

## AdCD40L Gene Therapy Counteracts T Regulatory Cells and Cures Aggressive Tumors in an Orthotopic Bladder Cancer Model

Angelica S.I. Loskog, Moa E. Fransson, and Thomas T.H. Totterman

**Abstract Purpose:** The aim of this study was to develop an immunostimulating gene therapy for the treatment of orthotopic bladder carcinoma by transferring the gene for CD40L into the tumor site. CD40L stimulation of dendritic cells induces interleukin-12 expression that drives Th1 type of immune responses with activation of cytotoxic T cells.

**Experimental Design:** The gene for murine CD40L was transferred into bladders of tumor-bearing mice using an adenoviral vector construct. To facilitate viral uptake, the bladders were pretreated with Clorpactin. Survival of mice as well as transgene expression and immunologic effect, such as resistance to tumor challenge and presence of T regulatory cells, were monitored.

**Results:** On viral vector instillation, CD40L expression could be detected by reverse transcription-PCR. As a sign of transgene function, interleukin-12 (IL-12) expression was significantly increased. *AdCD40L* gene therapy cured 60% of mice with preestablished tumors. The cured mice were completely resistant to subcutaneous challenge with MB49 tumor cells, whereas the growth of a syngeneic irrelevant tumor was unaltered. Furthermore, the mRNA expression level of the T regulatory cell transcription factor Foxp3 was evaluated both in tumor biopsies and lymph nodes. There were no differences within the tumors of the different treatment groups. However, Foxp3 mRNA levels were down-regulated in the lymph nodes of AdCD40L-treated mice. Correspondingly, T cells from AdCD40L-treated mice were not able to inhibit proliferation of naive T cells as opposed to T cells from control-treated, tumor-bearing mice.

**Conclusions:** *AdCD40L* gene therapy evokes Th1 cytokine responses and counteracts T regulatory cell development and/or function.

Malignancy of the urinary bladder often presents as a superficial tumor that can be removed by transurethral resection. However, new tumors are constantly arising, and to prolong the tumor-free intervals, Bacillus Calmette-Guerin (BCG) can be instilled repeatedly after tumor resection (1). BCG is a crude bacterial suspension causing a local inflammation that can be monitored by the release of a number of cytokines into the urine (2–4). Moreover, BCG has been ineffective in activating tumor-specific CTLs (2, 5). Failure of BCG treatment could be a reflection of the inability of this therapy to activate CTLs. Bladder cancer is an attractive candidate for innovative therapies, such as gene

therapy and immunotherapy, due to its accessibility for transurethral noninvasive inspection and surgery. In our previous work, we have shown that immunostimulating gene therapy potently regresses s.c. growing tumors by initiating strong Th1-like responses with induction of tumor-specific CTLs (6). In that study, the gene for murine CD40 ligand (CD40L) was transferred into the tumors by adenoviral vectors. CD40L is a type II transmembrane protein belonging to the tumor necrosis factor superfamily. CD40L is up-regulated on CD4<sup>+</sup> T cells upon stimulation but can as well be expressed under certain conditions on other cell types (7). Upon binding of CD40L to its counter receptor CD40 on dendritic cells, the latter will mature and enhance their capacity as antigen-presenting cells. Moreover, CD40L stimulation of dendritic cells yields a strong IL-12 production and release, which is needed for potent CTL induction (8). Both CD40L and IL-12 have been used separately in bladder cancer models with promising results (6, 9–12). However, novel therapies as well as BCG often fail to completely cure mice with orthotopic bladder cancer (12–14). In the work presented here, we have successfully treated mice with aggressive orthotopic tumors with *AdCD40L* gene therapy. *AdCD40L* induced high expression of IL-12 and generated systemic tumor-specific responses. In parallel, the development and/or function of T regulatory cells in the lymph nodes was suppressed.

