# Captopril, an Angiotensin-Converting Enzyme Inhibitor, Promotes Growth of Immunogenic Tumors in Mice

Piotr J. Wysocki, Eliza P. Kwiatkowska, Urszula Kazimierczak, Wiktoria Suchorska, Dariusz W. Kowalczyk, and Andrzej Mackiewicz

**Abstract**

**Purpose:** Antitumor potential of angiotensin-converting enzyme inhibitors has been shown in different preclinical settings, which always involved immunocompromised organisms or nonimmunogenic tumor models. In our study, we wanted to evaluate the effect of captopril on growth of immunogenic tumors in immunocompetent animals.

**Experimental Design:** We used different murine tumor models to evaluate the effect of captopril on tumor take and survival of tumor-bearing immunocompetent and immunocompromised mice. We used an orthotopic renal cell cancer model and highly immunogenic tumor model, which were based on kidney subcapsular injection of RenCa cells or s.c. injection of MethA cells, respectively. To show the influence of captopril on antigen-specific immune responses, we have used two model antigens (green fluorescent protein and β-galactosidase).

**Results:** Captopril decreased survival of RenCa-bearing, immunocompetent mice in a dose-dependent manner and in adjuvant setting. In nephrectomized mice, captopril shortened their survival. Captopril promoted formation of immunogenic MethA sarcoma tumors but had no effect on nonimmunogenic melanoma cells (B78-H1). Treatment of immunocompromised mice bearing MethA tumors or RenCa kidney tumors with captopril did not affect tumor formation nor survival, respectively. Captopril-treated mice immunized with AdLacZ or AdGFP vectors did not generate or generated decreased numbers of antigen-specific $CD8^+$ T cells, respectively. However, they showed B-cell responses represented by infiltration of MethA tumors with activated B cells and dramatically increased serum level of β-galactosidase-specific antibodies.

**Conclusions:** Our results show a novel role of captopril in tumor biology and the tumor-promoting properties of captopril seem to be associated with its immunomodulatory potential.

Captopril (D-3-mercapto-2-methylopropanoyl-L-proline), an orally active inhibitor of angiotensin-converting enzyme (ACE) that converts angiotensin I into angiotensin II, is widely used in the management of hypertension and heart failure (1). In addition to its ability to reduce blood pressure, captopril has also several other biological activities. It can ameliorate arthritis (2), reverse diabetic retinopathy (3), enhance insulin sensitivity (1), lower thrombotic risk (4, 5), decrease atherosclerosis and renal failure (6–8), and lower the incidence of radiation-induced pulmonary damage and radiation-induced fibrosarcomas in rats (9, 10).

Moreover, ACE inhibitors are believed to decrease the risk of cancer due to reduction of angiotensin II activity, which in experimental studies was shown to stimulate neoangiogenesis by increasing vascular endothelial growth factor production (11–14), potentiate the mitogenic effects of epidermal growth factor (15), and induce cellular oncogenes c-myc, c-fos, c-N-ras, and growth factors, such as transforming growth factor-β (16, 17).

A retrospective cohort study of 5,207 patients with a follow-up of 10 years showed that long-term use of ACE inhibitors may protect against cancer, especially lowering the relative risk of gynecologic cancers (18). Long-term captopril intake decreased also the risk of prostate cancer (19). Experimental studies *in vivo* showed that captopril inhibited tumor growth and extended survival of tumor-bearing mice by inhibition of angiogenesis and tumor metastatic potential (20). However, antitumor potential of ACE inhibitors has only been shown in immunocompromised organisms or nonimmunogenic tumor models. Captopril is also known to display significant immunomodulatory functions (21).

In our study, we evaluated the efficacy of captopril in treatment of immunogenic tumors and showed that it promoted growth of these tumors and significantly impaired generation of functional antigen-specific $CD8^+$ T cells.

## Materials and Methods

***Animals.*** Female BALB/c, C57BL/6, and C.B-17 *scid/scid* F1 mice, ages 8 to 12 weeks, were used. BALB/c and C57BL/6 mice were

**Authors' Affiliation:** Department of Cancer Immunology, University of Medical Sciences at GreatPoland Cancer Center, Poznan, Poland

Received 11/15/05; revised 4/18/06; accepted 4/27/06.

**Grant support:** State Committee of Scientific Research (Warsaw, Poland) grant PBZ-KBN 091/P05/54/2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Piotr J. Wysocki, Department of Cancer Immunology, University of Medical Sciences at GreatPoland Cancer Center, Garbary Street 15, 61-866 Poznan, Poland. Phone: 48-603-119-271; Fax: 48-061-852-8502; E-mail: pwysocki@plusnet.pl.



doi:10.1158/1078-0432.CCR-05-2489

purchased from the Polish Academy of Sciences (Warsaw, Poland) and C.B-17 *scid/scid* mice were obtained from Harlan UK Ltd. (Oxon, United Kingdom). Animals were kept under constant pathogen-free conditions in rooms with 12-hour day/night cycle with unlimited access to food and water. All experiments were done according to the guidelines approved by the Ethical Committee at the University of Medical Sciences (Poznan, Poland).

***Tumor cells.*** Highly immunogenic murine RenCa (renal cell carcinoma) cells, MethA (cholantrene-induced sarcoma) cells, and low immunogenic B78-H1 melanoma cells were used throughout experiments. Cells were maintained in DMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics, and 2 mmol/L L-glutamine (all from Invitrogen), hereafter called culture medium. Cells were cultured in 78 $cm^2$ culture plates at 37°C in a fully humidified atmosphere of 5% $CO_2$/95% air and passaged every 3 to 4 days.

***Peptides.*** The H-2$K^b$-restricted β-galactosidase 96-103 (DAPIYTVN) CTL epitopic peptide (22) and an unrelated, H-2$K^b$-restricted, control peptide (VMCYSPPL; ref. 23) were synthesized by Polygen (Lodz, Poland). The H-2$K^d$-restricted enhanced green fluorescent protein (EGFP) 200-208 (HYLSTQSAL) CTL epitopic peptide was synthesized by ProImmune Ltd. (Oxford, United Kingdom; ref. 24).

***Adenoviral recombinants.*** An E1-deleted adenoviral recombinant of the human strain 5 expressing β-galactosidase, termed AdLacZ, has been purchased from Invitrogen. An E1-deleted adenoviral recombinant of the human strain 5 expressing EGFP, termed AdGFP, was kindly provided by Dr H. Ertl (Wistar Institute, Philadelphia, PA). The viruses were propagated and titrated on E1-transfected 293 cells as described previously (25).

**In vivo *experiments.*** All study groups comprised eight animals. All experiments were repeated thrice. MethA and B78-H1 cells mice were inoculated s.c. into left hip of mice on day 0 ($5 \times 10^5$ cells suspended in 100 μL PBS).

