Human γδ T lymphocytes induce robust NK cell mediated antitumor cytotoxicity through CD137 engagement

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Abstract.

Natural killer (NK) cells are innate effector lymphocytes which control the growth of MHC class I negative tumors. We show here that \(\gamma\delta\) T lymphocytes, expanded \textit{in vitro} in the presence isopentenylpyrophosphate (IPP), induce NK cell mediated killing of tumors which are usually resistant to NK cytolysis. The induction of cytotoxicity towards these resistant tumors requires priming of NK cells by immobilized hIgG1 and costimulation through CD137L expressed on activated \(\gamma\delta\) T lymphocytes. This costimulation increases NKG2D expression on the NK cell surface, which is directly responsible for tumor cell lysis. Moreover, culturing PBMC with zoledronic acid, a \(\gamma\delta\) T lymphocyte activating agent, enhances NK cell direct cytotoxicity and ADCC against hematopoietic and nonhematopoietic tumors. Our data reveal a novel function of human \(\gamma\delta\) T lymphocytes in the regulation of NK cell mediated cytotoxicity and provide rational for the utilization of strategies to manipulate the CD137 pathway to augment innate antitumor immunity.
Introduction:

Natural killer (NK) cells contribute to innate immune responses against virally infected and neoplastic cells. NK cells usually recognize and attack tumor cells that lack major histocompatibility complex (MHC) class I. Our previous studies in murine tumor models clearly demonstrated that gamma delta (γδ) T lymphocytes play an important role in the regulation of antitumor NK cell function. Specifically, we have shown that γδ T lymphocytes are required for the antitumor activity of NK cells in vivo. More recently, we have demonstrated that culturing human PBMC with agents which activate γδ T lymphocytes induce NK cell mediated cytotoxicity against tumors that normally resist NK killing. These findings are concordant with other studies which show that γδ T lymphocytes regulate the early phase of NK cell mediated antibacterial responses in mice. Taken in concert these data strongly suggest that γδ T lymphocytes are important in the regulation of NK cell functions.

γδ T cells are characterized by the expression of a T cell receptor (TCR) consisting of both gamma and delta chains, and account for 1-10% of CD3+ cells in the peripheral blood of healthy adults. Approximately 70% of γδ T lymphocytes express the Vγ2Vδ2 TCR and can be expanded and activated by phosphoantigens such as the cholesterol biosynthesis intermediate, isopentenylpyrophosphate (IPP), or synthetic bisphosphonates (e.g. pamidronate disodium and zoledronic acid). Upon stimulation, γδ T lymphocytes acquire the capacity to destroy solid tumors of diverse origins such as squamous cell carcinoma of the head and neck (SCCHN), melanoma, colon cancer and breast carcinoma, suggesting that γδ T lymphocytes are important antitumor effector cells. The validity of this antitumor function is further supported by mouse models.
demonstrating that mice deficient in $\gamma\delta$ T cells have increased sensitivity to the development of methylcholanthrene (MCA)-induced tumors$^{14}$. In addition a recent pilot clinical study showed that $\gamma\delta$ T lymphocyte adoptive therapy for patients with advanced renal cell carcinoma was well tolerated and induced antitumor immune responses$^{15}$.

The antitumor effects of $\gamma\delta$ T lymphocytes are recognized to result from both direct killing of tumor targets and trans-activation of adaptive immune responses. For example, recent data demonstrate that activated $\gamma\delta$ T lymphocytes cause the maturation of DC which promote development of acquired immunity$^{16}$. In addition, $\gamma\delta$ T cells are known to cross-present tumor antigens (Ags) to CD8$^+$ cytolytic T lymphocytes$^{17,18}$. Despite their well characterized role in mediating adaptive immune responses, the mechanisms by which $\gamma\delta$ T cells regulate cells of the innate immune system, such as NK cells, are unclear.

In this report we demonstrate that $\gamma\delta$ T lymphocytes provide a costimulatory function for NK cells stimulated with suboptimal doses of immobilized human IgG1. Costimulated NK cells display upregulation of the activation markers CD25, CD54 and CD69 and effectively kill solid tumors which are traditionally resistant to NK mediated lysis. These costimulatory effects are partially regulated by the interaction of CD137L, expressed on activated $\gamma\delta$ T lymphocytes, with CD137, present on activated NK cells. CD137/CD137L engagement increases NKG2D expression on NK cells which augmented tumor killing. In addition, ex vivo culture of PBMC with zoledronic acid induces $\gamma\delta$ T lymphocyte activation, resulting in enhanced NK cell mediated tumor cytotoxicity. Our data define a novel mechanism through which $\gamma\delta$ T lymphocytes enhance the cytolytic function of NK cells and provide a clear opportunity to enhance
existing cancer treatment strategies combining ADCC and killing of non-opsonized tumor targets.
Materials and Methods

Tumor cell lines. Squamous cell carcinoma head and neck tumor cell lines TU167, TU159 and MDA1986 were graciously provided by Dr. Gary Clayman (M.D. Anderson Cancer Center). 012SCC was provided by Dr. Bert O’Malley (University of Pennsylvania). The K562 cell line was purchased from American Type Culture Collection (ATCC CCL-213). CD137L transfected and mock transfected P815 cell lines were established in our laboratory as previously described. All tumor cell lines and peripheral blood mononuclear cells (PBMC) were cultured in complete RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10 mM HEPES (all purchased from Gibco). To ensure the purity of original and cultured tumor cell lines we performed haplotyping using a PCR kit (One Lambda Inc., Canoga Park, CA) and/or flow cytometry staining with antibodies against HLA class I (BD Biosciences, San Jose, CA).

