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A Human NK Cell Activation/Inhibition Threshold Allows Small Changes in the Target Cell Surface Phenotype To Dramatically Alter Susceptibility to NK Cells

Tim D. Holmes,* Yasser M. El-Sherbiny,* Adam Davison,* Sally L. Clough,* G. Eric Blair, † and Graham P. Cook*

NK cell activation is negatively regulated by the expression of target cell MHC class I molecules. We show that this relationship is nonlinear due to an NK cell activation/inhibition threshold. Ewing’s sarcoma family tumor cell monolayers, which were highly susceptible to NK cells in vitro, developed a highly resistant phenotype when cultured as three-dimensional multicellular tumor spheroid structures. This suggested that tumor architecture is likely to influence the susceptibility to NK cells in vivo. Resistance of the multicellular tumor spheroid was associated with the increased expression of MHC class I molecules and greatly reduced NK cell activation, implying that a threshold of NK cell activation/inhibition had been crossed. Reducing MHC class I expression on Ewing’s sarcoma family tumor monolayers did not alter their susceptibility to NK cells, whereas increased expression of MHC class I rendered them resistant and allowed the threshold point to be identified. This threshold, as defined by MHC class I expression, was predictive of the number of NK-resistant target cells within a population. A threshold permits modest changes in the target cell surface phenotype to profoundly alter the susceptibility to NK cells. Whereas this allows for the efficient detection of target cells, it also provides a route for pathogens and tumors to evade NK cell attack.

The response of NK cells to infected cells and tumors is regulated by both activating and inhibitory receptors.

Killing of target cells only occurs when activating signals outweigh inhibitory ones (1–3). The missing-self model and the identification of inhibitory receptors for classical MHC class I molecules, such as the killer cell Ig-like receptors (KIR) and the CD94/NKG2A receptor for HLA-E, has revealed that MHC class I negatively regulates NK cell activation (4–6). The subsequent discovery of NK cell activation receptors such as NKG2D, DNAX accessory molecule-1 (DNAM-1), and the natural cytotoxicity receptors (NCRs) provided evidence that it is the net balance of activating and inhibitory signals that determines the outcome of NK cell–target interactions (1, 3). This implies the existence of an activation/inhibition threshold; signals from numerous activating and inhibitory receptors converge on the guanine nucleotide exchange factor Vav1 and compete to phosphorylate or dephosphorylate this molecule and determine whether activation or inhibition results (7–9). Despite detailed knowledge of these signaling pathways, few studies have considered the role of the threshold in determining the outcome of interactions between NK cells and populations of tumor cells.

We have analyzed the interactions between human NK cells and Ewing’s sarcoma family tumors (ESFT). These are solid tumors caused by chromosome translocations, most frequently between the EWS gene on chromosome 22 and the Fli1 gene on chromosome 11 (10). ESFT cells are highly sensitive to NK cell attack in vitro via the action of NKG2D and DNAM-1 (11), yet evade NK cells in vivo. Thus, tumor progression in vivo must be accompanied by NK cell evasion mechanisms that are not evident using conventional cell cultures and NK cell cytotoxicity assays. We have investigated the role of solid tumor architecture in NK cell recognition of ESFT using a multicellular tumor spheroid model (MCTS). Our results indicate that solid tumor architecture is highly resistant to NK cell attack and were suggestive of an NK cell activation threshold. We identify this threshold and demonstrate that it allows small changes in the tumor cell-surface phenotype to profoundly influence the susceptibility of target cells to NK cell attack. This has implications for NK cell recognition and immune evasion of both tumors and infected cells.