Mice (BALB/c or C.B-17 *scid/scid*) receiving renal subcapsular injection of RenCa cells were anesthetized with Avertin anesthesia according to a standard protocol (26). Briefly, mice received i.p. injection (0.7 mg/g) of Avertin working solution (2-2-2 tribromoethanol diluted in tert-amyl alcohol). Skin of the anesthetized mice at left lumbar region was shaved with an electric shaver. Next, the skin and s.c. tissue were cut with a scalpel and the left kidney was exposed. Using a tuberculin syringe, $1 \times 10^4$ RenCa cells suspended in 10 μL PBS were injected subcapsularly into the exposed kidney. Finally, the wound was closed with two to three surgical stitches. In a set of experiments, 10 days after tumor implantation, mice were again anesthetized, the tumor-bearing kidney was exposed, and following ligation of renal artery and vein a nephrectomy was done according to uro-oncological guidelines. In all animals that received subcapsular injections of RenCa cells, an autopsy was done to determine the cause of death.

Tumor-bearing mice were receiving captopril dissolved in drinking water in doses of 25 or 60 mg/kg/d. Twice weekly tumor growth and mice survival were monitored.

***Flow cytometry analysis of tumor-infiltrating lymphocytes.*** Tumor-bearing control and captopril-treated mice inoculated with MethA cells were sacrificed 20 days after inoculation (mean tumor diameter, 1 cm). Tumors were excised, minced, and pooled (eight mice per group), and tumor-infiltrating mononuclear cells were isolated by gradient centrifugation. The single-cell suspension was stained with anti-CD8, anti-CD4, anti-CD40 (FITC), anti-CD25, anti-CD62L (phycoerythrin), and anti-B220 (allophycocyanin) monoclonal antibodies (PharMingen/Becton Dickinson, San Diego, CA).

***Immunization protocol.*** Captopril-treated and control groups of eight 8- to 12-week-old BALB/c mice were immunized s.c. with $1.5 \times 10^7$ plaque-forming units of recombinant AdGFP diluted in 100 μL saline. Captopril-treated and control groups of C57BL/6 mice were immunized s.c. with $1.5 \times 10^7$ plaque-forming units of recombinant AdLacZ diluted in 100 μL saline. Eight days after immunization, splenocytes were harvested and processed for CTL and T-helper assays. In some experiments, 14 days after AdGFP immunization, mice were boosted i.p. with $1.5 \times 10^6$ RenCa cells expressing EGFP and splenocytes were harvested 7 days after second immunization.

For antibody response measurement, 14 days after immunization, blood was collected by retro-orbital puncture for serum preparation.

***Detection of antigen-specific CD8$^+$ T cells.*** Freshly isolated splenocytes obtained from nonimmunized, AdGFP-immunized nontreated, and captopril-treated mice ($2 \times 10^6$ cells) were incubated with allophycocyanin-labeled Pro5 MHC pentamer H-2$K^d$-HYLSTQSAL (ProImmune) at room temperature for 15 minutes in the dark and washed with PBS. Cells were then incubated with phycoerythrin-conjugated anti-mouse CD8 antibody (PharMingen) at 4°C for additional 20 minutes in the dark. After completing the staining process, cells were washed again and then analyzed immediately using a FACSCan flow cytometer (BD Biosciences, San Diego, CA).

***Intracellular IFN-γ staining of CD8$^+$ T cells.*** Freshly isolated splenocytes obtained from nonimmunized, AdGFP- or AdLacZ-immunized nontreated, and captopril-treated mice ($10^6$ cells) were stimulated with epitopic peptide (0.2 μg/mL) overnight in 1 mL RPMI with 10% FBS and L-glutamine supplemented with 25 ng/mL human recombinant interleukin (IL)-2 (Bender MedSystems, Vienna, Austria) and 50 μmol/L 2-mercaptoethanol. On the next day, cells were incubated with 1 μL/mL brefeldin A (GolgiPlug, PharMingen) for 6 hours at 37°C, then washed with PBS, and surface stained with allophycocyanin-conjugated anti-mouse CD8 antibody. After washing, cells were fixed at 4°C for 20 minutes with Cytofix/Cytoperm (PharMingen), washed with PermWash (PharMingen), and stained for IFN-γ with phycoerythrin-conjugated rat anti-mouse IFN-γ monoclonal antibody (PharMingen). Background staining and thresholds between positive and negative populations were determined by staining with a phycoerythrin-conjugated isotype control antibody (rat IgG1; PharMingen). Cells were then analyzed using FACSCan flow cytometer.

***Cytokine release assay.*** Splenocytes obtained from nonimmunized, AdLacZ-immunized nontreated, and captopril-treated mice ($10 \times 10^6$ cells per well) were cultured in 1.5 mL RPMI with 10% FBS and L-glutamine in 24-well plates with or without 2.5 μg/mL recombinant β-galactosidase (Sigma-Aldrich, St. Louis, MO). After 72 hours of stimulation, supernatants were tested for IL-2, IL-4, IL-10, and IFN-γ by standard ELISA or Mouse Th1/Th2 Cytokine Cytometric Bead Array (BD Biosciences).

***T-helper proliferation assay.*** Splenocytes obtained from nonimmunized, AdLacZ-immunized nontreated, and captopril-treated mice ($1 \times 10^6$ cells per well) were cultured in 200 μL RPMI with 10% FBS and L-glutamine in 96-well plates with or without 2.5 μg/mL relevant recombinant proteins for 3 to 5 days. Subsequently, [$^3$H]thymidine (1 μCi/well) was added for 10 hours. Cells were then harvested and the radioactivity was measured using a scintillation counter.

***Detection of antigen-specific antibodies by ELISA.*** Specific anti-β-galactosidase antibodies were measured by ELISA on 96-well plates (Maxisorp, Nunc, Greiner, Germany) coated with recombinant β-galactosidase (2.5 μg/mL in PBS, 100 μL/well) and blocked with 3% bovine serum albumin in PBS. Sera obtained from nonimmunized and AdLacZ-immunized, control, and captopril-treated mice were added in serial dilutions in PBS, starting with 1:200 dilution. After 1-hour incubation, plates were washed four times with PBS-Tween 20 and then incubated with 1:5,000 dilution of horseradish peroxidase–conjugated goat anti-mouse immunoglobulin (Dako Cytomation, Glostrup, Denmark) for 1 hour. After extensive washing with PBS-Tween 20, substrate (OPD tablets, Sigma-Aldrich) was added for 20 minutes at room temperature. After stopping the reaction with 3 mol/L HCl, plates were read in an automated ELISA microplate reader (Bio-Tek Instruments, Denkendorf, Germany) at 490 nm. Each sample was tested at eight log 2 dilutions set up in duplicate wells. Specific titers were determined as the reciprocal of the highest serum dilution giving absorbance value at least 2-fold higher than background.