**Authors' Affiliation:** Rudbeck Laboratory, Clinical Immunology Division, Uppsala University, Uppsala, Sweden

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**Requests for reprints:** Angelica Loskog, Rudbeck Laboratory C11, Clinical Immunology Division, Dag Hammarskjolds vag 20, 751 85 Uppsala, Sweden. Phone: 46-18-611-9181; Fax: 46-18-611-0222; E-mail: angelica.loskog@klinimm.uu.se.

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## Materials and Methods

**Adenoviral vectors.** Vectors were constructed and produced as described previously (6). Briefly, murine CD40L cDNA was inserted into the AdEASY adenoviral vector system (kind gift from Dr. B. Vogelstein, Johns Hopkins, Baltimore, MD). E1- and E3-deleted, replication-deficient recombinant adenoviruses were produced by four rounds of infection of 293 cells. Virus titers were determined by plaque assay. The AdLacZ vector was a kind gift from Canji, Inc. (San Diego, CA).

**Experimental mouse model.** Mouse bladder 49 (MB49) cells (15) were a kind gift from Dr. Esuvaranathan (National University Hospital, Singapore). The Lewis lung cell carcinoma 1 (LLC1) cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM supplemented with penicillin-streptomycin (1%), sodium pyruvate (0.1%), and fetal bovine serum (10%; Invitrogen, Paisley, Scotland). The orthotopic model using poly-L-lysine described elsewhere was used (16). Briefly, C57BL/6 mice were anesthetized and catheterized (INSYTE.w24Gx3/4, BD Biosciences, San Diego, CA). To enhance tumor take, the bladders were preincubated with poly-L-lysine (Sigma, St. Louis, MO) before instillation of  $1 \times 10^6$  MB49 cells per mouse. *In vivo* transduction of the bladders was done with  $1 \times 10^9$  plaque forming units of adenoviral vector per mouse after pretreatment with Clorcompactin WCS-90 (0.1% solution; United Guardian, Inc., Hauppauge, NY) as transduction enhancer. Pretreatments and vector instillations were given on days 1, 7, and 14. Twenty mice per group were used. Ten mice were sacrificed at different time points to take biopsies and collect lymph nodes. The remaining mice were monitored for survival. All animal experiments were approved by the local animal ethics committee (Dnr:C43/2).

**Reverse transcription-PCR.** RNA was isolated using TRIzol reagent, and cDNA was obtained with Superscript II Reverse Transcriptase (Invitrogen). The following primers were used for PCR: forward 5'-GAGCAAGAGAGGCATCCTCA, reverse 5'-AGACAGCACTGTGTGGC-GT ( $\beta$ -actin); forward 5'-CCATGGATGATAGAAACATACAGCCAACT, reverse 5'-GCGGCCGCTCAGAGTTTGAGTAAGCCA (CD40L). The PCR reactions were carried out with Taq Polymerase from Invitrogen, and the PCR products were analyzed on 1% agarose containing ethidium bromide.

**Quantitative PCR.** Biopsies from tumor and lymph nodes were taken and snap-frozen in liquid nitrogen before storage at  $-80^\circ\text{C}$ . The biopsies were mechanically pulverized, and the RNA was purified using TRIzol (Invitrogen). The cDNA was synthesized as described above. Quantitative PCR was done using the Taqman system (iCycler, Bio-Rad Laboratories, Inc., Hercules, CA). The reaction was done with Taq polymerase AmpliTaq Gold (Applied Biosystems, Foster City, CA). Probes and primer pairs for  $\beta$ -actin and Foxp3 were designed as follows: forward 5'-TGAAGATCAAGATCATTGCT, reverse 5'-CTGCTT-GCTGATCCACATCTG, probe 5'-CCTGGCCTCAGTGTCCACCTTCC ( $\beta$ -actin); forward 5'-TTCCTTCCCAGAGTCTTCCAC, reverse 5'-CCAG-GATGGCCCATCGGATAAG, probe 5'-ATGGACTACTTCAAGTACCA-CAATATGCGACC (Foxp3). IL-12 primers were designed according to described sequences (ref. 17; Genset Oligos, Paris, France). To correct for the differing amounts of cDNA content between the samples, all copy numbers were corrected to  $\beta$ -actin. The mRNA copy number in 5  $\mu\text{L}$  cDNA is shown in the figures.

