Immunoprevention of HER-2/neu Transgenic Mammary Carcinoma through an Interleukin 12-Engineered Allogeneic Cell Vaccine

Carla De Giovanni, Giordano Nicoletti, Lorena Landuzzi, Annalisa Astolfi, Stefania Croci, Alberto Comes, Silvano Ferrini, Raffaella Meazza, Manuela Iezzi, Emma Di Carlo, Piero Musiani, Federica Cavallo, Patrizia Nanni, and Pier-Luigi Lollini

1Cancer Research Section, Department of Experimental Pathology, University of Bologna, Bologna; 2Istituti Ortopedici Rizzoli, Bologna; 3Istituto Nazionale per la Ricerca sul Cancro, IST, Genoa; 4Istituto Scientifico G. Gaslini, Genoa; 5Aging Research Centre, “G. D’Annunzio” University Foundation, Chieti; and 6Department of Clinical and Biological Sciences, University of Turin, Ospedale S. Luigi Gonzaga, Orbassano, Italy

ABSTRACT

This study evaluated the ability of cytokine-engineered allogeneic (H-2q) HER-2/neu-positive cells to prevent tumor development in mammary cancer-prone virgin female BALB/c (H-2q) mice transgenic for the transforming rat HER-2/neu oncogene (BALB-neuT mice). Repeat vaccinations with cells engineered to release interleukin (IL)-2, IL-12, IL-15, or IFN-y showed that IL-12-engineered cell vaccines had the most powerful immunopreventive activity, with >80% of 1-year-old BALB-neuT mice free of tumors. On the contrary all of the untreated mice and all of the mice vaccinated with IL-12-engineered cells lacking either HER-2/neu or allogeneic antigens developed mammary carcinomas within 22 or 33 weeks, respectively. Whole mount, histology, immunohistochemistry, and gene expression profile analysis showed that vaccination with IL-12-engineered cells maintained 26-week mammary glands free of neoplastic growth, with a gene expression profile that clustered with that of untreated preneoplastic glands. The IL-12-engineered cell vaccine elicited a high production of IFN-y and IL-4 and a strong anti-HER-2/neu antibody response. Immune protection was lost or markedly impaired in BALB-neuT mice lacking IFN-y or antibody production, respectively. The protection afforded by the IL-12-engineered cell vaccine was equal to that provided by the systemic administration of recombinant IL-12 in combination with HER-2/neu H-2q cell vaccine. However, IL-12-engineered cell vaccine induced much lower circulating IL-12 and IFN-y, and therefore lower potential side effects and systemic toxicity.

INTRODUCTION

In the forthcoming years, postgenomic medicine will allow for the enlightenment of the individual genetic predisposition to different diseases, including cancer. People at risk of cancer should benefit from appropriate, effective, and safe prevention approaches if available. Tumor immunoprevention, an obvious option for virus-related tumors, could also become a way to avoid development and progression of genetically determined tumors, provided that relevant tumor antigens will be identified and targeted with no or acceptable side effects (1–5).

Mice transgenic for oncogenes are models of human cancer natural history and of genetic cancer predisposition (6, 7), and can help in assessing whether immunoprophylactic approaches of cancer are effective (1, 3, 8). Studies on HER-2/neu transgenic murine models have shown that soluble factors (such as cytokines or other immunostimulating agents; Refs. 9, 10) and vaccines based on DNA (11–13), peptides (14, 15), proteins (16), or cells expressing tumor antigens (17–19) have a significant preventive activity. Effective immune targeting of HER-2/neu was found in several human and experimental systems (20–26). Cancer immunoprevention could also be designed for different tumor antigens (27). An impressive immunoprevention of HER-2/neu transgenic mammary cancer was obtained through the systemic administration of recombinant interleukin (rIL)-12 combined with a cell vaccine presenting both HER-2/neu antigens and allogeneic MHC H-2 glycoproteins (19). Such multivalent vaccination strategy required the presence of the three components (rIL-12, HER-2/neu, and allogeneic MHC) to maintain most HER-2/neu transgenic mice free from mammary tumors up to at least 1 year of age, whereas all of the untreated mice succumbed to mammary cancer within about 20–25 weeks. Such results encourage pursuing immunoprophylactic approaches for cancer.

