Article

4-1BB (CD137) Ligand Enhanced Anti-Tumor Immune Response against Mouse Forestomach Carcinoma *In Vivo*

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Cancer occurrence and development has been demonstrated to be associated with escape from immune surveillance, and low costimulatory molecules expression has been considered as one of the important reasons for cancer evading the immune system. 4-1BB (CD137) is a costimulatory molecule expressed on the surface of activated T cells. Interaction of 4-1BB with its natural ligand (4-1BBL) expressed on antigen presenting cells (APCs) has been shown to amplify T-cell mediated immunity. We therefore examined whether murine cancer cells expressing 4-1BBL could produce anti-tumor effects in inoculated mice. Mouse forestomach carcinoma (MFC) cells were transfected with 4-1BBL gene (MFC/4-1BBL). The proliferation of the transduced cells in vitro was not different from that of parental cells. However, MFC/4-1BBL cells developed small tumors and induced higher cytotoxicity of tumor infiltration lymphocyte (TIL). Production of cytokines (IFN-γ, TNF-α and IL-2) in serum and cytotoxic T lymphocyte (CTL) activity of splenocytes from mice immunized with mitomycin C (MMC)-treated MFC/4-1BBL cells were significantly higher than that from mice immunized with MMC-treated parental MFC and MFC/pMKITneo cells. These results suggest that modification of cancer cells with 4-1BBL gene can produce anti-tumor immune responses. *Cellular & Molecular Immunology*. 2008;5(5):379-384.

Key Words: 4-1BBL, CTL, anti-tumor, TIL

Introduction

The prognosis of cancer patients is disappointing, although the curative resection and adjuvant chemo-radiotherapy are carried out (1). In order to solve this problem, many biologic therapies have been performed.

It is well known that T cell-mediated response plays a very important role in anti-tumor immunity. An effective T cell response can destroy tumor cells only after T cell receives two key signals from the TCR and co-stimulatory molecules. First, Ag-specific T cells receive a TCR signal after binding of the TCR to antigen-presenting cells (APCs) expressing peptide-bound MHC molecules. These T cells then receive a second set of signals on binding of their co-stimulatory molecules to the corresponding molecular pairs on the APCs, and the T cells become activated. Without co-stimulation, T cells will undergo apoptosis or become anergic (2-6). The fact that tumor cells are found to have low co-stimulatory molecule expression may explain how tumor cells evade the immune system.

4-1BB (CD137) is a member of tumor necrosis factor (TNF) receptor superfamily of type I membrane proteins and has been originally identified as an inducible gene in activated T cells. 4-1BB ligand (4-1BBL) is shown to be a type II surface glycoprotein belonging to the TNF superfamily. Expression of 4-1BBL is restricted to APCs, such as dendritic cells, macrophages, and activated B cells (7-9). Members of the TNF-TNF receptor superfamily have been shown to play critical roles in regulating cellular activation, differentiation and apoptosis. Several *in vitro* studies have reported that 4-1BBL/4-1BB interaction provided a co-stimulatory signal to T cells, and increased T cell proliferation and cytokines production (10-12). However, few relatived investigations of 4-1BBL/4-1BB interaction to T-cell-mediated anti-tumor immunity *in vivo* have been conducted. Therefore we have chosen 4-1BBL as a co-stimulatory molecule in order to reinforce anti-tumor efficacy of effector cells *in vivo*.

Materials and Methods

Cell culture and infection procedure

Mouse forestomach carcinoma (MFC) cell line purchased from Chinese Academy of Medical Science Shanghai was

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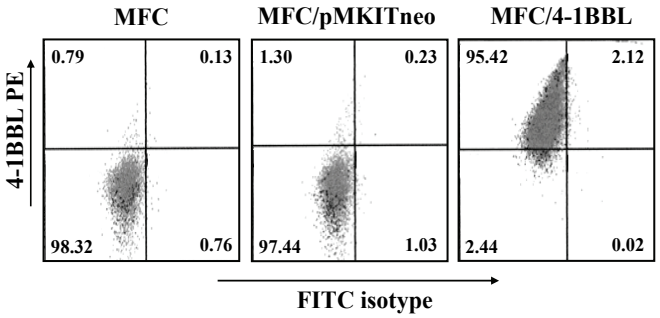

