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Epithelial-mesenchymal transition leads to NK cell-mediated metastasis-specific immunosurveillance in lung cancer

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During epithelial-mesenchymal transition (EMT) epithelial cancer cells transdifferentiate into highly motile, invasive, mesenchymal-like cells, giving rise to disseminating tumor cells. Few of these disseminated cells successfully metastasize. Immune cells and inflammation in the tumor microenvironment were shown to drive EMT, but few studies investigated the consequences of EMT for tumor immunosurveillance. In addition to initiating metastasis, we demonstrate that EMT confers increased susceptibility to natural killer (NK) cells and contributes, in part, to the inefficiency of the metastatic process. Depletion of NK cells allowed spontaneous metastasis without affecting primary tumor growth. EMT-induced modulation of E-cadherin and cell adhesion molecule 1 (CADM1) mediated increased susceptibility to NK cytotoxicity. Higher CADM1 expression correlates with improved patient survival in 2 lung and 1 breast adenocarcinoma patient cohorts and decreased metastasis. Our observations reveal a novel NK-mediated, metastasis-specific immunosurveillance in lung cancer and present a window of opportunity for preventing metastasis by boosting NK cell activity.

Introduction
Significant progress has been made in determining and defining the key regulators of immunosurveillance in primary tumors, wherein the balance of protumor and antitumor responses dictates tumor progression. Targeting these key regulators led to the successful development of checkpoint inhibitors that boost T cell–mediated immunity against multiple tumor types, including non–small cell lung cancer (NSCLC) (1, 2). It is now increasingly clear that the microenvironment and the conditions tumor cells encounter in the primary tumor are disparate from what they encounter during metastasis. This suggests that mechanisms of immunosurveillance in the primary tumor may also be different from those that regulate metastatic spread (3), indicating the existence of metastasis-specific immunosurveillance (3–8).

Epithelial-mesenchymal transition (EMT) involves dissolution of cell-cell adhesions, downregulation of epithelial markers, induction of mesenchymal markers, and breach of the basement membrane to migrate and invade (9, 10). In addition, EMT also endows cancer cells with stem–cell–like properties, resistance to targeted therapies, and the ability to evade host immunosurveillance (11, 12). The multifunctional cytokine transforming growth factor-β (TGF-β) is a potent inducer of EMT and promotes tumor progression in late-stage tumors (13). Consistently, expression of TGF-β is frequently upregulated in cancers, including NSCLC (14), and is correlated with enhanced invasion, metastasis, and poor prognosis for patients with lung cancer (15). In NSCLC, we have shown that TGF-β-induced EMT confers a migratory and invasive phenotype promoting metastasis (16, 17); inhibition of EMT blocked tumor metastasis (18, 19), and an EMT-associated secretory phenotype was predictive of outcomes in NSCLC patients (20), demonstrating the biological and clinical significance of EMT in lung tumor progression.

Given the extensive molecular and cellular changes that occur during EMT, it is likely that tumor cell interactions with both innate and adaptive immune cells in the tumor microenvironment will be altered. Several studies implicated both innate and adaptive immune cells as drivers of EMT (21). However, few studies have investigated the immunomodulatory consequences of EMT (22). Studies to date have shown evasion of T cell responses and suppression of dendritic cell functions by cells undergoing EMT, suggesting an important role for EMT in immune editing (23, 24). Consistent with this, we demonstrated an immune-evasive consequence of EMT by which tumor cells escape complement-dependent cytotoxicity by modulating the complement regulatory protein CD59 (25). In contrast, here we report a novel and unexpected consequence of EMT that confers enhanced susceptibility to NK cell–mediated killing.