In view of a translation to clinics, potential toxicity problems caused by the systemic administration of rIL-12 must be overcome (28–31). Other immunostimulatory cytokines, such as IFN-γ, IL-2, and IL-15, could be candidates for immunoprophylactic studies, but they also showed high systemic toxicity (32). Cytokine gene transduction could be a strategy to obtain cell vaccines able to present both the appropriate antigens and the adjuvant cytokine effect (26, 33). To verify this approach, in the present study we evaluated the immunopreventive ability of allogeneic HER-2/neu cell vaccines engineered with various cytokines against HER-2/neu transgenic mammary carcinoma.

MATERIALS AND METHODS

Mice. BALB-neuT virgin female mice (H-2q), overexpressing the activated rat HER-2/neu oncogene driven by the mouse mammary tumor virus promoter, and IFN-γ gene knockout BALB-neuT mice were bred and maintained as reported (9, 19). A female mμT mouse (knockout for the immunoglobulin μ chain gene; Ref. 34) on BALB/c genetic background, a kind gift from Dr. Thomas Blankenstein (Max-Dellbruck Center for Molecular Medicine, Berlin, Germany), was crossed with a BALB-neuT male mouse; F1 male mice were backcrossed with female μMT to obtain mice homozygous for the μ chain knockout allele and heterozygous for the HER-2/neu transgene. B220-positive B cells were routinely monitored by flow cytometry using monoclonal antibody RA3–6B2 (BD PharMingen, San Diego, CA). Experiments were authorized by the local animal use and care committee. Individually tagged virgin females used in the experiments were treated and inspected for mammary tumors as reported previously (9, 19).

Cells and Transfections. Cells used as allogeneic cytokine-engineered vaccines were derived from mammary carcinomas of FVB-neuM202 mice (H-2q), transgenic for the rat HER-2/neu proto-oncogene (35). Two HER-2/neu-positive cell clones (N202.1A and TT12.E2, here referred to as Neu/H-2q/B), derived from independent tumors, were used as recipient. The following plasmid vectors were used as described previously: BCMGneoIFN-γ (carrying the murine IFN-γ cDNA; Refs. 36, 37); BCMG-neoIL-2 (murine IL-2; Ref. 38), kindly provided by A. Gulino (University La Sapienza, Rome, Italy); pVKL/IL-15RESneo (human IL-15, modified to obtain secretable biologically active cytokine; Ref. 39); and pIL-12-RES1neo or pIL-12-RESShygro polycistronic expression plasmids (murine IL-12 genes; 36, 37).

Received 9/22/03; revised 2/26/04; accepted 3/5/04.

Grant support: Italian Association for Cancer Research (AIRC), the Italian Foundation for Cancer Research (FIRC), the Italian Ministry for Education, University and Research, and the University of Bologna. A. Astolfi and S. Croci are recipients of fellowships from FIRC, Milan, Italy.

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.

Note: Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org).

Requests for reprints: Carla De Giovanni, Cancer Research Section, Department of Experimental Pathology, viale Filopanti 22, I-40126 Bologna, Italy. Phone: 39-051-241110; Fax: 39-051-242169; E-mail: carla.degiovanni@unibo.it.
Ref. 39). Stable transfectants were obtained by selection in culture medium containing 500 μg/ml of genetin or 250 μg/ml of hygromycin B (Boehringer/Roche, Milan, Italy) and cloned by limiting dilution. Cytokine levels released in the supernatant by 10⁶ transfectant cells in a 72-h culture, evaluated with the indicated ELISA assays, were the following: Neu/H-2²/A/IFNγ: 727 ± 57 ng/ml of IFN-γ (Endogen, Woburn, MA); Neu/H-2²/A/IL12: 1473 ± 806 ng/ml of IL-2 (Endogen); Neu/H-2²/A/IL15: 1325 ± 269 pg/ml of IL-15 (R&D Systems Inc., Minneapolis, MN); and Neu/H-2²/A/IL12 and Neu/H-2²/A/IL12: 50 ± 12 and 285 ± 87 ng/ml of IL-12, respectively (R&D Systems Inc.). Cytokine production by the selected clones was stable for >3 months of continuous culture. The chosen cytokine-engineered cells showed a decreased s.c. growth in syngeneic hosts compared with nonengineered cells, but not a total rejection, with tumors still occurring in 40–100% of mice (data not shown). A limited in vivo persistence of the engineered cells was found previously to be a requisite to obtain a good antitumor immune response (40).

