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Increased expression of IL-21 reduces tumor growth by modulating the status of tumor-infiltrated lymphocytes

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ABSTRACT

The role of interleukin (IL)-21 in influencing tumor growth or enhancing anti-tumor immunity is somewhat controversial. To further understand the potential regulatory effects of IL-21, we utilized an IL-21-secreting EG7 tumor model to demonstrate the direct effects of IL-21 on host tumor-infiltrating lymphocyte (TIL) profiles. Vector control EG7 cells (EG7-Vec) produced very low amounts of IL-21 and were highly tumorigenic. In contrast, IL-21-expressing EG7 cells, EG7-IL-21L and EG7-IL-21H, secreted relatively and extremely high levels of IL-21, respectively. Most importantly, both IL-21-expressing EG7 cells' control of tumor growth was not due to increased proliferative ability of tumor cells, but resulted from the induction of cytotoxic cellular responses in immunocompetent mice. To identify the effects of cancer immunoediting, tumor-infiltrating lymphocyte profiles were analyzed. NK cell populations appeared to be increased in EG7-IL-21H tumor sites at days 6–8 (progression stage), though this phenomenon did not persist at days 10–12 (regression stage). However, at both days 6–8 and 10–12, a higher frequency of $CD8^+$ T cells was observed at the tumor site in EG7-IL-21H-inoculated mice than in EG7-Vec-inoculated mice. These findings suggest that NK cell-mediated tumor rejection may efficiently drive the development of tumor-specific cytotoxic T cell responses with the help of elevated IL-21 expression. These results also suggest the therapeutic potential of IL-21.

Introduction

Interleukin (IL)-21 is a cytokine with a four-helix-bundle structure that was first discovered in 2000 (Parrish-Novak et al. 2000). IL-21 is a member of the IL-2 cytokine family. Members of the IL-2 family, including IL-2, IL-4, IL-7, IL-9, and IL-15, all employ the common γ-chain in their receptor complexes. IL-2 family members play pivotal roles in the regulation of lymphoid cell development and function (Asao et al. 2001; Habib et al. 2002; Kovanen and Leonard 2004; Leonard and Spolski 2005; Leonard et al. 2008). At first, IL-21 production was believed to be limited exclusively to activated $CD4^+$ T cells. Currently, it is known that IL-21 is also produced by natural killer T cells (Coquet et al. 2007). Recently, follicular helper T cells were found to be a major source of IL-21 as well (Chtanova et al. 2004). The IL-21 receptor (IL-21R) is expressed on numerous cell types within the immune system, including macrophages, B, T, NK, NKT, and dendritic cells. The multiple origins of IL-21 and wide expression of IL-21R lead to the pleiotropic effects of IL-21on both innate and adaptive immune responses (Mehta et al. 2004; Parrish-Novak et al. 2002; Spolski and Leonard 2008).

IL-21 has been shown to be a novel growth and survival factor in multiple myeloma through an autocrine insulin-like growth factor 1 loop (Brenne et al. 2002; Menoret et al. 2008). However, because of the fact that IL-21 possesses extensive immunomodulating effects, it has recently attracted attention as a tool to generate anti-tumor responses for cancer immunotherapy (di Carlo et al. 2007; Sondergaard and Skak 2009; Spolski and Leonard 2008). Several reports demonstrated that administration of recombinant IL-21 (Frederiksen et al. 2008; Moroz et al. 2004; Sondergaard et al. 2010, 2007), IL-21 gene expression vectors (Brady et al. 2004; Kishida et al. 2003; Wang et al. 2003), or IL-21 gene-modified cells (Croce et al. 2008; Dou et al. 2009; Fang et al. 2008; Furukawa et

Abbreviations: DT, doubling time; EG7-IL-21H, EG7 clone with high IL-21 expression; EG7-IL-21L, EG7 clone with low IL-21 expression; EG7-Vec, EG7 clone with vector transfected; IL-21, interleukin-21; NK, natural killer; Treg, regulatory T; TIL, tumor-infiltrating lymphocytes.

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al. 2006; Kumano et al. 2007; Ugai et al. 2003a,b; Zhao et al. 2010) elicits significant anti-tumor responses. In addition, IL-21 also has the ability to inhibit tumor angiogenesis (Castermans et al. 2008). These advantageous effects on the survival of tumor-bearing hosts provide further impetus for the use of IL-21 in cancer treatment.

The notion of cancer immunoediting explains how the immune responses defend the host and modulate tumor behavior (Dunn et al. 2002). Cancer immunoediting is delineated as a three-step process: elimination, equilibrium and escape (Dunn et al. 2004). The status of cancer development is susceptible to the profile of immune cells, particularly tumor-infiltrating lymphocytes (TIL). The role of TIL can be either beneficial or harmful (Yu and Fu 2006). For example, effector cells restrain tumor growth, whereas regulatory T (Treg) cells encourage tumor growth. Cytokines and related molecules present in the tumor milieu are key factors in controlling TIL profiles. In this study, we utilized an IL-21-secreting EG7 tumor model to demonstrate the direct effects of IL-21 on host TIL profiles.