Natural killer (NK) cells are innate lymphoid cells known for their ability to recognize and rapidly eliminate infected or transformed cells (26–28). In humans, there is a correlation between low NK cell cytotoxicity in peripheral blood and increased cancer risk (29). Conversely, NK cell infiltration into tumor tissue is associated with better patient prognosis in multiple malignancies, including NSCLC (29). However, studies investigating the role of NK cells in tumor progression and metastasis are very limited. NK cells express an array of germline-encoded receptors that enable them to detect and respond to their targets while sparing normal...
**Figure 1.** EMT differentially regulates NK ligands and promotes susceptibility to NK-mediated cytotoxicity. (A) Heatmap (blue: downregulation; red: upregulation) representing fold changes, from 0 hours to 72 hours, time course of differentially expressed EMT markers and NK ligand genes during TGF-β–induced EMT, from a previously published gene expression profile data set (GEO GSE17708) (36). (B) Representative flow cytometric plot of cytotoxicity assay showing locations of effector, NK92mi (fluorophore-null cells), and target cells (fluorophore positive) and their exclusion DNA-binding dye status (viability indicator). PI, propidium iodide. (C–G) NK92mi-mediated cytotoxicity plots after 4 hours of coculture at indicated E/T ratios per cell type and treatment. Cell lines were treated with TGF-β (5 ng/ml) for 3, 6, 12, 6, and 6 days to induce optimum EMT, as assessed by complete E-cad downregulation and induction of vimentin or N-cadherin, in A549 (C), H460 (D), H358 (E), MCF7 (F), and DLD-1 (G), respectively. Data represent triplicate mean ± SEM, and 2-tailed unpaired *t* tests were performed. All experiments were repeated at least twice. (H) Freshly isolated human peripheral blood–derived NK cells were used as effector cells (E/T, 10:1) against A549 cells. K562 cells were used as a positive control for cytotoxicity. Data represent mean ± SEM, and 2-tailed, unpaired, *t* tests were performed. (I) To assess experimental metastasis, A549 cells were treated with TGF-β(5 ng/ml) in vitro and injected through the tail vein into RAG−/− mice. After 8 weeks, lungs were harvested to assess tumor burden. NRS, normal rabbit serum. Data represent 2 independent experiments, *n* = 3–4 for each group, and pooled results are shown. Error bars are SEM; Mann-Whitney *U* test was performed, *P* < 0.05, **P* < 0.01, ***P* < 0.001, ****P* < 0.0001.
cells. Depending on the signal they transmit, these receptors are classified into inhibitory and activating receptors (30). Integration of signaling from these receptors determines whether or not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules on potential target cells. NK cells also express other non-MHC-dependent inhibitory receptors, including killer lectin-like receptor G1 (KLRG1) (31). The type I, epithelial cadherin (E-cad) has been identified as an inhibitory ligand that engages KLRG1 in both mice and humans (32). Cell adhesion molecule 1 (CADM1) has been identified as an activating NK ligand and binds to the cytotoxic and regulatory T cell–associated molecule (CRTAM) receptor on NK cells (33). CADM1 is identified as a tumor suppressor toxic and regulatory T cell–associated molecule (CRTAM) receptor on potential target cells. NK cells also express other non–MHC-receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated. NK cells use their inhibitory receptors to detect the presence of MHC class I as self-molecules not an NK cell becomes activated.

Results

EMT differentially regulates NK ligands and confers enhanced susceptibility to NK cytotoxicity. NK cell reactivity is regulated by the balance of activating and inhibitory receptor engagement by ligands expressed on the target cells. Analysis of a previously obtained time course gene expression profile during TGF-β-induced EMT (17, 36) identified differential modulation of several NK-activating ligands, in addition to the modulation of epithelial and mesenchymal markers. We observed a significant time-dependent induction of mRNA for the NK-activating ligands PVR, CADM1, ULBP2, and ULBP4 (Figure 1A). Similarly, among the known inhibitory ligands, E-cad expression was significantly suppressed, but there was no significant change in the expression of MHC I in response to EMT. Collectively, the increased expression of activating ligands and decreased expression of inhibitory ligands in tumor cells undergoing EMT suggest a potential increased susceptibility to NK-mediated cytotoxicity. To test this, we assessed the susceptibility of a panel of human cancer cell lines to NK-mediated cytotoxicity before and after EMT. All cell lines were stimulated with TGF-β to induce EMT and then cocultured with the human NK cell line NK92mi to assess tumor cell–specific killing by flow cytometry (Figure 1B). Consistent with the ligand expression profile, we observed a significantly increased susceptibility to NK-mediated cytotoxicity in EMT cells compared with the controls at all tumor/NK cell ratios tested (Figure 1, C–G). The EMT-induced susceptibility to NK cells was not specific to lung cancer cells but was also observed in breast (Figure 1F) and colon (Figure 1G) cancer cells, suggesting that it may be a more general phenomenon in response to EMT. Similarly, murine lung cancer cells also demonstrated increased susceptibility to NK-mediated cytotoxicity after EMT (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI97611DS1). In this case, CD45+, NK1.1+, CD3ε– NK cells isolated from total splenic cells after overnight culture were used as effector cells against 344SQ murine lung cancer cells as targets.