Materials and Methods

Cells, reagents, and flow cytometry

The ESFT cell lines and the patient samples all contain the EWS-Fli1 translocation and were provided by S. Burchill and the Cytogenetics Department, Leeds Teaching Hospitals National Health Service Trust. Abs for flow cytometry were as follows (clone; supplier): CD56 (AF12-7H3; Miltenyi Biotec), CD3 (OKT3; BD Biosciences), MHC class I (W6/32; Sigma-Aldrich), HLA-E (3D12; ebioscience), CD107a and CD107b (H4A3&H4B4; BD Biosciences), MIC-A/B (6D4; Cambridge Biosciences), and NKG2A (Z199; Beckman Coulter). Abs/proteins for receptor blocking experiments were: NKG2D (1D11; BD Biosciences), NKP30 and

Abbreviations used in this article: DNAM-1, DNAX accessory molecule-1; ESFT, Ewing’s sarcoma family tumor; KIR, killer cell Ig-like receptor; KLRG1, killer cell lectin-like receptor G1; EWS, Ewing’s sarcoma family tumor; ESFT, Ewing’s sarcoma family tumors; KIR, killer cell Ig-like receptor; KLRG1, killer cell lectin-like receptor G1; β2m, β2-microglobulin; β2m-siRNA, short interfering RNA molecules targeting β2-microglobulin mRNA; MCTS, multicellular tumor spheroid; NCR, natural cytotoxicity receptor; siRNA, short interfering RNA; SKES-1 MCTS, SKES-1 corresponding spheroid structure; SKES-1 mono; SKES-1 monolayer; TC32 MCTS, TC32 corresponding spheroid structure; TC32 mono; TC32 monolayer.

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NKp44 (p30-15 and p44-8; both gifts of Dr. C. Watzl, University of Heidelberg, Heidelberg, Germany), and NKp46 (9E2/nkp46; BD Biosciences). Chimeric FcR fusion proteins of NKG2D and the NCR molecules were purchased from R&D Systems. Flow cytometry was performed using FACS caliber or LSRII flow cytometers (BD Biosciences) and cell sorting using a MoFlo (Dako Cytomation).

**Cell culture and transfection**

ESFT monolayer and MCTS culture were performed as previously described (12). The cell lines A673 (and HeLa) were cultured in DMEM, SK-N-MC cells were cultured in DMEM/F12, and SK-ES-1 cells grown in McCoy’s 5A, TC32, RD-ES, and patient-derived samples (and K562) were grown in RPMI 1640 medium. Monolayers were cultured using Falcon Primera flasks (BD Biosciences). For spheroids, cells were propagated as monolayers and then seeded onto agar-coated flasks for 72 h to prevent attachment before transfer to stirrer flasks (Integra Biosciences) and grown for 7–14 d (12). For RNA interference, short interfering RNAs (siRNAs) targeting β2-microglobulin, HLA-E, or controls were transiently transfected into either TC32 or HeLa cells using Oligofectamine and Opti-MEM media (Invitrogen). Cells were used in cytotoxicity experiments 72 h posttransfection. The β2-microglobulin (β2-m) and control siRNA duplexes were synthesized by Eurogentec (with 3′ TT extensions). For the β2-m mRNA (Genbank Accession: NM_004048.2; http://www.ncbi.nlm.nih.gov/genbank/), we used siRNA duplex targeting positions 144–162. Two additional siRNA duplexes were evaluated targeting the β2-m mRNA at nt 219–237 and 402–420. The 144–162 duplex gave the best inhibition of expression as judged by cell-surface expression of MHC class I molecules using the W6/32 Ab and flow cytometry. The control siRNA duplex had the sequence UUUCCUGAACGGUCAGCU (with 3′ TT extensions), and for HLA-E, we used a Dharmacon siGENOME SMARTPool M-012106-01-0005 comprising four separate oligonucleotides.