**Flow cytometry.** Cells were analyzed by flow cytometry using monoclonal antibodies against CD4 conjugated to FITC and directed against CD25 conjugated to phycoerythrin. The cells were stained with antibodies and subsequently analyzed by flow cytometry (FACSCalibur, BD Biosciences).

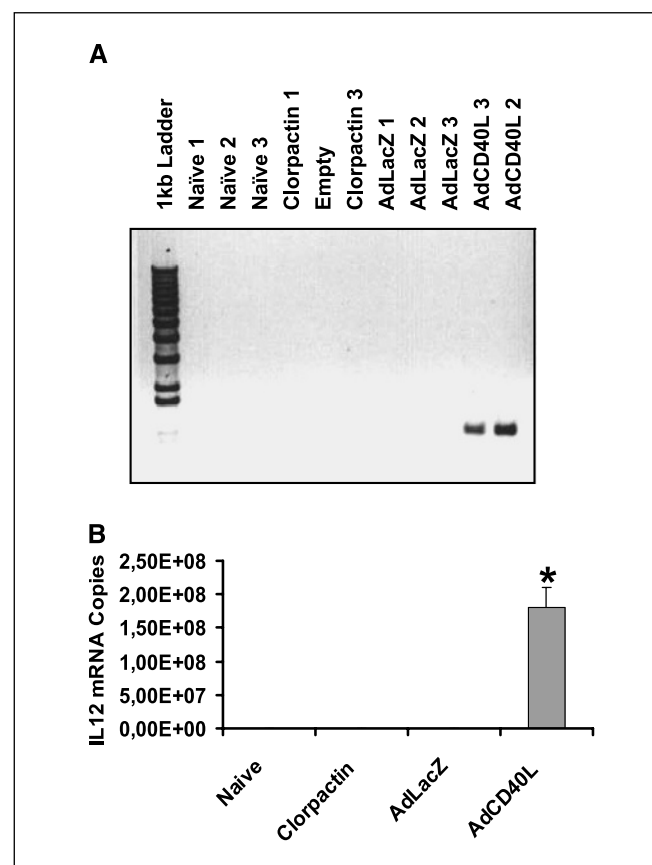
**Proliferation assay.** Lymph node cells in the different treatment groups were irradiated by 60 Gy and cocultured with anti-CD3/IL-2-stimulated naive lymph node cells at different ratios. The cells were cultured in 96-well flat-bottomed wells in a total volume of 200  $\mu\text{L}$  of RPMI supplemented with penicillin-streptomycin (1%), glutamine (1%), sodium pyruvate (0.1%), fetal bovine serum (10%),  $\beta$ -mercaptoethanol (1%), HEPES (1%), and MEM (1%; Invitrogen).

Twenty microliters of Alamar Blue were added per well, and the absorbance was measured at different time points according to the manufacturer's protocol (Biosource International, Camarillo, CA).

**Statistical analysis.** The results were statistically evaluated using Microsoft Analyseit by Excel. The methods of analyses were calculations of SE of mean, Student's *t* test, and Mann-Whitney *U* test.

## Results

**AdCD40L efficiently transduces bladder epithelium and initiates interleukin-12 expression.** Mice were implanted with tumor using the poly-L-lysine method as described (16). Treatments started at day 1 and were given once weekly. After three treatments, the mice were monitored for a total of 50 days. Clorcompactin was used as a transduction enhancer because the bladder wall is otherwise fairly resistant to adenovector gene transfer (16). CD40L expression was examined 24 hours after treatment by reverse transcription-PCR in three mice per group run in parallel to the 10 monitored mice. CD40L was strongly expressed in the bladders treated with AdCD40L vector (Fig. 1A). Upon CD40L interaction with CD40 on dendritic cells, dendritic cells mature and release IL-12 (8). Correspondingly, the CD40L-expressing bladders coexpressed high levels of IL-12 mRNA as shown by quantitative PCR (Fig. 1B).