**In vitro *expansion of CTLs.*** Splenocytes obtained from AdGFP-immunized, control, and captopril-treated mice were cocultured

($6 \times 10^6$ cells) with irradiated (8 Gy) RenCa cells expressing GFP ($1.5 \times 10^4$) in RPMI with 10% FBS and L-glutamine supplemented with 50 μmol/L 2-mercaptoethanol and 25 ng/mL recombinant human IL-2 for 3 days. In some experiments, splenocytes were stimulated with epitopic peptide (0.2 μg/mL) in RPMI with 10% FBS and L-glutamine supplemented with 50 μmol/L 2-mercaptoethanol and 25 ng/mL recombinant human IL-2 for 5 to 7 days.

***Statistical analyses.*** Survival curves were analyzed by log-rank test. For the comparison of tumor take rate between groups, a $\chi^2$ test was used. Differences between samples in immunologic test were analyzed for significance by Student's *t* test (two-tailed) or one-way ANOVA test.

## Results

***Captopril decreases survival of renal cell cancer–bearing, immunocompetent mice in a dose-dependent manner.*** To evaluate the effect of captopril on survival of mice with immunogenic tumors, RenCa cells were implanted subcapsularly into left kidney. Fourteen days after initiation of the experiment, mice receiving high-dose captopril (60 mg/kg/d) started to die and all animals in this group died after 63 days (median survival, 56 days). In mice receiving low-dose captopril (25 mg/kg/d), the survival time was longer (median, 63 days). The longest survival was observed in the control group (median, 70 days). A significant difference ($P = 0.02$) in median survival was observed between the high-dose captopril and control groups (Fig. 1A). In all animals, autopsy revealed massive tumors of left kidney, ascites, and lung metastases.

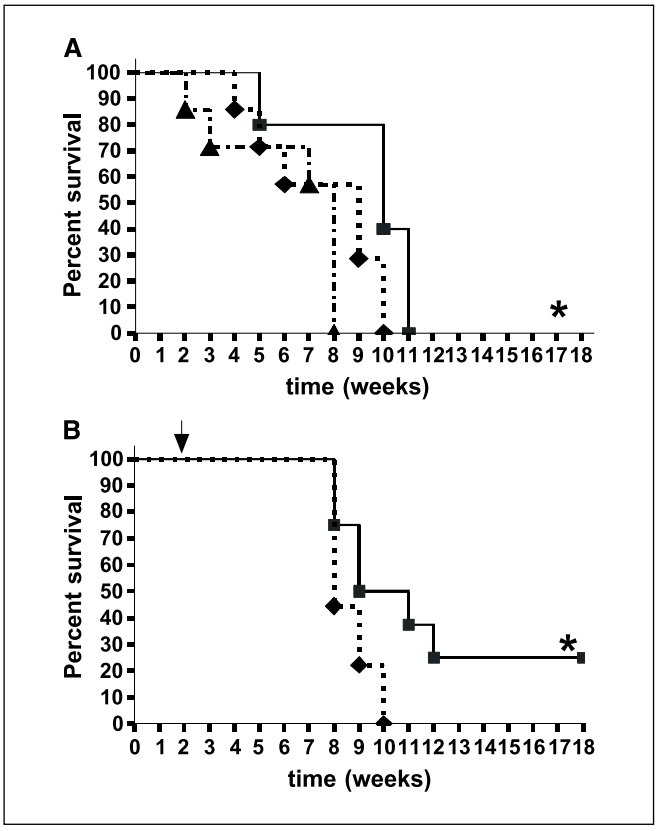


**Fig. 1.** Effect of captopril on survival of mice with orthotopically implanted renal cell cancer cells (RenCa). *A,* captopril decreases survival of BALB/c mice with orthotopic renal cell cancer in a dose-dependent manner. *, $P < 0.02$. *B,* adjuvant administration of captopril (25 mg/kg/d) after nephrectomy of kidneys containing renal cell cancer tumors decreases survival of surgically treated mice. *Arrow,* time of nephrectomy (10 days after implantation of RenCa cells). ■, control (plain water); ♦, low-dose captopril (25 mg/kg/d); ▲, high-dose captopril (60 mg/kg/d).

***Captopril in an adjuvant setting decreases survival of mice with resected primary tumor.*** Nephrectomy or partial nephrectomy is a standard treatment in renal cell cancer patients. Accordingly, we analyzed the effect of captopril in an adjuvant setting on the survival of mice that underwent resection of the primary kidney tumor 10 days after implantation of RenCa cells. One day after the surgical procedure, mice in a good condition were randomized into two groups: one received plain drinking water and the other received drinking water with captopril (25 mg/kg/d). Seventy days after the nephrectomy, all captopril-treated animals died, whereas in the control group 25% of animals were still alive at the end of the experiment (130 days after resection of the primary tumor; Fig. 1B). A significant difference ($P < 0.05$) in median survival was observed between the experimental groups. In all animals that died during the experiment, autopsy revealed numerous lung metastases and ascites due to neoplastic infiltration of peritoneum.

***Captopril promotes growth of immunogenic tumors in immunocompetent mice.*** To determine whether the tumorigenic effect of captopril depended on the immunogenicity of tumor cells, highly immunogenic MethA and low immunogenic B78-H1 cells were used. Mice that received a suboptimal dose ($5 \times 10^5$) of MethA cells (inducing tumors in ~50% of animals) were divided into two groups: control and low-dose captopril (25 mg/kg/d). Within 28 days, all captopril-treated mice developed tumors, whereas in the control group ~50% of animals remained tumor-free until the end of the experiment (Fig. 2A). There was a statistically significant difference in tumor take between both groups ($P < 0.05$).

Subsequently, C57BL/6 mice received s.c. injection of non-immunogenic murine melanoma cells (B78-H1). There were no significant differences in the dynamics of tumor take between both control and low-dose captopril groups. However, a slight delay was visible in the captopril-treated group: 25% of animals developed tumors 7 days later than animals in the control group (Fig. 2B).

To evaluate whether the observed tumorigenic effect of captopril was associated with an impairment of specific immune mechanisms, we analyzed its influence on growth of MethA tumors in *scid/scid* mice and survival of the *scid/scid* mice with implanted RenCa tumors. Both control and captopril-treated (25 mg/kg/d) *scid/scid* mice developed s.c. MethA tumors much faster than immunocompetent animals. On day 17 in all *scid/scid* mice of both groups, palpable tumors were detected (Fig. 2C) and the dynamic of tumor formation in both study groups was comparable. Similarly, there were no statistically significant differences in the survival between control and captopril-treated *scid/scid* mice after subcapsular implantation of RenCa cells (Fig. 2D).

