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Prevention of Lethal Tumor Growth and Generation of Long-lasting Immunity *In Vivo* Using CD137L and Interleukin-12 Gene Transfer

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Abstract. *In a wide range of solid tumors, overexpression of CD137L has been shown to induce tumor immunity partly due to the stimulation of CD8+ CTL, which was even increased when immunotherapy with interleukin-12 (IL12) was additionally employed. However, little in known regarding hematologic neoplasias in this respect. Of the 8 animals receiving IL12-secreting tumor cells, 2 died. Animals treated with CD137L-expressing tumor cells and the combination group, all animals survived. Interestingly, re-challenge with wild-type tumor cells was rejected by all animals in the CD137L group and all remaining animals in the IL12 group, while these in the control group died. IL12- and CD137L-transfected plasmocytoma cells prevented tumor growth and induced long-lasting immunity. Our results warrant follow-up for future clinical use in patients with myeloma.*

Many efforts have been made to induce an antitumor response based on the immune system. *In vitro* and *in vivo*, relevant effector cell activity, *e.g.* CD8+, natural killer (NK) cell or CTL response, has been shown to be crucial. To overcome T-cell anergy or to augment an existing but insufficient immune response, many strategies have been applied: i) direct stimulation of effector cells, ii) up-regulation of co-stimulatory factors, and iii) improvement of antigen presentation (1-3). Interleukin-12 (IL12) and CD137-CD137L interaction are promising ways in inducing an effective antitumor response (4).

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CD137 and its ligand, CD137L, are members of the tumor necrosis factor (TNF) receptor and TNF superfamily, respectively. Members of this family are important signalling molecules ultimately linking surface receptor stimulation with gene regulation (5, 6). The CD137 receptor was first described as being expressed on T-cells (CD4+ and CD8+). However, recent findings described expression on dendritic cells (DCs), follicular DCs, activated NK cells, neutrophils, eosinophils, and osteoblastic cells. This widespread occurrence may play an important role for tumor cell killing, since some mechanisms may be interrelated (7).

CD137L is a 34 kDa type II transmembrane glycoprotein containing TNF-homology domains (8). It is constitutively expressed on activated antigen-presenting cells (APC) such as B cells, macrophages and DCs. In B-cells, expression is enhanced after CD40 ligation, anti-IgM treatment and cyclic adenosine monophosphate (AMP) exposure as well as in the presence of lipopolysaccharides (LPS) (9). In macrophages, interferon (IFN)-gamma leads to CD137L expression (10). In CD8+ cells, CD137-CD137L interaction enhances proliferation, cytokine production and cytotoxic killing activity. T-cell activation has been found to depend on some other known co-stimulatory effects (*e.g.* CD28), but it seems that CD137-CD137L interaction is independent of other interactions (11); this may be due to the fact that some co-stimulatory effects deploy at a particular time in the development of a T-cell response (12).

Interleukin 12 (IL12), a heterodimeric cytokine coding for two separate genes p35 and p40 connected with a linker protein, is naturally produced by dendritic cells, macrophages and human B-cells in response to antigenic stimulation. IL12 is involved in the differentiation of naive T-cells into Th1 cells and it is known as a T-cell stimulating factor, mediating the enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T-lymphocytes. While CD137L is a protein bound to the cell surface, IL-12 is secreted into the culture media of the cells.

In a wide range of solid tumors, IL12 and CD137L have been shown to induce antitumor immunity. Yet, little information is available for hematological malignancies (13, 14). In a wide range of solid tumors, overexpression of CD137L as well as systemic anti-CD137 antibody administration have been shown to induce tumor immunity. This was at least partly due to the stimulation of CD8+ CTL. Costimulation by other known interactions is not unconditionally necessary. Antitumor effects were even increased when immunotherapy with IL12 was additionally employed, either systemically or locally (15, 16). Yet, there is rare data on the effect of CD137L immunotherapy in hematological malignancies (17). Here, we present data obtained in a murine plasmocytoma model to evaluate the effect of CD137L and IL12, either as mono- or combinational therapy.

