Published in final edited form as:

Cancer Immunol Immunother. 2010 March ; 59(3): 409–417. doi:10.1007/s00262-009-0760-1.

Intratumoral DNA electroporation induces anti-tumor immunity and tumor regression

Olga Radkevich-Brown, Marie P. Piechocki, Jessica B. Back, Amy M. Weise, Shari Pilon-Thomas, and Wei-Zen Wei

Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201

Abstract

In situ expression of a foreign antigen and an immune-modulating cytokine by intratumoral DNA electroporation was tested as a cancer therapy regimen. Transgene expression in the tumors sustained for 2–3 wks after intratumoral electroporation with mammalian expression plasmid containing firefly luciferase cDNA. Electroporation with cDNA encoding tetanus toxin fragment C (TetC) induced tetanus toxin-binding antibody, demonstrating immune recognition of the transgene product. Intratumoral electroporation with TetC and IL-12 cDNA after mice were treated with CD25 mAb to remove regulatory T cells induced IFN- γ producing T cell response to tumor associated antigen, heavy inflammatory infiltration, regression of established tumors and immune memory to protect mice from repeated tumor challenge. Intratumoral expression of immune modulating molecules may be most suitable in the neoadjuvant setting to enhance the therapeutic efficacy and provide long term protection.

Keywords

neoadjuvant immunotherapy; intratumoral DNA electroporation; tumor microenvironment

Introduction

The response to neoadjuvant or pre-operative chemotherapy has been correlated with overall survival, showing the importance of minimizing tumor load before definitive surgery [1, 9, 21]. It would be most advantageous to amplify anti-tumor immunity during neoadjuvant therapy to enhance long-term disease-free survival.

Tumor tissue is a rich reservoir of tumor-associated antigens (TAA), but endogenous immunity to TAA fails to reject tumors in cancer patients [14, 22, 26]. To amplify anti-tumor immunity, immunostimulatory molecules may be introduced systemically, but drug toxicity and induction of autoimmunity are dose limiting. There have been prior attempts to directly modulate tumors to avoid systemic side-effects. Glycolipid containing α -gal (Gal α 1-3Gal β 1-4GlcNAc-R) was inserted into tumor cell membrane for recognition by the abundant natural anti- α -gal Ab in humans [6]. CCL20 chemokine has been delivered to the tumor to recruit inflammatory cells [5], and MHC class II transactivator was expressed in the tumor to enhance antigen presentation to CD4 T cells [18]. Other immune modulating molecules, such as CpG-ODN [29], LIGHT [36], IL-12 [19], CD40L [17], or GM-CSF [4] have been introduced in the form of nucleotides, proteins, plasmid DNA, or

viral vectors. Some anti-tumor activity was achieved with these regimens, but the results have been variable.

We previously showed that removal of CD4⁺CD25^{hi} Treg by CD25 mAb resulted in the regression of established TUBO tumors that expressed rat neu and the induction of sustained anti-neu immunity in wt BALB/c, but not in neu-tolerant BALB NeuT transgenic mice [12, 33]. Therefore, the foreign neu expressed by the tumor cells facilitated tumor rejection in the Treg depleted environment. Tetanus toxin fragment C (TetC), a non-toxic, immunogenic fragment of tetanus exotoxin [10] that contains a universal CD4 T cell epitope [23], is a candidate molecule to facilitate immune priming in the tumor. In the US population, tetanus toxin is a recall antigen due to prophylactic immunization and may be exploited in cancer immunotherapy [15, 30].

TetC DNA has functioned as an adjuvant in specific cancer vaccines [3, 30]. Here, we tested the hypothesis that intratumoral expression of TetC in Treg-depleted mice would induce immune priming to endogenous tumor associated antigens. Plasmid DNA was used to deliver exogenous genes because it is free of confounding foreign entities and can be administered repeatedly, allowing continuous expression of the encoded molecules. Although only a fraction of the tumor cells was transfected *in vivo*, expression of secreted TetC (SecTetC) induced immune priming to tumor associated antigens. When combined with IL-12, the treatment resulted in tumor regression or growth retardation and protective immune memory.