***Captopril augments antigen-specific CD4$^+$ T-cell responses.*** To assess a magnitude of specific CD4$^+$ T-cell responses *in vitro*, we studied proliferative responses after stimulation with the whole β-galactosidase protein. Splenocytes from AdLacZ-immunized mice were cultured *in vitro* with β-galactosidase for 5 days followed by a pulse with [$^3$H]thymidine. Mice immunized with AdLacZ alone had a weak but measurable antigen-specific proliferative response against β-galactosidase. However, captopril treatment during immunization process led to a significant β-galactosidase proliferative response *in vitro*. The proliferation

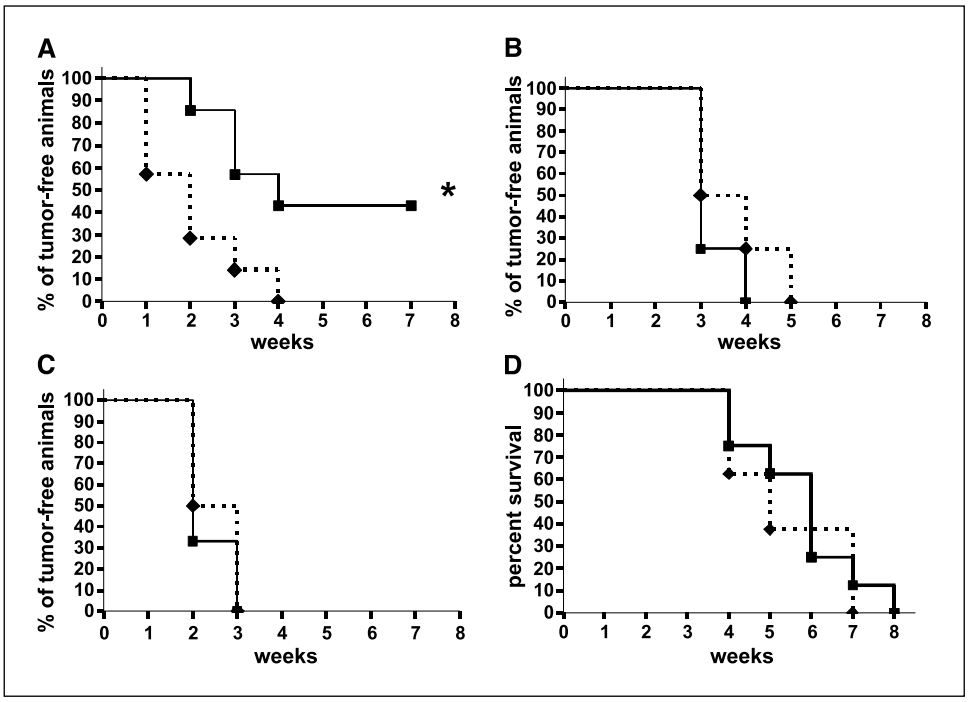


**Fig. 2.** Effect of captopril on the formation of s.c. implanted tumors. *A,* captopril promotes growth of immunogenic tumors in BALB/c mice after s.c. injection of suboptimal (inducing tumors in 50% of animals) dose of MethA cells. *B,* captopril slightly retards the onset of nonimmunogenic murine melanoma tumors in C57BL/6 mice. *C,* captopril does not influence the growth of immunogenic MethA tumors in *scid*/*scid* mice. *D,* captopril does not influence the survival of *scid*/*scid* mice with orthotopic renal cell cancer. ■, control (plain water); ♦, low-dose captopril (25 mg/kg/d).

rate of splenocytes from captopril-treated group was 3-fold higher compared with control, AdLacZ-immunized mice ($P < 0.001$; Fig. 3A).

To evaluate the immunomodulatory effect of captopril on $CD4^+$ T cells in qualitative terms, splenocytes from AdLacZ-immunized mice were stimulated *in vitro* with β-galactosidase for 48 hours. After stimulation, culture supernatant was collected and analyzed for IL-2, IL-4, IL-10, and IFN-γ by ELISA. In both control and captopril-treated mice, *in vitro* restimulation with β-galactosidase induced a comparable cytokine production in splenocytes. Although there was a slightly higher level of cytokine production in captopril-treated group, the difference seemed to be insignificant. These results indicate that captopril does not modulate considerably Th1/Th2 balance in responding lymphocytes in this particular setting (Fig. 3B).

***Effect of captopril on the humoral immune responses.*** Fifteen days after immunization with recombinant AdLacZ, blood samples from control and captopril-treated animals were analyzed for β-galactosidase-specific antibodies. In the captopril-treated group, there was a 16-fold increase ($P < 0.001$) of anti-β-galactosidase antibodies compared with the control group (Fig. 3C).

***Captopril impairs development of antigen-specific $CD8^+$ T cells.*** To evaluate the effect of captopril on mounting of antigen-specific $CD8^+$ T-cell responses, two model antigens (GFP and β-galactosidase) were used.

In the first set of experiments, control and captopril-treated mice were immunized with the recombinant adenoviral vector encoding GFP. The population of GFP-specific, $CD8^+$ splenocytes of captopril-treated animals 14 days after immunization followed by 7 days of *in vitro* restimulation was ~2-fold smaller than of control mice (Fig. 4A).

In the next set of experiments, to evaluate the effect of captopril on formation of antigen-specific, IFN-γ-secreting $CD8^+$ T cells, another model antigen (β-galactosidase) was used in C57BL/6 mice. As shown on Fig. 4B, AdLacZ immunization induced weak but measurable antigen-specific CTL responses. However, in contrast to control mice, where ~0.6% of circulating $CD8^+$ T cells displayed the β-galactosidase specificity, captopril-treated animals did not mount any antigen-specific CTL response (Fig. 4B).

***Captopril increases infiltration of immunogenic tumors by B cells.*** Immunophenotypic analysis of tumor-infiltrating lymphocytes was carried out to shed more light on the possible mechanisms involved in promotion of tumor growth in captopril-treated animals. Flow cytometry analysis of tumor-infiltrating lymphocytes did not reveal significant differences in the T-cell infiltrate from each group studied (Fig. 5A-C). However, the MethA tumors in the captopril-treated animals were more densely (+30%) infiltrated by B cells as shown by anti-CD40 and anti-B220 staining (Fig. 5D).

## Discussion

There are five major findings of these studies. Captopril (*a*) promotes growth of immunogenic tumors, (*b*) promotes tumor recurrence in minimal residual disease, (*c*) impairs generation of antigen-specific $CD8^+$ T cells, (*d*) enhances infiltration of immunogenic tumors by B cells, and (*e*) increases production of antigen-specific antibody.

We employed an orthotopic animal model of renal cell cancer, which, in contrast to the s.c. model, resembles clinical course of renal cancer in human in terms of growth pattern, metastatic potential, and responsiveness to systemic treatment (27). Other orthotopic tumor models, such as a prostate cancer model, have also shown importance and specificity of a microenvironment on tumor biology (28). In various *in vivo* studies, a broad range of captopril doses have been evaluated (19-94 mg/kg/d; refs. 13, 29, 30). We have concentrated on the

effect of captopril on tumor growth at doses that were well tolerated by animals in our study.