Materials and Methods

Vector construction. The cDNAs coding for the membrane-bound murine CD137L and soluble murine IL-12 were kindly obtained from Shu-Hsia Chen, Mount Sinai School of Medicine, New York, USA, and Richard Mulligan, Children's Hospital, Boston, USA, respectively.

To construct a vector including the cytokine cDNA, a NotI and HindIII restriction site were attached to the CD137L and IL12 gene by PCR, respectively. cDNA was cloned into the mammalian expression vector pcDNA3.1(–) (5,427 kb; Invitrogen GmbH, Germany) containing a CMV promoter, a multiple cloning site, and an ampicillin as well as a neomycin resistance gene. The cytokine cDNA was ligated downstream of the CMV promoter. Confirmation of correct insertion was obtained by gel electrophoresis (Figure 1) and sequencing (GATC Biotech AG, Konstanz, Germany).

We chose not to insert both cDNAs into one single vector so as to be able to differentiate between the antitumor effects of each single cytokine.

Cell culture. MPC11 (DMSZ, Braunschweig, Germany) is a murine plasmocytoma cell line derived from the Balb/c strain expressing IgG2b. Cells were cultured in RPMI 1640 medium (PAA Laboratories GmbH, Austria) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine (both from PAA, Cölbe, Germany), 100 U/ml penicillin/100 U/ml streptomycin (both from Seromed, Jülich, Germany) at 37˚C in a humidified 5% CO_2 atmosphere. For transfection, an Amaxa Nucelofection system (Amaxa, Cologne, Germany) was used. A 5 μg circular plasmid was added to 100 μl Amaxa Nucelofection Solution containing 10^6 MPC11 cells; nucleofection was then performed. Subsequently, cells were plated on 96-well dishes at a density of 10^4 cells per well. After 48 h, selection was initiated by addition of G418 (neomycin) in increasing doses up to a final concentration of 0.8 mg/ml.

Enzyme-linked immunosorbent assay/ELISA. Culture supernatants of the of IL12-producing MPC11 cells (10^6 cells were incubated in 1 ml RPMI-1640 medium for 24 h) were tested by ELISA to quantify the amount of IL-12 secreted by the nucleofected MPC11 cells. IL-12 ELISA kits (R&D Systems, Germany) were used according to the manufacturer's instruction. Plates were measured at a wavelength of 405 nm in an ELISA reader (Rosys Anthos Photometer 2010, Krefeld, Germany).