Material and Methods

Mice

All animal procedures were conducted in accordance with accredited institution guidelines and the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (<http://grants.nih.gov/grants/olaw/olaw.htm#pol>). C57BL/6 and BALB/c (age 6–12 wks) mice were purchased from Charles River Laboratory (Frederick, MD) or bred in our animal facility.

Cell Lines and Reagents

Cell lines were cultured as previously described [11, 13]. BALB/c mouse mammary tumor lines D2F2 [20], D2F2/E2 [34], TUBO [2, 28], and C57BL/6 TC-1 lung cell line [16] have been described previously. TUBO and TC-1 were provided by Dr. Guido Forni, University of Turin, Orbassano, Italy and Dr. T.C. Wu, the Johns Hopkins University, Baltimore, MD, respectively. D2F2 cells were co-transfected with pGL3 (Promega, Madison, WI) and pRSV2/neo to generate D2F2/Luc cells that stably express luciferase. C57BL/6 mammary tumor line E0771 was obtained from the late Dr. Gordon Ross (University of Louisville, Louisville, KY).

Plasmid DNA and *in vivo* DNA electroporation

pGL3 encoding firefly luciferase under CMV promoter was purchased from Promega. pNGVL3-mIL-12 encoding murine IL-12 subunits p35 and p40 separated by IRES was purchased from National Gene Vector Lab (Ann Arbor, MI). pSecTag2B was from Invitrogen. pEFBos/GM-CSF (pGM-CSF) encoding murine GM-CSF was provided by Dr. N. Nishisaka (Osaka University, Osaka, Japan).

pLPCX/TetC containing human codon-optimized cDNA of TetC was obtained from Dr. Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore, MD). A 1.37-kb HindIII/BamHI fragment encoding full length TetC, aa 1–452, was isolated from pLPCX/TetC and

ligated into pSecTag2B. The Ig κ leader sequence is in frame with the 5' end of TetC. The orientation and integrity of the inserted sequences were verified by restriction analysis and DNA sequencing.

For intratumoral DNA electroporation, mice were inoculated subcutaneously (s.c.) with $1-2 \times 10^5$ indicated tumor cells. When tumors reached 2×2 to 3×3 mm, mice were anesthetized with ketamine/xylazine, and 100 μ g of each indicated plasmid DNA was injected into the tumor in a total volume of 50 μ l of sterile saline using a tuberculin syringe with a 26-gauge needle. Needle was inserted into the tumor mass, and DNA solution was slowly dispensed into the tumor along the needle track, while the needle was being withdrawn. The injection was followed immediately by square wave electroporation using BTX830 apparatus (BTX Harvard Apparatus, Holliston, MA). Tweezers applicator was placed around the tumor to deliver eight pulses at 100V for 20msec. Intratumoral treatment was repeated after 3–6 days. Tumor growth was monitored by weekly palpation and tumor volume was calculated by $X^2Y/2$, where X and Y represent the short and long dimension, respectively. Comparison of tumor volume at a given time point was analyzed by 1-tail Student's *t* test. Differences in tumor incidence were analyzed by log-rank test.

Western blot analysis of recombinant SecTetC protein

Serum-free culture supernatants were collected from BALB/c NIH 3T3 fibroblasts transfected with SecTetC DNA, concentrated 13 fold using Millipore Centricon YM-10 (Millipore, Bedford, MA) and analyzed by Western blot as described previously [24]. Membranes were hybridized with goat anti-tetanus exotoxin IgG (Cortex Biochem, Concord, MA) or sera from mice immunized with pSecTetC, followed by labeling with HRP-conjugated donkey anti-goat or goat anti-mouse Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were developed with enhanced SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) and imaged with IS4000 imager (Kodak, New Haven, CT). Recombinant TetC was the positive control.

Measurement of anti-TetC Ab by ELISA

The 96-well flat bottom microtiter ELISA plates were pre-coated by overnight incubation with 50 μ L of 0.25 flocculation units/ml adsorbed tetanus toxoid (Sanofi Aventis, Bridgewater, NJ). The plates were incubated with immune sera and bound IgG was detected with goat anti-mouse-HRP (Santa Cruz Biotechnology) and ABTS substrate. Differences in Ab concentration were analyzed by Student's *t* test.

