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IL-21 Enhances Tumor Rejection through a NKG2D-Dependent Mechanism

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IL-21 is a cytokine that can promote the anti-tumor responses of the innate and adaptive immune system. Mice treated with IL-21 reject tumor cells more efficiently, and a higher percentage of mice remain tumor-free compared with untreated controls. In this study, we demonstrate that in certain tumor models IL-21-enhanced tumor rejection is NKG2D dependent. When engagement of the NKG2D receptor was prevented, either due to the lack of ligand expression on the tumor cells or due to direct blocking with anti-NKG2D mAb treatment, the protective effects of IL-21 treatment were abrogated or substantially diminished. Specifically, IL-21 only demonstrated a therapeutic effect in mice challenged with a retinoic acid early inducible-16-bearing lymphoma but not in mice bearing parental RMA tumors lacking NKG2D ligands. Furthermore, treatment with a blocking anti-NKG2D mAb largely prevented the therapeutic effect of IL-21 in mice challenged with the 4T1 breast carcinoma, the 3LL lung carcinoma, and RM-1 prostate carcinoma. By contrast, IL-21 did mediate beneficial effects against both the parental DA3 mammary carcinoma and DA3 tumors transfected with H60, a NKG2D ligand. We also observed that IL-21 treatment could enhance RMA-retinoic acid early inducible-16 tumor rejection in RAG-1−/− deficient mice, thereby demonstrating that the IL-21-induced protective effect can be mediated by the innate immune system and that, in this case, IL-21 does not require the adaptive immune response. Collectively, these findings suggest that IL-21 therapy may work optimally against tumors that can elicit a NKG2D-mediated immune response. The Journal of Immunology, 2005, 175: 2167–2173.

A n activating type II disulfide-linked homodimeric receptor, NKG2D is expressed on NK cells, γδ-TCR+ T cells, and CD8+ αβ-TCR+ T cells. In association with adapter proteins DAP10 (in humans and mice) or DAP12 (in mice only), NKG2D signals through the PI3K pathway or the Syk-ZAP70 tyrosine kinase pathway, respectively (1). In mice, NKG2D binds to several cell surface glycoproteins, including the retinoic acid early inducible-1 (RAE-1) proteins (2, 3), a minor histocompatibility Ag H60 (2, 3), and murine UL-16-binding protein-like transcript-1 (4, 5). There are at least five RAE-1 genes (RAE-1α, β, γ, δ, and ε) currently identified, and these are differentially expressed in various mouse strains. In humans, NKG2D binds to MHC class I-related chains A and B (6), which are highly polymorphic non-classical MHC molecules, and to the UL-16-binding proteins (also known as RAE-T1) (7–9), which are the human orthologs of the mouse RAE-1 molecules. Expression of the NKG2D ligands on healthy tissues is limited; however, on tissues that have undergone transformation or have been infected by viruses, expression of these ligands can become up-regulated. Many tumor cell lines and primary tumor cells display increased surface expression of RAE-1 (2, 3) or MHC class I-related chains A and B (10–12). Susceptibility of a tumor cell to NK cell-mediated lysis is enhanced by the presence of these ligands, endogenous (6) or induced (13, 14), and the enhanced killing can be inhibited by blocking the NKG2D receptor with mAb against NKG2D or its ligand (15).

IL-21 is a cytokine produced by activated T cells, and its receptor (IL-21R) is expressed in lymphoid tissues, in particular on NK, B, T, and dendritic cells, as well as macrophages (16). IL-21 is structurally related to the cytokines IL-2 and IL-15 and signals through a receptor complex requiring the common γ-chain (17), similar to the IL-2R and IL-15R. IL-21 has been reported to promote the maturation of NK cell progenitors from the bone marrow (16); however, in contrast to IL-2 and IL-15, treatment with IL-21 limits mature NK cell proliferation (18, 19). Although IL-21 does not increase the overall NK cell population, it has been shown to augment the functional maturation of NK effector functions, such as cytokine production and cytotoxicity (20, 21). IL-21 also activates CD8+ T cells and promotes tumor Ag-specific activation and clonal expansion of specific CD8+ T cells (22, 23). IL-21-stimulated CD8+ T cells have enhanced survival and cytotoxic responses compared with CD8+ T cells stimulated with IL-2 (23). By potentiating the effector functions of NK and CD8+ T cells, IL-21 enhances both the innate and adaptive components of tumor immunity and promotes the tumor-free survival of treated mice.