Antibodies and fusion proteins. Fluorochrome-conjugated mAbs against the following Ags were purchased from the vendors indicated and used according to the manufacturers instructions: γδ TCR, CD56, CD3, CD69, CD54, CD40L, CD80, CD86, CD28, CD94, CD161, CD16, CD152, CD278, CD279, CD134, CD137, CD252, CD137L, IFN-γ, TNF-α (BD Biosciences); CD44, CD46, NKG2D (Biolegend). Blocking experiments with NKG2D and CD54 were purchased from R&D System USA. Human IgG1 was obtained from Sigma Aldrich. Human soluble recombinant CD137Ig, CD134Ig, CD152Ig fusion proteins were purchased from R&D System USA.
**Flow cytometry.** All Ab staining for cell surface markers was performed according to the following protocol. The cells were washed once in PBS containing 1% FBS and 0.05% NaN3, incubated with appropriate amounts of mAb at 4°C for 30 min and re washed in PBS. For intracellular cytokine analysis, cells were cultured with various stimuli and 3µM monensin (Golgi stop) was added during the last 4 hours of culture. The cells were stained with mAb against cell surface molecules (e.g. γδ TCR, CD3, CD56), fixed and permeabilized using the BD Cytofix/Cytoperm Kit as described by the manufacturer (BD Biosciences). After permeabilization, the cells were stained with PE-conjugated mAb specific to IFN-γ and TNF-α. To determine granzyme A and B expression, permeabilized cells were stained with PE-conjugated anti-human Granzyme A and B or the appropriate isotype control (BD Biosciense).

In most flow cytometry samples, at least 3x10⁴ gated NK cells or γδ T lymphocytes (defined as CD3- CD56+ and CD3+ and γδ TCR+, respectively) were acquired using a BD LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ). All samples were analyzed using FACS Diva software (Becton Dickinson).

**γδ T cell expansion and activation.** Buffy coats from healthy donors were purchased through Biologic Specialty Corp. (Colmar, PA) as approved under the University of Maryland IRB exempt. For expansion of γδ T cells, whole PBMC were separated on a Ficoll gradient (Amersham Biosciences, Piscataway, NJ) and 1x10⁶ cells/ml were cultured in complete media with 15 µM isopentyl pyrophosphate (IPP) (Sigma) and 100 U/ml human recombinant IL-2 (Tecin, Biological Resources Branch, National Institutes
of Health, Bethesda, MD). Fresh complete medium and IL-2 supplement at 100 U/ml was added every 3 days. After 14 days of culture, cells were harvested and the percentage of γδ T cells was measured by flow cytometry. The percent of γδ T lymphocytes in IPP expanded cultures varied from 60% to 90%, and the range for individual experiments is reported in the figure legends. γδ T lymphocytes were not purified prior co-culture with NK cells.

Alternatively, PBMC isolated from buffy coats (3x10^6 cells/ml) were cultured with 15 μM Zometa (Novartis) alone or in the presence of 10 μg/ml Rituximab (Genentech). Activation of NK cells was verified by FACS or NK cells were purified from the cultures using magnetic beads and used in cytotoxicity assay.

**Immunomagnetic bead purification of NK cells.** NK cells were isolated from fresh PBMC by negative selection using MACS NK cell isolation kit (MiltenyiBiotec) according to the manufacturer’s instructions. The purity of the resulting cell populations was checked routinely by flow cytometry. NK cell purity generally exceeded 97%.

**NK cell and γδ T lymphocyte coculture.** Human IgG1 (hIgG1) was immobilized on plastic culture plates by incubating hIgG1 (2.5 μg/ml) in PBS at 4°C overnight, a condition that provides stable attachment of Igs on neutral plastic substrates. Purified NK cells (2x10^6 cells/well) and IPP-expanded γδ T lymphocytes (1x10^6 cells/well) were co-cultured in 1 ml of RPMI in 24 well cell plates (Falcon) precoated with 10 μg/ml of hIgG1. After 48 hours of culture, NK cells were assessed by flow cytometry and/or purified using MACS negative isolation kits for analysis of cytolytic activity. In some
experiments purified NK cells (2x10^6 cells/well) were cocultured with mock or CD137L transfected P815 cells (1x10^6 cells/well). In blocking experiments, human soluble recombinant Ig fusion proteins or mAbs (i.e. CD137Ig, CD152Ig, CD134Ig) at 10 μg/ml were included at the onset of the NK and γδ T cell cocultures. After 48 hours, NK cells were purified and tested for cytotoxicity against SCCHN targets.

In some experiments purified NK cells (1x10^6 cells/ml) were cultured with live TU167 (0.5x10^6 cells/ml) alone, in the presence of 10 μg/ml hIgG1 (isotype control) or Cetuximab (Bristol-Myers). Activation of NK cells was confirmed by FACS.

