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Immunotherapy for murine K1735 melanoma: Combinatorial use of recombinant adenovirus expressing CD40L and other immunomodulators

Isabelle Peter,[1,†] Michael Nawrath,[2,†] Jivko Kamarashev,[3] Bernhard Odermatt,[4] Anna Mezzacasa,[1] and Silvio Hemmi[1]

[1]Institute of Molecular Biology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland; [2]Institute of Medical Virology, University of Zürich, Gloriastrasse 30, CH-8028 Zürich, Switzerland; and Departments of [3]Dermatology and [4]Experimental Immunology, University Hospital, Gloriastrasse 31, CH-8091 Zürich, Switzerland.

We have constructed and tested five recombinant adenoviruses (Ads) that express a variety of immunomodulators, including CD40 ligand (CD40L), a potent costimulator of several components of the immune system. We demonstrate that CD40L expressed from Ad in K1735 mouse melanoma cells leads to a strong reduction in tumorigenicity and to efficient protective immunity in a vaccination setting. Subsequently, using a therapeutic approach, we found that local, intratumoral coinjection of CD40L- and IL-2–expressing Ads was superior to any other agents tested and resulted in an at least 1.9-fold increase in mean survival time, in contrast to systemic application of recombinant CD40L or GM-CSF proteins, which had no significant effects. When using vaccination as a therapeutic approach, the combinations of CD40L plus IL-2 or GM-CSF plus IL-2 from Ad gave rise to an extended (2.8-fold) increase in mean survival time. A detailed analysis of immune cells present within regressing tumors indicated that mainly $CD4^+$ and $CD8^+$ T cells, and to a lesser extent dendritic cells, infiltrated the tumor mass, but not NK cells, macrophages, or granulocytes. These results propose that a combination of CD40L plus IL-2 has an improved efficacy over the use of single agents when applied for direct *in situ* therapy or vaccination therapy.
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Once uncommon, melanoma now accounts for 4% of all diagnosed cancers.[1] Surgical removal of melanoma at an early stage is curative; however, once disseminated, no medical therapy has been shown to improve the outcome for patients with melanoma in randomized trials. Thus, alternative treatment options like immunotherapy and gene therapy are being actively explored. The majority of these efforts aim to induce an effective systemic CTL response that will target melanoma cell antigens[2] and eventually lead to control of metastatic tumors.

To artificially "jump-start" an effective immune response against tumor antigens,[3] numerous immunomodulators have been tested for their efficacy to induce an antitumor response *in vivo*. Trials in animal models as well as clinical trials included, for example, vaccines consisting of tumor cells transduced with genes for cytokines like IL-2[4] or IL-12.[5] In addition, growth factors like GM-CSF[6,7] or costimulator protein molecules B7-1 and B7-2[3,8] have also been used. Many of these approaches led to regression or even eradication of tumors in animal models; however, most clinical studies have produced disillusioning results with only a limited number (10–30%) of patients developing T-cell–specific antitumor immunity. The accompanying clinical response rate was even lower.[9]

Since the realization that communication through the costimulatory molecules CD40–CD40 ligand (CD40L) expressed on immature antigen presenting cells (APCs) such as DCs and activated $CD4^+$ helper T cells, respectively, induces a profound activation of the APCs and ultimately a powerful cytotoxic T-cell[10] immune response *in vivo*, CD40L has been added to the list of promising immunomodulators for use as experimental tumor treatments. CD40L (CD154) is a type II membrane protein of 33 kDa and is a member of the tumor necrosis factor gene family, whereas the receptor CD40 represents a member of the TNF receptor family. Based on the hypothesis that *in vivo* expression of CD40L on malignant cells will trigger local APCs to present tumor antigens to the cellular immune system, several studies have been reported in which introduction of the CD40L gene into murine cells *ex vivo* and subsequent implantation to syngeneic hosts results in reduced tumor establishment.[11-13] In the last study,[13] it was demonstrated

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Address correspondence and reprint requests to: Dr Silvio Hemmi, Institute of Molecular Biology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. E-mail: hemmi@molbio.unizh.ch
†Both authors contributed equally to this work.

that only a small percentage of tumor cells is needed to express the costimulatory molecule to produce significant protection. *In vivo* transfer (*via* adenovirus [Ad] vectors) of the gene encoding CD40L resulting in antitumor immunity was demonstrated for B16 melanoma, CT26 colon cancer, and Lewis lung carcinoma cells.[14,15]

The objective of the present study was to compare the antitumor effects of Ad-mediated CD40L expression on K1735 mouse melanoma cells along with other immunomodulators in experiments analyzing tumorigenicity, vaccination potential and therapeutic efficacy on preestablished tumors. Tumorigenicity was clearly most efficiently reduced by expression of CD40L, whereas for vaccination purposes, CD40L and GM-CSF had similar efficacy. For all therapeutic treatment modalities, our results propose that a combination of Ad vectors expressing CD40L plus IL-2 has an improved efficacy over the use of single agents when applied for direct *in situ* therapy or vaccination therapy.

Materials and methods

Cell culture

K1735-M2 cells were provided by IJ Fidler, Department of Cancer Biology, University of Texas. The Ag104A sarcoma cell line was provided by H Schreiber, Department of Pathology, University of Chicago. The A549 human lung carcinoma cell line was received from P Sonderegger, Institute of Biochemistry, University of Zürich. The human embryonic retina cell line 911 was obtained from F Fallaux (Medical Genetic Center, Leiden, the Netherlands). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS; ICN, Costa Mesa, CA) and were routinely screened for the absence of mycoplasma contamination.

Flow cytometric analysis

For flow cytometric analysis of K1735 cells and macrophages, cells were washed with PBS and detached by treatment with PBS/20 mM EDTA. Approximately 10^6 cells were incubated with either 50 μl of CTLA4-Ig supernatant or 1 μg of CD40L-specific antibody in 250 μl BSS/5% FCS (BSS: 0.14 M NaCl, 1 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, pH 6.9) for 30 minutes on ice. Cells were washed by pelleting in BSS/2% FCS and, when stained for B7 expression, were further incubated with 1 μg of phycoerythrin-labeled secondary conjugate and washed again before cytofluorometric analysis (Epics XL; Coulter, Miami, FL). FACS measurements consisted of 10,000 viable cells per sample.