Figure 1. Flow cytometric analysis of the expression of 4-1BBL in parental and transduced cells. Cells (1×10^6) were incubated with PE-anti-mouse 4-1BBL for 30 min, and then detected by flow cytometry and analyzed by Expo32 ADC software. The expression of 4-1BBL in MFC/4-1BBL cells were more than 95%, while little expression in the other cells.

cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/L streptomycin. Expression plasmids pMKITneo and pMKITneo/4-1BBL were kindly provided by Professor H.Y (Juntendo University School of Medical, Tokyo, Japan). Expression plasmids were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer's protocol. In brief, 5 μg of deoxyribonucleic acid and 15 μl of Lipofectamin2000 were diluted in 250 μl Opti-MEM (Invitrogen), and incubated for 5 min at room temperature. Then the diluted DNA was combined with the diluted Lipofectamin2000, and incubated for 20 min. The DNA-Lipofectamine2000 complexes were added to each well containing cells and medium, and incubated at 37°C in a CO_2 incubator for 24 h. The cells were harvested and selective medium containing G418 (1 mg/ml, Invitrogen) was added in the following day. G418 resistant MFC/pMKITneo and MFC/4-1BBL cells were used for experiments.

Flow cytometric analysis

To determine 4-1BBL expression, cells (1×10^6) were incubated with PE-anti-mouse 4-1BBL (Biolegend) for 30 minutes at room temperature and washed by PBS twice. Cells were then filtered through 500 mesh copper screen before analyzed by flow cytometry (Epics-XLII, Beckman Coulter, USA) and PE-rat IgG (Biolegend) was as the isotype control.

Cell proliferation assay in vitro

Target cells were detached with 0.01% EDTA-0.25% trypsin solution. Cells (3×10^4/well) were seeded in 24-well plates and incubated at 37°C in a CO_2 incubator. Proliferation was determined daily in triplicate by hemocytometer and trypan blue exclusion.

Tumorigenicity of cells in vivo

The right dorsal of 6-week-old 615 mice (Chinese Academy of Medical Science Animal Center, Beijing, Animal Admission Number 017) was injected subcutaneously with 1×10^6 parental and transduced cells, respectively. Tumor volume was measured with callipers and was calculated according to the formula: $1/2 \times \text{length} \times \text{width}^2$.

Isolation of TIL (tumor infiltrating lymphocyte) and cytotoxicity assay

Fresh tumor tissues were dissected from mice injected parental and transduced cells. The samples were washed in medium to remove blood and minced completed with ground edged slides, and then the samples were filtered through an 100 μm filter. The cell suspensions were centrifuged with 2 superimposed layers of 100% and 75% Ficoll-Hypaque. The enriched mononuclear cell population fractions were collected at the 75%-to-100% interface. Cells were then incubated at 37°C in presence of rhIL-2 for 9 days. The Cytotoxicity of TIL against parental MFC cells was determined by microculture tetrazolium dye (MTT) assay as previously described (13).

Immunization

Parental and transduced cells were washed twice with PBS and incubated with 100 μg/ml mitomycin C (MMC) at 37°C for 1 h. Cells (5×10^6/mouse) were injected into mice (n = 15) as whole tumor cell vaccines. The same injection was repeated at day 7 and 14. On day 21 after primary injection, production of cytokines in serum and CTL activity of splenocytes were measured from 5 mice of each group. Fatal dose of parental MFC cells (5×10^6/mouse) was injected intraperitoneally into another 10 mice of each group to monitor survival daily.

CTL assay

Spleen cells from immunized mice were restimulated with MMC-treated MFC cells for 5 days in the presence of 50 IU/ml of rIL-2 and served as effector cells. Target cells were co-cultured with effector cells at different E:T ratios in 96 round bottom plates. After a 4-hour incubation at 37°C, the amount of released lactate dehydrogenase was determined by using CytoTox96 (Promega, USA) according to the manufacturer's instructions.

Enzyme linked-immunosorbent assay

The contents of cytokines in serum from immunized mice were measured by ELISA according to the manufacturer's instructions (BD Pharmingen).