As experimental controls, two other cell lines were transfected with IL-12 genes as reported above. Cell clone N202.1E (here referred to as Neu/H-2²) was derived from the same mammary cancer originating N202.1A but lacked HER-2/neu expression (35). HER-2/neu-positive cell clone TUBO (here referred to as Neu/H-2²) was derived from a mammary carcinoma of BALB-neuT mice (19, 41). IL-12 transfectants obtained from these cells were named Neu/neu²/H-2²/IL12 and Neu/²/H-2 IL12, respectively.

Conditions for cell cultures and mitomycin C treatment (to block cell proliferation, when required) and the evaluation of the surface expression of HER-2/neu and class I H-2 alleles were performed as reported (19, 41).

**Vaccination Protocol.** Starting at the sixth week of age, BALB-neuT mice entered the vaccination protocol, consisting of 4-week cycles: in the first 2 weeks of rest. Unless otherwise specified, vaccination cycles were repeated lifelong; in indicated experiments, mice received only the first three vaccination cycles and then were observed for tumor appearance. Vaccination with nonengineered cells, performed with the same schedule as above, was combined to five daily i.p. administrations (50 ng in the first course and 100 ng thereafter) of mouse rIL-12 (kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA) in the third week, according to the protocol reported previously (19). Control mice received only the vehicle 0.01% mouse serum albumin (MSA; Sigma Chemical Co., St. Louis, MO). Their tumor progression mirrored that of untreated mice.

**Morphological Analysis.** Groups of 3 mice were killed at the indicated times. Tissue samples were processed as described previously (42) for histological examination or immunohistochemistry. The following antibodies were used: anti-p185/neu (C-18; Santa Cruz Biotechnology, Inc.) and antiproliferating cell nuclear antigen (Ylem, Rome, Italy). Pelt preparation for mammary gland whole mount was performed as described. ⁷

**In Vitro Restimulation, Cytotoxicity Assay, and Cytokine Release.** Mixed lymphocyte-tumor cell cultures (MLTC) were performed with spleen mononuclear cells cocultured at a 50:1 ratio with proliferation-blocked Neu/H-2² cells for 6 days in RPMI 1640 supplemented with 10% fetal bovine serum and with 20 units/ml of recombinant IL-2. The ability of MLTC-derived lymphoblasts to lyse Neu/H-2², Neu/H-2² tumor cells or allogeneic H-2² concanavalin A-induced lymphoblasts was evaluated by a standard ⁵¹Cr release assay and percentage of lysis was calculated as described (39, 43). Supernatants from MLTC performed as above with a 10:1 splenocyte:tumor cell ratio were assayed for IFN-γ and IL-4 by ELISA assays (R&D Systems Inc.) as described (19).

**Antibody Response and Immunoprecipitation Assay.** Sera were collected from individual mice in experimental groups at different time points and stored at −80 °C. Anti-Her-2/neu total antibodies and subclasses were studied by flow cytometry as reported previously (19). For immunoprecipitation studies, Neu/H-2²/A or Neu/neu²/H-2² tumor cells were metabolically labeled for 6 h with 100 μCi of ³⁵S-labeled Cysteine + Methionine mixture (Amersham) in DMEM without methionine and cysteine supplemented with 10% glucose and 3% diazyl fetal bovine serum. Cells were washed three times in PBS at 4 °C and then lysed in lysis buffer containing 1% NP40 for 30 min in ice. After preclearing with protein A-Sepharose, lysates were immunoprecipitated with protein A-Sepharose (Pharmacia) and 15 μl of preimmune or vaccinated mice serum or 10 μl of anti-HER-2/neu Ab4 monoclonal antibody (Onogene). Immunoprecipitated molecules were washed and eluted in 1× loading buffer containing 2% SDS, resolved by standard SDS-PAGE on a 10% polyacrylamide gel, and visualized by autoradiography.