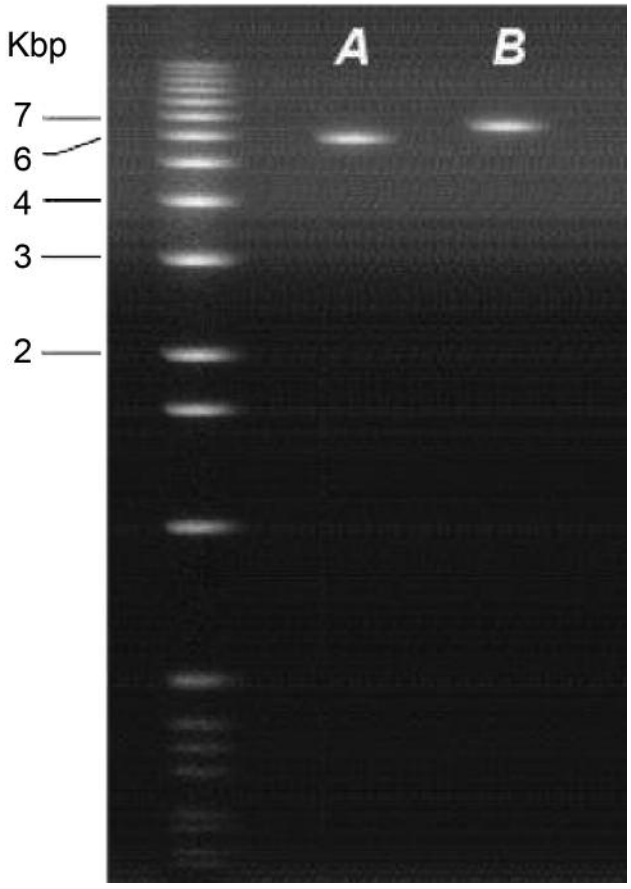

Figure 1. *Gel electrophoresis of the constructed plasmids. After linearization of the constructed plasmids by restriction digestion, gel electrophoresis was performed to estimate the size of the constructed plasmids. Kbp: kilo base pair, A: linearized pcDNA-CD137L vector, B: linearized pcDNA-IL12 vector after restriction digestion.*

FACScan. CD137L-PE antibody was obtained from eBioscience (Kranenburg, Germany) and 5 μl were added to 5×10^5 CD137L cells in a volume of 500 μl PBS in full/1% bovine serum albumin. The incubation time was 30 minutes on ice. Measurements were taken with a Beckton Dickinson FACS (Heidelberg, Germany). Analysis was performed with WinMDI (The Scripps Research Institute, La Jolla/California/USA).

Tumor protection experiments. All animal experiments were performed at least in duplicate with groups of 6 BALB/c mice (Charles River, Sulzfeld, Germany. A lethal tumor dose (5×10^5 cells) of IL12- or CD137L-transfected tumor cells was injected subcutaneously, respectively. In the combination group 2×10^5 IL12- and 2×10^5 CD137L-transfected tumor cells were injected simultaneously. Surviving animals were challenged with an additional lethal dose of wild-type (wt) tumor cells. In addition to that, transfected cells were subcutaneously injected in combination with wild-type tumor cells according to Table I.

When wild-type tumor cells and transfected tumor cells were administered simultaneously, cells were injected on either side of

Table I. *Experiments of subcutaneous injection of transfected cells in combination with wild-type tumor cells.*

No	Group	Injected cells	Place and time of application
1	Control group	5×10^5 wild-type tumor cells	Left, day 1
	IL12	5×10^5 IL12-transfected tumor cells	Left, day 1
	CD137L	5×10^5 CD137L-transfected tumor cells	Left, day 1
	Combination	2.5×10^5 CD137L- and 2.5×10^5 IL12-transfected tumor cells	Left, day 1
2	Control group	5×10^5 wild-type tumor cells	Left, day 1
	IL12	5×10^5 wild-type tumor cells	Left, day 1
		5×10^5 IL12-transfected tumor cells	Right, day 1
	CD137L	5×10^5 wild-type tumor cells	Left, day 1
		5×10^5 CD137L-transfected tumor cells	Right, day 1
	Combination	5×10^5 wild-type tumor cells	Left, day 1
		5×10^5 combination tumor cells	Right, day 1
3	Control group	5×10^5 wild-type tumor cells	Left, day 1
	IL12	5×10^5 wild-type tumor cells	Left, day 1
		5×10^5 IL12-transfected tumor cells	Right, day 8
	CD137L	5×10^5 wild-type tumor cells	Left, day 1
		5×10^5 CD137L-transfected tumor cells	Right, day 8
	Combination	5×10^5 wild-type tumor cells	Left, day 1
		5×10^5 combination tumor cells	Right, day 8

the animals' back. Tumor volume was calculated as follows: volume = length x width^2 x 0,52. Animals were killed when tumor volume reached 2000 mm^3. Statistical survival analyses with the software GraphPad InStat, Version 3.0.0, applied the Mann-Whitney test (non-paired, non-parametric).