Reagents used for flow cytometry included: human CTLA4-IgG_1 fusion protein specific for human and mouse B7-1/-2 (CD80/CD86),[16] which was used in the form of cell culture supernatant of stably transfected J558L myeloma cells; R-PE–labeled anti-mouse CD40L (CD154) (09025B) and appropriate isotype controls, which were purchased from Pharmingen, San Diego, CA; secondary fluorochrome conjugated $F(ab')_2$ rabbit anti-human IgG, which was purchased from Serotec, Oxford, UK.

Recombinant Ad vectors

Recombinant E1/E3-deleted Ad vectors were constructed by homologous recombination in 911 cells between a plasmid containing the transgene under the control of the RSV or CMV promoter and a genomic *Cla*I DNA fragment isolated from AdMLP–lacZ.[17] Recombinant Ad was plaque purified three times, amplified, and CsCl purified. Viral titers were determined by plaque assay using 911 cells. Biological titers varied between 3×10^9 and 3×10^{10} pfu/mL, when determined in a standard assay using 2 mL of medium and the cell layers contained in six-well plates. Virion concentrations were determined according to Maizel et al.[18] Bioactivity was found to vary between 0.01 and 0.029 for the different viruses; however, all experiments were performed with one and the same batch of individual viruses. Replication-competent Ad (RCA) content was found to be $<10^{-8}$ when determined in a plaque assay using A549 cells.

The following promoters were used for Ad-mediated transgene expression: the Rous Sarcoma virus (RSV) enhancer/promoter (pRcRSV; Invitrogen, Carlsbad, CA), and the human cytomegalovirus (CMV) immediate early promoter.[17] The plasmids encoding both subunits of mouse IL-12[19] were kindly provided by U Gubler, Hoffmann-La Roche, Nutley, NJ. The sequences of both subunits were subcloned and linked by an internal ribosomal entry site derived from encephalomyocarditis virus to allow bicistronic expression. The bioactivity of IL-12 resulting from this construct has been documented elsewhere.[20] The plasmid encoding the mouse IL-2 was provided by W Fiers, Ghent, Belgium. The plasmid encoding the mouse GM-CSF was provided by N Gough, Victoria, Australia. The plasmid encoding the mouse B7-1 was received from J Pavlovic, Experimental Virology, Zürich, Switzerland. The plasmid encoding the mouse CD40L was provided by Immunex, Seattle, WA.

In vitro *assays and ELISA*

Peritoneal macrophages were isolated from C3H mice by peritoneal lavage. The cells were plated at 2×10^5 cells/well and admixed with 10^5 irradiated K1735 cells transduced for 2 days with AdCMV–mCD40L using an MOI of 200. After 24 hours, the mix of cells was stained with CTLA4-IgG_1 followed by PE-conjugated $F(ab')_2$ rabbit anti-human IgG. Individual cells alone were, in addition to the mix of cells, analyzed to determine basal B7 expression of each cell type.

Enzyme-linked immunosorbent assays (ELISAs) were used to determine mouse IL-2, IL-12, and GM-CSF. Supernatants of transduced cells were collected every 24 hours over 10 days and frozen in aliquots at -80°C. For determination of IL-2, specific reagents were acquired from Genzyme, Cambridge, MA. The detection of GM-CSF was performed using a commercial immunoassay EM-GMCSF from Endogen, Boston, MA. For determination of IL-12, an ELISA was established using two rat monoclonal antibodies, together with recombinant mouse IL-12, generously provided by U Gubler, Hoffmann-La Roche: the rat anti–IL-12 p70 antibody 9A5 was used as the coating antibody, whereas the HRPO-labeled rat anti–IL-12 p40 antibody 5C3 was used for detection.

Immunohistological staining

Tumor tissues were removed, immersed in Hank's balanced salt solution, and snap frozen in liquid nitrogen. For the staining of cell differentiation markers, frozen tissue sections of 5-μm thickness were cut in a cryostat, placed on siliconized glass slides, air dried, fixed in acetone for 10 minutes, and stored at $-70°C$. Rehydrated tissue sections were incubated with primary rat monoclonal antibodies against MHC class I (H-2K^{bkds}, M1/42), MHC class II (Ia^{bda}, IE^{dk}; M5/114.15.2; TIB-120, American Type Culture Collection, Rockville, MD; CD45R/B220 (RA3-6B2; PharMingen), CD3 (KT3), CD4 (YTS 191), CD8, macrophages/F4/80 (M; Cl:A3-1; HB-198, ATCC), splenic marginal zone macrophages (MZM; ER-TR9), splenic marginal metallophilic macrophages (MM; MOMA 1; Biomedicals, Augst, Switzerland), follicular DCs (FDC; 4C11), interdigitating DCs (IDC; NLDC-145; Biomedicals), and CD11b (M1/70). CD11c was stained with primary monoclonal hamster antibodies (N418). NK cells were stained using a polyclonal rabbit anti–asialo-GM1 antiserum (Wako Chemicals, Osaka, Japan). To stain for mouse Ig, sections were incubated with monoclonal rat anti-mouse IgM (R6-60.2) or a mixture of rat anti-mouse IgG$_1$ (G1-6.5), IgG$_{2a/2b}$ (R2-40) and IgG$_3$ (R40-82; all from PharMingen). Primary antibodies were revealed by sequential incubation with goat antibodies against species-specific Igs, followed by alkaline phosphatase–labeled donkey antibodies against goat immunoglobulins (Jackson ImmunoResearch Laboratories, West Grove, PA). Dilutions of anti-Ig reagents were made in Tris-buffered saline (TBS) containing 5% normal mouse serum. Alkaline phosphatase was visualized using naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as substrate, yielding a red color reaction product. Endogenous alkaline phosphatase was blocked by levamisole. Color reactions were performed at RT for 15 minutes with reagents from Sigma Chemical, St. Louis, MO. Sections were counterstained with hemalum, and coverslips were mounted with glycerol and gelatin.