**Transwell coculture.** Purified NK cells (2x10^6 cells/well) were resuspended in 1 ml of RPMI and placed in 24-well plates pre coated with 10 μg/ml hIgG1. IPP-expanded γδ T lymphocytes were resuspended at 0.5x10^6 cells/ml and 0.5 ml of cells were added into the Transwell (Costar) with a polycarbonated membrane (pore diameter 0.4 μM) permeable for soluble factors. Cells, separated by a transwell, were cultured for 48 hours as previously described and expression of activation markers was analyzed by flow cytometry.

**Cytotoxicity assay.** NK cell cytotoxicity was measured using a standard ^51^Cr-release assays, as described previously^33^. Briefly, target cells (2x10^6 in 0.3 ml of complete media) were incubated for 90 min at 37°C in 5% CO₂ with 150 μCi of ^51^Cr (GE Healthcare, Piscataway, NJ). The labeled cells were then washed twice with media and incubated for an additional 30 min to reduce background radioactivity. Cells were then washed two more times and adjusted to a concentration of 5x10^4 cells/ml in complete
media. Labeled targets cells were cultured for 30 min with 4 μg/ml Retuximab or Cetuximab. Effector NK cells were purified from γδ T lymphocyte co culture or from cultured PBMC by immunomagnetic MACS NK negative selection kit (MiltenyiBiotec). Serial dilutions of effector cells (100 μl/well) were added into each well of 96-well V-bottomed plates (Corning, NY). Aliquots of 51Cr-labeled target cells (100 μl/well) were dispensed into wells containing effector cells. The plates were centrifuged at 200 rpm for 2 min and incubated at 37°C in 5% CO2. After 4 hours of incubation, the plates were centrifuged again at 13,000 rpm for 5 min and 100 μl aliquots of the supernatants from each well were transferred to a new plate containing 100 μl/well of Optiphase Supermix scintillation fluid (Perkin Elmer, Boston, MA). Radioactivity was measured using 1450 Microbeta counter (Wallac, Turku Finland). In some experiments anti-NKG2D or isotype control mouse IgG were added at 10 μg/ml, 15 min before the addition of labeled target cells. The percentage of specific cytotoxicity was calculated as (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100. Spontaneous release was determined by incubating the targets with 100 μl of complete media. Maximum release was determined by incubating the target cells with 100 μl of 0.5% Triton-X.

**Statistical analysis.** All cytotoxicity data were analyzed by using the Student’s test, whereby p<0.05 indicated that the value of the test sample was significantly different from the relevant control.
RESULTS:

γδT lymphocytes activate hIgG1 primed NK cells.

We have previously shown that long term (14 day) culture of PBMC with IPP + IL-2 results in the induction of NK cell mediated cytotoxicity against tumors that are normally resistant to NK cell killing. We also observed that IPP did not stimulate NK cells directly and γδ T lymphocytes were critical for IPP-induced NK cell cytotoxicity. Usually NK cells do not survive for a long period of time without cytokine support. Therefore, in order to understand the mechanisms of γδ T lymphocyte mediated NK cell activation in a more physiological model, in this study we used a short term culture of fresh NK cells purified from PBMC of healthy donors and γδ T lymphocytes expanded in vitro. As shown in Figure 1a, fresh NK cells cultured with media or γδ T lymphocytes alone do not express CD69, a molecule associated with NK cell activation. In contrast, consistent with our previously published data, after incubating in wells pre-coated with hIgG1, 25% of NK cells express CD69 on their surface. Adding γδ T lymphocytes to hIgG1 coated wells further increases the expression of CD69 on NK cells to 45.9% (Fig. 1a). A similar pattern of expression was seen for CD54, another marker of NK cell activation (Supplementary Fig. 2). Neither immobilized hIgG1 alone nor in combination with NK cells induced activation of γδ T lymphocytes, as measured by the expression of activation markers (Supplementary Fig. 3). Since short term (48 hours) culture of γδ T lymphocytes without hIgG1 did not result in the activation of NK cells, plastic immobilized hIgG1 was included in all subsequent experiments as a putative initial signal for NK cell priming.
We also determined an optimal ratio for NK cell activation by γδ T lymphocytes. As shown in Figure 1b culturing 2x10^5 NK cells with 10^5 or 5x10^4 T lymphocytes (2:1 and 4:1 ratio, respectively) induced significant increases in CD69 and CD54 expression on NK cells primed with hIgG1, while ratios of 20:1 (10^4 IPP expanded γδ T lymphocytes) failed to increase the expression of activation markers on NK cells. Therefore, in subsequent experiments we used a ratio of 4:1. These results indicate that in vitro expanded γδ T lymphocytes stimulate the activation of hIgG1 primed human NK cells.

γδ T lymphocytes enhance NK cell mediated antitumor cytotoxicity.