**NK cell purification and functional assays**

NK cells (>95% pure) were purified from healthy donors using indirect magnetic immunoselection (Miltenyi Biotec) and activated using 50 U/ml IL-2 for 7 to 8 d. Cytotoxicity assays were performed using an FACS-based assay, as described (13). For receptor blocking, NK cells were treated with 15 μg/ml Ab prior to addition of target cells. To compare NK cell degranulation in response to monolayers or MCTS, an equal surface area of target cells was calculated for MCTS using an eye-piece graticule, to measure MCTS radius (r) and the surface area calculated using the formula: area = 4πr². The degranulation assays were performed as previously described (14, 15). For gross analysis of spheroid death, similar sized spheroids were individually placed in separate wells of an agar-coated 96-well plate. NK cells (or other populations) were added at the appropriate E:T ratio and cocultured on a rocker platform within a tissue culture incubator for up to 96 h. Individual spheroids were visualized under the microscope or isolated, fixed with 3% paraformaldehyde, and stained according to the surface area of tumor cells presented to the NK cell according to the surface area of tumor cells presented to the NK cell according to the surface area of tumor cells presented to the NK cell. For RNA interference, short interfering RNAs (siRNAs) targeting β2-microglobulin, HLA-E, or controls were transfected into either TC32 or HeLa cells using Oligofectamine and Opti-MEM media (Invitrogen). Cells were used in cytotoxicity experiments 72 h posttransfection. The β2-microglobulin (β2-m) and control siRNA duplexes were synthesized by Eurogentec (with 3′ TT extensions). For the β2-m mRNA (Genbank Accession: NM_004048.2; http://www.ncbi.nlm.nih.gov/genbank/), we used siRNA duplex targeting positions 144–162. Two additional siRNA duplexes were evaluated targeting the β2-m mRNA at nt 219–237 and 402–420. The 144–162 duplex gave the best inhibition of expression as judged by cell-surface expression of MHC class I molecules using the W6/32 Ab and flow cytometry. The control siRNA duplex had the sequence UUUCCUGAACGGUCAGCU (with 3′ TT extensions), and for HLA-E, we used a Dharmacon siGENOME SMARTPool M-012106-01-0005 comprising four separate oligonucleotides.

**Results**

**ESFT trigger NK cell activation via NKGD2 and the NCRs**

Under conventional monolayer culture conditions, ESFT cell lines are highly susceptible to NK cells via the action of NKGD2 and DNAM-1 (11). The NCR molecules also play a major role in NK cell activation by tumors (16–18), suggesting that the high susceptibility of ESFT to NK cells might be due to coengagement of NKGD2 and NCRs. ESFT cell lines were sensitive to killing by both resting and IL-2–activated NK cells when cultured as monolayers (Fig. 1A). Cytotoxicity assays using receptor-blocking Abs revealed that all three NCR molecules were involved in ESFT recognition. Furthermore, blocking multiple NCRs or combining NCR and NKGD2 blocking reduced ESFT killing considerably (Fig. 1B–D). Three ESFT cell lines analyzed in detail showed nearly identical responses to NK cells treated with blocking Abs. This suggested that they expressed very similar repertoires of NK cell activation ligands and staining with soluble NKGD2 and NCR molecules supported this (Supplemental Fig. 1). Expression of MHC class I molecules varied across the ESFT panel, but there was no apparent relationship between MHC class I expression and susceptibility to NK cells (Fig. 1A, Supplemental Fig. 1). For example, SKES-1 and TC32 expressed very different levels of cell-surface MHC class I but were both susceptible and had similar responses to receptor blocking Abs (Fig. 1A–C).

**ESFT with a solid tumor-like architecture are highly resistant to NK cells**

Despite the high degree of susceptibility in vitro, ESFT, like other tumors, evade NK cells in vivo. However, ESFT is a solid tumor, and NK cells must recognize and presumably penetrate this structure to effect tumor clearance, a feature that is not represented in model systems that use cells cultured in monolayers.