Animal experiments

Female C3H (H-2^k) 6- to 9-week-old mice were obtained from Charles River, Germany or from BRL Füllinsdorf, Switzerland. For analysis of tumorigenicity of Ad-transduced K1735 cells, tumor cells were transduced using an MOI of 500 for the different Ads. Two days post infection, the cells were harvested, washed, and suspended at 10^7 cells/mL in FCS-free DMEM medium, and 10^6 cells were injected subcutaneously (s.c.) into the right flank of each animal. Tumor growth was monitored every third day by measuring two perpendicular tumor diameters using calipers. The tumor volume (v) was calculated as follows: $v=(d_1 d_2^2)/2$, where d_1 corresponds to the longer and d_2 to the shorter diameter. Tumor-bearing animals were sacrificed if tumor volumes exceeded 3000 mm^3. For statistical evaluation, tumor appearance was analyzed as a factor of treatment using one-way analysis of variance (ANOVA), followed by paired contrasts.

For the evaluation of induction of protective immunity by vaccination with Ad-transfected tumor cells, K1735 cells were transduced as described above. Two days post infection, the transduced K1735 cells were collected and irradiated with 5000 rad, a dose previously determined to abrogate tumor induction, and 10^6 or alternatively 10^7 cells were injected intraperitoneally (i.p.) at day -10 or day -31, respectively. For tumor challenge, 10^6 non-irradiated K1735 were injected s.c. as described above (day 0). Tumor-free animals were rechallenged on day 91 or 94 to evaluate the longevity of the immune response. For statistical analysis, the data were first analyzed using three-way factorial ANOVA, again followed by paired contrasts.

For therapeutic treatment of preexisting tumors using exogenous recombinant proteins, tumors were inoculated as described above, and treatment started with s.c. injections at day 10. Treatments consisted of: PBS/1% mouse serum for 14 days, mouse CD40L trimer at 2.5 mg/kg/day for 14 days, mouse GM-CSF at 60 μg/kg/day for 11 days, and GM-CSF at 60 μg/kg/day for the first 9 days, then continued with GM-CSF at 60 μg/kg/day for another 2 days, and with CD40L at 1.25 mg/kg/day for another 5 days. All recombinant proteins were generously provided by Immunex, Seattle, WA. Statistical analysis was performed as described for the tumorigenicity experiment.

For therapeutic treatment of preexisting tumors using intratumoral (i.t.) injection of Ad vectors, tumors were inoculated as described above. Animals groups were randomly chosen before tumors appeared. Treatment, consisting of i.t. injection of Ad (10^8 pfu in 100 μl) expressing various immunomodulators, started either at day 21, when all animals had large palpable tumors, and was continued every third day for a total of five treatments, where possible, or started as soon as tumors became palpable, and was continued until tumors completely regressed or animals were sacrificed. For therapeutic treatments of preexisting K1735 tumors using i.p. inoculation of Ad-transduced tumor cells, treatment started at day 10, consisted of injections of 5×10^6 transduced and irradiated (5000 rad) K1735 cells, and was continued every third day for a total of five treatments. Statistical analysis was performed as described for the tumorigenicity experiment.

Results

In vitro characterization of Ad-mediated expression of immunomodulators

Five recombinant Ad vectors: AdCMV–mCD40L, AdCMV–mB7-1, AdCMV–mIL-2, AdRSV–mIL-12, and AdRSV–mGM-CSF were constructed that expressed the mouse costimulatory molecules CD40L, B7-1, and the cytokines IL-2, IL-12, and GM-CSF, respectively. Expression of the individual transgenes was confirmed by transducing mouse K1735 cells at different MOIs followed by either performing flow cytometric analysis of K1735 cells for CD40L and B7-1 detection, or by determination of secreted cytokines using specific ELISAs. Whereas untransduced K1735 cells expressed no detectable CD40L or B7-1, dose-dependent expression of both proteins was found in

cells transduced with the appropriate Ad constructs. Using an MOI of 500, more than 95% of tumor cells expressed CD40L and B7-1, with mean fluorescence intensity values rising from 0.3 to 65 for mock-transduced and CD40L-transduced cells, and 0.3 to 86 for mock-transduced and B7-1–transduced cells, respectively. In addition, when expression was analyzed 1, 7 or 10 days postinfection, expression levels were lower on day 1 and 10, yet similar on day 7. As for the expression of CD40L and B7-1, expression of the cytokines was found to peak between days 4 and 7. The values determined for day 4 using an MOI of 500 were 84 ng of IL-2/10^6 cells/24 hours, 2.6 ng of IL-12/10^6 cells/24 hours, and 37 ng of GM-CSF/10^6 cells/24 hours, which are in the range of reported effective expression levels for IL-2[21] and GM-CSF,[6,21] but rather lower than that reported for IL-12[5].

Following stimulation with CD40L, upregulation of B7 molecules represents one of the known responses of APCs such as DCs or macrophages.[22] To demonstrate that Ad-mediated CD40L expression on K1735 cells induced B7 upregulation, peritoneal macrophages from C3H mice were stimulated *in vitro* with CD40L-expressing K1735 cells for 24 hours. As shown in Figure 1, upregulation of B7 molecules on macrophages was observed when cocultured with CD40L-expresssing K1735 cells, but not when cocultured with K1735 transfected with AdCMV–lacZ or with untransfected K1735 cells, thus confirming biological functionality of the membrane-bound CD40L molecules.

