction of more effective and long-lasting clinical responses (45). A yet unexplored possibility for enhancing the susceptibility to NK cell-mediated killing is by modulating the redox balance of tumor cells. Because selenite is known to induce oxidative stress, which alters the set of proteins expressed by the cell to promote cell survival (46), we hypothesized that this drug could sensitize tumor cells to NK cell-mediated killing by modulating their surface expression of NK cell receptor ligands.

In this study, we show that selenite treatment of tumor cells results in a dose-dependent blockade of the posttranscriptional synthesis of HLA-E, leading to reduced cell surface expression and augmented killing by CD94/NKG2A-positive NK cells. Our results suggest that selenite may be used to potentiate the antitumor cytotoxicity in settings of NK cell-based immunotherapies.

Materials and Methods

Cells

This study was approved by the Regional Ethics Review Committee of Stockholm (approval nos. 2006/229-31/3, 2009/1138-31/3, and 2007/1089-32). Lymphocytes from the peripheral blood of healthy donors were enriched by density gradient centrifugation (Ficoll-Hypaque; Amersham Biosciences, Uppsala, Sweden) (21). Cells were frozen in 10% DMSO (Sigma-Aldrich, St. Louis, MO) and 90% heat-inactivated FBS (Life Technologies, Grand Island, NY) and then stored in liquid nitrogen. NK cells were isolated from frozen PBMCs using the NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and resuspended in complete medium (RPMI 1640 containing 100 μg/ml L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin) supplemented with 1000 IU/ml IL-2 (Proleukin; Chiron, Emeryville, CA) and incubated overnight (16 h) at 37°C before use. Overnight IL-2–activated NK cells were used throughout the study unless otherwise specified. Freshly isolated lung adenocarcinoma cells were obtained from a pleural effusion from the Department of Clinical Pathology and Cytology, Karolinska University Hospital Huddinge, Sweden. The erythroleukemia cell line K562, the cervix cancer cell line HeLa, the AML cell line NB4, the ovarian cancer cell lines Skov-3 and CaOV4 (all from the American Type Culture Collection, Manassas, VA), the lymphoblastoid cell line 721.221 (a gift from Drs. Peter Parham and Eric Long), the multiple myeloma cell lines OPMI and EJM (gifts from Dr. Richard Childs), and the mouse mastocytoma cell line P815 (a gift from Dr. Eric Long) were all maintained in complete medium at 37°C and 5% CO_2. K562 cells transfected with HLA-E, 721.221-Cw3, and 721.221-Cw4 were maintained in complete medium supplemented with 0.5 mg/ml G418 (Life Technologies). The STAV-AB malignant mesothelioma cell line, provided by Dr. Julius Klominek (47), was maintained in RPMI 1640 containing 10% heat-inactivated human AB serum (Sigma-Aldrich), 100 μg/ml L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C and 5% CO_2.

Chemicals and treatment of cells

Sodium selenite (Na_2SeO_3), selenocystein (SeCys), selenomethionin (SeMet), and tris(2-carboxyethyl)phosphine (TCEP) were all purchased from Sigma-Aldrich. Cells were reseeded in medium supplemented with either compound and cultured for 24 h followed by washing in PBS before being harvested with 5 mM EDTA (Sigma-Aldrich). The HLA-G leader sequence peptide (VMAPRTLFL), purchased from GenScript (Piscataway, NJ), was used to stabilize the expression of HLA-E on K562-E cells during overnight incubation at 26°C.

Abs and reagents for flow cytometry

The following reagents and fluorescent-labeled mAbs were used: anti–CD56-PE-Cy7 (B159), anti–CD107a-FITC (H4A3), anti–CD14-allophycocyanin-Cy7 (MØP9), anti–CD112-PE (R2.525), IgG1-PE (X40), IgG2a-allophycocyanin (G155-178), and rat anti–mouse IgG1-allophycocyanin (X56) were all purchased from BD Biosciences (Franklin Lakes, NJ). The anti-CD3 Cascade Yellow (UCHT1) and the anti–BerEp4-FITC (BerEp4) mAbs were purchased from DakoCytomation (Glostrup, Denmark). IgG1 isotype control PE (679.1Mc7) and anti–NKG2A-PE (Z199) were purchased from Beckman Coulter (Fullerton, CA). The anti–HLA-ABC-allophycocyanin (W6/32) mAb was from Serotec (Oxford, U.K.). The following nonconjugated Abs were used: anti–HLA-E (3D12) from eBioscience (San Diego, CA), anti-CD155 (PV404.19) from Beckman Coulter, and IgG1 (MOPC-21) from BioLegend (San Diego, CA). The anti-UL16 binding protein (ULBP) 1 (M295), anti-ULBP2 (M311), anti-ULBP3 (M551), anti-ULBP4 (M476), anti-MHC class I chain (MIC) A (M673), and anti-MICB (M362) Abs were provided by Amgen (Seattle, WA). The Live/Dead cell stain kit (Invitrogen, Carlsbad, CA) was used for exclusion of dead cells in all experiments.

