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Super natural killer cells that target metastases in the tumor draining lymph nodes

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ABSTRACT

Tumor draining lymph nodes are the first site of metastasis in most types of cancer. The extent of metastasis in the lymph nodes is often used in staging cancer progression. We previously showed that nanoscale TRAIL liposomes conjugated to human natural killer cells enhance their endogenous therapeutic potential in killing cancer cells cultured in engineered lymph node microenvironments. In this work, it is shown that liposomes decorated with apoptosis-inducing ligand TRAIL and an antibody against a mouse natural killer cell marker are carried to the tumor draining inguinal lymph nodes and prevent the lymphatic spread of a subcutaneous tumor in mice. It is shown that targeting natural killer cells with TRAIL liposomes enhances their retention time within the tumor draining lymph nodes to induce apoptosis in cancer cells. It is concluded that this approach can be used to kill cancer cells within the tumor draining lymph nodes to prevent the lymphatic spread of cancer.

1. Introduction

It has been estimated that 29–37% of cancer patients with breast, colorectal and lung cancers are diagnosed with metastases in their tumor draining lymph nodes (TDLN) [1]. These patients are at a higher risk for distant organ metastases through the vast network of lymphatic capillaries. Lymph nodes (LN) are oval-shaped organs filled with immune cells where the lymphatic capillaries converge, providing an opportunity for the resident immune cells to act against virus infected host cells and cancer cells that may be present in the lymph fluid. An important subset of immune cells patrolling the LN is the natural killer (NK) cell population, representing about 1–5% of mononuclear cells [2]. Despite evidence of NK cell mediated anti-tumor immune response in the LN of experimental animals [3], the LN still remains the first site of metastasis in melanomas and carcinomas [4]. Tumor cell survival is promoted by primary tumor-induced NK cell abnormalities in the TDLN [5]. It has been shown that human NK cells in secondary lymphoid organs such as LN display a different phenotype [6], allowing distinction from the bulk of the NK cells present in the blood. This presents an opportunity to engineer endogenous NK cells in the TDLN to specifically kill cancer cells to prevent the lymphatic spread of cancer. NK cell mediated anti-tumor immune response often involves the expression of Tumor necrosis factor-α Related Apoptosis Inducing Ligand (TRAIL) on its surface, which initiates apoptosis by interacting with death receptors on cancer cells [7]. TRAIL has been widely investigated as a potential therapeutic agent since its discovery in 1975 [8]. Despite the selectivity of TRAIL in inducing apoptosis in cancer cells, there are yet few promising reports on its clinical efficacy [9]. This has motivated several groups to design new formulations to address the limited clinical efficacy of TRAIL.

The advent of use of host immune cells to fight cancer has led to some innovative immunotherapeutic approaches. Several of these efforts have progressed to the clinic in the past decade, such as IL-2 therapy [10], ipilimumab [11] (anti-CTLA4 antibody) and chimeric antigen-receptor engineered T-cell therapy [12]. This has motivated the design of new immunotherapeutic approaches as an alternative to side-effect prone chemotherapy and radiation therapy often used in the treatment of advanced stages of cancer [13]. Nanotechnology-based immunotherapeutic approaches have received attention as a potential route of administering immunomodulatory cytokines/antibodies because of their ability to reduce systemic toxicity compared to conventional formulations [14]. The delivery of immunotherapeutic agents via nanoscale carriers has the advantages of (i) penetrating through the smallest capillary dimensions, (ii) specifically targeting tumors when engineered

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with targeting moieties and (iii) eliciting a localized stimuli-sensitive response because of their capacity to release the immunotherapeutic drug in response to specific cues within the tumor such as pH, hypoxia etc. Nanoscale carriers such as liposomes have been FDA approved for use in chemotherapy and marketed as DOXIL (liposomal doxorubicin) [15] and Abraxane (albumin bound paclitaxel) [16]. Taking a cue from the body's natural defense mechanism, we previously showed that "super" natural killer cells formed through attachment of TRAIL-coated liposomes to NK cells via targeting antibody can effectively induce apoptosis in human cancer cell lines in engineered *in vitro* LN microenvironments [17]. When cocultured with human cancer cell lines that are known to metastasize to LN in experimental animal models, engineered super natural killer cells were able to induce apoptosis in cancer cells to a significantly higher degree compared to unmodified NK cells.

The goal of the present study was to determine if TRAIL liposomes targeted to NK cells that traffic to the tumor draining inguinal LN of mice bearing a subcutaneous human xenograft tumor could effectively prevent the metastasis of a primary tumor to the TDLN. Orthotopic models are also used for studying cancer metastasis in experimental animal models [18]. While orthotopic models have the advantage of providing a more realistic microenvironment for the primary tumor to metastasize, subcutaneous models are often used in studies investigating the lymphatic spread of cancer [19]. Here, we describe a therapeutic approach to target and kill cancer cells in the subcutaneous tumor draining inguinal LN by functionalizing natural killer cells with liposomes conjugated with the apoptosis-inducing ligand TRAIL, and an antibody against NK1.1 antigen expressed on murine NK cells (Fig. 1A). The functionalization of NK in the TDLN, creating "super" natural killer cells with sustained retention time, effectively prevents the lymphatic spread of the primary tumor.