Treg depletion

To deplete CD25^{hi} Treg, mice were injected i.p. with 60 μ g PC61 (ATCC, Manassas, VA) mAb, one day after tumor implantation. Depletion of CD25^{hi} T cells was verified by flow cytometry, as previously described [27, 33].

In vivo and *in vitro* luciferase assays

At indicated times following intratumoral pGL3 electroporation, mice were anesthetized with ketamine/xylazine and injected i.v. with 1.6 mg D-luciferin (BD Biosciences, San Jose, CA). Luminescence and X-ray images were obtained with an IS4000MM multimodal imager (Kodak) and the levels of bioluminescence were analyzed using MetaMorph software (Molecular Devices). As positive control, D2F2/Luc tumor cells stably transfected with luciferase were injected s.c. into the opposite flank. As an additional control, 50 μ g of pGL3 in 25 μ l PBS was injected into quadriceps of mice followed by electroporation [11, 13].

For *in vitro* activity assay, tumors were electroporated twice, 3 days apart with saline or pGL3 before they were removed 7 or 10 days after initiation of DNA electroporation. Tumor tissue was weighed, and dissociated into single cell suspension prior to lysis with 1× CCLR buffer (Promega). Luciferase activity was measured using a Promega *in vitro* luciferase assay system, and protein concentration was calculated using QuantiLum recombinant luciferase as the standard.

Measurement of T cell response by ELISpot Assay

All ELISpot reagents were purchased from BD Biosciences. Her-2 reactive T cells in tumor-draining lymph nodes were enumerated by IFN- γ and IL-5 ELISpot assay as we previously described [13, 27]. Data were analyzed using the 1-tail Student's *t* test.

Immunohistochemistry

Tissue sections of the tumors and draining lymph nodes were stained with Hematoxylin and Eosin and histological images were collected with a Zeiss microscope using a Sony 970 CCD camera interfaced with the MCID7 imaging software package.

Results

Kinetics of protein expression after intratumoral DNA electroporation

To evaluate the level and duration of gene expression by intratumoral DNA electroporation, BALB/c mice were inoculated s.c. in the right flank, with D2F2 mammary tumor cells. When the tumors were 2×2 mm or greater, pGL3 DNA encoding firefly luciferase was injected into the tumor, followed immediately by electroporation over the injection site. The procedure was repeated in 3 days. To measure luciferase activity, the enzyme substrate D-luciferin was injected i.v. 1 wk after DNA electroporation. Luminescence (Fig. 1a, left panel) and X-ray (Fig. 1a, middle panel) images were acquired and superimposed (Fig. 1a, right panel) to illustrate the concordance between the light emitted from cleavage of D-luciferin and the location of tumor (Fig. 1a, “1”), thus demonstrating luciferase activity in the treated tumor. Each mouse also received pGL3 electroporation into the right quadriceps muscle with corresponding luminescence (Fig. 1a, “2”). Therefore, luciferase is expressed whether luciferase cDNA was electroporated into the tumor or muscle. As a positive control, D2F2/Luc cells stably transfected with luciferase were injected into the opposite flank, and luminescence of the tumor was evident (Fig. 1a, “3”).

To measure the kinetics of recombinant luciferase expression, five BALB/c mice were inoculated s.c. with D2F2 tumors, followed by 2-time intratumoral pGL3 electroporation. Relative luminescence intensity was recorded at 6 hrs and on days 2, 4, 7, 10, 14, 17, 21, 24, 30, and 37 after the initial DNA electroporation (Fig. 1b). Luminescence was detected in all five test mice at 6 hrs. With some fluctuation, luminescence at $100\text{--}250 \times 10^3$ relative luminescence units sustained for 2 to 3 wks before declining. Luciferase activity was also detected after intramuscular DNA electroporation for 3–4 wks (data not shown). Therefore, DNA electroporation resulted in localized gene expression at the site of injection.