Materials and methods

Mice and cell lines

C57BL/6 mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. SCID mice (NOD.CB17-Prkdc^{scid}/Tcu) were bred and all mice were housed at the Laboratory Animal Center of the National Health Research Institutes. All animal studies were approved by the Animal Committee of the National Health Research Institutes and performed according to their guidelines. The cell lines used in the study include EG7 (American Type Culture Collection, CRL-2113) and IL-21-expressing EG7 clones.

In vitro tumor cell growth assay

Cells were cultured at a density of 5×10^4 cells/2 mL/well for EG7-Vec, EG7-IL-21L (low expression), and EG7-IL-21H (high expression) in 24-well plates. Cells were counted from one well every day to establish cell growth curves. The population doubling time (DT) was calculated using the following formula: $DT = (t - t_0)\log 2/\log(C/C_0)$, where t and t_0 are times of cell counting and C and C_0 are cell counts at t and t_0, respectively.

Animal models

C57BL/6 or SCID mice received subcutaneous injections of 5×10^5 tumor cells in 0.2 mL of PBS in the left flank. The presence or absence of tumors was assessed by visual inspection and palpation. Tumor size was measured three times a week with a caliper and mice were sacrificed when tumor volume reached 3000 mm^3. Tumor volume was estimated by the formula $V = \text{width} \times \text{length} \times (\text{width} + \text{length})/2$.

Quantification of IL-21 in culture media

EG7-Vec, EG7-IL-21L, or EG7-IL-21H cells were cultured at 2.5×10^6/0.5 mL/well in 48-well plates. Culture supernatants were collected after 4 h and stored at −80 °C until use. Serial dilutions of samples were tested for IL-21 levels using a DuoSet ELISA kit (R&D Systems, Inc., Minneapolis, MN). All assays were performed according to the manufacturer's protocols.

Cytotoxicity assay

Standard ^{51}Cr release assays were used. Splenocytes (2.5×10^6 cells/well) were cultured with 10,000-rad-irradiated EG7 cells (0.5×10^6 cells/well) for 5 days in 24-well plates and were used as effector cells. Cultures were supplemented with 20 U/mL rIL-2. Parental EG7 cells were labeled with $Na_2[^{51}Cr]O_4$ at 37 °C for 1 h and used as target cells after washing. Target cells (5×10^3 cells/well) were cultured with effector cells at different effector/target ratios in 96-well round-bottom plates. After a 5-h incubation at 37 °C, the amount of released [^{51}Cr] in supernatants was counted and the percentage of specific target cell killing was calculated according to the formula [(sample release − spontaneous release)/(total release − spontaneous release)] × 100.

Preparation of tumor single cell suspension

Resect the tumor and cut it into small pieces of approximately 5 mm. Transfer the tumor pieces into the 70-μm cell strainer. Gently triturate and strain cells just before use.

Tumor-infiltrating lymphocyte profiles

Tumor nodules were harvested and prepared as single cell suspensions at days 6–8 or days 10–12 after injection of tumor cells. Nonspecific staining was blocked by incubation with anti-CD16/CD32 antibody (BD Biosciences) in PBS for 10 min at 4 °C. Cells were stained with PE-conjugated anti-NK1.1 antibody (BD Biosciences) and APC-conjugated anti-CD3ε antibody. NK1.1$^+$/CD3$^-$ cells were considered to be NK cells. For intracellular FoxP3 staining, cells were first surface-stained with FITC-conjugated anti-CD4 antibody and APC-conjugated anti-CD25 antibody. After washing, cells were treated with fixation and permeabilization buffer (eBiosciences, San Diego, CA) according to the manufacturer's directions. FoxP3-positive cells were stained with PE-conjugated anti-FoxP3 antibody. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (1 μg/mL), and Brefeldin A (3 μg/mL) at 37 °C in 5% CO_2 for 4 h. Cells were surface stained with PECy5-conjugated anti-CD4 antibody and APC-conjugated anti-CD8 antibody. After washing, cells were treated with fixation and permeabilization buffer (eBiosciences, San Diego, CA) according to the manufacturer's directions. IFN-γ-positive cells were stained with FITC-conjugated anti-IFN-γ antibody. Isotype-matched antibodies conjugated with FITC or PE were used as negative controls in all intracellular staining. All antibodies were purchased from eBiosciences unless otherwise noted. Stained cells were analyzed on a FACSCalibur machine (BD Biosciences) and data analysis was performed with CELLQuest software (BD Biosciences).

Statistical analyses

Statistical analyses were carried using GraphPad Prism version 5.02 (GrpahPad Software, Inc.). Statistical significance of differences between groups was assessed using a one-tailed Student's *t*-test. The log-rank test was used in survival analyses. Differences with a P value of less than 0.05 were considered statistically significant.

Results

Generation and characterization of IL-21-expressing EG7 clones

Plasmids encoding murine IL-21 (pcDNA4-IL-21) were constructed and introduced into EG7 cells by electroporation. A total of 38 successfully transfected clones were obtained with a variety of IL-21 expression levels (data not shown). Two representative clones with low and high IL-21 expression (EG7-IL-21L and EG7-IL-21H, respectively) were selected. They had virtually iden-