2. Materials and methods

2.1. Reagents and antibodies

Bovine serum albumin (BSA), Paraformaldehyde (PFA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), NaCl, 2-Mercaptoethanol and chloroform (ACS grade) were all obtained from Sigma–Aldrich. Leibovitz's L-15, Dulbecco's Modified Eagle's Medium (DMEM) and Hybri-care cell culture media were obtained from ATCC. RPMI 1640 cell culture media, penicillin-streptomycin (PenStrep), Fetal Bovine Serum (FBS), Ultra-low IgG FBS, Hank's Based Salt Solution (HBSS), Phosphate Buffered Saline (PBS), $NaHCO_3$, Non-Essential Amino Acids (NEAA), Traut's reagent and DAPI stain were all purchased from LifeTechnologies. Recombinant soluble human tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL), recombinant murine interleukin-2 (IL-2) and IL-15 were obtained from Peprotech. L-α-phosphatidylcholine from egg (Egg PC), ovine wool cholesterol (Chol), 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DSPE-mPEG2000) and 1,2-distearoyl-*sn-glycero*-3-phospho ethanolamine-*N*-[maleimide (polyethylene glycol)-2000] (DSPE-Mal-mPEG2000) were purchased from Avanti Polar Lipids. Mouse anti-human DR4 and DR5 conjugated to PE, Mouse anti-human TRAIL (primary Ab), FITC anti-mouse IgG (secondary Ab), mouse IgG1 isotype control, goat anti-mouse IgG and PE mouse IgG1 isotype control were all purchased from BioLegend. Anti-mouse CD3, CD11c, CD335, B220 and CD11b conjugated to APC along with isotype controls were purchased from eBioscience. Annexin-V FITC apoptosis detection kit was purchased from Trevigen. Liver activity detection kits for measuring serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from BioVision. Mouse NK cell isolation kit was purchased from Stemcell Technologies. Human TRAIL ELISA kit and mouse interferon-γ (IFN-γ) ELISA kit were obtained from R&D systems and eBioscience, respectively. Protein G columns and buffers for isolating anti-NK1.1 antibody secreted by mouse hybridoma cell line were purchased from GE Healthcare Lifesciences. 7-Aminoactinomycin/carboxyfluorescein succinimidyl ester (7-AAD/CFSE) cell-mediated cytotoxicity assay kit were obtained from Cayman Chemicals. Luciferase used for *in vivo* bioluminescent imaging was purchased from Gold Biotechnology.

2.2. Cell lines and cell culture

The SW620 cell line established from cancer cells isolated from the tumor draining LN of a human patient with primary colon cancer (ATCC number CCL-227) was obtained from ATCC and cultured in L-15 medium supplemented with 10% (vol/vol) FBS and 100 U/mL PenStrep under humidified conditions at 37 °C with 5% CO_2. Murine melanoma cell line B16F0 (ATCC number CRL-6322) was obtained from ATCC and cultured in DMEM medium supplemented with 10% (vol/vol) FBS and 100 U/mL PenStrep under humidified conditions at 37 °C with 5% CO_2. Mouse hybridoma cell line PK136 (ATCC number HB191) secreting anti-NK1.1 antibody against murine NK cells was purchased from ATCC and cultured in Hybricare medium supplemented with 10% ultralow IgG FBS and 1.5 g/L of $NaHCO_3$. Hybridoma cell culture was maintained at a concentration between 1×10^5 and 1×10^6 cells/mL. For all experiments, cell viability was assessed by trypan blue dye exclusion before counting. Isolated mouse NK cells were cultured in RPMI media supplemented with 10% FBS (vol/vol), 1% NEAA, 50 μM 2-mercaptoethanol, 100 U/mL murine IL-2 and 10 U/mL murine IL-15.

2.3. Mice and in vivo tumor model

Cornell University's Institutional Animal Care and Use Committee (IACUC) approved all the experimental protocols and methods performed in mice. 6- to 8-week-old male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed at the Transgenic Mouse Core Facility at Cornell University in filter-top cages under pathogen-free conditions with free access to water and food. These mice were used for toxicology and pharmacokinetics experiments. 6- to 8-week-old male B6.129S7-*Rag1*^{tm1Mom}/J mice (B6Rag1) were purchased from Jackson Laboratory. B6Rag1 mice have a "non-leaky" immunocompromised phenotype. They lack mature T-cells and B-cells allowing human cancer cell lines to propagate but have its innate immunity intact (high NK cell activity). The high NK cell activity is advantageous for evaluating the therapeutic efficacy of engineered liposomes directed towards NK cells in the tumor draining LN. The animals were weighed weekly and monitored for signs of distress by the Cornell Center for Animal Resources and Education (CARE) facility staff.

For spontaneous metastasis to the inguinal LN, luciferase-expressing SW620 cells (2×10^6 cells/100 μL of PBS) were injected subcutaneously into the lower left abdominal flank. After cancer cell injection, animals were monitored weekly for primary tumor growth and metastasis to the inguinal lymph node using an *in vivo* luciferase-based reporter assay. Luciferin was administered at 150 mg/kg per animal intraperitoneally using a 30G insulin syringe needle. Animals were placed under anesthesia using 2% isoflurane and imaged 10 min post-injection for maximum bioluminescence signal. Images were acquired at 10 s exposure time using a Xenogen IVIS 200 Imaging System. For quantification of total flux wherever reported, in-house developed region of interest (ROI) measurement