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Original article

CD137 enhances cytotoxicity of $CD3^+CD56^+$ cells and their capacities to induce $CD4^+$ Th1 responses☆

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Abstract

CD137 (4-1BB) is a TNFR superfamily member that mediates the costimulatory signal resulting in T cells and NK cells proliferation and cytokines production, but the effects of CD137 signaling on $CD3^+CD56^+$ cell subpopulation have not been well-documented. The aim of this study was to investigate the effects of CD137 signaling on regulation of $CD3^+CD56^+$ cell function. Anti-CD137 mAb or mouse IgG1 isotype control was added to CIK cell culture to determine the effects of proliferation and anti-tumor effects on $CD3^+CD56^+$ cells. We observed that anti-CD137 mAb could dramatically promote proliferation of CIK cells. And CD137−CIK cells and $CD3^+CD56^+$ cell subpopulation within them possessed higher ability to kill tumor cell line A549. The SCID mice engrafted with A549 cells and treated with CD137−CIK cells have prolonged survival. Further studies revealed that the percentages of $CD3^+CD56^+$ cells were elevated significantly in CD137−CIK cells. The expression of NKG2D was up-regulated on $CD3^+CD56^+$ cells from CD137−CIK cells. The expression of IFN-γ, IL-2 and TNF-α increased significantly whereas the production of TGF-β_1, IL-4 and IL-10 decreased in $CD3^+CD56^+$ cells from CD137−CIK cells. In addition, anti-CD137 mAb can elevate the capacity of $CD3^+CD56^+$ cells to induce $CD4^+$ Th1 responses. We further showed that the anti-CD137 mAb also had the same effects on $CD3^+CD56^+$ cells expanded from the PBMCs of patients with NSCLC. We concluded that CD137 signaling could enhance the abilities of CIK cells to kill tumor cells in vitro and in vivo via increasing the proportion of $CD3^+CD56^+$ cells and their cytotoxicity. Furthermore, CD137 signaling can elevate the capacity of $CD3^+CD56^+$ cells to induce $CD4^+$ Th1 responses which may enhance their anti-tumor activity indirectly. Taken together, our studies could be considered as valuable in CIK cells-based cancer immunotherapy.

Keywords: CD137; CIK cells; $CD3^+CD56^+$ cells

1. Introduction

Cytokine-induced killer cells (CIK cells) are non-major histocompatibility complex (MHC)-restricted cytotoxic cells generated by incubation of PBMCs with anti-CD3 mAb, IL-2 and IFN-γ [1,2]. It has been also generally accepted that adoptive immunotherapy of CIK cells holds promise for improving the outcome of solid tumors [3]. The greatest lytic activity in CIK cells cultures is found in the population of $CD3^+CD56^+$ cells. This population was initially described in 1986 by Ritz [4] and Lanier [5] and further characterized by Ortaldo [6] who showed that $CD3^+CD56^+$ cells are capable of lysing a broad array of tumor cell targets in a non-MHC-restricted manner. These studies lead to the suggestion that increasing anti-tumor effects of subpopulation of $CD3^+CD56^+$ cells may be an efficient approach to enhance the anti-tumor capacity of CIK cells.

CD137 (4-1BB) is a TNFR superfamily member that is expressed by activated natural killer (NK) cells, T cells and dendritic cells (DCs) [7,8]. The natural ligand for CD137 (CD137L) is found on activated B cells, macrophages and DCs. CD137L binding to CD137 mediates the costimulatory signal that results in T cells and NK cells proliferation and

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cytokines production. In addition, CD137 signaling can protect T cells from activation-induced cell death [9,10]. However, the effect of CD137 signaling on $CD3^+CD56^+$ cell subpopulation is still not clear. In this paper, we showed that CD137 signaling mediated by anti-CD137 mAb could enhance anti-tumor activity of $CD3^+CD56^+$ cells within CIK cells from healthy donor and patients with NSCLC. We further investigated the mechanism by which CD137 signaling regulated the function of $CD3^+CD56^+$ cells.