To demonstrate that this increased cytotoxicity is not specific to the NK92mi cell line, untouched human NK cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors as effectors against EMT and control A549 cells as targets. The differential susceptibility between EMT and control A549 cells was also observed when primary PBMC-derived NK cells were used as effectors (Figure 1H). K562 cells were used as positive controls to ensure that cytotoxic-cells were isolated. Next, to demonstrate the direct link between EMT and NK-mediated immunosurveillance in vivo, we induced EMT in A549-GFP cells as described earlier (19) and injected them into the tail vein of 2 groups of Rag1–/– mice. In one group, NK cells were depleted with weekly anti-asialo GM1 (anti-ASGM1) treatment, and mice were sacrificed after 6–8 weeks to assess lung metastasis. We observed metastatic lung nodules only in the mice treated with ASGM1 and not in the control group (Figure 1I), demonstrating EMT-induced susceptibility to NK-mediated immunosurveillance, in vivo.

NK cell depletion allows spontaneous metastasis without affecting primary tumor growth. In earlier studies we demonstrated that TGF-β-induced EMT in A549 cells triggers a migratory and invasive phenotype in vitro and promotes metastasis in vivo (16, 19). Inhibition of TGF-β signaling, which prevents EMT, blocked metastasis of A549 cells (19), indicating an EMT-dependent metastasis model. To assess the role of NK cells in the above model, we implanted 10^6 A549 cells in 2 groups of Rag1–/– mice on either side of the dorsal flank. Rag1–/– mice do not have T and B cells but have functional NK cells. One group of mice was treated weekly with ASGM1 antibody to deplete NK cells. Interestingly, depletion of NK cells had no effect on the primary tumor growth (Figure 2A). In contrast, we observed spontaneous lung metastasis only in the mice that were depleted of NK cells (Figure 2B), suggesting the presence of an NK-mediated metastasis-specific immunosurveillance. Together with the observations in Figure 1, this also suggests an EMT-dependent mechanism. To assess the extent of depletion, splenic cells were isolated and analyzed by flow cytometry. We observed nearly a 90% reduction in the number of NK cells (CD45+NK1.1–CD3ε– cells) with ASGM1 treatment (Supplemental Figure 2B).

To assess the role of NK cells in metastasis in an immunocompetent host, we used a syngeneic murine model of Lewis lung carcinoma (LLC) cells. We subcutaneously implanted LLC cells in C57BL/6 hosts with and without NK cell depletion using ASGM1 antibody. LLC cells grow very aggressively in the syngeneic host but do not metastasize. As with the xenograft model above, depletion of NK cells had no effect on the primary tumor growth (Figure 2C), but we observed spontaneous lung metastasis only in mice that were treated with ASGM1 antibody (Figure 2D). In a parallel experiment, we used NK1.1 antibody instead of ASGM1 to deplete NK cells and observed similar results (Supplemental Figure 3, A and B). Since there is no single method that can exclusively deplete only NK cells, our data with ASGM1 (which is also known to affect basophil populations) and NK1.1 antibody (which is also known to deplete NK-T cells) showing similar effects demonstrate an NK cell–dependent phenomena.

The potential contribution of T and B cells to the observed metastasis-specific immunosurveillance was assessed by implanting of LLC cells in T and B cell–deficient Rag1/– mice. Notably, we observed similar spontaneous metastasis only upon NK deletion (Figure 2F), and there was no effect on the growth of the pri-
primary tumor with and without NK cell depletion (Figure 2E). We also observed similar results when S44SQ cells were implanted in C57BL/6 mice with and without ASGM1 NK depletion (Supplemental Figure 1, C and D). Collectively, similar observations in 4 different models above demonstrate the presence of an NK cell–dependent, metastasis-specific immunosurveillance mechanism.

Loss of E-cad expression sensitizes tumor cells to NK cytotoxicity through KLRG1. Classically, inhibitory signals on NK cells are mediated by receptors that recognize MHC I molecules. However, recent studies demonstrate that NK cells also express other receptors like KLRG1 that can recognize MHC-independent inhibitory signals such as E-cad (32). Since E-cad is downregulated during EMT with a concomitant increase in the susceptibility to NK cell–mediated cytotoxicity, we reasoned that E-cad may be an important inhibitory signal that protects epithelial cells from NK cell–mediated cytotoxicity. To test this, we inhibited E-cad expression by 3 different siRNA molecules in A549 cells (Figure 3A), before assessing their susceptibility to NK cell cytotoxicity using NK92mi cells as described for Figure 3. We observed that E-cad inhibition increased susceptibility of A549 cells to NK-mediated cytotoxicity (Figure 3B). However, the magnitude of cytotoxicity did not reach the levels observed after EMT. Next, we determined whether E-cad–induced inhibitory signaling is indeed mediated through KLRG1 on NK cells. siRNA-mediated inhibition of KLRG1 expression in NK92mi cells (Figure 3C) enhanced their cytotoxicity against the non-EMT control as well as EMT-induced tumor cells (Figure 3D), demonstrating that KLRG1 mediates E-cad–induced inhibitory signaling. Inhibiting the E-cad/KLRG1 axis did not fully recapitulate the enhanced cytotoxicity against A549 cells undergoing EMT, suggesting that it may also require the induction of an activating ligand.

