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Adoptive transfer of natural killer cells promotes the anti-tumor efficacy of T cells

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ABSTRACT

The density of NK cells in tumors correlates positively with prognosis in many types of cancers. The average number of infiltrating NK cells is, however, quite modest (approximately 30 NK cells/sq.mm), even in tumors deemed to have a "high" density of infiltrating NK cells. It is unclear how such low numbers of tumor-infiltrating NK cells can influence outcome. Here, we used ovalbumin-expressing tumor cell lines and TCR transgenic, OVA-specific cytotoxic T lymphocytes (OT-I-CTLs) to determine whether the simultaneous attack by anti-tumor CTLs and IL-2-activated NK (A-NK) cells synergistically increases the overall tumor cell kill and whether upregulation of tumor MHC class-I by NK cell-derived interferon-gamma (IFNγ) improves tumor-recognition and kill by anti-tumor CTLs. At equal E:T ratios, A-NK cells killed OVA-expressing tumor cells better than OT-I-CTLs. The cytotoxicity against OVA-expressing tumor cells increased by combining OT-I-CTLs and A-NK cells, but the increase was additive rather than synergistic. A-NK cells adenovirally-transduced to produce IL-12 (A-NK^{IL-12}) produced high amounts of IFNγ. The addition of a low number of A-NK^{IL-12} cells to OT-I-CTLs resulted in a synergistic, albeit modest, increase in overall cytotoxicity. Pre-treatment of tumor cells with NK cell-conditioned medium increased tumor MHC expression and sensitivity to CTL-mediated killing. Pre-treatment of CTLs with NK cell-conditioned medium had no effect on CTL cytotoxicity. In vivo, MHC class-I expression by OVA-expressing B16 melanoma lung metastases increased significantly within 24–48 h after adoptive transfer of A-NK^{IL-12} cells. OT-I-CTLs and A-NK^{IL-12} cells localized selectively and equally well into OVA-expressing B16 lung metastases and treatment of mice bearing 7-days-old OVA-B16 lung metastases with both A-NK^{IL-12} cells and OT-I-CTLs lead to a significant prolongation of survival.

Thus, an important function of tumor-infiltrating NK cells may be to increase tumor cell expression of MHC class-I through secretion of IFNγ, to prepare them for recognition by tumor-specific CTLs.

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1. Introduction

It was the ability of natural killer (NK) cells to lyse tumor cells within minutes after first contact that led to their discovery in 1975 [1,2]. Since then, the importance of NK cell-mediated cytotoxicity in the setting of cancer and infection, has been extensively investigated [3–8]. The relatively low density of infiltrating NK cells found in most solid tumors has made it difficult to accept NK cell cytotoxicity as an effector function of major importance for immune-mediated control of established malignancies. In contrast, many studies have clearly demonstrated that NK cells play an important role in the defense against hematogeneous metastasis via their ability to eliminate circulating tumor cells as they navigate through the capillaries of the lungs [9–15] and possibly other organs as well [16]. Lately, an increasing number of studies have shown that NK cells play a major role in immune-regulation, bridging the gap between innate and adaptive immune responses [17–20]. This includes cross-talk, mediated by cytokines as well as membrane-bound receptors, with dendritic cells (DCs) in the periphery, leading to DC activation and maturation [21–27]. NK/DC cross-talk can also

Abbreviations: Ab, Antibody; Adv-IL-, Adenoviral vector with gene for interleukin-; A-NK, Activated natural killer cells; A-NK^{IL-12}, A-NK cells transduced with Adv-IL-12; A-NK^{mock}, A-NK cells transduced with empty Adv-; CM, Complete Medium; CPM, Counts per minute; CTL, Cytotoxic T lymphocyte; DAMP, Damage-associated molecular pattern; DCs, dendritic cells; E:T, Effector-to-target ratio; GFP, Green fluorescent protein; i.v., intravenous; IFNγ, Interferon-gamma; IL-, Interleukin-; IU, International unit; M05, Ovalbumin-expressing B16 melanoma; MHC, Major histocompatibility complex; MHC, Major histocompatibility complex; mr−/hr.-, Murine recombinant−/human recombinant-; NKCM, NK cell-conditioned medium; OT-I-CTLs, CTLs derived from OT-I transgenic mice; Ova, ovalbumin; PAMP, Pathogen-associated molecular pattern; PE, Phycoerythrin; PEG-IL-2, IL-2 complexed with polyethylene glycol; PHA, Phytohemagglutinin; Sq.mm, Square millimeter; TCR, T cell receptor; Th1/Th2, T-helper-1/T-helper-2; T-LAK cells, PHA and IL-2 stimulated bulk splenocytes; TNFα, Tumor necrosis factor α.