Results

Expression of membrane-bound CD137L and IL12 in MPC11 after electroporation. MPC11 plasmocytoma cells were transfected with CD137L and IL12 using as electroporation technique. After transfection and selection, 95% of MPC11-CD137L cells were measured as positive for membrane-bound CD137L as assessed in FACS analysis (Figure 2). In ELISA, MPC11-IL-12 cells expressed high amounts of murine IL-12 (2 μg/ml/10^6 cells / 24h). Multiple transfections were performed in order to select the clone with high expression rates.

Administration of transfected tumor cells. When CD137L secreting tumor cells were administered, all animals survived, though transient tumors formed in 6/8 animals (Figure 3). Compared to the control group, this difference was significant ($p<0.0001$). In comparison, treatment with IL12-secreting tumor cells led to lethal tumor growth in 2/8 animals ($p<0.0001$). Coadministration of IL-12 and CD137L led to transient tumor growth in 1/8 animals, but all treated animals survived ($p<0.0001$). Survival of animals treated with engineered tumor cells did not differ significantly. The administration of wild-type tumor cells in control animals led to the death of all animals (Figure 4).

Wild-type tumor rechallenge of surviving animals. Animals who had survived the treatment with transfected tumor cells were then challenged with wild-type tumor cells (Figure 4, broken line; day 60). Here, lethal tumor growth was observed in 2/8 animalsof the IL12-CD137L combination group. All remaining animals in the IL12 group (6/6) and the CD137L group (8/8) survived. All control animals (8/8) treated with wild-type MPC11 cells died (Figure 4).

Simultaneous administration of wild-type and transfected tumor cells. Simultaneous administration of wild-type tumor cells and transfected tumor cells was performed as tumor application on contralateral sides (Figure 5). In animals treated with CD137L and wild-type tumor cells, lethal tumor growth was prevented in 5/8 animals ($p=0.0415$ compared to control). When IL12-secreting cells were administered simultaneously with wild-type tumor cells, 6/8 animals died ($p=0.2209$). Moreover, 6/8 animals died when a combinational of IL12- and CD137L-expressing tumor cells and wild-type tumor cells were administered ($p=0.1641$). There was no significant difference between the three treatment groups, nevertheless the CD137L monotherapy appeared to allow better survival.

Therapy of established wild-type tumors with transfected tumor cells. Transfected tumor cells were also administered contralaterally after establishing a lethal dose of wild-type tumor cells (growth of a tumor nodule >0.8 cm). When either CD137L- or IL12-producing tumor cells were used, 6 out of 7 animals in each group died (insignificant compared

