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# LAG-3 Enables DNA Vaccination to Persistently Prevent Mammary Carcinogenesis in HER-2/*neu* Transgenic BALB/c Mice[1]

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## ABSTRACT

**Within 33 weeks of life, all 10 mammary glands of virgin BALB/c mice transgenic for the transforming rat HER-2/*neu* oncogene under the mammary tumor virus promoter (BALB-neuT mice) progress from atypical hyperplasia to invasive palpable carcinoma. Repeated DNA vaccination with plasmids coding for the extracellular and transmembrane domain of the protein product of rat HER-2/*neu* (r-p185$^{neu}$) delayed tumor onset and reduced tumor multiplicity, but this protection eventually declined, and few mice were tumor free at 1 year of age. Association of plasmid vaccination with administration of soluble mouse LAG-3 (lymphocyte activation gene-3/CD223) generated by fusing the extracellular domain of murine LAG-3 to a murine IgG2a Fc portion (mLAG-3Ig) elicited a stronger and sustained protection that kept 70% of 1-year-old mice tumor free. Moreover, this combined vaccination, which was performed when multiple *in situ* carcinomas were already evident, extended disease-free survival and reduced carcinoma multiplicity. Inhibition of carcinogenesis was associated with markedly reduced epithelial cell proliferation and r-p185$^{neu}$ expression, whereas the few remaining hyperplastic foci were heavily infiltrated by reactive leukocytes. A stronger and enduring r-p185$^{neu}$-specific cytotoxicity, a sustained release of IFN-$\gamma$ and interleukin 4, and a marked expansion of both CD8$^+$/CD11b$^+$/CD28$^+$ effector and CD8$^+$/CD11b$^+$/CD28$^-$ memory effector T-cell populations were induced in immunized mice. This combined vaccination also elicited a quicker and higher antibody response to r-p185$^{neu}$, as well as an early antibody isotype switch. These data suggest that the appropriate costimulation provided by mLAG-3Ig enables DNA vaccination to establish an effective protection, probably by enhancing cross-presentation of the DNA coded antigen.**

## INTRODUCTION

The concept of using specific immune responses to hamper early preclinical stages of tumor progression has been recently endorsed by several experimental observations (1). Engineered cell vaccines, protein, peptides, and DNA vaccines have been shown to effectively protect transgenic mice genetically predestined to develop mammary and prostate carcinomas (reviewed in Ref. 2). Whereas these studies provide significant proofs of concept, the model adopted markedly influences the weight of protection afforded because the kind and intensity of tolerance to the transgene product and the aggressiveness of the carcinogenesis are the variables that most critically affect the outcome of such protection (2).

HER-2/*neu* oncogene encodes a tyrosine kinase growth factor receptor (p185$^{neu}$) homologous to other members of epidermal growth factor receptor family (3). Its overexpression is frequent in human epithelial tumors and correlates with particular aggressiveness (4). In the rat, a single point mutation replacing the valine residue at position 664 in the TM[3] of r-p185$^{neu}$ with glutamic acid favors r-p185$^{neu}$ homo- and heterodimerization that converts the rat Her-2/neu proto-oncogene into a dominant transforming oncogene (5).

BALB/c virgin female mice transgenic for the transforming rat HER-2/*neu* oncogene (referred to as BALB-neuT mice) provide one of the most aggressive models of rat HER-2/*neu* multifocal mammary carcinogenesis (6, 7). In 3-week-old BALB-neuT mice, r-p185$^{neu}$ is markedly overexpressed on the surface of the cells of the rudimental mammary gland (6). At 6 weeks, the r-p185$^{neu}$ cells give rise to a widespread mammary atypical hyperplasia. Multiple microscopic masses somewhat equivalent to multiple carcinomas *in situ* are evident in all 10 mammary glands around week 10. These enlarge and converge in a rapidly growing, invasive, and metastasizing carcinoma that becomes palpable in all 10 glands between the 25th and 30th week of age (6–8). In these mice, repeated vaccination with plasmids coding for distinct portions of r-p185$^{neu}$ alone (9, 10) or in combination with the immunomodulatory 163–171 nonapeptide of IL-1$\beta$ (11) delayed tumor onset and reduced tumor multiplicity. However, this early protective response eventually declines as time progresses, and none or very few mice are tumor free at 1 year of age (Ref. 11; data not shown). This study reports the efficacy of DNA vaccination combined with the administration of soluble mouse LAG-3 (lymphocyte activation gene-3/CD223) generated by fusing the ECD of murine LAG-3 to a murine IgG2a Fc portion [mLAG-3Ig (12, 13)] in lengthening the protection offered by anti-r-p185$^{neu}$ DNA vaccination in BALB-neuT mice.

LAG-3 is a type I transmembrane protein associated with the T cell receptor-CD3 complex and binds MHC class II molecules in a manner similar to CD4. It is expressed in all subsets of T and natural killer cells after activation (14), and its expression is up-regulated by IL-2 and IL-12 (15). Soluble mLAG-3Ig engages MHC class II glycoprotein more efficaciously than CD4 (16). The manner in which class II molecules of DCs are loaded with peptides and engaged may have consequences for the immune responses they induce (17). The presence of LAG-3 on CD4 T cells binding certain forms of class II-associated peptides may more efficiently license DCs for class I peptide presentation to CD8 T cells (17). For this reason, soluble mLAG-3Ig has been used as vaccine adjuvant for both conventional (18) and tumor antigens (13).

The additive effect stemming from combining the vaccination with DNA plasmids coding for the extracellular domain and TM of r-p185$^{neu}$ (referred to as p185 plasmids) with mLAG-3Ig keeps most 1-year-old BALB-neuT mice tumor free and markedly extends their

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[3] The abbreviations used are: TM, transmembrane domain; DC, dendritic cell; ECD, extracellular domain; SPC, spleen cell; PCNA, proliferating cell nuclear antigen, IL, interleukin; dThd, thymidine; DPBS, Dulbecco's PBS; Mit-C, mitomycin C; mAb, monoclonal antibody; sbp, serum binding potential; L.U., lytic unit(s).

disease-free survival and reduces tumor multiplicity when multiple *in situ* carcinomas are already present.

## MATERIALS AND METHODS

**Mice.** BALB-neuT female mice overexpressing the transforming activated rat HER-2/*neu* oncogene under control of the mouse mammary tumor virus promoter (6) were bred for us under specific pathogen-free conditions by Charles River (Calco, Italy). Individually tagged virgin BALB-neuT mice were used and treated according to the European Union guidelines. Mammary glands were inspected weekly, and each tumor mass was measured with calipers in the two perpendicular diameters. Progressively growing masses with a mean diameter of >2 mm were regarded as tumors. Growth was monitored until all 10 mammary glands displayed a tumor or until a tumor exceeded a mean diameter of 10 mm, at which time mice were sacrificed for humane reasons.

**Cells.** N202.1A (r-p185$^{neu}$ positive) and N202.1E (r-p185$^{neu}$ negative) cell clones were derived from mammary carcinomas of a FVB-neuN #202 mouse (H-2$^{q}$) transgenic for the rat HER-2/*neu* proto-oncogene (19), whereas TUBO (r-p185$^{neu}$ positive) cell clones were obtained from a mammary carcinoma of a BALB-neuT mouse (H-2$^{d}$; Ref. 9). F1-F (H-2$^{d}$) is a r-p185$^{neu}$-negative skin fibroblast line that spontaneously transformed after the 15th *in vitro* passage (9). Cells were cultured in DMEM (BioWittaker, Walkerville, MD) supplemented with 20% (or 10% for F1-F) fetal bovine serum (Life Technologies, Inc., Milan, Italy) at 37°C in a humidified 5% $CO_2$ atmosphere.

