Anti-CD137 Monoclonal Antibody Promotes the Direct Anti-Tumor Effect Mediated by Peripheral Blood-Derived Human Dendritic Cells *in vitro*

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CD137, a costimulatory factor of TNFR family, is expressed on activated T cells and freshly isolated mouse dendritic cells (DCs). To date, there are only limited data dealing with the expression and the effect of CD137 on human DCs. We report in this work that CD137 can coexpress with CD137L on immature peripheral blood-derived human DCs (9.77%). The mature DCs stimulated by LPS showed a much higher level of CD137 expression (36.06%). Ligation of CD137 on the surface of DCs with anti-CD137 monoclonal antibody (mAb) could enhance the direct anti-tumor effect mediated by human DCs *in vitro.* The agonistic anti-CD137 mAb was able to elevate by about 20% of the DC-mediated inhibition of tumor growth in five tumor cell lines. These results indicate that the appliance of anti-CD137 mAb might be used as a new strategy for tumor immunotherapy. *Cellular & Molecular Immunology*. 2004;1(1):71-76.

Key Words: dendritic cell, tumor, costimulatory molecule, CD137

Introduction

CD137 is a member of tumor necrosis factor receptor (TNFR) superfamily, which is expressed on activated T lymphocytes (1, 2). The ligand for CD137 (CD137L) is detected on antigen presenting cells (APCs), such as dentritic cells (DCs), activated macrophages and B cells (3-5). Studies *in vitro* have demonstrated that agonistic mAb against CD137 and its ligand costimulate proliferation and cytokine secretion in both $CD4^+$ and $CD8^+$ T cells (6-9). However, studies performed *in vivo* suggest that CD137 plays a more prominent role in the generation of CTL response than that of Th cell response (10-14). The systemic administration of mAbs to CD137 or gene transfer of CD137L into tumor cells induces potent cell-mediated immune responses against tumors (15-18). Injection of anti-CD137 mAb in tumor-bearing mice leads to regression of well-established tumors in various mouse models (15, 16, 19, 20). Provision of antigen and CD137 signaling can break immunological ignorance, promoting regression of poorly immunogenic tumors (21). CD137 also regulates graft-versus-host disease, graft-versus-leukemia, and graft rejection in allogeneic bone marrow transplant recipients (22-24). However, the administration of an agonistic anti-CD137 antibody dramatically reduces the incidence and severity of experimental autoimmune encephalomyelitis (EAE) or systemic lupus erythematosus (SLE) (25, 26). Thus, the accumulating reports suggest a crucial role for CD137 costimulation in T cell or B cell responses.

The recent observations have demonstrated that CD137 could coexpress with CD137L on antigen presenting cells, such as splenic DCs and bone marrow- derived DCs of mice, follicular DCs of human (27-30). Whether peripheral blood-derived human DCs express CD137 is unknown.

DCs, as a kind of professional APCs, are widely used in the experimental immunotherapy of cancer (31-33). Although in these approaches DCs mediate their anti-tumor effect by stimulating tumor-specific T lymphocytes, recent observations suggest that DCs themselves have effector cell functions in anti-tumor activity (34, 35). Thus, DCs have been shown to inhibit growth and to induce apoptosis of tumor cell lines *in vitro* (36).

In the present research, we report that the peripheral blood-derived human DCs can express CD137. More importantly, the agonistic anti-CD137 mAb can enhance the direct anti-tumor effect mediated by human DCs.

Materials and Methods

Reagents

The recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF, $\geq 1\times10^7$units/mg), Interleukin 4 (IL-4, $\geq 5\times10^6$units/mg) were purchased from Peprotech

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Abbreviation: DC, dendritic cell; GM-CSF, granulocyte-macrophage colony stimulating factor; IL-4, interleukin 4; LPS, lipid polysaccride; TNFR, tumor necrosis factor receptor.

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Company (USA). The FITC-labeled anti-HLA-DR and PE-labeled anti-CD137, anti-CD137L, anti-CD14 were purchased from BD PharMingen. FITC-labeled and PE-labeled mouse IgG isotype control Abs were also purchased from BD PharMingen. MTT, LPS, DMSO were obtained from Sigma (St. Louis, MO).