Solid tumors are highly organized and complex structures consisting of malignant cells and many interacting cell types, including immune cells, stromal cells, and blood vessels. We have analyzed the effect of solid tumor-like architecture on NK cell activation using a highly simplified model. ESFT were cultured as MCTS, three-dimensional structures with an outer rim of live cells (~100 μm deep) and a central core of apoptotic and dead cells caused by a lack of oxygen and nutrient diffusion to this depth. Although lacking the complexity of a solid tumor, MCTS represent a convenient starting point to dissect solid tumor–NK cell interactions, and, despite their limitations, MCTS more closely resemble solid tumor architecture than conventional monolayer cultures (12, 19, 20). The presence of apoptotic/dead cells in MCTS structures prevented the use of conventional NK cell cytotoxicity assays due to the high background of cell death in the absence of NK cells. Instead, we used NK cell degranulation, as determined by the presence of granule-derived CD107 molecules on the NK cell surface, as a direct measure of NK cell activation (14). We compared the ability of TC32 and SKES-1 monolayers (denoted TC32 Mono and SKES-1 Mono, respectively) and their corresponding spheroid structures (denoted TC32 MCTS and SKES-1 MCTS, respectively) to cause degranulation of IL-2–activated NK cells (Fig. 2A, 2B). The ratio of NK cells to targets was adjusted according to the surface area of tumor cells presented to the NK cells in both the monolayer and MCTS, such that the tumor cells were in excess. The spheroid structures of both cell lines induced much less NK cell degranulation than their corresponding monolayers (Fig. 2A, 2B), indicating that the architecture of these structures conferred greatly increased resistance to NK cell attack. Thin sections derived from MCTS that had been cocultured with different effector cell populations for 48 h exhibited little or no damage in the presence of unstimulated NK cells. However, SKES-1 MCTS were destroyed by IL-2–activated NK cells, whereas the TC32 MCTS remained intact (Fig. 2C). This differential sensitivity of SKES-1 MCTS and TC32 MCTS structures to IL-2 activated NK cells was visible by microscopy of whole spheroids (Fig. 2D) and mirrored the differential NK responses to the MCTS structures detected by the degranulation assay (Fig. 2B; p = 0.004). These results suggested that the MCTS structures exhibited weaker engagement of NK cell activation receptors and/or stronger engagement of inhibitory receptors compared with the monolayers. In support of this, comparison of the surface phenotype of spheroid-derived cells and monolayer cells revealed increased expression of MHC class I molecules on cells from the spheroid structures of both cell lines (Fig. 2E). In contrast, soluble NKp44 bound less to spheroid-derived cells than those cultured as monolayers, whereas there was little difference in binding of soluble NKGD2 by these populations. Thus, increased resistance of the MCTS structures was associated with the increased expression of MHC class I as well as the reduced expression of potential NKp44 ligand(s).
The role of ESFT MHC class I molecules in NK cell inhibition

The missing-self model predicts that reduction of MHC class I expression will result in increased susceptibility to NK cells and vice versa. SKES-1 Mono cells expressed much less MHC class I expression compared with the other ESFT cells analyzed, yet exhibited similar susceptibility to NK cell attack (Fig. 1), as shown previously (11). This suggested that MHC class I levels did not strongly influence the susceptibility of ESFT, yet elevated MHC class I levels on the MCTS was associated with inhibition of NK cell activation. This apparent paradox could be explained by the existence of an NK cell activation threshold regulated by MHC class I; expression below the threshold would allow NK cell activation, whereas expression above the threshold level would inhibit activation and render the tumor cell resistant to attack. To test this hypothesis, we manipulated MHC class I levels on TC32Mono cells.

Cell-surface expression of MHC class I molecules requires a ternary complex of MHC class I H chain, the antigenic peptide, and β2m, and loss of β2m expression prevents cell-surface expression of this complex (21). Transient transfection of siRNA molecules targeting β2m mRNA (β2m-siRNA) inhibited the cell-surface expression of MHC class I molecules in TC32Mono and HeLa cells, whereas expression of the NKG2D ligands MICA and MICB were unaffected by the β2m-siRNA or control siRNA (Fig. 3A). We also increased expression of MHC class I molecules. IFN-γ induces the cell-surface expression of MHC class I and protects target cells against NK cells (22, 23); SKES-1Mono, TC32Mono, and HeLa cells all increased expression of cell-surface MHC class I molecules in response to this cytokine (Fig. 3B).