**p185 Plasmids.** The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, CA) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A). The sequences for the ECD and TM of transforming r-p185$^{neu}$ were generated from the PCR product using the primers 3′-CGCAAGCTTCATCATGGAGCTGGC-5′ and 3′-CGGAATTCGGGCTGGCTCTCTGCTC-5′ and the primers 3′-CGCAAGCTTCATGGAGCTGGC-5′ and 3′-ATGAATTCTTTCCGCATCGTGTACTTCTTCCGG-5′, respectively, as described previously (9, 20). PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hin*dIII and *Eco*RI, and cloned into the multiple cloning site of the pCMV plasmid to obtain the p185 plasmid used in this work. *Escherichia coli* strain DH5α was transformed with p185 or the empty plasmid (pcDNA3) and then grown in Luria-Bertani medium (Sigma, St. Louis, MO; Ref. 20). Large-scale preparation of p185 plasmids was carried out by alkaline lysis, using Endofree Quiagen Plasmid-Giga kits (Qiagen, Chatsworth, CA). DNA was then precipitated, suspended in sterile saline at 1 mg/ml, and stored in aliquots at −20°C for subsequent use in immunization protocols.

**mLAG-3Ig.** Recombinant mLAG-3Ig molecules were generated by fusing the ECD of murine LAG-3 to a murine IgG2a Fc portion (13, 16) to form a recombinant protein that was then produced in Chinese hamster ovary cells and purified on protein A columns (Ares Advanced Technology, Randolph, MA). The total protein purity was >95% soluble LAG-3Ig by Coomassie Blue SDS-PAGE densitometry. Potential contamination of the purified protein with bacterial endotoxin (lipopolysaccharide) was determined by using the chromogenic Limulus amebocyte lysate assay (BioWittaker). A calibration curve based on enzymatic activity *versus* lipopolysaccharide was constructed to determine endotoxin units in the test sample, and values of <1 EU/mg were obtained for mLAG-3Ig.

**Vaccination Schedules.** At the specified time points, BALB-neuT mice received a single injection of 100 μg of p185 plasmids in 0.1 ml of sterile saline solution (0.9% NaCl; SALF, Bergamo, Italy) in the exposed left quadriceps through a 28-gauge needle syringe, followed a few seconds later by 0.1 ml of DPBS (Sigma) alone (DPBS controls) or containing 1 μg of either mLAG-3Ig or a nonspecific isotype-matched purified control mouse Ig (mIgG2a; PharMingen, San Diego, CA). Subsequent boosts with the same combinations were performed contralaterally.

**Whole Mount Analysis, Histology, and Immunohistochemistry.** Whole mount preparations were performed as reported by Medina (21). Briefly, the skin of euthanized BALB-neuT mice was fixed overnight in 10% buffered formalin. The mammary fat pads were scored into quarters and gently scraped from the skin. The quarters were immersed in acetone overnight and then rehydrated and stained in ferric hematoxylin (Sigma), dehydrated in increasing concentrations of alcohol, cleared with histo-lemon, and stored in methyl salilcylate (Sigma). Digital photos were acquired with a Nikon Coolpix 995 (Nital SpA, Turin, Italy) mounted on a stereoscopic microscope (MZ6; Leica Microsystems, Milan, Italy). Whole mounts were then embedded in paraffin, sectioned at 4 μm, and stained with H&E. For immunohistochemistry, these sections were incubated for 30 min with anti-r-p185$^{neu}$ antibodies (C-18-G; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-PCNA antibodies (Ylem, Rome, Italy). After washing, they were overlaid with biotinylated goat antirat or antirabbit immunoglobulin (Vector Laboratories, Burlingame, CA) for 30 min. Unbound antibodies were removed, and the slides were incubated with avidin-biotin complex/alkaline phosphatase (Dako, Milan, Italy).

**Cytotoxic Activity.** A single-cell suspension of SPCs obtained from the spleen of each of five mice from each treatment group was washed in DPBS and resuspended in RPMI 1640 (BioWittaker) supplemented with 10% fetal bovine serum as described previously (22). SPCs ($2 \times 10^7$) from each suspension were cultured with $5 \times 10^5$ Mit-C (Sigma)-treated stimulator TUBO cells (22) for 6 days at 37°C. Recovered lymphocytes were admixed with $5 \times 10^3$ [$^3$H]dThd-labeled TUBO or F1-F target cells at various E:T ratios in round-bottomed 96-well microtiter plates in triplicate. The percentage of specific lysis was then evaluated after 48 h and recorded, and the data were expressed as L.U. ($L.U._{.20}/10^7$) calculated according to the equation of Pross *et al*., as previously described in detail (22).

**Cytokine Production.** The amount of IFN-γ and IL-4 released by $2 \times 10^6$ SPCs/ml cultured for 2 days at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum in the absence (fresh SPCs) or presence of 1 μg/ml anti-CD3 and anti-CD28 mAb [PharMingen (stimulated SPCs)] was assayed by ELISA (IFN-γ, PharMingen; IL-4, R&D System Inc., Minneapolis, MN).

**Flow Cytometry.** SPCs obtained at progressive time points from five mice from each treatment group were incubated individually or pooled at first with rat anti-CD8 antibody (53.6.72 hybridoma, anti-Lyt 2; America Type Culture Collection, Manassas, VA) and then with anti-r-Ig-coated immunomagnetic beads (Miltenyi Biotec, Calderara di Reno, Italy). Recovered $CD8^+$ cells (>96% purity) were stained with anti-CD28-FITC (Cedarlane Laboratories, Celbio, Pero, Italy) and anti-CD11b-phycoeritrine (PharMingen) antibodies. Two-color analysis of both surface molecules was performed by FACScan (Becton Dickinson, Mountain View, CA). The cells were gated on living cells by cell size and granularity. Data analyzed through CellQuest (Becton Dickinson) software were displayed as bivariate dot plots.

**Antibody Response.** At the specified time points, sera were collected from mice in each group, and their binding to r-p185$^{neu}$-positive N202.1A and r-p185$^{neu}$-negative N202.1E cells was determined by flow cytometry after 1:200 dilution (9). The antibody titer is expressed as sbp $\times 10^{-3}$/ml, calculated as previously described in detail (9, 22). Isotype determinations were carried out by an indirect immunofluorescence procedure on cells preincubated with mouse sera diluted 1:20 by adding a secondary biotin-conjugated mAb directed against mouse IgG1, IgG2a, IgG2b, IgG3, and IgM, all from CALTAG Laboratories (Burlingame, CA). FITC-streptavidin (Dako) was used as the last step.

**Statistics.** Differences in tumor incidence were evaluated with the Mantel-Haenszel log-rank test; differences in tumor multiplicity, L.U., IFN-γ and IL-4 production by SPCs, and antibody titer were evaluated with Student's *t* test.

## RESULTS

**Inhibition of the Progression of Mammary Lesions.** To evaluate whether the association of mLAG-3Ig enhanced the protective efficacy of DNA vaccination, BALB-neuT mice were vaccinated on weeks 4 and 7 with plasmids plus control mIgG2a or mLAG-3Ig, and their mammary pad was inspected weekly to monitor the appearance of palpable tumor masses of increasing size. The combination of empty plasmids plus mLAG-3Ig did not affect the time of first appearance of a palpable carcinoma as compared with DPBS controls, and no mice were tumor free at week 25 (Fig. 1, *left panel*). Only a slight, temporary reduction in carcinoma multiplicity was found (Fig. 1, *right panel*). By contrast, in mice vaccinated with p185 plasmids plus either control mIgG2a or mLAG-3Ig, the first appearance was greatly delayed, and carcinoma multiplicity was markedly reduced (Fig. 1, *right panel*). Around week 40, whereas carcinomas

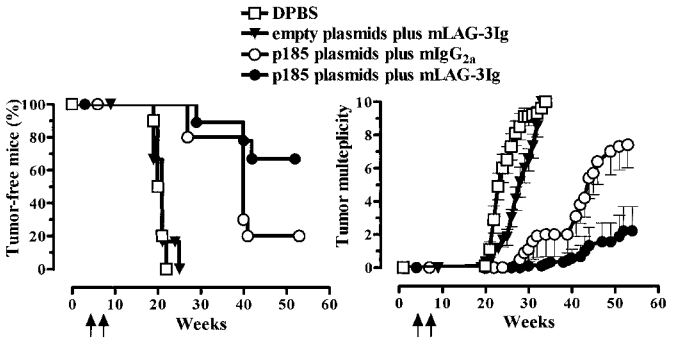


Fig. 1. Inhibition of mammary carcinogenesis in BALB-neuT mice vaccinated on the 4th and 7th week (*arrows*) with p185 plasmids plus mLAG-3Ig. Percentage of tumor-free mice (*left panel*) and tumor multiplicity (*right panel*) calculated as the cumulative number of incident tumors/total number of mice and shown as mean ± SE are represented. Mice received DPBS alone, empty pcDNA3 plasmids plus mLAG-3Ig, and p185 plasmids plus control mIgG2a or mLAG-3Ig. Each group consisted of 9 or 10 mice. The tumor-free survival curve of mice vaccinated with p185 plasmids plus LAG-3Ig is significantly different ($P = 0.033$ by the Mantel-Haenszel test) from that of mice vaccinated with p185 plasmids plus control mIgG2a. From week 29, the mean tumor multiplicity in mice vaccinated with p185 plasmids plus control mIgG2a or mLAG-3Ig is significantly different ($P = 0.01$, Student's $t$ test).

had become palpable in several glands of the great majority of mice vaccinated with p185 plasmids plus control mIgG2a, almost 70% of those vaccinated with p185 plasmids plus mLAG-3Ig were tumor free and remained so until week 52, when the experiment was ended (Fig. 1, *left panel*). The mean tumor multiplicity in this group was about 2 and was significantly lower than that of mice vaccinated with p185 plasmids plus control mIgG2a (Fig. 1, *right panel*).

