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CD40L-Transfected Myeloma Cells Transfer Prolonged Immunity *In Vivo*

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Abstract. *Background: In a large number of patients with multiple myeloma, chemotherapy is the only therapeutic option. During recent years, major effort has been put into immunotherapeutic approaches for this malignancy. Materials and Methods: In this study, wild-type (wt) myeloma cells (5×10^5) were injected subcutaneously into Balb/c mice. CD40L-transfected myeloma cells (5×10^5) were subsequently injected intratumorally into the established (>100 mm^3) wt tumor nodules. Overall survival and tumor growth were measured. Results: Out of eight animals receiving wt tumor cells, one died prior to the formation of a solid tumor nodule. Following the CD40L-transfected myeloma cell injection, stable complete remission at day 60 with all the animals surviving resulted. On day 60, a re-challenge was performed with wt myeloma cells. No tumor growth was observed after 120 days out of seven remaining animals, one died. Conclusion: Intratumoral injection of CD40L-transfected myeloma cells induces complete tumor remission and long lasting immunity against tumor recurrence.*

Major effort has been put into the research of hematological neoplasias and their treatment. In spite of huge progress being made during the last few years (1-3), still little is known about the immunotherapeutic approach in this respect (2, 4).

Here, data obtained in a murine plasmocytoma model evaluating the antitumor effect of CD40L (CD154) are presented.

CD40 ligand (CD40L), a member of the tumor necrosis factor (TNF) superfamily (5), is a type II membrane glycoprotein consisting of 261 amino acids, whose expression is mainly limited to the CD4-T-cell subset (6, 7). CD40L plays a key role in the activation of antigen-presenting cells (APC), such as macrophages, B-lymphocytes and dendritic cells (8-10). It serves as a co-stimulatory factor which is primarily expressed on activated T-lymphocytes and induces the increased differentiation of B-lymphocytes into plasma cells and the prolonged activation of macrophages (11, 12), thus promoting the specific orientation of the immune system against the antigenic target (13). In the current literature, CD40L has been shown to have promising antitumor effects in a number of solid tumors (14) and it plays an important role in tumor vaccination (15, 16). Furthermore, the CD40-CD40L interaction plays an important role in enhancing the antigen-specific T-cell response by activating dendritic cells and thus inducing the production of interleukin (IL)-12 (17). This response remains active as long as the antigenic target stays in the system. Additionally, this interaction induces the activation of antigen presenting cells and the production of cytokines, thus promoting further immune response (18). Moreover, great efforts have been made to facilitate existing antitumor responses by intensifying co-stimulatory mechanisms and thus inducing a more potent antigen presentation on the professional antigen presenting cells such as dendritic cells (19). Although soluble CD40L agonists are growth inhibitory, membrane-presented CD40 ligand induces apoptosis in carcinoma cells (20).

Multiple myeloma, a treatable but still incurable disease (21), is a well known hematological neoplasm derived from plasma cells with the presence of paraproteins (22). In a wide range of different neoplasias, overexpression of CD40L has been shown to induce increased tumor immunity (6, 7). However, there is little information available concerning the immunotherapeutical application of CD40L in a murine plasmocytoma model.

Materials and Methods

Vector construction. The cDNA coding for the membrane-bound murine CD40L was obtained from Invivogen (Cayla SAS, Toulouse, France). After amplification of CD40L-DNA by PCR and the introduction of a specific digestion enzyme (*HindIII*) restriction site, the DNA was cloned into the mammalian expression vector

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pcDNA3.1(–) (5,427 kb; Invitrogen GmbH, Karlsruhe, Germany) containing a CMV promoter which induces the expression of proteins and an ampicillin as well as a neomycin resistance gene for the following selection process. The cytokine cDNA was ligated downstream of the CMV promoter. Confirmation of correct insertion and sequence was obtained by gel electrophoreses after DNA digestion and sequencing (GATC Biotech AG, Konstanz, Germany).

Cell culture. MPC11 (DMSZ [German Collection of Microorganisms and Cell Cultures], Braunschweig, Germany) is a murine plasmocytoma cell line derived from the Balb/c strain expressing IgG2b. The cells were cultured in RPMI-1640 medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine (both from PAA, Cölbe, Germany) and 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 Nucleofection system (Amaxa, Cologne, Germany) was used. A 5 μg circular plasmid was added to 100 μl Amaxa Nucleofection Solution containing 1×10^6 MPC11 cells to achieve nucleofection. Subsequently, the cells were plated on 96-well dishes at a density of 1×10^4 cells per well. After 48 h, selection was initiated by the addition of G418 (neomycin) in increasing doses up to a final concentration of 0.8 mg/ml.