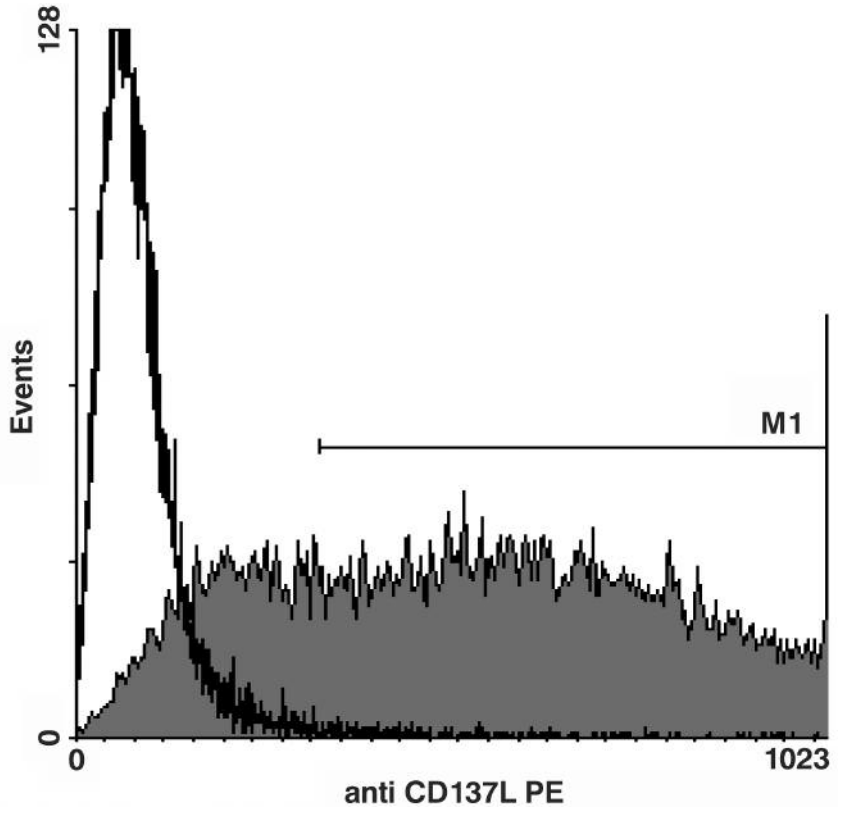

Figure 2. *After transfection with CD137L cDNA, membrane bound expression of CD137L was determined in FACS analysis. Compared to wild-type MPC11 cells (black line), 81.9% (M1) of MPC11 cells transfected with CD137L cDNA (grey adrea) expressed CD137L. Results from a representative experiment are shown.*

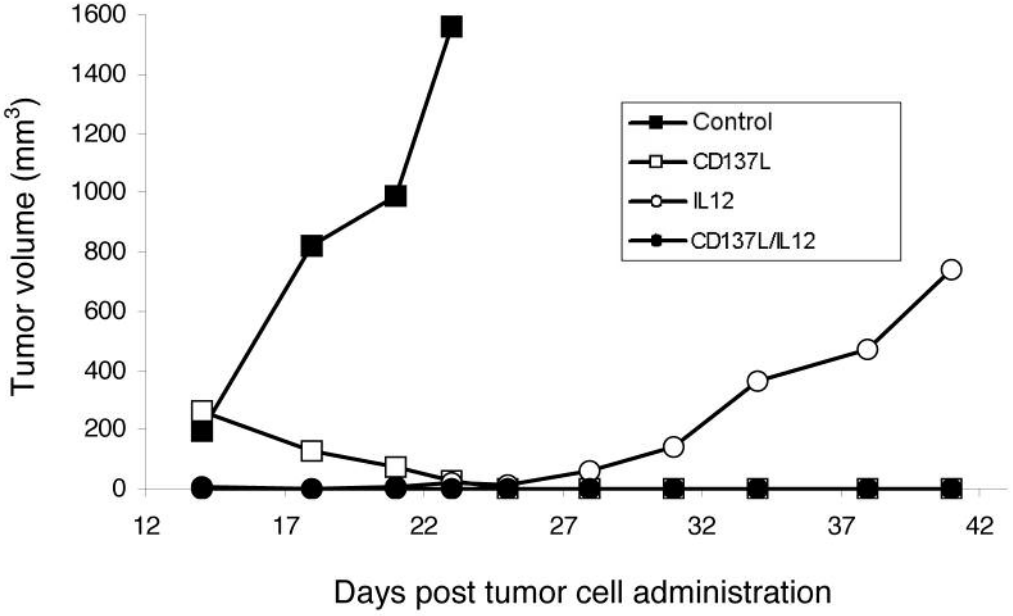

Figure 3. *Tumor growth after the administration of transfected tumor cells. Transfected tumor cells were administered subcutaneously to the back of the animals. Tumor volume was calculated by the formula length x (width)^2 x 0.52. Observation of control tumors (closed squares) ended at death of the first animal. Transient tumors formed after CD137L-expressing cells were administered (open squares), but both in the CD137L- and the CD137L/IL-12 (closed circles) group, all animals survived. In contrast, progressive tumor growth was observed in IL-12 producing tumors (open circles).*