In studies of Small et al. and Hii et al., high doses of captopril (80 and 94 mg/kg/d, respectively) markedly reduced tumor growth and its metastatic potential and extended survival of treated mice. However, Small et al. used a low immunogenic mammary carcinoma cells (MA-16), whereas Hii et al. employed a xenogeneic model of human renal cell cancer cells implanted into immunocompromised *scid/scid* mice (29, 30). Moreover, low doses of captopril (20 mg/kg/d) were also shown to inhibit growth of particular tumors (e.g., murine hepatoma; ref. 14). In contrast, in our studies, captopril promoted growth of renal cell cancer in an orthotopic model in mice. Moreover, adjuvant administration of captopril to mice with resected renal tumors shortened their survival, which indicated that captopril might also enhance progression of a minimal residual disease. The 25% survival rate of nontreated animals after resection of primary kidney tumor resembles clinical conditions, where 10-year survival rate of localized renal cancer patients who underwent nephrectomy varies between 20% and 50% (31). Our studies employing immunocompetent and immunocompromised mice and high and low immunogenic tumors (MethA and B78-H1 cells, respectively) clearly showed that the tumor-promoting properties of captopril were associated with its influence on the immune system. We have not observed such spectacular antitumor effects of captopril in *scid/scid* mice as described by Hii et al. most likely due to a significantly lower dose of captopril used. The antitumor effect of captopril in the above-mentioned studies was only observed in mice receiving the highest dose of

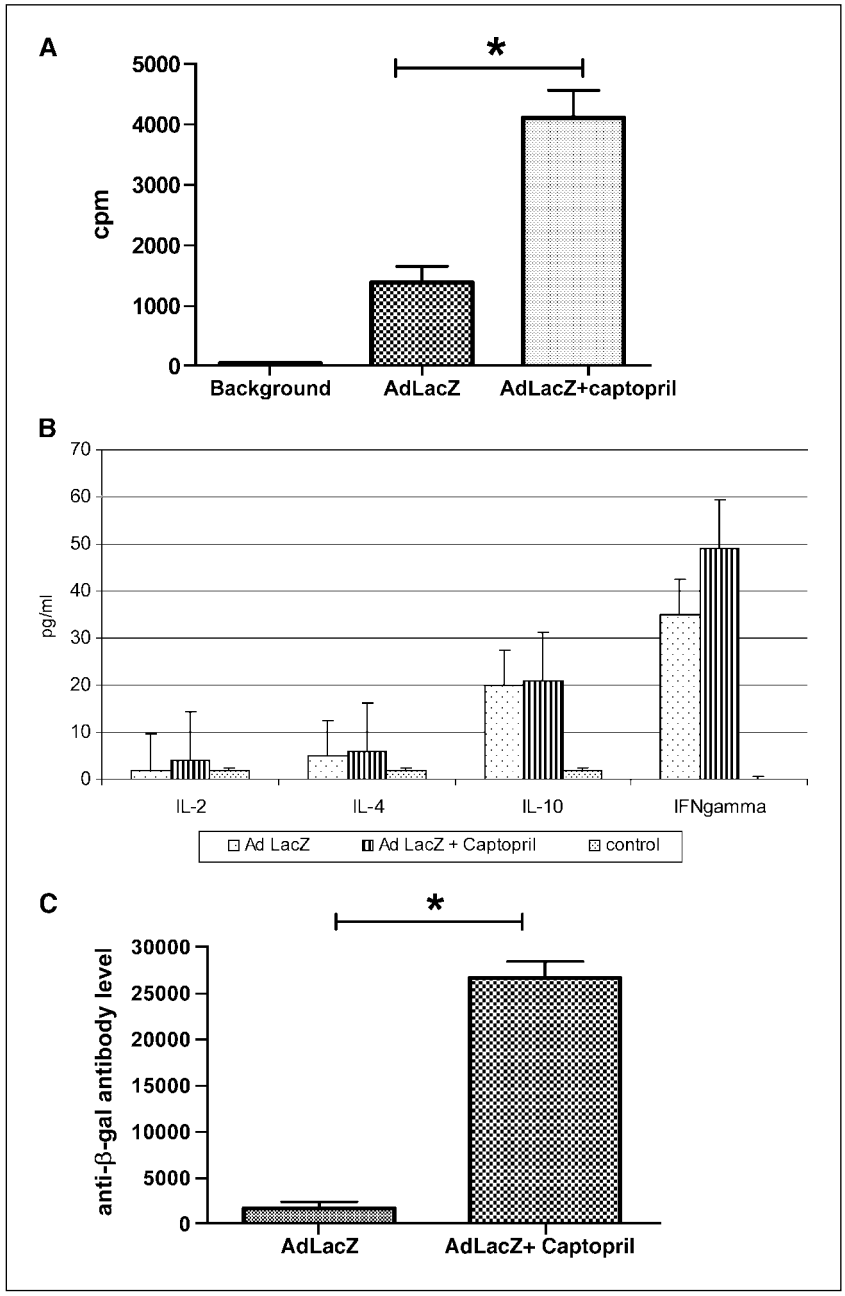


**Fig. 3.** Effect of captopril on particular variables of immune response. *A,* T-helper immune responses *in vivo* are increased by captopril. Splenocytes from AdLacZ-immunized control or captopril-treated mice were stimulated *in vitro* with β-galactosidase in 5 days [$^3$H]thymidine proliferation assay. Splenocytes from mock-immunized mice were used as control. Captopril is able to enhance T-helper immune responses induced by AdLacZ vaccine by a factor of 2 to 3. *, $P < 0.001$. *B,* captopril does not modify Th1/Th2 balance. Splenocytes from AdLacZ-immunized, control, and captopril-treated mice were stimulated with purified β-galactosidase. After 48 hours of culture, supernatant was harvested and assayed for cytokine content by ELISA. Splenocytes from naive mice were used as control. *C,* captopril increases production of β-galactosidase-specific antibodies in AdLacZ-immunized mice. Groups of mice were immunized with AdLacZ and fed with captopril in drinking water or plain water. After 2 weeks, serum was collected and tested on ELISA plates coated with β-galactosidase. Data are end point titers defined as the inverse of the highest dilution that resulted in an absorbance value twice greater than that of the preimmune serum with a cutoff value of 0.05. *, $P < 0.001$.