Considering the previous findings showing that many primary tumors and tumor cell lines up-regulate NKG2D ligand expression, which sensitizes tumor cells to NK cell lysis, we set out to investigate whether the NKG2D pathway is involved in IL-21-enhanced tumor rejection. In this study, we have addressed whether...
NKG2D-dependent NK cell-mediated rejection of tumors can be augmented by IL-21 therapy.

Materials and Methods

**Mice**

Inbred C57BL/6 (B6) mice were purchased from the National Cancer Institute, Charles River Laboratories, or The Walter and Eliza Hall Institute of Medical Research. Inbred BALB/c mice were purchased from The Walter and Eliza Hall Institute of Medical Research. RAG-1-/- mice were kindly provided by Dr. J. Baron (University of California, San Francisco, CA) and were maintained on a C57BL/6 background. Perforin +/-, IFN-γ +/-, perforin +/- × IFN-γ +/-, and gld mice, all backcrossed onto the B6 or BALB/c background, were bred at the Peter MacCallum Cancer Centre. All mice were maintained and treated in accordance with the University of California, San Francisco, Committee on Animal Research and the Peter MacCallum Cancer Centre Animal Ethics guidelines.

**Cell lines and transfectants**

The RMA (H-2b-positive) T cell lymphoma cell line (kindly provided by Dr. D. Ryan, University of California, San Francisco) was cultured in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Stable transfectants of RMA-RAE-1 or RMA cells transfected with the same vector used for RAE-1, but without a cDNA insert (mock transfected), were made as previously described (14). The sensitivities of 3LL Lewis lung carcinoma (perforin- and FasL-sensitive, H-2d), RM-1 prostate carcinoma (perforin- and FasL-sensitive, H-2b), 4T1 mammary carcinoma (perforin-, TRAIL-insensitive, H-2b), and Renca renal carcinoma (perforin- and TRAIL-sensitive and FasL-insensitive, H-2b) and their maintenance has been previously described (24–27). DA3-m (mock vector alone infected) mammary carcinoma (perforin-, FasL- and TRAIL-sensitive, H-2d) and DA3-H60 (H60 infected) mammary carcinoma (perforin-, FasL- and TRAIL-sensitive, H-2b) cells were prepared and selected by flow cytometry as previously described (28).

**51Cr release assay**

Effector cells were isolated from B6 mice injected i.p. on day −4, −2, 0 and with 75 μg of IL-21 (Zymogenetics) or PBS. No tumor cells were injected into the mice. On day +1, spleens were harvested, lysed with ACK buffer (BioWhittaker), and stained with anti-CD4 mAb (GL1.5) and anti-CD8 mAb (53.6.7). The mAb-coated cells were then incubated with goat anti-rat IgG-coated and goat anti-mouse IgG-coated magnetic beads (Quagen). Subsequently, T and B cells were negatively depleted by magnetic cell sorting. The purified cells were then immediately used as effector cells in a standard 4-h cytotoxicity assay, as previously described (29).

**Tumor experiments**

**RMA T cell lymphoma.** Groups of 10 B6 wild-type (WT) or RAG-1−/− mice were injected (i.p.) with 75 μg of IL-21 or PBS on day −4, −2, 0, 2, and 4. On day 0, mice were injected i.p. with 1 × 10⁵ RMA-RAE-1 transfectants or RMA mock-transfected cells. For depletion of NK cells, mice were injected i.p. on day −1, 1, 7, and weekly thereafter with 200 μg of anti-NK1.1-depleting mAb (PK136; purified from ascites grown by Harlan Bioproducts; hybridoma kindly provided by Dr. W. Seaman, University of California, San Francisco). Effectiveness of the NK cell depletion was confirmed by flow cytometry, as indicated by the absence of DX5 mAb (BD Pharmingen) positively staining cells in the peripheral blood of anti-NK1.1 mAb-treated compared with control-treated mice. Mice were monitored every other day for tumor ascites development, indicated by swelling of the abdominal region, and were sacrificed when tumor burden became excessive, to avoid pain and suffering.