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occur in lymph nodes, where NK cells play an important role in the Th1/Th2 biasing of the mature immune response [20].

Interestingly, the density of NK cells in tumor tissue has been found to correlate positively with prognosis. This was first shown in colorectal cancer by Coca et al. [28] and has later been found to also be in case in many other types of cancers as well, including gastric and hepatocellular carcinoma, adenocarcinoma of the lungs, renal cancer, and squamous cell cancers of the esophagus, lungs, and vulva [29–36]. It is, however, hard to envision that the NK cells' cytotoxic activity alone is able to influence outcome, since even in tumor tissues found to have the "high" density of infiltrating NK cells associated with better prognosis, these high densities appear to be very modest, i.e., usually well below 100 cells per sq.mm when counted in 2–10 μm thick tissue sections. These low densities are not supportive of NK cell mediated lysis of tumor cells as the sole mechanism behind the positive correlation between NK density and prognosis. It is more likely that NK cells provide the initial source of antigens via their tumor cell killing and then establish cross-talk with nearby DCs, to support their maturation and production of IL-12 [25,37], as these activities are critical in the development of a Th1-biased anti-tumor T cell response [38,39].

Here, we present evidence that NK cells may help anti-tumor CTLs at the tumor sites by killing MHC-negative tumor cells and, more importantly, by preparing the tumor tissue for recognition by CTLs via IFNγ-induced upregulation of tumor cell MHC class-I expression.

2. Materials and methods

2.1. Animals

Female C57BL/6 and B6·129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb (OT-I) mice, 8–12 weeks of age, were obtained from Taconic Biosciences, Inc. Congenic B6·Pl–Thy-1aCy (Thy1.1) male and B6·SJL-Ptprca Pep3b/BoyJ (CD45.1) female mice, 8–12 weeks of age, were obtained from Jackson (Bar Harbor, ME, USA). The use of animals for the experiments described below was approved by the Institutional Animal Care and Use Committee, University of Pittsburgh.

2.2. Tumor cell lines

The subline F10·*P*1 of the B16 melanoma (C57BL/6 origin) was established in our laboratory from a B16-F10 lung metastasis. The chicken OVAlbumin-transduced M05 variant of the B16 melanoma cell line (expressing the SIINFEKL peptide in H-2Kb) was a kind gift from Dr. Louis Falo, University of Pittsburgh [40]. Lewis lung carcinoma (3*LL*) and Panc02 adenocarcinoma cells were purchased from The American Type Culture Collection (ATCC). The MC38 colon carcinoma was a gift from Dr. M. Shurin, University of Pittsburgh. MC38 and Panc02 tumor cells were transfected to produce OVA-expressing variants, MC38^{OVA} and Panc02 ^{OVA}, respectively. All cell-lines were maintained in RPMI-1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 20 mM Hepes buffer, 0.8 g/l streptomycin and 1.6x10^5 U/l penicillin (from here-on referred to as complete medium, CM). Adherent cells were detached by exposure to 0.02% EDTA for 2–3 min and washed three times in RPMI-1640. Cell viability, judged by trypan blue dye exclusion test, was always >95%. Murine pulmonary metastases were established by tail vein injection of 0.2–0.4x10^6 cells in 0.3 ml of RPMI-1640 into C57BL/6 mice, pretreated on day −1 with 40 μl anti-asialoGM1 antiserum (Wako Pure Chemicals, Wako, TX, USA).