**Fig. 1.** Detection of CD40L and IL-12 in bladder tissue. AdCD40L-treated bladders expressed CD40L in contrast to the other groups. *B*, screening for IL-12 mRNA by quantitative PCR. Columns, mean ( $n = 3$  mice per group); bars, SE.  $\beta$ -Actin levels were used to correct for different amounts of RNA in the samples. IL-12 mRNA copies were significantly increased in bladders from AdCD40L-treated mice (Student's *t* test:  $\alpha = 0.01$ ,  $P = 0.0011$ ). Low amounts of IL-12 could be detected in the other groups (too low to appear).

AdCD40L gene therapy cures mice with orthotopic bladder tumors. The MB49 orthotopic model is an aggressive tumor model. Because the adaptive immune system needs ~7 days to build up responses, we initiated treatments soon after implantation. Hematuria is an early sign of tumor growth, both in human bladder cancer and the orthotopic MB49 murine model. Already after 1 week of tumor growth, hematuria could be detected in >50% of all mice independently of treatment course (Fig. 2A). At day 14, AdLacZ mice and the

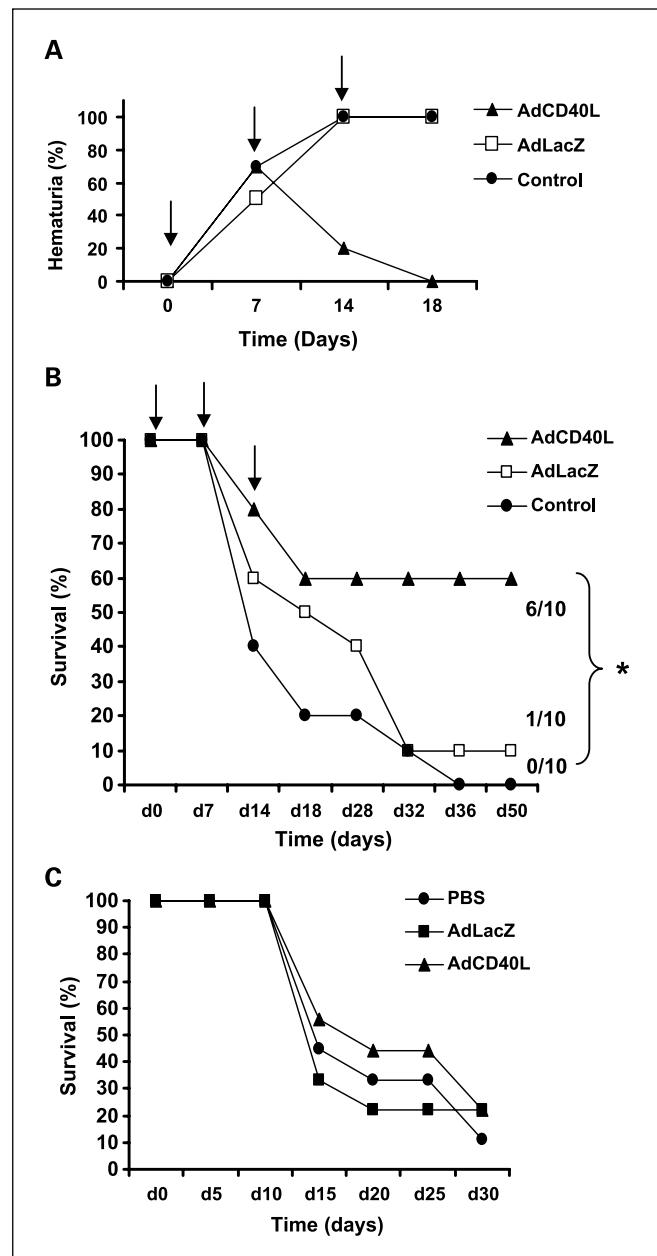


Fig. 2. *In vivo* antitumor effects of AdCD40L. Mice were implanted with tumor and subsequently treated three times with adenoviral vectors (days 1, 7, and 14). Clorpectin was used as a transduction enhancer in (A) and (B). Control mice received either PBS or Clorpectin. These mice showed equal results and are together as a control group. Each group consisted of 10 mice except for (C) that shows nine mice per group. A, presence of hematuria during the first 2 weeks of monitoring. B, survival of mice. The AdCD40L group was significantly different from control and AdLacZ mice (Mann-Whitney *U* test:  $\alpha = 0.05$ ,  $P = 0.0232$ ). C, tumor-bearing mice receiving adenovector treatments without the enhancer Clorpectin.

