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Intramammary Application of Non-methylated-CpG oligodeoxynucleotides (CpG) Inhibits both Local and Systemic Mammary Carcinogenesis in Female BALB/c Her-2/neu Transgenic mice

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ABSTRACT

CpG are powerful drugs activating the innate immune system. In this study, the ability of their intramammary administration in impeding the devastating progression of carcinogenesis in all the mammary glands of female BALB/c mice transgenic for the rat *neu* transforming oncogene was assessed. Starting when in situ carcinomas were scattered over all their mammary glands (week 10), mice received CpG injections in the stroma of the fourth left gland. Local neoplastic progression was inhibited by six monthly administrations. CpG not only delayed the onset of carcinomas in the injected gland, but also hampered their progression. Extended latency was observed for tumors in glands both close to and far from the injection site. When the experiment ended (week 45), no tumors were palpable in 67% of the injected glands and a markedly impaired tumor growth was evident in the others. An impressive local infiltrate of CD11b+ cells with the morphologic features of macrophages, plasma cells, B220+ B cells, and CD4+ and CD8+ T cells was quickly recruited to the CpG-treated glands. High quantities of IFN-? producing cells were only presented in the ipsilateral axillary draining lymph nodes of the treated glands. Enhanced natural killer (NK) lytic activity was also detected in the spleens. Inhibition of progression was weaker when only four injections were given, and abolished by *in vivo* depletion of NK cells. CpG monotherapy is thus effective in an aggressive model of autochthonous cancer. The results strongly support the administration of CpG as a local monotherapy of multiple invasive microscopic lesions.

INTRODUCTION

CpG act as adjuvants and powerful activators of the innate immune system by triggering of the Toll-like receptor 9 (TLR9) signaling pathway [1, 2]. They induce maturation of dendritic cells (DC) and activate them to secrete interleukin (IL)-6, IL-12, granulocyte-monocyte colony stimulating factor (GM-CSF), several chemokines, tumor necrosis factor (TNF)-a and interferon (IFN)-? [3, 4, 5]. These cytokines then stimulate IFN-? production and the cytotoxicity of natural killer (NK) cells [6], which are also directly activated by CpG [7]. A novel cell subset that comprises and is activated by CpG stimulation has been described [8, 9]. Strong induction of IL-12 and IFN-? accompanied by almost negligible secretion of T helper (TH) 2 type cytokines indicates that CpG promote the stimulation of a dominant TH1 adaptive response [10].

These properties suggested the employment of CpG to establish immune interventions against cancer. Their use to promote activation of DC *in vitro* has resulted in enhanced anti-tumor T cell responses upon DC adoptive transfer [11, 12]. Co-administration of irradiated tumor cells or tumor antigens with CpG as adjuvant also improves their anti-tumor effect, particularly in prophylactic settings [13, 14, 15]. Stimulation of anti-tumor immunity by intratumoral injection of CpG is an even more attractive approach since it does not require identification of tumor-specific antigens. Even so, it has certain limitations: i) it requires prior knowledge of tumor location; ii) it is confined to accessible tumors; iii) it is not practical for widely disseminated tumors. Indeed, CpG has been used in experimental animal models either as monotherapy, by peritumoral injection of already established transplantable tumors [16, 17, 18], or for the treatment of minimal residual disease following chemotherapy or resection [19, 20].

Most of the experimental data and beliefs, about the potential of CpG in cancer immunotherapy stem from challenges of young and healthy mice with transplantable tumors or cell lines. However, these models represent an extremely artificial setting. Tumors arising in genetically engineered mice provide more accurate and reliable models of autochthonous cancer, which recapitulates many of the molecular and genetic changes that occur during the stepwise progression of human cancer [21, 22]. Encouraging results have been reported in FVB mice transgenic for the *neu* proto-oncogene (FVB-NeuN mice, #N202), which develop mammary carcinomas characterized by an indolent progression [23]. Intraperitoneal administration of CpG before tumor onset reduced tumor incidence, whereas the growth rate was not significantly affected [24].