CD107a assay

NK cells were coincubated with target cells at a ratio of 1:1 in a final volume of 200 μl in a 96-well plate at 37°C and 5% CO_2 for 2 h. After 1 h coincubation, GolgiPlug (BD Biosciences) was added in a 1:1000 dilution. At the end of the assay, cells were stained with the Live/Dead dye and an Ab mixture, labeling the NK cells and its expression of CD107a, for 15 min on ice. Before analysis on the flow cytometer, the cells were washed and resuspended in CellFix (BD Biosciences).

Flow cytometry-based cytotoxicity assays

Target cells were prelabeled with the fluorescent membrane dye TFL4 (Oncoimmunin, Gaithersburg, MD). NK cells were coincubated with target cells at a ratio of 10:1 in a final volume of 200 μl for 2 h at 37°C and 5% CO_2. At the end of the assay, cells were stained with 7-aminoactinomycin D (BD Biosciences) to determine apoptosis before acquisition on a flow cytometer. Percentage specific cytotoxicity was calculated as follows: $100 \times$ [(NK cell-induced apoptosis − spontaneous apoptosis)/(100 − spontaneous apoptosis)].

Flow cytometry

All flow cytometry samples were stained on ice and washed twice before being analyzed on a CyAn ADP LX 9 color flow cytometer (DakoCytomation). The data were subsequently analyzed with FlowJo software (Tree Star, Ashland, OR).

Measurement of oxidative stress

Cells were resuspended in buffer (50 mM Tris-HCl [pH 7.6] and 1 mM EDTA) on ice, followed by sonication and centrifugation at $25{,}000 \times g$ for 10 min at 2°C. The protein concentration was determined using a protein assay (Bio-Rad, Hercules, CA) and a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA) (48). The concentration of free thiols was measured after the addition of 50 μl 5′,5′-dithio-bis(2-nitrobenzoic acid) (0.4 mg/ml) and 500 μl 6 M guanidine-HCl in 0.2 M Tris-HCl (pH 8.0) to a cell homogenate in a 1-cm quartz cuvette. The absorbance at 412 nm was measured within 20 min and the concentration was calculated using $\varepsilon 412 = 13.6$/mM.

Western blot

The cells were concentrated and resuspended in 1× SDS sample buffer (Invitrogen) supplemented with 50 μM DTT followed by repetitive heating (90°C) and freezing (−20°C) to degrade DNA. The isolated protein fraction was separated on a 4–20% Novex SDS-PAGE gel (Invitrogen) and transferred to nitrocellulose membranes. The membranes were blocked

with 5% BSA in TBS before overnight incubation with the mouse anti-human HLA-E mAb at 4˚C. After washing, the membrane was incubated with biotinylated goat anti-mouse antisera (Vector Laboratories, Burlingame, CA) followed by incubation with HRP-conjugated rabbit anti-goat antisera (Jackson ImmunoResearch Laboratories, West Grove, PA). The blot was developed with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and the chemiluminescent signal was acquired with LAS 4000 (Fuji Film Life Science). Finally, the signal density was analyzed with ImageJ (http://rsbweb.nih.gov/ij/).

Real-time RT-PCR

Total RNA was extracted by the use of the RNeasy Mini kit (Qiagen, Stockholm, Sweden) and converted to cDNA by using the cDNA reverse transcription kit from Applied Biosystems (Foster City, CA). Amplification of cDNA was performed using the TaqMan Gene Expression Master Mix and a 7500 Fast real-time PCR system (both from Applied Biosystems). The primers and probes for HLA-E (Hs00428366_m1) and 18S rRNA (4310893E) were purchased as pre-developed TaqMan gene expression assays (Applied Biosystems). 18S rRNA served as an endogenous control to normalize the amount of sample cDNA. Relative amounts of HLA-E were calculated using the comparative threshold cycle method (49).

KIR and HLA genotyping

Genomic DNA was isolated from 100 μl peripheral blood by using the DNeasy blood and tissue kit (Qiagen). The *KIR* genotyping kit from Olerup SSP (Stockholm, Sweden) was used for KIR genotyping. KIR ligands were determined by using the *KIR HLA* ligand kit (Olerup-SSP) for detection of the *Bw4*, *Cw3* (C1), and *Cw4* (C2) motifs and the *HLA-A* low-resolution kit (Olerup-SSP) for detection of the *HLA-A3/A11* motif.

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA) using the Wilcoxon and the Mann–Whitney *t* tests for paired and unpaired groups, respectively. Two-way ANOVA tests with Bonferroni posttests were used where appropriate.

Results

Enhanced NK cell-mediated killing of tumor cells pretreated with selenite

To assess whether selenite could be used to sensitize tumor cells to NK cell killing, we first studied NK cell-mediated recognition of the previously characterized mesothelioma cell line STAV-AB upon treatment with selenite (35, 47, 50, 51). Degranulation by short-term (16 h) IL-2–activated NK cells, as assessed by CD107a expression (52), increased 2-fold ($p < 0.05$) following coincubation with tumor cells pretreated with selenite compared with untreated tumor cells (Fig. 1*A*, 1*B*). A previously described flow cytometry-based killing assay (53) that detects the apoptosis of tumor targets was used to determine whether the observed increase in NK cell degranulation was associated with an increased lysis of the tumor cells. Indeed, pretreatment with selenite led to significantly ($p < 0.01$) increased lysis of STAV-AB cells (Fig. 1*C*), suggesting that selenite sensitizes STAV-AB cells to killing NK cells.