**Morphological Analyses.** Sequential whole mounts of the mammary glands illustrated the steps of carcinogenesis in the DPBS control and DNA vaccinated mice. The numerous hyperplastic foci and neoplastic side buds evident in mammary glands of 8-week-old mice progressed to confluent and well-established carcinomas by week 17 in both DPBS controls (data not shown) and empty vector plus mLAG-3Ig-injected mice (Fig. 2, *top row*), whereas they were dramatically and similarly reduced at these times in mice that received p185 plasmids plus either control mIgG2a or mLAG-3Ig. However, by week 52, carcinoma masses of different size and invasiveness were evident in the few surviving mice immunized with plasmids plus control mIgG2a (Fig. 2, *middle row*), whereas those that received mLAG-3Ig were almost all free from both palpable masses (Fig. 1)

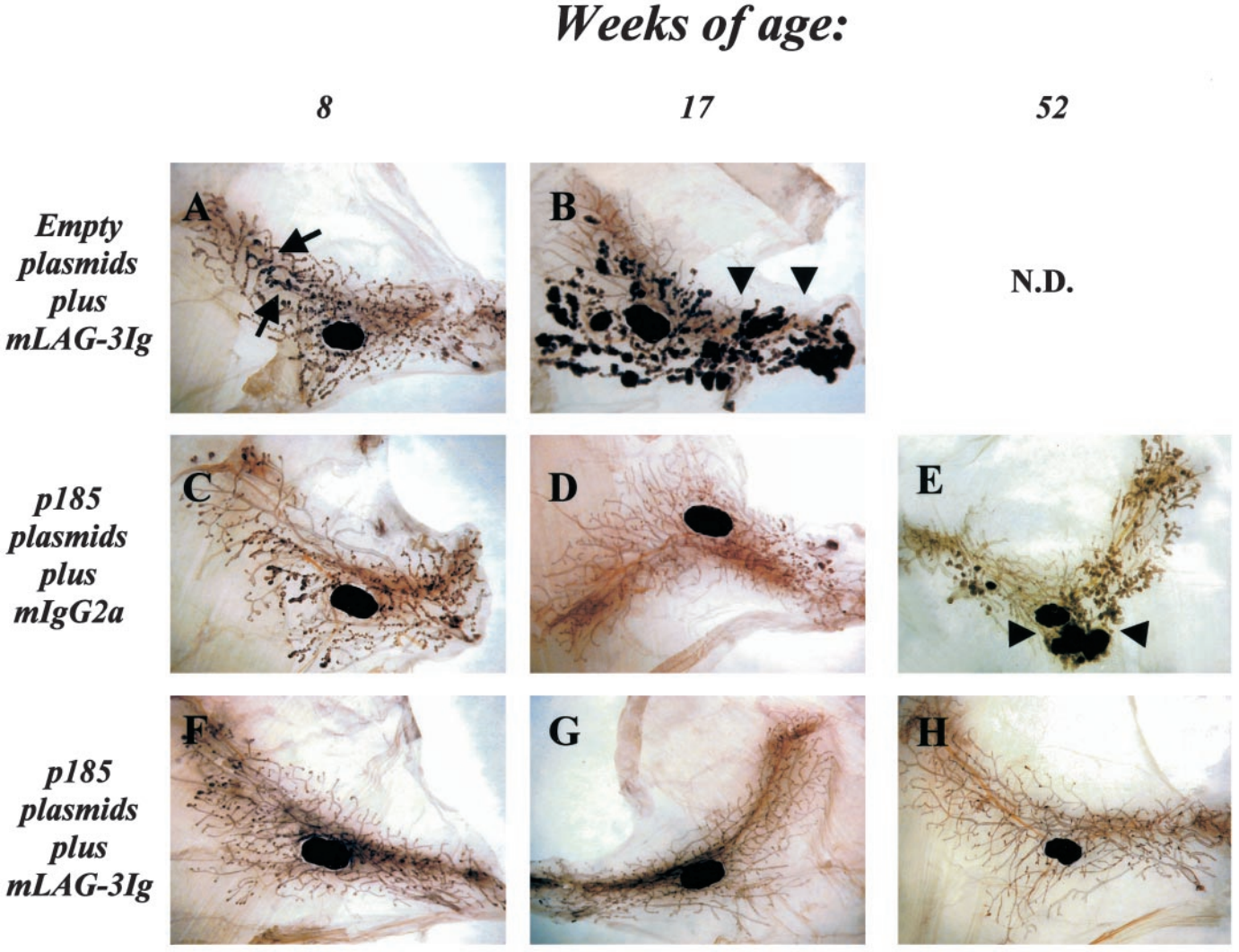


Fig. 2. Sequential whole mounts of the mammary glands showing progression of carcinogenesis in vaccinated BALB-neuT mice. At the 8th week of age, mice vaccinated with empty pcDNA3 plasmids plus mLAG-3Ig showed numerous side buds and hyperplastic foci (*A, arrows*) that progressed to confluent and well-established carcinomas (*B, arrowheads*) by the 17th week. An almost identical progression pattern was displayed by DPBS controls (data not shown), whereas a progressive dramatic reduction of side buds and hyperplastic foci was displayed by mice vaccinated with p185 plasmids plus control mIgG2a (*C* and *D*). At week 52, however, these mice display renewal of the neoplastic growth, resulting in the development of hyperplastic foci and carcinoma masses (*E, arrowheads*). In the mammary glands of the majority of mice vaccinated with p185 plasmids plus mLAG-3Ig, the clearance of side buds evident at week 8 (*F*) was complete at week 17 (*G*) and persisted until week 52 (*H*). The *central oval black area* in all panels is the draining lymph node. N.D., not done.