Luciferase gene expression was verified *in vitro* in a repeated experiment. On days 7 and 10 after intratumoral pGL3 electroporation, tumors were removed and dissociated. Luciferase activity in tumor lysates was measured as described in Materials and Methods, and enzyme concentration calculated using QuantiLum recombinant luciferase as the standard. Luciferase activity was detected in all four treated tumors (Fig. 1c, filled circles), but not five control tumors (Fig. 1c, open circles).

To test if DNA electroporation is applicable to other solid tumors, pGL3 expression was tested in BALB/c mice bearing TUBO tumor and C57BL/6 mice bearing E0771 or TC-1 tumor. Luminescence was detected when measured on days 7 or 10 after intratumoral pGL3 electroporation (Fig. 1d). Therefore, consistent gene expression was achieved in all test tumors, showing the feasibility of using intratumoral DNA electroporation as a standard treatment regimen.

Construction and characterization of pSecTetC encoding a secreted tetanus toxin fragment C (TetC)

TetC contains a promiscuous helper epitope, p30, which can be presented by several mouse and human MHC class II alleles, and Ab to TetC recognize tetanus toxin [10]. Because the bacterial coding sequence does not translate efficiently in mammalian cells, TetC sequence optimized for mammalian expression [31] was used to construct pSecTetC that encodes a secreted protein for antigen dispersion in the tumor after *in vivo* transfection (Fig. 2a).

NIH 3T3 fibroblasts were transiently transfected with pSecTetC, and a ~64 kDa band was detected in the culture supernatant by Western blotting using goat anti-tetanus exotoxin Ab (Fig. 2b), showing secretion of SecTetC by transfected cells. The 50 kDa recombinant bacterial TetC (rTetC) was the positive control. The higher molecular weight of SecTetC produced by transfected cells was likely the result of protein glycosylation in mammalian cells.

To test the immunogenicity of SecTetC, BALB/c mice were electrovaccinated once, i.m. with pSecTetC and pGM-CSF. Anti-TetC Ab was detected by Western blotting against SecTetC from the culture supernatant (Fig. 2c). Binding to the 64 kDa SecTetC was detected with immune but not control mouse sera, showing the induction of anti-TetC Ab by pSecTetC electrovaccination.

To test whether immune sera also recognized the native bacterial tetanus protein, binding of immune sera to tetanus toxoid was measured by ELISA (Fig. 2d). pSecTetC immune sera but not control sera bound to tetanus toxoid. Therefore, Ab induced by pSecTetC recognized both SecTetC and native bacterial tetanus toxoid, demonstrating the immunogenicity of pSecTetC.

Immune responses to TetC were induced by intratumoral DNA electroporation

To enhance immune response, mice were treated with 60 µg CD25 mAb one day after tumor injection, which was previously shown to deplete CD25^{hi}Foxp3⁺ cells for about 3 wks [27]. In mice inoculated with D2F2 tumor, 60 µg CD25 mAb and intratumoral electroporation of blank vector reduced tumor growth in 1 of 7 mice (Supplementary Fig. S1) showing minimal impact of Treg depletion and blank vector treatment.

To test if pSecTetC administered into the tumor induces immune response, BALB/c mice inoculated with D2F2 tumor were electroporated with pSecTetC in the tumor when the tumors were 2–3 mm in diameter (Fig. 3, left panel). Humoral immune response to TetC was detected in the immune sera 2 weeks later at 1:200 dilution by the binding to tetanus toxoid using ELISA (Fig. 3, right panel). Treatment with CD25 mAb further enhanced the antibody response (Fig. 3, right panel). Therefore, SecTetC was expressed by intratumoral DNA electroporation to mount a significant immune response.

Inhibition of D2F2 tumor growth by expressing SecTetC and IL-12 in the tumor

To test the anti-tumor efficacy, BALB/c mice inoculated with D2F2 tumor received CD25 mAb and two-time intratumoral electroporation with pSecTetC and pIL-12. All mice treated

with blank vector showed progressive tumor growth (Fig. 4a). In mice treated with pSecTetC, one tumor (25%) regressed after wk 3. In mice receiving pIL-12, 2 of 5 (40%) tumors regressed after wk 3. When mice received both pSecTetC and pIL-12, 5 of 8 (63%) tumors regressed after transient growth (Fig. 4a). Therefore, significant tumor rejection was achieved by low-dose CD25 mAb and intratumoral electroporation with pSecTetC and pIL-12. This experiment has been repeated twice with similar results, i.e. pSecTetC + pIL-12 > pIL-12 > pSecTetC in tumor growth inhibition. But, statistically significant difference was not always observed between the pSecTetC + pIL-12 and pIL-12 group.