Monolayer cells with altered levels of cell-surface MHC class I molecules were then analyzed for their susceptibility to NK cell-mediated killing. HeLa cells obeyed the predictions of the missing-self hypothesis, and killing was inversely related to cell-surface MHC class I expression (Fig. 3C). However, TC32Mono cells demonstrated a threshold of MHC class I expression controlling susceptibility to NK cells; inhibition of MHC class I expression did not increase the susceptibility of TC32Mono to NK cells, indicating that MHC class I expressed by the TC32Mono was not restraining NK cell activation. However, the IFN-γ-mediated induction of MHC class I on TC32Mono protected them against NK cell attack (Fig. 3D). This protection was dependent on MHC class I expression, as shown by the loss of IFN-γ-mediated protection in the presence of the β2m-siRNA (Fig. 3D, 3E). In the case of SKES-1Mono, IFN-γ-induced expression of MHC class I molecules (Fig. 3B) but did not protect them against NK cell attack (Fig. 3F). However, cell-surface MHC class I expression on IFN-γ-induced SKES-1Mono cells did not reach the level of MHC class I expression exhibited by the uninduced TC32Mono cells; this level of MHC class I expression did not inhibit attack by NK cells (Fig. 3B, 3D). Thus, expression of MHC class I on IFN-γ-induced SKES-1Mono cells remained below the threshold required to inhibit NK cells.

The IFN-γ stimulation of TC32Mono cells also induced expression of the MHC class Ib molecule HLA-E (Fig. 4A). This suggested that the IFN-γ-mediated inhibition of killing might result from interactions between HLA-E and inhibitory NKG2A/CD94 heterodimers. However, siRNA-mediated inhibition of HLA-E expression in IFN-γ-treated TC32Mono cells did not
restore the susceptible phenotype (Fig. 4A, 4B). Furthermore, killing assays using NKG2Aα and NKG2Aββ populations of NK cells generated by FACs were equally inhibited by IFN-γ–treated TC32Mono cells, revealing that this inhibition was not mediated by interactions between HLA-E and NKG2Aα/CD94 heterodimers (Fig. 4C, 4D).

A threshold for NK cell inhibition/activation regulated by MHC class I

The above experiments identified a threshold for NK cell activation relative to target-cell MHC class I expression. Expression of TC32Mono MHC class I molecules above the threshold point (as mediated by IFN-γ) resulted in NK cell inhibition, whereas expression below the threshold (as mediated by β2m-siRNA) did not affect activation. We analyzed the threshold in detail by treating TC32Mono cells with differing amounts of IFN-γ, β2m-siRNA, or a combination of the two to generate populations of cells with varying levels of cell-surface MHC class I molecules (Fig. 5A). These populations were used in cytotoxicity assays with IL-2–activated NK cells. The protection against killing conferred by these treatments was calculated relative to a control siRNA-transfected TC32Mono population and plotted according to the level of MHC class I expression (Fig. 5A, 5B). These results show that the transition from a resistant target to a sensitive one occurs over a relatively narrow range of MHC class I expression, identifying the threshold. For a given cell line, a threshold implies that the proportion of target cells within a population that express MHC class I above the threshold should equal the proportion of cells that are resistant to NK cell-mediated killing. When MHC class I expression is altered in these cells, it should be possible to predict the proportion of cells in this population that are resistant to killing. A population of TC32Mono cells in which 26% of the cells were resistant to killing was used to assign an MHC class I expression level to the threshold. Using a flow cytometry plot, we interpolated the MHC class I expression level that placed 26% of cells above the threshold (resistant) and 74% below (susceptible). This MHC class I expression value (the threshold) was then applied across the different TC32Mono populations shown in Fig. 5A to calculate the proportion of each population, which showed MHC class I expression above the threshold. The predicted size of the resistant population was then compared with the experimentally defined resistant population. As expected, the two values were in very close agreement (Fig. 5C). We then repeated this experiment with monolayer cultures of the SK-N-MC cell line (SK-N-MCMono). Unlike TC32Mono, we were unable to induce the very high levels of MHC class I expression in SK-N-MCMono required to reach a plateau of NK cell killing. However, like TC32Mono, SK-N-MCMono cells showed a sharp transition from a susceptible to a resistant phenotype consistent with a threshold level of MHC class I protecting against NK cell attack (Fig. 5D).

