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Evasion of Innate Immunity Contributes to Small Cell Lung Cancer Progression and Metastasis

Mingrui Zhu^{1,2}, Yi Huang^{1,2}, Matthew E Bender^{1,2}, Luc Girard^{2,3}, Rahul Kollipara⁴, Buse Eglenen-Polat^{1,2}, Yujiro Naito⁵, Trisha K Savage⁶, Kenneth E Huffman^{2,3}, Shohei Koyama⁵, Atsushi Kumanogoh⁵, John D Minna^{2,3,7,8}, Jane E Johnson^{2,6,7}, Esra A Akbay^{1,2,*}

¹Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX

²Simmons Comprehensive Cancer Center, Dallas, TX

³Hamon Center for Therapeutic Oncology Research University of Texas Southwestern Medical Center

⁴McDermott Center for Human Growth and Development

⁵Department of Respiratory Medicine and Clinical Immunology, Graduate School of medicine, Osaka University

⁶Department of Neuroscience, University of Texas Southwestern Medical Center

⁷Department of Pharmacology

⁸Department of Internal Medicine, University of Texas Southwestern Medical Center

Abstract

Small cell lung cancer (SCLC) is a pulmonary neuroendocrine cancer with very poor prognosis and limited effective therapeutic options. Most patients are diagnosed at advanced stages, and the exact reason for the aggressive and metastatic phenotype of SCLC is completely unknown.

Despite a high tumor mutational burden, responses to immune checkpoint blockade are minimal in SCLC patients. This may reflect defects in immune surveillance. Here we illustrate that evading NK surveillance contributes to SCLC aggressiveness and metastasis, primarily through loss of NK cell recognition of these tumors by reduction of NK-activating ligands (NKG2DL). SCLC primary tumors expressed very low level of NKG2DL mRNA and SCLC lines express little to no surface NKG2DL at the protein level. ChIP-Seq showed NKG2DL loci in SCLC are inaccessible compared to NSCLC, with few H3K27Ac signals. Restoring NKG2DL in preclinical models suppressed tumor growth and metastasis in an NK cell-dependent manner. Likewise, HDAC inhibitor treatment induced NKG2DL expression and led to tumor suppression by inducing infiltration and activation of NK and T cells. Among all the common tumor types, SCLC and

*Corresponding author: **Address correspondence to:** Esra Akbay, esra.akbay@utsouthwestern.edu and phone: 2146484159, Address: 6000 Harry Hines Blvd, Dallas Texas, 75235.

Authors' contributions:

MZ: conceptualization, data curation, formal analysis, investigation, writing original draft, writing review and editing; YH: formal analysis, investigation; MEB: investigation; LG: data curation, formal analysis; RK: formal analysis; BEP: investigation; YN: formal analysis, investigation; TSK: investigation; KEH: formal analysis; SK: formal analysis, investigation; AK: resources, funding acquisition; JDM: supervision, writing review and editing, funding acquisition, resources; JEJ: supervision, funding acquisition, writing review and editing, resources; EAA: conceptualization, supervision, formal analysis, writing original draft, writing review and editing, funding acquisition, data curation, resources.

neuroblastoma were the lowest NKG2DL-expressing tumors, highlighting a lineage dependency of this phenotype. In conclusion, these data show that epigenetic silencing of NKG2DL results in a lack of stimulatory signals to engage and activate NK cells, highlighting the underlying immune avoidance of SCLC and neuroblastoma.

Introduction:

Small cell lung cancer (SCLC) has a very poor prognosis, with little change in treatment over decades causing the NCI and US Congress to designate it a “recalcitrant cancer” (1). Treatment for SCLC for the last 30 years has involved use of cisplatin and etoposide with most tumors relapsing within one year (2). Recently nivolumab (anti-PD1 antibody) was approved for third-line treatment and combination of atezolizumab (anti-PDL1 antibody) with carboplatin and etoposide was approved for frontline treatment for metastatic SCLC, however the survival gains were measured in only a few months and it is not yet clear such immune checkpoint blockade impacts long term survival of SCLC patients (3).

The majority of the SCLCs (over 90%) have inactivating mutations in *RB1* and *TP53* but do not have targetable driver mutations. A smaller subset (about 8%) has inactivation of *RBL2* (P130)(4). Small cell lung cancer has been classified into subtypes based on expression of 4 transcription factors: *ASCL1*, *NEUROD1*, *POU2F3*, and *YAP1*. *ASCL1*^{high} and *NEUROD1*^{high} groups make up of 80% of all SCLC and, with expression of a panel of neuroendocrine genes, are referred to as “neuroendocrine” SCLCs. By contrast, the other 2 transcription factor subtypes do not express this neuroendocrine gene panel and are referred to as “non-neuroendocrine” SCLCs (5). Some of the low neuroendocrine SCLCs have a different morphology referred to as “variant.” Despite these biological differences among the SCLC subtypes, currently there are no treatments tailored for each of these subtypes and the immune landscape of these subtypes has not been fully explored.

Other neuroendocrine tumors such as neuroblastomas share similar features with SCLC such as expression of neural transcription factor *ASCL1*. Neuroblastoma has two subtypes according to super-enhancer-associated differentiation states, adrenergic (ARDN) and mesenchymal (MES). The two states can exist in one tumor and interconvert to each other (6). A recent study showed that *ASCL1* is higher in ARDN neuroblastoma compared to MES neuroblastoma (7). Besides these primary neuroendocrine tumors, neuroendocrine transformation has been observed after acquisition of resistance to targeted therapies such as with EGFR inhibitors (8) or checkpoint blockade (9) in lung adenocarcinomas, and resistance to castration therapy in prostate cancer (10).

Responses to checkpoint blockade were shown to positively correlate with the total number of somatic mutations or potential neo-antigens in several cancers (11). Despite the high mutational burden in SCLC (12), for reasons we do not fully understand, SCLCs respond poorly to immunotherapies. PD-1 immune checkpoint blockade has been approved as first-line in combination with cisplatin and etoposide in SCLC even though it is only effective in a small subset (~10%) patients (13). There is little known for SCLC about tumor immune interactions and innate immunity. Both in experimental models and patient tumors, SCLC

tumors exhibit fewer total immune cells within the tumor microenvironment, compared to NSCLCs potentially accounting for poor responses to immune checkpoint blockade (14).

