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

*J Immunol* 2011; 186:1538-1545; Prepublished online 29 December 2010; doi: 10.4049/jimmunol.1000951

http://www.jimmunol.org/content/186/3/1538

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/12/29/jimmunol.1000951.DC1

**References**

This article cites 45 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/186/3/1538.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
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 Journal of Immunology, 2011, 186: 1538–1545.

The online version of this article contains supplemental material.

© 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00

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 NKG2A (Z199; Beckman Coulter).
The Journal of Immunology

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 FACSCalibur or LSRII flow cytometers (BD Biosciences) and cell sorting using a MoFlo (DakoCytomation).

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 (β2m) and control siRNA duplexes were synthesized by Eurogentec (with 3′ TT extensions). For the β2m mRNA (Genbank Accession: NM_004048.2; http://www.ncbi.nlm.nih.gov/), we used siRNA duplex targeting positions 144–162. Two additional siRNA duplexes were evaluated targeting the β2m 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 UUCUCCGAACGUGUCACGU (with 3′ TT extensions), and for HLA-E, we used a Dharmacon siGENOME SMARTpool M-012106-01-005S 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 FACSBased 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 eyepiece graticule to measure MCTS radius (r) and the surface area calculated using the formula: area = 4πr

Results

ESFT trigger NK cell activation via NKG2D and the NCRs

Under conventional monolayer culture conditions, ESFT cell lines are highly susceptible to NK cells via the action of NKG2D 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 NKG2D 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 NKG2D 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 NKG2D 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 expression 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 TC32MCTS and SKES-1MCTS, respectively) and their corresponding spheroid structures (denoted TC32Spheroids and SKES-1Spheroids, 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-1MCTS were destroyed by IL-2–activated NK cells, whereas the TC32MCTS remained intact (Fig. 2C). This differential sensitivity of SKES-1MCTS and TC32MCTS 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 NKG2D 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 potent 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 TC32 Mono 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 TC32 Mono 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-1 Mono, TC32 Mono, 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, TC32 Mono 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 TC32 Mono to NK cells, indicating that MHC class I expressed by the TC32 Mono was not restraining NK cell activation. However, the IFN-γ-mediated induction of MHC class I on TC32 Mono 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-1 Mono, IFN-γ induced expression of MHC class I molecules (Fig. 3B) but did not protect them against NK cell attack (Fig. 3D). However, cell-surface MHC class I expression on IFN-γ-induced SKES-1 Mono cells did not reach the level of MHC class I expression exhibited by the uninduced TC32 Mono 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-1 Mono cells remained below the threshold required to inhibit NK cells.

The IFN-γ stimulation of TC32 Mono 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 TC32 Mono cells did not

![Figure 1](http://www.jimmunol.org/DownloadedFrom/1540/AN-NK-CELL-Activation-Inhibition-Threshold/1540.1.png)

**FIGURE 1.** ESFT are highly susceptible to NK cells via the action of NKG2D and NCR molecules. A, Killing of ESFT cell lines by unstimulated NK cells (gray bars) or IL-2–activated NK cells (black bars); killing of the prototype target cell line K562 is shown for comparison. All assays were performed at an E:T ratio of 1:1. Error bars indicate SD based on triplicates. Cell lines are arranged from left to right according to increasing expression of cell-surface MHC class I (geometric mean of fluorescence [GMF]; see also Supplemental Fig. 1). Cytotoxicity assays, performed at an E:T ratio of 5:1, in the presence of blocking Abs against the NK cell activation receptors indicated, or an appropriate isotype control Ab (Iso), using the ESFT cell lines SKES-1 (B), TC32 (C), and SK-N-MC (D). Results are expressed as the percent inhibition of killing observed using the specific blocking Ab compared with the isotype control. These assays are representative of a minimum of three separate experiments. Receptors were blocked individually (gray bars) or in combination (black bars), as indicated. SKES, SKES-1; SKN, SK-N-MC.
restore the susceptible phenotype (Fig. 4A, 4B). Furthermore, killing assays using NKG2A<sup>+</sup> and NKG2A<sup>−</sup> populations of NK cells generated by FACS were equally inhibited by IFN-γ-treated TC32<sub>Mono</sub> cells, revealing that this inhibition was not mediated by interactions between HLA-E and NKG2A/CD94 heterodimers (Fig. 4C, 4D).

**FIGURE 2.** Increased resistance of ESFT MCTS structures to NK cells. A, Degranulation of IL-2–activated NK cells in response to no tumor targets, monolayer cultures of SKES-1 (SK2D) or TC32 (TC2D), or MCTS cultures of SKES-1 (SK3D) or TC32 (TC3D). Degranulation of NK cells was determined by exposure of CD107 at the cell surface of CD56<sup>+</sup> cells. The percentage of CD107<sup>+</sup> NK cells is shown. B, Summary of degranulation using monolayers (black bars) or MCTS (gray bars); error bars show SD based on triplicates (P1 = 0.006; P2 = 0.004, using the Student t test). C, H&E-stained sections of SKES-1 and TC32 spheroids cocultured with medium alone (Medium), PBMC depleted of NK cells (NK), or SK-N-MC monolayer cultures (SC-N-MCMono). Unlike TC32 Mono, we were unable to induce the killing of monolayer cultures of the SK-N-MC cell line (SK-N-MCMono). 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 the proportion of cells above the threshold (resistant) and 74% below (susceptible). This MHC class I expression value (the threshold) was then applied across the different TC32<sub>Mono</sub> 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-MC<sub>Mono</sub>). Unlike TC32<sub>Mono</sub>, we were unable to induce the very high levels of MHC class I expression in SK-N-MC<sub>Mono</sub> required to reach a plateau of NK cell killing. However, like TC32<sub>Mono</sub>, SK-N-MC<sub>Mono</sub> 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 TC32<sub>Mono</sub> and SK-N-MC<sub>Mono</sub> 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 a range of MHC class I expression that presumably spans the inhibitory threshold and leads to greater numbers of cells being killed. How-ever, in the case of TC32 Mono, all of the cells within the target cell population express MHC class I below the threshold level, and further reductions cannot increase the number of susceptible cells. Resistant TC32Mono cells were only evident when MHC class I expression was induced beyond the inhibitory threshold via IFN-γ stimulation. Verhoeven et al. (11) treated SKEs-1 cells with IFN-γ and noted that elevated MHC class I expression did not confer resistance to NK cells; the data presented in this study suggest that this was because the threshold point was not reached. Surprisingly, the IFN-γ-induced expression of HLA-E on TC32Mono cells did not confer resistance to NK cell attack. This may be because expression of potent activating ligands outweighs inhibition by HLA-E. Alternatively, peptides presented by HLA-E on TC32 cells may not efficiently engage the NKG2A/CD94 heterodimer. 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 B2m-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 Log10 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 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 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 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 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.
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 can 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 activation 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 minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. Blood 114: 2657–2666.