Results

Establishment of MFC cells expressing 4-1BBL

MFC cells were transfected with pMKITneo and pMKITneo/4-1BBL, and the G418-resistant cells (MFC/pMKITneo, MFC/4-1BBL) were selected. Flow cytometric analysis showed the expression of 4-1BBL in parental and transduced cells respectively (Figure 1). The proliferation rate of transduced cells *in vitro* was not significantly different from that of parental cells ($p > 0.05$, Figure 2)

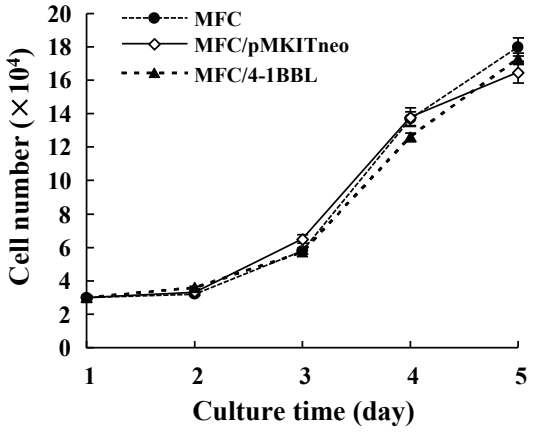

Figure 2. 4-1BBL did not inhibit the proliferation of MFC cells *in vitro*. For cell proliferation assay *in vitro* each cell line (3×10^4 cells/well) was seeded in 24-well plate. Number of cells was determined daily in triplicate by trypan blue staining. No significant differences were found among each groups.

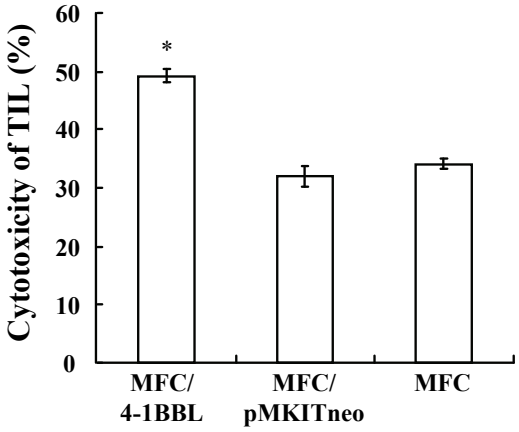

Figure 4. 4-1BBL upregulated the cytotoxicity of TIL. TIL was isolated by discontinuation density gradient centrifugalization and incubated in medium containing recombination IL-2 for 9 days. Parental MFC cells were used as targets for lysis by TIL at an E:T ratio of 40:1. *$p < 0.01$ *vs* MFC or MFC/pMKITneo group.

4-1BBL decreased the tumorigenicity of tumor cells in vivo

To determine the effect of 4-1BBL expression on tumor growth *in vivo*, parental MFC, MFC/pMKITneo and MFC/4-1BBL cells were injected into the right dorsal of 615 mice. Tumor growth in mice injected with MFC/4-1BBL cells was much slower than that in mice injected with parental MFC or MFC/pMKITneo cells. The appearance of tumor node in mice injected with MFC/4-1BBL cells was also obviously later than that in mice injected with parental or MFC/pMKITneo cells (Figure 3).

4-1BBL upregulated cytotoxicity of TIL

To determine the anti-tumor effect of 4-1BBL *in vivo*, cytotoxicity of TIL from mice injected with parental and transduced cells was examined by MTT assay. The cytotoxicity of TIL from mice inoculated with MFC/4-1BBL cells was much higher than that from mice with parental MFC and MFC/pMKITneo cells (Figure 4).

The whole tumor cell vaccine modified with 4-1BBL increased production of cytokines and CTL activity

To determine the immune function of the whole tumor cell vaccine *in vivo*, CTL activity of splenocytes from immunized mice was evaluated. As shown in Figure 5, CTL activity of splenocytes from mice immunized with MMC-treated MFC/4-1BBL cells was dramatically higher than that from

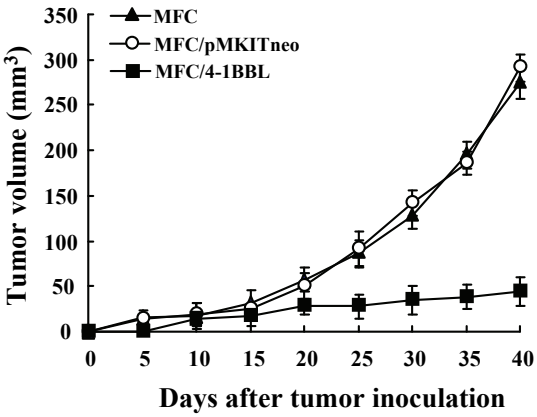

Figure 3. 4-1BBL decreased the tumorigenicity of tumor cells *in vivo*. Mice were injected subcutaneously with parental MFC, MFC/pMKITneo and MFC/4-1BBL cells (1×10^6 cells/mouse) in right dorsal on day 0, respectively. Data represent the mean ± SD of five mice.