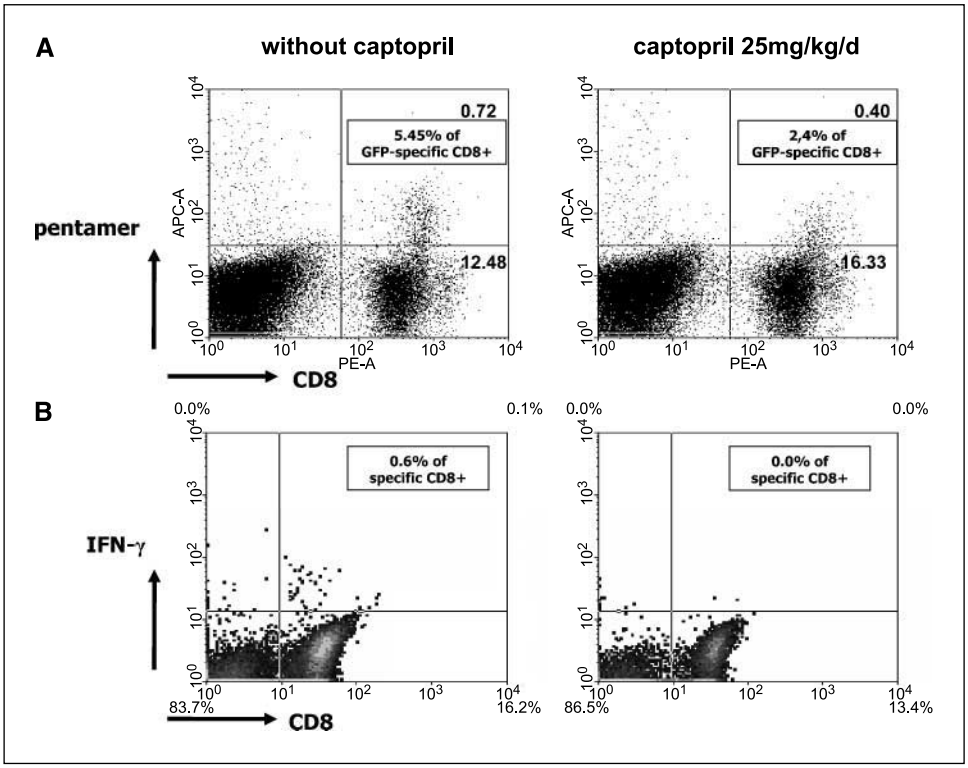


**Fig. 4.** Captopril inhibits cytotoxic T-cell responses *in vivo*. *A,* splenocytes from AdGFP-immunized, control, or captopril-treated mice were stimulated with GFP peptide followed by anti-CD8 plus pentamer staining and then analyzed by flow cytometry. Captopril-treated mice generated 50% smaller population of GFP-specific $CD8^+$ T cells compared with controls. *B,* splenocytes from AdLacZ-immunized, control, or captopril-treated mice were stimulated with β-galactosidase peptide followed by anti-CD8 plus intracellular anti-IFN-γ staining and then analyzed by flow cytometry. Whereas AdLacZ-immunized mice show β-galactosidase-specific $CD8^+$ T cells producing IFN-γ, captopril treatment inhibits such $CD8^+$ T-cell response *in vivo*.

the drug (94 mg/kg/d). In our experiments, similar doses of captopril were toxic and caused a significant mortality of treated animals (data not shown). However, the slightly delayed development of nonimmunogenic B78-H1 murine melanoma tumors in captopril-treated, immunocompetent mice may confirm the existence of antitumor potential of captopril in particular conditions.

The behavior of immunogenic tumors suggests that captopril may promote tumor growth through regulation of the immune system. Captopril is known for its immunomodulatory properties (32). It inhibits T-cell reactivity (33, 34), suppresses IL-12 production (35), induces immune unresponsiveness, and prolongs allograft survival (36). Results of our studies indicated that the tumor-promoting activity of captopril depended on a modulation of host immune system. First, indirect evidence came from observations of highly immunogenic tumors, such as MethA and low immunogenic B78-H1 melanoma tumors. Enhanced formation of tumors in all captopril-treated animals after injection of suboptimal dose of MethA cells strongly indicated its tumor-promoting activity. However, captopril did not affect MethA tumor growth in immunodeficient mice. Thus, in an organism, which is unable to mount an immune response against tumor antigens due to either a host immune incompetence or low tumor immunogenicity, captopril is not showing its tumor-promoting properties and may display antitumor activity associated with inhibition of tumor vessel formation.

Assuming that captopril may influence the immune system, which in turn controls growth of immunogenic tumors, we evaluated immunomodulatory functions of captopril *in vivo*. Because precise quantitative analyses of immune responses to undefined tumor antigen are problematic, we decided to use GFP and β-galactosidase as model antigens. Therefore, to assess the immunomodulating activity of captopril *in vivo*, we immunized groups of control and captopril-treated mice with an adenoviral vector encoding either β-galactosidase or GFP. Immunization of control mice with an AdGFP vector generated ~10% of antigen-specific $CD8^+$ T cells. However, treatment of immunized mice with captopril decreased the GFP-specific population of $CD8^+$ T cells by 50%. By using another model antigen (β-galactosidase), we confirmed the observed inhibitory effect of captopril on generation of functional, antigen-specific CTLs. In nontreated animals, AdLacZ immunization induced measurable T-cell-mediated responses against adenovirus-derived β-galactosidase. The relatively low frequency of β-galactosidase-specific, IFN-γ-secreting $CD8^+$ T cells generated by immunization of mice with AdLacZ (0.6%) was similar to frequencies of specific CTLs observed in human after immunization with vaccinia or variola viruses (37). Moreover, we observed that administration of captopril significantly augmented infiltration of immunogenic tumors by activated B cells and boosted specific, anti-β-galactosidase antibody response. Such a behavior could indicate a shift of the ongoing immune reaction toward Th2-type response. However, to our surprise, we did not observe any changes in cytokine profiles, which would indicate on Th2 shift.

Tumors employed in our studies did not express MHC II molecules. Accordingly, induction of tumor immunity depended exclusively on priming of $CD4^+$ T cells by MHC $II^+$ antigen-presenting cells and then activation of CTL by MHC class I–restricted antigens (38, 39). It is possible that the mechanism of inhibition of T-cell-dependent immunity by captopril was associated with an increased number and activity of tumor-infiltrating B cells (40). It is acknowledged that B cells can compete with dendritic cells for tumor antigens. Although

B cells can also present antigens to T cells, they are far less efficient than dendritic cells (41). It is known that B cells are very inefficient in cross-priming and thus are not able to stimulate T cells against tumor antigens (42). The MHC product and MHC-peptide complexes are 10 to 100 times lower on B cells than on dendritic cells (43). Moreover, captopril was shown to directly impair immunostimulatory functions of dendritic cells by inhibition of the their endogenous renin-angiotensin system (44). An increased proliferation of antigen-specific $CD4^+$ T cells from captopril-treated mice does not exclude the possibility that some functions of these cells were impaired by captopril during the induction phase of immune response, which could not be detected by *in vitro* assays. Qin et al. have clearly showed that B cells can impair functions of