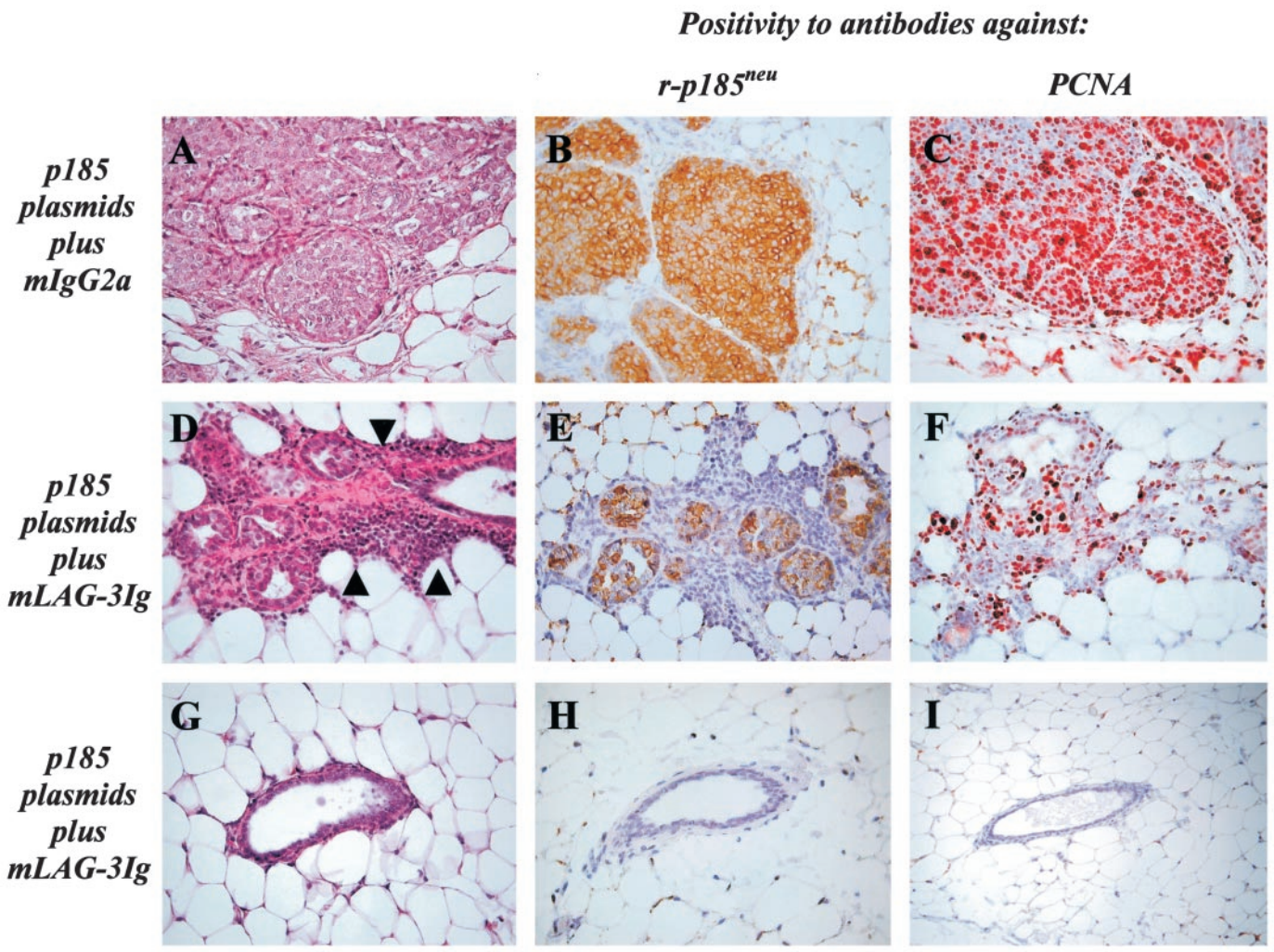


Fig. 3. Histological features of carcinogenesis progression, expression of r-p185$^{neu}$, and nuclear expression of PCNA in the mammary glands of vaccinated BALB-neuT mice. The late invasive carcinomas (*A*) that eventually grew in the mammary glands of 52-week-old mice vaccinated with p185 plasmids plus control mIgG2a also displayed high r-p185$^{neu}$ (*B*) and PCNA expression (*C*). In the mammary glands of two of six 52-week-old mice vaccinated with p185 plasmids plus mLAG-3Ig, foci of atypical hyperplasia were surrounded and invaded by a prominent reactive cell infiltrate (*D*, *arrowheads*). In these lesions, the expression of r-p185$^{neu}$ was markedly reduced and mostly confined to the cytoplasm (*E*), whereas few cells displayed PCNA positivity (*F*). The mammary glands of the other four 52-week-old mice were formed of ducts and ductules (*G*) lined by a single layer of epithelial cells without evident r-p185$^{neu}$ (*H*) and PCNA (*I*) expression.

and side buds (Fig. 2, *bottom row*). This inhibition of progression was evident at week 8 and 17 and became more evident by week 52.

These progression and inhibition patterns were fully endorsed histologically at week 52. Multiple invasive lobular carcinomas expressing both membrane r-p185$^{neu}$ and nuclear positivity to PCNA (Fig. 3, *A*−*C*) and foci of atypical hyperplasia and neoplastic side buds surrounded by a scanty reactive necrotic and hemorrhagic cell infiltrate were evident in mice vaccinated with p185 plasmids plus control mIgG2a (Fig. 3*A*). Lobular carcinomas and foci of atypical hyperplasia were also found in two of six survivor mice vaccinated with p185 plasmids plus mLAG-3Ig (Fig. 3*D*), although a few of their glands displayed a pronounced reactive cell infiltrate in close contact with neoplastic and hyperplastic epithelial cells, in which the expression of r-p185$^{neu}$ was markedly reduced and mostly confined to the cytoplasm (Fig. 3*E*). This limited expression was associated with reduced PCNA positivity (Fig. 3*F*). By contrast, all of the glands of the other four survivors were formed of ducts and ductules lined with a single layer of epithelial cells without r-p185$^{neu}$ and PCNA expression (Fig. 3, *G*−*I*).

**Cellular Reactivity Associated with Carcinogenesis Inhibition.** To evaluate *in vitro* cellular immune responses to r-p185$^{neu}$ associated with the impressive and long-lasting inhibition of carcinogenesis, SPCs were obtained at progressive times after vaccination, stimulated with r-p185$^{neu}$-positive TUBO cells, and assayed against [$^{3}$H]dThd-labeled target cells. Whereas no significant cytotoxicity was found 1 week after the last boost (Fig. 4), SPCs obtained 10 and 28 weeks after the last boost with p185 plasmids plus mLAG-3Ig displayed a significant r-p185$^{neu}$-specific cytotoxicity (Fig. 4). At 10 weeks after the last boost (mice were 17 weeks of age), the cytotoxic response was much stronger in mice immunized with p185 plasmids plus mLAG-3Ig than in mice immunized with p185 plasmid plus mIgG2a. Twenty-eight weeks after the last boost (mice were 35 weeks of age), the cytotoxic response was almost nil in mice immunized with p185 plasmid plus control IgG2a. By contrast, a low but significant cytotoxicity was still evident in mice immunized with plasmids plus mLAG-3Ig (Fig. 4). The cytotoxicity of SPCs from mice treated with DPBS or empty plasmids plus mLAG-3Ig was always marginal (data not shown).

The kinetics of cytokine production by fresh and anti-CD3 and anti-CD28-stimulated SPCs from mice immunized with p185 plasmids plus control mIgG2a and mLAG-3Ig was then evaluated. No differences in the amounts of IFN-$\gamma$ and IL-4 released were found 1 week and 10 weeks after the last boost (Fig. 5). However, 28 weeks after the last boost, stimulated SPCs from mice that received p185

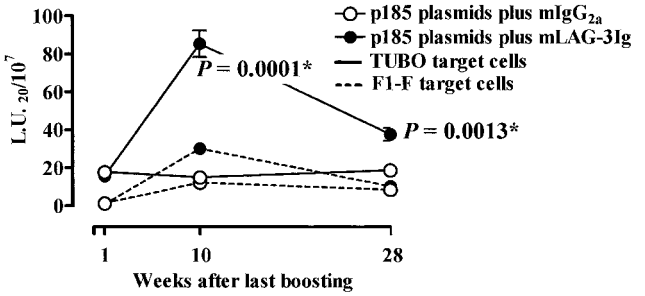


Fig. 4. Comparison of the cytotoxic activity of SPCs from BALB-neuT mice vaccinated in the presence or absence of mLAG-3Ig. SPCs from five vaccinated mice 1 week (8-week-old mice), 10 weeks (17-week-old mice), and 28 weeks (35-week-old mice) after the last boost with p185 plasmids plus control mIgG2a or mLAG-3Ig were stimulated with Mit-C-treated TUBO cells and assayed in a 48-h [$^3$H]dThd release assay against r-p185$^{neu}$-positive TUBO and r-p185$^{neu}$-negative F1-F target cells. Cytotoxic activity is expressed as L.U. (L.U.$_{20}$/10$^7$), mean ± SE. Where vertical bars are not appreciable, the SE is smaller than the size of the symbol. ∗, L.U. from mice immunized with p185 plasmids plus mLAG-3Ig were significantly different from those of mice immunized with p185 plasmids plus mIgG2a at both 10 and 28 weeks.