Decreased tumorigenicity of K1735 melanoma cells that express CD40L, B7-1, IL-2, IL-12, or GM-CSF from Ad

To examine the effects of CD40L on tumor growth *in vivo*, K1735 murine melanoma cells were transduced with AdCMV–mCD40L at an MOI of 500, and C3H mice were inoculated with 10^6 of these transduced cells s.c. 2 days later. Under these conditions, the in-culture growth rate of such transduced cells was not altered when

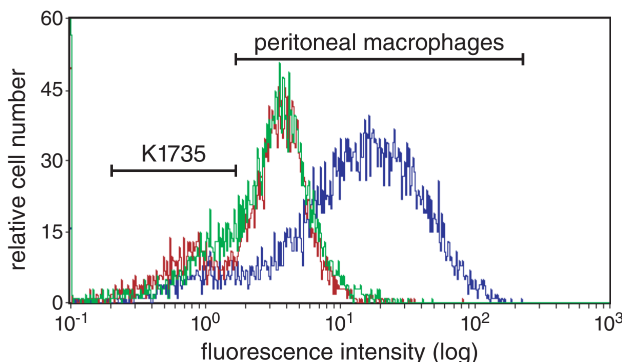

Figure 1 Ad-mediated CD40L expression on K1735 cells upregulates B7-1/-2 expression on peritoneal macrophages. Flow cytometric analysis of B7-1/-2 expression of cocultures of 2×10^5 peritoneal macrophages mixed either with 10^5 irradiated untransduced (green), AdCMV–lacZ–transduced (red) or AdCMV–mCD40L–transduced K1735 cells (blue histogram). Bars indicating expression assignments to individual cell populations were determined by measuring expression on K1735 cells or macrophages alone, in combination with backgating.

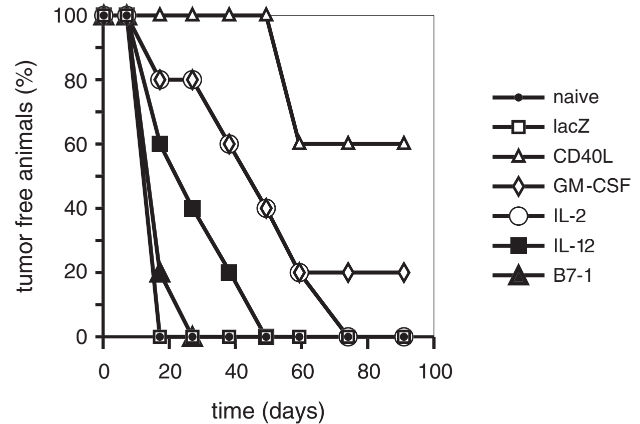

Figure 2 Tumorigenicity of Ad-transduced K1735 cells. K1735 murine melanoma cells were transduced *ex vivo* with Ad vectors expressing the indicated immunomodulators using an MOI of 500. Two days post infection, 10^6 transduced cells, or as control, 10^6 untransduced (naive) K1735 cells were inoculated s.c. into C3H mice (n=5). The day of tumor appearance in mice was recorded (tumor incidence).

compared to untransduced cells (results not shown). As we wanted to compare the effects of CD40L with the efficacy of already established immunomodulators, K1735 cells transduced with AdCMV–mB7-1, AdCMV–mIL-2, AdRSV–mIL-12, and AdRSV–mGM-CSF were included in this experiment. As controls, untransduced cells or cells transduced with AdCMV–lacZ were also included. Compared to these controls, tumorigenicity was strongly reduced for all melanoma cells transiently expressing the immunomodulators, except for B7-1–expressing cells (Fig 2). The mean number of days to detectable tumor was 17±3 for untreated mice and 17±5 for lacZ mice (no statistical difference). The strongest effect was found for CD40L-expressing cells: 3 of 5 mice were still tumor-free 90 days after inoculation, whereas using AdRSV–mGM-CSF transduced K1735 cells, 1 of 5 mice remained tumor free for the same time period. When AdCMV–mIL-2, AdCMV–mIL-12, or AdCMV–mB7-1 were used, none of the mice remained tumor free. The mean number of days to detectable tumor for B7-1–expressing cells was 19±5 days, not statistically different from the lacZ-expressing cells (P=.37), whereas for the other treatments, statistically significant higher numbers were obtained: for IL-12–expressing cells 31±10 days (P=.016), for IL-2–expressing cells 43±21 days (P=.034), for GM-CSF–expressing cells >65±31 days (P=.021), and for CD40L-expressing cells >97±42 days (P=.036). These results also qualitatively suggest that the introduction of CD40L exerted a more potent antitumor effect than the response resulting from expression of GM-CSF, IL-12, or IL-2 (P=.24).

Vaccination using Ad-transduced K1735 cells

Several reports have demonstrated induction of antitumor immunity in vaccination models, in particular using GM-CSF.[6,23] We performed tumor protection experiments to investigate if K1735 mouse melanoma cells transduced with

Ad encoding the immunomodulator CD40L elicit protective immunity against subsequent challenge with parental K1735 cells, and how this response compared with the use of GM-CSF alone, or a combination of CD40L plus GM-CSF. Thus, K1735 cells were transduced at an MOI of 500, and 2 days later, cells were harvested, irradiated and either 10^6 or 10^7 cells were inoculated i.p. into C3H mice (Fig 3). Challenge with unmodified K1735 cells was performed either 10 or 31 days after vaccination. Tumor-free animals were rechallenged either 91 or 94 days later, respectively.

Whereas all naïve animals developed tumors within 21 days, single vaccinations with cells expressing any of the immunomodulators induced a delay or abrogation of tumor growth. The effect was dose dependent, as 10^6 cells showed a rather marginal protection, in contrast to 10^7 cells, which conferred protection, e.g., for 5 of 5 animals when using a CD40L-expressing inoculum, within at least the first 100 days after challenge ($P<.001$). Any of the treatments involving an immunomodulator resulted in a better outcome when compared to β-galactosidase ($P=.002$). The combination of GM-CSF plus CD40L did not clearly differ in inducing immune protection when compared to GM-CSF or CD40L alone ($P=.12$). Remarkably, the protective response seemed to be relatively long-lived, as the primary challenge at day 31 compared to day 10 did not result in significant differences in protection ($P=.90$). In addition, a rechallenge after an additional 91–94 days left most animals immunoprotected.