Cell lines

HepG2 (human hepatocellular carcinoma), HepG2.2.15 (human hepatocellular carcinoma containing integrated HBV DNA), HT29 (human colon adenocarcinoma) were purchased from Shandong Academy of Medical Sciences (Jinan, Shandong, China) and stored in our laboratory. Lovo (human colon adenocarcinoma), Hela (human cervical carcinoma) were originally purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

Generation of peripheral blood-derived human DCs

DCs were generated from human peripheral blood monocytes as described previously with minor modifications. Briefly, PBMCs were obtained from buffy coats from heparinized whole blood of healthy donors (Blood Center of Shandong, Jinan, Shandong, China) by density gradient centrifugation. These cells were resuspended in RPMI1640 and 1×10^7 cells in 3ml medium were added to each well of six-well plates (Costar, Cambridge, MA), then allowed to adhere for 2h at 37°C, in 5%CO_2. Non-adherent cells were gently washed out with warm medium (serum-free RPMI1640) at 37°C. The resulting adherent cells were cultured in medium supplemented with GM-CSF (1000U/ml) and IL-4 (500U/ml) in 5%CO_2, at 37°C. Every 2 days, half of the medium was replaced by fresh medium containing double concentration of GM-CSF and IL-4 as indicated above. Cell suspensions were collected for analysis of surface phenotype at different stages of development. After 5 days of culture, DCs were harvested for subsequent experiments.

Flow cytometry

Phenotypic analysis was performed by flow cytometry. DCs (5×10^5/ml) were washed and resuspended in cold PBS containing 0.1% sodium azide (Sigma). Subsequently, they were incubated with FITC-labeled or PE-labeled mAbs specific for human CD14, HLA-DR, CD137, CD137L or isotype-matched controls for 30 min at 4°C in PBS. After 2 washes, stained cells were analyzed by FACS Caliber flow cytometry (Becton Dickinson, Mountain View, CA) and CellQuest analysis software (Becton Dickinson).

Determination of effector (E) to Target (T) ratio

Human immature DCs induced by rhGM-CSF and rhIL-4 were harvested and then diluted into four different concentrations: 1.25×10^4, 2.5×10^4, 5×10^4 and 1×10^5 DCs in 200µl medium, were added into each well of 96-well plates, respectively and were cultured in the presence of 5µg/ml LPS, 24 hours later, 100µl medium was withdrawn and 1×10^4 tumor cells in 100µl medium were added. There were three groups: group1 (DCs alone), group2 (tumor cells alone), group3 (DCs plus tumor cells). Each group was performed in triplicate. After culture at 37°C for 24h, 20µl MTT (5mg/ml) was added and the DCs were cultured for another 4h. Plates were centrifuged at 1000rpm for 5 min, and then supernatants were gently removed. 100µl of 100%DMSO was added to dissolve formazan. The OD of each well was read using a microplate reader (model550, Bio-Rad, Hercules, CA) at 570nm. The percentage of growth inhibition of tumor cells was calculated as following: growth-inhibition (%)=$[1-(OD_{E+T}-OD_E)/OD_T]\times100\%$, OD_{E+T} represented the OD mean value of group3 (DCs plus tumor cells), OD_E represented the OD mean value of group1(DCs alone), OD_T represented the OD mean value of group2 (tumor cells alone). Data were expressed as the mean ± SD of triplicate wells.

Growth-inhibition of various tumor cell lines mediated by human DCs

Immature DCs (2.5×10^4) in 200µl medium were cultured for 24h in 96-well plates in the presence of LPS (5µg/ml), and supernatant was harvested as DCs-cultured supernatant control. According to 5:1 of E to T ratio, 1×10^4 cells tumor cells (HT29, Hela, Lovo, HepG2 and HepG2.2.15) in 100µl medium were added to each well above, respectively. The growth-inhibition of tumor cells mediated by DCs and that of the DC-cultured supernatant were detected by MTT method as above.

Impact of anti-CD137 mAb on the direct inhibition effect mediated by human DCs on tumor cells

Human immature DCs (2.5×10^4) in 200µl medium were cultured in 96-well plates in the presence of LPS (5µg/ml). 24 hours later, 100µl medium was withdrawn, 1×10^4 tumor cells or 1×10^4 tumor cells with anti-CD137 mAb (5µg/ml) were added to above wells. The growth-inhibition effect of tumor cells was detected by MTT method as above.

Statistics

The Student *t* test was used for analyzing whether the differences between the values of the test groups and those of the relevant controls were significant. $p<0.05$ was regarded as statistical significance. Data were shown as the mean ± SD of triplicate wells.

