OncoImmunology 2:12, e27440; December 2013; © 2013 Landes Bioscience

Invariant NKT cells
Killers and conspirators against cancer

Hamid Bassiri[1,*], Rupali Das[2], and Kim E Nichols[2,*]

[1]Division of Infectious Diseases; Children's Hospital of Philadelphia; Philadelphia, PA USA; [2]Division of Oncology; Children's Hospital of Philadelphia; Philadelphia, PA USA

Keywords: glycolipids, immunosurveillance, invariant natural killer T cells, perforin, tumor

Although invariant natural killer T (iNKT) cells influence antitumor responses *indirectly* by secreting cytokines and promoting the cytolytic functions of T and NK cells, we find that iNKT cells mediate direct tumoricidal activity in vitro and significantly inhibit tumor growth in vivo, even in the absence of other cytotoxic lymphocytes.

Described over 2 decades ago, invariant natural killer T (iNKT) cells are innate-type T lymphocytes that bear "invariant" T-cell receptors (TCRs) comprised of a limited number of Vα and Vβ chains that recognize glycolipid antigens presented by the CD1d molecule.[1] Upon TCR activation, iNKT cells rapidly secrete large amounts of a variety of cytokines, including interferon γ (IFNγ), interleukins (IL)-4, IL-13, IL-17, tumor necrosis factor α (TNFα), and granulocyte macrophage colony-stimulating factor (GM-CSF). Along with the mediators produced by the CD1d-expressing antigen-presenting cells with which iNKT cells interact, these cytokines recruit and stimulate the antitumor functions of cytotoxic lymphocytes such as natural killer (NK) and $CD8^+$ T cells (**Fig. 1**).[2] In so doing, iNKT cells act as cellular adjuvants that boost innate as well as adaptive antitumor responses. While this *indirect* anti-tumor mechanism has been extensively characterized in murine tumor models, evidence in support of a *direct* role for iNKT cells in the control of tumor growth is not as robust. In fact, many of the published reports highlight the indirect role of iNKT cells or do not adequately exclude such a mechanism.

To exclusively assess the tumor-directed functions of iNKT cells themselves, we examined the lytic activity of highly purified primary murine iNKT cells against a variety of cancer cells in vitro. iNKT cells killed malignant cells via a mechanism that depended on the TCR, CD1d, and glycolipid antigens, such as the prototypical iNKT cell-stimulating glycosphingolipid α-galactosylceramide (α-GalCer), or its synthetic analogs PBS44 and PBS57. Employing CD1d-expressing EL4 murine T lymphoma cells as our model tumor target, we demonstrate that: (1) murine iNKT cells kill glycolipid-loaded but not unloaded tumor cells in vitro; (2) the cytolytic activity of iNKT cells is reduced in the presence of CD1d-blocking antibodies; and (3) iNKT cells lacking SLP-76, which is critical for TCR-induced signaling, fail to kill EL4 cells. Together, these findings highlight the critical role of TCR/CD1d/glycolipid interactions in the cytolytic responses of iNKT cells against malignant cells. Interestingly, OCH, an analog of α-GalCer with a truncated sphingosine chain that stimulates iNKT cells to secrete IL-4,[3] fails to promote the killing of EL4 cells, suggesting a possible dichotomy in the signals that are required for the cytolytic and secretory functions of iNKT cells.

To elucidate the killing mechanisms employed by iNKT cells in vitro, we used iNKT cells from mice lacking interferon γ (IFNγ), FAS ligand (FASL), perforin (PRF1), or tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10, best known as TRAIL). While a previous study implicated FAS/FASL signaling in the killing of neoplastic B cells by iNKT cells,[4] we found that FASL-, as well as IFNγ- and TRAIL-deficient iNKT cells killed EL4 cells normally. In contrast, perforin-deficient iNKT cells displayed a partial reduction in cytotoxicity, which was fully abolished in the absence of both FASL and perforin. These observations reveal that iNKT cells employ multiple cytotoxic mechanisms, the choice of which likely depends upon the presence of specific death receptor ligands on the target cells.

Finally, we examined the capability of iNKT cells to inhibit tumor growth in mice. To exclude the indirect effects of iNKT cells on other cytolytic effectors, we employed NOD-SCID *Il2rg*$^{-/-}$ (NSG) mice, which lack endogenous lymphocytes. Remarkably, the adoptive transfer of purified iNKT cells to NSG mice resulted in protection from a challenge with glycolipid-loaded EL4 cells. Consistent with our in vitro findings, FASL- and TRAIL-deficient iNKT cells protected NSG mice from tumor challenge, whereas perforin-deficient iNKT cells failed to do so. Interestingly, the adoptive transfer of purified iNKT cells significantly extended the survival of tumor-bearing NSG mice, irrespective of whether or not the inoculated EL4 cells had been pre-loaded with glycolipid antigens. While this latter observation pointed to a TCR-independent

*Correspondence to: Hamid Bassiri; Email: bassiri@email.chop.edu; Kim E Nichols; Email: nicholsk@email.chop.edu
Submitted: 12/03/2013; Accepted: 12/04/2013; Published Online: 01/03/2014
Citation: Bassiri H, Das R, Nichols KE. Invariant Natural Killer T cells: Killers and Conspirators Against Cancer. OncoImmunology 2013; 2:e27440; http://dx.doi.org/10.4161/onci.27440