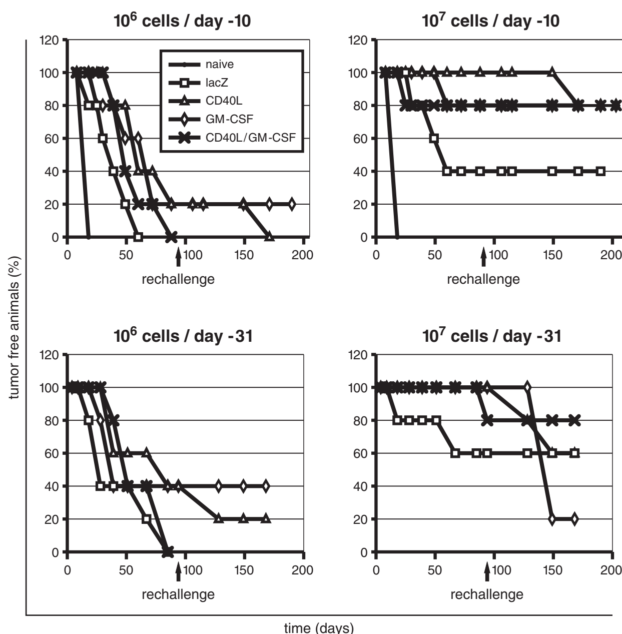

Figure 3 Tumor-free survival of mice vaccinated with transduced K1735 cells. Groups of C3H mice ($n=5$) received i.p. either 10^6 (left panels) or 10^7 (right panels) irradiated K1735 cells transiently expressing β-galactosidase, CD40L, GM-CSF, or CD40L+GM-CSF. The mice were challenged (day 0) with 10^6 unmodified K1735 cells either 10 days (upper panels) or 31 days (lower panels) after vaccination. Tumor-free animals were rechallenged either 91 or 94 days after the first challenge. Appearance of tumors in naïve animals is indicated in the upper panels as controls.

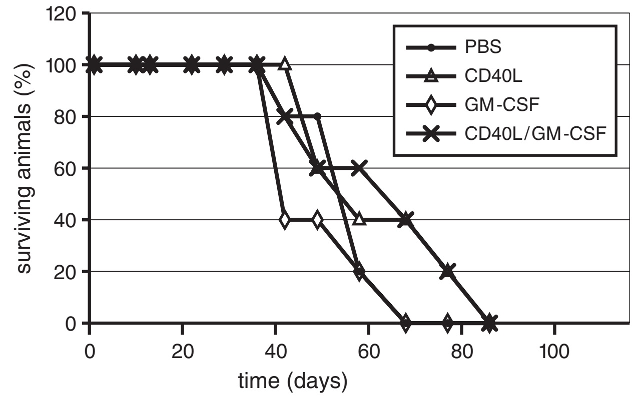

Figure 4 Therapeutic treatment of preexisting tumors using recombinant proteins. Tumors were inoculated s.c. using 10^6 cells. At day 10, treatment started with daily s.c. injections ($n=5$). Treatments consisted of: PBS/1% mouse serum for 14 days, mouse CD40L trimer for 14 days, mouse GM-CSF for 11 days, and GM-CSF for the first 9 days, then continued with GM-CSF for another 2 days and with CD40L for another 5 days.

Immunoprotection was tumor specific, as all CD40L-vaccinated animals challenged with a different but syngeneic tumor type (sarcoma Ag104A) developed tumors at a similar time as the control mice (data not shown). Furthermore, *in vivo* cell depletion with cognate antibodies indicated that both $CD8^+$ and $CD4^+$ T cells were required for inducing protective immunity using CD40L-expressing cells (data not shown), a finding that has also been reported for the antitumor effect of CD40L in the P815 model.[12]

Of note, expression of β-galactosidase led to protection in 2 of 5 or 3 of 5 animals, respectively, which is higher than the protective effect of vaccinating with naïve K1735, giving rise to protection in 1 of 10 animals at this higher dosage (M Nawrath, unpublished results). It has been reported that bacterial β-galactosidase can function as a neo-antigen,[24] which may explain the adjuvant effect found in our tumor vaccination settings.

Taken together, these results confirm earlier findings of a potent and long-lived immune protection induced by GM-CSF–secreting tumor vaccine cells, and demonstrate similar efficiency for CD40L-expressing cells in the K1735 melanoma model.

Therapy on preestablished K1735 tumors

Having demonstrated a significant protective effect by expressing CD40L in tumor cells in the K1735 vaccination model, we wanted to assess whether similar results could be obtained in a therapeutic extension of this tumor model.

In our first experiment, we tested whether systemic application of recombinant proteins including CD40L, GM-CSF, or the combination of both would have a therapeutic effect on tumor growth (Fig 4). Starting on day 10 after tumor cell administration, mice were injected daily for 10 days with the indicated recombinant proteins or with PBS. Using this treatment schedule, we could detect some variation in animal survival that was not statistically significant ($P=.5$). Treatment with recombinant CD40L resulted in a mean survival time of 64 ± 17 days; the

combination of CD40L plus GM-CSF, 64±19 days; compared to PBS-treated animals, 57±9 days, or GM-CSF alone, 52±11 days.

In a second set of therapy experiments we wanted to evaluate two treatment modalities involving either local gene expression mediated by i.t. injection of Ad, or alternatively, using a vaccination strategy that consisted of i.p. injection of irradiated tumor cells expressing immunomodulators at an "early" time point, day 10, before tumors became apparent.