**Microarray.** Mammary glands and tumors were collected from vaccinated and untreated mice at different time points of progression. Total RNA was extracted with TRIzol reagent (Invitrogen) from snap-frozen, pulverized tissue, and its integrity was checked on agarose gel. Biotin-labeled antisense cRNA was prepared according to Affymetrix (Santa Clara, CA) protocol, as reported (44). Raw data from MAS 5.0 were analyzed with Gene Spring software (Silicon Genetics, Redwood City, CA). Briefly, each measurement was normalized to the median of all of the measurements in that sample, and each gene in each sample was normalized to the median of the measurements of that gene in 6-week samples. Only genes called present with and with a signal intensity >20 in at least 3 samples were retained for additional analysis. Average linkage hierarchical clustering was performed with Genesis software⁸ on genes that showed at least a 4-fold difference in the expression level in at least one comparison.

**Statistical Analysis.** Differences in tumor-free survival curves were analyzed by Mantel-Haenszel test. Tumor multiplicities, and cytokine and antibody levels were compared by Student’s t test.

**RESULTS**

**Immunoprevention of HER-2/neu Transgenic Mammary Cancer by Cytokine-Engineered Cell Vaccines.** To investigate the possibility to obtain an effective cancer preventive vaccine combining three components (HER-2/neu antigens, allogeneic MHC, and cytokine) in the same cell, we transfected the genes for IFN-γ, IL-2, IL-12, or IL-15 in Neu/H-2²/A cells. Transfectants (designated Neu/H-2²/A/IFNγ, Neu/H-2²/A/IL12, Neu/H-2²/A/IL15, and Neu/H-2²/A/IL12) were used as an immunoprophylactic vaccine in mammary cancer prone BALB-neuT females (Fig. 1). Vaccination with Neu/H-2²/A/IL12 determined a strong inhibition of mammary carcinogenesis: most vaccinated females remained tumor-free up to at least 1 year of age. Cell vaccines releasing IFN-γ or IL-2 induced a statistically significant delay in tumor latency, but the percentage of tumor-free mice progressively fell and reached 0% at 1 year. Only a slight, not significant, delay was induced by IL-15-engineered cell vaccine. Although the low efficacy of the IL-15-engineered cell vaccine may relate to the low secretion rate of this cytokine, it should be underlined that in previous studies IL-15-engineered mammary carcinoma TSA cells, secreting 0.4–1.6 ng/ml of IL-15, induced immune-mediated rejection responses when implanted into syngeneic mice, and were active both as prophylactic and therapeutic vaccine against a TSA challenge (45). Moreover, the existence of multiple post-transcriptional mechanisms, which down-regulate IL-15 protein synthesis and secretion (46, 47), prevented the possibility to achieve higher IL-15 secretion rates without altering the mature protein structure. These data show that IL-12 expression engineered into allogeneic HER-2/neu cells provides a powerful immunopreventive cell vaccine and that IL-12 has the most effective adjuvant activity in comparison to other immunostimulatory cytokines.

**IL-12-Engineered Neu/H-2² Cell Vaccines.** To study the variability between independently obtained cell vaccines, Neu/H-2²/A/IL12 cell vaccine was compared with Neu/H-2²/B/IL12, an IL-12 transfectant derived from an independent HER-2/neu mammary tumor of the same H-2² transgenic FVB-neuN mouse origin (35). Both engineered cell vaccines highly efficiently halted mammary carcinogenesis, maintaining 83–90% of mice up to 52 weeks of age free from mammary tumors (Fig. 2, A and B). Vaccines also caused a significant decrease in the number of tumors. IL-12-engineered vaccines ap-

---

⁷ Internet address: http://ccm.ucdavis.edu/tgmouse/HistoryOfLab/wholhtm1.htm.

⁸ Internet address: http://genome.tugraz.at.
peared slightly more effective than allogeneic HER-2/neu cells combined to systemic rIL-12; however, the difference was not statistically significant. As statistical analysis did not show significant differences between the two IL-12-engineered clones, data obtained with Neu/H-2^d^/A/IL12 and Neu/H-2^d^/B/IL12 will be pooled hereafter.

Vaccinated tumor-free mice showed a strong resistance to a challenge with syngeneic Neu/H-2^d^ mammary carcinoma cells: 65% of vaccinated mice were still tumor-free 30 weeks after challenge, whereas all of the nonvaccinated BALB-neuT mice developed tumors within 4 weeks. Vaccinated mice were not resistant to a challenge with unrelated, MHC-compatible TS/A mammary carcinoma cells (data not shown).