Anti-tumor activity of the immune system largely depends on cytotoxic cells: T and NK cells. While T cells are an important component of adaptive immunity and depend on specific antigens, NK cells are part of innate immunity and recognize tumors by germline encoded patterns (15). NK cells are critical in preventing lung tumor growth, as depletion of NK cells was shown to facilitate lung cancer initiation in experimental models (16). NK cells are widely circulating lymphocytes, specialized in eliminating virus-infected cells and malignant cells. They attack cancer cells by secreting cytotoxic proteins, such as perforin and granzymes, and exosome membrane-bound death ligands. Anti-tumor activity of NK cells is stimulated by NKG2D ligands (NKG2DL) present on the surface of cancer cells (17,18). In humans, NKG2DLs can be classified into 2 subsets, MICA/B and RAET1 (also known as ULBP). While mice do not have genes corresponding to human MICA/B, mice do have orthologs of human RAET1 genes including *Rae1 $\alpha/\beta/\gamma/\delta/\epsilon$* , *MULT1* and *H60a/b/c* which were also shown to bind to NKG2D and are considered mouse NKG2DLs(19). Cytotoxic T cells also express NKG2D receptor, and NKG2D ligands can stimulate cytotoxicity of T cells (20).

Previous reports suggested lower expression of major histocompatibility complex (MHC-1) expression in SCLC (21). While low MHC expression would make SCLC resistant to adaptive immunity, this state should make SCLCs susceptible to NK killing (22) yet these tumors grow aggressively in mice and patients. Here we studied SCLC-NK and T cell interactions and report lack of surface ligands for NK activating receptor NKG2D in both preclinical and clinical samples of neuroendocrine SCLC and neuroblastoma. In our preclinical models, restoration of NKG2D ligand expression restored immune responses and clearance of primary SCLC tumors and metastasis highlighting this critical immune evasion mechanism by SCLCs as well as a roadmap to new immune treatment strategies.

Materials and Methods:

Computational analysis of data:

Analysis of cell line expression data: RNA samples were submitted to paired-end RNA sequencing (RNA-seq). Reads were aligned to the human reference genome GRCh38 using STAR-2.7 corrected text: (<https://github.com/alexdobin/STAR>) and FPKM values were generated with cufflinks 2.2.1 (<http://cole-trapnell-140lab.github.io/cufflinks/>). These were then normalized (top quartile normalization) and log transformed. Data were deposited to dbGaP under accession: phs001823.v1.p1 (23).

Analysis of patient tumor data: Individual genes were compared by log ratio (mean expression difference on a log scale) and T test between George SCLC(4) and Lung TCGA datasets (24). These datasets were normalized together prior to this comparison.

CCLE: Log2 mRNA values were obtained from the CCLE database (25) and were graphed.

Chip sequencing data: Super-enhancers were determined using published H3K27Ac ChIP-seq data (H69 data is from GSE62412; and A549, 3122 and PC9 data are from GSE89128; and H2087 data is from GSE72956) and unpublished data (for H510 and H2107) and calculated by HOMER using bound regions that were significant at Poisson p value threshold of 1×10^{-9} for the super-enhancer prediction.

RNA Sequencing:

RNA was extracted using Qiagen RNeasy Mini Kit (Cat# 74106). RNA was submitted to UTSW Mcdermott Sequencing Core for library preparation and sequencing. Samples were tested for integrity and concentration before library preparation. 1µg of DNase treated total RNA was prepared with the TruSeq Stranded mRNA Library Prep Kit from Illumina. Poly-A RNA was purified and fragmented before strand specific cDNA synthesis. cDNA were then A-tailed and indexed adapters were ligated. After adapter ligation, samples were PCR amplified and purified with beads. Samples were quantified then run on the Illumina NextSeq 500 using V2.5 reagents. Raw FASTQ files were analyzed using FastQC v0.11.2 and FastQ Screen v0.4.4, and reads were quality-trimmed using fastq-mcf. (ea-utils/1.1.2–806). The trimmed reads were mapped to the hg19 assembly of the human genome (the University of California, Santa Cruz, version from igenomes) using STAR v2.5.3a. Duplicated reads were marked using Picard tools (v1.127; <https://broadinstitute.github.io/picard/>). Differential expression analysis was performed using edgeR with statistical cutoffs of FDR 0.05 and log2CPM 0. Gene ontology analysis was done by PantherGO classification system. Data was deposited to GEO under accession number GSE161313.

ELISA for MICA

All patient samples were obtained from subjects providing written informed consent for blood in accordance with the Declaration of Helsinki and studies were approved by an Institutional review board at Osaka University (no.11122, 16450). Sampling was performed during routine clinical procedures before the initiation of treatment. 69 serum samples from 25 small-cell lung cancer (SCLC) and 44 NSCLC patients, who initiated the treatment in Osaka University Hospital between Oct 2012 and Mar 2017, were investigated. SCLC patients are consisted of 12 extensive disease (ED)-SCLC and 13 limited disease (LD)-SCLC. NSCLC patients are consisted of 15 squamous cell cancer (Sq) and 29 adenocarcinoma (Ad) with respective clinical stage: stage IIA (n=2), IIB (n=2), IIIA (n=4), IIIB (n=4) and IV (n=32). 11 Ad cases had EGFR mutations and 2 Ad cases had ALK translocation. Detailed information related to patient samples are in Supplementary table 1.

MICA levels in the blood from lung cancer patients, healthy people, or cell lines were quantified using the Human MICA DuoSet ELISA kit (R&D systems). For cell lines 1×10^6 cells were plated and media was collected for ELISA 24 hours after plating.

Cell culture

All human lung cell lines were obtained from Hamon Center for Therapeutic Oncology Research lines (University of Texas Southwestern Medical Center). Cell lines were DNA fingerprinted using PowerPlex 1.2 kit (Promega) and confirmed to be mycoplasma-free using e-Myco kit (Boca Scientific). Cells were maintained in RPMI-1640 (Life

Technologies) with 5% FBS at 37°C in a humidified atmosphere containing 5% CO₂. Outside of the Hamon Center the cells were maintained in RPMI supplemented 10% fetal bovine serum and with 1% Penicillin-Streptomycin (10000U/ml). Human neuroblastoma cell lines SK-N-BE (CRL-2271) & SK-N-SH (HTB-11) were obtained from ATCC and maintained in DMEM (ThermoFisher catalog #10569010) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Human neuroblastoma cell lines IMR32 (CCL-127) was also obtained from ATCC and maintained in EMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Mouse SCLC cell line RP984 was maintained in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. HEK293FT was maintained in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. RPP cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Human NK92 cell line (ATCC, Cat# CRL-2407) was purchased from ATCC. NK92 was maintained in α -MEM (Gibco, Cat# 12571063) without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine (Gibco, Cat# 25030081) and 1.5 g/L sodium bicarbonate (Gibco, Cat# 25080094). To make the complete growth medium, following components were added to the base medium: 0.2 mM inositol (Sigma, Cat# I7508); 0.1 mM 2-mercaptoethanol (Gibco, Cat# 21985023); 0.02 mM folic acid (Sigma, Cat# F8758); 10U/ml recombinant IL-2 (PerpoTech, Cat# GMP200-02); adjust to a final concentration of 12.5% horse serum (Gibco, Cat# 16050122) and 12.5% fetal bovine serum (Gibco, Cat# 16000044).