For the first therapy model, two independent experiments were performed, in which tumors were allowed to grow to detectable sizes. In the first of these experiments, the tumor sizes ranged from 100 to 1500 mm^3 with a mean volume of 449 mm^3 (Fig 5A). The treatment consisted of five consecutive i.t. injections of Ad expressing CD40L, IL-12, or β-galactosidase or PBS alone as controls, starting at day 21. In the PBS- and AdCMV–lacZ–treated animals, most animals were euthanized before the fifth treatment, as tumor volumes exceeded tolerable sizes (mean survival time 31±3 and 29±5 days for PBS treatment and AdCMV–lacZ treatment, respectively, with no significant difference). Intratumoral treatment using CD40L-expressing virus demonstrated a transient tumor regression leading to extended survival of all five animals (mean survival time 55±18 days, P=.023), with one tumor that regressed completely for 25 days. For AdRSV–mIL-12 treatment, mean survival time amounted to 44±14 days (P=.072).

In the second of these experiments, a similar, but more aggressive i.t. treatment schedule was performed using Ad vectors expressing CD40L, IL-2, or a combination of both, starting as soon as tumors became palpable (tumor volumes ranged between 23 and 138 mm^3, with a mean volume of 74 mm^3, Fig 5B). Intratumoral treatment was repeated every third day as often as possible until either the tumors regressed or the animals were sacrificed. Transient, but this time incomplete regression was induced with the i.t. treatment using CD40L-expressing virus, resulting in a mean survival time of 51±9 days (P=.093). Similarly, for treatment with IL-2–expressing virus, a mean survival time of 58±20 days was found (P=.066). In comparison, treatment with PBS alone resulted in a mean survival time of 42±7 days. Treatment using IL-2–expressing virus resulted in transient, complete tumor regression for 1 of 8 animals, whereas the treatment using CD40L plus IL-2–expressing vectors resulted in 3 of 5 transiently tumor-free animals and 1 long-term tumor-free animal. The mean survival time for the latter treatment was determined to be >81±19 days (P=.005), which represents more than a >1.9-fold increase.

The vaccination treatment consisted of five consecutive i.p. injections of Ad-transduced, irradiated K1735 cells that expressed β-galactosidase, CD40L, the combination of CD40L plus IL-2, GM-CSF or B7-1, or the combination of GM-CSF plus IL-2, starting at day 10 after tumor inoculation. The mean survival times of animals treated with untransduced, lacZ-transduced or CD40L plus GM-CSF–transduced K1735 cells were quite similar with 42±5, 42±7, and 41±7 days, respectively, with no significant difference. However, the mean survival time increased when the transduced cells expressed either the combination of CD40L plus B7-1 (52±10 days, P=.063), CD40L alone (>89 days, P=.018), the combination of CD40L plus IL-2 (>118 days, P=.0004), or the combination of IL-2 plus GM-CSF (>113 days, P=.004). Tumor-free animals were found for the treatment using CD40L alone (1 of 5), the combination of CD40L plus IL-2 (2 of 5) and for the combination of IL-2 plus GM-CSF (1 of 5).

In summary, for therapy by vaccination, combinations of Ad-infected cells expressing CD40L plus IL-2 or GM-CSF plus IL-2 resulted in an at least 2.8-fold increase of mean survival time, whereas for direct i.t. injection of virus, the combination of CD40L plus IL-2 appeared superior to single treatments, resulting in an at least 1.9-fold increase of mean survival time. These results stand in contrast to those attained through use of exogenously supplied CD40L and GM-CSF

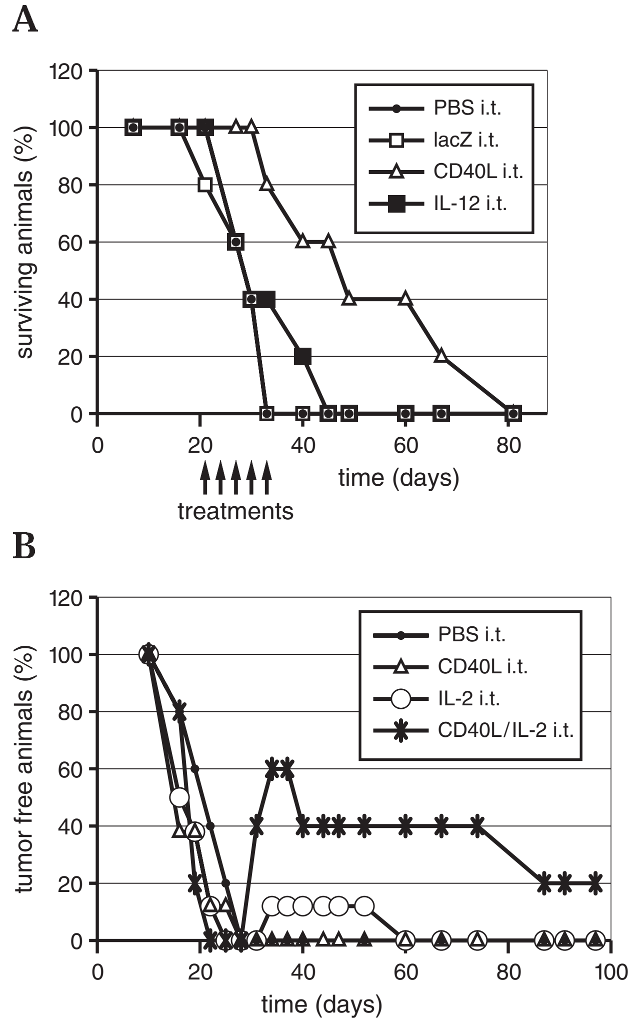

Figure 5 Therapeutic treatment of preexisting K1735 tumors using i.t. injection of Ad vectors. A total of 10^6 parental K1735 cells were inoculated s.c. into C3H mice at day 0. Treatment, consisting of i.t. injection of Ad vectors (10^8 pfu in 100 μl) expressing the indicated immunomodulators, started at day 21 (n=5), and was continued every third day for five treatments in total **(A)**, or started as soon as tumors became palpable (n=5 for PBS and CD40L/IL-2 treatment, n=8 for CD40L and IL-2 treatment), and was continued until tumors completely regressed or animals were sacrificed **(B)**.

recombinant proteins, which had no significant effect on animal survivability.