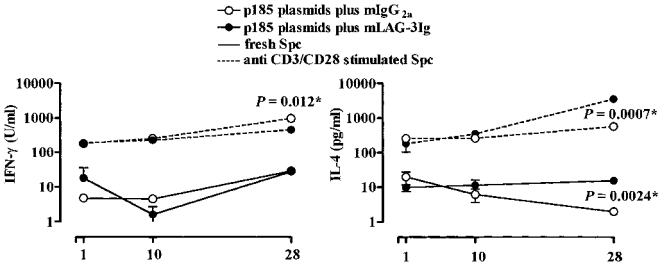


Fig. 5. Comparison of IFN-γ (*left panel*) and IL-4 (*right panel*) production by SPCs from BALB-neuT mice vaccinated in the presence or absence of mLAG-3Ig. SPCs from vaccinated mice 1 week (8-week-old mice), 10 weeks (17-week-old mice), and 28 weeks (35-week-old mice) after the last boost with p185 plasmids plus control mIgG2a or mLAG-3Ig. The amount of cytokine released by fresh and anti-CD3 and anti-CD28 mAb-stimulated SPCs was shown as a mean titer released by SPCs from 5 individually tested mice/group ± SE. ∗, values from mice immunized with p185 plasmids plus mLAG-3Ig are significantly different from those of animals immunized with p185 plasmids plus mIgG2a at both 10 and 28 weeks.

plasmids plus mLAG-3Ig released greater amounts of both cytokines. A marked amount of IL-4 was also released at this time by fresh SPCs from mice immunized with p185 plasmids plus mLAG-3Ig, whereas at all time points the titers released by fresh SPCs from mice treated with DPBS or empty plasmids plus mLAG-3Ig were marginal, and those released by stimulated cells were no higher than 100 units/ml IFN-γ and 150 pg/ml IL-4 (data not shown).

Assessment of effector/memory CD8 cells in the spleen showed that CD11b$^+$/CD28$^+$ double-positive lymphocytes were more numerous in SPCs from five mice vaccinated with p185 plasmids plus mLAG-3Ig (Fig. 6). This difference was already evident 1 week after the last boost and doubled after 10 weeks. This increase may be correlated with the higher reactivity of mice immunized with p185 plasmids plus mLAG-3Ig because their effector T cells are still able to proliferate and produce IFN-γ after stimulation (23). Furthermore, 10 weeks after the last boost, mice vaccinated with p185 plasmids plus mLAG-3Ig presented a markedly expanded population of CD8$^+$/CD11b$^+$/CD28$^-$ cells. These are thought to comprise memory effector cells that kill target cells and migrate to inflammatory sites but no longer proliferate (23, 24). No expansion of these two T-cell populations was found in SPCs from mice treateds with DPBS or empty plasmids plus mLAG-3Ig (data not shown).

**Anti-r-p185$^{neu}$ Antibody Response Associated with Carcinogenesis Inhibition.** After p185 plasmid immunization, the antibody response to r-p185$^{neu}$ increased during the following 28 weeks. Both the speed of appearance and the titer of anti-r-p185$^{neu}$ antibodies were markedly higher in mice vaccinated with p185 plasmids plus mLAG-3Ig (Fig. 7). One week after the last boost, whereas the response in mice immunized with p185 plasmids plus control mIgG2a was mostly due to IgM, a T helper cell switch signal was suggested by the presence of anti-r-p185$^{neu}$ IgG of all subclasses in the sera of mice immunized with p185 plasmids plus mLAG-3Ig (Fig. 7). The quick appearance and persistent high production of IgG1 in these sera pointed to the influence of IL-4 in the B-cell isotype switch (25). The dominance of IgG1, in fact, was still evident 28 weeks after the last boost with p185 plasmids plus mLAG-3Ig (Fig. 7).

**Delay of Mammary Carcinogenesis.** In the next series of experiments, BALB-neuT mice were vaccinated and boosted much later (the 14th and 16th weeks), when their mammary glands already displayed multiple lesions somewhat similar to carcinoma *in situ* (Fig. 8, *A* and *B*). At this stage, vaccination with plasmids plus control mIgG2a was no longer able to delay the appearance of the first tumor or reduce tumor multiplicity (Fig. 8, *C* and *D*). By contrast, the time at which one tumor was palpable was markedly extended to week 36 in all mice vaccinated with p185 plasmids plus mLAG-3Ig compared with around week 21 in the other treatment groups. Tumor multiplicity, too, was strongly reduced from week 21 until week 52, when the experiment ended.

Two weeks after the last boost, a stronger SPC cytotoxic activity, higher levels of IFN-γ and IL-4 release, and higher titers of anti-r-p185$^{neu}$ antibodies were found in mice vaccinated in the presence of mLAG-3Ig (Fig. 9).

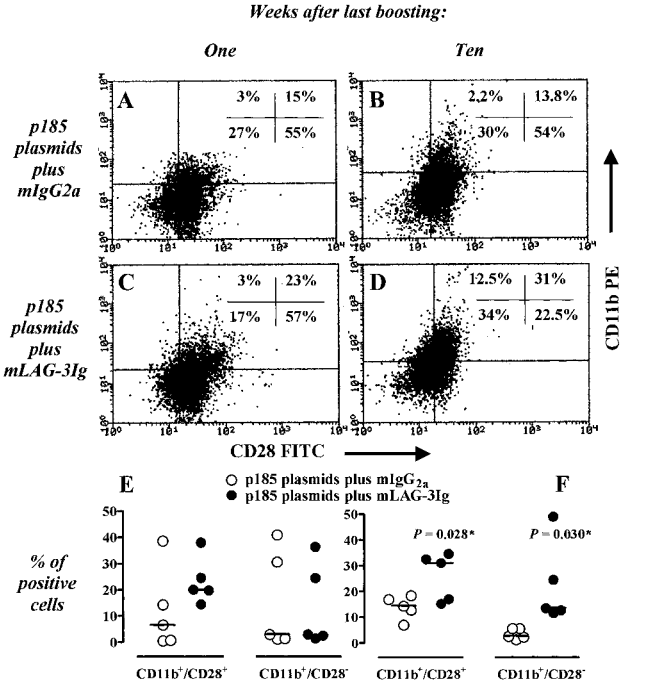


Fig. 6. Expansion of CD8$^+$/CD11b$^+$/CD28$^+$ and CD8$^+$/CD11b$^+$/CD28$^-$ cell populations in the SPCs of vaccinated BALB-neuT mice. SPCs from mice vaccinated with p185 plasmids plus control mIgG2a (*A* and *B*) or plus mLAG-3Ig (*C* and *D*) were obtained 1 and 10 weeks after the last boost and processed (both pooled and individually) for CD8 enrichment. CD8$^+$-enriched cells (>96%) were stained with anti-CD28-FITC (*abscissa*)- and anti-CD11b-PE (*ordinate*)-specific antibodies. *A*–*D* show representative cytofluorometric analysis with pools of CD8$^+$-enriched cells from five mice analyzed with CellQuest software (Becton Dickinson). The percentage of cells in each quadrant is given in the *top right corner* of each panel. *E* and *F* show the percentages of CD11b$^+$/CD28$^+$- and CD11b$^+$/CD28$^-$-positive cells present in CD8$^+$-enriched cells obtained from five mice tested independently. *Horizontal bars* mark the median values. ∗, values from mice immunized with p185 plasmids plus mLAG-3Ig are significantly different from those of mice immunized with p185 plasmids plus mIgG2a.