Stable expression cell lines

MICA and Rae1 delta cDNA were purchased from Origene (MICA: Cat# SC1200303, Rae1 delta: Cat# MR221464). MICA and Rae1 delta fragments were cloned from plasmids by Phusion High-Fidelity DNA Polymerase (ThermoFisher, Cat# F530). The fragments and backbone (pLv_x-EF1a-IRES-puro, Addgene Cat# 85132) were double-digested with restriction enzyme and then ligated by T4 DNA ligase (ThermoFisher, Cat# EL0012). Ligation products were transformed to NEB Stable Competent *E. coli* (Cat# C3040) and then spread on selection LB agar plate. After overnight culture at 37°C, clones were chosen for culture in LB broth. Plasmids were extracted from the cells and validated by sanger sequencing. Validated plasmids (pLv_x-EF1a-IRES-MICA-puro/pLv_x-EF1a-IRES-Rae1d-puro), pMD2.G (Addgene Cat# 12259) and psPAX (Addgene Cat# 12260) or empty vectors were transfected to HEK293FT cells by lipofectamine 2000 transfection reagent (ThermoFisher, Cat# 11668030). After transfection HEK293FT was maintained in opti-MEM (ThermoFisher, Cat# 31985088) overnight. Then change the medium to virus production medium (DMEM, 10% fetal bovine serum, Glutamax, 1% Penicillin-Streptomycin and 1% BSA). Media were collected at 24h and 48h post production. Then media was filtered through 0.45 μ M PVDF filter. Both empty vector control or MICA/Rae1d over-expressing virus medium supplemented with polybrene (Sigma Cat# TR-1003-G, 2 μ g/ml for human SCLC and 8 μ g/ml for mouse SCLC) was added to cells. Cells were cultured with virus for 48h and then selected with complete culture medium containing 1 μ g/ml puromycin for 72h. Selected cells were stained with PE conjugated flow antibodies and fluorescence associated cell sorting (FACS) sorted.

NK92/Tumor cell co-culture

NK92 cells were activated with 1000U/ml of recombinant human IL-2 (PeproTech catalog # 50813175) for 48h. SHP77 cells were seeded into 96-well plate at the density of 5000 cells/well. H69 cells were treated with vehicle (DMSO (Sigma catalog #D2650)) or 1 μ M TSA (MedChemExpress cat # HY-15144) for 24h and then were dissociated with Accumax (Innovative Cell Technologies, Cat#AM105-500) and then seeded into 96-well plate at the density of 5000 cells/well. NK92 and tumor cells were co-cultured at the ratio of NK92/Tumor cells=10:1 for 4h in the presence of TSA. After 4h co-culturing, 50 μ l of medium was harvested for cytotoxicity assay using CyQUANT™ LDH Cytotoxicity Assay Kit (ThermoFisher, Catalog # 20300) following manufacturer's instructions. Final percentage of lysis was calculated by the formula %lysis=100*(Release-Tumor spontaneous release-PBMC spontaneous release)/(Tumor maximum release- Tumor spontaneous release). Cells were later harvested for flow cytometry analysis as described. NK92 cells were gated as CD45+ population.

Mouse xenografts and allografts.

All mice were housed in a barrier facility and maintained on standard chow. Human cell lines were implanted into athymic nude mice (The Jackson Laboratory, Cat#002019) of 4-8 weeks and mouse cell lines RP984 were implanted into hybrid mice of C57B6/J and 129S1 (C57B6/J(The Jackson Laboratory, Cat#000644) and 129S1(The Jackson Laboratory, Cat#002448) of 6-8 weeks. Both male and female mice were included in all studies. 5 million human SCLC cells or 1 million mouse SCLC cells were injected subcutaneously with Matrigel (Corning, Cat#354230) and injected into both sides of mouse flanks. Width (shorter dimension) and length (longer dimension) of tumor were measured by digital caliper and volume was calculated using following formula. Volume (mm³) = width (mm) x width (mm) x length (mm)/2. Mice were randomized for treatments. In wildtype mice, NK cells were depleted by i.p. injecting 200 μ g isotype control or mouse anti-NK1.1 antibody every 3 days. CD4+ T cells were depleted i.p. injecting 200 μ g isotype control or mouse anti-CD4+ antibody every 3 days. CD8+ T cells were depleted i.p. injecting 200 μ g isotype control or mouse anti-CD8+ antibody every 3 days. In nude mice, NK cells were depleted by i.p. injecting 200 μ g isotype control antibody or mouse anti-sialoGM1 antibody every 3 days. For metastasis studies 5 $\times$ 10⁴ mouse cells were injected by intravenous (IV) route. All animal work described in this manuscript has been approved by the University of Texas Southwestern Institutional Animal Care and Use Committee.

Drug Treatment

For in vitro assays, all drugs were dissolved in 100% DMSO. For TSA, human SCLCs were treated with 0, 1, 5 μ M for 48h and mouse SCLC was treated with 0, 0.1, 0.2, 0.5 μ M for 48h. For vorinostat, human SCLCs human SCLCs were treated with 0, 0.5, 1, 5 μ M for 48h and mouse SCLC was treated with 0, 0.5, 1M for 48h. For pracinostat, human SCLCs were treated with 0, 1, 5 μ M for 48h and mouse SCLC was treated with 0, 0.25, 0.5, 1 μ M for 48h.

For in vivo treatment, a stock solution of TSA was prepared in DMSO (Sigma Cat#D2650) at 5mg/ml concentration. 10% TSA stock solution in DMSO, 5% Tween-80 (MedChemExpress Cat#HY-Y1891), 40% PEG300 (MedChemExpress Cat#HY-Y0873)

and 45% PBS were mixed for final administration. The vehicle control and drug was administrated by oral gavage at 5mg/kg 5 times a week.