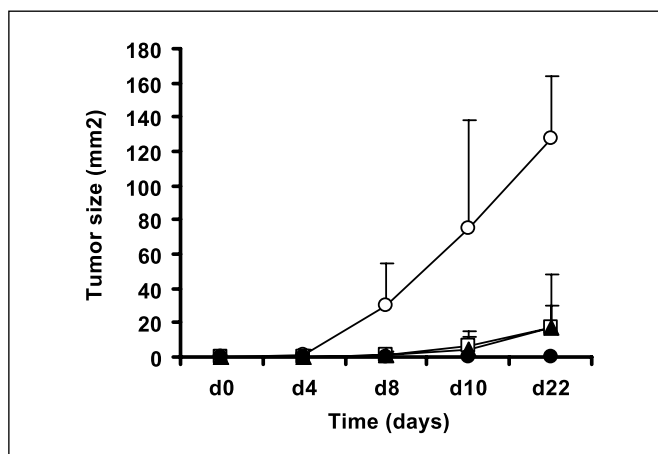


Fig. 3. Induction of protective immunity by AdCD40L. At day 50, the surviving AdCD40L/Clorpectin mice from Fig. 2B received s.c. challenge with native MB49 cells and syngeneic LLC1 cells. Tumor growth in naive versus AdCD40L cured mice. MB49 cells grew aggressively in naive mice (○) but not in the cured mice (●), whereas the slowly growing LLC1 cells formed tumors in both naive (□) and cured mice (▲).

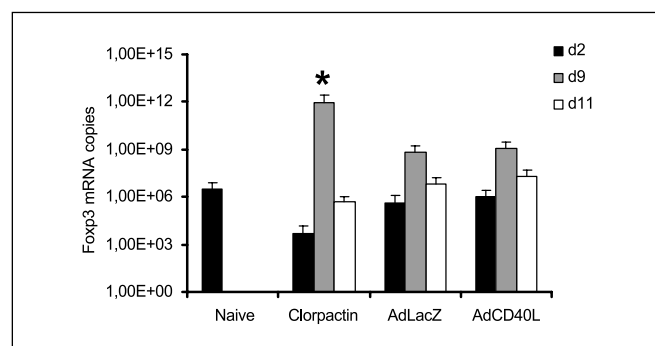
control mice all presented with hematuria. However, in the AdCD40L group, only 20% of mice were positive. Four days later, none of the AdCD40L mice had hematuria. A severe sign of tumor growth is rapid weight loss. If the mice lose 20% of body weight or show other signs of discomfort, they need to be immediately sacrificed. At day 14, half of the mice in the control group (6 of 10) and AdLacZ group (4 of 10) had to be euthanized. Also in the AdCD40L group, two mice lost weight and were euthanized (Fig. 2B). Tumor growth was confirmed in all sacrificed mice by macroscopy. At day 18, the survival in all groups decreased further. However, during examination of the bladders, it was revealed that the two AdCD40L mice did not have growing bladder tumors. A white hard necrotic mass was seen that blocked the urethra, which probably caused the signs of distress leading to euthanization. All of the remaining six AdCD40L-treated mice were completely cured from tumor. All control mice had large tumor loads and did not survive after day 36. The AdLacZ treatment had some effect on survival up to day 28, but at day 50, only one mouse was still alive. To confirm that Clorpectin was needed for the treatment effect in the AdCD40L group, mice were treated by adenovectors alone. In Fig. 2C, it is shown that AdCD40L instillation alone without Clorpectin preincubation of the bladders of tumor-bearing mice had no effect on survival.