Results

Induction and identification of peripheral blood-derived human DCs

DCs were generated from adherent human PBMCs of healthy donors in the presence of GM-CSF and IL-4. The majority of cells in the culture at day 5 showed morphology of DC-like cells. Examination of surface markers of these cells by flow cytometry analysis with specific mAbs demonstrated the expression of HLA-DR on 64.01% of these cells, and less than 5% of these cells were stained with anti-CD14, indicating a low content of monocytes.

Expression of CD137 and CD137L on human DCs

The results from flow cytometry showed human immature DCs expressed a moderate level of CD137L (about 41.03%) and a low level of CD137 (about 9.77%). The expression of CD137 increased to 36.06% on mature DCs induced by LPS (Figure 1).

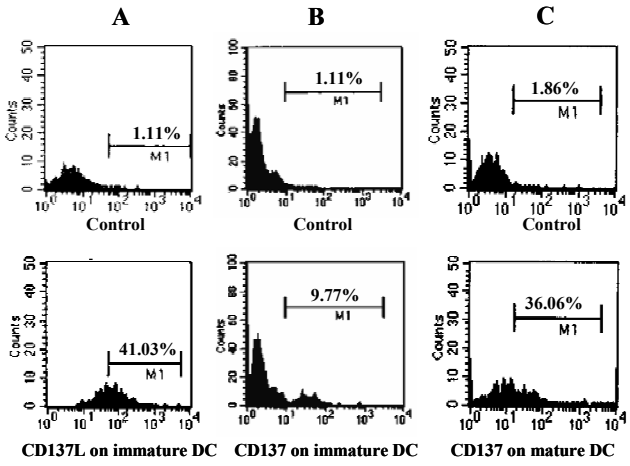

Figure 1. The expression of CD137 and CD137L on DCs derived from human peripheral blood monocytes. Human DCs were induced by IL-4 and GM-CSF. DCs (5×10^5) were resuspended in cold PBS containing 0.1% sodium azide, and then they were incubated with 1μg PE-labeled mAbs specific for human CD137, CD137L or isotype-matched controls for 30 min at 4°C in PBS. After 2 washes, stained cells were analyzed by FACS Caliber Flow Cytometry. 41.03% of immature DCs expressed CD137L (A) and 9.77% expressed CD137 (B). The expression of CD137 increased to 36.06% on mature DCs (C).

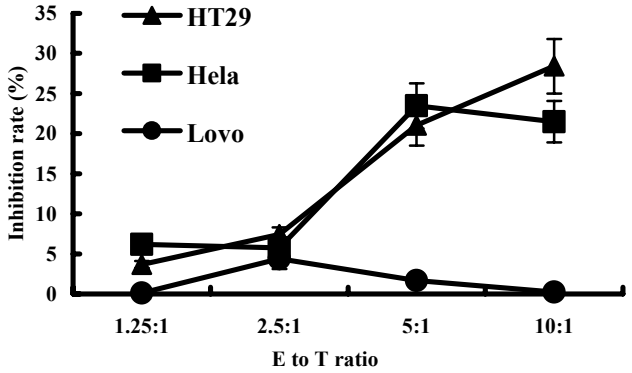

Figure 2. The inhibition of tumor cells mediated by human DCs at various E to T ratio. DCs (1.25×10^4, 2.5×10^4, 5×10^4, 1×10^5) in 200μl medium were added into each well of 96-well plates, respectively and were cultured in the presence of 5μg/ml LPS, At 24 hours, 100μl medium was withdrawn and 1×10^4 tumor cells in 100μl medium waere added. After cultured for 24h at 37°C, 20μl MTT (5mg/ml) was added and the DCs were cultured for additional 4h. Plates were centrifuged at 1000rpm for 5 min, and then supernatants were gently removed. 100%DMSO was added to dissolve formazan. The OD value of each well was read using microplate reader at 570 nm. The growth-inhibition rate of tumor cells was calculated. It was showed that human DCs had a significant inhibition effect on tumor cell lines HT29, Hela. The inhibition rate of tumor cells was higher at 5:1 of E to T ratio. DCs had no significant inhibition on tumor cell line Lovo at any E to T ratio.