To analyze the role played by the various components of the vaccine, mice were vaccinated with IL-12-engineered cells lacking either HER-2/neu expression (Neu^{neo}/H-2^d^/IL12) or allogeneic MHC antigens (Neu/H-2^d^/IL12). After vaccination with Neu^{neo}/H-2^d^/IL12 or Neu/H-2^d^/IL12 only a slight delay in the onset of mammary carcinoma was found, and all of the mice developed tumors within 32–33 weeks of age (Fig. 2, A and B).

In some groups of mice vaccination with Neu/H-2^d^/IL12 cells was concluded after the first three cycles, to evaluate the degree of prevention of a “short” vaccination schedule (Fig. 2, C and D). This treatment still determined a highly significant delay in mammary carcinogenesis (P < 0.001 versus MSA control group), but the proportion of tumor-free mice progressively decreased over time, with a quarter of mice remaining tumor-free at 1 year of age. The comparison of short vaccination protocols including systemic rIL-12 or engineered IL-12 did not yield significantly different results.

**Effects of Vaccination on Tumor Progression.** A microscopic analysis of mammary glands was first performed by the whole mount technique (Fig. 3). Untreated controls at 15 weeks of age showed a diffuse hyperplasia with some focal in situ carcinoma that led to the appearance of multiple mammary tumors at 22 weeks (Fig. 3, A and B), eventually affecting almost all of the mammary glands (see Fig. 2). Vaccination with Neu^{neo}/H-2^d^/IL12 did not significantly modify hyperplasia and tumor onset (Fig. 3, C and D). On the contrary, vaccination with Neu/H-2^d^/IL12 led to mammary glands almost devoid of hyperplastic or neoplastic lesions (Fig. 3, E and F). Similar results were also shown by histological and immunohistochemical analysis. In vaccinated mice, mammary gland ducts were formed by a single layer of epithelial cells lacking the expression of both proliferating cell nuclear antigen and HER-2/neu oncogene product, p185^{neu} (Fig. 4, D–F). On the contrary, age-matched untreated mice revealed multifocal mammary carcinomas showing an expansive and invasive growth pattern and formed by highly proliferating (proliferating cell...
nuclear antigen-positive) cells strongly expressing surface p185 neu (Fig. 4, A–C).

Mammary glands from untreated controls and from mice vaccinated with Neu<sup>+</sup>/H-2<sup>Q</sup>/IL12 cells were collected at 6, 15, 19, and 26 weeks of age, and gene expression profiles were studied. Hierarchical clustering of the differentially expressed genes (Fig. 5; Supplementary Data) showed the overexpression in tumor-bearing samples of two large clusters (denoted as A and C) that mainly group genes induced by HER-2/neu expression or related to tumor progression, and one cluster of genes (B) overexpressed in tumor-negative vaccinated samples, almost exclusively composed by genes related to the immune response. Clusterizing of samples with respect to genes showed that mammary glands bearing multiple focal carcinomas (19 weeks) and macroscopic lobular tumors (26 weeks) clustered together and far apart from 6-week and 15-week (hyperplastic/early neoplastic) mammary glands, whereas mammary tissue from vaccinated mice even at
Fig. 5. Hierarchical clustering of mammary gland gene expression patterns of BALB-neuT mice at different time points of progression. Treatment of mice: CTRL, none; vax/A, vaccination with Neu/H-2^d^/A/IL12; vax/B, vaccination with Neu/H-2^d^/B/IL12. Genes with at least a 4-fold difference in at least one comparison (466 genes) were selected for clustering. Clusters A and C contain genes regulated by HER-2/neu or related to tumor progression, cluster B groups immune response genes. Gene symbols and names are shown in Supplementary Data as reported in Locus Link. Clusters A–C are indicated by colored bars.
Fig. 6. In vitro proliferation and cytokine release by spleen cells of BALB-neuT mice vaccinated with the indicated treatment. Spleen cells were collected after three vaccination cycles and incubated in vitro for 6 days alone or together with mitomycin C–treated cells expressing p185 neu (Neu/H-2d), allogeneic MHC glycoproteins (Neu/neu/H-2d), or both (Neu/H-2d). Supernatants were collected for cytokine ELISA assay and splenocytes were recovered and counted. Data are the mean from 3–8 mice per group; bars, ±SEM. Statistical significance versus control: *P < 0.05; **P < 0.01.