In order to determine if γδ T lymphocyte stimulation of hIgG1 primed NK cells enhances their cytolytic activity, we stimulated purified NK cells with media, hIgG1 or IPP expanded γδ T lymphocytes in the presence of immobilized hIgG1. After 48 h, NK cells were re-purified by negative selection, enabling us to obtain a highly pure population of “untouched” NK cells (>99%) for functional analysis (Fig. 2a). Use of these NK cells as effectors against various tumor cell lines revealed that cells cultured with media or immobilized hIgG1 alone did not kill SCCHN (TU167, 012SCC, MDA1986), melanoma (Mel526), breast cancer (MDA MB231 and MCF-7), B cell lymphoma (Daudi), or T cell lymphoma (Jurkat) tumor cell lines. However γδ T lymphocytes significantly increased the lytic activity of hIgG1 primed NK cells against the above cell lines (Fig. 2b). The killing of colon cancer lines (HCT 116), that appears to be sensitive to NK cell mediated cytotoxicity, was also significantly increased by γδ T lymphocytes. The activation of NK cells was independent of donor HLA-type, since both autologous and allogeneic γδ T
lymphocytes enhanced cytolytic activity. This lack of HLA restriction in NK cell activation by \( \gamma\delta \) T lymphocytes was very reproducible and observed in more than 20 independent experiments. Based on these findings, in subsequent studies we used NK cells and \( \gamma\delta \) T lymphocytes derived from the PBMC of different donors, enabling us to obtain sufficient numbers of NK cells for functional and phenotypic analysis. Overall these data suggest that IPP activated \( \gamma\delta \) T lymphocytes enhance direct NK cell mediated cytolytic activity against hematopoietic and nonhematopoietic tumors.

**Cell-to-cell contact is essential for NK cell activation by \( \gamma\delta \) T lymphocytes.**

We have previously shown that soluble factors produced by \( \gamma\delta \) T lymphocytes are responsible for activation of NK cells in long term culture\(^4\). To ascertain whether the enhanced NK cell activation by \( \gamma\delta \) T lymphocytes in short term culture is mediated by cell-to-cell contact or soluble factors, we used a transwell system. Purified NK cells were placed in lower wells coated with hIgG1 and IPP-expanded \( \gamma\delta \) T lymphocytes were added to either the lower or the upper wells. As expected, NK cells co-cultured with immobilized hIgG1 and \( \gamma\delta \) T lymphocytes, placed in lower wells, showed increased expression of the CD69 and CD54 activation markers (**Fig. 3**). When NK cells were separated from \( \gamma\delta \) T lymphocytes by a membrane with 0.4 \( \mu \)m pore size there was no increase in the expression of activation markers. These data indicate that, in contrast to our long-term exposure data, cell-to-cell contact is required for NK cell activation by \( \gamma\delta \) T lymphocytes during short term interaction.

**Expression of costimulatory molecules on activated \( \gamma\delta \) T lymphocytes and NK cells.**
It is known that ligands belonging to the B7 and TNF super families are essential for costimulation of immune cells\textsuperscript{21,22}. Having demonstrated the activation of hIgG1 primed NK cells by γδ T lymphocytes requires cell-to-cell contact, we characterized the expression of costimulatory molecules on these cells. First, we analyzed the expression of known costimulatory ligands on γδ T lymphocytes. As shown in Figure 4a, γδ T lymphocytes in unstimulated PBMC did not express CD80, CD86, CD252 (OX40L) or CD137L (4-1BBL) on their surface. However, stimulation of PBMC with IPP and IL-2 for 14 days induced the expression of CD86 (86%), CD252 (12%), and CD137L (58%). Moreover, CD134 (OX40) and CD137 (4-1BB) expression were enhanced on the surface of NK cells cultured with immobilized hIgG1 and this expression was further augmented by the addition of γδ T lymphocytes (Fig. 4b). No expression of CD28, CD152 (CTLA-4), CD278 (ICOS) and CD279 (PD-1) were observed on NK cells even after coculture with γδ T lymphocytes in the presence of immobilized hIgG1 (Fig. 4b and Supplementary Fig. 4).

**Activation of NK cells by γδ T lymphocytes is partially mediated by CD137/CD137L interactions.**

The fact that NK cell costimulation with γδ T lymphocytes and IgG1 induces CD137 and CD134 suggested that some of the observed antitumor effects might be mediated by TNF superfamily members. To determine if CD134 and/or CD137 are involved in the activation of NK cells by γδ T lymphocytes we used fusion proteins to block engagement of CD134 and CD137 with their cognate ligands. Addition of CD152Ig (used as negative control) or CD134Ig fusion proteins into the culture did not inhibit the activation of NK
cells by γδ T lymphocytes (Supplementary Fig. 5a). However CD137Ig partially inhibited CD54 expression on NK cells (Fig. 5a). These results suggest that stimulation of hIgG1 primed NK cells by γδ T lymphocytes involves CD137.

To confirm the role of CD137 in the activation of NK cells, purified NK cells were cultured with irradiated P815 cells expressing CD137L (Supplementary Fig. 6). Culturing purified NK cells with mock transfected P815 cells in the presence of immobilized hIgG1 did not induce the expression of CD54 or CD25 (Fig. 5b). In contrast CD137L expressing P815 tumors significantly increased the expression of activation markers on NK cells. The inclusion of the CD137Ig fusion protein to cultures containing NK cells and CD137L transfected P815 tumors, completely abrogated the expression of CD54 and CD25 (Fig. 5b), indicating that CD137Ig fusion protein blocks CD137/CD137L engagement. Overall, these results demonstrate that CD137/CD137L interactions are at least partially involved in the activation of NK cells by γδ T lymphocytes.