Infiltration of tumors with DCs and CD4$^+$/CD8$^+$ T cells following intratumoral treatment with recombinant Ads

As i.t. injection of Ad vectors expressing CD40L, IL-2 or the combination of both had resulted in transient or even complete regression in several of the treated animals, we were interested to determine which type of immune cells were infiltrating regressing tumor masses. Thus, tumor tissues were removed when tumor volumes had regressed by approximately 50% following i.t. injections of Ad vectors, and were snap frozen. Figure 7 depicts frozen sections of K1735 tumors stained for various cell markers such as CD4 and CD8 for T cells, CD11c for DCs or asialo-GM1 for NK cells. Cell nuclei were counterstained with hematoxylin. Other markers used included one for T cells (CD3), B-cell markers (IgM, CD45R/B220), markers for general macrophages (F4/80), splenic marginal zone macrophages (ER-TR9), or splenic marginal metallophilic macrophages (MOMA 1), follicular DCs (4C11), interdigitating DCs (NLDC-145), and granulocytes (CD11b) (results not shown). In addition, MHC I and II expression was analyzed, which is mainly a marker for infiltrating cells and tumor cells exposed to IFN-γ, as K1735 cells express very little MHC I and no MHC II *in vitro*.[25] By careful judging of at least three different tumor sections of each treatment type, we found that in particular CD4$^+$ and CD8$^+$ T cells, and to a weaker extent also DCs, infiltrated regressing tumors of mice injected with vectors expressing IL-2, CD40L or the combination of the immunomodulators, but to a much lower extent in lacZ-treated mice or untreated mice (not shown, as identical to lacZ treatment). However, we could not find a significant increase of infiltration for other types of immune cells such as NK cells, B cells, different subsets of macrophages, or granulocytes.

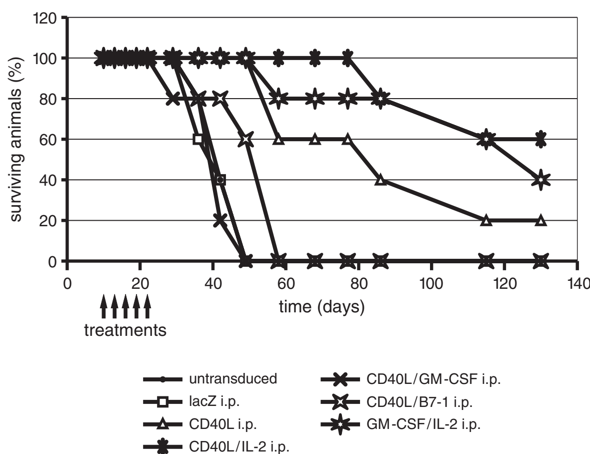

Figure 6 Therapeutic treatment of preexisting K1735 tumors using i.p. inoculation of Ad-transduced tumor cells. A total of 10^6 parental K1735 cells were inoculated into C3H mice at day 0 (n=5). Intraperitoneal treatment consisting of injection of 5×10^6 transduced and irradiated K1735 cells expressing the indicated immunomodulators was started at day 10, and was continued every third day for a total of five treatments.

Discussion

In this study we demonstrate that expression of CD40L (a) significantly inhibited the tumorigenicity of K1735 melanoma cells in C3H mice (H-2^k), (b) allowed induction of protective immunity in a vaccination setting, and (c) induced in combination with IL-2 efficient tumor regression following *in vivo* transduction. Our results extend similar findings reported for leukemia cells,[11,26] Neuro-2a cells,[13] P815 mastocytoma cells,[12] B16 melanoma (H-2^b), CT26 colon cancer, and Lewis lung carcinoma[14,15] with respect to the combinatorial use of immunomodulators to improve antitumor efficiency. The findings reported here add further weight to the generalized efficiency of the CD40L immunomodulator. As for melanoma, our data indicate that inhibition of tumor growth and the induction of protective immunity by tumor-derived CD40L is not restricted to H-2^b melanomas and H-2^b strains of mice, but can also be observed in the genetically distinct H-2^k melanomas and mice.

Our results of the vaccination experiments indicate that CD40L has a similar efficacy in inducing protective immunity as GM-CSF. Since the study of Dranoff et al,[6] multiple studies in other tumor models have confirmed the potent systemic immunity induced by GM-CSF–expressing cell vaccines (for a review see Ref. [27]). The biological activity of GM-CSF is based on the induction of proliferation and maturation of hematopoietic cells, including stimulation of DC accessory properties.[28] In a study by Vereecque et al,[26] the systemic and local effects induced by using GM-CSF– and CD40L–expressing cells have been compared. Using DA1-3b leukemia cells, expression of either GM-CSF plus B7-1 or CD40L alone induced the best protection, followed by GM-CSF alone. Consistent with our experiment, the combination of CD40L plus GM-CSF did not result in an additive nor synergistic effect over the single modulators.

No therapeutic effects could be determined with systemically injected recombinant CD40L and GM-CSF as single agents or in combination. Of note, recombinant, soluble CD40L is being tested in phase I trials, however, only for the specific treatment of CD40-positive cancer cells,[29] as recombinant CD40L is directly cytotoxic to CD40-expressing tumors.[30] In contrast, the mouse K1735 cells used in this report did not exhibit a cytopathic effect when incubated with recombinant CD40L *in vitro* (not shown).