RNA Extraction & qRT-PCR

RNA was extracted by Zymo Direct-zol RNA Miniprep(Cat#R2051). 1µg of total RNA was reverse transcribed to cDNA by Applied Biosystems TaqMan High-Capacity RNA-to-cDNA Kit(fisher, Cat#43-874-06) in 20µl system. 1µl product was used for qPCR in 10µl system by Taqman mastermix (Thermofisher Cat#4369016). Amplification was assessed by QuantStudio 3 Real-Time PCR System.

Flow cytometry analysis

Cell lines were first stained with fixative live/dead cell stain (Fisher Cat#50-112-1528) Room Temperature (RT) for 8min to stain the dead cells. They were then stained with fluorophore conjugated antibodies (in FACS buffer) for 20min on ice. Samples were washed with FACS buffer (2% FBS in PBS). Stained samples were analyzed using BDFACS Canto. Data was analyzed using FlowJo.

Mouse tissues were minced and digested at 37°C for 1hr (100 units/ml collagenase Fisher Cat#17104019, 10µg/ml DNase I Sigma Cat#DN25, 10% heat-inactivated FBS in RPMI) to dissociate cells. Red blood cells were lysed in the dissociated tissue with ACK lysis buffer (Fisher Cat#A1049201). Then tissues were passed through 70µm cell strainer to generate single cell suspension. Cells were stained with fixative live/dead cell stain at RT for 8min. Then they were incubated with CD16/32 antibody (Biolegend, Cat#101320) for 20min on ice. Next, they were incubated with fluorophore conjugated antibodies diluted in FACS buffer for 20min on ice to stain for surface markers. Samples were then washed with FACS buffer after every incubation with fixative dye or antibody. For the intracellular markers intracellular staining protocol was followed after surface staining.

Intracellular staining was performed by eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermofisher, Cat#00-5523-00). Samples were permeabilized with fixation/permeabilization buffer at 4°C overnight. Then they were incubated with fluorophore conjugated antibodies (IFNγ, Ki67, granzyme) diluted in FACS buffer for 20min on ice. Samples were washed with permeabilization buffer after every incubation with antibody. All antibodies were diluted in permeabilization buffer.

To determine T cell activation lymphocytes were enriched using Ficoll-Paque (GE healthcare) following the protocol. Enriched samples were incubated with PMA/ionomycin/ Golgi plug for *ex vivo* stimulation for 6 hours. Cells were then first stained with surface markers (for lineage markers) then intracellular markers (for IFNγ, Ki67 and Granzyme b) as detailed above. Samples were run on BDFACS Canto and flow data was analyzed using FlowJo. Source and catalog numbers of all antibodies used for flow are listed in Supplementary table 2.

Results:

SCLCs have reduced *MICA/B* RNA and lack *MICA/B* on cell surface

To begin to understand the SCLC tumor and innate immune cell interactions, we analyzed antigen presentation by MHC-1 expression in NSCLC vs SCLC cell lines at the protein level. As reported by several studies, neuroendocrine (NE)-SCLC lines (SCLC-A (ASCL1 high) or N (NeuroD1)) tested showed reduced MHC-1 expression compared to NSCLC lines as determined by flow cytometry (Supplementary Figure 1). Lack of MHC expression is one of the reasons for reduced immunogenicity despite the high tumor mutational burden. Next, we explored SCLC-innate immune cell interactions. We analyzed expression of ligands for the most potent NK cell activating receptor NKG2D (*MICA*, *MICB*, *ULBP1*, 2 and 3) in clinical and preclinical samples. We analyzed NKG2DL expression from RNA-seq data of human lung adenocarcinomas from TCGA (24) and SCLC tumors from George et al(4), *MICA/B* and *ULBP1,2,3* expression was significantly decreased in primary human SCLCs compared to NSCLC (Figure 1A, Supplementary figure 2). Shed *MICA* has also been reported to be found in the circulation of cancer patients with a potentially distinct role than surface *MICA* (26). We found that while late stage NSCLCs patients show significantly elevated *MICA* protein in blood, SCLC patients with late stage or extensive disease have similar levels of *MICA* in blood as healthy controls (Fig 1B) (n=44 NSCLC patients, 25 SCLC patients, and 16 healthy controls). SCLC lines showed a similar pattern of reduced NKG2DLs (*MICA*, *MICB*, *ULBP2* and 3) (All SCLC subtypes included) (Figure 1C and Supplementary figure 2). We also determined protein expression of NKG2DLs and found human SCLC-A lines lacked detectable surface *MICA/B* expression (Figure 1D) and soluble *MICA/B* (Figure 1E) while NSCLCs expressed these proteins. Overall, patient derived SCLC compared to NSCLC lines showed a significantly lower level of total NKG2DLs (Supplementary figure 3). To determine if the observations in patient samples and patient derived materials are applicable to mouse models, we examined NKG2DL expression in genetically-engineered mouse models (GEMMs), the NSCLC *Kras*^{G12D} lung adenocarcinoma model, and the SCLC *Rb1/p53/p130* (RPP) triple knockout model. Epcam positive tumor cells isolated by flow cytometry from SCLC GEMMs showed very little or no surface NKG2DL proteins as compared to the NSCLC GEMMs (Figure 1F, G). These findings argue that SCLCs, particularly SCLC-A's, have a reduced "visibility" to adaptive and innate immunity. There was also a reduction in overall immune cell infiltration (Figure 1H), and NK cell infiltration in the SCLC tumors as compared to NSCLCs (Figure 1I) in the syngeneic tumors of *Kras*^{G12D} *p53*^{-/-} NSCLC or *Rb1/p53* SCLC GEMMs implanted into wild type animals. NK cell activation was also reduced in SCLCs as compared to NSCLC tumors as indicated by CD107 staining (Figure 1J).

Restoration of *MICA* in SCLC caused NK cell activation and promoted tumor regression in an NK cell dependent manner

To determine the functional role of NKG2DL expression in SCLC tumor growth, shaping the tumor immune environment, and preventing metastasis, we expressed NKG2DL in SCLC models (Figures 2 and 3). To determine the changes in direct killing of tumor cells we first co-cultured *MICA* (human NKG2DL)-expressing stable human SCLC line (SHP77) (Figure 2A) and the corresponding vector control with human NK cell line NK92. *MICA*

expression caused increased cytotoxicity and activation as indicated by Interferon gamma (IFN γ)+ staining (Figure 2B).