NKG2D receptor is not involved in the EMT-induced susceptibility to NK-mediated cytotoxicity. Since we observed a robust induction of the NKG2D ligands ULBP2 and ULBP4 during EMT, we tested the potential involvement of NKG2D as an activating NK cell receptor mediating tumor cell killing after EMT. We assessed the involvement of NKG2D by independently blocking its expression, using siRNA, and function, using neutralizing antibodies, in NK92mi cells. K562 cells, whose NK-mediated cytotoxicity is partly NKG2D dependent, were used as a positive control (37). Interestingly, inhibition of expression or function of NKG2D receptors had no effect on A549 tumor cell killing before or after EMT (Figure 4, A and B). This suggests that alternative activating receptors may be critical for NK cell recognition of A549 cells after EMT.

CADM1 expression is modulated by EMT-MET cycling and mediates tumor cell susceptibility to NK cytotoxicity. Since we observed a robust induction of the NK2G2D ligands ULBP2 and ULBP4 during EMT, we tested the potential involvement of NK2G2D as an activating NK cell receptor mediating tumor cell killing after EMT. We assessed the involvement of NK2G2D by independently blocking its expression, using siRNA, and function, using neutralizing antibodies, in NK92mi cells. K562 cells, whose NK-mediated cytotoxicity is partly NK2G2D dependent, were used as a positive control (37). Interestingly, inhibition of expression or function of NK2G2D receptors had no effect on A549 tumor cell killing before or after EMT (Figure 4, A and B). This suggests that alternative activating receptors may be critical for NK cell recognition of A549 cells after EMT.
expression of CADM1 strongly correlated with prolonged patient survival (Figure 8A). CADM1 expression inversely correlated with higher tumor stage and positive nodal status (Figure 8, B and C). We further validated the survival analysis in an independent lung adenocarcinoma data set (n = 720) (Figure 8D) (42), and also in an estrogen receptor–positive breast cancer data set (n = 548) (Figure 8E) (43), as we saw an enhanced EMT-induced NK cytotoxicity in MCF7, an estrogen receptor–positive breast cancer cell line (Figure 1F). In all cases we observed similar survival benefit with increasing CADM1 expression. Additionally, we investigated the correlation between E-cad expression and patient survival in the same cohorts and found no effect on patient outcome (Supplemental Figure 8). This observation is consistent with the modest effect of E-cad/KLRG1 inhibition (Figure 3) on NK cytotoxicity, indicating the need of an activating ligand for optimum NK activation.

Discussion

Studies thus far have focused on understanding how a small proportion of disseminating cells escape host surveillance and metastasize. Unfortunately, very little attention is paid to the understanding of the mechanisms that successfully eradicate more than 99% of tumor cells. It is now increasingly appreciated that the immune system plays an important role in the surveillance against metastasis (3-7). Since EMT is critical for metastasis, exclusive focus on evasive or resistance mechanisms that cells acquire after EMT may have promoted an unintended bias: that cells undergoing EMT must be resistant to host antitumor responses. On the contrary, it is equally feasible that metastatic cells after EMT are also vulnerable to host immunosurveillance. In other words, when cancer cells exit the immunosuppressive primary tumor microenvironment it is possible that they may pay a toll to metastasize by becoming more susceptible to host immunosurveillance. Strongly supporting the above notion, our studies show that in addition to promoting an invasive phenotype (18, 19, 44), EMT renders cancer cells more susceptible to NK-mediated killing, in vitro, by modulating activating and inhibitory ligands. This is consistent with a singular in vitro study that showed increased susceptibility of a colon cancer cell line to NK cells (45). More importantly, for the first time to our knowledge, we have demonstrated the effect of this enhanced susceptibility on tumor metastasis in vivo in multiple mouse models. In addition, we also observed this phenomenon with breast cancer cell lines, indicating the broader significance of this study. Together, this suggests that the NK cell–mediated immunosurveillance mechanism may contribute in part to the inefficiency of the metastatic process.

