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Interleukin-21 restrains tumor growth and induces a substantial increase in the number of circulating tumor-specific T cells in a murine model of malignant melanoma

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ABSTRACT

New strategies of immunotherapy are currently being evaluated, and the combination of chemo- and immunotherapy has shown promising results. The cytokine interleukin-21 (IL-21) is known to enhance immune function, and in this study we have investigated its ability to boost the efficacy of chemoimmunotherapy—cyclophosphamide and adoptive cell transfer (ACT)—in the B16-OVA/OT-I murine model of malignant melanoma. Subcutaneous B16-OVA tumors were established in C57BL/6J mice 8 days before adoptive transfer of tumor-specific OT-I T cells. In addition to cyclophosphamide and ACT, one group of mice received daily injections of murine IL-21 (mIL-21). Mice treated with mIL-21 had more tumor-specific T cells in the circulation 4 and 7 days following ACT ($P = 0.004$ and $P = 0.002$, respectively). Importantly, mIL-21 and ACT controlled tumor growth instantly and more effectively than ACT alone ($P = 0.001$, day 4)—an effect that persisted up to 5 days after the last mIL-21 injection. We conclude that mIL-21 enhances chemoimmunotherapy: it amplifies the number of tumor-specific T cells in the circulation and also stunts early tumor growth.

1. Introduction

Successful adoptive immunotherapy of tumors is dependent on T cell specificity towards the tumor and the ability of transferred cells to survive and proliferate following transfer. Adoptive cell transfer (ACT) in combination with systemic chemotherapy has recently shown promising results in malignant melanoma [1–3]. The use of low doses of cyclophosphamide, a chemotherapeutic, has demonstrated immunomodulatory properties such as (1) creating a niche in the immune system, giving rise to enhanced homeostatic expansion of antigen-specific T cells [4,5], (2) stimulating the innate immune system [6], (3) inducing type I interferons [7], and (4) increasing the probability of tumor reactivity by decreasing the proportion and inhibitory function of $CD4^+CD25^+$ regulatory T cells [6,8,9]. The synergistic effect of chemotherapy in combination with ACT is, however, still not always powerful enough to ensure complete tumor elimination.

Dudley and colleagues obtained promising results when they attempted to enhance the efficacy of chemoimmunotherapy in patients by the exogenous administration of IL-2 [10,11]. IL-2, however, can lead to activation-induced cell death and is known to support development of suppressive regulatory T cells [12,13]. Therefore, other cytokines given to support the adoptively transferred cells are also of potential interest, including IL-7, IL-12, IL-15, and IL-21 [3].

IL-21 is the most recently identified member in the family of cytokines that share the common γ-chain receptor [14]. This cytokine is primarily produced by activated $CD4^+$ T cells and natural killer T (NKT) cells [15,16] and can regulate T, B, and NK cell proliferation, survival and differentiation as well as effector function. Moreover, unlike IL-2, IL-21 does not support proliferation of activated regulatory T cells nor activation-induced cell death. Due to direct anti-tumor activity as well as immunomodulating effects, IL-21 has recently attracted attention in cancer immunotherapy [17–20].

In this study, we have investigated the ability of mIL-21 to enhance the efficacy of a regimen of chemoimmunotherapy consisting of cyclophosphamide and ACT in the murine B16-OVA/OT-I tumor model system. We established subcutaneous tumors of the transgenic, ovalbumin-expressing murine melanoma cell line B16-OVA in C57BL/6J mice. Eight days later, we adoptively

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transferred 15–20 $\times$ 10^6 OT-I spleen cells stimulated for 72 h with the ovalbumin-derived peptide SIINFEKL ($OVA_{257-264}$). Our OT-I mouse is homozygous for Thy1.1 and transgenic for a Vα2, Vβ5 T cell receptor, recognizing SIINFEKL in association with H-$2K^b$. Mice were sacrificed 1, 4, or 7 days following ACT and tumor, spleen, and peripheral blood samples were evaluated by multiparameter flow cytometry and immunohistochemistry.

2. Materials and methods

2.1. Animals

Female C57BL/6JBomTac mice, 8–10 weeks old (Taconic M&B, Ry, Denmark), female C57BL/6JOla-Hsd, 9–10 weeks old (Harlan Laboratories, Horst, The Netherlands), and 9–19 weeks old female hemizygous OT-I and homozygous Thy1.1 mice (B6.PL-Thy1^a/CyJ Tg(TcraTcrb) 1100Mjb, in-house breeding program and Charles River, Sulzfeld, Germany) were housed under standard conditions at the animal facility, the Faculty of Health Sciences, University of Aarhus. For survival studies mice were killed before the tumor volume reached 1 cm^3 according to ethical standards. The Danish Experimental Animal Inspectorate approved all animal experiments.

2.2. Cell cultures

The B16-OVA cell line [21] was cultured in UltraCulture (BioWhittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/ml glutamine (Gibco, Life Technologies, Paisley, Scotland) and 0.5 mg/ml G418 (Calbiochem, San Diego, CA). The cells were maintained at 37 °C in a 5% CO_2 atmosphere.