**CD137 mediates the induction of NK cell cytotoxicity by γδ T lymphocytes.**

We next investigated whether CD137 engagement enhances the cytolytic potential of NK cells cultured with γδ T lymphocytes. Reproducibly, and consistent with our previous findings20, hIgG1 alone did not induce cytolytic function of NK cells while addition of γδ T lymphocytes significantly increased killing of SCCHN targets (Fig. 5c). The addition of soluble CD137Ig fusion protein decreased the cytolytic activity of NK cells cultured in the presence of immobilized hIgG1 and γδ T lymphocytes by 40%, suggesting that CD137 engagement is important for the regulation of NK cell cytolytic function.
CD152Ig fusion protein containing the same Fc portion did not inhibit the induction of NK cell cytotoxicity (Supplementary Fig. 5b). To confirm the role of CD137 signaling in the activation of NK cell mediated cytotoxicity, purified NK cells were cultured with CD137L transfected P815 cells (Supplementary Fig. 6). Data presented in Figure 5d indicate that NK cells cultured with hIgG1 and γδ T lymphocytes induced 29% cytotoxicity against SCCHN cells at 20:1 effector:target ratio. Thirteen percent cytotoxicity was observed in NK cells cultured with CD137L transfected P815 while only 6% cytotoxicity was mediated by NK cells cultured with mock P815. These data suggest that CD137 is at least partially involved in the regulation of NK cell cytolytic activity costimulated by γδ T lymphocytes.

**hIgG1 primed, CD137 costimulated NK cells utilize NKG2D for tumor cytolysis.**

We next sought to understand the mechanism of tumor killing by NK cells cultured in the presence of γδ T lymphocytes. It is well known that NKG2D regulates NK cell cytotoxicity against many tumors. Resting NK cells express considerable amount of NKG2D on their surface (Supplementary Fig. 7). We observed reproducible increases in the expression of NKG2D on the surface of NK cells from 11 different donors cultured with immobilized hIgG1 and γδ T lymphocytes (Fig. 6a). Furthermore, the cytolytic activity of NK cells cultured with γδ T lymphocyte correlated with the levels of NKG2D expression (Supplementary Fig. 8). In contrast, we found no expression of other well-characterized NK cell receptors (i.e. CD16, NKp30, NKp44, NKp46, CD94 CD161) on stimulated NK cells (Supplementary Fig. 9).
We have found that many SCCHN tumors express ULBP-2 and ULBP-3, NKG2D ligands (Supplementary Fig. 10), suggesting a role of NKG2D in SCCHN killing by NK cells. Results presented on Figure 6b indicate that anti-NKG2D mAb blockade reduced the cytolysis of TU167 tumors by NK cells cultured with γδ T lymphocytes from 27% to 17% at 20:1 E:T ratio. These observations indicate that NK cells co-cultured with γδ T lymphocytes may kill SCCHN tumors by recognizing NKG2D ligands.

To verify the role of CD137 engagement in the induction of NKG2D expression on NK cells cultured with γδ T lymphocytes we used CD137Ig fusion protein to block CD137/CD137L interaction. The addition of CD137Ig decreased γδ T lymphocyte induced expression of NKG2D on NK cells from 82.8% (MFI 19,522) to 30.5% (MFI 3,872) (Fig. 6c), indicating that CD137 engagement is important for the induction of NKG2D expression. Experiments utilizing CD137L transfected P815 cells further confirmed the involvement of CD137 signaling in the NKG2D expression (Fig. 6d). Overall these data indicate that CD137 engagement plays a significant role in the control of NKG2D expression which is important for tumor killing by NK cells cultured with expanded γδ T lymphocytes.

Zoledronic acid enhances both direct NK cytotoxicity and ADCC against SCCHN and lymphoma.

Our data suggest that priming of NK cells by immobilized hIgG1 induces CD137 expression which is important for γδ T lymphocyte induced activation. In order to provide a platform for translating these observations, we next sought to develop a
clinically applicable system for immobilizing human IgG in vivo and providing simultaneous γδ T lymphocyte activation. First, we evaluated if opsonized tumor could serve as a platform for IgG immobilization. The results shown on Figure 7a indicate that the EGFR positive SCCHN cell line, TU167, when opsonized with the anti-EGFR mAb (Cetuximab), used clinically for the treatment of patients with SCCHN, induces expression of CD137 on NK cells. These data suggest that mAbs which target tumor associated antigens, when composed of the appropriate IgG isotype, can also provide a first signal for NK activation.

In order to build on this finding, we next evaluated whether clinically relevant agents which activate γδ T lymphocytes can be used in combination with mAb opsonized tumors to enhance tumor killing through direct cytolysis and ADCC. Co-culture of PBMC with Rituximab (a clinical grade mAb recognizing CD20 on B cells) and Zometa (zoledronic acid), a bisphosphonate approved for clinical use which induces activation of γδ T lymphocytes, results in notable upregulation of CD69 on NK cells (Fig. 7b). Interestingly, culturing PBMC with zoledronic acid alone also induces NK cell activation (Fig. 7b).

To verify the effects of γδ T lymphocytes activation on NK cell cytotoxicity in physiological conditions we cultured whole PBMC with Zometa. NK cells were purified from stimulated cultures and their direct cytotoxicity and ADCC was evaluated in standard 4 hour Cr-release assays. As shown in Figure 7, incubation of PBMC with Zometa significantly increases direct cytolytic activity of NK cells against SCCHN (TU167) and B cell lymphoma (Ramos) targets. Interestingly depletion of γδ T
lymphocytes prior culturing PBMC with Zometa significantly reduced but did not completely abrogate the effects of Zometa on direct NK cytotoxicity.