Fig. 7. Antibody production by BALB-neuT mice vaccinated with the indicated treatment. A, cytofluorometric analysis of serum binding to Neu/H-2d cells. Mean from 3–10 mice bled after 3–4 vaccination cycles (sera diluted 1:65); bars, ±SEM. Statistical significance: **P < 0.01 at least between vaccinations. Controls, Neu/neu/H-2d; Neu/neu/H-2d/IL12; Neu/H-2d + rIL-12; Neu/H-2d/IL12; Neu/H-2d alone. Mean of 5–6 mice per group as above. Statistical significance: **P < 0.01 at least between vaccinations. C, SDS-PAGE analysis of molecules immunoprecipitated from Neu-positive or -negative cells by immune or preimmune mice sera. Pooled sera from 2 mice bled after four vaccination cycles with Neu/H-2d/IL12 cells (lanes 1, 4, 5, and 6) or from preimmune mice (2, 6, and 9) were used for immunoprecipitation of protein extracts from [35S]methionine- and [35S]cysteine-labeled NeuH-2d/A, Neu/neu/H-2d, or NeuH-2d cells. The Ab4 anti-p185 neu monoclonal antibody was also used for immunoprecipitation from cell extracts (lanes 3, 7, and 10). In lane 4 the Neu/H-2d/A cell lysate was preabsorbed with Ab4 monoclonal antibody and protein A-Sepharose before immunoprecipitation with immune serum. Arrow indicates the expected size of the p185 neu protein.

26 weeks of age clustered together and closer to the tissues from 6- and 15-week-old mice. Therefore, vaccination appeared to block, even at the molecular level, the carcinogenetic program. The main difference between untreated mice at early stages of progression and vaccinated mice was the strong induction of immune system-related genes by vaccination (cluster B). No difference was observed in the expression pattern between mammary glands of mice treated with either IL-12-engineered cell vaccine or between the two time points analyzed (15 and 26 weeks).

Cell-Mediated and Humoral Responses. To study the immune activities associated with cancer prevention, we examined both cell-mediated and humoral responses elicited in BALB-neuT mice by IL-12-engineered allogeneic cell vaccines in comparison to the systemic administration of rIL-12. Activation of spleen cells was studied both by a cytotoxicity assay and by proliferative and cytokine-releasing activity in culture after three cycles of treatment. After in vitro MLTC restimulation, splenocytes from mice vaccinated with Neu/H-2d/IL12 cells displayed very low cytotoxic activity (<10%; data not shown) against Neu/H-2d or Neu/H-2d tumor cells, in agreement with data obtained previously with the systemic rIL-12-including protocol (19). However, the MLTC-derived lymphoblasts efficiently lysed allogeneic H-2d splenocytes, indicating that alloreactive CTLs were generated.

Spleen cells from mice vaccinated with Neu/H-2d cells engineered to release IL-12 (Neu/H-2d/IL12) or with systemic rIL-12 displayed significantly increased spontaneous in vitro proliferation and cytokine production compared with control mice (Fig. 6) and mice treated with systemic rIL-12 alone (19). Vaccines increased production of IFN-γ and IL-4. IFN-γ was released both by CD4 and CD8 cells, whereas IL-4 was only produced by CD4 cells (data not shown). Proliferation and cytokine release were additionally increased by in vitro restimulation with cells presenting p185 neu (Neu/H-2d), allogeneic MHC glycoproteins (Neu/neu/H-2d), or both (Neu/H-2d); therefore, a polyclonal activation of the immune response occurred, as can be expected based on the multiple antigenic stimulation. The comparison of the vaccination protocols including systemic or engineered IL-12 showed similar in vitro proliferation activity and cytokine release. It should be noted that similar levels of in vitro proliferation and cytokine release were also found in mice.
Fig. 8. Levels of interleukin (IL)-12 (A and B) and IFN-γ (C and D) in sera of mice vaccinated with Neu/H-2q/IL12 cells or Neu/H-2q cells + recombinant (r)IL-12. A and C, kinetics of serum cytokines induced by cell vaccines. Mice were bled the day before vaccination (day 0), then 2 h after cell injection (day 1) and every 24 h thereafter. Neu/H-2q cells, ○; Neu/H-2q/IL12 cells. B and D, kinetics of serum cytokines induced by treatment with rIL-12 (●). Mice were bled the day before starting treatment (day 0), 2 h after rIL-12 injection from day 1 to day 5, then every 24 h thereafter. Mean from 3–9 mice per point are shown; bars, ±SEM. Sensitivity levels of ELISA assays were 10 pg/ml. Untreated mice mostly showed negligible (=10 pg/ml) cytokine levels.

vaccinated with Neu\textsuperscript{neg}/H-2q cells both engineered to release IL-12 or combined with systemic rIL-12 (data not shown).