Furthermore, the proportion of cells within the population that expressed MHC class I above the threshold was strongly correlated with the size of the resistant population (Fig. 5E). Interestingly, the threshold level of MHC class I that protects TC32Mono and SK-N-MCMono cells is very similar. A threshold value that is predictive of susceptibility to NK cell-mediated killing indicates that relatively small changes in MHC class I expression can profoundly affect the susceptibility of target cells to NK cell-mediated attack.

Discussion

The key observation that mouse NK cells killed tumors lacking MHC class I molecules (4) was accompanied by the finding that increased MHC class I expression was inversely proportional to
NK cell susceptibility (4–6, 22, 24). These results were closely followed by similar findings using human cells (25–28) and argue for a quantitative outcome of interactions between MHC class I and KIR molecules, as supported by our manipulation of MHC class I expression levels using HeLa cells. However, the ESFT cell lines tested in this study did not appear to obey these rules.

Instead, our results demonstrate the existence of a threshold of NK cell activation that is regulated by target cell MHC class I. Definition of the threshold allows predictions to be made regarding the susceptibility of a target cell population to NK cells. For example, the HeLa cell population used in this study exhibits the differential expression of HLA-E and associated protective peptides in the different cell types used in these studies. Indeed, TC32 expresses HLA-B alleles with leader sequences previously shown to be nonprotective (29). In the case of TC32 cells, we demonstrate that the protective effect of IFN-γ is β2m dependent but HLA-E independent. Although some studies have suggested that IFN-γ protects tumor cells from NK cells via increased MHC class I expression (22, 23), others have suggested that such protection might be an MHC class I-independent event (30, 31). These apparently contradictory results may result from the differential expression of HLA-E and associated protective peptides in the different cell types used in these studies.

Expression of MHC class I on TC32MCTS cells exceeded the threshold identified using IFN-γ–treated TC32Mono, and thus resistance of TC32MCTS to IL-2–activated NK cells might be
FIGURE 5. A threshold of NK cell inhibition/activation mediated by MHC class I. A. Populations of TC32Mono cells expressing different levels of MHC class I were generated (using Bm-siRNA/IFN-γ) and used in killing assays with IL-2–activated NK cells. MHC class I expression was analyzed using the W6/32 Ab; isotype control staining is also shown (iso). The dotted line bordering the left of the gray shaded area is the threshold level of MHC class I expression that confers protection of TC32Mono cells to IL-2–activated NK cells. The position of the threshold was fixed from a single experiment in which 26% of a population of TC32Mono cells was resistant to killing; the flow cytometry plot of MHC class I expression of this population was interpolated to define the MHC class I expression level that split this population into 74% (below threshold, sensitive to killing) and 26% (above threshold and resistant). The gray shaded area shows MHC class I expression above the threshold; TC32Mono cells that express MHC class I within this region were predicted to be protected from NK cell attack. This was then determined using killing assays and the actual degree of protection conferred by these treatments expressed as the percentage inhibition of killing compared with TC32Mono cells treated with a control siRNA. B, Resistance to killing and MHC class I expression. The proportion of NK cell-resistant TC32Mono cells (expressed as percentage inhibition of killing compared with control siRNA-transfected control cells) versus the log_{10} geometric mean (GM) of fluorescence of MHC class I molecules, for the data shown in A. C, The threshold is predictive of the number of resistant cells in a population. Correlation between the proportion of cells expressing MHC class I molecules above the threshold (calculated from A) versus the proportion of cells resistant to attack (compared with controls, % inhibition). Correlation (goodness-of-fit) was assessed by linear regression (r^2). D and E show the same analysis as B and C, respectively, performed on populations of SK-N-MCMono cells.