To date, a significant number of studies in mouse models have implicated NK cells in the control of metastasis (46) and also showed that evasion of NK-mediated immunosurveillance plays a critical role in cancer progression. However, the clinical relevance of these findings is not fully understood. To address this, we next examined the clinical significance of CADM1 expression in human lung adenocarcinoma data sets.

CADM1 expression in primary tumor correlates with improved patient survival and decreased metastasis. To further demonstrate clinical significance, gene expression of CADM1 was assessed in a primary human lung adenocarcinoma data set (n = 442) (41). Univariate Cox overall survival analysis revealed that increased
role in maintaining metastatic latency (47). Supporting this, clinical studies in multiple solid tumors demonstrated an inverse correlation between the number of circulating or tumor-infiltrating NK cells and the presence of metastasis at the time of diagnosis (46). Similarly, higher expression of NK cell–activating receptors was shown to correlate with better prognosis in patients with or at risk of metastasis. However, mechanisms involved in this metastasis-specific control are unknown. Our current observations are not only consistent with the above notion but also provide a mechanistic basis by determining the activating and inhibitory axis involved. Moreover, we did not observe any effect of NK cell depletion on the growth of the primary tumor microenvironment, thereby allowing metastasis-specific control.

The direct interaction between KLRG1 and E-cad has been established. It is implicated in the recognition of non-MHC ligand-mediated inhibitory signaling in NK cells (32). Even though the role of this interaction has been proposed in tumor progression, to our knowledge it has never been demonstrated in a physiologically relevant context, until this study. Our data suggest that the ratio between E-cad and CADM1 expression may dictate the susceptibility of tumor cells to NK-mediated cytotoxicity as they abandon the immunosuppressive primary tumor microenvironment, thereby allowing metastasis-specific control.

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metastasis. This novel metastasis-specific immunosurveillance mechanism presents a potential window of opportunity to prevent metastasis by boosting NK cell functions. Multiple NK-based therapeutic strategies, at both the preclinical and the clinical stage, are under development for cancer immunotherapy with promising outcomes (57, 58). Approaches that can induce or restore activating ligands or that can neutralize inhibitory ligands or receptors may boost NK cell functions. Analogous to chimeric antigen receptor T cells, these approaches can also aid in the design of “super-NK” cells for cancer immunotherapy.

Methods

Cell lines and culture conditions

Murine Lewis lung carcinoma (LLC) and 344SQ and human A549, NK92mi, H1299, H460, H358, K562, and MCF7 cells were grown in 95% air/5% CO2 at 37°C. Culture medium for LLC cells was DMEM supplemented with 10% heat-inactivated FBS, 0.3 mg/ml l-glutamine, 50 U /ml penicillin, and 50 μg/ml streptomycin. Human cell lines were cultured according to the guidelines from the American Type Culture Collection. For EMT experiments, cells were grown in 6-well plates to 30%–40% confluence (50,000 cells per well) in complete medium. They were serum-starved for 24 hours and then treated with TGF-β (5 ng/ml) for 72 hours.

Mice

Eight-week-old male or female C57BL/6J, B6.129S7-Rag1tm1Mom/J (RAG1−/−) mice were purchased from The Jackson Laboratory and housed in specific pathogen–free conditions. All animal experiments were conducted in accordance with procedures approved by the University Committee on Use and Care of Animals and conformed to the policies and procedures of the Unit for Laboratory Animal Medicine at the University of Michigan.

Tumor models

Cancer cells were grown in their respective media. Cells were lifted using EDTA and removed with complete media. Cells were spun at 250 g in a tabletop centrifuge and washed 2 times with plain serum-free media. Cells were then resuspended in plain media at 10 × 10⁶ cells per milliliter and stored on ice until injection.

Subcutaneous. Cells were loaded into a 1-ml syringe fitted with a 26-gauge needle, and 100 μl of this cell suspension was injected subcutaneously in the right and left dorsal flanks of mice, resulting in 2 injections of 1 × 10⁶ cells each per mouse. Tumors were measured twice weekly with manual calipers, and a modified ellipsoidal volume equation, \( V = \frac{L \times W^2}{2} \), was used to determine tumor sizes. Mice were euthanized upon reaching a 2,500-mm³ tumor volume limit.