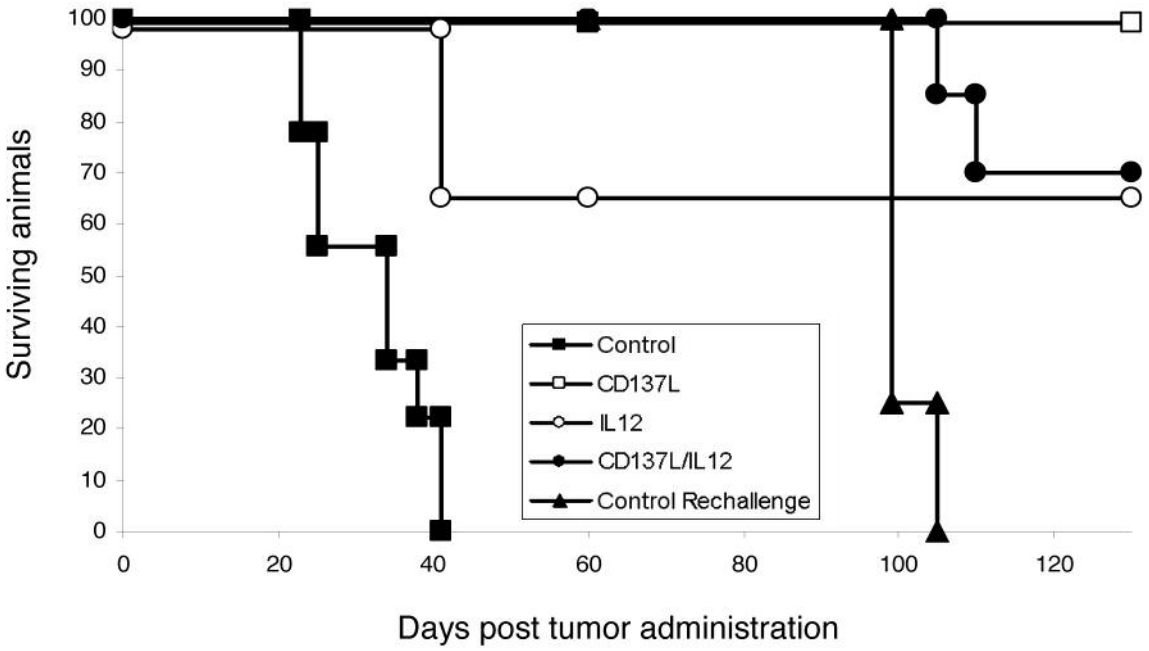

Figure 4. *Survival after the administration of transfected tumor cells and wild-type cell rechallenge. Transfected tumor cells were administered subcutaneously to the back of the animals. Control animals died after administration of a lethal tumor dose of tumor cells (closed squares). All animals treated with CD137L (open squares) and CD137L/IL-12 coproducing (closed circles) tumor cells survived, whereas 2/8 of animals treated with IL-12 producing tumor cells (open circles) died. Tumor cell re-challenge (broken line, day 60; control: triangles). Wild-type tumor cells were administered subcutaneously to the contralateral side of the back of the animals on day 60. This tumor re-challenge showed that protective immunity persisted in all animals of the CD137L (open squares) and the IL-12 group (open circles), whereas 2/8 animals treated with CD137L/IL-12 co-producing tumor cells died. Data from 8 animals per group are shown.*