In this study, the ability of monthly intramammary GpG administrations to impede the devastating progression of carcinogenesis in

all their mammary glands to which female BALB/c mice transgenic for the rat *neu* transforming (ErbB-2, Her-2/neu) oncogene (BALB-neuT664V-E mice) are genetically predestined was assessed. In these mice the multiple foci of atypical hyperplasia present in all mammary glands by week 4 progress to a diffuse atypical hyperplasia (week 6) and multifocal *in situ* carcinoma around week 10. These foci then expand and merge to form invasive lobular carcinomas that become palpable around week 16-17 [25].

MATERIALS AND METHODS

Mice and cells. Virgin BALB-neuT664V-E female mice (H-2d) transgenic for the transforming activated rat *neu* oncogene under the transcriptional control of the mouse mammary tumor virus promoter [25] were bred for us under specific pathogen free conditions at Charles River Laboratories (Calco, Italy). Individually tagged mice of the same age were treated according to the European Community guidelines. They were randomly assigned to the control and treated groups and all groups were treated concurrently. Mammary pads were inspected weekly to monitor tumor appearance. Progressively growing masses of 1.5 mm mean diameter were regarded as tumors. Tumor multiplicity was calculated as the cumulative number of incident tumors / total number of mice, and is reported as mean ± SE [23]. Each neoplastic mass was measured with calipers in the two perpendicular diameters until all ten mammary glands displayed a tumor or until a tumor exceeded a mean diameter of 10 mm, when mice were euthanized to avoid unnecessary suffering. The concordant results always obtained from each experiment performed three times were cumulated. TUBO cells are a rat *neu*+ cell line derived from a carcinoma arising in a BALB-neuT664V-E mouse [26]. They were cultured in DMEM with 20% fetal bovine serum, and 100 U/ml penicillin and 100 µg/ml streptomycin all purchased from GIBCO, Paisley,UK.

CpG administration. Synthetic CpG 1826 (TCCATGACGTTCCTGACGTT) oligodeoxynucleotides (ODN) and non-CpG-containing ODN 1745 (TCCATGAGCTTCCTGAGCTT) (non-CpG 1745) purchased from TIB BIOMOL (Berlin, Germany) as phosphorothioate-modified to increase their resistance to nuclease degradation were dissolved in PBS (Sigma-Aldrich, St. Louis, MO). Starting from the 10th week of age, 100 µl PBS alone or containing 100 µg of non-CpG 1745 or CpG 1826 were injected in the pad of the IV left mammary gland every four weeks.

Measurement of cell proliferation. TUBO cells (1.5 x 104) in 100 µl were seeded in U-bottomed wells of a 96-well microtiter plate (Sarstedt Inc., Newton, NC) in quadruplicate and cultured for 24, 48 or 72 hours in the presence of 3 or 6 µg/ml of CpG 1826, non-CpG 1745 or medium alone. Eighteen hours before harvesting, 1 µCi of [3H]thymidine (Amersham International, Freiburg, Germany) was added to each well. Cells were harvested on paper filters (Filtermat A; Wallac, Freiburg, Germany) using a cell harvester (Inotech, Wohlen, Switzerland) and the uptake of [3H]thymidine into the DNA of proliferating cells was determined with a scintillation counter (Wallac 1450, Micro-Trilux, Perkin Elmer, Rodgau-Juegesheim, Germany). The results are expressed as counts per minute (cpm).