To confirm our observations in patient derived models, we utilized human SCLC lines representing the SCLC-A transcription factor subtype (SHP77, NCI-H510 & NCI-H69). Expression of MICA suppressed the growth of subcutaneous xenografts in athymic nude mice with functional NK cells (Figure 2C, supplementary fig 4A, B) owing to the fact that human MICA interacts with mouse NKG2D ligands (27). MICA expression does not affect the growth rate of these cells in vitro (Supplementary Figure 4C). Inhibition of SCLC tumor growth in nude mice was dependent on NK cells as, the tumor rejection phenotype was partially reversed when NK cells were depleted (Figure 2D). To determine the immune mediated mechanism of tumor clearance after restoration of MICA expression, we generated single cell suspensions of control and MICA expressing tumors and analyzed by flow cytometry (Supplementary figure 5A). In xenografts formed by SCLC SHP77, we saw an increase in infiltrating total immune cells (CD45+) and NK cells (Figure 2E). In addition, NK cells in MICA-expressing tumors were more activated as determined by CD107a and Interferon gamma (IFN γ) production (Figure 2E). Similar results were seen in xenografts formed by SCLC models NCI-H510 and NCI-H69 (Supplementary Figure 5B & 5C). These results demonstrate that restoring MICA expression on SCLCs can boost the anti-tumor effect of NK cells.

To confirm our observations in a fully immunocompetent setting, we used a syngeneic line RP984, that was derived from SCLC tumors from a Rb1/p53 double mutant mouse (28). This model when implanted into strain matched wild type animals allows us to study the tumor immune microenvironment in a fully immunocompetent context. Expression of a mouse NKG2DL-Rae1d in RP984 syngeneic SCLC model caused significant reduction in the growth of primary tumors in mouse flanks (Figure 3A–C) while it did not change their in vitro growth rate (Supplementary Figure 4C). One of the common clinical features of SCLCs is the ability to metastasize. When RP984 cells were implanted intra-venously into mice, tumors metastasized to livers and lungs with prominent macroscopic tumors apparent in livers (Figure 3D and 3E). Expression of Rae1d significantly reduced the metastatic ability of mouse SCLCs to livers (Figure 3E). Inhibition of tumor growth in this setting was dependent on NK cells as tumors in the Rae1 expressing group grew significantly larger in NK cell depleted mice while non-Rae1d expressing SCLC tumors did not show a significant difference in growth in either NK depleted vs non-depleted tumors (Figure 3F). Depletion of CD4 T cells did not influence the growth of Rae expressing tumors while depletion of CD8T cells partially reduced tumor rejection (Figure 3G).

In the immunocompetent syngeneic model using the RP984 SCLC model, we observed a significant increase in T cell infiltration with Rae1d expression (Figure 3H). Proliferation of both cytotoxic T cell (CD3+CD8+) and NK cells were increased in the Rae1d-expressing group, which was defined by Ki67+ staining (Figure 3H). Additionally, IFN γ production of NK cells was increased (Figure 3H). Since the NKG2D receptor is also expressed on T cells and T cells can be activated by NKG2D-NKG2DL interaction, both T cell and NK cells were activated in an immunocompetent host. These observations demonstrate that restoring

NKG2D ligands in SCLC preclinical models with initial low levels of NKG2DL expression promotes anti-tumor immunity.

SCLCs downregulate MICA/B epigenetically

NKG2DLs are regulated at epigenetic, transcriptional, posttranscriptional, and posttranslational levels. Chemotherapy is a known transcriptional inducer of NKG2DL through activation of the cGAS/STING pathway (29,30). We treated SCLCs with the standard of care chemotherapy agents, cisplatin, etoposide or a PARP inhibitor (olaparib) that was previously shown to induce the STING pathway in SCLC. However, these treatments did not induce NKG2DL expression in human SCLC lines (Supplementary Figure 6A and B). We thus speculated that these genes may be epigenetically silenced. We analyzed H3K27Ac-ChIP-seq data from multiple NSCLC and SCLC cell lines and found that MICA/B enhancers are hypoacetylated in ASCL1 high SCLC lines as compared to NSCLC lines (Figure 4A and 4B). Since H3K27ac usually marks active enhancers and promoters, it appeared that MICA/B was downregulated epigenetically in SCLCs. To see if we could reverse this epigenetic inhibition, we utilized commonly used pan-histone deacetylase (HDAC) inhibitors—trichostatin (TSA) and Vorinostat (SAHA). Treatment with these HDAC inhibitors resulted in an increase in *MICA/B* mRNA (by RTqPCR) in human SCLC lines H510 and H69 (Figure 4C). Since HDAC inhibitors affect gene expression globally, we performed RNA-Seq in H69 cells treated with TSA to determine changes across the transcriptome and also validated findings with RTqPCR for *MICA/B*, *ASCL1* and *INSM1*. The most significantly affected pathways included genes involved in neural transcription (*ASCL1*, *INSM1*) and cytotoxic T cell regulation (*MICA*, *MICB*) (Figure 4D, 4E). Looking more broadly at the significantly changed genes, interestingly, we observed loss of expression of neuroendocrine genes *ASCL1*, *INSM1*, *CHGB*, *BEX1*, and *DDC* (Figure 4F and Supplementary table 3). After TSA treatment, there was an increase in expression of genes involved in recognition of cancer cells by T cells: all NKG2DLs, structural subunit of MHC-1 (*B2M*), and MHC-1 genes were significantly elevated. Pro-survival genes (*BCL2*, *MCL1*) were decreased and pro-apoptotic genes (*BAK*, *BAX*) were significantly increased in the treated cells (Figure 4F). E2F family of transcription factors are upregulated in SCLCs due to inactivation of RB and promotes tumor proliferation and the expression of these E2F transcription factors was downregulated by TSA treatment (31). Finally, Notch genes were increased (Figure 4F) in TSA treated cells, potentially contributing to shutdown of the expression of a neuroendocrine program. Treatment of human neuroendocrine SCLC lines (H69, H510, H82 and H209) with TSA increased MICA/B protein expression (Figure 4G and H). Additional HDAC inhibitors SAHA and pracinostat also induced MICA in H69 and H510 at the protein level (Figure 4H). All these experiments suggest that epigenetic regulation contributes to neuroendocrine differentiation, deregulated proliferation and reduced immune visibility in SCLCs and that epigenetic targeting agents such as HDAC inhibitors can reverse this.