As for the i.t. treatment using Ad vectors, the repeated i.p. vaccination treatment also revealed a better efficacy when the combination of CD40L plus IL-2 was used, compared to CD40L alone, and resulted in an almost 3-fold increase of the mean survival time. These findings confirm data by Dilloo et al, who used the 4-day therapeutic model of mouse CD40-positive A20 leukemia cells.[11] In our therapeutic vaccination system, the combination of GM-CSF plus IL-2 demonstrated similar efficacy as CD40L plus IL-2. Interestingly, with the combination of CD40L plus GM-CSF, the therapeutic effect of CD40L is totally abrogated, and in the combination with B7-1, strongly reduced. A negative effect of B7-1 coexpression on antitumor immune responses has been reported by Chong et al using the K1735 melanoma model.[31] It is unclear why coexpression

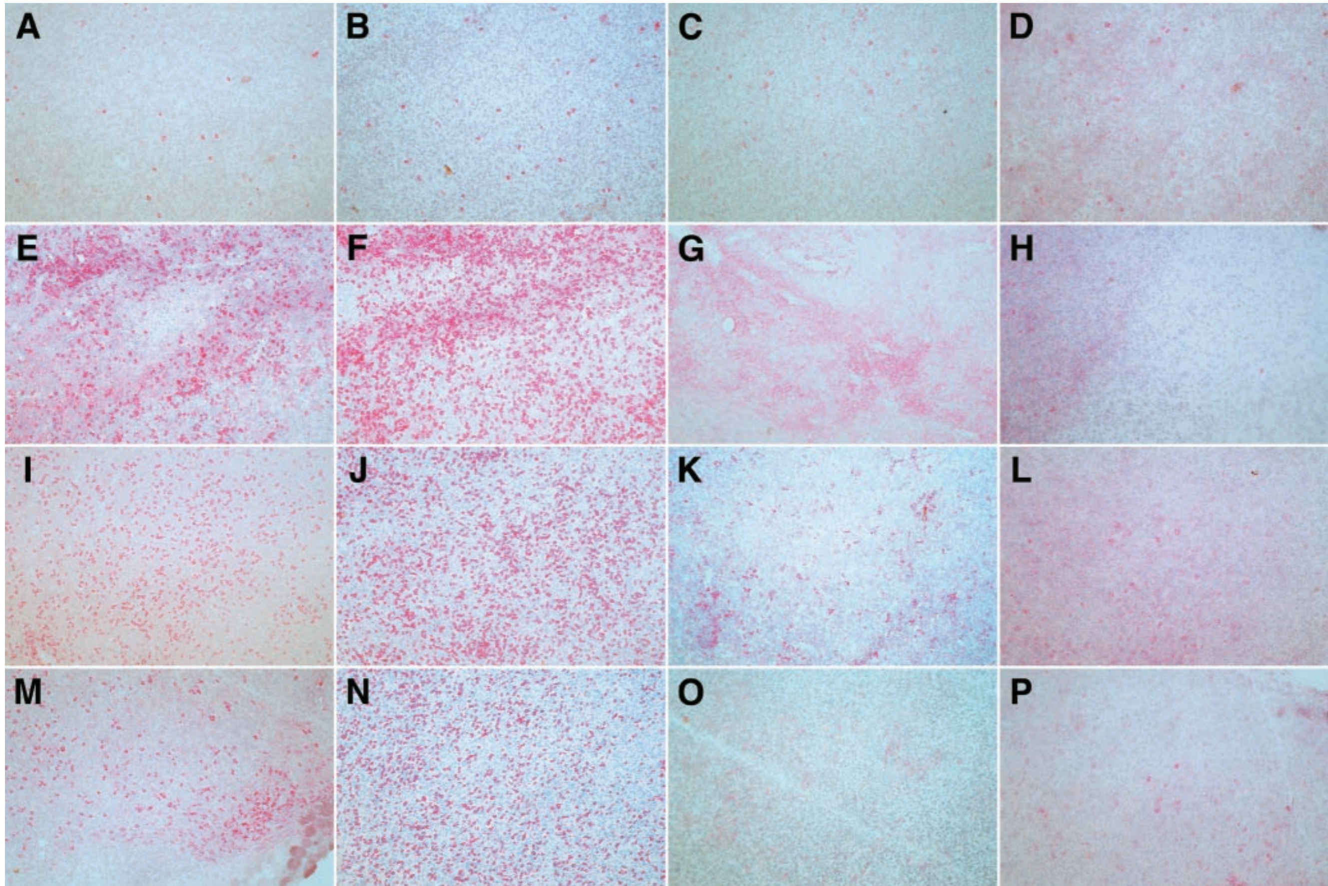

Figure 7 Immunohistological staining of tumor sections from nonregressing and regressing K1735 tumors. Sections from tumors injected with Ad expressing lacZ **(A–D)**, IL-2 **(E–H)**, CD40L **(I–L)**, and the combination of IL-2 plus CD40L **(M–P)** were prepared and stained as described in *Materials and methods*. Markers used included: CD4 **(A, E, I, M)** and CD8 **(B, F, J, N)** for T cells, CD11c for DCs **(C, G, K, O)**, and asialo-GM1 for NK cells **(D, H, L, P)**. Cell nuclei have been counterstained with hematoxylin and are blue. Magnification: ×125.

of GM-CSF plus CD40L in our therapeutic vaccination approach (Fig 6) resulted in a drastic reduction in the antitumor effects of CD40L, compared to the results of the vaccination experiment (Fig 3). One difference is a treatment schedule with five repeated inoculations versus a single inoculation, respectively, resulting in a extended expression of the combination of these modulators, and suggesting that molecules in the CD40 pathway need tight regulation, rather than unbalanced expression.

By our analysis of regressing tumors, we confirmed that (at least at this particular time point) tumors are massively infiltrated with $CD4^+$ and $CD8^+$ T cells when treated with CD40L, IL-2, or the combination of both, confirming results by others.[14,15] In addition, we also found a moderate increase in infiltration of DCs. IL-2 has been reported to induce on its own tumor infiltration by mainly T cells and NK cells. However, we could not observe enhanced infiltration of NK cells, nor of granulocytes and macrophages, suggesting that at this particular time point, cells of the innate immune system do not become activated, which has been proposed for the initial immune response against P815 cells secreting CD40L.[12]

It has been suggested that *in situ* immunological gene therapy locally renders tumor cells "mock" APCs that directly interact with and activate tumor-specific lymphocytes.[32] Due to the complexity of immune responses, the coordinated delivery of multiple immunostimulatory signals will be necessary to optimize the response. The results presented here suggest that the combination of CD40L plus IL-2 may offer valuable clinical benefits for *in situ* and vaccination treatments of melanomas.

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