RT-PCR for TLR9. Total RNA was extracted from TUBO cells (5x 106) with Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized with 1 µg of total RNA in a total reaction volume of 20 µl with the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen). cDNA (2 µl) was amplified with *Taq* DNA polymerase (Promega, Milan, Italy). PCR for mouse TLR9 and ß-actin cDNA was performed for 40 cycles, each consisting of denaturation (95°C for 50 s), annealing (56°C for 50 s) and an elongation step (72°C for 50 s), followed by an additional extension step (72°C for 7 min). The following primer sequences (Invitrogen) were used to amplify mouse TLR9 and ß -actin respectively: 5'-GCT TTG GCC TTT CAC TCT TG-3' (TLR9 forward), 5'-AAC TGC GCT CTG TGC CTT AT-3' (TLR9 reverse), 5'-CAC CCT GTG CTG CTC ACC GAG GCC-3' (ß -ACTIN forward), and 5'-CCA CAC AGA GTA CTT GCG CTC AGG-3' (ß -actin reverse). PCR products were separated on 2% agarose gels containing ethidium bromide (Sigma-Aldrich), visualized and photographed under ultraviolet (UV) light.

Whole-mount, histological and immunohistochemical analysis. Groups of six treated or control mice were sacrificed at week 16. Mammary tissue was fixed in paraformaldehyde / lysine / periodate solution (all from Sigma-Aldrich) and used for whole-mount preparation [25], histology, and immunohistochemistry. Four-micrometer frozen sections were stained with hematoxylin / eosin (Bio Optica, Milan, Italy). For immunohistochemistry, cryostat sections were incubated for 30 min with antibodies that specifically detect the following: CD4 T cells (Chemicon, Milan, Italy), CD11b and CD8 T cells (both from Oxford Biotech, Milano, Italy), granulocyte-1 (GR-1) (clone RB6-8C5), B220 (PharMingen, San Diego, CA), cyclo-oxigenase-2 (COX-2) and microsomal prostaglandin E2 synthase 1 (mPGES1) (both from Cayman, Milan, Italy). Primary antibodies were incubated with tissue sections and then with biotinylated secondary antibodies and detected with avidin / biotin complex / alkaline phosphatase (DAKO, Milan, Italy).

Whole-mount image analysis. Images of whole-mount preparations were taken with a Nikon Coolpix 950 digital camera (Nital Spa, Turin, Italy) mounted on a stereoscopic Leica Mz 6 microscope (Leica Microsystems, Milan, Italy). A 0.63 objective was used to obtain images with a total magnification of x 6.3 and a resolution of 2.048 x 1.536 pixels (giving 17.34 x 13 cm images). Images were acquired within Adobe Photoshop version 6.0 graphic software (Adobe Systems, San Jose, CA). Spatial calibration was determined by photographing a 1 mm stage using the same parameter values. The distance drawn on the 1 mm calibration image was divided by 1,000 to find the number of pixels per micrometer. A grid with 2.5 mm distance between lines was superimposed and any lesions on the duct surface at the grid intercepts were measured perpendicular to duct direction. Points with no lesions were ranked as zero. At least 250 points were measured on each mammary gland. Lesion measurements were recorded on an excel spreadsheet and mean and se were calculated for each treatment group.

Flow cytometric identification of infiltrating immune cells. The relative numbers of immune cells from mammary glands and draining lymph nodes were evaluated by flow cytometry. mice were injected twice and two weeks after the second injection of PBS alone or containing CpG 1826, cells were isolated by mechanical desegregation from the dissected IV left mammary gland, after removal of the associated lymph node, and from the left axillary lymph nodes draining the injected gland. Cellular suspensions were counted using a cell counter and size analyzer Z2 (Beckman Coulter, Krefeld, Germany), and lymphocyte concentration was determined using a cell size range of 4.0-8.6 µm. Cells were treated with Fc receptor blocker (CD16/CD32) for 15 min at 4°C. PE-conjugated anti-mouse leukocyte common antigen 5 (Ly-5) CD45 (clone 30-F11), PerCP-conjugated anti-mouse CD4 (clone L3T4), PE-conjugated anti-mouse CD8???

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REFERENCES

ADDIN EN.REFLIST

[ADDIN EN.REFLIST 1]Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S. Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408*, 740-745.