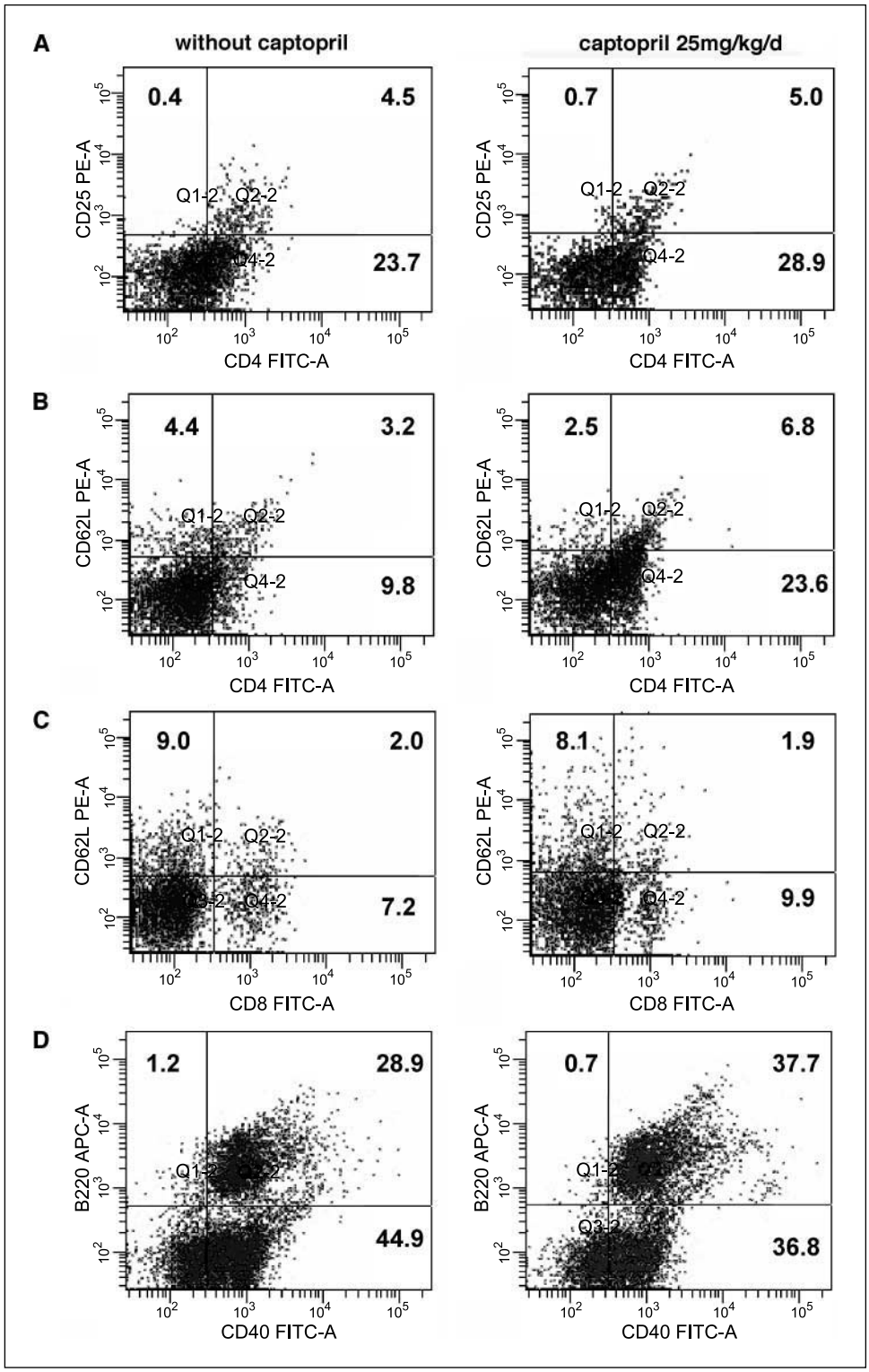


**Fig. 5.** Flow cytometry analysis of lymphocytes infiltrating s.c. MethA tumors in control and captopril-treated mice. There were no significant differences between both control and captopril-treated mice in $CD4^+CD25^+$ T cells (*A*), $CD4^+CD62L$ T cells (*B*), and $CD8^+CD62L$ T cells (*C*). However, in the captopril-treated mice, MethA tumors contained a significantly larger population of active $CD40^+B220^+$ B cells (30%) compared with control animals (*D*).

$CD4^+$ T cells in the generation of T-cell-dependent tumor immunity (40).

Both AdLacZ-immunized control and captopril-treated animals produced comparable and balanced amounts of IL-10 and IFN-γ in response to *in vitro* stimulation with the β-galactosidase. These data clearly showed that captopril had potent immunomodulatory functions that could affect immune responses. However, because we did not observe any changes in Th1/Th2 balance, the question why captopril inhibits antigen-specific CTL development still remains open.

As a potential anticancer treatment modality, captopril most likely displays two different opposing pharmacologic effects: (*a*) inhibition of T-cell responses with a potential to promote tumor growth and (*b*) antitumor activity due to the inhibition of tumor neoangiogenesis. Needless to say, in clinical settings, depending on the tumor type and patient immune status, captopril-based antitumor treatment should be properly considered.

From a perspective of our findings, the proposed tumor protection activity of ACE inhibitors deserves consideration. We think the observation that long-term use of ACE inhibitors may protect against cancer does not mean that captopril may be used as an anticancer agent. In prophylactic settings, it is still unknown whether captopril acts directly on tumor cells or cells in a premalignant state. Agents that could be applied in prophylactics do not necessarily display an antitumor activity and vice versa.

Moreover, obtained results and accumulated data indicate that management of hypertension in cancer patients based on administration of ACE inhibitors, such as captopril, may require reconsideration.