Treg depletion was critical in this regimen because tumor-bearing mice which received pSecTetC and pIL-12 without prior treatment with CD25 mAb showed only modest reduction in tumor growth (not shown), demonstrating the importance of reducing negative regulation in this therapy regimen.

Histological analysis of control D2F2 tumors (Fig. 4b, left panel) showed a poorly differentiated carcinoma that consisted of solid nests of polygonal to spindle-shaped tumor cells undergoing frequent central necrosis and separated by a delicate stromal network. Tumor cells had sharply defined borders, a moderate amount of eosinophilic cytoplasm, a high nuclear to cytoplasm ratio, and hyperchromatic and pleomorphic round to elongated nuclei with frequent nucleoli and abundant mitotic figures. Tumors treated with intratumoral pSecTetC and pIL-12 DNA electroporation (Fig. 4b, right panel), however, showed massive lymphocytic infiltration surrounding a core of necrotic debris, indicating immune-mediated destruction of treated tumors.

Immune memory induced by tumor regression following DNA electroporation

In a repeated experiment to test if immune memory was induced, BALB/c mice were inoculated with D2F2 tumor, treated with CD25 mAb and electroporated with pSecTetC and pIL-12. The tumors in the 4 control mice that received CD25 mAb and blank vector grew progressively (Fig. 5a, top panel). In contrast, 5 of 9 (56%) tumors that received pSecTetC and pIL-12 regressed completely by wk.6 (Fig. 5a, bottom panel). Mice that rejected D2F2 tumors were rechallenged at wk 12 in the opposite flank with the same tumor (Fig. 5b), and all were protected from a second D2F2 challenge, while progressive tumors developed in control mice. Taken together, these data showed that electroporation of pSecTetC and pIL-12 in the tumor following Treg depletion resulted in anti-tumor immunity and long-term memory.

Immune response to TAA induced by intratumoral DNA electroporation

To test immune priming to a specific TAA by intratumoral DNA electroporation, BALB/c mice were inoculated with Her-2 positive D2F2/E2 tumor cells, treated with 60 µg of CD25 mAb and intratumoral electroporation with pSecTetC, pIL-12, or both. Tumor-draining lymph nodes were removed 2 wks after DNA treatment and lymph node cells were pooled from 2–3 mice in each group. Her-2-specific T cell response was measured by ELISpot assay. Anti-Her-2 IFN- γ -producing T cells were detected in immune lymph nodes by incubation with engineered antigen presenting cells 3T3/EKB which expressed Her-2, K^d and CD80 (B7.1). Her-2-specific IFN- γ T cells were induced by pSecTetC ($212 \pm 19/10^6$ lymph node cells), pIL-12 ($147 \pm 72/10^6$) and pSecTetC + pIL-12 ($370 \pm 89/10^6$), with pSecTetC + pIL-12 showing the highest activity ($p < 0.01$ compared to mice receiving single treatment) (Fig. 6). IL-5 producing T cells were not detected (data not shown), showing preferential induction of Th1 response. Therefore, a strong Th1/Tc1 response to tumor-associated Her-2 was induced. Induction of antitumor immunity by *in situ* immune priming via intratumoral expression of a foreign antigen and IL-12 may be very beneficial in the neoadjuvant setting.