Experimental metastasis. Cells were grown and lifted via EDTA, washed twice in Dulbecco’s PBS (DPBS), and resuspended at 5 × 10⁶ cells per milliliter of DPBS. Two hundred microliters of this cell suspension was injected via lateral tail vein of mice. Mice were monitored, and after 8 weeks mice were euthanized and lungs excised for lung module counts.

siRNA transfection

CDH1, KLRG1, NKG2D, and CADM1 siRNA molecules were purchased from Integrated DNA Technologies. A scrambled sequence from the same company was used as control. Cells at 30%–40% confluence (50,000 cells per well) were transfected with siRNA using Lipofectamine 2000 and Opti-MEM medium (Thermo Fisher Scientific).
tific). After 6 hours of transfection, cells were allowed to recover overnight from transfection in RPMI-1640 medium with 10% FBS before induction of EMT or further assessments as indicated. One million NK92mi cells were transfected in 5-ml round-bottom tubes.

**Figure 5. CADM1 expression is modulated by EMT-MET cycling and mediates tumor cell susceptibility to NK cytotoxicity.** (A) A549 cells were treated with TGF-β (5 ng/ml) to induce EMT, and total proteins were extracted at the indicated times. After 72 hours, cells were washed 3 times and replaced with fresh media to induce mesenchymal-epithelial transition (MET), and total proteins were extracted at the indicated times. Protein expression of E-cad, CADM1, vimentin, and GAPDH was assessed by Western immunoblotting. (B) A549 cells treated with and without TGF-β for 72 hours were fixed and assayed for E-cad and CADM1 expression by immunofluorescence staining. Scale bars: 100 μm. (C) To stably knock out CADM1, Cas9-expressing A549 cells were transduced with lentiviruses expressing 3 different CADM1-specific CRISPR sgRNAs and a nontargeting (NT) control sgRNA. CADM1 knockout was assessed by Western immunoblotting using 2 different CADM1 antibodies raised against C-terminal (CADM1-c) and N-terminal (CADM1-n) portions. (D) and (E) Susceptibility of CADM1-KO A549 cells to NK cytotoxicity was assessed by coculturing with either NK92mi cells or primary human blood-derived NK cells from 4 different donors. In (D), mean ± SEM is shown; 2-way ANOVA with Tukey’s post hoc analysis was performed, **P < 0.01, ****P < 0.0001. In (E), EMT controls are from Figure 1H, as these experiments were performed simultaneously. Mean ± SEM is shown; 2-tailed, unpaired t tests were performed, *P < 0.05, **P < 0.01.
Genecopiea. CADM1 (catalog HCP206321-LvSG03-3-B) and NT (catalog CCPCTR01-LvSG03-B) bacterial stocks were plated, and a single colony was selected for plasmid growth and subsequent virus production. Lentiviral particles containing the specific targeting constructs were then used to infect A549-Cas9 cells. These new cell lines were verified by puromycin selection (1 μg/ml) and mCherry expression validated by flow cytometry.

Western blot analysis

Cells were washed with DPBS, pH 7.4, after treatment and lysed in radiocommunoprecipitation assay buffer (RIPA) containing NaF, Na3VO4, and protease inhibitor. Samples containing 10-20 μg of total protein were electrophoresed on SDS-polyacrylamide gels and transferred onto a PVDF membrane by electroblotting. Membranes were probed with the following primary antibodies: anti-CADM1 (3E1; catalog CM004-3, MBL International; 1:1,000), anti-CADM1 (polyclonal; catalog ABT6, EMD Millipore; 1:500), anti–N-cadherin (polyclonal; catalog 54945, Sigma-Aldrich; 1:1,000), anti-E-cadherin (36/E-Cadherin; catalog 610182, BD Transduction Laboratories; 1:1,000), anti-N-cadherin

CRISPR/Cas9-mediated deletion

A549 cells expressing Cas9 were purchased from Genecopiea. CADM1 (catalog HCP206321-LvSG03-3-B) and NT (catalog CCPCTR01-LvSG03-B) bacterial stocks were plated, and a single colony was selected for plasmid growth and subsequent virus production. Lentiviral particles containing the specific targeting constructs were then used to infect A549-Cas9 cells. These new cell lines were verified by puromycin selection (1 μg/ml) and mCherry expression validated by flow cytometry.