**Tumor metastases models.** 3LL, RM-1, Renca, and DA3 cell lines were inoculated i.v. as indicated, at a dose previously shown in wild-type (WT), gene-targeted, or NK cell-depleted mice to result in similar numbers of lung metastases. For all experimental metastases models, mice were injected i.v. with tumor cells (on day 0) and were sacrificed 14 days later; the lungs were removed, and surface metastases were counted with the aid of a dissecting microscope. Mice were treated with recombinant mouse IL-21 (50 μg i.p.) or PBS on days 0, 1, 2, and 3. Some groups of mice were depleted of NK cells, but not NK T cells, using 100 μg of anti-asialo-GM1 antiserum (WAKO) i.p. on day −1, 0, and 7, as described (30). Some WT mice were treated with neutralizing anti-Fasl (250 μg i.p.) on days 0, 1, 7, and 4. On day 0, mice were injected i.p. with 1 × 10⁵ RMA-RAE-18 cells. Data are representative of four independent experiments.

**Results**

**IL-21 treatment enhances the in vivo rejection of RAE-1-transfected RMA cells**

Ectopic expression of RAE-1 on RMA T cell lymphoma cells renders the NK cell-sensitive tumor cells susceptible to NKG2D-mediated killing (13, 14). The rejection capability of the host immune system, however, is not limitless, and mice injected with higher cell dosages (1 × 10⁶, 10⁷) of RMA-RAE-1 cells succumb to tumors (14) (Fig. 1A). Based on these observations, we set out to investigate whether treatment with IL-21 could enhance the host immune system and prolong tumor-free survival of mice injected with a higher tumor-inducing cell dosage. B6 WT mice were treated with a regimen of IL-21 or PBS (control) and injected i.p. with 2 × 10⁶ RMA-RAE-18 cells.
were incubated with 51Cr-labeled RMA or RMA-RAE-1, and 0. On day 0, mice were treated i.p. with 75 μg of IL-21 or PBS on days -4, -2, 0, 2, and 4. On day 0, mice were injected i.p. with 1 × 10^5 RMA parental cells and monitored for tumor growth. Data are representative of one experiment. B, 51Cr-release assay measuring the lytic activity of IL-21-activated effector cells. C57BL/6 mice were treated i.p. with 75 μg of IL-21 or PBS on days -4, -2, and 0. On day +1, spleens were harvested and negatively depleted of T and B cells (see Materials and Methods). The remaining effector cells were incubated with 51Cr-labeled RMA or RMA-RAE-18 cells. Data are representative of three experiments and shown with error bars indicating SEM. Groups in which IL-21 treatment significantly reduced that group’s number of lung metastases compared with control PBS-treated effects (Fig. 2B).

To investigate whether the efficacy of IL-21-mediated rejection could be modulated by the presence of a NKG2D ligand in another tumor model, we examined the DA3 mammary carcinoma cell line. Negative for endogenous NKG2D ligand expression, this cell line was either mock infected or infected with a retrovirus to express H60, another NKG2D ligand (33). WT BALB/c or BALB/c mice genetically deficient in perforin or TRAIL were injected i.v. with either 1 × 10^5 parental DA3 cells or 1 × 10^6 DA3-H60 cells, treated with a single injection of IL-21 or PBS i.p., sacrificed 14 days later, and analyzed for lung metastases (Fig. 2C). Even in mice injected with parental DA3 cells, which lacked H60 expression, IL-21 treatment provided some protective effect that was perforin dependent, but TRAIL and FasL independent. Suppression of DA3-H60 tumor metastases in the lung was comparatively strongly enhanced by IL-21 treatment, with treated WT, TRAIL-/-, and FasL-neutralized WT mice all displaying only a few residual metastases compared with the same mice treated with PBS. Thus, even though DA3-H60 cells were injected at a 10-fold astastic tumor nodules were counted. Data are representative of two independent experiments. Experimental metastases were recorded as the mean number of metastases ± SEM. *, groups in which IL-21 treatment significantly reduced that group’s number of lung metastases compared with PBS treatment; ***, groups in which treatment with anti-NKG2D mAb significantly increased that group’s number of lung metastases compared with treatment with control Ig (by Mann-Whitney U test, p < 0.05).
injected i.p. with 50 µl livers were harvested on day 25 after tumor inoculation, and metastatic tumor nodules were counted. Data are representative of one experiment.