Discussion

In the current study, reduction of tumor growth and *in situ* priming to TAA indicates that intratumoral DNA electroporation after Treg depletion may be a plausible neoadjuvant regimen. Profound inhibition of primary tumor growth was observed. Importantly, immune response to TAA was induced to protect against tumor rechallenge, supporting the potential efficacy of intratumoral gene therapy in neoadjuvant setting. In a neoadjuvant trial, patients with locally advanced breast cancer who achieved complete clinical response to preoperative chemotherapy had a 75% nine-year overall survival, compared to 65% in non-responders [35]. In a bladder cancer trial, median survival was 77 months versus 46 months in patients receiving neoadjuvant chemotherapy compared to surgery alone [8]. In such neoadjuvant setting, induction of immune memory by intratumoral DNA electroporation may enhance long-term protection.

TetC has been described as a vaccine adjuvant by Buchan, et. al. [3] who generated DNA vaccines encoding fusion protein of TetC and tumor antigen AH1 or idiotypic Ab for intramuscular electroporation. TetC enhanced immune response against these designated antigens, resulting in reduced tumor growth. However, when administered intramuscularly without antigen, TetC DNA did not induce anti-tumor immune response. We showed the induction of significant T cell response to tumor associated Her-2, when pSecTetC was injected into the tumors where it functions as an *in situ* adjuvant with the potential to amplify the immune response to all tumor associated antigens. Anti-tumor efficacy of pSecTetC may be further amplified in pre-immunized hosts such as Tetanus vaccinated individuals in US. It is possible that intratumoral electroporation with other immune modulating cytokines or increased doses of IL-12 may result in tumor rejection. But, the potential to exploit a recall antigen in humans combined with greater efficacy observed with pSecTetC + pIL-12 shows the benefit of combined treatment.

Both innate and adaptive immunity may contribute to the observed tumor growth inhibition. IL-12 is a potent NK activator and directs Th1 cell differentiation [32]. It is also associated with the inhibition of angiogenesis [32]. Intratumoral delivery of IL-12 by DNA electroporation can reduce tumor growth via immunological and anti-angiogenic mechanisms [19]. TetC is a strong bacterial antigen as demonstrated by the induction of TetC Ab after one-time DNA electroporation. Continuous liberation of both SecTetC and IL-12 results in the strongest immune priming to TAA, such that treated tumors regress or are reduced in size, and demonstrate heavy inflammatory infiltration. The microenvironment modified by expressing SecTetC, IL-12, or both favors Th1/Tc1 activation because IFN- γ , but not IL-5 T cell response was induced.

Luciferase expression lasted about 3 wks after the gene was electroporated into the tumor or muscle, demonstrating the feasibility of sustained *in vivo* transgene expression. Luciferase expression was detected only after electric pulses were applied (not shown). This localized transgene expression further increases the safety of this treatment. Because plasmid vectors are free of pathogenicity and immunogenicity, the treatment can be repeated as needed with the same or alternative immune modulating molecules to amplify immune responses [7, 25].

We have previously shown that mice undergoing tumor regression following Treg depletion had an enhanced immune reactivity to self-antigens, such as thyroglobulin [12, 33]. The approach of intratumoral gene expression focuses the immune response to antigens in the tumor, thus minimizing the need for systemic immune modulation and reducing the risk of autoimmunity.

In conclusion, a new immunotherapy regimen that combines Treg depletion and intratumoral delivery of pSecTetC and pIL-12 has demonstrated potential utility in cancer therapy by inhibiting tumor growth and by *in situ* immune priming to endogenous TAA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NIH CA76340 and CA125680 (WZW).

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Fig. 1.

Expression of luciferase by *in vivo* DNA electroporation. **a** Luciferase activity *in vivo*. BALB/c mice were inoculated s.c. with D2F2 tumor cells in the right flank ("1") and with D2F2/Luc cells in the left flank ("3"). When D2F2 tumors reached 2×2 – 3×3 mm, pGL3 luciferase DNA was injected into D2F2 tumor ("1") or muscle ("2"). On d. 7 after the first pGL3 injection, mice received D-luciferin i.v., and luminescence (left panel) and X-ray (middle panel) images were acquired. The two images were superimposed (right panel) to define the location of luminescence. **b** Kinetics of luciferase expression. Five D2F2-bearing BALB/c mice received intratumoral pGL3 electroporation. Luminescence images were