In order to determine whether the interplay of Zometa and opsonized tumor can enhance killing of SCCHN, we employed a combinatorial approach. As expected we observed higher levels of NK mediated cytotoxicity against TU167 and Ramos targets in the presence of appropriate Abs (Cetuximab and Rituximab, respectively). Nevertheless, culturing PBMC with Zometa significantly enhanced NK mediated ADCC. Moreover depletion of \( \gamma^\delta \) T lymphocytes from PBMC before the addition of Zometa reduced NK killing to the level of cytotoxicity observed in NK cells purified from PBMC cultured without Zometa (Fig. 7). This observation indicates that \( \gamma^\delta \) T lymphocytes are critical for the Zometa induced increase of NK killing of SCCHN and B cell lymphoma. Furthermore, these results define the physiological and clinical relevance of the interaction between \( \gamma^\delta \) T lymphocytes and NK cells for the regulation of direct and antibody dependent NK cytotoxicity.
DISCUSSION

Although the role of CD137 signaling in the activation of cytolytic αβ T lymphocytes is well established \(^{24}\) our results provide the first evidence that CD137 engagement costimulates antitumor function of hIgG1 primed NK cells. These findings suggest that two signals are required for the optimal activation of NK cells in a manner similar to the two signal model established for αβ T lymphocyte activation. This idea is relevant to the improvement of mAb-based cancer immunotherapy where a combination of mAb specific to tumor Ag(s) with CD137 cross-linking, induces direct NK cell mediated tumor lysis. Specifically, our findings suggest that combination of Ab based cancer immunotherapies with either adoptive transfer of \(\textit{in vitro}\) expanded γδ T lymphocytes or systemic injection of γδ T lymphocyte stimulating agents (zoledronic acid) \(^{8-10}\) may improve clinical outcome through the enhancement of direct and ADDC tumor killing by NK cells.

While CD137/CD137L interactions partially account for the activation potential of γδ T lymphocytes on hIgG1 stimulated NK cells, other costimulatory molecules are likely to also play a role. Although it is reported that NK cell activation can be triggered by CD80 and CD86 \(^{25,26}\), our study did not reveal known receptors for these ligands on the surface of stimulated NK cells. Similarly, CD152Ig did not block the activation of NK cells by γδ T lymphocytes, suggesting that CD80 and CD86 are not involved in the activation of NK cells. Furthermore, in contrast to previous reports describing the presence of functional ICOS on murine NK cells, we found no expression of ICOS on human NK cells \(^{27}\). Taken in concert, our data suggest that other costimulatory and/or adhesion molecules expressed on the surface of activated γδ T lymphocytes are involved...
in the activation of NK cells antitumor properties. The identification of these costimulatory molecules may improve Ab and NK cell based cancer immunotherapy.

Activated NK and CD8+ αβ T cells express the NKG2D receptor which recognizes specific ligands (ULBPs and MIC A/B) presented on tumors\(^{28}\). It has been shown that CD137 regulates the expression of NKG2D in human cord blood CD8 T lymphocytes\(^{24}\). Our data indicate that CD137 engagement is important for the induction of NKG2D receptor expression on NK cells by γδ T lymphocytes. Moreover anti-NKG2D mAb significantly inhibits the cytolytic potential of γδ T lymphocyte stimulated NK cells against tumor cell lines, suggesting that the increased NKG2D expression mediated by CD137, augments the cytolytic potential of NK cells. However it is conceivable that other molecules expressed on γδ T lymphocyte activated NK cells are also involved in the killing of tumors, since anti-NKG2D mAb blocking did not completely abrogate cytolytic activity.

Our previous data indicate that long term culture (14 days) of PBMC with agents specifically activating γδ T lymphocytes (i.e. IPP + IL-2) increases the cytolytic activity of NK cells\(^4\). This NK cell activation was mediated by soluble factors released by γδ T lymphocytes during the culture. In contrast, our current results indicate that activation of NK cells in short term culture (48 hours) with expanded γδ T lymphocytes requires cell-to-cell contact and priming with immobilized hIgG1. These findings suggest pleiotropic mechanisms of NK cell functions regulation by activated γδ T lymphocytes. The direct physiological role of NK and γδ T cells interaction in diseases and the maintenance of immune responses remain to be determined.
The clinical significance of γδ T lymphocytes and NK cell interaction were confirmed by experiments utilizing PBMC cultured with clinically applicable reagents for the treatment of patients with SCCHN and lymphoma. Our data indicate that culturing PBMC with Zometa, increases direct and Ab dependent NK cytotoxicity against SCCHN and lymphoma targets. It is conceivable that γδ T lymphocytes are more important for the regulation of ADCC since γδ T cell depletion had only partial impact on zoledronic acid induced direct NK cell cytotoxicity. However other molecular and cellular targets of zoledronic acid which are involved in the regulation of direct NK cell cytotoxicity remain to be determined. Overall our data suggest that administration of γδ T cell activating agents may improve antitumor effects of Cetuximab and Rituximab used for the treatment of patients with SCCHN and B cell lymphoma, respectively.