To determine the functional role of NKG2DL restoration by HDAC inhibitors, we first performed a co-culture assay with NK cell line NK92 and human SCLC line H69 in presence of TSA. Cancer cells were pre-treated and NK cells were added in the culture with TSA present. TSA-treatment caused increased cytotoxicity and increased activation of NK92

cells as compared to vehicle treated control (Figure 5A). For the mouse SCLC model RP984, we performed flow cytometry for all NKG2DLs and found that HDAC inhibitors increased NKG2DL expression (Figure 5B, 5C). *In vivo*, we tested the HDAC inhibitor TSA in immunocompetent mice with RP984 allografts. TSA treatment (5mg/kg) caused significant decrease in tumor growth (Figure 5D), associated with increased NKG2DL expression on the surface of tumor cells (Figure 5E). TSA treatment also increased NK cell recruitment into the tumor and activation of cytotoxic T cells (IFN γ + Granzyme β +) (Figure 5E). Depletion of NK cells significantly reduced the therapeutic effect of TSA *in vivo* (Figure 5F) indicating that this particular HDAC inhibitor functions through activating NK cells given in this dose and schedule to this model. Our results indicated that NKG2DL expression can be induced by HDAC inhibitors in SCLC models and this can suppress SCLC growth *in vivo* and modulate tumor microenvironment to trigger the anti-tumor immunity by NK cells and T cells.

Low neuroendocrine SCLC subtypes express MICA, while neuroblastomas also exhibit reduced or no MICA expression

We wished to further understand whether these observations in neuroendocrine SCLCs are common to all SCLC subtypes and additionally whether these observations can be applied to other neuroendocrine tumors. MICA/B expression is significantly negatively associated with ASCL1 in all cancer lines and specifically SCLC lines (Supplementary Figure 7A). The majority of SCLC lines express high ASCL1 (70%). While SCLCs in general have lower levels of MICA/B compared to NSCLC, there is heterogeneity between the SCLCs. Neuroendocrine SCLCs expressed significantly lower levels of MICA mRNA as compared to non-neuroendocrine SCLCs (Figure 6A). When classified into different subtypes driven by transcription factors (32), YAP1 high SCLCs have significantly higher levels of MICA as compared to ASCL1 high, NEUROD1 high or POU2F3 lines (Figure 6B). Expression level of MICA/B in additional cell lines (NeuroD^{high} line H82 and YAP^{high} H841) were validated at the protein level (Supplementary Figure 7B).

To determine whether there are other tumors which lack NKG2DL expression we analyzed the cancer cell line encyclopedia (CCLE) dataset (25). SCLCs were second lowest NKG2DL expressing cells while neuroblastoma had the lowest among all cancer lines used in this study (Figure 6B). Interestingly, these tumor types were the top two tumor types expressing ASCL1 indicating a potential lineage dependency of this NK cell avoidance phenotype. We examined ASCL1 and MICA/B expression in 3 neuroblastoma lines. CCLE data showed that neuroblastoma line SK-N-SH had low ASCL1 and high MICA/B while the 2 other ASCL1^{high} neuroblastoma lines (IMR32, SK-N-BE) expressed low levels MICA/B, which was validated by flow cytometry (Figure 6C). When treated with HDAC inhibitors these two ASCL1^{high} neuroblastoma lines which had no detectable surface MICA/B showed increased MICA mRNA, and protein levels (Figure 6D, 6E). This suggest that an increase in innate immune visibility induced by HDAC inhibitors can be applicable to both SCLC and Neuroblastoma.

Finally, we isolated high and low neuroendocrine phenotype exhibiting isogenic cell pairs using the GEMM RPP model. It was previously shown that in mixed suspension cultures of

SCLC lines adherent population represents the variant phenotype (33). This led us to separate suspension(S) and adherent(A) tumor cell populations, from the mouse RPP SCLC line (derived from Rb1/p53/p130 triple knockout SCLC GEMM). RPP-A expressed less ASCL1 mRNA compared to RPP-S, and higher levels of NKG2DLs at the protein level (Supplementary Fig 8A, 8B). Demonstrating the plasticity of SCLC, we found the non-attached RPP-S SCLC subtype always gave rise to an adherent population (Supplementary Fig 8C), explaining the residual NKG2DL expression in the RPP-S cells. Together, these observations support the hypothesis that low NKG2DL expression is associated with high ASCL1 expression and neuroendocrine phenotype, while high NKG2DL expression is associated with the low ASCL1, low neuroendocrine expression phenotype.

Discussion:

We show here that human and mouse SCLCs expressing ASCL1, exhibiting neuroendocrine phenotype, lack expression of surface NKG2DL providing a mechanism for their escape from NK immune surveillance. Another neuroendocrine cancer type- neuroblastoma showed similar lack of NKG2DL expression in ASCL1 high cell lines. Restoring NKG2DL expression in SCLC tumor cells that lack NKG2DL was able to suppress tumor growth. We found that epigenetic changes through histone deacetylation led to lack of NKG2DL expression and thus appears to be a mechanism of deregulated innate recognition in SCLCs. Therapeutically, pan-HDAC inhibitors induced NKG2DL expression in SCLC *in vitro* and *in vivo*, and SCLC tumors were sensitive to HDAC inhibition *in vivo*, triggered anti-tumor immunity by recruiting and activating NK cells and T cells in the preclinical models. Interestingly, HDAC inhibitor also reduced expression of lineage oncogene *ASCL1* and several other neuroendocrine genes expression in SCLCs further highlighting a role of epigenetic regulation of neuroendocrine gene program.

Paucity of total immune infiltrates in SCLC tumor microenvironment (14) and as we show here specifically paucity of NK cells combined with reduced NK and T cell visibility of the tumor cells may contribute to immune resistance of SCLCs. Increasing NK cell recognition of tumors by pharmacologic means such as epigenetic regulators, may sensitize them to standard treatments and T cell killing. Our studies argue for determination of MICA expression as a prognostic factor in SCLC patient tumors and a subset of SCLCs may be considered for HDAC inhibitor treatments. HDACs are potential therapeutic targets in both NSCLC and SCLC and have effects beyond modulating NKG2DLs. An HDAC inhibitor suppressed cell proliferation, induced cell cycle arrest and activated Notch signaling in SCLCs *in vitro* (34). Inhibitor of HDAC6, a cytoplasmic HDAC was shown in a prior study to cause NK cell dependent cytotoxicity in a preclinical SCLC model though the phenotype was not related to NKG2DLs(35).