[2]Krieg, A. M. CpG motifs: the active ingredient in bacterial extracts? *Nat. Med.* **2003**, *9*, 831-835.

[3]Jakob, T.; Walker, P. S.; Krieg, A. M.; Udey, M. C.; Vogel, J. C. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by

immunostimulatory DNA. *J. Immunol.* **1998**, *161*, 3042-3049.
[4]Sparwasser, T.; Koch, E. S.; Vabulas, R. M.; Heeg, K.; Lipford, G. B.; Ellwart, J. W.; Wagner, H. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **1998**, *28*, 2045-2054.
[5]Hartmann, G.; Weiner G. J.; Krieg, A. M. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci.* **1999**, *96*, 9305-9310.
[6]Ballas, Z. K.; Rasmussen, W. L.; Krieg, A. M. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **1996**, *157*, 1840-1845.
[7]Roda, J. M.; Parihar, R.; Carson, W. E. CpG-containing oligodeoxynucleotides act through TLR9 to enhance the NK cell cytokine response to antibody-coated tumor cells. *J. Immunol.* **2005**, *175*, 1619-1627.
[8]Pillarisetty, V. G.; Katz, S. C.; Bleier, J. I.; Shah, A. B.; Dematteo, R. P. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12. *J. Immunol.* **2005**, *174*, 2612-2618.
[9]Taieb, J.; Chaput, N.; Ménard, C.; Apetoh, L.; Ullrich, E.; Bonmort, M.; Péquignot, M.; Casares, N.; Terme, M.; Flament, C.; Opolon, P.; Lecluse, Y.; Métivier, D.; Tomasello, E.; Vivier, E.; Ghiringhelli, F.; Martin, F.; Klatzmann, D.; Poynard, T.; Tursz, T.; Raposo, G.; Yagita, H.; Ryffel, B.; Kroemer, G.; Zitvogel, L. A novel dendritic cell subset involved in tumor immunosurveillance. *Nat. Med.* **2006**, *12*, 214-219.
[10]Chu, R. S.; Targoni, O. S.; Krieg, A. M.; Lehmann, P. V.; Harding, C. V. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* **1997**, *186*, 1623-1631.
[11]Brunner, C.; Seiderer, J.; Schlamp, A.; Bidlingmaier, M.; Eigler, A.; Haimerl, W.; Lehr, H. A.; Krieg, A. M.; Hartmann, G.; Endres, S. Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. *J. Immunol.* **2000**, *165*, 6278-6286.
[12]Pilon-Thomas, S.; Li, W.; Briggs, J. J.; Djeu, J.; Mule, J. J.; Riker, A. I. Immunostimulatory effects of CpG-ODN upon dendritic cell-based immunotherapy in a murine melanoma model. *J. Immunother.* **2006**, *29*, 381-387.
[13]Weiner, G. J.; Liu, H. M.; Wooldridge, J. E.; Dahle, C. E.; Krieg, A. M. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10833-10837.
[14]Kim, T. Y.; Myoung, H. J.; Kim, J. H.; Moon, I. S.; Kim, T. G.; Ahn, W. S.; Sin, J. I. Both E7 and CpG-oligodeoxynucleotide are required for protective immunity against challenge with human papillomavirus 16 (E6/E7) immortalized tumor cells: involvement of CD4+ and CD8+ T cells in protection. *Cancer Res.* **2002**, *62*, 7234-7240.
[15]Stern, B. V.; Boehm, B. O.; Tary-Lehmann, M. Vaccination with tumor peptide in CpG adjuvant protects via IFN-gamma-dependent CD4 cell immunity. *J. Immunol.* **2002**, *168*, 6099-6105.
[16]Heckelsmiller, K.; Rall, K.; Beck, S.; Schlamp, A.; Seiderer, J.; Jahrsdörfer, B.; Krug, A.; Rothenfusser, S.; Endres, S.; Hartmann, G. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J. Immunol.* **2002**, *169*, 3892-3899.
[17]Furumoto, K.; Soares, L.; Engleman, E. G.; Merad, M. Induction of potent antitumor immunity by in situ targeting of intratumoral DCs. *J. Clin. Invest.* **2004**, *113*, 774-783.
[18]den Brok, M. H.; Sutmuller, R. P.; Nierkens, S.; Bennink, E. J.; Toonen, L. W.; Figdor, C. G.; Ruers, T. J.; Adema, G. J. Synergy between in situ cryoablation and TLR9 stimulation results in a highly effective in vivo dendritic cell vaccine. *Cancer Res.* **2006**, *66*, 7285-7292.
[19]Heckelsmiller, K.; Beck, S.; Rall, K.; Sipos, B.; Schlamp, A.; Tuma, E.; Rothenfusser, S.; Endres, S.; Hartmann, G. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. *Eur. J. Immunol.* **2002**, *32*, 3235-3245.
[20]Weigel, B. J.; Rodeberg, D. A.; Krieg, A. M.; Blazar, B. R. CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma. *Clin. Cancer Res.* **2003**, *9*, 3105-3114.