In summary, it has been shown that in vitro expanded γδ T lymphocytes can improve adaptive immune responses against tumor Ags, by effectively presenting tumor Ags to conventional αβ T lymphocytes\textsuperscript{17,18}. Our data indicate that in vitro culture with γδ T lymphocyte activating agents (IPP or Zometa) can also improve antitumor innate function, as determined by increased NK cell cytotoxicity. Thus activation of γδ T lymphocytes in vivo or adoptive transfer of in vitro expanded γδ T lymphocytes has the potential to improve existing strategies for cancer immunotherapy. In particular a combination of tumor specific mAbs that engage Fc receptors on NK cells (Cetuximab or Rituximab)\textsuperscript{29} and γδ T lymphocytes activating agents approved for clinical use (e.g. Zometa)\textsuperscript{30,31}, may improve existing cancer immunotherapy by stimulating both the adaptive and innate antitumor immunity. Of perhaps greater import, this activation
strategy may overcome pre-existing defects in NK cell function recognized to exist in patients with large tumor burdens, further augmenting the clinical utility of this strategy.
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AUTHOR CONTRIBUTIONS

A.M. performed the experiments, analyzed the data and wrote the manuscript; X.Z. and L.W. helped perform individual experiments; B.R.G. and C.D.P., designed the study and analyzed data; S.E.S. and A.I.C. designed the study, analyzed data and wrote the manuscript.

CONFLICT OF INTEREST DISCLOSURES

S.E.S. is a cofounder and major stockholder in Gliknik, a Biotechnology company. He also receives royalties from the Mayo Clinic College of Medicine for IP related to CD137 which is licensed to 3rd parties. The other authors have no financial conflict of interest.


Figure 1. γδ T lymphocytes increase hIgG1 induced activation of NK cells. (a) Purified NK cells and IPP+IL-2 expanded γδ T lymphocytes were co-cultured at 4:1 ratio in the presence or absence of plate-immobilized hIgG1 (2.5 μg/ml) for 48 hours. The expression of CD69 was analyzed by flow cytometry. Histograms represent gated CD3-CD56+ NK cells. An example of gating strategy is shown in Supplementary figure 1. (b) Purified NK cells (2x10⁵ cells/well) were cultured with indicated numbers of IPP+IL-2 expanded γδ T lymphocytes in the presence of immobilized hIgG1 (2.5 μg/ml) for 48 hours. Expression of activation markers CD69 and CD54 were assessed by flow cytometry and plotted as a percentage of CD69 and CD54 positive gated NK cells. Representative data from one of the 10 independent experiments is shown.

Figure 2. γδ T lymphocytes induce NK cell mediated cytotoxicity against various tumor cell lines. (a) Purified NK cells were cultured with bulk IPP expanded PBMC at a 2:1 ratio for 48 hours (i.e. 2 NK cells/1 IPP expanded PBMC). IPP expanded PBMC in these experiments contained 70-80% γδ T lymphocytes, so the actual ratio of NK cells to IPP expanded γδ T lymphocytes was approximately 2/0.8. NK cells were re purified from the cultures by immunomagnetic depletion of non-NK cells. Representative dot plots of NK + γδ T cells (right) and NK cells purified after 48 hours of culture (left) are shown. (b) Cytolytic activity of NK cells purified after co-culture with expanded γδ T cells and immobilized hIgG1 (2.5 μg/ml) for 48 hours was analyzed in a standard 4 hour ⁵¹Cr-release assay against indicated tumor targets. Data are presented as mean±SD of triplicate samples and are representative of 7 independent experiments. * - p<0.05 compared with NK cells cultured with hIgG1 alone.
Figure 3. Cell-to-cell contact is required for the activation of NK cells by γδ T lymphocytes. Trans-well experiments were performed by culturing purified NK cells in lower wells coated with hIgG1 (2.5 μg/ml). Expanded γδ T lymphocytes were added either to the lower (cell-to-cell contact) or to the upper wells (soluble factors). The ratio of NK to γδ T cells was 4:1. After 48 hours of culture, the expression of CD69 and CD54 was analyzed by flow cytometry. The bar diagrams depict the percentage of CD69 and CD54 expressing cells in gated NK populations. Representative data from 1 of 3 independent experiments is shown.

Figure 4. Expression of costimulatory ligands and receptors on γδ T lymphocytes and NK cells. (a) Fresh γδ T lymphocytes from normal donors (top histograms) or γδ T lymphocytes expanded in the presence of IPP+IL-2 (lower histograms) were stained with mAb specific for CD80, CD86, CD252 (OX40L) and CD137L (41BBL). The expression of indicated costimulatory ligands on gated CD3+γδTCR+ cells is shown. (b) NK cells cultured with media alone or immobilized hIgG1 with or without in vitro expanded γδ T lymphocytes for 48 hours were stained with mAbs specific for CD28, CD152 (CTLA-4), CD134 (OX40) and CD137 (4-1BB). Overlays of histograms representing gated CD3-CD56+ NK cells are shown. Depicted data represent 1 of 5 independent experiments.