acquired at 6 hrs and on days 2, 4, 7, 10, 14, 17, 21, 24, 30, and 37, and relative luminescence intensity of individual animals is shown. Arrows indicate times of DNA electroporation. **c** Luciferase activity measured *in vitro*. BALB/c mice implanted with D2F2 tumors received pGL3 electroporation. On days 7 or 10, luciferase activity in tumor lysates was measured by *in vitro* luminescence assay, and luciferase protein concentration was determined. Open circles: tumors treated with saline (n=5); closed circles: tumors treated with pGL3 (n=4). **d** pGL3 expression in other tumors. BALB/c mice bearing TUBO tumors and C57BL/6 mice bearing E0771 or TC-1 tumors received pGL3 as described. Luciferase activity was measured by luminescence imaging. A representative animal from each group is shown (n=5–7 mice/group). Intramuscular electroporation of pGL3 was the positive control

Fig. 2.

Construction and expression of pSecTetC. **a** 1.371-kb HindIII/BamHI fragment containing TetC aa 1–452 was isolated from pLPCX/TetC and ligated into the HindIII/BamHI site of pSecTag2B. **b** Recombinant SecTetC protein. Serum-free culture supernatant was collected from BALB/c NIH 3T3 fibroblasts transfected with blank pSecTag2B vector or pSecTetC. Recombinant bacterial TetC (rTetC) was the positive control. **c** Anti-TetC antibodies. Control or SecTetC containing supernatants were resolved as described. SecTetC immune serum was collected from BALB/c mice after one i.m. electrovaccination with 50 µg each of pSecTetC and pGM-CSF. Membranes were hybridized with normal or immune sera at 1:200 dilution, and anti-SecTetC antibodies were detected with goat anti-mouse HRP. **d** Recognition of tetanus toxoid by immune sera. Levels of tetanus toxoid-binding IgG from control (open circles, n=9) or immune sera (filled circles, n=5) were measured by ELISA

Fig. 3.

Immune response to TetC after intratumoral DNA electroporation. BALB/c mice were inoculated with D2F2 tumor cells s.c., and indicated groups were treated with 60 μ g of CD25 mAb i.p. one day later. pSecTag2B vector or pSecTetC were injected into the tumor as described followed by electroporation. Sera were collected 2 wks after DNA treatment. The level of tetanus toxin-binding Ab was measured by ELISA

b

Fig. 4. Inhibition of D2F2 tumor growth by intratumoral electroporation with pSecTetC and pIL-12 in Treg-depleted mice. **a** Tumor growth inhibition. BALB/c mice bearing D2F2 tumor were treated with CD25 mAb and DNA electroporation. Tumor volumes of individual mice are shown. Arrows indicate times of DNA electroporation. * $P < 0.05$, ** $P = 0.001$, *** $P < 0.001$ at wk. 7 after tumor challenge, compared to mice treated with blank vector. **b** Inflammatory infiltration and tumor destruction. On d. 5 after DNA electroporation, tumors from animals treated with blank vector (left panel) or SecTetC and IL-12 DNA (right panel) were removed for histological analysis. Images were collected at 40X magnification

Fig. 5.

Immune memory after tumor regression. **a** D2F2 tumor bearing mice were treated with 60 μ g of CD25 mAb and blank vector or pSecTetC and pIL-12. **b** Immune memory following tumor regression. BALB/c mice which rejected D2F2 tumor as described in **a** received a second challenge of 2×10^5 D2F2 tumor cells in the opposite flank 6 wks after tumor regression (filled circles). Naïve BALB/c mice were used as controls (open triangles). Tumor-free survival is shown. * $P < 0.001$ by log-rank test

Fig. 6.

Immune response to tumor-associated antigen in tumor-draining lymph nodes. BALB/c mice implanted with D2F2/E2 tumors were treated with CD25 mAb and DNA electroporation as described. Tumor-draining inguinal and axillary lymph nodes were harvested 2 wks later, and cells from each group were pooled. Her-2-specific T cells were measured by IFN- γ ELISpot assay by incubation with engineered antigen presenting cells 3T3/KB (open bars) or 3T3/EKB (solid bars). * $P=0.01$ compared to individual treatment