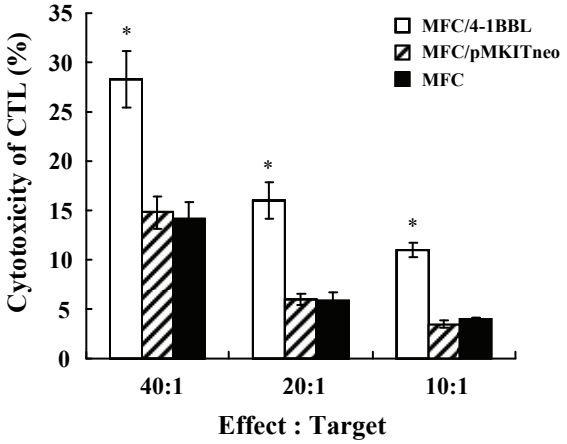

Figure 5. The whole tumor cell vaccine modified with 4-1BBL increased CTL activity. Splenocytes from mice injected with MMC-treated parental and transduced cells were co-cultured with MMC-treated parental MFC cells *in vitro* for 5 days. Cytotoxic activity was assayed using CytoTox 96 nonradioactive cytotoxicity assay. Data represented the mean ± SD of five mice.

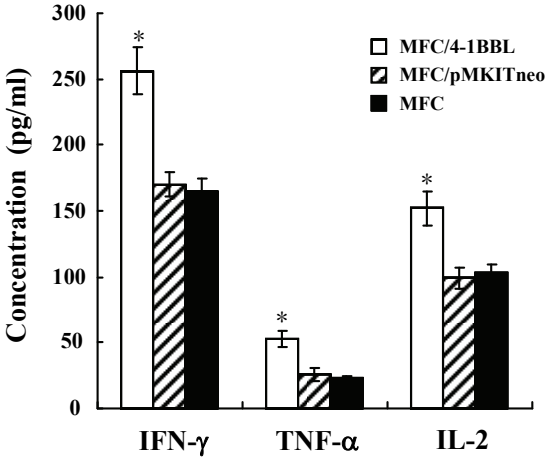

Figure 6. The whole tumor cell vaccine modified with 4-1BBL increased production of cytokines. Production of cytokines in serum from mice injected with MMC-treated parental and transduced cells was detected. The levels of IFN-γ, TNF-α and IL-2 in serum from mice injected with MMC-treated MFC/4-1BBL cells were increased obviously. *$p < 0.01$ compared with that from mice injected with MMC-treated parental MFC and MFC/pMKITneo cells.

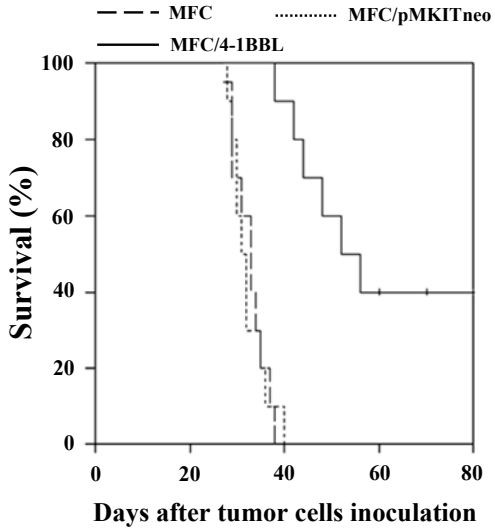

Figure 7. Kaplan-Meier survival curve of mice. According to the log-rank test, there were significant differences among three groups ($p < 0.01$). Compared with those of the other two groups, the survival rate of mice immunized with MMC-treated MFC/4-1BBL cells was significant higher ($p = 0.004$ *vs* MFC group and $p = 0.001$ *vs* MFC/pMKITneo group).

mice injected with MMC-treated parental MFC and MFC/pMKITneo cells. Also, the level of cytokines in serum from mice was examined. The results showed that the contents of cytokines (IFN-γ, TNF-α and IL-2) in serum from mice injected with MMC-treated MFC/4-1BBL cells were much more than that from mice injected with MMC-treated parental MFC and MFC/pMKITneo cells (Figure 6).