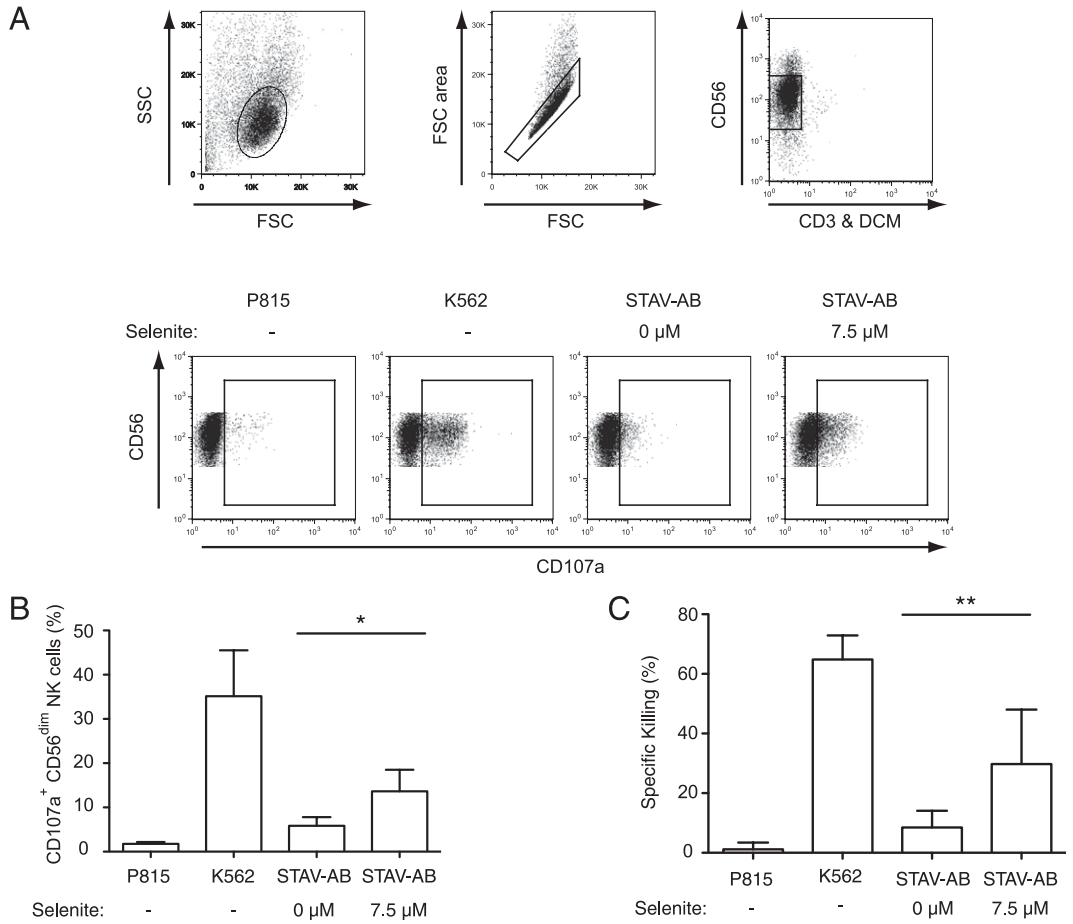

FIGURE 1. Selenite sensitizes tumor cells to killing by overnight IL-2–activated NK cells. Degranulation and killing were monitored after coincubation with overnight IL-2–activated NK cells and target cells treated with selenite. *A*, Representative FACS plots showing CD107a expression on $CD56^{dim}$ NK cells after stimulation with the indicated target cells. *B*, The CD107a expression on NK cells after stimulation with untreated or selenite-treated (7.5 μM) STAV-AB cells ($n = 10$). Columns show mean of CD107a expression (%); bars indicate SD. *C,* specific NK cell killing of untreated or selenite-treated (7.5 μM) STAV-AB cells ($n = 10$). P815 and K562 cells were used as negative and positive controls, respectively. Columns show mean specific killing (%); bars indicate SD. $*p < 0.05$, $**p < 0.01$.

Selenite induces a dose-dependent downregulation of HLA-E expression on tumor cells

Because selenite is known to induce oxidative stress and thereby alter the cellular protein expression (26, 46), we next monitored the expression of a broad repertoire of NK cell receptor ligands on STAV-AB cells after exposure of 7.5 μM selenite for 24 h. Interestingly, selenite induced a distinct loss of HLA-E and MICA expression, whereas the expression of HLA class I, MICB, ULBP1–4, CD155, CD112, CD54, CD58, and CD102 was either only marginally affected or unaffected (Fig. 2*A*, 2*B* and data not shown). Of note, none of the stress-induced NKG2D ligands MICB and ULBP1–4 (54) was upregulated following exposure to selenite. The observed downregulation of HLA-E and MICA expression was dose-dependent and occurred gradually over time, and it reached its maximum after ~24 h treatment (Fig. 2*B* and data not shown).