Figure 6. Inhibition of CADM1 in tumor cells allows spontaneous metastasis without affecting primary tumor growth. (A) mCherry-expressing CADM1 KO and control A549 cells were subcutaneously implanted into the dorsal flanks of RAG1-/- mice. Primary tumor growth was monitored, and mean tumor volumes are plotted; mean ± SEM shown. Data are representative of 1 experiment (n = 4–5 mice per group). (B) Overt lung nodules were counted on the excised lungs to assess spontaneous metastasis. Data represent 2 independent experiments, and pooled data are shown; error bars are SEM, and Mann-Whitney U test was performed. ****P < 0.0001. (C) Presence, or lack thereof, of metastatic spread was further confirmed by visualization of mCherry-positive tumor cells in the cross sections of the lungs by immunofluorescence. Top row scale bars: 500 μm; lower row: 50 μm, 100 μm, and 50 μm, respectively.
(32/E-Cadherin; catalog 610921, BD Transduction Laboratories; 1:1,000), anti-GAPDH–HRP (GAPDH 71.1; catalog G9295, Sigma-Aldrich; 1:1,000), and anti-vimentin (13.2; catalog V5255, Sigma-Aldrich; 1:1,000). Secondary antibodies, containing HRP, included anti-mouse–HRP (polyclonal; catalog A9044, Sigma-Aldrich; 1:80,000) and anti-rabbit–Alexa Fluor 488 FITC (BD Transduction Laboratories) or with CADM1 (ABT66;catalog 130-092-657). This was performed to the manufacturer’s specifications. Briefly, donated peripheral blood samples from healthy donors were subjected to ACK red blood lysis, and white blood cells were collected by centrifugation. Cells were counted and appropriate amounts of antibody cocktail were added from the NK isolation kit.

Cytotoxicity assay

Target cells were cultured and treated with TGF–β (5 ng/ml) in 1% serum media for 3–12 days, depending on cancer cell type, as previously described. These cells were then lifted with EDTA, washed in DPBS, and labeled with CFSE at 0.5 μM concentration for 10 minutes. The staining was stopped with ice-cold complete media for 5 minutes, spun at 250 g, and washed twice. Cells that were already GFP or mCherry positive were not stained and were immediately used. These cells were counted and cocultured for 4 hours with NK92mi killer cells in complete media corresponding to the cancer cells. These reaction tubes were then placed on ice for 4 hours, and propidium iodide or Sytox Blue (Thermo Fisher Scientific, catalog S34857) was added and incubated for 5 minutes and then analyzed via flow cytometry. Cells that were labeling dye or fluorophore positive and lacking NK cells was subtracted.

Immunocytochemistry/immunohistochemistry

Cells were grown on glass coverslips; after treatments, cells were fixed with ice-cold methanol for 15 minutes at –20°C. Cells were spun and washed in FACS buffer. Primary antibody incubations were performed at the following dilutions for 30 minutes on ice: CD3e (145-2C11), 1:200; NK1.1 (PK136), 1:100; CD45 (30-F11), 1:200 (BD Biosciences); KLRG1 (13F12F2), 1:100 (eBioscience). Cells were analyzed with an Attune Acoustic Flow cytometer (Applied Biosystems). Sorting was performed on a FACSAria IIIu at the University of Michigan Flow Cytometry Core. Analysis was performed with FlowJo version 10 (FlowJo LLC).

Human NK cell isolation

Human NK cells were isolated in an untouched manner using a commercially available MACS NK isolation kit (Miltenyi Biotec, catalog 130-092-657). This was performed to the manufacturer’s specifications. Briefly, donated peripheral blood samples from healthy donors were subjected to ACK red blood lysis, and white blood cells were collected by centrifugation. Cells were counted and appropriate amounts of antibody cocktail were added from the NK isolation kit.

Figure 7. Restoring CADM1 expression in tumor cells is sufficient to confer susceptibility to NK cytotoxicity. (A) Stable A549 cell lines expressing empty vector (EV) or vector with doxycycline-inducible (DOX-inducible) human CADM1 overexpression (CADM1 OE) were developed. Expression of TGF–β–induced CADM1 was assessed in the presence and absence of doxycycline by Western immunoblotting, and susceptibility to NK cytotoxicity was assessed using NK92mi cells as effectors, as described for Figure 1. Mean ± SEM is shown; 1-way ANOVA with Tukey’s post hoc analysis was performed, **P < 0.05, ***P < 0.001, ****P < 0.0001. (B) H1299 cells harboring CADM1 promoter hypermethylation were cultured in the presence and absence of a pan–DNA methylase inhibitor, 5-azadeoxycytidine (5-Aza). CADM1 expression and NK cytotoxicity were assessed as described above. Mean ± SEM is shown; 2-tailed, unpaired t tests were performed, ****P < 0.0001.

