**Killing the Killer: Natural Killer Cells to Treat Ewing’s Sarcoma**

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**Summary**

Cho and coworkers show that Ewing’s sarcoma cells are highly sensitive to expanded allogeneic natural killer (NK) cells, partially through a NKG2D and DNAM-1 dependent mechanism. Using an orthotopic murine model, they also demonstrate that irradiation significantly enhances the NK cell killing of these tumors.
In this issue of *Clinical Cancer Research*, Cho et al. demonstrate that immunotherapy using *ex vivo* expanded natural killer (NK) cells may be an effective treatment for Ewing sarcoma (EWS) (1). EWS are neural ectodermal tumors with a peak incidence in the second decade of life. While the majority of patients with localized disease can be successfully treated with multimodality therapy (chemotherapy, radiation and surgery), approximately 30% will ultimately relapse. These relapsed patients, as well as those who present with metastatic disease, are unlikely to be cured despite modern therapy, hence, there is a need for new treatments.

Immunological therapies are now being explored for a number of diseases not responsive to standard chemotherapy, including EWS. Allogeneic NK cells are emerging as a promising treatment strategy. In contrast to T cell- and dendritic cell-based therapies, NK cells do not require the identification of tumor antigens, antigen priming or vaccination strategies. Because allogeneic NK cells do not cause graft vs. host disease (GVHD), healthy siblings or parents could be used as donors. Moreover, allogeneic NK cells may be better at killing tumors than autologous NK cells (2).

NK cytotoxicity is determined by the ‘balance’ of signaling though numerous activating or inhibitory receptors. In general, the mechanisms that control NK cytotoxicity are skewed to favor inhibition (i.e., tolerance). Inhibitory receptors recognize MHC class I expression and when engaged, cytotoxicity is inhibited. Reductions in MHC class I expression occur during viral infection and malignant transformation, allowing NK cells to recognize “stressed” cells. The two main inhibitory receptors are CD94/NKG2A and killer inhibitory receptors (KIR). The CD94/NKG2A complex recognizes the non-polymorphic protein, HLA-E, while KIR recognize specific epitopes on HLA-A, -B and -C.

To trigger NK cytotoxicity activating receptors must also be engaged. Over the past 10 years, intensive research has lead to the discovery of NK cell activating receptors. One of the most studied activating receptors is NKG2D, which is expressed on resting NK cells and is up-regulated following activation and cytokine stimulation. This receptor binds to the MHC class I like proteins MICA and MICB, as well as ULBP 1-4. These NKG2D ligands are induced on malignant cells via DNA-damaging signals (irradiation and chemotherapy) (3). Therefore, a main strategy for NK cell based immunotherapy might be to activate NK cells *ex vivo* (to up-regulate NKG2D), to induce NKG2D ligand expression on tumor tissue *in vivo* (2) or both.

Other NK activating receptors include 2B4 (recognizing CD48), TRAIL (recognizing DR4/5), several natural cytotoxicity receptors (NCRs: Nkp30, Nkp44, Nkp46, and Nkp80) and DNAx accessory molecule-1, (DNAM-1, CD226). Resting NK cells
express NKp30, NKp46, and NKp80, but not NKp44. Like NKG2D, NCRs are up-regulated by NK cell activation and are considered to have important roles in NK-tumor interactions, although the ligands for NCRs (and their biology) require further elucidation. Finally, DNAM-1 is also expressed by both resting and activated NK cells as well as other components of the hematopoietic system (platelets, T cells, and monocytes). The ligands for DNAM-1 are the poliovirus receptor, PVR (CD155) and Nectin-2 (CD122).

Previously, Verhoeven and coworkers demonstrated that EWS cell lines are sensitive to NK cell cytotoxicity (4). They found that NK killing of EWS was mainly mediated through the activating receptors NKG2D and DNAM-1 since antibody blocking significantly attenuated NK cytotoxicity. Moreover, several EWS lines and patient samples were found to express NKG2D and DNAM-1 ligands. Compared to controls, the cytotoxicity of NK cells from EWS patients was decreased, but could be restored following rhIL-15 stimulation in vitro.