**Cured mice show systemic and MB49-specific immunity.** The mice cured by Clorpectin-AdCD40L were s.c. challenged with naive MB49 tumor cells. Syngeneic LLC1 cells were injected in the opposite flank. All mice rejected the MB49 tumor cells but LLC1 cells formed tumors (Fig. 3). Naive mice were injected with both MB49 and LLC1 as a control of tumor take. LLC1 cells grew slowly compared with MB49 cells, and the experiment reached a natural end point when control mice carried large MB49 tumors and had to be euthanized. The AdCD40L-cured mice could be monitored further to verify that the LLC1 cells continued to grow. These experiments showed that the antitumor effect by *CD40L* gene therapy was associated with a MB49-specific and systemic immune response.

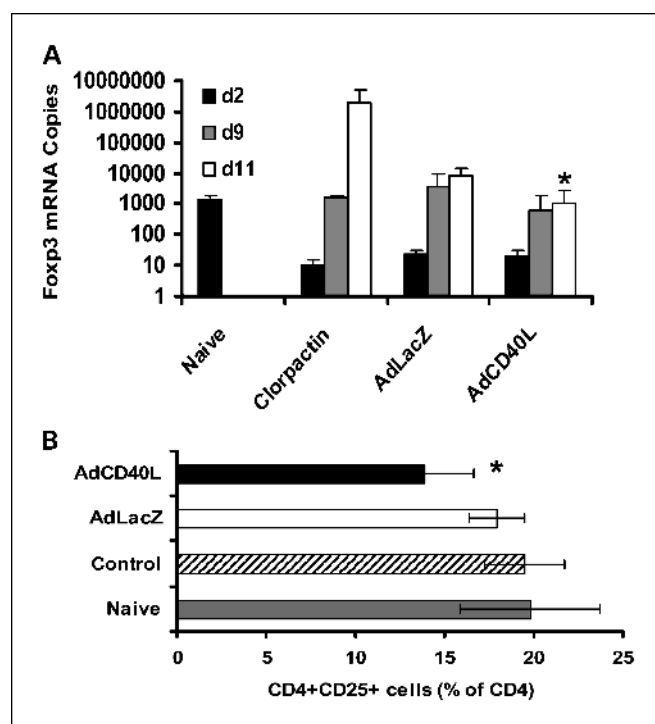
**CD40L gene therapy counteracts T regulatory cells.** During the recent years, many tumors have been shown to be infiltrated by T regulatory cells. These cells effectively inhibit CTLs; thereby, the tumor evades immune destruction (18). T regulatory cells coexpress surface CD4 and CD25 as well as the transcription factor Foxp3 (19). Bladders from naive mice and mice with different treatments were screened for Foxp3 mRNA expression. On day 9, mice receiving Clorpactin alone had higher Foxp3 levels than the other groups. Besides this day, there were no significant differences among the groups (Fig. 4). However, upon tumor inoculation the number of Foxp3 mRNA copies increased in control treated mice (Clorpactin) at day 11, whereas the Foxp3 levels were significantly lower in AdCD40L-treated mice (Fig. 5A, in log scale to visualize the Clorpactin group together with the other groups). Moreover, the lymph nodes of AdCD40L-treated mice showed a lower number of CD4<sup>+</sup>CD25<sup>+</sup> cells as shown by flow cytometry (Fig. 5B). The AdLacZ vector also had some effect on Foxp3 levels although that did not seem to effect survival of the mice. To investigate these T regulatory-like cells further, their capacity of inhibiting proliferation of other T cells was examined. Figure 6 shows that cells from Clorpactin and AdLacZ mice could significantly inhibit the growth of stimulated naive cells at a 1:1 ratio compared with AdCD40L-treated mice. This experiment points out that CD40L gene therapy may systemically abrogate T regulatory cells even if the Foxp3 levels within the tumors were not different from controls.