FIGURE 3. IL-21 enhances rejection of tumor cells that endogenously express NKG2D ligand, and this rejection is dependent upon NKG2D receptor engagement. A, Groups of five BALB/c WT mice were injected into the mammary fat pad with 2.5 × 10⁴ 4T1 tumor cells on day 0. Primary tumors (~10 mm²) were surgically resected on day 8, and mice were injected i.p. with 50 µg of IL-21 on days 10, 11, and 12. All BALB/c mice were treated with either anti-mouse NKG2D mAb (250 µg i.p.) or control Ig (250 µg i.p.) on days 0, 1, 7, and 8 after tumor inoculation. Lungs and livers were harvested on day 25 after tumor inoculation, and metastatic tumor colonies were plated and counted as described in Materials and Methods. Data are representative of two independent experiments. B, Groups of five B6 WT, pfp⁻/⁻, and gld (FasL mutant) mice were injected i.v. with 1 × 10⁵ RM-1 cells. Mice were injected i.p. with 50 µg of IL-21 on days 0, 1, 2, and 3 where day 0 is the day of tumor inoculation. All B6 mice were treated with either anti-mouse NKG2D mAb (250 µg i.p.) or control Ig (250 µg i.p.) on days 0, 1, 7, and 8 after tumor inoculation. Lungs were harvested 14 days after tumor inoculation, and metastatic tumor nodules were counted. Data are representative of one experiment. C, Groups of five B6 WT, pfp⁻/⁻, IFN-γ⁻⁻, IFN-γ⁻⁻, IFN-γ⁻⁻, and gld mice were injected i.v. with 5 × 10³ 3LL tumor cells. Mice were injected i.p. with 50 µg of IL-21 on days 0, 1, 2, and 3 where day 0 is the day of tumor inoculation. All B6 mice were treated with either anti-mouse NKG2D mAb (250 µg i.p.) or control Ig (250 µg i.p.) on days 0, 1, 7, and 8 after tumor inoculation. Lungs were harvested 14 days after tumor inoculation, and metastatic tumor nodules were counted. Data are representative of two independent experiments. * Groups in which treatment with anti-NKG2D mAb significantly increased that group’s number of lung or liver metastases compared with treatment with control Ig (by Mann-Whitney U test, p < 0.05).
NK cells mediate IL-21-induced anti-tumor response

To investigate whether the NKG2D-dependent IL-21 enhanced killing of RMA-RAE-16 cells was being mediated by NK cells, we examined tumor rejection in NK cell-depleted mice. RAG-1−/− mice were given the IL-21 regimen used in the previous RMA experiments, treated with the NK cell-depleting anti-NK1.1 mAb, injected with 1 × 10^5 RMA-RAE-16 cells, and monitored for tumor growth (Fig. 4A). Even in the absence of an adaptive immune response, IL-21 conferred a protective effect, and IL-21-treated mice rejected the RMA-RAE-18 cells more efficiently than PBS-treated mice. The IL-21 protection was NK cell dependent, and the IL-21-treated RAG-1−/− mice depleted of NK cells developed tumors at a rate mirroring PBS-treated mice.

We also examined NK cell involvement in the rejection of a tumor metastases model by using Renca renal carcinoma cells. Like the other lung metastases models, rejection of Renca tumor cells was enhanced by IL-21 treatment (Fig. 4B). Notably, depletion of NK cells with anti-asialo-GM1 antisera, however, abrogated the IL-21-mediated protection, and the depleted mice developed as many lung metastases as PBS-treated mice. An equivalent loss of protection was also observed in IL-21-treated mice injected with a neutralizing (nondepleting) anti-NKG2D mAb, thereby providing further support that the NKG2D-dependent protection in IL-21-treated mice was mediated by NK cells.

Discussion

In this study, we have shown that, in the tumor models examined, IL-21-mediated rejection of tumors and suppression of metastases is NKG2D dependent. Tumor cells expressing NKG2D ligands were rejected with a significantly higher efficiency in IL-21-treated mice than in control mice. As documented by use of the RMA lymphoma transfected with RAE-1, this protection was dependent on the presence of the NKG2D ligand and did not occur in IL-21-treated mice injected with parental tumor cells, which lacked NKG2D ligand expression. The involvement of the NKG2D receptor in IL-21-enhanced tumor rejection was also directly implicated in the recognition process by blocking the receptor with a mAb. Preventing the engagement of the NKG2D receptor abrogated the protective effects of IL-21, and mice developed tumors similarly to mice that did not receive IL-21 treatment.