## References


1. Materson BJ, Preston RA. Angiotensin-converting enzyme inhibitors in hypertension. Arch Intern Med 1994;154:513–23.
2. Martin MFR, Surrall KE, McKenna F, Dixon JS, Bird HA, Wright V. Captopril: a new treatment for rheumatoid arthritis? Lancet 1984;8390:1325–8.
3. Jackson WE, Holmes DL, Garg SK, Harris S, Chase HP. Angiotensin-converting enzyme inhibitor therapy and diabetic retinopathy. Ann Opthalmol 1992;24: 99–103.
4. Collins R, Peto R, Flather M, et al. ISIS-4A randomized factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected acute myocardial infarction. Lancet 1995;345:669–85.
5. Young JB. Angiotensin-converting enzyme inhibitors in heart failure: new strategies justified by recent clinical trials. Int J Cardiol 1994;43:151–63.
6. Chobanian AV, Haudenschild CC, Nickerson C, Drago R. Antiatherogenic effect of captopril in the Watanabe heritable hyperlipidemic rabbit. Hypertension (Dallas) 1990;15:327–31.
7. Ashab I, Peer G, Blum M, et al. Oral administration of L-arginine and captopril in rats prevents chronic renal failure by nitric oxide production. Kidney Int 1995;47: 1515–21.
8. Lewis EJ. Captopril and diabetic nephropathy. J Am Med Assoc 1995;273:1831.
9. Ward WF, Kim YT, Molteni A, Solliday NH. Radiation-induced pulmonary endothelial dysfunction in rats: modification by an inhibitor of angiotensin converting enzyme. Int J Radiat Oncol Biol Phys 1988; 15:135–40.
10. Ward WF, Molteni A, Ts'ao C, Hinz JM. The effect of captopril on benign and malignant reactions in irradiated rat skin. Br J Radiol 1990;63:349–54.
11. Le Noble FA, Schreurs NH, van Straaten HW, et al. Evidence for a novel angiotensin II receptor involved in angiogenesis in chick embryo chorioallantoic membrane. Am J Physiol 1993;264:460–5.
12. Le Noble FA, Hekking JW, Van Straaten HW, Slaaf DW, Struyker Boudier HA. Angiotensin II stimulates angiogenesis in the chorio-allantoic membrane of the chick embryo. Eur J Pharmacol 1991;195:305–6.
13. Yoshiji H, Kuriyama S, Kawata M, et al. The angiotensin-I-converting enzyme inhibitor perindopril suppresses tumor growth and angiogenesis: possible role of the vascular endothelial growth factor. Clin Cancer Res 2001;7:1073–8.
14. Yoshiji H, Yoshii J, Ikenaka Y, et al. Suppression of the renin-angiotensin system attenuates vascular endothelial growth factor-mediated tumor development and angiogenesis in murine hepatocellular carcinoma cells. Int J Oncol 2002;6:1227–31.
15. Norman J, Badie-Dezfooly B, Nord EP, et al. EGF-induced mitogenesis in proximal tubular cells: potentiation by angiotensin II. Am J Physiol 1987;253: 299–309.
16. Wolf G, Neilson EG. Angiotensin II induces cellular hypertrophy in cultured murine proximal tubular cells. Am J Physiol 1990;259:768–77.
17. Wolf G, Ziyadeh FN, Helmchen U, Zahner G, Schroeder R, Stahl RA. ANG II is a mitogen for a murine cell line isolated from medullary thick ascending limb of Henle's loop. Am J Physiol 1995;268:940–7.
18. Lever AF, Hole DJ, Gillis CR, et al. Do inhibitors of angiotensin-I-converting enzyme protect against risk of cancer? Lancet 1998;352:179–84.
19. Ronquist G. Association between captopril, other antihypertensive drugs and risk of prostate cancer. Prostate 2004;58:50–6.
20. Molteni A, Ward WF, Ts'ao CH, et al. Cytostatic properties of some angiotensin I converting enzyme inhibitors and of angiotensin II type I receptor antagonists. Curr Pharm Des 2003;9:751–61.
21. Godsel LM, Leon JS, Engman DM. Angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists in experimental myocarditis. Curr Pharm Des 2003;9:723–35.
22. Heeger PS, Valujskikh A, Lehmann PV. Comprehensive assessment of determinant specificity, frequency, and cytokine signature of the primed CD8 cell repertoire induced by a minor transplantation antigen. J Immunol 2000;165:1278–84.
23. Vierboom MP, Nijman HW, Offringa R, et al. Tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. J Exp Med 1997;186:695–704.
24. Gambotto A, Dworacki G, Cicinnati V, et al. Immunogenicity of enhanced green fluorescent protein (EGFP) in BALB/c mice: identification of an H2-Kd-restricted CTL epitope. Gene Ther 2000;7:2036–40.
25. Kowalczyk DW, Wlazlo AP, Shane S, Ertl HC. Vaccine regimen for prevention of sexually transmitted infections with human papillomavirus type 16. Vaccine 2001;19:3583–90.
26. Weiss J, Zimmermann F. Tribromoethanol (Avertin) as an anaesthetic in mice. Lab Anim 1999;33:192–3.
27. Ahn KS, Jung YS, Kim J, Lee H, Yoon SS. Behavior of murine renal carcinoma cells grown in ectopic or orthotopic sites in syngeneic mice. Tumor Biol 2001; 22:146–53.
28. Vieweg J, Heston WD, Gilboa E, Fair WR. An experimental model simulating local recurrence and pelvic lymph node metastasis following orthotopic induction of prostate cancer. Prostate 1994;6:291–8.
29. Hii SI, Nicol DL, Gotley DC, Thompson LC, Green MK, Jonsson JR. Captopril inhibits tumour growth in a xenograft model of human renal cell carcinoma. Br J Cancer 1998;77:880–3.
30. Small W, Jr., Molteni A, Kim YT, Taylor JM, Ts'ao CH, Ward WF. Mechanism of captopril toxicity to a human mammary ductal carcinoma cell line in the presence of copper. Breast Cancer Res Treat 1999; 55:223–9.
31. Linehan WM, Shipley WU, Parkinson DR. Cancer of the kidney and urether. In: DeVita VT, Hellman S, Rosenberg SA, editors. Cancer: principles and practice of oncology. 5th ed. Philadelphia: Lippincott-Raven; 1997. p. 858–911.
32. Delfraissy JF, Galanaud P, Balavoine JF, Wallon C, Dormont J. Captopril and immune regulation. Kidney Int 1984;6:925–9.
33. Odaka C, Mizuochi T. Angiotensin-converting enzyme inhibitor captopril prevents activation-induced apoptosis by interfering with T cell activation signals. Clin Exp Immunol 2000;3:515–22.
34. Yeung JH. A comparison of the effects of angiotensin-converting enzyme inhibitors with bradykinin, angiotensin II and their specific antagonists on concanavalin A-induced proliferation of mouse T-lymphocytes. Methods Find Exp Clin Pharmacol 1994;3: 163–72.
35. Constantinescu CS, Goodman DBP, Ventura ES. Captopril and lisinopril suppress production of interleukin-12 by human peripheral blood mononuclear cells. Immunol Lett 1998;62:25–31.
36. Olausson M, Mjornstedt L, Wramner L, Herlitz H. Effect of captopril treatment on allograft survival and induction of unresponsiveness in heart-transplanted, ATG-treated rats. Nephrol Dial Transplant 1992;11: 1130–2.
37. Terajima M, Cruz J, Raines G, et al. Quantitation of $CD8^+$ T cell responses to newly identified HLA-A*0201-restricted T cell epitopes conserved among vaccinia and variola (smallpox) viruses. J Exp Med 2003;197:927–32.
38. Huang AY, Golumbek P, Ahmadzedeh M, Haffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science 1994;264:961–5.
39. Cayeux S, Richter G, Noffz G, Dorken B, Blankenstein T. Influence of gene-modified (IL-7, IL-4 and B7) tumor cell vaccines on tumor antigen presentation. J Immunol 1997;158:2834–42.
40. Qin Z, Richter G, Schuler T, Ibe S, Cao Z, Blankenstein T. B cells inhibit induction of T cell-dependent tumor immunity. Nat Med 1998;4:627–30.
41. Croft M. Activation of naive, memory and effector T cells. Curr Opin Immunol 1994;6:431–7.
42. Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18: 767–811.
43. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:242–52.
44. Lapteva N, Ide K, Nieda M, et al. Activation and suppression of renin-angiotensin system in human dendritic cells. Biochem Biophys Res Commun 2002; 296:194.