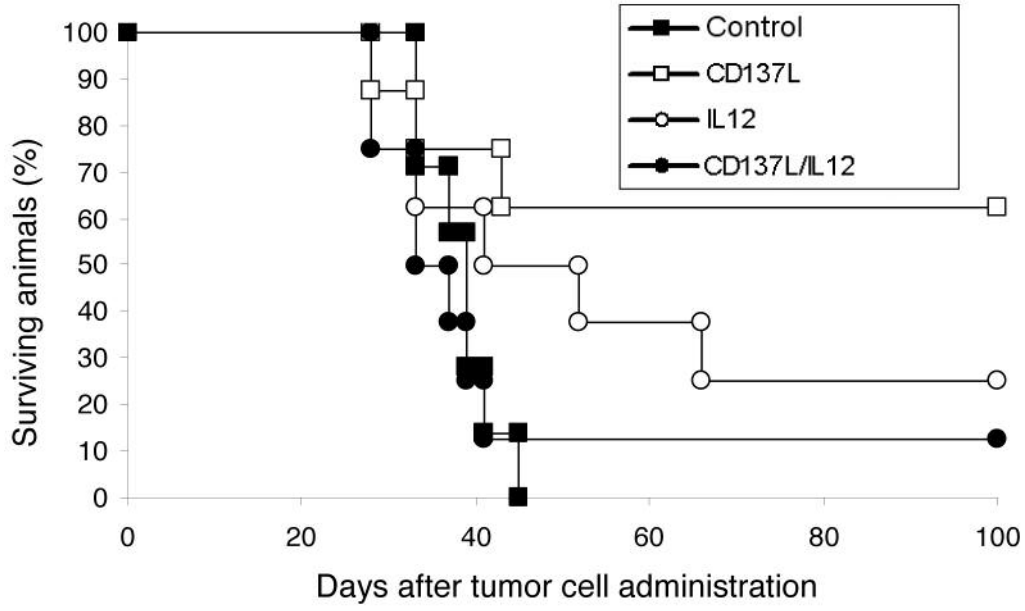

Figure 5. *Survival after simultaneous, contralateral administration of transfected tumor cells and wild-type tumor cells. Transfected tumor cells were administered subcutaneously to the left side of the back of the animals with simultaneous administration of wild-type tumor cells to the right side. Control animals died after administration of a lethal dose of tumor cells (filled squares). Im comparison, 3/8 and 6/8 treated with CD137L- (open squares) and IL12-producing tumor cells died from contralateral wild-type tumor growth, respectively. Interestingly, 7/8 of animals treated with CD137L/IL-12 co-producing tumor cells died from wild-type tumor growth. Data from 8 animals per group are shown.*

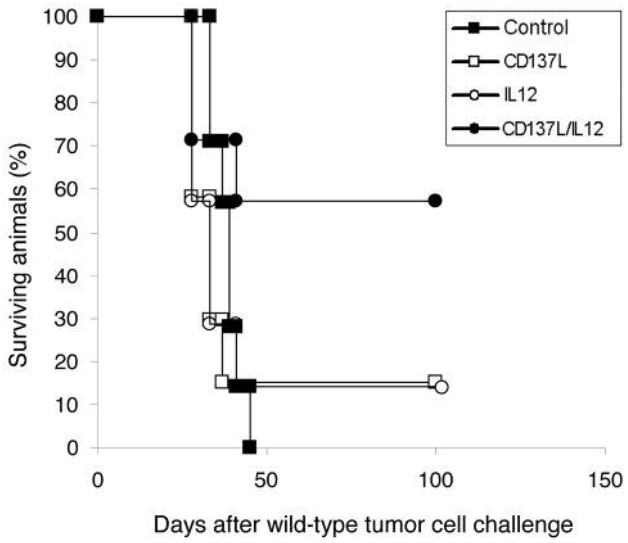

Figure 6. *Survival after delayed, contralateral administration of transfected tumor cells after tumor growth of wild-type tumor cells. Wild-type tumoe cells were administered subcutaneously to the left side of the back of the animals: After growth of a tumor nodule >0.8 cm, transfected tumor cells were administered to he right side. Control animals died after administration of a lethal dose of tumor cells (filled squares). A total of 3/8 animals treated with CD137L/IL12-co-producing tumor cells died from wild-type tumor growth compared to 7/8 animals in the CD137L and IL-12 group. Data from 8 animals per group are shown.*

to control). When a combination of IL12- and CD137L-secreting cells was administered, lethal tumor growth was prevented in 4 out of 7 animals (p=0.0103; Figure 6).