Previous trials have shown that NKG2DL stimulating drugs improved responses in patients receiving NK cell infusion (36). Thus, an HDAC inhibitor could be combined with adoptive NK cell transfer for patient treatment. Even though we observed increased NK cell cytotoxicity with HDAC inhibitors in our *in vitro* and *in vivo* models, others have shown that HDACs can negatively impact NK cell viability (37). HDACs also can increase (38) or

decrease (39) NKG2D receptor expression on NK cells and modulate NK cell cytotoxicity or other immune cell functions independent on direct effect on tumor cells.

NKG2DLs are not only found on the surface of tumor cells but also in the circulation in cancer patients. We observed similarly low levels of circulating NKG2DLs in SCLC patients while NSCLC patients showed increased NKG2DL in blood. The role shed NKG2DL plays in mediating NK responses was shown to be highly variable. Some reports indicated that presence of soluble NKG2DLs were shown to cause internalization of NKG2D receptor and suppression of NK cells (40–42). There are also reports about lack of NK cell suppression with NKG2DLs (43) or activation of NK cells with soluble NKG2DLs(44). SCLCs show reduced levels of soluble NKG2DLs. While in mouse SCLCs this did not result in increased NK activity possibly due to lack of surface NKG2DL and fewer NK cells in the tumor. The lack of shed NKG2DLs can be exploited in future studies as part of NK cell activation therapies in SCLCs.

Neuroendocrine tumors are also detected after transformation of other tumor types such as lung adenocarcinoma and prostate cancer. About 5% of the EGFR mutant, tyrosine kinase inhibitor resistant, lung adenocarcinomas acquire targeted therapy resistance by transforming to SCLC (45). There are also several reported cases on NSCLC to SCLC transformation after PD1/PD-L1 blockade. Selective pressure imposed by treatments may drive the transformation or allow the rare subpopulations of neuroendocrine clones to expand under such treatment pressure. SCLC has an immune cold tumor microenvironment with few infiltrated cytotoxic lymphocytes compared to NSCLC. Thus, transformation to SCLC with reduced immune visibility may protect the tumor cells from immune cytotoxic T cells and NK cells contributing to treatment resistance. Our findings indicate the need to explore pharmacologic epigenetic regulation of SCLC with monitoring for re-expression of NK ligands such as NKG2DL and potentially other less characterized NK cell activating ligands (46) and determining their effect on cellular and innate immune responses in clinical samples for this hard to treat lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest Disclosure:

J.D.M. receives licensing fees from the NCI and UT Southwestern to distribute cell lines. Other authors declare no relevant conflicts of interests.

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Significance:

This study discovers in SCLC and neuroblastoma impairment of an inherent mechanism of recognition of tumor cells by innate immunity and proposes that this mechanism can be reactivated to promote immune surveillance.

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Figure 1: SCLCs lack MICA/B expression

(A) *MICA* and *MICB* mRNA levels extracted from the RNA-sequencing data of human lung tumors from TCGA (lung Adeno) and George et al dataset (SCLC tumors).

(B) Quantification of MICA in the blood of SCLC patients, NSCLC patients, and healthy people by ELISA (n=44 for NSCLC patients, 25 for SCLC patients and 16 for healthy people).

(C) *MICA* and *MICB* RNA levels in NSCLC vs SCLCs from UTSW cell line collection (23).

(D) MICA/B expression on human NSCLC (H1792, H2030, H460, SKLU) vs SCLC lines SCLC lines (H69, H510, SHP77, and H2081) was determined by flow cytometry.

Representative flow images and quantification of flow signal (Mean fluorescent intensity (MFI)) are shown. Average of the two measurements per cell line is graphed.

(E) Quantification of soluble MICA in the media from 1×10^6 NSCLC vs SCLC lines in panel D.

(F) Representative NKG2DL expression and (G) Quantification of NKG2DL expression in the dissociated Epcam+ tumors cells from NSCLC (*Kras*^{LSL-G12D}, n=8) and SCLC GEMM (*Rb*^{-/-} *p53*^{-/-} *p130*^{-/-}, n=6) lung tumors. Epcam+ tumor cells determined by flow cytometry.

(H-J) Quantification of CD45+ immune cells (H), NK cells (NK1.1+) (I), and activated NK cells (NK1.1+CD107a+) (J) in NSCLC(n=5) and SCLC (n=4, 8) allograft models. Marker positive cell count per gram of tumor tissue is graphed. (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001) All experiments were done at least for 3 times and representative results were shown.

Figure 2: Expression of MICA in human SCLC causes activated NK cell response and tumor regression in athymic nude mice.

(A) Expression of MICA in MICA-expressing stable human SCLC SHP77 line determined by flow cytometry. Empty backbone of lentiviral plasmid served as control vector. All control in the figure stands for control vector.

(B) NK92 cytotoxicity against SHP77 with or without over-expressing MICA determined by LDH release. Activation of NK92 cells were indicated by IFN γ ⁺ NK92 cells determined by flow cytometry.

(C) *In vivo* growth of xenografts formed by stable MICA-expressing SHP77(n=6) or control vector SHP77(n=6) in nude mice.

(D) *In vivo* growth of xenografts formed by stable MICA-expressing SHP77 or control vector SHP77 in nude mice with or without NK cells. Iso stands for isotype control for the NK cell-depleting antibody.(n=6 for each group)

(E) Flow cytometry analysis of SHP77 xenografts. NK cells were gated as CD3-NKp46+CD49b+, denoted by black polygon on the left. Immune cells were gated as CD45+. NK cell activation was indicated by CD107a+ and IFN γ +. (*, p<0.05; **, p<0.01; ****, p<0.0001). All experiments were done at least for 3 times and representative results were shown. (For tumors, n=9 for control, n=11 for MICA-expressing. Two tumors from each mouse were harvested; For mice n = 6 for each group, some tumors were excluded from immune analysis because of dead animals or insufficient material).

Figure 3: Expression of Rae1d in mouse SCLC RP984 causes activated NK and T cell response and tumor regression in wildtype mice.

(A) Schema of in vivo experiment with mouse cells.

(B) Expression of Rae1d in Rae1d-expressing stable mouse SCLC RP984 line determined by flow cytometry. Empty backbone of lentiviral plasmid served as control vector. All control in the figure stands for control vector.