Because pretreatment with selenite sensitized STAV-AB cells to NK cells, we hypothesized that the observed loss of HLA-E could be a possible mechanism for the augmented killing of the tumor cells. This would fit with the observation that selenite treatment did not alter the susceptibility of HLA-E-negative K562 cells to NK cell-mediated killing (Supplemental Fig. 1). To address whether the observed selenite-induced loss of HLA-E expression on STAV-AB cells could be extrapolated to other tumor cells, we assessed the HLA-E expression on a panel of tumor cells following treatment with selenite. Indeed, selenite induced a complete loss of HLA-E expression on several tumor cell lines of various histological origins as well as on freshly isolated HLA-E–expressing tumor cells from a patient diagnosed with adenocarcinoma of the lung (Fig. 2*C*).

Taken together, these results demonstrate that selenite induces dose- and time-dependent alterations of the NK cell receptor ligand repertoire with a nearly complete loss of HLA-E expression on the cell surface of tumor cells of different origins.

Inhibition of HLA-E protein synthesis occurs at the posttranscriptional level

The fact that the reduction of cell surface expression of HLA-E occurred several hours after selenite treatment (data not shown) excluded the possibility that selenite induced a rapid conformational change of the epitope recognized by the HLA-E binding mAb. Instead, this observation suggested that the loss of HLA-E expression could be attributed to abrogated protein synthesis. To delineate how selenite affects the expression of HLA-E, we next studied its effects on protein synthesis by measuring the mRNA level, the total protein level, and the cell surface expression of HLA-E on STAV-AB cells following treatment with increasing doses of selenite for 24 h. The expression of total and cell surface HLA-E proteins decreased gradually with increasing doses of selenite, whereas the mRNA level was unaffected (Fig. 3), suggesting that selenite inhibits the HLA-E protein synthesis at a posttranscriptional level.

Reduced HLA-E expression is associated with intracellular oxidative stress

To examine whether the observed effect of selenite was a consequence of increased intracellular oxidative stress, we monitored the expression of HLA-E and thiol oxidation following treatment with selenite, SeCys, and SeMet. Selenite and SeCys, but not the negative control SeMet, are known to effectively induce intracellular oxidative stress (26). The prime biological effect of SeMet is a delayed selenium source that induces the expression of antioxidant enzymes (26). Selenite and SeCys pretreatment led to a loss of HLA-E expression on STAV-AB cells, whereas no such effect was observed following treatment with SeMet (Fig. 4*A*). To examine whether the loss of HLA-E expression was a consequence of reduction of disulfide bridges and disruption of the tertiary structure of HLA-E molecules expressed on the cell surface, we next treated the cells with TCEP, a cell-impermeable highly reducing compound that breaks disulphide bridges in the extracellular compartment. However, TCEP did not induce any NK cell receptor ligand modifications on the cell surface, thus indicating that reduction of disulfides in cell surface proteins did not cause the loss of HLA-E (Fig. 4*A*). These results suggest that selenite-induced intracellular oxidative stress might be a key mechanism underlying the observed loss of HLA-E expression. Furthermore, the data also exclude the involvement of a shared property by selenium compounds, including the nonoxidating SeMet, as means of loss of HLA-E expression.

To further examine the role of intracellular oxidative stress, we also assessed the levels of free thiols in tumor cells after exposure to selenite, SeCys, SeMet, or TCEP (Fig. 4*B*). Treatment with selenite and SeCys, but not SeMet or TCEP, led to a reduction in free thiols. In summary, these results demonstrate that selenite affects HLA-E expression through a mechanism involving increased intracellular oxidative stress.

CD94/NKG2A-positive NK cells display increased recognition of tumor cells treated with selenite

Reduced surface expression of HLA-E following selenite treatment should facilitate the recognition of tumor cells by CD94/NKG2A-positive NK cells but not by CD94/NKG2C-positive NK cells. To test this prediction, the relative responses of these two NK cell subsets were analyzed by gating on $NKG2A^+$ $NKG2C^-$, $NKG2A^-NKG2C^+$, and $NKG2A^-NKG2C^-$ subsets within the cytotoxic $CD56^{dim}$ NK cell population. Indeed, degranulation by CD94/NKG2A single-positive NK cells was increased following coincubation with selenite-treated STAV-AB cells (Fig. 5*A*). However, responses by CD94/NKG2C-positive NK cells were low when incubated with untreated tumor cells and, as expected, remained low when NK cells were stimulated with selenite-treated tumor cells (Fig. 5*A*). Furthermore, the response by the double-negative NK cell subset was unaffected. The experimental system was validated by coincubating NK cells with HLA-E–transfected K562 cells that were either unpulsed or pulsed with the HLA-G leader sequence peptide (Gsp) to stabilize the cell surface expression of HLA-E (Supplemental Fig. 2*A*). Importantly, selenite treatment also sensitized tumor cells to resting CD94/NKG2A single-positive NK cells (data not shown).