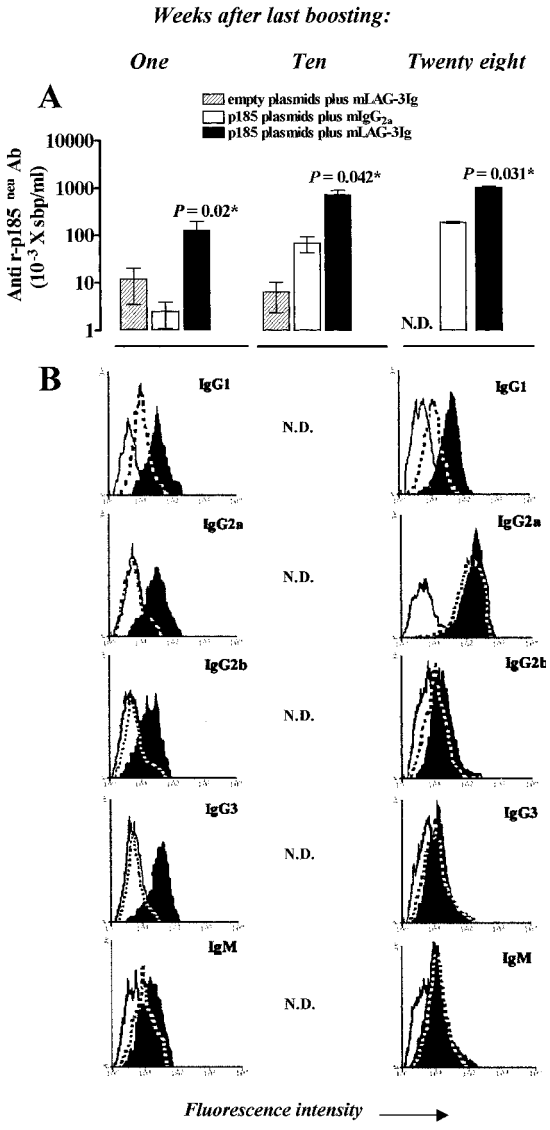


Fig. 7. Production of antibodies to r-p185$^{neu}$ by vaccinated BALB-neuT mice. One, 10, and 28 weeks after the last boost, sera were collected from five mice from the group vaccinated with empty or p185 plasmids plus mLAG-3Ig or with p185 plasmids plus control mIgG2a and individually tested. *A*, the specific binding of each serum to r-p185$^{neu}$-positive N202.1A cells was evaluated and expressed as the mean sbp ± SE. *, values from mice immunized with p185 plasmids plus mLAG-3Ig are significantly different from those of mice immunized with p185 plasmids plus mIgG2a at all of the times tested. *B*, influence of mLAG-3Ig on isotype switch. N202.1A (r-p185$^{neu}$-positive) cells were stained after pooling the sera collected as specified above. *Open profiles*, cells stained with secondary antibody alone; *dotted profiles*, cells incubated with sera from mice vaccinated with p185 plasmids plus control mIgG2a; *solid black profiles*, mice vaccinated with p185 plasmids plus mLAG-3Ig. In each panel, the *ordinates* represent the number of cells.

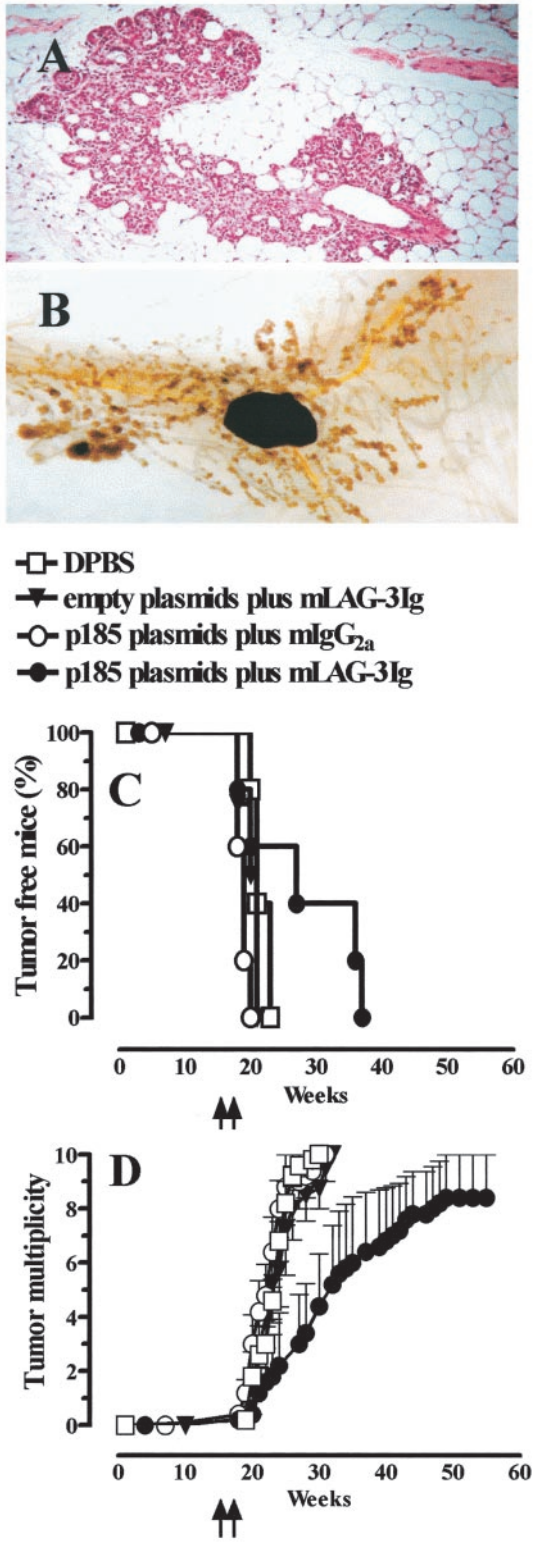


Fig. 8. Delay in appearance of multifocal *in situ* carcinomas by p185 plasmid vaccination plus mLAG-3Ig. Vaccination was performed on week 14 and 16, when histological analysis (*A*) and whole mounts (*B*) showed that multiple foci of atypical hyperplasia, large side buds, and *in situ* carcinomas were already present in all of the mammary glands of BALB-neuT mice. Percentage of tumor-free mice (*C*) and tumor multiplicity (*D*) calculated as the cumulative number of incident tumors/total number of mice and shown as mean ± SE are represented. Mice received DPBS alone, empty pcDNA3 plasmids plus mLAG-3Ig, p185 plasmids plus control mIgG2a, and p185 plasmids plus mLAG-3Ig. Each group consisted of 10 mice. As compared with all of the other groups of mice, the group immunized with p185 plasmids plus mLAG-3Ig displayed a significantly higher number of tumor-free mice (from week 21 to week 35, $P < 0.003$ by Mantel-Haenszel test) and a significantly reduced tumor multiplicity (from week 21 until week 52) as shown by Student's *t* test ($P < 0.01$).

## DISCUSSION

BALB-neuT virgin female mice are genetically predestined to die because multiple invasive mammary carcinomas develop in all 10 of their mammary glands (6, 7). This multifocal carcinogenesis is temporarily inhibited by p185 plasmid vaccination. Whereas all control mice display one or more palpable tumors by week 20–25, about 80% of those immunized with p185 plasmids plus control mIgG2a at week 4 and 7 are still tumor free 32 weeks after the last immunization. This protection, however, quickly fades, and only about 20% of mice remain tumor free at 1 year of age. Multiple DNA boosting (9) did not increase this percentage (data not shown). By contrast, association of mLAG-3Ig with DNA vaccination kept 70% of mice tumor free until

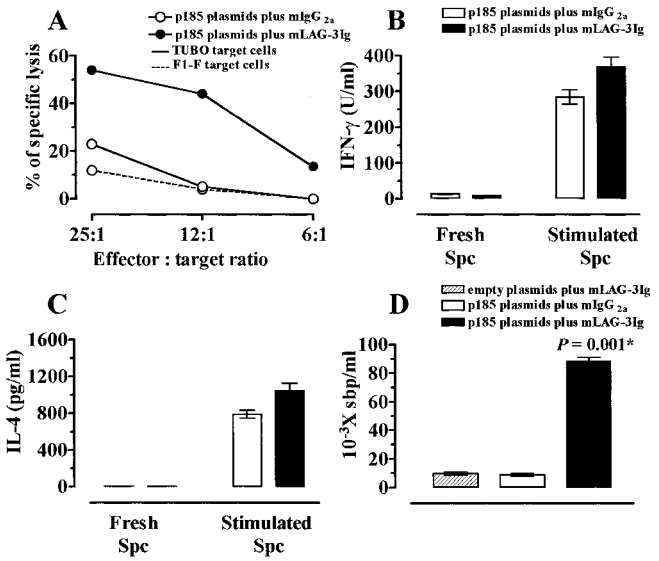


Fig. 9. Cellular reactivity associated with the delay of carcinoma growth. SPCs were obtained on week 18 from mice vaccinated on week 14 and 16 with p185 plasmids plus control mIgG2a or mLAG-3Ig. *A*, SPCs were assayed in a 48-h [$^3$H]dThd release test against r-p185$^{neu}$-positive TUBO and r-p185$^{neu}$-negative F1-F target cells after *in vitro* stimulation with Mit-C-treated TUBO cells. The representative values of one of five tested mice are shown. *B* and *C*, IFN-γ (*B*) and IL-4 (*C*) production by SPCs from mice vaccinated with p185 plasmids plus control mIgG2a or mLAG-3Ig fresh or stimulated with mAb anti-CD3 and anti-CD28, for 48 h. The *bars* represent mean values obtained from five individually tested mice ± SE. *D*, 2 weeks after the last boost, sera were individually collected from five mice from the group vaccinated with empty or p185 plasmids plus mLAG-3Ig or with p185 plasmids plus control mIgG2a. The specific binding of each serum to r-p185$^{neu}$-positive N202.1A cells was evaluated and expressed as the mean sbp ± SE. ∗, values from mice immunized with p185 plasmids plus mLAG-3Ig were significantly different from those of mice immunized with p185 plasmids plus mIgG2a and with empty plasmids plus mLAG-3Ig.