Discussion

In the present study, we demonstrated the efficacy of CD137L gene-modified murine plasmocytoma cells to induce a long-lasting antitumor response against wild-type tumor challenge. In comparison to IL12 or even IL12/CD137L gene-modified tumor cells, CD137L-expressing tumor cells seem to have a more potent antitumor activity, which was demonstrated during the wild-type rechallenge. Although a transient tumor formed in 6/8 animals of the CD137L group, all animals survived, which could be contributed to a delayed but effective antitumor effect. Interestingly, when a preestablished tumor had to be cured, the combined activity of CD137L- and IL12-expressing tumor cells was superior to that of both CD137L- or IL12-expressing tumor cells, respectively. What has to be looked at in further experiments is the question why there is such a difference between the simple administration of modified tumor cells and the therapy of established wild-type tumors in the CD137L group. One explanation could be the number of modified tumor cells administered: the antitumor effect may not be strong enough with this number of cells, which could be increased. It may also be due to the delayed effect of the established tumors finally leading to the animals' death.

Thus, future experiments should be performed with different number of cells being administered to confirm these data and address the role played by different cells of the innate or acquired immune system. CD137L is capable of preventing tumor growth but for an established tumor, this antitumor effect could not prevent the animals' death.

The combination of CD137L and IL12 has already been successfully used as given in previous reports (*e.g.* in (3)). However, little is known about haematological neoplasias in this respect. The uniqueness of the report presented here is that we used a plasmocytoma tumor model as plasmocytome cells may function as antigen-presenting cells.

Gene modified tumor cells expressing different cytokines have been tested for induction of a significant antitumor response both *in vitro* and *in vivo* (4). This applies for both solid and hematological malignancies (18, 19). Cytokines with direct effects on Th1 lymphocytes, cytotoxic T lymphocytes (CTL) and NK cells were more potent than cytokines with effects on B lymphocytes and Th2 lymphocytes. Moreover, great efforts were made to facilitate existing antitumor responses by intensifying co-stimulatory mechanisms and the induction of a more potent antigen presentation on professional antigen presenting cells (APC) such as dendritic cells (DC) (20).

Because of the widespread distribution of its receptor CD137, CD137L is an attractive molecule for cancer immunotherapy. CD137 is constitutively expressed on CD4+ and CD8+ T-cells (21). In these cells, CD137 activation may break immune tolerance and outweighs T-cell anergy. Preferentially CD8+ T-cells are expanded, independently from IL-2.

CD4+ T cells are also relevant for the generation of a potent antitumor response in CD137 activation: They enhance proliferation and the cytolytic function of CD8+ cells (13, 22-24). Depletion of NK cells expressing CD137 led to abrogation of antitumor effects of CD137 activation, hinting that there is substantial crosstalk between T-cells and NK cells (20).

Stimulation of CD137 also leads to DC IL12 and IL6 production and enhances antigen presentation as well as up-regulation of co-stimulatory molecules such as B7-1 and B7-2. Moreover, activation of the CD137 receptor increases the number of tumor infiltrating lymphocytes (TIL) in an IFN-γ-dependent manner. *In vivo* experiments have shown that the migration capabilities of TIL vanish when IFN-γ is blocked or treated animals are IFN-γ deficient (25).

Different strategies to generate CD137 receptor activation and a subsequent antitumor response have been applied. Most

published data rely on agonistic antiCD137 antibodies that were administered systemically. Other strategies included CD137L gene transfer in tumor cells: either CD137L was secreted in a soluble or a membrane-bound form.

In conclusion, IL12- and CD137L-transfected plamocytoma cells prevented tumor growth and induced longlasting immunity. This strategy should be followed-up for future clinical use in patients with myeloma.

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