## Discussion

The MB49 orthotopic bladder cancer model is aggressive, and few experimental therapies of established tumors have shown complete tumor regression or long-term survival. The current treatment of human bladder cancer is instillation of BCG as described. In the MB49 mouse model, BCG may increase survival of treated mice, but complete responses are rare even with four to six treatments. The mice survive as long as treatment is given, but then the tumor progresses and eventually kills the mice (12–14). Therefore, we aimed to develop an alternative treatment for BCG-resilient tumors.



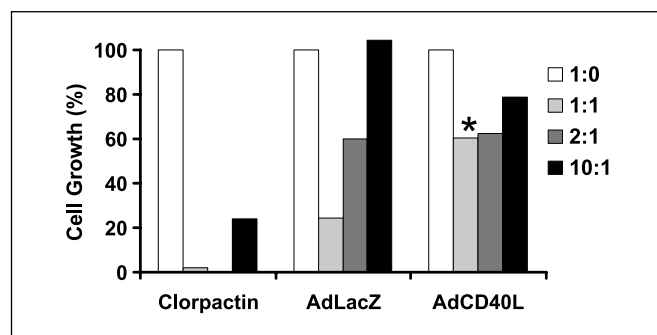
**Fig. 4.** Foxp3 expression in bladder tissue. At different time points, mice in Fig. 2B were sacrificed, and tumor biopsies were analyzed for expression of Foxp3 mRNA by quantitative PCR.  $\beta$ -Actin levels were used to correct for different amounts of RNA in the samples. Columns, mean; bars, SE. There were no significant differences in Foxp3 mRNA levels among the different groups, except for the Clorpactin group day 9 that exhibited higher levels (Student's *t* test:  $\alpha = 0.01$ ,  $P < 0.0001$ ). The naive group represent bladders without tumor.



**Fig. 5.** Foxp3 expression in lymph nodes. The lymph nodes from mice in the different treatment groups were analyzed for the presence of CD4<sup>+</sup>CD25<sup>+</sup> cells by flow cytometry (A) and Foxp3 mRNA by quantitative PCR (B). Columns, mean; bars, SE. AdCD40L-treated mice had lower amounts of CD25<sup>+</sup> cells in the CD4<sup>+</sup> population compared with the other groups as well as naive mice without tumor (Student's *t* test:  $\alpha = 0.01$ ,  $P = 0.0008$ ). Correspondingly, the Foxp3 mRNA copy number were lower in AdCD40L-treated mice compared with Clorpactin-treated control mice at day 11 (Student's *t* test:  $\alpha = 0.01$ ,  $P < 0.0001$ ). However, the levels in naive mice were similar to those of AdCD40L-treated mice.

CD40L gene therapy has proven extremely potent in different mouse models, including our s.c. MB49 model (6, 9), where all tumors regressed upon repeated i.t. AdCD40L injections. However, when instilling AdCD40L into the bladders, the transduction levels were poor, and no significant antitumor effects were seen. Because the bladders are naturally protected from viral and bacterial infection by physical barriers, such as the gag-mucin layer, viral vector gene transfer may be blocked as well. We have previously described methods for breaking this barrier using transduction enhancers (16). By far, the best enhancer was Clorpactin, which was subsequently used in this study. Clorpactin did not affect tumor growth compared with PBS but was necessary for aiding AdCD40L treatment. This drug has been used to treat patients with interstitial cystitis (20). Therefore, we do not foresee difficulties in using the compound for clinical trials.