week 52, when the experiment ended. The absence of a palpable tumor was usually accompanied by complete clearance of neoplastic side buds and r-p185$^{neu}$-expressing cells from the mammary glands, as shown by whole mounts and pathological observations, although residual neoplastic lesions were still evident in a few glands.

In BALB-neuT mice, the target transforming oncogene is embedded in the genome (5). This builds up a dynamic relationship between the oncogenic rat HER-2/*neu* signals and the inhibitory potential of the immune reactions activated by p185 plasmid vaccination. In the absence of the continuous onset of r-p185$^{neu}$ neoplastic cells, the strong and sustained inhibition of carcinogenesis that follows vaccination with p185 plasmids plus mLAG-3Ig would seem to coincide with a definitive cure. These marked preventive effects of DNA vaccination were lost when it was applied in older mice with already evident neoplastic lesions similar to multiple hyperplastic foci and carcinoma *in situ*. Even here, however, p185 plasmids plus mLAG-3Ig enhanced the immune response and significantly extended tumor latency.

These findings show that DNA vaccination is an efficient way to prevent the progression of early lesions but is much less effective when begun in mice with advanced lesions (1). The critical part played by mLAG-3Ig in allowing p185 plasmid vaccination to elicit enduring protection is not surprising. Addition of mLAG-3Ig as adjuvant in vaccination with a particulate or soluble protein antigen effectively stimulates the priming of cytotoxic T lymphocytes, activates the proliferative response of spleen T cells along with their Th1-type cytokine production, and induces much higher antibody titers than a conventional adjuvant (18). Enhancement of these immunological activities and the induction of antitumor immune responses and tumor regression (13, 18) appear to rest on the ability of the LAG-3-induced MHC class II signal to activate macrophages and immature DCs. Cross-linking of certain MHC class II molecules expressed on their membrane and probably contained in lipid rafts (17) contributes to both their activation and the commitment of T cells toward Th1/T killer activity (26, 27).

Activation of CD4 and CD8 T-cell functions after DNA vaccination rests on cross-presentation of antigenic peptides released by transfected myocytes through bone marrow-derived macrophages and DCs (28, 29). The protection against carcinogenesis associated with the presence of mLAG-3Ig correlates with a marked enhancement of both the cellular and the humoral immune response (18). In effect, mLAG-3Ig appears to enhance a mixed Th2-Th1 immune response, rather than a Th1 response only. In immunized mice, it induced a persistent cellular cytotoxicity, a sustained release of IFN-γ, expansion of both CD8$^+$/CD11b$^+$/CD28$^+$ effector and CD8$^+$/CD11b$^+$/CD28$^-$ effector memory T-cell populations (23, 24), and a faster shift toward Th1-type IgG2a. However, mLAG-3Ig also induced a shift toward IgG1, enhanced IgG2a production, and persistently enhanced the titer of anti-r-p185$^{neu}$ antibody. These latter activities may be directly related to the much higher IL-4 production observed in the spleen (25).

The anti-r-p185$^{neu}$ antibodies in the sera of immunized mice may induce a functional block of the r-p185$^{neu}$ receptor (30), down-regulate its expression on the cell membrane (30, 31), impede its ability to form the homo- or heterodimers that spontaneously transduce proliferative signals to the cells (31, 32), and block its ability to bind ligands (33), as has been observed with anti-Her-2/neu mAb. They also significantly suppress the growth of transplantable p185$^{neu}$-positive tumors (34, 35) and the onset of mammary carcinomas in HER-2/*neu* transgenic mice (31) and delay tumor growth in patients with HER-2/*neu*-positive tumors (36). The morphological features of the inhibited mammary cell proliferation associated with marked membrane down-modulation of r-p185$^{neu}$ and diminished nuclear positivity of PCNA, characterizing the regression of both preneoplastic lesions and incipient carcinomas, point to direct inhibition by these antibodies.

The success of DNA vaccination in the inhibition of r-p185$^{neu}$-positive carcinomas is apparently associated with a high and sustained antibody response to a growth factor receptor, whose down-regulation slows the preneoplastic cell proliferation and tumor development. This inhibition mechanism is different from immunological destruction of the malignant cells (7–9). However, a massive tumor cell killing also takes place because these antibody isotypes activate polymorphonuclear leukocytes and other cells to mediate antibody-dependent cell cytotoxicity (35, 37, 38) and complement-dependent cytotoxicity and inhibit the growth of r-p185$^{neu}$-positive tumors *in vivo* (35). Moreover, in immunized mice, cytotoxic T cells as well as T cells releasing IFN-γ and mediating intratumor delayed-type hypersensitivity reactions also appear to play an important effector and regulatory role in the inhibition of BALB-neuT mice mammary carcinogenesis (7–9, 39).

The association of mLAG-3Ig with DNA vaccination halted the progression of multifocal carcinogenesis in all 10 mammary glands of BALB-neuT mice for a long period. mLAG-3Ig may thus be supposed to provide a costimulatory signal that allows vaccination to establish longer and more effective protection, probably by enhancing the cross-presentation of the DNA-coded protein (27). The effect of mLAG-3Ig administration is to sustain the persistence of a responsive status instead of increasing an actively ongoing immune reaction. The result of its coadministration becomes more and more significant as the mice age. A similar form of combined vaccination could be considered as a noninvasive option in the management of patients diagnosed with early HER-2/*neu* (c-*erb*B-2)-expressing neoplastic lesions.