OT-I cell cultures were generated from single-cell suspensions of spleens from OT-I mice prepared by homogenization in Hanks' Balanced Salts Solution (HBSS, Gibco), followed by lysis of erythrocytes with ammonium chloride. The splenocytes were cultured at 0.5 $\times$ 10^6 cells/ml in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/ml glutamine (Gibco), 1% non-essential amino acids (Gibco) and 50 μM ß-mercaptoethanol (Sigma, St. Louis, MO). Splenocytes were stimulated with 0.2 μg/ml SIINFEKL peptide (Inbios, Naples, Italy), and cultured for 72 h at 37 °C, 5% CO_2. At the end of culture, the cells had divided between 5 and 6 times (measured by CFSE staining) and had a viability between 84% and 94% (measured by trypan blue exclusion).

2.3. Subcutaneous tumors

B16-OVA cells were harvested using trypsin–EDTA (Gibco), centrifuged, and washed once in UltraCulture. The cells were adjusted to 10 $\times$ 10^6 cells/ml in UltraCulture, and 50 μl of this suspension (5 $\times$ 10^5 cells) were injected subcutaneously into the right side of the lower back of C57BL/6 J mice, anesthetized with isoflurane (Abbott, Scandinavia AB, Solna, Sweden).

At the time of adoptive transfer (8 days post injection of B16-OVA cells), mice were inspected for tumor development, and the tumor size was determined using a digital caliper. Assuming an ellipsoid tumor shape, the volume was calculated as length $\times$ width^2 $\times$ $\pi/6$. Mice with established medium-large sized tumors were selected as recipients (mean volume = 92 mm^3, range 32–163 mm^3, n = 104).

2.4. Injection of ovalbumin-specific T lymphocytes

OT-I cells cultured for 72 h in the presence of the SIINFEKL peptide were washed twice and resuspended in HBSS. Between 15 and 20 million cells in 300 μl HBSS were injected intravenously into mice bearing 8-day old B16-OVA tumors through the tail vein using a 23G or 27G syringe (Terumo, Leuven, Belgium). The number of infused cells was determined based on titration experiments (data not shown).

2.5. Injection of cyclophosphamide and/or mIL-21

Mice were injected intraperitoneally with 100 mg/kg cyclophosphamide (Baxter Oncology GmbH, Halle/Westphalia, Germany) suspended in 300 μl isotonic saline 2 h before adoptive transfer of OT-I cells using a 23G syringe (Terumo), assuming an equal weight of 20 g for all mice. Control mice were given mock injections of 300 μl isotonic saline.

Mice receiving mIL-21 immunotherapy were injected daily intraperitoneally until organ harvest with 50 μg recombinant mIL-21 diluted in 200 μl PBS using a 27G or 30G syringe (Terumo). The 50 μg dose was chosen on the basis of dose–titration experiments by Søndergaard et al. [19]. Control mice were given mock injections of 200 μl PBS. mIL-21 was kindly provided by Kresten Skak, Novo Nordisk A/S, Bagsvaerd, Denmark.

2.6. Organ processing for flow cytometric analysis

Before organ extraction and at the time of analysis, blood was obtained from the orbital plexus by a skilled lab technician and then stabilized with EDTA. Subsequently the mice were sacrificed, tumors and spleens were extirpated and weighed. Spleens were mechanically homogenized to prepare a single cell suspension, while tumors were cut into small pieces and transferred to a 15 ml tube containing 9 ml RPMI (Gibco) supplemented with 175 U/ml collagenase IA (Sigma). Tubes were placed in a rotating rack at 37 °C. After 30, 40, 50 and 60 min the tumor suspension was gently mixed by pipetting. Following a total incubation time of 70 min the tumor cell suspension was centrifuged, washed once in PBS/BSA/Az (PBS, pH 7.4, with 0.5% BSA and 0.09% NaN_3) and subsequently resuspended in the same buffer.

2.7. Flow cytometry

Known fractions of the blood, tumor- and spleen cell suspensions were stained with PE-Cy7-conjugated anti-CD45 (eBioscience, San Diego, CA), APC-conjugated anti-CD8a, PE-conjugated anti-CD4, and FITC-conjugated anti-Thy1.1 (BD Pharmingen, Franklin Lakes, NJ) for 15 min at room temperature in the dark. In addition, some samples were stained with PE-conjugated anti-CD107a (eBioscience). Cells were washed in PBS/BSA/Az and resuspended in PBS (pH 7.4) with 0.9% formaldehyde. Immediately before analysis, CytoCount beads (DAKO, Glostrup, Denmark) were added to each sample to allow subsequent enumeration of selected cell populations.

To determine the purity of the cells to be injected as well as expression of the IL-21 receptor, OT-I cells at baseline and after 72 h stimulation with SIINFEKL peptide, were stained with FITC-conjugated anti-CD8 (own production), and either PE-conjugated SIINFEKL tetramer (Immunomics) or anti-mIL-21R (eBioscience) or rat IgG2a (BD Pharmingen), and PerCP-conjugated anti-Thy1.1 (BD Pharmingen), for 30 min at room temperature in the dark. The cells were washed twice in PBS/BSA/Az before analysis.

Samples were analyzed the same day on a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA), collecting up to 80,000 crude-gated lymphocytes with a maximum run-time of 5 min per sample. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).