Despite significant reduction in HLA-E expression, selenite did not affect the total level of HLA class I at the cell surface on STAV-AB cells. Consequently, KIR–HLA interactions that determine NK cell reactivity should remain intact following selenite treatment. To test this prediction, we monitored the degranulation of single KIR-positive NK cells following incubation with STAV-AB cells (harboring the HLA-Bw4 and HLA-C2 KIR-ligands, but lacking HLA-C1 and HLA-A3/A11) treated with increasing concentrations of selenite (Fig. 5*B*). NK cells were isolated from HLA-C1/C2–positive healthy donors homozygous for the group A *KIR* haplotype to avoid cross-reactive binding of anti-KIR mAbs to activating KIRs present in donors with group B *KIR* haplotypes (7). Overnight, IL-2–activated NK cells coexpressing NKG2A together with KIR2DL3 (sensing missing HLA-C1 and HLA-E) gradually displayed increased degranulation against STAV-AB tumor cells pretreated with increasing doses of selenite (Fig. 5*B*). In contrast, the KIR2DL1 single-positive NK cell subset was totally inhibited by its cognate ligand HLA-C2, despite treatment of tumor cells with 7.5 μM selenite. Furthermore, KIR and NKG2A double-negative NK cells, known to be of an immature and hyporesponsive phenotype (5), were unresponsive to stimulation with either untreated or