explained, at least in part, by this protective effect. In addition, expression of putative Nkp44 activating ligands were reduced on the TC32MCTS compared with TC32Mono, suggesting that alterations in both inhibitory and activating ligands contribute to the reduced susceptibility of the spheroids. Similar alterations in expression of MHC class I and Nkp44 ligands were observed on the SKES-1MCTS compared with SKES-1Mono. However, SKES-1MCTS remained susceptible to IL-2–activated NK cells. In this regard, the level of MHC class I expression on SKES-1MCTS cells remained below the inhibitory threshold identified using TC32Mono. Superimposing the threshold value between cell types and monolayers/ spheroids is speculative for several reasons. Firstly, NK cell activation is not solely determined by MHC–KIR interactions but by the balance of expression of numerous inhibitory and activating ligands. Furthermore, the KIR and HLA loci are highly polymorphic, and the ability of MHC class I to inhibit NK cells depends not only on the level of MHC class I expression but also on the ability of a particular HLA molecule to functionally interact with the KIR repertoire. In this respect, SKES-1 and TC32 both express HLA-C1, C2, and Bw4 epitopes, allowing them to interact with KIR2DL2, KIR2DL3, KIR2DL1, and KIR3DL1, which are common among KIR haplotypes (32). In addition, recent data indicates that different HLA-bound peptides can profoundly alter the outcome of inhibitory KIR–HLA interactions (33). Despite this complexity, the degree of NK cell degranulation induced by the different spheroids and monolayers analyzed was consistent with their expression of MHC class I molecules relative to the threshold identified using TC32Mono. The identified threshold was very similar in TC32Mono and SK-N-MCMono, and both the cell-surface phenotype and the use of receptor-blocking Abs in killing assays suggested that the different ESFT cell lines expressed similar repertoires of NK cell-activating ligands. Thus, resistance of the spheroid structures to IL-2–activated NK cells is consistent with the expression of MHC class I relative to the identified threshold. Improved models in which MHC class I levels can be manipulated within a given spheroid are required to properly address the contribution of MHC class I expression to spheroid resistance. The role of other ligands in determining the resistance of spheroids to NK cells, as well as the importance of other immune evasion strategies (such as release of immunosuppressive cytokines), requires further analysis. The role of Nkp44 ligands in this process awaits definition of these ligands at the molecular level.

Thresholds defined by target cell killing were previously observed using NK cell clones and 721.221 target cells expressing a single cognate MHC class I molecule (34). In this case, the threshold of individual clones differed. Similarly, individual mouse NK cells vary in their responsiveness to targets according to the number of inhibitory receptors for MHC class I that they express (35). At first sight, our data appear to be inconsistent with these results. However, we have defined the threshold according to the susceptibility of target cells to a polyclonal population of NK cells exhibiting a normal distribution of killing activity; weak effector cells may spare target cells with subthreshold MHC class I expression levels, but these targets will eventually be detected and killed by strongly responsive effectors. The enhanced cytotoxic activity of IL-2–activated NK cells results from potent induction of the killing apparatus (13, 15) and may also result from the lowering of the NK cell activation threshold by alterations in the relative expression of inhibitory and activating receptors and/or their associated signaling components.
Malignant cells from human solid tumors are frequently susceptible to NK cells when cultured as monolayers in vitro, yet by definition evade NK cells (and other immune effector functions) in vivo. The in vivo setting clearly provides an environment under which efficient immune evasion operates and many mechanisms of tumor evasion of NK cells have been described (36–41). Our results suggest that solid tumor architecture may play an important role in determining the susceptibility to NK cells in vivo. Reduced NK cell activation due to the expression of inhibitory ligands above a threshold level is likely to be just one facet of this resistance. It is tempting to speculate that architecture itself contributes directly to the resistance of the MCTS; for example, by limiting the infiltration of NK cells due to the presence of tight junctions (19). Interestingly, expression of E-cadherin, a ligand of the NK cell inhibitory receptor killer cell lectin-like receptor G1 (KLRG1), is upregulated on ESFT MCTS structures (20). However, expression of N-cadherin, which is also a ligand of KLRG1, was similarly reduced, and the role of KLRG1–cadherin interactions in ESFT killing remains unknown. Spheroid models or similar three-dimensional culture approaches will provide a useful tool to begin to dissect the contribution of human solid tumor architecture to immune evasion. It will be interesting to determine the molecular basis of the differential expression of NK cell activating and inhibitory ligands under different growth conditions, and these investigations are underway.