Figure 5. Blocking of CD137L partially inhibits γδ T lymphocyte induced cytolytic activity of NK cells. (a) Purified NK cells were co-cultured with IPP+IL-2 expanded γδT lymphocytes at 4:1 ratio in the presence of immobilized hIgG1 (2.5 μg/ml) for 48 hours.
In some groups, soluble CD137Ig fusion protein at 10 μg/ml was added to block CD137 receptor and ligand interactions. The bar diagram represents the percentage of cells expressing CD54 in gated CD3-CD56+ NK cell population. Representative data from 1 of 4 independent experiments is shown. (b) Purified NK cells were co-cultured with either mock (left histograms) or CD137L transfected P815 (central histograms) at a 4:1 ratio in the presence of immobilized hIgG1 (2.5 μg/ml). In some wells containing NK cells and CD137L transfected P815 tumors, soluble CD137Ig fusion protein (10 μg/ml) was added (right histograms). After 48 hours of culture, cells were stained for CD54 and CD25. Histograms represent cells gated on CD56+CD3- NK population. (c) Soluble CD137Ig fusion protein (10 μg/ml) was included during the culture of purified NK cells and γδ T lymphocytes (4:1 ratio) in hIgG1 pre-coated plates for 48 hours. Cytotoxicity of NK cells re purified after culture was analyzed in a standard 4 hour ⁵¹Cr-release assay against the TU167 SCCHN cell line. Data are presented as mean±SD of triplicate samples and are representative of 4 independent experiments. * - p<0.05 compared with NK cells cultured in the presence of CD137Ig blocking. (d) NK cells were purified from PBMC of healthy donors and co-cultured with irradiated mock or CD137L transfected P815 cells at a 4:1 ratio. Expanded γδ T lymphocytes were used as a positive control for NK cell activation. After 48 hours of culture, NK cells were repurified and used as effectors against TU167 SSCHN target cells. Data are presented as mean±SD of triplicate samples and representative of 3 independent experiments. * - p<0.05 compared to NK cells cultured with mock transfected P815.
Figure 6. CD137 ligation on NK cells results in enhanced NKG2D expression which is involved in tumor cell killing. (a) NK cells purified from PBMC of 11 individual donors were co-cultured in the presence of expanded γδ T lymphocytes (4:1 ratio) on plates pre-coated with hIgG1. After 48 hours of culture, the expression of NKG2D was analyzed on NK cells. Dots represent individual values of NKG2D expression on gated NK cells. Horizontal lines represent average values of NKG2D expression in indicated groups. (b) Cytotoxic activity of NK cells purified after 48 hours of culture with in vitro expanded γδ T lymphocytes was measured in a standard 4 hours ⁵¹Cr-release assay against TU167 SCCHN. Blocking anti-NKG2D antibodies or isotype control IgG were added into the wells containing purified NK cells and TU167 targets for the duration of the cytotoxicity test. Data are presented as mean±SD of triplicate samples and are representative of 2 independent experiments. * - p<0.05 compared with isotype control. (c) CD137Ig (10 μg/ml) was added to wells containing NK cells and IPP+IL-2 expanded γδ T lymphocytes. After 48 hours of culture, cells were stained with anti-NKG2D mAb. The histograms depict NKG2D expression on gated CD56+CD3- NK cells. Numbers in brackets indicate MFI of NKG2D expression. (d) Purified NK cells were cultured with irradiated mock or CD137L transfected P815 cells (4:1) on plates pre-coated with hIgG1. After 48 hours the expression of NKG2D was analyzed by FACS. Expanded γδ T lymphocytes were used as a positive control for NK cells activation. Numbers in brackets indicate MFI of NKG2D expression.

Figure 7. Zoledronate, a γδ T lymphocyte activating agent, enhances NK cell activation and cytotoxicity. (a) Purified NK cells were cultured for 96 hours in the
presence media, TU167 cells alone, TU167 + 10 μg/ml hIgG1 (isotype control) or TU167 + 10 μg/ml Cetuximab. CD137 expression on CD56+ NK cells was analyzed by FACS. Two representative experiments are shown. (b) Whole PBMC were incubated in the presence of media, 10 μg/ml Rituximab, 15 μM Zoledronate or a combination of Rituximab with Zoledronate. The expression of CD69 on gated CD3-CD56+ NK cells was analyzed by FACS 96 hours after initiation of the cultures. A representative of 3 independent experiments is depicted. (c) Whole PBMC were cultured with media (circles) or Zoledronate (squares). Alternatively, γδ T lymphocyte depleted PBMC was cultured with Zoledronate (triangles) for 96 hours. NK cells were purified from the groups described above. NK cell direct cytotoxicity (left plots) or ADCC (right plots) was measured in a standard 4 hour 51Cr-release assay against TU167 SCCHN or Ramos B cell lymphoma targets. Data are presented as mean±SD of triplicate samples and are representative of 2 independent experiments. * - p<0.05 compared with NK cells purified from γδ T lymphocyte depleted cultures.
Figure 1
Figure 2

(a) Depletion of non-NK cells (magnetic beads) from NK + IgG1 + γδT cells.

(b) Cytotoxicity of NK cells against different cell lines at various E:T ratios.

- TU167
- 012SCC
- MDA1986
- Mel526
- MDA MB231
- MCF-7
- Daudi
- Jurkat
- HCT 116
Figure 3
Figure 4
Figure 5
**Figure 6**

(a) Percentage of NKG2D+ NK cells after treatment with Media, hlgG1, or hlgG1 + γδ T cells.

(b) Cytotoxicity assay showing Isotype control and anti-NKG2D treatment at varying E:T ratios.

(c) Flow cytometry analysis of NKG2D expression under different conditions: Media, hlgG1, hlgG1 + γδT, hlgG1 + γδT + CD137lg.

(d) Flow cytometry analysis of NKG2D expression under different conditions: Media, hlgG1, hlgG1 + Mock, hlgG1 + CD137L, hlgG1 + γδ T cells.
Figure 7.
Human γδ T lymphocytes induce robust NK cell mediated antitumor cytotoxicity through CD137 engagement

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