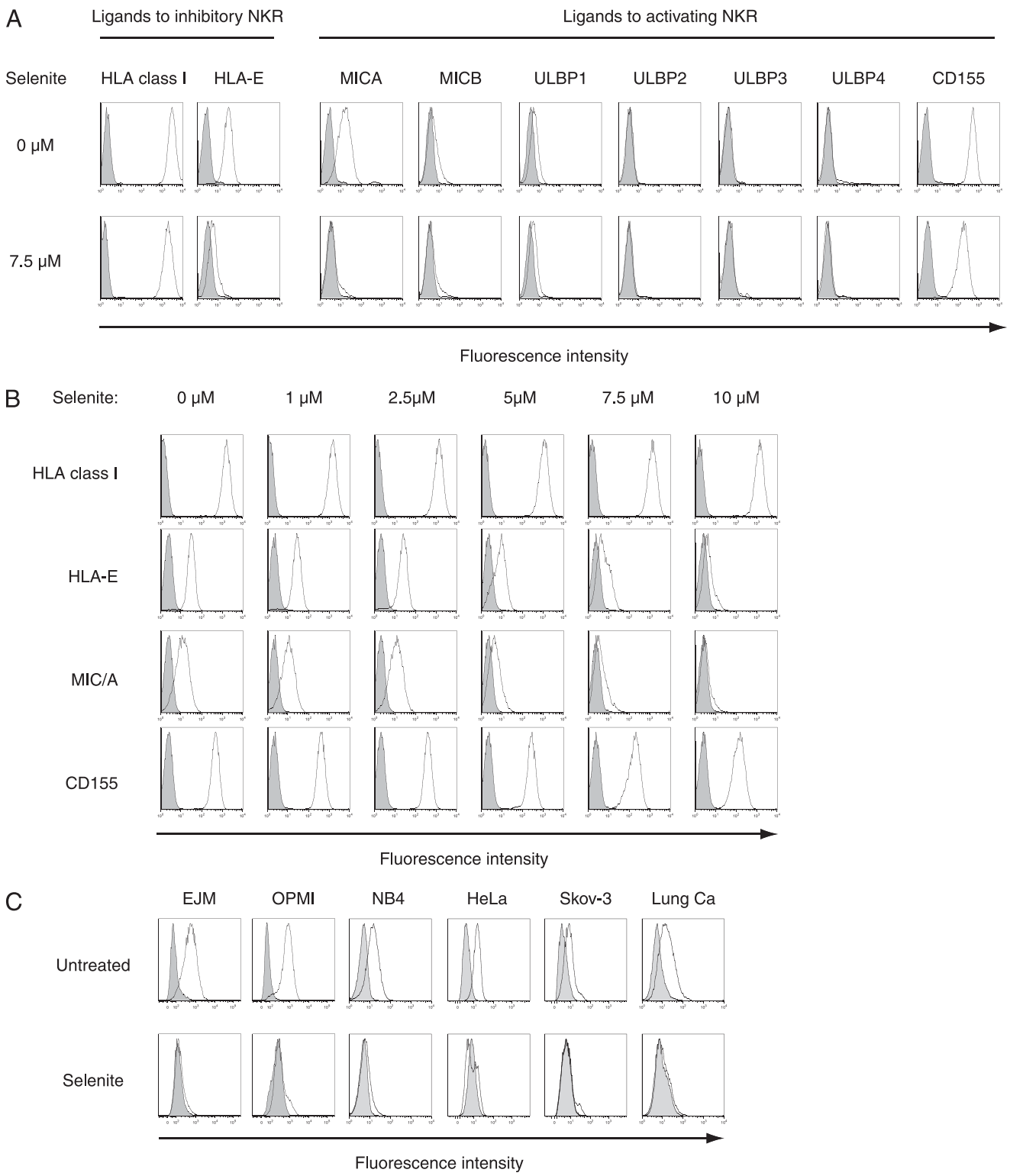

FIGURE 2. Dose-dependent loss of HLA-E after selenite treatment. *A*, Histograms show the expression of the major NK cell receptor ligands on untreated or selenite-treated STAV-AB cells. *B*, Histograms show the expression of NKR ligands on the tumor cells following treatment with selenite at the indicated doses. *C*, HLA-E expression on tumor cell lines and freshly isolated adenocarcinoma from one patient with lung cancer following 24 h after treatment with selenite. Lines indicate ligand expression; filled histograms show isotype control. One representative experiment of three is shown.

selenite-treated targets. The specificity of the distinct KIR-expressing subsets was verified by using a panel of target cells transfected with various KIR ligands (Supplemental Fig. 2*B*). Overall, these results demonstrate that selenite specifically modulates the CD94/NKG2A–HLA-E interaction due to its selective downregulation of HLA-E on the target cells.

Discussion

In this study we show that the oxidative agent selenite sensitizes human tumor cells to lysis by CD94/NKG2A-positive NK cells by downmodulating the expression of HLA-E. The loss of HLA-E expression was caused by oxidative stress-induced protein reduction at a posttranscriptional level. Because the CD94/NKG2A

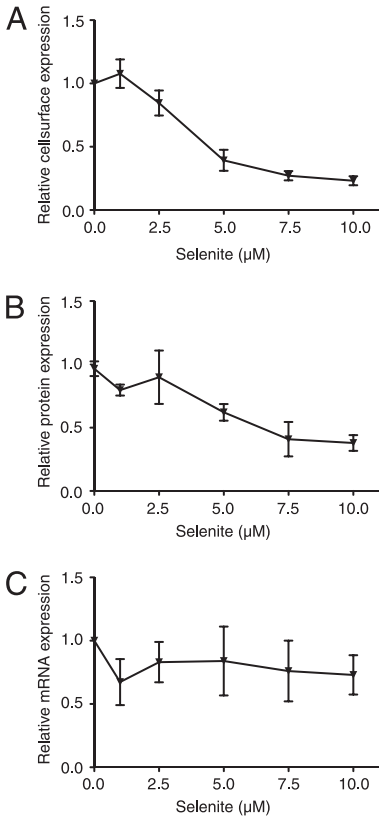

FIGURE 3. Abrogation of HLA-E protein synthesis following selenite treatment. *A*, Cell surface expression of HLA-E relative to isotype control following selenite treatment ($n = 7$). *B*, The protein expression of HLA-E in STAV-AB cell homogenates relative to actin following selenite treatment ($n = 3$). *C*, The mRNA levels of HLA-E relative to the constitutively expressed mRNA of 18S in STAV-AB cells following selenite treatment ($n = 3$). Lines show mean; bars indicate SD.

receptor controls the activity of a large proportion of human NK cells, selenite-mediated suppression of HLA-E expressed on tumor cells may represent an attractive strategy to promote anti-cancer immunity.

The central role for inhibitory HLA-E and CD94/NKG2A interactions in restraining tumor targeting by NK cells was recently highlighted in a study by Nguyen et al. (24) in which Ab-mediated blockade of the CD94/NKG2A receptor augmented NK cell-mediated killing of primary AML cells ex vivo. Ab-mediated abrogation of the inhibitory interactions between CD94/NKG2A and HLA-E could potentially also be useful in the treatment of several other HLA-E–expressing tumor types, including lymphoma, ovarian carcinoma, glioma, colon carcinoma, and melanoma (20–23, 25). However, a potential risk of administrating antagonistic anti-NKG2A Abs may be NK cell targeting of normal HLA-E–expressing cells. Moreover, Ab-mediated blockade of the CD94/NKG2A receptor could potentially also reduce the cytotoxic capacity CD94/NKG2A-expressing NK cells due to retuning of the responsiveness in the periphery (55). In this regard, drugs that specifically suppress the expression of HLA-E on the target cells may be an attractive approach to abolish the inhibitory interactions between CD94/NKG2A and HLA-E.

The reduced HLA-E expression observed following selenite treatment was associated with increased levels of oxidative stress in the exposed targets. Similar to all other selenium compounds entering cells, selenite is metabolized through the intermediary metabolite selenide that, in high concentrations, causes oxidative stress through redox cycling (26, 46). Although enzymatically regulated by a rate-limiting step, selenite is also metabolized and incorporated into SeCys, which in itself can be metabolized to selenide and thus induce oxidative stress (26, 46). Consistently, our data showed that treatment of tumor cells with either selenite or SeCys induced powerful oxidative stress associated with loss of HLA-E, whereas the redox status and the HLA-E expression were completely unaffected by treatment with SeMet, which only slowly metabolizes to selenide and does not cause oxidative stress.