Fig. 9. Lack of inhibition of mammary carcinogenesis in immuno-deficient knockout BALB-neuT transgenic mice. A and B. IFN-γ knockout BALB-neuT mice; C and D, µMT-antibody negative BALB-neuT mice (monitored for the lack of antibody response). Groups of 5–10 mice received the indicated treatment. A and C, tumor-free survival curve. Statistical significance of difference between Neu/H-2q/IL12 and murine serum albumin (MSA) group: P < 0.01 in µMT-antibody negative BALB-neuT mice. B and D, tumor multiplicity per mouse (mean; bars, ±SEM). Statistical significance of difference between Neu/H-2q/IL12 and MSA group: P < 0.05 at least from week 18 onwards in µMT-antibody negative BALB-neuT mice.

Systemic Cytokine Levels Induced by Vaccination. A rationale for the use of IL-12-engineered cell vaccines was to avoid potential systemic toxicity by administered rIL-12 or by IL-12-induced IFN-\gamma; therefore, we examined serum levels of IL-12 and IFN-\gamma in vaccinated mice (Fig. 8). For both cytokines, levels induced by IL-12-engineered cells showed rapidly transient peaks, approaching at most 100 pg/ml for 1–2 days. Such levels were about 1 order of magnitude lower than those obtained with exogenous rIL-12, which caused cytokine serum concentrations at ng/ml levels for 4–5 consecutive days. Even considering that vaccination cycles with engineered cells consisted of four injections at 3–4 day intervals, the calculated cumulative cytokine levels and exposure times for the whole treatment were still lower than those of the rIL12-including protocol. These data show that IL-12-engineered vaccines could have a decreased potential toxicity without loss of vaccine efficacy.
Lack of Inhibition of Mammary Carcinogenesis in Immunodeficient Knockout BALB-neuT Mice. The immunopreventive efficacy of the vaccination with Neu/H-2\(^\delta\)/IL12 cells was completely lost in HER-2/neu transgenic mice lacking IFN-\(\gamma\) (Fig. 9, A and B) and was severely decreased in IgM knockout \(\mu\)MT HER-2/neu transgenic mice unable to mount an antibody response (Fig. 9, C and D). The latter mice were still able to produce IFN-\(\gamma\) in response to the allogeneic stimuli and to IL-12 released by the engineered cell vaccine (data not shown), which explain the residual immunopreventive activity. The results obtained in knockout mice indicate that both IFN-\(\gamma\) and antibody responses played central roles in the immunopreventive efficacy of IL-12-engineered allogeneic HER-2/neu cell vaccines.

**DISCUSSION**

Data reported in this article show that a potent immunopreventive cell vaccine against HER-2/neu mammary cancer can be obtained through IL-12 gene transduction into allogeneic HER-2/neu cells. A comparison among various engineered cytokines showed that IL-12 is much more effective than IFN-\(\gamma\), IL-2, or IL-15. Moreover, engineered IL-12 can substitute for the systemically administered rIL-12, without loss of efficacy and with a lower toxic potential.

Cancer immunoprevention is a fresh and intriguing notion that arose from experimental data showing that immunoprophylactic approaches protect healthy individuals from cancer growth, even in models with a low immunogenicity, compared with the low efficacy of immunotherapeutic approaches in hosts bearing established tumors (1–5). The knowledge of individual genetic predisposition to defined cancers will provide the basis to develop tailored immunoprevention treatment. Of course immunoprophylaxis in healthy patients should take in the most consideration the risk:benefit ratio of the treatment, developing strategies with no or very limited potential side effects (3).

IL-12 is a promising adjuvant for cancer and other vaccines (28–31, 48). This cytokine polarizes the immune response toward the Th1 type and activates potent antiangiogenic mechanisms, mainly through the induction of IFN-\(\gamma\) and a cascade of other secondary and tertiary cytokines (29).