[21]Green, J. E.; Hudson, T. The promise of genetically engineered mice for cancer prevention studies. *Nat. Rev. Cancer* **2005**, *5*, 184-198.
[22]Lollini P. L.; Cavallo, F.; Nanni, P.; Forni, G. Vaccines for tumour prevention. *Nat. Rev. Cancer* **2006**, *6*, 204-216.
[23]Boggio, K.; Nicoletti, G.; Di Carlo, E.; Cavallo, F.; Landuzzi, L.; Melani, C.; Giovarelli, M.; Rossi, I.; Nanni, P.; De Giovanni, C.; Bouchard, P.; Wolf, S.; Modesti, A.; Musiani, P.; Lollini, P. L.; Colombo, M. P.; Forni, G. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. *J. Exp. Med.* **1998**, *188*, 589-596.
[24]Sfondrini, L.; Besusso, D.; Rumio, C.; Rodolfo, M.; Menard, S.; Balsari, A. Prevention of spontaneous mammary adenocarcinoma in HER-2/neu transgenic mice by foreign DNA. *Faseb J.* **2002**, *16*, 1749-1754.
[25]Pannellini, T.; Forni, G.; Musiani, P. Immunobiology of her-2/neu transgenic mice. *Breast Dis.* **2004**, *20*, 33-42.
[26]Curcio, C.; Di Carlo, E.; Clynes, R.; Smyth, M. J.; Boggio, K.; Quaglino, E.; Spadaro, M.; Colombo, M. P.; Amici, A.; Lollini, P. L.; Musiani, P.; Forni, G. Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas. *J. Clin. Invest.* **2003**, *111*, 1161-1170.
[27]Rolla, S.; Nicoló, C.; Malinarich, S.; Orsini, M.; Forni, G.; Cavallo, F.; Ria, F. Distinct and non-overlapping T cell receptor repertoires expanded by DNA vaccination in wild-type and HER-2 transgenic BALB/c mice. *J. Immunol.* **2006**, *177*, 7626-7633.
[28]Slamon, D. J.; Clark, G. M.; Wong, S. G.; Levin, W. J.; Ullrich, A.; McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **1987**, *235*, 177-182.
[29]Peoples, G. E.; Gurney, J. M.; Hueman, M. T.; Woll, M. M.; Ryan, G. B.; Storrer, C. E.; Fisher, C.; Shriver, C. D.; Ioannides, C. G.; Ponniah, S. Clinical trial results of a HER2/neu (E75) vaccine to prevent recurrence in high-risk breast cancer patients. *J. Clin. Oncol.* **2005**, *23*, 7536-7545.
[30]Hortobagyi, G.N. Trastuzumab in the treatment of breast cancer. *N. Engl. J. Med.* **2005**, *353*, 1734-1736.
[31]Ambrosino, E.; Spadaro, M.; Iezzi, M.; Curcio, C.; Forni, G.; Musiani, P.; Wei, W.Z.; Cavallo, F. Immunosurveillance of Erbb2 carcinogenesis in transgenic mice is concealed by a dominant regulatory T-cell self-tolerance. *Cancer Res.* **2006**, *66*, 7734-7740.
[32]Noll, B.O.; McCluskie, M.J.; Sniatala, T.; Lohner, A.; Yuill, S.; Krieg, A.M.; Schetter, C.; Davis, H.L.; Uhlmann, E. Biodistribution and metabolism of immunostimulatory oligodeoxynucleotide CPG 7909 in mouse and rat tissues following subcutaneous administration. *Biochem. Pharmacol.* **2005**, *69*, 981-991.
[33]Cifaldi, L.; Quaglino, E.; Di Carlo, E.; Musiani, P.; Spadaro, M.; Lollini, P.L.; Wolf, S.; Boggio, K.; Forni, G.; Cavallo, F. A light, nontoxic interleukin 12 protocol inhibits HER-2/neu mammary carcinogenesis in BALB/c transgenic mice with established hyperplasia. *Cancer Res.* **2001**, *61*, 2809-2812.
[34]Nanni, P.; Nicoletti, G.; De Giovanni, C.; Landuzzi, L.; Di Carlo, E.; Cavallo, F.; Pupa, S.M.; Rossi, I.; Colombo, M.P.; Ricci, C.; Astolfi, A.; Musiani, P.; Forni, G.; Lollini, P.L. Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2/neu transgenic mice. *J. Exp. Med.* **2001**, *194*, 1195-1205.
[35]Quaglino, E.; Iezzi, M.; Mastini, C.; Amici, A.; Pericle, F.; Di Carlo, E.; Pupa, S.M.; De Giovanni, C.; Spadaro, M.; Curcio, C.; Lollini, P.L.; Musiani, P.; Forni, G.; Cavallo, F. Electroporated DNA vaccine clears away multifocal mammary carcinomas in her-2/neu transgenic mice. *Cancer Res.* **2004**, *64*, 2858-2864.
[36]Quaglino, E.; Mastini, C.; Iezzi, M.; Forni, G.; Musiani, P.; Klapper, L.N.; Hardy, B.; Cavallo, F. The adjuvant activity of BAT antibody enables DNA vaccination to inhibit the progression of established autochthonous Her-2/neu carcinomas in BALB/c mice. *Vaccine* **2005**, *23*, 3280-3287.
[37]Quaglino, E.; Rolla, S.; Iezzi, M.; Spadaro, M.; Musiani, P.; De Giovanni, C.; Lollini, P.L.; Lanzardo, S.; Forni, G.; Sanges, R.; Crispi, S.; De Luca, P.; Calogero, R.; Cavallo, F. Concordant morphologic and gene expression data show that a vaccine halts HER-2/neu preneoplastic lesions. *J. Clin. Invest.* **2004**, *113*, 709-717.
[38]Sakai, Y.; Morrison, B.J.; Burke, J.D.; Park, J.M.; Terabe, M.; Janik, J.E.; Forni, G.; Berzofsky, J.A.; Morris, J.C. Vaccination by genetically modified dendritic cells expressing a truncated neu oncogene prevents development of breast cancer in transgenic mice. *Cancer Res.* **2004**, *64*, 8022-8028.
[39]Tegerstedt, K.; Lindencrona, J.A.; Curcio, C.; Andreasson, K.; Tullus, C.; Forni, G.; Dalianis, T.; Kiessling, R.;