The whole tumor cell vaccine modified with 4-1BBL prolonged the life span of mice rechallenged tumors

To determine the immune protection effect of the whole tumor cell vaccine, mice immunized with MMC-treated parental and transduced cells were inoculated intraperitoneally with lethal dose of parental MFC cells to monitor survival daily. The survival rate of mice immunized with MMC-treated MFC/4-1BBL cells was significantly higher than that of mice immunized with MMC-treated parental MFC and MFC/pMKITneo cells (Figure 7).

Discussion

Cancer occurrence and development has been demonstrated to be associated with escape from immune surveillance. Cancer cells may evade the immune system in the following ways: 1) low-level expression of the major histocompatibility complex molecules; 2) poor co-stimulatory molecule expression; 3) absence of recognized tumor Ags; 4) the secretion of immunosuppressive substances such as TGF-β. Therefore, the aim of tumor immunotherapy has been reported to generate long-lasting functionally active $CD8^+$ T cells specific for the cancer cells to overcome the ways in which cancer cells evade the immune system (14-16). Two signals are thought to be the requisite of activating a naïve T cell. One signal occurs through the TCR:MHC:Ag complex while the other is provided through co-stimulation. T cell will be anergic in response to one signal alone if co-stimulation is absent (2, 3, 5). A number of studies have demonstrated effective anti-tumor targeting in clinical trials using the co-stimulatory molecules but to date these have almost utilized the B7:CD28 pathway (17).

4-1BB/4-1BB ligand is another pair of co-stimulatory molecules in addition to B7/CD28. The interaction of 4-1BB/4-1BBL provides a very important co-stimulatory signal independent of CD28, and is becoming the focus in present immune researches (18, 19). In this study, MFC cells were transfected with 4-1BBL gene and cells with high expression of 4-1BBL was established. The proliferation and tumorigenicity assay showed that 4-1BBL did not inhibit cancer cell growth *in vitro*, but suppressed cancer growth *in vivo*. This result suggested that 4-1BBL had no direct toxicity to cancer cells, and its anti-tumor effect *in vivo via* indirect ways. Enhancement of cytotoxicity of TIL might be a way for 4-1BBL to execute anti-tumor effect.

Bertram et al. demonstrated that the T-cell immune responses of $4\text{-}1BB^{-/-}$ mice, as measured by cytokine production and $CD8^+$ T-cell cytotoxic T lymphocyte activity, were diminished (20). It has been confirmed that in conjunction with "signal one", the interaction of 4-1BB/4-1BBL provides co-stimulatory signals to T cells through the activation of NF-κB, c-Jun and p38 downstream pathway (21). Melero reported that the 4-1BB signaling pathway was shown to lead to a preferential stimulation of $CD8^+$ compared

with $CD4^+$ T cells, however, selective depletion of $CD4^+$ T-cell subpopulation *in vivo* with mAb resulted in the loss of the anti-tumor effect (22). In our previous study, we demonstrated that the number of $CD8^+$ and $CD4^+$ T cells in mice immunized with MMC-treated MFC/4-1BBL cells were increased simultaneously (data not shown). This result was similar to Cannons' report that there were no differences between $CD8^+$ and $CD4^+$ T cells in differentiation, maintenance of survival and enhancement of effective activity after 4-1BBL stimulation (21). Our present study showed that CTL activity of splenocytes and cytokine production in serum from mice immunized with MMC-treated MFC/4-1BBL cells were dramatically increased compared with those from mice immunized with MMC-treated parental MFC and MFC/pMKITneo cells. The main effector cells performing CTL activity are $CD8^+$ T cells, while the main cells producing cytokines such as IL-2, IFN-γ and TNF-α are $CD4^+$ Th1 cells. Th1 cells play a critical role in cellular immunity by their cytokines activating $CD8^+$ T cells. In addition, IL-2, IFN-γ and TNF-α themselves possess anti-tumor effect. Long-term survival of mice immunized with MMC-treated MFC/4-1BBL cells when rechallenged lethal dose of parental MFC cells indicated that the whole tumor cell vaccine modified with 4-1BBL excute anti-tumor immune response by activating $CD8^+$ and $CD4^+$ T cells.

In summary, we concluded that the whole tumor cell vaccines modified with 4-1BBL have potential anti-tumor effect. Enhancement of the cytotoxicity of TIL and CTL contributed to 4-1BBL against cancer.

Acknowledgements

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