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## REFERENCES

1. Forni, G., Lollini, P-L., Musiani, P., and Colombo, M. P. Immunoprevention of cancer: is the time ripe? Cancer Res., *60:* 2571–2575, 2000.
2. Finn, O. J., and Forni, G. Prophylactic cancer vaccines. Curr. Opin. Immunol., *14:* 172–177, 2002.
3. Yarden, Y., and Sliwkowski, M. X. Untangling the ErbB signalling network. Nat. Rev. Mol. Cell. Biol., *2:* 127–136, 2001.
4. Gullick, W. J., Love, S. B., Wright, C., Barnes, D. M., Gusterson, B., Harris, A. L., and Altman, D. G. C-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br. J. Cancer, *63:* 434–438, 1991.
5. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell, *54:* 105–115, 1988.
6. 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., and Forni, G. Interleukin-12 mediated prevention of spontaneous mammary adenocarcinomas in two lines of HER-2/*neu* transgenic mice. J. Exp. Med., *188:* 589–596, 1998.
7. Di Carlo, E., Diodoro, M. G., Boggio, K., Modesti, A., Modesti, M., Nanni, P., Forni, G., and Musiani, P. Analysis of mammary carcinoma onset and progression in HER-2/*neu* oncogene transgenic mice reveals a lobular origin. Lab. Investig., *79:* 1261–1269, 1999.
8. Cavallo, F., Di Carlo, E., Quaglino, E., Iezzi, M., Strasly, M., Bussolino, F., Colombo, M. P., Nanni, P., Lollini, P-L., Musiani, P., and Forni, G. Prevention by delay: nonspecific immunity elicited by IL-12 hinders HER-2/*neu* mammary carcinogenesis in transgenic mice. J. Biol. Regul. Homeost. Agents, *15:* 351–358, 2001.
9. Rovero, S., Amici, A., Di Carlo, E., Bei, R., Nanni, P., Quaglino, E., Porcedda, P., Boggio, K., Smorlesi, A., Lollini, P-L., Landuzzi, L., Colombo, M. P., Giovarelli, M., Musiani, P., and Forni, G. DNA vaccination against rat Her-2/neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. J. Immunol., *165:* 5133–5142, 2000.
10. Di Carlo, E., Rovero, S., Boggio, K., Quaglino, E., Amici, A., Smorlesi, A., Forni, G., and Musiani, P. Inhibition of mammary carcinogenesis by systemic IL-12 or p185neu DNA vaccination in HER-2/*neu* transgenic BALB/c mice. Clin. Cancer Res., *7* (Suppl)*:* 830s–837s, 2001.
11. Rovero, S., Boggio, K., Di Carlo, E., Amici, A., Quaglino, E., Porcedda, P., Musiani, P., and Forni, G. Insertion of the DNA for the 163–171 peptide of IL-1$\beta$ enables a DNA vaccine encoding $p185^{neu}$ to inhibit mammary carcinogenesis in HER-2/neu transgenic BALB/C mice. Gene Ther., *8:* 447–452, 2001.
12. Huard, B., Prigent, P., Pagès, F., Bruniquel, D., and Triebel, F. T cell major histocompatibility complex class II molecules down-regulate $CD4^+$ T cell clone responses following LAG-3 binding. Eur. J. Immunol., *26:* 1180–1186, 1996.
13. Prigent, P., El Mir, S., Dreano, M., and Triebel, F. Lymphocyte activation gene-3 induces tumor regression and antitumor immune responses. Eur. J. Immunol., *29:* 3867–3876, 1999.
14. Triebel, F., Jitsukawa, S., Baixeras, E., Roman-Roman, S., Genevee, C., Viegas-Pequignot, E., and Hercend, T. LAG-3, a novel lymphocyte activation gene closely related to CD4. J. Exp. Med., *171:* 1393–1405, 1990.
15. Bruniquel, D., Borie, N., and Triebel, F. Genomic organization of the human LAG-3/CD4 locus. Immunogenetics, *47:* 96–98, 1997.
16. Huard, B., Tournier, M., Hercend, T., Triebel, F., and Faure, F. Lymphocyte activation gene-3/major histocompatibility complex class II interaction modulates the antigenic response of $CD4^+$ T lymphocytes. Eur. J. Immunol., *24:* 3216–3221, 1994.
17. Machy, P., Serre, K., Baillet, M., and Leserman, L. Induction of MHC class I presentation of exogenous antigen by dendritic cells is controlled by $CD4^+$ T cells engaging class II molecules in cholesterol-rich domains. J. Immunol., *168:* 1172–1180, 2002.
18. El Mir, S., and Triebel, F. A soluble lymphocyte activation gene-3 molecule used as a vaccine adjuvant elicits greater humoral and cellular immune responses to both particulate and soluble antigens. J. Immunol., *164:* 5583–5589, 2000.
19. Nanni, P., Pupa, S., Nicoletti, G., De Giovanni, C., Landuzzi, L., Rossi, I., Astolfi, A., Ricci, C., De Vecchi, R., Invernizzi, A. M., Di Carlo, E., Musiani, P., Forni, G., Menard, S., and Lollini, P-L. $p185^{neu}$ protein is required for tumor and anchorage independent growth not for cell proliferation of transgenic mammary carcinoma. Int. J. Cancer, *87:* 186–194, 2000.
20. Amici, A., Venanzi, F. M., and Concetti, A. Genetic immunization against neu/erbB2 transgenic breast cancer. Cancer Immunol. Immunother., *47:* 183–190, 1998.
21. Medina, D. Preneoplastic lesions in mouse mammary tumorigenesis. Methods Cancer Res., *7:* 3–53, 1973.
22. Giovarelli, M., Musiani, P., Modesti, A., Dellabona, P., Castrati, G., Allione, A., Consalvo, M., Cavallo, F., Di Pierro, F., De Giovanni, C., Musso, T., and Forni, G. The local release of IL-10 by transfected mouse mammary adenocarcinoma cells does not suppress but enhances antitumor reaction and elicits a strong cytotoxic lymphocyte and antibody dependent immune memory. J. Immunol., *155:* 3112–3123, 1995.
23. Fiorentini, S., Licenziati, S., Alessandri, G., Castelli, F., Caligaris, S., Bonafede, M., Grassi, M., Garrafa, E., Balsari, A., Turano, A., and Caruso, A. CD11b expression identifies $CD8^+CD28^+$ T lymphocytes with phenotype and function of both naive/memory and effector cells. J. Immunol., *166:* 900–907, 2001.
24. McFarland, H. I., Nahill, S. R., Maciaszek, J. W., and Welsh, R. M. CD11b (Mac 1): a marker for $CD8^+$ cytotoxic T cell activation and memory in virus infection. J. Immunol., *149:* 1326–1333, 1992.
25. Snapper, C. M., Finkelman, F. D., and Paul, W. E. Differential regulation of IgG1 and IgE synthesis by interleukin-4. J. Exp. Med., *167:* 183–196, 1988.
26. Avice, M-N., Sarfati, M., Triebel, F., Delespesse, G., and Demeure, C. E. Lymphocyte activation gene-3, a MHC class II ligand expressed on activated T cells, stimulates TNF-$\alpha$ and IL-12 production by monocyte and dendritic cells. J. Immunol., *162:* 2748–2753, 1999.
27. Andreae, S., Piras, F., Burdin, N., and Triebel, F. Maturation and activation of dendritic cells induced by lymphocyte activation gene-3 (CD223). J. Immunol., *168:* 3874–3880, 2002.
28. Corr, M., Lee, D. J., Carson, D. A., and Tighe, H. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. J. Exp. Med., *184:* 1555–1560, 1996.
29. Kumar, V., and Sercaz, E. Genetic vaccination: the advantages on going naked. Nat. Med., *2:* 857–858, 1996.
30. Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A., and Greene, M. I. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell, *41:* 697–706, 1985.
31. Katsumata, M., Okududaira, T., Samanta, A., Clark, D. P., Drebin, J. A., Jolicoeur, P., and Greene, M. I. Prevention of breast tumors development *in vivo* by downregulation of the p185neu receptor. Nat. Med., *1:* 644–648, 1995.
32. Klapper, N. L., Vaisman, N., Hurwitz, E., Pinkas-Kramarski, R., Yarden, Y., and Sela, M. A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. Oncogene, *14:* 2099–2109, 1997.
33. Xu, F., Lupu, R., Rodriguez, G. C., Whitaker, R. S., Boente, M. P., Berchuck, A., DeSombre, K. A., Boyer, C. M., and Bast, R. C., Jr. Antibody-induced growth inhibition is mediated through immunochemically and functionally distinct epitopes on the extracellular domain of c-erbB-2 (HER-2/*neu*) gene product p185. Int. J. Cancer, *53:* 401–408, 1993.
34. Drebin, J. A., Link, V. C., Winberg, R. A., and Greene, M. I. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. Proc. Natl. Acad. Sci. USA, *83:* 9129–9133, 1986.
35. Drebin, J. A., Link, V. C., and Greene, M. I. Monoclonal antibodies specific for the neu oncogene product directly mediate anti-tumor effects *in vivo*. Oncogene, *2:* 387–394, 1988.
36. Pegram, M. D., and Slamon, D. J. Combination therapy with trastuzumab (Herceptin) and cisplatin for chemoresistant metastatic breast cancer: evidence for receptor-enhanced chemosensitivity. Semin. Oncol., *26:* 89–95, 1999.
37. Shen, L., Lydyard, P. M., Roitt, I. M., and Fanger, M. W. Synergy between IgG and monoclonal IgM antibodies in antibody-dependent cell cytotoxicity. J. Immunol., *127:* 73–78, 1981.
38. Huls, G., Heijnen, I. A., Cuomo, E., van der Linden, J., Boel, E., van de Winkel, J. G., and Logtenberg, T. Antitumor immune effector mechanisms recruited by phage display-derived fully human IgG1 and IgA1 monoclonal antibodies. Cancer Res., *59:* 5778–5784, 1999.
39. 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., and Lollini, P-L. Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2/*neu* transgenic mice. J. Exp. Med., *194:* 1195–1205, 2001.