IL-12 is a key cytokine in cancer immunoprevention. Systemic rIL-12 administered alone in healthy mice during the induction of chemical carcinogenesis (49) or the development of HER-2/neu transgenic mammary tumors (9) was able to delay the carcinogenic process. Antiangiogenic IFN-\(\gamma\)-mediated mechanisms played an important role in delaying both chemical and transgenic carcinogenesis (9, 29, 49, 50). The combination of systemic IL-12 with an allogeneic antigen-expressing cell vaccine led to an almost complete prevention of HER-2/neu-induced mammary tumorigenesis (19). In this combined approach the maximal prevention required all of the three components (HER-2/neu, allogeneic MHC antigens, and rIL-12) and was due to the induction of both cellular and humoral immune responses. Although Th1 and Th2 responses were both increased by our vaccine approach, the relative importance of IFN-\(\gamma\)-dependent antibody subclasses for successful cancer prevention indicates that type-1 responses were more important than type-2 (Ref. 19 and present article). Therefore, rIL-12 seems a crucial component for the induction of a full immunoprevention of carcinogenesis. Mice subjected to a lifelong treatment with the three-component vaccine were healthy, with no evident toxicity, and their lifetime almost approached that of nontransgenic parental mice. Such an outstanding result, however, was not amenable to clinical development, because doses of rIL-12 used in murine models are well above the maximum tolerated dose in humans.

The maximum tolerated dose of rIL-12 in humans ranges between 200 and 500 ng/kg for i.v. or s.c. administration (29, 30). Severe toxic effects that included fever and flu-like symptoms, nausea, fatigue, oral stomatitis, and elevation in liver enzymes were correlated with the induction of IFN-\(\gamma\)-\(\beta\) in serum. A slightly higher tolerability was reported for i.p. administration of rIL-12, likely due to the prolonged confinement of IL-12 and IFN-\(\gamma\) in the peritoneal cavity (51). Attempts to decrease rIL-12 dose in the prevention of HER-2/neu transgenic mammary cancer showed that minimal doses of systemic rIL-12 that delay tumor formation were still higher than human maximum tolerated dose (52).

The major activity of IL-12 in our three-component vaccine appeared to be its adjuvant role in the induction of the immune response (19); thus, we hypothesized that an IL-12-engineered cell vaccine, able to release the cytokine locally, could attain the same result with a lower toxicity risk. We found that IL-12-engineered allogeneic HER-2/neu cell vaccines elicit the same high level of prevention of HER-2/neu mammary carcinogenesis obtained previously with cells plus systemic rIL-12. The high efficacy of IL-12-engineered cell vaccine with an overall tumor-free survival at 1 year higher than 80% was illustrated by morphological studies and confirmed at the molecular level by the analysis of gene expression profiles. Mice vaccinated with IL-12-engineered cells showed strongly decreased levels of circulating IL-12 and IFN-\(\gamma\) as compared with mice receiving rIL-12, thus suggesting that a major risk of systemic toxicity (i.e., cytokine serum level) could be avoided or significantly reduced by this gene therapy approach without loss of efficacy.

Data from both morphological and molecular studies indicated that mammary gland “normalization” was an early event, already detectable after three to four vaccination cycles; therefore, we also tested the possibility of shortening the vaccination protocol to the first three cycles, to additionally decrease the potential toxicity of the immunoprophylactic treatment. However, this short protocol only afforded a delay in mammary tumor development; also in this case systemic versus engineered IL-12-including vaccinations showed similar efficacy. Such data also show that periodic boosts of the immune response are necessary to obtain a high-level protection. The possibility, however, that a lighter lifelong vaccination schedule (i.e., by decreasing the number of cell vaccine injections per cycle) could attain the same result remains to be explored.

In conclusion, we showed here for the first time that IL-12-engineered tumor cells can be successfully used as an immunoprophylactic multicomponent vaccine to protect mice from spontaneous genetically determined carcinogenesis.

**ACKNOWLEDGMENTS**

We thank Guido Forni (University of Turin, Turin, Italy) for helpful discussions and critical reading of the manuscript.

**REFERENCES**


Immunoprevention of HER-2/neu Transgenic Mammary Carcinoma through an Interleukin 12-Engineered Allogeneic Cell Vaccine

Carla De Giovanni, Giordano Nicoletti, Lorena Landuzzi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/11/4001

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2004/06/08/64.11.4001.DC1

Cited articles
This article cites 51 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/11/4001.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/64/11/4001.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.