In this issue of Clinical Cancer Research, Cho and colleagues use their previously published method to expand NK cells with a K562 feeder cell line that is genetically modified to express membrane-bound IL-15 and 4-1BB ligand (5-6). These cell lines potently activate and expand NK cells over 2-3 weeks in culture. Using these expanded NK cells, Cho tested the cytotoxicity against EWS, rhabdomyosarcoma, neuroblastoma, and osteosarcoma (1). Among these, a number of EWS lines were extremely sensitive to allogeneic NK cells from healthy donors (median cytotoxicity is 87.2% at 1:1 E:T). Similar to Verhoeven (4), Cho presents some data supporting the involvement of NKG2D and DNAM-1 in tumor recognition since blocking these receptors attenuated killing (1). However, there was a poor correlation between NKG2D and DNAM-1 ligand expression and cytotoxicity; perhaps suggesting that other activating receptors may also be operational in EWS killing, but the differences in the results between the two laboratories may also reflect varying techniques of NK cell activation and expansion.

One of the major clinical goals in the upcoming years will be to determine how to integrate immunotherapy into current treatment regimens. As described above, NK cells recognize stressed or abnormal cells. One form of cell stress is chemotherapy and irradiation induced tissue injury which, on malignant tissue, may increase the expression of NK activating ligands. Thus, research has started to focus on whether tumors can be “sensitized” to NK killing. Unfortunately, Cho demonstrates that EWS lines treated with relevant chemotherapy agents (doxorubicin or vincristine) did not induce expression of the ligands for NKG2D and DNAM-1 (1). In contrast, this approach was successful in multiple myeloma and increased the susceptibility to NK mediated attack (7). Therefore, elucidation of other chemotherapeutic agents that might sensitize EWS cells to NK killing may be useful for combination chemo-immunotherapy.
While the attempts at “sensitizing” EWS cells to chemotherapy were not successful, Cho and coworkers demonstrated the survival of tumor-bearing mice treated with irradiation followed by NK cells had significantly improved survival compared to irradiation controls (not treated with NK cells) (1). These results are likely explained by the findings of Gasses, who demonstrated the relationship between irradiation, genotoxic stress, cell cycle arrest and the upregulation of NKG2D ligands (3). Thus, the combination of irradiation and NK cell therapy may be promising for EWS.

So how might NK cell infusions potentially be integrated into the care of EWS patients and what are the steps that are needed to test this therapy (figure 1)? First, is a myriad of regulatory issues that would need to be satisfied to move to the clinic. Assuming this to be successful, the next step would be to obtain sufficient amounts of NK cells for therapy. Considering that the number of NK cells in the peripheral blood is relatively small and that NK cells show poor in vitro proliferation, this is not a trivial hurdle. In this regard, data from the Campana laboratory is highly encouraging. Next is determining the source of NK cells. Whether or not patient-derived NK cells can be expanded to the same extent as healthy donors was not tested. Moreover, whether partially MHC matched sibling (or parental) derived NK cells are better than patient-derived (and expanded) NK cells is unknown. However, some caution is required here since there are potential concerns with residual allogeneic T cell contamination which could result in GVHD. In this regard, it is not entirely certain that NK expansion is even needed, since it may negatively influence the homing and trafficking of NK cells to tumor sites. While these issues will take time to be resolved, we are confident that NK cells may offer hope for EWS patients and we applaud Cho for taking the next step in making immunotherapy a real possibility for patients with EWS.
References


Figure 1:

**Conceptualized Schema of NK Cell Therapy For Patients With Ewing’s Sarcoma.** Peripheral blood lymphocytes are collected from a healthy donor. The NK cells are expanded over 2-3 weeks using the K562-feeder cell line (K562-mb15-41BBL) and low dose IL-2 (left). The patient receives irradiation therapy to the site(s) of disease, which increases the expression of NKG2D ligands. The infused NK cells traffic to the sites of disease and kill the tumor tissue.
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