Tumor protection experiments. The animal experiments were performed with groups of 8 Balb/c mice (Charles River, Sulzfeld, Germany. A 'lethal' tumor dose (5×10^5 cells) of wt tumor cells was injected subcutaneously into the back of all the mice producing a solid tumor nodule after a few days. After tumor growth of more than 100 mm^3 (~d7) of the mice of the treatment group, 5×10^5 CD40L-transfected tumor cells were administered into the preformed tumor nodule. The surviving animals were challenged with an additional 'lethal' dose (5×10^5 cells) of wt tumor cells administered subcutaneously at the same site as the previous injections on day 60. Tumor volume was calculated as follows: volume=length× $width^2\times0.52$. The animals were killed when tumor volume reached 2000 mm^3.

The control group of eight mice received no further treatment.

Statistical analysis. For statistical analysis, the results were expressed as the mean±standard error of the mean (SEM) and Students' *t*-test was used for statistical analysis. A *p*-value below 0.05 was considered significant. Statistical survival analysis with the software GraphPad InStat, Version 3.0.0, applied the Mann-Whitney-*U*-test (non-paired, non-parametric).

Results

Expression of membrane-bound CD40L after nucleofection. After transfection and selection, 89% of the MPC11-CD40L cells were measured as positive for membrane-bound CD40L as assessed by FACS analysis (Figure 1). Multiple transfections were performed in order to select the clone with high expression rates.

Tumor development. All the animals in the control group developed a tumor larger than 2000 mm^3 and therefore had to be sacrificed by day 34 (Figure 2).

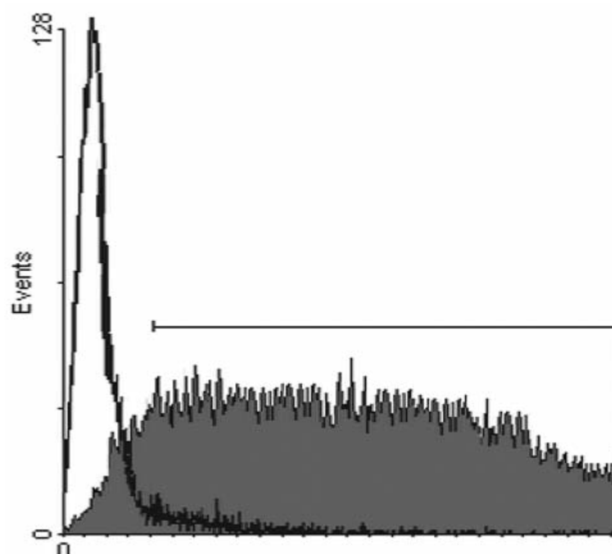

Figure 1. *Membrane-bound expression of CD40L was determined by FACS analysis after transfection with CD40L cDNA. Eighty nine percent of transfected MPC11 cells (shadowed area) expressed CD40L in comparison to 2% of wt MPC11 cells (black line).*

A solid tumor nodule formed after a few days. One out of eight animals in the treatment group died prior to the formation of a solid tumor nodule. After the MPC11-CD40L cells were injected into the tumor nodules (~d7) of the remaining seven animals, all showed a complete remission by day 60 (Figure 2). Compared to the control group, this difference was significant (p=0.01).

Wild-type tumor rechallenge. All seven animals of the CD40L-treatment group survived for 60 days and were then challenged with the second 'lethal' dose of wt tumor cells. Lethal tumor growth was observed only in 1/7 animals, all the remaining animals (6/7) survived without a new formation of a tumor nodule until day 120 (Figure 3).