2.3. Preparation of A-NK cells

Spleens were removed aseptically from C57BL/6 and CD45.1 congenic B6·SJL-Ptprca Pep3b/BoyJ mice and a single-cell suspension was prepared in RPMI-1640. Erythrocytes were lysed by incubation with ammonium chloride–potassium buffer at room temperature for 3 min and the spleen cells were subsequently washed twice in RPMI-1640. CD3 and B220 positive cells were magnetically removed following incubation of the cell culture with rat anti-CD3 and rat-antiB220 antibody and subsequently with anti-rat coated magnetic beads (Dynal Biotech, Lake Success, NY, USA). The CD3/B220-depleted cells were resuspended in fresh CM containing 6000 IU/ml rhIL-2 (kindly provided by Novartis Pharma AG, Basel, Switzerland) to a final concentration of 1x10^5 cells/ml and cultured in tissue culture flasks (Falcon, B&D, Franklin Lakes, NJ, USA) at 37 °C in an atmosphere of 5% CO_2. Fresh CM containing 6000 IU/ml IL-2 was added every 2–3 days as needed. After 5–7 days of culture, non-adherent cells and adherent cells were harvested after a brief treatment with 0.02% EDTA and washed twice in RPMI-1640 before use. Routinely, on day 5 of culture, the A-NK cells were >95% CD45.1+ (or CD45.2+), >95% Thy1.2+, >95% asGM1+, >90% NK1.1+, >90% NKp46+ <2% CD8+, <2% CD4+.

2.4. Preparation of anti-tumor CTLs

To produce Thy1.1 and Thy1.2 double-positive anti-tumor CTLs specific for B16-M05 cells, splenocytes from *F*1[B6·Pl–Thy-1aCy x B6·129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)] mice were prepared and, after red blood cell lysis, transferred to T150 plastic flasks at 2x10^5 cells/ml of CM supplemented with 100 IU/ml of rhIL-2 and 8 μg/ml of PHA-P (phytohemagglutinin-P; DIFCO, Detroit, MI, USA). After 24–48 h aggregated splenocytes were isolated by light centrifugation and transferred to new culture flasks with fresh CM supplemented with 100 IU/ml of rhIL-2/ml. Fresh CM with IL-2 was added every 2–3 days as needed. After 5–7 days of culture, non-adherent cells and adherent cells were harvested after a brief treatment with 0.02% EDTA and washed twice in RPMI-1640 before use.

2.5. NK cell-conditioned medium (NKCM)

On day 5–6 of culture with IL-2, A-NK cells were harvested, adjusted to 1 million cells/ml in CM with 6000 IU of IL-2 and cultured for 24 h. After 24 h of culture, the supernatant was harvested and centrifuged at 3000 rpm for 15 min to remove cells and debris. The supernatant was then passed through a 0.4 μm filter, aliquoted and stored at —80C.

2.6. Cytotoxicity assays

Standard 51Cr-release assays were performed in 96-well, round-bottomed microtitre plates. Approximately 5×10^6 target cells were re-suspended in 100 μl sodium chromate (Na^{51}CrO4: DuPont, Germany) for 45 min at 37C. After labelling cells were washed three times in cold RPMI-1640. Effector cells were mixed with 1×10^4 target cells at various effector: target ratios. Plates were incubated at 37C, 5% CO_2 atmosphere for 4 h. After centrifugation for 5 min at 600 g, 100 μl of supernatant from each well was removed and counted in a gamma counter. Spontaneous release was estimated by culturing target cells in medium alone. Maximum release was estimated by adding 100 μl of 10% Triton X-100 to target cells. All experiments were performed in triplicate, and the percentage of cytotoxicity was calculated as: % Specific lysis = (cpm of test − cpm spontaneous release) / (cpm maximum release − cpm spontaneous release) × 100.

2.7. Live-cell microscopy

OT-I-CTLs were incubated with Cell Tracker Orange (Invitrogen, cat.no. C2927) at 1 microM in RPMI1640 for 30 min. After wash, the CTLs were resuspended in complete medium with 100 IU IL-2/ml and added (3:1) to 8-well chamber slides (Ibidi, cat.no. 80826) with GFP+ B16-M05 tumor cells, which had been pre-exposed to A-NK cell-conditioned medium for 18–24 h or with non-treated (control) GFP+ B16-M05 tumor cells. All NKCM and control medium was removed from the chamber wells before addition of the CTLs. Using an Olympus

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