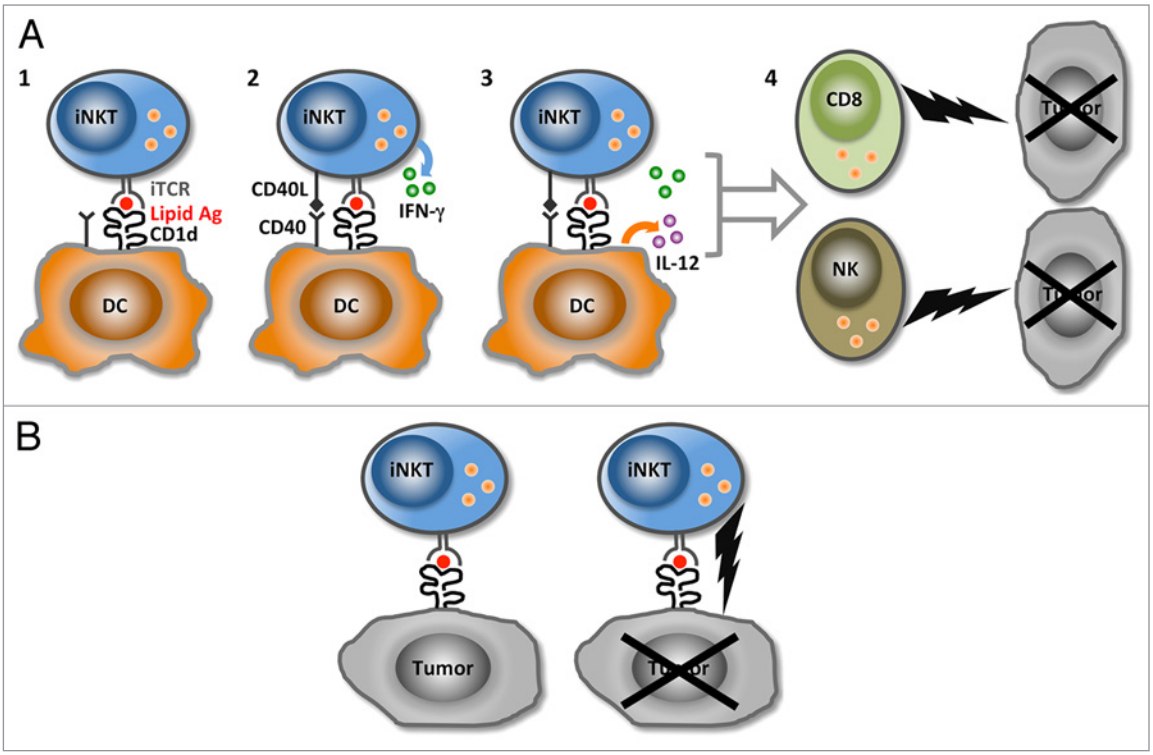

Figure 1. Indirect and direct mechanisms underlying invariant NKT cell antitumor responses. (**A**) The interactions between invariant natural killer T (iNKT) cells and professional-antigen presenting cells (1) such as dendritic cells (DC) stimulate iNKT cells to produce IFNγ (2) and express CD40 ligand (CD40L), which results in DC activation and production of interleukin 12 (IL-12) (3). In this feed-forward loop, IL-12 further stimulates IFNγ production by iNKT cells. Ultimately, the combination of these cytokines *indirectly* boosts the cytolytic activity of $CD8^+$ T cells and NK cells (4). (**B**) iNKT cells may exert *direct* antitumor effects through cytolysis, ligand-induced killing, or the production of soluble mediators (e.g., antiangiogenic factors) that negatively influence the growth and/or survival of neoplastic cells..

antineoplastic activity for iNKT cells in vivo, the administration of anti-CD1d antibodies partially reduced the ability of these cells to control tumor growth. Overall, these findings are in line with those of a recent study in which Epstein–Barr virus (EBV)-infected lymphoblastoid cell lines treated with a retinoic acid receptor agonist—to upregulate and maintain the expression of CD1d—were found to stimulate iNKT cells even in the absence of exogenous antigens.[5]

Collectively, our data demonstrate that iNKT cells can effectively and directly control the growth of $CD1d^+$ tumors, but they also generate several questions that are the subject of ongoing investigation. First, does the protection afforded by iNKT cells involve their activation via TCR-independent mechanisms? A previous report suggests that the engagement of the activating NK-cell receptor killer cell lectin-like receptor subfamily K, member 1 (KLRK1, best known as NKG2D) can stimulate the cytotoxic potential of iNKT cells.[6] The precise contribution of the TCR, NKG2D, cytokines and/or other factors to iNKT cell-mediated protection from tumors remains unknown. Second, if iNKT cells are recognizing neoplastic cells in situ via a TCR-mediated mechanism, does this interaction involve the presentation of endogenous glycolipids, such as the recently described β-D-glucopyrannosylceramides (β-GlcCers)?[7] If so, can malignant cells alter their presentation of endogenous glycolipids to selectively influence the activation of iNKT cells under specific circumstances? We demonstrate that EL4 cells produce endogenous β-GlcCers; however, the ways in which the composition or expression level of these endogenous lipids may be altered are currently unknown. Third, can iNKT cells serve as a platform for cellular immunotherapy? Although several trials have attempted to address this question, many of these studies involved the administration of α-GalCer or α-GalCer-pulsed dendritic cells,[8] which would be expected to induce indirect iNKT-dependent antineoplastic effects. Furthermore, most of these studies targeted $CD1d^-$ tumors, thereby further reducing the possibility of direct iNKT cell-mediated antitumor effects. The answers to these questions will improve our understanding of how to best harness the immunostimulatory and cytolytic activities of iNKT cells as a novel and potentially more effective therapy for CD1d-expressing tumors.

Disclosure of Potential Conflicts of Interest

KEN has received a commercial research grant from Vaccinex, Inc. The other authors have no potential conflicts on interest to disclose.

Acknowledgments

This work was supported by grants from The Clinical Immunology Society and Talecris Biotherapeutics (to HB), and the NIH (T32AI007634, K12HD04335, K08CA166184 to HB, and R01HL089745 to KEN).

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