Ramqvist, T. A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors. *Cancer Res.* **2005**, *65*, 5953-5957.
[40]Varesio, L.; Landolfo, S.; Giovarelli, M.; Forni, G. The macrophage as the social interconnection within the immune system. *Dev. Comp. Immunol.* **1980**, *4*, 11-19.
[41]Chen, Y.; Zhang, J.; Moore, S.A.; Ballas, Z.K.; Portanova, J.P.; Krieg, A.M.; Berg, D.J. CpG DNA induces cyclooxygenase-2 expression and prostaglandin production. *Int. Immunol.* **2001**, *13*, 1013-1020.
[42]MacDermott, R.P. Alterations in the mucosal immune system in ulcerative colitis and Crohn's disease. *Med. Clin. North. Am.* **1994**, *78*, 1207-1231.
[43]Anderson, G.D.; Hauser. S.D.; McGarity, K.L.; Bremer, M.E.; Isakson, P.C.; Gregory, S.A. Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. *J. Clin. Invest.* **1996**, *97*, 2672-2679.
[44]Portanova, J.P.; Zhang, Y.; Anderson, G.D.; Hauser, S.D.; Masferrer, J.L.; Seibert, K.; Gregory, S.A.; Isakson, P.C. Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *J. Exp. Med.* **1996**, *184*, 883-891.
[45]Needleman, P.; Isakson, P.C. The discovery and function of COX-2. *J. Rheumatol.* **1997**, *49*, 6-8.
[46]Amin, A.R.; Attur, M.; Abramson, S.B. Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis. *Curr. Opin. Rheumatol.* **1999**, *11*, 202-209.
[47]Betz, M.; Fox, B.S. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J. Immunol.* **1991**, *146*, 108-113.
[48]Wu, C.Y.; Wang, K.; McDyer, J.F.; Seder, R.A. Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. *J. Immunol.* **1998**, *161*, 2723-2730.
[49]Cheng, X.; Lopez, D.M. CD4+, but not CD8+, T cells from mammary tumor-bearing mice have a down-regulated production of IFN-gamma: role of phosphatidyl serine. *J. Immunol.* **1998**, *160*, 2735-2741.