Despite the clear association between loss of HLA-E expression and induction of intracellular oxidative stress found in this study, the exact mechanism for reduced HLA-E expression following selenite treatment remains unclear. Our data demonstrating that treatment of STAV-AB cells with the highly reactive but cell-impermeable agent TCEP did not result in loss of HLA-E expression excluded the possibility that loss of HLA-E was a consequence of extracellular reduction of disulfide bridges. Instead, our data show that selenite induces intracellular oxidative stress that reduces cellular levels of the HLA-E protein without affecting its transcription. This observation is in line with the current literature indicating that oxidative stress can cause global reduction of

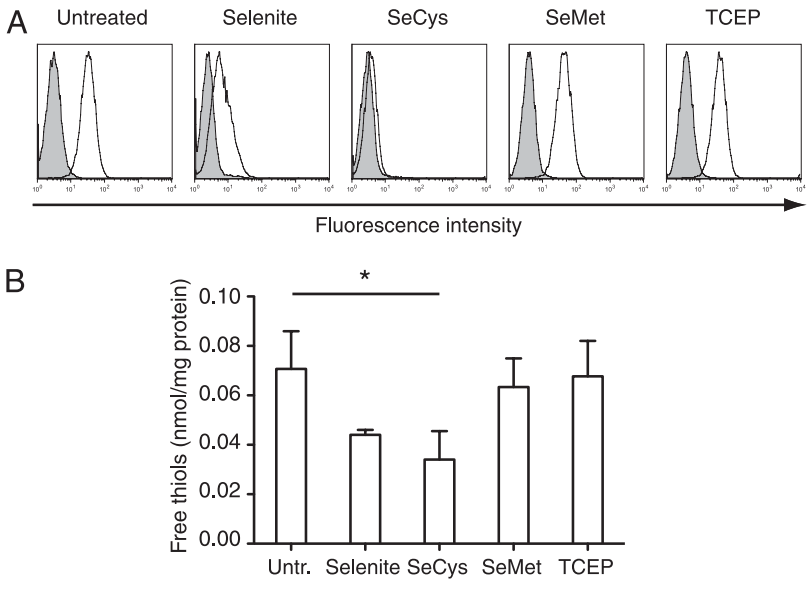

FIGURE 4. The loss of HLA-E is associated with intracellular oxidative stress. STAV-AB cells were treated with either 7.5 μM selenite, 333 μM SeCys, 10 μM SeMet, or 1 mM TCEP. *A*, Histograms show the expression of HLA-E on tumor cells after treatment with the indicated compound. One representative experiment of three is shown. *B*, The relative content of free thiols to total protein content in the cells after treatment ($n = 3$). Columns show mean of free thiols to total protein content (%); bars indicate SD. *$p < 0.05$.

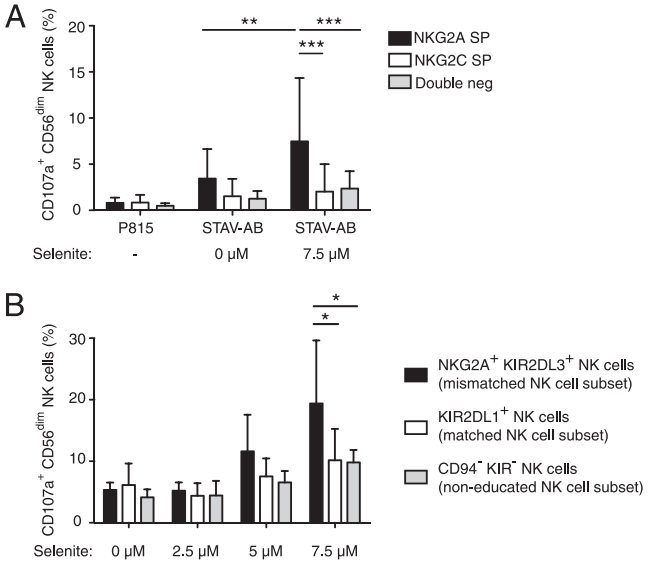

FIGURE 5. Loss of HLA-E results in enhanced recognition by alloreactive overnight IL-2–activated CD94/NKG2A-positive NK cells. The expression of CD107a was assessed on specific subsets of overnight IL-2–activated $CD56^{dim}$ NK cells. *A*, CD107a expression on $NKG2A^+NKG2C^-$ (NKG2A SP), $NKG2A^-NKG2C^+$ (NKG2C SP), and $NKG2A^-NKG2C^-KIR^-$ (Double neg) NK cell subsets ($n = 10$) after stimulation with selenite-treated STAV-AB cells. P815 cells were used as negative control. *B*, CD107a expression on alloreactive $NKG2A^+KIR2DL3^+$ (KIR-ligand mismatched), non-alloreactive $KIR2DL1^+$ (KIR-ligand matched), and hyporesponsive $CD94^-KIR^-$ (noneducated) NK cell subsets after stimulation with STAV-AB cells pretreated with increasing doses of selenite ($n = 4$). Columns show mean of CD107a expression (%); bars indicate SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