IL-21 is a potent activator of both the innate and adaptive anti-tumor responses (18, 22, 23, 34); as a result, mice treated with the cytokine have an enhanced ability to reject tumor cells (20, 35–37). In concurrence with these past observations, mice treated with IL-21 were able to reject substantially higher tumor doses of RMA-RAE-16 or DA3-H60 cells than PBS control mice (Fig. 1). The IL-21-enhanced rejection of RMA tumor cells was completely dependent on the presence of the RAE-1 ligand, whereas the rejection of DA3 tumor cells was to some extent able to occur independently of the NKG2D pathway. This protective effect of IL-21 in the absence of NKG2D ligand has also been observed using the B16F10 melanoma model (20, 38, 39) (and our unpublished data). Due to the low MHC class I expression of DA3 and B16, it was possible that these tumor cells were more susceptible to effector lysis, and, as a result, IL-21-activated NK cells were able to lyse these tumor cells without being activated through the NKG2D pathway. This rejection process was most likely mediated predominantly by NK cells given that IL-21 treatment could still enhance rejection of DA3 and B16 cells in RAG1−/− mice, which lack a functional adaptive immune response, and this protective effect was abrogated when mice were depleted of NK cells (20).

We also observed that IL-21-mediated suppression of the DA3 tumor metastases could occur through a NKG2D-dependent mechanism. IL-21-treated mice were able to suppress a 10-fold higher dose of DA3-H60 cells compared with mice injected with DA3 parental cells; and treatment with a blocking anti-NKG2D mAb abrogated this protection. To verify that endogenously expressed NKG2D ligands could also stimulate the IL-21-enhanced rejection of tumor cells, we examined the rejection of the 4T1, RM-1, 3LL, and Renca tumor cell lines (Figs. 3 and 4B). Suppression of metastases of all of these tumor lines was enhanced by treatment with IL-21 and was observed to be NKG2D dependent. Taken together, these results demonstrate that activation of effector cells through the NKG2D pathway can enhance the potency of IL-21-mediated activation.
IL-21 can modulate the surface expression of various NK cell markers, such as NK1.1 and CD94 (20). We examined whether IL-21 affected the surface expression of NKG2D by staining freshly isolated peripheral NK cells from mice treated with IL-21 in vivo. The expression level of NKG2D on IL-21-activated NK cells was comparable to the level on NK cells isolated from PBS control mice, as determined by using flow cytometry (data not shown). Thus, IL-21 treatment did not enhance NK cell killing activity by increasing expression of the activating NKG2D receptor.

IL-21-enhanced tumor rejection has been shown to be dependent on perforin (20). A major component of NK cell effector function, perforin plays a critical role in the NKG2D-mediated tumor rejection process (26, 33, 40) and has been shown to play an important role in IL-2-, IL-12-, and IL-18-enhanced tumor rejection (33). In this study, we have demonstrated that the IL-21 protective effect, which is mediated through NKG2D, is dependent on perforin (Figs. 2B, 3, and 4B). Without perforin, mice treated with IL-21 and injected with DA3-H60 cells were not protected, and these mice developed tumor metastases in a quantity comparable to PBS-treated control mice. In contrast, in these tumor models, we found that the IL-21 protective effect mediated through NKG2D was not affected by the absence of Fas or IFN-γ pathways.

Although studies have shown that IL-21 can augment the anti-tumor responses of CD8+ T cells (18, 33, 38), we propose that the NKG2D-mediated tumor rejection of the tumors used in our study was mediated primarily by NK cells. Moreover, IL-21 could enhance tumor rejection independently of the adaptive immune response. IL-21-treated RAG-1−/− mice rejected RMA-RAE-1 cells more efficiently, and a higher percentage remained tumor-free compared with PBS-treated control mice. This protection was abrogated when mice were depleted of NK cells with anti-NK1.1 mAb treatment (Fig. 4A). Similarly, depletion of NK cells in the Renca lung metastasis model protected the protective effects of IL-21, and mice developed metastases in a quantity similar to PBS-treated control mice (Fig. 4B). Taken together, these data have demonstrated that NK cells activated through the NKG2D pathway are a major contributor to IL-21-activated anti-tumor immunity.

Disclosures
A. Nelson is a prior employee of, and S. Hughes and P. V. Sivakumar are current full-time employees of, Zymogenetics, a biotech company performing clinical trials with IL-21.

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