Table 1. Flow cytometric analysis of the lymphoid cells recovered from mammary glands and draining lymph nodes of BALB-neuT664V-E mice following intrammary injection of PBS alone or supplemented with CpG 1826

Recovered lymphoid cells	Mammary gland injected with:	Draining lymph node
	PBS alone	CpG 1826
	PBS alone	CpG1826
CD45+		
500 ± 129a	100%b	
5 050 ± 469 a	100%	(x 10)c
2037 ± 325 a		

100%
10075 ± 1567 a
100%
(x 5)

CD4+

11 ± 2.9
2 %

1060 ± 181
21 %
(x 96)

1200 ± 86
59 %

4350 ± 550
44 %
(x 4)

CD4+ and Foxp3+

2 ± 1
(0.4 %)

65 ± 10
1.3%
(x 33)

Ndd

Ndd

CD8+

10 ± 2.5
2 %

500± 97
10 %
(x 50)

364 ± 80
18 %

1795 ±459
18 %)
(x 5)

CD49b+

150 ± 26
30 %

1410 ± 124
28 %
(x 9)

16 ± 4
0.8 %

60 ± 14
0.6 %
(x 4)

B220+

300 ± 35
60 %

1020 ± 153
20 %
(x 3)

480 ± 107
24 %

1860 ± 337
19 %
(x 4)

CD11b+

6 ± 1.1
1.2 %

1565 ± 236
31%
(x 260)

52 ± 13
3 %

750 ± 109
7 %
(x 14)

CD11c+

4 ± 1
0.8 %

90 ± 21
2 %
(x 22)

28 ± 5.2
1 %

240 ± 49
23 %
(x 9)

a,b,c,Total number (x 10-3) (a), percentage (b) and fold increase (c) of reactive lymphoid cells recovered from the IV mammary gland and ipsilateral draining lymph node.
dNot done