Direct growth-inhibition effect of human DCs on tumor cell lines

Human immature DCs induced by rhGM-CSF and rhIL-4 were harvested and stimulated by LPS for 24h, then the growth-inhibition effect of DCs on tumor cell lines (HT29, Hela, Lovo) was detected by MTT. It was shown that human DCs had no significant direct inhibition effect on tumor cell line Lovo at any E to T ratio. But DCs had a significant inhibition effect on tumor cell lines HT29, Hela and HepG2.2.15. When E to T ratio was 1.25:1, 2.5:1, 5:1, 10:1, respectively, the growth-inhibition rate of HT29 was 3.66%, 7.42%, 21.04%, 28.41%, respectively, and that of Hela was 6.17%, 5.75%, 23.46%, 21.48% respectively. It demonstrated that the growth-inhibition rate of two tumor cells was higher at 5:1 of E to T ratio (Figure2).

In order to compare the DC-mediated growth-inhibition of various tumor cells (HT29, Hela, Lovo, HepG2 and HepG2.2.15), the growth-inhibition effects were detected at 5:1 of E to T ratio. The results showed that human DCs had a significant inhibition effect on HT29, Hela and HepG2.2.15, the inhibitory rate was 20.16%, 25.44%, 75.41%, respectively. However, DCs had no obvious inhibition effect on Lovo and HepG2 (Figure 3). These results indicated that human DCs could only inhibit the growth of some tumor cells, but their role was various for different tumor cells.

In order to see whether the growth-inhibition effect of human DCs on tumor cells was caused by the direct contact of tumor cells to DCs, or by cytokines secreted by DCs, we explored further the mechanism of the inhibition effect. In the study, tumor cells were cocultured with DCs supernatant, then the inhibition effect was detected. The result showed that DCs supernatant had no obvious inhibition effect on above five tumor cells lines, the inhibition rate was lower than 10% (Figure 3).

Impact of anti-CD137 mAb on the direct inhibition effect on tumor cells mediated by human DCs

Our research demonstrated DCs could directly inhibit the growth of tumor cell lines. The results from flow cytometry showed that human DCs expressed CD137. In order to study whether CD137 on human DCs can affect DC-mediated growth-inhibition of tumor cells, we use agonistic anti-CD137 mAb to trigger off the CD137 on DCs, then to detect change of DC-mediated growth-inhibition of tumor cells. The results showed that administration of agonistic anti-CD137 mAb could significantly promote the direct anti-tumor effect mediated by human DCs alone ($p<0.05$). When DCs were used alone, the growth-inhibition rate of HT29, Hela, HepG2, HepG2.2.15 and Lovo was increased from 20.16%, 25.44%, 75.41%, 0.15%, 1.86%, respectively to 40.31%, 57.07%, 97.49%, 17.81%, 21.6%, respectively when DCs were treated with anti-CD137 mAb. Interestingly, anti-CD137 mAb could also elevate direct anti-tumor effect of DCs on HepG2 and Lovo that were not inhibited by DCs alone. Anti-CD137 mAb elevated by about 20% DC-mediated growth-inhibition rate of all five tumor cells lines studied (Figure 4).

Discussion

It has been demonstrated that CD137 is expressed on activated T lymphocytes. Its ligand is expressed on DCs, activated macrophages, B cells, and activated T cells. CD137 on activated T cell binds to CD137L on APCs to

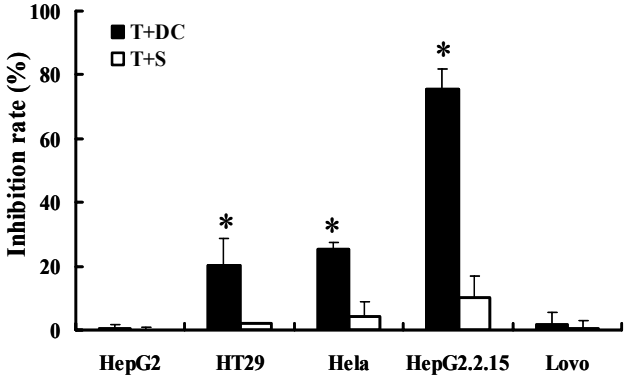

Figure 3. The inhibition of tumor cells mediated by human DCs. 2.5×10^4 immature DCs in 200μl medium were cultured for 24h in 96-well plates in the presence of LPS (5μg/ml), and supernatant was harvested as DC-cultured supernatant control. According to 5:1 of E to T ratio, 1×10^4 cells tumor cells (HT29, Hela, Lovo, HepG2 and HepG2.2.15) in 100μl medium were added to above each well, respectively. The growth-inhibition of tumor cells mediated by DCs and DC-cultured supernatant was detected by MTT method. Figure 3 showed human DCs had a significant direct inhibition effect on HT29, Hela and HepG2.2.15, but no inhibition effect on Lovo and HepG2 (black bars). DC-cultured supernatant (S) had no obvious inhibition effect on above five tumor cell lines (light bars).