2. Materials and methods

2.1. Generation of CIK cells

Human PBMCs were obtained from healthy donors (from 20 to 40 years old) or NSCLC patients (Cancer Center, the First Affiliated Hospital, Nanjing Medical University, Nanjing, PR China) (Table 1) by Ficoll–Hypaque density centrifugation and washed thrice with PBS. The non-adherent population of PBMCs was resuspended at the concentration of 10^6/ml in complete medium containing RPMI 1640 (American, GIBCO), 10% fetal calf serum (FCS) (American, GIBCO), 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C, 5% CO_2. To generate CIK cells, human recombinant IFN-γ (American, Stem Cell Technologies) was added to a final concentration of 1000 U/ml on day 0. After 24 h of incubation, IL-2 and anti-CD3 mAb (American, BD Bioscience), respectively, at a final concentration of 1000 U/ml and 50 ng/ml were added. At the same time, 2 μg/ml of anti-CD137 mAb (4B4-1, American, BD Bioscience) or mouse IgG1 isotype control was, respectively, added to culture medium. Fresh medium containing 1000 U/ml of IL-2 and 2 μg/ml of anti-CD137 mAb or mouse IgG1 isotype control was added every other day.

Table 1
Clinical details of the patients included in this study.

NSCLC patient[a]	Admission number	Age	Gender[b]	Previous treatment
1	0526077	74	M	Operation
2	0531902	75	M	No
3	0529023	68	M	No
4	0526074	61	M	Operation
5	0530408	37	M	Operation
6	0531017	49	F	No
7	0527713	75	M	Operation
8	0527667	67	F	No
9	0532560	60	M	Operation
10	0535586	50	M	Operation
11	0544286	39	M	Operation
12	0528531	59	F	Operation
13	0528072	67	F	No
14	0545100	71	M	Operation
15	0509043	51	F	No
16	0540058	65	F	Operation
17	0551704	54	F	Operation
18	0543428	65	M	Operation
19	0509186	55	F	No
20	0536207	76	M	Operation

[a] Patients weren't given any radio-chemotherapy and were diagnosed in pathology.
[b] Male was represented by M; female was represented by F.

2.2. Cell lines

The human lung adenocarcinoma cell line A549 was purchased from American Type Culture Collection (CCL no. 185) and maintained in HEPES-buffered RPMI 1640 with 10% FCS and antibiotics. A549 cells were inoculated into 25 cm^2 culture flasks (American, Corning Costar). The medium was replaced every other day and cells were passaged when they reached 80% confluence.

2.3. Proliferation assay

On alternate days, the proliferation of the CIK cells was assayed by cell counting with trypan blue exclusion test.

2.4. Cytotoxicity assay

The cytotoxicity was estimated by quantification of LDH activity in the culture medium [11]. Cell sorting was performed with FACSVantage SE (Becton Dickinson) to collect double-positive cells ($CD3^+CD56^+$ cells). Cytotoxicity assays were carried out in round-bottomed 96-well plates with a final sample volume of 100 μl/well. Tumor cells (A549, 2×10^5/ml cells) in 50 μl/well were co-cultured with effector cells (CIK cells or $CD3^+CD56^+$ cells) at various effector-to-target ratios (20:1, 10:1 and 5:1). All samples were run in triplicate. The LDH activity was quantified by measuring the absorbance at 490 nm with spectrophotometer (Switzerland, Tecan Sunrise). Use the following formula to compute percent cytotoxicity for each effector:target ratio:

$$\%\text{Cytotoxicity} = \frac{\left(\text{LDH}_{\text{experimental}} - \text{LDH}_{\text{effectorcells}} - \text{LDH}_{\text{spontaneous}}\right)}{\left(\text{LDH}_{\text{maximal}} - \text{LDH}_{\text{spontaneous}}\right) \times 100\%}$$

$LDH_{experimental}$: release resulting from the co-culturing of effector cells and target cells; $LDH_{effector\ cells}$: release resulting from the culturing effector cells separately; $LDH_{spontaneous}$: release resulting from the culturing A549 cells (low control); and $LDH_{maximal}$: add 10 μl of lysis solution (10×) to 100 μl/well of target cells (high control).

2.5. Hu–CIK–SCID therapeutic trial

On day 21, CIK cells were collected and resuspended in PBS at the concentration of 2×10^7/ml. Recipient CB.17 severe combined immunodeficient (SCID) male mice, which were 7–10-week-old, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (China). A total of 30 mice were injected intraperitoneally with A549 cells (1×10^6/ml, 0.5 ml/injection) on day 0 and treated on day 1 with CD137–CIK cells or control IgG1–CIK cells (2×10^7/ml, 0.5 ml/injection, E/T = 20:1) or PBS (0.5 ml/injection). There were 10 mice in