CD40L was strongly expressed in AdCD40L-treated bladders but was not detected in the other groups. To investigate the function of transgene expression, we did quantitative PCR for the Th1 cytokine IL-12. IL-12 was strongly expressed by CD40L-expressing bladders, indicating that dendritic cells in the bladder were activated due to CD40 stimulation. The vector backbone carrying an irrelevant transgene (LacZ) did not increase IL-12 mRNA copies. Hence, CD40L gene therapy initiates Th1-related responses in the tumor milieu. CD40L gene therapy cured 60% of mice with orthotopic tumors. Because this group initially had hematuria, a clear sign of



**Fig. 6.** AdCD40L therapy counteracts T regulatory cell function. Lymph node cells from mice in the different treatment groups were irradiated with 60 Gy and cocultured at different ratios with lymph node cells from naive mice stimulated with anti-CD3 antibodies and IL-2. Proliferation was measured by Alamar Blue. Naive cells proliferated vigorously upon anti-CD3/IL-2 stimulation (1:0, 100%) but could be inhibited by cells from Clorpactin-treated mice. One of three experiments with similar results. The AdCD40L group significantly differed from the Clorpactin group at a 1:1 ratio (Student's *t* test:  $\alpha = 0.01$ ,  $P < 0.0001$ ).

tumor growth, CD40L not only prevented tumor take but actually regressed established tumors. Tumor take was easily confirmed in euthanized mice by macroscopy. The tumor-bearing bladders were heavily infiltrated with blood vessels already at early tumor stages. Two of the four sacrificed mice in the AdCD40L group did not have growing tumors. Instead, white necrotic tumor debris blocked the urethra causing discomfort, and the mice had to be sacrificed. There were no visible signs of autoimmunity with bladder destruction neither in these mice nor in the other mice. The cured AdCD40L mice resisted s.c. challenge with MB49 cells but could not resist syngeneic irrelevant tumor cells (LLC1). This experiment showed that the therapy-induced immunity was MB49 specific as well as systemic.

Apart from induction of Th1-related responses confirmed by IL-12 expression and systemic tumor-specific immunity, CD40L seemed to abrogate T regulatory cell development and/or function. More specifically, T cells in the lymph nodes from AdCD40L-treated mice had reduced Foxp3 levels at day 11 compared with mice receiving Clorpactin alone. The levels were reduced to levels seen in lymph nodes of naive tumor-free mice. Moreover, T cells from CD40L-treated mice did not inhibit the proliferation of mitogen-stimulated naive T cells as potently

as did T cells from Clorpactin- or AdLacZ-treated mice. We speculate that the activation/maturation of dendritic cells by CD40L reverts the suppressive milieu that promotes T regulatory cell differentiation and function. However, the Foxp3 levels within bladder tumors were not different from the levels in naive mice with the exception of Clorpactin-treated mice at day 9 after tumor implantation. At day 9, AdLacZ and AdCD40L had similar Foxp3 levels. The backbone vector may have had some stimulatory effect by itself. By day 11, the Foxp3 were present at similar levels in all groups. Still, the systemic reduction of T regulatory cells in AdCD40L-treated mice may have allowed potent antitumor responses to be generated. The presence of Foxp3 in CD40L-treated bladders may be a natural mechanism to prevent autoimmunity following local inflammation. In other murine tumor models, tumor rejection as well as inhibition of tumor growth has been achieved by systemic depletion of T regulatory (CD4<sup>+</sup>CD25<sup>+</sup>) cells before tumor inoculation (21, 22). Human bladder carcinomas are infiltrated by Foxp3<sup>+</sup>CD4<sup>+</sup> T cells. Moreover, patient peripheral T cells were unresponsive to anti-CD3/IL-2 stimulation but were able to inhibit proliferation of T cells from healthy donors.<sup>1</sup> Hence, human bladder cancer patients seem to share several features with the MB49 mouse model, and patients may also benefit from CD40L gene therapy.

In conclusion, in this article, we showed that adenoviral vectors carrying the CD40L gene could efficiently transduce the murine bladder epithelium, provided it was used together with the transduction enhancer Clorpactin. Furthermore, AdCD40L gene therapy cured 60% of the mice with aggressive orthotopic tumors and stimulated a systemic tumor-specific immune response. The current immunotherapy boosts Th1 responses via activation of dendritic cells (IL-12 production) and further inhibits the development and/or function of T regulatory cells. Based on our published work and the results presented here, a clinical phase I/II trial of AdCD40L and the enhancer Clorpactin is under way.

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<sup>1</sup> Loskog et al., submitted for publication.

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