Immunodepletion

The following antibodies were administered i.p. to deplete NK cells: 25 μl per mouse of stock rabbit polyclonal anti-asialo GM1 (anti-ASGM1) (catalog 986-10001, Wako) diluted to a final volume of 100 μl in DPBS; and 200 μg per mouse of murine monoclonal anti-NK1.1 (clone PK136; catalog BP0036, BioXcell) diluted to a final volume of 100 μl in double-distilled H₂O (ddH₂O). One hundred microliters of undiluted normal rabbit serum (catalog A9044, Sigma-Aldrich; 1:80,000) and anti-chicken–HRP (polyclonal; catalog 31401, Thermo Fisher Scientific; 1:10,000), and anti-vimentin (13.2; catalog V5255, Sigma-Aldrich; 1:10,000), and anti-GAPDH–HRP (GAPDH 71.1; catalog G9295, Sigma-Aldrich; 1:1,000). Secondary antibodies, containing HRP, included anti-mouse–HRP (polyclonal; catalog A9044, Sigma-Aldrich; 1:80,000) and anti-rabbit–Alexa Fluor 488 FITC (BD Transduction Laboratories) or with CADM1 (ABT66; catalog 130-092-657). This was performed to the manufacturer’s specifications. Briefly, donated peripheral blood samples from healthy donors were subjected to ACK red blood lysis, and white blood cells were collected by centrifugation. Cells were counted and appropriate amounts of antibody cocktail were added from the NK isolation kit.

Cytotoxicity assay

Target cells were cultured and treated with TGF–β (5 ng/ml) in 1% serum media for 3–12 days, depending on cancer cell type, as previously described. These cells were then lifted with EDTA, washed in DPBS, and labeled with CFSE at 0.5 μM concentration for 10 minutes. The staining was stopped with ice-cold complete media for 5 minutes, spun at 250 g, and washed twice. Cells that were already GFP or mCherry positive were not stained and were immediately used. These cells were counted and cocultured for 4 hours with NK92mi killer cells in complete media corresponding to the cancer cells. These reaction tubes were then placed on ice for 4 hours, and propidium iodide or Sytox Blue (Thermo Fisher Scientific, catalog S34857) was added and incubated for 5 minutes and then analyzed via flow cytometry. Cells that were labeling dye or fluorophore positive and lacking NK cells was subtracted.

Immunocytochemistry/immunohistochemistry

Cells were grown on glass coverslips; after treatments, cells were fixed with ice-cold methanol for 15 minutes at –20°C. Cells were spun and washed in FACS buffer. Primary antibody incubations were performed at the following dilutions for 30 minutes on ice: CD3e (145-2C11), 1:200; NK1.1 (PK136), 1:100; CD45 (30-F11), 1:200 (BD Biosciences); KLRG1 (13F12F2), 1:100 (eBioscience). Cells were analyzed with an Attune Acoustic Flow cytometer (Applied Biosystems). Sorting was performed on a FACSAria IIIu at the University of Michigan Flow Cytometry Core. Analysis was performed with FlowJo version 10 (FlowJo LLC).
supervised the study. DGB, GC, and TJS gave reagents, analyzed data, and reviewed the manuscript. GC helped with in silico analysis.

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Figure 8. Reduced CADM1 expression is correlated to worse patient survival and metastasis. (A) The lung adenocarcinoma patient cohort of Shedden et al. (41) (Lung n = 442) was stratified into groups expressing low and high CADM1 and was assessed for overall survival. (B and C) The Shedden et al. data set is further classified into subgroups based on tumor stage and nodal status recorded at the time of diagnosis. Mean CADM1 expression and its distribution are depicted in the violin plots with box-and-whisker overlays in white. Two-tailed, unpaired t tests were performed, *P < 0.05; **P < 0.01. (D) The lung adenocarcinoma patient cohort of Györfy et al. (42) (Lung n = 720) was stratified into groups expressing low and high CADM1 and was assessed for overall survival. (E) The breast carcinoma patient cohort of Györfy et al. (43) (estrogen receptor–positive (ER+) breast n = 548) was stratified into groups expressing low and high CADM1 and was assessed for overall survival. (A, D, and E) Data sets shown here are Kaplan-Meier survival curves with log-rank P values comparing the groups.


with MAGuK-family proteins MPP3 and Dlg.