(C) *In vivo* growth of allografts formed by stable Rae1d-expressing(n=6) RP984 or control vector(n=6) in wildtype mice.

(D) Representative image of SCLC metastasis in the lung of mice implanted with RP984 cells intra-venously(IV). Yellow arrows point to SCLC lesions.

(E) Representative image of SCLC metastasis in the liver of mice implanted with RP984 cells by intravenously(IV) injection and quantification of tumor burden in the liver as represented by total and % tumor area in the livers. Representative tumor area was outlined by orange boundary and yellow arrows. (n=11 for control, n=9 for Rae1d-expressing)

(F) *In vivo* growth of allografts formed by stable Rae1d-expressing RP984 or control vector RP984 in wildtype mice with or without NK cells. Iso stands for isotype control for the NK cell-depleting antibody, n=6 for each group.

(G) *In vivo* growth of allografts formed by stable Rae1d-expressing RP984 or control vector RP984 in wildtype mice with or without CD8+ or CD4+ T cells. Iso stands for isotype control for the CD8+ or CD4+ T cell-depleting antibody, n=5 for each group.

(H) Flow cytometry analysis of RP984 allografts. Tumor infiltrated T cells were gated as CD3+. Cytotoxic T cells were gated as CD3+CD8+. NK cell activation was indicated by IFN γ +. NK cell and T cell proliferation was indicated by Ki67+. (n=9 for control, n=11 for Rae1d+ tumor tissue). 2 tumors from each mouse were harvested; For mice n=6 for each group, some tumors were excluded from immune analysis because of dead animals or insufficient material.) (n.s. not significant, *, p<0.05; **, p<0.01***, p<0.001; ****, p<0.0001). All experiments were done at least for 3 times and representative results were shown.

Figure 4: SCLCs downregulate MICA/B epigenetically

(A) Chip-seq on H3K27ac at *MICA/B* loci of human NSCLC and ASCL1^{high} SCLC. NSCLC (A549, H2087, H3122, PC9). SCLC-A (ASCL1^{high}, H2107, H510, H69). (B) Quantification of normalized H3K27 signals. Each column represents a single cell line. (C) *MICA/B* mRNA in human SCLC cell lines H69 and H510 after TSA (1 μ M) or SAHA (5 μ M) treatment after 48 hours.

(D) Significantly upregulated or downregulated pathways relevant neuroendocrine differentiation or tumor innate recognition after treating H69 with TSA as determined by RNA-Seq.

(E) *ASCL1* and *INSM1* mRNA in human SCLC cell lines H69 and H510 after TSA or SAHA treatment.

(F) Heatmap showing changes in the expression of selected genes: in lineage transcription factors, NKG2D ligands, pro-survival or apoptosis genes, E2F family of genes and Notch signaling genes from the RNA-Sequencing results. All genes shown here were statistically different between treated and vehicle treated controls.

(G) Flow cytometry for MICA/B in H209 treated with HDAC inhibitor TSA for 48h.

(H) Quantification of MICA/B expression determined by flow cytometry of human SCLC cell lines H69, H510, H82, and H209 after treatment with pan-HDAC inhibitors (TSA, SAHA & Pracinostat). (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$) All experiments were done at least for 3 times and representative results were shown.

Figure 5: HDAC inhibitor treatment enhances cytotoxicity of lymphocytes against SCLC.

(A) NK92 cytotoxicity against H69 treated with vehicle control or TSA determined by LDH release assay. Activation of NK92 cells were indicated by IFN γ + NK92 cells determined by flow cytometry.

(B) Flow cytometry for NKG2DL in RP984 treated with HDAC inhibitor TSA.

(C) Quantification of NKG2DL expression determined by flow cytometry of mouse SCLC cell line RP984 after treatment with pan-HDAC inhibitors (TSA, SAHA & Pracinostat).

(D) *In vivo* growth of allografts formed by mouse SCLC cell line RP984 treated with HDAC inhibitor TSA at 5mg/kg/5 times a week by oral gavage. Treatment starts 7 days post tumor inoculation until the last day mice were sacrificed. (n=4 for each group) Representative images of SCLC tumors from panel D and quantification of final dissected tumors.

(E) Flow cytometry analysis of TSA-treated RP984 allografts. Cytotoxic lymphocyte activation was indicated by IFN γ +Granzyme β +. (For tumors, n=4, 11 for vehicle control, n=4, 11 for TSA-treated; For mice, n=4, 6 for vehicle control, n=4, 6 for TSA-treated, some tumors were excluded from immune analysis because of dead animals or insufficient material.)

(F) *In vivo* growth of allografts formed by mouse SCLC cell line RP984 treated with TSA and isotype control or NK depleting antibodies. Quantification of final dissected tumor weights was shown on the left. Iso stands for isotype control for the NK cell-depleting antibody. (n=4 for Veh-Iso and TSA-Iso, n=3 for Veh-NK depletion and n=5 for TSA NK-depletion. 2 tumors from each mouse were harvested). (*, p<0.05; **, p<0.01) All experiments were done at least for 3 times and representative results were shown.

Figure 6: High ASCL1 expressing Neuroendocrine SCLCs and Neuroblastoma express low level of NKG2DLs

(A) MICA expression across non-neuroendocrine vs neuroendocrine SCLCs, and expression in different subsets of SCLCs classified by lineage transcription factors (ASCL1, NEUROD1, POU2F3, and YAP1) (data extracted from CCLE and classification is based on (32)).

(B) Expression of *ASCL1* and *MICA* in all cancer types from CCLE.

(C) *ASCL1* and *MICA* and *MICB* mRNA levels in 1 ASCL1 low and 2 ASCL1 high neuroblastoma cell lines (SK-N-SH, IMR32 & SK-N-BE) (left). MICA/B expression at the protein level determined by flow cytometry (right).

(D) *MICA* expression in ASCL1^{high} neuroblastoma lines IMR32 and SKNBE at mRNA level (by RTqPCR, *MICA* mRNA levels were normalized to house-keeping genes *beta-actin* or *r18S*) after treatment of HDAC inhibitors (TSA & Vorinostat).

(E) MICA/B and overall NKG2DL expression in 2 ASCL1^{high} neuroblastoma lines after treatment of HDAC inhibitors (TSA & Vorinostat) determined by flow cytometry. (n.s, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001) All experiments were done at least for 3 times and representative results were shown.