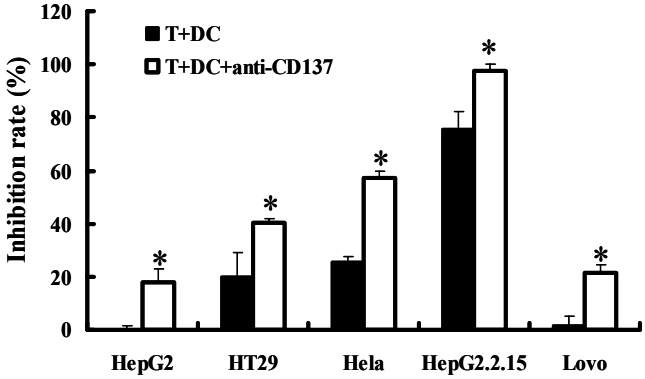

Figure 4. Anti-CD137 mAb enhanced the growth inhibition of tumor cells mediated by human DCs. 2.5×10^4 human immature DCs in 200μl medium were cultured in 96-well plates in the presence of LPS (5μg/ml). 24 hours later, 100μl medium was withdrawn, 1×10^4 tumor cells or 1×10^4 tumor cells with anti-CD137 mAb (5μg/ml) were added to above wells. The growth-inhibition effect of tumor cells was detected by MTT method. The agonistic anti-CD137 mAb could elevate by about 20% inhibition rate of five detected tumor cell lines mediated by DCs.

stimulate T cell proliferation and differentiation. Many reports demonstrate that CD137 has an important role in costimulatory pathways of T cells (37, 38). However, recent reports indicate that CD137 is also expressed on a variety of cell types in addition to activated T cells (39, 40). Wilcox RA reported the mouse splenic DCs and bone marrow-derived DCs expressed CD137 constitutively (27) which was down-regulated by anti-CD40 stimulation. Triggering CD137 increases the secretion of IL-6 and IL-12, indicating that CD137L can directly activate DCs. The results from Pauly S (28) demonstrated human CD137 was expressed on follicular DCs and could costimulate B lymphocyte activation in germinal centers. In this study, it was found that immature DCs from human monocytes of peripheral blood could coexpress CD137 (9.77%) with CD137 ligand (41.03%). The expression of CD137 increased from 9.77% to 36.06% after stimulating DCs by LPS. Triggering off CD137 on the surface of DCs through administration of agonistic anti-CD137 mAb *in vitro* enhanced the direct anti-tumor effect mediated by human DCs, the average inhibition rate increased by about 20%. The results demonstrated CD137 on human DCs from peripheral blood monocytes was functional.

It has been known that injection of anti-CD137 mAb in tumor-bearing mice leads to regression of well-established tumors in various mouse models (15, 16, 19, 20), but its mechanism is unclear. Our results suggested that agonistic anti-CD137 mAb could trigger off CD137 on the surface of DCs and further enhance the direct anti-tumor effect mediated by human DCs. This may be one of mechanisms that anti-CD137 mAb regresses tumor *in vivo*. However, we don't know how anti-CD137 mAb enhances direct anti-tumor effect mediated by DCs. Liu S et al. (35) reported that human $CD14^+$ monocyte-derived DCs expressed TNF-α-related apoptosis-inducing ligand (TRAIL) and exhibited cytotoxicity to some types of tumor cells partially through TRAIL. So we suppose that anti-CD137 mAb may enhance direct anti-tumor effect of DCs by highly expressed TRAIL.

As professional APCs, DCs present antigens to T cells to stimulate T cell activation. Since DCs are widely distributed in most tissues, they are able to participate in the growth inhibition of tumors, especially metastases. DCs pulsed with tumor antigen peptides are widely used in the experimental immunotherapy of cancer. Human DCs pulsed with tumor antigen peptides or mRNA have been performed in a phase I to phase II study of patients with cancer. Although in these approaches DCs mediate their anti-tumor effect by stimulating tumor-specific T lymphocytes, recent observations suggest that DCs themselves have multiple effector cell functions in anti-tumor activity. Our studies demonstrate that agonistic anti-CD137 mAb could enhance the direct anti-tumor effect of DCs. It suggests that the DCs pulsed with tumor antigen peptides and administration of agonistic anti-CD137 mAb will be a new strategy to immunotherapy of tumor.

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