A threshold for NK cell activation allows relatively small changes in MHC class I expression (or other regulatory ligands) to profoundly influence the susceptibility of tumor cells or virus-infected cells to NK cell attack. Small reductions in cell-surface MHC class I expression that cross the threshold favor NK cell activation, providing a sensitive detection mechanism for NK cells. Many tumors (including Ewing’s sarcoma) downregulate MHC class I expression during disease progression, presumably reflecting cytotoxic T cell evasion (42, 43). A threshold might allow reduction of MHC class I expression to reduce recognition by T cells without compromising resistance to NK cells. Furthermore, an NK cell activation threshold explains the in vivo persistence of tumor cells that continue to express both NK cell activating and inhibitory ligands, because selection by the immune system will favor outgrowth of tumors that fail to trigger NK cells (44–46).

Despite the increased resistance of the spheroid structures to NK cells, it was encouraging that IL-2–activated NK cells showed some efficacy against the SKES-1 MCTS. This suggests that cytokine-based strategies remain a viable option for the treatment of some solid tumors. However, the striking differences in the NK cell susceptibility of ESFT when cultured using the two- or three-dimensional systems shown in this study highlights the need for improved models to better define the interactions between human NK cells and solid tumors.

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The authors have no financial conflicts of interest.

References
ACTION/INHIBITION THRESHOLD

AN NK CELL ACTIVATION/INHIBITION THRESHOLD

NKC and solid tumors.

of tumor of NK cells have been described (36–41). Our results suggest that solid tumor architecture may play an important role in determining the susceptibility to NK cells in vivo. Reduced NK cell activation due to the expression of inhibitory ligands above a threshold level is likely to be just one facet of this resistance. It is tempting to speculate that architecture itself contributes directly to the resistance of the MCTS; for example, by limiting the infiltration of NK cells due to the presence of tight junctions (19). Interestingly, expression of E-cadherin, a ligand of the NK cell inhibitory receptor killer cell lectin-like receptor G1 (KLRG1), is upregulated on ESFT MCTS structures (20). However, expression of N-cadherin, which is also a ligand of KLRG1, was similarly reduced, and the role of KLRG1–cadherin interactions in ESFT killing remains unknown. Spheroid models or similar three-dimensional culture approaches will provide a useful tool to begin to dissect the contribution of human solid tumor architecture to immune evasion. It will be interesting to determine the molecular basis of the differential expression of NK cell activating and inhibitory ligands under different growth conditions, and these investigations are underway.

A threshold for NK cell activation allows relatively small changes in MHC class I expression (or other regulatory ligands) to profoundly influence the susceptibility of tumor cells or virus-infected cells to NK cell attack. Small reductions in cell-surface MHC class I expression that cross the threshold favor NK cell activation, providing a sensitive detection mechanism for NK cells. Many tumors (including Ewing’s sarcoma) downregulate MHC class I expression during disease progression, presumably reflecting cytotoxic T cell evasion (42, 43). A threshold might allow reduction of MHC class I expression to reduce recognition by T cells without compromising resistance to NK cells. Furthermore, an NK cell activation threshold explains the in vivo persistence of tumor cells that continue to express both NK cell activating and inhibitory ligands, because selection by the immune system will favor outgrowth of tumors that fail to trigger NK cells (44–46).

Despite the increased resistance of the spheroid structures to NK cells, it was encouraging that IL-2–activated NK cells showed some efficacy against the SKES-1 MCTS. This suggests that cytokine-based strategies remain a viable option for the treatment of some solid tumors. However, the striking differences in the NK cell susceptibility of ESFT when cultured using the two- or three-dimensional systems shown in this study highlights the need for improved models to better define the interactions between human NK cells and solid tumors.

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References
 ACTION/INHIBITION THRESHOLD

AN NK CELL ACTIVATION/INHIBITION THRESHOLD

NKC and solid tumors.