Discussion

CD40L is a member of the TNF superfamily (5). The expression of CD40L is a highly regulated process which takes place on the surface of activated T-cells (23). As shown from former studies, additional cytokine signals enhance the up-regulation of CD40L. The CD40L mRNA expression peaks up to 2 hours after T-lymphocyte activation, and is fully expressed within 6 hours, followed by rapid disappearance from the cell surface, as CD40L is barely detectable after 16 hours (11, 16). This transient expression of CD40L offers only a short time to deliver helper signals interacting with B-cells (19), macrophages, or dendritic cells

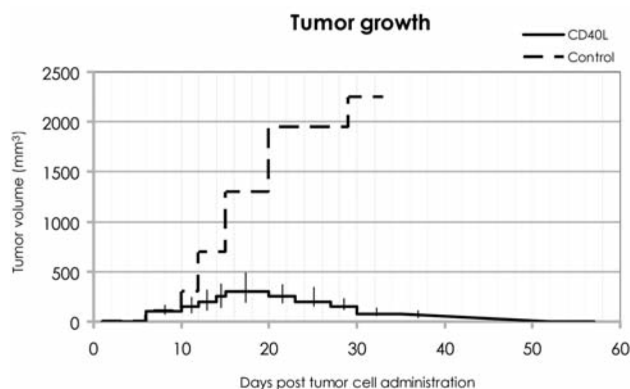

Figure 2. *Tumor growth. Tumor volume was calculated by the formula length×(width)^2×0.52. Observation of control tumors ended at death of the first animal. Treatment group: CD40L-expressing cells administered when tumors exceeded 100 mm^3.*

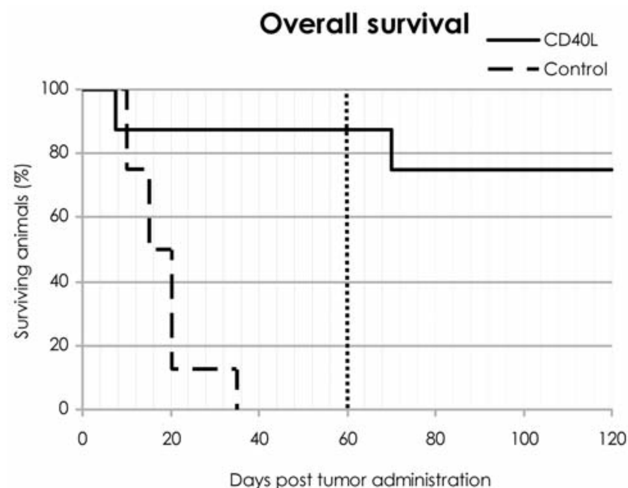

Figure 3. *Overall survival. Wild-type (wt) tumor cells were administered subcutaneously on day 0. Control animals received no further treatment and all died within 34 days. Treated animals (n=7), injected intratumorally (~d7) with CD40L-transfected tumor cells and with a second wt tumor cell challenge on day 60. Data from 8 animals per group are shown.*

(23-26). Thus, an up-regulation of CD40L followed by higher CD40/CD40L-interaction at the site of antigen presentation results in a higher antigen-specific response of T-lymphocytes, dendritic cells, B-lymphocytes and macrophages (27-29).

In this study, the CD40L-transfected myeloma cells were shown to be capable of inducing a highly effective long-lasting antitumor response to established wt myeloma nodules. Within four weeks after the subcutaneous intratumoral administration of the modified tumor cells (MPC11-CD40L), all of the preformed wt tumor nodules disappeared in all the surviving animals. During this time, no further restriction of the animals' physical constitution was apparent. Furthermore, there was no growth of additional tumor nodules in 6/7 animals during the 60 days following the rechallenge with a second 'lethal' dose of wt tumor cells. These results showed that even after the first successful immunological response, an effective and long-lasting anti tumor environment was established.

Due to the fact, that both the wt tumor cells and subsequently, the modified tumor cells were administered at the same location, this antitumor response could be explained by the high concentration of CD40L at the site of the tumor nodules. This would have increased the up-regulation of local CD40/CD40L interactions inducing the enhancement of the antigen-specific T-cell response. Thus, the intratumoral injection of CD40L-transfected myeloma cells was followed by activated acquired and innate immunity, inducing complete regression of the established tumor nodules and resisting a tumor cell rechallenge.

Future experiments could be performed with different numbers of wt, as well as CD40L-transfected tumor cells at different times, in order to find out whether this acquired immune response would still be effective. The unique feature of the present study was the use of a plasmocytoma tumor model, as plasmocytoma cells themselves may function as antigen-presenting cells. Most published data rely on agonistic CD40 antibodies that were administered systemically. Other strategies have included CD40L gene transfer in other antigen-presenting cells, such as dendritic cells (29). In conclusion, CD40L-transfected plasmocytoma cells effectively prevent tumor growth and serve as a tumor vaccine, inducing long-lasting immunity. This strategy should be followed up for future clinical use in patients with myeloma.

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