protein synthesis in favor of selective production of specific proteins that are required for cell survival, without affecting transcription (46). Previously, such responses proved to be mediated by inhibitory phosphorylation of the eukaryotic initiation factor 2α and a simultaneous switch to internal ribosome entry site-mediated translation (46). However, changes in global protein synthesis would also reduce the expression of all the other NK cell receptor ligands assessed in our study. One explanation for the selective loss of HLA-E on the cell surface following selenite treatment of STAV-AB cells could relate to its relatively short half-life due to an unstable tertiary structure. Unlike classical HLA class I molecules, HLA-E has a very limited repertoire of peptides that could stabilize the complex and thereby regulate its expression (56). The general reduction of protein synthesis during cellular stress could further limit the peptide pool for HLA-E and thereby also cause reduced expression. Finally, selenite may exert its effect on HLA-E expression by increasing protein degradation due to misfolding caused by perturbation of the critical disulfide bridges in the peptide-loading complex. Involvement of the latter mechanism is supported by data showing that selenite can induce apoptosis via caspase-8 and caspase-9, both of which are activated upon the unfolded protein response initiated by endoplasmatic reticulum stress following accumulation of unfolded and misfolded proteins in the endoplasmatic reticulum (34, 57). Hence, the exact mechanism for selenite-induced loss of HLA-E, but also the basis for its selective targeting of the HLA-E molecule, needs further delineation.

One attractive aspect of selenite is that it has selective effects on tumor cells compared with normal tissue (39). This phenomenon is explained by the intracellular accumulation of selenite caused by its efficient uptake following cysteine recycling through the cystine/glutamate antiporter and multidrug resistance protein, both of which are frequently overexpressed on tumor cells (38). In addition to this selective uptake, tumor cells have also been shown to be more sensitive to selenite per se, since the intracellular redox cycles between selenide, thiols, and oxygen that cause formation of reactive oxygen species (26) are more pronounced in tumor cells due to their increased levels of thiols compared with normal cells (58). Because of these selective effects on tumor cells, it would be exciting to explore whether selenite can induce specific downregulation of HLA-E on tumor cells in vivo, while preserving its expression on normal cells and thereby preventing NK cell targeting of normal HLA-E–expressing tissues. Besides its effects on HLA-E, selenite is also an attractive drug candidate because of its other anti-tumor properties, including inhibition of cellular growth and induction of DNA strand breaks leading to apoptosis (35–38, 59). In fact, one recent study showed that low concentrations of selenite were more cytotoxic to primary AML blast cells than were conventional drugs (60), and several other studies have indicated that selenite is especially cytotoxic in tumor cells that are resistant to conventional drugs (34, 40). Moreover, selenite has also been shown to potentiate the effect of conventional chemotherapeutic drugs (43).

Thus, selenite could be used to target tumor cells via multiple pathways including direct cytotoxicity and synergies with conventional chemotherapeutic drugs, as well as by augmenting NK cell-mediated killing. The possibility of combining selenite and adoptive NK cell therapy in the clinic merit further attention especially since data from an on going phase I clinical trial (M. Björnstedt, personal communication) and previous trials (61, 62) demonstrate that selenite could be administrated safely to patients in milligram doses yielding plasma concentrations of 5–20 μM. However, because selenite may be toxic to NK cells and hamper their responsiveness in some donors (Supplemental Fig. 3), it will be important to carefully examine the settings of this approach before it can be translated into a clinical trial. In vivo experiments using mouse models would not only provide a better understanding of the potential need for a temporal delay between selenite administration and NK cell infusion, but also what preparation of NK cells is most optimal to use with selenite. Although our data show that resting and short-term IL-2–activated NK cells respond to selenite-exposed tumor cells, we cannot exclude that expanded NK cells with a high expression of the CD94/NKG2A receptor (16, 63) are even more potent effectors in this setting. Given that the CD94/NKG2A receptor educates NK cells to react to targets lacking HLA-E expression, one may speculate that expanded NK cells would mediate a more prominent response in vivo to selenite-exposed tumor cells as compared with NK cells that have been stimulated overnight with IL-2 to augment their responsiveness without affecting the proportion and intensity of the CD94/NKG2A receptor expression (64).

Another important aspect to consider is what tumor types would be most susceptible to this regimen. Tumors that are primarily recognized by NK cells through signaling via the DNAM-1 receptor, including melanoma, ovarian carcinoma, neuroblastoma, myelodysplastic syndromes, and leukemias (65–69), may be good candidates since the DNAM-1 ligand CD155 was unaffected by selenite treatment at concentrations that completely abolished HLA-E expression. In contrast, the role for selenite in augmenting NK cell targeting of HLA-E–expressing tumors for which the recognition primarily depends on MICA-mediated triggering of the NKG2D receptor may not be as prominent, since our data show that also MIC/A is lost following exposure of STAV-AB cells to selenite.

In conclusion, we report that selenite suppresses HLA-E expression on tumor cells and thereby renders them susceptible to

CD94/NKG2A-positive NK cells. Although further studies are warranted on the exact mechanism of selenite-induced HLA-E suppression and the potential benefits of this in clinical settings, this study provides a new principle for sensitization of tumor cells to NK cells.

Disclosures

The authors have no financial conflicts of interest.

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